Investigation of immune-suppressive genes expressed by the *Cotesia rubecula* bracovirus (CrBV)

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For my mother, Janet (1946-2002), who provided immeasurable love and support but was not able to see the result. I miss you mum.

So we gathered us 'round some Hakea flowers
You can watch them die by hours, by hours
Sun fall, sun rise
Cold mallee skies

See the beauty of them all, ma
For I could give you back but little
For all of that stuff you've done
Slowly we're returning to where we came from, ma
I know I came from you
I know I am your son

See the beauty of them all
From your final place in the mallee sands
All we can do is carry you
With my medal and your violets in your hands
Two by two, on the mallee sands
We will carry you with our hands
And lay you down upon the land
Because you carried us
You carried us

The inchworm song

Inchworm, inchworm
Measuring the marigolds
You and your arithmetic
Will probably go far

Two and two are four
Four and four are eight
Eight and eight are sixteen
Sixteen and sixteen are thirty two

Inchworm, inchworm
Measuring the marigolds
Seems to me you'd stop and see
How beautiful they are

Two and two are four
Four and four are eight
Eight and eight are sixteen
Sixteen and sixteen are thirty two

(Frank Loesser)

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Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other 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.

Richard Vernon Glatz

January 2004

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Abstract

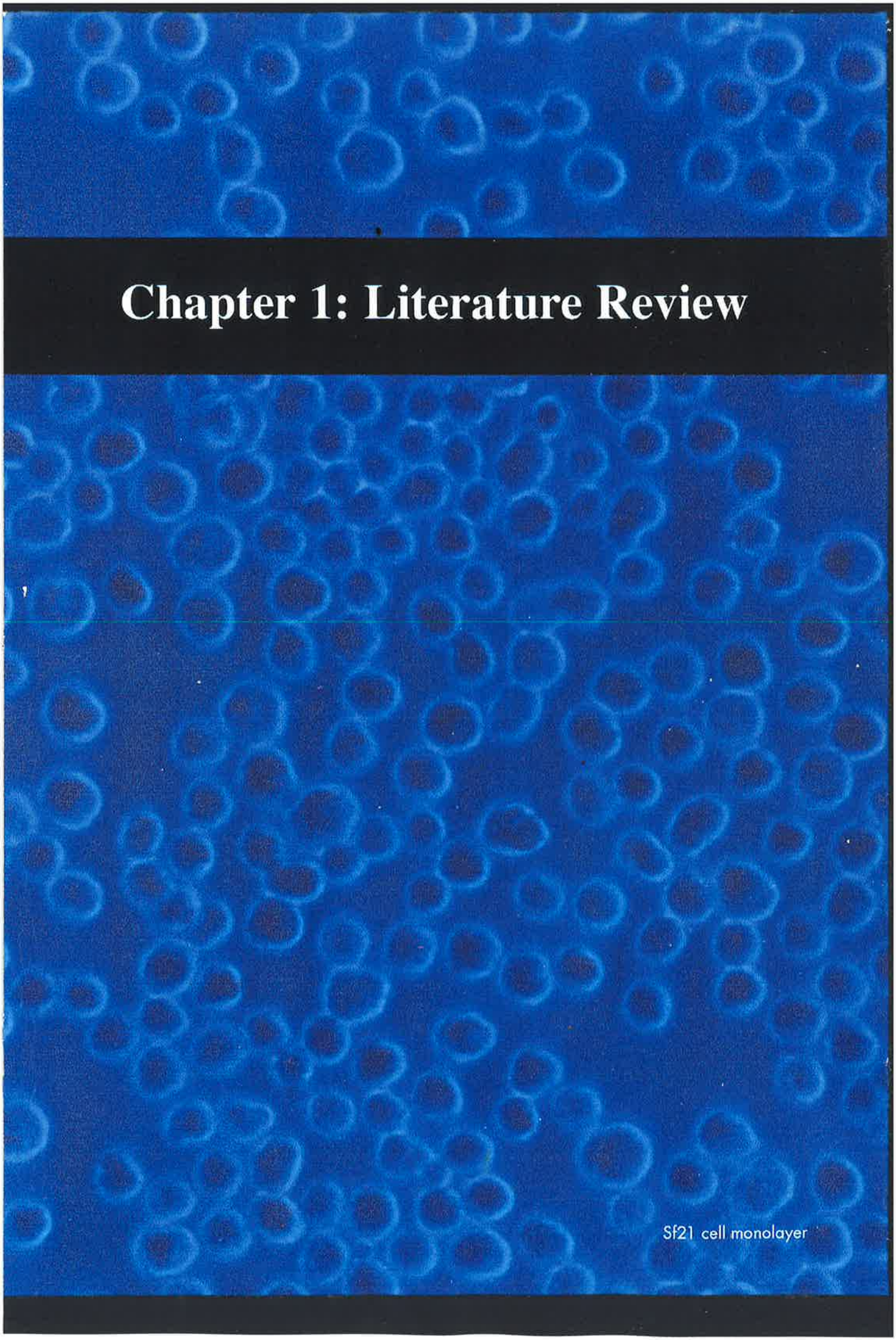
The hymenopteran endoparasitoid, *Cotesia rubecula,* employs integrated forms of active and passive immune-suppression in overcoming the defences of its host, *Pieris rapae*, a cosmopolitan pest of cruciferous crops. The immune-suppressive activity arises from a complex of maternally secreted proteins and polydnavirus (PDV) particles, which are injected in a host larva with the parasitoid egg at oviposition. The PDV associated with *C. rubecula* (CrBV) is unusual in that only four main viral genes (CrV1-CrV4) are expressed in *P. rapae* tissues and that expression is transient, remaining at high levels only in the period between four and eight hours post-parasitisation (hpp). Previously, CrV1 was characterised and found to inactivate host haemocytes by causing disruption of their cytoskeleton, leading to abrogation of immune-associated processes such as spreading. In this study, a cDNA library was constructed from parasitised *P. rapae* larvae and screened with total CrBV DNA, leading to isolation of CrV2 and CrV3. The open reading frame of each gene was cloned in a bacterial expression vector and the resultant recombinant proteins were used to produce antibodies against CrV2 and CrV3.

CrV2 has an open reading frame of 960 bp (with no introns) and encodes a glycoprotein of ≈ 40 kDa, which is secreted from infected haemocytes and fat body. Comparison of CrV2 deduced amino acid sequence with other known sequences revealed no significant homologies. CrV2 protein was detected in host larvae at 6 hpp, remaining in large amounts for at least a day and was declining by 48 hpp. A putative coiled-coil region at the C-terminus of CrV2 is suspected of involvement in formation of CrV2 trimers that were detected under non-denaturing conditions. CrV2 was visualised within haemocytes in large endosomes at 24 hpp. Although the function of CrV2 remains unclear, it appears to interact with host haemocytes presumably to suppress their immune function.

The CrV3 gene contained and intron and was found to encode a C-type lectin (CTL) homologue, which is secreted from infected host haemocytes and fat body into haemolymph. Two CrV3 monomers (of ≈ 14 and 17 kDa) were detected in parasitised larvae with the larger monomer being an N-glycosylated form of the smaller monomer. CrV3 dimers and tetramers were also detected *in vivo*. Recombinant CrV3 forms larger complexes and was shown to agglutinate ovine red blood cells, an activity that was Mn^{2+}- and Mg^{2+}-dependent but was independent of Ca^{2+}. CrV3-mediated hemagglutination was inhibited by EDTA but not by biological concentrations of 29 potential ligands tested. Interestingly, CrV3 is similar to invertebrate CTLs associated with humoral defence but not with previously isolated viral lectins. Further, CrV3 homologues were recently detected in bracoviruses from *C. ruficrus* and *C. karyai*, indicating that a novel CTL family is expressed by some *Cotesia*-associated PDVs. CrV3 probably interacts with a soluble host haemolymph component associated with host humoral immune defences.

CrV1 and Crp32 (an immune-suppressive *C. rubecula* calyx protein) were used to produce recombinant *Autographa californica* multiple nucleopolyhedrosis viruses (AcMNPVs), pathogens with putatively enhanced virulence in *P. rapae*. Bioassays were undertaken to investigate the pathogencity of wild-type AcMNPV in *P. rapae* (previously unreported) and the effect of insertion of Crp32. Although the proportion of larval deaths due to wild-type AcMNPV was significant, the slow rate of mortality indicated that *P. rapae* is only semi-permissive to AcMNPV. Crp32 insertion proved insignificant in terms of the proportion and rate of larval mortality. Given the semi-permissive nature of *P. rapae*, recombinant AcMNPVs expressing immune-suppressive and appropriate reporter genes may be useful for elucidating mechanisms of insect immunity and more specifically, how CrBV acts to subvert these mechanisms in *P. rapae*.

Chapter 1: Literature Review



Sf21 cell monolayer

Chapter 1: Literature Review

1.1 Introduction to polydnaviruses

Polydnaviruses (PDVs) are a unique group of double-stranded DNA insect viruses, so named because of the polydisperse nature of their genomes, which are organised as a series of different circular DNAs (or segments). Only several PDV genomes have been studied in detail, with these limited data showing a range in segment number from less than ten to greater than 25 (Webb, 1998). These viruses are replicated within certain Ichneumonoid endoparasitoids with which they share an association that is both obligate and symbiotic, causing no known deleterious effects in the parasitoid (Beckage, 1998). PDVs are atypical of viral symbionts in that PDV DNA is integrated into the genome of the parasitoid (Xu & Stoltz, 1991; Fleming & Summers, 1991; Gruber *et al.*, 1996) and vertically transmitted between parasitoids, in Mendelian fashion (Stoltz *et al.*, 1986; Stoltz, 1990).

Virus particles are produced in association with the reproductive tract of female parasitoids and are injected into mainly lepidopteran hosts, accompanying parasitoid eggs (Webb, 1998). Virions then infect host tissues and a host-specific sub-set of viral genes is expressed, the products of which modify development, behaviour and immune response of the lepidopteran host (Fleming, 1992). PDVs are in most cases required for survival/development of the parasitoid egg and larva within a given host (Edson *et al.*, 1981; Stoltz & Guzo, 1986; Guzo & Stoltz, 1987) and female wasps are needed for virus production and transference, thus the symbiotic relationship existing between PDV and parasitoid. The unique nature of this relationship and the marked effect of PDV-derived proteins on lepidopteran host physiology (many species of which are agricultural and food-storage pests) have ensured that PDVs have been extensively studied. Much of this work has focussed on identity and action of biologically active PDV proteins within lepidopteran

hosts. It should be noted that the bulk of knowledge about PDVs derives from studies performed on the PDV associated with *Campoletis sonorensis* (CsIV). Very few other PDVs have been highly characterised and no PDV genome has been completely mapped or sequenced (Webb, 1998). Therefore, generalised statements about PDV biology should be considered in this light.

PDVs comprise the viral family Polydnaviridae based on their polydisperse DNA genome and their association with a defined group of parasitic hymenoptera (Webb *et al.*, 2000). The polydnaviridae are further subdivided into two genera, *Ichnovirus* and *Bracovirus*, based on differential morphology and morphogenesis, envelope properties, sub-cellular site of replication, number of enveloped virions and host range (Beckage, 1998). Vertical transmission ensures that each parasitoid species carries a virus that is genetically isolated from other viruses and which can therefore be considered as a unique virus species (Webb, 1998). Table 1-1 (from Webb, 2000) provides a list of officially described PDV species and their abbreviations, split into genera. The diversity of ichneumonoid wasps suggests that many more PDVs exist than are presently described. Figure 1-1 illustrates the phylogenetic relationship between PDV genera and their ichneumonoid hosts.

1.1.1 Genus *Ichnovirus*

Ichnovirus (IV) particles, found in the reproductive tract of some female ichneumonid parasitoids, were first detected by Rotheram (1967). Most IVs have been isolated from wasps in the subfamily Campopleginae although virus has also been detected in several genera from the Banchinae and Ctenopelmatinae (Turnbull & Webb, 2002 – see Fig. 1-1). It is thought that all wasp species in these subfamilies contain IVs, whilst the remaining 22 ichneumonid subfamilies do not have species associated with IVs (Webb, 1998).

Ichnovirus	
Campoletis aprilis ichnovirus	CaIV
Campoletis flavicincta ichnovirus	CfIV
*Campoletis sonorensis ichnovirus**	CsIV*
Casinaria arjuna ichnovirus	CarIV
Casinaria forcipata ichnovirus	CfoIV
Casinaria infesta ichnovirus	CiIV
Diadegma acroyctyae ichnovirus	DaIV
Diadegma interruptum ichnovirus	DiIV
Diadegma terebrans ichnovirus	DtIV
Eriborus terebrans ichnovirus	EtIV
Enytus montanus ichnovirus	EmIV
Glypta fumiferanae ichnovirus	GfIV
Hyposoter annulipes ichnovirus	HaIV
Hyposoter exiguae ichnovirus	HeIV
Hyposoter fugitivus ichnovirus	HfIV
Hyposoter lymantriae ichnovirus	HlIV
Hyposoter pilosulus ichnovirus	HpIV
Hyposoter rivalis ichnovirus	HrIV
Olesicampe benefactor ichnovirus	ObIV
Olesicampe geniculatae ichnovirus	OgIV
Synetaeris tenuifemur ichnovirus	StIV

Bracovirus	
Apanteles crassicornis bracovirus	AcBV
Apanteles fumiferanae bracovirus	AfBV
Ascogaster quadridentata bracovirus	AaBV
Ascogaster argentifrons bracovirus	AqBV
Cardiochiles nigriceps bracovirus	CnBV
Chelonus altitudinis bracovirus	CalBV
Chelonus blackburni bracovirus	CbBV
Chelonus nr. curvimaculatus bracovirus	CcBV
Chelonus inanitus bracovirus	CinaBV
Chelonus insularis bracovirus	CinsBV
Chelonus texanus bracovirus	CtBV
Cotesia congregata bracovirus	CcBV
Cotesia flavipes bracovirus	CfBV
Cotesia glomerata bracovirus	CgBV
Cotesia hyphantriae bracovirus	ChBV
Cotesia karyai bracovirus	CkBV
Cotesia marginiventris bracovirus	CmaBV
*Cotesia melanoscela bracovirus**	CmeBV*
Cotesia rubecula bracovirus	CrBV
Cotesia shaeferi bracovirus	CsBV
Diolcogaster facetosa bracovirus	DfBV
Glyptapanteles flavicoxis bracovirus	GflBV
Glyptapanteles indiensis bracovirus	GiBV
Glyptapanteles liparidis bracovirus	GlBV
Hypomicrogaster canadensis bracovirus	HcBV
Hypomicrogaster ecdytolophae bracovirus	HecBV
Microplitis croceipes bracovirus	McBV
Microplitis demolitor bracovirus	MdBV
Phanerotoma flavitestacea bracovirus	PfBV
Pholetesor ornigis bracovirus	PoBV
Protapantales paleacritae bracovirus	PpBV
Tranosema rostrale bracovirus	TrBV

Table 1-1. List of official polydnaviruses, split into genera *Bracovirus* and *Ichnovirus*, and their abbreviations (from Webb *et al.*, 2000). * type species for relevant genus.

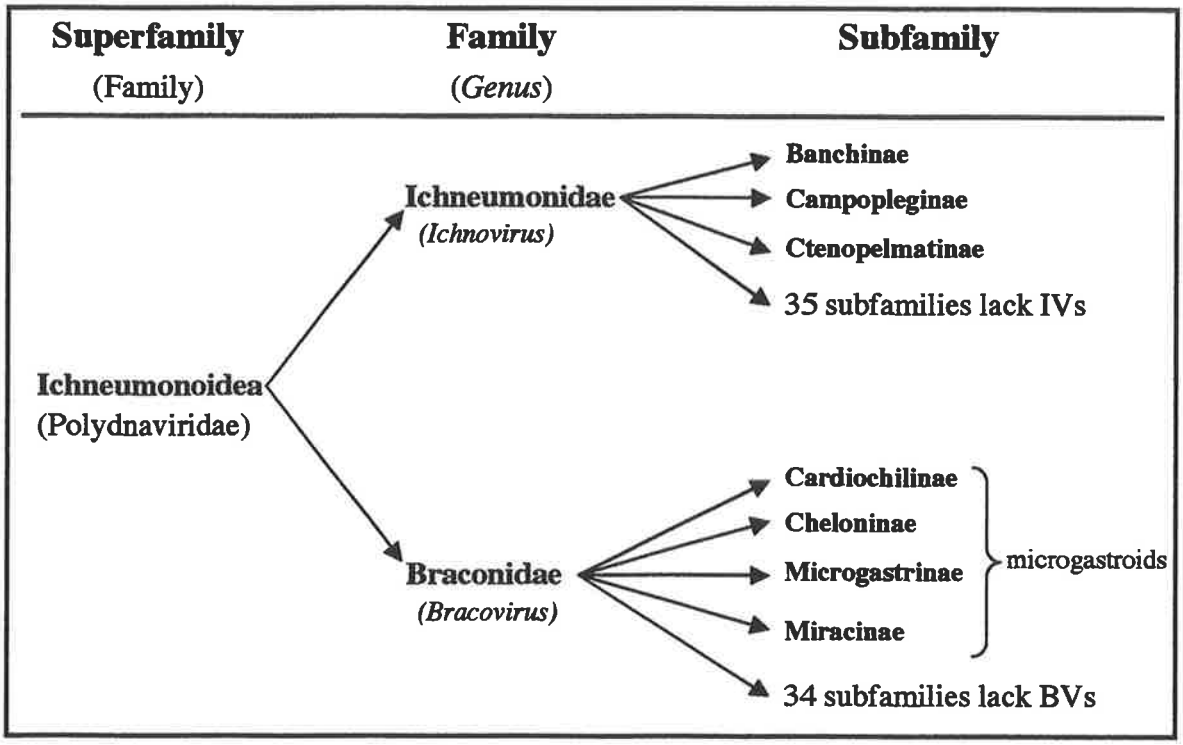


Figure 1-1. Phylogenetic relationships between polydnaviruses (*brackets*) and Ichneumonoid wasps (*bolded*), adapted from Turnbull & Webb (2002). All species in listed sub-families are thought to be associated with polydnaviruses. Ichnoviruses are apparently restricted to three inchneumonid subfamilies whilst bracoviruses are restricted to four microgastroid subfamilies. It is thought that ancestral viruses (lepidopteran pathogens) began symbiotic relationships with hymenopteran ancestors common to the listed subfamilies (in both braconidae and ichneumonidae) but possibly not to the subfamilies without polydnaviruses.

The lenticular nucleocapsids of IVs appear to be of similar size (≈ 85 nm × ≈ 330 nm) and are enclosed by two separate membranes (Stoltz & Vinson, 1979). PDV virions are generally complex, comprised of 20-30 structural proteins ranging in molecular weight from 10-200 kDa (Webb *et al.*, 2000). Some IV structural proteins appear to be formed from peptides encoded on multiple genome segments as even larger segments have too little DNA to encode mature structural proteins (Krell *et al.*, 1982). Some IV proteins have been shown to be of high antigenic similarity to wasp-derived proteins contained in venom, which is injected into the host with the parasitoid egg (Webb & Summers, 1990).

Aggregate genome size has been estimated as ranging from ≈ 75-250 kbp with individual segments ranging from 2-28 kbp (Webb, 1998). Virus particles from *Hyposoter exiguae*, are shown in figure 1-2. It appears that individual IV genome segments are heritably maintained in nonequimolar ratios, a phenomenon that may potentially derive from differential segment replication and/or packaging efficiencies (Webb, 1998). The packaging mechanism for viral genome segments is poorly characterised and although the nucleocapsids could potentially hold the largest of IV genomes, it is not clear if the entire genome is ever packaged within single virions (Webb, 1998).

1.1.2 Genus *Bracovirus*

The demonstration of DNA in reproductive fluids of the parasitoid *Cardiochiles nigriceps* by Vinson & Scott (1975) was the first evidence for the existence of bracoviruses (BVs). Most BVs have been detected in the two braconid subfamilies Cheloninae and Microgastrinae although it is suspected that all species in the four largest microgastroid subfamilies contain BVs (Whitfield, 1997; see Fig. 1-1).

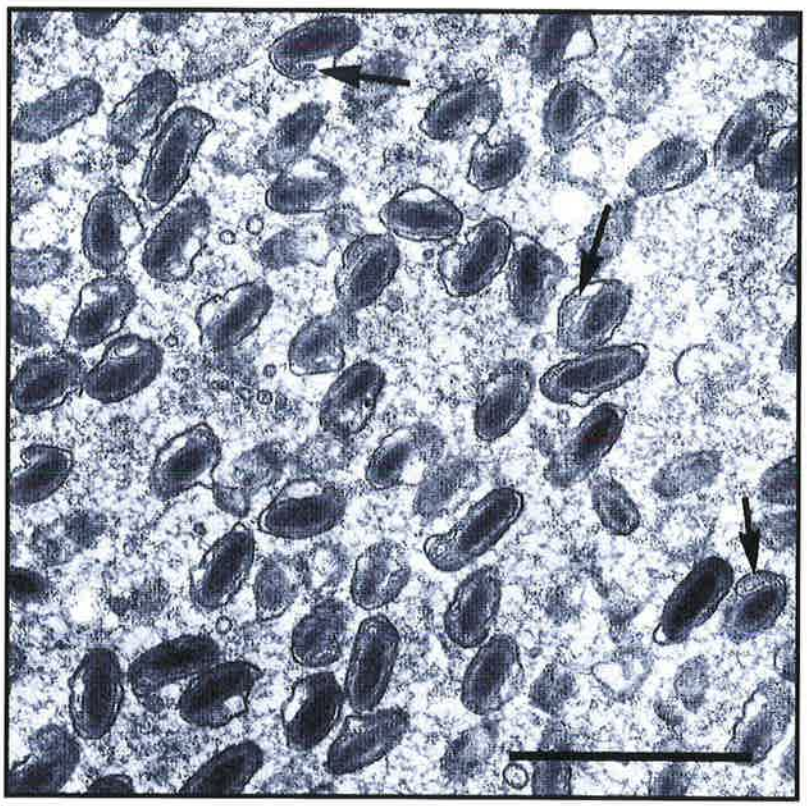


Figure 1-2. Virions of *Hyposoter exiguae* ichnovirus suspended in calyx fluid in the parasitoid oviduct lumen (from Stoltz & Vinson, 1979). *Arrows* indicate protrusions of the inner virion envelope, which may be involved in entry of virions into lepidopteran host cells. *Bar* = 1 μm.

In contrast to IVs, BVs have cylindrical (rod shaped) nucleocapsids, one or several of which are enclosed by a single membrane only, depending on the virus species (Stoltz & Vinson, 1979). Nucleocapsids vary in length from 30-150 nm but generally have a uniform diameter of ≈ 30-40 nm (Webb, 1998). Singly and multiply enveloped viruses are shown in figures 1-3*A* and 1-3*B*, respectively. Mature capsids of some BVs have a characteristic tail appendage, the suspected function of which is to penetrate host nuclear pores and to allow release of viral DNA into the nucleus (Stoltz & Vinson, 1979). Figure 1-4 shows examples of different BV virions and nucleocapsids showing characteristic tail appendages.

BV genome size has been estimated as ranging from above 100 kbp to 200 kbp (Strand *et al.*, 1992; Albrecht *et al.*, 1994; Soller & Lanzrein, 1996). Histograms of variation in nucleocapsid length and DNA contour length for *Cotesia melanoscela* bracovirus (CmeBV) showed correlation between frequencies of various size classes for both parameters (Fig. 1-5). This suggests that BV nucleocapsids each contain a single genome segment and further that nucleocapsid length and frequency of that length occurring correlate with the length of the encapsidated segment and its frequency of occurrence, respectively (Krell, 1991). This is supported by Albrecht *et al.* (1994), who found each capsid of *Chelonus inanitus* BV (CinaBV) released one DNA species when treated with osmotic shock and argued that such BVs exist as a population of discrete virion types.

Although not as highly characterised as for IVs, protein composition of BVs appears to be of similar complexity. The peptide profile (SDS-PAGE) for CmeBV showed at least 18 different sized peptides, five of which form the two major capsid proteins of 38 kDa and 32 kDa in size (Krell, 1991). As for IVs, several BV structural proteins display high antigenic similarity to

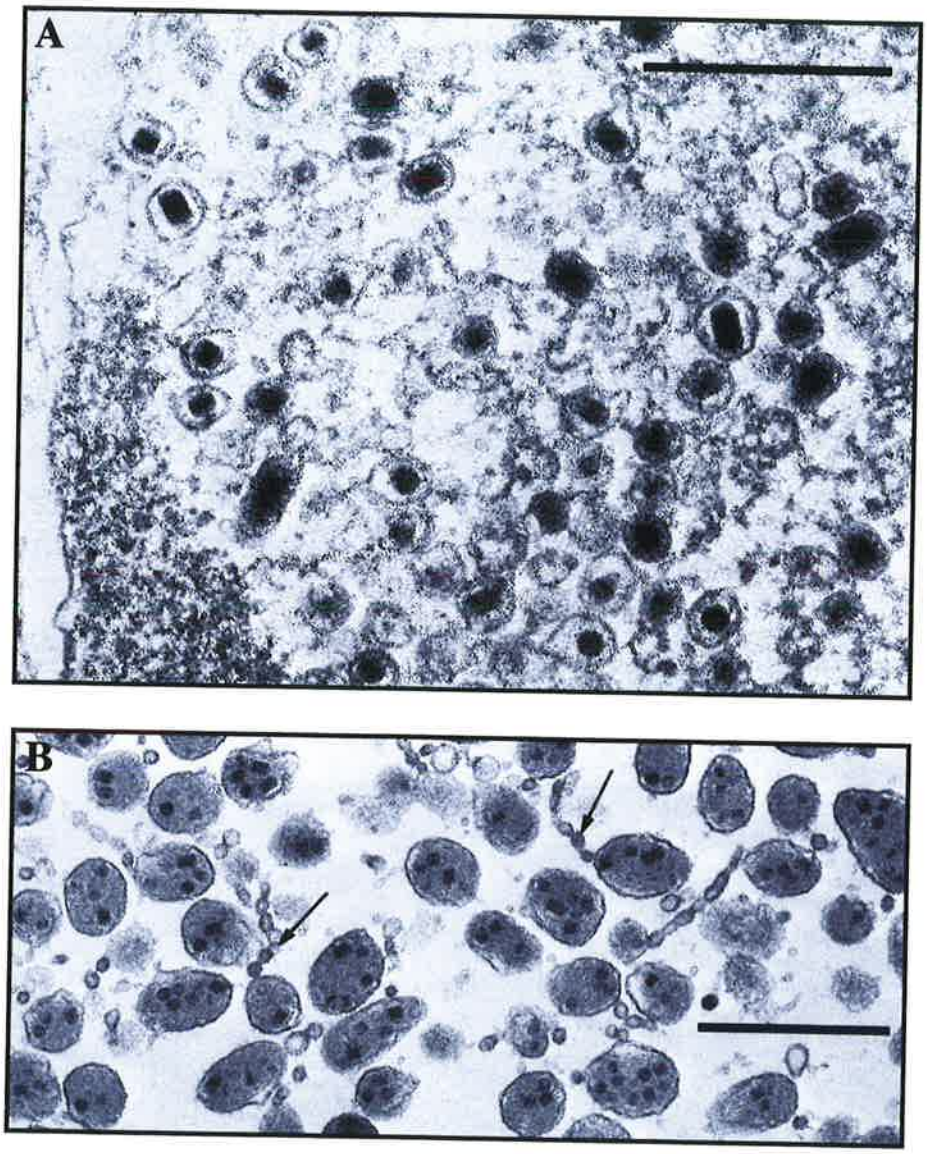


Figure 1-3. Examples of singly and multiply enveloped bracovirus particles (from Stoltz & Vinson, 1979). *A*, intranuclear bracovirus in calyx cells of *Chelonus texanus*. Note that each virion consists of one cylindrical nucleocapsid within each membrane. *B*, *Cotesia melanoscela* bracovirus particles suspended in calyx fluid. Multiple capsids are contained within each membrane and membrane protrusions are visible on most particles (*arrows*). *Bars* = 0.5 μm.

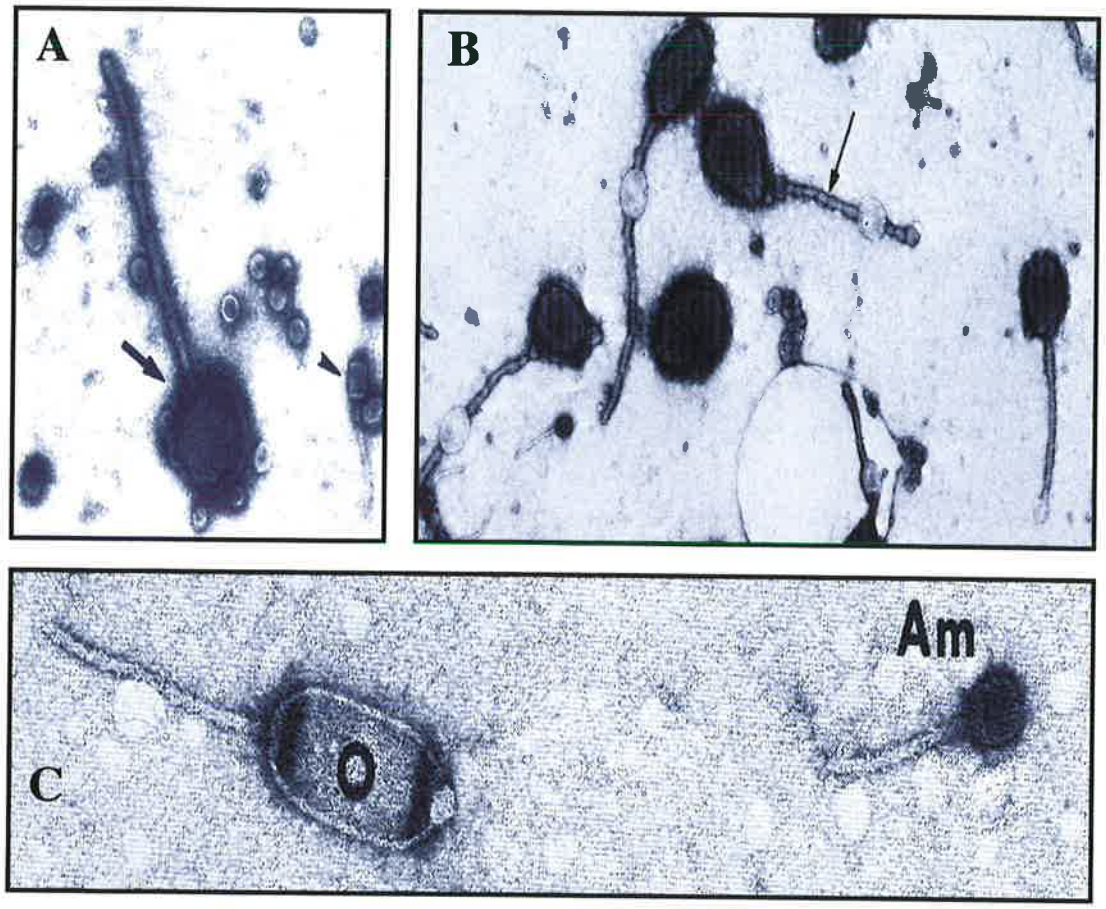


Figure 1-4. Electron microscopy showing negatively stained bracovirus virions and nucleocapsids. *Arrows* denote envelope protrusions. *A*, single bracovirus particle isolated from *Cotesia congregata* (from Beckage *et al.*, 1994). The *arrowhead* indicates a naked virion. *B*, bracovirus virions isolated from calyx fluid of *Cotesia melanoscela* (from Stoltz & Vinson, 1979). *C*, comparison of nucleocapsids with tail appendages, from *Oryctes sp.* (*O*) and *Cotesia melanoscela* (*Am*) (from Stoltz & Vinson, 1979).

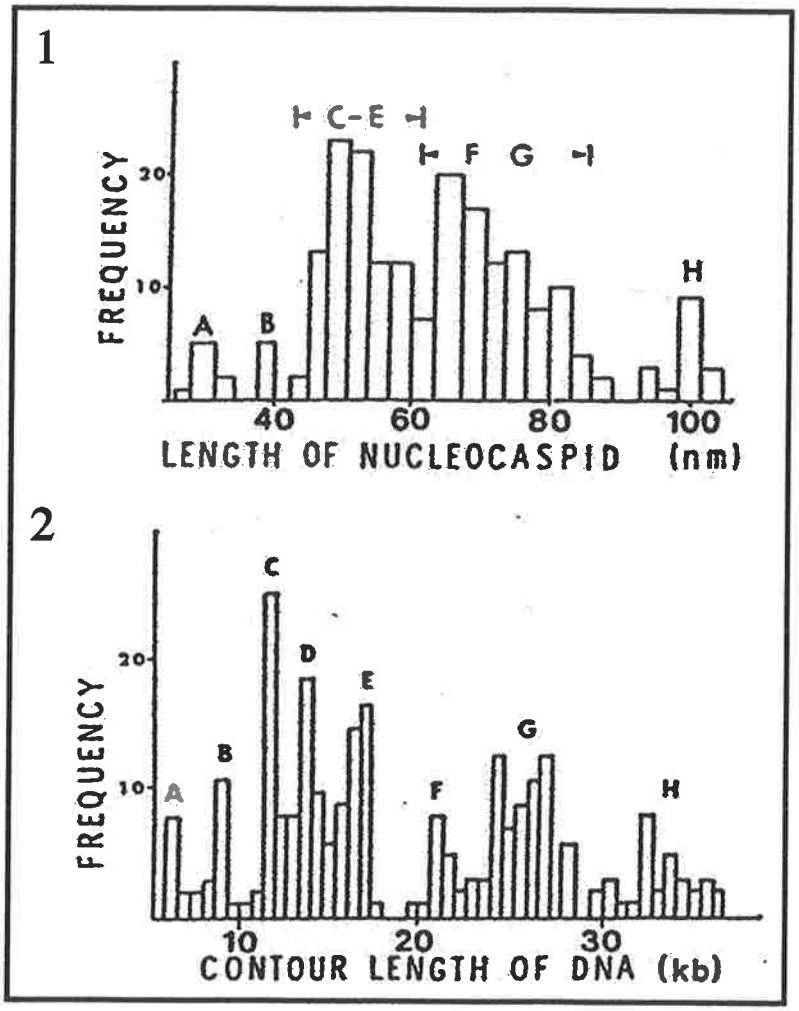


Figure 1-5. Distribution of nucleocapsid lengths (*1*) and viral DNA lengths (*2*) measured by electron microscopy for the *Cotesia melanoscela* bracovirus (from Krell, 1991). Resultant histograms could be superimposed, suggesting that each capsid contains a single DNA species and that capsid length and frequency of occurrence of that capsid length correlate with the length of encapsidated DNA and the frequency of occurrence of that DNA length. *A-H*, correlating nucleocapsid and DNA length size classes.

parasitoid venom proteins and were in fact recognised by monoclonal antibodies raised against the venom proteins (Strand *et al.*, 1994).

1.1.3 Relationship between *Ichnovirus* and *Bracovirus*

The most obvious evolutionary explanation for PDVs is that they are evolved from lepidopteran viral pathogens encountered by parasitoid larvae and this is supported by their morphological resemblance to nudiviruses, ascoviruses and baculoviruses (Webb, 1998). Indeed, BVs were originally classified as a baculovirus subgroup due to the similar capsid morphology and sharing singly or multiply enveloped capsids within a single membrane (Krell, 1991). Further evidence arises from the presence an intermediate viral form, which may represent a transitional phase from lepidopteran pathogen to wasp symbiont (Whitfield & Asgari, 2003). Parasitoids are known to act as vectors for insect pathogens such as ascoviruses, which are transmitted poorly by ingestion alone (Federici, 1993).

Although the two PDV genera are placed into one family based on numerous similarities, there is much evidence indicating that the Polydnaviridae is not a monophyletic grouping. Firstly, BV and IV virions differ in morphology, morphogenesis and structure (Fleming, 1992) whilst showing lower levels of biochemical and genetic similarity than would be expected for closely related viral genera (Cook & Stoltz, 1983; Stoltz & Whitfield, 1992). Secondly, the distribution of BVs and IVs within their respective hymenopteran families is restricted to a sub-set of sub-families (Whitfield, 1997; see Fig. 1-1), which are separated by ancestors that seemingly did not carry PDVs (Webb, 1998). Indeed, basal ichneumonid groups lack IVs indicating that the association with IVs arose subsequent to the ichneumonoid families themselves (Turnbull & Webb, 2002). Thus, the *Bracovirus* and *Ichnovirus* may actually constitute separate families.

The obligate, symbiosis between PDV and wasp would suggest coevolution of the two parties (within a given PDV genus/wasp family). This idea was initially supported by DNA hybridisation and banding patterns as well as extensive Western blot analyses, which suggested that IVs from the genus *Hyposoter* were more closely related to each other (and to those from *Diadegma*) than to those in other ichneumonid genera (Krell, 1991). Belshaw *et al.* (1998) suggested that the IV-associated subfamilies are paraphyletic, thus indicating several independent wasp-virus lineages have arisen after a putative initial association.

In contrast, cladistic analysis based on molecular and morphological characters suggests that BVs are a monophyletic lineage (Whitfield, 1997). Therefore, it seems likely that PDV genera arose from different parasitoids, (one ichneumonid and one braconid) whose larvae encountered a pathogenic insect virus with which they subsequently formed an obligate association, presumably due to a selective advantage conferred on both parties by the association.

1.2 The polydnavirus infection-cycle

The obligate, symbiotic nature of the parasitoid-virus relationship means that the life-cycle of each organism is unable to be considered in isolation. The life-cycle of each organism, in relation to the other, is shown in figure 1-6.

1.2.1 Polydnavirus replication and morphogenesis

An interesting aspect of PDV biology is the fact that although proviral DNA is present in all cells of PDV-associated parasitoids of both sexes, production of particles only occurs in calyx cells of female adult wasps (Stoltz & Vinson, 1979). Thus, replication is regulated by specificities in parasitoid sex, developmental-stage and tissue-type. Even within the calyx, highly replicative

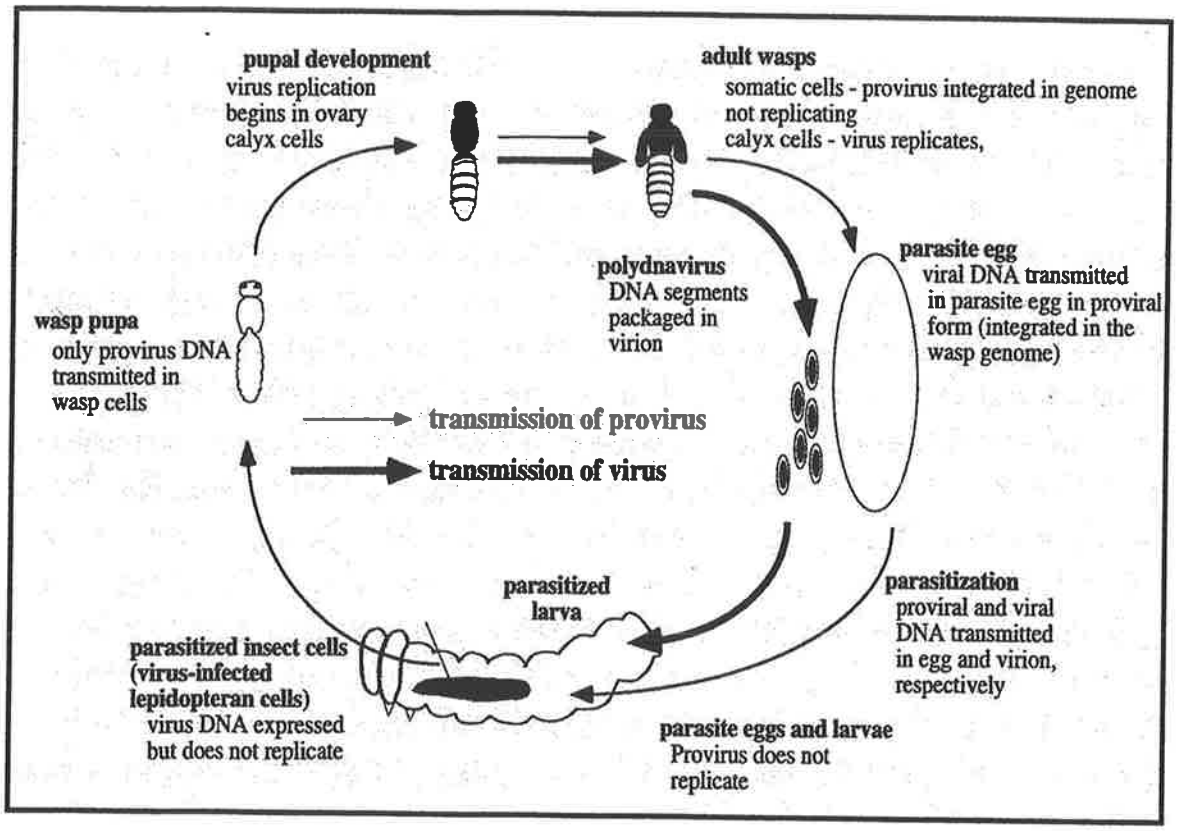


Figure 1-6. Generalised life-cycle of a polydnavirus and its hymenopteran endoparasitoid host (from Webb, 1998). Viral DNA is transmitted as proviral (integrated) DNA (*thin arrows*) and also as circularised viral segments within virions (*thick arrows*). In replicative endoparasitoid cells (adult and pupal calyx cells) and infected lepidopteran cells, viral DNA is present in a circularised, unpackaged form. Viral particles are produced only in replicative endoparasitoid cells. In all other (non-replicative) endoparasitoid cells, viral DNA is present as the integrated form. Transference of virus between endoparasitoids is via vertical transmission of chromosomal DNA.

cells appear to be interspersed with those that are not (Krell, 1987). Figure 1-7 shows the female reproductive tract of the endoparasitoid wasp, *Cotesia rubecula*. Volkoff *et al.* (1995) state that calyx cells of *Hyposoter didymator* (Ichneumonidae) are particularly suited to virus production and secretion in that they have abundant ribosomes and endoplasmic reticulum, a nucleus with high surface area and many pores in its membrane, and a cell membrane with high surface area in the region where virions bud through. It should be noted that the detection of episomal viral DNA (via hybridisation experiments) in tissues other than the calyx, in both male and female *C. sonorensis* wasps, suggests that most adult tissues are probably weakly replicative (Fleming & Summers, 1986; Webb, 1998).

When ready to pupate, last instar endoparasitoid larvae usually emerge from the lepidopteran larval host, leading to the demise of the host shortly thereafter. During pupation the developing adult becomes increasingly melanised in an ordered process. PDV replication has been correlated with specific pupal developmental stages, soon after initiation of cuticular melanisation, characterised by melanisation of specific body parts (Norton & Vinson, 1983; Albrecht *et al.*, 1994; Pasquier-Barre *et al.*, 2002; Tanaka *et al.*, 2002). A link also exists between initiation of virus replication and increasing ecdysteroid titer, an event known to occur in the later stages of pupal development (Webb & Summers, 1992). Replication is continued in the adult female at varying levels depending on various individual wasp parameters, for example the number of ovipositions performed (Norton & Vinson, 1983; Fleming & Summers, 1986; Theilmann & Summers, 1986; Fleming, 1992).

It was initially postulated that replication is initiated when individual virus DNA segments are excised from the chromosomal DNA of the developing endoparasitoid (Gruber *et al.*, 1996; Savary *et al.*, 1997; Webb, 1998). However, recent evidence suggests that controlled localised

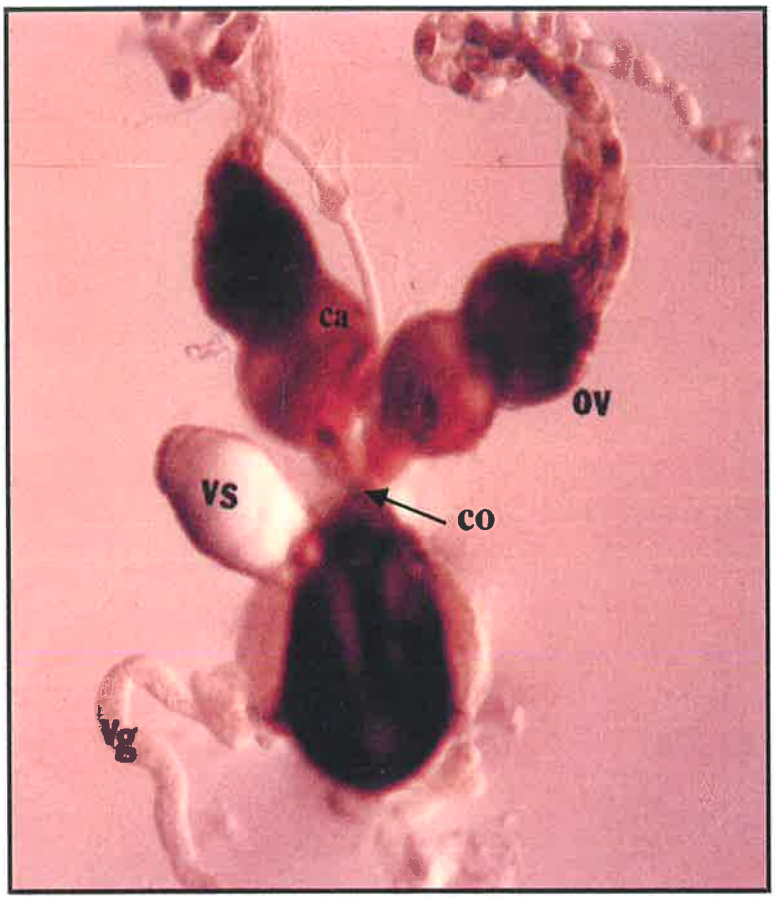


Figure 1-7. Female reproductive tract removed from the hymenopteran endoparasitoid, *Cotesia rubecula* (Braconidae: Microgastrinae). Eggs are produced in the ovary (*ov*) before passing into the lumen of the calyx (*ca*), a neck-like region at the ovaries' base. Here they are exposed to calyx fluid consisting of a high concentration of mature polydnavirus virions as well as calyx- and ovary-related proteins. Wasp venom is produced in the venom gland (*vg*) and is stored in the venom sack (*vs*) before release into common oviduct (*co*) lumen. Eggs and calyx fluid from both ovaries then pass through the common oviduct (*co*) and are subsequently oviposited into lepidopteran hosts along with venom proteins. (Photograph by Sassan Asgari).

chromosomal amplification occurs prior to excision of virus segments (Pasquier-Barre *et al.*, 2002). Gruber *et al.* (1996) analysed four integration loci from CinaBV and found that each contained short tandem repeats flanking proviral elements, which act as sites of excision. PCR analysis showed that parasitoid sequences outside of the tandem repeats were joined after excision of viral DNA.

Nucleocapsids, assembled in large nuclei of calyx cells, are associated with a virogenic stroma and are enveloped by a single membrane when mature (Stoltz & Vinson, 1979). *H. exiguae* IV (HeIV) virions within the nucleus of a calyx cell are shown in figure 1-8. How DNA segments are packaged remains unclear (Webb, 1998). Virions then accumulate in the cytoplasm before entering the calyx lumen by either budding through the cytoplasmic membrane (acquiring a second virion membrane) for IVs (Stoltz & Vinson, 1979) or by cell shedding and subsequent lysis for BVs, supported by the presence of cellular debris in calyx fluid (Stoltz & Vinson, 1977; de Buron & Beckage, 1992).

IVs appear to bud through abundant microvilli at the apical pole of the cell, aligning themselves at a specific orientation to the membrane prior to the budding process (Volkoff *et al.*, 1995). Figures 1-9*1* and *2* show passage of immature virions from the nucleus of a calyx cell, into the calyx lumen. Calyx tissue appears to show a gradient of morphological states ranging from having an absence of virus particles in the outer calyx regions to having cell nuclei almost full of particles in regions close to the calyx lumen (Stoltz & Vinson, 1979). Each PDV particle shows a membrane protrusion (inner membrane for IVs) which is suspected of involvement with the basal membrane of the subsequently parasitised host (see figures 1-3 and 1-4*A* and *B*) (Stoltz & Vinson, 1979).

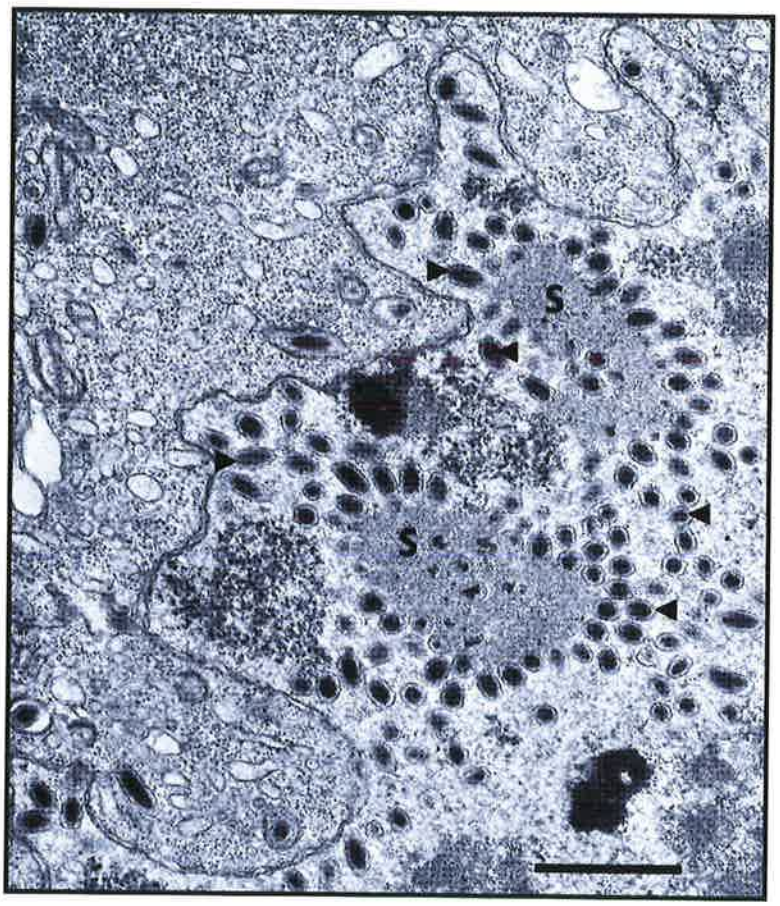


Figure 1-8. Replication of typical ichnovirus particles from *Hyposoter exigue* in a calyx cell nucleus (from Stoltz & Vinson, 1979). Particles (some denoted by *arrowheads)* are associated with a virogenic stroma (*s*) and are enveloped by single membrane. *Bar* = 1 μm.

1.2.2 Transmission of polydnavirus to the parasitised host

Calyx fluid, wasp venom and eggs are passed through the ovipositor of the parasitoid into the hemocoel of the host insect. Calyx fluid and venom appear to play at least a partial role in protecting the egg from host immune responses (Feddersen *et al.*, 1986; Asgari & Schmidt, 1994; Webb & Luckhart, 1996; Asgari *et al.*, 2003). Thus PDV DNA is introduced to the parasitised host as both integrated DNA within chromosomes of the wasp egg and as infective virus DNA within nucleocapsids of injected virions (Stoltz & Whitfield, 1992).

Virions quickly move to host basal membrane and then infect tissues such as fat bodies, muscle and trachea (Stoltz & Vinson, 1979a). Haemocytes are also heavily infected soon after oviposition (Li & Webb, 1994; Asgari et al, 1996; Yamanaka *et al.*, 1996). An infected haemocyte is shown in figure 1-10. It was shown for CsIV that the outer membrane of virions is lost during an interaction with the host basal membrane (see Fig. 1-9*3* and *4*) (Stoltz & Vinson, 1979). Further, the protrusion of the inner viral membrane penetrated the basal lamina and may have been involved in a suspected fusion event with the underlying cellular membrane (see Fig. 1-9*4*), allowing virions into the cell.

Similar events are suspected for BVs although they are singly enveloped. BV particles of *Cotesia congregata* and *C. melanoscela* were observed in association with host basal membrane (and underlying cells) less than 1 hpp, with electron microscopy showing no evidence of phagocytosis, indicating probable entry of virions into cells via membrane fusion (Stoltz & Vinson, 1979a). Capsids were then seen associating with nuclear pores such that the capsid tails penetrated into the nucleus. Empty capsids were witnessed at about two hours after oviposition, suggesting that BV DNA was uncoated at the nuclear pores. The function of the BV capsid tail in these

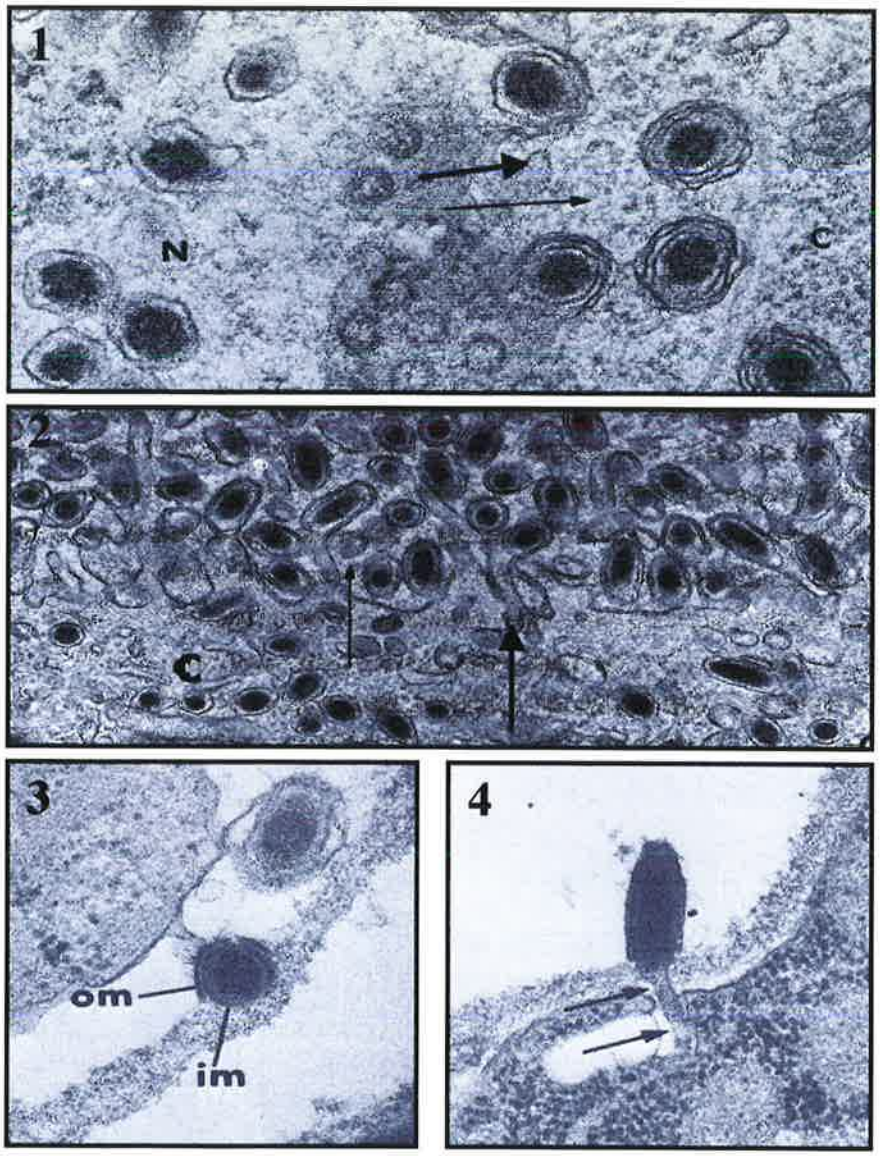


Figure 1-9. Release of *Campoletis sonorensis* ichnovirus from replicative cells and entry into lepidopteran host cells (adapted from Stoltz & Vinson, 1979). *1*, release of immature virions from the nucleus (*N*) of a calyx cell into the cytoplasm (*C*). Particles appear to bud through both membranes (in direction of *arrow*s) of the nuclear envelope but subsequently appear in the cytoplasm with a single envelope. *2*, virions then bud (in direction of *arrows*) through the cell membrane (from the cytoplasm (*C*) to the calyx lumen) acquiring a second virion membrane. *3 & 4*, simultaneous penetration of *Heliothis viriscens* basal membrane by inner virion membrane (*im*) and loss of outer virion membrane (*om*) at 1.75 hours post-oviposition. Penetration is presumably via membrane fusion and may involve a tubular protrusion associated with inner particle membranes (*arrows*).

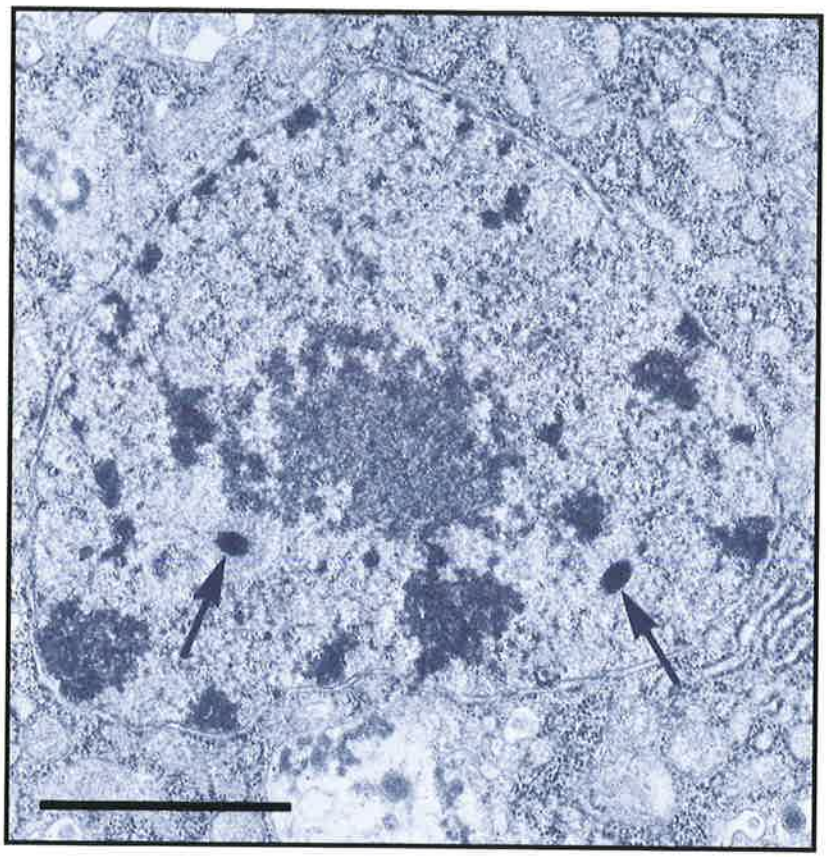


Figure 1-10. Nucleocapsids of the *Campoletis sonorensis* ichnovirus (*arrows*) in the nucleus of a *Heliothis viriscens* haemocyte, at 2.25 hours post-oviposition (from Stoltz & Vinson, 1979). *Bar* = 1 μm.

interactions, if any, is unknown (Stoltz & Vinson, 1979a). In contrast, virions of CsIV entered the nucleus of infected cells intact (usually within two hours of oviposition), via enlarged nuclear pores, with uncoating of DNA occurring within the nucleus (Stoltz & Vinson, 1979).

