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Detection of DNAs homologous to betanodavirus genome RNAs in barfin flounder *Verasper moseri* and Japanese flounder *Paralichthys olivaceus*

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ABSTRACT: I investigated the presence of DNA homologous to genome RNA1 and RNA2 (RNA1 DNA and RNA2 DNA) of betanodaviruses — the causative agent of viral nervous necrosis (VNN) — in eggs, sperm, ovarian cavity fluid, larvae, and juveniles of barfin flounder *Verasper moseri* and larvae and juveniles of Japanese flounder *Paralichthys olivaceus* collected at 6 sites in Hokkaido, Japan, from 1994 to 2001. RNA1 DNA and RNA2 DNA were detected by PCR in 13 and 33% of barfin flounder samples and 0 and 69% of Japanese flounder samples, respectively. No infectious virus was detected by cell culture or by successive immunoblot against coat protein (genome RNA2 product) using an E-11 cell line, except for a virus present in 1 dead fish collected during an outbreak of VNN in 1995. Nucleotide sequence analysis showed that RNA1 DNA had a 82 to 96% similarity to betanodavirus genome RNA1, and that RNA2 DNA had a 69 to 98% similarity to RNA2. The detection rate of RNA2 DNA after intraperitoneal injection of betanodavirus strain HCF-1 into larvae and juveniles of the 2 flounder species was higher in samples from surviving fish than in the uninfected controls, whereas the detection rate of RNA1 DNA did not show a clear trend. Infectious virus was only detected in samples from fish that died subsequent to injection. Transfection assays of the viral genome RNAs into the barfin flounder cell line MK-1 and Japanese flounder cell lines H-1 and H-2 resulted in production of RNA2 DNA in all 3 cell lines. Quantitative measurement by ELISA revealed reverse transcriptase (RTase) activity. These results suggest that the DNA forms are produced and persist in the 2 flounder species as both clinical and subclinical infections, and do not lead to virion production.

KEY WORDS: DNA form · Betanodavirus · Viral nervous necrosis · VNN · Barfin flounder · Japanese flounder

INTRODUCTION

Betanodaviruses, which belong to the family *Nodaviridae*, are the causative agents of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy in more than 30 marine fish species including Japanese parrotfish *Oplegnathus fasciatus* (Yoshikoshi & Inoue 1990), barramundi *Lates calcarifer* (Glazebrook et al. 1990, Munday et al. 1992), striped jack *Pseudocaranx dentex* (Mori et al. 1992), Japanese flounder *Paralichthys olivaceus* (Nguyen et al. 1994), barfin flounder *Verasper moseri* (Muroga 1995), and others (Munday & Nakai 1997, Muroga et al. 1998, Muroga 2001, Munday et al. 2002). The genome has 2 single-stranded positive sense RNAs, RNA1 and RNA2, which encode 2 non-structural proteins and a coat protein (Mori et al. 1992, Nagai & Nishizawa 1999, Balls et al. 2000).

DNA forms homologous to the genomic RNA of the measles virus *Paramyxoviridae*, Sindvis virus *Togaviridae*, tick-borne encephalitis virus *Flaviviridae* (Zhdanov 1975), and lymphocytic choriomeningitis virus *Arenaviridae* (Klenerman et al. 1997) in human and mouse tissues and in cell lines infected with the viruses have been reported, whereas the presence of DNA forms in fish RNA viruses remains unknown.

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The presence of DNA forms in samples would affect the detection of RNA viral genomes by reverse-transcription (RT)-PCR when investigating subclinical infection in epidemiological studies. Furthermore, the implications of DNA forms for the production of infectious virus remain to be resolved.

The purpose of this study was to detect DNA forms homologous to betanodavirus genome RNAs in barfin flounder and Japanese flounder, and to investigate the relationship between the presence of DNA forms and production of infectious virus in the 2 flounder species.

MATERIALS AND METHODS

Fish samples. Tissue samples (n = 134) from sperm, unfertilized eggs, ovulated eggs, fertilized eggs, ovarian cavity fluid, whole larvae (100 mg pooled), and brain and eyes of juveniles (100 mg pooled tissue from 10 fish) of barfin flounder were collected at the Hokkaido Institute of Mariculture (Shikabe, Hokkaido, Japan) in 2000 and 2001 (Appendix 1, Table A1; available at www.int-res.com/journals/supplementary-material/d072p225_app.doc). In 1995 some barfin flounder larvae reared at this facility died of VNN, and all fish in these enclosures (presumably diseased) were culled. Since then, VNN has not occurred at the facility. Thirteen Japanese flounder samples including 11 larval samples (100 mg pooled) and 2 samples from wild juveniles (100 mg pooled tissue from 10 fish) were collected from 5 sites in Hokkaido from 1994 to 2000 (Appendix 1, Table A2). In 1995, VNN occurred at 1 site (Site B; Appendix 1;Table A2), but VNN has never occurred at the other 4 sites. Samples were stored at –80°C until use. None of the larvae and juveniles showed clinical signs of VNN except for 1 dead Japanese flounder larval sample (Sample 2 collected at Site B; Appendix 1; Table A2) collected during the outbreak of VNN in 1995. Cell culture and successive immunoblot assays using an E-11 cell line did not detect any infection by the virus except for this 1 dead Japanese flounder sample (Sample 2; Appendix 1; Table A2).

Experimental fish. Barfin flounder larvae (25 mm total length [TL]) were transferred from the Hokkaido Institute of Mariculture to the wet laboratory of the Hokkaido Central Fisheries Experimental Station (Yoichi, Hokkaido) in 2000, reared for 1 yr and used as juveniles (90 mm TL) for experimental infections. In 2001, barfin flounder larvae (29 mm TL) were again transferred from the same facility to the wet laboratory and reared until use. Japanese flounder juveniles (112 mm TL) were artificially inseminated in 2000 and reared for 1 yr at the wet laboratory until use, and this procedure was repeated again in 2001 (total length of juveniles in 2001: 30 mm). For each of the 2 flounder species, the experimental fish were the progeny of the same adult fish group. The experimental fish were normal in appearance and showed no clinical signs of VNN. Ten individuals collected from each fish group at random showed no cytopathic effects (CPE) in cell culture, and no virus coat protein was detected by successive immunoblot assay using an E-11 cell line, as described below.

Cell line. The E-11 cell line (Iwamoto et al. 2000) was grown at 25°C in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) (final concentration), 100 U ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin sulfate, and adjusted to pH 7.4 with 0.1 N HCl.

The barfin flounder larval cell line MK-1 and Japanese flounder larval cell lines H-1 and H-2 were grown in primary cultures at the Hokkaido Central Fisheries Experimental Station. The 3 cell lines were grown at 20°C in Eagle's minimum essential medium (Invitrogen) supplemented with 20% fetal bovine serum (Sigma), 100 units ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin sulfate, and adjusted pH to 7.2 with 7.5% sodium bicarbonate. The 3 cell lines were non-permissive for betanodavirus, including strain HCF-1.

Cytopathic effects (CPE) assay. Sperm, eggs, larvae, and juveniles were ground with a mortar and pestle. The preparation was mixed with 9 volumes of Leibovitz's L-15 medium supplemented with 1000 U ml^{-1} penicillin G and 1000 µg ml^{-1} streptomycin sulfate at pH 7.4, and incubated at 4° for 20 h. Samples were then centrifuged at 3000 × *g* for 15 min at 4°C. The supernatant was diluted to 10^{-3} by 10-fold serial dilutions with Leibovitz's L-15 medium. The E-11 cells were grown in 0.1 ml Leibovitz's L-15 medium on 24-well plates to 80% confluency, then 50 µl of each dilution was inoculated into a monolayer of E-11 cells at 80% confluency on 24-well plates. The cells were incubated at 20°C for 10 d (1st culture) to observe CPE. Thereafter, 50 µl of the culture medium in each well was passaged to prepared E-11 cells on 24-well plates, and the cells were incubated at 20°C for 10 d (2nd culture) to observe CPE. Betanodavirus was identified by immunoblot assay with rabbit anti-betanodavirus strain HCF-1 coat protein serum.

Virus. Betanodavirus strain HCF-1 was isolated from dead Japanese flounder larvae collected during an outbreak of VNN in 1995 (Sample 2; Appendix 1; Table A2) using the E-11 cell line as described above. The virus-containing culture medium was centrifuged at 3000 × *g* for 15 min at 4°C, and the supernatant was stored at –80°C until use.

Virus propagation for experimental infections. The virus was propagated by culturing the stored betanodavirus strain HCF-1 using the E-11 cell line. For titra-

tion of the virus, the virus-containing culture medium was diluted to 10^{-6} by 10-fold serial dilutions with Leibovitz's L-15 medium (Invitrogen). From each dilution, 50 µl was added to the E-11 cells at 80% confluency in 96-well plates and incubated for 10 d at 20°C. Four wells were used for each dilution. After 10 d, the 50% tissue culture infectious dose ($TCID_{50}$ ml^{-1}) was calculated using the method of Reed & Muench (1938).

