

Characterization of grouper nervous necrosis virus (GNNV)

S C Chi, B J Lo and S C Lin

Department of Zoology, National Taiwan University, Taipei, Taiwan

Abstract

Grouper nervous necrosis virus (GNNV) was isolated from moribund grouper larvae, *Epinephelus* sp., using a fish cell line GF-1. The present study describes the biochemical and biophysical properties of GNNV and the expression of GNNV in diseased grouper larvae. Viral protein was detectable in most of the GNNV-infected GF-1 cells by the fluorescent antibody technique (FAT) after 12 h post-infection (p.i.), although no cytopathic effect (CPE) appeared at that time. Clear CPE developed on the third day, and complete disintegration of the monolayer occurred over the subsequent two days. The infectivity of GNNV can be blocked following treatment at 60 °C for 1 h. GNNV was sensitive to pH 3 and pH 10–12 with a 4 log₁₀ drop in infectivity. Purified GNNV was analysed by SDS-PAGE, and then stained with periodic acid silver. The positive staining indicated that its two capsid proteins were glycoproteins. Genomic RNAs of GNNV were extracted from purified virions and analysed. The molecular weights of genomic RNAs were 1.02 × 10⁶ and 0.50 × 10⁶ Da. The T2 region of the coat protein gene of GNNV was amplified by polymerase chain reaction (PCR), and the multiple alignment of the T2 sequence of two GNNV isolates with four genotypes of fish nodaviruses revealed that these two isolates (GNNV9410 and GNNV9508) belong to the red-spotted grouper nervous necrosis virus (RGNNV) genotype. The tissue distribution of GNNV in naturally infected grouper larvae was investigated by *in situ* hybridization using a dig-labelled probe, which showed that GNNV was not only detected in the brain and retina, but also in the

gill, skeletal muscle, liver, pyloric gland, intestine and blood cells in the heart.

Keywords: NNV, fish nodavirus, grouper.

Introduction

Viral nervous necrosis (VNN) is a disease found world-wide in many species of marine fish, causing an extremely high mortality of affected larvae and juveniles (Munday, Langdon, Hyatt & Humphrey 1992; Nakai, Mori, Nishizawa & Muroga 1995; Breton, Grisez, Sweetman & Ollevier 1997; Chi, Lo, Kou, Chang, Peng & Chen 1997; Munday & Nakai 1997). The agent of VNN has been identified as a new member of the Nodaviridae because of the properties of the viral genome and its proteins (Mori, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992). Fish nodaviruses have now been classified into four genotypes: tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa, Furuhashi, Nagal, Nakai & Muroga 1997).

Nerve tissue is the target tissue for nervous necrosis virus (NNV) infection, but NNV can also infect other tissues. The distribution of NNV in other tissues varies with fish species and age. Although highly restricted to the nerve tissue in larval sea bass (Comps, Trindade & Delsert 1996), NNV has been detected in the epithelium of skin hyperplasia of striped jack larvae in acute infections (Nguyen, Nakai & Muroga 1996), and has been detected in many other organs in the infection of Atlantic halibut larvae (Grotmol, Totland, Thorud & Hjeltnes 1997; Grotmol, Bergh & Totland 1999).

Grouper aquaculture is a vital industry in Taiwan. In recent years, a disease symptomatically

Correspondence Dr S C Chi, Department of Zoology, National Taiwan University, Taipei, Taiwan.
(e-mail: shauchi@ccms.ntu.edu.tw)

identical to VNN has repeatedly occurred in hatchery-reared grouper (Chi 1997). Numerous non-enveloped, cytoplasmic viral particles (20–25 nm in diameter) were observed in the brain cells of diseased larval and juvenile groupers under electron microscope examination. The virions were isolated and identified to be fish nodavirus by polymerase chain reaction (PCR), and the viral isolates were designated as GNNV9410 and GNNV9508 (Chi *et al.* 1997; Chi, Lin, Su & Hu 1999). Vertical transmission was suggested as a major pathway for larval infection, because 100% of 17 lots of grouper eggs collected from different farms within 1 year revealed NNV contamination by two step PCR examination (Chi & Lo 1998). To amplify grouper nervous necrosis virus (GNNV) *in vitro*, a new cell line GF-1 was developed from the fin tissue of *Epinephelus coioides*, which showed high susceptibility to GNNV (Chi *et al.* 1999). The optimal temperature for GNNV replication in GF-1 cells is 24–32 °C, and virus titre increases with rising temperature. The VNN can be induced by bath exposure of healthy grouper larvae to the GNNV-containing cell culture supernatant with mortality reaching 100% within 3 days (Chi *et al.* 1999). In this study, the biochemical and biophysical properties of GNNV isolated by GF-1 cells were further characterized, and the expression of GNNV in the moribund grouper larvae was detected.

