Essential Role of the NV Protein of *Novirhabdovirus* for Pathogenicity in Rainbow Trout

Maria-Isabel Thoulouze,* Edwige Bouguyon, Catherine Carpentier, and Michel Brémont

Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique,
78352 Jouy-en-Josas Cedex, France

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Novirhabdovirus, infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV) are fish rhabdoviruses that, in comparison to the other rhabdoviruses, contain an additional gene coding for a small nonvirion (NV) protein of unassigned function. A recombinant IHNV with the NV gene deleted but expressing the green fluorescent protein (rIHNV-ΔNV) has previously been shown to be efficiently recovered by reverse genetics (S. Biacchesi et al., J. Virol. 74:11247–11253, 2000). However, preliminary experiments suggested that the growth in cell culture of rIHNV-ΔNV was affected by the NV deletion. In the present study, we show that the growth in cell culture of rIHNV-ΔNV is indeed severely impaired but that a normal growth of rIHNV-ΔNV can be restored when NV is provided in *trans* by using fish cell clones constitutively expressing the NV protein. These results indicate that NV is a protein that has a crucial biological role for optimal replication of IHNV in cell culture. Although IHNV and VHSV NV proteins do not share any significant identity, we show here that both NV proteins play a similar role since a recombinant IHNV virus, rIHNV-NV_{VHSV}, in which the IHNV NV open reading frame has been replaced by that of VHSV, was shown to replicate as well as the wild-type (wt) IHNV into fish cells. Finally, data provided by experimental fish infections with the various recombinant viruses strongly suggest an essential role of the NV protein for the pathogenicity of IHNV. Furthermore, we show that juvenile trout immunized with NV-knockout IHNV were protected against challenge with wt IHNV. That opens a new perspective for the development of IHNV attenuated live vaccines.

Infectious hematopoietic necrosis virus (IHNV) and Viral hemorrhagic septicemia virus (VHSV) are both salmonid rhabdoviruses belonging to the Novirhabdovirus genus of the Rhabdoviridae family. Similar to mammalian rhabdoviruses, their genomic RNA contain genes encoding structural proteins, the nucleoprotein N, the polymerase-associated protein P, the matrix protein M, the glycoprotein G, and the large RNA-dependent RNA polymerase L. In contrast, the novirhabdoviruses possess an additional gene, localized between the G and L genes, that encodes a small nonstructural protein “nonvirion” NV (2, 18). To date, the involvement of the NV protein in the viral replication cycle has been putative and has never been clearly evaluated. The conservation of a functional NV gene in some fish rhabdoviruses suggests that the NV protein could play a role either for the viral replication or for the pathogenicity of the virus. As an approach to demonstrate a biological role of NV, fish cells transiently transfected with a plasmid expressing the NV gene were found to undergo cell rounding, suggesting a possible interaction between NV protein and the cytoskeleton (6). However, several observations are contradictory for a biological role of NV. (i) The absence of amino acid sequence homology between NV from different novirhabdoviruses such as IHNV and VHSV is puzzling. (ii) The only common similarity between the novirhabdoviruses is the conserved location of the NV gene, situated between the G and L

cistrons, and the size of that gene (ca. 400 to 500 nucleotides long). (iii) Among fish rhabdoviruses, some of them, like the spring viremia of carp virus, do not possess a gene encoding NV. Thus, the question of NV involvement in IHNV multiplication and pathogenicity is still open.

Reverse genetics technology on negative strand RNA viruses offers the possibility to manipulate the viral genome proteins (for a review, see reference 8) and may thus allow elucidation of the biological role of viral proteins during the replication cycle, particularly for the nonstructural. As an example, studies on the role of the NS2 protein of the respiratory syncytial virus (RSV) have been undertaken by deleting the gene encoding that nonstructural protein (25, 26) and have shown that NS2 protein was not essential for RSV replication, although its presence greatly improves viral replication in cell culture. In vivo, recombinant RSV knockout for NS2 was shown to be attenuated, suggesting a role of NS2 in the pathogenicity (26).

Recently, Johnson et al., applying a reverse genetics system to the *Snakehead rhabdovirus*, a warm-water fish novirhabdovirus, have generated a recombinant virus containing a targeted nonsense mutation in the NV gene (15). In cell culture, this recombinant virus exhibited the same behavior as the wild-type (wt) virus, indicating that NV does not play a crucial role for in vitro replication. More recently, we have shown that recombinant IHNV knockout for NV was efficiently recovered by a reverse genetics system (4) and that reporter genes inserted in the NV locus were efficiently expressed. However, preliminary experiments had suggested that the replication of NV-knockout IHNV in cell culture might be altered.

In the present study, we show that the growth of rIHNV-ΔNV in cell culture is indeed severely impaired, suggesting a

* Corresponding author. Mailing address: Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Bat. 440, 78352 Jouy-en-Josas Cedex, France. Phone: 33(1)34-65-26-15. Fax: 33(1)34-65-26-21. E-mail: thoulouz@jouy.inra.fr.

