Functional analysis of replication determinantsin classical swine fever virus

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Preface

This thesis is the result of my three-year Ph.D. education at the National Veterinary Institute, Technical University of Denmark (DTU) and has been internally funded by DTU Vet.

The work presented in this thesis would not have been possible without the support from my colleagues, family and friends during the past three years. Creating a Ph.D. thesis is not an individual process; rather it takes place in a social context and includes several persons, whom I would like to thank. First, I wish to express my gratitude to my main supervisor Graham J. Belsham and co-supervisor Thomas Bruun Rasmussen, who have put their valuable experience and wisdom to my disposal. Your scientific guidance, discussions and constructive feedback have been of great value. Thank you for always keeping your doors open and supporting my research throughout this process.

I wish to acknowledge the support from all the fantastic people at Lindholm. The mentality and atmosphere is really unique and my years at the island will always remind me of a special time in my life. The list of names of people is too long to mention here but you know who you are. I would like to express my sincerest thanks to everyone around our little coffee table in the screening lab. We have had some great talks and your caring nature and creation of a relaxed atmosphere has made even Monday mornings fun. A special thank goes to my good friend and colleague Jonas Kjær. You have been by my side throughout this project, in good times and bad. I wish to thank both former and present Ph.D. students at Lindholm. When I started this project, we were only two students and at the moment the number has reached six allowing as good a forum for scientific as for non-scientific talks.

Thanks to Dirk Höper and Ilona Reimann for hosting me during my research stay at the Friedrich-Loeffler-Institut (Germany). Also, I wish to thank Maria Jenckel for giving me the opportunity to learn new techniques and for her hospitality. Furthermore, I would like to thank the Epizone network for financing this stay.

Finally, an everlasting thank to my family and friends for their support and patience. Thanks for understanding, strengthening and motivating me throughout this project. Even though you may not always understand my research, it is nothing compared to your love and kindness.

Johanne Hadsbjerg, November 2016

Abstract

The ability to efficiently produce new viral progeny is an essential process in the viral life cycle. Virus replication is a complex process involving viral proteins, interactions within the viral genome and assistance from host proteins. Identification of replication determinants of important human and animal pathogens should facilitate finding new approaches for efficient disease control. The principal aim of this thesis is to characterise determinants involved in the replication of classical swine fever virus (CSFV). Classical swine fever is a highly contagious virus disease of domestic pigs and wild boar with great socio-economic importance for countries, like Denmark, that have a significant trade in agricultural production. CSFV has, like other RNA viruses, a high mutation rate allowing the virus to adapt to changes in selection pressure. Hence obtaining a more thorough knowledge of sequence variation during virus replication is crucial for the design of effective vaccines and for diagnostic purposes.

This thesis is comprised of four parts: An overview consisting of a general introduction (part 1) and a discussion (part 4) which provides a review of CSFV biology with an emphasis on the viral life cycle and structural RNA elements important for virus replication. This establishes a theoretical background and provides perspectives for the work performed during this Ph.D., which is outlined in part 2 that summarises the project aims and findings. These findings are described in-depth in part 3 comprising the three manuscripts included in the thesis.

Manuscript 1 has focused on adaptations that occurred in the CSFV genome following introduction of mutations into the internal ribosome entry site (IRES) and after serial passaging in cell culture and when tested *in vivo*. Sequence adaptations occurred in the coding regions for NS2 and NS5B that restored the coding sequence to that of the parental field strain. When rescued viruses, containing mutant IRES elements, were introduced into pigs no induction of clinical disease could be observed and only limited levels of viral RNA were detected. In contrast, inoculation with the parental virus caused similar clinical symptoms as that observed with the parental field strain. Analysing the adaptation of the viruses throughout the entire viral genome during virus replication allowed a more comprehensive understanding of the virus properties beyond what can be explained by knowing just the consensus sequence.

Manuscript 2 examines replication determinants by analysing a sequence motif in the coding region of NS5B which has the possibility to form long-range RNA-RNA interactions. When synonymous mutations were introduced into this motif in a CSFV replicon (a self-replicating but non-infectious genome) reduced RNA replication was observed compared to a wild type replicon. However, this effect was not observed in the context of the infectious virus. Interestingly, an adaptive silent mutation in a conserved part of the structural CSFV protein E1 occurred upon virus passaging in cell culture. Knowledge of these sequence variations and putative long-range interactions will provide valuable insights into mechanisms underlying virus translation and replication.

In manuscript 3, a selection marker has been inserted into a CSFV-based replicon making it suitable for screening of antiviral drugs that block virus translation or RNA replication. The neomycin phosphotransferase coding sequence was chosen as a selection marker which confers resistance to neomycin. The neomycin-selectable replicon was demonstrated to be functional and designed with the aim of facilitating the maintenance of replicons by selection within mammalian cells. This might prove to be useful for the maintenance and expression of specific viral antigens in an efficient manner in order to produce more effective and safer prototype vaccine candidates.

Resumé (Danish summary)

En vigtig proces i den virale livscyklus er evnen til effektivt at producere nye virale afkom. Virus-replikation er en kompleks proces, der involverer virale proteiner, interaktioner i det virale genom og samspil med værtsproteiner. Identifikation af afgørende faktorer for genom-replikationen af vigtige humane og animalske patogener vil lette bestræbelserne på, at finde nye metoder til effektiv sygdomsbekæmpelse. Hovedformålet med denne afhandling er at karakterisere vigtige faktorer involveret i replikationen af klassisk svinepestvirus (CSFV). Klassisk svinepest er en særdeles smitsom virussygdom, der rammer tamsvin og vildsvin, og den har en stor samfundsøkonomisk betydning for lande, som Danmark, der har en væsentlig handel indenfor landbrugsproduktionen. CSFV har, ligesom andre RNA-virus, en høj mutationsrate, hvilket tillader virusset at tilpasse sig ændringer i selektionstryk. Derfor vil et mere indgående kendskab til sekvensvariationen, samt mekanismerne under virus-replikationen være afgørende for udformningen af effektive vacciner og til diagnostiske formål.

Denne afhandling består af fire dele: En oversigt bestående af en generel introduktion (del 1) og en diskussion (del 4), der beskriver biologien af CSFV med fokus på den virale livscyklus samt strukturelle RNA elementer, som er vigtige for virus-replikationen. Dette giver et teoretisk grundlag og en perspektivering til arbejdet udført i løbet af denne ph.d., som er skitseret i del 2, der opsummerer ph.d.-projektets mål og resultater. Disse resultater er beskrevet til bunds i del 3 indeholdende de tre manuskripter, der indgår i afhandlingen.

Manuskript 1 fokuserer på tilpasninger, der observeres i CSFV-genomet efter at mutationer er introduceret i det interne ribosom indgangssted (IRES), samt efter gentagne passager i cellekultur og ved *in vivo* forsøg. Tilpasninger i sekvensen ses i de kodende regioner for NS2 og NS5B, som gendanner den kodende sekvens til den som findes i den oprindelige virusstamme. Ved podning af grise, med virus indeholdende muterede IRES-elementer, blev der ikke observeret kliniske symptomer og der blev kun påvist begrænsede mængder af viralt RNA. I modsætning hertil forårsagede podning med vildtypevirus kliniske symptomer, som også observeres i den oprindelige virusstamme. Ved at undersøge tilpasningen af virus i hele dets

genom under virus-replikationen kan det give en mere omfattende forståelse af de virusegenskaber, der kan forklares ved mere end blot at kende konsensussekvensen.

I manuskript 2 undersøges faktorer afgørende for virus-replikationen ved at analysere et sekvensmotiv i den kodende region af NS5B, som har mulighed for at danne langtrækkende RNA-RNA interaktioner. Et CSFV replikon (et autonomt replikerende, men ikke-infektiøst genom) blev muteret med ikke-kodende mutationer i det ovennævnte motiv, hvilket førte til nedsat RNA-replikation sammenlignet med et vildtype-replikon. Denne effekt blev dog ikke observeret i infektiøse virus. Interessant nok opstod en ikke-kodende mutation i en bevaret del af det strukturelle CSFV-protein E1 efter passage af virus i cellekultur. Kendskab til disse sekvensvariationer og formodede langtrækkende interaktioner vil give indsigtsfuld viden i mekanismerne bag virus-translation og -replikation.

I manuskript 3 blev en selektionsmarkør sat ind i et CSFV-baseret replikon, hvilket gør det velegnet til screening af antivirale lægemidler, som blokerer virus-translation eller RNA-replikation. Den kodende sekvens for neomycin phosphotransferase blev valgt som en selektionsmarkør, hvilket giver resistens over for neomycin. Neomycin-selekterbare replikoner blev designet med henblik på at gøre det nemmere at opretholde replikoner ved selektion i cellekultur og resultaterne viser, at de er fuldt funktionelle og udtrykker virusprotein. Dette kan vise sig at være nyttigt til effektivt at opretholde og udtrykke specifikke virale antigener med henblik på at producere mere effektive og sikre prototype vaccinekandidater.

Part 1 • Introduction

Chapter 1

Classical swine fever virus

History and classification

Classical swine fever (CSF), formerly known as hog cholera, is a highly contagious and economically significant viral disease affecting domestic pigs and wild boar. Classical swine fever virus (CSFV) is a member of the *Pestivirus* genus, which belongs to the *Flaviviridae* family (Figure 1). Other members of the *Pestivirus* genus are bovine viral diarrhoea virus (BVDV) type 1 and 2 and border disease virus (BDV) (Becher *et al.*, 1999). While infection by CSFV is restricted to pigs, the other *Pestivirus* species have been found to infect a wide range of animals, i.e. bison, reindeer, giraffe and pronghorn antelope (Avalos-Ramirez *et al.*, 2001; Becher *et al.*, 1999; Vilcek *et al.*, 2005). Recently, several other porcine pestiviruses have been described, i.e. Bungawannah virus and atypical porcine pestivirus (Beer *et al.*, 2016; Hause *et al.*, 2015; Kirkland *et al.*, 2007; Postel *et al.*, 2016).

CSFVs can be classified into three genotypes with three or four sub-genotypes (Lowings *et al.*, 1996; Paton *et al.*, 2000). Genotype 1 mostly comprises the historical isolates, while the majority of CSF outbreaks during the past decade in Europe are caused by genotype 2 viruses

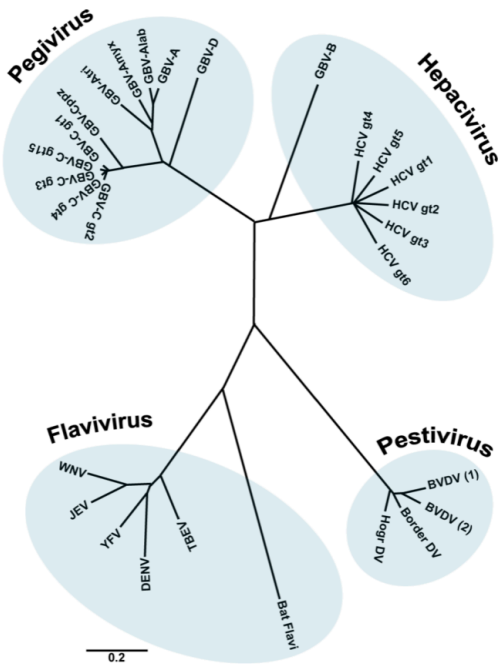

Figure 1. Phylogenetic tree of selected members of the *Flaviviridae* family. The tree is depicted based on analysis of conserved motifs within the RNA-dependent RNA polymerase (RdRp). The branch lengths are proportional to the genetic difference where the distance scale corresponds to amino acid substitutions per position. Classical swine fever virus (CSFV) is denoted its former name hog cholera virus (Hogr DV) (Romero-Brey & Bartenschlager, 2014).

(Postel *et al.*, 2012). Most of the isolates from genotype 3 are from Asia (Sakoda *et al.*, 1999). Despite the genetic diversity, no separate serotypes have been identified and there is no evidence for an impact of the sequence diversity on vaccine efficacy (Graham *et al.*, 2012).

An outbreak of CSF in areas with intensive pig farming can cause a great socio-economic challenge as seen during the latest outbreak in the Netherlands in 1997 – 1998 (Pluimers *et al.*, 1999). The total financial costs of the outbreak were calculated to be US $2.3 billion and during the 15 months that the outbreak lasted, more than 11 million pigs were destroyed (Meuwissen *et al.*, 1999). Understanding CSFV diversity and distribution is important for designing optimised control strategies. Even though the current vaccines strategies have proven to be effective against all known strains, CSFV exhibit a high mutation rate making the possibility for new strains to emerge likely and these might not be covered by the prevalent control policies. For countries free of CSF, reintroduction of the disease poses a constant threat, particular due to a strict non-vaccination policy. The non-vaccination policy was implemented in 1990 to allow free trade of pigs within Europe. However, the huge economic losses, due to stamping out and ethical considerations following large-scale culling of healthy animals, underline the requirement of alternative control strategies. Until now, control strategies are mainly based on vaccination or stamping out. Live attenuated vaccines, such as the Chinese C-strain vaccine, are highly efficacious and widely used for the control of CSF in endemic areas. The development of marker vaccines which allows to distinguish infected from vaccinated animals has been pivotal. Two subunit marker vaccines based on the CSFV E2 glycoprotein have been commercialised and have been shown to be highly effective and safe (Blome *et al.*, 2006). However, these have to be accompanied by use of discriminatory enzyme-linked immunosorbent assay (ELISA) tests, designed to detect antibodies against E^{rns}, for differentiation of infected from vaccinated animals (DIVA). A chimeric pestivirus based on the DIVA principle has recently been licenced within EU for use in outbreak situations in herds within restricted control zones (Dräger *et al.*, 2016; Goller *et al.*, 2015).

Understanding CSFV diversity and distribution is also imperative as the vast majority of CSF outbreaks in Europe occur through transmission from endemically infected wild boar to domestic pigs (Moennig, 2015). Hence, knowledge on the geographical pattern and genetic range of the disease is of key importance. CSFV is a listed disease of the World Organisation for Animal Health (OIE) requiring that all occurrences are reported. The disease is endemic in

much of Asia, Central and South America and in some parts of Africa. However, many of the European countries are declared free of CSF (OIE website[1]). The latest reported cases in Europe were found in the wild boar population in Latvia with 30 and 6 cases in 2014 and 2015, respectively. In Denmark, CSF has not been present since 1933 and it has, like the other Nordic countries, the status as "historically free" from CSF.

CSFV genome and proteins

CSFV is a positive-stranded RNA virus with a genome of approximately 12.3 kb containing a single large open reading frame (ORF) flanked by untranslated regions (UTRs). This ORF is translated into a polyprotein of about 3,900 amino acids, which is processed into at least twelve mature proteins by viral and host proteases (Rümenapf *et al.*, 1993). The CSFV RNA is uncapped unlike all eukaryotic messenger RNAs (mRNAs) but instead harbours an internal ribosome entry site (IRES) in the 5'-UTR that directs cap-independent translation of the viral RNA. Moreover, several structural elements involved in negative-stranded RNA synthesis are found in the 3'-UTR, which is non-polyadenylated (Deng & Brock, 1993). The high conservation of the nucleotide sequence in the UTRs implies that important signals for viral RNA replication are located in these regions (Fletcher & Jackson, 2002; Xiao *et al.*, 2004).

Following processing of the encoded polyprotein, four structural proteins and eight non-structural proteins are generated (Figure 2). The structural proteins consist of the three envelope glycoproteins termed E^{rns}, E1 and E2 located on the surface of the virion as well as the core protein lining the nucleocapsid (Stark *et al.*, 1993; Thiel *et al.*, 1991). The main functions of the structural glycoproteins involve; (i) protection of the RNA genome before infection, (ii) host cell interactions, in order to achieve virus entry, and (iii) interactions with the other viral proteins to form new virus particles. More specifically, E1 and E2 form an E1 – E2 heterodimer that mediates viral attachment and entry determining cell tropism (Fernández-Sainz *et al.*, 2011; Wang *et al.*, 2004). E2 also exists as a homodimer and is, along with E^{rns}, a neutralising antigen during CSFV infection (Konig *et al.*, 1995; Weiland *et al.*, 1992). Monoclonal antibodies against E2 and E^{rns} are used as diagnostic tools to distinguish between the different pestivirus species (Edwards *et al.*, 1991).

[1] http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasetimelines

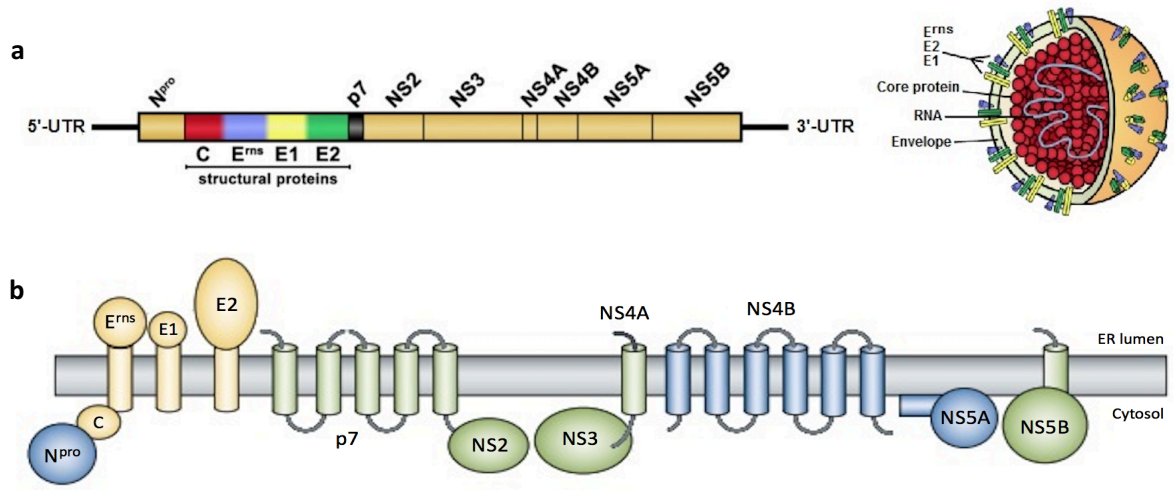

Figure 2. Schematic presentation of the genome organisation and topology of CSFV.
(a) Structure of the CSFV genome and depiction of the virion. Modified from (Beer *et al.*, 2007).
(b) Proposed organisation of the pestivirus viral proteins. Structural proteins are depicted in yellow and non-structural proteins are shown in green (required for infectious virus production) or blue (other functions). Modified from (Murray *et al.*, 2008).

The non-structural proteins include N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Five of these proteins (NS3, NS4A, NS4B, NS5A and NS5B) associate to form the replication complex (Moulin *et al.*, 2007). Of these, the RNA-dependent RNA polymerase (RdRp) NS5B is the enzyme responsible for synthesising the new RNA strands making it a central protein in the replication complex (Steffens *et al.*, 1999; Xiao *et al.*, 2002a). Except for the leader protein N^{pro}, all of the encoded proteins are essential for the virus life cycle in cultured cells (Tratschin *et al.*, 1998). N^{pro} interacts directly with interferon regulatory factor 3 (IRF3) mediating its degradation; thus it has an antagonistic effect on the interferon-α/β pathway and a role in early innate immune evasion by CSFV (Gottipati *et al.*, 2014, 2016; Ruggli *et al.*, 2003). Moreover, N^{pro}, along with NS2, is an autoprotease cleaving itself off the viral polyprotein. The timing of the self-cleavage of NS2 is essential for the viral life cycle as the uncleaved NS2-3 is important in CSFV particle formation and replication (Moulin *et al.*, 2007). NS3 contains an N-terminal serine protease domain that requires NS4A as a protein cofactor. The protease cleaves between leucine and small uncharged amino acids and its function is essential for viral RNA replication as substitutions eliminating the protease activity abolish the formation of new viral progeny, as shown for BVDV (Grassmann *et al.*, 1999). In addition, NS3 harbours nucleoside triphosphatase (NTPase) and helicase activity. The helicase domain of NS3 has been shown to interact with the IRES which promotes IRES-mediated translation (Gorbalenya *et al.*, 1989; Zhu *et al.*, 2010). Promotion of IRES-mediated viral translation may occur in a similar manner as has been

demonstrated for a well-characterised RNA helicase, the eukaryotic translation initiation factor eIF4A. eIF4A is believed to be involved in scanning of the 40S ribosome along the mRNA by disrupting secondary structures upstream of the initiation codon (Pause & Sonenberg, 1992; Rozen *et al.*, 1990). NS5A is the only protein of the replication complex that can be complemented in *trans* and viral RNA replication is inhibited when the concentration of NS5A exceeds that of NS5B (Chen *et al.*, 2012; Grassmann *et al.*, 2001). The CSFV p7 protein functions as a viroporin creating hydrophobic pores at the membrane of virus-infected cells (Gladue *et al.*, 2012; Guo *et al.*, 2013; Largo *et al.*, 2016). Furthermore, p7 induces the secretion of the pro-inflammatory cytokine interleukin-1β (IL-1β) that is important in the innate immune response to viral infection (Lin *et al.*, 2014).

Viral population dynamics

RNA viruses like CSFV have a high mutation rate due to the lack of proof-reading capabilities of the RdRp resulting in low fidelity replication (Drake, 1993). The estimated error rates among RNA viruses range from 10^{-3} to 10^{-5} substitutions per nucleotide, which are several magnitudes higher than that for cellular DNA replication (Domingo & Holland, 1997; Drake & Holland, 1999). As a consequence, RNA viruses replicate as a swarm of closely related viruses but with slightly different genomes, called a viral quasispecies. The high mutation rate can be beneficial for the virus making it capable of evading the immune response of the host due to changed antigenic determinants. If the mutation frequency becomes too high, it can be detrimental for the virus. This property has been utilised by researchers to develop a new antiviral strategy, termed lethal mutagenesis (Agudo *et al.*, 2016; Arias *et al.*, 2013; Perales *et al.*, 2009). The aim is to increase the replication error rates above the threshold level for virus viability and this approach has been shown to yield decreased virus infectivity and pathogenicity *in vivo* (Gnädig *et al.*, 2012; Sanz-Ramos *et al.*, 2012). In contrast, increasing the replication fidelity results in attenuation of the virus highlighting the finely-tuned relationship between the mutation rate and viral replication (Vignuzzi *et al.*, 2008; Zeng *et al.*, 2014).

Molecular cloning of CSFV cDNA

The ability to genetically modify virus genomes has provided a powerful tool for the study of the structure and function of the viral genome including viral replication, virus − host interactions and as a way to engineer genetically-defined vaccines. Reverse genetics has proven an efficient approach to do just that by making precise genetic alterations with the purpose of studying certain phenotypic traits.

