Rescue of synthetic salmonid rhabdovirus minigenomes

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Infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) are both serious salmonid pathogens of the Novirhabdovirus genus (Rhabdoviridae family). They cause an acute to chronic viscerotropic disease, mostly but not exclusively in fingerling and yearling rainbow trout, with significant mortality. Similar to mammalian rhabdoviruses, IHNV and VHSV virion structures are composed of roughly 12 kilobases of negative-sense single-stranded RNA tightly associated with a nucleoprotein (N), a polymerase-associated protein (P) and an RNA-dependent RNA polymerase (L), a matrix protein (M) and a glycoprotein (G) which induces the synthesis of neutralizing antibodies in infected fish (Lorenzen et al., 1990). An additional gene, located between the G and L cistrons, encodes a non-structural protein (Nv) (Kurath & Leong, 1985; Basurco & Benmansour, 1995), the role of which is unknown. The entire nucleotide sequences of both IHNV and VHSV genomes have been determined (Morzunov et al., 1995; Schütze et al., 1995, 1999).

Recent advances in the field of reverse genetics for the non-segmented and segmented negative-stranded RNA viruses (for reviews see Conzelmann, 1998; Pekosz et al., 1999) have opened the way for the generation of infectious viruses derived from cloned cDNA and, thus, the ability to introduce targeted mutations in the viral RNA genomes. As a first step towards the elaboration of a reverse genetics system for salmonid rhabdoviruses, an IHNV-derived cDNA plasmid construct, pHNV-CAT(−), in which all the IHNV coding regions were deleted and replaced by the chloramphenicol acetyltransferase (CAT) reporter gene, was engineered. A T7 promoter sequence and a hepatitis δ virus antigenome ribozyme sequence (Perrota & Been, 1991) were fused to the IHNV trailer/L-gene end and leader/N-gene start sequences respectively (Fig. 1A).

Plasmid pHNV-CAT(−) was linearized with SpeI, and 1 µg was used for the synthesis of RNA in a T7-driven transcription reaction with a Ribomax kit (Promega). Epiheliona papulosum cyprini (EPC) cell monolayers (Fijan et al., 1983) in 6-well plates (3 × 10^6 cells per well) were infected with IHNV (European strain 32-87, 5 p.f.u. per cell) for 5 h at 14 °C, washed with serum-free medium and transfected with 7 µg of in vitro-synthesized RNA for 5 h at 20 °C with Lipofectin reagent (Gibco-BRL). Cells were incubated at 14 °C until total cytopathic effect was observed (3 days post-infection). Supernatants were then harvested, clarified by low-speed centrifugation and used to infect fresh cells. At 24 h post-infection, the cells were monitored for CAT activity. Briefly, 200 µl of cell lysate was prepared from each 6-well monolayer and 80 µl of each sample, adjusted to contain an equal amount of proteins, was assayed for CAT activity with [14C]chloramphenicol as the substrate (Gorman et al., 1982). The reactions were incubated for 90 min and the products were analysed by ascending thin-layer chromatography (TLC).

**Synthetic T7-driven cDNA minigenomes containing the bacterial chloramphenicol acetyltransferase gene as a reporter were derived from the genome of two salmonid novirhabdoviruses, infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV). We showed that an exogenous IHNV RNA minigenome transfected into fish cells could be rescued following IHNV infection as it was replicated, encapsidated and transcribed. When cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), transfected with the plasmid carrying the IHNV minigenome (genomic- and antigenic-sense) and superinfected with IHNV, rescue of the minigenome was more efficient. Heterologous VHSV/IHNV rescue experiments failed. Finally, when the IHNV N, P and L proteins were expressed from cDNAs in cells, the minigenome was also successfully rescued, indicating that the nucleocapsid proteins were biologically functional. These data represent the first example of rescue experiments for non-mammalian rhabdoviruses replicating at a low temperature.**

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Fig. 1. Rescue of the pIHNV-CAT(−) minigenome following vTF7-3 and IHNV infections after one passage. (A) Structure of the cDNA encoding a vRNA IHNV-CAT(−) analogue. IHNV trailer/L-gene end (GE) and leader/N-gene start (GS) sequences are flanked by a T7 RNA polymerase promoter (T7) and the antigenome hepatitis δ virus ribozyme sequence (δ) followed by a T7 transcription terminator sequence (T7φ). The CAT reporter gene is inserted in an antisense orientation with respect to the T7 promoter. (B) The pIHNV-CAT(−) plasmid was transfected into vTF7-3-infected EPC cells and mock-infected or superinfected with IHNV. The supernatant was collected and used to infect fresh cells. CAT activity was checked on cell lysates at various times post-infection (bottom). A pCAT plasmid was used as the positive control for CAT activity. The plasmid encoding the N nucleoprotein, pT7-N (1 µg), was also transfected at P0 (right part of the figure).

