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Construction of nervous necrosis virus (NNV) genome-based DNA replicon vectors for the delivery of foreign antigens

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The advantages of replicon vectors of RNA viruses include a high ability to stimulate innate immunity and exponential amplification of target mRNA leading to high expression of foreign antigens. The present study aimed to construct a DNA-layered nervous necrosis virus (NNV) replicon vector system in which the capsid protein gene was replaced with a foreign antigen gene and to compare the efficiency of foreign antigen expression between the conventional DNA vaccine vector and the present replicon vector. We presented the first report of a nodavirus DNA replicon-based foreign antigen expression system. Instead of a two-vector system, we devised a one-vector system containing both an NNV RNA-dependent RNA polymerase cassette and a foreign antigen-expressing cassette. This single-vector approach circumvents the issue of low foreign protein expression associated with the low co-transfection efficiency of a two-vector system. Cells transfected with a vector harboring hammerhead ribozyme-fused RNA1 and RNA2 (with the capsid gene ORF replaced with VHSV glycoprotein ORF) exhibited significantly higher transcription of the VHSV glycoprotein gene compared to cells transfected with either a vector without hammerhead ribozyme or a conventional DNA vaccine vector expressing the VHSV glycoprotein. Furthermore, the transcription level of the VHSV glycoprotein in cells transfected with a vector harboring hammerhead ribozyme-fused RNA1 and RNA2 showed a significant increase over time. These results suggest that NNV genome-based DNA replicon vectors have the potential to induce stronger and longer expression of target antigens compared to conventional DNA vaccine vectors.

Key words: Nervous necrosis virus, DNA-layered replicon vector, One-vector system, Heterologous antigen expression

Introduction

Recently, research on the application of in vitro transcribed RNA or DNA layered vectors of RNA virus genomes for the development of effective delivery tools for foreign antigens has been actively conducted in mammals to enhance the protective efficacy of vaccines (Englezou et al., 2018; Ljungberg et al., 2007; Reap et al., 2007). While much of this research in mammals has focused on alphaviruses due to their versatility in host specificity and foreign gene expression (Kim et al., 2014; Dominguez et al., 2023), the same advantages cannot be easily applied to fish alphaviruses. Fish alphaviruses, represented by salmon alphavirus, replicate only at low temperatures, thus limiting the applicability of fish alphavirus-based replicon vectors in fish living in warmer environments (McLoughlin and Graham, 2007). Negative-sense RNA viruses such as rhabdoviruses, including viral

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hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), snakehead rhabdovirus (SHRV), and spring viremia carp virus (SVCV), exhibit temperature preferences according to species (Ahne et al., 2002; Kim et al., 2019; Lee et al., 2021). However, they are not suitable candidates for RNA or DNA layered replicon vectors due to the requirement for not only the full genome construct but also N-P-L ribonucleic protein complex-supplying vectors to express foreign antigens (Rouxel et al., 2016).

Given that nodaviruses are positive-sense RNA viruses and piscine nodaviruses prefer warm temperatures (20~30°C) (Panzarin, 2014; Toffan, 2016), they hold potential as replicon vectors for delivering antigens to warm-water fish. However, nodavirus genomes are bipartite - RNA 1 encoding RNA-dependent RNA polymerase and RNA 2 encoding capsid protein (Zhou and Routh, 2024). To construct foreign antigen-expressing DNA layered nodavirus replicon vectors, one cassette for expressing RNA 1 and another cassette harboring the entire sequence of RNA 2, in which the capsid protein gene ORF is replaced with the foreign antigen gene ORF, should be constructed in two separate vectors or one vector system. Additionally, ribozymes, one of several catalytic RNAs occurring in nature, are utilized for precise virus genome production in DNA-layered nodavirus replicons (Nolden et al., 2016; Atieh et al., 2018). Ball (1992) constructed flock house virus (FHV; an insect nodavirus) RNA replicon and verified a high synthesis rate of capsid proteins in cells transfected with the RNA replicon. To our knowledge, no reports are available on the replicons of piscine nodaviruses.

The advantages of replicon vectors of RNA viruses include a high ability to stimulate innate immunity and exponential amplification of target mRNA leading to high expression of foreign antigens (Atasheva et al., 2014; Näslund et al., 2011; Tonkin et al., 2010). The aim of the present study was to construct a DNA layered nervous necrosis virus (NNV) replicon vector system in which the capsid protein gene was replaced with a foreign antigen gene and to compare the efficiency of foreign antigen expression between the conventional DNA vaccine vector and the present replicon vector.

Materials and Methods

Cells and Virus

Baby hamster kidney (BHK-21) cells (KCLB-10010) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Welgene) at 37°C, and Epithelioma papulosum cyprini (EPC, ATCC) cells and E11 cells were cultured in Leibovitz medium (L15, Sigma) at 28 and 25°C, respectively. Each medium was supplemented with 10% fetal bovine serum (FBS, Welgene) and penicillin-streptomycin (100 U/ml penicillin, 100 μg/ml streptomycin; Welgene). VHSV KJ2008 of genotype IVa and red-spotted grouper nervous necrosis virus (RGNNV) were cultured using EPC and E11 cells at 15 and 25°C, respectively, in the presence of 2% FBS and antibiotics.

Construction of eGFP expressing vector

E11 cells were infected with RGNNV, and cDNA was synthesized using M-MLV Reverse Transcriptase (ELPIS) from total RNA extracted from cells showing 80% CPE. The NNV antigenome was detected using primers listed in Table 1 for the construction of the replicon vector.

To investigate the hammerhead ribozyme (HHR) effect on enhanced green fluorescence protein (eGFP) expression, the 5' UTR of the RNA2 gene fused to the 5' end of the eGFP ORF and the 3' UTR of the RNA2 gene fused to the 3' end of the eGFP ORF (UTR-eGFP-HDV) were amplified by overlapping PCR. Six nucleotides (nt), modified according to the 5' UTR of the RNA2 sequence at the beginning of the HHR region, and the HHR were synthesized using overhang PCR and fused to the upstream of the UTR-eGFP-HDV fragment by PCR amplification us-

Table 1. Primers used in this study

Primers	Sequence (5'-3')
For the construction of pCMV-UTR-eGFP-HDV	
eGFP-5UTR-SacI-F	GAGCTCTCTGGCTAACTAATCCATCACCGCTTTGCAATCAC
eGFP-5UTR-R	TCCTCGCCCTTGCTCACCATTGTGATTGCAAAGCGGTGAT
eGFP_F	ATGGTGAGCAAGGGCGAGGAGCTGTTCACCG
eGFP_R	TTACTTGTACAGCTCGTCCATGCC
eGFP-3UTR-F	TGGACGAGCTGTACAAGTAATGACCAATTTGAACAATTGA
RNA2_3UTR_HDV_R	GAGGTGGAGATGCCATGCCGACCCACCAGAGTTGAGAAGCGATCAGCGG
RNA2_3UTR_HDV_F	CCGCTGATCGCTTCTCAACTCTGGTGGGTCGGCATGGCATCTCCACCTC
eGFP-3UTR-HDV-NotI-R	GCGGCCGCTGGCTCTCCCTTAGCCATCCG
For the construction of pCMV-HHR- UTR-eGFP-HDV	
EGFP-HHR-5UTR-HindIII_F	AAGCTTGGATTACTGATGAGTCCGTGAGGAC
EGFP-3UTR-HDV-NotI-R	GCGGCCGCTGGCTCTCCCTTAGCCATCCG
For the construction of pRNA1-HDV	
RNA1-5UTR-SacI-F	GAGCTCTCTGGCTAACTAACATCACCTTCTTGCTCTGTTGAGTAAT
RNA1-HDV-R	TGGAGATGCCATGCCGACCCCGCCGAAGCGTAGGACAGCA
RNA1-HDV-F	TGCTGTCCTACGCTTCGGCGGGGTCGGCATGGCATCTCCA
RNA1-HDV-XbaI-R	TCTAGATGGCTCTCCCTTAGCCATCCGAGTGG
For the construction of pUTR-vG-HDV	
VHSV-G-5UTR-F1	CGCTTTGCAATCACAATGGAATGGAATACTTTTTCTTGGTGATT
VHSV-G-5UTR-F2	TAATCCATCACCGCTTTGCAATCACAATGGAA
VHSV-G-5UTR-SacI-F	GAGCTCTCTGGCTAACTAATCCATCACCGCTTTGCAATC
vG_1524_R	TCAGACCATCTGGCTTCTGGAGAAC
VHSV-G-3UTR-F1	TTCTCCAGAAGCCAGATGGTCTGATGACCAATTTGAACAATTGATTAA
VHSV-G-HDV-XbaI-R	TCTAGATGGCTCTCCCTTAGCCATCCG
For the construction of pHHR-RNA1-HDV	
VNNRNA1_gRNA-HHR_NheI_F	GCTAGCGCGATGTTACTGATGAGTCCGTGAGGA
HHR-RNA1_R	AACAGAGCAAGAAGGTGATGTTAGACTATAGGAATTCCTTTCCTAT
RNA1-5UTR-F	TAACATCACCTTCTTGCTCTGTTGAGTAAT
VHSV-G-HDV-HindIII-R	AAGCTTTGGCTCTCCCTTAGCCATCCGAGT
For the construction of pHHR-UTR-vG-HDV	
VHSV-G-5UTR-HHR-F1	GGAATTCCTATAGTCTAATCCATCACCGCTTTGCAATCACAATGG
VHSV-G-5UTR-HHR-F2	CGAAACTATAGGAAAGGAATTCCTATAGTCTAATCCATCACCGCT
VHSV-G-5UTR-HHR-F3	AGTCCGTGAGGACGAAACTATAGGAAAGGAATTCCTATAGTC
vG-gRNA-7nt-HHR10-BamHI-F	GATCCTGGATTACTGATGAGTCCGTGAGGACG
VHSV-G-HDV-NotI-R	GCGGCCGCTGGCTCTCCCTTAGCCATCCGAGTGG
For quantitative of plasmid copy number	
ori_353_RT_F	GCCACTGGTAACAGGATTAG
ori_467_RT_R	CAGAGCGCAGATACCAAATA
For quantitative of vG	
VG-realtime-641F	ATTGCCCTACCTCAGAAACG
VG-realtime-790R	CGGTCTTGATCCATTCTGTCC

ing primers listed in Table 1. The amplified fragment was designated as HHR-UTR-eGFP-HDV. Both fragments were cloned into the pGEX-T easy vector (Promega) using primers listed in Table 1. The fragments digested with SacI and NotI or HindIII and NotI were ligated into pcDNA3.1+, designated as pCMV-UTR-eGFP-HDV or pCMV-HHR-UTR-eGFP-HDV, respectively. EPC cells were transfected with 3 μg of pCMV-UTR-eGFP-HDV or pCMV-HHR-UTR-eGFP-HDV using Fugene HD (Promega) according to the manufacturer's instructions for the analysis of the HHR effect on eGFP translation levels. After 24 hours of transfection, cell fluorescence was observed under a microscope.

Construction of NNV replicon vector

The VHSV glycoprotein gene (vG) was PCR amplified using cDNA of VHSV antigenome collected from EPC cells infected with VHSV. The amplified product was cloned into the pGEX-T easy vector, and then the vG gene digested with NheI and HindIII was ligated to the pFC (PhiC31 Doner, SBI) vector after enzyme digestion, designated as pFC-vG.

The RNA1 gene corresponding to the RNA polymerase gene of NNV fused to the HDV ribozyme at the downstream region was amplified using overlapping PCR and cloned into the pGEX-T easy vector. The T vector was digested with SacI and XbaI, then the RNA1-HDV fragment was ligated to pcDNA3.1+, labeled as pRNA1-HDV. The 5' end of vG fused to the 5' UTR of the RNA2 gene and the 3' end of vG fused concurrently to the 3' UTR of the RNA2 gene and the HDV ribozyme (UTR-vG-HDV) were amplified using overlapping PCR with primers listed in Table 1 and cloned into the pGEX-T easy vector. The UTR-vG-HDV product digested with SacI and XbaI was inserted into pcDNA3.1+, designated as pUTR-vG-HDV. To construct two cassettes expressing vector, pRG was constructed with CMV-UTR-vG-HDV-bGH cassette and pRNA1-HDV after digestion with AgeI and BamHI. Six nt modified depending on the start sequence of the 5' UTR of the RNA2 gene were inserted at the beginning of the HHR region, and UTR-vG-HDV was fused to the end region of the HHR (HHR-UTR-vG-HDV) by overlapping PCR with primers listed in Table 1. The fragment was cloned into the pGEX-T easy vector and digested with BamHI and NotI for ligation to pcDNA3.1+. The vector was named as pHHR-UTR-vG-HDV. The fragment of HHR-RNA1-HDV was obtained by overlapping PCR from 6 nt modified according to the 5' UTR of the RNA1 gene overhang at the 5' end of HHR, RNA1 gene, and HDV ribozyme. The fragment was cloned into the pGEX-T easy vector and ligated to pcDNA3.1+ after digestion with NheI and HindIII, designated as pHHR-RNA1-HDV. pHRG was constructed with CMV-HHR-RNA1-HDV-bGH and pHHR-UTR-vG-HDV after digestion with SgrDI and BglII. As a control vector of pHRG, CMV-HHR-UTR-eGFP-HDV cassette was digested with SacI and XbaI and ligated to pHRG after digestion using the same restriction enzyme for replacement of vG with the eGFP gene.

Evaluation of NNV replicon system

BHK-21 cells in 6-well cell culture plates were transfected with 1.6×10^{11} copies of pFC-vG, pRG, or pHRG using Fugene HD (Promega) according to the manufacturer's instructions for the analysis of vG expression. After 24, 48, and 72 hours post-transfection, RNA was extracted from each group of cells using a Hybrid-R kit (GeneAll), and cDNA was synthesized using a specific primer listed in Table 1 for RT-qPCR according to absolute quantification using the standard curve method by LightCycler 480 (Roche). For the analysis of plasmid copy, transfected cells were collected 24 h after transfection, and total DNA was extracted for qPCR according to absolute quantification using SYBR Green Premix (Enzynomics). The PCR primers used for RT-qPCR and qPCR are in Table 1. The expression level of vG was calculated relative to the plasmid copy number in the cells.

Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$).

Since EPC cells exhibited low transfection efficiency, they were treated with G418 antibiotics (400 μg/ml, Sigma) for selection after transfection in 6-well plate with pFC-vG, pRG, or pHRG using Fugene HD (Promega) according to the manufacturer's instructions. Cells containing each vector were used to extract total RNA or DNA, and the analysis of vG expression level and plasmid copy number was performed by qRT-PCR or qPCR using the absolute quantification method with a standard curve generated by LightCycler 480 (Roche). The results were calculated following the above-mentioned process.

Results

Hammerhead ribozyme (HHR) on eGFP expression

Green fluorescence was observed in EPC cells transfected with pCMV-UTR-eGFP-HDV or pCMV-HHR-UTR-eGFP-HDV (Fig. 1A) at 24 hours post-transfection. There was no discernible difference in fluorescence intensity between the transfected cells (Fig. 1B).

Verification of VHSV G (vG) expression by NNV replicon vector

The plasmid copy number in BHK cells containing pFC-vG (Fig. 2A) was significantly higher than in cells transfected with pRG and pHRG, despite using the same copy number of each plasmid in the transfection process. The vG expression level, calculated relative to the plasmid copy number, indicated that the pHRG group exhibited higher expression than the G and RG groups at 48 hours post-transfection (Fig. 2B).

When transfection was performed in EPC cells and selected with G418 antibiotics, the plasmid copies in cells transfected with pFC-vG were significantly higher than in the RG and HRG groups (Fig. 2C). Additionally, the HRG group showed higher vG expression levels than the G and RG groups.

Discussion

In this study, we present the first report of a nodavirus DNA replicon-based foreign antigen expression

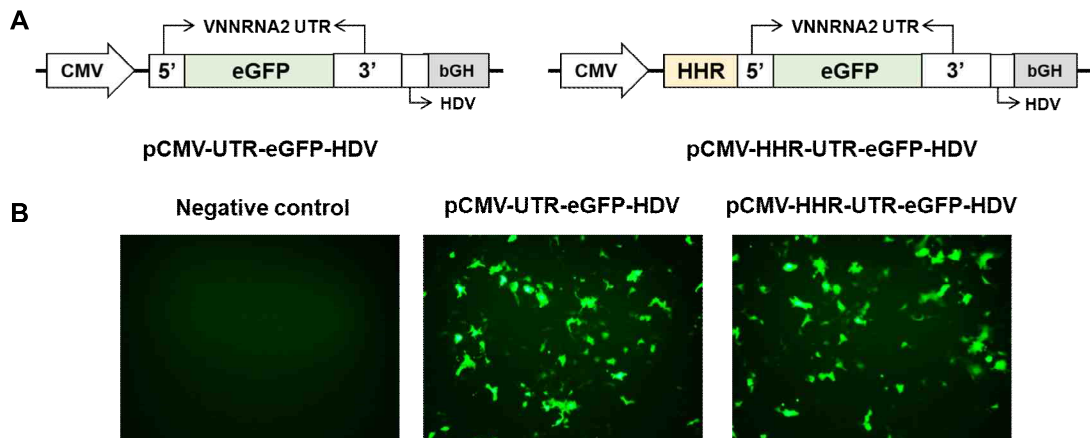

Fig. 1. (A) Construction of the eGFP expression vector containing the RNA2 leader and trailer of NNV on both 5' and 3' ends. The 5' end of the leader was fused to HHR (pCMV-HHR-UTR-eGFP-HDV) or not (pCMV-UTR-eGFP-HDV). (B) Fluorescence observation of EPC cells transfected with pCMV-HHR-UTR-eGFP-HDV or pCMV-UTR-eGFP-HDV at 24 hours post-transfection.

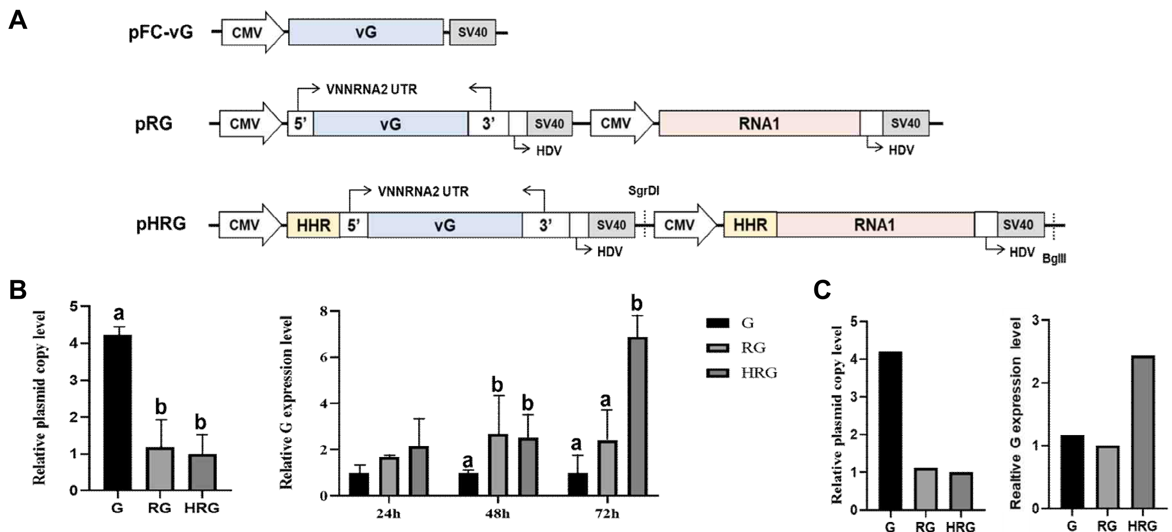

Fig. 2. (A) Construction of VHSV G protein expressing vectors. pFC-vG vector is driven by CMV promoter alone. pRG and pHRG are NNV replicon vectors with or without hammerhead ribozyme sequence (HHR). (B) BHK-21 cells (triplicates) transfected with each vector. Different letters on the bar represent statistical significance (p<0.05). (C) EPC cells transfected with each vector (two independent experiments were conducted and one experimental result is provided).

system. Instead of a two-vector system, we devised a one-vector system containing both an NNV RNA-dependent RNA polymerase cassette and a foreign antigen-expressing cassette. This single-vector approach circumvents the issue of low foreign protein expression associated with the low co-transfection efficiency of a two-vector system. Moreover, in RNA viruses with segmented genomes, maintaining balance in replication among each segment is crucial for viral propagation. Co-transfecting two vectors encoding RNA1 and RNA2 of NNV into a single cell may result in an imbalance in replication. Our one-vector system addresses this issue by arranging the bipartite full genome of NNV into a single vector, thereby mitigating replicative imbalance.

Nodaviruses possess 5’-capped RNA1 and RNA2 in viral particles, which is accomplished by the RNA-dependent RNA polymerase of nodaviruses (Odegard et al., 2010; Venter and Schneemann., 2008). However, in the present study, hammerhead ribozyme was used for precise trimming of the 5’ end of each RNA1 and RNA2, which would, eventually, be resulted in an uncapped 5’ end of each segment transcript. Despite this modification, cells transfected with a vector harboring hammerhead ribozyme before the RNA2 (with the capsid gene ORF replaced with eGFP ORF) exhibited strong fluorescence similar to cells transfected with a vector without hammerhead ribozyme, indicating that the ribozyme had no adverse effect on RNA2 translation. However, it's worth noting that the cutting efficiency of hammerhead ribozyme is not one hundred percent, potentially leading to the production of some 5’-capped hammerhead ribozyme-fused RNA2 transcripts that can be translated in a cap-dependent manner.

DNA replicon vectors of RNA viruses offer advantages over conventional DNA vaccine vectors, including the potential to stimulate stronger immune responses and achieve higher and longer expression of target antigens due to the exponential increase in antigen transcript facilitated by viral RNA-dependent RNA polymerase (Berglund et al., 1998; Hariharan

et al., 1998; Leitner et al., 2000; Näslund et al., 2011). In our study, cells transfected with a vector harboring hammerhead ribozyme-fused RNA1 and RNA2 (with the capsid gene ORF replaced with VHSV glycoprotein ORF) exhibited significantly higher transcription of the VHSV glycoprotein gene compared to cells transfected with either a vector without hammerhead ribozyme or a conventional DNA vaccine vector expressing the VHSV glycoprotein. Furthermore, the transcription level of the VHSV glycoprotein in cells transfected with a vector harboring hammerhead ribozyme-fused RNA1 and RNA2 showed a significant increase over time. These results suggest that NNV genome-based DNA replicon vectors have the potential to induce stronger and longer expression of target antigens compared to conventional DNA vaccine vectors, highlighting the importance of precise genome end trimming for the construction of virus genome-based replicon vectors.

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