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Generation and characterization of NV gene-knockout recombinant viral hemorrhagic septicemia virus (VHSV) genotype IVa

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ABSTRACT: A recombinant viral hemorrhagic septicemia virus (rVHSV-ΔNV-EGFP) containing the enhanced green fluorescent protein (EGFP) gene instead of the NV gene was produced using the reverse-genetics method. For use as a positive control, another recombinant virus (rVHSV-wild) was also generated, which had an identical nucleotide sequence to the wild-type VHSV genome except for a few artificially replaced nucleotides. The rVHSVs were rescued using a system controlled by T7 RNA polymerase supplied by a retroviral vector. Generation of rVHSV-ΔNV-EGFP and rVHSV-wild was confirmed by sequencing of RT-PCR products, and rescue of infectious rVHSVs was confirmed by observation of plaque formation. Replication efficiency of rVHSV-wild was distinctly lower than that of wild-type VHSV, suggesting that the artificially replaced nucleotides, especially when immediately preceding the G or NV gene start codons, might affect the replication of the virus. Replication of rVHSV-ΔNV-EGFP was slightly lower than that of rVHSV-wild when epithelioma papulosum cyprini cells were infected with multiplicity of infection (MOI) 1.0, but much lower when cells were infected with MOI 0.00001. These results suggest that the NV gene plays an important role in VHSV replication through interactions with host-cell responses, and the lower replication ability of rVHSV-wild compared to wild-type VHSV might be caused by replaced nucleotides just before the NV gene open reading frame (ORF) rather than the G gene ORF. In olive flounder *Paralichthys olivaceus*, rVHSV-wild produced slower-progressing mortalities than wild-type VHSV, whereas rVHSV-ΔNV-EGFP pathogenesis was highly attenuated. These results suggest that the NV protein of VHSV may play an important role not only in viral replication but also in viral pathogenesis.

KEY WORDS: Viral hemorrhagic septicemia virus · Reverse genetics · Recombinant VHSVs · NV gene · *In vitro* replication · *In vivo* virulence

INTRODUCTION

Viral hemorrhagic septicemia disease (VHSD) caused by infection with viral hemorrhagic septicemia virus (VHSV) is one of the major causes of morbidity and mass mortality in both cultured and wild fish worldwide (Schlotfeldt & Ahne 1988, Schlotfeldt et al. 1991, Mortensen et al. 1999, Isshiki et al. 2003, Skall et al. 2005). VHSV is an enveloped negative-strand RNA virus and belongs to the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Lenoir & de Kinkelin 1975, Walker et al. 2000, Tordo et al. 2005). The virus consists of an 11 to 12 kb nucleotide genome encoding 5 structural and structure-associated proteins: a nucleoprotein (N), a polymerase-associated phosphoprotein (P), a matrix protein (M), a glycoprotein (G), an RNA-dependent RNA polymerase (L); and a nonstructural protein (NV) that is the defining feature of *Novirhabdovirus* (Schütze et al. 1996, 1999, Tordo et al. 2005).

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The reverse-genetics approach to generate infectious negative-stranded RNA viruses from cloned cDNAs has helped to elucidate the function of viral genes and to develop prophylactic vaccines (Schnell et al. 1994, Palese et al. 1996, Neumann et al. 2002, Neumann & Kawaoka 2004, von Messling & Cattaneo 2004). In *Novirhabdovirus*, snakehead rhabdovirus (SHRV) was firstly generated by reverse genetics (Johnson et al. 2000). Thereafter, infectious hematopoietic necrosis virus (IHNV) (Biacchesi et al. 2000, 2002, Romero et al. 2005, Ammayappan et al. 2010a) and VHSV (Ammayappan et al. 2010b, Biacchesi et al. 2010) were generated by the reverse-genetics method.

VHSVs can be divided into 4 major genotypes based on the sequence of G and N genes (Snow et al. 1999, Einer-Jensen et al. 2004), and each genotype is closely correlated not only with the geographic location but also with the pathogenesis in host fishes (Snow et al. 2005, Brudeseth et al. 2008). The previously reported recombinant VHSVs were genotype Ia (Biacchesi et al. 2010) and genotype IVb (Ammayappan et al. 2010b). In Korea, only genotype IVa of VHSV has been reported as a pathogen of farmed olive flounder *Paralichthys olivaceus* since first reported in 2001 (Kim et al. 2003, 2009). Thus the recombinant VHSV rescued in the present study is the first recombinant VHSV made from an Asian VHSV strain, genotype IVa, and can be utilized as a prophylactic vaccine against VHSD in olive flounder.

The role of the transmembrane G protein has been well investigated. The G protein mediates the entry of the virion into cells and is the target of neutralizing antibodies (Engelking & Leong 1989, Lorenzen et al. 1990, Estepa et al. 1999). However, the role of the NV gene is controversial, ranging from no effect on proliferation of SHRV (Alonso et al. 2004) to significant positive effect on viral replication such as IHNV (Biacchesi et al. 2000, Thoulouze et al. 2004). Recently, Biacchesi et al. (2010) and Ammayappan et al. (2010b) reported that replication of recombinant VHSVs in epithelioma papulosum cyprini (EPC) cells was significantly inhibited by knockout of the NV gene.

In the present study, we rescued recombinant VHSVs having the enhanced green fluorescent protein (EGFP) gene instead of the NV gene (rVHSV-ΔNV-EGFP) or having the same nucleotide sequence as the wild-type VHSV KJ2008 genome except for a few artificially replaced nucleotides (rVHSV-wild) using the reverse genetics method, and analyzed the role of NV gene in viral replication and pathogenesis.

MATERIALS AND METHODS

Cells and virus

EPC cells were cultured in Leibovitz medium (L-15; Sigma) supplemented with penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and 10% fetal bovine serum (FBS; Gibco). VHSV KJ2008 strain isolated in 2008 from moribund olive flounder in a natural outbreak of VHSD on a commercial farm in Korea was propagated in monolayer of EPC cells at 15°C in the presence of 2% FBS and antibiotics. Cultures displaying extensive cytopathic effect (CPE) were harvested and centrifuged at 4000 × *g* for 10 min at 4°C, and the supernatants were stored at –80°C.