For CSFV, the first cDNA clone derived from the genomic RNA was reported in 1989 (Meyers *et al.*, 1989). By using four overlapping clones, a large fraction of the genome was obtained constituting a continuous sequence of 12,284 nucleotides. Later on, construction of a full-length cDNA clone (ca. 12.3 kb) of the highly virulent Alfort/187 strain was performed and, in the same year, an infectious cDNA corresponding to the avirulent CSFV C-strain was generated (Ruggli *et al.*, 1996). This formed the basis for the development of a C-strain marker vaccine (Moormann *et al.*, 1996). However, the early cDNA cloning procedure was tedious and problems occurred with maintaining the large cDNA in standard plasmid vectors in *E. coli*. Introduction of cDNA into bacterial artificial chromosomes (BACs) proved a way to circumvent this problem (Rasmussen *et al.*, 2010). The BAC cloning system was developed for the cloning of large eukaryotic DNA fragments (80 − 300 kb) and has now been applied to a large number of DNA viruses and some RNA viruses (Tischer & Kaufer, 2012). A major advantage of using BAC vectors to maintain virus genomes is the well-established mutagenesis techniques in *E. coli* and they have enabled the construction of novel RNA genomes by using targeted recombination-mediated mutagenesis and virus rescue (Rasmussen *et al.*, 2013).

Chapter 2

CSFV viral life cycle

The infectious cycle of CSFV can be considered in several steps including

(i) attachment and entry
(ii) release of the genome into the cytosol
(iii) translation of the genomic viral RNA into structural proteins and non-structural proteins
(iv) assembly of a replication complex on the surface of the endoplasmatic reticulum (ER)
(v) amplification of the viral genome via a double-stranded RNA intermediate
(vi) virion assembly, maturation and release

An overview of the CSFV life cycle can be seen in Figure 3.

Attachment and entry

The earliest stage of infection in the virus life cycle happens when the virus particle comes in contact with the host cell. Virus entry is specific for susceptible host cells depending on viral surface proteins and host cell receptors. Binding of the virus to a receptor on the surface of the cell is the first step in a cascade of interactions between the host cell and the virus required to initiate viral infection. Numerous viruses use the ubiquitously expressed cell surface polysaccharide heparan sulfate as a receptor in assisting viral binding and entry (Liu & Thorp, 2002). Enhanced binding to heparan sulfate during propagation in cell culture has been observed for CSFV (Eymann-Häni *et al.*, 2011). Adaptation of CSFV to heparan sulfate has been related to amino acid changes in the envelope protein E^{rns}, which caused altered replication characteristics both *in vitro* and *in vivo* but only minor effects on the virulence (Mayer *et al.*, 2003).

The binding of the CSFV particle to the host cell is facilitated by interactions between the surface-exposed viral envelope proteins E^{rns}, E1 and E2 and cellular receptors (Hulst &

Moormann, 1997; Wang *et al.*, 2004). Porcine complement regulatory protein CD46 has been demonstrated to be the major cellular receptor for CSFV mediating viral entry along with heparan sulfate (Dräger *et al.*, 2015). CD46 has been known as a cellular receptor for BVDV for some years (Maurer *et al.*, 2004). It is believed that BVDV and CSFV have a similar route of infection as both display decreased infectivity in inhibition experiments with the CSFV E2 protein (Hulst & Moormann, 1997). Recently, the mechanism by which CSFV gains entry into the cell is demonstrated to be similar to that of BVDV (Shi *et al.*, 2016). BVDV enters the cell by clathrin-dependent endocytosis and fusion takes place with an acidic endosomal compartment where the low pH triggers conformational changes exposing fusion peptides (Lecot *et al.*, 2005). Such a fusion motif is localised within the E1 structural protein whereas the E2 acts as a structural scaffold (El Omari *et al.*, 2013). In CSFV, putative fusion peptides are found in the E2 protein (Fernández-Sainz *et al.*, 2014; Holinka *et al.*, 2016). This triggers fusion of viral and endosomal membranes and the virus particle becomes internalised. However, BVDV and CSFV have one characteristic that does not fit well with this mechanism as they are both highly stable in acidic environments (Krey *et al.*, 2005). As pestiviruses are acquired via direct or indirect contact, the acid-resistant phenotype might have appeared to reflect a need for enhanced stability in harsh environments. This is unlike other members of the *Flaviviridae* family, such as some of the flaviviruses, where transmission occurs by arthropods making the requirement for protection against environmental hazards redundant. Besides the acidification, a maturation event is thought to occur in pestiviruses, where complex intermolecular disulphide bridges between the glycoproteins are destabilised allowing fusion between the virion and the endosome. After the nucleocapsid has been uncoated, which requires a non-degradative ubiquitination step, the viral genome is delivered to the cytosol for translation and replication (Byk *et al.*, 2016; Rajsbaum & García-Sastre, 2014).

Viral RNA translation and replication

Following release of the viral RNA into the cytosol, the genome serves as an mRNA for translation of the encoded proteins and as a template during viral RNA replication to produce new viral RNA genomes. These processes occur at intracellular membranes that become rearranged giving rise to viral replication complexes (VRCs) (Shulla & Randall, 2016). Formation of VRCs is a distinctive feature of positive-stranded RNA viruses. The RNA is

protected from innate immune sensors and the RNA degradation machinery, located in the cytosol, as the replication complexes serve as a barrier between viral RNA replication and the cytosol shielding the viral genome from host defences (den Boon *et al.*, 2010).

Positive-stranded RNA viruses can be classified into three superfamilies; picorna-like, flavi-like and alpha-like viruses based on the amino acid homology of their RdRps (Koonin *et al.*, 1993; Koonin, 1991). Among these viruses, two different modes to remodel the cellular membrane, during formation of VRCs, exist. The picorna-like viruses establish membranes with positive curvature forming double-membrane structures while the latter two superfamilies induce negatively curved membranes initiated by invaginations of the pre-existing membrane bilayer (Belov & Van Kuppeveld, 2012; Romero-Brey & Bartenschlager, 2014). These membrane-associated replication complexes are composed of viral proteins, replicating RNA and altered cellular membranes (Ahlquist *et al.*, 2003). Although CSFV has a limited number of proteins encoded by its genome, it can replicate very efficiently in infected host cells by using host proteins, membranes, lipids and metabolites.

Following cytosolic entry of the viral RNA, ribosomes are recruited to achieve translation. As CSFV is positive-stranded, the RNA can directly serve as a mRNA. Ribosomes bind to a highly

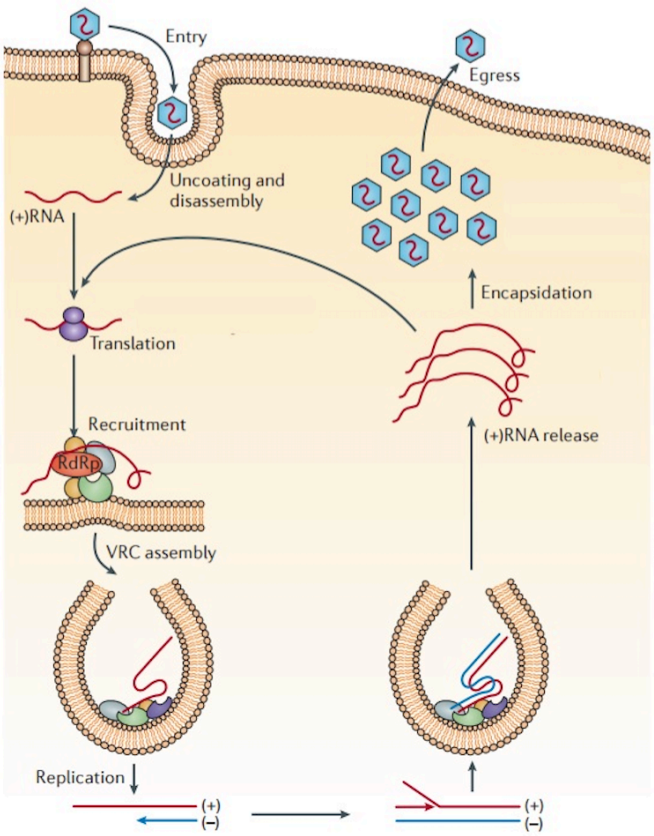

Figure 3. Overview of the CSFV viral life cycle.
The virus enters the cell by endocytosis and once inside the cell, the positive-stranded RNA ((+)RNA) genome is released into the cytosol. Here the RNA is translated by host ribosomes and the polyprotein is processed into structural and non-structural proteins. The non-structural proteins form a viral replication complex (VRC) and recruits the (+)RNA to the endoplasmatic reticulum (ER). Viral replication is initiated and (+)RNA is released that can either start a new cycle of translation and replication or become encapsidated and released by exocytosis. Modified from (Nagy & Pogany, 2011).

structured part of the genome termed the IRES to direct cap-independent translation initiation of protein synthesis (Fletcher & Jackson, 2002). As the viral genome is translated in the 5' – 3' direction, viral replication by the polymerase, that proceeds in the 3' – 5' direction, is blocked. Hence translation and RNA replication are mutually exclusive processes, because they proceed in opposite directions on the RNA template. Switching between these two mechanisms is an intricate process where both viral and cellular proteins play a critical role (Gamarnik & Andino, 1998). Moreover, regulatory RNA elements contribute in facilitating and regulating these genomic mechanisms.

The viral non-structural protein NS5A plays a pivotal role in the switch from translation to replication. A region in the C-terminal part of NS5A represses IRES-directed translation in a dose-dependent manner, which may lead to switching to viral replication when the level of NS5A exceeds a certain threshold (Xiao *et al.*, 2009). NS5A has also been shown to interact with the host protein FKBP8 to promote viral replication (Li *et al.*, 2016). Furthermore, interaction between NS5B and NS5A suppresses the inhibitory effect that NS5A has on viral RNA translation (Sheng *et al.*, 2012a). Three discontinuous fragments of NS5A (including amino acids 137 – 172, 224 – 268 and 390 – 414) are essential for the binding of NS5A to NS5B (Chen *et al.*, 2012). Small amounts of NS5A stimulate viral replication but when the concentration of NS5A exceeds that of NS5B, viral replication is inhibited stressing the role of NS5A in modulating viral replication through concentration changes. This suggests that NS5A can regulate viral RNA synthesis by binding to NS5B. Moreover, both NS5A and NS5B interact with the 3'-UTR but NS5A exhibits a stronger binding capacity to its cognate 3'-UTR (Sheng *et al.*, 2012b). When the concentration of NS5A exceeds that of NS5B, it outcompetes the binding of NS5B to the 3'-UTR resulting in an inhibition of viral RNA synthesis. In hepatitis C virus (HCV), another member of the *Flaviviridae* family, the small non-coding miRNA miR-122 has been shown to interact with the 5'-UTR which stimulates HCV replication (Jopling *et al.*, 2005). The dependence on host miRNAs for pestivirus replication has also been demonstrated (Scheel *et al.*, 2016).

CSFV genome replication consists of several consecutive processes. First, the replicase NS5B recognises and binds to the 3'-UTR starting RNA synthesis in which a minus-strand RNA is produced using the plus-strand genomic RNA as a template. Using the newly generated minus-strand RNA, a progeny plus-strand RNA is generated. For CSFV, the RdRp NS5B functions

as the replicase. NS5B has been found to form a complex with both the plus- and minus-strand of the genomic 3'-UTR, but binds with highest efficiency to the minus-strand RNA. Following deletion of the five 3'-terminal bases (CCCGG) initiation of minus-strand synthesis is blocked (Xiao *et al.*, 2004). In particular, the three continuous Cs at the 3'-terminus are essential for the initiation of RNA synthesis, which was tested by making mutants with consecutive deletions in the CCCGG motif. A fragment spanning the last 21 nucleotides of the 3'-terminus has been identified as a recognition site for the NS5B replicase responsible for viral replication (Xiao *et al.*, 2002b). Deletion of these 21 nucleotides abolishes the binding of NS5B and initiation of minus-stranded synthesis is lost (Xiao *et al.*, 2004). This suggests that RNA genome replication is initiated by binding of NS5B to the 21 nucleotide fragment located at the 3'-end of the plus-strand.

Assembly and release

Assembly of pestivirus virions is initiated at ER-derived membranes by budding of viral capsids (Grummer *et al.*, 2001). Once the virions are in the ER lumen, they are probably taken up into vesicles and budded from exit sites in the ER. This takes place in the ER-Golgi intermediate compartment, which mediates trafficking between the ER and Golgi apparatus. Pestivirus virions pass through the Golgi apparatus where sugar monomers are attached to the glycoproteins by glycosylation enzymes (Jordan *et al.*, 2002). The virions are then transported to the cell surface, where single-particle exocytosis occurs (Schmeiser *et al.*, 2014). However, pestivirus morphogenesis is still poorly understood.

Pestivirus infection is sensitive to brefeldin A, a drug that blocks protein export from the ER and causes disruption of the Golgi apparatus (Macovei *et al.*, 2006). Upon treatment with brefeldin A, infectious pestivirus particles accumulate within cells thus the release of virions is inhibited. This suggests that pestivirus assembly is completed within the ER and the Golgi apparatus without the requirement of further processing before maturation and egress.

Besides the involvement of NS5A in the translation/replication switch, it is also linked to virus assembly. The effect of NS5A on CSFV assembly and production is thought to be related to its interaction with the core protein (Sheng *et al.*, 2014). The same mechanism has been described for HCV (Appel *et al.*, 2008). NS5A has been shown to associate with the cellular protein

annexin A2 which is a lipid raft-associated scaffold protein that is involved in a number of cellular processes including membrane trafficking, endosome formation, aggregation of vesicles and exocytosis (Donnelly & Moss, 1997). Annexin A2 interacts with several viruses and mediates virus entry, replication, assembly, budding and release. A direct interaction has been found between NS5A and annexin A2 and this is necessary for CSFV assembly (Sheng *et al.*, 2015). The same relationship has previously been demonstrated for HCV (Masaki *et al.*, 2008). A proteomic analysis shows that annexin A2 is upregulated about 5-fold in CSFV-infected cells (Sun *et al.*, 2008). Furthermore, knockdown of annexin A2 significantly reduces infectious CSFV production but has no effect on viral RNA replication.

Studying viral replication using reporter proteins

Advances in molecular biology have allowed manipulation of RNA genomes and led to development of replicons, which are self-replicating but non-infectious RNAs. In these RNA replicons at least one essential structural protein has been deleted (Khromykh, 2000). Deletion of one or more of the structural proteins renders virion assembly impossible making it unable to escape the infected cell. Hence, replicons can be regarded as disabled viruses unable to produce infectious progeny. The presence of a reporter protein within the replicon allows quantitative assessment of the replication competence of a given RNA virus. The reporter protein is cloned in frame with the upstream protein and to ensure that the proteins downstream from the reporter are properly freed, a 2A peptide from e.g. foot-and-mouth disease virus (FMDV) or encephalomyocarditis virus (EMCV) can be inserted. The 2A peptide induces translational skipping (at a …NPG/P motif) resulting in a co-translational break in of the polypeptide chain after the NPG sequence by prevention of peptide bond formation (Donnelly *et al.*, 2001; Ryan & Drew, 1994). Translation resumes from the next codon (encoding the P residue) in the downstream protein sequence without the requirement for a normal translation initiation process.

Reporter proteins can be applicable in two ways; either for qualitative or quantitative assays. The qualitative reporters allow *in vivo* studies of protein localisation during different parts of the virus life cycle. This can be obtained using fluorescent proteins like the green fluorescent protein (GFP), DsRed or mCherry (David *et al.*, 2015; Isken *et al.*, 2014; Osborn *et al.*, 2005). By using

enhanced GFP (EGFP) a dark-to-bright fluorescent reporter has been generated for CSFV (Chen *et al.*, 2015). EGFP is non-fluorescent until the protein is properly folded due to fusion to a quenching peptide. Hence cleavage of the quenching peptide is required for light emission, which can be carried out by the NS3 protease during CSFV infection. A strong correlation between CSFV replication and fluorescence intensity is seen allowing an easy and rapid detection of CSFV growth.

The quantitative reporters are able to catalyse enzymatic reactions making it possible to determine translational or transcriptional activities. Examples of such reporters are luciferase and β-galactosidase (Greer & Szalay, 2002; Pai *et al.*, 2005). A reporter capable of stably expressing the *Gaussia* luciferase protein has been generated in a full-length West Nile virus cDNA clone (Zhang *et al.*, 2016). A linear correlation is observed between the luciferase signal and viral titers determined by a plaque assay, which allows a fast and sensitive quantification of viral replication. By coupling flow cytometry and GFP fluorescence measurements, GFP can also be used as a quantitative reporter (Soboleski *et al.*, 2005). There are several advantages in using GFP as a reporter; intracellular accumulation can be directly observed *in vivo* over time and GFP expression can be measured for individual cells.

The use of replicons has made it easier to study some aspects of virus replication. Deletion of most of the viral structural proteins has been applied in several replicon designs, including in BVDV and CSFV (Behrens *et al.*, 1998; Moser *et al.*, 1999). These replicon constructs demonstrate that NS3, NS4A, NS4B, NS5A and NS5B are essential for pestivirus replication. Replication determinants of CSFV have been studied by inserting the RdRp NS5B of the highly virulent Koslov strain into the backbone of the moderately virulent Paderborn strain (Risager *et al.*, 2013). The presence of the Koslov virus NS5B coding sequence significantly enhanced viral RNA replication while replacing the Paderborn NS5B with that of the avirulent C-strain decreased the replication. This demonstrates that replication efficiency is dependent on the viral replicase NS5B. A similar approach has been conducted for NS4B, where the N-terminal domain of NS4B from a low virulence strain was exchanged with the corresponding sequence from a highly virulent strain (Tamura *et al.*, 2015). The N-terminal domain of NS4B harbours five amino acid differences between the two strains and when the replication efficiency was tested, these five amino acids from the highly virulent strain markedly contributed to enhanced viral replication. Moreover, when this chimeric virus was inoculated into pigs, it resulted in clear

clinical symptoms thus showing that the N-terminal domain of NS4B contributes to pathogenicity in pigs.

Replicons have been used in vaccine development, thereby eliminating the risk of reversion when using conventional live attenuated vaccines. Viral structural proteins can be replaced with vaccine antigens allowing the viral vectors to replicate their genome without being infectious as they cannot make new virus particles (Lundstrom, 2005). Another strategy has been utilised for CSFV in which deletion mutants are *trans*-complemented after transfection. *Trans*-complementation can be achieved by expressing the viral proteins missing from the replicon in *trans*. The packaged replicons will only infect cells once and thereby deliver the viral RNA into the cytosol. Transmission of the virus particles is only made possible by introducing the replicons into a cell line that expresses the missing structural proteins. This has been done for CSFV; in some systems the structural proteins E^{rns} or E2 have been deleted and the replicons are later transfected into cells expressing E^{rns} or E2 (Van Gennip *et al.*, 2002; Widjojoatmodjo *et al.*, 2000). These replicons are denoted virus replicon particles (VRPs) and have the advantage of being non-transmissible, which make them safe as vaccines as they cannot revert to a virulent serotype. When pigs were infected with a highly virulent CSFV strain four weeks after immunisation with the VRPs, the pigs displayed protection against the virus challenge (Widjojoatmodjo *et al.*, 2000).

Chapter 3

Functional structural RNA elements

RNA structure and function

In 1956, Francis Crick formulates the central dogma where genetic information in the form of DNA becomes transcribed into RNA, which is then translated into proteins (Thieffry & Sarkar, 1998). As such RNA is merely considered as a messenger for translation (Jacob & Monod, 1961). Today, understanding of the biological functions of RNA have broadened to include various structural, regulatory and enzymatic functions to this ribonucleic acid (Garst *et al.*, 2011; Li *et al.*, 2012). This has led to the term 'the RNA world' hypothesising that RNA has a decisive role in the evolution of life by sustaining genetic continuity through RNA replication and catalytic properties (Gilbert, 1986).

Functional structural RNA elements are in general created by three-dimensional organisation of small structural motifs, formed by base pairing between self-complementary sequences from different parts of the RNA chain. This enables the RNA to adopt unique shapes allowing *cis*- and *trans*-actions, i.e. interactions within the RNA or with proteins. Changes in base pairing function is a simple yet powerful way to regulate viral processes by exposing or sequestering nucleotides that can interact with other parts of the RNA chain or with proteins.

Conserved RNA secondary structures in *Flaviviridae*

The nature of RNA facilitates the generation of complex three-dimensional structures despite being composed of only four chemically similar nucleotides. Although RNA can exist as a single-stranded molecule, it was in 1956 discovered to also form double-stranded structures (Rich & Davies, 1956). RNA can fold into several secondary structures including duplexes, hairpins, internal bulges, internal loops and junctions (Figure 4). Loops have shown to be critical for the formation of long-range RNA-RNA interactions and important in the recognition of small molecules, whereas the single-stranded regions often have a function as binding sites for proteins (Auweter *et al.*, 2006; Lunde *et al.*, 2007; Nicholson & White, 2014).

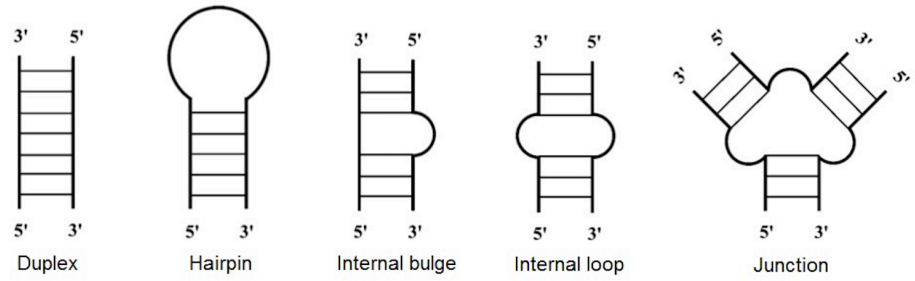

Figure 4. RNA secondary structure. Due to the single-stranded nature of RNA and its ability to base pair, it can fold into numerous structures. Adapted from (Ding & Lawrence, 2003).

Viral RNA genomes do not solely encode proteins but can also harbour RNA motifs that play a crucial role in the viral life cycle. An example of such a motif is the IRES located in the 5'-UTR. The IRES was discovered nearly 30 years ago in two picornaviruses describing an alternative translation initiation mechanism independent of the 5'-cap structure (Jang *et al.*, 1988; Pelletier & Sonenberg, 1988). Based on the secondary structure, the picornavirus IRES elements are divided into at least five types in addition to atypical IRES elements, e.g. present within dicistroviruses (Lozano & Martínez-Salas, 2015; Roberts & Groppelli, 2009). IRES-mediated translation is dependent on both canonical initiation factors and IRES trans-activating factors (ITAFs). ITAFs are cellular proteins which can serve as RNA chaperones which help stabilising the IRES configuration (Pacheco & Martinez-Salas, 2010). In addition, they may regulate IRES activity thereby mediating cell type specificity and consequently influence viral spread.