Materials and methods

Fish samples

Mass mortalities were encountered in grouper larvae with body length 0.8–1 cm reared in Kaohsiung hatchery in southern Taiwan in 1995. Diseased larvae lost their appetites, swam in a whirling manner and died within 3 days. Moribund larvae were collected for this study.

Healthy grouper larvae from another hatchery were collected and were determined to be NNV-free by PCR.

Immunofluorescent staining

The GF-1 cells were fixed by 10% formalin 3, 6, 9 and 12 h post-viral infection, incubated with 0.2% Triton-X 100 for several minutes, washed with PBST (phosphate buffer with 0.05% Tween 20), blocked with 3% skimmed milk, reacted with mouse anti-GNNV 9410 serum at room temper-

ature for 1 h, and stained by FITC conjugated goat antimouse antibodies.

Effect of temperature and pH on viral infectivity

A virus suspension with an original titre of $10^{6.5}$ TCID₅₀ mL⁻¹ was divided into four test tubes, separately incubated at different temperatures (40, 50, 60 and 70 °C) for 1 h, and then titrated.

The L15 medium was adjusted to five pH values (pH 3, 5, 7, 10 and 12). Aliquots of virus solution with an original titre of 10^7 TCID₅₀ mL⁻¹ were 10 fold diluted by each pH medium, incubated for 30 min, and then titrated. Titration was performed in 96-well plate seeded GF-1 cells cultured at 28 °C for 5 days.

Virus purification

GF-1 cells were scraped into the medium when cytopathic effect (CPE) was extensive and the cell debris was pelleted at 10 000 *g* for 30 min. The supernatant was transferred to a bottle and polyethylene glycol (PEG, molecular weight 20 000) and NaCl were added to reach a final concentration of 5 and 2.2%, respectively. The supernatant was stirred for 4–6 h at 4 °C, and the virus particles were pelleted by centrifugation at 10 000 *g* for 1 h. The pellets were re-suspended in a small amount of TNE buffer (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.3), and an equal volume of Freon 113 was added. The mixture was shaken vigorously for 5 min, and the emulsion was separated into the Freon and aqueous phase by centrifugation at 3000 *g* for 10 min. The aqueous phase was collected, layered on a pre-formed 10–40% (w/w) CsCl gradient and centrifuged at 60 000 *g* for 20 h. The visible virus band was collected, diluted with 10 mL TNE buffer, and then pelleted by centrifugation at 50 000 *g* for 1 h. The final pellet was resuspended in a small volume of TE buffer.

Periodic acid-silver stain

Periodic acid-silver stain is a sensitive method to detect carbohydrate. Purified virus and a pre-stained marker (Bio-Rad Laboratories, Hercules, CA, USA, cat. 1610305) were loaded in duplicate on 12% SDS–polyacrylamide gel. The pre-stained marker contains ovalbumin (OVA) which can be used as a

positive control for glycoprotein, and bovine serum albumin (BSA) which can be used as a negative control for glycoprotein. After SDS–polyacrylamide gel electrophoresis, the gel was cut into two pieces, each piece containing one copy of the prestained marker and the purified GNNV. One piece of gel was stained with Coomassie blue R250, and the other was processed for periodic acid – silver stain according to the method described by Dubray & Benzard (1982).

Extraction and electrophoretic analysis of GNNV genomic RNA

Purified virus suspension was treated with proteinase K at a final concentration of 20 $\mu\text{g mL}^{-1}$ for 1 h at 37 °C. The nucleic acid was extracted by adding 500 μL acid phenol (pH 4.0, Amresco) and 150 μL chloroform/isopropanol (24:1). Two volumes of cold absolute ethanol were added to the aqueous phase supplemented with 0.3 M sodium acetate, and the nucleic acid was allowed to precipitate at –70 °C for 30 min. The nucleic acid was then pelleted, washed with 70% ethanol, dried, resuspended in diethylpyrocarbonate (DEPC)-treated distilled water, and then stored at –70 °C. Electrophoresis analysis of GNNV 9508 genomic RNA was performed on 2% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA).

Sequencing of viral nucleic acid

The T2 region of the coat protein gene of GNNV was amplified by using a primer set (F1,R3). Primer sequences were based on those of Nishizawa, Mori, Nakai, Furusawa & Muroga (1994). The T2 PCR products of two GNNV isolates (GNNV 9410 and GNNV 9504) were cloned into pGEM-T easy vector (Promega Corp.) in order to determine the

nucleotide sequence using a Perkin Elmer 373 DNA sequencer.

Preparation of dig-labelled T4 probe

Viral RNA was extracted by acid phenol and chloroform, and then reverse-transcribed by MMLV reverse transcriptase. The T4 region of the coat protein gene of NNV was then amplified by using a primer set (F2,R3) according to the method described in Chi *et al.* (1997). The T4 probe was made with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations.