Establishment of T7 RNA polymerase (T7 RNAP)-expressing EPC cells

To establish a retroviral vector-based T7 RNAP-expression system, multiple cleavage sites (MCS; *Xho*I-*Apa*I-*Xho*I-*Sal*I-*Bam*HI-*Hind*III-*Bgl* II) were synthesized *in vitro* and inserted into the pLNHX retroviral expression vector (Clontech), resulting in pLNHX-MCS. A PCR fragment encoding the T7 RNAP open reading frame (ORF) that contains *Hind*III and *Bam*HI sites was cloned into pGEM T-easy vector (Promega), and inserted into the pcDNA 3.1(+) vector (Invitrogen) under the control of the early cytomegalovirus (CMV) promoter, resulting in pCMV-T7 RNAP. The T7 RNAP-expressing cassette including CMV promoter and the bovine growth hormone (BGH) polyadenylation signal in the pCMV-T7 RNAP was digested with *Bgl*II and *Sal*I, subcloned into the pLNHX-MCS vector, and named pLNHX-pCMV-T7 RNAP. GP2-293 cells were sub-cultured in a T_{25} flask (6×10^5 cells) at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% FBS and antibiotics. Cells were grown to about 80% confluence and transfected with 5 µg of the pLNHX-pCMV-T7 RNAP vector and 5 µg of the pVSV envelope vector (Clontech) using the calcium phosphate method. To increase transfection efficiency, the culture medium was replaced with medium containing 25 µM chloroquine at 3 to 4 h prior to transfection. The medium was changed 10 h post-transfection, and recombinant retroviruses that expressed T7 RNAP were collected 48 h post-transfection. EPC cells in culture medium containing 4 µg of polybrene (Sigma) were infected with the recombinant retroviruses. The culture medium was replaced at 24 h post-infection with culture medium containing G-418 (400 µg ml^{-1}; Sigma) for selection.

Construction of full-length and NV gene-deleted complementary DNA (cDNA) clones of VHSV

The plasmid harboring a full-length cDNA of the VHSV KJ2008 genome (pVHSV-wild) was constructed by multi-step cloning processes, and the sequences and locations of oligonucleotide primers used in the construction are shown in Table 1 and Fig. 1, respectively. Total RNA was extracted from VHSV KJ2008-infected EPC cells using RNAiso plus reagent (Takara). To obtain cDNA, 1 µg of the purified RNA was incubated with 0.5 µl (0.5 µg ml^{-1}) of random hexamers (Promega) at 80°C for 5 min, and further incubated at 42°C for 60 min in a reaction mixture containing 2 µl of each 2.5 mM dNTP mix (Takara), 0.5 µl of M-MLV reverse transcriptase (Promega), and 0.25 µl of RNase inhibitor (Promega) in a final reaction volume of 10 µl. To amplify VHSV cDNA fragments, PCR in a reaction volume of 20 µl was performed with 5× HiPi Plus PCR Premix (Elpis-Biotech), 1 µl of the cDNA template (10^{-1} diluted), and 1 µl (10 pmol $µl^{-1}$) of specific primers for each fragment. Thermal cycling conditions were 1 cycle of 3 min at 95°C (initial denaturation) followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 3 min at 72°C, with a final extension step of 7 min at 72°C. PCR products were purified using gel purification kit (Cosmo Genentech), and subcloned into the pGEM T-easy vector. Several clones were sequenced using an automatic sequencer (Applied Biosystems).

The plasmid pVHSV-wild was constructed by assembling of each fragment using unique restriction sites already present in the VHSV genome or artificially created by insertion of nucleotides. The *Nae*I/*Age*I PCR fragment 1 containing T7 RNAP promoter, nucleoprotein (N), phosphorylated protein (P), and matrix protein (M) was generated by ligation of *Nae*I/*Kpn*I fragment 1a and *Kpn*I/*Age*I fragment 1b using the *Kpn*I restriction site. The *Age*I/*Sac*II PCR fragment 2 contained only the glycoprotein (G) ORF. The *Sac*II/*Nar*I PCR fragment 3 containing the nonstructural protein (NV) ORF was generated by the assembling of 3a and 3b PCR products by overlapping PCR, and a *Cla*I restriction site was created between fragment 3a and 3b for convenient replacement of the NV ORF with a foreign gene ORF. The *Nar*I/*Spe*I PCR fragment 4 and *Spe*I/*Pst*I PCR fragment 5 contained RNA-dependent RNA polymerase (L). PCR fragment 5 was generated by assembling of 5a and 5b PCR products fused with the antigenomic sequence of hepatitis delta virus ribozyme (HdvRz) and the T7 transcription termination sequence. Each PCR fragment was gel purified, subcloned into pGEM T-easy vectors, and inserted step by step into pT7-MCS vectors containing multiple cleavage sites (*Nae*I-*Age*I-*Sac*II-*Cla*I-*Nar*I-*Aat*II) inserted into the pGEM T-easy backbone. Firstly, fragment 5 was digested with *Spe*I and *Pst*I, subcloned into the pT7-MCS, and ligated with fragment 4 after digestion with *Nar*I and *Spe*I. Fragment 1 was digested with *Nae*I and *Age*I, and subcloned into pT7-MCS containing fragments 4 and 5. Fragment 2 was digested with *Age*I and *Sac*II, and ligated to pT7-MCS containing fragments 1, 4, and 5. Finally, fragment 3 was digested with *Sac*II and *Nar*I, and cloned into pT7-MCS containing fragments 1, 2, 4, and 5, resulting in pVHSV-wild.

To generate the NV gene-deleted mutant rVHSV, the NV gene ORF in the full-length cDNA construct (pVHSV-wild) was removed by digestion with *Cla*I and *Nar*I, and the EGFP gene ORF was inserted into the digested plasmid, resulting in pVHSV-ΔNV-EGFP. The integrity of DNA sequence of the vectors was confirmed by sequencing of the full-length cDNA using the DNA sequencer.

