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Establishment and Characterization of the Epithelioma Papulosum Cyprini (EPC) Cell Line Persistently Infected with Infectious Pancreatic Necrosis Virus (IPNV), an Aquabirnavirus

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Infectious pancreatic necrosis virus (IPNV), a type species of aquabirnaviruses in the family *Birnaviridae,* is an etiological agent of infectious pancreatic necrosis and has been isolated from epizootics of cultured salmonids. In the present study, an epithelioma papulosum cyprini (EPC) cell line persistently infected with IPNV (PI-EPC) was experimentally established by subculturing EPC cells surviving IPNV infection, and was characterized. PI-EPC cells were morphologically indistinguishable from EPC, but continued to grow and yield IPNV. PI-EPC cells showed no cytopathic effect due to IPNV inoculation, and susceptibility of PI-EPC cells against heterologous viruses was not different from that of EPC cells. Only one cell of $10^{3.5}$ PI-EPC cells produced IPNV at approximately $10^{0.5}$ 50% tissue culture infectious dose ($TCID_{50}$)/cell/day, which was approximately 1,000 times lower than that of normal EPC cells. PI-EPC cells that did not yield IPNV (N-PI-EPC) were screened. The IPNV genome was detected from both PI-EPC and N-PI-EPC cells, and the IPNV VP2 structural protein was detected from both cell lines, but no other IPNV proteins were observed by Western blot analysis with anti-IPNV serum. Thus, multiplication of IPNV in PI-EPC cells was regulated by some host cell factors, except interferon.

Keywords: aquabirnavirus, infectious pancreatic necrosis virus, IPNV, persistent infection, virus productivity, EPC

Introduction

Infectious pancreatic necrosis virus (IPNV), a type species of the genus *Aquabirnavirus* in the family *Birnaviridae*, is the etiological agent of infectious pancreatic necrosis. IPNV has been isolated from epizootics in cultured salmonids and a variety of aquatic animals in freshwater and seawater environments all over the world (Reno, 1999; Munro and Midtlyng, 2011). In some cases, aquabirnavirus isolates lack any association with disease, although they are identified serologically as IPNV. Thus, aquabirnavirus isolates with no pathogenicity to salmonids are currently named aquabirnaviruses to be distinguished from IPNV (Reno, 1999; Munro and Midtlyng, 2011; Delmas *et al*., 2012). Aquabirnaviruses have a non-enveloped, icosahedral capsid of approximately 60 nm in diameter containing the bisegmented, double-stranded RNA genome (segments A and B) (Delmas *et al*., 2012). Segment B, with a size of 2.8 kb, encodes a minor internal polypeptide VP1 with Mr 94 k, the putative RNA dependent RNA polymerase, whereas segment A, with 3.1 kb, contains two open reading frames (ORFs) partially overlapped; a large ORF for the polyprotein with Mr 106 k (NH2-pVP2-NS-VP3-COOH) and a small ORF for VP5 with Mr 17 k. The polyprotein is cleaved into three polypeptides: pVP2, the precursor of the VP2 major capsid protein; NS, a non-structural protein with protease activity associated with cleavage of the polyprotein; and VP3, a minor capsid protein (Duncan and Dobos, 1986; Duncan *et al*., 1987; Håvarstein *et al*., 1990; Manning and Leong, 1990; Manning *et al*., 1990; Magyar and Dobos, 1994; Delmas *et al*., 2012). A molecular phylogenetic analysis based on the VP2/NS junction region of IPNV and other aquabirnaviruses revealed the existence of seven discrete genogroups (Nishizawa *et al*., 2005).