The presence of an IRES allows the virus to begin efficient translation without the need of 5'-capping. Moreover, the virus can block the cap-dependent translation initiation of the host without compromising the viral protein synthesis, thus hijacking the cellular translation machinery (Bushell & Sarnow, 2002). Within the *Flaviviridae* family, two types of translation initiation are reported. *Hepacivirus* and *Pestivirus* contain an IRES with a long 5'-UTR that might be derived from certain members of the picornavirus family (Belsham, 2009). On the contrary, viruses within the *Flavivirus* genus, which have an insect host, harbour a capped RNA and no IRES but instead requires an interaction between the two untranslated regions for translation.

The untranslated regions in *Pestivirus* have been shown to form extensive secondary structures (Brown *et al.*, 1992; Deng & Brock, 1993). About 70 % of the nucleotides in the 5'-UTR are involved in base pairing, where a large part (ca. 350 nucleotides) is constituted of the IRES. In

particular domains II and III of the 5'-UTR have been identified to be key elements for translation of the CSFV genome (Fletcher & Jackson, 2002; Friis *et al.*, 2012).

Secondary and tertiary structural motifs in the 3'-UTR are considered to be important functional elements for minus-strand RNA synthesis and might function as sites of promoter elements that controls this synthesis. The *Pestivirus* 3'-UTR consists of four stem loops with a variable AU-rich region and a conserved region (Vilček *et al.*, 1999). The AU-rich regions can bind the host protein HuR, which is known to regulate RNA stability (Brennan & Steitz, 2001). The binding of HuR to the AU-rich regions in the 3'-UTR promotes HCV RNA replication (Shwetha *et al.*, 2015). Upon knockout of HuR, viral RNA replication is almost abolished in HCV, while it has no effect on the replication of the alphavirus Sindbis virus (SINV) (Marceau *et al.*, 2016). However, SINV is shown to have a high binding affinity of HuR resulting in increased viral yield but this effect may be due to other factors than viral replication (Sokoloski *et al.*, 2010).

Cis-acting RNA elements in plus-stranded RNA viruses

RNA elements contributing to interactions within the genome are denoted *cis*-acting RNA elements (CREs). They do not only contribute to RNA-RNA interactions but can recruit viral and host factors, which are known as *trans*-acting factors. CREs impact several aspects of the viral life cycle including genome replication, translation and encapsidation (Liu *et al.*, 2009). Some CREs are involved in long-range interactions that are found within a wide range of positive-stranded RNA viruses. A long-range interaction spans distances ranging from a few hundred nucleotides up to several thousands. These interactions are often located in internal bulges or loop regions bringing distant parts of the RNA chain closer together. An overview of known long-range interactions within *Flaviviridae* can be seen in Table 1.

Most of the interactions involve sequences located in 5'-UTR and 3'-UTR. However, some RNA structural elements can be found in the protein coding region too, as seen in HCV. A stem loop, in the coding region for the non-structural protein NS5B, denoted 5BSL3.2, can interact with either the 3'-UTR, the IRES or with the stem loop structure SL9110 located approximately 200 nucleotides upstream of 5BSL3.2 (Diviney *et al.*, 2008; Romero-López *et al.*,

2012; Romero-López & Berzal-Herranz, 2012). This network of interactions suggests how the transitions between the different steps of the viral life cycle of HCV are regulated. Early in the infection, the IRES will interact with the ribosome favouring the interactions with SL9110 and the 3'-UTR. Upon accumulation of viral proteins, a transition will occur towards viral replication making the 3'-UTR accessible to the polymerase due to contact between 5BSL3.2 and the IRES and SL9110.

Genus	***Virus***	***RNA-RNA interaction***	***Step in the viral life cycle***
Pestivirus	CSFV	IRES – 3'-UTR	Translation
Hepacivirus	HCV	IRES – 5BSL3.2	Translation
		SL9110 – 5BSL3.2	
		5BSL3.2 – 3'-UTR	Genome replication
		DLS – DLS	
Flavivirus	DENV + WNV	5'-UAR – 3'-UAR	Genome replication
		5'-DAR – 3'-DAR	
		5'-CS – 3'-CS	

Table 1. Long-range RNA-RNA interactions within the *Flaviviridae* family.
Cis-acting RNA interactions influencing translation or replication found within the members of the *Flaviviridae* family. Adapted from (Nicholson & White, 2014).
Abbreviations: 5BSL3.2, NS5B stem loop 3.2; CS, cyclisation sequence; CSFV, classical swine fever virus; DAR, downstream of AUG region; DENV, dengue virus; DLS, dimer linkage sequence; HCV, hepatitis C virus; IRES, internal ribosome entry site; SL, stem loop; UAR, upstream of AUG region; UTR, untranslated region; WNV, West Nile virus.

A proper balance in these interaction networks is pivotal for accurate regulation of viral translation and replication. Identifying protein-protein interactions between viral and cellular proteins allows a more comprehensive understanding of the dynamics of viral translation, RNA replication and host specificity. Several host factors are recruited to viral genomes and have been shown to influence and stabilise interactions within the RNA. Recently, 55 cellular proteins were found to interact with an RNA motif located within the 3'-end of the ORF of HCV consisting of the stem loops 5BSL3.1-3.3 (Ríos-Marco *et al.*, 2016). Moreover, a number of host proteins have been found to bind the non-structural protein NS5A. For example, NS5A interacts with the host cell protein heat shock protein 70 (HSP70) in CSFV and HCV (Khachatoorian *et al.*, 2014; Zhang *et al.*, 2015). The cellular heat shock proteins have shown to play an important role in the replication of RNA viruses (Vasconcelos *et al.*, 1998; Weeks & Miller, 2008; Zheng *et al.*, 2010). Also an interaction between NS5A and the eukaryotic

elongation factor 1A (eEF1A) has been established in BVDV and CSFV (Johnson *et al.*, 2001; Li *et al.*, 2015).

Both cellular and viral proteins are involved in facilitating *cis*-acting interactions between the 5'-UTR and 3'-UTR. A cellular protein associated with bringing the two untranslated regions together is the human La protein. The La protein functions in the unwinding of double-stranded RNA and in the maturation of the RNA polymerase III (Wolin & Cedervall, 2002). Its function as a CRE has been seen in HCV, coxsackie B virus and Japanese encephalitis virus (Kumar *et al.*, 2013; Souii *et al.*, 2015; Vashist *et al.*, 2011). Furthermore, the La protein has been found to interact with the 5'-UTR of poliovirus and encephalomyocarditis virus stimulating viral translation (Kim & Jang, 1999; Meerovitch *et al.*, 1993). Also viral proteins within the *Flaviviridae* family play a role in end-to-end interactions. An example is the viral core protein in West Nile virus (WNV) and NS3 in Dengue virus (DENV) which chaperone 5'-UTR to 3'-UTR annealing (Gebhard *et al.*, 2012; Ivanyi-Nagy & Darlix, 2012). A *cis*-acting RNA element present within the CSFV 3'-UTR consisting of a hexamer of the terminal bases (CCCGGC) negatively regulates translation (Huang *et al.*, 2012). This is in concordance with a block in viral replication upon deletion of the pentamer CCCGG (Xiao *et al.*, 2004) Indications have been seen for a requirement of the 5'-UTR and 3'-UTR of CSFV to participate in a *cis*-interaction. This was observed when studying apoptosis; the severity of this process was increased when both UTRs were transfected together compared to either the 5'-UTR or 3'-UTR alone (Hsu *et al.*, 2014). In the DENV genome circularisation is proposed to be important during minus-strand synthesis (Filomatori *et al.*, 2006). Direct long-range RNA-RNA interactions are present at the 5'-end and 3'-end of the RNA genome (Alvarez *et al.*, 2005; de Borba *et al.*, 2015). Genome circularisation is also a part of the RNA replication in poliovirus, where the mechanism is mediated through a protein-protein bridge consisting of ribonucleoproteins (Herold & Andino, 2001). Many RNA binding proteins have multiple RNA binding domains allowing them to bring separate RNA elements together.

RNA secondary structure probing

Insight into the mechanisms of actions of RNA requires a thorough knowledge of its structure. Accurate probing using selective 2'-hydroxyl acylation analysed by primer extension (SHAPE)

provides quantitative reactivity information of the RNA nucleotides (Merino *et al.*, 2005; Wilkinson *et al.*, 2006). This is achieved as flexible RNA nucleotides show enhanced reactivity towards the SHAPE reagents. Hence, single-stranded nucleotides are more prone to react with SHAPE reagents, while base paired and structurally constrained nucleotides are less reactive. As the SHAPE technology is backbone specific, it is independent of base identity thus providing information on local structure. Typically, SHAPE reactions are performed together with a control reaction without a probing reagent. The data from the control experiment is used to normalise the data from the probed reaction to provide specific SHAPE reactivity for a given nucleotide position.

Before SHAPE reagents were widely used for structural probing, either chemical or enzymatic probing were applied. The chemical and enzymatic reagents rely on several different approaches depending on the RNA molecule. These can be (i) RNase digestion, (ii) RNA crosslinking, (iii) binding of complementary nucleotides to single-stranded RNA regions and (iv) nuclease treatment to determine scissions specific for single-stranded or double-stranded regions (Ehresmann *et al.*, 1987). These methods can be quite laborious and many are not generalizable giving biases in their reactivity towards some bases and showing a hierarchy of reactivity that is incompletely understood. Many of these shortcomings can be overcome by the SHAPE technology and this has now emerged as the gold standard in RNA secondary structure probing (Deigan *et al.*, 2008; McGinnis *et al.*, 2012; Weeks & Mauger, 2011).

Especially two SHAPE reagents are utilised with slightly different reaction profiles, which is useful in examining the presence of different structures. The two reagents are *N*-methylisatoic anhydride (NMIA) and 1-methyl-7-nitroisatoic anhydride (1M7) which are hydroxyl-selective electrophiles that react with the 2'-hydroxyl group of flexible nucleotides in the RNA (Weeks & Mauger, 2011). 1M7 has a reaction time of 14 seconds for one hydrolysis half-life whereas NMIA reacts about 18 times slower (Mortimer & Weeks, 2007; Weeks & Mauger, 2011). This means that NMIA can detect nucleotides in unusual conformations that have slow reaction dynamics. An alternative SHAPE reagent, denoted *N*-propanone isatoic anhydride (NPIA), allows removal of a large amount of the background signal and enriches for probed RNA by covalent coupling to a biotin molecule (Poulsen *et al.*, 2015a). The issue with high background signal is especially seen with whole virus genome structure probing. However, the ability to perform whole genome probing has allowed the study of the tertiary structure giving

information on long-range interactions that bring individual structural elements together to create higher-ordered structures. SHAPE has been used to map long-range interactions in a number of viruses i.e. HCV, human immunodeficiency virus (HIV) and Moloney murine sarcoma virus, which have provided a broader picture of the interaction networks during viral translation and RNA replication (Badorrek & Weeks, 2005; Legiewicz *et al.*, 2010; Tuplin *et al.*, 2012).

NGS and whole genome RNA structure probing

Over the past ten years, a shift has occurred from Sanger sequencing, considered as a first-generation technology, towards newer methods called next generation sequencing (Metzker, 2010). Compared to Sanger sequencing, NGS includes a cell-free system for DNA library preparation, its sequencing reactions produce thousands to millions of reads compared to hundreds and lastly is the sequencing output detected directly (van Dijk *et al.*, 2014). SHAPE chemistry can be combined with next generation sequencing (NGS) allowing multiplexed paired-end deep sequencing of primer extension products. This enables determination of location and frequency of modifications by the SHAPE reagents giving information regarding structural constraints such as base pairing or interaction patterns.

Combining RNA structure probing and NGS has revolutionised the knowledge about RNA by enabling transcriptome-wide structure probing giving information on the biological and chemical properties of RNA structure and function (Kwok *et al.*, 2015; Smola *et al.*, 2015). Two techniques to examine high-ordered structures have emerged using either SHAPE or chemical probing reagents. These are called SHAPE-mutational profiling (SHAPE-MaP) and RNA interaction groups by mutational profiling (RING-MaP) (Homan *et al.*, 2014; Siegfried *et al.*, 2014). At sites of SHAPE or dimethyl sulfate modifications, these methods incorporate non-complementary nucleotides and the modification sites are revealed by subsequent NGS analysis and mutational profiling. An addition to chemical probing and SHAPE is a high-throughput crosslinking-based structure analysis able to detect RNA duplexes (Weidmann *et al.*, 2016). This method crosslinks paired RNA strands and ligates the two strands together, which are then sequenced identifying interacting RNA sequences.

An improved RNA secondary structure model has been achieved by incorporating SHAPE reactivity data with algorithms based on pseudo-free energy and thermodynamic principles (Low & Weeks, 2010). Using this approach, the global organisation of two RNA viruses, the 1,058 nucleotide-long satellite tobacco mosaic virus and the 4,778 nucleotide-long tomato bushy stunt virus have been predicted (Archer *et al.*, 2013; Athavale *et al.*, 2013). The results have provided insights into long-range interactions in the context of the whole genome. Algorithms designed to study long-range interactions in full viral genomes have been developed, which have confirmed a number of already known interactions within various positive-strand RNA viruses as well as predicting additional interactions (Fricke & Marz, 2016). Having statistically strong and user friendly computational tools are essential for systematic analysis of large-scale NGS-based structure data.

Part 2 • Thesis outline

Chapter 4

Thesis outline

This PhD study has focused on characterisation of replication determinants of classical swine fever virus (CSFV) resulting in the following project aims:

i. Analysis of sequence adaptations after serial passaging in cell culture (manuscript 1)
ii. Identification of replication determinants using targeted modifications of genetically defined viruses and replicons (manuscript 2)
iii. Generation of a stable, selectable replicon within mammalian cells able to express viral proteins in a cytoplasmic setting (manuscript 3)

In manuscript 1 with the title "Sequence adaptations during growth of rescued classical swine fever viruses in cell culture and within infected pigs" were viruses with mutations in the IRES serially passaged in cell culture and introduced into their natural host, the pig. Using full genome sequencing, adaptations both in the IRES domain and in the rest of the genome were analysed. RNA viruses including CSFV evolve rapidly due to the high error rate and lack of proofreading mechanism of the RNA-dependent RNA polymerase. Hence, the high mutation rates make the virus highly adaptable. Upon introduction of mutations, the virus can undergo compensatory adaptations, which can provide new insights into the biology of the virus.

Manuscript 2 entitled "Analysis of potential RNA interactions with a motif in the NS5B coding region of classical swine fever virus that influence viral RNA replication" has focused on the examination of replication determinants. Genetically defined viruses and replicons (self-replicating but non-infectious genomes) encoding reporter proteins have been used for the analysis of virus replication. Targeted modifications of the viral RNA genome have shown to reduce the replication efficiency. Use of replicons permits a quantitative assessment of the replication efficiency of the mutant RNAs. RNA has been generated by *in vitro* transcription from cDNAs containing different modifications and tested for their capacity to replicate in porcine kidney cells as measured by luciferase activity. Based on these results, previous studies

and theoretical modelling, a potential motif within the viral genome important for replication was identified. RNAs from these replicons was tested for their capacity to replicate in comparison to wild type virus. When replication determinants were identified, similar modifications were introduced into an established full-length CSFV BAC clone, which can generate infectious RNA. RNA transcripts from these new constructs were characterised in porcine kidney cells to determine infectivity and growth rate.

Manuscript 3 denoted "Establishment of a cytoplasmic expression system using a classical swine fever virus-based replicon" focused on the generation of a replicon containing neomycin phosphotransferase (NPT) conferring resistance to neomycin. This will facilitate the maintenance of replicons by selection within mammalian cells. The NPT selectable marker was inserted downstream from *Gaussia* luciferase functioning as a reporter protein. This will allow for establishment of a cytoplasmic expression system. Such a system makes it possible to stably express proteins, encoded by other positive-sense RNA viruses, without problems with nuclear RNA processing. Engineering of reporter sequences into the viral genome will enable the study of the virus life cycle in more detail and the production of more efficient and safer prototype vaccine candidates. Furthermore, insertion of a selection marker into a replicon should make it suitable for screening antiviral drugs that block virus translation and/or RNA replication.

Part 3 • Papers

Chapter 5

Manuscript 1

Sequence adaptations during growth of rescued classical swine fever viruses in cell culture and within infected pigs

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Sequence adaptations during growth of rescued classical swine fever viruses in cell culture and within infected pigs

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ABSTRACT

Classical swine fever virus (CSFV) causes an economically important disease of swine. Four different viruses were rescued from full-length cloned cDNAs derived from the Paderborn strain of CSFV. Three of these viruses had been modified by mutagenesis (with 7 or 8 nt changes) within stem 2 of the subdomain IIIf of the internal ribosome entry site (IRES) that directs the initiation of protein synthesis. Rescued viruses were inoculated into pigs. The rescued vPader10 virus, without modifications in the IRES, induced clinical disease in pigs that was very similar to that observed previously with the parental field strain and transmission to in-contact pigs occurred. Two sequence reversions, in the NS2 and NS5B coding regions, became dominant within the virus populations in these infected pigs. Rescued viruses, with mutant IRES elements, did not induce disease and only very limited circulation of viral RNA could be detected. However, the animals inoculated with these mutant viruses seroconverted against CSFV. Thus, these mutant viruses were highly attenuated *in vivo*. All 4 rescued viruses were also passaged up to 20 times in cell culture. Using full genome sequencing, the same two adaptations within each of four independent virus populations were observed that restored the coding sequence to that of the parental field strain. These adaptations occurred with different kinetics. The combination of reverse genetics and in depth, full genome sequencing provides a powerful approach to analyse virus adaptation and to identify key determinants of viral replication efficiency in cells and within host animals.

1. Introduction

Classical swine fever virus (CSFV) is the causative agent of the highly contagious, and economically important, disease termed classical swine fever (CSF). CSFV is a member of the *pestivirus* genus of the *Flaviviridae*. The CSFV genome is a single stranded, positive sense, RNA of approximately 12.3 kb (see Fig. 1a). The genome includes a single large open reading frame flanked by a 5′-untranslated region (UTR) of ca. 373 nt and a 3′-UTR of ca. 228 nt (Deng and Brock, 1993). The 5′-UTR of CSFV, like certain other members of the family *Flaviviridae* such as hepatitis C virus (HCV) and bovine viral diarrhea virus (BVDV), does not have the 5′-terminal cap structure found on all eukaryotic cellular mRNAs and on the genomes of the members of the *flavivirus* genus within this virus family. However, the pestivirus and HCV 5′-UTRs contain an internal ribosomal entry site (IRES) that directs the cap-independent initiation of protein synthesis (Wang et al., 1993; Rijnbrand et al., 1997; Chon et al., 1998). Structural analysis of the similar HCV and CSFV IRES elements has identified two major structural elements; these are a single large stem-loop (domain II) and a complex domain III (see Fig. 1b) (reviewed in Kieft et al., 2001; Fraser and Doudna, 2007; Lukavsky, 2009). The latter, in the CSFV IRES, contains several stem-loop structures (subdomains IIIa, IIIb, IIIc, $IIId_1$, $IIId_2$ and IIIe) together with a pseudoknot (subdomain IIIf) that comprises stem 1a, stem 1b and stem 2 (Deng and Brock, 1993; Fletcher and Jackson, 2002). The HCV IRES structure has an extra element (termed domain IV) compared to the CSFV IRES while the former lacks subdomain $IIId_2$.

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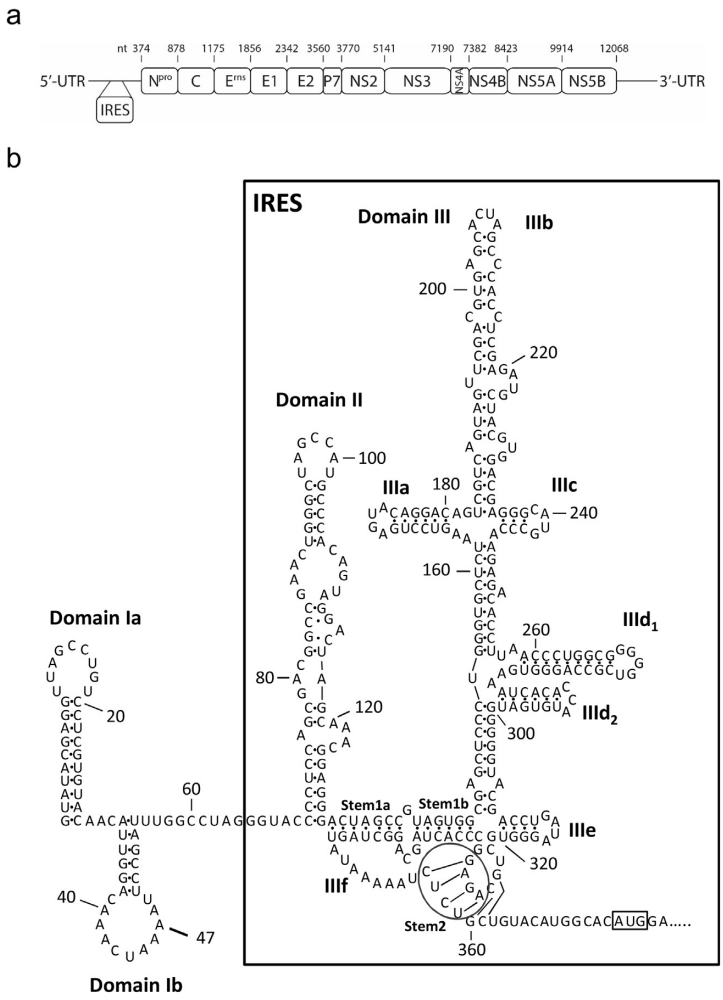


Fig. 1. Structure of the CSFV genome. (a) Genome organisation of CSFV indicating the location of the IRES and the individual protein coding regions. (b) Secondary structure and sequence of the CSFV IRES. The location of stem 2 within the pseudoknot (subdomain IIIf) is marked by an oval.

Extensive work has been performed to define the structures and functions of the different domains within the HCV and related IRES elements (reviewed in Lukavsky, 2009). Domain II is known to adopt a bent configuration; this structure induces a conformational change in the 40S ribosomal subunit needed for proper RNA docking and allows for the eIF5-mediated hydrolysis of the eIF2-bound GTP that precedes the formation of the 80S ribosomal complex (Spahn et al., 2001; Locker et al., 2007; Pestova et al., 2008). Domain III binds directly to the ribosomal 40S subunit through interactions with the subdomains $IIId_1$ and IIIf (Kieft et al., 2001; Lytle et al., 2002; Berry et al., 2010). The apical region of this domain, including a four-way junction formed by subdomains IIIa-c, binds to the translation initiation factor eIF3 (Kieft et al., 2001; Hashem et al., 2013).