FIG. 1. Construction of pIHNV-ΔNV and pIHNV-NV_{VHSV} plasmids. The pIHNV-ΔNV and pIHNV-NV_{VHSV} plasmid constructs were generated by insertion into the pIHNV backbone of an AgeI/NsiI fragment containing the GFP or NV_{VHSV} gene.

crucial role of NV for the optimal IHNV replication. To get more insight into the role of NV in IHNV replication, we generated additional recombinant viruses, which have been studied both *in vitro* and *in vivo*. Fish cells constitutively expressing the NV protein have also been generated and used to replicate a recombinant IHNV knockout for NV. Although both IHNV and VHSV express NV proteins of no significant similarity, we engineered a recombinant IHNV virus, rIHNV-NV_{VHSV}, in which the NV gene of IHNV has been replaced by that of VHSV. Viral replication was compared both *in vitro* and *in vivo*. The role of NV protein of IHNV and VHSV during the replication of IHNV in cell culture and the involvement of NV genes in the pathogenicity of IHNV in trout are discussed.

MATERIALS AND METHODS

Viruses and cell cultures. IHNV 32/87 French strain was propagated in monolayer cultures of *Epithelioma papulosum cyprini* (EPC) cells at 14°C as previously described (10). Chinook salmon embryo cells (CHSE₂₁₄) were used to amplify some of the recombinant viruses.

Recombinant vaccinia virus expressing the T7 RNA polymerase, vTF7-3 (12), was kindly provided by B. Moss (National Institutes of Health, Bethesda, Md.).

EPC cells constitutively expressing NV. The NV_{IHNV} gene was subcloned into the pCDNA1.1amp (Stratagene) eukaryotic expression vector under the control of the early cytomegalovirus (CMV) promoter, leading to the pCMV-NV plasmid. pCMV-NV (4.8 μg) was transfected together with 1.2 μg of pSV2-neo (a plasmid coding for neomycin resistance; Clontech) into 8×10^6 EPC cells (in 60-mm-diameter petri dish) by using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. At 3 days posttransfection, cell monolayers were treated with trypsin, split into two 100-mm-diameter petri dishes, and incubated in culture medium containing Geneticin (G418; 500 μg/ml, final concentration). One month later, single cell clones were isolated and grown in the presence of selective medium. Expression of the NV protein was monitored by indirect immunofluorescence assay using a rabbit antiserum directed against *Escherichia coli*-expressed NV_{IHNV} protein (kindly provided by H. Schutze) (23).

pIHNV-ΔNV and pIHNV-NV_{VHSV} plasmid constructs. Full-length IHNV-derived antigenomic cDNA constructs containing the open reading frame (ORF) of the gene of either the enhanced green fluorescent protein (GFP) or the NV from VHSV instead of the NV_{IHNV} ORF (pIHNV-ΔNV) are depicted in Fig. 1. In these constructs, the reporter gene is bordered by two unique SpeI and SmaI restriction enzyme sites that were used to remove the reporter gene and used to insert the NV_{VHSV} ORF, leading to pIHNV-NV_{VHSV}. NV_{VHSV} ORF was gen-

erated by reverse transcription-PCR (RT-PCR) from RNA extracted from EPC VHSV-infected cells by using specific primers (24).

Recovery of recombinant virus. Transfection experiments were carried out as described previously (4). Approximately 4×10^6 EPC cells per well were grown in six-well plates and infected with the recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase at a multiplicity of infection of 5. After 1 h of adsorption at 37°C, the cells were washed twice and transfected with a plasmid mixture containing 0.25 μg of pT7-N, 0.2 μg of pT7-P, 0.2 μg of pT7-L, and 0.12 μg of pT7-NV with 1 μg of the pIHNV-ΔNV full-length cDNA construct by using the Lipofectamine reagent (Gibco-BRL) according to the supplier's instructions. Alternatively, a plasmid mixture containing 0.25 μg of pT7-N, 0.2 μg of pT7-P, and 0.2 μg of pT7-L without any pT7-NV was used with 1 μg of the pIHNV-NV_{VHSV} full-length cDNA construct. The cells were incubated for 7 h at 37°C and then shifted at 14°C for 6 days. Subsequently, the cells were suspended by scratching the plates with a rubber policeman and then subjected to two cycles of freezing and thawing. The cell culture supernatant (called P0 supernatant) was clarified by centrifugation at $6,000 \times g$ for 10 min in a microcentrifuge and used to inoculate fresh EPC cell monolayers in 24-well plates at 14°C.

Enrichment of virus mutants. Supernatants obtained at the recovery step (P0 inocula) were subjected to three passages on EPC cells, one passage on CHSE cells, and a final passage on EPC cells. At each step, viral inoculum was concentrated 25- to 100-fold (depending on the passage number) by ultracentrifugation with a SW28 rotor at 25,000 rpm for 45 min at 4°C.

Plaque assay. EPC cells grown in 6- or 12-well plates were infected with the supernatants of each time point postinfection (p.i.) (for the growth curve) of each virus (wt rIHNV, rIHNV-ΔNV, and rIHNV-NV_{VHSV}). After 1 h of adsorption at 14°C, cells were overlaid with agarose (0.35% in GMEM-Tris culture medium). Five days p.i., cell monolayers were fixed with 10% Formal, the agarose overlay was removed, and cells were stained with 2% crystal violet. Titers were expressed in PFU per milliliter.

Immunofluorescence and fluorescence-activated cell sorting assays. Intracellular NV_{IHNV} protein expression in EPC-NV cell clones was checked by an immunofluorescence assay. Cells (in 96-well plates) were fixed with a mixture of alcohol-acetone (1:1 [vol:vol]) at -20°C for 15 min. NV immunodetection was performed by incubation with a rabbit NV-specific antiserum diluted to 1:400 in phosphate-buffered saline (PBS)-Tween for 45 min at room temperature. Cells were then washed incubated with fluorescein-conjugated anti-rabbit immunoglobulins (P.A.R.I.S., Compiègne, France), washed again, and examined for staining.

