Salmonid alphavirus infection in Atlantic salmon - viral properties and host responses to infection

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Thesis for the degree of Philosophiae Doctor (PhD)

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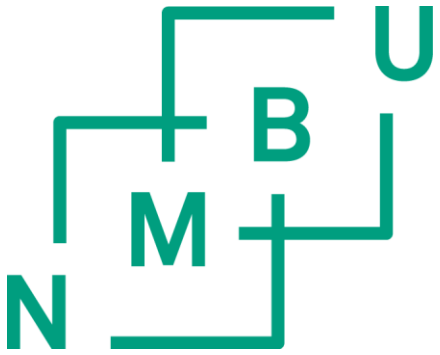
Adamstuen 2015



Series of dissertations at the

Norwegian University of Life Sciences

Thesis number 2015:2

ISSN 1894-6402
ISBN 978-82-575-1937-7

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ACKNOWLEDGEMENTS

The work in this thesis was carried out during the year of 2007 and 2011. The manuscript and thesis writing were continued since then and completed in spring, 2014. It has taken long time to reach the end, but certainly I have learned a lot from many people in different aspects.

First of all, I would like to thank my co-supervisor, Øyvind Haugland, who has been very supportive during my PhD. Thank you for the critical review of manuscripts and helpful solutions with many technical problems. I would also like to thank my project partner, Cheng Xu, who has been together with me building up the basis of research for the project.

Special thanks go to my cooperators, SFV group in Karolinska Institute in Sweden, including Professor Peter Liljeström and Senior scientist Daniel X. Johansson who provided us many valuable ideas, technical aids, and critical review of manuscripts. Thanks extend to other lab members in SFV group for your kindness during my stay at Karolinska.

I would like to give many thanks to all my colleagues in AKVA group, including the present and previous members. It has been very enjoyable for me to work in this lab and became friends with many of you. Thanks to those of you who have been listening to me and discussing with me regarding all kinds of problems. Professor Stephen Mutoloki, thank you for your kind help with the first paper and your daily concern about me and my family.

Finally, I would like to give biggest thank to my boss, Professor Øystein Evensen, who has brought me into this field by accepting me as master student in 2005. I have learned a lot from you during each brain storming and sorry for those days I stopped your leave for kindergarden and started discussing about the experiments. Now I realized that was not a good timing but thank you for being patient with me all the time. Also thank you for being encouraging throughout the study period no matter I was in good or bad condition.

Last but not least, thanks to my dearest family for all your support. My little boy, Hikaru, thank you for coming to us in 2012 and letting me learn how to be a mother. Your smile has given me a lot of energy to move our life forward. My husband, Naoki, thank you for sacrificing your job in Japan and moving to live with me in Norway. Your love, support and

patience have helped me reach the end of this thesis. My parents and brothers, thank you for your each warm concern and always being there when I need you even though we are so far away. All my friends in Norway, Taiwan and elsewhere in the world, thank you for being part of my life and bringing me a lot of energy during each gathering.

SUMMARY

Pancreas disease (PD) is a contagious viral disease in salmonid aquaculture in Europe and North America. PD is caused by salmon pancreas disease virus also referred to as salmonid alphavirus (SAV), which belongs to the genus alphavirus within the family *Togaviridae*. Up to now, at least six subtypes of SAV have been reported. In Norway SAV3 was the only subtype detected in PD diseased fish, but from 2010, a marine variant of subtype 2 has been on an increase, particularly in the Møre and Romsdal and Sør-Trøndelag counties. A better understanding of host-pathogen interactions will provide the basis for improved disease control and vaccine development. Currently, there is one commercial vaccine licensed in Norway and UK/Ireland against PD, based on inactivated whole virus. The efficacy of the vaccine under field conditions has been questioned.

In this thesis, the focus was first on the elicited immune response induced by SAV3 infection both *in vivo* and *in vitro*. Fish challenged in lab-experiments with SAV3 developed classical, pathological changes as seen under a natural infection. The sequence of pathological changes in the primary target organs, pancreas and subsequently heart, coincides with virus replication levels. Despite a strong innate immune response was detected in the affected organs, the virus infection progressed. The same phenomenon was observed when SAV3 was inoculated onto susceptible cell lines. However, when cells were pre-treated with recombinant IFN-α from 24 to 4 hours prior to infection, viral replication was halted, suggesting the timing of initiation of IFN system is imperative for the antiviral activity.

The work was followed by the development of an infectious SAV3 cDNA clone by use of reverse genetics. The constructed cDNA clone containing the full-length genome can be manipulated by deletion, insertion or substitution via genetic engineering. This constitutes a powerful tool for studying viral pathogenesis and developing nucleic acid based vaccines. The recovery of recombinant SAV3 was successful in three different cell lines (CHH-1, CHSE-214 and BF-2), though the recovery was dependent on the IFN response in the transfected cells. The 6K-deleted cDNA clone failed to generate infectious virus despite production of viral proteins was detected in the cytoplasm. RNA recombination was observed when the 6K-deleted cDNA was co-transfected into cells together with a helper cDNA encoding all structural genes, resulting in rescued, full-length viral RNA. A SAV3 replicon based vector

vaccine was developed and modified by inserting a hammerhead (HH) ribozyme sequence upstream the 5'UTR sequence and incorporating N-terminal 102 nt of capsid gene downstream the internal UTR and upstream the foreign gene. This construct confers a significant increase of the foreign antigen expression.

In conclusion, we have established an *in vivo* infection model of SAV3 and documented the antiviral role of IFN-α against SAV3 replication. We have also produced SAV3 recombinant virus using reverse genetics and a replicon construct for expression of heterologous proteins in fish cells. These tools can be used in future studies of viral pathogenesis and development of future virus vaccine.

SAMMENDRAG (In Norwegian)

Pancreas disease (PD) er en smittsom virussykdom hos laksefisk i akvakultur i Europa og Nord-Amerika. PD er forårsaket av salmon pancreas disease virus (SPDV) også betegnet salmonid alfavirus (SAV), tilhørerende slekten alfavirus i familien *Togaviridae*. Hittil har minst seks subtyper av SAV (SAV1-6) blitt rapportert, og innenfor disse er SAV3 funnet utelukkende i Norge. Bedre forståelse av vert-agens interaksjoner vil gi grunnlag for bedre sykdomskontroll og danne grunnlaget for vaksineutvikling. For tiden er det en kommersielt tilgjengelig vaksine mot PD, basert på inaktivert hel-virus, men den gir ikke tilfredsstillende beskyttelse i felt.

I denne avhandlingen fokuserte vi først på immunresponsen ved SAV3 infeksjon både *in vivo* og *in vitro*. Fisk eksperimentelt smittet med SAV3 utviklet klassiske patologiske forandringer lik de vi finner ved naturlige infeksjoner. Rekkefølgen av patologiske forandringer i primære målorganer, bukspyttkjertel og senere hjerte, sammenfalt med nivå av virusreplikasjon. Til tross for at infeksjonen induserte sterke medfødte, immunresponser i affiserte organer, begrenset ikke dette infeksjonen. Det samme så vi når SAV3 ble inokulert på mottakelige cellelinjer. Imidlertid, når cellene ble forbehandlet med rekombinant IFN-α (fra 24 til 4 timer før infeksjon), hindret det replikasjonen av virus. Dette tyder på at tidspunkt for aktivering av IFN-systemet er avgjørende for antiviral aktivitet.

Vi etablerte deretter et revers genetikk system for SAV3 slik at vi kunne produsere rekombinant virus fra en cDNA klon som inneholdt (kodet for) hele SAV3 genomet. Dette muliggjør at man senere kan modifisere sekvensen ved å endre, slette eller legge til elemenenter i virusgenomet, for slik å lage virusmutanter. Dette utgjør et svært viktig verktøy når en skal studere egenskaper hos virus, virus patogenese og fremtidig utvikling av DNA baserte vaksiner. Produksjon av rekombinant SAV3 var vellykket i tre forskjellige cellelinjer (CHH-1, CHSE-214 og BF-2), selv om effektiviteten varierte avhengig av i hvor stor grad de ulike cellelinjene produserte IFN. Vi forsøkte deretter å lage en attenuert SAV3 stamme uten genet som koder for 6K proteinet. Til tross for at virusets proteiner ble uttrykt i cellenes cytoplasma, klarte vi ikke å produsere rekombinant SAV3 uten 6K. Vi oppdaget derimot at SAV3 viste stor evne til å RNA rekombinasjon. Rekombinasjon ble observert når cDNA kodende for SAV3 uten 6K ble ko-transfektert inn i celler sammen med et hjelpe-cDNA som inneholder kun strukturelle gener (full-lengde). Dette resulterte i hyppig reversjon til full-

lengde virus. Med utgangspunkt i den infeksiøse cDNA klonene laget vi et SAV3 replicon for uttrykk av heterologe proteiner i cellekultur, og deretter ble effekt av ulike modifiseringer testet. Blant annet undersøkte vi effekten av å sette inn en «hammer-head» (HH) ribozyme sekvens oppstrøms for replikonet. Videre hvordan innlemming av N – terminale deler (102 nt) av kapsid genet nedstrøms for intern UTR og oppstrøms for innsatt fremmed gen gjorde at man fikk et signifikant høyere uttrykk av fremmed protein.

Som konklusjonen har vi etablert en *in vivo* infeksjon modell for SAV3 og dokumentert IFN-α sin viktige antivirale effekt mot SAV3. Vi har videre produsert rekombinant SAV3 virus ved hjelp av revers genetikk samt et SAV replikon konstrukt for uttrykk av heterologt protein i fiskeceller. Disse verktøyene kan brukes i videre studier av virusets patogenese og utvikling av fremtidige virusvaksiner.

LIST OF PAPERS

Paper I

Gene expression studies of host response to salmonid alphavirus subtype 3 experimental infections in Atlantic salmon

Authors: Xu C*, Guo TC*, Mutoloki S, Haugland Ø, Evensen Ø

Published: *Veterinary Research 2012, 43:78*

* Equal Contribution

Paper II

Alpha interferon and not gamma interferon inhibits salmonid alphavirus subtype 3 replication *in vitro*

Authors: Xu C, Guo TC, Mutoloki S, Haugland Ø, Marjara IS, Evensen Ø

Published: *Journal of Virology 2010, 84:8903-8912*

Paper III

A 6K deletion variant of salmonid alphavirus is non-viable but can be rescued through RNA recombination

Authors: Guo TC, Johansson XD, Haugland Ø, Liljeström P, Evensen Ø

Published: *PLoS ONE 2014, 9(7): e100184.*

Paper IV

Modification of a salmonid alphavirus replicon vaccine vector for high-level expression of foreign antigens

Authors: Guo TC, Johansson XD, Liljeström P, Evensen Ø, Haugland Ø

Published: *Journal of General Virology 2014, In press.*

INTRODUCTION

General background

Salmonid aquaculture at an industrial scale started in the 1970s (Karlsen *et al.*, 2013). During the 1980s, salmon farming in Norway experienced large losses due to bacterial infections which resulted in a strong increase in the use of antibiotics to control infections (Lillehaug *et al.*, 2003). As a consequence the emergence of antibiotic resistance among fish pathogens started to become a problem to the industry, created fish welfare concerns and had direct economic impact on production. Today we know that vaccination against bacterial diseases in Norwegian salmonid aquaculture has been a successful story (Hastein *et al.*, 2005), accompanied by the reduced use of antibiotics from 48,570 kg in 1987 to 1,051 kg in 2002 (NIFES, 2003). During the same period, the production of salmonids increased from ~55,300 tons to ~553,000 tons. In 2013 the total production of salmon and trout was more than 1.2 million tons with 972 kg antibiotics used (Mattilsynet, 2014). Commercially available vaccines are proved highly efficacious and conferred protection against several important fish pathogens including furunculosis (*Aeromonas salmonicida ssp. salmonicida*), vibriosis (*Vibrio anguillarum* – two serotypes), coldwater vibriosis (*Vibrio salmonicida*), yersiniosis (*Yersinia ruckeri*) and winter ulcers (*Moritella viscosa*) (Gudding & Van Muiswinkel, 2013; Press & Lillehaug, 1995). Most vaccines used today in Norwegian aquaculture are multivalent and administered by intraperitoneal (IP) injection with an oil adjuvant (water-in-oil emulsion) to increase the immune response and to prolong the duration of immunity. Despite the oil adjuvant is important for long term protection it also commonly causes side effects manifested as intra-abdominal granulomas and melanin pigmentation at the injection site (Haugarvoll *et al.*, 2010; Koppang *et al.*, 2008; Mutoloki *et al.*, 2004; Mutoloki *et al.*, 2006; Mutoloki *et al.*, 2010).

In spite of the remarkable success that commercial vaccines have had when it comes to controlling bacterial diseases, there are still a long way to go to obtain good protection against viral diseases (Biering *et al.*, 2005; Gomez-Casado *et al.*, 2011). Today, viral infections account for significant losses in Norwegian aquaculture. Viral vaccines against infectious pancreatic necrosis (IPN), infectious salmon anemia (ISA), and salmon pancreas disease (SPD) are commercially available as experimental vaccines or have marketing authorization (against IPN and SPD), but these vaccines do not confer full protection against mortality. Current viral

vaccines consist of either formalin-inactivated whole-virus antigens (IPNV and SPDV) or recombinant subunit antigens (IPNV-VP2), both being non-replicating and non-infective antigens (Christie, 1997; Karlsen *et al.*, 2012; Munang'andu *et al.*, 2013). The main advantage of these types of vaccines is the safety profile. However, they confer limited protection in the field, despite high protection levels can be obtained when these vaccines are tested in experimental trials. Non-replicating and non-infective antigens trigger mainly B-cell mediated humoral responses while cellular responses are weak (or absent). The latter is considered to be the most effective against intracellular pathogens, such as viruses (Woolard & Kumaraguru, 2010). To elicit cell-mediated defence mechanism in fish, several types of replicating vaccines, such as live-attenuated vaccines, replication deficient viral vaccines, and DNA vaccines are attractive candidates (Evensen & Leong, 2013; Gomez-Casado *et al.*, 2011).

Pancreas disease

Pancreas disease (PD) was first observed in Atlantic salmon (*Salmo salar*) in Scotland in 1976 (Munro *et al.*, 1984). It was subsequently discovered in other countries such as Norway, Ireland, France, USA, Italy and Spain (Boucher, 1994; Graham *et al.*, 2007; Kent, 1987; Murphy *et al.*, 1992; Poppe *et al.*, 1989). The disease has been endemic since 2003 and became notifiable in 2007 in Norway. In 2012 and 2013, outbreaks of PD are reported from 137 and 99 fish farms, respectively (Gjevre *et al.*, 2013). PD normally affects farmed Atlantic salmon and rainbow trout during the first year at sea and the incidence is highest during the summer and early autumn (Rodger & Mitchell, 2007). The clinical signs of PD include inappetence, slow growth, lethargy, and increased number of faecal casts in the cages. Histopathological changes in PD-diseased fish first appear in the pancreas and the heart tissue (ventricle), manifested as necrosis of the entire exocrine pancreas as well as degeneration and necrosis of cardiomyocytes (Lopez-Doriga *et al.*, 2001). Skeletal muscle degeneration with subsequent invasion of leukocytes is also commonly found in diseased fish and severe skeletal myopathy likely explains the abnormal swimming behaviour of affected fish. The mortality rates in Norwegian PD outbreak are found to be highly variable, ranging from 0 to 80% (Taksdal *et al.*, 2007).

Salmonid alphavirus (SAV)

The infectious agent causing pancreas disease in Atlantic salmon was first identified in 1995 as a “toga-like” virus (Nelson *et al.*, 1995) and was named salmon pancreas disease virus (SPDV). It was later assigned to the genus alphavirus within the family *Togaviridae* (Weston

et al., 1999). In 2000, the agent causing sleeping disease in rainbow trout (Sleeping disease virus – SDV) was identified and confirmed to be an atypical alphavirus (Villoing *et al.*, 2000). The agent causing pancreas disease in Norwegian aquaculture was characterized in 2005 and named Norwegian salmonid alphavirus (NSAV) as it differed slightly from SPDV and SDV (Hodneland *et al.*, 2005). It was subsequently proposed that the nomenclature of these viruses should be assigned to subtypes of salmonid alphavirus (SAV1-3) based on nucleotide sequence criteria (Weston *et al.*, 2005), SAV1 for SPDV, SAV2 for SDV, and SAV3 for NSAV. Until now, at least six SAV subtypes are distinguished based on genotype, and these subtypes are found in geographically defined areas (Fringuelli *et al.*, 2008). In addition to SAV3, which is exclusively found in Norway, a marine SAV2 variant was detected in mid-Norway from 2010 and has rapidly spread within mid-Norway (Hjortaas *et al.*, 2013).

Alphaviruses

Alphavirus is one of the two genera in the family *Togaviridae* and the other genus (Rubivirus) has rubella virus as its sole member (Strauss & Strauss, 1994). Alphaviruses are small, enveloped viruses that infect a broad range of insect and vertebrate hosts. Currently there are at least 24 recognized virus species within the genus (Powers *et al.*, 2001), where two virus species infecting marine animals are identified, southern elephant seal virus (SESV) and salmonid alphavirus (SAV) (La *et al.*, 2001; Weston *et al.*, 1999).

Alphaviruses are classified as arboviruses (arthropod-borne virus) and are transmitted to vertebrates by hematophagous insects such as mosquitoes and ticks, which are the arthropod vectors in the enzootic cycle. In the arthropod vectors, alphavirus replicates and causes a persistent infection in the salivary glands (Strauss & Strauss, 1994). Through the bite of the host, the infected mosquitos/ticks inoculate virus-containing saliva into the blood of the vertebrate host. In contrast to alphaviruses in general, SAV infections can be transmitted without an insect vector (Kongtorp *et al.*, 2010; Paper I in this thesis). A few studies have found SAV associated with sea lice, though it remains unclear whether sea lice contribute to the infection either directly or indirectly (Petterson *et al.*, 2009; Rodger & Mitchell, 2007).

Alphaviruses can be divided into two categories, Old World and New World viruses, based on the geographic area where they are present (Garmashova *et al.*, 2007b). The diseases caused by alphavirus infection differ between Old World and New World viruses. Many Old World viruses cause a disease characterized by rash and high fever and sometimes arthralgia

(Suhrbier *et al.*, 2012). In this category Semliki Forest virus (SFV) and Sindbis virus (SINV) are the two best-studied prototype alphaviruses. Both SINV and SFV can be grown to high titers in insect cells but are less infectious to humans (Laine *et al.*, 2004; Lundstrom, 2003b; Suhrbier *et al.*, 2012). Another Old World species, Chikungunya virus (CHIKV), has recently become a re-emerging public health concern in Africa, Asia, and Italy (Caglioti *et al.*, 2013). In contrast to the symptoms caused by Old World viruses, several New World viruses cause encephalitis, such as Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV). These are clinical important pathogens, albeit with low occurrence, which cause serious illness and sometimes high mortality rates in humans and animals (Nolen-Walston *et al.*, 2007; Quiroz *et al.*, 2009; Roy *et al.*, 2009).

Genomic structure

The alphavirus genome is a single positive-strand RNA of approximately 11.7 kb in length comprising two open reading frames (ORFs) (Strauss & Strauss, 1994). The first ORF encodes the replicase polyprotein while the second ORF encodes viral structural proteins (Figure 1). The RNA genome is capped at the 5' end and polyadenylated at the 3' end. The 5'-terminal two-thirds of the genomic RNA encodes four non-structural proteins (nsPs), designated nsP1 to nsP4. The 3'-terminal one-third of the genome encodes structural proteins including the capsid protein, two glycoproteins (E1 and E2), and two small peptides 6k and E3. Additionally, the genome contains three untranslated regions (UTRs) at 5' end (5'UTR), 3' end (3'UTR), and an internal untranslated region (I'UTR) between the non-structural and the structural genes. 5' and 3' UTR contain sequences important for transcription/replication of genomic RNA and I'UTR region contains a promoter for transcription of the subgenomic RNA (Frolov *et al.*, 2001; Levis *et al.*, 1990).

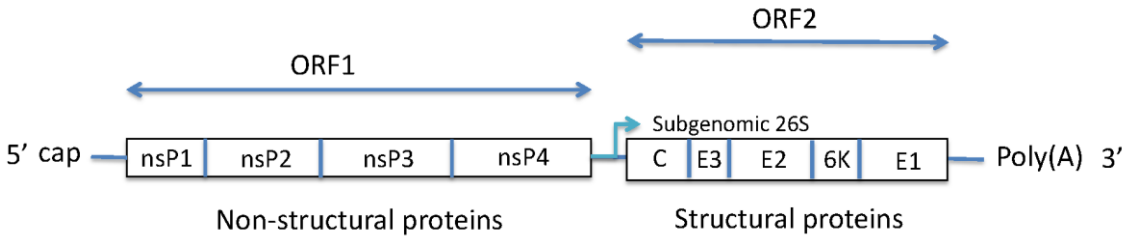


Figure 1. Genomic organization of alphavirus. The alphavirus genome is composed of two ORFs, ORF1 encodes four non-structural proteins (nsP1-nsP4) and ORF2 driven by a 26S subgenomic promoter, encodes the capsid protein (C), two glycoproteins (E1 and E2), and two small peptides (6k and E3).

Virion structure

Alphaviruses are small viruses about 65-70nm in diameter (Figure 2) (Jose *et al.*, 2009; Sherman & Weaver, 2010). The RNA genome is encapsulated by 240 copies of capsid protein arranged in a T=4 icosahedral shell forming the nucleocapsid. The nucleocapsid is enveloped by a tight-fitting lipid bilayer containing mainly two viral glycoproteins E1 and E2. The surface of the virion is made up of 80 trimers of E1/E2 heterodimers, also arranged in a T=4 icosahedral lattice.

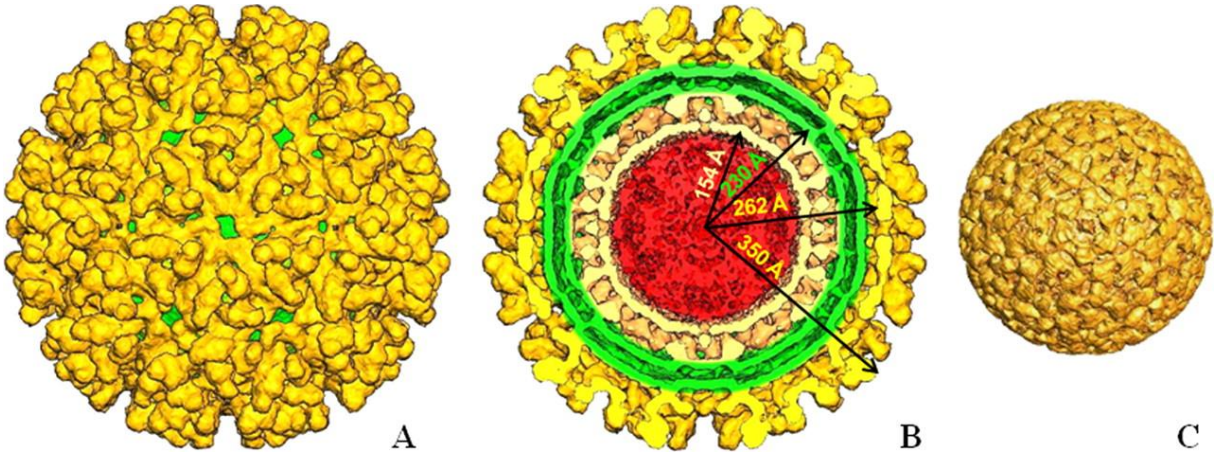


Figure 2. 3D map of recombinant WEEV revealed by Cryo-EM. (A) The trimer spikes are clearly visible. Green layers represent the outer leaflet of the lipid membrane separating the glycoprotein shell from the inner nucleocapsid. (B) The front half of the map is removed to reveal a radical arrangement of the virus particle. The viral RNAs are in red. (C) WEEV nucleocapsid, organized into an icosahedral shell with T=4 symmetry (Source: Sherman & Weaver, 2010). Reprinted with permission.

Virus Entry

Alphavirus infection is initiated by attachment of the viral enveloped protein to a cell surface protein that serves as its receptor on the host cell plasma membrane. The viral E2 glycoprotein is responsible for receptor binding; although E1 might also play some role in the interaction (Strauss *et al.*, 1994). One or several proteins might be utilized as functional receptors with varying affinity. After binding, the virus enters the cell by clathrin-mediated endocytosis and is transported to the endosome (Helenius *et al.*, 1980; Marsh & Helenius, 1980). The acidification within the endosome triggers fusion of the viral spike proteins with endosomal membranes. The nucleocapsid is then released into cytoplasm where it is subsequently uncoated by ribosomes and viral RNA is released and prepared for replication.

Replication

Once released into the cell, the genomic positive-strand RNA of alphaviruses directly serves as the messenger RNA for protein synthesis and as a template for the generation of minus-strand RNA (Strauss & Strauss, 1994) (Figure 3). For SINV, the 5' two-thirds of the genomic RNA is translated into two different polyproteins, P123 or P1234 (translational read-through), with the formal being the predominant product (Li & Rice, 1993). However, in some other alphaviruses such as SFV, an opal termination codon is not present and therefore only the polyprotein P1234 is produced (Takkinen, 1986). The resultant polyproteins are autocleaved into nsP1-4 by the function of nsP2 that recognizes conserved cleavage sites between the nsPs (Vasiljeva *et al.*, 2003). These four nsPs form the replication complex responsible for the amplification of the viral RNA. The initial cleavage occurs only between nsP3 and nsP4 and results in the early replication complex P123 and nsP4. This complex produces the minus-sense RNA as the template for the generation of positive-sense RNA and subgenomic RNA (Shirako & Strauss, 1994). At a later stage, irreversible conformational changes are required to the polyprotein processing at nsP1/2 and nsP2/3 sites. The fully processed replication proteins, nsP1-4, form the late replication complex which produce the positive-sense genomic and subgenomic RNA, but is no more able to produce negative-sense RNA (Gorchakov *et al.*, 2008; Lemm *et al.*, 1994). In SINV, transcription from the subgenomic promoter generates about threefold more subgenomic RNA relative to the full-length genomic RNA (Raju & Huang, 1991).

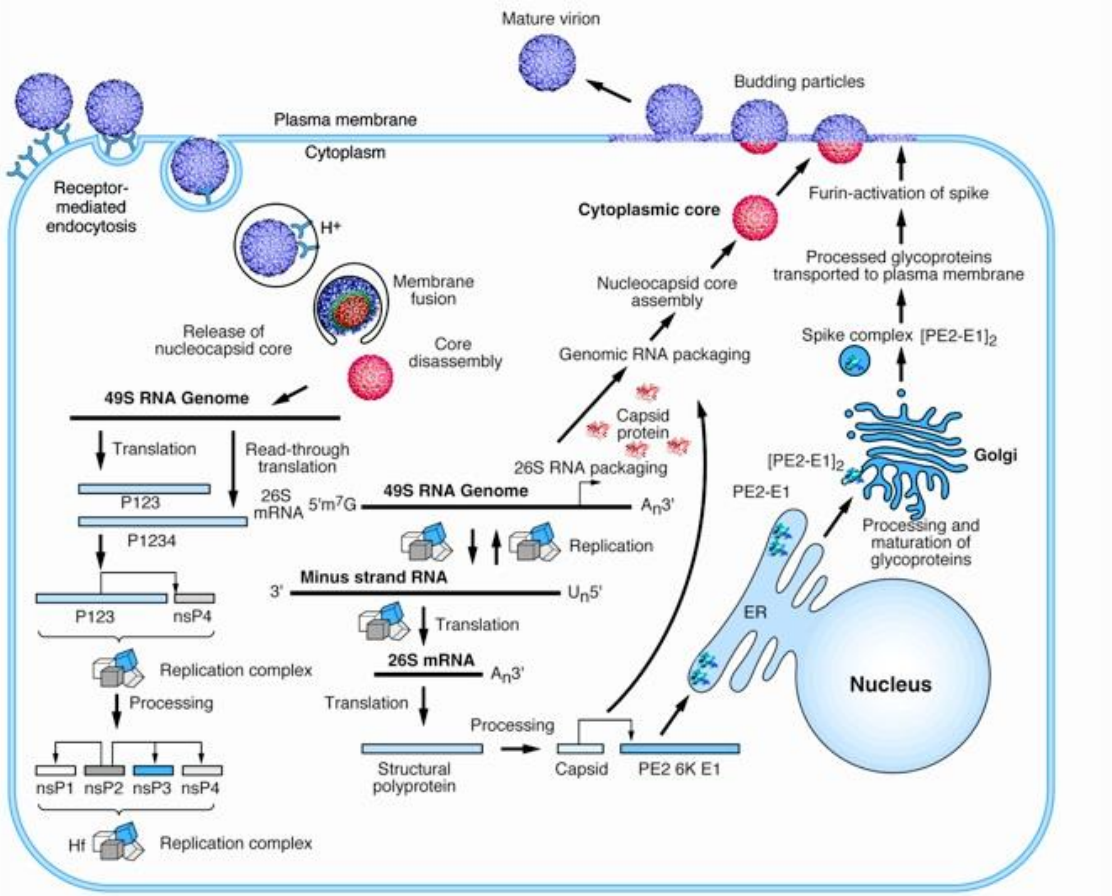


Figure 3. Alphavirus life cycle. This diagram illustrates the replication/propagation cycle of alphavirus including attachment, endocytosis, release of nucleocapsid, transcription, translation, post-translational polyprotein processing, genome packaging and assembly of nucleocapsid core and finally release/budding of infectious viral particles. We acknowledge the permission of using this figure original from Dr. Richard J Kuhn's webpage. (Available: http://bilbo.bio.purdue.edu/~viruswww/Kuhn_home/research.php)

Structural protein processing

The 26S subgenomic RNA encodes the structural proteins, including capsid (C), enveloped glycoproteins E1, pE2 (E2 and E3), and a small hydrophobic protein 6K. These proteins are initially translated as a 26S polyprotein and processed individually to the mature form by post-translational cleavage (Figure 4). At first, the capsid protein, located at N-terminus of the structural polyprotein, co-translationally releases itself from the polypeptide by its own serine protease activity at C-terminal (Skoging & Liljestrom, 1998). The rest polyprotein containing unprocessed pE2, 6K and E1 is directed by a signal sequence to translocate into the ER lumen where host signal peptidase liberates pE2, 6K and E1 from each other (Garoff *et al.*, 1990). pE2 and E1 undergo folding and post-translational modification and subsequently form heterodimers (pE2-E1) in the ER before trafficking via the secretory pathway to the plasma membrane where the pE2 and E1 further assemble into trimmers (commonly referred to as spikes), and pE2 is cleaved by a furin protease into the small E3 and E2 proteins. The function of individual viral protein is listed in Table 1.

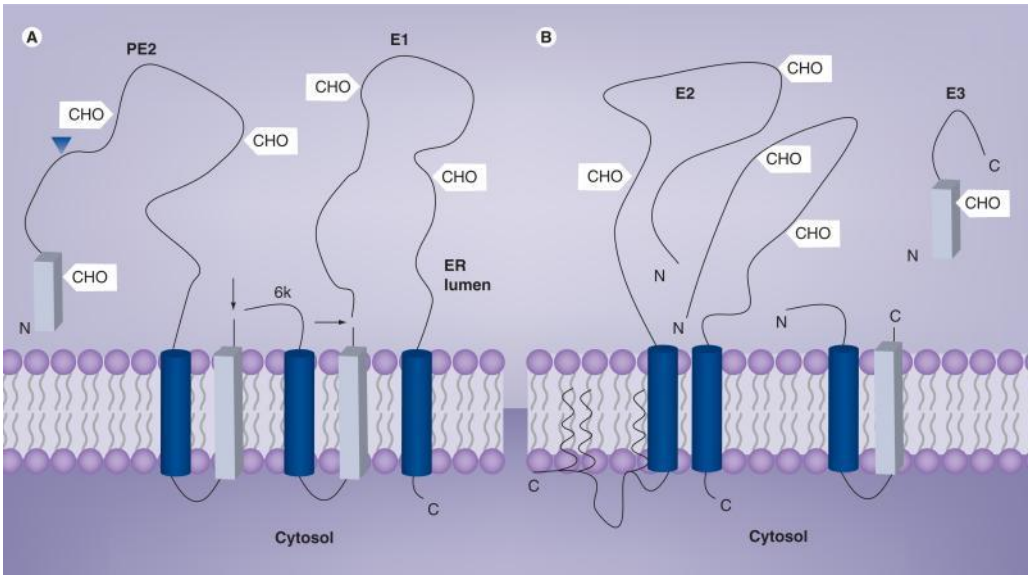


Figure 4. Model of glycoprotein configuration on the (A) ER lumen: host signal peptidase liberates pE2, 6K and E1 from each other, and (B) plasma membranes: pE2 is cleaved by a furin protease into the small E3 and E2 proteins (Source: Jose *et al.*, 2009). Reprinted with permission.

Table 1. Function of individual viral proteins

	Features and Functions	References
nsP1	• Possess capabilities of guanine-7 methyltransferase and guanyltransferase: responsible for capping of viral RNA at 5' end	(Ahola *et al.*, 1997)
nsP2	• An enzymatic protein • N-terminal : RNA binding associated with ATPase and GTPase activity, implicated in unwinding of intermediate double-stranded RNA during replication • C-terminal: functions as the non-structural proteinase	(Mayuri *et al.*, 2008)
nsP3	• Mutations in nsP3 affect negative strand RNA synthesis and subgenomic RNA synthesis • Interaction of nsp2/nsp3 is important for the function of replication complex	(LaStarza *et al.*, 1994)
nsP4	• Function as RNA dependent RNA polymerase (RdRp) • Its concentration is strictly regulated inside cells and excess nsp4 is rapidly degraded	(Kamer & Argos, 1984) (de Groot *et al.*, 1991)
Capsid	• A multifunctional protein • Viral RNA genome is encapsidated into nucleocapsid cores • C-terminal end: autoproteolytic activity, release itself from the nascent polypeptide chain • N-terminal peptide (amino acids 33-68) of VEEV and EEEV capsid protein: plays the most important role in the down-regulation of cellular transcription and development of CPE • Amino acids 1-34 of capsid in SFV: translational enhancer	(Skoging & Liljestrom, 1998) (Garmashova *et al.*, 2007a) (Sjoberg *et al.*, 1994; Yamanaka & Xanthopoulos, 2004)
E3	• A small cysteine-rich glycoprotein • Produced from the cleavage of PE2 to E3 and E2 • Carry signal for the translocation of pE2 into the lumen of ER	(Presely & Brown, 1989) (Lobigs *et al.*, 1990)
E2	• Involved in the virulence determinant • Bind to cell receptor	(Dropulic *et al.*, 1997; Glasgow *et al.*, 1994; Lee *et al.*, 2002; Santagati *et al.*, 1995)
6K	❖ Detailed in the paragraph below	
E1	• Trigger fusion between viral and cellular membrane	(Lescar *et al.*, 2001; Roussel *et al.*, 2006)

6K

Alphavirus 6K protein is a small and highly hydrophobic protein. The 6K proteins of SFV or SINV have been shown to be involved in membrane permeabilization at the late infection stage (Gaedigk-Nitschko *et al.*, 1990; Liljestrom *et al.*, 1991; Sanz *et al.*, 1994). Oligomerization of 6K proteins leads to ion channels formation in cell membranes and increases membrane permeability, which facilitates virus budding (Loewy *et al.*, 1995). An identified aromatic domain (rich in aromatic amino acids) at the N-terminal of 6K shows a

strong tendency to insert in the interfacial phase of the phospholipid bilayer, which also facilitates membrane destabilization. In addition to the role associated with membrane permeabilization, 6K also provides cleavage sites for polyprotein processing at its N-terminal and C-terminal ends (Liljestrom & Garoff, 1991b). A SFV mutant lacking the entire 6K is processed correctly between PE2 and E1 without altering glycoprotein formation, heterodimerization and intracellular transport. However, the budding process of the SFV 6K deletion mutant is impaired and virus titer is reduced (Liljestrom *et al.*, 1991; Loewy *et al.*, 1995). Deletion of 6K in SINV also causes budding problem and in addition the cleavage of polyprotein becomes less efficient. The sequence of 6K gene in Salmonid alphaviruses has been identified by sequence homology to other alphaviruses (Weston *et al.*, 2002) though the actual cleavages sites remain to be determined experimentally. In paper III, we showed the pivotal role of 6K protein in SAV3 infectivity as no viable virus was rescued from the SAV3 cDNA clone lacking the entire 6K transfected cells. Recently, a novel protein termed transframe (TF) occurring as a frame shift product during translation of the 6K gene was identified (Snyder *et al.*, 2013). It is demonstrated that TF is not absolutely required as the lack of TF does not affect genome replication, particle infectivity, or protein trafficking but leads to a decreased release of viral particles.

