Comparison of epizootic haematopoietic necrosis and Bohle iridoviruses, recently isolated Australian iridoviruses

Sandra G. Hengstberger¹, Alex D. Hyatt^{1,*}, Rick Speare², Barbara E. H. Coupar¹

¹ Australian Animal Health Laboratories, C.S.I.R.O., PO Bag 24, Geelong 3220, Victoria, Australia

² Anton Breinl Centre for Tropical Health and Medicine, James Cook University of North Queensland, Townsville 4811, Queensland, Australia

ABSTRACT: Epizootic haematopoietic necrosis virus (EHNV) and Bohle virus (BIV) are piscine and amphibian iridoviruses respectively which have been isolated in Australia from redfin perch *Perca fluviatilis* L., cultured rainbow trout *Oncorhynchus mykiss* (Walbaum) and the ornate burrowing frog *Limnodynastes ornatus* (Gray). To determine whether the viruses were similar they were examined at the ultrastructural, protein, antigenic and genomic levels. EHNV was found to differ from BIV in cytopathic effect on host cells, size, site of virus release, molecular weights of proteins and restriction endonuclease digest profiles of the respective DNA's. On the other hand both iridoviruses were also found to infect a similar range of tissue culture cells, exhibit a similar sequence of ultrastructural events and possess proteins of similar antigenic reactivity. DNA from EHNV and BIV cross hybridized to a high level. When the BIV polypeptides were compared to the published data for purified frog virus 3 (FV3) and with the appropriate restriction enzyme profiles, a similarity was noted between the 2 viruses. This implies that EHNV, which shares many structural and biochemical characteristics with BIV, is itself related to the *Ranavirus* genus of iridoviruses.

INTRODUCTION

Epizootic haematopoietic necrosis virus (EHNV), a piscine iridovirus (Eaton et al. 1991), has been isolated from redfin perch *Perca fluviatilis* L. and cultured rainbow trout *Oncorhynchus mykiss* (Walbaum) in Victoria, Australia (Langdon et al. 1986, 1988). Outbreaks reported since 1984 have involved mass mortalities of fish populations within Victoria, New South Wales and South Australia. EHNV is also known to cause disease in a range of fish species (Langdon 1989) and may be implicated in the population decline of at least 1 indigenous species, namely Macquarie perch *Macquaria australasia* (Curvier) (Langdon 1989). The presence of the virus in Australian rainbow trout hatcheries has resulted in the enforcement of restrictions in the sale and movement of fish from these hatcheries.

The family Iridoviridae encompasses 4 named genera including *Iridovirus* (represented by type 1 Tipula iridescent virus), *Chloriridovirus* (e.g. type 2 mosquito iridescent virus), *Ranavirus* (e.g. frog virus 3, FV3), *Lymphocystivirus* (e.g. fish lymphocystis disease virus, FLDV) and a fifth proposed 'goldfish group' (Francki et al. 1991). The piscine iridoviruses include FLDV and goldfish virus (GFV). These 2 groups are separated into different genera based upon ultrastructural and biochemical characteristics (Essani & Granoff 1989). EHNV has also been described as an iridovirus (Eaton et al. 1991) and, although it has not been compared directly with FLDV and GFV, it has been reported to be biochemically different from other piscine iridoviruses and ultrastructurally different from FLDV. Comparison of the polypeptide and DNA restriction enzyme digest profiles of EHNV with those described in the literature for other iridoviruses has indicated that EHNV is more closely related to the genus *Ranavirus* than to the 'goldfish virus group' (Eaton et al. 1991).

* Addressee for correspondence

Recently, an iridovirus termed 'Bohle virus' (BIV) has been isolated from the ornate burrowing frog *Limnodynastes ornatus* (Gray) in Queensland, Australia (Speare & Smith 1992). The virus was reported to vary in size from 137 to 172 nm, contain methylated double stranded DNA and replicate in a variety of piscine and mammalian cell lines (Speare & Smith 1992). Based on this information Speare & Smith (1992) speculated that BIV was similar to EHNIV and to ranaviruses such as FV3.

The isolation of BIV represents the second iridovirus isolated from aquatic species in Australia. Both viruses originated from organisms in environments with an overlapping geographical distribution. In this paper we compare the ultrastructural, protein and molecular characteristics of the above isolates. This information is used to ascertain whether both piscine and amphibian viruses belong to the same or different genera.

MATERIALS AND METHODS

Cells and viruses. Bluegill fry (BF-2, ATCC CCL 91) and chinook salmon embryo (CHSE-214, ATCC CRL 1681) cells were grown at 24 °C and Vero cells (ATCC CCL 81) at 37 °C in Eagle's minimum essential medium (EMEM) containing 10 % foetal calf serum (FCS) and 10 mM HEPES. *Aedes albopictus* (C636, Australian National University, Canberra, Australia) cells were grown at 24 °C in basal medium of Eagle (BME) containing 1 % FCS and 1 % non-essential amino acids. Baby hamster kidney (BHK-21) cells referred to hereafter as BHK (Commonwealth Serum Laboratories, Melbourne, Australia) were grown at 37 °C in EMEM containing 10 % FCS, 10 mM HEPES and 5 % tryptose phosphate broth. Madin-Darby bovine kidney (MDBK, ATCC CCL 22) cells were grown at 37 °C in EMEM containing 5 % FCS and 10 mM HEPES. Pig kidney 15 (PK15, ATCC CCL 33) cells were grown at 37 °C in EMEM containing 5 % FCS, 10 mM HEPES and 4 % sodium pyruvate.