Although the conversion of \(^{14}C\)chloramphenicol to its acetylated form was rather low (data not shown), it was, however, significant, indicating that the in vitro-synthesized IHNV RNA had been encapsidated and transcribed. It is likely that the very low level of CAT activity observed reflects the small number of mature RNA molecules which had reached the appropriate intracellular compartment to be encapsidated and released into the cell culture medium. A demonstration that the IHNV RNA minigenome is replicated in the infected cells was provided by additional passage onto fresh cells. Such rescue of exogenous RNA by a helper virus has been described for a number of single-stranded negative-sense RNA viruses belonging to the family Paramyxoviridae (Park et al., 1991; Collins et al., 1991; Yunus et al., 1999; Sidhu et al., 1995; De & Banerjee, 1993; Dimock & Collins, 1993; Randhawa et al., 1997). However, it has never been described for members of the family Rhabdoviridae such as vesicular stomatitis virus or rabies virus (Conzelmann, 1998), nor for rhabdoviruses replicating at a low temperature (14 °C) like IHNV or VHSV. The ability to rescue the T7-driven in vitro-synthesized RNA minigenome prompted us to investigate whether the T7 RNA polymerase could be provided in fish cells by infection with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986) and, thus, if it could be an alternative system for rescue experiments.

The pIHNV-CAT(−) plasmid was transfected in vTF7-3-infected EPC cells and superinfected with IHNV. Thus, EPC cell monolayers in 6-well plates (3 × 10⁶ cells per well) were infected with vTF7-3 (m.o.i. of 5) for 1 h at 37 °C. Cell monolayers were washed twice and transfected with 1 µg of pIHNV-CAT(−). These cells were incubated for 5 h at 37 °C. The mixture was then removed and the cells were infected with IHNV (5 p.f.u. per cell) and incubated overnight at 20 °C, then at 14 °C for 48 h. Cells and supernatants were harvested for further analysis and passaging experiments. As above, the recovery of the encapsidated IHNV minigenome was checked by monitoring CAT gene expression in infected cells after one passage. CAT activity was detected as early as 8 h post-infection and was optimal at 30 h post-infection (Fig. 1B). After 30 h, cells were too damaged due to the growth of the wild-type IHNV to accurately monitor CAT gene expression. CAT expression was directly related to the presence of IHNV proteins, since when IHNV superinfection was omitted (Fig. 1B) no CAT activity was detected. The rescue of the IHNV RNA minigenome synthesized in cells from the pIHNV-
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Fig. 2. (A) Structure of the cDNAs encoding mini-antigenome IHNV-CAT RNA (positive-sense). (B) The pIHNV-CAT(+) plasmid was transfected into vTF7-3-infected EPC cells and mock-infected (−) or superinfected (+) with IHNV and CAT activity was checked with no passage (P0) or after one passage (P1) of the virus supernatant.

CAT(−) plasmid proved to be more efficient than when exogenous RNA was provided. The encapsidated minigenome was replicated as, after two passages of the cell supernatant, CAT activity was detected in infected cells. CAT activity was even increased when the P1 supernatant was diluted (1:10, data not shown). Surprisingly, when a plasmid encoding the IHNV nucleoprotein (pT7-N, see below) was co-transfected with pIHNV-CAT(−), after one passage of the supernatant the CAT activity was drastically reduced (Fig. 1B, right lane), although the CAT activity at P0 was as high as when pT7-N was omitted (data not shown).

Although the leader and trailer sequences of both IHNV and VHSV are largely different, 10 out of the 12 extreme terminal nucleotides are conserved, and thus it was of interest to undertake the rescue of the IHNV minigenome with VHSV as the helper virus and vice versa. Thus, a pVHSV-CAT(−) construct, derived from the VHSV genome was engineered as for the pIHNV-CAT(−) minigenome. pVHSV-CAT(−) was transfected into vTF7-3-infected EPC cells and the RNA minigenome was shown to be encapsidated, replicated and propagated following VHSV infection, but not following IHNV infection. The IHNV minigenome was not propagated following VHSV infection (data not shown). This negative result indicated that the cis-acting elements in IHNV and VHSV genomes are not conserved.

