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The Nucleoprotein and Phosphoprotein Are Major Determinants of the Virulence of Viral Hemorrhagic Septicemia Virus in Rainbow Trout

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ABSTRACT Viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus, infects several marine and freshwater fish species. There are many strains of VHSV that affect different fish, but some strains of one genetic subgroup have gained high virulence in rainbow trout (*Oncorhynchus mykiss*). To define the genetic basis of high virulence in trout, we used reverse genetics to create chimeric VHSVs in which viral nucleoprotein (N), P (phosphoprotein), or M (matrix protein) genes, or the N and P genes, were exchanged between a trout-virulent European VHSV strain (DK-3592B) and a trout-avirulent North American VHSV strain (MI03). Testing of the chimeric recombinant VHSV (rVHSV) by intraperitoneal injection in juvenile rainbow trout showed that exchanges of the viral P or M genes had no effect on the trout virulence phenotype of either parental strain. However, reciprocal exchanges of the viral N gene resulted in a partial gain of function in the chimeric trout-avirulent strain (22% mortality) and complete loss of virulence for the chimeric trout-virulent strain (2% mortality). Reciprocal exchanges of both the N and P genes together resulted in complete gain of function in the chimeric avirulent strain (82% mortality), again with complete loss of virulence in the chimeric trout-virulent strain (0% mortality). Thus, the VHSV N gene contains an essential determinant of trout virulence that is strongly enhanced by the viral P gene. We hypothesize that the host-specific virulence mechanism may involve increased efficiency of the viral polymerase complex when the N and P proteins have adapted to more efficient interaction with a host component from rainbow trout.

IMPORTANCE Rainbow trout farming is a major food source industry worldwide that has suffered great economic losses due to host jumps of fish rhabdovirus pathogens, followed by evolution of dramatic increases in trout-specific virulence. However, the genetic determinants of host jumps and increased virulence in rainbow trout are unknown for any fish rhabdovirus. Previous attempts to identify the viral genes containing trout virulence determinants of viral hemorrhagic septicemia virus (VHSV) have not been successful. We show here that, somewhat surprisingly, the viral nucleocapsid (N) and phosphoprotein (P) genes together contain the determinants responsible for trout virulence in VHSV. This suggests a novel host-specific virulence mechanism involving the viral polymerase and a host component. This differs from the known virulence mechanisms of mammalian rhabdoviruses based on the viral P or M (matrix) protein.

KEYWORDS VHSV, fish virus, nucleoprotein, phosphoprotein, rhabdovirus, virulence determinants

Viral hemorrhagic septicemia virus (VHSV) causes severe disease and mortality in many marine and freshwater fish species worldwide (1–3). VHSV is an enveloped, nonsegmented, negative-sense RNA virus in the *Novirhabdovirus* genus of the *Rhab-*

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doviridae family, species *Piscine novirhabdovirus* (4, 5). The genome of VHSV is composed of approximately 11 kb of single-stranded RNA, which contains six genes that are located along the genome in the 3′-to-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 nonvirion protein (NV), and a large protein (L), which is a viral polymerase (6, 7). The virus replicates entirely in the cytoplasm by means of a combination of virus-encoded and host-derived factors. The functions of the five canonical rhabdovirus proteins have been defined largely based on studies of the mammalian rabies virus (RABV) and vesicular stomatitis virus (VSV), and their roles are considered universal for rhabdoviruses (4, 8). N is the major internal structural protein and encapsidates the RNA genome. Large protein is responsible for the virion-associated RNA transcription and genome replication activity (reviewed in reference 9). P protein is a cofactor responsible for binding L protein to the N protein-RNA template, and it also has a chaperone role for N protein. N, P, and L proteins together form the functional viral ribonucleoprotein (RNP) complex (9). The M protein condenses the RNP into a tightly coiled RNP-M protein complex, which gives the virion bullet-like shape. G glycoprotein forms trimer surface spikes that protrude through the cell-derived envelope and interact with host cell receptors to facilitate cell entry (10). An additional gene located between the G and L cistrons codes for a nonvirion protein, which is unique to novirhabdoviruses. The NV protein is required for efficient replication and pathogenicity in fish (11–13), and it also suppresses apoptosis and innate immune responses (14–16).

Viral hemorrhagic septicemia (VHS) disease was first described for freshwater-reared rainbow trout (*Oncorhynchus mykiss*) in Europe in 1938, and it has continued to cause severe losses in the European trout farming industry since the 1950s (1, 2, 17). In the late 1980s, VHSV was isolated for the first time in western North America from asymptomatic adult Pacific salmon and Pacific cod showing hemorrhagic lesions (18). Surveys of wild fish in the Northeast Pacific Ocean showed an extensive reservoir of VHSV in many marine species, and VHS epidemics have occurred in wild Pacific herring (*Clupea pallasii*) (19, 20). Extensive surveys in European waters also revealed a large VHSV reservoir in diverse marine fish (21). In the Asian Pacific Ocean, VHSV has caused disease outbreaks in cultured olive flounder (*Paralichthys olivaceus*) since 1996, and it is enzootic in wild marine flounder (22, 23). In 2005, VHSV emerged in the Great Lakes region of the North America, causing infection and major mortality events in several freshwater fish species (24–28).

VHSV isolates are classified into four genotypes (designated I to IV) based on phylogenetic analyses of the G and N sequences (29, 30). Each group is endemic to specific geographic regions in the northern Atlantic and Pacific Oceans, and each appears to infect regional fish species (30–35). Genotype I is divided into six sublineages (Ia to If), with the Ia strain being virulent and responsible for most outbreaks in European freshwater rainbow trout farms (29, 30, 35, 36). Genotype II and III viruses are endemic in wild marine fish in Europe (29, 30, 36–38). Genotype IV viruses are further divided into three sublineages: IVa, IVb, and IVc. The IVa lineage is widely endemic in Asia and along the West coast of North America, IVb has become endemic and causes major outbreaks among freshwater species in the Great Lakes region, and IVc occurs on the East coast of North America (22, 24, 25, 27, 32, 34, 39, 40). The virulence of VHSV isolates is host specific and varies generally with the viral genotypes and sublineages. Genotype IV isolates are virulent in several wild marine and freshwater fish species, but they are not virulent in rainbow trout (18, 22, 41, 42). European genotype Ia and Ic isolates are virulent for rainbow trout, whereas VHSV marine isolates of genotypes Ib, II, and III are avirulent or show low virulence in rainbow trout (1, 36, 43), with occasional exceptions representing adaptive host jumps (37).

