ORIGINAL ARTICLE

A Reverse Genetics System for the Great Lakes Strain of Viral Hemorrhagic Septicemia Virus: the NV Gene is Required for Pathogenicity

Arun Ammayappan · Gael Kurath · Tarin M. Thompson · Vikram N. Vakharia

Abstract Viral hemorrhagic septicemia virus (VHSV), belonging to the genus *Novirhabdovirus* in the family of *Rhabdoviridae*, causes a highly contagious disease of fresh and saltwater fish worldwide. Recently, a novel genotype of VHSV, designated IVb, has invaded the Great Lakes in North America, causing large-scale epidemics in wild fish. An efficient reverse genetics system was developed to generate a recombinant VHSV of genotype IVb from cloned cDNA. The recombinant VHSV (rVHSV) was comparable to the parental wild-type strain both in vitro and in vivo, causing high mortality in yellow perch (*Perca flavescens*). A modified recombinant VHSV was generated in which the NV gene was substituted with an enhanced green fluorescent protein gene (rVHSV-ΔNV-EGFP), and another recombinant was made by inserting the EGFP gene into the full-length viral clone between the P and M genes (rVHSV-EGFP). The in vitro replication kinetics of rVHSV-EGFP was similar to rVHSV; however, the rVHSV-ΔNV-EGFP grew 2 logs lower. In yellow perch challenges, wtVHSV and rVHSV induced 82–100% cumulative per cent mortality (CPM), respectively, whereas rVHSV-EGFP produced 62% CPM and rVHSV-ΔNV-EGFP caused only 15% CPM. No reversion of mutation was detected in the recovered viruses and the recombinant viruses stably maintained the foreign gene after several passages. These results indicate that the NV gene of VHSV is not essential for viral replication in vitro and in vivo, but it plays an important role in viral replication efficiency and pathogenicity. This system will facilitate studies of VHSV replication, virulence, and production of viral vectored vaccines.

Keywords Viral hemorrhagic septicemia virus · Reverse genetics · Synthetic biology · Pathogenesis · Yellow perch · Non-virion gene

Introduction

Viral hemorrhagic septicemia (VHS) was first described as a disease of freshwater-reared rainbow trout in Europe in 1938, and it has continued to cause severe losses in the European trout farming industry since the 1950s (Hoffmann et al. 2005; Skall et al. 2005; Smail 1999; Wolf 1988). Viral hemorrhagic septicemia virus (VHSV) causes acute systemic disease and hemorrhagic lesions among juvenile rainbow trout, with mortality rates as high as 90%. In the late 1980s, VHSV was isolated for the first time in western North America from asymptomatic adult Coho salmon and Pacific cod showing extensive hemorrhagic lesions (Meyers and Winton 1995). Surveys of wild fish in the North-east Pacific showed an extensive reservoir of VHSV in many marine species, and VHS epidemics have been documented in wild Pacific herring and sardines (Hedrick et al. 2003; Meyers and Winton 1995). VHSV has a broad host range and has been isolated from at least 48 fish species from different parts of the world, including North America, Asia, and Europe (Skall et al. 2005). Since 2005, VHSV has emerged in the Great Lakes region of the USA and Canada, where it has caused significant mortality in muskellunge,

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yellow perch, freshwater drum, round gobies, gizzard shad, and infected 28 different fish species (Elsayed et al. 2006; Groocock et al. 2007; Lumsden et al. 2007; Kane-Sutton et al. 2009). The invasion of the Great Lakes ecosystems by VHSV is a major event resulting in large-scale multi-species epidemics and strong regulatory restrictions on aquaculture industries. As part of the research response, yellow perch are being developed as an appropriate new laboratory model for study of Great Lakes VHSV. Sequence analysis of the glycoprotein gene of the Great Lakes VHSV isolate MI03 indicates that it is a new genotype, designated IVb, which is different from previous European and western American VHSV isolates (Elsayed et al. 2006). All VHSV isolates from the Great Lakes region analyzed to date are members of the genotype IVb sub-group (Groocock et al. 2007; Lumsden et al. 2007).

VHSV is a non-segmented, negative-stranded RNA virus and a member of the genus *Novirhabdovirus* in the family of *Rhabdoviridae* (Tordo et al. 2004). The genome of VHSV is composed of approximately 11-kb of single-stranded RNA, which contains six genes (Schütze et al. 1999) that are located along the genome in the 3′–5′ order: 3′-N-P-M-G-NV-L-5′, nucleocapsid protein (N), polymerase-associated phosphoprotein (P), matrix protein (M), surface glycoprotein (G), a unique non-virion protein (NV), and virus polymerase (L) (Basurco and Benmansour 1995; Schütze et al. 1999).

Reverse genetics is a powerful tool to investigate gene functions in RNA viruses. A method of recovering negative-strand RNA viruses from the full-length cDNA clones was first developed for rabies virus by Schnell et al. (1994). This method involved expressing a full-length positive-strand (anti-genomic) RNA copy of the virus genome under the control of a T7 RNA polymerase (T7 RNAP) promoter, along with the viral N, P, and L proteins. In these cells, T7 RNA polymerase was supplied by the recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst et al. 1986). After this recovery, many negative-sense RNA viruses have been recovered using similar technique, namely vesicular stomatitis virus (Lawson et al. 1995; Whelan et al. 1995), human respiratory syncytial virus (Collins et al. 1995), and two fish novirhabdoviruses, snakehead rhabdovirus (SHRV; Johnson et al. 2000), and infectious hematopoietic necrosis virus (IHNV; Biacchesi et al. 2000). All of these systems are based on expression of viral genomes and genes using T7 RNA polymerase provided by the helper vaccinia virus.

To date, a reverse genetics system for VHSV is not available. Therefore, we constructed a full-length cDNA clone of a Great Lakes strain of VHSV and assembled in an expression plasmid under the control of a cytomegalovirus (CMV) promoter. Transfection of this full-length plasmid along with supporting plasmids (N, P, NV, and L) into *Epithelioma papulosum cyprini* (EPC) cells resulted in the recovery of a viable VHSV. Here, we report the generation of a recombinant VHSV of genotype IVb entirely from cDNA plasmids without the helper vaccinia virus. To the best of our knowledge, this is the first report of recovery of the Great Lakes VHSV from cloned cDNA.

A defining characteristic of Novirhabdovirus genomes is the presence of a unique NV gene, which has no significant similarity with any other viral gene. The role of the NV was examined previously using deletion mutants of IHNV and SHRV generated by reverse genetics systems, but their results differed substantially both in vitro and in vivo (Alonso et al. 2004; Thoulouze et al. 2004). Therefore, to study the function of the NV gene of VHSV, we generated a recombinant VHSV in which the NV gene was substituted with a reporter EGFP gene. We also generated a recombinant VHSV by inserting an EGFP gene between the P and M genes to explore the vector potential of VHSV. In this report, we compare the biological characteristics of the recovered VHSV and recombinant VHSVs lacking the NV gene and/or expressing the EGFP gene in cell culture, and evaluate their pathological functions in a natural host species, yellow perch (*Perca flavescens*).