Experimental infections. For each species, 50 larvae (barfin flounder: 29 mm TL; Japanese flounder: 30 mm TL) and 50 juveniles (barfin flounder: 90 mm TL; Japanese flounder: 112 mm TL) were intraperitoneally injected with strain HCF-1 at 10^5 $TCID_{50}$ g^{-1} and reared at 20°C for 60 d. Whole heads of the larvae, and the brain and eyes of juveniles were collected from the dead fish and stored at –80°C until use as 'dead fish' samples. The surviving fish were then reared for a further 90 d at 20°C and observed for behavior and external signs of the disease. Whole heads of the larvae, and the brain and eyes of juveniles were then collected as 'surviving fish' samples. Non-infected fish served as an uninfected negative control. For both flounder species, 10 samples were randomly collected from each of the control, dead, and surviving fish samples and assayed.

Extraction of DNA and RNA. Both DNA and RNA were extracted from sperm, eggs, ovarian cavity fluid, larvae, juveniles, and cell lines using a SepaGene Kit (Sanko Junyaku). The extracted DNA and RNA contained DNA and RNA purified from 1 mg of tissue or 1 µl of ovarian cavity fluid per 1 µl of pure water. The extracted DNA and RNA of cells cultured in a 25 cm^2 flask was dissolved in 50 µl of pure water. The purity and concentration of DNA and RNA was determined spectrophotometrically. Extracted DNA and RNA had optical density OD_{260}/OD_{280} ratio values of 1.8 to 2.0. In order to detect DNA forms, extracted samples were assayed for PCR as described below. The presence of DNA forms was confirmed by DNase I (Takara Bio) digestion; part of the extracted sample was digested with DNase I, purified using a SepaGene Kit, and assayed for PCR, which failed to yield specific PCR products. Moreover, several samples were digested with RNase A (Takara Bio), purified, and assayed by PCR to confirm that DNA amplifications did not contribute RNA. To detect viral genome RNA, extracted samples were digested with DNase I, purified by SepaGene Kit, and assayed for RT-PCR as described below. The absence of DNA in DNase I-digested samples was confirmed by PCR, which failed to yield specific PCR products.

PCR. Two sets of primers were used: (1) CF24, 5′-ACCAGACCAAGCCGTTACAGC-3′, and CF25, 5′-AGCAAAGCCGAGAGAAGAAG-3′, to amplify the 669 bp partial betanodavirus genome RNA1, and (2) F2, 5′-CGTGTCAGTCATGTGTCGCT-3′, and R3, 5′-CGAGTCAACACGGGTGAAGA-3′, to amplify the 421 bp partial betanodavirus genome RNA2. Primers CF24 and CF25 were designed based on the published nucleotide sequence of striped jack nervous necrosis viral genome RNA1 detected in a striped jack (GenBank accession number AB025018), because no RNA1 genome sequences derived from barfin flounder and Japanese flounder are recorded in the GenBank. Primers F2 and R3 were reported by Nishizawa et al. (1994). PCR was performed in a 50 µl reaction mixture containing 1 µl of purified sample solution, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 0.4 µM of each primer, using the Gene Amp 2400 system (Applied Biosystems). Cycling conditions were: pre-heating at 95°C for 12 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at either 56.1°C (primer set CF24 and CF25) or 55°C (primer set F2 and R3) for 30 s, and extension at 72°C for 30 s. A 20 µl aliquot of the reaction mixture was electrophoresed at 100 V for 30 min in 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide. An All Purpose Hi-Lo DNA Marker (Bionexus) was used as a size marker. Pure water was used as a negative control. A pGEM-T Easy (Promega) vector that encodes the 421 bp nucleotide partial RNA2 genome or the 669 bp nucleotide partial RNA1 genome was used as a positive control.

RT-PCR. RNA was reverse transcribed with an RNA PCR Core Kit (Applied Biosystems) in a 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 U of RNase inhibitor, 1 µM antisense primer (R3 or CF25), and 2.5 U of MuLV reverse transcriptase. Heating conditions were: 42°C for 30 min, followed by 99°C for 10 min, and chilling at 4°C for storage. PCR was then performed in a 100 µl reaction mixture using a GeneAmp 2400 system (Applied Biosystems).

Sequencing of PCR and RT-PCR products. PCR and RT-PCR products were ligated to a pGEM-T Easy vector (Promega) and amplified in *Escherichia coli*-strain MV 1184. The vector was isolated with a FlexiPrep Kit (GE Healthcare Bio-Sciences) and sequenced by an ABI PRISM 310 automated DNA sequencer (Applied Biosystems) with a BigDye terminator cycle sequencing FS Kit (Applied Biosystems). Homology analysis of the nucleotide sequences was conducted using the CLUSTAL W program (Thompson et al. 1994). The sequence data determined in this study were submitted to the DDBJ database under accession numbers AB046376, AB046377, and AB045980.

Cloning of betanodavirus coat protein gene. The RNA of a dead Japanese flounder larval sample collected during an outbreak of VNN in 1995 in Hokkaido (Sample 2; Appendix 1, Table A2) was used as a template for first strand cDNA synthesis of the betano-

davirus coat protein gene. In order to obtain the double-stranded cDNA, including the complete open reading frame (ORF) of the gene, rapid amplification of the 5′ cDNA end (5′ RACE) was performed using a 5′ RACE system version 2.0 Kit (Invitrogen). First strand cDNA was synthesized by applying the gene-specific antisense primer CF5 (5′-CCCGGTTAGTTTTCCGAGTC-3′) to the 3′ untranslated region and SuperScriptII reverse transcriptase (Invitrogen). After purification by a GlassMAX DNA isolation spin cartridge (Invitrogen), a homopolymeric tail was added to the 3′-end of the first strand cDNA using terminal deoxynucleotidyl transferase and dCTP. PCR amplification was performed using a 5′ RACE abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) (Invitrogen) and a nested gene-specific antisense primer CF6 (5′-CGGATGACCCGGTTAGTTTT-3′). Primer CF6 was designed based on the nucleotide sequence of striped jack nervous necrosis viral genome RNA2 (GenBank accession number D30814), and contained the last 8 nucleotides in the 3′ terminal sequence of the ORF. The PCR product was re-amplified by PCR using an abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) (Invitrogen) and CF6. After amplification, the 5′ RACE products were ligated to a pGEM-T Easy (Promega) vector to form the pGEM-T Easy/coat protein gene ORF and amplified in *E. coli* strain MV 1184. The recombinant vector was isolated with a FlexiPrep Kit (GE Healthcare Bio-Sciences). Cloning of complete coat protein gene ORF was confirmed by sequencing (Fig. 4).

To remove the stop codon TAA in the 5′ untranslated region, the recombinant vector was double-digested by *Sph* I and *Sal* I, and the DNA fragment containing the complete coat protein gene ORF was subcloned into a pKF18k vector (Takara Bio) for site-directed mutagenesis to form the pKF18k/coat protein gene ORF. Three nucleotides in the 5′ untranslated region of the coat protein gene were substituted to form an *Eco* RI recognition site, GAATTC, using a Mutan-Super express Kit (Takara Bio).

Expression and affinity purification of fusion protein. After site-directed mutagenesis, the pKF18k/coat protein gene ORF was digested with *Eco* RI. The fragment containing the complete coat protein gene ORF was subcloned to an expression vector pASK-IBA6 (Genosys Biotechnologies) to form the pASK-IBA6/coat protein gene ORF and amplified in *E. coli* strain BL-21. The transformed *E. coli* strain BL-21 was then cultured while shaking at 25°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) with ampicillin sodium at 100 µg ml^{-1} until the optical density OD_{550} reached 0.5, and was induced by addition of anhydrotetracycline at a final concentration of 200 ng ml^{-1} and further cultured while shaking at 25°C for 3 h. The cells were harvested by centrifugation at 4500 × *g* for 12 min at 4°C, resuspended in pure water, ultrasonicated, and centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was collected and stored at 4°C. To avoid irreversible binding of any naturally covalently biotinylated protein or free biotin in *E. coli* (Schmidt & Skerra 1993) to the StrepTactin affinity matrix (Genosys Biotechnologies), which is streptavidine engineered for optimized binding affinity (Voss & Skerra 1997) to an octapeptide (NH_2-WSHPQFEK-COOH) *Strep*-tag II with streptavidine-binding characteristics (Schmidt & Skerra 1994, Schmidt et al. 1996), avidine was added to the supernatant at 100 µg ml^{-1}, incubated for 30 min at 4°C, and centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was used for affinity purification of the fusion protein (the coat protein with *Strep*-tag II) with StrepTactin affinity columns (Genosys Biotechnologies). To confirm expression of the fusion protein, a 20 µl aliquot of the affinity-purified protein solution was loaded on 10% SDS-polyacrylamide gel followed by immunoblot assay with streptavidine alkaline phosphatase conjugate (see Fig. 5), because streptavidine specifically binds to the octapeptide *Strep*-tag II of the fusion protein, as described above. The chromatic reaction was developed using nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl-phosphate (Genosys Biotechnologies). Approximately 3 mg of the fusion protein was harvested and used for production of rabbit anti-betanodavirus coat protein serum. Measurement of antibody titers in rabbit serum was performed by ELISA with the fusion protein and anti-rabbit immunoglobulin G alkaline phosphatase conjugated antibodies (Sigma). On Day 49 after initial immunization, the absorbance (A) readings of diluted serum (ratio 1/25 600) at A 405 nm reached 0.894 and the whole blood was collected. To remove cross-reacting antibodies present in the antiserum, adsorption was conducted using acetone-dried E-11 cells according to the method of Sambrook et al. (1989).