In situ hybridization

Tissues of grouper larvae were fixed in 10% formalin and embedded in paraffin. After rehydration, 5 μm paraffin sections were washed for 10 min in PBS buffer at room temperature, and treated with proteinase K (10 mg mL^{-1} in PBS) at 37 °C for 30 min. The reaction was stopped by washing for 10 min in PBS containing 0.2% glycine. Sections were post-fixed by 4% paraformaldehyde for 10 min, and washed by PBS. Hybridization buffer (50% formamide, 5X Denhart's solution, 5X SSC, 0.025 M EDTA) was then added to the sections which were incubated at 37 °C for 2 h and then dig-labelled T2 probe was hybridized with the paraffin sections according to the manufacturer's instructions (Boehringer-Mannheim, Mannheim, Germany).

Results

Immunofluorescent staining

Viral protein was initially detected in the GNNV-infected GF-1 cells 3 h after infection, and 80% of the infected cells showed a positive reaction 12 h

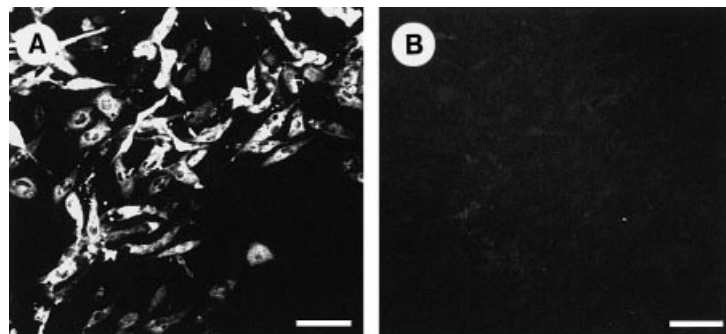


Figure 1 Immunofluorescent stain of (A) GNNV-infected GF-1 cells 12 h p.i.; (B) non-infected GF-1 cells, using rabbit antiserum against GNNV.

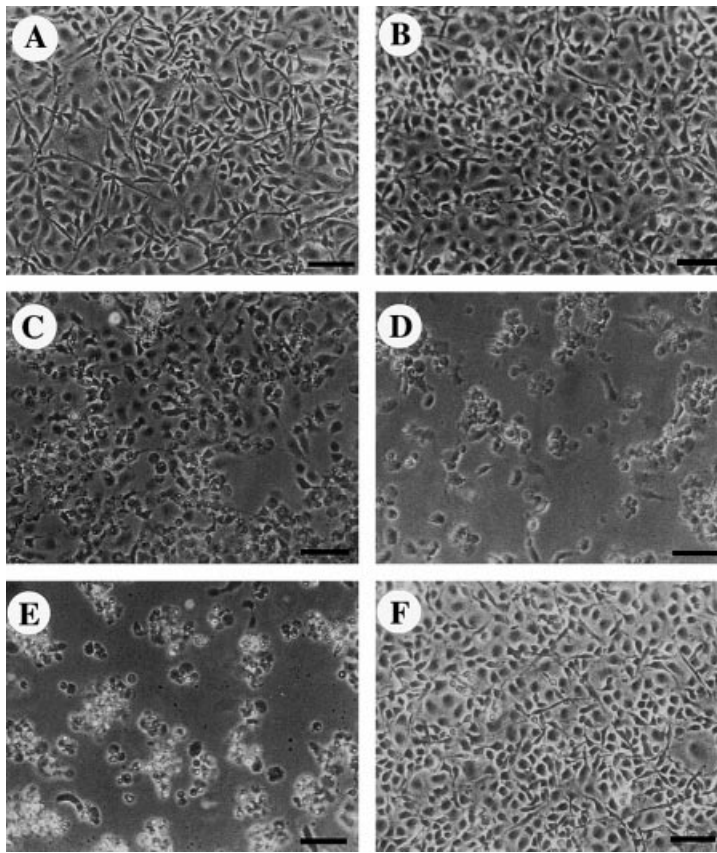


Figure 2 The CPE of GNNV-infected GF-1 cells at different periods p.i. (A) 1 day p.i. (B) 2 days p.i. (C) 3 days p.i. (D) 4 days p.i. (E) 5 days p.i.; (F) uninfected GF-1 cells (bar = 100 μ m).

post-infection, although no CPE appeared at that time (Fig. 1).

Development of CPE

After GNNV infection, only a small portion of GF-1 cells became refractile on the second day. More than half of the infected cells became rounded and swollen, detached and finally became lytic on the third and the fourth days post-infection (p.i.). Cells were completely disintegrated 5 days p.i. (Fig. 2).

Effect of temperature and pH on viral infectivity

Although GNNV is resistant to exposure to 40 and 50 °C for 1 h, its infectivity is totally blocked following treatment at 60 °C for 1 h.

