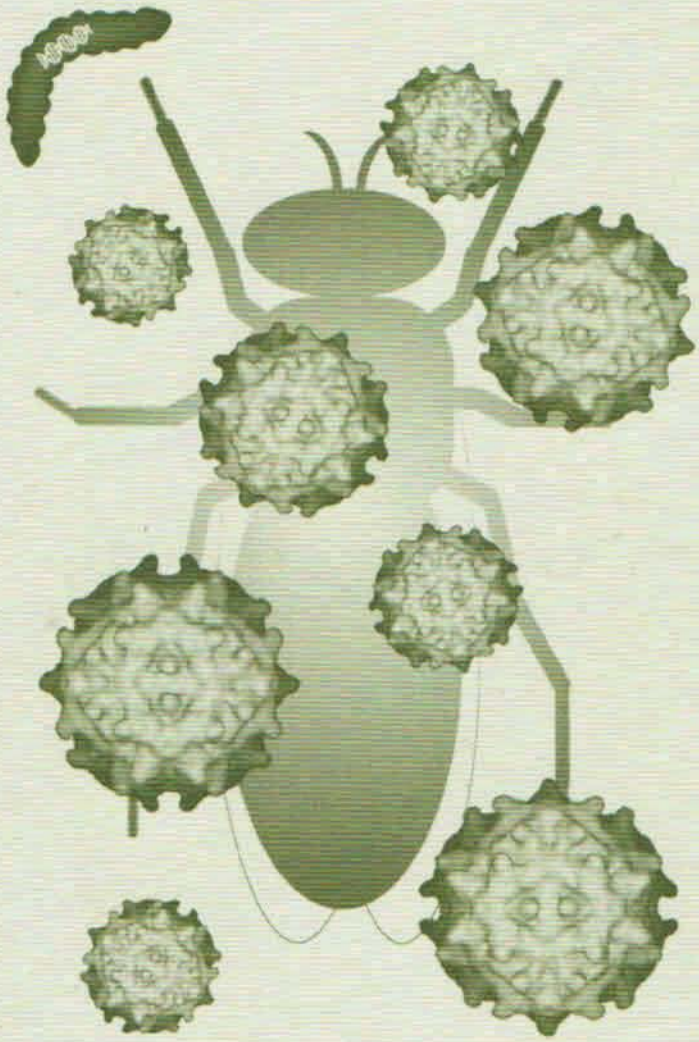
Insect Virology



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Contents

Iridoviruses

Trevor Williams and Vernon K. Ward

Abstract

Invertebrate iridescent viruses (IIVs) of the family *Iridoviridae* are icosahedral dsDNA viruses with large circularly permuted and terminally redundant genomes that infect a number of agricultural pests, medically important insect vectors and terrestrial isopods that live in damp or aquatic habitats. IIVs currently figure among the most neglected groups of entomopathogenic viruses, although they present a number of unusual and intriguing aspects in their physical properties, replication strategies and host–virus interactions. We present an overview of this group of viruses with emphasis on recent advances and on-going lines of research focussing on the current classification and progress in understanding the structure of IIV virions. We then outline the genomic characteristics and replication process followed by a brief description of their pathology and the ecological factors that determine the distribution and abundance of these viruses. Finally, we summarize future lines for research and identify potential novel applications in biotechnology and materials science.

Introduction

Invertebrate iridescent viruses (IIVs) are non-occluded icosahedral dsDNA viruses of the family *Iridoviridae* that infect invertebrates, particularly insects and terrestrial isopods that inhabit damp or aquatic environments. There can be little doubt that IIVs currently figure among the most neglected groups of entomopathogenic viruses. Generally perceived as being of little value as biocontrol agents and of minor economic importance, they have attracted the attention of virologists and insect pathologists more for their curiosity value and their intriguing relationships to other groups of vertebrate and invertebrate viruses rather than because of an inherent interest to understand IIV biology and ecology *per se*.

Whether or not that mindset is set to undergo a marked change remains to be seen, although as usually happens when working on viruses, recent advances in IIV research have generated a number of novel avenues of study that have the potential to significantly transform our view of these pathogens.

The *Iridoviridae* family has been the subject of two recent reviews (Williams *et al.*, 2005; Chinchar *et al.*, 2009). The older literature can be accessed through a number of reviews published in the 1980s and 1990s (Anthony and Comps, 1991; Devauchelle *et al.*, 1985a,b; Hall, 1985; Kelly, 1985; Darai, 1990; Ward and Kalmakoff, 1991; Williams, 1996, 1998). In the present work, we present an overview of this group with emphasis on recent advances and on-going lines of research. For this, we start by reviewing the current classification of the group, and then describe significant advances in understanding the structure of IIV virions. We then outline the genomic characteristics and replication process followed by a brief overview of pathology and an examination of the ecological factors that determine the distribution and abundance of these viruses. Finally, we summarize future lines for research and identify key issues that merit additional attention.

Classification

The classification of these viruses has been severely hampered by a lack of comparative molecular studies and a paucity of genome sequence information. The family *Iridoviridae* is currently divided into five genera (Table 6.1), of which three infect poikilothermic vertebrates (*Lymphocystivirus*, *Megalocytivirus* and *Ranavirus*) and two infect invertebrates, particularly insects in humid or aquatic habitats (*Chloriridovirus* and *Iridovirus*). The prototype species of the family is the ranavirus *Frog virus* 3 (FV-3) which represents the model virus for our understanding of key aspects of the replication process in these viruses (Chinchar *et al.*, 2005).

The IIVs, represented by the *Iridovirus* and *Chloriridovirus* genera, were originally named according to the host species and assigned a type number according to the sequence of their discovery (Tinsley and Kelly, 1970). However, a number of problems have been recognized with this system including a widespread lack of characterization data for many of the viruses, the absence of reference material for many of the isolates described several decades ago, and different type numbers assigned to the same virus isolated from different host species.

An alternative system of nomenclature was proposed for isolates for which some characterization information and reference material was available (Williams and Cory, 1994). This system involved assigning geographical descriptors to each virus based on the nearest large town to the site of isolation. More recently, an alternative nomenclature has been proposed to resolve the confusion that arises when a given virus displays a range of pathological symptoms, a broad host range and wide geographical distribution (Chinchar *et al.*, 2009). This approach is aimed at systematic naming of isolates rather than virus species. In the case of IIVs, this would involve indicating the host from which the isolate was obtained, the location of the isolate, the isolate number, and the year of isolation, e.g. (*Spodoptera frugiperda*/Tapachula/3/2002) for the third isolate from *S. frugiperda* larvae collected close to the town of Tapachula (Mexico) in 2002. Once the virus has been clearly identified, it could be referred to in an abbreviated form such as SF/T/3/02. However, these alternative proposals for nomenclature have yet to be adopted by researchers so that virus type numbers and host species names continue to be used.

The genus *Iridovirus* is the largest of the genera that infect invertebrates and currently comprises two species and 11 tentative species that require additional characterization in order to determine their species status (Table 6.2). The two virus species are *Invertebrate iridescent virus 1* (IIV-1) isolated from soil-dwelling larvae of the cranefly *Tipula paludosa* in Great Britain (Xeros, 1954), and the type species of the genus, *Invertebrate iridescent virus 6* (IIV-6), originally isolated from larvae of the rice stemborer moth, *Chilo suppressalis*, in Japan (Fukaya and Nasu, 1966). The members of this genus are sometimes referred to as the small IIVs, having a dehydrated virion diameter of 110–160 nm, more typically in the range 120–130 nm. The viruses in this genus can be further categorized into one of three complexes based on serological, DNA hybridization and partial major capsid protein

Table 6.1 Genera in the family *Iridoviridae*

Genus	Hosts infected	Type species (abbreviation)
Chloriridovirus	Diptera	*Invertebrate iridescent virus 3* (IIV-3)
Iridovirus	Insects and terrestrial isopods	*Invertebrate iridescent virus 6* (IIV-6)
Lymphocystivirus	Flat fish (worldwide)	*Lymphocystis disease virus 1* (LCDV-1)
Megalocytivirus	Fish (SE Asia)	*Infectious spleen and kidney necrosis virus* (ISKNV)
Ranavirus	Cold blooded vertebrates (mainly amphibians, fish and reptiles)	*Frog virus 3* (FV-3)

Table 6.2 Species and tentative species in the *Iridovirus* genus

Virus name (strain common names)	Accession numbers	Hosts infected
I. Species		
Invertebrate iridescent virus 1	M33542, M62953	*Tipula paludosa* (Diptera)
Tipula iridescent virus		
Invertebrate iridescent virus 6	AF003534, M99395	*Chilo suppressalis* (Lepidoptera)
Chilo iridescent virus		
Gryllus iridovirus		*Gryllus* spp. (Orthoptera)
II. Tentative species		
Invertebrate iridescent virus 2	AF042335	*Sericesthis pruinosa* (Coleoptera)
Sericesthis iridescent virus		
Invertebrate iridescent virus 9	AF025774	*Wiseana cervinata* (Lepidoptera)
Invertebrate iridescent virus 10		*Eudonia (Witlesia)* sp. (Lepidoptera)
Invertebrate iridescent virus 18		*Opogonia* sp. (Lepidoptera)
Invertebrate iridescent virus 16	AF025775	*Costelytra zealandica* (Coleoptera)
Costelytra zealandica iridescent virus		
Invertebrate iridescent virus 21		*Helicoverpa armigera* (Lepidoptera)
Invertebrate iridescent virus 28		*Hydrocyrius columbiae columbiae*[1] (Hemiptera)
Invertebrate iridescent virus 22	AF042341, M32799	*Simulium variegatum* (Diptera)
Simulium sp. iridescent virus		
Invertebrate iridescent virus 23	AF042342	*Heteronychus arator* (Coleoptera)
Heteronychus arator iridescent virus		
Invertebrate iridescent virus 24	AF042340	*Apis cerana* (Hymenoptera)
Apis iridescent virus		
Invertebrate iridescent virus 29	AF042339	*Tenebrio molitor* (Coleoptera)
Tenebrio molitor iridescent virus		
Invertebrate iridescent virus 30	AF042336	*Helicoverpa zea* (Lepidoptera)
Helicoverpa zea iridescent virus		
Invertebrate iridescent virus 31	AF042337, AJ279821	*Armadillidium vulgare*[2] (Isopoda)
Invertebrate iridescent virus 32		*Porcellio dilatatus* (Isopoda)
Anticarsia gemmatalis iridescent virus	AF042343	*Anticarsia gemmatalis* (Lepidoptera)

[1]Host identity originally reported as *Lethocerus columbiae* but more likely to be *Hydrocyrius columbiae columbiae* Spinola or *Lethocerus niloticus* Stal. Williams (2008).