For the comparative kinetic study of virus spread, EPC cells were infected with wt rIHNV, rIHNV-ΔNV, and rIHNV-NV_{VHSV} at different multiplicities of infection (MOIs) ranging from 0.03 to 3 for 48 h, and infected cells (in 96-well plates) were processed as described above, except that primary antibody was a mouse monoclonal antibody (MAb) directed against the IHNV-P protein (unpublished result) and the secondary antibody was a fluorescein-conjugated anti-mouse antibody (P.A.R.I.S.). Cells were examined for staining with a UV light

FIG. 2. Characterization of rIHNΔNV virus phenotype on EPC cells. (A) Photomicrographs of foci formed by rIHNΔNV and wt rIHNΔNV on EPC cells in six-well dishes. The cell monolayers were infected with rIHNΔNV and wt rIHNΔNV and then cultured under an agarose-containing medium. Cells were fixed at 5 days p.i. and stained with crystal violet. (B) Immunofluorescence microscopy photograph of rIHNΔNV-infected EPC cells (left column) compared to wt rIHNΔNV-infected EPC cells (right column) at different times p.i.. Cells were infected at the same MOI (0.3) and fixed at the indicated times. Staining was with an anti- $P_{\text{IHNΔNV}}$ MAb and a fluorescein isothiocyanate-conjugated goat anti-mouse MAb. Cell monolayers were examined with a UV light microscope.

microscope and photographed with a computer-coupled camera (Nikon, Champigny-sur-Marne, France).

Alternatively, intracellular IHNΔNV proteins were quantified by flow cytometric analysis. EPC cells (in 12-well plates) were mock infected or infected with wt rIHNΔNV, rIHNΔNV, or rIHNΔNV- ΔNV_{VHSV} . At 42 h p.i., cell monolayers were treated with trypsin and split into polypropylene tubes for further staining. Cell suspensions were first fixed for 15 min with 4% paraformaldehyde in PBS, washed in PBS, and incubated with a rabbit anti-IHNΔNV antiserum diluted to 1:400 in permeabilization buffer (PBS containing 1% fetal calf serum, 0.1% [wt/vol] sodium azide, and 0.1% [wt/vol] saponin) for 45 min at room temperature. Cells were then washed in permeabilization buffer, incubated with fluorescein-conjugated anti-rabbit immunoglobulins diluted in permeabilization buffer, and finally washed in PBS. Cells were then analyzed by flow cytometry with a FACScan cytofluorimeter and CellQuest software (Becton Dickinson Co., Mountain View, Calif.).

Western blot analysis. EPC cells were infected at an MOI of 2 with wt rIHNΔNV, rIHNΔNV, and rIHNΔNV- ΔNV_{VHSV} . At different times p.i., cells pellets were disrupted by addition of 2× sample buffer (100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2% bromophenol blue, 200 mM dithiothreitol, 1% (vol/vol) β-mercaptoethanol). Approximately 2.10^5 cells equivalents of each infected cell extract was subjected to electrophoresis on SDS-4 to 12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membrane (Schleicher & Schuell). The blots were incubated with rabbit antiserum raised against purified IHNΔNV. Viral proteins were visualized by secondary incubation with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G antibodies (P.A.R.I.S.) revealed with an enhanced chemiluminescence detection system (Amersham Pharmacia).

Experimental fish infection and subsequent challenge. Experimental fish infections were conducted either by immersion or by injection as follows. Seventy five virus-free juvenile *Oncorhynchus mykiss* rainbow trout (mean weight, 0.4 g) were infected by static immersion with wt rIHNΔNV, rIHNΔNV, and rIHNΔNV- ΔNV_{VHSV} (final titer, 5×10^4 PFU/ml) for 3 h at 14°C in aquariums filled with 3 liters of freshwater. The aquariums were then filled up to 30 liters with freshwater. Alternatively, 75 virus-free juvenile trout were infected by intraperitoneal injection of 10^5 PFU per animal with a 100-μl inoculum of the various recombinants IHNΔNV. The controls were fish mock infected with cell culture medium and fish infected with wt IHNΔNV 32/87 French strain under the same conditions. Mortalities were monitored daily. The spleens, kidneys, and brains of dead or sampled fish were homogenized in a mortar with a pestle and sea sand in 9 volumes of GMEM medium containing penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). After centrifugation at $2,000 \times g$ for 15 min at 4°C, the supernatant was inoculated onto EPC cells, and virus-induced cytopathic effect was checked.

For subsequent challenge. Groups of 50 rainbow trout (mean weight, 0.4 g) were inoculated by immersion on day 0 with 5×10^4 PFU of rIHNΔNV virus/ml or were mock infected with cell culture medium. On day 30, both groups of trout were inoculated by immersion as described above with 5×10^4 PFU of the wt IHNΔNV 32/87 French strain/ml, and mortalities were monitored for 30 days.

Nucleotide sequencing. All of the nucleotide sequences of the plasmid constructs used in the present study were checked by sequencing reactions carried out on an ABI 373A DNA automatic sequencer by using the DyeDeoxy Terminator Prism kit (Applied Biosystem, Division Perkin-Elmer) and specific primers.