Packaged viral genes are then expressed by infected host cells (Theilmann & Summers, 1986; Stoltz *et al.*, 1988) and although viral genome segments may be maintained in the host throughout parasitoid development (Stoltz *et al.*, 1986; Theilmann & Summers, 1986; Strand *et al.*, 1992), expression of some genes is likely to be highly transient (Asgari *et al.*, 1996). The function of PDV genes expressed within the host is discussed in section 1.3.3.

No IV or BV replication has been detected in parasitised host tissues either by DNA hybridisation or morphological studies (Webb, 1998). Indeed, complete virions of CsIV had apparently disappeared as little as 24 hours post-oviposition (Stoltz & Vinson, 1979a; Webb, 1998). DNA associated with viral particles is quickly degraded when the host insect dies, however, integrated PDV DNA is present in the emergent parasitoid larva. The excision of integrated segments and subsequent production of virions in the developing parasitoid pupa, begin the next generation of the PDV life-cycle.

Thus, Stoltz (1993) described the PDV life-cycle as having two arms, one associated with the parasitoid and involving transmission and production of particles, and the other associated with the parasitised host and involving infection and pathogenicity. Webb (1998) describes this latter arm as a transient genetic colonisation event, which he defines as transient infection that benefits the colonising entity, in this case the endoparasitoid and the proviral DNA it contains.

Although PDVs are currently classified as viruses, several of their characteristics appear to be anomalous with those of other viruses. The polydisperse nature of their genome is a good example because a PDV 'species' does not apparently contain individual virions that house a complete set of viral genes. By the current definition of a virus (van Regenmortel, 2000) each PDV would be more correctly defined as a group of viral species with each species made up of virions containing the same DNA(s), as for the discrete virion types described by Albrecht *et al.* (1994). Indeed, differences between segment sequences (or groups of them) of a given PDV are far more than that between most currently accepted virus strains, even though segments may share some degree of homology.

Given that all known packaged PDV genes function directly in protecting the developing parasitoid, PDVs effectively function as a transport system for a sub-set of selected wasp-beneficial genes and other non-coding DNA. Thus, in recent times PDVs have been described as maternal secretions (Whitfield & Asgari, 2003) and as being analogous to wasp organelles (Federici & Bigot, 2003). However, such issues relate to taxonomic classification and may reflect shortcomings in the definition of viruses (already an arbitrary grouping) rather than the fact that PDVs are phylogenetically removed from viruses. It should be considered, however, that some terms associated with virology (e.g. infection, transmission, replication) may be misleading when interperating aspects of PDV biology.

1.3 Polydanavirus genes and gene expression

Theilmann & Summers (1988) placed PDV genes into three broad classes depending on where the genes were expressed. Class I genes are expressed in the parasitoid and are thought to have functions associated with PDV particle production whilst class II genes are expressed in the

lepidopteran host and thought to be associated with regulation of host immune response and development. Class III genes are of unknown function and expressed in both the parasitoid and its host. Most of the detailed characterisation of genes has dealt with class II genes (Webb, 1998), as these genes are linked to what is seen as the symbiotic activity of PDVs and may also have a practical use in pest management due to their potent effects on host regulation.

1.3.1 Polydnavirus gene families

To date, several PDV gene families have been reported (reviewed by Turnbull & Webb, 2002). Two of these families, denoted the cys and rep families, are known from CsIV (Cui & Webb, 1997a). Cys genes are characterised by a cysteine-rich motif and have a conserved structure (Dib-hajj *et al.*, 1993; Cui & Webb, 1996). Four cys genes have been characterised, these being WHv1.0, WHv1.6, VHv1.1 and VHv1.4 (Webb, 1998). Transcripts of these genes are abundant, appear within the first few hours post-parasitisation (hpp) (Blissard *et al.*, 1989; Cui & Webb, 1996) and have been linked to suppression of host immune response (Li & Webb, 1994).

Cys genes appear to encode glycosylated signal-peptides suggesting that gene products are secreted by infected cells (Webb, 1998) and all appear to be transcriptionally regulated by TATA box motifs (25-30 bp upstream from the site of transcript initiation) and putative cys-activating elements which are located several hundred nucleotides upstream of the TATA box motifs (Cui & Webb, 1997a). Cysteine residues in the motif of these genes are absolutely conserved whilst amino acids between the cysteine residues are hypervariable (Cui & Webb, 1996), a structural feature shared with conotoxin genes (Dib-hajj *et al.*, 1993). Summers & Dib-hajj (1995) postulated that hypervariable regions may function in providing the diversity required to overcome evolution of host immune responses, for a wide range of hosts.

The rep gene family is simply characterised by an imperfectly conserved repeated element of ≈ 540 bp in length, which hybridised to the majority of CsIV superhelical DNA segments (Theilmann & Summers, 1987; Theilmann & Summers, 1988). Sequence similarity between any two of the 540 bp elements was 60-70% (Theilmann & Summers, 1987). Only one rep gene has been sequenced, that being BHv0.9 (Theilmann & Summers, 1988; Webb, 1998). In general, CsIV class II genes are expressed rapidly and persistently throughout parasitoid development, with mRNA levels varying little between 12 hours and 8 days post-infection (Webb, 1998). Two other members of the rep family have been isolated from other viruses, one from *H. didymator* ichnovirus (HdIV) and one from *Tranosema rostrale* ichnovirus (TrIV) (Volkoff *et al.*, 2002).

Recent studies have identified another CsIV gene family, vinnexin genes (Turnbull & Webb, 2002). Four members were identified in CsIV, occurring on three different segments. It is postulated that vinnexins function by interfering with formation of gap-junctions between haemocytes during encapsulation. A fifth member of the vinnexin family has been isolated from HdIV (Turnbull & Webb, 2002).

Three genes, encoding proline- and glycine-rich proteins, were isolated from HdIV by infecting insect cell culture and subsequently producing a cDNA library (Volkoff *et al.*, 1999). The three cDNAs showed homology in tandem repeats and were linked to transcripts of 1.5, 1.6 and 2.3 kbp, respectively, which were also detected in *Spodoptera littoralis* larvae parasitised by *H. didymator*. The proteins appeared to be secreted but did not contain putative N-glycosylation sites associated with most other characterised PDV proteins (Volkoff *et al.*, 1999).

Two mRNA transcripts from MdBV (≈ 1 kbp and ≈ 1.5 kbp, respectively) with significant regions of homology, were identified in parasitised *Pseudoplusia includens* larvae (Strand *et al.*, 1997). Predicted peptide sequences showed a homologous cysteine-rich region at the 5' end of the genes, which also showed similarity with epidermal growth factor motifs, thus the family is denoted as containing EGF-like genes. Only one of the genes was shown to encode a signal peptide. Recently, a third member of the family was isolated (Trudeau *et al.*, 2000).

The early gene family (EP1-EP3) expressed by *C. congregata* bracovirus (CcBV) in *Manduca sexta* larvae, comprises three identified members whose products were predicted to be highly glycosylated (Harwood *et al.*, 1994). Expression of early genes was detected as little as 30 min post-parasitisation, with gene products comprising 10% of total haemolymph proteins at 24 hpp. Their function remains unclear although it is thought that EP1 may have a cytotoxic effect (Le *et al.*, 2003).

Three genes sharing similarities in some structural elements have been isolated from TrIV (Béliveau *et al.*, 2003). Two of these genes (TrV1 and TrV2) were assigned to a putative TrIV gene family of glutamate-rich proteins (apparently including other members not yet isolated).

Most other characterised genes, not assigned to putative gene families, are linked to inhibition of lepidopteran host immune response, as are those within the families (Webb, 1998). Interestingly, no cloned viral genes have a function that appears to directly alter host physiology leading to inhibited growth and development (Webb, 1998), even though it has been demonstrated that PDVs mediate such changes (Beckage *et al.*, 1987; Dover *et al.*, 1988; Dover *et al.*, 1988a; Dushay & Beckage, 1993; Shelby & Webb, 1994; Soller & Lanzrein, 1996; Shelby & Webb,

1997; Beckage, 1997). In general, the number of PDV genes detected remains small. Only 12 genes have been isolated from CsIV (Blissard *et al.*, 1987; Theilmann & Summers, 1988) and the number of genes isolated from other systems is usually less than ten.

1.3.2 Expression of polydnavirus genes in parasitoid wasps

Structural proteins of CsIV are the only class I genes to have been cloned and sequenced (Webb, 1998; Deng *et al.*, 2000). A cDNA clone encoding a 15 kDa structural protein was identified and showed no homology to known genes (Webb, 1998). Further, the protein was not secreted and appeared to be targeted to infected cell nuclei. A second structural protein, p44, was shown to reside on or between virion envelopes, however, interestingly the gene encoding this protein is not encapsidated but is located on the parasitoid genome (Deng *et al.*, 2000). Although this may indicate adventitious packaging of p44, copy number of the gene was shown to increase during viral replication in a manner indistinguishable from that of the encapsidated CsIV protein, p12.

These data support, in part, the hypothesis that excised viral segments are actually mosaics of virus and wasp genes (Stoltz, 1993). This idea suggests that there is selection pressure to lessen excision of genes that are only expressed in the host, whilst increasing the chance of excision and encapsidation of wasp-derived genes that may give the parasitoid-virus combination a selective advantage within the lepidopteran host.

Other putative class I genes have been located using viral genomic DNA probes in Southern hybridisation experiments (Webb, 1998). Blissard *et al.* (1989) reported three putative virus genes hybridising to a single excised CsIV segment (W), however none of these genes were shown to encode proteins (Webb, 1998). Sequence analysis of segment W revealed that only two

of the genes had putative open reading frames and that the third gene was associated with repetitive sequences in long terminal repeats flanking the segment (Cui & Webb, 1997). Such is the level of characterisation of most class I genes.

1.3.3 Expression of polydnavirus genes in lepidopteran hosts

Class II genes have been characterised for less than ten PDVs (Turnbull & Webb, 2002), the most data deriving from studies on CsIV (Blissard *et al.*, 1987; Theilmann & Summers, 1988; Dib-hajj *et al.*, 1993; Cui & Webb, 1996). The only other IV from which putative class II genes are described is HdIV (Volkoff *et al.*, 1999). As discussed earlier, HdIV genes were assigned to a putative family of glycine- and proline-rich proteins (see 1.3.1).

BVs for which putative class II genes have been characterised include *Cotesia congregata* bracovirus (CcBV) (Harwood & Beckage, 1994), *C. rubecula* bracovirus (CrBV) (Asgari *et al.*, 1996), *C. karyai* bracovirus (CkBV) (Yamanaka *et al.*, 1996) and MdBV (Strand *et al.*, 1997). As mentioned, all characterised class II genes appear to be associated with immune-suppression in the host (Webb, 1998).

1.3.3.1 Insect immune system components

For a parasitoid larva to develop successfully within a given host, the immune response of the host must be successfully challenged without killing the host prior to completion of parasitoid development. The insect immune response consists of humoral (or cell-free) components and cellular components, which tightly interact in the haemolymph to provide effective protection against foreign agents (Vinson, 1990).

Cellular immunity involves the action of haemocytes, which take part in three main defensive interactions, these being phagocytosis, encapsulation and nodule formation (Vinson, 1990). Phagocytosis involves the removal (from haemolymph) of small particulate objects by specialised haemocytes called granulocytes and plasmatocytes, or collectively immunocytes (Gupta, 1991).

Encapsulation and nodule formation both result in production of a multi-cellular capsule being formed around larger foreign objects or small objects not cleared by phagocytosis. In such reactions, granulocytes perform non-self recognition (Ratcliffe, 1993), before plasmatocytes encapsulate the object, with a final layer of granulocytes completing the capsule (Pech & Strand, 1996). Encapsulation is also usually accompanied by melanin deposition and the release of cytotoxic molecules (Carton & Nappi, 1997). Haemocytes may also play a more indirect role in immunity by secreting humoral factors involved in poison detoxification, coagulation and prophenoloxidase (an enzyme important for melanisation) activation (Gupta, 1991). Thus, cellular components play an important role in humoral immunity and as such, humoral and cellular components are closely linked.

Humoral immunity arises from the action of proteins (sometimes other molecules) that are already present or are inducible (Boman & Hultmark, 1987; Berg *et al.*, 1988), and is generally associated with infection by microorganisms (Boman, 1991). Gillespie *et al.* (1997) list three broad classes of innate humoral response, these being sentinel molecule binding (e.g. lectins), defensive melanisation and induction of antimicrobial peptides. Known inducible humoral agents include cecropins (Boman, 1991), insect defensins, proline-rich peptides such as apidaecin and glycine-rich peptides including attacins and sarcotoxins (Hetru *et al.*, 1994).

Many humoral immune molecules are classified as pattern recognition molecules (PRMs) that have evolved to recognise (and bind to) molecular patterns associated with pathogenic microorganisms (e.g. lipopolysaccharide (LPS) on bacterial surfaces). PRMs function by directly removing foreign objects from circulation or making them visible to immune system components, which then subsequently render them harmless (McGuinness *et al.*, 2003). One of the most important and diverse groups of PRMs are C-type lectins (CTLs), which are lectins (proteins which bind specific sugar determinants) whose action is dependent on the presence of divalent metal ions, most commonly Ca^{2+} (Kilpatrick, 2002).

Several CTLs have been identified as being associated with humoral immunity in insects and other invertebrates, with many being multimeric (Takahashi *et al.*, 1985; Kubo and Natori, 1987; Suzuki *et al.*, 1990; Haq *et al.*, 1996; Saito *et al.*, 1997; Arai *et al.*, 1998; Kakiuchi *et al.*, 2002; Yu & Kanost, 2003). Invertebrate CTL monomers are usually simple in structure, containing only a single functional domain, the carbohydrate recognition domain (CRD), which recognises and binds specific saccharides (Kilpatrick, 2002).

Two other important molecules, apparently associated with non-self recognition, are hemolin, which is known to attach to haemocytes and bacterial surface components (Schmidt *et al.*, 1993), and prophenoloxidase, which plays a direct role in melanisation (Ratcliffe *et al.*, 1984). Hemolin is a soluble protein already present in haemolymph and whose concentration is significantly increased upon bacterial infection (Sun *et al.*, 1990), whilst prophenoloxidase is present as an inactive form that is activated by a proteolytic cascade (Seybold *et al.*, 1975).

Antiviral immunity in insects is not well characterised and anomalous in that induction of humoral immune-related proteins, normally associated with infection by microorganisms, has not been reported (Shelby & Webb, 1999) although induction of scolexin, a plasma lectin, has been linked to baculovirus infection (Finnerty & Granados, 1997). Phenoloxidase (PO), an important enzyme in melanisation reactions, has been linked to antiviral immunity in that a PO inhibitor (phenylthiourea) was shown to remove virucidal activity from plasma of *Heliothis viriscens* larvae (Ourth & Renis, 1993, Shelby & Webb, 1999). Infection by CsIV was linked to reduced levels of several important molecules related to immunity including PO, DOPA, NADA and precursors of reactive quinones (Shelby & Webb, 1999 – see Fig. 1-11).

Recombinant baculovirus (expressing a LacZ reporter gene) has been used to detect encapsulation and melanisation of virus-infected tracheal cells in semi-permissive *H. zea* and *M. sexta* larvae (Washburn *et al.*, 1996; Washburn *et al.*, 2000), indicting that the immune response may primarily be directed against virus-infected cells rather than individual virions. These observations partly explain why parasitised insects are often more susceptible to viral infections (Brooks, 1993; Washburn *et al.*, 2000; Stoltz & Makkay, 2003). Obligatory hyperparasites also apparently utilise hosts with suppressed immune response to facilitate their own development, a phenomenon documented for the tussock moth, *Orgyia leucostigma* (Guzo & Stoltz, 1987). Interestingly, systemic infection of parasitised *M. sexta* larvae with recombinant baculovirus had no effect on development of *C. congregata* parasitoid larvae (Washburn *et al.*, 2000).

Several PDV class I and II gene products have been linked to disruption of the insect immune system, acting on various different components (see reviews by Beckage, 1998; Webb, 1998; Shelby & Webb, 1999; Turnbull & Webb, 2002). Class II genes are generally secreted by

infected cells into the haemolymph, where their immuno-suppressive role is manifest (Beckage, 1998). As in other areas of PDV biology, the immune-suppressive action of CsIV against *H. viriscens* is the most highly characterised, with disruption of both humoral and cellular elements reported (Shelby & Webb, 1999). Figure 1-11 summarises the known effects of CsIV in inhibiting the immune response *H. viriscens*.

1.3.3.2 Parasitism-induced changes to host cellular immunity

Edson *et al.* (1981) demonstrated that *C. sonorensis* eggs are encapsulated and melanised in the absence of CsIV and that these processes are abrogated by CsIV infection, however, infected haemocytes still appeared to maintain their phagocytic ability (Davies & Vinson, 1988). Encapsulation breakdown has been linked to cys family genes, which are mostly glycosylated and whose transcripts are more numerous than those of other CsIV genes. Cys-motif proteins arising from these genes bind to haemocytes, mainly granulocytes (Shelby & Webb, 1999). One of these proteins, VHv1.1, has a disruptive effect on the haemocyte cytoskeleton, leading to reduced levels of encapsulation (Li & Webb, 1994). The CrBV gene, CrV1, is similarly glycosylated, secreted and blocks encapsulation by disrupting the cytoskeleton of haemocytes (Asgari *et al.*, 1997). Site-directed mutagenesis of the single glycosylation site of VHv1.1 led to a loss of the ability to bind to haemocytes, indicting that binding is highly dependent on specific post-translational modification (Shelby & Webb, 1999). A related protein, VHv1.4, was tested for similar activity but did not, by itself, inhibit encapsulation significantly (Cui *et al.*, 1997).

Although the remaining three cys genes have not had their specific function elucidated, it has been shown that reducing the infective dose of virus by 50% leads to a proportional decrease in cys gene transcript titers in haemolymph and a measurable increase in encapsulation (Webb &

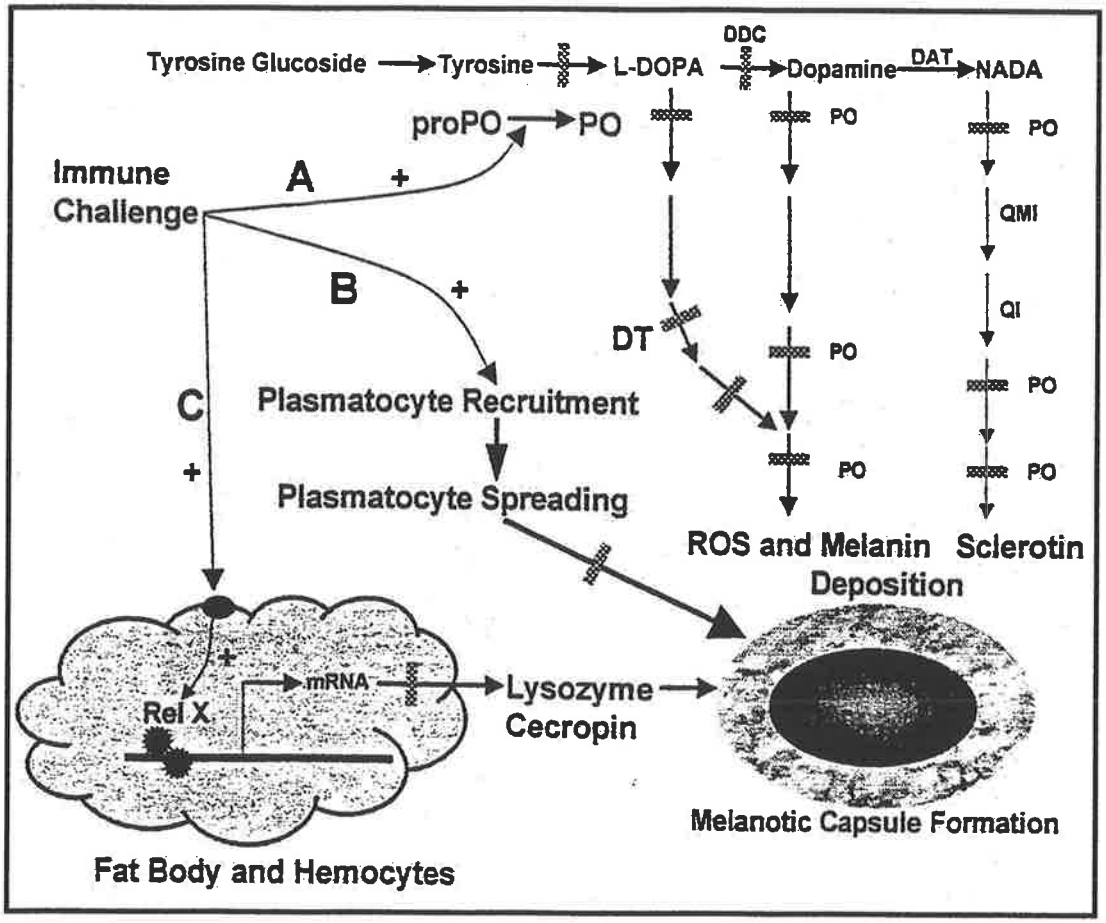


Figure 1-11. Summary of *Campoletis sonorensis* ichnovirus (CsIV) challenges to lepidopteran host cellular and immune response (from Shelby & Webb, 1999). *A*, effects on defensive melanisation cascade. *B*, effects on cell-mediated encapsulation response (including granulocyte binding and plasmatocyte recruitment, capsule formation by plasmatocytes, and capsule completion by granulocytes). *C*, effects on induction of antibacterial proteins by fat body and haemocytes. *Hatched bars* (right angles to pathways) represent points of inhibition by CsIV. Other indicators are as follows: *DAT*, dopamine acetyl transferase; *DDC*, DOPA decarboxylase; *DT*, dopachrome tautomerase; *NADA*, N-acetyl dopamine; *PO*, phenoloxidase; *QI*, quinone isomerase; *QMI*, quinone methide isomerase; *Rel X*, *rel*-like transcription factor, *ROS*, reactive oxygen species.

Cui, 1998). Shelby & Webb (1999) postulate that the four cys genes target different granulocyte sub-classes with the accumulative effect suppressing encapsulation.

Several BV proteins also appear to target haemocytes. Two of the EGF-like genes from MdBV were initially associated with loss of the ability of plasmatocytes to adhere to foreign surfaces and with apoptosis of granulocytes, characterised by cell surface blebbing, fragmentation of DNA and chromatin condensation (Strand, 1994; Strand & Pech, 1995; Strand *et al.*, 1997). Close to 90% of mRNA for EGF-like genes was associated with haemocytes (Strand *et al.*, 1992) and interestingly, only one of the genes appeared to be secreted (Strand *et al.*, 1997).

Later studies showed that MdBV infection of *P. includens* induced an increase in the total number of haemocytes but reduced the proportion of plasmatocytes (Strand *et al.*, 1999). Further, mRNA for a precursor of plasmatocyte spreading peptide (a protein interacting with plasmatocytes to allow adhesion to foreign surfaces) was shown to be under-expressed in haemocytes but not fat bodies. Thus, the observation that plasmatocytes lose the ability to adhere to foreign surfaces 4-6 hpp (Strand *et al.*, 1999). Recently, RNA silencing of the glc1.8 gene (from MdBV) was shown to reinstate the adhesive phenotype of infected culture cells, whilst silencing of egf1.0 did not (Beck & Strand, 2003).

One of five cDNA clones associated with CkBV encodes a protein that contains multiple N-linked glycosylation sites, is probably secreted and is heavily expressed (mainly in haemocytes) at a time period corresponding with disruption of encapsulation (Hayakawa *et al.*, 1994; Yamanaka *et al.*, 1996). The early genes associated with parasitisation of *M. sexta* by *C. congregata* are thought to cause haemocyte clumping and damage associated with apoptosis

(Lavine & Beckage, 1995). EP1 was isolated, sequenced and found to encode a secreted glycoprotein that was suspected of interacting with host haemocytes, although the mechanism of this interaction remains unknown (Beckage *et al.*, 1987; Harwood & Beckage, 1994). Recent evidence suggests EP1 may be linked to induction of apoptosis in infected haemocytes (Le *et al.*, 2003).

An interesting aspect of haemocyte-targetting PDV genes is that of variation in temporal expression patterns. The expression of the CrV1 protein has been shown to be strongly transient, with expression occurring in a period of four to eight hpp, after which transcripts decline (Asgari *et al.*, 1996). Haemocytes appear healthy and function correctly after several days. The mRNAs from CkBV were also abundantly expressed up to a period of 6 h but expression patterns after this time were not reported (Yamanaka *et al.*, 1996).

By contrast, EP1 transcripts could be detected after 6 days using *in vitro* assays, which is greater than half the time needed for larval development (Harwood & Beckage, 1994). Similarly, transcripts from the glycine- and proline-rich protein family expressed by HdIV could be detected 10 days after infection (Volkoff *et al.*, 1999). As an intermediate example, the MdBV EGF-like genes were highly expressed in haemocytes at 12 hpp, in decline at 24 hpp and almost undetectable at 96 hpp, which contrasts with the persistent expression of most other MdBV transcripts (Strand & Pech, 1995). Whilst the decline in CrV1 transcripts after 8 h is thought to be a regulated event (Asgari *et al.*, 1996), it has been postulated that reduction in transcripts of such mRNAs in other systems may be linked to PDV-mediated apoptosis (Strand & Pech, 1995).

Venom proteins have been shown to be essential for successful parasitism in some systems (Kitano, 1986; Tanaka, 1987). Venom from the braconid, *Pimpla hypochondriaca* causes deleterious effects on host haemocytes (Richards & Parkinson, 2000), possibly due to the presence of a cytotoxic protein (Parkinson & Weaver, 1999).

1.3.3.3 Parasitism-induced changes to host humoral immunity

The observation that CsIV infection leads to reduced larval plasma melanisation (Stoltz & Cook, 1983) indicated that polydnaviral infection may compromise humoral elements of the host immune response, however, the specific mode of action of any PDV gene, in this regard, is unknown (Beckage, 1998; Webb, 1998; Shelby & Webb, 1999).

Shelby *et al.* (1998) found that activity of the antibacterial proteins cecropin and lysozyme was reduced in three permissive hosts but not in a non-permissive host, when parasitised or injected with virus. Further, lysozyme mRNA in infected tissues was shown to be normally induced indicating that regulation of lysozyme activity is post-translational. Phospholipase C is an LPS-induced haemolymph protein identified in *Galleria mellonella* (Chui *et al.*, 1991), *H. zea* and *H. viriscens* (Shelby & Webb, 1999), whose specific defensive function is not known. Parasitisation by *C. sonorensis* reduced the level of Phospholipase C induction, potentially leading to lowered resistance against opportunistic bacterial infection (Shelby & Webb, 1999). Interestingly, the immune response of parasitised pupae, against gram negative bacteria, appeared to be enhanced by elements within *P. hypochondriaca* venom (Dani *et al.*, 2003), possibly to prevent ovipositor-associated bacteria from causing disease in the host and possibly preventing parasitoid development.

PO is an essential enzyme for melanisation, catalysing early steps of cascade reactions leading to deposition of eumelanin onto foreign particles (Marmaras *et al.*, 1996). Reduced plasma melanisation, attributed to reduced PO activity, is a characteristic of infection by several PDVs (Stoltz & Cook, 1983; Lavine & Beckage, 1995; Strand & Pech, 1995a; Carton & Nappi, 1997). CsIV infection reduces activity of PO as well as DOPA decarboxylase (DDC) and dopachrome tautomerase (DT), two enzymes involved in eumelanin formation, with DDC reduction occurring post-translationally (Shelby & Webb, 1999). Further, haemolymph concentrations of the eumelanin substrates tyrosine and tyrosine glucoside, are reduced by injection of CsIV calyx fluid. Thus, it is apparent that CsIV acts against the enzymes and substrates needed for melanisation cascades. See figure 1-11 for a summary of immune-suppressive effects attributed to CsIV. Decrease in enzyme activity appears to be attributable to post-translational regulation, (e.g. DDC and lysozyme (Shelby & Webb, 1999)), a means by which other viruses have been shown to regulate cell physiology (Pleogh, 1998).

Although all PDVs have genes that appear to actively suppress the lepidopteran immune system, the parasitoid egg must survive encapsulation in the lag period between oviposition and accumulation of immune-suppressive proteins to protective levels (Asgari & Schmidt, 1994). VHv1.1 needs to reach a concentration of $\approx$ 50 ng/μl to confer protection, which is not usually achieved until a day after oviposition (Shelby & Webb, 1999). During this lag period, parasitoid eggs are often protected by passive means. Eggs of the parasitoids *C. rubecula* and *C. karyai* are coated in proteins (Crp32 and IEP, respectively) that appear to share antigenic similarity with host proteins and thus prevent non-self recognition of the egg during the lag period (Asgari *et al.*, 1998; Hayakawa & Yazaki, 1997). Both proteins are apparently parasitoid-encoded PDV envelope-associated proteins, therefore may possibly protect virions as well.

Several maternal proteins have been associated with reduced host immunity during and after the lag period, including various ovarian and venom proteins, as well as virus-like particles (VLPs). Ovarian proteins are abundant in the oviduct lumen and transferred to the lepidopteran host during oviposition (Shelby & Webb, 1999). Ovarian proteins from CsIV are glycoforms of a 29 kDa protein synthesised in calyx cells and encoded by a single copy parasitoid gene (Luckhart & Webb, 1996). These proteins bind to both granulocytes and plasmatocytes, are taken up by endocytosis and disrupt the cytoskeleton, thus preventing encapsulation (see figure 1-11).

Ovarian proteins from CsIV could be detected in haemolymph for four days but lose their protective ability in the second day of infection (Shelby & Webb, 1999). Interestingly, ovarian proteins were first identified due to high antigenic similarity with viral structural proteins (Webb & Summers, 1990) and were only later associated with suppression of host immune response (Webb & Luckhart, 1994; Webb & Luckhart, 1996; Luckhart & Webb, 1996).

VLPs have been detected in reproductive tracts of many ichneumonid wasps (Feddersen *et al.*, 1986) and appear to provide advantages to the developing parasitoid via altering various aspects of host homeostasis (Beckage, 1993). *Venturia canescens* was the first parasitoid species in which a VLP was discovered (Rotheram, 1973) and its VLP is self-assembled from several proteins that are encoded on multiple parasitoid alleles (Hellers *et al.*, 1996). Some examples of VLP action include inactivation of host defences (Edson *et al.*, 1981) and alteration of host hormone titers (Zitnan *et al.*, 1995).

1.3.3.4 Parasitism-induced changes to host development

Apart from the immuno-suppressive effects discussed, PDV infection has also been correlated with altered host metabolism leading to developmental arrest of lepidopteran larvae, reduced feeding and growth, pigmentation anomalies, and precocious metamorphosis (Beckage, 1998; Shelby & Webb, 1999) although, as mentioned, no PDV genes have been directly linked to these functions.

CsIV has several pronounced and persistent effects on host larval metabolism (Shelby & Webb, 1999.) After parasitisation, concentrations of amino acids and trehalose in plasma are increased, whilst overall protein concentration is decreased (Vinson, 1990). Reduction in plasma protein concentration is due to post-translational down-regulation in fat body synthesis of storage proteins, arylphorin and riboflavin-binding protein (Shelby & Webb, 1994; Shelby & Webb, 1997). Larvae may still moult after CsIV infection but may not moult due to not attaining threshold size requirements (Shelby & Webb, 1999).

Dover *et al.* (1988) demonstrated that CsIV alone was responsible for developmental arrest in *H. viriscens* larvae and that the percentage of larvae arrested was proportional to the infective dosage. Larvae, injected with virus suspension, were found to temporarily arrest at the stage injected and then recovered to develop normally. During infection, ecdysteroid levels dropped and then rose again as larvae recovered. Such larvae showed partial degeneration of the prothoracic gland. Therefore, CsIV genes appear to target ecdysteroid regulation to halt development to the benefit of the developing parasitoid larva.

Synthesis of juvenile hormone esterase (JHE) is normally detected in *H. viriscens* on the third day of the fifth instar (Shelby & Webb, 1999). However, this does not occur in CsIV infected larvae due to post-transcriptional down-regulation by unknown viral proteins (Shelby & Webb, 1997). It is thought that the absence of plasma JHE leads to high plasma juvenile hormone concentrations and thus, host pupal development is abrogated (Shelby & Webb, 1999). *C. nigriceps* bracovirus (CnBV) also directly down-regulates ecdysteroid biosynthesis in early fifth instar *H. viriscens* prothoracic glands and damages cells within the gland (Pennacchio *et al.*, 1998). Further, a marked underphosphorylation of regulatory proteins was observed indicating that a protein kinase was inhibited, and genes similar to protein kinase inhibitors were detected on the CnBV genome.

It appears as though PDVs target specific proteins for post-transcriptional regulation, as CsIV infection does not significantly change the concentrations of other major plasma proteins, including lipophorin, transferrin and ferritin (Shelby & Webb, 1999). Thus, virus infection effectively transfers resources from lepidopteran host development to parasitoid larval development (Shelby & Webb, 1999).

The effect of *C. congregata* parasitism on *M. sexta* development has also been studied in some detail (Dushay & Beckage, 1993; Beckage & de Buron, 1993; Beckage *et al.*, 1994; Alleyne & Beckage, 1997). This system differs in that developmental arrest still occurs but is not apparent until several days or weeks after injection of virions and that the host's prothoracic glands do not degenerate and presumably, function normally (Dushay & Beckage, 1993; Beckage & de Buron, 1993).

M. sexta larvae injected with virus stopped developing at the pre-pupal stage ('wandering stage') and subsequently died (Dushay & Beckage, 1993; Beckage *et al.*, 1994). However, although infected larvae initially ate less (and hence weighed less) than controls, the delay in metamorphosis meant that infected larvae attained very large sizes before attempting to pupate and dying. This phenomenon also (as for CsIV) appeared to be dose-dependent in that more heavily parasitised larvae grew larger before death compared to less heavily parasitised larvae (Alleyne & Beckage, 1997). Infected larvae also turned pink rather than blue-green for control larvae (Beckage *et al.*, 1990; Beckage *et al.*, 1994). The significance of such metabolic regulation by the PDV remains unclear (Beckage, 1998) although may be related to the gregarious nature of *C. congregata.*

Altered host development has been noted in several other systems. Parasitism of *P. includens* by *M. demolitor* is linked to reduced JHE metabolism and developmental arrest, although the exact role of its PDV is unclear (Dover *et al.*, 1995; Balgopal *et al.*, 1996). *Microplitis croceipes* calyx fluid appeared to differentially reduce larval growth in its natural host, *H. zea,* and in an atypical host, *G. mellonella*, but had no effect on another atypical host, *Spodoptera exigua* (Gupta & Ferkovich, 1998).

Parasitism of *S. littoralis* by *Ch. inanitus* led to precocious onset of metamorphosis and developmental arrest in the precocious pupa and this effect was reliant on an apparent synergy between calyx fluid and wasp venom elements (Soller & Lanzrein, 1996). Another *Chelonus* species, *Ch. curvimaculatus*, caused precocious metamorphosis in its host, *Trichoplusia ni*, as well as an increase in arylphorin expression that was independent of decline in juvenile hormone titers (Jones & Wache, 1998). In this case, precocious metamorphosis did not require the

presence of a parasitoid larva although metamorphosis occurred earlier if a parasitoid larva was present.

Several other parasitism-related effects on growth and development have been briefly characterised. CkBV is linked to spermatogenesis in *Pseudaletia separata*, a process known as parasitic castration (Tanaka *et al.*, 1994; Tagashira & Tanaka, 1998). Degree of castration was dependent on which instar was parasitised, with later stages being less severely affected. Specific germ cells were targeted by both viral genes and wasp venom components such that abnormal chromosomes were manifest. Early CkBV gene expression was apparent in the testis and this expression peaked ≈ 12 hpp. CkBV has also been implicated in the post-transcriptional elevation of growth-blocking peptide (GBP) in fat bodies of *P. separata*, leading to elevated levels in haemolymph (Hayakawa *et al.*, 1998). Parasitised host fat bodies contained six times the levels of GBP pre-cursors found in healthy hosts and GBP-processing enzyme activity increased by 90% after parasitisation.

Thus, it is evident that parasitism causes many alterations to host metabolism, although it is often hard to determine the cause of any effect witnessed due to the complexity of the associated protein interactions. Several studies indicate that the emergent parasitoid larva also plays a role in host metabolic regulation (Beckage & de Buron, 1994; Dover *et al.*, 1995; Balgopal *et al.*, 1996; Jones & Wache, 1998). This is believed to be due to teratocytes, which arise from the serosa of the parasitoid egg, or to remnants of the amnion (Beckage & De Buron, 1994). Teratocytes may be important in both protection of the parasitoid larvae and in regulation of host metabolism but to date this has not been elucidated.

1.4 *Cotesia rubecula* and its associated bracovirus (CrBV)

This study deals with the endoparasitoid, *C. rubecula* (Braconidae: Microgastrinae), and its associated bracovirus. Data concerning CrBV are limited, with only one virus gene, CrV1, having previously been characterised (Asgari *et al.*, 1996; Asgari *et al.*, 1997). The morphology if CrBV is similar to other BVs in that singly or multiply enveloped nucleocapsids are cylindrical and have a tail structure (Asgari & Schmidt, 1994). Northern blot analysis indicated that four viral genes are transiently expressed in parasitised larvae (Fig. 1-12), CrV1 being the most abundantly expressed and appearing to target haemocytes (Asgari *et al.*, 1996).

C. rubecula itself is a solitary endoparasitoid of the cabbage-white butterfly, *Pieris rapae* (Pieridae: Pierinae). Photographs of various life stages of *C. rubecula* are shown in figure 1-13. Whilst considered to be specific to *P. rapae*, *C. rubecula* has been recovered from the closely related *P. brassicae*, although emergent adults were shown to be smaller, as presumably less fit (Harvey *et al.*, 1999). *P. rapae* is a serious pest of cruciferous crops in its native region of Europe and Northern Asia, as well as in regions where it has spread to, such as the USA and Australia (New, 1994). *C. rubecula* is therefore considered to be an important biological control agent and has been introduced into many countries, e.g. Australia in the 1940s (New, 1994).

Larvae parasitised by *C. rubecula* are known to show reduced growth (Harvey *et al.*, 1999), although no mechanism for this has been reported. Growth of the non-preferred host, *P. brassicae*, was arrested at an earlier stage (after parasitisation) and at a smaller larval body mass than for *P. rapae* (Harvey *et al.*, 1999). *P. rapae* was deemed to be a far better host in terms of parasitoid development and this was attributed to a differential ability of the parasitoid to regulate host physiology and biochemistry. Given that PDVs from closely related wasps, e.g. CcBV and

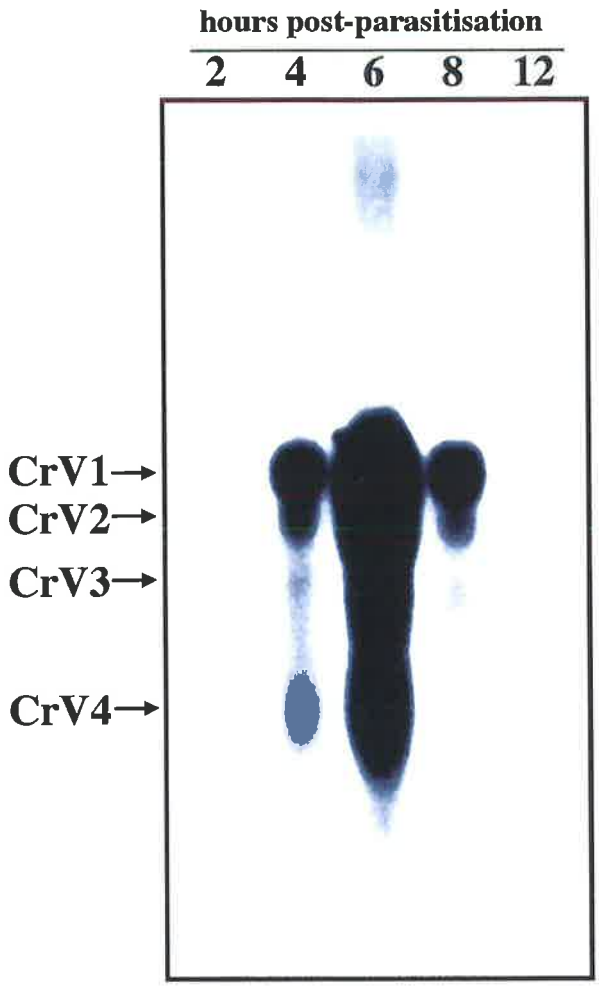


Figure 1-12. Temporal expression of *Cotesia rubecula* bracovirus (CrBV) genes in larvae of the lepidopeteran host, *Pieris rapae* (from Asgari *et al.*, 1996). Four different sized transcripts were detected by Northern blot analysis of RNA extracted from *P. rapae* larvae, at various times post-parasitisation, probed with total CrBV DNA. Transcripts were denoted CrV1-CrV4 by decreasing size.

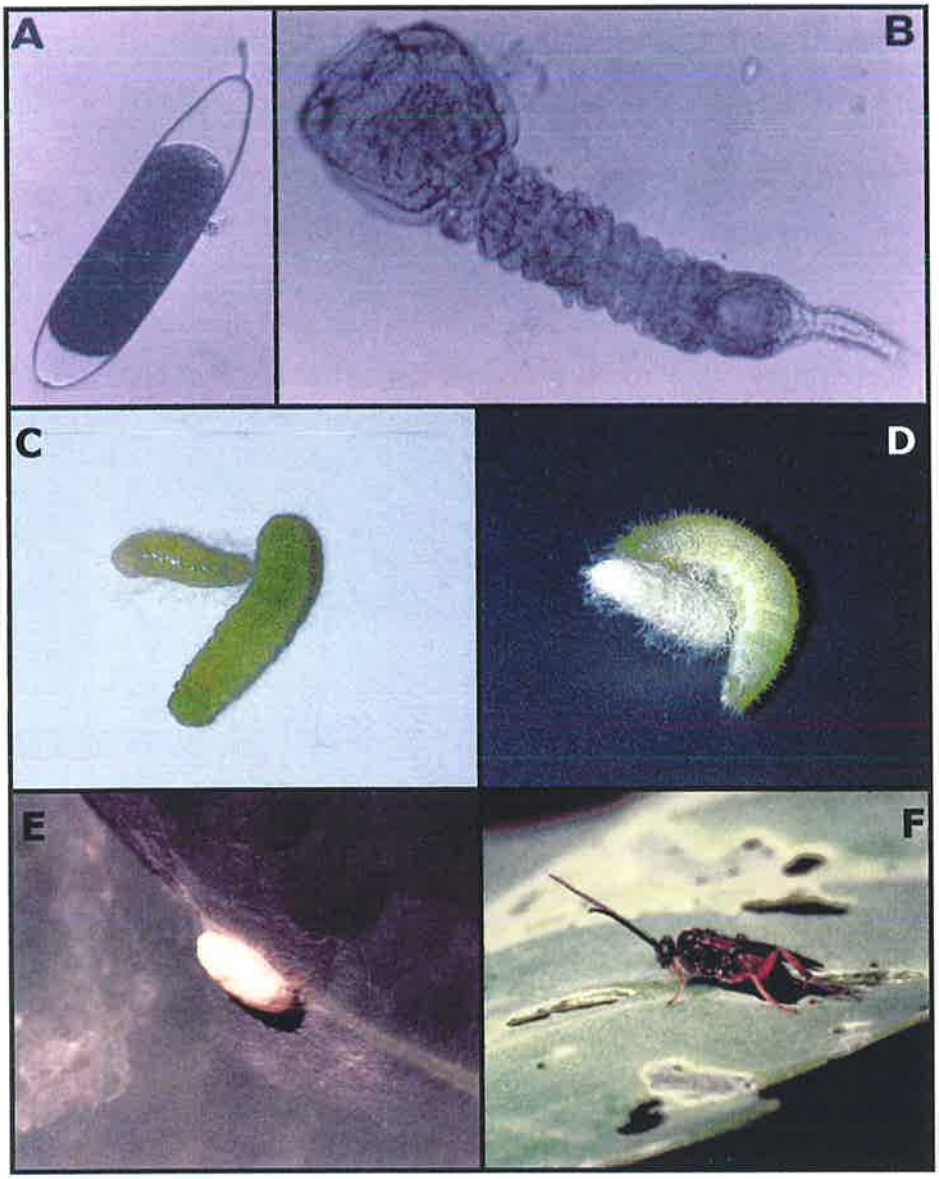


Figure 1-13. Life-stages of the solitary endoparasitoid, *Cotesia rubecula* (Braconidae: Microgastrinae). *A*, egg. *B*, first instar larva. *C*, last instar larva (*left*) after emergence from larva of the host, *Pieris rapae* (Pieridae: Pierinae - *right*). *D*, initial cocoon construction by last instar parasitoid larva, for pupation. The host is still alive but will perish within 24 hours. *E*, mature cocoon of parasitoid containing the developing pupa. Replication of the polydnavirus (CrBV) begins during pupation. *F*, adult. (Photographs by Sassan Asgari & Mike Keller).

CkBV, are known to target physiology and biochemistry, differential response of the lepidopteran hosts to CrBV genes could explain the higher suitability of *P. rapae* as a host for *C. rubecula.*

C. rubecula is known to inject a complex mixture of at least 11 venom proteins into *P. rapae* (Asgari, S.; unpublished data). Initial characterisation has revealed the presence of a serine proteinase homologue (Vn50) that interferes with activation of proPO (Asgari *et al.*, 2003). A small peptide has also been identified, which appears to enhance CrBV expression (Asgari, S.; unpublished data).

Thus, CrBV is not only an interesting subject in terms of its molecular biology and how this relates to other PDVs but also for more pragmatic reasons, such as its effect on *C. rubecula* as a biological control agent and how this can be manipulated. To date, molecular investigation has led to the characterisation of only two genes involved in *C. rubecula-P. rapae* interactions, CrV1 and Crp32, both of which suppress the lepidopteran immune system (Asgari & Schmidt, 1994; Asgari *et al.*, 1996; Asgari *et al.*, 1997; Asgari *et al.*, 1998).

1.4.1 CrV1

Asgari *et al.* (1996 & 1997) cloned and sequenced CrV1, demonstrating that it is an encapsidated gene expressed as a single transcript in parasitised host haemocytes and fat body. Sequence data showed the transcript to be polyadenylated, with an open reading frame of 912 bp coding for a deduced protein of 304 amino acids and ≈ 45 kDa in size. The deduced protein has a C-terminus signal peptide as well as a hydrophobic peptide at the N-terminus, and is secreted from infected cells. CrV1 dimers were detected in haemolymph of parasitised larvae, by non-denaturing PAGE.

Northern blot analysis indicated that CrV1 is heavily transcribed for a period ranging from four to eight hpp (Asgari *et al.*, 1996). Such a short period of high expression is uncommon amongst reported PDV genes (see 1.3.2.2) although some PDVs exhibit transient expression over longer periods. Reduced CrV1 expression at ≈ eight hpp is not effected by infective dose or addition of more virus via superparasitisation, suggesting the involvement of a regulated post-transcriptional event. The expression of CrV1 corresponds to various changes to the ultrastructure of haemocytes interacting with the protein and these changes were similar to those caused by cytochalasin D (Asgari *et al.*, 1998; see Fig. 1-14), a fungal toxin acting against the actin cytoskeleton of affected cells (Flanagan & Lin, 1980).

Although its exact mode of action is unknown, the presence of depolymerised actin in altered haemocytes suggests that CrV1 leads to abrogation of cell surface and cytoplasmic actin structural components (Asgari *et al.*, 1997). Virus-free calyx did not visibly alter haemocytes and recombinant CrV1 (expressed in baculovirus vectors and injected into larvae) elicited haemocyte alterations indistinguishable from parasitism (Asgari *et al*, 1996), suggesting that CrV1 expression may be solely responsible for the changes witnessed. Further, haemocytes appeared to recover and function normally, two to three days after parasitisation or injection with recombinant CrV1. A coiled-coil domain, containing a putative leucine zipper, is thought to be important for lipophorin binding and uptake of CrV1 by haemocytes (Asgari & Schmidt, 2002). No evidence of cell death or apoptosis was noted, a characteristic of haemocyte targetting by other PDVs (see 1.3.3.2). Interestingly, a CrV1 homologue identified in CcBV is thought to be involved in apoptosis of parasitised *M. sexta* larvae (Le *et al.*, 2003).

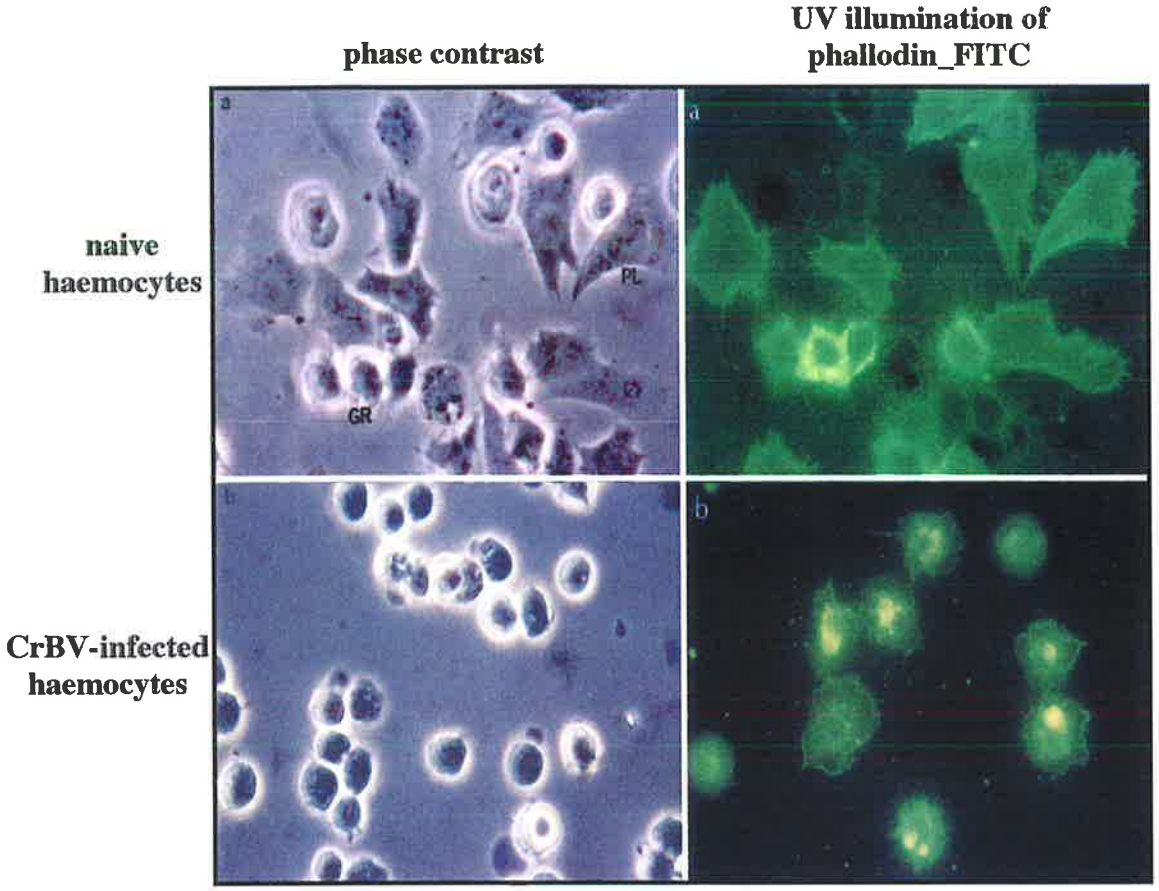


Figure 1-14. Effect of CrV1 on infected haemocytes (from Asgari *et al.*, 1998). Plasmatocytes (*PL*) and granulocytes (*GR*) were isolated from naive and parasitised *P. rapae* larvae, before being stained with FITC-labelled phallodin, in order to visualise the haemocyte cytoskeleton under UV illumination. The cytoskeleton of infected cells is depolymerised and has formed conglomerates in the cytoplasm whereas healthy cytoskeleton is distributed evenly around the cell surface. The same effect was noted when naive larvae were injected with recombinant CrV1. Treatment of haemocytes with the fungal toxin, cytochalasin D, produced similar effects.

Changes to haemocytes indicate that their ability to take part in cellular immune responses is compromised (Asgari *et al.*, 1996; Asgari *et al.*, 1997; see Fig. 1-14). Cell-surface glycoproteins recognised by *Helix pomatia* lectin (HPL), are not exposed on the surface of cells affected by CrV1. HPL binds predominantly to granulocytes in *P. rapae* and is implicated in immune activation of haemocytes in *Drosophila* (Theopold *et al.*, 1996), both of which indicate that cellular immune response may be inhibited by the loss of such a component. Indeed, over the time period corresponding with altered lectin binding properties, CrBV-infected haemocytes lose the ability to spread, attach to each other or bacteria and to remove foreign particles by phagocytosis.

It is thought that CrV1 is secreted from infected cells into serum, after proteolytic cleavage, going on to interact with the surface of haemocytes (Asgari *et al.*, 1997). Recombinant CrV1 produced similar effects on haemocytes only in the presence of haemolymph and not on purified haemocytes, indicating that CrV1 may undergo extracellular modification before interacting with haemocytes directly or requires a carrier molecule (Asgari *et al.*, 1996). It is unclear whether CrV1 interacts with a haemocyte surface receptor or is directly uptaken by haemocytes to interact with actin filaments. Without functional actin filaments haemocytes should not be able to undergo rearrangements of the cytoskeleton that are required for microparticle formation and degranulation, both of which are associated with immune activation (Yano *et al.*, 1994; Rosales *et al.*, 1994; Asgari *et al.*, 1996; Theopold & Schmidt, 1997).

It is clear that CrV1 plays a major role in the protection of the wasp egg and larvae from the time of its expression until haemocytes recover after several days. How the wasp larva is protected after this time is unknown and is an interesting point for investigation. Protection of the

parasitoid egg in the period before CrV1 expression is thought to be passive and associated with a parasitoid-derived gene, Crp32 (Asgari *et al.*, 1998).

1.4.2 Crp32

A layer (0.5-1.5 μm thick) composed of proteoglycan and glycoproteins is found on the surface of many microgastrine braconid wasp eggs (Kitano & Nakatsuji, 1978; Davies & Vinson, 1986). Asgari & Schmidt (1994) discovered a calyx fluid protein from *C. rubecula* attached to the surface of the parasitoid egg, and to the exterior of CrBV virions, which also formed part of the egg surface coating (Fig. 1-15*A* and *B*). Eggs dissected from within *C. rubecula* ovaries were encapsulated when injected into a host, as were SDS-treated eggs injected with virions (Fig. 1-15*C*), indicating that the protective mechanism is conferred within the lumen of the reproductive tract and is independent of CrBV infection.