[2]Described by Federici (1980) but may also infect *Porcellio laevis*, Grosholz (1992), *Porcellio scaber*, Cole and Morris (1980), and possibly a number of other isopod species. Wijnhoven and Berg (1999); Schultz *et al.* (1982), although this requires confirmation.

(MCP) gene sequence information (Williams and Cory, 1994; Webby and Kalmakoff, 1998). Group I comprises IIV-6 and possibly IIVs -21 and -28, although the identity of the two latter viruses requires confirmation. Group II comprises IIVs-1, -2, -9, -16, -22, -23, -24, -29, -30 and *Anticarsia gemmatalis* IIV that lacks a type number. Group III comprises the terrestrial isopod virus IIV-31 and a closely related isolate from the soil-dwelling scarab beetle *Phyllophaga anxia*.

The genus *Chloriridovirus* currently comprises a single species, *Invertebrate iridescent virus 3* (IIV-3), also known as Mosquito iridescent virus. Isolates from mosquitoes and midges tend to be larger than those of the *Iridovirus* genus. Dehydrated virion diameters of the so-called large IIVs range from 170 to 200 nm, although the true significance of virion size in defining chloriridoviruses is uncertain. Sequencing of the IIV-3 genome has revealed that the 190,132 bp genome has no obvious collinearity with IIV-6 (Delhon *et al.*, 2006). Of the 126 predicted genes, 52 had homologues in IIV-6 and 27 had homologues in all currently sequenced members of the family, although with low levels of deduced amino acid sequence identity. Phylogenetic analysis of a concatenated set of 11 conserved proteins indicated that the genetic distance between IIV-3 and IIV-6 was similar to that of members of other genera present within the family. However, as IIV infections have been reported in a total of 32 species of mosquitoes and midges (Williams, 2008), none of which have been characterized, the scope and validity of the genus is clearly in need of revision.

Relationships to other families

Iridoviruses are members of a monophyletic clade of nucleocytoplasmic large DNA viruses (NCLDVs) that includes the *Phycodnaviridae*, *Asfarviridae*, *Poxviridae* and the giant icosahedral *Mimiviridae* (Iyer *et al.*, 2001; Claverie *et al.*, 2009). These viruses encode their own machinery for transcription and RNA modification including RNA polymerase and transcription factors, many of which appear to have been acquired from their eukaryotic hosts (Tidona and Darai, 2000; Filée *et al.*, 2008), whereas in the case of the protist-infecting phycodnaviruses and mimivirus, most genes seem to have been acquired from bacterial sources (Filée *et al.*, 2008). Phycodnaviruses lack RNA polymerase genes and therefore depend on host transcriptional mechanisms in the nucleus (Wilson *et al.*, 2009). The clade shares a total of 31 ancestral genes including three genes for structural proteins: the capsid protein and two lipid membrane localized proteins. Of these ancestral genes, nine are core genes shared by all the families of the clade.

Based on the analysis of LCDV-1 and IIV-6, iridoviruses appear to have lost two ancestral genes: an ATP dependent DNA ligase and a capping enzyme required for RNA capping in the cytoplasm (Iyer *et al.*, 2001), but these viruses also differed from the ancestral group by possessing seven additional genes including thymidylate synthase, a cathepsin B-like cysteine protease and RNase III. This cladistic analysis did not include the more recently sequenced IIV-3 genome.

The similarities between iridoviruses, African swine fever virus (ASFV) and the algal phycodnaviruses extend beyond the architecture of the icosahedral capsid, a common inner lipid membrane and an electron dense core. Phycodnaviruses have a rather less prominent inner lipid membrane, a right-handed (dextro) skewed capsid lattice comprising doughnut-shaped capsomers with a smooth outer surface (Nandhagopal *et al.*, 2002), a high degree of similarity in the MCP primary and secondary structure, and instead of the fibrillar structures seen in iridoviruses, a single vertex spike has been observed recently in Paramecium bursaria Chlorella virus 1 (PBCV-1) (Cherrier *et al.*, 2009). In contrast, structural similarities between iridoviruses and the allantoid virions of ascoviruses are not immediately apparent whereas molecular evidence indicates a phylogenetic relationship between these viruses.

Ascoviruses differ from IIVs in having bacilliform virions and a circular genome with a high level of methylation of deoxycytidines, mostly at CpT and CpC residues (Cheng *et al.*, 1999; Bigot *et al.*, 2000). Evolutionary relationships were first proposed based on sequence information of the δ DNA polymerases of IIVs and the ascoviruses of lepidopteran insects (Knopf, 1998; Stasiak *et al.*, 2000), although caution is warranted when interpreting the

relatedness of morphologically distinct viruses based on the phylogeny of their replication proteins (Krupovic and Bamford, 2009). More recently, Bigot *et al.* (2009) identified 28 core genes that are shared by the symbiotic ascovirus DpAV-4a, three pathogenic ascoviruses and the invertebrate iridoviruses IIV-6 and IIV-3. Previous findings involving the analysis of multiple phylogenetic trees based on a selection of different genes from ascoviruses, iridoviruses, phycodnaviruses and ASFV had postulated that ascoviruses had evolved from iridoviruses (Federici and Bigot, 2003; Stasiak *et al.*, 2003). For the moment, it appears that ascoviruses and IIVs share a common origin but confirming that ascoviruses originated from IIVs will require additional IIV genome sequence information. However, based on the number of genes shared between DpAV-4a and IIV-6, Bigot *et al.* (2009) have suggested that DpAV-4a may have had a separate origin in the iridoviruses from that of the pathogenic ascoviruses. These relationships are discussed in detail in Chapter 1 of this book.

The virion

The icosahedral virions of IIVs are structurally complex and comprise an hydrated electron-dense core of DNA and associated proteins, an internal lipid membrane, an outer capsid and an external fringe of fibrils. Virions that bud out of cells may acquire an outer envelope, but this is not essential for infectivity.

One-dimensional SDS-PAGE of IIV-6 virions typically resolves 20–32 polypeptides in the range of 11 to 200 kDa (Krell and Lee, 1974; Barray and Devauchelle, 1979; Cole and Morris, 1980; Black *et al.*, 1981; Tajbakhsh and Seligy, 1990), whereas two-dimensional PAGE indicates the presence of more than 30 entities (Cerutti and Devauchelle, 1985). Most of this diversity is associated with the core and lipid membrane, although one surface labelling study on IIV-1 has indicated that the surface structure may be quite complex, with 10 or 11 polypeptides labelled under non-reducing and reducing conditions, respectively (Watson and Seligy, 1997).

The virion core comprises a 12.5 kDa major component and at least five additional polypeptides ranging in size up to a maximum of 81 kDa (Cerutti and Devauchelle, 1985). Freeze-etching studies of FV-3 have indicated that the DNA–protein complex may be organized as rods of ~10 nm in width arranged in a long coiled filament (Tripier-Darcy and Nermut, 1983), whereas the core structure of a mosquito isolate was described as bundles of parallel threads (Buchatsky, 1977). Lipid enveloped cores of IIV-3 were not infectious in cell culture or in mosquito larvae (Wagner *et al.*, 1975).

The internal lipid layer, 4 nm thick, constitutes 5–9% of the virion dry weight, of which 44–75% is phospholipid (Kalmakoff and Tremaine, 1968; Kelly and Vance, 1973; Balange-Orange and Devauchelle, 1982). The absence of microscopy evidence of virion cores budding through intracellular membranes (Stoltz, 1971) and the observations that the fatty acid and phospholipid composition of IIVs differ from that of the host cell (Balange-Orange and Devauchelle, 1982; Williams and Thompson, 1995) lead to the suggestion that the lipid layer may be generated *de novo* during the infection process. However, studies on other members of the NCLDV clade have revealed that their internal lipid envelopes are acquired from intracellular compartments (Schmelz *et al.*, 1994; Andrés *et al.*, 1998; Rouiller *et al.*, 1998), in a process facilitated by vimentin filaments concentrated in viral assembly sites (Risco *et al.*, 2002). To what degree IIV virion morphogenesis and lipid acquision mirrors that of poxviruses remains to be seen. As a case of history repeating itself, initial assertions that the lipid membrane was part of a double-layered structure (Stoltz, 1971; Yan *et al.*, 2000) were later rectified to indicate the single bilayer nature of this component (Stoltz, 1973; Yan *et al.*, 2009).

The formative electron microscope (EM) studies on IIV structure were performed by Wrigley (1969, 1970) and Stoltz (1971, 1973), and their findings have been broadly confirmed by the current model for IIV structure, based on detailed cryo-EM and image reconstruction studies on IIV-6 (Yan *et al.*, 2000, 2009). The vitrified IIV-6 virion has diameters of 162, 165 and 185 nm along the two, three and five fold axes of symmetry, respectively (Fig. 6.1A and B). Each particle is formed of 12 pentasymmetrons and 20 trisymmetrons arranged in an icosahedral, quasi-equivalent symmetry with a triangulation number of $T = 147$ ($h = 7$, $k = 7$). Both types of