Although aquabirnaviruses have been widely isolated from many fishes, mortality is low as age increases, and survivors become aquabirnavirus carriers. Carrier fish are asymptomatic, and cause either horizontal or vertical infections (Wolf, 1988). However, little is known about the mechanisms for establishing an aquabirnavirus carrier state. Aquabirnaviruses can replicate without cytolysis in some fish cell lines by persistent infection, such as chinook salmon embryo cell (CHSE-214), steelhead trout (STE-137), rainbow trout gonad cells (RTG-2) and black carp swim bladder cells (Hedrick *et al*., 1978; Hedrick and Fryer, 1981, 1982; Chen *et al*., 1993). All persistently infected cell lines have the following characteristics: 1) continued production of infectious virus, 2) continued presence of viral antigen in the majority of cells, 3) resistance to superinfection by a homologous virus and 4) continued cell division and growth of carrier cells, which are indistinguishable from normal uninfected cells (Rima and Martin, 1976; Hedrick *et al*., 1982). Examinations of persistently infected cells have revealed mechanisms that control viral persistence *in vitro* and may also operate on

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infections in the host (Hedrick and Fryer, 1981).

In the present study, an epithelioma papulosum cyprini (EPC) cell line persistently infected with IPNV (PI-EPC) was established by subculture of surviving cells from an artificial IPNV infection and characterized for morphology, susceptibility to other fish viruses, and viral productivity.

Materials and Methods

Viruses and cells

IPNV (ATCC VR299), IHNV (ChAb76) and viral hemorrhagic septicemia virus (VHSV, Obama 25) were used in the present study. IHNV ChAb76 was isolated from chum salmon, *Oncorhynchus keta*, in 1976 (Yoshimizu *et al.*, 1988), whereas VHSV Obama 25 was obtained from free-living Japanese flounder *Paralichthys olivaceus* in 1999 (Takano *et al.*, 2000; Nishizawa *et al.*, 2002). Both IHNV and VHSV are well-known fish pathogenic novirhabdoviruses in the family *Rhabdoviridae*. These three viruses were inoculated into EPC cells at a multiplicity of infection (m.o.i.) of 0.001 and cultured at 15°C with Eagle's minimum essential medium (MEM) (Nissui, Japan) supplemented with 10% fetal bovine serum (Gibco, USA), 1% L-glutamine, 100 IU/ml penicillin, and 10 μg/ml streptomycin (MEM_{10}-Tris). After centrifugation (12,000×g, 10 min, 4°C), viral culture supernatants were subdivided into small quantities and stocked at -80°C until use.

The titration of viral infectivity was performed using 96-well microplates seeded with EPC cells. After 14 days of culture, the appearance of cytopathic effect (CPE) was evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$) following a standard method.

Establishment of persistently infected EPC cells (PI-EPC) with IPNV

IPNV was inoculated to EPC cells at >1.0 of m.o.i. and cultured at 15°C for 14 days. After CPE appeared completely in the EPC cells, the surviving cells were washed three times with Hank's balanced salt solution (HBSS) (Nissui, Japan) and the culture medium was replaced with fresh MEM_{10}-Tris for subculture. The surviving cells eventually formed a new monolayer, and these cells were named PI-EPC, whereas the virus generated from the PI-EPC cells into culture medium was named $IPNV^{-PI}$. The monolayered PI-EPC cells were stained with May-Gründwald Giemsa stain for microscopic observation.

To estimate the ratio of cells that continuously yielded IPNV, PI-EPC cells were washed three times with HBSS, and then subcultured in 96-well plates at 10^3 or 10^4 cells/well. After culture of the cells at 15°C for a few days, 50 μl of the culture medium in each well was collected to inoculate normal EPC cells seeded in another 96-well plate to detect IPNV. The newly inoculated plates were incubated at 15°C for 2 weeks, and the ratio of the cells yielding IPNV was estimated by the number of wells exhibiting CPE. The PI-EPC cells that did not yield IPNV were collected for subculture as N-PI-EPC cells, meaning PI-EPC cells that did not produce IPNV.

Viral productivity of PI-EPC cells

Approximately 10^7 EPC and PI-EPC cells were cultured in 75 cm^2 flasks (Falcon, USA) and incubated at 15°C for 1 day. The adherent cells were washed three times with HBSS, inoculated with 10^2 $TCID_{50}$ IPNV, $IPNV^{-PI}$, IHNV, VHSV, and HBSS (mock infection), and cultured at 15°C for an additional 2 weeks. An aliquot of the culture medium was collected every 2–3 days to measure the viral infectivity titer.