The immune response following viral infections

The innate immune system plays a central role at the first line of defence against pathogens (Kawai & Akira, 2006). Upon infection, viral components such as single-stranded RNA, DNA and replication intermediate products double-stranded RNA (dsRNA), are recognized by the pattern-recognition receptors (PRRs) and subsequently activate the innate immune system (Diebold, 2010; Kawai & Akira, 2006; Kawai & Akira, 2007; Koyama *et al.*, 2008). There are three classes of PRRs which sense intracellular pathogens, namely Toll-like receptors (TLRs), retinoic acid-inducible gene I (Rig-I)- like receptors (RLRs), and nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) (Figure 5) (Takeuchi & Akira, 2009). TLRs and RLRs are involved in the production of type I interferons (IFNs), cytokines and chemokines, while NLRs are known to activate interleukin-1β (IL-1β) through activation of caspase-1 (Jacobs & Damania, 2012; Lamkanfi & Kanneganti, 2012). Genomic sequence analysis revealed that RLRs, such as RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), are highly conserved between mammals and fish (Rajendran *et al.*, 2012). Signalling pathway downstream of RLRs involves activation of IPS1 (also known as MAVS) and further phosphorylates IFN regulatory

factor 3 and 7 (IRF3/7). Nuclear translocation of these regulatory factors induces the transcription of cytokines and IFN genes.

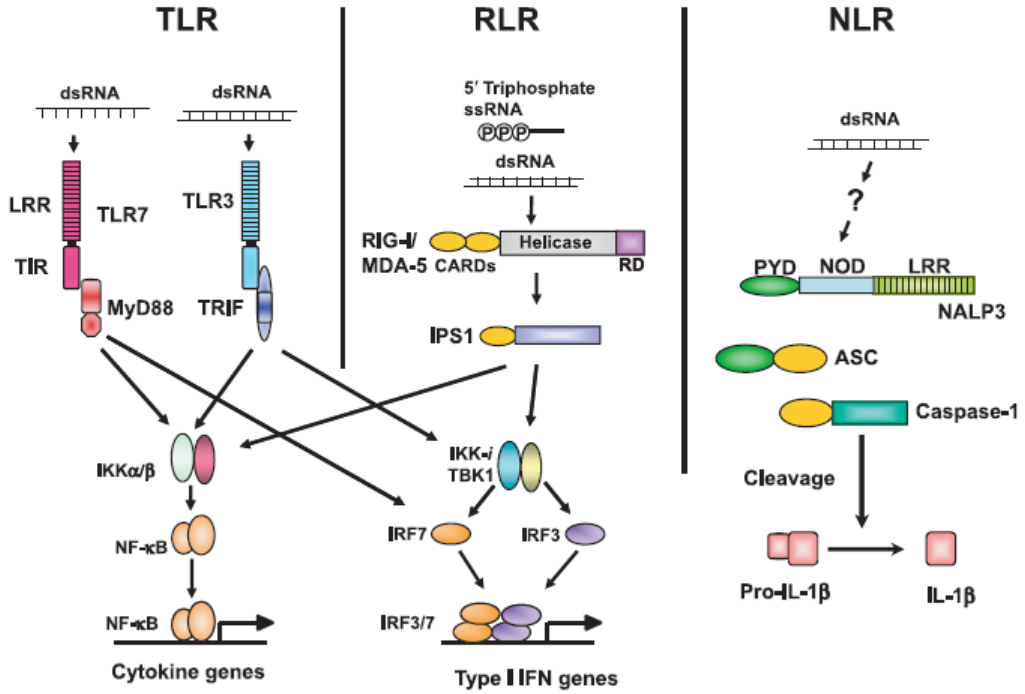


Figure 5. Three classes of pattern-recognition receptors (PRRs) and their signalling pathways. Upon infection, viral components are recognized by the PRRs and subsequently activate the innate immune system. These PRRs are classified into three classes: (I) TLR: Toll-like receptors. (II) RLR: retinoic acid-inducible gene I (Rig-I)- like receptors.(III) NLR: nucleotide oligomerization and binding domain (NOD)-like receptors. (Source: Takeuchi & Akira, 2009). Reprinted with permission.

The important role of IFNs in antiviral responses in mammals has been well documented. As viral diseases are important in salmonid aquaculture, many studies have been performed showing considerable conservation of IFN system between mammals and fish (Bergan *et al.*, 2010; Chang *et al.*, 2011; Collet, 2014; Langevin *et al.*, 2013). Mammalian IFNs have been classified as type I (α, β, ω, ε, and κ), type II (γ), and type III (λ). Within these, type I and type II are grouped as antiviral IFNs. In mammals, IFN binding to membrane receptors activate the JAK-STAT signalling pathway and downstream induce the transcription of IFN-stimulated genes (ISGs). Several hundreds of ISGs have been identified in human but only some of them such as Mx, ISG15, PKR, TRIM5, VIPERIN/VIG1 exert antiviral function (Langevin *et al.*,

2013). Several genes involved in this pathway are characterized in several fish species but their regulatory role remains unclear (Altmann *et al.*, 2004; Kibenge *et al.*, 2005; Rokenes *et al.*, 2007).

Innate immune response mediates the induction of adaptive immune response during viral infection (Aoshi *et al.*, 2011). Type I IFN regulates the expression of major histocompatibility complex (MHC) I in the infected cells, enhances histamine secretion by mast cells and activates NK cells (Blach-Olszewska, 2005; Finton & Strong, 2012). T cells can also be activated by type I IFN through up-regulation of both MHC I and MHC II and co-stimulatory molecules.

Immune responses elicited by different type of vaccines

The central dogma of vaccine development is to generate a safe and immunogenic vaccine. Today, commercially available vaccines against viral diseases in fish are (for the main part) based on non-infectious and non-replicating antigens, which confer good safety but marginal efficacy in controlling diseases. These non-replicating antigens are recognised as extracellular pathogens by PRRs on the cell surface. In higher vertebrates, the plasma membrane-associated PRRs are TLRs 1, 2, 4, 5, 6 and 10 whereas TLRs 3, 7, 8, and 9 are located intracellularly (Thompson *et al.*, 2011; Yokota *et al.*, 2010). Extracellular antigens are endocytosed into cytoplasm and presented through MHC class II to CD4+ T cells. This therefore skews the immunity towards humoral response generating neutralizing antibodies. In contrast, live-attenuated vaccines provide intracellular antigens mimicking natural viral infection resulting in induction of cytokines and co-stimulatory molecules which provide an adjuvant effect through recognition by intracellular PRRs and downstream, activate CD8+ T-cells which kill virus infected cells directly (Robert-Guroff, 2007).

Alphaviruses as vaccine vectors

Central to alphavirus research is the development of a vaccine or treatment against diseases caused by the different alphaviruses in a variety of host species (Roy *et al.*, 2014). Another important aspect of alphavirus research is to use the alphavirus as a vaccine vector, a tool for efficient production of recombinant proteins (Leitner *et al.*, 2000; Lundstrom, 1997; Rayner *et al.*, 2002). Scientists also express hope that these vaccine vectors can be used as tools for gene therapy and cancer treatment in the future (Lundstrom, 2001; Lundstrom, 2002; Lundstrom, 2003a; Lundstrom, 2012).

Alphavirus vectors are attractive tools in vaccine development; they are self-replicating RNA in the cytosol expressing heterologous antigen to high levels, and can be expressed in many cell types, including tumor cells (Lundstrom, 2002). In addition, they are cytopathic and greatly reduce the risk of integration of vector sequences into host genome and prevent persistence of infected cells. However, this feature results in transient gene expression and has limited its use in gene therapy where a long-term transgene expression is required. On the other hand, alphavirus vector-based vaccines gain more and more interest because of their superiority over other viral vectors in terms of the induction of innate and adaptive immune responses (Frolov *et al.*, 1996; Riezebos-Brilman *et al.*, 2006).

The alphavirus expression vectors can be constructed and delivered in three forms:

(I) Propagating vector

SINV was the first alphavirus being developed as replicon expression vector (Xiong *et al.*, 1989) by replacing the structural genes with the chloramphenicol acetyltransferase (CAT) gene (Figure 6). Similar replicon vectors were also developed using SFV and VEEV (Davis *et al.*, 1996; Liljestrom & Garoff, 1991a). Replicating vector-based system contains a full-length cDNA clone with a duplicated 26S subgenomic promoter driving expression of a heterologous antigen and can ultimately form infection-competent viral particles (recombinant virus). The gene of interest expressed by the second subgenomic promoter can be located either upstream or downstream of the genes encoding the structural proteins (Figure 6). This type of vector is replicating-competent and packaging-competent, but because of RNA size packaging constraints, the insert is generally limited up to 2kb. Although this is an efficient system to scale up protein production *in vitro*, the application as a vaccine *in vivo* can be limited for safety reason.

(II) Non-propagating vector

Alphavirus naked RNA and DNA based vaccines are packaging/propagating-defective but replicating-competent and have been shown to confer high immunogenicity, provoking both humoral and cytotoxic response (Berglund *et al.*, 1998; Zhou *et al.*, 1995). Alphavirus DNA or RNA replicons contain replicase (non-structural genes) and a heterologous gene instead of the structural genes (Figure 6). The cDNAs are cloned into one expression vector, which contains the eukaryotic promoter such as CMV. This vector is transfected into the cell and the cellular RNA polymerase II will transcribe the recombinant RNAs. These transcribed RNAs are transported into the cytoplasm and viral RNAs are amplified. In this case, the expression

level of heterologous proteins is dependent on the transfection efficiency. In addition, an expression vector with improved expression has been developed incorporating the enhanced translational signal located at 5' termini of the capsid gene (Sjoberg *et al.*, 1994).

Several advantages of alphavirus replicons have made it an attractive vaccine vector compared to other viral vectors. First, alphavirus vector provides both good safety (non-spreading) and high immunogenicity due to the replicating and cytopathic (induce apoptosis) features. Several studies have shown strong humoral and cellular responses induced by alphavirus replicon (Rayner *et al.*, 2002). Second, the expression of this vector is transient/lytic and thus decreases the risk of chromosomal integration. Third, since the antigen is encoded by self-replicating RNA where the required dose for immunisation is greatly reduced (100- to 1000-fold lower) and therefore is more cost-effective (Berglund *et al.*, 1998; Leitner *et al.*, 2000).

Alphavirus replication-induced apoptosis leading to transient antigen expression in the cells, confers not only safety but also seems to be an important feature for the activation of antigen presenting cells (Leitner *et al.*, 2003). However, this feature can also limit its use when long term expression is required. To broaden the range of its application, new SFV vectors with mutations in nsP genes have been engineered and these exhibit decreased cell death (Casales *et al.*, 2008).

(III) Suicide particles

Alphavirus replicon in combination with one helper vector encoding structural proteins *in trans* can form replication deficient viral particles, with one-round infection as a result (Tubulekas *et al.*, 1997; Zhou *et al.*, 1994). One key feature here is that the packaging signal is located in nsP1 for SINV and nsP2 for SFV while helper RNAs lack the packaging signal. Thus encapsidation into viral particles is not possible. The problem with this system is that RNA recombination could occur between replicon RNA and helper RNA during replication generating wild-type genome which again can form infectious viral particles. To resolve this problem, one helper vector has been split into two helper vectors (Smerdou & Liljestrom, 1999) (Figure 7). This greatly increases the safety as it is assumed that "double RNA" recombination occurs at very low frequency.

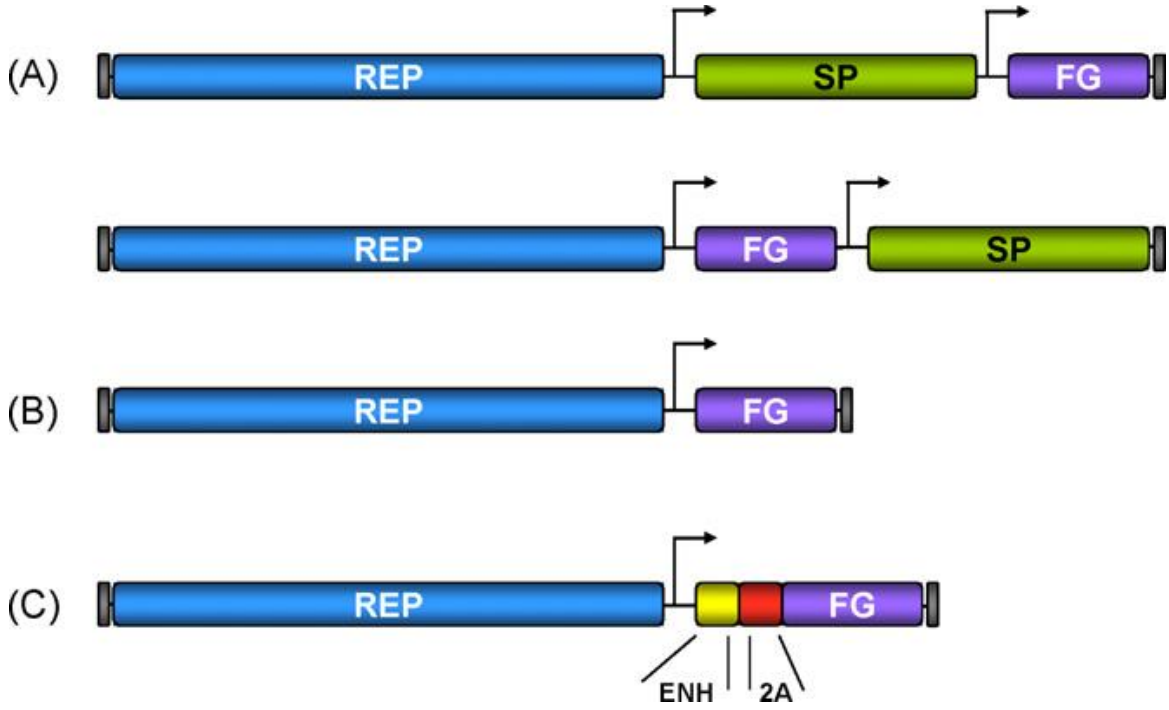


Figure 6. Construction map of replicon vaccine vectors. (A) Propagating vector: The gene of interest expressed by the second subgenomic promoter can be located either at upstream or downstream of structural protein genes. (B) Non-propagating vector: Naked RNA and DNA replicon based vaccines are packaging/propagating-defective but replicating-competent. (C) An enhancer and a 2A sequence derived from foot and mouth diseased virus (FMDV) are inserted prior to foreign gene to obtain an enhanced expression of antigens. REP: replicon; SP: structural protein; FG: foreign gene; ENH: enhancer; 2A: FMDV 2A sequence. (Source: Virus Research 153, 2010. 179-196). Reprinted with permission.

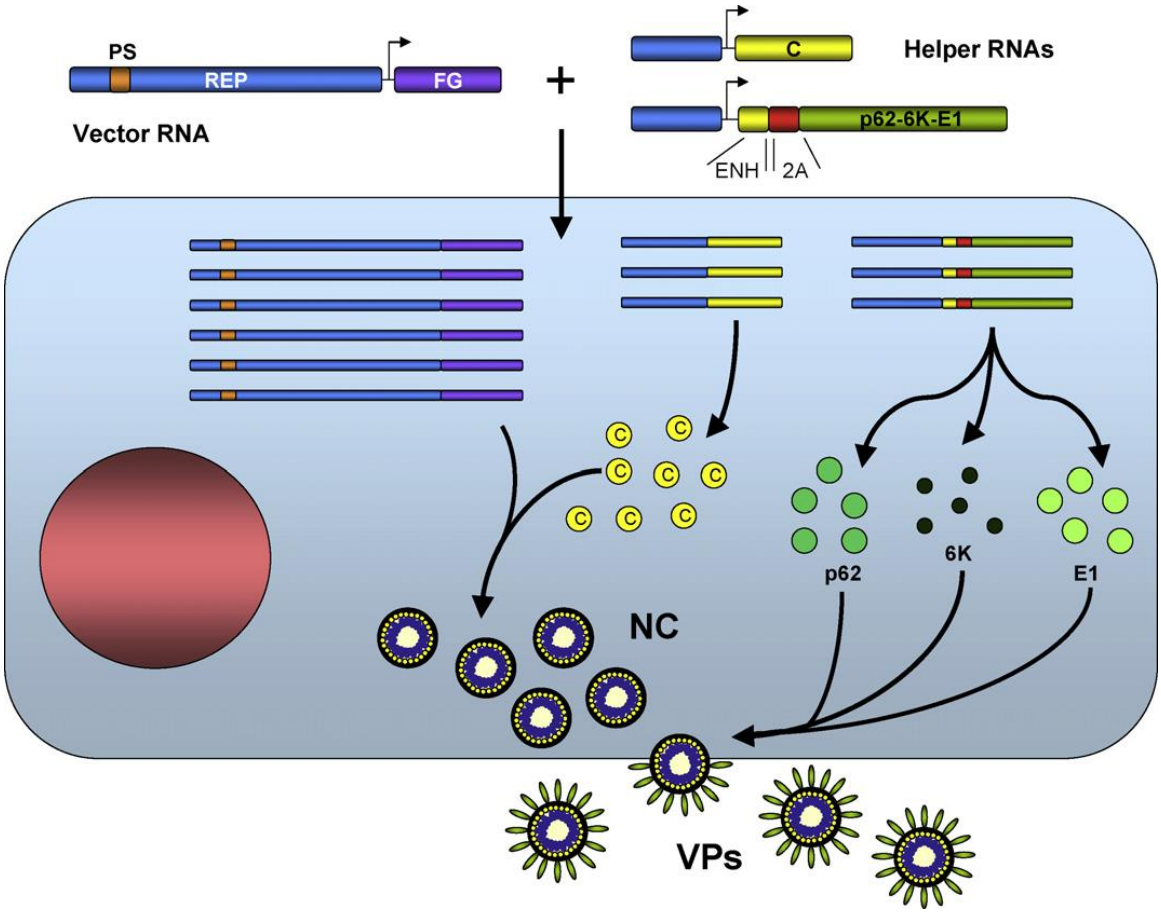


Figure 7. Construction of replication deficient viral particles. Alphavirus replicon vector in combination with one or two helper vectors encoding structural proteins *in trans* are co-transfected into host cells. Replicon RNAs and helper RNAs are replicated in cytoplasm; however, as the packaging signal is located within non-structural protein (nsP) gene, helper RNAs lack packaging signal and thus encapsidation will not occur. The second round of infection will not produce infectious progeny (non-propagating). PS: package signal; REP: replicon; C: capsid; SP: structural protein; FG: foreign gene; ENH: enhancer; 2A: FMDV 2A sequence; NC: nuclear capsid; VPs: viral particles. (Source: Virus Research 153, 2010. 179-196). Reprinted with permission.

Alphaviruses used as replicating vaccines – concerns with regard to RNA recombination

RNA viruses are known to undergo rapid genetic changes and thus gain the reputation as Nature's swiftest evolvers (Duarte *et al.*, 1994). The most common mechanism of genetic evolution is through nucleotide substitutions leading to the concept that RNA viruses occur as quasispecies. Some RNA viruses that contain segmented genomes, such as influenza virus, rotavirus, and bluetongue virus, can undergo genetic evolution by reassortment of the RNA segments. An additional mechanism, which involves the exchange of genetic information

between non-segmented RNAs, is RNA-RNA recombination (Lai, 1992;Simon-Loriere & Holmes, 2011). Several animal viruses, such as poliovirus and coronavirus (Jarvis & Kirkegaard, 1992; Keck *et al.*, 1987; Li & Ball, 1993), and plant viruses (Sztuba-Solinska *et al.*, 2011; White & Morris, 1994) have been proved to undergo RNA-RNA recombination at varying frequency.

During viral replication, RNA-dependent RNA polymerase (RdRp) switches the template strand while at the same time remain with ("hold on to") the nascent strand, thereby generating the hybrid RNA with mixed origins of molecules. This process is called "copy-choice recombination" and is the most widely accepted model of RNA recombination (Lai, 1992; Simon-Loriere & Holmes, 2011; Worobey & Holmes, 1999). Various factors can influence template switching by RdRps, including sequence identity between two RNA templates, the kinetics of transcription, and secondary structure in RNA template. Template switching occurs usually between two "homologous" RNAs at the crossover sites. RNA polymerases usually stall at sites of strong RNA secondary structure, which is the rate-limiting step during RNA synthesis and may promote template switching. The presence of strong secondary structure of two RNA templates may lead to homologous or non-homologous RNA recombination.

Three types of RNA recombination (Figure 8)

I. Homologous recombination

This type of recombination involves two similar or closely related RNA molecules with extensive sequence homology. The crossing over sites are perfectly matched between the two RNA templates so that the recombinant RNAs (the "outgoing copy") retain the exact sequence and structural organization of the parental RNA molecules.

II. Aberrant homologous recombination

In contrast to homologous recombination, crossovers occur not at homologous or comparable sites, but at unrelated although usually nearby sites on each parental RNA molecule. As a result, recombinant RNA contains sequence duplication or deletion and, in some cases, even insertion of nucleotides from unknown origin.

III. Non-homologous/ Heterologous recombination

Non-homologous recombination occurs on RNA molecules which do not show any sequence homology. The basis for selection of the recombination sites is

unclear. One possibility is that the crossover sites on the two RNAs share similar RNA secondary structures.

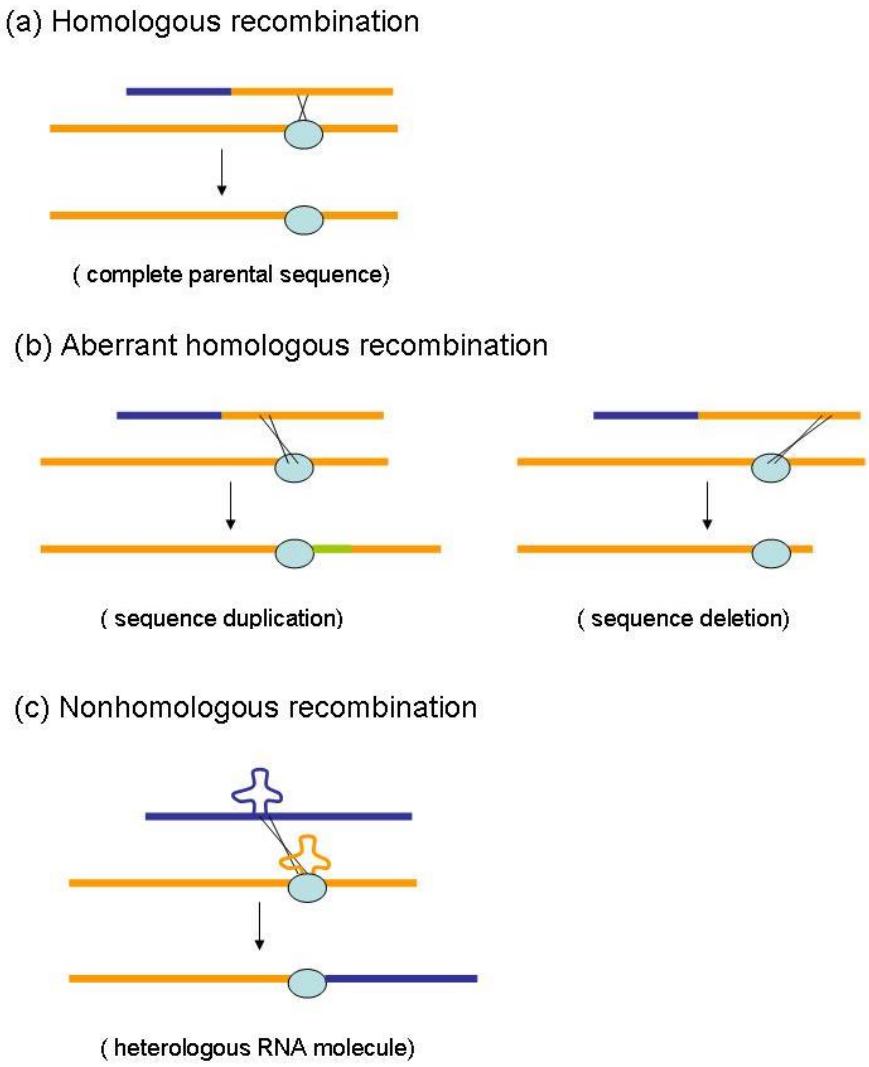


Figure 8. Mechanisms of viral RNA recombination. (a) Homologous recombination: involves two similar or closely related RNA molecules. (b) Aberrant homologous recombination: crossovers occur not at the homologous or comparable sites. (c) Non-homologous recombination: occurs on RNA molecules, which do not show any sequence homology. (Figure prepared by Tz-Chun Guo.)

AIMS OF THE STUDY

The overall objective of this thesis was to better understand the host-pathogen interaction for SAV3 infection *in vitro* and *in vivo.* We wanted to provide insight into host/cell immune responses post infection as well as to better understand aspects of virus replication phenomena, and thereby establish a scientific basis for development of novel viral vaccines.

The specific aims were to:

1. Profile the immune response elicited by SAV3 *in vivo* challenge in Atlantic salmon parr with focus on pathological changes, timing of the immune responses and the concurrent kinetics of viral replication in target organs
2. Document the importance of the innate immune response towards SAV3 infections *in vitro*, with a focus on interferon-alpha (IFN-α) and IFN-α induced responses
3. Construct a full-length infectious cDNA clone for production of recombinant SAV3 by reverse genetics, and to evaluate the ability of Δ6k SAV3 mutant to produce progeny and thereby serve as a potential attenuated vaccine
4. Construct a SAV3 replicon based vector and study the impact of modifications to the constructs for efficacious, heterologous antigen expression

METHODOLOGY

Reverse genetics of RNA viruses

Reverse genetics is an approach to discover the function of a gene by analysing the phenotypic effects from the altered gene sequences. The use of reverse genetics in virological studies allows rescue of infectious viruses from constructed cDNA clones encoding the viral genome. In contrast to virus isolated from infected animals which often contains a mixed population of genotypes (viral quasispecies), recombinant virus generated by reverse genetics can be made with a precise and well-defined single genotype. This approach opens the possibility of deleting genes for studying their function and introducing targeted mutations to determine genetic factors important in pathogenicity of virus and for interaction with the host immune system. The first engineered positive-strand RNA virus was poliovirus (*Picornaviridae* family) in 1981 (Racaniello & Baltimore, 1981), and subsequently a non-segmented negative-stranded RNA virus, rabies virus in 1994 (Schnell *et al.*, 1994). Reverse genetics systems have also been established for several fish RNA viruses (reviewed by Biacchesi, 2011). The first recombinant fish virus recovered from constructed full-length cDNA clone was established for IPNV in 1998 (Yao and Vakharia, 1998) and later for Norwegian IPNV isolates in 2005 (Santi *et al.*, 2005a; Santi *et al.*, 2005b). In addition to IPNV, reverse genetics systems of several fish viruses including infectious hematopoietic necrosis virus (IHNV) (Biacchesi *et al.*, 2000; Ammayappan *et al.*, 2010; Romero *et al.*, 2008), viral hemorrhagic septicemia virus (VHSV) (Biacchesi *et al.*, 2010; Ammayappan *et al.*, 2011), *Betanodavirus* NNV (Iwamoto *et al.*, 2001), salmonid alphavirus SAV2 (SDV) (Moriette *et al.*, 2006) and SAV3 (Karlsen *et al.*, 2010; Paper III) have been established.

Steps required for generating a SAV3 infectious cDNA clone

1. Isolate wild-type virus from field samples (tissue homogenates)
2. Propagate virus in susceptible cell lines
3. Sequence the full viral genome including precise 5’ and 3’ ends
4. Perform PCR amplification and assembly of PCR fragments to constitute a cDNA clone encoding the full viral genome
5. Subclone the full-length cDNA into an expression vector comprising necessary promoter sequence for transcription. The T7 promoter (for *in vitro* transcription) and CMV promoter (for expression in cells) are commonly used

6. Modify the full-length cDNA clone to allow precise expression of viral genome without incorporation of non-viral sequence
7. Transfect the constructed cDNA clone or *in vitro* transcribed RNA derived from the cDNA clone into cells for virus rescue
8. Confirmation and evaluation of rescued recombinant virus

Isolation and propagation of SAV from field outbreaks

Successful isolation from diseased fish depends on the infection stage and the viral loads (Petterson *et al.*, 2013). Diagnosis performed by ordinary PCR or real-time PCR (RT-PCR) is capable of detecting the presence of virus, though very often this does not guarantee the successful virus propagation in the cells. A recent study showing that numerous viral deletion mutants exist in naturally infected fish (Petterson *et al.*, 2013) may explain the difficulty of propagating virus. Several cell lines are used to propagate SAV including CHSE-214, BF-2, RTG-2, TO, and CHH-1. The choice of cell line for virus propagation is important, and we found CHH-1 cells to be the best suited for propagating virus and the SAV3-induced IFN response in CHH-1 was low compared to other cell lines (Paper III and Paper IV).

Rapid amplification of cDNA ends (RACE)

At the start of the study, complete sequences for SAV3 isolates were not available in GenBank. The full-genome sequence of the isolate (SAV3-H10) used throughout this work, was resolved based on previously published SAV3 sequences. As genome information covering the 5' and 3' ends were not available, RACE was performed to amplify and sequence the complete 5' and 3' viral genome end sequences. The procedure begins with isolation of total RNA from the organisms, either directly from infected animal tissues or virus cultured in cell culture. This is followed by first-strand cDNA synthesis using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT). The core advantage using this enzyme is its terminal transferase activity adding 3-5 residues of dC when it reaches the end of an RNA template (Chen & Patton, 2001). For 5' RACE cDNA, a **S**witching **M**echanism **A**t the 5'end of **R**NA **T**ranscript (SMART) oligo containing a universal primer sequence with 3-5 residues of dG allows the oligo to anneal to the extended first-strand cDNA tails (containing 3-5 residues of dC) (Figure 9). MMLV RT switches templates from the mRNA molecule to the SMART oligo, generating a complete cDNA copy of the original RNA with SMART oligo sequence at the end. The 3' RACE cDNA is synthesized using a conventional reverse transcriptional reaction, but with a special oligo (dT) conjugating with the universal primer

sequence anneals to the poly(A) tail of the mRNA (Figure 9). The requirement for RACE cDNA amplification is that at least 23-28 nucleotides of template sequence is known in order to design gene-specific primers (GSPs) for the 5' and 3' PCR reactions (Figure 10). GSPs together with the universal primer constitute the forward and reverse primers for PCR reactions. Nested GSPs, inner primers anneal to the first PCR product, might be needed in some cases where the level of background or nonspecific amplification in the first PCR reaction is too high with a single GSP. The obtained PCR products are subcloned into a vector for sequencing. Usually at least 10 colonies should be analysed for identification of each end sequence.

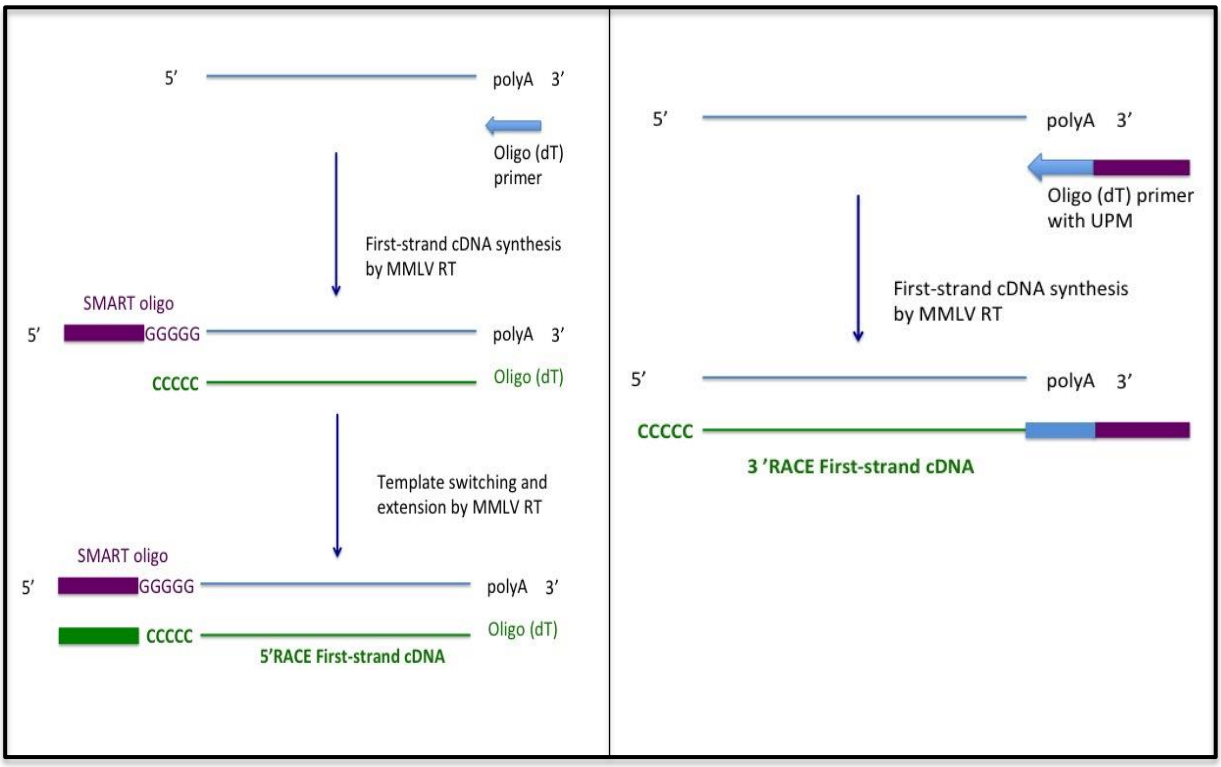


Figure 9. Mechanism of 5' (Left) and 3' (Right) RACE first-strand cDNA synthesis by MMLV RT. UPM: Universal primer. (Modified from Clontech SMARTer® RACE cDNA Amplification Kit user manual).

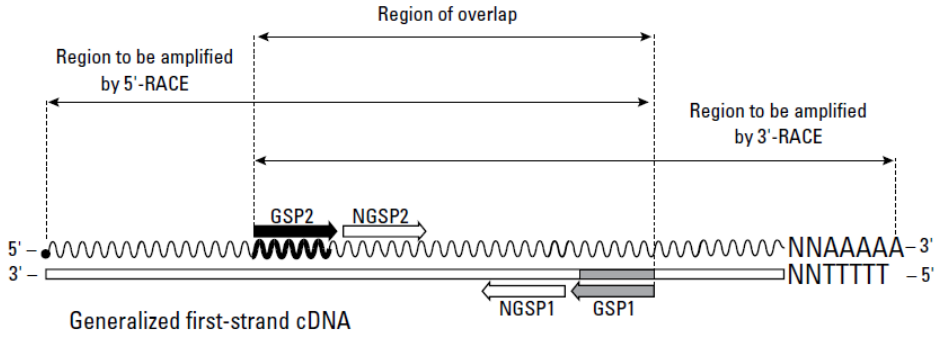


Figure 10. Gene-specific primers (GSP) and nested gene-specific primers (NGSP) used for 5' and 3' PCR reactions. (Source: Reprinted with permission from Clontech).

Gene manipulation by site-directed mutagenesis PCR

The construction of SAV3 full-length infectious cDNA clone is described in detail in paper III. Once the reverse genetic system is established it is possible to recover recombinant virus entirely from cDNA, and it is also possible to genetically manipulate the viral genome through site-directed mutagenesis. With this approach, deletions, insertions, and substitutions can be introduced into the viral genome.

a. Deletion

Two approaches can be applied for the deletion of gene sequences within a cDNA clone:

Two-step PCR:

First approach requires two single-cut restriction sites within the whole full-length construct. To perform this PCR, in total four primers are required. Two flanking (outward-to-inward) primers should contain two single-cut restriction sites at their 5' ends while the other two internal (inward-to-outward) primers should locate excluding the region where is going to be deleted. Furthermore, two internal primers should have overlapping sequences at their 5' ends for sequence annealing. This deletion procedure requires two runs of PCR. The first run is to amplify two fragments mediated by the four primers. The second run of PCR is to combine two fragments using the overlapping sequence at two internal primers (also called fusion PCR). After a second run of PCR, the amplicon should contain two single-cut restriction sites at its two terminal ends and at the same time the deletion has been made. At this stage the new amplicon can be cloned back to the original full-length cDNA clone using two single-cut restriction sites and the new circulized cDNA clone is a deletion-mutant. The drawback of this

method is that it is not always easy to find two single-cut restriction sites close to the deleted region and the larger the DNA construct size is, the less choices of single-cute restriction site will be available.

One-step PCR:

The concept of the second approach is one-step straightforward but the success relies on the purity of DNA template and the quality of DNA polymerase. As the total size of SAV3 cDNA clone is close to 17kb, long-range PCR is required with this method and therefore the selection of DNA polymerase is critical. The ideal DNA polymerase should possess high fidelity with processivity function to amplify the template accurately and should generate blunt-ended PCR products to avoid introducing any extra nucleotide. Another key point when the aim is to obtain a precise sequence after long-range PCR, is to run as few amplification cycles as possible since the efficacy of DNA polymerase declines dramatically after a certain number of cycles. Two primers oriented in the reverse direction running from two ends of the region to be deleted will generate a linearized PCR product with the deletion. The linearized PCR products should be re-circularized before transformation and propagation in bacteria. To re-circularize DNA, T4 polynucleotide kinase should be added to introduce 5'-phosphates at two ends of oligonucleotides and therefore allow subsequent ligation with DNA ligase.

b. Insertion

Short insert:

The insertion of a gene sequence into a cDNA clone can be achieved applying the same principle as described for deletion (above). If the "to be inserted sequence" is short such as a few base pairs, PCR primers can be designed to carry these nucleotides. Both two-step PCR and one-step PCR can be applied to achieve insertion of a few nucleotides. Through PCR amplification, the desired sequence will be introduced into the cDNA clone.

