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Interference of the life cycle of fish nodavirus with fish retrovirus

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Interference of the life cycle of grouper nervous necrosis virus (GNNV), a member of the *Nodaviridae*, genus *Betanodavirus*, by snakehead retrovirus (SnRV) has been studied *in vitro*. SGF-1, a new fish cell line that is persistently infected with SnRV, was induced by inoculating SnRV into the grouper fin cell line GF-1. Culture supernatants and cell pellets from both GNNV-infected SGF-1 and GF-1 cells were collected and employed for virus productivity analysis. The yields of GNNV RNA and capsid protein in GNNV-infected SGF-1 cells were similar to those in GNNV-infected GF-1 cells. However, when GF-1 cells were used for titration, the titre of the culture supernatant from GNNV-infected SGF-1 cells was much higher than that from GNNV-infected GF-1 cells. The titration result suggested that SnRV enhanced the infection or cytopathic effect (CPE) of GNNV during GNNV and SnRV coinfection of the GF-1 cell titration system, although SnRV cannot induce any CPE in GF-1 cells alone, nor can it increase the yield of GNNV after GNNV superinfection of SGF-1 cells. Moreover, GNNV cDNA was detected in both the pellet and the supernatant from GNNV-infected SGF-1 cells. This result indicated that SnRV reverse-transcribed the GNNV single-stranded genomic RNA into cDNA during GNNV superinfection of SGF-1 cells and created a new cDNA stage in the life cycle of the fish nodavirus.

Introduction

Viral nervous necrosis (VNN) is a worldwide disease of cultured marine fish which causes extremely high mortality at the larval and juvenile stages of the fish life cycle (Yoshikoshi & Inoue, 1990; Bloch *et al.*, 1991; Breuil *et al.*, 1991; Renault *et al.*, 1991; Mori *et al.*, 1992; Munday *et al.*, 1992; Comps *et al.*, 1994; Nakai *et al.*, 1995; Frerichs *et al.*, 1996; Le Breton *et al.*, 1997; Chi *et al.*, 1997; Grotmol *et al.*, 1997; Munday & Nakai, 1997; Bovo *et al.*, 1999). The causal pathogen of VNN disease has been characterized as a small, non-enveloped, bi-segmented, single-stranded, positive-sense RNA virus with a diameter of 25–30 nm (Mori *et al.*, 1992; Comps *et al.*, 1994; Chi *et al.*, 2001) and which belongs to the genus *Betanodavirus*, family *Nodaviridae* (Mori *et al.*, 1992).

Due to VNN, mass mortality of hatchery-reared grouper larvae and juveniles has occurred repeatedly in Taiwan (Chi *et al.*, 1997). The virions were isolated and identified by RT–PCR as a fish nodavirus, designated grouper nervous necrosis virus (GNNV) (Chi *et al.*, 1997). In order to amplify GNNV *in vitro*, the cell line GF-1 was developed from the fin tissue of a grouper, *Epinephelus coioides* (Hamilton) (Chi *et al.*, 1999), and was used to study the biochemical and biophysical properties of GNNV (Chi *et al.*, 2001). The SSN-1 cell line was derived from the whole fry tissue of Southeast Asian striped snakehead (*Ophicephalus striatus*) and proved to be persistently infected with a C-type retrovirus, snakehead retrovirus (SnRV) (Frerichs *et al.*, 1991). Notably, this cell line is permissive to NNV infection and has been employed for NNV isolation and amplification (Frerichs *et al.*, 1996; Iwamoto *et al.*, 1999). Fish nodaviruses amplified in SSN-1 cells typically have a high titre. Thus, SnRV may perform a significant role in fish nodavirus replication in SSN-1 cells (Iwamoto *et al.*, 2000). To understand the effect of SnRV on the life cycle of fish nodaviruses, the cell line SGF-1, which was persistently infected with SnRV, was established. Furthermore, the complex interactions between GNNV and SnRV during super- and coinfection of the host cells were investigated.