Subculture of PI-EPC cells with IPNV antiserum ($PI\text{-}EPC^{serum}$)

PI-EPC cells were cultured with MEM_{10}-Tris supplemented with 1% rabbit antiserum against IPNV; neutralization titer of the present antiserum was 1:2,560, meaning that the antiserum at 2,560-folds dilution completely neutralized 100 $TCID_{50}$ of IPNV (Takano *et al.*, 2000). After three subcultures with culture medium containing the antiserum, the culture medium was replaced with regular MEM_{10}-Tris and was subcultured an additional three times, and then cell-culture medium was harvested to identify the cells yielding IPNV.

RNA extraction and reverse-transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells using an RNA extraction kit (Isogen, Nippon Gene, Japan) according to the manufacturer's instructions, and were subjected to RT-PCR amplification with the primers ABV-P1 (5′-AGA GAT CAC TGA CTT CAC AAG TGA C-3′) and ABV-P2 (5′-TGT GCA CCA CAG GAA AGA TGA CTC-3′), targeting the VP2/NS junction region of the IPNV genome segment A (Heppell *et al.*, 1992; Nishizawa *et al.*, 2005). After heat denaturing at 95°C for 5 min, the extracted RNAs were incubated for reverse transcription at 42°C for 40 min in 10 μl of PCR buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl) containing 1 μl of extracted RNA solution, 10 U M-MLV reverse transcriptase (TaKaRa, Japan), 1 mM dNTP, 5 mM $MgCl_2$, and 1.0 μM each ABV-P1 and ABV-P2 primer. After heat denaturing with a 10 min 95°C incubation, the targeted DNA was amplified in 50 μl of PCR buffer containing 1.25 U of Ex-*Taq* DNA polymerase (TaKaRa), 0.2 mM dNTP, and 2 mM $MgCl_2$ using a thermal cycler programmed for 1 cycle at 72°C for 10 min and 95°C for 2 min; 30 cycles, each consisting of 95°C for 40 sec, 50°C for 40 sec, 72°C for 40 sec, and a final holding step at 72°C for 10 min. One microliter of the amplified product in the primary PCR was again amplified for 25 cycles in 25 μl of PCR buffer with the same PCR primers (secondary PCR) under the same conditions as for the primary PCR. The amplified products were analyzed by 1.5% agarose-Tris acetate gel electrophoresis (40 mM Tris-acetate; pH 8.0, 1 mM EDTA) and visualized under UV irradiation after ethidium bromide staining.

Immunofluorescence antibody technique (IFAT)

Targeted cells were fixed with 10% formalin at room temperature for 20 min and washed three times with PBS. After drying at 4°C overnight, cells were immunostained with anti-IPNV rabbit serum at 25°C for 1 h, and with FITC-conjugated goat anti-rabbit IgG (Dako, Denmark) at 25°C for 1 h. After

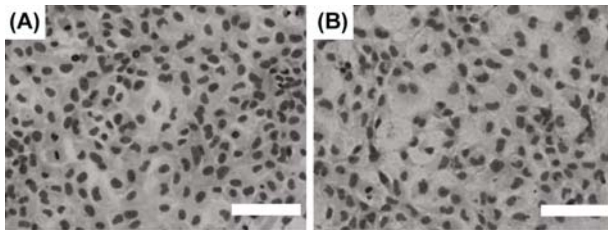


Fig. 1. Photomicrographs of the epithelioma papulosum cyprini (EPC) cell line persistently infected with infectious pancreatic necrosis virus (IPNV) (PI-EPC). PI-EPC and original EPC cells stained with May-Gründwald Giemsa. (A) normal EPC, (B) PI-EPC; Scale bar, 100 μm.

washing three times with PBS, cells were observed under a fluorescence microscope (Olympus, Japan).