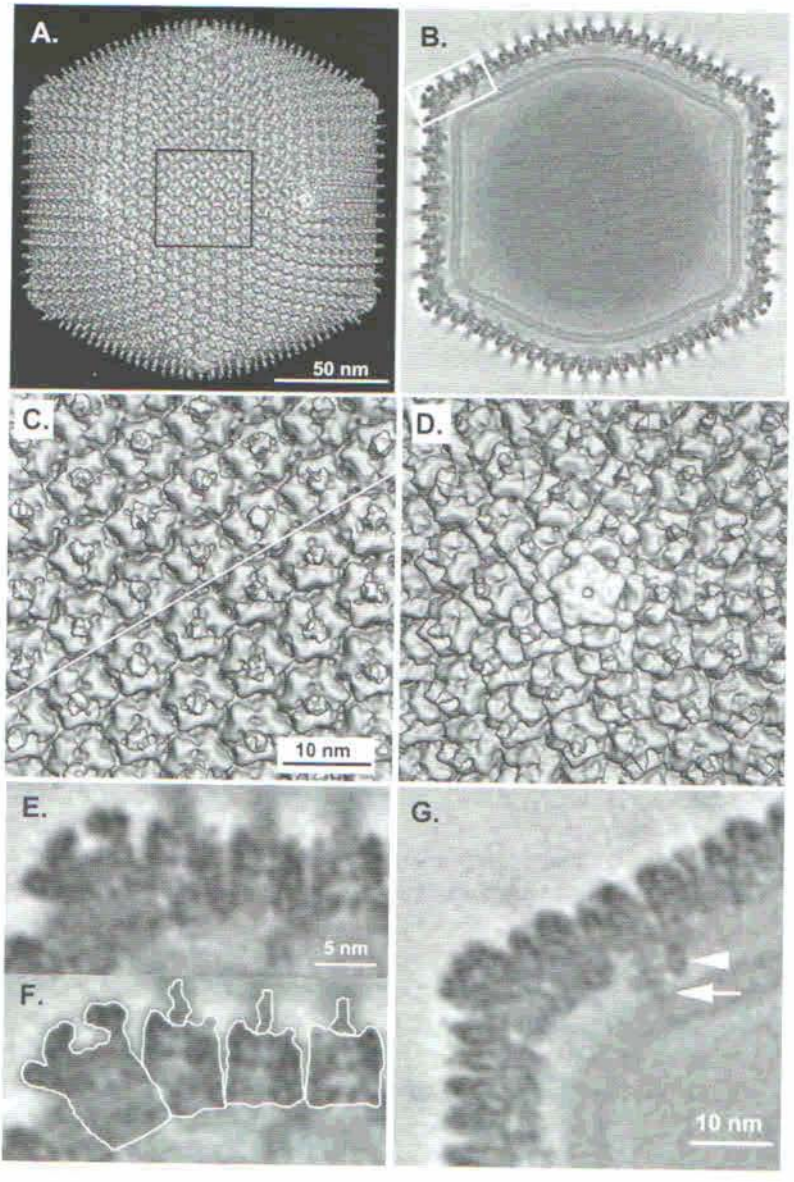

Figure 6.1 Three dimensional reconstruction of an IIV-6 particle at a resolution of 1.3 nm. (A.) 3-D density map of particle viewed along an icosahedral two-fold axis. The external fringe of filaments has been blurred away due to their varying position in relation to the symmetry of the capsid. (B) Central cross section, one pixel thick. A lipid bilayer follows the inner contour and icosahedral symmetry of the capsid shell whereas the core appears to lack any structures that are arranged following icosahedral symmetry. (C) Magnified view of the central region outlined in (A). The diagonal white line indicates the cleavage plane between adjacent trisymmetrons. (D) Magnified view of the propeller-like pentamer complex at the five-fold vertex. This complex differs from the trimeric capsomers in that it is larger, has a small axial hole, and lacks an external fibre. (E) Magnified view of boxed region in (B) showing transverse section of the pentamer complex and adjacent trisymmetrons. (F) Same as (E) but with the pentamer complex, three capsomers, and their fibres individually outlined. (G) Transmembrane anchor proteins beneath the pentasymmetrons showing two stick-like entities. The longer of the two (long arrow) crosses both leaflets of the bilayer, whereas the other (short arrow) stops at the outer leaflet of the internal lipid membrane. (Images reproduced from Yan *et al.* 2009 by permission of Elsevier.)

structure predominantly comprise hexavalent capsomers, a total of 1460 per virion, that are composed of the highly conserved MCP (Tidona *et al.*, 1998), that in IIV-6, comprises 467 amino acids (51.4 kDa) and represents ~40% of the total virion polypeptide (Moore and Kelly, 1980; Stohwasser *et al.*, 1993). Each hexavalent capsomer is formed by a non-covalent MCP trimer on the outer surface and a second MCP trimer linked by disulphide bonds on the inner surface (Cerutti and Devauchelle, 1985, 1990). Each MCP monomer contains two β-barrel domains, of the viral jelly-roll type, which are an ancient feature of icosahedral virus shell proteins (Khayat *et al.*, 2005; Krupovic and Bamford, 2008). The 55 capsomers in a trisymmetron are uniformly packed with an intercapsomer distance of 7.5 nm and in a single shared orientation, which is rotated by ~60° compared to the capsomers of the neighbouring trisymmetron (Fig. 6.1C). In addition, each pentasymmentron comprises 30 hexavalent (trimeric) capsomers and a single pentavalent capsomer at its centre, at the vertex of each pentasymmetron, a total of 12 in each virion. This pentavalent capsomer is significantly larger than the trimeric capsomers and has a five-bladed propeller-shaped external appearance and a small central pore below which a flask-shaped cavity is located (Fig. 6.1D–F).

A number of additional proteins have recently been identified in the capsid shell and in association with the lipid membrane (Yan *et al.*, 2009). These have been named zip monomers, zip dimers, finger proteins and anchor proteins (Fig. 6.2). The zip dimers appear as two halves of a clasp connecting the trimer capsomers along the edges of adjacent trisymmetrons, whereas zip monomers appear to be involved in linking trisymmetron capsomers with those of neighbouring pentasymmetrons. Three sets of nine inward-pointing finger proteins bind the capsomers along the first, second and third rows from the edge of each trisymmetron. Finally, an anchor protein connects each pentasymmetron with the lipid membrane at a distance of two capsomers from the pentavalent vertex (Figs. 6.1G and 6.2). It seems likely that this protein stabilizes the structure of the virion close to the vertices. Many other transmembrane proteins are present, but only the anchor protein can be visualized in image reconstruction studies due to its invariable position with respect to the icosahedral symmetry of the particle. Volume estimates, using the MCP as a reference, were used to estimate the molecular masses of these minor capsid proteins at 11.9, 19.7 and 32.4 kDa for the zip, finger and anchor proteins, respectively.

The outer surface of the capsid is covered by flexible fibrils or fibres. Conventional EM studies have suggested that these fibrils are often rather short (~2.5 nm in length) (Cole and Morris, 1980; Black *et al.*, 1981; Devauchelle *et al.*, 1985b) and may have terminal knobs (Stoltz, 1971, 1973), but this is clearly not the case in IIV-6 where a single fibril of 2 nm width and ~35 nm in length extends outwards from the centre of each trimeric capsomer (Figs. 6.1F and 6.3). The fragility of these structures is uncertain and they may have been damaged during sample preparation procedures involving dehydration and epoxy embedding for conventional EM studies. Cryo-EM studies that maintain the samples in a hydrated state have shown the presence of fibrils in IIV-6 and IIV-9 (Yan *et al.*, 2000, 2009; Juhl *et al.*, 2006) suggesting that this may be a feature of many IIVs. The presence of fibrils would be expected to affect the interparticle distance in quasi-crystalline arrays, which is influential in determining the iridescent phenomenon. Indeed, an isolate from the midge, *Chironomus plumosus*, was notable for the length of its well preserved fibrils that increased interparticle distance to such an extent that the isolate did not cause visible iridescence of infected host tissues (Stoltz *et al.*, 1968). The role of the fibrils remains elusive, but interactions with the host or improved stability in the environment have been postulated. Interestingly, the icosahedral mimivirus of amoeba is also surrounded by a long fringe of external fibrils that are partially digested by the host to achieve infection (Claverie *et al.*, 2009).

The genome

The IIV genome is a linear molecule of dsDNA. However, these genomes are characterized by being circularly permuted when mapped by restriction endonuclease analysis due to packaging of concatemeric DNA in a headful packaging system. The circular permutation and terminal redundancy of iridovirus genomes

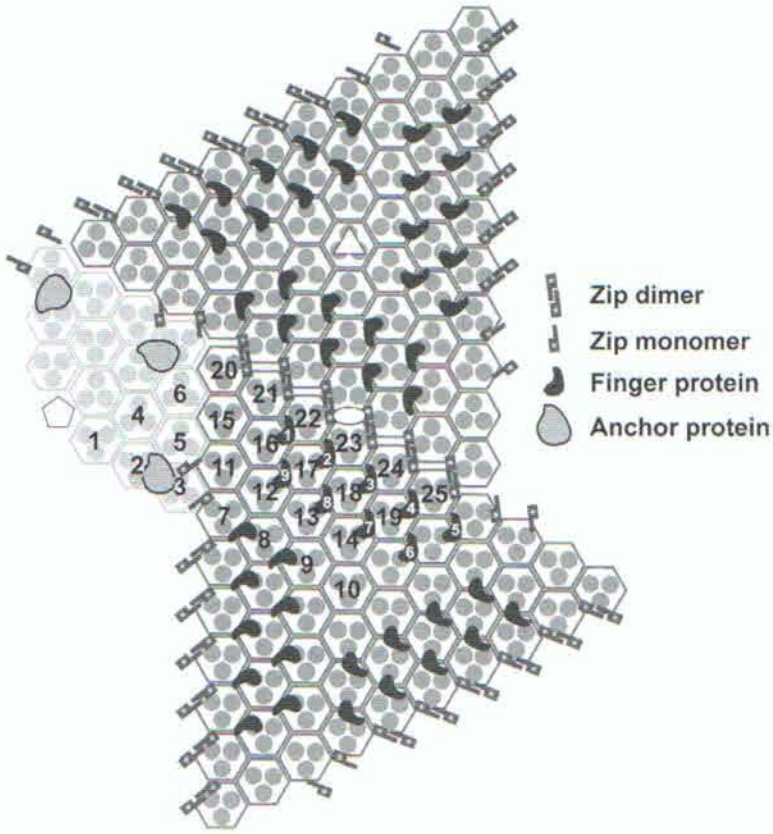

Figure 6.2 Schematic planar diagram of the IIV-6 capsid structure with the central pentamer complex (white pentagon) omitted for clarity. The centrally located white ellipse, triangle, and pentagon symbols highlight the positions of 2-, 3-, and 5-fold icosahedral axes, respectively. All major capsid protein trimers are shown as three small grey disks enclosed by a hexagon. The icosahedral asymmetric unit contains 24 and one third of these capsomers (numbered 1–25). Each trisymmetron contains 55 capsomers, all oriented similarly and rotated by 60° relative to those in the adjacent trisymmetron. Finger proteins bind to each trisymmetron (nine within one asymmetric unit are numbered in white), a total of 27 in each trisymmetron. A total of 18 Zip dimers are present at the interface between one trisymmetron and its adjacent trisymmetrons. Zip monomers are located at the interface between a trisymmetron and its neighbouring pentasymmetrons. The transmembrane anchor proteins (shown in Fig. 6.1G) are located under capsomers 2 and 3 beneath the pentasymmetron. (Image adapted from Yan *et al.* 2009, with permission from Elsevier)

was first proposed for FV-3 (Goorha and Murti, 1982), and subsequently confirmed by analysis of the genomes of IIV-6, IIV-9 and IIV-16 (Delius *et al.*, 1984; McMillan *et al.*, 1990; Ward and Kalmakoff, 1987; Webby and Kalmakoff, 1999). The terminal redundancy for IIV varies between species with pulse-field gel electrophoresis producing estimates ranging from 0% terminal redundancy for IIV-1 to 5.6% for IIV-6 and 7.8% for IIV-22 (Webby and Kalmakoff, 1999), which is somewhat lower than previous estimates for IIV-6 based on EM observations of terminal annealing (Delius *et al.*, 1984). The terminal redundancy is randomly distributed over the genome with all regions of the genome found at the termini of purified DNA (Delius *et al.*, 1984; McMillan *et al.*, 1990; Webby and Kalmakoff, 1999). This contrasts with FV-3 in which the termini are believed to be derived from a limited subset of the viral genome (Murti *et al.*, 1982).

