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PATHOLOGY, ISOLATION, AND PRELIMINARY MOLECULAR CHARACTERIZATION OF A NOVEL IRIDOVIRUS FROM TIGER SALAMANDERS IN SASKATCHEWAN

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ABSTRACT: An iridovirus was confirmed to be the cause of an epizootic in larval and adult tiger salamanders (*Ambystoma tigrinum diaboli*) from four separate ponds in southern Saskatchewan (Canada) during the summer of 1997. This organism also is suspected, based on electron microscopic findings, to be the cause of mortality of larval tiger salamanders in a pond over 200 km to the north during the same year. Salamanders developed a generalized viremia which resulted in various lesions including: necrotizing, vesicular and ulcerative dermatitis; gastrointestinal ulceration; and necrosis of hepatic, splenic, renal, lymphoid, and hematopoietic tissues. In cells associated with these lesions, large lightly basophilic cytoplasmic inclusions and vacuolated nuclei with marginated chromatin were consistently found. Virus was isolated from tissue homogenates of infected salamanders following inoculation of epithelioma papilloma cyprini (EPC) cells. The virus, provisionally designated Regina ranavirus (RRV), was initially identified as an iridovirus by electron microscopy. Subsequent molecular characterization, including partial sequence analysis of the major capsid protein (MCP) gene, confirmed this assignment and established that RRV was a ranavirus distinct from frog virus 3 (FV3) and other members of the genus *Ranavirus.* Intraperitoneal inoculation of $5 \times 10^{6.23}$ $TCID_{50}$ of the field isolate caused mortality in inoculated salamanders at 13 days post infection. Field, clinical, and molecular studies jointly suggest that the etiological agent of recent salamander mortalities is a highly infectious novel ranavirus.

Key words: Ambystoma tigrinum diaboli, iridovirus, molecular characterization, pathology, ranavirus, tiger salamander.

INTRODUCTION

Members of the family Iridoviridae are large enveloped viruses which contain a linear double-stranded DNA genome. Virions possess icosahedral symmetry and are formed in the cytoplasm within morphologically distinct virus assembly sites (Willis, 1990). Four genera are currently recognized: two genera, *Iridovirus* and *Chloriridovirus,* infect only invertebrates whereas members of a third genus, *Lymphocystivirus,* infects a variety of freshwater and marine fish causing self-limiting tumor-like masses in the skin (Williams, 1996). Viruses belonging to the fourth genus, *Ranavirus,* were originally thought to infect only amphibians (Granoff et al., 1965; Wolf et al., 1968) but recently iridovirus isolates from fish and reptiles have been shown to be more closely related to frog virus 3 (FV3), the type species of the genus *Ranavirus,* than to *Lymphocystivirus* (Hedrick et al., 1992a; Mao et al., 1997).

Iridoviruses have been recently identified as the cause of serious systemic disease in feral, cultured, and ornamental fish in Australia (Langdon et al., 1986), Europe (Ahne et al., 1989; Pozet et al., 1992), North America (Hedrick et al., 1992b; Plumb et al., 1996), and Asia (Armstrong and Ferguson, 1989; Tamai et al., 1997; Hedrick and McDowell, 1995). Iridoviruses have been also recognized as the cause of mortality in frogs in the United States (Granoff et al., 1965) and Australia (Speare and Smith, 1992; Cullen et al., 1995) and in the Sonoran tiger salamander (*Ambystoma tigrinum stebbinsi*) of the southwestern United States (Jancovich et al., 1997). Cunningham et al. (1996) identified an iridovirus-like particle in common

frogs (*Rana temporaria*) dying of "red-leg" in Britain and hypothesized that natural outbreaks of 'red-leg' may be caused by iridovirus, with or without infection by opportunistic pathogens such as *Aeromonas hydrophila.*

The apparent similarity of iridoviruses infecting amphibians, reptiles and fish has prompted speculation that one class of poikilothermic vertebrates might serve as a reservoir, alternate host, or amplifying host for viruses infecting another class of lower vertebrates (Granoff et al., 1965; Mao et al., 1997). Several observations support this view: (1) Bohle iridovirus, which was originally isolated from infected frogs in Australia, has been shown to be pathogenic to fish under experimental conditions (Moody and Owens, 1994); (2) although not linked to clinical disease, FV3 infection of sheatfish and rainbow trout led to the appearance of virions and viral antigen in the brain, gills, heart, kidney, and liver of experimentally infected fish (Enriquez, 1993, cited in Ahne et al., 1997); and (3) recently, Mao et al. (1999) described an iridovirus associated with infection of sympatric stickleback fish (*Gasterostelus aculeatus*) and red-legged frog tadpoles (*Rana auroa*) in the United States. These observations strengthen the suggestion that inter-class infections occur among sympatric species in the wild, and that fish may serve as reservoirs for amphibian viruses or vice versa.

Herein, we report the isolation of an apparently novel iridovirus responsible for high mortality among tiger salamanders in Saskatchewan, Canada. Infection led to systemic disease with all organ systems, except muscle and central nervous system, involved. Electron microscopy suggested that the agent was an iridovirus, and this observation was confirmed by molecular studies. Moreover, nucleotide sequence analysis of the 5′ end of the major capsid protein gene demonstrated that the virus responsible for disease in salamanders was a distinct member of the genus *Ranavirus.*

MATERIALS AND METHODS

Description of epizootic

Tiger salamanders were captured by dip net, seine net, and by hand from four ponds within a 60 km radius of Regina, Saskatchewan (Canada; 50°25′N, 104°35′W) as part of a mark-recapture program. Two ponds were man-made dugouts, the third was a natural marsh with a dugout at one end and the fourth was a man-made structure used for rearing fish. None were used by cattle or for household water in the past 10 yr. Histories and descriptions of ponds are given in Table 1. Pond B was monitored from April 1997, when ice was still present, until late October 1997; pond A and E were monitored from June until September, and Pond C was only visited in October. All sick or dead salamanders observed during this period were collected. Tiger salamanders captured in late May and early June were brought into the laboratory for marking at which time several were found to be diseased. Diseased and exposed salamanders were held in captivity and all eventually died, or became sick and were euthanized. Three of 10 tiger salamanders which had been in captivity for approximately 1 yr and held in the same room, but caged separately from the diseased salamanders, also became sick and died. Routine necropsies were performed on a total of 43 tiger salamanders. Ten salamanders were collected directly from the field while 30 had spent varying times in captivity ranging from four days to 35 days. The remaining three had been in captivity for approximately 1 yr. All sick salamanders were euthanized with an overdose of tricaine methanesulfonate (Finquel®, Argent Chemical Laboratories, Redmond, Washington, USA). An additional epizootic in salamanders near Vonda, Saskatchewan (52°19′N, 106°5′W) was investigated in July 1997, and seven tiger salamander larvae were submitted for necropsy.

Diagnosis

Tissues collected from carcasses for histological examination were fixed in neutral buffered 10% formalin and prepared routinely for microscopic examination. In addition to hematoxylin and eosin stain, Shorr's and Lendrum's phloxine tartrazine stains were used to better visualize cytoplasmic inclusions (Luna, 1992). Feulgen stain was used to demonstrate DNA in inclusions (Sheehan and Hrapchak, 1980). Tissue sections showing cytoplasmic inclusions were lifted from glass slides and prepared for electron microscopy as described by Blank et al. (1970). Tissues from two fresh carcasses collected from the field were plated at room tem-

TABLE 1. Information on ponds within Saskatchewan (Canada) where iridovirus infection was detected in tiger salamanders. Regina ranavirus was isolated from a salamander originating from pond B.