Immunoblot. After the CPE assay, the cells were harvested, suspended in Laemmli's sample buffer, and loaded on 10% SDS-polyacrylamide gels following immunoblot assay with rabbit anti-betanodavirus coat protein serum and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology). The chromatic reaction was developed using 3,3′-diaminobenzidine and H_2O_2. Prestained SDS-PAGE standards and Precision protein standards (Bio-Rad Laboratories) were used as size markers.

Transfection assay. In order to prepare viral genome RNAs for transfection assay, E-11 cells were infected with the virus strain HCF-1 with a multiplicity of infection (MOI) of 0.1 and incubated at 20°C for 10 d. CPE cells were then harvested aseptically. Both DNA and

RNA were extracted using a SepaGene Kit and digested with DNase I. The absence of DNA forms was confirmed by PCR, which failed to yield specific 669 and 421 bp products. The presence of viral genome RNA1 and RNA2 in the sample destined for transfection assays was confirmed by RT-PCR. RNA from E-11 cells without infection was prepared and served as a negative control.

H-1 cells, H-2 cells, and MK-1 cells at 80% confluency in 25 cm^2 flasks were each transfected with 1.8 µg RNA that contained the viral genome RNAs; 3 cell lines transfected with 1.5 µg RNA from E-11 cells without infection were used as negative controls. Both were incubated for 10 d at 20°C. DNA and RNA were extracted, digested with RNase A, and purified using a SepaGene Kit. DNA extracted from each 25 cm^2 flask was dissolved in 50 µl pure water. The presence/absence of RNA1 DNA and RNA2 DNA in DNase I-digested samples was analysed by PCR, which confirmed the absence of these DNA forms after failing to yield the specific 669 and 421 bp products.

In order to clarify whether DNA forms generated in cells from the transfected viral genome RNA persist for a longer incubation period, MK-1 cells were transfected with 1.5, 3.0, or 4.5 µg RNA that contained viral genome RNAs and incubated at 20°C for 5 wk with 5 successive passages (approx. 4-fold dilution of cell density per passage). Both DNA and RNA of the cells were extracted and treated as described above. DNA forms were detected by PCR. To compare the stability of generated DNA forms in each cell line with that of the cDNA of the coat protein gene ORF, the recombinant vector pGEM-T Easy/coat protein gene ORF was amplified in *E. coli* MV1184 and purified using a Flexi-Prep Kit. The purified recombinant vector was then digested with *Eco* RI and, after electrophoresis, a fragment containing the cDNA was purified from 0.8% agarose gels using Ultra-free DA (Millipore). MK-1 cells were transfected with 13.6, 27.2, or 54.4 µg cDNA and treated as described above. The purity and concentration of RNA and DNA in samples for transfection assays was determined spectrophotometrically.

Reverse transcriptase (RTase) assay. In order to quantitatively determine RTase activity through the incorporation of digoxigenin- and biotin-labeled dUTP into template/primer hybrid poly(A)-oligo$(dT)_{15}$, ELISA was conducted using a Reverse Transcriptase Assay, non-radioactive kit (Roche Applied Science). H-1, H-2, MK-1, and E-11 cells were lysed in 50 mM Tris-HCl buffer (pH 7.8) and centrifuged at 12 000 × *g* for 15 min at 4°C, and 40 µl cell-free lysate, which corresponded to approx. 1×10^6 cells, was applied per well. RTase activity of the sample was assayed at 20°C (which is optimal for the growth of the cells) for 15 h. Coloration was performed at 20°C for 30 min. RTase activity of the samples was determined according to a calibration curve of human immunodeficiency virus (HIV-1) RTase assayed at 37°C for 15 h, and expressed as pg HIV-1 RTase per well. The specific activity of HIV-1 RTase is more than 5 U μg^{-1}, where 1 unit is defined as the amount of enzyme required for the incorporation of 1 nmol of labeled dNTP within 10 min at 37°C; poly(A)-oligo$(dT)_{15}$ was used as the template/primer hybrid according to the instructions of the manufacturer.

RESULTS

Detection of DNA forms in fish

PCR products 669 and 421 bp in length were detected in sperm, unfertilized eggs, ovulated eggs, fertilized eggs, ovarian cavity fluid, larvae, and juveniles of barfin flounder (Fig. 1A,B) and in larvae and juveniles of Japanese flounder (Fig. 1C,D) collected at 5 sites using primer set CF24 and CF25 and primer set F2 and R3, respectively. The sizes of the products were identical to those deduced from corresponding regions of the viral genome RNA1 and RNA2 (Fig. 1). The DNA forms homologous to viral genome RNA1 and RNA2 were named RNA1 DNA and RNA2 DNA, respectively.

Barfin flounder samples collected in 2000 and 2001 were examined for the presence of RNA1 DNA and RNA2 DNA (Appendix 1, Table A1) and viral genome RNA1 and RNA2. RNA1 DNA and RNA2 DNA were detected in 17 and 44 samples, respectively, from a total of 134 samples (Appendix 1, Table A1), whereas viral genome RNA1 and RNA2 were detected in 5 and 12 samples, respectively. Only 5 samples were positive for both RNA1 DNA and RNA2 DNA. No sample contained both viral genome RNA1 and RNA2. The detection rate of RNA2 DNA was higher than that of RNA1 DNA. Neither CPE nor coat protein was detected in any sample of the E-11 cell line.

RNA2 DNA was detected in 9 samples of Japanese flounder larvae, from a total of 13, whereas no RNA1 DNA was detected (Appendix 1, Table A2). Neither CPE nor virus coat protein was detected except for 1 dead larval sample collected during an outbreak of VNN in 1995 (Sample 2; Appendix 1, Table A2), in which both RNA1 and RNA2 viral genomes were detected.

Nucleotide sequences of DNA forms

The nucleotide sequence of the 627 bp RNA1 DNA (RNA1 DNA [BF]) detected in barfin flounder (Sample

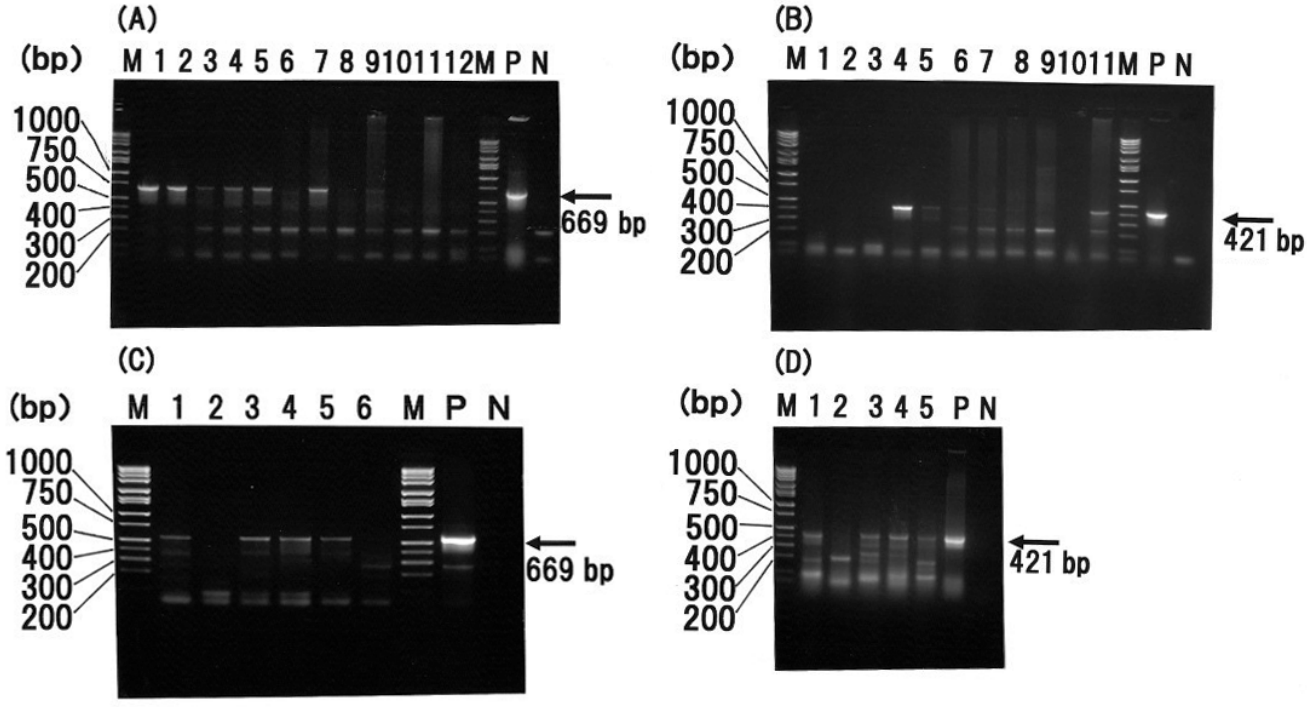

Fig. 1. PCR detection of DNA forms in barfin flounder and Japanese flounder. (A) RNA1 DNA in barfin flounder; 6 sperm samples collected in Mar 2001 (Lanes 1–6) and 6 juvenile samples collected in July 2001 (Lanes 7–12). Lanes 1, 2, 4, 5, and 7 are positive. Lane 3 is weakly positive. Other lanes are negative. (B) RNA2 DNA in barfin flounder; 6 unfertilized eggs (Lanes 1–6), 1 ovulated egg (Lane 7), and 4 sperm (Lanes 8–11) samples collected in March and April 2001. Lanes 4, 5, and 11 are positive. Lanes 6 and 7 are weakly positive. Other lanes are negative. (C) RNA1 DNA in Japanese flounder larvae (uninfected control in experimental infection). Lanes 1 and 3–5 are positive. Lanes 2 and 6 are negative. (D) RNA2 DNA in Japanese flounder; 5 larvae samples collected in June 2000 (Lanes 1–5). Lanes 1 and 3–5 are positive. Lane 2 is negative. M: DNA size marker; P: pGEM-T Easy vector encoding the 669 bp nucleotide partial RNA1 genome or the 421 bp nucleotide partial RNA2 genome (positive control); N: water (negative control)

61; Appendix 1, Table A1) was compared to 6 sequences of the 627 bp partial genome RNA1 of betanodaviruses detected in Atlantic halibut (GenBank accession number AJ401165), Japanese flounder (Sample 2; Appendix 1, Table A2, AB046376), greasy grouper (AF326776 and NC_004137), and striped jack (AB025018 and NC_003448) (Fig. 2). Sequence similarities between RNA1 DNA and viral genome RNA1 of the 6 strains ranged from 82 to 96% at the nucleotide level. In particular, RNA1 DNA showed a sequence similarity of 96% with the viral genome RNA1 detected in Atlantic halibut.

