Identification of the determinants of efficient Pestivirus replication

Risager, Peter Christian; Belsham, Graham; Rasmussen, Thomas Bruun

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Ph.D. Thesis · 2013

Peter Christian Risager

Technical University of Denmark

National Veterinary Institute, Lindholm

Supervisors:

Prof. Graham J. Belsham &
Dr. Thomas B. Rasmuseen
National Veterinary Institute, Technical University of Denmark, Kalvehave, Denmark

Assessment committee:

Prof. Lars E. Larsen
National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark

Prof. Paul Becher
University of Veterinary Medicine, Hannover, Germany

Prof. Michael Lindberg
Linnaeus University, Kalmar, Sweden

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Preface

This written work constitutes the end of three years of scientific research partly supported by DTU and by the Danish Research Council for Technology and Production Sciences (grant 274-08-0305).

The experimental part and daily work routines (plus supervision) have been carried out at the National Veterinary Institute, DTU, Lindholm. A three week short-term mission to the Animal Health Veterinary Laboratory Agency (AHVLA) in Weybridge, U.K. was granted by the EPIZONE network and carried out in February 2012.

The past 3 years have had a lot of memorable moments and milestone achievements. A lot of people have contributed both directly or peripherally for this Ph.D. to be successful. I can, with no doubt in mind, say that I could not have carried out this project alone. A lot of people need to be thanked in this regard.

First of all, the fantastic people at Lindholm are thanked for their great hospitality and for including me in the mentality of the island. I truly appreciate the time and the amusing events we have had together. The Lindholm atmosphere will always stay close to my heart, and remind me of a special time in my life.

I would like to thank my two supervisors, Thomas Bruun Rasmussen and Graham Belsham for their enthusiastic help and guidance through sometimes long and weary roads and for always helping me keep track of the overall goals and milestones. The permanent staff and visiting students in the "blå lagune" lab are thanked. Especially, Lone Nielsen is thanked for her 43 years of Lindholm lab. work expertise, and for in general being my extra two arms. Your sarcastic humor and (after all) friendly human being has been very enjoyable. Ph.D student Ulrik Fahnøe, is thanked for his valuable bioinformatics insights. This was very appreciated input to my thesis and it has been a pleasure to be working with you. Post. doc Martin Barfred Friis is thanked for his time in the lab and for CSFV knowledge sparring.

The FMDV group is thanked for including a CSFV guy like me in their morning bread gathering and social activities. Special thanks go to Maria Gullberg and Preben Normann for their cheerfulness and willingness to lend me a helping hand. I would also like to thank post.doc

researcher Bartosz Muszynski and visiting student Rosin Patel for their one year at Lindholm, and for some memorable events. Fellow Ph.D. student Desi Rangelova is thanked for her cheerfulness, for sharing the office and for making the long drive back and forth from Copenhagen enjoyable. Finally I would like to thank the wonderful cell culture staff for always being able to fulfill my sometimes last-minute decisions.

I would like to thank the European network, EPIZONE for financing a three week short-term mission to the Animal Health Veterinary Laboratories Agency (AHVLA) in Weybridge, U.K. Helen Crooke, Helen Everett, Nataliya Bunkova and Bentley Crudgington, in this regard are thanked for their hospitality and for including me in their research group. Through EPIZONE I was a member of network termed "young EPIZONE" which was a great way to interact and socialize with young scientists around the world. During my thesis I have visited and presented scientific results at the Friedrich Loeffler Institute (FLI) in Germany and also I had the opportunity to participate in the 8th pestivirus ESVV meeting in Hannover, Germany and the EPIZONE 6th annual meeting in Brighton U.K. This has been a great pleasure to be part of.

In the end, I would like to thank my family, my partner, Maria, for your support and willingness for letting me fulfill this amazing journey and my two after all most important achievements, Storm and Villads, who are constantly reminding me of the things most important in life.

I regard this not as an end but as the start of my independent research career, and I greatly appreciate DTU for providing me this opportunity.

In loving memory of my dad.

Peter Christian Risager, August 2013

Summary

The key for the survival of a virus is to copy its own genome into progeny genomes that allows continued reproduction. The mechanism behind this "copy function" or "replication" is a well-organized process that involves the formation of a replication complex in the cell and interactions between the viral proteins. The replication process in single-stranded RNA viruses of positive polarity requires a particular enzyme, an RNA dependent RNA polymerase, that has no direct counterpart elsewhere in nature. The variable nature of rapidly evolving viral genomes, pose a constant challenge to the host, and in depth knowledge of the traits that determine the fitness of the virus in this regard are highly valuable. Recent advances in the field of molecular virology with methods to manipulate viral genomes have significantly helped to uncover these core mechanisms responsible for exploitation of the host. This includes aspects of the infection, evasion from host antiviral defense, genome replication and viral assembly.

With special reference to a particular RNA virus, Classical swine fever virus (CSFV), this thesis deals with the elucidation of traits involved in replication of the viral genome. This is accomplished via the application of precisely bioengineered viral constructs and through the use of state-of-the-art virological methods. The presence of full-length cDNA sequences of RNA viruses within stable vectors has been the “holy grail” for the reverse genetics approaches, and for the rescue of bioengineered mutants. The availability, in our lab, of bacterial artificial chromosomes (BACs) containing full-length cDNA sequences which can be used to rescue three different CSFV strains with a spectrum of virulence, have been a central resource for this work.

The thesis is composed of four parts: Part 1, gives a general introduction to RNA viruses, with the focus on viruses classified within the *Flaviviridae*. Next, pestiviruses are described with special attention to classical swine fever virus and the disease it is responsible for. A brief history of types of viral vaccines is provided, finishing with a description of the molecular methods used for viral cDNA manipulation, bio-engineering approaches, description of viral reporters and so forth. **Part 2**, "Pestiviruses: Infection and requirements for viral RNA replication " is meant as a walk through the literature describing

Pestivirus/Classical swine fever virus replication determinants, including a thorough presentation of the viral proteins, and the involvement of these in the infection progress. **Part 3**, "The manuscripts", includes the papers published and submitted on this work. These describe the outcome of experiments performed during the three years. **Manuscript I** is a co-authored paper that describes a summary of the work I have been doing in my thesis dealing with the application of the Red/ET mediated homologous recombination method to modify viral cDNA. For proof of this method, CSFV/BDV chimeric clones were produced and characterized (Submitted paper, BMC genomics). **Manuscript II** describes the generation of replicons that express two different types of luciferases (Rluc and Gluc), and their application as a tool for easy monitoring of replication competence (published paper, Journal of General Virology (94), 1739-1748). **Manuscript III** describes the properties of chimeric replicons and infectious clones that include a RNA dependent RNA polymerase (NS5B) from one of three different CSFV strains with distinct virulence properties. The entire NS5B proved to influence replication competence and key residues for replication competence was identified as judged by reporter protein expression kinetics and from using infectious clones. Furthermore, evidence is provided that these specific single amino acid substitutions in the NS5B fingertip region, can influence the rate of viral RNA replication and virus spread. **Part 4**, is a summary and discussion of the general and overall conclusions and a walk through the milestones that have been achieved. Future perspectives and work that should be carried out are addressed as well.

Resumé (Danish summary)

Nøglen til overlevelse for en virus er at kopiere sit genom til tusindvis af nye kopier. Mekanismerne, der ligger til grund for denne "kopi-funktion" eller "replikation", er en velorganiseret proces, der involverer dannelsen af et "replikationskompleks" i cellen og en følgende interaktion virusproteinerne imellem. Positivt-enkeltstrengede RNA virus har til replikationsprocessen brug for et specifikt enzym, en RNA-afhængig RNA-polymerase (RdRp), hvis lige ikke findes andetsteds i naturen. Den variable natur af virale RNA genomer, der muterer hurtigt, er en konstant trussel for værten, og et dybdegående kendskab til de træk, der bestemmer virussets fitness, er derfor yderst vigtigt. Ny udvikling indenfor molekylær virologi har resulteret i effektive metoder til at manipulere virusgenomer, hvilket har hjulpet til at afklare kernemekanismerne i virussets udnyttelse af værtens celler. Dette inkluderer forskellige aspekter i infektionsforløbet, herunder omgåelse af værtens antivirale respons, genomreplikation og produktion af nye viruspartikler.

Denne ph.d.-afhandling har specielt fokus på genomreplikationen af pestiviruset Klassisk svinepest virus (eng. Classical swine fever virus (CSFV)). Forskningen er blevet udført via de nyeste molekylærvirologiske metoder baseret på revers genetik. Herved anvendes infektiøse kloner, hvor hele RNA virusgenomer kopieres til fuldlængde cDNA, indsættes i stabile plasmider og efterfølgende modificeres, hvorfra man igen kan gendanne nye virusgenomer og RNA virus, hvilket de senere år har revolutioneret forskningen i denne type virus. Etableringen i vores laboratorie af infektiøse kloner baseret på stabile plasmider indeholdende fuldlængde cDNA fra tre forskellige CSFV typer med meget forskellige virulenskarakteristika, har været et centralt omdrejningspunkt for denne afhandling.

Ph.D. afhandlingen er opdelt i fire dele: **Del 1**, "introduktion" giver en generel introduktion til RNA-virus, med specielt fokus på virus klassificeret indenfor *Flaviviridae*. Dernæst bliver *Pestivirus* beskrevet, her med specielt fokus på CSFV samt den sygdom det medfører. En kort gennemgang af virale vacciner bliver givet, og afsnittet afsluttes med en beskrivelse af de molekylære metoder brugt til at manipulere viralt cDNA. **Del 2**, "Pestivirus: Infektion og krav til RNA replikation", er ment som en gennemgang af den litteratur, der beskriver Pestivirus/CSFV replikationsforudsætninger, inkluderende en grundig præsentation af virusproteinerne og deres involvering i replikationsprocessen. **Del 3**, "Manuskripterne"

inkluderer publicerede og indsendte manuskripter i denne afhandling, der beskriver resultaterne fra de eksperimenter, der er blevet udført over de seneste tre år. **Manuskript I**, er et medforfatter-manuskript, der beskriver mit arbejde med anvendelsen af Red/ET medieret homolog rekombineringsmetoden til at modificere viralt cDNA. Som efterprøvning af metoden, er CSFV/BDV kimære kloner blevet produceret med denne metode og efterfølgende karakteriseret (indsendt manuskript, BMC genomics). **Manuskript II**, beskriver genereringen af replikons, der udtrykker to typer af luciferaser (Rluc og Gluc) og deres anvendelse som et værktøj for nem monitorering af replikationskompetencen (publiceret artikel, Journal of General Virology (94), 1739-1748). **Manuskript III**, beskriver egenskaberne for kimære replikons- og infektiøse kloner, der har en distinkt RdRp fra én af tre forskellige CSFV stammer med vidt forskellige virulensmønstre. Udskiftning af hele RdRp viste sig at influere på replikationskompetencen, og særligt vigtige aminosyrer for replikationen blev identificeret og vurderet ud fra reporterproteinudtryk samt ved at teste infektiøse kloner. Derudover viser resultaterne, at disse enkelt-aminosyreændringer i RdRp "fingerspids"-regionen, kan influere på RNA replikationsraten og virusspredningen. **Del 4**, er en opsummering og diskussion af de generelle konklusioner og en gennemgang af de opnåede milepæle. Fremtidige perspektiver bliver også adresseret.

Abbreviations

aa	Amino acid(s)
Ab	Antibody
BAC	Bacterial Artificial Chromosome
BDV	Border disease virus
bp	Base pair
BVDV	Bovine viral diarrhea virus
Cam	Chloramphenicol
cp	Cytopathogenic
CSF	Classical Swine Fever
CSFV	Classical Swine Fever Virus
DIVA	Differentiating Infected from Vaccinated Animals
DdRp	DNA dependent RNA polymerase
(c)DNA	(complementary) Deoxyribonucleic acid
ds	Double stranded
EMCV	Encephalomyocarditis virus
ER	Endoplasmatic reticulum
FMDV	Foot- and mouth disease virus
GAG	Glycosaminoglycan
Gluc	*Gaussia* luciferase
GFP	Green fluorescent protein
Hm	Homology
h.p.t/i./e.	Hours post transfection/infection/electroporation
HS	Heparan sulfate
IFN	Interferon
IRES	Internal ribosomeentry site
Kan	Kanamycin
Kb	Kilobase
mAb/pAb	Monoclonal/Polyclonal antibody
Ncp	Non-cytopathogenic
NGS	Next generation sequencing
NS/NSP	Non-structural/Non-structural protein
nt	Nucleotide
NTPase	Nucleoside triphosphatase
OIE	Office Internationale des Epizooties
ORF	Open reading frame
(q)PCR	(quantitative) Polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RdDp	RNA dependent DNA polymerase
RLU	Relative luminescence units
Rluc	*Renilla* luciferase
RNA	Ribonucleic acid
RT	Reverse transcriptase / room temperature
SP/ase	Signal peptide(ase)
SPP	Signal peptide peptidase
ss	Single-stranded
Strep	Streptomycin
Tet	Tetracycline
UTR	Untranslated region
VRP	Virus replicon particle
wt	Wild-type

PART 1, Introduction

General introduction

Classical swine fever (CSF) is a major threat to the pig farming industry in Denmark. and in worst case scenarios, an outbreak of Classical swine fever virus (CSFV) could have severe economic consequences for individual pig holdings as well as for national agriculture budgets. A severe example of this was seen in the outbreak of CSF in the Netherlands in 1997-98. Some 429 CSFV-infected herds were depopulated and nearly 1300 herds were slaughtered pre-emptively to prevent further spread. In total, about 11 million pigs were culled, including millions of uninfected pigs and the total cost was ca. € 1,7 billion (Meuwissen *et al*., 1999,Stegeman *et al*., 2000,Terpstra & de Smit., 2000). Although the EU countries are close to being free of CSF (EU CSFV reference laboratory website)[1], the virus is still present in some Eastern European countries, i.e. in certain wild boar populations, and thus pig industries in the EU countries are still very vulnerable to outbreaks, in part due to a "non-vaccination policy" for domestic pigs. The non-vaccination policy has been an approach to keep herds free of /naïve to CSFV, thereby only producing naïve and healthy pig herds (van Oirschot., 2003). In Denmark, CSFV is considered exotic and Denmark has been free of CSF since the last and only reported case in 1933[2]. The constant risk of a reintroduction of CSFV, have speeded up the effort to develop a vaccine which enables differentiation between infected and vaccinated animals, the so called DIVA-principle (Differentiating Infected from Vaccinated Animals) (van Oirschot., 1999). Live-attenuated vaccines continue to be most efficient approach for generating an efficient antibody response and achieving long-term protection against CSFV (Beer *et al*., 2007). A drawback of using live attenuated strains of a virus is the fact that reversion can produce a virulent strain and have severe consequences.

Unraveling the traits involved in viral replication is essential for our understanding of virus growth and pathogenicity in the host. RNA viruses show a high rate of mutation within their genomes (about 1 mutation per genome copy (Drake *et al*., 1998)) due, at least in part, to error-prone RNA polymerases. High mutation rates due to error-prone polymerase activity will generate advantageous escape mutations in a way that out-competes the cost of deleterious mutations, and thus eventually influence the pathogenicity, due to the escape from

[1] http://viro08.tiho-hannover.de/eg/
[2] http://www.foedevarestyrelsen.dk/Leksikon/Sider/Klassisk-svinepest.aspx

recognition. It is a general observation that replication competence and rate of mutagenesis influences the pathogenicity of RNA viruses, see e.g. (Regoes *et al*., 2013). A highly diverse virus population of genetically related viruses (quasispecies) allows a higher probability for some viruses to evade, for example, the host immune response while a virus with a higher fidelity polymerase would produce a more genetically stable viral genome which may be less adaptable to changing selection pressures (Vignuzzi *et al*., 2008).

Various approaches have been used to investigate replication determinants. Most of these studies have identified loci which, when modified, impair fitness of the virus. Strictly speaking, these factors can only be defined as such, if they enhance replication or virulence given that the majority of changes in the viral genome will decrease fitness. In spite of numerous indications, however, no "final" markers for the key traits involved in attenuation of replication or virulence of CSFV have been determined. Perhaps there is no simple answer to this; since these traits are not only present in the virus itself but, in the bigger picture, are also related to interactions with the host. Viruses have co-evolved and been adapted to their host during time in a “cat and mouse” chase, and through this have fine-tuned their lifecycle for their own successful reproduction, the so-called "red queens hypothesis". In fact, the most devastating strains are not necessarily the most virulent strains. In a small or low-density population, a longer incubation time or a well suited persistance strategy enables the virus to persist and not to die out with the infected host, and thus the less virulent virus that keeps the host alive will stay circulating for a longer period. This again however poses other problems for the virus to develop anti-host mechanisms for evasion of the host immune responses, if persisting in the animal for a longer period. The switch in a virus from virulence to avirulence or from a highly replicating to a low-replicating strain (or vice-versa) in this regard is interesting. Comparative analysis of genetically related viral strains that display different virulence characteristics or replication competences could be advantageous for elucidation of determinants involved in these core traits of the virus.

Classification of RNA viruses

Viruses are the most abundant biological entities in the world. The prevalence and diversification of RNA viruses out-numbers by far that of DNA viruses. The species concept in viral taxonomy is rather variable due to the rapidly evolving genomes, and certainly this is the case for RNA viruses. In general, RNA viruses have much smaller genome sizes, and have

error-prone polymerases, and as a consequence of this RNA viruses have a higher degree of diversification. Classification of viruses is complicated due to the lack of a known universal common ancestor, and thus the origin of viruses is still one of the major unanswered questions that are being disputed among virologists. Historically, viruses have been classified according to their host and their antigenicity/serotype plus their physical properties (e.g. size, density, nature of genome). Recent explosions in the availability of full genome sequences have allowed a more consistently defined classification system based on conserved parts/fragments or, if appropriate, on full-genome sequences of the viral nucleotide sequences. This approach can be used to establish the relatedness of different viruses independent of biological traits.

According to the latest International Committee on Taxonomy of Viruses (ICTV) online website[3], the virosphere can be assigned into 7 major orders (no viruses are ranked above the order level); namely: Caudovirales, Herpesvirales, Ligamenvirales, Mononegavirales, Nidovirales, Picornavirales and Tymovirales. An eighth group, contains 72 viral families not assigned to any order. Among these 72 families we find the *Flaviviridae* family. The word "flavi" is the latin word for "yellow", because one of the most famous flaviviruses is yellow fever virus. The *Flaviviridae* is comprised of three well established genera; hepaciviruses, flaviviruses and pestiviruses. A fourth genus, the pegiviruses has recently been incorporated within the family (Stapleton *et al*., 2011). The hepaciviruses had a sole member, a human pathogen, hepatitis C virus (HCV), but recently a non-primate HCV-like canine hepatitis C virus (CHV) was identified and is provisionally incorporated into this genus, which potentially could be an excellent model for future research (Bukh., 2011,Kapoor *et al*., 2011). The pestiviruses, hepaciviruses and pegiviruses only replicate in mammalian hosts and thus are distinct from the bulk of the flaviviruses which are insect vector-borne viruses (53 species incl. dengue virus, west nile virus, japanese encephalitis virus, tick-borne encephalitis virus and yellow fever virus), which replicate in both insect and mammalian hosts.

The pestiviruses

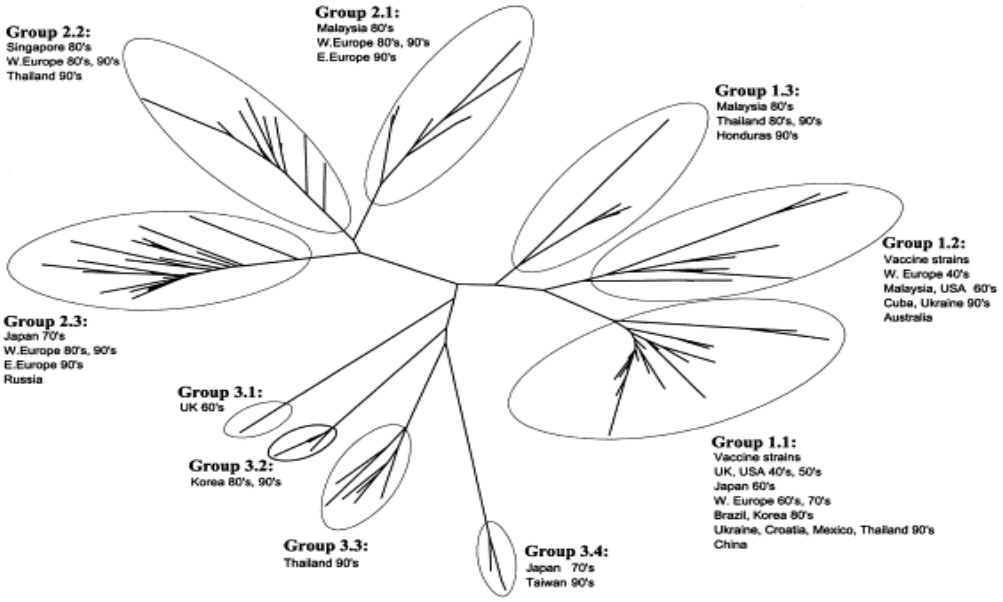
The *Pestivirus* genus is composed of four important pathogens of livestock; Bovine viral diarrhea virus 1 and 2 (BVDV-1 and BVDV-2), Border disease virus of sheep (BDV) and Classical swine fever virus (CSFV). Pestiviruses were sub-divided according to their host but

[3] http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1

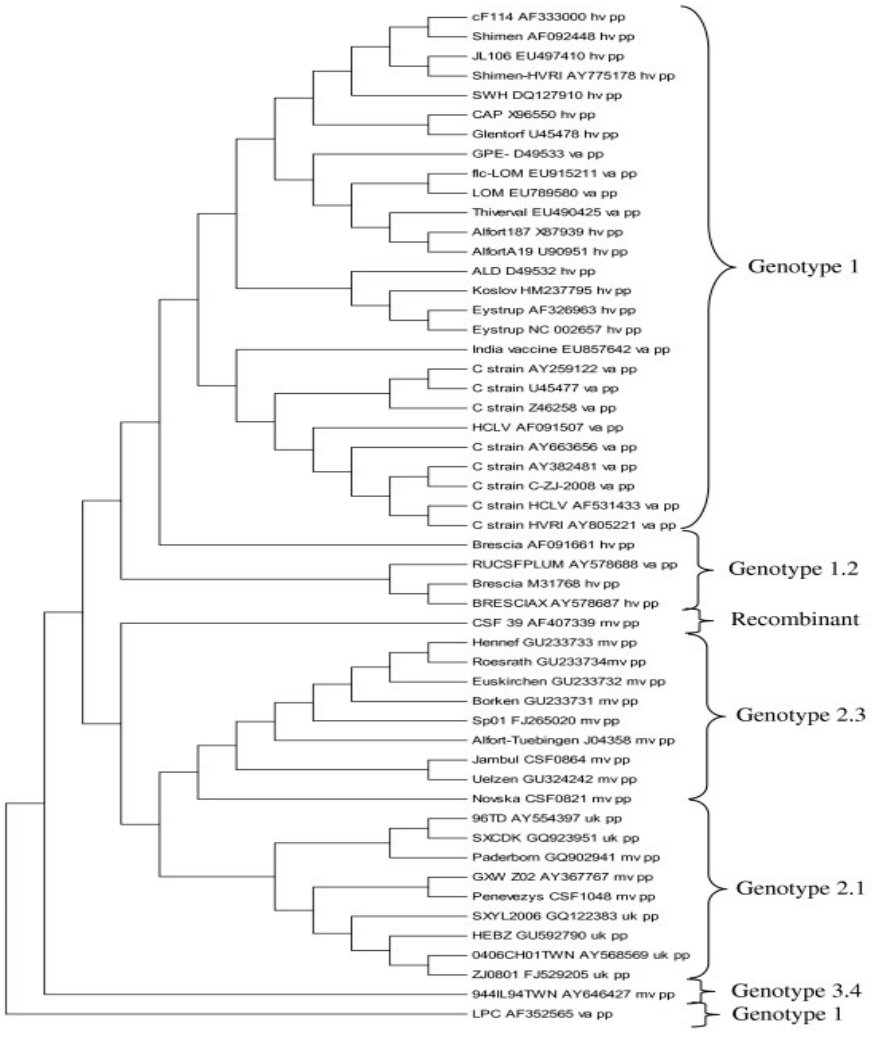
serological cross-reactivity has been demonstrated between all pestiviruses (Wieringa-Jelsma *et al*., 2006). While CSF viruses are predominantly restricted to pigs, the other *Pestivirus* species have been recovered from hosts of a greater variety. A number of atypical pestiviruses have been described originating from giraffe, reindeer, pronghorn antelope and from foetal calf serum ("HoBi" virus)(Schirrmeier *et al*., 2004). Recently, another pestivirus, "Bungowannah", was isolated from pigs (Kirkland *et al*., 2007). A recent study on metagenome analysis of bats revealed pestivirus-like sequences suggesting a broader host range for pestiviruses (Wu *et al*., 2012).The viruses were originally classified as a *Pestivirus* group within the family *Togaviridae* in 1982 but due to the discovery of the close antigenic relationships and insights into the nucleotide sequence similarities to *Flaviviridae* members, there was justification to include the pestiviruses together within the flavivirus family instead (Collett *et al*., 1988). Each species is subgrouped into strains partly according to their nucleotide sequence relationship and partly due to their antigenic characteristics. Typically the viral strains are named after the origin of the sampling site or outbreak case, e.g. CSFV strain Paderborn originated from the index case in the city of Paderborn (Germany).

CSFV phylogenetics

Based on the phylogenetic approach, CSFVs are divided into three phylogenetic groups (1, 2, and 3), with each group being divided into three or four subgenotypes (1.1 to 1.3; 2.1 to 2.3; 3.1 to 3.4) see fig. 1 (Greiser-Wilke *et al*., 2006,Paton *et al*., 2000,Postel *et al*., 2012). Genotype 1.1 contains strains as different as the avirulent Chinese "C"-strain and the highly virulent viruses i.e. Koslov, Shimen, Alfort 187 and Eystrup. The 1.1 genotype has been circulating in Europe for the last century (Paton *et al*., 2000). Until recently, genotype 2.3 viruses have been circulating in wild boars in e.g. Germany (Leifer *et al*., 2010). However, genotype 2.1 viruses have been the most prevalent virulent strains causing new introductions in Europe in recent years. e.g. in the U.K. outbreak in 2000 due to strain UK2000/7.1 (Graham *et al*., 2012,Sandvik *et al*., 2000) and the Paderborn strain from the devastating outbreaks in The Netherlands in 1997 (Stegeman *et al*., 2000). Genotype 3 viruses are mostly isolated from within Asia (see fig. 1).



A



B

Figure 1. A) The three major genotypes 1, 2 and 3, and their subgroups of CSFV based on nucleotide identities within 100 E2 coding sequences. Branch lengths are proportional to nucleotide differences.The geographical origin of the strains is included as well. From (Paton *et al.*, 2000). B) Specific strains of CSFV depicted in a phylogram based on full-genome sequences. Accession numbers and the polyprotein (pp) sequences and their virulence properties are depicted as well. Vaccine (va), moderately virulent(mv), highly virulent (hv) and unknown virulence (uk)(from (Leifer *et al.*, 2011))

History

Classical swine fewer has been known for many years, all around the world. Originally it was known as "swine fever" in England since 1862, "schweineseuche" in Germany, "swinpest" in Sweden, "pneumo-entérite infectieuse" in France, and in the United States it has been known since 1887 as hog cholera, pig fever or swine plague. In Denmark it was known as "swine-diphterie" (*in danish*, "svine difteritis" (Liess., 1987)). In 1903, Schweinitz and Dorset for the first time described the etiology of the disease and that indeed it was characterized as caused by an agent able to be passed through filters (which stopped bacteria), and thereby indicated that it might be of a viral origin (see (Edwards *et al*., 2000). Hog cholera virus, has for a long time been a name for the virus, but the abbreviation "HCV" (for hog cholera virus) could lead to confusion due to use of the same abbreviation for hepatitis C virus, and thus it is now more commonly known as classical swine fever virus "CSFV".

Pathogenesis

Infection with CSFV can result in an acute or chronic disease. The pathogenesis is determined by several factors such as the age of the animals and the virulence of the strain; whether it is low, moderate or the very severe cases with highly virulent strains, that causes up to 100% mortality within a week. The primary sites of replication are the tonsils and secondarily in endothelial cells, lymphoid organs and bone marrow. CSFV is immunosuppressive due to depletion of circulating B- and T-lymphocytes with a significant drop in leukocytes (leukopenia) and thrombocytopenia. Clinical symptoms during an acute infection include high fever, gastrointestinal disorders, respiratory failures, hemorrhages, central nervous system disorders; these symptoms can eventually result in moderate to high levels of mortality. Transmission is by oral and oronasal routes by direct or indirect contact and is not reported to be vector-borne as is the case for the majority of flaviviruses. Spread by feeding pigs with uncooked contaminated swill /garbage is also reported (Penrith *et al*., 2011). Virus is shed in blood and by all body secretions/excretions. The incubation period (time from virus transmission to when the first signs of the disease are typically apparent) is within 2-7 days and death occurs in the range from 4 days, in the most virulent strains, up to several weeks in moderately virulent strains. Mortality is also dependent on the age and condition of the animals involved (Floegel-Niesmann *et al*., 2009). Dependent on the stage of gestation, intrauterine infections by CSFV can give birth to persistently infected piglets or cause

abortions or severe abnormalities leading to death weeks after birth (Lohse *et al.*, 2012,Moennig *et al.*, 2003).

The virus

Pestiviruses are small enveloped animal RNA viruses (about 40 nm in diameter). The monopartite plus-sense single stranded RNA molecule of ca. 12.3 kilobases is protected by core (capsid) proteins (forming an internal capsid) and the three structural proteins (E^{rns}, E1 and E2) which are arranged as homo- and hetero dimers thereby creating a herringbone array-like appearance (fig. 2). The virion is further surrounded by a lipid bilayer, originating from the host cell, through which protrude the structural trans-membrane proteins E1 and E2, which are anchored in the virion, and the secreted multi-functional E^{rns} (see review in part 2). The host range of pestiviruses is restricted to cloven-hoofed animals (*Artiodactyla*, e.g., ruminants and pigs), and susceptible cell culture systems comprise cells from this order. CSFV tropism is limited to animals of the *Suidae* family.

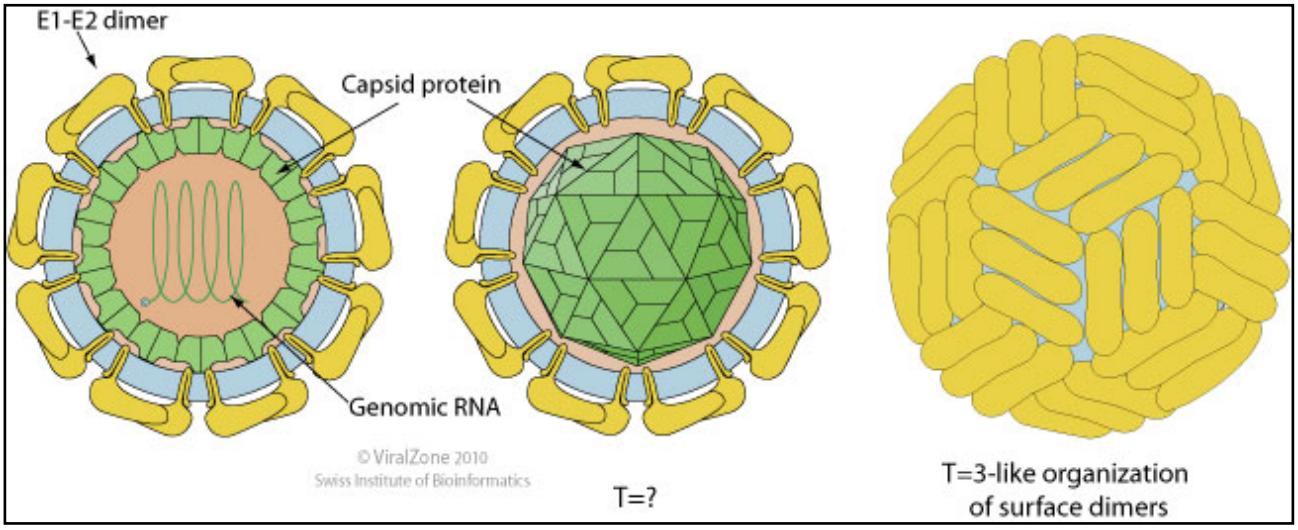


Figure 2. The morphology of the CSFV virion. The outer spherical appearance is arranged of homo- and hetero dimers of E1, E2 and also the E^{rns} proteins that are protruding the lipid membrane . The inner shell is arranged by the C protein that protects the single RNA molecule (from http://viralzone.expasy.org/).

CSFV is inactivated by heat, organic solvents and detergents, and can also be inactivated by UV light. The virus is stable at pH values between 5 and 10 (Edwards., 2000,Weesendorp *et al.*, 2008). Monoclonal antibodies recognizing CSFV, as initially described by (Wensvoort *et al.*, 1986) are primarily directed against E2 and to a lesser extent to E^{rns} and NS2/3 proteins.

Monoclonal antibodies directed against a variety of the nonstructural proteins have recently been described (Lamp *et al*., 2011).

CSFV virulence

The virulence of CSFV varies from the avirulent vaccine strains (e.g. C-strain, LPC, GPE-) over low virulent strains (e.g. Glentorf) to the moderately virulent strains (e.g. Paderborn, UK2000/7.1) and also to the highly virulent strains (e.g. Koslov, Brescia, Shimen, Eystrup, ALD). Virus proteins which may affect virulence include components which can determine tissue tropism (E^{rns}, E1 and E2), speed of replication (NS5B, NS5A, and NS2/3) and prevention of the induction of the IFN α/β system (Npro, E^{rns}). Also glycosylation of the proteins has been shown to influence the number of available antigens since it can hide the antigen targets and thus protect them from recognition and clearance by the immune system (Fournillier *et al*., 2001,Gavrilov *et al*., 2011). Although extensive research has been carried out trying to identify crucial virulence determinants, there is no complete answer to this. Mostly the studies have elucidated roles for virus components involved in attenuation of CSFV, rather than determining factors which increase the virulence of CSFV. Such studies with CSF viruses carrying modifications to potential virulence determinants have indicated that virulence determinants reside in N^{pro} (Mayer *et al*., 2004), E^{rns} (Meyers *et al*., 1999), the terminal domain of E1 (Risatti *et al*., 2005), or E2 (Risatti *et al*., 2006,Risatti *et al*., 2007,Van Gennip *et al*., 2004) and NS4B (Fernandez-Sainz *et al*., 2010). Clearly studies on attenuation of a virulent strain are more straightforward, given that it is easier to impair viral fitness rather than to identify factors that increase viral fitness. Recently, however, a study was based on the gain of virulence starting from an attenuated vaccine strain. It was shown that only three aa substitutions in the E2 and NS4A proteins of the Japanese CSFV vaccine strain GPE- (originally derived from the highly virulent ALD strain), could revert the vaccine strain into a virulent version (Tamura *et al*., 2012).

Quasispecies and adaptations to the host

Genome replication in RNA viruses is error-prone due to the lack of a proof-reading mechanism in the RNA dependent RNA polymerase (RdRp). Misincorporation rates of nucleotides by an RNA polymerase from an RNA virus is ca. 1 out of 10000 nucleotides (Drake *et al*., 1998,Sanjuan *et al*., 2010). In contrast, DNA polymerases have proof-reading systems to correct the errors, and thus misincorporation rates for these are as low as ca. 1 out of 10^6

nucleotides. Continuous nucleotide misincorporations ultimately lead to significant genetic variations within a viral population. This can select for variants with increased replication competence and variants that can evade the host immune response due to changed antigenic determinants. If the virus replication results in too high a mutation frequency then this will lead to too many adverse substitutions thereby decreasing the overall fitness. On the other hand, a high-fidelity RdRp could fail to produce enough escape mutants to enable the virus to propagate efficiently under altered selection pressures (Vignuzzi *et al*., 2008). Other selection pressures for antigenic shifts can be related to different hosts, resistance to antiviral agents etc. The variable nature of viral genomes continuously provides a pool from which the most well adapted viruses will be amplified most efficiently. The commonly used propagation method for pestiviruses in laboratories is based upon infection of continuous and stable cell lines. This method has a major drawback in that there is a continuous selection for the fastest growing variant. The use of primary cells for the propagation of CSFV could be a strategy to minimize adaptation biases, however lack of efficiency in the use of primary cells makes it unfavorable. One common bias from propagation in continous cell lines is a selection of variants that attach to the cell surface by interactions with membrane-associated heparan sulfate (HS). Although CSFV field isolates, in general, do not have significant affinity for HS, it has been demonstrated that *in vitro* cultivation of CSFV in swine kidney cells selects HS-binding viruses and that these variants had substitutions at two amino acids within the E^{rns} protein (residues 476 and 477) (Hulst *et al*., 2001).These substitutions resulted in viruses with a four-fold increased replication competence in cell culture (Mayer *et al*., 2003). HS is composed of unbranched glycosaminoglycans (GAG) or polysaccharide chains containing repeated disaccharide sequences which are usually covalently linked to proteins thereby forming a network of chains (proteoglycans) exposed on the surface of most types of cells as an extracellular matrix. Similar cell culture adaptations to HS have been reported for foot-and mouth disease virus (FMDV) and Sindbis virus (SV), and in summary as for CSFV animal experiments these HS-dependent variants were proven less virulent than their HS-independent counterparts (Klimstra *et al*., 1999,Neff *et al*., 1998,Van Gennip *et al*., 2004). Recently, a cell line has been developed that, when used in combination with DSTP 27 (a HS GAG blocker), does not select for viruses with affinity for HS, which could be an approach to circumvent cell adaptation biases (Eymann-Hani *et al*., 2011). Another bias in standard continuous cell line propagation is that there is no antibody selection. The fastest replicating

variant that is best adapted to the cell culture conditions will emerge and not necessarily the variant that is best fitted for *in vivo* conditions (see e.g. (Leifer *et al*., 2012)).