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Methods

■ **Cell culture and virus source.** The GF-1 (Chi *et al.*, 1997) and SSN-1 (Frerichs *et al.*, 1991) cell lines were used in the present study. The GF-1 cell line was maintained at 28 °C using L-15 medium (Gibco) supplemented with 5% foetal bovine serum (FBS) (Gibco); the SSN-1 cell

line was maintained at 25 °C using L-15 medium supplemented with 10% FBS.

One strain of GNNV was employed in this study. It was isolated from moribund grouper larvae and amplified in the GF-1 cell line at 28 °C (Chi *et al.*, 2001).

■ **Induction of GF-1 cells persistently infected with SnRV.** To establish a GF-1 cell line persistently infected with SnRV, the culture medium from SSN-1 cells was freeze-thawed three times, centrifuged at 1000 ***g*** for 10 min, filtered through a 0·22 μm membrane and inoculated onto GF-1 cells. After 1 h of adsorption at room temperature, the supernatant was discarded and the cells were washed three times with PBS. L-15 medium supplemented with 5% FBS was then added to the cells prior to incubation at 28 °C. The SnRV-infected GF-1 cells were named SGF-1 cells. After three subcultures, the cell pellets and culture supernatant of SGF-1 cells were collected for PCR examination using SnRV-specific primers.

■ **PCR for SnRV proviral DNA detection.** Genomic DNA was extracted from SSN-1 or SGF-1 cells by mixing 200 μl of cell-containing medium with 500 μl of GTC buffer (402 mM guanidine isothiocyanate, 250 mM sodium citrate, 17 mM sodium lauryl sarcosine and 46 mM 2-mercaptoethanol in DEPC-treated water) and 700 μl P:C:I [phenol (pH 8·0):chloroform:isoamyl alcohol at 25:24:1)]. After centrifugation at 10000 ***g*** for 10 min, the aqueous phase was removed for precipitation by mixing it with 60 μl 3 M sodium acetate and 600 μl isopropanol. The precipitate was washed with cold 70% ethanol and then centrifuged. The extracted DNA was re-dissolved in DEPC-treated water.

Proviral DNA was amplified by PCR using primers ML1 (5′ TGGTACCCATGGATACAGGTACCTCA 3′) and GPOL2 (5′ TGTCAGACATGGCCTGTACTTTAGCAGC 3′). These primers are specific to the *pol* gene, which encodes the reverse transcriptase of C-type retroviruses (Hart *et al.*, 1996). Amplification was performed by initial denaturation of 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. PCR products were examined by 1·5% agarose gel electrophoresis.

■ **Synchronous infection of GNNV in GF-1 and SGF-1 cells.** GF-1 cells and the fifth subculture of SGF-1 cells with the same cell numbers were separately inoculated into 25 cm^2 flasks and supplied with the same volume of L-15 medium supplemented with 5% FBS. The growth rates of both cell lines are very similar. Monolayers were 80% confluent in both cell lines by day 2 of growth. The culture media of GF-1 and SGF-1 cells were removed and GNNV at an m.o.i. of 0·1 was inoculated into each flask. Following 1 h of adsorption at room temperature, the cells were washed three times with PBS, supplied with same volume of culture medium and incubated at 28 °C. Following 5 days of incubation, culture supernatants from each of the GNNV-infected GF-1 and SGF-1 cells were collected and centrifuged at 1000 ***g*** for 10 min. The clarified supernatants were used for the subsequent RT–PCR, Western immunoblot and cross titration assays.

■ **RT–PCR for GNNV RNA detection.** Total viral RNA was extracted from 200 μl of clarified culture supernatant from each of the GNNV-infected GF-1 and SGF-1 cells using GTC buffer and acid (pH 4·0) phenol–chloroform extraction. Extracted RNA was dissolved in DEPC-treated water. The concentration of extracted RNA was determined using an RNA/DNA calculator (GeneQuant II, Pharmacia). RNA samples were serially diluted with DEPC-treated water and reverse-transcribed with M-MLV reverse transcriptase (Gibco) with reverse primer R3 for 1 h at 42 °C and then amplified using the primer pair (F1 and R3), according to the PCR conditions described by Chi *et al.* (1997). The primer sequences of R3 (5′ CGAGTCAACACGGGTGAAGA 3′) and F1 (5′ CGTGTCAGTCATGTGTCGCT 3′) were based on those described by Nishizawa *et al.* (1994). Amplification was performed by initial denaturation of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, with a final extension of 5 min at 72 °C. PCR products were analysed on a 1·5% agarose gel.