Cloning and sequencing of Crp32 revealed an open reading frame of 768 bp and a deduced protein of $\approx$ 32 kDa in size (Asgari *et al.*, 1998). Interestingly, Crp32 shows high antigenic similarity with a CrBV capsid protein of similar size, indicating that Crp32 is a viral structural protein or a parasitoid-derived protein released into the calyx lumen with virus particles (Asgari *et al.*, 1994). Further, Crp32 is antigenically similar to a 34 kDa protein located on *P. rapae* haemocytes, thus, the potential of Crp32 for mimicking host tissues and preventing non-self recognition and subsequent immune activation (Asgari *et al.*, 1998). This potential is demonstrated by the fact that resin beads coated with Crp32 and injected into healthy *P. rapae* larvae were not encapsulated, whereas uncoated beads were (Asgari *et al.*, 1998; Fig. 1.15*D* and *E*). Thus, Crp32 appears to confer a passive immune-protection on *C. rubecula* eggs in the time

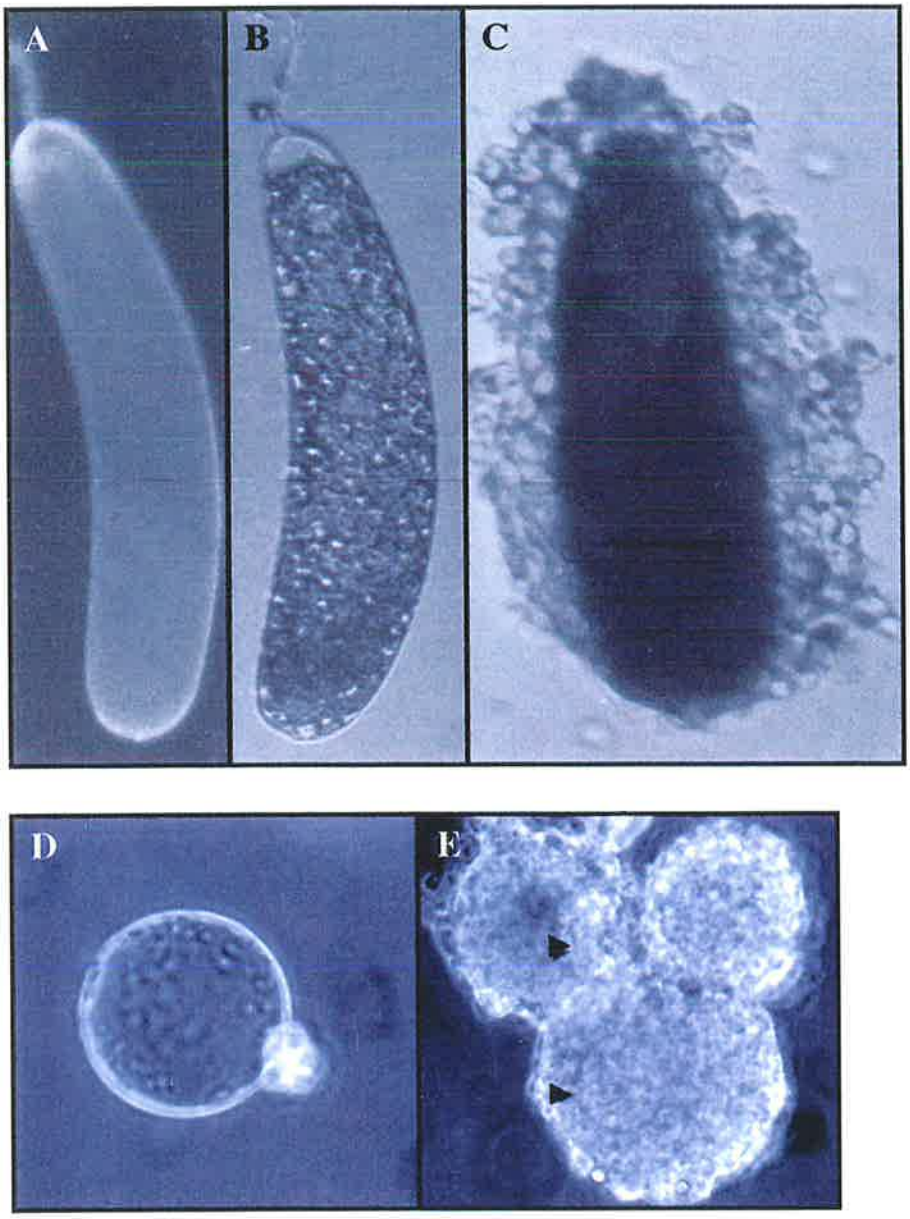


Figure 1-15. Presence of protective coating on *C. rubecula* eggs and passive protection of Crp32-coated foreign objects *in vitro* (from Asgari *et al.*, 1994 and 1988). *A*, UV illumination of anti-CrBV antibodies coating the surface of a non-encapsulated *C. rubecula* egg obtained from the calyx region, washed in PBS, injected into a naive larva and recovered after 12 hours. Crp32 is one component of the coating layer. *B*, phase contrast view of non-encapsulated *C. rubecula* egg treated as in *A*. *C*, encapsulation and melanisation of a manually injected parasitoid egg (obtained from the calyx gland) after removal of coating layer via washing with mild SDS. *D*, lack of encapsulation of resin bead coated with Crp32 and incubated with naive *P. rapae* haemocytes *in vitro*. *E*, encapsulation of beads (*arrowheads*) without Crp32 coating, when incubated with naive *P. rapae* haemocytes *in vitro*.

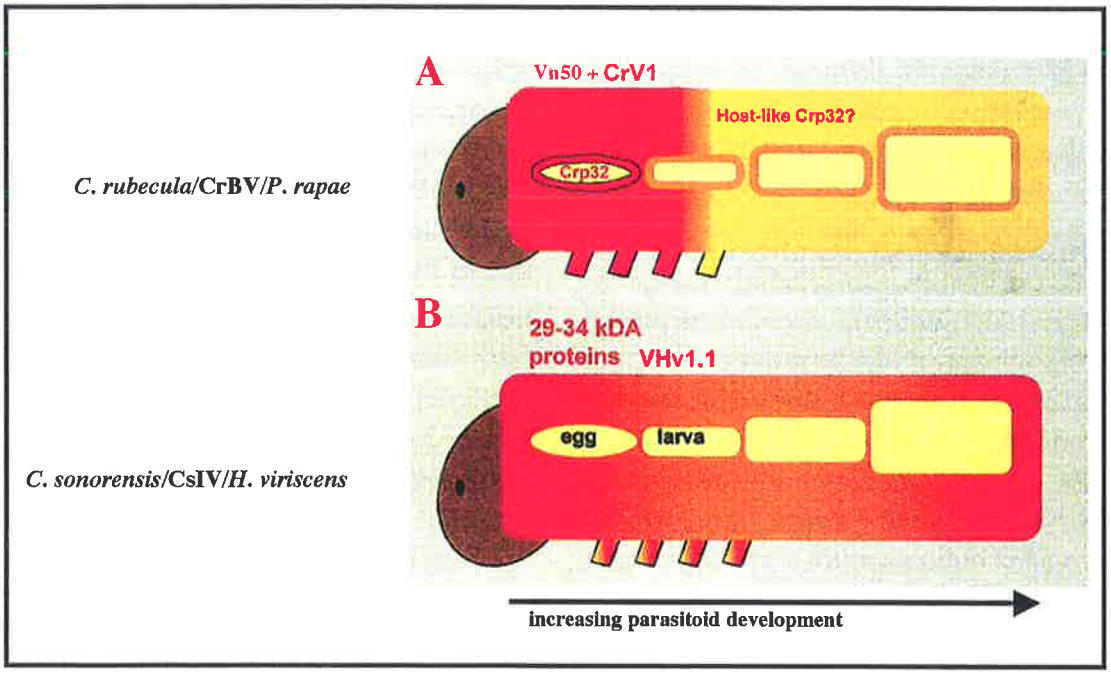


Figure 1-16. Putative suppression of lepidopteran host immune response by bracovirus- and ichnovirus-associated hymenopteran endoparasitoids (adapted from Schmidt *et al.*, 2001). Parasitoid development (i.e. time) increases moving *left to right*, where *red areas* indicate the temporal and spatial distribution of immune-suppressed haemocytes. *Yellow areas* indicate temporal and spatial distribution of active haemocytes (e.g. inside the *egg* and developing *larva*). *A, C. rubecula* applies active and passive means of immune-suppression against *P. rapae*. Vn50 is an injected venom protein known to inhibit activation of melanisation cascade reactions in parasitised larvae (Asgari *et al.*, 2003). Crp32 is a calyx protein present on the surface of the parasitoid egg and CrBV virions. It is thought Crp32 affords the egg passive protection by mimicking components of host tissues, prior to active suppression by CrBV gene products such as CrV1. CrV1 is expressed by *P. rapae* cells over a period of ≈ 4-10 hpp and provides systemic active suppression of host haemocytes by causing destabilisation of their cytoskeleton. Haemocytes recover after 2-3 days. The method of immune-suppression after this time is not understood. *B, C. sonorensis* actively and systemically suppresses the *H. viriscens* immune response over the total period of parasitoid larval development. A series of related 29-34 kDa calyx glycoproteins provide initial haemocyte inactivation. CsIV gene products, e.g. VHv1.1, are subsequently expressed and cause long-term inactivation. The calyx proteins and VHv1.1 both inactivate haemocytes by damaging their cytoskeleton.

period before CrBV transcripts build up, providing active suppression. Hayakawa & Yazaki, (1997) reported a similar situation for the closely related *C. karyai*, whereby a 50 kDa protein is described as being present on the CkBV envelope and sharing an epitope of 23 amino acids with the host, *P. separata*. As for CrBV, CkBV virions were detected on the surface of parasitoid eggs.

The similarity of Crp32 and the virus capsid may also mean that individual virions are protected from immune recognition, which could explain why virions introduced into haemolymph of an immune-competent host are not inactivated by immune cascades (Asgari *et al.*, 1994). Based on characterisation of CrV1, Crp32 and Vn50, it is apparent that *C. rubecula* employs integrated forms of active and passive immune-suppression. This is summarised in figure 1-16*A* and compared with mechanisms employed by *C. sonorensis* against *H. viriscens* (Fig. 1-16*B*).

1.5 Utilisation of baculoviruses for *in vitro* protein production

In the last 15 years, baculovirus expression vector systems (BEVS) have been developed to facilitate *in vitro* production of commercially important proteins in the fields of agriculture and medicine (Luckow & Summers, 1988). The vast majority of BEVS have involved the use of the *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) and *Spodoptera frugiperda* insect cell lines, Sf9 or Sf21 (Hink *et al.*, 1991), which are both readily available (Fraser, 1992). AcMNPV is the most utilised baculovirus as it is the most highly characterised (Kool & Vlak, 1993) and replicates in over 25 different cell lines (Du & Thiem, 1997). Upon infection of insect cell lines with recombinant virus, high yields of the foreign protein are produced. It is then a relatively simple process to purify the recombinant protein from the cells.

Various unique aspects of baculoviruses are utilised including highly active late gene promoters, an ability to accept large DNA inserts into the genome whilst maintaining replicative integrity and simple culturing procedures (Fraser, 1992). A generalised baculovirus infection-cycle is illustrated in figure 1-17.

Using BEVS, hundreds of proteins from viruses, bacteria, invertebrates, plants and mammals have been expressed (Jarvis, 1997), with most retaining their required biological activity due to processing of the mRNA transcript by eukaryotic host cell components (Luckow, 1991). The relative speed with which recombinants can generally be produced and isolated is an advantage of BEVS (Fraser, 1992). Interestingly, BEVS have been used to express several PDV proteins including VHv1.1, WHv1.6 (Soldevila & Webb, 1996) and CrV1 (Asgari et al., 1997), for the purpose of gene characterisation and functional analysis.

1.6 Utilisation of baculoviruses as bioinsecticides

Baculoviruses have long been exploited as bioinsecticides and provide a range of advantages compared to chemical controls. They are naturally occurring and have a limited host range, are harmless to non-target organisms, do not produce toxic chemical residues and can be effective at low doses (Vlak, 1993). Therefore, use of baculovirus-based insecticides is generally more environmentally friendly compared to chemical insecticides. These attributes also make baculoviruses ideal for incorporation into integrated pest management (IPM) strategies and sustainable agricultural systems.

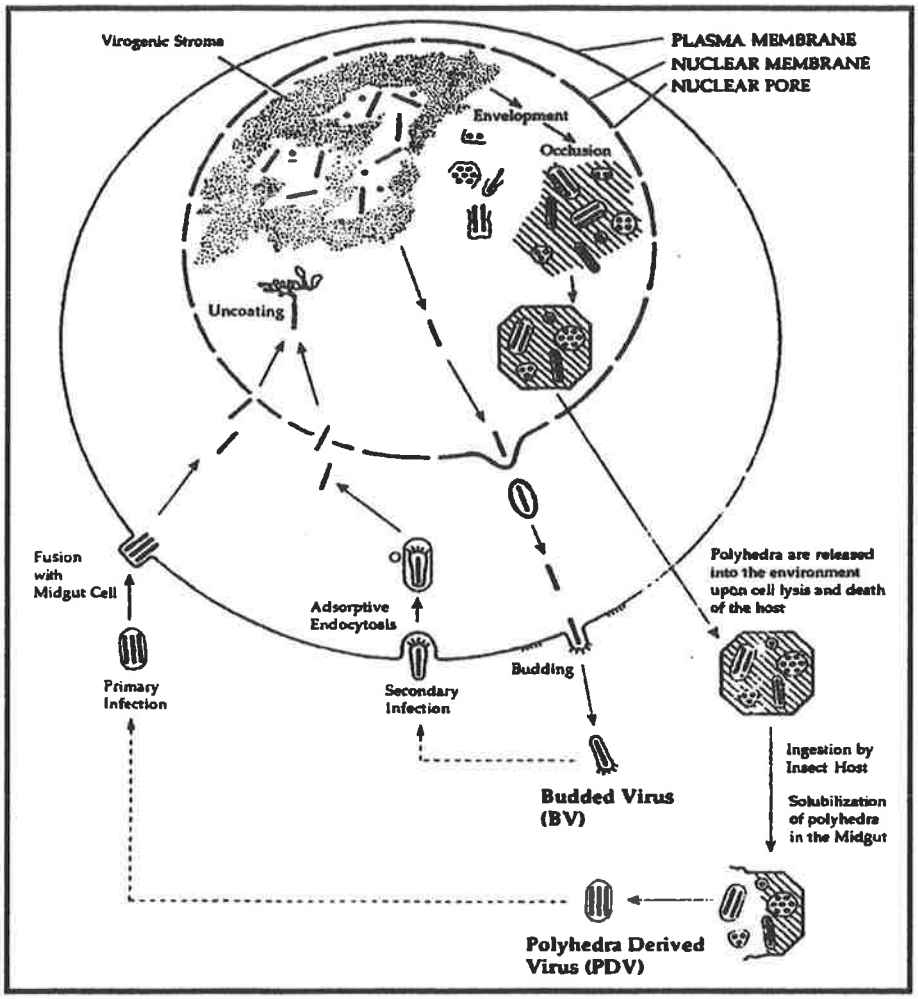


Figure 1-17. Typical infection-cycle of nucleopolyhedrosis viruses (from Blissard & Rohrmann, 1990). Polyhedra are ingested by a susceptible host insect and solubilised in the high pH (8-11) of the midgut. Virions of polyhedra-derived virus are released into the gut lumen and enter midgut epithelial cells by fusing with the microvillar membrane. Nucleocapsids are transported to the cell nucleus where viral DNA is uncoated, viral gene expression (and DNA replication) occurs and a dense mass of viral proteins (a virogenic stroma) forms. Progeny nucleocapsids assemble in and around the virogenic stroma before some bud through the nuclear membrane and make their way to the plasma membrane, apparently losing their nuclear derived envelope in the cytoplasm. Progeny nucleocapsids then bud through the nuclear membrane into the hemocoel, acquiring a budded virus specific envelope containing the virus-encoded envelope glycoprotein (gp64). Such virions are specialised to initiate secondary infection of other host cells. Other progeny nucleocapsids remain in infected cell nuclei before becoming enveloped by a *de novo* assembled membrane. These virions are subsequently occluded within polyhedrin matrices (polyhedra) that crystallise around them. A polyhedral envelope is added to the periphery of polyhedra at maturity. Upon insect death and cell lysis, polyhedra are released into the environment, forming the inoculum for a new round of infections.

1.6.1 The pros and cons of wild-type baculovirus bioinsecticides

Application of the wild-type baculovirus has been successful in a number of cases, the best example being the control of the soybean caterpillar, *Anticarsia gemmatalis*, with the nucleopolyhedrosis virus of that species (AgMNPV) (Moscardi, 1999). Application of the virus has reached one million hectares of cropping land per annum in Brazil. However, various limitations associated with the use of wild-type baculoviruses have meant that, in most cases, they do not afford adequate control within economic constraints.

Firstly, agrochemical companies consider that the commercial potential of wild-type baculoviruses is small compared to broad spectrum chemical controls, due to the small host range affected and the fact that later larval stages are less susceptible to infection (Vlak, 1993). In addition, baculovirus production is labour intensive and not easily automated on a commercial scale (Vlak, 1993). Indeed, use of wild-type AgMNPV in Brazil is only viable because farmers collect infected cadavers from treated crops for use as inoculum, effectively removing the cost of production (Moscardi, 1999). Other problems include poor application technology and low field persistence (Bonning & Hammock, 1996), variable potency (Black *et al.*, 1997), difficulties associated with registration and patenting (Wood & Granados, 1991), need for frequent monitoring to assess timing of application and farmer's attitudes (shaped by fast acting chemical insecticides) (Moscardi, 1999).

However, the greatest disadvantage when compared to chemical controls is the lag time between ingestion and the cessation of feeding (Vlak, 1993). In evolutionary terms, this lag time provides advantages for the virus, allowing large amounts of replication and possibly transport to new regions of the environment. However, it makes wild-type baculoviruses inefficient for use as

bioinsecticides in annual or intensive crops. The combination of these limitations saw wild-type baculoviruses account for only 0.2% of the less than 1% of world pesticide sales utilising biological controls, in the late 1980s (Jutsum, 1988).

1.6.2 Genetically modified baculoviruses as bioinsecticides

Recent advances in baculovirus molecular biology and the widespread use of BEVS have resulted in the generation of genetically modified baculovirus bioinsecticides (Vlak, 1993), based primarily and most successfully on AcMNPV (Black *et al.*, 1997). By inserting a foreign gene into the viral genome, the virus acts as an expression vector for the recombinant protein, expressing it as part of viral replication within infected host larvae (Wood & Granados, 1991). Thus, if the recombinant protein is harmful to the infected insect, it may die rapidly from the toxic effect of the protein rather than virus infection itself (Bonning & Hammock, 1996).

Figure 1-18 (from Vlak, 1993) illustrates the expected effect of using genetically enhanced baculoviruses (compared to wild-type) on the time between infection and cessation of feeding. If the rate of mortality can be increased such that larval death occurs earlier after infection, baculoviral bioinsecticides may become more economically viable.

As for protein expression in insect cell cultures, the foreign protein is usually placed under the control of the polyhedrin promoter in order to achieve maximum expression. A problem with this approach is that recombinant virus is negatively selected compared to wild-type virus, due to quicker death of the host (Bonning & Hammock, 1996) and poor environmental persistence due to removal of the polyhedrin gene. However, these aspects can be advantageous in terms of environmental safety and sustained commercial returns for the developer.

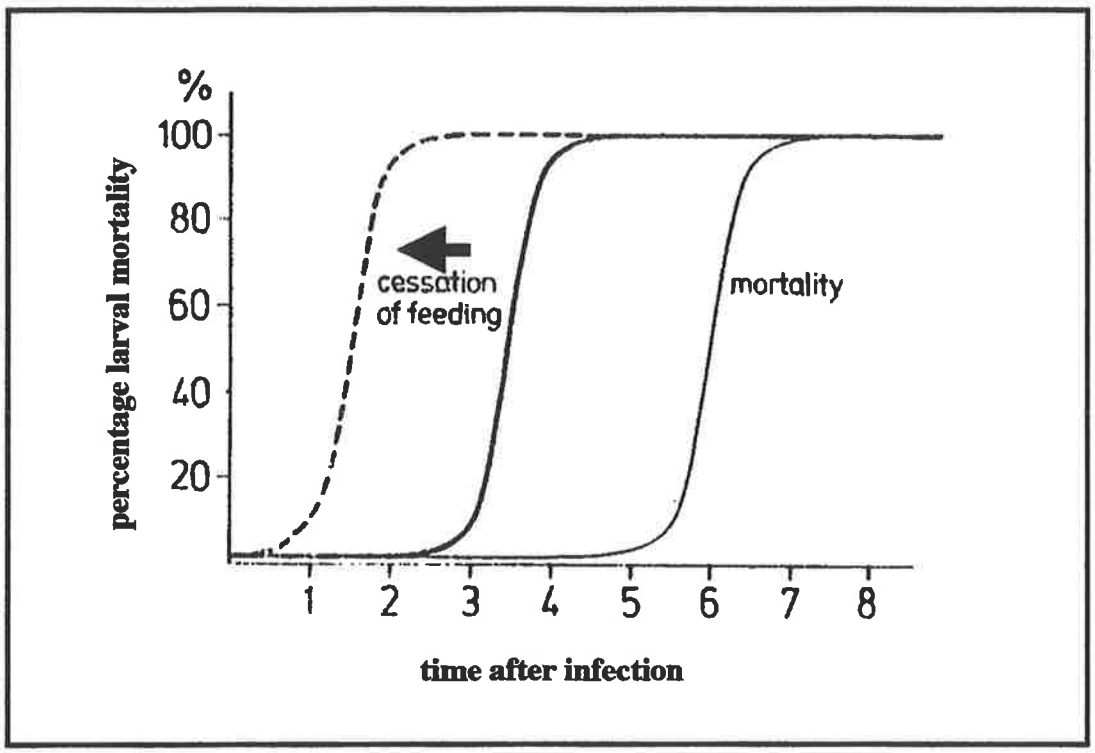


Figure 1-18. Schematic representation of the relationship between larval mortality (*thin line*) and cessation of feeding (*thick line*) in terms of percentage mortality versus time after infection (from Vlak, 1993). The *dotted line* (cessation of feeding and/or mortality) represents the anticipated effect of a recombinant baculovirus with enhanced insecticidal activity. Time to mortality/feeding cessation is reduced such that the mortality curve is shifted in the direction of the *arrow*.

Various characteristics are required in a recombinant protein if it is to be of use for modifying baculoviruses to enhance insecticidal ability. These include having an insect-specific targetting and/or mode of action and sufficient activity at low levels, being fast acting and lethal to the host but not cytotoxic to cultured cells and not requiring specialised processing to achieve activity (Black *et al.*, 1997). Generally, proteins acting systemically are preferable to those acting in infected cells alone (Bonning & Hammock, 1996).

1.6.3 Genetically enhanced baculoviruses expressing insect genes

One approach to genetic enhancement of baculoviruses, is to abnormally express insect genes that control important aspects of insect physiology (Black *et al.*, 1997). The insertion of diuretic hormone of *M. sexta* along with sequences needed for hormone secretion and alpha-amidation, into *B. mori* SNPV (BmSNPV), was the first attempt at such an approach (Maeda, 1989). Although the recombinant virus caused 30% reduction in hemolymph volume compared to wild-type BmSNPV, the rate of mortality was only marginally increased (Black *et al.*, 1997).

Other attempts utilised the insertion of various hormones and enzymes including prothoracicotropic hormone (O'Reilly *et al.*, 1995), juvenile hormone esterase (Bonning & Hammock, 1992) and eclosion hormone (Eldridge *et al.*, 1992) into various strains of AcMNPV. However, these bioinsecticides have proved largely unsuccessful in terms of commercial viability (Black *et al.*, 1997). The most promising of the recombinants containing insect genes has been a non-occluded form of AcMNPV expressing an insect chitinase, which killed *S. frugiperda* larvae 20% faster than wild-type AcMNPV (Gopalakrishnan *et al.*, 1995). Given the limited knowledge of insect development and endocrine systems, it has been difficult to produce recombinants that contain insect genes and provide predictable and/or controllable effects (Black *et al.*, 1997).

1.6.4 Genetically enhanced baculoviruses expressing insect-specific toxins

The strategy most likely to lead to a commercially viable baculovirus bioinsecticide is the insertion of insect-specific toxin genes into AcMNPV. In 1988, a gene coding for a scorpion toxin, *Buthus eupeus* insect toxin (BeIT), was inserted into the AcMNPV genome under control of the polyhedrin promoter (Carbonell *et al.*, 1988). However, only small amounts of biologically active toxin were produced, even though high levels of BeIT transcription occurred. Since that time, other scorpion toxin genes have been used to significantly enhance the efficacy of the recombinant baculoviruses.

The scorpion *Androctonus australis* produces a cysteine-rich peptide, 70 amino acids in length, which causes rapid paralysis in insects (Stewart *et al.*, 1991) but is non-toxic to vertebrates (Zlotkin, 1983). The gene coding for this toxin, AaIT (*A. australis* insect toxin), has been isolated and used to successfully paralyse insects via expression of the toxin *in vivo* by recombinant AcMNPV (and BmSNPV) vectors (Stewart *et al.*, 1991). When the gene coding for AaIT was placed under control of polyhedrin or p10 promoters, feeding damage from infected larvae was approximately half of that caused by wild-type infected larvae (Stewart *et al.*, 1991). This was due to a 30% reduction in the time to cessation of feeding, in larvae infected with the recombinant AcMNPV.

The gene for another scorpion toxin, LqhIT2 (*Leirus quinquestriatus hebreus* insect toxin), has been used in recombinant AcMNPV, under control of the IE1 (immediate early gene) promoter, to express active toxin in insects although information regarding relative effectiveness is not available (Black *et al.*, 1997). In contrast to AaIT, LqhIT2 induces complete relaxation of insect muscles after a short excitory period (Zlotkin *et al.*, 1991).

Recombinant AcMNPV has also been used to express an insect neurotoxin (TxP1) produced by the straw itch mite, *Pyemotes tritici*, utilising a modified polyhedrin promoter (Tomalski & Miller, 1991). Larvae infected with these non-occluding recombinants died almost two days before those infected with wild-type AcMNPV (Tomalski & Miller, 1991), corresponding to about a 35% reduction in time from to mortality. However, all of the virus-derived TxP1 proteins had reduced activity compared to the native protein (Black *et al.*, 1997).

Other toxins have been used in recombinant baculoviruses but have generally shown less promise. One novel approach was to use a maize mitochondrial gene (T-urf13) linked with male sterility (Korth & Levings, 1993), which produces a hydrophobic protein toxic to insects due to strong membrane binding (Bonning & Hammock, 1996). When *T. ni* larvae were infected with recombinant virus expressing T-urf13 under control of the polyhedrin promoter, a 40% decrease in killing time was achieved compared to wild-type AcMNPV (Korth & Levings, 1993). However, recombinants containing T-urf13 are also toxic to cell cultures, which would lead to difficulties with commercial production (Black *et al.*, 1997).

Toxins from *Bacillus thuringiensis* (BT) have also been utilised (Martens *et al.*, 1990; Merryweather *et al.*, 1990) but the inherently slow mode of action of the BT toxin means that recombinant AcMNPV is no faster at causing mortality than wild-type virus (Black *et al.*, 1997). Overall, the use of toxin genes, particularly neurotoxins, appears promising. Further reductions in time to mortalilty are necessary and may be achieved by the utilisation of more potent toxins and the use of multiple vectors to deliver combinations of toxins (Bonning & Hammock, 1996).

At present, the main factor limiting use of the more effective recombinant baculoviruses is their narrow host range. The efficiency of viral replication varies from host to host and may differ by several orders of magnitude, resulting in a range of effects from latent and sub-lethal to death (Cory *et al.*, 1997). Thus, economic host range, those species that can be controlled cost effectively, is usually far narrower than the total range of species which may become infected (biological host range) (Black *et al.*, 1997).

Host range may be widened by utilising co-occluded baculovirus species combinations, but it is unclear if this can be achieved within commercial guidelines (Black *et al.*, 1997). Another approach may be to genetically modify baculoviruses to increase their host range. Various genes have been identified as conferring the ability to replicate in a given host cell, e.g. hcf-1 in AcMNPV. Similarly, the *Lymantria dispar* multiple nuclear polyhedrosis virus (LdMNPV) has a gene (hrf-1) which allows recombinant AcMNPV to develop in a *L. dispar* cell line that is non-permissive to wild-type AcMNPV (Thiem *et al.*, 1996).

Generally, genetically modified baculovirus bioinsecticides show great promise for provision of greater flexibility in insect control, especially within the context of IPM. Most of the advantages of the wild-type bioinsecticides are retained, combined with a reduction in time before feeding cessation. Whilst there are several environmental concerns associated with the use of genetically modified organisms, and much care should be taken with any release, these should not be overstated given the major disadvantages associated with other means of pest control, e.g. chemical residues and non-target effects. It would be prudent to undertake further research on the effects of genetically modified baculoviruses on non-target hosts and parasitoids. Refinement of

formulations and application methods combined with increased rates of mortality should see the use of recombinant baculoviruses become increasingly common.

1.7 Summary and project aims

PDVs (Polydnaviridae) are unusual, obligate symbionts of certain ichneumonoid endoparasitoids. PDV genomes are integrated into that of the parasitoid and excised into numerous circular viral segments that are packaged into virions. Virions are replicated exclusively in calyx cells of the female parasitoid's reproductive tract and are injected into a lepidopteran host along with the parasitoid egg. Hence, when oviposition occurs, virus DNA is transferred to the host both as integrated DNA (within chromosomes of the unhatched parasitoid larva) and as polydisperse viral DNA in nucleocapsids.

Once inside the lepidopteran host, virions move into haemocytes and various host tissues, prior to expression of encapsidated viral genes. These genes function in both subverting the immune response of the host against virus and parasitoid components, and in regulating host growth and development to benefit the developing parasitoid. It is because of these benefits that PDV particles have been considered as being symbiotic in relation to the parasitoid. Last instar endoparasitoid larvae usually emerge from the host before pupating. During pupation, the regulated excision of viral segments from amplified regions of the parasitoid genome occurs and a new round of virus replication begins. The virus is transmitted vertically between parasitoids whilst imparting no adverse effects.

PDVs make interesting research subjects for many reasons including their obligate symbiosis, complex integrated genome and gene expression, activity in mediating host metabolism and

immune response, and evolutionary relationship with each other and their hosts. Much research has focussed on characterising the structure and function of viral genes. This study deals with two previously characterised immuno-suppressive genes associated with the parasitoid, *Cotesia rubecula*, and its bracovirus (CrBV). *C. rubecula* is an important biological control agent, parasitising *P. rapae*, a widespread pest of cruciferous crops.

CrV1 is one of four CrBV-encoded genes that are heavily expressed between 4-10 hpp in fat body and haemocytes of parasitised hosts. The CrV1 protein is secreted from infected cells into haemolymph where it undergoes modification before interacting with haemocytes. This interaction subsequently damages actin filaments within haemocytes, preventing the ability of the haemocytes to mount a cell-mediated attack on the parasitoid. Thus, CrV1 provides a transient, systemic, active suppression of the lepidopteran immune response and helps to facilitate parasitoid development.

The other previously characterised gene, Crp32, is encoded on the genome of *C. rubecula* and its product is released into the reproductive system of the female parasitoid. Crp32 forms part of a layer coating *C. rubecula* eggs and is associated with CrBV virion membranes. Because it shows high antigenic similarity to *P. rapae* biochemical components, Crp32 may passively protect the *Cotesia* egg from encapsulation by mimicking host tissue and preventing non-self recognition and subsequent immune activation.

Another useful and interesting group of insect viruses is the baculoviruses (Baculoviridae). Baculoviruses are pathogens of several orders of insects, most notably Lepidoptera, and have therefore been exploited as biological control agents. In recent years, baculoviruses have also

been utilised as *in vitro* and *in vivo* protein expression vectors by inserting a foreign gene into the baculovirus genome, under control of highly active virus promoters, and infecting insect cell cultures or larvae, respectively. Many proteins have been expressed in cell culture using recombinant baculoviruses, either to aid in functional analysis or for commercial production. Several PDV genes have been analysed using baculovirus expression vectors, including VHv1.1, WHv1.6 and CrV1. Most baculovirus expression systems utilise AcMNPV as this is the most characterised of the baculoviruses and has been commercially developed as an expression vector.

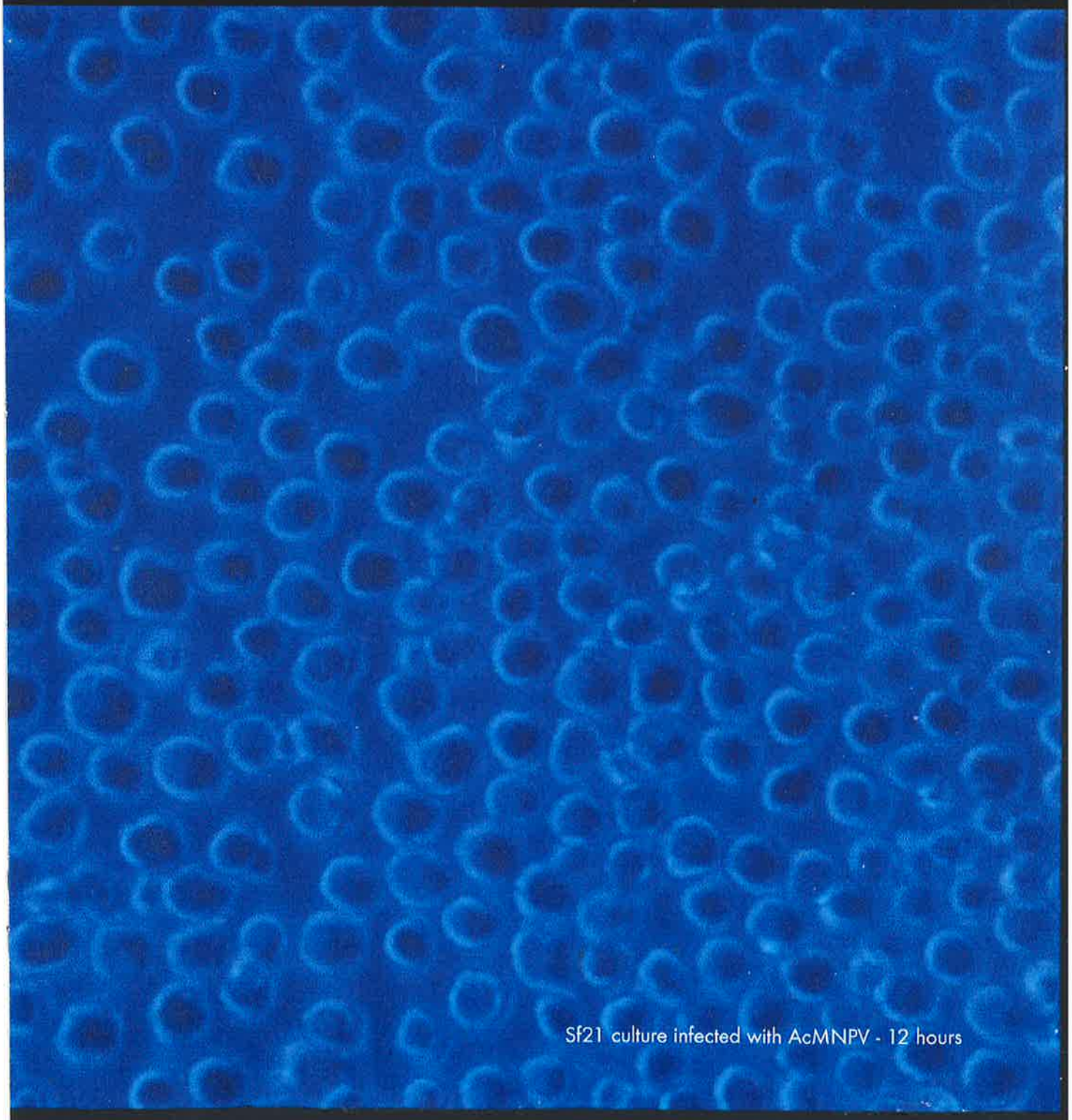
Another use of recombinant baculoviruses has been the production of improved baculovirus-based bioinsecticides. Wild-type viruses tend to kill too slowly to be of wide-spread use as biological control agents, so attempts have been made to improve their efficiency via insertion of foreign genes. Such genes have included insect-specific toxins, insect metabolism regulators and degradative enzymes such as chitinase. Improvements have been made to various baculoviruses, most often AcMNPV.

This study aims to isolate and characterise some of the remaining CrBV genes that are expressed in parasitised *P. rapae* larvae. Increased knowledge of molecular interactions in the *C. rubecula*/CrBV/*P. rapae* system may provide several benefits including increased characterisation of CrBV and PDVs in general, greater understanding of insect immune processes, identification of useful proteins that mediate metabolism or immunity and refinements to virus- or parasitoid-based biological control systems.

Further aims are to utilise the immuno-suppressive proteins, CrV1 and Crp32, to produce recombinant AcMNPVs, which can be tested for their insecticidal properties. Ideally, the effect

of wild-type AcMNPV on *P. rapae* (previously unreported) will be analysed and compared against that of the recombinant AcMNPVs. Given the immune-suppressive nature of CrV1 and Crp32 in *P. rapae*, it is possible that their addition to AcMNPV may lead to a reduced immune response of *P. rapae* against recombinant AcMNPVs containing them, compared to that mounted against wild-type virus. Such a system also provides a unique experimental model, which can be manipulated to try an elucidate some of the poorly understood mechanisms of insect immunity.

Chapter 2: Experimental Techniques



Sf21 culture infected with AcMNPV - 12 hours

Chapter 2: Experimental Techniques

2.1 Isolation and characterisation of bracovirus genes and production of antibodies against gene products

2.1.1 Construction and screening of a 6 hour parasitised larval *P. rapae* cDNA library. Total RNA was extracted from *P. rapae* larvae at 6 hpp by mated *C. rubecula* wasps (QuickPrep™ total RNA extraction kit - Amersham). mRNA was then isolated from total RNA (PolyATtract™ mRNA isolation system - Promega). The isolated mRNA was used for construction of the cDNA library containing clones packaged in pBluescript® SK(+/-) phagemids (cDNA synthesis kit, ZAP-cDNA® synthesis kit and ZAP-cDNA® Gigapak® III Gold cloning kit – Stratagene). The library was amplified and titred according to manufacturer's instructions before being probed with total CrBV DNA, previously digested with *Bam*HI and *Hind*III and labelled with ^{32}P. Probes were prepared as described (Ready-To-Go™ DNA labelling beads - Amersham), with hybridisation conducted at 65°C. Positive clones were rescreened resulting in isolation of partial CrV2 and complete CrV3 coding regions. CrV3 was sequenced using M13 forward and reverse primers directly from the phagemid vectors produced by the aforementioned protocols and subsequent automated sequencing (Applied Biosystems). 5' rapid amplification of cDNA ends (5' RACE) was used to complete the CrV2 sequence before the CrV2 cDNA fragment was cloned and sequenced (2.1.4).

2.1.2 Southern and Northern hybridisation. DNA samples were run on a 1% agarose gel and transferred to a nylon membrane (Amersham) as described (Sambrook *et al.*, 1989). RNA was isolated from 6 h parasitised *P. rapae* caterpillars according to Chomczynski & Sacchi (1987). RNA samples were run on 1% agarose gels under denaturing conditions, using formaldehyde,

and transferred to nylon membranes as described (Sambrook *et al.*, 1989). Probes were prepared as described (Ready-To-Go™ DNA labelling beads - Amersham) and hybridisation was carried out at 65°C. Slot-blots were carried out using the Bio-Dot® SF Microflitration Apparatus (BIO-RAD) as per manufacturers instructions.

2.1.3 5' amplification of CrV2 cDNA (5' RACE). Partial CrV2 cDNA (containing a poly-A tail and obtained via screening of the 6 h parasitised *P. rapae* library) was extended in the 5' direction utilising the 5' RACE System for Rapid Amplification of cDNA Ends (Version 2.0 - Life Technologies®). Three reverse primers were designed for this process, with the terminal reverse primer being CloneC-R (see 2.1.6). The sequence of two nested reverse primers were CloneC-R2 5'-GGAGATTCTTGAAGCAGGAAG-3' and CloneC-R3 5'-GGATCGAGGATTGCGACTG-3', with each binding site moving progressively along the clone towards the 5' end. Forward primers were random hexamer primers, supplied with the 5' RACE kit (Version 2.0 - Life Technologies®). The same PCR machines were used as for standard PCR of CrV2 and CrV3 (see 2.1.8).

2.1.4 Cloning and sequencing of 5' RACE CrV2 fragment. PCR product obtained from 5'RACE was ligated into the pGEM®-T Easy vector as described (Promega). The resultant recombinant plasmids were used to transform *Escherichia coli* cells (2.4.7). Sequencing PCRs were performed on purified plasmid, in both directions, using M13 primers. Sequence data were produced by automated sequencing (Applied Biosystems) and added to those obtained from the initial cDNA clone, to give the complete CrV2 open reading frame.

2.1.5 CrV2 and CrV3 nucleotide sequence analysis. Raw sequence data was refined and translated into the deduced amino acid sequence using SeqEd software (1.0.3; Applied Biosystems). This software was also utilised to align various sequences. Mapdraw software (3.0.9; DNASTAR Inc.) was used to identify restriction sites within the genes. Seqeunces were compared against those contained in the GenBank™ database, using a nucleotide BLAST search, accessed via the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.1.6 CrV2 primer design. For diagnostic purposes, primers were designed to amplify 290 bp of a CrV2-related cDNA clone (5' primer CloneC-F and 3' primer CloneC-R; see Fig. 3-5). Primer sequences were CloneC-F 5'-TGGAGCGGTAACCTATC-3' and CloneC-R 5'-CCCTGTCAATCAATTCACG-3'. In order to clone most of the CrV2 open reading frame (excluding signal peptide) into the pQE30 expression vector (Qiagen), specific primers were designed with 5' overhangs containing a stabilisation sequence and *Sph*I or *Hind*III restriction sites (5' primer CrV2-F and 3' primer CrV2-R, see Fig. 3-5). Primer sequences were CrV2-F 5'-CGCGGCATGCCCGTTGCAAGACAGAAG-3' and CrV2-R 5'-GCGCAAGCTTTTAGGGATGATCTCGAGC-3', with restriction sites underlined.

2.1.7 CrV3 primer design. Specific primers to the CrV3 open reading frame (5' primer CrV3-F and 3' primer CrV3-R, see Fig. 3-7*A*) were designed with 5' overhangs (as for CrV2 primers – see above) containing *Sph*I and *Pst*I restriction sites to allow for direct ligation of the amplified fragment into the pQE30 expression vector (Qiagen). The amplified fragment contained most of the open reading frame excluding the signal sequence at the 5' end (see Fig. 3-7). Primer

sequences were CrV3-F 5'-CGCGGCATGCAAAAACATAAGCATTCAG-3' and CrV3-R 5'-GCGCCTGCAGTCACTCCTTTGTGCAGAAG-3', with restriction sites underlined.

2.1.8 PCR amplification of CrV2 and CrV3. Approximately 30 ng of genomic DNA from female *Cotesia rubecula* wasps or 100-350 ng of plasmid DNA was used as template in PCR reactions. A 50 μl reaction was prepared by mixing 5 μl 10 × reaction buffer (Promega), 3 μl $MgCl_2$ (Promega), 1 μl forward primer (0.1 μg/ml), 1 μl reverse primer (0.1 μg/ml), 0.5 μl 15 mM dNTPs (Fisher Biotec), 0.4 μl *Taq* DNA polymerase (Promega) and 39.1 μl template DNA. After 5 min at 94°C, 30 amplification cycles were run including denaturing at 94°C for 1 min, annealing at 56°C for 30 seconds and extension at 72°C for 1 min. Final extension was carried out for 5 min at 72°C. Reaction products were electrophoresed on 1.2% agarose gels at 110 mA and visualised using ethidium bromide. Two different PCR machines were used: the PC-960 microplate thermal sequencer (Corbett Research) or the Eppendorf Mastercycler®.

2.1.9 Expression of CrV2 and CrV3 in bacteria. CrV2 and CrV3 gene-specific primers were designed to amplify the open reading frame of these genes, excluding a putative signal sequence at the amino termini of the proteins (see Figs. 3.5 and 3.7*A*). The primers were used in PCR of cloned, full-length genes to obtain the required fragment for ligation into the pQE30 bacterial expression vector (Qiagen). The desired PCR product was purified (Perfectprep® Gel Cleanup Kit - Eppendorf), precipitated and digested with relevant restriction enzymes (*Sph*I and *Hind*III for CrV2; *Sph*I and *Pst*I for CrV3) as was pQE30, before ligation of the digested DNAs using T4 DNA ligase (Promega). M15 strain of *E. coli* was transformed with the ligation reaction contents using heat-shock (see 2.4.7). Colonies containing desired recombinant vectors were identified by PCR of bacterial cells using vector-specific forward and reverse primers. Production of

recombinant CrV2 and CrV3 (containing 6 additional vector-derived histidine residues) were induced by addition of 1 mM IPTG to bacterial cultures, before incubation for 2 h at 37°C. The resultant fusion proteins were identified by Western blot analyses (2.2.3) and were mainly contained in the denatured insoluble fraction of total bacterial proteins, with only small amounts being present in the soluble fraction.

2.1.10 Purification of recombinant CrV2 and CrV3 proteins. Proteins were purified under denaturing conditions. 50 ml of induced bacterial culture was centrifuged at ≈ 7700 × g for 10 min at 4°C. Cells were then resuspended in a lysis buffer (6 M GuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8 .0) and gently rocked for 1 h. The sample was centrifuged at 12000 × g for 15 min at 4°C before incubation (1 h, RT) of the supernatant with 300 μl of Ni-NTA resin beads (Qiagen), previously equilibrated in 8 M urea (with 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0). Non-bound proteins were removed with buffers containing 8 M urea with pH > 6.3 and bound proteins eluted with buffers containing 8 M urea with pH < 6.0. Samples were diluted with 2 volumes of water before being dialysed overnight in TBS (0.15 M NaCl, 0.01 M Tris, pH 8.0) at 4°C to remove excess urea and renature the protein. Protein was concentrated by vacuum drying.

2.1.11 Production of antibodies against recombinant CrV2 and CrV3. Purified bacterial CrV2 and CrV3 were visualised on SDS-polyacrylamide gels (12% for CrV2; 15% for CrV3) by staining with 0.05% water-dissolved Coomassie Blue (Sigma) for 10-15 min. Recombinant protein bands were excised from the gels with sterile blades and crushed to fine pieces. Rabbits were used to produce antibodies by an initial injection of purified recombinant protein (≈ 5 mg) mixed with Freund's complete adjuvant (Sigma), followed by two booster injections with purified recombinant protein in Freund's incomplete adjuvant (Sigma) at four and six weeks,

respectively, after the initial injection. Antiserum was obtained a week after the final injection and was used to probe Western blot membranes at a dilution of 1:5000. Bound anti-CrV2 or anti-CrV3 were then visualised by alkaline phosphatase-labelled secondary anti-rabbit antibody (1:10000).

2.2 Characterisation of isolated bracovirus-expressed proteins

2.2.1 Computer analyses of CrV2 and CrV3 deduced amino acid sequences. All protein analysis tools were accessed through the ExPASy molecular biology server (http://us.expasy.org/tools). Theoretical isoelectric points and molecular weights were predicted using Compute pI/Mw software (http://us.expasy.org/tools/pi_tool.html). Subcellular localisation of proteins were predicted by PSORTII software (http://psort.nibb.ac.jp/form2.html). Putative signal peptide cleavage sites were identified by SignalP analysis (http://www.cbs.dtu.dk/services/SignalP; Nielson *et al.*, 1997). O-glycosylation sites were estimated using NetOGlyc software (http://www.cbs.dtu.dk/services/NetOglyc). N-glycosylation sites were predicted manually, and corresponded to amino acid triplets whereby asparigine (N) is the first amino acid, serine (S) or threonine (T) is the third and the middle amino acid is variable. Hydrophobicity profiles were produced by ProtScale software (http://us.expasy.org/cgi-bin/protscale.pl; Kyte & Doolittle, 1982). In addition, several analyses of protein primary structure were undertaken. Prediction of coiled-coil regions were undertaken by Coils software (http://www.ch.embnet.org/software/COILS_form.html). Putative classification of coiled-coil regions as dimeric or trimeric, were made by MultiCoil software (http://multicoil.lcs.mit.edu/cgi-bin/multicoil; Wolf *et al.*, 1997). Amino acid alignments were created using MegAlign software (3.1.3; DNASTAR Inc.).

2.2.2 Reverse transcription-PCR (RT-PCR) of CrV2 and CrV3. Gene specific forward and reverse primers (see 2.1.6 and 2.1.7 for design of primers) were used in RT-PCR of RNA isolated from 6 h parasitised *P. rapae* larvae, utilising AMV reverse transcriptase (AMV-RT, Promega). 1.5 μg RNA and 0.1 μg reverse primer, in a final volume of 10.7 μl, were heated at 95°C for 5 min to denature RNA, before being immediately cooled on ice. Reverse transcription was performed by adding 3 ml 5 × RT buffer (Promega), 0.3 μl RNasin (Promega), 0.5 μl AMV-RT and 0.5 μl 15 mM dNTPs (Fisher Biotec) before heating at 42°C for 1 h and then 95°C for 5 min. The total contents were then used in a PCR by adding 3.5 μl 10 × reaction buffer (Promega), 1 μl forward primer (0.1 μg/ml), 1 μl reverse (0.1 μg/ml), 0.5 μl 15 mM dNTPs, 0.4 μl *Taq* DNA polymerase (Promega) and 29 μl H_2O. Cycling, electrophoresis and visualisation protocols were as for standard PCR.

2.2.3 Collection of protein samples and Western blotting. *P. rapae* larvae were bled into PBS saturated with phenylthiourea (PTU - BDH), via removal of a proleg, and the haemolymph centrifuged at 2300 × g for 5 min at RT. Supernatant (cell-free haemolymph) was removed and the cellular pellet resuspended in PBS. Resuspended haemocytes were used directly (without storage) for uptake/encapsulation experiments. Gut tissue and head capsule were discarded before washing and homogenisation of fat body, and then centrifugation (9300 × g for 10 min) and collection of supernatant (fat-body proteins). Protein samples were stored at –20°C and electrophoresed on 15% (for CrV3) or 12% (for CrV2) SDS-polyacrylamide gels as described by Laemmli (1970). Standard conditions were denaturing, however, infrequently non-denaturing conditions were used, whereby SDS was absent from sample and running buffers. Proteins were generally not heated before electrophoresis unless testing the effect of heating. Samples were run in conjunction with SeeBlue™ pre-stained standard protein markers (Novex) to allow subsequent

estimation of sample protein sizes. Proteins were either stained within the gels using Coomassie Blue (Sigma) or alternatively, transferred to a nitrocellulose membrane (Amersham) as described (Sambrook *et al.*, 1989). Prior to obtaining antibodies against the recombinant proteins, blots were probed with 1:10000 dilution of an alkaline phosphatase-conjugated monoclonal anti-polyHISTIDINE antibody (clone His-1 - Sigma). Anti-CrV2 and -CrV3 antisera were used for Western blots and haemocyte staining, at a dilution of 1:5000.

2.2.4 Fluorescent labelling of CrV2 and CrV3 associated with infected *P. rapae* haemocytes. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody was used to visualise CrV2 and CrV3 associated with haemocytes. Haemocytes from parasitised *P. rapae* larvae and non-infected haemocytes treated with CrV2 or CrV3 were both stained as previously described (Asgari *et al.*, 1996). Briefly, larvae were bled into PBS saturated with PTU before centrifugation at 2300 × g for 5 min. The pellet was then resuspended gently in PBS before transfer of haemocytes to multi-well slides. Time was allowed for attachment before addition of recombinant protein. Cells were then fixed with PBS containing 4% paraformaldehyde and 0.05% Tween 20 (Sigma) before labelling of the recombinant protein with the relevant antiserum and FITC-conjugated secondary antibody. Cells were then viewed under UV illumination.

2.2.5 N-Glycosidase digestion of CrV3. Total proteins from cell-free haemolymph of 6 h parasitised *P. rapae* larvae were mixed with SDS-PAGE loading buffer containing ß-mercaptoethanol. Igepal CA-630 nonionic detergent (Sigma) was added to a final concentration of 0.8% before addition of 2 U of recombinant N-Glycosidase F (Roche Diagnostics) and incubation for 18 h at 37°C.

2.2.6 Characterisation of CrV3-mediated hemagglutination . Lectin activity was measured by mixing 25 μl of serially diluted bacterial CrV3 extract with 25 μl of 2% trypsinised and glutaraldehyde-stabilised ovine red blood cells (ORBCs - Sigma) in PBS and containing 2% BSA (Sigma). Samples were mixed well in U-bottom or tilted flat-bottom micro-titer wells before incubation at 37°C for 1 h. Complete agglutination caused ORBCs to form a diffuse layer over the bottom of the wells. Conversely unagglutinated cells formed a dot at the centre of the U-bottom wells or a crescent at the lower margin of tilted flat-bottom wells. Lectin titer was determined as the reciprocal of the maximum sample dilution causing complete ORBC agglutination. To test for inhibitory ligands, 5 μl of sugar solution (various concentrations) in PBS was added in place of 5 μl of PBS used to dilute ORBCs in the standard assay, before incubation. Lipopolysaccaride (*E. coli*, Serotype 055:B5A - Sigma) and laminari tetrose were added as for other sugars, up to a maximum concentration of 1 mg/ml. Comparison of concentrations causing 50% inhibition of lectin activity was made for all sugars tested. To test for dependence of lectin activity on divalent cations, 25 μl serial CrV3 sample dilutions were prepared in 1 mM cation (and 10 mM EDTA) and mixed with 25 μl 2% ORBCs as described above.

2.2.7 Cloning of *P. rapae* 18S ribosomal RNA gene fragment. Forward and reverse primers, as designed by Pollock *et al.* (1998), were used in RT-PCR of RNA from non-parasitised *P. rapae* larvae. RT-PCR conditions were the same as those used for CrV2 and CrV3 fragments (2.2.2) except that the PCR annealing temperature was lowered to 50°C. The resultant 865 bp amplification product was cloned and sequenced using the same method as for the CrV2 RACE fragment (see 2.1.4). The fragment was confirmed as being *P. rapae* 18S ribosomal RNA by comparison with known sequences contained in GenBank™ (as per 2.1.5). Primer sequences

were Pier-Rib-F (forward) 5'-ATGTTTTTGTTAAACAGGTGA-3' and Pier-Rib-R (reverse) 5'-AAACTAGGATTAGATACCCTATTAT-3'. The *Pieris* 18S rRNA gene fragment was used as a loading control in Northern slot-blots.

2.3 Construction and bioassays of recombinant baculoviruses containing immune-suppressive genes

2.3.1 Construction of recombinant baculovirus transfer vector containing CrV1. Primers were designed to amplify complete CrV1 from recombinant pBluescript® KS(+)_CrV1 (Asgari *et al.*, 1996). 5' overhangs, including *Bgl*II restriction site and stabilisation sequence, were added to allow for cloning of the amplified fragment into the intended baculovirus transfer vector, pAcPDIE1 (Novagen). Forward primer (CrV1-F) was 5'-CGCGAGATCTATGTCACTCGTCAAAAGTG-3'; reverse primer (CrV1-R) was 5'-CGCGAGATCTTCAAAAAAAAGTTTGCGATG-3'. The amplified CrV1 and pAcPDIE1 vector were digested with *Bgl*II endonuclease and then treated with calf intestinal phosphatase (Promega; see 2.4.5). Digestion reaction products were electrophoresed on a 1.2% low melting point agarose (Promega) gel and digested CrV1 and pAcPDIE1 were purified from the gel (Perfectprep® Gel Cleanup kit, Eppendorf). Both fragments were then ligated at 4°C overnight using T4 DNA ligase (Promega). Competent JM109 *E. coli* cells (Promega) were transformed with the ligation reaction products (via heat-shock; see 2.4.7). Single recombinant colonies containing desired recombinant transfer vector (pAcDIE1_CrV1) were identified via PCR utilising a vector-specific forward primer (IE1 promoter primer, Novagen) and a CrV1-R. Single recombinant bacterial colonies containing the pAcDIE1_CrV1 vector were cultured and the vector purified from the bacteria (QIAprep® Miniprep Kit - Quiagen).

2.3.2 Construction of recombinant baculovirus transfer vector containing Crp32. Complete Crp32 cDNA cloned in pBluescript® KS(+) (Asgari *et al.*, 1998) was amplified using gene specific primers, containing *Sac*II and *Spe*I restriction sites, allowing for subsequent cloning into the pTV3 baculovirus transfer vector (Novagen). Forward primer (pTV-p32-F) sequence was 5-CGCGCCGCGGATGGATAAGAAGATAATATG-3'; reverse primer (pTV-p32-R) sequence was 5'-CGCGACTAGTTTAGCCTTTTTTTGGCAG-3'. Amplified Crp32 and pTV3 vector were digested with *Sac*II and *Spe*I endonucleases before reaction products were electrophoresed and purified as for CrV1 (above). Ligation, bacterial transformation, screening (utilising IE1 promoter and pTV3-p32-R primers) and recombinant vector purification were conducted as for CrV1 (above).

2.3.3 Production of recombinant baculoviruses via transfection of insect cell cultures. *Spodoptera frugiperda* cell lines (Sf9 or Sf21) were co-transfected with recombinant baculovirus transfer vector (pAcPDIE1_CrV1 or pTV3_p32) and linearised *Autographa californica* baculovirus DNA (BaculoGold™ - PharMingen) as per Summers & Smith (1987). Recombinant baculoviruses were produced via homologous recombination of the two transfected elements. Recombinant baculoviruses were then isolated by plaque assays and propagated as described (Summers and Smith, 1987).

2.3.4 Isolation of polyhedra and budded virus from infected cell cultures. Sf21/Sf9 cells were separated from the culture medium by centrifugation in a desktop centrifuge at 12500 × g for five min. Supernatant was then removed and stored at 4°C for later use as inoculum or to obtain budded virions. To obtain polyhedra, the cell pellet was resuspended in 50 ml 0.5% SDS and incubated for 15 min at RT. The solution was again centrifuged as above. The pellet was twice

washed with distilled water, to remove SDS, and re-centrifuged (as above). The pellet was then finally resuspended in 1-2 ml distilled water. To isolate budded virus, supernatant from infected cell culture was centrifuged 53370 × g (Beckmann TL-100 centrifuge) to pellet budded virus particles, which could then be resuspended in PBS and stored at –20°C for later use.

2.3.5 Confirmation of production of desired transcript and protein by recombinant baculoviruses. Recombinant plaque amplified AcMNPVs were used to inoculate insect cell lines derived from *S. frugiperda* (Sf21 for CrV1-containing AcMNPV, Sf9 for Crp32-containing AcMNPV). Two days after inoculation, infected cells, budded virus and supernatant were collected for subsequent extraction of RNA or proteins. Production of recombinant transcript was confirmed via RT-PCR of extracted RNA, utilising primers to the relevant open reading frames (see 2.3.1 for CrV1 primers, 2.3.2 for Crp32 primers). Western blot analyses of extracted proteins were utilised to confirm recombinant protein production. Antiserum against CrV1 (Asgari *et al.*, 1996) and CrBV virions (Asgari *et al.*, 1994), both at a ratio of 1:5000, were utilised. Electrophoresis (12% SDS-PAGE) and Western blotting were carried out as described earlier (2.2.3).

2.3.6 Bioassay of recombinant baculovirus. 180 3rd instar *P. rapae* larvae were collected, assigned randomly into three treatment groups of 60, and individually housed. All larvae were starved for 3 h before being given seven mm leaf discs dipped into a 0.001% Tween 20 (Sigma) solution containing either 4×10^4 wild-type polyhedra/μl, 4×10^4 recombinant polyhedra/μl or no polyhedra. Larvae were left overnight to consume the inoculum discs. 48 larvae, that had entirely consumed their inoculum, were then chosen from each treatment before being placed together (in treatment groups) onto one of three cabbage plants. Larvae were then left until pupation with new

food being added as required. The number of larval deaths and the number reaching pupation was scored each day until all larvae were dead or pupated.