Western blot analysis

Cultured cells were washed three times with HBSS and harvested with sodium dodecyl sulfate (SDS)-denatured buffer (10% SDS, 0.5 M Tris-HCl; pH 6.8, β-mercaptoethanol), whereas virus particles in culture medium were concentrated by ultracentrifugation (150,000×g, 60 min, 4°C). After SDS-polyacrylamide gel electrophoresis analysis by the procedure of Laemmli (1970), separated proteins in the gel were electroblotted onto a nitrocellulose membrane by the procedure of Towbin *et al.* (1979). The blotted proteins were immunostained with an anti-IPNV rabbit serum and swine anti-rabbit IgG conjugated with alkaline phosphatase (Dako), and visualized with NBT-BCIP substrate buffer (0.34 mg/ml of nitroblue-tetrazolum, 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl-phoshate-toluidinium, 100 mM Tris-HCl; pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$).

Results

Morphology of PI-EPC cells

The PI-EPC and original EPC cells were stained with May-Gündwald Giemsa (Fig. 1). PI-EPC cells were morphologically indistinguishable from the original EPC cells. Moreover, PI-EPC cells were stably grown for more than 50 passages

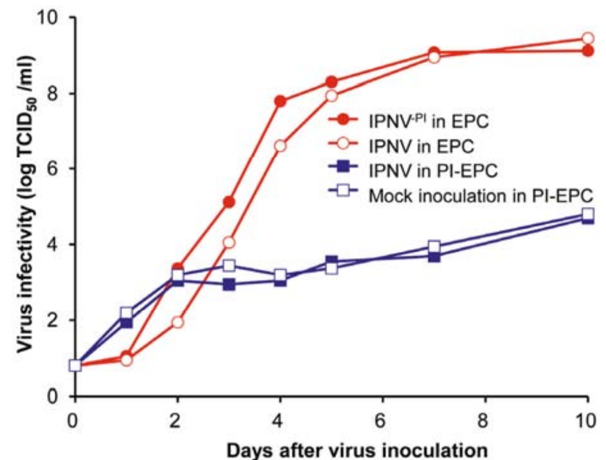


Fig. 2. Alteration in infectious pancreatic necrosis virus (IPNV) titer in the culture fluids of the epithelioma papulosum cyprini (EPC) cell line persistently infected with IPNV (PI-EPC). PI-EPC and EPC cell lines were inoculated with IPNV at 10^2 50% tissue culture infectious dose ($TCID_{50}$) or HBSS (mock infection) and incubated at 15°C for 10 days. The virus generated from the PI-EPC cells in culture medium ($IPNV^{-PI}$).

of subculture (data not shown).

Multiplication of IPNV in EPC and PI-EPC cells

Time-dependent changes in IPNV infectivity of PI-EPC and EPC cell culture media with or without inoculation of IPNV are shown in Fig. 2. The IPNV infectivity titer in the culture medium of PI-EPC cells increased slowly, and reached $10^{4.3}$ $TCID_{50}$/ml within 10 days of culture regardless of IPNV inoculation, indicating that multiplication of IPNV in PI-EPC cells was quite similar to each other. Furthermore, no CPE was induced in PI-EPC cells with and without an IPNV inoculation. IPNV titer in culture medium of EPC cells inoculated with IPNV began to be detected on day 2 of culture, and reached $10^{9.4}$ $TCID_{50}$/ml within 10 days of culture. In the EPC cell culture medium inoculated with $IPNV^{-PI}$ generated by PI-EPC, the $IPNV^{-PI}$ growth curve was almost the same as that of EPC cells inoculated with original IPNV. Typical CPE due to IPNV-infection were observed in both EPC cells with IPNV and $IPNV^{-PI}$ (data not shown).