The GNNV was resistant to pH 5, but sensitive to pH 3 and pH 10–12 for 30 min with titres reduced over 4 log₁₀ compared with titres (10^{6.0} TCID₅₀/0.1 mL) at pH 7.

Periodic acid-silver stain

Purified GNNV 9508 stained by Coomassie blue revealed two coat proteins with molecular weights

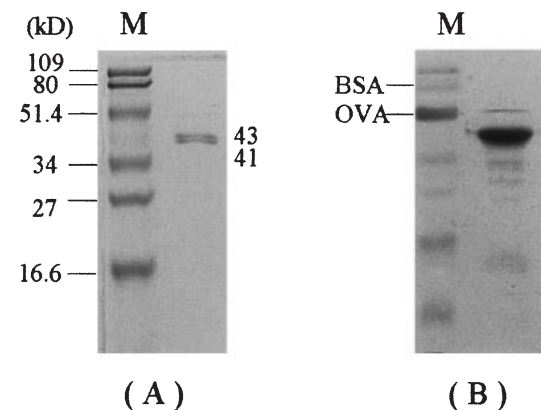


Figure 3 The protein profile of purified GNNV 9508 analysed by 12% SDS-PAGE and stained with (A) Coomassie blue and (B) periodic acid – silver. Glycoproteins stained brown following periodic acid-silver staining. M, prestained protein marker (Bio-Rad, Cat. 161–0305). BSA, bovine serum albumin; OVA, ovalbumin.

of 43 and 41 kDa (Fig. 3A). The two capsid proteins can be stained by periodic acid-silver, as is glycoprotein ovalbumin, the prestained marker (Fig. 3B). Therefore, GNNV coat proteins are glycoproteins.

Analysis of GNNV nucleic acid

Figure 4 displays agarose gel profiles of extracted purified virus RNA. The molecular weights of the two genomic pieces are 1.02×10^6 and 0.5×10^6 Da. The multiple alignments of the nucleotide sequence (T2 region) of the two GNNV isolates with four fish nodavirus genotypes are compared in Fig. 5. The similarity among these two isolates (GNNV 9410 and GNNV 9508) and RGNNV was up to 99%. Therefore GNNV isolated from larval and juvenile groupers reared in southern Taiwan belongs to the RGNNV genotype.

In situ hybridization

Sensitivity of the dig-labelled T4 probe was estimated by dot-blot analysis of serial diluted GNNV T4 PCR product, which detected 2.5 pg purified T4 PCR product.

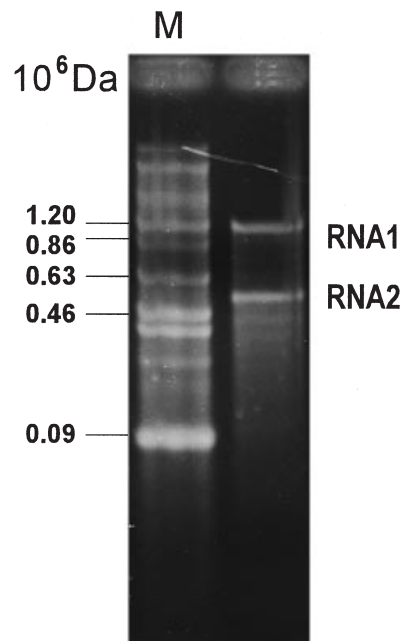


Figure 4 Electrophoretic pattern of purified GNNV genomic RNAs on 2% agarose gel. M, single strand RNA marker (Promega).

Specificity of the T4 probe was assayed by Northern blot analysis of GNNV genomic RNA, and T4 probe hybridized with genomic RNA of GNNV (data not shown). To further test the specificity of the T4 probe, we hybridized this probe with tissue sections of GNNV-free grouper larvae, and no positive reaction was found in the brain, retina, intestine and pyloric gland (Fig. 6).

The expression of GNNV in moribund grouper larvae was processed for *in situ* hybridization using the T4 probe, and a positive reaction was characterized by a dark precipitate in infected tissues. The results indicated in Figs 7 & 8 show that GNNV was widespread in the brain, retina, gill, skeletal muscle, liver, pyloric gland, stomach, intestine, and in blood cells of the heart.

Discussion

GNNV repeatedly causes acute infection in grouper larvae reared in southern Taiwan, and more than 80% of the infected fish die within 5 days after appearance of abnormal swimming behaviour and loss of appetite. The GNNV has been isolated from diseased grouper larvae and juveniles, and partially characterized (Chi *et al.* 1997; Chi, Lin, Su & Hu 1999). In this study, GNNV was further characterized both biophysically and biochemically and the expression of GNNV in diseased grouper larvae was investigated.