Isolates of EHNIV originally obtained from rainbow trout (EHNIV_T; Langdon 1989) and redfin perch (EHNIV_{RF}; Langdon et al. 1986) were plaque purified 3 times in BF-2 cells. Stock EHNIV was grown in BF-2 and CHSE-214 cell monolayers. Following infection at a multiplicity of infection (MOI) of ca 0.1 plaque forming units (PFU) per cell and incubation for 48 h at room temperature (RT), cells and debris were removed by centrifugation and released virus in the culture medium (approximate titre 10^5 PFU ml⁻¹) was stored at 4 °C. Following purification, as described below, stocks of intracellular (cell-associated) virus contained ca 10^9 PFU ml⁻¹.

An isolate of BIV obtained from *Limnodynastes ornatus* (Speare & Smith 1992) was passaged 6 times in BF-2 cells. Following infection and incubation for 48 h at RT, cells and debris were removed by centrifugation and released virus in the culture medium (approximate titre 10^6 PFU ml⁻¹) was stored at 4 °C. Following purification as described below, stocks of intracellular (cell-associated) virus contained ca 10^7 PFU ml⁻¹.

Purification of intracellular virus. Monolayers of CHSE-214 cells in sixty 150 cm² tissue culture flasks were infected with released virus (EHNIV or BIV) at a MOI of ca 0.1 PFU cell⁻¹. At 48 h post infection (p.i.) cells were removed from the flasks and pelleted by centrifugation at $2600 \times g$ in a bench-top centrifuge for 10 min. Pelleted cells were resuspended in 5 ml RSB (10 mM Tris (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂) and 0.5 ml 1,1,2-Trichlorotrifluoroethane (Genitron) and lysed. Virus was sheared from the cytoskeleton in a motor-driven Dounce homogenizer and purified as described by Eaton et al. (1991).

Cell susceptibility. Stocks of EHNIV_{RF}, EHNIV_T and BIV (100 µl) were added to monolayers of C636, BF-2, CHSE-214, Vero, BHK, MDBK and PK15 cells in 25 cm² tissue culture flasks. The cells were incubated for 1 h at RT and excess virus removed by washing 4 times in PBS before the appropriate medium (refer above) was added. At 1, 2, 3, 4 and 8 d p.i. the cytopathic effect (CPE) was recorded. At Day 8 p.i. culture supernatants were collected and analysed by enzyme-linked immunosorbent assays (ELISA) (refer below) for the presence of progeny viruses.

Enzyme linked immunosorbent assay (ELISA). The ELISA used in this study was based on that of Hyatt et al. (1991) and was used to determine if the virus isolates were replicating in the various cell types. The coating primary antibodies were sheep anti-EHNIV_T (raised to whole virus, 1:30 000) or sheep anti-EHNIV_{RF} (whole virus, 1:15 000). Secondary antibodies consisted of either rabbit anti-EHNIV_T (whole virus, 1:20 000), rabbit anti-EHNIV_T (capsid, 1:20 000, Eaton et al. 1991), rabbit anti-EHNIV_{RF} (whole virus, 1:10 000) or rabbit anti-BIV (whole virus, 1:500). The antigens consisted of EHNIV_T, EHNIV_{RF} or BIV at titers of: 4.5×10^7 , 2.2×10^7 and 1.0×10^7 PFU ml⁻¹ respectively (Expt a); and 5.3×10^6 , 1.8×10^7 and 5.2×10^5 PFU ml⁻¹ respectively (Expt b). When CPE was apparent the plates were read in a BIORAD Model 2550 EIA Reader at 450 nm and the data were recorded as optical densities (OD) of experimental sample less control. The control consisted of supernatants from uninfected cells which were processed as per experimental samples.

Electron microscopy. Negative contrast electron microscopy: Purified, cell-associated EHNIV_{RF}, EHNIV_T and BIV were adsorbed to copper grids and negatively stained with 2 % phosphotungstic acid (PTA) adjusted

to pH 6.8 with 1 M KOH. CHSE-214 cells were grown on carbon coated, filmed gold grids as described by Hyatt et al. (1987), infected with EHN_{RF}, EHN_T or BIV and stained at 24 h p.i. with 2 % PTA adjusted to pH 6.8 with 1 M KOH.

Thin section electron microscopy: BF-2 cells infected with EHN_{RF} or EHN_T were scraped and pelleted at 13 h p.i. CHSE-214 cells infected with EHN_{RF} or BIV were scraped and pelleted at 24 h p.i. Pellets were fixed in phosphate (Sorenson's) buffered 2.5 % glutaraldehyde, washed with Sorenson's buffer, post-fixed in 1 % osmium tetroxide, dehydrated in graded alcohols, infiltrated with propylene oxide and embedded in Polarbed 812 Epon resin. Ultra-thin sections were double stained in uranyl and lead citrate. Particles exhibiting a hexagonal outline were measured vertex to vertex. An Agar aids diffraction grating replica (2160 lines mm⁻¹) was used to calibrate the magnification of the electron microscope.

