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Recognition of *cis*-acting elements of infectious haematopoietic necrosis virus and viral hemorrhagic septicemia virus by homologous and heterologous helper proteins

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Abstract

Infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) are grouped in the *Novirhabdovirus* genus within the family *Rhabdoviridae*. There are many similarities between these two viruses including the lengths of the leader and trailer regions and the homologies of the terminal sequences. We have developed two systems in which IHNV and VHSV minigenomes encoding the marker green fluorescent protein (GFP) can be expressed from plasmids by T7 RNA polymerase. These negative sense minigenome RNAs can be replicated, transcribed and packaged into infectious particles when coinfected with homologous helper viruses. After infection of the minigenome transfected BHK-T7 cells by heterologous helper viruses GFP expression was observed, but packaging of the minigenome RNAs into virus particles did not occur. Packaging of chimeric minigenomes by IHNV and VHSV was also not observed. Cotransfections of the negative sense minigenome plasmids with plasmids encoding nucleoprotein (N), phosphoprotein (P) and RNA polymerase (L) of IHNV and VHSV were carried out in all combinations. Minigenome constructs were expressed only after cotransfection with a set of helper plasmids (N, P and L) all originated from one virus. These results indicate that the *cis*-acting elements responsible for the encapsidation and transcription were recognized by the homologous and heterologous helper proteins, but packaging of the minigenome RNAs required homologous helper viruses.

Keywords: IHNV; VHSV; Minigenome; Transcomplementation

1. Introduction

Infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) are a world wide problem in farmed rainbow trout responsible for substantial economic losses. Clinical signs include darkening of the skin, ascites, exophthalmus, lethargy and white pseudo faeces. The primary organs affected during an infection with both viruses are spleen and kidney, though most other tissues are also affected at the later stages of the diseases (Neukirch, 1986; Yasutake and Amend, 1972).

IHNV and VHSV are members of the genus *Novirhabdovirus* within viruses of the family *Rhabdoviridae*. Both possess a non-segmented negative strand RNA genome of approximately 11.1 kb with a coding capacity for five structural proteins (nucleoprotein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G), RNA polymerase (L)) and a nonstructural protein (NV) (Kurath and Leong, 1985). The gene order is 3′-leader-N-P-M-G-NV-L-trailer-5′ (Schütze et al., 1996). In non-segmented negative-strand RNA viruses genomic RNA is connected tightly with the nucleoprotein N to form an RNase-resistant nucleocapsid. This encapsidated genomic RNA is also associated with the phosphoprotein P and represents the template used by the polymerase protein L to produce the six mRNAs and the full-length antigenomic RNA. This full-length positive-sense copy of the genome is the intermediate

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in RNA replication and, like the genome, is encapsidated by N protein. N, P and L are the minimal set of proteins required for transcription and replication of the *Rhabdoviridae* (Stillman et al., 1995).

The genomic regions of IHNV and VHSV which contain the sequences for encapsidation, initiation of replication and transcription, and packaging are located at the 3′ and 5′ terminal ends of the genomes and are designated as leader and trailer, respectively. For vesicular stomatitis virus (VSV), the prototype rhabdovirus, Smallwood and Moyer (1993) and Li and Pattnaik (1999) demonstrated that optimal transcription of the genome requires the authentic 3′-terminal nucleotides which serve as a promoter region. The viral polymerase L binds at the genomic promotor at the 3′end (leader region) of the encapsidated negative-sense genomic RNA for synthesis of subgenomic mRNAs or complete antigenomic copies of the virus genome. This is the first step in virus replication. The second step, the production of the negative-sense genome, uses the antigenomic copy of the virus genome and the antigenomic promoter at the 5′end (trailer region). There are many similarities between the leader and trailer regions of IHNV and VHSV including the length and sequence homology of the termini. The leader regions of IHNV and VHSV are 174 and 167 bp in length, while the trailer regions comprise 154 and 151 bp, respectively. Sequence alignment shows 51.8% identity for the leader regions and, in particular, ten of the first 12 nucleotides are identical. Similar identities exist for the trailer regions of IHNV and VHSV. Here 43.3% identity for the complete sequence of the trailer regions was demonstrated and nine of the last ten nucleotides are identical.

Recently, it was shown that IHNV and VHSV were present in the same cells after double infection (Brudeseth et al., 2002). These authors also demonstrated that a single cell can support the simultaneous replication of both viruses. Because the proteins of the viruses colocalized in the cytoplasm they may influence replication of the heterologous virus. Furthermore, a series of recombinant chimeric IHNV with the VHSV glycoprotein, the VHSV matrixprotein, the VHSV matrixprotein and glycoprotein combined, and the SVCV glycoprotein were generated (Biacchesi et al., 2002). The data indicated that, e.g. the IHNV glycoprotein can be efficiently replaced with the glycoproteins of other fish rhabdoviruses, VHSV and SVCV (*Vesiculovirus*).

In this report, we investigate the recognition of *cis*-acting RNA sequences in the replication, transcription and packaging of IHNV and VHSV. For these studies we constructed negative-sense synthetic genome analogues from which all the viral coding sequences were replaced by a reporter gene. These minigenome plasmids and also the helper plasmids of the N, P and L proteins of IHNV and VHSV used the T7 RNA polymerase system for expression (for review see Conzelmann, 1998). Our studies show that the RNA sequences responsible for encapsidation and initiation of replication and transcription were accepted by homologous and heterologous helper proteins. However the packaging of the minigenomes by helper viruses succeeded only in the homologous system.