Materials and Methods

Virus and Cells

The Great Lakes MI03 strain of VHSV, isolated in 2003 from a diseased muskellunge (Elsayed et al. 2006), was propagated in EPC cells at 15°C (Fijan et al. 1983). The cells were grown at 25°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mML-glutamine. For preparation of recombinant virus stocks, confluent EPC cells grown at 25°C were infected at a multiplicity of infection (MOI) of 0.001 in MEM with 5% FBS. After 1 h of adsorption at 14°C, the inoculum was removed, and the cells were incubated at 14°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 4–5 days after post-infection, clarified and stored at −80°C for further processing.

RNA Extraction

Viral RNA used for initial cloning of the wild-type VHSV MI03 strain was extracted from viral stock using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For all subsequent work, viral RNA was extracted from infected cell culture supernatant using Qiagen RNeasy kit according to manufacturer's instructions and stored at −20°C for further processing. The complete nucleotide sequence of the Great Lakes strain of VHSV was determined recently in our

laboratory (Ammayappan and Vakharia 2009) and is available in the GenBank (accession number: GQ385941).

Construction of a Full-Length cDNA Clone of VHSV

The oligonucleotide primers used in the construction of various clones and their location are listed in Table 1. A full-length cDNA copy of the VHSV MI03 RNA genome was constructed by assembling six overlapping cDNA fragments generated through reverse transcription polymerase chain reaction (RT-PCR) by standard cloning techniques, as described (Ammayappan and Vakharia 2009). The clones were ligated serially by natural or artificially created unique restriction sites (Fig. 1). The cDNA sequences of hammerhead ribozyme (HHRz) cDNA (5′CTGATGAGTCCGT GAGGACGAAACTATAGGAAAGGAATTCCTA TAGTC3′) and hepatitis delta virus ribozyme (HdvRz) (5′ GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCC GACCTGGGCATCCGAAGGAGGACAGACGTC CACTCGGATGGCTAAGGGAGAGCCA 3′) were fused with fragment 1 (F1) and fragment 6 (F6), respectively, by overlapping PCRs. A KpnI restriction enzyme site present in the wild-type clone (at nucleotide position 10979) was destroyed by PCR-directed mutagenesis (silent mutation) for the ease of cloning (Fig. 1).

The subcloning of fragments was initially carried out in pSmart® vector (Lucigen Corp., Middleton, WI, USA), which was modified (TopoSmart) such that it would contain multiple cloning sites of TOPO® TA vector. From these subclones, a full-length clone of VHSV was assembled in the pCI vector (Promega Corp., Madison, WI, USA) as follows. The F1 fragment was digested with SpeI and NsiI and transferred to the TopoSmart vector. A portion of F2 was amplified with 2 F and PvuIIKpnR primers (this creates a PvuII site) and then ligated with F1 after digesting with NsiI and KpnI. This construct was digested with SpeI and KpnI restriction enzymes and was transferred to pCI vector between NheI and KpnI. The F2 and F3 fragments were ligated in vitro and then amplified with PvuIIF and SacIIKpnR primers. The amplified product was added to F1 and partial F2, which were already in pCI vector, by inserting between the PvuII and KpnI restriction sites. A small fragment from F3 was amplified with SacIIF and 5.3R primers and added to the above construct in pCI between SacII and KpnI sites to create a clone "A". The F5 was divided into two parts because of the existence of two NcoI sites. The 3′ part of F5 was ligated with F6 by digesting with SpeI and BsrBI in a separate TopoSmart vector to create a clone "C". The 5′ part of F5 was ligated with F4 by digesting with BsmBI and NcoI enzymes. The KpnI and BsmBI fragment from F3 was ligated to F4 and 3′ part of F5 by digesting with respective enzymes, which generated a clone "B". Then clones "B" and "C" were

ligated by digesting with SpeI restriction enzyme and to form a clone "BC". This clone was fused with clone "A" by digesting with KpnI and NotI restriction sites. Finally, a full-length plasmid of pVHSV was obtained (Fig. 1). Plasmid DNA was completely sequenced for its integrity using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). This plasmid is under the control of a CMV promoter. The pCI vector also has a T7 promoter downstream of CMV promoter, just before the multiple cloning sites. Although all the plasmids used in this study had a T7 promoter, it was not utilized for any purpose and it is not mentioned in the text.

Construction of Supporting Plasmids

Using the full-length clone of pVHSV as a template, the open reading frames (ORFs) of N (1,215 bp), P (669 bp), NV (369 bp), and L (5,955 bp) genes were amplified by PCR using respective primer pairs (Table 1). A Kozak consensus sequence was incorporated in front of the start codon of each ORF (Kozak, 1987). The N, P and NV ORFs were cloned between EcoRI and KpnI restriction sites; whereas the L ORF was cloned between NheI and NotI restriction sites of the pCI vector by digesting with appropriate restriction enzymes. The DNA from the resulting pN, pP, pNV, and pL support plasmids was sequenced using an automated DNA sequencer, as described earlier. All these plasmids are under the control of a CMV.

Construction of pVHSV-ΔNV and pVHSV-EGFP Plasmids

The NV gene deletion from the VHSV full-length clone (pVHSV-ΔNV) was made by replacing the NV ORF with the ORF of EGFP, using respective primers (Table 1). The EGFP ORF was amplified from the pIRES2-EGFP (Promega Corp., Madison, WI, USA) plasmid using EGFP-specific primers. Initially, three fragments were amplified separately (primers used to amplify the indicated fragments are in parentheses): (1) sequences between the stop codon of the G gene and the start codon of the NV gene (SacIIF and dNV-VHSstart-EGFPR); (2) sequences between the stop codon of the NV gene and part of the coding region of the L gene (dNV-VHSend-EGFPF and 5.3R) and; (3) EGFP ORF (dNV-VHSstart-EGFPR and dNV-VHSend-EGFPR). These three fragments were fused with each other by two-step overlapping PCR, and then amplified and used to replace the region between SacII and KpnI restriction sites in the full-length cDNA clone. This replacement created an NV deleted-EGFP substituted full-length cDNA clone of VHSV (pVHSV-ΔNV-EGFP), as shown in Fig. 2.