Scanning electron microscopy: Cells (CHSE-214) were grown on glass coverslips and infected with both piscine and amphibian iridoviruses. At 18 h p.i., cells were fixed in cacodylate buffered 2.5 % glutaraldehyde (30 min), washed in buffer (3 × 20 min) post-fixed in buffered 1 % osmium tetroxide (1 h), washed in double distilled water (3 × 5 min) and dehydrated in graded ethanol. Following dehydration, cells were critically point dried from carbon dioxide and sputter-coated with gold. Samples were viewed with a JEOL JSM 840 at 20 kV.

Immunoelectron microscopy: Purified, cell-associated EHN_{RF}, EHN_T and BIV were adsorbed to filmed nickel grids which were washed with PBS (3 × 1 min) and blocked with 5 % cold-water fish gelatin (f.g.) in PBS for 10 min. The grids were then placed in 1 % f.g. for a further 10 min and incubated in either rabbit anti-EHN_T (1:200), anti-EHN_{RF} (1:100) or anti-BIV (1:5) 1 h at RT. Grids were subsequently washed (6 × 3 min) with 1 % f.g. in PBS, incubated with protein A-gold (1:200 with 1 % f.g. in PBS) for 1 h at RT and then washed in PBS (3 × 1 min). The grids were post-fixed in 2.5 % glutaraldehyde (2 min), washed in water (3 × 3 min), and stained with 1 % aqueous uranyl acetate for 30 s.

Analysis of viral proteins from infected cells and purified virus. BIV-Infected cells: BIV was adsorbed to CHSE-214 cells at a MOI of 0.1 PFU cell⁻¹ in a 25 cm² tissue culture flask for 1 h at 24 °C. At 16 h the growth medium was removed and replaced with methionine-free EMEM containing 24 µCi ml⁻¹ [³⁵S]-methionine. After labelling for 1 h at 24 °C, the medium containing the radioactive methionine was removed and cells were washed with PBS, scraped into lysis buffer [125 mM Tris (pH 6.5), 2 % sodium dodecyl sulphate (SDS), 20 mM dithiothreitol, 10 % glycerol and 0.5 % bromophenol blue] and frozen at -20 °C.

[³⁵S]-methionine labelled virus: Cells (CHSE-214) were infected with each virus isolate as described above. At ca 20 h p.i., cells were washed with PBS and the medium replaced by methionine-free EMEM containing 50 µCi ml⁻¹ [³⁵S]-methionine. At ca 24 h p.i. cells were scraped from 4 labelled and 6 unlabelled flasks, pelleted and purified as described. Lysis buffer (7.5 µl) was then added to 30 µl of pelleted, dialysed virus.

All samples were boiled for 90 s prior to loading onto 10 % polyacrylamide gels (Laemmli 1970). Following electrophoresis, gels were dried and the [³⁵S]-labelled proteins detected by autoradiography. [¹⁴C]-labelled marker proteins included in each gel were purchased from Amersham.

Western blotting. Viruses were purified as described above. Prior to loading on 10 % polyacrylamide gel (Laemmli 1970), 10 µl of the virus (EHN_{RF}, EHN_T or BIV) from the supernatant of lysed and dounced CHSE-214 cells were mixed with 3 µl of a DTT mixture (325 mM dithiothreitol, 25 % bromophenol blue, 45 % glycerol in 0.25 M Tris HCl pH 6.5) and SDS to a final concentration of 2 % and then boiled for 5 min. Following electrophoresis proteins were transferred to nitrocellulose using a BIO-RAD transfer apparatus. The nitrocellulose was blocked overnight in 1 % skim milk in TBS (150 mM NaCl, 80 mM Tris-HCl pH 7.4) after which either rabbit anti-EHN_T (whole virus) diluted 1:300, rabbit anti-EHN_T (capsid) diluted 1:300, rabbit anti-EHN_{RF} (whole virus) diluted 1:200 or rabbit anti-BIV (whole virus) diluted 1:5 in 1 % skim milk was applied for 1 h at RT. After washing 3 times in TBST (150 mM NaCl, 80 mM Tris-HCl pH 7.4, 0.05 % Tween 20) the nitrocellulose was incubated with anti-rabbit IgG (Fc) alkaline phosphatase conjugate (Promega 1 mg ml⁻¹) diluted 1:7000 in 1 % skim milk for 1 h at RT. The nitrocellulose was washed in AP buffer (100 mM Tris pH 7.4, 100 mM NaCl, 5 mM MgCl₂) and developed with protoblot system substrate for 10 min in the dark. The reaction was stopped with water.

DNA purification and cross-hybridization. Dialysed, purified EHN_{RF}, EHN_T and BIV were adjusted to 0.1 M NaCl, 5 mM EDTA, 26 % (w/v) sucrose and 1 % SDS and mixed by rolling at 4 °C for 30 min. Proteinase K to a final concentration of 100 µg ml⁻¹ and ribonuclease A to 40 µg ml⁻¹ were added and the incubation continued at 37 °C for 18 h. Each solution was extracted twice with an equal volume of phenol:chloroform (1:1) and once with chloroform. Sodium acetate was added to 0.2 M followed by 2.5 volumes of ice cold ethanol. Precipitated DNA was spooled out, dried briefly under vacuum and resuspended with 10 mM Tris-HCl pH 7.5, to a concentration of 0.5 to 1.0 µg ml⁻¹. Restriction enzyme digests, agarose gel electro-

phoresis and field inversion gel electrophoresis were carried out as described in Coupar et al. (1990).