2. Materials and methods

2.1. Construction of minigenomes

First, a recombinant vector called pT7p-HDV-T7t was constructed on the basis of the pUC18 standard vector (Fig. 1). This modified vector contained the bacteriophage T7 RNA polymerase promotor (T7p), an artificial multiple cloning site (MCS), the hepatitis delta virus (HDV) ribozyme sequence and the T7 RNA polymerase terminator (T7t). The sequences for T7p and T7t were obtained from the plasmid pET-23a (Novagen, Madison). The HDV ribozyme sequence (Perrotta and Been, 1991) starts with three guanidine residues, which were integrated into a *Sma*I site, the final restriction site of an artificial MCS. Immediately after the T7p sequence the MCS starts with a *Stu*I restriction site followed by several restriction sites including *Nhe*I and *Nco*I. From plasmid pEGFP-N1 (Invitrogen) the ORF of the green fluorescent protein was amplified using the forward primer 5′-GATCCCATGG TGAGCAAGGG CGAGGAGC-3′ and the reverse primer 5′-GATCGC-TAGC TTACTTGTAC AGCTCGTCC-3′. The PCR product was digested with *Nco*I/*Nhe*I and cloned into

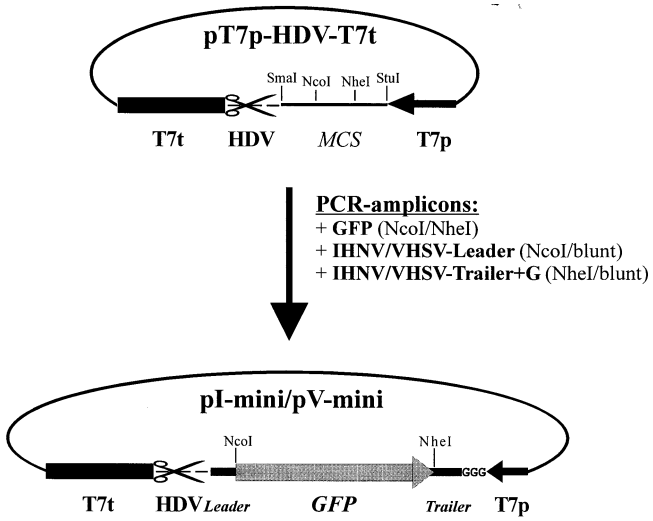


Fig. 1. Cloning strategy and schematic diagram of the IHNV and VHSV minigenome (pI-mini and pV-mini) constructs. The relevant regions for the construction and the function of the minigenomes are shown (T7p, T7 RNA polymerase promotor; T7t, T7 RNA polymerase terminator; HDV, hepatitis delta ribozyme sequence; MCS, multiple cloning site; GFP, open reading frame for the green fluorescent protein).

the *Nco*I/*Nhe*I sites of pT7p-HDV-T7t. The resulting plasmid containing the GFP ORF in inverse orientation relative to the T7p was called pT7p-GFP-HDV-T7t.

Finally, the 3′-leader and 5′-trailer sequences of IHNV and VHSV were inserted into the pT7p-GFP-HDV-T7t vector. The leader and trailer sequences were amplified by PCR using previously described cDNA clones (Schütze et al., 1995, 1999). For amplification of the IHNV leader the primer pair 5′-GTATAAAAAA AGTAACTTGA CTAAGC-3′ and 5′-GCTCACCATG GCGTCGTGAT CCGTTTC-3′ was used, and for the IHNV trailer the primer pair 5′-GGTATAAAAA AAGTAACAGA AGGGTTCTC-3′ and 5′-GATCGC-TAGC GTACACTCCA GCAGCACC-3′, respectively. The VHSV leader and trailer regions were amplified by the primer pair 5′-GTATCATAAA AGATGATGAG TTATG-3′ and 5′-GCTCACCATG GTTCTTCGAC TGAGTTC-3′, and the primer pair 5′-GGTATAGAAA ATAATACATA CCACAATCG-3′ and 5′-GATCGC-TAGC TGGAATCGCC CCGTTCATC-3′, respectively. The PCR products of the leader sequences were digested with *Nco*I and inserted into the *Nco*I/*Sma*I sites of the pT7p-GFP-HDV-T7t vector. The resulting plasmids were designated pI-T7p-GFP-L and pV-T7p-GFP-L, respectively. Similarly, the IHNV and VHSV trailer sequences were digested with *Nhe*I and inserted into the *Nhe*I/*Stu*I sites of the plasmids pI/V-T7p-GFP-L. The forward primers for the amplification of the trailer sequences contained an additional guanidine at the 5′end, so that after ligation of the PCR products into the *Stu*I site three guanidine nucleotides are located downstream from the T7 promotor region, which are recommended for optimal transcription initiation by T7 RNA polymerase (Ikeda et al., 1992; Sen and Dasgupta, 1993). The resulting negative-sense minigenomes of IHNV and VHSV were called pI-mini and pV-mini, respectively. Fig. 1 shows a schematic illustration of the construction of the minigenomes.

2.2. Construction of protein-encoding plasmids

The DNA sequences for the N, P, and L proteins of IHNV and VHSV were subcloned into the pCITE2a vector (Novagen, Madison) by PCR amplification and subcloning of cDNA (Schütze et al., 1995, 1999). The pCITE vector contains a cap-independent translation enhancer for increasing the translation efficiency of adjacent sequences. Each gene was first modified by PCR to place an *Nco*I or *Nco*I-compatible site at the translation start site and a *Not*I site at the downstream end.