Long insert:

If the "to-be-inserted sequence" is long such as several kilo bases, the sequence should first be amplified by PCR and the cloning should be performed with two-step PCR. In this case the vector is engineered so that it is flanked by two restriction sites used for cloning, and the primers used to amplify the insert should incorporate the corresponding restriction sites at each end. When the purpose is to obtain a fusion protein, the stop codon either on the insert or on the vector, depending on the N-terminal fusion or C-terminal fusion, should be deleted by mutagenesis.

c. Substitution PCR

Nucleotide substitution can be achieved by introducing mutations into PCR primers. After PCR amplification, the amplicons will contain mutated sequences. This can be easily performed by one-step PCR method, given that high processive DNA polymerase is utilized. In the case that DNA construct is very large, two-step PCR method will still be preferred.

Transfection

Recovery of recombinant virus from viral cDNA relies on the transfection technology. Transfection is the process by which nucleic acids are delivered into a host cell through the cell membrane. The technology includes lipid transfection and mechanical transfection or electroporation. Lipid transfection is performed by conjugating DNA to cationic lipid (liposomes) forming a complex, which facilitates fusion of the complex with the plasma membrane of the cells and eventually leads to DNA uptake. This method is simple, rapid and effective. Electroporation is the use of high-voltage electric shock to introduce DNA into cells, both *in vitro* and *in vivo*. When the electric pulse reaches the cell membranes, the phospholipid bilayers of the membrane is disturbed and a temporary hole or perforation of the membrane is created. DNA molecules can thus enter the membrane perforations and the cell membrane will self-seal after some time.

Transfection efficiency and cell viability are two important factors for successful transfection. Many commercial products are developed and optimised to obtain high transfection rate and low cytotoxicity in mammalian cells. The method of lipid transfection is simple and rapid, at the same time less number of cells and copies of DNA are required. In contrast, by electroporation DNAs are delivered straight into cell nuclei and therefore the onset of RNA transcription and protein production will be faster. While transfection into mammalian cell lines has become easier, it remains a challenge to acquire high transfection rate for fish cell lines. For recovery of SAV3 cDNA clones, both lipid transfection and electroporation were tested. Compared to lipid transfection, electroporation gives viral protein expression at earlier time points (post transfection) while at later time points, the transfection rate turn out to be comparable for the two methods.

SUMMARY OF SEPARATE PAPERS

Paper I

Salmonid alphavirus subtype-3 (SAV-3) infection in Atlantic salmon is exclusively found in Norway. The salmonid alphaviruses have been well characterized at the genome level but there is limited information about the host-pathogen interaction phenomena. This study was undertaken to characterize the replication and spread of SAV-3 in internal organs of experimentally infected Atlantic salmon and the subsequent innate and adaptive immune responses. In addition, suitability of a cohabitation challenge model for this virus was also examined. Groups of fish were infected by intramuscular injection (IM), cohabited (CO) or kept uninfected in a separate tank. Samples of pancreas, kidney, spleen, heart and skeletal muscles were collected at 2, 4 and 8 weeks post infection (wpi). Pathological changes were assessed by histology concurrently with viral loads and mRNA expression of immune genes by real time RT-PCR. Pathological changes were only observed in the pancreas and heart (target organs) of both IM and CO groups, with changes appearing first in the pancreas (2 wpi) in the former. Lesions with increasing severity over time coincided with high viral loads despite significant induction of IFN-α, Mx and ISG15. IFN-γ and MHC-I were expressed in all tissues examined and their induction appeared in parallel with that of IL-10. Inflammatory genes TNF-α, IL-12 and IL-8 were only induced in the heart during pathology while T cell-related genes CD3ε, CD4, CD8, TCR-α and MHC-II were expressed in target organs at 8 wpi. These findings suggest that the onset of innate responses came too late to limit virus replication. Furthermore, SAV-3 infections in Atlantic salmon induce Th1/cytotoxic responses in common with other alphaviruses infecting higher vertebrates. Our findings demonstrate that SAV-3 can be transmitted via the water making it suitable for a cohabitation challenge model.

Paper II

Salmonid alphavirus (SAV) is an emerging virus in salmonid aquaculture, with SAV-3 being the only subtype found in Norway. Until now, there has been little focus on the alpha interferon (IFN-alpha)-induced antiviral responses during virus infection in vivo or in vitro in fish. The possible involvement of IFN-gamma in the response to SAV-3 is also not known. In this study, the two IFNs were cloned and expressed as recombinant proteins (recombinant IFN-alpha [rIFN-alpha] and rIFN-gamma) and used for in vitro studies. SAV-3 infection in a

permissive salmon cell line (TO cells) results in IFN-alpha and IFN-stimulated gene (ISG) mRNA upregulation. Preinfection treatment (4 to 24 h prior to infection) with salmon rIFN-alpha induces an antiviral state that inhibits the replication of SAV-3 and protects the cells against virus-induced cytopathic effects (CPE). The antiviral state coincides with a strong expression of Mx and ISG15 mRNA and Mx protein expression. When rIFN-alpha is administered at the time of infection and up to 24 h postinfection, virus replication is not inhibited, and cells are not protected against virus-induced CPE. By 40 h postinfection, the alpha subunit of eukaryotic initiation factor 2 (eIF2alpha) is phosphorylated concomitant with the expression of the E2 protein as assessed by Western blotting. Postinfection treatment with rIFN-alpha results in a moderate reduction in E2 expression levels in accordance with a moderate downregulation of cellular protein synthesis, an approximately 65% reduction by 60 h postinfection. rIFN-gamma has only a minor inhibitory effect on SAV-3 replication in vitro. SAV-3 is sensitive to the preinfection antiviral state induced by rIFN-alpha, while postinfection antiviral responses or postinfection treatment with rIFN-alpha is not able to limit viral replication.

Paper III

Pancreas disease (PD) of Atlantic salmon is an emerging disease caused by Salmonid alphavirus (SAV), which mainly affects salmonid aquaculture in Western Europe. Although genome structure of SAV has been characterized and each individual viral protein has been identified, the role of 6K protein in viral replication and infectivity remains undefined. The 6K protein of alphaviruses is a small and hydrophobic protein which is involved in membrane permeabilization, protein processing and virus budding. Because these common features are shared across many viral species, they have been named viroporins. In the present study, we applied reverse genetics to generate SAV3 6K-deleted (Δ6K) variant and investigate the role of 6K protein. Our findings show that the 6K-deletion variant of salmonid alphavirus is non-viable. Despite viral proteins of Δ6K variant are detected in the cytoplasm by immunostaining, they are not found on the cell surface. Further, analysis of viral proteins produced in Δ6K cDNA clone transfected cells using radioimmunoprecipitation (RIPA) and western blot showed a protein band of larger size than E2 of wild-type SAV3. When Δ6K cDNA was co-transfected with SAV3 helper cDNA encoding the whole structural genes including 6K, the infectivity was rescued. The development of CPE after co-transfection and resolved genome sequence of rescued virus confirmed full-length viral genome being generated through RNA

recombination. The discovery of the important role of the 6K protein in virus production provides a new possibility for the development of antiviral intervention which is highly needed to control SAV infection in salmonids.

Paper IV

Salmonid alphavirus (SAV) replicon has been developed to express heterologous antigens but protein production was low to modest compared to terrestrial alphavirus replicons (Olsen *et al.*, 2013a). In this study, we have compared several modifications to a SAV replicon construct and analyzed their influence on foreign gene expression. We found that with an insertion of a translational enhancer consisting of the N-terminal 102 nucleotides of the capsid gene together with a nucleotide sequence encoding the FMDV 2A peptide caused a significant increase of the EGFP reporter gene expression. The importance of fusing a hammerhead (HH) ribozyme sequence at the 5' end of the viral genome was also demonstrated. In contrast, a hepatitis D virus ribozyme (HDV-RZ) sequence placed at 3'end did not augment expression of inserted genes. Taken together, we have developed a platform for optimized antigen production which can be applied for immunization of salmonid fish in the future.

RESULTS AND GENERAL DISCUSSIONS

Reverse genetics of salmonid alphavirus

The first infectious full-length cDNA of salmonid alphavirus (SAV2) was generated by Moriette *et al.* in 2006. A hammerhead ribozyme sequence placed immediately upstream of viral 5'UTR and downstream of CMV or T7 promoter functions as a self-cleavage enzyme which has shown to be crucial for the recovery of recombinant SAV2 (Moriette *et al*., 2006). A similar strategy has been applied to generate recombinant SAV3 (Karlsen *et al*., 2009; Karlsen *et al*., 2010; Paper III in this thesis), despite different backbone vectors were used to carry the viral genome. In the present study, we showed that rSAV3 can also be rescued from a full-length cDNA construct without incorporating the HH sequence at 5'end, although the recovery efficiency was significantly lower for this construct compared to HH construct (Paper IV). The explanation might be that non-HH cDNA construct contains a non-viral sequence between CMV promoter and 5' UTR (Paper III) which could impede the viral replication. If this is the case, removing the non-viral sequence at 5' end should improve virus recovery. However, it has been shown that recombinant SAV2 could not be recovered without a space sequence between the promoter and the 5'UTR (Leberre *et al*., 2011). The mechanism behind this remains unknown.

The recovered recombinant SAV2 (rSDV) was subjected to *in vivo* infection in juvenile rainbow trout and demonstrated to be fully attenuated and protective against subsequent challenge. Interestingly, the protective effect is seen only when the virus is recovered from CHSE-214 cells cultured at 10°C but not from BF-2 cells cultured at 14°C (Moriette *et al*., 2006). Further genome sequencing revealed six amino acid changes in the structural polyprotein (three in the E2, two in the 6K, and one in the E1). In this thesis, we demonstrated infectivity of rSAV3 *in vitro* and in addition we have also assessed the *in vivo* effect by intramuscular injection of full-length cDNA vectors into fish. At 8 weeks post injection, the target organs (pancreas and heart) were examined and shown to exhibit classical pathological changes indicating that pathogenic rSAV3 is formed *in vivo.* Mortality was not recorded (unpublished data) in line with other studies. To our knowledge, this is the first time that recombinant SAV was recovered from injecting cDNA into fish. The propagated virus in fish was reisolated from heart tissue and inoculated onto CHSE-214 cells where CPE was observed at 6 days post inoculation. Whether reducing the injection dose would reduce

pathological changes in internal organs and at the same time conferring protective immunity in fish remains to be evaluated.

Host-pathogen interaction *in vivo*

Characterization of immune gene expression post challenge is important for understanding mechanisms underlying host-pathogen interactions and for vaccine development. In paper I, fish were challenged with the SAV3-H10 isolate and then assessed for pathological changes, viral load, and expression of immune genes in different target organs over the course of infection. Pathological changes were first found in the pancreas and subsequently in the heart, in line with what has been described previously during natural infections (McLoughlin & Graham, 2007). Over the same period, IFN and ISGs were upregulated in association with the increase in viral load. It is noteworthy that ISG15 was upregulated 650-fold at two weeks post infection, before any pathological changes were evident. In conclusion, the results shed light on the importance of IFN system during SAV3 infection yet its antiviral role remains obscure as highly up-regulated IFN and ISGs in fish did not successfully hinder virus infection. This therefore brought us into the second study to investigate the role of IFN on viral replication (Paper II).

SAV and susceptibility to IFN system

It has been shown for other alphaviruses that IFN-α plays a pivotal role in protection against infection by limiting viral replication (Aguilar *et al.*, 2005;Grieder & Vogel, 1999;Stanton *et al.*, 1989). In agreement with this, our study showed that SAV3 is highly sensitive to IFN-α (Paper II), as cells pretreated with recombinant IFN-α before virus infection exhibited anti-SAV3 property. However, when virus infection and viral genome translation/replication occurred prior to treatment with recombinant IFN, the induced responses come too late to prevent viral replication and production of infectious progeny. This is in line with the observation that endogenous IFN increases concurrently with increase in viral RNAs copies, shown both *in vitro* and *in vivo* (Paper I, II, and III) but this is insufficient to hinder successful production of infectious progeny. In spite of this, results from Paper III and Paper IV suggest that IFN response regulates viral RNA replication to some extent. This was evidenced by early development of CPE and higher end-point virus titers when virus was propagated in CHH-1 cells (low IFN-competent) compared to CHSE-214 and in BF-2 cells (high IFN-competent). A similar study conducted previously on SAV1 infection in CHH-1 cells also showed earlier CPE compared with CHSE-214 and SHK-1 cells, yet no difference was found

with regard to the virus titers between these cell lines (Herath *et al.*, 2009). The discrepancy with our results might possibly be due to *in vitro* virulence differences between subtypes of SAV. The reduced IFN-α and ISGs (Mx and ISG15) response to SAV3 infection in CHH-1 cells could offer a superior environment for viral replication and assembly, which is further supported by the higher level of heterologous antigen expressed by the SAV3 replicon vector in CHH-1 cells compared to CHSE-214 and BF-2 cells (Paper IV). Taken together, these results suggest that CHH-1 cells might be well suited for generation of high titer SAV3 for inactivated vaccine development. Similarly, an IFN-deficient cell line, Baby hamster kidney-21 (BHK-21), has been widely used for the production of high titer alphaviruses (Atkins, 1979).

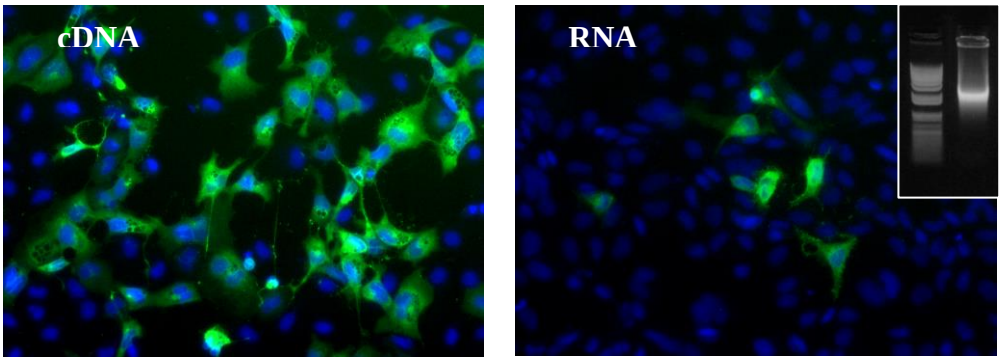
In paper II, we showed that recombinant salmon IFN-γ exhibited marginal antiviral effect against SAV3. In contrast to our result, a comparable study (Sun *et al*., 2011) suggested that recombinant trout IFN-γ had antiviral effect *in vitro*. Several factors might provide explanations for the differences seen. First, the amino acid sequence differs, around 10% between salmon and trout IFN-γ, and this might affect biological functions. This might be of importance since rainbow trout recombinant IFN-γ was used in the study by Sun *et al* (2011) and their main results for SAV3 are based on infecting TO cells (derived from Atlantic salmon). Furthermore, the temperature for IPTG induction (28°C in Sun *et al*'s study and 37°C in our study) and purification of IFN-γ from either soluble or insoluble phase might also influence on the function of purified proteins. Last, the SAV3 strains used in two studies possess different *in vitro* virulence. The strain used in the study by Sun *et al*. was non-lytic and they only see a 4 $\log_{10}$ increase (by real-time PCR) by day 14 post infection without any CPE. The strain we used is lytic and causing CPE at 8 days post infection in TO cells. On this basis it is questionable if the results are comparable. It should also be added that the authors conclude that the observed effects of IFN-γ on SAV3 replication is also partly dependent on IFNa induction (Sun *et al*. 2010).

Recombinant virus recovered from DNA and RNA based methods – impact from IFN susceptibility

In Paper III, we recovered rSAV3 from a full-length cDNA (pSAV3-HHFL) clone and the generated virus titre was comparable to what was found for wild-type virus. Furthermore, the rescued recombinant SAV3 (rSAV3) caused similar plaque morphology in infected cells as wild-type virus (data not shown). These results show the infectivity of rescued rSAV3 (plasmid-based) was identical to wild-type virus. A different approach, through transfection of

cells with *in vitro* transcribed RNAs, has been applied for rescue of other recombinant alphaviruses, like SFV and SINV (Liljestrom & Garoff, 1991a;Migliaccio *et al.*, 1989). The same approach was successfully tested for recovery of rSAV3 in CHSE-214 cells. However, despite the *in vitro* transcribed RNAs were confirmed to be intact prior to transfection, expression of viral proteins in cells transfected with *in vitro* transcribed RNAs was much lower compared to cells transfected with the infectious cDNA clone (Figure 11A). Further investigation revealed an immediate and strong induction of IFN-α and ISGs responses following transfection of *in vitro* transcribed RNA into cells (Figure 11B). In contrast, transfecting CHSE cells with the full-length cDNA clone did not provoke such an anti-viral response (Figure 11B), which likely explains the differences. Massive uptake of *in vitro* transcribed RNAs into cells is likely recognized by single-stranded viral RNA sensors, such as TLR7 and TLR8 (in endosomes) and RIG-I in the cytosol (Diebold, 2010;Dixit & Kagan, 2013;Koyama *et al.*, 2008;Yoneyama & Fujita, 2010), with subsequent IFN induction. This is in line with the results from Paper II, where the competition between the initiation of viral RNA replication and the induction of IFN response is critical for establishment of a successful infection. We further observed that virus recovery was augmented when *in vitro* transcribed RNAs were transfected into CHH-1 cells, a cell line with limited IFN expression (data not shown). In this regard, finding a SAV3 susceptible cell line completely lacking IFN expression (analogue to BHK-21 cells used for other alphaviruses) can facilitate virus recovery from the *in vitro* transcribed RNA.

(A)



(B)

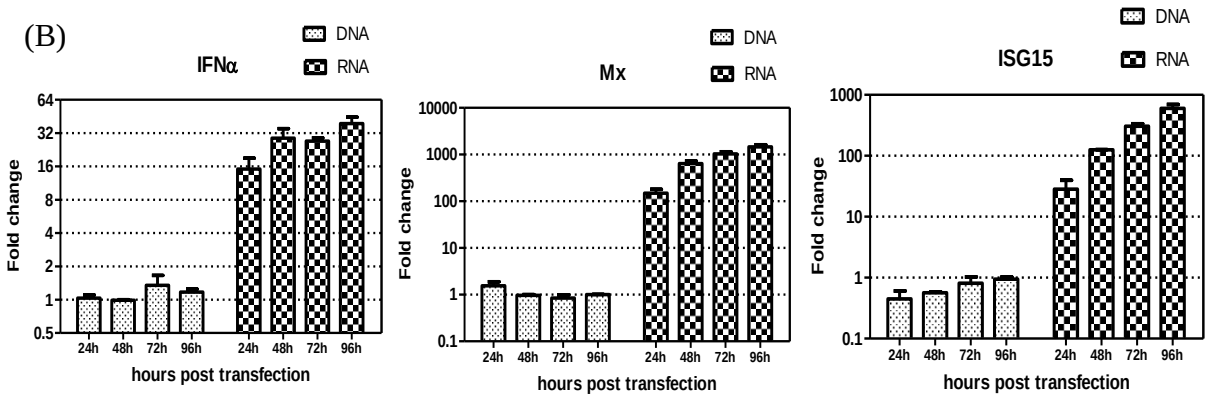


Figure 11. (A) Viral protein expression in CHSE-214 cells transfected with SAV3 full-length cDNA clone (Left) or *in vitro* transcribed RNAs generated from the same clone (Right). (B) The immune response in CHSE-214 cells elicited by transfection of either infectious cDNA or *in vitro* transcribed RNAs.

Deletion of the entire SAV3 6K gene is not a suitable strategy for generation of an attenuated replicating virus vaccine

The constructed full-length cDNA clone serves as a good basis for development of live-attenuated vaccines, which is expected to elicit both the humoral- and cell-mediated immunity, and hence conferring improved protection compared to inactivated or recombinant subunit vaccines. Deleting the entire 6K gene from SFV leads to successful production of attenuated virus. In contrast, complete deletion of 6K gene from the SAV3 cDNA construct (pSAV3-HHΔ6K cDNA) did not generate infectious particles (Paper III) and were therefore not a suitable strategy for developing an attenuated viral vaccine. Despite this, expression of viral proteins was detected, suggesting the RNA replication and translation were not impaired. Viral protein was however not detected on the cell surface, implying that translated viral proteins were somehow retained in the ER or Golgi and not transported to the cell membrane.

This can possibly be caused by an error in polyprotein cleavage/processing. However, to confirm this will require antibodies against each single viral protein and another challenge is the limited amount of viral protein expressed in pSAV3-HHΔ6K cDNA transfected cells, likely due to low transfection rate and non-spreading virus production. The use of a cell line with intact IFN production could also be an obstacle for viral protein expression. Nevertheless, the pull-down experiment revealed a larger size E2 protein produced in cells transfected with pSAV3-HHΔ6K cDNA (Paper III). Whether this is caused by the defect of polyprotein cleavage remains to be determined, but we cannot exclude the possibility of the erroneous post-translational modification on E2 protein resulting in a larger molecular weight. Another strategy to generate an attenuated variant could be the construction of a cDNA clone with partially deleted 6K gene preserving a few nucleotides on both ends which are thought to provide cleavage sites. In addition, a unique seven-amino acid sequence (GVRGWSA) was identified in the 6K protein of SAV subtypes 1-3, not found in other alphaviruses, possibly owing to fish are poikilothermic and also live at low water temperatures, leading to dissimilar lipid composition in the cell membrane. This unique sequence in SAV 6K therefore constitutes an interesting target for the future work with regard to its functional role and the potential discovery of an attenuated virus variant.

SAV3 RNA recombination is documented *in vitro*

Viral RNA recombination is important in evolution of viruses. Despite RNA recombination has been shown for other alphaviruses, to what extent this occurs for SAV remains unclear. A recent study suggested that RNA recombination might appear *in vivo* during SAV infection (Petterson *et al.*, 2013), as the authors detected various deletions within viral genomes from different field isolates. In paper III, we demonstrated that RNA recombination occurred in cells co-transfected with pSAV3-HHΔ6K cDNA and helper cDNA constructs containing complete structural genes. CPE was found in 14 out of 48 co-transfected wells indicating that restoration of a fully infectious virus occurred at a relatively high frequency. Further analysis of the cross-over site in more detail in one well with CPE revealed that several different recombinations had taken place at slightly different sites. This was evidenced by different restriction patterns when recombinant RNAs were RT-PCR amplified, cloned, and digested with restriction enzymes. However, after one round of virus passage in cells, restriction fragment analysis of the cross-over region became homogenous (Paper III), likely due to the selection of viable viral particles/viral clones able to infect cells in the next round of infection. It is expected that also non-viable truncated viral genomes generated in transfected cells can

be packaged into viral particles and passaged, and therefore a small amount of deletion variants might co-exists with full-length variants. It is however difficult to decide which of the generated deletion variants are viable. This could be confirmed by plaque purification and genome sequencing in future studies. Homologous analysis of full genome of SAV subtypes 1-3 revealed differences with regard to deletions/additions, which might be explained by RNA recombination occurring between subtypes. This remains a speculation and is (likely) in contrast to a recent study by Karlsen and co-workers (2013) showing that the six subtypes of SAV represent independent introductions to farmed fish populations. This is based on six full-genome sequence analyses and 71 partial sequencing of the structural ORF, which suggest that all six subtypes diverged prior to the twentieth century before rainbow trout was introduced into European aquaculture. The interpretation is that the different subtypes must have existed in wild populations or a reservoir. The ancestors of the strains found in aquaculture today thus likely represent independent introductions to farmed fish populations from the wild reservoir and each of the subtypes has developed into self-sustainable epizootics (Karlsen *et al*. 2013). As mentioned above, recombination is likely to occur in vivo (Petterson *et al*. 2013) and the possibility for mixed infections (with different subtypes) in the same population of fish cannot be ruled out. Recently, marine SAV2 has been found in areas (in Norway) where SAV3 is endemic (own observations). The molecular mechanism of RNA recombination for SAV remains to be understood, but the results presented in Paper III has delivered an important message regarding the safety of vaccines containing full-length or partially deleted viral genomes. There is a risk of RNA recombination between vaccine virus and field virus leading to reversion to virulence.

SAV3 Replicon based vaccine

The construction of SAV2 replicon containing all the non-structural genes plus GFP or Luciferase reporter gene was described previously by Moriette *et al*., 2006. Later, a similar replicon construct was developed for SAV3 (Karlsen *et al*., 2009) and demonstrated to be functional in fish, shrimp and mammalian cells in a temperature range of 4-37°C (Olsen *et al*., 2013), suggesting that despite the progeny of SAV is produced only at low temperature (10-15˚C), the replication machinery is not temperature restricted. This is supported by a recent study showing that RNA replication occurred at both 12 ˚C and 20 ˚C and further demonstrating that E2 glycoprotein is responsible for this low-temperature dependency and requires co-expressed E1 for virion formation (Hikke *et al*., 2014). Furthermore, SAV3 replicon encoding the infectious salmon anemia virus (ISAV) hemagglutinin-esterase (HE) is

proven to be an efficacious vaccine against ISA in fish (Wolf *et al*., 2013) using intramuscular immunization but no protection was induced when pSAV/HE was injected intraperitoneally (Wolf *et al*., 2014). Nevertheless, in contrast to other alphaviruses showing that the self-replicating RNA molecules (replicon) is capable of expressing its gene of interest to a level as high as 20% of total cellular protein production (Pushko *et al*., 1997), the level of antigen expression encoded by SAV replicon was in general low (Olsen *et al.*, 2013). As antigen dose correlates with protection, augmentation of the protein level expressed by a vaccine vector would be beneficial in vaccination. In Paper IV, we optimized antigen expression levels by incorporating a translational enhancer (the N-terminal 102 nucleotides of the capsid gene) into the expression vector. The expression level of a reporter gene, EGFP, in one cell unit was measured by flow cytometer and the result suggested this modified replicon vector containing the translational enhancer significantly enhanced antigen expression in the tested cell line (CHH-1). We also confirm that this vector was able to express the complete structural genes of IPNV (segment A) by examining expression of IPNV proteins in transfected cells *in vitro*. A recent study shows when Atlantic salmon was immunized with SAV3 replicon expressing IPNV segment A polyprotein and further challenged by cohabitation with IPNV shedder fish, low to moderate protection was obtained (Abdullah *et al*., 2015). However, it might still be interesting to evaluate whether the enhanced antigen expression obtained by the replicon generated in this study can improve the protection against IPNV.

In addition to the replicon-based vaccine, suicide viral particles, which undergo only one round of infection have been developed to be used as potential vaccines in the future. These particles go through a natural infection route in the host and replicate in the target sites (Zhou *et al.*, 1994). We have attempted to make suicide particles of SAV3, however without success. Co-transfection of a SAV3 replicon (carrying the EFGP gene) and a helper cDNA construct containing the complete structural genes did not lead to packaging of viral particles. Despite EFGP was expressed in transfected cells, CPE was not observed, and when supernatant from transfected cells was passaged to new cells, EGFP expression was no longer detected. It remains uncertain why SAV3 replicon together with helper cDNA was not able to form infectious virus particles as shown for other alphaviruses. One possible explanation might be the location of the packaging signal. For SFV, it is known that virus package signals are found within the nsP2 gene and for SINV it is found within nsP1 (Kim *et al.*, 2011;Kim *et al.*, 2013). If the packaging signal of SAV is found outside the non-structural genes, then capsid protein will not be able to pack the replicon. It is also likely that the transfected permissive cell lines

used in this study (CHSE-214 and CHH-1 cells) possess an interferon response sufficient to block the infection process if the recombinant virus is attenuated compared to wild-type virus.

MAIN CONCLUSIONS

- The pathological changes in SAV3 challenged Atlantic salmon were observed in pancreas and subsequently in heart, concomitant with high viral replication, which was detected alongside high up-regulation of IFN-α and ISGs. The earliest adaptive immune response was detected in the head kidney.

- SAV3 infection is sensitive to IFN-α. For successful inhibition of viral growth, timing of IFN-α induction is very important. Pre-treatment of rIFN-α at 24 to 4 hours prior to SAV3 infection in TO cells limited the development of CPE and reduced E2 gene expression from 40,000-fold (24 hr) to 2,000-fold (4hr).

- The recovery of recombinant SAV3 from a full-length cDNA clone was more efficient in CHH-1 cells than in CHSE-214 and BF-2 cells. This was likely due to the difference in IFN-expression levels between the cell lines. The recovered virus titres and the kinetics of viral growth were comparable to wild-type virus.

- rSAV3 full-length was successfully recovered from cDNA transfected cells but the SAV3 6K-deleted mutant was not viable. Further investigation revealed an E2 protein larger than the wild-type E2, possibly due to defects within polyprotein cleavage or errors occurring during post-translational modifications. Recombination occur frequently in vitro and rescue of replication deficient delta-6K variant strains occurs after co-transfection of delta-6K and SAV3 helper cDNA encoding the structural genes (including 6K).

- A modified SAV3 replicon vector incorporated with a translational enhancer, being the N-terminal 102nt of the capsid gene, lead to a significant increase of expression of heterologous protein levels. IPNV structural proteins were successfully expressed using this modified vector and therefore represents a potential future vaccine carrier.

FUTURE PERSPECTIVES

Pancreas disease (PD) has become one of the most challenging viral diseases in salmonid aquaculture. Development of an effective vaccine against PD is needed. Several approaches can be considered for future implications based on the current study.

- The constructed SAV3 full-length cDNA clone will be a valuable tool for the study of host-pathogen interactions. Point mutations can be made to evaluate different substitutions and roles for virulence.
- The role of entire 6K protein is important for infectivity. A unique domain containing seven amino acids, GVRGWSA, will be an interesting focus for future research.
- Improved design of SAV3 replicon vector leading to high expression levels of foreign antigens constitutes an optimal tool for a replicon based DNA vaccine.
- Finding an IFN deficient cell line is not only valuable for more efficient isolation of virus from field samples but can also be beneficial for virus propagation for ultimate vaccine production.
- Development of new types of vaccines such as RNA replicon vaccines might be considered in the future. Several advantages of RNA vaccines include: (i) no vector sequence in RNA vaccines as currently present in experimental DNA based vaccines; (ii) alphavirus RNA is self-replicating and therefore lower dosage is required for vaccination; (iii) long dsRNA eliciting IFN response could constitute a good vaccine adjuvant; (iv) RNA does not incorporate into the chromosomes of somatic cells.
- Chimeric viruses are often replication-competent but highly attenuated. Future studies of chimeric viruses combining two or more subtypes of SAV might be an interesting field to explore.

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VETERINARY RESEARCH

RESEARCH Open Access

Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon

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Abstract

Salmonid alphavirus subtype-3 (SAV-3) infection in Atlantic salmon is exclusively found in Norway. The salmonid alphaviruses have been well characterized at the genome level but there is limited information about the host-pathogen interaction phenomena. This study was undertaken to characterize the replication and spread of SAV-3 in internal organs of experimentally infected Atlantic salmon and the subsequent innate and adaptive immune responses. In addition, suitability of a cohabitation challenge model for this virus was also examined. Groups of fish were infected by intramuscular injection (IM), cohabited (CO) or kept uninfected in a separate tank. Samples of pancreas, kidney, spleen, heart and skeletal muscles were collected at 2, 4 and 8 weeks post infection (wpi). Pathological changes were assessed by histology concurrently with viral loads and mRNA expression of immune genes by real time RT-PCR. Pathological changes were only observed in the pancreas and heart (target organs) of both IM and CO groups, with changes appearing first in the pancreas (2 wpi) in the former. Lesions with increasing severity over time coincided with high viral loads despite significant induction of IFN-α, Mx and ISG15. IFN-γ and MHC-I were expressed in all tissues examined and their induction appeared in parallel with that of IL-10. Inflammatory genes TNF-α, IL-12 and IL-8 were only induced in the heart during pathology while T cell-related genes CD3ε, CD4, CD8, TCR-α and MHC-II were expressed in target organs at 8 wpi. These findings suggest that the onset of innate responses came too late to limit virus replication. Furthermore, SAV-3 infections in Atlantic salmon induce Th1/cytotoxic responses in common with other alphaviruses infecting higher vertebrates. Our findings demonstrate that SAV-3 can be transmitted via the water making it suitable for a cohabitation challenge model.

Introduction

Salmonid alphaviruses (SAV) are pathogens of salmonid fish causing pancreas disease (PD) and sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*), respectively. They represent species of single stranded, positive polarity RNA viruses belonging to the genus *Alphavirus* in the family *Togaviridae* where they are the only ones infecting fish [1,2]. At present, they have only been isolated in Europe [3] and are responsible for great economic losses in the farmed aquaculture industry [4]. SAV are grouped into 6 subtypes (SAV-1 to SAV-6) [5], with SAV-3 being the only subtype restricted to Norway [6].

Clinical signs and histopathology associated with SAV infections are detailed elsewhere [3,7] and include degeneration of the exocrine pancreas and myopathy of heart and skeletal muscles. Mortalities can range from 1% to about 48% [3,8]. Commercial vaccines in the form of injectable preparations are available despite that the protection offered is equivocal [3]. Indeed the number of PD epizootics has remained high over the years [9].

The development of efficacious vaccines depends on a good understanding of protective immune mechanisms. For SAV infections, this has not been achieved in detail and although several studies have been undertaken to examine host responses, very few have addressed in-vivo immune responses besides Desvinges and co-workers [10] who showed that phagocytic activity of head kidney leucocytes, levels of lysozymes and complement were significantly elevated following experimental infections,

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indicating an active immune reaction. These authors, however, failed to detect the interferon response probably due to the poor sensitivity of the method used. Interferons are the hallmark of antiviral responses in most living organisms [11] and have been shown to be important for the host response against alphaviruses in higher vertebrates [12]. They comprise three classes of cytokines (types I to III). Amongst the three types, types I (IFN α, β, ω, ε, κ) and III (λ) are directly induced by viruses [11,13-15]. For the remaining of this article, we will not differentiate between IFN subtypes.

Following entry into the host, virus nucleic acids are sensed by host pattern recognition receptors (PRR) including Toll-like receptors (TLR 3/7/8/9) in endosomes and retinoic acid inducible gene I (RIG-I), melanoma differentiation factor-5 (MDA5) and DNA-dependent activator of IFN regulatory factors (DAI) in the cytosol [16]. Once activated, the receptors signal via MyD88/TRIF adaptors (TLR) or through the mitochondrion-associated adaptor IPS-1 (RIG-I and MDA5), all culminating in the phosphorylation and translocation of interferon regulatory factors (IRF) into the nucleus where they induce transcription of IFNα and IFNβ genes resulting in the production of IFN [17-19]. Interferons exert their effects by binding to IFN receptors (IFNAR) on target cells thereby triggering signal transduction via the Janus kinase Signal transducer activator of transcription pathway [20]. This leads to the transcription of an array of antiviral genes such as Mx, ISG-15, double stranded protein kinase R (PKR) and 2'-5'oligoadenylate synthetase (OASs) [21-23]. It is also noteworthy that a positive feed-back loop exists whereby IFNα and IFNβ act through IFNAR to up-regulate virus sensing and enhance antiviral responses [16].

Through in-vitro studies, it has been shown that IFNα induces protection against SAV-3 induced-CPE in Atlantic salmon head kidney (TO) cells [24]. This is, however, dependent on the time of exposure to interferon prior to infection. Furthermore, a positive correlation between IFNα-stimulated gene Mx expression and protection of cells against SAV-induced CPE has also been demonstrated [25,26]. The situation in-vivo, however, remains poorly understood. The in-vivo environment represents a complex milieu that differs from that of in-vitro settings. For interferons, it has been shown that these environments can yield different effects on viruses [27,28]. While a recent study has shown that IFNα and its stimulated genes are up-regulated at early time (1–5 days) in the kidneys following SAV-1 infection of Atlantic salmon [29], antiviral responses in target organs remain unknown. The purpose of the present study therefore was to examine in-vivo host responses, especially IFNα and ISG, following experimental infection of Atlantic salmon with SAV-3 in target organs. Real-time PCR was used to assess gene expression changes. Although interferon expression is known to be important at early times following infection, sampling times of 2, 4 and 8 weeks following virus injection were chosen in the present study since this is when pathological changes are known to occur. Besides, interferon responses are known to play a role in the clearance of viruses even after the onset of adaptive immune responses [27]. Cohabitants were included in order to determine the suitability of such a model for fish challenges against SAV-3. Our findings demonstrate that the virus yield and pathology progress despite the expression of interferon and related genes, in conformity with earlier reports [24].

Materials and methods

Virus isolation and cell culture

Chinook salmon embryonic cells (CHSE-214; ATCC CRL-1681) were used for virus propagation. The cells were maintained at 20°C with L-15 media (Invitrogen, Paisley, UK) supplemented with 5% FBS, L-glutamin and gentamycin. The virus used in the present study has previously been described [24], is fully sequenced and shown to be a typical SAV-3 subtype [GenBank: JQ799139].