CSFV vaccines

Since the initial vaccine developments by Jenner in the late 18th century, and the efficacious live-attenuated rabies vaccine developed by Pasteur in 1885, virologists have made significant progress in the battle against viral agents. Many vaccines in use today are based on inactivated (killed) viruses. The drawback of this approach is that this type of vaccine mostly stimulates the humoral immune response rather than the cell-mediated. The action of antibodies induced by this type of vaccine enables the removal of circulating viruses and some of the virus-infected cells. Antibodies alone, however, cannot interact with viruses in all compartments of the body. Thus, a vaccine that induces both the humoral and cell-mediated immune responses, is more likely to provide a solid immune defense. Several approaches can be considered when designing the best vaccine. Killed vaccines have efficacy problems, whereas live-attenuated vaccines have safety problems. Live-attenuation is the oldest vaccination approach and still the best approach for generating the most efficient antibody response and long-term protection. Historically, an approach to develop a live-attenuated vaccine has been to perform serial passages of a virulent strain in a non-susceptible host and thereby eventually produce a virus which does not cause disease in its natural host, but still induces protection.

The gold-standard for vaccination against CSF in Europe is the live attenuated Chinese "C-strain" vaccine virus. The C-strain can be traced back to 1945, when a virulent CSFV, supposedly Shimen strain, was passaged several hundred times in rabbits (lapinized), and when the lapinized strain was inoculated into pigs then they were fully protected against CSF (Nie *et al*., 2003). Recent evidence though questions the Shimen strain as the true originator of C-strain vaccine strain (Xia *et al*., 2011). C-strain (and its derivatives) provide rapid and efficient protection against CSF (Beer *et al*., 2007,Graham *et al*., 2012,van Oirschot., 2003). There exist several versions of C-strain used for vaccination purposes (e.g. LPC, LC, Riemser, Riems). The drawback of live attenuation is the theoretical possibility of the avirulent virus reverting into a virulent strain and causing disease with severe consequences. Today, the use of reverse genetics allows molecular biologists to design and engineer a vaccine exactly as wished. Several CSFV vaccines with marker properties based on chimeric pestiviruses have

been developed over the years (Beer *et al*., 2007) . In particular, chimeric pestiviruses with substitution of the entire E2 protein have been described (Rasmussen *et al*., 2007,Reimann *et al*., 2004,van Gennip *et al*., 2000) but also mutants with more subtle modifications, such as the modification of the important TAV-epitope within the CSFV-E2 protein(Lin *et al*., 2000,Holinka *et al*., 2009,Reimann *et al*., 2010,Rasmussen et al., submitted) are promising marker vaccine candidates.

Full genome sequencing and the data generated by this, can provide information on why the historically used vaccines, that were generated blind, are in fact avirulent. Annotation of the sequence data and comparison with the wild-type field isolates should potentially indicate where the attenuation features are located.

Molecular virology

Manipulation of viral genomes by early virologists was made by exposures of the viral genome to mutagenic agents that introduced random mutations in the viral genome and was followed by the screening and selection for interesting phenotypic traits. This method is now known as forward genetics. In contrast to *forward genetics*, molecular virology today is dealing with aspects of *reverse genetics*. In this, precise modifications are made to the genome (e.g. sequence deletions, protein knockouts) and then the phenotypic effects are analyzed. This technological advance has opened up new doors for better understanding and characterization of viral proteins, and the persistence and evasion strategies. Improved *in vitro* reagents (e.g. DNA polymerases) have facilitated high-fidelity cloning efficiencies resembling that of *in vivo* methods. The availability of infectious clones, full length cDNA sequences of viral strains, and their propagation in stable plasmids has been the "holy grail" for reverse genetics. A crucial tool for the generation of complementary DNA from an RNA template was the discovery and application of the reverse transcriptase proteins, or RNA dependent DNA polymerases (RdDp) from the *Retroviridae* family (Baltimore., 1970,Temin & Mizutani., 1970). These polymerases are crucial in the various reverse transcription (RT) methods. Advances in the field of generating stable full-length RNA from a DNA template (using *DNA dependent RNA polymerases* or (DdRp)) for introduction into cells in order to initiate the infection cycle, has also been accomplished. For both RdDp and DdRp, these types of enzymes have limitations with respect to the maximum length of the RNA transcripts that can be generated in vitro, but a full length coronavirus (27,3 Kb) RNA transcript has been reported (Thiel *et al*., 2001). A widely used and efficient mammalian expression system is based on the vaccina virus T7 (VVT7) assay (Fuerst *et al*., 1986). In this, a modified *vaccinia* virus was engineered to express the T7 RNA polymerase in infected cells, and thus enable the expression of proteins from transfected plasmids containing a T7 promotor sequence upstream of a sequence of interest. Most infectious CSFV cDNA clones are designed to contain a T7 promoter upstream of the viral cDNA sequence and then run-off RNA transcripts are produced *in vitro* using T7 RNA polymerase for the downstream application such as transfection/electroporation into cells. Another approach based on the expression of DdRp protein that obviates the "virus infection" was the development of cells that express T7 DdRP

and these enabled rescue of viruses from a plasmid containing a full-length viral cDNA template (van Gennip *et al*., 1999) or a longPCR product (personal experience).

Another powerful, more recent tool, has been the introduction of Next Generation Sequencing (NGS), that allows in-depth (with high coverage) analysis of genome strands. The availability of sequences for annotation and correlations to analogous virus proteins, have allowed more precise protein predictions. Improved computing power and *in silico* analyses have allowed bioinformaticians to produce automated systems to manage the tremendous amount of sequence data and create new algorithms and software platforms to analyse the sequencing results. In contrast to standard Sanger sequencing, NGS data enables one to monitor a quasispecies population pool and (perhaps) predict beneficial mutations in the viral genome.

This project has dealt with the development and use of reverse genetics approaches for the manipulation of CSFV cDNA. In this section, there follows a description of the methods I have used in the context of bioengineering and in the evaluation of the constructs.

Virus rescue, infectious clones and BACs

Bioengineered manipulation of the genome of RNA viruses through reverse genetics is only possible if corresponding cDNA clones, preferably inserted in stable plasmids, are available for propagation of the genetic information in bacterial cells.

DNA viruses were the first to become available to genetic engineering techniques. The breakthrough was achieved with a small DNA virus simian virus 40 (SV40), that when a cloned DNA copy was transfected into cells, resulted in the formation of infectious viruses (Goff & Berg., 1976). Although small DNA viruses exist (e.g. polyomaviruses, parvoviruses etc.), RNA viral genomes have relatively small sizes of compared to many DNA viruses, and a lot of pioneering work and attention was given to these. As for many other important technological advances in virology, bacteriophages have been excellent model systems. The RNA genome of a small RNA bacteriophage "Qβ" (ca. 3500 nt), was inserted into a plasmid vector as cDNA (utilizing reverse transcriptase) that enabled the rescue of new viruses (Taniguchi *et al*., 1978). Rescue of poliovirus with a longer RNA genome (ca. 7440 nt) was subsequently reported (Racaniello & Baltimore., 1981). Employing *in vitro* transcribed RNA from a cDNA template containing an SP6 promotor was initially described for poliovirus, and proved to yield infectious plus- strands (Kaplan *et al*., 1985).

The first infectious cDNA clone and fully sequenced strain of CSFV (strain Alfort) was reported in 1989 (Meyers *et al*., 1989) and infectious clones of other CSFV strains followed next (e.g. strain "Brescia" and "C-strain"). Rasmussen *et al.* established a method for the generation of full-length RT-PCR pestivirus amplicons and subsequent cloning into bacterial artificial chromosomes (BACs) (Rasmussen *et al*., 2008,Rasmussen *et al*., 2010). BACs are ideally suited for the stable maintenance of large DNAs derived from viral genomes (Messerle *et al*., 1997). A considerable number of BAC systems have been established for large DNA viruses such as herpesviruses and poxviruses, in particular many herpesviruses have been cloned into BACs (for review see (Tischer & Kaufer., 2012)). Similarly, cDNAs corresponding to the full length genomes of members of the *Flaviviridae* family (Japanese encephalitis virus, (Yun *et al*., 2003)) and Dengue virus (Pierro *et al*., 2006) have been inserted into BACs. Recently, also BACs containing cDNAs of pestiviruses, including bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) have been established (Fan & Bird., 2008,Rasmussen *et al*., 2010). In our laboratory, we now also have BACs containing cDNA of CSFV strains "C-strain Riems" (Rasmussen et al. submitted (Manuscript 1)), "Koslov" (Fahnøe at al. in preparation) and "Rösrath" (unpublished) and BDV strain "Gifhorn" (unpublished). Single-copy BACs are convenient vectors for DNA insertions, and have proven to have genetic stability during propagation of DNA sequence inserts of more than 300 Kb (Shizuya *et al*., 1992). BAC vectors were the preferred choice for the propagation of clones and for setting up BAC libraries within the human genome project. The stability of BAC clones, though can vary with the choice of host. We have made studies regarding the genetic stability of CSFV cDNA in BACs inserted in *Escherichia coli* strain DH10B and in MD42. Although there is evidence that Paderborn cDNA seems to pick up an IS10 transposon element in a sequence dependent manner after 5 passages (unpublished data), in general the BAC system proves to be highly stable for propagation of pestivirus cDNA (Fan & Bird., 2008,Rasmussen *et al*., 2010). Stable maintenance of pBeloRiems cDNA was seen even after 15 passages in *E. coli*. (Rasmussen et al. submitted(Manuscript I)).

Cloning techniques - *in vitro* and *in vivo*

In early studies, standard cloning techniques have proven competent to generate full-length cDNA from, for example pestivirus RNA, which can be propagated within vectors and then be used to generate viable viruses through the introduction of RNA transcripts into cells.

Standard cloning strategies though have drawbacks that limit their application or at least make them more laborious and difficult. To construct modified full-length genomes it was necessary to amplify fragments of cDNA, then to clone these fragments and rely on convenient restriction sites before assembling the full length cDNA again with the possibility of introducing unforeseen mutations due to the complexity of these manipulations. Recent technological advances and availabilities of *in vitro* reagents for high-fidelity PCR amplifications have facilitated fast, efficacious and precise cloning and manipulations into viral cDNA. In contrast to random mutagenesis, made by exposure to chemical and physical mutagenic agents (mutagenic chemical, UV etc.), the availability of synthetic oligonucleotides has allowed the targeted modifications to the construct. This method, known as site-directed mutagenesis, covers many types of strategies, but ultimately frequently generates a PCR fragment to be ligated into the target. For long PCR product amplifications, a breakthrough was reported by Barnes *et al*. in 1994. In this, the most widely used, *Taq* DNA polymerase was mixed with a less error-prone polymerase and in combination with other improved conditions (buffers, thermal conditions etc.) PCR amplicons up to 45 Kb were reported (see (Barnes., 1994) and review by (Tellier *et al*., 2003)). The improved long-range PCR amplifications have led to *whole plasmid mutagenesis* using the whole (circular) plasmid as a template, and involving a post-PCR step utilizing particular restriction nucleases (e.g. *Dpn*I) that only cleave when the recognition sites are methylated, thereby removing the methylated plasmid template. DNA purified from a dam+ strain will be a substrate for these types of restriction nucleases. A well-established method was commercialized as the "Quickchange" method but others have used this in the cloning of viral genomes. A so-called target-primed plasmid amplification was developed for the cloning of the influenza virus genome (Stech *et al*., 2008). Our group has developed protocols for a modified method, known *in house* as mega-primer plasmid PCR (in short, megaPCR). This *in vitro* cloning approach is a convenient choice for cloning and introduces no/ or a minimum number of mutations into a CSFV genome (12,3 Kb) plus a 6,7 Kb BAC vector (Friis *et al*., 2012,Risager *et al*., 2013). For the application of mega-primer PCR, see also the work from our German collegues (Richter *et al*., 2011). Advances in gene targeting technologies, which utilize the homologous recombination system of cells or take advantage of temperate bacteriophage recombination proteins, have led to the development of a method that relies on *in vivo* methods to achieve targeted modifications, so called recombineering. This type of *in vivo* cloning approach benefits from using the host cells

own high-fidelity polymerases and proof-reading abilities, and thus should be a more accurate method. A streamlined method for this approach, has been commercialized as the Red/ET counter-selection method (Gene bridges). The method relies on bacteriophage encoded recombination proteins (red α, β, γ) (residing on the pRed plasmid) for homologous recombination with single-copy BAC´s. The method exploits the mechanisms behind a linear genomic lambda phage infection of an *E. coli* host. Alpha proteins remove homology regions in the dsDNA insert by a 5´-3´exonuclease mechanism. Beta proteins function as single strand binding proteins, enabling the binding and integration of a linear insert into the target region (situated on a circular plasmid) and gamma proteins enable the evasion of host cell defense mechanism proteins. The method is summarized in fig. 3. Briefly, a streptomycin resistant ($Strep^R$) strain of *E. coli* (strain DH10beta or alpha) is applicable as a host for the recombineering event. Initially a selection cassette (rpsL-neo) encoding an rpsL and a kanamycin/neomycin resistance gene and flanked by sequences which are homologous (termed "homology arms" or "Hm arms) to the target is inserted in place of region that is not necessary for the final construct. Incubation at 30 °C is necessary for maintaining pRed in the cells, linear products with Hm arms are integrated into the target. The method has provided high cloning efficiencies and is described in more detail in studies by (Friis *et al*., 2012,Risager *et al*., 2013) and Rasmussen *et al* (*submitted, Manuscript 1*).

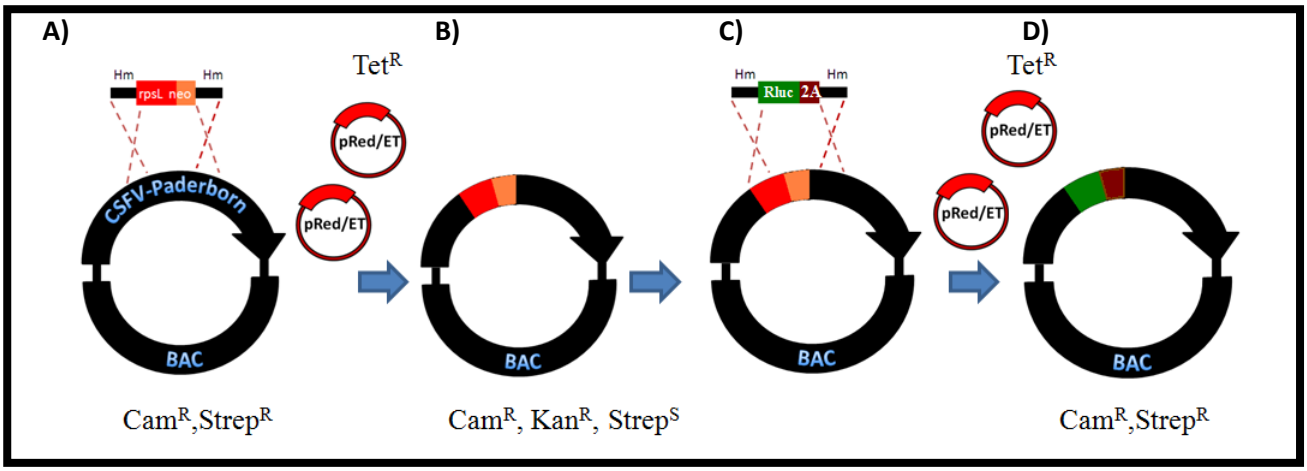


Figure 3. The counter-selection principle. A) Full-length CSFV cDNA is maintained in a BAC vector carrying a Cam resistance gene, and introduced into an *E.coli* strain ($Strep^R$). The introduction of pRed plasmids carrying Tet resistance genes, enables the production of recombination proteins for the integration of the linear insert that contain Hm arms to the target region. B) The linear insert, carrying a Kan resistance gene (the rpsL/neo casette), is now inserted into the target that changes the phenotype into streptomycin sensitive ($Strep^S$). C) The final *counter-selection*, is achieved by the swap of the rpsL/neo with the linear product, in this case containing a Rluc reporter sequence D) Again, the pRed derived recombination proteins facilitate the integration of the product and the final constructs can be selected on Cam, Strep containing media.

Recombinant viruses

Evidence has been found that sporadic recombination between *Pestivirus* strains can occur in nature and which ultimately contributes to their diversification (Becher *et al*., 2001,Becher & Tautz., 2011,He *et al*., 2007). Precise manipulation of the viral genomes can be facilitated by molecular biology techniques. The use and generation of recombinant viruses of mixed origin, so-called "chimeric" viruses is an approach to identify key determinants of the virus. This includes swapping sequences between closely related (or less related) strains, in order to identify regions of incompatibility (Binder *et al*., 2007,Richter *et al*., 2011,Risager *et al*., 2013) or virulence determinant investigations by combining strains displaying avirulent and virulent properties (Risatti *et al*., 2005,van Gennip *et al*., 2000), or lastly, for the generation of vaccine candidates with or without a marker (Beer *et al*., 2007,Rasmussen *et al*., 2007,Reimann *et al*., 2004,Wehrle *et al*., 2007)(Rasmussen et al. submitted (manuscript 1)). Recently, in an EU project, a chimera based on the BVDV CP7 backbone containing the E2 protein from CSFV strain Alfort has been tested and picked as a candidate for vaccination against CSFV in the EU. The vaccine termed CP7_E2alf fulfills the DIVA criteria and has proven efficacious for vaccination of piglets (Rangelova *et al*., 2012).

Replicons as reporters of viral replication

One method for quantitative or qualitative assessments of endogenous determinants of replication efficiency is by the use of self-maintained replicating constructs (replicons). Replicons represent, by definition, a molecule that replicates autonomously in host cells, and thus a full-length RNA genome of CSFV is in fact a CSFV replicon. Replicons, in this thesis context are referring to replication-competent viral genomes that are unable to generate progeny virus due to a functional defect of at least one structural protein but where all proteins/sequences required for genome replication are maintained. Importantly, the replicons should retain the ability to replicate when the RNA is introduced into mammalian cells. For viral constructs that are being used as reporters of key viral traits (e.g. replication), integration of foreign elements should be minimal and not interfere with the wild-type properties.

CSFV replicons engineered to lack the capacity to form infectious virus particles and also containing a foreign sequence (e.g. a selective marker or reporter gene) (see fig. 4), have proven able to act as a tool to monitor replication competence (Binder *et al*., 2007,Lohmann., 2009,Risager *et al*., 2013,Suter *et al*., 2011,Tong & Malcolm., 2006).

Like infectious viruses, replicons utilize the translation and metabolic machinery of their host cell for replication. Enhancement and/or maintenance of the reporter signal can be achieved by manipulations to the replicon by *i*) using an antibiotic resistance cassette, that can be inserted into the replicon to allow the replicons to be maintained, under selection, in a continuous cell line and *ii*) the insertion of an extra internal ribosome entry site (IRES), e.g. from encephalomyocarditis virus (EMCV) to allow internal-initiation on the RNA and hence translation of the residual viral polyprotein sequence. The latter, is also known as a "bi-cistronic" replicon, in contrast to "mono-cistronic" replicon where the ORF is translated as a single polyprotein. No infectious particles are produced from replicon infected cells and thus no horizontal cell-to-cell spread will occur, unless the replicons are introduced into cells that trans-complement the deleted proteins, thereby allowing the replicon to be packaged. Vertical transmission of replicons (without a resistance gene) can occur during cell divisions but will probably not be maintained for a long period without a selection system. Various reporter assay systems for viruses have been developed and used to monitor IRES activity, protein expression etc. Again, the aim of the study is important for the design of the assay. If using replicons for studies dealing with testing of anti-viral compounds that target the specific virus, it is advantageous to use an assay that expresses an immediate and high level signal, to monitor the decrease in signal intensity in a dose-dependent manner over time. For this, a bi-cistronic and selective marker containing assay would be appropriate. If in the other hand dealing with basic research in the viral genome, it is advantageous to only do minor manipulations to the viral genome, and to keep the background noise to a minimum.

Packaging of a replicon with structural virus proteins through, *trans*-complementation, is another approach that combines principles of killed and live vaccines. Such virus replicon particles (VRP) can fulfill the criteria of a safe vaccine and are also applicable as a protein delivery system and can be capable of inducing a protective immune response (Reimann *et al*., 2007,Steinmann *et al*., 2008,Suter *et al*., 2011). Trans-complementation can be achieved by expressing the viral proteins missing from the replicon (e.g. the structural proteins), *in trans* (hence "trans-complementation"). This trans-complementation should allow the replicons to infect cells once and thus deliver the RNA into the cytoplasm but further production and transmission of virus particles is impossible, unless a cell line that expresses the missing structural proteins is used. The VRP can express the carried foreign sequences as functionally

active and complete proteins and thus could be applicable as a safe vaccine (Frey *et al*., 2006,Suter *et al*., 2011).

Types of reporter proteins

An important aspect of viral gene expression studies is to choose an adequate type of reporter for the purposes of the experiment. Clearly a suitable reporter protein must not be toxic for the host or in any other way interfere with signaling cascades. Reporters such as luciferase and ß-lactamase, as well as a transactivator inducing secreted alkaline phosphatase (SEAP), have been used to monitor replication at early times after transfection.

Competing reporter systems based on various types of reporter genes for the detection of virus replication have proven applicable for this purpose. Reporters that have been used include fluorescent, bioluminescent, colorimetric and luciferase based systems. In general, reporter proteins can be applicable in two ways: as qualitative or quantitative reporters. Qualitative reporters that contain a fluorophore (e.g. green fluorescent protein (GFP), mCherry, dsRed etc.) excel in their ease of application for *in vivo* investigations and have been used in relation to investigations of protein localization, etc. i.e. localizing tagged proteins through live cell imaging. Quantitative reporter proteins can determine the translational or transcriptional activities of the gene/ORF/protein through measurement of an enzymatic activity (Fluc, Rluc, β-Gal etc.).

Early reporter proteins included enzymes, when incubated with specific colourless or non fluorescent substrates, could convert these substrates into coloured or fluorescent products. This includes, GUS-reporter systems (based on β-glucuronidase) (Sabesin *et al*., 1971) or the β-Gal assay (based on β-Galactosidase) (Bloom *et al*., 1995). CAT was widely used before luciferases, because it can readily monitor low levels of activity and had no background in mammalian cells (McInerney *et al*., 2000,Moser *et al*., 1999) .

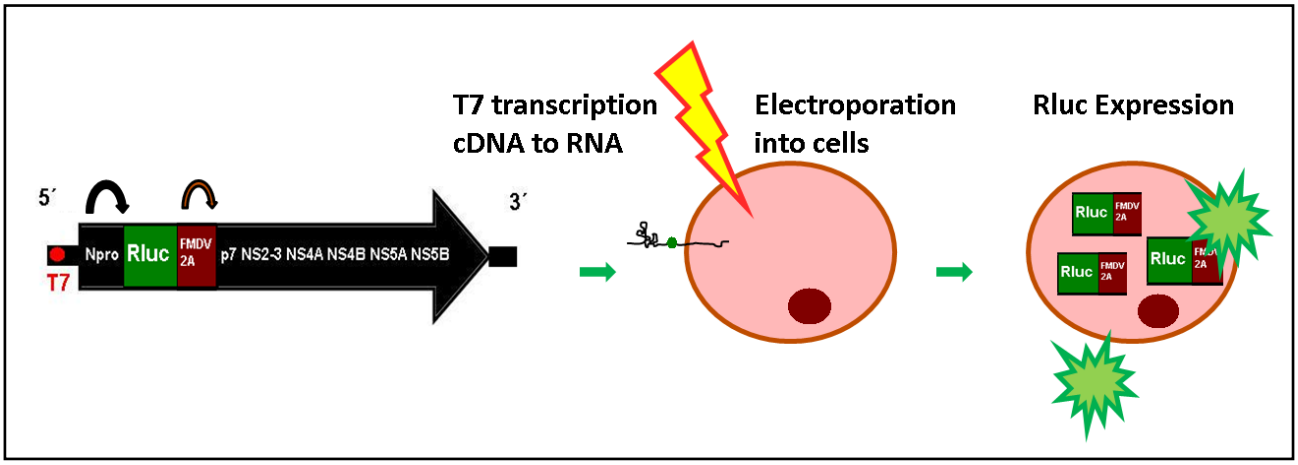


Figure 4. The principle behind a monocistronic CSFV replicon containing *Renilla* luciferase (Rluc) and foot-and-mouth disease virus 2A (FMDV2A) protein coding sequences. A T7 promotor sequence upstream the genome start enables the transcription of DNA into RNA. The introduction of the CSFV RNA into a permissive cell line e.g. by electroporation, is sufficient to initiate the infectious cycle and produce the Rluc enzyme. The action of the CSFV N^{pro} protein and the FMDV2A generates the freed Rluc2A product. The ability for the enzyme to cleave its substrate (in this case coelenterazine) can next be monitored in a luminometer. The signal can be interpreted as the replication competence of the replicon.

Commonly used reporter proteins today make use of proteins that produce bioluminescence. Bioluminescence involves the use of two different components. One is a luciferin (the substrate) and the other is a luciferase (that catalyzes the reaction). Some of the first luciferases were characterized some 40 years ago, see e.g. review by (Greer & Szalay., 2002). Luciferases (*latin* "light-bringer") are enzymes capable of emitting light in the presence of oxygen (and sometimes addition of ATP or magnesium) and a substrate (e.g. luciferin). Light is emitted because the reaction forms oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state (Fig 5). A variety of different molecules can act as luciferins and luciferases, depending on the species of the bioluminescent life form. Luciferin–luciferase systems include, among others, the bacterial *lux* genes of terrestrial *Photorhabdus* luminescens and marine *Vibrio harveyi* bacteria, as well as eukaryotic luciferase *luc* genes from firefly (*Photinus* sp.) species (for Fluc) and the sea pansy *Renilla reniformis* (for Rluc) respectively. Coelenterazine is a common luciferin used in marine animal bioluminescence systems (e.g. *Renilla, Gaussia, Cypridina* etc.), whereas D-Luciferin is the substrate for firefly luciferases. Some assays take advantages of the non-overlapping emisson spectral properties, i.e. a dual-luciferase assay has been developed using both Fluc (emission at 640 nm) and Rluc (emission at 525 nm) for simultaneous monitoring

of protein expression. This can be advantgeous in a di-cistronic reporter system for normalizing data.

In fluorescence, a substance absorbs light of one color and emits light of another color. The fluorophore has been exploited as qualitative reporters in various vectors and in fusion with other gene products of which the most famous is the GFP (from the jellyfish *Aequorea*). Numerous variants of GFP and GFP-like proteins exist such as blue, yellow, red, and cyan fluorescent proteins, and some of these variants have non-overlapping emission spectra which have allowed e.g. triple colour combinations (CFP, YFP, DsRed) for multi-color labeling and imaging of tagged proteins *in vivo*.

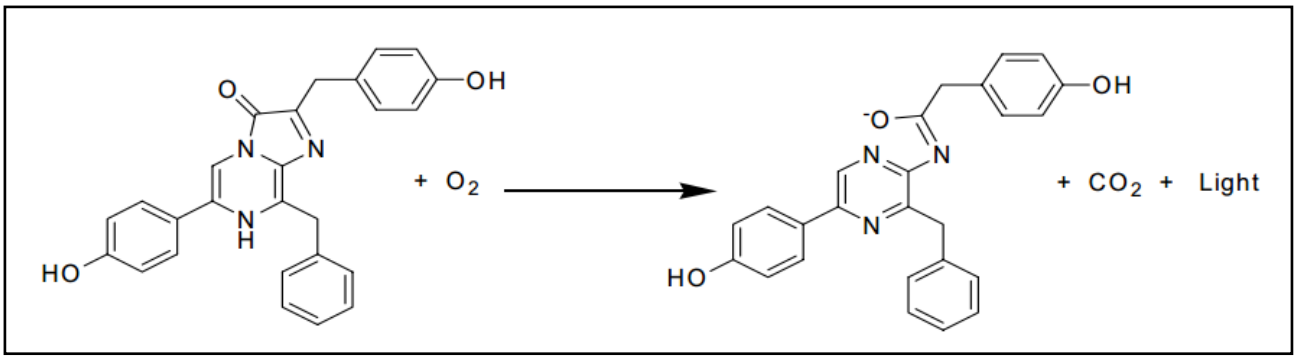


Figure 5. The chemical reaction in the Rluc assay. The Renilla luciferase enzyme oxidizes the substrate (coelenterazine) and converts it into coelenteramide and produces CO_2 and light. The light has a detectable emission peak at 525 nm (image from http://www.piercenet.com).

Spinach - A non-protein reporter

The above mentioned reporters are all based on enzymatic activity from expressed proteins. An interesting approach for tagging RNA, for doing live imaging and for tracing RNA dynamics and localization in living cells, has been the development of a sequence tag, functioning as an aptamer that can bind to a specific ligand (Paige *et al*., 2011). In this they designed an aptamer, that when bound to a fluorophore (termed DFHBI) had the same excitation and emission spectra as GFP, and they named this aptamer "spinach" (fig. 6). Verification for this was performed by the insertion of the tag into a cellular non-coding RNA. We reasoned, that if this tag could be inserted into a virus genome and not interfere with viral properties, it would potentially be an innovative and excellent method for tracing viral genome dynamics *in vivo* in the cell (see Conclusions & Future Perspectives).

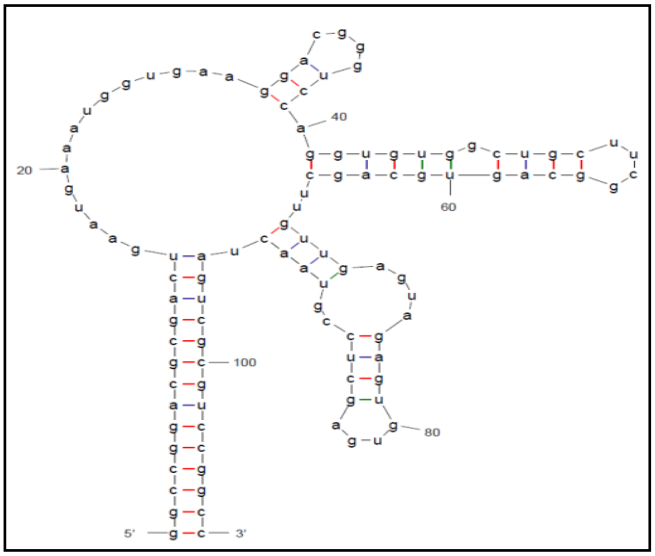


Figure 6. The secondary structure of the spinach aptamer sequence. Structure prediction was performed using mfold online software (http://mfold.rna.albany.edu)

PART 2. Pestiviruses: Infection and requirements for RNA replication

Overview

The positive sense pestivirus genomic RNA sequence is used like a messenger RNA (mRNA) to produce the encoded proteins. The replication of pestivirus RNA is a complex process involving multiple virus encoded proteins in concert with the 5´- and 3´- termini of both the positive and negative strands of the viral RNA. These processes require protein-protein interactions as well as interactions between proteins and cis-acting RNA elements (Liu *et al*., 2009). RNA replication of pestiviruses requires the viral RNA template, translation and hence cleavage of the nascent viral polyprotein into the functional proteins, including the nonstructural proteins 3, 4A, 4B, 5A, and 5B, and cellular proteins. It has been established that RNA replication can be achieved autonomously without the aid of structural proteins (Behrens *et al*., 1998,Moser *et al*., 1999). Virus assembly, to produce new viral progeny, in contrast does require both structural and non-structural viral proteins and also cellular factors such as host proteases (reviewed in (Murray *et al*., 2008a)).

In here, a summary of the proteins and important steps in the life cycle is given for *Pestivirus*es, and where appropriate, for analogous studies on other *Flaviviridae*.

Introduction

The replication of RNA viruses by RNA dependent RNA polymerases (RdRp) is a unique process that has no counterparts in the cell. The *Flaviviridae* family is placed within the Baltimore classification class IV, since it contains a single RNA molecule of plus-sense polarity. Other viruses in this class include picornaviruses, togaviruses, caliciviruses and coronaviruses. Due to their error-prone polymerases, RNA viruses have a general mutation rate ranging from 10^{-4} to 10^{-6} per nt, compared to that of DNA viruses that have much lower rates down to 10^{-8} per nt (Drake *et al*., 1998,Sanjuan *et al*., 2010). This suggests that CSFVs in general have mutation rates that result in substitution of 0.1 to 1 nt per synthesized genomic RNA molecule. The viral RNA genome is infectious, and thus the introduction of the RNA into cells is sufficient to initiate the infection cycle. An infection involves translation of the RNA for production of the viral proteins (structural and non-structural) and then replication of the genome in a replication complex, carried out on intracellular membranes in association with the ER (Diaz & Ahlquist., 2012,Egger *et al*., 2002,Miller *et al*., 2003,Moradpour *et al*., 2004,Stapleford & Miller., 2010), and finally the assembly and release of novel virions.

A great deal of work has been devoted to the human pathogens within the *Flaviviridae* (hepatitis C virus, Yellow fever virus, Dengue fever virus, West Nile virus etc.) but still relatively little attention has been given to the pestiviruses which have importance for agriculture. The sporadic outbreaks of diseases caused by pestiviruses through history with economically devastating impacts have reminded us of the need for an in-depth understanding of the properties of these viral pathogens. Furthermore, due to their close genetic similarity to flaviviruses and hepaciviruses, the pestiviruses provide an excellent, experimentally amenable, model for the related human pathogens.

During the past 20 years, infectious cDNA clones have been established for many viruses within the family *Flaviviridae*, including certain pestiviruses (Behrens *et al*., 1998,Mayer *et al*., 2003,Moormann *et al*., 1996,Rasmussen *et al*., 2008,Rasmussen *et al*., 2010,Ruggli *et al*., 1996). This breakthrough has enabled molecular virologists to generate infectious/replicating and bio-engineered viral constructs to analyse the determinants of viral replication and virulence.

The CSFV genome includes a single, large, open reading frame (ORF) which encodes a polyprotein of ca. 3900 amino acids. The ORF is flanked, at each end, by untranslated regions (UTRs). The genome is uncapped at its 5′-terminus and the 5′-UTR contains an internal ribosomal entry site (IRES) which directs cap-independent translation initiation on the viral RNA (Fletcher & Jackson., 2002,Friis *et al*., 2012). The 3′-UTR is unusual, compared to most eukaryotic mRNAs, in that it lacks a poly(A) tail but it is predicted to contain a variety of structural elements which are involved in initiating the synthesis of negative strands (Deng & Brock., 1993,Frolov *et al*., 1998,Pankraz *et al*., 2005,Yu *et al*., 1999). The viral polyprotein is co- and post-translationally cleaved, by cellular and virus encoded proteases, to produce four structural proteins (the core protein, C, and three envelope glycoproteins, E^{rns}, E1 and E2) plus eight major non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). See fig. 7A and 7B. These viral proteins function either as structural components of the virion or as components of the viral RNA replication complex, or as components of viral host evasion strategies (see N^{pro}, and E^{rns}).(Lindenbach *et al*., 2007,Meyers & Thiel., 1996,Rumenapf *et al*., 1993,Weiland *et al*., 1990a).

Protein synthesis is carried out on the rough ER and the majority of proteins synthesized here undergo glycosylation here as well as in Golgi apparatus. Correct protein arrangement in the cell i.e. sorting proteins into different intracellular compartments (organelles) or out of the

cell, is crucial for correct cell functioning. Intracellular trafficking of viral proteins in the cell can be governed on the micro filamentous cytoskeleton (actin filaments) by use of motor proteins. Many proteins have specialized signals that direct the proteins to their final destination. These sequences are known as "signal peptides"(SP) or signal sequences, leader sequences, homing signals etc. In the majority of newly synthesized proteins, SPs consist of a highly hydrophobic 5-30 aa peptide present at either the N-terminus or internally within the proteins that are destined towards the secretory pathway. The N-terminus of the protein, containing the signal, guides the protein to the ER and traverses the membrane of the ER through the protein-conduction channel, known as the the Sec61 channel (Zimmer *et al*., 2008). Signal peptides are removed by proteolysis through the action of signal peptidase or signal peptide peptidases. The CSFV structural proteins contain typical signal peptidase cleavage sites, only differing for E^{rns} that contains an atypical amphatic region (Bintintan & Meyers., 2010).