■ **Western immunoblot for GNNV capsid protein.** The same volume of virus-containing culture supernatant from each of the GNNV-infected SGF-1 and GF-1 cells was employed for 10% SDS–PAGE and analysed by Western immunoblotting, according to the method described by Chi *et al.* (1991). After SDS–PAGE, viral polypeptides were blotted onto a nitrocellulose (NC) membrane. The NC membrane was then soaked in 3% skimmed milk in Tris-buffered saline for 1 h, reacted with rabbit anti-GNNV serum for 1 h, incubated with goat anti-rabbit peroxidase-conjugate system for 1 h and finally stained with substrate containing 4-chloronaphthol.

■ **Cross titration of virus.** Titrations of virus-containing supernatant from each of the GNNV-infected GF-1 and SGF-1 cells were performed in two 96-well plates. One plate was pre-seeded with GF-1 cells and the other was pre-seeded with SGF-1 cells. The virus-containing supernatant was serially 10-fold diluted to 10^{11} with L-15 medium containing 2% FBS and inoculated into the pre-seeded 96-well culture plates. The last line of the 96-well plate was used as a negative control, in which only medium without any virus was inoculated. Eight wells were used for each dilution. Cytopathic effect (CPE) was observed for each day and the titre was determined on day 6. Virus titres are expressed as $TCID_{50}$/ml.

■ **Detection of GNNV cDNA.** The supernatants of GNNV-infected SGF-1 cells were collected at 4 h and 1, 3 and 5 days after GNNV inoculation. The cell pellet and supernatant were separated by centrifuging at 1000 ***g*** for 10 min. Total DNA was extracted from the cell pellets and supernatants of GNNV-infected SGF-1 cells by the method described above. Nucleic acids extracted from purified GNNV, GNNV-infected grouper larvae, SGF-1 cells and GNNV-infected GF-1 cells were used as multiple negative controls. Extracted DNA was amplified directly by PCR using GNNV-specific primers (F1 and R3). A sample of 1 μl of a 100-fold dilution of the PCR product was used in a nested PCR using forward primer P1 (5′ TCAGAGTAGTAAGCAACGCC 3′) and reverse primer N1 (5′ CAGGTATGTCGAGAATCTCC 3′). PCR and nested PCR amplifications comprised an initial denaturation for 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, with a final extension of 5 min at 72 °C. PCR products were checked by 1·5% agarose gel electrophoresis.

Results

Detection of SnRV in SGF-1 cells

No CPE was observed in either SnRV-infected GF-1 (SGF-1) cells, even after 1 week of incubation, or subcultures of SGF-1 cells. The morphology and growth-rate of SGF-1 cells are similar to those of GF-1 cells.

Fig. 1 illustrates the outcome of PCR detection of the SnRV *pol* gene in SSN-1, GF-1 and the third subculture of SGF-1 cells. Total DNA extracted from SSN-1 and GF-1 cells was serially diluted 10-fold to test the sensitivity of the PCR system used in the present study. The initial amounts of total extracted DNA from both cell lines were all 30 μg. According to these results, the PCR system used in the present study could detect