The helper plasmid pCITE-IN for the expression of the N protein of IHNV was obtained after digestion of the previously described plasmid pET-IN (Schütze et al., 1995) by *Nde*I and *Not*I. These 3′end *Nde*I/*Not*I fragment of the IN ORF was subcloned into the *Nde*I/*Not*I site of pCITE2a and called pCITE-IN1. For subcloning the 5′end a PCR product was amplified using a forward primer with a *Nco*I-compatible site (5′-GAGTCATGAC AAGCGCACTCA GAGAG-3′) and the reverse primer 5′-CTCCTCCGAC TTGACTCACC GCCCGCT-3′. The PCR product was digested by *Bsp*HI and *Bcl*I and the 140 bp fragment was cloned into the *Nco*I/*Bcl*I sites of pCITE-IN1 to obtain the helper plasmid pCITE-IN.

For cloning the P gene of IHNV the respective ORF was amplified by PCR using the forward primer 5′-GAGACATGTC AGATGGAGAA GGAGAACAG-3′ and the reverse primer 5′-GAGCTATTGA CCTTGCTTCA TGCGC-3′. The PCR product was cloned into pUC18*Sma*I standard vector. After digestion of the PCR product by *Afl*III and *Xho*I the fragment was cloned into the *Nco*I/*Xho*I sites of pCITE2a. The resulting helper plasmid was designated pCITE-IP.

The L gene expression plasmid of IHNV was cloned in three steps. For the amplification of the 5′end of the L gene a PCR was performed by using the forward primer 5′-GAGCCATGGA CTTCTTCGAT CTTGACA-TAG-3′ and the reverse primer 5′-CTAGACCAAG TGCCGTGCA TTCGTCGAGA ATTC-3′ followed by blunt end cloning of the ca. 1.9 kb PCR fragment (IHNV nt: 5025–6930) into a pUC18*SmaI* standard vector. After restriction with *Nco*I and *Bam*HI the 5′end of the IL-ORF was cloned into the pCITE2a *Nco*I/*Bam*HI sites to yield pCITE-IL1. The 3′end of the L polymerase gene was inserted after RT-PCR. The first strand primer was 5′-GCCAGGATCC ACCA-TATGCC CGTCC-3′ and the reverse primer was 5′-CTCGCGGCCG CTATTGTTCG CCTAGTGGAA AGAAGC-3′. The RT-PCR product of ca. 1.5 kb (IHNV nt: 9498–10985) was cloned into pUC18*SmaI.* After digestion with *Bam*HI and *Not*I the 3′end was subcloned into the *Bam*HI/*Not*I digested pCITE-IL1 to obtain the plasmid pCITE-IL2. In a last step, the main body of the IL-ORF (IHNV nt: 5342–9511) was removed from a cDNA clone by digestion with *Nsi*I and *Nde*I. This fragment was inserted into the *Nsi*I/*Nde*I sites of pCITE-IL2. The complete helper plasmid was designated pCITE-IL.

The primers used for amplification of the nucleoprotein (N) gene of VHSV were 5′-GATCCCATGG AAGGAGGACT TCGTGCAG-3′ (forward primer) and 5′-GATCGCGGCC GCTTAGTCTC AGAGTCCTCG-3′(reverse primer). The 1203 bp PCR product was digested with *Nco*I and *Not*I and cloned into the *Nco*I/*Not*I sites of pCITE2a. The resulting helper plasmid was designated pCITE-VN.

For cloning the phosphoprotein (P) gene of VHSV two internal *Nco*I sites were mutated by fusion PCR. The primers used to amplify three overlapping PCR products (A, B, C) were for fragment A 5′-GATCCC

ATGG CTGACATTGA GATGAGCGA-3′ (forward primer) and 5′-CTAGGGATCC GTGCGACAGG-3′ (reverse primer), for fragment B 5′-CCTGTCGCAC GGATCCCTAG-3′ (forward primer) and 5′-CCTTTTCCAT CGCGGAGAGA-3′ (reverse primer) and for fragment C 5′-TCTCTCCGCG ATG-GAAAAGG-3′ (forward primer) and 5′-GATCGCGGCC GCTACTCTAG CTTGTCCAGC-3′ (reverse primer). The substitutions of nucleotides for the elimination of the internal *Nco*I sites (underlined) did not influence the corresponding amino acid sequences. The final PCR amplification was performed using the fragments A, B and C as templates and the primer 5′-GATCCC ATGG CTGACATTGA GAT-GAGCGA-3′ (forward) and 5′-GATCGCGGCC GCTACTCTAG CTTGTCCAGC-3′ (reverse), respectively. The 685 bp PCR product was digested with *Nco*I and *Not*I, and cloned into the *Nco*I/*Not*I digested pCITE2a. The resulting helper plasmid was designated pCITE-VP.

The polymerase (L) gene of VHSV was cloned into pCITE2a in five steps. First, a 574 bp *Nco*I/*Nde*I restriction fragment of the 5′end of the L gene (VHSV nt: 5051–5625) was cloned into *Nco*I/*Nde*I digested pCITE2a (pCITE-VL1). As backbone for cloning the main body and the 3′end of the L gene a previously described cDNA clone pUC-V281 (VHSV nt: 10 040–11 121; Schütze et al., 1999) was used. Upstream of the VHSV sequence in pUC-V281 two restriction fragments of other cDNA clones were inserted. First, an upstream 1923 bp *Pst*I/*Sal*I restriction fragment (VHSV nt: 8582–10 505) was cloned into pUC-V281 *Pst*I/*Sal*I. The resulting plasmid pUC-VL2 was digested with *Nde*I and *Pst*I and a upstream 2957 bp *Nde*I/*Pst*I restriction fragment (VHSV nt: 5625–8582) was inserted (pUC-VL3). The final helper plasmid pCITE-VL was constructed by insertion the *Nde*I/*Not*I restriction fragment (VHSV nt: 5625–11 121) from pUC-VL3 into the *Nde*I/*Not*I digested pCITE-VL1 vector containing the first 574 bp of the L gene of VHSV.