To date, a total of 14 genomes have been fully sequenced in the family *Iridoviridae* (Table 6.3), but of these, the only viruses from invertebrates are those of IIV-3 (Delhon *et al.*, 2006) and

Table 6.3 Fully sequenced genomes from vertebrate and invertebrate iridoviruses

Genus, virus[a]	Size (bp)	ORF no.[b]	%GC	Accession no.	Reference
Chloriridovirus					
IIV-3	191,132	126	48	DQ643392	Delhon *et al.* (2006)
Iridovirus					
IIV-6	212,482	243[d]	29	AF303741	Jakob *et al.* (2001)
Lymphocystivirus					
LCDV-1	102,653	110	29	L63545	Tidona and Darai (1997)
LCDV-C	186,250	240	27	AY380826	Zhang *et al.* (2004)
Ranavirus					
TFV	105,057	105	55	AF389451	He *et al.* (2002)
ATV	106,332	96	54	AY150217	Jancovich *et al.* (2003)
FV-3	105,903	98	55	AY548484	Tan *et al.* (2004)
STIV	105,890	105	55	EU627010	Huang *et al.* (2009)
SGIV	140,131	162	49	AY521625	Song *et al.* (2004)
GIV	139,793	120	49	AY666015	Tsai *et al.* (2005)
Megalocytivirus					
ISKNV	111,362	124	55	AF371960	He *et al.* (2001)
RBIV	112,080	118	53	AY532606	Do *et al.* (2004)
RSIV	112,414	93[c]	53	BD143114	Kurita *et al.* (2002)
OSGIV	112,636	121	54	AY894343	Lu *et al.* (2005)

[a]IIV-3, invertebrate iridescent virus 3; IIV-6, invertebrate iridescent virus 6; LCDV-1, lymphocystis disease virus 1; LCDV-C, lymphocystis disease virus, China strain; TFV, tiger frog virus; ATV, *Ambystoma tigrinum* virus; FV-3, frog virus 3; STIV, soft-shelled turtle iridovirus; SGIV, Singapore grouper iridovirus; GIV, grouper iridovirus; ISKNV, infectious spleen and kidney necrosis virus; RBIV, rock bream iridovirus; RSIV, Red Sea bream iridovirus; OSGIV, orange spotted grouper iridovirus.

[b]Essentially non-overlapping ORFs encoding a minimum length of 40–62 amino acids.

[c]Minimum ORF of 86 amino acids.

[d]Revised annotation of Eaton *et al.* (2007).

IIV-6 (Jakob *et al.*, 2001), while the genome sequence of IIV-9 (genus *Iridovirus*) has been determined and is currently undergoing analysis (Wong, 2009). Genomes from different genera show substantial differences with no apparent collinearity between their genes unless they are very closely related (Jancovich *et al.*, 2003). The genomes have widely different GC contents at 48% and 29% for IIV-3 and IIV-6, respectively. Individual IIV genomes display less than 10% homology between genomes, reflecting the lack of collinearity and diversity of sequences found in these genomes (Webby and Kalmakoff, 1999). The codon bias of IIVs is thought to reflect this GC content (Tsai *et al.*, 2007). As mentioned previously, phylogenetic analyses of the MCP, for which many more sequences are available, suggests that IIV-3 may in fact be more closely related to the major complex of IIVs than IIV-6 (Delhon *et al.*, 2006).

IIV genomes possess extensive repeat sequences with 20% and 39% of repetitive DNA observed for IIV-3 (Delhon *et al.*, 2006) and IIV-16 (McMillan *et al.*, 1990), respectively. The characterization of a repeat region from IIV-6 revealed a complex repeat structure including overlapping and interspersed repeats (Fischer *et al.*, 1988). The role of these highly repetitive sequences is unknown, although a role in genome replication through branched chain concatemer formation is one possibility. The analysis of the genome of IIV-9 has shown a similar complex arrangement of repeats suggesting that this is a common feature of IIVs (Wong, 2009). One of the hallmarks of vertebrate iridovirus genomes is the high degree of methylation with a viral encoded DNA methyltransferase that, with one exception (Song *et al.*, 2004), results in the methylation of approximately 20% of the cytosines in the genome (Willis and Granoff, 1980). In contrast, IIV genomes are not methylated (<5% methylation at CpG residues).

The genomes of these viruses encode a range of genes with a surprising diversity. Analysis across all sequenced iridoviruses shows that only 26 genes are conserved across all members of the family, and when comparing IIV-3 and IIV-6, only 52 of the 126 IIV-3 genes are conserved in IIV-6 (Delhon *et al.*, 2006). Twelve of the 26 core genes are associated with DNA replication or transcription, indicating that the replication of the genome and viral gene transcription share a common basis between these viruses that are currently classified in different genera. Of the 52 genes shared between IIV-3 and IIV-6, only 12 have a predicted function, underlining the differences between these two viruses (Delhon *et al.*, 2006). The recent sequencing of IIV-9, which is in the main phylogenetic cluster of IIVs in which IIV-3 clusters by MCP analysis, may indicate whether IIV-3 or IIV-6 is the outlier of this group and if the current separation of *Iridovirus* and *Chloriridovirus* genera is appropriate (Wong, 2009).

The genes encoded by vertebrate and invertebrate iridoviruses have been described in detail in the respective publications (Table 6.3) and will not be covered in this chapter. However, a clear feature is the lack of co-linearity in gene order between the viruses unless they are essentially strains of the same species. This remarkable diversity in gene order indicates that there is no apparent requirement for clusters of genes with complimentary functions or clustering of transcripts. Whether this is a consequence of the replication strategy of hybridizing concatemers driving a high rate of recombination (Chinchar and Granoff, 1986), and hence a need for the control of individual genes to be independent of their neighbours, is unknown.

A number of interesting elements are being identified in iridovirus genomes. For example, an intein has been identified in the ribonucleotide reductase gene of IIV-6, representing the first report of such an element in an insect virus (Pietrokovski, 1998). Mini-inteins have also been identified in IIV-9 and IIV-16 (Goodwin *et al.*, 2006) suggesting that this may be a common feature of IIV genomes. In addition, the genome of the soft-turtle iridovirus was recently found to contain 12 putative precursor RNAs encoding 20 predicted microRNAs (miRNAs) (Huang *et al.*, 2009). Whether these miRNAs are produced was not determined, however, it offers an intriguing possibility for interaction with and control of host or viral gene expression (Cullen, 2009; also see Chapter 15). Whether these elements are also present in IIVs is not known, but given the high incidence of complex repeats in these genomes combined with the nuclear site of early gene expression, it would seem likely that candidates exist on these genomes.

Replication

Iridoviruses show very broad host ranges provided the temperature is kept relatively low (<30°C), indicating that they bind non-specifically to a wide range of cell surface receptors, or to a limited number of common receptor molecules (Williams, 1996). Both the naked and enveloped virions are infectious, and each presumably recognizes different receptors, although this has not been confirmed. Both types of virions appear to enter cells by receptor-mediated endocytosis (Hall, 1985), although the mechanisms by which these events occur are unclear. Once in the cytoplasm, particles may be surrounded by a membrane, in lysosome-like structures or as free virions that undergo uncoating. The DNA core

then migrates to the nucleus and initiates a two-stage replication cycle.

The replication of the genome is presumed to be essentially similar to that of FV-3, although the information supporting this is limited. The unit length genome of the infecting virus enters the nucleus where it is replicated into approximately unit or twice unit length progeny genomes in the first stage of genome replication (Goorha, 1982). The involvement of the nucleus is essential (Goorha *et al.*, 1977), emphasizing the importance of this step in viral replication. These nuclear genomes are the templates for early mRNA production and are transcribed by host RNApol II. The essentially unit length genomes are transported to the cytoplasm where the second stage of DNA replication occurs (Goorha *et al.*, 1978). Cytoplasmic replication leads to the formation of concatemeric replication complexes that are over 10 unit lengths in size (Goorha, 1982). Evidence for the formation of cross-hybridizing complexes includes the detection of single-stranded regions through the use of nuclease S1 and their subsequent repair (Goorha, 1982). Whether this process is the same in IIVs is not clear. Studies on IIV-9 showed that large complexes are indeed formed, although nuclear and cytoplasmic separation was not clearly defined as in FV-3 (Watson, 1997). In contrast, Cerutti *et al.* (1989) reported evidence for recombination in IIV-6 during replication, indicating that a similar process was occurring to that elucidated in FV-3. Further research is needed to confirm the presence and nature of the cytoplasmic stage of genome replication in IIVs.

Late in the infection cycle particle maturation occurs at cytoplasmic assembly sites. The exact relationship between assembly site formation, recruitment of concatemeric DNA, late gene transcription and assembly of virus particles is largely unknown. However, the accumulation of DNA and virion components leads to virion assembly and the presumed packing of DNA by a headful mechanism from concatemeric DNA that includes more than genome length DNA, thus leading to circular permutation and terminal redundancy (Goorha, 1982). An ATPase is one of the 26 core genes identified in all iridoviruses and may be a packaging ATPase involved in DNA packaging. A terminal redundancy of 10% through this mechanism would mean an extra ~20 kbp packaged into each particle and hence a significant gene duplication. Exactly how the network of cross-hybridizing concatemers is resolved into unit length genomes is unknown and the potential role(s) of these gene duplications representing all regions of the genome remain to be elucidated.

