Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Reverse genetic manipulation of the overlapping coding regions for structural proteins of the type II porcine reproductive and respiratory syndrome virus

Dandan Yu, Jian Lv, Zhi Sun, Haihong Zheng, Jiaqi Lu, Shishan Yuan *

Department of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, the Key Laboratory of Animal Parasitology, Chinese Ministry of Agriculture, Shanghai 200241, China

A R T I C L E   I N F O

Article history:
Received 29 May 2008
Returned to author for revision 28 June 2008
Accepted 7 September 2008
Available online 5 November 2008

Keywords:
PRRSV
Structural protein
Overlapping coding region
Reverse genetic manipulation
Transcription regulating sequence
TRS

A B S T R A C T

The overlapping genomic regions coding for structural proteins of porcine reproductive and respiratory syndrome virus (PRRSV) poses problems for molecular dissection of the virus replication process. We constructed five mutant full-length cDNA clones with the overlapping regions unwound and 1 to 3 restriction sites inserted between two adjacent ORFs (ORF1/2, ORF4/5, ORF5/6, ORF 6/7 and ORF7/3′ UTR), which generated the recombinant viruses. Our findings demonstrated that 1) the overlapping structural protein ORFs can be physically separated, and is dispensable for virus viability; 2) such ORF separations did not interrupt the subgenomic RNA synthesis; 3) the plaque morphology, growth kinetics, and antigenicity of these mutant viruses were virtually indistinguishable from those of the parental virus in cultured cells; and 4) these mutant viruses remained genetic stable in vitro. This study lays a foundation for further molecular dissection of PRRSV replication process, and development of genetically tagged vaccines against PRRS.

© 2008 Elsevier Inc. All rights reserved.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in pregnant sows and respiratory problems in all ages of pigs (Albina, 1997; Christianson and Joo, 1994; Done and Paton, 1995). PRRS was first reported in the United States in 1987 (Kelfaber, 1989), and it is still one of the most severe epidemic diseases threatening the swine industry worldwide. This is particularly true in China, where a highly pathogenic PRRS virus (PRRSV) variant strain has been causing devastating epidemic, so-called “porcine high fever disease (PHFD)”, for the past two to three years (An et al., 2007; Tian et al., 2007; Tong et al., 2007). Traditional vaccines displayed limited cross-protection efficacy, and better PRRS vaccine is in dire need to control PHFD.

PRRSV is further classified into two distinct genotypes, (European Type I (Wensvoort et al., 1991) and (North American type) II (Benfield et al., 1992). As a member of the Arteriviridae of the order Nidovirales (Snijder and Meulenberg, 1998), PRRSV is similar to lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) in terms of genomic structure, the mechanisms employed for replication and transcription. PRRSV genome is a single-stranded, positive-sense RNA of approximately 15 kb in length (Meulenberg et al., 1993), with a 5′ cap and 3′ poly(A) tail. The genomic coding region was flanked by terminal untranslated regions (UTR), 5′- and 3′-UTR, which play key regulatory roles in the viral life cycle (Shanmukhappa et al., 2000; Sun et al., 2007; Verheije et al., 2001, 2002a). The genome includes at least 9 open reading frames (ORFs), designated ORF1a, ORF1b, ORF2a, ORF2b, and ORF3–7. ORF2–7 encode at least 7 structural proteins including glycoprotein 2 (GP2), GP3, GP4, GP5, Membrane (M) protein, Nucleocapsid (N) protein, and the recently recognized ORF2b-encoded 73 amino acid nonglycosylated (E) protein (Mardassi et al., 1996; Meulenberg et al., 1993; Meulenberg, 2000; Nelsen et al., 1999; Saito et al., 1996; Thiel et al., 1993; Wootton et al., 2000; Wu et al., 2005). The functions of the structural proteins play in the virus replication process warrant further study. For instance, there is no experimental evidence supporting the assumption that GP5 is the viral attachment protein, while it is under debate if GP3 is a structural constituent of the PRRSV virion (Mardassi et al., 1996; Meulenberg et al., 1993; Meulenberg, 2000).