Construction of supporting plasmids encoding N, P, L proteins

The VHSV genes encoding N, P, and L proteins were amplified by PCR using the pVHSV-wild as a template and primer pairs specific for each gene (Table 1). The N and P genes were cloned with appropriate restriction enzyme cleavage sites; whereas the L gene was amplified as 2 fragments, L1 and L2, and cloned into pGEM T-easy vector as one fragment by ligation of *Spe*I and *Hind* III-digested fragment L2 with fragment L1. All the products were inserted into pcDNA3.1(+) vector under the control of a CMV promoter, resulting in pCMV-N, pCMV-P, and pCMV-L.

Rescue of recombinant VHSVs

EPC cells stably expressing T7 RNAP were grown to about 80% confluence and transfected with a mixture of pVHSV-wild or pVHSV-ΔNV-EGFP (2 µg), pCMV-N (500 ng), pCMV-P (300 ng), and pCMV-L (200 ng) using FuGENE 6 (Roche) according to the manufacturer's instructions. Transfected cells were incubated for 12 h at 28°C, and then coded to 15°C. When total CPE was observed, the cells were suspended by scraping the plates with a rubber policeman, submitted to 2 cycles of freeze-thawing and centrifuged at 4000 × *g* for 10 min. The resulting

Table 1. Primers used in the present study. Restriction sites are shown in **bold**. Underlined nucleotides indicate the T7 RNA polymerase promoter. Nucleotide position is given according to the nucleotide sequence order of the full VHSV KJ2008 genome (GenBank access. no. JF792424)

Primer name		Sequence (5′ to 3′)	Nucleotide position	Restriction enzyme
For construction of recombinant VHSV genomes				
T-MCS				
	F	GGCACCGGTCCGCGGATCGATGGCGCCGACGT		
	R	CGGCGCCATCGATCCGCGGACCGGTGCC		
T7VHSF1				
	F1-a	**GCCGGC**TAATACGACTCACTATAGGGGTATCATAAAATATGATGAG-TTATGTTACAGG	1–32	*Nae*I
	R1-a	**ACCGGTGGTACC**AGGATGGTGCGCTTTCTTTTGAATAGAGCCAT	2273–2316	*Kpn*I, *Age*I
	F1-b	**GGTACC**TTCTCCTCATCTTACCTCTAATGACGAGGACCGTGTCTCA	2305–2350	*Kpn*I
	R1-b	**ACCGGT**GACTTGTTGTGTACACAAACGTACC	2934–2964	*Age*I
VHSF2				
	F2	**ACCGGT**ATGGAATGGAATACTTTTTTCTTGG	2959–2989	*Age*I
	R2	**CCGCGG**TCAGACCATCTGGCTTCTGGAG	4467–4494	*Sac*II
VHSF3				
	F3-a	**CCGCGG**CCGCCTAAACCACCATAGTTTCC	4489–4517	*Sac*II
	R3-a	GTGCCGACTGGGTCGTCAT**ATCGAT**GTCTCACAGGGGTGCCATTT	4537–4581	*Cla*I
	F3-b	AAATGGCACCCCTGTGAGAC**ATCGAT**ATGACGACCCAGTCGGCAC	4537–4581	*Cla*I
	R3-b	**GGCGCC**TCATGGGGGAGATTCGGAGCCATTC	4907–4937	*Nar*I
VHSF4				
	F4	**GGCGCC**CCCCCTTCTCCCAGATAGAA	4932–4957	*Nar*I
	R4	**ACTAGT**GGGCCCCTGGTCGTGTGTGCTGTC	8471–8500	*Spe*I
VHSF5				
	F5-a	**ACTAGT**TCCTTACTTCGGGACTCAGACC	8495–8522	*Spe*I
	R5-a	CGAAGAACAAGAAATCCGAGGCAGGAGACTTGGGATCGGACGCC	11088–11131	
	F5-b	GGCGTCCGATCCCAAGTCTCCTGCCTCGGATTTCTTGTTCTTCG	11088–11131	
	R-T7Φ	**CTGCAG**CAAAAAACCCCTCAAGACCCG	end	*Pst*I
EGFP				
	F	**ATCGAT**ATGGTGAGCAAGGGCGAG		*Cla*I
	R	**GGCGCC**TTACTTGTACAGCTCGTCCATG		*Nar*I
For construction of supporting plasmids				
pCMV-N				
	F	**GGATCC**CGGCACTTAAGTAGCAAAAAGTTT		*Bam*HI
	R	**GCGGCCGC**TCCTTTTCTATCTATATGAGTTATGAGA		*Not*I
pCMV-P				
	F	**AAGCTT**CGGCACGATTATAGGAATTTTTC		*Hind*III
	R	**GCGGCCGC**TTTCTTTCTATCTATACGATGTGTTGTG		*Not*I
pCMV-L				
	F1	**AAGCTT**TGGCACTTTTGTTGTTTGTAGTC		*Hind*III
	R1	**GCGGCCGCACTAGT**GGGCCCCTGGTCGTGTG		*Spe*I, *Not*I
	F2	**ACTAGT**TCCTTACTTCGGGACTCAGACCAAACC		*Spe*I
	R2	**GCGGCCGC**GCTTTTTTTCAATCTAGTTGAGGAACAAG		*Not*I
For construction of T7 RNA polymerase-expressing plasmids				
pCMV-T7 RNAP				
	F	**AAGCTT**ATGAACACGATTAACATCGCTAAGAA		*Hind*III
	R	**GGATCC**TTACGCGAACGCGAAGTCCG		*Bam*HI
pLNHX-MCS				
	F	TCGAAGATCTAAGCTTGGATCCGTCGACCTCGAGGGGCCC		
	R	GATCGGGCCCCTCGAGGTCGACGGATCCAAGCTTAGATCT		
For detection of replication of recombinant VHSVs				
OF-18S				
	F	CAAGACGGACGAAAGCGAAAGCAT		
	R	TGGCATCGTTTACGGTCGGAACTA		
VHSV G				
	F	GTTTGTACAACATCACCCTGCCCA		
	R	TTGGAGACGAACACAATGCTCGG		