Iridovirus assembly sites are perinuclear electron translucent structures surrounded by rough ER membranes and polyribosomes. Netherton *et al.* (2007) have argued that these cytoplasmic inclusions resemble aggresomes as they sequester intermediate filaments and mitochondria that often appear damaged. In FV-3, vimentin filaments are phosphorylated and form cages around each assembly site that appear to act as a scaffold for virus replication and that simultaneously exclude cytoplasmic components (Chen *et al.*, 1986; Murti *et al.*, 1988). Consistent with these findings, polyribosomes and most newly synthesized viral proteins associate with intermediate filaments during infection (Murti and Goorha, 1989). A 108 kDa early protein is also recruited to viral assembly sites during FV-3 infection, although its role remains unclear (Chinchar *et al.*, 1984).

The processes that control virion assembly are also unclear. Proteins such as the MCP are required but what other factors are critical is largely unstudied. One potentially important gene is the myristylated membrane protein found in all sequenced iridoviruses, which may be analogous to the myristylated membrane protein of vaccinia virus (Huang *et al.*, 2009) that has been shown to be critical for virion assembly (Ravanello and Hruby, 1994). Once the virions have assembled there are two possible routes of final maturation for the virus particles. The majority exit the assembly site and accumulate a regular paracrystalline arrays, whereas some virions bud from the cell to become enveloped. The factors that define which pathway virions mature by are not known.

Gene expression

As for many aspects of IIV replication, transcript synthesis is presumed to be essentially similar to that of FV-3 and many similarities have been supported through studies of IIV-6 and IIV-9.

Parallel to the first stage of genome replication, viral gene transcription commences in the nucleus with the production of immediate early (IE) transcripts that initiate a temporal cascade characterized by delayed early (DE) and late (L) phases of gene expression. IE gene expression is defined as the events that occur prior to *de novo* protein synthesis and are expressed in the presence of the protein synthesis inhibitor cycloheximide. Subsequent DE genes are dependant upon one or more factors encoded by IE genes and are expressed in the presence of DNA synthesis inhibitors such as aphidicolin or AraC. Late gene expression is dependent upon factors encoded by DE genes and is also blocked by inhibitors of DNA synthesis (Barray and Devauchelle, 1985; Elliott and Kelly, 1980; D'Costa *et al.*, 2001; Nalcacioglu *et al.*, 2003).

Early gene transcription requires a functional nucleus and the use of α-aminitin during FV-3 infection has shown that host RNApol II is required (Goorha, 1981), whereas later phases of the transcription cascade are presumed to involve virus encoded subunit homologues of RNApol II designated vpol IIα and vpol IIβ. Indeed, the targeting of FV-3 vpol IIα with morpholino antisense oligonucleotides inhibits late gene transcripts (Sample *et al.*, 2007; Chinchar *et al.*, 2009). The transcription complex required for IIV transcription, particularly in the cytoplasm, is unknown. One feature of the large subunit of viral RNApol is the absence of a region termed the carboxyl-terminal domain that is associated with most RNA polymerases. This domain has been linked to transcription termination and polyadenylation as well as to interactions with the cap-synthesizing complex. It is interesting to note that this correlates with the absence of polyadenylation on iridovirus transcripts. It is not known what viral factors are associated with host RNApol II for IE gene expression other than they are virion associated transcriptional transactivators and that these transcripts are also non-polyadenylated (Chinchar *et al.*, 2009). The lack of a polyA tail combined with the absence of a domain usually associated with 5′ cap synthesis suggests that viral factors may be involved in translation of viral proteins.

Detailed analysis of IIV-6 transcripts by northern blotting using plasmid clones of the genome identified 137 transcripts ranging from 0.2 to 8.3 kb, of which 38 were classified as IE, 34 were DE and 65 designated as L (D'Costa *et al.*, 2001). The IE genes could be detected as early as 0.5 hours post infection, DE transcripts from 3 hours and L transcripts from 6 hours. This analysis complements earlier studies using ^{35}S methionine labelling to separate IIV-6 proteins produced in *Choristoneura fumiferana* cells into early and late phases, with early proteins detected as soon as 1 hour post infection and later proteins not detected before 5 hours post infection (Barray and Devauchelle, 1985). Many genes are transcribed across multiple phases (D'Costa *et al.*, 2004) and it is not clear if the switch to late gene expression is limited purely to the cytoplasmic concatemeric DNA generated during the second stage of replication, thus linking subcellular replication sites with the temporal control of replication, or if transcription continues within the nucleus, which is likely to require the nuclear translocation of IE and/or DE viral transcription factors from the cytoplasm. In this context, it is interesting to note that *bro*-like genes (baculovirus repeated ORF) have been identified in IIV-6 and IIV-31, as well as a number of other members of the NCLDV clade (Bideshi *et al.*, 2003). The BRO proteins have now been identified as nucleocytoplasmic shuttling proteins that use the CRM1(exportin 1/Xpo1p)-mediated nuclear export pathway in nucleopolyhedroviruses (Kang *et al.*, 2006).

Approximately 90% of the genome of IIV-6 is transcriptionally active and while many IE and DE genes appeared to occur in four and three physical clusters, respectively, the late gene transcripts are scattered across the entire genome (D'Costa *et al.*, 2004). A similar clustering of gene classes was also suggested for IIV-9, although the clustering was much less apparent (McMillan *et al.*, 1990). There are clear clusters of early and late genes in vertebrate iridoviruses (Lua *et al.*, 2005). Of particular interest to the observation of gene clustering is the apparent lack of conservation of gene order between disparate iridoviruses, which raises the question of the importance of clustering and whether temporal clusters are retained even when gene order is not. Combined with the observation that many transcripts are very large and that co-terminal 5′ and 3′ transcripts can be identified during infection (D'Costa *et al.*, 2004),

it is apparent that individual gene transcript analysis will provide valuable information on gene expression in these viruses.

Analysis of the early DNA polymerase (DNApol) and helicase genes and the late MCP gene was undertaken to identify promoter elements associated with different phases of the expression cascade in IIV-6 (Nalcacioglu *et al.*, 2003). As shown previously for FV-3, the promoters were only active in the presence of IIV-6 infection, presumably mediated by one or more proteins in the virus particle given that UV-irradiated virus can provide the necessary function (Cerutti *et al.*, 1989). A TATA-like element was identified, however, a subsequent study on the DNApol gene showed it to be a DE gene that was not produced in the presence of cycloheximide and an essential AAAAT promoter sequence was identified upstream of 20 DE genes and upstream of DNApol in eight iridoviruses for which sequence information was available, including IIV-3 (Nalcacioglu *et al.*, 2007). This provides the first information of a promoter sequence in IIV that may define a class of transcript. Intriguingly, the AAAAT promoter has also been identified in a single additional member of the NCLDV virus clade; namely the giant mimivirus where it appears as an octomer (AAAATTGA) identical to that of the IIV-6 DNApol promoter element, and is located 50 to 80 nucleotides upstream of almost half the mimivirus ORFs where it is believed to function as a signal for transcription of late-early genes by virus encoded transcription machinery present in the virion (Suhre *et al.*, 2005). How relevant the mimivirus model is to IIVs remains to be seen.

Recent studies on vertebrate iridoviruses using microarrays have shown similar results, with a classic temporal gene cascade, clusters of early and late genes from around the genome, the general separation of regulatory and nucleic acid metabolism associated genes occurring during the early phases and a link to DNA replication and virion proteins associated with late genes, together with a number of genes being expressed across the different temporal phases (Dang *et al.*, 2007; Lua *et al.*, 2005; Majji *et al.*, 2009; Teng *et al.*, 2008). In addition, the slow pace of IIV infection in insects compared to the rapid replication in cell culture has been echoed in the vertebrate viruses with a much slower progression of replication *in vivo* than that observed *in vitro* and distinct differences in gene expression in different cells and tissues (Dang *et al.*, 2007; Teng *et al.*, 2008).

Concomitant with viral macromolecular synthesis is the shutdown of host macromolecular synthesis (Cerutti and Devauchelle, 1980). There is a decrease in host RNA, DNA and protein synthesis and this effect is associated with the virion. UV-treated IIV-6 is able to inhibit host cell macromolecule production and this effect is also observed in cells treated with virion extracts. What factor(s) are causing these effects are unknown, although viral replication can continue, indicating a fundamental difference between viral and host cell macromolecule synthesis.

Apoptosis: induction and inhibition

The interactions between iridoviruses and the cells within which they replicate have received limited attention. An exception is in the induction and inhibition of apoptosis within infected cells. It is interesting to note that while the general principles of apoptosis are the same between vertebrates and invertebrates, they differ in a number of important details. This necessitates care when comparing vertebrate to invertebrate iridoviruses in the area of apoptosis. We start by considering apoptosis modulated by the vertebrate viruses and then signal how this process differs in IIV-infected cells.

Vertebrate iridoviruses and apoptosis

It is well known that infection by a number of vertebrate iridoviruses leads to apoptosis in cultured cells (Essbauer and Ahne, 2002; Chinchar *et al.*, 2003; Hu *et al.*, 2004; Imajoh *et al.*, 2004; Huang *et al.*, 2007; Lai *et al.*, 2008), following the well-characterized hallmarks of cellular apoptosis, including cell blebbing, chromatin condensation, DNA fragmentation, relocalization of phosphatidylserine to the cell surface and activation of caspases. The induction of apoptosis by FV-3 occurs following treatment with heat or UV-inactivated virus, indicating that gene expression is not required. This activation also occurs in BHK cells that do not support viral replication (Chinchar *et al.*, 2003).

The induction of apoptosis by other ranaviruses has been shown to involve mitochondrial morphological and distribution changes with a clear indication of a loss of mitochondrial membrane function as indicated by depolarization of membrane potential in the late stages of viral infection. The induction of apoptosis has been associated with an LPS-induced TNFα factor that has been linked to a collapse in mitochondrial membrane potential and consequent effects upon caspase-3 activity and Nf-κb dependant transcription (Huang *et al.*, 2008). Apoptosis induction has also been linked in part to the activation of a protein kinase in another fish iridovirus (Essbauer and Ahne, 2002). In all cases, central caspase-3 activity has been shown to increase during vertebrate iridovirus replication and where studied, the pancaspase inhibitor Z-VAD-FMK is able to block iridovirus-induced apoptosis, thus confirming the central role of caspase-dependant apoptosis in response to infection (Chinchar *et al.*, 2003; Imajoh *et al.*, 2004; Huang *et al.*, 2007, 2008; Chiou *et al.*, 2009).