For the insertion of an EGFP ORF before the M gene in the full-length pVHSV, first an NheI restriction site was introduced ahead of the M ORF by PCR mutagenesis

Table 1 Oligonucleotides used for cloning of the VSHV genome and construction of plasmids for virus recovery

Primers	Sequences[a,b]	Nucleotide no.[c]
VHSV genome primers		
VHSV 1 F	GTATCATAAAATATGATGAGT	1-21
VHSV 1R	CAACTTGAACTTCTTCATGGC	2028-2008
VHSV 2 F	AAGAAGACCGACAACATACTCT	1858-1879
VHSV 2R	GACGAAACTTTGAGAGGAGAAA	3993-3972
VHSV 3 F	ATCTCATTACCAACATGGCTCAAA	3892-3915
VHSV 3R	TTGTTCGCTTCTCCCCTAATTGT	5932-5910
VHSV 4 F	TGCCATAGACCTACTCAAGTTAT	5814-5835
VHSV 4R	CTGATCCATGGTGGCTATGTGAT	8042-8020
VHSV 5 F	AGATGATTGTCTCCACCATGAA	7846-7867
VHSV 5R	GAGATCCGCTCTCGTTCATCAA	10027-10006
VHSV 6 F	GACAAGAAAGCTGGGAAGAGA	9787-9807
VHSV 6R	GTGTATAGAAAATAATACATACCA	11183-11162
VHSV HHF1	AGTCTAGAACTAGTTTTTATGATACCTGATGAGTCCGTGAGGACGAAA	3′end
VHSV HHF2	AGGACGAAACTATAGGAAAGGAATTCCTATAGTCGTATCATAAAAGATG	3′end
VHSV HDVR	ATTATTTTCTATACACGGGTCGGCATGGCATC	5′end
VSHV HDVF	ATTATTTTCTATACACGGGTCGGCATGGCATC	5′end
Supporting plasmid primers		
VHSV NEcoF	CG*GAATTC*T<u>GCCACCATGG</u>AAGGAGGAATCCGTG	175-191
VHSV NKpnR	CC*GGTACC*TTAATCAGAGTCCTCGGGGTAGT	135-1331
VHSV PEcoF	CG*GAATTC*T<u>GCCACCATGA</u>CTGATATTGAGATGAG	1466-1481
VHSV PKPnR	CC*GGTACC*CTACTCTAACTTGTCCAACTCCGC	2158-2141
VHSV NVEcoF	CG*GAATTC*TGCCACCATGACGATCCAGCCGGCAC	4596-4616
VHSV NVKpnR	CC*GGTACC*TCATGAAGGAGACTCAGAGC	4931-4910
VHSV LNheF	CTA*GCTAGC*C<u>ACCATGG</u>AAATGTTTGAGCTAGA	5017-5037
VHSV LNotR	AA*GCGGCCGC*TCATTGCTCTCCAAATGGAA	10977-10962
Mutagenesis primers		
VHSV NheF	CCTCAGACAAGCTAGCACAAAAAAC	
VHSV NheR	GTTTTTTGTGCTAGCTTGTCTGAGG	
PvuIIKpnR	ACCGGTACCTTCAGCTGGTTGTGTACACAA	
PvuIIF	TGTACACAACCAGCTGAATCGAGAATG	
SacIIKpnR	ACCGGTACCTTCCGCGGGTGTGTTCAGACCA	
SacIIF	TGAACACACCCGCGGAAACCACCATAG	
VHSV5.3R	CCTGGATTCGTGTAGAGTCAA	
VHSVdKpnF	CAAGGGGTATCTCGGTGCCCTGCT	
VHSVdKpnR	GCACCGAGATACCCCTTGAGATCCT	
EGFP primers		
dNV-VHSstart-EGFPR	CTTGCTCACCATCTTTCTGTCTCACAGG	
dNV-VHSstart-EGFPF	ACAGAAAGATGGTGAGCAAGGGCGA	
dNV-VHSend-EGFPR	GGGGAGGAGTTCACTTGTACAGCTCGTC	
dNV-VHSend-EGFPF	CAAGTGAACTCCTCCCCCTTCTC	
VHSV P-EGFP-MF	GGGCTAGCACAGCCACCATGGTGAGCAAGGGCGA	
VHSV P-EGFP-MR	TGTTGTCTCTACTTGTACAGCTCGTCCATGC	
VHSV EGFP EndP/MF	AGCTGTACAAGTAGAGACAACACAATACATC	

[a] Restriction sites are in italics

[b] Kozak context is underlined

[c] The positions where the primers bind (nucleotide numbers) are according to the published sequence of the VHSV MI03 genome (Ammayappan and Vakharia 2009)
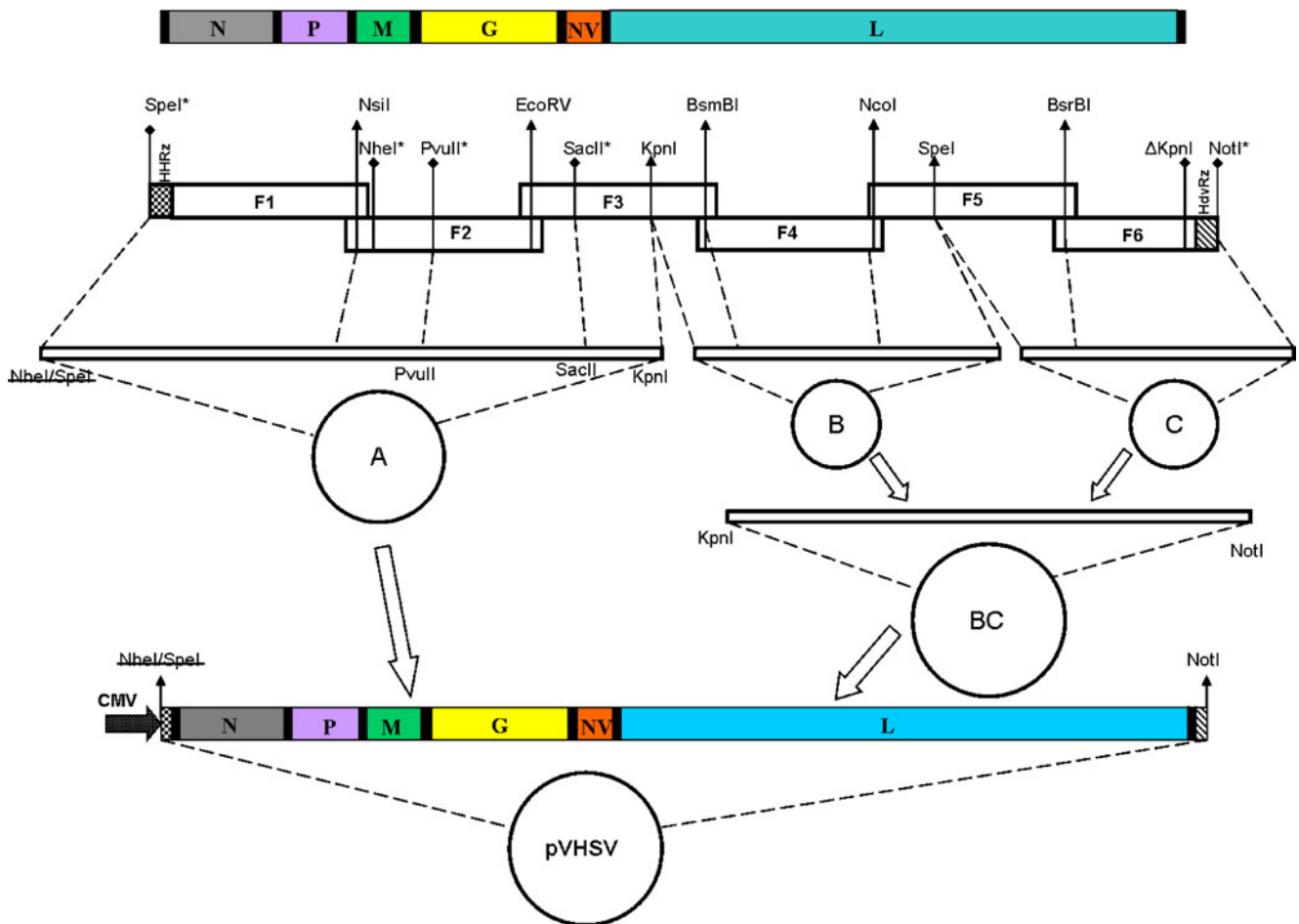
Fig. 1 Construction of a full-length cDNA clone of the VHSV MI03 genome. Six overlapping cDNA fragments covering the entire VHSV genome were assembled by ligation into a modified pSmart vector using the *SpeI*, *NsiI*, *EcoRV*, *BsmBI*, *NcoI*, *BsrBI*, and *NotI* restriction sites. Assembly was carried out in three steps; (1) cloning of six fragments (F) separately in a TOPO vector (see methods), addition of hammerhead ribozyme (HHRz) at the 5′-end of F1 and hepatitis delta ribozyme (HdvRz) at the 3′-end of F6; (2) construction of the A clone in the pCI vector and B and C clones in the pTOPOSmart vector (see "Materials and Methods"); (3) assembly of the full-length clone by ligating fragments from these three clones in the pCI vector. Restriction sites artificially created (*asterisk*) and naturally present in the genome are indicated. *CMV* cytomegalovirus immediate-early enhancer and promoter