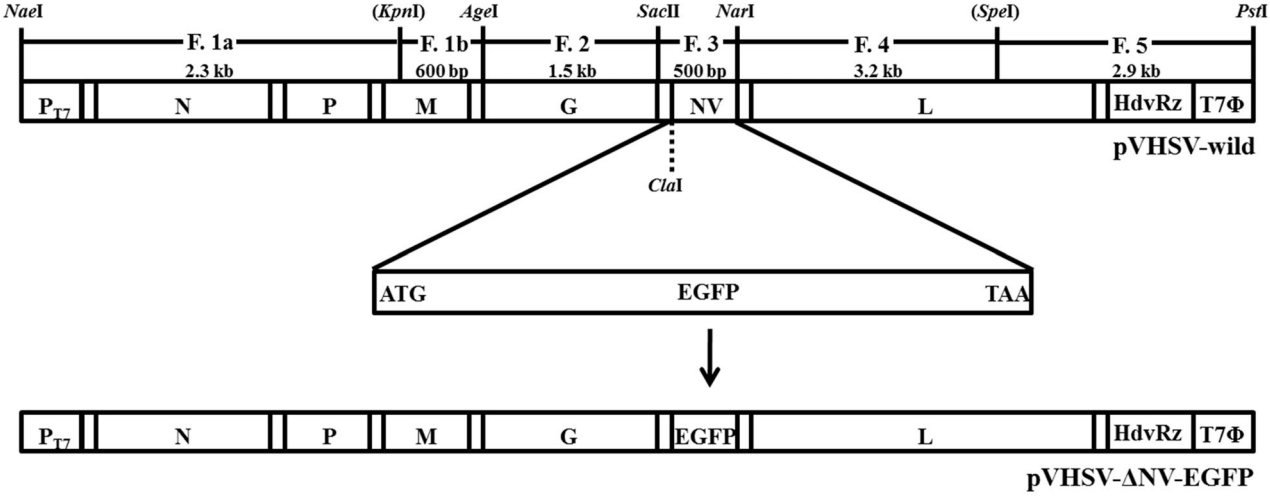

Fig. 1. Construction of the full-length cDNA (pVHSV-wild) and the mutated cDNA (NV gene replaced with EGFP gene) (pVHSV-ΔNV-EGFP) clones of VHSV KJ2008. The pVHSV-wild plasmid was constructed by assemblage of 5 fragments (F.1a,b to F.5) using restriction sites that are naturally present in the VHSV genome (in parentheses) or artificially inserted by replacement of several nucleotides. The N gene is flanked upstream by the T7 RNA polymerase promoter (P_{T7}), and the L gene is flanked downstream by the hepatitis delta ribozyme (HdvRz) and T7 transcription termination sequence (T7Φ). The pVHSV-ΔNV-EGFP was generated by replacement of the NV open reading frame (ORF) in the pVHSV-wild with the EGFP ORF using newly created *Cla*I/*Nar*I restriction enzyme sites

supernatant (named P0) was used to inoculate fresh EPC cell monolayers in a T_{25} flask at 15°C. At 7 to 10 d post-inoculation, the supernatant (P1) was harvested, aliquoted, and stored at –80°C.

Confirmation of recombinant VHSVs by RT-PCR and sequencing

Total RNA was extracted from the supernatant P1 using RNAiso plus reagent, and the N, P, G, NV, or EGFP genes were amplified by RT-PCR. The PCR products were analyzed on a 1% agarose gel, visualized with ethidium bromide, and, to verify the presence of the artificially inserted restriction sites, the G and NV gene fragments including regions of the replaced nucleotides were subjected to nucleotide sequencing.

Plaque assay

The titer of the wild-type VHSV and the recombinant VHSVs used in this study was determined by plaque assay (Burke & Mulcahy 1980). Briefly, EPC cell monolayers were inoculated with one of each viral stock, serially diluted from 10^{-5} to 10^{-7}. After 1 h of incubation at 15°C, the cells were overlaid with plaquing medium (0.7% agarose in L-15 containing 10% FBS and antibiotics). After 7 to 10 d of incubation to allow plaque formation, the cells were fixed by 10% formalin and stained with 3% crystal violet for 30 min at room temperature. After rinsing the cells with distilled water, the plaque-forming units (PFU) were counted and photographed.

Viral growth curve

The replication of wild-type VHSV and recombinant VHSVs in EPC cells was analyzed by plaque assay. EPC cells in 6-well plates were infected with the wild-type VHSV or recombinant VHSVs (rVHSV-wild and rVHSV-ΔNV-EGFP) at a multiplicity of infection (MOI) of 1.0 or 0.00001. The cells were incubated at 15°C and replication of viruses was analyzed at 1, 3, 5 and 7 d post-inoculation by the plaque assay. The experiment was conducted with triplicate groups.

Effects of NV protein expression on viral proliferation

The effect of NV protein on the proliferation of the wild-type VHSV and recombinant VHSVs (rVHSV-wild and rVHSV-ΔNV-EGFP) was analyzed using EPC cells that constitutively express NV protein by a vector equipped with a CMV promoter-driven NV gene expression cassette that was constructed by insertion of the NV gene ORF into pcDNA3.1(+) vector.

Naïve EPC cells and NV protein-expressing EPC cells were challenged with MOI 0.001 or 0.00001 of each virus type, and the progression of CPE was analyzed.

In vivo pathogenesis of recombinant VHSVs

A total of 50 olive flounder fingerlings (4 to 5 g; confirmed free from VHSV and showing no signs of illness) were intramuscularly (i.m.) injected with one of the recombinant VHSVs (rVHSV-ΔNV-EGFP or rVHSV-wild) at 1 of 2 doses (10^3 or 10^5 PFU per fish), and control fish were injected with an equal volume of L-15 alone. Experimental fish in each group (10 fish) were kept in five 30 l tanks at 15°C, and mortalities were recorded daily for 30 d post-injection.