Life cycle of CSFV: From adsorption to release of virions

The lifecycle of CSFV involves the following essential steps: adsorption, entry, translation, protein maturation, genome transcription (RNA replication), virus assembly and the release of virions. The host cell needs to be both susceptible (contain the right receptors) and permissive (with a suitable intracellular milieu) for an infection to be successful. Furthermore the virus must possess evasion strategies to overcome host defenses. Plus-strand RNA synthesis starts in between 3 to 6 h.p.i. The fastest increase in positive strand RNA synthesis is observed in between 9-15 h.p.i and a plateau in RNA accumulation is observed at 18-24 h.p.i (Gong *et al*., 1996,Mittelholzer1 *et al*., 2000). The infection steps are summarized in fig 8.

Adsorption and entry

Initiation of the infection is started by the attachment of surface exposed virus proteins to receptor molecules residing on the cell surface. The initial binding of the CSFV particle to the host is performed by an interaction between virion envelope proteins present of the virion surface E^{rns}, E1 and E2 and the cellular receptors (Rumenapf *et al*., 1993). An important receptor for BVDV attachment has been identified as CD46. The CD46 was shown to bind

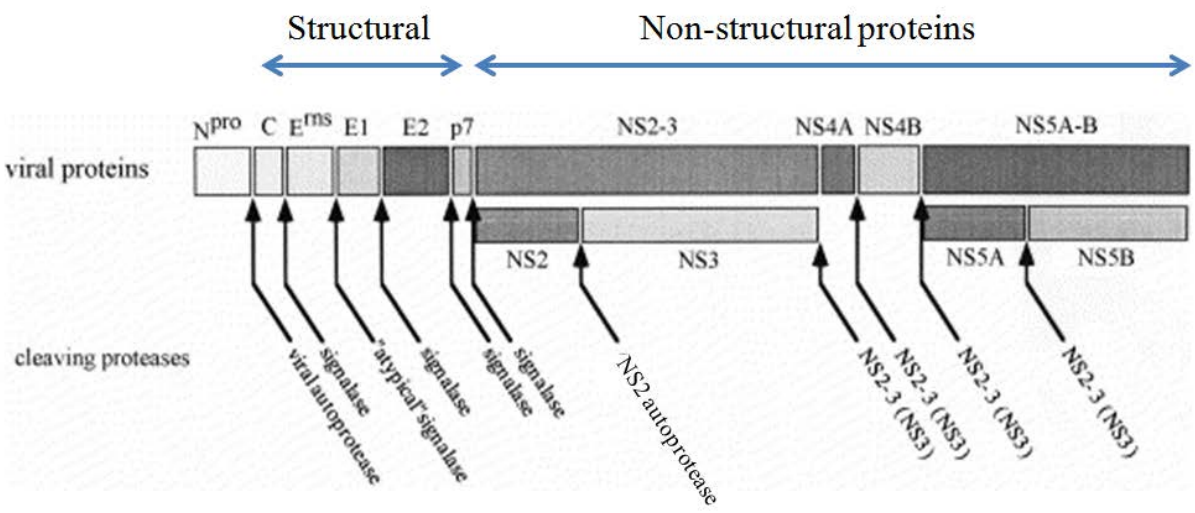
BVDV and subsequently promote virus entry. Blocking CD46 with mAbs led to a failure in virion adsorption in a dose-dependent manner (Maurer *et al*., 2004,Schelp *et al*., 1995). It is reasonable to believe that this is a similar receptor for CSFV but it has not been identified yet. Some studies have reported that heparan sulfate(HS) can act as a cellular receptor for tissue culture-adapted BVDV and CSFV, as removal of HS from the surface of swine kidney cells (SK6), by heparinase I treatment, almost completely abolished infection of these cells (Hulst *et al*., 2000,Hulst *et al*., 2001). HS binding prior to infection was also demonstrated for Dengue fever virus (Hilgard & Stockert., 2000), but is likely due to cell adaptation. Another essential receptor for adsorption is the low-density-lipoprotein (LDL) receptor (Agnello *et al*., 1999). Binding of the virus to cellular receptors is primarily accomplished by glycoprotein complexes composed of disulfide-linked E2-E2 homodimers, or secondarily as heterodimers composed of E2-E1 (Thiel *et al*., 1991,Weiland *et al*., 1990b) or as suggested by (Lazar *et al*., 2003) E2-E^{rns} heterodimers, which are also found on the virion envelope surface. Entry is accomplished by clathrin-dependent endocytosis as is seen with other enveloped viruses e.g. Semliki forest virus (SFV), Sindbis virus (SINV), vesicular stomatitis virus (VSV) and flaviviruses (Chu & Ng., 2004,Krey *et al*., 2005,Lecot *et al*., 2005). Enveloped viruses that invade the host cell via receptor-mediated endocytosis, require an acidic pH for fusion, but low pH is not sufficient to force adsorbed enveloped virus into fusion with the plasma membrane (Krey *et al*., 2005). The acidification triggers conformational changes that lead to exposure of the fusion peptides and thus membrane fusion. Fusion proteins can be divided into at least two different classes; flaviviruses use Class II fusion proteins. Pestivirus E2 proteins contain a fusion like peptide, though, it was unexpectedly found from a crystal structure of E2 that it does not contain a class II fusion protein fold, as was previously assumed. It seems that fusion is accomplished by a fusion protein not seen before (El Omari *et al*., 2013) and insertion of the fusion peptide into the target membrane, initiates an irreversible process. The whole virion is engulfed at clathrin-coated pits, and the capsid is internalized to form clathrin-coated vesicles inside the cell. Intracellular transport is accomplished on motor proteins (e.g. dynamin) that carry cargo such as macro molecules along the actin filaments. The low pH (due to the acidification inside the late endosomes) of the endosomal pathway triggers conformational changes in the viral glycoproteins that lead to the fusion of the viral and endosomal membranes Following uncoating of the nucleocapsid, the RNA genome is release into the cytoplasm (Krey *et al*., 2005,Lindenbach *et al*., 2007).

Proteolytic processing and the formation of a replication complex

Ribosomes can bind to directly to the IRES to direct cap-independent translation initiation at the polyprotein start position (AUG at nt. 374-376) for translation of the ORF into a precursor polyprotein. The processing of this polyprotein into smaller precursor peptides or proteins is then carried out in order to release the mature proteins that include both the structural and nonstructural components of the virus (fig. 7). The termini of the structural proteins contain signal proteins that are cleaved initially by signal peptidases and further internal signal sequences are recoqnised and cleaved by signal petide peptidases in the ER lumen(Weihofen *et al*., 2002). Non-structural proteins are processed in the cytoplasm primarily by the action of the N^{pro} and NS3 proteases (fig. 7).

Associations between the viral NS proteins and cellular components result in the formation of a replication complex that is associated with altered intracellular membranes on the surface of the endoplasmic reticulum (Miller *et al*., 2003). Important for the formation of a replication complex is a hydrophobic tail-anchoring mechanism at the extreme C-terminus of the polyprotein (in NS5B) and in NS4B that mediates association to the ER membrane (Brass *et al*., 2010,Gouttenoire *et al*., 2009a,Lee *et al*., 2011,Miller *et al*., 2003,Moradpour *et al*., 2004,Schmidt-Mende *et al*., 2001). The formation of such a replication complex compartmentalizes the production of viral proteins and RNA into small cytoplasmic viral factories localised on ER membranes in the perinuclear site of infected cells, which allows a coupling of functions residing in different polypeptide chains (fig. 7B). Although interactions seem to exist between the proteins in the replication complex of CSFV, it is not exactly clear how the RNA template is organized into the complex.

A



B

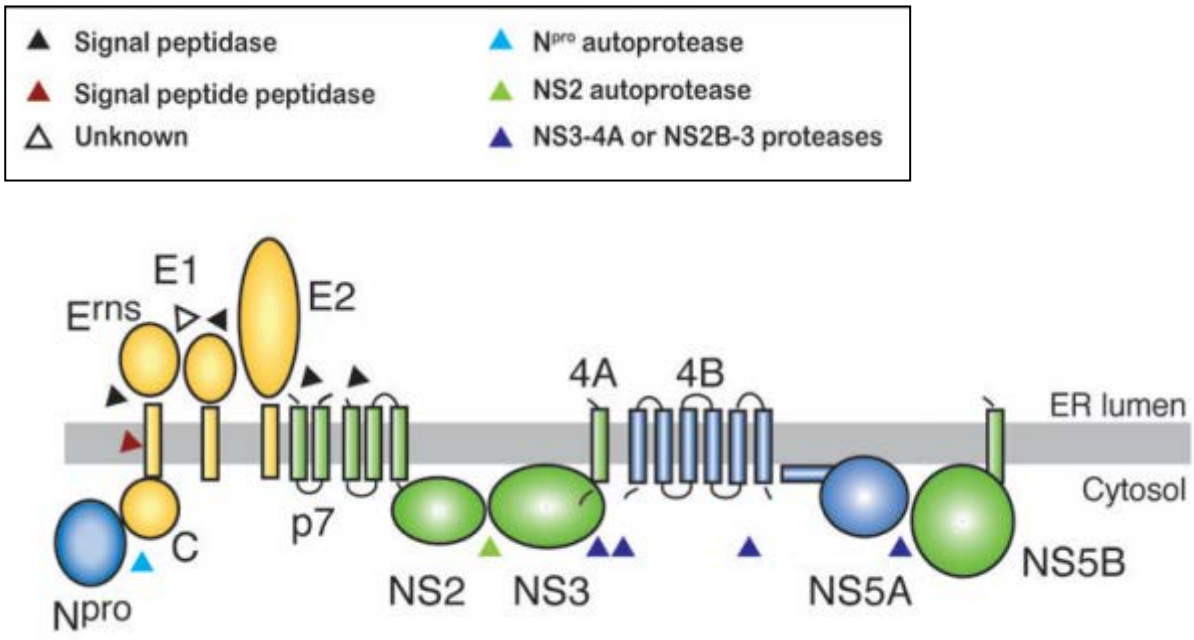


Figure 7. A) Overview of the the CSFV polyprotein, and the processing into at least 12 mature proteins by the action of cellular and viral proteases. Modified from (Kummerer *et al.*, 2000). B) Proposed topology of Pestivirus viral proteins with respect to the ER. Modified from(Murray *et al.*, 2008a) .

Replication

By analogy with other members of the *Flaviviridae* the first step of viral RNA replication is initiated by synthesizing a full-length negative-strand RNA complementary to the genomic positive-strand RNA. One hypothesis is that the replication complex preferentially replicates by a *cis*-acting mechanism in which, it replicates the viral RNA from the strand it was translated from. The 5'UTR and the 3' end of the negative-strand intermediate contribute to the formation of a positive-strand promoter for the second replication step in which the negative strand serves as the template for synthesis of additional positive-strand RNA molecules (Behrens *et al*., 1998). The NS5B protein facilitates binding of the template ((+) strand) 3´UTR and incorporation of a priming GTP that acts as the primer and hence replication is initiated (Choi *et al*., 2004). The closed hand conformation of the protein completely encircles around the active site in the palm domain (the GDD motif) and by this forms a channel wherein binding of the single-stranded RNA templates and the priming GTP nucleotides are facilitated and hence synthesis of minus strand is initiated (Curti & Jaeger., 2013,Xiao *et al*., 2004). The fingertip region contains the conserved motifs I and II and it has been proposed that these are important for binding the RNA template and the incoming NTPs. Interaction with the NS3 helicase protein is important for unwinding tertiary structures of template RNA, and the NS3 NTPase for hydrolysis of incoming NTPs. For detailed reviews on the NS5B, see (Butcher *et al*., 2001,Choi *et al*., 2004,Choi & Rossmann., 2009,Ferrer-Orta *et al*., 2006,O'Farrell *et al*., 2003). Since RNA replication has to initiate from the 3'- end of the RNA template of each strand, the corresponding 5' and 3' UTRs of the CSFV RNA genome contain the sequences required for the initiation and/or regulation of RNA replication. In order for *Flaviviridae* viruses to replicate successfully they must have complementary binding regions on both the 3' and the 5' ends of the genome for long range RNA-RNA interactions. A highly conserved "5´-GUAU" termini motif interaction with a corresponding "3´UTR -AUAC sequence appears to be crucial for cyclization of the genome, and thus a mutation in these sequences severely impairs replication (Becher *et al*., 2000,Frolov *et al*., 1998). Several recent studies show a close interaction between the 5´ and 3´ UTRs. In Dengue virus, the interaction between a cycling sequence (CS) and an upstream AUG region (UAR) with complementary regions in the 3' UTR are needed for replication (Polacek *et al*., 2009). Similarly, in foot-and-mouth disease virus two elements, the S fragment and the IRES, have been shown to interact with

elements in the 3'-UTR. One interaction promotes translation whereas the other interaction is necessary for replication (Serrano *et al*., 2006). For HCV, the *cis*-acting replication element (CRE) located in the 3'-UTR has been shown to form a kissing-loop RNA-RNA interaction with domain IIId within the IRES, this interaction is crucial for viral replication(Diviney *et al*., 2008,Romero-Lopez & Berzal-Herranz., 2009).

The newly synthesized positive-strand RNA can then be translated into polyprotein, or packaged into virions. The formation of dsRNA intermediates in the replication complex is a potent trigger of anti-viral defense mechanisms. Toll-like receptors (TLR) and the cytosolic RIG-I and MDA5 proteins recognize the viral RNA and respond by activating a signaling network that culminates in the induction of type I IFN and establishes an antiviral state. Central players of this signaling cascade and the involvement of the innate immune response are interferon regulatory factors (IRF) 3 and IRF7 (Fiebach *et al*., 2011). CSFV in response to this has evolved strategies to counteract this by e.g. the action of N^{pro} and E^{rns} proteins (see protein descriptions). The amount of dsRNA intermediates are kept at a minimum and is probably degraded immediately after template synthesis of positive-strands due to the action of E^{rns}. Positive-strand RNAs thus are present in large excess (100-fold) over the negative-strand RNA (Mittelholzer *et al*., 2000).

The genome is not used simultaneously for both translation and replication, which would cause collisions between the ribosomal subunits moving in the 5´- 3´ direction and the NS5B protein transcribing in the 3´-5´direction, translation and replication are tightly regulated during the viral life cycle. To avoid this, a switch mechanism between translation and transcription activities is important. There are indications that this switch may occur after the accumulation of NS5A and NS5B proteins, since these have been shown to inhibit the BVDV IRES-dependent translation *in vitro* (Gong *et al*., 1996,Li & McNally., 2001,Zhang *et al*., 2002).

As indicated above, RNA replication of pestiviruses requires viral RNA templates, the viral nonstructural proteins 3, 4A, 4B, 5A, and 5B, and cellular proteins. Cellular proteins include both proteases but also cellular factors for the translation and transcription activities. Members of the cellular protein group NF90/NFAR have been shown to associate with both the the 5´ and 3´ termini (Isken *et al*., 2003). It was suggested for BVDV that recruitment and association with these proteins were essentially involved in regulating replication

coordination through genome circularization, and also involved in determining translation activities (Isken *et al*., 2003,Isken *et al*., 2004).

Viral assembly and release

Virion assembly of viral proteins, RNA genome, and a lipid membrane to build an infectious particle and the final release from the host cell constitute the final stages of the viral life cycle. Little information is available on the assembly, the origin of the lipid envelope and the release of pestiviruses from infected cells. Assembly of virions is assumed to take place in the ER lumen where cellular enzymes required for processing and folding of viral proteins are present. Viral assembly is presumably commenced by the C-proteins encapsidation around the RNA genome (Kunkel *et al*., 2001,Kunkel & Watowich., 2002,Murray *et al*., 2008b). Envelope proteins have concentrated on the inside of the ER membrane, and the nucleocapsid is then enclosed by these envelope proteins in the ER lumen (Murray *et al*., 2008a). Next, secretion through the vesicle transport pathway to the Golgi apparatus, where final glycosylations and packaging in membrane systems of the host cell secretory pathway before transit to the cell surface and release by exocytosis takes place. An important protein for destabilising the membrane prior to exocytosis is the p7 protein due to is function as a viroporin (Chandler *et al*., 2012,Harada *et al*., 2000). Hepaciviruses and pestiviruses seem to mature rapidly after their formation, in that viable infectious viruses can be released intracellular in contrast to flaviviruses that have a late maturation stage at the site of budding (Macovei *et al*., 2006,Murray *et al*., 2008a). See fig. 8.

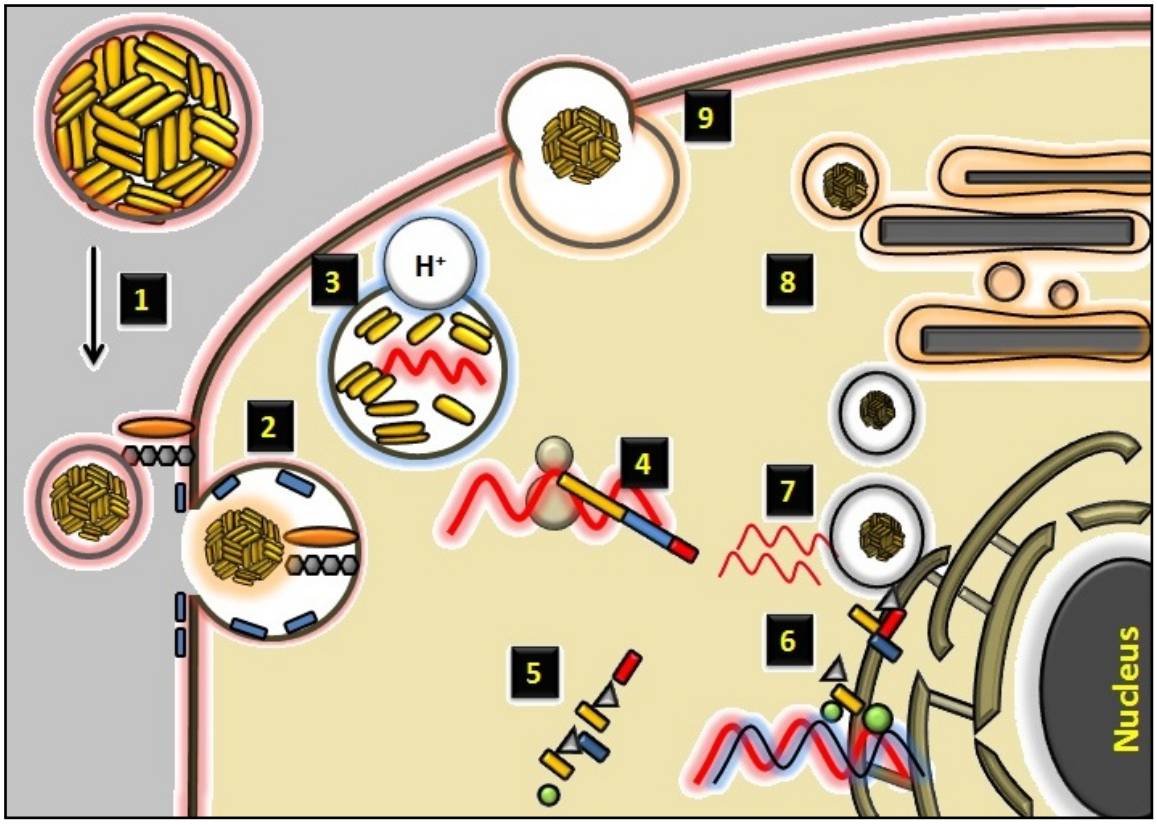


Figure 8. Overview of the infection and the intracellular steps involved in the CSFV lifecycle. 1) The attacment of the viral envelope proteins to host receptors mediates the 2) internalization into the host cell by clathrin-dependent endocytosis. 3)Fusion of membranes with host endosomes and pH acidification mediates the uncoating and release of the RNA genome into the cytosol. 4)The positive-sense genomic ssRNA is then recognized by the ribosome translation complex through the internal ribosome entry site (IRES) and is translated in the cytosol into a polyprotein. 5) The polyprotein is processed into structural and non-structural proteins(NSP). 6) NSP form and anchors a replication complex at the ER and initiates genome replication. Positive-strands are either used as template for negative-strand synthesis; translated into a polyprotein or packaged into new viruses. 7) Viral assembly is commenced in the ER lumen and virions are transported to the 8) Golgi apparatus where final maturation and packaging is carried out. 9) New viruses are transported via the secretory pathway and released by exocytosis.

Viral proteins and their function

The N-terminal protein (N^{pro}) is the leader protein of the polyprotein and is unique to the genus *Pestivirus*. It contains a cysteine-like autoprotease activity which cleaves itself off as the initial cleavage event in the nascent viral polyprotein thereby generating the amino-terminus of the C protein (Ruggli *et al.*, 2003,Stark *et al.*, 1993). It has been considered an accessory protein not required for virus replication, since it can be genetically manipulated without affecting the functionality (genome replication or virus assembly) of the virus in cell culture (Tratschin *et al.*, 1998). A role in the pathogenesis has been attributed to the protein due to its capacity to interfere with the type 1 IFN-induction pathway and by promoting the proteasomal degradation of interferon regulatory factor 3 (IRF3) thereby antagonizing the early innate antiviral immune response by preventing IFN-α/β induction (Bauhofer *et al.*, 2007,Gil *et al.*, 2006,Mayer *et al.*, 2004,Ruggli *et al.*, 2003,Ruggli *et al.*, 2009,Tratschin *et al.*, 1998). It was later shown, however, that a specific minimal elimination of the IRF 3-degrading function of N^{pro} did not affect the virulence of the virus, indicating that this activity of N^{pro} in itself is not a critical factor for virulence (Ruggli *et al.*, 2009). Simultaneously it was discovered that N^{pro} contains a zinc-binding motif in the C-terminal part, which coordinates one zinc atom per molecule and is essential for virus-mediated degradation of IRF3 (Szymanski *et al.*, 2009).

The C protein or "core"-protein (sometimes referred to as the capsid protein), is a small basic protein of ca. 99 amino acids. It is excised from the polyprotein by enzymatic cleavages to form the mature proteins for incorporation as structural units into the virions (Heimann *et al.*, 2006,Murray *et al.*, 2008b). The N-terminus is initially formed by the activity of N^{pro} and the C-terminus is released by the action of SP (Rumenapf *et al.*, 1993,Stark *et al.*, 1993). The resulting released protein is further internally cleaved by a SPP close to the C-E^{rns} junction (Heimann *et al.*, 2006). CSFVΔC mutants are avirulent *in vivo* and produce virions or subviral particles that contains no RNA. This could indicate a functional role of C to bind the RNA, (in a histone-like manner) thereby segregating the RNA and core complex from making contact with the envelope. Although morphogenesis or virion assembly normally is expected to commence with a packaging signal in the genome, this has not been identified for any members of the *Flaviviridae.* The N-terminal domain of the alphavirus C protein is similar to

the flavivirus C protein, and it has been speculated that the N-terminal domain of the C protein from alphaviruses is structurally related to flavivirus C protein in that they are approximately the same size (100 residues) and both contain a large percentage of basic residues, and thus they are likely to exhibit same function (Kofler *et al*., 2002). Mutants of Semliki Forest virus (an alphavirus belonging to the *Togaviridae*) that had deletions in C was severely impaired in RNA packaging (Forsell *et al*., 1995,Murray *et al*., 2008b). Interestingly the C-protein of HCV has been shown to efficiently self-assemble *in vitro* into nucleocapsid-like particles (Kunkel *et al*., 2001,Kunkel & Watowich., 2002). This together indicates that C proteins play roles in binding the viral RNA, and it is likely that RNA packaging is dependent on the C protein.

The E^{rns} protein (227 aa) previously known as E0, is the initial encoded envelope protein and is displayed on the surface of the virion. Together with the envelope glycoprotein E2, E^{rns} is capable of eliciting neutralizing antibody responses, however E^{rns} alone is only sufficient to induce partially protective immunity (Maurer *et al*., 2005). The protein forms a disulfide-linked homodimer with more than 50% of the molecular mass contributed by N-linked glycans (Rumenapf *et al*., 1993) and initially interacts with carbohydrates on the cell surface, but has no apparent role in cell entry (Fetzer *et al*., 2005,Hulst *et al*., 2000,Tautz., 2011). Signal peptidase generates the N-terminus of the E^{rns} glycoprotein, whereas the C-terminus is released by a SPase that recognizes a new type of SP cleavage site and generates an amphipathic signal protein (Bintintan & Meyers., 2010,Tews & Meyers., 2007). Critical for the formation of the homodimer is a cysteine disulfide bond between residues 171 in each monomer. Viruses with deletions of cys171 failed to dimerize and were attenuated in pathogenicity but not in replication competence (Tews *et al*., 2009). The protein lacks a typical trans-membrane domain for membrane anchoring as found in the E1 and E2 proteins. E^{rns} is only attached weakly to the virion through a non-typical membrane amphipathic helix anchor (Fetzer *et al*., 2005,Tews & Meyers., 2007). A unique feature of the E^{rns} protein is an intrinsic ribonuclease (rns) activity involving two catalytic active sites. The rns sequence displays homology with members of the single stranded RNA degrading T2 RNase superfamily (Schneider *et al*., 1993). A considerable proportion of the E^{rns} produced in infected cells is secreted into the extracellular environment and circulates in the body fluids of infected animals (Magkouras *et al*., 2008,Rumenapf *et al*., 1993,Tews & Meyers., 2007). *In vitro* and *in*

vivo studies have indicated that the enzymatic activity of E^{rns} plays a role in the regulation of RNA synthesis in infected cells and in the evasion of the immune defense system early in the infection by binding to dsRNA complexes and inhibiting dsRNA induced IFN-β production (Van Gennip *et al*., 2004,Xia *et al*., 2007). Presumably E^{rns} binds to ssRNA, but initially does bind the dsRNA intermediate complex, and that the nuclease activity is active when bound to dsRNA (Iqbal *et al*., 2004). Up to 95% of the E^{rns} protein is retained inside the cell and a recent study has identified a retention signal in the C-terminal part of E^{rns}, that is responsible for this (Burrack *et al*., 2012).

The E1 protein is the second and the smallest (195 aa) of the envelope proteins. The role of E1 is co-attachment to host receptors, and is present as E1-E2 dimers on the virion surface (Weiland *et al*., 1990a). E1 proteins are also modified with N-linked glycans at aa residues 500, 513 and 594. Modification of all of these three sites led to non-viable viruses, whereas removal of individual glycosylation sites led to attenuation of a CSFV strain Brescia (Fernandez-Sainz *et al*., 2009). The C-terminal region serves as a membrane spanning domain for anchoring in the lipid membrane of the virus.

The E2 protein, is the immunodominant pestiviral protein, and thus, the protein has been an interesting target for bioengineered vaccine candidates (Beer *et al*., 2007,Rasmussen *et al*., 2007,Reimann *et al*., 2004,van Gennip *et al*., 2000)(Rasmussen et al. submitted (manuscript 1)) and for genotype classification based on full-length E2 encoding sequences (Postel *et al*., 2012). The 373 aa protein is present as homodimers and as E2-E1 heterodimers and is essential for binding to cell receptors and thus in the initiation of infection. The C-terminal part serves as a membrane spanning domain. In the N-terminal region, E2 contains four antigenic domains (A to D) (El Omari *et al*., 2013). Most important is a very immunogenic, linear epitope located in the A domain, consisting of amino acids TAVSPTTLR (at aa 829 to 837 in the CSFV polyprotein). This motif is highly conserved among CSFV strains but divergent in BVDV and BDV strains. The TAVSPTTLR epitope is an important determinant for escape mutants evading the host defence mechanisms and involvement in virulence (Leifer *et al*., 2012,Risatti *et al*., 2006). The E2 protein is released in an ordered fashion. Initially a fusion protein consisting of E^{rns}, E1 and E2 is formed due to a cleavage at the C/E^{rns} junction by a SPase that generates the N-terminus. The C-terminus of this fusion protein is generated in differing forms, some in part with E2 and others in part with p7, since E2-p7 cleavage is

incomplete (Elbers *et al*., 1996,Rumenapf *et al*., 1993). The E2 is next released and the N-terminus is generated by host cell signalases, and thus a highly hydrophobic seven residues sequence "WLLLVTGA" is found directly upstream of the cleavage site.

The P7 protein. Deletion of p7 from BVDV does not affect RNA replication but blocks an early event in virus assembly and completely hinders production of infectious virus particles (Harada *et al*., 2000).The exact role of p7 has not been settled but the protein has a very similar structure to viroporins and hence it is thought to function in membrane permeabilisation and for releasing of infectious progeny (Harada *et al*., 2000). For HCV the p7 protein was found to self-assemble into stable hexameric or heptameric complexes capable of conducting ions and thus function as an ion channel (Chandler *et al*., 2012). The uncleaved E2-p7 fusion proteins may also be incorporated into virions, but this has not been detected yet (Murray *et al*., 2008a).

The NS2 and NS3 proteins are initially generated as one fusion protein due to the protease activity of NS3 that generates the C terminus. NS2 contains a cysteine autoprotease activity that generates the N-terminal part of NS3 (Lackner *et al*., 2004). The NS2 protein alone is not accredited to any function so far and in principle NS2 is functioning only in the context of uncleaved NS2/3 protein. The NS2 proteolytic processing is slow, and this timing is essential to the viral life cycle (see below). NS3 possesses three enzymatic activities essential for virus replication and thus is essential in the replication complex along with the NS5B protein: a serine protease located in the N-terminus, an NTPase and an RNA helicase activity located in the C-terminus, but NS3 has also been credited with a role in enhanced translation activity when bound to the IRES (Zhu *et al*., 2010). NS3 requires NS4A as a cofactor for efficient serine protease activity. The NS3 (plus 4A co-factor) protease mediates the processing steps at the C-terminus of NS3 and at all downstream cleavage sites of the viral polyprotein. The NS3 helicase specifically was shown to interact and unwind both plus- and minus template strands. The NTPase activity is related to hydrolyzation of NTP's in the nascent strand (Tamura *et al*., 1993). Interestingly the NS3 protein induces the production of antibodies, although these are not sufficient to protect against viral infection for flaviruses (Morozova *et al*., 1999). Uncleaved NS2/3 is required for virion production (Agapov *et al*., 2004,Moulin *et al*., 2007), whereas cleaved NS2/3 and thus the active NS3 protease is responsible for downstream proteolytic processing and RNA replication. This could indicate that the cleavage

event in NS2/3 is a regulator of a switch mechanism between RNA replication and viral assembly (Lackner *et al*., 2004). CSFV mutants lacking the NS2 protease replicated more efficiently and induced a cytopathic(cp) effect (Moser *et al*., 1999). In contrast to BVDV, cpCSFV strains have rarely been identified (Gallei *et al*., 2008,Laude., 1978). RNA replication is also here faster in cp-biotypes (that have increased levels of NS3) and thus it is likely that NS3 is a central regulator of RNA replication (Gallei *et al*., 2008,Lackner *et al*., 2004,Lamp *et al*., 2011,Li & McNally., 2001). Overexpression of NS3 protein, have been shown to induce apoptosis (Yamane *et al*., 2006). Cp variants produce high levels of NS3 as a free protein after the early phase of infection where in contrast, ncp BVDV variants produce largely NS2-3 and small amounts of NS3 during the early phase of infection. A cellular chaperone protein "Jiv" (J-domain protein interacting with viral protein) of limited availability has been shown to act as a regulating cofactor for NS2 in the cleavage event. When all available Jiv-proteins were depleted, NS2 protease activity ended and thus blocked replication (Lackner *et al*., 2004). This is an excellent example of how the viruses have evolved strategies for persistence in the host.

The NS4A protein is a small 64 aa zinc-binding phosphorylated protein with a hydrophobic N-terminus followed by a highly charged and acidic C-terminal domain. NS4A acts as cofactor and an indispensable factor for the NS3 serine protease activity (Moulin *et al*., 2007,Tautz *et al*., 2000). In other *Flaviviruses* another role attributed to the protein is to recruit other viral and cellular proteins to the replication complex (Lindenbach & Rice., 1999). In HCV, NS4A can activate the NS3 protease when provided *in trans* (Failla *et al*., 1994). Its exact function remains however largely unknown.

The NS4B protein is a highly hydrophobic integral membrane protein with membrane associated hydrophobic domains. A shared topology among NS4Bs in the *Flaviviridae* suggests that they have a shared function (Lundin *et al*., 2003). Since it can be cross-linked with NS5A and NS3 a role for NS4B in the replication complex was suggested (Qu *et al*., 2001,Zhu *et al*., 2010). A recent study demonstrated that aa 40 to 69 in the N-terminal portion of NS4B are essential for the formation of a functional replication complex and indicating a role in anchoring the replication complex to the ER (Gouttenoire *et al*., 2009b). The N-terminal part trans-locates to the lumen of the ER due to a signal peptide. For BVDV, a single amino acid mutation in NS4B could render the BVDV strain NADL noncytopathogenic and thus has been

suggested to have a role in pathogenesis (Qu *et al*., 2001). In HCV, it has primarily been associated with the formation of ER-derived membrane vesicles (Egger *et al*., 2002,Rai & Deval., 2011). When expressed alone NS4B mainly colocalizes with Golgi markers (Weiskircher *et al*., 2009).

The NS5A protein is a large hydrophilic protein (497 aa) involved in virus replication. NS5A is the only protein required for RNA replication that can be complemented *in trans*, indicating that unlike the other proteins involved in the replication complex NS5A membrane association might occur post-translationally (Grassmann *et al*., 2001,Sheng *et al*., 2010,Tong & Malcolm., 2006). Cleavage between NS5A and NS5B is slow and is accomplished by the action of the NS3 protease (Becher *et al*., 1998,Tautz *et al*., 1997), and thus NS5A-5B precursor proteins can easily be detected in cell extracts (Lamp *et al*., 2011). NS5A binds to the IRES in the same place as NS3 (Zhu *et al*., 2010) .

The **NS5B** protein is of particular significance in the replication process due to its role as the RNA dependent RNA polymerase (RdRp) which synthesizes the new RNA strands and is a central protein in the replication complex (Lohmann *et al*., 1999,Steffens *et al*., 1999,Xiao *et al*., 2002). The CSFV NS5B is the largest (containing 718 aa) of the 12 mature proteins. It has been shown that the protein initiates synthesis of both the positive and negative strands. Essential for synthesis of the negative-strand is the 3´ "CCCGG" sequence of the positive-strand, and similarly essential for the synthesis of new positive-strands is the 3´- CAUAUGCUC sequence of the minus-strand (Xiao *et al*., 2004). The crystal structure of the CSFV NS5B protein has still not been reported, but the data for the NS5B from BVDV crystal structure determined at 2.9 Å resolution (Choi *et al*., 2006), with ca. 70 % amino acid identity to CSFV NS5B, provides a good model. Using the protein prediction online tool (Nielsen *et al*., 2010) and Genious visualization software vR6.1.4 (Biomatters Ltd.), the CSFV NS5B protein structure was predicted (s**ee fig. 11** and manuscript III). All RdRps are similar in overall structure and contain several conserved structural motifs. Essential are three domains denoted as “fingers,” “palm,” and “thumb”. A fourth "N-terminal" domain is a unique feature for pestiviruses and is located over the thumb and interacts with the fingers and thumb domains through β-hairpin motifs, also termed "the fingertips" (Bressanelli *et al*., 1999,Choi *et al*., 2004). The N-terminal domain is highly positively charged, and might be involved in

opening up complex RNA hairpin structures or binding proteins in the replication complex (Choi *et al*., 2004,Shirako *et al*., 2000). Although sequence similarities between the RdRps of diverse positive strand RNA viruses are low, they share at least eight sequence motifs designated I-VIII (Choi *et al*., 2004). Like other RdRps, the pestivirus protein is folded into a closed formation that resembles a closed right hand. The "closed hand" or “o”-shape topology forms a channel and provides the correct structure and metal ions at the active site, including the GDD-motif, which is located in the palm-domain motif VI. This arrangement is crucial for facilitating *de novo* initiation of genome replication (Ferrer-Orta *et al*., 2006), and mutations in the GDD motif are detrimental for RNA replication (Wang *et al*., 2007). Genome replication is achieved by a primer-independent initiation of RNA synthesis. GTP binds close to the catalytic GDD-containing motif inside the RNA template-binding channel. A high concentration of GTP but not ATP, CTP, UTP, GDP, or GMP, was shown to stimulate RNA synthesis by the HCV RdRp (Lohmann *et al*., 1999). The polymerase activity of NS5B is stimulated by NS3 in a dose dependent manner by binding to NS5B

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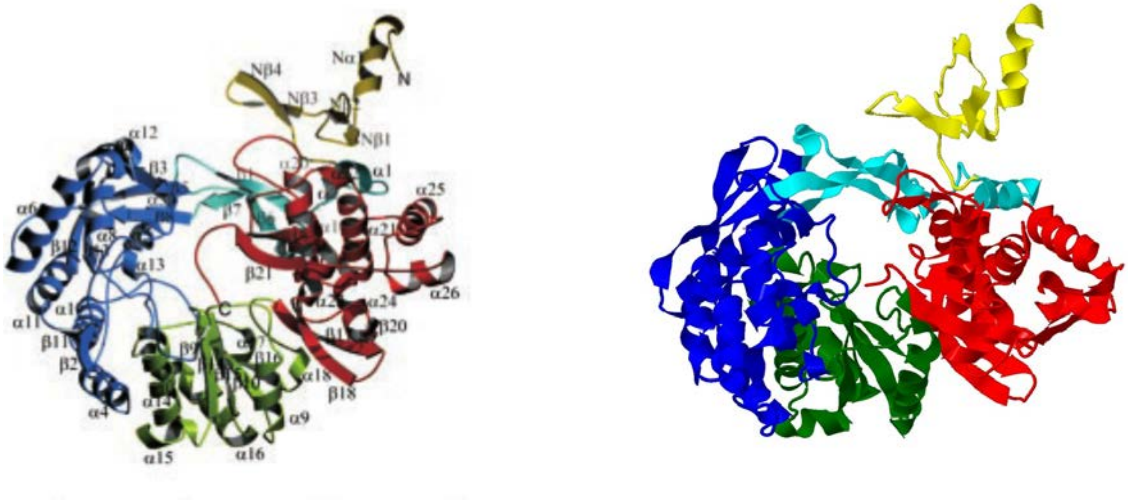

Figure 11. The NS5B protein structure of BVDV is shown in the left panel (from (Choi *et al*., 2004)) . The NS5B protein of CSFV strain Riems (to the right), was predicted using CPHmodels-3.2 server and visualized in Geneious v6.1.4 software tool. Depicted in colours are the N-terminus (yellow), the fingertips (cyan), the fingers (blue), the palm (green) and the thumb (red) domains.