The observation that high doses of virions induced rapid apoptosis whereas lower doses did not induce apoptosis until late in infection suggested the presence of an apoptosis inhibitor in vertebrate iridoviruses. The induction of apoptosis in fish cells exposed to UV light was rapidly inhibited by infection with a fish iridovirus (Lin *et al.*, 2008). Expression of the inhibitory factor occurred in the presence of the protein synthesis inhibitor cycloheximide and the DNA synthesis inhibitor aphidicolin, indicating that the anti-apoptotic protein was an IE gene. This was subsequently shown to be a virally encoded Bcl2-like protein that contained the signature BH1, 2, 3 and 4 motifs of this group of proteins. Furthermore, this protein was located in the virion, indicating that it is delivered as part of the infection process. Heat or UV-inactivation of the virus did not prevent the inhibition of apoptosis induced by UV-treatment of host cells (Lin *et al.*, 2008). Moreover, vertebrate iridoviruses encode initiation factor eIF2α homologues (Essbauer *et al.*, 2001) that are known to function as pseudo-substrate inhibitors of protein kinase R (PKR) in poxviruses. In the absence of the inhibitor, PKR phosphorylates host eIF2α leading to translational shut-off, but this mechanism has not been fully characterized in vertebrate iridoviruses and candidate eIF2α genes have not been identified in IIV-3 or IIV-6.

Overall then, studies on a variety of vertebrate iridoviruses have identified a combination of an antiapoptotic factor present in the virion, a Bcl2-like IE gene and a putative pseudosubstrate inhibitor of PKR. However, differences in the biology of vertebrate and invertebrate cells and the gene content of the viruses mean that some of the observations on vertebrate iridoviruses will not translate to IIVs, emphasizing that caution is required when extrapolating between these groups of viruses.

Invertebrate iridoviruses and apoptosis

Studies on apoptosis in IIVs are largely limited to IIV-6. Paul *et al.* (2007) investigated the observation that IIV-6 infections induce apoptosis in insect cells as indicated by cell blebbing and DNA fragmentation. Treatment of cells with a virion protein extract resulted in shutdown of host cell protein production and apoptosis, indicating that the inducing factor was associated with the virion. In addition, the use of the pancaspase inhibitor Z-VAD-FMK prevented the induction of apoptosis, indicating that a caspase dependant response was involved, as observed for vertebrate iridoviruses. However, this did not affect protein synthesis, suggesting that these two responses are not linked in IIV-6 infection.

In a follow up study, the delivery of a high dose of virus to cells was observed to trigger a massive apoptotic response; however, at lower doses the apoptotic response was inhibited, confirming that IIV-6 causes apoptosis, and that IIV-6 must possess an antiapoptotic factor that blocks apoptosis (Chitnis *et al.*, 2008). The entry of virions or virion proteins induced apoptosis and blocking this entry through the use of ammonium chloride to prevent endosome acidification, bafilomycin to block endocytosis, or attachment to polystyrene beads to prevent uptake, blocked apoptosis induction. Therefore, the uptake of virus or viral proteins is a critical step in induction of apoptosis by IIV-6. Furthermore, the use of cycloheximide to block *de novo* protein synthesis shows that protein expression is not required to

induce apoptosis and hence the inducing factor must be part of the virion.

Of particular interest was the observation that the Janus Kinase (JNK) signalling pathway was activated by IIV-6 infection and that the SP600125 inhibitor of JNK blocked IIV-6 apoptosis induction; the first indication that JNK is involved in an insect virus infection. Consistent with this was the identification of a Dronc-like apical caspase designated Cf-caspase-1 in *C. fumiferana* cells that was activated by the JNK pathway, confirming a caspase-dependant apoptotic response for an IIV. UV inactivated virus, protein extracts and cycloheximide strongly induced apoptosis. In contrast, IIV-6 infection showed muted apoptosis and the use of aphidicolin to block late gene expression indicated that the antiapoptotic factor was an early gene (Chitnis *et al.*, 2008).

The apoptosis inducing polypeptide has now been identified as a 49-kDa serine-threonine kinase with an ATP binding motif (Bilimoria, 2009). This is likely to be the same virion-associated kinase that was characterized by Monnier and Devauchelle (1980) from solubilized extracts of IIV-6. A 37-kDa fragment derived from this protein has been patented for its apoptotic and insecticidal properties. The insecticidal fragment has been named iridoptin; no doubt the details of the function of this protein will appear shortly in the scientific literature.

In a separate study, Ince *et al.* (2008) analysed the genome of IIV-6 and identified three open reading frames with homology to the baculovirus inhibitors of apoptosis (IAP). Of these, only ORF193R contained the RING finger and baculovirus inhibitor repeat (BIR) motifs found in functional IAPs. This was the only iridovirus protein identified with a BIR motif. ORF193R was demonstrated to be an IE gene that was capable of blocking actinomycin D-induced apoptosis and represents the first functional *iap* gene identified in an IIV.

IIV apoptosis induction and inhibition has many parallels to vertebrate iridovirus interactions with this pathway. One or more inducing factors are clearly associated with the particle and an IE gene(s) limits the apoptotic response during infection. However, the specific details appear to be different. Studies of more IIV apoptosis interactions will be required to determine if the observations in IIV-6 are universal, particularly given that IIV-6 and its close relatives appear to be phylogenetically separated from the largest complex of IIVs. However, apoptosis is one area where the IIV investigations are in many respects equal with, or leading, our overall understanding of these viruses.

IIV disease: signs and pathology

Signs of disease

The most evident symptom of patent infection is the iridescent hues, which arise from the quasicrystalline arrangement of virus particles in host cells or purified pellets of virus. Light reflected from the planes of regularly packed particles causes interference with light in similar phase that has been reflected from a neighbouring plane, a phenomenon known as Bragg diffraction, that results in a range of colours from lavender blue, to turquoise or green (Klug *et al.*, 1959). Iridescent colours are highly directional so that changes in viewing geometry can dramatically alter the hue and/or intensity of the observed colours (Doucet and Meadows, 2009). The larger particle size and greater interparticle spacing of viruses from mosquitoes results in iridescent colours of yellow-green, orange and red (Hemsley *et al.*, 1994), although there are a number of records of inconsistencies between observed iridescent colours and particle sizes that may be related to the degree of hydration of the infected cell, the length of capsid fibrils or some other optical property of the virus arrays (Williams, 2008). The biological function or adaptive significance of iridescence is not known and may be a trivial characteristic that arises from the physical structure and assembly characteristics of IIV particles (Stoltz, 1971; Kelly, 1985).

Natural patent infections are mainly observed in immature stages (larvae, nymphs), although adults are susceptible to lethal infection in terrestrial isopods and bees. Infection by an IIV is believed to be the causative agent of 'Clustering disease' in the Asian bee, *Apis cerana*. Diseased colonies become inactive, form small groups of flightless individuals that crawl on the ground, and the colony quickly perishes (Bailey

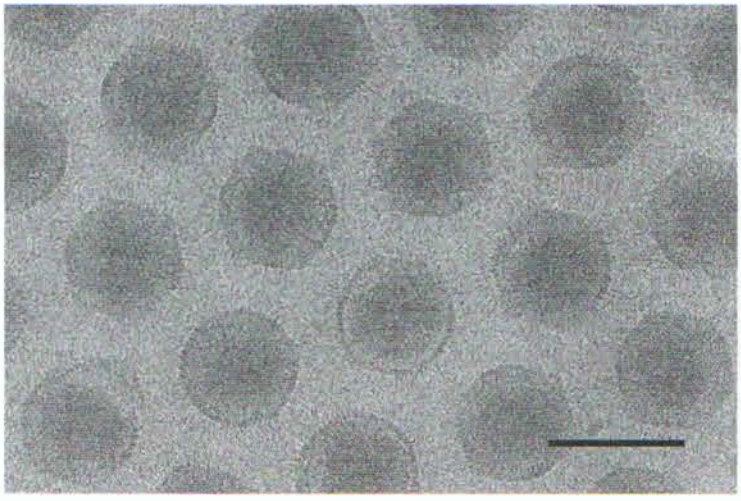

Figure 6.3 Photomicrograph of vitrified virions of IIV-6 showing external fringe of fibrils extending from the surface of the capsid (scale bar indicates 200 nm). Image courtesy of Xiaodong Yan and Tim Baker.

et al., 1976; Bailey and Ball, 1978; Singh, 1979; Mishra *et al.*, 1980). Reductions in fecundity and longevity have been reported in an aphid (*Toxoptera citricida*) that became patently infected after feeding on crude suspensions of IIV-6 (Hunter *et al.*, 2001b).

Sublethal (covert) infection is becoming increasingly recognized as a viral infection strategy with an important impact on the dynamics of certain insect populations (Sorrell *et al.*, 2009). Covert infections by IIVs have been observed in natural populations of the blackfly *Simulium variegatum* (Williams, 1993) and the mayfly *Ecdyonurus torrentis* (Tonka and Weiser, 2000), and laboratory populations of *Helicoverpa zea*, *Aedes aegypti* and *Galleria mellonella* (Sikorowski and Lawrence, 1997; Marina *et al.*, 1999; Constantino *et al.*, 2001). Covertly infected mayflies were observed to be infected by a low density of particles in the haemocytes and fat body with no obvious signs of disease. A large number of tubular structures typical of aberrant capsid formation were observed in cytoplasmic viral assembly sites (Tonka and Weiser, 2000), suggesting that *E. torrentis* may not be an optimal host for this virus.

In *Ae. aegypti*, adult mosquitoes that were covertly infected by IIV-6 in the larval stage suffered reduced body size (wing length of females) and reduced longevity with an early peak in the death rate and very few long-lived individuals. Covert infection was associated with a decrease in fecundity ranging from 22% on a single gonotrophic cycle to 36–39% on multiple (lifetime) gonotrophic cycles; the average number of female offspring produced by each mosquito (R_0) was reduced by 22–50%. No effects on egg fertility were detected (Marina *et al.*, 1999, 2003b).

The effects observed in mosquitoes may be due to the diversion of host resources to repair cells and tissues damaged by the pathological effects of sublethal disease, or the cost of mounting an immune response to control the infection. An alternative hypothesis, that sublethal effects were a consequence of a toxic virus protein associated with the internal lipid membrane (Cerutti and Devauchelle, 1980; Ohba *et al.*, 1990), was not supported in experimental studies; heat- or UV-inactivated IIV-6 did not significantly affect the reproductive capacity or longevity of mosquitoes and caused only minor changes in adult body size compared to controls (Marina *et al.*, 2003c).

Pathology

IIVs replicate extensively in the majority of host tissues, especially the fat body, haemocytes, and

epidermis. Infection of the midgut epithelial cells is rarely observed and it seems that the midgut is an unlikely route of entry for these viruses, which may account for their low prevalence in most host populations. IIV-1 caused the formation of epidermal tumours in silkworm larvae (Hukuhara, 1964), but such pathology is not observed in other hosts. Patently infected individuals that survive to pupate often show deformations of the pupa (Smith *et al.*, 1961; Carter, 1974; Stadelbacher *et al.*, 1978).

