BACULOVIRUS INHIBITORS OF APOPTOSIS

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This research programme was carried out in collaboration with the
NERC Institute of Virology and Environmental Microbiology, Oxford,
and Zeneca Agrochemicals plc, Bracknell

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I dedicate this thesis to my parents

Abstract

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A putative inhibitor of apoptosis gene was located in the *Heliothis zea* nucleopolyhedrovirus (HzSNPV) genome, at map units 76 to 77. Alignment of the predicted amino sequence encoded by this gene with reported inhibitor of apoptosis (*iap*) sequences identified a RING finger and baculovirus IAP repeat (BIR) conserved in all the members of this protein family. The predicted sequence of the HzSNPV *iap* was found to be 42% identical to that of *Orgyia pseudosugata* (Op) MNPV *iap* and 39% identical to that of *Cydia pomenella* (Cp) GV *iap*.

Primer extension analysis of the HzSNPV *iap* mRNA identified two transcription start sites typical of early (CAGT) and late (TAAG) baculovirus promoters. Activity from the early promoter motif was detected from 12 hours post infection, whilst activity from the late baculovirus promoter was detected from 24 hours post infection.

The *p35*-deficient mutant of AcMNPV (Ac*p35lacZ*) induces apoptosis in *Spodoptera frugiperda* (Sf21) cells, but not in *Trichoplusia ni* (*T. ni*) cells. In complementation assays with Ac*p35lacZ* in Sf21 cells, both OpMNPV *iap* and CpGV *iap* are capable of complementing P35 function to produce a normal infection, characterised by the formation of occluded virus from 18 hours post infection. The HzSNPV *iap* was unable to produce this complementation effect and is therefore unique amongst the *iap* homologues identified in baculoviruses other than AcMNPV.

Recombinant HzSNPV deficient in the production of *iap* was unstable and could not be isolated from the parental virus. The role of this gene in the infection process of HzSNPV remains unclear.

Recombinant AcMNPV deficient in the synthesis of IAP1 (Ac*iap1lacZ*) was derived. In addition, a virus deficient in both *p35* and *iap1* was constructed (Ac*p35*Δ*iap1lacZ*). Both viruses replicated normally in *T. ni* cells, suggesting that IAP1 is not responsible for inhibiting apoptosis in *T. ni* cells. In subsequent studies the host range of Ac*p35lacZ*, Ac*iap1lacZ* and Ac*p35*Δ*iap1lacZ* was examined in seven Lepidopteran cell lines. These results indicated that all three viruses replicated normally in *T. ni*, *Mamestra brassicae* or *Panolis flammea* cells, thus discounting a role for AcMNPV IAP1 in inhibiting apoptosis in the cells tested.

Publications and Presentations

Much of the information and results presented in this thesis have been presented or are in preparation for publication, in the following:

Anna Barnett, Robert Possee, Linda King and John Windass (1995) Functional analysis of an *iap* gene homologue from the baculovirus *Heliothis zea* single nuclear polyhedrosis virus. Poster Presentation, Oxford Brookes University Student's Day, Oxford, UK.

Anna Barnett, Caroline Griffiths, Martin Ayres, John Windass, Linda King and Robert Possee (1996) *In vitro* host range of AcMNPV deficient in *p35* and *iap1*. Poster Presentation: Annual Meeting, American Society of Virology, University of Western Ontario, London, Ontario, Canada.

Rachel Stephens, Anna Barnett, Carole Thomas, Robert Possee and Linda King. (1996) Identification of baculovirus *iap* genes. Poster Presentation: 10th International Congress of Virology, Jerusalem, Israel.

Anna Barnett, Linda King, John Windass and Robert Possee. (1996) Functional analysis of an inhibitor of apoptosis gene characterised from the baculovirus *Heliothis zea* SNPV NC-1. Poster Presentation: Zeneca Student's Day, Jealott's Hill Research Station, Bracknell, UK.

Anna Barnett, Petrina Smith, Linda King, John Windass and Robert Possee (1996) Identification of an inhibitor of apoptosis from *Heliothis zea* nucleopolyhedrovirus. Manuscript in preparation (*Virology*).

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Abbreviations

AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
Amp	ampicillin
APS	ammonium persulphate
ASFV	African swine fever virus
ATP	adenosine triphosphate
BHK	baby hamster kidney
BmNPV	*Bombyx mori* nucleopolyhedrovirus
bp	base pairs
BSA	bovine serum albumin
BV	budded virus
CAT	chloramphenicol acetyl transferase
ced	cell death gene
CIP	calf intestinal phosphatase
CNS	central nervous system
CpGV	*Cydia pomonella* granulovirus
cpm	counts per minute
*crm*A	cytokine response modifier A
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythimidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EBV	Epstein-Barr virus
ECV	extracellular virus
EDTA	ethylene-diamine-tetra acetic acid
EEC	European economic community
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain protein
FasL	Fas ligand
FCS	foetal calf serum
g	gram
hid	head involution defective
hILP	human IAP-like protein
hpi	hours post infection
hr	homologous repeat
HSV-1	Herpes simplex virus type 1
H. zea	*Heliothis zea*
HzSNPV	*Heliothis zea* single nucleopolyhedrovirus
iap	inhibitor of apoptosis
ICE	interleukin -1β-converting enzyme
IE	immediate early
IL-1β	interleukin-1β
JHE	juvenile hormone esterase
kbp	kilobase pairs
kDa	kilo Dalton
LB	Luria broth
LD	lethal dose
LGT	low gelling temperature

LMP	latent membrane protein
µCi	micro Curie
µg	microgram
µl	microlitre
µm	micrometre
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
min	minute
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
NAIP	neuronal apoptosis inhibitory protein
NF-κB	nuclear factor κB
ng	nanogram
nm	nanometre
NOV	non-occluded virus
NPV	nucleopolyhedrovirus
nt	nucleotide
OB	occlusion body
OD	optical density
OpMNPV	*Orgyia pseudotsugata* multiple nucleopolyhedrovirus
OV	occluded virus
p	page
pp	pages
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDV	polyhedrin derived virus
PE	polyhedrin envelope
PEG	polyethyleneglycol
pfu	plaque forming units
pi	post infection
PNK	polynucleotide kinase
psi	pounds per square inch
RFLP	restriction fragment length polymorphism
RIP	receptor-interacting protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RPV	rabbit pox virus
SDS	sodium dodecyl sulphate
serpin	serine protease inhibitor
Sf	*Spodoptera frugiperda*
SMA	spinal muscular atrophy
SSC	standard saline citrate
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N'-N'-N'-N'tetramethylethylenediamine
TRADD	TNF-R1-associated death domain protein
TRAF	TNF receptor-associated factor
TNF-R	Tumour necrosis factor receptor

Tris	Tris (hydroxymethyl)-aminoethane
T. ni	*Trichoplusia ni*
U	units
UV	ultra violet
V	volts
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indolyl-B-galactosidase
°C	Celsius (centigrade)

Chapter One

Introduction

1.1. General introduction.

The detrimental effects of chemical pesticides, both on individuals and the environment, have been well documented (Iyaniwura, 1991). Chemical pesticides cause pollution of ground water (Ritter, 1990) and contamination of foodstuffs by residues (Picer *et al.*, 1978; Kannan *et al.*, 1994). Pesticide residues can become concentrated in the tissues of organisms at the top of the foodchain (Kaphalia *et al.*, 1981; Simmonds *et al.*, 1993). Chemical pesticides are also indiscriminate in action, leading to the demise of target and non-target organisms alike (Leisy and van Beek, 1992). Records of insect resistance to existing chemical pesticides, and the details of the biochemical basis for the observed resistance, are continuing to emerge (Oppenoorth, 1984; Mouches *et al.*, 1986; Ffrench-Constant, 1993; Mutero *et al.*, 1994). There are a limited number of biochemical pathways suitable for chemical insecticide targets. The limited number of sites in the insect for insecticide action has resulted in few novel chemical insecticides being commercially developed, with which to combat resistant pests.

Increasing concern about such issues has renewed interest in microbial control agents, which are a selective alternative (Burges, 1981) and pose a minimal threat to non-target organisms (Summers *et al.*, 1975; Heinz *et al.*, 1995), the operator and the environment. Microbial control agents can be successfully incorporated into integrated pest management (IPM) programmes. Many microbial agents, including viruses, bacteria, fungi, nematodes and parasitic insects, have been examined as potential pest control agents (Miller *et al.*, 1983; Khachatourians, 1986), though only a few are in widespread use. Most insect pathogens, like other biological control agents, require a certain host density to be able to maintain themselves in a population. Epizootics of these pathogens can occur when there is a host population explosion, but in many cases considerable crop damage has been sustained before population levels are suppressed. Thus, a frequent criticism of microbial pathogens is that their effects on a pest population are usually too little too late. Since it is

unrealistic to expect naturally occurring pathogens to maintain pest populations consistently below economic thresholds, the issue becomes one of manipulating and utilising them more effectively, by inundative or inoculative approaches as a component of the IPM system.

Viruses pathogenic to insects have been described from several virus families, including the Iridoviridae, Poxviridae, Reoviridae and Baculoviridae. Most of these families have representatives which infect vertebrates and plants, in addition to the class Insecta (Huber, 1990). Baculoviruses are an exception, infecting only arthropods, and are thus considered the safest prospect for economic use as insect pest control agents. The majority of baculovirus isolates are from the Lepidoptera; over 500 species of this order are known to be hosts (Matthews, 1992). In addition, baculovirus isolates have been recorded from the Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichoptera (Granados and Frederici, 1986) and Crustacea (Couch, 1974; Anderson and Prior, 1992; Chang *et al.*, 1993). Baculoviruses have been successfully used as insecticides to control pests of agricultural crops, forests and plantations (Payne, 1982).

The high degree of host specificity of many isolates reduces their impact on non-target species but may also limit their use, particularly on crops where a complex of pests is established. A disadvantage of baculovirus insecticides is their slow speed of action. The time period between the initial infection by baculovirus to the death of diseased larvae varies and is affected by many factors, including larval age, temperature, virus dose, virulence of virus isolate, and nutrition of the larval host. The more virulent strains may kill larvae within 2 to 5 days (Ignoffo, 1966), but less virulent strains may take 2 to 3 weeks to kill their host. This inherent characteristic of baculoviruses permits crop damage to continue for some time after treatment.

Baculoviruses have been developed for use as vectors for the high level expression of heterologous proteins in insect cells (reviewed in Bishop, 1992).

The baculovirus expression system is capable of producing large amounts of recombinant proteins.

Advances in biotechnology have provided methods with which to address the unfavourable properties of baculoviruses with regard to their use as insecticides, as well as allowing the genetic basis of biological action to be unravelled. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most extensively studied baculovirus at the molecular level. Most of our current understanding of baculovirus gene structure and regulation has been derived from the study of AcMNPV over the past decade.

Genetic engineering has been the main focus for the improvement of baculovirus insecticides. The environmental release of genetically modified baculoviruses is currently under study and highlights the need for a greater understanding of the natural epizootiology of baculoviruses (Cory and Entwistle, 1990).

1.2. History of insect virus research.

Scientific reports from the sixteenth and seventeenth centuries contain detailed descriptions of the "wilting" disease of silkworms, indicating that insect viruses have been of interest for a long time. In the 1940s, Bergold (1947) made the crucial discovery of rod-shaped virions, characteristic of baculoviruses (baculo = rod), embedded within the crystalline protein matrix of the occlusion bodies. In the 1960s many new types of insect viruses, including iridoviruses, nodaviruses, polydnavirus-like particles and entomopoxviruses, were discovered. Progress in the establishment of insect cell lines (Grace, 1962) lead to the development of techniques for *in vitro* baculovirus culture. In the 1970s, the first insect virus was registered for use as a biological control agent. The mid-1970s marked the beginning of the molecular biology era. Molecular research on baculoviruses lead to their application as foreign gene expression vectors during the 1980s, and the development of genetic

engineering techniques has also advanced the improvement of baculoviruses to produce new biological pesticides.

1.3. Classification of baculoviruses.

The family Baculoviridae are divided into two genera based on structural criteria; the Nucleopolyhedroviruses (NPVs) and the Granuloviruses (GVs) (Murphy *et al.*, 1995). The occlusion bodies of NPVs range in size from 0.15 to 15 μm and contain many virions, which are either singly enveloped (SNPV) or multiply enveloped (MNPV). Occlusion bodies of viruses from the genus Granulovirus may be ovicylindrical and are 0.3 x 0.5 μm in size, containing one, or more rarely, two virions. The virions within the occlusion bodies consist of one or more rod-shaped nucleocapsids within an envelope derived by *de novo* synthesis and assembled in the nucleus (NPVs) or in the nuclear-cytoplasmic milieu after rupture of the nuclear membrane (GVs).

The nomenclature of baculovirus species is based on the host from which the virus was first isolated (Tinsley and Kelly, 1985). GVs and NPVs may infect the same host species, as has been observed for *Agrotis segetum* (Kozlov *et al.*, 1981) and *Trichoplusia ni* (Martignioni *et al.*, 1973). AcMNPV is the type baculovirus species.

1.4. Baculovirus structure.

1.4.1. General features.

Baculoviruses have a bi-phasic replication cycle that produces two distinct forms of infectious virus; budded virus (BV) and occluded virus (OV). The two virion types are genetically identical, but differ in morphology, protein composition, antigenicity, tissue tropism and time of appearance in the virus infection cycle (Summers and Volkman, 1976; Volkman and Keddie, 1990; Braunagel and Summers, 1994). BVs are involved in secondary infection

within the insect whereas OVs are involved in the horizontal transmission of the virus between insect larvae and are responsible for the primary infection of the host. OVs are contained within a crystalline structure which serves to stabilise the virus in the environment.

The baculovirus is composed of a rod-shaped nucleocapsid with an average size of 30 - 35 nm in diameter and 250 - 300 nm in length, surrounded by a membrane envelope. Baculovirus nucleocapsids are composed of a proteinaceous sheath known as the capsid, and a nucleoprotein core (Rohrmann, 1990). The proteins encoded by the genes *p39* (Pearson *et al.*, 1988; Blissard *et al.*, 1989; Thiem and Miller, 1989a), *p24* (Wolgamott *et al.*, 1993) and *p87* (Mueller *et al.*, 1990; Lu and Carstens, 1991) have been identified as major components of the nucleocapsid, but additional minor proteins such as that encoded by ORF 1629 of AcMNPV are also associated (Vialard and Richardson, 1993). The nucleoprotein core consists of a single molecule of double-stranded DNA associated with protein, the majority of which is a small basic protein (6.9 kDa), rich in arginine (Wilson *et al.*, 1987; Russell and Rohrmann, 1990; Maeda *et al.*, 1991). It has been suggested that condensation of the DNA and basic DNA-binding protein complex is necessary for encapsidation (Burley *et al.*, 1982).

1.4.2. Baculovirus genome structure.

The genome of baculoviruses consist of a single, large, double-stranded DNA molecule which is circular and supercoiled. The size of the genome varies, but is between 88 and 160 kilobasepairs (kbp) (Arif, 1986) and the A+T nucleotide content also varies widely (40-70%). Extensive sequencing and transcriptional analysis of several baculovirus genomes is providing a representation of gene organisation among the baculoviruses - the 133.9 kbp complete DNA sequence of AcMNPV has been published (Ayres *et al.*, 1994); in addition, the 128.4 kbp complete DNA sequence of *Bombyx mori* (Bm) NPV is available in the National Institute of Health (NIH) database. Regions of *Orgyia pseudotsugata* (Op)

MNPV (complete sequence finished, Rohrmann *et al.*, 1996), and of *Choristoneura fumiferana* (Cf) MNPV, *Cydia pomonella* (Cp) GV and *Heliothis zea* (Hz) SNPV are also accessible in the NIH databases. The relatedness of baculoviruses at the DNA level has been studied by comparative restriction enzyme mapping and hybridisation analyses (Summers *et al.*, 1980; Smith and Summers, 1982; Possee and Kelly, 1988; Gombart *et al.*, 1989). These studies showed a wide variation in the gross organisation and DNA sequence of baculovirus genomes although some gene regions, such as polyhedrin, are highly conserved (Zanotto *et al.*, 1993). Overall, baculoviruses appear to contain genes of similar types, although the homology between genes from different baculovirus species varies.

A diagrammatic representation of the AcMNPV genome is shown in Figure 1.1. The genome of AcMNPV is predicted to encode at least 154 potential open reading frames (ORFs) of >150 nucleotides (Ayres *et al.*, 1994), interspersed with several repeat regions containing two to eight 30-bp imperfect palindromic sequences, centred around *Eco*RI sites, known as homologous repeats (*hr*s) (Cochran and Faulkner, 1983).

Baculovirus ORFs are closely spaced and are found on both strands of the DNA; several ORFs overlap. Most ORFs, however, are separated by 2 to 200 bp of DNA rich in A+T content, constituting the promoter and termination regions of genes. Some promoters are located within neighbouring ORFs, for example, the *cg30* gene promoter of AcMNPV is located within *vp39* (Thiem and Miller, 1989b). Transcripts of one gene frequently initiate within, or extend into or through, neighbouring ORFs. There are numerous cases in which genes are transcribed as bicistronic or multicistronic RNAs (Eldridge and Miller, 1992; Whitford and Faulkner, 1992; Passarelli and Miller, 1994). There is no apparent clustering of genes with regard to function in the genomes of NPVs. Genes with related functions, such as those encoding virion structural proteins or late gene expression factors, are distributed randomly throughout the genome.

1.4.3. Structure of budded viruses (BVs).

Nucleocapsids produced early in infection pass from the cell nucleus to the cytoplasm and bud from the plasma membrane of the infected cell. BV contains a virus-encoded N-glycosylated protein called gp64 in OpMNPV (Blissard and Rohrmann, 1989) or gp67 in AcMNPV and CfMNPV (Whitford *et al.*, 1989; Hill and Faulkner, 1994). The gp64 protein accumulates at the plasma membrane where it becomes incorporated into the BV envelope as the virion buds from the cell (Volkman *et al.*, 1984). BVs enter neighbouring cells by a process of endocytosis. Acidification of the endosome is thought to initiate fusion of the endosomal membrane and the viral envelope, causing release of the nucleocapids (Volkman and Goldsmith, 1985). The gp64 protein contains hydrophobic domains which are required for its pH-dependent membrane fusion activity (Monsma and Blissard, 1995). A 25 kDa protein (p25) is also associated with the BV envelope and may be responsible for the interaction with polyhedrin during OV formation (Russell and Rohrmann, 1993).

1.4.4. Structure of occluded viruses (OVs).

At late stages in the infection process, nucleocapsids are retained in the nucleus of the host cell, where they are enveloped and incorporated into occlusion bodies. The occlusion body functions to protect the virion nucleocapsids in the environment, enabling survival of the virus outside of the host for several years (Evans and Harrap, 1982). Single (SNPV), or several (MNPV) nucleocapsids are enveloped by a membrane to form the virion particle (Fraser, 1986a). Baculovirus-induced intranuclear microvesicles are the foci for the assembly of the OV envelope (Hong *et al.*, 1994). Three proteins are involved in the formation of these microvesicle structures and later become components of the OV envelope: ODV-E66 (Hong *et al.*, 1994), ODV-E56 (Braunagel *et al.*, 1996) and p25 (Russell and Rohrmann, 1993). The structural protein gp41, which contains O-linked N-acetylglucosamine, has

been identified in the OVs of BmNPV, AcMNPV and HzSNPV (Sugimori *et al.*, 1991; Whitford and Faulkner, 1992; Ma *et al.*, 1993). The gp41 protein is thought to be located between the envelope membrane and the nucleocapsid.

The matrix of occlusion bodies is composed primarily of a single polypeptide which forms a crystalline lattice around the virion(s) (Kelly *et al.*, 1983). The matrix proteins of NPVs and GVs, known as polyhedrin and granulin, respectively, are closely related. The polyhedrin gene encodes a protein of approximately 29 kDa (Hooft van Iddekinge *et al.*, 1983; Iatrou *et al.*, 1985; Leisy *et al.*, 1986; Cameron and Possee, 1989). The polyhedrin genes of NPVs are on average 80% similar (Blissard and Rohrmann, 1990), and in some cases exhibit as much as 98% homology (van Strien, 1992).

Mature occlusion bodies also have an additional covering, known as the polyhedron envelope (PE) or calyx, on the surface of the occlusion body (Harrap, 1972). This is a proteinase-sensitive membrane (Russell and Rohrmann, 1990) composed of polysaccharide (Minion *et al.*, 1979) and the virus-encoded 34 kDa protein, pp34 (Whitt and Manning, 1988; Gombart *et al.*, 1989). The polyhedron envelope probably serves to stabilise the virus particle, since OVs lacking the PE are fragile (Zuidema *et al.*, 1989). The 10 kDa protein, p10, and a 74 kDa protein, p74, are also associated with OV structure. The *p10* gene is non-essential for BV infectivity (Vlak *et al.*, 1988), however, cells infected with an AcMNPV *p10* deletion mutant do not possess the nuclear structures associated with a wild-type infection (Williams *et al.*, 1989). The *p74* gene has been shown to be required for infectivity *in vivo* (Kuzio *et al.*, 1989).

1.5. Baculovirus replication *in vitro*.

1.5.1. General features.

The development of insect cell lines to support the growth of baculoviruses *in vitro* has permitted the extensive study of baculovirus replication and gene expression. At least 39 cell lines from Lepidoptera have been established (Hink, 1980). Gene expression has been studied most comprehensively for AcMNPV infection of Sf21, a cell line derived from the pupal ovarian cells of *Spodoptera frugiperda* (Vaughn *et al.*, 1977). Sf21 is popular for expression work because it performs well in both monolayer and suspension culture. OpMNPV is studied using *Lymantria dispar* cells (Quant-Russell *et al.*, 1987), and BmNPV using *Bombyx mori* cells (Maeda, 1989a). In all cases, the BVs (but not the OVs) are highly infectious to cells in culture. In addition, baculovirus DNA is infectious *per se* (Bud and Kelly, 1980). BV infection in cell culture is thought to represent general secondary infection in the insect host.

Baculovirus infection of insect cells is associated with a characteristic cytopathic effect (CPE) which involves the distension of the nucleus and the formation of a virogenic stroma in the nucleus (Xeros, 1956), the site of viral DNA synthesis. The baculovirus phosphoprotein pp31, encoded by the *39K* gene, is known to be associated with the virogenic stroma (Guarino *et al.*, 1992). The pp31 protein is phosphorylated by both cellular and virus-encoded kinases (Broussard *et al.*, 1996).

In AcMNPV-infected cells, virus gene expression is characterised by four temporal phases (Kelly and Lescott, 1981; Blissard and Rohrmann, 1990), in which successive phases may be dependent upon the previous phase for activation. Analyses of viral mRNAs have identified temporal classes of viral gene transcription. In the immediate early phase, virus genes are transcribed using host-cell polymerases; expression of these genes does not require prior synthesis of other virus proteins. In the delayed early phase some prior

synthesis of immediate early proteins is required. The late phase of gene expression is accompanied by DNA replication and the production of baculovirus structural proteins. In the very late stage, two virus proteins, polyhedrin and p10, are produced in large amounts. Maximal expression of very late genes occurs around 24 hours p.i. and remains at a high level beyond 48 hours p.i.

Baculovirus infection affects the host cell markedly, altering the cytoskeletal and nuclear structure initially, and during the late phase of infection, decreasing the levels of host-derived RNAs (Ooi and Miller, 1988). Host protein synthesis is also shut down at this time (Carstens *et al.*, 1979). Actin polymerisation is induced by the entry of AcMNPV into Sf host cells (Charlton and Volkman, 1993).

1.5.2. Adsorption, penetration and uncoating.

BV enters cells by receptor-mediated adsorptive endocytosis (Volkman and Goldsmith, 1985; Charlton and Volkman, 1993). This pathway requires gp64, the major glycoprotein of BV (Volkman *et al.*, 1984), which is responsible for receptor interaction with, and fusion to, the endosomal membrane (Blissard and Wenz, 1992). Specific binding of BV to Lepidopteran cells, and the entry of BV into host cells by direct membrane fusion has been examined using fluorescence-activated cell sorting (FACS) analysis (Horton and Burand, 1993). In addition, the infectivity of BV is blocked if the gp64 protein is not N-glycosylated, suggesting that glycosylation is necessary for membrane fusion (Jarvis and Garcia, 1994). Although the cell receptor that binds BV/gp64 has not yet been identified (Tardieu *et al.*, 1982), optimal binding of BVs to the receptor occurs at alkaline pH (Horton and Burand, 1993). The approximate number of BV receptor sites per cell is estimated to be 10^6 (Wickham *et al.*, 1990; Horton and Burand, 1993).

After fusion of the virus envelope and cell membrane, nucleocapsids are released into the cytoplasm. Nucleocapsids then move through the cell cytoplasm, interact with the nuclear pore and release the nucleoprotein core from the capsid sheath of the virus into the nucleus (Tweeten *et al.*, 1980). A virus-encoded protein kinase may be involved in this release process (Wilson and Consigli, 1985).

1.5.3. The early and delayed early phases of gene expression.

Baculovirus immediate early genes do not require prior synthesis of other virus proteins for their expression, as evidenced by their transcription in the presence of cycloheximide (Rice and Miller, 1986). They are detectable in the host cell from 0-3 h.p.i. Six immediate early genes have been identified to date; *ie-1* (Guarino and Summers, 1986a; 1987; 1988; Chisholm and Henner, 1988; Thielmann and Stewart, 1991), *ie-n* or *ie-2* (Carson *et al.*, 1988; 1991; Thielmann and Stewart, 1992), *p35* (Nissen and Friesen, 1989; Kamita *et al.*, 1993), *me53* (Knebel-Mörsdorf *et al.*, 1993), *he65* (Becker and Knebel-Mörsdorf., 1993) and *pe-38* (Krappa and Knebel-Mörsdorf, 1991; Krappa *et al.*, 1992). These six genes are involved in the transcriptional regulation of genes expressed later in the infection cycle. The early and delayed early phases of gene expression, therefore, represent the beginning of the highly ordered cascade of viral replication.

The *ie-1* gene is the most extensively studied of the immediate early genes. It encodes a putative DNA-binding protein (Guarino and Dong, 1991), which is a multifunctional transactivator of many virus-encoded genes. Both delayed early (Guarino and Summers, 1986a) and late genes (Guarino and Summers, 1988) have been shown to be transactivated by IE-1. In addition, the BmNPV homolog of AcMNPV *ie-1* has also been shown to stimulate the promoter of AcMNPV *39K* (Huybrechts *et al.*, 1992). Conversely, the expression of two immediate early genes, *ie-0* and *ie-2*, is inhibited by the IE-1 protein (Carson *et al.*, 1991; Kovacs *et al.*, 1991). The expression of *ie-1* is regulated by *cis*-acting

elements which reside in the 5' non-coding region of the gene (Pullen and Friesen, 1994). The *ie-1* gene product also interacts with *hr* sequences in the genome (Kovacs *et al.*, 1992).

Transcriptional analyses of *ie-1* have shown that it is spliced to produce *ie-0* (Chisholm and Henner, 1988); *ie-0* is also involved in gene transactivation but does not transactivate delayed-early gene promoters in the absence of an enhancer element (Kovacs *et al.*, 1991). Functional analysis of the IE-1 protein revealed two functional domains, one for DNA binding and the inhibition of *ie-0* expression, and a second domain for the activation of enhancer-linked genes (Kovacs *et al.*, 1992). In addition, IE-1 is required for viral DNA replication in transient transfection assays (Kool *et al.*, 1994; Lu and Miller, 1995). The IE-1 protein has also been shown to interact with host cell genes, specifically causing an increase in the expression of reporter genes under the control of the *B. mori* cytoplasmic actin gene promoter (Lu *et al.*, 1996).

A further immediate early gene, *ie-2*, previously known as *ie-n*, predicts a protein product of 47 kDa. This gene was also identified as a factor which transactivates the delayed early gene *39K* (Carson *et al.*, 1988; Carson *et al.*, 1991). Expression of *ie-2* alone does not have the capacity to transactivate *39K*; *ie-2* stimulates expression when *ie-1* is limiting (Yoo and Guarino, 1994). The transcription of *ie-2* is known to be regulated by *hr* regions, IE-1 and the IE-2 protein itself (Carson *et al.*, 1991; Yoo and Guarino, 1992). It has been shown that *ie-2* differs in its temporal expression profile compared to *ie-1*; *ie-2* expression peaks early in infection then declines, whilst *ie-1* is expressed throughout the infection (Guarino and Summers, 1987; Carson *et al.*, 1991).

Transcription of *p35* mRNA occurs within the first hour of infection, peaks after 6 to 8 hours, and declines thereafter (Nissen and Friesen, 1989). P35 is known to inhibit virally-triggered host cell apoptosis (Clem *et al.*, 1991), which will be discussed in more detail below.

The coding region of *me53* is located approximately 300 bp upstream of the *ie-0* coding region, and its promoter forms a divergent unit with the *ie-0* promoter (Chisholm and Henner, 1988). Transcripts of *me53* are detectable in the insect cell from 1 h.p.i., but activity decreases from 26 h.p.i. (Knebel-Mörsdorf *et al.*, 1993). A zinc finger motif is present at the carboxy terminus of ME53, although its functional significance is not known. Additionally, ME53 contains a proline-rich region, indicating a putative transcriptional activator motif (Mermod *et al.*, 1989).

The *he65* promoter is active from 2 to 48 h.p.i. (Becker and Knebel-Mörsdorf, 1993), encoding a protein of predicted size 65 kDa. Transcription of *he65* shows a temporal delay of 1 hour compared with the other immediate early genes, suggesting differential regulation of the *he65* promoter during the early stages of infection. HE65 remains stable in the late stages of infection, although its function is uncharacterised.

The immediate early gene *pe38* contains zinc finger and leucine zipper motifs (Krappa and Knebel-Mörsdorf, 1991). Expression of *pe-38* resembles that of *ie-2* and *p35*, in that transcription peaks very early in the infection and then declines. OpMNPV *p34* is a homologue of AcMNPV *pe-38*, and has been shown to transactivate the OpMNPV *ie-2* promoter (Wu *et al.*, 1993). PE-38 localises to distinct nuclear regions (Krappa *et al.*, 1995), and it has been demonstrated that an insect-cell specific protein binds to upstream regions of the *pe-38* promoter (Krappa *et al.*, 1992).

The delayed early phase of viral gene expression requires the prior synthesis of immediate early virus proteins. However, low levels of delayed early gene transcription can be supported by uninfected-cell nuclear extracts (Glocker *et al.*, 1992), suggesting the boundary between immediate early and delayed early genes is a difference in the levels of promoter activity rather than an absolute distinction. The most well-studied delayed early gene is *39K*, encoding a 31 kDa phosphoprotein (pp31) which is localised to the nuclear

matrix of virus-infected cells (Wilson and Price, 1988), and is transactivated by IE-1 (Guarino and Summers, 1986a).

During the early phase of AcMNPV infection, the environment of the cell is altered in preparation for the replication and expression of viral DNA. In the first 6 h.p.i., AcMNPV produces proteins required for DNA replication, including DNA polymerase (*dnapol*) (Miller *et al.*, 1981, Tomalski *et al.*, 1988), DNA helicase (*p143*) (Lu and Carstens, 1991), and a proliferating nuclear antigen homologue (*pcna*) (O'Reilly *et al.*, 1989).

1.5.3.1. Early gene promoter structure.

Early baculovirus genes are transcribed by the host RNA polymerase II. Most early promoters require the presence of the regulatory protein IE-1 (or its spliced gene product IE-0) to be efficiently transcribed in transient transfection assays (Guarino and Summers, 1986a). Early baculovirus promoters have a similar structure to eukaryotic promoters (Hoopes and Rohrmann, 1991), consistent with the use of host-cell transcription factors for the expression of these genes.

The main early consensus promoter sequences are a CAGT motif (Blissard and Rohrmann, 1989), and a TATA box 25-30 bp upstream of the CAGT transcriptional start point. The TATA-box motif is recognised by the TATA-binding protein, a component of host transcription factor IID, which provides a nucleation site for the assembly of the host RNA polymerase II complex. While the TATA box influences transcription initiation and supports transcription at basal levels (Blissard *et al.*, 1992), it is subject to activation by upstream elements in the baculovirus promoters (Dickson and Friesen, 1991; Guarino and Smith, 1992; Kool *et al.*, 1994; Pullen and Friesen, 1995). The CAGT sequence is important for efficient basal transcription initiation but does not appear to be essential for transcription in the presence of a TATA box (Blissard *et al.*, 1992). A consensus sequence similar to CAGT has been described in

several insect genes (Hultmark *et al.*, 1986; Bucher, 1990; Cherbas and Cherbas, 1993). In addition, host cell transcription factors have been shown to bind to GATA and CACGTG motifs, resulting in the activation of basal promoter transcription (Krappa *et al.*, 1992; Kogan and Blissard, 1994). The GATA motif has been described as a transcription factor binding site in Lepidopteran RNA polymerase II-transcribed genes (Skeiky and Iatrou, 1991), suggesting that during the initial stages of the infection, insect transcription factors play a role in stimulating baculovirus early gene promoters in addition to the stimulation provided by virally-encoded transcription factors. Three early gene promoters which have been examined in detail (*p35*, gp64 and *p39*) also have a late promoter motif and are transcribed both early and late in the infection cycle (Blissard and Rohrmann, 1991; Dickson and Friesen, 1991; Guarino and Smith, 1992).

1.5.4. The late phase of gene expression.

The late phase of gene expression, which in AcMNPV infection of Sf21 cells begins at 6 h.p.i. and extends to 24 h.p.i., involves DNA replication, late gene expression and the production of BV. The proteins synthesised at this time include BV structural proteins (e.g., p6.9K, vp39 and gp64), the minor virion structural proteins, and a variety of others such as pp31, ubiquitin, superoxide dismutase and conotoxin. The ubiquitin gene homologue, (*vubi*) (Guarino and Smith, 1990) has been shown to be anchored to the viral membrane by a phospholipid protein mechanism (Guarino *et al.*, 1995), and is of unknown function, though it is known to be non-essential for virus replication both *in vitro* and *in vivo* (Reilly and Guarino, 1996). The superoxide dismutase (*sod*) homologue may have a role in limiting the damage caused by free radicals which are released by the cell late in the infection process (Tomalski *et al.*, 1991). The conotoxin homologue is a putative calcium ion channel regulator (Eldridge *et al.*, 1992).

In the late stage of the infection, the baculovirus DNA adopts a unique nucleoprotein structure (Wilson and Miller, 1986). This may be the prelude to viral DNA packaging or may be a specific structure for DNA replication. Late transcription is coupled to DNA replication; aphidicolin blocks both viral DNA replication and late viral gene transcription (Rice and Miller, 1986). Eighteen late expression factor (*lef*) genes have been identified in AcMNPV (Li *et al.*, 1993; Lu and Miller, 1994; Morris *et al.*, 1994; Passarelli and Miller, 1993a; 1993b; 1993c; 1994; Passarelli *et al.*, 1994a; 1994b; Todd *et al.*, 1995), and collectively they can support viral replication and late gene expression in Sf21 cells using a transient transfection assay (Li *et al.*, 1993; Passarelli and Miller, 1993a; 1993b; 1993c; 1994; Lu and Miller, 1994; 1995; Todd *et al.*, 1995; Todd *et al.*, 1996). This assay used an overlapping library of AcMNPV clones to transactivate expression from reporter plasmids containing early, late or very late gene promoters linked to the chloramphenicol acetyl transferase (CAT) gene (Passarelli and Miller, 1993a; 1993b; 1993c). Following transfection of Sf21 cells with each of these plasmids and the AcMNPV library lacking one (or more) clones, regions of the AcMNPV genome containing genes necessary for late gene expression were identified.

The LEF proteins include the early transactivators IE-1 and IE-2 (Passarelli and Miller, 1993a), which may be due to the roles of these proteins in the transactivation of early gene expression (Guarino and Summers, 1986a; Carson *et al.*, 1988). The helicase gene, *p143*, has been identified as a *lef* (Passarelli and Miller, 1993b) and is required for DNA replication (Gordon and Carstens, 1984). The *p143* gene has been shown to have an essential role in AcMNPV DNA replication using the temperature sensitive mutant ts8 (Brown *et al.*, 1979; Gordon and Carstens, 1984). Since late gene expression is dependent upon DNA replication, *p143* may have an indirect effect on late gene expression by controlling aspects of DNA replication.

Similarly, *dnapol* has been identified as a *lef*, since it is involved in DNA replication. The *p35* gene has been shown to have *lef* activity, presumably due

to its ability to suppress apoptosis early in infection, thereby allowing late gene expression to proceed. The *39K* gene also has *lef* activity, although its involvement in late gene expression has not been established. The product of *39K* expression, pp31, is associated with the virogenic stroma assembled in the nucleus of the host cell at late times post-infection (Guarino *et al.*, 1992). The functions of the remaining *lef* proteins (named *lef*s 1 to 11) are not yet established, although *lef*-8, p47 and *lef*-9 have been implicated as components of the virus-encoded RNA polymerase complex (Passarelli *et al.*, 1994b; Xu and Guarino, 1996). The remaining *lef*s may be involved in aspects of viral DNA replication.

A transient expression assay system has also been used to identify genes involved in the DNA replication (and by implication the late gene expression) of OpMNPV infection of *L. dispar* cells (Ahrens and Rohrmann, 1995a). In this assay, OpMNPV *ie-1*, *lef*-2, *lef*-1, DNA polymerase, helicase and *lef*-3 were found to be essential for DNA replication (Pearson *et al.*, 1993; Ahrens and Rohrmann, 1995a; 1995b). In addition, three genes, *ie-2*, *p34* and *iap*, stimulated DNA replication (Ahrens and Rohrmann, 1995b).

The *hr* sequences of AcMNPV have been implicated as origins of DNA replication (Kool *et al.*, 1993; Leisy and Rohrmann, 1993) based on a demethylation assay for plasmid DNAs transfected into baculovirus-infected insect cells. However, the presence of *hr* sequences is not necessary for expression from late reporter genes in transient expression assays; other sequences have also been assigned roles as origins of viral DNA replication (Kool *et al.*, 1994).

1.5.4.1. Late gene promoter structure.

Late baculovirus gene expression involves the change from transcription by host RNA polymerase II to transcription from a novel, virus-induced, α-amanitin resistant RNA polymerase activity. This has been confirmed by the

presence of an α-amanitin resistant peak in chromatographic analyses of infected cell lysates (Fuchs *et al.*, 1983; Huh and Weaver, 1990; Yang *et al.*, 1991). An RNA polymerase complex capable of initiating accurate transcription from late and very late gene promoters has recently been purified from AcMNPV-infected cells. The complex was shown to consist of five protein subunits; three of which have been positively identified as baculovirus gene products. The complex was shown to consist of a 53 kDa protein identified as *lef*-9, a 102 kDa protein identified as *lef*-8, a 47 kDa protein identified as p47 (Carstens *et al.*, 1993), and two unidentified proteins of 51 and 140 kDa (Xu and Guarino, 1996).

The virus-induced RNA polymerase is responsible for late gene transcription from a specific TAAG initiation motif, which is also the predominant element for very late promoter activity (Ooi *et al.*, 1989; Morris and Miller, 1994). The context of the TAAG appears to affect the level of transcription. Some late genes have been shown to have multiple TAAG motifs upstream of the ORF, each serving as an initiation site i.e., *vp39* is transcribed from three TAAG transcriptional start sites at -57, -105 and -321 relative to the ORF methionine start codon (Thiem and Miller, 1989b). However, the *p74* gene utilises a TATTG motif which functions as a late promoter (Kuzio *et al.*, 1989). The presence of distinct late gene promoter elements supports the observation that late gene transcription is not carried out by host cell RNA polymerase II (Grula *et al.*, 1981).

1.5.5. The very late phase of gene expression.

Occluded viruses are produced in the very late phase of the baculovirus replication cycle, which commences at approximately 18 to 24 h.p.i. and extends to 76 h.p.i. or until cell lysis occurs. This phase is recognised by the dramatic increase in the transcription of *polh*, the gene encoding the polyhedrin protein which forms the main constituent of the occlusion body. Hyperexpression of polyhedrin, which is non-essential for BV production, has

been exploited in the use of baculoviruses as successful vectors for foreign gene expression (Smith *et al.*, 1983; Pennock *et al.*, 1984) (discussed further in Section 1.7). The baculovirus expression system is now widely used and has been extended to include the use of other promoters active during the very late phase, such as that of *p10* (Kuzio *et al.*, 1984). Like polyhedrin, p10 is also non-essential for BV production; it has been implicated in microtubule assembly (Cheley *et al.*, 1992) and nuclear disruption at the end of the infection (Williams *et al.*, 1989).

1.5.5.1. Very late gene promoter structure.

Very late genes are also transcribed by the virus-induced RNA polymerase from TAAG motif-based promoters. The very late genes *polh* and *p10* both possess a 12 nucleotide consensus sequence around the TAAG core (Rohrmann, 1986), and a downstream A+T-rich region, corresponding to the 5' untranslated leader sequence of the mRNAs (Ooi *et al.*, 1989; Weyer and Possee, 1989). The temporal expression of polyhedrin and *p10* are dissimilar however, since the *p10* promoter is active at earlier times than the polyhedrin promoter (Roelvink *et al.*, 1992).

1.5.6. Virion assembly in the late and very late phases.

Viral DNA is packaged and nucleocapsids are assembled during the late and very late phases of gene expression. The nucleocapsids leave the nucleus, by budding or by exit through nuclear pores, and pass through the cytoplasm. By interacting end-on with gp64-rich sections of the plasma membrane, the nucleocapsids bud from the cell, forming extracellular BV.

Occlusion of virions occurs in the nucleus of AcMNPV-infected cells from approximately 20 to 76 h.p.i. Nucleocapsids are aligned along phospholipid membrane segments and acquire an envelope of the protein 25K, a prerequisite for their incorporation into an occlusion body. Baculovirus

mutants known as *fp* (few polyhedra) are defective in membrane envelopment and 25K production (Beames and Summers, 1989). Few nucleocapsids are packaged into occlusion bodies in *fp* mutants. It has been shown that *fp* mutants produce a higher titre of budded virus, suggesting that envelopment in the nucleus directs nucleocapsids to be occluded rather than released as BVs. Nuclear localisation of polyhedrin is also reduced in *fp* mutants (Jarvis *et al.*, 1992). The maturation of occluded viruses involves the deposition of the calyx (polyhedron envelope), and pp34 of AcMNPV (Whitt and Manning, 1988) or the homologous 32 kDa protein of OpMNPV (Gombart *et al.*, 1989).

1.6. Baculovirus infection *in vivo*.

The most common mode of entry of a baculovirus into its insect host is by ingestion during larval feeding on foliage contaminated with virus. Although the pupal and adult stages of some species may be found to be infected, these insects are considered to have acquired the virus as larvae (Vail and Gough, 1970). Other modes of entry and infection include transovarial (passage within the egg) and transovum (contamination of the egg surface) passage, through spiracles into the tracheal system, or by parasitism (Granados, 1980). However, these are not considered important mechanisms of virus transmission because of their relatively low frequency of occurrence.

The life cycle of a baculovirus in an insect larva is illustrated in Figure 1.2. The occluded form of the virus is involved in the process of horizontal infection. Following ingestion of virus, the occlusion bodies are dissolved in the high pH conditions (pH 8.5 to 11.0) of the insect midgut (Granados and Lawler, 1981; Pritchett *et al.*, 1981; 1984), releasing virus particles into the gut lumen. The foregut and hindgut of the insect are of ectodermal origin and are lined with cuticle, presenting a barrier to infection. Therefore, sites in the midgut epithelium, which are not lined with cuticle, are involved in primary virus attachment and entry. Released virions bind to the columnar epithelial cells (Harrap, 1970; Summers, 1971) and enter the tips of the microvilli on the

apical brush border of cells between 0.25 to 2 hours post ingestion (Summers, 1971). Following fusion with the cell membrane (Tanada *et al.*, 1975), the nucleocapsids are released into the cytoplasm and are transported to the nucleus, where viral DNA replication occurs (Granados and Lawler, 1981). Virus infected-cell morphology is evident at 8 h.p.i., when the nucleus of the cell becomes enlarged. Nucleocapsids pass into the cytoplasm at approximately 12 h.p.i. (Granados and Lawler, 1981).

From the cytoplasm, nucleocapsids are transported to the basal membrane of the midgut cells, and between 12 and 24 h.p.i., nucleocapsids bud through the basolateral membrane to the haemocoel, acquiring the host-derived membrane and virus encoded gp64 (Keddie *et al.*, 1989). Secondary infection is achieved by BV produced from the midgut cells. Haemocytes and the epithelial cells lining the trachae are responsible for initiating secondary infection (Keddie *et al.*, 1989; Engelhard *et al.*, 1994). BVs accumulate in the haemolymph at approximately 16 h.p.i. The infection then spreads to other insect tissues including the fat body, endodermis, muscle sarcolemma and nerve ganglia (Harrap, 1970).

Virus replication is considerable in all the insect tissues before death occurs. Prior to death (>100 h.p.i) larvae become creamy in colour, cease feeding, and show limited movement. The host tissues break down as a result of the expression of virus-encoded chitinase (Hawtin *et al.*, 1995) and cathepsin (Ohkawa *et al.*, 1994; Slack *et al.*, 1995) proteins. Occluded viruses are liberated into the environment following the rupture of the insect cuticle; 10^9 OVs may be released from a single larva, and may remain viable in the environment for several years, until ingestion by another host larva recommences the replication cycle (Evans and Harrap, 1982).

Not all baculoviruses cause a lethal infection. Recorded sub-lethal effects of baculovirus infection (reviewed in Rothman and Myers, 1996), include extended development time of larvae (Goulson and Cory, 1995), altered

population sex ratio (Santiago-Alvarez and Vargas-Osuna, 1986), reduced fecundity (Patil *et al.*, 1989) and reduced egg-viability (Vargas-Osuna and Santiago Alvarez, 1988). The presence of persistent or latent virus infections in outwardly healthy insects has been recorded. A laboratory colony of *Mamestra brassicae* insects is known to harbour latent *M. brassicae* NPV, but do not experience substantial mortality unless they are stressed by superinfection with a second baculovirus species (Hughes *et al.*, 1993).

1.7. The use of baculoviruses as foreign gene expression vectors.

Baculoviruses have proved useful over the past decade as helper-independent viral vectors for the high-level expression of foreign genes (reviewed in Miller, 1988; Luckow and Summers, 1988; Atkinson *et al.*, 1990; Kidd and Emery, 1993). The system takes advantage of the hyper-expression of polyhedrin and p10, which can constitute up to 50% of the total cell protein at the end of an infection course (Kelly, 1982; Cameron *et al.*, 1989). Both genes are non-essential for virus growth *in vitro*; thus either gene may be deleted and a foreign gene expressed *in lieu* of the resident gene without detriment to viral replication (Smith *et al.*, 1983; Matsuura *et al.*, 1987). Alternatively, copies of these and other promoters may be used for the synthesis of foreign proteins (Emery and Bishop, 1987), allowing the production of recombinant viruses which express *polh* and *p10* and therefore produce OVs (Weyer *et al.*, 1990).

Due to the large size of the baculovirus genome it is awkward to insert foreign DNA directly into the genome. For this reason, the construction of recombinant baculoviruses involves the use of a transfer vector. Briefly, regions of the virus genome (flanking regions surrounding the desired point of insertion) are inserted into bacterial plasmids for propagation in *E. coli*. The *polh* coding region is then replaced with the foreign gene of choice, positioned downstream of the *polh* promoter, to derive a recombinant transfer vector. Insect cells are cotransfected with infectious baculovirus DNA and the transfer vector, using liposome mediated transfection (Felgner *et al.*, 1987; Groebe *et*

al., 1990). A portion of the progeny viruses (<2%) will have undergone homologous recombination between the identical sequences flanking the *polh* gene in the virus, and those flanking the foreign DNA in the transfer vector, resulting in the insertion of the foreign gene into the baculovirus genome. Recombinants can be isolated by plaque assay if a marker gene distinguishes them from the parental virus.

Improvements to the baculovirus expression system (reviewed in Davies, 1994) have included the increased efficacy of recombinant virus isolation following linearisation of the baculovirus genome prior to the cotransfection of insect cells (Kitts *et al.*, 1990), as linear viral DNA is non-infectious (Kelly and Wang, 1981). Additionally, deletion of essential genes in the parental virus by restriction enzyme digestion at engineered sites, followed by the introduction of a transfer vector replacing these essential gene regions plus the foreign gene, can allow >85% of the progeny viruses to be recombinants (Kitts and Possee, 1993). The use of promoters other than those controlling *polh* and *p10* have been investigated e.g., the basic protein promoter (Hill-Perkins and Possee, 1990).

Post-translational modifications of foreign proteins can occur in the infected insect cell, including phosphorylation, amidation and O-and N-linked glycosylations (Bishop, 1992). In addition, foreign proteins may be secreted, or locate to particular compartments of the cell according to their normal capabilities. Specifically, insect cells can recognise and cleave mammalian signal sequences that direct proteins to the endoplasmic reticulum (ER), e.g., the expression of interleukin-2 in recombinant AcMNPV involves its modification and secretion (Smith *et al.*, 1985). Evidence suggests that the sites targeted for glycosylation in insect cells are the same as those in mammalian cells (Hsieh and Robbins, 1984), although complex carbohydrate side chains may be replaced by short mannose side chains (Kuroda *et al.*, 1986). Most importantly, many proteins have been extracted from recombinant AcMNPV infected-cells in a biologically active form, for example, human β

interferon (Smith *et al.*, 1983) and human tissue plasminogen activator (Furlong *et al.*, 1988).

1.8. The use of baculoviruses as biological pesticides.

Naturally occurring baculoviruses have been recognised as having great potential for the biological control of insect pests and several baculovirus species have been used commercially for this purpose (reviewed in Huber, 1986). In 1973, a joint WHO/FAO meeting (World Health Organisation, 1973) endorsed the use of baculoviruses as insect pest control agents.

European sales for microbial pesticides in 1990 amounted to less than 1% of the total European insecticide market, with sales of approximately $10 million; a minor niche within the context of the agrochemical industry. On a worldwide basis the figures are similar (Jutsum, 1988). The majority of this amount corresponds to sales of *Bacillus thuringiensis* (*B.t.*). However, the use of microbial insecticides in agricultural practices is predicted to grow within the next decade, with some analysts forecasting that they will account for 15% of the insecticide market by the year 2000. This growth is most likely to arise from developments in biotechnology which will enable microbial agents to approach the level of performance expected of a chemical product.

Baculoviruses have been used primarily on crops which can sustain damage without major economic losses, such as forests (Benz, 1986). Most commercially successful baculoviruses are therefore directed against forest pests such as the gypsy moth, *Lymantria dispar* (GYPCHEK), Douglas fir tussock moth, *Orgyia pseudotsugata* (TM Biocontrol 1: Virtuss) and pine sawfly, *Neodiprion sertifer* (VIROX). In Europe a few baculovirus products are produced commercially for use in field crops, notably those targeted against the codling moth, *Cydia pomonella* (MADEX) for use in orchards, and the cabbage moth, *Mamestra brassicae* (MAMESTRIN) for use on vegetable crops.

Due to their unique properties of specificity, safety and biodegradability, microbial agents are an attractive alternative to chemical insecticides in pest management systems. No resistance problems have been encountered with NPVs, despite intensive testing (Whitlock, 1977). They leave no toxic residues but may remain in the environment in a viable form with the ability to infect subsequent insect generations. Disease spread does occur naturally but this epizootic effect cannot be relied upon for effective control and an "insecticidal" approach has therefore been adopted. Baculoviruses can be mass-cultured, formulated, packaged, stored and marketed in a similar manner to chemical insecticides, and the equipment used for application of chemical pesticides is generally suitable for these agents (Falcon, 1985).

Clearly, the limited commercial penetration of the global market by viral insecticides suggests that major factors constrain their wider use. Several key reasons explain the restricted use of viral pesticides. These include inadequate formulation and application technology, the difficulty of production, the problems of registration and patentability, the slow speed of kill (in relation to user expectation), and finally, the limited host range of many isolates.

1.8.1. Optimisation of application procedures.

Baculoviruses must be ingested by the target pest. As a consequence, optimisation of application technology is of critical importance for successful economic control. Careful timing of applications and appropriate spray coverage are essential to maximise encounter of the insect with the virus on the leaf surface. The persistence and viability of OVs in the crop environment can greatly influence the efficiency of the control operation. Degradation by ultra-violet (UV) light and mechanical weathering are the major factors affecting the long term viability of OVs on foliage. Baculoviruses are inactivated by exposure to UV radiation (Bullock, 1967; Yearian and Young, 1974). To a certain extent, persistence depends on the substrate on which the baculovirus is located, and its form in the environment (Jaques, 1985). On cotton, OVs are

inactivated more quickly than on any other plant; estimates of the time period of inactivation are as short as 24 hours. This is assumed to be due to the high alkalinity of the cotton leaf surface, which has a pH value of 9.0 to 9.9 (Andrews and Sikorowski, 1973) due to the presence of calcium and magnesium salts in the cotton gland exudate (Elleman and Entwistle, 1982). Loss of viability of *Spodoptera littoralis* NPV was shown to occur on cotton leaves but not on cabbage leaves or insect diet (Elleman and Entwistle, 1985). It was confirmed that the products of cotton gland leaf exudate were the causal factor. Viruses have been shown to retain activity for longer periods in soil than when exposed on foliage (Thompson *et al.*, 1981).

Microbial insecticides are formulated as either sprayable liquids or wettable powders. Dry dust formulations using carriers such as kaolin and talc have also been investigated (Montoya *et al.*, 1966), and offer a possible alternative to liquid formulations (Ignoffo and Garcia, 1996). Formulation has been the major strategy used to increase the persistence of polyhedra on the plant surface (Hostetter *et al.*, 1982). Additives can improve the physical performance of the baculovirus spray deposit. This is achieved by the use of diluents to aid dispersal and suspension of the virus in the spray liquid and the use of wetting agents and anti-evaporants to aid deposition and spread of the spray droplets on the target (Killick, 1990). Persistence of the virus can be improved by the addition of UV protectants, such as starch encapsulation (Ignoffo *et al.*, 1991) and activated charcoal (Ignoffo, 1973). Feeding stimulants such as sucrose (Stacey *et al.*, 1980; Johnson, 1982) can be used to encourage ingestion of virus-contaminated food by the insect.

1.8.2. Production of baculovirus insecticides.

Baculoviruses were previously produced using *in vivo* techniques. This method, whilst adequate on a small-scale, is costly to scale-up and difficult to assess for quality control in a commercial environment. High manufacturing

costs can lead to expensive products whose use is ruled out for all but the highest value (and usually lowest volume) markets.

It is now possible to produce viruses in a more cost effective and controlled manner using *in vitro* cell culture, the feasibility of which has been demonstrated using batch culture in large, sterile airlift fermenters (Granados and Federici, 1986). However the process is still uneconomic on an agricultural scale due to the high cost of growth media. Serum-free media are available (Maiorella *et al.*, 1988; Goodwin, 1990), which will have a major impact on reducing the costs of production, as serum is the most costly component of cell culture media. In most cases cell densities in serum-free media are similar to those obtained in serum-supplemented media, and the OVs produced show equal virulence to OVs produced from cells growing in serum-supplemented media (Chen *et al.*, 1993; McIntosh *et al.*, 1995). It has also been shown that individual cell lines differ greatly in their capacity to support virus replication. Specialised Lepidopteran cell lines are thus being developed to optimise production (Hukuhara *et al.*, 1990).

1.8.3. Registration and patentability.

Elcar (HzSNPV) was the first viral pesticide to be registered. It underwent rigorous safety and specificity tests before being registered by the USA Environmental Protection Agency in 1975. At the present time, it appears that every virus strain would need to undergo the registration process on a "case by case" consideration. For wild type microbial agents the registration track is faster and cheaper than for conventional insecticide products e.g., the average development cost is $5 million over 3 years for wild type *B.t* in the USA, compared to $80 million and 8 to 12 years registration time for a conventional chemical pesticide. Regulatory changes in plant protection product registration were implemented by the EEC in 1993 under directive 91/414/EEC (commonly referred to as the Agrochemical Registration Directive). The development of microbial pesticides has been encouraged by the Directive,

which harmonised registration requirements within the European Community (Marshall, 1990).

Regulatory issues concerning genetically modified microbial agents are currently unresolved and this could prove to be a temporal stumbling block for the development of genetically modified strains. The limits of patentability for microbial control agents have yet to be fully defined through litigation in the courts but two developments have increased the expectation that full protection may be possible. Firstly, the agreement by a number of countries (notably including N. America, Japan and Europe) to accept deposition in a recognised culture collection as evidence of reproducibility, and secondly, the development of genetic techniques such as restriction fragment length polymorphism (RFLP) typing, which allow a given organism to be "fingerprinted" and its illegitimate usage proven.

1.8.4. The genetic modification of baculovirus insecticides.

Growers are often unwilling to accept the slower speed of kill associated with baculovirus use because they are accustomed to conventional chemical pesticides which provide rapid knock-down and kill combined with broad-spectrum activity. The main objective of the genetic modification of insect viruses, therefore, is to improve their speed of action. The introduction of appropriate genes into the baculovirus genome, which could deliver a deleterious gene product in the larva, can greatly increase the speed of kill (reviewed by Possee *et al.*, 1993; Miller, 1995).

Several studies have focused on the introduction of genes such as regulatory hormones, which affect insect metabolic processes. Diuretic and anti-diuretic hormones are involved in the control of water balance in the insect. The *Manduca sexta* (tobacco hornworm) diuretic hormone has been inserted into *Bombyx mori* NPV under the control of the polyhedrin promoter (Maeda, 1989b). Larvae infected with this recombinant BmNPV showed a reduced

survival time compared to controls infected with wild type BmNPV, and in addition exhibited a 30% decrease in haemolymph volume compared to controls.

Insects undergo hormonally controlled metamorphosis during development. This aspect of insect biology has also been exploited for the development of faster-acting recombinant baculoviruses. The enzyme juvenile hormone esterase (JHE) controls hydrolysis and inactivation of juvenile hormone within larvae. A recombinant AcMNPV expressing *Heliothis virescens* JHE was constructed (Hammock *et al.*, 1990) and its properties investigated. In infected *T. ni* insects, feeding was reduced, but only in first instar larvae. This disappointing result may reflect insufficient quantities of JHE produced from expression in the baculovirus, or interference by cellular hormones such as ecdysteroid UDP-glucosyl transferase.

A greater understanding of the role of specific genes involved in the replication cycle of baculoviruses has altered the approach to the construction of recombinant viruses towards the deletion of viral genes rather than the addition of genes. Baculoviruses with deletions in specific genes may have altered properties compared to the wild type virus which may be favourable for the use of the virus as an insecticide. Some aspects of insect development are subjected to control by baculovirus genes, e.g., EGT blocks moulting of the insect host by inactivating the hormones which trigger ecdysis (O'Reilly and Miller, 1989; Riegel, 1994; Barrett *et al.*, 1995). AcMNPV *egt* encodes an enzyme which initiates the transfer of glucose (or galactose) from UDP-glucose to ecdysteroids, thereby inactivating the ability of these hormones to trigger moulting and pupation of the infected host (O'Reilly and Miller, 1989; O'Reilly *et al.*, 1991; 1992). Fourth instar *Spodoptera frugiperda* larvae infected with an AcMNPV *egt* deletion mutant moulted earlier than insects infected with wild type AcMNPV, and therefore had reduced feeding activity compared to wild type AcMNPV-infected insects, which continued to feed throughout the period of infection (O'Reilly *et al.*, 1992). Insects infected with the *egt*-deletion virus

should cause less crop damage in the period between infection and death compared to wild type virus-infected insects. The *egt*-deleted recombinant AcMNPV is the first genetically modified virus to be field-tested in the United States (Miller, 1995).

The δ-endotoxin gene from *Bacillus thuringiensis* subspecies *kurstaki* (HD73) has been inserted into AcMNPV (Merryweather *et al.*, 1990; Ribeiro and Crook, 1993). Feeding inhibition was observed in bioassays using diet contaminated with *B.t.* endotoxin-expressing recombinant AcMNPV-infected cell extracts, indicating the effective action of the δ-endotoxin (Merryweather *et al.*, 1990). Insect-specific toxin genes have been extensively studied, and have resulted in the most significant improvement in baculovirus efficacy. Several toxin genes have been inserted into AcMNPV, including scorpion *Buthus eupeus* insect toxin 1 (BeIT) (Carbonell *et al.*, 1988), the scorpion *Androctonus australis* (North African scorpion) insect toxin (AaHIT) (Stewart *et al.*, 1991) and the female mite *Pyemotes tritici* insect toxin (TxP-I) (Tomalski and Miller, 1991; 1992). AcMNPV expressing AaHIT (AcAaHIT) was shown to reduce feeding damage from *T. ni* infected larvae by 50% compared to controls (Stewart *et al.*, 1991). Field trials with AcAaHIT showed a reduction in the damage caused to cabbage plants by 25% compared with the damage caused by larvae feeding on plants treated with wild type AcMNPV (Cory *et al.*, 1994). In addition, the AaHIT toxin has been shown to cause infected larvae of *Heliothis virescens* to fall off the plant prior to cessation of feeding, thereby reducing the crop damage caused between the ingestion of virus and the death of the insect (Hoover *et al.*, 1995).

Investigations to discover and characterise effective toxins such as black widow spider neurotoxins (Kiyatkin *et al.*, 1995; Pescatori *et al.*, 1995), parasitic wasp venom (Quistad *et al.*, 1994) and additional mite toxins (Tomalski *et al.*, 1993) are ongoing. The use of different baculovirus promoters, including late/very late hybrid promoters, to drive toxin expression, can allow faster and earlier accumulation of the toxin in the insect (Tomalski and Miller, 1992). The

ultimate aim is to produce a baculovirus with a speed of action equivalent to that of a chemical pesticide.

Viruses have evolved to be effective parasites, but not successful insecticides (Miller, 1995). Some viral genes may be involved in maximising virus production and survival, but may detract from their utility as pesticides. It should be possible to further improve the insecticidal properties of baculoviruses as more detailed knowledge of the genetic factors involved in the normal mode of infection of viruses becomes available (Volkman and Keddie, 1990).

1.8.5. Risk assessment of genetically modified baculovirus insecticides.

In order to assess the risk of the release of a genetically modified organism, genetic modifications must be evaluated in relation to ecological and environmental issues (Tiedje *et al.*, 1989). This process is termed ecological risk assessment. One of the most important aspects is whether or not the addition or deletion of DNA affects the host range of the recombinant virus insecticide (Possee *et al.*, 1993), or provides a general selective advantage to virus growth and survival in the environment (Miller, 1995). Laboratory studies concerning a recombinant AcMNPV containing a scorpion toxin gene confirm that no host range changes resulted from this gene insertion (Possee *et al.*, 1993).

A serious concern is whether or not a recombinant protein, such as a toxin produced in the virus-infected insect, will present a hazard to other species in the environment. This will have to be determined on a case-by-case basis using laboratory toxicology studies. Employing genes with high vertebrate toxicity is not acceptable despite the overall safety of baculoviruses to vertebrates. A virus carrying a toxin gene which promotes rapid insect death intrinsically has a disadvantage for growth and survival (Tomalski and Miller, 1992), since its potential for replication will be reduced by the earlier death of the host

compared to wild type virus-infected hosts. However, the possibility that toxins expressed in recombinant virus-infected insects might affect insect populations which interact closely with the pest species, such as beneficial predators, must be considered. During feeding on a pest species infected with a virus expressing a toxin gene, a predaceous insect ingests the toxin and both budded and occluded forms of the virus. The toxin must therefore have no oral activity to predators, although proteolytic breakdown of the toxin in the gut is likely to occur. AaHIT has been shown to exhibit low oral toxicity in Dipteran larvae (Zlotkin *et al.*, 1992), which may seriously compromise its selection as a candidate toxin for use in a field situation, although TxP-I has no recorded oral toxicity affects. Additionally, the recombinant virus itself must be unable to cause cell or tissue damage in the predator. Finally, the recombinant virus must not be able to express the toxin gene in the predator.

AcMNPV can enter a variety of insect cells which cannot support viral replication, resulting in limited expression of viral genes in a promoter-dependent manner (Carbonell *et al.*, 1985; Morris and Miller, 1992; 1993). These observations suggest that the use of immediate early baculovirus promoters or generalist promoters such as *Drosophila* HSP70, which is a constitutive insect promoter, may be unacceptable for the control of expression of toxin genes in recombinant viruses. Expression from mammalian promoters has been observed in human and rabbit hepatocytes treated with recombinant baculoviruses expressing reporter genes under the control of these promoters (Hofmann *et al.*, 1995). A positive aspect of this work could be the potential of baculoviruses in gene therapy, but it further highlights the need for careful consideration of promoter type when constructing toxin-expressing baculoviruses.

Experiments by McNitt *et al.*, (1995) have shown that colonies of the social wasp *Polistes metricus* fed with toxin-expressing recombinant AcMNPV-infected larvae were unaffected by the toxin in a number of development parameters. However, feeding of insects infected with viruses expressing toxin genes under

the control of baculovirus early or constitutive cellular promoters (*Drosophila* HSP70) did result in the detection of the toxin in the host tissues, despite its lack of effect on the wasps. No toxin was detected when the toxin gene was expressed under the control of a late baculovirus promoter. This suggests that the use of late baculovirus promoters for the expression of foreign genes is necessary to reduce the environmental risk to non-target organisms.

A further concern is whether or not genetically modified baculoviruses have the potential to recombine with indigenous baculoviruses during coinfection of insect larvae, thereby allowing the transfer of foreign DNA to the indigenous virus by genetic recombination. In order for this to occur, the viruses would have to be co-infecting the same cell in the host larva. A high degree of genetic recombination has been recorded between closely related baculoviruses e.g., AcMNPV and *Rachiplusia ou* (Ro) MNPV infection of *Galleria mellonella* (Croizier *et al.*, 1988), although it is unlikely that distantly related baculoviruses have the potential for recombination. It has been shown that rescue of a polyhedrin negative AcMNPV by the polyhedrin gene from *Panolis flammea* (Pf) MNPV did not occur *in vitro* (Possee *et al.*, 1992). However, it is important to establish the genealogical relationships between baculovirus species so that testable hypotheses can be proposed for risk assessment experiments (Zanotto *et al.*, 1993). Other issues such as the genetic stability of the recombinant virus and its persistence in the environment must also be considered. Field testing of genetically modified forms of AcMNPV began in 1986 in an attempt to evaluate potential hazards, using baculoviruses expressing marker proteins rather than toxins (Cory *et al.*, 1994), and continues to date.

1.9. *Heliothis* spp.

1.9.1. The biology of *Heliothis*.

The generic status of *Helicoverpa*, first applied to *Heliothis zea*, *H. armigera*, *H. punctigera* and *H. assulta* by Hardwick (1965) is receiving increasing support

on taxonomic grounds. However, since this nomenclature is not yet widespread in the literature, the broader traditional interpretation of *Heliothis* is retained in this thesis.

Lepidoptera of the noctuid genus *Heliothis* (cotton bollworm/tomato fruitworm) are agricultural pests of worldwide significance. Of the 80 species currently recognised (Todd, 1978), *H. armigera*, *H. zea* and *H. virescens* have reached major pest status (Fitt, 1989). In the USA, for example, the estimated revenue loss associated with damaged cotton lint caused by *Heliothis* has averaged $84 million per year since 1980 (Menn, 1990). In addition, the estimated annual cost of insecticides to control *Heliothis* in pre-bloom cotton in Texas is approximately $2 million (Pair *et al.*, 1995). *H. armigera* (Hb) has one of the widest distributions of any agricultural pest, occurring throughout Africa, the Middle East, southern Europe, India, central and south-eastern Asia, Australia, New Zealand and many eastern Pacific Islands. Other *Heliothis* species are more restricted - *H. zea* (Boddie) and *H. virescens* (F.) occur in both North and South America whereas *H. punctigera* is endemic to Australia.

Heliothis larvae pass through six instars before pupation, which usually occurs in the soil. The adult moths are active at night and the females lay 90% of their eggs adjacent to the terminal buds of a plant. Eggs hatch in about 3 days in warm weather (25°C), but may take 9 days to hatch at a mean temperature of 17°C. Newly-hatched larvae frequently feed on nitrogen-rich areas of the crop plant. In a cotton crop, the larvae move down the branch towards the main stem, damaging the cotton squares (Ramalho *et al.*, 1984). The larvae confine themselves to the reproductive parts of the plant, feeding on flower buds and meristematic tissues. On cotton, the contents of the bud are eaten completely, leaving a hollow shell, and this often causes the bracts to flare open (flared squares are a reliable indication of bollworm damage). During feeding, the larvae often move about the plant, feeding on a number of bolls. This habit increases the economic damage caused to the crop, since the boll will be lost by shedding or attacked by fungi, which can enter the plant through the hole

made by the larva. As the larvae age, they are more difficult to control with chemical insecticides, due to their larger size and protected situation near the plant's base or within enclosed structures such as cotton bolls. Insecticide sprays must be timed to kill the maximum number of first and second instars, since these instars are more likely to be exposed on the plant surfaces.

Temperature affects the length of development between larval hatching and pupation; this can range from 15 to 60 days. The pupal period is about 16 days but may be extended in cooler weather, which can also induce diapause. The length of adult life is greatly affected by the availability of food, in the form of nectar or its equivalent. In the absence of food, the ovaries do not develop in the female moth and death occurs within 3 to 6 days. Consequently, moth activity is largely confined to the flowering phase of crops. The female moth is normally longer lived, having a lifespan averaging about 15 days compared to 9 days for the male.

1.9.2. Economic status of *Heliothis*.

Species of the genus *Heliothis* collectively attack a wide range of food, fibre, oil and fodder crops. Relative to the areas grown, cotton, soybeans, tobacco and pulses, all of which are high value crops or staple foods, account for most economic loss due to *Heliothis*. Many crops such as cotton have low damage tolerance, due to larval preference for plant structures high in nitrogen; principally the reproductive and meristematic tissues, such as cotton buds. Thus the insect is able to influence yield directly. Considerable economic losses are therefore common, and where control is attempted, reliance is traditionally placed on pesticide use. For example, 358,000 ha were treated against *H. virescens* and *H. zea* infestation in Texas, USA, and 432,000 ha were treated in Mississippi.

1.9.3. The control of *Heliothis* on cotton.

Cotton is the most important natural fibre in the world. It provides at least 80 countries with not only a basic raw material for their textile industries, but also with a means of earning foreign currency. During the pre-flowering phase, the cotton plant can tolerate levels of defoliation of up to 50% without serious effects on yield, providing there is enough time for compensatory growth. Once flowering begins however, the control criteria become more rigorous. The most critical period of crop development takes place 10 weeks after the first flower buds have been formed. During this period of rapid growth there is an increasing number of buds and bolls which attract *Heliothis*. For control to be effective, insecticide applications must be administered when the larvae are small. Field-scouting (a highly labour-intensive process) is required to determine spraying requirements. This is typically carried out every 3 to 5 days, noting pest numbers and crop damage.

1.9.4. Chemical insecticide products for the control of *Heliothis* on cotton.

The world market for cotton insecticides is estimated to grow by 8% per year. Much of the increase is accounted for by the USA and China where production is growing constantly. In the 1960s, the cotton insecticide market was well catered for by a selection of organochlorine and organophosphorus products. This was overturned upon introduction of the pyrethroids, which have proved successful until recently. A consequence of the excessive use of pyrethroid insecticides has been the development of resistance problems in important target species. For example, cypermethrin was bioassayed against strains of *H. armigera* (2nd to 6th instars) collected in South India during 1989-1991 and high levels of resistance were recorded (Armes *et al.*, 1992). A similar situation is being reported from the major cotton growing areas of the world. Resistance has been documented in 6 countries (Riley, 1990), including Australia (Wilson, 1974; Daly and Murray, 1988) and the USA (Luttrell *et al.*, 1987), both of

which are key market areas for pyrethroids. In *Heliothis*, pyrethroid resistance has been linked to sodium channel modification (Taylor *et al.*, 1993).

To date, the problem of pyrethroid resistance has been tackled by limiting usage, by defined "window" applications to minimise exposure of an insect generation to pyrethroids, and the use of synergists. The Pyrethroid Efficacy Group, a subcommittee of the Insecticide Resistance Action Committee, currently address this problem by providing technical advice for researchers, growers and governments. The extent to which new products will be developed for and introduced to the cotton insecticide market will largely depend on the performance of the pyrethroids over the next few years. Of the other products which are available to control *Heliothis*, the benzoylphenyl urea insecticides are limited in their efficacy and have inherent formulation problems. Recently, organophosphates such as dimethoate and chlorpyriphos have suffered resurgence. Experiments with a laboratory strain of *H. virescens* demonstrated acquired resistance to *B.t.* toxins (Gould *et al.*, 1992).

1.9.5. Prospects for the classical biological control of *Heliothis* on cotton.

The need to grow cotton as an annual crop, and the diverse complex of pests which attack it in any particular region, make it a difficult crop on which to practise classical biological control. Parasitoids such as *Bracon kirkpatricki* and *Trichogramma brasiliensis* have been released in the USA against *Heliothis* but are reported not to have exercised control on their own (Greathead, 1989). The only encouraging classical biological control result is the successful establishment of *Apanteles kazak* Telenga from S.E. Europe on *H. armigera* in New Zealand. However, parasitism was not sufficient to preclude the need for chemical control (Cameron, 1991).

Heliothis are not introduced pests and the opportunities for classical biological control are therefore limited (King and Coleman, 1989). Also, dispersal to and exploitation of a succession of food sources occurs rapidly with *Heliothis*,

because the adults are capable of long range movement. As a consequence, predatory insects are unable to be able to effect timely biological control. The greatest potential for classical biological control in the short term lies in maximising the impact of existing natural enemies through modifications in cultural practices and the use of selective insect pest control agents (King and Coleman, 1989).

Populations of *Heliothis* are prone to infection by a wide variety of naturally occurring microbial pathogens, some of which have been recognised as important factors in the suppression of these pests (Carner and Yearian, 1985). Efforts have been concentrated on *Bacillus thuringiensis* (*B.t.*) Berliner and baculoviruses, specifically the nucleopolyhedroviruses (NPVs). *B. thuringiensis* is the only commercially available microbial agent. It was initially registered in the USA in 1961. A number of products are available based on several different *B.t.* strains, notably subspecies *kurstaki* strain HD1 which has 20-200x greater activity than subspecies *thuringiensis*. The majority of biopesticide sales (92%) are of products containing *B.t.* Berliner as the active agent (Rodgers, 1993).

1.9.6. The *Heliothis zea* SNPV.

Viruses isolated from *Heliothis* species include a non-occluded virus from the family Iridoviridae (Stadelbacher *et al.*, 1978) and three types of occluded viruses from the cytoplasmic polyhedrosis virus (family Reoviridae) (Sikorowski *et al.*, 1971), the Granulovirus (Gitay and Polson, 1971) and the Nucleopolyhedrovirus (Ignoffo, 1965) families. The Nucleopolyhedrovirus, HzSNPV, is considered to have the most potential for use as a biological control agent. An electron micrograph of an HzSNPV particle is shown in Figure 1.3.

Approximately 200 reported field tests have been conducted using HzSNPV, of which 60% took place on cotton crops. It was determined that the optimum rate of treatment for cotton was $10x10^{10}$ OV/0.4 ha. This level of treatment was shown to give as effective control as chemicals insecticides.

A commercial product based on the HzSNPV was developed in the late 1970s by Sandoz Inc. under the trade name Elcar, but achieved limited commercial success due to a combination of factors. The HzSNPV Elcar formulation was registered prior to the 1976 growing season (Ignoffo *et al.*, 1976). When used properly, Elcar was effective and produced cotton yields equivalent to those obtained with existing insecticides. However, the product required a higher level of grower education and field management for successful use. Production of the virus was achieved *in vivo*, using a semi-automated insect rearing facility.

In 1982, Elcar, which had previously only been registered for use on cotton, was granted a broad-label expansion for use on other crops such as sorghum, soybean and tomatoes. However, the newly developed pyrethroids became available at this time, affording highly effective control of *Heliothis* on many of these crops, improved speed of kill, and broad spectrum control, at lower cost. As a consequence, the demand for Elcar was drastically reduced, and commercial production ceased in 1984. Increasing *Heliothis* resistance to pyrethroids and the success of genetic techniques for the manipulation of AcMNPV have renewed interest in the use of HzSNPV as a biological control agent.