The untranslated regions (UTRs)

Unlike the majority of cellular mRNAs, CSFV RNA lacks a 5´ cap-structure and a 3´ poly(A) tail. However, the 5´ and 3´ termini contain highly structured 5´and 3´ UTRs which are also known as non-translated regions or non-coding regions (NTR/NCR). The 5′ UTR is ca. 373 nt in length while the 3´UTR comprises ca. 228 nt. Within the CSFV 5'-UTR a number of structural folding elements have been identified. The 5´UTR starts with ca. 70 nucleotides which includes two small domains (Ia and Ib). Next, follows two highly structured regions, termed domains II and III, that constitute the internal ribosome entry site (IRES), that directs cap-independent translation of the viral polyprotein (See fig. 9). IRES elements were initially discovered and described for poliovirus and EMCV in 1988 (Jang *et al*., 1988,Pelletier & Sonenberg., 1988) and subsequently described for CSFV (Brown *et al*., 1992). A single type of IRES is found within viruses belonging to the *Flaviviridae* (but only in the viruses which do not replicate in an insect host as well), which is closely related to that found within certain picornaviruses but many members of this family have other types of IRES element (Belsham., 2009).

Key elements important for CSFV translation have been identified by introduction of mutations within domain II and III of the IRES (Fletcher & Jackson., 2002,Friis *et al*., 2012,Rijnbrand *et al*., 1997). The highly conserved 5′-terminal sequence motif 5′-GUAU probably represents a minimal *cis*-acting element essential for synthesis of viral genomic RNA (Becher *et al*., 2000,Frolov *et al*., 1998) and for efficient pestivirus replication, the remainder of the pre-IRES nucleotides (incl. domain Ia and Ib) are also required (Frolov *et al*., 1998).

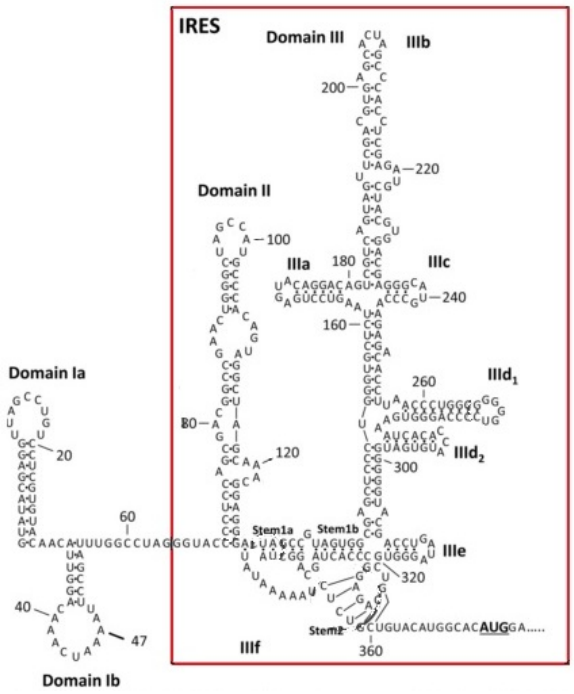

Figure 9. The detailed secondary structure of Paderborn 5´UTR. The highly structured IRES region is highlighted in the red box.
(Modified from Friis *et al*., 2012).

The 3'-UTR contains a variable AU-rich region plus a conserved region (Deng & Brock., 1993) but no poly(A) tail. A 12 nt insertion "CUUUUUUCUUUU" in the 3´UTR of the vaccine strain HCLV has been speculated to be responsible for its avirulence (Xiao *et al*., 2004) and this insertion was further found to be deleterious for binding between the 3´UTR and the NS3 helicase (Sheng *et al*., 2007). The CSFV 3´UTR contains 4 terminal stem–loops (SLI-IV), whereas the BVDV 3'UTR contains only three (SLI-III). The extreme end of the 3′ UTR represents a highly conserved element of which the last ca. 56 nt form a stable stem-loop structure denoted SLI. This outermost stem-loop and part of the single stranded region between SLI and SLII were shown to be essential for RNA replication in both replicons and infectious viruses (Liu *et al*., 2009,Pankraz *et al*., 2005,Yu *et al*., 1999). The remaining 3´UTR region in CSFV has variable sequences and is predicted to form three less-stable stem-loop structures SLII, SLIII and SLIV. Studies with BVDV RNAs indicated that deletion of SLII and SLIII together were found to result in a lethal growth phenotype. However, deletions of either SLII or SLIII individually did not have a significant effect on viral replication (Pankraz *et al*., 2005). For CSFV, analogous studies to those performed by Pankraz *et al* are lacking. A pilot study on deletions in CSFV 3´UTR was carried out during this PhD. SLII+III or only SLIII in the 3´UTR of CSFV strain Paderborn were deleted (see fig.10). These manipulations were made in both the replicon rPadRL2 (Risager *et al*., 2013) and in full length cDNAs. Unfortunately, both deletions abolished the viral replication, since luciferase signals did not increase in a 24 h assay (Risager, unpublished). In a study by Baroth *et al*. an insertion site was

identified in between SL II and SLIII of BVDV, which could tolerate a 300 nt insert without impairing replication (Baroth *et al*., 2010). Inspired by this, the spinach sequence (see elsewhere in the thesis) was inserted in between SLII and SLIII in CSFV Paderborn, but again this led to drastically reduced RNA replication (personal note).

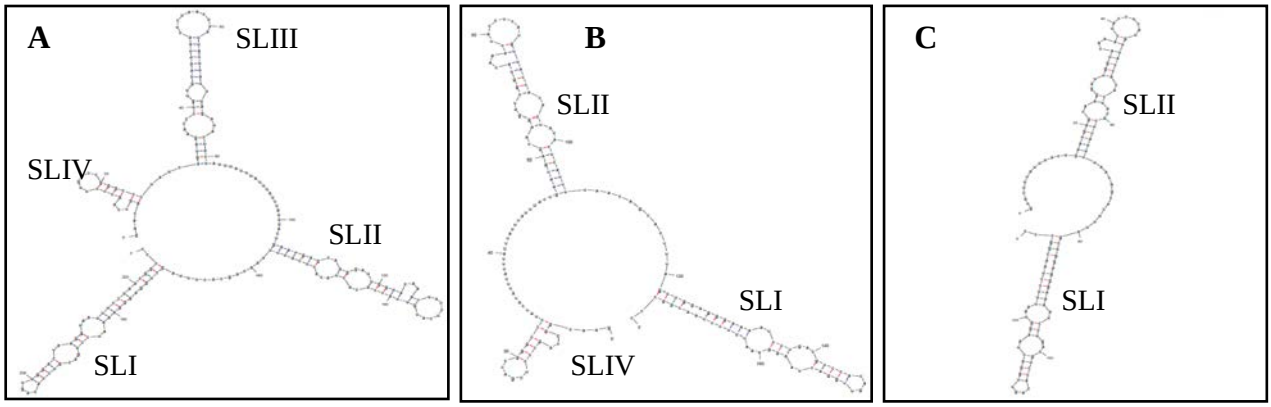

Figure 10. Secondary structure of CSFV Paderborn 3´UTR. A) wt 3´UTR consists of 226 nt arranged into 4 stem loops (SLI-IV) . B) Deletion of SLIII (176 nt). C) Deletion of SLIII+IV, 136 nt. Folded using mfold online software tool (http://mfold.rna.albany.edu/).

PART 3. The manuscripts

Manuscript I

"Efficient generation of recombinant RNA viruses using targeted recombination-mediated mutagenesis of bacterial artificial chromosomes containing full-length cDNA"

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Efficient generation of recombinant RNA viruses using targeted recombination-mediated mutagenesis of bacterial artificial chromosomes containing full-length cDNA

Thomas Bruun Rasmussen[1,*], Peter Christian Risager[1], Ulrik Fahnøe[1], Martin Barfred Friis[1], Graham J Belsham[1], Dirk Höper[2], Ilona Reimann[2] and Martin Beer[2]

[1] DTU National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark

[2] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems, Germany

* To whom correspondence should be addressed. Tel: +45 35887850; Fax: +45 35887901; Email: tbrur@vet.dtu.dk

Abstract

Background: Infectious cDNA clones are a prerequisite for directed genetic manipulation of RNA viruses. Here, a strategy to facilitate manipulation and rescue of classical swine fever viruses (CSFVs) from full-length cDNAs present within bacterial artificial chromosomes (BACs) is described. This strategy allows manipulation of viral cDNA by targeted recombination-mediated mutagenesis within bacteria.

Results: A new CSFV-BAC (pBeloR26) derived from the Riems vaccine strain has been constructed and subsequently modified in the E2 coding sequence, using the targeted recombination strategy to enable rescue of chimeric pestiviruses (vR26_E2gif and vR26_TAV) with potential as new marker vaccine candidates. Sequencing of the BACs revealed a high genetic stability during passages within bacteria. The complete genome sequences of rescued viruses, after extensive passages in mammalian cells showed that modifications in the E2 protein coding sequence were stably maintained. A single amino acid substitution (D3431G) in the RNA dependent RNA polymerase was observed in the rescued viruses vR26_E2gif and vR26, which was reversion to the parental Riems sequence and increased the growth rates of the rescued viruses.

Conclusions: These results show that targeted recombination-mediated mutagenesis provides a powerful tool for expediting the construction of novel RNA genomes and should be applicable to the manipulation of other RNA viruses.

Keywords: RNA, Genome, Targeted recombination, Bacterial artificial chromosome, Genetic stability, RNA virus, Pestivirus, Classical swine fever virus

Background

Bacterial artificial chromosomes (BACs) are ideally suited for the stable maintenance of large DNA sequences derived from viral genomes [1]. A considerable number of BAC systems have been established for large DNA viruses; in particular many different herpesvirus genomes have been cloned into BACs (for review see [2]). The first BAC systems using RNA virus cDNAs were described for coronaviruses [3-6] and recently the first BAC containing a full-length cDNA for a negative-stranded RNA virus was described [7]. Similarly, cDNAs corresponding to the full-length genomes of members of the *Flaviviridae* family (Japanese encephalitis virus [8] and Dengue virus [9]) have been inserted into BACs.

BACs containing full-length cDNAs of pestiviruses (also within the *Flaviviridae*), including bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) have recently been established [10, 11]. Infectious pestiviruses can be rescued using RNA transcripts derived from these BACs. The pestiviruses have single stranded positive sense RNA genomes, about 12.3 kb in length, which includes a single long open reading frame, encoding a large polyprotein, flanked by 5′ and 3′ untranslated regions (UTRs) that are critical for autonomous replication of the genome [12, 13]. The polyprotein is cleaved by cellular and viral proteases into four structural proteins (nucleocapsid protein C, envelope glycoproteins E^{rns}, E1 and E2) and eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The availability of genetically defined and stable pestivirus BACs facilitates the functional study of viral proteins or RNA structures and also the development of new marker vaccine candidates. Several CSFV vaccines with marker properties based on chimeric pestiviruses have been developed over the years [14]. In particular, chimeric pestiviruses with substitution of the entire E2 protein have been described [15-17] but also mutants with more subtle modifications, such as the modification of the important TAV-epitope [18] within the CSFV-E2 protein [19, 20] are promising marker vaccine candidates.

Manipulation of BACs using traditional cloning procedures can be difficult (e.g. because of a lack of convenient restriction enzyme sites) and thus a range of methodologies that apply bacterial genetics, including homologous recombination (e.g. Red/ET homologous recombineering) within the *E. coli* host, have been developed (for review, see [21]). The use of homologous recombination allows site-directed mutagenesis of BACs [22] and, by employing a counter-selection scheme, specific modifications can be obtained without leaving residual “foreign” sequences [23]. The main advantage of this method is that there are no target limitations (e.g. based on size or location) and no need for suitable restriction sites. The integration of the modified sequence is performed *in vivo* (within *E. coli*) thereby potentially being more accurate than *in vitro* approaches like PCR-based methods. Although *in vitro* cloning approaches based on the use of high-fidelity polymerases for PCR amplification have significantly improved in recent years, the use of *in vivo* approaches should allow a more accurate method of mutagenesis due to the use of the cells own high-fidelity replication system which includes proof reading. Whereas BAC recombination has been commonly used for modifying DNA viruses, there are only very few reports about the use of this technology for RNA viruses [7, 24, 25].

Here, a generally applicable strategy for the manipulation and rescue of chimeric pestiviruses from BACs is described as a model, and the flexibility of this approach is demonstrated by generating different modifications in the viral cDNA of the new CSFV-BAC, pBeloR26, derived from the modified live vaccine strain “C-strain Riems”. The targeted recombination-mediated mutagenesis described here includes the substitution of the 9 amino acid (aa) linear TAV-epitope (TAVSPTTLR) present in the E2 protein with the corresponding region (TTVSTSTLA) of a heterologous pestivirus (border disease virus, BDV, strain “Gifhorn”) and also the replacement of the entire CSFV E2 protein coding region with the whole E2 coding region from the same BDV, to generate marked

vaccine viruses that can be discriminated using specific anti-E2 monoclonal antibodies. The genetic stabilities of both the BAC constructs (within *E. coli*) and the rescued viruses have also been assessed.

Methods

Cells and viruses

Porcine kidney (PK15) and sheep fetal thymoid (SFT-R) cells were grown at 37°C (with 5% CO_2) in Dulbecco's minimal essential medium (DMEM) supplemented with 5% pestivirus-free fetal calf serum. Virus from a bait containing the modified live vaccine CSFV "C-strain Riems" was propagated once in PK15 cells and termed vRiemser. RNA obtained from BDV strain "Gifhorn" [26] was used for amplification of the Gifhorn E2-coding sequence.

DNA oligonucleotides

Oligonucleotide primers used are listed in Supplementary Table S1.

BAC constructs

The BAC construct, pBeloR26, was constructed using the long RT-PCR method as previously described [11] using RNA derived from the "C-strain Riems". Briefly, full-length viral cDNAs flanked by NotI sites were amplified by long RT-PCR using primers 5′Cstrain_T7_Not1 (which includes a T7 promotor for *in vitro* transcription, a NotI site and a region corresponding to the first 44 nt of the genome) and 3′CSFV_Not1 (that contains a Not1 site and sequence complementary to the 3'-terminal 35 nt of the genome that are conserved among many CSFVs including the C-strain). The product (ca. 12.3 kbp) was digested with NotI and inserted into similarly digested pBeloBAC11 (New England Biolabs, GenBank accession U51113). All BACs were modified and maintained in *E. coli* DH10B cells (Invitrogen) grown at 37°C in LB medium containing chloramphenicol (Cam, 15 µg/ml). The electroporation of bacteria was performed in 0.1 cm cuvettes using 1 pulse at 1800 V, 25 µF and 200 Ω in a Gene Pulser Xcell (Bio-Rad). BACs to be used as templates for long PCR or for screening by restriction enzyme digestion were purified from 4 ml overnight cultures of *E. coli* DH10B using the ZR BAC DNA Miniprep Kit (Zymo Research). BACs required for direct genome sequencing were purified from 500 ml cultures using the Large-construct kit (Qiagen).

Modification of the CSFV cDNA by Red/ET recombination

Modifications to the full-length CSFV cDNA were accomplished in *E. coli* DH10B (streptomycin resistant, $Strep^R$) using the Counter Selection BAC Modification Kit (Gene Bridges, Heidelberg, Germany). Initially, an intermediate construct was generated, pBeloR26_E2rpsLneo (Figure 1), by insertion of the rpsL-neo cassette (Gene Bridges, 1319 bp) with an extra NotI site for screening purposes which was amplified using primers Criems-TAVfor and Criems-TAVrev (Supplementary Table S1) in place of the TAVSPTTLR coding sequence (27 nt). Secondly, the rpsL-neo cassette in this intermediate construct was then replaced using counter-selection Red/ET recombination using a single-stranded oligonucleotide, Riems_TAV_Gifhorn (Supplementary Table S1) with the same homology arms as used for the rpsL-neo cassette, to introduce the coding sequence for the BDV "Gifhorn" epitope sequence (TTVSTSTLA). The resulting construct was named pBeloR26_TAV (Figure 1). The initial intermediate construct (with rpsL-neo) was then used to produce the pBeloR26_E2gif construct (Figure 1). For this, the E2 coding sequence was amplified from cDNA prepared from BDV "Gifhorn" RNA using two different primer pairs, one set with 50 nt homology arms (Criems_E2_gifFlong/Criems_E2_gifRlong) and another with 30 nt homologous sequences (Criems_E2_gifF/Criems_E2_gifR). The Red/ET recombination involved three steps (*i-iii*). Step *i*) the temperature-

sensitive pRedET expression plasmid (Gene Bridges) was introduced into electroporation-competent *E.coli* DH10B cells containing the parental BAC. The pRedET expresses the phage lambda proteins redα, redβ and redγ, under control of the arabinose-inducible pBAD promoter, allowing homologous recombination to occur. Immediately after electroporation, pre-warmed LB medium without antibiotics (1 ml) was added to the cells which were then incubated at 30°C for 1 hour, prior to spreading onto agar plates containing Cam (15 µg/ml) and tetracycline (Tet) (3 µg/ml) and then incubated at 30°C overnight to maintain the pRedET. The presence of the pRedET plasmid (conferring Tet^R) was verified by visual inspection of BAC-DNA preparations from the Cam^R / Tet^R colonies using agarose gel electrophoresis. Step *ii*) counter-selection marker cassettes (rpsL-neo) were amplified by PCR using primers with 30 nt or 50 nt extensions that were homologous to the target site in the BAC using the rpsL-neo plasmid (Gene Bridges) as template and the Phusion hot start II HF DNA polymerase (Thermo Scientific) with cycling conditions as follows: 98°C for 30s, followed by 35 cycles of 98°C for 10s, 60°C for 20s, 72°C for 60s, 1 cycle for 72°C 4 min. The PCR products (ca. 1400 bp) were isolated on a 1% TBE gel and purified using a GeneJET gel extraction kit (Thermo Scientific). Samples (30 µl), from an *E. coli* culture containing pRedET and the parental BAC grown overnight at 30°C in LB media (Cam, Tet), were used to inoculate 1.4 ml of fresh LB media with the same antibiotics to obtain exponentially growing bacteria at 30°C. Red/ET recombination proteins were induced by adding 50 µl of 10% L-arabinose (Sigma). The PCR product (200 ng) containing the rpsL-neo cassette was introduced into these bacteria using electroporation (as above). Following electroporation, the cells were grown at 37°C for 70 min (to allow recombination) and then selected on plates containing Cam (15 µg/ml), Tet (3 µg/ml) and Kan (15 µg/ml) overnight at 30°C to maintain the pRedET. Note, the rpsL cassette confers Streptomycin sensitivity ($Strep^S$) onto the resistant DH10B strain. The correct phenotype of the resulting colonies was confirmed by streaking the colonies onto plates containing Cam (15 µg/ml), Tet (3 µg/ml) and Kan (15 µg/ml) and grown at 30°C. Importantly, for the third step, the replacement of the rpsL-neo cassette (using counter-selection), the selected colonies were also streaked onto plates containing Cam (15 µg/ml) plus Strep (50 µg/ml) and shown to be $Strep^S$ indicating incorporation of a functional rpsL gene. The structures of the intermediate BACs were verified by restriction enzyme analysis and sequencing around the inserts. Step *iii)* the replacement of the rpsL-neo selection cassettes from the intermediate constructs using linear DNA fragments was achieved through counter-selection and Red/ET recombination. Again, the homologous sequences at the ends of the DNA fragment were used for Red/ET mediated recombination events to replace the rpsL-neo cassette with the sequence of interest. For generation of BACs with substitution of the entire E2 coding sequences, PCR products consisting of the sequence of interest flanked with homology arms identical to the target area were generated by PCR (as for the rpsL-neo cassette). For making constructs with substitution of shorter sequences (e.g. the TAV-epitope), the recombination was achieved using synthetic single stranded oligonucleotides rather than PCR products. Pre-heating of single stranded oligonucleotides at 95°C for 2 min followed by snap-freezing, prior to electroporation, empirically showed the best results. In each case, the DNA molecules were introduced into *E. coli* containing the BAC derivatives including the rpsL-neo cassettes together with the pRedET plasmid by electroporation as described above. Counter-selection against the rpsL-neo cassette (phenotype Cam^R, Tet^R, Kan^R) was employed using media containing Cam (15 µg/ml) and Strep (50 µg/ml) to isolate the required derivatives. The structures of the modified BACs were verified by restriction enzyme analysis and subsequent full-genome sequencing (see below).

Rescue of viruses and virus growth curves

BAC DNA (1 µg) was linearised with NotI or 1 µl BAC DNA was used as template for long PCR amplification using primers 5′C-strain_T7_Not1 and 3′CSFV (Supplementary Table S1). Linearised BACs or PCR products were purified with the GeneJet PCR purification kit (Thermo Scientific) and transcribed *in vitro* using a Megascript T7 kit (Invitrogen). Viruses were rescued from RNA transcripts (1 to 5 µg) by electroporation of porcine (PK15) or ovine

(SFT-R) cells essentially as described previously [24]. Cells were analysed using immunofluorescence microscopy (typically after 3 days) for the expression of NS3 and E2 proteins using specific monoclonal antibodies (mAbs), these were anti-NS3 (WB103/105, pan-pestivirus), anti-CSFV E2 (WH211, WH303, both CSFV specific) and anti-BDV E2 (WB166, BVDV/BDV specific) (AHVLA Scientific, United Kingdom) together with Alexa 488 conjugated goat anti-mouse IgG antibody (Molecular Probes, Invitrogen). The nuclei of cells were visualized using DAPI (Vector Laboratories) and images were recorded using a BX63 fluorescence microscope (Olympus). For peroxidase staining, cells were fixed and stained for the presence of pestivirus antigens using biotinylated pig anti-CSFV/BVDV polyclonal IgG followed by avidin-conjugated horseradish peroxidase (eBioscience) as previously described [27]. The same staining procedure was also performed using the anti-E2 mAbs. Samples containing virus-positive cells were passaged onto new cells. Virus growth curves were generated as previously described [24]. Briefly, PK15 or SFT-R cells were infected at a multiplicity of infection (MOI) of 0.1 pfu/cell and grown for three days. At 3, 12, 24, 48 and 72 hours post infection, cell samples were harvested for virus titration. Cell samples containing virus from each time point were assayed on PK15 or SFT-R cells by limiting dilutions and grown for three days to determine the virus titre (as $TCID_{50}$/ml).

Genome sequencing

BAC DNAs (5 µg), purified using the Large-construct kit (Qiagen), or PCR products (1 µg) amplified from viral cDNA or from BACs using the long PCR method (as above) were consensus sequenced using a 454 FLX (Roche) or an Ion Torrent PGM (Life Technologies). Both Newbler (Roche) and the bwa.bwasw alignment algorithm [28] were used for mapping the reads to the expected sequence. A combination of Samtools [29] and Lo-Freq-snp-caller [30] was used for downstream single nucleotide variant (SNV) analysis. Finally, clone consensus sequences were aligned using MAFFT in the Geneious software platform (Biomatters).

Results

Generation of a BAC containing full-length cDNA corresponding to the modified live vaccine “C-strain Riems”

BACs containing the full-length cDNA corresponding to the parental vRiemser (“C-strain Riems”) have been constructed according to the method described previously for the “Paderborn” strain of CSFV [11]. BACs containing the complete CSFV cDNAs were identified by restriction digest analysis and following linearization by NotI, RNA transcripts were produced and electroporated into PK15 cells. This screening resulted in the identification of a BAC containing a cDNA insert of 12316 nt, pBeloR26 (Figure 1), which yielded infectious virus, termed vR26, that could be propagated in PK15 cells (Figure 2, upper panels). The rescued vR26 displayed similar growth kinetics and virus yield in PK15 cells as the parental vaccine virus (Figure 3). Full-genome sequencing of the cloned BAC template, pBeloR26, revealed a number of differences throughout the genome when compared to the full-length consensus sequence of the cDNA used for the cloning procedure (see Table 1). These differences are non-representative variants within the cDNA. Overall, the BAC sequence differed from the cDNA sequence in 18 positions, 9 of these lead to predicted amino acid substitutions within the polyprotein; one in each of N^{pro}, E^{rns}, E1, E2 and NS3 and four amino acid substitutions in NS5B (Table 1). When compared to the published reference sequence (GenBank accession AY259122.1), the pBeloR26 BAC sequence differed at an additional 11 positions, 1 of these lead to a predicted amino acid substitution and there was one large insertion (27 nt) in the hypervariable region of the 3′-UTR (Supplementary Table S2).

Homologous recombination to obtain CSFV E2 chimeric constructs

To determine the utility of the targeted recombination-mediated mutagenesis system for pestiviruses, two different modifications of the E2 protein coding sequence within pBeloR26 were generated using the Red/ET recombination methodology. Initially, the sequence encoding the linear TAV-epitope (TAVSPTTLR) within the CSFV-E2 was substituted with the sequence encoding the corresponding region (encoding TTVSTSTLA) from the BDV strain "Gifhorn" as described in the Materials and Methods section. More than 90% of the colonies obtained using this procedure contained the required BAC structure. The complete genome sequences of the CSFV cDNA within two selected BACs, designated pBeloR26_TAV have been verified (data not shown). In addition, the complete coding sequence (1119 nt) for the CSFV-E2 protein was substituted by the corresponding sequence from BDV "Gifhorn". Again more than 90% of the colonies obtained contained the required BAC and the same proportion of correctly recombined BACs was obtained using either 30 nt or 50 nt homology arms. The chimeric BAC was designated, pBeloR26_E2gif and the complete virus genome sequence (cDNA) was verified (data not shown).

Rescue of modified virus from recombined BACs

After electroporation with RNA transcripts derived from either pBeloR26_TAV or pBeloR26_E2gif a large number of CSFV NS3-positive cells could be observed (data not shown) and chimeric virus stocks, termed vR26_TAV and vR26_E2gif, were generated after further passages in cells. Cells infected with these viruses and with the parental vR26 and vGifhorn strains were all stained with mAbs directed against the NS3 protein (Figure 2)., However, in contrast to the parental vR26 virus, the chimeric viruses rescued from the recombined BACs were not recognized by anti-E2 mAbs specific for the CSFV-E2 proteins (Figure 2) and thus, consistent with their structure, displayed the same antibody reaction pattern as vGifhorn. Two different anti-CSFV E2 mAbs, WH211 and WH303, were used for the staining and the latter has been shown previously to target the TAV-epitope [18]. As anticipated, cells infected with either the vGifhorn or with the chimeric vR26_E2gif could be shown to express the "Gifhorn" E2 protein using staining with an anti-BDV mAb (Figure 2). The presence of the BDV epitope TTVSTSTLA in vR26_TAV was insufficient to permit efficient recognition by this anti-BDV mab.

Genetic stability of the BACs in the bacterial host

The BAC constructs pBeloR26 and pBeloR26_E2gif were analysed for the genetic stability of the cDNA to determine the suitability of the BAC vector for maintaining full-length pestivirus cDNAs. *E. coli* DH10B cells containing the BACs were passaged 15 times, by overnight growth, and the complete viral cDNAs within the BACs were sequenced after the 1st and the 15th passage. No mutations were observed within the 12316 nt virus cDNA sequences after this extensive propagation of the BACs in the bacterial host, indicating a highly stable system for the maintenance of complete pestivirus cDNA sequences.

Genetic stability of viruses rescued from the BACs

The viruses, vR26 and vR26_E2gif, rescued from their respective BAC constructs, were also tested for their genetic stability within mammalian cells. Linearized BAC DNA was transcribed *in vitro* and the RNA was electroporated into PK15 cells. Three days after electroporation the cells were stained with the anti-NS3 antibody to detect the presence of replicating virus. Samples containing virus positive cells were passaged onto new cells, this process was repeated for 12 separate passages (each of three days). The virus titre (as $TCID_{50}$/ml) was determined for each passage. Passage of the rescued vR26_E2gif chimeric virus in PK15 cells resulted in rapidly decreasing virus titers and was discontinued after the 2nd passage (Figure 4A). Instead, further passage of this chimeric virus was performed in ovine SFT-R cells (the preferred cell type for BDV) and resulted in much higher titers of the chimeric virus. Virus titers reached more than 10^6 $TCID_{50}$/ml after the 1st passage and remained stable for 12 passages (Figure 4A). The

rescued vR26 was also efficiently propagated on the SFT-R cells but maintained a slightly lower titer than the vR26_E2gif chimeric virus (Figure 4A). To check that the viruses retained their antibody reaction properties (Figure 2) after these passages, cells were infected with viruses from the 12th SFT-R cell culture passage (termed vR26/P-12 and vR26_E2gif/P-12) and stained with a polyclonal anti-pestivirus serum and with specific mAbs directed against the CSFV-E2 and BDV-E2 proteins (Figure 4B). Cells infected with either the vR26/P-12 or the chimeric vR26_E2gif/P-12 were each detected by the polyclonal anti-pestivirus serum as expected. The anti-CSFV-E2 mAb specifically detected cells infected with vR26/P-12 but not cells infected by the chimeric virus containing the BDV-E2 protein (consistent with the results shown in Figure 2). In contrast, the anti-BDV-E2 mAb specifically detected infection by the vR26_E2gif/P-12 and did not recognize cells infected with vR26/P-12. Each result is in accord with the structure of the viruses.

The 4th passage of vR26 (vR26/P-4) displayed a slower growth rate than the virus obtained after 12 passages (see figure 5A). It also had a reduced growth rate compared to both the vR26_E2gif/P-4 and vR26_E2gif/P-12. The full-length sequence of pBeloR26 had revealed ten non-silent mutations compared to the reference sequence (AY259122.1) for this virus (Supplementary Table S2). Any of these mutations could be responsible for the impaired growth acting alone or in concert. For further investigation of this issue, full length cDNAs prepared from vR26 (P-4 and P-12) and vR26_E2gif (P-4 and P-12) were deep-sequenced using both the 454 FLX and Ion torrent PGM platforms for comparison and to determine the quasi-species distribution (Supplementary Figure S1 and S2). Sequencing data from both platforms revealed that both the vR26/P-12 and vR26_E2gif/P-12 were close to 100% changed at nt position A10665G compared to the BAC clones (resulting in the predicted amino acid substitution D3431G within the NS5B protein, the RNA-dependent RNA polymerase, see figure 5B). This adaptation is a reversion back to the consensus cDNA sequence of the parental vaccine virus, vRiemser (Table 2). Additionally, vR26/P-4 and vR26_E2gif/P-4 already showed evidence for this reversion being present within the population. For vR26/P-4, the level of reversion was 50%, while for vR26_E2gif/P-4 the extent of change was close to 80% (see figure 5B).

Discussion

In this study, we have established the first BAC containing the full-length cDNA of a CSFV vaccine strain. To demonstrate the utility of the Red/ET mediated recombination method we have generated a series of modified BACs derived from this CSFV full-length cDNA. These include BACs with substitution of the linear TAV-epitope present in the E2 protein and also BACs with substitution of the complete E2 protein with heterologous pestivirus sequences. We have also used the same approach for a range of different targeted modifications within CSFV BACs including specific deletions and substitutions in the 5'UTR of CSFV [24] and for insertions of heterologous reporter sequences into CSFV replicons [25]. Using Red/ET recombination-mediated mutagenesis for the targeted design, the work can be expedited and focused, in principal, on any sequence within the viral genome and is not dependent on the use of internal restriction sites. The results demonstrate that Red/ET recombination-mediated mutagenesis of pestivirus BAC cDNAs provides a useful tool for advancing the construction of modified pestiviruses.

Cells infected with the parental vR26 virus were recognized by the two anti-E2 mAbs (WH211 and WH303) specific for the CSFV-E2 proteins, in contrast cells infected with the modified viruses vR26_TAV and vR26_E2gif, rescued from the recombined BACs, were not detected by these mAbs. Furthermore, as expected, cells infected with the vR26_E2gif were recognized by the anti-BDV mAb (WB166) whereas no staining was observed with this antibody in vR26 infected cells or in cells with vR26_TAV. The mAb WH303 recognizes the CSFV TAV-epitope [18] and the difference in 4 aa between the TAV-epitope and the corresponding sequence from BDV strain “Gifhorn” is enough to

completely abolish the recognition by this mAb. The lack of staining of vR26_TAV infected cells by the WH211 indicated that the TAV-sequence is also important for the epitope recognized by this mAb. Thus, the chimeric pestiviruses, vR26_TAV and vR26_E2gif, containing heterologous E2 sequences can be readily discriminated from the vR26 using specific anti-E2 monoclonal antibodies. These new chimeric pestiviruses represents C-strain based marked vaccine candidates with the characteristics desired for safe and efficacious DIVA vaccines against CSFV. Indeed, Riems26_E2gif vaccinated pigs could be efficiently discriminated from C-strain vaccinated pigs and from CSFV infected pigs using CSFV-E2 specific antibody ELISAs (Rasmussen et al., unpublished results).

Genetic stability in the bacterial host

Nucleotide sequence data for the pBeloR26 showed a number of changes from the published reference sequence for "C- strain Riems". Some of these differences are present in the cDNA derived from the vaccine stock at a detectable level whereas others may represent low-level variants within the cDNA or errors introduced by the RT-PCR amplification. Full-length sequencing revealed that no changes occurred in the cDNA during extensive propagation in *E. coli* DH10B of the pBeloR26 and the E2-chimeric derivative, pBeloR26_E2gif, indicating a very high stability of these BAC-cloned CSFV cDNAs. This is essential if this system is to be useful for cloning and sequence manipulation, and contrasts with stability problems encountered with conventional plasmids containing full-length pestivirus cDNAs [31]. The stability of these BACs is consistent with previous reports on the stability of BACs containing other viruses of the family *Flaviviridae* in *E. coli* [8, 10].

Genetic stability of rescued viruses

Extensive passaging of the rescued vR26 and the chimeric virus derivative, vR26_E2gif, resulted in a change at nucleotide position A10665G (resulting in the predicted aa change D3431G) within the NS5B coding region. The same reversion to the consensus sequence for the "C-strain Riems" virus was observed in two independent virus populations, derived from independent BACs, and indicates that this substitution is important for efficient growth of the "C-strain Riems" virus in SFT-R cells. In contrast, all other nucleotide positions remained unchanged during virus propagation including the heterologous E2 sequences. The glycine (G) residue at position 3431 in the polyprotein is highly conserved amongst CSFV strains; indeed, alignment of CSFV sequences retrieved from GenBank did not reveal any variation at that position (data not shown). Thus, the difference in growth kinetics (Figure 5A) seemed to be determined by this single aa change, which probably leads to an impaired RNA-dependent RNA polymerase and a lower replication rate in early cell passage. However, the reversion in 50% of the vR26/P-4 genomes may be expected to give at maximum a 2-fold difference in growth rate compared to vR26/P-12, but a 10-fold lower production of virus was actually observed (Fig 5A) indicating that the modified NS5B may act as a dominant negative mutant. Whether this mutation was derived from the cloning procedure or reflects a low-level variant present in the parental vaccine stock is unclear.

Conclusions

In summary, the present study shows that targeted recombination-mediated mutagenesis of BACs containing full-length CSFV cDNA facilitate manipulation and rescue of chimeric pestiviruses. The system shows high genetic stability of the BAC constructs (within *E. coli*) and the chimeric viruses rescued from the BACs can be efficiently and stably propagated in SFT-R cells and this represents a suitable system for the production of virus stocks for future marked vaccine experiments. The strategies employed in this study have applicability not only for CSFV (and other pestiviruses) but should be adaptable to the study of other RNA viruses.