Nucleotide sequences of the 381 bp RNA2 DNA (RNA2 DNA [BF] and RNA2 DNA [JF]) detected in a barfin flounder sample (Sample 1; Appendix 1, Table A1) and a Japanese flounder sample (Sample 1; Appendix 1, Table A2) were compared to 11 sequences of the 381 bp partial genome RNA2 of betanodavirus detected in barfin flounder (GenBank accession numbers D38635 and AB046377), Atlantic halibut (AF160473), Japanese flounder (AB045980 and D38527), red-spotted grouper (D38636), greasy grouper (AF281657 and NC_004136), tiger puffer (D38637), and striped jack (D30814 and NC_003449) (Fig. 3). Sequence similarities between RNA2 DNA and the viral genome RNA2 of 11 betanodaviruses ranged from 69 to 98% at the nucleotide level. In particular, the sequence similarity between RNA2 DNA (BF) and the viral genome RNA2 detected in barfin flounder and Atlantic halibut was 98% at the nucleotide level; the sequence similarities between RNA2 DNA (JF) and viral genome RNA2 from barfin flounder and Atlantic halibut were also 98%.

Experimental infections

In barfin flounder, the cumulative mortalities of larvae and juveniles 60 d after infection were 38 and 6%, respectively, whereas cumulative mortalities of 2 and 0% were observed in uninfected controls. Although the cumulative mortality of larvae and juveniles of Japanese flounder reached 40 and 4% after infection, uninfected controls showed no mortality. Infected fish exhibited abnormal swimming behavior with hemorrhaging of the brain and darkening of body color. Sur-

viving larvae and juveniles were reared for a further 90 d, and no mortality was observed during that period.

In barfin flounder, RNA1 DNA was detected in 70% of larval and 0% of juvenile uninfected controls, in 0% of larval and 33% of juvenile fish that died subsequent to injection, and in 70% of larval and 80% of juvenile fish that survived. RNA2 DNA was detected in 10% of larval and 0% of juvenile uninfected controls, in 0% of larval and 33% of juvenile fish that died subsequent to injection, and in 80% of larval and 90% of juvenile fish that survived (Appendix 1, Table A3). The detection rates of RNA2 DNA in surviving fish samples were higher than those in uninfected controls of both larvae and juveniles; in contrast, in the respective surviving fish samples, the detection rate of RNA1 DNA was higher than that in juvenile uninfected controls, and equal to that in larval uninfected controls. Both viral genome RNA1 and RNA2 were detected in all dead

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RNA1 DNA (BF)  gggctcgcaa cgatgctaac cgatatcacg atgagttcac tatgatcgtc aaggcgttcc  60
AJ401165       .......... .......... .......... .......... .......... ..........  60
AB046376       .......g.. t......... .......... g......... ......t... ..a.......  60
AF326776       .......g.. t......... .......... .......... ......g... ..a.......  60
NC_004137      .......g.. t......... .......... .......... ......g... ..a.......  60
AB025018       ....c..g.. t........t .......... .......... .......... ..........  60
NC_003448      ....c..g.. t........t .......... .......... .......... ..........  60

RNA1 DNA (BF)  agaagaaaga agcatacaat gccccaaatt atccccggaa tatttcgacc gttccgcaca  120
AJ401165       .a........ .......... .......... .......... .......... ..........  120
AB046376       .a........ .........c ...g...... ....g..... c.....a..t ..........  120
AF326776       .a........ .........c .......... .....a.... c.....a... ........t.  120
NC_004137      .a........ .........c .......... .....a.... c.....a... ........t.  120
AB025018       .a........ .......... ........c. ....g..... c..a..a..t ..c.......  120
NC_003448      .a........ .......... ........c. ....g..... c..a..a..t ..c.......  120

RNA1 DNA (BF)  accaaaatgt caagctgtcc agctacacct acgctttcaa agctagtact ctccgacatg  180
AJ401165       .......... ....t..... .......... .......... .......... ....a.....  180
AB046376       c......c.. ....t..... ....t..... .......... ...c....tc ....ag....  180
AF326776       c......c.. ....t.a... .......... .......... ...c...gt. ....ag....  180
NC_004137      c......c.. ....t.a... .......... .......... ...c...gt. ....ag....  180
AB025018       c......... t......... .......... .......... ..aagcagtc ....ag....  180
NC_003448      c......c.. t......... .......... .......... ..aagcagtc ....ag....  180

RNA1 DNA (BF)  ttccgtggta catgccaacg cacacaccgg ctgaaatagc cgacgcagtg caaaacttgg  240
AJ401165       .......... .......... .......... .......... .......... ..........  240
AB046376       .......... .......... ........a. .......t.. t......... .....t..a.  240
AF326776       .......... .......... ........a. .......c.. t......... ..........  240
NC_004137      .......... .......... ........a. .......c.. t......... ..........  240
AB025018       .......... .........t .......... .......t.. ...g..t... ..g.gt....  240
NC_003448      .......... .........t .......... .......t.. ...g..t... ..g.gt....  240

RNA1 DNA (BF)  ctgccagtac cactgagttg gttgagactg actacagcaa attcgatggc acatttttgc  300
AJ401165       .......... .......... .......... .......... g.....c... .....c....  300
AB046376       .a..t...t. .......c.. .......... .......... g.....c... .....c....  300
AF326776       ....a...t. .......c.. .....a..c. .......... g......... .....c....  300
NC_004137      ....a...t. .......c.. .....a..c. .......... g......... .....c....  300
AB025018       .c..a...t. .......c.. .......... ....t..... ......c... ..g..cc.c.  300
NC_003448      .c..a...t. .......c.. .......... ....t..... ......c... ..g..cc.c.  300

RNA1 DNA (BF)  gctttatgcg tgagtgcgtc gagtttgcaa tctacaagcg gtgggtgcat ctggaccact  360
AJ401165       .......... .......... .......... .......... .......... ..........  360
AB046376       .......... ..g.a....t ........t. .......... t........c t.........  360
AF326776       .......... .......... ..a.....t. ....t..... c.....t..c ..........  360
NC_004137      .......... .......... ..a.....t. ....t..... c.....t..c ..........  360
AB025018       .t..c..... g...aat... ..a.....c. .t........ t........c ..c.....t.  360
NC_003448      .t..c..... g...aat... ..a.....c. .t........ t........c ..c.....t.  360

RNA1 DNA (BF)  tggccgagtt atctcaacta ttggcaaatg aactccaagc cccagctgtt acccgtctcg  420
AJ401165       .......... .......... .......... .......g.. .......... ..........  420
AB046376       ..c.a..a.. ga.aacct.. .....t.... ..a.t..... a..g..... .....at.g.  420
AF326776       ..c.a..... .a.aactt.. .....t.... .ga....... a..t..... ..a..a..g.  420
NC_004137      ..c.a..... .a.aactt.. .....t.... .ga....... a..t..... ..a..a..g.  420
AB025018       .aa.a..a.. g..cac.... ....g..... .......... a..t.....c .....g..g.  420
NC_003448      .aa.a..a.. g..cac.... ....g..... .......... a..t.....c .....g..g.  420
```

Fig. 2. Comparison of partial nucleotide sequences of RNA1 DNA from barfin flounder (BF) sperm and genome RNA1 from 6 betanodavirus strains (identified by GenBank accession number). AJ401165: Atlantic halibut; AB046376: Japanese flounder; AF326776 & NC_004137: greasy grouper; AB025018 & NC_003448: striped jack. .: nucleotides identical to that at the same position in RNA1 DNA (BF) sequence

```
RNA1 DNA (BF)  gtctaaagta tgaccctgac tgcagccgcc tcagcggctc ggcactcaca actgacggta  480
AJ401165       ....g..a.. .......... .......... .......t.. .......... ..........  480
AB046376       ..a.t..a.. ...t.....t .....t.... .t..t..... t..t.....g ..a..t....  480
AF326776       .ca.c..... .........t .....t.... ....t..t.. t..t...... ..c.....a.  480
NC_004137      .ca.c..... .........t .....t.... ....t..t.. t..t...... ..c.....a.  480
AB025018       .ca.c..a.. ......a... .....t..t. .t.....t.. c..t.....g ........g.  480
NC_003448      .ca.c..a.. ......a... .....t..t. .t.....t.. c..t.....g ........g.  480