2.3. Cell lines and viruses

Propagation of IHNV and VHSV in monolayer cultures of EPC and RTG-2 cells has been described by Schütze et al. (1995, 1999). For expression of the bacteriophage T7 RNA polymerase a baby hamster kidney (BHK21) cell line with a stable insertion of the T7 RNA polymerase gene (BHK-T7) (Buchholz et al., 1999; kindly provided by Schnell and Conzelmann) was used. BHK-T7 monolayer cultures were maintained at 37 °C in 5% CO_2 with DMEM supplemented with 2% FCS and 500 μg/ml geneticin.

2.4. Transfection and virus infection

Approximately 8×10^6 BHK-T7 cells were grown in 6-well plates and transfected with a plasmid mixture containing 0.75 μg of pCITE-IN/-VN, 0.50 μg of pCITE-IP/-VP, 0.25 μg of pCITE-IL/-VL and 2.5 μg of the minigenome constructs pI-mini and pV-mini. Transfection procedures were carried out using the Superfect reagent (Qiagen). Briefly, one hour before transfection the growth medium was replaced by serum-free medium (OptiMem, Gibco). After removing the serum-free medium, the plasmid DNA/Superfect mix in 1 ml OptiMem medium was added and cells were incubated at 37 °C in 5% CO_2. One hour post transfection (p.t.) 1 ml serum-supplemented growth medium was added to the cells. After a further hour the transfected cells were shifted to 15 °C and 2.5% CO_2 for 30 min. For virus infections transfection mixtures were removed and cells were infected with IHNV or VHSV at a multiplicity of infection of 10 and incubated at 15 °C. Four hours p.t. viral inoculum or transfection mixtures were removed and 2 ml fresh growth medium were added. After incubation for 5 days at 15 °C, fluorescent cells were first observed. The final analysis of the cells were carried out after 2 weeks. The transfection experiments were repeated at least four times.

2.5. FACS analysis

Co-transfections with or without viral infection were performed in 24-well plates using a quarter of the transfection mixture as described above. Cells were incubated at 15 °C for 14 days. For FACS analysis the growth medium was removed and cells were detached from the bottom by trypsinization. Cells dissolved with 500 μl fresh growth medium were used for FACS analyses. Each experiment was repeated four times and analyses were carried out using a FACSCalibur unit (Becton Dickinson). Negative controls (BHK-T7 cells either nontransfected, or transfected only with pI-mini or pV-mini) exhibited between 0.5 and 1% fluorescent cells which probably resulted from autofluorescence of the cells.

3. Results

3.1. Transfection of IHNV/VHSV-minigenomes and infection by homologous or heterologous helper virus

First, the cross-recognition of *cis*-acting sequences of the leader and trailer regions of IHNV and VHSV responsible for encapsidation and transcription was investigated to analyse which proteins of the RNP complex of IHNV can be replaced by proteins of the RNP complex of the VHSV and vice versa. For these

studies negative-sense minigenomes of IHNV and VHSV were constructed (Fig. 1). The pI-mini and pV-mini constructs were transfected and scored with or without subsequent superinfection with the homologous or heterologous helper virus. Results for transfection assays are shown in Table 1. When the pI-mini and pV-mini plasmids were transfected into BHK-T7 cells in the absence of helper viruses, GFP expression was never observed. When the minigenomes of IHNV and VHSV were transfected into BHK-T7 cells and infected with the homologous or heterologous helper virus, GFP fluorescence of the BHK-T7 cells ensued. These experiments were repeated at least four times and similar results were obtained.

Because different amounts of GFP expressing cells after transfection were observed, a quantitation of fluorescent BHK-T7 cells by FACS analysis was performed. The results of the FACS analyses are depicted in Fig. 2A and B. Cells transfected with pI-mini and infected by IHNV produced 14% GFP expressing BHK-T7 cells, while the IHNV minigenome transfected cells infected by the heterologous VHSV produced 5.8% fluorescent cells. Thus, the values of fluorescent pI-mini transfected BHK-T7 cells were different for the homologous and heterologous infection. When the mean value of fluorescent cells of pI-mini transfected cells following homologous infection was taken as 100%, then the percentage of fluorescent cells after transfection of pI-mini and superinfection by VHSV amounted to only 41.4% of the fluorescent value of the homologous infection.

The situation with the VHSV minigenome was different. Here, on average 25.5% GFP expressing BHK-T7 cells were observed after infection by the homologous VHSV. A similar level of fluorescent cells of 23.7% were shown for pV-mini transfected cells, which were infected with the heterologous virus (IHNV). Thus, the level of fluorescent cells after heterologous infection of pI-mini transfected cells achieved 93% of the efficiency of the homologous infection.