In previous attempts to identify the molecular basis of VHSV virulence in rainbow trout, several researchers sequenced the VHSV genomes of virulent and avirulent isolates from Europe and compared the amino acid sequences of all six VHSV proteins (44, 45). As few as 14 amino acid substitutions were identified among the selected

virulent and avirulent isolates, of which 5 occurred in the N protein, 3 each in the P and L proteins, 2 in the G protein, and 1 in the NV protein (44, 45). Other researchers have suggested specific amino acids in the N protein as candidate determinants for virulence in rainbow trout by comparing the genome sequences of genotype Ib and III isolates (46, 47). In addition, using reverse genetics studies, Romero et al. reported that a chimeric recombinant infectious hematopoietic necrosis virus (IHNV) containing the G gene of virulent VHSV was viable and virulent in rainbow trout (48), and Einer-Jensen et al. showed that recombinant IHNVs which contained G or NV genes from low- or high-virulence VHSV strains had no difference in virulence when tested in rainbow trout (49). Thus, to date, the individual VHSV protein(s) involved in virulence have not been identified.

To study the genetic basis of VHSV virulence, we used a reverse genetics approach to generate recombinant viruses that contained strategically designed substitutions of VHSV protein(s) in two selected VHSV strains that differ in virulence for rainbow trout. The Danish genotype Ia isolate DK-3592B (here noted as the "dk" strain) is highly virulent in rainbow trout (43). In contrast, the North American genotype IVb isolate MI03 from Michigan in the Great Lakes region (here noted as the "mi" strain) is avirulent in rainbow trout (41, 42). We previously described generating infectious clones of these two VHSV strains (pVHSVdk and pVHSVmi, respectively) and demonstrating that the recombinant VHSV (rVHSV) produced by each clone had the anticipated contrasting virulence when tested in rainbow trout, referred to here as trout virulence (13, 50). We use this qualified term because virulence in fish rhabdoviruses is host specific and there are many VHSV strains, including the MI03 strain used in this study, that are highly virulent in other fish species but have low virulence in rainbow trout (41, 42). The basic strategy involved making reciprocal exchanges of the protein-coding regions of selected genes, or combinations of genes, between the two infectious VHSV clones.

We recently reported testing of rVHSVs generated from chimeric infectious clones in which the G, NV, G and NV, or G, NV, and L genes of the trout-avirulent pVHSVmi clone were replaced with the homologous genes from the trout-virulent pVHSVdk clone (50). Reciprocal chimeras were made using the pVHSVdk clone backbone and replacing the G, NV, G and NV, or G, NV, and L genes with the homologous genes from the pVHSVmi clone. The resulting eight chimeric recombinant viruses were viable *in vitro* and *in vivo*, but they did not exhibit any change in trout virulence relative to the parental clones. Therefore, we concluded that the VHSV G, NV, and L genes, comprising 70% of the viral genome, do not contain genetic determinants of trout virulence (50).

In this study, we investigated the possibility of virulence determinants in the N, P, and M genes that make up the 3′ region of the viral genome. We also tested the N and P genes together because we reasoned that the viral N and P proteins of mammalian rhabdoviruses have been shown to work closely together as chaperone partners and components of the viral polymerase (51–54). Thus, we generated eight new recombinant viruses based on the pVHSVmi and pVHSVdk clones. The first four are the rVHSVmi series of chimeric viruses containing the N, P, M, or N and P genes from the trout-virulent VHSVdk strain, whereas the other four are the reciprocal rVHSVdk series of chimeric viruses containing the N, P, M, or N and P genes from the trout-avirulent VHSVmi strain. In this report, we describe the characteristics of these recovered chimeric rVHSVs and the parental rVHSVdk and rVHSVmi *in vitro* and the role of VHSV N, P, M, or N and P proteins in trout virulence as assessed by conducting challenge studies with juvenile rainbow trout.

RESULTS

Chimeric cDNA clones and recombinant VHSV. Construction of infectious clones of the VHSV MI03 and DK-3592B strains has been described previously, and the full-length clones were designated pVHSVmi and pVHSVdk, respectively (13, 50). These two parental clones were used to construct VHSV chimeric clones. Figure 1 shows a schematic presentation of eight full-length chimeric VHSV cDNA clones constructed by

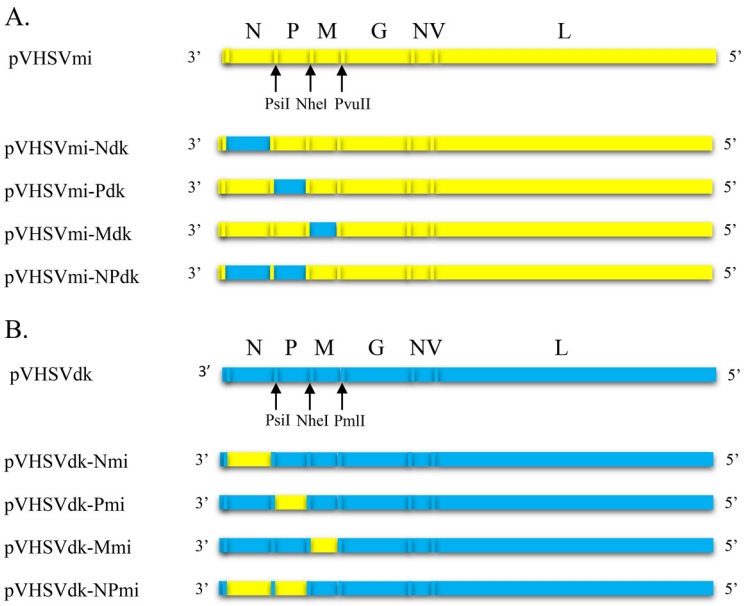

FIG 1 Schematic presentation of full-length cDNA constructs of VHSV strains MI03 (mi) and DK-3592B (dk) and various chimeric VHSVs. A map of the VHSV genome is shown at the top of panels A and B, depicting coding regions of the N, P, M, G, NV, and L genes that are separated by the flanking regulatory untranslated and intergenic sequences (vertical bars). Selected restriction sites, important for the construction of chimeric cDNA clones, are shown. These unique sites are present in the intergenic regions of the clones, which does not affect the coding regions of viral proteins. All these constructs contain a cytomegalovirus promoter at the 3′ end. (A) Chimeric cDNA clones derived by substituting VHSV DK-3592B gene(s) into the pVHSVmi full-length clone. (B) Chimeric cDNA clones derived by substituting VHSV MI03 gene(s) into the pVHSVdk full-length clone. Yellow boxes depict the coding regions of VHSV MI03, whereas the blue boxes represent the coding regions of VHSV DK-3592B.

exchanging the individual N, P, or M genes or the N and P genes between the pVHSVmi and pVHSVdk clones. These plasmid constructs were used to recover a series of recombinant VHSVs (rVHSVmi and rVHSVdk). To verify the chimeric nature of each rVHSV, RNA was extracted from these viruses and used to obtain reverse transcription-PCR (RT-PCR) products. Sequence analysis of the RT-PCR products confirmed the chimeric nature of the rVHSVs as designed.