Experimental challenge

Approximately 70 Atlantic salmon (*Salmo salar* L.) presmolts purchased from Sørsmolts AS in Sannidal, Norway and weighing 35 ± 10 g were used. The fish were healthy and the hatchery from which they were purchased had had no previous records of PD outbreaks. The fish were transported to the Norwegian School of Veterinary Science/Veterinary Institute shared wetlab by road in oxygenated bags. After 1 week acclimatization, the fish were treated with formalin (diluted 1:4000 in water) against ectoparasites for 30 min. The fish were then kept for a further week prior to the start of the experiment.

Challenging of the fish was done by first anaesthetizing them with 0.5 mL chlorobutanol per 1L of water. Thirty fish were injected intramuscularly (IM) with 0.2 mL of the virus (2×10^6 $TCID_{50}$/mL). One group of 15 uninfected fish were fin-clipped and cohabitated with the virus-injected group to document virus replication to a level that will result in virus shedding and spread through water. The control group consisted of 15 fish that were injected with L-15 medium and were kept in a separate tank from the SAV-infected fish.

Sample collection

At 2, 4 and 8 weeks post-infection (wpi), 10 SAV-3 injected fish, 5 cohabitants and 5 control fish were sacrificed. Parallel tissues including head kidney, spleen, heart, pancreas and muscle were collected in 10%

phosphate buffered formalin for histopathology and RNAlater (Sigma, St. Louis, USA) for gene expression analysis. Tissue samples preserved in formalin were fixed for a minimum of 4 days while those kept in RNAlater were stored at −80°C until required.

Histopathology

Paraffin-embedding, sectioning and staining with hematoxylin and eosin (H&E) were done according to standard histological procedures.

RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment according to the manufacturer's instructions. The concentration of RNA was determined by spectrophotometry using the Nanodrop ND1000 (Nanodrop Technologies, Wilmington, USA). For each sample, 1 μg of total RNA was subjected to cDNA synthesis using the SuperScript III reverse transcriptase system (Invitrogen, Paisley, UK) and oligo$(dT)_{20}$ primers in a total volume of 20 μL. The synthesized cDNA was diluted 5 times by adding 80 μL distilled water and stored at −20°C until further use.

Quantitative real-time PCR

Quantitative real-time PCR was performed using the LightCycler® 480 (Roche, Mannheim, Germany) instrument. For each gene, 2 μL of cDNA was used as template in a mixture of specific primers (10 μM) and the LightCycler 480 SYBR Green I Master mix (Roche) in a final volume of 20 μL. The mixtures were first incubated at 95°C for 10 min, followed by 40 amplification cycles (10 s at 95°C, 20 s at 60°C and 8 s at 72°C). The sequences of all primers used in this study are provided in Table 1.

For the viral E2 gene, the reaction mix containing 10 μL of Probe Master, 1 μL of primer-probe mix (final concentration of each primer (0.9 μM, probe 0.25 μM), 2 μL of cDNA template and 7 μL water was incubated for 10 min at 95°C, followed by 45 amplification cycles (10 s at 95°C, 30 s at 60°C and 1 s at 72°C). To calculate the absolute quantity of the virus, recombinant pGEM-T easy (Promega, Madison, USA) plasmid containing the E2 gene of SAV-3 was used to make a standard curve in nine orders of magnitude from 10^0 to 10^8, thus the copy number or viral cDNA was determined. The specificity of the PCR products from each primer pair was confirmed by the melting curve analysis and subsequent agarose gel electrophoresis.

The relative expression of the following genes was examined: IFN-α, Mx, ISG-15, IFN-γ, TNF-α, IL-12, IL-10, IL-8, CD3ε, CD4, CD8, TCR-α, MHC-I, and MHC-II. To calculate the gene products, the 2-CT method was used as described elsewhere [31]. All quantifications were normalized to β-actin.

Statistical analysis

One-way ANOVA with the Bonferroni post test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). The significant level for rejection of Ho was set at $p < 0.05$.

Results

Pathological changes, viral load and innate immune responses

Salmonids challenged with SAV do not develop clinical signs and mortalities are absent [3,32,33] but intramuscular or intraperitoneal injection of virus has been shown to result in an infection with high replication levels in target organs [32] with reproducible pathology. These infections are typically observed from 3 weeks post challenge and onwards, but with some differences between subtypes [34]. In order to understand the dynamics of infection we studied the interaction between viral proliferation of SAV-3 and host responses. This included, firstly, the description of pathology in primary target organs (pancreas and heart) and concomitantly, an assessment of virus replication levels in these organs; secondly, an assessment of the innate host responses following infection by examination of certain cellular markers and cytokines at transcription level before attempting to characterize the ensuing adaptive immune response. Tissues tested included primary target organs of SAV-3 and also the secondary replication site (skeletal muscle). In addition we also examined virus replication and also innate and adaptive immune genes in the head kidney and spleen. The expression of genes in the group injected intramuscularly (IM) as well as cohabitants (CO) were reported as mean fold changes relative to the control group.

Fish intramuscularly injected with SAV-3

In the IM group, pathological changes were first observed at 2 weeks post infection (wpi) in the pancreas characterized by acinar cell necrosis (Figure 1a), concomitant with high virus replication (Figure 2a). This was found despite high up-regulation of IFNα expression at 2 wpi (Figure 3a), increased expression of interferon-stimulated genes (ISGs) Mx (Figure 3b) and ISG-15 (Figure 3c). At 4 wpi, advanced degeneration and necrosis of acinar cells as well as inflammatory cell infiltration was observed in this group (Figure 1b), and correspondingly higher virus replication at this time point, increasing slightly from 2 to 4 wpi, albeit non-significantly (Figure 2a). Despite a higher inflammatory index in the pancreas at 4 wpi, there was no additional increase in expression of IFN-α; the expression was not different at

Table 1 Primers and probe sequences used for quantitative real-time PCR

Genes		Primer sequence	GenBank accession no.
β-actin	Fwd	CCAGTCCTGCTCACTGAGGC	AF012125
	Rev	GGTCTCAAACATGATCTGGGTCA	
SasaIFN-α	Fwd	TGGGAGGAGATATCACAAAGC	AY216594
	Rev	TCCCAGGTGACAGATTTCAT	
Mx	Fwd	TGCAACCACAGAGGCTTTGAA	U66475
	Rev	GGCTTGGTCAGGATGCCTAAT	
ISG15	Fwd	AAGTGATGGTGCTGATTACGG	AY926456
	Rev	TTGGCTTTGAACTGGGTTACA	
IFN-γ	Fwd	CTAAAGAAGGACAACCGCAG	AY795563
	Rev	CACCGTTAGAGGGAGAAATG	
TNF-α (1&2)	Fwd	AGGTTGGCTATGGAGGCTGT	NM_001123589
	Rev	TCTGCTTCAATGTATGGTGGG	NM_001123590
IL12-β	Fwd	CTGAATGAGGTGGACTGGTATG	BT049114
	Rev	ATCGTCCTGTTCCTCCG	
IL-10	Fwd	CGCTATGGACAGCATCCT	EF165029
	Rev	AAGTGGTTGTTCTGCGTT	
IL-8	Fwd	GGCCCTCCTGACCATTACT	NM_001140710
	Rev	ATGAGTCTACCAATTCGTCTGC	
CD3-ε	Fwd	TCAGGGCTCGGAAGAAGTCT	NM_001123622
	Rev	GCCACGGCCTGCTGA	
CD4	Fwd	GAGTACACCTGCGCTGTGGAAT	NM_001124539
	Rev	GGTTGACCTCCTGACCTACAAAGG	
CD8-α	Fwd	CACTGAGAGAGACGGAAGACG	NM_001123583
	Rev	TTCAAAAACCTGCCATAAAGC	
TCR-α	Fwd	GCCTGGCTACAGATTTCAGC	BT050114
	Rev	GGCAACCTGGCTGTAGTAGC	
MHC I	Fwd	CTGCATTGAGTGGCTGAAGA	AF508864
	Rev	GGTGATCTTGTCCGTCTTTC	
MHC II	Fwd	TCTCCAGTCTGCCCTTCACC	BT049430
	Rev	GAACACAGCAGGACCCACAC	
NSAV-E2*	Fwd	CAGTGAAATTCGATAAGAAGTGCAA	EF675594
	Rev	TGGGAGTCGCTGGTAAAGGT	
E2 Probe*		FAM-5'- AGCGCTGCCCAAGCGACCG- 3'-MGB	

*From Hodneland & Endresen [30].

2 wpi compared to 4 wpi (Figure 3a). The same was observed with Mx and ISG-15 (Figure 3b and c). By 8 wpi, these pathological changes progressed to the extent that most of the exocrine pancreatic tissue had been lost (Figure 1c). This resulted in a further decline in the amount of virus (Figure 2a; $p < 0.001$), as would be expected when the tissue supporting virus replication is lost. IFN-α expression at 8 wpi (Figure 3a) had been reduced to background levels ($p < 0.01$), also in conformity with the loss/destruction of most of the exocrine pancreatic tissue at this time point. Mx and ISG-15 also fell to background levels (Figure 3b and c).

In the heart, no histopathological changes were observed at 2 wpi (Figure 1d) and the first lesions were observed at 4 wpi, seen as necrotic myocardial cells in the spongy layers (Figure 1e). IFN-α was markedly upregulated by 2 wpi but unlike the pancreas, the expression in the heart remained fairly constant ($p = 0.05$) from 2 to 4 wpi (Figure 3a). Similarly, Mx and ISG-15 was markedly upregulated by 2 wpi, 50-fold and 640-fold, respectively. Despite this, there was a sharp increase in viral load during this period (Figure 2a, $p < 0.001$). By 8 wpi, the severity of lesions in terms of necrotic cardiomyocytes increased and there was a marked infiltration of inflammatory cells not seen at 4 wpi, firstly in the ventricular spongy layer, then extending to the compact layers (Figure 1f). Occasionally, necrotic cells were also found in the atrium. The viral load remained unchanged

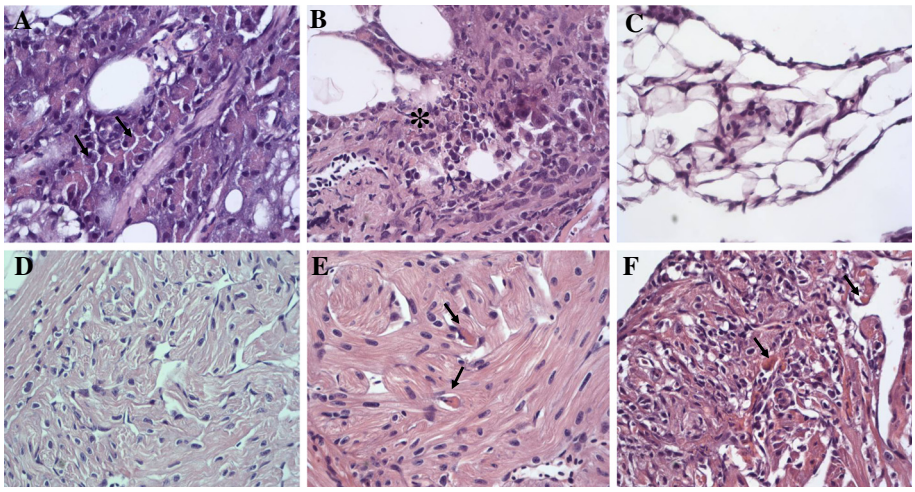

Figure 1 Pathological changes in different tissues of Atlantic salmon following infection with salmonid alphavirus subtype 3. (**A**) Pancreas, 2 wpi. Arrows = Multifocal necrosis in exocrine pancreatic cells.); (**B**) Pancreas, 4 wpi. Star = necrotic areas; (**C**) Pancreas, 8 wpi. Note depleted exocrine tissue; (**D**) Heart (ventricle), 2 wpi, normal; (**E**) Heart (ventricle), 4 wpi. Arrow = necrotic myocardial cell; (**F**) Heart (ventricle), 8 wpi, extensive cardiomyocytic necrosis and infiltration of inflammatory cells in the compact and spongious layers.

from 4 to 8 wpi (Figure 2a). At 8 wpi there was a moderate increase of IFN-α expression (non-significant) consistent with the increase in inflammatory response at this time point (Figure 1f). The expression of Mx was lower (non-significant) and markedly down for ISG-15 ($p < 0.001$) in this organ (Figure 3b and c).

While no histopathological changes were found in the skeletal muscle over the course of the experiment, there was a steady increase in viral load from 2 to 8 wpi (Figure 2a, $p < 0.001$; 2w versus 8w and 4w versus 8 wpi). In the skeletal muscles, IFN-α was not differentially expressed at 2 and 4 wpi although both Mx and ISG-15 were significantly up-regulated at these time points (Figure 3b and c). At 8 wpi however, IFN-α expression was significantly up-regulated ($p < 0.001$), coinciding with high expression levels of Mx and ISG-15 (Figure 3b and c; $p < 0.001$).

While there was a relatively high replication of virus (1 $\log_{10}$ less than the target organs) in head kidney and spleen (Figure 2a), no lesions were observed in these organs throughout the study period.

Cohabitant fish

In these fish, the virus was detected in all organs at all sampling times except at 2 wpi in the skeletal muscle (Figure 2b). At 2 and 4 wpi, low viral loads (about 10^2 to 10^3 copy numbers/μg of total RNA) were detected in tissues (Figure 2b). This translated to 2–3 $\log_{10}$ times lower than the virus detected in the IM group and these viral loads were associated with no pathology. At 8 wpi however, the virus load had increased to above 10^6 copy numbers/μg of total RNA in the pancreas and heart, coinciding with histopathological changes. The lesions in these organs were similar to those observed in the IM group at an earlier sampling time point (Figure 1).

The expression of IFNα in all organs sampled followed a similar trend i.e. induced at 2 wpi followed by a slight drop at 4 wpi and an increase again at 8 wpi (Figure 3d).

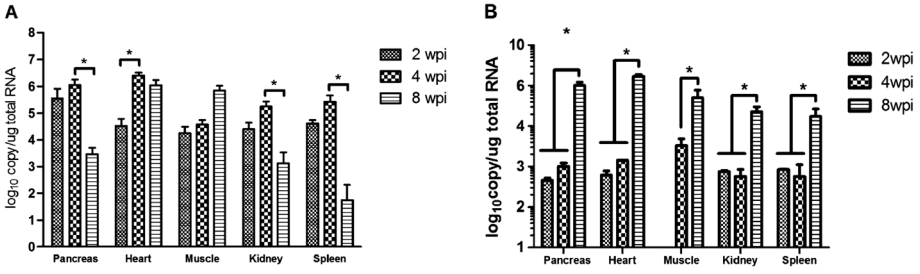

Figure 2 Salmonid alphavirus subtype 3 replication in different tissues of Atlantic salmon. A) Viral replication in different organs in intramuscularly-injected fish (average +SEM; $n = 6$ except for muscle where $n = 3$) (*$p < 0.001$). **B**) Viral replication in different organs of cohabitant fish at indicated time points. (average +SEM; $n = 2$ to 5) (*$p < 0.001$). wpi = weeks post infection. The virus was measured by the detection of copies of the E2 gene by real-time RT-PCR, expressed as $\log_{10}$ of total RNA.

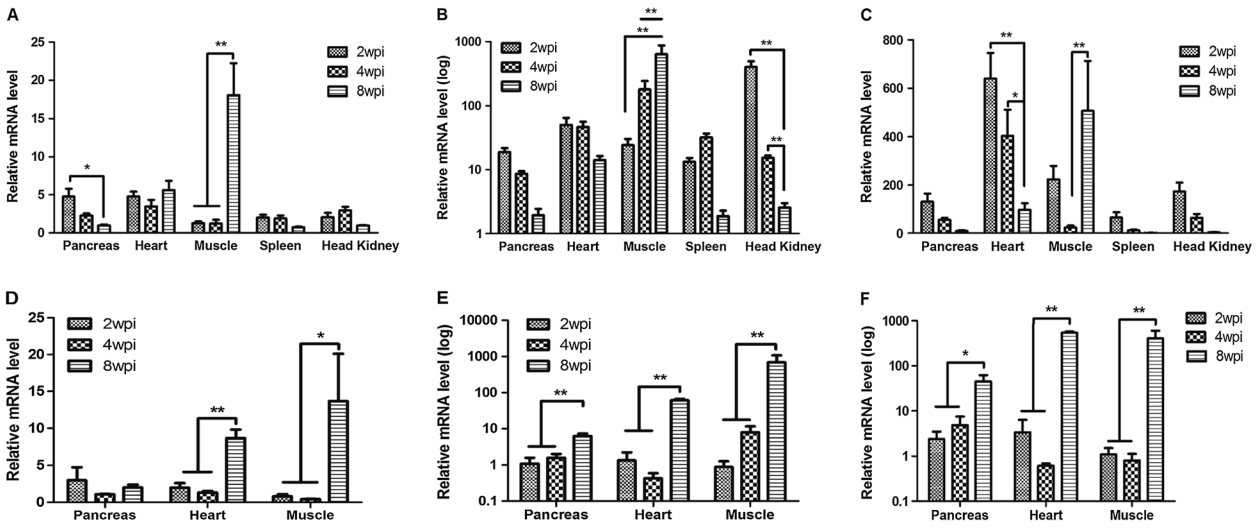

Figure 3 Expression of interferon alpha and ISG in different tissues of Atlantic salmon following injection and cohabitation challenge. IFN-α (**A**), Mx (**B**) and ISG-15 (**C**) gene expression measured at different times post challenge in injected fish. Mean values ± SEM relative to the mean of the control group (n = 9–10, except for muscle n = 3). Similar responses are presented in cohabitant fish, IFN-α (**D**), Mx (**E**) and ISG-15 (**F**) in the pancreas, heart and muscle tissues. Mean values ± SEM relative to the mean of the control group (n = 3–5). *$p < 0.01$, **$p < 0.001$.

This pattern was the same as that of IFN-induced genes Mx and ISG-15 in the heart. In the pancreas and skeletal muscles however, the expression of IFN-induced genes increased with time from 2 to 8 wpi (Figure 3e and 3f). It is noteworthy that the expression of IFNα, Mx and ISG-15 increased in the presence of pathology, consistent with the observations in the IM group.

Control fish

No mortalities were observed in any of the groups of fish in this study. No viruses were detected from the control fish, nor were any lesions observed.

Expression of immune-related genes of adaptive immunity

The mRNA expression of IFN-γ, TNF-α, IL-12, IL-10, IL-8, CD3ε, CD4, CD8, TCR-α, MHC-I, and MHC-II were examined in the spleen, head kidney, pancreas and heart in addition to IFN-α and ISG described in the previous section.

The earliest immune response to the infection was observed in the head kidney, with IFN-γ, IL-10 and MHC-I being significantly up-regulated at 2 wpi indicating a pro-inflammatory response (Table 2). At the same time point, MHC-I was also up-regulated in the pancreas.

By 4 weeks, the head kidney and spleen showed a similar pattern with IFN-γ, IL-10 and MHC-I being up-regulated (Table 2). For the head kidney, MHC-II was also found to be up-regulated, although moderately while there was a down-regulation of CD8 and TCR-α (Table 2), possibly as an indication of export of these cells to the site of infection (pancreas and heart). Several pro-inflammatory genes were up-regulated in the pancreas and heart at this time point including IFN-γ (in both organs), TNF-α (heart), and MHC-I in the heart. In addition IL-10 was markedly up-regulated in the pancreas (Table 2).

At 8 wpi, fewer genes were differentially regulated in the spleen and head kidney. In the former, the expression of IL-10 continued to be induced probably to dampen the immune response while IL-8, a chemoattractant was up-regulated at this time point (Table 2). In the head kidney, only MHC-I and IL-10 were induced at 8 wpi. In contrast, more immune-related genes were induced in the primary target organs (pancreas and heart) at 8 wpi compared to earlier time points. In the pancreas, there was a marked up-regulation of T cell markers/T cell responses (CD3, CD4, CD8, TCR, MHC-I) plus IL-10 while in the heart, all these genes as well as pro-inflammatory markers (IFN-γ, TCR-α, IL-12, IL-8 and MHC-II) were induced (Table 2).

Discussion

In this study we show that SAV-3 infection of Atlantic salmon cause pathology in target organs alongside high viral replication despite high expression levels of IFNα mRNA and interferon-stimulated genes, ISG-15 and Mx, at early time points post challenge. Type 1 interferons are well known for the establishment of an antiviral state in neighboring uninfected cells following viral invasion in vertebrates [11,13]. This is most important during the early stages of an infection, prior to the onset of the adaptive immune response. The increase in viral loads

Table 2 Relative expression of immune related genes in different tissues of Atlantic salmon after SAV-3 infection

Tissues	Spleen			Head kidney			Pancreas			Heart		
Genes	Weeks											
	2	4	8	2	4	8	2	4	8	2	4	8
IFN-γ	0.7 ± 0.2	**2.4 ± 0.5***	0.8 ± 0.1	**3.0 ± 0.7***	**4.1 ± 1.1***	0.8 ± 0.1	3.4 ± 0.7	**7.2 ± 0.9***	1.5 ± 0.4	1.1 ± 0.2	**6.3 ± 3.0***	**89.3 ± 23.2***
TNF-α	0.5 ± 0.1	0.7 ± 0.1	1.4 ± 0.4	1.2 ± 0.3	1.0 ± 0.3	0.6 ± 0.2	1.3 ± 0.3	1.4 ± 0.2	1.2 ± 0.1	2.2 ± 0.9	**2.5 ± 0.3***	**8.1 ± 1.5***
IL-12	0.7 ± 0.1	1.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	2.2 ± 0.3	**0.6 ± 0.1****	1.7 ± 0.4	1.0 ± 0.3	0.9 ± 0.2	**3.3 ± 0.7***
IL-10	0.9 ± 0.1	**7.5 ± 1.4***	**2.8 ± 0.5***	**10.0 ± 1.6***	**21.2 ± 4.1***	**4.7 ± 1.1***	3.1 ± 0.6	**23.9 ± 6.5***	**3.2 ± 0.8***	1.1 ± 0.3	4.1 ± 1.5	**37.3 ± 7.6***
IL-8	1.5 ± 0.3	1.5 ± 0.6	**3.0 ± 0.6***	1.0 ± 0.4	2.3 ± 0.8	1.9 ± 0.5	1.3 ± 0.2	1.7 ± 0.2	1.4 ± 0.2	1.0 ± 0.1	1.7 ± 0.3	**5.7 ± 1.2***
CD3-ε	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.4	1.5 ± 0.3	1.4 ± 0.1	**1.7 ± 0.2***	1.0 ± 0.2	1.3 ± 0.2	**10.4 ± 1.9***
CD4	0.7 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.2	**1.9 ± 0.3***	1.1 ± 0.5	2.5 ± 0.7	**11.1 ± 1.8***
CD8	1.2 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	**0.5 ± 0.1****	0.7 ± 0.1	**0.4 ± 0.0****	0.7 ± 0.1	**2.1 ± 0.2***	1.0 ± 0.2	0.8 ± 0.3	**51.6 ± 9.7***
TCR-α	1.5 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	**0.6 ± 0.1****	0.7 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	**2.0 ± 0.3***	0.5 ± 0.1	0.8 ± 0.2	**16.4 ± 2.5***
MHC I	1.7 ± 0.2	**3.1 ± 0.2***	1.5 ± 0.2	**1.7 ± 0.2***	**2.4 ± 0.2***	**1.5 ± 0.1***	**3.7 ± 0.7***	1.4 ± 0.2	**2.1 ± 0.2***	1.5 ± 0.3	**4.4 ± 0.5***	**8.2 ± 0.8***
MHC II	1.1 ± 0.1	1.6 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	1.7 ± 0.2*	1.4 ± 0.2	1.8 ± 0.3	1.4 ± 0.2	1.6 ± 0.3	**0.5 ± 0.1****	1.5 ± 0.3	**5.1 ± 0.8***

The results are shown as mean fold change ± standard error of virus infected group relative to the mean of the control group. Key: *Significantly up-regulated; **Significantly down regulated, otherwise not differentially induced ($P > 0.05$).

over time in target organs (pancreas, heart and skeletal muscles) and the progression of pathology in the pancreas and heart despite the up-regulation of IFN-α, Mx and ISG-15 (Figure 3) suggest that the onset of the innate response comes too late to limit virus replication. This fits well with a previous report where treatment of cells with IFN-α at the same time as SAV-3 infection failed to protect the cells against CPE in an in-vitro model [24].

All tissues examined in the present study contained SAV-3 and the kinetics of viral loads were in general consistent with the trends of expression of IFN-α, in common with reports of previous studies done in Atlantic salmon-derived cells [24] and also with other viruses [35]. The anticipation is that SAV-3 was sensed by host cells via pattern recognition receptors such as MDA-5 and LGP2 [36] leading to the expression and induction of IFN-α and consequently ISG [28]. The trends of induction of Mx and ISG-15 by IFN-α were, on average, consistent with previous reports [24,25] while the relationship between the expression profiles was not always proportional and for ISG-15, a somewhat different expression pattern was observed in some tissues of the IM group. In one study using an in-vitro model to assess the induction of ISG by IFN-α, similar inconsistencies were observed [37]. These findings probably reflect the complexity of the interferon signaling pathways or the diversity in fish since most fish ISG are often duplicated [13], as well as the effect of IFN-α independent stimulation of ISG [38,39].

In higher vertebrates, down-stream effects of IFN-α/β induction include the increased expression of MHC I molecules [40] and activation of NK cells [41,42]. From the genes examined in the present study (Table 2), MHC I was one of the earliest genes to be induced in each organ following increased virus expression and up-regulation of IFN-α. This was consistent with previous reports where a strong association was found between IFN induction and the transcription of MHC I gene [43]. MHC I is expressed in all nucleated cells and its transcription is elevated during viral infections as a result of IFN-α/β induction and more especially, IFN-γ [44]. It is noteworthy that in the present study, MHC I was also induced in almost all tissues where IFN-γ was up-regulated (Table 2), suggesting an association between the two genes in SAV infections in Atlantic salmon as also reported by others [37].

IFN-γ is a powerful pro-inflammatory cytokine produced by cells of the lymphocyte lineage and is required for the control of intracellular pathogens [45]. Its target cells are mainly those of the monocytic origin but $CD4^+$ Th1 cells are also activated [46,47]. In the present study, the expression of IFN-γ at 2 and/or 4 wpi in all organs analyzed suggests the involvement/activation of NK-like cells as part of the innate response since at these time points, there was no accompanying expression of T cell-related genes ($CD4^+$, $CD8^+$, TCR, CD3ε) (Table 2). This was consistent with the report that NK cells are the primary source of IFN-γ during the innate immune response [11,48]. However at 8 wpi, the expression of different genes (MHC I, CD8, TCR, MHC II, IL-12, CD4, TCR and CD3ε genes as well as the augmentation of IFN-γ) in the heart suggests a combined cytotoxic and Th1 mediated response. The pathological changes observed and the infiltration of inflammatory cells (Figure 1) fit very well with the expression of TNF-α and IL-8. It is noteworthy that the up-regulation of genes suggestive of a Th1/cytotoxic response was associated with inflammation/pathology at 8 weeks, with the reaction in the pancreas being greatly down-scaled suggesting a contraction phase. Even though IFN-γ has been shown to have a mild direct effect on SAV-3 [24], it appears to play an important role in shaping the cell mediated response or possibly contributes to the pathology seen in the target organs.

In conformity with the latter notion, it has been shown from studies of higher vertebrates that the expression of IFN-γ requires tight control since it can lead to immunopathology [49]. Furthermore, it has also been demonstrated that IFN-γ producing cells are suppressed by IL-10. IL-10 on the other hand, is itself produced by a large number of immune cells including regulatory and IFN-γ producing T cells [45,50,51]. In the present study IL-10 was consistently induced alongside IFN-γ, with the two genes showing similar trends (Table 2) that also rhymed with viral loads in individual tissues. These findings suggest the conservation of the regulation of these genes in vertebrates.

In the present study, no samples were collected prior to day 14 in the IM group, therefore, data showing the initial distribution of virus before this time is lacking. However, the viral loads of cohabitants at 2 wpi represent infection at an earlier time point compared to 2 wpi in the IM group. These results suggest that the pancreas, heart, kidney and spleen are probably all infected about the same time although the virus ultimately replicates to different levels in the different organs, with the highest load being reached in the pancreas and heart in the cohabitant group (Figure 2b). In the IM group, the virus was administered via skeletal muscle injection and it is not unlikely that an initial replication of virus occurred at the injection site, probably followed by the "draining" of the virus to other organs. The association between high viral loads and pathology in the pancreas and heart (Figures 1 and 2) suggest that the virus threshold for pathology in these organs is just above 10^6 virus RNA copy numbers/μg of total RNA. The presence of SAV-3 in all tissues examined was consistent with previous

reports that the virus has a wide range of tissue tropism in Atlantic salmon [32]. The finding of the highest viral load and pathology in the pancreas at 2 wpi in the IM group compared to other tissues is interesting especially since the viral loads culminated in all organs except the skeletal muscle at 4 weeks. This suggests that the pancreas is the most preferred site of SAV-3 replication. Several other reports allude to the pancreas as the first organ in which pathology is observed following SAV infection [3,33] and this fits with the definition of virus tropism, that being the ability of a virus to infect or cause damage to cells or tissues. On the contrary the slow and protracted increase in viral load in the skeletal muscles suggests that the organ is a site for viral persistence, in agreement with previous studies that have reported virus in this organ long after infection [32].

No lesions were observed in the skeletal muscles in the present study, in contrast with previous reports [3,7]. The viral load during the final sampling of the study was on the increase suggesting that termination at 8 weeks was probably too early, which would explain the lack of lesions. For mice infected with Sindbis virus fatalities occur when the virus invades the neurons [12]. For SAV-3 infections in Atlantic salmon, it is not clear which organs or the degree of pathology correlate with mortalities and should be a subject for further studies.

As already stated, a relationship exists between the viral load and tissue pathology, i.e. a viral load threshold has to be reached before pathology is caused. The delay in this threshold and also in the appearance of pathological changes in cohabitants in the present study compared to the IM group is consistent with a previous report where pathological changes in the former were not observed until 3 weeks following challenge [34]. These findings demonstrate that SAV-3 can spread via water, making the cohabitation challenge a possibility. The IM route of infection for SAV-3 is not natural since it is expected that fish get infected either through vectors or the water itself. Challenge studies using the cohabitation model have previously been described although they have not performed according to expectations firstly because of the difficulty to induce mortalities experimentally for SAV in general [32,34,52] and secondly because the strength of virus challenge seems to be somewhat attenuated compared to IM challenge [53]. In the present study, the presence of virus at low titers in cohabitants (Figure 2b, 2 and 4 wpi) probably allowed the fish to mount a protective immune response resulting in the delay/down regulation of pathology. Cohabitation challenge models for this virus should therefore aim to produce high quantities of infectious virus by shedders in order to enhance pathology in cohabitants or increase the number of shedders and thereby raise the infection pressure.

Finally, the rational development of vaccines offering protective immunity against pathogens relies on knowledge of basic immune responses to particular infections. This is not known in detail for SAV-3 infections in Atlantic salmon although a recent study performed by our group points to antibody responses playing a role [54]. In the present study, we demonstrate that SAV-3 infections induce mRNA transcripts of genes including IFN-α and its stimulated genes (ISG) at early time, followed by IFN-γ, TNF-α, IL-12, IL-10, IL-8, CD3ε, CD4, CD8, TCR-α, MHC-I, and MHC-II as the infection progresses. This is similar to what has been observed in other alphavirus infections in higher vertebrates [12,55], and suggests that the protection of fish against SAV-3 should be aimed at protocols that include eliciting both Th1 polarized and/or cytotoxic responses.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors designed the experimental challenge and contributed to the data analysis. XC, TG and SM took part in sampling. XC and TG did the gene expression experiments. ØE graded the histopathological changes. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by a grant from the Research Council of Norway "Immunisation strategies against viral pathogens of Atlantic salmon," project no. 182035.

Received: 27 July 2012 Accepted: 17 October 2012
Published: 1 November 2012

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doi:10.1186/1297-9716-43-78
Cite this article as: Xu *et al.*: Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon. *Veterinary Research* 2012 **43**:78.

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JOURNAL OF VIROLOGY, Sept. 2010, p. 8903–8912 Vol. 84, No. 17
0022-538X/10/$12.00 doi:10.1128/JVI.00851-10

Alpha Interferon and Not Gamma Interferon Inhibits Salmonid Alphavirus Subtype 3 Replication *In Vitro*▽

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Received 21 April 2010/Accepted 13 June 2010

Salmonid alphavirus (SAV) is an emerging virus in salmonid aquaculture, with SAV-3 being the only subtype found in Norway. Until now, there has been little focus on the alpha interferon (IFN-α)-induced antiviral responses during virus infection *in vivo* or *in vitro* in fish. The possible involvement of IFN-γ in the response to SAV-3 is also not known. In this study, the two IFNs were cloned and expressed as recombinant proteins (recombinant IFN-α [rIFN-α] and rIFN-γ) and used for *in vitro* studies. SAV-3 infection in a permissive salmon cell line (TO cells) results in IFN-α and IFN-stimulated gene (ISG) mRNA upregulation. Preinfection treatment (4 to 24 h prior to infection) with salmon rIFN-α induces an antiviral state that inhibits the replication of SAV-3 and protects the cells against virus-induced cytopathic effects (CPE). The antiviral state coincides with a strong expression of Mx and ISG15 mRNA and Mx protein expression. When rIFN-α is administered at the time of infection and up to 24 h postinfection, virus replication is not inhibited, and cells are not protected against virus-induced CPE. By 40 h postinfection, the alpha subunit of eukaryotic initiation factor 2 (eIF2α) is phosphorylated concomitant with the expression of the E2 protein as assessed by Western blotting. Postinfection treatment with rIFN-α results in a moderate reduction in E2 expression levels in accordance with a moderate downregulation of cellular protein synthesis, an approximately 65% reduction by 60 h postinfection. rIFN-γ has only a minor inhibitory effect on SAV-3 replication *in vitro*. SAV-3 is sensitive to the preinfection antiviral state induced by rIFN-α, while postinfection antiviral responses or postinfection treatment with rIFN-α is not able to limit viral replication.

Salmon pancreas disease virus (SPDV) is the causative agent of pancreas disease (PD) in Atlantic salmon and rainbow trout and is an emerging pathogen in Europe and North America (21). SPDV belongs to the genus *Alphavirus* within the family *Togaviridae* and is phylogenetically related to arthropod-borne alphavirus groups such as the Semliki Forest virus (SFV) group, the Sindbis virus (SINV) group, and Venezuelan equine encephalitis virus (VEEV)/Eastern equine encephalitis virus (EEEV) (24). SPDV was later termed salmonid alphavirus (SAV) and has been divided into subtypes (14), and six subtypes have now been recognized, SAV-1 to SAV-6 (9). SAV-1 was first isolated from farmed Atlantic salmon in Ireland and Scotland (37). Subsequently, sleeping disease virus (SDV) (and later SAV-2), which affects mainly rainbow trout, was discovered in the United Kingdom and France (35, 38). A third and new subtype, SAV-3, is found exclusively in Norway and affects both Atlantic salmon and rainbow trout (14, 33). Three additional subtypes of SAV from Scotland and Ireland have been described (9). Diseased fish are clinically characterized by inappetence, fecal casts, and emaciation, with main pathological changes found in pancreas, heart, and skeletal muscle (21, 25). Immunity to alphavirus infections in salmonids is poorly understood and has not been studied in any detail. In a previous study, interferon (IFN) responses were not detected following SAV-1 infection in salmon parr, possibly due to the low sensitivity of the detection method used (7). A more recent study showed Mx mRNA upregulation in response to SAV infection *in vitro* by using a macrophage cell line from salmon and suggested that Mx has an antiviral function against SAV (11). However, so far, no studies have been carried out to examine the functional aspects of type I IFN responses related to SAV infections of salmonids *in vivo* or *in vitro*.

Type I interferons (alpha/beta IFN [IFN-α/β]) and interferon-stimulated genes (ISGs) are known to play a pivotal role in innate immune responses against viral infections in all living organisms (18) and, therefore, also in fish (42). Mice devoid of type I IFN receptors are extremely susceptible to alphavirus infections, whereas there is no apparent role for IFN-γ (22). Studies of infections in higher vertebrates have shown that the IFN-α/β pathway represents the primary protective response against alphavirus infection involving the limitation of virus replication (1, 13). The antiviral effect of mammalian type I IFN is exerted through binding to the receptor of IFN-α/β, triggering the JAK-STAT pathway, which results in the expression of ISGs, some of which encode antiviral proteins, including Mx, ISG15, and double-stranded RNA (dsRNA)-dependent protein kinase R (PKR) (29). Numerous ISGs such as 2′,5′-oligoadenylate synthetase (OAS)/RNase L, zinc finger antiviral protein (ZAP), Mx, and ISG15 likely execute a direct or indirect inhibition of alphavirus replication in higher vertebrates (5, 16, 19), although the detailed mechanisms are not understood. The importance of ISGs in relation to alphavirus infections has not been studied for fish. Type I IFN and several of its stimulated genes have been cloned from Atlantic salmon,

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▽ Published ahead of print on 23 June 2010.

and their analogous antiviral functions are also partly characterized, which allows a detailed characterization of the responses at the transcript level. Furthermore, the antiviral nature of Atlantic salmon IFN was recently characterized by neutralizing IFN with specific antibodies *in vitro* (3, 23). Mx and ISG15 have been cloned from Atlantic salmon (26, 27), and their functional antiviral activity has been demonstrated against another salmon virus, infectious pancreatic necrosis virus (17). Atlantic salmon PKR protein has been cloned, but no functional studies have been performed. However, PKR from Japanese flounder (*Paralichthys olivaceus*) was recently characterized and shown to increase the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) and inhibit the replication of rhabdovirus in flounder embryonic cells (41). Atlantic salmon PKZ has a direct inhibitory effect on protein synthesis after transient expression in Chinook salmon embryo (CHSE) cells (4).