RNA1 DNA (BF)  acagcaaagc taacgcattc gtctcctact tggccaaccg attggctggg atggacgatg  540
AJ401165       ......tt.. .......... .......... .......... .......... ..........  540
AB046376       ......tt.. ...t..t... .....a..tc .c..tggt.. ta.......c .....t....  540
AF326776       ......tt.. ...t..t... .....a...c .t..tggt.. ca.......c .....t....  540
NC_004137      ......tt.. ...t..t... .....a...c .t..tggt.. ca.......c .....t....  540
AB025018       ......tt.. a..t..t... .....a..tc .t...gg... ccaa.....c .....t....  540
NC_003448      ......tt.. a..t..t... .....a..tc .t...gg... ccaa.....c .....t....  540

RNA1 DNA (BF)  gcgaagcatg gtcttggatt ggcattgttt acggtgacga tggtctccgt tccggcaacg  600
AJ401165       a......... .......... ........g. .......... .......... ..........  600
AB046376       a......t.. .a........ ..t.....c. .......t.. ...g...a.a ..t.......  600
AF326776       at.....t.. .........c ........g. .......t.. ...g.....a ..t..t..t.  600
NC_004137      at.....t.. .........c ........g. .......t.. ...g.....a ..t..t..t.  600
AB025018       a...g..... .a........ ..t.....c. .......... c......a.a .....a....  600
NC_003448      a...g..... .a........ ..t.....c. .......... c.....ta.a .....a....  600

RNA1 DNA (BF)  tgtctgacgc gttgctgtcc aaaactg 627
AJ401165       .t..g...a. cc.c..ca.t g.t.... 627
AB046376       .t..aa.t.g .c.c..ca.. g..... 627
AF326776       .t..aa...a .c.c..ca.. ..c.... 627
NC_004137      .t..aa...a .c.c..ca.. ..c.... 627
AB025018       .......... .......... ....... 627
NC_003448      .......... .......... ....... 627
```

Fig. 2 (continued)

larval and juvenile samples. Viral genome RNA1 and RNA2 were detected in 10% of juveniles and 20% of larvae from other samples, respectively.

In Japanese flounder, RNA1 DNA was detected in 50% of larval and 0% of juvenile uninfected controls, in 0% of larval and juvenile fish that died subsequent to injection, and in 10% of larval and 0% of juvenile fish that survived. RNA2 DNA was detected in 10% of both larval and juvenile of uninfected controls, in 100% of larval and 0% of juvenile fish that died subsequent to injection, and in 80% of larval and 100% of juvenile fish that survived (Appendix 1, Table A4). The detection rate of RNA2 DNA was higher in surviving fish samples than in uninfected controls (for both larvae and juveniles), whereas that of RNA1 DNA in surviving fish samples was lower than or equal to that in uninfected controls. Both viral genome RNA1 and RNA2 were detected in all dead fish samples. No viral genome RNA was detected in any other sample.

CPE and virus coat protein were detected only in fish that died subsequent to injection (Appendix 1, Tables A3 & A4, see Fig. 6C).

Cloning of complete coat protein gene ORF

Cloning of the complete coat protein gene ORF was confirmed by nucleotide sequencing of the recombinant vector pGEM-T Easy/coat protein gene ORF. A total of 1036 bp including 27 bp of the complete 5′ untranslated region and 1009 bp of the ORF were determined (Fig. 4). The complete ORF sequence (1017 bp) contained 8 bp deduced from the nucleotide sequence of striped jack nervous necrosis viral genome RNA2, including the stop codon TAA in the 3′ terminal sequence (see 'Materials and methods') that encodes 338 amino acid residues. Deduced molecular weight was 37 119 Da. The predicted amino acid sequence of the coat protein gene contains a D-75 residue and 2 adjacent stretches of arginines that are conserved in all nodaviruses (Delsert et al. 1997).

Expression and affinity purification of fusion protein

The fusion protein consisted of the octapeptide *Strep*-tag II (deduced molecular weight 1058 Da), 15 additional amino acid residues (NH_2-IEGRRRNRGPE-FQIT-COOH, deduced molecular weight 1829 Da), and the coat protein. The total deduced molecular weight was 40 006 Da. The expression of the fusion protein with the pASK-IBA6/coat protein gene ORF in *E. coli* BL-21 was confirmed. The affinity-purified fusion protein (Fig. 5, Lane 1) and the lysate of *E. coli* BL-21 with the recombinant vector (Lane 2) after

```
RNA2 DNA (BF)  ggagtgtgcg tctcagtgtt ccatctctcg agacacctga agacacattc gctccaatcc  60
RNA2 DNA (JF)  .......... .......... .......... .......... ...t...... ..........  60
D38635         .......... .......... .......... .......... ...t...... ..........  60
AB046377       .........a .......... .......... .......... .......... ..........  60
AF160473       .......... .......... .......... .......... ...t...... ..........  60
AB045980       .......t.. ...g..c... ..g.....t. .......... ...g..gact .....c...a  60
D38527         .......t.. a..g..c... ..g.....t. .......... ...g..cac. .....c...a  60
D38636         .......t.. at.g..c... ........t. .......... ...g..cac. .....c...a  60
AF281657       .......t.. a..g..c... ........t. .......... ...g..cac. .....c...a  60
NC_004136      .......t.. a..g..c... ........g. .......... ...g..cac. .....c...a  60
D38637         .......... c..t.....c ..t...t.g. .a........ g..a..... .........a  60
D30814         ....c..t.. c..t.....c ..g..c..t. .......... g.....cac. ........ta  60
NC_003449      ....c..t.. c..t.....c ..g..c..t. .......... g.....cac. ........ta  60

RNA2 DNA (BF)  taaccttggg accactctac aacgactccc ttg~~~~~~~ cagccagtga tttcaaatca  113
RNA2 DNA (JF)  .......... .......... .......... .......... ......a... ..........  113
D38635         .......... .......... .......... .......... ......a... ..........  113
AB046377       .......... .......... .......... .......... .......... ..........  113
AF160473       .....c.... .......... .......... .......... ......ac.. ..........  113
AB045980       .g..acaa.. tt.c..g... .....t.... .c........ .t..a..ca. c.....g..c  113
D38527         .g..acaa.. t..c..g... .....t.... ..t....... .ca.a.ac.. c.....g..c  113
D38636         .g..acaa.. tt.c..g... .....t.... ..t....... .ca.a.a... c.....g..c  113
AF281657       .g..acaa.. tt.c..g... .....t.... ..t....... .ca.a.c... c.....g..c  113
NC_004136      .g..acaa.. tt.c..g... .....t.... ..t....... .ca.a.a... c.....g..c  113
D38637         c..g.ca... ...g..g... .....t...a .ca.caactg .ca.ttc..g g..tcgt..c  119
D30814         ct...ca..c g......c.. .....t...a ..aacaacgg tta.a~c..g a..tcgt..c  119
NC_003449      ct...ca..c g......c.. .....t...a ..aacaacgg tta.a~c..g a..tcgt..c  119

RNA2 DNA (BF)  atacttcttg gctctaccca gcttgacatc gcccctgaag gagccgtctt ttcattagat  173
RNA2 DNA (JF)  .......... .......... .......... .......... .......... ..........  173
D38635         .......... .......... .......... .......... .........a ..........  173
AB046377       .......... .......... .t........ .......... .........a ..........  173
AF160473       .......... .......... .......... .......... .........a ..........  173
AB045980       ..c..c..g. .g..c..a.. .t.g.....t ..t..c..t. .g..aa.... .ca...g...  173
D38527         ..c..c..a. .a..c..a.c a..g.....t ........t. .c..aa.... ccagc.g..c  173
D38636         ..c..c..a. .a..c..a.c a..g.....t ........t. ....a..... ccagc.g..c  173
AF281657       ..c..c..a. .a..c..a.c a..g.....t ........t. ....a..... ccagc.g..c  173
NC_004136      ..c..c..a. .a..c..a.c a..g.....t ........t. ....a..... ccagc.g..c  173
D38637         ..c..c.... .....ggt.. .......... ..t...cc.. .ca.t....a ...ga.t..c  179
D30814         ..t..ct.g. ....g..... a..c...c.. ..t....c.a ac..t..... .gtcact..c  179
NC_003449      ..t..ct.g. ..g.g..... a..c...c.. ..t....c.a ac..t..... .gtcact..c  179