RESULTS

Rescue of recombinant VHSVs

The present VHSV KJ2008 strain was confirmed as the genotype IVa through sequence analysis, and the full genome sequence of the virus was registered in GenBank (Accession no.: JF792424). To rescue NV gene-knockout VHSV, a genomic mutant cDNA clone of VHSV in which the NV gene ORF was replaced with the EGFP gene ORF (pVHSV-ΔNV-EGFP; Fig. 1) was constructed. In addition, a full-length cDNA clone (pVHSV-wild) having a few different nucleotides from the wild-type VHSV KJ2008 genome was constructed for generation of a positive control recombinant virus (Fig. 1). The T7 RNAP-expressing EPC cells were established by transfection of cells with the retroviral vector carrying the polymerase gene, and the expression of T7 RNAP was verified by a clear RT-PCR band (data not shown). The evident CPE in EPC cells was induced by co-transfection with plasmids expressing the N, P, and L proteins and pVHSV-ΔNV-EGFP or pVHSV-wild (Fig. 2).

The presence of recombinant viruses in the stock supernatants was confirmed by RT-PCR, sequencing, and plaque assay. In RT-PCR analysis, the bands corresponding in size to viral genes were amplified from both pVHSV-ΔNV-EGFP- and pVHSV-wild-transfected cells (Fig. 3). To further verify that the rescued virus was derived from the transfected cDNAs, the PCR products of G and NV genes were sequenced, and the artificially replaced nucleotide sequences were detected. In plaque assays to confirm the presence of infectious recombinant viruses, 3×10^7 and 9×10^7 PFU of recombinant viruses were generated in cells inoculated with the stock supernatants of rVHSV-ΔNV-EGFP and rVHSV-wild, respectively (Fig. 4). The rVHSV-ΔNV-EGFP in-

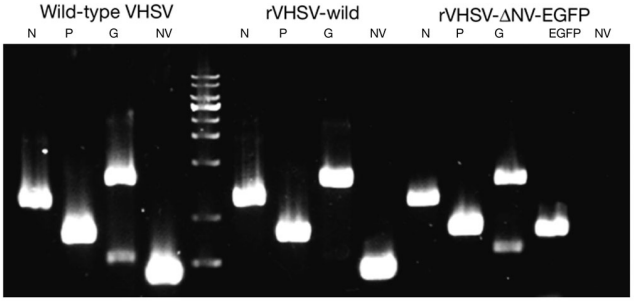

Fig. 3. Confirmation of the individual viral genes in rescued recombinant viruses by RT-PCR. Each viral genomic RNA extracted from supernatants of cells infected with wild-type VHSV, rVHSV-wild, or rVHSV-ΔNV-EGFP was amplified by RT-PCR with specific N, P, G, NV, or EGFP primers. PCR products were analyzed on a 1% agarose gel, and visualized with ethidium bromide. In the rVHSV-ΔNV-EGFP, the NV primers had no effect. The marker is a 1 kb DNA ladder (Bioneer)

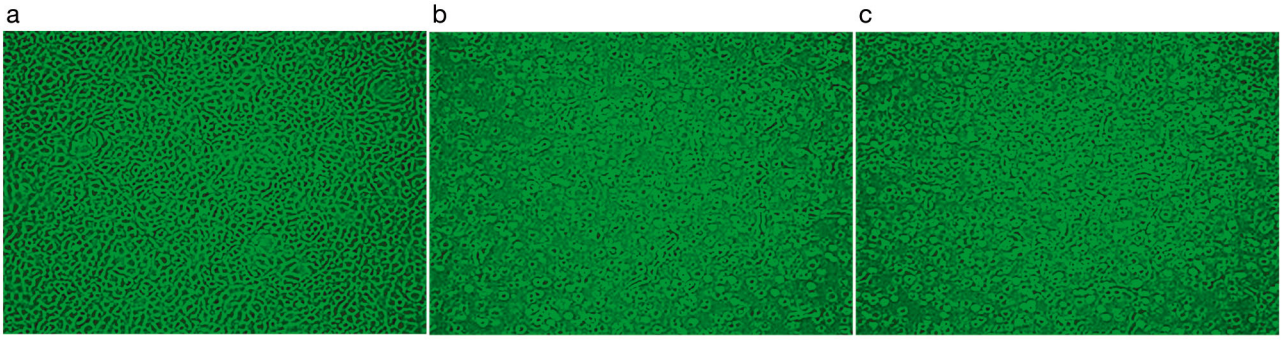

Fig. 2. Cytopathic effect (CPE) induced by co-transfection of epithelioma papulosum cyprini (EPC) cells. (a) Control cells showed no CPE. (b,c) Cells transfected with plasmids expressing (b) the N, P, L proteins and pVHSV-wild or (c) pVHSV-ΔNV-EGFP showed evident CPE

duced extensive CPE on EPC cells, which was irrespective to inoculated MOI (Fig. 5).

Growth of recombinant VHSVs

The growth of wild-type VHSV, rVHSV-wild, and rVHSV-ΔNV-EGFP in EPC cells was determined by plaque assay. The replication cycle and the final titers of the rVHSV-ΔNV-EGFP were distinctly lower than those of the wild-type VHSV, and slightly lower than those of the rVHSV-wild, when challenged with MOI 1.0 (Fig. 6a). However, the growth of the rVHSV-ΔNV-EGFP was distinctly lower than that of the rVHSV-wild, when challenged with MOI 0.00001 (Fig. 6b).

