ADVANCES IN VIRUS RESEARCH, VOL 65

A DECADE OF ADVANCES IN IRIDOVIRUS RESEARCH

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0065-3527/05 $35.00
DOI: 10.1016/S0065-3527(05)65006-3

I. Introduction: Impact of Iridoviruses on the Health of Ectothermic Vertebrates

The serendipitous discovery of *Frog virus 3* (FV-3), the best characterized member and type species of the family *Iridoviridae*, resulted from attempts by Allan Granoff and his coworkers to identify cell lines that would support the *in vitro* growth of Lucke herpesvirus (Granoff, 1984; Granoff *et al.*, 1966). This amphibian herpesvirus was causally connected with tumor development in frogs, but had proven intractable to propagation in cell culture. Reasoning that cultured amphibian cells might prove useful for Lucke herpesvirus propagation, primary cultures from normal and tumor-bearing kidneys were prepared from *Rana pipiens*. Surprisingly, a fraction of the primary cultures displayed cytopathic effects (CPE) consistent with viral infection, and several, likely identical, viruses were isolated. FV-3, which was derived from the kidney of a tumor-bearing frog, became the focus of early studies since it was initially thought that the virus might play a role in tumor development. While the association between FV-3 infection and tumor development turned out to be incorrect, continued study of FV-3 revealed a unique replication cycle and established iridoviruses as members of a distinct taxonomic family.

Early studies highlighted an interesting dichotomy between FV-3 growth *in vitro* and *in vivo*. Whereas infections *in vitro* occurred in a wide variety of mammalian, amphibian, and piscine cell lines and resulted in marked CPE, infections *in vivo* appeared to be sporadic, subclinical, and confined to only anurans, i.e., frogs and toads (Chinchar, 2002; Chinchar and Mao, 2000; Willis *et al.*, 1985). The observation that adult animals were relatively resistant to infection was confirmed and extended by Tweedel and Granoff (1968) who showed that adult frogs easily withstood inoculation with 10^6 plaque-forming units of FV-3, but that injection of as few as 900 plaque-forming units into frog embryos was lethal. Moreover, while localized outbreaks of FV-3-related disease were sometimes noted and virus could be isolated from cultured kidney cells, infections did not become widespread or involve large numbers of animals (Clark *et al.*, 1968; Wolf *et al.*, 1968). The clinical impression that developed from these and other studies suggested that FV-3 was neither very virulent nor a major threat to amphibian populations. This benign view of vertebrate iridoviruses persisted until the mid-1980s, when it became apparent that iridoviruses were responsible for widespread clinical illness in a variety of fish species (reviewed in Ahne *et al.*, 1997; Chinchar, 2000, 2002). Since that time, iridovirus disease has become increasingly

apparent as infections have been noted in various species of freshwater and marine fish, amphibians, and reptiles. However, while the number of reported disease outbreaks attributed to iridoviruses has increased markedly, it is not clear whether this is due to an actual increase in the prevalence of iridovirus disease, or in our collective ability to detect iridovirus infections. By 2004, iridoviruses have been linked to disease in wild and cultured frogs in Asia, North America, South America, Australia, and Europe (Cullen and Owens, 2002; Cunningham *et al.*, 1996; Wolf *et al.*, 1968; Zhang *et al.*, 2001; Zupanovic *et al.*, 1998b), salamanders in western North America (Bollinger *et al.*, 1999; Docherty *et al.*, 2003; Jancovich *et al.*, 1997), wild and cultured fish species in Asia, Australia, Europe, and North America (Ahne *et al.*, 1989, 1997; Langdon *et al.*, 1986; Nakajima *et al.*, 1998; Plumb *et al.*, 1996), and in reptile populations in Europe, North America, Africa, and Asia (Chen *et al.*, 1999; Drury *et al.*, 2002; Hyatt *et al.*, 2002; Mao *et al.*, 1997; Marschang *et al.*, 1999; Telford and Jacobson, 1993).

Most of the serious infections noted previously have been attributed to members of two genera within the family *Iridoviridae*: *Megalocytivirus* and *Ranavirus*. Megalocytiviruses (i.e., red seabream iridovirus [RSIV], rock bream iridovirus [RBIV], and infectious spleen and kidney necrosis virus [ISKNV]) infect a wide variety of marine fish in Southeast Asia, including several species that are widely used in aquaculture (Nakajima *et al.*, 1998). Outbreaks in these settings can be extensive, with mortalities approaching 100% (He *et al.*, 2000). The reasons for the explosive nature of recent outbreaks are not known, but may reflect one or more events, including the introduction of a novel pathogen into populations of susceptible fish species, ease of disease transmission among intensively cultured fish, or increased susceptibility to viral disease in immunologically stressed populations. Megalocytiviruses have been isolated from more than twenty species of marine fish and, although variously designated by the species which they infect or the disease they cause (e.g., infectious spleen and kidney necrosis virus), recent evidence suggests they are very similar (Do *et al.*, 2004, 2005). Analysis of individual viral genes (e.g., the major capsid protein [MCP] and ATPase genes) from a large number of viral isolates and whole genome analysis of three selected isolates (RSIV, RBIV, and ISKNV), indicates that these viruses share extensive sequence identity, yet differences are noted, and it is not yet clear whether these viruses represent strains/isolates of the same viral species or different species (Do *et al.*, 2004; He *et al.*, 2001; Sudthongkong *et al.*, 2002). Recent efforts have focused primarily on genetic analysis of

various megalocytivirus isolates, identification of new isolates, and development of diagnostic techniques and a viral vaccine. Little is known about the molecular events in megalocytivirus-infected cells; similarities to the replication cycle of the better characterized ranaviruses have yet to be determined.

In contrast to megalocytiviruses, members of the genus *Ranavirus* appear to be more genetically diverse and, in addition, infect cold-blooded vertebrates of three different taxonomic classes: bony fish, amphibians, and reptiles (Chinchar, 2002). While many ranaviruses share high levels of sequence similarity within the MCP gene and other viral genes, differences in host range, restriction fragment length polymorphism (RFLP), and protein profiles, and gene content suggest that they represent different viral species, rather than strains or isolates of the same viral species (Chinchar *et al.*, 2005; Hyatt *et al.*, 2000). Since the identification of epizootic hematopoietic necrosis virus (EHNV) from redfin perch (*Perca fluviatilis*) by Langdon *et al.* (1986), ranaviruses have been detected in sheatfish and catfish in Europe (Ahne *et al.*, 1997), ica (Plumb *et al.*, 1996), ornamental fish imported into the United States from Southeast Asia (Hedrick and McDowell, 1995), frogs on all continents except Africa and Antarctica (most likely for lack of study in the former and lack of hosts in the latter), and also in salamanders, turtles, and snakes (Bollinger *et al.*, 1999; Hyatt *et al.*, 2002; Jancovich *et al.*, 1997; Marschang *et al.*, 1999). In a few cases it appears that viruses infecting animals of one taxonomic class, e.g., fish, also infect animals of a different taxonomic class, e.g., amphibians (Mao *et al.*, 1999a; Moody and Owens, 1994). Whether cross-class infections are common in nature is not known, although the isolation of strains of FV-3 from fish and frogs indicates that interclass transmission is possible. Such events may explain the sudden appearance of disease in previously healthy populations.

The third genus of vertebrate iridoviruses, *Lymphocystivirus*, has been known by the disease it causes since the early 1900s, and was the first iridovirus genome to be sequenced (Tidona and Darai, 1997). However, because of its inability to be grown easily in culture, its replication cycle has not been studied. Infection with lymphocystis disease virus (LCDV) leads to the development of wart-like lesions, generally on the external surface of infected fish (Weissenberg, 1965). However, unlike papilloma virus infections that involve cellular hyperplasia, LCDV results in the massive enlargement of individual epidermal cells. Infections are not believed to be fatal, but large numbers of wart-like lesions may impair the mobility and feeding of infected fish and indirectly contribute to morbidity. Collectively, vertebrate

iridoviruses are a cause of localized die-offs in wild and farmed fish, reptile, and amphibian species, and are recognized as key factors in wildlife disease throughout the world (Daszak *et al.*, 1999, 2003).

II. Taxonomy

A. Definition of the Family Iridoviridae

Iridoviruses are large, icosahedral viruses with a linear, double-stranded DNA genome (Chinchar *et al.*, 2005) (Table I). Unlike other DNA viruses, viral particles can be either enveloped or nonenveloped, depending upon whether they are released from the cell by lysis, or bud from the plasma membrane. Although both enveloped and naked virions are infectious, the specific infectivity of the former is higher, suggesting that one or more viral envelope proteins play an important role in virion entry. In addition to the outer envelope found in viruses that bud from the plasma membrane, iridoviruses also possess a lipid membrane that lies between the viral DNA core and the capsid. Iridovirus infections result in the appearance within the cytoplasm of large, morphologically distinct viral assembly sites (AS). AS serve as a concentration point for viral protein and DNA and are the site of virion assembly. Mature virions accumulate within the cytoplasm in large paracrystalline arrays or bud from the plasma membrane. The appearance within the cytoplasm of arrays of icosahedral particles usually 120–200 nm in diameter is pathognomic for iridovirus infections. LCDV and white sturgeon iridovirus (WSIV) infections may involve much larger particles, exceeding 300 nm diameter (Adkison *et al.*, 1998; Madeley *et al.*, 1978; Paperna *et al.*, 2001).

In addition to their distinctive size and cytoplasmic location, iridoviruses are distinguished from other virus families by their genomic organization. The iridovirus genome is circularly permuted and terminally redundant, and the unique region ranges in size from ~100 kbp for ranaviruses and megalocytiviruses, to ~200 kbp for lymphocystiviruses and iridoviruses (Goorha and Murti, 1982). The terminal repetition adds another 10–30% to the size of the genome.

B. Features Distinguishing the Genera

Members of the family *Iridoviridae* infect only cold-blooded vertebrates (bony fish, amphibians, and reptiles) or invertebrates (primarily insects), but also crustaceans and mollusks (see Table II). Aside from

TABLE I
TAXONOMY OF IRIDOVIRUSES

Category	Designation	Distinguishing criteria
Family	***Iridoviridae***	Naked or enveloped icosahedral virions, 120–300 nm in diameter
		Genome is a single molecule of linear, dsDNA (102 – 212 kbp)*
		Genome is terminally redundant and circularly permuted
		Virions assemble and accumulate within the cytoplasm
Genus	***Ranavirus***	**Genome:** DNA is methylated†; ∼105 kbp
		GC content: 49–55%
		Virion diameter: ∼150 nm
		Host species: bony fish, amphibians, and reptiles worldwide
		Clinical illness: Systemic infection involving multiple internal organs; infections range from subclinical to fulminant with mortalities approaching 100%
	Lymphocystivirus	**Genome:** DNA is methylated; 103–186 kbp
		GC content: 27–29%
		Virion diameter: 200–300 nm; may possess a fibril-like fringe
		Host species: Marine and freshwater fish worldwide.
		Clinical illness: Wart-like, primarily external, lesions. Infected cells undergo massive enlargement
	Megalocytivirus	**Genome:** DNA is methylated; 105–118 kbp
		GC content: 53–55%
		Virion diameter: ∼150 nm
		Host species: marine fish in SE Asia.
		Clinical illness: Systemic infection involving multiple internal organs; mortality approaching 100%
	Iridovirus	**Genome:** DNA is not methylated (no viral DNA methyltransferase); 212 kbp
		GC content: 27%
		Virion diameter: 120–130 nm
		Host species: Insects, crustaceans, possibly mollusks

(*continues*)

TABLE I (*continued*)

Category	Designation	Distinguishing criteria
		Clinical illness: Massive levels of virus replication result in a change in color of infected insects; covert infections may be common
	Chloriridovirus	**Genome:** DNA is not methylated; ~135 kbp
		GC content: 54%
		Viirion diameter: ~180 nm
		Host species: Mosquitoes and midges (Diptera)
		Clinical illness: Massive levels of virus replication result in a change in color of infected insects; covert infections may be common

*Genome size indicates unique region and does not include the terminal repeats that add an extra 10–30%.

†Singapore grouper iridovirus (SGIV) appears to lack a DNA methyltransferase. As a result, it is the only known vertebrate iridovirus that lacks a methylated genome.

this difference in host range, one nearly universal feature distinguishing the genera infecting vertebrates (*Megalocytivirus, Ranavirus, Lymphocystivirus*) from those infecting invertebrates (*Iridovirus, Chloriridovirus*) is the presence of a DNA methyltransferase in the former (Willis and Granoff, 1980; Willis *et al.*, 1984). The viral DNA methyltransferase targets cytosine residues within the sequence CpG, and as a result, ~20% of cytosines within the viral genomic are methylated (Willis and Granoff, 1980). While the role of DNA methylation is not clear, methylation is required for virus replication, since drugs that inhibit DNA methylation, such as azacytidine, reduce viral yields by 100-fold and markedly impair viral DNA synthesis (Goorha *et al.*, 1984). The family *Iridoviridae* is currently organized into five genera based on particle size, host range, presence of a DNA methyltransferase, GC content, similarity within the MCP gene, clinical disease, and other criteria listed in Table I (Chinchar *et al.*, 2005). Cladistic analysis generally supports division of the *Iridoviridae* family into four genera (Wang *et al.*, 2003b). Differences between the trees generated therein are due to the tree building method used (maximum parsimony) and the choice of sequences that were analyzed.

The two invertebrate genera include invertebrate iridescent viruses (IIVs) that typically infect insects in moist or aquatic habitats (Table II). The genus *Chloriridovirus* comprises a single species, *Invertebrate*

TABLE II
SPECIES, TENTATIVE SPECIES, AND STRAINS REFERRED TO IN THIS CHAPTER

Genus *Species name (type species in bold)* Names of strains (abrreviations used)	Tentative species
Iridovirus	
Invertebrate iridescent virus 1 (IIV-1)	Anticarsia gemmatalis iridescent virus (AGIV)
***Invertebrate iridescent virus 6* (IIV-6)**	Invertebrate iridescent virus 2 (IIV-2)
	Invertebrate iridescent virus 9 (IIV-9)
	Invertebrate iridescent virus 16 (IIV-16)
	Invertebrate iridescent virus 21 (IIV-21)
	Invertebrate iridescent virus 22 (IIV-22)
	Invertebrate iridescent virus 23 (IIV-23)
	Invertebrate iridescent virus 24 (IIV-24)
	Invertebrate iridescent virus 29 (IIV-29)
	Invertebrate iridescent virus 30 (IIV-30)
	Invertebrate iridescent virus 31 (IIV-31)
Chloriridovirus	
***Invertebrate iridescent virus 3* (IIV-3)**	
Ranavirus	
Ambystoma tigrinum virus (ATV)	Rana esculenta iridovirus (REIR)
Regina ranavirus (RRV)	Testudo iridovirus (THIV)
Bohle iridovirus (BIV)	Singapore grouper iridovirus (SGIV)
Epizootic haematopoietic necrosis virus (EHNV)	
European catfish virus (ECV)	
European sheatfish virus (ESV)	
***Frog virus 3* (FV-3)**	
Box turtle virus 3	
Bufo bufo United Kingdom virus	
Bufo marinus Venezuelan iridovirus 1	
Lucké triturus virus 1	
Rana temporaria United Kingdom virus	
Redwood Park virus	
Stickleback virus	
Tadpole edema virus (TEV)	
Tadpole virus 2	
Tiger frog virus (TFV)	
Tortoise virus 5 (TV-5)	

(*continues*)

TABLE II (*continued*)

Genus *Species name (type species in bold)* Names of strains (abrreviations used)	Tentative species
Santee-Cooper ranavirus (SCRV)	
Largemouth bass virus (LMBV)	
Doctor fish virus (DFV)	
Guppy virus 6 (GV-6)	
Lymphocystivirus	
***Lymphocystis disease virus 1* (LCDV-1)**	Lymphocystis disease virus 2 (LCDV-2) Lymphocystis disease virus China (LCDV-C)
Megalocytivirus	
***Infectious spleen and kidney necrosis virus* (ISKNV)**	Rock bream iridovirus (RBIV)
Red Sea bream iridovirus (RSIV)	Olive flounder iridovirus (OFIV)
Sea bass iridovirus (SBIV)	
African lampeye iridovirus (ALIV)	
Grouper sleepy disease iridovirus (GSDIV)	
Dwarf gourami iridovirus (DGIV)	
Taiwan grouper iridovirus (TGIV)	
Unclassified	
White sturgeon iridovirus (WSIV)	

iridescent virus 3 (IIV-3) from the mosquito *Ochlerotatus* (*Aedes*) *taeniorhynchus*. The wild isolate has been referred to as "regular" mosquito iridescent virus in the literature to differentiate it from a turquoise-colored laboratory strain (Wagner and Paschke 1977). The genus status is based on large particle size (~180 nm diameter), host range (restricted to mosquito and possibly midge larvae), and studies on serological relationships, RFLP and DNA hybridization (reviewed by Williams, 1996). Surprisingly, given the global importance of mosquitoes as vectors of human disease, chloriridoviruses are among the least studied members of the family. Fortunately, the IIV-3 genome has recently been sequenced and is presently being analyzed (C. Alfonso, personal comment). The results of the analyses are eagerly awaited.