The molecular weights of genomic RNAs and capsid proteins of GNNV resemble those of striped jack NNV. The coat protein gene of SJNNV has been reported to contain a single open reading frame of 1023 bases encoding a protein of 340 amino acids with a calculated molecular weight of 37 kDa (Nishizawa, Mori, Furuhashi, Nakai, Furusawa & Muroga 1995). The value is approximately 5 kDa less than the molecular mass of the SJNNV coat protein (42 kDa) (Mori *et al.* 1992). In this study, the capsid proteins of GNNV were proven to be glycoproteins by periodic acid-silver stain. Therefore, the post-translational glycosylation of coat protein may explain why the purified NNV coat protein mass exceeds the calculated coat protein mass.

The GF-1 cells initially displayed fluorescence in a fluorescent antibody technique (FAT) assay 3 h after GNNV infection, and most of cells revealed positive staining 12 h p.i., before CPE appeared. Clear CPE appeared from the 3rd day p.i. and the entire cell sheet disintegrated 4–5 days p.i. These

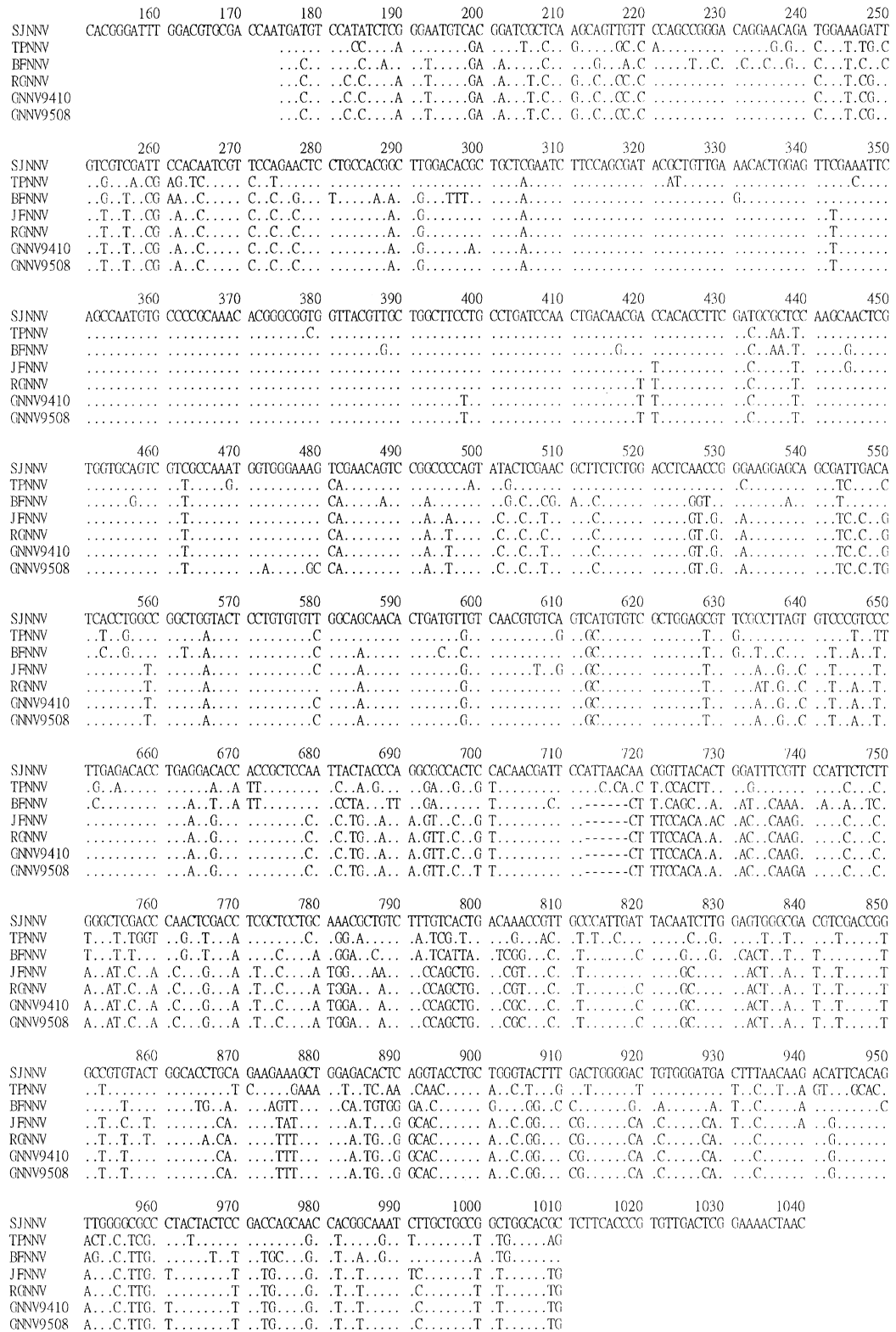


Figure 5 Multiple alignment of nucleotide sequences of PCR product from the coat protein genes of SJNNV, TPNNV, BFNNV, RGNNV and two GNNV isolates (GNNV 9410 and GNNV 9508).