(primers NheF, 5′- CCT CAG ACA <u>AGC TAG</u> CAC AAA AAA C-3′; NheR, 5′- GTT TTT TGT <u>GCT AGC</u> TTG TCT GAG G-3′) (nt position 2272–2277) (see Fig. 2). An additional fragment, comprised of the untranslated region between the P and M ORFs, was fused at the C-terminus of EGFP ORF by PCR. The *NheI* restriction sites were created by PCR at both ends of this cassette and it was inserted at the newly created *NheI* site in pVHSV to create a full-length clone of VHSV with an EGFP gene between the P and M genes (pVHSV-EGFP), as shown in Fig. 2.

DNA Transfection and Virus Recovery

DNA transfection was carried out according to the method described earlier (Ammayappan et al. 2010). Briefly, plasmids pVHSV or its derivatives (1 μg), pN (0.5 μg),

pP (0.2 μg), pL (0.2 μg), and pNV (0.15 μg) were diluted in 500ul μl Opti-MEM® medium (Invitrogen, Carlsbad, CA, USA). Next, Lipofectamine™ LTX reagent (Invitrogen, Carlsbad, CA, USA) was added, according to manufacturer's instructions, and incubated for 30 min at room temperature. The plasmid–Lipofectamine reaction mixture was added to the EPC monolayer in a six-well plate without replacing the growth medium. The transfection mixture was removed after 8 h of incubation at 28°C, and the transfected cells were washed and maintained in Eagle's MEM (ATCC, Manassas, VA, USA) containing 10% FBS at 14°C for 5 days. Cell monolayer was observed for the development of virus-induced CPE and the expression of EGFP was viewed by a fluorescent Axioplan® microscope at 405 nm (Carl Zeiss, Germany). After 5 days of incubation, the cells were submitted to three
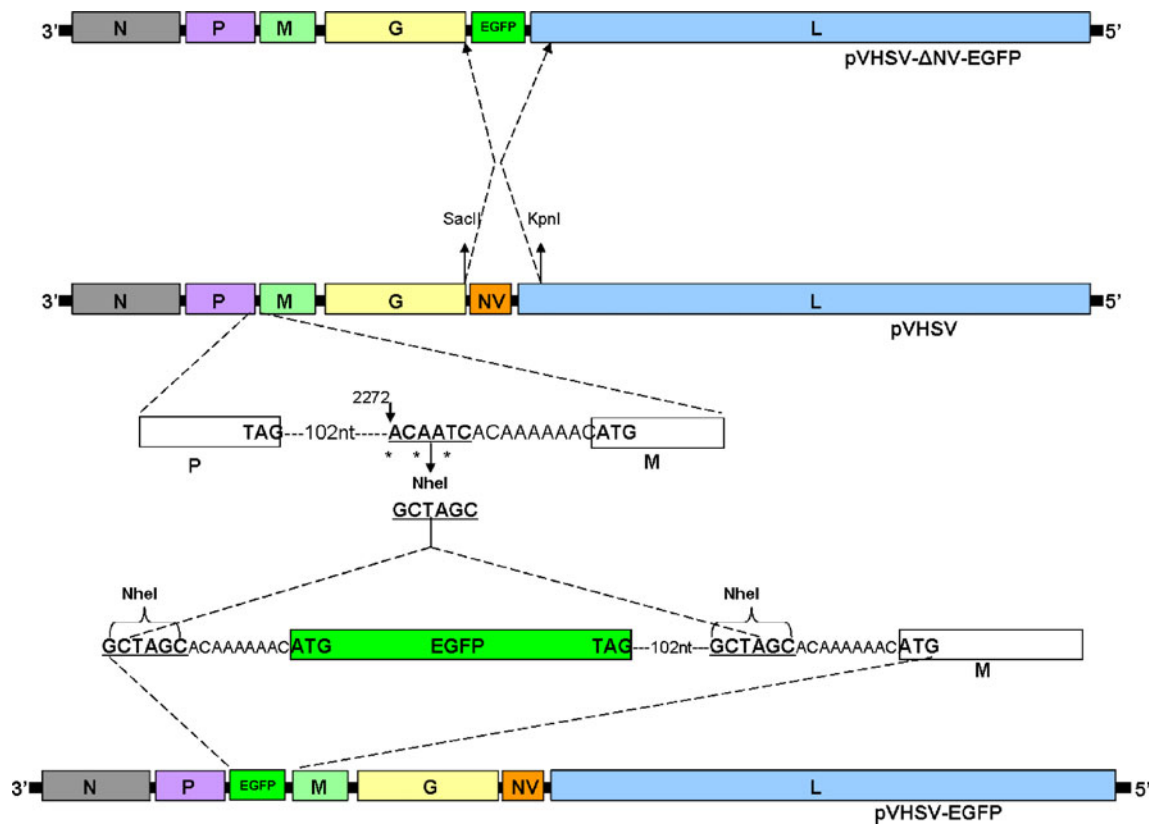
Fig. 2 Construction of pVHSV-NV and pVHSV-EGFP plasmids. The pVHSV-NV plasmid was constructed by replacing the VHSV NV ORF with an EGFP ORF. The pVHSV-EGFP plasmid was made by insertion of an EGFP ORF, fused with a copy of the untranslated region between the P and M ORFs at the 3′ end, into the pVHSV backbone at a newly created *NheI* restriction site

cycles of freeze thawing. Supernatant was clarified by centrifugation at 8,000×*g* in a microcentrifuge, and used to inoculate fresh cell monolayers in T-25 flasks at 14°C. The supernatant was harvested and clarified for further processing of the recombinant viruses.