FIG. 3. NV *trans*-complementation of rIHNV- Δ NV virus in EPC clones. Several EPC cell clones expressing NV gene or EPC control cells were infected with rIHNV- Δ NV virus. (A) Infectious viral particle production was measured by comparing the percentages of infected cells at the same dilution of the supernatant 4 days p.i. for each virus. Infection was revealed by immunofluorescence assay with an anti-P_{IHN} MAb. The results are expressed as the relative increase of viral yield produced by NV-expressing clone compared to EPC control cells. Values were calculated as follows: (the percentage of infected cells in NV-expressing clone – the percentage of infected cells in EPC control cells)/(100 – the percentage of infected cells in EPC control cells). (B) Plaque size of two EPC clones expressing NV gene or EPC control cells infected with rIHNV- Δ NV virus. Cells were infected with each virus and cultured in an agarose-containing medium. At 5 days p.i., cell monolayers were fixed and stained.

RESULTS

Growth of NV-knockout viruses is strongly affected in fish cells. Recombinant IHNV knockout for the NV gene has been previously shown to be affected by their growth in cell culture (4). In the present study we investigated more in details regarding that observation.

Recombinant IHNV viruses are routinely amplified by two or three successive passages on EPC cells. However, attempts to produce stocks of rIHNV- Δ NV by using this procedure were unsuccessful. Indeed, although the recovery efficiency did not significantly differ between rIHNV- Δ NV and wt rIHNV (750 and 1,370 PFU/ml, respectively), the successive passages on EPC cells monolayers resulted in a decrease in virus titer for rIHNV- Δ NV viruses, leading to the loss of the virus stock at passage 3 ($<10^3$ PFU/ml for rIHNV- Δ NV versus $\sim 10^8$ PFU/ml for wt rIHNV). Therefore, all further studies were performed with rIHNV- Δ NV virus stocks produced by a protocol that includes an ultracentrifugation concentration step (see Materials and Methods). This observation indicates that the replication of rIHNV- Δ NV viruses was severely affected by the NV deletion.

NV-knockout IHNV induces pinpoint plaques. To further investigate that observation, plaque assay was developed, and the plaque sizes were compared. EPC cells were infected with wt rIHNV or rIHNV- Δ NV viruses and incubated for several

days until the appearance of plaques. The cells were then fixed and stained with crystal violet, and the plaque sizes were compared (Fig. 2A; data are shown for rIHNV- Δ NV only). Although the plaques for wt rIHNV appear as soon as 3 days p.i., plaques formation for all of the three NV-knockout rIHNV was delayed. In addition, the plaque sizes for these viruses were drastically reduced compared to wt rIHNV plaques. Thus, rIHNV- Δ NV viruses are small plaque mutants, confirming a biological activity of NV. Titration of virus stocks indicates that the titer of rIHNV- Δ NV obtained by an enrichment procedure (see Materials and Methods) was roughly identical to the wt rIHNV titer ($\sim 10^8$ PFU/ml). To assess whether the spread of recombinant virus from cell to cell was affected by NV deletion, a comparative study on the kinetics for the propagation of the recombinant viruses on cell monolayers was performed. EPC cells were infected with either rIHNV- Δ NV or wt rIHNV at the same multiplicity of infection (MOI = 0.3) and incubated for 24, 36, 48, and 60 h p.i. Infected cells were revealed by immunostaining with a MAb directed against IHNV-P protein (Fig. 2B). At 24 h p.i., the sizes and numbers of foci of infected cells were similar for rIHNV with an NV deletion and wt rIHNV (Fig. 2B, upper panels). From 36 to 60 p.i., drastic differences appeared between rIHNV- Δ NV- and wt rIHNV-infected cell foci. The numbers and sizes of foci of cells infected with wt rIHNV evolved with respect to the

FIG. 4. Recovery and characterization of rIHN-NV_{VHSV} virus phenotype on EPC cells. (A) Gel analysis of the RT-PCR product to identify rIHN-NV_{VHSV}. Total RNA extracted from cells infected with wt rIHN, rIHN-ΔNV, or rIHN-NV_{VHSV} was amplified by RT-PCR with NV_{VHSV}-specific derived primers (see Materials and Methods). PCR products were analyzed on a 1% agarose gel. (B) The sizes of foci formed by rIHN-NV_{VHSV} on EPC cells (upper wells) are compared to these wt rIHN-induced foci (lower wells) at the same dilution (10^{-5} and 10^{-6}). At 5 day p.i., infected cells were fixed for both viruses and stained with crystal violet. (C) rIHN-NV_{VHSV}-infected EPC cells (left column) or wt rIHN-infected EPC cells (right column) were infected at the same MOIs. Infection was monitored as described for Fig. 2B.

total spreading of the virus to the cell monolayer; in contrast, the sizes and numbers of foci of cells infected with NV-knockout IHN increased extremely slowly. When the fluorescence confluence in wt rIHN-infected monolayers was observed (60 h p.i.; Fig. 2B, lower panels), only small fluorescent foci were detected in rIHN-ΔNV-infected cultures. This indicates that rIHN-ΔNV infection is still restricted in a few cells. In addition, light microscopic observation suggested that the intensity of cell fluorescence was reduced in rIHN-ΔNV-infected cells compared to wt rIHN-infected cells. This observation was confirmed by flow cytometric analysis (see Fig. 5B), indicating that the total amount of viral antigens per cell was slightly decreased in rIHN-ΔNV-infected cells. Similar results on comparative viral growth and comparative kinetic of viral dissemination on cell monolayers were observed with RTG₂ cells (data not shown), showing that the impact of NV deletion on rIHN phenotype described on EPC cells is not cell type specific.