For cross-hybridization studies 5-fold dilutions of sonicated DNA (from EHN_{RT}, EHN_T, BIV, vaccinia virus and calf thymus) in 10× SSC were denatured at 95 °C for 10 min, cooled on ice and loaded onto duplicate GeneScreenPlus (NEN) membranes using a 96 well manifold. The initial amount of DNA added was 0.5 µg. Prehybridized membranes were hybridized with nick translated ³²P-labelled DNA (from EHN_{RT} or BIV) overnight at 42 °C in buffer containing 50 % formamide. Membranes were washed with 2× SSC/0.1 % SDS followed by 0.1× SSC/0.1 % SDS at 60 °C and exposed to X-ray film.

RESULTS

Cell susceptibility

BF-2 and CHSE-214 cells were infected with EHN_{RT}, EHN_T and BIV. Although advanced CPE could be observed in all infected cultures by 3 d p.i., there was a discernable difference between that of piscine and amphibian virus-infected cells (Fig. 1). EHN_{RT} produced defined plaques which were circular and punctate in appearance (Fig. 1A). Cells associated with the perimeter of these plaques frequently possessed long cellular processes extending from a rounded cell body which contained characteristic large inclusion bodies detectable by phase light microscopy (data not shown). Following cell rounding and formation of the cellular processes, cells detached from the substrate forming the characteristic plaques. The plaques formed in BIV-infected cultures differed from those produced by EHN_{RT} in that they were diffuse (Fig. 1B). Within these cultures, infected cells became round, but did not produce the same long cellular processes. Inclusion bodies were also apparent, but the cells did not always detach from the substrate and produced only indistinct plaques. The differences in CPE were consistent

over a range of MOI and are therefore considered to represent fundamental differences in virus replication as opposed to differences in the kinetics of virus replication.

Of the mammalian cell lines which were infected with the 3 virus isolates, Vero and BHK cells exhibited the most advanced CPE by Day 3 p.i. CPE was less dramatic in the MDBK and PK15 cell lines. Insect cells

Fig. 1. Scanning electron micrographs of monolayers (cm) of CHSE cells infected with EHN_V (represented by EHN_T) and BIV. (A) The punctate CPE (*) is obvious in cells infected with EHN_V, the long cellular processes are indicated by short arrows and the rounded cell by long arrows. Scale bar = 100 µm. (B) CPE incurred by BIV. The CPE is more diffuse, most of the cells have rounded (large arrows) and few if any long cellular processes can be seen. Scale bar = 10 µm. [Cracks in the cell monolayers (bent arrows) are artifacts of specimen preparation]

(C636) infected with EHN_{V_{RF}}, EHN_{V_T} or BIV failed to show any signs of CPE. The ability of the viruses to infect the different cell lines was assessed by ELISA using antiserum raised against whole EHN_{V_T}. Fig. 2 shows optical densities obtained with the supernatants of cells infected with the different virus isolates. With the exception of cell line C636, all samples were positive suggesting that the CPE was due to virus replication.

Fig. 2. ELISA results from the supernatant of cells infected with the different virus isolates: (■) BIV; (▨) EHN_{V_{RF}}; (□) EHN_{V_T}. The supernatants were collected after advanced CPEs were observed. Results are expressed as optical densities of infected minus control supernatants. Plates were coated with sheep anti-EHN_{V_T} (whole virus)

Antigenicity

ELISA results. CHSE-214 cells were inoculated with EHN_{V_{RF}}, EHN_{V_T} and BIV. At 48 h p.i. the supernatants were removed and stored at 4 °C prior to analysis by ELISA. The supernatants were added to ELISA plates which had been previously coated

with sheep polyclonal antibodies directed against (1) EHN_{V_T} and (2) EHN_{V_{RF}}. The secondary antibodies comprised rabbit polyclonal antisera directed against (3) EHN_{V_T} (whole virus), (4) EHN_{V_T} (capsid), (5) EHN_{V_{RF}} (whole virus) and (6) BIV (whole virus). All isolates yielded positive results (Table 1) with their homologous and heterologous antisera. In both assay systems EHN_{V_{T,RF}} isolates gave stronger positive results against anti-EHN_V than anti-BIV antibodies (Table 1). The results indicate a high degree of cross-reactivity between any one antiserum and the different Australian iridoviruses.

Immunoelectron microscopy. Purified EHN_{V_{RF}}, EHN_{V_T} and BIV were adsorbed to carbon-coated, filmed gold grids and incubated with antisera generated against EHN_{V_T}, EHN_{V_{RF}} and BIV. Representative results are shown in Fig. 3. All isolates reacted with the 3 antisera. No difference in the pattern and/or intensity of immunostaining could be discerned between the isolates incubated in the presence of any one antiserum.