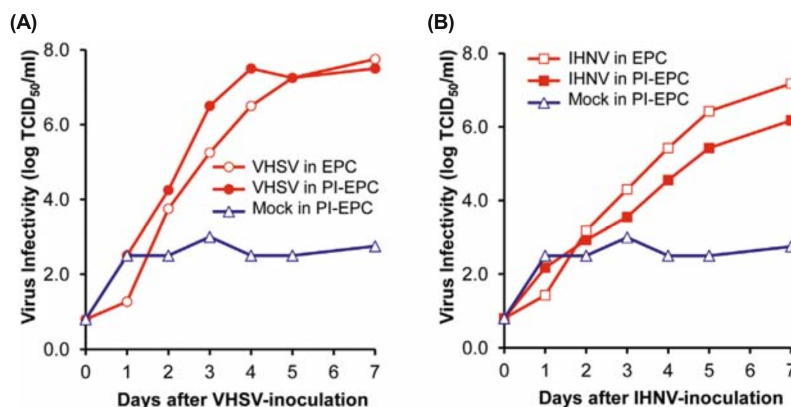


Fig. 3. Alterations of the virus titer in the culture media of epithelioma papulosum cyprini (EPC) cells persistently infected with infectious pancreatic necrosis virus (IPNV) (PI-EPC) cell lines inoculated with VHSV and IHNV. Viruses with 10^2 50% tissue culture infectious dose ($TCID_{50}$) and HBSS (mock-infection) were inoculated into each cell line and incubated at 15°C for 1 week. (A) VHSV inoculation, (B) IHNV inoculation.

Table 1. Estimated number of PI-EPC cells yielding IPNV

Passage No. of PI-EPC	Cells / well	Positive wells / Total wells (Positive rate)	Estimated number of cells yielding IPNV
Pass 18	10^4	91/96 (95%)	1 / 10,500 – 1 / 4,400 cells
	10^3	22/96 (23%)	
Pass 20	10^4	91/96 (94.8%)	1 / 10,500 – 1 / 1,800 cells
	10^3	53/96 (55.2%)	
Average			1 / 3,100 cells

PI-EPC, epithelioma papulosum cyprini (EPC) cell line persistently infected with infectious pancreatic necrosis virus (IPNV).
For calculation of average, we used data only for the wells containing 10^3 cells but not for those containing 10^4 cells, because of high possibility that double or triple PI-EPC cells yielding IPNV were contained in the wells showing high positive rate (more than 90%).

Multiplication of VHSV and IHNV in EPC and PI-EPC cells

Time-dependent changes of virus infectivity in EPC and PI-EPC cell culture media inoculated with 10^2 $TCID_{50}$ of VHSV and IHNV are shown in Fig. 3. VHSV titers in culture media of PI-EPC and EPC cells were detected from day 2 of VHSV inoculation, and reached $10^{7.5}$ and $10^{7.8}$ $TCID_{50}$/ml within 7 days of culture, respectively (Fig. 3A). Viral titers in the culture fluid of PI-EPC and EPC cells that were inoculated with IHNV reached $10^{6.1}$ and $10^{7.1}$ $TCID_{50}$/ml within 7 days of culture, respectively (Fig. 3B). Typical CPE due to novirhabdovirus infection were observed in both PI-EPC and EPC cells inoculated with VHSV or IHNV, and no significant difference was observed in the productivity of each novirhabdovirus between PI-EPC and EPC cells. In culture medium of PI-EPC cells that were mock-infected, $10^{2.8}$ $TCID_{50}$/ml of $IPNV^{-PI}$ was detected (Fig. 3), but no CPE were observed.

IPNV-producing ratio of PI-EPC cells

IPNV infectivity in the culture medium of each plate well was evaluated after PI-EPC cells were cultured at 10^3 and 10^4 cells/well in 96-well plates for 1 week (Table 1). After 18 passages of PI-EPC cells, IPNV was detected in 91 of 96 wells seeded at 10^4 cells/well (positive rate: 95%), and 22 of 96 wells seeded at 10^3 cells/well (positive rate: 23%). After 20 passages of PI-EPC cells, IPNV was detected in 91 of 96 wells seeded at 10^4 cells/well (positive rate: 95%), and 53 of 96 wells seeded at 10^3 cells/well (positive rate: 55%). When 22 of 96 wells seeded at 10^3 cells/well were IPNV positive, it suggested that 96×10^3 cells contained at least 22 cells yielding IPNV; the estimated number of cells yielding IPNV was 1 in 4,364 cells. Here, we used data only for the wells containing 10^3 cells but not for those containing 10^4 cells, because of high possibility that double or triple PI-EPC cells yielding IPNV were contained in the wells showing high positive rate (more than 90%). Based on this method, it was calculated that the estimated number of PI-EPC cells yielding IPNV was only one cell in approximately 3,100 cells, indicating that not all PI-EPC cells generated IPNV. PI-EPC cells in wells without IPNV were subcultured as N-PI-EPC cells but no different features were observed between N-PI-EPC and PI-EPC cells, except IPNV production (data not shown).