Pond	Location	Description	Other amphibians present[a]	Fish species present[b]	Estimate of salamander numbers	Proportion of salamanders diseased (estimate)	Basis of diagnosis[c]
A	50°47′N 104°16′W	—6 man-made fish-rearing ponds, filled each May, drained in July, ~10 yr old	WF, CF, LF adults only	pike, walleye	high	very low, only one sick adult found	CD, H
B	50°31′N 104°30′W	—man-made dugout, contains water all year, freezes solid in winter, >25 yr old	WF, CF adults and tadpoles	stocked with rainbow trout: 1995, 1996	very low	very high, all adults & larvae found were sick/dead	CD, H, EM, VI
C	50°5′N 104°37′W	—man-made dugout, contains water all year, freezes solid in winter, >15 yr old	CF adults and tadpoles	none	Oct 1997 high number of larvae	Oct 1997, all larvae found were sick/dead	CD, H, VI
D	50°19′N 106°5′W	—shallow depression filled with rain and run-off in wet years	unknown	none	unknown	unknown	CD, H, EM
E	50°57′N 104°30′W	—man-made dugout and natural pond, contains water all year, freezes solid in winter, >15 yr old	WF, CF adults only	pond 300 m away stocked 8–10 yr ago with rainbow trout	high	low, all sick animals were adults	CD, H

[a] WF = wood frog (*Rana sylvatica*); LF = leopard frog (*Rana pipiens*); CF = chorus frog (*Pseudacris triseriata*).
[b] Pike = northern pike (*Esox lucius*); walleye (*Stizostedion vitreum*); rainbow trout (*Oncorhynchus mykiss*).
[c] CD = clinical disease; H = histological lesions; EM = electron microscopy; VI = virus isolation.

perature on blood agar and MacConkey's agar and bacterial species were identified using the api 20 E system (bioMérieux Vitek, Inc., Hazelwood, Missouri, USA). Tissues, typically lung, liver, and spleen, were collected from the remaining carcasses and frozen at −20 C for virus isolation and further study; tissues from some carcasses were frozen at −70 C. To isolate virus, tissues were thawed, pooled and homogenized in 2 ml of virus transport media containing 77.5% (volume/volume) phosphate-buffered saline (PBS), 12.5% (volume/volume) trypticase soy broth, 2.5 μg/ml amphotericin B (ICN Biomedicals Inc., Aurora, Ohio, USA), 750 IU/ml penicillin (Ayerst Laboratories, Montreal, Quebec, Canada) and 750 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO, USA). The homogenate was centrifuged at 2,000 *g* for 10 min and the supernatant was inoculated onto epithelioma papulosum cyprini (EPC) cells in Leibovitz's L15 media (Life Technologies, Grand Island, New York, USA) containing 2.0 mM L-glutamine, 5% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin. EPC cells were grown in 25 cm^2 tissue culture flasks at room temperature (~20 C).

Tissue cultures demonstrating cytopathic effect (CPE) were freeze-thawed three times at −70 C, centrifuged to remove solids, and the supernatant was layered over 10% (weight/volume) sucrose and spun for 11 min in an ultracentrifuge (Beckman Airfuge, Beckman Instruments Inc., Palo Alto, California) at 90,700 *g*. The pellet was resuspended in water, placed on grids, and stained with 0.5% phosphotungstic acid for negative transmission electron microscopy, using standard techniques. A cytospin of culture media from infected flasks was prepared and incubated for 30 min with a monoclonal antibody to FV3 (BG11; Chinchar et al., 1984). The cytospin was rinsed three times with PBS, incubated for 30 min with goat anti-mouse fluorescein-isothiocyanate-labeled antibody, rinsed, dried and examined with a fluorescent microscope.

Characterization of virus

An isolate of RRV, grown initially in EPC cells, was passaged in fathead minnow (FHM) cells at 18–20 C in Eagle's minimal essential medium containing Hank's salts (HMEM) and 5% FCS. Subsequently, low titer, early passage, FHM-grown RRV stocks were used to infect confluent monolayers of FHM cells, grown in 25 cm^2 flasks, at a multiplicity of infection (MOI) of ~300 $TCID_{50}$/6 × 10^6 cells (i.e., 5 × 10^{-5} $TCID_{50}$/cell). At 6 and 7 days post infection—a time when focal necrosis was marked—cells were radiolabeled for 6 hr in methionine-deficient Eagle's minimal essential medium with Earle's salts (EMEM; Sigma Chemical Co., St. Louis, Missouri, USA) containing 25 μCi/ml [^{35}S]methionine (Amersham Life Science Inc., Cleveland, Ohio, USA). After labeling, the cells were lysed in 600 μl 125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.02% 2-mercaptoethanol, and 0.01% bromophenol blue and boiled for 3–5 min. Radiolabeled proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970) and visualized by autoradiography.

Confluent 25 cm^2 flasks of FHM cells were infected with FHM-grown RRV as described above and at 7 and 8 days post infection were labeled with growth medium containing 20 μCi/ml [^{3}H]methyl thymidine (Amersham Life Science Inc., Cleveland, Ohio, USA) for 6 hr. Infected cells were lysed in buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, and 0.5% SDS and digested overnight at 37 C with 100 μg/ml Proteinase K. Subsequently, DNA, isolated by phenol-chloroform extraction, was digested with *Hin*dIII, *Xba*I, *Hpa*II, and *Msp*I, as directed by the manufacturer, and analyzed by electrophoresis on 0.7% agarose gels (Mao et al., 1997).

To prepare DNA for PCR analysis, approximately 6 × 10^6 RRV infected FHM cells were lysed in buffer containing 50 mM KCl, 10 mM Tris, HCl, pH 9.3; 3 mM $MgCl_2$, and 0.5% Tween 20 and digested overnight with 100 μg/ml Proteinase K at 37 C. DNA from infected cells was used as a template and amplifed by PCR using primers targeted to highly conserved regions within the amino-terminal end of the major capsid protein gene of FV3 and other iridoviruses. The forward primer is 5′ GACTTGGCCACTTATGAC 3′ and corresponds to amino acids 15–20 within the FV3 MCP, whereas the reverse primer is 5′ GTCTCTGGAGAAGAAGAA 3′ and corresponds to amino acids 186–191 within the FV3 MCP. Conditions for the PCR reaction were those detailed in Mao et al. (1997). Amplified DNA, visualized by electrophoresis on 0.7% agarose gels, was isolated and cloned into the vector pCRII (Invitrogen Corp., Carlsbad, California, USA). The nucleotide sequence of both strands of the cloned fragment was determined by the method of Sanger et al. (1977) using the Sequenase Quick Denature Plasmid Sequencing Kit (Amersham Life Science Inc., Cleveland, Ohio, USA). The amino acid sequence was deduced and a multiple alignment generated using the program MEGALIGN (DNASTAR Inc., Madison, Wisconsin, USA). Based on the alignment generated by MEGALIGN, a

TABLE 2. Distribution of histological lesions observed in Regina ranavirus infected tiger salamanders in Saskatchewan (Canada).

Tissues	Intracytoplasmic inclusions	Pyknotic cells	Focal necrosis	Number examined
Lung	10	10	1	21
Heart	7	5	0	19
Skin	15	15	13	20
Liver	11	12	12	20
Stomach	14	14	10	19
Pancreas	7	8	6	15
Kidney	12	12	4	19
Intestine	16	16	11	21
Skeletal muscle	0	0	0	20
Spleen	12	12	3	15
Thymus	4	4	3	6
Brain	0	0	0	4
Spinal cord	0	0	0	9
Hematopoietic tissue	11	12	3	14
Gonads	2	2	0	2
Oral cavity	4	4	3	5

phylogenetic tree was constructed using the CLUSTAL V alogrithm within DNASTAR (Mao et al., 1997).

Experimental infection of salamanders

EPC-grown RRV was used to inoculate two adult tiger salamanders collected in British Columbia and housed at the Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon, Saskatchewan, Canada) for 9 wk prior to inoculation. Salamanders were caged individually in 18 × 32 × 10 cm clear plastic containers covered with screening. They were fed mealworms dusted with a vitamin and mineral supplement (Reptovit, Tetra, Tetra Werke, Germany) three times per week. The salamanders were inoculated intraperitoneally (i.p.) with 0.5 ml of stock viral solution containing $5 \times 10^{6.23}$ $TCID_{50}$ RRV. The stock solution, derived from a second passage of RRV on EPC cells, was sonicated for 3 sec prior to use. Salamanders were monitored daily for signs of disease. Upon death of the salamanders necropsies were performed and tissues were collected for histopathology and processed as described above. A homogenate of liver, spleen and stomach from each salamander was used for virus isolation.