Analysis of recombinant viruses by immunostaining. To detect the expression levels of VHSV structural proteins synthesized in epithelioma papulosum cyprini (EPC) cells infected with the rVHSVdk series or rVHSVmi series of chimeric viruses, the cellular proteins were separated by denaturing polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with polyclonal antibody made against the MI03 strain. Figure 2 shows the bands produced by reactivities of VHSV structural G, N, P, and M proteins with VHSV polyclonal antibody after immunostaining. The reason for low-intensity signal in some bands is that the antibody was made against the VHSV MI03 strain and it does not react strongly with the structural proteins of the DK strain. The results clearly show that substitutions of N and NP genes of rVHSVmi into the rVHSVdk series of chimeric viruses give strong signals but do not affect the expression of G or other viral proteins. Therefore, the loss of virulence observed in the constructs with N or NP substitutions in the virulent virus backbone (rVHSV-dk) is not due to lack of viral protein expression.

Replication kinetics of chimeric viruses *in vitro*. To analyze the *in vitro* growth characteristics of the chimeric rVHSVs harboring substitutions(s) of the N, P, M, or N and P genes, a multiple-step growth curve study was carried out in EPC cells. Figure 3 shows the growth curves of the parental rVHSVmi and rVHSVdk and their derivatives at different time points between 24 and 120 h postinfection (p.i). All rVHSVs were viable and replicated *in vitro*. Chimeric viruses of the rVHSVmi series (Fig. 3A) grew to final titers very similar to those of the parental rVHSVmi (approximately $1 \times 10^{7.0}$ PFU/ml) at

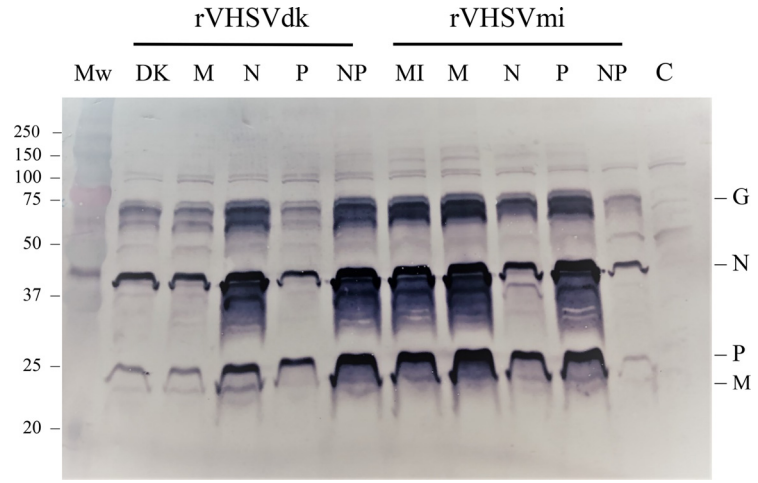

FIG 2 Western blot analysis of VHSV proteins synthesized in virus-infected cells. EPC cells were mock infected or infected with different rVHSVdk series or rVHSVmi series chimeric viruses and harvested at 72 h postinfection. After lysis, cellular proteins were fractionated by 12% SDS-PAGE, blotted onto nitrocellulose membrane, reacted with polyclonal rabbit anti-VHSV serum (prepared against the MI03 strain), and detected with streptavidin-alkaline phosphatase and BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt)/NBT (*p*-nitro blue tetrazolium chloride) color development reagents. Lane 1 (Mw) contains prestained low-molecular-weight protein markers from Bio-Rad. Lanes 2 to 6 contain cell lysates of rVHSVdk series chimeric viruses containing VHSV MI03 gene substitution(s), as follows: DK, parental rVHSVdk; M, rVHSVdk-Mmi; N, rVHSVdk-Nmi; P, rVHSVdk-Pmi; and NP, rVHSVdk-NPmi. Lanes 7 to 11 contain cell lysates of rVHSVmi series chimeric viruses containing VHSV-DK gene substitution(s), as follows: MI, parental rVHSVmi; M, rVHSVmi-Mdk; N, rVHSVmi-Ndk; P, rVHSVmi-Pdk; and NP, rVHSVmi-NPdk. Lane 12 (C), control EPC cell lysate (mock infected). The positions of marker proteins, in kilodaltons (left), and G, N, P, and M proteins of VHSV (right) are indicated.

120 h p.i., although the rVHSVmi-Ndk and rVHSVmi-NPdk viruses replicated slightly more slowly and had a titer ~0.5 log lower than that of rVHSVmi at 96 h p.i. Similarly, chimeric viruses of the rVHSVdk series (Fig. 3B) replicated to final yields very similar to those of the parental rVHSVdk (approximately $1 \times 10^{7.5}$ PFU/ml) at 120 h p.i., although rVHSVdk-NPmi lagged behind at 24 and 48 h p.i.

Trout virulence of chimeric viruses *in vivo*. Trout virulence was assessed by intraperitoneal injection challenge of triplicate groups of 20 juvenile rainbow trout. During 21 days postchallenge, control groups of fish challenged with parental wild-type VHSVmi (wtVHSVmi) or rVHSVmi had either no mortality or extremely low mortality, as expected (Fig. 4A and Table 1). Chimeric rVHSVmi viruses with the P or M gene from pVHSVdk also caused no mortality. However, in the group challenged with chimera rVHSVmi-Ndk, containing the N gene of pVHSVdk, there was mortality in each of the three replicate tanks beginning on day 4 and continuing to day 9, reaching a final average cumulative percent mortality (CPM) of 22% (Fig. 4A and Table 1). Even more notable, the group challenged with chimera rVHSVmi-NPdk, which contained both the N and P genes from pVHSVdk, had higher mortality beginning on days 3 to 4 and continuing through days 8 to 9, reaching a final average CPM of 82% (Fig. 4A and Table 1). Thus, gain-of-function increases in trout virulence were observed for both rVHSVmi-Ndk and rVHSVmi-NPdk.

In the reciprocal VHSVdk series, the positive-control group challenged with wtVHSVdk had 100% CPM and the rVHSVdk group had an average of 85% CPM. Chimeric rVHSVdk viruses with the P or M gene from pVHSVmi caused similarly high levels of mortality, averaging 83 and 98% CPM, respectively (Fig. 4B and Table 1). However, the group challenged with chimeric rVHSVdk-Nmi had almost no mortality (1 fish in one of three replicate tanks), and rVHSVdk-NPmi caused no mortality. Thus, the chimeras in which the N or N and P genes were exchanged had loss-of-function effects on rVHSVdk which were reciprocal to the gain-of-function effects observed for these genes in rVHSVmi.