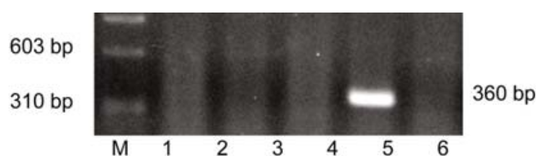
PI-EPC cells cultured with IPNV antiserum

No IPNV was detected in the culture medium after PI-EPC cells were subcultured three times with MEM_{10}-Tris supplemented with IPNV antiserum. Furthermore, IPNV productivity by those PI-EPC cells never recovered after an additional few subcultures with regular MEM_{10}-Tris. PI-EPC cells lacking IPNV following treatment with the anti-IPNV serum were denoted as $PI\text{-}EPC^{serum}$.

Detection of IPNV from PI-EPC cells by RT-PCR and IFAT

Approximately 360 bases of PCR product, the same size as the targeting region, were amplified from extracted nucleic

(A) Primary RT-PCR (30 cycles)



(B) Secondary PCR (55 cycles)

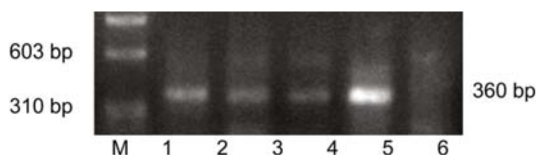


Fig. 4. Detection of the infectious pancreatic necrosis virus (IPNV) genome from epithelioma papulosum cyprini (EPC) cells persistently infected with IPNV, PI-EPC cells that did not yield IPNV (N-PI-EPC), and PI-EPC cells cultured with IPNV antiserum ($PI\text{-}EPC^{serum}$) by RT-PCR targeting the VP2/NS junction region with the ABV-P1 and ABV-P2 primers. (A) 1.5% agarose gel electrophoresis for RT-PCR products with 30 cycles of amplification, (B) PCR products with 55 cycles of amplification, Lanes: 1, amplified PCR products from PI-EPC cells; 2, N-PI-EPC cells; 3, $PI\text{-}EPC^{serum}$ cells; 4, IPNV (positive control); 5, EPC cells (negative control).

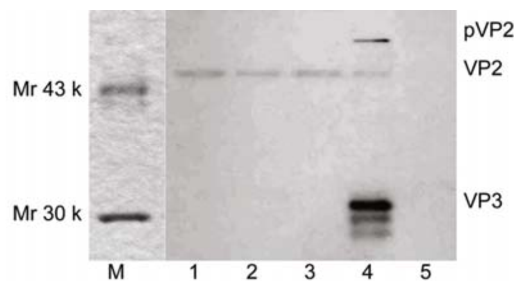


Fig. 5. Detection of structural proteins of infectious pancreatic necrosis virus (IPNV) in epithelioma papulosum cyprini (EPC), EPC cells persistently infected IPNV (PI-EPC), N-PI-EPC, and cells cultured with IPNV antiserum ($PI\text{-}EPC^{serum}$) by Western blot analysis with anti-IPNV rabbit serum. Lanes: M, protein marker; 1, PI-EPC cells; 2, N-PI-EPC cells; 3, $PI\text{-}EPC^{serum}$ cells; 4, EPC cells infected with IPNV; 5, normal EPC cells.

acids of PI-EPC, N-PI-EPC, and PI-EPC^{serum} cells by secondary PCR, although no PCR product was observed by primary RT-PCR (Fig. 4). The IPNV-inoculated EPC cells infected with IPNV were positive by primary PCR, but the mock-infected EPCs were negative. Furthermore, no positive IFAT reaction using the IPNV antiserum was observed in PI-EPC, N-PI-EPC, or PI-EPC^{serum} cells, although a clear positive-reaction was observed in EPC cells infected with IPNV (data not shown).