RESULTS

Field observations, clinical signs, and gross pathology

Carcasses of large tiger salamander larvae were first noticed in April, frozen in the ice of pond B. From late May through July sick or dead tiger salamanders were found in ponds B and E. Pond C was not visited until October 1997, at which time only larval salamanders were present and all were sick or dead. Only one sick salamander was found in pond A. Subjective estimates of populations at risk and mortality are given in Table 1.

Clinical signs of disease in captive tiger salamanders were variable and occasionally animals were found dead without premonitory signs. Typical clinical signs included a combination of loose feces, bloody stools, anorexia, vomiting which was occasionally bloody, small pale raised foci in the skin, and cutaneous erosions and ulcers. Within 48 hr of developing bloody feces, death occurred. The skin of infected salamanders became dark and speckled, and shed skin was fragmented. A few salamanders produced thick sticky mucus from the dorsum and tail which adhered to their skin, and several became stuck to the sides of their cages.

At necropsy, the coelomic cavity often contained clear fluid and there was mild to moderate generalized petechiation of serosal surfaces. Livers frequently had a mottled brown color and the stomach wall

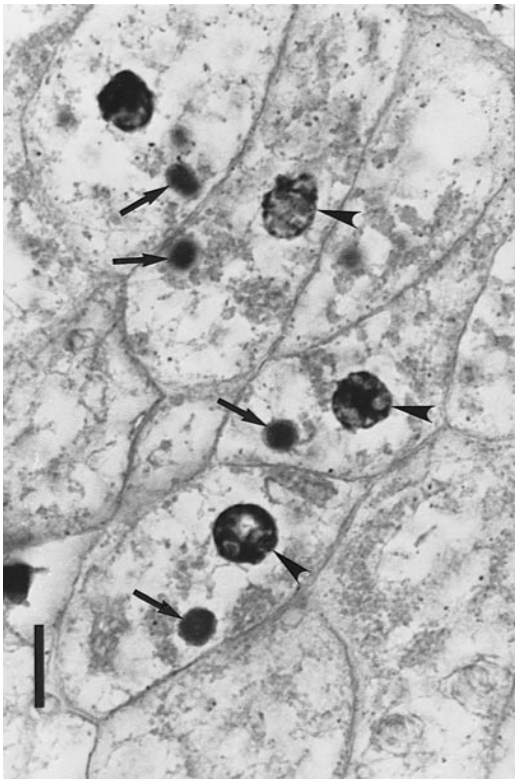

FIGURE 1. Hepatocytes infected with Regina ranavirus (RRV). Hepatocytes contain one to several intracytoplasmic viral inclusions (arrows). Nuclei contain condensed chromatin with amphophilic or clear inclusions (arrowhead). H&E. Bar = 15 μm.

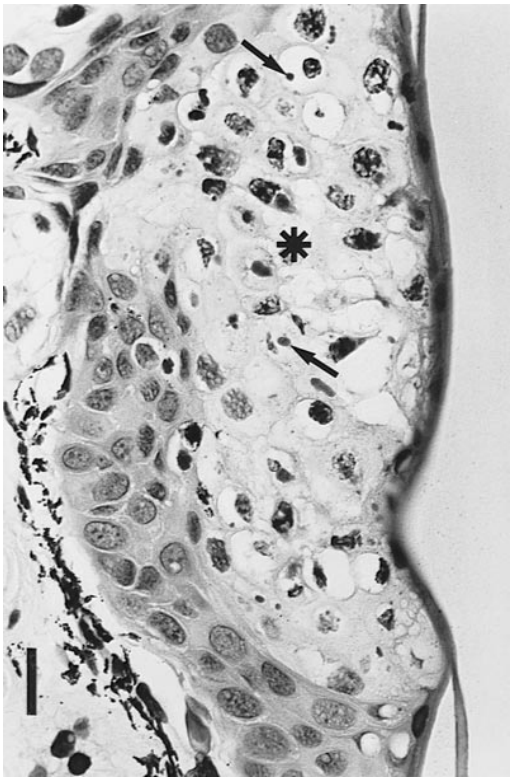

FIGURE 2. Early RRV induced skin lesions are characterized by intracellular edema of the stratum spinosum (asterisk). Cytoplasmic viral inclusions are indicated with an arrow. H&E. Bar = 30 μm.

was occasionally edematous and hemorrhagic.

Histopathology, electron microscopy, and virus isolation

Histological lesions were observed in all tissues except for nervous system and skeletal muscle (Table 2). Lesions consisted of scattered areas of single cell necrosis and variably sized areas of focal necrosis. Necrotic cells, and cells adjacent to areas of necrosis, commonly contained large cytoplasmic inclusions which stained basophilic to amphophilic with hematoxylin and eosin stain. Nuclei contained condensed chromatin and irregularly shaped vacuoles which frequently contained small amounts of amphophilic amorphous material (Fig. 1). The cytoplasmic inclusions stained red to red-blue with Lendrum's phloxine-tartrazine stain, stained blue with Shorr's stain, and stained light to dark red-purple with Feulgen stain. These inclusions correspond to viral assembly sites, the putative sites of viral DNA synthesis and virion formation (Willis et al., 1984).

Skin lesions could be separated into three types which reflected a continuum from early to late stages of infection. Early lesions consisted of intracellular and intercellular edema of the stratum spinosun frequently associated with areas of ballooning degeneration (Fig. 2). Nuclei were condensed and vacuolated. Cytoplasmic inclusions were numerous. More advanced lesions consisted of subcorneal and intraepidermal clefts and vesicles containing necrotic and degenerate acanthocytes (Fig. 3). Both types of lesions are the histological counterpart of the pale foci seen at

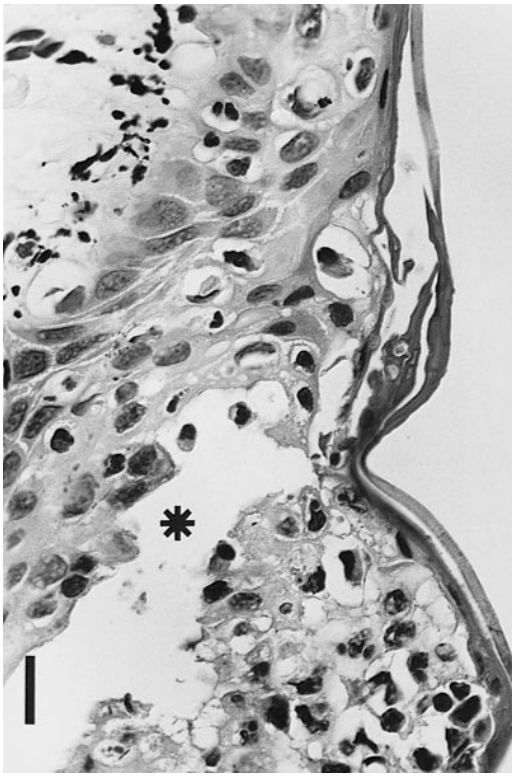

FIGURE 3. With time, RRV induced skin lesions develop to clefts and vesicles within the epidermis (asterisk). The epidermis overlying the vesicle eventually ruptures resulting in erosion and ulceration. H&E. Bar = 30 μm.

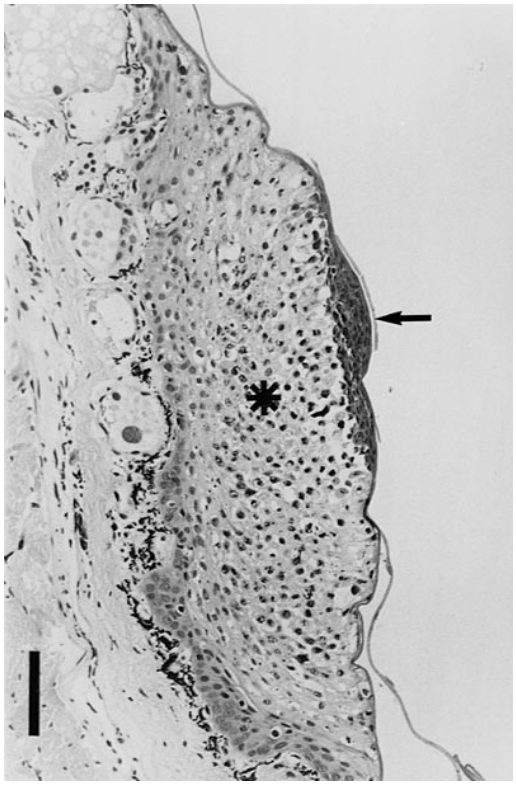

FIGURE 4. Focal area of epidermal hyperplasia (asterisk) and parakeratotic hyperkeratosis (arrow) thought to represent normal epidermal structures frequently infected with RRV. Cytoplasmic viral inclusions are numerous. H&E. Bar = 150 μm.

necropsy. These lesions progress to foci of erosion and ulceration. Small raised foci of hyperplastic and hypertrophic epidermis containing viral inclusions and areas of parakeratotic hyperkeratosis were occasionally seen, especially over the head (Fig. 4). These foci were frequently bilaterally symmetrical and were interpreted to be normal tubercles which had become infected.