2.4 Generally applied techniques

2.4.1 Insect cultures. *Cotesia rubecula* (Hymenoptera: Braconidae) endoparasitoid wasps were reared on cabbage-fed *Pieris rapae* (Lepidoptera: Pieridae) as described previously (Asgari & Schmidt, 1994).

2.4.2 Polydnavirus and genomic DNA isolation. Calyx fluid from 50 female wasps was collected in PBS (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 7.3 mM Na_2HPO_4, pH 7.6) by cutting the oviducts. The suspension was passed through a 0.45 μM syringe filter (Minisart®) and centrifuged at 15800 × g in a desktop centrifuge for 15 min. Pelleted virus particles were resuspended in 180 μl PBS and DNA was isolated from this suspension as described previously (Stoltz *et al.*, 1986). DNA was isolated from ovaries, female and male wasps (or *P. rapae* larvae) by homogenising them in a buffer made up of 10 mM Tris, 10 mM EDTA, 1% SDS, pH 8.0. Proteinase K was added to 250 μg/ml final concentration and samples incubated at 40°C overnight. Samples were treated with RNase A (10 μg/ml final concentration) at 37°C for 30 min before phenol/chloroform extraction. DNA was precipitated by adding two volumes of ethanol, 0.2 volume of 3 M CH_3COONa, pH 5.3, and centrifugation at 15800 × g for 20 min. Pellet was washed with 70% ethanol, dried at 37°C and resuspended in water before storage at 4°C.

2.4.3 Detemination of nucleic acid concentrations. The concentration of purified nucleic acid was calculated using absorption spectrophotometry (Varian DMS 100S UV visible spectrophotometer) whereby purified nucleic acid was diluted and the optical absorbance was

measured at 260 nm (ABS_{260}). If absorbance was measured at > 1.0, then nucleic acid was diluted further and ABS_{260} remeasured. Concentration was calculated using the following formula, where the nucleic acid constant is 50 for double stranded DNA and 40 for RNA:

$$[\text{nucleic acid}]\ (\mu g/\mu l) = \frac{ABS_{260} \times \text{dilution factor} \times \text{nucleic acid constant}}{1000}$$

2.4.4 Removal of hybridised probe from Southern or Northern membrane to allow reprobing. Used membrane was washed for 30 min in 0.4 M NaOH at 45°C. The membrane was then washed three times for 10 min in probe-removal solution (0.1 × SSC, 0.1% SDS, 0.2 M Tris pH 8.0) at 45°C. Washed membranes were then wrapped in plastic and stored at 4°C for later reuse.

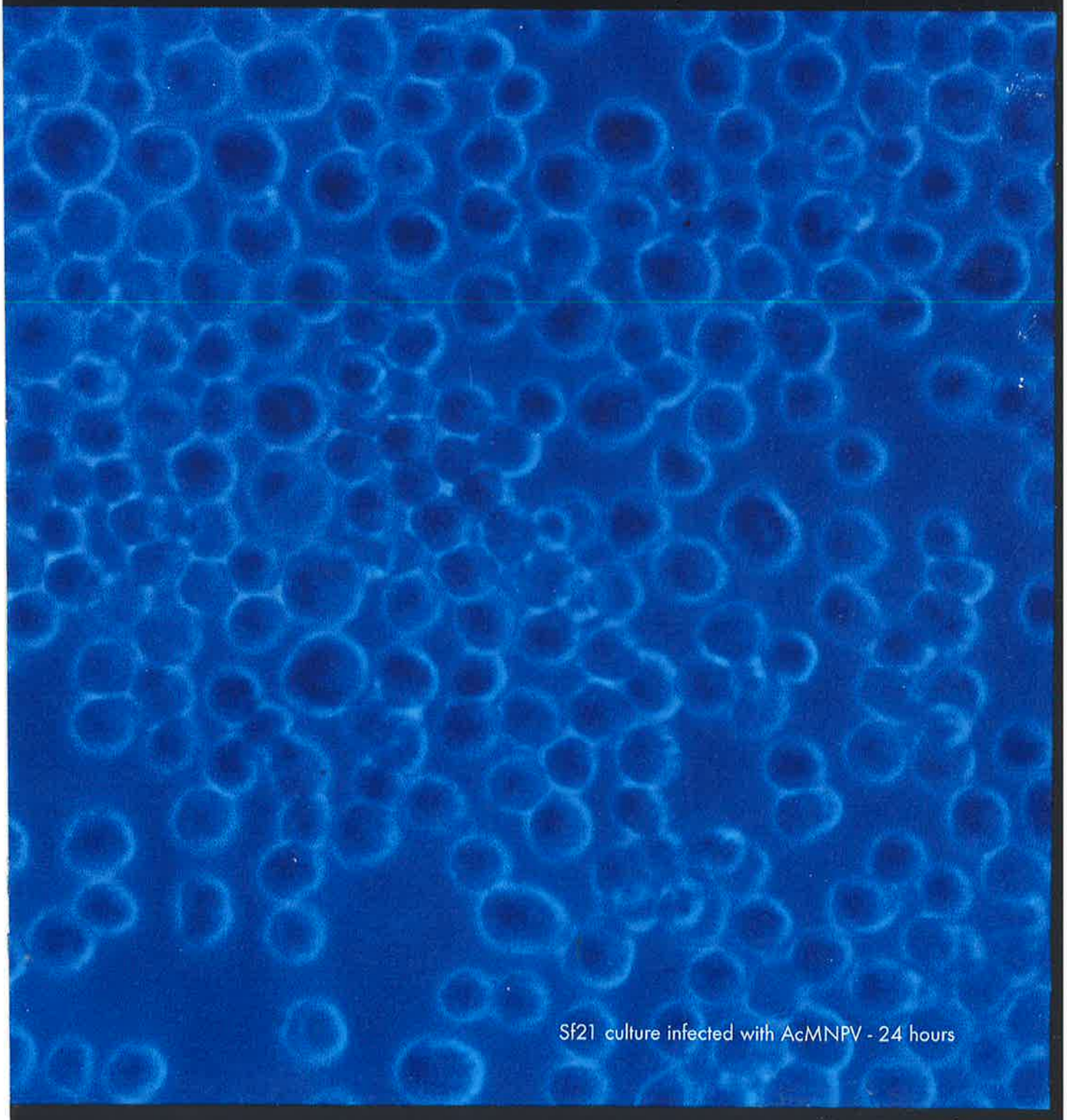
2.4.5 Calf Intestinal Phosphatase (CIP) reaction. 1 μl calf intestinal phosphatase (Promega) was added to 9 μl phosphatase buffer (Promega). 1 μl of diluted phosphatase was further diluted in 10 μl phosphatase buffer and the total volume increased to 100 μl via addition of the target DNA mixed in distilled water. The mixture was incubated at 37°C for 30 min, before addition of a further 1 μl of the original 10 × dilution of phosphatase and further 30 min incubation. 2 μl 0.5 M EDTA (pH 8.0) was added and the mixture incubated at 65°C for 15 min to stop the reaction. DNA was then extracted using phenol/chloroform.

2.4.6 Preparation of competent bacterial cells for heat-shock transformation. 5 ml of sterile LB medium (10 g/l Bacto™ tryptone - BDH, 5 g/l yeast extract - Oxoid, 5 g/l NaCl) was inoculated with bacterial cells and incubated overnight (with antibiotic) at 37°C. 100 ml of sterile LB medium was inoculated with the 5 ml overnight culture and cells were allowed to proliferate

to a density whereby ABS_{600} was ≈ 0.5 (Varian DMS 100S UV visible spectrophotometer). The cells were then cooled on ice for 30 min before being pelleted by centrifugation at 3000 × g for 5 min at 4°C. Cells were resuspended in 30 ml of 4°C TFBI (2.94 g/l 30 mM CH_3COOK, 9.9 g/l 50 mM $MnCl_2$, 12.09 g/l 100 mM RbCl, 1.47 g/l 10 mM $CaCl_2$, 150 ml/l 15% glycerol – adjusted to pH 5.8 by addition of 0.2 M CH_3COOH and autoclaved) before being repelleted as before. Cells were resuspended in 4 ml TFBII (2.09 g/l 10 mM MOPS pH 7.0 - Sigma, 11.03 g/l 75 mM $CaCl_2$, 1.21 g/l 10 mM RbCl, 150 ml/l 15% glycerol – autoclaved), separated into 100 μl aliquots, frozen in liquid nitrogen and then stored at –80°C.

2.4.7 Heat-shock transformation of competent bacterial cells. 100 μl aliquot of competent bacterial cells were removed from –80°C storage and placed on ice until just thawed. DNA (either purified plasmid or ligation reaction contents) was added to cells and gently mixed by flicking the tube before the mixture was placed on ice for 20 min. Cells were heat-shocked by placing them in a 42°C water-bath for 90 seconds, before they were placed immediately on ice for 2-5 min. 1 ml of sterile LB medium (see 2.4.6) was added to cells before incubation for 90 min at 37°C. Cells were then plated onto relevant medium (containing antibiotics) and incubated at 37°C overnight.

Chapter 3: Results



Sf21 culture infected with AcMNPV - 24 hours

Chapter 3: Results

3.1 Isolation and characterisation of bracovirus genes and production of antibodies against gene products

Polydnaviruses from *C. rubecula* parasitoid wasps are introduced into the haemocoel of the host, *P. rapae*, at parasitisation. Viral genes are transiently expressed following infection of host tissues (Asgari *et al.*, 1996). When total CrBV genomic DNA was used as a probe in a Northern blot analysis containing total RNA isolated from parasitised *P. rapae* caterpillars, four different sized viral transcripts were detected and referred to as CrV1-CrV4 by decreasing transcript size (Asgari *et al.*, 1996; see Fig. 1-12). CrV1 was previously isolated and identified by screening a cDNA library made from 6 h parasitised caterpillars using total CrBV DNA as a probe (Asgari *et al.*, 1996). The same method was employed here to isolate two cDNA clones, of ≈ 450 bp and ≈ 700 bp in length, encoding putative CrBV genes.

To confirm the cDNAs as being particle-derived, the fragments were cloned and used as probes in Southern blots of digested CrBV DNA (Fig. 3-1) and Northern blots of RNA from non-parasitised larvae and 6 h parasitised larvae (Fig. 3-2). The ≈ 450 bp and ≈ 700 bp cDNAs hybridised to CrBV restriction fragments of ≈ 15 kbp and ≈ 4 kbp, respectively, and to parasitism-specific transcripts of ≈ 1.2 kbp and ≈ 1.1 kbp, respectively. Size comparison of the hybridised transcripts indicated that the cDNAs originate from CrBV genes, CrV2 and CrV3, identified previously (Asgari *et al.*, 1996; see Fig. 1-12). These data and the fact that the same probes bound to genomic DNA from *C. rubecula* adults but not to genomic DNA from *P. rapae* larvae (Fig. 3-3), indicated that the cDNA originated from particles introduced to the larvae at

oviposition. Binding of the cDNAs to only one RNA band in each Northern blot revealed that the two clones are not significantly homologus with each other or with other CrBV-related genes.

Both cDNA clones were then sequenced and found to contain poly-A tails (Figs. 3-5 and 3-7*A*). Using these sequence data, primers were designed to amplify ≈ 300 bp of each cDNA (see Figs. 3-5 and 3-7*A* for primer sites) and these primers were used in RT-PCR of RNA from 6 h parasitised and non-parasitised *P. rapae* larvae. The presence of parasitism-specific amplification products, for each primers set, re-confirmed the cDNAs as being derived from CrBV (Fig. 3-4).

5' RACE was then employed to extend the ≈ 450 bp (CrV2-related) cDNA at the 5' end and complete the open reading frame. This procedure produced a fragment of ≈ 850 bp (data not shown), which was subsequently sequenced. Sequence data from the 5' RACE fragment was combined with those from the ≈ 450 bp cDNA clone, to give the total CrV2 open reading frame of 960 bp and flanking sequences (Fig. 3-5). A methionine codon (ATG) at the beginning of the open reading frame was identified as the only possible codon with a nucleotide sequence environment predicted for functional initiation codons (Cavener & Ray, 1991).

Computer analyses of the deduced amino acid sequence revealed a putative signal peptide encompassing the first 20 amino acids of the protein, with a cleavage point predicted at the end of the signal peptide (Fig. 3-5), indicating that CrV2 protein is probably secreted from CrBV-infected cells. Four putative N- and six putative O-glycosylation sites were predicted in the open reading frame, as well as a polyadenylation signal ≈ 120 bp downstream of the stop codon (Fig. 3-5). These data were used to generate specific primers to the CrV2 open reading frame (CrV2-F

and CrV2-R; Fig. 3-5), excluding the signal peptide. Comparison of RT-PCR and genomic DNA PCR products, utilising these primers, revealed no sequence differences indicating that genomic CrV2 DNA does not contain an intron. A hydrophobicity profile is shown in figure 3.6*A*. The presence of highly hydrophobic residues at the N-terminus of CrV2 is consistent with predictions of a secretion signal, targeted to the cell membrane. Coils software predicted the presence of a coiled-coil region, near the C-terminus of CrV2, comprised of amino acids 266-313 (Fig. 3.6*B*).

In contrast to CrV2, the complete CrV3 open reading frame was present in the ≈ 700 bp cDNA isolated during library screening (Fig. 3-7*A*). Computer analyses revealed that CrV3 also contained a putative signal peptide (first 14 amino acids) and corresponding cleavage point, allowing for secretion of the CrV3 protein (Fig. 3-7). As for CrV2, the CrV3 hydrophobicity profile (Fig. 3-8) supported this prediction in that highly hydrophobic residues were present at the N-terminus. Three putative N-glycosylation sites were predicted in the open reading frame and a polyadenylation signal located ≈ 150 bp downstream of the stop codon was identified (Fig. 3-7*A*). Primers were subsequently designed to amplify the CrV3 open reading frame, excluding the putative signal peptide (CrV3-F and CrV3-R; Fig. 3-7*A*). Unlike CrV2, analysis of genomic CrV3 sequence data revealed the presence of a 186 bp intron (Fig. 3-7). A schematic representation of the CrV3 gene is shown in figure 3-7*B*.

The open reading frames (excluding signal peptides) for both CrV2 and CrV3 were cloned into the pQE30 expression vector and used to transform *E. coli* cells, in which the expression of the corresponding proteins were subsequently induced. Molecular weights of 33.7 kDa for secreted CrV2 and 17.6 kDa for secreted CrV3, were predicted by PSORT II analysis. Analysis of Coomassie Blue-stained polyacrylamide gels, containing proteins from induced and non-induced recombinant bacterial cells, showed the presence of proteins that were highly up-regulated in

induced cells (Fig. 3-9). In CrV2-containing bacteria, the up-regulated protein was ≈ 37 kDa in size (Fig. 3-9*A*). The larger size of the expressed CrV2, compared to the predicted size (33.7 kDa), could be due to additional His residues fused to the expressed protein or altered mobility within the gel. In CrV3-containing bacteria the protein was ≈ 16 kDa in size (Fig. 3-9*B*), which was just below the predicted molecular weight of 17.6 kDa.

Both recombinant proteins were detected mainly in the insoluble (made soluble by denaturation) portion of total bacterial proteins (Fig. 3-9). PSORT II analyses predicted pI values for secreted CrV2 and CrV3, of 8.94 and 9.13, respectively. Ni-NTA resin beads were used to purify the recombinant proteins from the denatured soluble fraction. The identity of purified recombinant proteins was confirmed by Western blot analysis utilising anti-polyHISTIDINE as a probe (Fig. 3-10).

The purified proteins were then separated by SDS-PAGE and homogenised bands injected into rabbits, allowing for production of putative anti-CrV2 and anti-CrV3 antibodies. Serum from treated rabbits was used to probe cell-free haemolymph from non- and 6 h parasitised *P. rapae* larvae. In each case, the serum hybridised to the relevant induced recombinant protein (used for injection of rabbits) that was not recognised by rabbit pre-serum (Fig. 3-11), confirming successful production of CrV2- and CrV3-specific antibodies. Thus, specific primers and antibodies were successfully developed for each gene, providing diagnostic tools for further characterisation of CrV2 and CrV3.

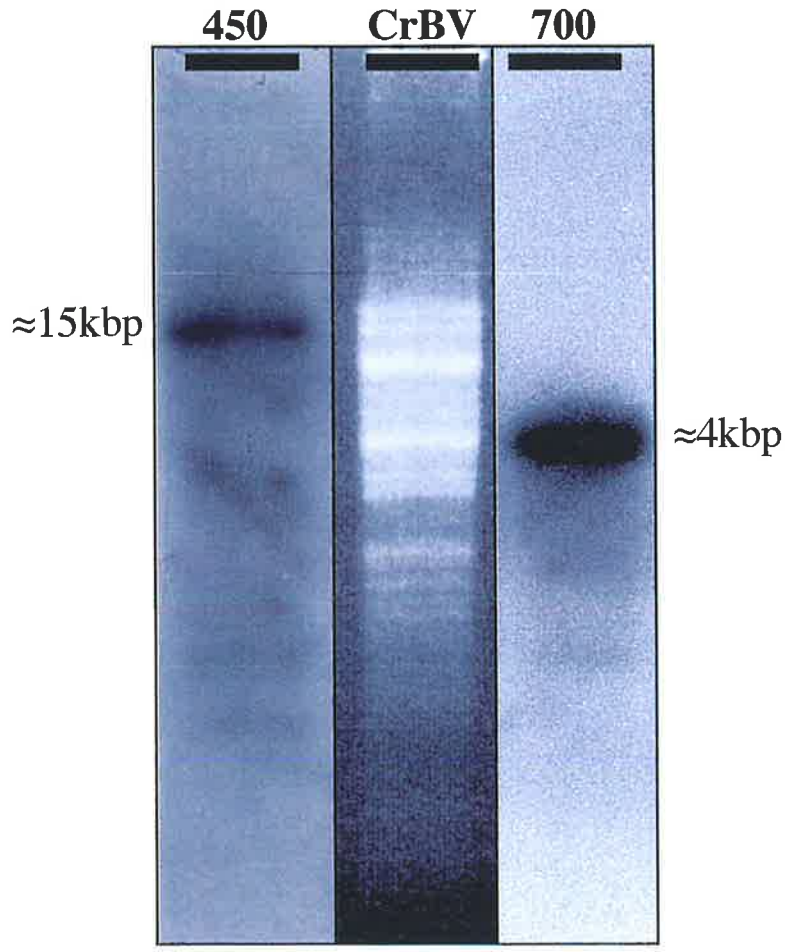


Figure 3-1. Southern hybridisation of ≈ 450 bp cDNA clone (*450*) and ≈ 700 bp cDNA clone (*700*) to CrBV DNA digested with *Eco*RI (*CrBV*). cDNA clones hybridised to restriction fragments of ≈ 15 kbp and ≈ 4 kbp, respectively. cDNA clones were obtained by screening a cDNA library constructed from 6 hour parasitised *P. rapae* larvae, probed with digested total CrBV DNA (as per Asgari *et al.*, 1996).

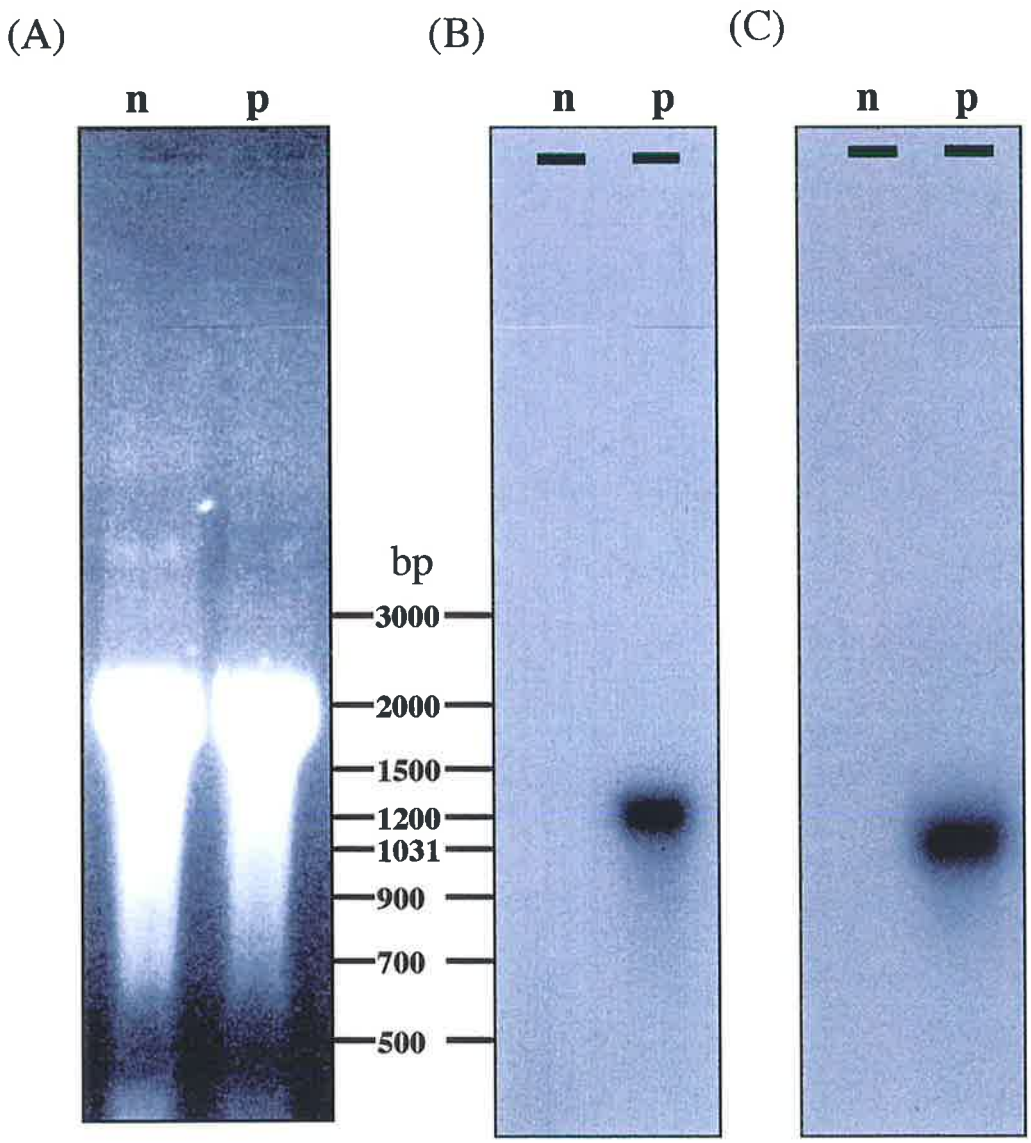


Figure 3-2. Northern blot analyses of RNA from *P. rapae* larvae. *A*, 20 μg RNA from non-parasitised (*n*) and 6 h parasitised (*p*) *P. rapae* larvae, electrophoresed on a 1% agarose gel containing ethidium bromide. *B*, corresponding autoradiograph when RNA was transferred to a membrane and probed with ^{32}P-labelled ≈ 450 bp cDNA clone. *C*, corresponding autoradiograph when RNA was transferred to a membrane and probed with ^{32}P-labelled ≈ 700 bp cDNA clone.

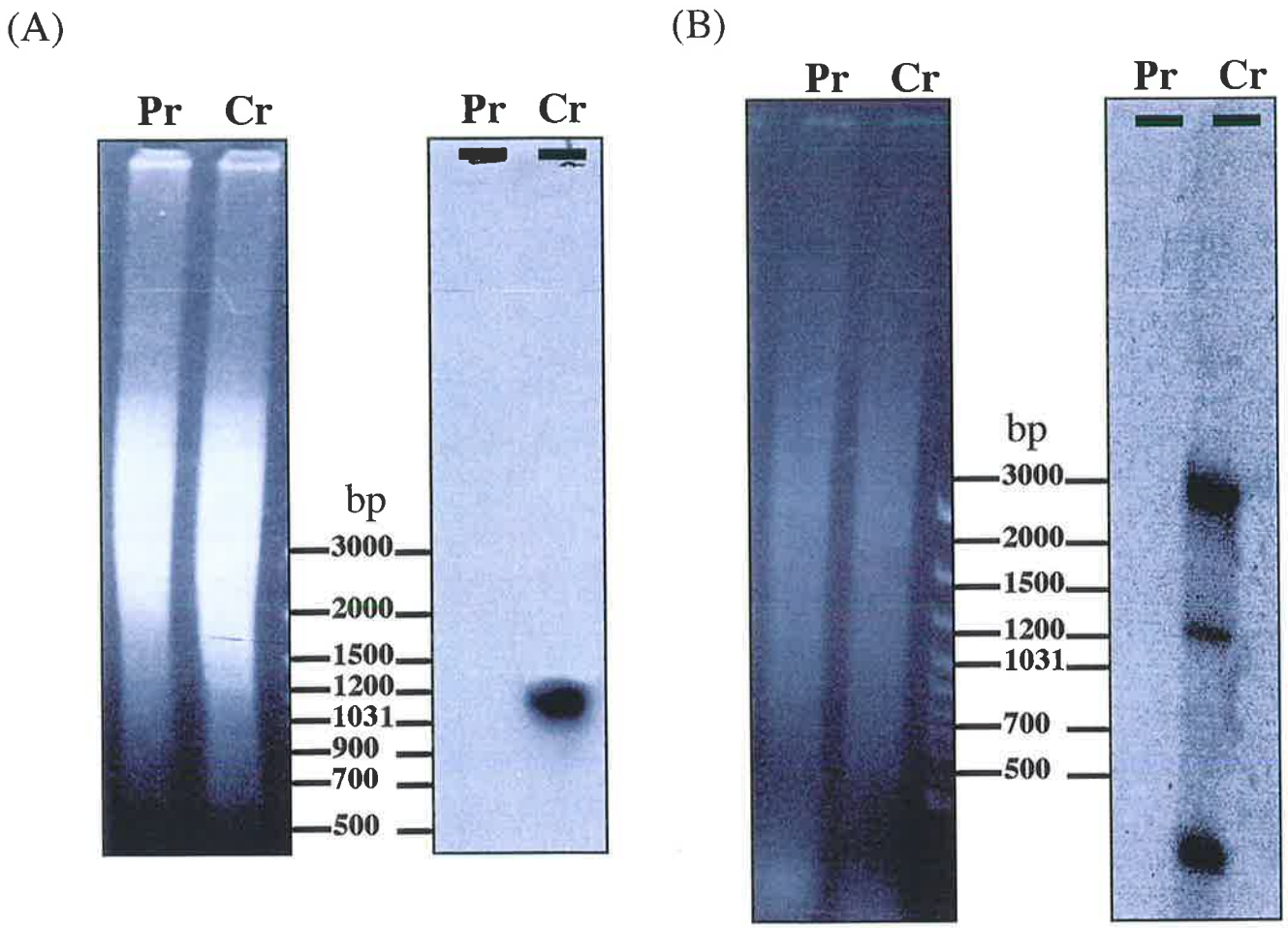


Figure 3-3. Southern blot analyses of genomic DNA from *Pieris rapae* and *Cotesia rubecula*. *A left and B left*, 1% agarose gels containing ethidium bromide were used for electrophoresis of genomic DNA from non-parasitised *P. rapae* larvae (*Pr*) and adult *C. rubecula* wasps (*Cr*), digested with *Bam*HI and *Hind*III. Corresponding autoradiographs (*A right and B right*) show hybridisation of ≈ 450 bp cDNA clone (*A right*) and ≈ 700 bp cDNA clone (*B right*) to *C. rubecula* DNA only.

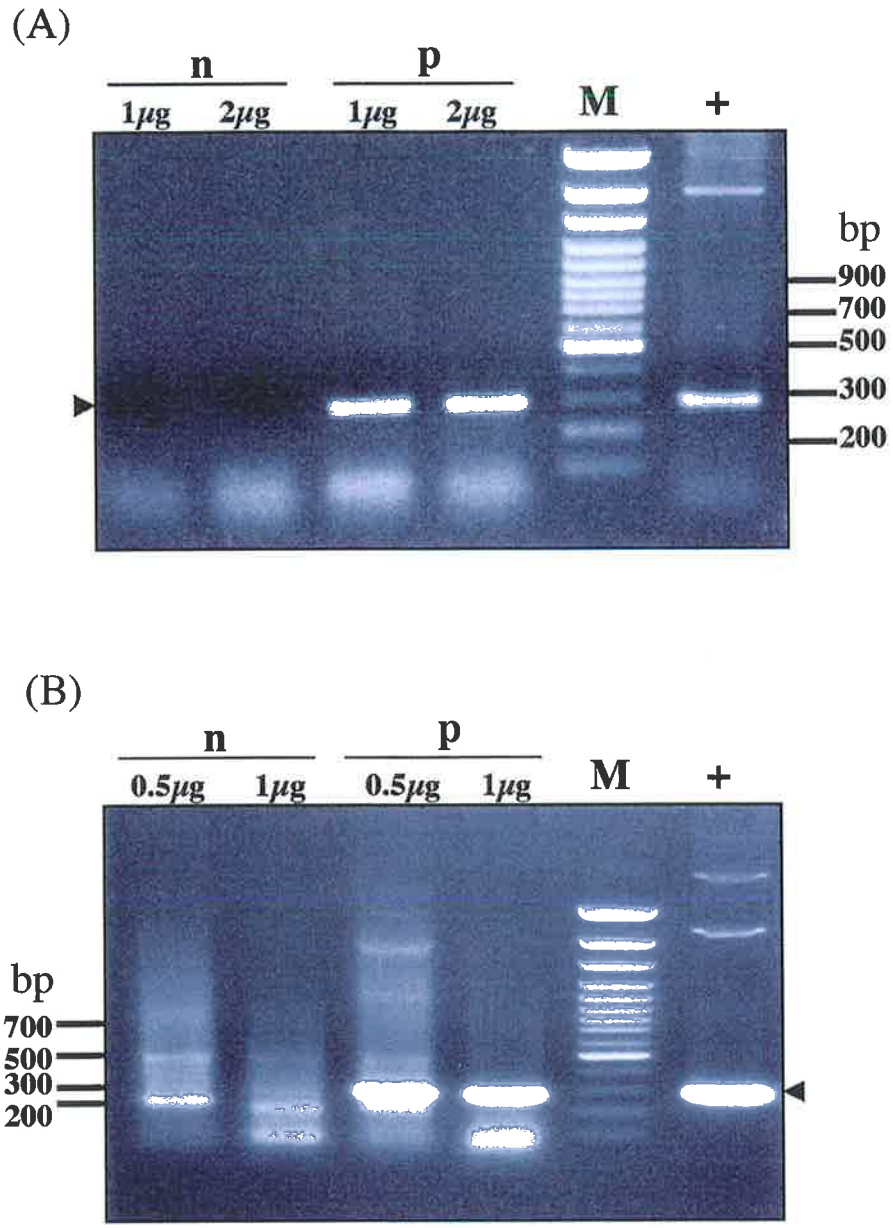


Figure 3-4. Electrophoresis of RT-PCR amplification products, using template RNA from non-parasitised (*n*) and 6 h parasitised (*p*) *P. rapae* larvae. *A*, RT-PCR utilising specific primers to CrV2 (CloneC-F and CloneC-R). *B*, RT-PCR utilising specific primers to CrV3 (CrV3-F and CrV3-R). Relative amounts of template RNA is shown (*μg*) and *arrowheads* indicate correctly amplified fragments that were obtained only from parasitised larvae (*p*) and recombinant plasmid containing the relevant gene (+). DNA size markers are shown (*M*).

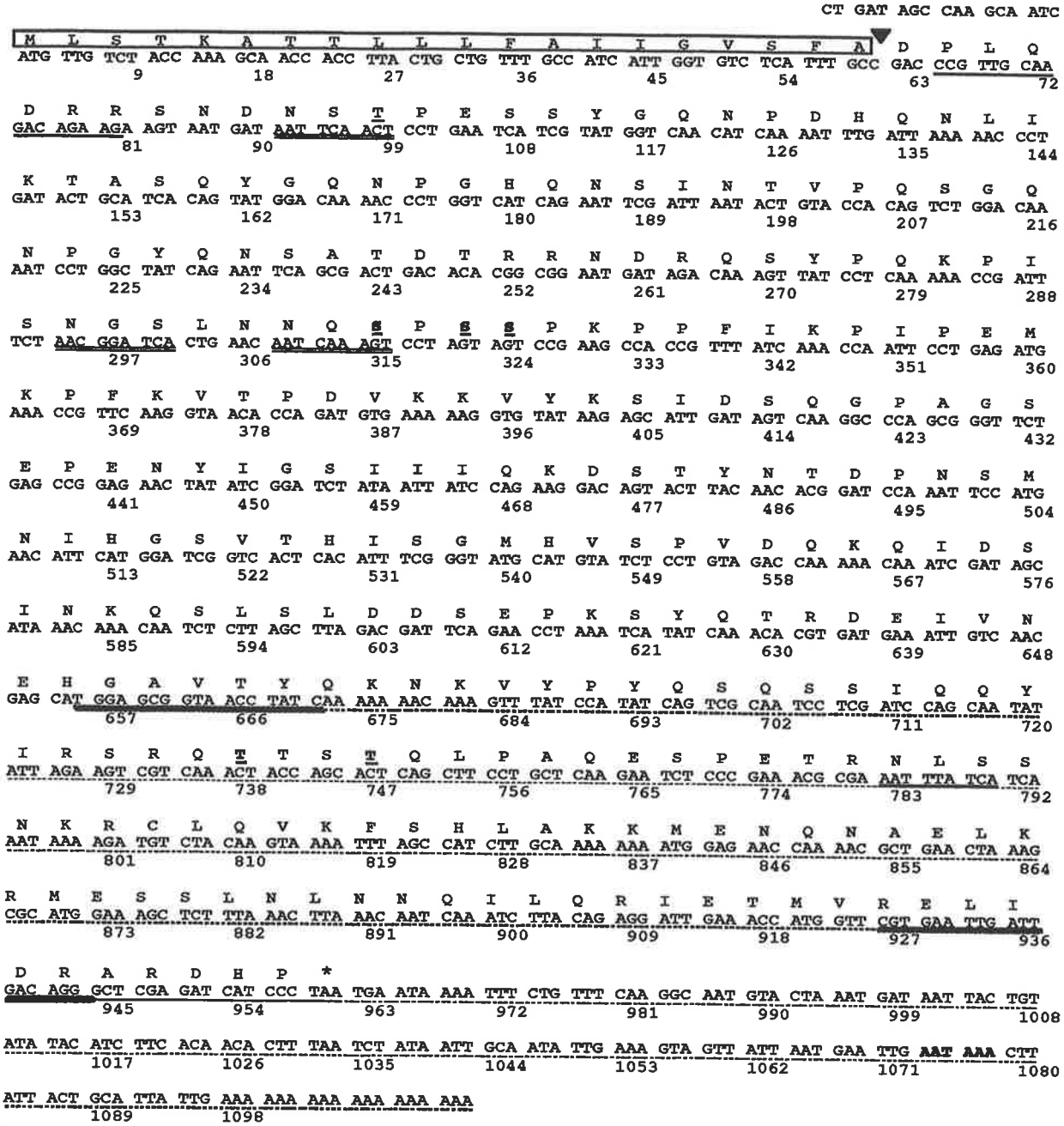
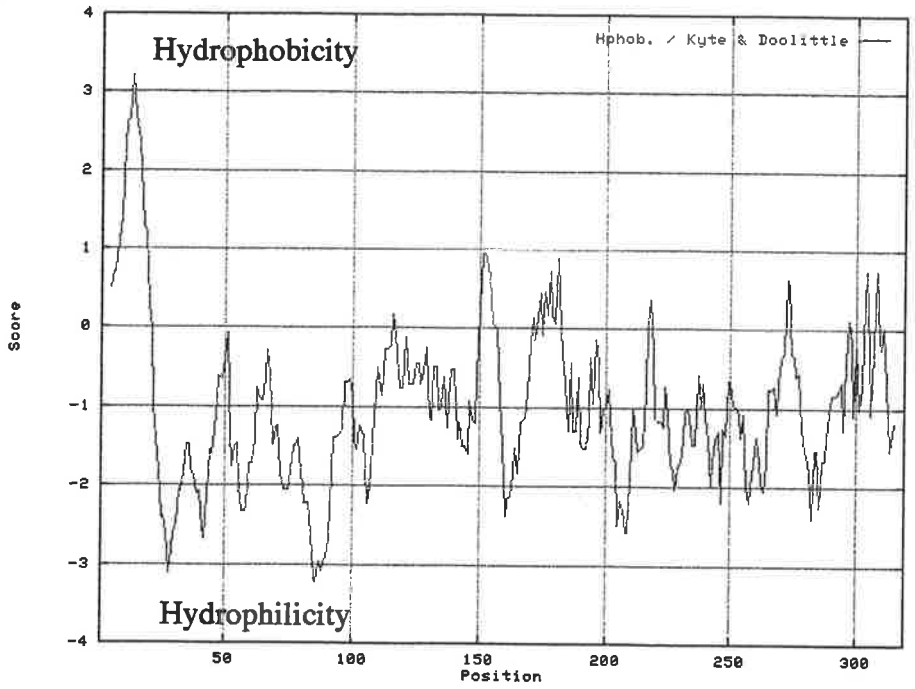


Figure 3-5. DNA nucleotide sequence and deduced amino acid sequence for CrV2 from the *Cotesia rubecula* bracovirus. The putative signal peptide is *boxed* and contains the methionine start codon as the first amino acid in the signal. The predicted signal peptide cleavage point is denoted with an *arrowhead*. Single amino acid (serine (S) and threonine (T)) residues identified as putative O-glycosylation sites are in ***bold and underlined*** whilst nucleotides representing putative N-glycosylation sites are *double underlined*. The stop codon is indicated by an *asterisk* and the polyadenylation signal is in *bold*. CrV2-F and CrV2-R primer binding sites are *underlined* and are located at the 5' and 3' ends of the gene respectively. The original ≈ 450 bp cDNA fragment (obtained by screening cDNA library) is *dotted underlined* and the CloneC-F and CloneC-R primers used to amplify 290 bp of the cDNA fragment are *wavy underlined*. Note that CloneC-R and CrV2-R share one base pair (primer nucleotide C), binding at position 943.

(A)



(B)

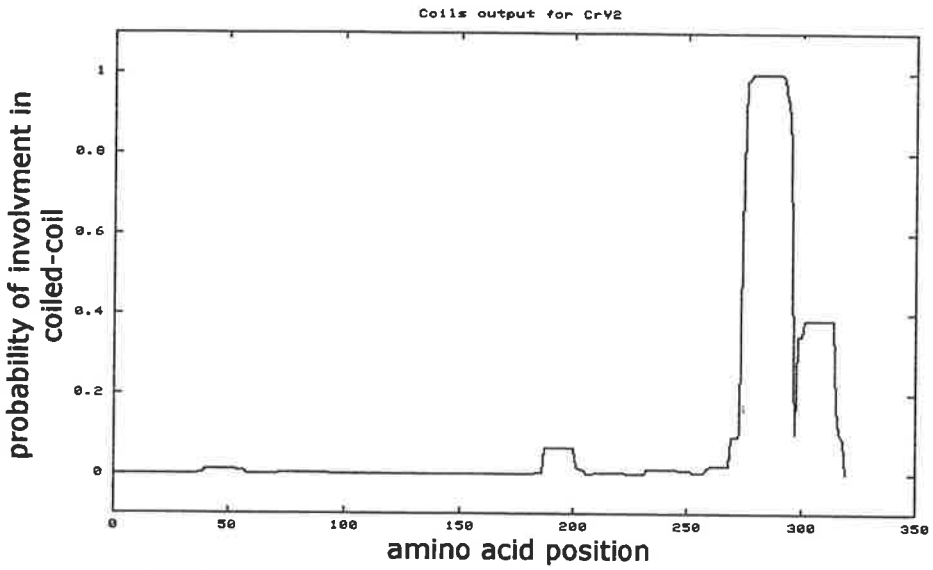


Figure 3-6. *A*, hydrophobicity profile of CrV2. Distribution of hydrophobic and hydrophilic domains were determined using ProtScale software. Amino acid positions are represented on the X-axis and a score of relative amino acid hydrophobicity is represented on the Y-axis. *Positive scores* indicate hydrophobic residues and *negative scores* indicate hydrophilic residues. A highly hydrophobic domain predicted at the N-terminus of CrV2 is consistent with a putative signal peptide at the same region. *B*, CrV2 amino acid sequence was analysed by Coils software. A putative coiled-coil region is predicted near the C-terminus of the protein (amino acids 266-313).

(A)

```
C AAT TGA TAG CCG TTA GCC GTA CGA GCT TGA CAG TTA TTC CGA AAA AAA AAC TAT TAT TCA AGA AAA

 M   N   K   L   I   C   L   M   V   L   P   A   V   L   S   K   N   I   S   I   Q   R   K   G
ATG AAT AAA TTA ATT TGT TTA ATG GTT TTA CCC GCG GTG CTG AGT AAA AAC ATA AGC ATT CAG AGG AAA GGT
          9          18          27          36          45          54          63          72

 R   L   T   I   G   S   S   E   S   Y   T   F   H   S   T   P   A   T   F   N   E   A   I   S
CGA CTA ACA ATC GGA TCC AGC GAA TCG TAC ACA TTC CAT TCA ACT CCA GCA ACC TTC AAT GAA GCC ATA AGT
         81          90          99         108         117         126         135         144

 I   C   K   Q   E   G   G   S   L   A   V   V   T   S   Q   K   A   E   D
ATT TGC AAA CAA GAA GGT GGA AGT CTT GCT GTC GTG ACC TCC CAG AAA GCA GAA GAT gtg agt att cat tat
        153         162         171         180         189         198         207         216

ttt tag tgt att aaa ttc agg agt gga tga ggc aaa tca aga ttc aaa tgt aaa att tcg cta agc cct gtt
        225         234         243         252         261         270         279         288

agt aaa cta aac tgt tat tct tca tta tca aaa aaa gga cat ttt atc tgc tag tga ccc ata aat gct aat
        297         306         315         324         333         342         351         360

                                     E   M   L   K   I   W   K   H   S   S   P   I   L   N   S
tgt act aaa ata atc gtt tct ctc tag GAA ATG CTG AAG ATT TGG AAA CAT TCA AGC CCT ATT CTA AAC TCA
        369         378         387         396         405         414         423         432

 T   N   G   L   T   S   Q   A   F   I   G   I   H   S   L   N   K   K   G   H   W   E   T   I
ACA AAT GGA TTG ACT TCA CAA GCT TTC ATC GGG ATC CAT AGC TTA AAT AAA AAA GGT CAC TGG GAA ACA ATC
        441         450         459         468         477         486         495         504

 D   G   E   S   P   K   Y   I   N   W   S   Q   H   W   S   G   G   R   K   P   S   T   S   S
GAT GGA GAA TCT CCA AAA TAC ATC AAT TGG AGT CAA CAC TGG TCA GGC GGA CGA AAA CCG AGC ACC TCT AGC
        513         522         531         540         549         558         567         576

 V   Q   K   C   G   S   L   L   K   H   G   G   L   D   N   V   E   C   Y   F   K   L   A   F
GTT CAA AAG TGT GGT AGT TTA TTA AAG CAC GGA GGA TTG GAT AAT GTA GAA TGT TAC TTC AAG CTC GCT TTC
        585         594         603         612         621         630         639         648

 F   C   T   K   E   *
TTC TGC ACA AAG GAG TGA CAT TGA AAA ACG TAT CGA TAA TTA TAG AGA TTA CTA CCT AAA AAT AAG TAA TGT
        657         666         675         684         693         702         711         720

TAC TTT TAG CTT CAA GTA TTA TTC CTA ATT ATG CTA TAA TAT TAT AAT ATA TAG ACT AAG TAA TAA ATT TCT
        729         738         747         756         765         774         783         792

TTA TTC AAA AAA AAA AAA AAA AAA AAA AA
        801         810         819
```

(B)

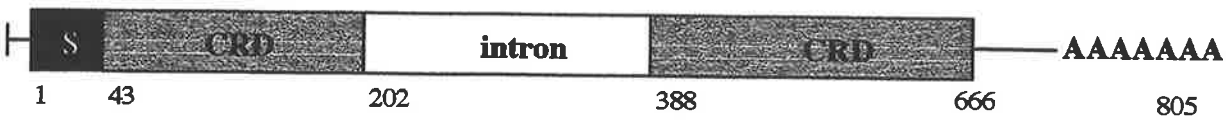


Figure 3-7. *A*, DNA nucleotide sequence and deduced amino acid sequence for CrV3 from the *Cotesia rubecula* bracovirus (GenBank™ Accession AY234855). The putative signal peptide is *boxed* and contains the predicted methionine start codon as the first amino acid. The predicted signal peptide cleavage point is denoted with an *arrowhead*. Amino acids representing putative N-glycosylation sites are *underlined*. The stop codon is indicated by an *asterisk* and the polyadenylation signal is in *bold*. CrV3-F and CrV3-R primer binding sites are *double underlined* and are located at the 5' and 3' ends of the open reading frame, respectively. Intron nucleotides are in *lower case letters*. *B*, schematic representation of genomic CrV3 showing relative position of signal (*S*), carbohydrate recognition domain (*CRD*) and *intron* sequences. *Numerals beneath* the gene indicate nucleotide position, where *1* corresponds to the first nucleotide of the start codon.

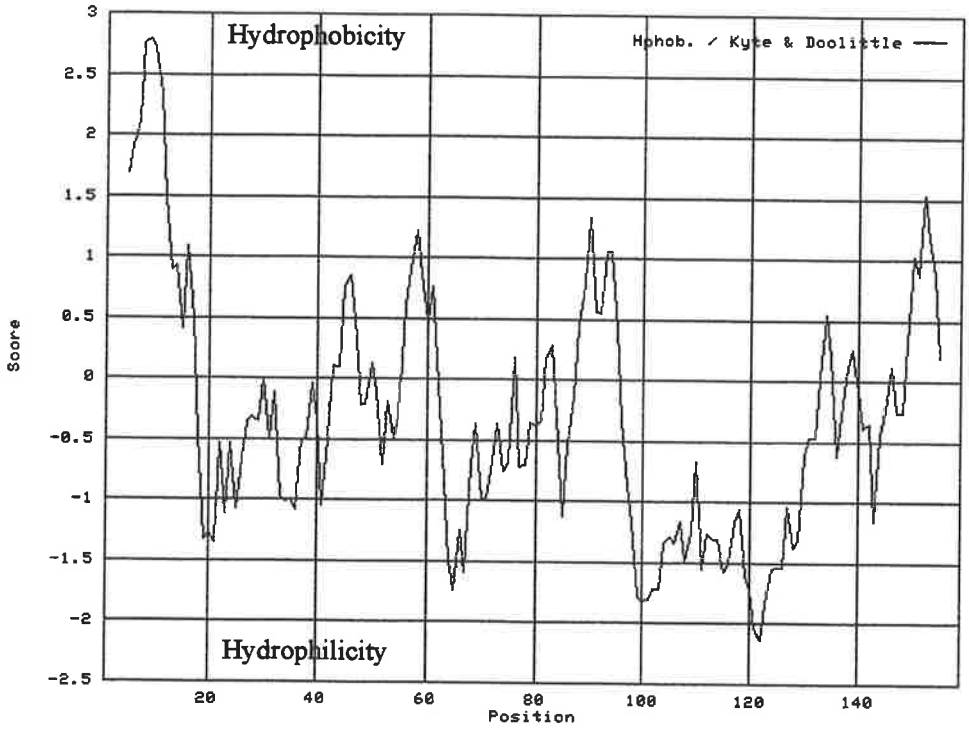


Figure 3-8. Hydrophobicity profile of CrV3. Distribution of hydrophobic and hydrophilic domains were determined using ProtScale software. Amino acid positions are represented on the X-axis and a score of relative amino acid hydrophobicity is represented on the Y-axis. *Positive scores* indicate hydrophobic residues and *negative scores* indicate hydrophilic residues. A highly hydrophobic domain predicted at the N-terminus of CrV3 is consistent with a putative signal peptide at the same region.

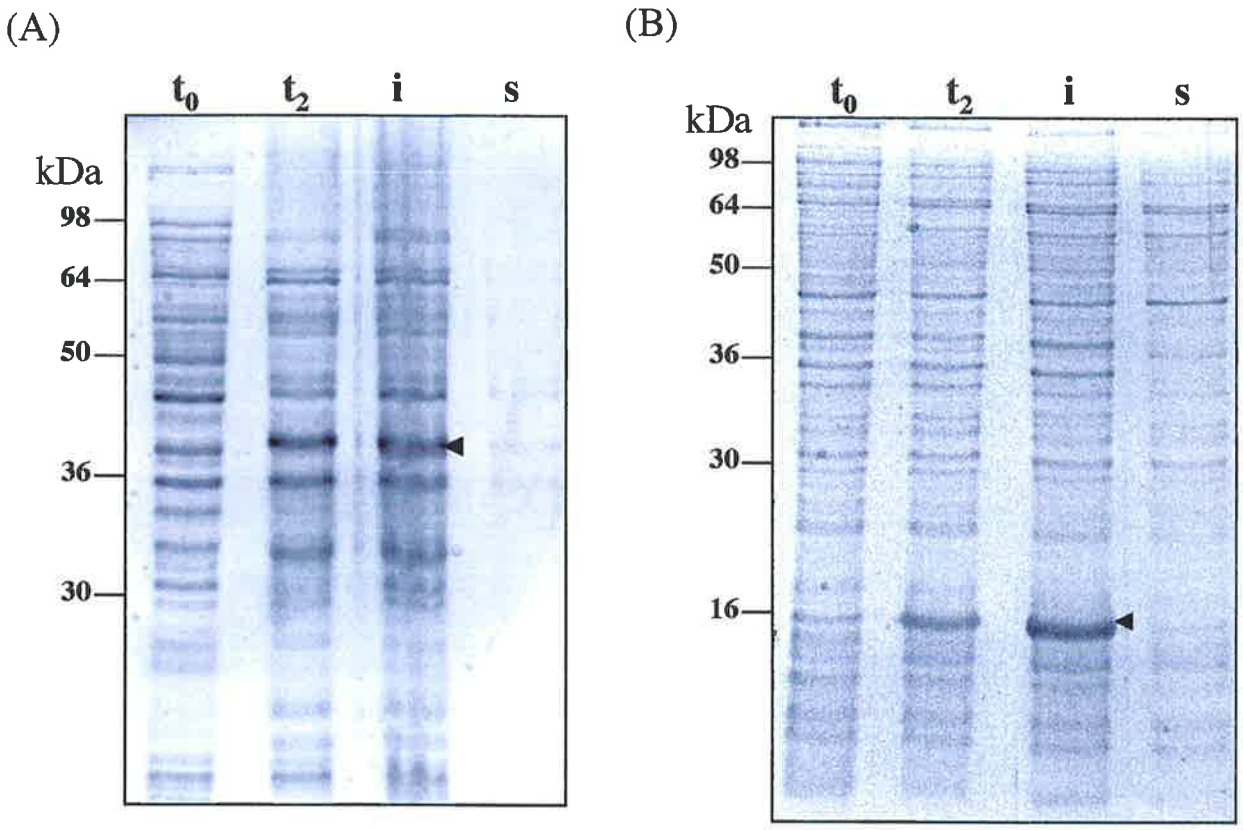


Figure 3-9. Induction and solubility of recombinant CrV2 and CrV3 proteins. Proteins from non-induced recombinant bacteria (t_0), 2 h IPTG-induced recombinant bacteria (t_2), as well as insoluble (i) and soluble (s) fractions of the induced bacteria, were separated by electrophoresis on SDS-polyacrylamide gels and stained with Coomassie Blue. *A*, 12% gel showing proteins from induced recombinant bacteria containing CrV2 open reading frame. *B*, 15% gel showing proteins from induced recombinant bacteria containing CrV3 open reading frame. Position of the relevant induced recombinant protein is shown by an *arrowhead*. In both cases, the recombinant protein was abundant in the insoluble fraction of induced bacteria.

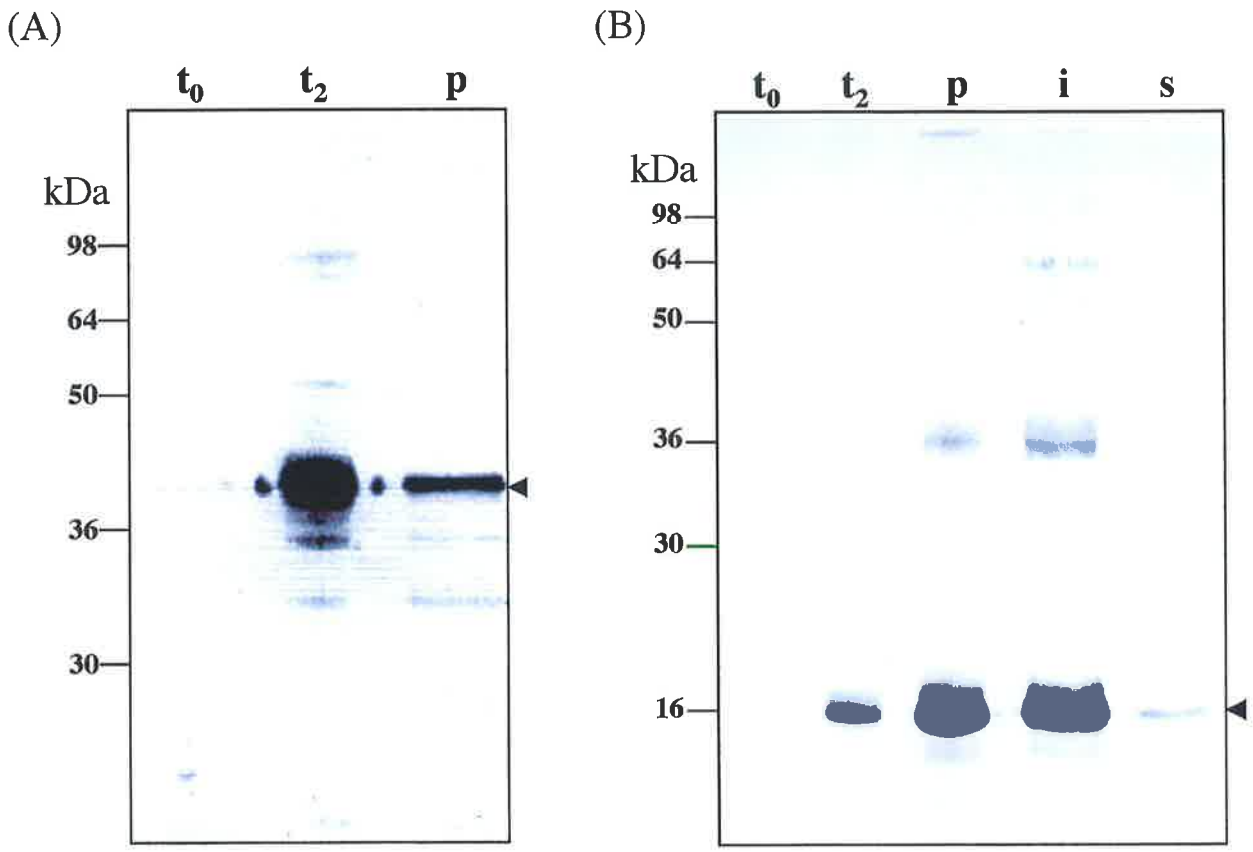


Figure 3-10. Purification of recombinant CrV2 and CrV3 proteins. Western blot analyses (utilising anti-polyHISTIDINE, 1:10000) showing recombinant CrV2 and CrV3 proteins (*arrowheads*) in induced recombinant bacterial cells (t_2) and in the purified fraction (p) but not in non-induced cells (t_0). *A*, purification of ≈ 37 kDa recombinant CrV2. *B*, purification of ≈ 16 kDa recombinant CrV3 and re-confirmation of most CrV3 being present in the insoluble fraction (i) of bacterial proteins compared to the soluble fraction (s).

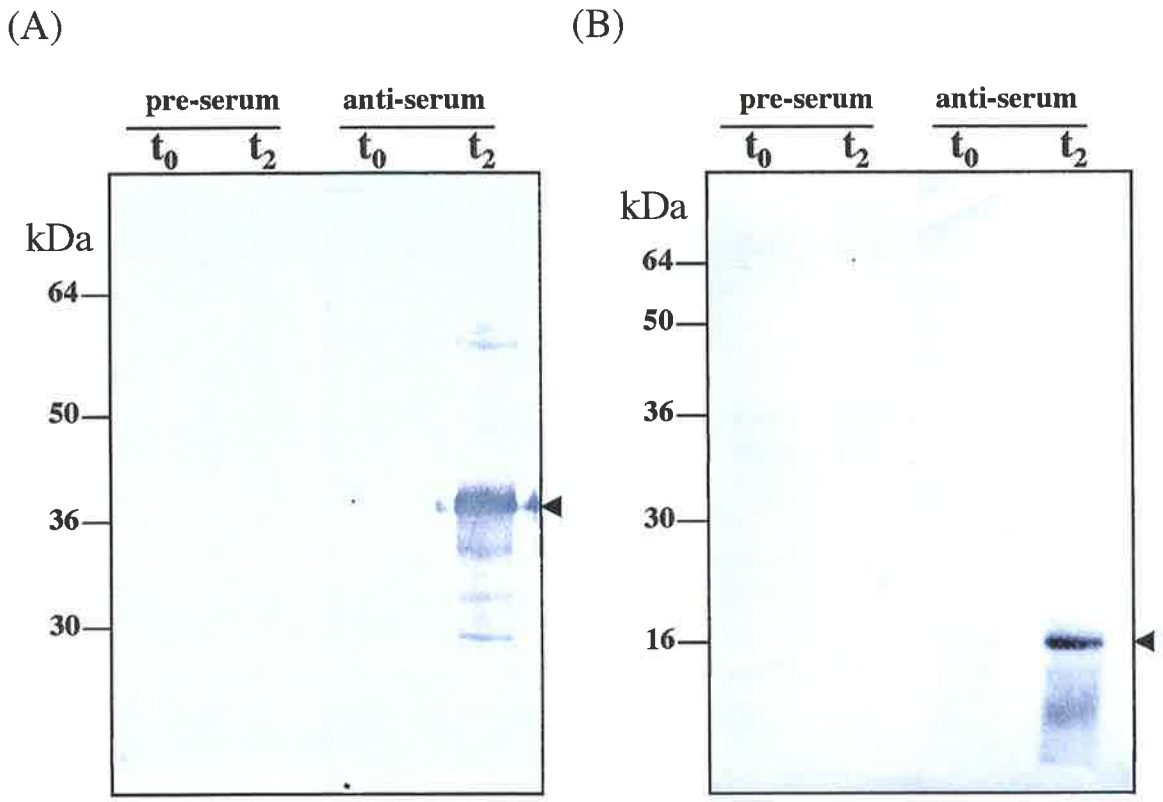


Figure 3-11. Confirmation of successful antiserum production against recombinant CrV2 and CrV3, using Western blot analyses of 2 h induced (t_2) and non-induced (t_0) recombinant bacteria, containing CrV2 or CrV3 partial open reading frames. Rabbit pre-serum was used as control probe to compare against probing with antiserum produced by rabbits injected with the relevant recombinant protein. *A*, detection of ≈ 37 kDa recombinant CrV2 protein (*arrowhead*) by antiserum only. *B*, detection of ≈ 16 kDa recombinant CrV3 protein (*arrowhead*) by antiserum only. Probes are indicated at *top*.