1.10. Baculovirus host range determination.

Naturally occurring baculoviruses have a high host specificity, which is seen as advantageous in conserving beneficial insects, but is regarded as a disadvantage when treating a crop where a complex of pest species is found. With the exception of AcMNPV, which has been demonstrated to infect 28 Lepidopteran species (Miller *et al.*, 1983), most baculoviruses infect only one or two host species, often members of a single genus. The property of specificity limits the market potential for baculovirus use, which is a major disincentive to the commercial development of baculoviruses by large agrochemical companies. However, it does mean that selected insect pests in a crop system

can be targeted. With increasing adoption of IPM philosophy this type of approach to insect pest control should become more acceptable.

The speed of action of baculovirus pesticides is being successfully addressed by genetic engineering. An understanding of the limitations to baculovirus host range is the next logical research step. If it were possible to modify baculovirus host range to include a complex of common pests, the attractiveness of baculoviruses to industry and user could be greatly enhanced. Additionally, from a safety perspective, it is critical that a greater understanding of the factors controlling host range are obtained, so that evaluations of how readily that property might be transferred between viruses can be assessed.

1.10.1. The genetic basis of baculovirus host range.

Virus host range determination can be considered at each of the different levels of the infection process. The initial interaction involves the ability of a virus to bind to and enter a particular cell type. In contrast to conventional theories concerning the importance of cell surface receptors in virus/host cell recognition (Johnson and Rosner, 1986; Neurath *et al.*, 1986; Lentz, 1990; Nemerow *et al.*, 1990; Bass *et al.*, 1991; Malvoisin and Wild, 1994; Thaker *et al.*, 1994; Gilbert *et al.*, 1995), it has been demonstrated that BV of AcMNPV enter a much wider range of cell lines than can support a productive infection, including non-Lepidopteran cell lines such as *Drosophila* (Carbonell *et al.*, 1985). Viral entry into a host cell is therefore not considered to be a critical factor in baculovirus host range determination. Once the virus is inside the cell it must activate early gene expression, DNA replication, late gene expression and budded virus formation and release in order to complete a productive life cycle *in vitro*. For environmental survival, very late gene expression and occluded virus formation are necessary. Interruptions to prevent a productive replication cycle may occur during any of these processes.

AcMNPV early gene transcription has been detected in both permissive and non-permissive Lepidopteran cell lines, indicating that the virus DNA is effectively delivered to the nucleus in these insect cells (Morris and Miller, 1992; 1993). However, AcMNPV was unable to enter mammalian cell nuclei (Carbonell and Miller, 1987). Late gene expression was shown to be very poor in non-permissive Lepidopteran cells compared to permissive cells such as Sf21, with a successive decline in the ability of non-permissive cells to support the later stages of gene expression (Morris and Miller, 1993). Therefore, it seems that the genes specific to the control of late gene expression may be the fundamental basis for baculovirus host range determination.

1.10.2. Baculovirus host range alteration.

Two of the eighteen late expression factor (*lef*) genes required for the expression of AcMNPV late genes in Sf21 cells, *p143* and *p35*, have been directly implicated in the control of AcMNPV host range. AcMNPV normally replicates in the cell lines *T. ni*-368 and Sf21 but not *Bombyx mori* cells (BmN), while BmNPV replicates in BmN but not *T. ni*-368 or Sf21. Coinfection of AcMNPV and BmNPV has been shown to result in the production of recombinant viruses with broader host ranges (Kondo and Maeda, 1991). Experiments to expand the host range of AcMNPV to allow successful replication in BmN cells have been performed (Maeda *et al.*, 1993). A 572 bp fragment from BmNPV responsible for the host range expansion was localised to the coding region of the BmNPV helicase gene (*p143*). The helicase gene may therefore allow DNA replication of AcMNPV to occur in *B. mori* cells, permitting late gene expression. Similar results were reported by Croizier *et al.*, (1994), where insertion of the helicase *p143* gene from BmNPV into AcMNPV resulted in a recombinant AcMNPV which was capable of full replication, culminating in the production of occluded viruses, in *B. mori* Bm5 cells. The region responsible was mapped to a minimal 79 nucleotide area comprising nucleotides 1651-1729 of the BmNPV *p143* gene. In this region of *p143*, AcMNPV and BmNPV possess different nucleotides at 6 positions,

corresponding to 4 amino acid substitutions. Substitution of 3 of the amino acids in the *p143* gene of AcMNPV to BmNPV-specific amino acids (valine to leucine at position 556, serine to asparagine at position 564 and phenylalanine to leucine at position 557) was sufficient to effect the host range expansion of AcMNPV to include infection of *B. mori*.

BmNPV and AcMNPV are very closely related at the genetic level (nucleotide similarity approximately 70%) and are probably host range variants of the same virus (Croizier *et al.*, 1994). The initial identification of the *p143* gene as a host range determinant was effected by coinfection of AcMNPV and BmNPV and the subsequent selection of variants which had undergone homologous recombination. It is likely that a high degree of homogeneity between the two virus genomes is required to allow successful random recombination events to occur between baculovirus genomes. This method is therefore not suitable for studies with viruses with widely different genetic properties.

P35 is responsible for blocking apoptosis in *Spodoptera frugiperda* (Sf21) cells infected with AcMNPV (Clem *et al.*, 1991). This observation was made while characterising a mutant AcMNPV lacking a functional *p35* during routine expression vector screening. The mutant, named the annihilator (vAcAnh), was isolated as a small plaque lacking occlusion bodies from Sf21 cells, as a consequence of apoptosis 12 to 24 hours post infection. In contrast, infection of *T. ni*-368 cells with vAcAnh did not cause apoptosis and resulted in normal replication of the virus, including occlusion body formation (Clem *et al.*, 1991). The vAcAnh baculovirus can, therefore, be propagated using the *T. ni*-368 cell line. Both Sf21 and *T. ni*-368 are fully permissive cell lines for wild type AcMNPV. These experiments have provided evidence to support the proposal that *p35* is a baculovirus host range factor, since this gene is required to maintain the host range of AcMNPV in the Sf21 cell line.

In addition, *lef-7*, *hcf-1* and *ie-2* have been shown to be differentially required for AcMNPV late gene expression in *T. ni* compared to Sf21 cells. The

requirement of *lef*-7, *hcf-1* and *ie-2* was observed during comparisons of *lef* requirements in Sf21 compared to *T. ni* cells using transient transfection assays (Lu and Miller, 1995). Firstly, a previously uncharacterised *lef* gene, host cell-specific factor-1 (*hcf-1*), corresponding to AcMNPV ORF 70, has been shown to be required for late gene expression in *T. ni*-368 cells but not Sf21 cells (Lu and Miller, 1996). Secondly, *ie-2*, *lef*-7 and *p35* were required for optimal late gene expression as part of a library of eighteen *lef* genes in Sf21 cell, but they did not contribute to transient late gene expression in *T. ni*-368 cells. It is unknown whether these differential requirements for late gene expression *in vitro* extend to the context of a viral infection *in vivo*.

Although AcMNPV does not infect *Lymantria dispar* (Gypsy moth) insects some *L. dispar* cell lines will support AcMNPV infection (Goodwin *et al.*, 1978). However, the cell line Ld652Y does not support AcMNPV replication; it is described as semi-permissive, demonstrating a cytopathic effect, viral DNA replication, and expression of all temporal classes of viral mRNAs (Guzo *et al.*, 1992), but no production of infectious virions following infection (McClintock *et al.*, 1986). However, AcMNPV is able to replicate in Ld652Y cells superinfected with LdMNPV (McClintock and Dougherty, 1987), suggesting that LdMNPV provides a factor which promotes complete replication of AcMNPV in this cell line. The gene *hrf-1*, that promotes AcMNPV replication in Ld652Y cells, has subsequently been identified from LdMNPV (Thiem *et al.*, 1996). A recombinant AcMNPV encoding the LdMNPV *hrf-1* gene was shown to be capable of replication in Ld652Y cells, overcoming the block in protein synthesis observed in wild type AcMNPV-infected Ld652Y cells. *Hrf-1* comprises a 218-codon ORF with a predicted molecular mass of 25.7 kDa, and has unusually highly charged and acidic regions, a feature similar to mammalian Gadd proteins (Zhan *et al.*, 1994). A herpes simplex virus type 1 (HSV-1) gene with homology to Gadd45, γ1 34.5, has been identified (Chou and Roizman, 1992; 1994), which can overcome a block in protein synthesis for HSV-1 (+17), a variant mutated in this gene. The function of *hrf-1* is unknown, but may be related to the role of γ1 34.5 in HSV-1 infection, overcoming a block in protein synthesis

rather than having an implication for DNA replication as is the case for *p143*, *p35* and *hcf-1*.

It is generally accepted that there are specific genes in the baculovirus genome which are implicated in the control of baculovirus host range. At the start of this project, it was decided to focus on one class of genes, namely inhibitors of apoptosis, since the *p35* gene of AcMNPV is involved in host range determination both *in vitro* and *in vivo* (Clem *et al.*, 1991; Clem and Miller, 1993). Research into the mechanism of apoptosis spans many different areas of biological science, including virology, and it is therefore of relevance to describe current knowledge regarding the mechanism of programmed cell death.

1.11. Programmed cell death.

Two common forms of cell death have been described in vertebrate and invertebrate tissues: necrosis refers to the morphology of cells which die from injury whereas programmed cell death is an active process of cellular self-destruction. Programmed cell death is important for development (e.g., metamorphosis), homeostasis (e.g., in the immune system B and T lymphocytes are removed if they fail arrange their antigen receptors correctly) and as a form of host defence (e.g., to limit viral infection).

The cellular morphology resulting from the activation of programmed cell death is described as apoptosis. Apoptotic cell death follows a specific pattern of ultrastructure morphology which is similar in most organisms and involves DNA degradation by nucleases, nuclear condensation (pyknosis) and plasma membrane blebbing (Kerr *et al.*, 1972); although not all forms of apoptosis are dependent upon nuclear DNA breakdown (Ucker *et al.*, 1992). Cellular fragments are rapidly phagocytosed by neighbouring cells or macrophages - there is no inflammatory response, in contrast to necrosis which involves rapid cell swelling and lysis and induces an inflammatory response. Apoptosis

requires *de novo* gene expression through the activation of a cell-intrinsic suicide program (Steller, 1995) whereas necrosis is a passive process (Cohen, 1993).

The initiation of apoptosis is carefully regulated by many different signals originating from both within and outside of the cell. These include lineage information, cellular damage inflicted by ionising radiation, other DNA-damaging agents (Clarke *et al.*, 1993) or viral infection, extra-cellular survival factors, cell interactions and hormones. These diverse signals may act to either suppress or promote activation of the cell death program. Inappropriate control of apoptosis is known to play a major role in many disease states (reviewed in Carson and Ribeiro, 1993; Thompson, 1995), including cancer (Hoffman and Liebermann, 1994), acquired immunodeficiency syndrome (Laurent-Crawford *et al.*, 1991; Banda *et al.*, 1992), neurodegenerative disorders and ischemic stroke (Raff *et al.*, 1993; Martinou *et al.*, 1994).

Increasing evidence suggests that the primary cell death pathway, or at least some components of it, are conserved among insects and vertebrates (Vaux, 1993). Therefore, results obtained from the study of experimentally more accessible insect systems may be directly relevant to the understanding of the mechanism of apoptosis in vertebrates. The study of virus-controlled programmed cell death *in vitro* is particularly important, due to the absence of complications caused by antibody-mediated immune responses which are found in mammalian *in vivo* systems. The isolation and characterisation of putative cell death inducing and cell death inhibiting factors is increasing gradually (for summary see Table 1.1 and 1.2). The scientific challenge lies in the determination of the mechanism of programmed cell death.

1.11.1. Properties of the cell death program.

Programmed cell death can be suppressed by inhibitors of RNA or protein synthesis (Martin *et al.*, 1988; Oppenheim *et al.*, 1990; Schwartz *et al.*, 1990).

However, in certain circumstances these biochemical agents may induce apoptosis, suggesting that the effector molecules for apoptosis are constantly present in cells (Raff *et al.*, 1993). Indeed, enucleated cells (cytoplasts) which are deprived of growth factors or are treated with the protein kinase inhibitor staurosporine will still undergo apoptosis (Jacobson *et al.*, 1994). If the apoptotic effectors are present in living cells, it follows that these effectors must be constantly suppressed in cells that normally survive. The model that cells continuously require signals from other cells for survival (Raff, 1992; Raff *et al.*, 1993) is receiving increasing support. Many different mammalian and insect cells do require extracellular factors produced by other cells in order to survive (Raff, 1992; Steller and Grether, 1994). Survival factors may prevent induction of apoptosis by reducing the amount or activity of cell death effector proteins, or they may inhibit cell death by boosting the activity of anti-apoptotic proteins.

1.11.2. Apoptosis and the cell cycle.

Numerous overall similarities between apoptosis and the cell cycle have been recorded, and it has been suggested that apoptosis and mitosis may be mechanistically related or coupled: an extreme view is that apoptosis may be an aberrant mitosis (Ucker, 1991). Genes which have a role in cell cycle proliferation, such as p53, c-*myc*, Rb-1, E1A, cyclin D1, c-*fos* and $p34^{cdc2}$ kinase have also been implicated in the control of apoptosis (Yonish-Rouach *et al.*, 1991; Debbas and White, 1993; Smeyne *et al.*, 1993; Freeman *et al.*, 1994; Meikrantz *et al.*, 1994; Shi *et al.*, 1994). Some of these genes affect apoptosis in specific situations, e.g., p53 is predominately required for mediating the apoptotic response to chromosomal damage (Lowe *et al.*, 1993). Some genes, such as c-*myc*, which is usually involved in cell transformation, differentiation and cell cycle progression, are capable of inducing apoptosis when they are aberrantly expressed (Evan *et al.*, 1992). It has been suggested that c-*myc* functions normally to induce both cell proliferation and apoptosis, although

the occurrence of apoptosis under these circumstances may equally be due to a conflict of incompatible growth signals (White, 1993).

1.11.3. Apoptosis in the nematode *Caenorhabditis elegans*.

Much of the current knowledge about specific cell death genes has been derived from genetic studies in the nematode *Caenorhabditis elegans* (Hengartner and Horvitz, 1994). The genetic basis of the cell death pathway in *C. elegans* has been fully characterised (Hengartner and Horvitz, 1994). Of the 1090 somatic cells formed during development, 131 die in a predictable, apoptotic manner (Hedgecock *et al.*, 1983; Sulston *et al.*, 1983). A number of mutations that affect specific stages of programmed cell death have been isolated, and the corresponding genes have been ordered into a genetic pathway. Fourteen genes have consequently been identified as having a role in the mechanism (reviewed in Horvitz *et al.*, 1994), which follows a single genetic pathway (Ellis *et al.*, 1991). The *ced-3* (cell death) gene, encoding a cysteine protease (Yuan *et al.*, 1993) and *ced-4* gene, encoding a 63 kDa novel protein of unknown function (Yuan and Horvitz, 1992), are both required for the activation of apoptosis; they act cell-autonomously (Yuan and Horvitz, 1990). Programmed cell deaths are not seen in mutants lacking *ced-3* or *ced-4* (Ellis and Horvitz, 1986). The action of the *ced-3* and *ced-4* genes is antagonised by expression of *ced-9*; cell death also fails to occur in activating mutants of *ced-9* (Hengartner *et al.*, 1992). Nematodes lacking a functional *ced-9*, however, or involving overexpression of *ced*-3 or *ced*-4, cause cells that normally survive to undergo apoptosis (Hengartner *et al.*, 1992; Shaham and Horvitz, 1996). Thus, *ced-9* is required to prevent *ced-3* and *ced-4* from causing cell death. In the absence of *ced-9* activity, nematodes display widespread ectopic death. In *C. elegans*, cell death occurs by default in the absence of protective functions (Hengartner and Horvitz, 1994). The *ced-9* gene is therefore an inhibitor of apoptosis for this organism.

1.11.4. The role of cysteine proteases in apoptosis.

In *C. elegans*, *ced-3* and *ced-4* are required for all somatic cell deaths (Hengartner and Horvitz, 1994). Inactivation of either gene results in the survival of cells that normally die. The *ced-3* gene encodes a protein that is similar to an emerging family of cysteine proteases: human interleukin-1β-converting enzyme (ICE) (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Yuan *et al.*, 1993); ICH-1 and its murine homologue, NEDD-2 (Kumar *et al.*, 1992; Wang *et al.*, 1994); CPP32 (also known as Yama or apopain) (Fernandez-Alnemri *et al.*, 1994); TX (also known as ICH-2 or ICE_{rel}II) (Faucheu *et al.*, 1995); TY (ICE_{rel}III) (Faucheu *et al.*, 1996); MCH-2 (Fernandes-Alnemri *et al.*, 1995); MCH-3 (also known as ICE-LAP3 or CMH-1) (Fernandes-Alnemri *et al.*, 1995); MCH-5 (MACH or FLICE) (Fernandes-Alnemri *et al.*, 1996); MCH-6 (ICE-LAP6) (Duan *et al.*, 1996) and MCH-4 (Fernandes-Alnemri *et al.*, 1996). These ten ICE homologues have recently been renamed caspase 1 to 10, respectively (Alnemri *et al.*, 1996), in order to overcome the confusion caused by the use of multiple names for these proteins.

Much of the structural information known about ICE-like proteases is based on studies performed with ICE. ICE is well characterised as the activator of the inflammatory cytokine interleukin-1β (IL-1β) through the cleavage of the inactive pro-interleukin-1β (pro-IL-1β) (31 kDa) into the active form, IL-1β (17 kDa), between residues Asp116 and Ala117 (Lin *et al.*, 1993). ICE is produced as an inactive 45 kDa precursor, which is itself cleaved at Asp residues (see below). ICE is required for the induction of apoptosis through the Fas antigen cell signalling pathway (Enari *et al.*, 1995) and appears to be activated near the top of an ICE-related protease cascade. One of the many targets of this pathway is the ICE-related protease, CPP32 (Enari *et al.*, 1996). The cascade ultimately results in the proteolysis of cellular substrates, as discussed below.

Substantial direct evidence supports the notion that ICE members are central to the effector mechanism of apoptosis. ICE-specific inhibitors (*p35* and *crmA*,

discussed below) can block apoptosis induced by a variety of signals in a range of biological systems. In addition, peptidyl inhibitors of ICE such as YVAD-CMK, YVAD-CHO and Z-VAD-FMK are potent inhibitors of apoptosis induced by a variety of different agents (Los *et al.*, 1995; Zhivotovsky *et al.*, 1995). This indicates that apoptosis mediated by different signals shares a common effector phase, involving one or more ICE-related proteases. Overexpression of various ICE proteases results in apoptosis (Wang *et al.*, 1994; Miura *et al.*, 1993; Kumar *et al.*, 1994; Faucheu *et al.*, 1995; Duan *et al.*, 1996).

ICE-deficient transgenic mice appear to possess normal developmental apoptotic function (Kuida *et al.*, 1995; Li *et al.*, 1995) but are defective in the production of mature IL-1α and IL-1β and are resistant to apoptosis induced by Fas antibody. Although these results suggest that ICE is not essential for apoptosis, given that there are multiple ICE-like proteases in humans, many with overlapping expression patterns, functional redundancy is a likely scenario.

It is currently thought that ICE, or related cysteine proteases, function in mammalian and invertebrate cells to bring about programmed cell death (Martin and Green, 1995). This may be induced by processing of substrates other than pro-IL-1β since some cell types can undergo apoptosis without producing IL-1β. Alternatively, the apoptotic process may require the activity of more than one member of the ICE protease family.

1.11.5. Cleavage of ICE-like proteases.

All the members of the ICE-related protease family are synthesised as pro-enzymes that are activated by proteolytic cleavage at a consensus sequence of 4 Asp-X bonds (where X is any amino acid) (Thornberry *et al.*, 1992). ICE-related protease processing releases an N-terminal pro-domain and cleaves the remaining polypeptide to 10 kDa and 20 kDa subunits. ICE-like proteases are the principal effectors of apoptosis through their proteolytic action on specific

cellular targets. Characterised targets include the nuclear proteins, poly(ADP-ribose) polymerase (PARP) (Kaufman *et al.*, 1993), lamins (Oberhammer *et al.*, 1994), topoisomerases (Voelkel-Johnson *et al.*, 1995) and ribonucleoprotein (U1-70 kDa) (Casciola-Rosen *et al.*, 1994). Degradation of these nuclear proteins may contribute to the collapse and fragmentation of chromatin during apoptosis. The cytoskeletal changes observed during apoptosis might be partly explained by the proteolytic cleavage of the cytoplasmic proteins actin (Kayalar *et al.*, 1996) and fodrin (Martin *et al.*, 1995) by ICE-related proteases.

The unusual substrate specificity of ICE-related proteases, involving cleavage at Asp-X bonds, is a property shared by one other eukaryotic protease called granzyme B, which has also been implicated in the control of apoptosis (Shi *et al.*, 1992; Caputo *et al.*, 1994). In addition, ICE and granzyme B can be inhibited by similar types of reagents (synthesised peptide aldehyde inhibitors) (Thornberry *et al.*, 1992). Granzyme B is a serine protease that is responsible for apoptosis caused by cytotoxic T lymphocytes (Shi *et al*, 1992; Heusel *et al.*, 1994). Although ICE itself is not a substrate for granzyme B cleavage (Darmon *et al.*, 1994), other ICE-related proteins may be processed by granzyme B.

1.11.6. Inhibition of apoptosis and ICE-like proteases.

Increasing evidence suggests that the different apoptotic signalling pathways ultimately converge to activate a common apoptotic program. Several proteins, aside from CED-9, have been shown to exhibit anti-apoptotic properties.

In mammals the *bcl-2* proto-oncogene, which was first identified at the site of translocations common to follicular lymphomas (Tsujimoto *et al.*, 1984), has been found to regulate cell death. BCL-2 can prevent apoptosis of myeloid and lymphoid cells when certain growth factors are withdrawn (Vaux *et al.*, 1988), and of neurons deprived of nerve growth factor (Bissonette *et al.*, 1992). It also prevents or delays apoptosis induced by γ-irradiation, glucocorticoids, heat shock and chemotherapeutic drugs (Sentman *et al.*, 1991; Miyashita and

Reed, 1992; Cuende *et al.*, 1993; Dole *et al.*, 1994). Human BCL-2 inhibits cell death in a variety of biological systems including *C. elegans* (Vaux *et al.*, 1992). It can extend the survival of Sf9 cells infected with AcMNPV when overexpressed by the virus (Alnemri *et al.*, 1992), and prevents death of lymphocyte B cells cultured *in vitro* (Nunez *et al.*, 1990). Downregulation of *bcl-2* is a common feature of different stages of B cell development characterised by extensive cell death and clonal selection (Merino *et al.*, 1994). However, overexpression of *bcl-2* does not block actinomycin D-induced apoptosis in Sf21 cells (Clem and Miller, 1994a). It also fails to protect cells against cytotoxic T cell killing (Vaux *et al.*, 1992).

In addition to *bcl-2*, a related family of genes, including *bcl-x* (Boise *et al.*, 1993), *mcl-1* (Kozopas *et al.*, 1993), murine A1 (Lin *et al.*, 1993) and *bax* (Oltvai *et al.*, 1993) appear to control cell death and BCL-2 function (Boise *et al.*, 1993). The BCL-2-related proteins BAX and BAK, share two highly conserved regions, referred to as BCL-2 homology 1 and 2 domains (BH-1 and BH-2) (Yin, 1994). BCL-2 has been connected with the c-*myc* proto-oncogene in regulating certain forms of apoptosis and has been shown to be capable of blocking apoptotic death induced by c-*myc* (Bissonnette *et al.*, 1992); it is currently thought to be one of the primary regulators of c-*myc*.

1.11.7. The role of the tumour necrosis family (TNF) cell signalling molecules in apoptosis.

In the immune system, cytotoxic T lymphocytes (CTLs) and natural killer cells (NKs) actively kill other cells by inducing them to undergo apoptosis. In these cases, an effector molecule expressed at the surface of, or a soluble cytokine produced by the CTL and NK effector cells, is thought to be responsible for death of the target cell.

The cell surface protein Fas (APO-1 or CD95) is recognised by cytotoxic monoclonal antibodies, which induce rapid apoptosis in lymphocyte cells (Itoh

et al., 1991), and is normally a receptor for the Fas ligand (FasL) (Trauth *et al.*, 1989). When FasL binds to Fas the target cell undergoes apoptosis, as a result of the transduction of the cell suicide signal through an intracellular signalling cascade. Fas is a type I membrane protein belonging to the tumour necrosis factor (TNF) and nerve growth factor (NGF) cytokine receptor family (Itoh *et al.*, 1991). This family of receptors includes TNF-R1, TNF-R2, CD40, OX40, CD27, 4-1BB and CD30 (reviewed in Smith *et al.*, 1994).

The TNF-R1 (55 kDa) and TNF-R2 (75 kDa) receptors are known to mediate inflammatory and immunoregulatory responses via binding of the TNF ligand, and are expressed on most cell types (reviewed in Tartaglia and Goeddel, 1992). The extracellular regions of this receptor family consist of 3 to 6 cysteine-rich domains which are involved in ligand binding, with the extracellular amino acid sequence relatively conserved. A cytoplasmic domain of 70 amino acids designated the death domain, is necessary for transduction of the apoptotic signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993).

Intracellular signal transducers couple the TNF receptor family to downstream signalling cascades. These signal transducers were identified through yeast two-hybrid interaction cloning (Fields and Song, 1989), by virtue of their interaction with the cytoplasmic death domains of the TNF and Fas receptors, and include the TNF receptor-associated factors (TRAFs) TRAF1 and TRAF2 (Rothe *et al.*, 1994), TRAF3 (or CRAF1) (Cheng *et al.*, 1995), TRAF4 (Regnier *et al.*, 1995), TRAF5 (Nakano *et al.*, 1996), TRAF6 (Cao *et al.*, 1996), TRADD (Hsu *et al.*, 1995) FADD (Chinnaiyan *et al.*, 1995) and receptor-interacting protein or RIP (Stanger *et al.*, 1995).

The TRAF family of signal-transducing proteins, of which six members have been defined to date (Rothe *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995; Regnier *et al.*, 1995; Cao *et al.*, 1996; Nakano *et al.*, 1996), are characterised by a conserved carboxy-terminal TRAF-C domain of 170 amino acids, an α-helical TRAF-N domain and an amino-terminal RING finger motif.

The TRAFs represent an expanding family of signal transducing proteins. Overexpression of TRADD, FADD or RIP mimics the effects of TNF-R1 or Fas activation, leading to programmed cell death (Hsu *et al.*, 1995; Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995). Similarly, the overexpression of TRAF2, TRAF5 and TRAF6 leads to the activation of transcription factors and subsequent apoptosis (Rothe *et al.*, 1995; Cao *et al.*, 1996; Nakano *et al.*, 1996).

Diverse signals converge to activate the ICE-related proteases that cause programmed cell death, as illustrated in Figure 1.4. Latent transcription factors, such as nuclear factor κB (NF-κB), are activated following signal transducer molecule interaction with the TNF receptors (Hill and Treisman, 1995; Rothe *et al.*, 1995). Three of the six TRAF family members have been shown to be specifically involved in NF-κB activation (Rothe *et al.*, 1995; Cao *et al.*, 1996; Nakano *et al.*, 1996). The NF-κB transcription factor may activate the changes in gene expression necessary to bring about apoptosis, specifically the activation of ICE-related proteases (Martin and Green, 1995). The activation of NF-κB can be achieved via independent signalling pathways since TRAF2 mediates NF-κB activation by TNF-R2 (Rothe *et al.*, 1995), whereas TRADD mediates NF-κB activation by TNF-R1 (Hsu *et al.*, 1995). Recently, TRADD has been shown to interact directly with both TRAF2 and FADD (Hsu *et al.*, 1996), suggesting that the signalling pathways induced by different receptors may overlap.

Extensive oligomerisation of TRAF/FADD occurs following ligand binding to the TNF or Fas receptor to result in the activation of ICE-like proteases. The ICE-like protease MCH-5 may be involved in the initial mediation of this interaction (Muzio *et al.*, 1996; Boldin *et al.*, 1996). Multiple isoforms of MCH-5 have been detected, which have varying effects on apoptosis, from the activation of cell death to repression of the Fas and TNF signalling pathway (Muzio *et al.*, 1996). Despite this complication, it is clear that ligand-activated Fas and TNF-R1 induce apoptosis by the recruitment of ICE-related

protease(s) to the receptor signalling complex formed by the oligomerisation of TRAF/FADD.

1.11.8. Programmed cell death in *Drosophila*.

In *Drosophila*, as in other organisms, the onset of apoptosis is influenced by many different intra- and extracellular signals that either promote or suppress cell death (Steller and Grether, 1994). Genetic analyses have led to the isolation of a gene called *reaper*, encoding a unique small polypeptide of 65 amino acids (White *et al.*, 1996), thought to share similarities with the "death domain" of the Fas/TNF-R1 protein family (Golstein *et al.*, 1995). Expression of *reaper* induces apoptosis in cells that normally survive (White *et al.*, 1994). Deletion mutants of *reaper* suppress apoptosis in response to many normally apoptosis-inducing stimuli, including irradiation with X-rays (Steller *et al.*, 1994). A role for *reaper*, or as yet unidentified homologues of *reaper*, in the cell deaths of other organisms has yet to be established.

A second gene in *Drosophila* has a role in the control of apoptosis. Embryos with mutations in the gene *hid* (head involution defective) have decreased levels of programmed cell death and contain extra cells in the head (Grether *et al.*, 1995). A novel 410 amino acid protein is encoded by *hid*, and its mRNA is expressed in regions of the *Drosophila* embryo where cell death occurs. Ectopic expression of *hid* in the *Drosophila* retina causes eye ablation.

Recently, a third gene, *grim*, has been shown to initiate apoptosis in *Drosophila* (Chen *et al.*, 1996). The *grim* gene encodes a protein of 138 amino acids, with an amino terminal region which shares similarity to that of *reaper*. It has been shown that transfection of *grim* cDNA into *Drosphila* SL2 cells induces apoptosis, and *grim* expression in the developing eye causes eye ablation, in a similar manner to *hid* (Chen *et al.*, 1996).

1.11.9. Inhibition of apoptosis in response to viral infection.

In addition to being significant in organismal and cellular functions, apoptosis is an important factor in the resistance strategy of a host cell infected with a virus. A number of viruses are known to trigger apoptosis during infection, and members of at least four different virus families, *Herpesviridae*, *Adenoviridae*, *Poxviridae* and *Baculoviridae*, encode genes which can prevent apoptotic cell death in their host cells. This viral mechanism allows the avoidance of host-cell apoptosis and increases the efficiency of virus replication, ensuring a successful infection and ultimately resulting in higher yields of virus progeny (Hershberger *et al.*, 1992). The inhibition of apoptosis therefore provides a strategic advantage for the virus.

Epstein-Barr virus (EBV) encodes a 191-amino acid protein, BHRF1, which is abundantly expressed early in the lytic replication cycle. The gene can efficiently suppress apoptosis induced by various DNA-damaging agents (Tarodi *et al.*, 1994). Studies carried out to investigate the function of this protein have indicated that it is not essential for viral replication (Marchini *et al.*, 1991). BHRF1 has some sequence similarities to BCL-2 and it also has a common function in that it can protect human B cells from programmed cell death (Henderson *et al.*, 1993).

The E1B 19 kDa protein was originally identified as an inhibitor of DNA fragmentation and cell death in human cells infected with adenovirus. Adenovirus mutants which fail to express functional E1B 19 kDa induce fragmentation of cellular and viral DNA (Pilder *et al.*, 1984), causing premature death of the host cell. This finding established a role for the adenovius E1B 19kDa protein as a cell survival maintenance factor required to prevent cell death triggered by adenovirus infection; establishment of an effective adenoviral infection therefore depends on the function of the E1B 19 kDa protein (Rao *et al.*, 1992). The adenovirus E1B protein does not contain significant sequence similarity with BHRF1 or BCL-2, although there are three

short regions of homology which may be important for their common function (Tarodi *et al.*, 1994). A viral gene product, E1A, has been found to be responsible for apoptosis in E1B mutant virus infected cells (White and Stillman, 1987); the E1B 19 kDa protein acts to compensate for the cytotoxic consequences of E1A function. The function of the E1B 19 kDa protein can be replaced in adenovirus by BCL-2. Sequence and mutational analyses suggest that there may be some structural similarities between these two protein (Boyd *et al.*, 1994). BCL-2 will also substitute for adenovirus E1B in a transformation assay with E1A (Rao *et al.*, 1992).

Poxviruses, specifically cowpox, inhibit apoptosis by producing an inhibitor of apoptosis called cytokine response modifier A (*crm*A). The *crm*A gene product is a member of the serine protease inhibitor (serpin) family and can specifically inhibit ICE (Ray *et al.*, 1992; Komiyama *et al.*, 1994) and granzyme B (Quan *et al.*, 1995). CRMA inhibits apoptosis in response to a variety of stimuli, including Fas- and TNF-induced apoptosis (Tewari and Dixit, 1995; Tewari *et al.*, 1995) and has been shown to limit the inflammatory response of an infected cell (Gagliardini *et al.*, 1994).

In addition, rabbitpox virus (RPV) encodes three members of the serpin family referred to as SPI-1, SPI-2 (CRMA) and SPI-3. No direct evidence is available to confirm the involvement of CRMA in apoptosis in the context of a poxvirus infection. However, disruption of SPI-1 (which is 47% identical to CRMA at the amino acid level) in RPV, leads to reduced host range of the virus in cell culture (Ali *et al.*, 1994). This reduction in host range has been shown to be associated with apoptosis (Brooks *et al.*, 1995). Although the cellular target of SPI-1 is unknown, it is likely that this target will be a member of the ICE-like protease family (Brooks *et al.*, 1995). The poxvirus serpins are therefore involved in the control of apoptosis, and as for *p35* in baculovirus, the deletion of serpin genes in poxvirus results in the loss of apoptosis inhibition, and directly influences the host range of the virus.

A further gene involved in the inhibition of apoptosis during virus infection is the γ1 34.5 gene of herpes simplex virus type 1 (HSV-1) (Chou and Roizman, 1992; 1994).

The 170 kbp double-stranded DNA virus, African swine fever virus (ASFV), encodes two genes which may be involved in the inhibition of apoptosis. Firstly, A179L encodes a polypeptide which has sequence similarity to the BCL-2/BAX family of apoptosis regulators (Williams and Smith, 1993; Neilan *et al.*, 1993). Secondly, A224L encodes a polypeptide with a predicted molecular mass of 27 kDa that is homologous to the IAP protein family. A224L contains a C-terminal zinc finger region ($CX_2CX_{11}CX_2C$) which differs from the IAP RING finger motif, and also contains only one BIR motif. Primer extension analysis revealed that the abundant mRNA transcript of this gene initiated at a late transcriptional initiation motif. The protein was shown to be incorporated into the ASFV particles (Chacon *et al.*, 1995). ASFV replicates in both mammalian (pigs) and arthropod hosts (ticks). It has been suggested that A224L could be specifically responsible for anti-apoptotic function in tick cells, whilst A179L could be responsible for anti-apoptotic function in mammalian cells (Chacon *et al.*, 1995).

The prevention of apoptosis is also important for the establishment of viral latency. The adenovirus latent membrane protein, LMP-1, is expressed during viral latency in B cells. LMP-1 may act by up-regulating cellular *bcl-2* expression, to provide a survival advantage for latently infected cells (Henderson *et al.*, 1991; Martin *et al.*, 1993).

In addition, poliovirus (Picornavirus) has been shown to induce apoptosis in non-permissive strains of HeLa cells (Tolskaya *et al.*, 1995), although the genes involved in anti-apoptotic function in permissive cells have not yet been characterised.

In order to prevent baculovirus infection, insect cells have the ability to undergo apoptosis, which may be triggered by viral expression of *ie1* (Prikhod'ko and Miller, 1996). Two types of baculovirus genes, *p35* and *iap*, are able to block programmed cell death in Sf21 cells. AcMNPV mutants deficient in *p35*, termed annihilator or vAcAnh, cause apoptosis in Sf21 cells but not *T. ni* 368 cells; *p35* has thus been implicated in the control of host range (Clem *et al.*, 1991). AcMNPV (but not vAcAnh) blocks apoptosis triggered by a non-viral signal such as the RNA dependent DNA polymerase inhibitor actinomycin D, suggesting that the P35 protein interacts directly in the host apoptotic pathway, rather than acting by preventing viral triggering of apoptosis (Crook *et al.*, 1993). When actinomycin D is added to AcMNPV infected-Sf21 cells prior to 5 hours post infection, apoptosis results, indicating that viral gene expression is needed for inhibition of cell death. Stably transfected cell lines expressing *p35* are protected from low doses of actinomycin D (Cartier *et al.*, 1994). Consistent with its function in the inhibition of apoptosis, *p35* is transcribed early and late in the infection process (Friesen and Miller, 1987).

The *p35* coding region is located within the *Eco*RI-S genome fragment of AcMNPV (86.8 to 87.9 map units) and encodes a protein 299 amino acids in length with a molecular mass of 34.8 kDa. The P35 protein migrates at 35 kDa in SDS-PAGE, suggesting it is not extensively modified by glycosylation or phosphorylation (Hershberger *et al.*, 1994). The P35 protein has a high lysine content (10.7%); with the lysine residues clustered in two regions. As a result, the carboxy terminus of the protein is highly basic. P35 has no sequence motifs for nuclear localisation or transmembrane domains; the P35 protein appears to be targeted to the cytoplasm of cells (Hershberger *et al.*, 1994). Recent data obtained using the yeast two hybrid system suggest that P35 interacts with itself, forming homodimers (Friesen, 1996).

Characteristic of immediate early AcMNPV genes, mRNA transcripts for *p35* are detected within the first hour after infection, accumulating through 6 to 8

hours and slowly declining thereafter (Nissen and Friesen, 1989). The *p35* gene is flanked by the early gene *94K* and by the *hr*5 enhancer element, which has been shown to be a *cis*-acting enhancer of the early *p35* promoter (Rodems and Friesen, 1993). The early and late transcriptional activities of *p35* are controlled by different regions of its promoter (Dickson and Friesen, 1991).

A *p35* homologue has also been characterised in BmNPV (Kamita *et al.*, 1993), in which it performs a similar function to AcMNPV *p35*, except that mutants lacking BmNPV *p35* result in a mixed virus phenotype, with some cells undergoing apoptosis and others supporting virus replication (Kamita *et al.*, 1993). However, the baculovirus OpMNPV, the genome of which is largely colinear with AcMNPV (Blissard and Rohrmann, 1990), appears to lack both *p35* and *p94* homologues (Gombart *et al.*, 1989). The genes flanking *p35* and *p94* in AcMNPV are present in OpMNPV, however.

A *Cydia pomonella* granulovirus (CpGV) gene capable of blocking apoptosis has also been identified (Crook *et al.*, 1993). The CpGV inhibitor of apoptosis (*iap*) gene was able to provide a functional complement for AcMNPV *p35*, allowing the vAcAnh phenotype to be propagated in Sf21 cells. The observation that OpMNPV did not contain a *p35* homologue also led Birnbaum *et al.*, (1994) to investigate which gene(s) in OpMNPV fulfil the *p35* gene function for the virus. By using the complementation assay, they were able to demonstrate that OpMNPV has an *iap* gene which is functionally, but not structurally, homologous to AcMNPV *p35*. In addition, AcMNPV contains two *iap*-like genes designated *iap1* (ORF 27) and *iap2* (ORF 71) (Ayres *et al.*, 1994). The AcMNPV *iap* genes appear to be non-functional as inhibitors of apoptosis, since *p35* mutants induce cell death in virus-infected cells, and they are unable to protect cells against actinomycin D-induced apoptosis (Clem *et al.*, 1994a).

The baculovirus IAPs all contain carboxy terminal Cys_3 His Cys_4 (C3HC4) zinc (or RING) finger-like motifs (Freemont *et al.*, 1991), found in a number of transcriptional regulatory proteins. Additional cysteine/histidine motifs which

form repeat regions termed baculovirus IAP repeats (BIRs) are present in the N-terminal regions of the IAP polypeptides.

Zinc finger motifs of cysteine and histidine residues suggest a DNA binding protein function (Berg, 1986), but are most commonly found at the amino terminus of a protein. Zinc fingers also exist in the early baculovirus genes *ie-1* (Passarelli and Miller, 1993c), *cg30* (Thiem and Miller, 1989b), *me53* (Knebel-Mörsdorf *et al.*, 1993) and *pe38* (Krappa and Knebel-Mörsdorf, 1991) of AcMNPV. In addition, zinc fingers have been characterised in other virus regulatory genes such as Herpes simplex virus ICP0 (Perry *et al.*, 1986). The zinc finger motif is also found in a number of cellular polypeptides that may have a role in regulating apoptosis. Several of these are encoded by proto-oncogenes, such as *mel*-18, which is involved in cell proliferation (Tagawa *et al.*, 1990), *pml* (de The *et al.*, 1991), *c-cbl*, a proto-oncogene that causes lymphomas when mutated (Blake *et al.*, 1991) and *bmi*-1, which is implicated in tumorigenesis (Haupt *et al.*, 1991). In addition, several insect regulatory genes, including the *Drosophila* segmentation genes *su(z)*2 (Brunk *et al.*, 1991) and *psc* (Brunk *et al.*, 1991) which are homologues of human *bmi*-1, also contain zinc finger motifs. The intimate involvement of apoptosis in both lymphocyte proliferation and embryonic development (Walker *et al.*, 1988; Williams *et al.*, 1990) suggest that at least some of the zinc finger-containing genes may be involved in the positive or negative regulation of apoptosis.

The nucleotide sequence of OpMNPV-*iap* predicts a 30 kDa polypeptide product with 58% amino acid sequence identity to the product of CpGV-*iap* but only 28% amino acid sequence identity to AcMNPV-*iap1*. The subcellular localisation of the IAP proteins is unknown, although the genes contain no recognisable signal sequences, transmembrane domains or nuclear localisation signals.

It has been demonstrated that both *p35* and the *iap*s of CpGV and OpMNPV act directly on the cellular apoptotic pathway independently of other viral

factors, since these genes will prevent actinomycin D-induced apoptosis when uninfected Sf21 cells are transfected with a plasmid expressing these genes under the control of a constitutive promoter (e.g., *Drosophila* heat shock promoter) prior to treatment with the drug (Clem and Miller, 1994a) .

1.11.10. Mammalian and insect homologues of baculovirus *iap*.

Homologues of the baculovirus *iap* genes have been identified in the human genome. A human gene implicated in a class of diseases termed spinal muscular atrophies (SMAs) has recently been characterised (Roy *et al.*, 1995). A central role for apoptosis has been established in the normal development of the central nervous system (CNS) throughout embryogenesis (Oppenheim, 1991). As many as 50% of the cells in the developing eukaryotic CNS, including motor neurons, undergo apoptosis. Spinal muscular atrophy (SMA) invokes inappropriate persistence of neuronal apoptosis resulting in a neurodegenerative disorder, due to the deletion of motor neurons in the CNS. It is a common monogenic cause of death in infancy. The gene leading to this phenotype has been mapped to human chromosome 5 using genetic linkage experiments with markers (Brzustowicz *et al.*, 1990) by comparing the DNA from SMA families to normal human DNA. This gene product has been designated neuronal apoptosis inhibitory protein (NAIP) (Roy *et al.*, 1995). The coding region of NAIP spans a 3.6 kbp region and encodes a 1232 amino acid protein. Analyses of Southern blots containing DNA from SMA families with NAIP cDNA revealed deletion areas, thereby demonstrating that NAIP abnormality is a contributing factor to spinal muscular atrophy disease (Roy *et al.*, 1995). The gene encoding NAIP, shares a region of homology with the baculovirus IAPs, specifically CpGV IAP (33% over 189 amino acids) and OpMNPV IAP (33% over 180 amino acids) (Roy *et al.*, 1995). NAIP contains 3 BIR motifs, although it contains no zinc finger motif.

Recently, six additional *iap*-like ORFs have been identified, including d*iap1* and d*ilp* (d*iap2*) from *Drosphila* (Hay *et al.*, 1995; Duckett *et al.*, 1996), murine

m*iap*, human h*ilp* (X-linked *iap*) (Liston *et al.*, 1996; Duckett *et al.*, 1996; Uren *et al.*, 1996) and human h*iap1* and h*iap2* (Rothe *et al.*, 1995; Uren *et al.*, 1996). All these genes contain zinc finger and BIR motifs. The h*iap1* and h*iap2* genes were identified due to their ability to interact with the TRAF2 signal transducer, and therefore exhibit the ability to affect the apoptotic signalling cascade (Rothe *et al.*, 1995). Low levels of h*iap1* and h*iap2* transcripts were detected in all the human tissue types examined (Rothe *et al.*, 1995), indicating the ubiquitous nature of the protein products of h*iap1* and h*iap2*. These two genes are located on chromosome 11 and exhibit a high degree of amino acid conservation (72% for the predicted protein sequences), suggesting they may be gene duplications.

1.11.11. The role of baculovirus *p35* in the inhibition of apoptosis.

The P35 protein has been shown to act upon the TNF/Fas antigen cell signalling pathway. Specifically, P35 blocks the cleavage of poly(ADP-ribose) polymerase (PARP) (Biedler *et al.*, 1995), a substrate normally processed by ICE-like proteases. P35 is an irreversible inhibitor of ICE, ICH-1, ICH-2 and CPP32, becoming cleaved to a C-terminal 25 kDa fragment that remains associated with the protease and an N-terminal 11 kDa fragment (Bump *et al.*, 1995). The cleavage of P35 occurs between residues Asp87 and Gly88, within the highly charged region of the protein, which is predicted to be exposed on the outer surface of the protein. P35 is a stoichiometric inhibitor of ICE-like proteases. When quantities of P35 are insufficient to block all ICE-like proteases, apoptosis proceeds, e.g., a baculovirus expressing ICE under the control of the polyhedrin promoter causes apoptosis in infected cells at late times in the infection (S. Chapple, pers. comm.). However, unlike CRMA, P35 has no effect on the proteolytic activity of granzyme B.

During baculovirus infection, P35 inhibits the action of the ICE-like protease produced in Sf21 cells (Sf protease) (Bertin *et al.*, 1996). This protease cleaves P35 and is potently inhibited by it, in an identical manner to ICE, ICH-1, ICH-

2 and CPP32. Site-directed mutagenesis analysis of the P35 protein has identified several residues required for anti-apoptotic function in Sf21 cells, including Asp87 and Asp84. Mutation of Asp87 resulted in a lack of cleavage by the Sf protease, and the loss of anti-apoptotic function. Mutation of Asp84 also caused a loss of apoptotic suppression but did not eliminate cleavage of the P35 protein, suggesting that cleavage itself is not sufficient for anti-apoptotic ability (Bertin *et al.*, 1996).

1.11.12. The apoptosis-inhibiting genes of baculoviruses are active in diverse systems.

P35 has the ability to block programmed cell death in a variety of phylogenetically diverse organisms and in response to a wide range of apoptotic inducers. The *p35* gene can rescue a *ced-9* mutant nematode, which normally produces a lethal phenotype in the nematode embryo (Sugimoto *et al.*, 1994). The capacity of *p35* to inhibit developmentally programmed cell death in *C. elegans* shows that it can suppress apoptosis directly and does not require the interaction of other baculovirus genes or factors specific to the host cell. This result also provides evidence that the cell death pathway operated by *C. elegans* and by insect cells as a result of baculovirus infection are essentially similar.

In *Drosophila*, transgenic expression of *p35* prevents programmed cell death normally occurring in the developing eye and embryo and in response to X-ray irradiation (Hay *et al.*, 1994). In addition, *p35* can block the effects of the *Drosophila* genes *reaper*, *hid* and *grim* (Grether *et al.*, 1995; Chen *et al.*, 1996; Pronk *et al.*, 1996; White *et al.*, 1996), suggesting that these genes activate a cell death program mediated by ICE-like proteases (White *et al.*, 1996).

In addition to protecting invertebrate cells from programmed cell death, *p35* can inhibit mammalian neuronal cell death induced by serum withdrawal,

growth factor deprivation or the addition of calcium ionophore (Rabizadeh *et al.*, 1993; Martinou *et al.*, 1995).

The *iap*s of baculoviruses do not appear to have the universal anti-apoptotic activity of *p35*. CpGV *iap* prevents apoptosis induced by adenovirus E1A in rat kidney cells (Clem *et al.*, 1996). However, expression of CpGV *iap* in rat neural cells does not affect cell survival (Clem *et al.*, 1996). The human *iap*s protect Rat-1 cells against apoptosis induced by serum withdrawal, but not against apoptosis induced by the protein kinase inhibitor staurosporine (Liston *et al.*, 1996). Experiments involving OpMNPV *iap* indicate that this gene, when introduced into a Sindbis virus vector and expressed under the control of a duplicated copy of a late viral promoter, can prevent Sindbis virus-induced apoptosis of baby hamster kidney (BHK) cells (Duckett *et al.*, 1996). In addition, both OpMNPV *iap* and human *iap* (h*ilp*) can promote the survival of cells transfected with an expression plasmid encoding the pro-ICE protease (Duckett *et al.*, 1996).

These observations provide further evidence to suggest that some aspects of the cell death pathway are evolutionarily conserved. The ability of *p35* to block apoptosis in different pathways and in distantly related organisms suggests a central and conserved role for ICE-like proteases in the induction of apoptosis. In addition, the conservation of IAP proteins in baculoviruses, humans, *Drosophila* and mice suggests that they are important for the regulation of apoptosis (Liston *et al.*, 1996). The differences in activity between *p35* and *iap* suggests that they act at different points in the apoptotic cascade. Both *p35* and *iap* have been implicated in the control of ICE-mediated apoptosis, though it is likely that these genes act at different points in the programmed cell death pathway.

The characterisation of *p35* and *iap* genes in the baculovirus system may have direct implications for the elucidation of the programmed cell death pathway in mammalian systems, thereby contributing to our understanding of this

fundamental cell mechanism and the diseases associated with its dysregulation.

1.12. Aims of thesis.

P35 is known to affect the host range of AcMNPV by preventing the virus-induced apoptotic response in infected cells. Recombinant AcMNPVs with point mutations, deletions, frameshift mutations or marker gene insertions in *p35* cause apoptosis in Sf21 cells, and suffer reduced production of BVs *in vitro* (Clem *et al.*, 1991). By implication, the *iap*s in other baculoviruses (OpMNPV and CpGV) which have a similar function to *p35 in vitro* (Crook *et al.*, 1993; Birnbaum *et al.*, 1994) may also have the potential to affect the host range of those viruses.

Inhibitor of apoptosis genes may have the capacity to act in a cell line-specific manner through interactions with both cell signalling receptors and transducers. In studies involving human cells transfected with the murine TNF receptors mTNF-R1 and mTNF-R2, mTNF-R1 demonstrated a similar affinity for murine TNF-α and human TNF-α, however, mTNF-R2 exhibited specificity for murine TNF-α (Lewis *et al.*, 1991). If the action of baculovirus *iap*s in some manner involves species specificity, as is the case in different insect species for AcMNPV *p35*, these genes may be generally involved in cell line-specific interactions.

In this study, an isolate of the baculovirus *Heliothis zea* single nucleopolyhedrovirus (HzSNPV NC-1), with the potential for commercial development, was examined in order to investigate the nature of its inhibitor of apoptosis genes, with a view to examining the genetic factors involved in the control of its host range.

Figure 1.1.

Circular map of the genome of AcMNPV.

The inner circle represents the *Hind*III map of AcMNPV and the outer circle the *Eco*RI map. The positions of the putative 154 methionine-initiated genes encoding proteins of 50 amino acids or more are represented as arrows, which indicate the direction of transcription. The ORFs are closely spaced and, in several cases, overlap. The locations of the *hr* sequences and the characterised transposon integration sites are shown inside the circles. The scale of the inner circle represents 100 map units.

Figure 1.2.

A diagrammatic representation of the life cycle of a baculovirus infection.

Occluded viruses ingested by Lepidoptera larvae dissolve in the alkaline conditions of the midgut, to release the virus particles. The virus particles then fuse with the membranes of the midgut epithelial cells and the nucleocapsids are released into the cytoplasm and pass into the cell nulcei, where uncoating and viral DNA replication occur.

Nucleocapsids bud from the cell, acquiring the virus-encoded glycoprotein, gp64, and spread the secondary infection in the insect. Nucleocapsids produced later in the infection process are enveloped in the nucleus and occluded within a crystalline matrix of polyhedrin protein. The occluded viruses are released during cell lysis, following death of the insect. Occluded viruses remain in the environment and bring about the horizontal transmission of the baculovirus infection.

(P = polyhedra, ECV = extracellular virus (BV), PDV = polyhedra derived virus (OV), NC = nucleocapsid).

Reproduced from King and Possee, 1992.

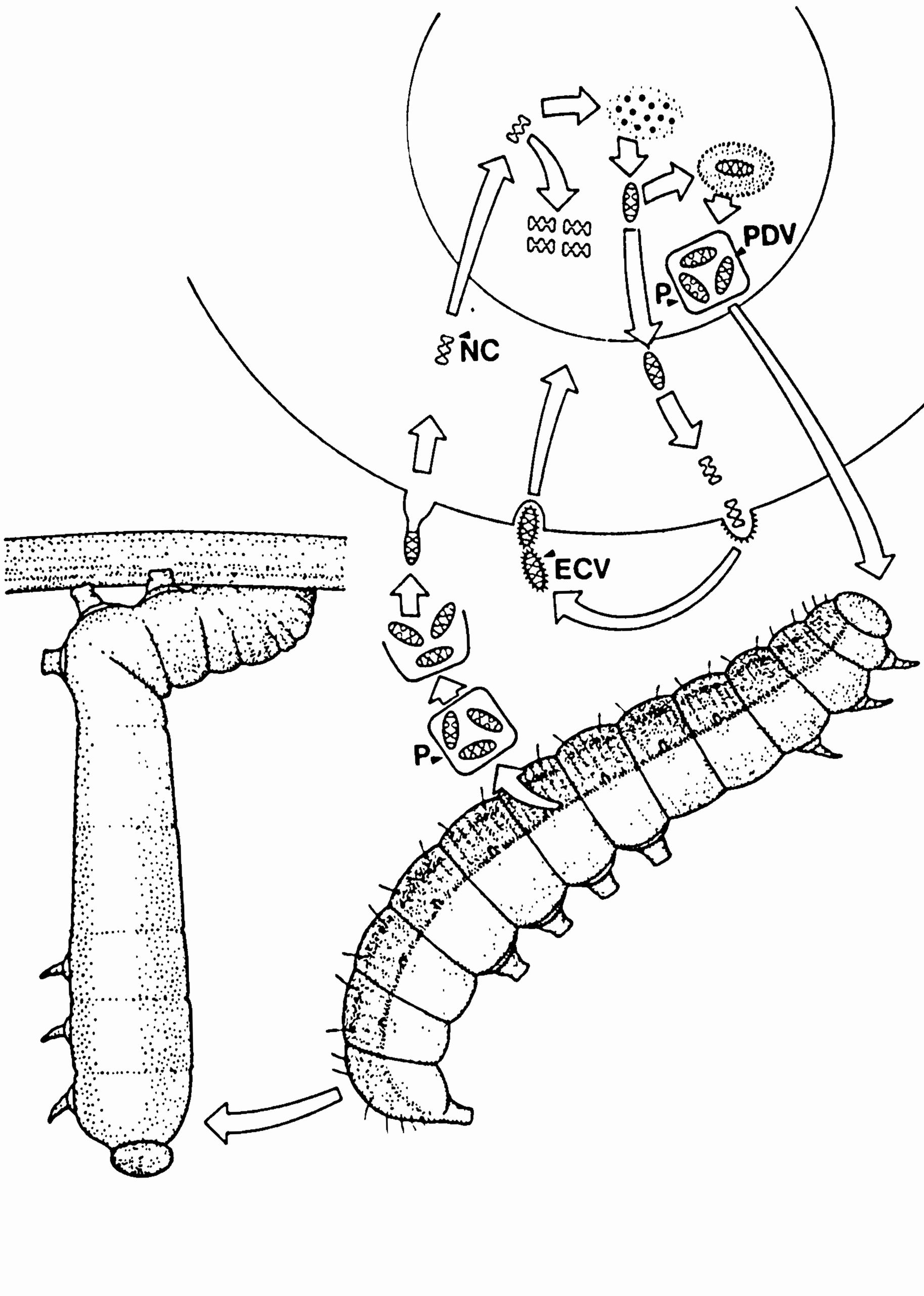

PDV
P
NC
ECV
P

Figure 1.3.

Electron micrograph of an occluded virus of HzSNPV NC-1.

A section of the HzSNPV NC-1 OV particle was examined by transmission electron microscopy. The HzSNPV NC-1 nucleocapsids are singly enveloped within the matrix of polyhedrin protein. The magnification of the picture is 160000 x.

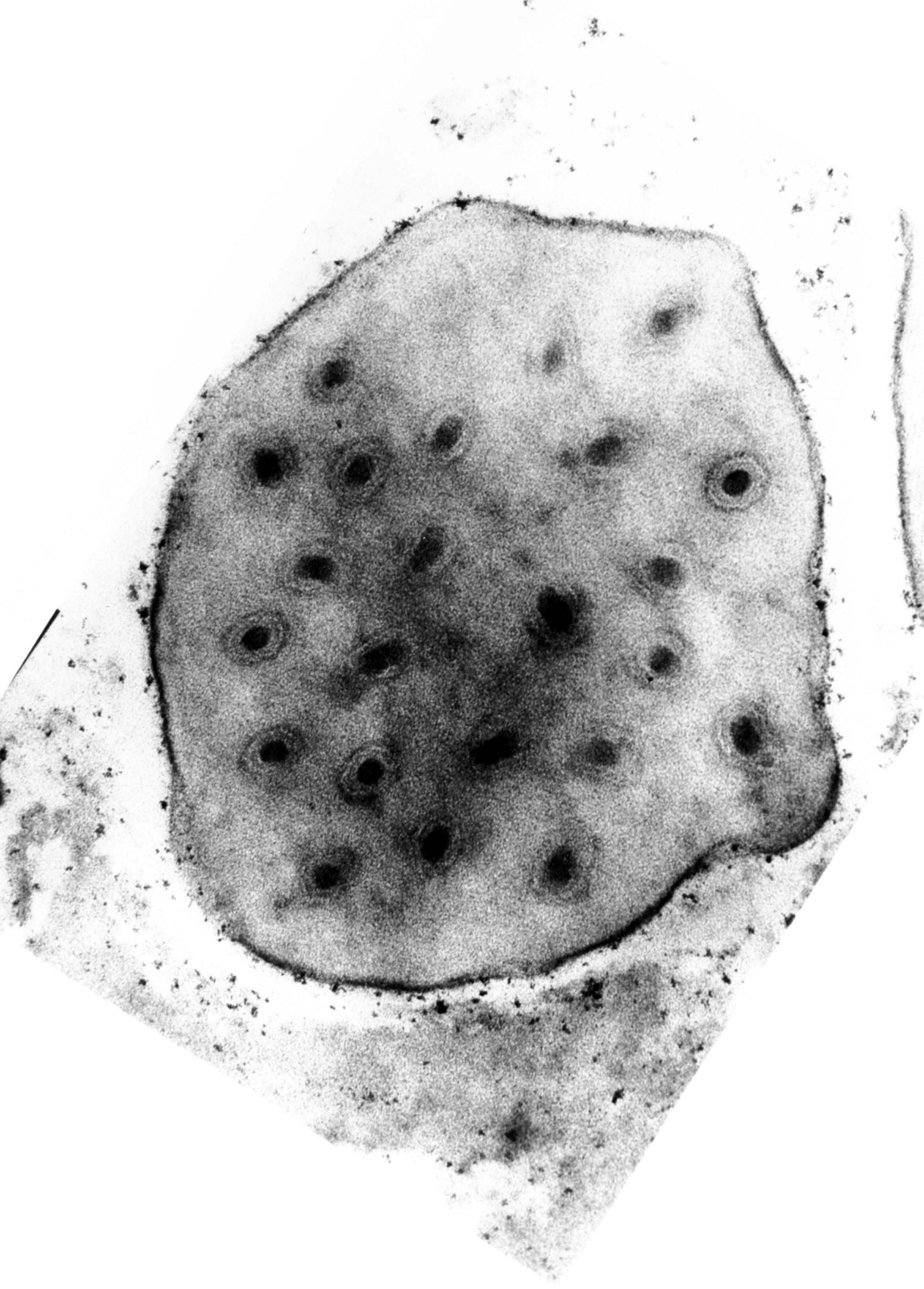


Figure 1.4.

Interaction between the cell surface receptors and signal transducers implicated in the control of programmed cell death.

The Figure represents a model of apoptosis showing members of the ICE family of proteases as effectors of cell death. Protein targets of the ICE-like proteases are indicated. Proteins such as PARP, proIL-1β, nuclear lamins and actin are known to be cleaved by ICE-like proteases. Inhibitors of apoptosis, such as the cowpoxvirus CRMA protein, baculovirus P35 and baculovirus IAP are also shown. The cell surface receptors Fas, TNF-R1 and TNF-R2, and their associated signal transduction proteins, are also indicated.

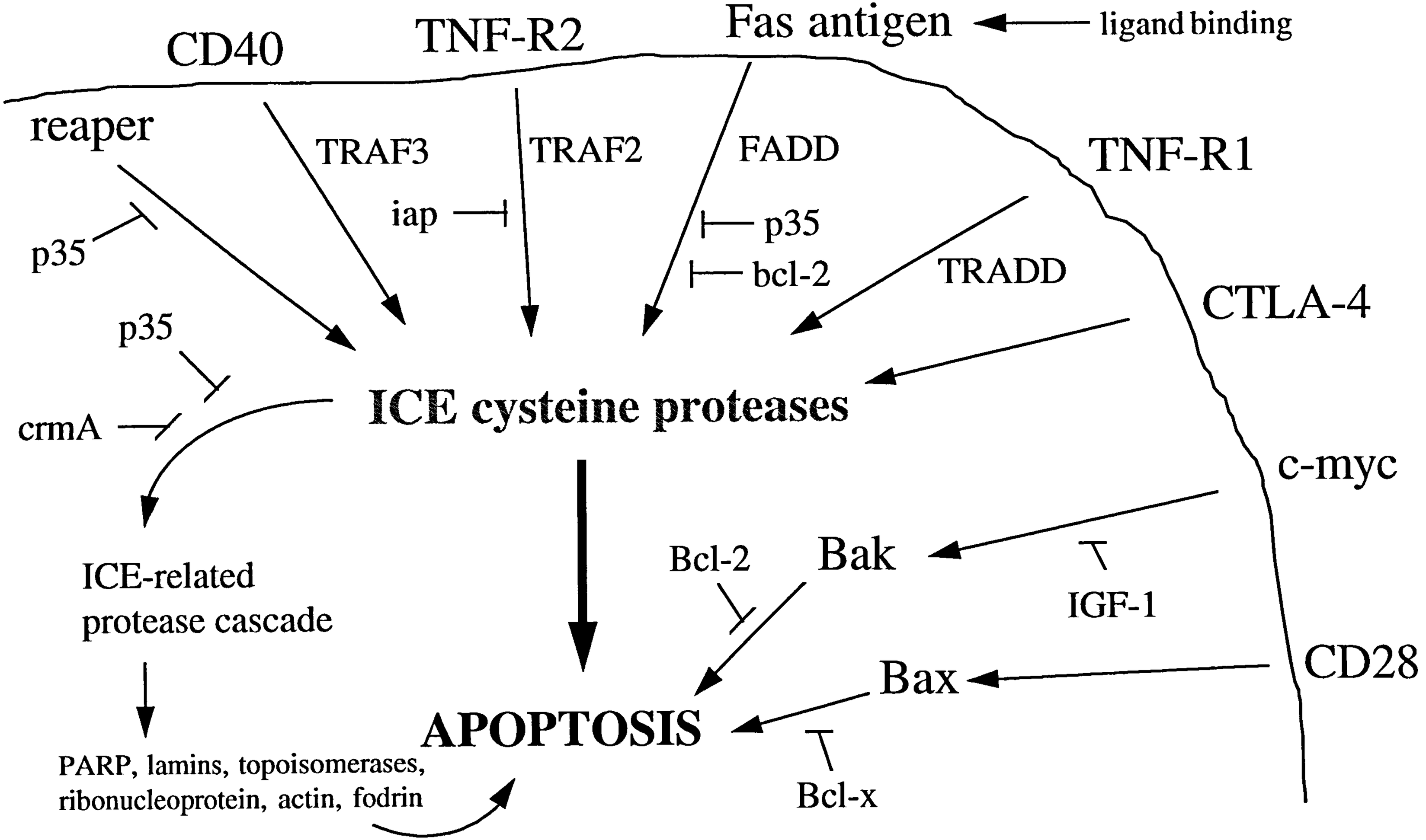

CD40
TNF-R2
Fas antigen
ligand binding
reaper
TRAF3
TRAF2
FADD
TNF-R1
iap
p35
p35
bcl-2
TRADD
CTLA-4
p35
crmA
ICE cysteine proteases
c-myc
Bcl-2
Bak
IGF-1
ICE-related
protease cascade
CD28
Bax
APOPTOSIS
PARP, lamins, topoisomerases,
ribonucleoprotein, actin, fodrin
Bcl-x

Table 1.1. Inhibitors of apoptosis.

A list of the agents that have been reported to inhibit apoptosis.

Physiological inhibitors	*Viral genes*	*Pharmacological agents*
growth factors	Adenovirus E1B	calpain inhibitors
extracellular matrix	**Baculovirus *p35***	cysteine protease inhibitors
CD40 ligand	**Baculovirus *iap***	tumour promoters
neutral amino acids	Cowpox virus *crm*A	PMA
zinc	Epstein-Barr virus BHRF1	Phenobarbital
oestrogen	and LMP-1	α-Hexachlorocyclohexane
androgens	African swine fever virus	
	LMW5-HL	
	Herpesvirus γ1 34.5	

Table 1.2. Inducers of apoptosis.

A list of the agents that have been reported to induce apoptosis.

Physiological activators	*Damage-related inducers*	*Therapy-associated agents*	*Toxins*
TNF family (FasL, TNF)	Heat shock	Chemotherapeutic	Ethanol
Transforming growth factor β	Viral infection	drugs	β-amyloid peptide
Neurotransmitters (Glutamate, Dopamine. N-methyl-D-aspartate)	Bacterial toxins	γ-radiation	
	Oncogenes - (*myc*, *rel*, E1A)	UV radiation	
Growth factor withdrawal	Tumour suppressors (p53)		
Loss of matrix attachment	Cytolytic T cells		
Calcium	Oxidants		
Glucocorticoids	Free radicals		
	Nutrient deprivation		

Chapter Two

Materials and Methods

2.1. MATERIALS.

2.1.1. Chemicals.

Chemicals were purchased from the following companies:

General chemicals were purchased from BDH Chemicals Limited (UK), Life Technologies Limited (UK) and Sigma Chemical Company Limited (UK). Chemicals required for media were purchased from Difco Laboratories Limited (UK), Life Technologies Limited (UK) and Oxoid (UK).

All solutions were made as weight per volume (w/v) unless otherwise stated. Solutions were sterilised where necessary either by autoclaving (15 pounds per square inch (p.s.i.) for 15 minutes or 10 p.s.i. for 20 minutes) or by filtration.

2.1.2. Radioisotopes.

Radioisotopes were purchased from DuPont or Amersham International plc. The following isotopes and specific activities were used:

[α-32P]dATP	>6000 Ci/mmol (>2.2x10^{14} Bq/mmol)
[α-35S]dATP	>1000 Ci/mmol (>3.7x10^{13} Bq/mmol)
[γ-32P]ATP	>5000 Ci/mmol (>1.8x10^{14} Bq/mmol)

2.1.3. Enzymes.

Restriction endonucleases were purchased from Amersham International plc, Gibco-BRL and Boehringer Mannheim (UK) Limited, and were used according to the manufacturers instructions. Calf intestinal phosphatase (CIP) was purchased from Boehringer Mannheim (UK) Limited; Klenow polymerase, T4 DNA Ligase and T4 DNA polymerase were purchased from Gibco-BRL; T4 polynucleotide kinase (PNK) from New England Biolabs; AMV reverse transcriptase from NBL Gene Sciences Ltd; Sequenase from United States Biochemicals; *Taq* DNA polymerase from Bioline; *Tbr* DNA

polymerase from NBL Gene Sciences Ltd and *Pfu* DNA polymerase from Stratagene. The random priming kit "Prime-a-Gene" was purchased from Promega Corporation.

2.1.4. Nucleic acids.

The following nucleic acids were used:

Salmon sperm DNA (type IV)	Sigma Chemical Company Ltd
λ DNA	Gibco-BRL
pUC118	Gibco-BRL
pBluescript II KS+	Stratagene
pT7T3-*Bgl*II	gift of R. Possee

2.2. INSECT CELL CULTURE AND BACULOVIRUS PROCEDURES.

2.2.1. Insect cell lines.

The following cell lines were used for propagating viruses:

Mamestra brassicae	gift of Oxford Brookes University, (King *et al.*, 1991)
Spodoptera frugiperda (APLB-Sf-21-AE)	Vaughn *et al.*, 1977
Lymantria dispar (Ld-652)	gift of Oxford Brookes University
Heliothis zea (parental)	gift of Zeneca Agrochemicals plc
Heliothis zea (BCIRL-Hz-AM3)	gift of Zeneca Agrochemicals plc (Lenz *et al.*, 1991)
Panolis flammea	derived by C. Allen, Institute of Virology and Environmental Microbiology (IVEM), Oxford
Spodoptera littoralis	derived by C. Allen, IVEM
Trichoplusia ni (TN-368)	gift of D. Knebel-Mörsdorf, University of Cologne, Germany (Hink, 1970)

2.2.2. Cell culture media.

The cell culture media TC100 (Gibco), TC199 MK (Gibco) (see Appendix 1) and Sf900 II (Gibco) were routinely used for the propagation of insect cells. TC100 was also prepared from basic ingredients as described by King and Possee (1992). Foetal calf serum (FCS) (Gibco) was used to supplement media where required.

2.2.3. Cell culture methods.

a.) General cell culture methods.

Stocks of cells were stored in liquid nitrogen in TC100 media containing 10% dimethyl sulphoxide (DMSO). Frozen cells were revived by thawing at 37°C, and transferred to a cell culture flask (25cm^3) containing 5ml of TC100 media. The culture was incubated for 1 hour at 28°C to allow the cells to settle on the plastic flask, following which the medium was replaced with fresh TC100 to remove the dimethyl sulphoxide. The cells were then returned to the incubator until required.

Following incubation, the cells were allowed to become confluent to a monolayer, then washed and harvested into fresh medium. Cell density and viability was determined using trypan blue staining (final concentration of 0.05% (w/v)) and counting in a haemocytometer chamber.

Suspension cultures were grown in round, flat-bottomed glass flasks which were agitated using a magnetic stirrer and bar follower. Cultures of up to 250ml were seeded with 1x10^5 cells/ml and incubated at 28°C. Cells were passaged when the cell density reached approximately 1.5x10^6 cells/ml.

b.) *Heliothis zea* cells.

The *Heliothis zea* cell line was derived from the ovarian cells of *Heliothis* pupae by McIntosh and Ignoffo, (1981). Cells derived from a clone of the parental *H. zea* cell line, named BCIRL-Hz-AM3 (Lenz *et al.*, 1991), were

routinely used. The cells were passaged in a modified TC199 MK medium (McIntosh *et al.*, 1973) supplemented with 10% foetal calf serum (FCS), penicillin (50 units/ml) and streptomycin (50µg/ml). Cultures were seeded with cells at between $1x10^5$ and $5x10^5$ cells/ml and maintained at 28°C until the cells became confluent (approximately 6-7 days). To passage the cells, the old media was removed and the cells were given two washes in 1x calcium and magnesium free (CMF) buffer. Trypsin (1x in dilution buffer) (1ml) was then added to the cells for 3 minutes to loosen them from the plastic cell culture flask. New media was added to the suspension and the cells were counted and dispensed into new culture flasks.

c.) Sf21 cells.

Spodoptera frugiperda (Sf21) APLB-Sf-21AE cells (Vaughn *et al.*, 1977) were maintained in homemade TC100 medium supplemented with 10% FCS. As detailed above, the cells reached maximum density after approximately 5 days. At 28°C, the optimum temperature for the growth of Sf21 cells, the population doubling time was approximately 24 hours. Sf21 cells were employed between passage numbers 150-180 only.

d.) *T. ni*, *P. flammea*, *M. brassicae* and *S. littoralis* cells.

The *T. ni* and other cell lines were passaged by gently loosening the cells into TC100 media with a plastic pastette. A fresh plastic culture flask was inoculated with 1/10 of the harvested cells with fresh medium. Cells were incubated at 28°C until they became confluent again.

e.) *L. dispar* cells.

L. dispar cells were grown in Sf900 II media supplemented with 5% FCS. The cells were passaged by gently loosening into fresh Sf900 II media using a plastic pasteur pipette. A fresh plastic culture flask was inoculated with 1/6 of the harvested cells with fresh medium. Cells were incubated at 28°C until they became confluent again.

2.2.4. Baculoviruses.

The *Heliothis zea* single nucleopolyhedrovirus (HzSNPV) isolate NC-1 used in this study was originally obtained from a cotton growing region in North Carolina, USA, by Zeneca Agrochemicals plc. The cloned isolate was derived by P. Smith (Oxford Brookes University) using a technique of repeated plaque assay. This isolate (clone 3) infects *Heliothis* cells in culture to produce high titre virus.

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) used in these studies was the C6 strain (Possee, 1986).

2.2.5. Virus infection of insect larvae.

All insect larvae (*T. ni* and *H. zea* species) were taken from disease-free cultures maintained at IVEM, Oxford. Larvae were maintained at 24°C, on semi-synthetic diet (King and Possee, 1992) which is a modification of Hoffman's Tobacco hornworm diet (Hunter *et al.*, 1984).

Individual larvae (3rd instar) were infected *per os* using small plugs of diet containing defined numbers of polyhedra (Hughes and Wood, 1981). A single larva was placed into a microtitre plate (24 well) containing the diet plug. The plate was subsequently covered with tissue and clingfilm (Saranwrap), and the lid securely fastened with tape. Plates were then incubated at room temperature (RT) for 24-36 hours, after which time those larvae which had eaten the entire diet plug were transferred to individual polypots containing a layer of fresh diet. The polypots were incubated at RT. Larvae were examined daily for signs of virus-induced mortality or pupation.

2.2.6. Larval bioassays: LD_{50} analysis.

Laboratory reared *H. zea* were used in all the assays. The virus stocks (AcMNPV C6, Ac*iap1*Δ and AcHz*iap*) were prepared as detailed in section 2.2.7. The concentration of occluded virus in the purified stocks was

determined using a haemocytometer counting chamber. The method of infection of larvae was by droplet feeding (Hughes *et al.*, 1986). Five virus concentrations, ranging from $1x10^8$ to $1x10^3$ polyhedra/ml were used. The data were analysed by backwards stepwise regression using GLIM (a statistics package for Generalised Linear Interactive Modelling, version 3.77, Royal Statistical Society, 1985). A model was constructed by sequentially removing the parameters (virus type and virus dose) being tested in the model, to determine the contribution of each factor to the deviance, and thereby examine its significance. A second series of models were derived using Probit modelling (Finney, 1971), using the S-Plus computer package (version 3.4) (Venables and Ripley, 1994).

2.2.7. Purification of polyhedra from virus-infected insect larvae.

Polyhedra were purified from infected insect larvae as described by Harrap *et al.*, (1977). Infected larvae were macerated in a 0.1% SDS solution in a sterile plastic bag and subsequently filtered through muslin layers into a 100ml beaker. Polyhedra were pelleted from the suspension at 4000 *g*, 4°C for 15 minutes (Beckman GS-15R centrifuge), and resuspended in 2-3mls TE (10mM Tris-HCl pH 8.0, 1mM EDTA). The polyhedra were dispersed by stirring at RT for 30 minutes and then layered onto a 50-60% (w/w) discontinuous sucrose gradient (in TE) in Ultraclear tubes (14 x 89 mm, Beckman). The gradients were centrifuged at 75000 *g*, 4°C for 30 minutes (SW41 or TH641 rotor, Beckman L8-70M ultracentrifuge). The pellets were drained and soaked overnight at 4°C in 1ml TE to allow resuspension. Polyhedra were stored at 4°C. The concentration of polyhedra present was assessed by counting in a 0.1mm haemocytometer.

2.2.8. Purification of virus particles from polyhedra.

The polyhedra suspension was treated with an equal volume of 0.1M Na_2CO_3 for 30 minutes at 28°C (in a water bath) with stirring to assist dissolution. Undissolved polyhedra were pelleted (3000 *g*, 10 minutes, Beckman GS-15R centrifuge) and the released virus particles were layered onto 30-60% (w/w) continuous sucrose gradients (in TE) which had been

allowed to form at 4°C overnight. After centrifugation at 75000 *g*, 4°C for 1 hour (SW41 or TH641 rotor, Beckman L8-70M ultracentrifuge), a band of virus particles was observed. The virus particles were harvested by removing with a plastic pastette and were diluted 1:5 in TE. Virus particles were then pelleted by centrifugation at 75000 *g*, 4°C for 1 hour (SW41 or TH641 rotor, Beckman L8-70M ultracentrifuge), and the pellets soaked overnight in TE (1ml) to allow gentle resuspension. Suspensions of virus particles were stored at 4°C.

2.2.9. Isolation of infectious virus DNA from purified virus particles.

Virus DNA was extracted from purified virus particles as described by King and Possee (1992). Pure virus particles were added to an equal volume of TE and a 1:4 volume of 20% sarkosyl solution (sodium lauryl sarcosinate in 10mM Tris, 10mM EDTA, pH 7.8) and incubated at 60°C for 1 hour. The lysed virus particles were layered onto a 6ml cushion of 46% (w/w) caesium chloride in TE containing 25µg/ml ethidium bromide. The tubes were filled, equilibrated by weight with liquid paraffin and centrifuged at 116000 *g*, 20°C for 16-20 hours (SW41 or TH641 rotor, Beckman L8-70M ultracentrifuge). The DNA was harvested by puncturing the bottom of the tube and collecting the DNA band by downward displacement. Ethidium bromide was removed by repeated extraction with TE-saturated butanol. The DNA was then dialysed against TE at 4°C, for 16-20 hours (with 2-3 changes of dialysis buffer). DNA concentrations were estimated by UV spectrophotometry (Cecil 6000 Series Spectrophotometer). An absorbance (A_{260}) of 1 corresponds to 50 µg/ml for double-stranded DNA. The ratio of readings at 260nm and 280nm (A_{260}/A_{280}) was calculated to provide an estimate of the purity of the DNA (pure preparations give a ratio value of 1.8). DNA solutions were stored in plastic bijous at 4°C until required.

2.2.10. Cotransfection of insect cells with virus DNA and transfer vector.

a.) Circular virus DNA.

Sf21, *T. ni*-368 and *H. zea* cells were cotransfected with circular virus DNA (0.1-4.0μg) and a 5-fold excess of plasmid DNA (purified on caesium chloride gradients, as described in section 2.3.5), using the liposome-mediated transfection method (Lipofectin™, Gibco-BRL) as described in King and Possee (1992). Following incubation at 28°C for 2 (AcMNPV transfection of Sf21 cells) or 14 days (HzSNPV transfection of *H. zea* cells), the media was harvested for titration by plaque assay.

b.) Linearised virus DNA.

Recombinant AcMNPV DNA containing the *lacZ* gene (3μg) was linearised by digestion with 10 units of the restriction enzyme *Bsu*36I (Kitts *et al.*, 1990; King and Possee, 1992) for 6 hours at 37°C. The reaction was then heat-inactivated by incubation at 65°C for 5 minutes. Cotransfections were then performed as detailed above, using the linearised viral DNA instead of circular DNA. For cotransfections, 100ng of linearised viral DNA and 500ng of transfer vector was routinely used.

2.2.11. Complementation assay for AcMNPV P35 function.

Complementation assays were carried out in a similar manner to the method for a cotransfection experiment detailed in section 2.2.10. Virus DNA purified from *T. ni*-368 cells infected with the *p35* deletion mutant of AcMNPV (Ac*p35lacZ*), was cotransfected into Sf21 cells using Lipofectin™ reagent, in combination with the viral or plasmid DNA being tested in the assay. The concentration of DNA used in the assay was 1μg of Ac*p35lacZ* DNA, and 5μg of test plasmid DNA or 2μg of test viral DNA, as appropriate. The cells were incubated at 28°C for 3-4 days, after which time they were examined by light microscopy. A complementation event occurred if the test DNA encoded a gene with the ability to function in place of AcMNPV *p35*.

This complementation event was identified by the presence of occluded virus in the cells.

2.2.12. Titration of virus by plaque purification.

Plaque assays were performed to determine the titres of recombinant and wild-type virus stocks, and also to allow the purification of recombinant viruses. The protocol used is based upon the method described by Brown and Faulkner (1977). Routinely, infectious virus stocks were diluted in ten-fold steps up to 10^{-7}; virus produced in cotransfection experiments (section 2.2.10) was diluted in a similar manner to 10^{-4}. Aliquots (100µl) of each dilution were gently pipetted onto duplicate 35mm dishes containing $1x10^6$ Sf21 cells. Dishes were incubated at RT for 1 hour. The inoculum was then removed using a Gilson pipette. Infected cells were overlaid with 2ml of TC100 medium/10% FCS containing 1% low gelling temperature (Seaplaque) agarose (Sigma Chemical Company Ltd). After allowing the overlay to set (30 minutes, RT), 1ml of TC100/10% FCS was added to each dish. The dishes were incubated at 28°C for 4 days before the plaques were visualised by staining with neutral red (0.05% in PBS) for 3 hours.

Plaques from cotransfections with viruses expressing *lacZ* were initially stained by adding 10µl 2.5% 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-gal) (Gibco BRL) to the media overlay for 12-18 hours prior to staining with neutral red, as detailed above. Well-isolated plaques were removed from the plaque titration using sterile plastic or glass pasteur pipettes, and washed into 0.5 ml of TC100/10% FCS.

2.2.13. Amplification of virus stocks.

Petri dishes (35mm diameter) were seeded with $5x10^5$ Sf21 cells and incubated at 28°C for 2 hours. The medium was removed and 100µl of the plaque isolate was applied to the centre of the dish. The virus was left to absorb into the cells for 1 hour, 2ml of TC100/10% FCS was added, and the dishes were incubated until the cells were well infected (4-5 days). The

virus-infected cell culture medium from each dish was harvested into a sterile plastic tube (Nunc) and stored at 4°C.

For the production of large stocks, viruses were propagated in suspension culture. Suspension cultures of Sf21 cells were seeded at $1x10^5$ cells/ml and were incubated until they reached a density of $5x10^5$ cells/ml. Virus was added at a multiplicity of infection (m.o.i.) of 0.1 plaque forming units (p.f.u.) /cell, and the culture incubated at 28°C for 4-5 days until 100% of the cells were infected. The culture was harvested and clarified by low speed centrifugation, and stored at 4°C. The virus titre was determined by plaque assay (section 2.2.12).

2.2.14. Extraction of virus DNA from virus-infected insect cells.

A 250-500 ml suspension culture was established ($5x10^5$ cells/ml) and infected with virus at an m.o.i. of 0.1, and incubated at 28°C for 4-5 days. The suspension was harvested and centrifuged at 1200 *g* (30 minutes, 4°C, Beckman JS-HS centrifuge) to pellet the infected-cell debris. The media (containing BVs) was centrifuged overnight in the Beckman L8-70M ultracentrifuge using a Type 19 rotor (54000 *g*, 4°C). The media was removed and the BV pellet soaked overnight in 1ml TE to resuspend the virus particles. The virus suspension was then loaded onto a 30-60% (w/w) sucrose gradient and centrifuged for 1 hour at 75000 *g*, 4°C, using the Beckman L8-70M ultracentrifuge (TH641 or SW41 rotor). The band of budded virus particles was harvested, diluted 1:5 with TE buffer and re-centrifuged under identical conditions to pellet the particles. The supernatant was removed and 1ml TE added. The pellet was allowed to soak overnight at 4°C. Virus DNA was extracted from the pure virus particles following the method described in section 2.2.9.

2.2.15. Extraction of RNA from infected insect cells.

Sf21 ($3x10^6$ in 60mm diameter dishes) or *H. zea* ($1x10^6$ in 60mm diameter dishes) monolayers were inoculated with virus at an m.o.i. of 2 - 5, and maintained at RT for 1 hour with gentle rocking. The inoculum was then

removed, replaced with 3ml of appropriate media and the dishes incubated at 28°C. Monolayers were harvested at various timepoints during the infection, and the cells were pelleted (6000 *g*, 10 minutes, microfuge) and washed twice in PBS. Cell pellets could be stored at -20°C until required. To extract the total cell RNA, the cell pellet was resuspended in 250µl guanidinium isothiocyanate solution (4M guanidinium isothiocyanate, 0.05M Tris-HCl pH 7.6, 0.001M EDTA, 2% SDS, 1% β-mercaptoethanol) to which 250µl of water-saturated phenol and 250µl sodium acetate/EDTA (0.1M sodium acetate pH 5.2, 1mM EDTA) were subsequently added. Samples were vortexed and incubated at 60°C for 10 minutes, with mixing every minute. 250µl chloroform was then added, and the solutions mixed and cooled to RT. The phases were separated by centrifugation (12000 *g*, 10 minutes) and the aqueous phase removed and extracted with an equal volume of phenol/chloroform, followed by chloroform alone. The RNA was precipitated at -20°C overnight with ethanol, pelleted by centrifugation at 12000 *g* for 30 minutes, washed with 75% ethanol and dried. Samples were resuspended in 20µl water and stored at -70°C.

2.3. BACTERIA.

2.3.1. Bacterial strains.

The following bacterial (*E. coli*) strains were used for the amplification of plasmids:

HB101:	F', *thi*-1, *hsd* S20, (r_B-, m_B-), sup E44, *rec*A13, *ara*14, *leu* B6, *pro* A2, *lac* Y1, *gal* K2, *rps* L20 (str^r), *xyl*-5, *mtl*-1, λ-. (Promega)
NM522:	*sup*E, *thi*-1, Δ(*lac-pro*AB), Δ(*hsd*SM-*mcr*B)5, (r_K-, m_K-), [F' *pro*AB, *lac*I^{q}ZDM15]. (Pharmacia)

2.3.2. Culturing and storage of *E. coli* strains.

Liquid cultures of bacteria in Luria broth (LB) medium (15% bactopeptone, 0.5% NaCl, 0.5% yeast extract, pH 7.5) were grown at 37°C with shaking. Strains were maintained on LB-agar plates (LB plus 1.5% bactoagar). NM522 cells were maintained at 4°C on M-9 minimal agar plates (Sambrook *et al.*, 1989). For long term storage, freshly grown cultures of *E. coli* were resuspended in LB plus 15% glycerol, and were stored at -70°C.

2.3.3. Preparation and transformation of competent *E. coli*.

a.) Preparation of competent *E. coli* cells.

A single colony was picked from a fresh plate of *E. coli* cells, and used to inoculate 10ml of LB. The culture was incubated for 16 hours at 37°C with shaking. A volume (2ml) of the overnight culture was used to inoculate 100ml of fresh LB and the cells were grown at 37°C with shaking until they reached a cell density corresponding to a spectrophotometer reading (OD_{650}) of 0.4 to 0.5. Cultures were chilled on ice for 30 minutes, and the cells were pelleted by centrifugation in a pre-chilled rotor at 4000 *g* for 15 minutes (Beckman GS-15R centrifuge). The supernatant was removed and the cells were resuspended in ice-cold 50mM $CaCl_2$ and chilled on ice for 1 hour. The cells were then re-pelleted by centrifugation at 4000 *g* for 30 minutes (Beckman GS-15R centrifuge). The supernatant was removed and the pellet resuspended to a final volume of 5ml in ice-cold 50mM $CaCl_2$, 15% glycerol. The cells were dispensed into 200µl aliquots, and stored at -70°C.

b.) Transformation of competent *E. coli*.

Competent cells were thawed on ice and 100µl of competent cells was mixed with one half of a ligation reaction (10µl), or 0.01ng of plasmid DNA. The mixture was cooled on ice for 10 minutes prior to heat shock for 2 minutes at 42°C. Luria broth (0.5ml) was added to the mixture, and the cells were incubated at 37°C for 30 minutes, to permit expression of the

plasmid drug resistance gene. One tenth and nine tenths of the cells were spread onto separate plates of LB-agar containing ampicillin (100µg/ml) (Sigma). For blue/white colony screening, 100mM IPTG (Gibco) (final concentration 0.1mM) and 2% X-gal (Gibco) in dimethyl formamide (final concentration 0.002%) were also added to the LB agar. The plates were incubated at 37°C overnight.

2.3.4. Colony hybridisation.

The method of colony hybridisation allows the selection of clones with specific inserts using α^{32}P-dATP-labelled DNA probes (Grunstein and Hogness, 1975). Bacterial colonies produced after transformation of host cells with ligation reactions (section 2.3.3) were streaked onto nitrocellulose filters positioned on agar plates, and replica plated onto "master" plates for storage at 4°C following incubation overnight at 37°C. Colonies on the nitrocellulose filters were grown for 6 hours at 37°C. Four large Petri dishes (diameter 15cm) were filled with the required solution and a circle of Whatman 3MM filter paper was floated on the solution. The nitrocellulose filters were placed colony-side uppermost onto the soaked Whatman paper in the first dish (0.2M NaOH) for 8 minutes to lyse the cells. The filters were then transferred to two dishes of 0.5M Tris-HCl pH 7.4 for 2 minutes each to neutralise the NaOH. Finally, the filters were placed on the fourth dish, containing 0.5M Tris-HCl pH 7.4, 1.5M NaCl for 4 minutes. The nitrocellulose filter was then air-dried at RT for 30 minutes prior to baking at 80°C for 2 hours.

Hybridisations were carried out as described in section 2.4.12. Positive clones detected by autoradiography were picked from the master plate and plasmid DNA was extracted for further analysis.

2.3.5. Purification of plasmid DNA.

a.) Small scale purification (miniprep).

Luria broth (2.5ml) containing ampicillin (100µg/ml) was inoculated with a single bacterial colony and incubated for 6-15 hours at 37°C with shaking. Cells were pelleted (6000 *g*, 5 minutes) and resuspended in 100µl glucose solution (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA). Alkaline lysis buffer (0.2M NaOH, 1% SDS, freshly made) was added (200µl), the reaction gently mixed, and the mixtures incubated on ice for 5 minutes. Potassium acetate solution (made by the addition of 11.5ml glacial acetic acid and 28.5ml water to 60ml 5M potassium acetate) was then added, and the reaction mixed gently and incubated on ice for 10 minutes. The cellular debris was pelleted by centrifugation (10000 *g*, 10 minutes, microfuge), and the supernatant fractions removed to a fresh eppendorf tube. Ribonuclease A (RNaseA) was added (6µl of a 10mg/ml stock, heat treated) and the mixture incubated at 37°C for 30 minutes. The preparation was then extracted with phenol/chloroform and the plasmid DNA was precipitated with ethanol (-70°C, 30 minutes). The DNA was pelleted by centrifugation at 12000 *g* for 15 minutes, washed twice with 70% ethanol, dried and resuspended in TE buffer (20µl). The DNA was stored at -20°C. RNaseA (final concentration 0.2mg/ml) was included in restriction enzyme digestions using plasmid DNA prepared by this method, to remove contaminating RNA.

b.) Medium scale purification (Qiagen prep).

Luria broth (30-50ml) containing ampicillin (100µg/ml) was inoculated with a single transformed bacterial colony and incubated for 15-18 hours at 37°C with shaking. Cells were pelleted (6000 *g*, 20 minutes, Beckman GS-15R centrifuge) and the pellet resuspended in 4ml P1 buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 100µg/ml RNaseA). Buffer P2 (200mM NaOH, 1% SDS) (4ml) was added and the solution mixed gently and incubated at RT for 5 minutes. Buffer P3 (3M potassium acetate pH 5.5) (4ml) was then

added, the solution mixed and incubated on ice for 15 minutes. The tubes were centrifuged at 12000 *g*, 4°C, for 30 minutes (JA-20 rotor, Beckman JS-HS centrifuge) to remove the cell debris. The supernatant was removed to a fresh plastic tube and the centrifugation repeated. A Qiagen tip-100 was equilibrated by applying 4ml QBT buffer (750mM NaCl, 50mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100) and the column allowed to empty by gravity flow. Subsequently, the cell supernatant was applied to the Qiagen column and the DNA allowed to enter the resin by gravity flow. The DNA/resin complex was washed twice with 10ml QC buffer (1M NaCl, 50mM MOPS pH 7.0, 15% ethanol) and the DNA eluted from the Qiagen column with 5ml QF buffer (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% ethanol). The DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 17000 *g*, 4°C, for 30 minutes (JA-20 rotor, Beckman JS-HS centrifuge). The DNA pellet was washed twice with 70% ethanol, dried, resuspended in TE (200µl) and phenol/chloroform treated to remove protein debris. The aqueous phase was removed to a fresh Eppendorf tube, and the DNA precipitated with ethanol, washed in 75% ethanol, dried and resuspended in 100µl TE. DNA was stored at -20°C.

c.) Large scale purification (maxiprep).

A starter culture of bacteria was produced by inoculation of 2ml of LB containing ampicillin (100µg/ml) with a colony of transformed bacteria. The culture was incubated overnight at 37°C with shaking. A 1 litre flask containing 250ml LB (plus 100µg/ml ampicillin) was inoculated with 1ml of the starter culture and incubated overnight at 37°C with shaking. The bacterial cells were pelleted (3000 *g*, 30 minutes, 4°C), and resuspended in 7.5ml of lysis buffer (50mM Tris-HCl pH 8.0, 25% sucrose). The suspension was placed on ice, 1.5ml lysozyme (10mg/ml) added, and maintained on ice for 10 minutes. Ribonuclease A (125µl of a 10mg/ml stock solution) and 3ml Tris/EDTA solution (0.25M EDTA, 0.25M Tris-HCl pH 8.0) were added and the solution mixed and left on ice for a further 5 minutes. Triton buffer (2% (v/v) Triton X-100, 50mM Tris-HCl pH 8.0, 10mM EDTA) was added (12.5ml), the solution gently mixed and the tubes left on ice for 15 minutes. The cellular debris was pelleted (40000 *g*, 4°C, 30 minutes, Beckman L8-

70M ultracentrifuge) and the supernatant extracted with phenol/chloroform in the presence of 1% SDS. The DNA was precipitated with ethanol, pelleted (12000 *g*, 4°C, 20 minutes, Beckman GS-15R centrifuge) and resuspended in 7ml TE containing 8g of caesium chloride and ethidium bromide (final concentration of 63µg/ml). The mixture was loaded into a Beckman "Quickseal" ultracentrifuge tube, filled to the top with liquid paraffin, and heat sealed. Tubes were centrifuged using a Ti50 or Ti70 rotor in the Beckman L8-70M ultracentrifuge at 100000 *g*, 20°C for 18-24 hours.

Plasmid DNA was harvested from the gradient using a 19 gauge needle. The ethidium bromide was extracted with butanol equilibrated in TE, and the DNA/caesium chloride mixture diluted 1/3 with TE prior to the precipitation of the DNA with ethanol. The DNA was pelleted (12000 *g*, 20 minutes, microfuge), resuspended in TE (500µl) and extracted with phenol/chloroform in the presence of 1% SDS. Ethanol precipitation of the DNA was performed, followed by two washes in 75% ethanol. The DNA was then dried and resuspended in TE (200-500µl).

The DNA concentration was estimated by measuring the absorbance at 260nm in a UV spectrophotometer, and agarose gel electrophoresis was used to confirm the concentration and the restriction enzyme digestion profile of the purified plasmid. DNA was stored at -20°C.

2.4. DNA AND RNA MANIPULATIONS.

Autoclaved MilliQ water was employed in all reactions requiring water.

2.4.1. Restriction endonuclease digestion.

For analysis of DNA by restriction fragment length polymorphism (RFLP), up to 1µg DNA was digested using 5-10 units (U) of enzyme in the appropriate buffer (as recommended and supplied by the manufacturer). Incubations were carried out for 1-6 hours at 37°C. For preparative digests, the amount of DNA and enzyme was increased as required. Analytical

digests of baculovirus genomic DNA were performed using 1-2μg DNA and 5 units of enzyme in an appropriate buffer, and were incubated for 3 hours at 37°C.

Multiple restriction enzyme digests were carried out in a buffer compatible for the enzymes used (as detailed in the manufacturer's instructions). If the buffer requirements of the enzymes were incompatible, digestions were performed sequentially with phenol/chloroform extraction and ethanol precipitation between each digestion.

2.4.2. Dephosphorylation of vector DNA.

Following restriction digestion of vector DNA, CIP was added directly to the reaction mix (>1 U/μg DNA) and the incubation continued for a further 15 minutes at 37°C. An aliquot of the reaction was removed prior to the addition of CIP, for use as a control. Enzymes were inactivated by the addition of EDTA to a final concentration of 25mM, and SDS to 0.1%, followed by subsequent phenol/chloroform extraction. The DNA was then concentrated by ethanol precipitation. The dephosphorylated vector was resuspended in TE (10-50μl), visualised by agarose gel electrophoresis, and the concentration of the prepared vector DNA assessed.

2.4.3. Phosphorylation of DNA.

T4 polynucleotide kinase (PNK) (New England Biolabs) was employed to phosphorylate the 5' ends of DNA molecules. Up to 10μg of DNA were phosphorylated in a total reaction volume of 100μl, containing 100mM Tris-HCl (pH 7.6), 20mM $MgCl_2$, 5mM dithiothreitol (DTT), 0.2mM EDTA and 0.2mM spermidine. A final concentration of 1U/μl reaction of PNK and 1mM ATP was added to the reaction. Reactions were incubated at 37°C for 1 hour, and terminated by incubation at 65°C for 10 minutes.

2.4.4. Ligation of DNA.

Ligations of DNA fragments into vector DNA (50-100ng) were performed using a range of concentrations of insert fragment (50 to 500ng/µl), including vector DNA:insert DNA ratios of 50ng:50ng, 50ng:150ng and 50ng:250ng. Reactions were performed in a total volume of 20µl, containing 50mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 1mM DTT, 1mM ATP and 2.5 units of T4 DNA ligase per reaction. The ligations were incubated either for 3-4 hours at RT, or overnight at 15°C, including appropriate control ligations.

2.4.5. In-filling of 3' recessed ends.

Recessed 3' ends of double-stranded DNA, generated by restriction enzyme digestion, were repaired to produce blunt ends using the large Klenow fragment of *E. coli* DNA polymerase I. Following digestion with the appropriate restriction enzyme, dNTPs (final concentration of 0.25mM each for dATP, dCTP, dGTP and dTTP) and Klenow fragment (1 unit/µg DNA) were added. After incubation at RT for 10 minutes, reactions were terminated either by heating to 65°C for 10 minutes or by extraction with phenol/chloroform (1:1), followed by precipitation of the DNA with ethanol.

2.4.6. Primer extension analysis.

The oligonucleotide to be used as a primer was end-labelled as described in section 2.4.11, and 5ng was mixed with 5µg of the total RNA extracted from infected cells in a 10µl annealing reaction, comprising 250mM KCl, 10mM Tris-HCl pH 8.3. The mixtures were heated for 3 minutes at 80°C, then annealed at 50°C for 45 minutes.

Primer extension reactions employed 2µl of the annealing reactions in a 6µl reaction volume comprising 12mM Tris-HCl pH 8.3, 8mM $MgCl_2$, 4mM DTT, 0.2mM dATP, 0.2mM dCTP, 0.2mM dTTP, 0.4mM dGTP and 10 units of AMV reverse transcriptase (NBL Gene Sciences Ltd). The reactions were incubated at 50°C for 45 minutes and were terminated by the addition of

2µl formamide. Reactions were analysed on a 6% polyacrylamide sequencing gel with an appropriate sequencing ladder, as described in section 2.4.13.

2.4.7. Agarose gel electrophoresis.

Horizontal agarose gels were routinely used for the analysis of DNA concentration and RFLP profiles using apparatus supplied by Bioline and Northumbria Biologicals Ltd. High gelling temperature agarose concentrations (UltraPure BRL) of between 0.6% to 1.3% were employed, depending upon the sizes of the DNA fragments to be analysed. Submerged gel electrophoresis was performed in 1x TBE buffer (0.089M Tris, 0.002M EDTA, 0.08M boric acid) containing 0.5µg/ml ethidium bromide. Prior to electrophoresis, samples were mixed with 1:4 volumes of 5x loading buffer (40% sucrose, 5x TBE, 2.5% SDS, 0.1% bromophenol blue dye).

Electrophoresis separations were performed at 20-100 volts (V) for 1 to 18 hours as required. Following electrophoresis, DNA was visualised using an Electronic Instant Photo Camera (UltraViolet Products, Cambridge). Photographs for publication were taken using a Fotodyne UV transilluminator, a Polaroid MP4 camera, and positive/negative film (Polaroid 55, 9x12 cm).

2.4.8. Isolation of DNA fragments from agarose gels.

DNA fragments were separated in 0.7-1.0% low gelling temperature (LGT) agarose gels containing ethidium bromide (0.5µg/ml), using electrophoresis in TBE buffer at 50-70 volts (V), with the apparatus on ice. The DNA was visualised under UV light and the required fragments excised from the gel.

a.) Gene clean method.

This method was employed for the isolation of DNA fragments of >500 bp in size. The DNA was purified from the gel using a "Gene Clean" method (GeneClean II Kit: supplier Bio 101 Inc.), in the following manner:

1.5ml 6M NaI solution was added to the gel slice with 250µl TBE modifier™. The gel/solution mixture was incubated at 55°C for 5 minutes to dissolve the agarose and release the DNA into suspension. GLASSMILK® suspension (silica matrix in water) (10-15µl) was added and a 5 minute incubation then followed, with gentle shaking, to allow the DNA to complex with the GLASSMILK® beads. This complex was pelleted using a short centrifugation in a high speed microfuge. The supernatant was removed and discarded, and the pellet washed 3 times with a New Wash solution (proprietary mixture of NaCl, Tris, EDTA and EtOH). The DNA was eluted into a 20-50µl volume of TE buffer by resuspending the beads in TE and incubating for 5-30 minutes at 50°C.

b.) phenol method.

For small DNA fragments (<500 bp), phenol extraction was used to isolate the DNA, as described in Possee and Kelly (1988). The gel slice was melted at 65°C, diluted to 0.2% agarose in water at 37°C and Tris-HCl pH 8.0 was added to a final concentration of 100mM. An equal volume of water-saturated phenol was added and the mixture was shaken at regular intervals during incubation at 37°C for 30 minutes. The organic and aqueous phases were separated by centrifugation (12000 *g*, 10 minutes) and the aqueous phase was re-extracted with phenol/chloroform. The DNA was precipitated with ethanol, pelleted, washed twice with 75% ethanol, dried and resuspended in TE (10-50µl).

2.4.9. Preparation of synthetic oligonucleotides.

Oligonucleotides were synthesised in the Department of Organic Chemistry, University of Oxford, using an Applied Biosystems (ABI) 380B DNA synthesiser. The oligonucleotides were deprotected by heating at 55°C for 16-18 hours. After cooling to RT, 500µl aliquots were vacuum-dried and resuspended in 200µl dH_2O. Oligonucleotide concentrations were estimated by UV spectrophotometry, measuring their absorbance at 260nm. Oligonucleotides to be used as primers in polymerase chain reaction (PCR)

experiments were diluted to a 50μm stock solution in water. Oligonucleotide pairs to be used as linkers were phosphorylated with PNK (section 2.4.3) and then annealed. Equal volumes of each oligonucleotide, containing approximately 100ng of DNA were mixed and heated to 70°C for 10 minutes. The mixture was cooled to RT and used directly in ligation reactions.

2.4.10. Polymerase chain reaction (PCR).

Reactions were performed using a RoboCycler Gradient 40 PCR machine (Stratagene). The reaction mixture consisted of 0.5μg each of the forward and reverse primers, 10ng of DNA, 2μl dNTPs (2.5mM), 2.5 units of *Taq* (Promega), *Tbr* (NBL Gene Science Ltd) or *Pfu* (Stratagene) DNA polymerase in the supplied buffer, in a total volume of 20μl. The reaction mix was overlaid with approximately 20μl of liquid paraffin to prevent evaporation. All PCR amplifications included negative controls consisting of the amplification cocktail with water replacing the template DNA.

Reactions were carried out using the following step program:

10 cycles:	
Denature	95°C 2 minutes
Anneal	50°C 2 minutes
Polymerase	72°C 1 minute
15 cycles:	
Denature	95°C 2 minutes
Anneal	65°C 2 minutes
Polymerase	72°C 1 minute
1 cycle:	
Denature	95°C 2 minutes
Anneal	65°C 2 minutes
Polymerase	72°C 10 minutes

One tenth of each reaction was then analysed by agarose gel electrophoresis (section 2.4.7), to identify a successful reaction. These reactions were extracted with phenol/chloroform and the DNA precipitated with ethanol and resuspended in water. The PCR products were then digested with appropriate restriction enzymes (section 2.4.1) and purified from LGT agarose gels (section 2.4.8) prior to their incorporation in ligation reactions (section 2.4.4). Alternatively, PCR products were labelled by random priming (section 2.4.11) and used in Southern hybridisation reactions (section 2.4.12).

2.4.11. Preparation of radiolabelled DNA probes.

a.) Random priming.

Probes were labelled to high specific activity (>10^9 cpm/μg DNA) by random priming (Feinberg and Vogelstein, 1983), following the protocol of the Prime-a-Gene Labelling System (Promega). Briefly, DNA (25ng) was incubated for 1 hour at RT in a final volume of 50μl with 10μl 5x Labelling Buffer (250mM Tris-HCl pH 8.0, 25mM $MgCl_2$, 10mM DTT, 1M HEPES pH 6.6 and 26 units random hexadeoxynucleotides), 1μl each of dCTP, dGTP and dTTP (1.5mM), 2.5μl α-^{32}P dATP (10μCi/μl) and 5 units of DNA polymerase I (Klenow). The radiolabelled DNA was separated from the unincorporated nucleotides by Sephadex G-50 column chromatography. Labelled DNA fractions were pooled and denatured at 90°C prior to adding to the hybridisation mixture.

b.) End-labelling of DNA.

End-labelling was carried out using PNK (section 2.4.3). The ATP used in the reaction was replaced with 20μCi γ-^{32}P ATP. Radioactive products were separated from unincorporated nucleotides by Sephadex G50 column chromatography.

2.4.12. DNA transfer and hybridisation.

DNA samples digested with appropriate restriction enzymes were size fractionated by electrophoresis through 0.7-1.0% agarose gels. The DNA was denatured by soaking the gel in 1.5M NaCl, 0.5M NaOH at RT for 30 minutes, and subsequent neutralisation in 0.5M Tris-HCl, pH 7.5, 1.5M NaCl at RT for 30 minutes. The DNA was then transferred to a nitrocellulose filter (Hybond, Amersham) using 20x SSC (3.0M NaCl, 0.3M Tri-sodium citrate) (Southern, 1975). Transfer proceeded for 12-18 hours, after which the filter was baked at 80°C for 2 hours.

Prior to hybridisation, filters were pre-treated by boiling in 1mM EDTA (pH 8.0) for 5 minutes. Hybridisations were carried out in sealed plastic bags submerged in a water bath. Filters were hybridised with denatured, α-^{32}P dATP labelled DNA probes in 50% formamide, 6x SSC, 0.1% SDS, 50mM phosphate buffer (pH 6.5), 5x Denhardt's solution with 100µg/ml denatured sonicated salmon sperm DNA at 37-42°C for 12-18 hours. After hybridisation the unbound probe was removed by 3 washes in 6x or 2x SSC (according to the required stringency), 0.1% SDS at 37-60°C (according to the required stringency) for 30 minutes with shaking. The filters were heat-sealed in a plastic bag and exposed to autoradiograph (Kodak X-Omat film) with enhancing screens (Laskey, 1980) at -70°C for 12-72 hours.

2.4.13. DNA sequencing.

a.) Sequencing reactions.

DNA sequencing reactions were carried out using the chain termination method (Sanger *et al.*, 1977). DNA templates were sequenced using modified T7 DNA polymerase (Sequenase Version 2.0, US Biochemicals; Tabor and Richardson, 1987) according to the protocols recommended by the supplier. The M13 primer (5' GTAAAACGACGGCCAGT) was used to sequence the end regions of cloned DNA fragments. Oligonucleotide primers were used to obtain the internal sequences of fragments.

b.) Polyacrylamide gel electrophoresis.

Sequencing and primer extension reactions were analysed using denaturing polyacrylamide gel electrophoresis. Gels of 6% polyacrylamide were prepared by mixing 50ml Ultrapure Sequagel-6 (National Diagnostics) to 12ml Ultrapure Sequagel complete buffer reagent (National Diagnostics). Ammonium persulphate (APS) (500µl of a 10% stock solution) was added to the mixture before pouring to promote polymerisation.

Reaction samples were heated to 80°C in a heating block for 3 minutes and placed on ice prior to loading onto the gel. Electrophoresis was performed in 1x TBE at constant power (40-45W) for 2-6 hours. Following electrophoresis the gels were dried onto Whatman 3MM paper and exposed to Kodak X-Omat S film at RT for 6-72 hours.

2.4.14. Sequence data analysis.

Sequence assembly and analysis was performed using the University of Wisconsin GCG package (versions 7.3 and 8) using the GelEnter, GelMerge and GelAssemble programs (Devereux *et al.*, 1984). Searches for sequence identities were made using the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990). For amino acid sequence analysis the PIR and GenPEP databanks were searched, while for nucleic acid sequence searches the GenBank/EMBL databases were used. The pairwise alignment program GAP was employed to produce an optimal alignment between amino acid sequences using the global algorithm of Needleman and Wunsch (1970). Multiple alignments were created in the PileUp program and graphically displayed using Prettyplot. Phylogenetic trees were produced using ClustalW (Thompson *et al.*, 1994) and displayed using Phylip.

Chapter Three

Analysis of the HzSNPV genome to detect an inhibitor of apoptosis (*iap*) homologue.

3.1. Introduction.

The initial remit of this project was to identify a potential host range factor in HzSNPV. On reviewing the literature pertinent to baculovirus host range, a directed approach to identify host range genes was chosen. The procedure involved attempting to isolate and characterise genes previously implicated in the control of this phenomenon. Data published by Clem *et al.*, (1991) suggests a host range function for AcMNPV *p35*, and by implication for the *iaps* which have been shown to be functional substitutes for *p35* in baculoviruses lacking *p35* (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). In order to investigate this hypothesis, it was necessary to identify an HzSNPV "inhibitor of apoptosis" gene.

Three strategies to isolate an *iap* in HzSNPV were considered. The first method involved the screening of HzSNPV genomic DNA using hybridisation with a radioactive DNA probe. The second method involved the screening of a plasmid library of HzSNPV cloned fragments using the same approach. The third strategy was the use of a functional assay to identify proteins which have the ability to suppress apoptosis in a biological system. Experiments involving the first two strategies are presented in this Chapter, and those involving a functional assay are presented in Chapter Four.

3.2. Results.

3.2.1. Hybridisation screening of HzSNPV NC-1 genomic DNA to detect inhibitor of apoptosis homologues.

HzSNPV NC-1 DNA was purified from virus particles, and 500 ng aliquots were digested with various restriction enzymes. The digested DNA was analysed by gel electrophoresis and transferred to a nitrocellulose membrane for hybridisation analysis with an AcMNPV *p35*-specific radioactive probe. A sample of pAc*Eco*RI-S (116039 to 117498 bp of the AcMNPV genome), which contains *p35* (Ayres *et al.*, 1994), was included as a positive control. A DNA fragment of the region 116492 to 117389 bp in

the circular genome of AcMNPV, containing the *p35* coding region, was generated by PCR (Figure 1a). This 955 bp DNA fragment was radiolabelled with α^{32}P-dATP using the Prime-a-Gene Labelling System (Promega). The radiolabelled DNA fragment was used to probe the nitrocellulose filter containing digested HzSNPV NC-1 DNA, using hybridisation conditions of 2x SSC and 42°C. Only the positive control, pAc*Eco*RI-S DNA, hybridised to the probe (data not shown).

A similar hybridisation analysis was performed using the AcMNPV *iap1* region as a probe. HzSNPV NC-1 DNA (500 ng) was digested with various restriction enzymes (as above), analysed by gel electrophoresis, and transferred to a nitrocellulose membrane. A sample of pAc*Sal*I-P (21827 to 23558 bp of the AcMNPV genome), containing *iap1*, was included as a positive control for the hybridisation. A 879 bp copy of the AcMNPV *iap1* gene (22600 to 23458 bp) was generated using PCR (Figure 1b). The reaction product was radiolabelled with α^{32}P-dATP, as described above, to derive a specific gene probe for *iap1*. The radiolabelled DNA fragment was used in a hybridisation experiment to probe digested HzSNPV NC-1 DNA. Only the positive control, pAc*Sal*I-P DNA, hybridised to the probe (data not shown).

The HzSNPV NC-1 DNA did not demonstrate hybridisation to AcMNPV *p35* or *iap1* DNA fragments in these analyses. The 500 ng of digested viral DNA produced a very weak pattern of ethidium bromide-stained restriction fragments; the DNA may have been too dilute to enable a hybridisation signal to be identified on the autoradiograph. Alternatively, the hybridisation conditions (2x SSC, 42°C) may have been too stringent to enable the detection of weakly homologous sequences. Therefore, in further experiments, a genomic library was used to increase the concentration of the DNA in the analyses, and the stringency of the hybridisation conditions was reduced.

3.2.2. Construction of an HzSNPV NC-1 genomic library.

Fragments from restriction enzyme digests (*Eco*RI or *Hind*III) of HzSNPV NC-1 DNA were ligated into a pUC118 vector using standard protocols (Sambrook *et al.*, 1989), to produce a genomic library. A proportion of the viral fragments were not isolated, due to their large size (>8 kbp) which made it difficult to insert them into the plasmid vector.

In order to identify the cloned *Eco*RI and *Hind*III fragments relative to the published restriction map of HzSNPV Elcar (Knell and Summers, 1984), comparative restriction enzyme profiling of each cloned fragment, using the enzymes *Sac*I, *Xho*I and *Kpn*I, was performed. The plasmid clones were positioned on the genetic map of HzSNPV strain Elcar (Knell and Summers, 1984), as shown in Figure 3.2, in order to assess the genome coverage available in the library.

The region between map units 71.3 and 92.7 of the HzSNPV genome was found not to be represented by the *Hind*III and *Eco*RI cloned fragments (Figure 3.2), due to the large sizes of the *Eco*RI-A (11.8 kbp), *Hind*III-A (19.1 kbp) and *Hind*III-D (9.5 kbp) fragments, which are located in this region. This region of the HzSNPV genome does not contain many restriction enzyme sites; in the published restriction map it is covered by *Bam*HI-A/B, *Sac*I-D/F, *Xho*I-B and *Kpn*I-C (Knell and Summers, 1984). These viral fragments range in size from 8.9 kbp (*Sac*I-F) to 34.2 kbp (*Bam*HI-A). To ensure that cloned fragments were available in this region of the HzSNPV map, the *Sal*I enzyme was used to digest the HzSNPV NC-1 DNA, and the resulting fragments were cloned into a pUC118 vector at the unique *Sal*I site. The *Sal*I fragments generated by digestion of HzSNPV NC-1 DNA were less than 8 kbp in size. Even the largest fragments could therefore be satisfactorily cloned into the plasmid vector. In this way, maximum representation of the genome of HzSNPV was obtained. The sizes of the purified *Sal*I clones produced are shown in Table 3.1.

3.2.3. Hybridisation screening of an HzSNPV NC-1 plasmid library to detect *iap* homologues.

The HzSNPV genomic library clones were digested with the appropriate restriction enzyme and transferred to a nitrocellulose membrane following gel electrophoresis. The *p35*-specific PCR product was radiolabelled and employed in several hybridisation experiments using stringent conditions (hybridising in 2x SSC and at temperatures of 65°C). However, only positive controls (pAc*Eco*RI-S, containing the AcMNPV *p35* gene) produced a hybridisation signal (data not shown). The various plasmid library clones (*Eco*RI, *Hind*III and *Sal*I) were all negative for *p35* homology. Hybridisation experiments performed at lower salt stringencies of 6x SSC and lower temperature (37°C) gave similar results (data not shown), suggesting that HzSNPV does not contain a gene homologous to the *p35* gene of AcMNPV.

Genomic libraries were also screened using hybridisation techniques for sequences with homology to AcMNPV *iap1*. The *Eco*RI and *Hind*III genomic clones did not demonstrate any hybridisation to AcMNPV *iap1*. However, a positive clone from the *Sal*I library, named pHzSNPV*Sal*I-3, demonstrated hybridisation to AcMNPV *iap1*, using hybridisation conditions of 6x SSC and 37°C (Figure 3.3). This cloned fragment was unique in its ability to hybridise to AcMNPV *iap1*.

DNA of pHzSNPV*Sal*I-3 was digested with various restriction enzymes to select those suitable for further analysis of the DNA fragment apparently containing an *iap* homologue. Restriction enzyme digestion with *Bgl*I, *Cla*I and *Pvu*II, and subsequent hybridisation to the radiolabelled AcMNPV *iap1* probe, was used to elucidate the exact region of *iap* homology in pHzSNPV*Sal*I-3. The two fragments produced by *Cla*I digestion of pHzSNPV*Sal*I-3 fragment (approximately 1.4 kbp and 7.6 kbp) both demonstrated hybridisation to AcMNPV *iap1* (Figure 3.4), indicating that at least a part of the *iap*-hybridising region of pHzSNPV*Sal*I-3 was present in the 1.4 kbp fragment. The 1.4 kbp *Cla*I fragment was subsequently isolated from pHzSNPV*Sal*I-3 and ligated into the unique *Cla*I site of a pBluescript

vector. The 1.4 kbp *Cla*I fragment represents a minimal region of the HzSNPV NC-1 genome which exhibited hybridisation to AcMNPV *iap1*.

3.2.4. DNA sequencing analysis of pHzSNPV*Sal*I-3.

DNA sequence analysis was performed on the 1.4 kbp *Cla*I fragment. The data obtained were used in a Blast search of the viral sequences in the Genbank and EMBL databases. This search showed that a translation frame of the DNA sequence of the 1.4 kbp *Cla*I fragment demonstrated homology to the baculovirus *iap*s. The *Cla*I fragment did not contain the 5' and promoter region of the putative ORF, consistent with the low levels of hybridisation to the larger fragment produced by *Cla*I digestion of pHzSNPV*Sal*I-3 which were observed (Figure 3.4). The 5' region of HzSNPV *iap* was sequenced using the larger *Sal*I fragment as a template. In order to examine the genes flanking *iap*, the DNA sequence of the entire HzSNPV *Sal*I-3 cloned fragment was determined, and the data are presented in Figure 3.5.

The putative HzSNPV *iap* encodes a protein composed of 268 amino acids. The DNA sequence preceding the *iap* ORF contains a TATA box, a CAGT early transcription initiation motif and a TAAG late transcription initiation motif (Figure 3.5). A polyadenylation signal was identified 26 nucleotides downstream of the TAA stop codon.

The HzSNPV *Sal*I-3 fragment, containing the *iap*-homologous sequence, was radiolabelled and used to probe restriction enzyme digests of HzSNPV strains Elcar and NC-1 (Figure 3.6), in order to examine whether the location of this restriction fragment differed in the genomes of these virus isolates. The *Sal*I-3 fragment (approximately 6 kbp) hybridised to the viral fragments *Hind*III-A/D, *Bam*HI-A/B and *Eco*RI-D/L in both Elcar and NC-1 (Figure 3.6), indicating that this gene locus was highly conserved between the two HzSNPV isolates. A 1.4 kbp *Cla*I fragment also hybridised to the probe. The positions of the hybridising fragments are identical for HzSNPV strain Elcar and NC-1. However, some differences in the *Bam*HI and *Eco*RV restriction profiles between the two HzSNPV strains were observed.

In Figure 3.6, the *Sal*I-3 fragment was mapped to the *Hind*III-A and *Hind*III-D fragments of HzSNPV NC-1 and Elcar. The fragment can thus be positioned on the genetic map of HzSNPV. Four ORFs were identified in the sequence analysis of the *Sal*I-3 fragment (Figure 3.5); an *iap* homologue (*iap*), a gene with similarity to ORF 2 of AcMNPV (orf 2), a superoxide dismutase gene homologue (sod) and a gene with similarity to the ORFs 106 and 107 of AcMNPV (orf 106/107). The genetic organisation of the sequenced region of the *Sal*I-3 clone is presented in Figure 3.7. The *iap* gene is encoded on the opposite DNA strand to the polyhedrin gene of HzSNPV (*Eco*RI-I fragment).

3.2.5. Comparison of HzSNPV *iap* with other *iap* homologues.

To date, five baculovirus *iap*s have been described; from CpGV, OpMNPV, BmNPV and AcMNPV (*iap1* and *iap2*). The human gene, *naip*, which encodes neuronal apoptosis inhibitory protein (NAIP), has also been implicated as a member of the *iap* group, since it demonstrates homology to the baculovirus *iap*s (Roy *et al.*, 1995). In addition, two putative *iap*s from *Drosophila*, one murine gene and a further three human genes contain motifs which implicate them to be homologues of baculovirus *iap* (Hay *et al.*, 1995; Rothe *et al.*, 1995; Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996).

An alignment of the baculovirus IAPs was constructed using the PILEUP programme in GCG. PILEUP creates a multiple sequence alignment using a simplification of the progressive alignment method of Feng and Doolittle (1987). The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences to produce a cluster. This cluster can then be aligned to the next most related sequence and so on. The sequences are first clustered to produce a dendrogram, which directs the order of the subsequent pairwise alignments. The result of this analysis is presented in Figure 3.8. Two motifs are characteristic of IAP proteins; the RING finger and repeat region (baculovirus IAP repeat or BIR). These motifs are maintained in HzSNPV IAP (Figure 3.8). The BIR motif consists of repeats of the sequence $GX_2YX_4DX_3CX_2CX_6WX_9HX_{6\text{-}10}C$, which are present in

the N-terminal and central portion of baculovirus IAPs (Birnbaum *et al.*, 1994).

A second alignment was generated incorporating the amino acid sequences of the baculovirus, human, mouse and *Drosophila* IAPs. The result of this analysis is presented in Figure 3.9. The human, mouse and *Drosophila* IAPs contain three repeats of the BIR sequence, compared to two repeats in the baculovirus IAPs (see Figure 3.9).

The RING finger motif (Lovering *et al.*, 1993) is found in approximately 30 other proteins (mainly involved in the control of transcription), however, the IAP motifs are C-terminal rather than N-terminal as in the other proteins of this group. The motif has also been identified in a number of cellular proteins which may be involved in the control of apoptosis, such as c-*pml* (de The *et al.*, 1991), TRAF2 (Rothe *et al.*, 1994) and TRAF3 (Hu *et al.*, 1994; Cheng *et al*, 1995). An alignment of the RING finger motifs of the IAPs and the cellular TRAF proteins is presented in Figure 3.10. The cysteine and histidine residues characteristic of this motif are conserved in all the sequences. The NAIP protein does not contain the RING finger motif and is not included in Figure 3.10.

The percentage amino acid identity and similarity between the members of the baculovirus IAP protein family, calculated using the GAP algorithm, are presented in Table 3.2. It can be seen that HzSNPV IAP is most closely related to OpMNPV IAP (60.2% similarity, 41.7% identity). However, CpGV IAP and OpMNPV IAP are the most related pair (73.7% similarity, 57.6% identity). The least related protein pair are OpMPNV IAP and AcMNPV IAP1 (49.8% similarity and 28.6% identity).

The significance of the similarity between the inhibitor of apoptosis protein sequences was determined using Monte Carlo analysis (Barton, 1990) and is shown in Table 3.3. The scores indicate that the baculovirus IAP family are significantly similar in the protein alignments, but that P35 is unrelated to this group of proteins despite its recorded anti-apoptotic activity.

The IAP proteins were introduced into ClustalW for neighbour-joining phylogenetic tree analysis. The trees were displayed using the program Phylip. Figure 3.11a and 3.11b show the radial dendrograms created from this program. Diagram a has gaps included but does not include corrections for multiple substitutions. Diagram b has gaps included and does include corrections for multiple substitutions. Figure 3.11c shows a bootstrapped tree (including gaps and corrections for multiple substitutions). In all 3 dendrograms the proteins hIAP2, hIAP1 and mIAP1 are closely grouped. Additionally, the proteins OpIAP, CpIAP and dIAP1 are grouped together. In Figure 3.11a the HzSNPV IAP is associated with the *Drosphila* IAPs, CpIAP and OpIAP. However, in Figures 3.11 b and c the HzSNPV IAP is associated with the AcMNPV IAP proteins. The distance matrices from ClustalW are shown in Table 3.4 (a and b). The matrices indicate the relative distances between protein pairs from the ClustalW calculation. Low values indicate that the proteins are closely related in the analysis. The matrix can be used to verify relationships observed in the dendrograms. If closely grouped proteins (e.g., mIAP1, hIAP1) have lower pair values in the matrix than any of the other pairs, their close position in the tree diagram is verified. However, pair relationships may be obscured in the dendrogram since it is often simplified by grouping together proteins at pre-determined levels of similarity.

The structural profile of the HzSNPV IAP protein was examined, in comparison to the CpGV IAP protein (Figure 3.12). Both these proteins have similar hydrophobicity/hydrophilicity profiles. The amino acid sequence of HzSNPV IAP was also submitted to Swiss-Model for 3D crystallographic matching (Peitsch, 1995; 1996). No homologous proteins have been crystallographically determined and no suitable matches were therefore reported.

3.2.6. Primer extension analysis of HzSNPV *iap* transcripts.

Primer extension analysis of viral mRNA produced over a time course of infection was performed to determine whether or not the HzSNPV *iap* ORF identified by hybridisation analysis was transcriptionally active. An

oligonucleotide primer (5' GTACAGGCCAATTTGCA), complementary to a sequence 79 nucleotides downstream of the putative translation start site within the mRNA, was end-labelled with γ^{32}P-dATP as described in Chapter Two (section 2.4.11). *Heliothis zea* cell monolayers were infected at an m.o.i. of 2, or mock-infected with medium. Monolayers were harvested at timepoints ranging from 12 to 72 hours p.i., whole cell RNA was purified (section 2.2.15) and the primer extension reactions were performed as detailed in Chapter Two (2.4.6). To enable the precise mapping of the primer extension product, the reaction mixtures were electrophoresed on a 6% polyacrylamide gel with a sequencing ladder produced using the same primer and pHzSNPV*Sal*I-3 template DNA. The resulting autoradiograph is shown in Figure 3.12. No product was detected in the mock-infected (negative control) sample. However, the HzSNPV-infected cell samples indicated that the *iap* of HzSNPV was transcribed at early and late stages of the infection (see Figure 3.13). Transcripts were first detected at 12 hours p.i., activated from the CAGT motif of the promoter; these continued until 48 hours p.i. Late transcripts were activated from the TAAG motif, commencing 24 hours p.i., and continuing until 72 hours p.i.

3.2.7. Alignments of the genes flanking *iap* in the HzSNPV NC-1 genome.

Four ORFs of >150 nucleotides were identified from the sequencing analysis of the HzSNPV NC-1 *Sal*I-3 fragment. These ORFs encoded homologues of *iap*, AcMNPV ORF 2, superoxide dismutase (SOD) and AcMNPV ORFs 106 and 107. Homologous ORFs were identified using the BLAST programme within GCG to search the NIH databases.

Alignments of these genes with their homologues were performed using PILEUP, and are presented in Figures 3.14 (ORF 2), 3.15 (ORF 106) and 3.16 (SOD). Analyses to determine the degree of amino acid similarity and identity between the proteins were performed, and these are presented in Table 3.5 (ORF 2), Table 3.6 (ORF 106) and Table 3.7 (SOD).

The crystallographic structure of several SOD proteins have been determined. The amino acid sequence of HzSNPV SOD was submitted to Swiss-Model (Peitsch, 1995; 1996) and the protein structure was modelled, based on the crystallographic structure of other SODs. The results of this analysis are presented in Figure 3.17.

3.3. Discussion.

Baculoviruses have been isolated from many species of insects, mainly from the order Lepidoptera. HzSNPV NC-1 was isolated from an infected *H. zea* larva, found in a cotton growing area in North Carolina, USA. HzSNPV NC-1 is therefore a regional variant of HzSNPV. The restriction map of HzSNPV NC-1 largely agrees with that previously published for the Elcar strain (Knell and Summers, 1984; Cowan *et al.*, 1994). Some differences are apparent in the *Bam*HI and *Eco*RV restriction enzyme profiles of strain NC-1 compared to strain Elcar. Previous studies with HzSNPV Elcar and an HzSNPV isolate from Brownsville, Texas, indicated that the *Eco*RI and *Hind*III restriction enzyme profiles of these isolates were conserved (McIntosh and Ignoffo, 1986), although some differences in the *Xho*I profile were observed. Similar results were reported by Gettig and McCarthy (1982) with HzSNPV Elcar and a different HzSNPV isolate from the USA. Therefore, we assume that US regional isolates of HzSNPV generally maintain the *Eco*RI and *Hind*III restriction profiles of Elcar. The published Elcar restriction map (Knell and Summers, 1984) can therefore be used as a suitable reference for regional variants of HzSNPV.

A genomic plasmid library of HzSNPV DNA was produced. The *Eco*RI and *Hind*III clones derived represented 79% of the HzSNPV genome (Figure 3.2). In order to obtain cloned DNA fragments of the remainder of the genome, *Sal*I clones were derived. Hybridisation techniques were employed to identify clones with similarity to AcMNPV *p35* and *iap1*. A cloned HzSNPV DNA fragment, which demonstrated hybridisation to an AcMNPV *iap1* probe, was shown to contain an *iap* homologue by sequence analysis. No putative *p35*-like genes were detected.

Homologues of the AcMNPV *p35* gene have not been identified in CpGV and OpMNPV (the entire genome sequence of OpMNPV is now available; Rohrmann *et al.*, 1996). It was not unexpected, therefore, that Southern blots with an AcMNPV *p35* probe failed to identify this gene in HzSNPV, although this may have been due to the lack of representation of *p35*-containing regions in the genomic library. It may be that *p35* is a recent addition to the genomes of AcMNPV and BmNPV. These baculoviruses have been shown to be very closely related in phylogenetic analyses (their *p35* genes share 90% amino acid sequence identity (Kamita *et al.*, 1993)). Following the acquisition of *p35*, its function may have superseded that of *iap*, allowing the AcMNPV *iap* gene to mutate without functional constraints on its structure. This may explain why AcMNPV *iap1* shares less homology to the other baculovirus *iap*s and is non-functional as an apoptosis inhibitor. However, since this gene is maintained as a complete ORF in AcMNPV, it may perform an important function not related to the inhibition of apoptosis in Sf21 cells. P35 is unique to the baculoviruses AcMNPV and BmNPV. The origin of *p35* is undetermined, although it is known that baculoviruses are capable of acquiring DNA from their hosts and associated sources e.g., the chitinase gene of AcMNPV bears strong resemblance to the chitinase A gene of *Serratia marcescens*, a bacterium resident in the insect gut (Hawtin *et al.*, 1995).

A single ORF (804 nt) encoding a putative inhibitor of apoptosis of 268 amino acids was located at map units 76 to 77 (approximately) in the HzSNPV NC-1 genome, in the reverse orientation to the polyhedrin gene. The HzSNPV NC-1 *iap* promoter region contains a TATA box, CAGT and TAAG motifs upstream of 3 potential ATG start sites. Kozak's rules (Kozak, 1986; 1987) which suggest that the utilised start codon is preceded by an A residue at the -3 or -2 position relative to the ATG translation start site, were used to indicate the most probable methionine start codon. Following Kozak's rules, it seems most likely that the second methionine is the true start site since it has an A residue at the -3 position. The TAAG baculovirus consensus late transcription start site detected upstream of the ATG is also present in the OpMPNV *iap* gene (Birnbaum *et al.*, 1994).

Transcription of HzSNPV *iap* was demonstrated by primer extension analysis, using an oligonucleotide primer homologous to a region within the putative mRNA sequence (Figure 3.13). The 5' ends of the mRNAs were mapped to the CAGT and TAAG motifs upstream of the translation start site. These motifs are characteristic of early and late baculovirus promoters (Rohrmann, 1986). The detection of early *iap* transcripts at times later than would be expected for AcMNPV early genes (12 to 48 h.p.i. for *H. zea* cells infected with HzSNPV, compared to 3 to 18 h.p.i. for Sf21 cells infected with AcMNPV) is consistent with the slower growth of HzSNPV compared to AcMNPV (Granados *et al.*, 1981).

The sequence of the protein encoded by HzSNPV *iap* shares 42%, 39% and 31% sequence identity at the amino acid level with OpMNPV IAP, CpGV IAP and AcMNPV IAP1 respectively, when calculated using the pairwise alignment program GAP. This suggests that HzSNPV IAP is more closely related to OpMNPV IAP and CpGV IAP than to AcMNPV IAP1, but not as closely related as OpMNPV IAP and CpGV IAP are to each other (57% identity). A Monte Carlo analysis indicates that the IAP proteins form a discrete family, and that the relationships are considered statistically significant. Although *p35* is a functional apoptotic inhibitor, it does not appear to be significantly related to the *iap*s, and shares no common motifs. AcMNPV IAP2 appears to be connected with this group but the scores from the alignment are more tenuous - in fact human NAIP is as closely related to AcMNPV IAP1 (score = 6.55) as AcMNPV IAP2 is to AcMNPV IAP1 (score = 7.46).

The IAP protein family contain two distinct structural features; RING fingers and BIR motifs. While RING finger domains have been found in several DNA binding proteins, they have not been shown to bind DNA, although they have been implicated in the control of protein-protein binding (Borden *et al.*, 1995). The RING finger structure for c-PML has been solved by NMR methods (Borden *et al.*, 1995). The RING finger motif of c-PML is required for the formation of PML oncogenic domains, which are essential for its role in the suppression of cell growth (Le *et al.*, 1996). The presence of RING finger motifs in several proteins known to be

involved in the apoptotic signal transduction cascade (e.g., TRAF2 and TRAF3) suggests that IAPs may act as mimics of these signal transducers. Human h*iap1* and h*iap2* were originally identified due to their ability to interact with TRAF2. Thus, the IAP proteins are evolutionary conserved cell components, suggesting that they may mediate an ancestral mechanism in the programmed cell death pathway. Given the close structural and functional relationship between viral IAPs and cellular IAPs, it is possible that baculovirus *iap* may have been acquired from its insect host to aid viral pathogenicity, since this gene is encoded by at least one insect species (*Drosophila*).

A gene with homology to AcMNPV ORFs 106 and 107 was detected downstream from the HzSNPV *iap* gene using the BLAST program in GCG to detect alignments at the amino acid level. This represents a major rearrangement between the viruses since this gene is not positioned near *iap1* in AcMNPV. The AcMNPV ORFs 106 and 107 have not been ascribed a function to date (Ayres *et al.*, 1994). Homologues of ORFs 106/107 were identified in the genomes of BmNPV and OpMNPV and, like the HzSNPV gene, these ORFs are combined to encode a larger ORF. Examination of the AcMNPV sequence data indicates it may be necessary to reconfirm the nucleotide sequence of this gene region, since a series of frameshifts would restore the AcMNPV ORFs 106 and 107 to a single ORF with close homology to the BmNPV gene.

Upstream of the HzSNPV *iap* gene, a gene with similarity to the ORF 2 of AcMNPV (Ayres *et al.*, 1994) was detected. In AcMNPV, this gene encodes a protein of 328 amino acids (Possee *et al.*, 1991), of unknown function. Three similar ORFs were detected at different locations in the genome of BmNPV. These repeat sequences have not been ascribed a function in BmNPV. The HzSNPV ORF 2 contains amino acid insertions, which may represent variably sized loops in the protein.

Upstream of HzSNPV ORF 2, a superoxide dismutase (SOD) homologue was identified. In HzSNPV, this gene encodes a protein of 159 amino acids with strong similarity to the superoxide dismutases of a variety of organisms.

The crystal structure of the *Bos taurus* SOD has been solved (Djinovic *et al.*, 1992). The redox reaction which occurs at the active site is rate-limited by diffusion and is enhanced by electrostatic guidance of the superoxide substrate into the active site channel by a conserved arginine residue (arginine 141 of *Bos taurus* SOD). The active site has been characterised, and is highly conserved between SOD proteins; it is defined by two motifs termed Prosite I and Prosite II. The possible function of this gene product during baculovirus infection is the catalytic conversion of reactive oxygen species produced by the host cell, to limit the damage caused by these metabolites, in order to prolong cell life for the purpose of viral replication.

Studies involving the deletion of AcMNPV *sod* indicate that it is not essential for normal virus replication in cell culture and larvae (Tomalski *et al.*, 1991); the role of *sod* in baculovirus infection is therefore undetermined. It is possible that *sod* has a role in virus replication or survival at extremes of temperature or UV light, and that its effects cannot be readily determined in the laboratory. Mutation of *sod* in *E. coli*, yeast and *Drosophila* results in the production of viable organisms which are sensitive to aerobic conditions or oxygen anion stress. Thus, SOD does not appear to be essential for viability in organisms, but may provide a growth advantage in certain environmental conditions. Transcription mapping of AcMNPV *sod* demonstrated the production of two mRNA transcripts, initiating at two different TAAG motifs which were contained within the promoter of the gene. The HzSNPV *sod* promoter overlaps that of ORF 2, and contains two TAAG and two TATA box motifs. Experiments to demonstrate *in situ sod* activity in AcMNPV-infected cells, by the inhibition of SOD protein activity bands with 5mM H_2O_2, were inconclusive (Tomalski *et al.*, 1991). SOD activity could be confirmed using SOD deficient yeast cells, which are sensitive to oxidative stress induced by 50μM paraquat. The addition of a heterologous *sod* gene to the *sod* yeast mutant enables the cells to tolerate in excess of 1000μM paraquat (Rabizadeh *et al.*, 1995). Thus, further studies on baculovirus SOD function may be warranted.

The data presented in this section reveal the presence of a transcriptionally active *iap* within the HzSNPV NC-1 genome, which encodes a protein

sharing significant homology with the IAPs of other baculoviruses. It follows that since no *p35* gene has been detected in HzSNPV, and in view of the close homology of the IAP of this virus to CpGV and OpMNPV IAPs, it might be expected that HzSNPV IAP is functionally active as an inhibitor of apoptosis. Experiments involving the complementation of a *p35* deficient mutant of AcMNPV by HzSNPV *iap* are presented in the following chapter of this thesis (Chapter Four). Further investigations into the function of HzSNPV IAP may indicate whether or not it has a role in controlling the narrow host range of HzSNPV.

Figure 3.1.

PCR generation of DNA fragments of AcMNPV *p35* and *iap1* for use as specific DNA probes.

The sequence of the *Eco*RI-S fragment of AcMNPV (116039 to 117498 bp of the genome) is shown (panel a). Primers synthesised complementary to the 5' and 3' regions of *p35* are shown in italics. Restriction enzyme sites were included in the primer sequences (underlined), preceded by a GC-rich anchor. The ATG start codon and TAA stop codon of *p35* are also shown in bold. Amplification of DNA by PCR using these primers and AcMNPV C6 template DNA resulted in the production of a 955 bp DNA fragment. PCR amplifications were carried out using the *Taq* polymerase enzyme (Promega) with an annealing temperature of 65°C.

Panel b shows the sequence of the *Sal*I-P fragment of AcMNPV (21827 to 23558 bp of the genome). Primers synthesised complementary to the 5' and 3' regions of *iap1* are shown in italics. Restriction enzyme sites were included in the primer sequences (underlined). The ATG start codon and TAA stop codon of *iap1* are also shown in bold. PCR amplification using these primers and AcMNPV C6 template DNA resulted in the production of a 879 bp DNA fragment.

Figure 3.1.a.

AcMNPV bp

*Eco*RI

116039 GAATTCGCTG GGCCTGGTGT CAGTACCCTC GCCATTGCGG CGCAAATAAC
116089 GACTCTTGAC GTCTCCGATT TCTTTTTGGC GGCAATAAGC ACTCCAATGC
116139 AAATACAAAA CTTTGTCGCA ACTACTGATG TTTTCGATTT CATTCTGAAA
116189 TTGTTCTAAA GTTTGTAACG CGTTCTTGTT AAAGTAATAG TCCGAGTTTG
116239 TCGACAAGGA ATCGTCGGTG GCGTACACGT AGTAGTTAAT CATCTTGTTG
116289 ATTGATATTT AATTTTGGCG ACGGATTTTT ATATACACGA GCGGAGCGGT
116339 CACGTTCTGT AACATGAGTG ATCGTGTGTG TGTTATCTCT GGCAGCGCGA
116389 TAGTGGTCGC GAAAATTACA CGCGCGTCGT AACGTGAACG TTTATATTAT

*Bam*HI
GCGAGGA

116439 AAATATTCAA CGTTGCTTGT ATTAAGTGAG CATTTGAGCT TTACCATTGC

primer 1 ⇒
TCCATGTGTG TAATTTTTCC GGT

116489 AAA**ATG**TGTG TAATTTTTCC GGTAGAAATC GACGTGTCCC AGACGATTAT

116539 TCGAGATTGT CAGGTGGACA AACAAACCAG AGAGTTGGTG TACATTAACA
116589 AGATTATGAA CACGCAATTG ACAAAACCCG TTCTCATGAT GTTTAACATT
116639 TCGGGTCCTA TACGAAGCGT TACGCGCAAG AACAACAATT TGCGCGACAG
116689 AATAAAATCA AAAGTCGATG AACAATTTGA TCAACTAGAA CGCGATTACA
116739 GCGATCAAAT GGATGGATTC CACGATAGCA TCAAGTATTT TAAAGATGAA
116789 CACTATTCGG TAAGTTGCCA AAATGGCAGC GTGTTGAAAA GCAAGTTTGC
116839 TAAAATTTTA AAGAGTCATG ATTATACCGA TAAAAAGTCT ATTGAAGCTT
116889 ACGAGAAATA CTGTTTGCCC AAATTGGTCG ACGAACGCAA CGACTACTAC
116939 GTGGCGGTAT GCGTGTTGAA GCCGGGATTT GAGAACGGCA GCAACCAAGT
116989 GCTATCTTTC GAGTACAACC CGATTGGTAA CAAAGTTATT GTGCCGTTTG
117039 CTCACGAAAT TAACGACACG GGACTTTACG AGTACGACGT CGTAGCTTAC
117089 GTGGACAGTG TGCAGTTTGA TGGCGAACAA TTTGAAGAGT TTGTGCAGAG
117139 TTTAATATTG CCGTCGTCGT TCAAAAATTC GGAAAAGGTT TTATATTACA
117189 ACGAAGCGTC GAAAAACAAA AGCATGATCT ACAAGGCTTT AGAGTTTACT
117239 ACAGAATCGA GCTGGGGCAA ATCCGAAAAG TATAATTGGA AAATTTTTTG
117289 TAACGGTTTT ATTTATGATA AAAAATCAAA AGTGTTGTAT GTTAAATTGC
117339 ACAATGTAAC TAGTGCACTC AACAAAAATG TAATATTAAA CACAATTAAA
117389 **TAA**ATGTTAA AATTTATTGC CTAATATTAT TTTGTCATTG CTTGTCATTT

117439 ATTAATTTGG ATGATGTCAT TTGTTTTTAA AATTGAACTG GCTTTACGAG
AACTTGAC CGAAATGCTC
⇐ primer 2

*Eco*RI
117489 TAGAATTC
ATCTTAATCTAGAGCC
*Bgl*II

Figure 3.1.b.

AcMNPV bp

*Sal*I

21827 GTCGACAAAA TTCGTTTTTC AAAAATCTGC CTTCGAAACA ACTACAATTC
21877 AGTATTGAAA AGTTGCCTCG TTTCACATTA ATCGCCATCT GCTCCTGCCA
21927 CAACATCTTC GTCAACTCGT GTGGCTCCAA TTGAATGGAC GACGGCGTAA
21977 AATAGCACAT TACGCCCGTT TCGTCGTGTT TCACGTTAAA AGCGCCGCTG
22027 TTGTACGGCA CCAGCTGCTG GTCCTCACCA CCTTCCGATC TTTCCCGCTT
22077 CGGCTGGTTG TCGTCGCTGC TCGAATATCC ATCGCCAATC TTGCGTTTAG
22127 TTGCCATGCT ACCGACGTGC GCTGTCTGCT GTGGTTCAAG TCTAATTGAA
22177 GTGTTTCACA GAATATAAGA TATATAATAA ATATGGACGA CTCTGTTGCC
22227 AGCATGTGCG TAGACAACGC GTTTGCGTAC ACTACTGACG ATTTATTGAA
22277 AAATATTCCT TTTAGTCATT CCAAATGCGC CCCTTTCAAG CTACAAAATT
22327 ACACCGTTTT GAAGCGGTTG AGCAACGGGT TTATCGACAA GTATGTGGAC
22377 GTGTGCTCTA TCAGCGAGTT GCAAAAGTTT AATTTTAAGA TAGATCGGCT
22427 AACCAACTAC ATATCAAACA TTTTCGAGTA CGAGTTTGTA GTTTTAGAAC
22477 ACGATTTGTC CACAGTGCAC GTCATTAACG CCGAAACAAA AACCAAACTG
22527 GGCCATATAA ACGTGTCGCT AAACCAAAAC GACGCAAACG TGCTCATTTT

*Bam*HI primer 1 ⇒

GGCCGG ATCCTGAACG AGGACACG

22577 GACCGTAACT TTAACGAGCT AAA**ATG**AACG AGGACACGCC CCCGTTTTAT
22627 TTTATCAGCG TGTGTGACAA CTTTCGCGAC AACACCGCCG AACACGTATT
22677 CGACATGTTA ATAGAAAGAC ATAGTTCGTT TGAAAATTAT CCCATTGAAA
22727 ACACGGCGTT TATTAACAGC TTGATCGTTA ACGGGTTTAA ATACAATCAA
22777 GTTGACGATC ACGTTGTGTG CGAGTATTGC GAAGCAGAAA TAAAAAATTG
22827 GTCCGAAGAC GAGTGTATTG AATATGCACA CGTAACCTTG TCGCCGTATT
22877 GCGCGTATGC TAACAAGATC GCCGAGCGTG AATCGTTTGG CGACAACATT
22927 ACCATCAACG CTGTACTAGT GAAAGAAGGC AAACCCAAGT GTGTGTACAG
22977 ATGCATGTCC AATTTACAGT CGCGTATGGA TACGTTTGTT AACTTTTGGC
23027 CTGCCGCATT GCGTGACATG ATTACAAACA TTGCGGAAGC GGGACTTTTT
23077 TACACGGGTC GCGGAGACGA AACTGTGTGT TTCTTTTGCG ACTGTTGCGT
23127 ACGTGATTGG CATACTAATG AAGACACCTG GCAGCGACAC GCCGCCGAAA
23177 ACCCGCAATG TTATTTTGTA TTGTCGGTGA AAGGTAAAGA ATTTTGTCAA
23227 AACTCAATTA CTGTCACTCA CGTTGATAAA CGTGACGACG ACAATTTAAA
23277 CGAAAACGCC GACGACATTG AGGAAAAATA TGAATGCAAA GTCTGTCTCG
23327 AACGCCAACG CGACGCCGTG CTTATGCCGT GTCGGCATTT TTGCGTTTGC
23377 GTTCAGTGTT ATTTTGGATT AGATCAAAAG TGTCCGACGT GTCGTCAGGA
23427 CGTCACCGAT TTTATAAAAA TATTTGTGGT G**TAA**TAAAAT GGTGTTCAAC

TA CCACAAGTTG

⇐ primer 2

23477 GTGTACTACA ACGGCTATTA TGTGGAAAAA AAATTCTCCA AGGAGTTTTT

CACACCTAGG CGCG

*Bam*HI

*Sal*I

23527 AATTCATATT GCGCCTGATT TGAAAAACAG CGTCGAC

Figure 3.2.

Restriction enzyme map of HzSNPV illustrating the positions of the cloned fragments in the *Eco*RI and *Hind*III plasmid libraries.

The genetic map of HzSNPV was previously established for strain Elcar (Knell and Summers, 1984). Strain NC-1 is very closely related; it has identical *Eco*RI and *Hind*III restriction enzyme profiles, but has additional *Eco*RV and *Bam*HI enzyme sites, compared to strain Elcar (see Figure 3.6).

The diagram shows the *Hind*III map (upper line) and the *Eco*RI map (lower line) of the HzSNPV genome. The restriction enzyme fragments are labelled alphabetically according to their size. The fragments which were obtained as plasmid clones are shown as horizontal lines, and the corresponding labels indicate the names of these clones. The region between map units 71.3 and 92.7 of the HzSNPV genome, which was not represented by fragments cloned in the *Eco*RI and *Hind*III libraries, is indicated by a dashed line.

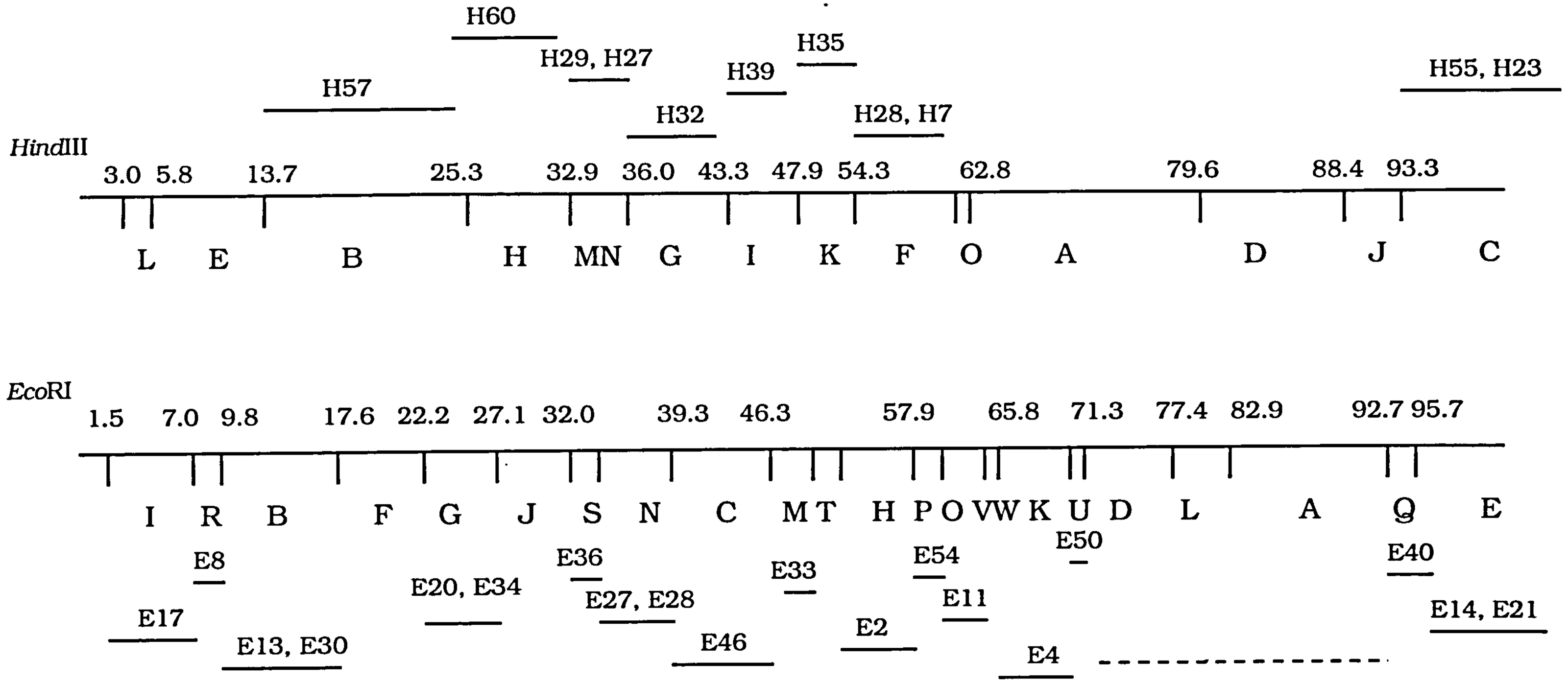

H60
H29, H27
H35
H39
H57
H55, H23
H32
H28, H7
HindIII
3.0 5.8 13.7 25.3 32.9 36.0 43.3 47.9 54.3 62.8 79.6 88.4 93.3
L E B H MN G I K F O A D J C
EcoRI
1.5 7.0 9.8 17.6 22.2 27.1 32.0 39.3 46.3 57.9 65.8 71.3 77.4 82.9 92.7 95.7
I R B F G J S N C M T H P O VW K U D L A Q E
E8
E36
E54
E50
E40
E33
E20, E34
E27, E28
E11
E14, E21
E17
E2
E13, E30
E46
E4

Table 3.1.

The *SalI* library of HzSNPV NC-1 fragments.

DNA fragments generated by *SalI* restriction enzyme digestion of HzSNPV NC-1 DNA were cloned into a pUC118 plasmid vector. Plasmid DNA was extracted from the resulting bacterial colonies and analysed by restriction enzyme digestion with *SalI* and gel electrophoresis in order to assess the size of the inserts obtained. The table shows the name and size of the viral fragments which were cloned in the *SalI* library.

Size (kbp)	Name in *SalI* library
7.5	S53, S61, S16
6.0	S31, S3
5.0	S13, S15
4.9	S22
4.8	S25
4.6	S19, S28
4.0	S52
3.3	S30
3.2	S37
2.9	S5, S71
2.8	S74
2.65	S39
2.1	S9, S34
2.0	S4, S14
1.9	S45
1.8	S1
1.65	S23, S44
1.4	S32, S64
1.35	S70
1.2	S81
1.15	S42
1.0	S48
0.55	S33
0.3	S36, S63

Figure 3.3.

Southern blot of DNA extracted from samples of the *Sal*I fragment library of HzSNPV NC-1.

DNA of the cloned HzSNPV *Sal*I fragments was purified using the miniprep method, digested with *Sal*I, and resolved on a 1% agarose gel containing 0.2µg/ml ethidium bromide. The DNA was transferred by to a nitrocellulose membrane and probed using a α^{32}P-dATP-labelled fragment of AcMNPV containing *iap1*, produced by PCR (see Figure 3.1b).

Panel a shows the restriction profiles obtained on digestion of pUC118 (lane 1), *Sal*I-3 (lane 2), *Sal*I-4 (lane 3), *Sal*I-5 (lane 4) and *Sal*I-6 (lane 5) with the *Sal*I restriction enzyme.

Panel b shows the Southern blot of the same gel probed with the 879 bp fragment of AcMNPV. The *Sal*I-3 clone hybridised to the probe (indicated by an arrow).

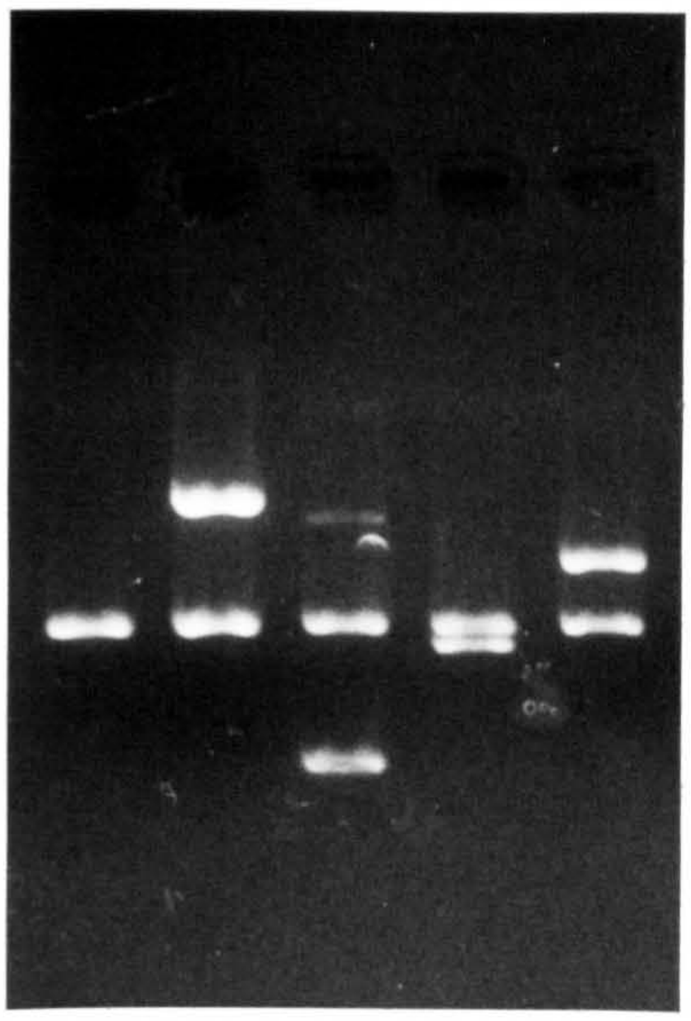
a.

Size (kbp) 1 2 3 4 5

5.5-

3.0-

2.0-



b.

1 2 3 4 5

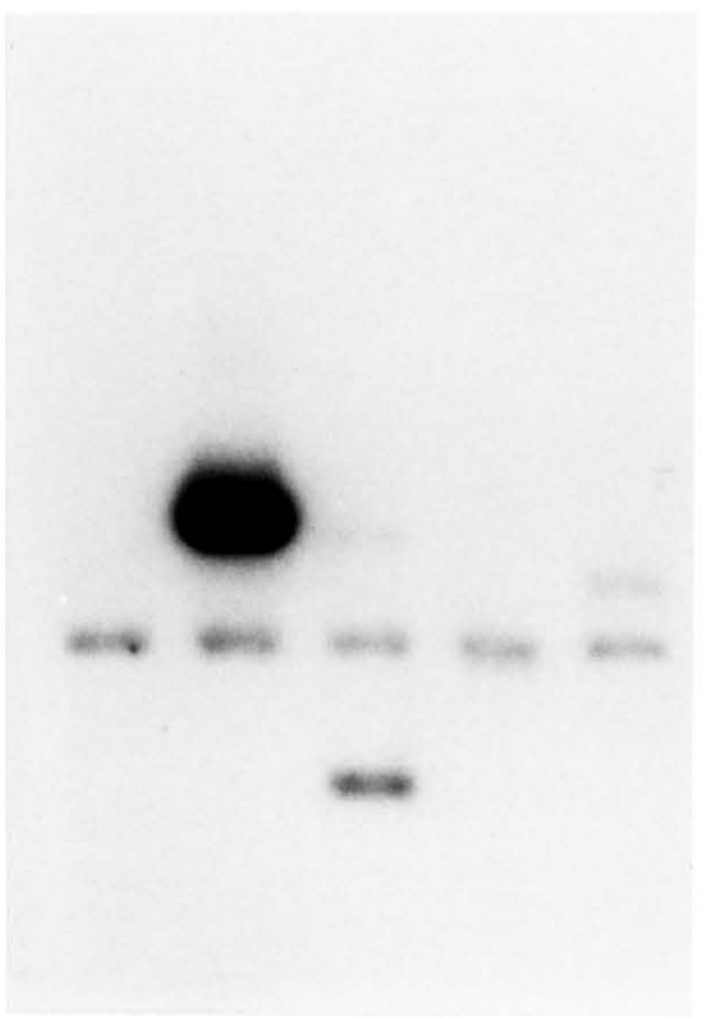


Figure 3.4.

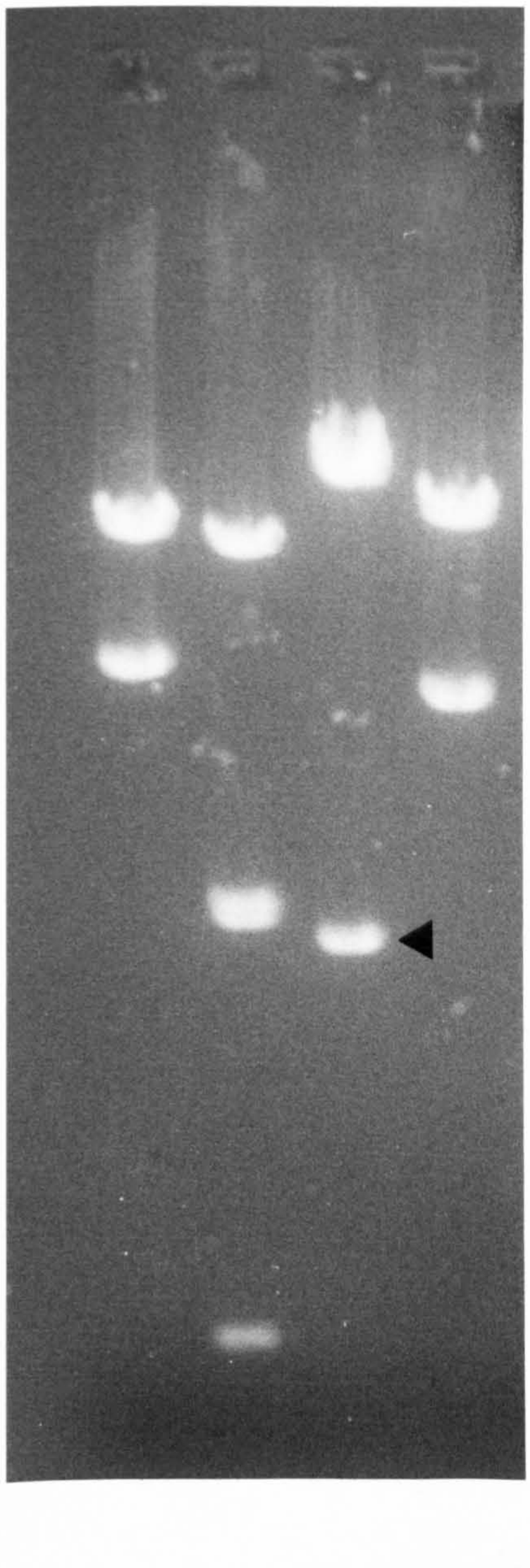
Southern blot of DNA extracted from the *Sal*I-3 HzSNPV NC-1 fragment to localise the DNA region encoding the *iap*-homologous sequence.

Plasmid clone pHzSNPV*Sal*I-3 DNA was digested with *Sal*I (lane 1), *Bgl*I (lane 2), *Cla*I (lane 3) and *Pvu*II (lane 4) and resolved on a 1% agarose gel containing 0.2µg/ml ethidium bromide. Panel a shows the electrophoresis of the DNA.

The DNA was subsequently transferred to a nitrocellulose membrane. Panel b shows the Southern blot of the gel, probed with a α^{32}P-dATP-labelled 879 bp fragment of AcMNPV, containing the *iap1* gene. A 1.4 kbp *Cla*I fragment hybridised to the probe (indicated by the arrow).

a.

Size (kbp) *Sal*I *Bgl*I *Cla*I *Pvu*II



b.

Figure 3.5.

Nucleotide sequence of the putative ORFs in the HzSNPV NC-1 *Sal*I-3 fragment.

The nucleotide sequence of the HzSNPV *Sal*I-3 cloned fragment was determined using standard techniques. Four ORFs of >150 nucleotides were identified in this 5.9 kbp region of the HzSNPV genome. In the figure, ATG start codons and TAA stop codons are shown in bold, early (CAGT) and late (TAAG) baculovirus promoter motifs are represented by asterisks and TATA motifs and polyadenylation signals are underlined. The numbers on the left hand side of the sequence indicate the position of the sequence in the HzSNPV *Sal*I-3 fragment. The location of the *iap* gene in this sequence representation is from 1937 to 2740.

SalI

```
1    GGTCGACAAG CGATTAGGCA GCGACACCGT CTTGACCGAT TCGATGACCT
     CCAGCTGTTC GCTAATCCGT CGCTGTGGCA GAACTGGCTA AGCTACTGGA

51   ATGAAGATAT AATGTTTTTG CGTTTTCCAG AATTGTACGC GGCCATGTAC
     TACTTCTATA TTACAAAAAC GCAAAAGGTC TTAACATGCG CCGGTACATG

101  TTTGATTCTC GCGATTTGGG CGATTGCGAT TCATTGTGTG TCCGCGACGT
     AAACTAAGAG CGCTAAACCC GCTAACGCTA AGTAACACAC AGGCGCTGCT

151  GGTAAAGTTT AACACAGTTT TAGGAACGGC GGGGGCGCCA AAATTTGTCG
     CCATTTCAAA TTGTGTCAAA ATCCTTGCCG CCCCCGCGGT TTTAAACAGC

201  AATCCATATT AGACACGGCC GGGTTCGTGT ACATCAATAT TTTGGCGTTA
     TTAGGTATAA TCTGTGCCGG CCCAAGCACA TGTAGTTATA AAACCGCAAT

251  GAATCGTGTC ATTTGAAAAA TAATGTAGGC AGTGCCAACA GCGATTTAGC
     CTTAGCACAG TAAACTTTTT ATTACATCCG TCACGGTTGT CGCTAAATCG

301  ATACTTAGAC ATGTCCATTA ATCGTTTACA AACTCCGTTG ATAGCCAATC
     TATGAATCTG TACAGGTAAT TAGCAAATGT TTGAGGCAAC TATCGGTTAG

351  GTTTGTCCAT TCCGTCAACG GGCAACGGCG GCAAACCCAC ACTATATTCG
     CAAACAGGTA AGGCAGTTGC CCGTTGCCGC CGTTTGGGTG TGATATAAGC

401  TCATTTTGGG GATGTCCAGA AGAATCGAGA CCGTTCAGAA TGCTAGTAGA
     AGTAAAACCC CTACAGGTCT TCTTAGCTCT GGCAAGTCTT ACGATCATCT

451  ATTGATGACG TGCGCCGTTG CCGATTACAA TATGGTTTAT ATTGCTAGCG
     TAACTACTGC ACGCGGCAAC GGCTAATGTT ATACCAAATA TAACGATCGC

501  ATTCGGAAAC TCAATTCGAA ATGGAAGATA CCATTTTGAT ACTAAACGAT
     TAAGCCTTTG AGTTAAGCTT TACCTTCTAT GGTAAAACTA TGCAAAGCTA

551  AATTTCACAG TTCGTGAAAT ATATAATATG TTGACCAATT ACAAGTTTAA
     TTAAAGTGTC AAGCACTTTA TATATTATAC AACTGGTTAA TGTTCAAATT

601  CAATTCAATT CGCTACAACG TTTTAACTCT AAACGAAAAA CAATCCAAAT
     GTTAAGTTAA GCGATGTTGC AAAATTGAGA TTTGCTTTTT GTTAGGTTTA

                                                  ** **
651  CTAAACGAAA CAGAAAACAA ACTAGTATCA ATTTAGATTA AGTTTACATT
     GATTTGCTTT GTCTTTTGTT TGATCATAGT TAAATCTAAT TCAAATGTAA

                          ****
701  TGTGTATTTT ACAATAAATA TAAGCGCTAC ATTCATGCGG CTATTTGTCG
     ACACATAAAA TGTTATTTAT ATTCGCGATG TAAGTACGCC GATAAACAGC

                    start orf 106/107
751  TTGTGCTCGT TTACACATAA TGGAGTCGAT TGATGTTGAC GATTTCGCTA
     AACACGAGCA AATGTGTATT ACCACAGCTA ACTACAACTG CTAAAGCGAT

801  AACAGCTAAT AGCGGACAAA TGTAGCGCTT TGATAGAATC AAACAAGATG
     TTGTCGATTA TCGCCTGTTT ACATCGCGAA ACTATCTTAG TTTGTTCTAC

851  CTTTCGCCCG ACATGATGGC GATGGTGAAA TTGGCCCGCG ACGAATATTT
     GAAAGCGGGC TGTACTACCG CTACCACTTT AACCGGGCGC TGCTTATAAA

901  CAAAGACCCA TCGTCGAAAA ATTACGAAAT ATTAAAAAAA CTGATTGGTC
     GTTTCTGGGT AGCAGCTTTT TAATGCTTTA TAATTTTTTT GACTAACCAG

951  ACACAAAATA CGTGGACGAT TCCATCGACT GCAAAGATTT CAATCGCCGC
     TGTGTTTTAT GCACCTGCTA AGGTAGCTGA CGTTTATAAA GTTAGCGGCG

1001 ATGTTACTTA TCGCCATCAA AGTGAGCGCT TCACGTGCGC GAGACTATTT
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1051 TAACAAATAC AAAACTGTAT TCGAATTGGC TTTGAAACGT TTGGACAGCA
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1101 TCAATCCCGA TATACGAAGT TCGCCTAGCG CTCTGCTACA ACACTATAAA
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1301 CGTAACCAAT GCGCGTCGTC CTTATCGGCA AACTATTTAT CAAATCGTAA
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1351 AGCAACTAGT ACGGACAGTC TTGCTAGCGA AAACATTACA GTTGAACGCG
     TCGTTGATCA TGCCTGTCAG AACGATCGCT TTTGTAATGT CAACTTGCGC

1401 TCTCGCAAGC GACAACACAA GCGGAAAAAC AGTACAACTT TATTAGACAG
     AGAGCGTTCG CTGTTGTGTT CGCCTTTTTG TCATGTTGAA ATAATCTGTC

                end orf 106/107

1451 CAAAGTTAAT TCTTTCGTGT ACAAGGCACA GATACACGAT CCGCCCAAAT
     GTTTCAATTA AGAAAGCACA TGTTCCGTGT CTATGTGCAA GGCGGGTTTA

1501 ATTACGTTGC AAGAGCTCTG TTCACATTGT AGAGCCAGTT GTTATCATGG
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1551 AAAAACACCA AATGGACTTG TACAAAGCGT TGATGCAGCA CAAAACTAAA
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1651 CGACACCGTC TACAGATACC CAAACATACT GTGAATGTTT GTGTGAACGA
     GCTGTGGCAG ATGTCTATGG GTTTGTATGA CACTTACAAA CACACAAGCT

1701 CGAAACGACG GTTTCAGTAT TGTGCTATCC TAATTCTCAA ACAAAACACG
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1751 GTTTGTTGAT TCGGAAACCT GTTAAAGATC TATTCTTCGA CAACGATCAC
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1801 GATTGTGTAC AGTGTATAAT ACCTAGTTGT GTAAACAATG ATGTTTGTAA
     CTAACACATG TCACATATTA TGGATCAACA CATTTGTTAC TACAAACATT

1851 TAATATAGTT TTAAATCATT GGCAATAAAA CAATACATAA AAAATGCAAA
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1901 AATTTTTTAT TTATCTCATT AGTCAAATAC TTTTATTTAA CTGATAAAAA
     TTAAAAAATA AATAGAGTAA TCAGTTTATG AAAATAAATT GACTATTTTT
                                        end iap

1951 CTTTTGTCAT ATCGTCGATT GATCTACGAC ACACAACACA TTTTTTTACT
     GAAAACAGTA TAGCAGCTAA CTAGATGCTG TGTGTTGTGT AAAAAAATGA

2001 TTGAAAGCAC ATTCTTCACA ACAGGCCAAA TGATGACACG GTAAAAACAT
     AACTTTGCTG TAAGAAGTGT TGTCCGGTTT ACTACTGTGC CATTTTTGTA

2051 GTAATTGCGT TCGTTCACGA AGCAAACTTT ACATGTACGT ATGTCACATT
     CATTAACGCA AGCAAGTGCT TCGTTTGAAA TGTACATGCA TACAGTGTAA

2101 CAGTAGTTTG ATTGTCGGAA TTACCGCCTT CTTTTTCGAC ACAGGCTTCG
     GTCATCAAAC TAACAGCCTT AATGGCGGAA GAAAAAGCTG TGTCCGAAGC

2151 GTGATTACCG TCTGCACGAA ATCTTTGCCT TTTTCCGATA GTACAAAATC
     CACTAATGGC AGACGTGCTT TAGAAACGGA AAAAGGCTAT CATGTTTTAG

2201 ACAATTTCTG TACCAGCGTG CGTGTTCTCG CCATGGTTCA TGCGTAAGCG
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2251 TCCAATTGCT TAATTTTCCG CCGCAATGAA AACATATTGT AATATCATCT
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2301 TTACCCGTAT ATACCCAACC AGCTTCTGCT AATTTACTCT TCAAGATTAT
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2351 CAGTGTTTGC GGCCAATTGT CAAACGATTT TAAACGATTT TCATAAGTTG
     GTCACAAACG CCGGTTAACA GTTTGCTAAA ATTTGCTAAA AGTATTCAAC

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     TTTTATTATA ACTAGTCCGG TTAAACGTTT GCATTGTATA GCTATACTAA

2701 CAGTTTTTAA TAATTCCAAA TCGGATTCCA TATAGGACAT CATCGCACAA
     GTCAAAAATT ATTAAGGTTT AGCCTAAGGA TATACCTGTA GTAGCGTGTT
                                        start iap

2751 GCGGCGAAGG ACAACGCTCT ACTGAATTCT CTATCGACAA GACAGGCTTT
     CGCCGCTTCC TGTTGCGAGA TGACTTAAGA GATAGCTGTT CTGTCCGAAA
                           ****

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     AAATATAGAT TGTATTTTCT CGAATGATTT GATAACGCAG CATAAAATGC
                            ****

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2901 CCTTTGATGT TACTTTGGTA AACACAAAAT GAATAAAAAA AAGGGTTAAT
     GGAAACTACA ATGAAACCAT TTGTGTTTTA CTTATTTTTT TTCCCAATTA

2951 AAAAAACCAA CAAACCGTAA AGGAAATTTA TTGCTCACAC AAATAACATT
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3001 ACAGATTTGT TGACGTCGTT GCTTCTGTAG CAGATGTTAT ATCTTTTTGA
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3051 GTAGTGACGT TTTCAATAGC CGGCACATTC CCTGGTATTA TGTTTGATTC
     CATCACTGCA AAAGTTATCG GCCGTGTAAG GGACCATAAT ACAAACTAAG
                                           end orf 2

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     TAGCATTTTA GCTTGTAATG TATGTAAGAA CTGTTTCATT AAAACTGTTA

3151 TATTCATGGC GTGCACAACT TGTTGTGCCG ATACACGTCG CCTGCATTGG
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     TTACTGCTGC AACACCGCAC CTTCCGTGCT CAAGAAACCG GTAAAAAAGC

3251 ACAATATTCT CCACGATAGC ATTTATACGA TCCTTGGCAT CGACGCTTTG
     TGTTATAAGA GGTGCTATCG TAAATATGCT AGGAACCGTA GCTGCGAAAC

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     GCAGTTTGTA AACCGCTGTG TTAGCAGAAG CAGCTAGTTT AGATTGCGAA

3351 TAAACTCTTC AATTAGTTTT GTATTAACAG TTTTGTTACG TTGACACATT
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3401 TCGACATCGG CGCGATATTT GGCACGTAAT TCAGTTTCGT CGAGCACCTC
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3551 AGTTGTAAAA ATTTGGTCGA ATCGCACAAC CACGCGTAAC GAGGCGACGG
     TCAACATTTT TAAACCAGCT TAGCGTGTTG GTGCGCATTG CTCCGCTGCC

3601 CTTAAATCTT TTTGGAGTAC ACAAAGTGTC ACGGTAGCGT TTTGCCACTT
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3701 CGTTTGCCAT TGACTATTCG TTCGTAACCG GTTATGTATT CTTCCTTTTC
     GCAAACGGTA ACTGATAAGC AAGCATTGGC CAATACATAA GAAGGAAAAG

3751 GGGCTGTTTT GTCAGGACCG GTACGACGCG ATTGCTTATC TTTTCGAGCG
     CCCGACAAAA CAGTCCTGGC CATGCTGCGC TAACGAATAG AAAAGCTCGC

3801 TTTGACGCAG TCGATGATTT TCGTCAATGT TATCTTTGGC GAGGAGTGCG
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3851 TTCGCTGCAA ACTGAAACAT TGACATGTTA GCTTGATGTG CCATGTCCTT
     AAGCGACGTT TGACTTTGTA ACTGTACAAT CGAACTACAC GGTACAGGAA

3901 CATTTGTAGT TGCATTTTGA ACTCTCGCTC TTTGTACTCA GACATTTGCT
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3951 GTTCGTAATT GCGCTTCATT TCCGACATAT TCGTGTTCCA TTCCGCGATT
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4051 AACATTCGCC AATTTTTGGT CGTAACTCAC TACTTCAGTA GAATTGTCTG
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4101 TGGACGATTG TCGCCTGTTT TCTATACTAT ATTTTCCAGT TCGTCTCAGT
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4151 TCGGGCAAGA CCTCTTCGAA TAGCCAACTT TGAAATTCCT CGGCTGCGGG
     AGCCCGTTCT GGAGAAGCTT ATCGGTTGAA ACTTTAAGGA GCCGACGCCC

4201 TAGCTTAGAA CGCATAATTA AAGCGTAAAT ACCGGCTTCG GTGATGAAAA
     ATCGAAGCTT GCGTATTAAT TTCGCATTTA TGGCCGAAGC CACTACTTTT

4251 GCGTATTCGG TTGCCAATTT AATGGCATTT CTATAGAATC TGATGACGTC
     CGCATAAGCC AACGGTTAAA TTACCGTAAA GATATCTTAG ACTACTGCAG

4301 ACAAGGGAGT GTTGATTCAA CACCCCCTTT ATTTCCGCCC ACGTTTTGCG
     TGTTCCCTCA CAACTAAGTT GTGGGGGAAA TAAAGGCGGG TGCAAAACGC

4351 CCATTGCGGT TTCACGTGAT CGTACAGTGC TCTTCTGGGA CATTTGTAAC
     GGTAACGCCA AAGTGCACTA GCATGTCACG AGAAGACCCT GTAAACATTG

4401 CCAAAGCTTC GGCGACACCG TGACCCGAAC ACAGAAATCG GTTTTCTTCG
     GGTTTCGAAG CCGCTGTGGC ACTGGGCTTG TGTCTTTAGC CAAAAGAAGC

4451 ATTTCAGTAA TCCAAACTTC ACCCAATTTA CATTTGCGAT TTACAAGATA
     TAAAGTCATT AGGTTTGAAG TGGGTTAAAT GTAAACGCTA AATGTTCTAT

4501 CATCTCTAAA ACAGTGCGAC AACTTCAAAG TGTAGACTTA AAAGTAAGCA
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     start orf 2                                  ****

4551 AATATTAACA CGTTACAATT GAAAGCCATA CATACATCGA AATTGTCCTA
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4601 TACATCGAAA TCGTCCTATA CATCGAAATT GTCGATGTGA CTAACAACAA
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          ****                start sod
4651 AAATAAGATC GAATATCATA ATGAAAGCTA TTTGTATTTT GAGCGGTGAC
     TTTATTCTAG CTTATAGTAT TACTTTCGAT AAACATAAAA CTCGCCACTG

4701 ATCAGCGGCG AAATTTGTTT CAGTCAAGAA TCGCCTTTAC ATTTAATCAA
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4751 AATCACCGGA TTCATACTTA ATTTGCCGCG TGGATTGCAC GGTATACACG
     TTAGTGGCCT AAGTATGAAT TAAACGGCGC ACCTAACGTG CCATATGTGC

4801 TTCACGAGTT CGGCGACACC AGCAACGGAT GTACGTCCGC CGGGGAACAT
     AAGTGCTCAA GCCGCTGTGG TCGTTGCCTA CATGCAGGCG GCCCCTTGTA

4851 TTCAATCCTA CGGGCCAAAC GCACGGGGCG CCAAACGCGA CCGTGCGTCA
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4901 CGTCGGCGAC TTGGGCAACG TCGAATCTTT CGGTATAAAT TCTTTGACAG
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5001 TTAGGTCGCA GTCTTGTCGT GCACACGGAC CGCGACGATC TCGGTTTGAC
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5051 TGATCATCCG TTAAGTCGTA TAACCGGTAA TTCCGGCGGC CGTCTCGGAT
     ACTAGTAGGC AATTCAGCAT ATTGGCCATT AAGGCCGCCG GCAGAGCCTA

                                                      end sod
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     GGCTTAGTTA CTTCGTCTAG TCGACGCGCA GGTGTTGTTG ATAAGGTGGC

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5751 AAGTTTTGTA TTGGCCATAG TGGCGTTAAA TATTATGACT CCAGCACATA
     TTCAAAACAT AACCGGTATC ACCGCAATTT ATAATACTGA GGTCGTGTAT

5801 TGTTTATGGA TGATTATAAT GTTAGCGACA TAATTGAAAC ATATCGAGCC
     ACAAATACCT ACTAATATTA CAATCGCTGT ATTAACTTTG TATAGCTCGG

5851 GTTCCGTTCG ACAACGATGT GAACATCTAC CGACCGACAA CATTAGTACA
     CAAGGCAAGC AGTTGCTACA CTTGTAGATG GCTGGCTGTT GTAATCATGT

          SalI
5901 ATCGTCGAC
     TAGCAGCTG
```

Figure 3.6.

Location of the *Sal*I-3 fragment in the genomes of HzSNPV NC-1 and HzSNPV Elcar using Southern blotting techniques.

DNA was purified from occluded virus using standard techniques. The DNA was digested with various restriction enzymes and resolved on a 0.7% agarose gel containing 0.2μg/ml ethidium bromide. The DNA was subsequently transferred to a nitrocellulose membrane and probed using a α^{32}P-dATP-labelled sample of pHzSNPV*Sal*I-3, which contains a 5.9 kbp region of HzSNPV NC-1 DNA.

Panel a shows the ethidium bromide-stained DNA.

Lane 1: Lambda DNA digested with *Eco*RI and *Hind*III (DNA ladder)
Lane 2: HzSNPV Elcar DNA digested with *Bam*HI
Lane 3: HzSNPV NC-1 DNA digested with *Bam*HI
Lane 4: HzSNPV Elcar DNA digested with *Cla*I
Lane 5: HzSNPV NC-1 DNA digested with *Cla*I
Lane 6: HzSNPV Elcar DNA digested with *Eco*RI
Lane 7: HzSNPV NC-1 DNA digested with *Eco*RI
Lane 8: HzSNPV Elcar DNA digested with *Eco*RV
Lane 9: HzSNPV NC-1 DNA digested with *Eco*RV

Panel b shows the Southern blot of the same gel probed with α^{32}P-dATP-labelled pHzSNPV*Sal*I-3 DNA. The restriction enzyme fragments of the HzSNPV genomes which hybridise to the probe are labelled with arrows on panel a.

a. **b.**

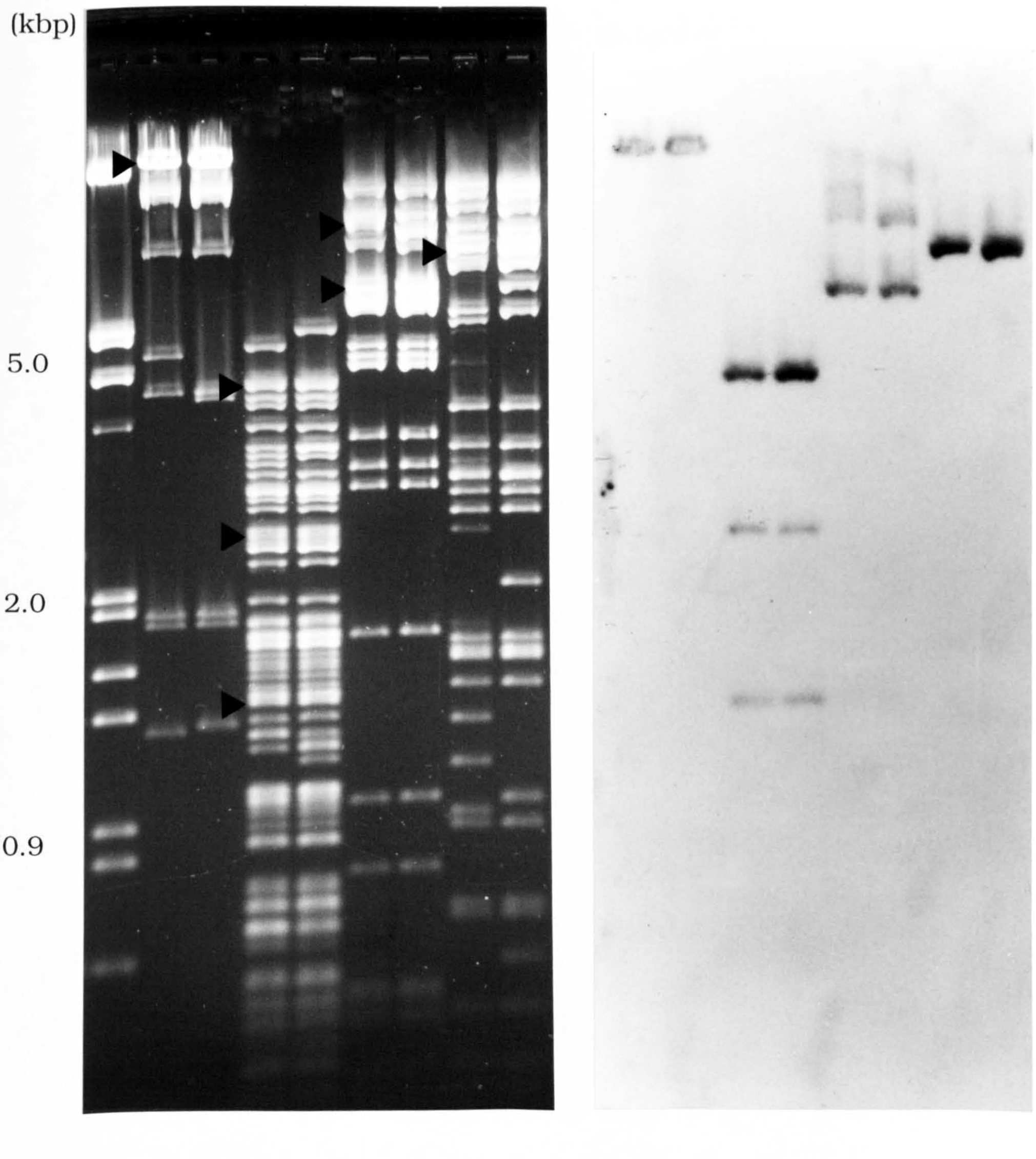


Figure 3.7.

Organisation of the ORFs within the *Sal*I-3 fragment of HzSNPV NC-1.

The upper line indicates the *Hind*III map for the complete genome of HzSNPV (Knell and Summers, 1984). The left of this scale corresponds to 0 m.u. and the 128 kbp circular genome has been linearised at this point (within the *Hind*III C fragment).

The 5.9 kbp *Sal*I-3 fragment is represented as a shaded horizontal line with *Sal*I, *Cla*I, *Sac*I, *Eco*RI and *Hind*III restriction endonuclease sites shown. The fragment is orientated relative to the published *Hind*III map of HzSNPV Elcar (upper line). The *Sal*I-3 fragment is located in the region from 75 to 80.9 map units of the HzSNPV genome. ORFs are shown as open arrows, the orientation of which represents the direction of transcription.

Previously characterised HzSNPV ORFs are marked on the diagram; polyhedrin (*polh*), ORF 1629 (1629), *ie-1* (Cowan *et al.*, 1994) and *p40* (Ma *et al.*, 1993).

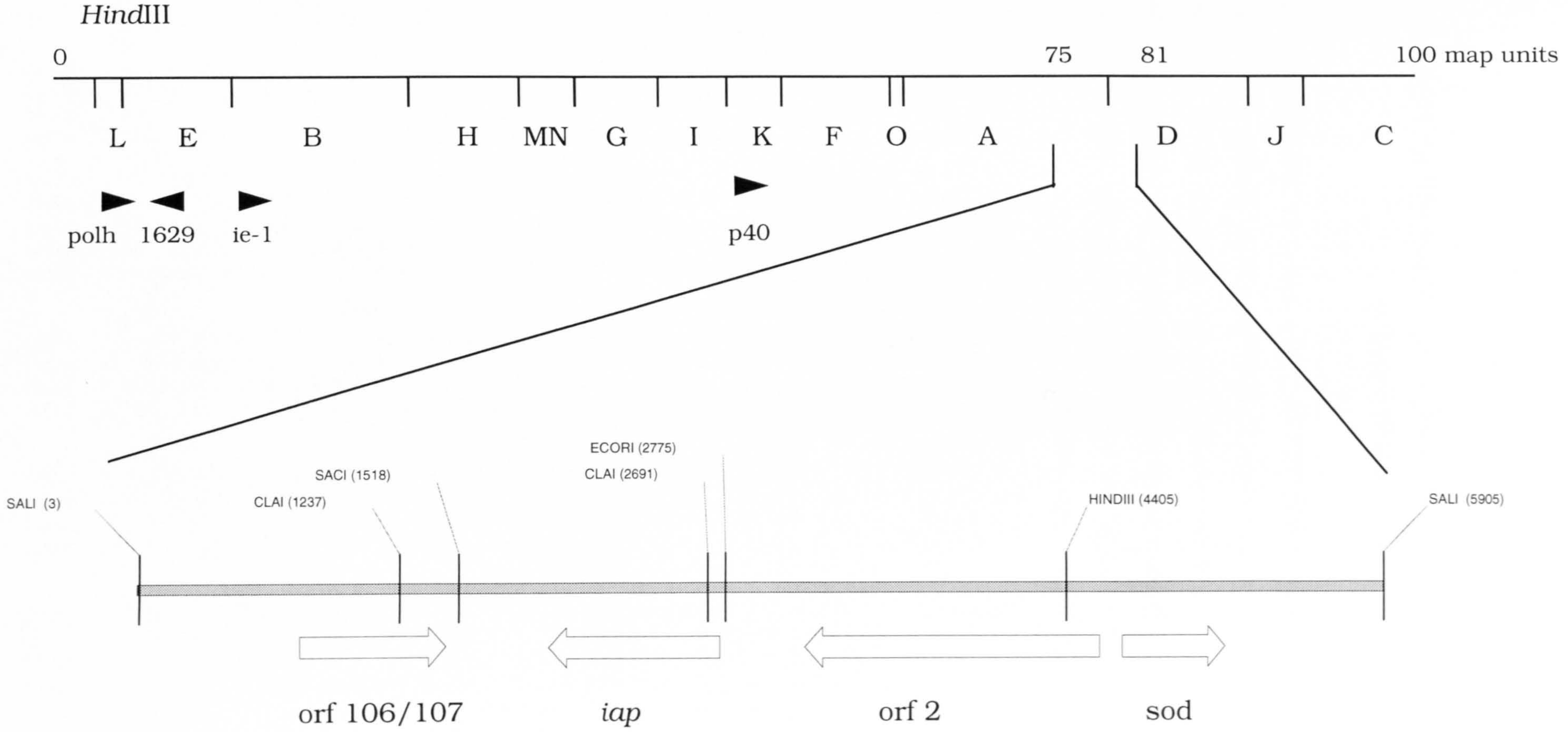

*Hind*III
0
75
81
100 map units
L
E
B
H
MN
G
I
K
F
O
A
D
J
C
polh
1629
ie-1
p40
SALI (3)
CLAI (1237)
SACI (1518)
ECORI (2775)
CLAI (2691)
HINDIII (4405)
SALI (5905)
orf 106/107
iap
orf 2
sod

Figure 3.8.

Alignment of the amino acid sequence of the IAP proteins of OpMNPV, CpGV, HzSNPV NC-1 and AcMNPV (IAP1).

The GCG program PRETTYPLOT was used to display the alignment following its generation in the PILEUP program. Identical residues and/or conservative changes are boxed. The BIR regions (BIR1 and BIR 2) are defined by dashed lines above them. The RING finger motif is underlined by an unbroken line. Gaps are introduced within the coding regions by the alignment program, to align the conserved motifs.

BIR 1

```
Cpiap  ----------------------------MSDLRLEEVRLNTFEKWPVS--FLSPETMAKNGFYYLG  36
Opiap  ----------MSSRAIGAPQEGADMKNKAARLGTYTNWPVQ--FLEPSRMAASGFYYLG  47
Hziap  ----------------MMSYMESDLELLKTESYRYVTFANWPVQYYFMDCAKMAQAGFYYLN  46
Aciap  MNEDTPPFYFISVCDNFRDNTAEHVFDMLIERHSSFENYPIENTAF-INSLIVNGFKYNQ  59

       ----------------------------------------
Cpiap  RSDEVRCAFCKVEIMRWKEGEDPAADHKKWAPQCPFVKGIDVCGSIVTTNNIQNTTTHDT  96
Opiap  RGDEVRCAFCKVEITNWVRGDDPETDHKRWAPQCPFVRN---------NAHDTPHDRAPPA  99
Hziap  KDDHVKCAFCKVEMMNWQHEDDPLEEHARWAPQCSYVKSIMSDANVCSEQKYIADQEF-Y  105
Aciap  VDDHVVCEYCEAEIKNWSEDECIEYAHVTLSPYCAYANKIAERESFGDNITINAVLVKEG  119
```

BIR 2

```
Cpiap  IIGPAHPKYAHEAARVKSFHN-WPRCMKQRPEQMADAGFFYTGYGDNTKCFYCDGGLKDW  155
Opiap  RSAAAHPQYATEAARLRTFAE-WPRGLKQRPEELAEAGFFYTGQGDKTRCFCCDGGLKDW  158
Hziap  KNKSKLSSYSTYENRLKSFDN-WPQTLIILKSKLAEAGWVYTGKDDITICFHCGGKLSNW  164
Aciap  KPKCVYRCMSNLQSRMDTFVNFWPAALRDMITNIAEAGLFYTGRGDETVCFFCDCCVRDW  179

       ------------------------
Cpiap  EPEDVPWEQHVRWFDRCAYVQLVKGRDYVQKVITEACVLPGENTTVSTAAPVSEPIPETK  215
Opiap  EPDDAPWQQHARWYDRCEYVLLVKGRDFVQRVMTEACVVRDADN----------EPH  205
Hziap  TLTHEPWREHARWYRNCDFVLSEKGKDFVQTVITEACVEKEGGNS----------  209
Aciap  HTNEDTWQRHAAENPQCYFVLSVKGKEFCQNSITVTHVDKRDDDNLNENAD---------  230

Cpiap  IEK---EPQVEDSKLCKICYVEECIVCFVPCGHVVACAKCALSVD-KCPMCRKIVTSVLK  271
Opiap  IERPAVEAEVADDRLCKICLGAEKTVCFVPCGHVVACGKCAAGVT-TCPVCRGQLDKAVR  264
Hziap  -DNQTTECDI---RTCKVCFVNERNYMFLPCHHLACCEECAFKVK-KCVVCRRSIDDMTK  264
Aciap  --------DIEEKYECKVCLERQRDAVLMPCRHFCVCVQCYFGLDQKCPTCRQDVTDFIK  282

Cpiap  VYFS-  275
Opiap  MYQV-  268
Hziap  VFIS-  268
Aciap  IFVV-  286
```

Table 3.2.

Percentage amino acid similarity and identity, calculated using a GAP algorithm, for the baculovirus IAP proteins.

The calculations were performed using a gap weight of 3 and a length weight of 0.1.

Sequence Pair	*Quality*	*Ratio*	*% Similarity*	*% Identity*
HzIAP x CpIAP	187.6	0.69	57.1	39.4
HzIAP x OpIAP	191.5	0.71	60.2	41.7
HzIAP x AcIAP1	152.1	0.56	52.8	31.4
CpIAP x OpIAP	248.9	0.92	73.7	57.6
CpIAP x AcIAP1	147.4	0.53	52.9	31.3
OpIAP x AcIAP1	144.9	0.54	49.8	28.6

Figure 3.9.

Alignment of the IAP protein family.

The protein sequences used in this analysis are:

Code	Organism/Source	Accession Number
Miap1	mouse	L49433
Hiap1	human	L49431/U45878
Hiap2	human	L49432/U45879
Hilp (X-iap)	human	U32974/U45880
Cpiap	CpGV	L05494
Opiap	OpMNPV	L22564
Diap1	*Drosophila*	L49440/U45881
Hziap	HzSNPV	from Figure 3.5.
Dilp (Diap2)	*Drosphila*	U32373/L49441
Aciap	AcMNPV	L22858
Naip	human	U19251
Aciap2	AcMNPV	L22858

The sequences were aligned using PILEUP and displayed via PRETTYPLOT. The BIR motifs and RING finger region are indicated in the Figure by dashed lines and an unbroken line respectively.

Table 3.3.

Significance of the similarity between the inhibitor of apoptosis protein family.

The IAP proteins were compared by Monte Carlo analysis. The scores of pairwise alignments were produced in Multalign and represent the number of standard deviations from the mean obtained when pairwise comparisons of the two sequences are compared to a random sequence comparison distribution. A score of six or more standard deviations from the mean is considered to be a significant similarity (Barton, 1990).

The protein sequences used in the analysis are:
Cydia pomonella GV IAP (CpIAP), *Orgyia psuedotsugata* MNPV IAP (OpIAP) *Heliothis zea* SNPV NC-1 IAP (HzIAP), *Autographa californica* MNPV IAP1 (AcIAP1), p35 (p35) and IAP2 (AcIAP2), and human NAIP (NAIP).

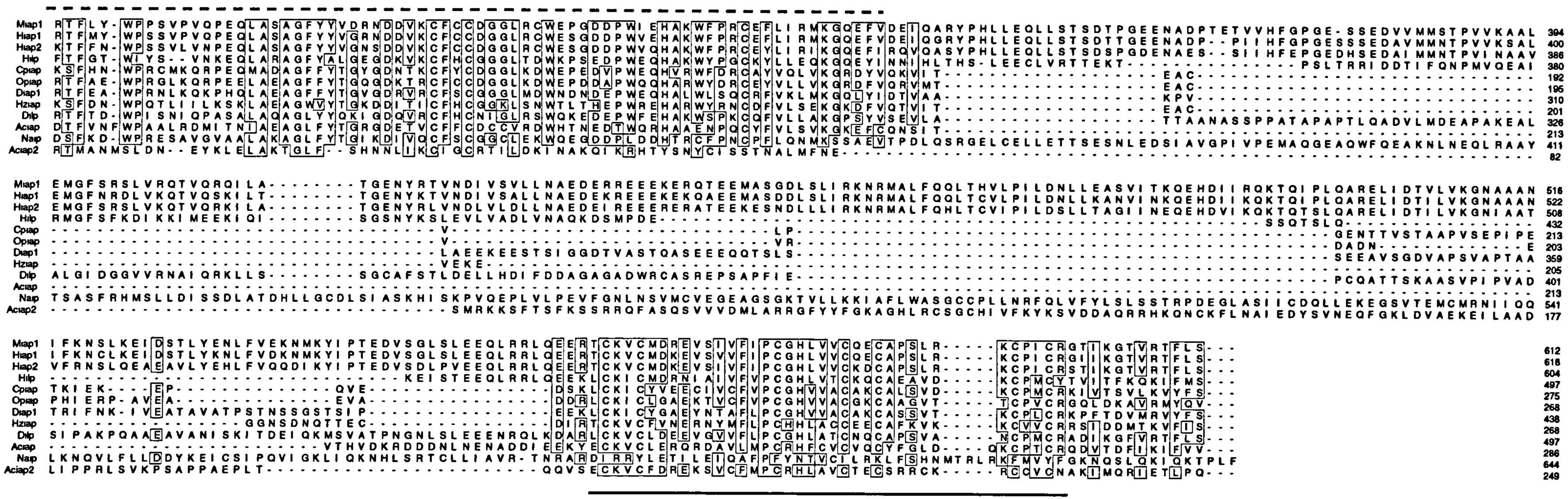
*******	CpIAP	OpIAP	HzIAP	AcIAP1	p35	AcIAP2	NAIP
CpIAP		39.96	26.96	17.17	0.76	9.22	15.64
OpIAP			29.87	19.00	0.86	9.29	15.59
HzIAP				17.24	0.33	10.14	14.13
AcIAP1					-0.90	7.46	6.55
p35						0.64	0.40
AcIAP2							3.16
NAIP							

BIR 1

BIR 2

BIR 3

Miap1	MDKTVSQRLQQGTLHQKLKRIMEKSTILSNWTKESEE-KMKFDFSCELYRMSTYSAFPRGVP-V	62
Hiap1	MHKTASQRLFPGPSYQNIKSIMEDSTILSDWTNSNKQ-KMKYDFSCELYRMSTYSTFPAGVP-V	62
Hiap2	MNIVENSIFLSNLMKSANTFELKYDLSCELYRMSTYSTFPAGVP-V	45
Hilp	MTFNSFEGSKTCVPADINKEEEFVEEFNRLKTFANFPSGSP-V	42
Cpiap	MSDLRLEEVRLNTFEKWPVS--FL	22
Opiap	MSSRAIGAPQEGADMKNKAARLGTYTNWPVQ--FL	33
Diap1	MASVVADLPSYGPIAFDQVDNNTNATQLFKNNINKTRMNDLNREETRLKTFTDWPLD--WL	59
Hziap	MMSYMESDLELLKTESYRYVTFANWPVQYYFM	32
Dilp	MTELGMELESVRLATFGEWPLNAPVSAEDLVANGFFATGNWLEAECHFCHVRIDRWEYGDQVAAGHRRSSPICSMVLAPNHCGNVPRSQESDNEGNSVVDSPESCSCPDLLLEANRLVTFKDWPNPN--I	128
Aciap	MNEDTPPFYFISVCDNFRDNTAEHVFDMLIERHSSFENYPIENTAF	46
Naip	MATQQKASDERISQFDHNLLPELSALLGLDAVQLAKELEEEEQKERAKMQKGYNSQMRSEAKRLKTFVTYEPYSSWI	77
Aciap2		0
Miap1	SERSLARAGFYYTGVNDKVKCFCCGLMLDNWKQGDSPVEKHRQFYPSCSFVQTLLS-ASLQSPSKNM--SPVKSRFAH--SSPLERGGIHSNLCSS----PLNSRAVEDF-----------SSRMDPC	170
Hiap1	SERSLARAGFYYTGVNDKVKCFCCGLMLDNWKLGDSPIQKHKQLYPSCSFIQNLVS-ASLGSTSKNT--SPMRNSFAHSLSPTLEHSSLFSGSYSSLSPNPLNSRAVEDIS-----------SSRTNPY	177
Hiap2	SERSLARAGFYYTGVNDKVKCFCCGLMLDNWKRGDSPTEKHKKLYPSCRFVQSLNSVNNLEATSQPTFPSSVTNS-THSLLPGTENSGYFRGSYSNSPSNPVNSRANQDFS-----------ALMRSSY	162
Hilp	SASTLARAGFLYTGEGDTVRCFSCHAAVDRWQYGDSAVGRHRKVSPNCRFINGFY----------LENSATQSTNSGIQNGQYKVENYLGSRDHFALDRPSETHADYLLRTGQVVDISDTIYPR	156
Cpiap	SPETMAKNGFYYLGRSDEVRCAFCKVEIMRWKEGEDPAADHKKWAPQCPFVK----------	74
Opiap	EPSRMAASGFYYLGRGDEVRCAFCKVEITNWVRGDDPETDHKRWAPQCPFVR----------	85
Diap1	DKRQLAQTGMYFTHAGDKVKCFFCGVEIGCWEQEDQPVPEHQRWSPNCPLLRR----------RTTNNVPINAEALDRILPPISY	134
Hziap	DCAKMAQAGFYYLNKDDHVKCAFCKVEMMNWQHEDDPLEEHARWAPQCSYVKS----------	85
Dilp	TPQALAKAGFYYLNRLDHVKCVWCNGVIAKWEKNDNAFEEHKRFFPQCPRVQM----------	181
Aciap	I-NSLIVNGFKYNQVDDHVVCEYCEAEIKNWSEDECIEYAHVTLSPYCAYANKIAERESF----------	105
Naip	-PQEMAAAGFYFTGVKSGIQCFCCSLILFGAGLTRLPIEDHKRFHPDCGFL----------LNKDVGNIAKYDIRVKNLKSRLRGG	152
Aciap2		0
Miap1	SYAMSTEEARFLTYSMWP-LSF-LSPAELARAGFYYIGPGDRVACFACGGKLSNWEPKDDAMSEHRRHFPHCPFL----------ENTS-ET----------QRFSIS-----NLSMQTHSARL	266
Hiap1	SYAMSTEEARFLTYHMWP-LTF-LSPSELARAGFYYIGPGDRVACFACGGKLSNWEPKDDAMSEHRRHFPNCPFL----------ENSL-ET----------LRFSIS-----NLSMQTHAARM	273
Hiap2	HCAMNNENARLLTFQTWP-LTF-LSPTDLAKAGFYYIGPGDRVACFACGGKLSNWEPKDNAMSEHLRHFPKCPFI----------ENQLQDT----------SRYTVS-----NLSMQTHAARF	259
Hilp	NPAMYSEEARLKSFQNWPDYAH-LTPRELASAGLYYTGIGDQVQCFCCGGKLKNWEPCDRAWSEHRRHFPNCFFVLGRNLNIRSESDAVSSD----------RNFPNSTNLPRNPSMADYEARI	269
Cpiap	GIDVCGSIVTTNNIQNTTTHDTIIGPA----------	112
Opiap	N--------NAHDTPHDRAPPARSAAA----------	115
Diap1	DICGANDSTLEMREHAYAEGVI-PMSQLIQSIGMNAVNAAGSVTGTAAPQPRVTVATHASTATQ----------ATGDVQPETCR----------PSAASGNYFPQYPEYAIETARL	230
Hziap	IMSDANVCSEQKYIADQEFYKNKSKLSS----------	121
Dilp	GPLIEFATGKNLDELGIQPTT----------	216
Aciap	GDNITINAVLVKEGKPKCVYRCMSN----------	135
Naip	KMRYQEEEARLASFRNWPFYVQGISPCVLSEAGFVFTGKQDTVQCFSCGGCLGNWEEGDDPWKEHAKWFPKCEFLRSKKSSEEITQYIQSYKGFVDITGEHFVNSWVQRELPMASAYCNDSIFAYEELRL	282
Aciap2	MNLMQFNFLILSTDGRF	17



RING finger motif

Figure 3.10.

Alignment of the RING finger domains of baculovirus IAPs and other proteins.

Baculovirus IAPs and IAPs from other organisms share two conserved domains: BIR repeats and RING finger motifs. The RING finger motif (C_3HC_4) has characteristic conserved cysteine and histidine residues, shown as a consensus sequence in the diagram.

The protein sequences grouped at the top of the alignment are from the baculovirus IAPs. Identical residues are shown in bold. Residues which differ between functional baculovirus IAPs (CpGV IAP and OpMNPV IAP) and non-functional IAPs (AcMNPV IAP1 and HzSNPV IAP) are denoted by X.

The second group of the alignment are the non-viral IAPs (excluding NAIP which does not contain the RING finger motif).

The third group of the alignment are the non-IAP proteins which have also been implicated in the control of apoptosis. These include the TRAF signal transducer proteins (excluding TRAF1 which does not contain a RING finger motif).

The numbers at the bottom of the alignment refer to the amino acid position within the motif. The numbers adjacent to the name of the protein indicate the position of the motif within the protein sequence.

```
                          X  XX XXXX  X  X XXX XX     XX
CpGV iap          228  CKICYVEECIVCF.VPCGHVVACAKC..A.LSVD..KCPMCR
OpMNPV iap        221  CKICLGAEKTVCF.VPCGHVVACGKC..A.AGVT..TCPVCR
HzSNPV iap        221  CKVCFVNERNYMF.LPCHHLACCEEC..A.FKVK..KCVVCR
AcMNPV iap1       238  CKVCLERQRDAVL.MPCRHFCVCVQC..Y.FGLD.QKCPTCR

Miap1             565  CKVCMDREVSIVF.IPCGHLVVCQEC..A.PSLR..KCPICR
Hiap1             571  CKVCMDKEVSVVF.IPCGHLVVCQEC..A.PSLR..KCPICR
Hiap2             557  CKVCMDKEVSIVF.IPCGHLVVCKDC..A.PSLR..KCPICR
Hilp              451  CKICMDRNIAIVF.VPCGHLVTCKQC..A.EAVD..KCPMCY
Diap1             391  CKICYGAEYNTAF.LPCGHVVACAKC..A.SSVT..KCPLCR
Dilp              451  CKVCLDEEVGVVF.LPCGHLATCNQC..A.PSVA..NCPMCR

hTRAF2             34  CSACKNILRRPFQ.AQCGHRY.CSFCLTSILSSGPQNCAACV
hTRAF3             53  CEKCHLVLCSPKQ.TECGHRF.CESCMAALLSSSSPKCTACQ
hTRAF4             18  CPLCGKPMREPVQVSTCGHRF CDTCLQEFLSEGVFKCPEDQ
hTRAF6             70  CPICLMALREAVQ.TPCGHRF.CKACIIKSIRDAGHKCPVCN
c-Pml              58  CQQCQAEAKCPKL.LPCLHTL.CSGCLEA..SGM.Q.CPICQ

consensus          C..C.....C.H...C..C.....C..C.
                   |       |       |       |       |
                   1       10      20      30      40
```

Figure 3.11.

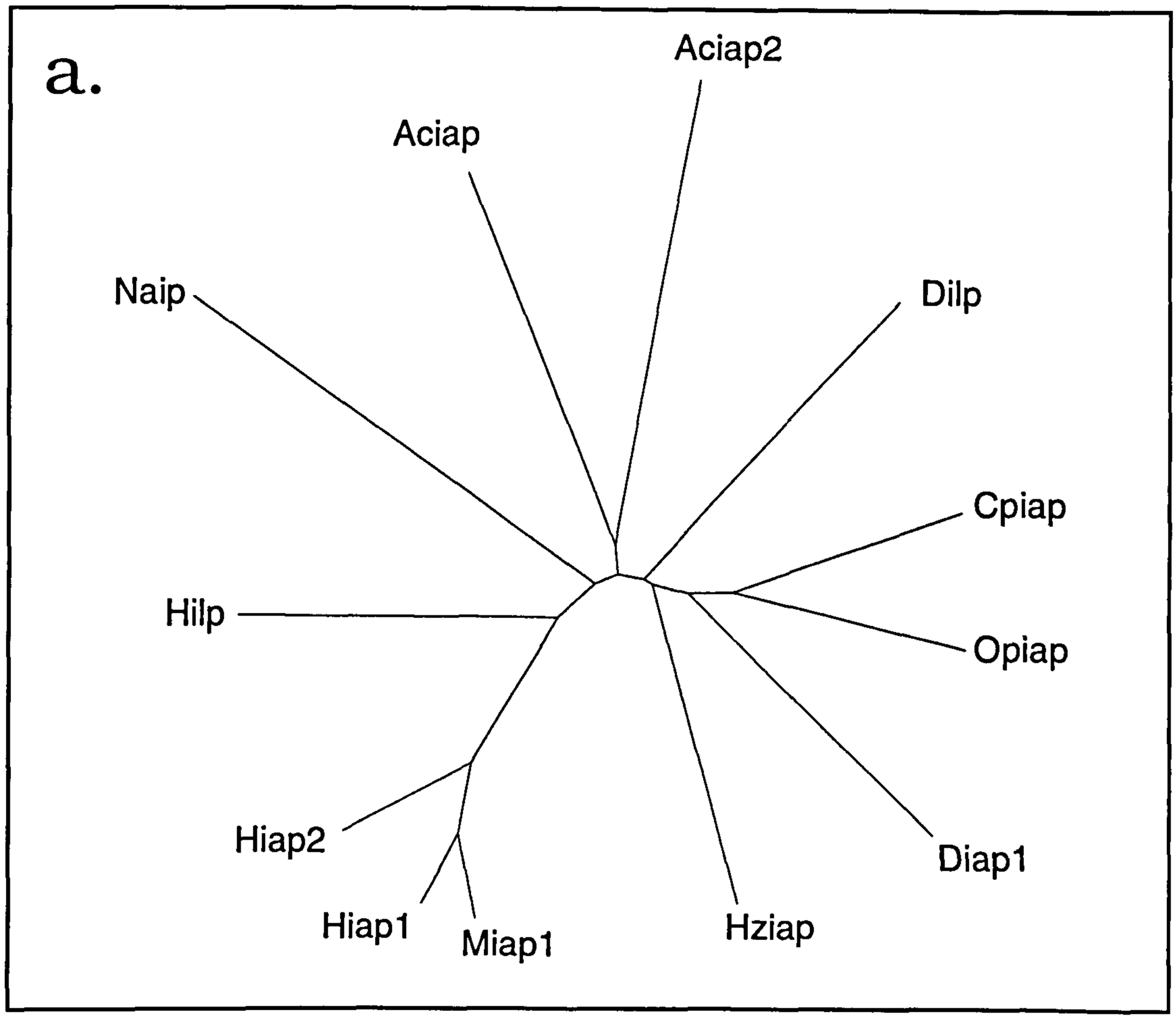
Dendrograms of the IAP protein family.

Panel a shows a radial phylogenetic tree created using Phylip drawtree. Gaps were included in the analysis, but no corrections for multiple substitutions were introduced.

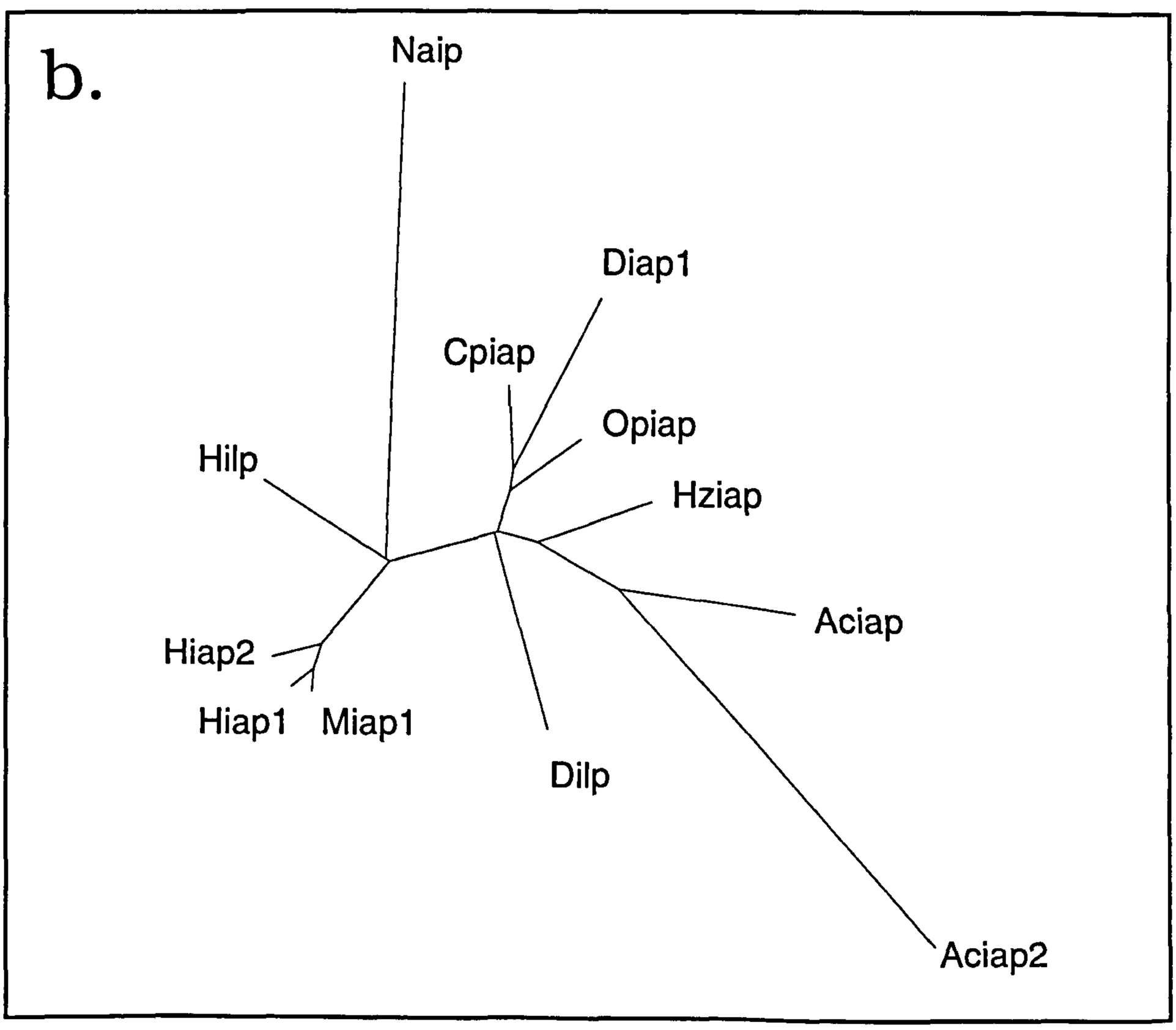
Panel b shows the same data represented as a radial phylogenetic tree (created using Phylip drawtree), but includes corrections for multiple substitutions.

Panel c shows a bootstrapped tree (created using TreeView), including corrections for multiple substitutions. The tree was iterated, and the numbers on the branches indicate the number of times (out of 1000) that the branch was included in the tree. This gives an indication of the reliability of the branch pattern.

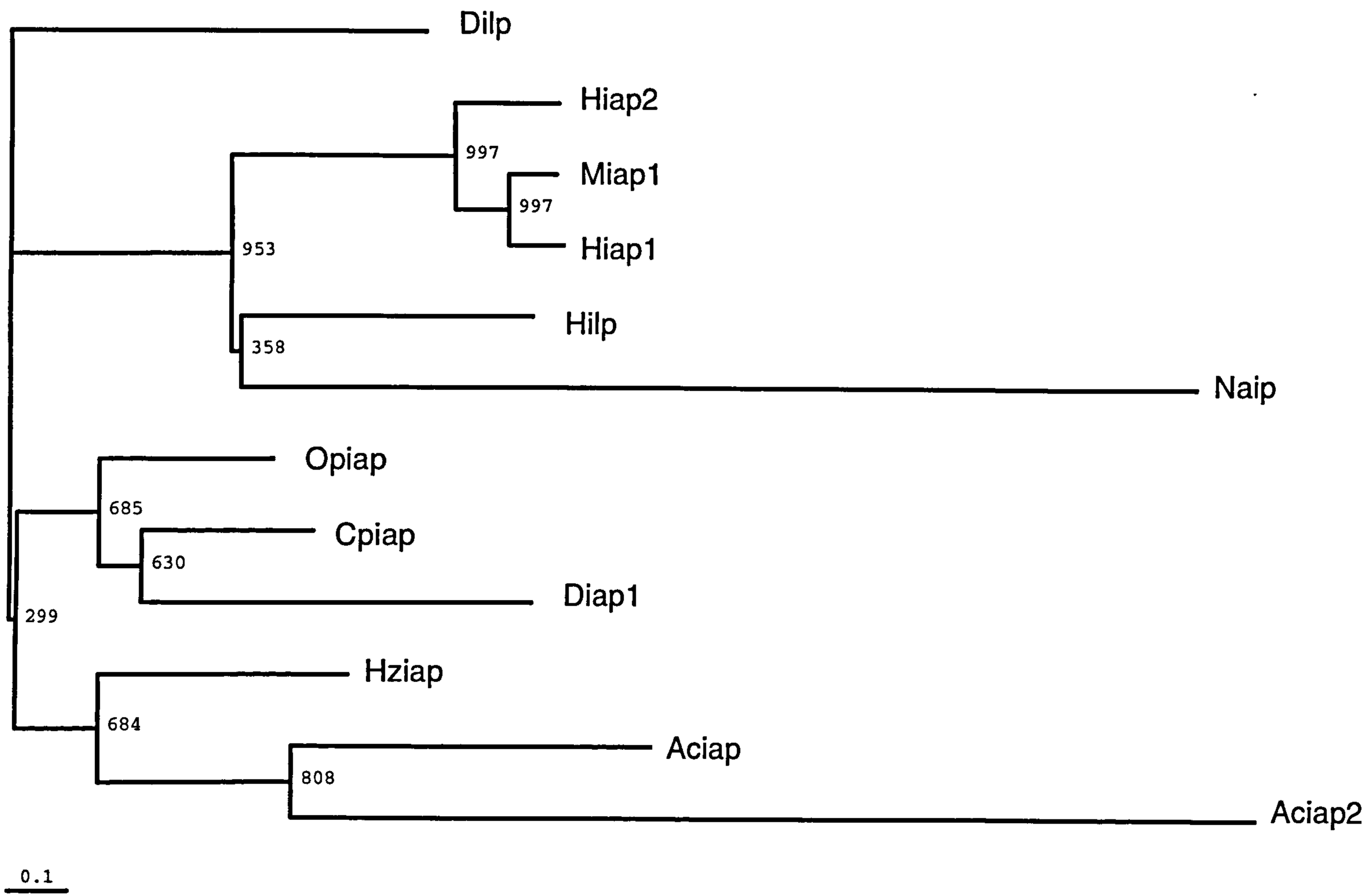
a.


Aciap2
Aciap
Naip
Dilp
Cpiap
Hilp
Opiap
Hiap2
Diap1
Hiap1
Miap1
Hziap

b.


Naip
Diap1
Cpiap
Opiap
Hilp
Hziap
Aciap
Hiap2
Hiap1
Miap1
Dilp
Aciap2

C.


Dilp
Hiap2
997
Miap1
997
Hiap1
953
Hilp
358
Naip
Opiap
685
Cpiap
630
Diap1
299
Hziap
684
Aciap
808
Aciap2
0.1

Tables 3.4 a and b.

Distance matrices from the IAP dendrograms.

The pairwise distance matrices calculated in the ClustalW program (used for the creation of phylogenetic trees) are shown below. Table a gives the data with no corrections for multiple substitutions (Figure 3.11a) and Table b. includes corrections for multiple substitutions (Figure 3.11b).

Table 3.4a.

Miap1	Hiap1	Hiap2	Hilp	Cpiap	Opiap	Diap1	Hziap	Dilp	Aciap	Naip	Aciap2
0											
0.155	0										
0.287	0.267	0									
0.577	0.578	0.568	0								
0.687	0.676	0.655	0.633	0							
0.652	0.648	0.638	0.663	0.424	0						
0.755	0.758	0.741	0.732	0.540	0.572	0					
0.682	0.693	0.683	0.701	0.582	0.566	0.640	0				
0.695	0.708	0.694	0.688	0.616	0.619	0.675	0.640	0			
0.727	0.720	0.729	0.707	0.701	0.704	0.738	0.693	0.753	0		
0.767	0.769	0.775	0.761	0.763	0.772	0.835	0.786	0.814	0.833	0	
0.799	0.811	0.824	0.818	0.832	0.779	0.810	0.762	0.793	0.774	0.904	0

Table 3.4b.

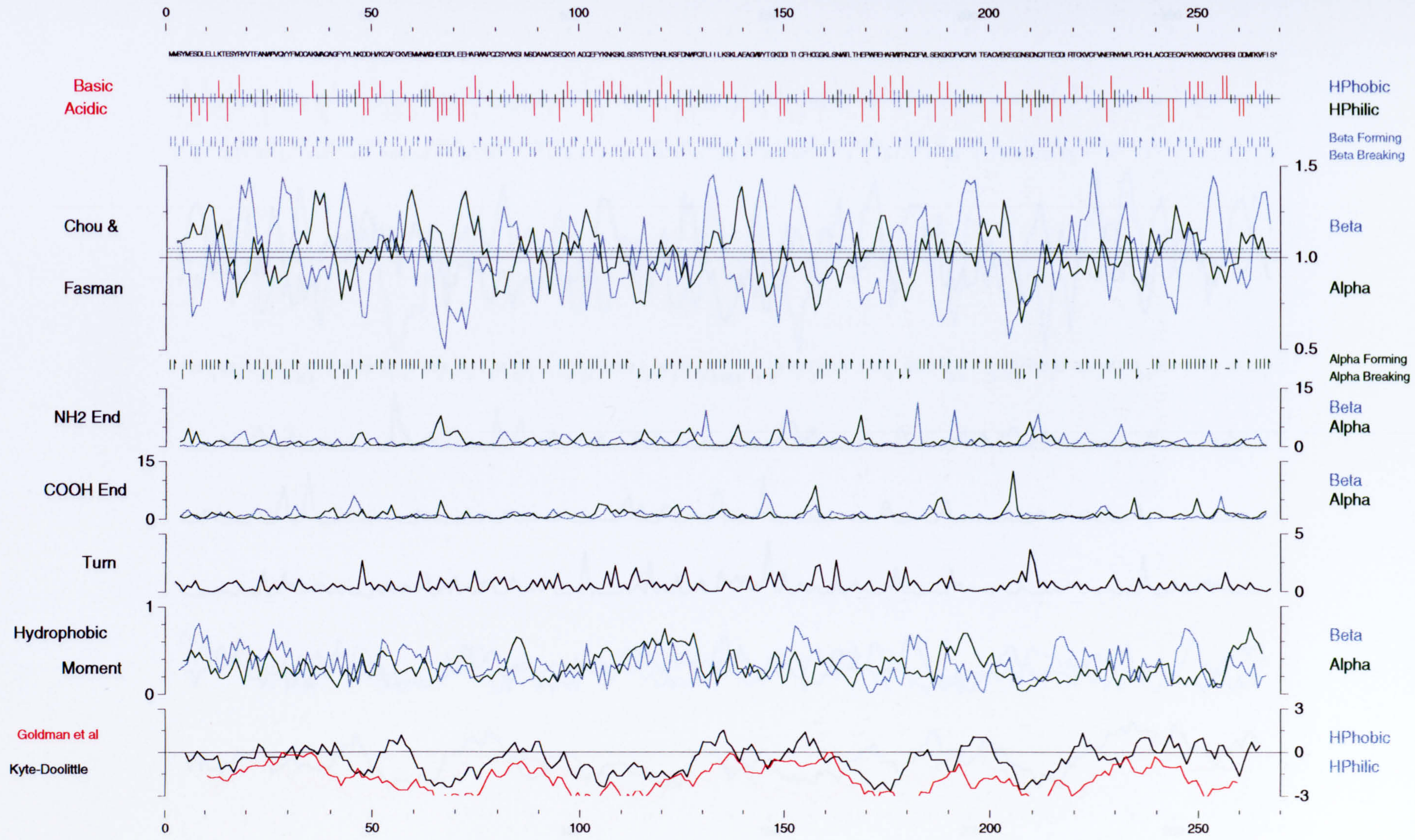
Miap1	Hiap1	Hiap2	Hilp	Cpiap	Opiap	Diap1	Hziap	Dilp	Aciap	Naip	Aciap2
0											
0.175	0										
0.362	0.330	0									
1.033	1.036	1.002	0								
1.522	1.460	1.348	1.250	0							
1.334	1.316	1.271	1.388	0.615	0						
2.000	2.020	1.900	1.826	0.912	1.016	0					
1.490	1.555	1.497	1.607	1.049	0.993	1.282	0				
1.567	1.653	1.560	1.523	1.176	1.189	1.454	1.278	0			
1.788	1.734	1.803	1.643	1.605	1.626	1.881	1.554	1.970	0		
2.090	2.110	2.170	2.050	2.060	2.140	2.980	2.290	2.640	2.940	0	
2.450	2.610	2.790	2.700	2.940	2.220	2.600	2.050	2.370	2.170	5.380	0

Figure 3.12.

The structural arrangement of the HzSNPV NC-1 IAP protein compared to CpGV IAP.

The structural profiles were generated using the GCG program PEPPLOT. The top line shows the amino acid sequence of the IAP protein. The second line represents the basic/acidic nature of the residues and below the beta-forming/breaking propensity of each residue. The Chou and Fasman plot represents the propensity of each window (size = 7 residues) to form α-helix or β-sheet. Turns in the peptide chain are predicted. The Goldman (solid line) and Kyte-Doolittle (broken line) plots predict the hydrophilicity/ hydrophobicity of the protein over a window of 7 residues (Kyte and Doolittle, 1982).

a. HzSNPV *iap*


0
50
100
150
200
250
Basic
Acidic
HPhobic
HPhilic
Beta Forming
Beta Breaking
Chou &
Fasman
1.5
1.0
0.5
Beta
Alpha
Alpha Forming
Alpha Breaking
NH2 End
15
0
Beta
Alpha
COOH End
15
0
Beta
Alpha
Turn
5
0
Hydrophobic
Moment
1
0
Beta
Alpha
Goldman et al
Kyte-Doolittle
3
0
-3
HPhobic
HPhilic
0
50
100
150
200
250

b. CpGV *iap*

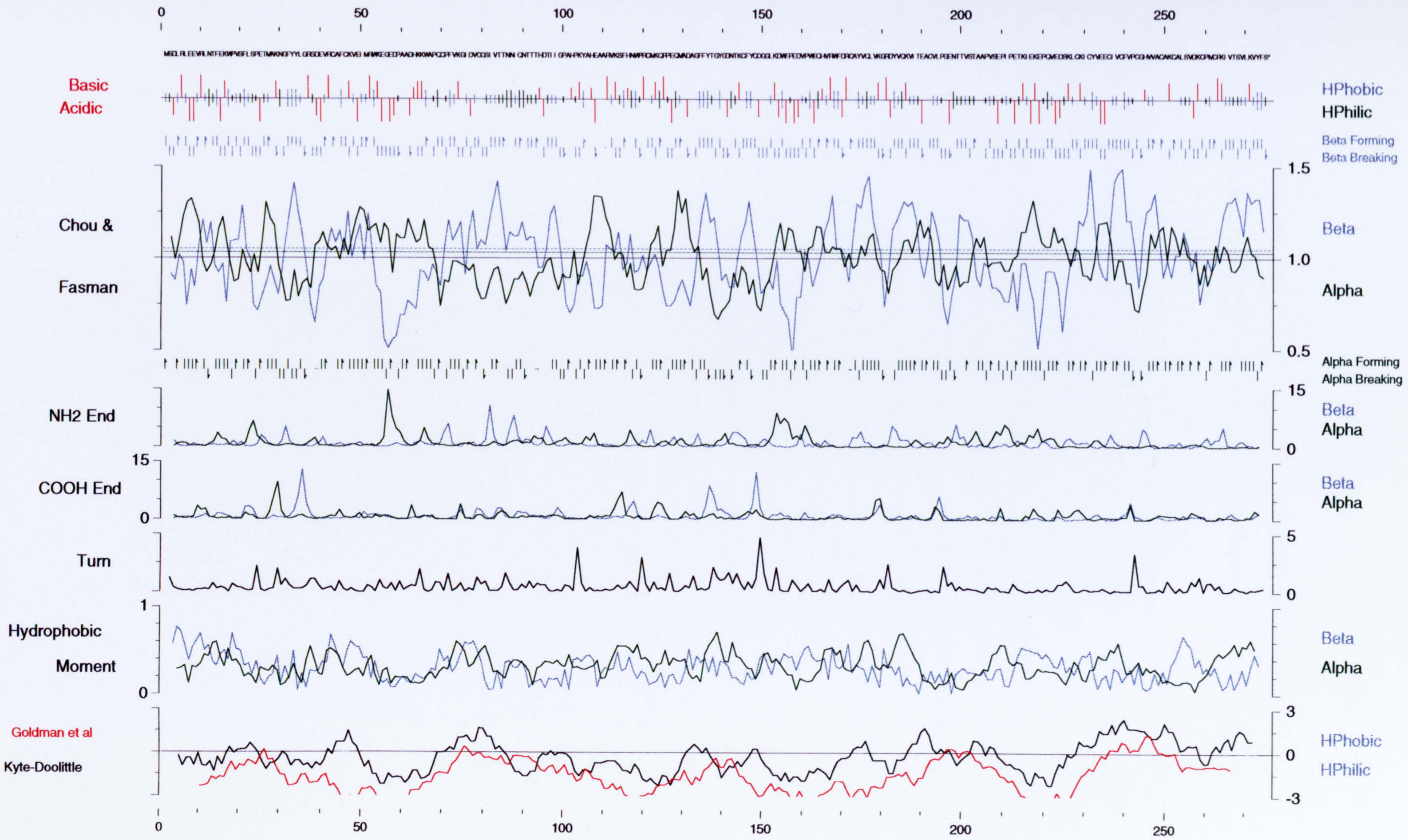

0
50
100
150
200
250
Basic
Acidic
HPhobic
HPhilic
Beta Forming
Beta Breaking
Chou &
Fasman
1.5
1.0
0.5
Beta
Alpha
Alpha Forming
Alpha Breaking
NH2 End
15
0
Beta
Alpha
COOH End
15
0
Beta
Alpha
Turn
5
0
Hydrophobic
Moment
1
0
Beta
Alpha
Goldman et al
Kyte-Doolittle
3
0
-3
HPhobic
HPhilic
0
50
100
150
200
250

Figure 3.13.

Mapping the transcription start site(s) of the mRNA transcribed by the HzSNPV NC-1 *iap* gene using primer extension analysis.

Total RNA was purified from mock-infected *H. zea* cells or cells infected with HzSNPV NC-1 for various times. Primer extension analysis was performed on the RNA preparations, using an oligonucleotide with the 5' nucleotide complimentary to a sequence 79 nucleotides downstream of the translation start site (taken as the second methionine start codon, underlined) within the putative *iap* mRNA (detailed below). The sequence motif TATA and the putative early and late start transcription initiation sites, CAGT and TAAG respectively, are also underlined.

The products of the reactions were analysed on a 6% polyacrylamide gel, adjacent to a corresponding sequencing ladder derived from the same primer, to enable the exact location of the transcription start site or sites to be determined.

```
CATTATTTGTCAAATCTAATAAACAAAATTTACGTAAAATACGACGCAATAGTTTAGTAA

                                                    EcoR1
GCTCTTTTATGTTAGATATAAAAAAGCCTGTCTTGTCGATAGAGAATTCAGTAGACGTTG

                    ⇒ IAP
TCCTTCGCCGCTTGTGCGATGATGTCCTATATGGAATCCGATTTGGAATTATTAAAAACT
SerPheAlaAlaCysAlaMetMetSerTyrMetGluSerAspLeuGluLeuLeuLysThr

                                PRIMER
          ClaI        3' ACGTTTAACCGGACATG 5'
GAATCATATCGATATGTTACGTTTGCAAATTGGCCTGTACAATATTATTTTATGGATTGT
GluSerTyrArgTyrValThrPheAlaAsnTrpProValGlnTyrTyrPheMetAspCys

GCTAAAATGGCTCAGGCCGGTTTCTATTATTTGAACAAAGACGACCATGTAAAATGTGCG
AlaLysMetAlaGlnAlaGlyPheTyrTyrLeuAsnLysAspAspHisValLysCysAla
```

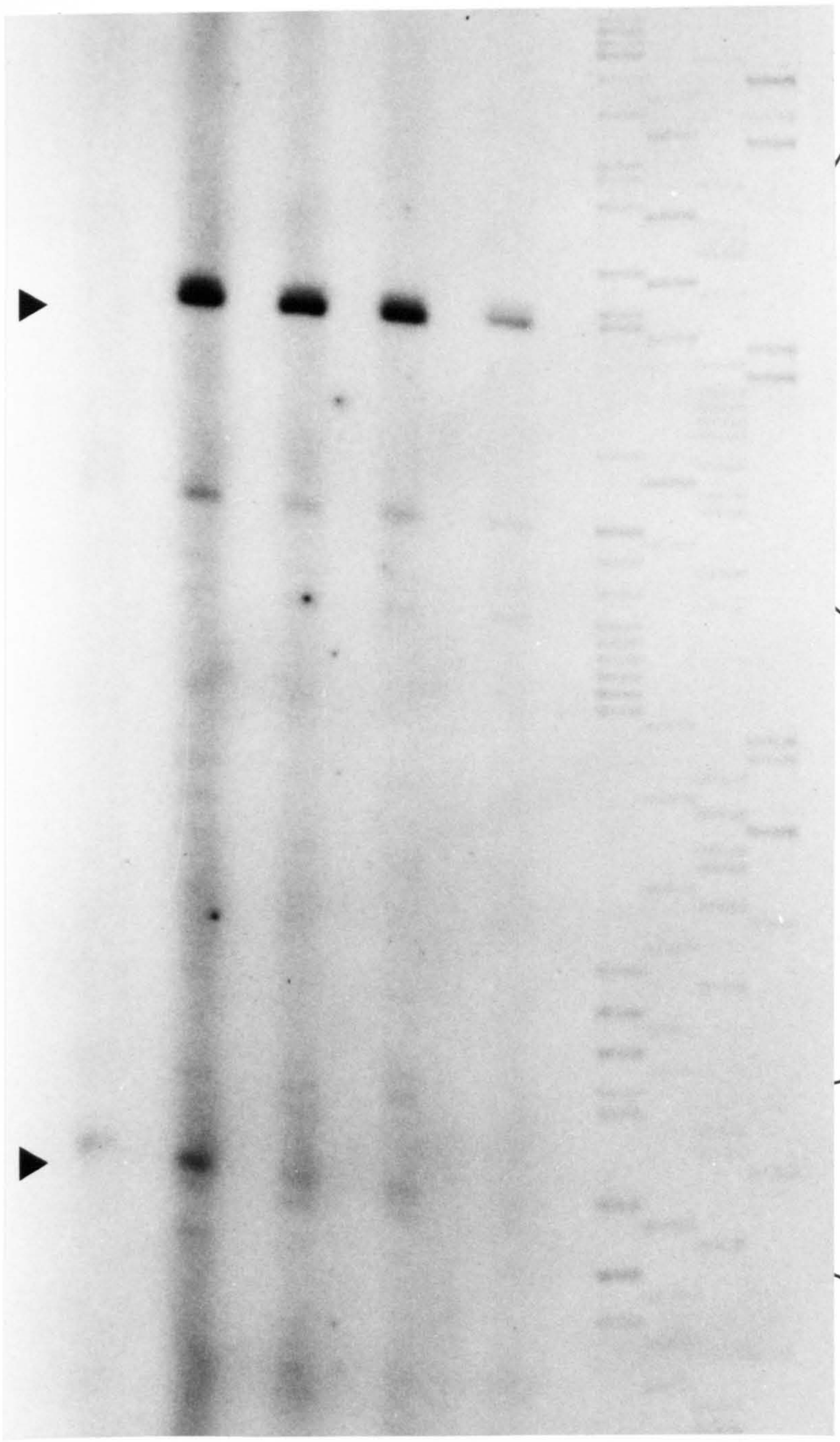

hours post infection
12 24 36 48 72 T C A G
A
A
A
T
C
AT
TA
TA
CG
G
A
G
A
A
A
A
T
A
C
A
A
T
C
T
A
A
A
GC
TA
CG
AT
T
C
T

Figure 3.14.

Alignment of the amino acid sequences of the ORF 2 proteins of HzSNPV NC-1, AcMNPV and BmNPV.

The GCG program PRETTYPLOT was used to display the alignment created in the PILEUP program. Identical residues and/or conservative changes are boxed. The BmNPV genome (Accession number: L33180) contains three repeats of this ORF; Bmorf2-1 (position 125983 - 124934 bp), Bmorf2-2 (position 77570 - 78526 bp) and Bmorf2-3 (position 21805 - 20852 bp).

```
Acorf2    --MARVKIGEFKFGEDTFNLRYVLERDQ-QVRFVAKDVANSLKYTVCDKAIRVHVDNKYKSLF-E-QTIQ  65
Bmorf2-1  --MAQVKIGQFKFGQDTFTLRYVLEQGNPQVKFVAKDIASSLKYGNCKDAVSRHVDKKYKYTYSE-SGAR  67
Bmorf2-2  --MAQVKIGEFKFGEDTFTLRYVLGDEQ-PVRFVAKDIASSLKYVNCERAIRVHVDGKYKSTFEH-ADQI  66
Bmorf2-3  --MAQVKIGEFKFGEDTFTLRYVLEQGNLQVKFVAKDIASSLKYVNCKQAVIVNVDKKYKTTYSE-SGSI  67
Hzorf2    MYLVNRKC---KLGEV-----WITEIENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVL  62

Acorf2    NGGPTSNSVVKRGDPLYLQPHTVLITKSGVIQLIMKSKLPYAIELQEWLLEEVIPQVLCTGKY-------  128
Bmorf2-1  LPPSAPNSVAKQGDPLYLQPHTVLITKSGVIQLIMKSKLPYAIELQEWLLEEVIPQVLCTGKY-------  130
Bmorf2-2  QH-HAPDSVAKQGDPLYLHPHTVLITKSGVIQLIMKSKLPYAIELQEWLLEEVIPQVLCTGKY-------  128
Bmorf2-3  PYTPAPDNVVKQGDPLYLQPHTVLITKEGVIQLIMKSKLPYAVELQAWLLEEVIPQVLCTGKY-------  130
Hzorf2    NQHSLVTSSDSIEMPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQ  132

Acorf2    ----------------------DPAIKQREEESKQLVTKLIATFTEHTNALQAVVAQKTEELVKKQEFIE  176
Bmorf2-1  ----------------------DPAIKQQEEKNKQLVTKLIATFTEHTNALQAVVAQKTEELFKKQEFIE  178
Bmorf2-2  ----------------------APAVEMDTNDVIAKIDDLTQKLTVANADLAE--ANRSLILFANEMIVA  174
Bmorf2-3  ----------------------APAVKMDTNYGV--IEELNKKLAFASESLAE--ANEKIIHFANALVTA  174
Hzorf2    SSTDNSTEVVSYDQKLANVQMEALQLLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMK  202

Acorf2    TDLVAKVVDLSDR----------------------AVQYPADKRKHPVLCVTRDGTTFTAITGQKTYV-----  271
Bmorf2-1  SNLIAKMIDLSDR----------------------AVQYPADKRKHPVLCVTRDGTTFTAITGQKTYV-----  280
Bmorf2-2  CHSLA-VCDVGNN----------------------EFAFLRPQKRSLGRSLKRLGSNDVIFSSD--YV-----  258
Bmorf2-3  LHSLA-VCALGGE----------------------KYAFLRAQKRSLNRSIKRLGSSDVVFSSD--YV-----  258
Hzorf2    CRSQLYAIEMQDKVAKRYRDTLCTPKRFKPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLR  342

Acorf2    ----------------------ENQKHKRNINVANIVVENIRPNPTVDWNNATDRLQAKRSKRSIVL--  316
Bmorf2-1  ----------------------ESQKHKRNIDAANIVVENIRPNPTVDWNNATDRLQSKRSKRSISFDS  327
Bmorf2-2  ----------------------PN-----SMNVLNKVKEAIPRNKFKAKHNRITLLED--YTREELMNV  298
Bmorf2-3  ----------------------PN-----AMNVLNKVKETLPRNQYKAKHNKITLLQN--LTRELLIDA  298
Hzorf2    YTNKLCTEMEVLDETELRAKYRADVEMCQRNKTVNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINA  412

Acorf2    ----------VRWKKRNNLKIG----------------  328
Bmorf2-1  LEEAQQFENRIKYLLKNANNIN----------------  349
Bmorf2-2  IGSTMTDRQIARMNSLRNAQ----------------  318
Bmorf2-3  VQSSMTERQIARLNNNFNL----------------  317
Hzorf2    IVENIVEKMAKELVPSTPQRRHSNAGDVYRHNKLCTP-  449
```

Table 3.5.

Percentage amino acid similarity and identity, calculated using a GAP algorithm, for the AcMNPV ORF 2 homologues in BmNPV and HzSNPV NC-1.

The calculations were performed using a gap weight of 3 and a length weight of 0.1.

Sequence Pair	*Quality*	*Ratio*	*% Similarity*	*% Identity*
Acorf2 x Bmorf2-1	409.8	1.25	87.2	80.9
Acorf2 x Bmorf2-1	226.8	0.71	61.8	46.2
Acorf2 x Bmorf2-3	208.8	0.65	58.2	43.4
Acorf2 x Hzorf2	142.7	0.43	44.1	25.6
Bmorf2-1 x Hzorf2	148.9	0.43	42.9	25.6
Bmorf2-1 x Bmorf2-2	222.6	0.69	61.4	44.6
Bmorf2-1 x Bmorf2-3	231.5	0.73	60.3	46.9
Bmorf2-2 x Bmorf2-3	356.4	1.12	80.4	69.3
Bmorf2-2 x Hzorf2	148.5	0.46	52.2	25.8
Bmorf2-3 x Hzorf2	144.2	0.45	50.8	28.5

Figure 3.15.

Alignment of the amino acid sequences of the ORF 106/107-homologues of HzSNPV NC-1, BmNPV, OpMNPV and AcMNPV.

The GCG program PRETTYPLOT was used to display the alignment created in the PILEUP program. Identical residues and/or conservative changes are boxed.

The stop codon present at the 3' end of AcMNPV ORF 106 is indicated by an arrow, and the methionine start codon of AcMNPV ORF 107 is indicated by an asterisk.

```
Bm106 --MTSNNFVDVDTFARQLITDKCSALIKNADLLPANILEIVEKARDRYFNEPTQKNYEYI  58
Op106 -MDAKYKVVDVDTFARQLITDKCSELIETENLLPANILHVVKQARDKYFEDPSVKNYEYV  59
Hz106 -----MESIDVDDFAKQLIADKCSALIESNKMLSPDMMAMVKLARDEYFKDPSSKNYEIL  55
Ac106 NIMTSNNFVDVDTFARQLITDKCSALIKV-RICCRQTF-RL-RRPETSILRANSKKL-IH  56

Bm106 KKLFLRTKYMDDSIDYKDFNRRILLIVFKFALNKSASYFPSYKEIIEVAIKRLNKINPDL 118
Op106 KNLFLRTKYMDDSIDYKNFNRRVLLIVFKFALNRGSGYFPSYRELIEVAVKRLNKINPDL 119
Hz106 KKLIGHTKYVDDSIDCKDFNRRMLLIAIKVSASRARDYFNKYKTVFELALKRLDSINPDI 115
Ac106 -KIIFTKKYMDDSIDYKDFNRRILLIVFKFALNKS-TILSIVQRDHRVAIKRLNKINPDL 114

Bm106 KSSPRAMLQHYNECLENLDNPVTDEHHLLT--FGKEVATKIFIEAFE-YSYTNTNAISMD 175
Op106 KSSPRAMLQHYNECLENLDNPVTDEHHLLT--FGKEVATKMFIEAFE-FSYASNNEINLT 176
Hz106 RSSPSALLQHYKECLDNLDNPRKDEHHLVT--FAKEIATKIFIDTIDVYSYTNKSSIQMT 173
Ac106 KSSPRN-----ASALQ-MFGKSRQSSHGRTSFVDKRVATKIFIEAFE-YSYTNTNAISMD 167
                  ▲          *

Bm106 KTD-EFDFIKQPTLKSLPDARPPSLLTSVMNERKRKLQ---NTNSKAKCLLPAPPS---- 227
Op106 TNKRGSDLFDPIPMPAPAPAPSASLLDNVMNERKRKLQASVTTTPPKRCKLADRPAQTTQ 236
Hz106 TT-----------------STRNQCASSLSANYLSNRKATSTDSLASENITVERVSQATTQAEK 220
Ac106 KTD-EFDFIKP-ALKPLPDARPPSLLANVMNERKRKLQ---NTNSTAKCLLPAPP----- 217

Bm106 -PPPPPQLRKLEKKNHLLPLFSL- 249
Op106 DTPRAPQPAPVRAQRPLFTL---- 256
Hz106 QYNFIRQQS--------------- 229
Ac106 -----PQLRKLEKKNHLLPLFSL- 235
```

Table 3.6.

Percentage amino acid similarity and identity for the baculovirus homologues of HzSNPV ORF 106, calculated using a GAP algorithm.

AcMNPV ORF 106/107 is not represented due to the likely presence of sequence errors. The calculations were performed using a gap weight of 3 and a length weight of 0.1.

Sequence Pair	*Quality*	*Ratio*	*% Similarity*	*% Identity*
Hz106 x Op106	197.8	0.86	67.7	48.5
Hz106 x Bm106	196.8	0.86	67.8	49.0
Op106 x Bm106	262.9	1.05	75.7	64.4

Figure 3.16.

Alignment of the amino acid sequences of superoxide dismutase (SOD) proteins.

Superoxide dismutase sequences were incorporated into a multiple amino acid sequence alignment using the PILEUP program within GCG. The data was displayed via PRETTYPLOT. Boxed regions represent amino acid residues which are identical or similar. The signature patterns characteristic of Cu/Zn superoxide dismutases are marked as I and II (Prosite - PS00087, Prosite - PS00332) (Bairoch, 1993).

The protein sequences used in this analysis are:

Code	**Organism**	**Accession Number**
Acsod	AcMNPV (baculovirus)	L22858
Hzsod	HzSNPV NC-1 (baculovirus)	from Figure 3.5.
Rnsod	*Rattus norvegicus* (rat)	PIR - JC1192
Btsod	*Bos taurus* (cow)	PIR - DSBOCZ
Ocsod	*Oryctolagus cuniculus* (rabbit)	PIR - S33162
Dssod	*Drosophila simulans* (fruitfly)	PIR - S05498
Casod	*Chymomyza amoena* (fruitfly)	PIR - S48117
Ccsod	*Ceratitis capitata* (Mediterranean fruitfly)	PIR - A45171
Ossod	*Oryza sativa* (rice)	PIR - S26354
Zmsod	*Zea mays* (maize)	PIR - A29077
Npsod	*Nicotiana plumbaginifolia* (tobacco)	PIR - JQ1334
Pssod	*Pinus sylvestris* (Scotch pine)	PIR - S20511
Smsod	*Schistosoma mansoni* (fluke)	PIR - A49241
Cesod	*Caenorhabditis elegans* (nematode)	PIR - A48256
Scsod	*Saccharomyces cerivisiae* (yeast)	PIR - DSBYC

Prosite I

```
Acsod  - - - MKAICILSG- - DVHGKIYFQQESANQPLKISGYLLNL-PRGLHGFHVHEYGDTSNGCTSAGEHFNPTNEDHG  69
Hzsod  - - - MKAICILSG- - DISGEICFSQESPLHLIKITGFFILNL-PRGLHGIHVHEFGDTSNGCTSAGEHFNPTGQTHG  69
Rnsod  - MAMKAVCVLKGDGPVQGVIHFEQKASGEPVVVSGQITGLTE-GEHGFHVHQYGDNTQGCTTAGPHFNPHSKKHG  73
Btsod  - - ATKAVCVLKGDGPVQGTIHFE- - AKGDTVVVTGSITGLTE-GDHGFHVHQFGDNTQGCTSAGPHFNPLSKKHG  70
Ocsod  - MATKAVCVLKGDGPVEATIHFEQKGTG-PVVVKGRITGLTE-GLHEFHVHQFGDNRQGCTSAGPHFNPLSKKHG  72
Dssod  - MVVKAVCVING- - DAKGTVFFEQESSGTPVKVSGEVCGLAK-GLHGFHVHEFGDNTNGCMSSGPHFNPYGKEHG  71
Casod  - MVAKAVCVING- - DAKGTVYFEQEDACAPVKVCGEITGLNK-GQHGFHVHEFGDNTNGCMSSGPHFNPLNKEHG  71
Ccsod  - MVVKAVCVING- - DVKGTVHFEQQDAKSPVLVTGEVNGLAK-GLHGFHVHEFGDNTNGCTSAGPHFNPYGNSHG  71
Ossod  - - MVKAVAVLASSEGVKGTIFFSQEGD-GPTSVTGSVSGLKP-GLHGFHVHALGDTTNGCMSTGPHFNPTGKEHG  71
Zmsod  - - MVKAVAVLAGTD-VKGTIFFSQEGD-GPTTVTGSISGLKP-GLHGFHVHALGDTTNGCMSTGPHFNPVGKEHG  70
Npsod  - - MVKAVAVLSSSEGVSGTIFFTQDGD-APTTVTGNVSGLKP-GLHGFHVHALGDTTNGCMSTGPHYNPAGKEHG  71
Pssod  MGLLKAVVVLNGAADVKGVVQFTQEGD-GPTTVTGKISGLSP-GLHGFHVHALGDTTNGCMSTGPHFNPLGKEHG  73
Smsod  - - - MKAVCVMTGTAGVKGVVKFTQETDNGPVHVHAEFSGLKA-GKHGFHVHEFGDTTNGCTSAGAHFNPTKQEHG  71
Cesod  - MSNRAVAVLRGE-TVTGTIWITQKSENDQAVIEGEIKGLTP-GLHGFHVHQYGDSTNGCISAGPHFNPFGKTHG  72
Scsod  - - MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPFKKTHG  73

Acsod  APDAEIRHVGDLGNIKSAGGYNSLTEVNMMDNVMSLYGPHNIIGRSLVVHTDKDDLGLT- - - DHPLSKTTGNSGGR  141
Hzsod  APNATVRHVGDLGNVESFGINSLTEVNIVDNVMSLFGPHSILGRSLVVHTDRDDLGLT- - - DHPLSRITGNSGGR  141
Rnsod  GPADEERHVGDLGNVA-AGKDGVANVSIIEDRVISLSGEHSIIGRTMVVHEKQDDLGKG- - - GNEESTKTGNAGSR  144
Btsod  GPKDEERHVGDLGNVT-ADKNGVAIVDIVDPLISLSGEYSIIGRTMVVHEKPDDLGRG- - - GNEESTKTGNAGSR  141
Ocsod  GPKDEERHVGDLGNVT-AGSNGVADVLIEDSVISLSGDMSVIGRTLVVHEKEDDLGKG- - - GNDESTKTGNAGSR  143
Dssod  APVDENRHLGDLGNIE-ATGDCPTKVNITDSKIITLFGADSIIGRTVVVHADAADDLGQG- - - GHELSKSTGNAGAR  142
Casod  APTDENRHLGDLGNIE-APGDGPTKVCINDSKITLFGENSIVGRTVVVHADPDDLGKG- - - GHELSKSSTGNAGAR  142
Ccsod  APSDLNRHLGDLGNIE-ASGDGATKVEISDKLITLFGENSIVGRTIVVHADPDDLGKG- - - GHELSKTTGNAGAR  142
Ossod  APQDENRHAGDLGNIT-AGADGVANVNVSDSQIIPLTGAHSIIGRAVVVHADPDDLGKG- - - GHELSKTTGNAGGR  142
Zmsod  APEDEDRHAGDLGNVT-AGEDGVVNVNITDSQIIPLAGPHSIIGRAVVVHADPDDLGKG- - - GHELSKSTGNAGGR  141
Npsod  APEDEVRHAGDLGNIT-VGEDGTASFTLTDKQIIPLAGPQSIIGRAVVVHADPDDLGKG- - - GHELSKTTGNAGGR  142
Pssod  APTDDNRHAGDLGNVT-VGTDGTVEFSITDSQIIPLSGPHSIVGRAVVVHADPDDLGKG- - - GHELSKSTGNAGGR  144
Smsod  APEDSIRHVGDLGNVV-AGADGNAVYNATDKLISLNGSHSIIGRSMVIHENEDDLGRG- - - GHELSKVTGNAGGR  142
Cesod  GPKSEIRHVGDLGNVE-AGADGVAKIKLTDTLVTLYGPNTVVGRSMVVHAGQDDLGEGVGDKAEESKKTGNAGAR  146
Scsod  APTDEVRHVGDMGNVK-TDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKG- - - DTEESLKTGNAGPR  144

                                                                                  Prosite II
Acsod  LGCGIIAICK- - - - - - - -  151
Hzsod  LGCGIIGVTNSYKEASVK  159
Rnsod  LACGVIGIAQ- - - - - - - -  154
Btsod  LACGVIGIAK- - - - - - - -  151
Ocsod  LACGVIGISP- - - - - - - -  153
Dssod  IGCGVIGIAKV- - - - - - -  153
Casod  IGCGVIGICKI- - - - - - -  153
Ccsod  LGCGVIGICKI- - - - - - -  153
Ossod  VACGIIGLQG- - - - - - - -  153
Zmsod  VACGIIGLQG- - - - - - - -  152
Npsod  VACGIIGLQG- - - - - - - -  151
Pssod  LACGVVGLQG- - - - - - - -  152
Smsod  LACGVVGLAAE- - - - - - -  154
Cesod  AACGVIALAAPQ- - - - - -  153
Scsod  PACGVIGLTN- - - - - - - -  158
                                  154
```

Table 3.7.

Percentage amino acid similarity and identity, calculated using a GAP algorithm, for AcMNPV and HzSNPV NC-1 SOD.

The calculations were performed using a gap weight of 3 and a length weight of 0.1.

Sequence Pair	*Quality*	*Ratio*	*% Similarity*	*% Identity*
Hzsod x Acsod	190.5	1.26	89.4	74.8
Hzsod x Btsod	128.4	0.85	64.0	48.0

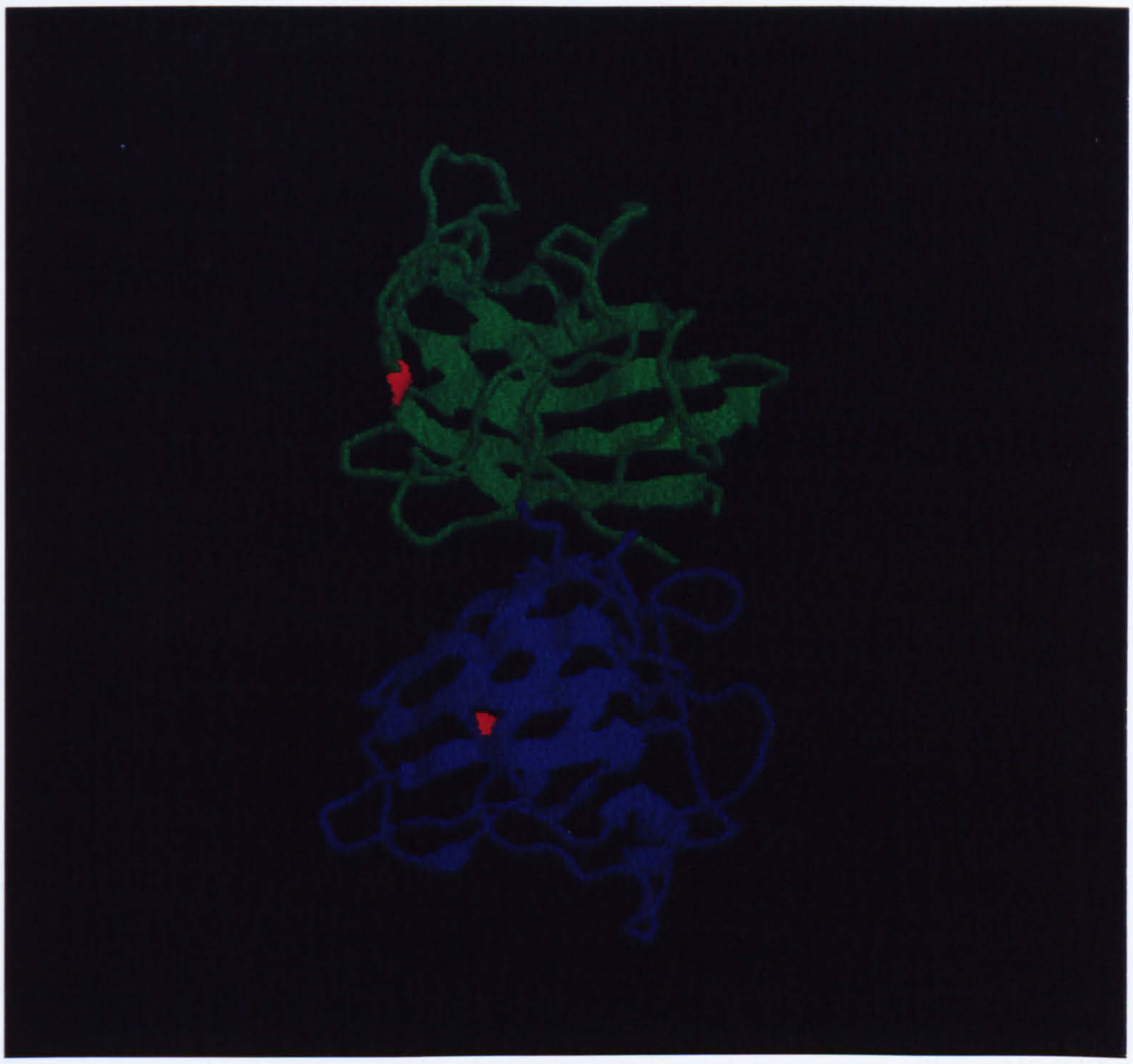
Figure 3.17.

Protein crystallography model of HzSNPV NC-1 superoxide dismutase.

The protein model was generated using co-ordinates supplied by Swiss-Model (Peitsch, 1995; 1996) and is based on the determined crystal structure of several SOD proteins. The co-ordinates were deciphered using Rasmol to produce images of the proteins. The figure shows (panel a) the crystal structure of *Bos taurus* SOD (PIR - DSBOCZ) (Djinovic *et al.*, 1992), which is represented as a dimer with the monomers coloured green and blue. The HzSNPV SOD model is shown in panel b (monomeric form). The N terminal region of the protein is shown in red and the C terminal region in blue, with a gradation in colour spectrum through the protein sequence.

X-ray crystallographic studies have identified several amino acids that are critical for superoxide dismutase activity (Djinovic *et al.*, 1992). The arginine residue at position 141 is marked in red on the crystal structure of the *Bos taurus* SOD (panel a).

a.



b.

Chapter Four

Analysis of the function of HzSNPV *iap*.

4.1. Introduction.

Previous studies conducted with the baculovirus *iap* family have focused on their ability to function in place of AcMNPV *p35* during infection of Sf21 cells with this virus. Indeed, both CpGV *iap* and OpMNPV *iap* were originally identified due to this functional ability (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). Complementation experiments were carried out using a mutant of AcMNPV deficient in *p35*, which causes apoptosis in Sf21 cells but replicates normally in *T. ni*-368 cells (Clem *et al.*, 1991).

The data described in this chapter have been separated into three sections, all of which have the common aim to characterise the function of HzSNPV *iap*. Firstly, in order to investigate whether or not HzSNPV NC-1 IAP can inhibit apoptosis, a *p35* deficient mutant of AcMNPV was constructed (Ac*p35lacZ*). Rescue experiments were performed by cotransfection of Sf21 cells with Ac*p35lacZ* DNA and plasmid DNA containing *iap*, and subsequent analysis of cells for signs of productive virus infection.

The classical approach to study gene function in baculoviruses is the generation of conditional lethal mutants, or the deletion or inactivation of specific genes by insertional mutagenesis. The consequences of the mutation on virus replication or pathogenicity can then be determined. An experiment was designed to construct a mutant of HzSNPV deficient in *iap*. The feasibility of recombinant HzSNPV production has been previously demonstrated by deletion of the polyhedrin gene of HzSNPV strain Elcar (Cowan *et al.*, 1994).

4.2. Results.

4.2.1. Complementation of *p35*-deficient AcMNPV with *iap*.

Experiments to investigate the function of baculovirus IAPs have employed an AcMNPV mutant deficient in *p35*, and examined the ability of IAP to substitute for P35 function in Sf21 cells (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). This virus was based on the AcMNPV L1 strain. In order to conduct similar experiments with HzSNPV *iap*, we devised a strategy to isolate a *p35*-deficient AcMNPV strain C6.

4.2.1.1. Construction of AcMNPV deficient in *p35*.

The *p35* coding region is located in the *Eco*RI-S fragment of AcMNPV, which had previously been cloned into the plasmid vector pUC7 by M. Ayres (Ayres *et al.*, 1994). The transfer vector, p*p35lacZ*, was constructed with the *E. coli* β-galactosidase gene (*lacZ*) inserted at the *p35* locus. Figure 4.1 illustrates the assembly of this transfer vector. The *Hind*III restriction site located 393 bp downstream of the *p35* ATG translation initiation codon in pAc*Eco*RI-S (position 116885 bp of the AcMNPV genome), was altered to *Bgl*II by the introduction of a synthetic oligonucleotide adaptor, 5' AGCTAGATCT, to generate the plasmid pAc*Eco*RI-S-*Bgl*II.

In order to achieve maximum expression of *lacZ* in the *p35*-deficient virus, so that this recombinant virus could be easily identified, the *lacZ* coding region employed was under the control of the AcMNPV polyhedrin promoter. The vector pAcRP23+8.*lacZ* (Possee and Howard, 1987) which contains the *lacZ* coding region preceded by the AcMNPV polyhedrin promoter, was employed to obtain a polyhedrin promoter/*lacZ* cassette. This 3.9 kbp DNA cassette was extracted from the vector by digestion of pAcRP23+8.*lacZ* with the restriction endonucleases *Bam*HI and *Bgl*II, gel electrophoresis of the reaction mixture and the purification of the 3.9 kbp DNA fragment from an agarose gel. This

DNA fragment was subsequently inserted into the unique *Bgl*II site of pAc*Eco*RI-S-*Bgl*II to produce the transfer vector p*p35lacZ* (Figure 4.1).

T. ni-368 cells were cotransfected with the transfer vector p*p35lacZ* and AcMNPV C6 DNA. The cotransfection medium was titrated by plaque assay and, following the addition of X-gal, blue plaques were identified. Six blue plaques were identified, and purified by further plaque assay. A homogenous stock of the AcMNPV *p35*-deficient mutant, Ac*p35lacZ*, was subsequently derived and an amplified stock of the virus was produced. Viral DNA was extracted from BV produced by Ac*p35lacZ*-infected *T. ni* cells, as described in Chapter Two (section 2.2.9.) and used for analysis by restriction enzyme digestion and for complementation assays.

4.2.1.2. Confirmation of the phenotype of Ac*p35lacZ*.

DNA fragment profiles generated using restriction enzyme digestion of Ac*p35lacZ* DNA and AcMNPV C6 DNA were compared. The DNA was transferred to a nylon membrane for Southern blot analysis, to detect the presence and position of the *lacZ* gene in Ac*p35lacZ*. A radiolabelled *lacZ* probe comprising a 3.2 kbp *Bsu*36I-*Bam*HI fragment from pAcRP23+8.*lacZ*, containing the majority of the *lacZ* gene coding region, hybridised to restriction enzyme digested DNA fragments of Ac*p35lacZ*, but did not hybridise to any fragments of AcMNPV C6 (Figure 4.2). The *Eco*RI-S fragment of Ac*p35lacZ* was modified in size due to the addition of an *Eco*RI site present at the 3' end of the *lacZ* coding region. The *lacZ* sequence co-migrates with the viral fragment *Eco*RI-L in the *Eco*RI restriction profile of Ac*p35lacZ*. An additional *Eco*RV site is introduced into Acp35*lacZ*, due to the presence of this restriction enzyme site in the *lacZ* coding region. Thus, two fragments hybridised to the *lacZ* probe in the *Eco*RV restriction profile of Ac*p35lacZ*. Therefore, the pattern of hybridisation was consistent with the correct introduction of *lacZ* at the *p35* locus (see Figure 4.2).

The phenotype of Ac*p35lacZ* in Sf21 cells was examined. Previous studies have shown that disruption of the *p35* gene of AcMNPV, by point mutation or deletion of the whole or part of the gene, results in a phenotype in Sf21 cells described as "annihilator" (Clem *et al.*, 1991). The infection of Sf21 cells with the annihilator virus results in cellular apoptosis 12-24 hours post infection. To confirm that the phenotype of Ac*p35lacZ* was identical to that of the annihilator virus, Sf21 cells were infected with Ac*p35lacZ* using an m.o.i. of 1, and incubated at 28°C. The cells exhibited apoptosis from 10 hours p.i. (Figure 4.3), characterised by the formation of cell surface protrusions which blebbed from the cell, and eventually resulted in cell breakdown. However, the cotransfection of Sf21 cells with 0.5μg of Ac*p35lacZ* DNA and 2.5μg of pAc*Eco*RI-S, and subsequent incubation of the cells for 3 days, resulted in a productive virus infection with polyhedra visible in the cells. Thus, the addition of *p35* restored the phenotype of Ac*p35lacZ* to that of wild type AcMNPV.

4.2.1.3. Complementation studies with Ac*p35lacZ* and HzSNPV NC-1 *iap*.

Studies involving OpMNPV *iap* and CpGV *iap* have shown that both these genes can substitute for AcMNPV P35 function in the annihilator mutant. This enables rescue of the virus in Sf21 cells to produce a normal virus infection, culminating in the production of occluded virus (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). The AcMNPV *iap1* and *iap2* genes do not have this ability. The HzSNPV *iap* gene demonstrates only 41% amino acid identity to OpMNPV *iap*, 39% to CpGV *iap* and 31% to AcMNPV *iap1*, suggesting that the gene may not be able to rescue the annihilator mutant of AcMNPV.

Following the methodology of Birnbaum *et al.*, (1994), complementation assays were performed with a variety of plasmid constructs. Assays were conducted in Sf21 cells, using 0.5μg of Ac*p35lacZ* viral DNA and 2.5μg of plasmid DNA (section 2.2.11). In all cases, Lipofectin™ reagent was used to effect the transfer of DNA into the cells. After incubation for 3 days, the dishes were

examined for signs of productive virus infection, characterised by the presence of occluded virus in the cells.

The ability of the HzSNPV *iap* gene to rescue Ac*p35lacZ* was investigated using different plasmid constructs: i.) the original HzSNPV fragment clone pHzSNPV*Sal*I-3 (Figure 4.4a), ii.) an *Eco*RI-*Cla*I fragment (pECHz*iap*) derived from pHzSNPV*Sal*I-3 (Figure 4.4b), iii.) with the HzSNPV *iap* coding region positioned between flanking regions of AcMNPV *p35* (p*p35*PHz*iap*-Q) to effect homologous recombination at the *p35* locus. In the latter construct, the HzSNPV *iap* gene was under the control of the *p35* promoter (Figure 4.4f).

The plasmid construct pECHz*iap* was assembled in the following manner: The plasmid pHzSNPV*Sal*I-3, which contained *iap* (Chapter Three), was partially digested with *Cla*I and digested to completion with *Eco*RI. The resulting 1538 bp DNA fragment was ligated into pBluescript SK+ previously digested with *Eco*RI and *Cla*I, to give pECHz*iap*. The restriction profile of pECHz*iap* was confirmed by RFLP analysis. M13 forward and reverse sequencing primers complementary to the vector sequences surrounding the multiple cloning site were used to determine the nucleotide sequence of the ends of the fragment (data not shown), to ensure that the 5' region of the *iap* gene was present (i.e., that the partial *Cla*I digest was successful).

The plasmid p*p35*PHz*iap*-Q was constructed in the following manner (Figure 4.4):

The AcMNPV *p35* promoter region was amplified by PCR using the primers 5' GCTCTAGAGTCGACAAGGAATCGTCGGTGGGCG (incorporating an *Xba*I restriction site, underlined) and 5' CCGGAAAAAAGATCTTTTGCTATGG (incorporating a *Bgl*II restriction site, underlined). The plasmid pAc*Eco*RI-S was used as a template for the amplification reaction. The DNA obtained from the PCR was digested with *Xba*I and *Bgl*II and the 263 bp fragment was ligated into the vector pT7T3-*Bgl*II (which has the *Hind*III restriction enzyme site modified to *Bgl*II), to produce the plasmid pT7T3-*p35*P. Sequencing analysis of

the cloned fragment confirmed there were no errors introduced by the PCR amplification. The *p35* promoter region was isolated from pT7T3-*p35*P by digestion of the vector with *Xba*I and *Bgl*II. This fragment was inserted into the vector pECHz*iap* at the *Xba*I and *Bam*HI sites to produce the vector p*p35*PECHz*iap* (Figure 4.4).

The plasmid p*p35*PECHz*iap* was digested with *Sal*I to release a 1790 bp fragment from the pBluescript vector. This fragment was then ligated into the *Sal*I site of pUC118 and orientated so that the *Hind*III site present in the pUC118 vector was positioned downstream of *iap*, to produce the construct pUC*p35*PECHz*iap*. The fragment Ac*Hind*III-Q, which contains the 3' coding sequence and downstream region of *p35*, was inserted into this *Hind*III site and orientated so that the 3' region of *iap* was located adjacent to the 3' region of *p35*. This plasmid, p*p35*PHz*iap*-Q, contained the HzSNPV *iap* gene under the control of the *p35* promoter and flanked downstream by the *Hind*III-Q region of AcMNPV. Following the cotransfection of Sf21 cells with this plasmid and Ac*p35lacZ* DNA, the *p35* promoter and *Hind*III-Q regions recombined with the corresponding regions of Ac*p35lacZ* and directed the HzSNPV gene to the *p35* locus.

The plasmids pHzSNPV*Sal*I-3, pECHz*iap* and p*p35*PHz*iap*-Q were examined for their ability to complement Ac*p35lacZ* in cotransfection experiments. As a positive control in the experiments, a plasmid containing a complete copy of the CpGV *iap* gene (pCpGV*Sal*I-B6) was obtained from N. Crook (HRI, Wellesbourne). The *p35* gene, in the form of pAc*Eco*RI-S, was also used as a positive control. HzSNPV and MbNPV DNAs were also tested in the experiment (Table 4.1). In complementation experiments, the addition of pAc*Eco*RI-S or pCpGV*Sal*I-B6 DNA enabled Ac*p35lacZ* to prevent apoptosis and a productive virus infection developed in the Sf21 cells. Similarly, the addition of MbNPV DNA enabled Ac*p35lacZ* to prevent apoptosis.

In similar experiments, cotransfection of Sf21 cells with Ac*p35lacZ* DNA and pHzSNPV*SalI*-3, pECH*ziap* or pp35PH*ziap*-Q DNA did not enable Ac*p35lacZ* to prevent apoptosis. In cotransfections involving Ac*p35lacZ* and these plasmids, the Sf21 cells underwent apoptosis, and those cells which were not transfected by viral DNA grew normally. Therefore, after the 3 day incubation period, the cells formed a monolayer, and the debris of cells which had undergone apoptosis was dispersed in the media. HzSNPV NC-1 DNA was unable to rescue Ac*p35lacZ*, and did not reproduce the result observed with MbNPV DNA. The medium from this cotransfection experiment was harvested and used to infect a 60mm dish containing $2x10^6$ Sf21 cells. If rescue had occurred, this would enable a low level of virus progeny present in the harvested media to multiply and produce virus plaques. Some cells in these dishes became apoptotic due to infection by Ac*p35lacZ*, however, no signs of productive AcMNPV infection were observed.

4.2.1.4. Construction and functional analysis of CpGV and HzSNPV *iap* hybrids.

Hybrids between the CpGV and HzSNPV *iap*s were constructed in order to examine whether the BIR motif or RING finger motif of the HzSNPV *iap* could actively substitute for the corresponding region of the CpGV *iap* in the complementation assay for P35 function. The putative protein sequences of CpGV *iap* and HzSNPV *iap* share 39% amino acid identity. The CpGV *iap* is an active inhibitor of apoptosis, as demonstrated by its ability to function in place of *p35* in a complementation assay. In an identical assay, the HzSNPV *iap* could not complement for *p35* function. This was also the case when the HzSNPV *iap* was positioned between flanking regions of *p35* to direct homologous recombination of HzSNPV *iap* into the genome of Ac*p35lacZ*.

The amino- and carboxy-encoding regions of HzSNPV *iap* were amplified by PCR. These DNA fragments were cloned into pCpGV*SalI*-B6 after digestion of the plasmid to remove either the 5' or 3' portion of CpGV *iap*. The plasmid

CpGV*Sal*I-B6, comprised a *Sal*I-*Sac*I 1612 bp fragment of CpGV, which contained *iap* and a portion of the downstream gene, ODV (a homologue of AcMNPV ORF 148). The experimental strategy was facilitated by the *Sty*I site present in both CpGV *iap* and HzSNPV *iap* due to the conserved amino acid residues, proline and tryptophan, at amino acid position 163/164 of CpGV IAP, and 170/171 of HzSNPV IAP (Figure 4.5). However, although the amino acid residues at this position were conserved, the nucleotide sequence in this region was not; the *Sty*I restriction site was CCTTGG for CpGV *iap* and CCATGG for HzSNPV *iap*. The Pro/Trp motif provided an ideal cloning site since these amino acids are centrally positioned in both proteins, separating the BIR and RING finger motifs (Figure 4.5).

The plasmid pHzCp*iap*, which contained the 5' region (encoding the BIR motif) of HzSNPV *iap* and the 3' region (encoding the RING finger motif) of CpGV *iap*, was constructed by PCR amplification of the HzSNPV *iap* 5' region and the subsequent ligation of this DNA fragment into pCpGV*Sal*I-B6, which had been previously digested with *Asp*718 and *Sty*I (Figure 4.6). The primers used in the PCR amplification of the 5' HzSNPV *iap* region were 5' GGCGGTACCG-GGAATGTGCCGGCTATTGA, incorporating an *Asp*718 restriction enzyme site (underlined), and the 3' primer, GTGTTCTCGCCTTGGTTCATG, incorporating a *Sty*I restriction enzyme site where an A residue was altered to T (underlined) in order to make it compatible to the *Sty*I site present in the CpGV *iap* gene sequence. The template for the reaction was HzSNPV NC-1 DNA. The structure of this hybrid, pHzCp*iap*, was confirmed by RFLP analysis, and the DNA sequence of the PCR-derived HzSNPV *iap* region was confirmed by sequencing analysis using primers previously generated for the sequencing of the *Sal*I-3 HzSNPV DNA fragment (Chapter Three).

Similarly, to construct the CpHz*iap* hybrid, the 3' region of HzSNPV *iap* was derived by PCR. The primers employed were 5' GCATGAACCAAGGCGAGAACA, incorporating a *Sty*I restriction enzyme site where a T residue was altered to A (underlined), and 3' GCAAGAGCTCTGTTCACATTG, incorporating a *Sac*I restriction enzyme site (underlined). Amplification by PCR of the 3' region of

HzSNPV *iap* produced a 723 bp product, which was digested with *Sty*I and *Sac*I. The CpGV*Sal*I-B6 vector was prepared by digestion to completion with *Sac*I, and partial digestion with *Sty*I, and the PCR-generated 3' region of HzSNPV *iap* was subsequently ligated into this plasmid, to construct the *iap* hybrid pCpHz*iap* (Figure 4.6). The construction of pCpHz*iap* was confirmed by RFLP analysis, and the sequence of the PCR-derived HzSNPV 3' *iap* region was confirmed by sequencing analysis using primers previously generated for the sequencing of the *Sal*I-3 HzSNPV DNA fragment (Chapter Three).

The ability of the hybrid constructs pHzCp*iap* and pCpHz*iap* to inhibit apoptosis was examined using the complementation assay for AcMNPV P35 function, as described previously in section 4.2.1.3. The hybrid construct pHzCp*iap* did not have the ability to complement for AcMNPV P35 function; cotransfection of Sf21 cells with pHzCp*iap* and Ac*p35lacZ* resulted in apoptosis of some cells, and no signs of productive virus infection. Experiments using pCpHz*iap* gave similar results. However, the unmodified CpGV *iap* did complement Ac*p35lacZ* in the assay, although, as previously observed, the unmodified HzSNPV *iap* was unable to do so. The results of this assay are presented in Table 4.2. In summary, neither hybrid gene maintained the anti-apoptotic ability of CpGV *iap*.

4.2.2. Derivation of an *iap* deletion mutant of HzSNPV.

4.2.2.1. Construction of the transfer vector p*iaplacZ*.

The transfer vector p*polhlacZ* was obtained from P. Smith (Oxford Brookes University). This transfer vector contains the 5' (700 bp) and 3' (400 bp) regions of HzSNPV NC-1 polyhedrin with the *lacZ* coding region replacing the polyhedrin gene coding region. The p*polhlacZ* transfer vector was partially digested with *Bam*HI and a synthetic oligonucleotide adaptor (5' GATCAAGCTT) was inserted into the *Bam*HI site in order to modify this restriction site to *Hind*III. The resulting plasmid constructs were analysed by RFLP and the plasmid p*polhlacZ-Hind*III, with a modified *Bam*HI at the 3' end

of the *lacZ* coding region, was selected. From this plasmid, the 5' polyhedrin gene region and *lacZ* coding region could be extracted as a 4.4 kbp *Hind*III cassette.

The plasmid pECHz*iap* (Figure 4.4) was digested with *Sty*I. A synthetic oligonucleotide adaptor (5' CATGAAGCTT) was inserted into the *Sty*I site in order to modify the *Sty*I restriction site to *Hind*III. The resulting plasmid was digested with *Hind*III and the 5' polyhedrin region and *lacZ Hind*III cassette was inserted to produce the transfer vector p*iaplacZ*, which contained *lacZ* (driven by the HzSNPV polyhedrin promoter) inserted at the modified *Sty*I site, interrupting HzSNPV *iap* (Figure 4.7). The organisation of this transfer vector was confirmed by restriction enzyme analysis and sequencing of the regions where genetic modifications had been introduced.

4.2.2.2. Cotransfection of *H. zea* cells with p*iaplacZ* and HzSNPV NC-1 DNA.

Cotransfections of *H. zea* cells with HzSNPV NC-1 DNA and either p*polhlacZ* or p*iaplacZ* were performed. The DNA mixtures (4μg HzSNPV NC-1 DNA, 20μg transfer vector) were cotransfected into *H. zea* cells using Lipofectin™ reagent. The cells were incubated at 28°C for 7 days, transferred to fresh medium, and incubated for a further 7 days. The medium was then harvested from the cotransfections and used in neat and ten-fold dilutions in a plaque assay. The plaque assay dishes were incubated for 6 days to allow for the development of viral plaques.

After staining with X-gal for 24 hours, a blue colouration was visible in plaques derived from recombinant virus produced in the cotransfection involving the p*polhlacZ* transfer vector (Figure 4.8). Some blue plaques were observed derived from virus produced in the cotransfection involving the p*iaplacZ* transfer vector (Figure 4.8), although the intensity of staining of the

cells in these plaques was less than that observed from recombinant viruses produced in the cotransfection involving the p*polhlacZ* transfer vector.

Plaques from the cotransfection using the p*polhlacZ* transfer vector were selected and subjected to repeated plaque purification. A recombinant virus deficient in the polyhedrin gene and expressing *lacZ* was successfully purified from the harvested media of the cotransfection of the p*polhlacZ* transfer vector with HzSNPV NC-1 DNA (P. Smith, unpublished results).

Blue plaques from the cotransfection using the p*iaplacZ* transfer vector expressed very low levels of β-galactosidase (see Figure 4.8). These plaques were isolated and subjected to repeated plaque assay. However, no blue plaques were identified from this second plaque assay, although many wild type HzSNPV NC-1 plaques were visible.

4.3. Discussion.

This chapter describes experiments to characterise the putative *iap* of HzSNPV NC-1, identified in Chapter Three. Once a positive clone had been analysed and shown to maintain the highly characteristic motifs common to the *iap* gene family, it was necessary to test the function of the gene as an inhibitor of apoptosis. In complementation assays, the HzSNPV *iap* gene was incapable of rescuing a *p35* deletion mutant of AcMNPV, Ac*p35lacZ*, and is therefore unique amongst the non-AcMNPV *iap* genes tested in this assay to date. Studies in our laboratory failed to identify a *p35* gene homologue in the HzSNPV virus using Southern blotting techniques (Chapter Three). Additionally, the observation that intact HzSNPV viral DNA does not rescue Ac*p35lacZ* supports the assumption that HzSNPV does not contain a functional apoptosis-inhibiting gene.

Three possible explanations for the lack of complementation of the AcMNPV *p35* deletion mutant by the HzSNPV *iap* gene arise: 1.) The HzSNPV *iap* gene

acts upstream of AcMNPV *p35* in the apoptosis cascade and the assay is therefore inappropriate as a test for the ability of the gene product to inhibit apoptosis. 2.) It may be that the mechanism of apoptosis plays a limited role in the infection process of HzSNPV. The existence of an *iap* gene homologue in the virus may suggest that apoptosis was important at one time in the evolutionary pathway of HzSNPV. 3.) The function of *iap* in CpGV and OpMNPV may have been misinterpreted; the fact that these gene products are functional inhibitors of apoptosis may be a unrelated to another unidentified process. To date, the deletion of the CpGV- and OpMNPV *iap* genes have not been reported, and it is therefore inappropriate to speculate about the function of the *iap* genes in their native situation with insufficient evidence *in loco*.

The hybrid *iap*s pCpHz*iap* and pHzCp*iap* were constructed, and tested in the *p35* complementation assay. Neither gene retained the anti-apoptotic ability of CpGV *iap*. The anti-apoptotic ability of AcMNPV *iap1* and CpGV *iap* hybrids has been previously examined (Clem and Miller, 1994). These hybrids were constructed by PCR-mediated gene splicing, and involved the exchange of both the BIR and RING finger domains. None of the five AcMNPV *iap1*/CpGV *iap* hybrids examined maintained the anti-apoptotic function of CpGV *iap*, although a hybrid containing the 5' region of CpGV *iap* and the 3' region of OpMNPV *iap* was functional in the complementation assay (Clem and Miller, 1994). The inability to replace either the BIR or the RING finger motifs in the CpGV *iap* with homologous equivalent motifs from AcMNPV *iap1* or HzSNPV *iap* shows that some of the non-conserved amino acids in these proteins must be critical for anti-apoptotic function. In the RING finger domain, 11 of the 37 amino acids are shared by all four *iap*s, but 15 amino acids are different between the functional (CpGV and OpMNPV *iap*) and non-functional (AcMNPV and HzSNPV *iap*) genes (Figure 3.10). In addition to these substitutions, AcMNPV *iap1* has an extra amino acid residue at position 31 that is not present in the other sequences. A detailed analysis of *iap* anti-apoptotic function could be examined by site-directed mutagenesis of the residues in functional *iap*s which differ from those present in non-functional *iap*s. The

identification of novel *iap*s, and the determination of their anti-apoptotic ability in the *p35* complementation assay, should aid the identification of residues for site-directed mutagenesis.

A transfer vector with the *E. coli* β-galactosidase gene interrupting the HzSNPV *iap* gene was constructed. However, the isolation of a recombinant HzSNPV deficient in *iap* proved unsuccessful, although the feasibility of the method for this virus-cell combination has been demonstrated by the isolation of a polyhedrin gene deletion virus (P. Smith, unpublished results). These data, combined with the fact that transcripts of the gene are detectable both early and late in the baculovirus infection process (see Chapter Three), indicate that the *iap* gene performs a critical, though unknown, role in the replication of HzSNPV.

Essential baculovirus genes cannot be studied by production of a mutant virus deficient in a specific gene, using standard techniques. However, a novel system employing replication of the baculovirus genome in yeast, can allow manipulations to be made in the baculovirus genome without relying on growth of the virus in insect cells. In order to replicate baculovirus DNA in yeast, the viral genome must contain three elements (Patel *et al.*, 1992); an autonomous replicating sequence (ARS), a sequence encoding centromere function (Cen) and a selectable marker e.g., tryptophan, uracil or sup-4o. The yeast is initially transfected with this recombinant virus, and yeast colonies containing the viral genome are selected. A transfer vector containing the gene of choice interrupted by a second yeast selectable marker is used to transfect yeast cells containing the baculovirus genome. Recombination occurs to incorporate the marker gene into the baculovirus genome in a small proportion of cases, thereby producing a virus with the selected gene deleted. Selection on plates deficient in two yeast markers allows for selection firstly of yeasts incorporating viral DNA and secondly for yeasts incorporating viral DNA with the selected gene replaced by a second marker. The yeast DNA is then extracted and used to cotransfect insect cells. The phenotype of the

recombinant virus can thus be examined. This strategy for the production of recombinant viruses could be used to delete *iap* from HzSNPV. However, recovery of baculovirus DNA from yeast cells is difficult, yielding very low amounts. The difficulty of transfecting *H. zea* cells may preclude the method.

It is likely that baculovirus IAP interacts to control apoptosis in insect cell lines, and this may occur in a cell-line specific manner if the cellular targets of IAPs from different baculovirus species are themselves significantly different. In order to examine this hypothesis, the cellular targets of baculovirus IAPs need to be fully defined. The cell interactions of baculovirus IAP are unknown, although the human IAPs are known to interact with the TNF receptor-associated factors (TRAFS) (Rothe *et al.*, 1995). This observation suggests that affinity purification of proteins in insect cells which interact with baculovirus IAP may provide important information towards the characterisation of the anti-apoptotic function of baculovirus IAP.

AcMNPV infection of *H. zea* cells does not result in the production of OV (McIntosh *et al.*, 1985). The activity of a reporter gene under the control of an AcMNPV very late promoter is very low in *H. zea* cells infected with recombinant AcMNPV (Morris and Miller, 1992), indicating that AcMNPV very late gene expression is unlikely to occur during infection of *H. zea* cells with AcMNPV. However, low levels of BV are produced from the infection. This suggests that the early and delayed early phases of gene expression proceed, but to a lesser extent than in permissive host cells such as Sf21 and *T. ni*.

Figure 4.1.

Construction of the transfer vector p*p35lacZ*.

The transfer vector used for the modification of AcMNPV *p35*, contained *E. coli* *lacZ* under the control of the AcMNPV polyhedrin promoter. The *lacZ* cassette was inserted at the *Hind*III site of *p35*, to interrupt transcription.

Panel a of the figure represents the organisation of *Eco*RI-S fragment of AcMNPV (1453 bp). The arrow shows the position of the *p35* ORF and indicates the direction of transcription. The restriction enzyme sites are detailed.

The construction of p*p35lacZ* involved the insertion of an oligonucleotide adapter into the unique *Hind*III site of pAc*Eco*RI-S, to alter the restriction site to *Bgl*II. This genetic modification is indicated by a dashed arrow in panel a.

The *lacZ* gene cassette was isolated from the plasmid pAcRP23.*lacZ* by digestion with *Bam*HI and *Bgl*II, and this DNA fragment is represented in panel b (not drawn to scale). The *lacZ* coding region is represented by an arrow, and the restriction enzyme sites present in the *lacZ* coding region are detailed. The *lacZ* coding region was inserted, as indicated by the dashed arrows, into the *Bgl*II site of pAc*Eco*RI-S-*Bgl*II to produce the transfer vector p*p35lacZ*.

a.

p35

*Eco*RI (2) *Sal*I (201) *Bst*XI (535) *Hind*III (846) *Sal*I (878) *Eco*RI (1454)

pAc*Eco*RI-S

Insertion of oligonucleotide adaptor to modify *Hind*III site to *Bgl*II and produce pAc*Eco*RI-S-*Bgl*II

AGCTAGATCT

*Bgl*II

b.

lacZ cassette extracted from pAcRP23+8.*lacZ*

polyhedrin promoter

lacZ

*Bgl*II *Eco*RV *Eco*RI *Bam*HI

Insertion of *lacZ* cassette into *Bgl*II site of pAc*Eco*RI-S-*Bgl*II.

polyhedrin promoter

5' *p35* *lacZ* 3' *p35*

*Eco*RI *Sal*I *Eco*RI *Eco*RV *Bgl*II *Eco*RI

p*p35lacZ*

Figure 4.2.

Southern blot hybridisation of Ac*p35lacZ* DNA compared to AcMNPV C6 DNA.

DNA was purified from BV isolated following the infection of Sf21 cells with AcMNPV C6, or *T. ni*-368 cells with Ac*p35lacZ*. The DNA (2µg) was digested with the restriction enzymes *Bgl*II, *Eco*RI or *Eco*RV and resolved on a 0.6% agarose gel containing 0.2µg/ml ethidium bromide (panel a). The DNA was transferred to a nitrocellulose membrane and probed using a α^{32}P-dATP-labelled DNA fragment encoding the *E. coli* β-galactosidase gene (*lacZ*).

Panel a shows the restriction profiles obtained:

Lane 1 = Lambda DNA digested with *Hind*III and *Eco*RI (DNA ladder)
Lane 2 = AcMNPV C6 DNA digested with *Bgl*II
Lane 3 = Ac*p35lacZ* DNA digested with *Bgl*II
Lane 4 = AcMNPV C6 DNA digested with *Eco*RI
Lane 5 = Ac*p35lacZ* DNA digested with *Eco*RI
Lane 6 = AcMNPV C6 DNA digested with *Eco*RV
Lane 7 = Ac*p35lacZ* DNA digested with *Eco*RV

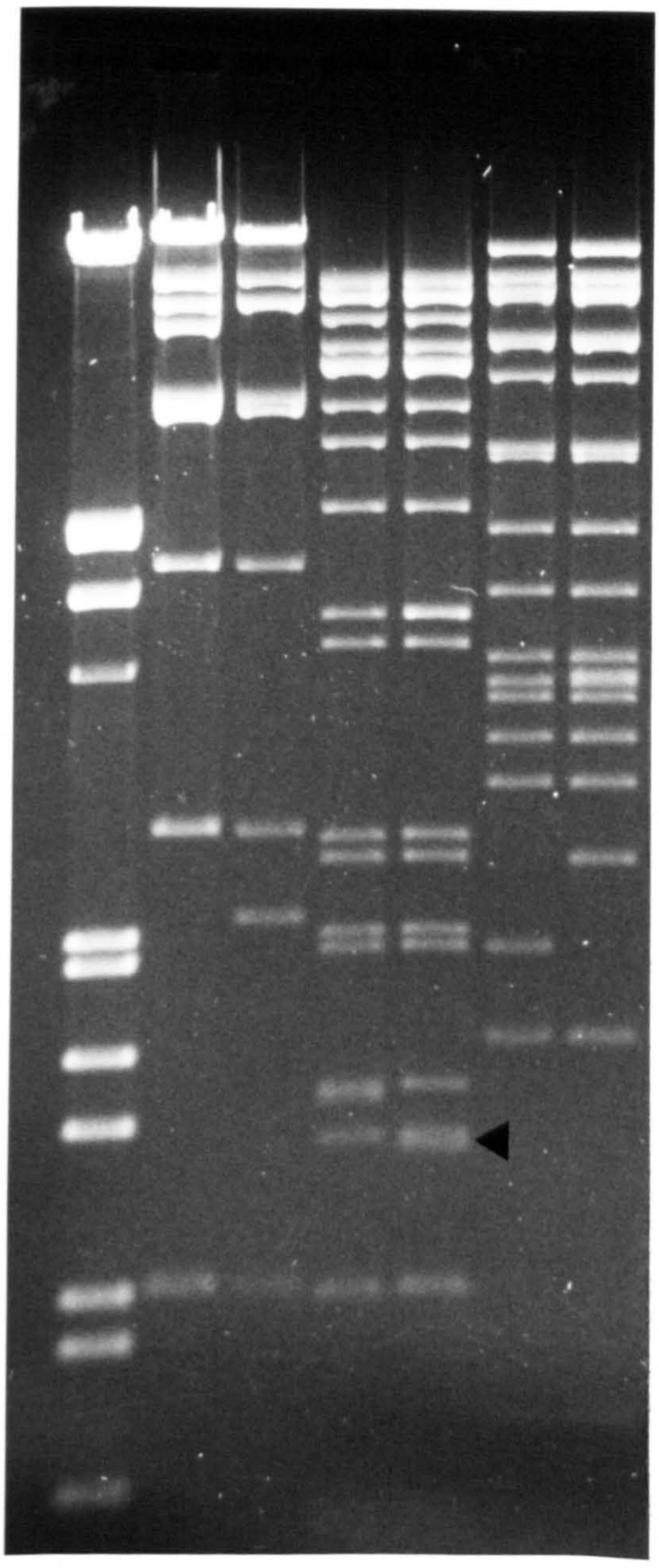
Panel b shows the corresponding Southern blot probed with the *lacZ* DNA fragment. The *lacZ* DNA fragment hybridises to Ac*p35lacZ* but not AcMNPV C6. The *Eco*RI-S fragment of Ac*p35lacZ* is reduced in size in the *Eco*RI profile (indicated by an arrow), due to the presence of an *Eco*RI site in the *lacZ* insertion. The addition of an *Eco*RV site in Ac*p35lacZ* positioned in the middle of *lacZ*, resulted in an extra *Eco*RV fragment in the restriction enzyme profile of Ac*p35lacZ*, compared to AcMNPV C6. The *lacZ* probe therefore hybridises to two fragments in the *Eco*RV restriction enzyme profile of Ac*p35lacZ*.

a.

b.

*Bgl*II *Eco*RI *Eco*RV

Size (kbp) 1 2 3 4 5 6 7



5.0

2.0

0.9

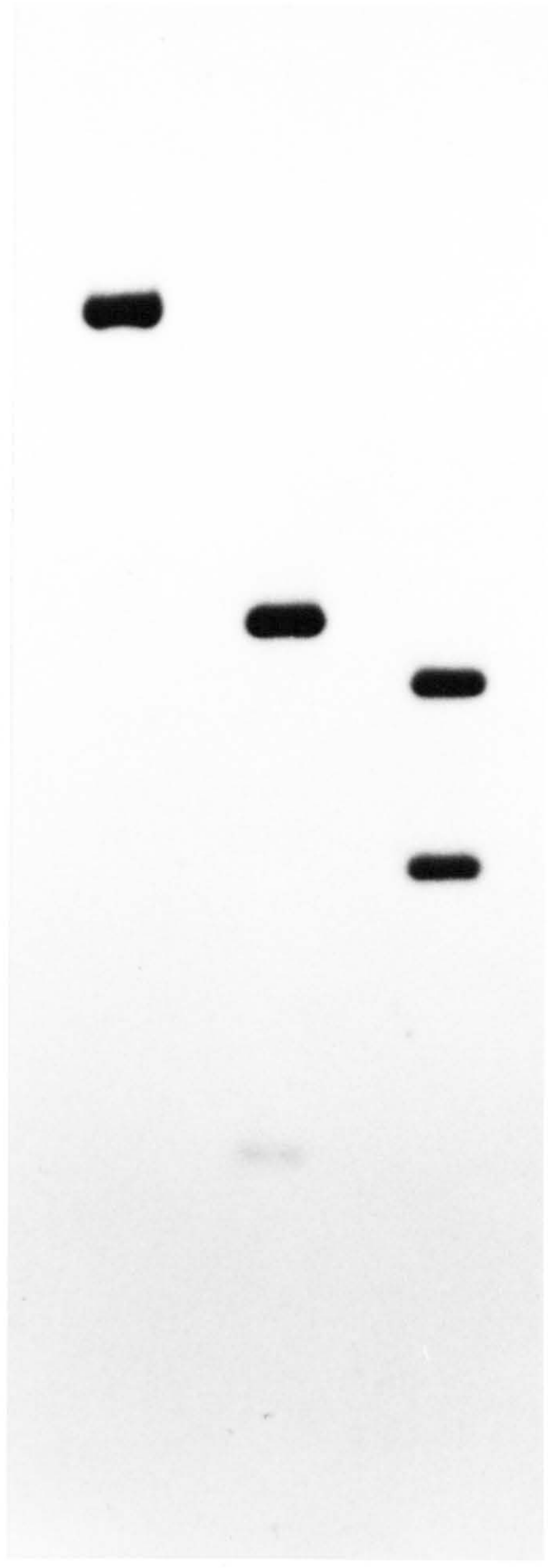


Figure 4.3.

Confirmation of the phenotype of Ac*p35lacZ* in Sf21 and *T. ni*-368 cells.

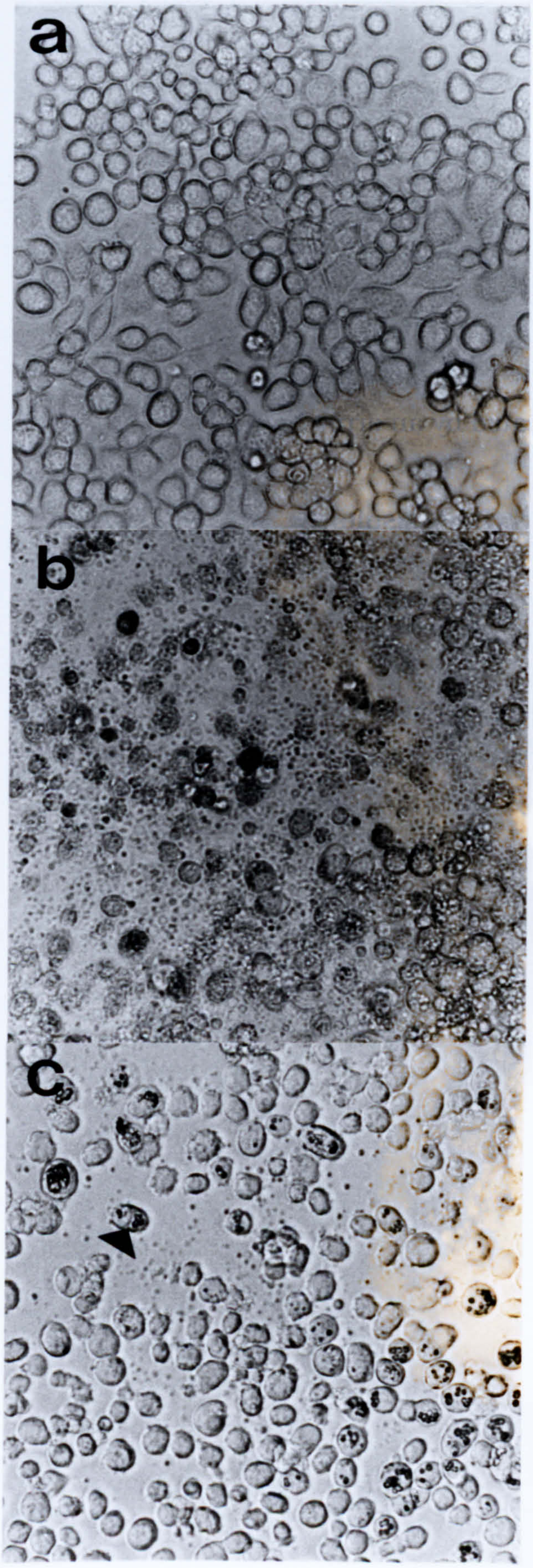
Sf21 cells infected with Ac*p35lacZ* (Sf21 panel b) show the distinctive features of apoptosis, characterised by cell blebbing, compared to uninfected Sf21 cells (Sf21 panel a). Infected cells were photographed 24 h.p.i. Cotransfection of Sf21 cells with Ac*p35lacZ* DNA and the plasmid pAc*Eco*RI-S, which contains a copy of the *p35* gene, results in the rescue of the virus and culminates in the presence of occlusion bodies (indicated by an arrow, Sf21 panel c) in the cells.

In *T. ni*-368 cells, Ac*p35lacZ* infection results in the production of viral occlusion bodies (indicated by an arrow) 3 days post infection (*T. ni* panel b). The appearance of mock-infected *T. ni*-368 cells is shown in *T. ni* panel a.

Cells were photographed using an Olympus OM-2 camera mounted above a Leitz Wetzlar light microscope. The photographs are reproduced at 200x magnification of the subject.

Sf 21 cells

T. ni-368 cells



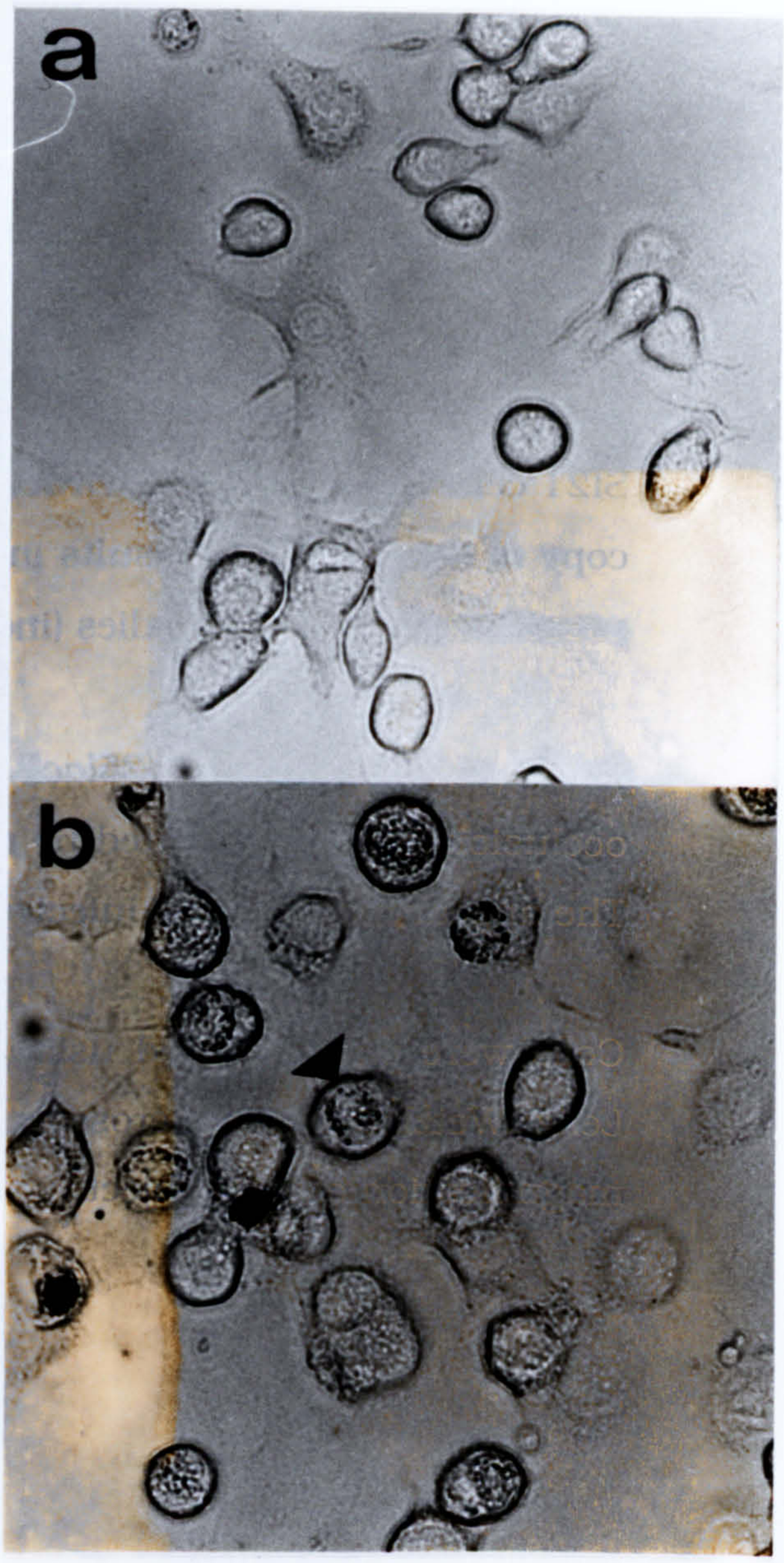


Figure 4.4.

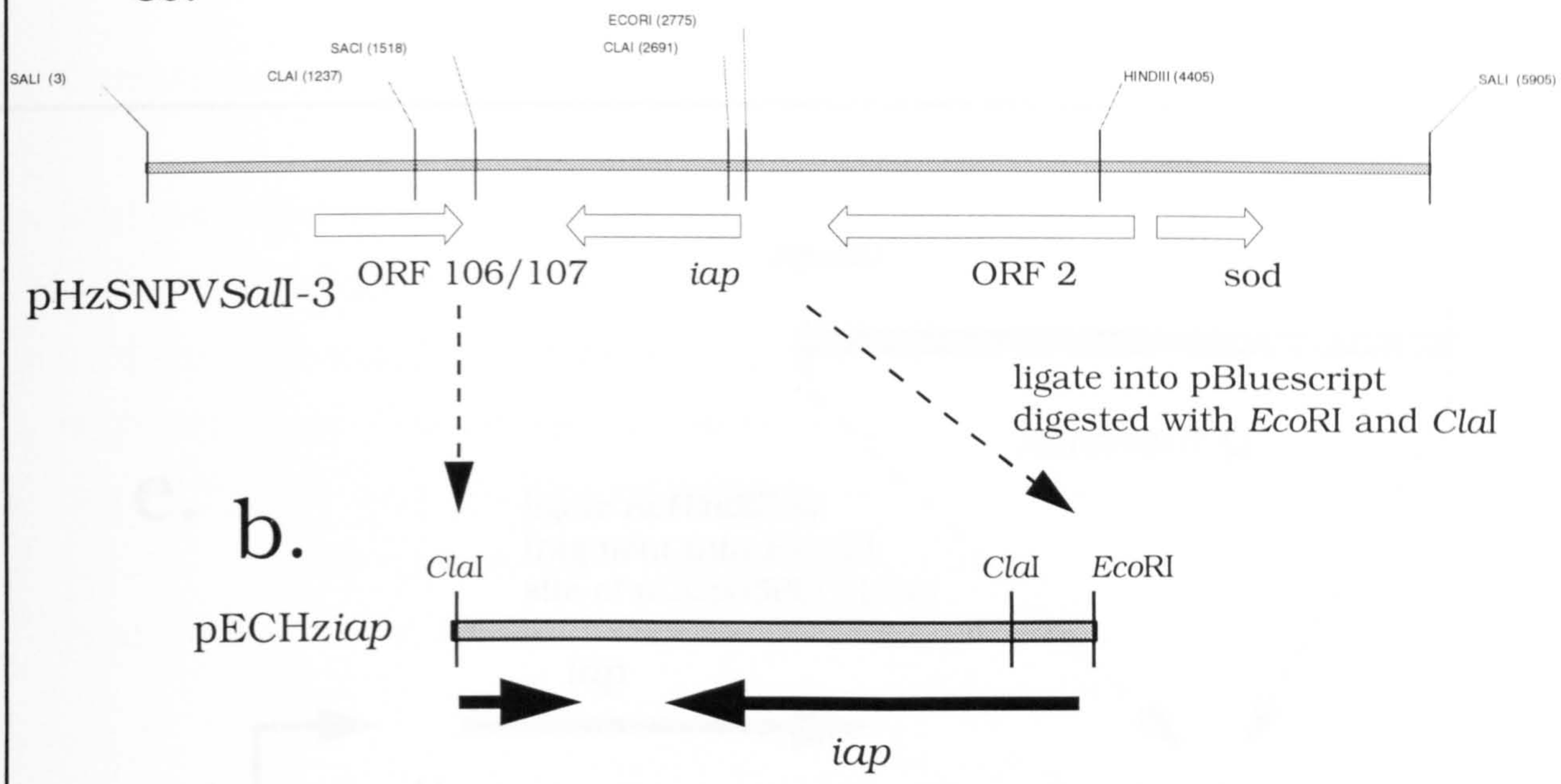
Construction of pECHz*iap* and p*p35*PHz*iap*-Q.

The plasmid pECHz*iap*, containing an *Eco*RI-*Cla*I fragment derived from pHzSNPV*Sal*I-3, was constructed by digestion of pHzSNPV*Sal*I-3 (panel a) with *Cla*I (partial) and *Eco*RI, represented by dashed arrows in the Figure. The fragment (panel b) was ligated into the unique *Eco*RI and *Cla*I restriction sites in the multiple cloning site of pBluescript SK+.

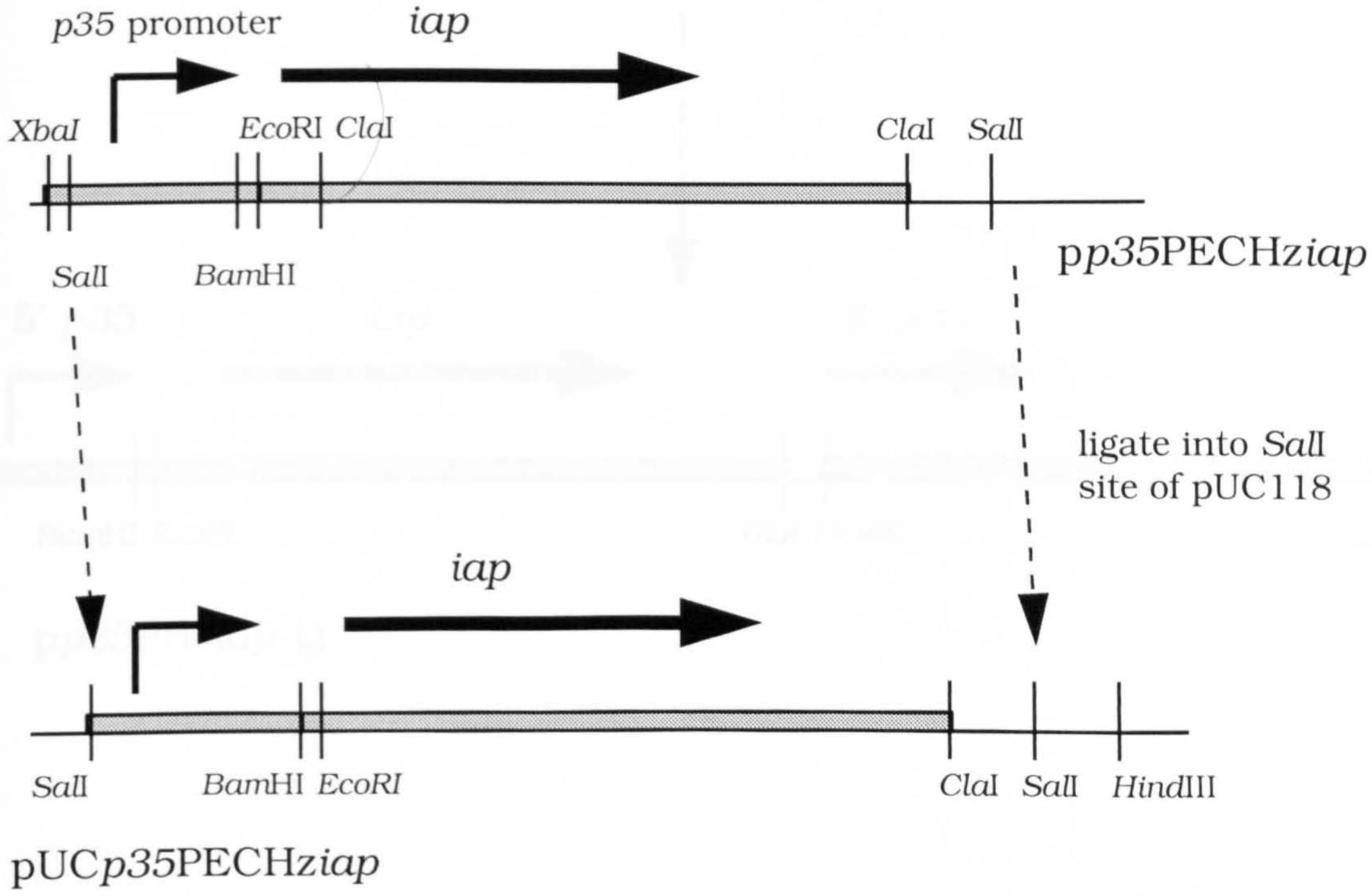
A PCR-derived DNA fragment encoding the *p35* promoter was subsequently ligated into the *Xba*I and *Bam*HI sites of pECHz*iap* (panel c). The *p35* promoter region and *iap* coding region were then extracted from this vector using *Sal*I (panel d, represented by dashed arrows), and ligated into pUC118.

The Ac*Hind*III-Q fragment was then ligated downstream of *iap* (represented by dashed arrows in panel e) into the *Hind*III site present in pUC*p35*PECHz*iap*, to derive the plasmid p*p35*PHz*iap*-Q (panel f).

a.



c.



pUC*p35*PECHz*iap*

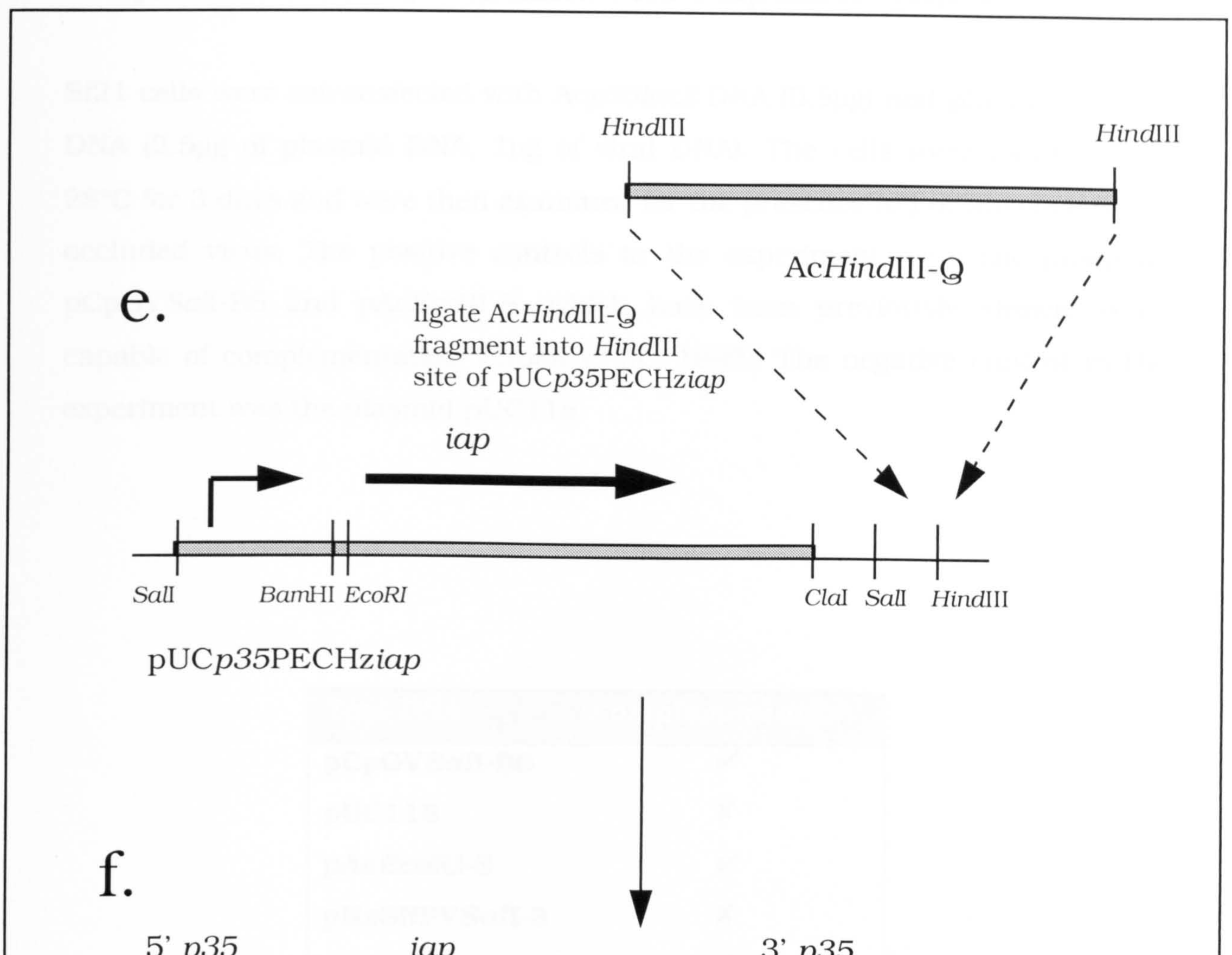

HindIII
HindIII
AcHindIII-Q
e.
ligate AcHindIII-Q
fragment into HindIII
site of pUCp35PECHziap
iap
SalI
BamHI EcoRI
ClaI SalI HindIII
pUCp35PECHziap
f.
5' p35
iap
3' p35
SalI
BamHI EcoRI
ClaI HindIII
HindIII
pp35PHziap-Q

Table 4.1.

Complementation assay with HzSNPV NC-1 *iap* and its derivatives.

Sf21 cells were cotransfected with Ac*p35lacZ* DNA (0.5μg) and plasmid or viral DNA (2.5μg of plasmid DNA, 1μg of viral DNA). The cells were incubated at 28°C for 3 days and were then examined for the presence (✔) or absence (✘) of occluded virus. The positive controls in the experiment were the plasmids pCpGV*Sal*I-B6 and pAc*Eco*RI-S, which have been previously shown to be capable of complementation (Crook *et al.*, 1993). The negative control in the experiment was the plasmid pUC118.

Test DNA	*Complementation*
pCpGV*Sal*I-B6	✔
pUC118	✘
pAc*Eco*RI-S	✔
pHzSNPV*Sal*I-3	✘
pECHz*iap*	✘
pp35PHz*iap*-Q	✘
HzSNPV DNA	✘
MbNPV DNA	✔

Figure 4.5.

Amino acid alignment of CpGV *iap* and HzSNPV *iap*.

The amino acid sequences of the CpGV and HzSNPV *iap* genes were aligned using the GCG programme PILEUP. This figure shows a PRETTYPLOT representation of that alignment. The conserved proline-tryptophan residues (PW) are marked with asterisks. The nucleotide sequence of CpGV *iap* and HzSNPV *iap* differs by 1 bp in this region, (CCTTGG compared to CCATGG), although the restriction site *Sty*I (recognition sequence CCXXGG, where X = A or T) is not altered. The BIR and RING finger motifs are also labelled and are represented by dashed and unbroken lines respectively.

BIR 1

```
Cpiap  - - - - - - - - M S D L R L E E V R L N T F E K W P V S - - F L S P E T M A K N G F Y Y L G R S D E  40
Hziap  M M S Y M E S D L E L L K T E S Y R Y V T F A N W P V Q Y Y F M D C A K M A Q A G F Y Y L N K D D H  50

Cpiap  V R C A F C K V E I M R W K E G E D P A A D H K K W A P Q C P F V K G I D V C G S I V T T N N I Q N  90
Hziap  V K C A F C K V E M M N W Q H E D D P L E E H A R W A P Q C S Y V K S I - M S D A N V C S E Q K Y I  99
```

BIR 2

```
Cpiap  T T T H D T I I G P A H P K Y A H E A A R V K S F H N W P R C M K Q R P E Q M A D A G F F Y T G Y G  140
Hziap  A D Q E F Y K N K S K L S S Y S T Y E N R L K S F D N W P Q T L I I L K S K L A E A G W V Y T G K D  149

Cpiap  D N T K C F Y C D G G L K D W E P E D V P W E Q H V R W F D R C A Y V Q L V K G R D Y V Q K V I T E  190
Hziap  D I T I C F H C G G K L S N W T L T H E P W R E H A R W Y R N C D F V L S E K G K D F V Q T V I T E  199
                                  * *

Cpiap  A C V L P G E N T T V S T A A P V S E P I P E T K I E K E P Q V E D S K L C K I C Y V E E C I V C F  240
Hziap  A C V E K E G G N S - - - - - - - - - - - - - - - - D N Q T T E C D I R T C K V C F V N E R N Y M F  233

Cpiap  V P C G H V V A C A K C A L S V D K C P M C R K I V T S V L K V Y F S -  275
Hziap  L P C H H L A C C E E C A F K V K K C V V C R R S I D D M T K V F I S -  268
```

RING finger motif

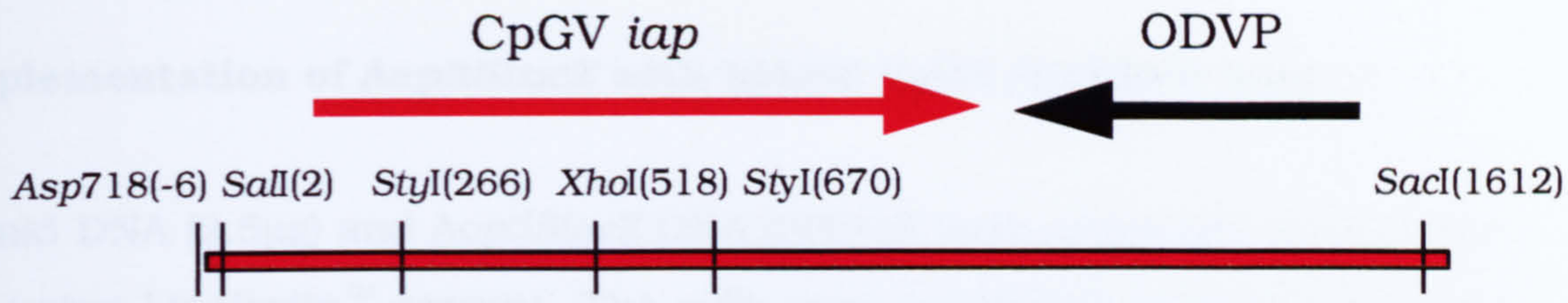
Figure 4.6.

Construction of CpGV *iap* and HzSNPV *iap* hybrids.

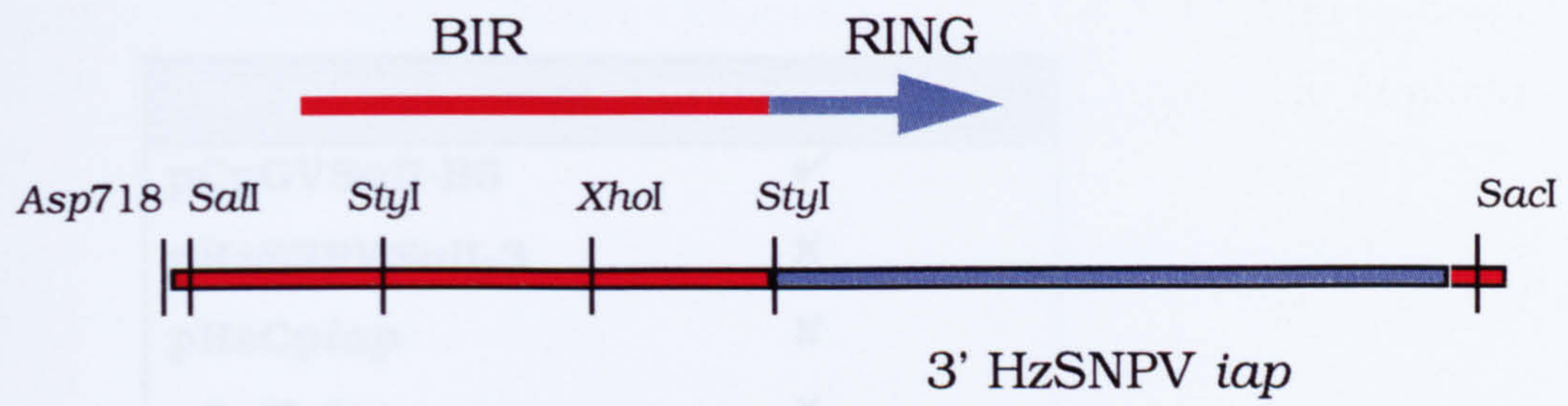
Hybrid *iap* constructs based on the pCpGV*Sal*I-B6 plasmid (panel a) were produced. The hybrid pCpHz*iap* was constructed by digestion of pCpGV*Sal*I-B6 with the restriction enzymes *Sty*I (partial) and *Sac*I. A PCR-generated fragment of the 3' region of the HzSNPV *iap* gene was ligated into the *Sty*I and *Sac*I sites to produce the plasmid pCpHz*iap* (panel b).

The hybrid pHzCp*iap* was constructed by digestion of pCpGV*Sal*I-B6 with the restriction enzymes *Asp*718 and *Sty*I. A PCR-generated fragment of the 5' region of HzSNPV *iap* was ligated into the *Asp*718 and *Sty*I sites to produce the plasmid pHzCp*iap* (panel c).

a.



b. pCpHz*iap*



C. pHzCp*iap*

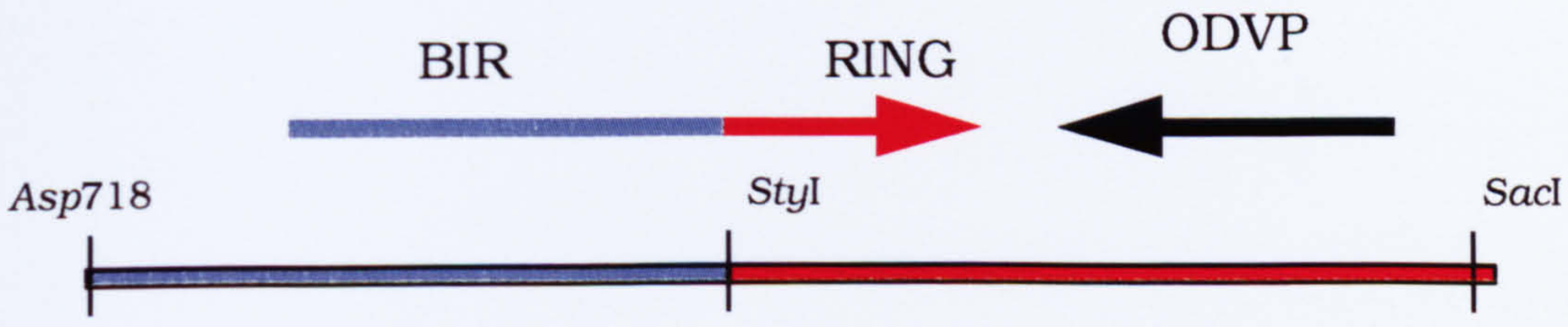


Table 4.2.

Complementation of Ac*p35lacZ* with hybrid CpGV/HzSNPV *iaps*.

Plasmid DNA (2.5µg) and Ac*p35lacZ* DNA (500ng) were cotransfected into Sf21 cells using Lipofectin™ reagent. The cells were incubated at 28°C for 3 days and were then examined for the presence (✔) or absence (✘) of occluded virus in the cells.

Test plasmid	*Complementation*
pCpGV*Sal*I-B6	✔
pHzSNPV*Sal*I-3	✘
pHzCp*iap*	✘
pCpHz*iap*	✘
pUC118	✘

Figure 4.7.

Construction of a transfer vector to facilitate the deletion of *iap* from the genome of HzSNPV NC-1.

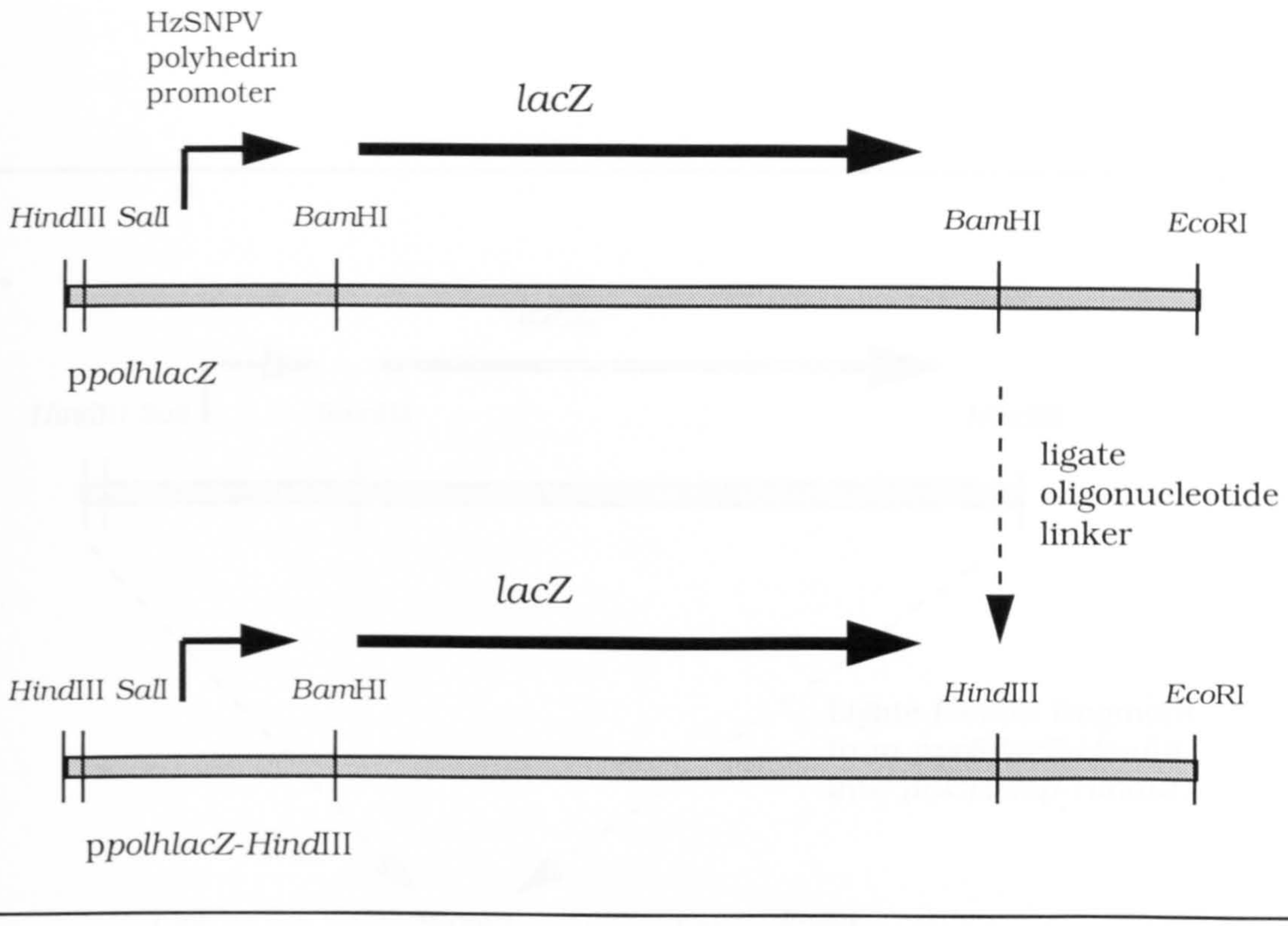
The transfer vector was p*iaplacZ* was constructed from the plasmid pECHz*iap* using a three-step cloning process.

In panel a the upper shaded line represents the transfer vector p*polhlacZ*, detailing the restriction enzyme sites present in the DNA fragment. An oligonucleotide linker was ligated into the *Bam*HI site at the 3' end of the *lacZ* coding region in the plasmid p*polhlacZ* to produce the plasmid p*polhlacZ-Hind*III. The 5' HzSNPV polyhedrin promoter and *lacZ* coding region could then be extracted from this vector as a *Hind*III cassette.

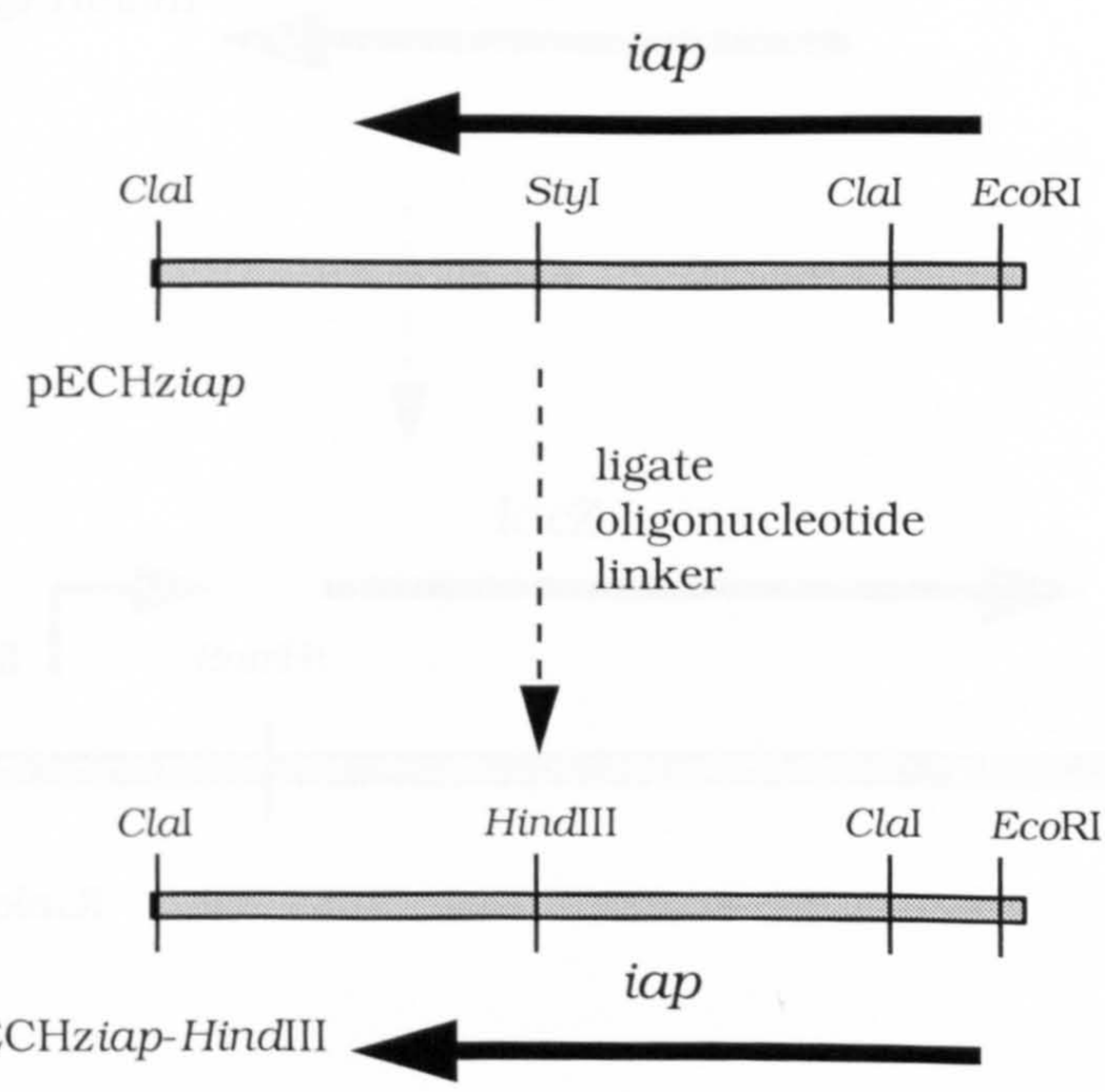
The *Sty*I site in the plasmid pECHz*iap* was altered to *Hind*III by the insertion of an oligonucleotide adaptor (panel b) to produce the plasmid pECHz*iap-Hind*III.

The DNA *Hind*III cassette containing the polyhedrin promoter driving *lacZ* (extracted from p*polhlacZ-Hind*III) was ligated into pECHz*iap-Hind*III to produce the transfer vector p*iaplacZ* (panel c).

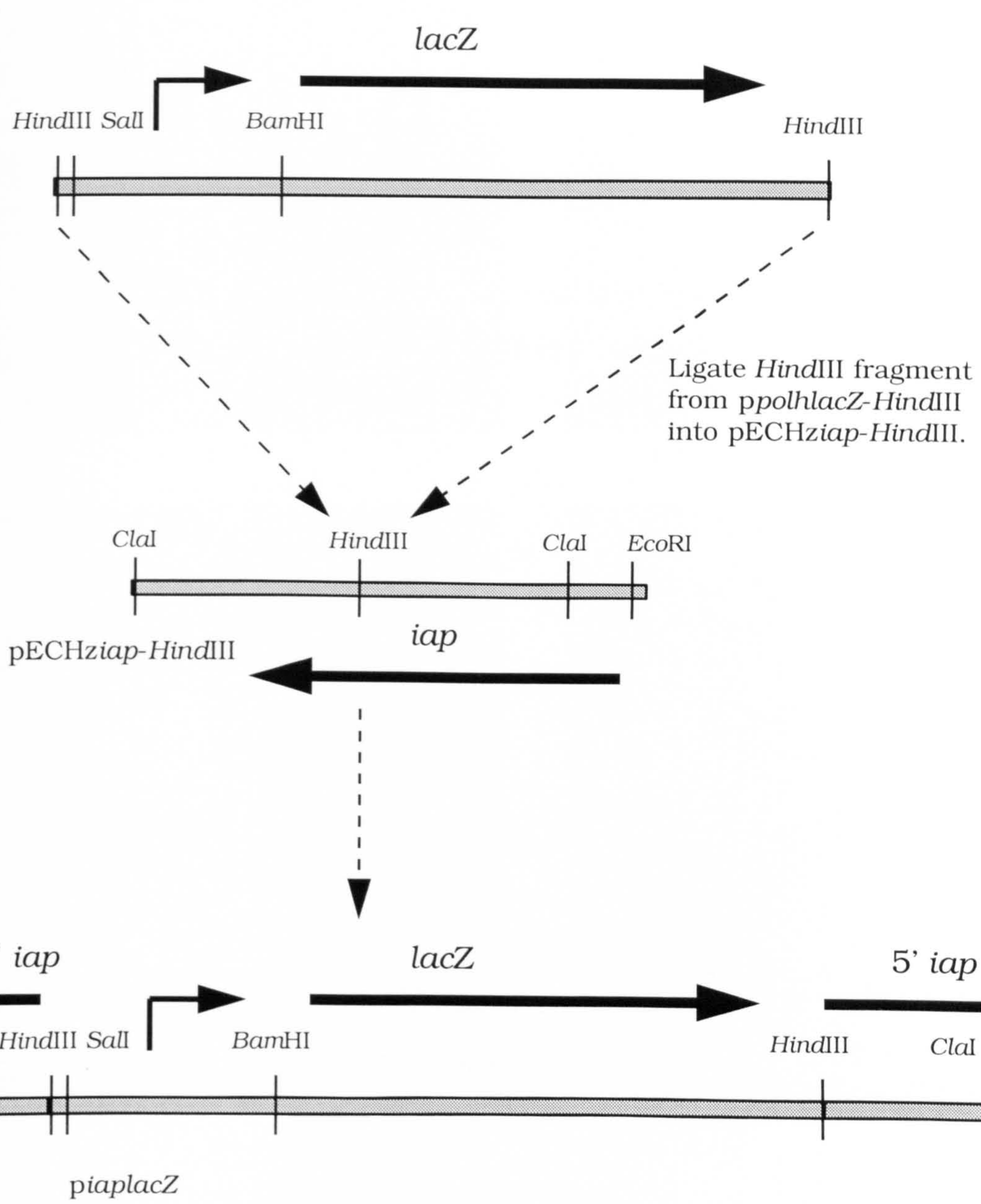
a.


HzSNPV
polyhedrin
promoter
lacZ
HindIII SalI
BamHI
BamHI
EcoRI
ppolhlacZ
ligate
oligonucleotide
linker
lacZ
HindIII SalI
BamHI
HindIII
EcoRI
ppolhlacZ-HindIII

b.


iap
ClaI
StyI
ClaI
EcoRI
pECHziap
ligate
oligonucleotide
linker
ClaI
HindIII
ClaI
EcoRI
iap
pECHziap-HindIII

c.


lacZ
HindIII SalI
BamHI
HindIII
Ligate HindIII fragment
from ppolhlacZ-HindIII
into pECHziap-HindIII.
ClaI
HindIII
ClaI
EcoRI
pECHziap-HindIII
iap
3' iap
lacZ
5' iap
ClaI
HindIII SalI
BamHI
HindIII
ClaI
EcoRI

piaplacZ

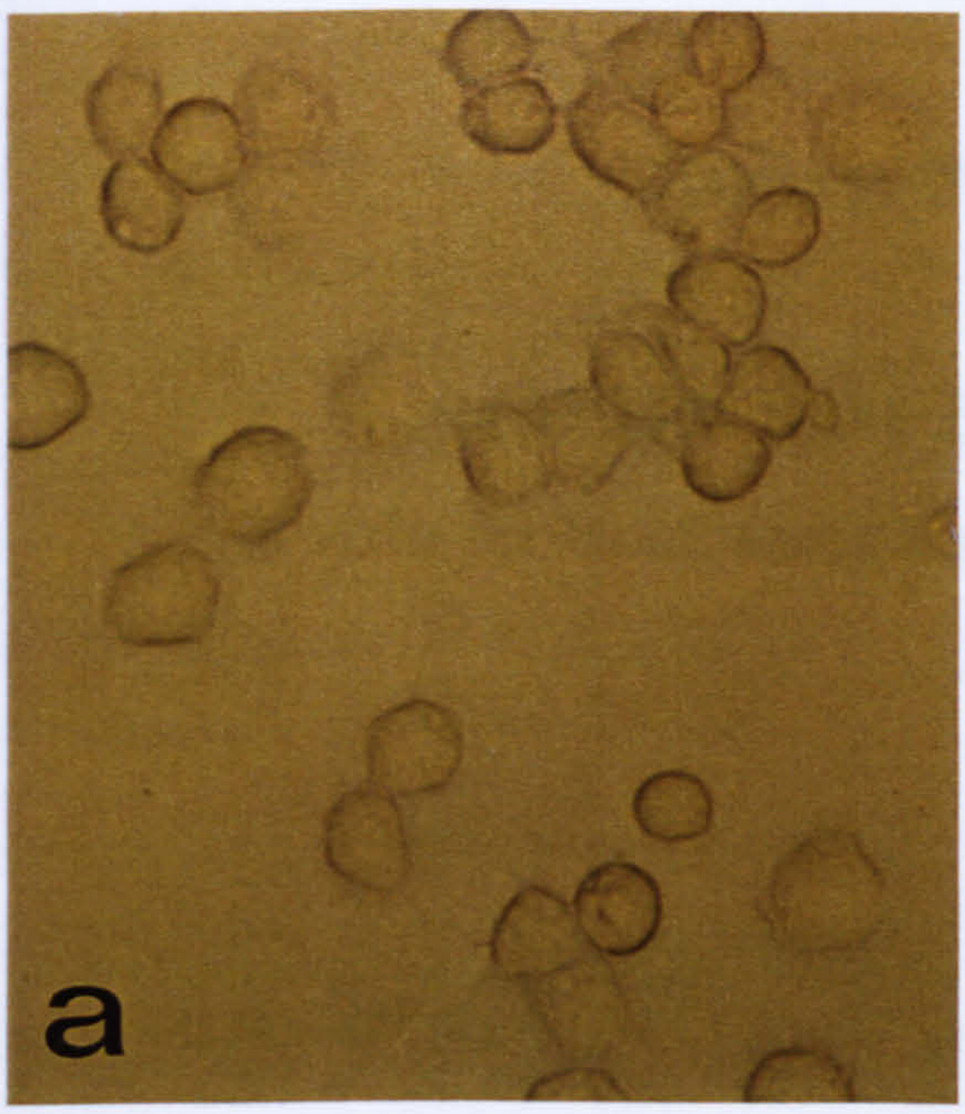
Figure 4.8.

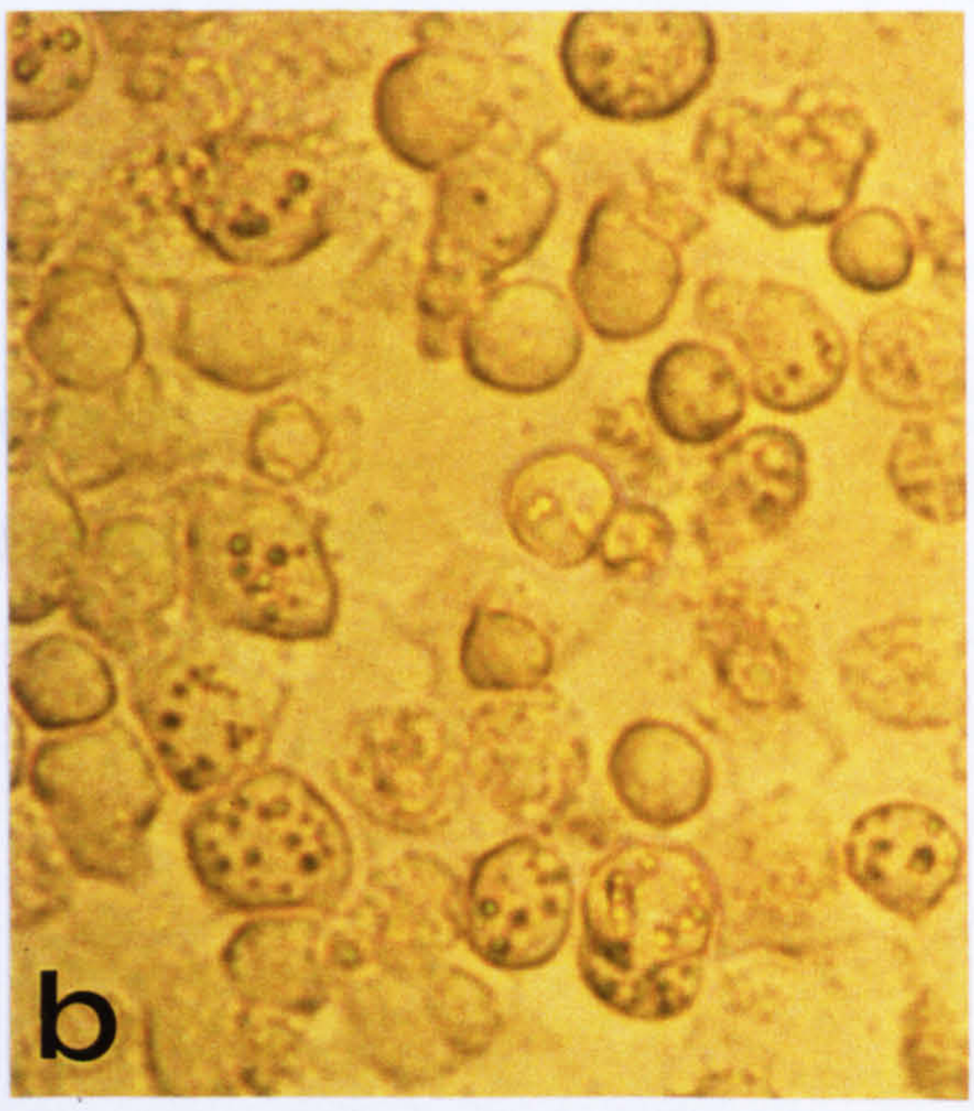
Plaque phenotype of the *iap* deletion HzSNPV in *H.zea* cells.

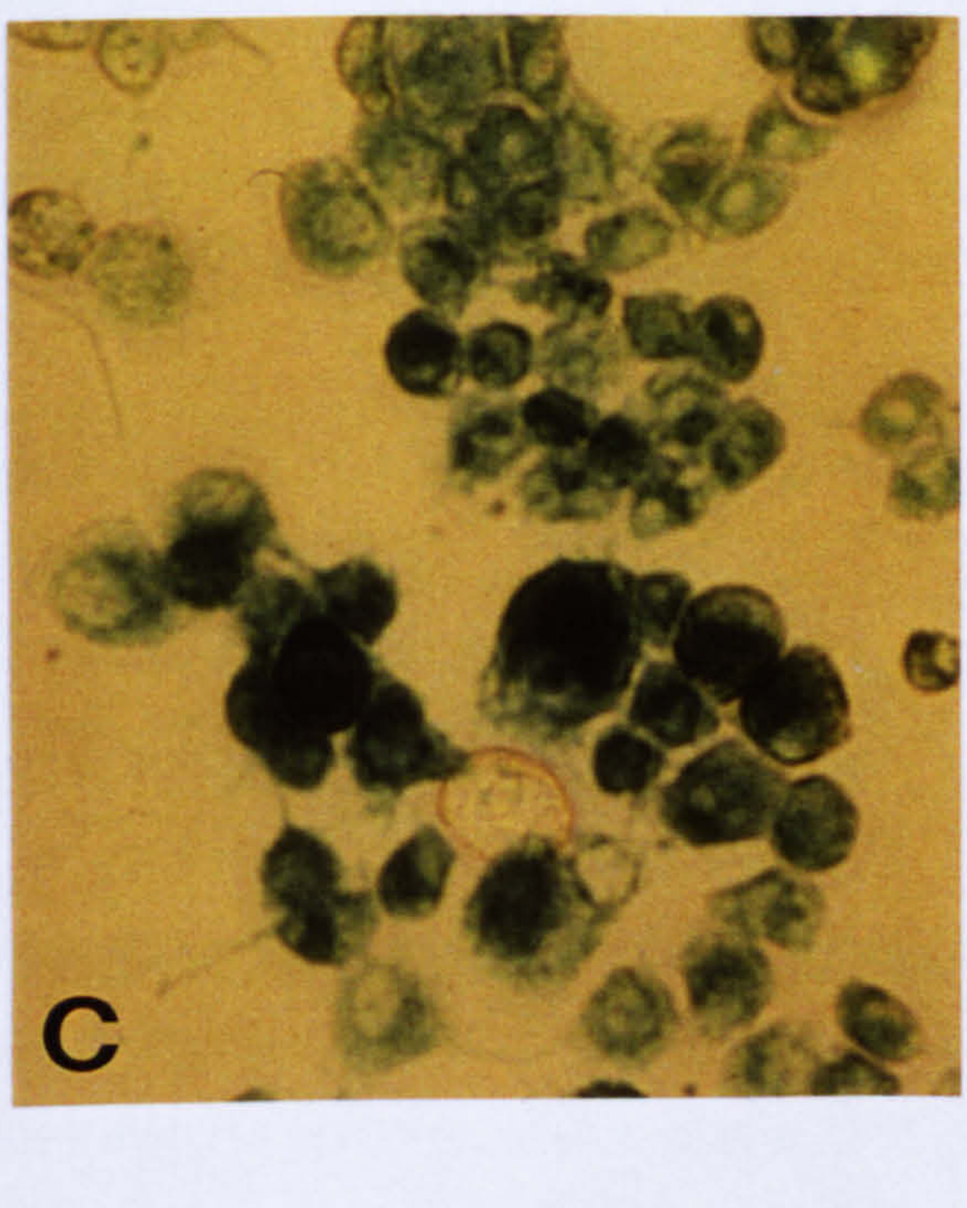
H. zea cells were cotransfected with HzSNPV-NC1 DNA (4μg), and HzSNPV NC-1 DNA (4μg) in combination with either p*polhlacZ* transfer vector (20μg) or p*iaplacZ* transfer vector (20μg).

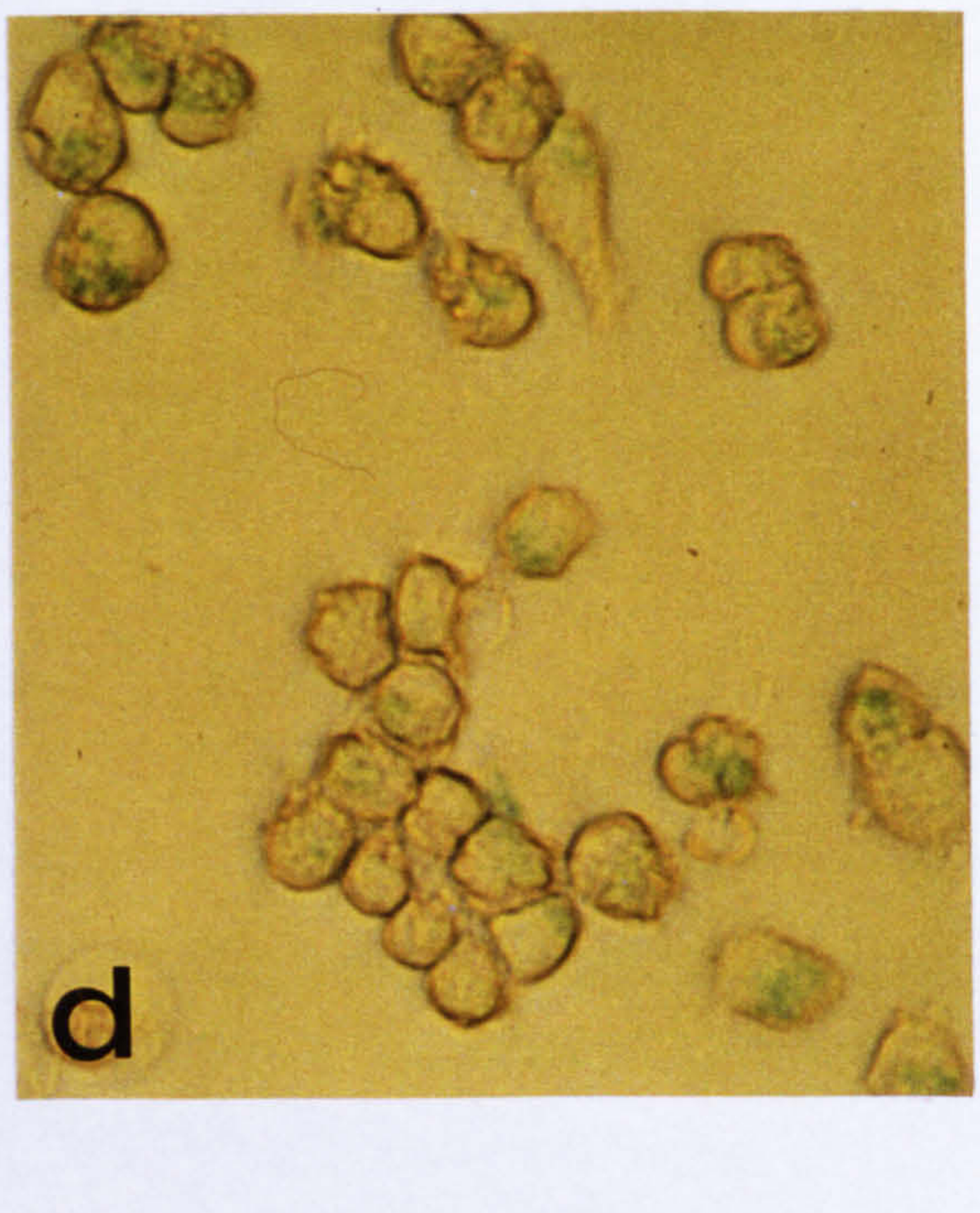
Panel a shows the appearance of uninfected *H. zea* cells. Panel b shows the appearance of *H. zea* cells infected with wild type HzSNPV NC-1.

Panel c shows the appearance of a blue plaque obtained from the cotransfection using the p*polhlacZ* transfer vector. Panel d shows the appearance of a blue plaque obtained from the cotransfection using the p*iaplacZ* transfer vector.


a


b


c


d

Chapter Five

AcMNPV IAP1 function;
a role in host range maintenance?

5.1. Introduction.

Although the function of P35 in AcMNPV infection has been extensively studied (Clem *et al.*, 1991; Clem and Miller, 1994a; 1994b), no work has been reported concerning the role of the IAP homologues, IAP1 and IAP2, in this virus. AcMNPV *iap1* (ORF 27) is 31.3% identical at the amino acid level to CpGV *iap*, and 28.6% identical to OpMNPV *iap* (Chapter Three). The extended homology in the rest of the protein distinguishes this gene product from the other AcMNPV proteins that contain a similar zinc finger motif (encoded by *cg30*, *pe38*, *ie-0* and *iap2*). Unlike the CpGV *iap* however, AcMNPV *iap1* and *iap2* (ORF 71) do not block AcMNPV *p35* deletion mutant-induced apoptosis in Sf21 cells, and also lack the ability to inhibit actinomycin D-induced apoptosis.

It has been proposed that the AcMNPV IAP may be required, in addition to P35, to block apoptosis in cells (Clem and Miller, 1994b). IAP may be the primary apoptosis-inhibiting protein encoded by some baculoviruses. However, AcMNPV and BmNPV may have acquired *p35*, to be active in conjunction with *iap*, to inhibit apoptosis in a wider range of hosts (Clem and Miller, 1994b). This could explain the relatively broad host range of AcMNPV compared to other baculoviruses, and also the apparent lack of *p35* in a closely related baculovirus with a narrower host range, OpMNPV (Gombart *et al.*, 1989).

The experiments described in Chapter Five aim to investigate the role of *iap1* in AcMNPV infection, specifically to examine whether this gene provides anti-apoptotic function during infection of *T. ni*, or other hosts, with Ac*p35lacZ*. An AcMNPV mutant deficient in *iap1* has been constructed (Chapter Four). Previous work (Clem and Miller, 1994b), indicated that *iap1* was essential in Ac*p35lacZ*-infected cells. Therefore, a strategy was devised to isolate AcMNPV deficient in both *p35* and *iap1*, and to study the properties of this recombinant virus, with an emphasis on detecting any host range differences compared to the single gene deletion mutants and wild type AcMNPV.

5.2. Results.

5.2.1. Construction of a recombinant AcMNPV deficient in *p35* and *iap1*.

The recombinant virus Ac*p35lacZ* (Chapter Four) was used as the basis for the production of a double mutant of AcMNPV deficient in both *p35* and *iap1*. The recombinant virus, Ac*iap1lacZ*, was constructed previously (M. Ayres, unpublished results). This virus contains the *lacZ* coding region inserted at the *iap1* locus, but it should be noted that Ac*iap1lacZ* has other detected changes in its genome.

In order to construct a *p35* deficient AcMNPV which produced colourless plaques in the presence of X-gal, a transfer vector with a deletion, rather than an interruption, in the *p35* coding region was produced. The plasmid pAc*Eco*RI-S was digested with *Bst*XI (position 116574 bp of the AcMNPV genome) and *Hind*III (position 116885 bp of the AcMNPV genome) and the ends repaired with Klenow enzyme. The vector DNA was then ligated to produce pAc*Eco*RI-S-FI, which has 311 bp of the *p35* coding region removed (see Figure 5.1). The structure of this transfer vector was confirmed by restriction enzyme analysis and sequencing of the flanking regions of the deletion. *T. ni*-368 cells were cotransfected with pAc*Eco*RI-S-FI DNA and linearised Ac*p35lacZ* DNA. Plaques which remained colourless in the presence of X-gal were selected and further purified to homogeneity to produce a stock of the virus Ac*p35*Δ.

Ac*p35*Δ DNA was prepared and analysed by restriction enzyme digestion, in comparison with Ac*p35lacZ* (Figure 5.2). Correct reassembly of the virus genome would result in the construction of Ac*p35*Δ, which should not contain *lacZ* sequences but should lack 311 bp of the *p35* coding region. The expected *Eco*RI digestion profile of Ac*p35*Δ would be identical to AcMNPV C6 apart from the loss of 311 bp from the *Eco*RI-S fragment, reducing the size of this fragment from 1458 bp to 1147 bp. However, the *Eco*RI profile of the Ac*p35*Δ isolate contained a 3 kbp fragment which was inconsistent with the expected profile of Ac*p35*Δ (Figure 5.2a). In order to

ascertain whether this DNA fragment was of viral origin, the Ac*p35*Δ DNA was digested with *Eco*RI, separated by electrophoresis prior to its transfer onto a nitrocelluolose membrane, and probed with α^{32}P-labelled AcMNPV C6 DNA (Figure 5.2b). The extra DNA fragment did not hybridise to the probe, indicating that the DNA was not of AcMNPV origin. The extra DNA fragment could be a transposon element or could represent residual *lacZ* sequence at the *p35* locus. The risk of genome rearrangement from host transposon action through propagation of viruses in *T. ni* cells has been well characterised (Cary *et al.*, 1989; Bauser *et al.*, 1996).

In order to examine the nature of the extra DNA band present in the *Eco*RI restriction profile of Ac*p35*Δ, this fragment of DNA was isolated by colony hybridisation, following the ligation of Ac*p35*Δ *Eco*RI digested DNA into pUC118. The DNA from two of these plasmids was purified and sequenced using the M13 forward and reverse primers. Sequence obtained using the forward primer on the 3 kbp fragment corresponded to the *p35* locus of the AcMNPV genome. The sequence obtained with the reverse primer corresponded to *lacZ* sequence. Further sequencing analysis, employing primers complementary to regions of the *p35* gene, enabled the characterisation of the remaining *lacZ* sequences present at the *p35* locus of Ac*p35*Δ. The result of this analysis is presented in Figure 5.3. The *p35* locus of Ac*p35*Δ contained a 780 bp deletion compared to Ac*p35lacZ*. This deletion was positioned from 116935 bp of AcMNPV to 732 bp of the inserted *lacZ* cassette. In addition, a 14 bp insertion of AT-rich DNA was located between the *p35* and *lacZ* sequences.

Four other virus isolates of Ac*p35*Δ were examined by dot bot analysis, using a radiolabelled *lacZ* probe (data not shown). However, all four viruses contained residual *lacZ* sequences. These viruses were not characterised further.

The phenotype of the Ac*p35*Δ isolate was examined in Sf21 cells. Apoptosis of the cells was observed from 10 h.p.i., indicating that the *p35* gene in the Ac*p35*Δ isolate was non-functional. This result was comparable to that

obtained following infection of Sf21 cells with Ac*p35lacZ*. Although the Ac*p35*Δ isolate did not have the expected restriction enzyme profile, it was decided to continue with the planned experimental programme, since the virus was a.) deficient in *p35* function and b.) produced colourless plaques in the presence of X-gal, and had identical properties to those required. Therefore, we decided to progress with the characterised Ac*p35*Δ isolate to produce an AcMNPV double mutant deficient in *p35* and *iap1*.

Genomic DNA of Ac*p35*Δ and p*iap1lacZ* DNA (described in section 4.2.2.1.) were used to cotransfect *T. ni*-368 cells. After incubation for 3 days, the cotransfection media were harvested and diluted to 10^{-3} for use in a plaque assay. Plaques which stained blue in the presence of X-gal were selected for further plaque purification. A double mutant deficient in *p35* and *iap1*, named Ac*p35*Δ*iap1lacZ*, was subsequently purified using *T. ni* cells.

5.2.2. Confirmation of the structure of the *p35/iap1* double deletion mutant by Southern blotting and PCR analysis.

To ensure that the anticipated recombination events had occurred in the double mutant, the DNA was analysed by two methods. Firstly, Southern blot hybridisation analysis was performed (Figure 5.4), using a probe for *lacZ* sequences. DNA isolated from AcMNPV C6, Ac*iap1lacZ*, Ac*p35lacZ*, Ac*p35*Δ and Ac*p35*Δ*iap1lacZ* was digested with *Eco*RI and separated by gel electrophoresis prior to probing with a ^{32}P-labelled *lacZ* probe (Figure 5.4). The *lacZ* probe comprised a 3.9 kbp *Bgl*II-*Bam*HI fragment from pAcRP23+8.*lacZ*, which incorporated the polyhedrin promoter. The hybridisation analysis indicated that, in addition to the lacZ fragment present at the *p35* loci of Ac*p35*Δ and Ac*p35*Δ*iap1lacZ*, Ac*p35*Δ*iap1lacZ* also contained a *lacZ* DNA fragment corresponding to that present in Ac*iap1lacZ*.

PCR analysis of the *iap1* and *p35* loci of the viral DNA was also used. This was achieved using primers described in Chapter Three. This analysis confirmed that Ac*p35*Δ*iap1lacZ* contained *lacZ* sequences at both the *p35* and *iap1* loci of its genome (Table 5.1). AcMNPV C6 DNA contains *p35* and

iap1 coding regions which, when amplified using the primers, produce fragments of 955 bp and 879 bp in size, respectively. Ac*p35lacZ* DNA contain a *lacZ* insertion at the *p35* locus and PCR with primers upstream and downstream of *p35* produced a DNA product of 4.7 kbp, however, this virus does not contain any modification to its *iap1* gene. Ac*iap1lacZ* has *lacZ* inserted at the *iap1* locus, and PCR of this region with primers upstream and downstream of *iap1* gives a product of 4.27 kbp, however, this virus does not contain any modification to its *p35* gene. The double mutant, Ac*p35Δiap1lacZ*, contains *lacZ* sequences at both the *p35* and *iap1* loci. Therefore, products produced by PCR amplification of these gene regions are comparable to those obtained from the PCR of *p35* in Ac*p35lacZ* (a reduction in the size of the fragment obtained is observed, due to the 780 bp deletion in this region) and PCR of *iap1* in Ac*iap1lacZ*.

The molecular analysis of Ac*p35Δiap1lacZ* DNA by both Southern blot and PCR confirms that both *p35* and *iap1* contained *lacZ* sequences which interrupted their coding regions.

5.2.3. The *in vitro* host range of AcMNPV *iap1/p35* deletion viruses.

Insect cells ($5x10^5$) were infected at an m.o.i. of 20 p.f.u./cell with AcMNPV C6, Ac*iap1lacZ*, Ac*p35lacZ* or Ac*p35Δiap1lacZ*. After 48 hours incubation at 28°C, the cells were examined under the light microscope for evidence of occluded virus production or the occurrence of apoptosis, characterised by severe cell blebbing. The results of this analysis are presented in Figure 5.5. (plates 1 to 7) and are summarised in Table 5.2. It can be seen that Ac*iap1lacZ* had the same phenotype as AcMNPV C6 in all the cell lines, resulting in the production of occluded virus following infection of Sf21, *T. ni*, *M. brassicae*, *S. littoralis*, *L. dispar*, *M. disstria* and *P. flammea* (Figure 5.5c). Ac*p35lacZ* and Ac*p35Δiap1lacZ* induce apoptosis in Sf21, *M. disstria*, *S. littoralis* and *L. dispar* 652 cells (Figure 5.5d and 5.5e). Cell blebbing induced by Ac*p35lacZ* and Ac*p35Δiap1lacZ* was observed from 10 h.p.i. in all the affected cell lines. *L.dispar* cells were totally fragmented at 48 h.p.i., however *M. disstria* cells displayed only partial fragmentation.

The effects of Ac*p35lacZ* and Ac*p35Δiap1lacZ* in this study were similar, in that productive infections were induced in *T. ni*, *M. brassicae* and *P. flammea* cells. However, the study did not provide any quantitative information and would not indicate if the additional deletion of *iap1* from Ac*p35Δ* caused any other effect on viral replication. Therefore, a quantitative study to analyse replication ability in the different cell lines was undertaken.

5.2.4. Replication of the *p35*, *iap1* and *iap2* deletion mutants in seven Lepidopteran cell lines.

The replication of the double mutant (Ac*p35Δiap1lacZ*) was assessed in five Lepidopteran cells lines, namely Sf21, *T. ni*, *M. brassicae*, *L. dispar* and *P. flammea*. Cells were infected with the viruses BacPAK6, Ac*p35lacZ*, Ac*iap1lacZ* and Ac*p35Δiap1lacZ* at an m.o.i. of 5. BacPAK6 was selected to represent "wild type" virus, in place of AcMNPV C6, because it contains *lacZ*, as do the *iap1/p35* deletion mutants used in this study. In addition, the virus Ac*iap2lacZ* was included in the analysis. This virus contains the *lacZ* coding region inserted in-frame with the *iap2* promoter and interrupting the *iap2* coding region (M. Ayres, unpublished results).

The virus-infected cells were incubated for 48 hours, before harvesting the medium. This was titrated using *T. ni* cells to assess virus yield. Three independent replicates of each experiment were performed. The data obtained are presented in Figure 5.6. Examination of the growth characteristics of the mutant viruses reveals that in cell lines where apoptosis was not induced by either Ac*p35lacZ* or Ac*p35Δiap1lacZ*, the virus yields were not significantly different compared to BacPAK6, for all the mutants tested. In the cell lines Sf21 and *L. dispar*, in which the deletion of AcMNPV *p35* function resulted in apoptosis, a subsequently lower yield of virus progeny was observed. The observed reduction was dramatic in Sf21 cells (100-fold lower) but was marginal in *L. dispar* cells, in which BV yields were low for all the viruses examined (approximately $1x10^5$ p.f.u./ml). The significance of the reduction was examined using

Student's T-test (with Welch's correction for data with unequal variances). The data indicated a significant difference between Ac*p35lacZ* and Ac*p35Δiap1lacZ* infection of Sf21 compared to infection by BacPAK6, Ac*iap1lacZ* and Ac*iap2lacZ* (mean of x = 2262.5, mean of y = 23, t = 6.2636, degrees of freedom = 7, p value = $4x10^{-4}$), however, a similar test using the data for *L. dispar* cells does not indicate a statistically significant difference (mean of x = 13.83, mean of y = 9.25, t = 1.8768, degrees of freedom = 10, p value = 0.09). For *P. flammea*, no differences between the yields of the five viruses were observed. In *T. ni*, Ac*iap2lacZ* displayed a slightly reduced yield compared to the other viruses. In *M. brassicae*, Ac*iap1lacZ* and Ac*p35Δiap1lacZ* displayed a slightly reduced yield compared to the other viruses, but this difference was not statistically significant (mean of x = 140, mean of y = 77.5, t = 1.6944, degrees of freedom = 13, p value = 0.114).

The deletion of *iap1* function, whether singly or in combination with *p35*, did not cause any significant reduction in virus yield compared to viruses containing an active *iap1*. Similarly the deletion of *iap2* had no effect on the virus yield from the various cell lines. These data suggested that the deletion of the *p35* gene was the main factor controlling replication ability in the different hosts. The additional deletion of *iap1* did not have any further effect on the ability of AcMNPV to maintain its host range. From these experiments, we concluded that *iap1* had no apparent role in the host range determination of AcMNPV and does not prevent apoptosis in cell lines which do not require *p35* to inhibit apoptosis.

5.3. Discussion.

Extensive changes in the genome can occur when baculoviruses are propagated in cell culture over long periods of time (Kumar and Miller, 1987). In order to construct the double mutant Ac*p35Δiap1lacZ*, two recombinant AcMNPVs, Ac*p35lacZ* and Ac*p35Δ*, were sequentially generated. These viruses could only be propagated in *T. ni* cells, and therefore, multiple passage of AcMNPV in *T. ni* cells was necessary to derive Ac*p35Δiap1lacZ*. The unexpected genetic structure of Ac*p35Δ* at the *p35*

locus, incorporating a 780 bp deletion of DNA and an insertion of 14 bp of DNA of unknown origin, may represent the occurrence of a transposon event during the propagation of this recombinant virus.

Serial propagation passage of AcMNPV using low m.o.i. can result in the selection of viruses with deletions or insertions of DNA. Insertions are usually found at a single locus affecting the 25K protein, giving rise to fp phenotypes (Fraser *et al.*, 1983; Beames and Summers, 1989). A strong selective advantage for BV production at the expense of occluded virus production can result; fp mutants have been shown to produce a higher titre of BV than wt AcMNPV and have a selective advantage for growth in serial passage *in vitro* (Potter *et al.*, 1978). Insertions are usually host-transposable elements. Transposons or mobile genetic elements were first described in maize in the 1950s (McClintock, 1958) and have since been recorded in many different types of organisms. Several transposons have been recorded at the 25K locus, and also at other loci in AcMNPV (Miller and Miller, 1982; Friesen and Nissen, 1990). Cell lines derived from *T. ni* are particularly prone to cause transposon insertion; a number of transposon types have been characterized from this cell line including the 2.7 kbp IFP2 transposon (Cary *et al.*, 1989) and *hitchhiker* (Bauser *et al.*, 1996). However, this phenomenon has additionally been recorded in serial passage of CpGV in insects (Jehle *et al.*, 1995). The potential involvement of transposon mutagenesis in the evolution of baculoviruses is only beginning to be appreciated (Fraser, 1986b; McDonald, 1993).

Insect transposable elements have characteristic target sites at the point of insertion (Beames and Summers, 1990), often targeting the tetranucleotide site TTAA (Schetter *et al.*, 1990; Wang and Fraser, 1993; Fraser *et al.*, 1995), and all terminate with 2 or 3 cysteine residues at the 5' ends of their inverted repeats. The construction of Ac*p35Δ* resulted in the addition of 14 bp of AT-rich sequence, which included the sequence TTAA (Figure 5.3). This acquired sequence may represent a unique transposon, or may be the remnants of a transposon event which occurred during the propagation of Ac*p35Δ*.

The data presented in Chapter Five indicate that both *iap1* and *iap2* are non-essential for AcMNPV infection *in vivo*. The replication of AcMNPV is unaffected by the removal of *iap1* or *iap2* from the genome in all the Lepidopteran cell lines tested. However, the removal of the *p35* gene has a significant effect on the ability of AcMNPV to replicate in certain cell types. Studies by Clem and Miller (1993) reported a two-log difference between the titre of a *p35* deletion mutant and AcMNPV L1 stocks harvested 48 h.p.i. (Sf21 cells infected at an m.o.i. of 20 p.f.u./cell).

The successful production of a recombinant AcMNPV deficient in both *p35* and *iap1* by propagation in *T. ni* cells suggests that *iap1* is not required for the control of apoptosis in *T. ni* cells, as suggested by Clem and Miller (1994b). Our study indicates that *iap1* has no significant influence on the *in vitro* host range of AcMNPV (Table 5.2). Viruses deficient in both *iap1* and *p35* maintain the same host range as viruses deficient solely in *p35*.

In order to detect subtle effects resulting from the deletion of *iap1*, a detailed examination of the growth characteristics of single and double mutants in different cell lines was undertaken. This revealed no detectable differences in the levels of virus progeny of Ac*p35lacZ* and Ac*p35Δiap1lacZ* produced in the different cell types (Figure 5.6). Further studies *in vivo* may aid the elucidation of the role of *iap1* in AcMNPV infection.

It has been previously reported that AcMNPV E2 causes apoptosis following infection of *Spodoptera littoralis* SL2 cells (Chejanovsky and Gershburg, 1995). The data also suggested that infection of *S. littoralis* SL2 with AcMNPV E2 did not result in the production of occluded virus in the cells. Although the levels of occluded virus were lower than those observed in other cell lines in our study, apoptosis of *S. littoralis* cells was not observed after infection with AcMNPV C6. In addition, occluded virus was detected in *S. littoralis* cells infected with AcMNPV C6 and Ac*iap1lacZ*. However, infection with either Ac*p35lacZ* or Ac*p35Δiap1lacZ* caused apoptosis in these cells (Figure 5.6). The origin of the *S. littoralis* cell line used in our experiments is not well documented. The cells were revived from liquid nitrogen stocks dated 1975, derived at IVEM from a culture of *S. littoralis*

(SL-A) insects maintained at IVEM at that time. Therefore, it is likely that the strain of cells we used was different to SL2. In addition, the strain of virus used in our experiments (C6) differed from that employed in previous studies (E2).

Although we have shown that it is possible to delete both *p35* and *iap1* from AcMNPV using *T. ni* cells, attempts to isolate a mutant AcMNPV deficient in *p35*, *iap1* and *iap2* proved unsuccessful (data not shown). However, recent work in our laboratory has resulted in the production of a virus deficient in both *iap1* and *iap2* (C. Griffiths, unpublished data). It is not known whether a mutant of AcMNPV deficient in *p35* and *iap2* is viable in *T. ni* cells. The theory that apoptotic-inhibition in *T. ni* cells is performed by *iap* may be valid if AcMNPV *iap2* has anti-apoptotic function in *T. ni* cells. Further studies will be required to test this hypothesis.

Figure 5.1.

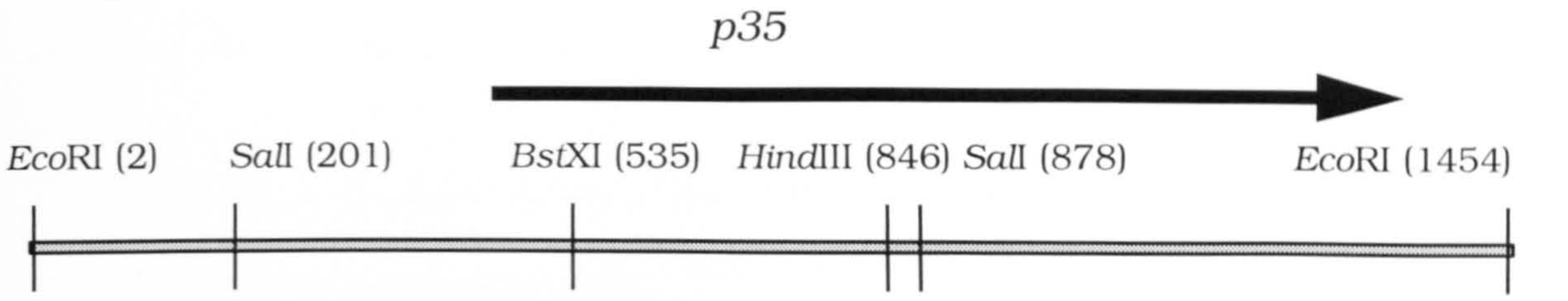
Construction of a transfer vector for the production of Acp35Δ.

Panel a shows the structure of pAc*Eco*RI-S. The *p35* gene is represented by an arrow, and the restriction enzyme sites are marked.

Panel b indicates the position of the deletion of a *Bst*XI to *Hind*III fragment in pAc*Eco*RI-S.

Panel c shows the structure of the transfer vector pAc*Eco*RI-S-FI, which has 311 bp removed from the *p35* coding region compared to pAc*Eco*RI-S.

a.



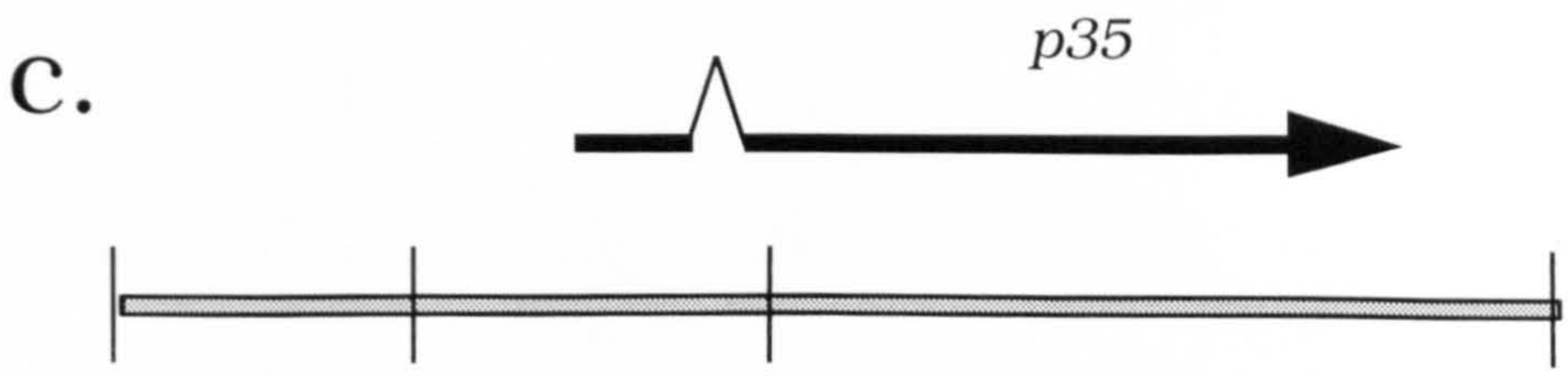
pAc*Eco*RI-S

digest with *Bst*XI and *Hind*III

b.

Klenow fill-in and religate

c.



pAc*Eco*RI-S-FI

Figure 5.2.

Southern blot hybridisation analysis of Ac*p35*Δ DNA.

The ethidium bromide stained agarose gel is pictured on the left (panel a).

Lane 1. Lambda DNA digested with *Eco*RI and *Hind*III (DNA ladder)

Lane 2. AcMNPV C6 DNA digested with *Eco*RI

Lane 3. Ac*p35lacZ* DNA digested with *Eco*RI

Lane 4. Ac*p35*Δ DNA digested with *Eco*RI

The autoradiograph of the same gel probed with α^{32}P-dATP-labelled AcMNPV C6 DNA is shown on the right (panel b). The arrow indicates the unexpected DNA fragment in the restriction enzyme profile of Ac*p35*Δ. It does not hybridise to AcMNPV C6 DNA and is therefore not of viral origin.

a. **b.**

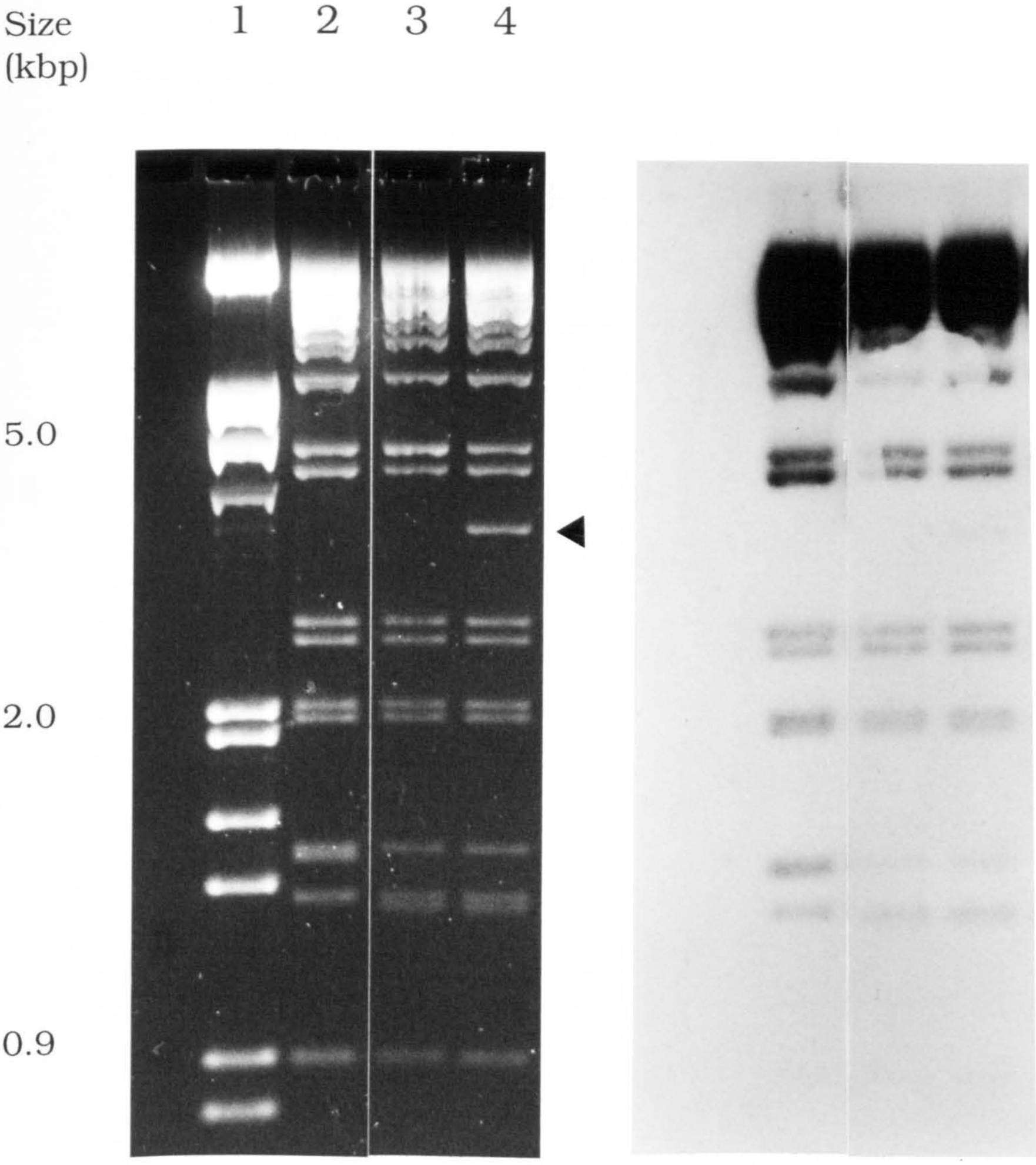


Figure 5.3.

Structure of Ac*p35*Δ at the *p35* locus.

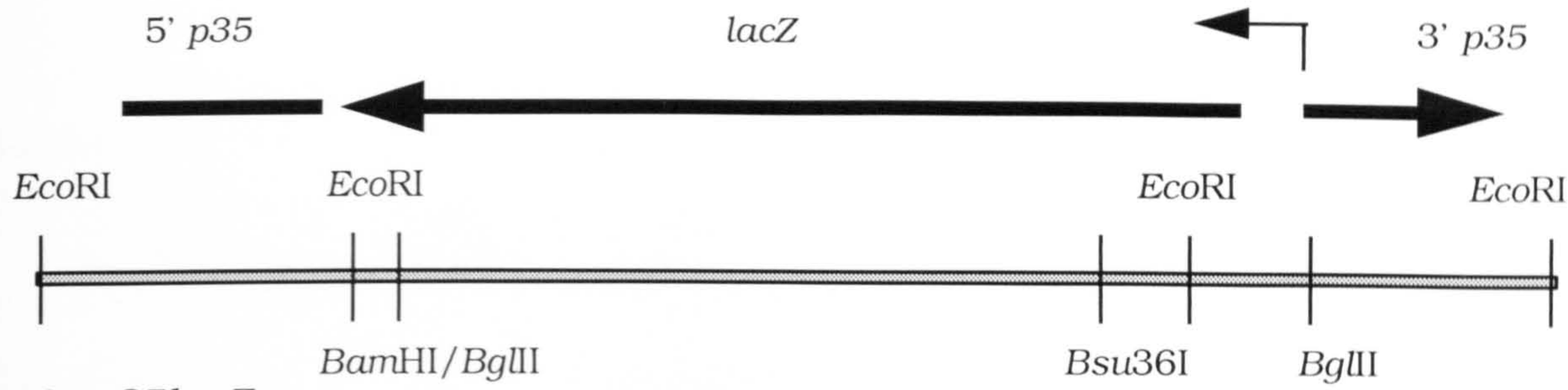
The unexpected DNA fragment in the *Eco*RI profile of Ac*p35*Δ was isolated by colony hybridisation to identify plasmids containing this DNA fragment. Sequencing of the DNA region enabled the characterisation of this locus of the Acp35Δ genome.

Panel a. The *p35* locus of Ac*p35lacZ* is represented as a horizontal shaded line, showing the *lacZ* coding region (arrow) interrupting the *p35* coding region (arrow). The restriction enzyme sites in this region are indicated.

Panel b shows the *p35* locus of Ac*p35*Δ, which has a deletion of 780 bp in the 5' region of the *lacZ* coding region and the 3' region of *p35*.

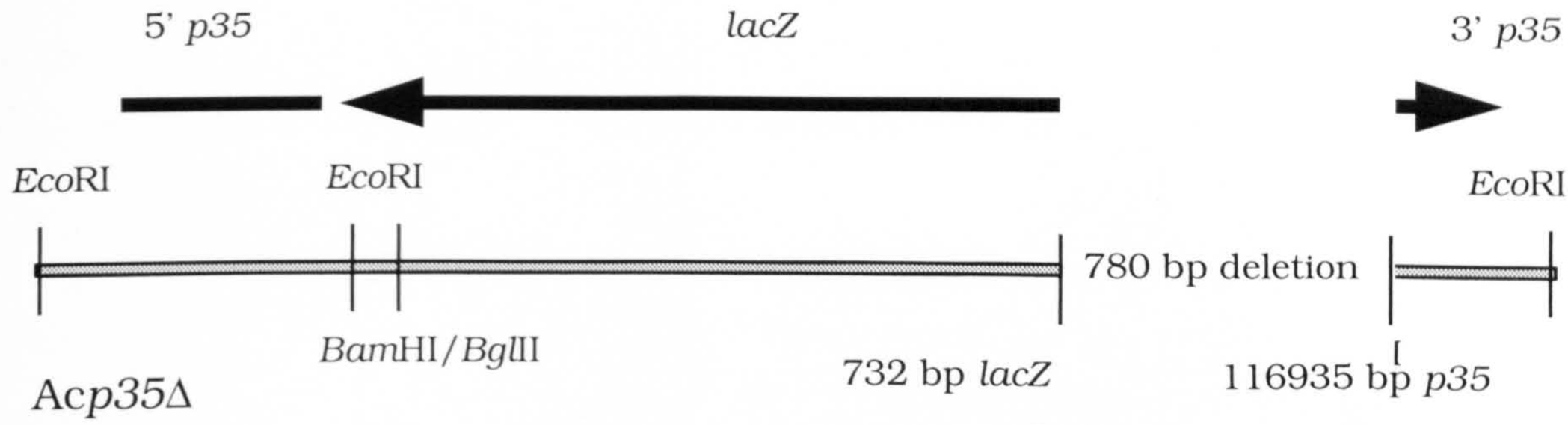
Panel c shows the deleted region in detail. A 14 bp insertion of AT-rich sequence is inserted between position 116935 bp of AcMNPV and position 732 bp of the *lacZ* coding region. The deletion reduces the size of the *Eco*RI fragment to 3.1 kbp from the 3.9 kbp fragment present in Ac*p35lacZ* (shown by a double arrowed horizontal line).

a.



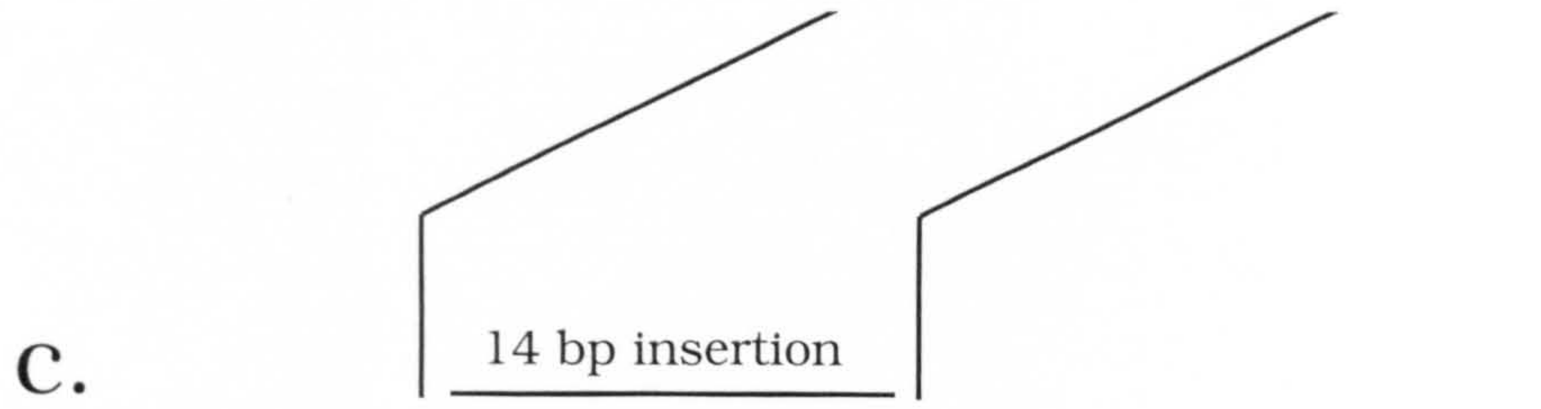
Ac*p35lacZ*

b.



Ac*p35Δ*

c.



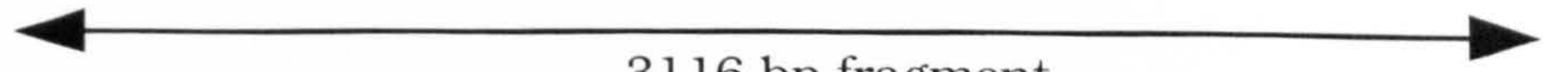


Figure 5.4.

Southern blot hybridisation analysis of Ac*p35Δiap1lacZ* DNA, probed with *lacZ*.

The agarose gel is shown in panel a. The DNA lanes are as follows: AcMNPV C6 (lane 1), Ac*iap1lacZ* (lane 2), Ac*p35lacZ* (lane 3), Ac*p35Δ* (lane 4) and Ac*p35Δiap1lacZ* (lane 5). The DNA samples were digested with *Eco*RI. Panel b shows the autoradiograph of the same gel probed with α^{32}P-dATP-labelled *lacZ* DNA.

The pattern of hybridisation is consistent with the expected profile of Ac*p35Δiap1lacZ*. Two bands in the *Eco*RI profile of this virus hybridise to *lacZ* - the first is identical to that in Ac*p35Δ* and the second identical to that in Ac*iap1lacZ*, indicating that both the *p35* and *iap1* genes of this virus are interrupted by *lacZ*.

a.

b.

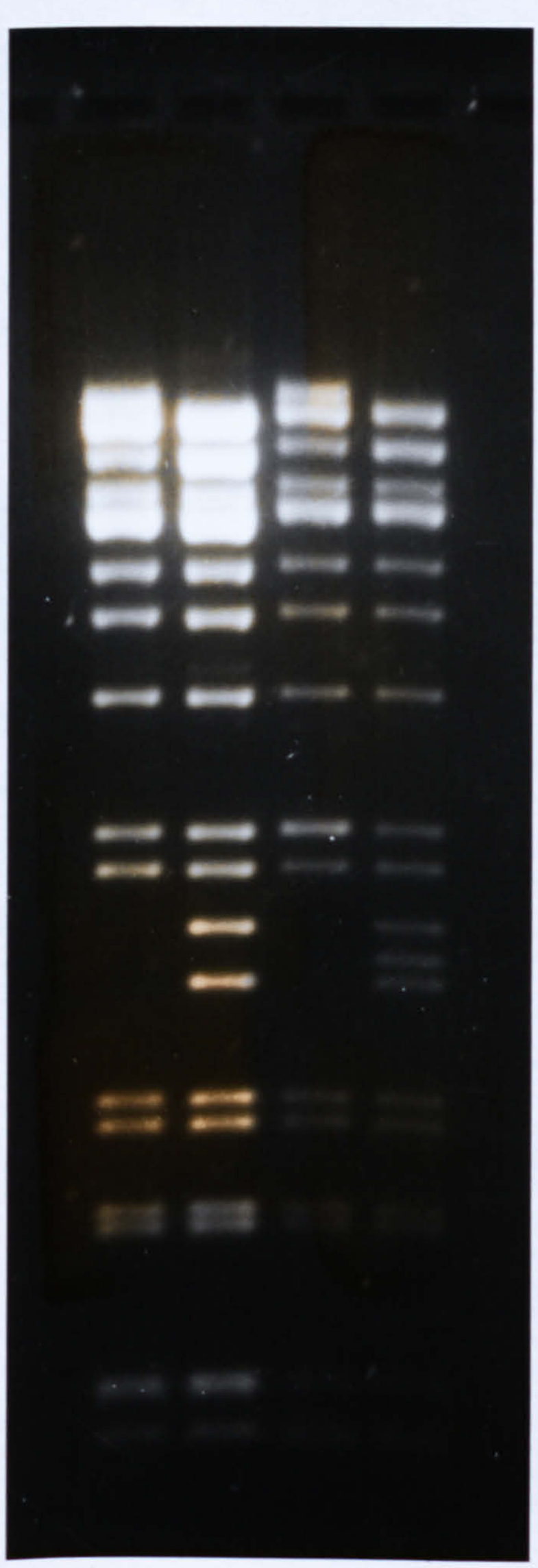


Table 5.1.

Analysis of Ac*p35*Δ*iap1 lacZ* DNA by PCR amplification.

Viral DNA was analysed by PCR using primers designed to amplify the *p35* and *iap1* coding regions (described previously in Chapter Three). The reactions were performed using *Taq* DNA polymerase, with an annealing temperature of 55°C. The reaction products (one tenth) were analysed by gel electrophoresis, and sized by comparison to lambda DNA markers.

The sizes of the DNA fragments obtained are shown in the table below:

Virus	*product size with p35 primers (bp)*	*product size with iap1 primers (bp)*
AcMNPV C6	955	879
Ac*p35lacZ*	4703	879
Ac*iap1lacZ*	955	4273
Ac*p35*Δ	3923	879
Ac*p35*Δ*iap1lacZ*	3923	4273

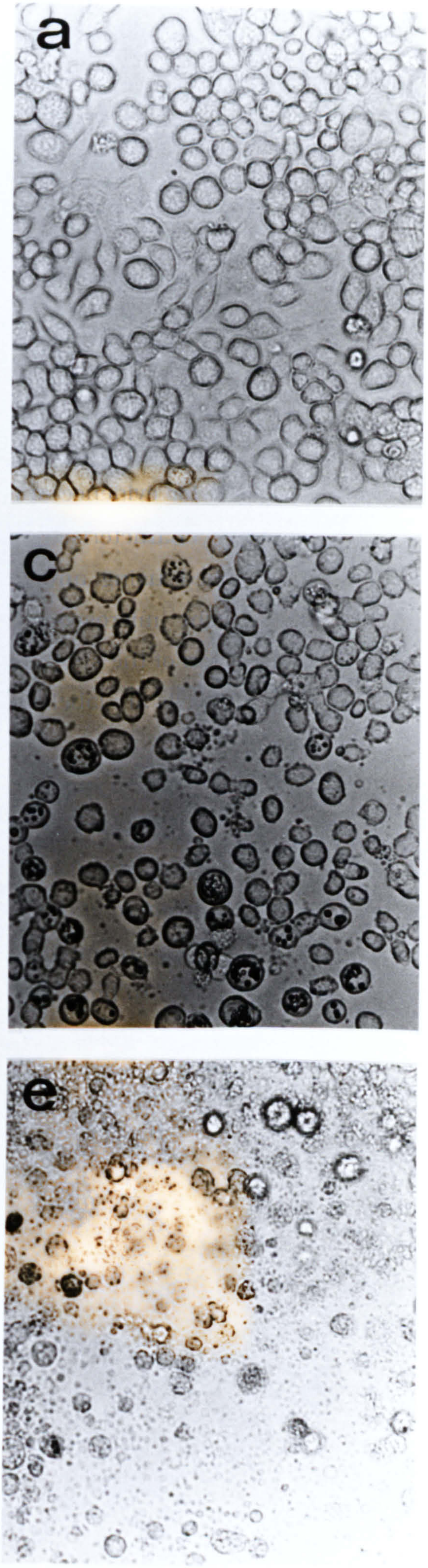
Figure 5.5.

Morphology of cells from seven insect species infected with AcMNPV C6, Ac*iap1lacZ*, Ac*p35lacZ* and Ac*p35Δiap1lacZ*.

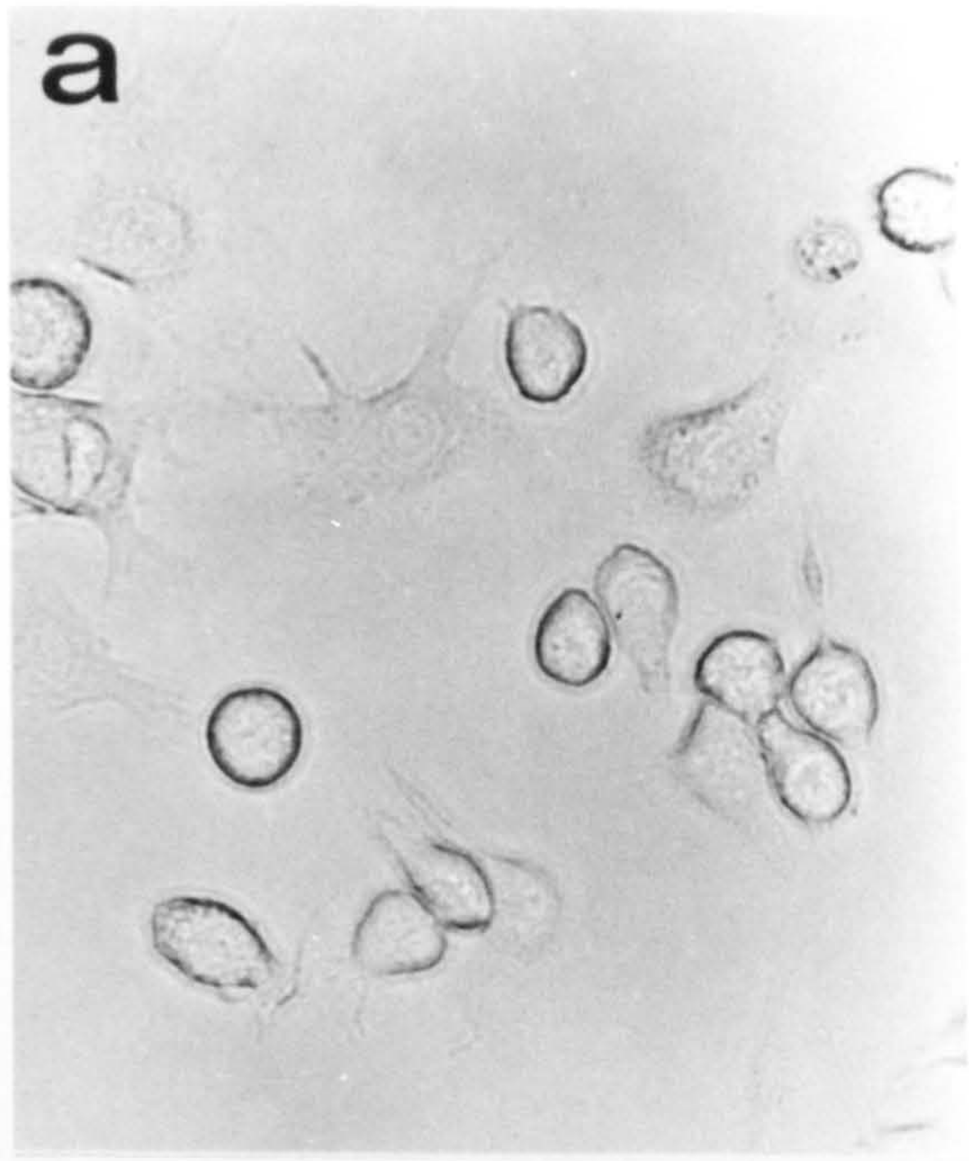
Cells ($5x10^5$) were infected at an m.o.i. of 20 p.f.u./cell with the viruses AcMNPV C6 (b), Ac*iap1lacZ* (c), Ac*p35lacZ* (d) and Ac*p35Δiap1lacZ* (e). After 48 hours incubation at 28°C, the cells were examined under the light microscope for evidence of occluded virus production or the occurrence of apoptosis, characterised by severe cell blebbing. The dishes were photographed using an Olympus OM-2 camera mounted above a Leitz Wetzlar light microscope. The photographs are reproduced at 200x magnification of the subject.
Uninfected cells are labelled (a).

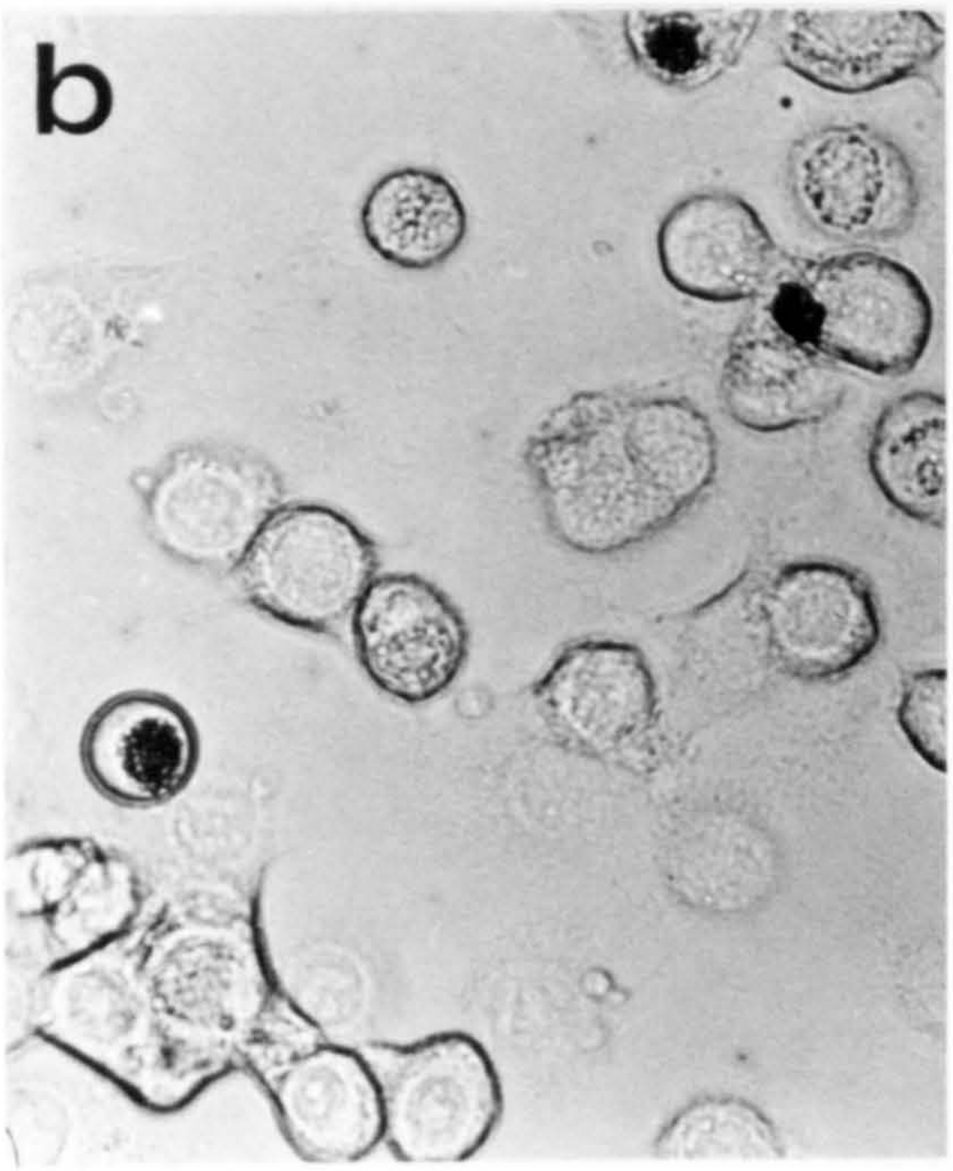
Panel 1. *Spodoptera frugiperda* 21
Panel 2. *Trichoplusia ni* 368
Panel 3. *Malacosoma disstria*
Panel 4. *Spodoptera littoralis*
Panel 5. *Panolis flammea*
Panel 6. *Mamestra brassicae*
Panel 7. *Lymantria dispar* 652

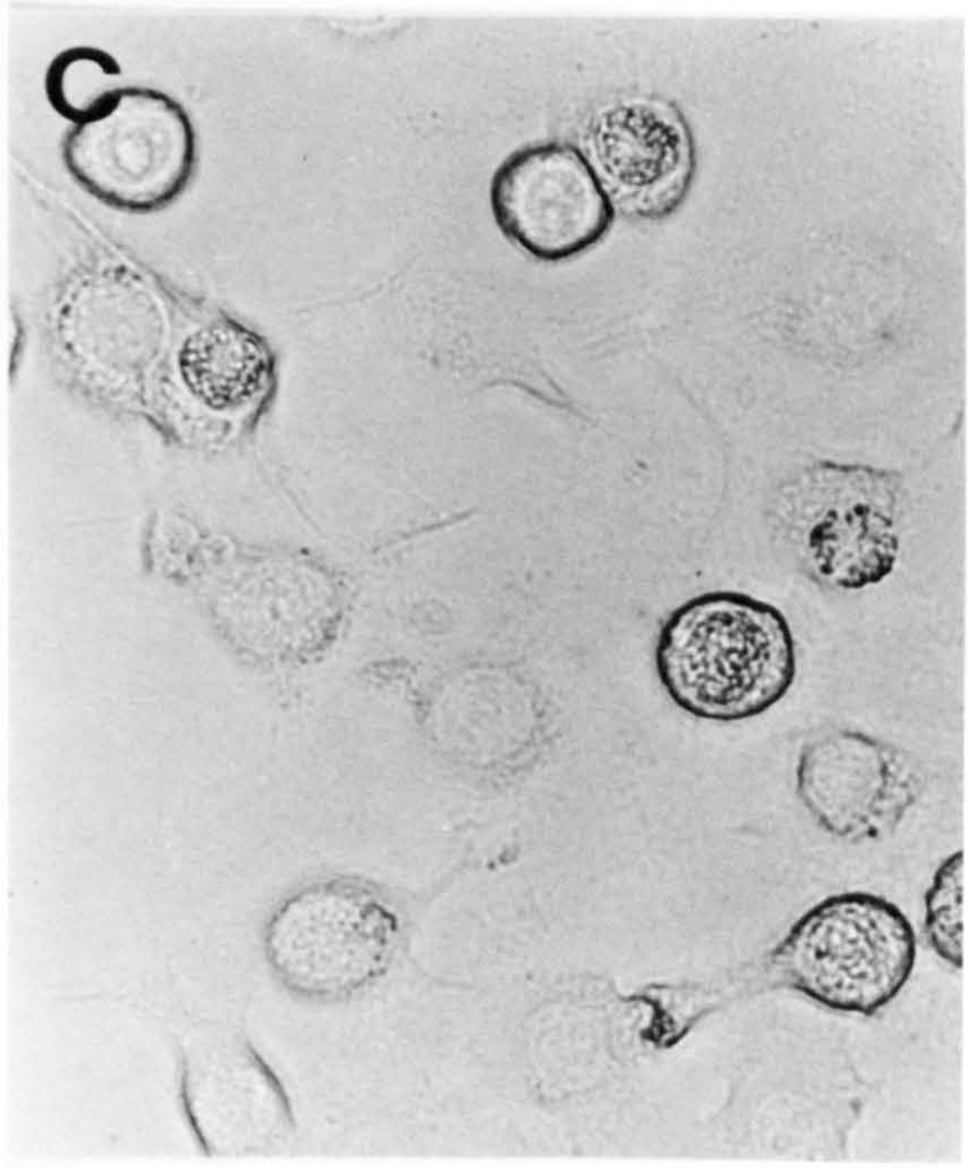
***Spodoptera frugiperda* 21**

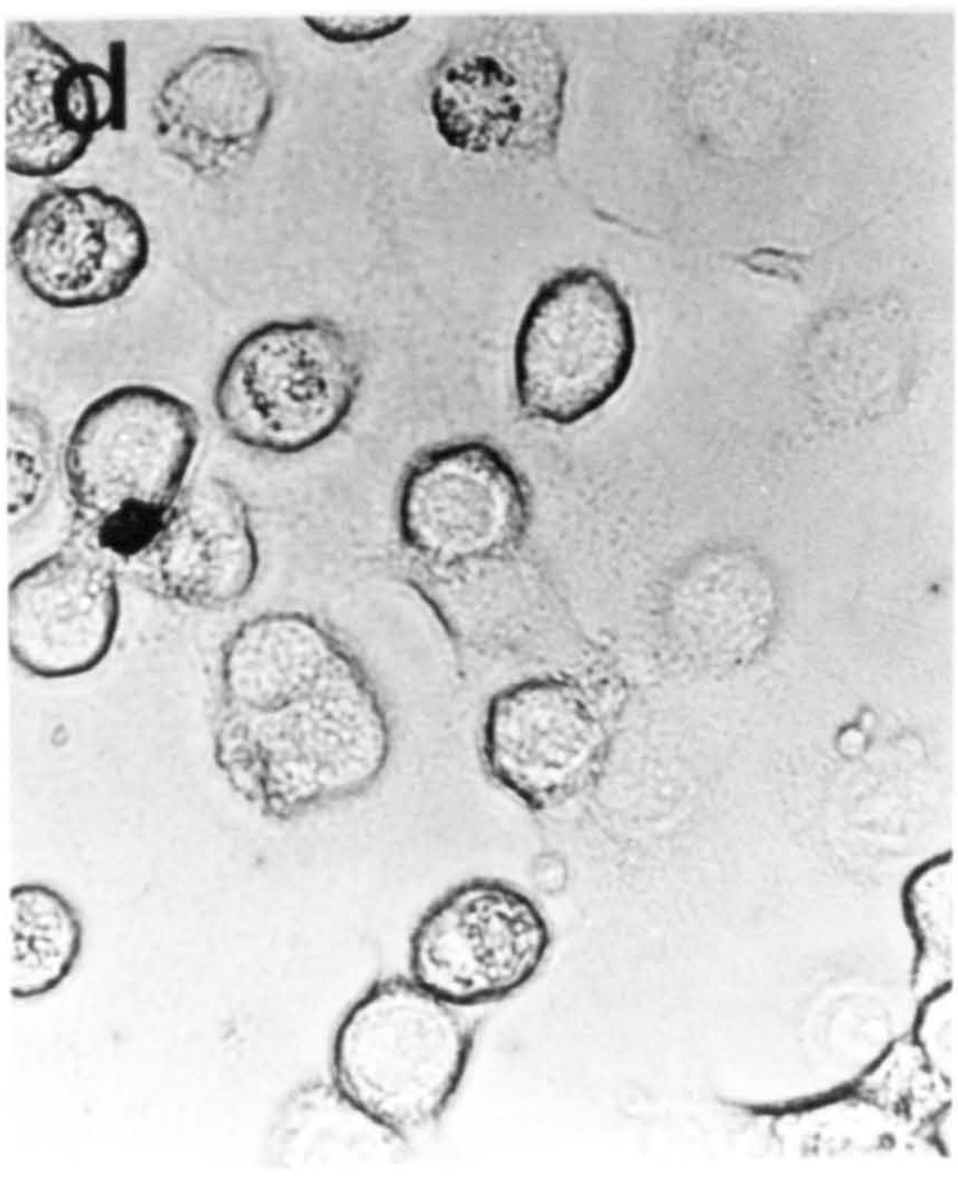


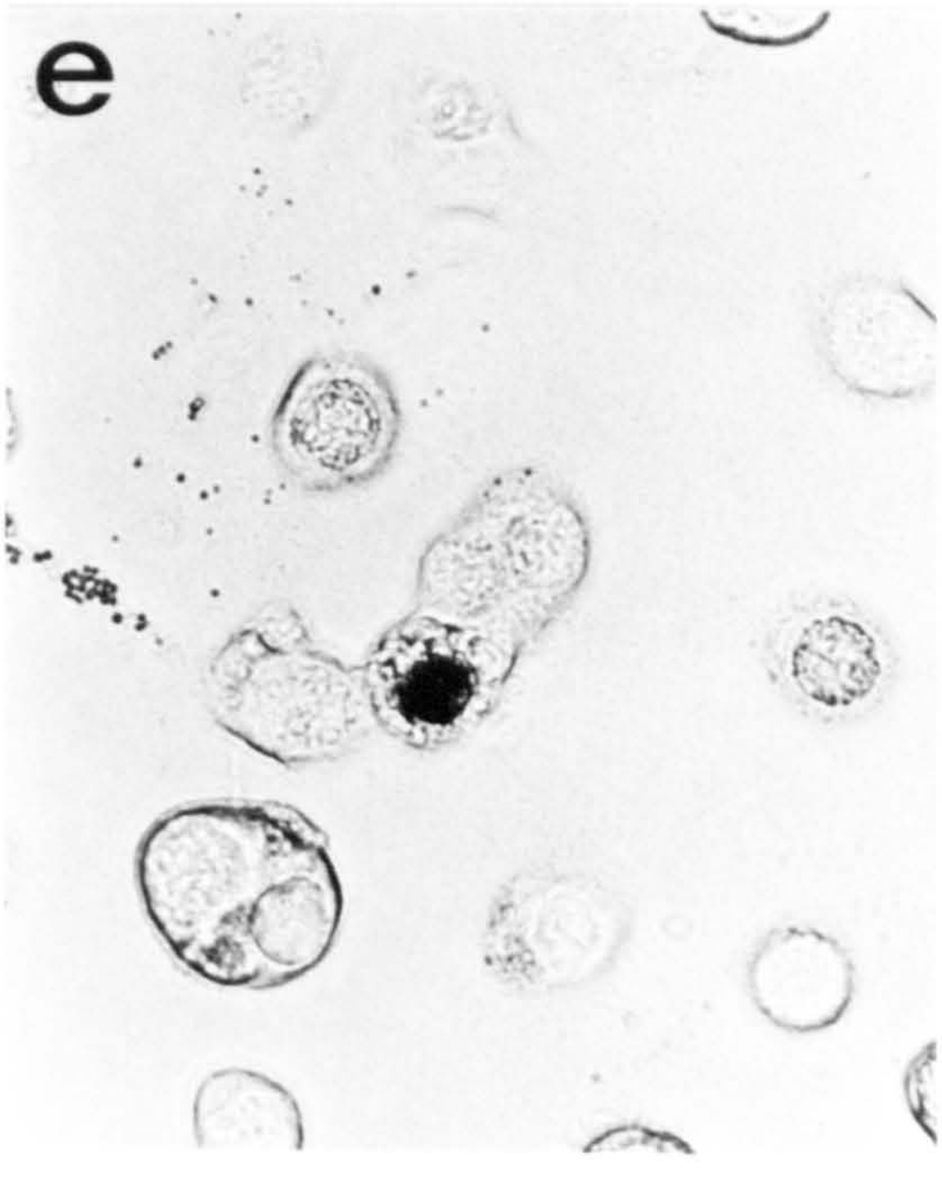
Trichoplusia ni 368



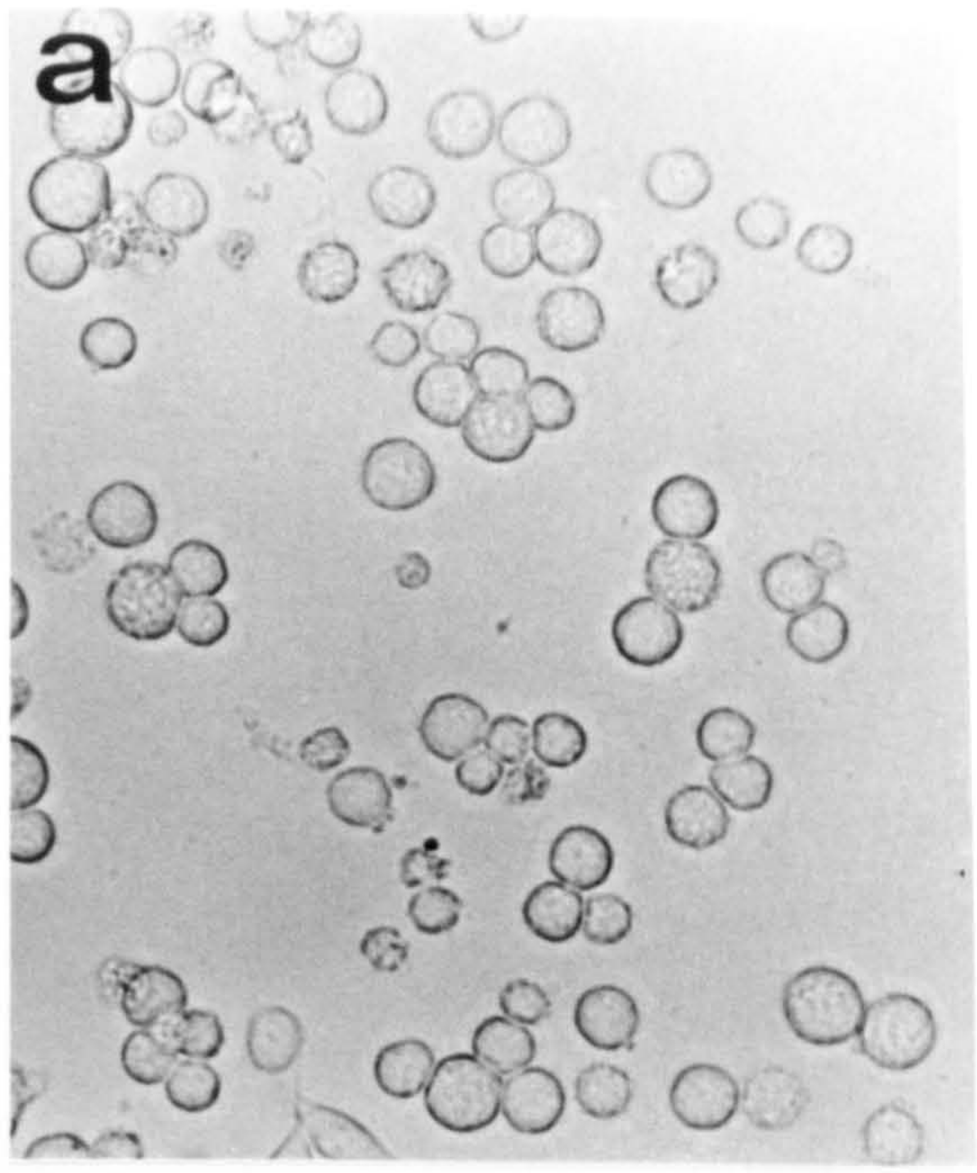


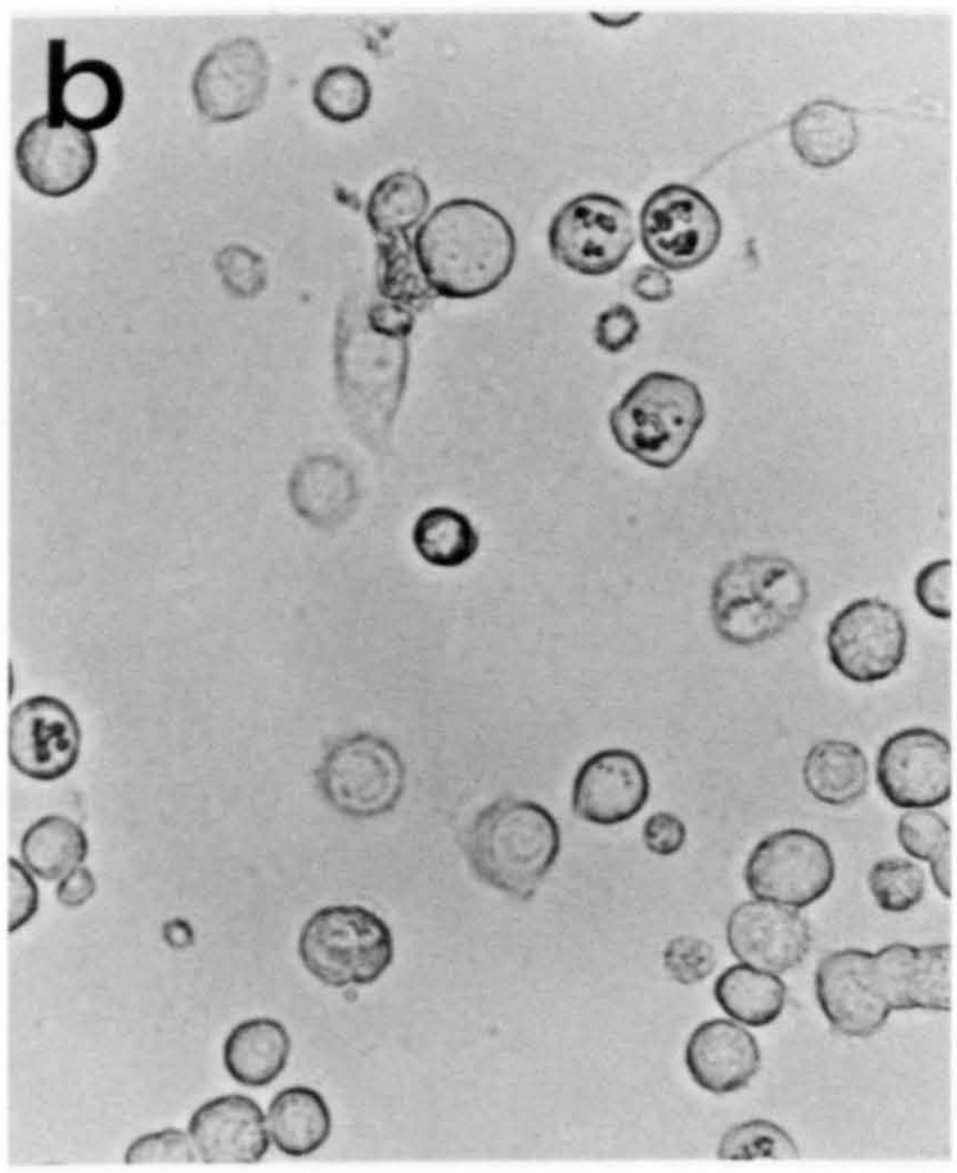


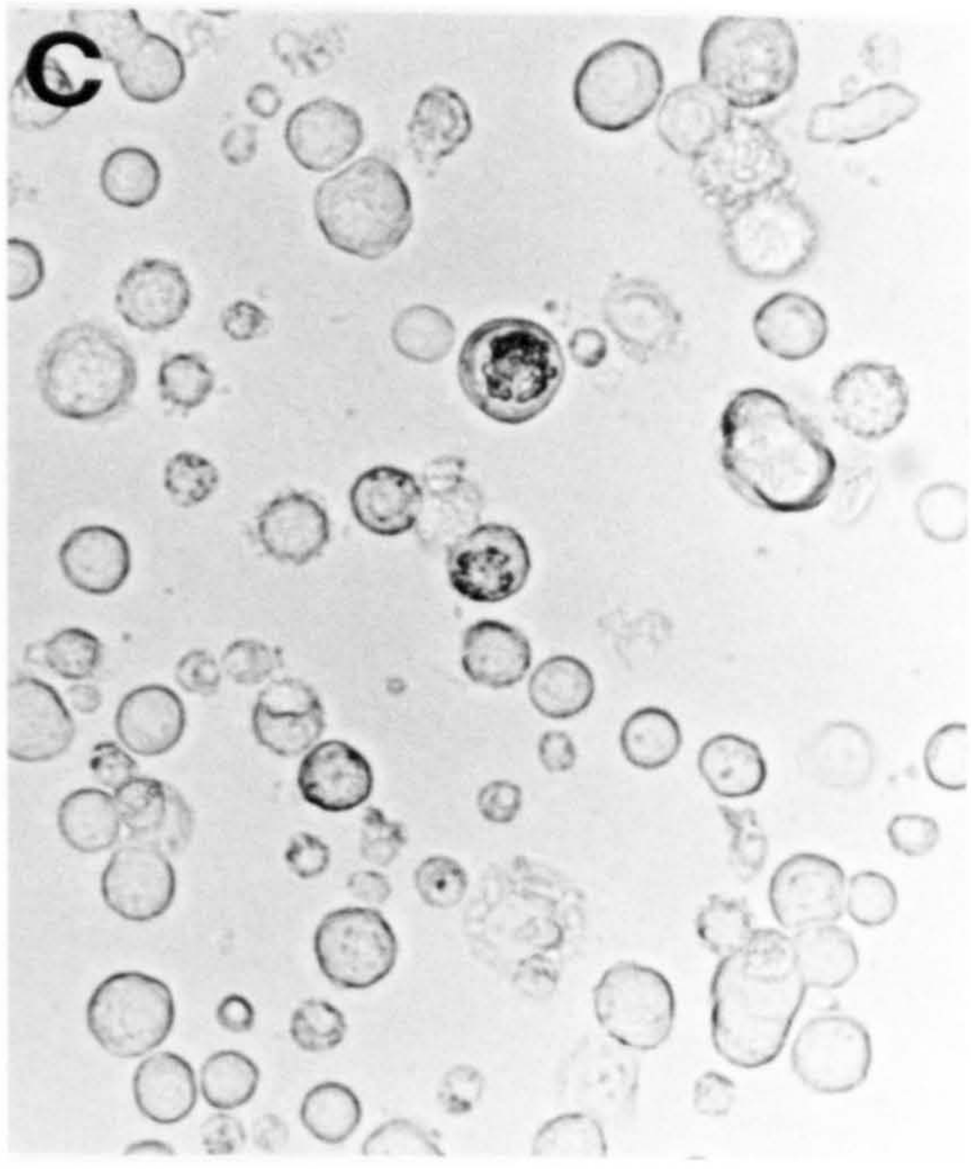


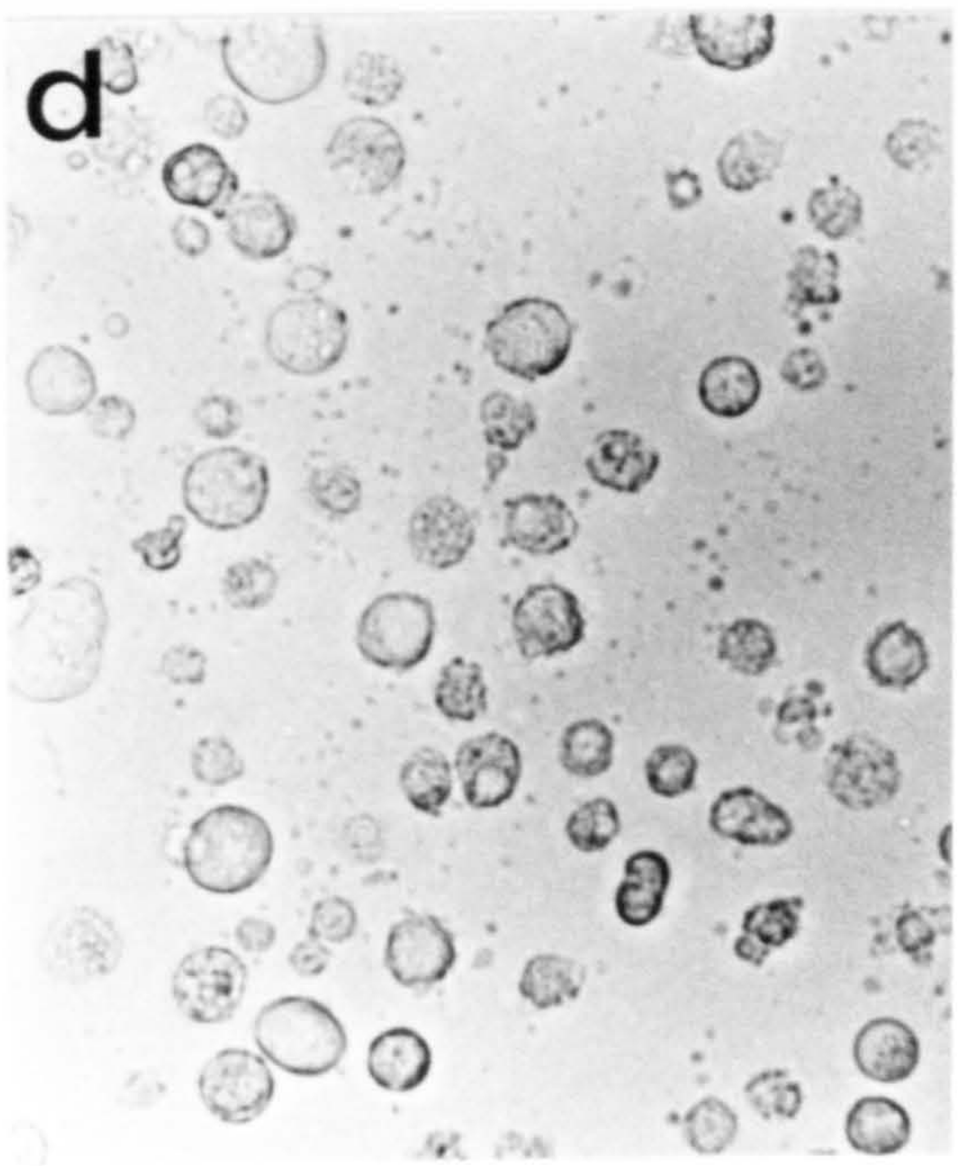


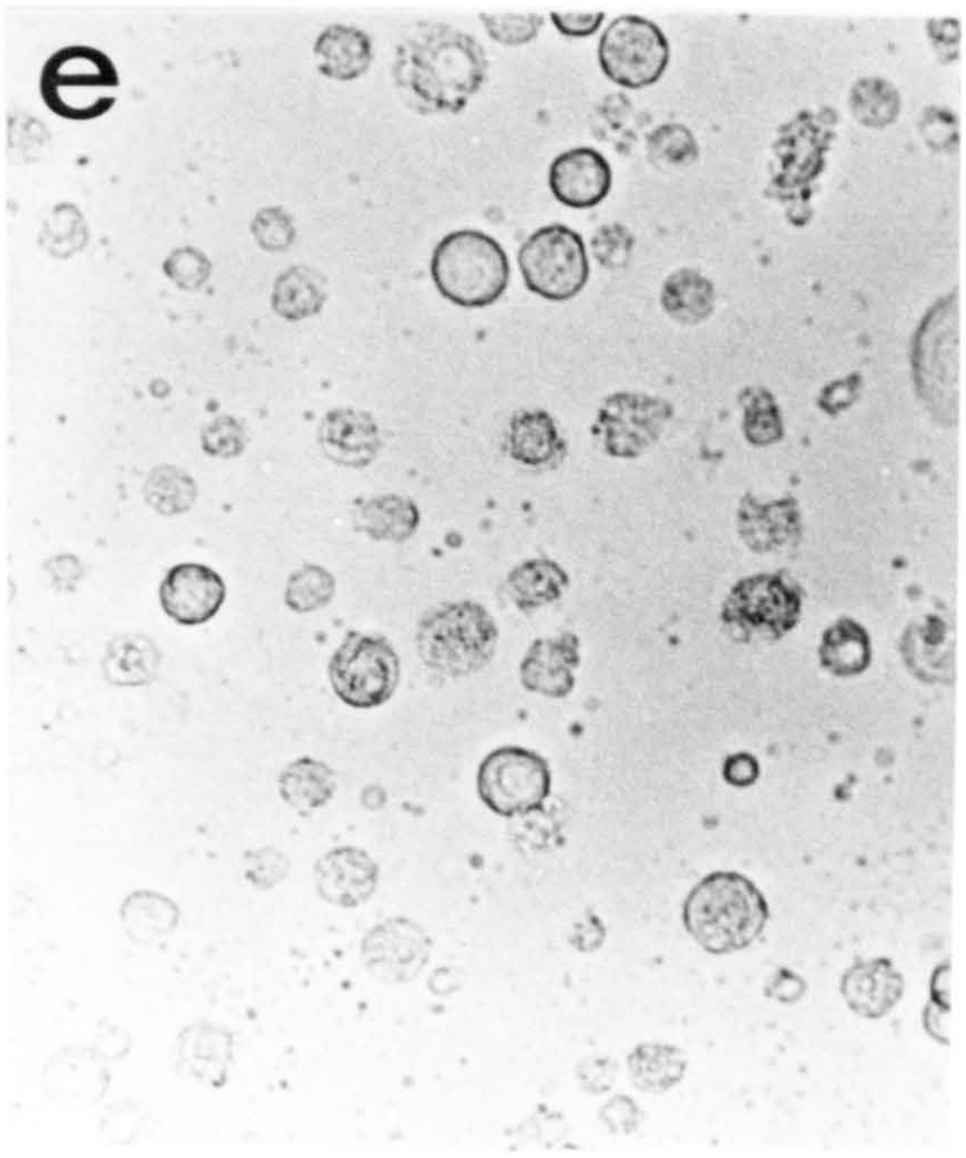
Malacosoma disstria



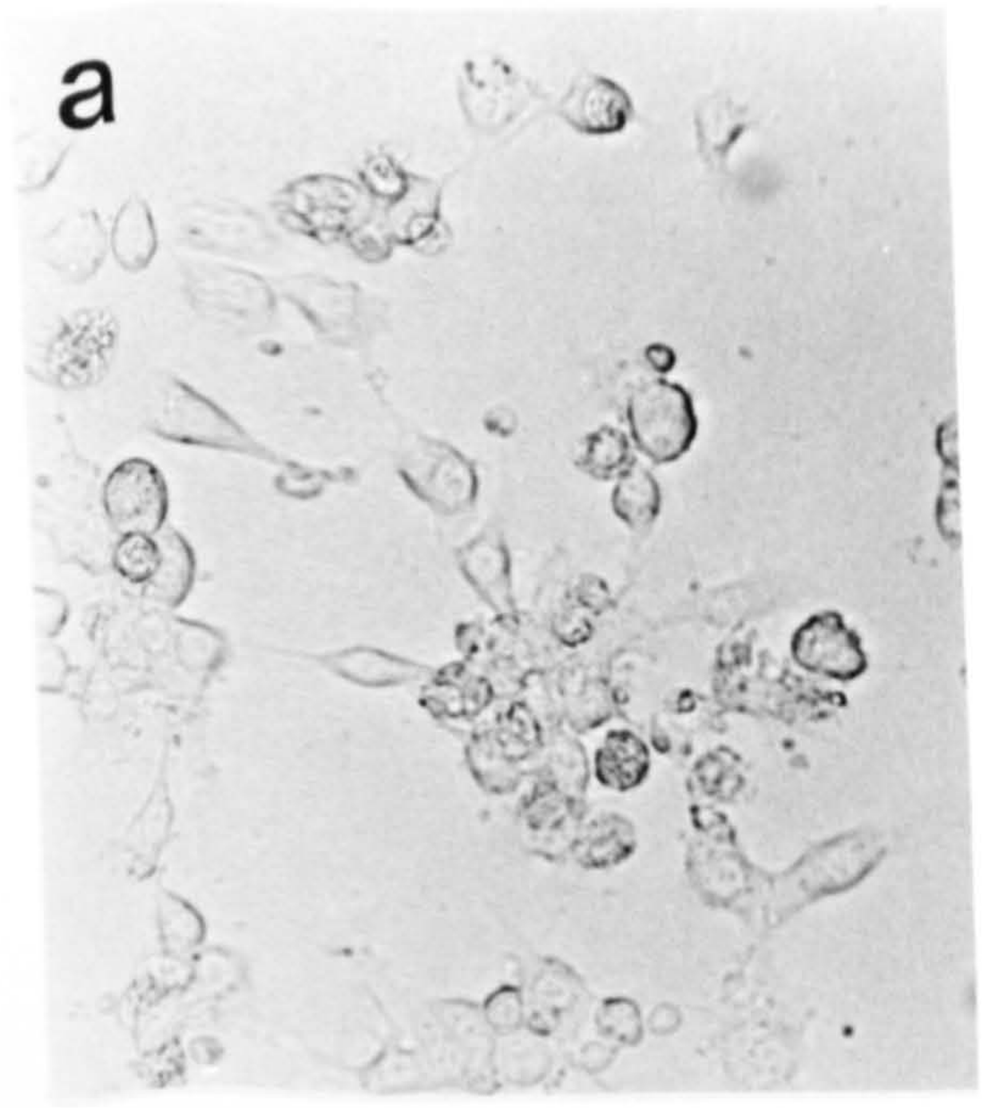


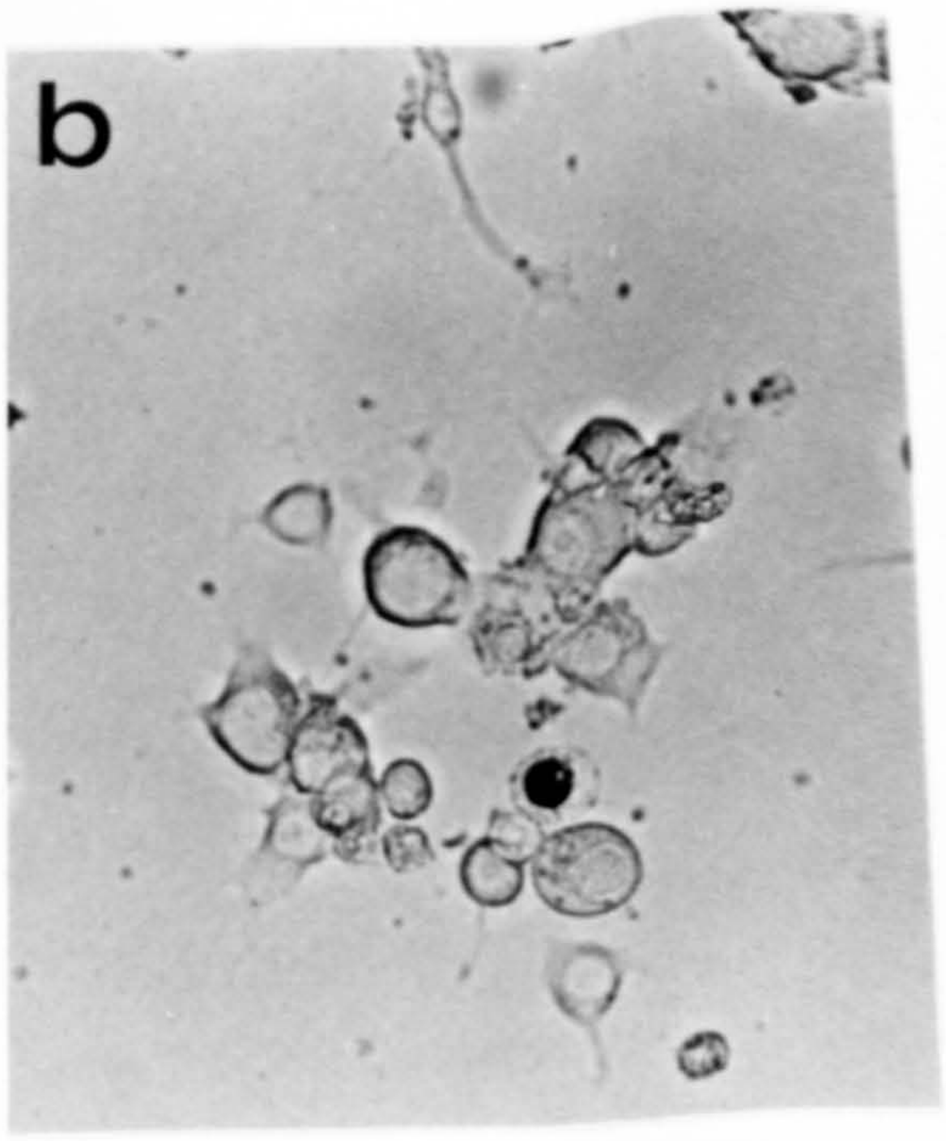


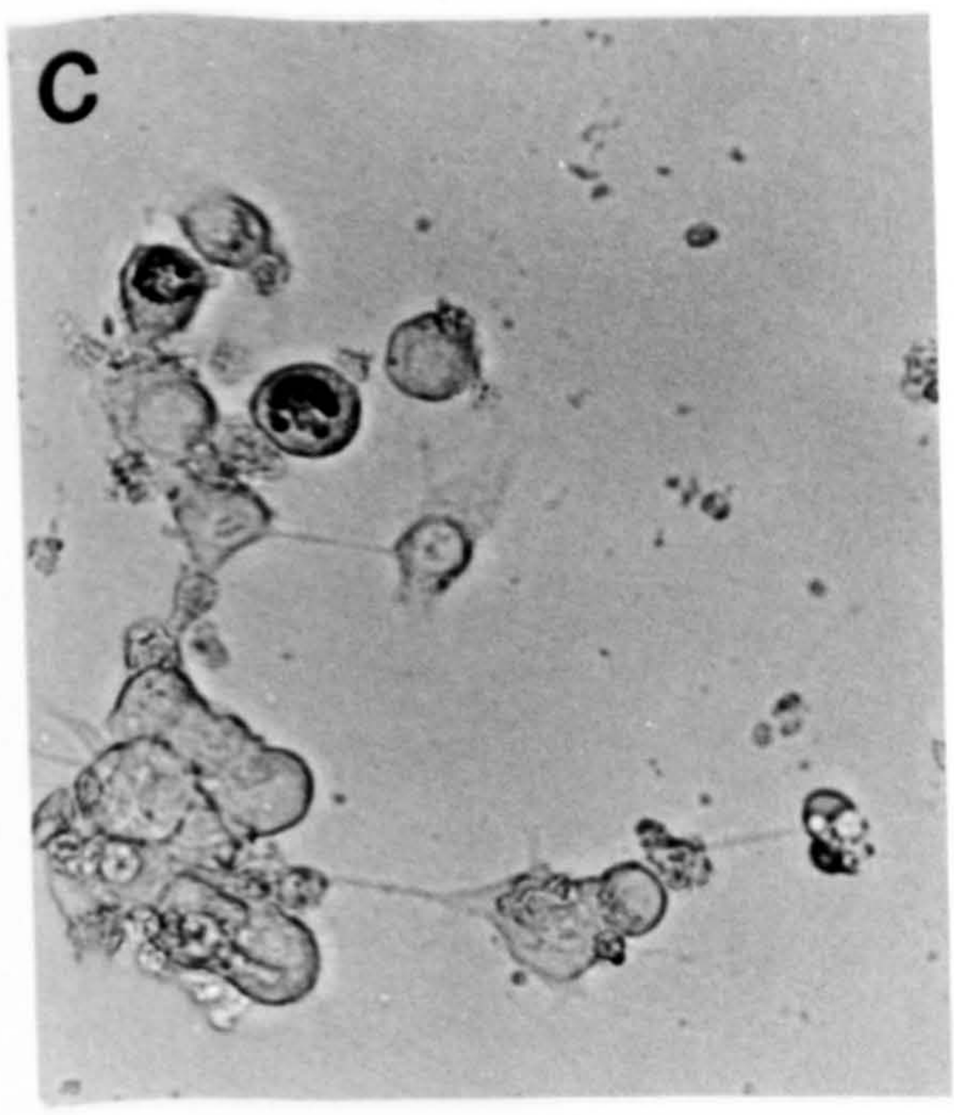


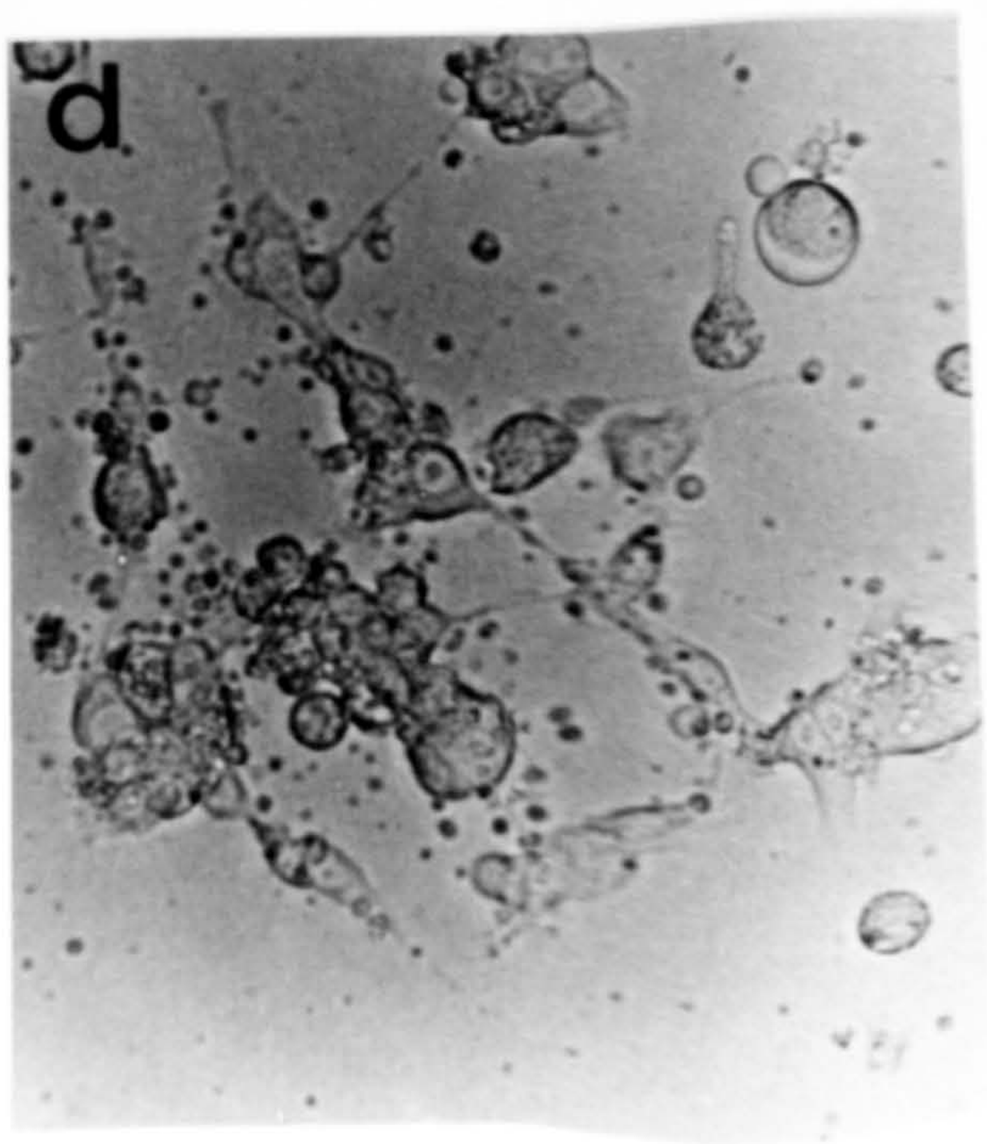


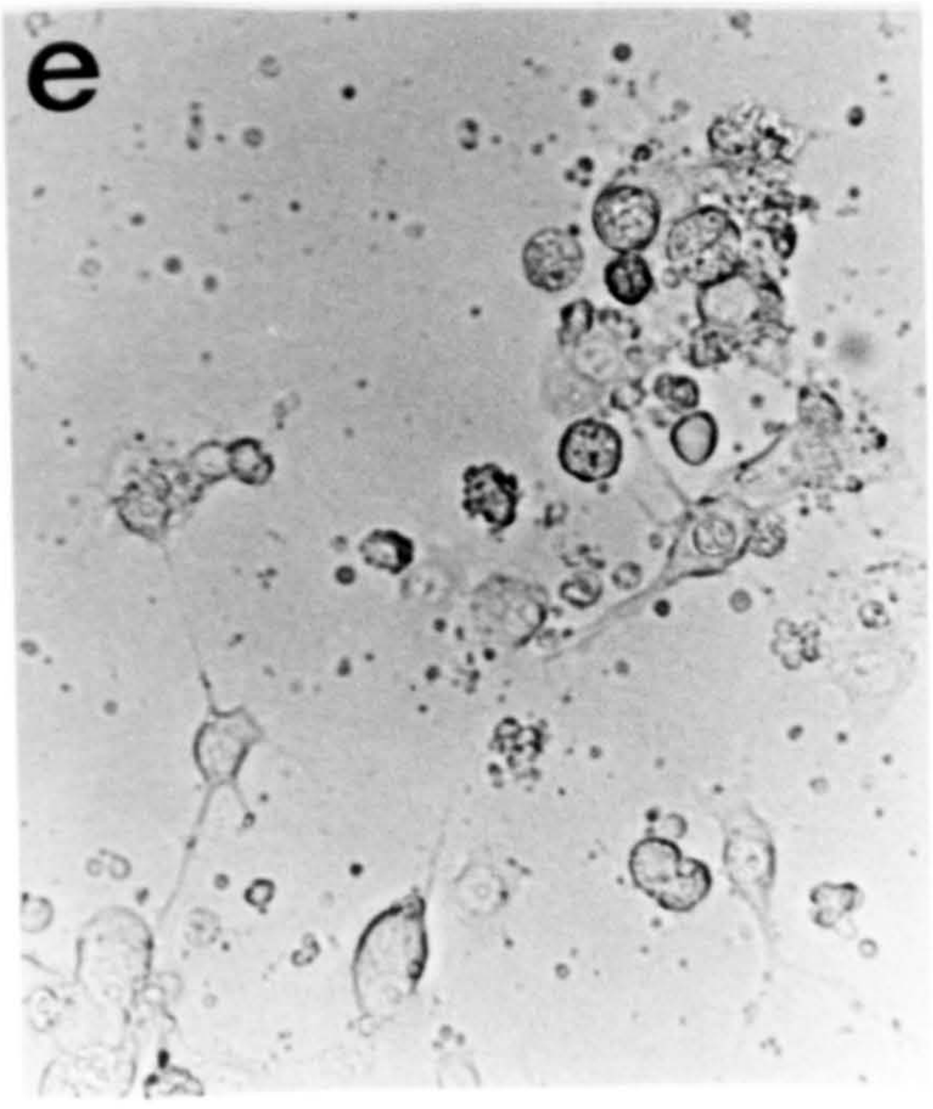
Spodoptera littoralis



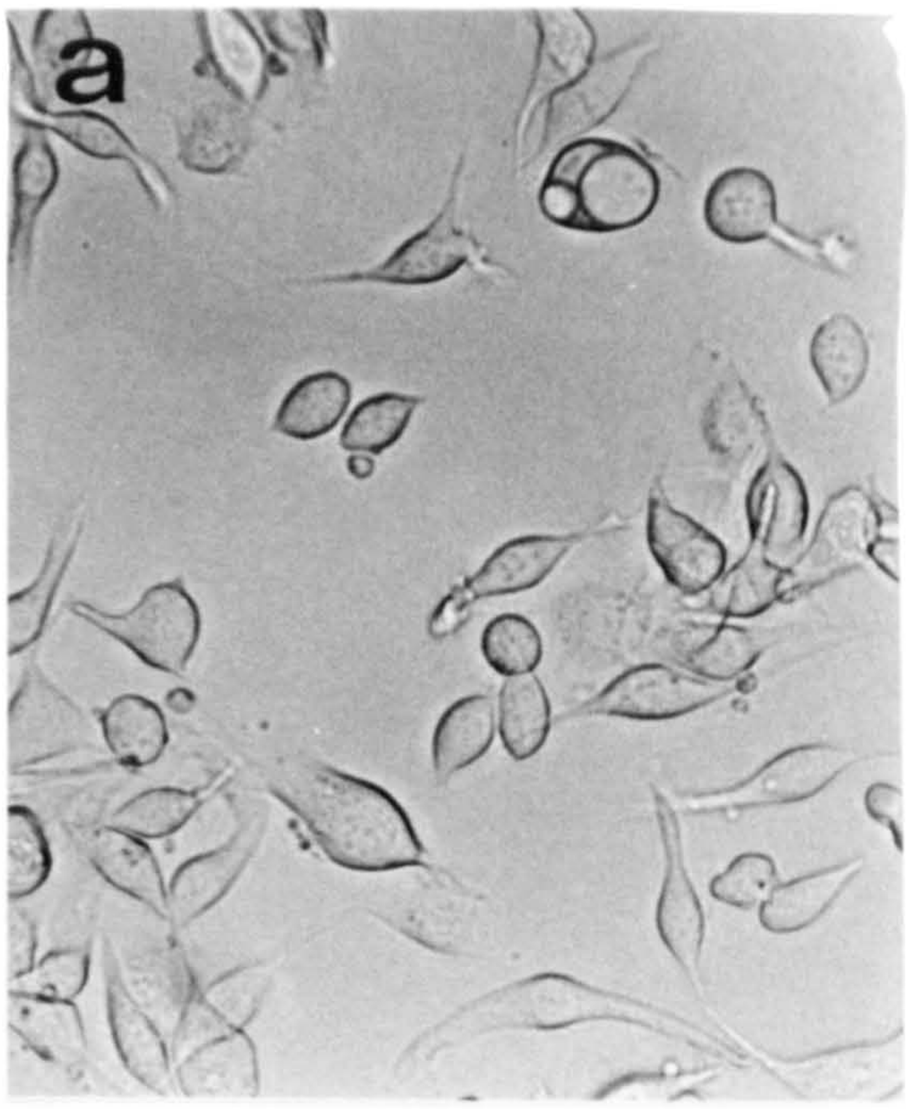


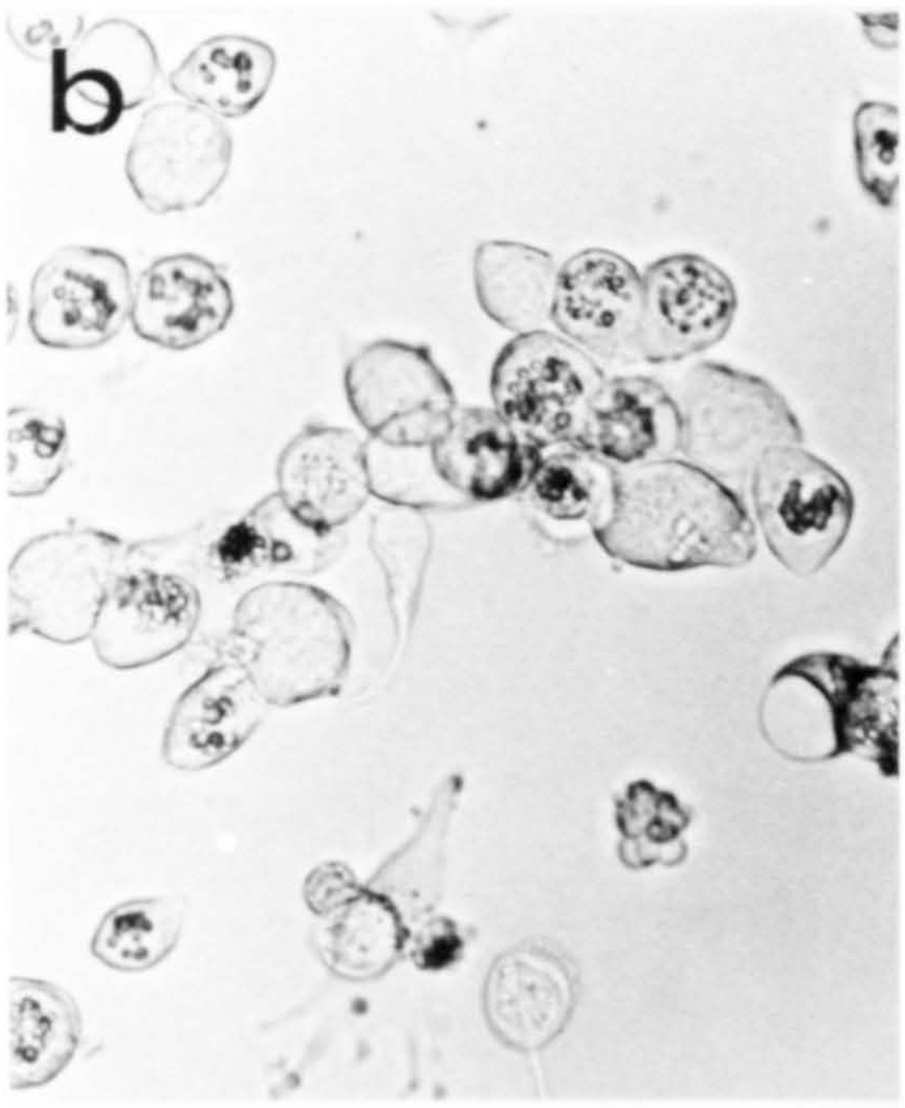


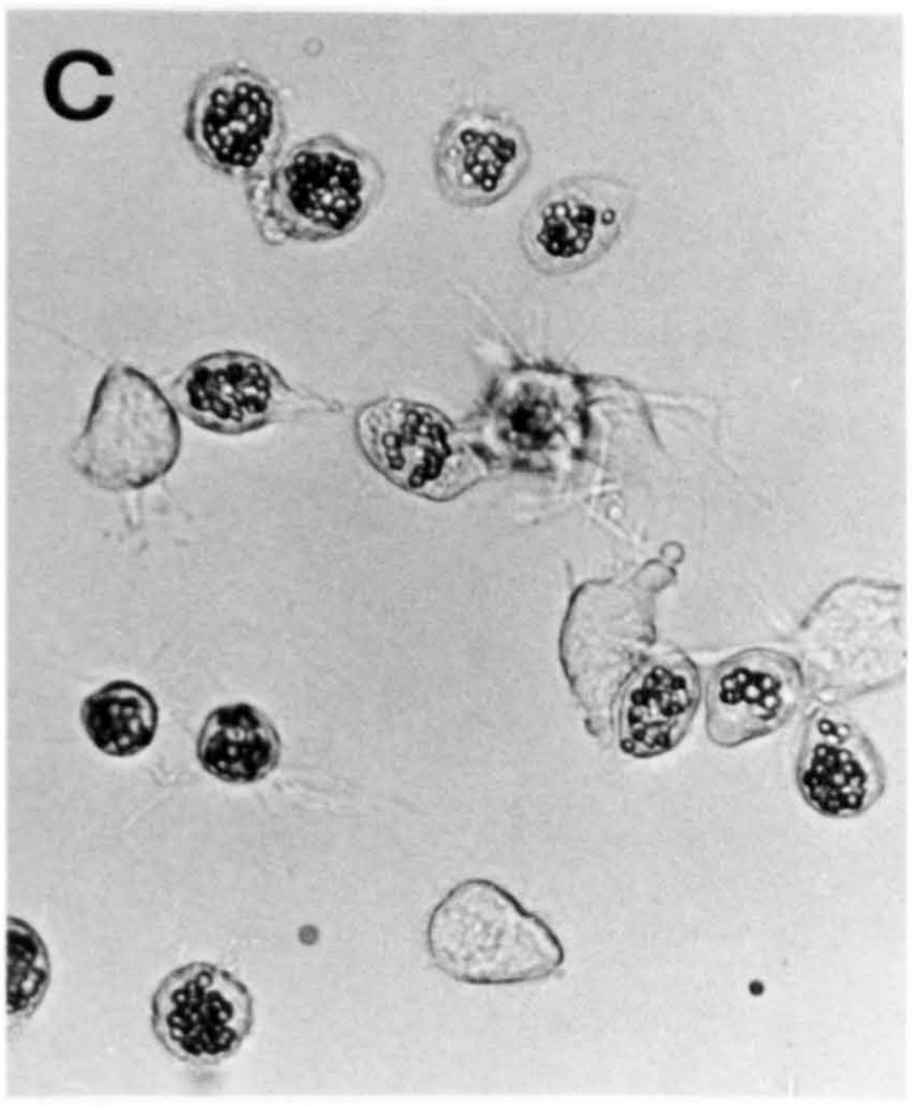


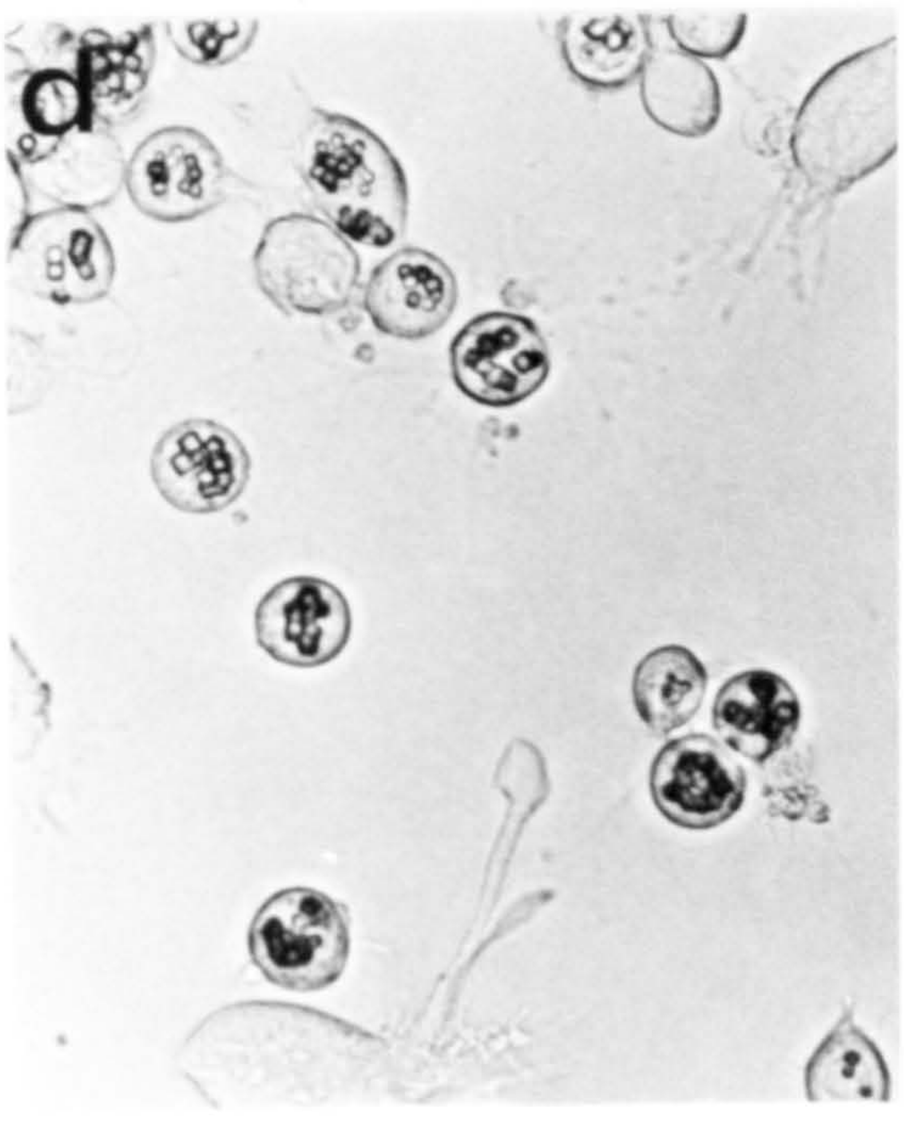


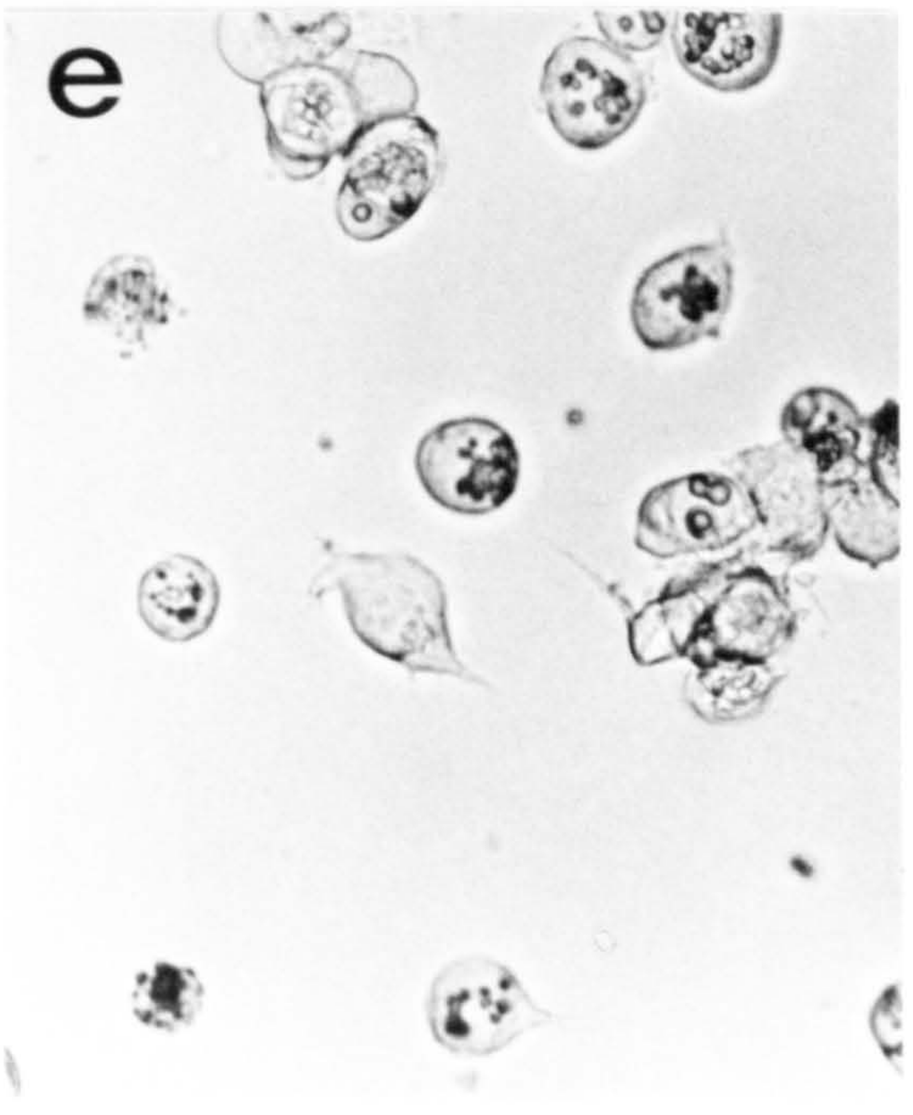
Panolis flammea



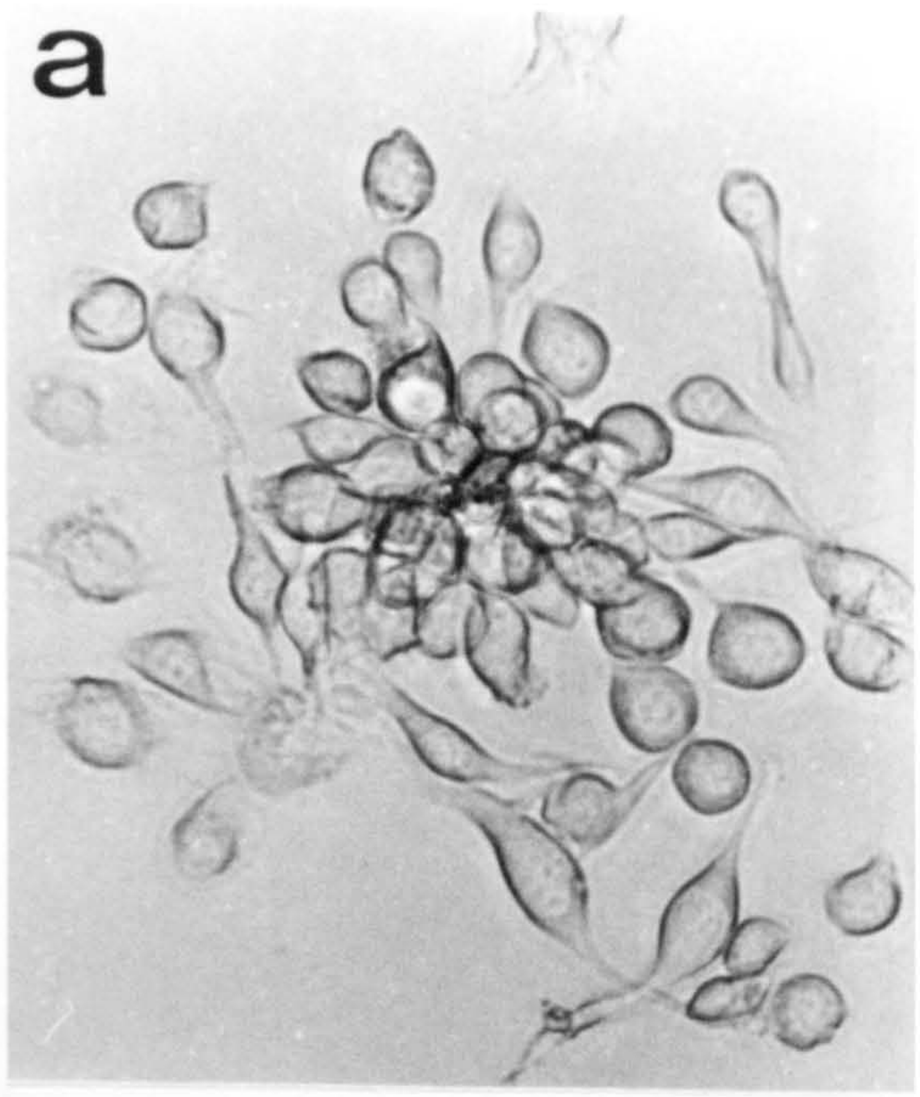


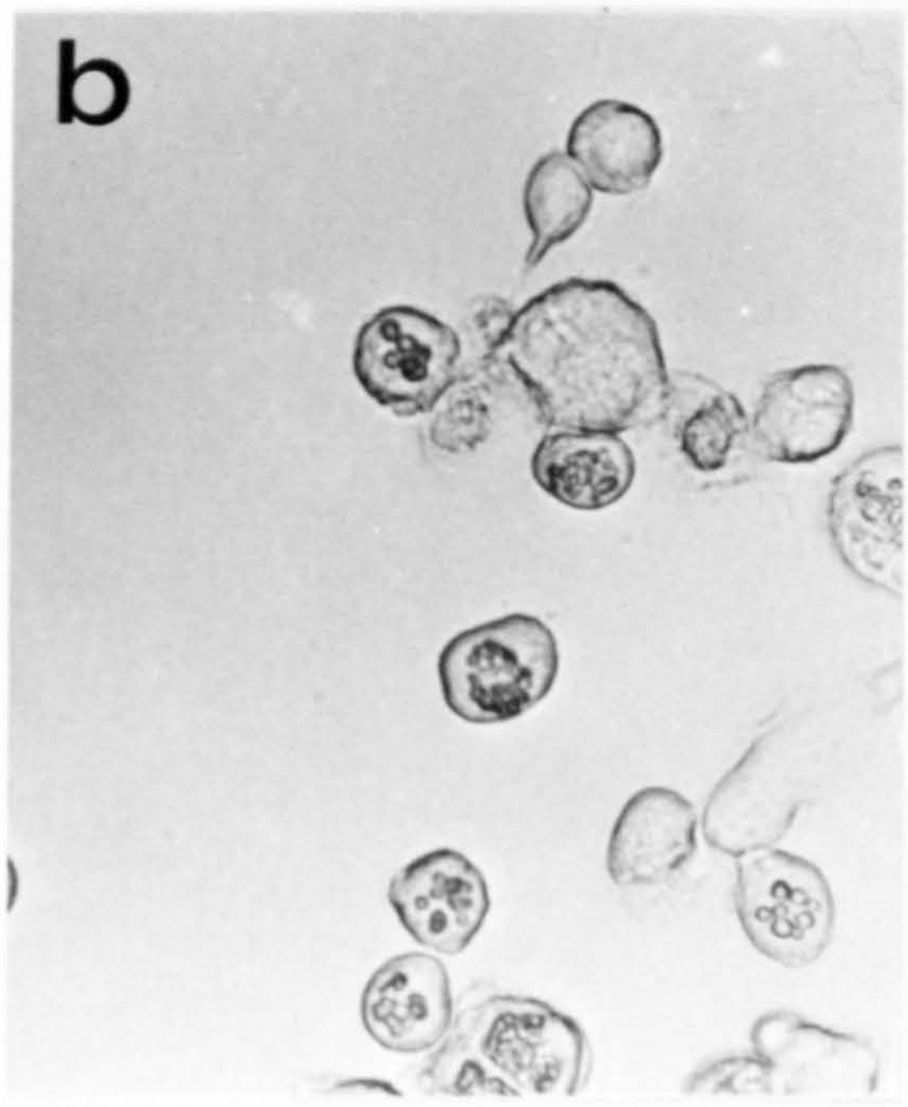


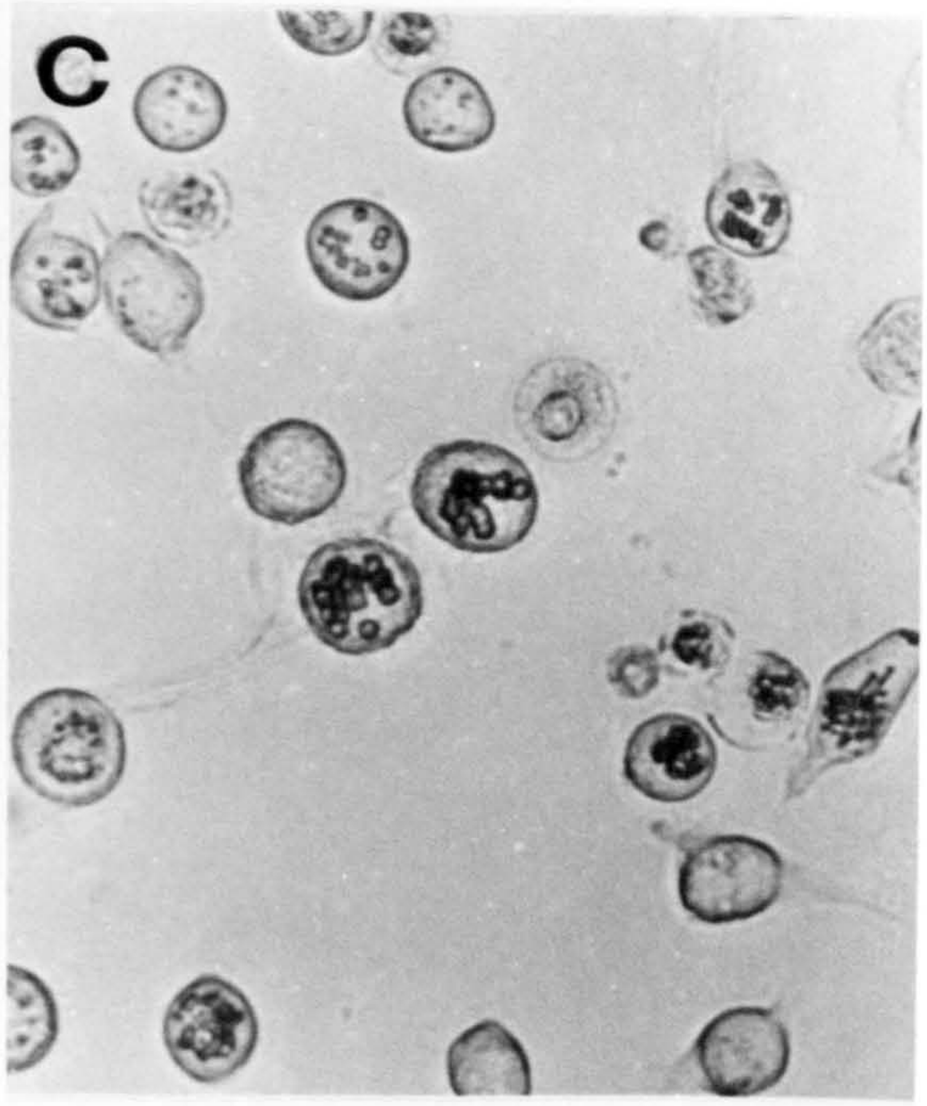


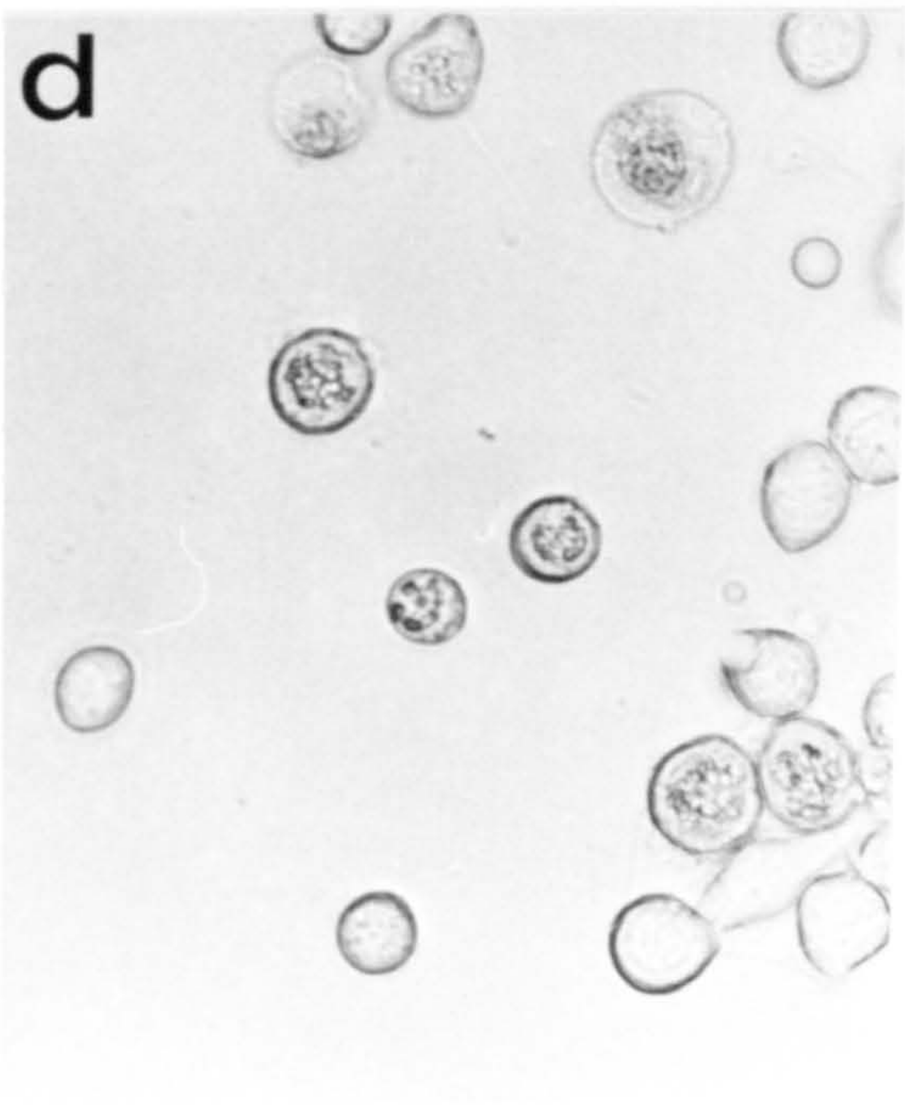


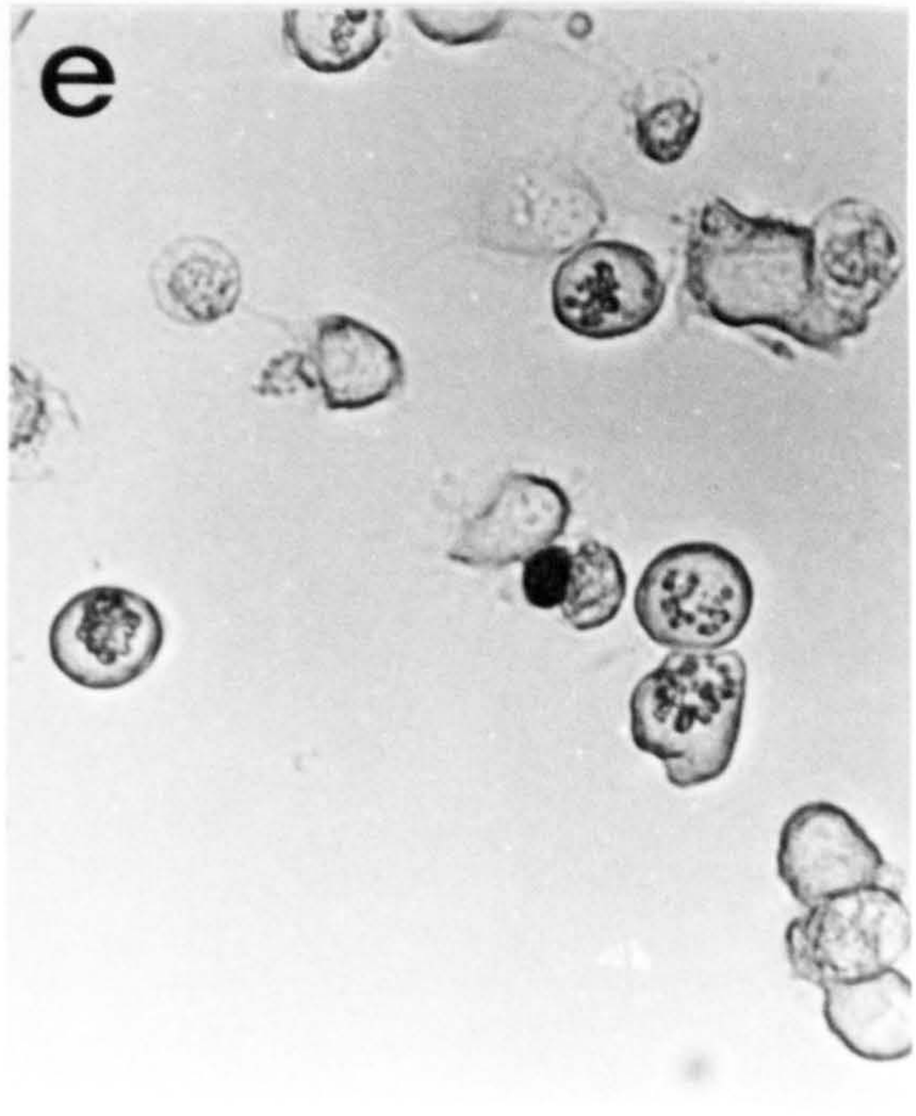
Mamestra brassicae



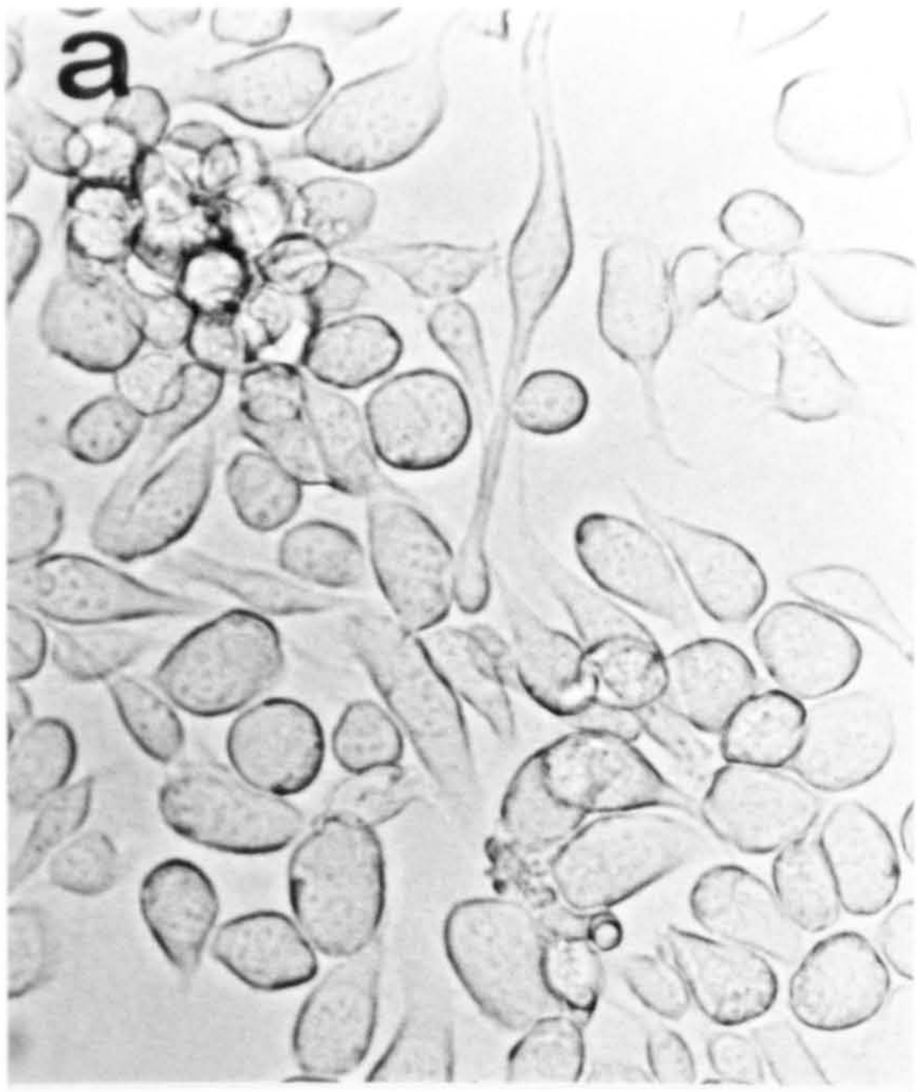


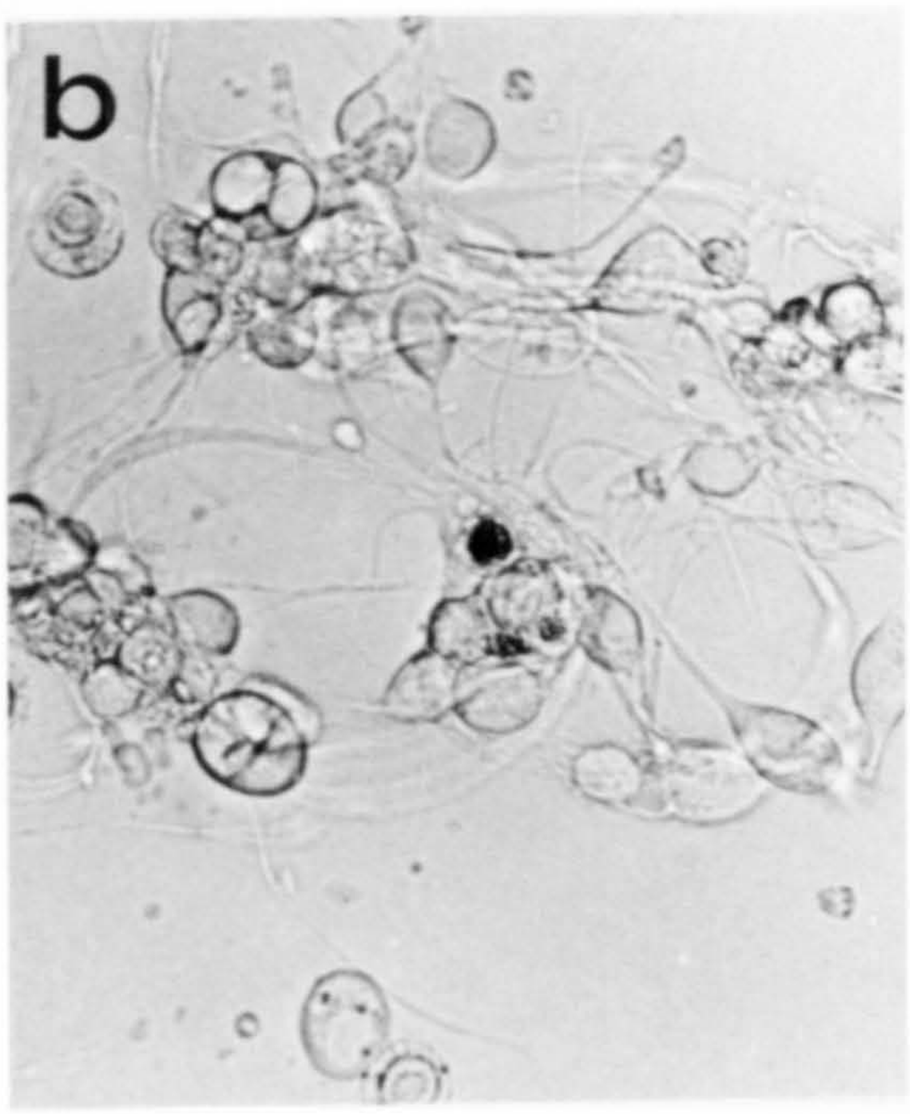


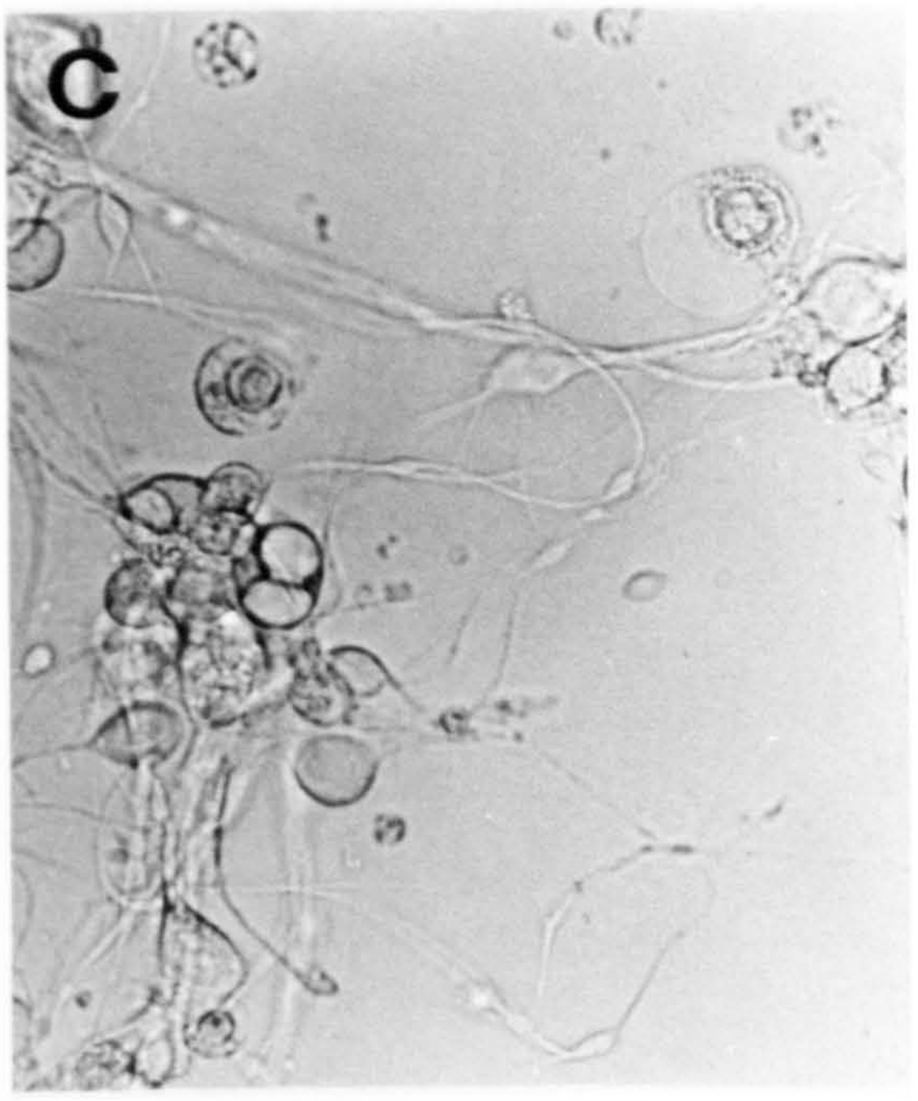


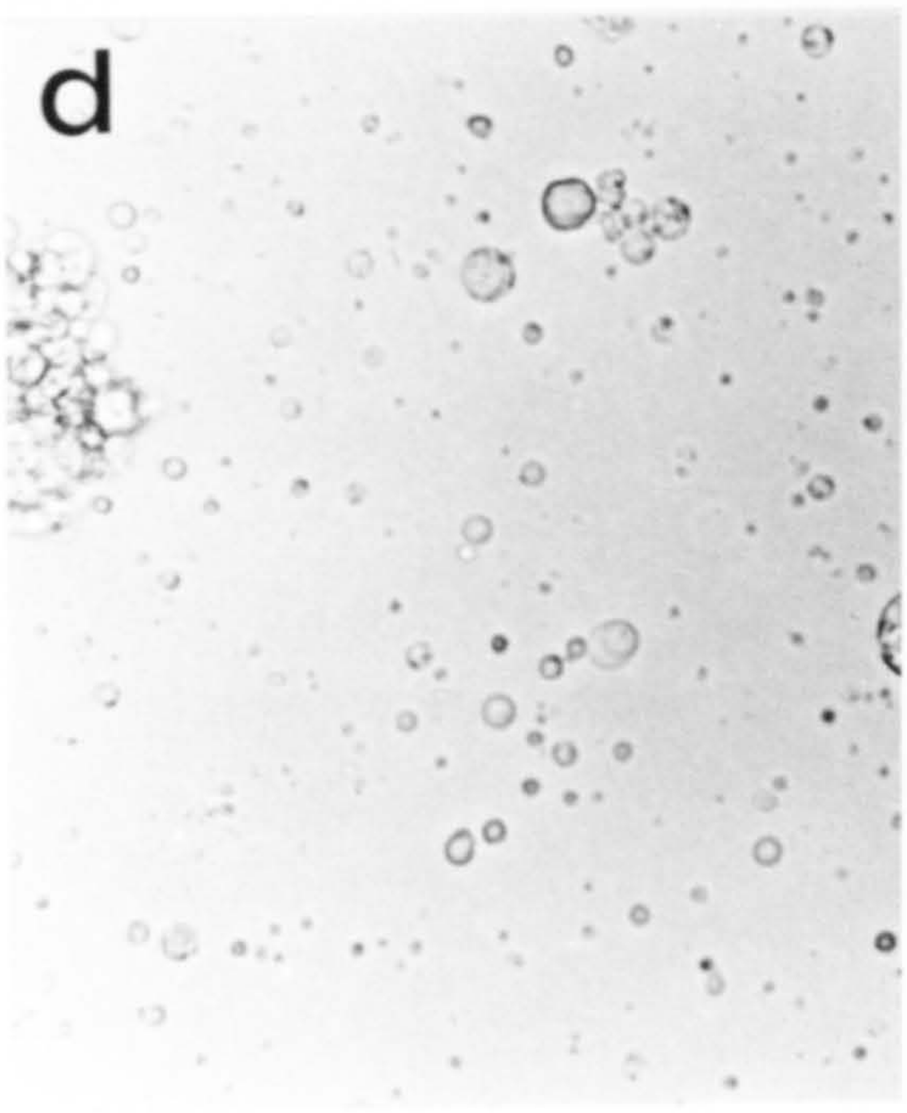


Lymantria dispar 652









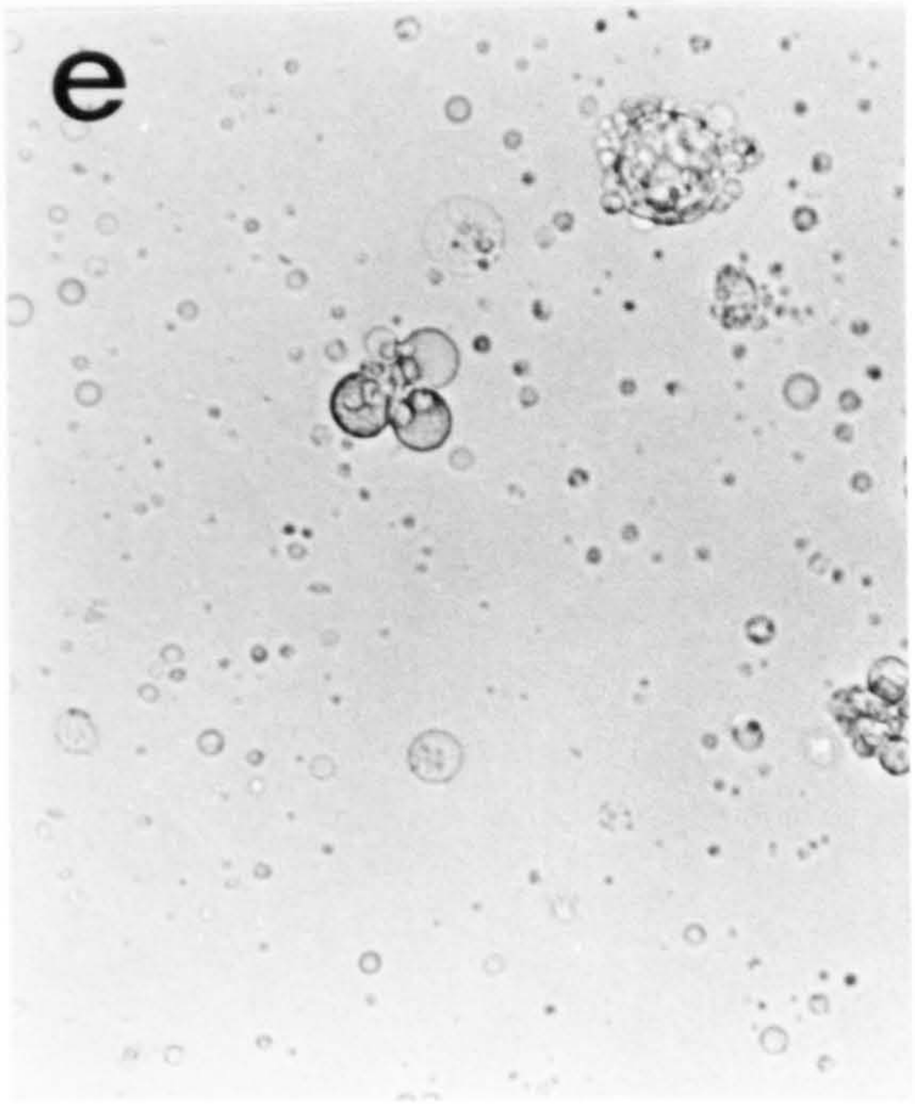


Table 5.2.

The *in vitro* host range of AcMNPV *p35/iap1* deletion viruses.

The results shown in Figure 5.5. are summarized in tabular form below. The ocurrence of apoptosis (abortive infection) is indicated by ✘ and the appearance of occluded virus in the cells (productive infection) is indicated by ✔.

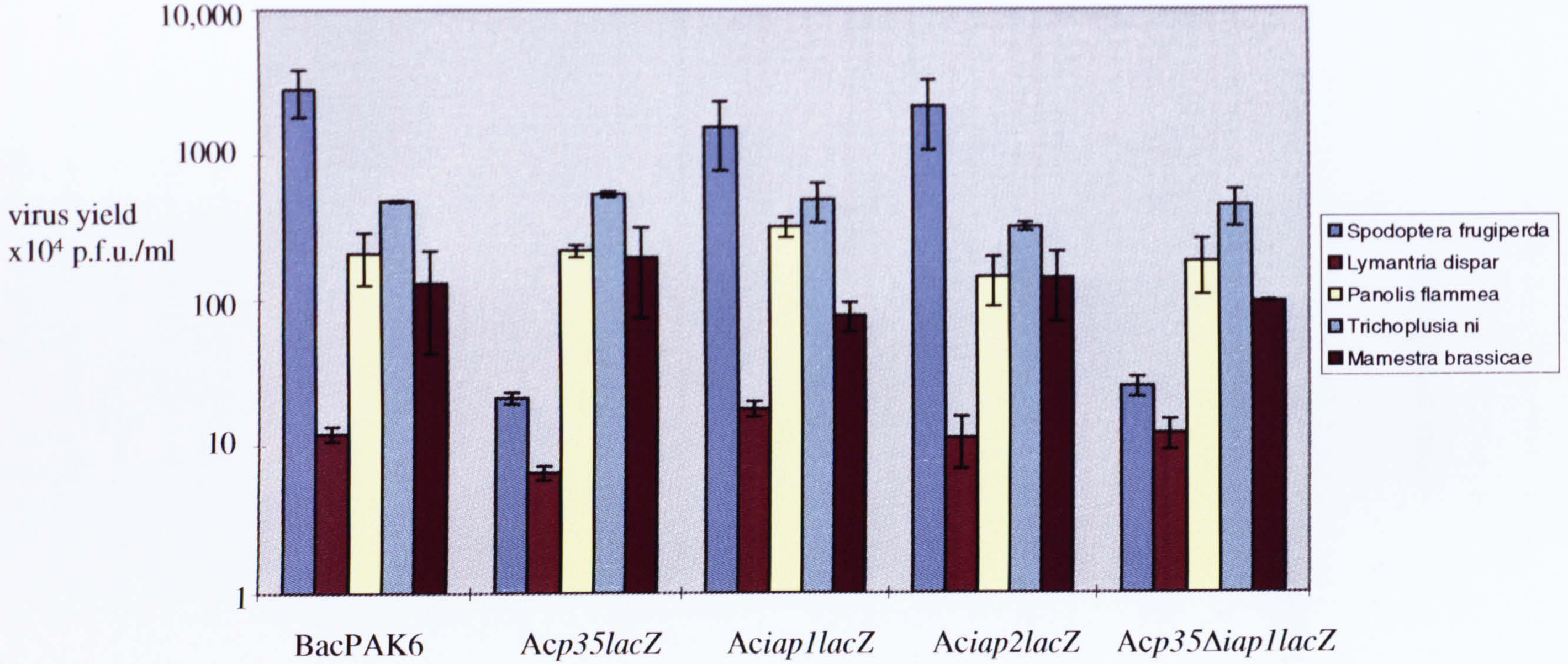
Cell line	*AcMNPV C6*	*Aciap1lacZ*	*Acp35lacZ*	*Acp35Δiap1lacZ*
Spodoptera frugiperda	✔	✔	✘	✘
***Trichoplusia ni* 368**	✔	✔	✔	✔
Malacosoma disstria	✔	✔	✘	✘
Spodoptera littoralis	✔	✔	✘	✘
Panolis flammea	✔	✔	✔	✔
Mamestra brassicae	✔	✔	✔	✔
***Lymantria dispar* 652**	✔	✔	✘	✘

Figure 5.6.

Infectious virus yield from cells infected with recombinant AcMNPVs deficient in *iap2*, *p35*, *iap1* and both *p35* and *iap1*.

Cells were infected at an m.o.i. of 5 p.f.u./cell with Ac*p35lacZ*, Ac*iap1lacZ*, Ac*iap2lacZ*, Ac*p35Δiap1lacZ* or BacPAK6 (Kitts and Possee, 1993). The media from the infection was harvested 48 h.p.i. and titrated using *T. ni* cells. The results are based on three independent replicates.

The data is represented as a horizontal bar chart. The x axis indicates the virus investigated, and the y axis represents the virus yield obtained ($x10^4$ p.f.u./ml). The height of the bar (p.f.u./ml) is the mean of 3 independent replicates, and the error bars indicate 1 standard deviation from the mean in both directions.


10,000
1000
100
10
1
virus yield
x10⁴ p.f.u./ml
Spodoptera frugiperda
Lymantria dispar
Panolis flammea
Trichoplusia ni
Mamestra brassicae
BacPAK6
Acp35lacZ
Aciap1lacZ
Aciap2lacZ
Acp35Δiap1lacZ
Virus

Chapter Six

Final discussion and future work

6.1. The importance of apoptosis in baculovirus infection.

The usefulness of baculoviruses as insecticides is limited by their speed of action compared to conventional chemical insecticides, and by the host specificity of the virus. Speed of action is currently being improved by the introduction of toxin genes into the virus genome using genetic engineering techniques. If it was also possible to modify a baculovirus to allow the infection of a complex of common pests, the attractiveness of baculoviruses to industry and user could be greatly enhanced, although this would also provoke concerns about their safety for non-target organisms. A greater understanding of the factors controlling baculovirus host range is therefore required.

Little is understood about the genetic control of baculovirus host range. However, certain genes, which regulate host cell apoptosis, have been implicated in the control of baculovirus host range (Clem *et al.*, 1991). This project focused on the characterisation of apoptotic inhibitors, with a view to increasing an understanding of the genetic basis of baculovirus host range.

The infection of many different cell species with different viruses can induce apoptosis and limit viral infection; for example, infection of Vero cells with varicella-zoster virus (VZV) (Sadzot-Delvaux *et al.*, 1995) or measles (Esolen *et al.*, 1995), infection of mouse alveolar cells with influenza virus (Mori *et al.*, 1995), and infection of human peripheral blood mononuclear cells with Sendai virus or Herpes virus type I (Tropea *et al.*, 1995). Programmed cell death is considered to be a defence mechanism employed by many, if not all, cell types to prevent infections by viruses.

Insects lack lymphocytes, the major source of vertebrate immunity to virus infections. An apoptotic response may be a mechanism to provide an organism lacking an antibody response with protection from virus infection; the death of a single infected cell could protect the rest of the organism provided that the virus replication cycle was blocked by this cell suicide event. In common with several other virus families, baculoviruses

encode genes which have the ability to prevent host cell apoptosis, allowing the successful replication of the virus in its host. This family of proteins includes P35, uniquely encoded by AcMNPV and BmNPV (Clem *et al.*, 1991; Kamita *et al.*, 1993). AcMNPV deficient in P35 had reduced replication ability, compared to wild type AcMNPV, in Sf21 and *L. dispar* cells, as demonstrated by results presented in Chapter Six. In these cell lines, the *p35* deficient mutant, Ac*p35lacZ*, caused apoptosis, which probably accounted for the observed lower BV yield of Ac*p35lacZ*. *In vivo*, P35 also contributed to the effectiveness of the virus, since AcMNPVs deficient in P35 have increased LD_{50} values in *Spodoptera frugiperda* larvae (Clem *et al.*, 1993). In conclusion, the evolution of anti-apoptotic genes by baculoviruses may represent a viral strategy to avoid host defences. The presence of apoptosis-inhibitors in the genome of AcMNPV has been shown to contribute to virus infectivity *in vivo*.

6.2. Interaction of P35 and IAP with cellular proteins.

P35 has also been shown to block apoptosis in diverse biological situations (Rabizadeh *et al.*, 1993; Hay *et al.*, 1994; Sugimoto *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996), suggesting its substrate, ICE-like protease, has a central and conserved role in the induction of apoptosis (Bump *et al.*, 1995; Xue and Horvitz, 1995). Baculoviruses from some insect species, which do not encode *p35*, rely on the anti-apoptotic action of *iap* gene products, which in complementation assays can substitute for P35 (Crook *et al.*, 1993; Birnbaum *et al.*, 1994).

The IAP protein family have been shown to be phylogenetically diverse, and homologues of the baculovirus proteins have so far been identified in humans, mice and *Drosophila*. The human IAP proteins, hIAP1 and hIAP2 have been shown to interact with the tumour necrosis receptor associated factor TRAF2 (Rothe *et al.*, 1995). TRAF2 is one of an expanding family of proteins that interact with members of the TNF receptor superfamily to initiate intracellular signal transduction events. These signalling proteins fall into two structural classes, containing either death domains (TRADD, FADD and RIP) or carboxy-terminal TRAF domains and amino-terminal

RING fingers (TRAFs 1 to 6) (Rothe *et al.*, 1995; Cao *et al.*, 1996; Hsu *et al.*, 1996, Nakano *et al.*, 1996; Takeuchi *et al.*, 1996). The death domain-containing proteins are involved in signalling by TNF-R1 and Fas antigen, two receptors which themselves contain death domains. In contrast, the TRAF domain proteins interact with receptors (TNF-R2 and CD40) that have no recognisable domains or motifs. The two classes of signal transducers are capable of direct interaction (Hsu *et al.*, 1996), suggesting the signalling pathways may overlap to some extent.

A biological property shared by the CD40, TNF-R1 and TNF-R2 signal transduction pathways is the activation of the transcription factor NF-κB (Kruppa *et al.*, 1992; Rothe *et al.*, 1994; Berberich *et al.*, 1994). Overexpression of TRAF2 (but not TRAF1 or TRAF3) also results in the activation of NF-κB (Rothe *et al.*, 1995). It appears that TNF-R1, TNF-R2 and CD40 all employ TRAF2 to transduce the signal for NF-κB activation (Takeuchi *et al.*, 1996). NF-κB consists of p50 and p65 subunits that are normally associated with I-κB (Liou and Baltimore, 1993). The phosphorylation of I-κB to release NF-κB is thought to be the cytokine-regulated step which results in apoptosis (via ICE protease activation). In this regard, TNF has been shown to activate protein kinase activity (VanArsdale and Ware, 1994), which is required for I-κB degradation and consequent NF-κB activation.

The TRAF proteins are all able to homodimerise through the conserved 150 amino acid carboxy-terminal TRAF domain (Hu *et al.*, 1994; Rothe *et al.*, 1994; Cheng *et al.*, 1995; Sato *et al.*, 1995; Nakano *et al.*, 1996), which associates them to the TNF receptor. These domains function as adaptors that can recruit additional signalling molecules, such as IAP (Rothe *et al.*, 1995), to the receptor complexes, as well as allowing TRAFs to associate to non-cognate receptors via other signalling molecules, such as RIP and TRADD (Hsu *et al.*, 1996). The TRAF proteins, with the exception of TRAF1, also contain an amino-terminal RING finger structure followed by five zinc fingers. The RING finger motif is required to trigger NF-κB activation in both TRAF2 and TRAF5 (Rothe *et al.*, 1995; Nakano *et al.*, 1996). A possible

role for the RING finger domain of TRAF proteins may be to regulate protein kinase activity, by recruiting kinase to the TNFR signalling complex.

The ability of human IAPs to prevent apoptosis induced by the overexpression of p32 ICE and overexpression of FADD has been examined. Two of the four human IAP genes, (hILP and hIAP1), and, in addition, OpMNPV IAP, can protect HeLa cells against the action of ICE. However, hIAP2 did not provide protection against ICE-mediated apoptosis (Uren *et al.*, 1996). In independent experiments, OpMNPV IAP and hILP could also protect human embryonic kidney cells from pro-ICE-induced apoptosis (Duckett *et al.*, 1996). The enforced expression of FADD also causes cell death in HeLa cells (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1995). OpMNPV IAP provided partial protection against FADD-induced apoptosis, but no reduction in the levels of apoptosis were observed by addition of the hIAP1, hIAP2 or hILP (Uren *et al.*, 1996). OpMNPV IAP and hILP also prevent apoptosis of baby hamster kidney (BHK) and mouse neuroblastoma cells induced by Sindbis virus (Duckett *et al.*, 1996).

In yeast two hybrid assays, hIAP1 and hIAP2 interact with TRAF1 and TRAF2, but hILP and OpMNPV IAP do not. None of the human IAP proteins interacts with TRAF3 (CRAF-1), however (Uren *et al.*, 1996). The BIR domain, though not the RING finger motif, was shown to be essential for hIAP1 binding to TRAF1 and TRAF2 (Rothe *et al.*, 1995). The interaction of hIAP1 with TRAF2 was shown to be mediated through the TRAF2 carboxy-terminal TRAF domain, though not the TRAF2 amino-terminal RING finger domain (Rothe *et al.*, 1995). Human IAPs are thus recruited to TNFR2, and prevent apoptosis, by binding to the TRAF complex.

The human IAPs, hIAP1, hIAP2 and hILP, also demonstrated an ability to suppress apoptosis caused by growth factor deprivation (Liston *et al.*, 1996). However, human IAP overexpression did not confer protection against the action of the protein kinase inhibitor staurosporine. This contrasts with the action of BCL-2, which was shown to be protective against staurosporine action (Liston *et al.*, 1996). This may reflect either the inability of IAP to prevent Fas ligand-induced apoptosis, or may

indicate that BCL-2 acts upstream of IAP in the apoptotic cascade. The viral *iap*s, CpGV and OpMNPV *iap*, have been shown to suppress apoptosis induced by actinomycin D in insect cells (Clem *et al.*, 1994a). However, the overexpression of h*iap1* and h*iap2* did not antagonise actinomycin D induced cell death in human HL60 cells (Rothe *et al.*, 1995).

The differing behaviour of the human IAP proteins in these analyses, suggests these proteins may be involved in different processes in cells. hILP and OpMNPV IAP did not interact with TRAF1, TRAF2 or TRAF3 in yeast two hybrid assays. This suggests that either the interaction of IAPs with TRAF does not correlate with their anti-apoptotic ability, or that other cellular targets mediate interactions with hILP and OpMNPV IAP. Although OpMNPV IAP protected cells against ICE-induced apoptosis, it was less capable of protecting cells against apoptosis caused by FADD. Also, hILP and hIAP1 did not block FADD-induced apoptosis, though they did block ICE-induced apoptosis. This result indicates that the FADD-induced apoptotic pathway may not contain substrates which IAP can satisfactorily control to prevent apoptosis.

The differing functional abilities of the four human IAP proteins indicates that some aspects of their function overlap, whereas other aspects do not. A possible implication of this is that the human IAPs demonstrate functional redundancy.

6.3. Structure and function of HzSNPV IAP.

In Chapter Three, a novel baculovirus *iap*, from HzSNPV, which retained the structural motifs common to the IAP protein family, was identified. These motifs included a carboxy-terminal RING finger motif and an amino-terminal baculovirus *iap* repeat (BIR). The transcription of HzSNPV *iap* was examined (Chapter Three) by primer extension analysis. Transcripts of the *iap* gene were detected from a baculovirus consensus early promoter motif until 24 h.p.i.; thereafter transcription switched to a consensus late promoter motif. Transcripts of *iap* were present throughout the course of the virus infection.

Studies were carried out to characterise the function of the HzSNPV *iap* gene product (Chapter Four). The gene was interrupted by the *lacZ* coding region under the control of the HzSNPV polyhedrin promoter by insertion mutagenesis, initially in a transfer vector. However, it was not possible to isolate a recombinant HzSNPV deficient in this gene product. Mutants of CpGV and OpMNPV deficient in *iap* have not been reported in the literature. The essential nature of HzSNPV IAP and other baculoviral IAPs, indicates that they perform important functions in their native situation.

Experiments in *Drosophila* to construct DIAP1 loss-of-function clones (Hay *et al.*, 1995) also failed to isolate loss-of-function homozygous mutants. This result is interpreted as indicating that DIAP1 is required for cell survival. Similarly, mutations in DIAP2 have not been detected.

Although the interaction of hIAP1 and hIAP2 with TRAF implies an important role for these proteins in the control of programmed cell death, their specific role remains to be determined. It has been shown that different TNF receptor types use different signalling systems (Lewis *et al.*, 1991). Therefore, it may be difficult to draw conclusions about the action of IAPs in heterogeneous systems. However, all IAPs display conserved structural features; a common architecture which predicts equally conserved biochemical properties intrinsic to IAPs. Aspects of the cell death pathway have been determined in different biological systems. It may be necessary to isolate the elements involved in a single system. This would be particularly important if some aspects of the system are species specific.

The implication that certain *iap* gene products interact closely with TRAF proteins, suggests a starting point for the characterisation of further proteins with the ability to bind to IAP. This could be achieved by expression of baculovirus IAP as a glutathione-S-transferase fusion protein and the affinity purification of proteins from cells and insect homogenates, with the ability to bind to IAP. It is possible that AcMNPV IAP1 and HzSNPV IAP may interact with novel TRAF-like proteins. Novel cellular proteins involved in the apoptotic process could be characterised and these proteins may differ according to the baculovirus gene examined and the species of

cell line or insect analysed. Differences between baculovirus *iap*s could also be investigated in this way, thus improving the evidence for a role of IAPs in host range determination. The inability of HzSNPV *iap* to act in place of AcMNPV *p35* suggests this virus-encoded protein is either temporally different from CpGV *iap*, that it is inactive as an inhibitor of apoptosis in the Sf21 cell line, or that a combination of gene products from HzSNPV are required to elicit an anti-apoptotic response. Although HzSNPV IAP did not contribute towards the control of apoptosis in Sf21 cells, it may be that the ability of this IAP to display anti-apoptotic ability is specific to *H. zea* cells.

6.4. The role of IAP1 in AcMNPV.

AcMNPV IAP1 does not appear to contribute to the inhibition of apoptosis in virus-infected insect cells, in the cell lines considered (Chapter Five). It is possible that the cell lines selected for study did not include species in which IAP1 is required. The apparent lack of activity could suggest functional diversity among viral IAPs. It is also possible that some IAPs have no role in the control of apoptosis. In view of the functional diversity displayed by mammalian IAPs however, the apparent lack of anti-apoptotic activity of AcMNPV IAP1 and HzSNPV IAP may simply reflect the action of these proteins in a specific assay - it may not be appropriate to gauge activity on the basis of a single assay. The function of HzSNPV could be further examined by testing the ability of the protein to inhibit apoptosis in a variety of assay systems, for example, the inhibition of actinomycin D-induced apoptosis in insect cells or whether HzSNPV IAP can protect mammalian cells from pro-ICE-induced apoptosis.

The data presented in Chapter Five indicate that AcMNPV *iap1* does not provide a back-up to AcMNPV *p35* in cell lines which do not require *p35* for the inhibition of apoptosis. The phenotype of Ac*p35*Δ*iap1lacZ* was identical to that of Ac*p35lacZ* in the host range studies, and in cell lines in which Ac*p35lacZ* does not induce apoptosis (*T. ni*, *M. brassicae* and *P. flammea*), no differences in virus replication were observed. Although the double mutant (*p35*/*iap1*) was viable and had comparable properties to Ac*p35lacZ*, the successive deletion of *iap2* to produce a triple mutant deficient in *p35*,

iap1 and *iap2* did not result in the production of a viable recombinant (data not shown). At least three hypotheses arise from these observations: 1.) The AcMNPV *iap1* we selected for our studies is not the true inhibitor of apoptosis in *T. ni* cells and this role is in fact performed by *iap2*. This could be tested by examining the properties of a double mutant deficient in *p35* and *iap2*. 2.) Although both *iap1* and *iap2* can be deleted separately, one of these genes may be required to perform a critical function in AcMNPV. The genes may represent duplications with complementary functions. This theory could be tested by examining the properties of a double mutant deficient in *iap1* and *iap2*. 3.) At least one inhibitor of apoptosis gene is required in *T. ni* cells, and this can be either *p35*, *iap1* or *iap2*. Therefore, although it would be possible to delete various combinations of these genes, it may not be possible to delete all three. To obtain a definitive result on the viablity of the AcMNPV *p35/iap1/iap2* triple mutant it will be necessary to construct this virus using the baculovirus yeast system (Patel *et al.*, 1992).

The genomic region in AcMNPV containing *p35* and *p94* may have been derived from an insertion of foreign DNA, since the flanking regions of *p35* and *p94* are conserved in OpMNPV, a virus which is largely colinear with AcMNPV (Gombart *et al.*, 1989) and yet lacks both these genes. In addition, only BmNPV has been shown to contain *p35* and *p94*. It is not known whether *p35* and *p94* were acquired from another virus or from host DNA. Homologues of viral apoptosis-inhibitors may be present in the insect genome. Developing an understanding of the origins of *p35* and *p94* will aid the characterisation of baculovirus evolution.

6.5. The importance of IAP structural motifs.

In over 60% of patients with the severe form of spinal muscular atrophy, there are deletions in the first two coding exons of *NAIP* (Roy *et al.*, 1995). These deletions result in the loss of the first BIR and most of the second BIR domains. Thus it appears that the BIR domains are essential to the function of NAIP in maintaining motor neuron survival. Similarly, these domains are required by CpGV to prevent apoptosis (Clem and Miller,

1994a). The BIR probably plays a critical role in the ability of IAP-related proteins to modulate cell survival. The conservation of histidine and cysteine spacing in the BIR motif suggests that this domain may be a novel form of zinc finger.

Previous experiments involving the interchange of BIR or RING finger motifs between CpGV IAP and AcMNPV IAP1 revealed that the sequences of both termini of CpGV IAP are crucial for its anti-apoptotic function (Clem and Miller, 1996). Experiments in Chapter Four investigated the exchange of domains between CpGV IAP and HzSNPV IAP. Two constructs, pHzCpIAP, consisting of the 5' region of HzSNPV IAP and the 3' region of CpGV IAP, and the opposing construct pCpHzIAP, were examined for their anti-apoptotic ability. Neither hybrid construct maintained the anti-apoptotic function of CpGV IAP. Both the BIR and RING finger domains of CpGV IAP are required for anti-apoptotic function in these experiments. Thus, the results presented in Chapter Four support those obtained by Clem and Miller.

NAIP encodes no RING finger motif. It is possible that this motif performs a regulatory function for CpGV and OpMNPV IAP. It is also possible that the redundancy of human IAP proteins means that one of the other human IAPs contributes RING finger motif action to NAIP, or that this putative regulatory function is performed by a different protein.

6.6. Future studies with *H. zea* and HzSNPV.

Previously, HzSNPV Elcar was successfully used to control *Heliothis* species in field conditions. Although the pathogenicity of this baculovirus has been widely studied, little information is available regarding its genetic composition. The complete genome sequence of AcMNPV is now available, and this information has aided the rational design of improved baculovirus insecticides. The sequencing data presented in Chapter Three corresponds to approximately 5% of the HzSNPV genome. Although this represents a small fraction of the entire genome sequence, the information enables hypotheses relating to the genetic modification of HzSNPV to be developed.

Four novel genes have been characterised in the 6 kbp *iap* locus presented in Chapter Three; *iap* was shown to be essential for virus replication, superoxide dismutase (*sod*) is probably non-essential since a superoxide dismutase deletion mutant has been successfully constructed in AcMNPV. ORF 106 and ORF 2 are uncharacterised in HzSNPV and other baculoviruses. In the AcMNPV genome, *iap1* (ORF 27) and *sod* (ORF 31) are positioned relatively close to each other on the circular map of the genome (22000 to 27000 bp), although ORF 2 (1000 bp) and ORF 106 (95000 bp) are distantly located (Ayres *et al.*, 1994). CpGV and OpMNPV have conserved *odvp* (occlusion derived virus protein) genes downstream and in antisense orientation to *iap* (Theilmann *et al.*, 1996; N. Crook, pers. comm.), suggesting the CpGV and OpMNPV viral genomes display colinearity at this gene locus. A partial sequence of HzSNPV *odvp* is available in Genbank, although this sequence is not located at the *iap* locus presented in Chapter Three.

It would be interesting to investigate whether the addition of *p35* to the genome of a virus which lacks this gene (e.g., HzSNPV or OpMNPV) could result in recombinant viruses with modified host ranges. This strategy would be greatly facilitated by the DNA sequence presented in Chapter Three of this report, which would allow strategies for the construction of transfer vectors to be devised.

Alternative approaches involving the introduction of HzSNPV genes into AcMNPV and vice versa may aid the identification of genes which have an impact on host range control. Future work is necessary to address fundamental questions on the control of baculovirus host range.

Recent evidence concerning AcMNPV infection of *H. zea* insects suggests that haemocytes may mediate an immune response in the larvae to halt the spread of AcMNPV infection (Washburn *et al.*, 1996). It has been suggested that this haemocyte response contributes to the refractive nature of AcMNPV in *H. zea* larvae. This observation may explain why AcHz*iap* exhibited increased replication in *H. zea* cells compared to AcMNPV C6, but did not contribute to improved infectivity of *H. zea* larvae (Chapter Four).

Further studies on the role of the haemocyte response *in vivo* are required to ascertain the importance of this mechanism in baculovirus host range.

6.7. The control of programmed cell death in insects.

In holometabolous insects such as *Drosophila* and *Manduca sexta*, the nervous system undergoes extensive remodelling during metamorphosis. Programmed cell death is widely exploited as a developmental mechanism for this purpose. Metamorphic cell deaths in these and other insects are controlled by changes in the level of ecdysone. However, evidence suggests that regardless of the source of the signal to initiate programmed cell death, the pathway is a common one. Three genes in *Drosophila* have been implicated in the activation of programmed cell death, *reaper*, *hid* and *grim* (Steller and Grether, 1994; Chen *et al.*, 1996). In *Drosophila*, *in situ* hybridisation studies indicate that *reaper* is expressed in cells which are programmed to die or are in the process of apoptosis. A constitutive promoter can be used to express *reaper* in cells which do not usually die, causing apoptosis of these cells. The protein encoded by *reaper* is known to multimerise *in vitro*, though it is not known whether this property is related to the function of the protein (White *et al.*, 1996). If programmed cell deaths in *Drosophila* occur by a common mechanism, on which multiple signalling pathways converge to induce the expression of *reaper*, we might expect this protein to be conserved in other insects, such as *Manduca sexta*. It would be interesting to investigate this hypotheses by screening insect genomic libraries with *reaper*-specific DNA probes.

It has been shown that AcMNPV *p35* blocks *reaper*-, *hid*- and grim-activated apoptosis (Chen *et al.*, 1996; White *et al.*, 1996). The overexpression of baculovirus *iap* (CpGV *iap*) also blocks some *reaper*-dependent cell death, although not as efficiently as the *Drosphila iaps*, D*iap1* and D*iap2* (Hay *et al.*, 1995). It is possible that *reaper*, *hid* and *grim* could negatively regulate cell death inhibitors such as *iap*. This model of *reaper* action warrants further investigation.

Infection of Sf9 cells by AcMNPV can block apoptosis induced by okadaic acid, suggesting that a viral protein or proteins may be responsible for this apoptotic block (Bergqvist and Magnusson, 1994). Okadaic acid is a polyether of C_{38} fatty acid, originally isolated form the black sponge *Halichondira okadai* (Suganuma *et al.*, 1988). It specifically inhibits protein phosphatase 2A, known to be involved in the control of cell cycle regulatory genes such as cdc2 (Jaramillo-Babb *et al.*, 1996), suggesting a stimulatory role for phosphatase 2A in aspects of cellular growth. Treatment of cells with okadaic acid has been shown to activate transcription factor NF-κB (Schmidt *et al.*, 1995; Singh and Aggarwal, 1995), suggesting that phosphatase 2A functions to inhibit NF-κB in growing cells. The altered levels of protein phosphatases induced by okadaic acid treatment have also been shown to activate ICE-related proteases in human ML-1 cells, resulting in subsequent apoptosis (Morana *et al.*, 1996), presumably as a result of the upregulation of NF-κB. In summary, these data suggest that protein phosphatase 2A mediates activation of NF-κB, leading to the activation of ICE-related proteases, which bring about cellular apoptosis. The inhibition of okadaic acid-induced apoptosis in baculovirus-infected cells implies that specific baculovirus gene products can prevent this cascade of events. In view of the ability of *p35* to bind diverse ICE-related proteases, the prevention of okadaic acid-induced apoptosis by baculovirus is likely to be achieved by P35. However, since the events initiating the apoptotic response commence upstream of direct ICE activation, it would be interesting to see whether baculovirus *iap* could prevent okadaic acid-induced apoptosis. Experiments designed to test the ability of recombinant viruses to prevent okadiac acid-induced apoptosis could be performed using the deletion viruses Ac*p35lacZ*, Ac*iap1lacZ*, Ac*iap2lacZ* and Ac*p35Δiap1lacZ*.

6.8. Methods for examining apoptosis.

The apoptotic process involves a sequence of cell shrinkage, increased cytoplasmic density, chromatin compaction, and segregation of the cell contents into masses that form protrusions from the nuclear membrane. These then separate to form membrane-bound apoptotic bodies. Light

microscopy was used to identify the cellular fragmentation typical of apoptosis in the work presented here. This technique is qualitative but does not give a quantitative measurement of the number of cells undergoing apoptosis. Several methods can be used to give quantitative evaluations of apoptosis, including flow cytometry and TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end-labelling). Cell shrinkage and condensation of chromatin and cytoplasm during apoptosis are reflected by the altered ability of the cell to scatter light (Darzynkiewicz *et al.*, 1992). This feature can be directly analysed by flow cytometry. TUNEL takes advantage of the DNA strand breaks which occur in the apoptotic cell as a result of endonuclease activity (Gavrieli *et al.*, 1992). The 3'-hydroxyl ends of DNA fragments are labelled, using biotin, dioxigenin or fluorescein (Li *et al.*, 1995).

6.9. Conclusion.

Programmed cell death plays either a major role, or an initiating role, in many diseases. There are two types of control over cell death which are of pharmacological importance: 1.) the ability to induce cell death when elimination of tissue is required, and 2.) the ability to prevent cell death when tissue proliferation or tissue loss results in disease. Examples of diseases in which knowledge about mechanisms of cell death could have a major impact on prevention and treatment are Huntingdon's, Parkinson's and Alzheimer's diseases, temporal lobe epilepsy and numerous other neurological syndromes. Pharmacological intervention would require an inhibitor of one important step in the cell death cascade to be effective. The study of programmed cell death has important consequences. The characterisation of inhibitor of apoptosis genes aids our understanding of the process and may produce candidate inhibitors, or aid the rational design of peptides, for the control of cell death. Virus proteins that mediate the suppression of host cell death are valuable probes for the characterisation of programmed cell death signal transduction pathways.

Baculovirus infection of insect cells is a unique *in vitro* study system for the analysis of basic cellular mechanisms such as that resulting in

programmed cell death, since it is devoid of any complications arising from the immune system of mammalian models. The identification of *iap* genes in diverse organisms, and the implication of *naip*, a human *iap* gene homologue, in the pathogenesis of a neurodegenerative disease (Roy *et al.*, 1995), suggest the further dissection of the action of these gene products in the cell is warranted. The baculovirus system can provide a basis for this field of study.

The cell death program appears to have been remarkably well conserved during metazoan evolution. Consequently, further studies on the mechanism of programmed cell death induced by baculovirus infection of insect cells should contribute to a better understanding of the mammalian process. Defining the interaction of baculovirus anti-apoptotic genes at the molecular level could also provide insight into other biological processes thought to involve apoptosis, such as cell cycle regulation, oncogenesis, tissue differentiation and development. It is anticipated that developments in the scientific research of virus anti-apoptotic gene function will be of enormous importance to the elucidation of the programmed cell death pathway, in particular, in understanding how signalling events at the plasma membrane impact on the machinery that executes programmed cell death.

Appendix 1.

Preparation of TC199MK tissue culture media (2 litres).

M199 powder
(Gibco 071-02100A, with Hanks and glutamine, without $NaHCO_3$)

H_2O	1560ml
$NaHCO_3$	0.7g
10x Hanks* (without $CaCl_2$)	100ml
$CaCl_2$ (1.4g/10ml)	1ml
100x glutamine	20ml
50x LAH	40ml
100x MOPS	20ml

1M NaOH (for pH adjustment)

Method:

1. Add 1 container of M199 powder to 950ml distilled H_2O.
2. Add 0.7g $NaHCO_3$
3. Add distilled H_2O to 1 litre and adjust the pH of the solution to 6.95 with 1M NaOH.
4. Add 560ml distilled H_2O, 100ml 10x Hanks, 1ml $CaCl_2$, 20ml 100x glutamine and 40ml 50x LAH and stir gently.
5. Add 20ml 100x MOPS.
6. Adjust the pH of the solution to 7.05 using 1M NaOH.
7. Filter the solution through a 2µm filter.
8. Store at 4°C.
9. Add FCS to 10% prior to use.

*10x Hanks	1 litre
NaCl	80g
KCl	4g
$MgSO_4$	1g
$MgCl_2.6H_2O$	2.1g
Na_2HPO_4	0.6g
KH_2PO_4	0.6g
dextrose	10g

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