In this study we were interested in understanding IFN-α- and IFN-γ-induced responses to SAV-3 infection *in vitro* and the possible role of these cytokines in controlling virus replication during the early stages of infection. The two IFNs were cloned and expressed as recombinant proteins and used for *in vitro* studies using a salmon macrophage cell line (36) as a model for SAV infection (11). We show that in SAV-3-infected cell cultures, IFN-α and ISG mRNAs are upregulated and Mx protein expression is strongly induced. By 40 h postinfection, eIF2α is phosphorylated, which coincides with a strong expression of the E2 protein of SAV-3. There is no clear arrest of macromolecular synthesis at early times postinfection (first 24 h postinfection), while from 36 h, there is a moderate downregulation of protein synthesis. The treatment of cell cultures with recombinant IFN-α (rIFN-α) 4 to 24 h prior to infection (preinfection treatment) induces an antiviral response that markedly inhibits the replication of SAV-3 and protects against cytopathic effects (CPE). Treatment with rIFN-α at time of infection and up to 24 h postinfection has a time-dependent effect on limiting SAV-3 replication: the later the treatment, the less efficient, and later treatment will not protect against CPE. Preinfection treatment (24 h) with IFN-γ has only a minor inhibitory effect on the replication of SAV-3 *in vitro*.

MATERIALS AND METHODS

Virus isolation and cell culture. Chinook salmon embryonic cells (CHSE-214; ATCC CRL-1681) were maintained at 20°C with L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS), L-glutamine, and gentamicin. TO cells (macrophage cell line), originating from salmon head kidney leukocytes (36), were grown at 20°C in HMEM (Eagle's minimal essential medium [MEM] with Hanks' balanced salt solution [BSS]) supplemented with L-glutamine, MEM nonessential amino acids, gentamicin sulfate, and 10% FBS. The SAV-3 isolate (named H10) used in this study was isolated from heart of an Atlantic salmon with clinical symptoms of PD. The isolate was propagated by inoculating 80% confluent CHSE-214 cells maintained with growth medium supplemented with 2% FBS. The isolate was identified as a SAV-3 variant by sequencing (14) and was passaged nine times in cell culture before being used in this study.

Protein synthesis shutdown. TO cells were infected with SAV-3 at a multiplicity of infection (MOI) of 20. At different time points postinfection, the cells were washed three times with phosphate-buffered saline (PBS) and then incubated for 30 min in Dulbecco's modified Eagle's medium lacking methionine and supplemented with 0.1% FBS and 20 μCi [^{35}S]methionine/ml. The cells were then harvested sequentially at 12, 24, 36, 48, 60, and 72 h and lysed by using CelLytic M reagent (Sigma). Protein was separated by SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was exposed in a PhosphorImager cassette and then scanned by using a Typhoon imager (GE Healthcare). The protein amount measured by densitometry was quantified with ImageJ software, and the value was expressed relative to a mock-infected control and corrected for the protein amount loaded into each lane.

RNA isolation and cDNA synthesis. Total RNA was isolated by using the RNeasy Plus minikit (Qiagen), and the concentration of RNA was determined by spectrophotometry (Nanodrop ND1000). For each sample, 500 ng of total RNA was subjected to cDNA synthesis using a Transcriptor first-strand cDNA kit (Roche) in a total volume of 20 μl. The synthesized cDNA was stored at −20°C until further use.

Quantitative real-time PCR. Quantitative PCR was performed by using LightCycler 480 SYBR green I master mix and the LightCycler 480 system (Roche). For each gene, 2 μl of cDNA was used as a template in a mixture of specific primers (10 μM) in a final volume of 20 μl. The mixtures were first incubated at 95°C for 10 min, followed by 40 amplification cycles (10 s at 95°C, 20 s at 60°C, and 8 s at 72°C). The sequences of primers used to assess the *in vivo* and *in vitro* expressions of Mx, ISG15, γIP10, and viral E2 are given in Table 1. For the viral E2 gene, the reaction mix contained 10 μl of LightCycler 480 Probes Master, 1 μl of primer-probe mix (final concentrations, 0.9 μM each primer and 0.25 μM probe), 2 μl of cDNA template, and 7 μl water and was incubated for 10 min at 95°C, followed by 45 amplification cycles (10 s at 95°C, 30 s at 60°C, and 1 s at 72°C). The specificity of the PCR products from each primer pair was confirmed by melting-curve analysis and subsequent agarose gel electrophoresis. The $2^{-\Delta\Delta C_T}$ method was used to calculate the gene products as described elsewhere previously (20). $2^{-\Delta\Delta C_T}$ is the relative mRNA expression representing the fold induction over the control group. All quantifications were normalized to β-actin (endogenous gene).

Cloning, expression, and purification of recombinant IFN-α and IFN-γ. Total RNA from the head kidney of Atlantic salmon was used as a template for cDNA synthesis by using a Transcriptor first-strand cDNA synthesis kit (Roche). For initial cloning, two pairs of primers, IFNα-F1 and IFNα-R1, and IFNγ-F1 and IFNγ-R1, were designed according to the Atlantic salmon salmo salar IFN-α1 (SasaIFN-α1) mRNA sequence (GenBank accession no. AY216594) and the Atlantic salmon SasaIFN-γ mRNA sequence (GenBank accession no. AY795563). For IFN-α, we amplified a region from 42 bp downstream of the start codon of the open reading frame (ORF) to 27 bp downstream of the stop codon. For IFN-γ, a region from 107 bp upstream of the start codon of the ORF to 75 bp downstream of the stop codon was amplified. The PCR products were purified by using the QIAquick gel extraction kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega). The region coding for the predicted full-length forms without the signal peptide was subcloned from pGEM-T into the prokaryotic vector pET-32c (Novagen) by using primer sets pET-IFNα-F1 and pET-IFNα-R1, and pET-IFNγ-F1 and pET-IFNγ-R1, containing NdeI and XhoI restriction sites. Truncated forms of IFN-α (trIFN-α) and IFN-γ (trIFN-γ), with deletions of the C-terminal 28 amino acids (aa) and 29 aa, respectively, were also constructed by using different reverse primers (pET-IFNα-R2 and pET-IFNγ-R2). The vectors were designed to express a C-terminal 6×His fusion protein to facilitate the purification of recombinant proteins. Full-length and truncated forms of IFN-α and IFN-γ were designated rIFN-α and trIFN-α and rIFN-γ and trIFN-γ, respectively, which will be used throughout. The recombinant vectors pET-rIFNα and pET-trIFNα, and pET-rIFNγ and pET-tr IFNγ, were confirmed by DNA sequencing and were transformed into the bacterial host, BL21(DE3), for expression driven by the T7 polymerase. Induction was carried out at 37°C for 2 h with 1 mM isopropyl thiogalactopyranoside (IPTG). The purification of 6×His-tagged rIFN was performed under denaturing conditions by using a His-Bind purification kit (Novagen). Protein concentrations were determined by using the Quick Start Bradford protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. The expression and the purity of recombinant IFN-α and IFN-γ were checked on a 12% Bis-Tris precast SDS-PAGE gel (Invitrogen Life Technologies) stained with PageBlue protein staining solution (Fermentas). Western blot analysis was performed to confirm the identity of the recombinant IFN-α by using a monoclonal anti-polyhistidine antibody (Sigma).

Cloning, expression, and purification of E2 and preparation of E2 antiserum. Viral RNA was extracted from the cell supernatant by using a QIAamp viral RNA minikit (Qiagen) and used as a template for cDNA synthesis by using a Transcriptor first-strand cDNA synthesis kit (Roche). A pair of primers, pET14b-E2-F and pET14b-E2-R, was designed according to the E2 gene of the Norwegian salmonid alphavirus isolate (GenBank accession no. AY604236). The cloning, expression, and purification of recombinant E2 proteins were performed as described above. The purified recombinant E2 protein was sent to PickCell Laboratories BV (Netherlands) for rabbit immunization and subsequently char-

TABLE 1. Primer and probe sequences for cloning and quantitative real-time PCR[a]

Primer	Sequence	Use	GenBank accession no.
IFNα-F1	CAGTATGCAGAGCGTGTGT	pGEMT cloning	AY216594
IFNα-R1	CGTAGCTTCTGAAATGAGTCTGG		
pET-IFNα-F1	GCGCATATGTGTGACTGGATCCGACAC	pET32 cloning	
pET-IFNα-R1	GCGCTCGAGGTACATCTGTGCTGCAAG		
pET-IFNα-R2	GCGCTCGAGGTTCATTTTTCTCAGAAC		
IFNα-F2	TGGGAGGAGATATCACAAAGC	qPCR	
IFNα-R2	TCCCAGGTGACAGATTTCAT		
Mx-F	TGCAACCACAGAGGCTTTGAA	qPCR	U66475
Mx-R	GGCTTGGTCAGGATGCCTAAT		
pET14b-E2-F	GCGCATATGGCTGTGTCTGCGTCGCCT	pET14b cloning	
pET14b-E2-R	GCGCTCGAGTTACGCACGAGCCCCAGG		
SAV-3 E2-F	CAGTGAAATTCGATAAGAAGTGCAA	qPCR	EF675594
SAV-3 E2-R	TGGGAGTCGCTGGTAAAGGT		
E2 probe	FAM-5′-AGCGCTGCCCAAGCGACCG-3′-MGB		
IFNγ-F1	AGGCGGTCTCGTTAAGTCAA	pGEMT cloning	AY795563
IFNγ-R1	TAAACTGACCCAAGATCAGC		
pET32-IFNγ-F	GCGCATATGGCTCAGTACACATCAATT	pET32 cloning	
pET32-IFNγ-R1	GCGCTCGAGCATGATGCTTGATTTGAG		
pET32-IFNγ-R2	GCGCTCGAGTTTCTCGTAGATGGTAAT		
IFNγ-F4	CTAAAGAAGGACAACCGCAG	qPCR	
IFNγ-R4	CACCGTTAGAGGGAGAAATG		
γIP10-F	TGCCAGAACATGGAGATCAT	qPCR	EF619047
γIP10-R	TTTACTGCACACTCCTTTGGTT		
ISG15-F	AAGTGATGGTGCTGATTACGG	qPCR	AY926456
ISG15-R	CACCGTTAGAGGGAGAAATG		
β-actin-F	CCAGTCCTGCTCACTGAGGC	qPCR	AF012125
β-actin-R	GGTCTCAAACATGATCTGGGTCA		

[a] Restriction sites are underlined. qPCR, quantitative PCR; FAM, 6-carboxyfluorescein; MGB, MGB-TaqMan probe.

acterized by Western blotting and immunofluorescence antibody test (IFAT) staining of SAV-3-infected cultures.

Induction of ISG expression by recombinant IFN-α and IFN-γ *in vitro*. TO cells were seeded in 24-well plates and cultured until confluent. For IFN-α, the cells were stimulated with 2.5 μg/ml rIFN-α and trIFN-α, nontreated cells were included as negative control, and samples were harvested at 3, 6, 12, and 24 h poststimulation. The cells were also treated with 10-fold serial dilutions of rIFN-α with an initial starting concentration of 0.47 mg/ml; at 24 h, samples were taken, and RNA was extracted. The induction of ISGs by IFN-α was documented by studying the gene upregulation of Mx and ISG15 by using real-time PCR. For IFN-γ, purified rIFN-γ was serially diluted from 0.33 mg/ml in cell medium and incubated with TO cells for 24 h. The induction of ISGs by IFN-γ was documented by studying the gene upregulation of γIP10 (gamma-IP CXCL10-like chemokine) using real-time PCR. The data are expressed as the mean fold changes in gene expression ± standard errors of different dilutions of the interferon-treated group relative to the nontreated control group after normalization to β-actin.

Antiviral assays. A CPE reduction assay was used to measure the protective effect of IFN-α against cytopathic effects in pretreated and infected cells (8). TO cells grown in 96-well plates were treated with a serial dilution of rIFN-α and trIFN-α, and rIFN-γ and trIFN-γ, for 24 h and subsequently infected with 1 MOI of SAV-3. Virus was left on the cells for 2 h to adsorb (15°C), after which the cells were washed three times with PBS. Untreated cells, infected and noninfected, were included as controls. The cell viability of the cell cultures subjected to the different treatments was assayed by using the CellTiter 96 AQueous One solution cell proliferation assay kit (Promega) at day 10 postinfection when strong CPE developed in untreated cells. The cell cultures were incubated at 15°C for 4 h, and the absorbance was measured at 490 nm by using a microplate reader (Tecan). For the virus yield reduction assay, culture supernatant and cell total RNA from infected cells in 24-well plates were collected. The titration of virus was done with CHSE-214 cells by the 50% tissue culture infective dose ($TCID_{50}$) method as described previously by Kärber (15), and viral RNA was quantified by real-time PCR.

Detection of Mx and E2 expression by IFAT. Cells seeded into 24-well culture plates were fixed with 4% paraformaldehyde for 30 min. After being washed with PBS, the cells were permeabilized with 0.1% Triton X-100 for 5 min on ice. The cells were washed once in PBS and blocked with 5% dry milk in PBS for 2 h before being incubated for 1 h with primary antibody. Anti-salmonid Mx (diluted 1:400; kindly provided by Jo-Ann Leong, Hawaii Institute of Marine Biology) and anti-E2 polyclonal antibody were used to detect Mx and E2 protein expressions, respectively. The cells were washed and incubated with Alexa 594 or Alexa 488 Fluor goat anti-rabbit IgG (Molecular Probes, Invitrogen) diluted 1:200 for 1 h. Finally, the cells were washed and examined by using a fluorescence microscope (Olympus). When nuclear counterstaining was used, it included Hoechst 33324 dye at 5 μg/ml.

Protein analysis of E2, Mx, and p-eIF2α in virus-infected cells. TO cells were infected with SAV-3 (MOI of 1) or left uninfected. At days 1, 2, 3, and 4 postinfection, uninfected and infected cells were treated with a 10^3 dilution of IFN-α for 16 h or left untreated. The cells were then lysed by using CelLytic M reagent (Sigma) and scraped from the dish. Expressions of the E2 (antiserum prepared as described above), phosphorylated eIF2α (p-eIF2α) (Cell Signaling), Mx, and actin (Sigma-Aldrich) proteins were detected by Western blotting.

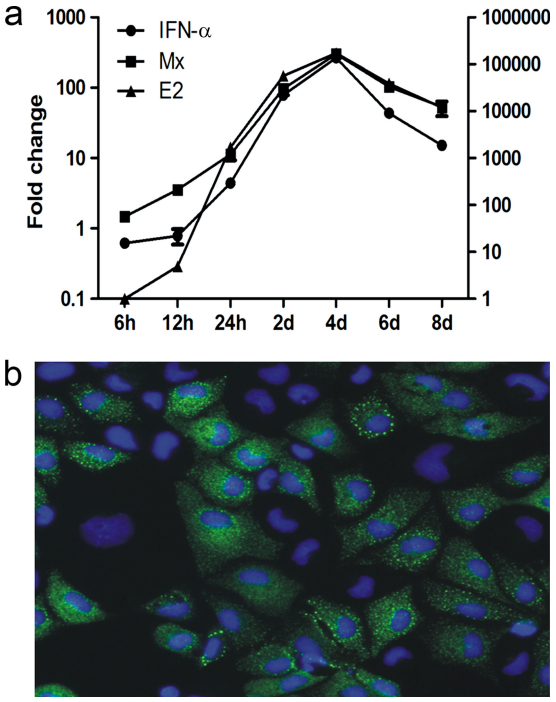

FIG. 1. (a) Expression levels of IFN-α, Mx, and viral E2 mRNA in TO cells infected with 1 MOI of SAV-3. The left *y* axis shows the fold increase of IFN-α and Mx mRNA relative to the nontreated control, and the right *y* axis shows the fold increase of viral E2 transcript levels relative to infected cells at 6 h. The results are expressed as means ± standard errors of the means (SEM) ($n = 2$). (b) Cytoplasmic Mx protein expression shown by indirect immunofluorescence in TO cells infected with SAV-3. Cells were fixed at 8 days postinfection. Nuclear staining was done with Hoechst 33324 dye.

Statistical analysis. All statistical analyses of gene expression results were performed with the help of GraphPad Prism 5.0 (GraphPad Software Inc.). Two-way analysis of variance (ANOVA) was used to calculate differences in the CPE protection assay at different concentrations. The significant level for rejection of hypothesis 0 (*Ho*) was set to a *P* value of <0.05.

RESULTS

Responses to SAV-3 infection in TO cells. The initial studies were designed to characterize the expression of IFN-α and Mx mRNA in response to SAV-3 infection in TO cells. The kinetics of the virus infection were monitored by real-time reverse transcription (RT)-PCR analysis of the expression of the protein E2-encoding gene, which was detected as early as 6 h postinfection and increased 1,600-fold from 6 h to 24 h postinfection (Fig. 1a). Over the same time period (6 h to 24 h), IFN-α transcript levels increased 4-fold, while Mx expression levels increased 10-fold (Fig. 1a). From 24 h postinfection to 4 days postinfection (dpi), there was a marked and parallel increase in levels of IFN-α, Mx, and E2 transcripts, all peaking at 4 dpi and declining at later times (Fig. 1a). Staining for the Mx protein at 8 days postinfection showed widespread expression in the infected cell culture (Fig. 1b). These findings provided a strong indication that SAV-3 infection will not result in a shutoff of interferon or interferon-induced responses.

rIFN-α induces expression of ISGs in salmon cell lines. With the purpose to conduct functional studies with recombinant IFN-α using a macrophage cell line of Atlantic salmon (TO cells) (36), we first cloned and expressed full-length IFN-α (rIFN-α) and tested its functional activity. The obtained sequence was aligned with SasaIFN-α1 (GenBank accession no. AY216594) and SasaIFN-α2 (accession no. AY216595) sequences, and the amino acid similarities were 96.1% and 99.3%, respectively. As a negative control, we constructed a truncated form of IFN-α (trIFN-α), with a deletion of a 28-aa stretch at the C-terminal end (31). Both full-length and truncated IFN-α were expressed in *Escherichia coli* BL21(DE3) cells using the pET prokaryotic expression system. The protein yield in the cell soluble fraction was much lower than that of the inclusion bodies (data not shown); thus, the purification of proteins was performed under denaturing conditions. A 6×His tag added at the C-terminal end of the protein facilitated purification with a His-Bind column. The expression and purification of recombinant proteins were identified by SDS-PAGE and Western blot analysis using an anti-His monoclonal antibody (Fig. 2a and b). The concentrations of purified full-length and truncated IFN-α were 0.47 mg/ml and 0.48 mg/ml, respectively. Dilutions of rIFN-α and trIFN-α refer to these initial concentrations in all experiments except when stated otherwise. A similar characterization was performed for IFN-γ by SDS-PAGE for the full-length and the truncated forms of purified samples (Fig. 2c). The protein concentration for purified full-length rIFN-γ was 0.33 mg/ml.

The biological function and activity of rIFN-α were assessed by measuring the expression of the interferon-induced Mx and ISG15 genes in TO cells using real-time RT-PCR after stimulation with rIFN-α. At 24 h poststimulation, Mx mRNA was found to be strongly expressed after rIFN-α treatment, while

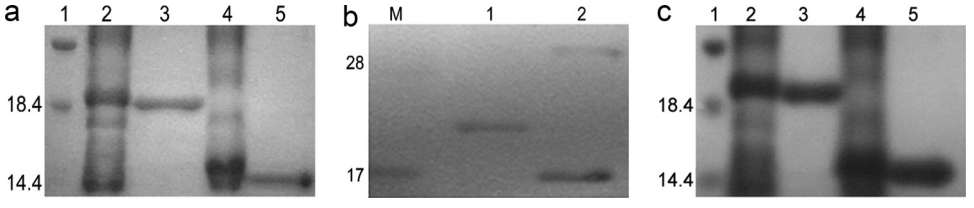

FIG. 2. (a) Expression and purification of mature and truncated IFN-α (SDS-PAGE). Lanes: 1, protein marker; 2, full-length recombinant IFN-α (rIFN-α); 3, purified rIFN-α; 4, truncated IFN-α (trIFN-α); 5, purified trIFN-α. (b) Western blot analysis of rIFN-α (lane 1) and trIFN-α (lane 2) in bacterial lysates. The predicted molecular masses of full-length and truncated IFN-α are approximately 19.4 and 16.0 kDa, respectively (M, marker lane). (c) Expression and purification of recombinant and truncated IFN-γ. Lanes: 1, protein marker; 2, full-length IFN-γ (rIFN-γ); 3, purified rIFN-γ; 4, trIFN-γ; 5, purified trIFN-γ. The predicted molecular masses of full-length and truncated IFN-γ are approximately 19.7 and 16.3 kDa, respectively.

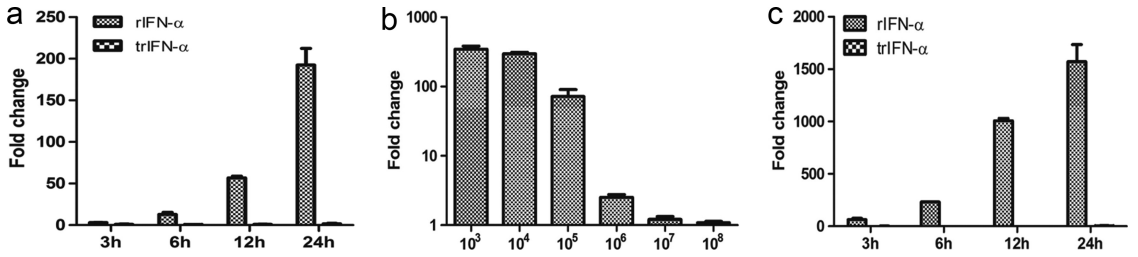

FIG. 3. Induction of Mx and ISG15 expression in TO cells after treatment with full-length recombinant and truncated IFN-α. (a and b) Time course expression levels of Mx (a) and dose-dependent induction of Mx mRNA by rIFN-α (b). Purified rIFN-α was diluted serially 10-fold from 0.47 mg/ml and incubated with TO cells for 24 h. The data are expressed as the mean fold changes in gene expression ± SEM of different dilutions for the rIFN-α-treated group relative to the nontreated control group after normalization to β-actin ($n = 2$). (c) ISG15 mRNA transcripts of TO cells stimulated with 2.5 μg/ml rIFN-α and trIFN-α. The data are expressed as the mean fold changes in gene expression ± standard errors for the IFN-α-treated group relative to the nontreated control group after normalization to β-actin ($n = 2$).

the truncated form was nonfunctional, and no expression of Mx was seen (Fig. 3a). By serial dilution we found that 0.47 ng/ml of rIFN-α still induced the expression of Mx (2.5-fold upregulation) (10^6 dilution) (Fig. 3b). The expression of ISG15 mRNA by 12 h post-rIFN-α treatment was 1,000-fold upregulated, increasing to >1,500-fold by 24 h (Fig. 3c). The expression of the Mx protein was confirmed by indirect immunofluorescence 24 h after rIFN-α stimulation. TO cells were stained positive with an anti-Mx antibody (Fig. 4a), while cells without treatment showed no staining (Fig. 4b).

rIFN-α induced a preinfection antiviral state that inhibits SAV-3 replication. We then went on to study the antiviral effects after pretreatment (24 h) of TO cells with rIFN-α. All studies were carried out using cell cultures originating from Atlantic salmon (*Salmo salar* L.), while we also included a limited study using CHSE cells (of Chinook salmon origin [*Oncorhynchus tshawytscha*]). We used the CPE reduction assay and the virus yield reduction assay (virus titer estimation) to measure the antiviral activity of rIFN-α on SAV-3 replication. TO cells were pretreated with a serial dilution of full-length and truncated IFN-α for 24 h and subsequently infected with 1 MOI of SAV-3. rIFN-α and trIFN-α were added to the cell cultures in culture medium, and the cultures were incubated for 24 h. At 0 h, medium was aspirated, virus was added, and the cultures were incubated for up to 10 days without changing the medium. Controls included infected cells not treated with rIFN-α or cells left uninfected. At 10 dpi when strong CPE occurred in the infected, non-IFN-α-treated cells, viability was assayed by using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. Protection from CPE in rIFN-α-pretreated cells was dose dependent, with significant differences ($P < 0.05$) between nontreated, infected cells and cells pretreated with rIFN-α at 10^3-, 10^4-, 10^5-, and 10^6-fold dilutions (Fig. 5a), corresponding to rIFN-α concentrations from 0.47 μg to 0.47 ng ml^{-1}. Conversely, trIFN-α gave no protection against CPE (Fig. 5a). Antiviral activity was also demonstrated for CHSE cells, and significant protection ($P < 0.05$) was seen for cells treated with rIFN-α at 10^3-, 10^4-, and 10^5-fold dilutions (Fig. 5b).

Morphologically, no CPE was observed for TO cells pretreated with rIFN-α at 10^3-, 10^4-, and 10^5-fold dilutions, while at a 10^6-fold dilution, CPE were observed (Fig. 6). Cultures pretreated with trIFN-α were not protected against CPE induced by SAV-3 infection even at the highest concentration used (Fig. 6).

The purity of the cloned E2 protein (Fig. 7a) and the specificity of the anti-E2 rabbit antiserum were documented by Western blotting (Fig. 7b) and IFAT staining (Fig. 7c) of SAV-3-infected cultures, and the inhibition of viral replication in IFN-α-treated cells was further demonstrated by an analysis of virus replication and viral protein (E2) synthesis. TO cells

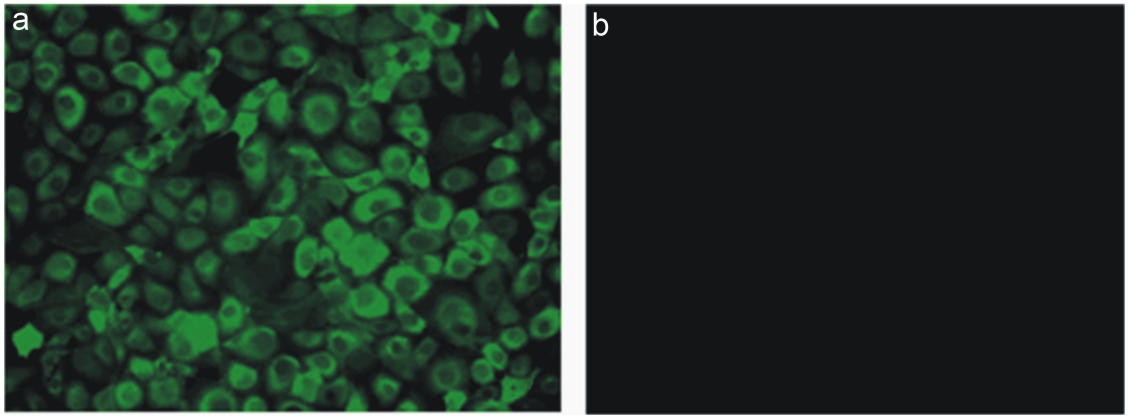

FIG. 4. (a) Detection of Mx protein expression by indirect immunofluorescence in TO cells stimulated with 2.5 μg/ml rIFN-α for 24 h and then fixed and stained. (b) Nontreated control cells.

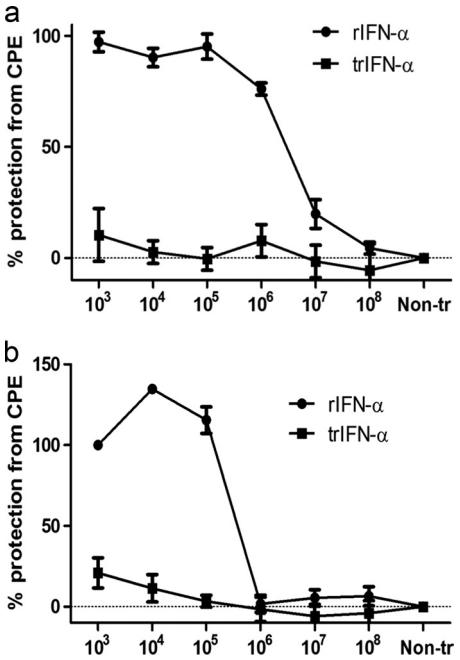

FIG. 5. Cytopathic effect (CPE) reduction assay expressed as percent protection from CPE in TO cells (a) and in CHSE cells (b). Cell cultures were stimulated with 10-fold serial dilutions of rIFN-α and trIFN-α 24 h prior to infection with 1 MOI of SAV-3. The lowest concentration with a biological effect (10^6 dilution) corresponds to 0.47 ng/ml, while the highest concentration used (10^3 dilution) was 0.47 μg/ml for IFN-α. For trIFN-α, the highest concentration used was 0.48 μg/ml ($n = 3$) (means ± SEM). Non-tr, nontreated cultures.

treated with 0.47 μg/ml rIFN-α totally inhibited the expression of the E2 protein at 2 days postinfection (Fig. 7e), while nontreated cells showed positive staining for E2 at the same time point (Fig. 7f). The antiviral activity of rIFN-α was confirmed by the virus yield reduction assay: the virus titer was reduced in TO cells treated with rIFN-α diluted 10^3-, 10^4-, and 10^5-fold (10^3- to 10^5-fold reduction) compared with nontreated control cells (Fig. 7d). These results showed that the pretreatment of cells with rIFN-α strongly inhibited SAV-3 replication and protected cells against virus-induced cell lysis (CPE).

Timing of rIFN-α treatment of SAV-3-infected cells. We then explored the effect of the timing of rIFN-α treatment (0.47 μg/ml) relative to when the cells were infected, starting IFN-α treatment 24 h prior to infection and ending 24 h postinfection. Virus replication was assayed by examining the relative expression of E2 by real-time RT-PCR (Fig. 8), and at the same time, we evaluated the development of CPE in pre- and posttreated infected cell cultures. Treatment 24 h prior to infection inhibited SAV-3 replication 40,000-fold relative to nontreated controls (Fig. 8). Preinfection treatment (−4 h) gave close to a 2,000-fold reduction in E2 gene expression and protection against CPE in infected cultures (Fig. 8). Treatment closer to the time of infection (−2 h and 0 h) and postinfection did not protect against CPE; however, there was a marked reduction in E2 gene expression levels relative to those of nontreated cell cultures. Treatment 24 h postinfection gave a 3-fold reduction in replication levels and strong CPE. The results clearly show that the timing of the IFN-α treatment is critical for the induction of the antiviral state, and its magnitude is dependent on the dose (Fig. 5a) and time (Fig. 8). While an antiviral state can be established through preinfection treatment, concurrent or postinfection treatment with rIFN-α will not protect infected cells against CPE, indicating that the virus has developed a strategy to evade the IFN-induced antiviral response at later stages of the infection cycle.

SAV-3 infection results in phosphorylation of eIF2α cells and moderate protein synthesis downregulation. These findings led us to further characterize the expression of viral protein (E2) and Mx and the phosphorylation of eIF2α by Western blotting over a 96-h period postinfection. TO cells were infected at an MOI of 1 with SAV-3 (0 h), and one parallel culture was also treated with rIFN-α (0.47 μg/ml) at 24, 48, 72, and 96 h postinfection. Control treatments included noninfected/rIFN-α-treated and infected/non-rIFN-α-treated cells (Fig. 9). After rIFN-α treatment, the cells were left to grow for an additional 16 h (after each treatment time) to assess responses to treatment. For simplicity, we refer to time points when rIFN-α was added. E2 expression was seen at 24 h (faint staining) postinfection in cultures treated with rIFN-α and left untreated, while Mx was not detected at this time point (Fig. 9). Faint staining for p-eIF2α was also seen. From 48 h on-

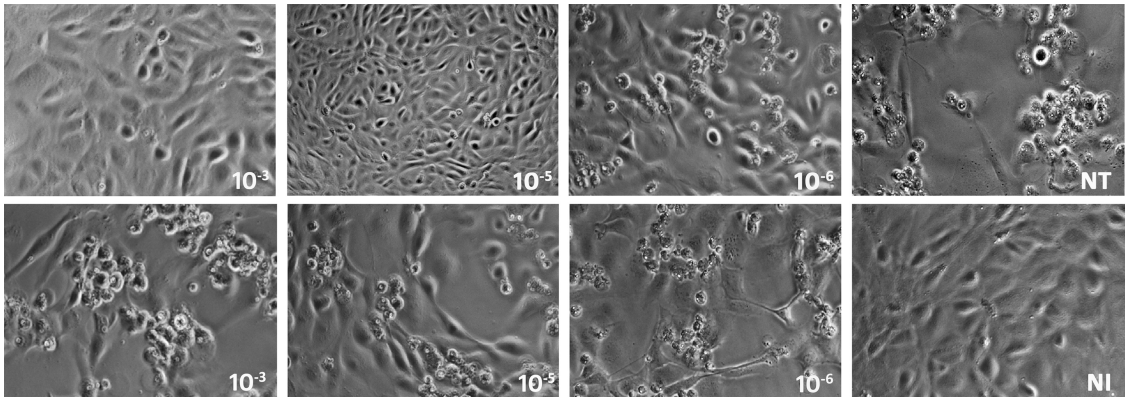

FIG. 6. Protection against CPE observed by morphology. The top row shows cells treated with rIFN-α, which showed dose-dependent protection against CPE at 10^3 and 10^5 dilutions, while initial CPE were seen at a 10^6 dilution. Conversely, trIFN-α did not show any protection against CPE (bottom row). Shown are representative findings for two independent experiments. NT, not treated; NI, not infected.

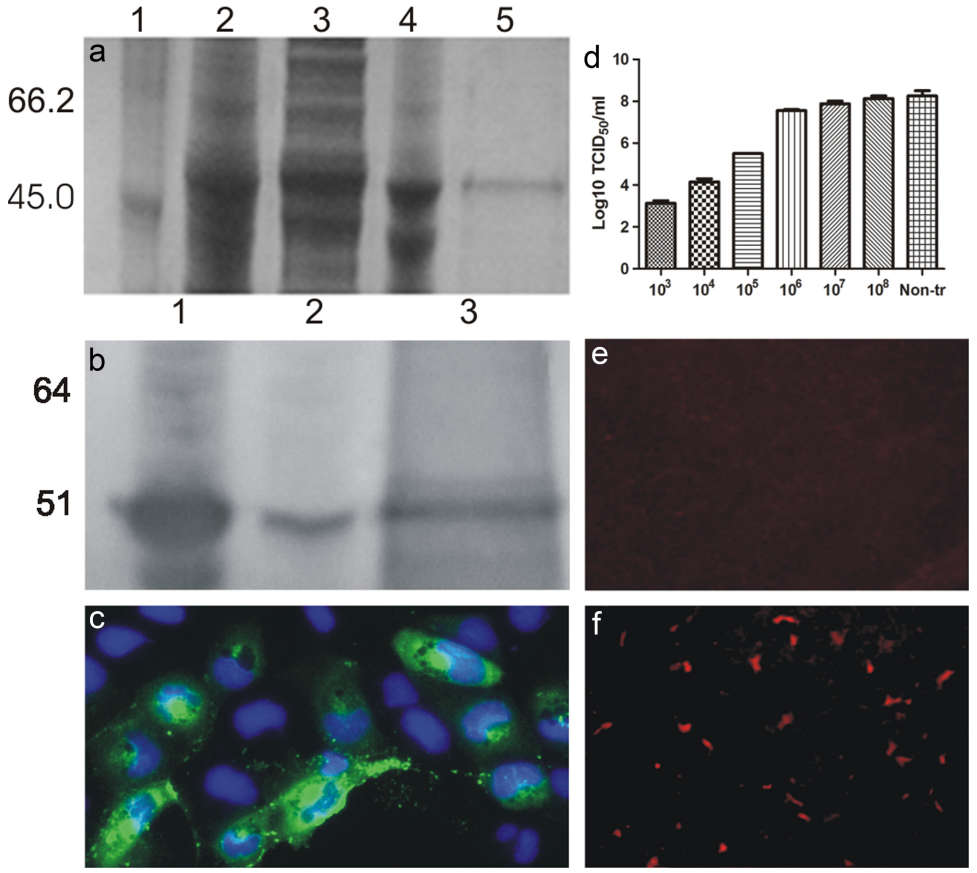

FIG. 7. (a) Purification of recombinant pET14b-E2 protein analyzed by SDS-PAGE. Lanes: 1, protein marker; 2, whole bacteria after induction; 3, soluble fraction after sonication; 4, insoluble fraction after sonication; 5, purified recombinant protein. (b) Characterization of polyclonal antibody against E2 by Western blotting. Lanes: 1, pET14b-E2 after induction; 2, pET14b-E2 without induction; 3, purified E2. (c) Reactivity of rabbit antibody against E2 by IFAT of CHSE cells infected with SAV-3 using a 1:400 dilution of the serum. (d) Virus yield reduction assay. TO cells in 24-well plates were stimulated with 10-fold serial dilutions of rIFN-α for 24 h and then infected with 1 MOI of SAV-3. Cell culture supernatants were collected when strong CPE were observed for the control infected cells. The virus titer of the supernatants at 10 days postinfection was determined by the $TCID_{50}$ method ($n = 2$) (means ± SEM). (e) Inhibition of E2 protein synthesis in interferon-treated TO cells. TO cells were treated with 0.47 μg/ml IFN-α for 24 h and subsequently infected with 1 MOI of SAV-3. At 2 days postinfection, cells were stained with anti-E2 antibody, and no cells were found to express virus protein. (f) TO cells without IFN treatment infected as described in the text and stained by IFAT for E2, showing distinct cytoplasmic staining in infected cells.