RNA2 DNA (BF)  cggccgctat ccattgacta cagtctgggc actggtgatg tcgaccgtgc cgtttactgg  233
RNA2 DNA (JF)  .......... .......... .......... .......... .......... ..........  233
D38635         ........g. .......... .......... .......... .......... ..........  233
AB046377       ........g. .......... .......... .......... .......... ......t...  233
AF160473       ........g. .......... .......... .......... .......... ..........  233
AB045980       ..a..at.g. .......... t..c..a..a .......... .......... t..c......  233
D38527         ..t.....g. .......t.. ...c..t..a .....a.... .t........ t..c..t...  233
D38636         ..t.....g. .......... ...c..t..a .....a.... .t........ t.....t...  233
AF281657       ..t.....g. .......... ...c..t..a .....a.... .t........ t.....t...  233
NC_004136      ..t.....g. .......... ..tc..t..a .....a.... .t........ t.....t...  233
D38637         a.a..a..g. .t..c..t.. ..ac.....a gt......c. .t........ t..g......  239
D30814         aaa...t.gc .......t.. ..a...t..a gtg..c..c. .......g.. ...g......  239
NC_003449      aaa...t.gc .......t.. ..a...t..a gtg..c..c. .......g.. ...g......  239
```

Fig. 3. Comparison of partial nucleotide sequences of RNA2 DNA from barfin flounder (BF) sperm and Japanese flounder (JF) juveniles and genome RNA2 from 11 betanodavirus strains (identified by GenBank accession number). AB046377 & D38635: barfin flounder; AF160473: Atlantic halibut; D38527 & AB045980: Japanese flounder; D38636: red-spotted grouper; D38637: tiger puffer; AF281657 & NC_004136: greasy grouper; D30814 & NC_003449: striped jack. .: nucleotides identical to that at the same position in RNA2 DNA(BF) sequence; ~~~: nucleotide gap present in upper 10 sequences

induction of expression produced a band of approx. 40 kDa, which was identical to the deduced molecular weight of the fusion protein; in contrast, the 40 kDa band was not detected in the lysate of *E. coli* BL-21 with (Lane 3) or without (Lane 4) the pASK-IBA6 vector after induction of the expression.

Immunoblot

Immunoblot assays revealed the presence of a 37 kDa protein in E-11 cells, 3 to 7 d after inoculation of the virus (Fig. 6A). Extra bands with lower molecular weights were also detected. This 37 kDa band was detected in E-

```
RNA2 DNA (BF)  catgtgaaga aagttgctgg caatgtggga acacctgcgg ggtggttcca ctgggggcta  293
RNA2 DNA (JF)  .......... .......... .......... .......... .......... ..........  293
D38635         .......... .......... .......... g......... .......... ..........  293
AB046377       .......... .......... .....c.... .......... .......... ..........  293
AF160473       .......... ...c...... .....c.... .......... .......... ..........  293
AB045980       ..cc.c.... .gt.....a. catcttcc .......... .c.....t.g t.....ca.c  293
D38527         ..cc.c.... .gta.....a ...act..c ........a. .c.....t.g ......ca.c  293
D38636         ..ca.c.... .gt.....a. ....ct..c ........a. .c.....t.g ......ca.c  293
AF281657       ..cc.c.... .gt.....a. ....ct..c ........a. .c.....t.g ......ca.c  293
NC_004136      ..cc.t.... .gt.....a. ....ct..c ........a. .c.....t.g ......ca.c  293
D38637         ..cc..ctc. .gaagaaa.. tg..ccaaac .ac.....a. .c.tc..gg. t.....at.g  299
D30814         ..cc..c... .gaaa..... ag.cactcag gt......t. ...ac..tg. ......a..g  299
NC_003449      ..cc..cg.. .gaaa..... ag.cactcag gt......t. ...ac..tg. ......a..g  299

RNA2 DNA (BF)  tgggataatt tcaacaaaac attcacgcag ggcgctgcct actattctga tgcgcagcct  353
RNA2 DNA (JF)  .......... .......... .......... ....t..... .......... ..........  353
D38635         .......... .......... ......a... ....t..... .......... ..........  353
AB046377       .....c.... .......... ......a... .......... .......... ..........  353
AF160473       .......... .......... ......a... .......... .......... ..........  353
AB045980       .....c..c. .......g.. g......g.t ....tg..t. ....c..... c.........  353
D38527         .....c.... .......... g.....ag.t ....t...t. ....c..... ..a.......  353
D38636         .....c..c. .......g.. g.....ag.t ....t..... ....c..... ..a.......  353
AF281657       .....c..c. .......g.. g.....ag.t ....t...a. ....c..... ..a......c  353
NC_004136      .....c..c. .t..t..g.. g.....ag.t ....t...t. ....c..... ..a......c  353
D38637         ......g... ....t...gt .......act ....tc.... .t..c..c.. cca.......  359
D30814         ......g.c. .t.....g.. ......agtt ..g..gc... ....c..c.. cca...a..a  359
NC_003449      ......g.c. .t.....g.. ......agtt ..g..ac... ....c..c.. cca...a..a  359