trans-complementation of rIHN knockout for NV. The finding that the growth of rIHN-ΔNV was strongly altered on EPC cells prompted us to test whether rIHN-ΔNV-altered growth phenotype could be reverted by NV *trans*-complementation. For that, EPC cells were stably transformed with a plasmid encoding the NV gene under the control of the early CMV promoter (pcDNA-NV). Several EPC-NV cell clones were selected, and the yield of expression of the NV protein was monitored by indirect immunofluorescence assay by using a rabbit polyclonal antiserum directed against NV (data not shown). NV expression was shown to be variable depending on the considered EPC-NV cell clone. Compared to EPC cells, the only notable phenotypic feature of the EPC-NV cell clones was a decreased adherence of the cells to the plastic wall. EPC-NV cell clones and EPC control cells were infected with rIHN-ΔNV and incubated for 5 days p.i., until the development of a total cytopathic effect (Fig. 3A). Parallel infections were performed with wt rIHN. Supernatants were harvested,

FIG. 5. Expression of IHNV proteins by rIHNV-ΔNV and rIHNV-NV_{VHSV}. (A) Time course of IHNV protein expression by recombinant wt IHNV, rIHNV-ΔNV, and rIHNV-NV_{VHSV}. EPC cells were infected with wt rIHNV, rIHNV-ΔNV, and rIHNV-NV_{VHSV} at an MOI of 2. Total cell extracts were harvested at the indicated times p.i. and subjected to Western blot analysis with an antiserum directed against purified IHNV. Viral proteins revealed are indicated on the right. (B) Flow cytometry was used to compare the level of total IHNV antigens synthesized in rIHNV-ΔNV-infected cells (lower panel), wt rIHNV-infected cells (upper panel), and rIHNV-NV_{VHSV}-infected cells (middle panel). For each infected culture, the fluorescence intensity obtained in EPC cells 42 h after infection (bold line) and the background fluorescence of uninfected EPC cells (curve on the left) were determined by using a rabbit anti-IHNV serum and a fluorescein isothiocyanate-conjugated goat anti-rabbit MAb. The results are representative of three independent experiments. (C) Release of recombinant wt IHNV, rIHNV-ΔNV, and rIHNV-NV_{VHSV} by infected cells. Supernatants from EPC cells infected with wt rIHNV, rIHNV-ΔNV, or rIHNV-NV_{VHSV} were harvested 72 h p.i. After they were clarified, supernatants were submitted to ultracentrifugation to pellet the viral particles. Samples were then subjected to Western blot analysis with an antiserum directed against purified IHNV. Viral proteins revealed are indicated on the left.

and titers were determined by endpoint dilution, and samples were revealed by immunofluorescence with a polyclonal antibody directed against purified IHNV. The results are expressed as the “relative increase in viral yield” as given by the formula: (the percentage of infected cells in NV-expressing clone – the percentage of infected cells in EPC control cells)/(100 – the percentage of infected cells in EPC control cells). Interestingly, the relative increase of the viral production of rIHNV-ΔNV was between 13- and 50-fold higher in rIHNV-ΔNV-infected EPC-NV cell clones than in rIHNV-ΔNV-infected EPC control cells. As a result, the titer of rIHNV-ΔNV produced in EPC-NV cells was roughly similar to the titer of wt rIHNV produced in EPC control cells ($\sim 10^8$ PFU/ml). However, no direct relationship could be established between the viral yield production into rIHNV-ΔNV-infected EPC-NV cell clones and the level of expression of the NV protein. In addition, the viral titer of wt rIHNV was ~ 10 -fold increased when EPC-NV cell clones were used for infection instead of EPC cells (data not shown). Thus, infection of EPC-NV cell clones with rIHNV-ΔNV resulted in an increased productive growth of the recombinant virus.

The sizes of the plaques induced by rIHNV-ΔNV infection onto EPC-NV clones and EPC control cells were compared (Fig. 3B). In contrast to the small-plaque phenotype observed

on EPC control cells, the plaque sizes of rIHNV-ΔNV on EPC-NV clones were very similar to those of wt rIHNV on EPC cells, indicating that NV *trans* expression in EPC cells caused reversion of the rIHNVΔNV plaque phenotype. Thus, the NV protein can be provided in *trans* to restore a normal growth of rIHNV knockout for NV and even to overproduce the wt rIHNV. The latter aspect suggests a viral replication regulatory role of the NV protein in infected-cell cultures. These data provide evidence that NV gene product is not only a structural “spacer” in IHNV genome that allows an optimal processivity of the viral polymerase during transcription or replication of IHNV. This result demonstrates that NV protein has a biological function necessary for efficient IHNV replication.

VHSV-NV protein can functionally replace the IHNV-NV protein. Although both IHNV and VHSV NV proteins do not exhibit significant amino acid residues identity, the similar locations of the two NV genes within their homologous genomes and the conservation of a functional gene prompted us to investigate whether both NV could have a similar impact on virus growth. To investigate that, a recombinant IHNV expressing the VHSV NV gene was engineered (Fig. 1). A pIHNV-NV_{VHSV} was constructed by replacing the GFP gene with the VHSV NV gene in pIHNV-ΔNV construct. After