3.2 Characterisation of isolated bracovirus-expressed proteins

3.2.1 Characterisation of CrV2

The CrV2 nucleotide and deduced amino acid sequences were compared against known sequences in GenBank™, however, no significant homology was detected. Western blot analysis of serum from non-parasitised and 6 h parasitised *P. rapae* larvae, probed with anti-CrV2, allowed visualisation of ≈ 37 kDa CrV2 in parasitised larvae only (Fig. 3-12*A*). Previous data showed the presence of a parasitism-specific glycoprotein in the haemolymph of *P. rapae* larvae, the production of which was initiated at ≈ 6 hpp (Asgari, 1997; Fig. 3-12*B*). Using anti-CrV2 to probe seral proteins from 6 h parasitised larvae, it was determined that the previously unidentified parasitism-specific glycoprotein is CrV2 (Fig. 3-12*B* and *C*).

RT-PCR, utilising primers to the CrV2 open reading frame, was used to test for the presence of CrV2 transcripts in haemocytes and fat body cells, at 6 hpp (see Fig. 3-13*A*). CrV2 transcripts were detected in both cell-types, with significantly greater amplification produced from fat body RNA. Western blot analysis, using anti-CrV2, was performed on proteins from the same larval cell-types (and cell-free haemolymph) at 6 hpp (Fig. 3-13*B*). CrV2 protein was present in fat body and haemocytes with a large amount in the cell-free haemolymph. These data suggest that CrV2 production occurs in infected fat body and haemocytes, with the protein being subsequently secreted into the cell-free haemolymph.

The RT-PCR experiments showing presence of CrV2 transcripts in parasitised larvae, indicated that CrV2 transcription was higher in fat body cells, compared to haemocytes (Fig. 3-13*A*). To test this inference quantitatively, Northern slot-blot analysis was employed. In order to measure that similar amounts of total RNA were loaded in each slot, a control gene was required for use as

a probe. Primers were previously designed to amplify a fragment containing 12S and 16S ribosomal RNA (rRNA) from *Colias* sp. butterflies (Pollock *et al.*, 1998), which are from the Pieridae family, as is *P. rapae*. When these primers were used in RT-PCR of RNA from non-parasitised *P. rapae* larvae, an amplification product of 817 bp was produced (excluding primer binding sites). This fragment was cloned, sequenced and compared to sequences contained in the GenBank™ database. Nucleotide comparisons identified the amplified RNA as originating from 18S rRNA and was shown to be 98% similar to 18S genes from several other lepidopteran species. Sequence data for the 817 bp *P. rapae* 18S gene fragment are shown in figure 3-14.

Probes for the slot-blot were produced by ^{32}P-labelling of PCR products obtained by utilising primers to the CrV2 open reading frame (CrV2-F & CrV2-R; Fig. 3-5) and primers to the cloned *P. rapae* 18S fragment. When these probes were applied against $\approx 2\ \mu g$ of RNA from haemocytes and fat body of 6 h parasitised larvae, there was no significant difference in the amount of CrV2 transcripts detected in the two samples (Fig. 3-15). Western blot analyses of larval serum, haemocytes and fat body at various points after parasitisation, showed that CrV2 was present in each sample at 6 hpp, reached a maximum level at $\approx$ 24 hpp, and was declining at 48 hpp (Fig. 3-16).

When cell-free haemolymph proteins (taken from 6 h parasitised and naive larvae) were analysed under non-denaturing conditions, a putative CrV2 trimer of $\approx$ 98 kDa was detected (Fig. 3-17*A*). MultiCoil software predicted a high probability of trimer formation due to the presence of the C-terminus coiled-coil region, based on the deduced amino acid sequence of CrV2 (Fig. 3-17*B*).

Haemocytes were isolated from larvae at different times post-parasitisation (and non-parasitised controls) and tested for CrV2 presence via staining with FITC-linked secondary antibody (Fig. 3-18). Although an increase in staining (compared to control) was observed at 6 hpp (data not shown), the maximum amount of staining occurred at 24 hpp (Fig. 3-18*A*). At this point, much of the CrV2 appeared to be internalised within the haemocytes, in large endosomes (Fig. 3-18*B*).

The number of healthy haemocytes (those showing spreading ability) appeared to be increased at 24 hpp, when compared with 6 hpp (data not shown), indicating that haemocytes were starting to recover at 24 hpp. Bacterially-expressed CrV2 was added to naive haemocytes in order to determine if these cells take up recombinant CrV2, in a similar manner to other polydnavirus proteins, including CrV1 (Asgari *et al.*, 1997). Uptake was not observed by fluorescent antibody labelling of CrV2, however, it is not known whether the CrV2 was inactivated due to denaturation caused by the purification process or the lack of native glycosylation.

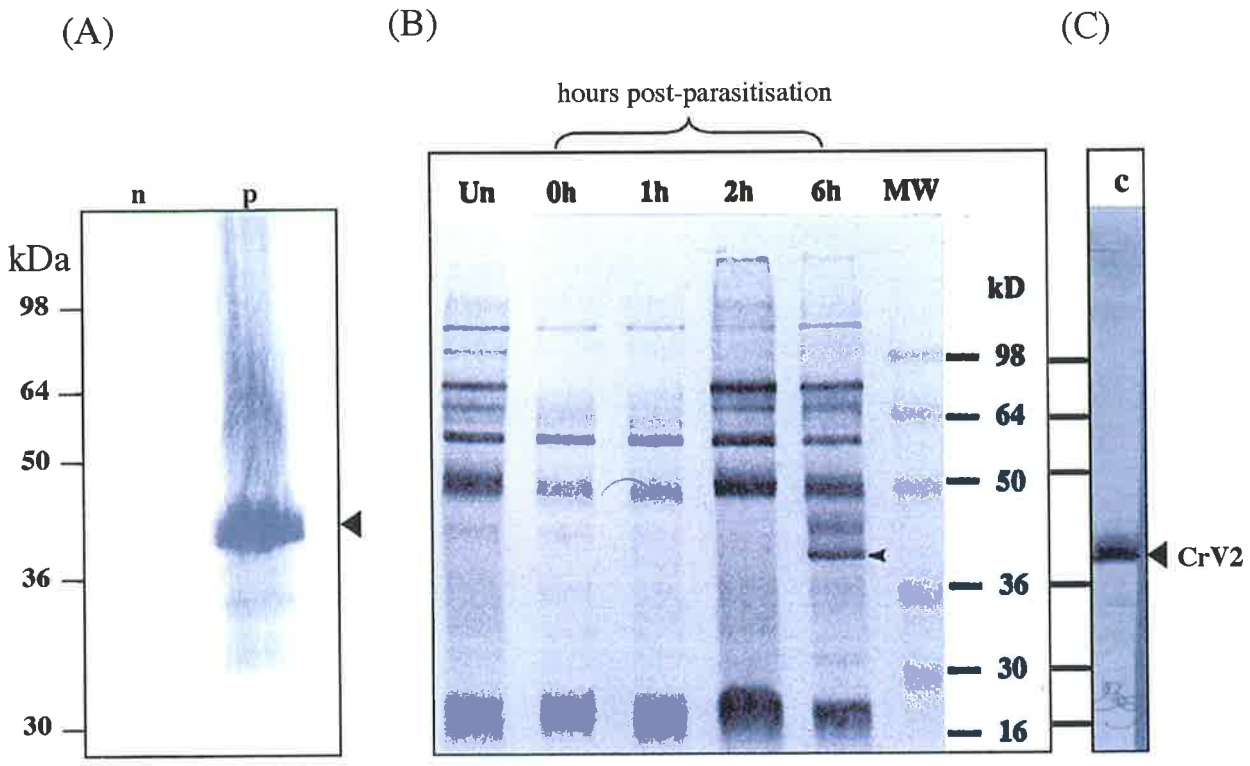


Figure 3-12. Western blot analyses (12% SDS-PAGE) showing expression of the CrV2 glycoprotein. *A*, detection of CrV2 (*arrowhead*) in cell-free haemolymph of 6 h parasitised *P. rapae* larvae (*p*) but not in naive larvae (*n*). Anti-CrV2 antiserum (1:5000) was used as a probe. *B*, analysis of cell-free haemolymph from *P. rapae* larvae at 0 hpp, 1 hpp, 2 hpp, and 6 hpp, using peroxidase-conjugated *Helix pomatia* lectin as a probe (from Asgari, 1997). Cell-free haemolymph from un-parasitised larvae (*Un*) was used as a negative control and molecular weight markers are shown (*MW*). An ≈ 37 kDa glycoprotein was detected in serum of 6 h parasitised larvae (*arrowhead*). *C*, analysis of cell-free haemolymph of 6 h parasitized *P. rapae* larvae, utilising anti-CrV2 antiserum (1:5000). The CrV2 protein corresponds to the previously identified ≈ 37 kDa parasitism-specific glycoprotein, detected at 6 hpp.

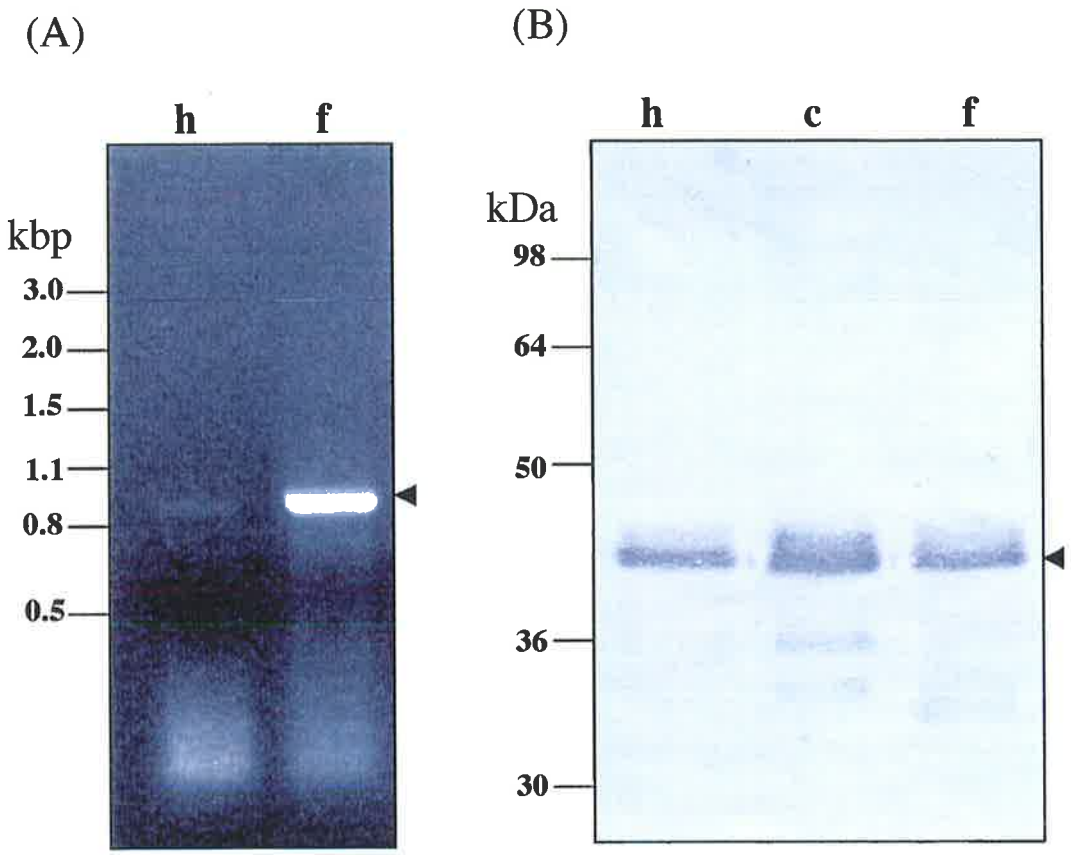


Figure 3-13. Transcription and expression of CrV2 in haemocytes (*h*), fat body (*f*) and cell-free haemolymph (*c*) of 6 h parasitised *P. rapae* larvae. *A*, RT-PCR of RNA from 6 h parasitised larvae, utilising primers to the CrV2 open reading frame (CrV2-F & CrV2-R). A strong signal was detected in fat body and a weak signal in haemocytes (*arrowhead*). *B*, Western blot analysis (12% SDS-PAGE; anti-CrV2 antiserum, 1:5000) of 6 h parasitised larvae showed presence of CrV2 in each sample (*arrowhead*).

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1       TCATTACCTC GGAGTTCTGA AAACCAACAA AATAGAACCG AGATCATATT CTATTATTCC    60
61      ATGCACGAAA TATTCAAGCG GCATTTTGAG CCCGCTTTGA GCACTCTAAT TTGTTCAAAG   120
121     TAAAATTGTC GGCCCACCTC GACACTCACC GAAGAGCACC GCGATAGGAT TTTGATATTG   180
181     AACCGGCGTT TTACCGCCGG CTCACCGACG ATATGCTCCG CAGACGTGTC AGTATCACCG   240
241     CGGATGCGGT GCACCGACAG CGCGGCGCAC AAATGCAACT ACGAGCTTTT TAACCGCAAC   300
301     AATTTTAGTA TACGCTATTG GAGCTGGAAT TACCGCGGCT GCTGGCACCA GACTTGCCCT   360
361     CCAATTGTTC CTCGTTAAAA TATTTAAAGT GTACTCATTC CGATTACGAG GCCTCGTAAG   420
421     AGTCCCGTAT CGTTATTTTT CGTCACTACC TCCCCGGTGC CGGGAGTGGG TAATTTGCGC   480
481     GCCTGCTGCC TTCCTTGGAT GTGGTAGCCG TTTCTCAGGC TCCCTCTCCG GAATCGAACC   540
541     CTGATTCCCC GTTACCCGTG ACAACCATGG TAGTCGCAGA AACTACCATC GAAAGTTGAT   600
601     AAGGCAGACA TTTGAAAGAT GCGTCGCCGG TACTGGGACC ATGCGATCGG CAAAAGTTAT   660
661     CCAGATTCAT CAAAATTAAC GACTTCGGAC GCTAGGCCCT CCGCCGATTG GTTTTGATCT   720
721     AATAAAAGCA CTCATCCCAT CACTGGTCAG AGTTCTGATT GCATGTATTA GCTCTAGAAT   780
781     TACCACAGTT ATCCAAGTAA CTGGGTAAGA TCTAAGG                            817
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Figure 3-14. Nucleotide sequence of a portion of 18S ribosomal RNA gene from *Pieris rapae* (Lepidoptera: Pieridae). Nucleotide position relative to the start of the fragment is indicated by *numerals* to the *left* and *right* of the sequence, with *1* indicating the first nucleotide at the 5' end and *817* indicting the last nucleotide at the 3' end.

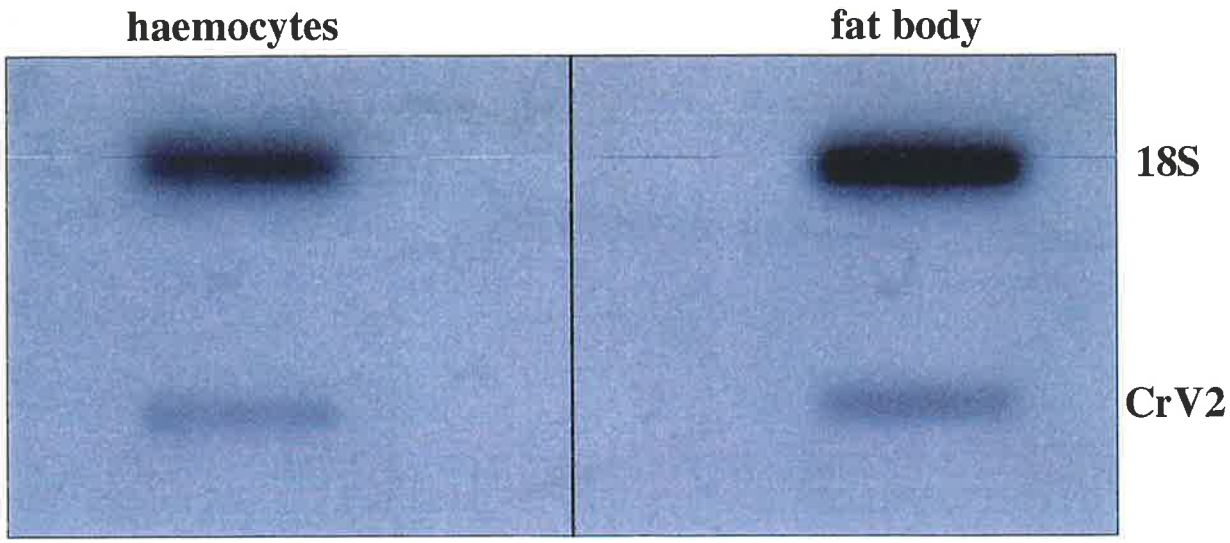


Figure 3-15. Northern slot-blot analysis of relative levels of CrV2 transcripts in ≈ 2 μg of RNA from haemocytes and fat body of 6 h parasitised *P. rapae* larvae. ^{32}P-labelled CrV2 fragment was used to detect CrV2 transcripts and ^{32}P-labelled *P. rapae* 18S rRNA was used as a control probe to test RNA loading. RNA source is shown at *top* and probes are identified at *right*. Haemolymph from six 4th instar larvae was pooled to provide haemocytes yielding ≈ 2 μg RNA. Similar levels of CrV2 transcripts were detected in each cell-type.

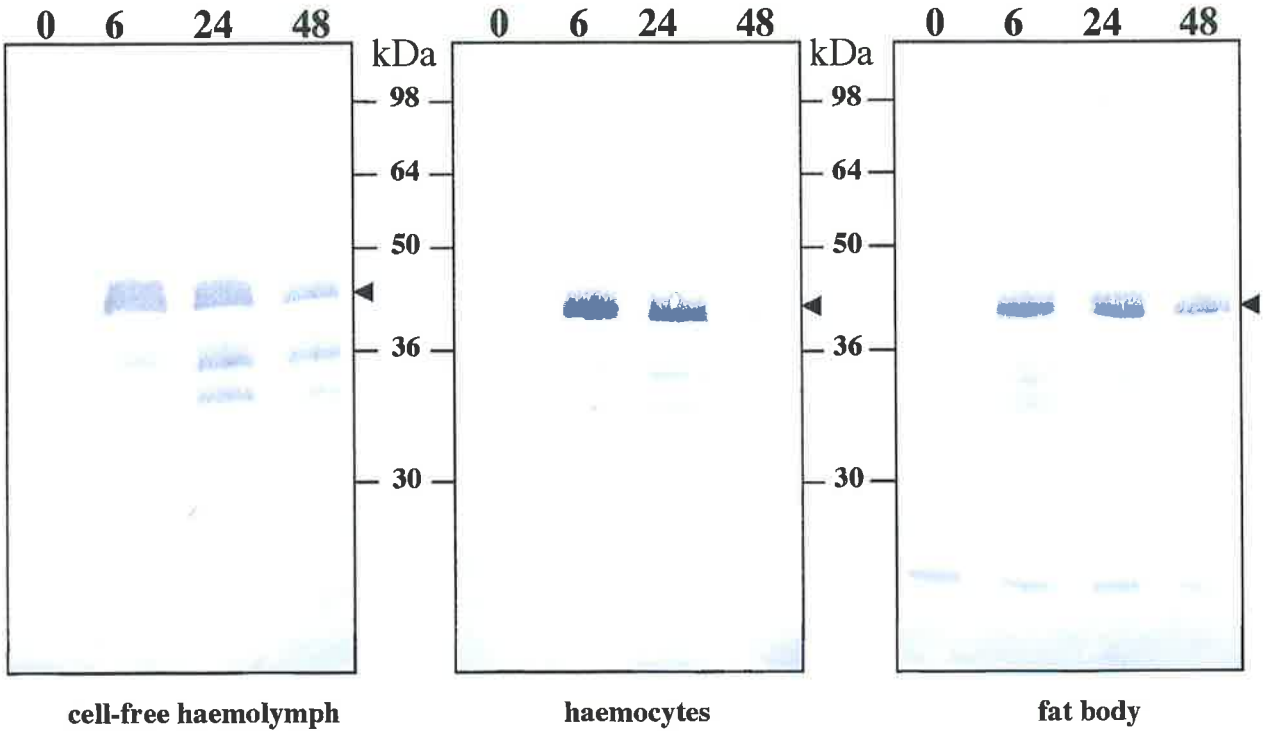


Figure 3-16. Western blot analyses (12% SDS-PAGE; anti-CrV2 antiserum, 1:5000) of *P. rapae* cell-free haemolymph, haemocytes and fat body. Proteins were collected at various time-points after parasitisation, which are indicated by *numerals above* the blots (units are hours). Protein source is shown *below* the blots. For each protein source, expression is detected at 6 hpp, large amounts of CrV2 are still present at 24 hpp and CrV2 levels are in decline at 48 hpp.

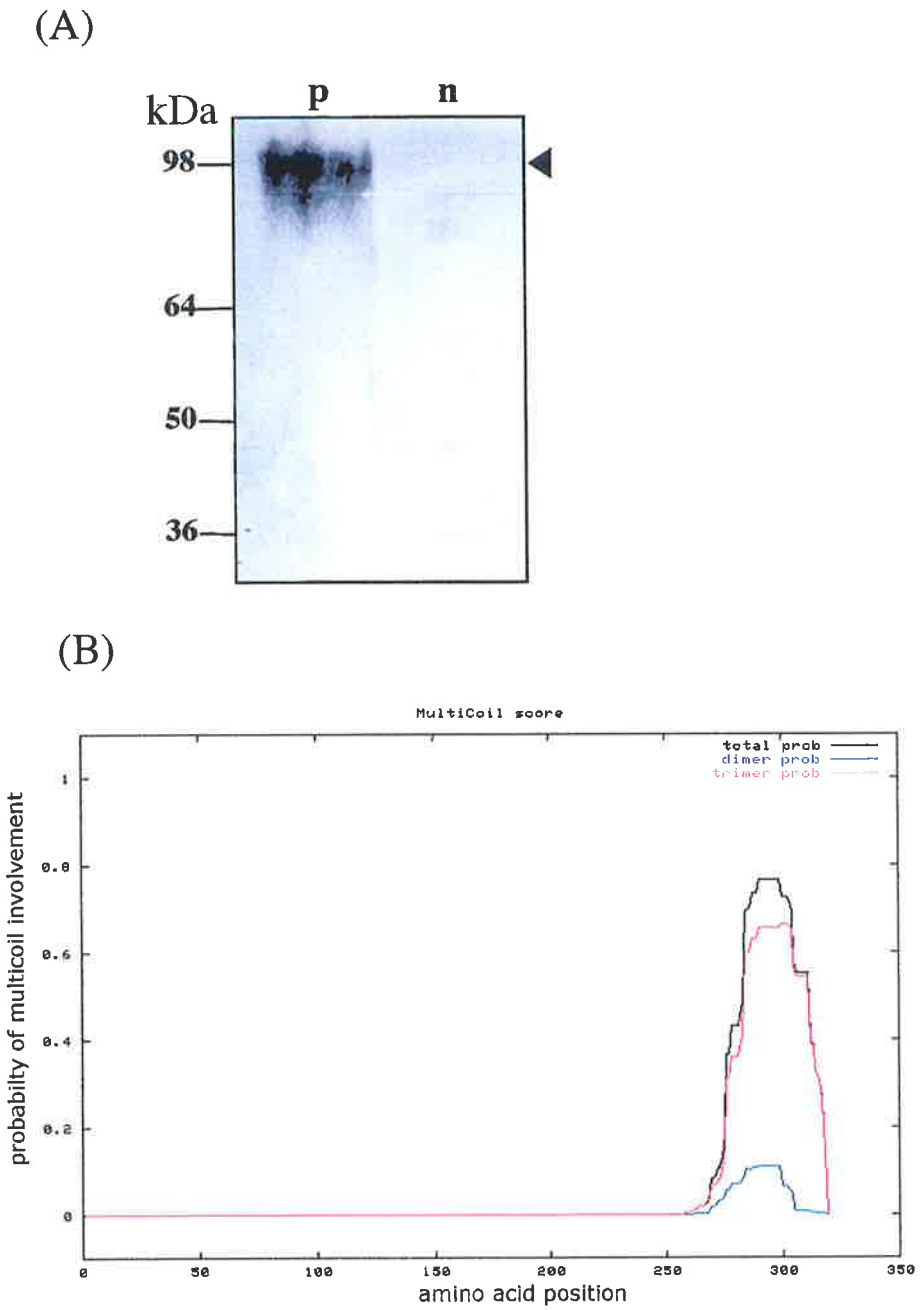


Figure 3-17. Trimerisation of CrV2. *A*, non-denaturing Western blot analysis of proteins from cell-free haemolymph from 6 h parasitised (*p*) and naive (*n*) *P. rapae* larvae, using anti-CrV2 antiserum (1:5000) as a probe. A putative CrV2 trimer of ≈ 98 kDa in size, was visualised (*arrowhead*). *B*, analysis of CrV2 deduced amino acid sequence by MultiCoil software indicates a high probability of trimer formation due to presence of the coiled-coil region near the C-terminus (amino acids 266-313). Probabiltiy of dimer formation was low.

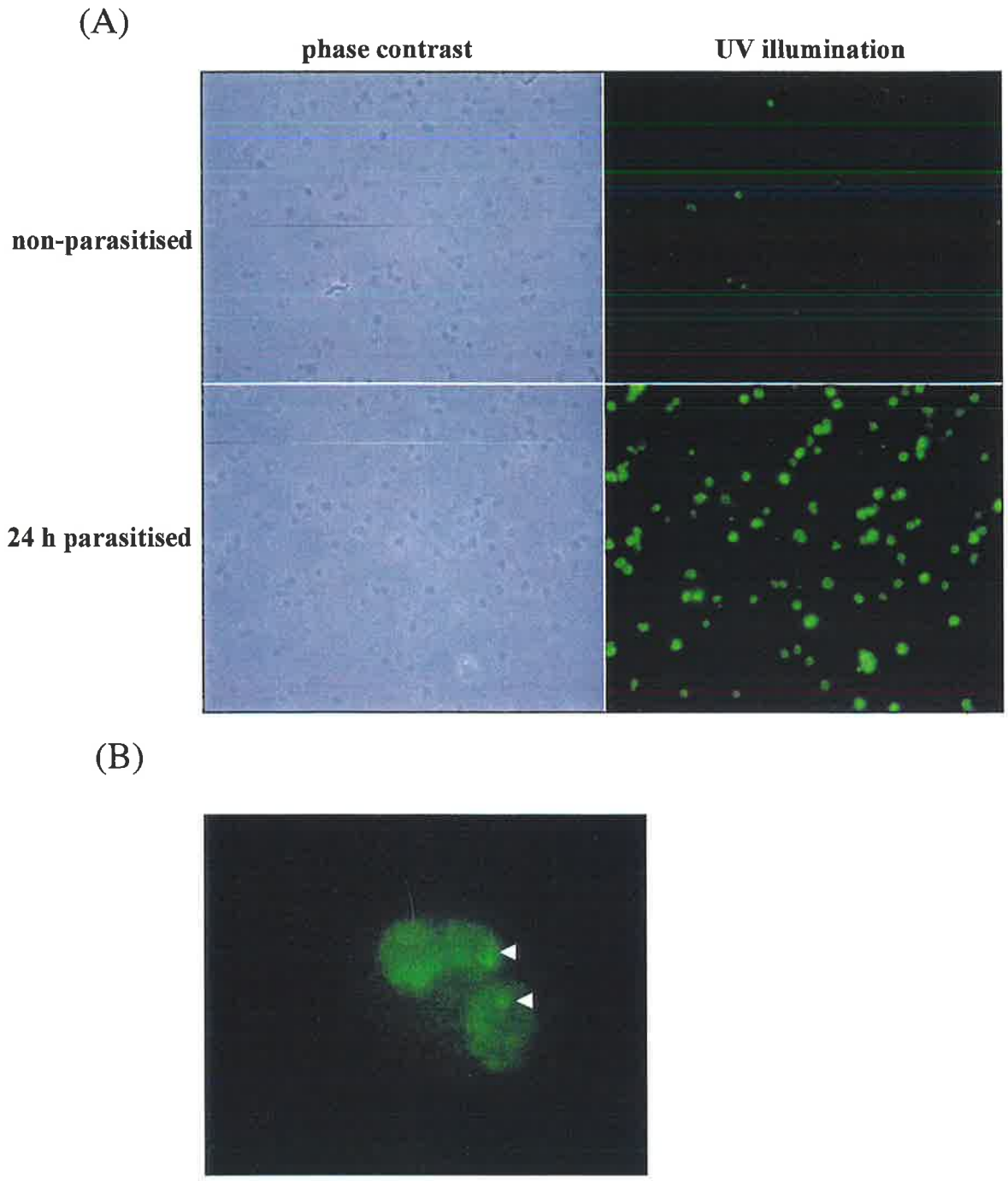


Figure 3-18. Visualisation of CrV2 in haemocytes from 24 h parasitised *P. rapae* larvae using antibodies against recombinant CrV2 and FITC-conjugated secondary antibody. *A*, fluorescent labelling of CrV2 associated with haemocytes from naive larvae and 24 h parasitised *P. rapae* larvae. Background labelling was low (see non-parasitised control) indicating that most staining results from labelled CrV2, detected in infected haemocytes. *B*, magnified view of several infected haemocytes showing CrV2, which is apparently internalised in large endosomes (*arrowheads*).

3.2.2 Characterisation of CrV3

In contrast to CrV2, comparison of CrV3 deduced amino acid sequence with those from the GenBank™ revealed significant homology with various C-type lectins (CTLs). Significantly, the spatial arrangement of key functional amino acids is conserved in CTLs from invertebrates and mammals, and also in CrV3 (Fig. 3-19*A*). Interestingly, the highest levels of similarity were observed with CTL homologues from *C. ruficrus* and *C. karyai* bracoviruses (Teramoto & Tanaka, 2003), 67% and 61% respectively (Fig. 3-19*B*). The next closest lectins are LPS-binding proteins from *Periplaneta americana* and *Bombyx mori*, although sequence similarity with these lectins is approximately half that of the bracovirus lectins. CrV3 was also found to be similar to a suite of *P. americana* lectins (data not shown). Overall, CrV3 has a simple structure, consisting of only a signal peptide and carbohydrate recognition domain (Fig. 3-7*B*).

A key test of whether a protein is a lectin, is to demonstrate the ability of the protein to agglutinate red blood cells and the inhibition of this process by small concentrations of a specific sugar ligand, bound by the CRD of the lectin (Sharon & Lis, 1989). Ovine red blood cells (ORBCs), prepared for agglutination assays by being trypsinised and gluteraldehyde-fixed, were used to test for CrV3 lectin activity. When added to ORBCs, recombinant CrV3 caused agglutination, whereas other co-purified bacterial proteins were shown to have no activity (Fig. 3-20*A* and *B*). Assays were conducted in either U-bottom micro-titer wells or by tilting flat-bottom micro-titer wells. ORBCs that were not agglutinated were influenced by gravity and thus formed a dot in the centre of U-bottom wells (Fig. 3-20*A*) or a crescent on the lower edge of tilted, flat-bottomed micro-titer wells (Fig. 3-20*B* and *C*). Agglutinated ORBCs formed a diffuse mat over the bottom surface of either type of well, and were not influenced by gravity (Fig. 3-20). Utilising

this assay, HPL was used to agglutinate ORBCs and was completely inhibited by its hapten sugar, N-acetyl-D-galactosamine (Hammarström & Kabat, 1971; Fig. 3-20*C*).

In order to test for inhibitory ligands of CrV3, various saccharides were added to agglutination assays, at final concentrations of ≈ 100 mM or ≈ 1 mg/ml, respectively. A typical inhibition assay is shown in figure 3-21. CrV3-mediated agglutination was not significantly inhibited by any of 29 potential ligands tested (Table 3-1), including common mono- and disaccharides as well as lipopolysaccharide from *E. coli*. The hapten sugar of a lectin would be expected to cause 50% inhibition at biological concentrations. For example, a galactose-specific *Drosophila melanogaster* lectin was inhibited by 50% in the presence of 0.1 mM galactose (Haq *et al.*, 1996).

A characteristic that differentiates CTLs from other lectins, is the dependence on divalent metal ions (most commonly Ca^{2+}) for lectin activity (Kilpatrick, 2002). Three experiments were carried out in order to test the effect of divalent metal ions on CrV3-mediated agglutination. Firstly, requirement for metal ions was demonstrated by abolishment of agglutination by addition of 1 mM of the chelator EDTA (Fig. 3-22*A*). Secondly, lectin activity was shown to be restored by addition of 0.5 mM Mg^{2+} and by 1 mM Mn^{2+} but was not restored by Ca^{2+} concentrations up to 5 mM (Fig. 3-22*B*). The effect of Mn^{2+} had a marked CrV3 concentration-dependent threshold, whereas the effect of Mg^{2+} gradually decreased as the relative CrV3 concentration decreased. Thirdly, serial dilutions of CrV3 were used to agglutinate ORBCs in the presence of 1 mM divalent metal ions (Fig. 3-23). Results (summarised in Table 3-2) indicate that agglutination was enhanced by divalent ions of manganese and magnesium (in order of relative effectiveness) but was independent of calcium ions.

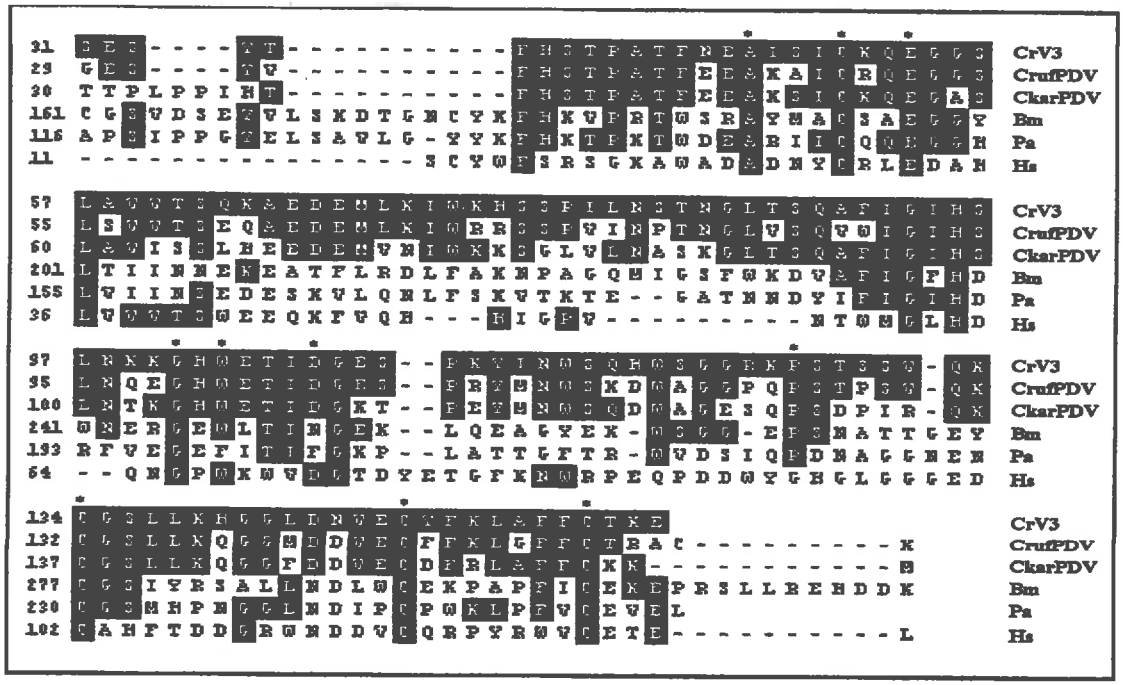
Western blots, utilising anti-CrV3 antibodies, showed two CrV3-related monomers, which were present only in parasitised larvae. These monomers were ≈ 17 kDa and ≈ 14 kDa in size, and were present in a ratio of ≈ 2:1 as judged by the relative intensity of electrophoresed bands (Fig. 3-24). CrV3 serum concentration was at a maximum at ≈ 6 hpp but was almost undetectable in haemolymph by Western analysis at 24 hpp (Fig. 3-24). RT-PCR, utilising primers to the CrV3 open reading frame, was used to test for production of CrV3 transcript in fat body and haemocytes from 6 h parasitised larvae (Fig. 3-25*A*). These data indicate that CrV3 is produced by infected haemocytes and fat body cells.

Western blot analysis (using anti-CrV3 antibodies) was performed on total proteins from larval fat body, haemocytes and cell-free haemolymph at 6 hpp (Fig. 3-25*B*). The presence of a large amount of CrV3 in the cell-free haemolymph compared to fat body or haemocytes, confirmed that the protein is secreted. The relative amount of each CrV3 monomer appears to vary depending on their location within parasitised larvae (Fig. 3-25*B*). In cell-free haemolymph, the ratio of larger monomer to smaller monomer is usually ≈ 2:1 (see Figs. 3-24 and 3-25*B*), whilst in fat body the ratio is reversed (Fig. 3-25*B*).

CrV3 hexamers, and smaller oligomers, were detected in purified bacterial CrV3, under denaturing conditions. Heating recombinant CrV3 to 65°C resulted in a breakdown of multimers into their components (Fig. 3-26*A*). However, boiling resulted in an increase of detectable multimers (Fig. 3-26*B*). A similar phenomenon was noted when cell-free haemolymph proteins, from 6 h parasitised larvae, were boiled. This treatment resulted in an increase of CrV3 tetramers and a decrease in dimers (Fig. 3-26*C*).

Treatment of cell-free haemolymph from 6 h parasitised larvae with a recombinant N-glycosidase, resulted in removal of the larger CrV3 monomer and an increase in the smaller monomer, suggesting that the larger monomer is an N-glycosylated form of the smaller monomer (Figs. 3-27*A* and *B*). No putative O-glycosylation sites were predicted to be present in CrV3. Dimer and tetramer CrV3 molecules were detected in small amounts, under denaturing conditions, in parasitised larvae and were both shown to contain glycosylated monomers (Figs. 3-27*A*, *B* and *C*). The relative amount of different oligomers appeared to vary with individual larvae and often only one type was detected (compare Figs. 3-26*C*, 3-27*A* and *B*).

(A)



(B)

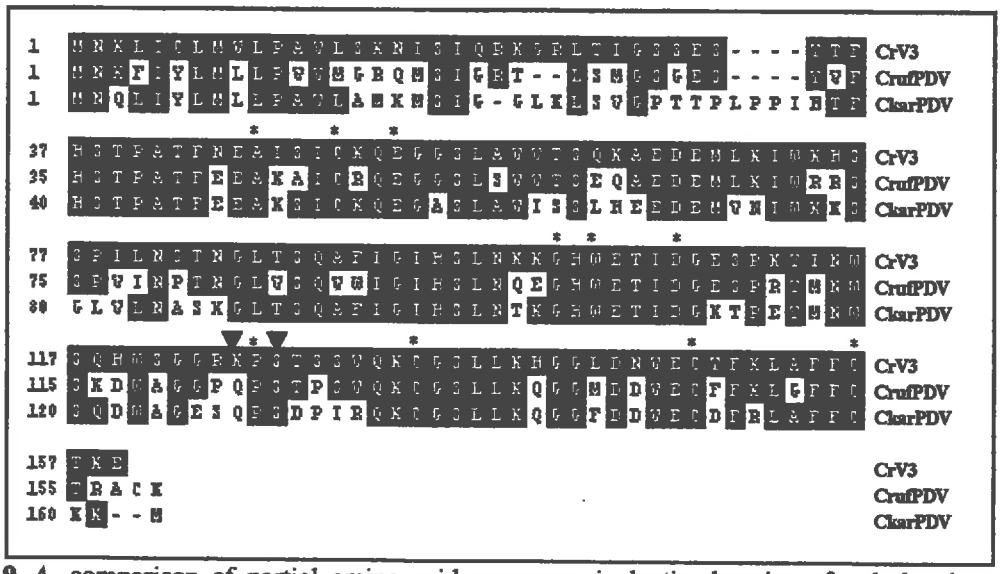


Figure 3-19. *A*, comparison of partial amino acid sequences in lectin domains of polydnavirus, insect and mammalian CTLs. Homologous residues with CrV3 are *boxed* in *black* and residues conserved among various CTLs are indicated by *asterisks*. *B*, comparison of complete amino acid sequences from CrV3 and CTL homologues from *Cotesia ruficrus* and *Cotesia karyai* bracoviruses. Residues considered important for metal ion and sugar binding are indicated with *arrowheads* and other indicators are as for *A*. Sequences are from CrV3 (*CrV3*, Genbank™ Accession AY234855), *C. ruficrus* bracovirus CTL homologue (*CrufPDV*; GenBank™ Accession BAC55179), *C. karyai* bracovirus CTL homologue (*CkarPDV*; GenBank™ Accession BAC55180), *Bombyx mori* lipopolysaccharide binding protein (*Bm*; GenBank™ Accession CAB38429), *Periplaneta americana* haemolymph lipopolysaccharide binding protein (*Pa*; GenBank™ Accession BAA00616) and *Homo sapiens* asialoglycoprotein receptor of hepatic lectin H1 (*Hs*; GenBank™ Accession NP00162).

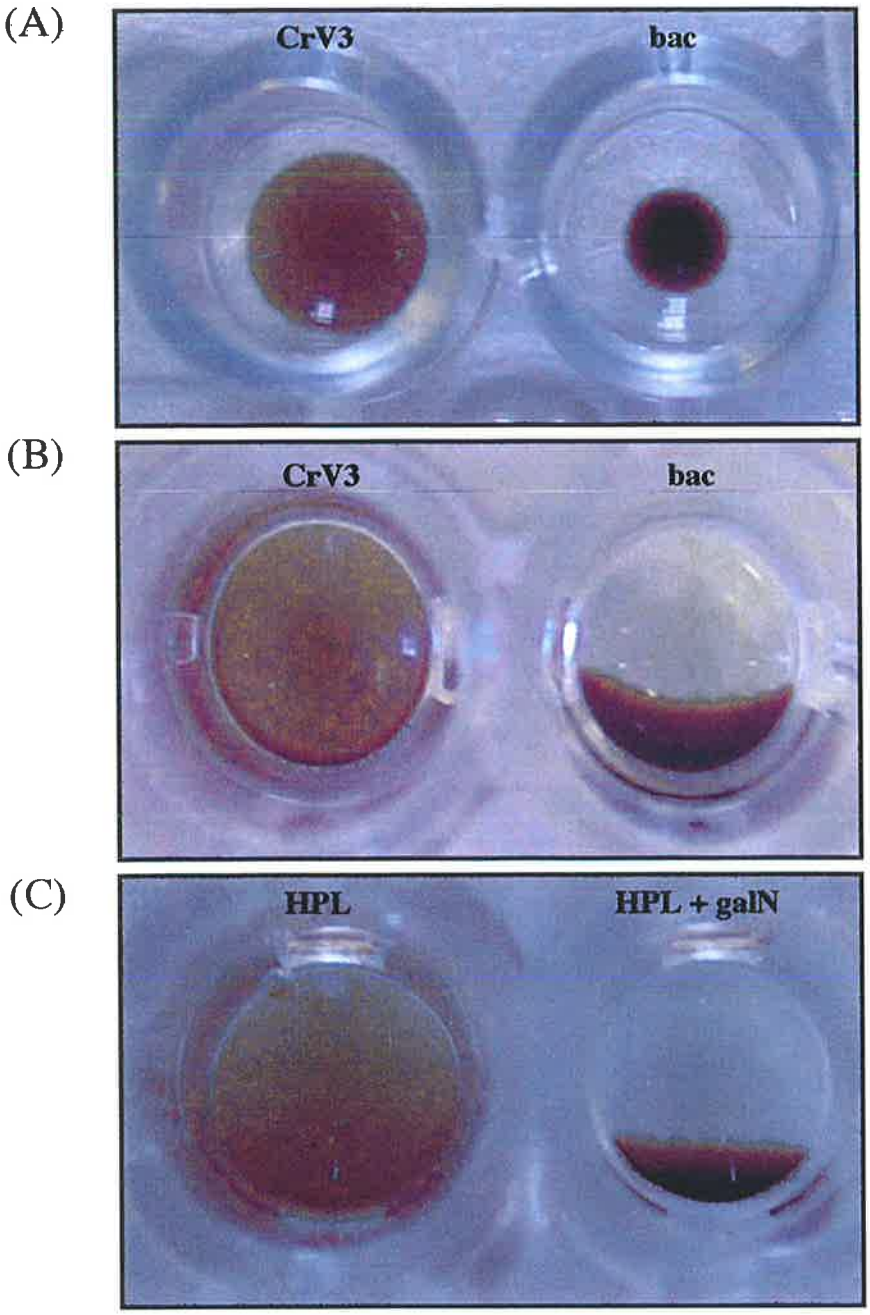


Figure 3-20. *A and B*, agglutination of ovine red blood cells (ORBCs) by bacterially-expressed CrV3 *(CrV3)* versus control proteins (*bac*), which were obtained using the same purification process (as for CrV3-containing bacteria) on bacteria containing empty expression vector. Assays were performed in U-bottom micro-titer wells (*A*) or tilted flat-bottom micro-titer wells (*B*), such that non-agglutinated cells (*bac*) sunk to the lowest point, forming a dot at the bottom of U-bottom wells (*A*) or a crescent at the lower margin of tilted flat-bottom wells (*B*). *C*, ORBCs agglutinated by lectin from *Helix pomatia* (*HPL*) and the inhibition of agglutination by addition of 100 mM N-acetyl-D-galactosamine (*HPL + galN*), the hapten sugar for *H. pomatia* lectin. In all cases, agglutinated cells (*CrV3 or HPL*) formed a diffuse layer over the surface of the well bottom and were not influenced by gravity.

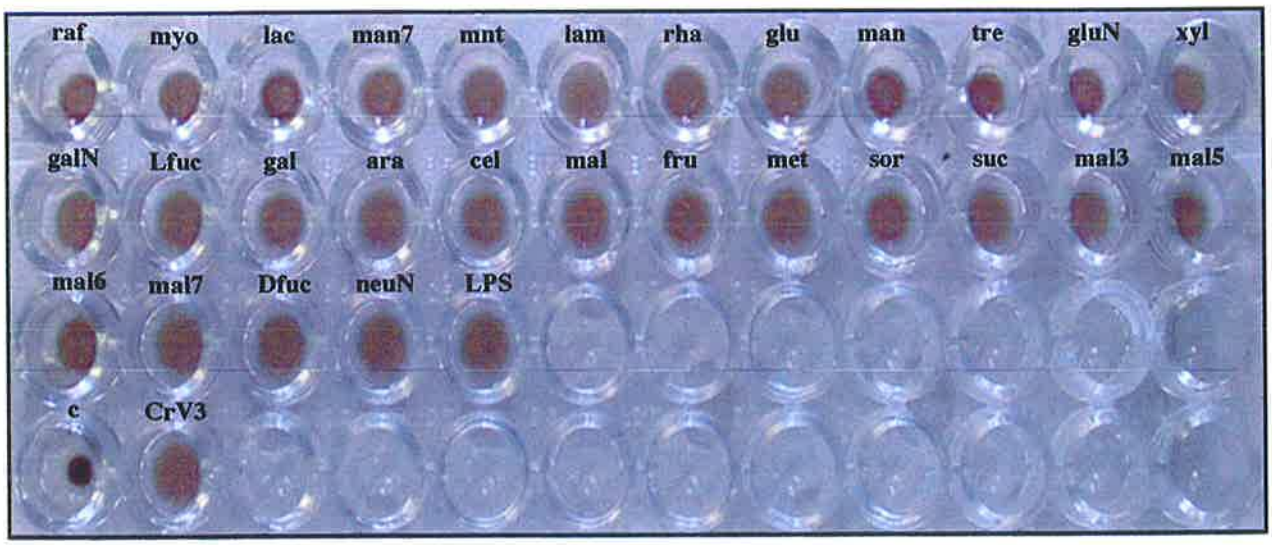
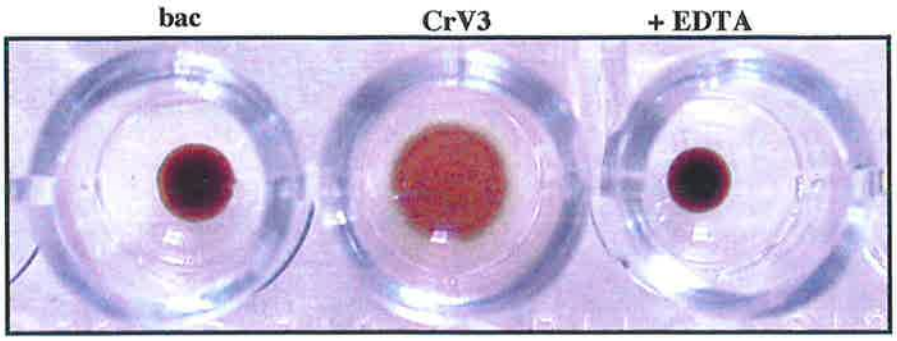


Figure 3-21. Typical CrV3 agglutination-inhibition assay. Various ligands were added to standard CrV3 agglutination mixtures (*CrV3*) to test for inhibition of CrV3-mediated agglutination. Saccharides were added to a final concentration of 100 mM, whilst lipopolysaccharide and laminari tetrose (a mixture of different laminari sugar polymers) were added to a final concentration of 1 mg/ml. Co-purified bacterial proteins (from bacteria containing empty expression vector) were used as a negative control (*c*). Agglutination was not significantly inhibited by any of the ligands tested. Ligand abbreviations are (from left to right and top to bottom) D-raffinose (*raf*), myo-inositol (*myo*), C-lactose (*lac*), D-mannoheptose (*man7*), D-mannitol (*mnt*), laminari tertrose (*lam*), α-L-rhamnose (*rha*), D-glucose (*glu*), D-mannose (*man*), D-trehalose (*tre*), N-acetyl-D-glucosamine (*glaN*), D-xylose (*xyl*), N-acetyl-D-galactosamine (*galN*), L-fucose (*Lfuc*), D-galactose (*gal*), L-arabinose (*ara*), D-cellobiose (*cel*), maltose (*mal*), β-D-fructose (*fru*), methyl-α-D-manno-pyranoside (*met*), D-sorbitol (*sor*), sucrose (*suc*), maltotriose (*mal3*), maltopentaose (*mal5*), maltohexaose (*mal6*), maltoheptaose (*mal7*), D-fucose (*Dfuc*), N-acetyl-D-neuraminic acid (*neuN*) and lipopolysaccharide from *Escherichia coli* serotype 055:B5 (*LPS*).

Ligand	Ligand concentration producing 50% inhibition
	mM
α-Lactose	>100
α-L-Rhamnose	>100
β-D-Fructose	>100
D-Cellobiose	>100
D-Galactose	>100
D-Glucose	>100
D-Fucose	>100
D-Mannitol	>100
D-Mannose	>100
D-Mannoheptose	>100
D-Raffinose	>100
D-Sorbitol	>100
D-Trehalose	>100
D-Xylose	>100
L-Arabinose	>100
Maltose	>100
Maltotriose	>100
Maltotetraose	>100
Maltopentaose	>100
Maltohexaose	>100
Maltoheptaose	>100
Methyl-α-D-manno-pyranoside	>100
Myo-inositol	>100
N-Acetyl-D-Glucosamine	>100
N-Acetyl-D-Galactosamine	>100
N-Acetyl-Neuraminic acid	>100
Sucrose	>100
	mg/ml
Laminari tetrose	>1
Lipopolysaccharide (*E. coli* , serotype 055:B5)	>1

Table 3-1. Inhibitory effect of various potential ligands on recombinant CrV3-mediated agglutination. Inhibition was assayed in the presence of ligands up to maximum concentrations of 100 mM or 1 mg/ml.

(A)



(B)

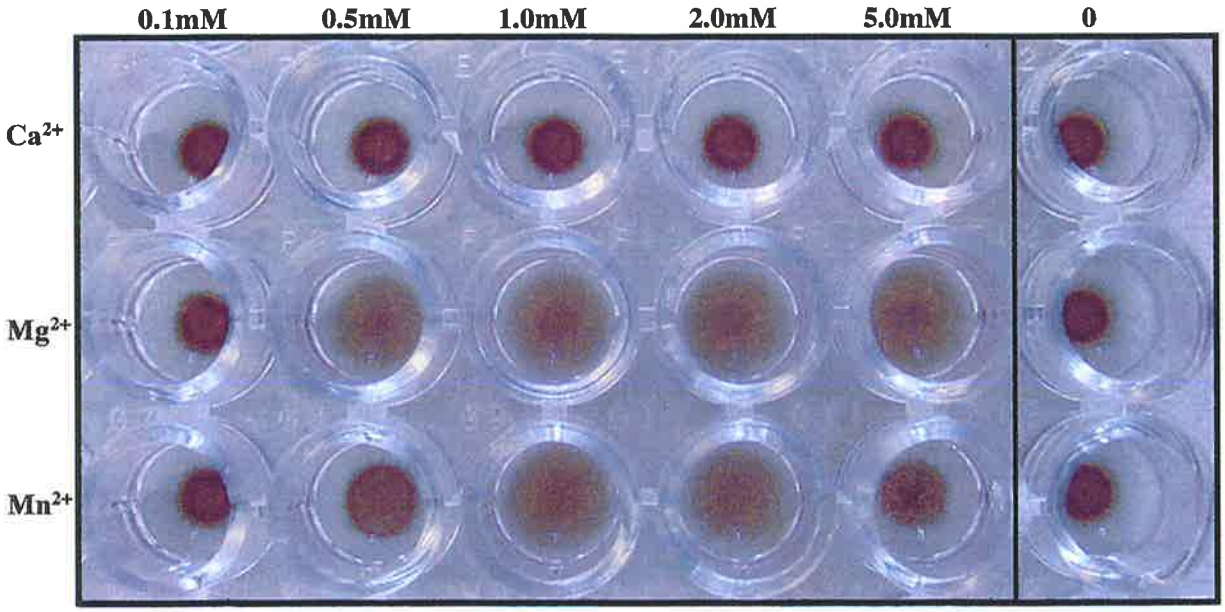


Figure 3-22. *A*, CrV3-mediated agglutination of ovine red blood cells *(CrV3)* was abolished by addition of 1 mM EDTA (+ *EDTA*.). Control proteins (*bac*) were obtained using the same purification process (as for CrV3-containing bacteria) on bacteria containing empty expression vector and did not agglutinate cells. *B*, agglutination assay showing abolishment of CrV3-mediated agglutination by addition of 1 mM EDTA (*0*) and reinstatement of agglutination by addition of increasing concentrations (shown at *top*) of various divalent metal ions (shown at *left*). Agglutination was reinstated by 0.5 mM Mg^{2+} and 1 mM Mn^{2+}.

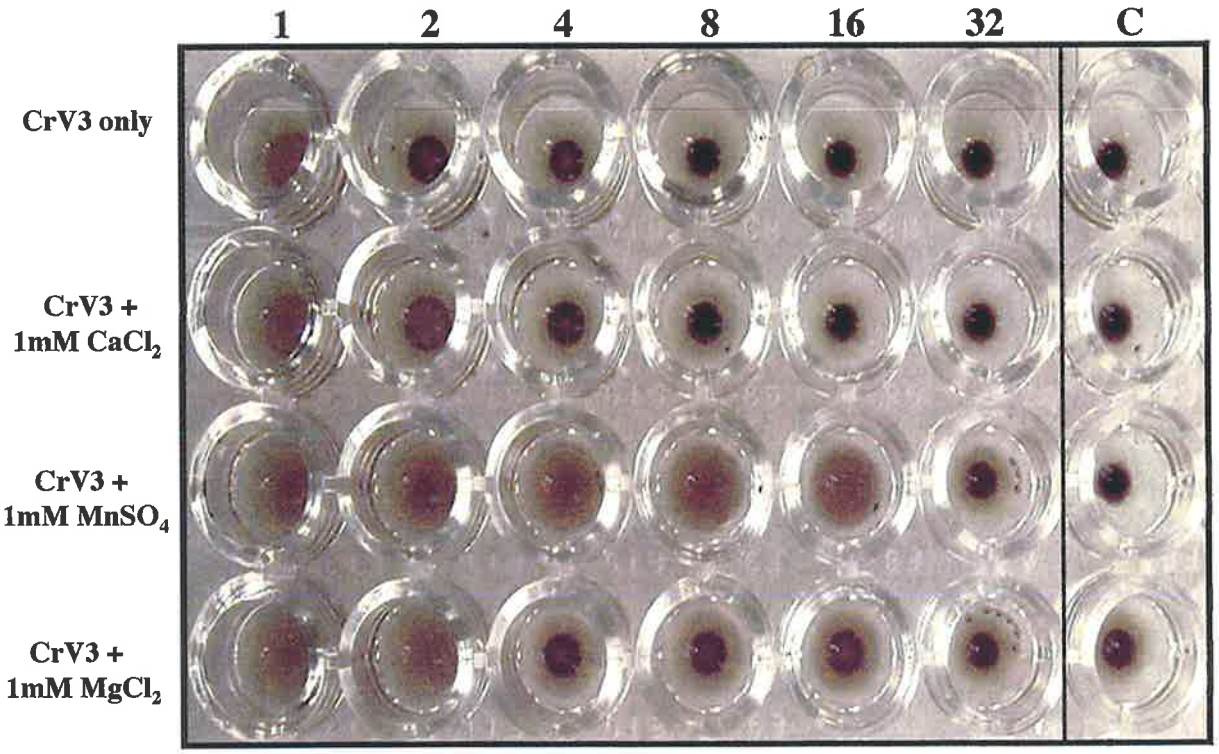


Figure 3-23. Typical agglutination assay showing effect of various 1 mM divalent cations on CrV3-mediated agglutination. Treatments are shown at *left*, and CrV3 titer (representing a serial dilution) is shown at *top*. Control treatments (*C*) contain no CrV3. Lectin titer was determined as the reciprocal of the maximum sample dilution causing complete ORBC agglutination.