RNA2 DNA (BF)  cgacagatct tgctgccagt gggcacgc 381
RNA2 DNA (JF)  .......... .......... ........ 381
D38635         .......... .......... ........ 381
AB046377       .......... .......... ........ 381
AF160473       .......... .......... ........ 381
AB045980       ..t..a..tc .c.....c.. t.....cg 381
D38527         ..t..a..tc .......t.. t.....tg 381
D38636         ..t..a...c .......t.. t.....tg 381
AF281657       ..t..a...c .......t.. t.....tg 381
NC_004136      ..t..a...c .......t.. t.....tg 381
D38637         ..g.....t. .......t.. ......ag 387
D30814         ..g..a.... .......g.c t....... 387
NC_003449      ..g..a.... .......g.c t....... 387
```

Fig. 3. (continued)

11 cells inoculated with samples from fish that died subsequent to injection (Fig. 6C), but not in those inoculated with uninfected controls (Fig. 6B) or with samples from surviving fish (Fig. 6D). An extra band of approx. 50 kDa was observed in all samples (Fig. 6).

Transfection

The RNA samples used for transfection assays were negative by PCR and positive by RT-PCR. RNA2 DNA was detected in all 3 cell lines (H-1, H-2, and MK-1) transfected with RNA that contained viral genome RNAs 10 days after transfection (Fig. 7A). No RNA2 DNA was detected in negative controls. RNA1 DNA was not detected in any of the 3 transfected cell lines or in the negative controls (Fig. 7B). No CPE was observed in any of the 3 transfected cell lines. E-11 cells that were infected with the virus and cultured for 10 d exhibited CPE and were assayed by PCR, which detected both RNA1 DNA and RNA2 DNA (Fig. 7A,B).

After 5 wk incubation with 5 successive passages, RNA2 DNA was detected in the MK-1 cell line transfected with 1.5 and 3.0 µg RNA that contained the viral genome RNAs, but not in the cell line transfected with 4.5 µg RNA (Fig. 7C). The cDNA of RNA2 was detected in cells transfected with 27.2 µg DNA. No CPE was observed in the cells.

RTase assay

When the average absorbance reading (from duplicates) at 405 nm with a reference wavelength of 492 nm was plotted against the HIV-1 RTase serial dilution, a linear calibration curve was produced with a range of 31.3 to 1000 pg HIV-1 RTase per well (Fig. 8). According to the manufacturer, the detection limit was defined to be a signal level twice that of the background, or 8.6 pg HIV-1 RTase per well. The RTase activities of H-1, H-2, MK-1, and E-11 cells were equivalent to 55, 35, 103, and 62 pg HIV-1

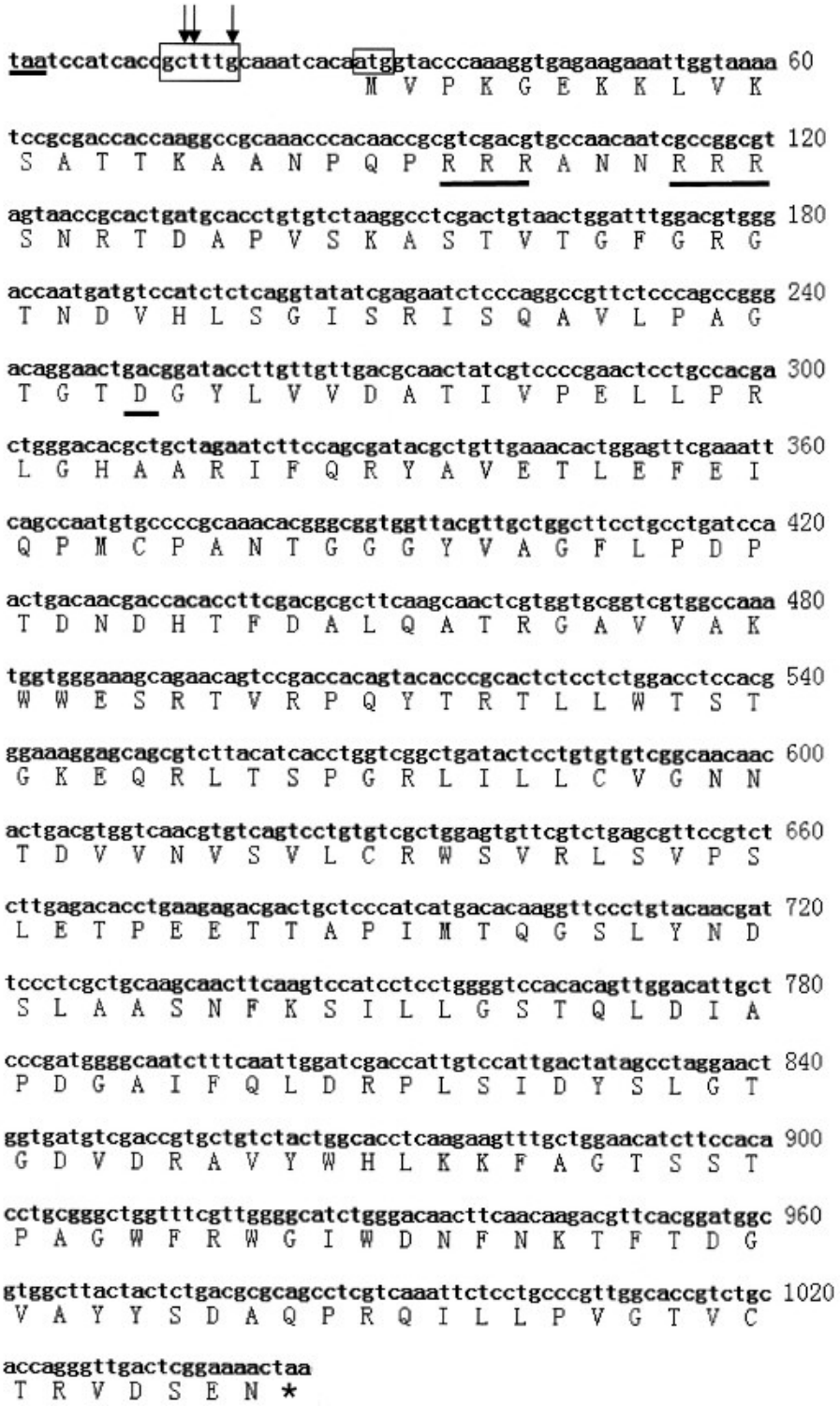

Fig. 4. Nucleotide and predicted amino acid sequences of coat protein gene of betanodavirus strain HCF-1 isolated from Japanese flounder. Nucleotide sequence consists of 27 bp from complete 5′ untranslated region and 1017 bp from ORF. The last 8 nucleotides (including stop codon taa in 3′ terminal sequence of ORF) were deduced from nucleotide sequence of striped jack nervous necrosis virus (NNV) RNA2 (GenBank accession number D30814). Stop codon taa present in 5′ untranslated region is underlined. Nucleotides indicated by ↓ were converted by site-directed mutagenesis (c→a, t→a, g→c) to form the *Eco* RI recognition site gaattc for subcloning to expression vector pASK-IBA6. atg (enclosed): start codon of coat protein gene; *: stop codon of coat protein gene. D-75 residue and 2 adjacent stretches of arginines (conserved in all nodaviruses) are underlined

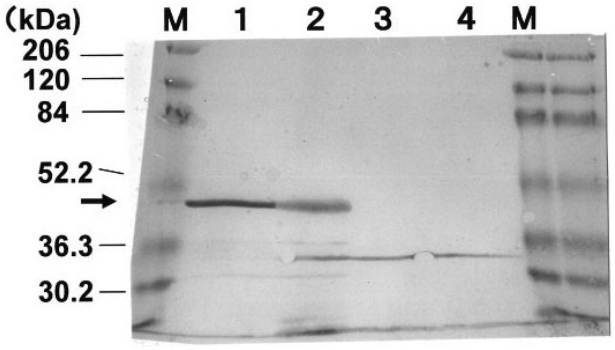

Fig. 5. Confirmation of expression of fusion protein in *E. coli* BL-21 (indicated by →) by immunoblot assay. Lane 1: affinity-purified fusion protein (deduced molecular weight 40006); Lane 2: *E. coli* BL-21 lysate with recombinant vector pASK-IBA6/coat protein gene ORF after induction of expression; Lane 3: *E. coli* BL-21 lysate with vector pASK-IBA6 after induction; Lane 4: *E. coli* lysate after induction. M: prestained SDS-PAGE standards

RTase per 10^6 cells at 20°C, respectively. Values from all 4 cell lines were within the linear portion of the calibration curve.

DISCUSSION

The DNA forms detected in this study were probably derived from viral genome RNAs. This conclusion is based on 3 findings. Firstly, experimental infections showed that the detection rates of the DNA forms RNA1 DNA and RNA2 DNA in the surviving fish samples were generally higher than those in uninfected controls (Appendix 1, Tables A3 & A4). Secondly, nucleotide sequence analysis revealed that sequence similarities between the DNA forms and viral genome RNAs were high (Figs. 2 & 3): sequence similarity between RNA2 DNA detected in the 2 fish species and viral genome RNA2 ranged from 69 to 98%; similarly, sequence similarity between RNA1 DNA and viral genome RNA1 ranged from 82 to 96%. Nishizawa et al. (1995) reported that a portion of the coat protein gene of 5 betanodaviruses, including barfin flounder and Japanese flounder nervous necrosis virus (NNV), has a sequence similarity of ≤28.6% with insect nodaviruses, but of ≥75.8% among the betanodaviruses themselves. Moreover, Nishizawa et al. (1997) reported that 25 isolates of betanodaviruses from 16 fish species were classified into 4 types; sequence similarity was ≥95% between each type. These studies analyzed the same region of the coat protein gene as this study. These findings imply close relationships between the DNA forms and the betanodaviruses. Thirdly, transfection assays showed that when transfected with the viral genome RNAs (not infectious virus), barfin flounder larval cell line MK-1 and Japanese flounder larval cell lines H-1 and H-2 contained RNA2 DNA 10 d after transfection (Fig. 5A). In addition, the 3 cell lines showed RTase activities.

A relationship between infection by RNA viruses and production of DNA forms has been reported (Zhdanov 1975, Klenerman et al. 1997). DNA forms with nucleotide sequences homologous to glycoprotein gene RNA and nucleocapsid gene RNA of lymphocytic choriomeningitis virus, which belongs to the family *Arenaviridae*, were detected in mouse splenocytes more than 200 d after infection with the virus (Klenerman et al. 1997). Furthermore, DNA forms with nucleotide sequences homologous to RNA genomes of measles virus (*Paramyxoviridae*), Sindvis virus (*Togaviridae*), and tick-borne encephalitis virus (*Flaviviridae*) were detected in chick-embryo fibroblasts, mouse L cells, and human Hep-2 cells infected with these viruses through 80 to 600 passages (Zhdanov 1975). Those studies indicated that DNA forms are produced and persist in the host after infection, but it was unclear whether infectious virus and its propagation, or viral genome RNAs only, were necessary for the production of DNA forms. This study showed that the E-11 cell line infected with betanodavirus strain HCF-1 produced both RNA1 DNA and RNA2 DNA (Fig. 7A,B). From results described above and transfection assays conducted in this study, production of DNA forms occurs both through the propagation of infectious virus and when viral genome RNAs only are present.