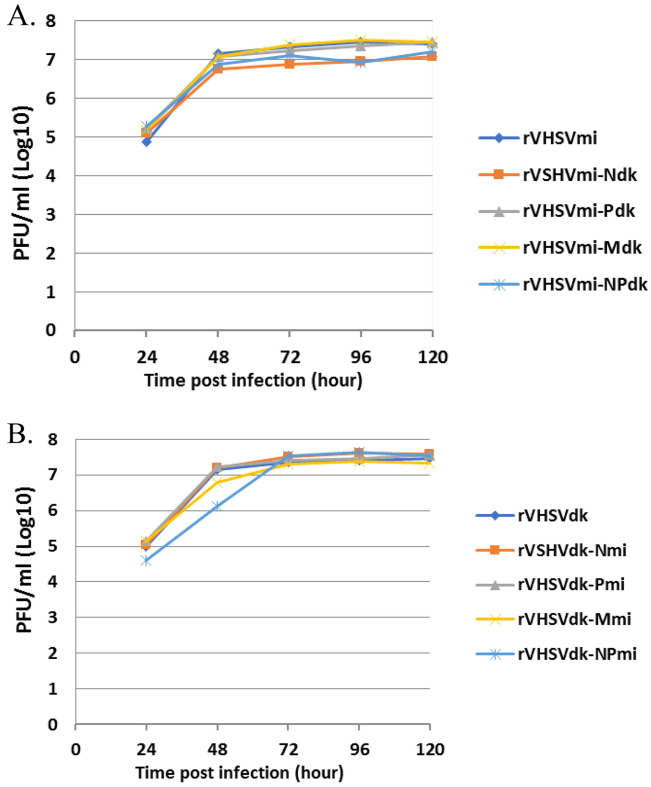

FIG 3 Replication kinetics of parental rVHSVmi, rVHSVdk, and chimeric rVHSVs *in vitro*. Monolayers of EPC cells were infected at an MOI of 0.01 with the chimeric viruses harboring substitutions for specific VHSV gene(s) in the parental rVHSVmi (A) or rVHSVdk (B) viruses derived from respective pVHSVmi or pVHSVdk plasmids. The viruses were harvested at the indicated time points, and virus titers were determined by plaque assay. Virus titers are the averages of viral plaques obtained in duplicate wells and from two independent experiments.

The mean day to death (MDD) in the gain-of-function groups of rVHSVmi-Ndk and rVHSVmi-NPdk was 6.1 to 7.1, which was within the range of 5.3 to 7.3 observed in the VHSVdk series groups that had high mortality (Table 1). Clinical signs were observed in approximately 29% of the fish that died, with exophthalmia, ascites, and external hemorrhage lesions being most common. These signs were observed for fish in the gain-of-function groups of rVHSVmi-Ndk and rVHSVmi-NPdk, as well as in the rVHSVdk series groups.

Statistical analyses of final CPM data for triplicate groups of 20 fish per treatment were first conducted by analysis of variance (ANOVA). In the rVHSVmi series (Fig. 4A), all low-virulence groups and the partial gain of function in the rVHSVmi-Ndk group fell within the same statistical group, and only the higher-virulence gain of function in rVHSVmi-NPdk group was significantly different from that of the parental rVHSVmi (Table 1). However, analysis of survival kinetics by Kaplan-Meier estimation of survival curves followed by log rank tests showed that the rVHSVmi group differed significantly from both the rVHSVmi-Ndk ($P = 0.00259$) and rVHSVmi-NPdk ($P = 7.8 \times 10^{-16}$) groups. In the rVHSVdk series (Fig. 4B), the rVHSVdk group differed significantly from both of the groups that showed loss of function, i.e., rVHSVdk-Nmi ($P = 7.8 \times 10^{-16}$) and pVHSVdk-NPmi ($P = 7.8 \times 10^{-16}$). These two loss-of-function groups were not significantly different from the rVHSVmi group by either statistical method, indicating a complete loss of function. The high virulence rVHSVdk group was not significantly different from that of the rVHSVmi-NPdk group by either statistical method, confirming complete gain of function for rVHSVmi-NPdk. rVHSVdk-Mmi was in a separate statistical group with even higher virulence than rVHSVdk by either statistical method (Table 1), but the wtVHSVdk group had even faster mortality (Fig. 4B).