Several studies have shown that mutations within domains II and III of the CSFV IRES lead to a reduction in translation initiation when measured using reporter gene assays (Rijnbrand et al., 1997; Fletcher and Jackson, 2002; Friis et al., 2012). In contrast, rather little is known about the effect of such mutations within the 5′-UTR on virus viability and growth in cell culture or susceptible host animals. For BVDV, it has been shown that mutations within domains Ia and Ib (upstream of the IRES) lead to a reduction in virus replication within cells in culture and within infected calves (Becher et al., 2000; Makoschey et al., 2004). Studies on the CSFV strain Alfort have shown that insertion of a 44 nt non-viral sequence into the loop of subdomain $IIId_2$ (at nt 294) had no major impact on virus growth in cells, however subsequent passage of the virus lead to generation of a deletion mutant in which 29 of these inserted nt were lost (Moser et al., 2001).

In a previous study (Friis et al., 2012), we introduced a range of mutant IRES elements into a full-length cDNA of the Paderborn strain of CSFV (Rasmussen et al., 2010). This genotype 2.1 strain has been established to be of moderate virulence (Uttenthal et al., 2003; Durand et al., 2009; Weesendorp et al., 2011). Viable viruses were rescued from three different mutants containing modifications within subdomain IIIf of the IRES. These mutant viruses each showed reduced growth characteristics in PK15 cells (Friis et al., 2012). We have now analysed the effect of such modifications within subdomain IIIf on the replication and pathogenicity of this strain of CSFV in swine, the native host. To complement these

studies, we assessed the genetic stability of these mutant viruses during serial passages in cells.

2. Materials and methods

2.1. Cells

Porcine kidney cells (PK15) cells were maintained in Dulbecco's Modified Eagles medium (DMEM) supplemented with 5% fetal calf serum.

2.2. Bacterial artificial chromosomes (BACs)

The construction of a BAC (termed pBeloPader10) containing the full-length CSFV Paderborn strain cDNA (Rasmussen et al., 2010) and mutant BACs with modifications within the IRES region of the CSFV genome have been described previously (Friis et al., 2012). The coding sequence of pBeloPader10 (GenBank Accession number GQ902941) differs from the parental CSFV strain Paderborn sequence (GenBank Accession number AY072924) at 6 nt positions. These nt substitutions result in the following changes (aa substitution, protein): C4136U (H1255Y, NS2); U7238C (Y2289H, NS4A); U7853A (Y2494N, NS4B); C10582U (silent, NS5B); G10989A (G3539E, NS5B) and G11864A (G3831R, NS5B). Three mutant BACs derived from pBeloPader10 containing additional modifications within subdomain IIIf (the pseudoknot) of the IRES element (termed PaderS2m6, PaderS2m7 and PaderS2m8) were also used in this study. Viruses rescued from two of these mutants, vPaderS2m6 and vPaderS2m7, were described previously by Friis et al. (2012). The PaderS2m8 clone was prepared as for the other mutants (as described by Friis et al., 2012). No analysis of the IRES activity of this mutant has been performed.

2.3. Preparation and passaging of viruses in cell culture

Recovery of infectious virus from the wt and mutant BACs and the determination of virus titers were performed as previously described (Friis et al., 2012). Virus rescued from the pBeloPader10 was termed vPader10 and the viruses with mutations in the stem 2 of the pseudoknot that were rescued from the modified BACs were termed vPaderS2m6, vPaderS2m7 and vPaderS2m8 respectively. Viruses at passage 4 (P4) were used for the animal inoculation (see below).

The vPader10 and mutant viruses were passaged a total of 20 times in PK15 cells using the same procedure in each case, i.e. cells in a 24-well plate were infected with 150 μl of the previous virus supernatant and harvested after 3 days and stored at −80 °C. RNA was extracted from 1 ml of cell supernatant generated after 4, 5, 10 and 20 passages (P4, P5, P10 and P20) using TRIzol LS reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) as previously described (Rasmussen et al., 2010). Total RNA was eluted from the column three times with 30 μl nuclease free water.

2.4. cDNA synthesis and sequencing

Genome length cDNA was generated from the RNA by RT-PCR essentially as described previously (Rasmussen et al., 2010) using an adapted forward primer (5′-TAATACGACTCACTATAGTATACGAGGTTAGCTCGTCCTCGTGTACAACATTGGACAAACTAAAATTCCGATTTGG-3′). The sequences of the 5′-UTRs were determined by Sanger sequencing, using PCR products of 381 bp, that were generated using the primers Pader47F and Pader427R (Friis et al., 2012). In addition, the genome length amplicons, corresponding to the complete genome of CSFV, were consensus sequenced by Next Generation Sequencing (NGS) as described previously (Fahnøe et al., 2014). The consensus sequence of the pBeloPader10 (GenBank Accession number GQ902941) or a subdomain IIIf mutant BAC was used as the reference for mapping of reads. Samtools, VarScan 2 (Koboldt et al., 2012) and VCFtools (Danecek et al., 2011) were used, in succession, in order to generate consensus sequences from the mapped reads. Subsequently, consensus sequences were aligned using MAFFT in Geneious (Biomatters, Auckland, New Zealand). Finally, a combination of Samtools (Li et al., 2009), Lo-Freq (Wilm et al., 2012) and SnpEff (Cingolani et al., 2012) was used for downstream SNP analysis.

2.5. Animal inoculation and sampling

A total of 15 cross-bred weaner pigs (nine to ten weeks old) were divided into three equal groups and housed in separate sections of BSL3 animal isolation facilities. Experimental procedures and animal management protocols were carried out in accordance with the requirements of the Danish Animal Experimentation Inspectorate. Three pigs from each group were exposed to approximately 7×10^6 $TCID_{50}$ of the virus preparation (P4) in 2 ml of DMEM via the intranasal route. Groups 1 (pigs 1–3) and 2 (pigs 6–8) were inoculated with the mutant viruses vPaderS2m6 and vPaderS2m8 respectively, whereas pigs in group 3 (pigs 11–13) were inoculated with vPader10. In addition to the three inoculated pigs, each group also included two non-inoculated pigs (group 1: pigs 4–5; group 2: pigs 9–10 and group 3: pigs 14–15) that were susceptible to virus transmission from the inoculated animals. Blood, serum samples and nasal swabs were collected on post-infection day (PID) 0, 3, 4, 5, 6, 7, 10, 14, 18, 21, 25, 28 and 33. Serum samples were used to detect the presence of CSFV-specific antibodies whereas EDTA-stabilised blood samples were used to determine changes in the B-cell population size (see below) and viral RNA accumulation. Nasal swabs were dissolved in 0.85% NaCl and used to determine virus shedding by measuring the level of viral RNA (as described below).

2.6. Clinical scoring and temperature measurements

Individual pigs were subjected to daily examination; body temperature and clinical signs were scored throughout the entire experimental period (PID −3 to 34). Pigs with a body temperature ≥40 °C were considered to have a fever. In order to obtain a semi-quantitative measure of clinical signs between the 3 groups, all pigs were scored using a modified version of the clinical scoring (CS) system developed by Mittelholzer et al. (2000). Clinical parameters were evaluated and scored on a graded 0–3 point scale, where 0 reflects the absence of clinical symptoms and 3 represents a severe level of CSF symptoms. A maximum score of 30 could be obtained. Moribund animals were euthanised according to predetermined humane endpoints.

2.7. Counting of B-cells

Counting of B-cells in EDTA-stabilised blood samples was carried out by single colour flow cytometry, as described previously (Nielsen et al., 2003). All samples were counted twice and data are presented as mean values.

2.8. Quantitative analysis of viral RNA load from EDTA-stabilised blood and nasal swabs

RNA was extracted from 100 μl of EDTA-stabilised blood samples or the dissolved nasal swab samples using a MagNA Pure LC robot with a MagNA Pure LC Total Nucleic Acid kit (Roche) and eluted in nuclease free water (50 μl). Two distinct quantitative RT-PCR (RT-qPCR) assays, to determine the level of CSFV RNA, were performed as described previously (Rasmussen et al., 2007;

Hoffmann et al., 2005). Samples with a Ct value lower than a threshold value of 40 were considered positive. Data are presented as 40 – sample Ct values.

2.9. Serological analysis of serum samples

The presence of CSFV-specific antibodies in serum samples was detected using a blocking enzyme-linked immunosorbent assay (ELISA) as described (Have, 1984). Absorbance was measured at 450 nm on a Multiscan RC instrument (Thermo Scientific) and data presented as percentage optical density (ODp) calculated in accordance with the following formula: ODp = (sample OD × 100)/(mean OD_{max}). The ODp values, lower than a threshold value of 70, were considered positive for antibodies against CSFV.

3. Results

3.1. Properties of the mutant CSF viruses within infected pigs

Our previous studies on the rescued CSFV mutants vPaderS2m6 and vPaderS2m7 showed that these mutant viruses, with modifications in the pseudoknot (subdomain IIIf), displayed reduced growth rates in PK15 cells compared to the parental vPader10 (Friis et al., 2012) even though the apparent differences in IRES activity were not significant (>90% of wt activity). To characterise the effect of these modifications on the replication of the viruses within the natural host of CSFV, we tested two of the rescued mutant viruses (vPaderS2m6 and vPaderS2m8) in parallel with the unmodified vPader10, all at P4, within pigs. Body temperatures and clinical signs were monitored on a daily basis for each pig in all three groups (see Fig. 2). None of the pigs exposed to either of the mutant viruses had any increase in body temperature during the experimental period and they showed no other clinical signs of infection (data not shown). In contrast, pigs 12 and 13, inoculated with the vPader10, had fever at PID 10 that declined over the following three days (Fig. 2a). However, at PID 14 a second increase in body temperature was detected in the same two pigs. This increase in body temperature was followed by the appearance of other typical clinical signs of CSF beginning from days 16 and 18 for pigs 13 and 12, respectively (Fig. 2b). These signs developed over the next few days to include lack of appetite, depression, petechiae on the ears, body and legs or larger haemorrhagic patches in the skin, diarrhea and ataxia. The elevated body temperature along with the other clinical signs persisted until pig 13 died on PID 21 while pig 12 died on PID 23. A similar biphasic increase in temperature was observed in pig 14 (a contact pig) with an initial onset of fever on PID 23 (Fig. 2a) and recurrence from PID 25 until euthanised (due to animal welfare reasons) on PID 32. As with the inoculated pigs, pig 14 displayed characteristic clinical signs of CSF but their occurrence was already apparent at the time of the initial increase in body temperature and persisted until death (Fig. 2b). From these results, it appears that the rescued vPader10 has a clinical incubation period of ca. 10 days before an initial increase in body temperature is observed (pigs 12 and 13). Furthermore, a similar increase in body temperature in the contact pig 14 occurred about 10 days later suggesting that the shedding and transmission of vPader10 from the inoculated pigs (pigs 12 and 13) occurred from about the time of the onset of fever.

3.2. Determination of the replication and shedding of CSFV in pigs

To follow the replication of the CSF viruses in the pigs, RT-qPCR assays were performed on RNA isolated from EDTA-stabilised blood. Each of the animals (pigs 11–13) inoculated with vPader10 had significant levels of viral RNA in the blood for several days (Fig. 2c). Indeed, viral RNA could be detected in the blood as early as PID 3 in these pigs. Transmission of this virus to the contact pigs occurred and RNA was also detected in the blood of these contact animals. The viremia was maintained until death for pigs 12, 13 (inoculated) and 14 (contact) while clearance of the virus from the blood occurred in pigs 11 (inoculated) and 15 (contact) after a period of viremia which lasted for about 10 days (Fig. 2c). In contrast, CSFV RNA was only detectable, at very low levels, in the blood of individual animals (pigs 3 and 6) from each of the groups

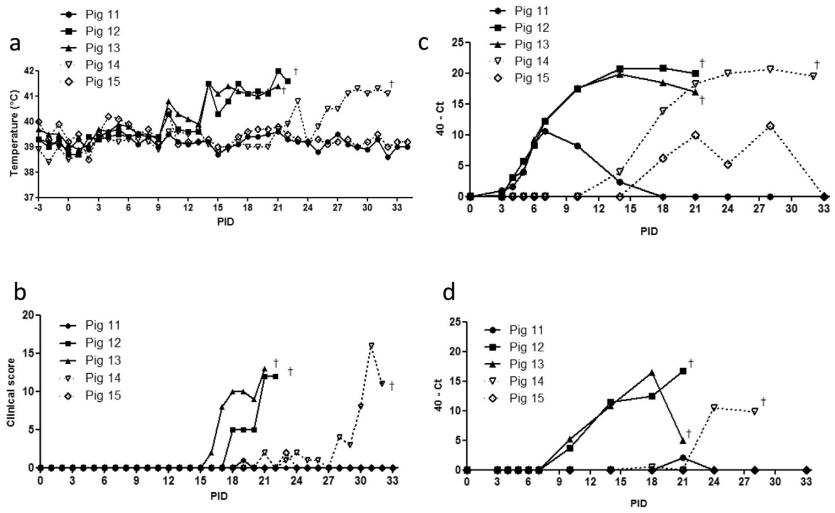


Fig. 2. Characterisation of the rescued vPader10 infection in pigs. The rescued vPader10 was inoculated into three animals (pigs 11–13, filled symbols) that were in contact with two others (14 and 15, open symbols). Rectal temperatures were monitored on a daily basis (a) and clinical scores determined (b). The clinical scores were assessed, using a modified version of the scoring system described previously (Mittelholzer et al., 2000), on a daily basis until death or euthanasia (indicated by †) or the end of the experiment (PID 35). Blood (c) and oral swab (d) samples were collected on the indicated days and assayed for the presence of CSFV RNA using RT-qPCR (presented as 40-Ct values). Similar results were obtained using the assays described by Rasmussen et al. (2007) (as presented) and by Hoffmann et al. (2005) (data not shown). Note the animals inoculated with vPaderS2m6 (pigs 1–3) and vPaderS2m8 (pigs 6–8) did not display any clinical signs of infection and only occasional, low level viremia (see text).

inoculated with the vPaderS2m6 or vPaderS2m8 mutants respectively (data not shown). In pig 6, vPaderS2m8 was detected on PIDs 14 and 18 and in pig 3 (inoculated with vPaderS2m6) viral RNA was detected on PID 28 only. It should be noted that viral RNA may have been present on days in between samplings. No viral RNA was detected in the pigs kept in contact with those inoculated with either of the mutant viruses (data not shown). In pigs 12, 13 and 14, the presence of viral RNA in the blood was observed for five to seven days prior to the increase in body temperature (compare Fig. 2a and c). During the period of elevated body temperature, these pigs had high levels of viral RNA in the blood, which persisted until they died or were euthanised.

To determine the period of virus excretion from the pigs, total RNA from nasal swabs was isolated and analysed for the presence of CSFV RNA by RT-qPCR. Nasal excretion from animals inoculated with either of the modified viruses was only detected, at a very low level, from a single animal, pig 8 (inoculated with vPaderS2m8) on PID 10 (not shown). In contrast, nasal excretion of the vPader10 was readily detected in pigs 12 and 13 from PID 10 through to PID 21 (Fig. 2d); as indicated above, these two animals died on PID 23 and 21, respectively. Furthermore, in the contact pigs, virus excretion was observed at a low level from pig 11 on PID 21 and at a higher level in pig 14 on PID 24 and 28 (prior to euthanasia on PID 32).

3.3. Serological responses in pigs infected with vPaderS2m6, vPaderS2m8 and vPader10

The serological response to infection with the viruses vPaderS2m6, vPaderS2m8 and vPader10, was determined using a blocking ELISA (Fig. 3). Among the pigs inoculated with vPaderS2m8, the presence of CSFV-specific antibodies could be detected on PID 14 in pig 6 (Fig. 3b). On PID 18, pig 7 had also seroconverted as had pig 8 by PID 21. Interestingly, one contact pig in this group (pig 9) also seroconverted against CSFV, this was detected from PID 21 through to PID 33 indicating that transmission of the vPaderS2m8 between pigs had occurred even though this virus failed to induce any clinical signs and there was no detectable viremia in this pig. In all pigs inoculated with vPaderS2m6 seroconversion against CSFV was detected by PID 24. However, no CSFV-specific antibodies could be detected in any of the contact pigs in the vPaderS2m6 group during the experimental period (Fig. 3a) indicating a lack of transmission of this mutant virus. In contrast, in pigs inoculated with vPader10 only one inoculated animal (pig 11) showed significant levels of CSFV-specific antibody in the serum (Fig. 3c), this pig also cleared the infection as judged by the loss of viremia (Fig. 2c). The lack of seroconversion in the other animals in this group corresponds well with the fact that pigs 12, 13 and 15 all had severely depressed levels of circulating B-cells (see below) and died relatively quickly.

3.4. Depletion of B-cells in CSFV-infected pigs

The level of circulating B-cells in the pigs was determined by flow-cytometry (Fig. 4). The pigs inoculated with vPader10 showed a rapid decline (by PID 3) in circulating B-cells (Fig. 4c). In pigs 12 and 13, the levels of B-cells in the blood remained low until the animals died on PID 23 and PID 21, respectively. However, recovery of the B-cell numbers occurred in pig 11 that survived the infection (and seroconverted (Fig. 3c)). Similarly, the in-contact animals in this group also had a low level of B-cells by PID 18 consistent with the later appearance of viral RNA in the blood of these animals (Fig. 2c). Recovery of B-cell numbers in one of these animals (pig 15) was also observed in accord with the clearance of the virus from the blood (see Figs. Fig. 2c and Fig. 4c). There were transient declines in circulating B-cells in pigs inoculated with vPaderS2m6 and vPaderS2m8 at about PID 6 (Fig. 4a, b) but the levels returned to normal and remained relatively constant in the in-contact animals.

3.5. Stability of the vPader10 genome sequence during replication in pigs

The vPader10, at P4, was used for the infection of pigs as described above. Viral RNA was extracted from the inoculum and also from the blood of pigs 12 (at PID 21), 13 (at PID 21) and 14 (at PID 28) when high viremia had been detected; full-length amplicons were generated by RT-PCR and then sequenced using NGS (Tables 1 and 2). Substitutions at nt 4136 and 11864 relative to the pBeloPader10 cDNA, were present in nearly 100% of the genome populations present in all three pigs at either PID 21 or PID 28 (Table 1). In the inoculum (P4), the G11864A was present in 97% of the genomes. However, in contrast, the C4136U change was only present in 8% of the genomes present in the P4 virus stock (Table 2). The C4136U change, results in the amino acid substitution H1255Y in the NS2 protein while the G11864A change results in the amino acid substitution G3831R within the NS5B protein (the RNA-dependent RNA polymerase). These two amino acid substitutions are reversions to the parental CSFV strain Paderborn sequence (GenBank Accession number AY072924) from the cloned sequence present within the pBeloPader10 cDNA (GenBank Accession number GQ902941). Insufficient viremia occurred in pigs inoculated with the mutant viruses to permit sequencing of the virus present within these animals.

3.6. Genetic stability of IRES mutations during serial passage in cell culture

Having identified two sequence changes in the virus recovered from the vPader10-infected pigs (12–14) compared to the parental cDNA, we wanted to determine if these, and/or other, adaptations occurred during growth of this virus and the mutant viruses in cell culture. The ability of the mutant viruses to grow in cell culture, but not in pigs, clearly extended the range of the analysis that could be performed. To explore the genetic stability of the viral genomes during virus replication, serial passages of mutant viruses were performed, in parallel, in PK15 cells. The viruses used for these experiments were vPader10, vPaderS2m6, vPaderS2m7 and vPaderS2m8 respectively (see below). At passage P5, P10 and P20, RNA was extracted from cell supernatants and the level of CSFV RNA was determined by RT-qPCR (Fig. 5). At P10 and P20 very similar levels of CSFV RNA were detected in each virus harvest but at P5 the mutant viruses, vPaderS2m6 and vPaderS2m7, produced less viral RNA than vPaderS2m8 and vPader10. Amplicons corresponding to the complete 5′-UTR were generated by RT-PCR from the P4, P5, P10 and P20 virus stocks and sequenced. The sequences of the region including the pseudoknot are shown in Fig. 6a–d. Each of the mutant viruses were designed to have 7 or 8 nucleotide substitutions within the stem 2 of the pseudoknot while retaining at least 4 of the 5 base pairs indicated in Fig. 6e. The rescued mutant viruses, even by P4, had adapted from the transcribed sequence at up to 3 positions within stem 2 of the pseudoknot. The resultant sequences kept the predicted secondary structure although the base pairing is different from the parental structure (Fig. 6e). In addition, vPaderS2m6 has 2 additional nt changes just upstream of the initiation codon but the significance of these changes is not known. The sequences of vPader10, vPaderS2m6 and vPaderS2m7 remained constant in this non-coding region from P4 through to P20. In contrast, a single nt substitution occurred within the pseudoknot of vPaderS2m8 between passage 10 and 20 (see Fig. 6d). This sequence change is predicted to restore the single base pair that has been disrupted

within the pseudoknot (see Fig. 6e). The activity of the IRES within the PaderS2m8 has not been determined but it is also expected to be close to wt since the rescued virus grew efficiently. However, it should be noted that within the rescued vPaderS2m8, the disrupted base pair (G A at P0) had adapted to a G-U base pair at P4 and changed again to a G-C base pair between P10 and P20 (the wt sequence is an A-U base pair).