The produced DNA forms, in particular RNA2 DNA, could potentially persist in fish for a relatively long time. In this study, barfin flounder collected at the Hokkaido Institute of Mariculture (Appendix 1; Table A1) and uninfected controls of the 2 fish species used in experimental infections at the Hokkaido Central Fisheries Experimental Station (Appendix 1; Tables A3 & A4) contained the DNA forms. Samples were derived from the same respective adult stocks held at the 2 facilities (i.e. barfin flounder: Hokkaido Institute of Mariculture; Japanese flounder: Hokkaido Central Fisheries Experimental Station) that spawned in 2000 and 2001, and contained RNA2 DNA in the ovarian cavity fluid (Appendix 1; Table A1). Hence, RNA2 DNA might persist for at least 1 yr. In experimental infections, surviving fish contained the DNA forms 150 d after infection. Moreover, transfection assay indicated that RNA2 DNA was detected in the transfected MK-1 cell line 5 wk and 5 successive passages after transfection (Fig. 5C). Results from field samples (Appendix 1, Tables A1 & A2), experimental infections (Appendix 1, Tables A3 & A4), and transfection assays showed that, in general, the detection rate of RNA2 DNA was higher than that of RNA1 DNA. RNA2 DNA

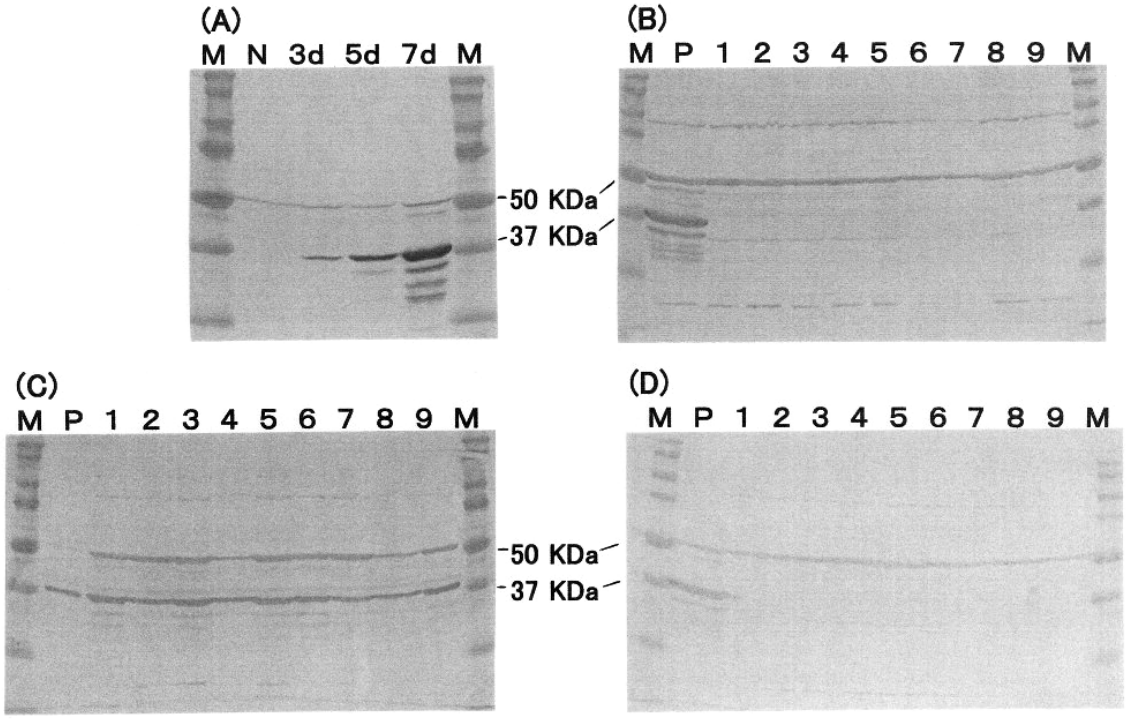

Fig. 6. Immunoblot of betanodavirus coat protein in E-11 cells. (A) E-11 cell line infected with betanodavirus strain HCF-1 at MOI 0.1. Samples collected 3, 5, and 7 d after infection are shown. N: negative control (uninfected E-11 cell line). (B) E-11 cell line inoculated with barfin flounder larvae uninfected controls. (C) E-11 cell line inoculated with barfin flounder larvae samples that died subsequent to injection of HCF-1. (D) E-11 cell line inoculated with surviving barfin flounder larvae samples. M: Precision protein standards; P: 5 or 7 d samples from (A) (positive control)

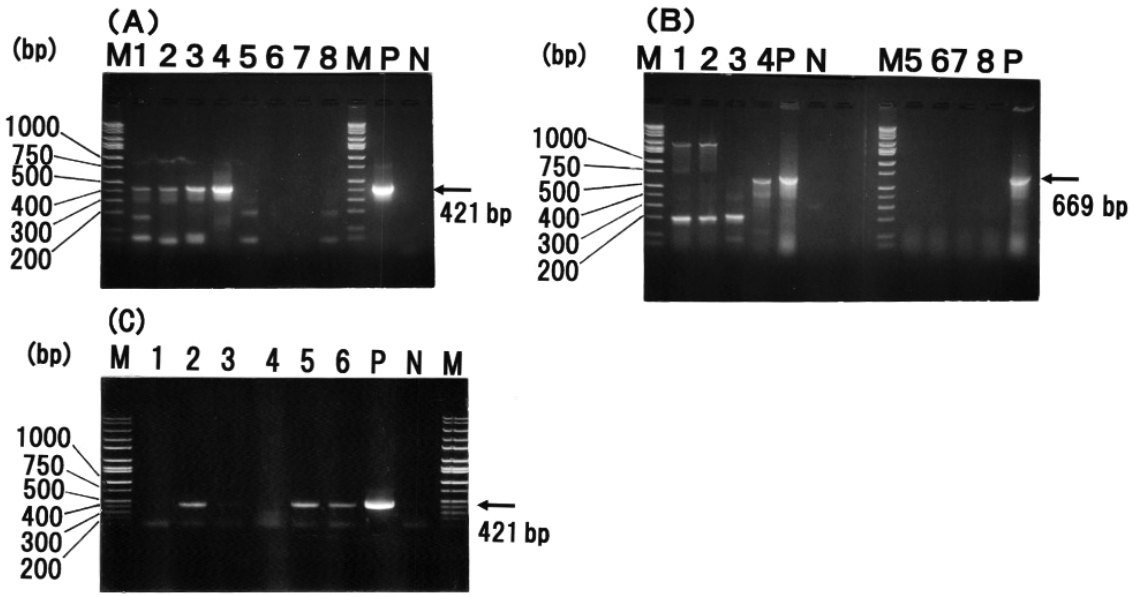

Fig. 7. PCR detection of RNA1 DNA and RNA2 DNA in cell lines transfected with RNA that contained viral genome RNA1 and RNA2, which was extracted from E-11 cells infected with betanodavirus strain HCF-1. (A) Detection of RNA2 DNA in cell lines H-1 (Lane 1; negative control = Lane 5), H-2 (Lane 2; negative control = Lane 6), and MK-1 (Lane 3; negative control = Lane 7). Negative controls: cells transfected with RNA from E-11 cells without infection of HCF-1. Lane 4: E-11 cells infected with HCF-1. Lane 8: E-11 negative control (cells not infected with HCF-1). Lanes 1–4 are positive. Lanes 5–8 are negative. (B) Detection of RNA1 DNA in H-1, H-2, MK-1 and E-11 cell lines. Cell lines were applied to each lane (1–8) as in (A). Lane 4 is positive. Other lanes are negative. (C) Detection of RNA2 DNA in MK-1 cells transfected with cDNA of viral genome RNA2 (Lane 1: 54.4 µg; Lane 2: 27.2 µg; Lane 3: 13.6 µg) and in MK-1 cells transfected with RNA of E-11 cells infected with HCF-1 (Lane 4: 4.5 µg; Lane 5: 3.0 µg; Lane 6: 1.5 µg). M: DNA size marker; P: pGEM-T Easy vector encoding the 669 bp nucleotide partial RNA1 genome or the 421 bp nucleotide partial RNA2 genome (positive control); N: water (negative control)

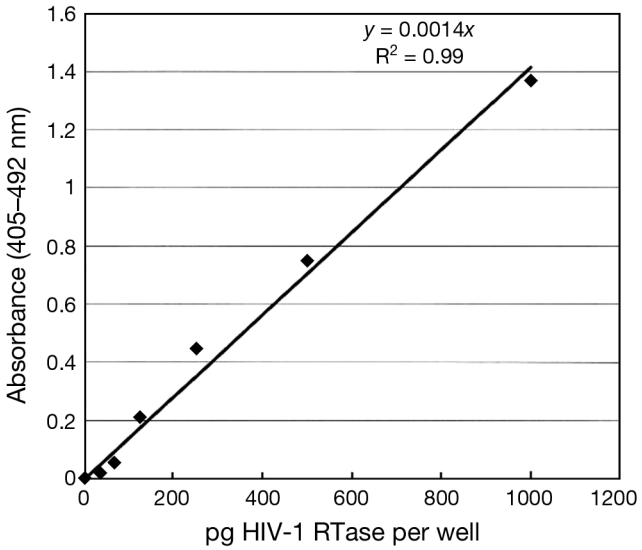

Fig. 8. Calibration curve of HIV-1 RTase activity (absorbance readings at 405 nm with a reference wavelength of 492 nm vs. HIV-1 RTase concentration)

may be produced more easily, may be more stable, or may persist for a longer time in fish as well as in the cell lines. My findings perhaps indicate that sample fish or their parents were subclinically infected at least once, and also that they retained the produced DNA forms for at least 1 yr: outbreaks of VNN (the causative agent being betanodavirus) were not observed in sample fish or their parents, and subclinical infections would not be noticed.

This study showed that the presence of DNA forms does not lead to production of infectious virus in these 2 fish species. In field samples, most of the samples positive for the DNA forms contained either RNA1 DNA or RNA2 DNA (Appendix 1, Tables A1 & A2): few samples contained both RNA1 DNA and RNA2 DNA. Moreover, samples contained either viral genome RNA1 or RNA2 but not both. Furthermore, in experimental infections, the surviving fish collected 150 d post-infection contained either viral genome RNA1 or RNA2 but not both, even though a few samples contained both RNA1 DNA and RNA2 DNA. These results may account for the fact that no detectable infectious virus was detected in the samples, because the presence of both viral genomes RNA1 and RNA2 is a prerequisite for virion production. Nevertheless, this does not exclude the possibility that production of infectious virus from the DNA forms occurs under other conditions. Johansen et al. (2004) reported that Nodavirus was reisolated using the SSN-1 cell line from subclinically infected Atlantic halibut *Hippoglossus hippoglossus* 1 yr after an acute outbreak of viral encephalopathy and retinopathy. This finding is very different from that of the present study. In order to clarify the contribution of DNA forms to the production of infectious virus, detection of the presence and frequency of DNA forms in subclinically infected fish will be required .

The presence of DNA forms in the 2 fish species should not be overlooked when RT-PCR assays are conducted. In this study, DNA forms and viral genome RNAs were detected separately (Fig. 1; Appendix 1, Tables A1 to A4). The presence of DNA forms was detected by PCR using untreated samples, and confirmed by the absence of PCR products when using DNase I-digested samples and presence of PCR products when using RNase A-digested samples. In contrast, the presence of viral genome RNAs were detected via the presence of positive RT-PCR products in DNase I-digested samples, and confirmed by the absence of specific PCR products in DNase I-digested samples. Thus, detection and confirmation of the presence of viral genome RNAs by RT-PCR required that samples be digested by DNase I before the assay. In this study, cDNA synthesis (reverse-transcription) of viral genome RNAs by Ampli*Taq* Gold DNA polymerase (followed by amplification of the cDNA products at a level detectable by RT-PCR) did not occur. The viral genome RNAs present in samples digested with DNase I were not detected by PCR under any of the conditions used in this study (Appendix 1, Tables A1 to A4). Although the presence and concentration of DNA forms in extracted total RNA samples (or in extracted combined DNA and RNA samples) would vary depending on the samples and extraction method used, investigation of subclinical infections in the 2 fish species via detection of viral genome RNAs by RT-PCR probably require the detection of viral genome RNAs separate from that of DNA forms.

The function of DNA forms homologous to betanodavirus genome RNAs remains unresolved. The presence of the DNA forms at a higher frequency, the sequence similarities between DNA forms and viral genome RNAs, and the generation of DNA forms from viral genome RNA2 in cell lines of the 2 fish species probably imply that DNA forms play a role in the host. The detailed enzymatic properties of RTase activities in larval barfin and Japanese flounder cell lines are unclear, and future studies that include the effects of inhibitory reagents and RTase molecular identities are required.

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