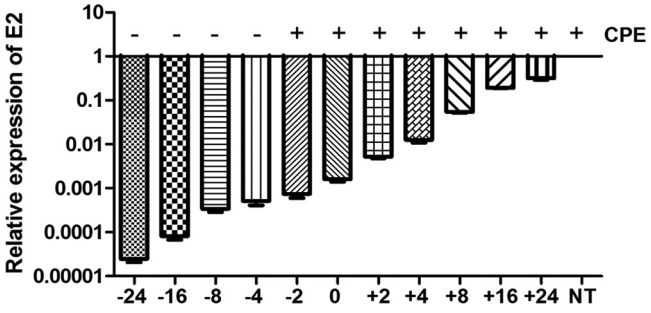

FIG. 8. Timing of IFN-α treatment of SAV-3-infected cells. Treatment 4 to 24 h prior to infection results in a marked reduction in virus replication (2,000- to 40,000-fold reduction) as measured by real-time PCR and protection against CPE. rIFN-α treatment at the time of infection or up to 24 h postinfection gives a reduction in virus replication but not protection against CPE. TO cells were infected with an MOI of 1, and collection was done at 4 days postinfection. Shown are representative data from two independent experiments, expressed relative to the nontreated control (NT). More details are given in Materials and Methods.

wards, there was marked E2 and Mx expression and staining for p-eIF2α in the SAV-3-infected cells not treated with IFN-α. The combination of infection and rIFN-α treatment gave the same results as those for the SAV-3-infected-only

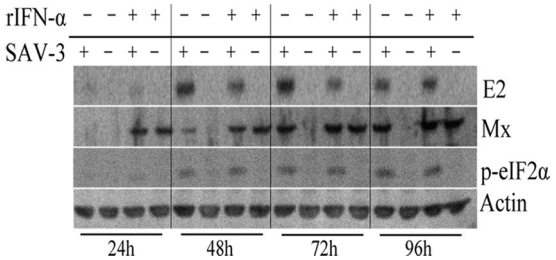

FIG. 9. Protein analysis of E2, Mx, and p-eIF2α in virus-infected cells. TO cells were infected with SAV-3 (MOI of 1) or left uninfected, and at 24, 48, 72, and 96 h postinfection, uninfected (−) and infected (+) cells were treated with 0.47 μg/ml of rIFN-α for 16 h or left untreated. Cells were lysed by using CelLytic M reagent (Sigma) and scraped from the dish. The expression of the E2, Mx, p-eIF2α, and actin proteins was detected by Western blotting.

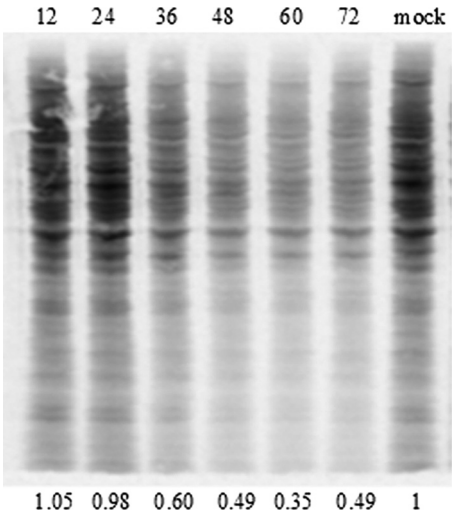

FIG. 10. Residual protein synthesis in TO cells infected with SAV-3. Confluent TO cells were grown in a six-well plate and infected as described in Materials and Methods. The membrane was exposed in a PhosphorImager cassette and then scanned by using a Typhoon imager. Numbers above the figure represent hours postpulsing. The protein amount was quantified by densitometry with ImageJ software as described in Materials and Methods. The results show 40, 51, and 65% reduction by 36, 48, and 60 h postinfection, respectively. Mock infection is shown at the far right.

cells although with a moderate reduction in levels of E2 expression at 48 and 72 h (Fig. 9). In noninfected cells treated with rIFN-α, there was marked Mx expression, while eIF2α was not found to be phosphorylated at any time point (Fig. 9). The finding that SAV-3 infection resulted in the phosphorylation of eIF2α prompted us to characterize the effect of virus infection on protein synthesis. We found a moderate reduction of protein synthesis in TO cells by 36 h (40% reduction), which was more prominent by 48 and 60 h postinfection, with 50% and 65% reductions, respectively (Fig. 10).

IFN-γ has a limited inhibiting effect on SAV-3 replication *in vitro*. On the basis that it was previously shown that rIFN-γ has a limited effect on alphavirus replication in mice (21), we wanted to understand any antiviral effects induced by rIFN-γ in fish cells. The functional activity of rIFN-γ was documented by its ability to induce the expression of γIP10 (Fig. 11a). The CPE reduction assay showed that rIFN-γ has no ability to rescue pretreated cells from CPE caused by SAV-3 infection (Fig. 11b), while there was an approximately 10-fold reduction of virus titers at the highest rIFN-γ concentration, determined by real-time PCR (1:200 dilution, corresponding to 1.65 μg/ml) (Fig. 11c).

DISCUSSION

This is the first report showing that IFN-α has a strong inhibitory effect on SAV-3 replication *in vitro* in permissive salmonid cell lines. This finding is in conformity with what was shown previously for other alphavirus species in higher vertebrates, where the stimulation of IFN-α/β pathways represents the primary protective response and plays a key role in protection against infection through the limitation of virus replication (1, 13, 32, 40). IFN-γ limits virus replication of SAV-3 marginally, again in concordance with what was found previously for higher vertebrates (22). These findings have implications for the understanding of host-pathogen interactions in SAV infections of salmonid fish and possibly provide new avenues for disease prevention.

The tools available to study the *in vivo* effects of type I IFN in infected fish at a functional level are very limited. *In vitro* methods using permanent cell lines permissive for virus infection are thus a good option for functional studies when this can be combined with treatment with functionally active cytokines. SAV-3 infection of TO cell was characterized by a rapid increase in viral E2 gene copy numbers from 12 to 24 h postinfection, representing a more-than-2-$\log_{10}$ increase over a 12-h period (Fig. 1). Over the same period, there was a strong upregulation of IFN-α and Mx transcripts, and these findings

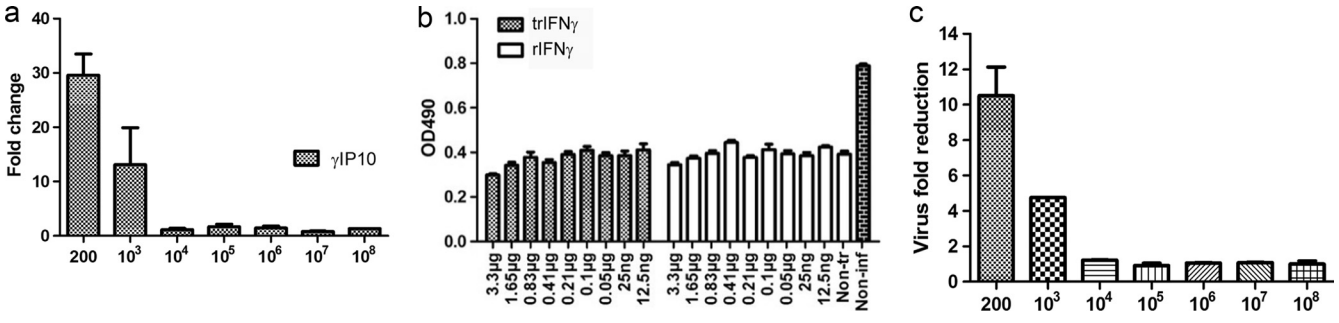

FIG. 11. (a) Induction of ISG mRNA by rIFN-γ in TO cells. Purified rIFN-γ was serially diluted from 0.33 mg/ml in cell medium and incubated with TO cells for 24 h. The data are expressed as the mean fold changes in gene expression ± standard errors of different dilutions of the IFN-γ-treated group relative to the nontreated control group after normalization to β-actin ($n = 2$) (±SEM). (b) Cytopathic effect reduction assay of IFN-γ-treated TO cells infected with SAV-3. TO cells were stimulated with serial dilutions of rIFN-γ and trIFN-γ for 24 h and subsequently infected with 1 MOI of SAV-3. There is no significant difference in protection against CPE between cell cultures pretreated with rIFN-γ and trIFN-γ ($n = 4$) (±SEM). Non-tr, nontreated cells; Non-inf, noninfected; OD490, optical density at 490 nm. (c) Real-time PCR quantification of virus in cell supernatants from TO cells stimulated with serial dilutions of mature IFN-γ for 24 h and subsequently infected with 1 MOI of SAV-3. Cell supernatants were collected when a strong CPE was observed in the control infected cells (8 days postinfection). The data are expressed as the mean crossing-point values ± standard errors using real-time PCR of different dilutions of an IFN-γ-treated group relative to the nontreated infected control group ($n = 2$) (±SEM).

are in conformity with data from a previous study (11) using a different subtype of SAV (SAV-1). To what extent the cell line used in this study reflects a true *in vivo* situation is difficult to determine, but TO cells have morphological and functional traits of salmon macrophages (35) and support the replication of a number of salmonid viruses, like salmon anemia virus (36) and infectious pancreatic necrosis virus (3). Furthermore, the treatment of TO cells with low concentrations of rIFN-α induced strong expression levels of IFN-induced genes, Mx and ISG15, and also a strong expression of Mx at the protein level (shown by IFAT), indicating that a functionally active cell line was used. The induction of an antiviral state in the pretreated cells was dependent on dose and time relative to infection, and protection against CPE was obtained when rIFN-α was added to the cells earlier than 4 h prior to infection and at a dose higher than 0.47 μg/ml. Treatment at the time of infection (or 2 h prior to infection) and postinfection gave no protection against CPE, although there was a reduction in viral copy numbers.

In a recent study by Gahlawat et al. (11), it was proposed that the suppression of Mx responses correlates with SAV replication; however, those studies did not include a functional assessment of the responses involved. Those researchers showed a difference in mRNA IFN-α and Mx expression levels in two cell lines of salmon origin, and they proposed a possible interference with IFN signaling and the suppression of Mx responses (11). Their observations were based solely on the observation that a cell line with low susceptibility had high Mx responses and vice versa. From our studies we have no indication that SAV-3-infected cells have impaired Mx responses (Fig. 1 and 9), and the treatment of TO cells with rIFN-α followed by SAV-3 infection did not influence the expression of Mx as assessed by Western blotting. Furthermore, a strong expression of Mx postinfection seems to have little influence on virus replication. On the other hand, the timing of the antiviral responses relative to infection is obviously of major importance. This is concordant with a recent study where it was shown that mRNA of the Old World alphaviruses encoded by subgenomic RNA is resistant to eIF2α phosphorylation, while genomic RNA replication is highly sensitive to eIF2α phosphorylation (34). It was also found that the replication of genomic RNA ceased with the onset of eIF2α phosphorylation, which again correlated with the appearance of structural proteins. We also find that the synthesis of the spike protein (E2) coincided with the phosphorylation of eIF2α in SAV-3-infected and rIFN-α-treated/SAV-3-infected cells (Fig. 9), with some reduction of staining intensity of E2 with increasing p-eIF2α staining, but still, there was a successful production of virus progeny (Fig. 1). The antiviral state induced through preinfection treatment results in strong ISG induction, a marked inhibition of virus replication, and no E2 staining (Fig. 7 and 8). Thus, in line with what was described previously for other alphaviruses (28), the exact antiviral mechanisms controlling viral replication are not known; it seems that protein translation from genomic viral RNA (incoming positive strand) is sensitive to IFN-α-induced responses. At the same time, it cannot be ruled out that the synthesis of spike mRNA driven by the 26S promoter is resistant to IFN-α-induced cellular responses, but further studies are needed to understand the details of the mechanisms involved.

A known strategy by which alphaviruses evade the IFN response is through global shutoff of host gene expression (28). The transcriptional shutoff caused by Old World alphaviruses depends on a nonstructural protein (nsP2), and in addition, nsP2 of Sindbis virus has an anti-interferon effect, likely through decreased IFN production, and mutations in nsP2 can attenuate Sindbis virus cytopathogenicity (10, 12). There is no protein shutoff in SAV-3-infected cells, but there is a downregulation of protein synthesis. This is more in line with New World alphaviruses that result in a more moderate downregulation of protein synthesis (2, 10, 12), where the capsid protein possibly plays a role in the arrest of macromolecular synthesis during early stages of infection, which will limit the formation of antiviral proteins in infected cells (2, 12). The New World alphavirus VEEV is also able to antagonize the IFN response using distinct mechanisms independent of host shutoff by inhibiting the phosphorylation of cytoplasmic signal transducer and activator of transcription 1 (STAT-1), which prevents the nuclear translocation of phosphorylated STAT-1/STAT-2 heterodimers (30). The result is a reduced formation of antiviral effector molecules like Mx and ISG15. More-recent studies have added complexity to the picture described above and have shown that VEEV nonstructural proteins arrested cellular translation (39). To what extent SAV-3 employs specific mechanisms to counteract the IFN response by targeting certain signaling proteins involved in the IFN pathway or IFN signaling pathway needs to be investigated. A better understanding of the induction of IFN-α and related responses and possible antiviral effects and regulation of host gene expression imposed by nonstructural and/or structural proteins of SAV-3 is strongly needed, and it is our understanding that this can best be studied by using reverse genetics methods.

The strong upregulation of IFN-α mRNA in response to SAV-3 infection is in contrast to what has been seen for SINV infection of permissive cell lines, while it is in conformity with what was observed for VEEV infection (in the same cell lines), where an upregulation of IFN-α mRNA transcripts postinfection was observed (39). The aquatic alphaviruses are defined as a separate clade within the genus *Alphavirus*, and they are phylogenetically different from Old World and New World alphaviruses (24). Their identification has raised interesting questions as to the origin of different variants of alphaviruses in general. While the Old World and New World alphaviruses have been classified into arthrogenic and neurotropic variants, the salmonid alphaviruses do not seem to fall into either of these categories, and they are not transferred by vectors (21). However, in a recent study (6), histopathological changes were found in the central nervous system following infection of Atlantic salmon with the SAV-3 subtype, while no such changes were found for SAV-1 variants. It remains to be decided if this is a general phenomenon of SAV-3 infections, but this observation combined with the similarities in cell responses to infection raises an interesting possibility that SAV-3 is functionally closer to the VEEV variants than the Old World viruses like SINV and Semliki Forest virus.

ACKNOWLEDGMENTS

This study was carried out with financial support from the Norwegian Research Council, project no. 182035, Immunization Strategies against Viral Pathogens of Atlantic Salmon.

We are also indebted to Jo-Ann Leong, School of Ocean, Earth Science Technology, Hawaii Institute of Marine Biology, for providing the antibody to the Mx protein.

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A 6K-Deletion Variant of Salmonid Alphavirus Is Non-Viable but Can Be Rescued through RNA Recombination

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Abstract

Pancreas disease (PD) of Atlantic salmon is an emerging disease caused by Salmonid alphavirus (SAV) which mainly affects salmonid aquaculture in Western Europe. Although genome structure of SAV has been characterized and each individual viral protein has been identified, the role of 6K protein in viral replication and infectivity remains undefined. The 6K protein of alphaviruses is a small and hydrophobic protein which is involved in membrane permeabilization, protein processing and virus budding. Because these common features are shared across many viral species, they have been named viroporins. In the present study, we applied reverse genetics to generate SAV3 6K-deleted (Δ6K) variant and investigate the role of 6K protein. Our findings show that the 6K-deletion variant of salmonid alphavirus is non-viable. Despite viral proteins of Δ6K variant are detected in the cytoplasm by immunostaining, they are not found on the cell surface. Further, analysis of viral proteins produced in Δ6K cDNA clone transfected cells using radioimmunoprecipitation (RIPA) and western blot showed a protein band of larger size than E2 of wild-type SAV3. When Δ6K cDNA was co-transfected with SAV3 helper cDNA encoding the whole structural genes including 6K, the infectivity was rescued. The development of CPE after co-transfection and resolved genome sequence of rescued virus confirmed full-length viral genome being generated through RNA recombination. The discovery of the important role of the 6K protein in virus production provides a new possibility for the development of antiviral intervention which is highly needed to control SAV infection in salmonids.

Citation: Guo T-C, Johansson DX, Haugland Ø, Liljeström P, Evensen Ø (2014) A 6K-Deletion Variant of Salmonid Alphavirus Is Non-Viable but Can Be Rescued through RNA Recombination. PLoS ONE 9(7): e100184. doi:10.1371/journal.pone.0100184

Editor: Pierre Boudinot, INRA, France

Received November 24, 2013; **Accepted** May 23, 2014; **Published** July 10, 2014

Funding: This study was carried out with financial support to ØE from the Norwegian Research Council, projects no. 182035 Immunisation strategies against viral pathogens of Atlantic salmon and no. 183204 Indo-Norwegian platform on fish and shellfish vaccine development, and from the Swedish Research Council to PL. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Salmonid alphavirus (SAV) is the causative agent of pancreas disease (PD) and sleeping disease in Atlantic salmon and rainbow trout, respectively. PD is a major problem in salmonid farming in Western Europe, causing high mortalities in the seawater stage. Diseased fish are clinically characterized by inappetence, fecal casts and emaciation with main pathological changes found in pancreas, heart and skeletal muscle [1]. To date, several subtypes of SAV sharing highly homogeneous genome sequences have been identified. Salmon pancreas disease virus (SPDV or SAV1) was first found in Ireland and Scotland in farmed Atlantic salmon [2]. Subsequently, sleeping disease virus (SDV or SAV2) which mainly affects rainbow trout was discovered in UK and France [3]. The third subtype of SAV (SAV3) is so far exclusively found in Norway affecting both Atlantic salmon and rainbow trout [4]. Additionally, another three discrete subtypes (SAV4–6) have been identified in Scotland and Ireland based on partial sequence (nsP3 and E2) analysis [5], and a marine SAV2-related virus is now also found in PD outbreaks in mid-Norway and Scotland [6]. All subtypes are geographically separated and distinguished based on phylogenetic analysis [7]. Only SAV 1–3 are fully sequenced, with a nucleotide identity of the three SAVs being above 90% over the entire genome.

SAV belongs to the genus alphavirus within the family *Togaviridae* [2]. Members of the alphavirus genus include many medically important pathogens such as Western equine encephalitis viruses (WEEV), Eastern equine encephalitis viruses (EEEV), Venezuelan equine encephalitis viruses (VEEV), Chikungunya virus, Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SINV). The diameter of an alphavirus virion is around 65–70 nm consisting of a tightly adherent lipid envelope surrounding an icosahedral capsid [8,9]. The genome is a positive-sense, single-stranded RNA of approximately 11–12 kb which is capped at the 5′ end and polyadenylated at the 3′ end and thus serves directly as mRNA for translation of the viral replicase upon entry into host cells. The genomic organization of alphavirus is divided into two open reading frames (ORFs) [10,11]. The first ORF encoding four non-structural proteins (nsP), designated nsP1-4, is responsible for transcription and replication of viral RNA. The second ORF, under the control of a 26S subgenomic promoter, codes for another polyprotein (capsid-PE2-6K-E1) making up the structural proteins (SP). PE2 and E1 are the envelope glycoproteins, associated as a heterodimer that migrates together with 6K through the secretory pathway to the plasma membrane. PE2 is cleaved to generate E3 and E2 glycoproteins in a post-Golgi compartment [12].

Alphavirus 6K protein is a small and highly hydrophobic protein. Alphavirus 6K together with several other viral gene

products, such as Poliovirus 2B, Influenza A virus M2, HIV-1 Vpu and HCV p7, are classified as viroporins which share a common feature of enhancing membrane permeability [13–15]. The 6K proteins of SFV or SINV have been shown to be involved in membrane permeabilization at the late stage of infection [16]. Oligomerization of 6K proteins leads to ion channels formation in cell membranes and increases membrane permeability, which facilitates virus budding [15]. An identified aromatic domain (rich in aromatic amino acids) at the N-terminal of 6K shows a strong tendency to insert in the interfacial phase of the phospholipid bilayer which also facilitates membrane destabilization [9]. In addition to the role associated with membrane permeabilization, 6K also provides cleavage sites for polyprotein processing at its N-terminal and C-terminal ends. Both the E2-6K and 6K-E1 cleavage sites are found on the luminal side of ER and cleaved by signal peptidase [17]. A SFV mutant lacking the entire 6K is processed correctly between PE2 and E1 without altering glycoprotein formation, heterodimerization and intracellular transport [18]. However, the budding process of the SFV 6K deletion mutant is impaired and virus titer is reduced [19]. Deletion of 6K in SINV also causes impaired budding and in addition the cleavage of polyprotein becomes less efficient [20]. The sequence of SAV3 6K has been identified but protein function and its role in virus formation have not been studied in any detail. We wanted to explore if deletion of the SAV3 6K gene would attenuate virus infectivity and thus could represent a strategy for development of a live-attenuated viral vaccine against PD. As the cleavage sites of the polyprotein including 6K remain to be proven experimentally for salmonid alphaviruses, these were deduced from amino acid sequence homology with other alphaviruses [21]. We examined the role of the 6K protein by deleting the entire gene using a reverse genetics approach and then studied generation of infectious progeny *in vitro*.

Materials and Methods

Cell culture and virus

Chinook salmon (*Oncorhynchus kituch*) embryonic cells (CHSE-214) were obtained from the American Type Culture Collection (ATCC). Chum salmon (*Oncorhynchus keta*) heart cells (CHH-1) and Bluegill fry cells (BF-2) were obtained from the European Collection of Cell Cultures (ECACC). All cell lines were maintained at 20°C in L-15 media (Invitrogen) supplemented with 5% FBS (Invitrogen), 2 mM L-glutamine with or without 50 µg/mL gentamycin. The SAV3 isolate (SAV3-H10) used in this study was obtained from a clinical outbreak of pancreas disease (PD) in Atlantic salmon from western Norway [22]. The isolate was propagated by inoculating homogenized heart tissue samples onto 80% confluent CHSE-214 cells maintained in growth medium supplemented with 2% FBS at 15°C. The isolate was confirmed as SAV3 by RT-PCR and DNA sequencing.

SAV3-H10 full genome sequencing

Virus was propagated in CHSE-214 cells until passage 11 at which time full genome sequencing was performed. Virus was harvested at nearly full CPE. The supernatant was first cleared by centrifugation at 1500 rpm for 10 minutes before virus sedimentation was performed by ultracentrifugation (Beckman, Optima Ultracentrifuge) in a volume of 1.5 mL with rotor TLA-45 at 65,000×g for 1.5 hours. After ultracentrifugation, most of the supernatant was carefully removed and discarded and the remaining 150 µl containing the virus pellet was subjected to viral RNA isolation using the QIAamp Viral RNA kit according to the manufacturer's instructions (Qiagen). cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with the mixture of random hexamers (1 µl) and oligo dT primers (1 µl). PCR was performed using Phusion High-Fidelity DNA Polymerase (Finnzymes) with primers (Table 1) based on a previously published sequence (GenBank no. AY604238). The rapid amplification of cDNA ends (RACE) technique was performed to obtain the full-length sequence including the exact 5′ and 3′ ends. The procedure was performed according to the manufacturer's instructions (BD SMART RACE cDNA Amplification Kit, Clontech). 5′ RACE-GSP and 3′ RACE-GSP primers were designed based on sequence information obtained in the first round (Table 1). RACE products were purified and subcloned into pGEM-T Easy vector, and for both 5′- and 3′-RACE, 10 colonies were picked up for sequencing (Eurofins MWG).

Construction of a full-length SAV3 cDNA clone

Viral RNA isolation and cDNA synthesis were performed as described above. The infectious clone was constructed by combining two clones containing roughly half of the genomic cDNA (Figure 1). The first fragment (6374 bp) was amplified with primers P1 and P2 flanked with *EcoR* I and *Afl* Π/*Not* I restriction sites respectively (Table 1). The second fragment (5527 bp) was amplified with primers P3 and P4 flanked with *EcoR* I/*Afl* Π and *Not* I sites respectively. PCR reactions contained 28.5 µl H_2O, 10 µl 5X Phusion HF Buffer, 3 µl 10 mM dNTPs, 6 µl 0.5 µM forward plus reverse primers, 2 µl viral cDNA and 0.5 µl Phusion High-Fidelity DNA Polymerase (Finnzymes). PCR was performed using the following conditions: 98°C 30 s, 35 cycles of 98°C 10 s, 60°C 30 s, 72°C 4 min, and finally 72°C 5 min. The two fragments constituting the entire viral genome were cloned separately into the pBluescript vector (Stratagene) at *EcoR* I and *Not* I sites following standard cloning procedures. pBluescript vectors containing the 6.5 kb and 5.5 kb fragments were subsequently digested with *Afl* Π and *Not* I and purified, before the full-length SAV3 cDNA clone without poly(A) was constructed by combining the two fragments at *Afl* Π/*Not* I site (Figure 1). A poly(A) tail was added by PCR at the 3′ end of the cDNA clone using primer P5 containing the poly(A) tail and flanked by *Bmt*I and *Not*I sites in combination with primer P3 (Table1). The PCR product was gel purified and combined with the pBluescript vector containing the 6.5 kb fragment at the *Afl* Π/*Not* I sites to yield the full-length SAV3 cDNA clone with poly(A). The resulting infectious cDNA clone was finally transferred from the pBluescript backbone and inserted into the pTurboFP635-N vector (Evrogen) at the *EcoR*I and *Not*I sites, and designated as pSAV3-FL.

Modification of the 5′ end, deletion of the 6K gene and generation of helper cDNA vector

To ensure precise cleavage at the 5′ end during transcription, a hammerhead (HH) ribozyme sequence [23] was inserted immediately upstream of the 5′ UTR region of the full-length cDNA construct. Furthermore, a T7 promoter was fused upstream to the HH sequence to obtain the capability of *in vitro* transcription. This was achieved by long-range PCR using the Phusion system as described above, with primers T7-HH-F and CMV-R (Table1) and *Not*I linearized pSAV3-FL DNA as template. The obtained PCR product was purified and treated with a T4 polynucleotide kinase (Promega) and subsequently self-ligated with T4 ligase (Promega). The full-length SAV3 cDNA construct containing the T7 promoter and HH sequences was named pSAV3-HHFL. Deletion of the 6K gene was obtained by amplifying pSAV3-HHFL using PCR with appropriate outward-facing primers (Δ6K-F and Δ6K-R), creating a PCR product of the entire plasmid but lacking the gene of interest (Table 1). The PCR product was

Table 1. Primers used for genome sequencing and vectors construction.

Used for	*Name*	*Primer sequence (5′→3′)*	*Orientation*	**Restriction site**
SAV3-H10 genome sequencing except 5′ and 3′ end	F1	TCACTGTAGATTTGCCCGCGG	For	
	R1	CCAACAGGTGTTACGCTTCCCGT	Rev	
	F2	ATAATGAGCTCATGACTGCGGCTGC	For	
	R2	GCTTCAGCACTGTGACCCGTTCC	Rev	
	F3	ACGGAGACGCTGTCCAGTTTCG	For	
	R3	TACACGGGGAAGGTGCTCTGTC	Rev	
	F4	AAGTGGAAAGCTGGTACAGAGTGGG	For	
	R4	GCACTTCTTCACCACGCAGTAGGTAA	Rev	
5′ RACE primer	5′ RACE-GSP	TGGAACACGAACGGCTCGAACCCGATCC	Rev	
3′ RACE primer	3′ RACE-GSP	CGCTTGGTGAAGTGGTGACGGCAGTCC	For	
Full-length SAV3-H10 cDNA clone construction	P1	GCTTGATATCGAATTCGATAAATCCAAAAGCATA	For	*EcoRI*
	P2	ACCGCGGTGGCGGCCGCAACGAGCTTAAGGTGGGG	Rev	*NotI/AflII*
	P3	CATGAATTCTAACTACCCCACCTTAAGCTCGTTCGGAGT	For	*EcoRI/AflII*
	P4	TTGCGGCCGCCTTATATTGAAAATTTTAAAACCAATAGATGACTCA	Rev	*NotI*
	P5	AAAAGCGGCCGCGCTAGC$(T)_{60}$CTTATATTGAAAATTTTAAAACCA	Rev	*BmtI/NotI*
Modification of full-length cDNA at 5′ end and construction of Δ6k and helper cDNA clones	T7-HH-F	TAATACGACTCACTATAGGGTGGATTTATCCTGATGAGT CCGTGAGGACGAAACTATAGGAAAGGAATTCCTATAGT CGATAAATCCAAAAGCATACA	For	
	CMV-R	GATCTGACGGTTCACTAAACC	Rev	
	Δ6k-F	TACGAACACACCGTGGTGGTCCCAATGGA	For	
	Δ6k-R	CGCACGAGCCCCAGGTATGCAGCACAATG	Rev	
	Helper-F	TGTAAACCATCTGCCGTTAGCCAC	For	
	Helper-R	TGATATATGTATGCTTTTGGATTTATCGA	Rev	
Construction of SFV-REP/SAV3-SP	CS-F	AAAACCCGGGATGTTTCCCATGCAATTCAC	For	*XmaI*
	E1-R	AAAAACTAGTTTAGCTCTTGACTATCCGGATTC	Rev	*SpeI*

doi:10.1371/journal.pone.0100184.t001

turned into a plasmid as described above and designated pSAV3-HHΔ6K. A SAV3 helper cDNA vector was constructed by deleting the replicon (nsP1, nsP2, nsP3, and nsP4) from the full-length cDNA clone except for 100 nt at carboxy-terminal of nsP4 that was preserved. This was achieved by PCR using the primers Helper-F and Helper-R (Table 1). The sequences of all final constructs were confirmed by DNA sequencing (Eurofins MWG).

Generation of polyclonal antibodies against SAV3 structural proteins (mouse anti-SAV3-SP)

The DNA vector used for immunization (pSFV-REP-SAV-SP) was constructed by inserting the structural protein genes of SAV3-H10 (SAV3-SP) amplified by PCR using the primers CS-F and E1-R (Table 1) into the Semliki Forest virus (SFV) replicon system [24]. Five Balb/C mice were immunized with pSFV-REP-SAV-SP (10 μg DNA/20 μl PBS per mice) via intradermal injection near the base of the tail followed by intradermal electroporation (Cyto Pulse Sciences, Inc., Glen Burnie, MD) as previously described [25]. DNA immunization was repeated using the same DNA vector at 3 and 10 weeks after the first injection. Additionally a booster immunization with recombinant SAV3-E2 protein (10 μg/50 ul PBS) expressed in E. coli [22] and mixed with 50 μl Freund's incomplete adjuvant (Sigma F5506) were given intramuscularly divided equally between the hind legs at week 6. Sera from the five immunized mice and two non-immunized controls were then harvested at week 13. After confirming specific staining in SAV3 infected cells by immunofluorescence (IF) assays, sera from all mice were pooled and stored at −20°C. All mice were bred and housed at the animal facility of the Department of Microbiology, Tumor and Cell Biology at the Karolinska Institutet, Stockholm, Sweden. Mice were anaesthetized with 4% isoflurane (Baxter Medical AB, Kista, Sweden) during all intradermal injections and electroporations. All mice experiments were approved by the Committee for Animal Ethics in Stockholm, Sweden, and performed according to given guidelines. Another antibody used in this study, rabbit anti-SAV3-E2, was generated previously [22].

Determination of anti-SAV3-SP IgG levels in immunized mice

ELISA plates (Nunc-immuno plate MaxiSorp F96) were coated with recombinant SAV3-E2 protein diluted in PBS. 50 μl of E2 protein (10 μg/mL) was added into each well and the plates were incubated at 4°C overnight. The plates were washed three times

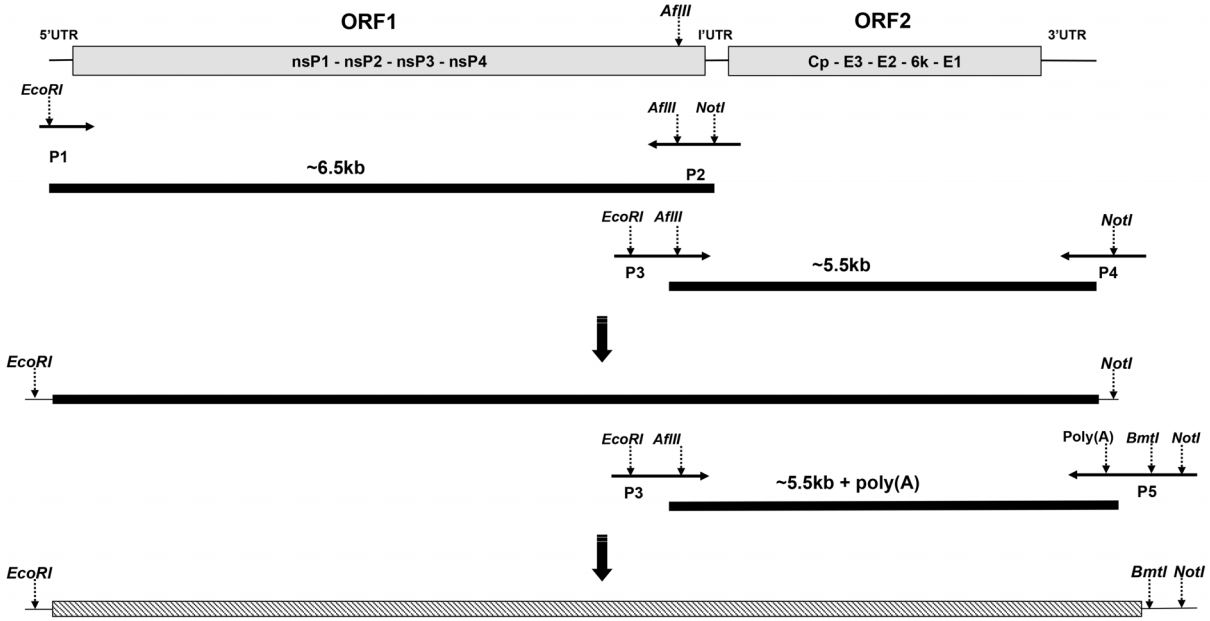

Figure 1. Schematic illustration of the full-length SAV3 cDNAs constructed. AflII is a unique restriction site residing within SAV3 nsp4 gene. The first 6.5 kb fragment and the second 5.5 kb fragment was amplified by primers P1/P2 and P3/P4, respectively. The purified PCR products were subcloned into the pBluescript vector with *EcoRI* and *NotI* sites. The 5.5 kb fragment was thereafter subcloned into the pBluscript vector containing the 6.5 kb fragment vector at *AflII* and *NotI* sites, to make the full-length SAV3 cDNA construct without poly(A). Primer P5 containing poly(A) was used in combination with primer P3 to introduce poly(A). The final insert constituting full-length SAV3 cDNA including poly(A) was finally subcloned into pTurboFP635-N at *EcoRI* and *NotI* sites. Fragments were inserted in pBluescript vector (solid, black line) and in pTurboFP635-N (hatched line).
doi:10.1371/journal.pone.0100184.g001

with 250 µl of phosphate-buffered saline with 0.1% Tween20 (PBST) and then blocked with 5% non-fat dry milk diluted in PBST for 2 hours at room temperature. Washing was performed as described above. Two-fold serial dilutions of sera in PBST (1:20 to 1:40960) from individual mouse (1–5) were then added to the wells and incubated for 1 hour at room temperature. The wells were subsequently washed and incubated for another 1 hour at room temperature with 100 µl of a 1:1000 dilution of peroxidase-conjugated goat anti-mouse IgG (Dako), followed by color development with 1,2-phenylenediamine dihydrochloride (OPD) tablets (Dako). 100 µl OPD solution was added to each well and plates were incubated for 30 min at room temperature before enzyme reactions were stopped by addition of 0.5 M H_2SO_4. Optical density (OD) was read as absorbance at 490 nm by an ELISA plate reader.

In situ detection of SAV3 by immunohistochemistry

Sections were made from paraffin embedded tissue of pancreas from SAV3 infected fish and blocked with 5% bovine serum albumin (BSA) for 20 min before incubating with mouse anti-SAV3-SP antibody at a dilution of 1:1000 for 30 min at room temperature. This was followed by the secondary antibody (biotinylated anti-mouse immunoglobulin diluted 1:300) for 30 min at room temperature. After incubation with Streptavidin–biotin alkaline phosphatase (1:500 dilution) for 30 min, sections were stained with Fast Red substrate (Sigma) and counterstained with hematoxylin.