To determine if other adaptations occurred within these viruses during these serial passages, full-length cDNA amplicons were generated from the viruses grown in PK15 cells and sequenced using NGS to determine both the consensus sequence and, in addition, the population structure within the virus stocks. It should be noted that the terminal sequences of the genome (both in the 5′-UTR (nt 1–59 and 3′-UTR (nt 12262–12296)) are determined by the primers used in the RT-PCR and may not correspond exactly to the viral RNA sequence. This sequence analysis confirmed the sequence changes identified within the 5′-UTR within the mutant viruses (see Table 2). In addition, it also revealed shared

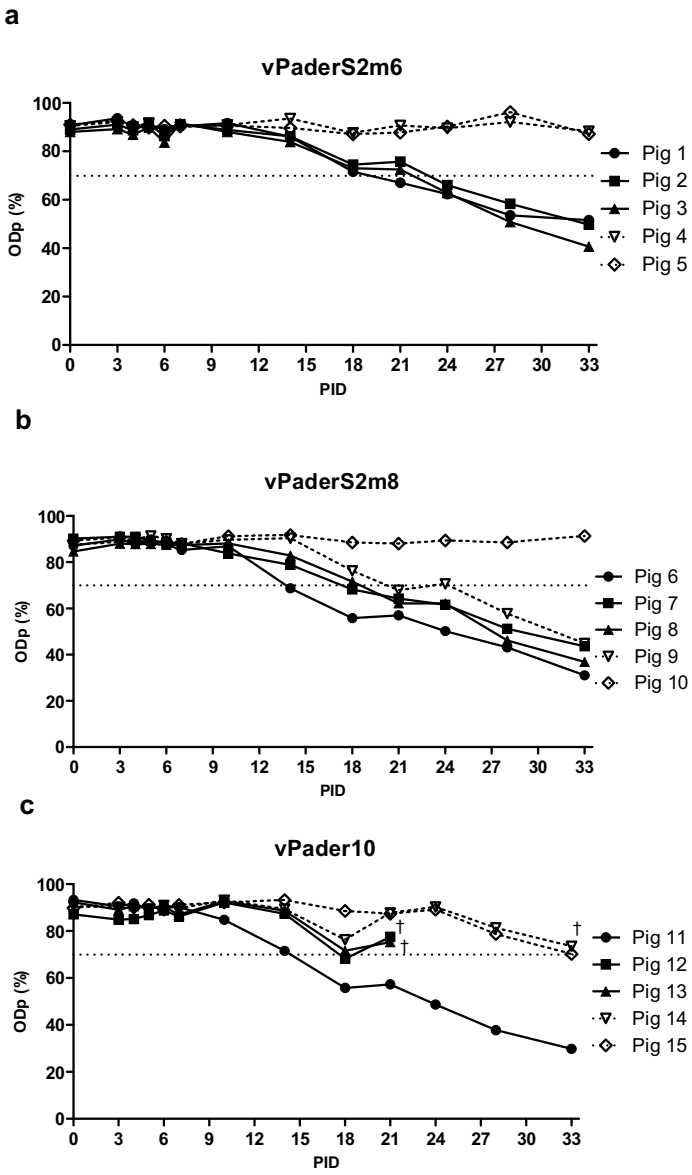


Fig. 3. Seroconversion in CSFV-infected pigs. Serum samples from inoculated pigs (filled symbols) or in-contact pigs (open symbols) collected on the indicated days were assayed for antibodies against CSFV using a blocking ELISA. ODp (%) values lower than 70% (threshold indicated by dotted line) are considered positive. The viruses used for inoculation were vPaderS2m6 (a), vPaderS2m8 (b) and vPader10 (c). The experiment was terminated on PID 35 but some animals died or were euthanised earlier (indicated by †).

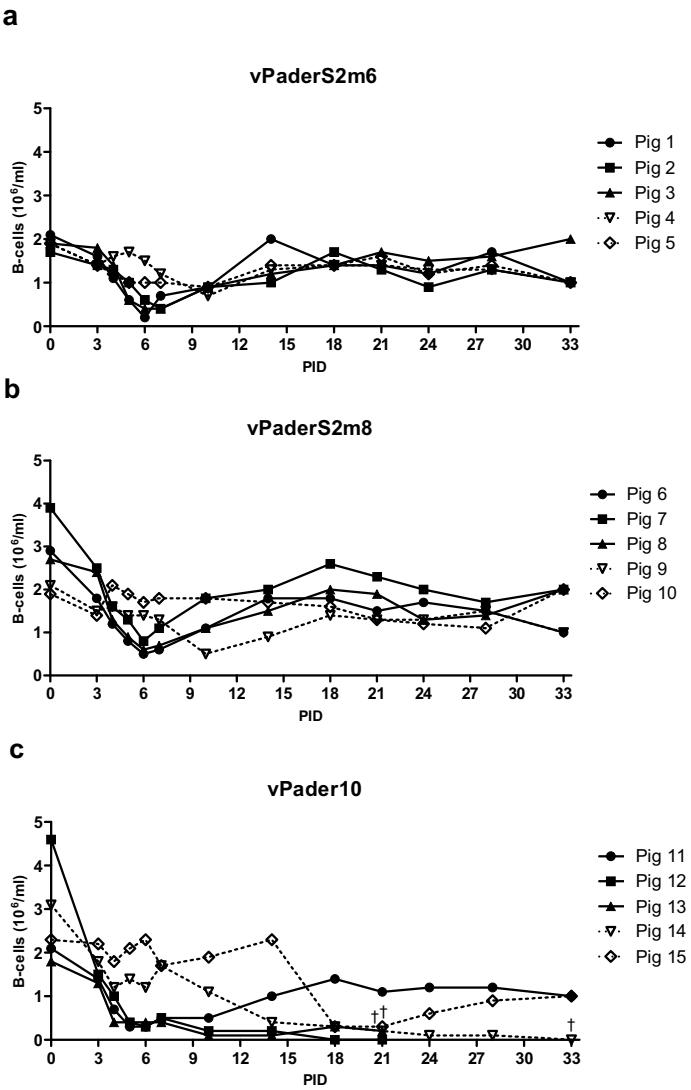


Fig. 4. Depletion of B-cells in CSFV-infected pigs. B-cells were counted (by flow cytometry) in EDTA-stabilised blood samples collected on the indicated days from inoculated pigs (filled symbols) or in-contact pigs (open symbols). The viruses used for inoculation were vPaderS2m6 (a), vPaderS2m8 (b) and vPader10 (c). The experiment was terminated on PID 35 but some animals died or were euthanised earlier (indicated by †).

Table 1
Sequence adaptations in vPader10 during replication of rescued CSFV in pigs.

vPader10 in infected pigs								
Region	nt position	pBeloPader10 (GQ902941)	Variant	Pig 12 PID21 (%)[a]	Pig 13 PID21 (%)[a]	Pig 14 PID28 (%)[a]	aa change	Paderborn wt (AY072924)
NS2	4136	C	U	99	99	99	H1255Y	U
NS5B	11864	G	A	100	100	100	G3831R	A

[a] The percentage (%) of each variant at each nt is only given if the level of this variant was present at a level of 10% or higher at some stage. Values are given to the nearest integer.

Table 2
Sequence adaptations during sequential passages of rescued CSFVs in PK15 cells.

vPader10 in PK15 cells

Region	nt position	pBeloPader10 (GQ902941)	Variant	P4 (%)[a]	P5 (%)[a]	P10 (%)[a]	P20 (%)[a]	aa change	Paderborn wt (AY072924)
NS2	4136	C	U	8	25	78	77	H1255Y	U
NS5B	11864	G	A	97	99	99	99	G3831R	A

vPaderS2m6 in PK15 cells

Region	nt position	pBeloPader10 (GQ902941)	Variant	P4 (%)	P5 (%)	P10 (%)	P20 (%)	aa change	Paderborn wt (AY072924)
5′-UTR	326	A	U	99	100	100	99	*	A
	327	G	C	100	99	100	100	*	G
	328	A	U	99	95	99	100	*	A
	329	G	C	99	99	98	99	*	G
	356	C	G	100	100	100	100	*	C
	357	U	A	99	96	100	100	*	U
	358	C	G	100	100	100	100	*	C
	359	U	A	99	100	99	100	*	U
	371	C	A	99	95	99	99	*	C
	372	A	U	98	95	99	99	*	A
NS2	4136	C	U	3	3	–	70	H1255Y	U
NS5A	9082	C	U	–	–	–	13	—	C
NS5B	11864	G	A	–	16	100	99	G3831R	A

vPaderS2m7 in PK15 cells

Region	nt position	pBeloPader10 (GQ902941)	Variant	P4 (%)	P5 (%)	P10 (%)	P20 (%)	aa change	Paderborn wt (AY072924)
5′-UTR	326	A	U	100	100	100	100	*	A
	327	G	C	100	100	100	100	*	G
	328	A	U	94	100	100	100	*	A
	329	G	C	94	100	100	100	*	G
	330	G	A	99	99	99	98	*	G
	356	C	G	100	100	100	100	*	C
	357	U	A	99	99	99	99	*	U
	358	C	G	100	100	100	100	*	C
	359	U	A	100	99	100	100	*	U
	372	A	U	99	99	99	99	*	A
C	1027	G	A	16	14	–	–	—	G
E^{rns}	1325	C	U	14	16	–	–	—	C
E1	2164	G	U	–	–	18	3	—	G
	2194	A	G	–	–	51	59	—	A
NS2	4136	C	U	–	–	–	13	H1255Y	U
NS5B	10913	G	U	14	13	–	–	D3514Y	G
	11864	G	A	–	–	99	99	G3831R	A

vPaderS2m8 in PK15 cells

Region	nt position	pBeloPader10 (GQ902941)	Variant	P4 (%)	P5 (%)	P10 (%)	P20 (%)	aa change	Paderborn wt (AY072924)
5′-UTR	207	G	A	–	–	13	65	*	G
	326	A	U	97	91	97	98	*	A
	327	G	C	96	90	94	97	*	G
	328	A	G	96	90	94	97	*	A
	329	G	C	96	90	94	97	*	G
	356	C	G	100	93	100	99	*	C
	357	U	C	9	5	27	79	*	U
	358	C	G	99	93	100	99	*	C
	359	U	A	99	92	99	99	*	U
N^{pro}	398	U	C	86	87	71	18	—	U
	668	C	U	–	–	–	16	H99Y	C
C	1115	G	A	8	81	61	15	E248K	G
E1	2164	G	U	–	19	3	9	—	G
NS2	4136	C	U	–	6	3	77	H1255Y	U
NS3	6488	G	U	–	–	1	48	A2039S	G
NS5B	11864	G	A	92	98	100	99	G3831R	A

*In the column 'aa change' indicates the change is in the non-coding region.
–In the column 'aa change' indicates the nt substitution is synonymous.
[a] The percentage (%) of each variant at each nt is only given if the level of this variant was present at a level of 10% or higher at some stage. Values are given to the nearest integer.

adaptations at nt 4136 (C to U, resulting in the amino acid substitution H1255Y within the NS2 protein) and at nt 11864 (G to A, resulting in the substitution G3831R within the NS5B protein) (see Fig. 7 and Table 2). These two amino acid substitutions occurred in all four virus populations but with different kinetics and, as indicated above, are reversions to the parental CSFV strain Paderborn sequence.

The reversion in the NS5B coding region of the vPader10 sequence was present in >90% of the virus population at P4 and P5 and by P10 it was found in essentially 100% of the virus population

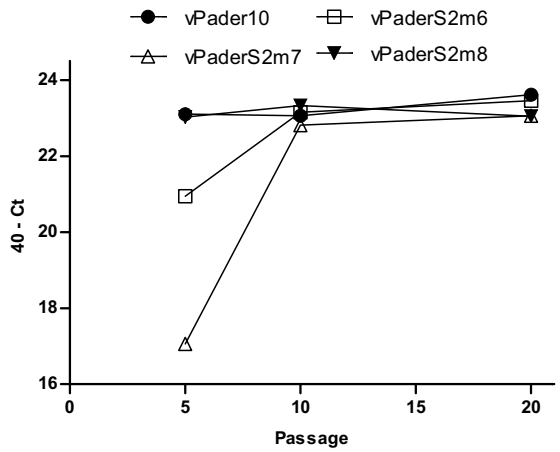


Fig. 5. Analysis of CSFV RNA production during passage in cell culture. The vPader10 and mutant CSFVs were passaged in PK15 cells. RNA was extracted from the indicated samples and the level of CSFV RNA was measured by RT-qPCR. Results (presented as 40-Ct values) were obtained using the assays described by Hoffmann et al. (2005).

(Fig. 7b and Table 2). The adaptation occurred with similar kinetics in vPaderS2m8, as for vPader10 (with ≥98% change at P5), but the change took more passages to reach high levels within vPaderS2m6 and vPaderS2m7 (Fig. 7b). In both cases, less than 20% of the virus populations had this adaptation at P5 but by P10 it had become dominant.

The C to U substitution at nt 4136 occurred more slowly than the nt 11864 change in all of the viruses and it also occurred more slowly in each of the sub-domain IIIf (pseudoknot) mutant viruses than in vPader10 (Fig. 7a, Table 2). In vPader10, the C4136U change reached a level of about 80% by P10 and remained at that level at P20 whereas the IRES mutant viruses retained the initial sequence at P10 but about 80% of the vPaderS2m6 and vPaderS2m8 populations had acquired the nt change by P20. In contrast, less than 20% of the vPaderS2m7 population had this substitution by P20.

Several other nt changes occurred at a low level but did not accumulate during passage in cell culture, e.g. nt 6682 was changed from A to G in about 2–3% of the vPaderS2m8 genomes both at P5 and P10 (not shown). In contrast, in the vPaderS2m8, a G to A substitution was present at nt 1115 in about 80% of the reads at P5 but this had declined to about 60% by P10 and 15% by P20. Thus, it seems that this change (resulting in an E to K amino acid substitution at residue 248 of the C protein) was unfavoured but clearly the selection is not very strong. The significance of this mutation is not known. It should be noted that the three other aa residues that differ between the rescued vPader10 and the field strain virus did not revert (see Methods section).

4. Discussion

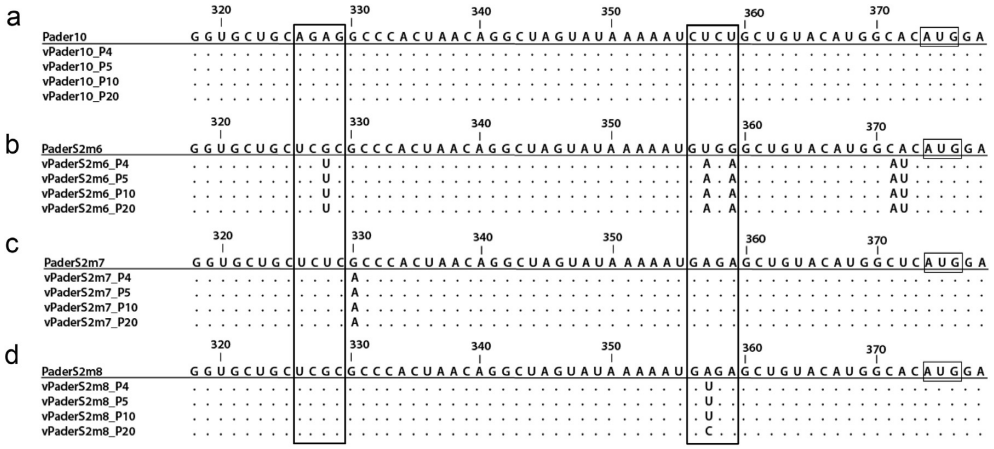
The CSFV IRES has a key role in virus replication since it directs the initiation of protein synthesis on the viral RNA. It is apparent that certain mutations in the pseudoknot, which have little or no effect on the translational efficiency of the IRES in cell culture, can diminish the ability of the virus to replicate in cells (e.g. see Friis et al., 2012). In this study, mutant CSFVs, which have multiple mutations within subdomain IIIf of the IRES, have been tested for their ability to replicate and cause disease within their natural host animal, the pig. The viruses containing these mutations in the IRES can be grown successfully in cell culture but they failed to replicate significantly in pigs. Thus, it was not possible to determine the selection pressure operating on the mutations in the IRES in infected animals.

However, by analysing the virus populations, using NGS, during growth of the virus in cell culture, it was possible to identify adaptations that occurred during this process. Only one sequence change was observed to occur within the IRES between the rescued viruses at P4 and P20. This single change was at nt 357 within the vPaderS2m8. This change occurred between P10 and P20, is predicted to enhance the stability of the base-pairing within the stem 2 of the pseudoknot (see Fig. 6e). However, certain nt changes occurred between the steps of RNA transcription and the analysis of the rescued virus at P4. It is apparent that the vPaderS2m6 contained, at P4, two substitutions at nt 371 and 372 plus 3 nt changes within the stem 2 (at nt 328, 357 and 359) while vPaderS2mut7 had a change at nt 330 compared to the parental sequence. In addition, within the coding region, non-synonymous mutations occurred at nt 4136 and nt 11864 in each of the mutant viruses and also in the rescued vPader10 strain. These amino acid changes correspond to reversions to the consensus sequence of the parental Paderborn strain of CSFV from the variant sequence present within the pBeloPader10 clone. These coding changes (within the NS2 (H1255Y) and NS5B (G3831R) coding regions) occurred independently in all 4 rescued viruses in cell culture, thus there appears to be significant selection pressure for them, especially for the change in NS5B (Table 2). In vPader10 and vPaderS2m8 the reversion at nt 11864 was present in >92% of the virus population at P4 (as used for the animal experiment) but was essentially absent in vPaderS2m6 and vPaderS2m7 at P4 (Fig. 7b, Table 2). However, by P10 when vPaderS2m6 and vPaderS2m7 had gained the ability to replicate more efficiently (Fig. 5), the G11864A change is present within ≥99% of the genomes (Fig. 7b, Table 2). Interestingly, the change within the NS2 coding region accumulated more slowly and reached a maximal level within the populations of around 70–80% for each of the rescued viruses at P20 except it only reached 13% of the population in vPaderS2m7 (Fig. 7a and Table 2). These coding changes in NS2 and NS5B appeared at very high levels in the vPader10-infected pigs (Table 1) (and only the G3831R change in NS5B was present at significant levels in the inoculum) indicating that these reversions are important for growth in the native host. A different coding change within the NS5B (D3431G) of rescued viruses based on the Riems vaccine strain of CSFV has previously been observed leading to a reversion to the parental form in two independent rescued CSFV populations (Rasmussen et al., 2013). However, the effect of these specific reversions on the function of these proteins is not currently known.

Since the vPader10 is rescued from a unique cDNA clone, it is genetically defined and thus represents a good starting point for determining specific changes to the virus sequence that affect virus replication and pathogenicity. However, it is apparent that, by the time the virus was rescued from the RNA transcripts, it already represents a population of closely related viruses.

The multiple mutations within stem 2 of the pseudoknot (subdomain IIIf) within the IRES were clearly sufficient to attenuate the virus in animals, at least for vPaderS2m8 (since the NS5B change had already occurred in this virus inoculum), but did not produce a significant block on replication efficiency in cells (Fig. 5). It is not clear what factors are suppressing the replication *in vivo*. It may be that this region within the IRES has functions unrelated to translation initiation.

A single nt adaptation occurred in the IRES during passage of vPaderS2m8 in cell culture, between passage 10 and 20. This change is predicted to enhance the stability of the stem 2 within the subdomain IIIf; a G A mismatched pair in vPaderS2m8 adapted to make a G-C base pair (this is stable but is different from the A-U



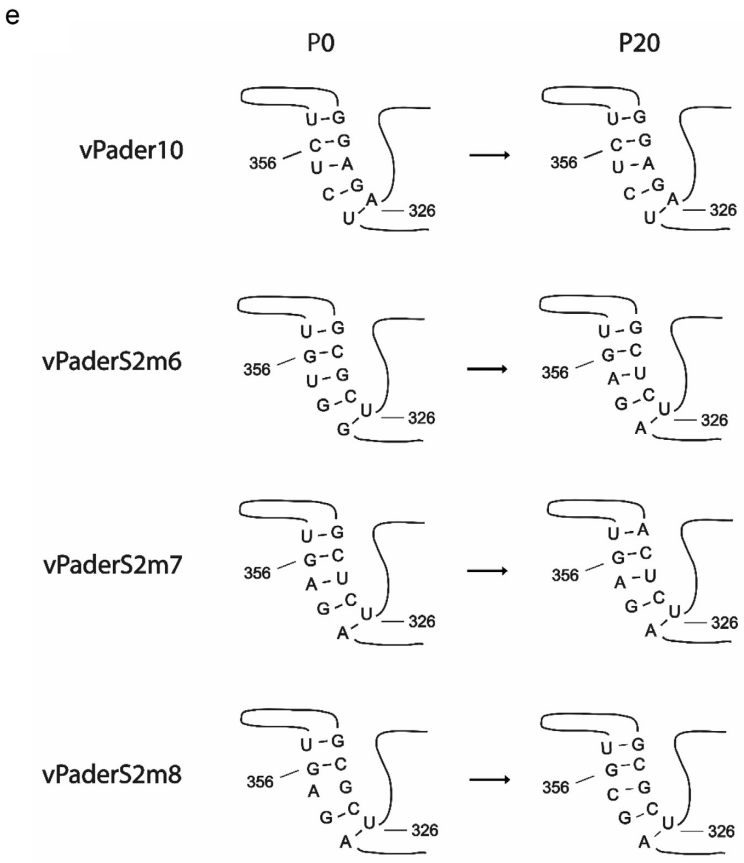


Fig. 6. Sequences of IRES elements following multiple virus passages in cell culture. The IRES sequences of vPader10 (a), vPaderS2m6 (b), vPaderS2m7 (c) and vPaderS2m8 (d) following passage in cell culture were identified by Sanger sequencing and confirmed by NGS (see below). The sequence differences from wt are shown whereas identical nt are indicated by a (.). The predicted secondary structures of the stem 2 within subdomain IIIf of these different viruses are shown (e).

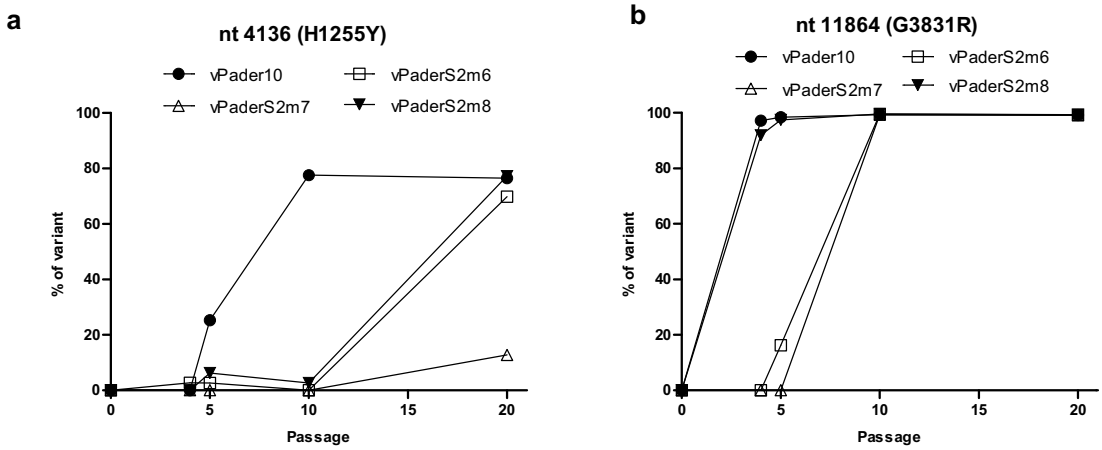


Fig. 7. Sequence adaptations in CSFV genomes during passage of viruses in cell culture. The complete genome sequences of vPader10 and the indicated mutant viruses at P4, P5, P10 and P20 were determined by NGS of full-genome length amplicons generated by RT-PCR. The proportion of the mapped reads containing the non-synonymous changes at nt positions 4136 (panel a) and 11864 (panel b) are presented. These nt changes are reversions to the consensus sequence of the parental Paderborn strain (GenBank Accession number AY072924) from the variants present in the pBeloPader10 clone (GenBank Accession number GQ902941). Note the P0 sequences are based on sequencing of the cloned DNA templates.

base pair present in the wt IRES, see Fig. 6e). It can clearly be difficult for a virus to adapt to multiple nt changes since intermediates in the reversion process may be very defective and hence will not survive long enough to adapt further. It is interesting to note that when a 44nt insertion was introduced into the subdomain $IIId_2$ the resultant virus appeared to grow as well as the wt virus (Moser et al., 2001). This subdomain is clearly not essential for IRES activity; indeed the HCV IRES lacks this feature. However, during passage in cells a deletion of 29nt occurred within this added sequence indicating that this insertion mutant is unstable. It appears that the repeated passage of mutant viruses within cells is an efficient system for identifying sub-optimal sequences within the viruses that can be selected against even when the selection pressure appears rather modest.