Supporting data

The data sets supporting the results of this article are included within the article and its additional file (including Supplementary Tables S1 and S2 and Supplementary Figures S1 and S2).

Competing interests

The authors declare they have no competing interests.

Authors' contributions

TBR, IR and MB conceived the study and developed the approach. TBR, PCR, MBF carried out and optimised the experiments. TBR, UF and DH carried out sequence analyses. All authors contributed to the interpretation of results. Funding was obtained by TBR and GJB. All authors contributed to the drafting and revision of the manuscript. All authors read and approved the final manuscript.

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Figures

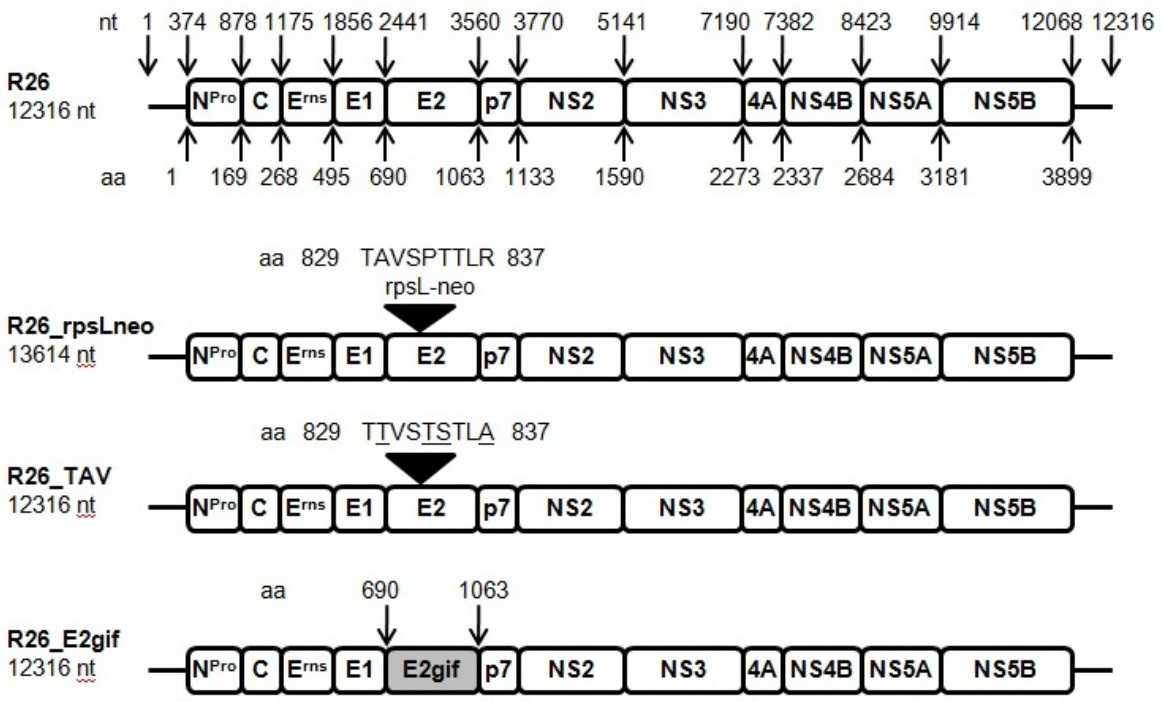


Figure 1. Schematic representation of the CSFV genome organization and the BACs constructed and used in this study. Nucleotide (nt) and amino acid (aa) positions within R26 for the 5' and 3' termini together with the translational start and stop codons of the polyprotein coding region plus cleavage sites used to make the individual proteins (Npro, C, Erns, E1 and E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) are indicated. Insertion of the rpsL-neo in place of the TAV-epitope within CSFV E2 for the intermediate construct (R26_rpsLneo) and the subsequent replacement with the TTVSTSTLA sequence (R26_TAV) and the complete substitution of the E2 sequence (R26_E2gif) are shown. Names of BAC constructs begin with “pBelo” and rescued viruses with “v” (e.g. pBeloR26 and vR26). Cell culture passage no. of virus is indicated with “/P” (e.g. vR26/P-4).

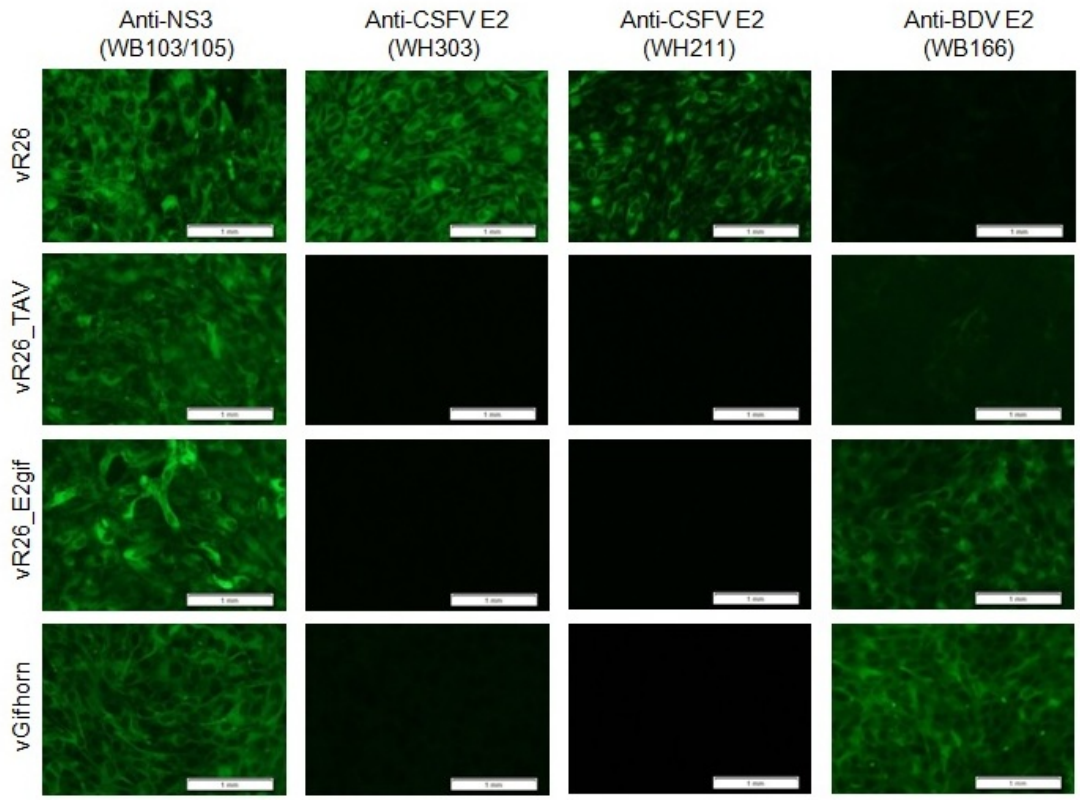


Figure 2. Antibody reaction patterns of pestivirus infected cells. SFT-R cells were infected with vR26 and its two derivatives vR26_E2gif and vR26_E2TAV plus vGifhorn [26]. After 72 h, the cells were fixed and stained with monoclonal antibodies against the NS3 protein (WB103/105, left column), the CSFV E2 protein (WH303 and WH211, middle columns) and the BDV E2 protein (WB166, right collumn) as indicated and viewed using a fluorescence microscope.

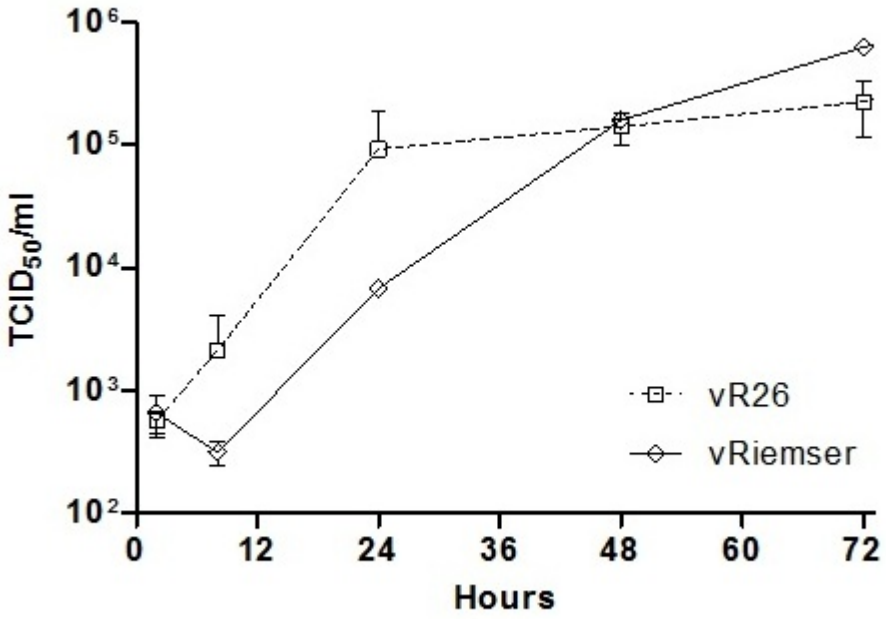


Figure 3. Growth characteristics of vR26 compared to the parental vRiemser. The growth of the rescued vR26 and the parental vRiemser strains was evaluated in SFT-R cells using an MOI of 0.1 pfu/cell. Virus titers were determined from harvests prepared at 3, 24, 48, and 72 h post infection. Data are represented as mean +SD (n = 3).

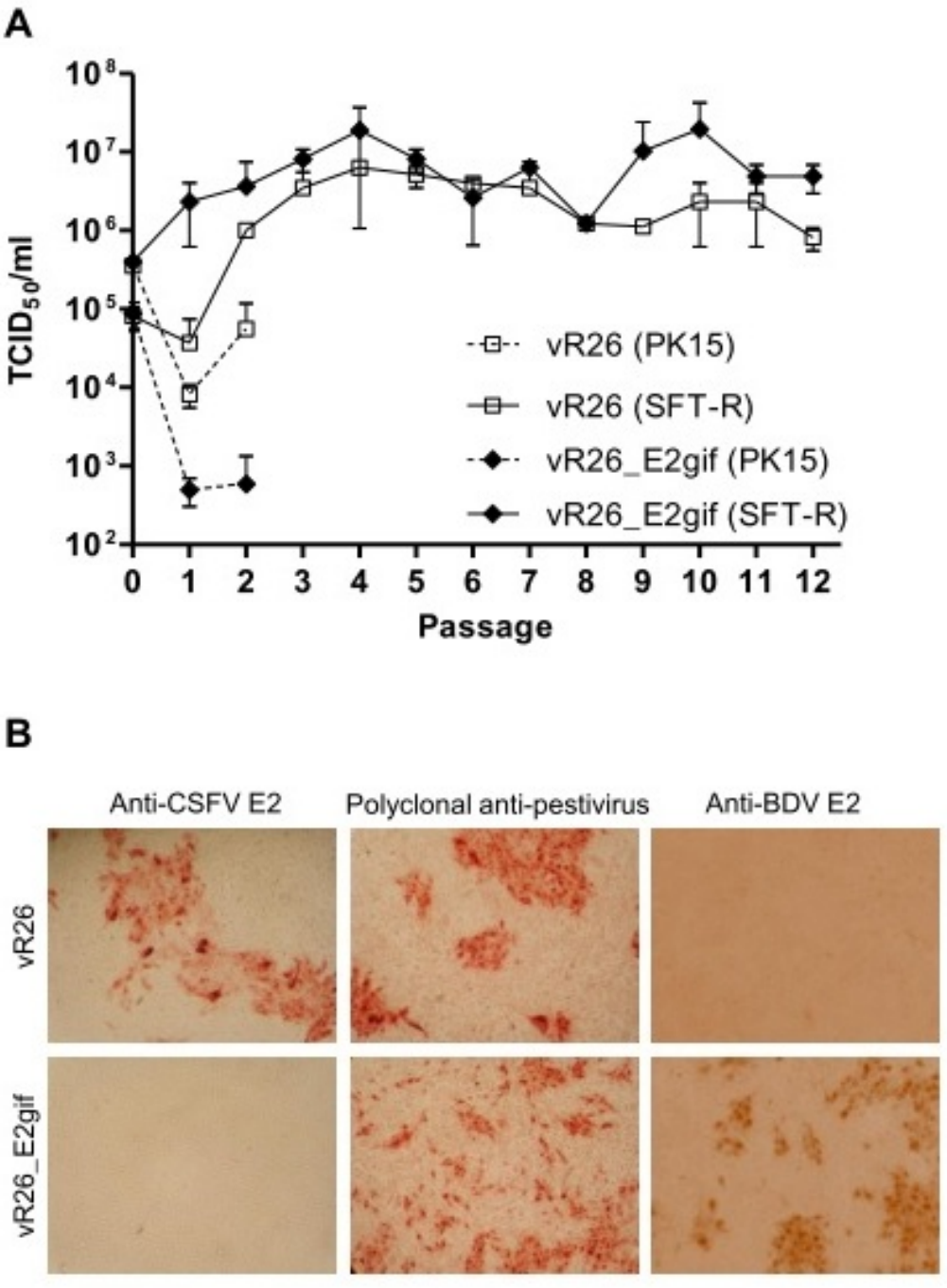


Figure 4. Characteristics of the vR26 and the chimeric vR26_E2gif in cells. (**A**).Virus yield assays were performed during passage (from passage 1 to 12) of vR26 and vR26_E2gif in PK15 and SFT-R cells as indicated. For each passage, cells were harvested after 3 days and the virus titres determined. Data are presented as mean +SD (n = 2). (**B**) Cells (SFT-R) infected with the vR26 (P-12) or with the vR26_E2gif (P-12) viruses were stained using a polyclonal anti-pestivirus serum (which recognizes both BVDV and CSFV proteins) and with specific mAbs directed against the CSFV E2 (WH211) and BDV E2 (WB166) proteins as indicated.

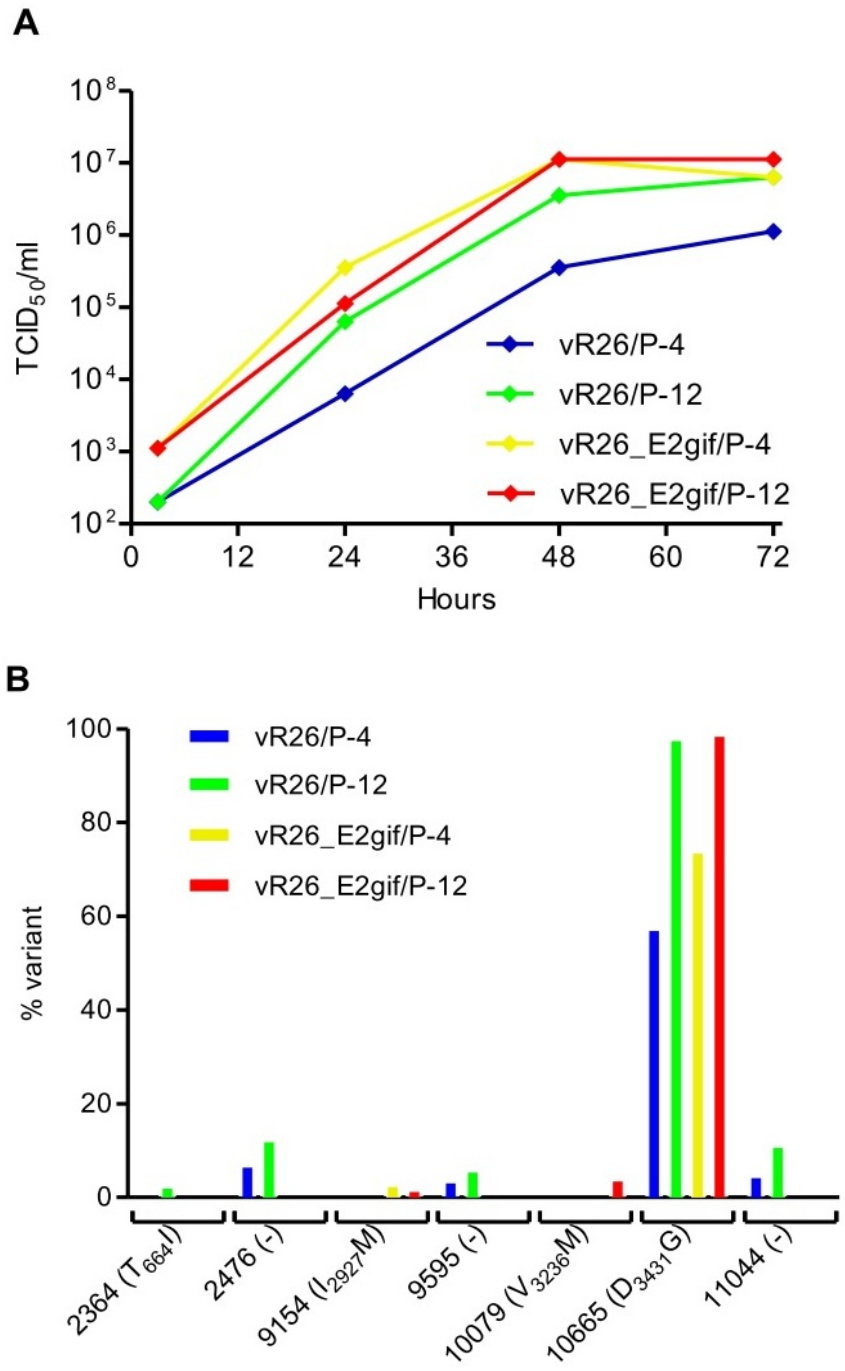


Figure 5. (**A**) Time courses of virus yields after infection of SFT-R cells with vR26 (P-4 and P-12) and vR26_E2gif (P-4 and P-12). Cells were infected with the viruses (MOI of 0.1 plaque-forming units per cell) and harvested after 3, 24, 48 and 72 h, as indicated, and the virus titres determined. (**B**) Single nucleotide variant (SNV) data obtained from cDNA generated from the rescued viruses vR26 (P-4 and P-12) and vR26_E2Gif (P-4 and P-12). The bar diagram depicts the variation distribution (as % variant) in the data set from the 454 FLX by Lofreq SNV-caller above 2% in frequency. SNVs (nt position in the cDNA) leading to silent mutations (-) or non-silent mutations (e.g. D3431G) are illustrated.

Tables

Table 1 Nucleotide and amino acid differences between the consensus cDNA sequences of the parental vaccine virus (vRiemser), the cloned BAC cDNA (pBeloR26) and cDNA of the rescued vR26/P-12.

Region	nt position	vRiemser (cDNA)	pBeloR26 (BAC cDNA)	vR26/P-12 (cDNA)	aa change (in pBeloR26)
N^{pro}	695	G	A	A	E108K
E^{rns}	1427	C	T	T	H352Y
E1	2364	T	C	C	I664T
E2	3068	G	A	A	D899N
NS2	4441	A	G	G	-
NS3	5500	A	G	G	-
	5530	A	G	G	-
	6043	T	C	C	-
	6201	T	C	C	V1943A
NS5A	9589	T	C	C	-
NS5B	10079	A	G	G	M3236V
	10134	A	G	G	K3254R
	10272	A	G	G	K3300R
	10665	G	A	G*	G3431D
3´ UTR	12128	T	A	A	
	12136	T	C	C	
	12137	C	T	T	
	12152	T	A	A	

***** Nt position 10665 in vR26/P-12 is reverted from A to G as in the parental cDNA. Positions leading to aa changes are indicated by shading.

Supplementary tables and figures

Supplementary Table S1. Oligonucleotide primers used in this study.

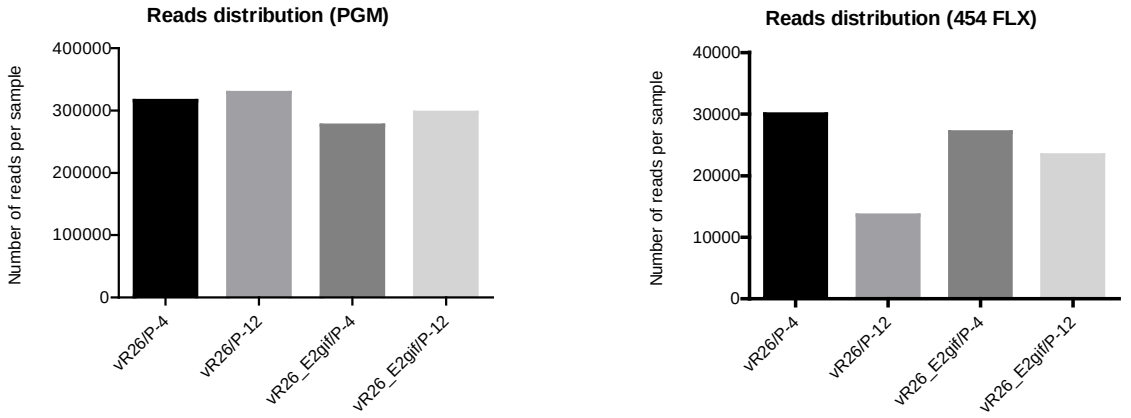
Primer:	Sequence (5'-3'):
5'Cstrain_T7_Not1	ATAT*GCGGCCGC*TAATACGACTCACTATAGTATACGAGGTTAGTTCATT CTCGTATACACGATTGGACAAATC
3'CSFV_Not1	TATA*GCGGCCGC*GGGCCGTTAGGAAATTACCTTAGTCCAACTGTGGA
3'CSFV	GGGCCGTTAGGAAATTACCTTAGTCCAACTGTGGA
Riems_TAV_Gifhorn	GTG CTT TCT ATC TTG TCT GCC CAA TAG GGT GGA CGG GTG TCA TAG AGT GC**A CCA CTG TTA GCA CTA GCA CCC TGG CC**A CAG AAG TGG TAA AGA CCT TCA GGA GAG ACA AGC CCT TTC CGC ACA GAA T
Criems_E2_gifF	ATG GCT GTT ACT AGT AAC TGG GGC ACA AGG CCA ATT TGC CTG CAT CGA GAA TTA CA
Criems_E2_gifFlong	AGA TCG TGC AAG GTG TGG TAT GGC TGT TAC TAG TAA CTG GGG CAC AAG GCC AAT TTG CCT GCA TCG AGA ATT ACA
Criems_E2_gifR	TCA ACA CTA CCT CGC CCT GGC CCA ATG GTA ATG CTG ATG CCA TTT GCT CTG TCA GTA C
Criems_E2_gifRlong	GGT GAT TAA GTT CCC TAT CAA CAC TAC CTC GCC CTG GCC CAA TGG TAA TGC TGA TGC CAT TTG CTC TGT CAG TAC
Criems-TAVfor	GTG CTT TCT ATC TTG TCT GCC CAA TAG GGT GGA CGG GTG TCA TAG AGT *GCG GCC GC***G GCC TGG TGA TGA TGG CGG GAT CG**
Criems-TAVrev	ATT CTG TGC GGA AAG GGC TTG TCT CTC CTG AAG GTC TTT ACC ACT TCT GT**T CAG AAG AAC TCG TCA AGA AGG CG**

NotI sites are in italics and the T7 promoter is underlined. Nucleotide sequence encoding (TTVSTSTLA) is bold and underlined, whereas the rpsL-neo sequences are displayed in bold.

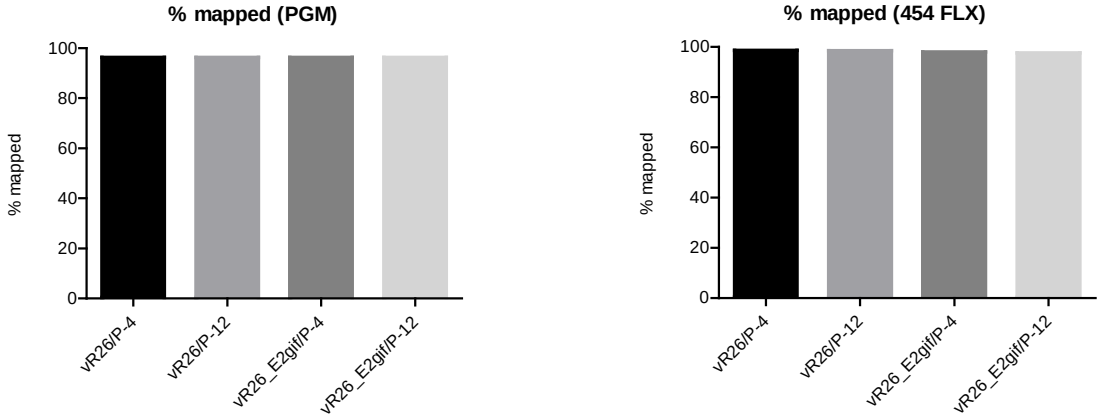
Supplementary Table S2 Nucleotide and amino acid differences between the published C-strain "Riems", the consensus cDNA sequence of the parental vaccine virus (vRiemser) and the cloned BAC cDNA (pBeloR26).

Region	nt position	C-strain "Riems" (AY259122.1)	vRiemser (cDNA)	pBeloR26 (BAC cDNA)	aa change (from AY259122.1)
5'UTR	137	G	A	A	
N^{pro}	695	G	G	A	E108K
E^{rns}	1427	C	C	T	H352Y
E1	2364	T	T	C	I664T
E2	3068	G	G	A	D899N
NS2	4111	C	T (75%)	T	-
	4441	A	A	G	-
NS3	5500	A	A	G	-
	5530	A	A	G	-
	6043	T	T	C	-
	6070	C	T (83%)	T	-
	6201	T	T	C	V1943A
	6478	T	C	C	-
NS4B	8305	A	G (76%)	G	-
NS5A	9328	C	T (80%)	T	-
	9475	T	C (79%)	C	-
	9589	T	T (C 8%)	C	-
NS5B	10079	A	A	G	M3236V
	10134	A	A	G	K3254R
	10272	A	A	G	K3300R
	10665	G	G	A	G3431D
	11696	G	A	A	V3775I
3'UTR	12128	T	T	A	
	12136	T	T	C	
	12137	C	C	T	
	12148	-	TTTTATTTATT TAGATATTATT ATTTA	TTTTATTTATT TAGATATTATT ATTTA	
	12152	T	T	A	
	12173	T	C	C	
	12185	C	T	T	

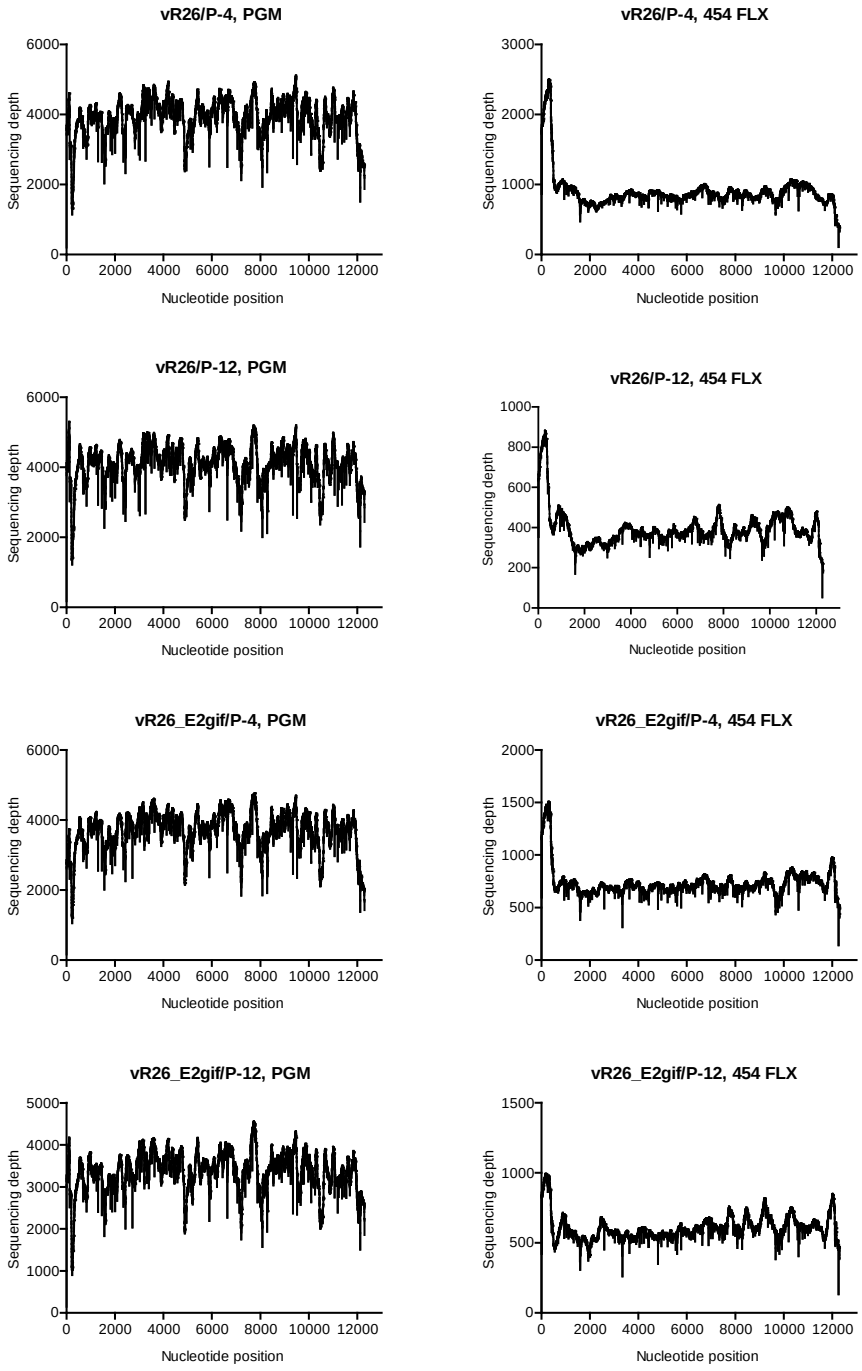
A



B



Supplementary Figure S1. Comparison of vR26/P-4, vR26/P-12, vR26E2gif/P-4 and vR26E2gif/P-12 run on the Iontorrent PGM and the 454 FLX sequencing platforms. (**A**)The sequence read distribution per sample shown as number of reads for both platforms. (**B**) The percentage of reads mapped to the pBeloR26 reference sequence by the bwa.bwasw alignment algorithm for all four samples on both platforms.



Supplementary Figure S2. Sequence depth per nucleotide position in the genome for vR26/P-4, vR26/P-12, vR26E2gif/P-4 and vR26E2gif/P-12 run on the Iontorrent PGM and the 454 FLX sequencing platforms. The horizontally aligned graphs compare the sequencing depth between the sequencing platforms for each sample analyzed by BEDTools (32). The x-axis depicts the nucleotide position in the viral genome and the y-axis shows the sequencing depth.

Manuscript II

"Analysis of classical swine fever virus RNA replication determinants using replicons "

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Analysis of classical swine fever virus RNA replication determinants using replicons

Peter Christian Risager, Ulrik Fahnøe, Maria Gullberg, Thomas Bruun Rasmussen and Graham J. Belsham

National Veterinary Institute, Technical University of Denmark, Lindholm, Kalvehave 4771, Denmark

Correspondence
Graham J. Belsham
grbe@vet.dtu.dk

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Self-replicating RNAs (replicons), with or without reporter gene sequences, derived from the genome of the Paderborn strain of classical swine fever virus (CSFV) have been produced. The full-length viral cDNA, propagated within a bacterial artificial chromosome, was modified by targeted recombination within *Escherichia coli*. RNA transcripts were produced *in vitro* and introduced into cells by electroporation. The translation and replication of the replicon RNAs could be followed by the accumulation of luciferase (from *Renilla reniformis* or *Gaussia princeps*) protein expression (where appropriate), as well as by detection of CSFV NS3 protein production within the cells. Inclusion of the viral E2 coding region within the replicon was advantageous for replication efficiency. Production of chimeric RNAs, substituting the NS2 and NS3 coding regions (as a unit) from the Paderborn strain with the equivalent sequences from the highly virulent Koslov strain or the vaccine strain Riems, blocked replication. However, replacing the Paderborn NS5B coding sequence with the RNA polymerase coding sequence from the Koslov strain greatly enhanced expression of the reporter protein from the replicon. In contrast, replacement with the Riems NS5B sequence significantly impaired replication efficiency. Thus, these replicons provide a system for determining specific regions of the CSFV genome required for genome replication without the constraints of maintaining infectivity.

INTRODUCTION

Classical swine fever virus (CSFV) causes a severe, highly contagious disease in swine (family Suidae). Infection can lead to a fatal outcome and outbreaks of disease result in important socioeconomic losses worldwide (Terpstra & de Smit, 2000). CSFV is a member of the genus *Pestivirus*, as are bovine viral diarrhea virus (BVDV) and border disease virus. The pestiviruses are related to members of the genera *Flavivirus* (e.g. yellow fever virus) and *Hepacivirus* (e.g. hepatitis C virus, HCV) and together form the family *Flaviviridae* (Lindenbach *et al.*, 2007). The CSFV genome is a positive-sense, ssRNA molecule of approximately 12 300 nt. The genome includes a single, large ORF, which encodes a polyprotein of approximately 3900 aa, that is flanked by UTRs. The genome is uncapped at its 5′ terminus and the 5′ UTR contains an internal ribosomal entry site (IRES), which directs cap-independent translation initiation on the viral RNA (Fletcher & Jackson, 2002; Friis *et al.*, 2012). The 3′ UTR is unusual, compared with most eukaryotic mRNAs, in that it lacks a poly(A) tail but is predicted to contain a variety of structural elements that are involved in initiating the synthesis of negative-sense strands (Deng & Brock, 1993; Frolov *et al.*, 1998; Pankraz *et al.*, 2005; Yu *et al.*, 1999). The viral RNA genome is infectious, and thus introduction of the RNA into cells is sufficient to initiate the infection cycle; this involves translation of the RNA to produce the viral proteins and then subsequent replication of the genome mediated by some of these proteins.

The viral polyprotein is co- and post-translationally cleaved, by cellular and virus-encoded proteases, to produce four structural proteins (the core protein, C, and three envelope proteins, E^{rns}, E1 and E2) plus eight non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Meyers & Thiel, 1996; Rümenapf *et al.*, 1993; Weiland *et al.*, 1990).

Replicons are replication-competent RNA molecules that are incapable of generating infectious progeny virus due to loss of one or more structural proteins (e.g. E1 or E2). Early work on subgenomic pestivirus replicons was performed using transcripts based on BVDV (Behrens *et al.*, 1998; Reimann *et al.*, 2003; Tautz *et al.*, 1999; Yu *et al.*, 1999) and CSFV (Mittelholzer *et al.*, 1997; Moser *et al.*, 1999) genomes. In these studies, it was shown that the CSFV and BVDV structural proteins C, E^{rns}, E1 and E2 and also the non-structural proteins N^{pro}, p7 and NS2 are non-essential for genome replication. Thus, the genome sequences encoding NS3–NS5B together with the 5′ and 3′ UTRs are the minimal elements required for autonomous pestivirus RNA

One supplementary table is available with the online version of this paper.

replication. The NS3 and NS5B proteins have key roles in replication, including processing of the polyprotein and as the RNA-dependent RNA polymerase (RdRp), which synthesizes new viral RNA, respectively.

The presence of a reporter protein coding sequence within a replicon facilitates quantitative assessment of the replication competence of a particular RNA species. Reporter proteins such as *Renilla reniformis* luciferase (Rluc) and firefly luciferase have been widely used for the quantitative assessment of protein expression (reviewed by Greer & Szalay, 2002). Recently, another luciferase protein from *Gaussia princeps* (Gluc) has been described (Tannous *et al.*, 2005); this protein is secreted from cells and can be assayed using the cell supernatant without cell lysis. The Gluc protein, in tandem with a downstream foot-and-mouth disease virus (FMDV) 2A peptide, has been shown to yield a functional luciferase from an HCV-derived replicon (Jones *et al.*, 2007).

Swapping corresponding protein coding sequences between related viral strains, thereby forming chimeric RNAs, provides a strategy to identify important interactions between different components of the replication machinery. Indeed, previous studies using HCV replicons showed that exchanging the RdRp from strains with different replication efficiencies could greatly enhance or impair replication (Binder *et al.*, 2007).

The Paderborn strain of CSFV has been characterized as being moderately virulent (Uttenthal *et al.*, 2003); the virus was isolated from the devastating outbreak of classical swine fever (CSF) in the Netherlands in 1997 (Oleksiewicz *et al.*, 2003; Widjojoatmodjo *et al.*, 1999). A full-length cDNA amplicon, derived from the Paderborn virus, was introduced into a stable, single-copy, bacterial artificial chromosome (BAC). Following *in vitro* transcription and introduction of the RNA into cells, recovery of infectious CSF viruses from this cDNA was achieved (Rasmussen *et al.*, 2010).

Using this CSFV cDNA as a backbone, multiple replicons either with or without reporter protein coding sequences have now been produced. In addition, chimeric replicons have been made that contain coding sequences, for selected viral proteins, exchanged between CSFV strains of distinct virulence and the effect on the replication efficiency has been determined.