Severe lesions also were observed in the gastrointestinal tract and were responsible for the vomiting and bloody diarrhea which were observed clinically in some animals. Lesions ranged from foci of mucosal cells containing viral inclusions and pyknotic nuclei to larger areas of mucosal epithelial necrosis and ulceration (Fig. 5). The ulceration was occasionally associated with hemorrhage into the lumen and, in some cases, numerous protozoan and metazoan parasites. The submucosa and lamina propria were inconsistently congested and edematous. Viral inclusions were occasionally seen in endothelial cells of the submucosa.

Hepatocytes also frequently contained viral inclusions which were either associated with single degenerate cells or larger areas of focal necrosis (Fig. 6). Hematopoietic tissue immediately subjacent to the hepatic capsule contained cells with inclusion bodies and there were varying degrees of single cell necrosis, focal necrosis and hypocellularity (Fig. 7). Similar lesions were seen in spleen, thymus and other lymphoid structures throughout the body. Inclusions and necrotic cells were seen both in glomeruli and renal tubular epithelium.

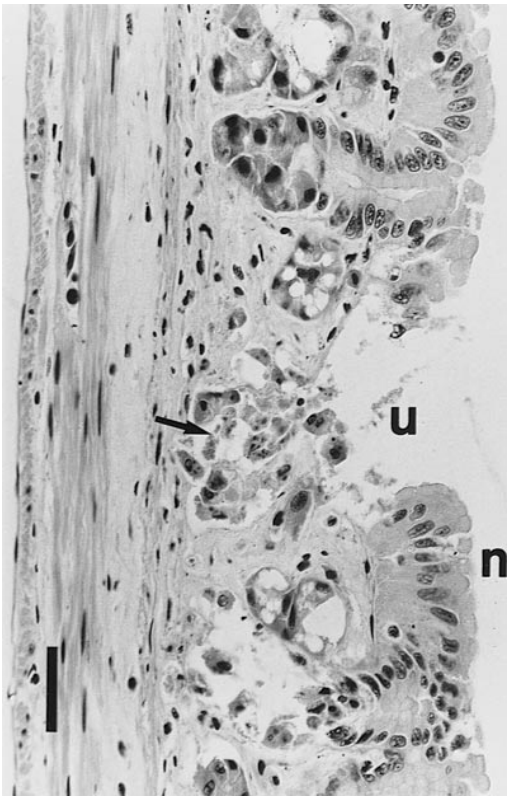

FIGURE 5. Gastric mucosa of a tiger salamander infected with RRV. Normal mucosal epithelium (n) surrounds a focal ulcer (u). The gastric gland at the base of the ulcer contains necrotic cells (arrow) with dark, pyknotic nuclei. H&E. Bar = 75 μm.

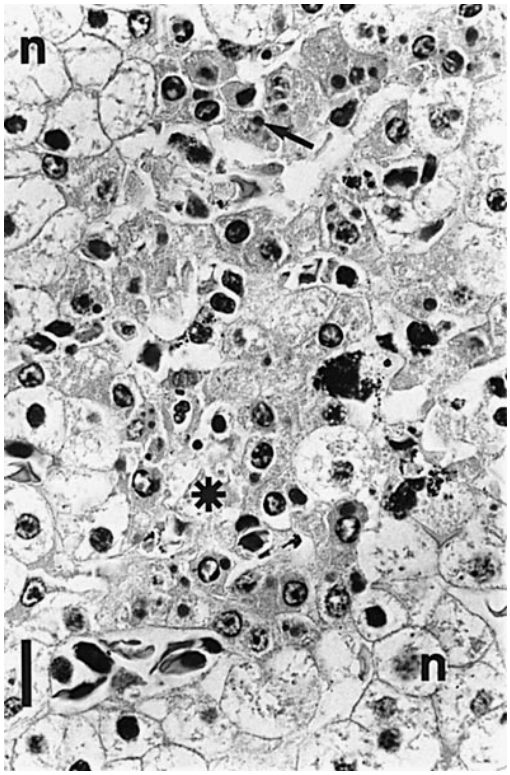

FIGURE 6. Liver from a tiger salamander infected with RRV. Focal areas of hepatocellular necrosis (asterisk) are distinguished by hypereosinophilic cytoplasm and pyknotic nuclei. Areas containing normal hepatocytes are marked with an 'n'. Cytoplasmic viral inclusions are numerous (arrow). H&E. Bar = 30 μm.

Electron microscopy of stained tissue sections lifted from glass slides revealed the cytoplasmic inclusions to be viral assembly sites with paracrystalline arrays of icosahedral virions (Fig. 8). The viruses in tissue sections lifted from glass slides were approximately 140 nm in diameter whereas negatively stained virions in EPC cells measured approximately 190 nm in diameter. Paracrystalline arrays of icosahedral virions (120–300 nm in diameter) within the cytoplasm of infected cells is pathognomonic for iridovirus infection and provides strong presumptive evidence that the etiological agent is a member of the family *Iridoviridae* (Willis et al., 1984). No discernible structures were evident in the vacuoles within the nucleus.

Aeromonas hydrophila, Aeromonas spp. and *Enterobacter* spp. were isolated in low numbers from lung, liver, kidney and spleen of two salamanders. High numbers of the same bacteria were isolated from the intestines. Given the consistent association of viral inclusions with lesions and the frequent isolation of these bacteria from aquatic species, with or without disease, the isolates were considered contaminants or secondary invaders.

Most larval salamander carcasses from pond D, collected during an outbreak in July 1997, were autolyzed, making histological interpretation difficult. However, cytoplasmic inclusions were observed in the liver and kidney of one carcass, and there were scattered areas of possible cel-

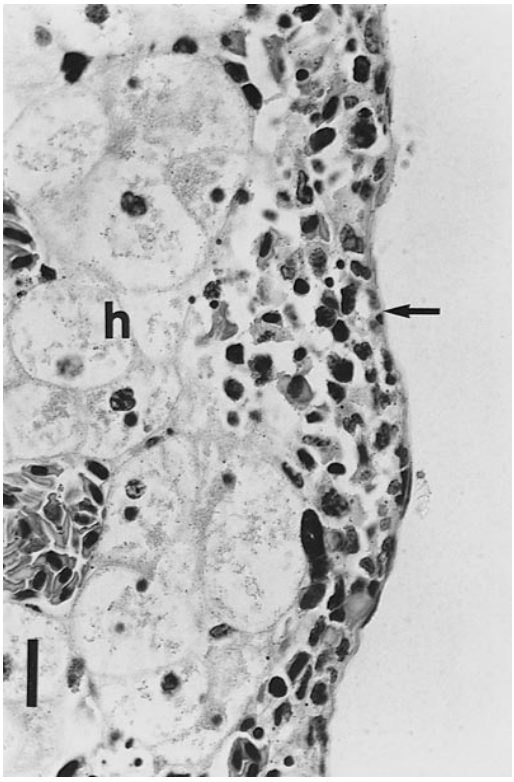

FIGURE 7. Liver from a tiger salamander infected with RRV. Hematopoietic tissue is found between hepatocytes (h) and the hepatic capsule (arrow). Hematopoietic tissue is hypocellular and several cells contain cytoplasmic viral inclusions. Some nuclei are pyknotic and others contain marginated chromatin. H&E. Bar = 30 μm.