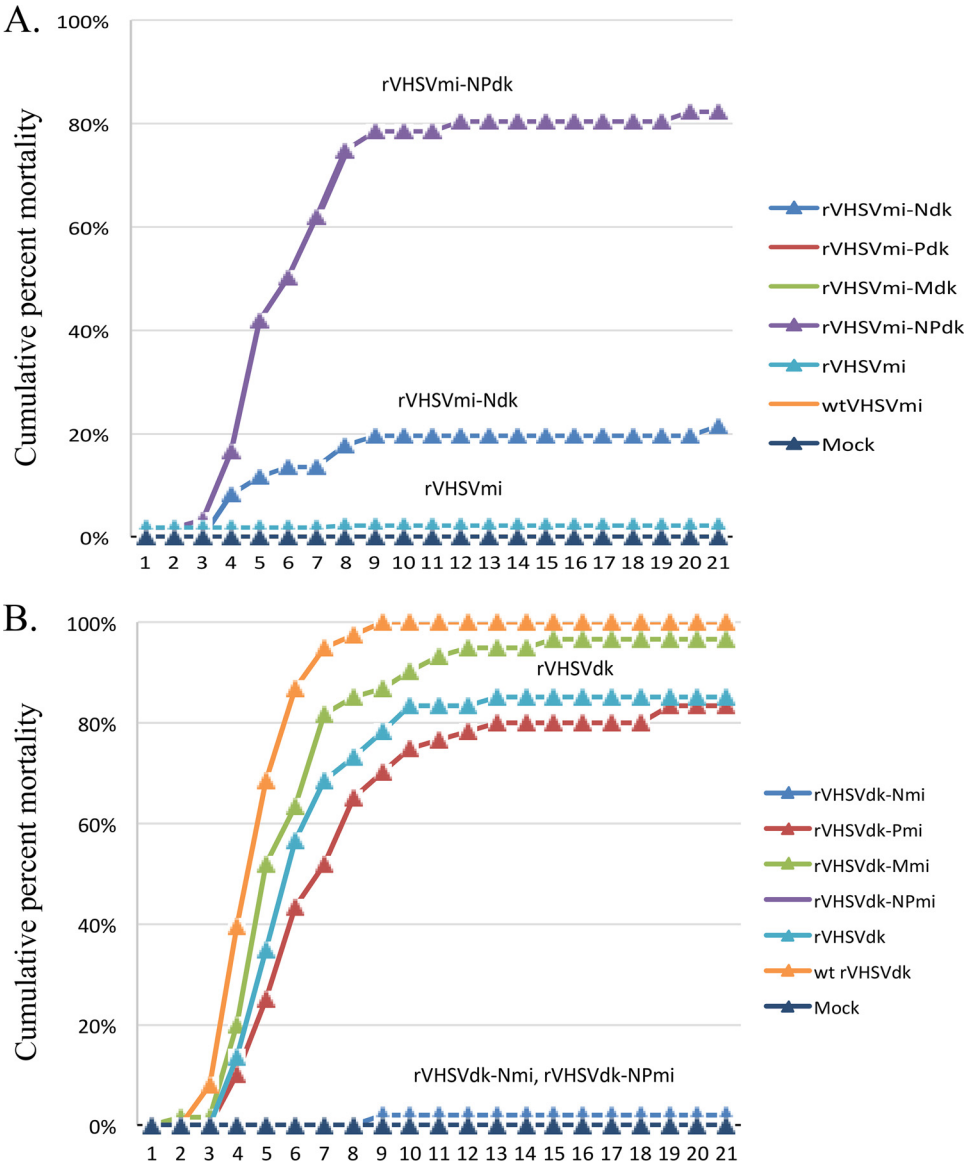

FIG 4 Virulence of parental rVHSVmi, rVHSVdk, and chimeric rVHSVs in juvenile rainbow trout. Juvenile rainbow trout were challenged as described in Table 1 by intraperitoneal injection with parental rVHSVmi or rVHSVdk, chimeric viruses with reciprocal exchanges of the N, P, M, or N and P genes, or wild-type MI03 or DK-3592B viruses or were mock challenged with PBS. Mortality of fish after challenge was recorded daily and expressed as the average cumulative percent mortality (CPM) for triplicate subgroups of 20 fish. Variation among subgroups is provided in Table 1. (A) rVHSVmi series groups; (B) rVHSVdk series groups.

DISCUSSION

To elucidate genetic determinants of VHSV host-specific virulence in rainbow trout, we systematically exchanged the six viral genes, either individually or as selected gene combinations, between the trout-avirulent pVHSVmi clone (representing VHSV strain MI03, genotype IVb) and the trout-virulent pVHSVdk clone (representing VHSV strain DK-3592B, genotype Ia). All recombinant viruses were viable, replicated in cell culture, and expressed viral N and P proteins. Previous challenge experiments testing of chimeric recombinant VHSVs with reciprocal exchanges of the G, NV, G and NV, or G, NV, and L genes together did not show any change of the virulence phenotypes for the contrasting parental recombinant viruses in juvenile rainbow trout (50). In the subsequent study presented here, there was a partial gain of the trout virulence function when the N gene of pVHSVdk was substituted into the trout-avirulent parental clone

TABLE 1 Results of the *in vivo* challenge study in juvenile rainbow trout

Virus treatment group	Individual tank final CPM[a]	Avg final CPM	Statistical group[b]	Mean day to death ± SD[c]
wtVHSVmi	0, 0	0		NA
rVHSVmi	0, 0, 6	2	a	NA
rVHSVmi-Ndk	**24, 29, 12**	**22**	a*	7.1 ± 0.78
rVHSVmi-Pdk	0, 0, 0	0	a	NA
rVHSVmi-Mdk	0, 0, 0	0	a	NA
rVHSVmi-NPdk	**77, 82, 88**	**82**	b*	6.1 ± 0.33
wtVHSVdk	100, 100	100		5.34*
rVHSVdk	95, 85, 80	85	b	6.2 ± 0.19
rVHSVdk-Nmi	**0, 6, 0**	**2**	a*	9.0*
rVHSVdk-Pmi	80, 85, 85	83	b	7.31 ± 0.70
rVHSVdk-Mmi	95, 100, 95	98	c*	6.1 ± 0.56
rVHSVdk-NPmi	**0, 0, 0**	**0**	a*	NA
Mock	0, 0	0		NA

[a]All treatment groups were tested as triplicate subgroups of 20 juvenile rainbow trout, with the exception of the wild-type VHSVmi positive-control treatment and the mock challenge negative-control treatment that were tested as duplicate groups (as indicated in column 2) due to space limitations in the aquatic biosafety level 3 wetlab. Bold is used to highlight the most informative results showing gain or loss of virulence phenotype in rainbow trout.

[b]Statistical analyses are shown as letters indicating sets of treatment groups that do not differ significantly in an ANOVA on final CPM data. Asterisks indicate treatment groups that differed from their respective parental rVHSV groups (rVHSVmi or rVHSVdk) by survival analyses.

[c]Mean day-to-death (MDD) for individual tanks was used to calculate the group average MDD and SD. Asterisks indicate MDD for groups that had mortality data from only 1 or 2 tanks, due to either tanks with no mortality or fewer than 3 tanks in the treatment. In these cases, no SD could be calculated. NA, all tanks in the treatment group had no mortality.

pVHSVmi to create rVHSVmi-Ndk virus, resulting in a moderate 22% CPM. Substitution of both the N and P genes together resulted in a complete gain of virulence function in rVHSVmi-NPdk virus to a level comparable with that of the virulent parental rVHSVdk virus (82% CPM for rVHSVmi-NPdk virus and 85% CPM for rVHSVdk virus). It is important to recognize that trout virulence was tested in this study using delivery of virus by intraperitoneal injection, which bypasses natural infection barriers associated with viral entry into the host. Therefore, the virulence observed in the gain-of-function chimeras may be higher than would have been observed by a more natural immersion exposure, and there may be additional virulence determinants associated with viral entry that were not assessed in this study (55). Interestingly, substitution of the P gene alone in rVHSVmi-Pdk virus did not have any effect on the avirulence of the parental rVHSVmi virus. This suggests that the N gene is an essential virulence determinant and the P gene is a strong enhancer of the trout virulence determinant in the N gene, but this P enhancement is not expressed without the N gene. These results were strongly supported by the complete loss of virulence function observed for the reciprocal rVHSVdk series of viruses in which the N gene or the N and P genes from pVHSVmi clone were substituted into the virulent pVHSVdk clone to create rVHSVdk-Nmi and rVHSVdk-NPmi viruses, respectively. Again, substitution of the VHSVmi P gene alone into the virulent pVHSVdk clone had no effect on the high trout virulence of the rVHSVdk-Pmi virus, suggesting that the effect of the P gene is dependent on the presence of the homologous N gene for loss of virulence function.