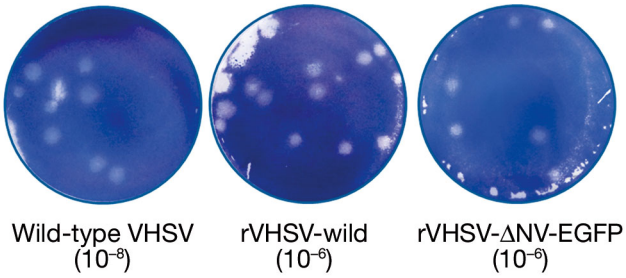

Fig. 4. Plaque formation in EPC cells infected with 10^{-6} to 10^{-8} diluted wild-type VHSV, rVHSV-wild, and rVHSV-ΔNV-EGFP. Cells were cultured under 0.7% agarose-containing plaquing medium, fixed at 7 d post-infection, and stained with crystal violet

Effects of cell-derived NV protein expression on cytopathic effect

At 3 d post-infection, in the naïve EPC cells, CPE was observed only in cells infected with MOI 0.001 of the wild-type VHSV and MOI 0.001 of the rVHSV-wild (Fig. 7a). However, in the NV protein-expressing EPC cells, distinct CPE was observed even in cells infected with MOI 0.001 of the rVHSV-ΔNV-EGFP (Fig. 7b). At 5 d post-infection, more severe CPE was observed in the NV protein-expressing EPC cells than the naïve cells in all infected cell groups (data not shown).

In vivo pathogenesis of recombinant VHSVs

The cumulative mortalities of fish i.m. injected with the rVHSV-wild were 90 to 100%, whereas

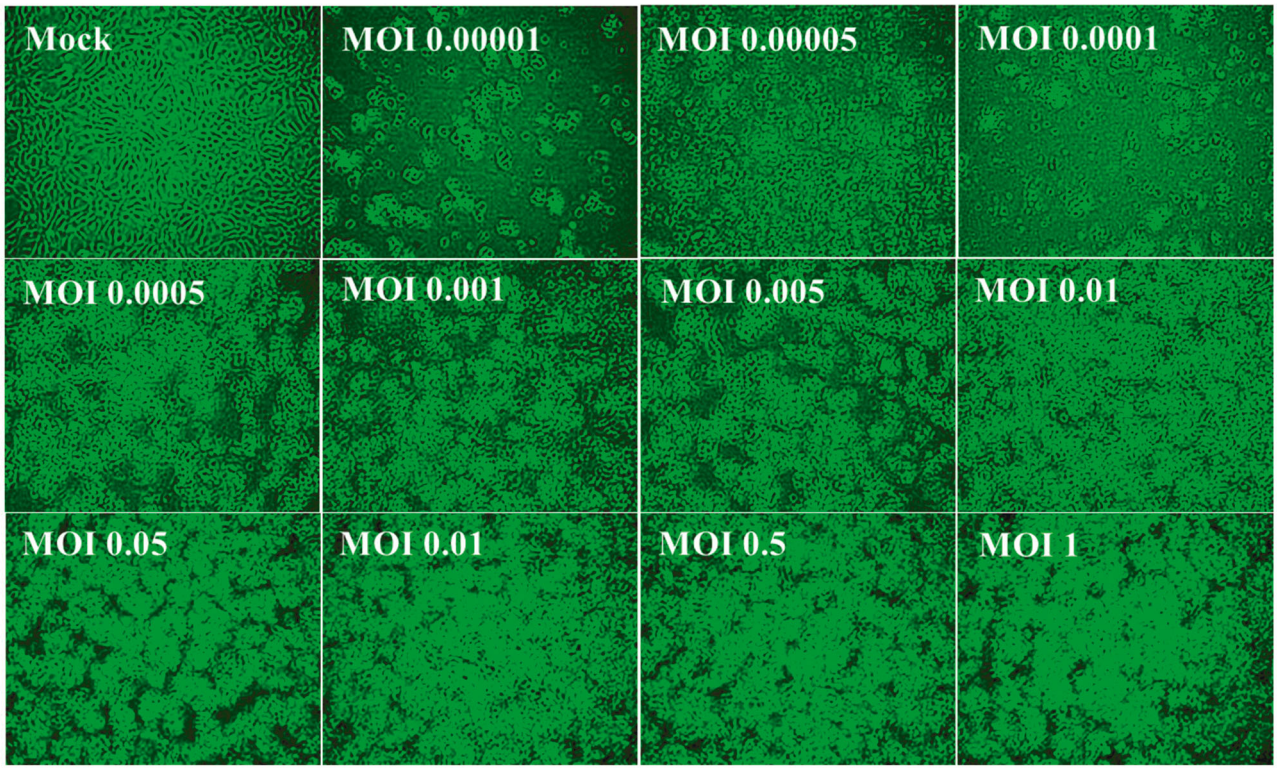

Fig. 5. Induction of CPE by rVHSV-ΔNV-EGFP infection at various multiplicities of infection (MOI) in EPC cells in 6-well plates at 7 d post-infection

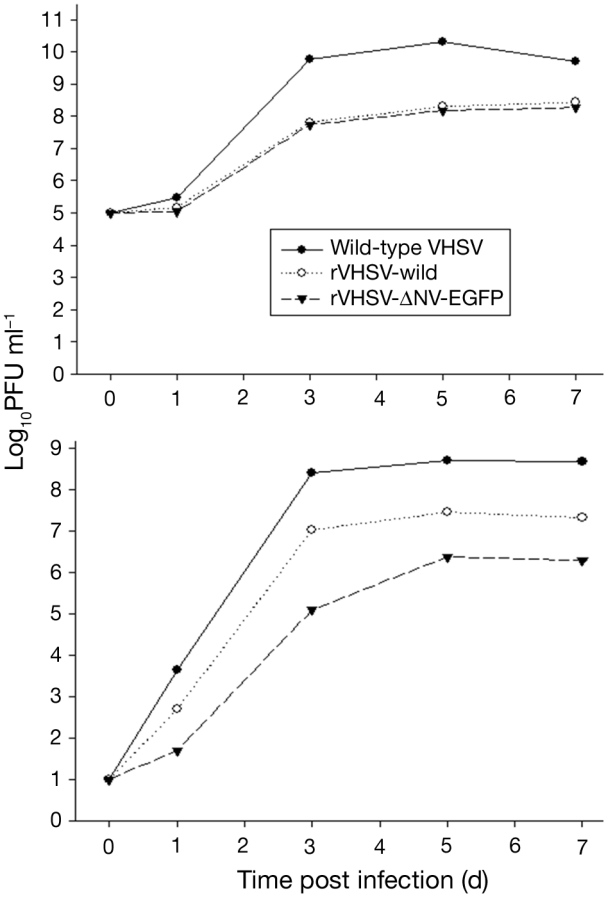

Fig. 6. Growth curves of wild-type VHSV, rVHSV-wild, and rVHSV-ΔNV-EGFP. EPC cells were infected with (a) multiplicities of injection (MOI) 1.0 or (b) MOI 0.00001 of each virus, and the replication of viruses was analyzed by plaque assay after 1, 3, 5 and 7 d post-inoculation. The viral titers are averages of the triplicate groups

no fish died by i.m. injection with rVHSV-ΔNV-EGFP (Fig. 8).