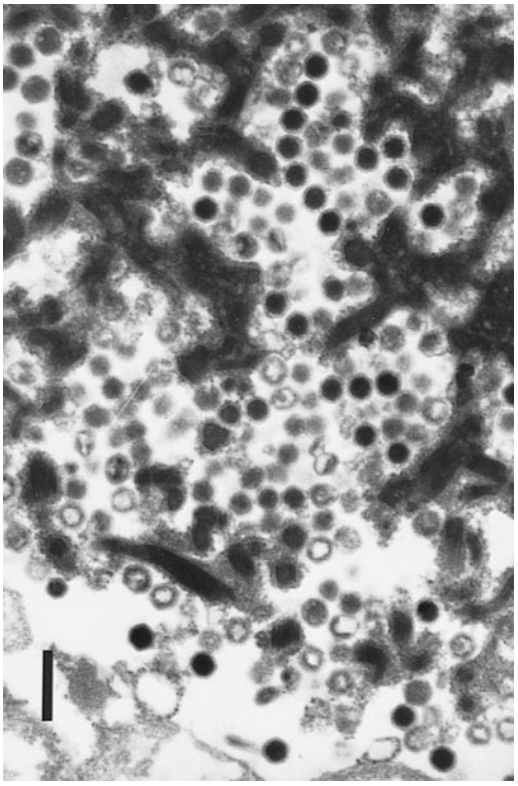

FIGURE 8. Transmission electron micrograph of liver tissue, lifted from a glass slide, showing a viral assembly site within the cytoplasm of a hepatocyte. Note the hexagonal outline of the virus and central electron-dense nucleocapsid. Bar = 0.3 μm.

lular degeneration which could not be confirmed due to autolysis. Paracrystalline arrays of viral particles similar to confirmed cases of iridovirus infection were identified by electron microscopy in hepatocytes and renal tubular epithelial cells (data not shown). Virus isolation was not attempted from these carcasses.

The diagnosis of iridovirus infection at each of the five ponds was based on a combination of clinical signs, characteristic histological lesions, transmission electron microscopy and virus isolation (Table 1). Virus was isolated from two tiger salamanders which were originally captured from ponds B and E but had been in captivity 28 and 12 days, respectively, before dying. Virus from the pond B salamander was used for molecular characterization. Virus was isolated from a diseased salamander which had been collected from pond C and frozen at −20 C for 7 mo prior to isolation attempts. All three virus isolates reacted with the FV3 monoclonal antibody.

Virus characterization

Although the studies cited above suggest that an iridovirus was responsible for the salamander epizootics, it is not known which iridovirus species is the etiological agent. Since FV3 and LT-1 through LT-4 (an iridovirus isolated from a frog tumor after passage in the red eft and designated *Lucke-Triturus* virus) replicate to high titers in red efts and in some adult newts, it is possible that one of these viruses, or

a novel iridovirus agent, is responsible for salamander mortalities (Clark et al., 1968). To identify the iridovirus agent responsible for salamander mortalities, we characterized the agent using a panel of molecular assays: SDS-polyacrylamide gel electrophoresis of viral protein synthesis, analysis of restriction enzyme digests of viral DNA, and sequence analysis of the major capsid protein.

EPC cultures were inoculated with tissue homogenates from infected salamanders and observed for the development of CPE. When cultured at ~20 C, CPE was readily detected by 9 days post infection. For molecular characterization, RRV was grown in FHM cells, a cell line shown to support the growth of FV3 and a variety of other ranaviruses (Mao et al., 1997). Initial attempts to grow RRV in FHM cells at 26 to 28 C, a temperature permissive for other ranaviruses (Mao et al., 1997), were unsuccessful. However, as in EPC cells, virus grew well at lower temperatures, i.e., 19 to 23 C. Infection of FHM cells with an early passage, low titer stock resulted in progressive CPE accompanied by focal necrosis (plaques). Infected cultures were radiolabeled 6 (early, E) and 7 (late, L) days post infection for 6 hr with [^{35}S]methionine, and radiolabeled proteins were analysed by SDS-polyacrylamide gel electrophoresis. As shown in Figure 9, multiple novel bands, not detected in mock-infected FHM cells were observed in RRV infected cultures. In day 6 cultures (E) inhibition of host cell protein synthesis was not yet complete, whereas by day 7 (L) only virus specific proteins were detected. Moreover, a major protein band, co-migrating with the major capsid protein (MCP) of FV3, was readily detected in RRV infected cultures. The presence of a ~48 kDa MCP in RRV is consistent with the suggestion that the infectious agent is an iridovirus (Mao et al., 1997).

To determine whether the agent responsible for salamander mortality was FV3, an FV3-like agent such as LT-1, or a novel virus, radiolabeled DNA was extracted from RRV infected cells and subjected to restriction endonuclease (REN) analysis. Because iridovirus infection inhibits host cell DNA synthesis, radiolabeled (i.e., newly synthesized) DNA, isolated from infected cells when CPE is marked, is predominantly of viral origin. Digestion of DNA from RRV infected cells with *Hin*dIII and *Xba*I showed profiles (Fig. 10) distinct from FV3, LT1, and other known ranaviruses (Essani and Granoff, 1989; Mao et al., 1997). To strengthen the suggestion that RRV is an iridovirus, DNA from RRV infected cells was digested with *Hpa*II or *Msp*I. Although both enzymes

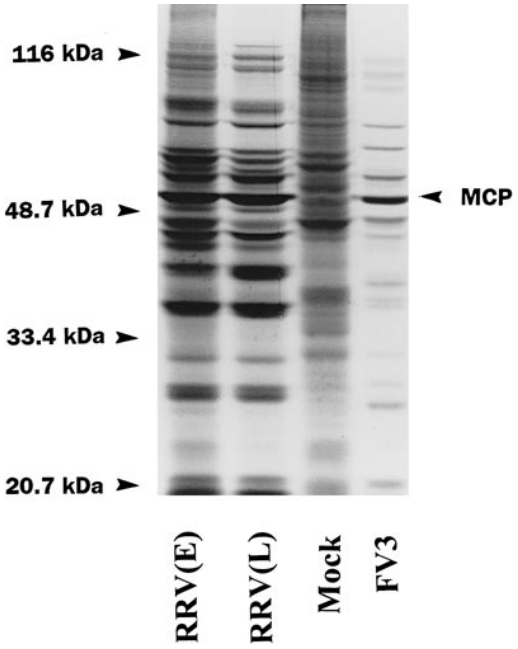

FIGURE 9. Protein synthesis in RRV infected cells. FHM cells were infected with RRV at a low multiplicity of infection (~5×10^{-5} $TCID_{50}$/cell) and at 6 and 7 days post infection, a time when CPE was marked, FHM cells were radiolabeled with methionine-deficient EMEM containing 25 μCi/ml [^{35}S]methionine for 6 hr. As controls, mock-infected and FV3-infected FHM cells were similarily labeled. Radiolabeled proteins were separated on 10% SDS-polyacrylamide gels and visualized by autoradiography. RRV(E) and RRV(L) denote proteins labeled in RRV infected cells at 6 (E) and 7 (L) days post infection. Molecular weight markers are shown to the left of the autoradiogram, and the position of the FV3 major capsid protein (MCP) is indicated.