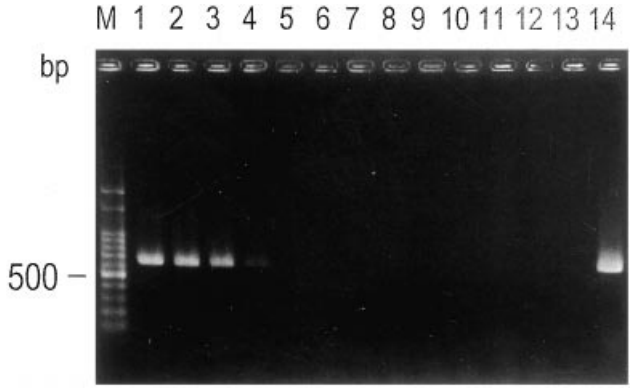

Fig. 1. PCR detection of SnRV nucleic acid in SSN-1, GF-1 and SGF-1 cells. Lanes: M, DNA marker (100 bp ET ladder, TOPBIO); 1–9, DNA extracted from SSN-1 cells and serially diluted to 30, 3 and 10^{-1} to 10^{-7} μg; 10, no DNA template; 11–13, DNA extracted from GF-1 cells and serially diluted to 30, 3 and 10^{-1} μg; and 14, 30 μg DNA extracted from SGF-1 cells.

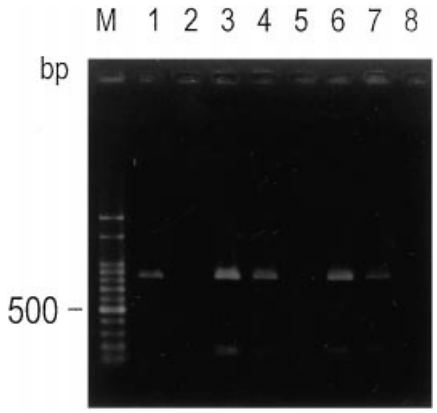

Fig. 2. Detection of GNNV nucleic acids by RT–PCR in GNNV-infected GF-1 and SGF-1 cells. Lanes: M, DNA marker (100 bp ET ladder); 1, RNA extracted from purified GNNV as a positive control; 2, DEPC-treated water as a negative control; 3–5, RNA extracted from GNNV-infected GF-1 cells and serially diluted to 10^{-2} to 10^{-4} μg; and 6–8, RNA extracted from GNNV-infected SGF-1 cells and serially diluted to 10^{-2} to 10^{-4} μg.

at least 10^{-2} μg total DNA extracted from SSN-1 cells. Although 30 μg total DNA extracted from GF-1 cells was employed in PCR amplification, no target band appeared, accounting for why GF-1 cells are SnRV-free. The SnRV *pol* gene could be amplified using the DNA extract of SGF-1 cells. Furthermore, SnRV was also detected in the twentieth subculture of SGF-1 cells (data not shown). Thus, the SGF-1 cell line became persistently infected with SnRV.

CPE of GNNV-infected GF-1 and SGF-1 cells

GNNV was synchronously inoculated with the same m.o.i. into GF-1 cells and the fifth subculture of SGF-1 cells in 25 cm^2 flasks. CPE in both cell lines appeared 3 days post-infection and completed on day 5. GNNV-infected cells first became as round as a swelled ball, then detached and finally lysed. The development and characteristics of CPE in both cell lines were very similar to those of the CPE described in our previous paper (Chi *et al.*, 1999).

RT–PCR of nucleic acids extracted from GNNV-infected GF-1 and SGF-1 cells

Total RNA was extracted from the same volumes of the clarified culture supernatants of GNNV-infected GF-1 and SGF-1 cells 5 days post-infection. The RNA was purified and then dissolved in the same volume of DEPC-treated water. The concentration of total RNA was 0·023 μg/μl in the supernatant from GNNV-infected GF-1 cells and 0·016 μg/μl in the supernatant from GNNV-infected SGF-1 cells. The concentration of total RNA extracted from GNNV-infected GF-1 was a bit higher than that from GNNV-infected SGF-1. The same volumes (6 μl) of these two RNA templates were used for reverse transcription and one-sixth of the volume of the reverse-transcribed products was applied for the following PCR amplification.

A serial 10-fold dilution of the original RNA templates was designed to reduce the saturation effect of RT–PCR end-products. The amounts of RT–PCR products were proportional to the amounts of input RNA templates (Fig. 2) and similar densities of RT–PCR products were revealed in the agarose gel when the two RNA templates with the same dilution factor were amplified. This result indicated that the production of GNNV RNA from GNNV-infected GF-1 cells was similar to that of GNNV-infected SGF-1 cells.