IIVs can replicate *in vitro* in cell lines from many insects albeit with different degrees of efficiency (Cerutti *et al.*, 1981; Charpentier *et al.*, 1986; Constantino *et al.*, 2001). Some insect cell lines appear not to support replication but suffer marked cytotoxic effects following inoculation (Cerutti and Devauchelle, 1979; Belloncik *et al.*, 2007), that are related to the recognized toxic effects of virions and virion extracts (Ohba and Aizawa, 1979a; Cerutti and Devauchelle, 1982; Ohba *et al.*, 1990). IIVs are also unusual in being capable of non-genetic reactivation. In this process, viral DNA alone is not infectious to cells but infection is achieved when cells are inoculated with mixtures of DNA and solubilized virion extract or UV-inactivated virus indicating that one or more inner membrane-associated components of the virion are required to initiate a productive infection (Cerutti *et al.*, 1989).

Cytopathic effects associated with IIV infection include cell rounding and the appearance of extensive areas of finely granulated material devoid of cell organelles that are the cytoplasmic viral assembly sites. Large numbers of virions form quasicrystalline arrays in close association but not generally within this zone (Fig. 6.4). Marked cell contraction and detachment are also commonly observed. Blebbing of cell membranes, cell to cell fusion (syncytia) and related apoptotic changes are observed at high multiplicities of infection (Cerrutti and Devauchelle, 1979; Mathieson and Lee, 1981; Charpentier *et al.*, 1986; Czuba *et al.*, 1994). Changes in the position and morphology of mitochondria have been reported (Hess and Poinar, 1985), consistent with the aggresome-like nature of these cytoplasmic inclusions (Netherton *et al.*, 2007).

Ecology

Host range

IIVs have been isolated from infected hosts on all continents except Antarctica. Examination of confirmed and suspected species records from

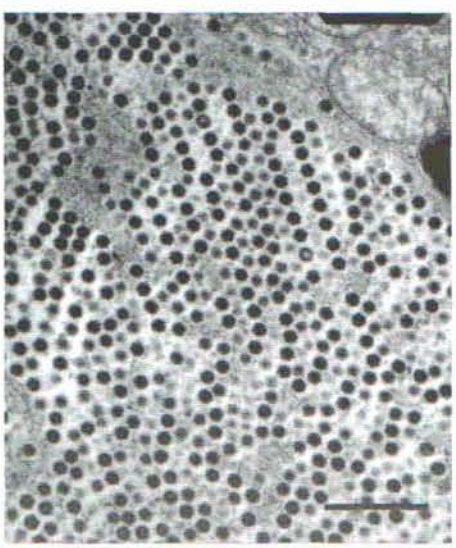

Figure 6.4 Quasicrystalline array of IIV-3 particles and granular material from the viral assembly site in infected cells of *Ochlerotatus taeniorhynchus* (scale bar indicates 1 μm). Image courtesy of James Becnel.

insects and isopods shows the greatest interest in these viruses in North America (41 species) and Europe (36 species) followed by Australia/Oceania (10 species), Africa (six species), South America (three species) and Asia (three species) (Williams, 2008). This represents a 50-fold difference between Europe and Asia in terms of host species per area and suggests that many host species remain to be discovered in Africa, South America and Asia. Indeed, this relationship may be set to change given the rapid growth in insect virology in China over the past decade.

IIVs have been reported to infect 99 species of terrestrial and aquatic invertebrates (Hall, 1985; Williams, 2008), although a number of these records are based on the characteristic signs of patent infection rather than the observation of virus particles or molecular evidence. Of these, 70 are insect species and 18 are species of terrestrial isopods commonly known as woodlice, sowbugs or pillbugs. The insect records are dominated by Diptera with aquatic immature stages (44 species), followed by Coleoptera (nine species, of which eight have soil-dwelling larvae), Lepidoptera (nine species), Orthoptera (four species), and four species from other orders. There are also nine species records from invertebrates such as daphnids, molluscs, crabs, and shrimp among others, in freshwater and marine habitats for which the relationships with IIVs are uncertain. A record from the marine mollusc *Nautilus* sp. suggested that this isolate was more closely related to vertebrate ranaviruses than to IIVs (Gregory *et al.*, 2006), whereas an iridovirus isolated from the sergestid shrimp *Acetes erythraeus* showed ~80% similarity in the MCP sequence to those of IIVs (Tang *et al.*, 2007). As mentioned at the beginning of this chapter, perhaps the principal feature that unites the large majority of these host species is the damp or aquatic nature of the habitats they occupy during part, or all, of their life cycle.

The host range of many IIVs in the laboratory depends on the route of inoculation. In general, the range of hosts infected by peroral inoculation is markedly more restricted than the host range defined by studies in which the inoculum is injected. For instance, the best studied example is that of IIV-6, that can infect numerous (>100) insect species of agricultural and medical importance including Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Homoptera, Diptera and Orthoptera (Mitsuhashi, 1967; Hama, 1968; Fukuda, 1971; Jensen *et al.*, 1972; McLaughlin *et al.*, 1972; Ohba, 1975; Kleespies *et al.*, 1999; Henderson *et al.*, 2001; Hunter *et al.*, 2001b) and non-insect species such as terrestrial isopods and a centipede (Ohba and Aizawa, 1979b). A strain of IIV-6 was also observed to cause lethal infection of hymenopteran endo- and ectoparasitoids that developed on infected lepidopteran larvae (López *et al.*, 2002).

The majority of these viruses can be easily grown in very large quantities by intrahaemocoelic inoculation of wax moth larvae, *G. mellonella*, which is the standard laboratory host. However, a few IIVs do not replicate in this host, including the mosquito virus IIV-3, IIV-16 from *Costelytra zealandica* and the bee virus, IIV-24. A few studies have attempted to determine host range given asymptomatic infection. Ohba (1975) observed an increase in the titre of IIV-6 in insects that showed no signs of patent disease. Ward and Kalmakoff (1991) reported that dot-blot DNA hybridization could be used to detect replication of IIV-9 in heterologous host species prior to, or in the absence of iridescence of host tissues. Similarly, an indirect fluorescent antibody technique was employed to demonstrate the presence of IIV-22 antigen in the tissues of mosquitoes, sandflies and a triatomine bug, with the quantity of antigen proportional to the interval between inoculation and testing (Tesh and Andreadis, 1992). As is the case with most insect viruses, it is now widely accepted that the appraisal of host range requires diagnosis of infection using techniques other than observation of the characteristic signs of patent disease.

A steadily growing body of evidence indicates that some IIVs naturally infect multiple species of hosts, sometimes across different orders or even different classes. Three of the best examples are as follows. First, strains of IIV-9, originally isolated from soil-dwelling larvae of *Wiseana cervinata* (Lepidoptera) (Fowler and Robertson, 1972; Ward and Kalmakoff, 1987) were also found infecting sympatric larvae of *Eudonia* (*Witlesia*) *sabulosella* (Lepidoptera) and *Opogonia* sp. (Coleoptera) in New Zealand (Williams and Cory, 1994; Webby and Kalmakoff, 1998). Second,

strains of IIV-6 have been reported naturally infecting lepidopteran larvae of *C. suppressalis* and *S. frugiperda* (Williams and Hernández, 2006) and commercial colonies of the crickets *Gryllus bimaculatus, G. campestris* and *Acheta domesticus* (Kleespies *et al.*, 1999; Just and Essbauer, 2001). A record of IIV-6 naturally infecting whiteflies (Homoptera) requires confirmation (Hunter *et al.*, 2001a). Lastly, the woodlouse virus IIV-31 naturally infects at least four and probably more species of terrestrial isopod (Crustacea), a mermithid nematode (Hess and Poinar, 1985) and a closely related strain has been isolated from larvae of the soil-dwelling beetle *Popillia japonica*, possibly via the consumption of infected isopods by the beetle (Lacey and Adams, 1994; Webby and Kalmakoff, 1998).

Single host species may also be infected by different IIVs in different locations or at different times. A population of blackfly larvae (*S. variegatum*) was infected by three different IIVs in different months and at different sites along the River Ystwyth in Wales (U.K.), which lead to speculation concerning the potential role of alternative hosts in the lotic environment for the survival and transmission of each of these viruses (Williams, 1995).

Finally, IIV-6 can productively infect reptile cells *in vitro* (McIntosh and Kimura, 1974) and there is evidence that it may be able to infect reptiles (Just *et al.*, 2001), but not amphibians (Ohba and Aizawa, 1982). A chameleon believed to have consumed infected crickets died and post-mortem tissue samples were used to inoculate two reptilian cell lines that subsequently developed cytopathic effects. Injection of the cell culture supernatant resulted in patent disease in crickets, and the sequence of a PCR product from inoculated crickets was identical to the IIV-6 strain sequence reported from commercially reared crickets (Weinmann *et al.*, 2007). However, it remains to be seen whether the virus is capable of replication in the chameleon host and whether transmission occurs in natural reptile populations that prey on infected insects.

Transmission

In general, the mechanisms and route of transmission of these viruses are poorly understood. As stated previously, transmission is highly dependent on the route of infection because of the marked differences in IIV infectivity by ingestion compared to intrahaemocoelic injection. The reasons for the low *per os* infectivity may reside in the virion's susceptibility to host digestive enzymes. EM studies have shown that virions of IIV-3 are degraded shortly after entering the mosquito larval midgut (Stoltz and Summers, 1971), so that infection of midgut cells may only be possible at the anterior section of the midgut where the newly formed peritrophic matrix is relatively thin or fragile (Hall and Anthony, 1971; Hembree and Anthony, 1980), although this has yet to be demonstrated.

Mixing virus inoculum with silicon carbide fibres or ground sand has been shown to result in significant increases in the prevalence of infection of mosquito larvae, presumably due to physical damage to the peritrophic matrix by these substances that facilitated access to the midgut cells by virions (Undeen and Fukuda, 1994; Marina *et al.*, 1999). In contrast, mixtures of virus with a stilbene derived optical brightener, known to degrade the chitin structure of the peritrophic matrix, or small silicon carbide grains, did not result in a significant increase in infection of mosquito larvae (Marina *et al.*, 2003a).