Detection of IPNV structural proteins by Western blot

The detected IPNV structural proteins in PI-EPC, N-PI-EPC, PI-EPC^{serum}, and IPNV-infected EPC cells by Western blot with the IPNV antiserum are shown in Fig. 5. Five proteins, including pVP2 with Mr 56 k, VP2 with Mr 49 k, major VP3 with Mr 32 k, and two minor VP3s with Mr 30 k and Mr 28 k were detected with IPNV (Fig. 5, lane 4). VP2 with Mr 49 k was detected in PI-EPC, N-PI-EPC, and PI-EPC^{serum} cells; however, no VP3s with Mr 28-32 k or pVP2 with Mr 56 k was detected in those cells (Fig. 5, lanes 1–3). No IPNV structural protein was observed in mock-infected EPC cells (Fig. 5, lane 5).

Discussion

In the present study, a PI-EPC cell line, which was persistently infected with IPNV, was experimentally established by subculturing EPC cells surviving an artificial IPNV infection. The established PI-EPC cells were morphologically indistinguishable from original EPCs (Fig. 1) but continued to grow and generate IPNV. PI-EPC cells showed no CPE from the IPNV infection, and PI-EPC cells continually produced IPNV regardless of IPNV inoculation (Fig. 2), suggesting that the established PI-EPC cells were resistant to superinfection with a homologous virus. It was thus confirmed that the established PI-EPC cells completely satisfied the characteristics for persistent infection described previously by Rima and Martin (1976) and Hedrick *et al.* (1982). However, the PI-EPC cells and the original EPC cells were susceptible to IHNV and VHSV and productivities of IHNV and VHSV by PI-EPC were the same as those of the original EPC cells (Fig. 3). An epidemiological survey revealed that isolation of IHNV and/or VHSV from mixed infected fishes with aquabirnaviruses are often limited due to powerful multiplication of aquabirnaviruses *in vitro* (Takano *et al.*, 2001; Watanabe *et al.*, 2002). Thus, PI-EPC cells are convenient and useful to isolate viruses other than IPNV from fishes with a mixed infection.

Approximately 10^5 $TCID_{50}$ ($10^{4.3}$ $TCID_{50}$/ml ×5 ml) of IPNV was eventually generated in the culture medium of PI-EPC cells from $10^{7.0}$ cells until day 10 of culture (Fig. 2). It was estimated that only one of $10^{3.5}$ PI-EPC cells yielded IPNV (Table 1), suggesting that the PI-EPC cells generated IPNV at approximately $10^{0.5}$ $TCID_{50}$/cell/day [10^5 $TCID_{50}$/($10^{7.0}$ cells/$10^{3.5}$ cells)/10 days]. In a cytolytic infection with IPNV, EPC cells were calculated to produce approximately $10^{3.7}$ $TCID_{50}$/cell, because approximately $10^{10.7}$ $TCID_{50}$ of IPNV ($10^{9.4}$ $TCID_{50}$/ml ×$10^{1.3}$ ml) were generated from $10^{7.0}$ cells (Fig. 2). Thus, IPNV productivity by PI-EPC cells was approximately 1,000 times lower than that of the original EPC cells. This is strongly supported by the results that the IPNV genome in PI-EPC cells was detected only by secondary PCR but not by primary RT-PCR (Fig. 4). Moreover, PI-EPC cells were negative by IFAT with anti-IPNV rabbit serum. It was incidentally confirmed that the detection limit of the present RT-PCR targeting IPNV genome was approximately 10^3/reaction by 30-cycle amplification and a few copies/reaction by 55-cycle amplification, respectively (data not shown). Furthermore, it was difficult to detect PI-EPC cells yielding $10^{0.5}$ $TCID_{50}$/cell/day of IPNV by IFAT under the present conditions.