Immunofluorescence (IF) assay

Detection of SAV3 viral proteins in cultured cells was performed by IF assay. Virus infected cells and cells transfected with SAV3 cDNA clones were fixed in 4% formaldehyde for 15 min at room temperature followed by permeabilization with 0.1% Triton-X100 for 5 min on ice. Cells were then blocked with 5% non-fat dry milk (Bio-Rad) for 30 min before incubated sequentially with the primary and secondary antibodies. Cells were incubated at room temperature for one hour with primary antibody (mouse anti-SAV3-SP) diluted 1:1000 in 5% BSA. After washing four times with PBS, secondary antibody diluted 1:400 in 5% BSA (Alexa Fluor 488- goat anti-mouse IgG, Invitrogen) was incubated for another 30 min at room temperature. Cells were again washed four times with PBS and counterstained with Hoechst 33324 (5 µg/ml) for 2–5 minutes at room temperature before washing with PBS and analyzed using a fluorescence microscope (Olympus, IX81).

Transfection of cells and recovery of recombinant virus in three susceptible cell lines

CHSE-214, CHH-1 and BF-2 cells grown to 80% confluence in 24-well plates were transfected using the Fugene HD transfection reagent (Roche) following the manufacturer's protocol. Briefly, 2 µg of each SAV3 infectious cDNA clone was diluted in 100 µl Opti-MEM I reduced serum medium (Invitrogen) before 6 µl of transfection reagent was added. The transfection mix was incubated at room temperature for 15 min before 25 µl was added into each well. Transfected cells were incubated at 15°C and harvested at 2, 4, 7, 10 and 13 days post infection. The collected virus supernatant was subjected to virus titration by $TCID_{50}$ in CHSE-214 cells using the Spearman–Kärber method [26].

Real-time PCR

Real-time PCR was performed using the LightCycler LC480 instrument and LightCycler 480 SYBR Green I Master mix (Roche) in a final volume of 20 µl. The PCR reactions were first incubated at 95°C for 10 min, followed by 40 amplification cycles

(10 s at 95°C, 20 s at 60°C and 8 s at 72°C). Final concentration of primers in the reaction mix was 0.5 μM and sequence information of primers used to assess *in vitro* expression of IFNα, Mx, and ISG15 were as previously described [22]. The specificity of the PCR product from each primer pair was confirmed by melting curve analysis and subsequent agarose gel electrophoresis. The $2^{-\Delta\Delta}CT$ method was used to calculate expression levels as described elsewhere [27]. $2^{-\Delta\Delta}CT$ is the relative mRNA expression representing fold induction over the control group. All quantifications were normalized to β-actin (endogenous gene). Statistical analyses and graphs were performed with the help of GraphPad Prism 5.0 (GraphPad software Inc., USA).

Total protein analysis and radioimmunoprecipitation assay (RIPA)

CHH-1 cells were seeded in the 6-well plate one day prior to infection with wt SAV3 or transfection with either pSAV3-HHFL or pSAV3-HHΔ6K cDNA clones. Virus infected cells were incubated at 15°C for 48 hours while transfected cells were incubated for 96 hours. Cells were then starved in methionine-free MEM medium (Mpbio) for 30 minutes followed by radiolabeling with 50 μCi [^{35}S] methionine/ml (Montebello Diagnostics) in starvation medium for 6 hours prior to harvest. For total proteins analysis, cells were lysed using CelLytic^{TM} M solution (Sigma) and clarified by centrifugation as described in the manufacturer's manual. 15 μg of total proteins was loaded per lane (wt SAV3 infected cells, pSAV3-HHFL transfected cells, pSAV3-HHΔ6K transfected cells, and non-treated control cells), separated by SDS-PAGE and blotted onto a PVDF membrane (Bio-Rad). The membrane was exposed in a phosphorImager cassette for at least 16 hours before image was scanned using the Typhoon imager (GE healthcare). For the analysis of membrane proteins, cells were lysed with phase separation lysis buffer and separated into membrane-bound detergent phase and soluble phase following a protocol described previously [28]. Proteins in the detergent phase were diluted 50-fold in PBS containing 1% Triton X-100 and a protease inhibitor cocktail (Sigma) before being added to pre-incubated complex of IP matrix (Santa Cruz) and mouse anti-SAV3-SP antibodies. The protein-antibody-IP mixture was incubated overnight at 4°C on a rotator. The reaction mixes were then carefully washed three times with PBS, resuspended in protein buffer and boiled for 5 minutes. Protein samples were then analyzed on SDS-PAGE, blotted and scanned as described above.

Results

Construction of an infectious SAV3 cDNA clone

The isolate (SAV3-H10) used in this study has previously not been fully characterized. With the purpose to obtain a functional infectious cDNA clone, the first step was to determine the full genome sequence including the 5′ and 3′ UTR regions by RACE. Genome sequencing except for the 5′ and 3′ end was performed with primers based on known sequences from other SAV3 strains deposited in GenBank. To ensure functionality of the recombinant virus, the exact sequences of the viral ends were revealed by 5′ and 3′ RACE. To obtain the full-length SAV3 cDNA construct, two fragments with the sizes of 6374 bp and 5527 bp was combined, using the internal restriction site *AflII* and external restriction sites *EcoR I* and *Not I* for ligation (Figure 1). At this stage the full-length SAV3 cDNA excluding 3′ poly(A), 11877 bp in length, was inserted into the pBluescript vector. The addition of a poly(A) tail sequence at the 3′ end was made by PCR and the resulting full-length SAV3 cDNA containing poly(A) was subcloned into the pTurboFP635-N vector (Figure 2A). Two different cDNA clones were fully sequenced and the sequencing result showed nucleotide sequences identical to the parental virus sequences. This suggests an efficient and predictable method for construction of full-length SAV cDNA clones has been established. In order to ensure precise cleavage at the 5′ end during transcription, a HH ribozyme sequence was inserted immediately upstream of the 5′ UTR as described by others [23]. The construct was further engineered by fusing a T7 promoter in front of the HH sequence. Finally, the non-viral sequences between the CMV and T7 promoter were removed, generating the final infectious cDNA clone (Figure 2B) for rescue of recombinant virus.

Production and characterization of polyclonal antibodies against SAV3 structural proteins

To evaluate the functionality of the constructed full-length cDNA clone and modified constructs, specific anti-SAV3 antibodies are needed. The SFV replicon system has proven to be a useful vector for expression of heterologous genes and the vector design for enhanced immunogenicity in mice [24,29] was employed to generate anti-SAV3 antibody. ELISA results confirmed high levels of antibodies generated from immunized mice with minimal background, while non-immunized mice were negative (Figure 3A). The anti-SAV3 mice serum also allowed specific detection of SAV3 in infected pancreatic tissue by immunohistochemistry (Figure 3B). When mouse anti-SAV3-SP serum was incubated with proteins from wt SAV3 infected CHH-1 cells blotted on a PVDF membrane, four bands were clearly revealed. No specific bands were detected in uninfected control cell samples (Figure 3C). Two bands at around 50 kDa and 57 kDa were most likely E2 and pE2 (E2+E3), respectively, although we cannot rule out that one of them might as well be E1. Additionally, two larger bands with the size of around 100 kDa and 110 kDa were possibly corresponding to uncleaved polyproteins. Further analysis revealed that the antibody provided strong and highly specific detection of SAV3 in cell culture by immunostaining, making it possible to evaluate the expression kinetics of SAV3 viral proteins during the course of viral infection. As shown in Figure 3D–I, following inoculation with wt SAV3, no viral protein was detected at 16 and 24 hours post inoculation (hpi), while some cells expressed viral proteins at 40 hpi. At 48 hpi, an increasing number of positive staining cells were detected concomitant with a slight change in morphology. CPE consisting of cell shrinkage was observed from 72 hpi, progressing into more severe CPE being observed at 96 hpi.

rSAV3 is predominantly recovered in CHH-1 which have a feeble IFN response

In order to rescue infectious SAV3 virus by reverse genetics in a virus-favored environment, we first sought for an optimal cell line for virus replication. The full-length cDNA clone was transfected into three susceptible cell lines, CHSE-214, BF-2, and CHH-1. Extensive CPE was observed in CHH-1 cells appearing at 5 days post transfection (dpt). Comparable CPE was observed at 10 dpt in CHSE-214 and BF-2 cells (data not shown), suggesting that these cell lines were more resistant to SAV3 infection. In line with these results, infectious virus was rescued as early as 2 dpt in CHH-1 cells and increased to a viral titer of 4.7×10^7 $TCID_{50}$/ml at 7 dpt and 6.8×10^7 $TCID_{50}$/ml at 13 dpt (Figure 4A). The rescue of infectious virus was significantly delayed in CHSE-214 and BF-2 cells (rescued from 4 dpt) and the resulting peak titers were 1–1.5 log_{10} lower than what was obtained in CHH-1 cells (Figure 4A). In a previous study we have demonstrated the effect of IFN-α on limiting SAV3 viral replication [22] and this prompted us to

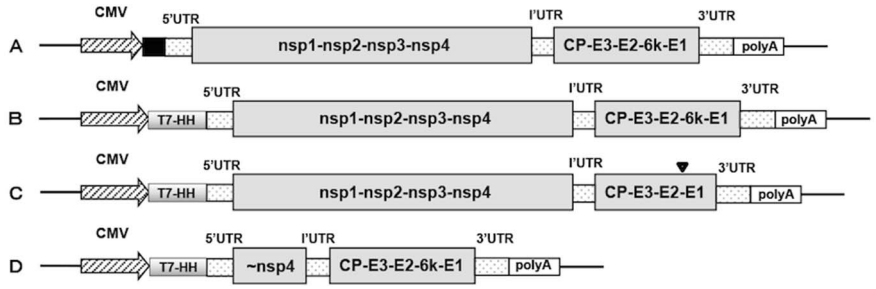

Figure 2. Organization map of full-length SAV3 and deletion variants constructed. (A) Full-length SAV3 cDNA cloned in pTurboFP635-N vector without modifications. (B) Full-length SAV3 cDNA with modification at the 5′ end. Non-viral sequences (▮) were removed and a T7 promoter was inserted right after the CMV promoter. In addition, a hammerhead ribozyme (HH) sequence was added in front of 5′ UTR in order to generate the accurate 5′ end. (C) SAV3 Δ6k mutant: the entire 6k gene was deleted (▼) from the full-length construct (B) by PCR. (D) Organization of the helper cDNA vector containing sequences starting about 100 bases upstream of the nsP4 C-terminal throughout the structural proteins including poly(A).
doi:10.1371/journal.pone.0100184.g002

associate the discrepancy of CPE and viral replication with capabilities of IFN induction in different cell lines. We therefore measured innate immune responses in these three cell lines post SAV3 infection. The induction of IFNα and two interferon-stimulated genes (ISGs), Mx and ISG15, was quantified at RNA expression level by real-time PCR. Indeed, the result showed that responses in both IFNα and the two ISGs were low in CHH-1 while significantly stronger responses were detected in CHSE-214 and BF-2 cells (Figure 4B). At 1 and 2 dpi, the induction of IFNα and ISGs was in general low in all examined cell lines. At 4 dpi, markedly high levels of IFNα and ISGs were induced in CHSE-214 and BF-2 cells (Figure 4B). IFNα was upregulated ~120-fold in CHSE-214 and ~150-fold in BF-2 cells. In contrast, upregulation of IFNα was only 5-fold in CHH-1 cells. For the ISGs, Mx was upregulated ~400-fold in CHSE-214, ~1450-fold in BF-2, and 90-fold in CHH-1 cells, while ISG15 was upregulated ~135-fold in CHSE-214, 215-fold in BF-2 cells, and 23-fold in CHH-1 cells. The same pattern was obtained for Mx protein expression in CHSE-214 and CHH-1 cells by western blot (Figure 4C), confirming differential induction of IFN responses in different SAV3 susceptible cell lines. From the results above, we conclude that CHH-1 is the optimal cell line for rescue of

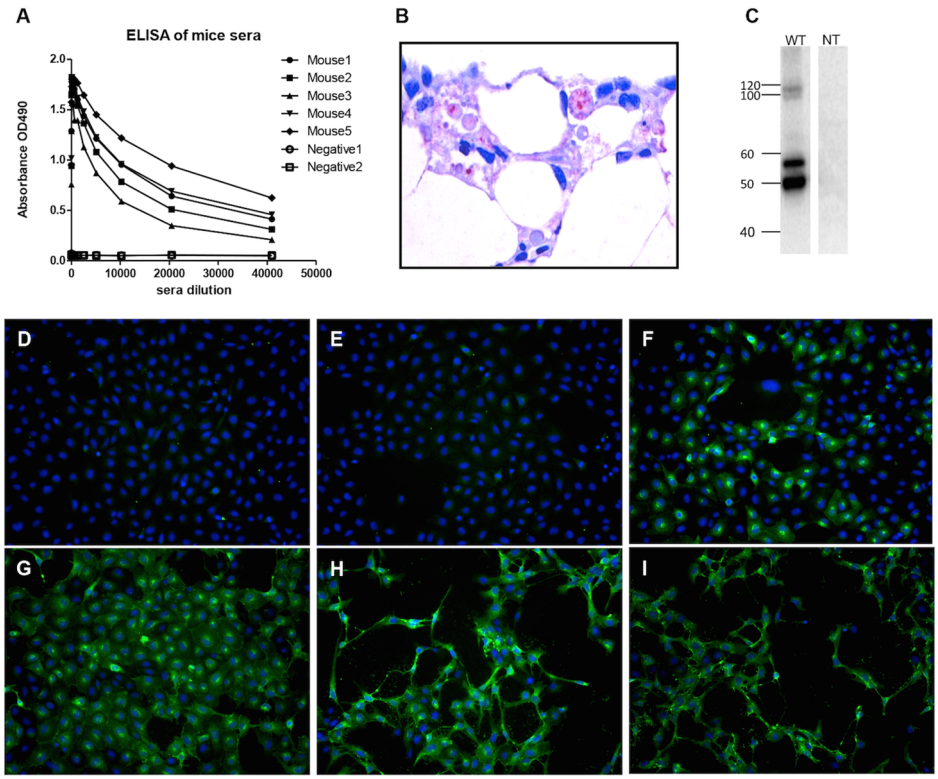

Figure 3. Characterization of the mouse anti-SAV3-SP antibody against structural proteins (SP). (A) All mice (no. 1–5) immunized with pSFV-Rep-SAV3-SP yielded high titer antibodies by ELISA. (B) Detection of SAV3 by immunohistochemistry in necrotic, exocrine pancreas cells of Atlantic salmon infected with wild-type SAV3. (C) Detection of SAV3 viral proteins by western blot. WT: wild-type SAV3. NT: Non-treated cells used as negative control. (D–I) Dynamics of SAV3 viral protein expression by immunostaining in CHSE-214 cells at different time points post infection using wild-type SAV3 (MOI = 1). (D) 16 hpt (E) 24 hpi (F) 40 hpi (G) 48 hpi (H) 72 hpi (I) 96 hpi.
doi:10.1371/journal.pone.0100184.g003

infectious SAV3 virus from full-length cDNA clones, and thus the following studies were performed in CHH-1 cells.

SAV3 6K contains a unique insertion not found in other alphaviruses

The 6K protein of SAV3 or other salmonid alphaviruses has not been characterized in any detail. In the present study, we first aligned the SAV3 6K sequence with sequences from other alphaviruses (Figure 5A) and performed hydrophobicity scale characterization (Figure 5B). Results showed that SAV3 6K shares 38–51% similarity to other alphaviruses, while being highly conserved when compared to SAV1 (94.1%) and SAV2 (95.1%). Although sequence similarities were found, differences between SAV and other alphaviruses were detected. First, the 6K protein of SAV3 possesses 68 amino acids, being slightly larger than 6K proteins of other alphaviruses, manifested with a unique insertion of 7 amino acids (GVRGWSA) located in the transmembrane domain 2 (TMD 2) (Figure 5A). Second, two hydrophobic residues in the aromatic domain of 6K, Trp-11 and Trp-19, are fully conserved among all alphaviruses (Figure 5A). The first corresponding residue in SAV3 6K, Trp-12, is conserved with other alphaviruses. However, the second corresponding hydrophobic residue in SAV3 6K is Gly-20. Hydrophobicity analysis of the 6K protein using the Kyte-Doolittle method, showed two potential transmembrane domains (TMD) in SAV3, as seen in other alphaviruses (Figure 5B). These two putative TMDs were further analyzed by TMpred program [30]. The first TMD starts from Val-17 to Leu-37, with a predicted outside to inside orientation and the second TMD is from Phe-47 to Cys-66, with the orientation inside to outside membranes (Figure 5C). A cysteine rich domain between the two TMDs was found in SAV3 and conserved with other alphaviruses, possibly forming a cytosolic loop connecting the two transmembrane domains of 6K. Aligning 6K among SAV subtypes 1–3 showed that only five amino acids were not conserved between them (Figure 5C), while the aromatic domain and the unique insertion (GVRGWSA), were conserved among three SAV subtypes.

SAV3 lacking the entire 6K is deficient in producing infectious particles

We then went on to investigate the role of 6K for production of viable progeny by constructing recombinant SAV3 lacking the entire 6K gene. Despite the fact that multiple attempts with the Δ6K cDNA clone (pSAV3-HH Δ6K) were made to recover virus both from transfected CHH-1 and CHSE-214 cells, CPE was not observed and no virus was recovered. We next examined whether viral proteins were expressed in transfected cells by immunostaining (Figure 6). CHH-1 cells were transfected with either full-length SAV3 (pSAV3-HHFL) or SAV3 lacking 6K (pSAV3-HHΔ6K) cDNA clones and expression of viral proteins was examined at 2, 3, 4, 6, 8, 10 dpt. Due to complete CPE present at day 8 following transfection with full-length SAV, these cells were examined for 6 days. At 2 dpt, viral protein expression was observed both in full-length (Figure 6A) and Δ6K cDNA transfected cells (Figure 6E). At

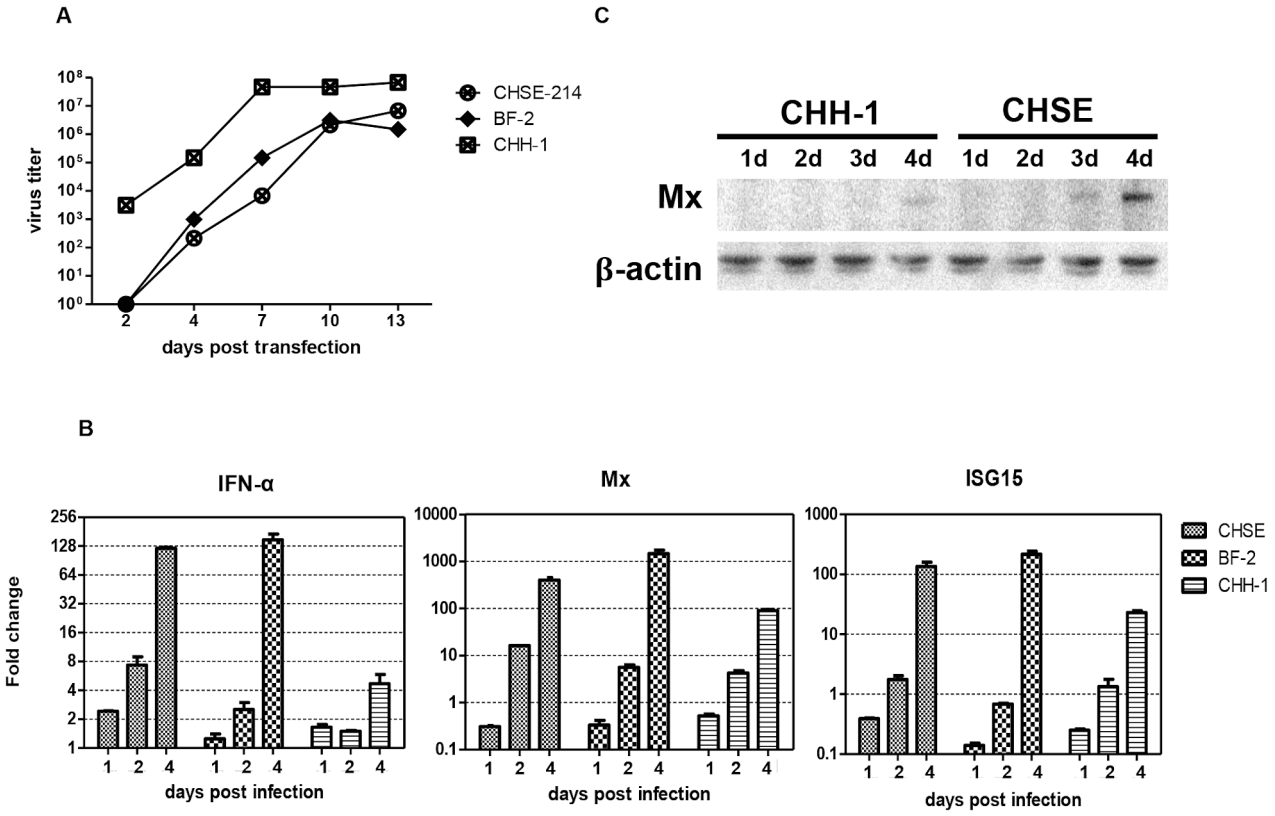

Figure 4. Recovery of rSAV3 in three susceptible cell lines and detection of IFN and ISGs response in each cell line. (A) The constructed infectious SAV3 cDNA clone was transfected into CHH-1, CHSE-214, and BF-2 cell lines. Supernatant from each cell line was collected at indicated time points and subjected to virus titration by $TCID_{50}$. (B) Elicited IFN and ISG mRNA expression (Mx and ISG15) following SAV3 infection (MOI = 1) was evaluated by real-time PCR. Each time-point represents three biological replicates. (C) Expression of the Mx protein was detected in CHH-1 and CHSE-214 cell lines by western blot at 1 to 4 days post infection with wtSAV3 (MOI = 1). β-actin expression was used as internal control.
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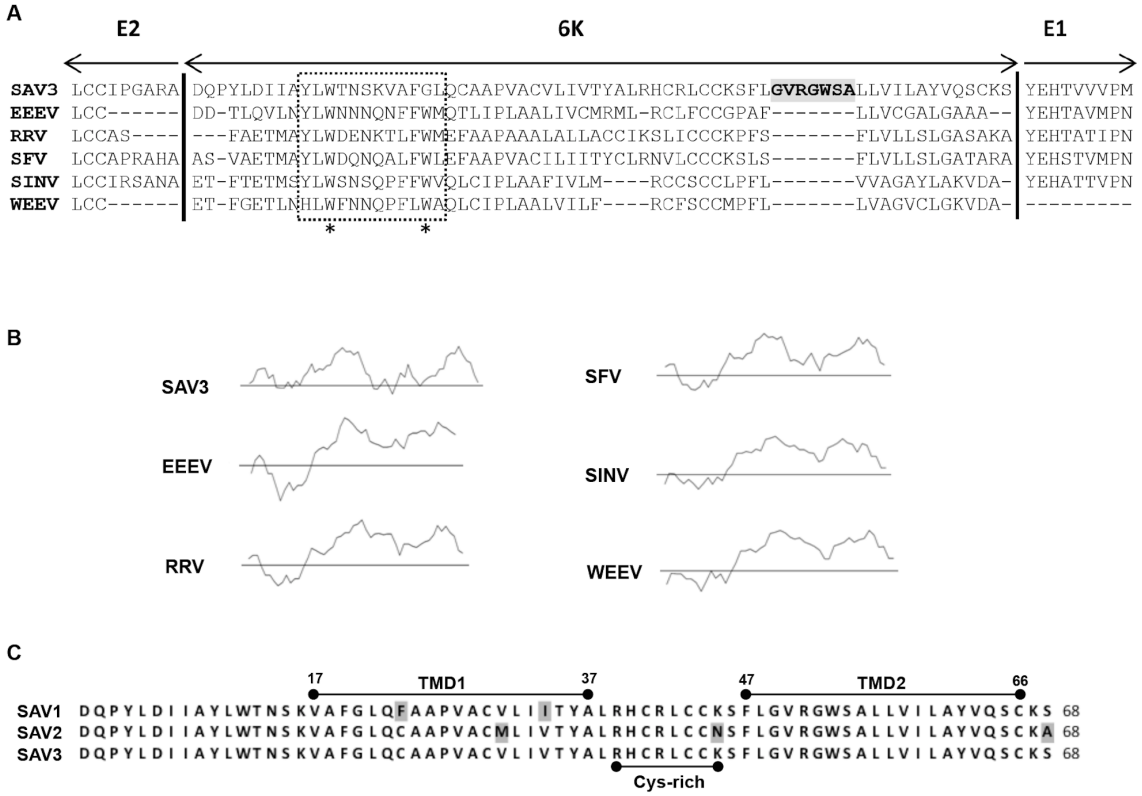

Figure 5. Analysis of SAV3 6K protein. (A) The SAV3 6K protein sequence aligned with other alphaviruses. The C-terminal end of E2 and N-terminal end of E1 were included to show the conservation around the (E2-6K) and (6K-E1) signal peptidase cleavage sites. The aromatic domain is shown inside the rectangular square. The unique seven amino-acid domain in SAV, GVRGWSA, is highlighted in grey. EEEV: Eastern Equine Encephalitis; RRV: Ross River; SFV: Semliki Forest; SINV: Sindbis; WEEV: Western Equine Encephalitis. (B) Hydrophobicity scale analysis of 6K amino acids by Kyte-Doolittle plots. The central line is the hydrophobicity scale at zero. Above zero are hydrophobic regions. (C) SAV1–3 6K protein sequences were aligned and two transmembrane domains were predicted. Between two TMDs, there is a cysteine rich domain.
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3 dpt, a distinct difference of viral protein expression was seen between full-length SAV3 (Figure 6B) and Δ6K mutant (Figure 6F). The number of positive cells was higher for CHH-1 cells transfected with full-length SAV, and the staining of virus-positive cells was shown in a multi-focused pattern indicating spread of virus from primary infected cells to neighboring cells. At 4 dpt, a large number of virus-positive cells were seen for pSAV3-HHFL transfected culture and correspondingly, changes in cell morphology started to appear (Figure 6C). By day 6 the virus infection had spread to all cells and evident CPE was present (Figure 6D). In contrast, the number of virus positive cells increased slightly in CHH-1 transfected with pSAV3-HHΔ6K cDNA up to 4 dpt, waning at 8 and 10 dpt (Figure 6G–J). Correspondingly, no CPE was observed (Figure 6E–J) and the cell cultures grew to higher density at late time points (Figure 6E versus 6J). These results show that viral proteins were expressed in pSAV3-HHΔ6K cDNA transfected cells but infectious virus was not recovered spreading from the primary transfected cells.

Structural viral proteins expressed in pSAV3-HHΔ6K transfected cells were retained in the cytoplasm and could not be detected on the cell membrane

To explain the results above, we assumed that the spread of virus was impaired due to a budding defect of virus lacking 6K, which has been shown for other alphaviruses [19]. If this was the case, viral proteins should be detected on the plasma membrane of pSAV3-HHΔ6K transfected cells. To test this, transfected cells were immunostained under both permeabilized and non-permeabilized conditions. In pSAV3-HHFL transfected cells, viral proteins were clearly observed both in the cytoplasm (Figure 7A) and on the plasma membrane (Figure 7B), indicating that viral proteins were transported to the cell surface. Although viral proteins were detected in the cytoplasm of cells transfected with pSAV3-HHΔ6K (Figure 7C), we were not able to detect any viral proteins on the cell surface (Figure 7D). The finding suggests that the structural viral proteins in pSAV3-HHΔ6K transfected cells were retained in the cytoplasm and were not transported to the cell membrane.

SAV3 lacking the entire 6K yielded an E2 protein of larger size

Since alphaviruses produce polyproteins which are subsequently cleaved into separate proteins, we wanted to know whether viral proteins were properly processed in pSAV3-HHΔ6K transfected cells. To study viral protein synthesis, we metabolically labeled the newly synthesized proteins with S^{35} methionine. Characterization of total proteins by SDS-PAGE and autoradiography showed a partial shutdown of protein translation in both wt SAV3 infected (85% shutdown) and pSAV3-HHFL cDNA transfected cells (70% shutdown). In contrast only a moderate shutdown (33%) was seen in pSAV3-HHΔ6K cDNA transfected cells (Figure 8A). Newly synthesized SAV viral proteins, one being the putative capsid protein with the size of around 37 kDa was also visualized by autoradiography, which is in agreement with the size of the capsid

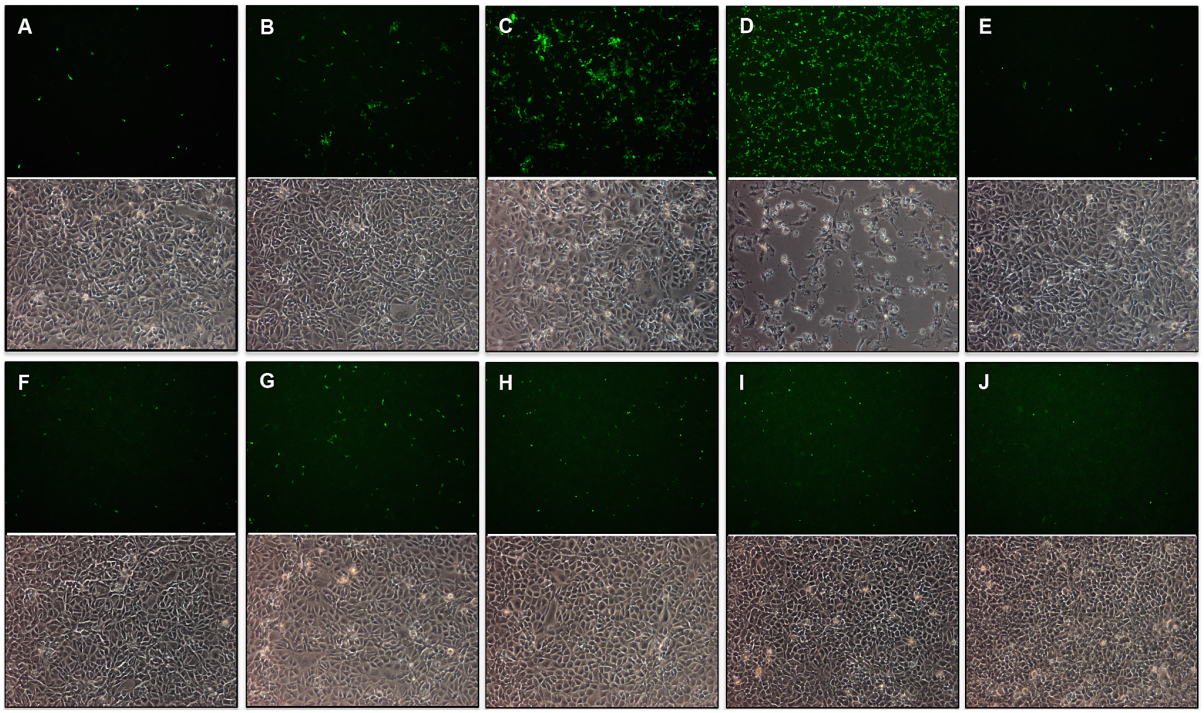

Figure 6. Immunostaining showing expression of viral proteins in CHH-1 cells. Cells transfected with pSAV3-HHFL clone were stained at 2, 3, 4, 6 dpt. (A–D), while cells transfected with the pSAV3-HHΔ6K construct were stained at 2, 3, 4, 6, 8, 10 dpt (E–J). Cell morphology at each corresponding time point is shown below the fluorescence photo.
doi:10.1371/journal.pone.0100184.g006

protein reported earlier by Moriette et. al [31]. However, in contrast to other alphaviruses, the fact that SAV does not induce total shutdown of host protein translation makes it difficult to identify each single viral protein or polyprotein. In order to enrich viral proteins over host proteins, S^{35} labeled total proteins were separated into an aqueous and a detergent phase and radio-immunoprecipitated with mouse anti-SAV3-SP antibody against structural proteins. The separated detergent phase was applied to RIPA as it is assumed that most SAV3 viral structural proteins will be located either on ER/Golgi or plasma membranes. The results showed virus-specific bands (Figure 8B), similar to the viral proteins detected by western blot using mouse anti-SAV3-SP antibody (Figure 3C). For the wt SAV3 and full-length construct a faint capsid protein band was still present in the membrane fraction, very likely representing preassembled virus particles found on membranes [32]. As mentioned above, proteins corresponding to approximately 50 kDa and 57 kDa were possibly E2, E1 and pE2 (E2+E3). It is noticeable that the viral protein patterns for wt SAV3 and rSAV3 were identical (Figure 8B). The protein pattern in pSAV3-HHΔ6K cDNA transfected cells was however different. One strong band with a molecular mass slightly larger than 60 kDa was found only in pSAV3-HHΔ6K transfected cells and not present in wt SAV3 infected and pSAV3-HHFL cDNA transfected cells (Figure 8B). Western blot analysis using the rabbit anti-SAV3-E2 specific antibody [22] confirmed that this protein was indeed virus specific (Figure 8C), likely corresponding to a precursor of the E2 protein, but cannot be the non-cleaved E2–E1 as the size was too small.

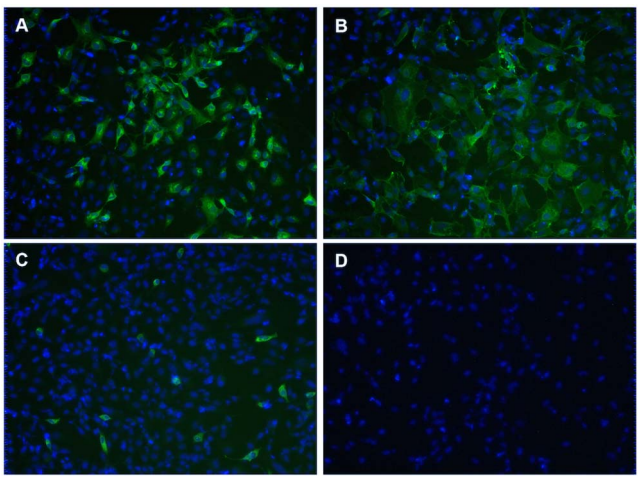

Figure 7. Examination of viral protein trafficking to the plasma membrane. CHH-1 cells were transfected with pSAV3-HHFL cDNA (A and B) or pSAV3-HHΔ6k cDNA (C and D). At 4 dpt, cells were immunostained under permeablized (A and C) or non-permeablized (B and D) conditions. Green fluorescence indicates localization of expressed viral proteins while blue fluorescence shows cell nucleus counterstained by DAPI. The image is representing at least three repeated experiments.
doi:10.1371/journal.pone.0100184.g007

The infectivity of SAV3 lacking the entire 6K was rescued through viral RNA recombination

To investigate whether deficient production of progeny from SAV3 lacking 6K could be rescued by providing a helper plasmid, the pSAV3-HHΔ6K cDNA was co-transfected with a helper cDNA containing the whole structural gene region in CHH-1 cells. At 12 dpt, CPE was detected in 14 out of 48 transfected wells (Figure 9A&B). To examine whether viruses obtained from these CPE-positive wells were fully infectious or propagation-defective

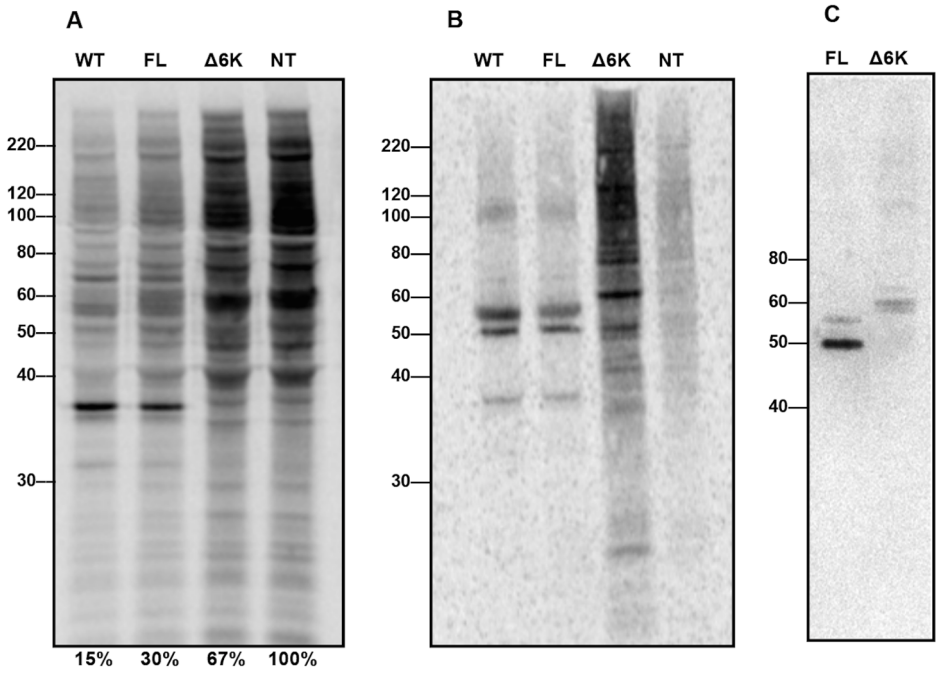

Figure 8. Analysis of viral proteins expression by RIPA and western blot. (A) SDS-PAGE and autoradiography of S^{35} labeled total proteins from cell lysates of CHH-1 cells infected with wtSAV3 (WT), transfected with pSAV3-HHFL cDNA (FL), or transfected with pSAV3-HHΔ6k cDNA (Δ6k). Non-treated (NT) cells were used as protein expression control. The protein density of each transfected group relative to the control group (set as 100%) is noted under the graph. (B) Total proteins from cell lysates were further immunoprecipitated using the anti-SAV3-SP antibody and revealed several bands corresponding to SAV3 viral proteins. The pattern of viral proteins from wtSAV3 infected and pSAV3-HHFL transfected cells was identical while being different in pSAV3-HHΔ6k transfected cells, where a strong band slightly larger than 60 kDa was identified. (C) Immunopreciptated viral proteins were also examined by Western blot using rabbit anti-SAV3-E2 antibody and again one band slightly larger than 60 kDa was found in line with the result from (B) indicating suboptimal processing of E2.
doi:10.1371/journal.pone.0100184.g008

that undergo only one round of infection, virus supernatant derived from the transfected cells was passaged to new CHH-1 cells. Development of CPE was consistently seen for three passages, demonstrating successful formation of infectious virus likely through RNA recombination during viral replication. To prove this, supernatant was collected from a well showing CPE and from which viral RNA was isolated and the SAV structural genes were amplified from the capsid protein gene to E1. After cloning and transformation, plasmids were extracted from 12 colonies and digested with the restriction endonucleases *EcoR* I and *BsrG* I. As *BsrG* I is a unique site within 6K, digested plasmids will contain three fragments if 6K is present, otherwise only two fragments will appear (Figure 9C). The results showed that 5 out of 12 examined clones contained the 6K gene, which was further confirmed by DNA sequencing. Notably, these clones contained fragments of different sizes, suggesting that recombination did not occur at precise sites. We therefore went on to study what was present in the following passage. Viral RNAs isolated from the first passage were amplified by PCR targeting the region between nsP4 and E1. Following cloning and digestion with *EcoR* I, *EcoR* V, and *BsrG* I, plasmids will contain four fragments if the 6K gene was present. The result showed that all digested clones contained 6K in the viral genome (Figure 9D). One of the cloned viral genomes was fully sequenced and the result revealed the genome sequence identical to that of parental virus (SAV3-H10).