Arteriviral structural proteins are expressed through a set of co-terminal subgenomic mRNAs (sgmRNA), which are believed to be generated via a Nidovirus-specific, not yet fully-understood discontinuous transcription. Several lines of evidence support two models: a leader-primed transcription model, with another involving the discontinuous extension of minus-strand RNA. Based on these models, the transcription-regulating sequence (TRS, UUAACC for PRRSV) of the Leader (5′ UTR in PRRSV) and the downstream Body TRS (TRS-B), preceding the ORF coding region of the structural protein in the viral genome play key roles in discontinuous transcription (Pasternak et al., 2006). Base-pairing between the
Leader TRS (TRS-L) and the complementary sequences in the minus-strand of the TRS-B mediates discontinuous jumping during synthesis of the minus-strand templates for EAV sgRNAs (van Marle et al., 1999). TRS-flanking sequence could also affect nidooviral transcription (Curtis et al., 2004; Nelsen et al., 1999; Pasternak et al., 2004, 2006; Sola et al., 2005; Yount et al., 2006). In addition, Pasternak et al. (2004) engineered several copies of a body TRS-flanking sequence cassette inserted into an EAV full-length clone-derived replicon, and observed that sgRNA abundance progressively favor smaller RNA species, implying that downstream TRS-B could exert negative effect on the upstream one.

The specific mechanism(s) underlying subgenomic RNA transcription of PRRSV have not been elucidated clearly. For instance, numerous TRS-like sequences exist in PRRSV genome, and non-canonical TRSs were detected for all individual PRRSV sgRNAs (Nelson et al., 1993; Meng et al., 1995; Zheng et al., unpublished data). In addition, the role of TRS-flanking sequence plays for PRRSV sgRNA synthesis remains to be known.

The successful development of infectious clones of EAV (de Vries et al., 2000; van Dinten et al., 1997) and PRRSV (Calvert et al., 2002, 2003; Fang et al., 2006a; Meulenberg et al., 1998; Nielsen et al., 2003; Truong et al., 2004; Yuan and Wei, 2008) opens new avenue for molecular dissection of the arteriviral RNA replication and transcription. These infectious clones have also provided powerful tools for the development of genetically engineered vaccines that can be differentiable with PRRSV field isolates, a desirable trait of vaccine for ultimate eradication of PRRS. Furthermore, such reverse genetic system (RGS) provides an excellent platform for functional dissection of arteriviral structural proteins. However, it is known that variable length of overlapping coding sequence shared by the adjacent ORFs of the structural proteins. For example, an overlapping region of 255 nt exists between ORF3/4 of Type I PRRSV, while 15 and 10 nt span the coding regions between ORF5/6 and ORF6/7, respectively (Meng et al., 1995; Meulenberg and Petersen-den Besten, 1996; Morozov et al., 1995; Nelsen et al., 1999). The biological significance of these overlapping regions on PRRSV replication is unknown. On the other hand, such overlapping nature poses problem for genetic manipulations of an individual structural protein ORF without affecting the adjacent one. Aiming to define the cell tropism determinant, Verheije et al. (2002b) constructed chimeric arteriviruses using Type I PRRSV infectious clone as backbone expressing the terminal domains of EAV GP5 and M protein, and found that these assumed viral attachment protein GP5 or M failed to change cell tropism as expected. In addition, these authors found that separation of the overlapping regions was critical for viral viability for such chimeric viruses, implying that arterivirus gene expression is tightly regulated. de Vries et al. (2000) demonstrated that while the precise arrangement of the overlapping regions for EAV structural proteins was also necessary for viral infection, it was possible to rescue virus from transfected cells when overlapping regions between ORF4/5, ORF5/6, and ORF4/5/6 in EAV infectious clones were separated by inserting polylinkers. Although numerous PRRSV infectious clones have been successfully constructed (Calvert et al., 2002, 2003; Fang et al., 2006a; Meulenberg et al., 1998; Nielsen et al., 2003; Truong et al., 2004; Yuan and Wei, 2008), the issue of overlapping coding regions of the structural proteins still impedes functional studies.

To facilitate further reverse genetic manipulation of PRRSV, we investigated the importance of the arrangement of overlapping genes of the structural proteins of a North American PRRSV (Yuan and Wei, 2008). We separated the overlapping sequences existing between the beginning and end of ORF5/6 and ORF6/7, and inserted the restriction enzyme sites (Pac I, Ssa I, and Asc I) through a reverse genetic system (RGS) approach based on an attenuated, PRRSV infectious clones pAPRRS (Yuan and Wei, 2008). We also directly inserted Pac I, Ssa I, and Asc I sites between ORF1/2, and inserted a unique restriction enzyme site (Nde I) between ORF4/5, and ORF7/3' UTR. A series of mutation viruses were recovered after transfection of cells using in vitro RNA transcripts of the constructed full-length mutant DNA clones. Our findings demonstrated that 1) the overlapping nature of the coding regions for structural proteins is dispensable for the PRRSV viability; 2) the insertion of foreign nucleotide sequence is feasible for physical separation of the ORFs coding for structural proteins, and such separation did not interrupt the subgenomic RNA synthesis; 3) the plaque morphology, growth kinetics, and antigenicity of these mutant viruses were virtually indistinguishable from those of the parental virus in cultured cells; and 4) these mutant viruses remained genetic stable for at least five passages in vitro culture system. This study lays a foundation for further molecular dissection of PRRSV replication process, and development of genetically tagged vaccines against PRRS.

**Results**

**Rescue of the PRRSV mutant virus with unwound ORF overlapping region**

To facilitate PRRSV reverse genetic manipulation, and investigate the significance of the overlapping coding sequences of PRRSV structural proteins, we generated a series of mutant plasmids with overlapping regions separated by inserting 1 to 3 restriction sites. As shown in Fig. 1, pORF12 had Pac I, Ssa I, and Asc I sites inserted between ORF1/2. Meanwhile, an Nde I restriction site was created 3′ to ORF5/6, and ORF7/3′ by inserting ATG between ORF7/3′ UTR in similar manner. pORF56c and pORF673 were constructed by inserting restriction sites (Pac I, Ssa I, and Asc I) in the tandem overlapping regions of ORF5/6 and ORF6/7. Sequencing analysis revealed that these constructs were all consistent with the original design, demonstrating that we had obtained a series of full-length mutant plasmids with ORF overlapping regions separated and inserted with restriction sites.

Typical PRRSV cytopathic effect (CPE) developed at day 4 post-transfection in Marc-145 cells transfected with in vitro RNA transcripts derived from all pORF12, p5ND7, pORF673, and pORF7/3′ UTR, while not in mock-transfected control. The CPE developed in all ORF-separation mutants resembled to that of the parental infectious clone, pAPRRS. The supernatant of the cell culture containing the rescued viruses were harvested and aliquotted, kept at −80 °C, and designated the initial generation (P₀). An aliquot (200 μl) of 1000 fold diluted P₀ viral liquid was used to infect fresh Marc-145 cells. The supernatant of cell culture was harvested at 5 dpi, designated P₁, aliquoted and frozen. Serial passage was conducted to generate passage P₂−P₅.

To prove that the rescued viruses were indeed from the transfected synthetic RNAs, IFA were conducted. As shown in Fig. 2, the supernatant of the transfected cells contains PRRSV viruses, evidenced by positive staining of the infected cells against Nsp2 monoclonal antibody of PRRSV. In addition, the result indicated that all mutant viruses including vORF12 (Panel A), v5ND7 (B), vORF56c (C), vORF673 (D), and vORF7/3′ (E) shared the similar Nsp2 distribution pattern with the parental virus (data not shown). These results demonstrated that the rescued viruses from all full-length mutant cDNA clones were PRRSV-specific, as no staining was detected in negative control (Fig. 2, Panel F).

**The mutant PRRSV viruses remained genetically stable in vitro**

To further identify if the mutant viruses retained the designed mutations and remained genetically stable during serial passage, P₁ and P₅ of the rescued viruses were used for RT-PCR and nucleotide sequence analysis with primer pairs flanking the mutation sites. The correct size of RT-PCR products were amplified from all of the rescued viruses for both passage level (data not shown), indicating there is no dramatic disturbance in the insertion regions. Nucleotide sequencing...
results revealed that the foreign insertion sequences were retained in P₁ viruses of all rescued viruses (data not shown), indicating the latter were indeed generated from the transfection of the synthetic RNAs from the designed full-length mutant cDNA clones. Moreover, nucleotide sequence comparisons between the corresponding regions of the mutant plasmid, P₅, and P₇ viruses showed that there were no other mutations in the flanking region (1–2 kb) of the inserted restriction sites. These results demonstrated that the overlapping nature of coding sequences between adjacent ORFs of the structural proteins is not essential for virus viability, and is genetically stable in...
at least the 1–2 kb flanking region of the mutation site for at least five passages in cultured cells.

The separation of overlapping regions does not alter the wild-type virological characteristics of the mutant viruses

Genetic manipulation of the PRRSV structural protein coding regions could affect virological properties. To address this, we performed viral plaque morphological analysis of the fifth passage of the mutant viruses rescued from full-length mutant plasmids pORF12, p5ND7, pORF56c, pORF673, pORF7Ua, and pAPRRS (Fig. 3A). Virus plaque morphology remained essentially the same with that of the parental virus, vAPRRS. Multiple-step growth curves of the mutant viruses were measured by determining the virus titres (PFU/ml) of the supernatant collected at the indicated time points. As shown in Fig. 3B, the titer of the parental virus vAPRRS (□) was consistently slightly higher than those of the five mutant viruses, and peaked at 72 hpi. In contrast, the mutant viruses lagged 12 h in reaching maximum titer. In particular, the peak titer of vORF56c (▽) was 7.58×10⁴ PFU ml⁻¹ at 84 h, considerably lower than the 1.51×10⁵ PFU ml⁻¹ at 72 hpi of wild-type virus, indicating that the insertion between ORF 5 and 6 could adversely affect the virus growing ability. However, the overall pattern of the growth curve was consistent, which suggests that insertion of 3–23 nt between the ORFs did not lethally affect viral growth.

Discussion

As a relatively small RNA virus, arterivirus utilized a rather sophisticated gene expression strategy in terms of genomic organization, genomic and subgenomic RNA synthesis, mRNA translation, and downstream virus replication process. Unlike its cousins of arteriviruses and coronavirus, the viral replication process and control thereof is poorly understood. It is not yet known if PRRSV adopts the same transcriptional regulatory mechanisms with other nido- or arteriviruses. It also remains further investigation what roles the structural proteins play in the process of PRRSV infection. As the most economically significant swine disease, a better vaccine based on reverse genetic manipulation would be of particular interest to combat the ever-evolving pathogen.

To overcome the tight genomic organization of arterivirus, de Vries et al. (2001) engineered recombinant EAV containing separated ORF5/6 with overlapping sequences unwound as restriction sites, in which a GFP was inserted with an EAV-specific transcription regulation sequence. However, the recombinant EAV-GFP virus was gradually lost during passage on cell culture. In the similar manner, Groot Bramel-Verheije et al. (2000) successfully separated ORF6 and 7 of the prototypic European LV strain, sharing a mere 64% nucleotide identity with North American type. These authors further

The rescued viruses maintain the PRRSV subgenomic RNA profile

To further define if the mutant viruses retained the viral RNA profiles in infected cells, northern blotting analysis was conducted using a synthetic biotin-labeled probe complementary to the 3' UTR, shared by all viral RNA species. Consistent with the parental virus vAPRRS, all mutant viruses produced at least seven sgRNAs in the Passage 5 virus infected cells (sgmRNA2–7, Fig. 4A). Additionally, two subgenomic RNAs (Fig. 4A, black arrow) were also detected between mRNNA2 and genomic RNA, which are similar in size to some heterocline RNA molecules associated with PRRSV VR2332 strain described previously (Yuan et al., 2000). These results indicated that genetic manipulation of the structural gene-encoding region did not impose visible changes to the viral mRNA pattern. However, the abundance of individual mRNA species, especially sgmRNA2 and 3 (Fig. 4A, arrow head) of vORF56c is visibly lower than those of other viruses, while the downstream sgmRNA7 and genomic RNA displayed the same abundance.

We next assessed the impact of restriction site insertion on mutant viral mRNA transcription, especially the utilization of the respective TRS-B, to which the local flanking sequence and secondary structure could be altered because of the insertional mutations. To do so, we designed the forward primer in the genomic leader region and the reverse primer located in the individual ORF of the structural protein, such that specific discontinuous jumping site of the Body part to the Leader can determined. As shown in Fig. 5, The Leader–Body junction of sgRNAs for v5ND7 (Fig. 5A), sgmRNA6 (Fig. 5B) and sgmRNA6.1 (Fig. 5C) for vORF56c, and sgmRNA7.1 (Fig. 5D) and sgmRNA7.2 (Fig. 5E) for vORF673 were determined by subgenomic RNA-specific RT-PCR. Nucleotide sequence analysis of each RT-PCR product revealed that the same TRS-B was employed for individual sgmRNA synthesis by the respective mutant virus and the parental virus (data not shown). Meanwhile, the inserted nucleotide sequences were observed in their corresponding locations (Fig. 5, boxed), except for vORF56c, of which the noncanonical TRS-B6.1 (Fig. 5C) located 255 nucleotides downstream the ORF6 start codon. However, the same noncanonical TRS-B6.1 was also found in the parental virus infected cells (Yuan et al., unpublished observation). It remains further investigated if the TRS-B6.1 mediated sgmRNA encodes, if any, a truncated form of M or extended sgmRNA7 and/or nucleocapsid. Overall, these results suggesting that these mutations did not deleteriously affect viral subgenomic transcription.
fused a 9 amino acids hemagglutinin (HA) tag of the avian influenza virus. The insertion was identified by the fourth generation of the recombinant virus, implying that the gene expression strategy is highly regulated. In the present study, all of the designed mutant cDNA clones of a North American PRRSV generated viable viruses, demonstrating that the overlapping nature of the structural ORFs is dispensable for virus replication. This is the first report on whole separation of structural protein coding regions of a North American type PRRSV, and provided a platform for further discussion of virus replication and developing genetically engineered vaccine.

Although the mutant viruses shared the similar characteristics with their parental virus, it was apparent that the growth rate of vORF56c is decreased comparing with vORF673, in which the same three restriction sites were inserted between ORF5/6 and ORF6/7. Viral plaque assay and IFA results revealed no significant virological differences between ORF56c and other viruses. However, as shown in Fig. 4A, the insertion between ORF5/6 might adversely affected synthesis of the upstream sgRNA2 and 3. Paradoxically, the sgRNA4 abundance was increased during the ORF5/6 insertion. Though quantitative study is warranted to measure the relative mRNA abundance, it appeared that insertion into certain region of the PRRSV may disturb the relative sgRNA levels and thus virus production. These results indicating that the inserted site and/or sequence nature of the inserted gene has to be selective for attempt to use PRRSV as an expression vector.