Divalent cation/chelator	Lectin activity
	$titer^{-1}$
Bacterial extract	1
10 mM EDTA	n.d.
1 mM Calcium	1
1 mM Magnesium	2
1 mM Manganese	16

(*n.d.*, not detected)

Table 3-2. Effect of addition of various 1 mM divalent cations or 1 mM EDTA on bacterial CrV3-mediated agglutination. Lectin titer was determined as the reciprocal of the maximum sample dilution causing complete ORBC agglutination.

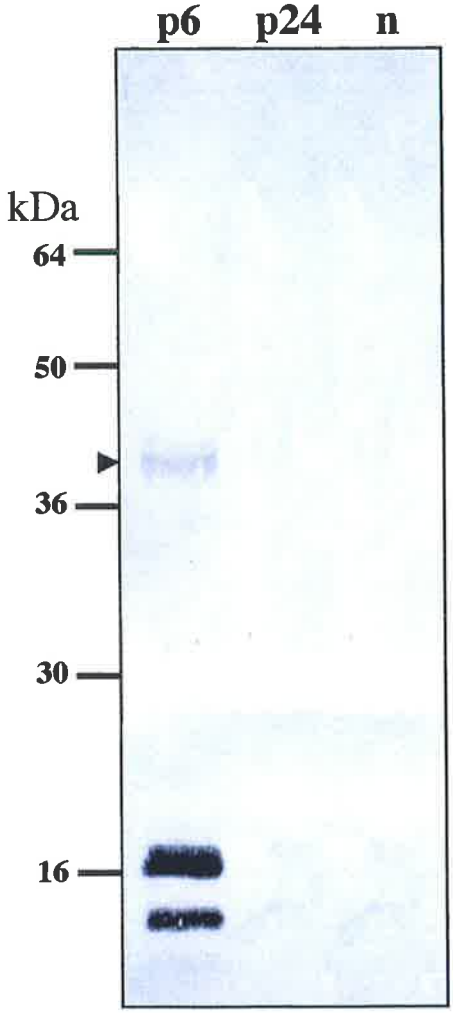


Figure 3-24. Western analysis (15% SDS-PAGE; anti-CrV3 antiserum, 1:5000) of cell-free haemolymph from 6 h parasitised (*p6*), 24 h parasitised (*p24*) and non-parasitised (n) *P. rapae* larvae. Parasitism-specific CrV3 monomers of ≈ 14 kDa and ≈ 17 kDa in size are detected at 6 hpp, in a ratio of 1:2, but are almost undetectable at 24 hpp. A small amount of CrV3 dimer (*arrowhead*) is also detected at 6 hpp.

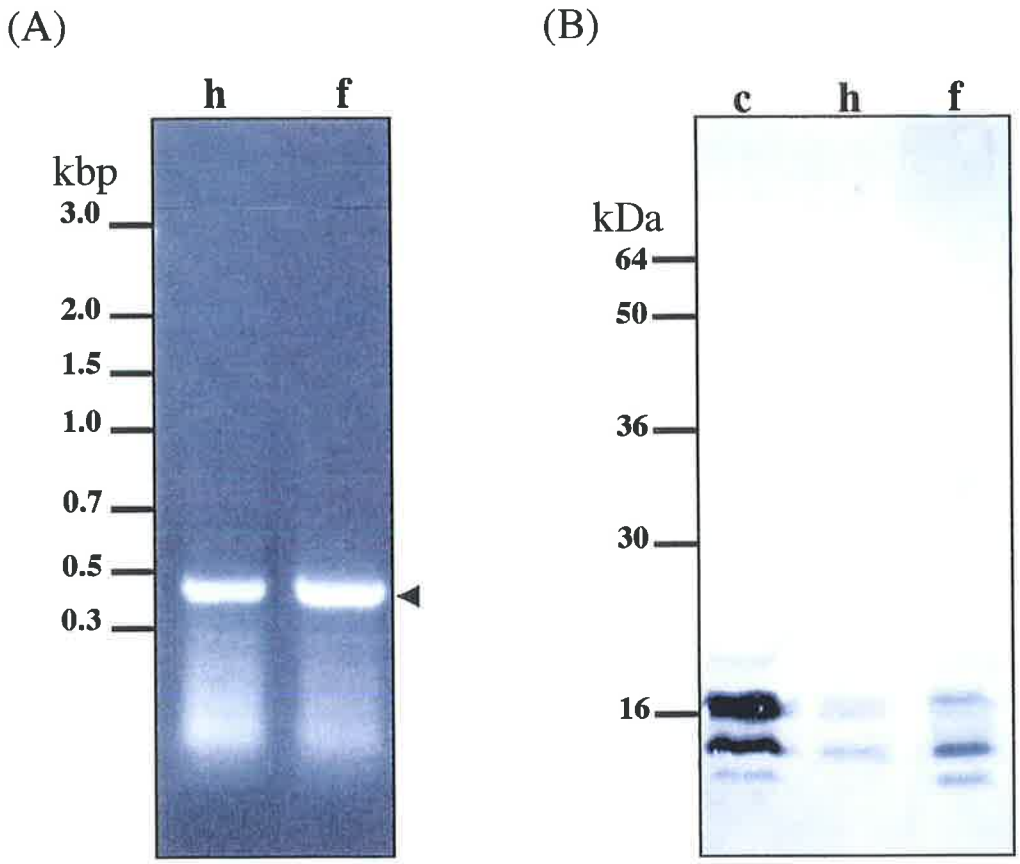


Figure 3-25. Analysis of CrV3 transcripts and protein in haemocytes (*h*) fat body (*f*) and cell-free haemolymph (*c*) from 6 h parasitised *P. rapae* larvae. *A*, RT-PCR of total RNA utilising primers specific to the CrV3 open reading frame (CrV3-F and CrV3-R). Strong signals were detected in haemocytes and fat body indicating transcription of CrV3 in these cells. *B*, Western blot analysis (15% SDS-PAGE; anti-CrV3 antiserum, 1:5000) showed presence of both CrV3 monomers in each sample. The majority of CrV3 is located in cell-free haemolymph indicating that CrV3 is secreted from infected haemocytes and fat body, into the serum.

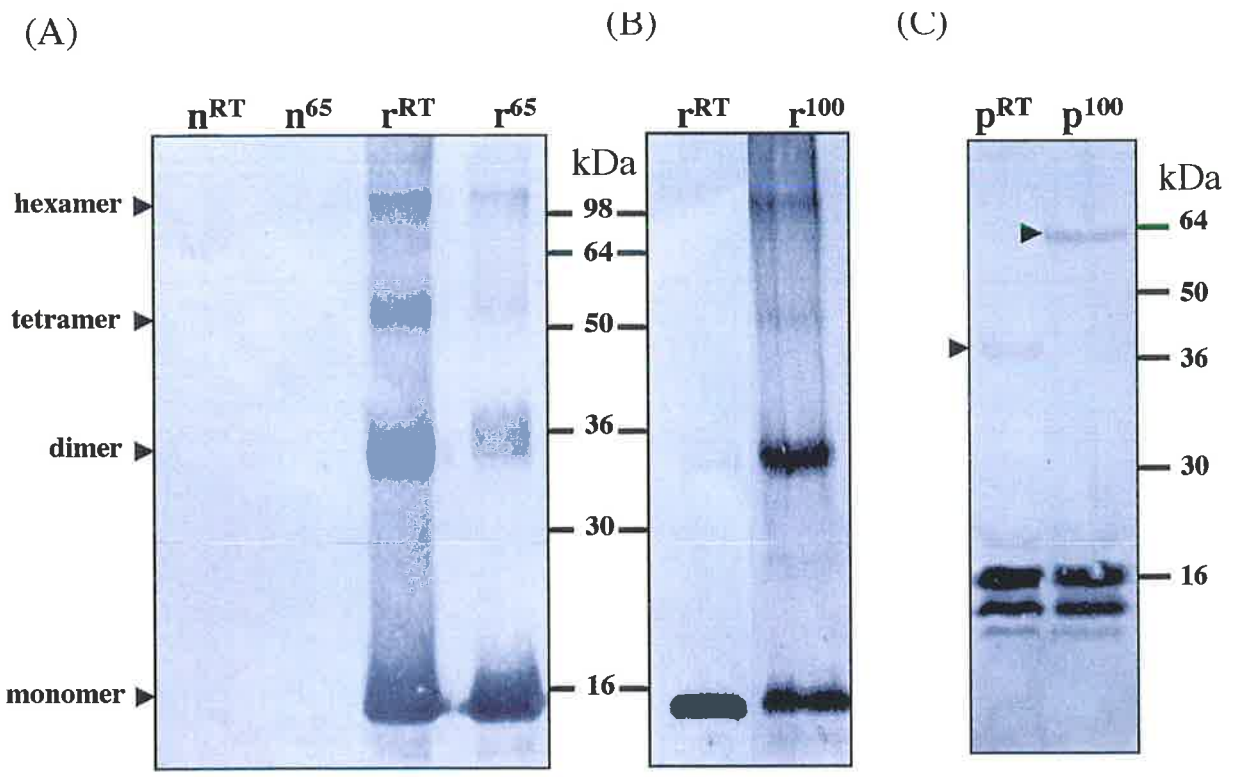


Figure 3-26. Western blot analyses (15% SDS-PAGE; anti CrV3 antiserum, 1:5000) illustrating the oligomerisation of recombinant and native CrV3. *A*, analysis of purified extract from induced bacteria containing empty expression vector (*n*) or recombinant vector containing CrV3 open reading frame (*r*). No CrV3-related molecules were detected in cells containing empty expression vector, that were left at room temperature (n^{RT}) or those heated at 65°C for 10 minutes (n^{65}). CrV3 monomer and a range of multimers (*arrowheads* in *A*) were detected in recombinant cells that were left at room temperature (r^{RT}). Multimers were denatured into smaller components when recombinant cells were heated at 65°C for 10 minutes (r^{65}). *B*, analysis of induced recombinant bacteria containing CrV3 open reading frame (*r*). Boiling of CrV3 sample (r^{100}) resulted in denaturation of a larger CrV3 complex, allowing detection of multimers not seen in the same amount of protein left at room temperature (r^{RT}). *C*, analysis of cell-free haemolymph from 6 h parsasitised *P. rapae* larvae (*p*), which was either left at room temperature (p^{RT}) or boiled for 10 minutes (p^{100}) prior to electrophoresis. Boiling resulted in dimer (*lower arrowhead* in *C*) denaturation into monomers and release of tetramer (*upper arrowhead* in *C*) from a putative larger complex.

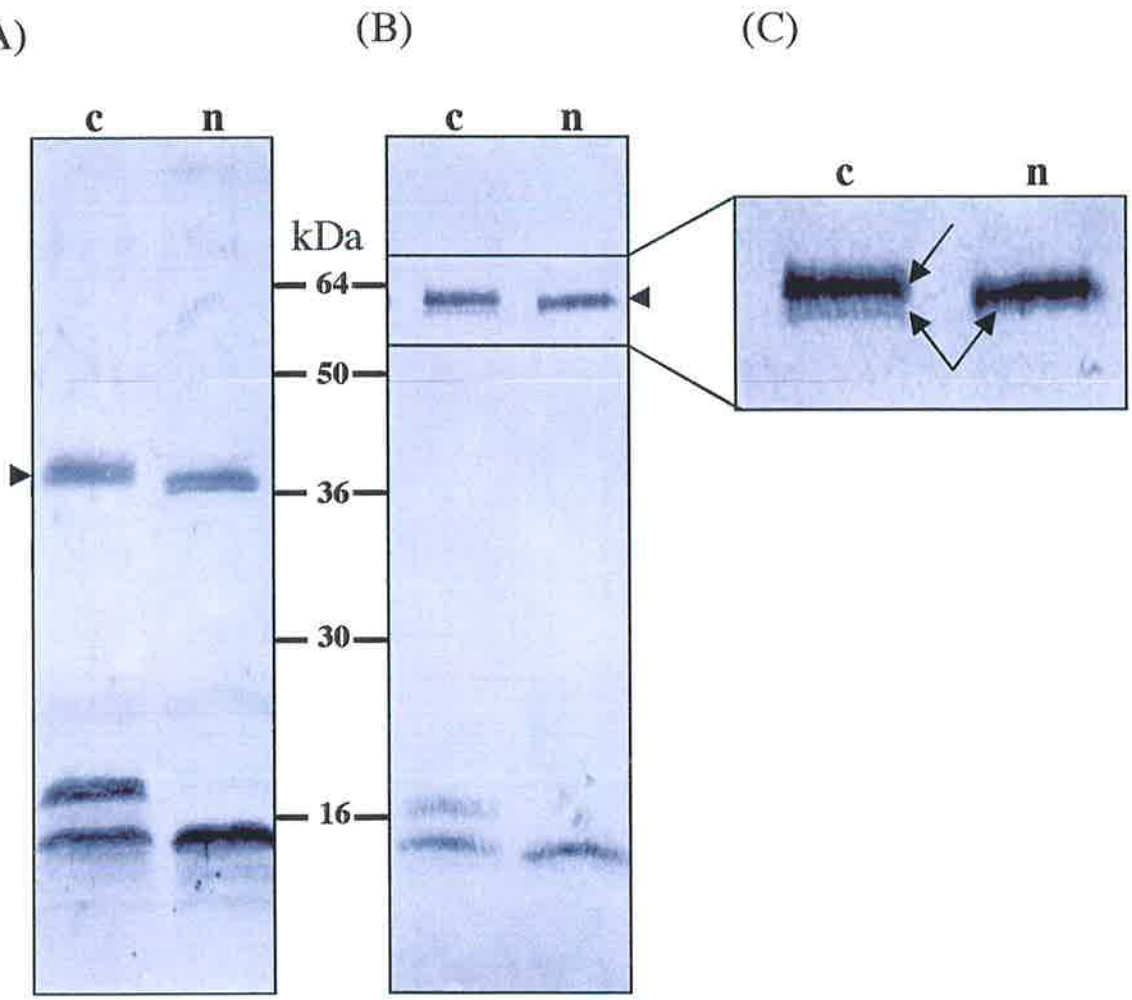


Figure 3-27. Western analyses (15% SDS-PAGE; anti CrV3 antiserum, 1:5000) illustrating deglycosylation of CrV3 monomres and oligomers found in cell-free haemolymph of 6 h parasitised *P. rapae* larvae. *A* and *B*, analysis of untreated (*c*) and N-glycosidase F treated (*n*) proteins. In both blots, the larger (≈ 17 kDa) monomer is deglycosylated such that it is identical to the smaller (≈ 14 kDa) monomer. Decrease in size of CrV3 dimer (*arrowhead* in *A*) and tetramer (*arrowhead* in *B*) indicate that these multimers contain at least one glycosylated monomer. *C*, magnified view of untreated (*c*) and N-glycosidase F treated (*n*) CrV3 tetramer bands (*arrows*), showing removal of larger tetramer by N-deglycosylation.

3.2.3 Comparison of CrV2 and CrV3 expression

During isolation and characterisation of CrV2 and CrV3, anecdotal evidence pointed to large differences in the amount of each transcript and protein detected in parasitised larvae. Therefore, several experiments were undertaken in order to directly compare relative levels of transcription and protein production in cell-free haemolymph, haemocytes and fat body of parasitised larvae. Previous work showed that each of the four identified CrBV genes expressed in parasitised larvae was expressed transiently (with maximum expression near 6 hpp) and that the up- and down-regulation of each gene occurred over similar time frames (Asgari *et al*, 1996; see Fig. 1-12). Therefore, 6 h parasitised larvae were used for the comparison.

Northern slot-blot analysis was used to compare relative CrV2 and CrV3 transcript levels contained in RNA extracted from non- and 6 h parasitised *P. rapae* larvae (Fig. 3-28). The *P. rapae* 18S fragment (Fig. 3-14) was used as control probe to determine RNA loading. Probing similar amounts of RNA with CrV2 and CrV3 probes indicated that CrV2 transcript made up a significantly greater proportion of total transcripts, than CrV3 transcript. Western blot analyses of similar amounts of protein from haemocytes, serum and fat body, probed with both anti-CrV2 and anti-CrV3, indicated that the CrV2 protein is present at significantly greater levels than CrV3, for each sample tested (Fig. 3-29). In the analyses shown, CrV2 was located in large amounts in each sample, whilst CrV3 was hardly visible, given the same amount of total loaded protein (Fig. 3-29).

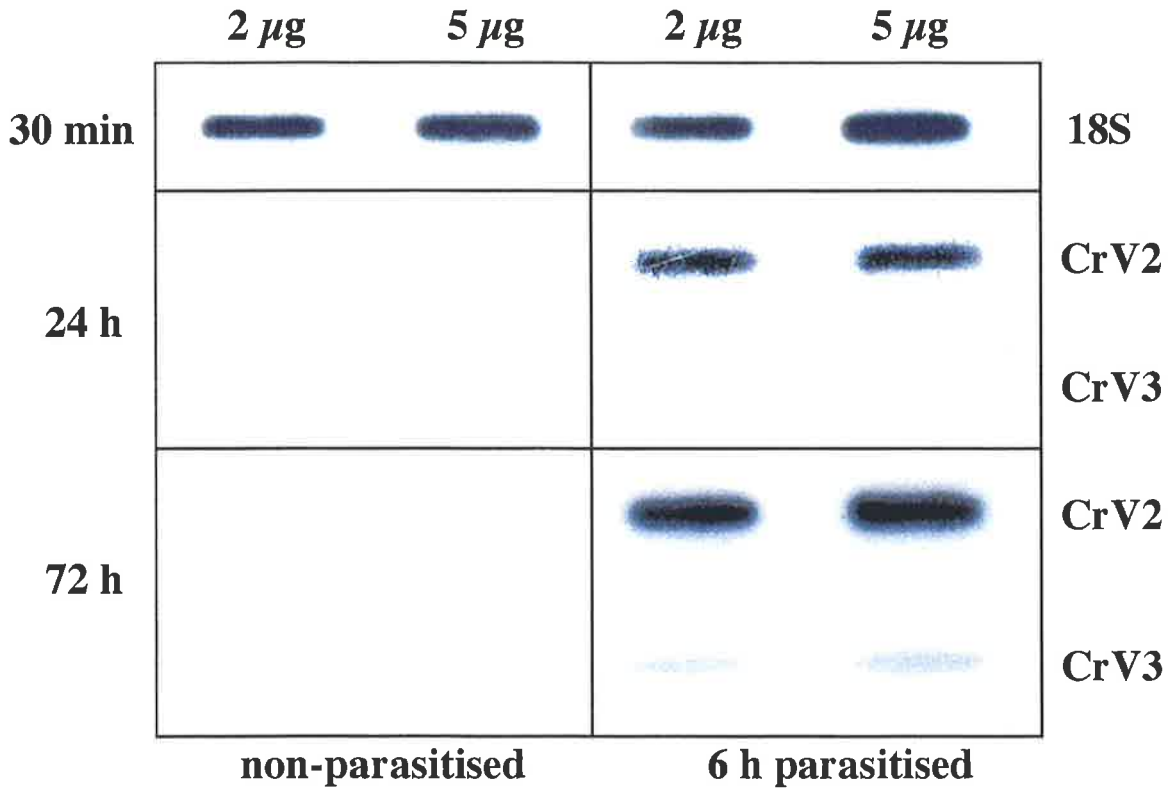


Figure 3-28. Slot-blot comparing relative levels of CrV2 and CrV3 transcripts in total RNA from non- and 6 h parasitised *P. rapae* larvae. Probes consisted of ^{32}P-labelled CrV2 and CrV3 fragments to measure viral transcripts, and a ^{32}P-labelled *P. rapae* 18S rRNA fragment to measure RNA loading. Putative amounts of loaded RNA are shown at *top*, the source of the RNA (i.e. naive versus parasitised larvae) is indicated at *bottom*, autoradiograph exposure period is shown at *left* and probe type is indicated at *right*. CrV2 transcripts comprised a significantly greater proportion of total transcripts, than CrV3 transcripts.

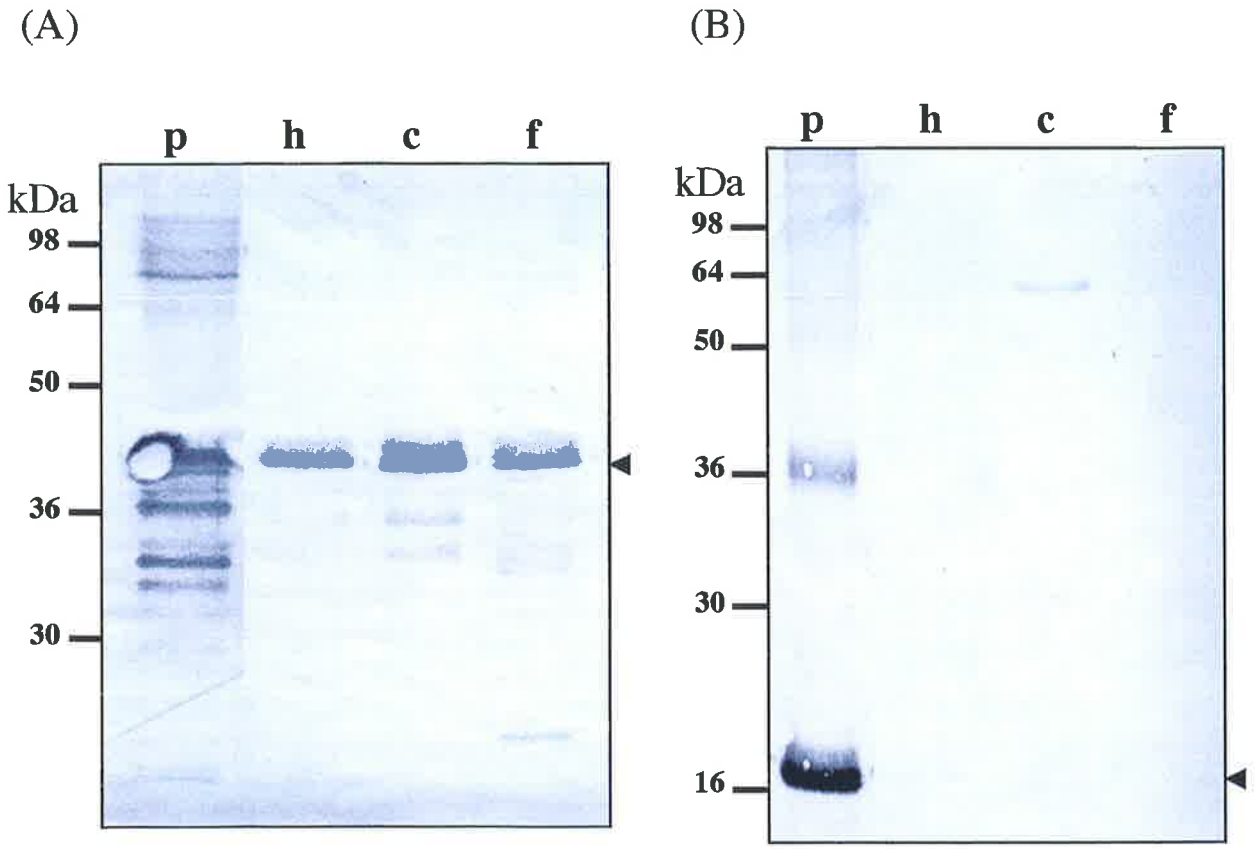


Figure 3-29. Comparison of relative CrV2 and CrV3 levels in haemocytes (*h*), cell-free haemolymph (*c*) and fat body (*f*) from 6 h parasitised *P. rapae* larvae. In each case, purified recombinant protein (*p*) was used as a positive control. *A*, Western blot analysis (12% SDS-PAGE; anti-CrV2 antiserum, 1:5000) of CrV2 in various samples. *B*, Western blot analysis (15% SDS-PAGE; anti-CrV3 antiserum, 1:5000) of CrV3 in various samples. The same amount of each protein sample was used for each analysis allowing direct comparison of CrV2 and CrV3 levels within a given sample.

3.3 Construction and bioassays of recombinant baculoviruses containing immune-suppressive genes

Two genes, which are associated with immunity in *P. rapae*, were previously isolated and characterised. The first, Crp32, is one of the *C. rubecula* calyx proteins and is suspected of passively protecting the *C. rubecula* egg by mimicking *P. rapae* tissue components (Asgari & Schmidt, 1994; Asgari *et al.*, 1998). Secondly, CrV1, expressed by CrBV in the *P. rapae* host, actively suppresses the host immune response by disrupting the actin cytoskeleton of haemocytes that uptake CrV1 (Asgari *et al.*, 1996; Asgari *et al.*, 1997). Because of their immune-disruptive properties, these genes were candidates for insertion into the genome of AcMNPV (a pathogenic virus of over 30 lepidopteran species – Du & Thiem, 1997) in order to increase its pathogenicity in *P. rapae*. Such a virus would potentially be a valuable biological control against *P. rapae*, as the inserted genes have evolved to cause immune-suppression in this species.

Construction of recombinant baculoviruses was begun by PCR amplification of each gene (previously cloned) and subsequent insertion of the amplified product into an AcMNPV transfer vector. Lepidopteran cell lines derived from *Spodoptera frugiperda* (Sf21 & Sf9) were co-transfected with transfer vector containing the immune-suppressive transgene (see 2.3.1 and 2.3.2 for construction of transfer vectors), and linearised partial AcMNPV DNA (Fig. 3-30). Linearised AcMNPV (Fig. 3-30*B*) is not infective due to its linearisation and the absence of part of two genes, an essential gene for infectivity and the polyhedrin gene. The transfer vector (Fig. 3-30*A*) contained missing parts of these genes as well as homologous regions with those contained in the linearised AcMNPV. These homologous regions acted as sites for intracellular recombination, producing an infective circularised AcMNPV containing an immune-suppressive transgene (Fig. 3-30*C*). Figure 3-30 schematically illustrates a generic recombination event leading to production

of recombinant baculovirus *in vitro*. Putative recombinant AcMNPVs were selected based on the presence of polyhedra and were isolated by plaque assay, before being amplified in order to obtain large amounts of budded virus and polyhedra.

In order to confirm that recombinant AcMNPVs had been successfully produced, putative recombinant plaque amplified virus was used to infect healthy cell cultures. Two days after inoculation, infected cultures were separated into cells, supernatant and budded virus (pelleted from supernatant). RNA was extracted from infected cells and was used as template in RT-PCRs utilising primers to the open reading frame of the relevant transgene. For each infected cell culture, transcription of the transgene was confirmed (Fig. 3-31). Transcription of recombinant protein was not detected in cell cultures infected with wild-type AcMNPVs (data not shown). Recombinant AcMNPVs containing CrV1 and Crp32 were denoted as AcMNPV_CrV1 and AcMNPV_Crp32, respectively.

Western blot analyses of proteins from infected cells, non-infected cells, culture medium and budded virus, were then employed to confirm production of each recombinant protein (Fig. 3-32). CrV1 was detected mainly in association with the culture medium, as it was secreted by cells inoculated with AcMNPV_CrV1, as is the case in *P. rapae* cells infected with CrBV. Only a small amount of CrV1 was detected in the cells themselves (Fig. 3-32*A*). Crp32 was detected equally in cells inoculated with AcMNPV_Crp32 and in association with the budded virions produced by such cells (Fig. 3-32*B*). Since Crp32 has a transmembrane domain without a cleavage site (Asgari *et al*, 1998), it was expected that Crp32 would be detected on the surface of budded virions, as they acquire an envelope whilst budding through the membrane of infected cells. Recombinant proteins were not detected in healthy cells or cells infected with wild-type

AcMNPV. Thus, production of recombinant AcMNPVs, expressing either CrV1 or Crp32, was confirmed.

AcMNPV_Crp32 was chosen as the first candidate for pathogenicity testing via bioassay. Several test bioassays and two full-scale bioassays were undertaken whereby leaf discs were dipped into solutions containing polyhedra from wild-type and recombinant AcMNPV, along with 0.001% Tween 20 to aid with spreading of inoculum on the leaf disc surface. Control leaf discs were prepared by dipping into a 0.001% Tween 20 solution with no polyhedra. Larvae were randomly assigned to one of the three groups and housed individually while they consumed their leaf discs.

Prior to the bioassays undertaken in this study, no published data could be found characterising the pathogenic effect of wild-type AcMNPV against *P. rapae* and no other papilionoid host has previously been reported for AcMNPV. Data comparing the percentage of larvae successfully reaching pupation for control and wild-type AcMNPV treatments, for the two full-scale bioassays, were pooled (Fig. 3-33) and subjected to chi square analysis. A p value of < 0.0009 indicates that the effect of the wild-type AcMNPV on *P. rapae* was significant in terms of the number of larvae successfully reaching pupation and hence, in terms of the percentage of larval deaths. An average of $\approx 45\%$ of larvae successfully reached pupation for AcMNPV treated larvae with an average of $\approx 86\%$ of larvae reaching pupation in the control treatment (i.e. 55% and 14% of larvae died, respectively) – see Fig. 3-33.

Figure 3-34 shows the time-course of larval deaths for a typical full-scale bioassay (i.e. larval mortality curves). Trends indicate that the effect of the Crp32 transgene was insignificant in terms of the number of larval deaths or the timing of the deaths. It is clear that many more larvae

died in both AcMNPV treatments but that larval deaths appear delayed in that the vast majority occurred after the point at which most control-treated larvae had successfully entered the pupal phase (Fig. 3-34). This is indicative of the most obvious symptoms which were that the larvae generally stopped eating after 3-5 days and then became moribund and lay on the food plant or the ground, long after a healthy larva would have pupated. Such larvae became lighter in colour and their faeces became liquid. Eventually the larvae died, becoming liquefied and dark brown in colour about 24 h after death. Polyhedra were visible in the liquefied larval tissues when examined under light microscope. The relatively low death rate (for such a high dose of inoculum) and the large amount of time required for most larval deaths, indicate that both AcMNPVs were only semi-permissive in *P. rapae*. Due to time constraints and targetting of research towards the characterisation of CrV3, testing of the recombinant baculoviruses was halted to be continued in a later study.

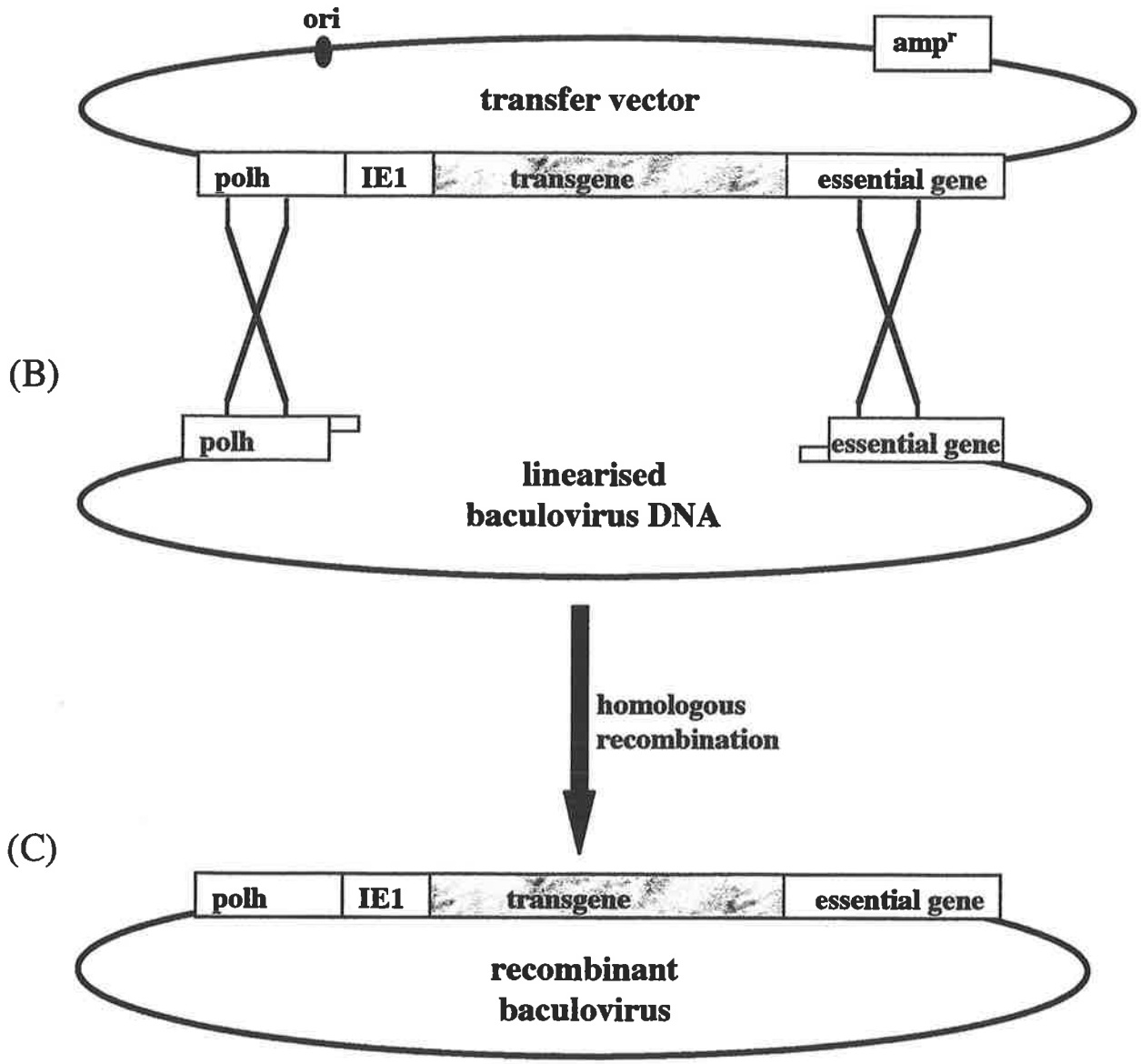


Figure 3-30. Schematic diagram of typical recombination events leading to production of recombinant *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV). Sf9 or Sf21 insect cell cultures were co-transfected with a transfer vector (*A*) containing the *transgene* (either CrV1 or Crp32, respectively) under control of the immediate-early promoter (*IE1*), and linearised AcMNPV DNA (*B*) that is non-infective due to its linearity and presence of only part of an *essential gene* for viral infection. Homologous recombination occurs intracellularly between the polyhedrin gene (*polh*) and the *essential gene*, which are contained in both transfected genetic elements. Sites of homologous recombination are indicated by *crossovers*, linking the polyhedrin and essential genes of each transfected element. The result of homologous recombination is the production of infective, recombinant AcMNPV (*C*) that can be selected by the presence of polyhedra in infected cells. Other indicators relate to the origin of replication (*ori*), ampicilin resistance gene (*amp*^{r}), both originating from the transfer vector.

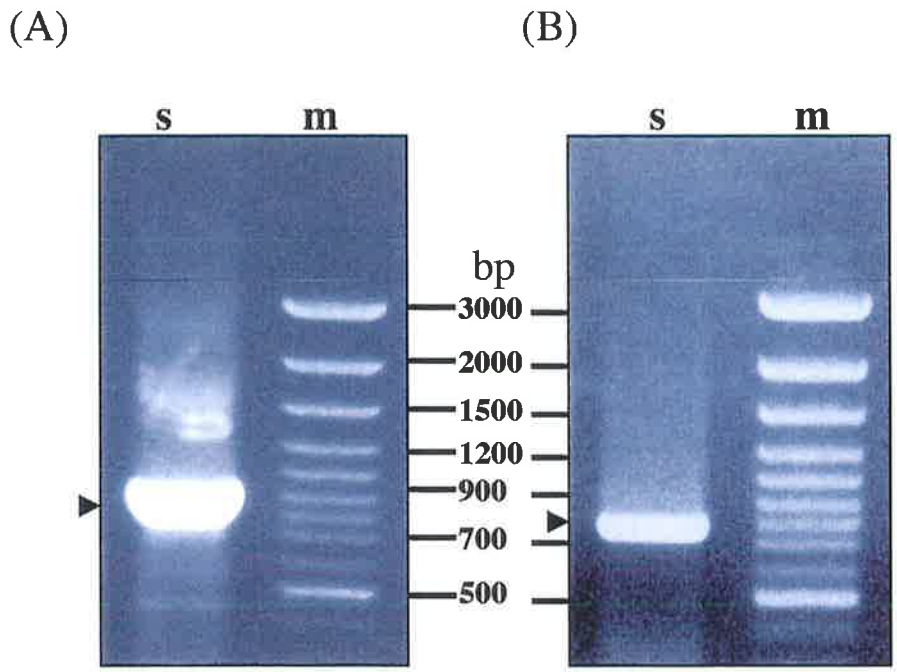


Figure 3-31. RT-PCRs detecting transcription of the transgenes CrV1 (*A*) and Crp32 (*B*) in lepidopteran cell lines (Sf21 and Sf9, respectively) infected with plaque-amplified recombinant *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV). In each case the correctly amplified transcript is denoted by an *arrowhead*. Recombinant transcript was not detected in cell cultures infected with wild-type AcMNPV (data not shown).

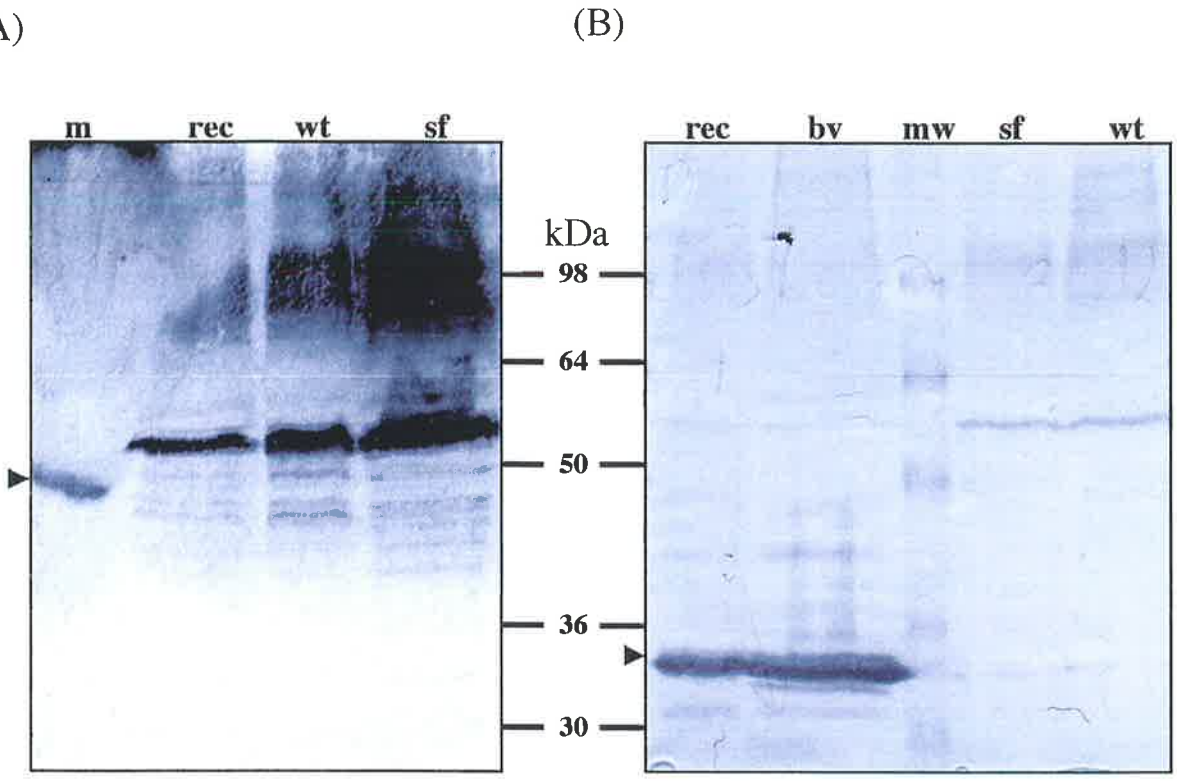


Figure 3-32. Western blot analyses (12% SDS-PAGE) showing production of recombinant proteins by lepidopteran cell lines infected with plaque-amplified recombinant *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV). Proteins from cells infected with recombinant AcMNPV (*rec*), budded virions originating from these cells (*bv*) and culture medium (*m*) were tested for presence of recombinant protein. Naive cells (*sf*) and those infected with wild-type AcMNPV (*wt*) were used as negative controls. *A*, recombinant CrV1 was detected mainly in the culture medium (*m*), using anti-CrV1 antiserum (1:5000) as a probe. *B*, detection of recombinant Crp32 in infected cells (*rec*) and budded virions (*bv*), using anitserum against *C. rubecula* calyx fluid (1:5000) as a probe. Molecular weight protein marker is shown (*mw*).

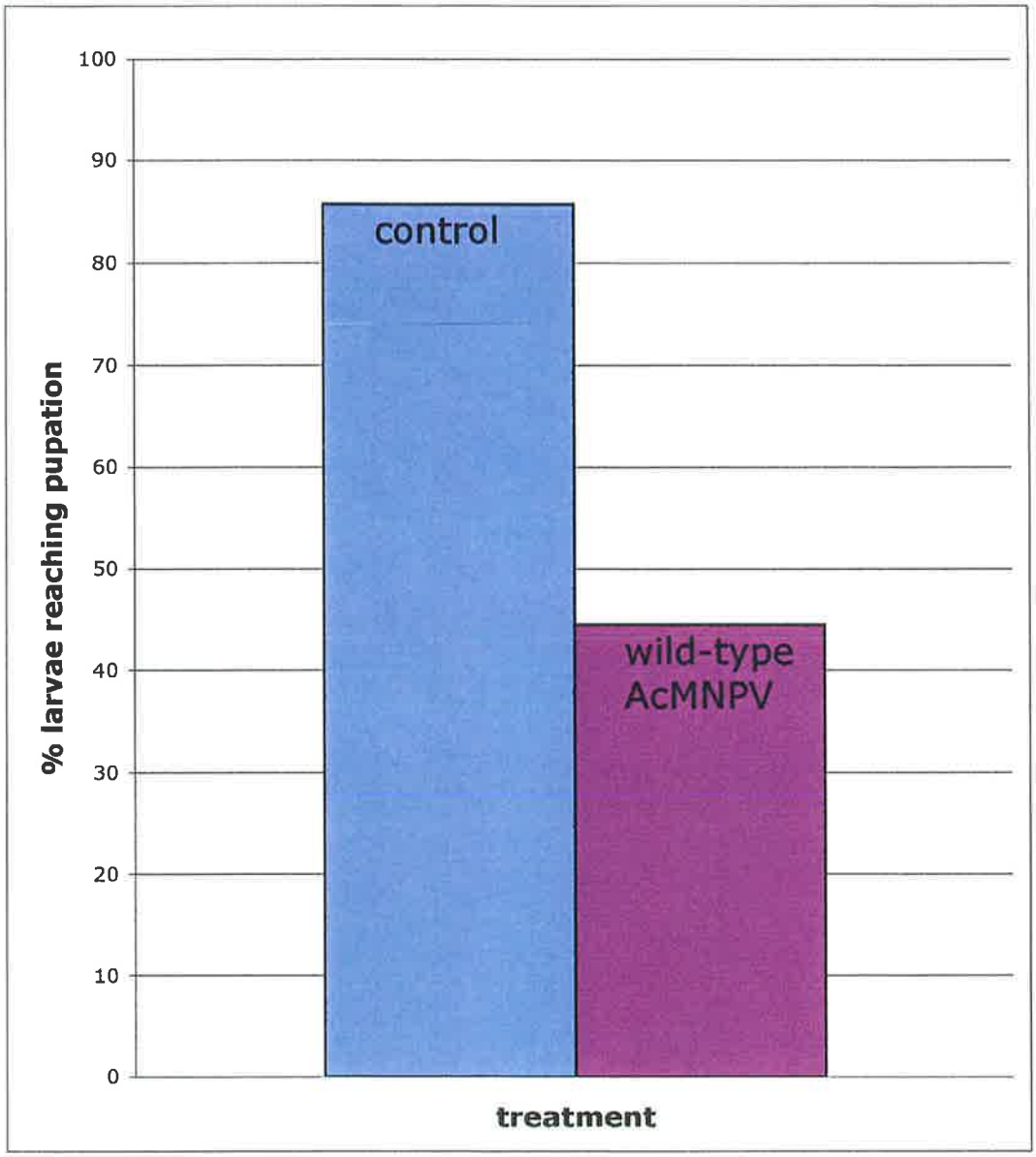


Figure 3-33. Effect of wild-type *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) on the percentage of third instar *P. rapae* larvae successfully reaching pupation. Larvae were fed leaf discs coated with wild-type polyhedra or with control leaf discs (no polyhedra) before being placed on a normal diet. Chi squared testing on pooled data suggested that the effect of AcMNPV was significant, with $p < 0.0009$.

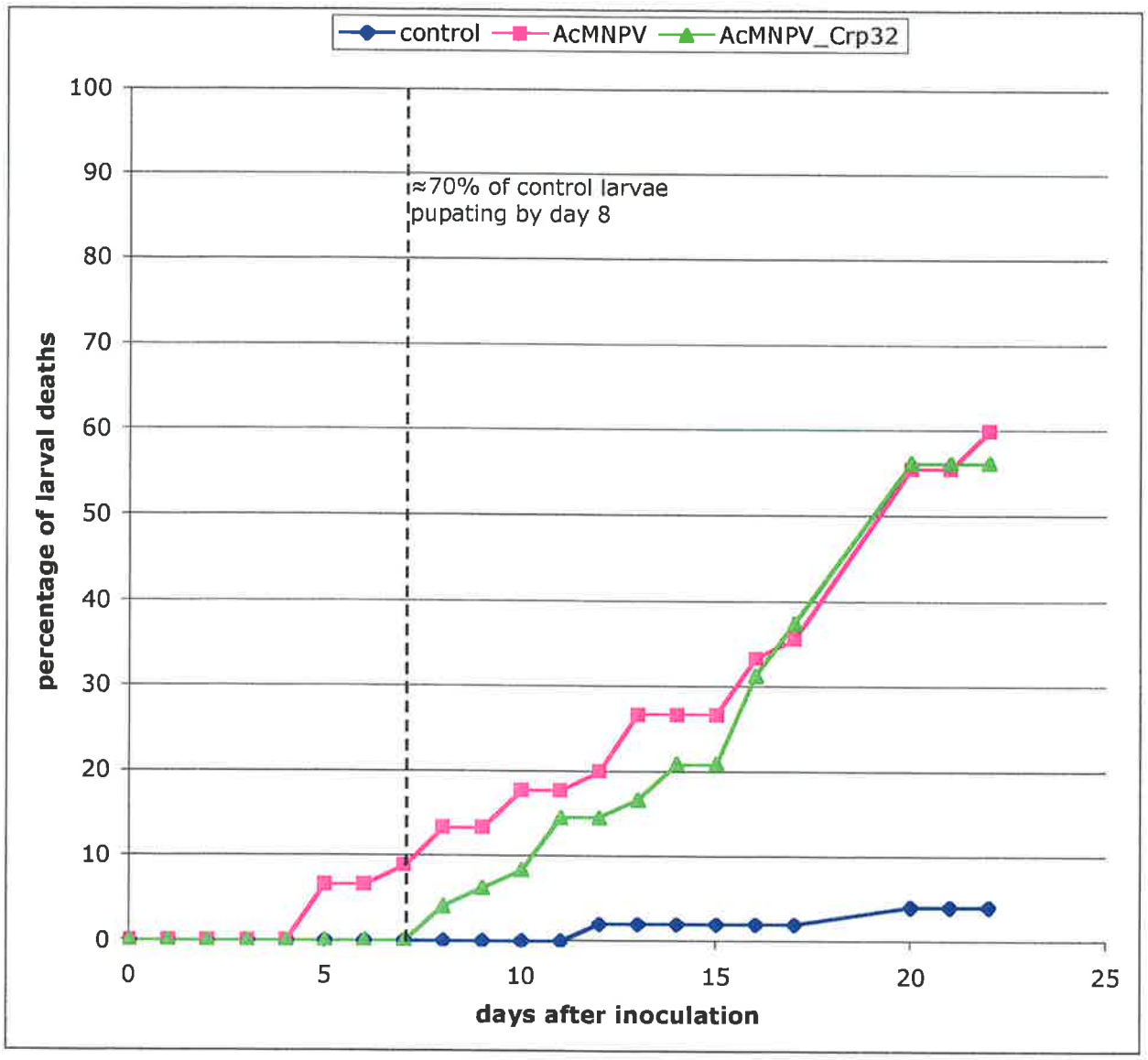
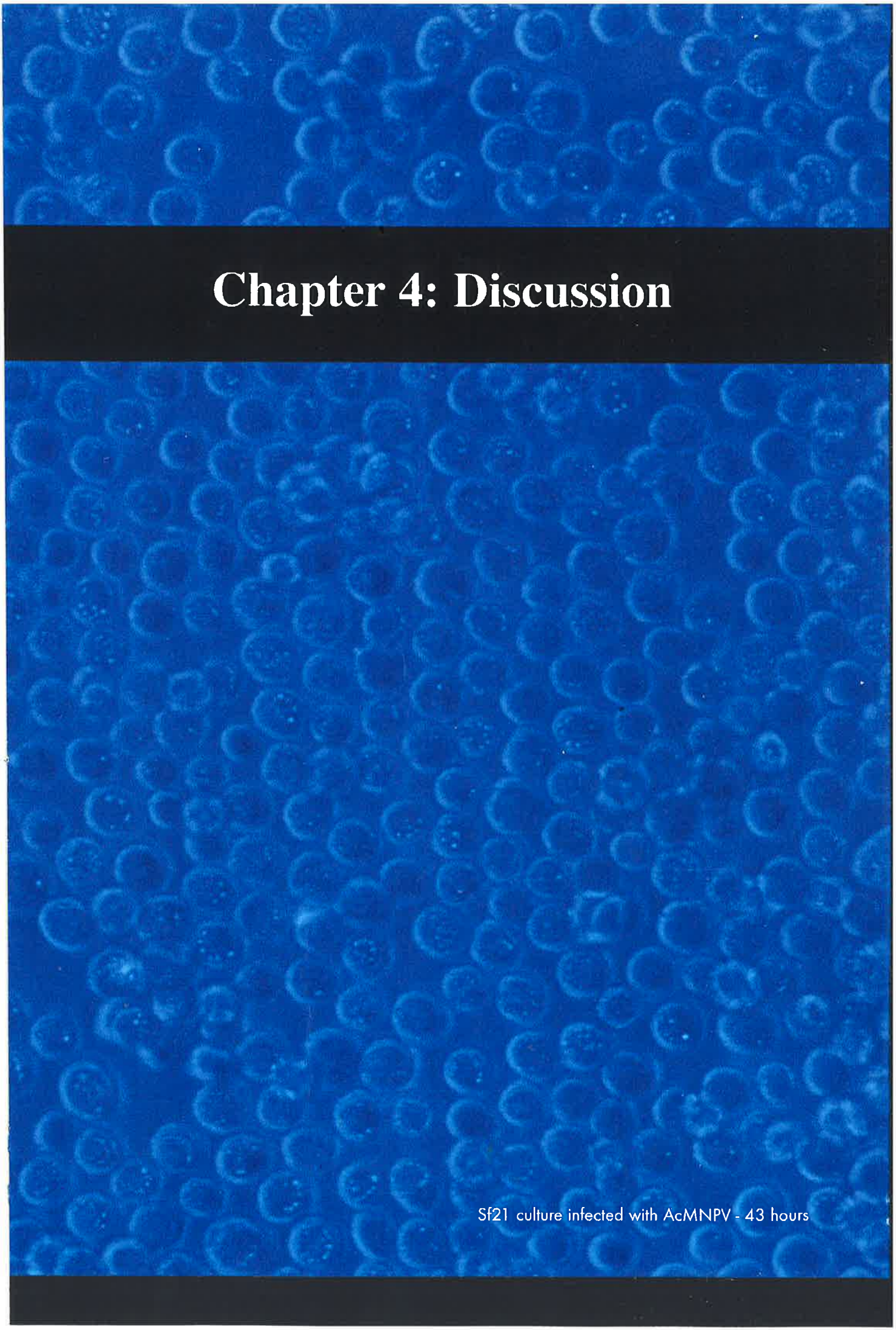


Figure 3-34. Effect of wild-type and recombinant *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) on the timing and percentage of third instar *P. rapae* larval deaths. Larvae were fed leaf discs coated with wild-type polyhedra or recombinant polyhedra, or alternatively with control leaf discs. The graph illustrates one typical bioassay where the number of dead larvae were counted each day and the percentage of larval deaths plotted against time after inoculation. The *dotted line* indicates the time point (for both full-scale bioassays) at which ≈ 70% of control treated larvae had reached pupation. Wild-type AcMNPV appears to be only semi-permissive in *P. rapae* larvae and the effect of adding the Crp32 transgene was insignificant.

Chapter 4: Discussion



Sf21 culture infected with AcMNPV - 43 hours

Chapter 4: Discussion

C. rubecula bracovirus genes are transiently expressed following infection of *P. rapae* cells (Asgari *et al.*, 1996). Previously, total CrBV genomic DNA was used as a probe against RNA from 6 h parasitised *P. rapae* caterpillars and four different sized viral transcripts were detected (Asgari *et al.*, 1996; Fig. 1-12). These were referred to as CrV1-CrV4 by decreasing size. CrV1 was previously isolated by screening a cDNA library made from 6 h parasitised caterpillars, using total CrBV DNA as a probe (Asgari *et al.*, 1996). In order to isolate and investigate the role of other CrBV-expressed genes in the host-parasitoid interaction, a similar approach was employed here to isolate CrV2 and CrV3. In addition, previously characterised immune-related genes (Crp32 and CrV1) were individually inserted into the genome of AcMNPV in order to investigate the effect of the genes in relation to the virulence of AcMNPV in *P. rapae*.

4.1 Isolation and characterisation of CrV2

Several screens of the cDNA library led to the isolation of an ≈ 450 bp cDNA fragment encoding part of a putative CrBV gene and including a poly-A tail (Fig. 3-5). The cDNA fragment was cloned and sequenced, facilitating the design of primers to amplify 290 bp of the 5' end (CloneC-F and CloneC-R; Fig. 3-5). The amplified fragment was shown to be parasitism-specific, in that it hybridised to RNA extracted from parasitised larvae but not to RNA from naive larvae (Fig. 3-2*A* and *B*), and this was confirmed using RT-PCR of RNA from parasitised and naive larvae (Fig. 3-4*A*).

The size of the hybridised transcript (≈ 1.2 kbp) corresponded to that of CrV2, detected previously (Asgari *et al.*, 1996). Further, the clone hybridised to digested viral DNA (≈ 15 kbp

fragment; Fig. 3-1) and to genomic DNA from *C. rubecula* adults but not to *P. rapae* genomic DNA (Fig. 3-3*A*). Thus, it is clear that the cDNA fragment originated from CrBV particles introduced to *P. rapae* larvae at parasitisation. Binding of the cDNA to only one transcript in the Northern blot (Fig. 3-2*A* and *B)* revealed that CrV2 shows no significant homology with other CrBV-related genes or *P. rapae* genes.

5' RACE was then employed to extend the cDNA towards the 5' end, producing a fragment containing the complete the open reading frame. The total CrV2 open reading frame of 960 bp and flanking sequences are shown in figure 3-5, and important sites in the gene (including initial cDNA clone and all primer binding sites) are highlighted. Analysis of transcribed and genomic CrV2 sequences indicated that CrV2 does not contain an intron.

Comparison of CrV2 nucleotide sequence against other known sequences revealed no significant homology. Computer analyses of the deduced amino acid sequence predicted a N-terminus signal peptide followed by a cleavage site (Fig. 3-5). This was supported by the presence of highly hydrophobic residues in the same region (Fig. 3-6*A*), indicating that the signal peptide may function in anchoring the protein to the membrane, prior to cleavage, and subsequent release of the protein from infected cells into the extracellular space. N- and O-glycosylation sites were also predicted (Fig. 3-5), suggesting that the CrV2 protein undergoes post-translational glycosylation.

Computer analysis also predicted the presence of a coiled-coil region near the C-terminus of CrV2 (Fig. 3-6*B*), which has a high probability of being involved in trimer formation (3-17*B)*. Western blot analysis utilising anti-CrV2 antiserum, under non-denaturing conditions, showed the presence of what appears to be a CrV2 trimer of ≈ 98 kDa in size (Fig. 3-17*A*). Coiled-coil

regions are commonly associated with the multimerisation of proteins (Alber, 1992), however, a coiled-coil region found in CrV1 was shown to be essential for lipophorin-binding and uptake by haemocytes but was not required for dimerisation of CrV1 (Asgari & Schmidt, 2002). Based on observed CrV2 trimer formation under non-denaturing conditions and prediction of high probability of trimer formation due to the coiled-coil region, it appears that the coiled-coil region is likely to function in this way. However, a role associated with binding to transport proteins (which may be a prerequisite for entry into target cells) or cell-surface receptors, cannot be ruled out.