Electron microscopy

Negative contrast electron microscopy (NCEM). Ultrastructurally, EHN_{V_{RF}}, EHN_{V_T} and BIV were very similar. Complete virions possessed outer envelopes (Fig. 3). This outer envelope encompassed an inner capsid, which has been shown previously (Eaton et al. 1991) in EHN_{V_T} to be comprised of capsid subunits containing a 51 kD protein and a lipid membrane. When infected cells were extracted with a non-ionic detergent (NP40), viruses were observed to be associated with the filamentous framework of the cytoskeleton (data not shown). Grid-cell-cultures of infected cells showed that EHN_V isolates budded from the plasma membrane (Fig. 4A), particularly along the long cellular processes of infected cells. In BIV-infected cells where extended cell processes were absent, budding

Table 1. Representative ELISA optical densities at 450 nm for the various isolates captured by antibodies against EHN_{V_T} and EHN_{V_{RF}}. Values represent (sample – uninfected control)

1° antibody	2° antibody	Optical density		
		EHN _{V_{RF}}	EHN _{V_T}	BIV
Sheep anti-EHN _{V_T} (whole virus)	Rabbit anti-EHN _{V_T} (whole virus)	1.532	1.321	0.742
	Rabbit anti-EHN _{V_T} (capsid)	1.462	1.277	0.743
	Rabbit anti-EHN _{V_{RF}} (whole virus)	1.432	1.744	1.132
	Rabbit anti-BIV (whole virus)	0.220	0.271	0.122
Sheep anti-EHN _{V_{RF}} (whole virus)	Rabbit anti-EHN _{V_T} (whole virus)	1.114	1.382	1.272
	Rabbit anti-EHN _{V_T} (capsid)	1.384	1.216	0.979
	Rabbit anti-EHN _{V_{RF}} (whole virus)	1.651	1.754	1.479
	Rabbit anti-BIV (whole virus)	0.231	0.264	0.235

Fig. 3. Negatively stained and gold-labelled BIV and EHNIV. Each of the isolates was labelled with each antiserum. (A) EHNIV_T (representative of both piscine isolates) incubated in the presence of anti-BIV antibodies and protein A-gold. Inset: negative stained preparation of EHNIV_T. The host-cell-derived envelope (large arrow) and inner core surrounded by capsid subunits (short arrow) are indicated. (B) BIV incubated in the presence of anti-EHNIV_T antibody and protein A-gold. A similar result was obtained when the virions were incubated with anti-EHNIV_{RF} antibody. Inset: negative stained preparation of BIV. The host cell membrane (large arrow) and capsid subunits (short arrows) are indicated. Scale bars = 100 nm

occurred from the body of the host cell (Fig. 4B). The sizes (diameters) of the virions were not measured in NCEM samples as this could lead to spurious results due to degeneration of viruses and variation in size due to air drying.

Thin section electron microscopy. The ultrastructure of EHNIV_{RF} and EHNIV_T-infected cells was very similar. EHNIV_{RF} and BIV-infected cells were compared. Infected cells possessed lobate nuclei with marginated heterochromatin and a disorganised cytoplasm. Within the cytoplasm, non-membrane bound viral inclusion bodies (VIB) appeared as rarified areas. These electron translucent structures were devoid of cellular organelles (Fig. 5). Another prominent feature of the cytoplasm was the aggregation of mitochondria around the perimeter of the

VIB. Within the VIB, areas of condensation, presumably DNA (Murti et al. 1985b), were frequently observed (data not shown). These electron dense areas were usually associated with developing virus particles. Crystalline arrays of virions (Fig. 5) were observed in all infected cells and these were located both within the VIB and the cytoplasmic matrix. In addition to crystalline arrays, individual virus particles were observed in the cytoplasm in lysosomes and budding from the host cell plasma membrane. Within the inclusion bodies, virions were observed in different stages of assembly (e.g. capsid fragments, incomplete and complete capsids). No major differences in replication strategies were noted for any one virus in different host cells, namely BF-2 and CHSE-214 cells.

Fig. 4. Grid-cell-cultures of BIV and EHNIV-infected CHSE cells. (A) EHNIV_T (representative of EHNIV isolates) budding from extended cellular processes of an infected cell (C). (B) BIV budding from an infected cell. No long cellular processes are present. Scale bars = 100 nm

Fig 5 Transmission electron micrograph of a BIV-infected CHSE cell Only 1 micrograph is included as it is representative of the overall ultrastructure of cells infected with all 3 isolates Nu: nucleus, m: mitochondria, IB: inclusion body (viroplasm); *: paracrystalline array of IB-associated viruses, c*: paracrystalline array of cytoplasmic viruses; c: individual cytoplasmic viruses, B: viruses budding from the host cell membrane Scale bar = 1 µm

Table 2. Size of EHN_V and BIV calculated from intracellular arrays

	Size of EHN _V _{RF} in CHSE cells (nm)	Size of BIV in CHSE cells (nm)
No. of values	100	102
Mean $\pm$ SD	172 $\pm$ 6	159 $\pm$ 6
<i>t</i> -value (df = 200)	14.708	

The electron micrographs of virus particles in the cytoplasmic crystalline arrays were used to calculate the diameters of the virions for each of the 3 isolates. No major differences were observed between EHN_V_{RF} and EHN_V_T. However, when EHN_V_{RF} was compared to BIV (Table 2) the virions of BIV were found to be significantly smaller.