In reverse genetics strategies to identify genetic determinants of any viral phenotype, a gain of function is more convincing than a loss of function, and demonstration of reciprocal gain and loss of function is most conclusive. Thus, the results presented here show clearly that the N and P genes contain trout virulence determinants for the VHSV strains tested in this study. These strains are members of VHSV genotypes Ia and IVb. Within each of these genotypes, many viral strains have been tested experimentally and the trout virulence phenotypes are consistent within each genotype (41–43, 56). Therefore, we suggest that the MI03 and DK-3592B strains tested in this study are likely representative of VHSV genotypes Ia and IV in general, such that the host-specific virulence mechanism revealed here is likely to apply in general to these VHSV

genotypes. However, it is interesting that MI03 and DK-3592B represent evolutionarily divergent genotypes, and VHSV phylogenies do not suggest that genotype IV is ancestral to genotype I (29–31, 34, 36). This suggests that the N and P gene roles here may be applicable for VHSV in general.

Phylogeny and molecular evolution studies have shown that trout virulence in VHSV is a trait derived by adaptation after host jumps of trout-avirulent ancestral viruses into aquaculture populations of rainbow trout (1, 29, 30, 57). This was first described as VHSV host jumps in the 1950s that resulted in evolution of trout virulence for VHSV genotypes Ia and 1c (30), and more recent host jumps have occurred in Northern Europe involving VHSV genotypes Ib, Id, and III (1, 37, 58). It would be interesting to repeat this reverse genetics study by exchanging the N and P genes between trout-avirulent and trout-virulent strains with a more direct evolutionary link, such as those involved in the recent disease outbreaks in marine net-pen rainbow trout. It is also interesting to consider whether the finding of trout virulence determinants in the N and P genes applies even more broadly to other fish rhabdovirus species that exhibit host-specific virulence in rainbow trout, such as IHNV (59, 60).

The discovery that the VHSV N and P genes contain determinants of trout virulence represents an important step toward understanding how these viruses have adapted to rainbow trout and elucidating the mechanism(s) of their high virulence. In construction of the chimeric viruses tested in this study, only the open reading frames (ORFs) for the genes were exchanged, suggesting that the changes in phenotype were due to differences within the viral N and P proteins and not the flanking regulatory sequences. Based on general rhabdovirus molecular biology, the involvement of both the N and P proteins suggests possible mechanisms involving either the viral RNA-dependent RNA polymerase complexes or the role of P as a chaperone for N protein. For model mammalian rhabdoviruses, it has been shown that the viral N, P, and L proteins, together with host components, comprise the viral RNA polymerase complexes that accomplish transcription and replication of the viral genome during the viral infection cycle in host cells (4, 8, 9, 53, 54, 61, 62). Therefore, it is possible that the trout virulence determinants in the N and P proteins of rVHSVdk result in increased speed or efficiency of viral transcription and/or replication in rainbow trout, allowing the virus to outpace the host innate immune response and resulting in high virulence. The host specificity of the trout virulence phenotype suggests that this mechanism involves interaction with one or more rainbow trout host components. An alternative to a polymerase-based mechanism is that the trout virulence determinants in the N and P proteins of rVHSVdk may involve the P protein chaperone role as it interacts with N protein to keep it soluble and prevents N from nonspecific association with cellular RNA (51, 52). However, the involvement of host components in this interaction has not been reported. These hypothetical mechanisms for how the VHSV N and P proteins might determine the trout virulence are speculative at present and remain to be tested experimentally. Regardless of the molecular interactions involved, the finding of an essential VHSV virulence determinant in the N protein, enhanced by the P protein, indicates a novel host specificity mechanism for rhabdoviruses. This is because, to date, the determinants of host and tissue specificity defined for rhabdoviruses involve the P protein as a suppressor of interferon signaling for RABV and the M protein as a mediator of global inhibition of host gene expression for VSV (reviewed in reference 63).

The predicted N and P proteins of VHSV DK-3592B and MI03 differ by 29 and 15 amino acids, respectively (Fig. 5). However, alignment of these sequences with homologous sequences of additional VHSV genotype Ia (trout virulent), Ib (trout avirulent), and IVa and IVb (trout avirulent) strains reveals a much smaller number of amino acids that correlate consistently with the trout virulence phenotype. In the N protein, these include the three amino acids R/K46G, T83M/A, and G392E (Fig. 5A). In the P protein, a single amino acid, T39P/S, differs consistently with trout virulence. We suggest one or more of these N protein residues, and the single P protein residue T39, as the most likely molecular determinants responsible for differential trout virulence.