DISCUSSION

There have been several reverse genetics systems for rescue of negative-strand RNA viruses, which are distinguished by the T7 RNA polymerase-supplying methods and the ribozymes for generation of the intact viral genome without extra nucleotides at the termini that hamper the recovery efficiency of recombinant viruses (Schnell et al. 1994, Radecke et al. 1995, Schneider et al. 1997, Buchholz et al. 1999, Inoue et al. 2003). In the genus *Novirhabdovirus*, a recombinant vaccinia virus was used as a helper virus to supply T7 RNAP to generate recombinant SHRV and pT7 expression plasmid as well (Johnson et al. 2000), IHNV (Biacchesi et al. 2000, 2002, 2010), and VHSV (Biacchesi et al. 2010). Alonso et al. (2004) rescued a recombinant SHRV using EPC cells that stably expressed T7 RNAP by transfection with the polymerase-expressing plasmids instead of the vaccinia virus. Ammayappan et al. (2010a,b) produced recombinant IHNV and recombinant VHSV by transfection of EPC cells with a plasmid-based rescue system in which viral genes were expressed under the control of a cytomegalovirus (CMV) immediate-early promoter, and the transcript was trimmed by ribozymes of hammerhead virus and hepatitis delta virus, which paved the way for production of recombinant viruses without using the T7 RNAP expression system. In the present study, we generated recombinant VHSVs using a system controlled by T7 RNAP that was supplied by a retroviral vector not previously used for production of recombinant novirhabdoviruses. The retroviral vector system was advantageous because a long-term stable cell line could be established, and the use of an antibiotic was not required subsequently after selection of transfected cells. In the present study, generation of the rVHSV-ΔNV-EGFP and the rVHSV-wild was confirmed by sequencing of the RT-PCR products, and the rescue of infectious rVHSVs was confirmed by observation of plaque formation.

The role of the NV gene in replication of novirhabdoviruses has been controversial. Johnson et al. (2000) reported that the infectivity and proliferation in EPC cells and virion morphology of the NV gene mutant SHRV generated by attering the 23rd amino acid codon (arginine) of the NV gene into a stop codon, were not different from those of the wild-type SHRV. Alonso et al. (2004) further demonstrated that a recombinant SHRV lacking the whole NV gene ORF was not different from wild-type SHRV in replication *in vitro* and *in vivo* pathogenesis. However, no recombinant SHRVs were generated by deletion of the whole NV gene ORF plus the G/NV gene junction. Biacchesi et al. (2000) observed that the growth of recombinant IHNVs lacking the NV gene ORF or carrying the GFP gene ORF (or the chloramphenicol acetyltransferase gene ORF) instead of the NV gene ORF was much slower than that of recombinant IHNV-wild or wild-type IHNV. Later, Thoulouze et al. (2004) reported that a rIHNV lacking the NV gene ORF showed severely retarded growth in EPC cells and highly reduced virulence in rainbow trout. Similarly, a recombinant VHSV lacking whole NV gene including the gene start and gene end signals showed severely lower (about 10^5× lower) viral titers in EPC cell cultures compared to recombinant VHSV-