Recombinant bacteria were used to express CrV2 (Figs. 3-9*A* and 3-10*A*) and recombinant CrV2 was used to generate antibodies in rabbits (Fig. 3-11*A*). The anti-CrV2 antiserum was then utilised to detect the presence of large amounts of CrV2 in the cell-free haemolymph of parasitised larvae (Fig. 3-12*A*). Anti-CrV2 antiserum also allowed a previously detected parasitism-specific glycoprotein, found in the cell-free haemolymph of 6 h parasitised larvae (Asgari, 1997), to be identified as CrV2 (Figs. 3-12*B* and *C*). The presence of CrV2 in cell-free haemolymph of parasitised larvae, confirms that CrV2 is secreted from infected cells into the surrounding serum. Further, CrV2 must be a glycoprotein containing N-acetyl-D-galactosamine residues, as it was previously detected by HPL-binding (Asgari, 1997; Fig. 3-12*B*). The detection of CrV2 at 6 hpp, but not at 2 hpp (Fig. 3-12*B*), is consistent with the timing of expression of CrV1-CrV4 (Asgari *et al.*, 1996).

RT-PCR was used to detect CrV2 transcripts in fat body and haemocytes, with haemocytes giving a very weak signal (Fig. 3-13*A*). It was initially suspected that fat body supported significantly greater CrV2 transcription compared to haemocytes, however, this was refuted by

quantitative Northern slot-blot that showed similar transcript levels in both cell-types (Fig. 3-15). These data are consistent with secretion of CrV2 into cell-free haemolymph, from infected haemocytes and fat body cells. The protein was already present in large amounts (in each sample) at 6 hpp, remained at high levels for at least 24 h and was in decline by 48 hpp (Fig. 3-16). Thus, it is apparent that although transcription of CrV2 (and other CrBV genes) is transient, and is much reduced at 12 hpp (Asgari *et al.*, 1996), the CrV2 protein persists for several days *in vivo*.

The 18S rRNA gene fragment isolated from *P. rapae* (Fig. 3-14) proved to be useful for quantitative Northern blot experiments, allowing measurement of relative levels of total RNA used as a target for hybridisation (Fig. 3-15). Attempts were initially made to utilise *Drosophila melanogaster* rRNA for this purpose but a lack of homology with *P. rapae* sequences meant that this probe was not useful at the required hybridisation temperature. The *P. rapae* 18S fragment hybridised at 65°C, the same hybridisation temperature used for CrV2 and CrV3 probes. The high level of similarity of the *P. rapae* 18S sequence with those from other lepidopteran insects (closest species were ≈ 98% identity - data not shown) means that this probe would probably be useful to determine RNA loading for these organisms also.

Most of the previously characterised PDV genes target haemocytes of parasitised larvae, e.g. CrV1 (Asgari *et al.*, 1996; Asgari *et al.*, 1997), EP1 from CcBV (Beckage & Kanost, 1993; Beckage *et al.*, 1994), VHv1.1 from CsIV (Dib-Hajj *et al*, 1993) and EGF-like genes from MdBV (Strand *et al*, 1997; Trudeau *et al.*, 2000). To investigate if haemocytes take up CrV2, FITC-labelled antibody was used to label CrV2 in infected haemocytes (Fig. 3-18*A* and *B*). Haemocytes were recovered from larvae at different times post-parasitisation (0 h, 6 h, 24 h) and tested for CrV2 presence. Although an increase in staining (compared to control) was observed at 6 hpp

(data not shown), the maximum amount of staining occurred at 24 hpp. Most of the visualised CrV2 appeared to be internalised in endosome-like structures (Fig. 3-18*B*).

The large amount of CrV2 internalised in haemocytes at 24 hpp and corresponding low level of CrV2 transcription in haemocytes at the same time point (Asgari *et al.*, 1996; Fig. 1-12), may indicate that haemocytes are the target of CrV2. In such a scenario, haemocytes and fat body cells would secrete CrV2 into the serum, from where haemocytes would acquire CrV2, as is the case for CrV1 (Asgari *et al.*, 1996; Asgari *et al.*, 1997). Indeed, CrV1 and CrV2 are similar in terms of their size and being oligomeric, secreted glycoproteins. It cannot be ruled out that some of the CrV2 visualised in haemocytes at 24 hpp is manufactured by the cells, although such high levels would not be expected for a secreted protein. Another complication that makes quantitative assessments difficult is that fat body and serum still contain relatively large amounts of CrV2 at this time point, however, CrV2 associated with fat body may be largely extracellular due to homogenisation of fat body tissue during purification.

Interestingly, the number of haemocytes showing spreading ability appeared to increase at 24 hpp, compared to 6 hpp (data not shown), indicating that haemocytes start to recover, even though large amounts of CrV2 are present within cells and in circulation. It is possible that CrV2 may not act directly in haemocyte inactivation but may be involved with enhancement of other CrBV genes, such as CrV1, or with developmental regulation.

Attempts were made to investigate the interaction of recombinant CrV2 with naive *P. rapae* haemocytes *in vitro*, however, uptake or binding were not be detected. It is suspected that the recombinant CrV2 used in these experiments may have been inactive due to denaturation

associated with the purification process. The fact that CrV2 trimers are only detected via non-denaturing Western blot analysis, whilst CrV2 monomers are only detected under denaturing conditions (caused by low levels of SDS), indicates that the active CrV2 molecules are highly sensitive to such conditions. This is in contrast to CrV3 multimers, which were visible under denaturing conditions and required heating to cause dissociation of CrV3 oligomers (see below). Further studies are required to investigate conditions required to obtain active purified CrV2, the mode of action of CrV2 and its exact role in parasitism. Experiments should aim to determine any interactions between CrV2 and haemocytes *in vitro*, both alone and in the presence of other CrBV proteins. Injection of active recombinant CrV2 into naive larvae may also reveal if CrV2 interacts with haemocytes and if *P. rapae* seral proteins are required for haemocyte uptake.

4.2 Isolation and characterisation of CrV3

CrV3 was detected and isolated using the same procedures as for CrV2. CrV3 was shown to originate from CrBV particles by using its cDNA clone as a probe in the same series of hybridisation experiments as for CrV2 isolation, with similar results. Briefly, the CrV3-related clone hybridised to RNA from parasitised larvae but not to RNA from naive larvae (Fig. 3-2*A* and *C*), confirmed using RT-PCR utilising clone-specific primers (Fig. 3-4*B*). The size of the hybridised transcript (≈ 1.1 kbp) corresponded to that of CrV3, detected previously (Asgari *et al.*, 1996). Further, the clone hybridised to CrBV DNA (≈ 4 kbp restriction fragment; Fig. 3-1) and to genomic DNA from *C. rubecula* adults but not to *P. rapae* genomic DNA (Fig. 3-3*B*). The clone hybridised to three restriction fragments from *C. rubecula* genomic DNA as the *C. rubecula* DNA was digested with *Bam*HI and *Hind*III, and the CrV3 clone has three related restriction sites in its nucleotide sequence. Binding of the CrV3 cDNA to only one transcript in the Northern blot

(Fig. 3-2*A* and *C)* reveals that CrV3 shows no significant homology with other CrBV-related genes or *P. rapae* genes (as was the case for CrV2).

The CrV3 clone was sequenced, analysed and found to contain the full-length CrV3 open reading frame (Fig. 3-7). A putative signal peptide and corresponding cleavage site were located at the N-terminus of CrV3 protein, a characteristic supported by the presence of highly hydrophobic amino acid residues in this region (Fig. 3-8). Three putative N-glycosylation sites were identified but no O-glycosylation sites were predicted. Thus, sequence analysis predicted that CrV3 should be a secreted glycoprotein, as for CrV2 and CrV1.

In contrast to CrV2, an intron sequence (of 186 bp) was located in the genomic CrV3 fragment (Fig. 3-7) and no coiled-coil region was predicted for CrV3. Further, when the deduced amino acid sequence of CrV3 was compared to other known sequences, the spatial arrangement of key functional amino acid residues was conserved with those in carbohydrate recognition domains (CRDs) of C-type lectins (CTLs) across a wide range of organisms, from viruses to mammals (Fig. 3-19*A*). The overall structure of CrV3 is simple, with a signal peptide and conserved CRD, which is interrupted by an intron (Fig. 3-7*B*). No other functional domain appears to be present. Thus, sequence similarities suggest that CrV3 is a lectin whose activity is dependent on the presence of divalent metal ions.

Interestingly, the highest levels of similarity are with hypothetical CTLs from *C. ruficrus* (67% similarity) and *C. karyai* bracoviruses (61% similarity) (Teramoto & Tanaka, 2000), indicating that these proteins are homologous with CrV3 (Fig. 3-19*B*). The next closest lectins are *P. americana* and *B. mori* LPS-binding proteins, although similarity with these lectins is

approximately half that of the bracovirus lectins. CrV3 was also found to be similar to a suite of other *P. americana* lectins (data not shown).

It is of note that the *Cotesia*-associated PDV lectins show greater similarity with invertebrate lectins compared to those of other viruses. Virus lectins are generally surface proteins that are involved in attachment of the virion to specific sugar determinants on target cells (Sharon & Lis, 1989). However, polydnavirus particles enter host cells prior to lectin expression and probably express soluble lectins as part of immune-suppression, a function much closer to that of induced humoral invertebrate lectins. The simple structure of CrV3 (Fig. 3-7*B*) is also a feature shared with several invertebrate CTLs.

Very few parasite or parasitoid lectins are known, but those that are appear to show homology with host proteins that are important for immune responses against the parasite (Loukas & Maizels, 2000). Sequence similarities between CrV3-like lectins and invertebrate lectins, and similarities in parasite/host lectins, may indicate that some parasite/polydnavirus genes originate from host genetic material. Further, it appears that *Cotesia*-related bracoviruses express a novel polydnavirus gene-family of closely related lectins. Other PDV gene families have been identified (Turnbull & Webb, 2002) but no invertebrate virus protein has thus far been characterised as a CTL. Thus, CrV3 is unique in that it is associated with an invertebrate virus and that it appears to be involved in virus-mediated host manipulation, after the entry of virions into host cells. Much debate exists as to the ancestral form of PDVs. The bracovirus lectins may be important for evolutionary studies and appear to support hypotheses that a bracovirus was present in a common *Cotesia* ancestor and that some bracovirus genes originated from insects.

In order to test if CrV3 is indeed a CTL, recombinant CrV3 was used in a series of hemagglutination experiments. Purified recombinant CrV3 agglutinated trypsinised and glutaraldehyde-fixed ovine red blood cells (ORBCs), whereas other copurified proteins (i.e. from bacteria containing empty expression vector) failed to cause agglutination (Figs. 3-20*A* and *B*).

The hapten sugar of CrV3 would be expected to significantly inhibit CrV3-mediated hemagglutination, when present in the agglutination assay at biological concentrations. The agglutination assay proved to be suitable for determining the hapten sugar, in that hemagglutination caused by HPL was able to be inhibited by addition of its hapten sugar, N-acetyl-D-galactosamine (Hammerström & Kabat, 1971; Fig. 3-20*C*). Thus, the identity of the specific sugar ligand(s) of CrV3 was investigated by addition of a variety of individual saccharides to CrV3 agglutination mixtures. A typical agglutination-inhibition assay is shown in figure 3-21. CrV3-mediated agglutination was not significantly inhibited by any of 29 potential ligands tested at 100 mM (summarised in table 3-1).

The hapten sugar of a lectin commonly inhibits lectin-mediated agglutination at concentrations below 1 mM (e.g. galactose-specific *Drosophila* lectin; Haq *et al.*, 1996). None of the common mono- and disaccharides were significantly inhibitory (even at 100 mM), a result which was not expected, given the relatively simple specificities of the closest invertebrate lectins (commonly binding galactose or related saccharides). Given the similarities observed between parasite-derived CTLs and the host-derived CTLs important in targetting them (Loukas & Maizels, 2000), it is conceivable that carbohydrates on a *Pieris* lectin (or other glycoprotein), perhaps similar to CrV3, may be the primary ligand of CrV3. Thus, CrV3 would suppress immune-activity of the hypothetical *Pieris* protein by removing it from circulation. The primary ligand may also be an

immune-induced form of a commonly occurring molecule, acquiring an unusual glyco-modification or proteolytic cleavage as part of immune induction.

As would be expected for a CTL, activity was completely abolished in the presence of 1 mM EDTA (Fig. 3-22*A*), which chelates any available metal ions. Further, abolishment of lectin activity by EDTA was able to be reversed by addition of 0.5 mM Mg^{2+} and 1 mM Mn^{2+} but was not reversed by Ca^{2+} concentration up to 5 mM (Fig. 3-22*B*). Also, activity of purified recombinant CrV3 extract (without EDTA addition) was enhanced in the presence of 1 mM Mg^{2+} and 1 mM Mn^{2+} but not by 1 mM Ca^{2+} (Fig. 3-23). Again, Mg^{2+} gave the strongest enhancement with Mn^{2+} providing slightly weaker enhancement. The effect of Mn^{2+} had a marked CrV3 concentration-dependent threshold, whereas the effect of Mg^{2+} gradually decreased as CrV3 levels were reduced (Fig. 3-23). This may indicate that Mn^{2+} may be important for tight regulation of CrV3 activity *in vivo*. The enhancement of recombinant CrV3-mediated hemagglutination by divalent metal ions is summarised in table 3-2. It is important to consider that metal dependence of native CrV3 may differ from that of recombinant protein.

Although, CrV3 shows homology with other CTLs and has displayed divalent ion-dependent lectin activity, its calcium independence and lack of binding to common galactose-derived sugars is atypical among other characterised CTLs. It is possible that recombinant CrV3 has altered specificity compared to wild-type CrV3, due to differences in post-translational modifications, however this is unlikely given that lectin activity is readily demonstrated in ORBC aggregation and its dependence on divalent ions. It seems more likely that CrV3 requires a complex sugar or amino acid residues for its binding, or is highly discerning in relation to which sugar anomer is encountered or what accessory elements are attached to the basic sugar monomer.

Amino acid residues on each side of the conserved proline (Pro^{126} in CrV3, Fig. 3-19*B*) are known to be important determinants of carbohydrate specificity (Drickamer & Taylor, 1993). The closest invertebrate lectins to CrV3 mostly exhibit galactose-type binding and are characterised by the sequence Gln-Pro-Asn, whereas the equivalent CrV3 sequence is Lys^{125}-Pro^{126}-Ser^{127} (Fig. 3-19*B*). Whilst other galactose-type binding lectins have a serine residue following the conserved proline (Drickamer & Taylor, 1993), as do the other hypothetical bracovirus lectins, the occurrence of the preceding lysine residue (as in CrV3) is rare among such lectins. Thus, the unusual CrV3 sequence may possibly explain why simple galactose-derived sugars do not inhibit CrV3-mediated agglutination and may indicate that CrV3 specificity is atypical. It is perhaps intuitive that CrV3 may have highly specific binding requirements as it presumably targets an individual element associated with host immunity.

Preliminary *in vitro* experiments suggest that CrV3 may lessen the ability of naive host haemocytes to spread on a foreign surface and may cause agglutination of these cells at high concentrations when present in the surrounding medium but does not seem to attach to these cells. It is possible that CrV3 interacts with a soluble haemolymph component that is required for activation of cellular defence. Without knowledge of CrV3 specificity, purification of native CrV3 to homogeneity from parasitised *P. rapae* larvae remains problematic.

Recombinant CrV3 was used to generate anti-CrV3 antiserum (Fig. 3-11*B*). Western blots utilising anti-CrV3 antiserum allowed identification of two CrV3-related monomers, which are present mainly in the cell-free haemolymph (Figs. 3-24 and 3-25*B*). Thus, it was confirmed that CrV3 is secreted from infected cells, into cell-free haemolymph. The two monomers were $\approx$ 17

kDa and ≈ 14 kDa in size, and were present in a ratio of ≈ 2:1 as judged by the intensity of electrophoresed bands (Fig. 3-24).

RT-PCR, utilising primers to the CrV3 open reading frame, was used to test for production of CrV3 transcript in fat body and haemocytes from 6 h parasitised larvae (Fig. 3-25*A*). These data indicate that CrV3 is produced by haemocytes and fat body cells. Western blot analysis, using anti-CrV3 antibodies, was then performed on total proteins from 6 h parasitised larval fat body, haemocytes and cell-free haemolymph (Fig. 3-25*B*). The presence of a large amount of CrV3 in the cell-free haemolymph compared to fat body or haemocytes, again confirms that the protein is secreted and possibly interacts with soluble haemolymph components, rather than haemocytes.

It appears that the relative amount of each CrV3 monomer varies with its location within parasitised larvae (Fig. 3-25*B*). In cell-free haemolymph, the ratio of ≈ 17 kDa monomer to ≈ 14 kDa monomer is usually 2:1 (see Figs. 3-24 and 3-25*B*), whilst in fat body the ratio is reversed (Fig. 3-25*B*). These data are again consistent with CrV3 being secreted from fat body (and/or haemocytes) into the haemolymph as this is where most of the secreted (≈ 17 kDa) monomer is detected. CrV3 monomers of ≈ 14 kDa, detected in fat body and haemocytes, is probably intracellular. The smaller monomer detected in serum is presumably a deglycosylated form of the secreted monomer. However, some of the ≈ 14 kDa monomer in fat body may be intercellular as proteins are extracted from total fat body, not only from fat body cells.

CrV3 hexamers, and smaller oligomers, were detected in purified recombinant CrV3, under denaturing conditions (Fig. 3-26*A*). The small amount of CrV3 associated with haemocytes (relative to cell-free haemolymph) indicates that haemocytes are probably not the target of CrV3.

Indeed, CrV3 was not detected in haemocytes from 6 h parasitised larvae using FITC-labelled secondary antibodies (data not shown).

Heating recombinant CrV3 to 65°C resulted in a breakdown of smaller multimers into their components (Fig. 3-26*A*). However, boiling resulted in an increase of all detectable multimers (Fig. 3-26*B*), indicating that CrV3 is forming much larger homogeneous complexes that are denatured at temperatures above 65°C. These large complexes were probably not entering the acrylamide gel or were not transferred to the membrane. Formation of large multimers is characteristic of several of the invertebrate CTLs previously characterised (Marchalonis & Edelman, 1968; Giga *et al.*, 1985; Kubo & Natori, 1987; Muramoto & Kamiya, 1990; Saito *et al.*, 1997). The observation that CrV3 appears to only form multimers that are multiples of two, suggests that pre-formed CrV3 dimers are the minimum element required for polymerisation. The fact that bacterial CrV3 forms multimers indicates that sugar residues are not required for dimerisation/multimerisation.

Dimer and tetramer CrV3 molecules were also detected in small amounts, under denaturing conditions, in parasitised larvae (Figs. 3-26*C* and 3-27). The relative amount of different oligomers appeared to vary with individual larvae and often only one type was detected (compare Figs. 3-26*C*, 3-27*A* and *B*). The significance of this phenomenon is not clear. Boiling of cell-free haemolymph proteins from 6 h parasitised larvae resulted in an increase in CrV3 tetramers and a decrease in dimers (Fig. 3-26*C*). It seems likely that boiling denatures the dimers and releases the tetramers from a larger CrV3 multimer or was released from a complex formed with a soluble haemolymph component (or both).

Treatment of cell-free haemolymph from 6 h parasitised larvae with a recombinant N-glycosidase resulted in removal of the larger monomers and an increase in the amount of smaller monomers, suggesting that the larger monomer is an N-glycosylated form of the smaller monomer (Figs. 3-27*A* and *B*). No putative O-glycosylation sites were predicted by computer analysis. CrV3 dimers and tetramers, detected in small amounts under denaturing conditions in parasitised larvae, were both shown to contain glycosylated monomers (Fig. 3-27). Lectin monomers with similar characteristics to CrV3 (i.e. similar size, ratio and differing only by glycosylation) have been identified in *Drosophila melanogaster* (Haq *et al.*, 1996), although the biological significance of glycosylation (or deglycosylation) of the *Drosophila* lectin is not understood.

Presumably, only glycosylated CrV3 monomers are secreted from infected cells before wasp or host enzymes remove N-linked carbohydrates to produce ≈ 14 kDa monomers. A similar phenomenon has been reported for the CrV1 protein, which has N-acetyl-D-galactosamine residues removed by *P. rapae* haemolymph (Asgari *et al.*, 1997). CrV3 serum concentration was peaked at ≈ 6 hpp but was almost undetectable in serum by Western blot analysis at 24 h parasitisation (Fig. 3-24), an observation consistent with the transient expression of CrV3 (Asgari *et al.*, 1996).

Further research will aim to determine the CrV3 binding specificity, obtain purified native CrV3 and determine its mode of action in parasitisation. Using such information, CrV3 may possibly be developed for commercial uses e.g. as a diagnostic tool. If CrV3 has a unique specificity, CrV3 would potentially be an important tool for detecting the presence of its primary ligand in different biological systems. Lectins are widely used for such purposes, e.g. lectins from such diverse organisms as *H. pomatia* (a snail), salmon and soya bean are used routinely in automated blood

grouping machines as they agglutinate erythrocytes of one type, by binding to ligands unique to that type (Levene *et al.*, 1994). Lectins are also important for studies of epidemiology (Schalla and Morse, 1994), virology (Olofsson *et al.*, 1994) and microbiology in general (Slifkin, 1994; Lakhtin, 1994). CrV3 may be of similar value, especially if its primary ligand is associated with immune-specific modification of pre-existing proteins.

4.3 Comparison of CrV2 and CrV3 expression

Initial Western blot analyses of the CrV2 and CrV3 proteins, indicated large differences in the amount of each protein present in 6 h parasitised *P. rapae* larvae. Previous data also indicated that CrV3 transcript levels are generally lower than transcript levels of the other three major CrBV genes detected (Asgari *et al.*, 1996; Fig. 1-12).

Slot blot analysis (using *P. rapae* 18S rRNA as a control gene) showed that CrV2 transcript makes up a significantly higher proportion of total transcripts than did CrV3 transcripts (Fig. 3-28). Indeed, RNA probed with ^{32}P-labelled CrV2, produced a visible signal in just a few hours of autoradiograph exposure, whereas the same amount of RNA probed with ^{32}P-labelled CrV3 required nearly three days of autoradiograph exposure in order to produce a visible signal.

Western blot analyses indicated that the difference in the amount of CrV2 and CrV3 transcripts is maintained at the protein level (Fig. 3-29). When the same amount of total protein from haemocytes, serum and fat body were probed with antiserum against CrV2 or CrV3, the CrV2 protein was easily visualised as a strongly labelled SDS-PAGE band (Fig. 3-29A), whereas CrV3 could not be identified (Fig. 3-29*B*). In order to visualise CrV3, a much larger amount of total protein had to be loaded.

These data indicate that CrV2 is present in significantly higher amounts (than CrV3) in haemocytes, cell-free haemolymph and fat body of parasitised larvae, and that this difference is regulated at the transcriptional level. The biological significance of this differential expression is not known. It may be that the difference reflects that CrV2 targets a specific cell-type (e.g. haemocytes) and may be degraded once it has performed its function (as for CrV1), therefore larger amounts of active protein would be required in the serum. In contrast, CrV3 appears to function in cell-free haemolymph and may possibly be recycled after binding to a target molecule. Alternatively, the target molecule of CrV3 may be present in small amounts e.g. an immune-induced form of a molecule containing an unusual glycosylation or proteolytic cleavage.

4.4 Construction and bioassays of recombinant baculoviruses

The immune-suppresive action of the CrBV-derived CrV1 gene and the *C. rubecula*-derived Crp32 gene, were previously characterised. CrV1 is known to cause active immune-suppression by causing depolymerisation of the actin cytoskeleton of infected *P. rapae* haemocytes (Asgari *et al.*, 1996; Asgari *et al.*, 1997). In contrast, Crp32 provides passive protection for oviposited *C. rubecula* eggs by forming part of the egg coating layer, which mimics components of *P. rapae* cells or masks foreign determinants on the egg surface (Asgari & Schmidt, 1994; Asgari *et al.*, 1997). Because of the properties mentioned, these genes were considered as candidates for improving the virulence of pathogens of *P. rapae*, by inserting them into the genome of the pathogens. To achieve this end, two recombinant AcMNPVs were constructed, each containing one of the genes under control of an early virus promoter. AcMNPV was chosen as the pathogen because it has a wide host range compared to other baculoviruses (Du & Thiem, 1997). In addition, it is the most characterised of the baculoviruses and several transformation and recombination kits are available allowing easy production of recombinant viruses.

Recombinant AcMNPVs were used to infect cell cultures and were amplified in this way, to provide large amounts of inoculum. RT-PCR (Fig. 3-30) and Western blot analyses (Fig. 3-31) of infected cultures were used to confirm production of recombinant transcripts and proteins within infected cell cultures. Both CrV1 (Fig. 3-30*A*) and Crp32 (Fig. 3-30*B*) transcripts were successfully amplified from RNA purified from infected cultures. The resultant protein products of these transcripts were also successfully detected, although the bulk of each recombinant protein was associated with different fractions taken from infected cultures (compare Figs. 3-30*A* and *B*).

CrV1 protein was associated mainly with the supernatant of infected cultures, as was expected given that CrV1 is normally secreted from cells of origin as part of CrBV infection (Asagri *et al.*, 1996). The small amount of CrV1 detected in infected cells is believed to be due to the relatively weak action of the IE1 promoter (King & Possee, 1992). Traditionally, genes inserted into the AcMNPV genome have been placed under control of the very late promoters for polyhedrin and p10 genes (via replacement of the genes), because they are hyperexpressed and not required for viral replication within host cells (Fraser, 1992; Jarvis, 1997). CrV1 was placed under control of IE1 as it was considered that the best way to decrease time to mortalilty of infected larvae would be to produce the recombinant protein during primary infection of gut cells, rather than allowing up to 48 h for production of proteins under a very late, though strong, promoter. This approach was apparently successful in that relatively large amounts of secreted CrV1 were detected 48 h post-inoculation, as would be required for improved virulence of AcMNPV in *P. rapae*. The small amount of cellular CrV1 probably reflects that the levels produced under the IE1 promoter are generally low, compared to the amount produced as part of CrBV infection.

In contrast to CrV1, large amounts of Crp32 were associated not only with infected cells, but with budded virions that emerged from such cells. This is due to Crp32 being a non-secreted protein that is associated with the cell membrane (Asgari *et al.*, 1994) and therefore, is only removed from the cellular fraction when budded virions acquire part of the membrane during the budding process. Thus, a significant amount of Crp32 would be expected to remain associated with infected cells, especially if they have not yet produced large amounts of budded virus, whereas most CrV1 would be secreted from infected cells. The association of Crp32 with budded virions is desired since the 'visibility' of such virions to the *P. rapae* immune system should be reduced, as the virions have acquired a coating that possibly mimics *P. rapae* biochemical components. Therefore, two recombinant AcMNPVs were produced with potentially enhanced pathogenicity against *P. rapae*.

Several bioassays were conducted in order to test the pathogenicity of wild-type AcMNPV in *P. rapae* (previously unreported) and compare this with the pathogenicity of recombinant AcMNPV_Crp32. Results of these bioassays indicated that wild-type AcMNPV, administered at a high dosage, had a significant effect in terms of the proportion of *P. rapae* larvae that died compared with naive larvae (Figs. 3-33 and 3-34). However, *P. rapae* appeared to be only semi-permissive for AcMNPV, a phenomenon that has been reported in other species such as *Helicoverpa zea* (Washburn *et al.*, 2000). This was evidenced by the slow rate of larval deaths (Fig. 3-34).

In permissive hosts, most larval deaths would be expected to occur within several days of infection. However, inspection of the time-course of larval deaths in a typical bioassay (Fig. 3-34) revealed that infected larvae remained alive, and arrested in the larval phase, for much longer

than naive larvae. Seventy percent of naive larvae had reached the pupal phase eight days after mock inoculation, whereas the majority of deaths of AcMNPV-infected larvae occurred after this point (Fig. 3-34). Therefore, the effect of wild-type AcMNPV was mainly to arrest larval development and cause eventual death, apparently due to starvation as well as the effect of viral proteins.

As larvae (from individual treatment groups) were placed together on the same plant, there may have been some horizontal infection of healthy larvae due to the presence of inoculum in waste products or regurgitated fluids from infected larvae. However, given the apparently semi-permissive nature of *P. rapae* towards high doses of wild-type and recombinant AcMNPV, such a phenomenon would be expected to have negligible effect.

Symptoms such as liquefied faeces indicated that AcMNPV infection was damaging the larval gut, however, the fact that larvae remained alive for an extended period (though moribund) and eventually became light green in colour (due to lack of chlorophyll intake) indicates that effects of starvation were an important factor in producing mortality. In permissive larvae, death is not generally related to starvation (due to a relatively short time between inoculation and mortality) but is mainly due to the overwhelming effect of viral proteins. Dead *P. rapae* larvae contained significant amounts of polyhedra indicating that many cells were infected, but the efficiency of secondary infection in this system, may be poor. If used in the bioassays, it is possible that first or second instar larvae may have been affected more severely. However, it would be expected that the large amount of inoculum ingested by third instar larvae in the bioassays would have been enough to cause severe disease in a permissive species. The semi-permissive nature of *P. rapae* is not entirely unexpected in that no permissive papilionoid hosts have been previously reported.

One advantageous effect of AcMNPV-infection was that food consumption was much reduced (compared to controls) 4-5 days post-inoculation. This was not tested quantitatively but was apparent when having to provide new food plants for the control larvae only. However, the time period required for reduction of feeding would be too long for such a virus to be of commercial value.

It was hoped that infection by recombinant AcMNPV_Crp32 would lead to an increase in the proportion of larval deaths, a reduction in time to death and/or a reduction in the time until larval feeding is significantly reduced. However, the effect of the Crp32 transgene proved to be insignificant, as is shown by the time-course of larval deaths occurring in a typical bioassay (Fig. 3-34). A significant improvement in pathogenicity (due to Crp32 insertion) would lead to the larval mortality curve for AcMNPV_Crp32 being shifted to the left along the time axis (i.e. earlier in time), relative to the larval mortality curve produced by wild-type infection (see Fig. 1-18 for a generic example). However, larval mortality curves for wild-type and recombinant AcMNPVs proved to be almost identical, and certainly not significantly different (Fig. 3-34).

Previous studies of a semi-permissive lepidopteran species indicated that immune response was the primary cause of semi-permissiveness (Washburn *et al.*, 2000). The fact that the effect of insertion of Crp32 was insignificant, in terms of virulence, indicates that the semi-permissiveness of *P. rapae* is probably not entirely due to immuno-resistance, as this process should be compromised by the presence of Crp32, although this assumption requires confirmation. It appears that the immune system is probably not targetting individual virions due to differences between *Pieris* tissues and the virion surface. However, this cannot be ruled out because other proteins on the virion surface may be recognised as foreign by the *P. rapae* immune system. It

seems likely that the gut cells of infected larvae are damaged, leading to cessation of feeding, whilst further spread of infection throughout the larval body is slow. Secondary infection may be inefficient perhaps due to incompatibility of *P. rapae* cells for infection by budded virus or encapsulation of initially infected gut cells.

Experiments involving AcMNPV_CrV1 were terminated due to the semi-permissive nature of *P. rapae*, a lack of time and the need to characterise CrV3. AcMNPV_CrV1 may prove to be a more effective agent against *P. rapae* in that the transgene should actively target haemocytes (i.e. act as a systemic cellular toxin) rather than passively protecting budded virions, which may or may not be the target of the *Pieris* immune response. CrV2 and CrV3 are also possible candidates for insertion into AcMNPV, given their role as putative immune-associated molecules within *P. rapae*.

Although AcMNPV_Crp32 (and wild-type AcMNPV) were not demonstrated as being useful as commercial biocontrol agents, the system as a whole may be useful for studying how the immune system of *Pieris* responds to virus infection. When combined with reporter genes, such as LacZ, spatial and temporal information relating to viral infection, spread and possibly clearance, may be acquired. The presence of a reporter gene may allow elucidation of sub-lethal effects or differences in primary and secondary infection patterns associated with viruses containing different immune-suppressive transgenes. Such information would be invaluable in determining how and where the *P. rapae* immune system interacts with AcMNPV and any recombinant protein produced by recombinant AcMNPV infection. Thus, recombinant AcMNPVs expressing CrBV genes and reporter genes may provide a valuable experimental tool to further examine *in vivo*, the function of CrBV genes, including CrV2 and CrV3.

Such a system was successfully used to show that prior parasitisation of *H. zea* by CsIV resulted in increased distribution of AcMNPV infection within subsequently infected larvae, compared to those which were not parasitised, and further, that the cellular immune response of *H. zea* was significant in preventing AcMNPV infection, leading to the semi-permissive nature of *H. zea* (Washburn *et al.*, 2000). Semi-permissive species may potentially be more useful than highly permissive species for such studies, as the immune system is quickly overwhelmed in species that die quickly.

4.5 General discussion: the *C. rubecula*/CrBV/*P. rapae* system

The *C. rubecula*/CrBV/*P. rapae* system is unique among known endoparasitoid/virus/host interactions, in several ways. A single Northern blot (Fig. 1-12) illustrated that only four major CrBV genes are expressed and that the expression is transient, remaining strong for a period of 4-6 h (Asgari *et al.*, 1996). Three of these genes, CrV1-CrV3, have now undergone varying degrees of characterisation. Each are known to be glycoproteins that are secreted from infected cells, forming multimeric structures of varying complexity.

The small number of CrBV genes expressed in the host provides the opportunity to elucidate the complete role of CrBV in parasitisation, in a simpler and more focussed way than in other PDV systems. For example, CsIV is thought to express over 35 genes comprising several genes families (Turnbull & Webb, 2002), a factor that makes detailed determination of its role far more complex than for CrBV. The overall role of PDVs as active immune-suppressors is widely accepted. Nearly all of the characterised genes are known to target host haemocytes, however, other functions such as alteration of developmental regulation and behaviour, are also known (Fleming, 1992).

From investigations thus far, it appears that the main role of CrBV is immune-suppression of the larval host. CrV1 is known to enter host haemocytes, causing inactivation of their cytoskeleton and reducing the ability of infected haemocytes to carry out immune-related tasks, such as phagocytosis and spreading (Asgari *et al.*, 1996, Asgari *et al.*, 1997). Thus, CrV1 acts as a haemocyte-specific toxin, although its transient expression means that cells recover after a few days. Interestingly, a CrV1 homologue was identified in CcBV but differed in that expression was strong for 72 h and continued at low levels throughout development of the wasp larvae (Le *et al.*, 2003). It is believed that the CrV1 homologue affects haemocytes by mediating apoptosis (unlike for CrBV), perhaps a function of continuous expression. However, the level of complexity of the CcBV system means that linking apoptosis to a single gene is difficult.

The apparent simplicity of CrBV regulation of *P. rapae* immunity (involving only four main genes) implies that many functions performed by more complex PDVs have been lost or are being performed by wasp-derived proteins. Passive protection of the wasp egg by Crp32 (a calyx protein) may be one example of maternal protein secretions protecting the egg inside the host. It is known that *C. rubecula* injects a complex suite of at least 11 venom proteins into the host at oviposition and some of these are involved in regulation of CrBV gene expression (S. Asgari; unpublished data) and inhibition of activation of phenoloxidase, an immune-associated enzyme (Asgari *et al.*, 2003). It is possible that CrBV is involved entirely in active immune-suppression, with other functions (including some active and passive immune-suppression) being performed by calyx or venom proteins.

The function of CrV2 is as yet undetermined but given that it is a secreted protein that is detected within haemocytes in large amounts, at a time when overall CrBV expression has declined,

suggests that haemocytes are probably the target of CrV2 activity. Also, CrV2 is similar to CrV1 in terms of size, expression levels, presence of a coiled-coil region and formation of small oligomers. Such similarities, and the fact that most characterised class II PDV genes appear to target haemocytes may further indicate that the function of CrV2 is similar to CrV1. It is possible that CrV2 enhances or complements the activity of CrV1 but there is currently no evidence to support this. Although interaction of recombinant CrV2 with haemocytes *in vitro* was not demonstrated, the apparent fragility of the CrV2 trimer implies that purified recombinant CrV2 may have been inactive.

CrV3 was found to have a spatial arrangement of key functional amino acids that is conserved in CTLs from a wide variety of organisms (Fig. 3-19*A*). CrV3 was demonstrated as causing agglutination of ORBCs, i.e. displaying lectin activity, a process that was shown to be dependent on Mn^{2+} and Mg^{2+} but was independent of biological concentrations of Ca^{2+}. Apart from apparent CrV3 homologues detected in CkBV and CrBV (Teramoto & Tanaka, 2003), the closest CTLs to CrV3 were insect CTLs that are secreted into cell-free haemolymph, upon induction by foreign elicitors, such as lipopolysaccharide on bacterial surfaces. As such, these CTLs act as immune-molecules by binding to specific sugar moieties associated with foreign surfaces, thereby rendering them visible to the immune system and facilitating their removal from circulation.

Two abnormal aspects of CrV3 are the lack of binding to simple galactose-based sugars (as do most of simple invertebrate CTLs) and calcium independence. The two factors are probably related in that binding of divalent ions to CRDs causes conformational changes that allow binding of a specific sugar ligand (Drickamer, 1993). Ca^{2+} might not bind to the CrV3 CRD or it may cause a rearrangement leading to inefficient sugar binding. Alternatively, Mg^{2+} and Mn^{2+}

may cause a desired but unusual rearrangement upon binding, thereby producing an efficient saccharide binding site with unusual properties.

It seems probable that these unusual characteristics relate to the specific function of CrV3 in parasitisation. CrV3 is secreted and does not seem to be internalised by haemocytes, as evidenced by the small amount of CrV3 detected in haemocytes by Western blot analyses (Fig. 3-25*B*) and the low level of labelling of infected haemocytes by fluorescent antibodies. Therefore, it appears that CrV3 acts similarly to the closest insect lectins, in that it is soluble in the haemolymph but presumably targets an immune-associated molecule from the insect rather than a molecule foreign to the insect. The targeted molecule probably contains an unusual sugar modification that may actually represent a humoral response to CrBV infection. It may also be that CrV3 requires the presence of certain amino acids for binding, e.g. those exposed by proteolytic cleavage, or is highly sensitive to which sugar anomer it encounters or which chemical modifications are attached to the basic sugar unit contained in the ligand. Some of these features may be also specific to immune-induction associated with parasitism or virus infection.

The lack of dependence on calcium ions may be a mechanism by which CrV3 bypasses regulation of host lectins, allowing independent regulation of CrV3. It is possible that calcium independence indicates that the parasitoid drives down calcium levels as part of immune-suppression, leading to loss of function of host CTLs or other immune-associated molecules.

The fact that Mn^{2+} enhanced CrV3-mediated agglutination at concentrations near 1.0 mM, and displayed an upper threshold of activity (Fig. 3-22*B*), may indicate that Mn^{2+} is important for independent regulation of CrV3. The threshold of Mn^{2+} enhancement may be a means by which

CrV3 can be recycled, i.e. by causing release of CrV3 from a previous binding reaction when relative unbound CrV3 concentration is low. Mg^{2+} may also be important due to its strong enhancement of hemagglutination at only 0.5 mM, although this is not uncommon among CTLs. Thus, the homology of CrV3 with insect CTLs, but altered regulation, may reflect that CrV3 has evolved from an insect lectin but has undergone evolutionary changes to its regulation to make it independent of host regulation. Given similarities observed between parasite and host CTLs (Loukas & Maizels, 2000), it is conceivable that CrV3 targets a similar *P. rapae* CTL, which would presumably be Ca^{2+}-dependent and released as part of the *Pieris* immune response.

The molecules that are ultimately the closest to CrV3 are the CrV3 homologues (putative CTLs) found in bracoviruses from two other *Cotesia* wasps. Thus, these *Cotesia*-associated PDVs appear to express a unique family of unusual CTLs. Such a phenomenon suggests that these wasps originated from a common ancestor, which contained a virus with a gene homologous to CrV3. The fact that the next closest CTLs are from insects supports a theory that PDVs (or their ancestral form) have acquired some functions from their hosts. This similarity contrasts starkly with the lack of similarity of CrV3 with other known viral lectins, which are invariably attached to the virion surface and are involved with attachment to specific cell-types.

Homologues of CrV1 have been found to occur in six *Cotesia* species, and the matching phylogenetic trees created by analysis of wasp 16S rRNA and NADH1 genes, also matched that produced by analysis of CrV1 homologue sequences (Whitfield, 2000). Thus, CrV1 and CrV3 provide independent evidence of different groups of *Cotesia* wasps evolving from a common, PDV-containing, ancestor.

It is generally accepted that PDVs evolved from a pathogenic virus of an insect host, which became exposed to a parasitoid. Presumably, viral genetic material became integrated into the ancestral parasitoid genome and then began to 'lose' encapsidated genes such that eventually only those advantageous to the 'genetic host' (in this case a wasp), became packaged in virions. Thus, even though PDVs probably evolved from a pathogenic virus, it is probably more accurate to class them as a maternal secretion, an idea raised by Whitfield & Asgari (2003), rather than a traditional virus (which are usually defined as containing DNA for structure and replication, within individual virions that each contain total virus genetic information).

Evolution of PDVs from a pathogenic virus is also supported by the presence of possible intermediate forms. For example, an ascovirus associated with the ichneumonid *Diadromus pulchellus* is not pathogenic to the wasp carrier but still causes disease in lepidopetran larvae once injected (Whitfield & Asgari, 2003). The genome of this ascovirus exists as episomal DNA in wasp cells and is transferred vertically as for PDVs (Bigot *et al.*, 1997). Therefore, this ascovirus may represent an evolutionary step in the process from pathogen to wasp symbiont (Whitfield & Asgari, 2003). However, it is unlikely that the exact intermediate viral forms for PDVs are being observed and more likely that there is a continuum of genetic entities whose form may stabilise and then become diverse, once they provide a selective advantage to their host.

The loss or alteration of pathogenic genes in the lepidopteran host, is an example of a change that may confer a selective advantage to the parasitoid. It is conceivable that the high level of diversity associated with ichneumonoid endoparasitoids, and their associated PDVs, is a direct result of the selective advantage conferred by the evolution of PDVs as part of the wasp

'extended phenotype'. Given that reduction is generally considered as being an evolutionary refinement, it is tempting to speculate that CrBV is one of the most highly evolved of the known BVs. Such refinement is possibly evidenced by the very short period of high expression levels of few CrBV genes. Other PDVs also appear to show a transient period of high expression but this period generally extends for several days and viral proteins are usually produced throughout development of parasitoid larvae. Thus, even though CrBV proteins may be expressed at low levels for longer periods, the initial 4-6 h period of high expression may reflect a refinement of longer periods of high expression characteristic of other PDVs.

The relatively short period of strong CrBV gene expression may also reflect that the genes are no longer involved in developmental regulation as it would be expected that gene products would need to be continually produced to maintain required developmental alterations in the host. Thus, it appears that viral functions relating to developmental regulation are no longer undertaken by CrBV and may have been acquired by other wasp elements. Therefore, there may be a separation of functions between wasp and CrBV genes whereby CrBV genes are mainly involved in a short period of active suppression of haemocytes and their activation, whilst wasp-derived proteins may be mainly involved in developmental alteration and longer acting forms of immune-suppression. Venom proteins from exoparasitoids are involved mainly in regulation of host metabolism and immune-suppressive genes are not required as the parasitoid larvae do not directly encounter the immune system (Richards & Edwards, 1999). It is unknown how the developing *C. rubecula* larva is protected once levels of CrBV proteins decline.

It should be considered, however, that unique (possibly more highly evolved) aspects of CrBV may reflect unique characteristics of the wasp and/or lepidopteran host environments and/or

biologies, rather than the amount of evolutionary refinement or the period of time over which evolution occurred. For example, low numbers of genes may reflect the small host range of *C. rubecula* (only *P. rapae* and *P. brassicae*) or the fact that *C. rubecula* is a solitary parasitoid (compare with CcBV from a gregarious parasitoid).

Because of the specific action of CrBV against *P. rapae* immune elements, CrBV genes are potential candidates for improving the virulence of pathogens of *P. rapae* (e.g. AcMNPV). Although the addition of Crp32 to the AcMNPV genome caused no significant effect on pathogenicity, active immune-suppressors such as CrV1 (possibly CrV2 and CrV3) may be more useful, as they may 'seek and destroy' *P. rapae* immune elements, especially if expression is continuous. Such a pathogen would potentially be of use as specific biological control agent of *P. rapae*, a worldwide pest of crucifers.

Aside from their use as biocontrol agents, there are several other reasons for producing recombinant baculoviruses containing CrBV genes. Firstly, baculoviruses infect eukaryotic cells and as such, any post-translational modifications of recombinant proteins will be closer to those found *in vivo*. Thus, large amounts of eukaryotically modified CrBV protein can be produced by, and purified from, cell cultures.

Secondly, the semi-permissive nature of *P. rapae* for AcMNPV, means that the immune system of *P. rapae* must encounter the virus and must be able to mount some form of defence against AcMNPV, even though larvae became moribund and eventually died. Symptoms exhibited by infected larvae were consistent with primary AcMNPV infection causing damage to gut cells (thus causing cessation of feeding), with secondary infection being quite slow and resulting in

larvae living for long periods before succumbing to the disease. Thus, when combined with a reporter gene, temporal and spatial infection patterns, along with associated immune responses, can be determined for wild-type AcMNPV. These data could then be compared with those obtained when using recombinant AcMNPVs, expressing each CrBV gene, or wasp genes. It would be expected that the immune system would interact with each virus in a slightly different way, which may or may not affect the virulence of the virus. Such a system would allow investigation of elements of the *P. rapae* immune response and how they interact with viral or wasp proteins, and thus, may infer how the transgenes function and their role in parasitisation.

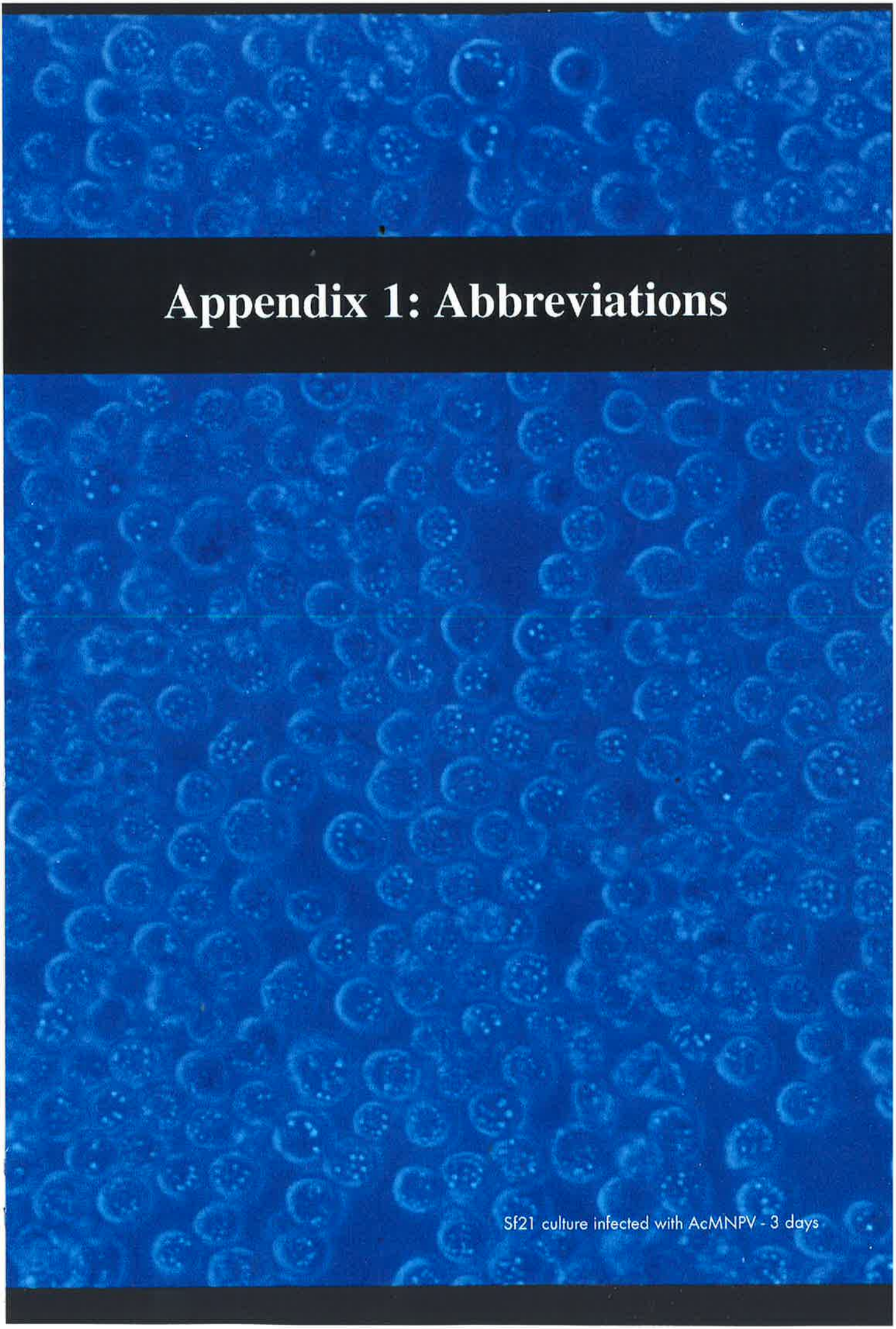
Another approach may be to use RNA silencing of individual CrBV genes expressed in infected cell culture, in order to detected any phenotypic differences resulting from differential CrBV gene silencing. Recently, RNA silencing was exploited successfully to show that glc1.8 (from MdBV) was associated with loss adhesive properties of infected cells (Beck & Strand, 2003).

Useful diagnostic tools, including antibodies and specific primers, have now been developed for three of the four main CrBV genes. Primers to part of the CrV4 gene were also previously developed (Asgari, S.; unpublished data) but isolation of the total gene has been difficult thus far. It is suspected that CrV4 does not contain a poly-A tail (Asgari, S., pers. comm., 2003) whereas the currently available cDNA library (made from 6 h parasitised *P. rapae* larvae) only contains transcripts that have been polyadenylated. However, it should be a relatively simple exercise to isolate and characterise CrV4. AcMNPVs containing CrV1 have been produced as part of this study and the production of other recombinant AcMNPVs containing CrV2-CrV4 can be easily constructed.

Given these tools, the *P. rapae*/CrBV/*C. rubecula* system represents a unique opportunity to develop a complete model of immune-suppressive activity carried out by CrBV and thus glean more general information relating to PDVs and insect immunity. No other PDV system is close to being fully characterised, in terms of the function of viral genes within the host. Genes such as CrV1 and CrV3 also raise interesting question about the origin of PDVs and their genes.

Future research will aim to further characterise elements of CrBV gene expression and determine the detailed function and regulation of the gene products. Evolutionary studies will further explore evidence relating to ancestral PDV forms and the way in which PDVs have apparently driven the successful radiation of certain ichneumonoid endoparasitioids. Further investigation of CrV3 should also provide useful data. If the unusual sugar specificity can be determined along with its cause, this will provide valuable information on how ligand specificity of CTLs is regulated and may give clues as to the target molecule of CrV3. Such a lectin could conceivably have commercial potential given its unusual specificity and regulation, e.g. may be developed as a diagnostic tool to detect an unusual ligand, related to some biological function or situation, such as infection. In general, the close biological interactions involved in parasitoid/PDV/host systems, may provide much information about the evolution of genetic entities and the way these entities acquire highly specific interactions with their host, apparently providing a selective advantage for both parties.

Appendix 1: Abbreviations



Appendix 1: Abbreviations

AaIT	*Androctonus australis* insect toxin
ABS_λ	absorbency of a solution at a given wavelength (λ) in nanometres
AcMNPV	*Autographa californica* multiple nucleopolyhedrosis virus
AcMNPV_Crp32	recombinant AcMNPV expressing Crp32 gene
AcMNPV_CrV1	recombinant AcMNPV expressing CrV1 gene
AgMNPV	*Anticarsia gemmatalis* multiple nucleopolyhedrosis virus
AMV-RT	Avian Myeloblastosis Virus reverse transcriptase
Asn	asparagine
BeIT	*Buthus eupeus* insect toxin
BEVS	baculovirus expression vector system
BmSNPV	*Bombyx mori* single nucleopolyhedrosis virus
bp	base pair(s)
BHv0.9	member of cys-motif gene family expressed by CsIV
BPB	bromophenol blue
BSA	bovine serum albumin
BT	*Bacillus thuringiensis*
BV	bracovirus
C	carbon
°C	degrees centigrade
Ca/Ca^{2+}	calcium/calcium ions
CcBV	*Cotesia congregata* bracovirus
CkBV	*Cotesia karyai* bracovirus
Cl	chloride (or chlorine)

CloneC-F	forward primer to amplify 303 bp of initial CrV2 cDNA clone
CloneC-R	reverse primer to amplify 303 bp of initial CrV2 cDNA clone
CloneC-R2	first nested reverse primer for 5' RACE of CrV2 cDNA clone
CloneC-R3	second nested reverse primer for 5' RACE of CrV2 cDNA clone
CmeBV	*Cotesia melanoscela* bracovirus
CnBV	*Cardiochiles nigriceps* bracovirus
CrBV	*Cotesia rubecula* bracovirus
CRD	carbohydrate recognition domain
Crp32	32 kDa *C. rubecula* calyx protein found on egg surface and CrBV virions
CrV1	encapsidated haemocyte-targetting gene expressed by CrBV
CrV2	encapsidated gene expressed by CrBV thought to target host haemocytes
CrV2-F	forward primer for amplification of the CrV2 open reading frame
CrV2-R	reverse primer for amplification of the CrV2 open reading frame
CrV3	encapsidated C-type lectin homologue expressed by CrBV
CrV3-F	forward primer for amplification of the CrV3 open reading frame
CrV3-R	reverse primer for amplification of the CrV3 open reading frame
CrV4	encapsidated gene of unknown function expressed by CrBV
CsIV	*Campoletis sonorensis* ichnovirus
CTL	C-type lectin
cys	polydnavirus gene family containing a cysteine-rich motif
DDC	DOPA decarboxylase
DNA	deoxyribose nucleic acid
dNTPs	deoxynucleotide triphosphates
DOPA	dihydroxyphenylalanine

dsDNA	double-stranded deoxyribose nucleic acid
DT	dopachrome tautomerase
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
EGF	epidermal growth factor
egf1.0	member of EGF-like gene family expressed by MdBV
EP1	member of early gene family expressed by CcIV
EP3	member of early gene family expressed by CcIV
et al.	*et alii* (and others)
FITC	flourescein isothiocyanate
g	gram(s) or one times the force of gravity
GBP	growth-blocking peptide
glc1.8	MdbV gene implicated in loss of cell adhesion properties
Gln	glutamine
gp64	64 kDa envelope glycoprotein of budded viruses
Gu	guanidine
h	hour(s)
H	hydrogen
HaGV	*Helicoverpa armigera* granulosis virus
HaSNPV	*Helicoverpa armigera* single nucleopolyhedrosis virus
hcf-1	AcMNPV gene encoding a host cell factor
HdIV	*Hyposoter didymator* ichnovirus
His	histidine
HPL	*Helix pomatia* lectin

hpp	hours post-parasitisation
hrf-1	LdMNPV gene encoding a host cell factor
HzSNPV	*Helicoverpa zea* single nucleopolyhedrosis virus
i.e.	*id est* (that is)
IE1	immediate early gene expressed by AcMNPV
IEP	50 kDa *C. karyai* calyx protein found on egg surface and CkBV virions
IPM	integrated pest management
IPTG	isopropyl-ß-D-thiogalactopyranoside
IV	ichnovirus
JHE	juvenile hormone esterase
K	potassium
kbp	kilobase pair(s)
kDa	kiloDalton(s)
LacZ	*Escherichia coli* gene coding for ß sub-unit of ß-galactosidase
λ	wavelength
LB	Luria-Bertani
LdMNPV	*Lymantria dispar* multiple nucleopolyhedrosis virus
LPS	lipopolysaccharide
LqhIT2	*Leirus quinquestriatus hebreus* insect toxin 2
Lys	lysine
M	molar
M13	promoter originating from M13 bacteriophage
mA	millampere(s)
MdBV	*Microplitis demolitor* bracovirus

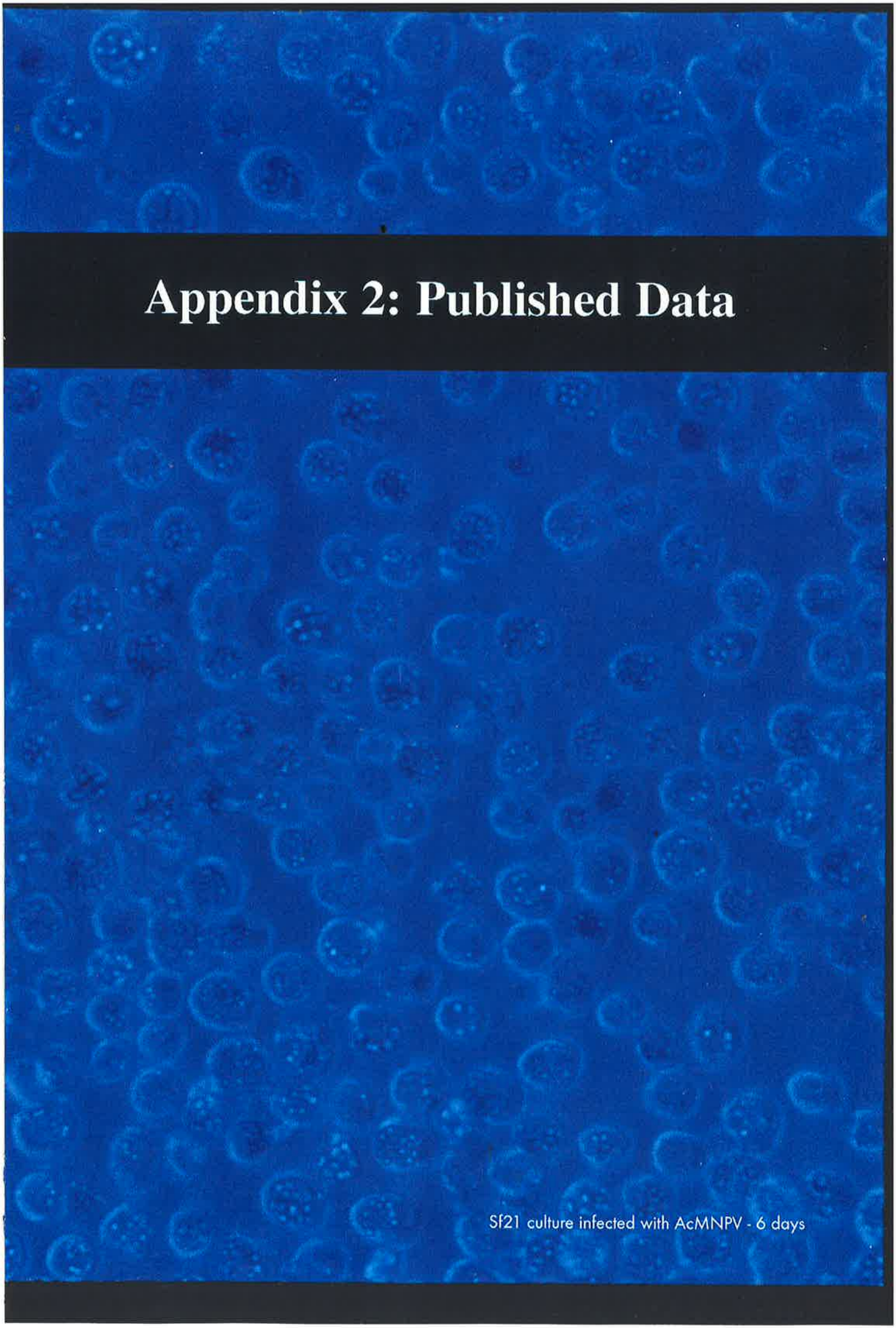
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
min	minute(s)
mg	milligram(s)
Mg/Mg^{2+}	magnesium/magnesium ions
mJ	millijoule(s)
ml	millilitre(s)
MNPV	multiple nucleopolyhedrosis virus
Mn/Mn^{2+}	manganese/manganese ions
MOPS	3-[*N*-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid
Na	sodium
NADA	N-acetyl dopamine
NCBI	National Centre for Biotechnology Information
ng	nanogram(s)
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre(s)
O	oxygen
ORBC	ovine red blood cell
P	phosphorous
p10	baculovirus p10 protein involved in host cell lysis
p12	encapsidated gene expressed by CsIV
p35	baculovirus gene encoding the p35 protein which inhibits apoptosis

p44	CsIV structural protein
^{32}P	α-^{32}P-labelled deoxycytosine triphosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDV	polydnavirus
pH	negative logarithm of hydrogen ion concentration in moles/litre
Pier-Rib-F	forward primer used to RT-PCR 865 bp fragment of *P. rapae* 18S rRNA
Pier-Rib-R	reverse primer used to RT-PCR 865 bp fragment of *P. rapae* 18S rRNA
PO	phenoloxidase
PRM	pattern recognition molecule
Pro	proline
PTU	phenylthiourea
RACE	rapid amplification of cDNA ends
Rb	rubidium
rep	polydnavirus gene family with 540 bp conserved repeated element
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature (and pressure)
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine

Sf9	insect cell line derived from *Spodoptera frugiperda*
Sf21	insect cell line derived from *Spodoptera frugiperda* ovaries
SNPV	single nucleopolyhedrosis virus
sp.	species
SSC	sodium-sodium-citrate
TAE	Tris-acetate/EDTA buffer
Taq	*Thermus aquaticus*
TBE	Tris-borate/EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
Tris	Tris-hydroxymethyl-aminomethane
TrIV	*Tranosema rostrale* ichnovirus
TrV1	glutamate-rich protein expressed by TrIV
TrV2	glutamate-rich protein expressed by TrIV
T-urf13	maize mitochondrial gene linked to male sterility
TxP1	insect neurotoxin produced by the straw itch mite, *Pyemotes tritici*
U	unit(s)
USA	United States of America
UV	ultra violet
V	volt(s)
VHv1.1	member of cys-motif gene family expressed by CsIV
VHv1.4	member of cys-motif gene family expressed by CsIV
VLP	virus-like particle
Vn50	*C. rubecula* venom protein thought to inhibit host melanisation cascades

W	DNA segment from CsIV
WHv1.0	member of cys-motif gene family expressed by CsIV
WHv1.6	member of cys-motif gene family expressed by CsIV
×	multiplied by or times concentration/volume
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

Appendix 2: Published Data



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Characterization of a Novel Protein with Homology to C-type Lectins Expressed by the *Cotesia rubecula* Bracovirus in Larvae of the Lepidopteran Host, *Pieris rapae**

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Polydnaviruses are essential for the survival of many Ichneumonoid endoparasitoids, providing active immune suppression of the host in which parasitoid larvae develop. The *Cotesia rubecula* bracovirus is unique among polydnaviruses in that only four major genes are detected in parasitized host (*Pieris rapae*) tissues, and gene expression is transient. Here we describe a novel *C. rubecula* bracovirus gene (*CrV3*) encoding a lectin monomer composed of 159 amino acids, which has conserved residues consistent with invertebrate and mammalian C-type lectins. Bacterially expressed CrV3 agglutinated sheep red blood cells in a divalent ion-dependent but Ca^{2+}-independent manner. Agglutination was inhibited by EDTA but not by biological concentrations of any saccharides tested. Two monomers of ~14 and ~17 kDa in size were identified on SDS-PAGE in parasitized *P. rapae* larvae. The 17-kDa monomer was found to be an *N*-glyscosylated form of the 14-kDa monomer. CrV3 is produced in infected hemocytes and fat body cells and subsequently secreted into hemolymph. We propose that CrV3 is a novel lectin, the first characterized from an invertebrate virus. CrV3 shows over 60% homology with hypothetical proteins isolated from polydnaviruses in two other *Cotesia* wasps, indicating that these proteins may also be C-type lectins and that a novel polydnavirus lectin family exists in *Cotesia*-associated bracoviruses. CrV3 is probably interacting with components in host hemolymph, resulting in suppression of the *Pieris* immune response. The high similarity of CrV3 with invertebrate lectins, as opposed to those from viruses, may indicate that some bracovirus functions were acquired from their hosts.