RESULTS

Design of CSFV replicons

The pBeloPader10 BAC, including the complete cDNA of the Paderborn strain of CSFV, can be used to make RNA transcripts that are able to replicate and produce infectious virus within cells (Rasmussen *et al.*, 2010). Modifications to this CSFV cDNA have been achieved using targeted recombination, which avoids the need for convenient restriction enzyme sites or subcloning and permits the modification of any region of the cDNA sequence. Two different types of in-frame deletion have been made within the Paderborn cDNA. In rPad1, nt 896–3541 of the CSFV cDNA were deleted and this removed most, or all, of the coding sequence for the C, E^{rns}, E1 and E2 proteins. A second, smaller deletion (in rPad2) removed nt 974–2329, which resulted in the loss of part, or all, of the C, E^{rns} and E1 coding sequences but left the E2 coding region intact (Fig. 1). In addition, using the same targeted recombination approach, the Rluc–2A coding sequence (a fusion of the Rluc with the FMDV 2A peptide coding sequences) has been inserted at the site of these deletions to produce the reporter replicons rPad1RL and rPad2RL, respectively (Fig. 1). In each case, an intact ORF has been maintained so that the non-structural proteins, which are required for RNA replication, can be produced. The FMDV 2A sequence (only 18 aa in length) generates a co-translational cleavage at its own C terminus at an NPG/P junction (Donnelly *et al.*, 2001). Thus, the protein sequences, downstream of the 2A sequence, are freed from the reporter protein and should be functionally unmodified [a slight caveat to this is that the N terminus of the downstream protein will be a proline (P) residue]. The complete sequences of the BACs containing these four different replicon cDNAs have been verified (data not shown).

Functional analysis of replicons

To assess the replication competence of these four replicons, RNA transcripts derived from the parental BAC (pBeloPader10) and from the replicon derivatives were produced, *in vitro*, and introduced into sheep fetal thymus (SFT-R) cells by electroporation. After 24 h, cells were stained for the presence of the CSFV E2 and NS3 proteins using immunofluorescence assays (Fig. 2). No expression of these proteins was detected in mock-treated cells. However, the transcripts derived from the parental pBeloPader10 expressed both the E2 and the NS3 proteins in the cytoplasm of the cells. Furthermore, if these cells were harvested and the material passaged onto fresh cells, then these cells also expressed the E2 and NS3 proteins (data not shown) due to the production of infectious virus (Friis *et al.*, 2012; Rasmussen *et al.*, 2010). Each of these four replicons expressed the NS3 protein in the cytoplasm of cells (Fig. 2) indicating that they were functional. In addition, both rPad2 and its derivative rPad2RL produced the E2 protein (Fig. 2), as these contain the E2 coding sequence (Fig. 1). However, in contrast, no signal indicative of E2 expression was detected from the rPad1 and rPad1RL transcripts as these both lack the E2 coding sequences. No spread of the NS3 or E2 protein expression to adjacent cells was observed (data not shown), as no infectious virus progeny are formed.

Expression of reporter proteins by replicons

The replicons rPad1RL and rPad2RL contain the Rluc–2A coding sequence. It was necessary to determine that the

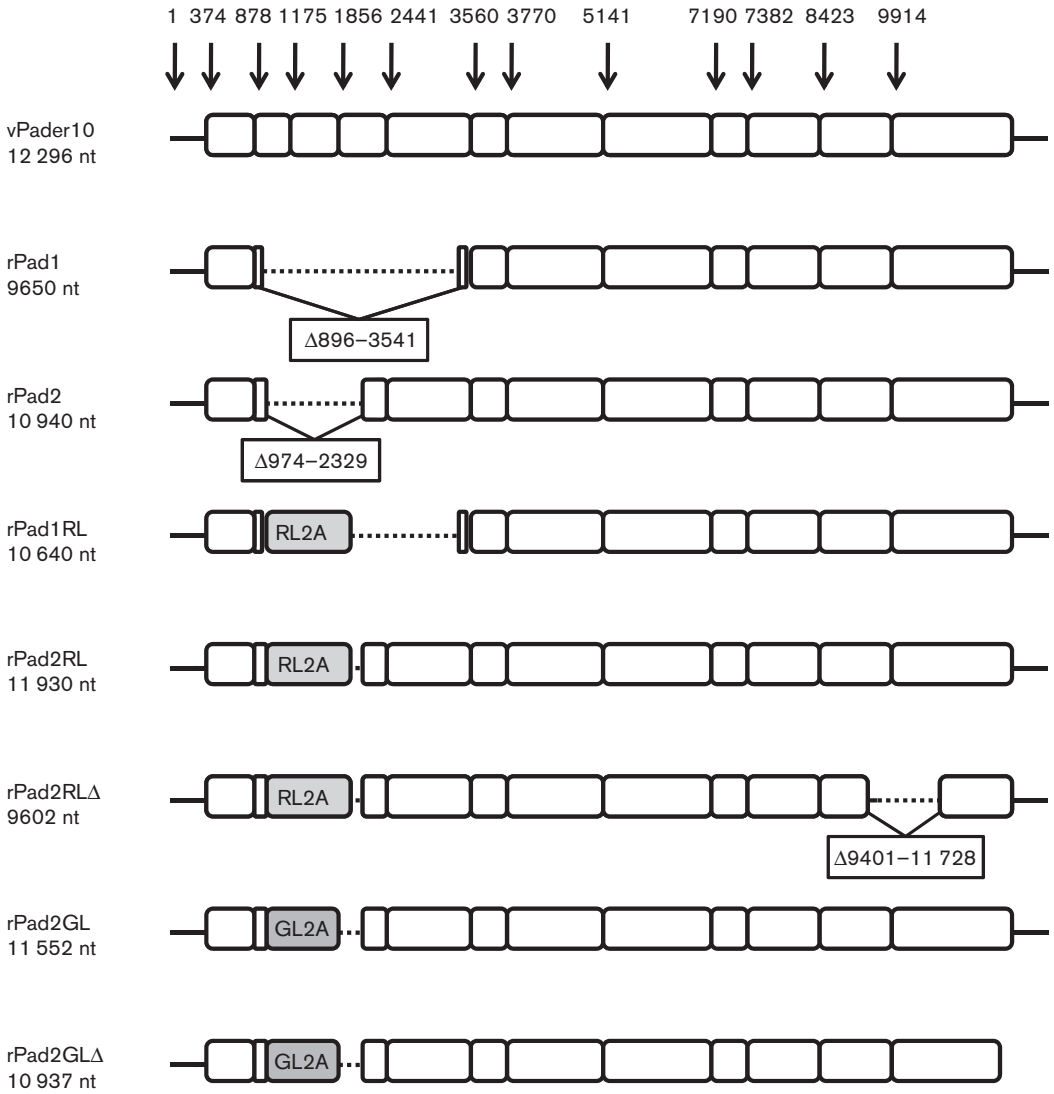

Fig. 1. Schematic representation of the CSFV genome organization and the replicons constructed and used in this study. The in-frame deletions within the Paderborn CSFV cDNA and the insertion of either the Rluc–2A (designated RL2A) or the Gluc–2A (designated GL2A) luciferase reporter sequences are indicated. The lengths of the CSFV RNA transcripts (including the reporter sequences where appropriate) are shown.

replicon sequences expressed a functional Rluc and that this expression reflected RNA replication. RNA transcripts were produced *in vitro* from amplicons generated from the BACs and introduced into SFT-R cells using electroporation. As a negative control, a derivative of rPad2RL was produced lacking a portion of the NS5A and NS5B coding sequence; this construct was termed rPad2RLΔ (Fig. 1). RNA transcripts were also produced from this construct and were assayed in the same way. Cell extracts were prepared 3, 12 and 24 h after electroporation and assayed for Rluc activity (Fig. 3). Luciferase activity was observed at 3 h from each of these RNAs, presumably reflecting translation of the input RNA. At this time, about a 100-fold increase in Rluc values compared with mock-treated cells (background) was observed. A further increase in luciferase expression was observed at 12 or 24 h in cells that received the rPad1RL and rPad2RL transcripts. In contrast, no increase in luciferase activity was observed from the rPad2RLΔ transcripts. Thus, the enhanced signal seen at 12 or 24 h was dependent on the ability of the RNA to be replicated. It was consistently found that higher levels of Rluc activity were obtained with the rPad2RL replicon than with the rPad1RL (Fig. 3). Packaging of the replicon rPad2RL into virus-like particles, which can initiate a single cycle of infection, can be achieved using the RNA from pBeloPader10 cDNA as a helper. The supernatant from cells electroporated with the replicon rPad2RL transcript together with the pBeloPader10 transcript was passaged

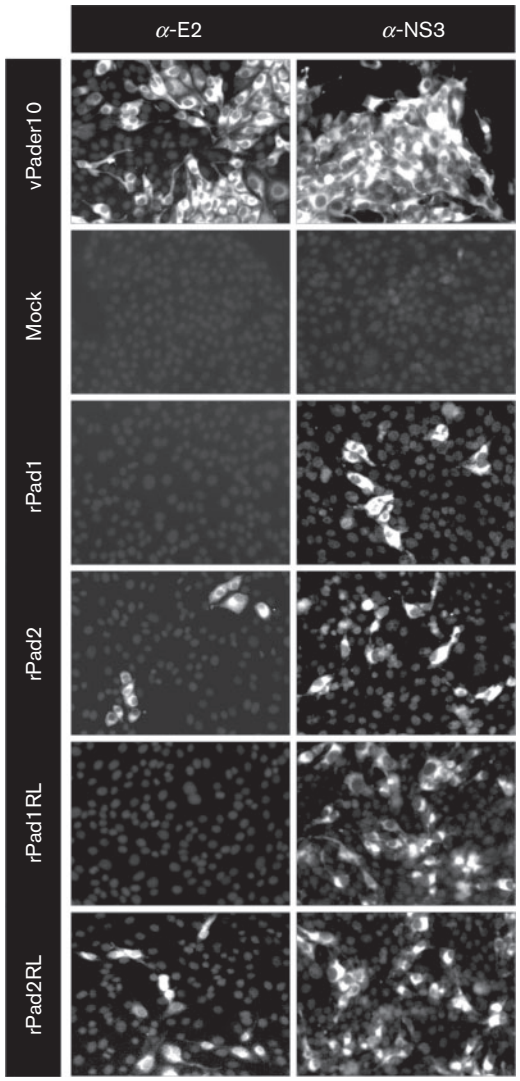

Fig. 2. Expression of CSFV proteins by the replicons. RNA transcripts derived from Paderborn virus cDNA and its indicated derivatives were introduced into SFT-R cells using electroporation. After 24 h, the cells were fixed and stained with antibodies against the CSFV E2 (left) and NS3 protein (right) as indicated and the nuclei (all images) were visualized using DAPI. Mock-treated cells were used as a negative control.

onto naïve cells and produced Rluc within these cells, thereby indicating spread of the packaged replicon (data not shown).

To characterize the nature of the Rluc protein expressed from the rPad1RL and rPad2RL replicons, cell extracts were prepared at 24 h post-electroporation and analysed by immunoblotting (Fig. 4). As a loading control, anti-actin antibodies were used and detected actin in all the cell extracts. In contrast, no products were detected using anti-Rluc antibodies in mock-treated cells or from cells that received the rPad1 and rPad2 replicons (without the Rluc sequence). However, Rluc-related products of 38 and

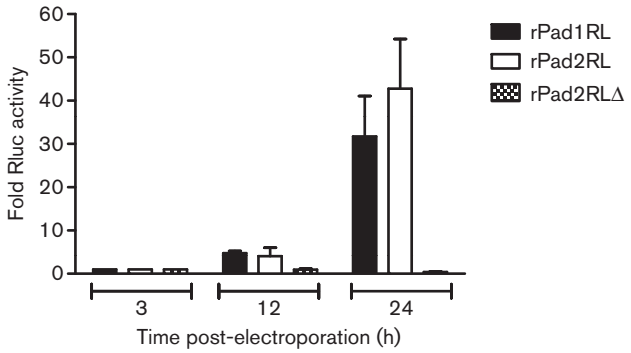

Fig. 3. Rluc reporter protein expression by replicons. As in Fig. 2, the indicated RNA transcripts were introduced into SFT-R cells by electroporation. At the indicated times, cell lysates were prepared and assayed for the expression of Rluc. Results are means (+SD) of three independent experiments. The signal detected at 3 h post-electroporation was set to 1 in each case and the values at other times calculated relative to this value.

41 kDa were detected within cells that received the rPad1RL and rPad2RL replicons, respectively (Fig. 4). The Rluc-related products expressed from these replicons ought to have the Rluc protein fused to the residual portions of the C protein but should be released from downstream sequences by the action of the FMDV 2A peptide (Fig. 1). The predicted sizes of the products were 335 aa (~37 kDa) and 361 aa (~40 kDa) for rPad1RL and rPad2RL, respectively, which corresponded well to the observed products (Fig. 4).

An alternative derivative of the rPad2 replicon was produced which contained the Gluc sequence and was termed rPad2GL (Fig. 1). This luciferase is secreted by cells and hence can be assayed within the cell medium, without harvesting the cells. RNA transcripts were produced from

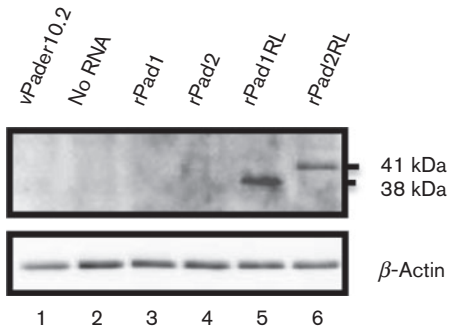

Fig. 4. Characterization of the Rluc reporter protein expressed by replicons. Cell lysates were prepared from SFT-R cells electroporated with the indicated transcripts and analysed by SDS-PAGE and immunoblotting using anti-Rluc (upper panel) or anti-actin (lower panel) antibodies. The β-actin served as a loading control. The sizes of the observed Rluc products are indicated.

the rPad2GL replicon and introduced into cells. Medium from the cells was collected at various time intervals (up to 96 h later) and assayed for Gluc activity (Fig. 5). Consistent with the results observed with the rPad2RL replicon (Fig. 3), detection of Gluc activity from the rPad2GL replicon was observed from 3 h but increased significantly at 48, 72 and even 96 h. A truncated RNA transcript, rPad2GLΔ, lacking the 3′ UTR and part of the NS5B coding sequence (Fig. 1), failed to replicate (Fig. 5). Thus, the nature of the reporter protein within the replicons did not affect their utility as markers for replication of the viral RNA.

Chimeric replicons as a tool for identifying determinants of CSFV RNA replication

To analyse the role of specific regions of the CSFV genome in determining virus replication efficiency, several chimeric replicons were generated. Modifications to the Paderborn-based replicon, rPad2RL, were made by introducing the homologous regions from either an attenuated vaccine strain of CSFV (Riems strain) or from the highly virulent Koslov strain. The regions of the replicon that were modified were the NS2 and NS3 coding sequences (as one block, as in rPad2RL.R2/3 and rPad2RL.K2/3; see Fig. 6a) or the NS5B coding sequence (as in rPad2RL.R5B and rPad2RL.K5B; Fig. 6a). The complete genome sequences of these chimeric constructs were verified (data not shown). There were 52 and 50 aa differences between the NS2/NS3 regions of Riems and Koslov strains (out of a total of 1140 aa), respectively, compared with the Paderborn sequence while there were 46 and 41 aa differences within the respective NS5B sequences (out of 718 aa). RNA transcripts derived from each of these chimeric BACs were introduced into cells and the expression of Rluc monitored (Fig. 6b). The chimeras containing the NS2 and NS3 sequences from the Riems and Koslov viruses were non-functional. However, the presence of the Koslov virus NS5B coding sequence (as in rPad2RL.K5B) significantly enhanced (approx. fourfold) the level of Rluc expression observed when compared with the parental rPad2RL (Fig. 6b). In contrast, replacement of the Paderborn strain NS5B sequence with the equivalent region from the Riems strain (as in rPad2RLR5B) resulted in decreased Rluc production (to ~30 %) compared with that obtained with the parental rPad2RL (Fig. 6b).

DISCUSSION

Targeted modification of CSFV sequences

The use of targeted modifications to sequences contained within BACs has been described by Muyrers *et al.* (1999). We recently described the use of this method to modify the IRES of the CSFV genome (Friis *et al.*, 2012) but the system is applicable to any region of the viral cDNA (T. B. Rasmussen *et al.*, in preparation). The main advantage is that there are no target limitations (based either on size or on location) and no need for suitable restriction sites in the vicinity of the targeted region (Fujimoto *et al.*, 2009). Integration of the modified cDNA sequence into the BAC containing the viral cDNA is performed *in vivo* (within *Escherichia coli*) taking advantage of the host-cell, high-fidelity DNA replication machinery, thereby making it less error-prone than *in vitro* PCR-based methods.

Design and properties of CSFV replicons

Many different approaches could be taken to the design of CSFV-based replicons, e.g. deletion of as much as possible of the viral sequence or a more conservative approach with maintenance of some of the structural proteins. Thus, one type of replicon (as in rPad1 and rPad1RL) had a deletion of most of the structural protein coding region (i.e. C to E2), only keeping 6 aa portions of the C and E2 proteins to enable processing of the protein junctions. The RNA transcripts were significantly shorter (either 9650 nt alone or 10 640 nt with the inclusion of the Rluc–2A sequence) than the full-length genome (12 296 nt). Analogous replicons, based on BVDV and CSFV, have been described (Behrens *et al.*, 1998; Moser *et al.*, 1999; Tautz *et al.*, 1999). A second type of replicon, as in rPad2 and rPad2RL, was based on earlier studies with BVDV replicons by I.

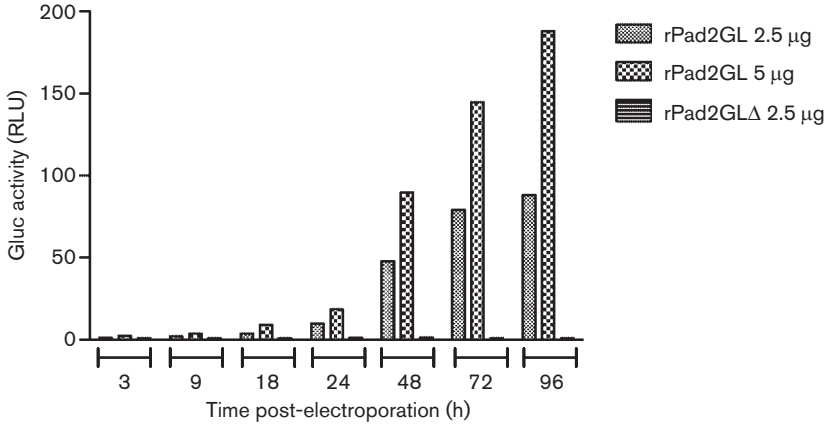

Fig. 5. Expression of the secreted luciferase reporter protein, Gluc, by a replicon. SFT-R cells were electroporated with RNA transcripts derived from the indicated cDNAs and, at the indicated times, samples of the medium were collected from above the cells and assayed for Gluc activity. RLU, Relative light units.

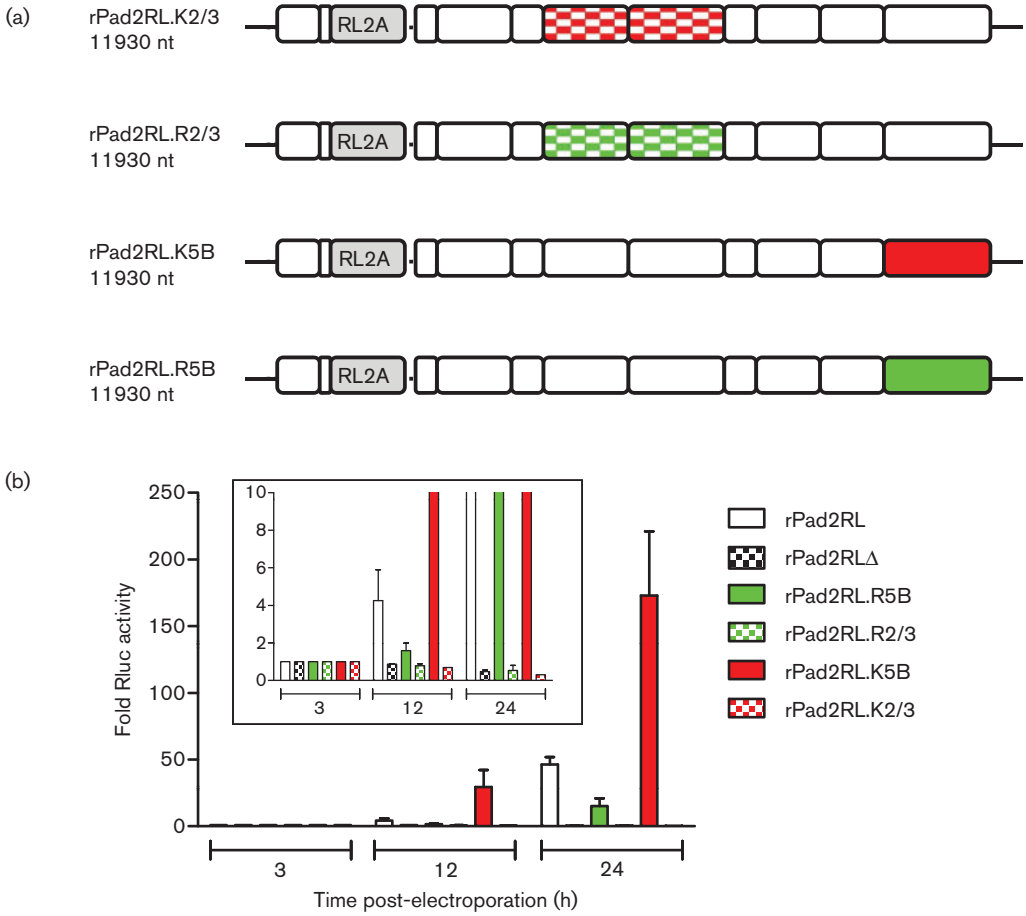

Fig. 6. Construction and analysis of chimeric CSFV replicons. (a) Derivatives of the rPad2RL replicon were constructed in which either the NS2/NS3 coding sequences or the NS5B sequences from the Paderborn strain were substituted by the corresponding regions of the Koslov (K, in red) or Riems (R, in green) strains of CSFV. (b) RNA transcripts derived from the parental rPad2RL (Fig. 1) or the indicated derivatives (Figs 1 and 6a) were introduced into SFT-R cells by electroporation. At the indicated times, cell lysates were prepared and assayed for Rluc activity. Results are presented as the mean values (± SD) from three independent experiments in which the expression level at 3 h post-electroporation was set to 1 in each case. Inset contains the same data with a different *y*-axis scale.

Reimann and M. Beer (personal communication) and these molecules retained a functional E2 sequence, but deleted all of E^{rns}. A similar E^{rns}-deficient CSFV replicon has been described by Frey *et al.* (2006). In addition, with the introduction of the Rluc–2A sequence, the resulting RNA genome (~11.9 kb) has a similar size as the complete virus genome (12.3 kb). The replicon including the E2 sequence (rPad2RL) replicated better than the replicon lacking this protein (rPad1RL) (Fig. 3). Recent studies have indicated that E2 and NS4B can act synergistically in determining virus replication (Tamura *et al.*, 2012).

The CSFV-derived replicons containing the Rluc reporter are suitable for following the translation and replication of the viral RNA. The system is sufficiently sensitive to detect initial translation from RNAs that are not able to replicate (e.g. rPad2RLΔ, which lacks a functional RdRp) but can also monitor the much greater signal from replication-competent RNAs. The replicons function in a variety of cell lines; all the data presented here were derived from ovine cells (SFT-R) as the highest luciferase signals were detected in this cell line. However, comparable results have also been obtained in swine cells (PK-15 and SK6; data not shown). The differences in signal may reflect the efficiency of introducing the RNA into cells rather than differences in the replication efficiency within the cells.

The production of chimeric replicons, containing portions of the genomes of other strains, demonstrated that the replication efficiency of these RNAs was dependent on the source of the RdRp (NS5B). Incorporation of the NS5B sequence from the highly virulent Koslov strain into the Paderborn backbone greatly enhanced the replication-dependent expression of the reporter protein. In contrast, introduction of the NS5B from the vaccine strain (Riems) had the opposite effect and resulted in decreased reporter

protein expression (Fig. 6b). This indicates that the activities of the RdRp and/or its interactions with other viral components are key determinants of replication efficiency. Chimeric replicon RNAs containing the NS2 and NS3 coding regions derived from infectious cDNAs corresponding to either the Koslov strain or the Riems26 strain within the Paderborn backbone proved to be replication defective (although the full-length sequences were verified). As NS2 is not required for RNA replication (Behrens *et al.*, 1998; Moser *et al.*, 1999), this suggests some incompatibility between the NS3 proteins from the Koslov and Riems strains with the Paderborn strain components. Such an interaction with NS3 is unlikely to be with NS5B directly because including the Koslov NS5B protein coding sequence did not adversely affect replication. However, other studies have indicated a requirement for a specific amino acid motif within NS4A of BVDV for the correct functioning of NS3 as a protease (Tautz *et al.*, 2000; Xu *et al.*, 1997). The presence of the non-homologous 3′ UTR sequences from the Paderborn strain may be incompatible with the Koslov and Riems NS2/3 proteins as an interaction between the NS3 helicase protein and the 3′ UTR has been demonstrated (Sheng *et al.*, 2007).

The ability to make replicons containing different reporter proteins could have utility in certain circumstances. We have shown that both Rluc and the secreted Gluc protein can each be used in this system. Other studies have used chloramphenicol acetyltransferase, within the N^{pro} coding sequence of CSFV (Moser *et al.*, 1999), or firefly luciferase (Suter *et al.*, 2011) and GFP within BVDV replicons (Reimann *et al.*, 2003). The luciferase reporter protein assays are highly sensitive and can cover a wide range of activities. Furthermore, the use of Gluc removes the need for harvesting the cells and allows results to be determined from a single batch of cells with a single introduction of RNA.

CONCLUSIONS

The replication of pestivirus RNA is a complex process involving multiple virus-encoded and cellular proteins in concert with the 3′ UTR of both the positive and the negative strands of the viral RNA. Replicons provide a useful tool to analyse key features that determine RNA replication efficiency. However, particular factors, e.g. the NS5B protein, may influence replication in multiple ways; its activity as the RdRp is clearly critical for the process of RNA replication but its interactions with other components (e.g. RNA or protein) of the replication machinery are also likely to be very important. Chimeric replicons based on the related HCV have been used to identify determinants of replication efficiency in cells (Binder *et al.*, 2007), but clearly studies on virulence are difficult for this human pathogen. The observations described here with CSFV showed that exchanging the NS5B from the Paderborn strain (which displays intermediate virulence) with that of the highly virulent Koslov strain increased replication significantly. Thus, these replicons may be a useful tool for identifying candidate features of the virus that determine replication in the natural host. There are 41 aa differences (out of a total of 718 aa) between the RdRp from the Koslov and Paderborn strains. Identifying which of these differences are involved in determining replication efficiency in cells and if these are sufficient to confer a difference in virulence within CSFV-infected pigs is clearly important.

In addition, the lack of replication observed with chimeric RNAs containing the NS2 and NS3 coding sequences from the Koslov and Riems strains can also be informative for identifying interactions between these sequences and other virus components. It is known that the pestivirus NS3 protein interacts with other virus proteins, e.g. NS4A (Tautz *et al.*, 1997; Xu *et al.*, 1997) and NS5B (Wang *et al.*, 2010), and thus the construction of further chimeras could be used to identify other interactions and to define them at the individual amino acid level.

METHODS

Cells. The SFT-R cell line was grown at 37 °C (5 % CO_2) in Dulbecco's minimal essential medium (DMEM) supplemented with 5 % FCS.

CSFV cDNA. The cDNA clone of the CSFV-Paderborn strain, designated pBeloPader10, has been described previously (Rasmussen *et al.*, 2010). It was maintained within *E. coli* strain DH10B $T1^R$ (phenotype streptomycin-resistant; Invitrogen) and grown on selective medium containing 15 μg chloramphenicol ml^{-1}.

Modification of the CSFV cDNA by targeted recombination. Modifications to the full-length CSFV cDNA were accomplished using a counter-selection BAC modification kit (GeneBridges) as described elsewhere (Friis *et al.*, 2012; T. B. Rasmussen *et al.*, in preparation). All primers for the targeted recombination are listed in Table S1 available in JGV Online. Detailed information about recombination procedures is available on request.

(i) Counter-selection of rpsL/neo cassettes to form replicons. To make the intermediate constructs pBeloPadrpsL1 and pBeloPadrpsL2, containing the rpsL/neo selection cassette, the primer sets NproCrpsLFor with E2P7rpsLRev1 and Erns1787rpsLFor with ErnsE1rpsLRev2 were used. The replacement with reporter gene coding sequence was also achieved through targeted recombination. PCR products, including the Rluc–2A coding sequence and with homology arms identical to the Paderborn genome target, were generated by standard PCRs. Deletion of the coding region for C to E2 (Δ nt 896–3541) and introduction of the Rluc sequence was achieved by production of a fragment (P10delCtoE2_RLuc2A, ~1100 bp) using primers Pader10NproCRLucFor and Pader10P7E2_2A_RLucRev with plasmid pRBRluc (Belsham *et al.*, 2008) as template. Similarly, introduction of the Rluc–2A, with deletion of the coding region for C to E1 (Δ nt 974–2329), was achieved using a PCR product (fragment P10delCtoE1_Rluc2A, ~1100 bp) which was produced using primers Pader10C974RLucFor and Pader10E12329_2A_RlucRev. Deletion mutants without the Rluc–2A insertions were made in the same way except that recombination was achieved using single-stranded oligonucleotides P10_DelCtoE2 and P10_DelCtoE1. A replication-deficient version of the rPad2RL replicon was made by making an in-frame deletion using two *Hin*dIII restriction sites in the Paderborn cDNA;

the sites (nt 9401 and 11 728) are located in the NS5A and NS5B coding regions.

(ii) Construction of replicon containing the Gluc coding sequence. The Gluc replicon was constructed using a combination of the counter-selection approach and a modified version of the protocol for target-primed plasmid amplification (Wei *et al.*, 2004; Stech *et al.*, 2008; Friis *et al.*, 2012). Megaprimer P10delCtoE1_Gluc2A (~700 bp) was made using primers P10C974GLuc_Fw and P10E12329GLuc2A_Rev in a standard PCR using pCMV-Gaussia Luc (Thermo Scientific) as template and Phusion hot start II HF DNA polymerase (Thermo Scientific). The megaprimer PCR used 200 ng megaprimer P10delCtoE1_Gluc2A and 100 ng Dam+ pBeloPadrpsL2 template with 10 µl HF buffer, 1 µl dNTPs (10 mM) and 1 µl Phusion hot start II HF DNA polymerase and water to 50 µl. PCR conditions were: 98 °C for 30 s, followed by 20 cycles of 98 °C for 10 s, 48 °C for 60 s and 72 °C for 20 min, one cycle of 72 °C for 20 min, and hold at 4 °C. The PCR products were digested with *Dpn*I, purified using a GeneJET PCR purification kit (Thermo Scientific) and electroporated into DH10B Electromax cells. Colonies were selected on agar containing 15 µg chloramphenicol ml^{-1} plus 50 µg streptomycin ml^{-1}, and the BAC DNAs were analysed. An amplicon corresponding to the replication-deficient version of rPad2GL, termed rPad2GLΔ (Fig. 1), was made with reverse primer Pan-CSF-11682-R and used to produce an RNA transcript lacking the 3′ UTR and part of the NS5B coding sequence.

(iii) Production of chimeric CSFV replicons. For the generation of chimeric replicons, cDNAs corresponding to the coding sequences for NS2/3 and NS5B from the Koslov and Riems virus strains were generated. The NS2/3 sequences from the Koslov and Riems strains were amplified using primers BbP10_PaKosRi_p7_NS2_F and BbP10_PaKosRi_NS3_4_R using BAC templates, containing full-length CSFV cDNAs, pBeloKos10 (U. Fahnøe *et al.*, in preparation) and pBeloRiems26 (T. B. Rasmussen *et al.*, in preparation), respectively. The pBeloKos10 sequence encodes NS2/3 and NS5B protein sequences, which are 100 % identical to that predicted from the Koslov sequence (GenBank accession no. HM237795) but RNA transcripts derived from this BAC are replication defective due to mutations elsewhere in the cDNA. The pBeloRiems26 is derived from a variant of the Riemser/C-strain vaccine strain and generates an infectious RNA transcript. The PCR products (~3500 bp) were generated using Phusion hot-start II HF DNA polymerase and cycling conditions were as follows: 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 59 °C for 20 s and 72 °C for 165 s, one cycle of 72 °C for 165 s, and hold at 4 °C. Similarly, the NS5B coding sequences (~2200 bp) were amplified from pBeloKoslov10 and pBeloRiems26 using primers BbP10_PaKosRi_NS5B_F and BbP10_KosRi _NS5B_R using similar PCR conditions. Introductions of the PCR amplicons into pBelorPad2RL were carried out using the MegaPCR approach as described above.

Template production and *in vitro* transcription. Amplicons corresponding to the replicon cDNAs were generated, using the BACs as templates, using the long PCR method (Rasmussen *et al.*, 2008, 2010) as follows: 5 µl PCR buffer I, 1 µl forward primer (5′Paderborn T7_NotI) and 1 µl reverse primer (3′Paderborn) (200 µM each), 2 µl BAC template (~25 ng), 0.5 µl AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen) and water to 50 µl. To increase the yield, each reaction was performed in triplicate and pooled prior to column purification. The PCR products (~9–12.3 kb) were purified using the GeneJET PCR clean-up kit and eluted in 2 × 20 µl diethylpyrocarbonate (DEPC)-treated water at 65 °C.

In vitro transcription reactions were carried out using the MEGAscript T7 RNA transcription kit (Ambion). For a 20 µl reaction, 0.5 µg of the PCR product was used. Incubation was performed at 37 °C for 3 h. RNA was purified using a MEGAclear kit (Ambion), eluted in 110 µl DEPC-treated water (95 °C). RNA was evaluated using gel electrophoresis in a 1 % Tris/borate/EDTA agarose gel containing 10 000-fold-diluted SYBRsafe gel stain (Life Technologies) and the yield was quantified using a NanoDrop. The RNA was stored at −80 °C until use.

Replicon assay procedure. For each replicon to be assayed, 1 ml SFT-R cells (2×10^6 ml^{-1}) was centrifuged and resuspended in the same volume of cold PBS and kept on ice. An aliquot (800 µl, 1.6×10^6 cells) was transferred to a 0.4 cm cuvette (Bio-Rad) and 2.5 µg RNA was added, briefly mixed and introduced into the cells by electroporation (950 µF and 180 V on a Gene Pulser XCell; Bio-Rad) essentially as described elsewhere (Gallei *et al.*, 2005). After electroporation, cells were allowed to settle at room temperature for 10 min before seeding into six-well plates [250 µl per well (~5.0×10^5 cells) with 3 ml DMEM containing 5 % FCS].

Alternatively, for simultaneous harvesting of samples for Rluc, Western blot analysis and immunofluorescence staining, the assay procedure for six-well plates was adapted to 24-well plates: using 50 µl (~1.0×10^5 cells) per well electroporated with 500 ng RNA and with 600 µl DMEM (5 % FCS). Adding the cells to the medium was considered the assay starting point, T_0. Plates were then incubated at 37 °C in 5 % CO_2. After 3, 12 and 24 h, the medium was removed and monolayers were washed once with 1 × Dulbecco's PBS (DPBS; Gibco) and either frozen at −80 °C or lysed immediately.

Rluc and Gluc assays. Plates were thawed at room temperature, the cells were lysed by adding 400/80 µl (for 6- or 24-well assays, respectively) of 1 × Renilla Luciferase Assay Lysis buffer (Promega) to each well and incubated at 37 °C for ~15–30 min. Lysates were harvested and luciferase activity was quantified in a Bio-orbit 1253 Luminometer (Aboatox) by adding 20 µl lysate into 100 µl coelentarazine substrate diluted 1 : 100 in Renilla Luciferase Assay buffer (Promega). Assays were performed in triplicate. For the Gluc replicon assays, at the indicated times, 50 µl of medium was removed from the cells and frozen at −20 °C. After thawing, 20 µl was assayed as described above.

Immunofluorescence imaging. At 24 h post-electroporation, cells were stained for the detection of E2 and NS3 antigens by immunofluorescence using mAbs WH303 and WB103/105, respectively (AHVLA Scientific) as previously described (Friis *et al.*, 2012; Reimann *et al.*, 2003). The presence of E2 and NS3 and the cell nuclei were visualized using a goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) and DAPI (VectaShield; Vector Laboratories), respectively. Images were taken using a BX63 fluorescence microscope (Olympus).

