Veterinary Microbiology 192 (2016) 123–134

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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

Johanne Hadsbjerg[4], Martin B. Friis[1,4], Ulrik Fahnøe[2], Jens Nielsen[3], Graham J. Belsham[*], Thomas Bruun Rasmussen

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

ARTICLE INFO

*Article history:*
Received 15 January 2016
Received in revised form 24 June 2016
Accepted 6 July 2016

*Keywords:*
Pestivirus
Next generation sequencing (NGS)
Attenuation
IRES
Reverse genetics

ABSTRACT

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



## 1. Introduction

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

* Corresponding author.
*E-mail address:* grbe@vet.dtu.dk (G.J. Belsham).
[1] Current address: Department of Clinical Microbiology, Herlev Hospital, Herlev Ringvej 75, Herlev 2730, Denmark.
[2] Current address: Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases and Clinical Research Centre, Hvidovre Hospital and Department of International Health, Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.
[3] Current address: Statens Serum Institut, Artillerivej 5, Copenhagen 2300, Denmark.
[4] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.vetmic.2016.07.004
0378-1135/

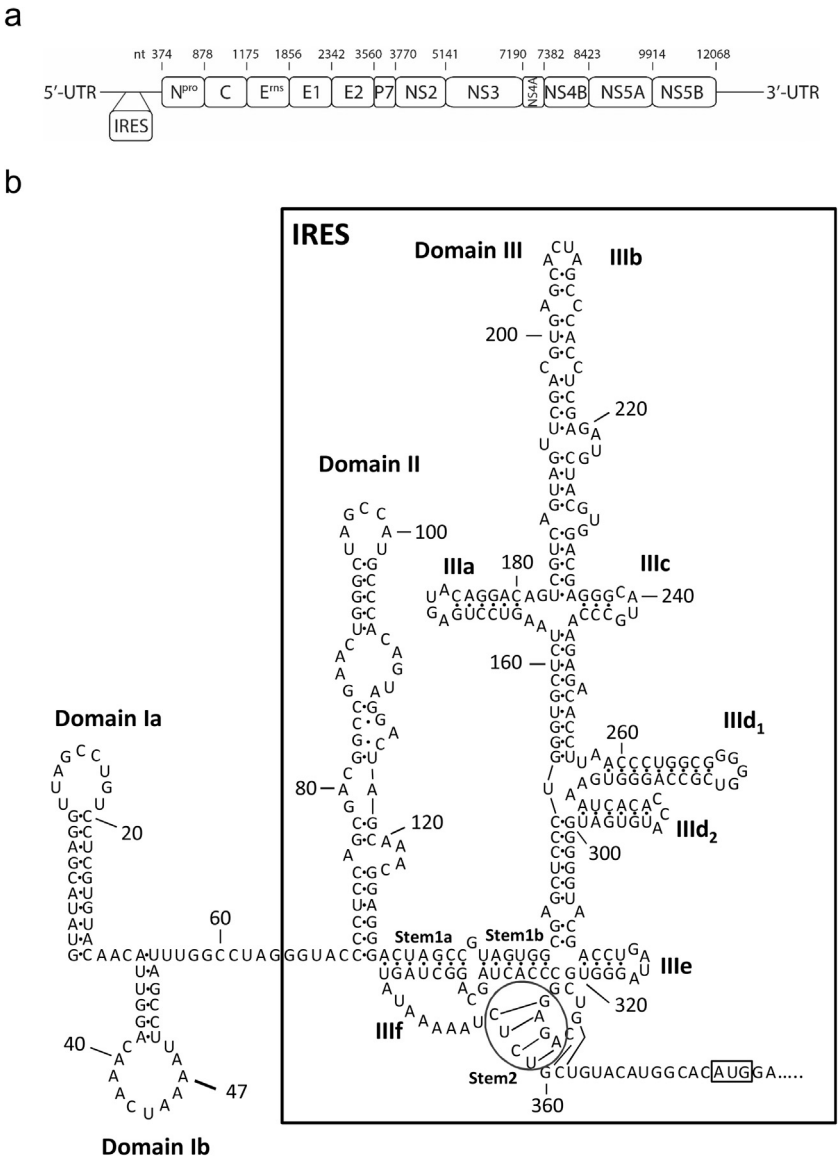


**Fig. 1.** Structure of the CSFV genome. (a) Genome organisation of CSFV indicating the location of the IRES and the individual protein coding regions. (b) Secondary structure and sequence of the CSFV IRES. The location of stem 2 within the pseudoknot (subdomain IIIf) is marked by an oval.

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

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

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