Discussion

This is the first report to document the importance of 6K for the generation of infectious progeny of a salmonid alphavirus, and SAV3 lacking the entire 6K protein was not able to form infectious particles. Although viral proteins were expressed within pSAV3-HHΔ6K transfected cells, viral envelope proteins were not detected on the cell surface and no viable virus was released from transfected cells that could spread to neighboring cells. In contrast, cell-to-cell spread from initial transfected cells was observed following transfection with the full-length SAV3 construct (pSAV3-HHFL).

Studies of other alphaviruses have shown that 6K is dispensable for infection as a variant lacking the entire 6K still formed infectious viral particles, although infectivity was attenuated and virus budding was affected leading to decreased virus titers [18,19]. In contrast, our results show that SAV is sensitive to 6K-deletion. Furthermore, we found that SAV contains a unique seven-amino acid domain "GVRGWSA", conserved within the three fully sequenced subtypes SAV1–3, and not found in other alphavirus species. The 6K protein is associated with membranes and it is not unlikely that this insertion adapts the 6K protein to function in cold-blooded animal species that carry a different membrane composition compared to warm-blooded animals. To maintain the viscosity of their membranes as poikilothermic animals, fish cell membranes contain a high content of unsaturated fatty acids to keep them fluid at low temperatures. What role this unique domain in SAV3 6K plays for infectivity will be investigated in the future.

Although there are functional differences, similarities exist between the 6K gene of SAV3 and other alphaviruses. This includes conservation of an aromatic domain, despite one key residue, a non-aromatic amino acid glycine in position 19 of SAV1–, differed from the aromatic amino acid tryptophan found

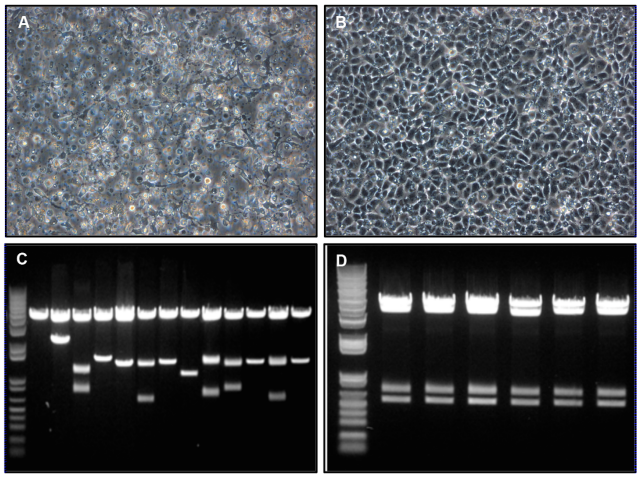

Figure 9. SAV3 RNA recombination and sequence variation. Extensive CPE appeared after co-transfection of pSAV3-HHΔ6k and the helper cDNA construct in CHH-1 cells. From a total of 48 parallel wells monitored, 14 wells were CPE-positive (A) and 34 wells were CPE-negative (B). (C) Supernatant from a well showing CPE were collected. SAV structural genes were amplified and cloned, before plasmids from 12 clones were enzyme digested with *EcoR*I and *BsrG*I (Lane 2–13). Lane 1 is the empty backbone plasmid. Three fragments per lane indicated by arrows show presence of the 6K gene (in 5 out of 14 clones). Fragments of different sizes suggested that recombination did not occur at precise sites. (D) Analysis of virus supernatant from the first passage. SAV structural genes were amplified and cloned, before plasmids from 6 clones were enzyme digested with *EcoR*I, *EcoR*V, and *BsrG*I. Four fragments (in all lanes) indicate the presence of 6K gene, and identical patterns among all clones suggest selection of viable recombinations in the first passage.
doi:10.1371/journal.pone.0100184.g009

in other alphaviruses (Figure 5A). Furthermore, hydrophobicity pattern and two putative transmembrane domains of SAV separated by a cysteine-rich region also share similarities with other alphaviruses. All these similarities theoretically imply that SAV3 6K functions as a viroporin in a similar way as for other alphaviruses, while being indispensable for SAV3 infectivity. This is comparable to the viroporin p7 protein of HCV which is also essential for infectivity [33,34] and viability [35]. Due to this, p7 has become an interesting target for development of antiviral compounds [36–39]. Likewise, our findings suggest that targeting 6K of SAV3 might be an antiviral approach.

Despite 6K provides cleavage sites for polyprotein processing, deletion of the entire 6K gene of SFV had no impact on polyprotein processing, E1/E2 heterodimerization, and intracellular trafficking [18]. Proper glycoprotein proteolytic processing was however hampered in SINV variants containing either a partially deleted 6K lacking the amino acid residuals 24 to 45 or an in-frame insertion of 15 amino acid within the 6K gene, resulting in reduced formation of viral particles and reduced plaque size [20,40]. We could not unequivocally determine whether the process of SAV polyprotein cleavage shares similarity to other alphaviruses, partly because protein shutdown was not 100% in the host cells, making it difficult to identify the viral proteins during protein synthesis. To resolve this problem, development of antibodies specifically recognizing each viral protein will be needed. When we compared the size of the E2 glycoprotein of the Δ6K mutant by radioimmunoprecipitation and western blot, we observed that the glycoprotein was of larger size than E2 of wild-type SAV3. Impaired polyprotein processing between E2 and E1 leading to the production of unauthentic E2 proteins could explain this. Another explanation is that the large size of E2 of the Δ6K mutant could result from incorrect post-translational modification, appearing as a heavily non-trimmed glycosylated protein stuck in the secretory pathway. Although immunofluorescent staining showed that viral proteins were expressed within pSAV3-HHΔ6K transfected cells, viral proteins were not detected on the cell surface, suggesting impaired trafficking of viral proteins from the ER or other intracellular compartments to the plasma membrane.

Non-segmented RNA viruses, such as picornaviruses, coronaviruses, alphaviruses and several plant viruses, accelerate genome exchange through RNA recombination [41]. In this study, we demonstrated frequent RNA recombination by SAV3 within cells transfected with pSAV3-HHΔ6K cDNA together with a helper cDNA clone containing all structural genes including 6K gene in CHH-1 cells. A recent publication by our group documenting that natural infection of SAV3 generates numerous viral deletion mutants through imprecise RNA recombination [42], is in agreement with the result of this study showing recombination in co-transfected cells in vitro at non-specific sites. After one round of passage in cells, we found that the structure of all plasmids analyzed by restriction enzyme digestion was identical, documenting strong selection for viable variants. The fact that RNA recombination occurs in alphaviruses therefore seems to address pivotal safety concerns regarding recombinant alphaviruses used as attenuated vaccines.

SAV subtypes 1–3 are closely related but differences within the nsP3 gene consists of several insertion/deletion sequences, likely have arisen through RNA recombination [4,43]. The mechanisms of recombination of non-segmented genomes are not fully understood, but occur during viral replication and likely involve switching between viral templates while holding on to the nascent viral strand [44]. More detailed studies of alphavirus RNA recombination has been performed in SINV [45–47], showing that RNA recombination of SINV gives rise to deletions, insertions and genome rearrangements [48]. Interestingly, many of these altered SINV RNAs are still infectious. Similarly, Hahn et al. [49] demonstrated that WEEV is a recombinant virus and suggested that WEEV appears to have arisen by recombination between a EEEV-like virus (capsid protein) and a SINV-like virus (glycoproteins), which underlines the importance of RNA recombination in virus evolution.

For more than one decade, CHSE-214 and BF-2 cells have been widely used for propagation of SAV [2,4,43,50]. The CHH-1 cell line develops CPE faster than CHSE-214 and Salmon head kidney-1 (SHK-1) cells after infection with SAV1 [51]. Based on the above, we compared recovery of recombinant SAV3 in these three cell lines, CHSE-214, BF-2 and CHH-1. In conformity with the published results, CPE occurred earlier after transfection with the full-length cDNA clone in CHH-1 cells compared to CHSE-214 and BF-2 cells. Moreover, a 10 times higher virus titer was obtained in CHH-1 cells. As SAV is highly sensitive to IFN-α responses [22], the difference was likely due to CHH-1 cells generating weak IFN-α and ISGs responses to SAV3 infection when compared to the other two cells lines (Figure 4). Similarly, Baby hamster kidney-21 (BHK-21), an IFN-deficient cell line, has been widely used for the production of high titer alphaviruses [52,53].

We have also shown that a SFV replicon construct containing the SAV3 structural genes (capsid-E3–E2-6K–E1) delivered as a DNA vaccine to mice for primary immunization and combined with E2 recombinant protein boosting elicited production of high titer anti-SAV3 antiserum, as shown by immunostaining of cells, immunohistochemistry, and western blot. However, chimeric

alphaviruses composed by a SFV replicon and SAV3 structural proteins failed to assemble into virus particles in transfected BHK-21 cells (no CPE) despite high levels of SAV3 structural proteins being detected in the cytoplasm (data not shown). It is possible that the SAV3 capsid protein does not recognize the SFV packaging signal located in nsP2 [54] or that surface proteins of SAV3 (E1 and E2) adapted for cold-water environment are not functional at higher temperatures.

Based on the finding of SFV lacking the entire 6K gene giving rise to the formation of the attenuated virus, we were interested in exploring if deletion of the SAV3 6K gene could similarly attenuate virus infectivity and constitute a strategy for a live-attenuated viral vaccine. This was not a viable strategy. Future studies addressing 6K partial deletion variants in detail or including co-transfection with a plasmid encoding 6K in trans for virus rescue should be conducted. Finally identification of an IFN-α deficient cell-line supporting growth of salmonid alphaviruses will be of great importance for rescue of attenuated SAV variants and for production of high virus titers.

Author Contributions

Conceived and designed the experiments: TG DJ PL ØE. Performed the experiments: TG DJ. Analyzed the data: TG DJ ØH PL ØE. Contributed reagents/materials/analysis tools: TG DJ ØH. Wrote the paper: TG DJ ØH PL ØE.

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IV

Modification of a salmonid alphavirus replicon vector for enhanced expression of heterologous antigens

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Contents category: Short communication

Summary word count: 150

Manuscript word count: 3267

Summary

Salmonid alphavirus (SAV) replicon has been developed to express heterologous antigens but protein production was low to modest compared to terrestrial alphavirus replicons (Olsen *et al.*, 2013a). In this study, we have compared several modifications to a SAV replicon construct and analyzed their influence on foreign gene expression. We found that with an insertion of a translational enhancer consisting of the N-terminal 102 nucleotides of the capsid gene together with a nucleotide sequence encoding the FMDV 2A peptide caused a significant increase of the EGFP reporter gene expression. The importance of fusing a hammerhead (HH) ribozyme sequence at the 5' end of the viral genome was also demonstrated. In contrast, a hepatitis D virus ribozyme (HDV-RZ) sequence placed at 3'end did not augment expression of inserted genes. Taken together, we have developed a platform for optimized antigen production which can be applied for immunization of salmonid fish in the future.

Sustainable development of aquaculture relies on preventing and controlling diseases, for which the use of vaccines has been of major importance. Despite vaccination efficiently controlling many bacterial diseases in salmonid aquaculture, viral infections still remain a major problem. Although commercial vaccines are available against infectious pancreatic necrosis (IPN) and pancreas disease (PD), all non-replicating vaccines based on inactivated whole virus (IPNV and PD) and recombinant subunit vaccines (IPNV-VP2), the general understanding has been that these vaccines confer sub-optimal protection (Salgado-Miranda *et al.*, 2013). A key question regarding development of protective vaccines against virus infections in fish and higher vertebrates is to understand the mechanisms underlying the difference between inferior protection attained by inactivated vaccines and the strong immunity often induced by replicating variants (Robert-Guroff, 2007). Replicating vaccines possess the advantage to mimic an actual infection and therefore induce T-cell mediated immunity, whereas inactivated (non-replicating) protein antigens elicit mostly B-cell mediated humoral responses (Lundstrom, 2012). Concerns regarding reversion to virulent virus and ecosafety have however hindered the use of live vaccines in aquaculture.

The use of alphaviruses as vector vaccines serves as an attractive technology for the generation of novel and improved vaccines as these vectors express high levels of recombinant proteins in a broad spectrum of hosts including many mammalian and insect cells (Lundstrom, 2001;Rayner *et al.*, 2002;Riezebos-Brilman *et al.*, 2006). The alphavirus genome is a positive-stranded RNA capped at the 5' end and polyadenylated at the 3'end with the size of approximately 11.7 kb (Strauss & Strauss, 1994). The genome structure of alphavirus is composed of two open reading frames (ORFs). The first ORF encodes a polypeptide containing four non-structural proteins (nsp1-4), which forms the replicase complex involved in RNA replication and transcription. The second ORF is driven by a 26S

62 subgenomic promoter and express the structural proteins (CP-E3-E2-6k-E1) responsible for
63 encapsidation of viral RNA and assembly into enveloped particles (Strauss & Strauss, 1994).
64
65 Compared to the classical live-attenuated form where the virus infects the animal without
66 causing disease, recombinant replicon vaccines have several advantages in terms of safety. By
67 removing the genes that encode for the viral structural genes, and replacing them by vaccine
68 antigens, these viral vectors can replicate their genomes but are not productively infectious as
69 they are unable to make new virus particles (Lundstrom, 2005). The use of plasmid-encoded
70 viral replication systems is believed to induce a strong immune response through intracellular
71 antigen expression and the ability of the replicating RNA to stimulate innate antiviral signals
72 (Leitner *et al.*, 2003), including generation of double-stranded RNA intermediates, which
73 cannot be achieved utilizing a RNA polymerase II-dependent conventional DNA vaccine
74 (Leitner *et al.*, 2000). Furthermore, the property of self-replicating RNA derived from
75 alphavirus DNA-based vectors allows lower dosage required for immunization compared to
76 conventional DNA vaccines, reducing the cost of vaccine production (Berglund *et al.*, 1998).
77 Several alphaviruses such as Sindbis virus (SINV), Semliki Forest virus (SFV) and
78 Venezuelan equine encephalitis virus (VEEV) have all been developed as potential replicon
79 based vaccine vectors carrying heterologous antigens and shown to elicit protective immune
80 response in animals (Bennett *et al.*, 2000;Frolov *et al.*, 1996;Lundstrom, 1997;Lundstrom,
81 2009;Zhou *et al.*, 1995). The SFV replicon has been developed to express antigens in fish
82 cells, however with limitations because of very low protein expression at low temperatures
83 (15°C and below) (Phenix *et al.*, 2000). In contrast, salmonid alphavirus (SAV) causes natural
84 infections in farmed salmonids at low temperatures and therefore represents an alternative
85 replicon based vector vaccine. Recently published studies have indeed shown that a SAV
86 replicon based vaccine was functional in cells originating from fish, mammals and insects and

87 at temperatures ranging from 4 to 37°C (Olsen *et al.*, 2013). Further the SAV based replicon
88 vaccine carrying the hemaglutinin-esterase (HE) gene of infectious salmon anemia virus
89 (ISAV) has been proven to protect Atlantic salmon against ISAV challenge when delivered by
90 intramuscular injections (Wolf *et al.*, 2013a), while intraperitoneal immunization with the
91 same dose induced no protection (Wolf *et al.*, 2014). However, SAV replicons give low to
92 modest protein production compared to what is reported from terrestrial alphavirus replicons
93 (Olsen *et al.*, 2013). Modifications to the construct for improved levels of protein expression
94 would therefore be valuable, as it is well known that antigen dose in fish vaccines are well
95 correlated with protection (Munang'andu *et al.*, 2013). The objective of the present study was
96 to develop a SAV3 replicon vaccine vector based on a previously constructed infectious full-
97 length cDNA clone (Guo *et al.*, 2014) and evaluate different modifications to the constructs
98 for enhanced expression of heterologous protein.
99
100 Initially, the effect of inserting a hammerhead (HH) ribozyme sequence as described
101 previously (Guo *et al.*, 2014) to ensure precise cleavage at the 5' end was evaluated. Chinook
102 salmon embryonic (CHSE-214) cells were transfected (Fugene HD transfection reagent,
103 Roche) with an infectious full-length SAV3 clone with or without the HH ribozyme sequence
104 fused at the 5' end (pSAV3-HHFL and pSAV3-FL respectively) and incubated at 15˚C. Cell
105 supernatant was collected at 2, 4, 8, 9, 11 and 13 days post transfection (dpt), followed by
106 titration of rescued virus on CHSE-214 cells by the method of Spearman and Kärber (Miller
107 & Ulrich, 2001). Recovery of recombinant virus in pSAV3-HHFL transfected cells started at
108 4 dpt ($1x10^3$ $TCID_{50}$/mL) and generated a peak titer of $2.1x10^7$ $TCID_{50}$/mL at 13 dpt (Fig.1a).
109 In contrast, infectious virus recovered from pSAV3-FL was delayed until 11dpt ($4.7x10^2$
110 $TCID_{50}$/mL) and end-point titers were reduced 1000-fold compared with the construct
111 containing the HH-ribozyme. It has previously been reported that inclusion of a HH ribozyme

112 sequence is indispensable for rescue of SAV2 by reverse genetics (Moriette *et al.*, 2006). Our
113 result confirmed the important role of HH ribozyme, yet we also observed that recovery of
114 virus from pSAV3-FL (non-HH) cDNA was feasible albeit giving lower titers.
115
116 Based on the findings above, the full-length cDNA clone with an inserted HH sequence at the
117 5' end was used as a template for construction of a SAV3 replicon vector. The structural
118 genes were deleted by PCR splicing using primers F1/R1 and F2/R2 (Table 1). At the same
119 time two restriction sites, *AgeI* and *AscI* were introduced downstream of the viral subgenomic
120 promoter and upstream of 3'UTR to facilitate cloning of insert DNA. To evaluate the
121 functionality of the SAV3 replicon, the enhanced green fluorescent protein (EGFP) marker
122 gene was inserted into the SAV3 replicon at the *AgeI* and *AscI* sites, generating pRep-EGFP
123 (Fig.1b). It has previously been shown for SFV replicons that the first 102 nt of the capsid
124 gene, including the initiating AUG, function as a translational enhancer. When the capsid
125 gene segment was fused to the heterologous gene, expression of the corresponding fusion
126 protein was found to be at the same level as viral structural proteins during wild type SFV
127 infection (Sjoberg *et al.*, 1994). Despite only 56% sequence similarity between SAV3 and
128 SFV within the N-terminal end of the capsid protein (data not shown), we decided to examine
129 whether this enhancing property is evolutionary conserved in SAV3. The 102 nt of N-terminal
130 SAV3 capsid gene were therefore inserted into pRep-EGFP. To obtain an accurate 5'end of
131 the expressed antigen, the 2A peptide sequence derived from foot-and-mouth disease virus
132 (FMDV) was placed immediately downstream of the translational enhancer sequence
133 generating the construct pRep-E2A-EGFP (Fig. 1b). The FMDV 2A peptide executes
134 translational skipping and thus prevents the formation of a peptide bond between enhancer
135 and antigen (Ryan & Drew, 1994) thus producing authentic EGFP protein. Two replicon
136 constructs, pRep-EGFP and pRep-E2A-EGFP, were then transfected into Chum salmon heart

(CHH-1) cells (Herath T. *et al.*, 2009) by electroporation (Neon transfection system, Invitrogen) and incubated at 15˚C. Expression of EGFP in the cells transfected with or without E2A in the construct was detectable from 2 dpt using fluorescence microscope (Olympus, IX81). At 4 dpt, a number of EGFP positive cells were observed yet the intensity of EGFP per cell could not be quantified by fluorescence microscopy. Therefore, in order to clarify the role of E2A on EGFP expression levels, cells electroporated with either pRep-E2A-EGFP or pRep-EGFP were trypsinized, spun down and suspended in PBS before analyed with a flow cytometer (Guava easyCyte HT system, Merk Millipore). The results showed that the transfection rate of two replicon constructs by electroporation was similar (data not shown), while the mean intensity of EGFP per cell was significantly higher in the E2A+ group (Fig. 1c) showing the benefit of introducing the N-terminal 102 bases of the SAV3 viral capsid gene as a translation enhancer.

Furthermore, it has been shown that a hepatitis D virus ribozyme (HDV-RZ) sequence placed immediately downstream of the poly(A) sequence enhances expression levels of the reporter gene in a SINV replicon vector (Yamanaka & Xanthopoulos, 2004). HDV-RZ self-cleaves at its 5' end and therefore generates authentic 3' termini of viral RNA molecules, which again facilitate the successful initiation of RNA replication in transfected cells. To evaluate whether HDV-RZ can improve expression levels of inserted genes in SAV3 replicon vectors, HDV-RZ sequence (Wen *et al.*, 1999) was introduced into the pRep-E2A-EGFP vector by stepwise fusion PCR using primers listed in Table 1. The constructed plasmid (Fig. 1b), pRep-E2A-EGFP-RZ, together with pRep-E2A-EGFP were separately transfected into CHH-1 cells using Fugene HD transfection reagent (Roche) and total fluorescence intensities of EGFP were measured by a microplate reader (Tecan GENios). The total intensities of EGFP were not different ($p>0.05$) for cells transfected with pRep-E2A-EGFP vs. pRep-E2A-EGFP-RZ (Fig.

1d), suggesting the exact 3'end sequence of SAV3 RNA molecules is not a crucial factor for viral replication.

We have previously documented that CHH-1 cells is the optimal cell line for the generation of recombinant SAV3 (Guo *et al.*, 2014) within three susceptible cell lines, Chinook salmon embryo (CHSE-214), Bluegill fry (BF-2), and CHH-1. To determine whether expression of foreign antigen using the SAV3 replicon system is cell-dependent, the pRep-E2A-EGFP vector was transfected into the same cell-lines. Compared to CHH-1 cells the EGFP expression in CHSE-214 and BF-2 cells was delayed, and in this regard we prolonged the incubation time up to 10 days before evaluating the intensity of fluorescence. The result showed that EGFP expression in CHH-1 cells was significantly higher compared to CHSE-214 and BF-2 cells (Fig. 1e). For validation of the results above, the EGFP gene was replaced with another reporter gene, luciferase. The activity of expressed luciferase was assayed with the luciferase reporter assay system (Promega) before luminescence was detected by a microplate reader (Tecan GENios). When comparing the ability of the three transfected cell lines to produce the gene of interest, luciferase activity with the results obtained for EGFP expression. These results further support our previous study (Guo *et al.*, 2014) where we found that virus infection of CHH-1 cells results in limited induction of interferon, Mx and ISG15, and consequently generate higher amount of viral products and interestingly, also luciferase post transfection. To what extent the same mechanisms apply remains elusive.

We then moved on to test the capability of the replicon system to express the complete structural genes from a heterologous virus of importance in salmonid aquaculture, infectious pancreatic necrosis virus (IPNV). The pRep-E2A-EGFP vector was engineered to carry the

complete structural proteins of IPNV, replacing the EGFP gene and designated pRep-E2A-IPNsegA. As IPNV infection in CHH-1 cells has previously not been described, IPNV was first inoculated onto CHH-1 cells. Significant CPE was evident from day 5 post infection by phase contrast microscopy (Fig. 2a). Immunostaining of cells infected by IPNV using a rabbit anti-IPNV primary antibody (Munang'andu *et al.*, 2013b) gave a strong signal in fluorescence microscopy confirming the successful expression of IPNV protein in CHH-1 cells (Fig. 2b). We finally tested the expression of IPNV structural proteins produced by the pRep-E2A-IPNsegA construct and the result showed evident expression of IPNV structural proteins at 5 dpt (Fig. 2d). As expected, the percentage of positive cells was lower compared to infected cells (Fig. 2b) in line with the replicon not being able to spread from initially transfected cells.

A recently published study has shown the potential of a similar SAV3-based replicon vaccine for protection against ISAV infection (Wolf *et al.*, 2013b). However levels of produced antigen are of pivotal importance for vaccine efficacy, and expression levels from this construct were low to moderate (Olsen *et al.*, 2013b). This study reports the importance of inserting a translational enhancer in the construct, as antigen expression levels were sub-optimal when the heterologous gene was located immediately after the viral subgenomic promoter. This important finding underscore the potential use of the improved SAV3 replicon vectors for fish vaccine development, though future immunization studies should be conducted to evaluate impact on protection against challenge.

Acknowledgement. This study was carried out with financial support to ØE from the Norwegian Research Council, projects no. 182035 *Immunisation strategies against viral pathogens of Atlantic salmon* and no. 183204 *Indo-Norwegian platform on fish and shellfish vaccine development,* and from the Swedish Research Council to PL.

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Figures and legends

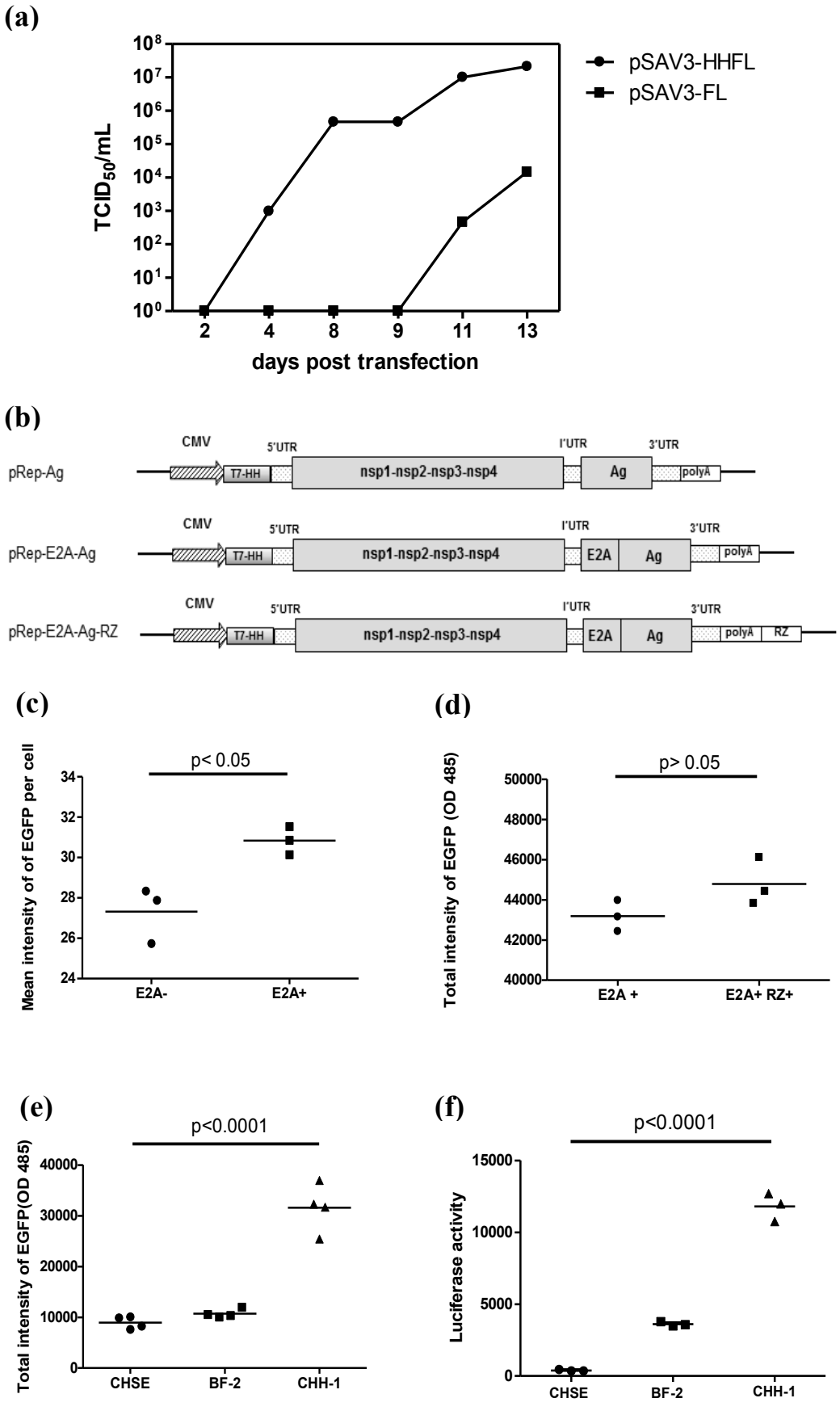

Fig. 1. (a) Supernatant from cells transfected with pSAV3-HHFL and pSAV3-FL was collected at indicated time points and viral titration was performed. (b) Design of modified SAV3 replicon constructs for improved protein expression. Abbreviations: Ag, antigen (in this study: EGFP / Luciferase / IPNV segA); E, translational enhancer; 2A, FMDV 2A peptide; RZ, Hepatitis D ribozyme (c) Expression of EGFP in CHH-1 cells transfected with either pRep-EGFP or pRep-E2A-EGFP was quantified by flow cytometer. Each data represents the mean intensity of EGFP per cell when 35,000 transfected cells from each individual transfection were analyzed. (d) Quantification of total EGFP expression in the cells transfected with either pRep-E2A-EGFP or pRep-E2A-EGFP-RZ. (e) Quantification of EGFP expression in three different cell lines following transfection with pRep-E2A-EGFP. (f) Quantification of Luciferase expression in three different cell lines following transfection with pRep-E2A-LUC. All transfections including replicates were performed independently and all statistical data were analyzed by unpaired t test using GraphPad 5.0 software.

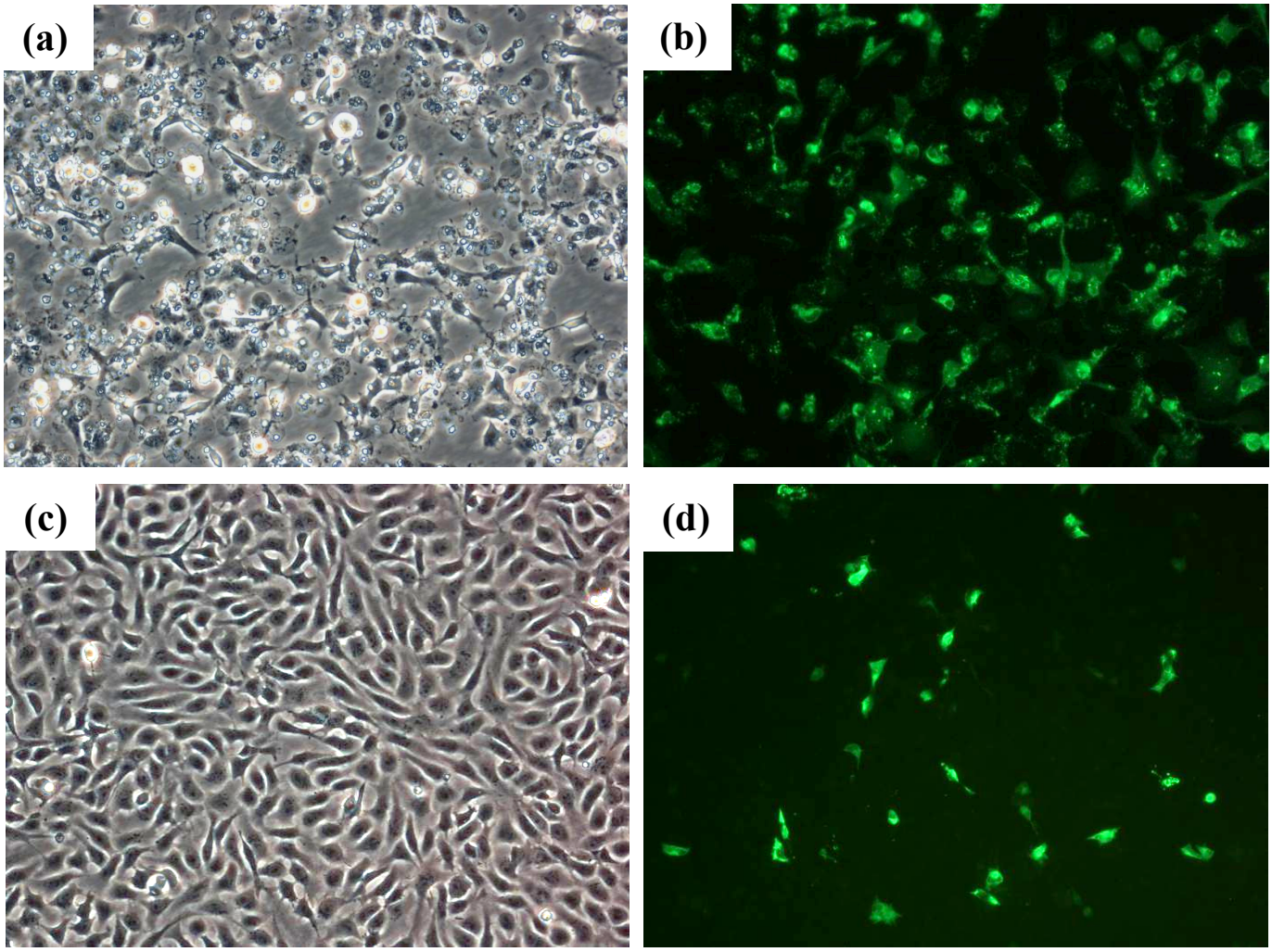

Fig. 2. (a) CPE induced by IPNV infection in CHH-1 cells at 5 days post infection (b) Infected cells were immunostained to document presence of IPNV structural proteins (c) CHH-1 cells transfected with pRep-E2A-IPNsegA showed no CPE at 5 dpt. (d) Immunostaining showing expression of IPNV structural proteins by pRep-E2A-IPNsegA at the same time point.

Table 1. Primers used for plasmids construction.

Designation	Primer sequence (5'→ 3') Sequences for restriction sites are underlined.	Restriction site
F1-F	ACCGGTAATATAATATGGCGCGCCATTCCGGTATATAAATTGCTCAC	AgeI / AscI
F1-R	AGGTTCAGGGGGAGGTGTGGGAGG	
F2-F	ACAGAGAACTAGTCCGAAGATTG	SpeI
F2-R	GGCGCGCCATATTATATTACCGGTGGTTGGTTGAGAGTATGATGCAGAAAATATTAAGG	AscI / AgeI
F2-E2A-R	GGCGCGCCATATTATATTACCGGTGGGCCCAGGGTTGGACTCGACGTCTCCCGCAAGCTTAAGAAGGTCAAAATTCTTAGTGCGCGGCCGGTACGG	AscI / AgeI
EGFP-F	ATATTACCGGTATGGTGAGCAAGGGCGAGGAG	AgeI
EGFP-R	ATATTGGCGCGCCTTACTTGTACAGCTCGTCCATG	AscI
LUC-F	ATATTACCGGTATGGAAGACGCCAAAAACAT	AgeI
LUC-R	ATATTGGCGCGCCTTACTTTCCGCCCTTCTTGGCC	AscI
SegA-F	ATATTACCGGTATGAACACAAACAAGGCAACCG	AgeI
SegA-R	ATATTGGCGCGCCTTACACCTCAGCGTTGTCTCC	AscI
RZ-F	GATCACATGGTCCTGCTGGAGTTCGTGACC	
RZ-R1	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTATATTGAAAATTTTAAAACCA	
RZ-R2	CCGCGAGGAGGTGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	
RZ-R3	CCGCGCTAGCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGG	BmtI
HDV-RZ	GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAG	