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

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
\textsuperscript{ms}, E1 and E2) plus eight non-structural proteins (N\textsuperscript{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
\textsuperscript{ms}, E1 and E2 and also the non-structural proteins N\textsuperscript{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
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, Ems, 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, Ems 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 cotranslational 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
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. 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.](http://vir.sgmjournals.org)
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 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. 2. Expression of CSFV proteins by the replicons.](image)

![Fig. 3. Rluc reporter protein expression by replicons.](image)

![Fig. 4. Characterization of the Rluc reporter protein expressed by replicons.](image)
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, rPad2GLΔ2, 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 strain (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 10640 nt with the inclusion of the Rluc–2A sequence) than the full-length genome (12296 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.
Reimann and M. Beer (personal communication) and these molecules retained a functional E2 sequence, but deleted all of Erns. A similar Erns-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...

**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.
Determinants of CSFV replication

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% CO₂) 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 T1R (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 NrpsLfor with E2P7rpsLRev1 and Erns1787rpsLFor with Erns1787rpsLRev2 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 986–3541) and introduction of the Rluc sequence was achieved by production of a fragment (P10delCtoE2_RLucz2A, ~1100 bp) using primers Pader10NproCrlucFor and Pader10P7E2_2A_RlucRev with plasmid pBBRLuc (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 pRluc2A replicon was made by making an in-frame deletion using two HindIII restriction sites in the Paderborn cDNA.
the sites (nt 9401 and 11728) 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). Megaprimers P10delCtoE1_Gluc2A (~700 bp) were made using primers P10C974Glu-fw and P10E12329Glu2A_Rev in a standard PCR using pCMV-Gaussia Luc (Thermo Scientific) as template and Phusion hot start II HF DNA polymerase (Thermo Scientific). The megaprimers were digested with DpuI, purified using a GeneJET PCR purification kit (Thermo Scientific) and electroporated into DH10B (Invitrogen) competent cells to obtain infectious RNA transcript. The PCR products (~3500 bp) were recovered using the GeneJET PCR clean-up kit and eluted in 2 μl DEPC-treated water (95 °C). DNA was amplificated 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 (Thermo Scientific). The RNA was stored at −80 °C until use.

Replicon assay procedure. For each replicon to be assayed, 1 ml SFT-R cells (2 × 10⁶ ml⁻¹) was centrifuged and resuspended in the same volume of cold PBS and kept on ice. An aliquot (800 μl, 1.6 × 10⁶ 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 XCcell; 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 × 10⁵ 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 × 10⁵ 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, T₀. Plates were then incubated at 37 °C in 5 % CO₂. 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 lysis into 100 μl coelenterazine 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 (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-lg secondary antibodies conjugated to HRP (PI061 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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