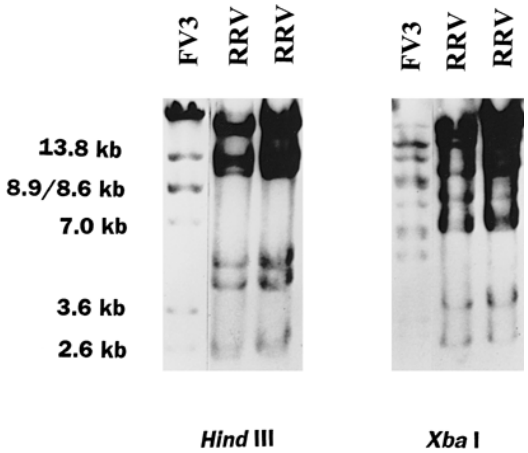

FIGURE 10. Restriction endonuclease digestion of viral DNA: *Hin*dIII and *Xba*I profiles. Viral DNA labeled *in vitro* as described in Methods was extracted, digested with the indicated restriction endonucleases, separated by agarose gel electrophoresis, and visualized by fluorography. Viral DNA was prepared from FV3-infected (FV3) as well as duplicate RRV infected cultures (RRV). RRV profiles were overexposed to show faint lower molecular weight bands. Note RRV profiles are distinctly different than those of FV3.

recognize the same target sequence (5′ CCGG 3′), the former will not cleave methylated DNA (i.e., 5′ CC®GG 3′), whereas the latter is insensitive to methylation. As seen in Figure 11, DNA from both RRV and FV3-infected cells was not cleaved by *Hpa*II, but was readily digested with *Msp*I indicating that RRV, like FV3, contains a highly methylated genome, a feature common to vertebrate iridoviruses (Willis et al., 1984). As a control for nuclease activity, DNA isolated from cells infected with *Ictalurus melas* herpesvirus (ImHV) (Alborali et al., 1996), a piscine virus which does not contain a highly methylated genome, was readily digested by both enzymes.

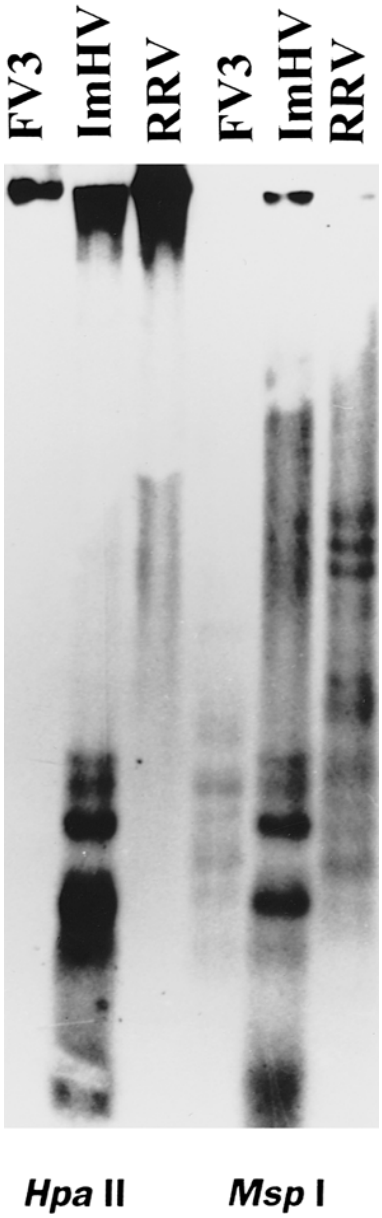

FIGURE 11. Restriction endonuclease digestion of viral DNA: *Msp*I and *Hpa*II. Viral DNA was prepared as in Figure 10 and digested with either *Msp*I or *Hpa*II. As a positive control for digestion with *Hpa*II, DNA was also prepared from cells infected with a newly isolated herpesvirus from *Ictalurus melas* (ImHV). Following digestion, viral DNA was analyzed as in Figure 10.

Sequence analysis of RRV

To verify that RRV is an iridovirus, DNA was amplified from RRV infected cells by PCR using oligonucleotide primers specific for highly conserved regions within the major capsid protein (MCP) gene of known iridoviruses (Mao et al., 1997). The RRV product co-migrated with a ~500 nu-

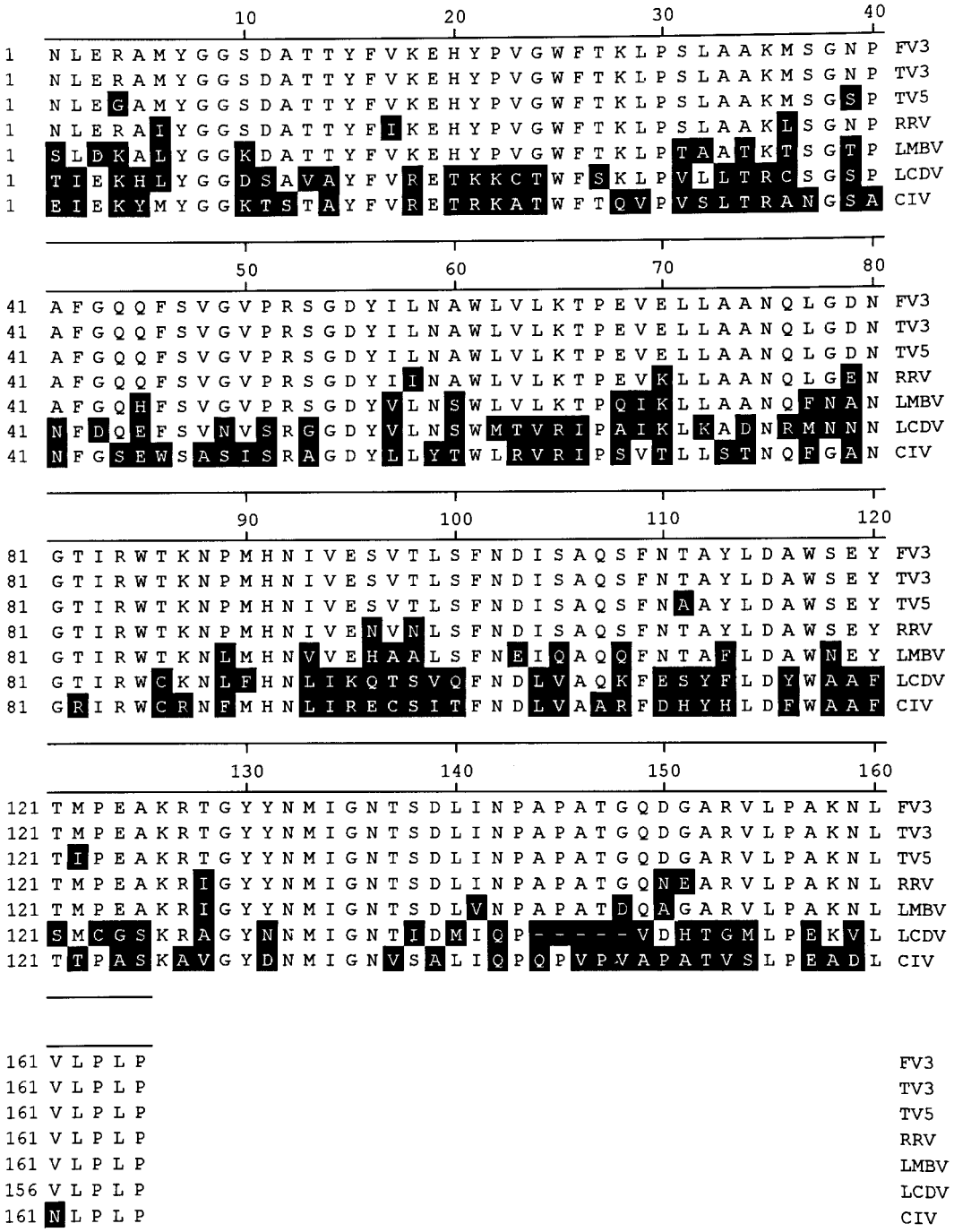

FIGURE 12. Multiple alignment of the deduced amino acid sequence of RRV and other vertebrate and invertebrate iridoviruses. The 5′ end of the MCP gene of RRV was amplified by PCR, cloned, and its sequence determined by the dideoxynucleotide chain termination method of Sanger et al. (1977). The amino acid sequence of the RRV MCP (amino acids 15–191) was deduced and aligned with representative iridovirus sequences using the program MEGALIGN within DNASTAR. The FV3 amino acid sequence is shown in line 1 and amino acid differences from that sequence are highlighted by filled squares. Amino acids encoded by the forward and reverse primers are not shown. The Accession Number for the RRV sequence is AF080218 and the Accession Number for the iridovirus isolated by Plum et al. (1996) from largemouth bass (LMBV) is