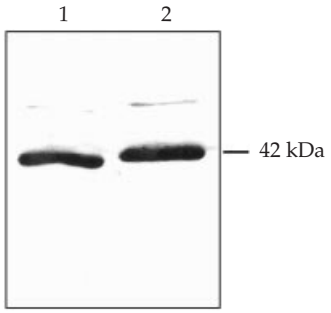

Fig. 3. Detection of GNNV protein by Western immunoblot with rabbit polyclonal antibodies against GNNV. Lanes: 1, GNNV-infected GF-1 cells; and 2, GNNV-infected SGF-1 cells.

Western immunoblot analysis of GNNV-infected GF-1 and SGF-1 cells

The cell supernatants of GNNV-infected GF-1 and SGF-1 cells were analysed by SDS–PAGE and then Western immunoblot staining with rabbit anti-GNNV polyclonal antibodies. Fig. 3 presents the experimental results, which indicate that

Table 1. Cross titration of the culture supernatants from GNNV-infected GF-1 and SGF-1 cells

Virus titres of the supernatants collected from GNNV-infected cell lines are expressed as log $TCID_{50}$/0.1 ml.

Titration system	Virus titre (log $TCID_{50}$/ml)	
	Supernatant from GNNV/GF-1 cells	Supernatant from GNNV/SGF-1 cells
GF-1	7.7	11.5
SGF-1	6.5	7.0

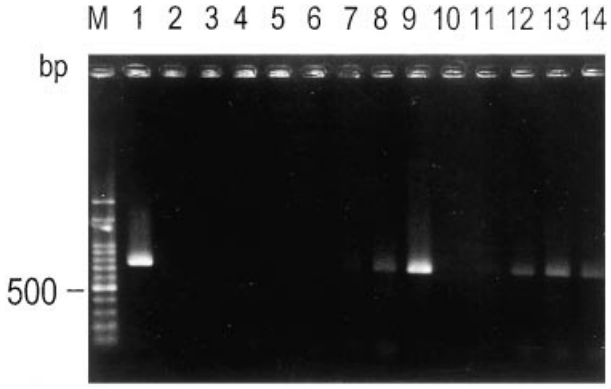

Fig. 4. Nested PCR detection of GNNV cDNA in GNNV-infected SGF-1 cells with GNNV-specific primers (P1 and N1). Lanes: M, DNA marker (100 bp ET ladder); 1, RNA extracted from purified GNNV and amplified by RT–PCR using primers P1 and N1; 2, DEPC-treated water as a negative control; 3, RNA extracted from purified GNNV particles and used for PCR without reverse transcription; 4, DNA extracted from GNNV-infected GF-1 cells and used for PCR directly; 5, DNA extracted from SGF-1 cells; 6–9, DNA extracted from the pellets of GNNV-infected SGF-1 cells collected 4 h and 1, 3 and 5 days after GNNV infection; 10, DNA extracted from the supernatant from SGF-1 cells; and 11–14, DNA extracted from the supernatants of GNNV-infected SGF-1 cells collected 4 h and 1, 3 and 5 days after GNNV infection.

GNNV capsid protein densities from these two samples are similar.

Cross titrations of the supernatants from GNNV-infected GF-1 and SGF-1 cells

Table 1 lists the virus titres of the supernatants from GNNV-infected GF-1 and SGF-1 cells in the two cell titration systems. When the supernatant from GNNV-infected SGF-1 cells was titrated in GF-1 cells, the titre was extremely high ($1 \times 10^{11.5}$ $TCID_{50}$/ml); this titre is about 4·5 logs higher than the titre of the same supernatant titrated in SGF-1 cells ($1 \times 10^{7.0}$ $TCID_{50}$/ml). It was also about 4–5 logs higher than the titres of the supernatant from GNNV-infected GF-1 cells, which were titrated either in GF-1 cells ($1 \times 10^{7.7}$ $TCID_{50}$/ml) or in SGF-1 cells ($1 \times 10^{6.5}$ $TCID_{50}$/ml).