Polydnaviruses are particles specifically associated with the ovaries of certain Braconid and Ichneumonid endoparasitoids (1). They are divided into genera, *Ichnovirus* and *Bracovirus*, based on differing host range and morphology (2). Polydnavirus genomes exist as a series of different circular DNA segments (3), which are packaged singly or in groups into individual polydnavirus particles (1). Particle-associated DNA segments are known to originate from wasp chromosomal DNA and are transferred in their integrated form to subsequent generations of wasps (4). Thus, polydnaviruses from different wasps are genetically isolated from each other and considered as separate "species" (5). "Transmission" of particles is exclusively vertical (4, 6), and particles are therefore not detected in males, although episomal polydnavirus DNA may exist (7).

Production of particles is restricted to specialized ovarian calyx cells (1) and is initiated in the pupal phase, soon after the onset of cuticular melanization, and continues in female adult wasps (8–11). Although the replication mechanism is not completely understood, recent evidence suggests that controlled localized chromosomal amplification occurs before excision of the particle segments (10). Larger chromosomal segments may have smaller segments nested within (12). Particles accumulate in the oviduct and are injected into the host hemocoel, together with the parasitoid egg and various maternal secretions, at oviposition. The presence of polydnavirus particles is essential for survival of the egg and/or developing parasitoid larva (13–15).

Polydnavirus DNA segments do not contain genes for particle replication, so no particles are produced in the lepidopteran host (1, 16). Particles enter most host cell types (17, 18), and viral transcripts are produced in the first few hours after parasitization. Transcripts are generated either transiently (19) or persistently (17) during parasitism. Relative levels of *Campoletis sonorensis* ichnovirus gene expression in *Helicoverpa virescens* larvae depend largely on gene copy number (16); therefore, segment nesting could conceivably function to increase the copy number of genes essential for parasitoid survival. Such genes presumably would encode abundantly expressed, secreted proteins rather than intracellular proteins (16).

Cotesia rubecula bracovirus (CrBV)[1] genes are expressed in the host larvae, *Pieris rapae*, over a relatively short time period, from 4 to 12 h after parasitization (19). CrBV appears to express only 4 major genes, which differs from other systems, such as *C. sonorensis* ichnovirus, which is suspected of expressing over 35 genes comprising several gene families (20). The products of particle-associated genes act to suppress the host immune response (19, 21–25), most often by targeting hemocytes. Gene products may also lead to physiological disorders (*e.g.* arrested development) by interfering with the host endocrine system (26–29).

Suppression of the host immune response appears to be the primary function of most polydnavirus genes expressed in lepidopteran larvae and is considered an important evolutionary

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY234855.

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[1] The abbreviations used are: CrBV, *Cotesia rubecula* bracovirus; CTL, C-type lectin; CRD, carbohydrate recognition domain; PBS, phosphate-buffered saline; RT, reverse transcription; ORBC, ovine red blood cell.

adaptation for an organism directly exposed to the immune system of its host. One of the four major CrBV genes, *CrV1*, encodes a glycoprotein that is abundantly expressed in host tissues and inactivates hemocytes by destabilizing the cytoskeleton (19, 30). As a result, infected hemocytes are unable to encapsulate the parasitoid egg. A 32-kDa wasp-specific protein (Crp32) produced in calyx cells is associated with particles and also covers the parasitoid's eggs, providing passive immune protection for the developing embryo (31). Whereas Crp32 appears to provide passive protection for the parasitoid, polydnavirus genes provide protection by actively suppressing host immune function. Both elements are required for survival and development of the *C. rubecula* parasitoid (31).

C-type lectins (CTLs) are proteins that bind to specific glycodeterminants and require the presence of divalent metal ions, most commonly Ca^{2+}, to exhibit binding (32). CTLs are defined by a series of conserved residues in their carbohydrate recognition domains (CRDs) (33). Amino acid sequence differences in various CRDs produce a range of carbohydrate binding specificities. CTLs are extremely diverse and have been subdivided into seven groups based on gene structure and nature of non-lectin domains (32). One class, simple CTLs, has been isolated from invertebrates and appears to function as part of induced humoral immune responses (32), presumably binding to carbohydrates on the surface of foreign bodies or damaged tissue. These lectins are generally multimeric, with each monomer containing one CRD, and most often bind galactose as the primary ligand (33). Here we report on a novel CrBV gene, *CrV3*, the product of which shows divalent ion-dependent lectin activity and has a conserved CTL domain similar to those isolated from invertebrates and mammals. Although CTLs have been isolated from a range of invertebrates, this is the first report of a CTL associated with invertebrate viruses.

EXPERIMENTAL PROCEDURES

Insect Cultures—C. rubecula (Hymenoptera: Braconidae) endoparasitoid wasps were reared on cabbage-fed *P. rapae* (Lepidoptera: Pieridae) as described previously (34).

Virus and Genomic DNA Isolation—Calyx fluid from 50 female wasps was collected in PBS (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, and 7.3 mM Na_2HPO_4, pH 7.6) by homogenization of ovaries. The suspension was passed through a 0.45 μM syringe filter (Minisart®) and centrifuged at 15,800 × *g* in a desktop centrifuge for 15 min (35). Pelleted virus particles were resuspended in 180 μl of PBS, and DNA was isolated from this suspension as described previously (4). DNA was isolated from ovaries and female and male wasps by homogenizing them in a buffer made up of 10 mM Tris, 10 mM EDTA, and 1% SDS, pH 8.0. Proteinase K was added to a final concentration of 0.25 μg/μl, and the samples were incubated at 40 °C overnight. Samples were treated with RNase A (125 μg/μl) at 37 °C for 30 min and then extracted with phenol/chloroform. DNA was precipitated by adding 2 volumes of ethanol and 0.2 volume of 3 M sodium acetate, pH 5.3, and centrifugation at 15,800 × *g* for 20 min. Pellet was washed with 70% ethanol, dried at 37 °C, and resuspended in water.

Southern and Northern Hybridization—DNA samples were run on a 1% agarose gel and transferred to a nylon membrane (Amersham Biosciences) as described previously (36). Total RNA was isolated from 6 h parasitized *P. rapae* caterpillars according to Chomczynski and Sacchi (37). RNA samples were run on 1% agarose gels under denaturing conditions, using formaldehyde, and transferred to nylon membranes as described previously (36).

Construction and Screening of a 6 h Parasitized Larval P. rapae Library—Total RNA was extracted from *P. rapae* larvae at 6 h after parasitization by mated *C. rubecula* wasps (QuickPrep™ total RNA extraction kit; Amersham Biosciences). mRNA was then isolated from total RNA (PolyATtract™ mRNA isolation system; Promega). The isolated mRNA was used for construction of the cDNA library containing clones packaged in pBlueskript® SK(±) phagemids (cDNA synthesis kit, ZAP-cDNA® synthesis kit, and ZAP-cDNA® Gigapak® III Gold cloning kit; Stratagene). The library was amplified and titered according to the manufacturer's instructions before being probed with total CrBV DNA previously digested with *Bam*HI and *Hin*dIII and labeled with ^{32}P. Probes were prepared as described (Ready-To-Go™ DNA labeling beads; Amersham Biosciences). Positive clones were rescreened, resulting in isolation of the complete CrV3 coding region. CrV3 was sequenced using M13 forward and reverse primers directly from the phagemid vectors produced by the aforementioned protocols and subsequent automated sequencing (Applied Biosystems).

PCR Amplifications—Specific primers to the CrV3 open reading frame (5′ primer CrV3-F and 3′ primer CrV3-R; see Fig. 1A) were designed containing *Sph*I and *Pst*I restriction sites to allow for direct ligation of the amplified fragment into the pQE30 expression vector (Qiagen). Primer sequences were as follows (restriction sites are *underlined*): CrV3-F, CGCGGCATGCAAAAACATAAGCATTCAG; and CrV3-R, GCGCCTGCAGTCACTCCTTTGTGCAGAAG. Approximately 30 ng of genomic DNA from female *C. rubecula* wasps or 100–350 ng of plasmid DNA was used as template in PCR reactions. A 50-μl reaction was prepared by mixing 5 μl of 10× reaction buffer, 3 μl of $MgCl_2$ (Promega), 1 μl of CrV3-F primer (0.1 μg/μl), 1 μl of CrV3-R (0.1 μg/μl), 0.5 μl of deoxynucleotide triphosphates (15 mM), and 0.5 μl of *Taq* DNA polymerase (Promega) and template DNA. After 5 min at 94 °C, 30 amplification cycles were run including denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. Final extension was carried out for 5 min at 72 °C. Reaction products were electrophoresed on 1.2% agarose gels at 110 mA and visualized using ethidium bromide.

Reverse Transcription-PCR (RT-PCR)—CrV3-F and CrV3-R primers were used in RT-PCR of RNA isolated from 6 h parasitized *P. rapae* larvae, utilizing avian myeloblastosis virus reverse transcriptase (Promega). 1.5 μg of RNA and 0.1 μg of CrV3-R primer, in a final volume of 10.7 μl, were heated to 95 °C for 5 min to denature RNA, before being cooled on ice. Reverse transcription was performed by adding 3 μl of 5× RT buffer (Promega), 0.3 μl of RNasin (Promega), 0.5 μl of avian myeloblastosis virus reverse transcriptase, and 0.5 μl of deoxynucleotide triphosphates (15 mM) before heating at 42 °C for 1 h and then heating at 95 °C for 5 min. The total contents were then used in a PCR by adding 3.5 μl of 10× reaction buffer, 1 μl of CrV3-F primer (0.1 μg/μl), 1 μl of CrV3-R (0.1 μg/μl), 0.5 μl of deoxynucleotide triphosphates (15 mM), 0.5 μl of *Taq* DNA polymerase, and 29 μl of H_2O. Cycling, electrophoresis, and visualization protocols were as performed for standard PCR of CrV3.

Collection of Protein Samples and Western Blotting—P. rapae larvae were bled into PBS saturated with phenylthiourea via removal of a proleg, and the hemolymph was centrifuged at 2300 × *g* for 5 min at room temperature. Supernatant (cell-free hemolymph) was removed, and the cellular pellet was resuspended in PBS. Gut tissue and head capsule were removed, and the fat body was washed and then homogenized in PBS before centrifugation (9300 × *g* for 10 min) and removal of supernatant (fat body proteins). Protein samples were stored at −20 °C and electrophoresed on denaturing 15% SDS-polyacrylamide gels as described by Laemmli (38). Proteins were generally not heated before electrophoresis unless testing the effect of heating. Samples were run in conjunction with SeeBlue™ pre-stained standard protein markers (Novex) to allow subsequent estimation of sample protein sizes. Proteins were either stained within the gels using Coomassie Blue (Sigma) or, alternatively, transferred to a nitrocellulose membrane (Amersham Biosciences) as described previously (36). Before obtaining anti-CrV3, blots were probed with a 1:10,000 dilution of an alkaline phosphatase-conjugated monoclonal anti-polyHistidine antibody (clone His-1; Sigma). Anti-CrV3 was used at a dilution of 1:5000 (see below).

Expression of CrV3 in Bacteria—Gene-specific primers were designed (CrV3-F and CrV3-R) to amplify the open reading frame of the CrV3 gene, excluding a putative signal sequence corresponding to the first 14 amino acids of the protein (see Fig. 1). These primers were used in PCR of phagemid vector produced during library screening to obtain the required fragment for ligation into the pQE30 bacterial expression vector (Qiagen). The desired PCR product was purified (Perfectprep® Gel Cleanup Kit; Eppendorf), precipitated, and digested with *Sph*I and *Pst*I, as was pQE30, before ligation of the digested DNAs using T4 DNA ligase (Promega). M15 strain of *Escherichia coli* was transformed with the ligation reaction contents, using heat shock. Colonies containing desired recombinant vectors were identified by PCR of bacterial cells using vector-specific primers. Production of bacterial CrV3 (containing 6 additional vector-derived histidine residues) was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to bacterial cultures before incubation for 2 h at 37 °C. The resultant fusion protein was identified by Western blotting and contained mainly in the insoluble fraction of total bacterial proteins, with only a small amount being soluble.

Purification of Insoluble Bacterial CrV3 Protein—50 ml of induced

(A)

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      C AAT TGA TAG CCG TTA GCC GTA CGA GCT TGA CAG TTA TTC CGA AAA AAA AAC TAT TAT TCA AGA AAA
                                                          ▼
 M  N  K  L  I  C  L  M  V  L  P  A  V  L  S  K  N  I  S  I  Q  R  K  G
ATG AAT AAA TTA ATT TGT TTA ATG GTT TTA CCC GCG GTG CTG AGT AAA AAC ATA AGC ATT CAG AGG AAA GGT
        9        18        27        36        45        54        63        72
 R  L  T  I  G  S  S  E  S  Y  T  F  H  S  T  P  A  T  F  N  E  A  I  S
CGA CTA ACA ATC GGA TCC AGC GAA TCG TAC ACA TTC CAT TCA ACT CCA GCA ACC TTC AAT GAA GCC ATA AGT
        81        90        99       108       117       126       135       144
 I  C  K  Q  E  G  G  S  L  A  V  V  T  S  Q  K  A  E  D
ATT TGC AAA CAA GAA GGT GGA AGT CTT GCT GTC GTG ACC TCC CAG AAA GCA GAA GAT gtg agt att cat tat
       153       162       171       180       189       198       207       216
ttt tag tgt att aaa ttc agg agt gga tga ggc aaa tca aga ttc aaa tgt aaa att tcg cta agc cct gtt
       225       234       243       252       261       270       279       288
agt aaa cta aac tgt tat tct tca tta tca aaa aaa gga cat ttt atc tgc tag tga ccc ata aat gct aat
       297       306       315       324       333       342       351       360
                                 E  M  L  K  I  W  K  H  S  S  P  I  L  N  S
tgt act aaa ata atc gtt tct ctc tag GAA ATG CTG AAG ATT TGG AAA CAT TCA AGC CCT ATT CTA AAC TCA
       369       378       387       396       405       414       423       432
 T  N  G  L  T  S  Q  A  F  I  G  I  H  S  L  N  K  K  G  H  W  E  T  I
ACA AAT GGA TTG ACT TCA CAA GCT TTC ATC GGG ATC CAT AGC TTA AAT AAA AAA GGT CAC TGG GAA ACA ATC
       441       450       459       468       477       486       495       504
 D  G  E  S  P  K  Y  I  N  W  S  Q  H  W  S  G  G  R  K  P  S  T  S  S
GAT GGA GAA TCT CCA AAA TAC ATC AAT TGG AGT CAA CAC TGG TCA GGC GGA CGA AAA CCG AGC ACC TCT AGC
       513       522       531       540       549       558       567       576
 V  Q  K  C  G  S  L  L  K  H  G  G  L  D  N  V  E  C  Y  F  K  L  A  F
GTT CAA AAG TGT GGT AGT TTA TTA AAG CAC GGA GGA TTG GAT AAT GTA GAA TGT TAC TTC AAG CTC GCT TTC
       585       594       603       612       621       630       639       648
 F  C  T  K  E  *
TTC TGC ACA AAG GAG TGA CAT TGA AAA ACG TAT CGA TAA TTA TAG AGA TTA CTA CCT AAA AAT AAG TAA TGT
       657       666       675       684       693       702       711       720
TAC TTT TAG CTT CAA GTA TTA TTC CTA ATT ATG CTA TAA TAT TAT AAT ATA TAG ACT AAG TAA TAA ATT TCT
       729       738       747       756       765       774       783       792
TTA TTC AAA AAA AAA AAA AAA AAA AAA AA
       801       810       819
```

(B)

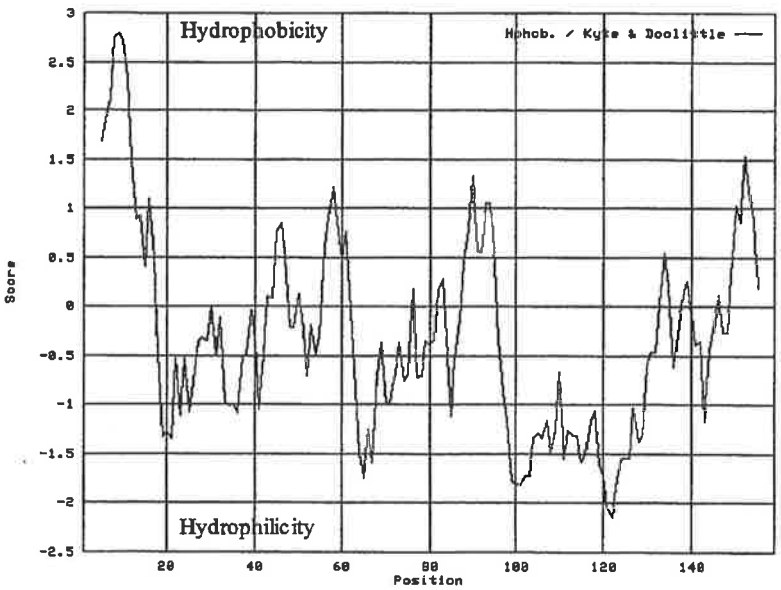

FIG. 1. *A*, DNA nucleotide sequence and deduced amino acid sequence for CrV3 from the *C. rubecula* bracovirus (GenBank™ accession number AY234855). The putative signal peptide is *boxed* and contains a predicted methionine start codon as the first amino acid. The predicted signal peptide cleavage point is denoted with an *arrowhead*. Amino acids representing putative *N*-glycosylation sites are *underlined*. The stop codon is indicated by an *asterisk*, and the polyadenylation signal is in *bold*. CrV3-F and CrV3-R primer binding sites are *double underlined* and located at the 5′ and 3′ ends of the open reading frame, respectively. Intron nucleotides are in *lower case letters*. *B*, hydrophobicity profile of CrV3. Distribution of hydrophobic and hydrophilic domains was determined using ProtScale software. Amino acid positions are represented on the *x* axis, and a score of relative amino acid hydrophobicity is represented on the *y* axis. *Positive scores* indicate hydrophobic residues, and *negative scores* indicate hydrophilic residues. A highly hydrophobic domain predicted at the N terminus of CrV3 is consistent with a putative signal peptide at the same region.

bacterial culture was centrifuged at ~7700 × *g* for 10 min at 4 °C. Cells were then resuspended in a lysis buffer (6 M GuHCl, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 8 .0) and gently rocked for 1 h. The sample was centrifuged at 12,000 × *g* for 15 min at 4 °C before incubation (1 h, RT) of the supernatant with 300 μl of nickel-nitrilotriacetic acid resin beads (Qiagen) previously equilibrated in 8 M urea (with 0.1 M NaH_2PO_4 and 0.01 M Tris, pH 8.0). Non-bound proteins were removed with buffers containing 8 M urea with pH > 6.3, and bound proteins were eluted with buffers containing 8 M urea with pH < 6.0. Samples were diluted with 2 volumes of water before being dialyzed overnight in Tris-buffered saline (0.15 M NaCl and 0.01 M Tris, pH 8.0) at 4 °C to remove excess urea to renature the protein. Protein was concentrated by vacuum drying.

Anti-CrV3 Antibody Production—Purified bacterial CrV3 was visualized on 15% SDS-acrylamide gels by staining with water-dissolved Coomassie Blue. CrV3 protein bands were excised from the gel with sterile blades and crushed. One rabbit was used to produce anti-CrV3 by an initial injection of the purified CrV3 (~5 μg) mixed with Freund's complete adjuvant (Sigma), followed by two booster injections with purified CrV3 with Freund's incomplete adjuvant (Sigma) at 2 and 4 weeks, respectively, after the initial injection. Antiserum was obtained 2 weeks after the final injection and used to probe Western blot membranes at a dilution of 1:5000. Bound anti-CrV3 was then visualized by alkaline phosphatase-labeled secondary anti-rabbit antibody (1:10,000).

N-Glycosidase Digestion of CrV3—Total proteins from cell-free hemolymph of 6 h parasitized *P. rapae* larvae were mixed with SDS-PAGE loading buffer containing β-mercaptoethanol. Igepal CA-630 nonionic detergent (Sigma) was added to a final concentration of 0.8% before addition of 2 units of recombinant *N*-glycosidase F (Roche Diagnostics) and incubation for 18 h at 37 °C.

Characterization of CrV3-mediated Hemagglutination—Lectin activity was measured by mixing 25 μl of serially diluted bacterial CrV3 extract with 25 μl of 2% trypsinized and gluteraldehyde-stabilized ovine red blood cells (ORBCs; Sigma) in PBS containing 2% bovine serum albumin. Samples were mixed well in U-bottomed microtiter wells before incubation at 37 °C for 1 h. Complete agglutination caused ORBCs to form a diffuse layer over the bottom of the wells, whereas unagglutinated cells formed a small dot at the center of the wells. Lectin titer was determined as the reciprocal of the maximum sample dilution causing complete ORBC agglutination. To test for inhibitory ligands, 5 μl of sugar solution (various concentrations) in PBS was added before incubation in place of the 5 μl of PBS used to dilute ORBCs in the standard assay. Lipopolysaccaride (*E. coli*, serotype 055:B5A; Sigma) and Laminari tetrose were added as described for the other sugars, up to a maximum concentration of 1 mg/ml. Comparison of concentrations causing 50% inhibition of lectin activity was made for all sugars tested. To test for dependence of lectin activity on divalent cations, 25 μl of serial CrV3 sample dilutions was prepared in 1 mM divalent cations (Mg, Mn, and Ca) or 1 mM EDTA and mixed with 25 μl of 2% ORBCs as described above. Increasing concentrations of divalent cations were also added to EDTA-inhibited CrV3 to restore lectin activity.

RESULTS AND DISCUSSION

Molecular Characterization and Expression of CrV3—*C. rubecula* parasitoid wasps inject polydnavirus particles into the hemocoel of *P. rapae* larvae at oviposition, leading to infection of host tissues by the particles and transient expression of particle-associated genes (19). CrV1 was previously isolated by screening a cDNA library constructed from 6 h parasitized caterpillars using total CrBV DNA as a probe (19). The same method was used here to isolate a ~700-bp cDNA encompassing the coding region of a putative CrBV gene and including a poly(A) tail (Fig. 1*A*).

To confirm the cDNA as particle-derived, the fragment was cloned and used as a probe in both a Southern blot of digested CrBV DNA (Fig. 2*A*) and a Northern blot of RNA from unparasitized and 6 h parasitized larvae (Fig. 2*B*). Hybridization occurred to a CrBV restriction fragment of ~4 kb and to a parasitism-specific transcript of ~1.1 kb. These data and the fact that the same probe bound to genomic DNA from female wasps but not to that from *P. rapae* (data not shown) indicate that the cDNA originated from particles introduced to the larvae at oviposition.

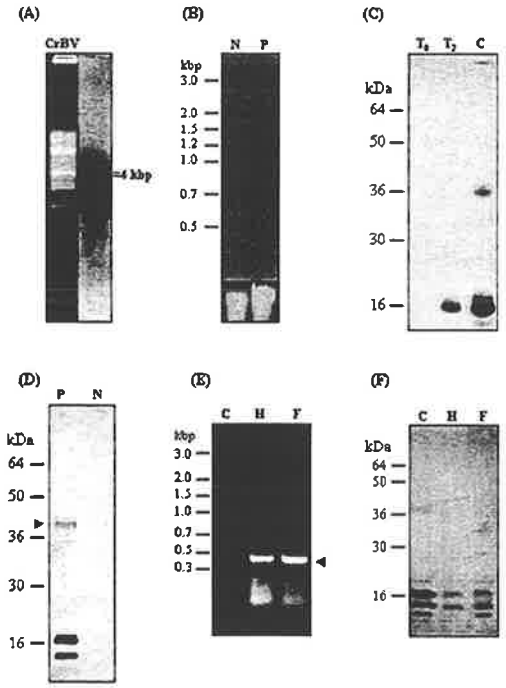

FIG. 2. *A*, Southern hybridization of ^{32}P-labeled CrV3 cDNA to a ~4-kb *Eco*RI restriction fragment of CrBV DNA (*CrBV*). The corresponding gel is shown to the *left*. *B*, Northern hybridization utilizing ^{32}P-labeled CrV3 cDNA fragment to probe total RNA extracted from non-parasitized (*N*) and 6 h parasitized (*P*) *P. rapae* larvae. Hybridization was to a parasitism-specific transcript of ~1.1 kb in size. Approximately 20 μg of RNA was loaded in each lane (see loading control at the *bottom*). *C*, Western blot analysis (15% SDS-PAGE; anti-polyHISTIDINE, 1:10,000) of total proteins from non-induced (T_0) and 2 h induced (T_2) recombinant M15 bacterial cells (containing partial CrV3 open reading frame) and bacterial CrV3 protein extract (*C*) purified from induced cells. *D*, Western blot analysis (15% SDS-PAGE; anti-CrV3 antiserum, 1:5000) of cell-free hemolymph from 6 h parasitized (*P*) and non-parasitized (*N*) *P. rapae* larvae. Parasitism-specific CrV3 monomers of ~17 and ~14 kDa in size are clearly visible (ratio, approximately 2:1). The *arrowhead* indicates a small amount of CrV3 dimer. *E*, RT-PCR of total RNA from cell-free hemolymph (*C*, negative control), hemocytes (*H*), and fat body (*F*) from 6 h parsasitized *P. rapae* larvae. Specific primers to the open reading frame of CrV3 (CrV3-F and CrV3-R) were utilized, resulting in detection of CrV3 transcript (*arrowhead*) in hemocytes and fat body. *F*, Western blot analysis (15% SDS-PAGE; anti-CrV3 antiserum, 1:5000) of cell-free hemolymph (*C*), hemocytes (*H*), and fat body (*F*) from 6 h parasitized *P. rapae* larvae. Note that most CrV3 is located in cell-free hemolymph, where the monomer ratio is the opposite of that in fat body.

Binding of the cDNA to only one site in the Northern blot reveals that CrV3 shows no significant nucleotide sequence homology with other CrBV-related genes. The cDNA was subsequently sequenced with data showing an open reading frame of 480 bp (Fig. 1*A*). A methionine codon (ATG) at the beginning of the open reading frame was identified as the only possible codon with a nucleotide sequence environment predicted for functional initiation codons (39). The predicted molecular mass of CrV3 is 17.6 kDa, with a pI of 9.13. Computer analyses (PSORT II; psort.nibb.ac.jp/form2.html) of the deduced amino acid sequence revealed a putative signal peptide encompassing the first 14 amino acids of the protein, with a cleavage point predicted at the end of the signal peptide (Fig. 1*A*), indicating that CrV3 protein is probably secreted from cells of origin. A hydrophobicity plot (Fig. 1*B*) was produced using ProtScale software (40). Highly hydrophobic residues near the N terminus support predictions of signal sequence composed of N-terminal amino acids. Three putative *N*-glycosylation sites were found in the open reading frame, as well as a polyadenyl-

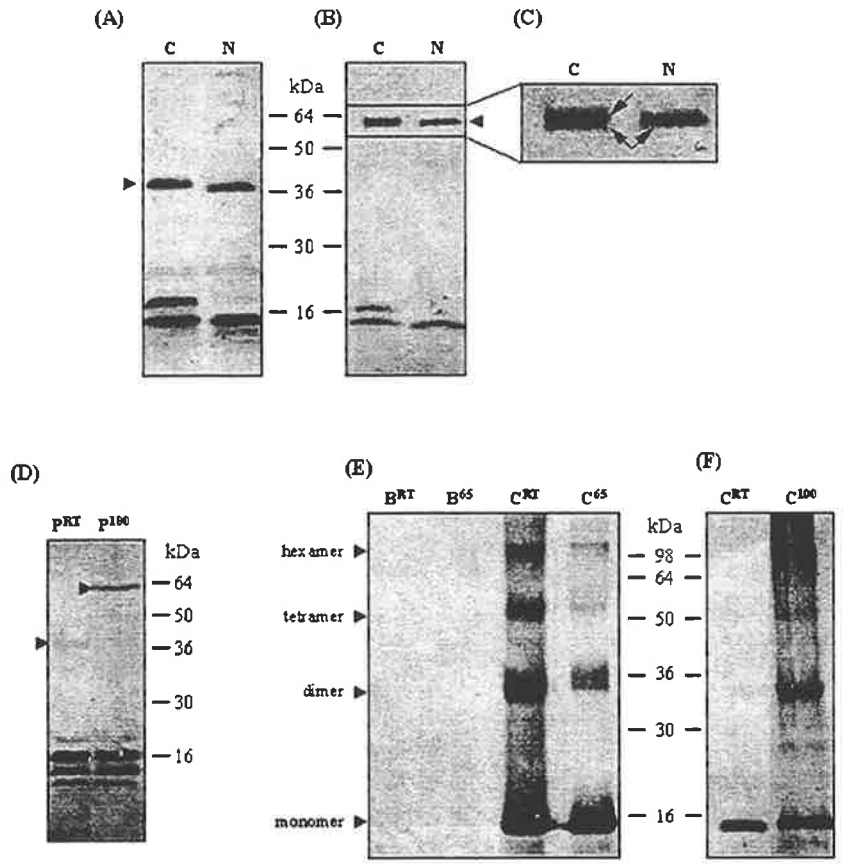

FIG. 3. **Multimerization of CrV3.** Anti-CrV3 antiserum (1:5000) was used in all Western analyses (15% SDS-PAGE). *A* and *B*, analysis of untreated (*C*) and *N*-glycosidae F-treated (*N*) proteins from cell-free hemolymph of 6 h parasitized *P. rapae* larvae. In both blots, the larger ~17-kDa monomer is deglycosylated such that it is identical to the smaller ~14-kDa monomer. Decreases in the size of CrV3 dimer (*arrowhead* in *A*) and tetramer (*arrowhead* in *B*) indicate that these multimers are also glycosylated. *C*, magnified view of untreated (*C*) and *N*-glycosidase F-treated (*N*) CrV3 tetramer bands. *D*, analysis of cell-free hemolymph from 6 h parsasitized *P. rapae* larvae, which was either left at room temperatute (P^{RT}) or boiled for 10 min (P^{100}) before electrophoresis. Heating resulted in dimer (*lower arrowhead*) denaturation into monomers and release of tetramer (*upper arrowhead*) from a putative larger complex. *E* and *F*, analysis of purified extract from induced bacteria containing empty plasmid or recombinant plasmid with CrV3 open reading frame. No CrV3-related molecules were detected in non-recombinant cells that were left at room temperature (B^{RT}) or heated to 65 °C for 10 min (B^{65}). CrV3 monomer and a range of multimers were detected in recombinant cells that were left at room temperature (C^{RT}). Multimers were denatured into components when recombinant cells were heated at 65 °C for 10 min (C^{65}). Boiling of CrV3 sample (C^{100}) resulted in denaturation of a larger CrV3 complex, allowing detection of multimers not seen in the same amount of protein left at room temperature (C^{RT}).

ation signal ~150 bp downstream of the stop codon (Fig. 1*A*).

Sequence data were used to generate specific primers to the CrV3 open reading frame (CrV3-F and CrV3-R; see Fig. 1*A*). Comparison of RT-PCR and genomic DNA PCR products, utilizing these primers, revealed the presence of a 186-bp intron in the genomic CrV3 DNA. The intron was located within the conserved lectin domain. The CrV3 open reading frame (excluding the putative signal peptide) was cloned into pQE30 vector and used to transform *Escherichia coli* cells in which the CrV3 protein was subsequently induced. Analysis of Coomassie Blue-stained SDS-polyacrylamide gels containing proteins from non-induced and induced cells showed the presence of a ~16-kDa protein that was heavily up-regulated in induced cells and present mainly in the insoluble portion of the total bacterial proteins (data not shown). Nickel resin beads were used to purify the protein. Confirmation of purification of the up-regulated protein was achieved by using Western blot analysis with anti-polyHistidine as a probe (Fig. 2*C*).

Purified protein from the insoluble fraction was used for injection into rabbits and production of putative anti-CrV3 antibodies. Serum from injected rabbits was used to probe cell-free hemolymph from non-parasitized and 6 h parasitized *P. rapae* larvae. The serum hybridized to a parasitism-specific protein that was not recognized by rabbit pre-serum (data not shown), confirming successful production of anti-CrV3 antibodies. Western blots utilizing anti-CrV3 antibodies showed two CrV3-related monomers, which are present mainly in the cell-free hemolymph (Fig. 2, *D* and *F*). These monomers were approximately 17 and 14 kDa in size and were present in a ratio of approximately 2:1 as judged by the intensity of electrophoresed bands (Fig. 2, *D* and *F*). Treatment of cell-free hemolymph from 6 h parasitized larvae with a recombinant *N*-glycosidase resulted in removal of the larger monomer and an increase in the smaller monomer, suggesting that the larger monomer is an *N*-glycosylated form of the smaller monomer (Fig. 3, *A* and *B*). No putative *O*-glycosylation sites were predicted by computer analysis. Similar lectin monomers (differing by glycosylation) have been identified in *Drosophila melanogaster* (41), although the biological significance of glycosylation (or deglycosylation) of the *Drosophila* lectin is not understood.

RT-PCR, utilizing primers from the CrV3 open reading frame, was used to test for production of CrV3 transcript in fat body and hemocytes from 6 h parasitized larvae (see Fig. 2*E*). These data indicate that CrV3 is produced by hemocytes and fat body cells. Western blot analysis, using anti-CrV3 antibodies, was performed on total proteins from larval fat body, hemocytes, and cell-free hemolymph at 6 h after parasitization (Fig. 2*F*). The presence of a large amount of CrV3 in the cell-free hemolymph compared with fat body or hemocytes confirms that the protein is secreted and possibly interacts with soluble hemolymph components. It appears that the relative amount of each CrV3 monomer varies with its location within parasitized larvae (Fig. 2*F*). In cell-free hemolymph, the ratio of 17-kDa monomer to 14-kDa monomer is usually 2:1 (see Fig. 2, *D* and *F*), whereas in fat body, the ratio is reversed (Fig. 2*F*). These data are consistent with CrV3 being secreted from fat body (and/or hemocytes) into the hemolymph because this is where most of the 17-kDa monomer is detected. The smaller monomer detected in fat body and hemocytes is probably intracellular. Presumably, only glycosylated monomers are secreted from infected cells before wasp or host elements remove *N*-glycosylated carbohydrates to produce the 14-kDa monomer. A similar phenomenon has been reported for the CrV1 protein, which has *N*-acetyl-D-galactosamine residues removed by

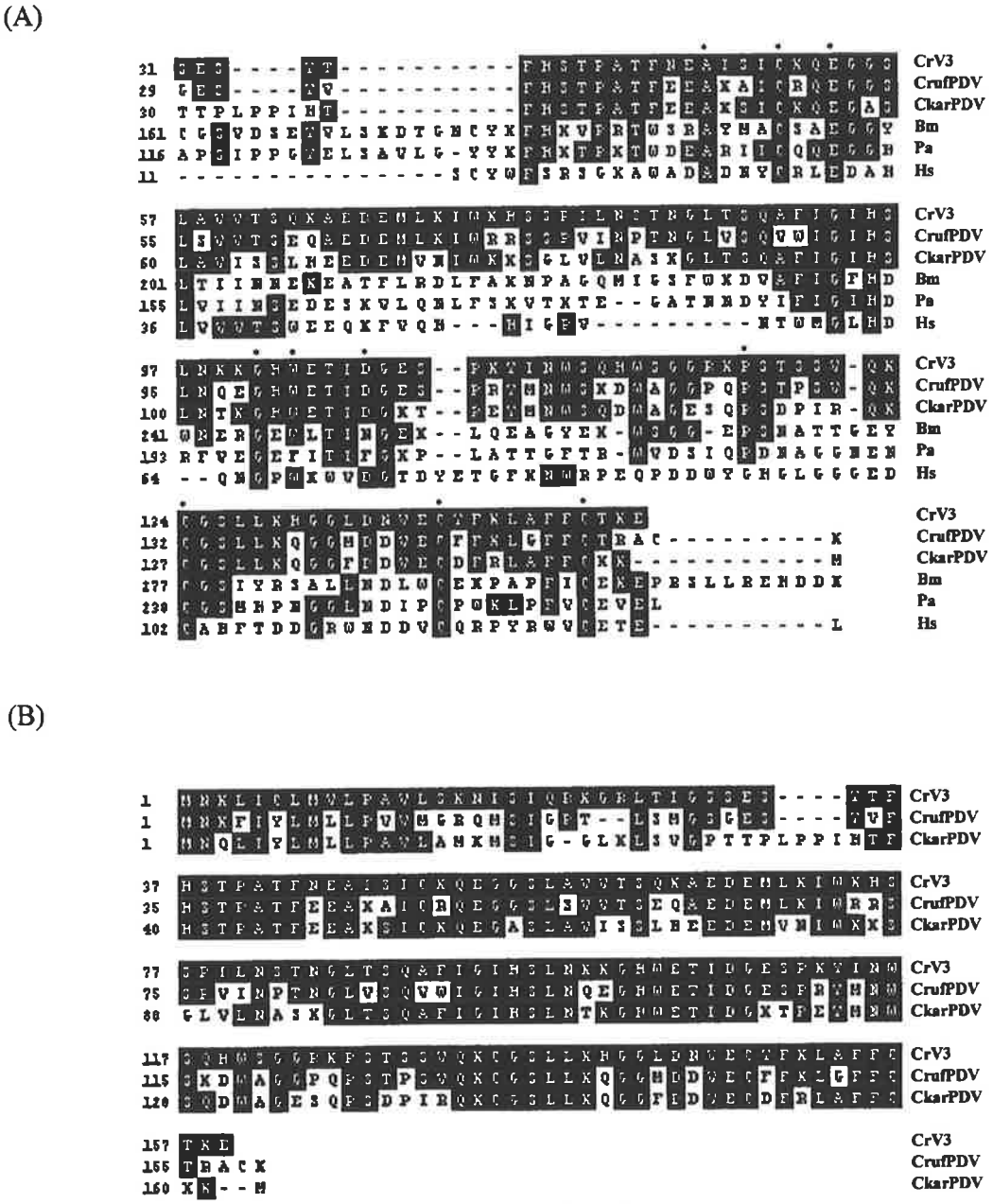

FIG. 4. *A*, comparison of partial amino acid sequences in lectin domains of polydnavirus, insect, and mammalian CTLs. Homologous residues with CrV3 are *boxed* in *black*, and residues conserved among various CTLs are indicated by *asterisks*. *B*, comparison of complete amino acid sequences from CrV3 and hypothetical proteins from *C. ruficrus* and *C. karyai* bracoviruses. Indicators are as described for *A*. Sequences are from CrV3 (*CrV3*; GenBank™ accession number AY234855), *C. ruficrus* bracovirus hypothetical protein (*CrufPDV*; GenBank™ accession number BAC55179), *C. karyai* bracovirus hypothetical protein (*CkarPDV*; GenBank™ accession number BAC55180), *B. mori* lipopolysaccharide-binding protein (*Bm*; GenBank™ accession number CAB38429), *P. americana* hemolymph lipopolysaccharide-binding protein (*Pa*; GenBank™ accession number BAA00616), and *Homo sapiens* asialoglycoprotein receptor of hepatic lectin H1 (*Hs*; GenBank™ accession number NP00162).

Pieris hemolymph (30). CrV3 hemolymph concentration was at a maximum at ~6 h parasitization but was almost undetectable in hemolymph by Western analysis at 24 h parasitization (data not shown), an observation consistent with the transient expression of CrV3 (19).

Dimer and tetramer CrV3 molecules were detected in small amounts under denaturing conditions in parasitized larvae and were both shown to contain glycosylated monomers (Fig. 3, *A*–*C*). The relative amount of different oligomers appeared to vary with individual larvae, and often only one type was detected (compare Figs. 2*D* and 3, *A* and *B*). The significance of this phenomenon is not clear. Boiling of cell-free hemolymph proteins from 6 h parasitized larvae resulted in an increase in CrV3 tetramers and a decrease in dimers (Fig. 3*D*). It seems likely that boiling denatures the dimers and releases the tetramers from a large complex formed with a soluble hemolymph component or CrV3 alone. CrV3 hexamers and smaller oligomers were detected in purified bacterial CrV3 under denaturing conditions (Fig. 3*E*). Heating bacterial CrV3 to 65 °C resulted in a breakdown of smaller multimers into their components. However, boiling resulted in an increase of all detectable multimers (Fig. 3*F*), indicating that the bacterial CrV3 is forming much larger homogeneous complexes that are denatured at temperatures near 100 °C. The observation that CrV3

TABLE I
Effect of addition of various 1 mM divalent cations or 1 mM EDTA on bacterial CrV3-mediated hemagglutination.

Divalent cation/chelator	Lectin activity
	$titer^{-1}$
Bacterial extract	1
1 mM EDTA	ND[a]
1 mM Calcium	1
1 mM Magnesium	2
1 mM Manganese	16

[a] Not detected.

TABLE II
Effect of various potential ligands on bacterial CrV3-mediated hemagglutination

Inhibition was assayed in the presence of various potential ligands up to maximum concentrations of 100 mM or 1 mg/ml.

Ligand	Ligand concentration producing 50% inhibition
	mM
α-Lactose	>100
α-L-Rhamnose	>100
β-D-Fructose	>100
D-Cellobios	>100
D-Galactose	>100
D-Glucose	>100
D-Fucose	>100
D-Mannitol	>100
D-Mannose	>100
D-Mannoheptose	>100
D-Raffinose	>100
D-Sorbitol	>100
D-Trehalose	>100
D-Xylose	>100
L-Arabinose	>100
L-Fucose	>100
Maltose	>100
Maltotriose	>100
Maltotetraose	>100
Methyl-α-D-manno-pyranoside	>100
Myo-inositol	>100
N-Acetyl-D-galactosamine	>100
N-Acetyl-D-glucosamine	>100
N-Acetyl-neuraminic acid	>100
Sucrose	>100
	mg/ml
Laminari tetrose	>1
Lipopolysaccharide (*E. coli*, serotype 055:B5)	>1

appears to only form multimers that are multiples of dimers suggests that pre-formed CrV3 dimers are the minimum element required for polymerization. The fact that bacterial CrV3 forms multimers indicates that sugar residues are not required for dimerization/multimerization. These large complexes were probably not entering the acrylamide gel or were not transferred to the membrane. Formation of large multimers is characteristic of several of the invertebrate CTLs characterized previously (42–46).

Similarity of CrV3 and Known CTLs—Comparison of the deduced amino acid sequence with those from the GenBank™ revealed that CrV3 shows significant conservation with various C-type lectins. Significantly, key amino acids are conserved in CTLs from invertebrates and mammals that are also found in CrV3 sequence (Fig. 4*A*). Thus, sequence similarities suggest that CrV3 is a lectin whose activity is dependent on the presence of divalent metal ions. Interestingly, the highest levels of similarity are with hypothetical proteins from *C. ruficrus* and *C. karyai* bracoviruses (67% and 61%, respectively) (Fig. 4*A*), indicating that these proteins may also function as CTLs. The next closest lectins are lipopolysaccharide-binding proteins from *Periplaneta americana* and *Bombyx mori*, although sequence similarity with these lectins is approximately half that of the bracovirus lectins. CrV3 was also found to be similar to a suite of *P. americana* lectins (data not shown).

It is of note that the *Cotesia*-associated polydnavirus lectins show greater similarity with invertebrate lectins compared with those of other viruses. Virus lectins are generally surface proteins that are involved in attachment of the virion to specific sugar determinants on target cells (47). However, polydnavirus particles enter host cells before lectin expression and probably express soluble lectins as part of immune suppression, a function much closer to that of induced humoral invertebrate lectins. CrV3 has a simple structure, consisting of only a signal peptide and CRD, another featured shared with several invertebrate lectins. No other functional domain appears to be present. The few known parasite or parasitoid lectins appear to show homology with host proteins that are important for immune responses against the parasite (48). It is conceivable that, having structural and sequence similarities to host lectins, CrV3 might compete with host lectins for binding sites that are involved in recognition or induction of the immune system. Sequence similarities between CrV3-like lectins and invertebrate lectins and similarities in parasite/host lectins support a hypothesis that some parasite genes originate from host genetic material.

CrV3 Lectin Activity: Hapten Sugars and Dependence on Divalent Metal Ions—Purified bacterial CrV3 agglutinated trypsinized and gluteraldehyde-fixed ovine red blood cells. Lectin activity was shown to be enhanced in the presence of 1 mM Mg^{2+} and Mn^{2+} but was independent of Ca^{2+} (Table I). Lectin activity was completely abolished in the presence of 1 mM EDTA and was restored by the addition of 0.5 mM Mg^{2+} or 1 mM Mn^{2+} but not by Ca^{2+} concentrations up to 5 mM. Surprisingly, this is in contrast to other described CTLs, which are invariably Ca^{2+}-dependent. The effect of Mn^{2+} had a marked CrV3 concentration-dependent threshold, whereas the effect of Mg^{2+} gradually decreased as CrV3 levels were reduced (data not shown). This effect and the strong enhancement of agglutination by Mn^{2+} may indicate that Mn^{2+} may be important for tight regulation of CrV3 activity *in vivo*. It is possible that metal dependence of native CrV3 differs from that of recombinant protein.

CrV3-mediated agglutination was not significantly inhibited by any of over 20 sugars tested at 100 mM (Table II). It would be expected that the hapten sugar should completely inhibit agglutination at concentrations near 1 mM. None of the common mono- and disaccharides were significantly inhibitory, a result that was not expected, given the relatively simple specificities of the closest vertebrate lectins (commonly binding galactose). To test the assay, lectin from *Helix pomatia* was used to agglutinate cells and was completely inhibited by its hapten sugar, *N*-acetyl-D-galactosamine (49). It is possible that bacterial CrV3 has altered specificity compared with wild-type CrV3 due to differences in post-translational modifications; however, this is unlikely, given that lectin activity is readily demonstrated by red blood cell aggregation and dependence on divalent ions. It seems more likely that CrV3 requires a complex sugar and/or amino acid residues for its binding or is highly discerning in relation to which sugar anomer is encountered or what accessory elements are attached to the basic sugar monomer. Amino acid residues on each side of the conserved proline (Pro^{126} in CrV3; Fig 4*B*) are known to be important determinants of carbohydrate specificity (33). The closest lectins to CrV3 mostly exhibit galactose-type binding and are characterized by the sequence Gln-Pro-Asn, whereas the equivalent CrV3 sequence is Lys^{125}-Pro^{126}-Ser^{127}. Whereas other galactose-type binding lectins have a Ser residue following the

conserved proline (33), as do the hypothetical bracovirus lectins, the occurrence of the preceding lysine residue in CrV3 is rare among such lectins. Thus, the unusual CrV3 sequence may possibly explain its Ca^{2+} independence and why simple galactose-derived sugars do not inhibit CrV3-mediated agglutination as expected and may indicate that CrV3 specificity is atypical. It is perhaps intuitive that CrV3 may have highly specific binding requirements because it presumably targets an individual element associated with host immunity. Lipopolysaccharide from *E. coli* (serotype 055:B5; Sigma) at 1 mg/ml also failed to significantly inhibit CrV3-mediated agglutination.

Preliminary *in vitro* experiments suggest that CrV3 may lessen the ability of healthy host hemocytes to spread on a foreign surface and may cause agglutination of these cells at high concentrations when present in the surrounding medium but does not seem to attach to these cells. It is possible that CrV3 interacts with a soluble hemolymph component that is required for activation of cellular defense. Without knowledge of CrV3 specificity, purification of native CrV3 to homogeneity from parasitized *P. rapae* larvae remains problematic.

In summary, the *CrV3* gene from CrBV has conserved amino acid residues consistent with known CTLs from invertebrates and mammals, and the recombinant protein shows divalent ion-dependent lectin activity. However, this CTL is unique in that it does not require Ca^{2+} for its lectin activity. In addition, lectin activity was not inhibited by common carbohydrates, implying that it may be specific to non-carbohydrate ligands or may require an accessory component(s). CrV3 monomers are composed almost entirely of a single C-type CRD and appear to aggregate into multimers. Thus, we propose that CrV3 is a novel multimeric CTL expressed as part of CrBV infection of host larval tissues. The most probable function of CrV3 is to interact with host hemolymph components to lessen immune reactions against the developing parasitoid. Of the characterized CTLs, CrV3 shows highest similarity with lipopolysaccharide-binding proteins from insects. However, CrV3 appears homologous to hypothetical proteins isolated from bracoviruses associated with two other *Cotesia* wasps. Therefore, it seems likely that these hypothetical proteins will also function as lectins and may have similar metal ion dependence/binding specificity to CrV3. Furthermore, it appears that *Cotesia*-related bracoviruses express a novel polydnavirus gene family of closely related lectins. Other polydnavirus gene families have been identified (20), but no invertebrate virus protein has thus far been characterized as a CTL. Much debate exists as to the ancestral form of polydnaviruses. The bracovirus lectins may be important for evolutionary studies and appear to support a hypothesis that a bracovirus was present in a common *Cotesia* ancestor and that some bracovirus genes originated from their insect hosts. Further research will aim to determine the CrV3 binding specificity, obtain purified native CrV3, and determine its mode of action.

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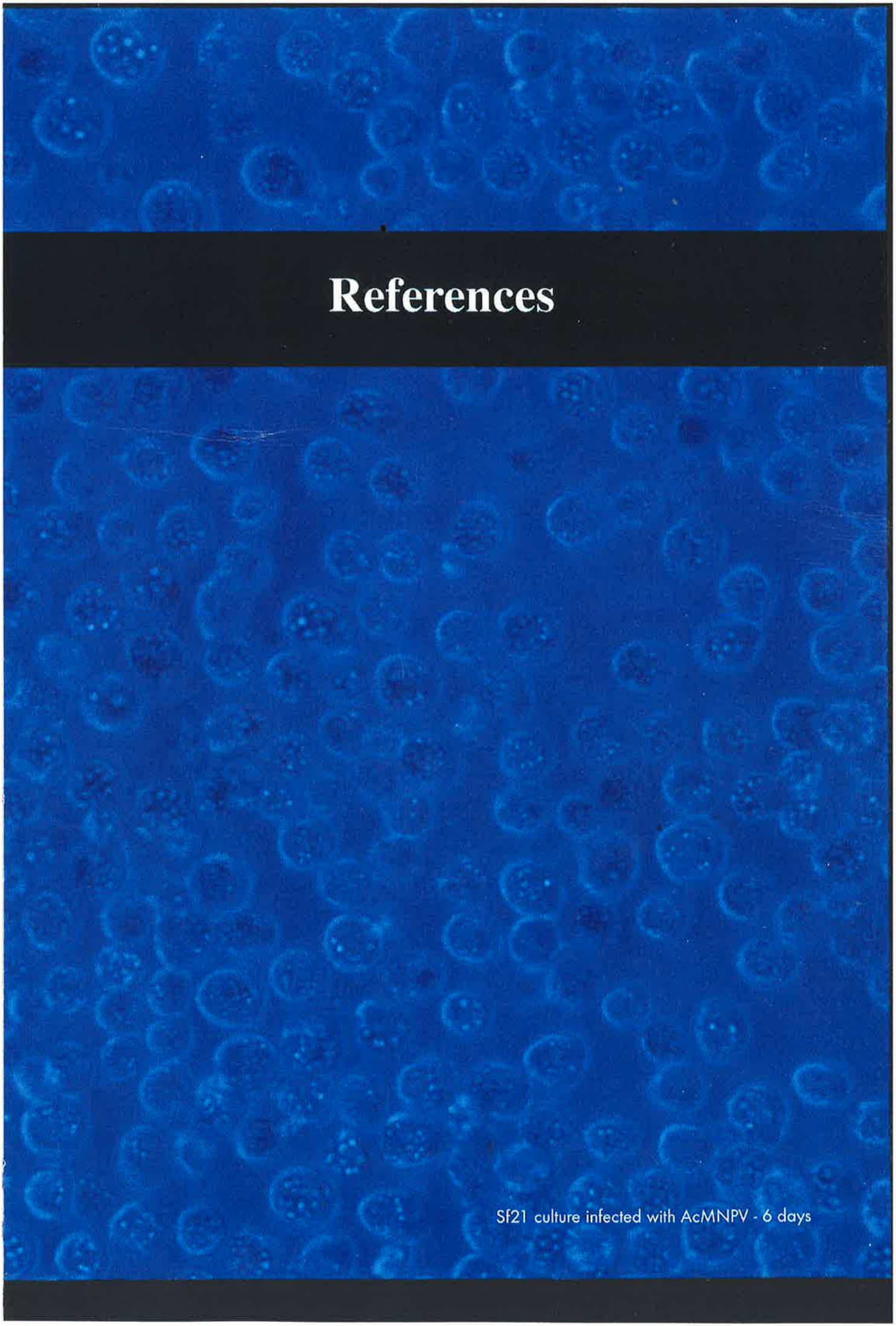

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