It has been suggested previously that the VHSV N gene contains trout virulence

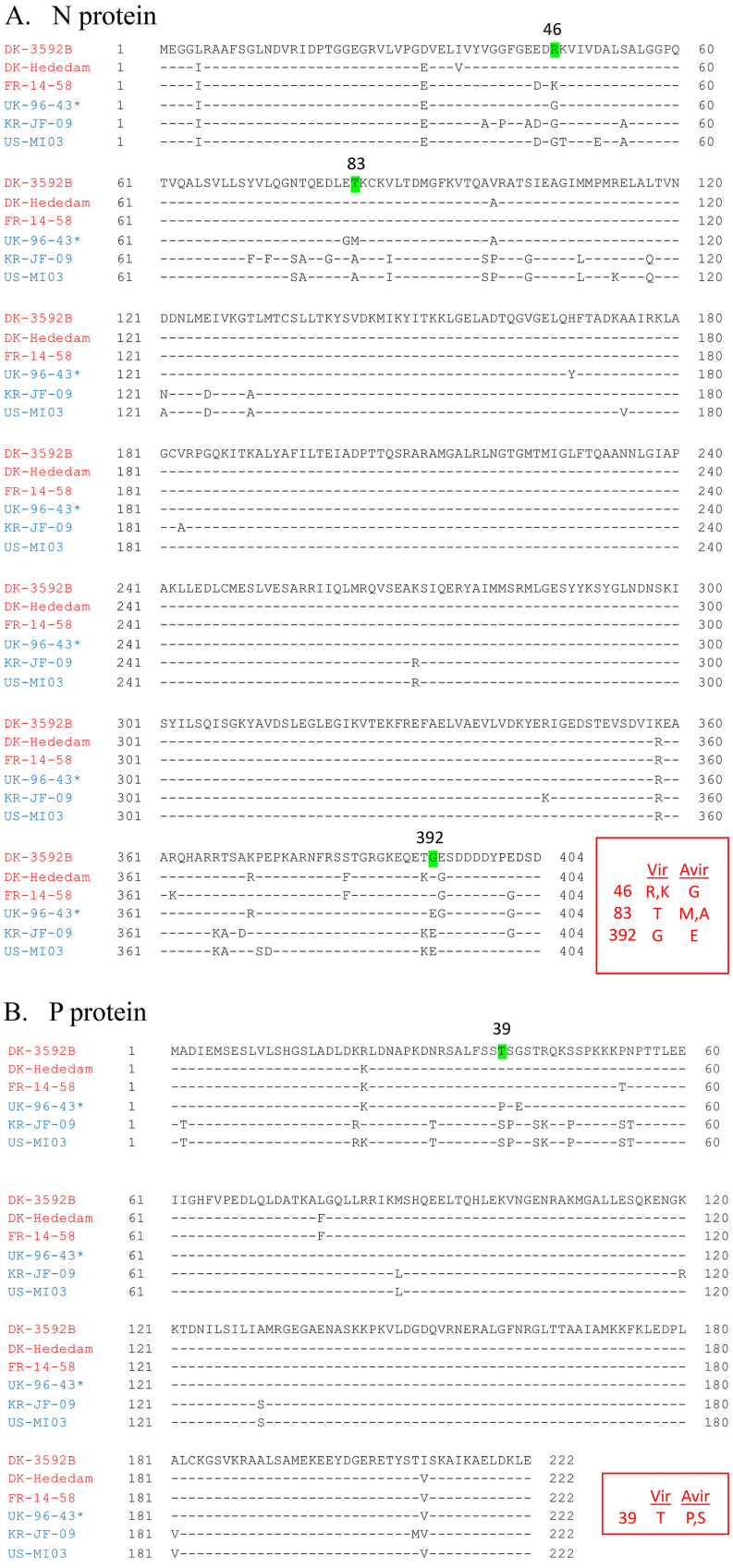

FIG 5 Comparison of the N and P protein sequences of selected VHSV strains from genotypes I and IV. Strains that are virulent in rainbow trout are labeled in red, and strains that are avirulent are labeled in

(Continued on next page)

determinants, based on comparison of sequences for VHSV genotype III strains and genotype Ib strains that differ in trout virulence (46, 47). Amino acid 46 of the N protein has been noted by previous authors as differing among VHSV strains that vary in trout virulence (45, 46), and this is consistent with our results in Fig. 5. However, the specific amino acid residues responsible for trout virulence in both the N and P proteins of VHSV remain to be confirmed in future work, likely by reverse genetics studies involving more refined exchanges of the N and/or P gene regions and *in vitro* mutagenesis of various trout-virulent and -avirulent strains of recombinant VHSV.

In this work, the level of virulence observed with the combined N and P gene chimeras suggests that these two genes are both necessary, and sufficient, to account for the high trout virulence observed in genotype Ia VHSV strains. This does not necessarily mean that other viral proteins are not involved in virulence functions but that they do not contain the genetic determinants that define the host specificity of the virulence for rainbow trout. In addition, there may be other combinations of genes not tested in this study that have an effect on virulence. In general terms, there may be more than one mutational pathway to virulence in rainbow trout, and it is possible that different trout virulence mechanisms have evolved for other VHSV strains or genotypes. If this is the case, perhaps there is not only one universal mechanism or genetic determinant for trout virulence in all VHSV genotypes. This could explain why it has not yet been possible to conclusively identify virulence determinants based on comparison of genome sequences of trout-virulent and -avirulent strains of VHSV from different genotypes. Beyond trout virulence, it is interesting to consider whether the N and P genes are also responsible for alternative host-specific virulence phenotypes for VHSV in other fish species, such as genotype IV strains in yellow perch (*Perca flavescens*) (28, 41, 42), and genotype IVa strains in herring (42, 64) and olive flounder (22, 65).

The work presented here comprises the first conclusive identification of VHSV viral genes that determine high virulence in rainbow trout. As noted above, this will likely lead to future studies testing the role of N and P genes in the trout virulence of other VHSV strains and genotypes, testing determinants for host-specific virulence in other fish species, defining the determinants at higher resolution to identify the specific nucleotides in the N and P genes that are responsible for the phenotype, and elucidating the mechanism by which the N and P genes affect host-specific trout virulence. This will advance our understanding of viral host jumps and the evolution of host specificity, with potential implications for fish health management. In addition, since the N protein has not previously been reported as a specific virulence determinant for any other rhabdovirus, this suggests a novel mechanism that expands our understanding of the basic virology of rhabdovirus virulence.

MATERIALS AND METHODS

Viruses and cells. The North American MI03 strain of VHSV (genotype IVb) (24) was originally from M. Faisal at Michigan State University, and the European DK-3592B reference strain of VHSV (genotype Ia) (43) was provided by K. Einer-Jensen and N. Lorenzen of the Danish Technical University. These parental virus strains and all recombinant VHSVs generated in this study were propagated in the epithelioma papulosum cyprini (EPC) cyprinid fish cell line (66) at 14°C as previously described (50).

Construction of chimeric cDNA clones of VHSV. Construction of infectious clones of the VHSV MI03 and DK-3592B strains has been described previously (13, 50). The full-length clones of the VHSV MI03 and DK-3592B strains were designated pVHSVmi and pVHSVdk, respectively (Fig. 1). These clones were modified and used as a backbone to introduce desired substitution(s) of structural protein genes of reciprocal VHSV strains. To construct chimeric cDNA clones of VHSV, synthetic DNA fragments were

FIG 5 Legend (Continued)
blue. (A) Alignment of the N protein sequences of the virulent DK-3592B strain with virulent genotype Ia strains DK-Hededam and FR-14-58 and avirulent strains UK-96-43* (genotype Ib), KR-JF-09 (genotype IVa), and US-MI03 (genotype IVb). (B) Alignment of the P protein sequences of the virulent DK-3592B strain with virulent and avirulent strains of genotypes Ia, Ib, IVa, and IVb described above. An asterisk indicates that the N and P protein sequences of the trout-avirulent UK-96-43 strain are identical to the N and P sequences of the trout-avirulent genotype Ib strain DK-4p37 (45). Amino acid residues in green differ consistently between trout-virulent and trout-avirulent VHSV strains, suggesting that they may be putative trout virulence determinants. The dashes indicate amino acid identity.

procured (Biomatik, Canada) which contained the ORF sequences of N, P, M, or N and P while maintaining original leader and intergenic sequences of the individual pVHSVmi or pVHSVdk backbone. These DNA fragments were cloned between the unique restriction sites (PsiI, NheI, and PvuII/PmlI) present in the noncoding regions of individual pVHSVmi or pVHSVdk plasmids to obtain desired chimeric clones containing the N, P, M, or N and P gene substitutions (Fig. 1). DNA from all the above-mentioned clones was sequenced by the dideoxy chain termination method to verify various gene substitutions in these clones.