Detection of GNNV cDNA in GNNV-infected SGF-1 cells

The size of the target PCR product of the GNNV-specific primer set (P1 and N1) is 795 bp. After DNA extraction, nucleic acids from different samples were applied directly for PCR. No target PCR product was amplified using the templates from purified GNNV, GNNV-infected GF-1 or SGF-1 cells. However, target PCR products were amplified using the DNA templates from either culture supernatant or cell pellets of GNNV-infected SGF-1 cells and the amount of target PCR product increased as the GNNV infection period increased (Fig. 4).

Discussion

The SSN-1 cell line is highly susceptible to fish nodavirus and NNV titres in this cell line can be as high as $1 \times 10^{9–10}$ $TCID_{50}$/ml (Iwamoto *et al.*, 2000). However, the SSN-1 cell line is persistently infected by the C-type retrovirus SnRV (Frerichs *et al.*, 1991). Successful production of GNNV in the SSN-1 cell line could be ascribed to the possibility that the receptor of GNNV can be induced by SnRV (Munday & Naikai, 1997; Iwamoto *et al.*, 2000). However, the role of SnRV in the infection or production of GNNV remains unknown. The aim of this investigation was to study the influence of fish retrovirus SnRV on the life cycle of fish nodavirus GNNV.

Iwamoto *et al.* (2000) have tried many times to clone an SnRV-free cell line from SSN-1 cells but SnRV still can be detected in every clone they obtained. SnRV is reported to be distinguishable from all known retrovirus groups due to the presence of an arginine tRNA primer-binding site (Hart *et al.*, 1996). This finding may be the cause of the difficulty of obtaining an SnRV-free clone from a cell line persistently infected with SnRV. The GF-1 cell line was proved to be SnRV-free (Fig. 1). Herein, SnRV-containing culture supernatant from SSN-1 cells was inoculated into GF-1 cells and a new cell line persistently infected with SnRV, which was designated the SGF-1 cell line, was induced. No CPE was observed in the SnRV-infected GF-1 cells or the subcultures of SGF-1 cells. Fig. 1 revealed that cDNA of the SnRV *pol* gene can be detected in the genomic DNA of the third subculture of SGF-1 cells. Moreover, cDNA of SnRV has also been detected in the fifth,

fifteenth and twentieth subcultures of SGF-1 cells (data not shown). These results imply that SnRV can infect the GF-1 cell line, reverse-transcribe the single-stranded genomic RNA into cDNA and integrate the cDNA into the genomic DNA of host cells. Furthermore, the genomic RNA of SnRV has been detected in the culture supernatant from SGF-1 cells by RT–PCR (data not shown); hence, SnRV can complete its life cycle in SGF-1 cells. The C-type retrovirus SnRV has been discovered in many fish species but grouper has not been reported (Frerichs *et al.*, 1991). Since SnRV can easily infect the grouper cell line GF-1, it is inferred that grouper is a possible natural host candidate for SnRV.

In order to compare the relative amounts of GNNV RNA and capsid proteins and the titres of the supernatants derived from GNNV-infected GF-1 and SGF-1 cells at the end of the development of CPE, we synchronized all the conditions between GF-1 and SGF-1 cells during GNNV infection. End-point RT–PCR was used in the present study to analyse the amounts of GNNV RNA produced in both cell systems. In general, the yield of RT–PCR is not always proportional to the amount of input RNA template, especially when the concentration of input RNA is high. Therefore, a serial 10-fold dilution of the original RNA template was designed to reduce the saturation effect of RT–PCR end-products. Fig. 2 indicates that the amounts of RT–PCR products were proportional to the amounts of input RNA templates. Therefore, quantitative comparison of GNNV RNA extracted from the clarified supernatants of GNNV-infected GF-1 and SGF-1 cells determined by the end-point RT–PCR remains meaningful.