As mentioned above, although IPNV^{-PI} was slowly generated from PI-EPC cells, it was possible for IPNV^{-PI} and the original IPNV to multiply in EPC cells (Fig. 2), suggesting no difference in multiplication ability between IPNV and IPNV^{-PI}; preferably some regulatory factors for IPNV multiplication exist in PI-EPC cells. One of the host cell factors to regulate viral multiplication is interferon (IFN), and previous studies demonstrated that multiplication of IPNV is regulated by inducing IFN in EPC cells and fishes (de Kinkelin *et al.*, 1992; Dorson *et al.*, 1992; Pakingking *et al.*, 2004). However, the present factor regulating IPNV multiplication in the PI-EPC cells could not be IFN, because heterologous viruses such as VHSV and IHNV multiplied well in PI-EPC cells as well as in the original EPC cells (Fig. 3).

Although no PI-EPC cells with the IPNV antigen were detected by IFAT, the IPNV genome was detected in PI-EPC cells after 55 cycles of PCR amplification (Fig. 4), suggesting that IPNV could exist in PI-EPC cells at a very low level. This is convincing, because only one of $10^{3.5}$ PI-EPC cells produced IPNV at approximately $10^{0.5}$ $TCID_{50}$/cell/day (Table 1) as mentioned above, indicating that >99.9% of PI-EPC cells could be N-PI-EPC cells without producing IPNV. Interestingly, the IPNV genome was detected in N-PI-EPC cells screened from PI-EPC cells following 55 cycles of PCR-amplification (Fig. 4), and the VP2 IPNV structural protein was also detected in N-PI-EPC cells by Western blot analysis (Fig. 5). These results indicate that N-PI-EPC cells were still infected with IPNV, although no IPNV was produced. In fact, we discovered that N-PI-EPC cells rarely began to produce IPNV in culture medium (data not shown). No IPNV VP3 structural protein was synthesized in N-PI-EPC cells (Fig. 5), which may indicate its role as a host cell factor regulating the yield of IPNV in N-PI-EPC cells. Incidentally, no VP3 was observed in PI-EPC cells, and no difference was observed in the pattern of IPNV structural proteins between PI-EPC and N-PI-EPC cells (Fig. 5). This could be due to the observation that >99.9% of PI-EPC cells were composed of N-PI-EPC cells, as mentioned above.

PI-EPC^{serum} cells were also obtained by subculturing PI-EPC cells in MEM_{10}-Tris supplemented with IPNV antiserum for blocking the infection of newly produced IPNV. The PI-EPC^{serum} cells showed the same characterization as the PI-EPC cells. For example, they were IPNV negative by both IFAT and 30 cycles of PCR amplification, IPNV positive by 55 cycles of PCR-amplification, and VP2-positive but VP3-negative by Western blot analysis (Figs. 4 and 5). These results suggest that PI-EPC cells producing IPNV disappeared following subculture with IPNV antiserum and that N-PI-

EPC cells remained. Unfortunately, it is unclear how PI-EPC cells producing IPNV disappeared in the subculture with anti-IPNV serum. It was reported that aquabirnaviruses including IPNV have been culture-isolated from many free-living fishes being apparently healthy (Takano *et al.*, 2001; Watanabe *et al.*, 2002; Yun *et al.*, 2008), suggesting that persistently infected cells with aquabirnabiruses could also exist *in vivo* frequently. Furthermore, Nishizawa *et al.* (2008) has established a novel fish cell line, PI-BF-2, which is persistently infected with SnRV, a fish retrovirus, and revealed that SnRV enhances propagation of heterologous virus in PI-BF-2 cells. Thus, we believe that these persistently infected fish cell lines could be a useful *in vitro* model for analyses of viral carrier states in fishes.

Acknowledgements

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