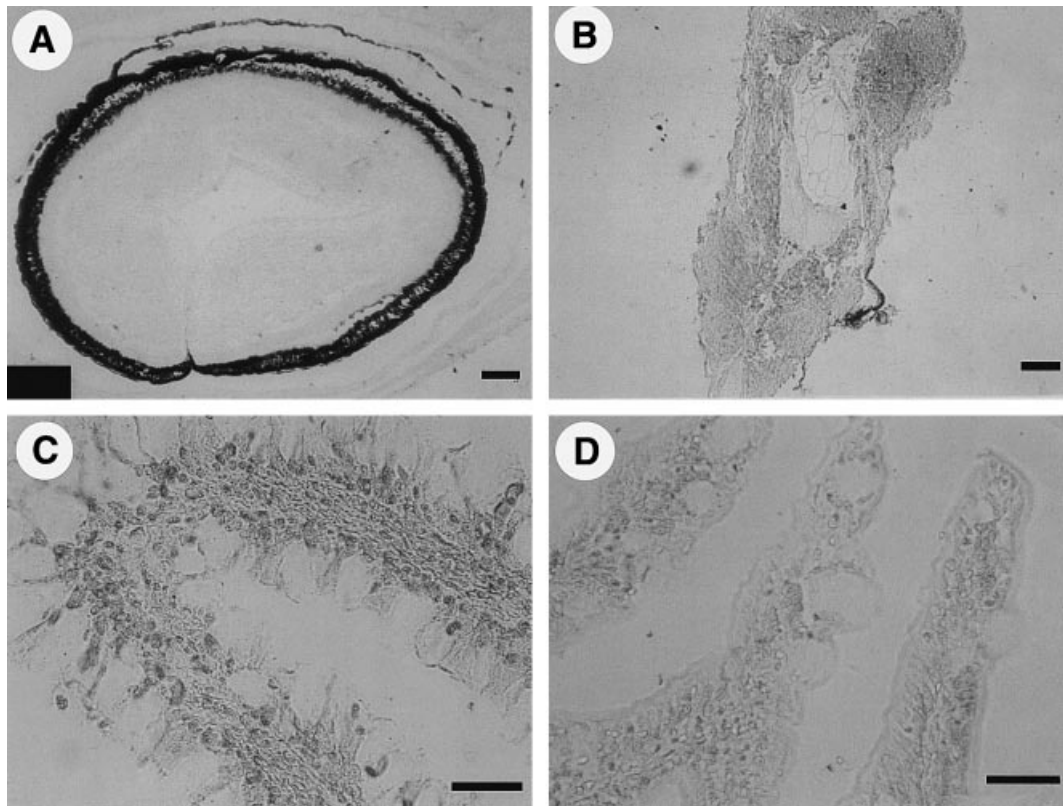


Figure 6 *In situ* hybridization of GNNV-free grouper larvae using dig-labelled T4 probe. Probe reacted negatively with histological sections of (A) retina, (B) brain, (C) intestine and (D) pyloric gland (bar = 1 μ m).

results reconfirmed that GF-1 cell line is highly permissive to GNNV, thus making it a good tool for detection or further study of the virus.