As shown using the grid-cell-culture technique, viruses were released from the cell by budding (Fig. 4). They appeared to approach areas of host membrane where evagination of a thickened trilaminar membrane indicated the beginning of the budding process. However, as was observed in the negative stained preparations, EHN_V frequently budded from extended cellular processes whereas BIV budded from the body of the cell.

Polypeptides synthesised in EHN_V- and BIV-infected cells

Cells infected with EHN_V and BIV induced a rapid inhibition of host cell protein synthesis (data not shown) and the synthesis of numerous virus-specific proteins. Intracellular BIV proteins determined by polypeptide gel electrophoresis are shown in Fig. 6A. The autoradiograph shows the presence of at least 20 polypeptides with molecular weights (MW) estimated to range from 22 to 123 kD. The number and size of BIV-specific proteins resembled that described for EHN_V and FV3-infected cells (Willis et al. 1977, Elliot & Kelly 1980, Eaton et al. 1991). Fig. 6B shows the polypeptides in purified BIV and EHN_V. Comparison of the BIV proteins in cells and purified viruses (Fig. 6A, B, Lane 4) reveals that specific proteins (e.g. 27, 35, 54, 57, 81 and 111 kD) were not present in purified virus and may represent non-structural proteins. Conversely a 92 kD protein was detected in purified BIV, but not amongst the intracellular proteins. The reason for this may reflect the amount of methionine in the protein and its comparative level amongst the cell-associated proteins or additional processing before incorporation into the virions.

Fig. 6B shows the polypeptide compositions of EHN_V_T (Lane 2) and EHN_V_{RF} (Lane 3). Overall there were only a few apparent differences between the profiles of the 2 piscine isolates. For EHN_V_T there were additional 37 kD and possible 82 kD proteins. However, these differences may be a reflection of the low amounts of proteins present and thus the inability to detect them may be a quantitative problem. These differences are also apparent in Fig. 7. In contrast EHN_V_{RF} differed from EHN_V_T in the presence of (1) additional 31 kD and 88 kD proteins and (2) the presence of a 72 kD compared to a 73 kD in EHN_V_T.

The differences in polypeptide profiles between BIV and the EHN_V isolates were more pronounced. The major differences were the presence of 99, 92, 61, 49, 43, 36, 30 and 26 kD proteins in purified BIV. Some of these proteins may be analogous to the 101, 88, 60, 51, 35 and 29 kD proteins from purified EHN_V isolates. There did not appear to be any analogous EHN_V proteins for the 43 or 26 kD proteins. The polypeptide profile of BIV also differed from that of the EHN_V profiles in the apparent absence of proteins analogous to the 86, 82, 37 and 31 kD proteins of EHN_V.

Western blot analyses

A coomassie-blue-stained mini-gel of SDS-treated, purified EHN_V_{RF}_T and BIV is shown in Fig. 7. This figure is representative of gels from which proteins were transferred to nitrocellulose and serves as a visual aid for the interpretation of the following blots. Minor discrepancies in calculated MW of specific polypeptides shown in Figs. 6, 7 or 8 for any one isolate can be attributed to differences in gel sizes and or running conditions.

Proteins from the 3 viruses reacted with homologous and heterologous antisera (Fig. 8). The antiserum used in Fig. 8A was raised to the 51 kD capsid protein (Fig. 6B) of EHN_V_T (Eaton et al. 1991). This antiserum reacted strongly with the homologous proteins of EHN_V_T, EHN_V_{RF} and with a 49 kD protein of BIV and to a lesser extent with a 44 kD protein (Fig. 8A, Lane 1). EHN_V proteins of MW 44 and 38 kD were also recognised by this antiserum. It is interesting to note that whilst the 38 kD protein of EHN_V was readily detected in Western blots of EHN_V, it was not apparent in stained gels of virus proteins (Figs. 6B & 7). Proteins from the 3 virus isolates which were probed with antiserum raised against purified EHN_V_T are shown in Fig. 8B. Using this serum, the 50 and 44 kD proteins were less intensely stained and the 38 kD protein was only

slightly apparent. Whilst there were subtle differences between the piscine isolates in Fig. 8B (e.g. 82, 76 and 38 kD proteins), more pronounced differences can be seen in Fig. 8C where the SDS-treated proteins have been reacted with antiserum raised against whole purified EHN_{RF}. The differences are highlighted by the comparatively intense 38 kD EHN_T and 45 kD EHN_{RF} protein. Whilst many BIV proteins reacted with antibodies to the piscine viruses, the EHN_V and BIV profiles differed in the presence of a more intensely stained 112 kD protein in BIV (Fig. 8B), the absence of many of the minor immunostained proteins (e.g. 76, 61, 42 and 38 kD)

and the absence of a major analogous 50 kD protein when incubated with antiserum raised against purified EHN_T. The blot in Fig. 8D shows the reaction of SDS-treated proteins with polyclonal antiserum raised against BIV. This antiserum produced a very weak homologous response but the blot is included as it illustrates the ability of the antiserum to react with heterologous EHN_V proteins. Anti-BV antiserum detected proteins of apparent MW 93 and 50 kD in BV, EHN_T and EHN_{RF}. Proteins from uninfected CHSE-214 cells failed to react with the antisera used in these experiments (data not shown) as did infected cells with normal (control) sera.

Fig. 6. Proteins in (A) BIV-infected cells and (B) purified EHN_T, EHN_{RF} and BIV with estimated molecular weights (kD). Radioactively labelled proteins in Lanes 1 to 4 were detected by autoradiography. Lane 1: markers; Lane 2: EHN_T; Lane 3: EHN_{RF}; Lane 4: BIV

Fig. 7. Proteins in purified viruses from infected cells. This coomassie-blue-stained mini-gel of SDS-treated virus is included as it is representative of the proteins transferred to nitrocellulose and facilitates the interpretation of the Western blots (Fig. 8). Molecular weights (kD) were estimated by reference to stained markers (Lane 4) run in the same gel. Lane 1: BIV; Lane 2: EHNVT_i; and Lane 3: EHNVR_F. (For the EHNVT_i profiles the 61, 92 and 116 kD proteins are non-structural; Eaton et al. 1991)

Restriction endonuclease analyses and cross hybridization

Comparison of restriction enzyme profiles for EHNVT_i and BIV DNAs showed different patterns for digests with *Kpn*I, *Bam*HI, *Hind*III and *Nco*I (Fig. 9) and for

Fig. 8. Western blot analyses of proteins illustrated in Fig. 7. Blots using uninfected CHSE cells as antigen with normal serum in lieu of antibody are not included as these did not generate any bands. Molecular weights were estimated by reference markers run in the original gel and transferred to nitrocellulose (not shown). The Western blots indicate proteins reactive with (A) antisera raised against the 51-52 kD capsid protein of EHNVT_i; (B) antisera raised against whole EHNVR_F; (C) antisera raised against whole EHNVT_i; (D) antisera raised against BIV. In all profiles, Lane 1 contains BIV; Lane 2, EHNVT_i; Lane 3, EHNVR_F.

Fig. 9. Restriction endonuclease digestion patterns for the DNA from (1) EHNVR_F, (2) EHNVT, and (3) BIV digested with *Kpn*I, *Bam*HI, *Hind*III or *Nco*I. Markers were (A) λ DNA digested with *Hind*III (23.1, 9.42, 6.68, 4.36, 2.32 and 2.03 kb); and (B) λ high-molecular-weight markers (48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.1, 15.0, 12.2, 10.1, 8.6 and 8.3 kb)

*Xba*I, *Bgl*II and *Pst*I (data not shown). Differences, however, between EHN_V isolates were relatively minor. With *Kpn*I, *Bam*HI and *Nco*I, most bands were common to both EHNVT and EHNVR_F DNA, however, some bands were present in only one of the EHN_V isolates. *Hind*III fragment sizes were indistinguishable between EHN_V isolates by conventional (Fig. 8) and field inversion gel electrophoresis (data not shown).

Estimates of genome size by addition of restriction fragment sizes (using enzymes *Kpn*I, *Xba*I, *Bam*HI, *Bgl*II, *Nco*I and *Hind*III) indicated that the 2 isolates of EHN_V had DNAs that were 125 ± 10 kb and

that of BIV was 109 ± 12 kb.

A cross-hybridization experiment using approximately equal concentrations of DNA from all 3 iridovirus isolates with vaccinia virus and calf thymus DNA as controls indicated a high level of sequence homology between the 3 iridoviruses (Fig. 10). No cross reactions with either vaccinia virus or calf thymus DNA were observed. The high stringency conditions used to wash the blots suggested a level of homology in the order of 90%. In each case a slightly higher level of hybridization, indicated by the intensity of the spots, was achieved with the homologous probe.

Fig. 10. Five-fold dilutions of DNA (initial amount added 0.5 µg) from EHNVRT, EHNVT, BIV, vaccinia virus and calf thymus were hybridized with ³²P-labeled DNA from either EHNVRT or BIV. Unbound probe was washed off with 0.1× SSC/0.1% SDS at 60 °C

DISCUSSION

Although the overall ultrastructural and morphogenic events appeared to be very similar for both BIV and EHNVT, the viruses differed in both size and the site of virus release from host cells.

EHNVT has been reported (Eaton et al. 1991) to bud from the host cell plasma membrane. Data from this study indicated that EHNVT was similar to FV3 (Tripier et al. 1974, Murti et al. 1985a) in that it buds from both the elongated cellular processes which are rich in microfilaments (Murti et al. 1985b) and from thickened areas of cell membrane associated with the body of the cell. Cells infected with BIV, however, did not produce long cellular protrusions. The significance of this difference is not known.

BIV was also found to be significantly smaller (159 nm) than EHNVT (172 nm). The size, measured vertex to vertex, was intermediate between that reported for non-enveloped FV3 (145 nm) (Lunger & Came 1966, Tripier & Kirn 1973) and EHNVT under the same conditions (i.e. vertex to vertex in thin sections). The icosahedral capsid of FV3 consists of 72 to 75 Å capsomers which contain a trimeric arrangement of a 49 kD protein (Darcy-Tripier et al. 1986). The analogous protein in EHNVT had a MW of 51 kD. Eaton et

al. (1991) found that this polypeptide, which is slightly larger than the cognate protein in FV3, constituted 75 to 90 Å capsomers which were themselves larger than those of FV3. The data suggested that BIV contained a 49 kD capsid protein which was antigenically related to that of EHNVT. Although the capsomeric subunits of BIV were not isolated and measured in this study, it would appear that the difference in size between EHNVT and BIV capsids could be the consequence of capsomeric proteins of different molecular weights.

Differences between the piscine and amphibian isolates become more pronounced at the protein level. The polypeptide profiles for the piscine isolates were very similar. To date only 1 EHNVRT and EHNVT isolate have been examined and the minor differences which exist must therefore be viewed with caution. Although differences between the piscine and amphibian viruses were more pronounced, the data indicated that EHNVT and BIV proteins exhibited varying degrees of antigenic cross-reactivity. In FV3, 48, 44, 43, and 38 kD proteins have been reported to be associated with the virion surface (Chinchar et al. 1984, Darcy-Tripier et al. 1986). Proteins of similar size were found in EHNVT and BIV and reacted with EHNVT-specific antiserum in Western blots. The presence of

antigenically related proteins in EHNV and BIV explains the cross-reactivity displayed by specific antisera in IEM and antigen capture ELISAs. The possession of similar antigenic epitopes in piscine and amphibian iridoviruses also indicates the necessity of developing further diagnostic tests to replace those currently available (Hyatt et al. 1991, Steiner et al. 1991). Such diagnostic assays utilise polyclonal antisera identical to those described in this paper and are therefore not appropriate for the specific detection of EHNV. The results in this report, however, indicate that it is possible to differentiate between EHNV_T, EHNV_{RF} and BIV by the use of Western blots. The specific labelling of 38 and 45 kD proteins by anti-EHNV_T antiserum would be sufficient to discriminate the isolates.

Although the origin of Australian iridoviruses is unknown, EHNV and BIV appear to be related. Comparison of polypeptides from BIV with those of FV3, as described by Willis et al. (1977) and Elliot & Kelly (1980), indicate that FV3 has many proteins of similar MW (e.g. 98, 93, 69, 61, 52, 50, 46, 44, 36, 31, 29 and 27 kD). The similarity of BIV to FV3 was also evident by comparing the restriction endonuclease (*Kpn*I and *Hind*III) profiles with those of FV3 (Essani & Granoff 1989) and the susceptibility of FV3 and BIV to different restriction enzymes. Both EHNV isolates and BIV were susceptible to digestion with *Xba*I. Other ranaviruses such as tadpole edema virus (TEV) and Lucke tritirus virus (LTV) are also digested with this restriction enzyme but GFV is not (Essani & Granoff 1989). These ranaviruses have been described to be closely related with homologies of 90 % or greater, however only 24 % homology was observed between FV3 and GFV (Essani & Granoff 1989). Results from this paper showed that the Australian iridoviruses were closely related with homologies of ca 90 %. The ability of restriction enzymes to detect differences between the genomic DNAs of the Australian isolates suggested that the piscine and amphibian isolates represent different strains of the same genus which is probably the *Ranavirus* genus. Data from Hedrick et al. (1992), which was published during the preparation of this manuscript, showed that there was a striking similarity between EHNV and the iridoviruses from the sheatfish *Silurus glanis* which was isolated in northwestern Germany (Ahne et al. 1989) and the catfish *Ictalurus melas* (Pozet et al. 1992) which was isolated in France and FV3. Collectively these data suggest that all these viruses should be grouped into the same genus, namely, the genus *Ranavirus*. Due to the geographical isolation of Australia, hybridization studies involving specific Australian aquatic iridoviruses and exotic viruses such as FV3 and GFV cannot be performed until virus

isolates and/or DNA can be obtained from North America or Europe. At present we are trying to acquire antisera and cloned DNA of the exotic viruses for future comparative studies.

As EHNV and BIV appear to be different strains of the same group of iridoviruses, it is interesting to note that EHNV has been transmitted, under laboratory conditions, to 7 other endemic and indigenous fish species (Langdon 1989). These included mosquito fish *Gambusia affinis* (Baird & Girard), mountain galaxias *Galaxias olidus* (Gunther), Macquarie perch *Macquaria australasica* (Cuvier), silver perch *Bidyanus bidyanus* (Mitchell), Murray cod *Maccullochella peelii* (Mitchell) and golden perch *Macquaria ambigua* (Richardson). Of these species, mosquito fish, silver perch, Murray cod and golden perch share a common geographical distribution along the eastern coast of Australia. Despite the geographical distribution of susceptible species, EHNV has, to date, been detected only in Victoria, southeast New South Wales and South Australia, and BIV only in Queensland. The isolations have, in the main, been from samples submitted for diagnosis and not from extensive national field surveys. The overall distribution of both isolates throughout the Australian continent is therefore largely unknown. The results presented in this report therefore raise many environmental questions. For example, what are the overall distributions of EHNV and BIV? Has BIV been transmitted (in the field) to *Bufo marinus* (cane toads) which are currently advancing across the Australian continent? Can BIV infect fish and EHNV infect frogs? Can frogs and cane toads survive infection and become carriers? If the distributions of these viruses are not ubiquitous and the answer to any of the preceding questions is yes, then what will be the environmental impact on Australia's indigenous piscine and amphibian fauna?

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