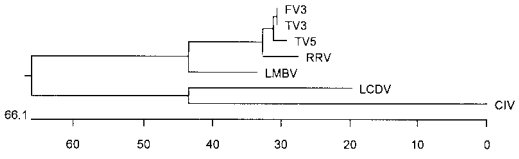

FIGURE 13. Phylogenetic analysis of the iridovirus major capsid protein: Taxonomic position of RRV. A phylogenetic tree was constructed using the multiple alignment shown in Figure 12 and the CLUSTAL V algorithm within MEGALIGN (DNASTAR). Branch length, indicated by the scale beneath the tree, is proportional to the number of amino acid substitutions.

cleotide product amplified using FV3 DNA as template (data not shown) and was subsequently cloned and sequenced. A multiple alignment of the deduced amino acid sequence of the RRV product along with corresponding sequences from representative invertebrate and vertebrate iridoviruses is shown in Figure 12. At the amino acid level, RRV is ~93% identical to FV3 and occupies an intermediate position between a cluster of closely-related isolates (FV3, TV3, TV5) and more distantly-related isolates (LMBV, LCDV, and CIV). These relationships are depicted graphically in Figure 13 in which a phylogenetic tree, based on data provided by the multiple alignment, is shown. Taken together, the sequence data provides unequivocal evidence that RRV is an iridovirus, and indicates that it is more closely related to FV3 (genus *Ranavirus*) than to LCDV (genus *Lymphocystivirus*). Furthermore, the data (Fig. 13) supports the division of the family Iridoviridae into at least three genera, i.e., genus *Iridovirus* (CIV), genus *Lymphocystivirus* (LCDV), and the genus *Ranavirus* (FV3, TV3, TV5, RRV, and LMBV).

Experimental challenge

Virus grown in EPC culture was used to infect two tiger salamanders i.p. Both experimentally infected animals died 13 days following inoculation with $5 \times 10^{6.23}$ $TCID_{50}$ of virus. Although neither animal showed obvious clinical signs, at necropsy lesions in both salamanders were similar to those described previously. Virus was isolated from both salamanders and identified as an iridovirus by electron microscopy. These observations indicate that the virus detected in tissue culture and by electron microscopy is the etiological agent of disease.

DISCUSSION

An iridovirus closely related yet distinct from FV3 was identified as the cause of an epizootic in tiger salamanders in southern Saskatchewan during the summer of 1997. Total mortality and mortality rates were difficult to estimate; however, based on the number of carcasses found in the field and the virtual absence of adult salamanders after the outbreak in pond B and C, mortality was assumed to be high. High mortality was observed in salamanders captured from these sites during the mortality event and in previously captive salamanders indirectly exposed to infected individuals. These observations, along with the death of both salamanders inoculated i.p. with the isolated virus, suggest the virus is highly infectious and virulent. Further studies using natural routes of exposure, such as ingestion or bath immersion, are needed to characterize the infectivity and virulence of this virus.

A concurrent epizootic in tiger salamanders over 200 km north of Regina also appeared to be caused by an iridovirus, although no attempt was made to characterize the virus. Further work is needed to determine the geographic range of RRV and determine if other iridoviruses are circulating in tiger salamander populations.

Lesions indicate tiger salamanders died

←

AF080250; accession numbers for the other listed sequences can be found in Mao et al. (1997). Turtle virus 3 (TV3), turtle virus 5 (TV5) lymphocystis disease virus (LCDV), *Chilo* iridescent virus (CIV).

of a generalized viremia affecting virtually all tissues except for the nervous system and musculoskeletal system. The most severe lesions were observed in the skin, gastrointestinal tract, liver, lymphoid, and hematopoietic tissue. Lesions, including the presence of cytoplasmic inclusions, were similar to those described for other iridovirus diseases such as epizootic haematopoietic necrosis virus (EHNV) infection which affects wild redfin perch and farmed rainbow trout in Australia (Reddacliff and Whittington, 1996) and Bohle iridovirus infecting 4 frog species in Australia (Speare and Smith, 1992; Cullen et al., 1995). Acute necrotizing myocarditis as seen with EHNV infections was not observed in RRV infections. Skin lesions also were not as prominent in EHNV infections and consisted only of occasional skin ulceration.

An epizootic in Sonora tiger salamanders (*Ambystoma tigrinum stebbinsi*) caused by an iridovirus has been recently described (Jancovich et al., 1997) which has some similarities to the die-off in Saskatchewan. The iridovirus, named *Ambystoma tigrinum* virus (ATV) was highly infectious and pathogenic to salamanders, similar to that observed with RRV infections. Skin lesions were the most obvious abnormality reported and were used to describe four stages of disease progression. Light microscopic observations characterized the skin lesions as polyps caused by proliferation of epidermal cells. Cells of the epidermis, gills and liver were described as having "enlarged nuclei with marginated chromatin and nuclear inclusions". Sloughed skin and mucus were described in salamanders captured in the field. This differs from RRV induced illness where skin lesions, although common, were not consistently seen. Histological lesions in RRV infected salamanders consisted of focal areas of intracellular and intercellular epidermal edema and necrosis leading to vesicle formation, erosion and ulceration. Single cell and focal necrosis of liver, gastrointestinal mucosa, spleen, hematopoietic tissue, kidney and other tissues were also common in RRV infected salamanders, but were not reported in ATV infected salamanders. The incubation period also differed between RRV and ATV infected salamanders, 13 days and 5 to 7 days, respectively. Differences in incubation periods may reflect the temperature at which the salamanders were housed after exposure. RRV exposed salamanders were housed at approximately 20 C while ATV exposed salamanders were held at 25 C. When ATV-infected salamanders were housed at 8 C they took over 21 days to develop clinical signs (Jancovich et al., 1997). Temperature differences also were noted *in vitro.* Whereas most of the ranaviruses studied previously replicated in FHM cells at 26 to 28 C (Mao et al., 1997), replication of RRV was restricted at this temperature. In contrast, ATV-infected cell lines were incubated at 24 C. Thus it appears that ATV and RRV differ in their sensitivity to temperature.

The molecular studies described above complement the electron microscopy and demonstrate that RRV is a member of the genus *Ranavirus.* Moreover, sequence analysis indicates that RRV, although distinct from FV3, is more closely related to FV3 and other ranaviruses than it is to LCDV-1 (genus *Lymphocystivirus*). The highly conserved nature of the iridovirus MCP (Mao et al., 1996; Mao et al., 1997; Tidona et al., 1998) reenforces the suggestion that definitive identification of an iridovirus requires more than simply demonstrating serological cross-reactivity with a given antiserum. Cells infected with FV3, EHNV, ECV (European catfish virus), and ESV (European sheatfish virus) cross-react with a mouse monoclonal antibody (Chinchar et al., 1984) directed against the FV3 MCP (V. G. Chinchar, unpubl. obs.), and the same antibody also cross-reacted with RRV infected cells. Thus, to distinguish among closely related ranavirus isolates, definitive identification of an unknown iridovirus requires sequence analysis of one or more viral genes.

The presence of highly conserved regions within the major capsid protein, coupled with considerable differences in less conserved regions, suggests that sequence analysis of the MCP gene may be an appropriate way to distinguish among different iridovirus strains and species. Based on the suggestion of Ward (1993) that strains within a given virus species show greater than 80% identity within specific genes, it appears as if RRV, TV3, TV5, and FV3 may be strains of the same viral species. However, differences in restriction endonuclease profiles, growth properties (i.e., the temperature restriction of RRV), and pathological findings may be sufficient to classify it as a distinct species within the genus *Ranavirus.* Finally, given controversy concerning the correct nomenclature applicable to iridoviruses—i.e., whether to name the virus after the first species in which it was isolated or to identify a given isolate by a geographical descriptor (Williams, 1996)—we have provisionally designated the isolate from Canadian salamanders as Regina Ranavirus (RRV). This name distinguishes it from the isolate made from a subspecies of tiger salamander in the southwestern United States (Jancovich et al., 1997), but does not imply that salamanders are the only species susceptible to infection, nor does it imply, without additional serological and molecular studies, that ATV and RRV are distinct viral species. Further experimentation will be required to determine the taxonomic relationship of ATV and RRV and to ascertain if other amphibian, reptilian, or piscine species are susceptible to RRV infection.