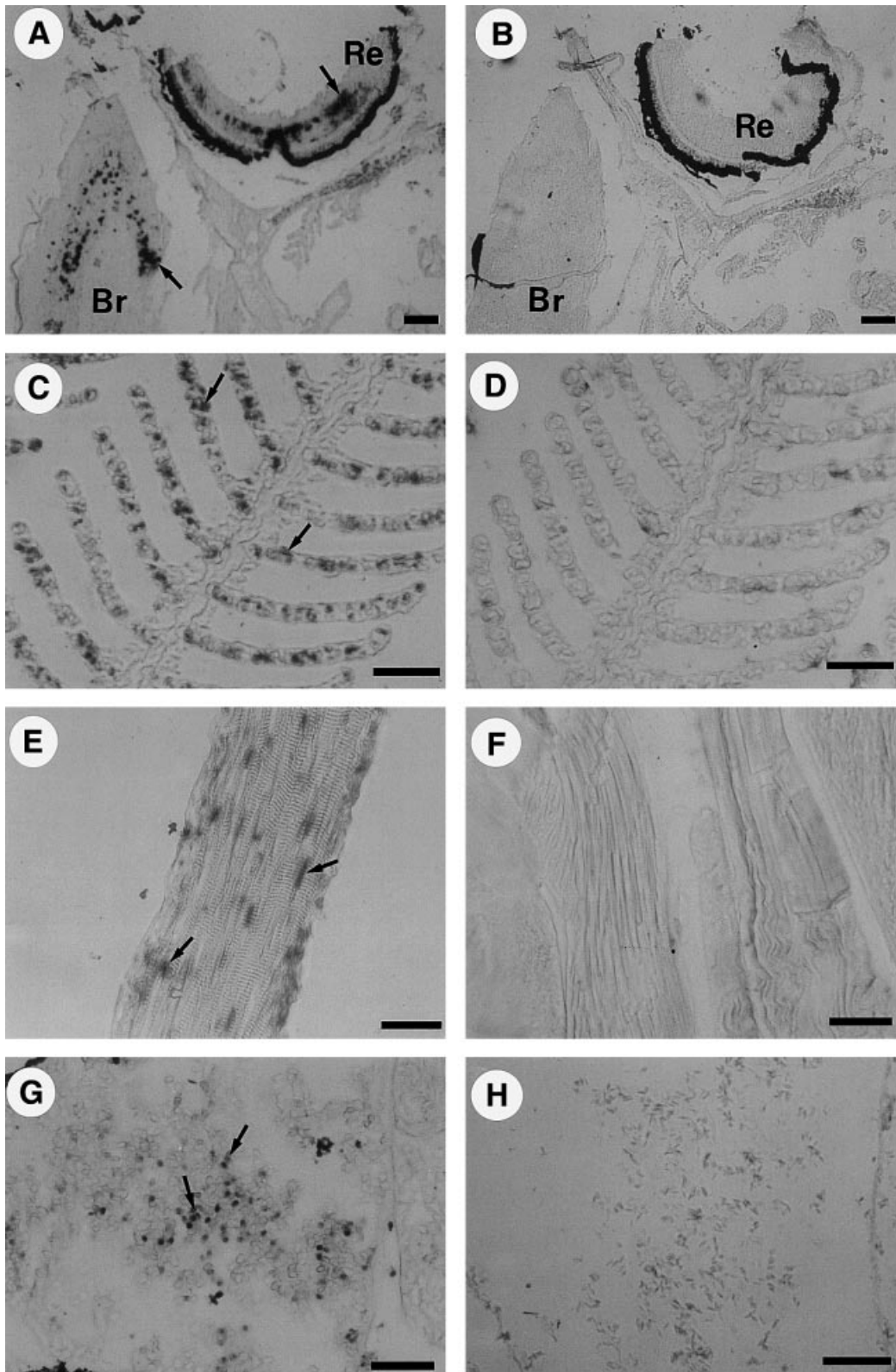
The SJNNV is resistant to pH 3, but can be inactivated by high alkalinity, i.e. pH 12 for 10 min (Arimoto, Sato, Maruyama, Mimura & Furusawa 1996). The NNV isolated from juvenile sea bass was resistant to pH 2–9 for 30 min (Frerichs, Rodger & Peric 1996). In this study, GNNV was resistant to pH 5, but sensitive to pH 3 and pH 10–12 after 30 min treatment with a 4 log decrease in infectivity. The sensitivity of GNNV to low pH differs from both SJNNV and NNV from sea bass juveniles but the reasons for this are unclear.

The result of the T2 sequence alignment of GNNV with four fish nodavirus genotypes indicated that the two GNNV isolates from Taiwan shared 99% similarity with the RGNNV isolate from Japan. Grouper is popular in Asia and is important for the aquaculture industry. In Taiwan, most grouper broodfish are originally captured

from the wild, with a small portion of larvae being imported from South-East Asia. As many NNV cases have been reported in East Asia (Munday & Nakai 1997), NNV may be transmitted between countries via carrier broodfish or larvae. Viral screening of captured broodfish and development of NNV-free broodfish from disinfected NNV-free eggs will be important in control of this disease among groupers.

Several reports have described the tissue tropism of NNV in different species of larval and juvenile fish, although the results are inconsistent. In larval sea bass, NNV was restricted to nerve tissue (Comps *et al.* 1996); in larval striped jack, NNV

Figure 7 *In situ* hybridization of infected grouper larvae with dig-labelled T4 probe. Histological sections include head (A,B), gill (C, D), skeletal muscle (E,F) and heart (G,H): Re, retina; Br, brain. Positive signals (arrows) were found in sections of (A) (C) (E) and (G). No signal was found in the negative controls (B,D,F,H) in which T4 probe was replaced by hybridization buffer (bar = 1 μ m).