It is clear that cannibalism or predation of infected individuals, involving the consumption of very large quantities of virus, is a viable route of transmission in isopods (Federici, 1984), tipulid larvae (Carter, 1973b), caterpillars (Williams and Hernández, 2006), mosquitoes (Linley and Nielsen, 1968a), and crickets (Fowler, 1989; Kleespies *et al.*, 1999). Similarly, density-modulated aggressive interactions, both intra and inter-specific, influence transmission rates in isopods (Grosholz, 1992, 1993) and mosquitoes (Marina *et al.*, 2005), presumably as wounding compromises the integrity of the cuticle and allows the entry of virions into the injured host. Rainfall and soil moisture positively influence the prevalence of infection in both isopods and tipulid larvae (Ricou, 1975; Grosholz, 1993). Few studies have considered the efficacy of transmission from alternative sources of IIV inoculum in the environment. Patently infected insects disseminate viable virions in their faeces but at concentrations insufficient to result in *per*

os transmission (Carter, 1973a; Ohba, 1975; Williams and Hernandez, 2006).

Diseased insects are reported to be more sluggish than healthy conspecifics but may continue to live for several weeks or months (Hama, 1968; Linley and Nielsen, 1968a; Sikorowski and Tyson, 1984; Poprawski and Yule, 1990). This sluggish response means that infected hosts are more prone to become the victims of cannibals than their healthy counterparts (Kleespies *et al.*, 1999; Williams and Hernández, 2006). In addition, infected soil-dwelling larvae have been reported to move upwards, close to the soil surface prior to death, where the probability of transmission by cannibalism or predation may be enhanced (Fowler and Robertson, 1972).

IIV-3 survives in populations of the mosquito *Ochlerotatus taeniorhynchus* by alternating cycles of horizontal and vertical transmission. Infected larvae tend to die in the final instar and are cannibalized by early and late instar conspecifics. Individuals that become infected in the early instars develop patent disease and die prior to pupation whereas individuals that acquire infection in the late instars pupate and emerge as adults. A significant proportion of the adult females transmit the infection to their progeny. These progeny develop normally until the third or fourth instar when they die of patent disease, leading to a further cycle of transmission (Linley and Nielsen, 1968a,b; Woodard and Chapman, 1968; Fukuda and Clark, 1975). Serial passage and prolonged exposure experiments have indicated that the dynamics of the virus–host relationship are highly stable in laboratory systems (Woodard and Chapman, 1968; Undeen and Fukuda, 1994). Both the transmission and replication of IIV-3 were reported to be enhanced by the presence of a picornavirus that persisted as a low level inapparent infection in the laboratory mosquito colony (Wagner *et al.*, 1974). The advent of molecular techniques would now permit a thorough dissection of the transmission cycles reported in this pathosystem.

Persistence

The persistence of IIVs in the environment is not well understood. IIVs are highly sensitive to desiccation with complete loss of infectivity in dry habitats in periods of less than 24 hours (Linley and Nielsen, 1968b; Reyes *et al.*, 2004), or a few days (McLaughlin *et al.*, 1972; Grosholz, 1993). In contrast, IIVs gradually lose infectivity over periods of weeks or months when incubated in water in the laboratory (Day and Gilbert, 1967; Mullens *et al.*, 1999), or moist soil or aquatic environments (Marina *et al.*, 2000; Reyes *et al.*, 2004). IIV-6 binds strongly to certain clay minerals found in soils but not to the degree observed in the occlusion bodies of nucleopolyhedroviruses (Christian *et al.*, 2006). Virus persistence is reduced in the presence of leaf exudates on cotton (McLaughlin *et al.*, 1972), the presence of soil microbiota (Reyes *et al.*, 2004), and rapidly lost following exposure to sunlight (Linley and Nielsen, 1968b; Hernández *et al.*, 2005), or elevated temperatures (Day and Mercer, 1964; Marina *et al.*, 2000).

In addition to the persistence of IIV-3 in populations of *O. taeniorhynchus*, described above, there is steadily growing evidence that the infection strategies adopted by IIVs vary with the host-virus system. The great majority of infections of isopods and *S. frugiperda* larvae were patent and lethal (Grosholz, 1992; López *et al.*, 2002), whereas covert sublethal infections were common in laboratory studies on *Ae. aegypti* and *G. mellonella* and *H. zea* (Sikorowski and Lawrence, 1997; Marina *et al.*, 1999; Constantino *et al.*, 2001). Covert infections have also been detected in natural populations of the blackfly, *S. variegatum* and the mayfly, *E. torrentis* (Williams, 1993; Tonka and Weiser, 2000). In the case of the blackfly, the prevalence of covert infection was quantified by bioassay of larval homogenates in *G. mellonella* and confirmed by a nested PCR targeted at the MCP gene. Covert infection was highest in the spring and autumn and preceded that appearance of patent infections in the larval blackfly population (Williams, 1993, 1995). Sublethal infections would allow the virus to exploit vertical routes of transmission to host offspring, which appears common in baculoviruses (Vilaplana *et al.*, 2008), but this has not been studied in IIVs, except for IIV-3 and some evidence from aphids and weevils infected by IIV-6 in the laboratory (Hunter *et al.*, 2001b, 2003).

Dispersal

The mechanisms by which IIVs achieve dispersal and factors that determine the rate of that dispersal are largely speculative. It is likely that the flights of sublethally infected adult insects in search of oviposition sites are a major route of virus dispersal to new habitats, whereas infected aquatic insects in rivers may disperse downstream by drifting. The incidence of disease in terrestrial isopods was highest during the wetter months of the year and was positively correlated with their tendency to disperse and negatively correlated with the distance between suitable patches of habitat (Grosholz, 1993). Endoparasitic wasps were capable of disseminating virus between *S. frugiperda* larvae on adjacent plants in field cages (López *et al.*, 2002), and potentially could disperse the virus over much larger distances in their search for suitable hosts.

Genetic diversity in IIV populations

The factors that generate and maintain genetic diversity in insect virus populations, particularly in baculoviruses, have become the subject of considerable interest over the past decade, not simply due to an increasing awareness of the prevalence and magnitude of this diversity (Graham *et al.*, 2004; Cory *et al.*, 2005), but also because of the implications that this has on virus phenotype (Hodgson *et al.*, 2004), interactions between different genotypes (López-Ferber *et al.*, 2003; Simón *et al.*, 2008), and the efficacy of virus-based insecticides (Muñoz and Caballero, 2000). In the context of IIVs, high levels of genetic variation have been observed in strains isolated from blackflies and *S. frugiperda* larvae. In blackflies collected from a river in Wales, each host larva appeared to be infected by a slightly different strain of IIV-22, as revealed by restriction endonuclease analyses (Williams and Cory, 1993; Williams, 1995). Similarly, almost all the diseased fall armyworm larvae collected from different maize plants in a field in southern Mexico were found to be infected with slightly different strains of an IIV-6 like virus, while one strain was found to be present in several different individuals (N. Hernández and T. Williams, unpublished data). The mechanisms generating this variation and the selective advantages that may, or may not, accompany it have not been the subject of study, although similar heterogeneity has been reported in iridovirus pathogens of fish (Goldberg *et al.*, 2003), suggesting that variation may be an intrinsic component in the replication or infection strategies of this family of viruses.

Concluding remarks

Iridoviruses have provided many surprises over the years, starting with the observation of a cytoplasmic virus that caused iridescence in *Tipula* larvae (Xeros, 1954) and the subsequent observations on massive replication and the Bragg diffraction of light by viral arrays (Williams and Smith, 1957). The circular permutation and terminal redundancy of the genome was also a surprise in an animal virus (Murti *et al.*, 1985). Now, advances in nanotechnology are opening new approaches to the study of these unusual viruses. Of particular interest is how the biophysical properties of the virus, such as iridescence, can be used to develop new biomaterials. Recent studies have shown that the regular and repetitive structures of virus particle biocolloids offer advantages over other colloidal materials, with viruses used to construct multilayered polymers (Yoo *et al.*, 2006), or electronic materials (Tseng *et al.*, 2006).

IIVs offer a number of properties for biomaterial construction. The 140–180 nm iridovirus virion is large for a virus and it is this size, combined with a regular array of particles providing spaced refractive centres, which brings Bragg refraction of light into the visible spectrum. This opens the possibility of developing novel photonic materials from IIVs. For example, IIV-9 has been assembled into crystals with defined optical properties that are determined by the hydration state of the crystal affecting interparticle spacing (Juhl *et al.*, 2006). Furthermore, these particles have a range of chemical functionalities on their surface from the constituent amino acids and have been used as biotemplates to build complete metal nanoshells with defined sizes (Radloff *et al.*, 2005). Biophysical analysis of IIV virions using Brillouin light scattering has shown that the virions are surprisingly hard with the DNA core dominating the rigidity, reflecting a high compression state of the genome within the core (Hartschuh *et al.*, 2008). These studies indicate that IIVs have potential as alternatives

to classic colloidal materials; they have low surface charges and multifunctionality that can be exploited in materials synthesis and prospective new technological applications.

The sequencing of additional IIV genomes will provide important information on the relationships among these viruses and to other virus families. At present, the analysis of the IIV-3 and IIV-6 genomes has supported the concept that these are very different viruses from different genera, yet MCP sequence analysis suggests that IIV-3 may be more similar to other members of the *Iridovirus* genus than IIV-6. The upcoming analysis of IIV-9 should help resolve this issue. Moreover, additional genome sequencing is urgently needed to support virus species definitions in the invertebrate genera.

The majority of IIV genes are of unknown function and even those that show domain homology to known proteins are largely based on theoretical activities with undefined roles in virus replication. Analysis of the viral proteome and its interaction with the host is an area that deserves considerable attention. Molecular tools for investigative genome manipulation are now widely employed and the application of a genetic modification system and the use of antisense or RNAi technologies offer a wealth of possibilities to improve our understanding of IIV infection and replication strategies.

IIVs naturally infect major insect pests and medically important vectors, but are not considered useful as biocontrol agents due to the often low prevalence of patent infections and their broad host range. However, IIVs have the potential to generate considerable scientific interest due to their potential biotechnological applications (Bilimoria, 2009), and their impact as pathogens of economically important invertebrates such as mopane worms (*Gonimbrasia belina*, Lepidoptera) that are a major food industry in southern Africa, and possibly oyster populations that periodically suffer putative iridovirus disease, although the proximity of the relationship between the iridoviruses of molluscs and the IIVs is unknown. Despite the enthusiasm of a small number of researchers, major advances in the study of these viruses will require a fundamental shift in our perception of the importance of IIVs as pathogens, or their potential applications in biotechnology or materials science.

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