FIG. 6. Phenotype of rIHN Δ NV and rIHN Δ NV_{VHSV} viruses in vivo and immunization with rIHN Δ NV against wt IHN challenge. Mortality of juvenile rainbow trout inoculated by immersion or by intraperitoneal injection. (A) Juvenile rainbow trout were inoculated by static immersion with 5×10^4 PFU of rIHN Δ NV, rIHN Δ NV_{VHSV}, wt rIHN, or wt IHN 32/87 French strain/ml or were mock infected. (B) Juvenile trout were inoculated by intraperitoneal injection with 10^5 PFU of rIHN Δ NV, rIHN Δ NV_{VHSV}, wt rIHN, or wt IHN 32/87 French strain. Cumulative mortalities recorded at 30 days p.i. are expressed. (C) Fifty virus-free juvenile trout were inoculated by immersion with 5×10^4 PFU of rIHN Δ NV virus/ml or were mock infected with cell culture medium. At day 30 p.i., both groups of trout were challenged by immersion as described above with 10^6 PFU of the wt IHN 32/87 French strain/ml. Mortalities were monitored daily. Cumulative mortalities at day 30 postchallenge are presented.

transfection of vTF7-3-infected EPC cells with a mixture of pIHN Δ NV_{VHSV}, pT7-N, pT7-P, and pT7-L, a recombinant rIHN Δ NV_{VHSV} was recovered and amplified through passages in cell culture. The demonstration that the NV_{VHSV} gene was expressed by the rIHN Δ NV_{VHSV} was provided by RT-PCR on RNA extracted from rIHN Δ NV_{VHSV}-infected EPC cells (Fig. 4A). The expected 395-nucleotide PCR product was observed only in the rIHN Δ NV_{VHSV}.

In the case described above, the production of virus stock did not require any centrifugation concentration step as described for rIHN Δ NV viruses. rIHN Δ NV_{VHSV} virus growth was indistinguishable from that of wt rIHN, since the titer after three passages onto EPC cells was roughly 10^8 PFU/ml. To compare the growth of rIHN Δ NV_{VHSV} and wt rIHN in EPC cells, the plaque sizes and the comparative kinetics of the virus spread were determined by immunostaining with an anti-P_{IHN} MAb. As shown in Fig. 4B, the plaque phenotype of rIHN Δ NV_{VHSV} was very similar to wt rIHN. The appearance of fluorescent foci (24 h p.i.) and the further propagation of infection were very similar for rIHN Δ NV_{VHSV} and wt rIHN (Fig. 4C), indicating that NV protein from VHSV and IHN have a similar effect on IHN replication cycle. In addition, in contrast to what was previously observed for

rIHN Δ NV-infected cells, the fluorescence intensity of rIHN Δ NV_{VHSV}- and wt rIHN-infected cells was not significantly different.

Altogether, these results show that rIHN Δ NV_{VHSV} and wt rIHN growth in EPC cell is similar, showing that rIHN Δ NV defects were efficiently counteracted by NV_{VHSV} *cis* expression in EPC cells. Thus, NV_{VHSV} protein is able to fulfill the function of NV_{IHN} that is important for efficient IHN replication. Furthermore, similar results were obtained with RTG₂ cells (data not shown), confirming that functional replacement of NV_{IHN} by NV_{VHSV} is not cell type specific.

Viral protein synthesis is not significantly affected by the lack of NV expression. Although the spread on cell monolayers was drastically different for rIHN Δ NV, rIHN Δ NV_{VHSV}, and wt rIHN, we sought to determine whether the rate of IHN protein synthesis could be altered by the absence of NV. Therefore, EPC cells were infected with rIHN Δ NV, rIHN Δ NV_{VHSV}, or wt rIHN at an MOI of 2 PFU per cell, and total proteins were harvested at several time points. Western blot analysis of the protein extracts with an antiserum directed against IHN showed that the accumulation of viral proteins was detectable under these conditions beginning between 6 and 14 h p.i. for wt rIHN and rIHN Δ NV_{VHSV} but only 18 h

p.i. for rIHNV- Δ NV (Fig. 5A). The global levels of expression of viral proteins detected by this serum, i.e., G, N, P, and M, were only transiently affected by NV deletion. The accumulation of viral protein in rIHNV- Δ NV and in rIHNV-NV_{VHSV}-infected cells appeared to proceed with a delayed kinetics (8 and <4 h, respectively, for rIHNV- Δ NV and rIHNV-NV_{VHSV}) and at a somewhat lower magnitude than in wt rIHNV-infected cells. However, by 26 h p.i., the level of viral proteins was only modestly higher in cells infected with wt rIHNV.

Flow cytometric analysis was performed to quantify this latter observation at a time point corresponding to a late stage of infection and before the total destruction of the cell monolayer (Fig. 5B). For that, EPC cell monolayers were infected with rIHNV- Δ NV, rIHNV-NV_{VHSV}, and wt rIHNV at the same MOI of 2, followed by incubation for 42 h. Infected cell cultures were then briefly treated with trypsin, and infection was revealed with an anti-IHNV polyclonal serum. Samples were then analyzed by flow cytometry. Parallel analysis was performed on mock-infected EPC cells. The fluorescence patterns revealed that the intensity of cells expressing IHNV antigens was comparable between rIHNV- Δ NV-infected cells (12 arbitrary units) and wt rIHNV-infected cells (16 arbitrary unit). Moreover, the global amount of IHNV antigens was slightly but significantly increased in rIHNV-NV_{VHSV}-infected cells compared to wt rIHNV-infected cells (26 versus 16 arbitrary units). This indicates that at 42 h p.i. the NV_{VHSV} *cis* expression counteracts the slight effect of the NV loss and is even more efficient at allowing viral protein synthesis in EPC cells than when NV_{IHNV} is naturally expressed.