DNA transfection and recovery of chimeric viruses. To generate recombinant chimeric viruses, EPC cells were transfected with eight different plasmid constructs (Fig. 1) along with the same supporting plasmids, pN, pP, and pL of the MI03 strain, using the protocol described earlier (13). After transfection, the cells were washed and maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) at 14°C for 5 days. Cell monolayers were observed for the development of virus-induced cytopathic effect (CPE). After 5 days of incubation, the cells were submitted to three cycles of freeze-thawing. The supernatant was clarified by centrifugation at 8,000 $\times$ *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 characterization 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 multiplicity of infection (MOI) of 0.01 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 to 5 days postinfection, clarified, and stored at −80°C. The titer of the virus was determined by plaque assay, as described previously (13). The titers of the recombinant parental viruses (rVHSVmi and rVHSVdk) and chimeric viruses ranged from 1.2×10^6 to 2.4×10^8 PFU/ml.

Confirmation of chimeric nature of the recombinant viruses. RT-PCR was performed on RNA extracted from the recovered viruses to confirm the chimeric nature of recombinant VHSVs in which the complete ORFs of the structural genes were substituted between the two VHSV backbones while maintaining the intergenic regions of the individual full-length clones. Viral RNA was extracted from the supernatants of VHSV-infected cell cultures, using an RNeasy minikit (Qiagen). RT-PCR was performed to verify the presence of the specific region(s) of VHSV gene sequences substituted between the two clones, including artificially created restriction sites (NheI, PvuII, and PmlI). The obtained RT-PCR products were directly submitted for DNA sequencing in the core facility of the Institute of Marine & Environmental Technology to confirm the presence of the VHSV gene fragments and genetic markers introduced into the genome.

Characterization of VHSV proteins synthesized in virus-infected cells by Western blotting. To detect viral protein expression by different recombinant viruses, EPC cells were infected with rVHSVdk series or rVHSVmi series chimeric viruses and harvested at 72 h postinfection. After lysis, the cellular proteins were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), transferred onto a nitrocellulose membrane, and incubated with polyclonal rabbit anti-VHSV serum against MI03 strain (14). VHSV proteins were detected with streptavidin-alkaline phosphatase and BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt)/NBT (*p*-nitro blue tetrazolium chloride) color development reagents.

Viral growth curves in cell culture. To analyze the *in vitro* growth characteristics of the recombinant VHSVs, confluent EPC cells were infected with the parental recombinant VHSVs of strains MI03 (rVHSVmi) and DK-3592B (rVHSVdk) or different chimeric recombinant VHSV stocks at an MOI of 0.01 in individual T-25 flasks. Virus present in the infected cell culture supernatant was collected at different intervals, clarified by centrifugation, and titrated on EPC cells by plaque assay, as described earlier (13).

Virus challenge experiments in juvenile rainbow trout. The *in vivo* virus challenge experiment was conducted in compliance with guidelines provided by the *Guide for the Care and Use of Laboratory Animals* (67) and the U.S. Public Health Service's *Policy on the Humane Care and Use of Laboratory Animals* (68). Research-grade naive juvenile rainbow trout were provided by Clear Springs Foods, Inc., of Buhl, ID. Fish were reared to appropriate size in the Western Fisheries Research Center aquatic biosafety level 2 (ABSL2) wetlab on flowthrough, sand-filtered, UV-irradiated lake water at 15°C, and given pelleted feed (Skretting) at 1.5% body weight per day. Feed was withheld for 24 h prior to the start of each experiment, and during this time fish were gradually acclimated to 12°C. Fish were then transferred to the aquatic biosafety level 3 (ABSL3) laboratory of Western Fisheries Research Center, where all *in vivo* virus experiments were conducted.

Juvenile rainbow trout with an average weight of 1.4 g were challenged with virus by intraperitoneal injection of a dose of 1.7×10^4 PFU per fish in 25 μl of phosphate-buffered saline (PBS). Negative-control groups were mock infected by injection with PBS containing no virus. The experiment had 13 treatment groups as shown in Table 1. Each treatment group was tested in triplicate subgroups of 20 juvenile rainbow trout, except for the positive-control wild-type parental virus strain wtVHSVmi and the mock-infected negative controls that were tested as duplicate groups of 20 fish due to limitations of tank space in the ABSL3 wetlab. After injection, the individual replicate subgroups of fish were held in 30-liter tanks with flowthrough water at 12°C and fed three times per week. Fish were monitored daily for morbidity and mortality for a period of 21 days. Fish that died during the challenge were collected daily, and disease signs were recorded in dead fish before storing them at −80°C. A subset of fish that died in each treatment group (between 1 and 11 fish per treatment group, depending on the number of fish that died) were tested for infectious virus by plaque assay (69) to confirm the presence of virus at titers that suggested viral infection as the likely cause of death.

Differences in arc-sine-transformed final cumulative percent mortality (CPM) values for all virus-challenged treatment groups were evaluated by ANOVA followed by Tukey's *post hoc* test to define significant differences among treatment groups (implemented with PASW Statistics vV.18; IBM Analytics). Kinetics of survival of the fish in different groups within each experiment was analyzed using Kaplan-Meier estimation of survival curves, followed by comparison of the survival curves by log rank test. A P value of <0.05 was considered significant for all tests. Mean day to death (MDD) was calculated for fish that died within each individual replicate tank and then reported as the average MDD and standard deviation for replicate tanks within each treatment. For MDD calculations, we excluded 2 fish that died on day 1 postchallenge, based on the likelihood that these fish died due to injury during injection rather than to virus infection.

Characterization of the chimeric viruses recovered from infected fish. For each treatment group that had mortality, viral RNA from one fish that died late in the monitoring period was sequenced to verify that recovered viruses contained the expected introduced substitutions of genes from two different genotypes of VHSV. Genomic RNA was extracted from partially purified virus using an RNeasy minikit (Qiagen) and subjected to RT-PCR amplification to obtain cDNA fragments of the VHSV genome, as described earlier. These DNA fragments were purified and sequenced to confirm the presence of introduced substitutions of different structural protein genes into the VHSV genomes.

Validation of resources and biosafety. The relevant sequences of the chimeric viruses were determined at the plasmid DNA level and by RT-PCR of virus in infected samples. Experiments were performed in an ABSL3 facility, which is appropriate for the viruses being studied. The gain-of-function chimeras did not have additional virulence beyond that of the wild-type trout-virulent strain and therefore do not represent agents of concern.

Data availability. The authors declare that all data supporting the findings of this study are available within the article. For reference, the complete nucleotide sequences of the VHSV MI03 strain and the VHSV DK-3592B strain are available in GenBank under accession no. GQ385941 and KC778774, respectively.

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