Preparation of Virus Stocks and Plaque Assay

To prepare recombinant virus stocks, confluent EPC cells grown in T-75 flasks at 25°C were infected at a MOI of 0.001 in MEM with 5% FBS. After 1 h of adsorption at 14°C, the inoculum was removed, and the cells were incubated at 14°C until extensive CPE was observed. The supernatant was collected 4–5 days post-infection, clarified, and stored at −80°C. The titer of the virus was determined by plaque assay, as described previously (Burke and Mulcahy 1980). Confluent monolayers of EPC cells, grown in six-well plates, were infected with the virus present in the supernatant. The virus samples were diluted serially and each sample was titrated in duplicate wells. After 1 h of incubation at 14°C, the cells were washed once with phosphate-buffered saline (PBS) and overlaid with 0.75% methylcellulose (Difco) in Eagle

MEM containing 10% FBS. After 5 days of incubation at 14°C, the overlays were removed and the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing of the cells with distilled water, the plaques were counted and photographed. The titer of the (1) rVHSV-MI03 was 3.8×10^8 PFU/ml; (2) rVHSV-EGFP was 5.8×10^8 PFU/ml; and (3) rVHSV-ΔNV-EGFP was 3.6×10^7 PFU/ml.

RT-PCR and Confirmation of the Genetic Tags

RT-PCR was performed on RNA extracted from the recovered viruses to confirm the presence of artificially introduced genetic markers. Briefly, the viral RNA was extracted from partially purified virus obtained after ultracentrifugation (collected from a 26% sucrose cushion), using an RNeasy® Mini Kit (Qiagen). RT-PCR was performed to verify the presence of *NheI*, *PvuII*, and *SacII* or *KpnI* restriction sites that were artificially introduced or destroyed, respectively, during the cloning process. Restriction analysis of the PCR products was carried out on a 1% agarose gel. The obtained RT-PCR products were also

subjected to DNA sequencing to confirm the presence of artificially introduced genetic tags.

Viral Growth Curves and Plaque Characteristics in Cell Culture

To analyze the growth characteristics of the recombinant VHSVs, confluent EPC cells were infected with the wild-type parental MI03 strain (wtVHSV) or recombinant virus stocks at an MOI of 0.01 in individual T-25 flasks. Virus present in the infected cell culture supernatant was collected at different time intervals, clarified by centrifugation, and titrated on EPC cells by plaque assay, as described earlier (Burke and Mulcahy 1980).

Viral Challenge Studies in Fish

Fish were reared and handled under the guidelines provided by the *Guide for the Care and Use of Laboratory Animals* and the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Pathogen-free yellow perch were obtained from a captive broodstock developed by F. Binkowski and F. Goetz at the University of Wisconsin, Milwaukee, WATER Institute. Fish stocks were reared in the Western Fisheries Research Center wet lab using flow-through, sand-filtered, UV-irradiated lake water at 12°C. Fish were given feed pellets (BioOregon) every other day at 1% total fish weight. Fish challenge studies were conducted in the Aquatic Biosafety Level 3 laboratory of Western Fisheries Research Center. For viral challenge, triplicate groups of 15 juvenile yellow perch, average weight 3.4 g, were injected intraperitoneally (IP) with 50 µl of PBS containing 10^5 PFU of virus, for each of four treatments: wtVHSV strain MI03; rVHSV; rVHSV-ΔNV-EGFP; or rVHSV-EGFP. As a fifth treatment, mock-infected control groups were injected with PBS only. Experimental groups were held separately in 30 L tanks at 12°C and fish were monitored daily for 30 days. Fish that died during the challenge were collected daily and disease signs were recorded in dead fish before saving them at −80°C. From each virus-challenged treatment group (45 fish total), six fish that died were titered for virus by plaque assay (Batts and Winton 1989) to confirm the presence of virus as the likely cause of death. At the end of the experiment, six surviving fish were also titered for virus from each group where there were survivors. Virulence was assessed and compared between groups using the Statistical Package for the Social Sciences, version 11.5. Cox regression analysis was used to verify that there were no significant differences between triplicate groups within each treatment, and differences between treatment groups were analyzed using Kaplan–Meier estimation of

mortality curves followed by comparison with a log-rank test (Statistical Package for the Social Sciences, version 11.5).

Results

Construction of a Full-Length cDNA Clone and Supporting Plasmids of VHSV

To develop a reverse genetics system for VHSV, we assembled a full-length clone of the Great Lakes VHSV strain MI03 in the pCI vector from six overlapping cDNA clones, as depicted in Fig. 1. The complete genome sequence of this VHSV strain has been determined recently (Ammayappan and Vakharia 2009). Three unique restriction sites (*NheI*, *PvuII*, and *SacII*) were introduced in the intergenic regions and one restriction site (*KpnI*) was destroyed by silent mutation in the L gene of the full-length clone. To facilitate the virus rescue and efficiency, the cDNA was fused with self-cleaving ribozyme (HHRz and HdvRz) sequences at both ends. This allows precise cleavage at the termini of RNA and leaves authentic 3′- and 5′- viral RNA ends.

The ORF of all the supporting plasmids (pN, pP, pNV, and pL) were amplified by PCR from the full-length VHSV clone using specific primer pairs and cloned into the pCI expression vector. The pCI vector was selected to express all the proteins because it contains CMV enhancer/promoter sequence, which is known to be active in fish cells (Lopez et al. 2001). A Kozak consensus sequence was included in front of the start codon of each ORF to provide an optimal sequence context for protein translation (Kozak 1987).

Rescue of the Recombinant VHSV from Cloned cDNA

To generate viable rVHSV, transfection experiments were carried out on EPC cell monolayers in six-well plates at 25°C. Cells were transfected with a mixture of full-length cDNA and supporting plasmids of VHSV, and then shifted to 14°C after 8 h. When the CPE was evident at 72 h post-transfection, the supernatant was collected and analyzed for presence of the recombinant virus. To verify that the rescued virus was derived from cloned cDNA plasmids, genomic RNA was subjected to RT-PCR and restriction enzyme digestion. Figure 3 shows the analysis of RT-PCR products obtained from viral RNA of wtVHSV and rVHSV that were digested with different restriction enzymes. No PCR product was obtained when the RT was omitted from the reaction before PCR. Digestion of the RT-PCR products of rVHSV with respective restriction enzymes yielded expected fragments (lanes 5–8), whereas the products of the wtVHSV remained undigested, except *KpnI* (lanes 1–4). In addition, sequence
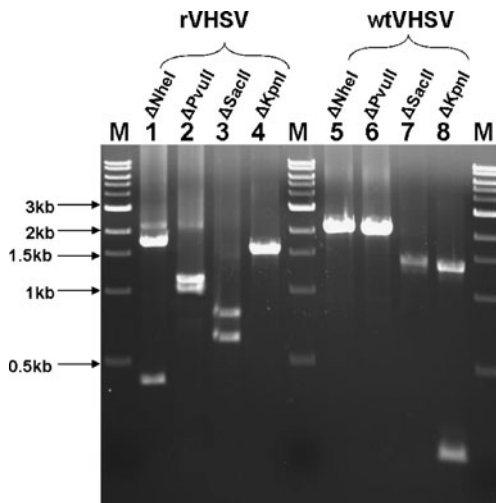
Fig. 3 Analysis of the RT-PCR products to identify the tagged sequences in the genome of recovered recombinant VHSV. Genomic RNA isolated from the wild-type VHSV MI03 and recombinant rVHSV was amplified by RT-PCR with specific primers covering the regions where restriction enzyme sites were introduced (*NheI*, *PvuII*, *Sac II*) or eliminated (*KpnI*) in the cloning process. Gel-purified RT-PCR products of the wild-type VHSV and rVHSV (approximate sizes 2,100; 2,100; 1,400; and 1,500) were digested with restriction enzymes *NheI* (*1* and *5*), *PvuII* (*2* and *6*), *SacII* (*3* and *7*), and *KpnI* (*4* and *8*). *M* molecular size markers, *1–4* rVHSV, *5–8* wild-type VHSV. All RT-PCR products of rVHSV were digested by the respective restriction enzymes, except for *Kpn1*. Conversely, the products from the wild-type remained undigested, except *KpnI*. The sizes of the products (approximate): *NheI* (1,721 and 415 bp), *PvuII* (1,113 and 1,023 bp), *SacII* (786 and 624 bp), *KpnI* (1,370 and 206 bp). The sizes of the molecular marker are indicated on the *left*

analysis of the amplified fragments confirmed the presence or absence of restriction sites that were introduced during cloning as genetic markers. These results demonstrate that the recovered virus was indeed derived from cloned cDNA plasmids.

Recovery of NV-Deficient Recombinant VHSV

To investigate the function of the NV gene in pathogenesis, we constructed a recombinant VHSV in which the ORF of NV was deleted and replaced it with EFGP ORF (Fig. 2). Transfection of EPC cells with a mixture of this modified cDNA and supporting plasmids of VHSV gave rise to a viable recombinant VHSV (rVHSV-ΔNV-EGFP). The CPE produced by this virus was clearly observed 72 h post-transfection.

Recovery of Recombinant VHSV Expressing Foreign Gene

To facilitate the insertion of foreign gene sequences into a full-length cDNA clone of VHSV, a unique *NheI* site was created by site-directed mutagenesis in the untranslated region between the P and M ORFs (Fig. 2). The EGFP

ORF was inserted at the *NheI* site to create a transcription cassette flanked by an additional copy of the untranslated sequences between the P and M ORFs. For virus recovery, the resultant modified pVHSV-EGFP plasmid was then cotransfected with the supporting plasmids. The CPE produced by this virus was clearly visible 72 h post-transfection (Fig. 4a). When transfected cells were examined under a fluorescent microscope, it gave a strong, positive green fluorescence signal, which could be easily visualized (Fig. 4b). RT-PCR was performed on the RNA extracted from the recovered virus and the obtained products were then subjected to DNA sequencing to confirm the presence of the EGFP ORF and flanking regulatory sequences. We also observed that rVHSV-EGFP stably expressed EGFP for at least ten serial passages in EPC cells. These results suggest that rVHSV could be used as a vector to express foreign genes.

In Vitro Growth Characteristics of Recombinant Viruses

Figure 5 depicts the growth curves of wtVHSV, rVHSV, rVHSV-ΔNV-EGFP, and rVHSV-EGFP to indicate the replication efficiency of these viruses in cell culture. The recombinant rVHSV grew at the same rate as wtVHSV for the first 36 h, and then leveled off from 48 to 96 h at a slightly lower titer ($\approx$0.5 logs) than the wtVHSV. After a lag period between 36 and 60 h, the rVHSV-EGFP virus reached a final titer similar to that of rVHSV. Thus wtVHSV, rVHSV, and rVHSV-EGFP, all replicated efficiently and grew to a high titer (10^7–10^8 PFU/ml) by 72 h post-infection. In contrast, rVHSV-ΔNV-EGFP replicated as well as the other viruses for the first 24 h, but then leveled off at a titer that was 2 logs lower than rest of the recombinant VHSVs. When rVHSV-ΔNV-EGFP infected cells were examined under a fluorescent microscope at 405 nm, it gave a very weak but positive green fluorescence signal (data not shown), suggesting that NV gene is not required for replication in cell culture.

To characterize the recombinant virus plaque phenotypes, wtVHSV, rVHSV, rVHSV-ΔNV-EGFP, and rVHSV-EGFP were subjected to plaque assays in EPC cells and analyzed 7 days post-infection. Although visible plaques were produced as early as 40 h post-infection, plaques produced by the wtVHSV and rVHSV were larger in size than those produced by rVHSV-ΔNV-EGFP and rVHSV-EGFP.

Pathogenicity Studies in Fish

To assess the pathogenicity and virulence phenotypes of wtVHSV and recombinant VHSVs in vivo, juvenile yellow perch were challenged by IP injection with equal amounts (1×10^5 PFUs per fish) of wtVHSV, rVHSV, rVHSV-ΔNV-EGFP, or rVHSV-EGFP. Mortalities were recorded daily for
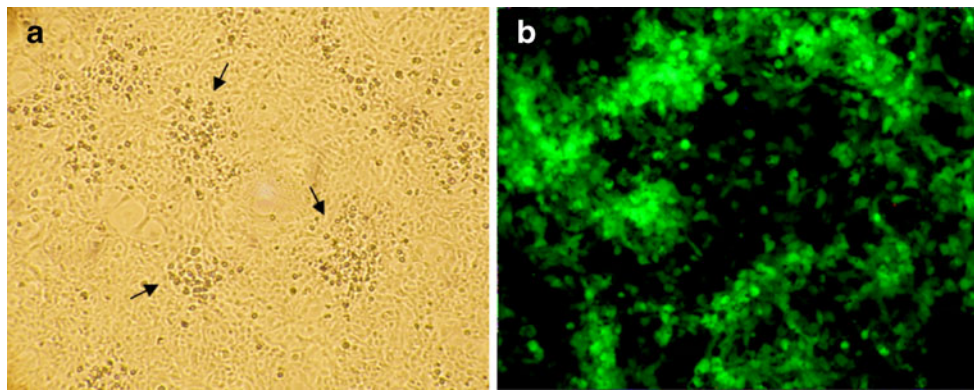
Fig. 4 Cytopathic effect induced by the recombinant VHSV in EPC cells. EPC cells were infected with recombinant VHSV expressing EGFP (rVHSV-EGFP) **a** virus-induced cytopathic effect in EPC cells 72 h post-infection; rounding of the cells and foci of dead cells (black arrows) in infected EPC monolayer. **b** Expression of the EGFP gene in EPC cells 48 h post-infection; cells infected with rVHSV-EGFP yielded green fluorescence when examined under a fluorescent microscope at 405 nm