Although the levels of proteins were not dramatically different between NV-knockout and NV-expressing viruses, it was possible that the lack of NV expression had an effect on the release of viral particle-infected cells. By 72 h p.i., when the cell monolayer was totally destroyed for both infected cultures, the supernatants were assayed for the presence of viral proteins (Fig. 5C). The level of viral proteins detected by Western blotting with a polyclonal anti-IHNV was clearly decreasing from wt rIHNV to rIHNV- Δ NV. This result indicates that the loss of NV expression has a significant effect on the release of viral particles that is partially restored in the presence of NV_{VHSV}.

NV is associated with pathogenicity in trout. The abilities of all of the viruses described here to replicate *in vivo* and to induce disease symptoms in trout were compared. Samples of 75 juvenile trout (mean weight, 0.4 g) were infected by bath immersion with 5×10^4 PFU of the wt rIHNV, rIHNV- Δ NV, or rIHNV-NV_{VHSV}/ml. As a positive control, wt IHNV 32/87 strain was included. Mortalities were recorded every day for 4 weeks after virus exposure. As shown in Fig. 6A, wt IHNV 32/87, wt rIHNV, and rIHNV-NV_{VHSV} are also highly pathogenic for trout since cumulative mortalities reached a plateau of between 80 and 100% by 1 month p.i. Trout infected with these viruses developed typical symptoms of IHNV infection, and fish began dying between days 5 and 6. In contrast, trout infected with the rIHNV- Δ NV did not develop any external signs of disease, and no mortality was recorded. Virus was recovered from all sampled survivors or dead fish infected with wt rIHNV and rIHNV-NV_{VHSV} (i.e., five of five tested) but from only one sample of five trout infected with rIHNV- Δ NV

at 2 weeks p.i.. This experiment shows that the NV gene is essential for IHNV pathogenicity in trout and that NV_{VHSV} protein can functionally replace the NV_{IHNV} protein both *in vitro* and *in vivo*.

To rule out the possibility that the absence of mortality observed when trout were exposed to the rIHNV- Δ NV could be due to the route of administration, experimental infections were performed by injection (Fig. 6B). Samples of 75 juvenile trout (mean weight, 1.5 g) were infected by injection with 100μ l of 10^6 PFU of wt IHNV 32/87, wt rIHNV, or rIHNV- Δ NV/ml, and mortalities were recorded every day for 4 weeks after the virus injection. As shown in the Fig. 6B, only 25% of death was recorded after 4 weeks for trout injected rIHNV- Δ NV compared to 90 to 100% of wt IHNV (rIHNV or IHNV 32/87)-infected animals. Thus, differences in virus pathogenicity were independent of the route of administration. These results were consistent with a loss of pathogenicity due to NV deletion, and this effect is reversed by NV_{VHSV} expression.

Altogether, the data demonstrated that both NV proteins from IHNV and VHSV play an essential and similar role for the pathogenicity of novirhabdovirus in trout.

The protection induced by rIHNV- Δ NV against wt IHNV in trout was evaluated (Fig. 6C). Samples of 50 juvenile trout were infected by bath immersion with either 5×10^4 PFU of the rIHNV- Δ NV/ml or with cell culture medium only (mock infected). At 30 days p.i., both samples were challenged with 10^6 PFU of the wt IHNV 32/87 strain/ml, and mortalities were recorded every day for 4 weeks after the challenge. As shown in Fig. 6C, the rIHNV- Δ NV provided a high level of protection against challenge with the wt IHNV 32/87 strain, since 92% of trout previously infected with rIHNV- Δ NV were resistant to wt IHNV administered by bath immersion. These results indicated that rIHNV- Δ NV viruses are attenuated live viruses which induce a protective immune response in trout.

DISCUSSION

The data presented here show that the NV gene product of the IHNV is necessary for efficient virus replication in cell culture and, more interestingly, that NV protein is essential for virus pathogenicity in trout. The essential role of NV for IHNV efficient replication was suggested by the observation that NV-knockout rIHNV have altered growth in cell culture, reflected by a small-plaque phenotype and a reduced spread on EPC cell monolayers. When cells constitutively expressing NV were infected with rIHNV- Δ NV, we found that the replication defect can be rescued by the *trans* expression of the NV gene. These data provide evidence not only that the NV gene acts as a structural "spacer" in the IHNV genome for the optimal processivity of the viral polymerase during transcription or replication of or IHNV but also that the NV gene product possesses an essential biological function for the IHNV growth in cell culture. The *trans*-complementation experiments provide evidence that the lack of NV_{IHNV} expression by itself is responsible of rIHNV- Δ NV phenotype.

Interestingly, it was found here that IHNV- and VHSV-NV gene products have a globally similar impact on the IHNV virus cycle *in vitro*. The growth of the chimeric virus rIHNV-NV_{VHSV} and of wt rIHNV were very similar, as demonstrated by the equal plaque sizes phenotype and by identical spreading

features. This result was unexpected since computer-assisted comparison analysis showed that NV_{IHN}V and NV_{VHSV} proteins are not closely related, exhibiting a low level of amino acid sequence similarities (47.6%) and no obvious conserved domains. These observations were supporting the hypothesis that NV gene would not be essential for the virus replication in fish per se or that the role of NV protein could have diverged among the novirhabdoviruses (17). The data presented here give new insight on NV importance for fish rhabdovirus biology. However, *in vitro* studies revealed that rIHN_V-NV_{VHSV} differs from wt rIHN_V in two minor aspects: (i) the cytopathic effect of rIHN_V-NV_{VHSV}-infected EPC cells was slightly different from that of wt rIHN_V-infected EPC cells (rounding cells aspect was more pronounced) and (ii) the amounts of viral proteins synthesized until 42 h p.i. were slightly increased in rIHN_V-NV_{VHSV}- compared to wt rIHN_V-infected cells (Fig. 5B). The molecular basis of these phenomena remains unclear. A complete demonstration would be given by the study of an rVHSV expressing NV_{IHN}V but a VHSV reverse genetic system is not available yet.