Western blot analysis. Lysates for Western blot analysis were prepared at 24 h post-electroporation. Cells were washed with DPBS and lysed by adding 400 or 80 µl (for six- and 24-well assays, respectively) of Buffer C [0.125 M NaCl, 20 mM Tris/HCl (pH 8.0) 0.5 % NP-40] and clarified by centrifugation (18 000 ***g*** for 5 min). Samples of the supernatants were mixed with Laemmli sample buffer, heated to 100 °C for 5 min and analysed using SDS-PAGE (12.5 % polyacrylamide) and electroblotting onto PVDF membranes (Millipore). After blocking in PBS containing 0.1 % Tween-20 and 5 % non-fat dry milk overnight, membranes were incubated for 3 h with primary antibodies [anti-Rluc (Millipore) and anti-actin (Abcam)] diluted in blocking buffer. Immunoreactive proteins were visualized on a Bio-Rad Chemi-Doc XRS system using species-specific anti-Ig secondary antibodies conjugated to HRP (P0161 and P0448; Dako) and a chemiluminescence detection system (ECL Select; Amersham).

DNA sequencing. Full-genome sequencing was performed on all the replicon cDNAs within the BACs and the expected sequences were

obtained. This was performed by full-length PCR amplification of each construct. The product was purified on a PCR clean-up spin column and measured on a NanoDrop machine. Subsequently, 500 ng of each product was sequenced on an Ion Torrent PGM machine. Fastq files were checked for quality control using FastQC and later trimmed by Prinseq-lite. The BWA aligner mapped reads to the expected consensus sequence and Samtools obtained construct sequences. Finally, sequences were aligned by the MAFFT algorithm in Genious software to confirm sequences.

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Manuscript III

"Determinants of replication efficiency within the NS5B RNA polymerase of classical swine fever virus"

Manuscript in preparation for Journal of Virology

"Determinants of replication efficiency within the NS5B RNA polymerase of classical swine fever virus"

Peter Christian Risager, Ulrik Fahnøe, Graham J. Belsham, Thomas Bruun Rasmussen*

National Veterinary Institute, Technical University of Denmark, Lindholm, Kalvehave DK-4771

Abstract

The NS5B protein of Classical swine fever virus (CSFV) is the RNA dependent RNA polymerase (RdRp). Swapping the NS5B protein coding sequences between related viral strains, thereby forming chimeric genomes, provides a strategy to identify and manipulate important interactions between different components of the RNA replication machinery. A replicon based on the vaccine "C-strain Riems" was established and used as a backbone to generate chimeric RNAs that encode the NS5B from CSFVs with different virulence characteristics. The properties of the replicons were determined to quantify the effect of the different RdRps on the rate of RNA replication. Replacement of the NS5B coding sequence from the vaccine strain with that of the highly virulent strain Koslov increased replication significantly. Out of the 9 aa substitutions between the Koslov and Riems NS5B proteins, the non-conservative substitutions at residues 140 and 162 were examined for their importance for replication efficiency. A variant of the Riems NS5B with S140 and P162 (SP), as predominantly found in vaccine strains, was modified to encode G140 and T162 (GT) as in the Koslov strain. Surprisingly, the GT variant did not exhibit increased replication. However, the variant with S140 and T162 (ST) displayed a 2.5-fold increase in replication rate compared to the (SP)/(GT) variants. The effect of the modified RdRps on the replication rates of the replicons was confirmed in chimeric viruses produced by swapping the NS5B coding sequences in the full-length CSFV cDNA. Evidence is provided that the virus spread/focus size observed in cell culture can be affected by substitution of single aa residues within the NS5B protein.

Introduction

Classical swine fever virus (CSFV) causes an economically important, severe and highly contagious disease of porcine animals (*Suidae* family). CSFV is classified within the *Pestivirus* genus of the family *Flaviviridae* and has high similarities to hepaciviruses (e.g. hepatitis C virus). The CSFV genome is a positive sense, single-stranded RNA molecule of ca. 12.3 kilobases. The genome includes a single, large, open reading frame (ORF) which encodes a polyprotein of ca. 3900 amino acids. The ORF is flanked, at each end, by untranslated regions (UTRs). The viral polyprotein is co- and post-translationally cleaved, by cellular and virus encoded proteases (1-3), to produce four structural proteins (the core protein, C, and three envelope proteins, E^{rns}, E1 and E2) plus eight major non-structural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B).

The non-structural viral proteins form a membrane-associated replication complex, and replication of the viral genome involves interactions between a combination of these viral proteins and host cell proteins (4). The NS5B protein is of particular significance in the replication process due to its role as the RNA dependent RNA polymerase (RdRp) which synthesizes the new RNA strands (5-7). The CSFV NS5B is the largest (containing 718 amino acids (aa)) of the 12 mature proteins.The structure of the CSFV NS5B protein has not been reported but structural information for the NS5B from another pestivirus, bovine viral diarrhea virus (BVDV) (8-10), provides a good model since BVDV and CSFV NS5B are closely related (ca. 70% amino acid identity). All RdRps are similar in overall structure that resembles a closed right hand and contain three domains denoted as “fingers”, “palm”, and “thumb”. A fourth, N-terminal, domain is a unique feature of the pestivirus proteins and is located over the thumb and interacts with the fingers and thumb domains through β-hairpin motifs (8). This N-terminal domain is highly positively charged and might be involved in opening up complex RNA hairpin structures or removing RNA binding proteins in the replication complex (8, 11). Several loops protruding from the fingers, the so-called "fingertips", bind to the thumb domain and completely encircle the active site (12). The “closed hand” or “o”-shape topology of the NS5B protein forms a channel that provides the correct arrangement for catalysis and for positioning of metal ions at the active site (including the GDD motif VI) and thus for facilitating *de novo* initiation of genome replication (8, 12). Mutations in the GDD motif are detrimental for RNA replication (13). Although sequence similarities between the RdRps of diverse positive strand RNA viruses are low, they share eight sequence motifs designated I-VIII (8). Synthesis of the negative strand RNA (the initial stage in viral RNA replication), is achieved in the channel by interaction between the single-stranded RNA template (the 3′UTR), a priming GTP and the NS5B protein (14).

Replicons encoding a luciferase reporter protein provide a convenient tool to analyze some of the key features of the virus which determine RNA replication efficiency. Swapping protein coding sequences between related viral strains, thereby forming chimeric genomes, provides a strategy to identify important interactions between different components of the replication machinery. Indeed previous studies using HCV

or CSFV constructs showed that swapping the RdRp from strains with different replication efficiencies, could greatly influence the level of replication (15-18).

The observations described here with CSFV show that exchanging the NS5B from the “C-strain Riems” (a vaccine strain) with that of the highly virulent “Koslov strain”, increased RNA replication significantly, as judged by reporter protein expression from replicons and from the kinetics of virus growth. It is demonstrated that single amino acid residues at positions 140 and 162 in the NS5B fingertip region are important for this difference in replication competence. Evidence is further provided that the viral spread/plaque size observed in cell culture monolayers is affected by modification of these individual amino acids.

Materials and methods

Cells, viruses and CSFV cDNA.

Sheep fetal thymus (SFT-R) cells were grown in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 5% FCS at 37°C with 5% CO_2.

Bacterial artificial chromosomes (BACs) containing the complete cDNA sequences of the CSFV vaccine “C-strain Riems” (pBeloR26) (Rasmussen et al., *submitted*), the CSFV strain “Paderborn” (pBeloPader10) (19) and the CSFV strain “Koslov” (pBeloKos10, Fahnøe et al. *in preparation*) were used as the basis for the generation of new constructs. Each of these BACs can be used to produce RNA transcripts which generate infectious virus following introduction into mammalian cells. The BACs were maintained within *E. coli* strain DH10B $T1^R$ (Invitrogen) and grown on selective media containing chloramphenicol (Cam) (15 µg/ml). For the virus growth experiments, the CSFV vaccine “C-strain Riems” (termed vRiemser) was used as a control (20).

Production of CSFV Riems Rluc2A replicon.

The deletion of the coding region for C to E1 (Δnt 974-2329) from the pBeloR26 and introduction of the Rluc+FMDV2A sequence was achieved in several steps. Initially, an amplicon (R26delCtoE2_RLuc2A) was made using primers KosRi_Rluc974F and Riems_Rluc2329_2A_R with the plasmid pRBRluc (21) as a template (note all oligonucleotides are listed in table 1). The PCR product (ca. 1100bp) was generated using Phusion hot-start II HF DNA polymerase (Thermo Scientific) with cycling conditions as follows: 98°C for 1 min, followed by 35 cycles of 98°C for 15 s, 58°C for 20 s, 72°C for 1 min, 1 cycle for 72°C 3 min, and hold at 4°C. Post PCR clean-up was performed using GeneJET PCR purification kit (Thermo Scientific) and the fragment was used as a megaprimer for generation of the Rluc replicon using a modified version of the

protocol for target-primed plasmid amplification (16, 22-25) that is termed here: "megaPCR". The megaPCR was carried out using 200 ng of megaprimer and 100 ng of pBeloR26 template with 10 µl HF-buffer, 1 µl of dNTPs (10mM) and 1 µl of Phusion hot start II HF DNA polymerase and water to 50 µl. PCR conditions were: 98°C for 30 s, followed by 20 cycles of 98°C for 10 s, 48°C for 60 s, 72°C for 20 min, 1 cycle for 72°C 20 min, and hold at 4°C. The PCR products were digested with *Dpn*I, purified as described above and the modified BACs were electroporated into *E. coli* as described elsewhere (16) and screened. The resulting BAC, with the required structure, was termed pBelo_rR26.

Generation of replicons and infectious clones containing chimeric NS5B.

The coding sequences for NS5B, from the Koslov and Paderborn viral strains respectively, were generated using the forward primer BbRi_PaKosRi_NS5B_F and reverse primers BbRi_KosRi_NS5B_R (using the pBeloKos10 template that encodes a functional Koslov NS5B) and BbRi_Pader_NS5B_R (with pBeloPader10 as template). The PCR products (ca. 2200 bp) were generated using Phusion hot-start II HF DNA polymerase (Thermo Scientific) with cycling conditions of: 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, 59°C for 20 s, 72°C for 2 min, 1 cycle for 72°C 2 min, and hold at 4°C. Post PCR, the two amplicons, BbR_NS5B_Kos and BbR_NS5B_Pad were purified and used as megaprimers in a MegaPCR reaction (as described above) with pBelo_rR26 as a template for the generation of replicon rR26.K5B (containing the Koslov NS5B) and rR26.P5B (containing the Paderborn NS5B). The same megaprimers were used together with pBeloR26 as a template for the generation of infectious clones pBeloR26.K5B and pBeloR26.P5B.

Single amino acid substitutions in NS5B.

pBelo_rR26 (now termed here rR26.SPD for clarity) was initially modified, using primers BbRi_PaKosRi_NS5B_F and BbRi_KosRi_NS5B_R with cDNA derived from vRiemser as template, to correct a mutation, that results in an amino acid substitution D3431G, to generate the clone rR26.SPG. Using the same cDNA as template and forward primers 1) R26.6_SPtoGT, 2) R26NS5B_Gly_F and 3) R26NS5B_Thr_F with the reverse primer BbRi_KosRi_NS5B_R, three PCR fragments were generated encoding within NS5B the indicated aa residues at positions 140 and 162 respectively 1) Gly (G) and Thr (T), 2) Gly (G) and Pro (P) and 3) Ser (S) and Thr (T). Using these fragments, megaPCRs were conducted for the generation of the variants rR26.GTG, rR26.GPG and rR26.STG as described above. Variants were also obtained as infectious clones based on pBeloR26 for R26.SPD, R26.SPG and R26.STD.

Long PCR template production and *in vitro* transcription.

Amplicons corresponding to the full length virus cDNAs were generated, using the BACs as templates, using the long PCR method, as described (19), briefly. Reactions included 5 µl PCR buffer I, 1 µl forward primer (5'C-strain_T7) and 1 µl reverse primer (3´UTR_R) (200 µM each), 2 µl BAC template (~25 ng), 0.5 µl AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen) and water up to a total volume of 50 µl. To increase the yield, each reaction was performed in triplicate and then pooled. The PCR products were purified using the GeneJET PCR clean up kit and eluted in 2 x 20 µl DEPC treated water at 65°C. The yield was evaluated using a Nanodrop (Thermo scientific). *In vitro* transcription reactions were performed using the MEGAscript®T7 RNA transcription kit (Ambion) in accordance with the manufacturer's protocol. For a 20 µl reaction, 0.5 µg of the longPCR product was used. Incubation was performed at 37°C for 3 h. RNA was analysed on a 1% TBE agarose gel containing 10.000x diluted SYBR®safe gel stain (Life technologies) and the yield was quantified using a Nanodrop. The RNA was stored in aliquots at –80°C until use.

Replicon assay sampling and luciferase assays

Replicon assay sampling and luciferase assays were performed as previously described (16). Briefly, for each replicon to be assayed, SFT-R cells (2 x 10^6/ml) in cold phosphate-buffered saline (PBS) were transferred to a 0.4 cm cuvette (Bio-Rad), mixed with 5µl RNA and electroporated (950 µF and 180 V on a Gene Pulser XCell (Bio-Rad)). The cells were allowed to recover at RT for 10 min before seeding into 3 wells of a 6 well plate (250µl /well (ca. 5.0 x 10^5 cells) with 3 mls DMEM plus 5% FCS). Adding the cells to the media was considered the assay starting point, termed T_0. Plates were then incubated at 37°C in 5% CO_2. After 3, 12 and 24 hrs, the cell medium was removed and the monolayers were washed once with 1x Dulbecco's PBS (DPBS) (Gibco) and the plates were frozen at - 80°C immediately.

Plates were thawed at room temperature and cells were detached and lysed by adding 400µl of 1x Renilla Luciferase Assay Lysis Buffer (Promega) to each well and incubated at 37°C for ca. 15-30 min. Lysates for all time points were harvested and luciferase activity was quantified in a single-tube luminometer Bio-Orbit 1253 Luminometer (Aboatox, Turku, Finland) by adding 20 µl lysate into 100 µl coelentarazine substrate diluted 1:100 in Renilla Luciferase Assay Buffer (Promega). The initial luciferase signals, determined in extracts prepared at 3 hours post electroporation (hpe), were within the range of ca. 0.5 to 1.5 in direct luminescence units (with a background of ca. 0.01 in the assay) reflecting the translation of the input RNA, and were set to 1 for the relative luminescence units (RLU). Data were collected from three independent assays.

Virus growth curves and Immunoperoxidase staining of cells

Virus stocks for the time course experiments were prepared by infection of 80-90% confluent monolayers of SFT-R cells in Falcon 10 flasks with viruses from a frozen "passage 0" (obtained after the initial electroporation of RNA transcripts). For vR26 a virus stock of 4th passage in which the D251G substitution

(within the NS5B protein) had occurred was used (termed vR26/P-4)(Rasmussen *et al, submitted* , Manuscript I) while vRiemser was used for comparison (20). After 3 days, the supernatants from the flasks were divided into aliquots after one freeze-thaw cycle and centrifugation and titrated in SFT-R cells. Briefly, titration was performed in triplicate in 96-well flat-bottom Costar plates using 50 µl supernatants (in ten-fold dilutions), 50 µl DMEM and 50 µl SFT-R cells ($4x10^5$ cells/ml in DMEM + 5% FCS). After 3 days the cell monolayers were stained for the production of pestivirus proteins using HRP immunostaining. Briefly, after fixation of plates in 99% EtOH for 45 min, visualization of the infected SFT-R cells was achieved using incubation with biotinylated pig anti-CSFV/BVDV polyclonal IgG followed by avidin-conjugated horseradish peroxidase (AV-HRP, eBioscience), and incubation for 25 min with 0.04% ethylcarbazole in EtOH (26). Virus titers were quantified as $TCID_{50}$/ml. To determine the growth characteristics, SFT-R cells were infected with the viruses at a multiplicity of infection (MOI) of 0.001. At 3 h post infection (hpi), the medium was removed from the cells and fresh DMEM with 5% FCS (1 ml) was added. At 3, 12, 24, 48 and 72 hpi, the infected cells were frozen at -80°C. Virus yield, at each time point, was determined by titration as described above.

RNA transcripts from the longPCR products were used for electroporation of SFT-R cells seeded in 6-well Nunc plates as described for the replicon assay. For vR26.SPG, RNA obtained from a longPCR product of vR26/P-12 (a 12th passage of vR26; Rasmussen *et al.* (*submitted*, Manuscript I) was used. At 48 hpe, cell monolayers were immunostained as described above.

DNA sequencing

Sequencing of DNA around new junctions was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) essentially in accordance with the manufacturer's instructions. Briefly, cycle sequencing was performed in 10 µl reactions (1 µl Ready Reaction Premix, 2 µl 5x BigDye sequencing buffer, 5 pmol primer, 100-150 ng longPCR product and water) under the following conditions: 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Electrophoresis was carried out on a 3500 genetic Analyzer (Applied Biosystems).

Full genome sequencing was performed on all the replicon cDNAs within the BACs shown in Figure 1 and Figure 3. This was performed by full-length PCR amplification of each construct. The products were purified using Thermo-Fisher PCR clean up spin columns and eluates were measured on a Nanodrop. Subsequently, 500 ng of each product was sequenced on an Ion Torrent PGM machine (as described (16); Rasmussen *et al*. (*submitted*, Manuscript I). For all constructs the expected sequences were obtained (data not shown).

NS5B protein fold prediction

A 3-dimensional (3D) structure of NS5B was predicted, this was initially generated as a pdb file using cphmodel server (27), and next folded and visualized in Geneious software tool vR6 (Biomatters Ltd.).

Statistical analysis

Statistical analysis was performed using GraphPad prism version 6.02 (GraphPad Software, San Diego, CA). Student's t-test was used to examine the significance of mean differences between the control group and variants. Differences were considered statistically significant with a probability of $p \leq 0.05$.

Results

The effect of distinct NS5B proteins on replicon function

An Rluc2A replicon based on the vaccine "C-strain Riems", termed rR26.SPD, was constructed and used as a backbone to generate chimeric, monocistronic, replicons expressing the NS5B protein from the virulent "Koslov" and "Paderborn" strains and these were termed rR26.K5B and rR26.P5B respectively (see fig.1). Initially, rR26.SPD, rR26.K5B and rR26.P5B were compared against each other. Figure 2A shows the significant increase in luciferase expression observed from rR26.K5B at 12 h (12-fold) and at 24 h (181-fold) compared to the initial signal from translation detected at 3 hpe. In contrast, expression from rR26.SPD and rR26.P5B did not increase before 24 h and then showed only 6 and 12-fold increases in RLU respectively (fig. 2A). Previous observations indicated that the replication efficiency of rR26.SPD was impaired due to a mutation at nt position 10665 leading to a G251D substitution in the NS5B protein compared to the consensus sequence (see Rasmussen et al., *submitted*). To evaluate if this mutation could explain the impaired replication rates observed here, another replicon (termed rR26.SPG) with the reversion of D251 to G was constructed. Assay conditions were changed slightly for these studies in that 5 µl of each RNA transcript was used in order to enhance the luciferase signal and for the ease of use. The rR26.SPG RNA displayed a 16-fold increase in signal at 12 hpe compared to the initial (3 h) value and a 233-fold increase in RLU value was apparent at 24 hpe (fig. 2B). However, although the rR26.SPG replicon replicated faster than the rR26.SPD it was only about 35 % as active as the rR26.K5B (fig. 2B). Following introduction of the rR26.K5B RNA into cells, a large increase in Rluc expression was observed at 12 hpe (166-fold compared to the initial value) and by 24 h the signal had increased by 666-fold (see fig.2B). Using the rR26.SPD transcript, no increase in luciferase signal was observed between 3 and 12 hpe, but after 24 h the Rluc expression had increased by 23-fold (fig 2B). The high level of Rluc expression observed with the rR26.K5B transcript indicates a substantially higher rate of replication achieved by the Koslov NS5B protein compared to the Riems NS5B within this replicon.

Influence of specific modifications of the NS5B coding sequence on virus growth

To investigate the influence of the different NS5B proteins in the context of infectious viruses, chimeric viruses were prepared containing the NS5Bs from the Paderborn (vR26.P5B) and Koslov (vR26.K5B) strains (fig. 3) and virus growth rates were determined (fig. 4). A stock of vR26 (vR26/P-4) which contains S140, P162 and in which ca. 50% of the D251 had reverted to G251 (fig. 3) (Rasmussen *et al. submitted*, manuscript I) was used to infect cells (at MOI of 0.001) and analyzed in parallel with the vRiemser vaccine strain, plus the rescued strains vR26.K5B and vR26.P5B (see fig. 3). Only the growth of vR26.K5B could be detected at 12 h but production of each of the viruses was observed by 24 h and titres increased through to 72 hpi (fig. 4). The vR26.K5B showed the highest virus yield and the titre reached $10^{5.5}$ $TCID_{50}$/ml after 48 h and ca. $10^{6.2}$ $TCID_{50}$/ml at 72 hpi. The growth of vR26.SPG was detected initially at 24 hpi and was present at a 100-fold lower level than the vR26.K5B at this time but reached a level close to 10% of the R26.K5B by the end of the assay (72 hpi). The Riemser vaccine strain was also first detected at 24 hpi and yielded levels of virus between that of R26.K5B and R26.SPG reaching ca. $10^{5.5}$ $TCID_{50}$/ml at 72 hpi (see fig. 4).

Comparison of NS5B variants primary and tertiary amino acid sequences

To identify specific amino acids that may be responsible for the increased replication competence conferred by the Koslov NS5B, a comparison of the NS5B sequences was carried out. The predicted amino acid sequences of the NS5B proteins were aligned using the “Koslov” (acc. no. HM237795.1) and “C-strain Riems” (acc. no. AY259122.1) sequences and the differences were mapped in relation to the 8 conserved sequence motifs within NS5B (I-VIII) (8). This comparison revealed 9 aa substitutions between the proteins (fig. 5A). Of these differences, 3 of them are located within the C-terminal region and are relatively conservative changes that do not significantly change the amino acid properties (I595V, M638I and V719A). Within the motif V, a conservative I423V change was also observed. In addition, at position 362 a switch between the positively charged residues K362 in Koslov and R362 in Riems was apparent and also between the negatively charged E to D at both residues 98 and 280. Two non-conservative aa substitutions were apparent at positions 140 and 162. At residue 140 the change is from glycine to serine while at residue 162 it is a substitution of threonine by proline. The presence of the latter residue is able to bring about a significant change in the local secondary structure as it “kinks” the polypeptide chain and disrupts helices. An alignment of the sequence including residues 140 and 162 from 54 different CSFV sequences available at Genbank was performed (supplementary fig. S1). This showed that S140 and P162 are present in most attenuated CSF vaccine strains, while G140 and T162 are predominantly found in the virulent strains. The combination of S140 and T162 (ST) was only present in one CSFV strain (Uelzen) and no published sequencess have the G140 and P162 (GP) combination.

To obtain further insights into the consequences of substituting specific amino acids within NS5B on its properties, the 3D structures of the NS5B variants were predicted (fig. 5B). Consistent with the BVDV NS5B structure (8) 580 aa of the 718 aa in CSFV NS5B, folded into a conformation that could be assigned into N-terminal (95-136), Fingers (165-259, 289-313 and 351-420), Palm (413-350, 411-499) and Thumb (500-674) domains. Some 8 of the 9 amino acid amino acid differences between the NS5B proteins of the Riems and Koslov strains could be located in the model (fig.5B). The ninth difference is present at the extreme C-terminus (pos. 718), and is not present in the predicted structure. The fingertip region including residues 137-164 and 260-288, contains the key residues 140 and 162 (fig. 6A). As shown in figure 6A, the two fingertip regions, including residue 162 and the motif II (including the key residues 283, 285 and 287), are predicted to be in very close proximity. The predicted tertiary structures for the NS5B of the Riems variants SPG, GTG, GPG, STG and also the Koslov and Paderborn strains were compared in the fingertip region (fig. 6). It was observed that the conformation of residues 153, 154 and 277-279 varied between these variants (marked in orange). The variants with G at residue 140 have an extended β-sheet-like structure at residues 153 and 154 whereas the variants with P at residue 162 have an additional β-sheet-like structure at residues 277-279. Thus, the GPG variant has β-sheet-like structures at both positions while the GTG derivative has the sheet-like structure at residues 153 and 154 but not at 277-279 while the SPG variant has the opposite arrangement. Variant STG and the Koslov NS5B (K5B) do not have sheets at these positions and interestingly the Paderborn protein (P5B) is predicted to have a similar structure as STG and K5B (fig. 6B).

Replication competence affected by single amino acid substitutions in NS5B.

To determine how the amino acids present at position 140 and 162 influence the rate of replication, the replicons rR26.GTG, rR26.GPG and rR26.STG (fig. 1) were produced and their replication was monitored using Rluc assays (see Fig. 2B). All of these replicons have the consensus G251 (in contrast to rR26.SPD). The replicon with the double amino acid substitution SP to GT, (rR26.GTG), showed similar replication efficiency as the parental rR26.SPG at 12 hpe (ca. 16 RLU for rR26.SPG and ca. 27 RLU for rR26.GTG) while at 24 hpe, both exhibited a 233-fold increase in expression. The rR26.GPG variant behaved in a similar manner with a 24-fold increase in expression at 12 hpe and 366-fold increase at 24 hpe. Strikingly, the rR26.STG variant showed the highest replication rate and produced an increase in luciferase activity of ca. 95-fold by 12 hpe and 728-fold at 24 hpe (see fig. 2B). Interestingly, this replicon (with just 1 amino acid change from the SPG triplet present in the consensus sequence of the parental vaccine strain) displayed similar enhanced replication rates as the rR26.K5B.

The effect of amino acid substitutions in NS5B on virus spread in cell monolayers

Vaccine strain derived viruses vR26.SPD and vR26.SPG, when used at low MOI or when inspected at passage 0 (following introduction of RNA), produce small defined islands (foci) of virus positive cells in the cell monolayer. It was observed that the various derivatives of vR26 with modifications in the NS5B protein displayed significantly different focus morphologies (see fig. 7). A slight increase in the number of virus positive cells was observed between vR26.SPD and vR26.SPG, with 10% and 20% of the cells infected respectively, although the size of infected cell foci appeared similar for these two strains. Substituting the full NS5B protein of the vaccine virus with that of the “Paderborn” and “Koslov” strains, significantly enhanced the size of the virus infected cell foci that were produced and the total number of cells infected also increased significantly (up to ca. 70% and 100% at 48 hpe respectively). Interestingly, the rescued virus containing the R26 NS5B with just one aa substitution P162T (vR26.STD) compared to the vR26.SPD, produced infected cell foci with significantly enhanced size and the number of infected cells was up to ca. 80-90% (c.f. vR26.SPD) (see fig. 7).

Discussion

The construction and analysis of chimeric CSFV genomes consisting of sequences derived from different strains is valuable for identifying the compatibilities (or not) between the different components of the virus. This is important for unraveling the protein-protein interactions or other *trans* and *cis*-acting elements that underlie important traits of the virus. It was shown previously, based on a CSFV replicon system, that the production of chimeric RNAs, i.e. with the RdRp coding sequence from different strains, could be used to demonstrate that the replication of these RNAs are affected by the origin of this enzyme (16). The NS5B protein can influence replication in multiple ways; its activity as the RdRp is clearly critical for the process of RNA replication but its interactions with other components (e.g. RNA *cis*-acting elements or proteins) of the replication machinery are also likely to be very important (28, 29), including the proper anchoring of the replication complex to the ER (30). It is now shown that incorporation of the NS5B coding sequence from the highly virulent "Koslov" strain into a replicon based on a C-strain vaccine backbone also enhanced the expression of the reporter protein (and thus replication)(see fig. 2A and 2B), and this result was confirmed within the context of the infectious virus since it also increased the virus growth rate (fig. 4) and increased the size of the infected cell foci (fig. 7).

The “C-strain Riems” is a live attenuated CSFV vaccine strain (31). The “Paderborn” and “Koslov” strains are, in contrast, moderately and highly virulent variants of CSFV respectively. Comparison of sequence data for the rescued vR26 with the consensus sequence for this strain (GenBank acc. no. AY163260) revealed a predicted amino acid substitution (D to G) at aa residue 3431 within the whole polyprotein, corresponding to residue 251 in NS5B. During virus passage it was shown that this substitution reverted, this was detected at the 4^{th} cell passage and full reversion to the consensus sequence (G251) was apparent at the 12^{th} passage

(Rasmussen et al., *submitted*). The substitution D251G led to significantly enhanced growth kinetics of the vR26 strain in cell culture, which indicated that this position was important for the rate of replication. G251 is present and conserved in most CSFV strains and is located, within the α-helices in the finger domain of NS5B, close to the nucleotide binding pocket in motif I and II (14). The D residue is negatively charged whereas G is uncharged. It seems that introduction of a negatively charged residue at this position could impair the integrity of the finger domain binding to the thumb and thus impair channel formation, and hence the proper and effective positioning of the negatively charged RNA template. Whether the initial mutation to the defective variant was generated during the cloning procedure or reflects a low-level variant present in the parental vaccine stock is not known (Rasmussen et al., 2013 *submitted*). This substitution was corrected in the replicon, designated rR26.SPG, and at 24 h this “corrected” replicon produced a 10-fold higher signal than rR26.SPD (fig. 2B). Likewise, strongly enhanced replication competence was observed when the replicon included the Koslov NS5B (as in rR26.K5B) rather than the Paderborn NS5B (as in rR26.P5B) (fig. 2A). Comparison of the full length vPader10 sequence to the consensus sequence (GenBank acc. no. AY072924.1) revealed a substitution at aa 651 in NS5B (residue. 3831 in the polyprotein) from the G in the consensus sequence to D in vPader10, and that this substitution reverted to parental aa during passage of vPader10 (data not shown). The replication competence of both the replicon and the virus were impaired due to this substitution and it is likely to be responsible, at least in part, for impaired replication of the replicon rR26.P5B and vR26.P5B compared to rR26.K5B (fig. 2A) and vR26.K5B (fig. 4) respectively.

To identify specific amino acids that may be responsible for the increased replication competence conferred by the Koslov NS5B, a comparison of the strains was performed. There are 41 amino acid differences (out of a total of 718 aa) between the Koslov RdRp and the Paderborn enzyme but only 9 amino acid differences between the Koslov and C-strain NS5B proteins (both genotype 1.1). Identifying which of these differences are involved in determining replication efficiency is clearly important. The core of the CSFV polymerase protein begins at residue 137, downstream of the N-terminal domain. The first region, comprising 28 aa (residues 137-164) and also the 29 aa at residues 289-313, form the “fingertips”. Out of the 9 amino acid substitution between Koslov and C-strain NS5B, 2 of them are of particular interest since they are non-conservative. These are located at positions 140 and 162 in the fingertip region (corresponding to residues 3320 and 3342 in the entire polyprotein) (see fig. 5A). Interestingly, virulent strains generally contain G140 and T162 at these positions whereas CSF vaccines contain S140 and P162 (see supplementary fig. S1). In order to investigate if the residues 140 and 162 could have a significant role in the enhanced replication rate observed with the Koslov NS5B, single amino acid substitutions in the C-strain NS5B were produced. The four combinations "SP" (the wt C-strain variant), "GT" (the Koslov/Paderborn variant) plus the" ST" and " PG" variants (containing single aa substitutions) were produced within the context of the rR26.SPG replicon (fig. 1). Since the Koslov NS5B protein contains the GTG pattern of residues, it had been assumed that this combination would be the most efficient of the variants. Unexpectedly, replicons containing the variant (GT) showed no difference in replication compared to the Riems variant (SP) (c.f. R26.SPG and R26.GPG in fig.

2B). However, the variant rR26.STG, showed a significant increase in replication level (fig 2B). This variant only differs from rR26.SPG at one position, S162T, The replication level of the rR26.STG was comparable to rR26.K5B which contains the entire Koslov NS5B coding sequence. The STG combination of residues is only found, to date, in the CSFV strain Uelzen (supplementary fig. S1). It has been suggested that the fingertip region and its flexibility is important for binding incoming NTPs and translocation of the template during elongation (9, 12). By analogy to observations on the NS5B protein of BVDV (9), the finger-tip residues 157-159 and 266-283 (containing motifs I+II) connect and establish binding to the thumb domain thereby forming a channel which is involved in RNA template and nucleotide triphosphate (NTP) binding (8). Substitution of motif II residues in the nucleotide binding pocket of BVDV NS5B RNA polymerase from R283, R285, and I287 to alanine led to both decreased NTP binding and RNA replication efficiency (14), indicating that the integrity of this motif is important for replication competence. Likewise, substitution of residues K141 (in motif I) and K155 or F162 (both within motif II) in the fingertip region of HCV NS5B (corresponding to residues K263, K282 and Y289 in the CSFV protein) drastically reduced polymerase activity (32). The introduction of a proline (P) at residue 162 (as seen in the C-strain strain) may be expected to result in significant change to protein function since proline has distinct properties from the other amino acids which may affect the local structure of the protein and alter its flexibility. From a model of the 3D/tertiary structure of the NS5B protein of CSFV strain Riems, it appears that the fingertip regions interact with each other, and that residue 162 is in close proximity to motif II (fig. 6A). The predicted structures for the NS5B variants SPG, GTG, GPG, STG plus the entire Koslov (K5B) and Paderborn (P5B) proteins are shown in fig. 6B. It is seen that the STG, P5B and K5B proteins each have a very similar structure in the fingertip regions, whereas the other four variants are significantly different. The differences are present in regions containing residues 153, 154 and 277-279 (marked in orange). While the STG, P5B and K5B variants do not have β-sheets at either of these positions, the other four do have β-sheets at one or more of these sites (see fig. 6B). Why the GTG residues in Riems result in the extended beta-sheet, in contrast to the STG, plus the P5B and K5B structures should be further elucidated in future studies to gain further insights about how the tertiary protein structure in NS5B influences replication competence.

In vitro virus growth experiments in cell culture have previously indicated that the replication speed of CSFV does not seem to be a specific trait correlating with virulence in a study that compared replication competence for a variety of strains with diverse virulence properties (33). However it was indicated that the virus spread/shedding might be more pronounced for the highly virulent strains compared to strains of lower virulence and thus could be distinguished in cell culture (33, 34). Similarly, vaccine strains of CSFV (e.g. GPE-) and attenuated strains of BVDV have been shown to produce smaller “islands” of infected cells compared to virulent strains in cell monolayers (34, 35). Decreased sizes of infected cell foci or plaques due to avirulent viruses in comparison with virulent strains have been noted in previous studies with other positive strand RNA viruses (35-38). It was observed that the different vR26 derivatives with variation in their NS5B sequences displayed significantly different patterns of infected cell foci formation (see fig. 7).

No significant change in foci formation could be attributed to the D251G substitution. The vR26.SPG showed the typical small infected cell foci, even though the replication competence increased 10-fold due to this adaptation (fig. 2B). Viruses containg the Paderborn (vR26.P5B) and Koslov polymerase (vR26.K5B) showed higher levels of infected cells, ca. 70% for vR26.P5B and up to 100% for vR26.K5B. To elucidate the influence of residue 162 on virus spread between cells, a virus was produced from the replication "impaired" vR26.SPD in which P was substituted with T (termed vR26.STD). This single amino acid substitution within the virus drastically modified the infected cell foci morphology and resulted in infection of up to 90% of the cells, in contrast to infection of just 10% of the cells with the parental strain (fig. 7) under the same infection conditions.

In summary, several conclusions can be drawn from this study. It has been shown that substitution of the RdRp from a CSFV strain of high virulence into an attenuated strain can significantly improve replication competence (fig. 2 and 4). From inspection of the nine aa substitutions between strain “Koslov” and “C-strain Riems” and analysis of their influence on structure and activity it is proposed that the introduction of a proline at residue 162 residing in the fingertip region is important for the impaired replication and viral spread of the attenuated strain. This was concluded on the basis of i) structural similarities in NS5B fingertip region between STG and K5B (and P5B) as judged by *in silico* modeling of NS5B (fig. 6B) ii) the higher levels of luciferase activity expressed by rR26.K5B and rR26.STG variants compared to the rR26SPG in a replicon assay and iii) infected cell foci obtained using vR26.K5B and vR26.P5B include up to 100% of the cells in the monolayer, whereas, in contrast, only about 10% of the cells were infected with the attenuated strain (vR26.SPG). Interestingly the P162T substitution alone greatly increased the size of the infected cell foci (from ca. 10% to ca. 90% for vR26.SPD and vR26.SPG, respectively, see fig. 7).

The results presented here, represent a starting point for the characterization of the determinants of replication competence. Ongoing work in our lab is focusing on further characterization of the relevance of increased viral shedding on virulence and the molecular determinants behind these traits.

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TABLES AND FIGURES.

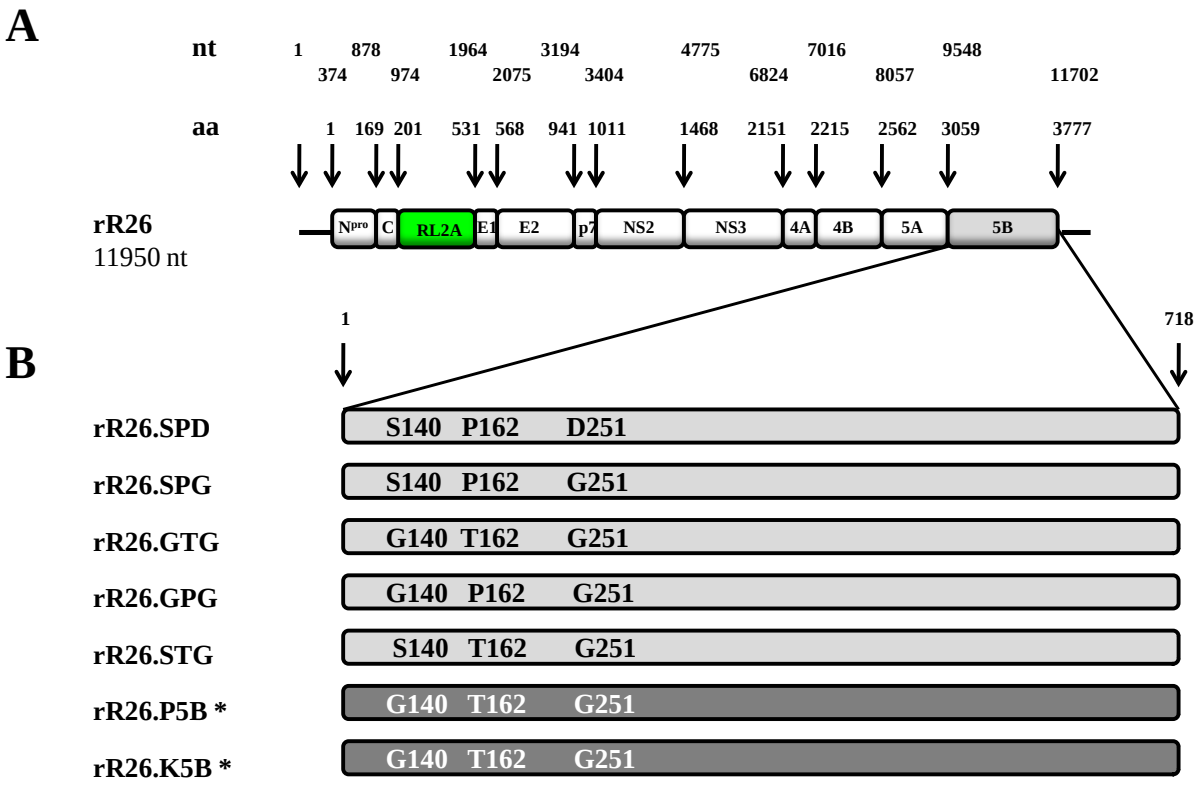


Figure 1. Replicons used in this study. A) The rR26 replicon, containing the Rluc2A protein (in green) and NS5B protein highlighted in light grey. Nucleotide (nt) or amino acid (aa) of the initial residue of the individual viral proteins position is indicated. B) Presentation of the replicons containing single amino acid substitutions (SPD, SPG, GTG, GPG, and STG) and replicons containing entire NS5B protein (718 aa) from "Paderborn" (rR26.P5B) and "Koslov" (rR26.K5B). *)Only the substitutions at the three highlighted positions in the Paderborn and Koslov NS5B (in dark grey) are shown.

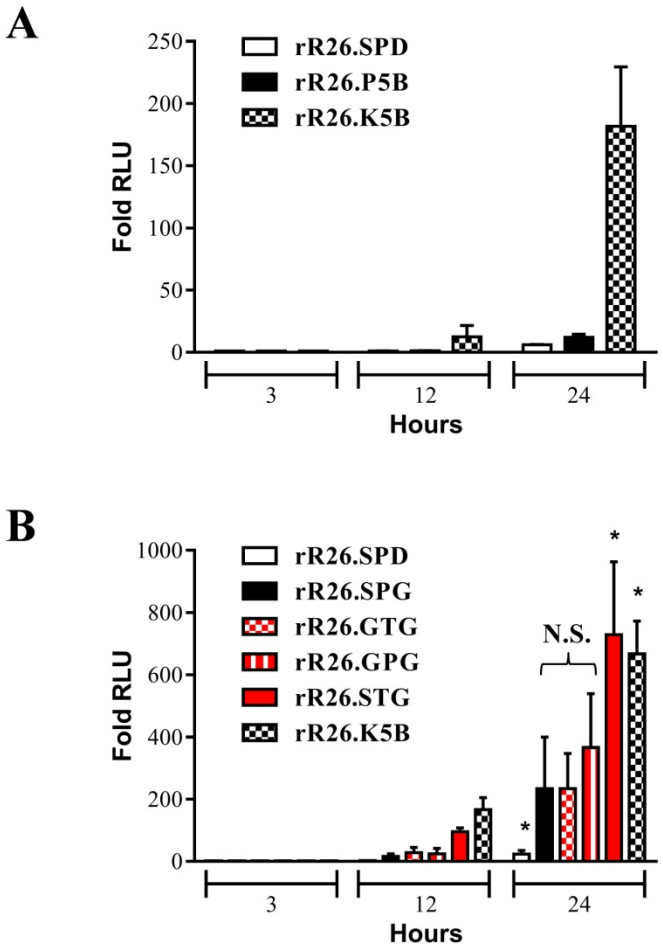


Figure 2. Replication of chimeric CSFV replicons as determined from reporter protein expression. RNA transcripts were electroporated into SFT-R cells and the cells were then frozen at the indicated times and cell extracts were assayed for Rluc activity. The expression level at t = 3 h (prior to significant replication) was set to 1 in each case and other values, determined for the same transcript at later times, were expressed relative to this. In panel A) the indicated transcripts were compared against each other using 2.5 µg of input RNA. In panel B) the indicated RNA transcripts (5µl in each case) were used but otherwise assayed as in panel A. Data are presented as means +SD (n ≥3). The significant difference in RLU between control (rR26.SPG) and the variants are indicated with $P < 0.05$; *. N.S; Not significant.

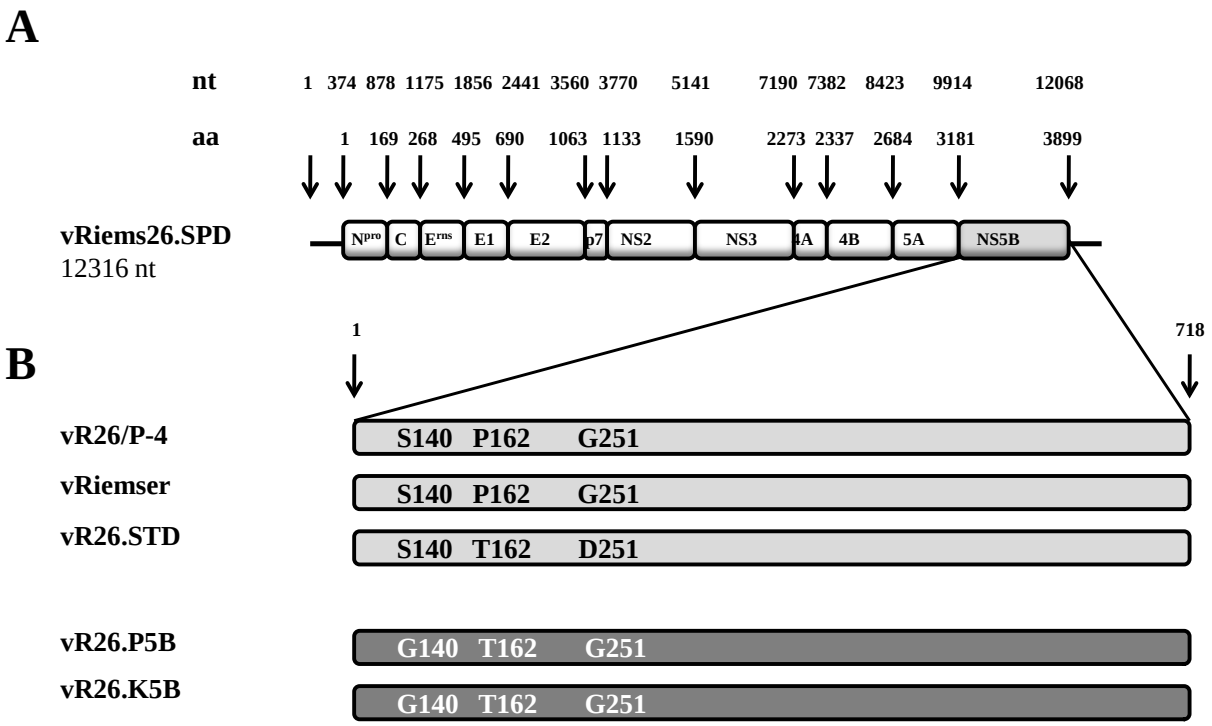


Figure 3. Infectious viruses used in this study containing Riems backbone only varying with substitutions in NS5B at positions 140,162 and 251 for vR26/P-4, vRiemser and vR26.STD, and containing destinct NS5Bs for vR26.P5B and vR26.K5B. For vR26.P5B and vR26.K5B aa positions are indicated only for the three positions. Nucleotide (nt) or amino acid (aa) positions of the initial residue of the individual virus proteins are indicated.

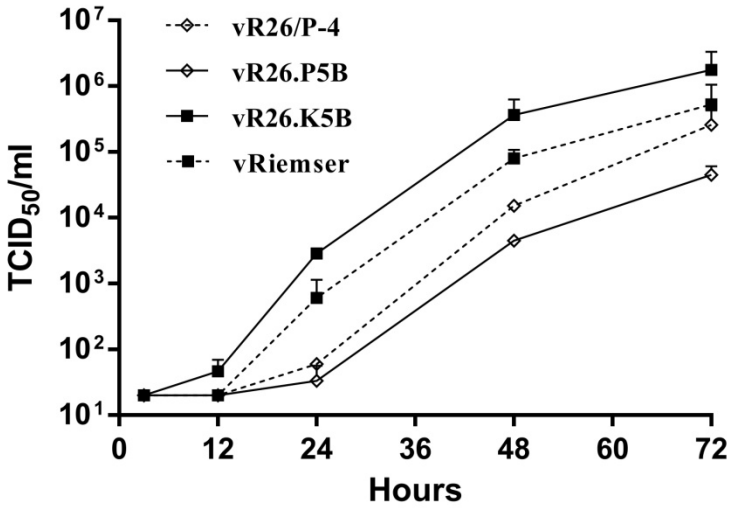


Figure 4. Growth characteristics of vR26, vRiemser and chimeric CSFVs in SFT-R cells. Virus titers for each of the indicated viruses were determined from harvests prepared at 3,12, 24, 48,and 72 hpi. Data is presented as mean +SD (n=3).

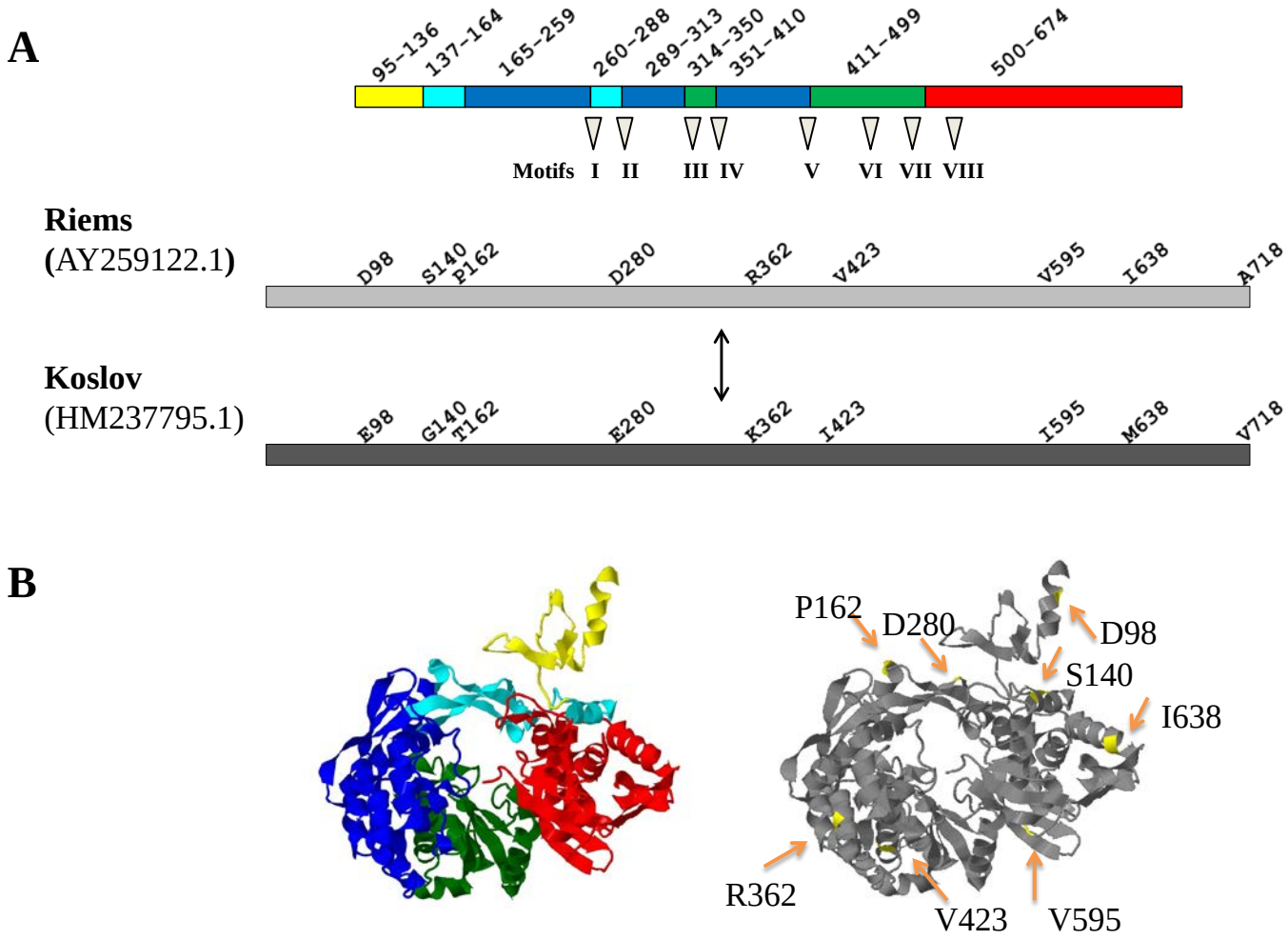


Figure 5. Structure of the CSFV NS5B protein. A) 580 of the 718 aa sequence is presented as a coloured bar and each domain is depicted as "the N-terminal domain" (yellow), "fingertips" (cyan), "fingers" (blue), "palm" (green) and "thumb"(red). In grey bars, the 9 aa substitutions between Riems and Koslov are shown. Localization of the 8 conserved sequence motifs are depicted as well. B) Predicted 3D structure of NS5B. Domains are represented by the same colours as described above (the left panel). The localization of 8 of the 9 aa substitutions are shown (right panel).

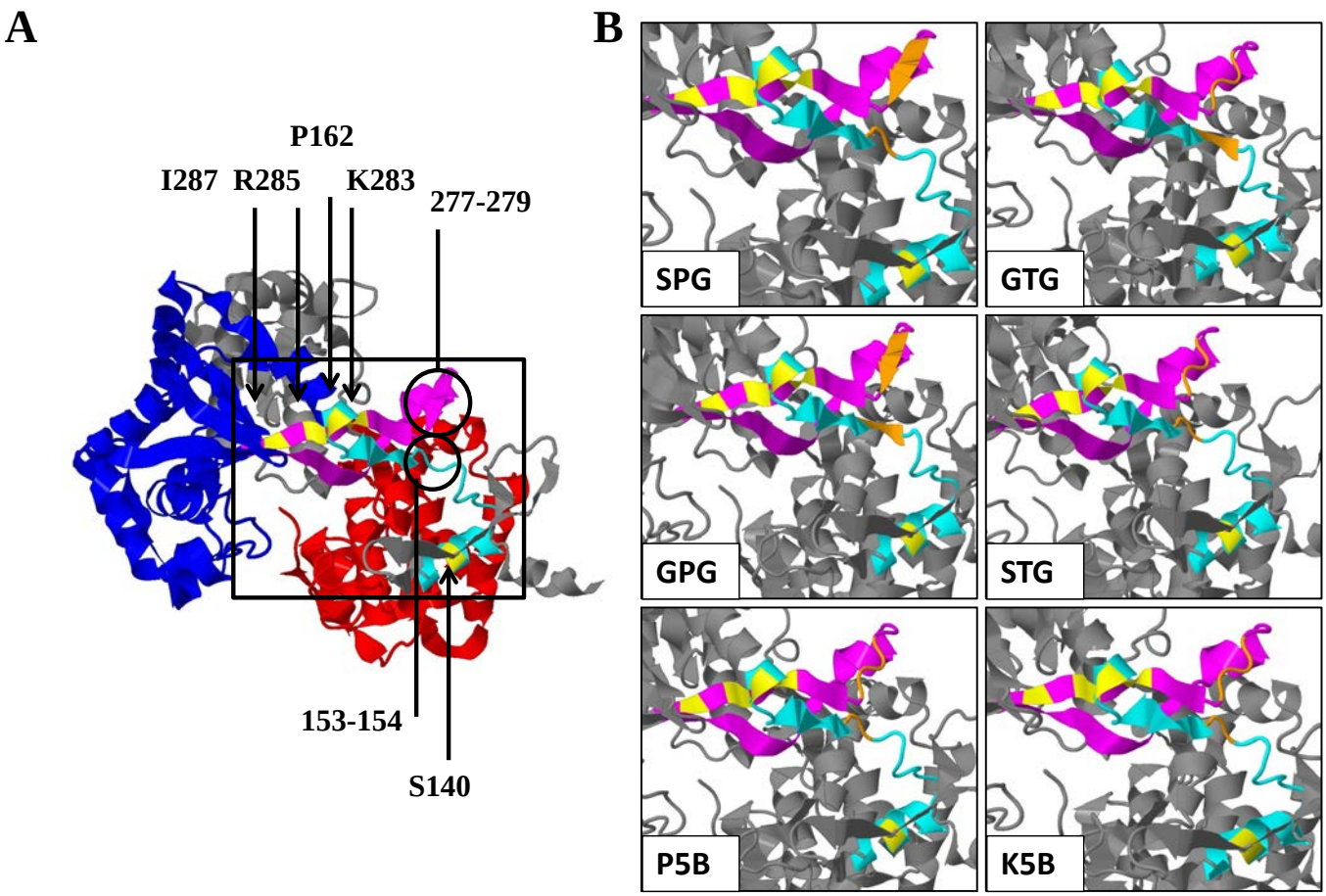


Figure 6. Predicted structure of CSFV NS5B viewed from above**.** A) The fingertip region comprising 137-164, is marked in cyan and fingertip region 260-288 is marked in magenta. Arrows indicate S140 and P162 and K283, R285 and I287. Residues 153-154 and 277-279 are highlighted as well. The fingers (blue) and thumb (red) domains are depicted for orientation purpose. B) Predicted 3D structure of variants of NS5B. Residues 153-154 and 277-279 are highlighted in orange.

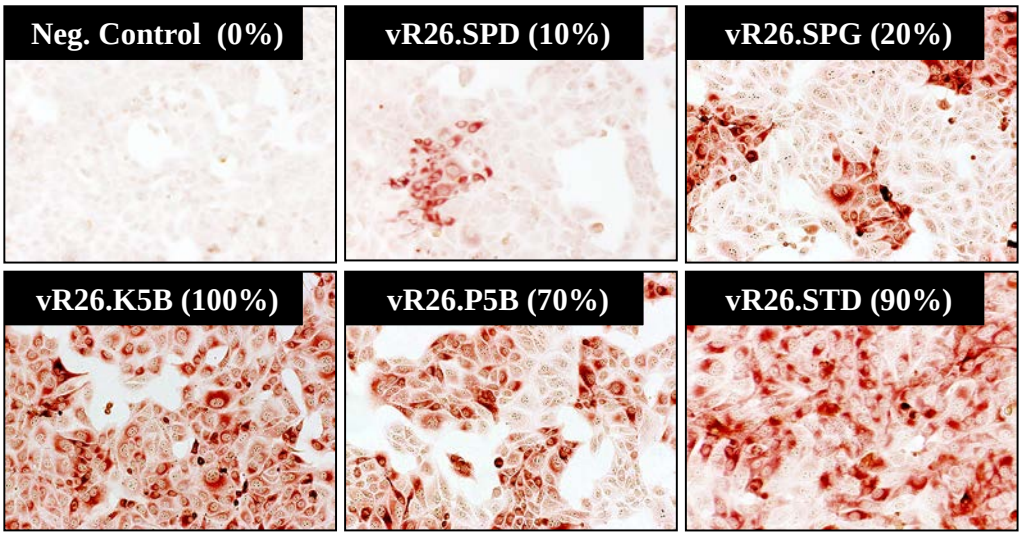
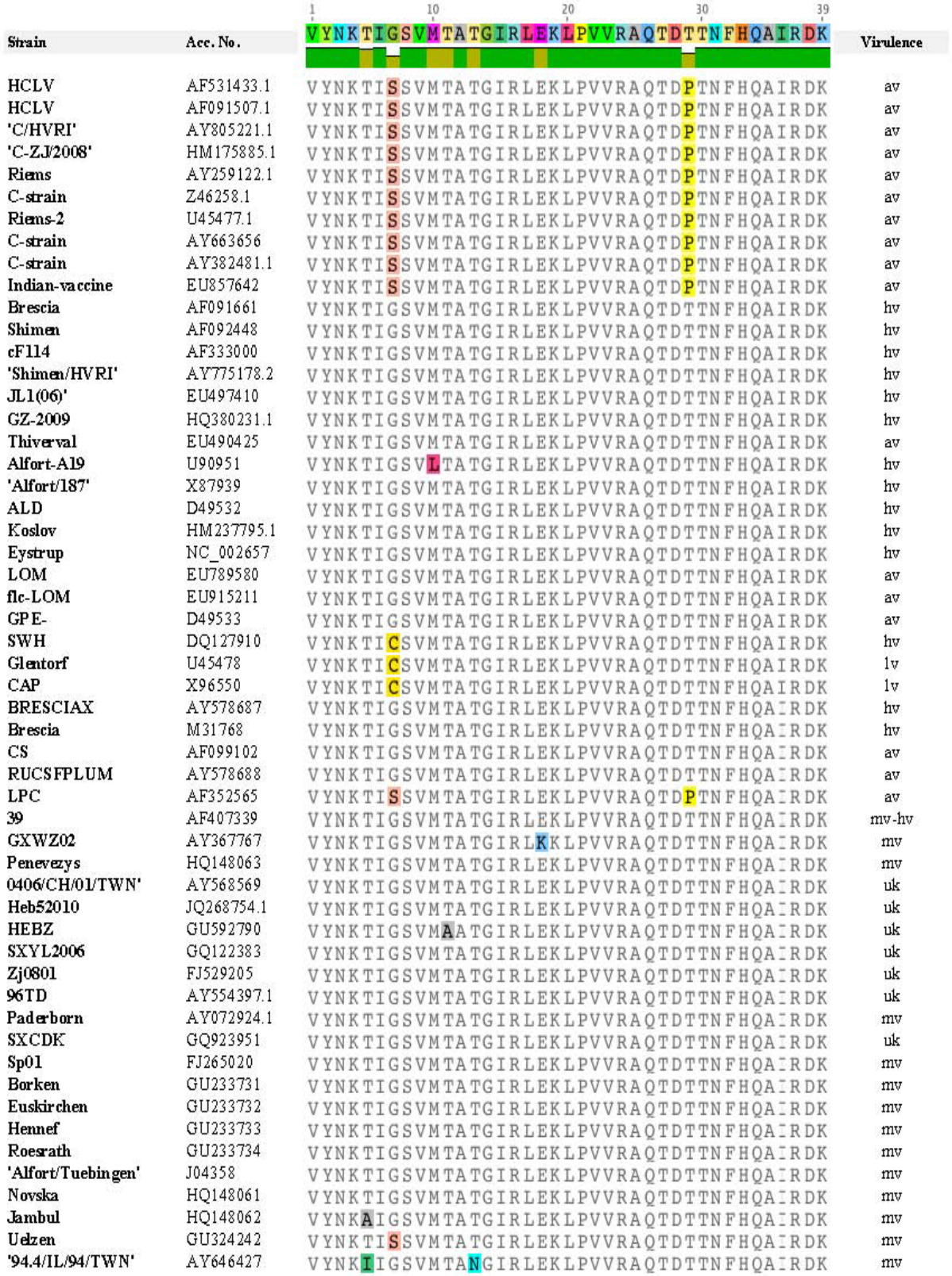


Figure 7. Virus spread in cell monolayers. Cells were electroporated with RNA and virus infected cells were visualised at 48 hpe by polyclonal immunoperoxidase staining using brightfield microscopy (10x magnification), for visual inspection of the infected cell foci .

Table 1. Oligonucleotides used in this study.

Primer name	Sequence 5´- 3´
5'C-strain_T7	TAATACGACTCACTATAGTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATC
3´UTR_R	GGGCCGTTAGGAAATTACCTTAGTCCAACTGTGGA
KosRi_Rluc974F	AAATTAAAAATAGCCCCAAAAGAGCATGAGAAGGACAGCATGACTTCGAAAGTTTATGATCCA
Riems_Rluc2329_2A_R	GCAGATGAGGAATGCAATGGTTGATGCGCTATTCCAGACGGGCCCAGGGTTGGACTCCACATCTCCA
	GCCAACTTGAGCAGGTCAAAGTTCAGCAGTTGTTCATTTTTGAGAACTCGC
BbRi_PaKosRi_NS5B_F	GGTGCTAAAGTGTATTCTATGAAACTGAGTAATTGGGTGATGCAAGAAGA
BbRi_KosRi_NS5B_R	TCATGCCCCTCTCCCTATCAGCGTCATCATCATGACTCTCAGCCTCCT
BbRi_Pader_NS5B_R	TCATGCCCCTCTCCCTATCAGCGTCATCATCATAACTCTCAGCCTCCT
R26.6_SPtoGT	GTGTATAACAAGACAATAGGCTCAGTAATGACAGCTACTGGTATCAGGTTGGAGAAGTTGCCCGTGG
	TTAGGGCCCAGACAGACACAACCAACTTCCA
R26NS5B_Gly_F	GTGTATAACAAGACAATAGGCTCAGTAATGACAGCTACTGGTATC
R26NS5B_Thr_F	AGGGCCCAGACAGACACAACCAACTTCCACCAAGCAATAAGG

Underlined letters indicate a T7 promotor site,



Supplementary figure 1 (S1). Alignment of a 39 aa region from pos. 134 to 172 in the CSFV NS5B protein derived from 54 full genome sequences retrieved from Genbank. The presence of residues S140 and P162 is observed in avirulent (av) strains, whereas the presence of G and T at these positions, are predominatly

found in highly virulent (hv) and moderately virulent (mv) and low virulent (lv) strains. uk=unknown virulence.

PART 4. Conclusions & future perspectives

General conclusions and future perspectives

A major breakthrough for the molecular characterization of CSFV was achieved with the availability of a full genome sequence and infectious clone (Meyers *et al*., 1989). The elucidation of cleavage events and the processing of the polyprotein into the mature viral proteins was another step closer to the understanding of how the viral proteins were involved in the replication process and in unlocking the host defence mechanisms (Rumenapf *et al*., 1993). Investigations related to the pathogenicity of CSFV peaked in the beginning of the 2000´s in the aftermath of the devastating outbreaks of CSF in the Netherlands 1997 and the UK in 2000. Among other things, efforts have been put into building a global sequence database of collected CSFV samples for keeping track of the outbreak situation globally and to aim at improved eradication strategies and for determination of the genetic traits in the CSFV genome from comparisons of the CSFV sequences[4]. Live-attenuated RNA viruses still make the most efficient vaccines and provide the best immunity against CSFV. Deeper knowledge and elucidation of traits involved in viral fitness is thus of high interest.

The data and experiments carried out during the three years of research have shed light on the subject and addressed answers to the milestones as contemplated in the original project description. In the thesis, two state-of-the art cloning approaches, one a PCR based method (*in vitro*) and another one utilizing homologous recombineering (*in vivo*), have been crucial for the generation of the viral constructs. Both methods proved efficient at producing successful constructs as verified by full-genome sequencing. The initial part of the PhD was focused on the application of the homologous recombineering approach also termed Red/ET recombination. The system is applicable to any modifications in a BAC driven system that contains the full-length cDNA of CSFV. In example the 9 aa TAVSPTTLR epitope was substituted with similiar epitope sequence of BDV "Gifhorn" (Manuscript I). The choice of using the single-copy BAC system was a necessity for proper use of the Red/ET system, but also due to its capability for maintaining the genetic stability of the insert. This method is described in details in manuscript I. These cloning methods facilitated efficient and precise manipulation of the viral cDNA. Of the two, the PCR based method in the end proved to be the fastest and still very efficacious cloning strategy. The drawback of using the megaPCR approach for the amplification of a 20Kb amplicon would be the concerns of introducing unintended mutations into the amplicon. This was circumvented by the use of high-fidelity proof reading polymerases, and full-genome sequencing showed that these concerns were not a problem. Limitations to the target-size and proof-reading activity could however very well pose a problem for larger amplicons. The

[4] http://ec.europa.eu/research/agriculture/projects_showcase09_en.htm

application of the Red/ET cloning method was optimized and thus would potentially be a preferred choice in the clonings and manipulations of larger inserts/genomes.

The analysis of the replication processes and efforts to identify the determinants for efficient replication has been the main subject of the thesis. Replication competence can be monitored using replicons in a closed cell system in which horizontal transfer of viruses between cells are prevented due to deletion of the structural virus proteins which are required for virus assembly and cell attachment, but where the replication machinery is still kept intact. It was contemplated initially to develop and implement a replicon-based assay containing a reporter protein to be applicable as a tool for easy monitoring of replication competence. Integration of reporter protein coding sequences were designed in a way that kept the constructs monocistronic and thus with a translation initiation mechanism unmodified from the original virus. New CSFV replicon constructs based on the "Paderborn" strain (Manuscript II) and the "C-strain Riems" (Manuscript III) were developed. In addition to these, more replicons were produced that contained other deletions, but as shown in manuscript II, inclusion of the E2 protein was advantageous for the replication. Bioluminescent reporter proteins *Renilla* and *Gaussia* luciferases (Rluc and Gluc) were evaluated for their utility in this regard, and were both proven applicable (see Manuscript II). Rluc replicons were the standard assaying method throughout this thesis work, and a 24 hour assay proved sufficient to do comparative evaluations of genome regions from different CSFV strains using the reporter construct as a backbone. The Gluc replicon excels in its ease of use due to the secreted nature of the reporter protein, and thus sample harvesting could be carried out directly from the media/supernant without the need for cell lysis (as in the Rluc reporter assay). The use of Gluc (or other secreted reporter proteins) to our knowledge has never been described for CSFV before, but it is a promising tool, due to its easy application. For the Gluc reporter assay, conditions should be further optimized and fine tuned for future applications. In manuscript II it was shown that increasing amounts of Gluc could be monitored over time, and that a doubling in RNA amounts caused a doubling of luciferase activity as expected..

Swapping regions between related viral strains, should be beneficial for the identification of non-compatible regions or other regions enhancing or impairing replication competence. Swapping the NS2/3 protein coding regions (encoding helicase, NTPase and serine protease activities) from a CSFV Paderborn (genotype 2.1) virus with the corresponding region from a genotype 1.1 virus led to the production of a non-replicating genome. The reason could be in relation to incompatibilities of a genotype 2.1 NS4A (co-factor for NS3 protease) or other cis-acting elements to a genotype 1.1 NS2/3 protein. On the other hand swapping the core enzyme for replication (the NS5B protein) in between the genotypes was compatible and indeed influenced replication competence. This was shown using

the Paderborn genome as a backbone in manuscript II and the "C-strain Riems" in manuscript III. In both studies, the Koslov RdRp increased replication significantly.

A central point of interest has been the particular features of genotype 1.1 viruses including both the avirulent vaccine “C-strain Riems” to the highly virulent strain “Koslov”. The reason for the special interest in the comparison between these genotype 1.1 strains is their limited sequence diversification. The "Koslov" and "C-strain Riems" are 96,2% identical at the nucleotide level and show 97,7 % aa identity. In the whole genome the two strains only differ by ca. 92 amino acids. An ideal approach would be to mutate each of these positions to identify which of these that could upregulate the properties of virulence or replication. It was obvious from the constructs containing destinct NS5Bs, that Koslov NS5B has a significant impact on replication competence (manuscript II+III). For manuscript III, special attention was given to this NS5B protein, that varies at nine aa positions between the two strains. Most of these changes were due to conservative substitutions that should have no impact on physical properties in the protein. Two residues however, at pos. 140 and 162 in the NS5B protein is due to non-conservative substitutions. "C-strain" derivatives entirely contain S140 and P162 (SP variant) whereas "Koslov", and also "Paderborn", contain G140 and T162 (GT variant). It was then speculated that if substituting S140G and P162T in the Riems NS5B protein it would lead to enhanced replication competence. Interestingly the GT variant in "C-strain Riems", and also the in between variant "GP", did not increase replication competence in the replicon containing these modified NS5Bs. Unexpectedly however, the "ST" variant (S140 and T162) replicated to same extend as was seen in "Koslov". For verification of these conclusions drawn from the replicon assay in manuscript III, analogous genome modifications as used in the replicon studies were introduced into the backbone of infectious clones and multistep growth curves were carried out to monitor the *in vitro* properties of these rescued viruses. Indeed, these studies produced similar results as obtained with the replicon tool. It was found that individual amino acid residues in NS5B were important for replication competence. Interestingly, it was found that especially aa residue at position 162 in NS5B seemed to influence both 1) the speed of replication and 2) the viral spread in cell culture (increased size of infected cell foci). By visual inspection of the monolayer staining it appears that the plaques are enlarged due to the activity of the Paderborn and Koslov strain RNA polymerases as compared to the activity of the viruses containing the native Riems enzyme and a single amino acid residue in this protein was shown to be key to determining the replication competence (see below).

In silico folding of variants of NS5B, interestingly showed variations of sheet structures at aa positions 153-154 and 277-279. An extended sheet structure at 153- 154 but not at 277-279 was present in the "GT" variant, while wt "SP" variant has the opposite. The "GP" variant has sheet structures at both positions while the "ST" variant in contrast has no sheet structure, and interestingly closely resembled the K5B and P5B NS5B structures (manuscript III). This structural similarity between "ST" variant

and K5B may explain the similar replication rates of the two, and indicate the impact of aa 162. Future studies should reveal if additional mutations in the GT variant also can produce a similar tertiary structure to prove the importance of this conformation in replication efficiency. Considering the few aa substitutions in between the genotype 1.1 NS5Bs, it should be amenable to produce clones carrying each of these intermediate NS5Bs and check for their properties in cell culture.

During the three years of research I have in addittion worked on several pilot studies, either not mentioned or just briefly mentioned in this thesis. One of the most promising methods was the works on spinach tagging. The idea was conceived during my research stay at AHVLA, Weybridge. Since the spinach sequence contains stop codons in all 6 reading frames, integration into a reading frame would be fatal and thus attempts to integrate the sequence into the Paderborn 3´UTR instead were made. Constructs were generated (both infectious clones and replicons) that contained the spinach sequence plus a tRNA scaffold (in total ca. 110 nt), and the inserts were verified by full genome sequencing. None of the manipulations, unfortunately, exhibited significant replication, and thus were not suitable for further investigation (personal note). Another interesting method was the application of the SK6.T7 cell line. This method enabled the rescue of live CSFV viruses from plasmids containing the entire cDNA CSFV genome and an upstream T7 promotor sequence. (van Gennip *et al*., 1999). The cell line was kindly shipped to me by Van Gennips lab, and I optimized a method for stable transfection (lipofection) of a longPCR product and showed that the efficiency of this method was up to 100 fold higher compared to our standard virus rescue method. A small-scale work on the trans-encapsidation/trans-complementation of the rPad2RL replicon with the structural proteins was carried out, and indicated that an enhanced luciferase signal was present in the trans-complemented VRP in contrast to the non-complemented replicon. The application of this kind of VRP could have potential for delivering "cargo" in to the cell in form of a replicon or other kinds of nucleic acid/proteins to the cell and thus obviating viral contamination in form of infectious viruses.

The research carried out in this thesis have shed some light on the requirements for efficient viral replication, but also raised some new questions in this regard. The results from replicons and the infectious clones showed that the NS5B of Koslov indeed had an increased replication rate compared to that of the vaccine strain. Future work should enlarge the span of of the various virulence types, in order to investigate if the enhanced replication and viral shedding is a trait of virulence, and also animal trials should be conducted testing the virulence of the different strains (e.g. variant STG). Future work could be addressed as well to check for other compatibilities (or not) between regions of the genome from different genotypes in order to investigate the properties of other cis-acting elements important for the viral fitness.

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National Veterinary Institute
Technical University of Denmark

Bülowsvej 27
1870 Frederiksberg C

www.vet.dtu.dk