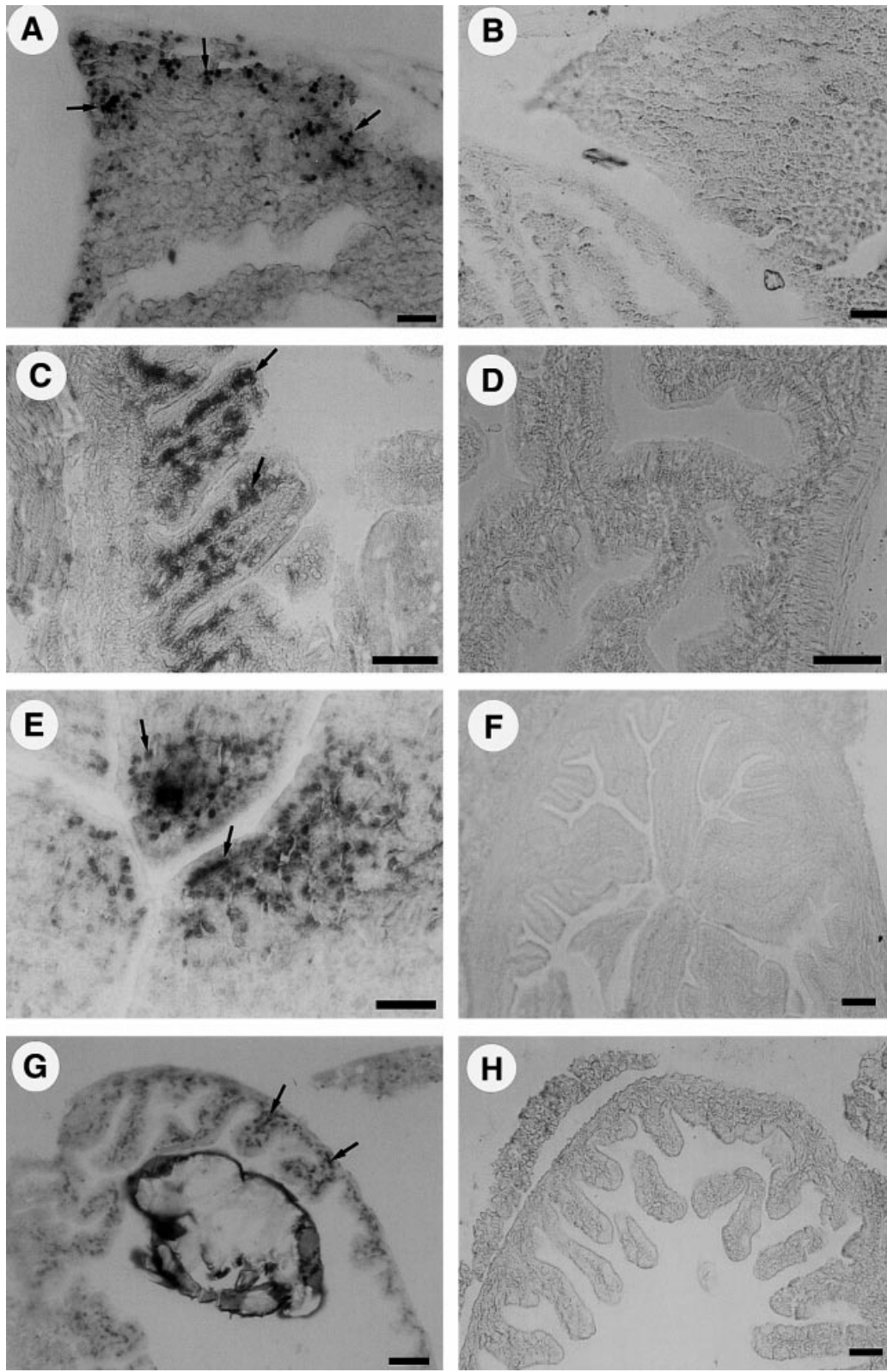


Figure 8 *In situ* hybridization of infected grouper larvae with dig-labelled T4 probe. Histological sections include liver (A,B), pyloric gland (C, D), stomach (E,F) and intestine (G,H). Positive signals (arrows) were found in sections (A) (C) (E) and (G). No signal was found in the negative controls (B,D,F,H) in which T4 probe was replaced by hybridization buffer (bar = 1 µm).

was always found in nerve tissue and sometimes in the epithelium of fish in acute infections (Nguyen *et al.* 1996). In larval and juvenile Atlantic halibut, it can infect nerve cells, macrophages, lymphocyte-like cells, myocardial cells, and the vascular and endocardial endothelium (Grotmol *et al.* 1997); in yolk-sac larval Atlantic halibut, NNV was initially found in the posterior brain and anterior intestine, and then spread widely to liver, olfactory epithelium, gills and pectoral fins (Grotmol *et al.* 1999). In this study, GNNV was detected not only in brain and retina, but also in gill, muscle, liver, pyloric gland, stomach, intestine, and the blood cells of the heart (Figs 7 & 8). The systemic distribution of GNNV in moribund grouper larvae resembles the situation in NNV-infected Atlantic halibut larvae. As NNV has been detected in the blood cells of grouper larvae by *in situ* hybridization and in blood samples of GNNV-infected juveniles by PCR (data not shown), we suggested that viraemia is an important factor in the pathogenesis of the infection.

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