30 days after virus challenge. As shown in Fig. 6, wtVHSV and rVHSV induced 82–100% average cumulative percent mortality (CPM), respectively, indicating that the virus generated by reverse genetics is fully virulent and successfully mimics the phenotype of the parental wt strain. The wtVHSV exhibited somewhat lower mortality than rVHSV, probably due to loss of titer during storage. The rVHSV-EGFP virus caused 62% CPM, indicating that it is also a pathogenic virus, but insertion of the foreign gene has reduced the level of virulence relative to rVHSV. The rVHSV-ΔNV-EGFP virus induced only 15% CPM, demonstrating that the NV-deficient VHSV is highly attenuated relative to rVHSV in yellow perch. Sequence analysis of
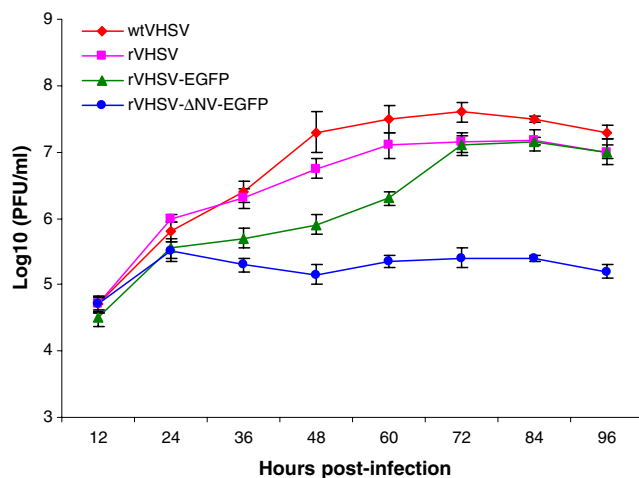
the recovered viruses from these fish revealed no reversion of the mutations and the recombinant viruses stably maintained the foreign gene after passage in fish. Among fish that died in each virus-infected group, those titered for virus were all positive with high titers ranging from 10^5 to 10^7 PFU/g. Among survivors titered for virus at the end of the experiment, there was no virus in fish from the mock-infected control group (0/6 fish positive), but virus was detected in fish from the virus-infected groups as follows: wtVHSV, 5/6 fish positive with titers 10^2–10^6 PFU/g; rVHSV-EGFP, 3/6 fish positive with lower titers 10^2–10^3; rVHSV-ΔNV-EGFP, 2/6 fish positive with titers 10^3–10^4. There were no survivors to titer in the rVHSV group.

Clinical disease signs typical of VHS were observed on nearly all fish that died during this challenge study (Fig. 7 and Table 2). The most common signs were exophthalmia (with or without hemorrhage) in 70–89% of the dead fish, and hemorrhage at the base of the pectoral fins in 75–86%. External hemorrhages on the top of the head were also commonly observed at 42–71%, and external hemorrhages were visible on the ventral, dorsal, and lateral surfaces of 18–86% of the fish. Slightly less common were hemorrhages at the vent in 14–36%, and around the jaw and/or gills in 11–43%. Importantly, there were no differences in frequency of various disease signs in fish that died from the different treatment groups. Thus, the small number of fish that died after challenge with the rVHSV-ΔNV-EGFP virus had disease signs equivalent to those that died after exposure to all the other viruses.

Discussion

In this report, we describe for the first time generation of a recombinant VHSV entirely from cloned cDNA without the helper vaccinia virus. This system is very efficient and

Fig. 5 Replication kinetics of the recombinant VHSVs. EPC cells were infected at an MOI of 0.01 with the wild-type VHSV (*filled diamond*), recombinant VHSV (rVHSV; *filled square*), NV-deleted VHSV (rVHSV-ΔNV-EGFP; *asterisk*) or recombinant VHSV expressing EGFP (rVHSV-EGFP; *filled triangle*), harvested at the indicated time points, and virus titers were determined by plaque assay. Average titers and standard deviation (*error bars*) from three independent experiments are shown

Fig. 6 Percent cumulative mortality caused by wild-type VHSV and recombinant VHSVs in yellow perch. Juvenile yellow perch were challenged by intraperitoneal injection with 10^5 PFUs of wtVHSV, rVHSV, rVHSV-ΔNV-EGFP, rVHSV-EGFP, or mock-infected with PBS. Mortalities were recorded daily for 30 days and are expressed as the average CPM for triplicate groups of 15 fish
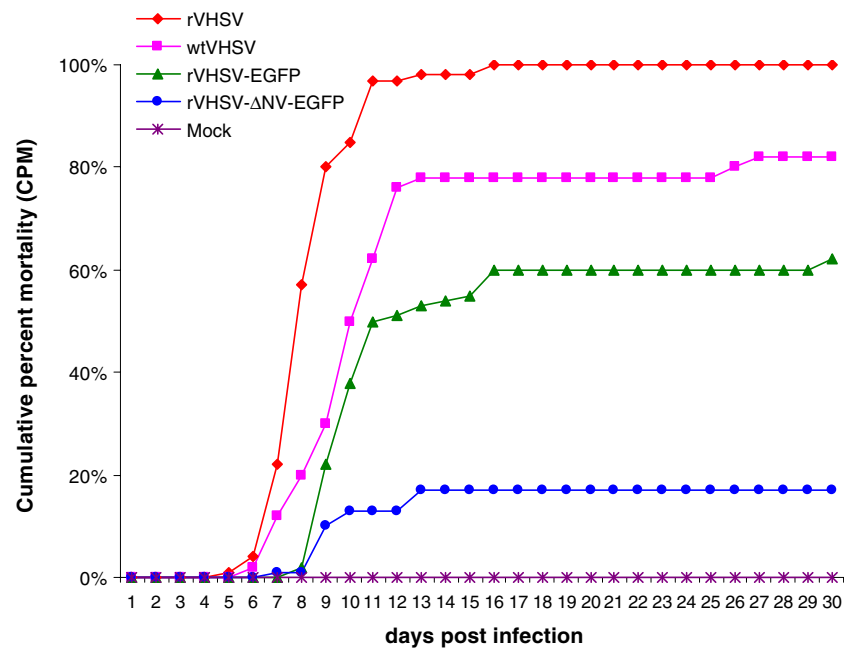
convenient for recovery of Novirhabdoviruses, which does not require the use of maintaining stable cell lines expressing T7RNA polymerase (T7RNAP' Alonso et al. 2004). The reverse genetics systems previously described for other Novirhabdobviruses, IHNV (Biacchesi et al. 2000) and SHRV (Johnson et al. 2000), are based on the recombinant vaccinia virus expressing T7RNAP. In this study, recombinant VHSV of the Great Lakes strain was

Fig. 7 Disease signs in yellow perch that died after infection with recombinant VHSV viruses. **a** rVHSV-EGFP, 11 days post-infection, arrow indicates severe exophthalmia with ocular hemorrhage; **b** rVHSV-EGFP, 11 days post-infection, arrows indicate hemorrhage at the base of the pectoral fin and ventral hemorrhages. Overall disease signs did not vary between treatment groups

generated after transfection of EPC cells with CMV-driven plasmids allowing simultaneous expression of an antigenomic copy of the VHSV RNA, and N, P, NV, and L proteins.