The results of RT–PCR and Western immunoblot assays showed that the productivity of GNNV RNA and capsid protein was similar in the supernatants from GNNV-infected GF-1 and SGF-1 cells (Figs 2 and 3). Therefore, the existence of SnRV in SGF-1 cells did not increase or decrease the production of GNNV. However, when both supernatants were titrated in GF-1 cells, the titre ($10^{11.5}$ $TCID_{50}$/ml) of culture supernatant from GNNV-infected SGF-1 cells was much higher than that of supernatant from GNNV-infected GF-1 cells (Table 1). This phenomenon is unusual because SnRV did not increase GNNV production in SGF-1 cells. Similar results were also observed several times in the pre-tests. Therefore, the high titre is not owing to the high concentration of GNNV in the supernatant from GNNV-infected SGF-1 cells.

Two possibilities arise for this phenomenon: first, although SnRV alone cannot induce CPE of the host cells, the CPE induced by GNNV in the titration system may be enhanced by SnRV competition for cell resources; second, through some unclear mechanisms, SnRV in the SnRV/GNNV-containing supernatant from GNNV-infected SGF-1 cells can aid GNNV infection of GF-1 cells during titration in GF-1 cells by increasing either the adsorption of GNNV particles to cells or the delivery of GNNV genome into cells.

If competition for cellular resources by SnRV and GNNV can enhance CPE in the titration system, the titre of the supernatant from GNNV-infected GF-1 cells titrated in SGF-1 cells (SnRV-PI cells) should also be higher than the titre ($10^{7.7}$ $TCID_{50}$/ml) of the same supernatant titrated in GF-1 cells (SnRV-free cells). However, the titre of the same supernatant titrated in SGF-1 cells was $1 \times 10^{6.5}$ $TCID_{50}$/ml. Therefore, the first possibility is unlikely.

If SnRV in the supernatant from GNNV-infected SGF-1 cells can aid GNNV infection of GF-1 cells (SnRV-free cells) during titration, the aid will be interfered by homologous SnRV in SGF-1 cells (SnRV-PI cells) when the same supernatant was titrated in SGF-1 cells. Table 1 presents the results that the homologous interference of SnRV was reflected in the lower titre ($10^{7.0}$ $TCID_{50}$/ml) titrated in SGF-1 cells than the titre ($10^{11.5}$ $TCID_{50}$/ml) titrated in GF-1 cells. Therefore, the second hypothesis is more likely.

Notably, the cDNA of GNNV was detected not only in the cell pellet but also in the supernatant from GNNV-infected SGF-1 cells, and the production of GNNV cDNA increased as the incubation time increased (Fig. 4). However, the GNNV cDNA has never been found in the culture supernatant or the pellets derived from GNNV-infected GF-1 cells. Fish nodaviruses are bi-segmented, single-stranded RNA viruses and their life cycle should not have a cDNA stage. The appearance of GNNV cDNA in GNNV-infected SGF-1 cells indicates that the reverse transcriptase of fish retrovirus SnRV in the SGF-1 cells could react with the proviral GNNV RNA and create a new cDNA stage in the life cycle of GNNV. Moreover, the detection of GNNV cDNA in the pellet and culture supernatant from GNNV-infected SGF-1 cells infers that newly created GNNV cDNA may be packed into virus particles and released into the culture supernatant.

Whether the cDNA of GNNV is packed into GNNV or SnRV particles requires further investigation. If this situation happened, when the supernatant from the GNNV-infected SGF-1 cells was titrated in GF-1 cells, the GNNV genome could be delivered either by the GNNV particles to GF-1 cells through GNNV receptors or by the re-constructed SnRV particles with the GNNV cDNA through SnRV receptors. This could then enhance the infection of GNNV to GF-1 cells. This re-constructed virus particle hypothesis is a novel consideration and may be proven in further experiments.

To our knowledge, this study addresses for the first time the cDNA stage of a fish nodavirus induced by a fish retrovirus within a cell culture system. The interactions between these two heterologous RNA viruses during super- or coinfection may be complex. Fish retroviruses and nodaviruses have been found in many species of fish and the host ranges of these two virus species possibly overlap (Frerichs *et al.*, 1991; Nakai *et al.*, 1995). Whether the interaction of fish nodavirus GNNV and fish retrovirus SnRV *in vivo* will be similar to that *in vitro* is an interesting topic for further study.

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