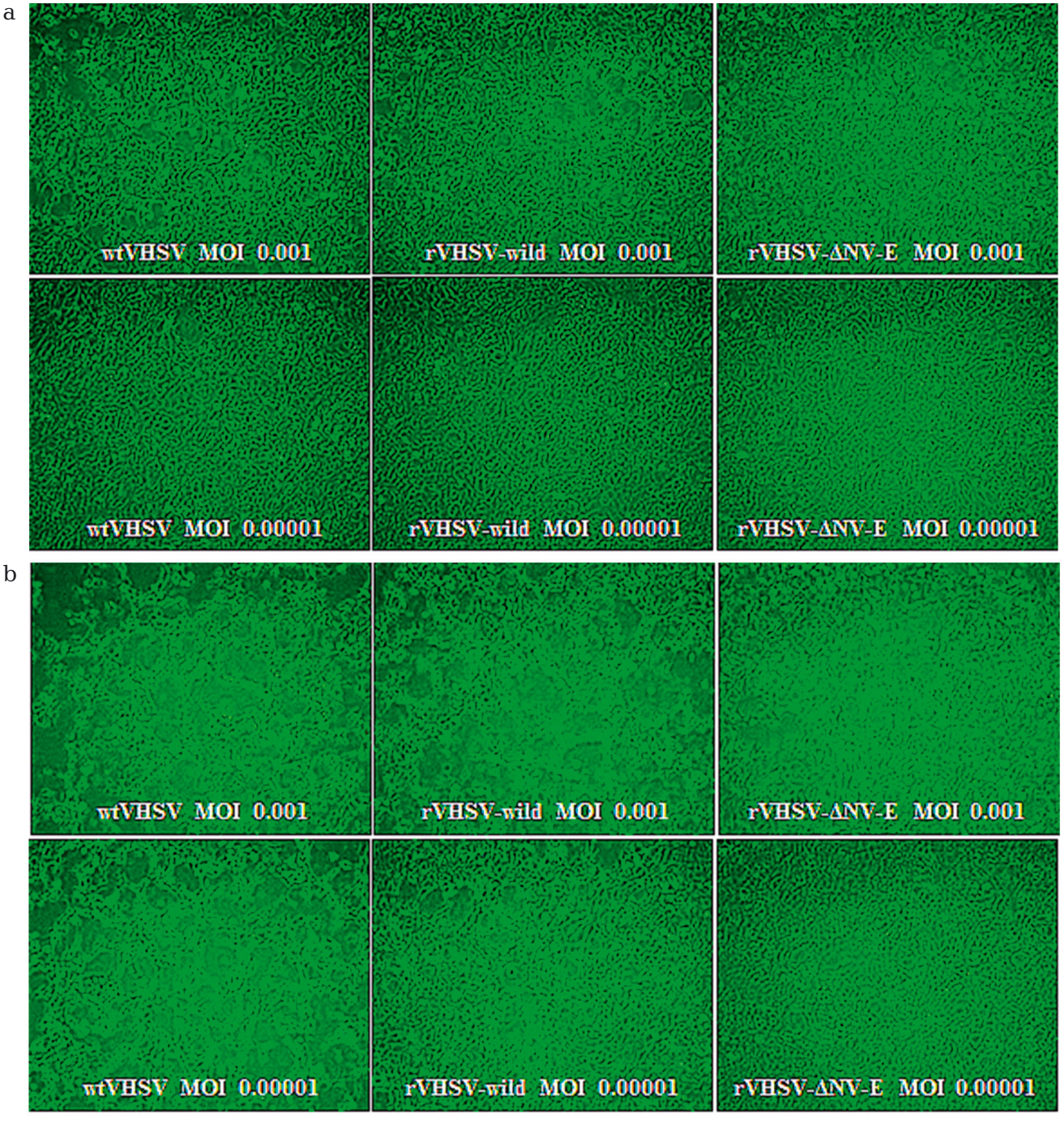

Fig. 7. CPE induced by infection of (a) naïve EPC cells or (b) the NV protein-expressing EPC cells with wild-type VHSV (wtVHSV), rVHSV-wild, and rVHSV-ΔNV-EGFP (rVHSV-ΔNV-E) at MOI 0.001 or 0.00001 at 3 d post-infection

wild or wild-type VHSV (Biacchesi et al. 2010). Ammayappan et al. (2010b) also reported that the titer of *in vitro* replicated rVHSV carrying the EGFP ORF instead of NV ORF was 2 orders of magnitude lower than that of other rVHSVs that had an intact NV gene, and a rVHSV lacking the NV gene was highly attenuated in yellow perch. In the present study, the *in vitro* replication cycle and the final titers of the rVHSV-wild were distinctly lower than those of the wild-type VHSV, suggesting that artificially replaced nucleotides in the genome of rVHSV-wild might affect the growth of the virus. The differences

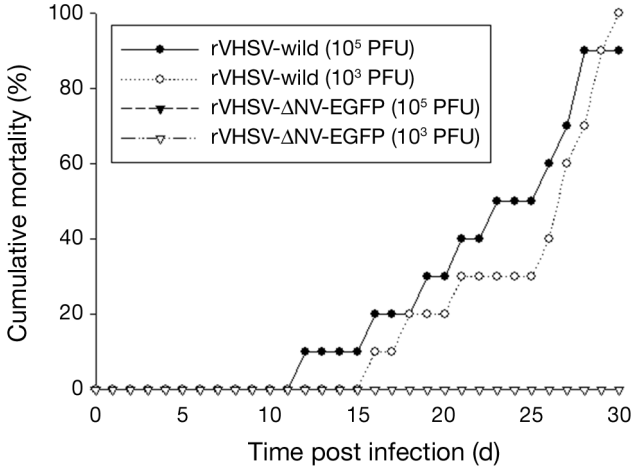

Fig. 8. *In vivo* pathogenesis of recombinant VHSVs. Olive flounder *Paralichthys olivaceus* were infected by intramuscular injection with the rVHSV-wild or rVHSV-ΔNV-EGFP at 2 doses (10^3 and 10^5 plaque-forming units [PFU] per fish). Cumulative mortalities were recorded daily for 30 d post-injection

of the rVHSV-wild genome sequence from the wild-type VHSV were the presence of *Age*I and *Sac*II restriction enzyme sites just before and after the G gene ORF, respectively, and *Cla*I and *Nar*I sites just before and after the NV gene ORF, respectively, by replacement of the original nucleotides. The replaced nucleotides just before the start codon of either the G gene or the NV gene are considered to be the cause of the slower growth of the rVHSV-wild. In the present study, growth of the rVHSV-ΔNV-EGFP was slightly lower than that of the rVHSV-wild when cells were infected with MOI 1.0, but became greatly lower when cells were infected with MOI 0.00001. These results suggest that the NV gene might play an important role in VHSV replication through interactions with host cell responses, and the lower replication ability of the rVHSV-wild compared to the wild-type VHSV might be caused by replaced nucleotides just before the NV gene ORF rather than the G gene ORF. In the present study, the role of the NV gene in VHSV replication was further confirmed by the increased proliferation of the recombinant VHSVs by supplementation of the NV protein in EPC cells. Interestingly, the time for CPE observation in wild-type VHSV was also reduced by expression of NV protein in EPC cells, suggesting the positive role of NV protein in VHSV replication efficiency.

In the present study, pathogenesis of the rVHSV-wild in olive flounder differed from virulence of the wild-type VHSV in that the rVHSV-wild produced much slower progressing mortalities (Kim & Kim 2011). However, the rVHSV-ΔNV-EGFP was highly attenuated in olive flounder, which was also previously demonstrated (Kim & Kim 2011). These results suggest that the NV protein of VHSV plays an important role in viral pathogenesis, which was similar to the results of Ammayappan et al. (2010b). Most negative-strand RNA viruses have the ability to antagonize the interferon responses in infected cells (García-Sastre 2001), and the nonstructural protein NS1 in influenza viruses is a well-known interferon antagonist (Talon et al. 2000, Hale et al. 2008). Further research on the role of the NV protein in modulation of host immune responses should be conducted.

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