From the information described above, it is apparent that at least in the case of vPaderS2m8, the block on replication in pigs was not due to the NS5B change since >90% of the virus had already reverted to wt at this position in the P4 virus (see Fig. 7b). It should be noted that there was not a complete block on virus replication since the animals inoculated with these mutant viruses did seroconvert against CSFV (Fig. 3a,b) and evidence was obtained that transmission of vPaderS2m8 occurred to one animal (pig 9) as judged by seroconversion in an in-contact animal (Fig. 3b). The mutant viruses failed to induce any clinical signs of disease and only transient, very low level, viremia was detected in single animals and thus they are considered attenuated.

The incubation period (of ca. 10 days) before an initial increase in body temperature and the observed clinical signs in pigs inoculated with the vPader10, at P4, are comparable to previous findings with wt Paderborn isolates (Uttenthal et al., 2003; Durand et al., 2009; Weesendorp et al., 2011). This demonstrates that the rescued virus, with the two reversions to wt in NS2 and NS5B (as described above), has properties that closely resemble the moderate virulence of the parental virus strain. The early loss of circulating B-cells (Fig. 4) seen in the vPader10-infected pigs is consistent with earlier studies using virulent strains of CSFV (Summerfield et al., 2001). The mutant viruses (vPaderS2m6 and vPaderS2m8) induced only a transient decline in circulating B-cells as observed previously in pigs with mild clinical disease (Nielsen et al., 2010).

The ability to make precise modifications within the virus genome, to rescue the modified viruses and then follow the adaptation of the viruses throughout the entire genome during the course of virus replication, at a high level of sensitivity (sub-consensus) using NGS, is a very strong combination of techniques. This enables the identification and characterisation of key determinants of virus replication in cells and pathogenicity in host animals.

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Manuscript 2

Analysis of potential RNA interactions with a motif in the NS5B coding region of classical swine fever virus that influence viral RNA replication

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Abstract

The RNA genome of classical swine fever virus (CSFV) contains multiple conserved structural elements directing protein synthesis and viral replication. Characterisation of such structural elements provides valuable insight into the virus life cycle and mechanisms underlying viral RNA replication. Genetically defined viruses and self-replicating (non-infectious) replicons encoding a luciferase reporter protein were used for the analysis of virus replication. Use of replicons permits a quantitative assessment of the replication efficiency of wt and mutant RNAs. The mutant RNAs contained targeted modifications in a motif located within the coding region for the RNA-dependent RNA polymerase (NS5B). Introduction of specific silent mutations into this motif resulted in a reduced replication capability compared to the wt replicon. However, when these same mutations were tested using full-length CSFV constructs, there was little difference in virus replication but an adaptive silent mutation in the structural CSFV protein E1 appeared upon passaging. Taken together, this study shows that a specific motif within the coding region of NS5B is important for viral RNA replication most likely through interactions with RNA elements within the virus genome or with host factors.

Introduction

Classical swine fever virus (CSFV) is a small, enveloped positive-sense RNA virus belonging to the *Pestivirus* genus within the *Flaviviridae* family. The genome consists of a single long open reading frame (ORF) flanked by untranslated regions (UTRs). The ORF is translated into a large polyprotein encoding 12 proteins comprising four structural proteins (C, E^{rns}, E1 and E2) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). An RNA element located in the 5'-UTR, denoted the internal ribosome entry site (IRES), directs cap-independent initiation of protein synthesis (Fletcher & Jackson, 2002; Rijnbrand *et al.*, 1997). Besides this IRES function, the 5'-UTR may also contain *cis*-acting RNA elements that are important, not only for viral translation and replication, but also in regulating the switch between these processes.

Genome replication of positive-sense RNA viruses is regulated by *cis*-acting RNA elements and *trans*-acting factors (Li & Nagy, 2011; Liu *et al.*, 2009). Regulatory *cis*-acting RNA elements are sequences or structures influencing recruitment of viral and cellular proteins; the proteins recruited to these structures are defined as *trans*-acting factors. Besides the local functions displayed by most *cis*-acting RNA elements, long-range interactions between distant RNA elements are known to regulate virus replication. Long-range interactions have been found in several members of the *Flaviviridae* family, including hepatitis C virus (HCV) (Diviney *et al.*, 2008; Romero-López & Berzal-Herranz, 2009) and dengue virus (DENV) (Alvarez *et al.*, 2005). In HCV, a domain termed 5BSL3.2, in the coding sequence for NS5B (the RNA-dependent RNA polymerase), has been shown to interact with IRES subdomain IIId (Romero-López & Berzal-Herranz, 2009). This interaction is believed to be pivotal for the translation/replication switch. A similar interaction within the RNA genome of CSFV, which contains an IRES element resembling that of HCV, is highly likely.

Introduction of mutations within the CSFV IRES domains II and III have been shown to reduce viral translation (Friis *et al.*, 2012; Kolupaeva *et al.*, 2000). IRES subdomain $IIId_1$ is known to interact with the 40S ribosomal subunit but might be important for functions besides translation such as RNA replication since some mutations introduced into subdomains II and $IIId_1$ caused inhibition of virus propagation with little apparent effect on IRES activity (Friis *et al.*, 2012).
The RNA secondary structure is strongly influenced by the primary sequence, since almost 10 % of sequence positions, when randomly mutated result in structure changes (Fontana *et al.*, 1993; Schuster *et al.*, 1994). Hence, introduction of synonymous (silent) mutations into the RNA genome might influence the secondary structure of the RNA concomitant with a change in *cis*- or *trans*-acting interactions.

In the present study, a sequence motif within the coding region for the NS5B protein of CSFV has been identified, from sequence analysis, to have the capability of interacting with the IRES. The effect of making synonymous mutations in this motif on replication efficiency has been examined in the context of the replicon compared to the wild type (wt) virus. Furthermore, the same mutations have also been examined within the context of the infectious virus.

Methods

Viruses and cells. Porcine kidney cells (PK15) were cultured in Dulbecco's Modified Eagles medium (DMEM, Gibco) with 5 % foetal calf serum (FCS). The CSFV strain Paderborn was propagated in PK15 cells and used for the construction of an infectious cDNA clone (IC). Titration of CSFV was performed in 96-well plates (Costar, Cambridge, MA). After 3 days in culture, viral infectivity was assessed using an immunoperoxidase cell staining assay using the CSFV monoclonal antibody WH303. Titers were calculated according to the method of Reed and Muench and expressed as 50 % tissue culture infective dose (TCID50)/ml. Serial passages were performed in PK15 cells as described previously (Hadsbjerg *et al.*, 2016) and RNA was extracted from 1 ml of cell supernatant generated after 1 and 5 passages (P1 and P5). RNA was extracted using TRIzol LS reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen) and eluted from the column three times with 30 µl nuclease free water as previously described (Rasmussen *et al.*, 2010).

Construction of CSFV mutants. The CSFV mutants were constructed in replicons and as full-length constructs. The replicons containing the *Gaussia* luciferase (Gluc) coding sequence were based on the previously described rPad2GL construct (Risager *et al.*, 2013). For the full-length constructs, an IC of the virulent strain Paderborn termed pBeloPader10 was used as a template to obtain all cDNA IC constructs described. Silent modifications in a motif of NS5B were introduced into the cDNA clone (at nucleotide (nt) position 11917) and the Gluc replicon (at nt position 11173). Also for both replicons and full-length ICs, a single point mutation within the NS5B protein (G11864A resulting in the amino acid substitution G3831R) was introduced which corresponds to reversion to the parental CSFV Paderborn sequence (GenBank Accession number AY072924). To test for possible codon bias, similar mutations were introduced into a second motif upstream within NS5B at nt position 11050 for the ICs and nt position 10306 in the replicon constructs, respectively.

The products were digested with *DpnI* (leaving only the newly amplified DNA) and then transformed into electro-competent DH10B *E. coli* cells. Colonies were grown on agar plates with chloramphenicol selection (15 µg/ml). Positive clones containing the introduced modifications were selected using sequence analysis around the NS5B motif using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI3500 Genetic analyzer (Applied Biosystems). Each of the IC constructs and replicons were subsequently full-length

sequenced to verify that only the site-directed mutagenesis-induced changes were present. This was performed on an Ion Torrent PGM (Life Technologies) and the sequences were analysed using the CLC Genomics Workbench software (CLC bio).

In vitro virus rescue and virus growth curves. Replicons and full-length genomic clones were *in vitro* transcribed using the MEGAscript T7 transcription kit (Ambion). RNA was transfected into PK15 cells by electroporation (180 V and 950 µF on a Gene Pulser XCell, Bio-Rad). Cells were seeded on 24-well plates and incubated at 37 °C with 5 % CO_2. After 3 days, stocks of rescued virus were stored at -80 °C. Virus growth curves were generated by infecting PK15 cells (300,000 cell/ml) with 2 x 10^{10} viral genome copies. At 3h to 96h post-infection, cell samples were harvested and the genome copy numbers were measured by RT-qPCR (Hoffmann *et al.*, 2005). For visualisation of virus-infected cells, using immunofluorescence for the NS3 protein was carried as previously described (Friis *et al.*, 2012). Briefly, at 72h post-infection cells were stained with the anti-CSFV NS3 monoclonal antibody WB103/105 (AHVLA Scientific) and Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Cell nuclei were visualised using DAPI (VectaShield) and images were taken with a BX63 fluorescence microscope (Olympus).

Luciferase assay. At indicated time points after electroporation (3h, 24h, 48h and 72h), 50 µl of the medium was removed and frozen at -20 °C. After thawing, 20 µl was assayed by addition of 100 µl coelentarazine substrate diluted 1:100 in Renilla Luciferase Assay buffer (Promega). Luciferase activity was quantified in a Bio-orbit 1253 Luminometer (Aboatox) and measurements were normalised to the 3h post-electroporation signal.

Results

Identification of a motif in CSFV that influences viral replication

To investigate whether a putative interaction in the CSFV RNA is important in viral replication, a triplet motif of three cytosines (CCC) within the NS5B coding sequence was modified to affect the possible interaction with the IRES domain $IIId_1$ that contains a GGG motif. This NS5B motif was chosen as it shared a sequence similarity with the *cis*-acting RNA element 5BSL3.2 of HCV that has been shown to interact with the HCV IRES (Figure 1). Specifically, silent mutations in the CCC motif (CAC, CGC and CUC) were generated by site-

directed mutagenesis of a CSFV replicon. Thus, any variation in replication efficiency should not be due to changes in RNA polymerase activity (as the amino acid sequence is unchanged) but rather due to interactions of the RNA with other parts of the viral genome or with *trans*-acting factors. The effect on replication was measured using the luciferase reporter system. RNA transcripts generated *in vitro* were introduced into porcine PK15 cells by electroporation and the replication efficiency was assessed by measurement of luciferase activity. The Gluc replicon rPad2GL has previously been described by Risager *et al.*, 2013 but it has a mutation in the NS5B coding region (A11864G resulting in the amino acid substitution R3831G) that negatively affects viral replication. To restore the replicative ability, this mutation was corrected in the replicon rPad to match that of the parental CSFV Paderborn strain. Luciferase activity of rPad2GL and the corrected rPad can be seen in Figure 2 and it is apparent that correction of the non-synonymous mutation in NS5B significantly increased the level of expressed luciferase. The rPad replicon was therefore used as a template to construct the CSFV mutants with modifications within the motif in NS5B.

All three NS5B mutants (with synonymous mutations at nt position 11173) displayed significantly reduced viral replication compared to the wt replicon (Figure 3a). To exclude that this result was due to any codon bias, an analogous motif ca. 900 nt upstream (nt position 10306), also containing three consecutive cytosines, was mutated in a similar manner. The luciferase measurements showed no difference in viral replication for these mutants indicating that it was not the motif in itself but rather the specific location in NS5B that was important for the replication change (Figure 3b).

Growth of full-length CSFV mutants with modifications in a motif in NS5B

To test whether the viral growth was also influenced by mutating the CCC triplet, the same silent mutations were introduced into full-length virus. After one passage in porcine cells, RNA accumulation was examined by RT-qPCR. The viral titers of all full-length constructs were comparable and can be seen together with the number of estimated virus genome copies in Figure 4c. Cells were infected with the same virus genome copies from each of the mutants and virus growth curves were performed. The virus rescued from the mutants located in the upstream motif (at nt position 11050) displayed the same growth characteristics as the wt virus vPad supporting the luciferase data (Figure 4b). No change in growth characteristics could be observed between vPad and the three NS5B mutants with a silent mutation (at nt position

11917) (Figure 4a). For the vPad.C11917U mutant, with a cytosine to uracil change (at nt position 11917) at the last base of the codon (resulting in no amino acid change within the protein), a higher level of viral RNA was present at 3h post-infection but as shown in Figure 4c, the level of viral RNA after one passage was comparable between the viruses giving no apparent reason for this difference.

Viral infectivity was also determined by staining for the CSFV non-structural protein NS3. All of the mutants were found to be infectious and NS3 was observed in the cytoplasm of infected cells (Figure 5). Both the vPad.C11917U mutant and the vPad.C11050G mutant appeared to be less infectious than the vPad wt as they exhibited slightly less NS3 staining compared to the other NS5B mutants but this effect was not supported by the viral growth curves (Figure 4a). However, it should be noted that the immunostaining for the NS3 protein is a more qualitative than a quantitative assay.

Genetic stability of full-length NS5B mutants during serial passage in cell culture

The genetic stability of the NS5B mutants was examined after serial passages in cell culture. No changes were observed in the NS5B motif (including nt position 11917) or in the upstream motif (including nt position 11050) after 5 passages (data not shown). After one passage in PK15 cells, a synonymous secondary mutation at nt position 2164 within the E1 protein coding region occurred. The G2164U mutation was present in 41 – 74 % of the virus population at passage 1 in mutants with changes at nt position 11050, whereas only the guanine mutant vPad.C11917G of the NS5B mutants had acquired this change by this time (Figure 6). After four additional passages, the majority of the virus population of both the wt virus vPad and all mutants exhibited the G2164U mutation. Hence, it seemed to be a cell culture adaptation. By performing single nucleotide polymorphism (SNP) analysis, no other nucleotide changes were present in the majority of the virus population.

Discussion

In recent years, significant advances have been made in identifying and characterising *cis*-acting RNA elements in positive-sense RNA viruses. These studies have revealed insights into the structure and function of these elements and their role in regulating the virus life cycle.

By introducing specific silent mutations into a motif within the NS5B coding region, three consecutive cytosines were shown to be required for efficient RNA replication (Figure 3). This effect is not associated with any change in the amino acid sequence of the RNA polymerase but is probably due to interactions within the RNA genome. A previous study has found that the extreme 3'-UTR of CSFV is involved in translation repression (Huang *et al.*, 2012). Moreover, a possible long-range interaction between the last six nucleotides of the genome and the apical loop of domain $IIId_1$ in the IRES was suggested. Using CSFV and bovine viral diarrhoea virus (BVDV), the $IIId_1$ loop was blocked by antisense oligonucleotides or RNA aptamers and resulted in inhibition of the IRES-mediated translation (Kikuchi *et al.*, 2005; Tallet-Lopez *et al.*, 2003). These results suggested that the $IIId_1$ apical loop in CSFV and BVDV could be a control element for regulating viral gene expression.

The mutants with modifications in the NS5B coding region were analysed for cell culture dependent adaptations. After 5 passages, the mutated positions in the NS5B motif (including nt 11917) and the upstream motif (including nt 11050) remained unchanged. The NS5B motif (including nt 11917) is quite conserved for CSFV and comparison between 85 CSFV strains revealed only one strain with uracil instead of cytosine at that position. The same was true for the motif at nt position 11050, where only 1/85 CSFV strains had uracil and not cytosine. In contrast to the stability of these mutations, an adaptive silent mutation occurred in the E1 protein coding region (G2164U) after only one passage in cell culture and this was present in a majority of the virus population for both wt and mutant CSFVs at passage 5 (Figure 6). The guanine at nt position 2164 is very highly conserved in CSFV. Notably, when 85 full-length CSFV strains were aligned 9 strains had an adenine at nt position 2164 instead of guanine. These 9 strains were all vaccine strains indicating that the guanine might be important for virulence. Furthermore, the position 2164 is close to the N-linked glycosylation site N3 in E1 (Fernandez-Sainz *et al.*, 2009).

Interestingly, the G2164U adaptation was also found upon serial passaging of mutant CSFVs with multiple modifications in subdomain IIIf of the IRES but at a much lower level (Hadsbjerg *et al.*, 2016). In vPader10 the G2164U adaptation was present in under 10 % of the virus population after 20 passages. The sequence of vPader10 differed from the parental CSFV Paderborn strain at nt position 11864 in NS5B (resulting in the amino acid substitution R3831G) causing a lower replication rate. However, nearly 100 % of the genomes had the

amino acid substitution G3831R present in the parental strain, when assayed after 4 passages. Hence, the lower presence of the G2164U adaptation in vPader10 compared to vPad and the mutant CSFVs with modifications in NS5B should not be caused by impaired replication. The G2164U adaptation was also present in the domain IIIf IRES mutants (Hadsbjerg *et al.*, 2016). For two of the mutants, the G2164U mutation reached its highest level after passages 5 and 10, where approximately 20 % of the virus population exhibited the adaptation. However, after 20 passages the adaptation was only present in 3 – 8 % of the virus genomes. Thus, the selection for G2164U was not as strong as seen in the present study. It is not known whether the G2164U adaptation is related to the mutations introduced into the coding region of NS5B.

The significant effect on RNA replication seen for the replicons was not apparent in the full-length constructs (Figure 3 and Figure 4). The lack of some of the structural proteins in the replicons might have an impact. It may be that the G2164U adaptation present in the full-length constructs influenced the growth but had no effect on the replicons as they did not contain that part of E1. The structural proteins can influence virus replication as has been demonstrated in rhinovirus, a single stranded positive-sense RNA virus within the *Picornaviridae* family. A conserved *cis*-acting replication element was present in the coding sequence for the capsid protein VP2 that is essential for virus replication (Cordey *et al.*, 2008).

The RNA structure is important for proper function of the virus. Probing of the RNA secondary structure, using for example selective 2'-hydroxyl acylation analysed by primer extension (SHAPE) provides a quantitative way of determining RNA structure at a single nucleotide resolution (Merino *et al.*, 2005; Wilkinson *et al.*, 2006). SHAPE mapping of long-range interactions has been performed in a number of viruses including HCV (Tuplin *et al.*, 2012), FMDV (Diaz-Toledano *et al.*, 2016) and Mason-Pfizer monkey virus (MPMV) (Kalloush *et al.*, 2016). Whole-genome structure probing was attempted on two of the replicon constructs using SHAPE. However, in an initial experiment, it was not possible to obtain SHAPE probing which might be due to premature termination of the reverse transcriptase only yielding structure information from the 3'-end (data not shown). Thus, optimisation of the protocol for mapping of long-range interactions is needed.

An extensive network of RNA-RNA interactions has been characterised for HCV but knowledge of RNA-RNA interactions within the CSFV genome is still scarce. Further understanding on these putative long-range interactions will provide valuable insight into mechanisms underlying viral RNA replication. The strategy employed here does not only apply to studies on CSFV but can be used to investigate the replication of a broad range of viruses.

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Figures

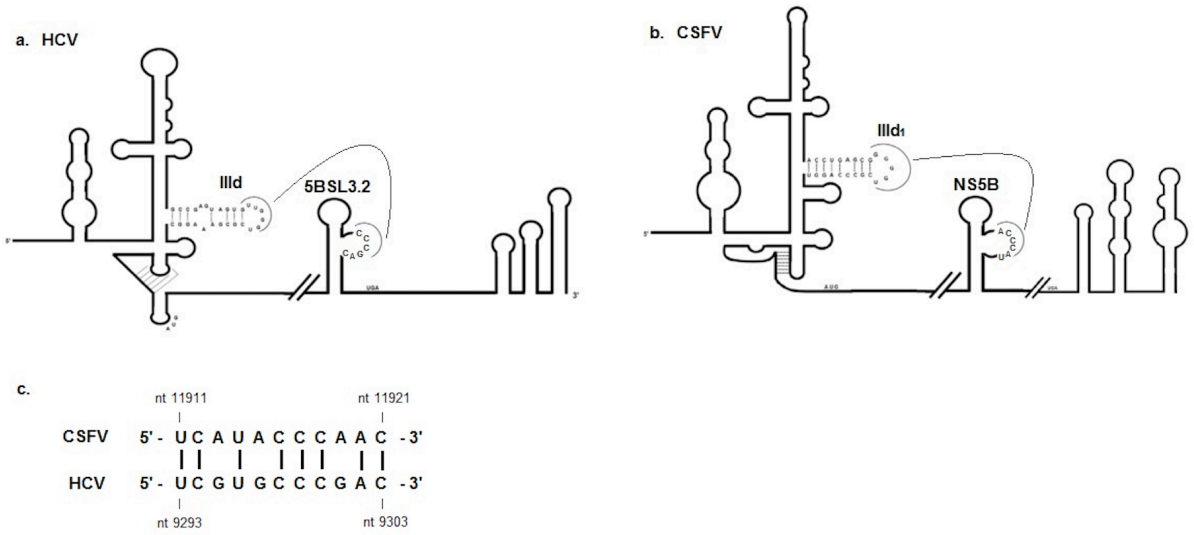


Figure 1. Proposed interactions between the IRES domain IIId$_1$ and a motif in the coding region for NS5B. (a) A long-range RNA-RNA interaction is present between 5BSL3.2 and the IRES IIId domain of HCV. (b). Hypothesised interaction between a motif in NS5B and the IRES IIId$_1$ domain of CSFV. In this study, mutations have been introduced into a CCC triplet in NS5B. Modified from (Huang et al., 2012). (c) Sequence similarity between the CSFV motif in the coding region of NS5B and the cis-acting RNA element 5BSL3.2 of HCV. Homology is observed within a motif consisting of 11 nt of which 8 nt are a direct match.

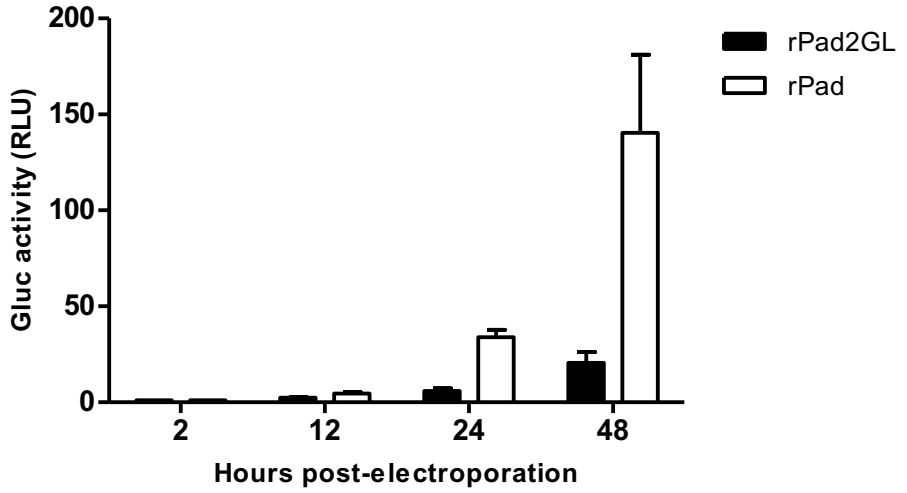


Figure 2. **Reversion of a secondary mutation in NS5B to the parental sequence restores CSFV replication.** A coding mutation in the NS5B protein of the Gaussia luciferase replicon rPad2GL (described by Risager et al., 2013) was corrected to match the parental CSFV Paderborn sequence (denoted rPad). Three independent experiments were carried out and measurements were normalised to the 2h post-electroporation signal. RLU, relative light units.

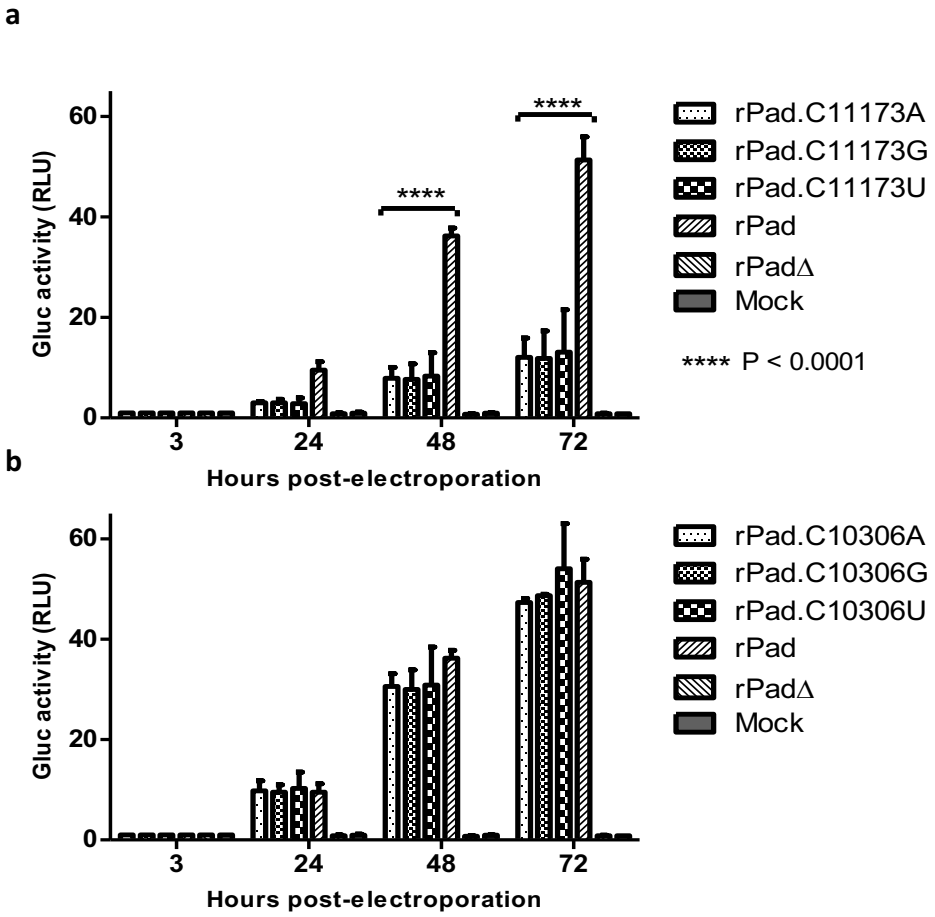
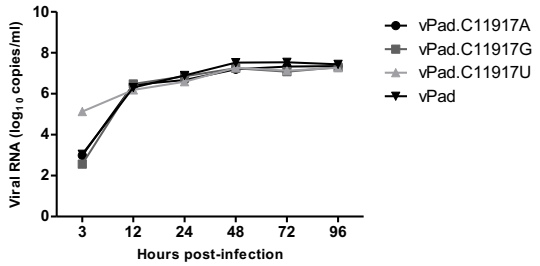


Figure 3. Viral replication of wt and mutant CSFV replicons within PK15 cells. PK15 cells were electroporated with RNA transcripts of wt and mutant CSFV replicons and at the indicated time points, samples of the medium were collected and assayed for Gluc activity. rPadΔ is a replication-defective mutant that lacks a part of the coding region of NS5B and the entire 3'-UTR. The mock sample is uninfected without an RNA transcript. The results are based on three independent experiments and the measurements were normalised to the 3h post-electroporation signal. (a) Gluc activity of the wt replicon (rPad) and mutants with specific silent mutations (at nt position 11173) in a motif in NS5B. (b) Gluc activity of the wt replicon (rPad) and mutants with specific silent mutations (at nt position 10306) in a motif in NS5B.

a



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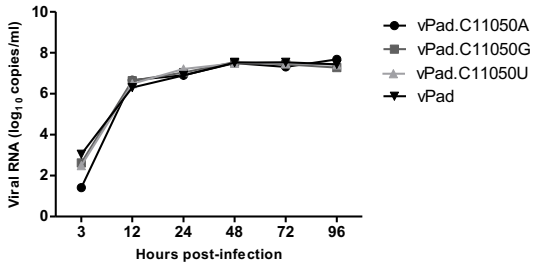

c

Construct	$TCID_{50}$/ml	Virus genome copies/ml
vPad	6.3×10^6	3.4×10^7
vPad_C11917A	3.6×10^6	4.1×10^7
vPad_C11917G	1.1×10^7	2.3×10^7
vPad_C11917U	1.1×10^7	2.5×10^7
vPad_C11050A	9.3×10^6	2.1×10^7
vPad_C11050G	6.3×10^6	2.8×10^7
vPad_C11050U	1.1×10^7	3.1×10^7

Figure 4. Growth characteristics of wt and mutant CSFV ICs within PK15 cells. PK15 cells were electroporated with RNA transcripts of wt and mutant CSFVs from passage 1. (a) The growth of rescued wt virus and mutants with specific silent mutations (at nt position 11917) in a motif in NS5B as measured by RT-qPCR. (b) The growth of rescued wt virus and mutants with specific silent mutations (at nt position 11050) in a motif in NS5B using one-step growth curves as measured by RT-qPCR. (c) Viral titers and copy numbers were determined in virus harvests collected at 72h post-infection with wt and mutant CSFVs from passage 1.

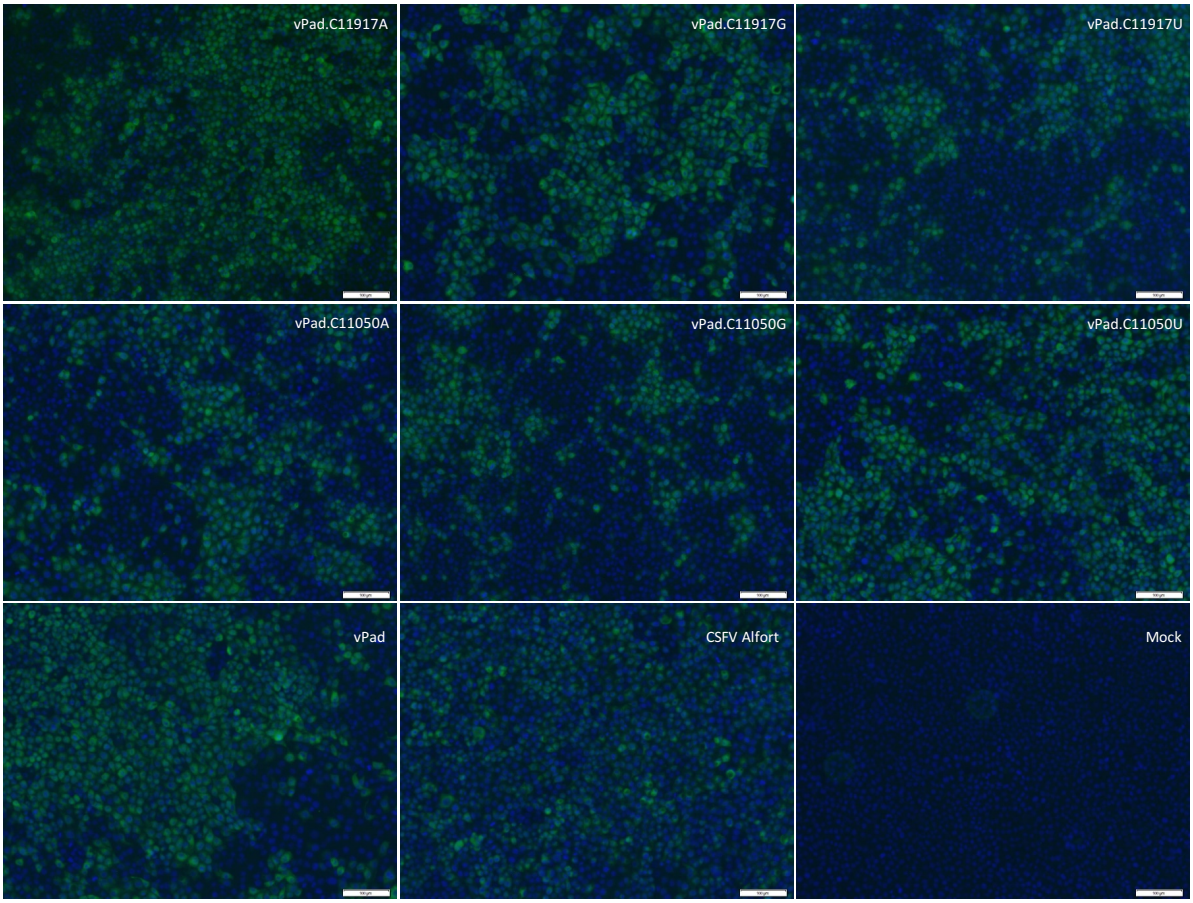

Figure 5. Immunostaining of NS3. Infectivity was tested in vitro by transfecting RNA transcripts of wt and mutant CSFV ICs into PK15 cells. The production of the non-structural protein NS3 (green) and the cell nuclei stained with DAPI (blue) were visualised after 72h using immunostaining. CSFV Alfort was used as a positive control and the mock sample is uninfected. Pictures were taken at 10x magnification.

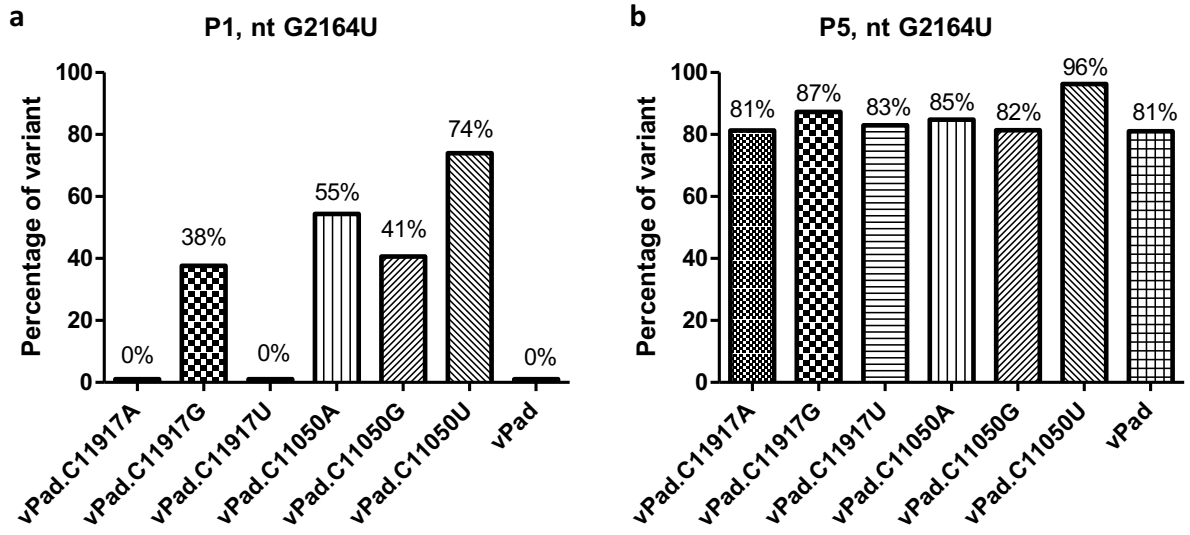

Figure 6. Sequence adaptation in wt and mutant CSFV ICs within PK15 cells. The complete genome sequence of vPad and the indicated mutants were determined by NGS at passage 1 and passage 5. Full-length amplicons were generated by RT-PCR and the proportion of mapped reads containing the synonymous mutation G2164U in the E1 structural protein is presented.

Manuscript 3

Establishment of a cytoplasmic expression system using a classical swine fever virus-based replicon

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Establishment of a cytoplasmic expression system using a classical swine fever virus-based replicon

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Abstract

The ability to efficiently express viral proteins or antigens is an invaluable tool for vaccine development and diagnostic purposes. Viral vectors can induce a high level of protein production by delivering and maintaining genetic material within mammalian cells. By using positive-sense RNA viruses, where the viral life cycle takes place in the cytoplasm of infected cells, an expression system can be established without a requirement for nuclear RNA processing. A genetically defined replicon, derived from classical swine fever virus (CSFV), has been used to construct a neomycin-selectable replicon. The neomycin phosphotransferase coding sequence (as cDNA) was introduced into a bacterial artificial chromosome (BAC) containing cDNA corresponding to a CSFV replicon. RNA transcripts were produced *in vitro* and transfected into porcine PK15 cells by electroporation. Viral translation and replication was assessed by the accumulation of the luciferase reporter protein and expression of the CSFV non-structural protein NS3. The neomycin-selectable replicon was demonstrated to be functional and retained similar levels of luciferase activity and NS3 protein production as a CSFV replicon without the neomycin phosphotransferase sequence. The selectable CSFV replicon might prove useful for maintenance and expression of viral antigens in an efficient manner.

Introduction

Classical swine fever (CSF) is caused by a highly contagious virus infection in pigs with high economic importance for countries with a significant trade of agricultural products. Classical swine fever virus (CSFV), classified as a member of the pestivirus genus, has a positive-sense RNA genome containing a single large open reading frame encoding a polyprotein that is processed into structural and non-structural proteins. The 5'-untranslated region (UTR) contains an internal ribosome entry site (IRES) that directs translation initiation on the viral genome (Fletcher & Jackson, 2002). The 3'-UTR is proposed to be involved in pestivirus genome replication, which takes place in the cytoplasm of infected cells (Isken *et al.*, 2003;

Pankraz *et al.*, 2005). The structural proteins of CSFV are not required for replication and maintenance of the viral RNA (Moser *et al.*, 1999). Hence, replicons which are self-replicating and non-infectious genomes can be generated by deleting the coding region for one or more of the structural proteins. Production of replicons relies on the availability of infectious cDNA clones, which permit genetic manipulation of viral genomes. cDNA clones containing pestivirus genomes (as cDNA) that are known to be replication competent are available (Meyers *et al.*, 1996; Moormann *et al.*, 1996; Rasmussen *et al.*, 2010; Ruggli *et al.*, 1996). Such cDNA clones have been used to construct replicons for CSFV and other pestiviruses (Reimann *et al.*, 2003; Risager *et al.*, 2013). For hepatitis C (HCV), full-length cDNA clones were shown to be capable of generating infectious HCV in chimpanzees, which was the only known animal model at that time, but these HCV cDNAs could not generate infectious virus in cell culture initially (Bartenschlager, 2006; Kolykhalov *et al.*, 1997).

By inserting a gene for a selectable marker into a cell, an artificial selection system can be achieved, hence allowing cells that express the inserted gene to be isolated. Commonly, genes conferring antibiotic resistance are used as selectable markers. A widely used selectable marker is neomycin phosphotransferase (NPT), which confers resistance (neo^r) to the cytotoxic drug neomycin sulfate (G418). Neomycin selection has been used for HCV as a way to study viral replication since the virus was found to grow poorly in cell culture until the establishment of a specific cell culture system in 2005 (Wakita *et al.*, 2005; Zhong *et al.*, 2005). Until then HCV replication could only be analysed using HCV-derived replicons, which expressed the NPT marker and hence conferred resistance to G418 on cells containing the replicon (Lohmann *et al.*, 1999). Thus, cell colonies with replicating HCV genomes could be isolated. However, it was found that the replicons acquired adaptive mutations affecting viral replication in certain HCV strains (Blight *et al.*, 2000). An alternative selection agent, puromycin N-acetyltransferase (PAC) has been used instead to study replication in a wider range of HCV genotypes (Liang *et al.*, 2005). This system has provided a powerful tool for studying mechanisms of HCV replication and selecting potential antiviral agents.

Viral vectors, based on modified viral genomes, can be suitable for achieving specific protein production as they can deliver and maintain genetic material inducing a high level of protein expression (Bentley *et al.*, 1994; Bleckwenn *et al.*, 2003; Campeau *et al.*, 2009; Cotten *et al.*, 2003).

Using positive-sense RNA viruses, which have their viral life cycle in the cytoplasm, an expression system without nuclear involvement can be established. This system will avoid issues with nuclear RNA processing, thus preventing unwanted splicing or other RNA modifications. Moreover, viruses such as pestiviruses and HCV are non-cytopathogenic, thus cells can maintain the positive-sense RNA virus replication for a prolonged period of time (Greiser-Wilke *et al.*, 1993).

Viral vectors constitute a convenient way to deliver antigens to a target cell or tissue. The majority of viral vectors used as vaccines are designed to induce cellular immune responses by activating T cells and B cells. Still, inoculation with a booster containing protein antigens is usually necessary to elicit broad and potent antibodies. However, some viral vectors such as those based on alphaviruses can be genetically modified to induce a potent antibody response, which might prove to be a useful alternative (Forsell *et al.*, 2007; Liljeström & Garoff, 1991; Lundstrom *et al.*, 2001). The selectable replicons can also provide a cell-based system to assay antiviral drugs.

The aim of this study was to develop a cell-based stable and selectable reporter system using a CSFV replicon. This system should allow maintenance and expression of antigens derived from other positive-sense RNA viruses that replicate exclusively in the cytoplasm of cells. These antigens can be difficult to express efficiently using conventional mammalian expression systems that generally express the mRNAs from within the nucleus. This cytoplasmic expression system, should be useful for the synthesis of antigens and protein products from important animal viruses could be used for vaccine development and diagnostic purposes.

Methods

Cells. The porcine kidney cell line PK15 was grown in Dulbecco's Modified Eagles medium (DMEM) containing 5 % foetal calf serum (FCS).

Replicon construction. A cDNA clone of the CSFV strain Paderborn termed pBeloPader10 (Rasmussen *et al.*, 2010) has previously been used to construct a replicon containing the *Gaussia* luciferase (Gluc) coding sequence (Risager *et al.*, 2013). A neomycin-selectable replicon was generated by inserting the coding sequence for neomycin phosphotransferase (NPT), conferring

G418 resistance, downstream of the coding sequence for the Gluc – FMDV 2A peptide (18 aa in length). A counter-selection cassette (rpsL/neo cassette; Gene Bridges) conferring streptomycin sensitivity and neomycin resistance was used as a template to construct a PCR product containing the NPT sequence flanked by coding sequences encoding the FMDV 2A peptide and the EMCV 2A peptide (18 aa in length) using standard PCR. This fragment, which maintained the single long open reading frame (ORF), was subsequently utilised as a megaprimer using a modified version of the protocol for targeted-primed plasmid amplification (Friis *et al.*, 2012; Wei *et al.*, 2004). The PCR products were treated with *DpnI*, purified using the GeneJET PCR purification kit (Thermo Scientific) and electroporated into DH10B *E. coli* cells. Colonies were selected on agar plates containing chloramphenicol at 15 µg/ml and plasmids were purified using the ZR BAC DNA miniprep kit (Zymo Research). Genome length amplicons were generated using PCR (as described by Rasmussen *et al.*, 2008, 2010) using AccuPrime *Taq* DNA polymerase, high fidelity (Invitrogen). A single point mutation, causing a lower replication rate, within the NS5B protein coding sequence (A11864G resulting in the amino acid substitution R3831G) was corrected causing reversion to the parental CSFV Paderborn sequence (GenBank Accession number AY072924). The Gluc replicon with this amino acid reversion is denoted rPad and the neomycin-selectable replicon is called rPad_neo. Insertion of the NPT coding region was verified by sequence analysis using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI3500 Genetic analyzer (Applied Biosystems). Moreover, the replicons were full-length sequenced using next generation sequencing (NGS) and analysed with the CLC Genomics Workbench software (CLC bio).

Generation and transfection of RNA transcripts. Using the MEGAscript T7 transcription kit (Ambion) the full-length amplicons were *in vitro* transcribed for 3h at 37 °C. The RNA was evaluated on a 1 % TBE agarose gel containing a 10,000x dilution of the SYBR Green II RNA gel stain (Invitrogen) and the yield was determined using a Qubit RNA BR assay kit (Invitrogen) on a Qubit 3.0 fluorimeter. Transfection was conducted essentially as described in Friis *et al.*, 2012. Briefly, the RNA was introduced into PK15 by electroporation using a Gene Pulser XCell (Bio-Rad) set at 180 V and 950 µF. After electroporation, the cells were allowed to settle on ice for 10 min before seeding on a 24-well plate; this was considered the assay starting point, T_0. Three hours after plating, the medium was replaced with fresh DMEM containing 5 % FCS and 50 µl aliquots of the medium was frozen at -20 °C at 3h, 24h, 48h and 72h post-electroporation.

Luciferase assay. The level of expressed luciferase was assayed as previously described (Risager *et al.*, 2013). In brief, 20 µl supernatant was added to 100 µl of Renilla luciferase assay reagent containing a 100-fold dilution of the coelentarazine substrate in Renilla luciferase assay buffer (Promega). Luciferase activity was quantified using a Bio-orbit 1253 Luminometer (Aboatox).

Detection of the CSFV NS3 protein within cells by immunofluorescence. Staining of PK15 cells for the presence of the CSFV non-structural protein NS3 was carried out as previously described (Friis *et al.*, 2012). Cells were incubated for 72h at 37 °C in 5 % CO_2 before staining with the anti-CSFV NS3 monoclonal antibody WB103/105 (AHVLA Scientific) and Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Cell nuclei were visualised using DAPI (VectaShield) and images were taken with a BX63 fluorescence microscope (Olympus).