The *Iridovirus* genus comprises two species: *Invertebrate iridescent virus 1* (IIV-1), and the type species of the genus *Invertebrate iridescent virus 6* (IIV-6), often referred to as Chilo iridescent virus (CIV). There are an additional 11 tentative species recognized, for which insufficient characterization information is available to determine

species status (Table II). Members of the genus are sometimes referred to as small iridescent viruses due to their particle size in thin section (120–130 nm diameter), which is smaller than the particle size of the chloriridovirus, IIV-3. There are several instances in which species and tentative species in the *Iridovirus* genus occur in the literature with host-derived names. This generates confusion, as there are clear examples of individual IIVs that infect different host species. For example, IIV-9 isolated from larvae of the moth *Wiseana cervinata* in New Zealand also naturally infects at least two other soil-dwelling insects in the same region (Williams and Cory, 1994). Originally, each of these isolates was given the host name and a type number, but this system was rationalized in the last ICTV report, and they are now united as the tentative species, invertebrate iridescent virus 9 (IIV-9). Levels of terminal redundancy in IIV genomes are typically 5–12%, depending on the method used (Delius *et al.*, 1984; Webby and Kalmakoff, 1999).

The species and tentative species within the *Iridovirus* genus can be assigned to one of three complexes based on serological findings: RFLP similarities, DNA hybridization, and partial sequence of the MCP (Webby and Kalmakoff, 1998; Williams and Cory, 1994). The largest complex comprises IIV-1 plus nine tentative species, the second comprises IIV-6 and strains thereof, and the third comprises IIV-31 from isopods and an isolate from the beetle *Popillia japonica*. The identification of these complexes has proved useful in the characterization of novel isolates (Just and Essbauer, 2001; Kinard *et al.*, 1995; Williams, 1994).

C. Features Delineating the Species

The delineation of iridovirus species is an ongoing process best described by several clear examples. Sequence analysis suggests that the genus *Megalocytivirus* may be composed either of a single virus species possessing a broad host range among marine fish, or a series of closely-related but molecularly distinct species from different hosts. For example, RBIV and ISKNV share 85 of 118 putative open reading frames (ORFs), but amino-acid identity varies among the shared genes from a high of 91–99% (53 ORFs), and 81–90% (25 ORFs), to a low of 60–80% (7 ORFs) (Do *et al.*, 2004). Recent genome sequence analysis of an Asian isolate of LCDV suggests that the genus *Lymphocystivirus* is composed of two species: a European/Atlantic Ocean species (LCDV-1) and a distinct Asian/Pacific Ocean species (LCDV-C) (Tidona and Darai, 1997; Zhang *et al.*, 2004a). LCDV-1 and LCDV-C are clearly members of the same genus, as indicated by their low GC

content (27–29%) and high sequence identity within the MCP gene (88%). In contrast, members of the *Megalocytivirus* and *Ranavirus* genera show GC contents of ~55% and share only 49–53% amino-acid identity with the MCPs of the LCDV species. However, LCDV-1 and LCDV-C can be differentiated as distinct species for several reasons. First, there is little colinearity between the genomes of LCDV-1 and LCDV-C, suggesting that they have undergone a high degree of rearrangement. Moreover, even in one of the more similar regions (nts 15,055-25,423), the eight putative ORFs only show 68% identity at the nucleotide level. In addition, LCDV-C has the lowest coding density of any iridovirus and the highest level of tandem and overlapping repeat elements. For example, within one ~500 bp region there were eight 66 bp repeats. Thus, there are likely at least two species recognized in the genus *Lymphocystivirus*.

The genus *Ranavirus* comprises six virus species based on analysis of host range, sequence identity, and protein and RFLP profiles (Hyatt *et al.*, 2000; Mao *et al.*, 1997). FV-3, EHNV, *Bohle iridovirus* (BIV), *Ambystoma tigrinum virus* (ATV), and *European catfish virus* (ECV) comprise five closely-related viral species that share over 90% sequence identity within the highly conserved MCP and other genes, but clearly differ from each other in host range and RFLP profiles. *Santee-Cooper ranavirus* (SCRV) and the recently identified Singapore grouper iridovirus (SGIV, a tentative species) are the most divergent members of the genus and show approximately 80% and 70% sequence identity within the MCP gene, respectively (Mao *et al.*, 1999b; Ting *et al.*, 2004). Moreover, preliminary data indicate that SGIV is unique among the vertebrate iridoviruses in lacking a DNA methyltransferase, and thus a methylated genome, and in possessing a purine nucleoside phosphorylase (Song *et al.*, 2004; Ting *et al.*, 2004). The absence of a DNA methyltransferase is surprising, as the presence of a methylated genome was assumed to be a hallmark characteristic of vertebrate iridoviruses. Furthermore, the earlier suggestion that the DNA methyltransferase was part of a restriction/modification system (Goorha *et al.*, 1984; Willis *et al.*, 1989) suggests that the long-standing rationale for genome methylation, namely protection of the viral genome from virus-induced attack by host nucleases, may be incorrect.

Phylogenetic analysis, based on sequence comparison of the MCP genes of 15 iridovirus species, tentative species, and strains, supports the taxonomic divisions discussed previously (Fig. 1). MCP sequence information is not yet available for any member of the genus *Chloriridovirus*, although DNA polymerase analysis of IIV-3 provisionally supports the concept of a distinct genus (Stasiak *et al.*, 2003). The four

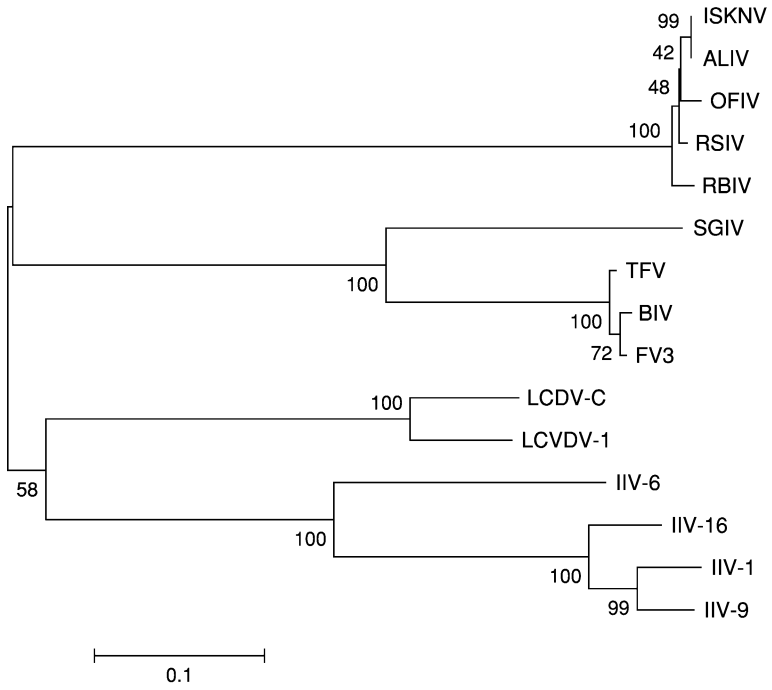

FIG 1. Phylogenetic relationships among iridoviruses. The complete inferred amino-acid sequence of the MCP of 15 iridoviruses representing four different genera were aligned using the CLUSTAL W program found within DNASTAR (Madison, WI). Based on that alignment, a phylogenetic tree was constructed using the Neighbor-Joining algorithm within Mega 2.1 (Kumar *et al.*, 2001). The tree was generated using the Poisson correction and was validated by 1000 bootstrap repetitions. Branch lengths are drawn to scale, and scale bar is shown. The numbers at each node indicate bootstrapped percentage values. The sequences used to construct the tree were obtained from GenBank: BIV (AY187046), FV-3 (U36913), TFV (AY033630); SGIV (AF364593), LCDV-1 (L63545); LCDV-C (AAS47819.1), IIV-1 (M33542); IIV-6 (AAK82135.1); IIV-9 (AF025774), IIV-16 (AF025775), ALIV (AB109368), ISKNV (AF370008), RBIV (AY533035), RSIV (AY310918), OFIV (AY661546).

genera for which extensive sequence information is available can be partitioned into four distinct taxonomic groups that share ~50% amino-acid similarity within the MCP. Within a given genus, sequence similarity is considerably higher, ranging from 64 to 100%. Moreover, the four extant genera differ markedly in their "within genus" variability. Megalocytiviruses show marked sequence similarity (97–100%) within the MCP, suggesting that they are isolates or strains of a single species.

The two species (LCDV-1, LCDV-C) of the genus *Lymphocystivirus* share 88% similarity between their MCP genes. Ranaviruses, on the other hand, show a cluster of viruses with high levels (>90%) of similarity (FV-3, ATV, BIV, TFV), which are distinguished from each other by host range and RFLP profiles, as well as two less-related members (SCRV and SGIV) with considerably greater sequence divergence. Finally, the one invertebrate iridovirus genus for which sequence data is available is comprised of a cluster of isolates with sequence similarities ranging from 64–93%. While most of these likely comprise separate viral species, those with the greatest similarity might also be considered strains of the same viral species. We recognize that phylogenetic trees generated by choosing a different gene (e.g., ATPase or DNA polymerase gene in place of the MCP gene) may yield trees with different topographies. While phylogenetic analysis of conserved viral genes such as the MCP supports the taxonomy described above, analysis of individual viral genomes highlights key similarities and differences among iridovirus genomes (Tidona *et al.*, 1998). The most accurate tree will likely be one based on whole genome sequence data (Herniou *et al.*, 2001), or a concatenated set of viral genes (Rokas *et al.*, 2003).

The two species recognized in the genus *Iridovirus*, IIV-1 and IIV-6, are quite dissimilar, and there is no doubt that they represent distinct species. Low levels of DNA annealing in solution, even under low stringency conditions, support the concept that certain members of the genus are quite distinct from one another (Webby and Kalmakoff, 1999). Perhaps the major challenge facing the classification of the IIVs is the ability to quantify heterogeneity within and between species. For the iridoviruses in general, and the IIVs in particular, species definitions are severely limited by the availability of data, particularly sequence information from a range of genes. This is probably due to the lack of interest in the IIVs as potential agents of biological control of insects, the low number of researchers with an active interest in IIVs, and difficulties in finding financial support for studies on these viruses.

For most of the tentative species presently recognized, there exists only a single isolate—an important obstacle to understanding within species variation. However, in the case of IIVs from the blackfly, *Simulium variegatum*, a number of isolates exist, each isolated from different infected individuals in Wales. Comparison of the RFLP profiles indicated that these isolates differed from one another in various degrees (Williams, 1995; Williams and Cory, 1993). Preliminary sequencing studies suggested that they could be divided into two groups: one group being IIV-22-like and another group being similar

to IIV-2 (R. Webby, personal communication). Variation among IIV isolates from a single host species was also observed in fall armyworm larvae (*Spodoptera frugiperda*) collected in southern Mexico. RFLP analysis suggested that the *S. frugiperda* isolate may be a strain of IIV-6 (N. Hernández and T. Williams, unpublished data).

The need to quantify within species heterogeneity in IIV-6 has been highlighted in several studies. Williams and Cory (1994) noted that there were at least two strains of IIV-6 being used in laboratories in different parts of the world. The isolate that has been completely sequenced by Jakob *et al.* (2001) differs from the isolates used in New Zealand, Australia, and the USA. Recently reported isolates from crickets (Just and Essbauer, 2001; Kleespies *et al.*, 1999) appear to be strains of IIV-6 that share an average of 95.2% sequence identity in a selection of seven proteins, including the MCP (Jakob *et al.*, 2002). Moreover, IIV-21 and IIV-28 may have been contaminated by IIV-6 at some point, because they were originally reported as being distinct isolates, but now are almost identical to the IIV-6 isolate from Australia and New Zealand (Webby and Kalmakoff, 1998).

As indicated above, the task of defining virus species is not straightforward. The International Committee on Taxonomy of Viruses (ICTV) defines a virus species as "a polythetic class … constituting a replicating lineage and occupying a particular ecological niche," but applying that definition to real virus populations is problematic (Van Regenmortel, 2000). For example, there are no accepted cut-off points for sequence similarity or RFLP identity that unambiguously define the boundaries between genera, species, and strains. Thus, virus species represent a class in which the presence or absence of no single character is sufficient to include (or exclude) membership in the group. Thus, ranavirus species are defined by a collection of sequence data, RFLP and protein profiles, host range, and physical characteristics. While these taxonomic distinctions may seem purely academic, they have potentially important commercial implications. For instance, if the ranavirus EHNV is strictly defined based on host species, RFLP profile, protein profile, and sequence analysis, then fish shipped from an EHNV-positive region, such as Australia, into an EHNV-free zone, such as North America, must be certified free of virus. However, if the species definition is more loosely applied, for example, if all ranaviruses with MCP sequence identity greater than 90% are considered to be members of the same virus species, then shipment of fish from Australia into the United States would not require certification, since the definition would include FV-3 and ATV that are already present in North America. Regardless of whether a given virus is classified as

a distinct species, or as a strain of an existing species, there is a critical need for diagnostic tests to distinguish between relatively innocuous iridoviruses and those responsible for mortal disease. Development of these tests is discussed later.

D. Relationships with Other Families of Large DNA Viruses

Iridoviruses are members of a monophyletic clade of large, nucleocytoplasmic DNA viruses that includes the families *Poxviridae, Phycodnaviridae* and *Asfarviridae* (Iyer *et al.*, 2001). Members of the *Iridoviridae, Asfarviridae,* and *Poxviridae* replicate in the cytoplasm or, after starting replication in the nucleus, complete it in the cytoplasm. These viruses encode their own machinery for transcription and RNA modification including subunits of RNA polymerase and transcription factors, many of which appear to have been acquired from eukaryotic host cells (Tidona and Darai, 2000). Phycodnaviruses lack RNA polymerase subunit genes, and must therefore depend on host transcriptional mechanisms in the nucleus (Van Etten *et al.*, 2002). The clade shares a total of 31 ancestral genes including three genes for structural proteins: the capsid protein and two lipid membrane localized proteins. The structure of the ancestral virus appears likely to have been icosahedral, based on gene content. A recently described giant icosahedral virus (400 nm diameter) from amoebae may also be a member of this clade as indicated by analysis of ribonuclease reductase and topoisomerase II sequences (La Scola *et al.*, 2003).

In poxviruses, the ancestral capsid protein is conserved in one domain of the D13L protein of vaccinia virus, believed to form a scaffold for the construction of viral crescents during particle assembly (Sodeik *et al.*, 1994). The complex poxvirus particle appears to represent a derived state compared to the icosahedral ancestor. Based on the analysis of LCDV-1 and IIV-6 as the only fully sequenced iridoviruses at that time, Iyer *et al.* (2001) identified two ancestral genes that had been lost by the iridoviruses (ATP dependent DNA ligase and a capping enzyme required for RNA capping in the cytoplasm). The iridoviruses differed from the ancestral group by possessing seven additional genes including Rnase III, thymidylate synthase, and a cathepsin B–like cysteine protease. In contrast, ASFV and the phycodnaviruses only acquired a single gene (lambda type restriction exonuclease), and each lost five different genes from the ancestral compliment. The cladistic analysis may now require updating given the increase in the number of iridovirus genomes that have been sequenced completely.

The architectural similarities between iridoviruses, African swine fever virus (ASFV), and phycodnaviruses extend beyond the icosahedral symmetry of the capsid, a common inner-lipid membrane and an electron dense core. High-resolution models now reveal the presence of a core shell and twin inner membranes originating from collapsed cisterna derived from the endoplasmic reticulum in ASFV (Andrés *et al.*, 1998; Rouiller *et al.*, 1998). Vaccinia virus inner lipid envelopes are derived from an intracellular compartment between the endoplasmic reticulum and the Golgi apparatus, a process apparently facilitated by vimentin intermediate filaments concentrated in the viral factories (Risco *et al.*, 2002; Schmelz *et al.*, 1994). These observations suggest that the hypothesis of *de novo* synthesis of the iridovirus lipid component proposed during early electron microscope studies (Stoltz, 1971) should be re-examined. Phycodnaviruses have a less prominent inner lipid membrane, a right-handed (dextro) skewed capsid lattice comprising doughnut-shaped capsomers with a smooth outer surface, and lack the fibrilar structures seen in iridoviruses (Nandhagopal *et al.*, 2002; Yan *et al.*, 2000). Structural similarities between iridoviruses and the allantoid particles of ascoviruses are not immediately apparent although molecular evidence indicates clear relationships between these viruses.

Sequence comparisons of the virus-encoded δ DNA polymerase indicated putative evolutionary relationships among these viruses and ascoviruses of lepidopteran insects (Knopf, 1998; Stasiak *et al.*, 2000) (Fig. 2A). The relationship between iridoviruses and ascoviruses appears particularly close. The genome of the ascovirus DpAV-4 was found to be circular, contained large (1–3 kbp) interspersed repeats, and 76% of deoxycytidines were methylated. Examination of dinucleotide frequency ratios indicated that methylation occurred mostly at CpT and CpC dinucleotides. In contrast, the linear but circularly permuted genomes of IIV-3, IIV-6, and IIV-31 lack large regions of repetitive DNA and possess very low levels of methylation, less than 5% (Bigot *et al.*, 2000; Cheng *et al.*, 1999). The IIVs were most likely methylated at CpT residues, rather than the CpG residues targeted by vertebrate iridoviruses. Homologs to about 40% of the proteins encoded by *Spodoptera frugiperda ascovirus 1a* are found in IIV-6 with lower percentages seen among vertebrate iridoviruses, phycodnaviruses and ASFV (Stasiak *et al.*, 2003). Analysis of the major capsid protein revealed seven conserved domains that were used for alignment, resulting in a tree very similar to that generated by analysis of the DNA polymerase (Fig. 2B). Additional analyses of thymidine kinase, ATPase III and several other proteins yielded similar results

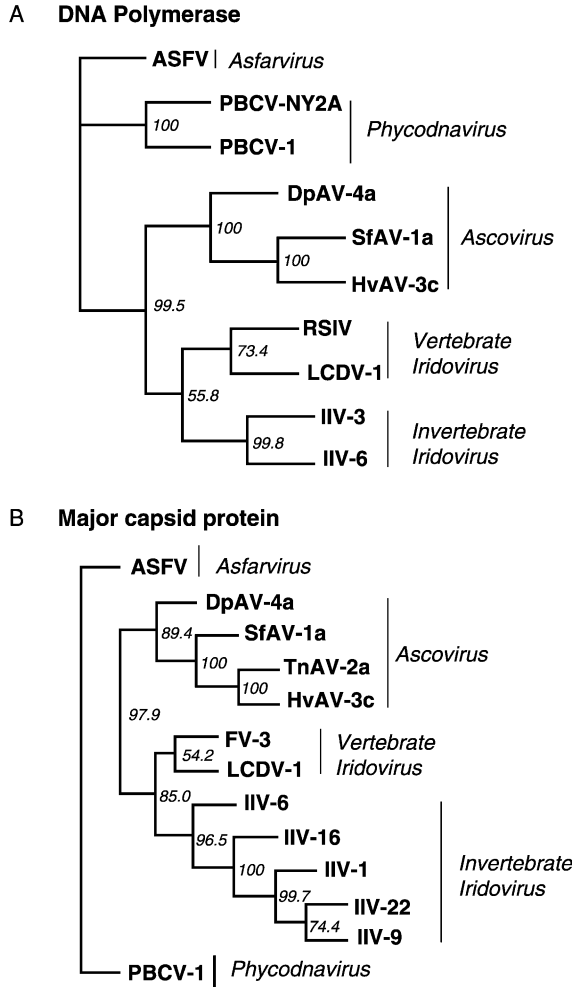

FIG 2. Consensus trees resulting from phylogenetic analyses of (A) δ DNA polymerase and (B) major capsid protein from one asfarvirus, *African swine fever virus* (ASFV); two phycodnaviruses, *Paramecium bursaria Chlorella virus 1* and *NY2A* (PBCV-1, PBCV-NY2A); four ascoviruses *Spodoptera frugiperda ascovirus 1a* (SfAV-1a), *Trichoplusia ni ascovirus 2a* (TnAV-2a) *Heliothis virescens ascovirus 3c* (HvAV-3c), *Diadromus pulchellus ascovirus 4a* (DpAV-4a); three vertebrate iridoviruses, *Frog virus 3* (FV-3), *Lymphocystis disease virus 1* (LCDV-1), Red sea bream iridovirus (RSIV); and six invertebrate iridescent viruses (IIV-1, IIV-3, IIV-6, IIV-9, IIV-16, IIV-22). Consensus trees based on parsimony procedure, numbers at each node indicate bootstrapped percentage values from 1000 repetitions. Modified with permission from Federici and Bigot (2003).

(Tidona *et al.*, 1998; Zhao and Cui, 2003). Multiple phylogenetic trees generated using different viral genes supported the conclusion that ascoviruses are more closely related to invertebrate iridoviruses than to vertebrate iridoviruses (Federici and Bigot, 2003; Stasiak *et al.*, 2003). Moreover, similarities in virus transmission support that view. Like insect iridoviruses, ascoviruses have very low *per os* infectivity, but are highly infectious by injection and depend on parasitoid wasps for transmission (Govindarajan and Federici, 1990). The recent observation of parasitoid mediated transmission of an IIV of Lepidoptera (López *et al.*, 2002) lends additional support to the hypothesis that ascoviruses evolved from invertebrate iridoviruses.

III. Genomic Studies

A. Genetic Content

Currently, nine iridovirus genomes have been sequenced in their entirety: IIV-6 (Jakob *et al.*, 2001), LCDV-1 (Tidona and Darai, 1997), LCDV-C (Zhang *et al.*, 2004a), TFV (He *et al.*, 2002), FV-3 (Tan *et al.*, 2004), ATV (Jancovich *et al.*, 2003), ISKNV (He *et al.*, 2001), RBIV (Do *et al.*, 2004), and SGIV (Song *et al.*, 2004) (Table III). Some of these viruses likely comprise distinct species within the same genera (e.g., LCDV and LCDV-C; ATV, SGIV, and FV-3), while others appear to be isolates of the same viral species (FV-3 and TFV; ISKNV, RBIV, and SGIV). Analysis of iridovirus genomes indicates that putative ORFs are closely packed, lack introns, and are generally nonoverlapping. Moreover, the few viral genes for which a 3′ terminus has been determined display terminal hairpins (i.e., an intragenic palindrome) that may play a role in transcriptional termination or in mRNA stability (Kaur *et al.*, 1995; Rohozinski and Goorha, 1992). Repeat regions and palindromes appear to be very common among iridoviruses. ATV contains 76 copies of a 14 bp palindrome while the closely-related TFV genome contains 52 copies and iridoviruses from other genera, LCDV, IIV-6, and ISKNV display three, one, and no copies of the same repeat, respectively (Jancovich *et al.*, 2003). The function of repeat regions is unknown. However, since they are often found within intergenic regions, they might play a role in viral gene transcription as initiation or termination signals. Alternatively, repeat regions through homologous pairing may be responsible for the high level of recombination seen among iridoviruses.

TABLE III
COMPARISON OF IRIDOVIRUS GENOMES

Genus	Virus	Genome size (kb)	Number of potential genes	GC content (%)	GenBank accession number
Ranavirus	FV-3	105,903	98	55	AY548484
	TFV	105,057	105	55	AF389451
	ATV	106,332	96	54	AY150217
	SGIV	140,131	162	49	AY521625
Lymphocystivirus	LCDV-1	102,653	110	29	L63515
	LCDV-C	186,247	176	27	AY380826
Megalocytivirus	ISKNV	111,362	105	55	AF371960
	RBIV	112,080	118	53	AY532606
Iridovirus	IIV-6	212,482	234	29	AF303741

Iridovirus genes can be classified into four categories: genes directly involved in virus replication, genes involved in immune evasion, genes homologous to those seen in other iridoviruses but not homologous to other genes in the database, and genes with no known homology (Table IV). Approximately 25–30% of the iridovirus genes encode polypeptides with homology to proteins of known, or presumed, replicative function, including DNA polymerase, the two largest subunits of DNA-dependent RNA polymerase II, the small and large subunits of ribonucleotide reductase, dUTPase, and DNA methyltransferase, or to known structural proteins (e.g., the major capsid protein) (Table IV). While it is reasonable to assume that viral homologs of cellular proteins of known function perform similar activities in virus replication, this hypothesis has not been verified in most situations. For example, although FV-3 encodes homologs of the two largest subunits of cellular DNA-dependent RNA polymerase II (Pol II), it has not been formally shown that they are involved in the transcription of late viral mRNA. Moreover, experience in other viral systems suggests that sequence homology does not always correlate with functional homology (Caposio *et al.*, 2004; Lembo *et al.*, 2004). Alternatively, some viral genes (e.g., those involved in the salvage pathway) may not be needed in all cell types and thus are dispensable for replication in those cells.

Among the 85 genes common to ISKNV and RBIV, 54 genes differ due to the insertion or deletion of amino acids within a given ORF or to alterations in the start or stop codon (Do *et al.*, 2004). Whether these

TABLE IV
CORE COMPLEMENT OF IRIDOVIRUS GENES

Category/gene product	Virus species and tentative species								
	FV-3	TFV	ATV	SGIV	LCDV-1	LCDV-C	IIV-6	ISKNV	RBIV
DNA/RNA Synthesis									
DNA polymerase	√	√	√	√	√	√	√	√	√
RNA polymerase II, α	√	√	√	√	√	√	√	√	√
RNA polymerase II, β	√	√	√	√	√	√	√	√	√
RAD2 repair enzyme	√	√	√	√	√	√		√	√
DNA methyltransferase	√	√	√		√	√		√	√
Rnase III	√	√	√	√	√	√	√	√	√
Helicase	√	√		√	√	√		√	√
Topoisomerase-like enzyme							√		
mRNA capping enzyme									√
Salvage Pathway									
Ribonucleotide reductase, α	√	√	√	√	√	√	√		
Ribonucleotide reductase, β	√	√	√	√	√	√	√	√	√
dUTPase	√	√	√	√			√		
Thymidylate synthase		√	√			√	√		
Thymidine kinase				√	√		√		
Thymidylate kinase	√		√			√	√		√
Purine nucleoside phosphorylase				√					
Immune evasion									
eIF-2α homolog	Δ*	√	√						
β-OH steroid oxidoreductase	√	√	Δ	√	√	√			
CARD-containing protein	√		√	√		√			
TNF receptor				√	√	√			
TRAF2-like protein									√
Bak-like protein				√					
Miscellaneous									
CTD-like phosphatase				√		√			
Reverse transcriptase-like						√			
ATP-dependent protease	√								
Inhibitor of apoptosis							√		
PCNA	√		√		√	√	√		
Major capsid protein	√	√	√	√	√	√	√	√	√

*Δ, gene present in a truncated form.

genetic changes are tolerated because they have little effect on protein function, or whether they reflect adaptation to a specific host species is not known. They are not, however, confined just to megalocytiviruses. Among the genomes of ATV, TFV, and FV-3, most viral genes show >95% sequence identity. However, inspection of those few genes that show <80% identity detects the presence of not only single amino-acid substitutions, most likely due to point mutation, but also the insertion or deletion of small blocks of amino acids. Interestingly, in some cases the indel occurs within a region of repeated amino-acid motifs. For example, at the C-terminus of ATV ORF 15L, there are 13 repeats of the sequence [Q_nRPV] where n = 4 – 8, whereas within the corresponding ORFs of TFV and FV-3 there are six and nine copies, respectively.

While most viral genes with homology to known cellular genes are involved in DNA and RNA metabolism, a smaller number, similar to the poxvirus immunomodulatory genes, might play roles in evading host immune responses (Alcami and Koszinowski, 2000; Johnston and McFadden, 2003; Nash *et al.*, 1999). For example, ranaviruses encode genes with homology to a β hydroxysteroid oxidoreductase (βSOR), the α subunit of eukaryotic initiation factor 2 (eIF-2α), and a caspase recruitment domain CARD-containing protein (vCOP) (Essbauer *et al.*, 2001; Jancovich *et al.*, 2003; Tan *et al.*, 2004). βSOR may be involved in steroid metabolism and regulate immune responses (Alcami and Koszinowski, 2000). The viral homolog of eIF-2α (vIF2α) may function in maintaining viral protein synthesis in infected cells by serving as a decoy for activated protein kinase R (PKR), an interferon-induced, dsRNA-activated kinase that phosphorylates eIF-2α and thus blocks translational initiation (Beattie *et al.*, 1991; Kawagishi-Kobayashi *et al.*, 1997; Langland and Jacobs, 2002). Finally, vCOP may modulate caspase activation and thus control the induction of apoptosis or regulate the inflammatory response (Bouchier-Hayes and Martin, 2002). Additional immune evasion genes (e.g., TNF receptor-like, Bak-like, and TRAF2-like proteins, as well as an inhibitor of apoptosis [IAP] protein) have also been detected in some iridoviruses (Table III). However, for the most part, gene identities have been assigned based on BLAST analysis, so caution must be exercised in the assignment of functions to these putative ORFs.

In addition to the abovementioned proteins, for which a function is inferred by homology, more than half of the iridovirus ORFs encode putative proteins that lack significant homology to known cellular proteins in the current databases. Surprisingly, despite the lack of homology to known nonviral proteins, the vast majority of these are

conserved among all iridoviruses, suggesting that they likely play key roles in viral replication, whether they function directly in virus replication by acting as transcription factors, assembly proteins, or structural components, or whether they downregulate immune responses by interfering with IFN, TNF, MHC, STAT, or other immune-related proteins, remains to be seen.

Viral genes involved with nucleic acid metabolism generally show marked sequence similarity compared to the corresponding genes of higher and lower vertebrates. For example, the identity/similarity between FV-3 Pol II α and the largest subunit of human Pol II is 28/45% over 707 amino acids. Similarities between cytokine genes of ectothermic vertebrates and mammals are, however, considerably lower, making it that much more difficult to decide if a given viral gene is a homolog of a cellular cytokine gene. For instance, the amino-acid sequence identity between fish TNF and interferon genes and those of mammals is only about 20% (Long *et al.*, 2004a; Zou *et al.*, 2003). If the same low level of identity exists between the TNF and interferon receptors of lower vertebrates and their mammalian counterparts, it would be extremely difficult to detect an iridovirus homolog of a cytokine receptor by comparison to its mammalian counterpart. However, the increasing availability of sequences of key immune-related genes in lower vertebrates, such as *Xenopus*, axolotl, zebrafish, etc., should greatly facilitate the identification of viral homologs. Since other large DNA viruses, including poxviruses, herpesviruses, and African swine fever virus, encode proteins that mediate viral evasion of host immunity (Johnston and McFadden, 2003; Tortorella *et al.*, 2000), it is likely that iridoviruses also contain such genes.

In IIV-6, five genes involved in DNA replication are clustered in shared orientation in one region of the genome: DNA polymerase, DNA topoisomerase II, a helicase, nucleoside triphosphatase I, and an exonuclease II (Muller *et al.*, 1999). Other notable putative genes include an NAD+ dependent DNA ligase, which contrasts with the ATP-dependent DNA ligase reported from PBCV-1 and ASFV, and a putative homolog of sillucin, a cysteine-rich peptide antibiotic. The genome contains six origins of replication (Jakob and Darai, 2002; Jakob *et al.*, 2001). The promoter regions of the MCP and DNA polymerase have recently been characterized (Nalcacioglu *et al.*, 2003). Two adjacent ORFs been detected in IIV-6 with truncated homology to the nuclear polymerizing (ADP-ribosyl) transferase from eukaryotic organisms (Berghammer *et al.*, 1999). Interestingly, the large subunit of the IIV-6 ribonucleotide reductase appears to contain an intein, a form of selfish genetic element that removes itself from the protein

posttranslationally by an autocatalytic splicing mechanism. This is the first reported insect virus intein (Pietrokovski, 1998).

Baculovirus repeated ORFs (*bro* genes), known as ALI motifs, in entomopoxviruses due to the invariant alanine-leucine-isoleucine residues (Afonso *et al.*, 1999), are a multi-gene family of unknown function. The amino terminal residues contain a DNA binding domain and are conserved across many families of viruses, whereas the C-terminus is also conserved among members of the nucleocytoplasmic large DNA virus clade. Certain BRO proteins may influence host DNA replication or transcription by regulating host chromatin structure (Zemskov *et al.*, 2000). Three *bro*-like genes have been identified in the invertebrate iridovirus IIV-6 and one in IIV-31, compared to 2–11 *bro*-like genes in ascoviruses. Apart from baculoviruses, *bro*-like genes are also present in entomopoxviruses, phycodnaviruses and a number of bacteriophages and bacterial transposons (Bideshi *et al.*, 2003). Sequence analysis indicates that *bro*-like genes in IIVs are most closely related to those of an ascovirus (DpAV-4a), although two of those in IIV-6 appear to be derived from fusion of N- and C-termini of different origins. However, *bro* diversity appears largely independent of the evolutionary history of invertebrate viruses. The *bro*-like genes are not found in viruses of vertebrates.

B. Gene Order and Elucidation of Gene Function

Despite similarity in their complement of core genes involved in DNA/RNA metabolism and other conserved functions (Table IV), iridovirus genomes show a marked lack of colinearity. Dot plot analyses, in which the nucleic acid sequence of one virus genome is compared position-by-position to that of another, indicate that strains of an iridovirus species (FV-3 vs. TFV), show a high level of sequence colinearity, whereas comparisons between species (ATV vs. TFV), show clear evidence of rearrangement. Moreover, dot plot analyses of viruses from different genera (ATV vs. IIV-6, LCDV-1, and ISKNV) showed no colinearity, indicating considerable rearrangement from the ancestral precursor (Jancovich *et al.*, 2003). Reduced colinearity likely reflects a high rate of recombination during virus replication. As the only genetic constraint may be the need to maintain the integrity of essential open reading frames, rearrangements that shuffle the genome without inactivating those genes would not be selected against. With time, those gene constellations that promote efficient growth in one or more host species would likely be selected resulting in the establishment of viral lineages with markedly different gene order, host preference, or tissue

tropism. Moreover, the high rate of recombination seen among various iridoviruses may reflect the presence of the terminal redundancies, the complex nature of concatameric DNA, or the presence of repeat elements throughout the genome.

Data from various iridovirus genome sequencing projects coupled with extensive sequencing studies involving cold-blooded vertebrates provides the basis for a systematic survey of the genetic complement of the family *Iridoviridae*. Earlier studies used temperature-sensitive and drug-resistant mutants to elucidate the function of a few replicative genes (Chinchar and Granoff, 1984, 1986). Contemporary approaches involving the use of small interfering RNA (siRNA) and antisense morpholinos (asMO) to knock down viral gene expression, and homologous recombination to knock out viral gene function, should allow us to link a specific viral gene to its function (Means *et al.*, 2003; Meister and Tuschl, 2004; Neuman *et al.*, 2004). Preliminary "proof of concept" experiments with FV-3 have successfully demonstrated that, as MO targeted against the MCP inhibited capsid protein synthesis and reduced viral yields (V. G. Chinchar, unpublished data). Continued progress using the above three methodologies should allow us to efficiently determine the function of key viral genes—surely one of the most exciting prospects in the study of these viruses.

IV. Viral Replication Strategy

A. General Features of Iridovirus Replication

Most of what is known about iridovirus replication has been elucidated using FV-3 as a model (Fig. 3). Because of this, viral replication will be described from the viewpoint of FV-3, and other iridoviruses will only be mentioned where their replication differs from that of FV-3. The virus particle binds to a common, but currently unknown, cellular receptor, since cells of mammalian, piscine, and amphibian origin are readily infected at temperatures $<32^{\circ}$C (Chinchar, 2002; Goorha and Granoff, 1979; Granoff, 1984; Williams, 1996). As both naked and enveloped virions are infectious, there are likely at least two different host receptor proteins. Following binding to host cells, enveloped virus is thought to enter via receptor mediated endocytosis, whereas naked virions uncoat by fusion at the plasma membrane. Subsequently, viral DNA cores make their way to the nucleus, where they engage in the first phase of FV-3 replication.

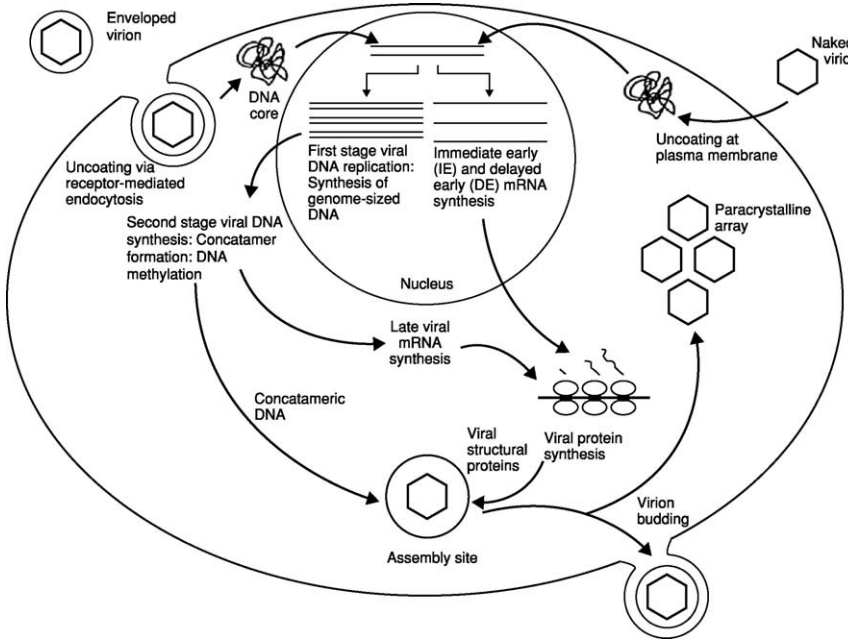

FIG 3. Iridovirus replication cycle. See text for details.

Viral gene expression begins in the nucleus, where two key events take place. In the first of these, early viral transcripts of two classes, immediate early (IE) and delayed early (DE), are synthesized using input virion DNA as a template. Early transcripts are thought to encode regulatory proteins and key catalytic enzymes such as the viral DNA polymerase and the viral homologs of cellular RNA polymerase II (Pol II). Following its translation in the cytoplasm, the viral DNA polymerase enters the nucleus where it synthesizes unit to twice unit size copies of the viral genome, representing first stage DNA synthesis (Goorha, 1982; Goorha *et al.*, 1978). Newly synthesized viral DNA is subsequently transported into the cytoplasm where further rounds of DNA replication result in the formation of large concatameric structures, representing second stage DNA synthesis (Goorha and Dixit, 1984). In addition, viral DNA is methylated in the cytoplasm through the enzymatic activity of a virus-encoded cytosine DNA methyltransferase (Willis *et al.*, 1984). Goorha *et al.* (1984) suggested that vertebrate iridoviruses contain a restriction-modification system similar to that seen in some bacteriophages. In this view, methylation protects

the viral genome from degradation by a virus-encoded endonuclease. Virions demonstrate endonuclease activity, but no endonuclease that targets unmethylated CpG sequences has been molecularly identified. While the restriction-modification scenario is plausible, the identification of at least one vertebrate virus (SGIV) that lacks a DNA methyltransferase calls this model into question (Song *et al.*, 2004). Either this virus lacks both the DNA methyltransferase and the endonuclease, or methylation plays another role in the virus life cycle. An alternative role for viral DNA methylation is suggested by the observation that bacterial DNA containing unmethylated CpG residues induces innate immunity following its interaction with toll-like receptor 9 (TLR9) (Bauer *et al.*, 2001). Perhaps FV-3 methylates its genome to prevent TLR9-mediated induction of innate immunity.

Viral DNA, presumably in its concatameric form, is found within cytoplasmic viral assembly sites (AS). However, it is not known if viral DNA is directed to developing AS, or whether viral proteins coalesce around concatameric DNA and result in AS formation. Circumstantial evidence (see following) suggests that late (L) viral mRNA synthesis takes place either within the cytoplasm of infected cells or within AS, and is catalyzed by a virus-modified cellular polymerase, or a novel viral Pol II. In support of the latter, FV-3 and other iridoviruses encode multiple subunits of Pol II, including the two largest subunits (Tan *et al.*, 2004). Clearly, the synthesis of L viral messages in the cytoplasm requires either a novel viral polymerase, or some mechanism that transports cellular Pol II from the nucleus into the cytoplasm. While both mechanisms are possible, the existence of virus-encoded subunits favors the former. Late viral transcripts encode viral structural proteins that make their way into AS and take part in virion formation. The specific steps in virion formation are not known, but it is thought to be a concentration dependent process that involves the packaging of viral DNA by a "headful" mechanism using concatameric DNA as an intermediate (Goorha and Murti, 1982). FV-3 is believed to utilize a headful mechanism, in which the unit length genome along with an additional 20–30 kbp of genomic DNA is packed into a preformed viral capsid. As a consequence of this mode of packaging, the viral genome is circularly permuted and terminally redundant. While headful packaging ensures that each virion receives at least one copy of every gene, the terminal redundancy may have an evolutionary advantage. Since 10–30% of the viral genome is effectively diploid, it is possible that mutations within one of the two genes allow the emergence of viral proteins with new functions. Newly synthesized virions exit the AS, and accumulate either in large, paracrystalline arrays

elsewhere in the cytoplasm, or bud from the plasma membrane and, in the process, acquire an envelope. Most FV-3 virions remain cell-associated, suggesting that accumulation within paracrystalline arrays is the predominant pathway. Both nonenveloped and enveloped forms of FV-3 are infectious, but the latter have a higher specific infectivity. Events in virus replication are summarized in Fig. 3.

B. RNA Synthesis: Virion-Associated and Virus-Induced Transcriptional Activators

FV-3 gene expression occurs in three coordinated phases leading to the synthesis of IE, DE, and L transcripts (Willis and Granoff, 1978; Willis *et al.*, 1977). As described above, IE and DE transcription takes place in the nucleus and are catalyzed by host Pol II, whereas L transcription is a cytoplasmic event catalyzed by a virus-modified or virus-encoded Pol II (Goorha, 1981). Biochemical studies, using inhibitors such as cycloheximide (CHX, which block translation and allow only IE viral mRNAs to be synthesized) and flurophenylalanine (which blocks the appearance of L viral mRNAs), support the notion that one or more IE proteins are required to switch on DE transcription, and that one or more DE proteins are needed to activate L mRNA synthesis (Willis *et al.*, 1977). In addition to DE proteins, full L gene expression is only seen in the presence of viral DNA replication (i.e., inhibitors of viral DNA polymerase such as phosphonoacetic acid or cytosine arabinoside, or temperature sensitive mutants that block DNA synthesis, result in markedly reduced levels of L transcripts and proteins) (Chinchar and Granoff, 1984, 1986). Events in the coordinated expression of viral genes are shown in Fig. 4. Compelling evidence supporting the role of host Pol II in the synthesis of IE transcripts comes from the observation that viral transcription is blocked in wild-type host cells by α-amanitin, an inhibitor of host Pol II, but not in cells that are α-amanitin resistant (Goorha, 1981). Data in support of the notion that IE and DE transcription takes place in the nucleus, whereas L mRNA synthesis occurs in the cytoplasm, is based on the observation that E, but not L, mRNAs possess 4–5 ^{m}6A residues/1000 nucleotides (Raghow and Granoff, 1980). Since the enzyme responsible for synthesis ^{m}6A methylation is thought to be a nuclear enzyme, it follows that only E mRNAs display this modification.

As indicated above, early viral transcription is catalyzed by host Pol II, whereas L viral mRNA synthesis is likely mediated by a virus-encoded enzyme. The largest subunit of vPol II is homologous to a wide range of eukaryotic Pol IIs and displays 28–30% sequence identity and

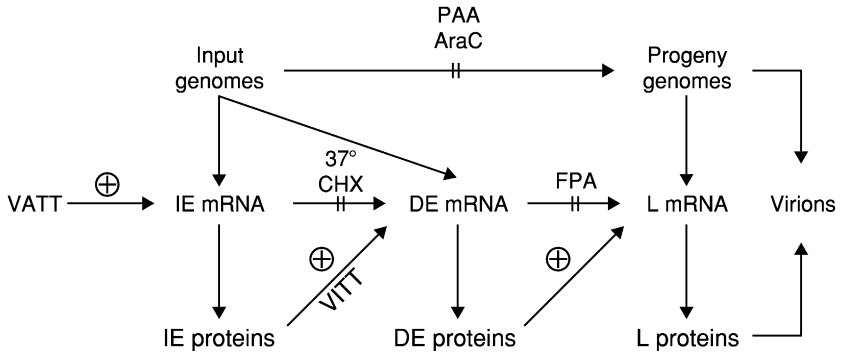

FIG 4. RNA synthesis in FV-3 infected cells. VATT and VITT are the putative transcriptional activators of IE (VATT) and DE/L (VITT) mRNA synthesis. The points at which various drugs or elevated temperatures act to block viral RNA synthesis are shown.

44–46% sequence similarity to various fungal, plant, mammalian, and arthropod Pol IIs. Despite the marked homology, the viral protein differs from host Pol II by the absence of the carboxyl-terminal domain (CTD). The CTD is composed of multiple repeats (~50 in man and mouse) of the sequence YSPTSPS that are phosphorylated by various kinases. The CTD may be viewed as a docking platform for factors that mediate transcription (Barilla *et al.*, 2001). Truncation of the CTD results in a drop in transcriptional initiation, and recent evidence suggests that factors that mediate transcriptional termination and polyadenylation bind to the CTD (Kim *et al.*, 2004; Meininghaus and Eick, 1999). As vPOL II lacks a CTD, it is anticipated that it may utilize novel mechanisms to catalyze viral RNA synthesis.

In contrast to herpes simplex virus type 1, which also uses host Pol II for its transcription, FV-3 DNA is noninfectious. However, FV-3 DNA extracted from virus particles or present within heat-inactivated virions can be nongenetically reactivated by coinfection with UV-inactivated FV-3 (Willis *et al.*, 1979). It is thought that functional proteins from UV-inactivated FV-3 drive IE transcription using functional genomic DNA as a template. Subsequently, one or more of the newly synthesized IE proteins are believed to activate DE transcription and allow for the eventual synthesis of L genes and the formation of infectious virions. Both IE and DE transcription are catalyzed by host Pol II. Furthermore, one or more DE proteins are thought to be required for L mRNA synthesis. Whether the DE protein requirement

for L mRNA synthesis reflects the need for a novel transcriptional transactivator, or whether it simply is due to the requirement for a virus-encoded RNA polymerase, is not known.

Willis and Granoff (1985) showed that a virion-associated transcriptional transactivator (VATT) is required for the synthesis of IE mRNA, whereas a newly synthesized virus-induced transcriptional transactivator (VITT) is needed to override the methylation-induced transcriptional block and synthesize DE mRNA (Willis *et al.*, 1989, 1990a,b). The molecular identities of VATT and VITT are unknown, as is their precise mechanism of action. VATT may alter the DNA template, modify cellular Pol II, or interact with cellular transcription factors and permit transcription of IE messages. Thompson *et al.* (1988) showed that a mutated IE promoter that was able to be methylated *in vitro* was still transcriptionally active, suggesting that IE genes do not contain methylatable sites in regions critical for transcription. They interpreted these results to suggest that the role of the VATT was not to override the inhibitory effect of methylation, but rather to permit IE transcription by a yet to be defined mechanism. In support of this model, a chloramphenicol acetyltransferase (CAT) reporter gene located downstream from an IE FV-3 promoter was only transcribed in FV-3 infected cells. CAT expression did not require full FV-3 gene expression as CAT mRNA synthesis was detected in cells infected in the presence of CHX, as well as in cells infected with UV-inactivated FV-3. CAT activity was, however, not seen in cells infected by heat-inactivated virus, suggesting that VATT was a heat-sensitive protein (Willis and Granoff, 1985). In contrast, a plasmid containing the CAT reporter gene downstream from a methylated adenovirus promoter (^{m}Ad-CAT) was only transcribed in productively FV-3-infected cells, suggesting that the role of the VITT is to override the inhibitory effect of a methylated promoter (Thompson *et al.*, 1986). As expected, ^{m}Ad-CAT was not expressed in cells infected with heat-inactivated or UV-inactivated FV-3, or in cells infected in the presence of CHX. Taken together, these results indicate that a virion-associated protein (VATT) is required to initiate IE transcription, while a newly synthesized viral protein (or virus-induced cellular protein, VITT), is required for DE transcription.

The patterns of transcription in IIV-6 and IIV-9 are similar to that described for FV-3. The invertebrate virus genomes are organized into distinct clusters of IE and DE genes, whereas the L genes are scattered throughout the genome (D'Costa *et al.*, 2001, 2004; McMillan and Kalmakoff, 1994). Of the 468 potential ORFs (234 of which are nonoverlapping) identified in the IIV-6 genome (Jakob *et al.*, 2001),

just 137 transcripts have been identified and classified by temporal expression (D'Costa *et al.*, 2004).

C. Inhibition of Host Macromolecular Synthesis

FV-3 infection is accompanied by a rapid and profound inhibition of host macromolecular synthesis (Goorha and Granoff, 1974; Raghow and Granoff, 1979). It is thought that shut-off of cellular transcription and translation is a direct effect of virus infection, whereas inhibition of cellular DNA synthesis is a consequence of the inhibition of RNA and protein synthesis (Willis *et al.*, 1985). In productively infected cells, the shut-off of cellular protein synthesis is accompanied by a switch to the selective synthesis of viral proteins. The transition from host to viral protein synthesis is likely the result of several interacting events: the inhibition of cellular transcription, the degradation of host messages, the synthesis of abundant amounts of highly efficient viral transcripts, and the presence of a viral homolog of eukaryotic initiation factor 2 alpha (eIF-2α) that is thought to play a role in maintaining viral translation in the face of a shut-off of host protein synthesis (Chinchar and Dholakia, 1989; Chinchar and Yu, 1990, 1992). A translational activator of L protein synthesis has been suggested based on biochemical experiments, but the putative factor has not been identified based on molecular or genetic data (Raghow and Granoff, 1983). It should be noted that the VATT is heat-sensitive, whereas the factor responsible for host translational shutoff is a heat-stable protein, as shutoff takes place not only in productively-infected cells, but in cells infected with heat- and UV-inactivated FV-3. However, the identity of the inhibitor remains unknown.

Most, but not all, ranaviruses examined to date encode a $\sim$260 amino-acid protein (vIF2α) that is homologous to eIF-2α at its amino terminus (Essbauer *et al.*, 2001; Jancovich *et al.*, 2003; Yu *et al.*, 1999). Interestingly, vaccinia virus, as well as other poxviruses, encodes a much shorter (88 amino acid) protein, designated K3L, that is also homologous to the amino terminus of eIF-2α (Kawagishi-Kobayashi *et al.*, 1997). Although sequence identity/similarity is limited, all three proteins share a highly conserved motif (KGYID[L/V]) thought to play a role in the interaction between PKR and eIF-2α. In the currently accepted model, K3L and vIF2α are thought to act as pseudosubstrates that bind activated PKR and prevent the phosphorylation, and thus inactivation, of eIF-2α. Surprisingly, ranavirus vIF2α shows no homology to eIF-2α or to any other protein in the database downstream of the KGYID motif. Whether the carboxyl-terminal two-thirds

of vIF2α plays an additional function in virus replication is not known. Surprisingly, in the FV-3 isolate sequenced by Tan *et al.* (2004), the amino-terminal two-thirds of the putative vIF2α protein has been truncated. However, despite the loss of the domain thought to be involved in PKR binding, this FV-3 isolate readily shuts off host translation and selectively synthesizes viral proteins. Since vaccinia virus has at least two proteins that prevent PKR activation, K3L that binds PKR and blocks phosphorylation of eIF-2α and E3L that binds dsRNA and inhibits PKR activation (Langland and Jacobs, 2002), it is possible that FV-3 also possesses another protein that maintains viral protein synthesis in the face of host translational shut-off.

D. Apoptosis

Ranavirus infections are accompanied by marked cytopathic effect *in vitro*. Recent work indicates that cultured cells infected with ranaviruses undergo apoptosis, as indicated by Hoechst staining, DNA laddering, TUNEL assay, and the appearance of phosphatidylserine on the outer leaflet of the plasma membrane (Chinchar *et al.*, 2003; Essbauer and Ahne, 2002; Zhang *et al.*, 2001). In addition, apoptosis is blocked by a pan-caspase inhibitor (Z-VAD-FMK), strengthening the suggestion that caspase activation played a role in this process (Chinchar *et al.*, 2003). Apoptosis has been described in GF cells infected by RSIV, and it was suggested that caspase-3 and caspase-6 may be involved in the morphological changes seen in the middle and late stages of apoptosis (Imajoh *et al.*, 2004). While these studies make a strong case for apoptosis *in vitro*, it is unclear whether cell death seen in infected animals is due to necrosis or apoptosis. A recent study with LCDV suggests that there may be differences between *in vivo* and *in vitro* responses. Hu *et al.* (2004) employed Hoechst staining, DNA laddering, and caspase activation techniques to demonstrate that LCDV infection of FG-9307 cells resulted in apoptotic death. However, since infected animals typically show wart-like growths resulting from enlargement of individual cells, there appears to be a discrepancy between cell death *in vitro* and cell fate *in vivo*. In view of that, the authors suggest that virus-host interactions *in vivo* likely play a key role in determining the mode and the timing of the apopototic response. A TNF receptor-like homolog (ORF 167L) was recently identified in LCDV with possible involvement in the apoptosis cascade (Essbauer *et al.*, 2004).

Apoptosis inhibitors that block the activation of caspases (Seshagiri and Miller, 1997) have been detected in IIV-6 (Jakob and Darai, 2002).

These inhibitors of apoptosis proteins (IAP) are characterized by the presence of a C_3HC_4 Zinc/RING finger and BIR (baculovirus inhibitor repeat) domains. The three copies of this putative gene identified in IIV-6 vary in length and show 17.5–19.5% identity and 22.9–40.6% similarity in amino-acid sequence to the IAP protein of *Cydia pomonella granulovirus,* a demonstrated functional inhibitor of apoptosis (Birnbaum *et al.*, 1994). Three additional ORFs in IIV-6 showed lower levels of homology to established *iap* genes.

V. Vertebrate Iridoviruses: Pathology and Diagnosis

Pathology associated with vertebrate iridoviruses ranges from inapparent subclinical to fulminant fatal infections. Infection of marine and freshwater fish by LCDV is distinctive, since it is accompanied by the presence of wart-like lesions on the dermal surfaces of infected fish (Weissenberg, 1965). Histological examination reveals the presence of greatly enlarged individual cells, each encapsulated by a hyaline matrix. The duration of cell growth and virus proliferation is variable and probably temperature-dependent. Lymphocystis lesions regress spontaneously and are usually not life-threatening. However, when lesions affect the eyes (Dukes and Lawler, 1975) or other internal organs (Russell, 1974), infected fish may die. Mortality may also occur under culture conditions, particularly if fish are debilitated or suffer secondary bacterial infection. In contrast, fish infected with megalocytiviruses are lethargic, have abnormal coloration of the body, petechia of the gills, and lesions involving the spleen, gills, and digestive tract (Gibson-Kueh *et al.*, 2003; He *et al.*, 2000; Jung *et al.*, 1997). RSIV, for example, causes cell hypertrophy in the spleen, heart, kidney, liver, and gills and leads to necrosis of the renal and splenic hematopoietic tissues (Nakajima *et al.*, 1998; Oshima *et al.*, 1998; Qin *et al.*, 2002). Cellular hypertrophy is accompanied by the appearance of inclusion body-bearing cells (IBC). IBC contain a unique inclusion body that may be sharply delineated from the host cell cytoplasm by a limiting membrane. The inclusions contain viral AS as well as abundant ribosomes, rough endoplasmic reticulum, and mitochondria.

Ectothermic animals infected with ranaviruses show variable pathology. LMBV infections appear to involve only the swim bladder and result in a build up of a wax-like material in the lumen of the bladder (Hanson *et al.*, 2001). Infections with EHNV, FV-3, and ATV cause systemic infections involving the liver, kidney, and gastrointestinal tract (Ahne *et al.*, 1997; Bollinger *et al.*, 1999; Jancovich *et al.*,

1997; Wolf *et al.*, 1968). Typical symptoms of infection in fish include focal or generalized necrosis of the hematopoietic tissues and other organs, hemorrhages of internal organs, and peticheal hemorrhages of the skin. Altered swimming behavior has been observed in some cases. ATV infection of salamanders causes lesions involving internal organs and also leads to skin sloughing and the development of dermal polyps (Jancovich *et al.*, 1997). BIV is highly pathogenic for tadpoles and juveniles of several species (*Limnodynastes ornatus, Litoria caerulea, L. alboguttata, Cyclorana brevipes, Pseudophryne* sp.), as well as juvenile and adult cane toads, *Bufo marinus*. Experimental challenges in juvenile frogs have resulted in high mortality (Cullen and Owens, 2002). Metamorphs and adults generally do not show overt signs of infection, although adults occasionally suffer weakness, and hemorrhages may occur. Histopathological changes include acute necrosis of hematopoietic and lymphoid tissues, as well as leucocytes in liver, kidney, and respiratory organs. Significant hemosiderosis may occur in chronically infected frogs (Cullen and Owens, 2002). BIV experimentally transmitted to barramundi fish caused focal or diffuse necrosis of the hematopoietic tissues of the kidney, spleen and the liver, resulting in 100% mortality (Moody and Owens, 1994).

Experimental infection of adult, immunocompetent *Xenopus* indicates that these animals readily clear FV-3 infection and that viral antigens are detected only transiently in the kidney. In contrast, immunocompromised adult *Xenopus* succumb to disease and show viral antigens in the kidney, liver, and spleen. *Xenopus* tadpoles are readily infected with FV-3 and show high levels of mortality (Gantress *et al.*, 2003; J. Robert, unpublished data). Histologically, there appears to be a difference between ranavirus and megalocytivirus infections. The latter are characterized by the appearance of IBCs, whereas ranavirus infections show no such structures. In place of inclusion bodies, ranavirus infections are accompanied by the presence of one or more AS within the cytoplasm. As infection progresses, AS may become quite large, but unlike inclusion bodies, they are not set apart from the cytoplasm by a limiting membrane and lack ribosomes and mitochondria. Moreover, AS appear to be surrounded by elements of the cellular cytoskeleton (Murti and Goorha, 1989, 1990; Murti *et al.*, 1988). Whether these elements contribute to the formation and integrity of the AS, or whether they are simply excluded as the AS enlarges is not known. In this respect, microtubules, intermediate filaments, and vimentin structures are integral components of viral factories in ASFV-infected cells (Heath *et al.*, 2001).

Gross lesions induced by megalocytivirus and ranavirus infections are not unique, so that definitive identification of the viral agent requires additional tests. The presence of 120–300 nm nonenveloped icosahedral particles within the cytoplasm of infected cells establishes probable infection by an iridovirus, but does not indicate either the genus or species of the infecting agent. Classical techniques of virus isolation using cell culture are laborious and require subsequent molecular analyses (Drury *et al.*, 2002). To this end, a number of PCR-based and antigen-based tests have been developed (Gould *et al.*, 1995; Marsh *et al.*, 2002; Office International des Epizooties, 2000; Oshima *et al.*, 1996, 1998; Whittington *et al.*, 1997, 1999; Zupanovic *et al.*, 1998a,b). Definitive identification of the etiological agent depends on PCR primers that amplify a specific size fragment from a known viral target, or that flank a region containing a unique DNA sequence (Chinchar and Mao, 2000; Hyatt *et al.*, 2000). PCR detection tests have frequently targeted the MCP (Bollinger *et al.*, 1999; Mao *et al.*, 1997, 1999a,b; Marsh *et al.*, 2002; Murali *et al.*, 2002), DNA methyltransferase (Mao *et al.*, 1999b), DNA polymerase (Kurita *et al.*, 1998), ATPase (Sudthongkong *et al.*, 2002), and ribonucleotide reductase genes (Oshima *et al.*, 1996, 1998), or other ORFs (Ahne *et al.*, 1998; Cullen and Owens, 2002; Gould *et al.*, 1995; Tamai *et al.*, 1997), or characteristic restriction fragments of the viral genome (Kim *et al.*, 2002; Miyata *et al.*, 1997). Nested PCR targeted at a *Pst*I fragment of RSIV appeared to offer no advantages over conventional PCR (Wang *et al.*, 2003a).

Serological tests have been developed for detecting antiranavirus antibodies (Zupanovic *et al.*, 1998a) and iridoviral antigens (Qin *et al.*, 2002; Shi *et al.*, 2003), but need to be interpreted with caution because of cross-reactivity between members of the same viral genus (Hedrick *et al.*, 1992a). Interestingly, the monomeric form of the MCP may not be the most important protein antigenically in EHNV or SGIV. Only 3 out of 20 mAbs precipitated the 50 KDa MCP of EHNV, whereas the majority of mAbs recognized polypeptides of 15 and 18 KDa (Monini and Ruggeri, 2002). Similarly, mAbs to SGIV reacted strongly with proteins of 100 and 117 KDa, but showed no affinity to the MCP (Shi *et al.*, 2003).

A number of more complex diagnostic techniques have also been developed. *In situ* hybridization targeted at MCP gene sequences has been successfully employed to identify SGIV infected tissues in formalin fixed samples of Malabar grouper (Huang *et al.*, 2004). PCR + RFLP was successfully used to differentiate between ranaviruses (Marsh *et al.*, 2002). Most recently, amplified fragment length

polymorphism (AFLP) and real-time quantitative PCR were applied to detect and differentiate between geographical isolates of LMBV (Goldberg *et al.*, 2003), and multiplex PCR has been employed for the diagnosis of different strains of RSIV using sets of primers targeted at three virus genes and a virus specific *Pst*I fragment (Jeong *et al.*, 2004). Loop-mediated isothermal amplification (LAMP) has also been shown to detect the presence of RSIV, using primers designed from the *Pst*I restriction fragment sequence (Caipang *et al.*, 2004). The LAMP procedure was ten times more sensitive than conventional PCR. A clear correlation between the number of copies of template DNA and the optical density of a magnesium pyrophosphate by-product of the reaction indicated that the LAMP technique could be used semi-quantitatively to estimate the concentration of RSIV in fish samples.

VI. Immune Responses to Vertebrate Iridoviruses

Our understanding of immune responses among lower vertebrates is incomplete, although experience with several species of bony fish and *Xenopus* indicate that fish and amphibians possess immune responses that are functionally and molecularly similar to those of higher vertebrates (DuPasquier, 2001; DuPasquier *et al.*, 1989; Miller *et al.*, 1998; Shen *et al.*, 2002). Lower vertebrates display many of the effectors associated with innate responses including antimicrobial peptides with antiviral activity (Chinchar *et al.*, 2001, 2004; Zhang *et al.*, 2004b), complement, Natural Killer cells (Hogan *et al.*, 1996), interferon (Long *et al.*, 2004a), and phagocytic cells. Amphibians and fish develop viral antibodies (Bengten *et al.*, 2000), and likely possess antigen-specific cytotoxic cells (Yoder, 2004). At the molecular level, genes for immunoglobulin, T cell receptor (TCR), and MHC class I and II have been identified (Antao *et al.*, 1999, 2001; Bengten *et al.*, 2000; Wilson *et al.*, 1998). Sequence analysis has revealed that lower vertebrates also possess antiviral cytokines such as TNF, interferon, and other immune-related molecules such as Fas Receptor and caspase 8, but the molecular identity between the genes from lower vertebrates and those from mammals is low (Long *et al.*, 2004a,b; Zou *et al.*, 2003). The low level of identity has frustrated efforts to identify CD4 and CD8 in some species (e.g., channel catfish) and interferon in amphibians. Moreover, several striking differences exist between lower and higher vertebrates. First, premetamorphic tadpoles lack MHC class I molecules and show impaired cytotoxic T cell (CTL) responses (DuPasquier

et al., 1989). Second, mammals possess five classes of immunoglobulin (IgA, IgD, IgE, IgG, and IgM), whereas channel catfish possess two (IgD and IgM), with the latter arranged as a tetramer, rather than the pentamer seen in mammals (Miller *et al.*, 1998). Third, mammalian NK cells possess two types of inhibitory receptors on their surface (Killer Cell Ig-like receptors [KIRs] and CD94/NKG2 in humans; Ly49 and CD94/NKG2 in mice) that recognize self MHC and prevent cell killing. In contrast, KIRs, Ly49-like molecules, or CD94/NKG2 have not yet been detected in fish. In their place, a large family of NITRs (novel immune-type receptors) has been identified that may serve as the functional equivalent of the inhibitory and activation receptors seen in mammals (Hawke *et al.*, 2001; Yoder *et al.*, 2004).

Little is known about the immune response in any lower vertebrate species against iridovirus infection. However, the ability to protect fish from megalocytivirus infection by vaccination suggests that immune responses play a role in protection from disease (Nakajima *et al.*, 1999). Interestingly, an iridovirus causing acute necrosis of the spleen and renal hematopoietic tissue in catfish (Pozet *et al.*, 1992) has been observed to suppress macrophage activity and lymphocyte proliferation in sheatfish cells *in vitro* (Siwicki *et al.*, 1999), although *in vivo* studies have yet to be reported. Furthermore, the widespread occurrence of subclinical infections with LMBV suggests that either innate or acquired responses control the virulence of iridovirus infections and prevent or moderate clinical disease (Hanson *et al.*, 2001). In *Xenopus*, Gantress *et al.* (2003) demonstrated that adult animals developed anti-FV-3 antibodies following intraperitoneal injection with FV-3. Furthermore, the ability of prior FV-3 infection to protect tadpoles and prevent clinical disease induced by a pathogenic ranavirus designated RGV-Z suggests that lower vertebrates can mount protective responses against iridoviruses (V. G. Chinchar and S. LaPatra, unpublished data). Whether protection involves both viral antibodies and antiviral cytotoxic T cells is presently unknown, but it is interesting to note that *Xenopus* tadpoles are much more sensitive to FV-3 infection than adults, and this may be due to the absence of MHC class I expression in premetamorphic animals (Gantress *et al.*, 2003). Somamoto *et al.* (2002) demonstrated the presence of cytotoxic T cell (CTL)-like effectors that lysed rhabdovirus-infected syngeneic crucian carp cells; however, whether anti-iridovirus CTLs are present in the various species of susceptible amphibians, reptiles, and fish remains to be determined. Hopefully, the *Xenopus*/FV-3 system will prove amenable to definitively identifying antiranavirus CTLs. If immunity to vertebrate iridovirus infection is similar to that

seen in mammals, we might expect protection to be a multi-faceted phenomenon involving antimicrobial peptides, cytokines, and NK cells acting early as a first line of defense, followed by the appearance of antiviral CTLs and antiviral antibodies. While circumstantial evidence supports a role for all of these, in no one system have all components been identified and characterized.

VII. Ecology of Vertebrate Iridoviruses

A. Infections in Natural Populations

Our understanding of the ecology of iridovirus infections is fragmentary. Two examples may serve to illustrate the state of our knowledge. First is the case of *Ambystoma tigrinum virus*. ATV is the virus responsible for localized mass mortality of various subspecies of tiger salamanders throughout western North America. ATV die-offs have been seen in manmade water holes in Arizona, farm ponds in Saskatchewan, and natural water courses in Utah and other western states (Bollinger *et al.*, 1999; Docherty *et al.*, 2003; Jancovich *et al.*, 1997). Mortality is high, and although some populations recover, others stay depressed for several years (D. Schock, personal communication). Iridoviruses are relatively stable in aquatic environments, but it is unclear whether subsequent die-offs reflect the reintroduction of the virus, the activation of latent or subclincial infections in the surviving host population, transmission from asymptomatic sympatric alternative hosts (e.g., infected frogs or fish), or persistence of the virus in the environment. It is not clear if ATV infects only salamander species or also fish and other amphibian species (Jancovich *et al.*, 2001). If these latter species can be infected, they may serve as a reservoir for the virus when transmission opportunities in salamander populations are limited (D. Schock, unpublished data). Natural modes of transmission include water-borne exposure or cannibalism of infected animals or carcasses (Jancovich *et al.*, 2001). It is hypothesized that the recrudescence of active infection, perhaps induced by spawning or other stresses, leads to the release of virus and infection of susceptible hatchlings. Moreover, the transport by recreational fishermen of ATV-infected bait salamanders may serve as an unintended but effective method of dispersing ATV throughout western North America (Jancovich *et al.*, 2005).

Second is the case of largemouth bass virus. Following its initial detection in the Santee-Cooper Reservoir (South Carolina, USA) in the

1996, LMBV has been shown to be present through the southeastern United States, as far north as Michigan, and as far west as Texas (Grizzle and Brunner, 2003; Grizzle *et al.*, 2002; Plumb *et al.*, 1996). Although LMBV is responsible for the death of adult largemouth bass (*Micropterus salmoides*), infection is not invariably fatal, as large numbers of virus-positive, subclinically-infected fish have been detected in some lakes (Hanson *et al.*, 2001; Woodland *et al.*, 2002). The reasons why LMBV infection triggers die-offs in one case and leads to subclinical infections in another are not known. Whether resistance reflects pre-existing immunity, an effective response against a new infection, or environmental insults that lead to immune suppression is not known. However, different geographical isolates of LMBV were recently shown to exhibit major differences in pathogenicity and virulence in largemouth bass juveniles; the number of fish that died from LMBV infection and the titer of virus in the survivors differed markedly according to the origin of the isolate (Goldberg *et al.*, 2003). Genotypic differences in LMBV isolates appear to have marked implications for key phenotypic characteristics. Aside from largemouth bass, other species of centrachids are susceptible to infection, but do not develop clinical signs of disease. Whether these species serve as reservoirs for LMBV is not clear. Interestingly, doctor fish virus and guppy virus 6 isolated from tropical fish imported from Southeast Asia share sequence similarity with LMBV within the MCP gene, and all are classified as strains of SCRV (Mao *et al.*, 1997, 1999b). Whether this indicates that LMBV-like viruses have a worldwide distribution or whether it suggests that the virus was introduced into North America via imported tropical fish is not known.

B. Infections in Farmed Populations

The number of reports of iridovirus infection of fish has increased rapidly over the past decade, with abundant reports from China, Japan, Korea, and Taiwan. Matsuoka *et al.* (1996) reported that systemic iridovirus disease occurred in 19 species from 3 taxonomic orders of cultured marine fish in Japan. A comparison of iridovirus isolates from long-spined sea bream (*Argyrops spinifer*), amberjack (*Seriola dumerili*), striped jack (*Caranx delicatissimus*), and albacore (*Thunnus thynnus*) indicated each was pathogenic for red sea bream (*Pagrus major*) (Nakajima and Maeno, 1998). Other economically important marine species affected by iridoviruses in this region

include sea bass, *Lateolabrax* sp. (Nakajima and Sorimachi, 1995), various species of grouper, *Epinephelus* spp. (Chou *et al.*, 1998; Chua *et al.*, 1994; Danayadol *et al.*, 1996; Murali *et al.*, 2002), red drum, *Sciaenops ocellata* (Weng *et al.*, 2002), large yellow croaker, *Larimichthys crocea* (Chen *et al.*, 2003), striped beakperch, *Oplegnathus fasciatus* (Jung and Oh, 2000) and turbot, *Scophthalmus maximus* (Shi *et al.*, 2004).

The white sturgeon iridovirus (WSIV) is a significant cause of mortality of juvenile white sturgeon (*Acipenser transmontanus*) in North America and among Russian sturgeon (*A. guldenstadi*) in northern Europe (Adkison *et al.*, 1998; Hedrick *et al.*, 1990; Watson *et al.*, 1998a). Other sturgeon species have been experimentally infected (Hedrick *et al.*, 1992b). WSIV has been detected in white sturgeon originating from wild-caught adults in California, Oregon, Washington, and Idaho (Hedrick *et al.*, 1990; LaPatra *et al.*, 1994, 1999). There is little antigenic relationship between WSIV, EHNV, or RSIV. The survival time of infected fish is negatively correlated with temperature (Watson *et al.*, 1998b). Pallid sturgeon (*Scaphirhynchus albus*) and shovelnose sturgeon (*S. platorynchus*) have suffered high levels of mortality in North and South Dakota, but the causative agent has yet to be characterized (MacConnell *et al.*, 2001).

It is unclear whether the mortality seen among cultured marine fish in Asia and among farmed frogs in China and the United States (V. G. Chinchar and S. LaPatra, unpublished data; Zhang *et al.*, 2001) is due to the introduction of virus into the affected species via an unknown animal reservoir, or represents an endemic pathogen of those species whose pathogenicity has been enhanced by environmental stress. Fish may succumb to endemic diseases, given the stress induced by intensive farming practices, as reported for WSIV infected sturgeon (Georgiadis *et al.*, 2001). Additionally, pathogens are presented with ample opportunities for transmission in high-density, immunologically compromised farmed animal populations. There is growing evidence that exposure to pesticides, increased UV irradiation, and other environmental insults can result in immune suppression that may render animals susceptible to parasite and pathogen loads that would not trigger disease in a healthy organism (Bly *et al.*, 1992; Carey *et al.*, 1999; Cheng *et al.*, 2002; Kiesecker, 2002; Quiniou *et al.*, 1998; Taylor *et al.*, 1999). Such effects may have major consequences at the population level, and much current attention is focused on the plight of amphibian populations globally (Blaustein *et al.*, 2003; Kiesecker *et al.*, 2001).

VIII. Structure of Invertebrate Iridescent Viruses

A highly detailed model of iridovirus particle structure has been defined by cryo-electron microscopy and three-dimensional image reconstruction (Yan *et al.*, 2000). Particles of IIV-6 were observed in closely packed quasi-crystalline hexagonal arrays with an interparticle distance of 4–6 nm. Particle diameter was calculated to be 162–165 nm along the two and threefold axes of symmetry, respectively. The outer capsid is composed of trimeric capsomers, each approximately 8 nm diameter and 7.5 nm high, arranged in a pseudo-hexagonal array. A thin fiber projects radially from the surface of each capsomer and is probably important in regulating interparticle distance (Fig. 5A). This would explain the results of Mercer and Day (1965), who reported remarkably stable interparticle distances (5–8 nm) of IIV-2 under radically different conditions of hydration, type of solvent, and the use of polymers and antibodies. Surface fibrils have been observed in a number of other iridoviruses from both vertebrates and invertebrates (reviewed by Williams, 1996).

At the base, the capsomers are interconnected, forming a contiguous icosahedral shell ~2.5 nm thick (Fig. 5B). The MCP exists externally as a noncovalent trimer and internally as a trimer linked by disulfide bonds (Cerutti and Devauchelle, 1985, 1990). The trimeric structures of the inner capsid shell are rotated ~60° relative to the outer capsomers (Fig. 5C). Surface labeling indicated that the surface structure of IIV-1 was fairly complex, with 10 or 11 polypeptides ^{125}I-labelled under nonreducing and reducing conditions, respectively (Watson and Seligy, 1997). A lipid bilayer, 4 nm thick, surrounds the DNA core, and is intimately associated with an additional inner shell beneath the fused layer of the capsid. The effect of lipid solvents and detergents on IIV infectivity appears to depend on the assay system used to measure virus titer; *in vitro* assays generally indicate higher sensitivity to solvents than *in vivo* assays (Martínez *et al.*, 2003). The capsomers are arranged into trisymmetron and pentasymmetron facets. Each particle consists of 20 trisymmetrons, composed of 55 capsomers, and 12 pentasymmetrons composed of 30 capsomers and one hexavalent capsomer of uncertain composition (Fig. 5D). Pentavalent capsomers are located at the vertices of the particle in the centre of each pentasymmetron. This gives a total of 1460 capsomers plus 12 pentavalent capsomers per particle, in agreement with previous calculations (Manyakov, 1977; Wrigley, 1970).

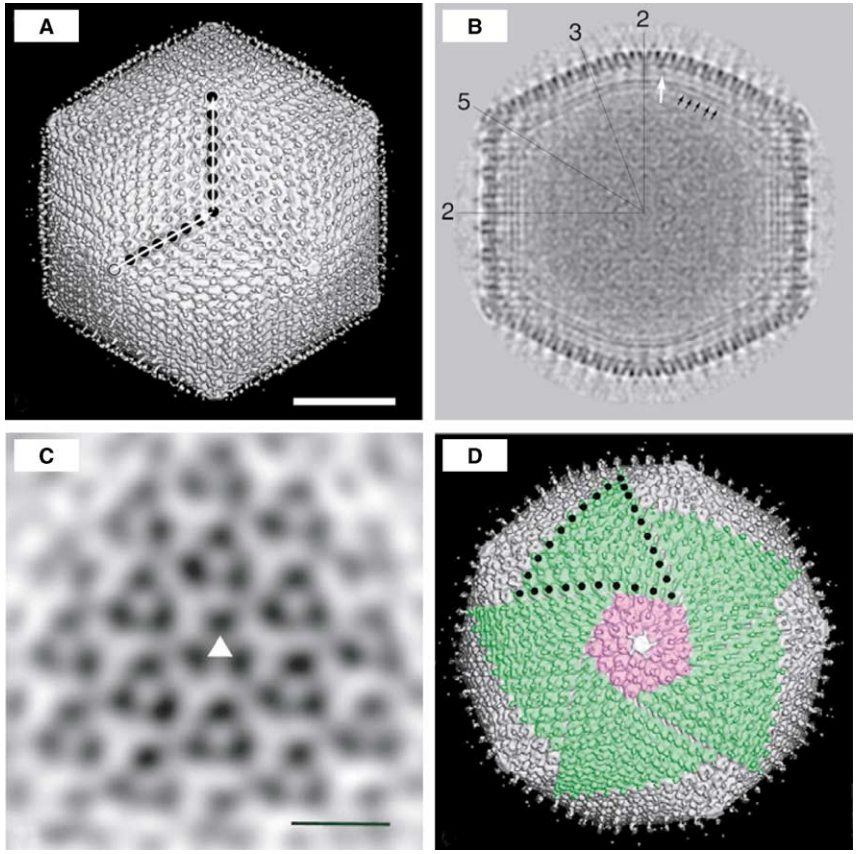

FIG 5. (A) Three dimensional reconstruction of IIV-6 particle viewed down a threefold axis of symmetry by cryo-electron microscopy. Circles indicate the position of capsomers along the h and k lattice used to calculate the triangulation number (T). The proximal ends of surface fibers are visible, whereas the distal ends have become lost during the reconstruction process because of their flexibility (white bar = 50 nm applies to images A, B, and D). (B) Central section of the reconstruction density map viewed along twofold axis indicating twofold, threefold, and fivefold axes of symmetry. A lipid bilayer is seen beneath the capsid shell (black arrows) with numerous connections to an additional shell (white arrow) beneath the outer shell. (C) Close-up view of the trimers comprising each capsomer shown as a planar section through a reconstruction density map viewed along threefold axis (triangle) (black bar = 10 nm). (D) Facets of capsid with five trisymmetrons (highlighted in green) arranged around one pentasymmetron (in pink). The central capsomer of the pentasymmetron (uncolored) is pentavalent. The edge of each trisymmetron comprises ten capsomers (black dots). Images reproduced with permission from Yan *et al.* (2000).

The triangulation number, T, which describes the relationship between the fivefold axes of rotational symmetry of the particle, is calculated as $T = h^2 + hk + k^2$, where h and k indicate the number of steps (capsomers) required in two directions to reach the next pentasymmetron, i.e. the distance between apices. As the values of h and k are both 7, T = 147 (Yan *et al.*, 2000) (Fig. 5D).

IX. Ecology of Invertebrate Iridescent Viruses

Since the last comprehensive reviews of iridoviruses of invertebrates (Williams, 1996, 1998), principal advances in our understanding of IIV ecology relate to recognizing the importance of inapparent infections for virus survival and the consequences of covert infection on the reproductive capacity of the host.

IIVs infect many species of insects and several other invertebrate taxa, notably terrestrial isopods. The massive proliferation of IIV particles and their paracrystalline arrangement in the cytoplasm of host cells result in the distinctive iridescent hues of infected insects from which the family derives its name. This type of infection has been labeled patent infection to differentiate it from covert infections that may be common but less visible (Williams, 1993). The color of patently diseased insects ranges from lavender, blue, and turquoise for the small IIVs (genus *Iridovirus*) to yellow-green, orange, and red in infected aquatic Diptera. The shift in the color spectrum is indicative of larger particle size and greater interparticle spacing among viruses infecting Diptera (Hemsley *et al.*, 1994). There is no known adaptive advantage arising from iridescence. Natural infections are mainly observed in the immature stages (larvae, nymphs) of insects, although adults may also be patently infected in bees, isopods, crickets, and flour beetles.

A. Patent IIV Infections Are Usually Rare

In patently infected animals, the majority of host tissues are infected by IIV particles with particularly abundant replication in the body fat and epidermis (Federici, 1980; Hall and Anthony, 1971; Kelly, 1985). Individuals with patent infections that survive to pupate may suffer marked deformations of the pupa, particularly of the wing buds (Carter, 1974; Smith *et al.*, 1961; Stadelbacher *et al.*, 1978). Cellular pathology has been reviewed elsewhere (Williams, 1996).

Despite their visibility, the prevalence of patent IIV infection in host populations is usually very low. The majority of reports mention

finding a few infected individuals, rarely exceeding 1% of the population, although abundant infections and even epizootics have occasionally been observed (Williams, 1998). However, the records of patent infections are probably not representative of their occurrence in nature, as frequent infections are far more likely to be reported than rare ones. Indeed, the low prevalence of infections in natural populations and difficulties in transmitting the virus to susceptible hosts are the principal factors that have deterred researchers interested in the biological control of insects from studying IIVs. These viruses are distributed globally across temperate and tropical regions and are most frequently observed in larval mosquito populations (Williams, 1998).

B. Seasonal Trends in Infections

An association of IIV infection with moisture is observed seasonally among soil-dwelling arthropods. The prevalence of patently infected terrestrial isopods (variously known as woodlice, sowbugs, and slaters) is highest in the coolest and wettest months of the year (December-March) in both northern Europe and the southern United States (Federici, 1980; Wijnhoven and Berg, 1999). Seasonal increases in the prevalence of IIV disease are concurrent with reductions in the spatial structure of the isopod population. In the drier months, the distribution of isopods is highly aggregated, within which patch densities are high and inter-patch distances are large, reducing the probability of dispersal. In the wetter months the situation is reversed—patches break down, isopods disperse more, and the prevalence of disease increases (Grosholz, 1993).

In southern Mexico, epizootics of patent infection were observed on an annual basis in multiple species of sympatric blackflies (Hernández *et al.*, 2000). The prevalence of patent infection was also seasonally affected, with 41–100% of blackfly larvae infected at the end of the dry season when river levels were at a minimum and larval populations were high. This was followed by a marked decrease in patent infections, concurrent with the start of the rainy season, that caused a decline in larval population densities. The same patterns were seen yearly thereafter. However, attempts at quantifying covert infections by insect bioassay failed, as the virus did not replicate in *Galleria mellonella* (Hernández *et al.*, 2000). Seasonal fluctuations in the prevalence of IIV infections of chironomid larvae have also been reported in the southern USA with infections only seen in March-May in Florida (Fukuda *et al.*, 2002) or February-April in Louisiana (Chapman *et al.*, 1968).

C. Covert Infections Can Be Common

Covert infections are not obvious to the naked eye, and infected hosts appear normal and may develop fully and reproduce as adults. Covert infections appear to be a low virulence infection rather than an early stage of a lethal disease that subsequently progresses to patent infection. An example of the latter is seen in terrestrial isopods from field populations that appear normal when collected, but develop patent infections shortly afterwards when observed under laboratory conditions, indicating that they were already infected when taken from the field (Federici, 1980). In contrast, covertly infected hosts do not spontaneously develop patent infections when incubated in the laboratory, at least in the case of blackflies (Williams, 1993, 1995).

The presence of abundant covert infections in blackfly populations (*Simulium variegatum*) in Wales, UK was originally detected using insect bioassay and PCR techniques (Williams, 1993). The prevalence of infection in the blackfly population was seasonally affected, with peaks of up to 37% infection during Spring and Autumn (Williams, 1995). However, the prevalence of infections was not correlated with host density. Patently infected individuals were only rarely observed during the study, but interestingly, they were observed immediately following the peak prevalence of covert infection. Sympatric blackfly species were not observed to suffer IIV infections.

Covert infections have been diagnosed by electron microscopic observation of low numbers of virus particles in the fatbody and hemocytes of mayfly larvae (Tonka and Weiser, 2000). Infected cells displayed abundant closed tubular structures of unknown function within viral assembly sites (Fig. 6). These closed tubular structures, although not commonly seen, are a result of aberrant assembly of capsid components (Buchatsky and Raikova, 1978; Darcy and Devauchelle, 1987; Devauchelle, 1977; Stoltz, 1973).

Laboratory studies on infection of *Aedes aegypti* by IIV-6 detected the prevalence of patent infection in the range 0.1–1.3%, whereas covert infections were approximately ten times more common (Marina *et al.*, 1999, 2003a,b,c). The nature of covert infection is poorly understood. The titer of virus particles in covertly infected hosts was shown to increase over time in both mosquitoes (Marina *et al.*, 1999) and *G. mellonella* larvae (Constantino *et al.*, 2001), but tissue tropisms and the density of particles in covertly infected insects remain unclear. IIV particles from covertly infected hosts can be manually transmitted to healthy hosts resulting in patent disease, indicating that the virulence of the pathogen is not a fixed characteristic and that genetically

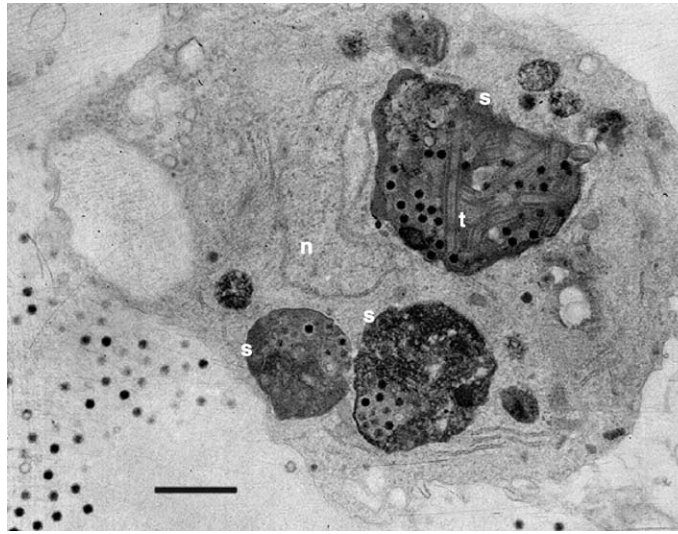

FIG 6. IIV particles in a hemocyte of a covertly infected mayfly larva showing abundance of tubular structures (t) and a low number of virus particles associated with viral assembly sites (s). In contrast, patent infection involves massive proliferation of IIV particles in infected insect cells. Cell nucleus (n), bar = 1 μm. Reproduced with permission from Tonka and Weiser (2000).

identical isolates can cause both types of infection. Doubtless, physiological stressor experiments and studies on covertly infected hosts inoculated with coinfecting pathogens or parasites have the potential to clarify the nature of covert infection and the mechanisms that trigger patent disease, as reported for Lepidopteran baculoviruses (Fuxa *et al.*, 1999; Hughes *et al.*, 1997).

The techniques used to detect covert infection include direct electron microscopic observation of infected tissues (Tonka and Weiser, 2000), PCR using primers targeted at the major capsid protein gene, endpoint dilution in cell culture and insect bioassay in larvae of the wax moth, *G. mellonella* (Constantino *et al.*, 2001). Immunological and DNA hybridization techniques have also been employed to determine the presence of infections in host range studies discussed below. The sensitivity of cell culture depends to a large extent on the cell line(s) employed. Of 12 different cell lines tested, the most sensitive were those of *Drosophila* (DR1, DR2) and the least sensitive were those of *Aedes aegypti*, *Ae. albopictus,* and *Plutella xylostella* (PX2-HNV3) (Constantino *et al.*, 2001). The *Ae. albopictus* cell line was previously

described as nonpermissive to IIV-6 (Cerutti and Devauchelle, 1980), but semipermissive to IIV-1 (Tajbakhsh *et al.*, 1990). Lepidopteran lines such as the commonly used *Spodoptera frugiperda* Sf9 and Sf21 were 10-fold less sensitive than the *Drosophila* lines, and those from other noctuid species were of intermediate sensitivity. Between 15 and 64 particles of IIV-6 represented an infectious unit in Sf9 cells (Constantino *et al.* 2001). This compares to a sensitivity of approximately ten particles of IIV-3 in *Ae. aegypti* cells (Webb *et al.*, 1975). Czuba *et al.* (1994) reported that the sensitivity of plaque assay of IIV-1 in Sf9 cells varied according to whether virus concentration was estimated by optical density or direct counting, as many particles in their preparations did not contain a genome.

Insect bioassay is a simple technique in which a test insect is homogenized, semi-purified, and injected into *G. mellonella*. Patent infections of *G. mellonella* larvae were used to indicate that virus particles were present in the test insect. This technique is highly sensitive (Constantino *et al.*, 2001). Most, but not all, IIVs replicate well in *G. mellonella*; IIV-3, IIV-16, IIV-24, and an isolate from blackflies (Hernández *et al.*, 2001) cannot be detected by this method.

Nested and conventional PCR has been used successfully to amplify conserved sequences in the MCP gene from covertly infected insects (Constantino *et al.*, 2001; Williams, 1993). The sensitivity of the amplification technique was calculated to be 1000 particles/insect, much of which reflects the loss or dilution of genome copies that occurs during DNA purification. The use of carrier DNA, such as salmon sperm DNA, proved useful when attempting to amplify virus sequences from very small insects or insect tissues contaminated by insect gut material (Williams, 1993).

D. Covert Infections Are Detrimental to Host Fitness

There is increasing awareness of the importance of sublethal diseases on the dynamics of insect populations (Boots *et al.*, 2003). Sublethal infections by baculoviruses, entomopoxviruses, and small RNA viruses are frequently detrimental to the development, reproduction, and survival of their insect hosts (Rothman and Myers, 1996). However, not all studies on the sublethal effects of virus infections demonstrate the presence of the pathogen in the survivors. This oversight leaves open the possibility that survivors represent a resistant subset of the population with different demographic characteristics, or that a tradeoff exists between the cost of mounting an immune response and future investment in reproduction or longevity. With the advent

of RT-PCR, the presence of the pathogen can now be confirmed with considerable certainty (see Burden *et al.*, 2003 for an example with baculovirus). To date, RT-PCR has not been employed to detect mRNA generated in covert IIV infections.

The influence of covert IIV infections on host fitness has been examined using the mosquito *Ae. aegypti* and IIV-6 as a model system (Marina *et al.*, 1999, 2003a,b,c). Mosquito larvae were briefly immersed in an IIV-6 suspension, subjected to repeated washing steps and then reared to adulthood, whereupon demographic parameters were determined. Insect bioassay was used to classify adult mosquitoes as (i) covertly infected, (ii) inoculated survivors that did not become infected or, (iii) controls. The findings have been remarkably consistent. Exposure to inoculum results in an extended immature development time. More importantly from the population viewpoint, covertly infected mosquitoes suffered a decrease in fecundity ranging from 22% on a single gonotrophic cycle to 36–39% on multiple (lifetime) gonotrophic cycles. Progeny production, reflected in the net reproductive rate (R_0) being the average number of female offspring produced by each mosquito, was reduced correspondingly by 22–50%. No effects on egg fertility have been detected, perhaps because the prevalence of infertility increases with age and covertly infected females die at younger ages than healthy conspecifics. Covert infection is also associated with reduced body size (wing length of females) and reduced adult longevity. The pattern of mortality also differs in covertly infected insects with an early peak in the death rate (d_x) and very few long-lived individuals (Marina *et al.*, 2003b). Reduced body size is likely to impact reproductive success negatively in natural mosquito populations, especially for males that have to compete for mates. A marked reduction in fecundity and reduced longevity have been reported in an aphid (*Toxoptera citricida*) fed crude suspensions of IIV-6. However, the replication of IIV particles in aphids has yet to be demonstrated explicitly (Hunter *et al.*, 2001b).

Why is covert infection costly to the host? The effects of sublethal infection may reflect the cost of mounting an immune response or because virus-induced cell death requires the diversion of host resources to repair damaged cells and tissues. Indeed, clear relationships between immune response (melanization) and larval developmental rate, adult body size, and fecundity have been reported in *Ae. aegypti* (Koella and Boëte, 2002; Schwartz and Koella, 2004). In contrast, the possibility that sublethal effects were a consequence of a toxic virus protein associated with the inner lipid membrane (Cerutti and Devauchelle, 1980; Ohba *et al.*, 1990) received little support from

experimental studies with heat- or UV-inactivated virus. Heat- or UV-inactivated IIV-6 did not affect mosquito reproduction or longevity and caused only minor changes in adult body size compared to control insects. This may have been due to cellular apoptosis or reduced feeding of inoculated insects (Marina *et al.*, 2003c). Indeed, UV-inactivated ASFV and FV-3 trigger apoptosis in the absence of gene expression or replication (Carrascosa *et al.*, 2002; Chinchar *et al.*, 2003).

E. Host Range

Accurate appraisal of host range evidently requires diagnosis of infection on grounds other than the development of an iridescent patent infection alone. Historical studies have established that IIVs differ markedly in their host range, from those that naturally infect just one or very few species, to those, such as IIV-6, with broad host ranges. However, the range of species in which IIVs replicate depends very much on the route of infection. Laboratory studies in which the inoculum is injected indicate that many IIVs can productively infect species of agricultural and medical importance, and can often infect different taxonomic orders or even classes (Fukuda, 1971; Henderson *et al.*, 2001; Ohba, 1975; Ohba and Aizawa, 1979).

Replication *in vitro* is even more catholic. IIV-6 replicates in many insect cell lines and can even infect in reptile cells (McIntosh and Kimura, 1974). However, IIV-6 does not replicate *in vivo* in frogs (Ohba and Aizawa, 1982). Following intraperitoneal injection into *Rana limnocharis*, the titer of IIV-6 declined 100-fold over a period of four days. The *in vitro* host range of IIV-6 was recently extended to include cells from a whitefly (Funk *et al.*, 2001), a leafhopper, a lacewing (Hunter *et al.*, 2001a), and a root weevil (Hunter and Lapointe, 2003). Replication is abolished at temperatures of $\sim$30°C (Tesh and Andreadis, 1992), so mammalian cells held at 37°C are not productively infected by IIV-6.

Laboratory studies involving the ingestion of high doses of IIV-6 also indicate a broad host range. An IIV-6-like isolate from commercial colonies of crickets (*Gryllus campestris, Acheta domesticus*) could be transmitted by feeding very high concentrations (2.2×10^{11} particles/ml) to other orthopteran species. Patent infection was subsequently observed in five of the eight species of locusts, crickets, and cockroaches tested (Kleespies *et al.*, 1999). Dipping of cricket nymphs in concentrated virus suspensions also resulted in infection. The possibility of covert infection was not examined. Also, brown citrus aphids

(*Toxoptera citricida*) that consumed a crude suspension of IIV-6 were believed to have been infected, although viral replication or cytopathological effects were not demonstrated (Hunter *et al.*, 2001b).

We take this opportunity to call for caution in the interpretation of host range with reference to two recent studies. First, evidence for the *in vitro* replication of an IIV-6 isolate from a New Zealand virus collection in a whitefly cell line from *Bemisia tabaci* biotype B (Funk *et al.*, 2001) was followed shortly afterwards by a report of IIV-6 infecting natural populations of *B. tabaci* in Florida (Hunter *et al.*, 2001a). However, although the isolation procedure involved applying whitefly homogenates to the cell line, no virus particles were observed in whiteflies from which the homogenates were prepared. Moreover, the MCP gene sequence of the New Zealand isolate and the supposed whitefly isolate differ from that of the published IIV-6 sequence by ~5% (Jakob *et al.*, 2001; Stohwasser *et al.*, 1993), suggesting that the virus isolated from the whitefly may have been a contaminant from the initial cell line study. Second, a strain of IIV-6 has been reported from diseased reptiles (Just *et al.*, 2001). Various tissues were used to inoculate viper heart cells (VH2) incubated at 28°C. An IIV was isolated from VH2 cells showing cytopathic effects, leading to the conclusion that the reptiles were infected by IIV. However, SDS-PAGE, restriction endonuclease and sequence analysis of a PCR amplified fragment of the MCP gene revealed that the isolate from VH2 cells was identical in every way to an IIV-6 like strain isolated from patently infected crickets in the same laboratory at about the same time (Just and Essbauer, 2001). Since IIVs are capable of persisting almost indefinitely as low-level infections in cell lines (Mitsuhashi, 1967), the inferred infections of whiteflies and reptiles by IIV-6 will require confirmation.

Several studies have attempted to determine host range based on the presence of both symptomatic and asymptomatic infection. The indirect fluorescent antibody technique demonstrated the presence of virus antigen in head and abdominal squashes of mosquitoes, sandflies, and a triatomid bug following injection of IIV-22 from *Simulium variegatum*. The quantity of antigen was proportional to the interval between inoculation and testing. Low densities of IIV particles were also observed in the cells of one mosquito species by electron microscopy (Tesh and Andreadis, 1992).

Ward and Kalmakoff (1991) reported that dot-blot DNA hybridization could be used to detect viral replication in several species of Lepidoptera and Coleoptera prior to, or in the absence of, iridescence

of host tissues, following injection of IIV-9 from *Wiseana* sp. (Lepidoptera). Similarly, Henderson *et al.* (2001) used dot-blot assays to demonstrate changes in the concentration of viral DNA signaling the replication of IIV-6 in injected cotton boll weevils (*Anthonomus grandis*).

While the host range of IIVs in nature is likely far more restricted than laboratory studies would lead us to believe, certain IIV species are capable of infecting a limited number of sympatric host species. Examples include IIV-9 isolated from soil dwelling Lepidoptera in New Zealand and others mentioned by Williams (1998).

During recent transmission studies, an IIV isolated from *Spodoptera frugiperda* (Lepidoptera) was observed to infect and kill the immature stages of hymenopteran endo- and ectoparasitoids that developed in or on infected hosts (López *et al.*, 2002). The interval between parasitism and virus infection was critical to parasitoid survival; parasitoids could only develop when hosts were infected shortly prior to parasitoid emergence and pupation. These studies were performed in the laboratory, but, as many species of Lepidoptera are host to parasitoids and IIV infections, it seems likely that IIVs may frequently infect and kill the developing stages of hymenopteran parasitoids in nature.

It has been suggested that at least six different genera of North American woodlice (Isopoda), and an isopod infecting nematode, are host to IIV-31 (Cole and Morris, 1980; Grosholz, 1993; Poinar *et al.*, 1980; Schultz *et al.*, 1982). However, to date, genetic similarities among isolates from different species indicating cross species transmission have only been demonstrated for certain species of *Porcellio* and *Armadillidium* (Cole and Morris, 1980; Federici, 1980). Similarly, eight different genera of terrestrial isopods were reported as hosts to IIV in northern Europe, based on iridescence of tissues (Wijnhoven and Berg, 1999) and electron microscope observations (Poinar *et al.*, 1985). In contrast, several reports of IIVs infecting natural populations of mosquitoes or blackflies suggest that patent infections are limited to one or a few sympatric species despite an abundance of alternative hosts (Anderson, 1970; Chapman *et al.*, 1971; Popelkova, 1982; Williams, 1995). It is intriguing that IIVs have never been reported from natural populations of *Anopheles* mosquitoes, despite successful infection in the laboratory. Given that many IIVs are capable of replicating in a range of invertebrate hosts but do not appear to infect more than a few sympatric species in nature, the key factors that determine the functional host range of these viruses are likely to be behavioral, or related to specific associations that favor transmission, such as parasitism, described later.

F. Transmission and Route of Infection

The route of infection remains unclear in many host-IIV systems. As *per os* infection almost invariably requires ingestion of very large doses of particles, cannibalism or predation of infected individuals is considered a likely mechanism of transmission in the mosquito *Ochlerotatus* (*Aedes*) *taeniorhynchus* (Linley and Nielsen, 1968a,b), terrestrial isopods (Federici, 1980; Grosholz, 1992), tipulid larvae (Carter 1973a,b), and the late instars of *Spodoptera frugiperda* (Chapman *et al.*, 1999; O. Hernandez and T. Williams, unpublished data).

As IIV-3 virions were degraded in large numbers in the gut of mosquito larvae, the peritrophic membrane appeared to be an effective barrier preventing infection of gut cells (Stoltz and Summers, 1971). By physically damaging the peritrophic membrane using an inoculum of IIV-3 containing silicon carbide "whiskers," a threefold increase in patent infection of mosquito larvae was observed (Undeen and Fukuda, 1994). Mixtures of IIV-6 and ground sand also resulted in an increase of patent and covert infection of *Ae. aegypti* (Marina *et al.*, 1999), but mixtures of IIV-6 with an optical brightener, know to degrade the chitin structure of the peritrophic membrane, or small silicon carbide grains, did not result in a significant increase in infection of mosquitoes, leading to the suggesting that IIVs depend on mechanisms other than ingestion to achieve infection, possibly wounding (Marina *et al.*, 2003a).

Intra- and interspecific aggression between individuals resulting in wounding has been identified as an important factor influencing IIV transmission in natural populations of isopods and laboratory populations of mosquitoes. Doubling the density of *Porcellio scaber* populations resulted in an insignificant change in the prevalence of infection by IIV-31, whereas introducing an equal density of *Porcellio laevis* was accompanied by a threefold increase in infection (Grosholz, 1992). Similarly, a 10-fold increase in the density of *Ae. aegypti* larvae resulted in a small but significant increase in nonspecific mortality, presumably due to increased aggression at high densities, and a concurrent threefold increase in the overall prevalence of infection (covert + patent infections combined) (Marina *et al.*, 2005). The transmission coefficient (υ), representing the probability of becoming infected, decreased over time at both host densities. This was interpreted as evidence of heterogeneity in the susceptibility of individuals, with the most susceptible insects acquiring an infection quickly and the remaining individuals experiencing a reduced probability of infection, or possibly a developmental effect, with an increased probability of

transmission in young larvae, and a reduced probability of infection in older larvae. Such nonlinearities in the transmission of insect pathogens are well recognized for Lepidopteran baculoviruses and have now been extended to include nonoccluded viruses in aquatic insects.

The very high pathogenicity seen following injection of IIV led to the suggestion that parasites and parasitoids may be able to transmit these viruses as they penetrate the host hemocoel. Enhanced transmission of an IIV from midge larvae (*Culicoides variipennis sonorensis*) by infected juvenile stages of a mermithid nematode was demonstrated in laboratory studies (Mullens *et al.*, 1999). Over 90% of infected midge larvae collected from the field in California were also infected by nematodes. In the laboratory, 40–100% of midge larvae became patently infected when incubated in a virus suspension containing juvenile nematodes, whereas almost no infections were observed in larvae incubated with virus suspension alone. In all cases, the virus killed the host larvae before the nematode could emerge. This is the first demonstration that nematodes can initiate infection during the act of host penetration.

Parasitoid mediated transmission was recently demonstrated in laboratory and field studies on *S. frugiperda* and a homologous IIV isolate from southern Mexico (López *et al.*, 2002). The ichneumonid endoparasitoid *Eiphosoma vitticolle* stung significantly more virus-infected than healthy larvae, apparently due to a lack of defense reactions in sluggish, virus-infected hosts. After stinging an infected larva, 100% of the female wasps transmitted the infection to healthy hosts during subsequent acts of stinging. Caged field experiments supported this result: virus transmission to healthy larvae only occurred in cages containing healthy and infected hosts and parasitoids. In contrast, a braconid ectoparasitoid was incapable of virus transmission, presumably because the parasitoid's ovipositor did not penetrate the hemocoel during oviposition. A report of IIV-like particles in the Varroa mite (Camazine and Liu, 1998), a parasite of honey bees, might represent an additional candidate for parasite-mediated transmission, as the original host to the mite *Apis cerana* suffers frequent IIV infections in northern India (Bailey *et al.*, 1976).

Evidence for vertical transmission has only been reported for IIV-3 infected mosquitoes. Adult females, infected as late instar larvae, appear capable of transmitting the virus to between 19 and 46% of their offspring (Woodard and Chapman, 1968). Progeny larvae develop normally until the third or fourth instar, when the signs of disease become manifest and the larvae become lethargic or die, and are consumed by conspecifics, resulting in horizontal transmission. The

pattern of vertical transmission was highly aggregated—patently infected progeny appeared in the broods of just 8% of females. However, all of the larvae from those broods were infected (Linley and Nielsen, 1968a), although others have asserted that vertical transmission may not be so efficient (Fukuda and Clark, 1975). This system merits reexamination with modern molecular techniques.

G. Moisture is Crucial to IIV Survival in the Environment

The ability of IIVs to persist outside of a host is not well understood. The stability of these viruses in water has been attributed to virus particle structure, particularly the internal lipid membrane (Kelly, 1985). The infectivity of IIV-2 fell by 50% after 32 days at 4°C (Day and Gilbert, 1967). Similarly, a 10-fold reduction in titer was seen in IIV-6 after 50 days at either at 4°C or 25°C. Loss of IIV-6 infectivity was faster and more variable when tubes of virus suspension were placed in a small pond with a average water temperature of 27°C. The stability of IIV-6 was sensitive to extremes of pH (<4.0 or >9.0), but not to the presence of metal ions (Marina *et al.*, 2000).

Laboratory and field observations indicate that IIVs are particularly sensitive to desiccation. An IIV from a midge retained infectivity for six weeks in water at unspecified laboratory temperatures, whereas infectivity was not observed in infected midge cadavers that had been left dry for eight weeks (Mullens *et al.*, 1999). IIV-6 incorporated into a boll weevil bait gradually lost infectivity over 14 days and was inactivated more rapidly when sprayed on cotton plants (McLaughlin *et al.*, 1972). Following the death of the host, IIV-31 in the cadavers of isopods remained infective for just five days at ambient laboratory temperatures (Grosholz, 1993).

Soil represents an important environmental reservoir for most entomopathogenic viruses. The infectivity of IIV-3 in mosquito larvae fell markedly after two days in fresh or brackish water at 27°C, but when inoculated onto damp soil, the virus failed to cause patent infections in mosquito larvae after 24 hours (Linley and Nielsen, 1968b). Virus extraction and insect bioassay techniques were recently applied to determine the effect of soil moisture and soil sterility on the persistence of IIV-6 at 25°C in the laboratory (Reyes *et al.*, 2004). Virus extraction was problematic due to the high affinity for clay minerals; an albumen solution was used as desorbant. Loss of activity in dry soil (6.4% moisture) was very rapid and was not studied beyond 24 hours. However, soil moisture did not affect the rate of inactivation of virus in damp (17% moisture) or wet soil (37% moisture). In contrast, soil

sterilization significantly improved the persistence of IIV-6 activity, both in damp and wet soil. These figures represent half-lives of 4.9 days in nonsterile soil, 6.3 days in sterilized soil (data pooled for 17% and 37% moisture treatments), compared to 12.9 days for aqueous virus suspensions incubated in the laboratory.

Exposure to solar UV light resulted in a very rapid inactivation of IIV-6 in water, with infectivity dropping by approximately nine logarithms after 24 hours' exposure to sunlight (A. Hernández, unpublished data). The presence of soil sediment greatly improved the persistence of the virus. Extra-host persistence in soil and aquatic habitats is likely to represent an important aspect of the ecology of these viruses.

Interestingly, a peptide from a diapausing chrysomelid beetle was identified with high homology to a putative peptide of unknown function identified in the genome of IIV-6 (Tanaka *et al.*, 2003). The diapause specific peptide was similar to antifungal peptides from plants and the conotoxin-like peptides from baculoviruses. When purified, the peptide inhibited growth of a dermatophyte pathogen, but not of the insect pathogen *Beauveria bassiana*. The possibility that IIV-6 produces this peptide to avoid opportunistic exploitation of IIV-infected insect cadavers by soil-dwelling fungi, thereby improving persistence and the probability of transmission, merits examination.

X. Iridoviruses in Noninsect Marine and Freshwater Invertebrates

Information on iridoviruses infecting noninsect aquatic invertebrates is scarce and consists almost entirely of descriptions of particle morphology, DNA content, cytoplasmic assembly, and cytopathology and histopathology. Putative iridovirus infections have been reported in a marine annelid worm, *Nereis diversicolor* (Devauchelle, 1977; Devauchelle and Dorchon, 1973), the marine crustaceans *Balanus eburneus* (Leibovitz and Koulish, 1989), *Sacculina carcini* (Russell *et al.*, 2000), *Protrachypene precipua* (Lightner and Redman, 1993), and *Macropipus depurator* (Montanie *et al.*, 1993), the freshwater snail, *Lymnaea truncatula* (Barthe *et al.*, 1984; Rondelaud and Barthe, 1992; Ruellan, 1992), and the marine cephalopod, *Octopus vulgaris* (Rungger *et al.*, 1971).

A number of iridovirus infections have been reported in oysters. Gill necrosis virus disease is an epizootic disease characterized by ulceration of the gills, labial palps, and the mantle concurrent with the slimming of the oysters followed by death (Arvy and Franc, 1968;

Alderman and Gras, 1969). This disease is believed to be involved in the disappearance of the Portuguese oyster from major culture areas, including the Atlantic coast of France (Comps, 1970; Comps and Duthoit, 1976). Hemocytic infectious virus disease appears to have affected Portuguese oysters since the 1970s, although clinical signs were not reported. Virus infection is systemic and targets hemocytes for destruction (Comps, 1983). This disease also causes mortality in Pacific oysters in France (Comps, 1980; Comps and Bonami, 1977; Comps *et al.*, 1976). Recently, iridovirus-like particles were observed in adult Japanese oysters, *Crassostrea gigas,* suffering gill erosion and abnormal mortalities in Mexico (Cáceres-Martínez and Vásquez-Yeomans, 2003).

Larval stages of Pacific oysters infected by oyster velar virus disease (OVVD) (Elston, 1979; Elston and Wilkinson, 1985) have been described in hatcheries in Washington State, USA. Loss of ciliated velar epithelial cells, which may appear as blebs on the periphery of the velum as they are sloughed, form the characteristic "blisters" associated with the disease. Larvae lose the ability to swim. OVVD can cause near 100% mortality in affected hatchery tanks.

The absence of DNA sequence information for noninsect invertebrate iridoviruses represents a serious hurdle for the development of molecular diagnostic tools. Recent studies aiming at establishing a PCR protocol for the detection of oyster iridoviruses focused on conserved sequences from vertebrate and insect iridoviruses (Barbosa-Solomieu, 2004). Three genes encoding essential proteins (ATPase, MCP, and the small subunit of ribonucleotide reductase) were selected for assays. Preliminary findings indicated that primers designed from MCP and ATPase genes may be useful for the detection of mollusk iridoviruses. Studies are currently underway to sequence the PCR products and determine their relationship to sequences registered in the databases.

XI. Conclusions

This review has mainly focused on the advances in iridovirus research over the past decade. Here we summarize salient features of the abovementioned studies, and discuss their importance to future work.

Iridoviruses are emerging as major mortality factors in natural populations of amphibians and reptiles, and in commercially important marine and freshwater fish. It is not clear if this is a result of

the introduction of exotic pathogens into new host species, or a consequence of alterations in the transmission or virulence of endemic pathogens in populations of physiologically stressed or immunocompromised hosts.

As our knowledge of iridovirus biology increases, so does our understanding of the taxonomic structure of the family. A new genus (*Megalocytivirus*) has recently been created to accommodate a number of viruses isolated from diseased fish in Southeast Asia, and an established one (*Chloriridovirus*) is about to be validated following the sequencing of the genome of the mosquito pathogen IIV-3.

The question of what defines an iridovirus species remains highly pertinent. A steadily increasing number of sequencing studies, together with a growing appreciation of the degree to which phenotypic characteristics can vary among strains of a virus, represent important advances in defining iridovirus species. However, we are still a long way from a quantitative understanding of intraspecies and interspecies variability and how it impacts the differentiation of one species from another.

Genomic sequencing projects have clarified evolutionary relationships between iridoviruses and other families of large, nucleocytoplasmic DNA viruses. Iridoviruses appear situated at the center of a clade of DNA viruses that include the poxviruses, phycodnaviruses, ASFV, ascoviruses, and possibly a new giant virus of amoebae (mimivirus). Further studies may shed light on the origins of these closely linked families.

We are beginning to understand the interactions between iridoviruses and the immune system of ectothermic vertebrates. Further studies on iridovirus pathobiology and the functioning of lower vertebrate immune systems will doubtless reveal intriguing viral strategies for overcoming host defenses and coevolutionary host responses that modulate the virulence of iridovirus infections and protect against clinical disease.

Not all iridovirus infections result in mortal disease. Indeed, the recognition that many iridovirus infections are chronic, especially among insects, should stimulate studies on the importance of such illnesses on host fitness. Sublethal effects have been demonstrated in infected insects, and theoretical studies suggest such effects have a major influence on the dynamics of host populations. However, the consequences of chronic disease on the demography (fecundity, fertility, death rate, etc.) of infected amphibian and fish populations are notable by their absence.

Our ability to detect iridovirus infections has advanced markedly with the adoption of highly sensitive LAMP and multiplex PCR

techniques for rapid diagnosis of infected individuals. However, the identification of novel isolates is still hampered by a lack of adequate sequence information for key iridovirus genes and the difficulty of propagating some viruses (e.g., LCDV) in cell culture.

Following three decades of neglect, the study of virion structure has returned to the spotlight with the construction of a high-resolution model that offers the most precise representation of an iridovirus particle to date. Additional studies are in progress to further improve the resolution of the model.

An entire group of putative iridoviruses infecting marine and freshwater invertebrates has been largely ignored, despite being suspected of being responsible for serious diseases and mass mortalities in economically important species, particularly oysters. Molecular studies are required to ascertain the relationships between these noninsect invertebrate isolates and those from the established genera.

Elucidation of the complete genetic sequence of FV-3 and other iridoviruses opens the way for a detailed biochemical and genetic study of iridovirus replication. Understanding the role of key replicative genes such as the viral homolog of Pol II and putative immune control proteins such as the viral homolog of eIF-2α should now be possible using knock-down technology (e.g., siRNA and antisense morpholinos) and knock-out (e.g., homologous recombination) technology. This work, coupled with studies of antiviral immunity, should lead to the development of better vaccines and control vectors. Moreover, since DNA methylation often results in gene silencing and plays a key role in development and carcinogenesis, determining how the highly methylated genomes of the vertebrate iridoviruses are transcribed will shed light on a fundamental process in higher eukaryotic systems.

After an extended period of quiescence, interest in the iridoviruses as pathogens of ectothermic animals has flourished over the past decade. This has been stimulated by a growing appreciation of their impact as agents of morbidity and mortality in natural and commercially important animal populations. Following marked advances in our understanding of the taxonomic relationships within the *Iridoviridae* and our ability to define the genetic identity of virus species, future possibilities for research on iridoviruses appear very encouraging.

ACKNOWLEDGMENTS

We thank Misha Sokolov for translating Russian texts and Thomas Tonka, Jaroslav Weiser, Brian Federici, Xiaodong Yan and Timothy Baker for providing figures. This

work was supported by the Spanish Ministerio de Ciencia y Tecnología (AGL2002-04320-C02-01 to TW) and the U.S. National Science Foundation (award 2002-35204-12211 to VGC).

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