The full-length recombinant virus recovered here, rVHSV, was equivalent by all measures tested to the parental virus strain from which it was cloned. In vitro measures included replication kinetics, final stock titer, and plaque size during growth in cell culture. Pathogenicity and virulence were tested in vivo using a natural host species, yellow perch, which has experienced large-scale mortality events in the Great Lakes due to VHSV. Challenge of yellow perch with rVHSV resulted in high virulence and clinical disease signs indistinguishable from the parental wtVHSV strain. Thus, this recombinant comprises an extremely useful tool for many future investigations of the unique VHSV genotype that has invaded the Great Lakes.

In addition to its value for studying the field emergence of Great Lakes VHSV, many nonsegmented negative-strand RNA viruses have been proven to be capable of functioning as vectors. The feasibility of this approach was originally demonstrated by introducing reporter genes into the genomes of mammalian and avian RNA viruses, including rabies virus, vesicular stomatitis virus, respiratory syncytial virus, and Newcastle disease virus (Bukreyev et al. 1996; Mebatsion et al. 1996; Nakaya et al. 2001; Schnell et al. 1996). The modular organization of rhabdovirus genomes allows easy manipulation for the expression of foreign genes. In addition, a major advantage of using nonsegmented negative-stranded RNA viruses for the expression of foreign genes is that they stably maintain foreign genes for several passages (Mebatsion et al. 1996; Schnell
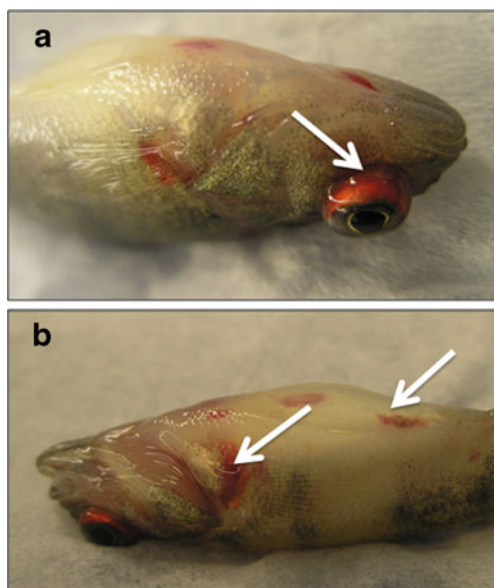
Table 2 Frequency of visible external disease signs in yellow perch that died after challenge with wild-type or recombinant VHSVs

External disease sign	wtVHSV MI03 (%)	rVHSV (%)	rVHSV-EGFP (%)	rVHSV-ΔNV-EGFP (%)
Exophthalmia	70	89	89	86
Pectoral fin base hemorrhage	86	80	75	86
Top of head hemorrhage	70	42	61	71
Ventral surface hemorrhage	65	56	68	86
Dorsal surface hemorrhage	30	29	29	57
Lateral surface hemorrhage	46	58	18	43
Hemorrhage at vent	30	36	25	14
Hemorrhage at jaw/gills	11	24	14	43
No Signs	0	2	0	0

Data is for fish that died in all replicate tanks within each treatment group. Due to variation in mortality levels (see Fig. 7); the number of dead fish assessed was 37 for wtVHSV MI03, 45 for rVHSV, 28 for rVHSV-EGFP, and 7 for rVHSV-ΔNV-EGFP

et al. 1996). It has been demonstrated that IHNV and SHRV could be used as vectors to express foreign genes (Alonso et al. 2004; Biacchesi et al. 2000). To demonstrate that recombinant VHSV could be used as a vector, we expressed the EGFP as a foreign gene inserted between the P and M genes. The recombinant VHSV expressing EGFP was recovered successfully and rVHSV-EGFP was very stable, maintaining the foreign gene even after ten passages. Compared with the full-length rVHSV and the wtVHSV strain, rVHSV-EGFP showed a transient delay in replication in vitro with approximately 1 log lower virus production at 2 days post-infection. It is interesting that this moderate impairment of replication in vitro correlated with a significant reduction in final mortality in vivo, where rVHSV-EGFP caused 62% CPM compared with 82–100% in the rVHSV and wtVHSV groups. Nevertheless, the fact that rVHSV-EGFP did replicate to high final titers in vitro and replicated sufficiently in vivo to retain pathogenicity, indicated that VHSV can be genetically manipulated through reverse genetics to stably express foreign proteins to relatively high levels, and thus has a great potential to serve as a viral vector.

The *Novirhabdovirus* genus was named after the unique NV (non-virion) gene (Kurath and Leong 1985), which is present in all the four species of Novirhabdoviruses (Tordo et al. 2004). Alonso et al. (2004) reported that a recombinant SHRV without the NV gene replicated in vitro to the same high levels as wild-type virus, and was equally virulent as the wild-type in a zebrafish (*Danio rerio*) challenge. In contrast, Thoulouze et al. (2004) demonstrated that a recombinant IHNV in which the NV gene was deleted and replaced with a reporter gene was viable, but it replicated poorly in vitro, had smaller plaques, and was highly attenuated in a rainbow trout, compared to wild-type IHNV. In this present study, we demonstrated that the NV gene was not essential for the production of viable VHSV, but without the NV gene the virus replicated poorly in vitro (Fig. 5), produced smaller

plaques, and was highly attenuated in the yellow perch (Fig. 6), compared to wtVHSV. Thus, our results are similar to those of IHNV finding that NV gene is required for efficient viral replication and pathogenesis (Thoulouze et al. 2004), but differ from SHRV finding that NV gene is nonessential for pathogenicity (Alonso et al. 2004).

The in vivo challenge work described here was novel in using the yellow perch host species that is relevant to the recent emergence of freshwater VHSV in the Great Lakes of North America. Although the level of mortality varied significantly between each different virus treatment group, the kinetics of mortality and the disease signs observed were typical for VHSV challenge studies described in the more commonly used rainbow trout host (Smail 1999). The high frequency of hemorrhages at the base of the pectoral fins in all groups was notable, and is reminiscent of the discovery made by bioluminescence imaging that the fin bases are a major portal of entry for IHNV in rainbow trout (Harmache et al. 2006). However, the perch described here were initially infected by IP injection rather than immersion, so this sign indicates either hemorrhage due to internal infection processes, or secondary infections from shed virus. It is particularly interesting that the severity of disease in those fish that died in each treatment group, as indicated by clinical disease signs, did not vary between virus challenge groups. This provides preliminary insight into the mechanism(s) by which these manipulations of the VHSV genome modify the virulence phenotype, suggesting that in vivo differences among the recombinant viruses lie in the proportion of fish that are able to resist or control the infection, rather than in the course of disease in those that succumb.

To conclude, development of a reverse genetics system for VHSV is a major breakthrough in the field of VHSV research. This not only paves the way to basic studies of gene function and viral pathogenesis, but may facilitate the production of attenuated viral vectored vaccines against various pathogens.

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