This is the first report of an iridovirus infecting tiger salamanders in Canada and only recently have iridoviruses been recognized as a cause of mortality in tiger salamanders in the United States (Jancovich et al., 1997). Whether this is a previously unrecognized virus, endemic in tiger salamander populations, or a virus endemic to sympatric poikilothermic species remains to be determined. Striped chorus frogs (*Pseudacris triseriata*), leopard frogs (*Rana pipiens*) and wood frogs (*Rana sylvatica*) along with several species of native and introduced (e.g., rainbow trout, *Oncorhynchus mykiss*) fish were found at these ponds (Table 1). Future studies will examine these populations to determine whether sympatric poikilothermic vertebrates, other than salamanders, are susceptible to infection with RRV.

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LITERATURE CITED

ALBORALI, L., G. BOVO, A. LAVAZZA, H. CAPPELLARO, AND P. F. GUADAGNINI. 1996. Isolation of an herpesvirus in breeding catfish (*Ictalurus melas*). Bulletin of the European Association of Fish Pathologists 16: 134–137.

AHNE, W., H. J. SCHLOTFELDT, AND I. THOMSEN. 1989. Fish viruses: Isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). Journal of Veterinary Medicine B 36: 333–336.

———, M. BREMONT, R. P. HEDRICK, A. D. HYATT, AND R. J. WHITTINGTON. 1997. Special topic review: Iridoviruses associated with epizootic haematopoietic necrosis (EHN) in aquaculture. World Journal of Microbiology and Biotechnology 13: 367–373.

ARMSTRONG, R. D., AND H. W. FERGUSON. 1989. Systemic viral disease of the chromide cichlid *Etroplus maculatus.* Diseases of Aquatic Organisms 7: 155–157.

BLANK, H., C. DAVIS, AND C. COLLINS. 1970. Electron microscopy for the diagnosis of cutaneous viral infections. British Journal of Dermatology 83: 69–80.

CHINCHAR, V. G., D. W. METZGER, A. GRANOFF, AND R. GOORHA. 1984. Localization of frog virus 3 proteins using monoclonal antibodies. Virology 137: 211–216.

CLARK, H. F., J. C. BRENNAN, R. F. ZEIGEL, AND D. T. KARZON. 1968. Isolation and characterization

of a virus from the kidneys of *Rana pipiens* with renal adenocarcinoma before and after passage in the red eft, *Triturus viridescens.* Journal of Virology 2: 629–640.

CULLEN, B. R., L. OWENS, AND R. J. WHITTINGTON. 1995. Experimental infection of the Australian anurans (*Limnodynastes terraereginae* and *Litoria latopalmata*) with Bohle iridovirus. Diseases of Aquatic Organisms 23: 83–92.

CUNNINGHAM, A. A., T. E. S. LANGSTON, P. M. BENNETT, J. F. LEWIN, S. E. N. DRURY, R. E. GOUGH, AND S. K. MACGREGOR. 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philosophical Transactions of the Royal Society of London: Biological Sciences 351: 1539–1557.

ENRIQUEZ, R. 1993. Charakterisierung dreier Fisch Iridovirus-Isolate vom Flußwels (*Silurus glanis*), Katzenwels (*Ictalurus melas*) und Flußbarsch (*Perca fluviatilis*). Veterinary Medicine Dissertation, University of Munich, Germany, 150 pp.

ESSANI, K., AND A. GRANOFF. 1989. Amphibian and piscine iridoviruses proposal for nomenclature and taxonomy based on molecular and biological properties. Intervirology 30: 187–193.

GRANOFF, A., P. E. CAME, AND K. A. RAFFERTY. 1965. The isolation and properties of viruses from *Rana pipiens:* The possible relationship to renal carcinoma. Annals of the New York Academy of Science 126: 237–255.

HEDRICK, R. P., T. S. MCDOWELL, W. AHNE, C. TORHY, AND P. DEKINKELIN. 1992a. Properties of three iridovirus-like agents associated with systemic infections of fish. Diseases of Aquatic Organisms 13: 203–209.

———, ———, J. M. GROFF, S. YUN, AND W. H. WINGFIELD. 1992b. Isolation and some properties of an iridovirus-like agent from white sturgeon *Acipenser transmontanus.* Diseases of Aquatic Organisms 12: 75–81.

———, AND ———. 1995. Properties of iridoviruses from ornamental fish. Veterinary Research 26: 423–427.

JANCOVICH, J. K., E. W. DAVIDSON, J. F. MORADO, B. L. JACOBS, AND J. P. COLLINS. 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi.* Diseases of Aquatic Organisms 31: 161–167.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

LANGDON, J. S., J. D. HUMPHREY, L. M. WILLIAMS, A. D. HYATT, AND H. A. WESTBURY. 1986. First virus isolation from Australian fish: An iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. Journal of Fish Diseases 9: 263–268.

LUNA, L. G. 1992. Histopathological methods and color atlas of special stains and tissue artifacts. American Histolabs, Inc. Gaithersburg, Maryland, 767 pp.

MAO, J., T. N. THAM, G. A. GENTRY, A. M. AUBERTIN, AND V. G. CHINCHAR. 1996. Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. Virology 216: 431–436.

———, R. P. HEDRICK, AND V. G. CHINCHAR. 1997. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229: 212–220.

———, D. E. GREEN, G. FELLERS, AND V. G. CHINCHAR. 1999. Molecular characterization of iridoviruses isolated from sympatric amphibians and fish. Virus Research 1999: In press.

MOODY, N. J. G., AND L. OWENS. 1994. Experimental demonstration of the pathogenicity of a frog virus, Bohle iridovirus, for a fish species, barramundi *Lates calcarifer.* Diseases of Aquatic Organisms 18: 95–102.

PLUMB, J. A., J. M. GRIZZLE, H. E. YOUNG, AND A. D. NOYES. 1996. An iridovirus isolated from wild largemouth bass. Journal of Aquatic Animal Health 8: 265–270.

POZET, F., M. MORAND, A. MOUSSA, C. TORHY, AND P. DEKINKELIN. 1992. Isolation and preliminary characterization of a pathogenic icosahedral deoxyribovirus from the catfish *Ictalurus melas.* Diseases of Aquatic Organisms 14: 35–42.

REDDACLIFF, L. A., AND R. J. WHITTINGTON. 1996. Pathology of epizootic haematopoietic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss* Walbaum) and redfin perch (*Perca fluviatilis* L). Journal of Comparative Pathology 115: 103–115.

SANGER, F., S. NICKLEN, AND A. R. COULSON. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science, USA 74: 5463–5467.

SHEEHAN, D. C., AND B. B. HRAPCHAK. 1980. Theory and practice of histotechnology, 2nd Edition. The C. V. Mosby Company, St. Louis, Missouri, 481 pp.

SPEARE, R., AND J. R. SMITH. 1992. An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. Diseases of Aquatic Organisms 14: 51–57.

TAMAI, T., K. TSUJIMURA, S. SHIRAHATA, H. ODA, T. NOGUCHI, R. KUSUDA, N. SATO, S. KIMURA, Y. KATAKURA, AND H. MURAKAMI. 1997. Development of DNA diagnostic methods for the detection of new fish iridoviral diseases. Cytotechnology 23: 211–220.

TIDONA, C. A., P. SCHNITZLER, R. KEHM, AND G. DARAI. 1998. Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? Virus Genes 16: 59–66.

WARD, C. W. 1993. Progress towards a higher taxonomy of viruses. Research in Virology (Institute Pasteur) 144: 419–453.

WILLIAMS, T. 1996. The iridoviruses. Advances in Virus Research. 48: 345–412.

WILLIS, D. B., R. GOORHA, AND V. G. CHINCHAR. 1984. Macromolecular synthesis in cells infected by frog virus 3. Current Topics in Microbiology and Immunology 116: 77–106.

———. 1990. Taxonomy of iridoviruses. *In* Molecular biology of iridoviruses, G. Darai (ed.). Kluwer Academic Publishers, Boston, Massachusetts, pp. 1–12.

WOLF, K., G. L. BULLOCK, C. E. DUNBAR, AND M. C. QUIMBY. 1968. Tadpole edema virus: Viscerotropic pathogen for anuran amphibians. Journal of Infectious Disease 118: 253–262.

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