The results indicate that IHNV and VHSV proteins are able to encapsidate and transcribe homologous and heterologous RNA minigenomes. Although higher values of fluorescent BHK-T7 cells were observed for the homologous infections of the minigenome transfected cells, the infection of pV-mini transfected cells by VHSV and IHNV showed nearly identical values of fluorescent BHK-T7 cells. In contrast, the values of fluorescent pI-mini transfected BHK-T7 cells differed significantly for the homologous and heterologous infection.

3.2. Cotransfection of IHNV/VHSV-minigenomes with homologous and heterologous helper plasmids

Since the transfection of the IHNV and VHSV minigenomes and infection by homologous and heterologous helper virus showed that sequences responsible for encapsidation and transcription were recognized, these results were confirmed by cotransfections of the minigenomes by helper plasmids. Six helper plasmids which expressed the N, P and L proteins of IHNV and VHSV (pCITE-IN, -IP, -IL; pCITE-VN, -VP, -VL) were constructed on the basis of the T7 RNA polymerase system.

When the plasmids pI-mini and pV-mini were transfected into BHK-T7 cells together with the homologous helper plasmids for N, P and L GFP fluorescence of the cells ensued. When the minigenomes were cotransfected with a complete set of heterologous helper plasmids,

Table 1
GFP expression after transfection of minigenomes into BHK-T7 cells

Transfection	Minigenome	Helper virus	Helper plasmids	GFP expression
1	pI-mini	∅[a]	∅	−
2	pI-mini	IHNV	∅	+
3	pI-mini	∅	pCITE-IN, -IP, -IL[b]	+
4	pI-mini	VHSV	∅	+
5	pI-mini	∅	pCITE-VN, -VP, -VL[c]	+
6	pI-mini	∅	pCITE-I/V[d]-plasmids in all combinations	−
7	pV-mini	∅	∅	−
8	pV-mini	VHSV	∅	+
9	pV-mini	∅	pCITE-VN, -VP, -VL	+
10	pV-mini	IHNV	∅	+
11	pV-mini	∅	pCITE-IN, -IP, -IL	+
12	pV-mini	∅	pCITE-I/V-plasmids in all combinations	−

−: only background levels of fluorescence (0.5–1.0% of cells) were observed. +: fluorescence above background levels (usually > 5% of cells) was observed.

[a] Not used.

[b] Helper plasmids for expression of N, P and L proteins of IHNV.

[c] Helper plasmids for expression of N, P and L proteins of VHSV.

[d] pCITE-IN, -IP, -IL and pCITE-VN, -VP, -VL.

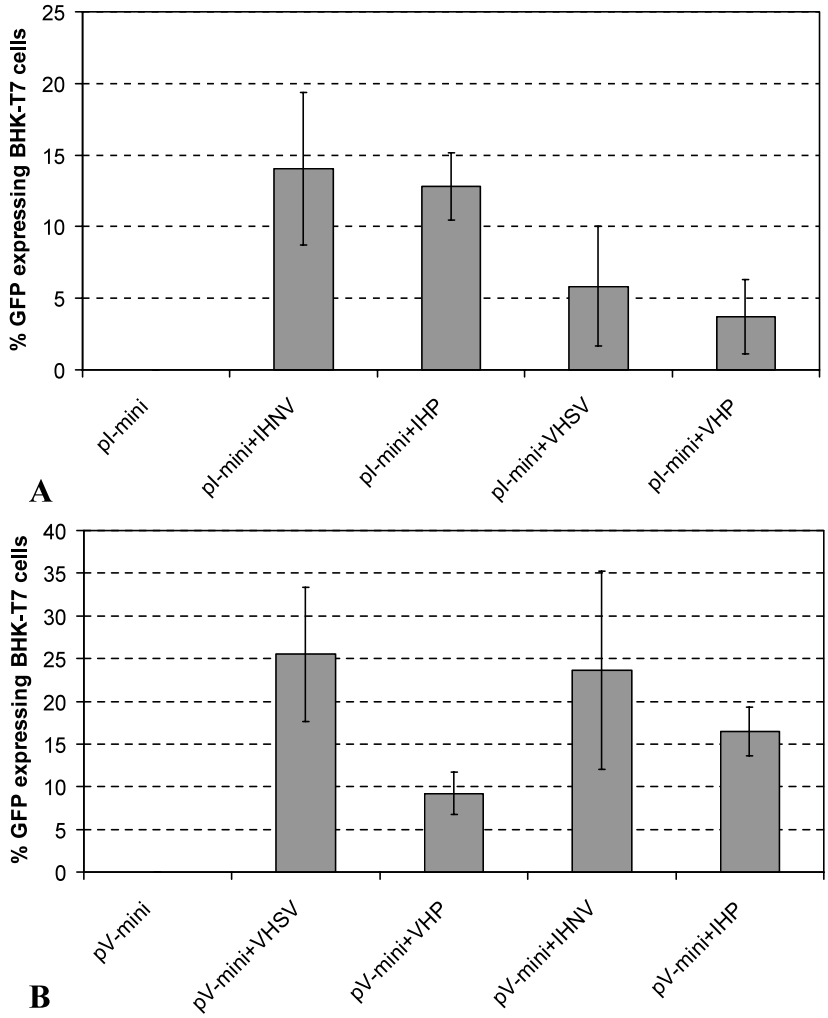


Fig. 2. FACS analysis of GFP expressing BHK-T7 cells. BHK-T7 cells were transfected with pI-mini (A) and pV-mini (B), respectively, and infected with the homologous or heterologous virus (IHNV and VHSV). Further cotransfections of the minigenomes with helper plasmids encoding the N, P and L proteins of IHNV (IHP) or VHSV (VHP) were carried out. Results from four independent experiments were averaged with the deviations shown by the error bars. Autofluorescence (0.5–1.0% of cells) has been subtracted.

GFP expressing BHK-T7 cells were also observed, which reflects the results of the homologous and heterologous infections of pI-mini and pV-mini transfected cells.

The amount of GFP expressing cells was again established by FACS analysis. The results are depicted in Fig. 2A and B. When BHK-T7 cells were cotransfected by pI-mini and a complete set of helper plasmids encoding N, P and L of IHNV 12.8% GFP expressing cells were observed. After cotransfection of BHK-T7 cells with pI-mini and a heterologous set of helper plasmids only 3.7% fluorescent cells were counted. The cotransfection of BHK-T7 cells with pV-mini and VHSV helper plasmids produced 9.2% fluorescent BHK-T7 cells, whereas the cotransfection with the heterologous IHNV helper plasmids resulted in 16.5% GFP expressing cells.

When the mean value of fluorescent cells of pI-mini transfected cells following homologous infection described above (14% absolute) was taken as 100%, then the fluorescent cells after cotransfection with pI-mini and the homologous IHNV helper plasmids (12.8%) amounted to 91.5%. When cells were cotransfected with pI-mini and the heterologous set of helper plasmids (3.7%), only 26.4% fluorescent cells compared to the infection of pI-mini transfected cells by IHNV were observed. The results for the transfection assays of the VHSV minigenome were different. When the mean value (25.5%) of fluorescent cells after transfection of pV-mini and superinfection with VHSV was defined as 100%, then the percentage of fluorescent BHK-T7 cells cotransfected with pV-mini and the homologous VHSV helper plasmids (9.2%) was only 36%. When heterologous IHNV helper plasmids were used for cotransfection with pV-mini (16.5%), a percentage of 64.7% fluorescent cells was observed. In general, the amounts of fluorescent BHK-T7 cells after cotransfections were lower as the amounts of GFP expressing cells after

transfection of the minigenomes and infection with IHNV and VHSV.

In further cotransfection assays the ability for transcomplementation of the IHNV and VHSV helper proteins (N, P and L) by the heterologous counterparts were investigated. Interactions of the helper proteins are essential for the encapsidation and transcription of the minigenome RNA, and fluorescent BHK-T7 cells cotransfected with the minigenomes and a mixed combination of the helper plasmids would indicate interactions of heterologous helper proteins. In the cotransfection assays each helper plasmid was singly replaced by the heterologous counterpart. Thus, all six combinations of heterologous helper plasmid exchange were tested for each minigenome construct. The results of the studies are summarized in Table 1. When BHK-T7 cells were cotransfected with minigenomes and a set of helper plasmids containing one heterologous plasmid GFP expression was never observed. All cotransfection assays were repeated at least four times and identical results were obtained.

The results indicate that the helper proteins N, P and L of IHNV and VHSV expressed by plasmids can recognize the sequences used for encapsidation and initiation of transcription of homologous and heterologous minigenome RNA. Cotransfections of the minigenomes from IHNV and VHSV with a mixed set of helper plasmids never showed fluorescent BHK-T7 cells. In conclusion, the prerequisite for encapsidation and transcription of the minigenomes is a set of helper proteins (N, P and L) of homologous or heterologous origin.

3.3. Rescue of IHNV and VHSV minigenomes by homologous and heterologous helper virus

Since the sequences for encapsidation and transcription of the minigenome RNAs were recognized by N, P, and L proteins of homologous and heterologous origin, we investigated the packaging of progeny minigenome RNA by the homologous and heterologous helper viruses. BHK-T7 cells were transfected with the minigenomes and infected with the homologous or heterologous helper virus. As negative control the plasmid pCITE-GFP was transfected instead of the minigenome plasmids. After 14 days BHK-T7 cells were lysed by freezing and thawing and cell debris was removed by centrifugation for $2000 \times g$ at 4 °C. Aliquots of 50, 100, 250 and 500 μl of the cleared supernatants were transferred on to fresh monolayers of EPC cells in 6-wells and incubated at 15 °C until a beginning CPE was observed. Cells were controlled regularly for the appearance of fluorescent plaques. If the minigenome RNAs were packaged by the helper viruses, fluorescent plaques of infected EPC cells should be observed. The results of three independent experiments are summarized in Table 2.

First, the pI-mini and pV-mini transfected BHK-T7 cells were infected with the homologous helper viruses. After freezing and thawing of the cells cleared supernatants were transferred on fresh monolayers of EPC cells and fluorescent plaques of GFP expressing EPC cells were regularly detected (Fig. 3A and B). When the transfected cells were infected with heterologous helper virus fluorescent plaques of EPC cells never occurred. Thus, minigenome RNAs were not packaged by heterologous helper viruses.

For further studies, chimeric minigenomes of IHNV and VHSV were constructed. If sequences of the leader or trailer regions alone were sufficient for the packaging of minigenome RNA, chimeric minigenomes should be packaged by IHNV or VHSV. The chimeric minigenomes pItr/Vle-mini and pVtr/Ile-mini containing the trailer region of IHNV and the leader region of VHSV or vice versa were subcloned using the *Nco*I- and *Nde*I-sites of pI-mini and pV-mini (Fig. 1). The sequences for encapsidation and transcription of the chimeric minigenomes were recognized by both viruses, since GFP expressing BHK-T7 cells were observed after transfection with the chimeric minigenomes and infection with IHNV and VHSV. However, when transfected and infected cells were lysed by freezing and thawing and supernatants were passaged on fresh EPC cells, fluorescent plaques were never observed (Table 2).

4. Discussion

In this report we describe the construction and analysis of subviral replication systems from IHNV and VHSV based on the T7 RNA polymerase expression system. These minigenomes were used for the investigation of sequences of the leader and trailer regions of both viruses which are responsible for initiation of encapsidation, transcription, and genome replication and subsequently for packaging. Expression of the inserted GFP reporter gene was used as a marker for replication of the minigenome RNA after forming a functional RNP complex.

For VSV it was shown that the exact 3′end of the viral RNA genome is crucial for transcription and replication, whereas extra GGG nucleotides added at the 5′end were tolerated (Pattnaik et al., 1992). For our studies negative sense minigenomes of IHNV and VHSV with a GGG nucleotide insertion between the T7 promoter and the 5′ trailer sequence were constructed. For cotransfection experiments helper plasmids encoding the proteins N, P and L of IHNV and VHSV were cloned. The transcription of the negative sense minigenomes and the helper protein genes N, P, and L were achieved by T7 RNA polymerase (Conzelmann, 1998).

Table 2
Packaging of minigenome RNA by helper viruses

Transfection	Minigenome	Helper virus	GFP expression in BHK-T7 cells	Packaging of minigenomes
1	pCITE-GFP[a]	IHNV	+	−
2	pI-mini	IHNV	+	+
3	pV-mini	IHNV	+	−
4	pVtr/Ile-mini[b]	IHNV	+	−
5	pItr/Vle-mini[c]	IHNV	+	−
6	pCITE-GFP	VHSV	+	−
7	pI-mini	VHSV	+	−
8	pV-mini	VHSV	+	+
9	pVtr/ Ile-mini	VHSV	+	−
10	pItr/Vle-mini	VHSV	+	−

−: only background levels of fluorescence (0.5–1.0% of cells) were observed. +: observed fluorescence above background levels.
[a] Plamid for expression of GFP under control of the T7 RNA polymerase promotor.
[b] Negative sense chimeric minigenome plasmid containing the leader region of IHNV and the trailer region of VHSV.
[c] Negative sense chimeric minigenome plasmid containing the leader region of VHSV and the trailer region of IHNV.

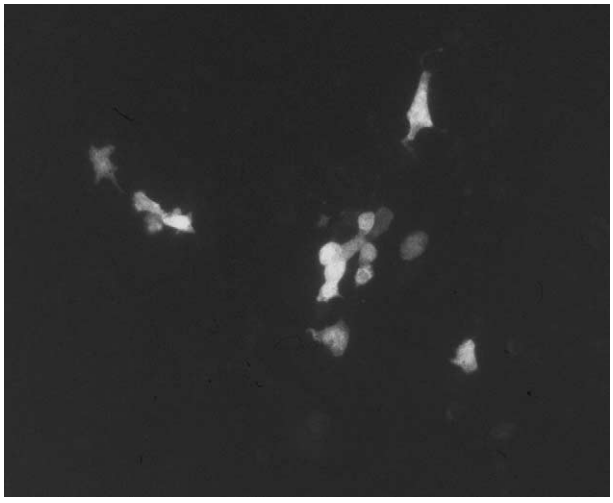
After transfection of minigenomic DNA into T7RNA polymerase expressing cells viral RNA of negative polarity (genomic orientation) is transcribed which is unable to lead to translation of this encoded protein. Only the respective RNA with positive polarity (antigenomic sense) which can only result from replication of the primary negative-sense transcript is able to be translated. Since the main prerequisite for genome replication is the formation of the RNP complex, consisting of genomic RNA and the proteins N, P and L, expression of the marker gene reflects recognition of the primary transcript by the supplied helper proteins. They are produced after infection of cells transfected with minigenome alone with the respective helper virus, or after cotransfection of the minigenome with N, P and L helper plasmids.

Replication, i.e. encapsidation and transcription, of minigenomic RNA was demonstrated by expression of GFP from the minigenome constructs. After transfection of T7 expressing cells with pI-mini or pV-mini alone, only background levels of GFP expression of ca. 0.5–1.0% fluorescent cells were observed. This is most likely due to autofluorescence of a very limited number of cells since it was also observed in nontransfected parallel cultures. After infection of pI-mini or pV-mini transfected BHK-T7 cells with homologous or heterologous helpervirus, a significant number of GFP expressing cells were observed in all cases. These results indicated, that the *cis*-acting elements responsible for encapsidation and transcription/replication were recognized by both viruses.

Thus, our data differ from those of Marburg virus (MBGV) and Ebola virus (EBOV), two other member of the order *Mononegavirales*, in which there was no mutual recognition of *cis*-acting elements of minigenomes (Mühlberger et al., 1999).

FACS analyses of pV-mini transfected BHK-T7 cells showed similar values of GFP expressing cells after homologous and heterologous infections. For the IHNV minigenome less GFP expressing cells were observed after infection by VHSV compared to the infection with IHNV. Interestingly, the rate of GFP expressing BHK-T7 cells after transfection of the VHSV minigenome and infection with VHSV or IHNV was higher compared to the IHNV minigenome. The first 12–16 nucleotides of the leader regions of IHNV and VHSV represent the genomic promoter for the transcription of the six subgenomic mRNAs and the antigenomic full length RNA (Morzunov et al., 1995; Schütze et al., 1995, 1999). Sequence alignment of the IHNV and VHSV leader regions shows that ten of the first 12 nucleotides are identical. Only the nucleotides in position 5 and 7 were different for both viruses. Possibly, the pyrimidines in these position of the VHSV genome create a stronger promoter for the viral polymerases. A similar exchange of nucleotides in the respiratory syncytial virus (RSV) leader region resulted in a large increase in RNA synthesis (Grosfeld et al., 1995). Also Brudeseth et al. (2002) reported a restriction of the distribution and a lower titer of IHNV after a double infection of fishes with IHNV and VHSV which indicated an in vivo growth advantage of VHSV.

The results of the transfection assays were verified by cotransfection of the minigenomes with expression plasmids of the N, P and L protein of IHNV and VHSV. In these studies GFP expression was observed after cotransfection of the minigenomes only with a complete set (N, P and L) of helper plasmids which, however, all had to originate from the same virus. The values of GFP expressing BHK-T7 cells after cotransfection experiments were lower than the values after transfection of the minigenomes and infection with



A: pI-mini + IHNV

B: pV-mini + VHSV

Fig. 3. Packaging of the minigenomes by the homologous helper viruses. BHK-T7 cells transfected with the minigenomes [pI-mini (A) and pV-mini (B)] were infected with the homologous helper viruses (IHNV and VHSV). After freezing and thawing of the cells, supernatants were transferred to fresh EPC cell monolayers and screened for the appearance of fluorescent plaques.

IHNV and VHSV. It can be assumed that infection of the transfected cells was more efficient than the co-transfection assays. The FACS analyses showed that the transcomplementation of VHSV by the IHNV helper proteins was more efficient than vice versa (Fig. 2). The efficiency of the recognition of the IHNV minigenome by the VHSV helper proteins was lower but also significant.

Several authors investigated a heterologous exchange of proteins of viruses from the order *Mononegavirales*. Conzelmann et al. (1991) reported that L protein deficient rabies virus serotype 1 (RV-1) defective interfering particle RNA was replicated and transcribed by heterologous RV helper virus (RV-serotypes 2–4) L proteins. The proteins of the RNP complex of the bovine respiratory syncytial virus (BRSV) and the human respiratory syncytial virus (HRSV) are highly conserved (greater 69%) and the HRSV proteins were transcomplemented by the helper proteins of BRSV and vice versa (Yunus et al., 1999).

Although the amino acid identities for the helper proteins of IHNV and VHSV are only 42% (N), 37% (P) and 60% (L), a functional exchange was investigated by cross-transcomplementation of single helper proteins. Cotransfection assays of pI-mini and pV-mini with a mixed set of helper plasmids of both viruses showed that a prerequisite for the recognition of sequences responsible for encapsidation and transcription of the minigenome RNAs is a set of helper proteins from one virus. Thus, N, P and L must have the same virus origin to allow the replication of either minigenome. We assume that proteins from different viruses are unable to form functional RNP complexes. Similar results were obtained for the MBGV and the EBOV (Mühlberger et al., 1999). So far, exchange of single nucleocapsid proteins of heterologous viruses of the *Mononegavirales* was only successful in a cell-free system (Chandrika et al., 1995). These authors reported that it was possible to replace the nucleoprotein of the Sendai virus by the measles virus nucleoprotein.

Biacchesi et al. (2002) used the reverse genetic system of IHNV for the recovery of chimeric viruses. A recombinant virus containing the N, P, L and NV protein of IHNV as well as the M and G protein of VHSV could be propagated. So far, recovery experiments of chimeric IHN and VHS viruses with an exchange of only the N, P or L protein were not published. In the light of our results it can be assumed that chimeric IHN and VHS viruses can only replicate if the N, P and L proteins all originate from the same virus.

In further studies the packing of the IHNV and VHSV minigenomes by homologous and heterologous helper viruses was investigated. BHK-T7 cells transfected with pI-mini or pV-mini were infected by the homologous and heterologous viruses. Supernatants of lysed cells were transferred to fresh EPC cell monolayer and cells were controlled regularly for the appearance of fluorescent plaques. Fluorescent plaques were observed only in experiments with the homologous virus. Thus, the minigenome RNAs were encapsidated, transcribed, and replicated by heterologous or homologous virus, but was packaged into virions only from homologous origin. Rescue experiments of the minigenomes with the heterologous virus were not successful and thus, it appears that the packaging is highly specific for the homologous RNA. This was confirmed by packaging experiments with chimeric minigenomes pVtr/Ile-mini and pItr/Vle-mini which were not packaged by either virus.

Rescue systems have been described for several members of the *Rhabdoviridae*. Moyer (1989) and Pattnaik and Wertz (1991) reported about rescue experiments by VSV and Conzelmann and Schnell (1994) showed the rescue of synthetic genomic RNA analogs of RV. Le Mercier et al. (2002) demonstrated that the RV minigenome was packaged by Mokola virus, another member of the genus *Lyssavirus*, while the more related European bat lyssavirus 1 did not package. Successful packaging of a VHSV minigenome RNA by the homologous helper virus VHSV was described by Betts and Stone (2001), and Biacchesi et al. (2000a,b) reported about rescue experiments of IHNV minigenome RNA by homologous and heterologous helper viruses (IHNV and VHSV). Parallel to our results the authors showed that the packaging of IHNV and VHSV minigenome RNA occurred after the homologous infection only. These authors postulated that the *cis*-acting elements in IHNV and VHSV genomes are not conserved. Our work uncovers significant differences regarding the requirement of heterologous helper proteins in encapsidation and initiation of transcription and replication, and in packaging.

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