Selection with G418. PK15 cells transfected with the neomycin-selectable replicons were seeded in a 24-well plate. The cells were allowed to settle for 3h after which the plate was washed to remove dead cells and the cells were replenished with fresh medium. After 24h, the cells were supplemented with DMEM containing 5 % FCS and neomycin sulfate G418 (0.8 mg/ml). The susceptibility of G418 in PK15 cells was established by seeding 5,000 cells/well in a 96-well while titrating with G418. Final concentrations of G418 used ranged from 0.2 – 1.4 mg/ml. The cells were incubated for up to 5 days at 37 °C with 5 % CO_2. The lowest G418 concentration showing complete cell death (0.8 mg/ml) was chosen for selection. The medium with G418 was replaced every third day. Luciferase activity in the cell medium was measured at 3h, 24h, 48h and 72h post-electroporation.

Results

Establishment of the selectable replicon

To generate a CSFV selectable replicon, the NPT coding region was inserted into a CSFV replicon containing *Gaussia* luciferase (Gluc) as described by (Risager *et al.*, 2013). The replicon designated rPad_neo is depicted in Figure 1. To ensure proper release of Gluc and NPT, the "self-cleaving" 2A peptide from foot-and-mouth disease virus (FMDV) and encephalomyocarditis virus (EMCV) was inserted downstream of the two coding sequences, respectively. Insertion of the 2A peptide in tandem with a protein ensures the yield of a

functional and unmodified reporter (Donnelly *et al.*, 2001; Jones *et al.*, 2007). This is a result of a co-translational break of the polypeptide chain within a conserved motif of the 2A peptide (DvExNPG/P) through ribosome skipping between the G and P residue. The Gluc-FMDV2A-NPT-EMCV2A cassette was inserted in-frame and only cells that had taken up the replicon should be resistant to the cytotoxic drug G418, which effectively blocks protein synthesis (Southern & Berg, 1982). The complete sequence of the rPad_neo replicon was verified by NGS.

In vitro characterisation of the selectable replicon

To determine the replicative ability of rPad_neo, RNA transcribed from full-length PCR products was transfected into porcine PK15 cells. Samples of medium were collected at 3h, 24h, 48h and 72h after electroporation and assayed for production of Gluc protein by measuring the luciferase activity (Figure 2). A truncated replication-defective replicon denoted rPadΔ, lacking a part of the coding region of NS5B and the 3'-UTR, was used as a non-replicating control (this should yield Gluc from translation of the input RNA but no replication will occur). Time-dependent expression of the Gluc was obtained with the intact replicons but little or no expression was achieved with the replication defective variant. The amount of secreted luciferase was equivalent for the Gluc replicon (rPad) and the neomycin-selectable replicon (rPad_neo) demonstrating that the replicative ability remains intact after insertion of the NPT coding sequence. To confirm this result, expression of the non-structural CSFV protein NS3 was examined by immunostaining (Figure 3). Both replicons expressed the NS3 protein in the cytoplasm of transfected PK15 cells, indicating that they were functional. No viral spread to adjacent cells was observed as infectious virus progeny cannot be formed due to the lack of structural proteins. Selection of cells containing the replicon with G418 was then attempted. The amount of G418 to obtain proper selection was determined using final concentrations of G418 ranging from 0.2 to 1.4 mg/ml. Complete cell death was observed using 0.8 mg/ml G418 (data not shown). Similar levels have been used for other studies with PK15 cells (Xu *et al.*, 2008; Zhou *et al.*, 2010). When cells were grown under G418-selection, extensive cell death was observed despite transfection with the rPad_neo replicon. G418 was added 24h after electroporation and it might be that the cells were more sensitive to selection and needed extra time to grow and express the NPT. As a consequence, no luciferase activity could be measured after selection with G418.

Discussion

To date, it has been possible to demonstrate that the replicon expresses the Gluc reporter protein and that the CSFV NS3 protein can be detected within the cytoplasm of cells. However, it has not yet been possible to achieve selection of cells based on the presence of the NPT coding region. The difficulties with achieving G418-selection for the rPad_neo replicon may be due to problems with obtaining adequate amounts of full-length RNA for transfection, as the structure of the construct has been confirmed by NGS. It may also be necessary to optimise the time when the G418 selection is applied. When selection has been established, the expression of the replicon RNA will be measured using RT-qPCR on a weekly basis for up to one month. Moreover, the replication competence will be analysed by assaying the luciferase expression of the Gluc reporter protein. When the stable and selectable reporter system has been developed, antigens from other positive-sense RNA viruses will be introduced. This could be non-infectious FMDV empty capsids consisting of processed products of P1-2A or the envelope glycoproteins E1 and E2 of HCV (Beaumont *et al.*, 2016; Gullberg *et al.*, 2013; Rweyemamu *et al.*, 1979). A number of replicon-based vectors derived from positive-sense RNA viruses have been established. Among these are the Australian flavivirus Kunjin virus that can stably express replicon RNA and heterologous proteins along with antibiotic resistance marker proteins (Pijlman *et al.*, 2006).

Further studies are needed to obtain a selection system that can be used for development of vaccines or antiviral drugs.

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Figures

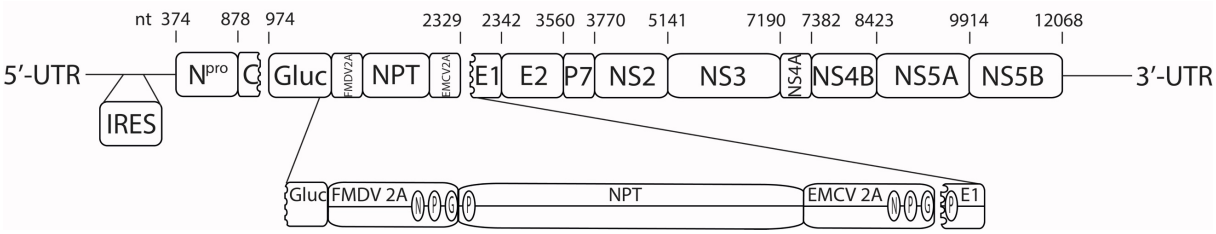

Figure 1. Schematic representation of the selectable replicon rPad_neo. The replicon is based on the previously described construct rPad2GL (Risager et al., 2013) with partial deletion of the structural proteins C and E1 as well as a complete removal of E^{rns}. A single point mutation within the NS5B protein coding sequence (A11864G resulting in the amino acid substitution R3831G), present in the rPad2GL replicon, was corrected causing reversion to the parental CSFV Paderborn sequence (GenBank Accession number AY072924). The neomycin phosphotransferase coding region (NPT) and 18 aa from the EMCV 2A protein were inserted in conjunction thus maintaining the open reading frame. The predicted amino acids within the conserved motif for the 2A coding sequences, resulting in a co-translational break, are shown.

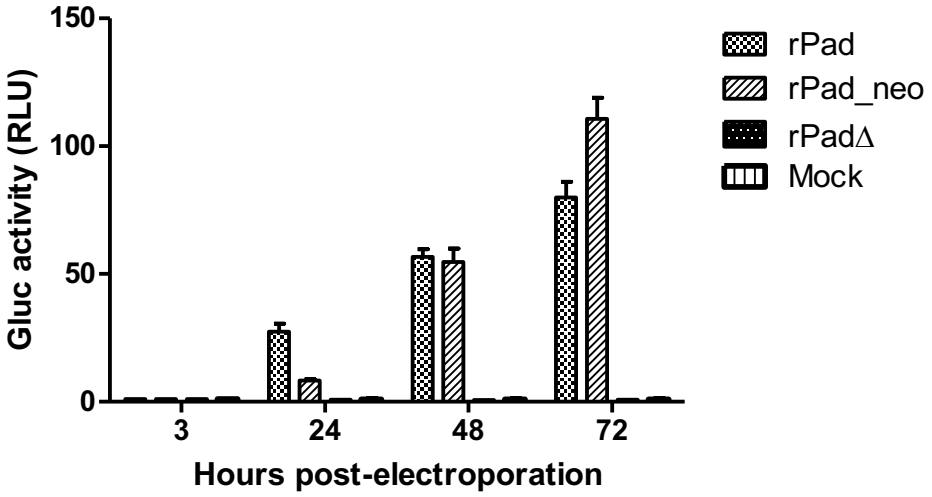

Figure 2. Luciferase activity of the neomycin-selectable replicon. The expression level of the secreted Gaussia luciferase reporter protein is comparable between the rPad replicon and the rPad_neo containing the NTP marker. rPadΔ is a truncated RNA transcript lacking a part of the NS5B coding region and the entire 3'-UTR. Three technical replicates were performed and measurements were normalised to the 3h post-electroporation signal. RLU, relative light units.

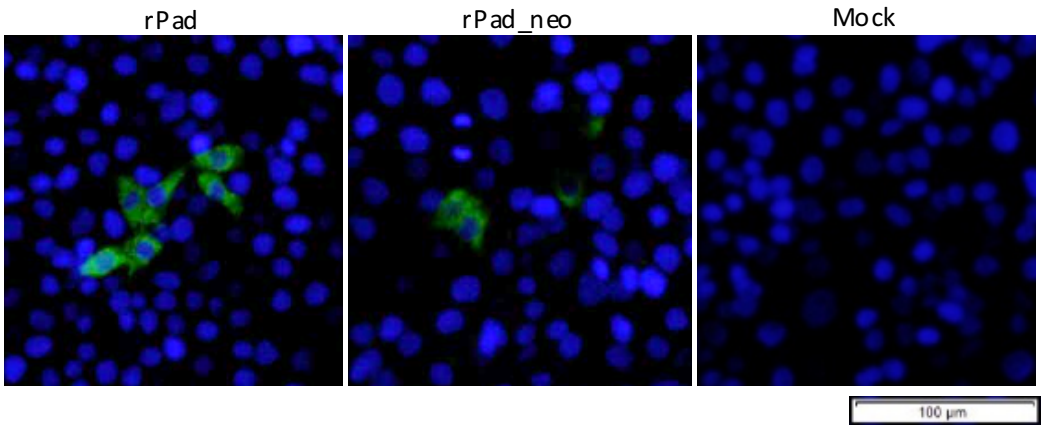

Figure 3. Expression of the non-structural CSFV protein NS3. RNA transcripts containing the Gluc replicon (rPad) and the neomycin-selectable replicon (rPad_neo) were transfected into PK15. The NS3 protein was detected with the pestivirus-specific monoclonal antibody WB103/105 and a secondary antibody labelled with Alexa Fluor 488 (green). The cell nuclei were stained with DAPI (blue). Staining for NS3 was found for both replicons but only few cells were found to be stained due to the lack of cell spread of the replicons.

Part 4 • Conclusions and perspectives

Chapter 6

Conclusions and perspectives

Positive-stranded RNA viruses comprise over one-third of known virus genera and include important human, animal and plant pathogens (den Boon *et al.*, 2010). RNA viruses have a very high mutation rate. This high error rate leads to genome differences ranging on average from 0.1 – 10 nucleotides compared to the parental genome. For FMDV, the consensus sequence of field strains changes 0.5 – 1.0 % each year corresponding to 40 – 80 nucleotides/year (Haydon *et al.*, 2001). Recently, the substitution rate for the complete CSFV genome was investigated using virus strains collected during a French outbreak in wild boar between 2003 and 2007 (Goller *et al.*, 2016). The overall substitution rate was 1.5×10^{-3} substitutions per site a year, which corresponds to 15 – 20 nucleotides/year. The error-prone replication results in the formation of a cloud of closely related viruses commonly referred to as a "quasispecies". Thereby heterogeneous virus populations of genetically closely related but distinct variants are formed that contain a spectrum of mutations that can be beneficial, neutral or deleterious for replication. For example, it has been shown that replication of CSFV produce a high proportion of genomes that contain deleterious mutations and only a minority of the genomes are infectious (Fahnøe *et al.*, 2015). Some defects could potentially be *trans*-complemented but this requires that the same cell is infected with multiple virus particles.

Due to this property of RNA viruses, different sequence variations can emerge during replication. An interesting aspect is the constraints on this variation. Many of the changes are silent as has been shown in manuscript 1. This manuscript also demonstrates the importance of RNA structure, since a stable structure, the pseudoknot in the IRES, is crucial. Not only single-point mutations have an influence on the ability of the virus to replicate but also more dramatic changes can happen as a result of RNA recombination. One of the factors behind the emergence of new viral strains and species is this recombination, where insertions, deletions or duplication occur. Most cytopathogenic pestivirus strains originate from non-cytopathogenic viruses by non-homologous RNA recombination (Gallei *et al.*, 2005). RNA recombination is also a central aspect for removing debilitating or lethal mutations and can contribute to viral pathogenicity. Not only viral sequences can be incorporated in a new way through

recombination but also various cellular protein coding sequences can be inserted, as has been seen for BVDV (Becher & Tautz, 2011). It is hypothesised that RNA recombination depends on a copy choice, or "polymerase jumping", mechanism where the polymerase switches template during negative-strand synthesis (Gallei *et al.*, 2004). Recombination sites were mainly located in single-stranded regions within the BVDV genome in line with the involvement of endoribonuclease cleavage and subsequent ligation during recombination (Austermann-Busch & Becher, 2012). However, this is different for the mechanism proposed for RNA recombination in poliovirus that was shown not to be site-specific (Kirkegaard & Baltimore, 1986).

It has become increasingly evident that intra-genomic interactions between distant RNA elements can mediate important aspects of the viral life cycle. These RNA elements form extensive structures through base pairing between complementary sequences contributing to genome structure stability. Furthermore, such RNA structures provide binding sites for viral or cellular proteins that together with RNA-RNA interactions influence virus replication. Interactions between RNA elements are also important in switching from translation to virus replication as has been seen for HCV and DENV (Palau *et al.*, 2013; Villordo *et al.*, 2010). Interactions between distant RNA elements have been investigated in manuscript 2, where introduction of mutations in a motif within the NS5B coding region capable of a putative interaction with the 5'-UTR resulted in decreased virus replication within a replicon. This demonstrates that sequence variations with no effect on the protein function (as the amino acid sequence was unchanged) can affect virus replication. However, evidence for a direct interaction between this NS5B motif and the 5'-UTR has yet to be established. A number of techniques have been used to show the presence of long-range RNA-RNA interactions including surface plasmon resonance, electrophoretic mobility shift assay and SHAPE mapping (Palau *et al.*, 2013; Souii *et al.*, 2013; Tuplin *et al.*, 2012). Many functional RNA structures in virus genomes have been described during the last 20 years. Many of those involved in intra-genomic interactions that are located within the untranslated parts of the genome. However, extensive RNA structures within the coding region are also found (Liu *et al.*, 2009). The function of many of these still has to be elucidated. Some could be required for virus replication, like the *cis*-acting replication element, or for RNA packaging within new virus particles. Others could be important for protein binding to either separate RNA structures, for bringing the RNA

structures together or to maintain RNA structure stability. Secondary structures within the coding sequence have been found for FMDV RNA using whole genome structure probing (Poulsen *et al.,* 2015b). 17 regions in the protein coding regions of the genomes containing previously experimentally un-probed structures were identified and several of these appeared to be functionally conserved.

By the use of computational methods, long-range RNA-RNA interactions can be predicted. By comparing 23 pestivirus strains with the *Gaussia* luciferase replicon rPad, twelve different long-range RNA-RNA interactions were identified based on a p-value below 0.05 (unpublished data). Of these, eight interactions are with the 5'-UTR; either within the UTR (two predicted interactions) or with sequences for the coding region for E2, NS2, NS3 and NS5B, respectively. Predicted interactions were also found solely within the protein coding regions with interactions spanning distances from 5,000 to 6,000 nucleotides between NS5B and NS2 or NS3. This data has been gathered as previously described by Fricke & Marz, 2016. No conserved interactions among the pestiviruses were predicted between the 5'-UTR and the motif in the NS5B coding region examined in manuscript 2. Moreover, it was not possible to examine for interactions within CSFV genomes alone as the sequences were too similar.

Different steps of the virus life cycle including receptor-mediated entry and delivering of the intact RNA genome to the cytosolic site of translation and replication can have an impact on viral infectivity. Mutations in regions of the viral genome mediating virus entry, translation or RNA replication might influence the viability of the virus population (Lauring & Andino, 2010). In picornaviruses, the number of RNA molecules capable of initiating an infection resulting in one plaque forming unit (pfu) has been estimated. In cells infected with FMDV virion RNA about 100 – 1000 genomes were required to induce one pfu and a microinjection study indicated that a high proportion of the genomes was viable (Belsham & Bostock, 1988). Approximately 100 RNA genomes have been shown to correspond to around one $TCID_{50}$ in the highly virulent CSFV Koslov strain (Fahnøe *et al.*, 2014). The relatively ratio between the viral titer and the virus genome copies, presented in manuscript 2, is even lower. However, calculating the number of virus genome copies is only an estimation and cannot be directly compared to a virus titer.

The expression of recombinant proteins using a viral vector has been well established. A widely-used system in the production of viral vaccines is the baculovirus expression system (Felberbaum, 2015). Among the viral vaccines produced are two CSFV subunit vaccines based on the E2 glycoprotein (Beer *et al.*, 2007). An expression system in the unicellular green alga *Chlamydomonas reinhardtii* has been used to produce the HIV antigen P24, a likely indispensable component of a future AIDS vaccine (Barahimipour *et al.*, 2016). Selection of transgenic algal clones using a selectable marker allowed high-level expression of the antigen. This alga is advantageous as it can be easily genetically modified concomitant with its ability to be cultured in large volumes giving favourable conditions for production of foreign antigens. To ensure continuous growth of replicon containing cells, antibiotic selection is applied. This allows the cells containing the selectable replicon, which might grow less efficiently than uninfected cells, to be maintained. Moreover, selection is sustained as mutations (e.g. in the polymerase) might occur affecting the replicative ability of the replicons. Usually, selection is applied for approximately one month in order to maintain the expression of the replicon.

The establishment of a selectable replicon, as has been performed in manuscript 3, can be adapted to other related viruses, e.g. the pestiviruses BVDV or BDV. Unlike CSFV that is restricted to domestic pigs and wild boar, BVDV and BDV can infect a wider range of hosts including cloven-hooved animals like cattle, sheep and goats. Hence, they are expected to grow better in a number of different cell types which is advantageous for large-scale production. However, as the RNA genome is introduced directly into cells through transfection there should be no need for appropriate cell receptors. A benefit in establishing the selectable replicon in another pestivirus than CSFV is the lower restrictions on biosafety contamination levels. CSFV is an OIE-listed virus and thus higher biosafety levels are needed than for other pestiviruses. Even though the CSFV replicon is non-infectious, it still contains the replicative parts of the CSFV genome. Thus, the precautions for working with replicons derived from CSFV are more restrictive than for BVDV replicons.

Different constraints on sequence variation can also operate in cell culture and within an infected animal. Tropism for different cells or tissues can differ between the various members of the virus population and this might affect virulence. Replication determinants are not only influenced by the virus itself but also related to its interactions with the host. Virus replication intrinsically relies on the replication machinery of the cell and adaptation to the host is essential

for virus propagation. The host animal will upon infection try to block the virus, e.g. by interferon induction and generation of antibodies. In turn, the virus has ways to obstruct this as seen with the viral protein N^{pro} that mediates proteasomal degradation of IRF3. The ability of the virus to overcome these host defense systems can also influence the nature of the virus population that circulates in an infected host animal. In addition, viruses that have to be transmitted between different animals have to achieve infection after a bottleneck, as not all of the virus genomes can survive such a selection event. Each of these systems might select for different characteristics.

Several strategies have been used to investigate replication determinants. Most of these have relied on indirect evidence through studies of impaired viral fitness upon introduction of mutations into a specific region. However, replication determinants should rather be identified based on the ability to enhance virus replication or increase virulence. Replication determinants can be delineated by systematic swapping of coding sequences between high, moderate and low virulence strains. Such a gain of function study was performed on CSFV, where the RNA polymerase of a highly virulent strain was inserted into a vaccine strain causing increased growth rates and *in vitro* viral spread (Risager *et al.*, 2013). In a separate study, three amino acid residues in the E2 and NS4B proteins, that are critical for CSFV virulence, were identified after serial passage of a CSFV vaccine strain in cell culture (Tamura *et al.*, 2012). Introduction of mutations that modified these three residues enhanced virus spread and replication in cell culture and increased the pathogenicity *in vivo.* By use of reverse genetics, functional viruses from non-functional cDNA clones have been created that were infectious in cell culture and virulent in pigs (Fahnøe *et al.*, 2015). These studies demonstrate how comparative analysis of genetically related viruses with different replication competences or virulence can be useful in unravelling determinants pivotal for virus replication.

Understanding the relationship between RNA genome structure and function is of great importance in gaining insights into virus replication determinants. Knowledge on the folding dynamics and transitions between different RNA structures is crucial. It will be interesting to investigate the presence of other *cis*-acting RNA elements and their role in virus replication. This could be expanded to more CSFV genotypes or other members of the pestivirus genus. These regions are highly conserved and might be considered excellent candidates for the design

of antiviral strategies. Moreover, investigating how viral and cellular proteins regulate long-range RNA-RNA interactions will be of interest. Clearly, the virus is influenced by its cellular environment but the exact mechanisms are unknown.

Abbreviations

BAC	Bacterial artificial chromosome
BDV	Border disease virus
bp	Base pair
BVDV	Bovine viral diarrhoea virus
cDNA	Complimentary DNA
CRE	*Cis*-acting RNA element
CSF	Classical swine fever
CSFV	Classical swine fever virus
DENV	Dengue virus
DIVA	Differentiating infected from vaccinated animals
DNA	Deoxyribonucleic acid
eEF1A	Eukaryotic elongation factor 1A
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmatic reticulum
FMDV	Foot-and-mouth disease virus
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSP70	Heat shock protein 70
IL-1β	Interleukin-1β
IRES	Internal ribosome entry site
IRF3	Interferon regulatory factor 3
ITAF	IRES trans-activating factor
kb	Kilobase
mRNA	Messenger RNA
NGS	Next generation sequencing
NPT	Neomycin phosphotransferase
NTPase	Nucleoside triphosphatase
OIE	Office International des Epizooties
ORF	Open reading frame
pfu	Plaque forming units
RING-MaP	RNA interaction groups by mutational profiling
RNA	Ribonucleic acid
SHAPE	Selective 2'-hydroxyl acylation analysed by primer extension
SHAPE-MaP	Selective 2'-hydroxyl acylation analysed by primer extension mutational profiling
SINV	Sindbis virus
UTR	Untranslated region
VRC	Viral replication complex
VRP	Virus replicon particle
WNV	West Nile virus
(+)RNA	Positive-stranded RNA

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