A construct encoding an IHNV mini-antigenome (positive-sense) containing the CAT gene was engineered (Fig. 2A). The results of the rescue experiment using pIHNV-CAT(+) are shown in Fig. 2(B). At 24 h following cell transfection and virus infections (IHNV and vTF7-3), cells were lysed and analysed for CAT activity (passage P0). A background of CAT activity was observed (Fig. 2B, left lane) when cells were infected only with vTF7-3 and transfected with pIHNV-CAT(+), due to the messenger sense of RNA synthesized by the T7 RNA polymerase; however, when cells were super-infected with IHNV, CAT activity was dramatically increased, demonstrating that the mini-antigenome construct was replicated (Fig. 2B, P0). CAT activity was detected at P1 as well, indicating that the construct was replicated and encapsidated (Fig. 2B, P1).

Sequences containing IHNV genes encoding the N nucleoprotein (1176 bp long), the P phosphoprotein (693 bp long), the Nv non-structural protein (336 bp long) and the L RNA polymerase (5958 bp long) were recovered by PCR from a full-length DNA copy of the IHNV genome (unpublished data) using specific respective primers (primers sequences are available from the authors upon request). PCR products were digested with the appropriate restriction enzymes and inserted into XbaI- and BpiI1102I-digested pET-14b vector (Novagen), resulting in plasmids pT7-N, pT7-P, pT7-Nv and pT7-L. In these constructs, the IHNV coding regions were inserted between the T7 promoter and T7 terminator sequences. For mammalian rhabdoviruses like rabies virus and vesicular stomatitis virus, it has been shown that minigenomes are...
Fig. 3. IHNV active replicative complex from cDNAs. (A) Various combinations of the pIHNV-CAT(−) minigenome construct and the plasmids encoding the nucleoprotein (pT7-N), the polymerase-associated protein (pT7-P), the RNA polymerase (pT7-L) and the non-virion protein (Nv) were used to transfect vTF7-3-infected cells. The amounts of the respective plasmids (bottom) are indicated in micrograms (µg). pBlueScript SK plasmid (pBS) was added in some experiments to keep the total amount of transfected DNA constant. CAT activity was checked at the indicated times (top) after transfection. (B) Quantification of CAT activity by phosphorimaging when pT7-Nv was added in the transfection mix (ng). TLC plates were scanned using a phosphorimager (Phosphorimager, Molecular Dynamics). CAT activity is expressed in arbitrary units. The values indicated are the means of duplicate experiments.

rescued when the viral nucleocapsid proteins are expressed from cDNAs in cells (Pattnaik et al., 1992; Conzelmann & Schnell, 1994). A test was developed to evaluate whether the three IHNV-derived replicative complex proteins are expressed and functionally active in EPC cells after transfection of the respective recombinant pT7-N, pT7-P and pT7-L plasmids. Based on the knowledge that the synthetic pIHNV-CAT(−) minigenome could be replicated and encapsidated when the replicative complex proteins were provided by IHNV infection, minigenome constructs were transfected into vTF7-3-infected EPC cells together with two different combinations of pT7-N, pT7-P and pT7-L. CAT activity was monitored at 24 and 48 h post-transfection (Fig. 3 A). Depending on the ratio of the four plasmid constructs used, CAT activity was variable but detectable in all cases, indicating that the three constructs encoding the replicative complex were functional and able to encapsidate, replicate and transcribe the IHNV minigenome. As previously mentioned, the addition of pT7-N plasmid in excess drastically reduced the level of CAT activity, contrasting with the results obtained with respiratory syncytial virus, a paramyxovirus, for which replication of a synthetic minigenome increased after the addition of a plasmid encoding the nucleoprotein (Fearns et al., 1997).

IHNV and VHSV are both rhabdoviruses which replicate at 14 °C in fish cells and encode a non-viral (Nv) protein. They belong to a genus called Novirhabdovirus. Using the optimal conditions determined above, the effect of adding increasing amounts of the pT7-Nv construct was determined (Fig. 3 A). Quantification of CAT activity was performed by phosphorimaging of the acetylated spots detected on TLC plates (Fig. 3 B). A roughly 5-fold increase in CAT activity was observed when the pT7-Nv expression plasmid was added to the transfection mixture in catalytic amounts (0–125 µg). Thus, the Nv protein may play a role at either replication or the transcription step, at least in this minigenome system. This result contrasts with those recently published for another novirhabdovirus, snakehead rhabdovirus (SHRV), which replicates at an elevated temperature of 31 °C (Johnson et al., 2000), and for which a reverse genetics system allowing the recovery of infectious virus entirely from cDNAs has been established. In the SHRV system, when the Nv gene is mutated to introduce a premature stop codon in the coding region no differences in the virus titre are observed compared to the wild-type SHRV. It will be of interest to examine the role of the IHNV Nv protein during virus replication in vitro and in vivo when a reverse genetics system for IHNV is established.

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