For both IHNV and VHSV, the function of the NV gene and/or NV protein is still unknown and there are no reports of genes analogous to NV in the genome of rhabdoviruses of nonfish hosts. The NV_{IHN}V protein is a small nonstructural protein (ca. 12.3 to 14.5 kDa for NV_{VHSV}) that is expressed in infected cells as soon as 24 h p.i. in EPC cells (23; unpublished data). No particular interaction with other viral components (IHNV proteins or genome) has been described. During infection, NV_{IHN}V is distributed diffusely in the cytoplasm of infected cells (23; unpublished data). The rIHN_V-ΔNV phenotype described in the present study can result from a decrease of infectious viral particles produced per cell and/or from a virus-induced refractory state of neighbor cells. We show that the virus titer of infectious rIHN_V produced per cell was indeed strongly reduced by NV deletion. A decrease in the rIHN_V titer in the absence of NV could result from the reduced production of IHNV proteins observed in rIHN_V-ΔNV-infected cells (as determined by flow cytometry and Western blotting experiments), suggesting that NV would directly play a role in the transcription-transduction process of IHNV. Alternatively, the absence of NV could result in a sequestration of virus components or of intracellular viral particles into inappropriate cell compartments or even in the inhibition of IHNV budding. The first hypothesis seems unlikely since the drastic decrease in viral particles produced in rIHN_V-ΔNV cell culture supernatants does not reflect the slight reduction of IHNV proteins synthesized in infected cells. In contrast, the latter hypothesis would be in agreement with the strong reduction in viral particle production observed for rIHN_V-ΔNV only (Western blotting experiment), without any drastic changes in IHNV structural protein synthesis in infected cells. Preliminary analysis of NV function in transfected cells suggests that it could interact with the cytoskeleton (6), explaining possibly the differences of cytopathic effect induced by rIHN_V viruses expressing NV_{IHN}V or NV_{VHSV}. Such hypotheses, involving a role for NV in virus morphogenesis, as previously suggested for bluetongue virus protein NS3 (3), require further studies. Alternatively, the limited spread of rIHN_V-ΔNV may be indirectly explained by an anti-interferon role for NV. As described for several mammalian viruses carrying nonstructural

protein-encoding genes, it can be hypothesized that NV could present activities interfering with interferon cascade, leading to the restriction of this infection (1, 5, 9, 11, 14, 16, 20, 21, 27; for a review, see reference 13). The study of a potential anti-interferon activity is difficult because the rainbow trout interferon-encoding gene has not been cloned yet. However, this hypothesis is supported (i) by the small-plaque phenotype of rIHN_V-ΔNV, which also characterizes interferon-deficient mutants; (ii) the fact that preliminary experiments based on ultracentrifugation suggest that an antiviral soluble molecule is differentially induced by rIHN_V-ΔNV and wt rIHN_V (unpublished observations); and (iii) by the behavior of rIHN_V-ΔNV in trout, which is immunogenic without being virulent (see below). The potential involvement of NV gene products in interferon cascade is currently under investigation.

The ability of the various recombinants IHNV to induce disease and mortality in trout has been evaluated. Interestingly, we found that NV deletion rIHN_V viruses are not pathogenic for juvenile trout, demonstrating that NV_{IHN}V is essential for the IHNV virulence. Similar results were obtained when viruses were administered either by bath immersion or by intraperitoneal injection, demonstrating that the lack of trout virus-induced pathogenicity is independent from the way of administration. As it is *in vitro*, the level of rIHN_V-ΔNV replication is likely to be reduced *in vivo*, but it is clear that rIHN_V-ΔNV viruses efficiently penetrate into the fish without inducing any signs of disease. We also demonstrated that the pathogenicity of recombinant virus was restored when NV_{VHSV} is expressed. Thus, NV from IHNV or VHSV may be considered as newly identified virulence factors with the same functional properties. The basis of the protective properties of viruses with NV deleted requires further characterization since rIHN_V-ΔNV viruses may be of therapeutic value for vaccine development. The challenge in developing live-attenuated virus vaccines is to eliminate residual virulence without compromising immunogenicity. By deleting a nonstructural encoding gene, the present method for attenuating IHNV is to reduce indirectly its level of replication. This process could, unfortunately, reduce its immunogenicity due to the reduction of viral antigens, as observed for rIHN_V-ΔNV *in vitro*. In the present study, we evaluated the rIHN_V-ΔNV viruses for protective efficiency in trout. When administrated by immersion, rIHN_V-ΔNV virus efficiently protects juvenile rainbow trout against challenge with IHNV 32/87, demonstrating that rIHN_V-ΔNV is still immunogenic. Thus, as described for defective NS1 mutants of influenza virus A (19) or for NS2 deletion mutants of RSV (7), the deletion of NV in the IHNV genome constitutes an attractive approach for the generation of live vaccines by attenuating virus virulence while retaining protective properties. Furthermore, deletion mutants such as rIHN_V-ΔNV viruses are irreversibly modified, making them attractive candidates for vaccine development.

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