Although TRS-L and complementary TRS-B base-pairing plays key roles in the discontinuous transcription process of CoV and Arterivirus sgRNA (Pasternak et al., 2005; van Marle et al., 1999), these are not the only factors affecting nidoviral transcription. Indeed, the flanking sequences of TRS influence sgRNA transcription (Curtis et al., 2004; Nelsen et al., 1999; Pasternak et al., 2004, 2006; Sola et al., 2005; Yount et al., 2006), de Vries et al. (2000) discovered an novel atypical sgRNAs,2 from a recombinant EAV containing foreign sequence between ORF5 and ORF6, indicating the landscape for certain sgRNA synthesis can be changed by insertional mutation and thus activate cryptic or new TRS. The recombinant PRRSVs described in this study did not produce detectable novel subgenomic RNA, but insertion between ORF5/6 might cause the down- or up-regulation of the upstream sgRNA2/3 and 4, respectively. Curiously, the same insertion sequence inserted between ORF6/7 barely affected virus replication. It implies that the 39 nt insertion of into ORF5/6 locus induces changes of local secondary structure and spatial relationship with some yet-unresolved upstream transcription-regulating elements. Using the energy minimization program of Mfold Web server (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1−2.3.cgi) (Zuker, 2003) and the secondary structure drawings of RNA molecules by RNAvis version 2 (De Rijk et al., 2003), we conducted local secondary structure prediction to all mutation sites including all TRS and flanking sequence. Compared with the native structure (Fig. 4B), ORF5/6 insertion induces a major branched stem-loop structure (Fig. 4C), among other minor changes. All other mutations did not induce major changes in terms of the overall structure, number and position of stem-loops, as well as the TRS position, which is always located on loop area (data not shown).

Infectious cDNA-based replicon vector is becoming a powerful tool for dissecting virus replication process, and as a development vector for gene of interest. Because of a series of sgRNAs are employed, nidovirus is attractive vector in that multiple GOIs can be express theoretically. Unlike coronaviruses containing numerous accessory genes for virus replication, arterivirus has a rigidicosahedral nucleocapsid and overlapping coding regions, it proves to be difficult to find an optimal locus for GOI expression. Numerous studies have been directed utilizing the nonstructural protein 2 (nsp2) coding region, the most variable part of PRRSV genome, as a GOI insertion site (Fang et al., 2006b; Han et al., 2007; Kim et al., 2007). In most cases, the inserted genes were modified and/or chewed out during further serial passage, maybe due to the fact that nsp2 and its fusion form have to be under proteolytic processing during nsp maturation. In our study, we developed a platform that can be potentially used to express GOI between ORFs coding for structural proteins. In addition, these mutant cDNA clones would be of great value for making chimeric PRRSV as vaccine candidate. Using an attenuated PRRSV derived infectious cDNA as the backbone, one can readily swap in the immuno-protective protein(s) easily. Such chimeric virus vaccine would be genetically tagged, and powerful to combat the ever-changing PRRSV (Wang et al., 2008).

Taken together, we conclude that 1) the overlapping structural protein ORFs can be physically separated, and is dispensable for virus viability; 2) such ORF separations did not interrupt the subgenomic RNA synthesis; 3) the plaque morphology, growth kinetics, and antigenicity of these mutant viruses were virtually indistinguishable from those of the parental virus in cultured cells; and 4) these mutant viruses remained genetic stable in vitro. This study lays a foundation for further molecular dissection of PRRSV replication process, and development of genetically tagged vaccines against PRRS.

Materials and methods

Cells and viruses

Marc-145 cells (ATCC, Manassas, VA) were propagated in DMEM with 6% fetal bovine serum (Gibco-BRL, Gaithersburg, MD) and maintained in DMEM with 2% fetal bovine serum at 37 °C with 5% CO₂. The Type II PRRSV virus, derived from the infectious clone pAPRRS (Gao et al., 2007; Sun et al., 2007; Yuan and Wei, 2008) was used as a wild-type (wt) control in all experiments.

Construction of mutant plasmid

The oligonucleotide primers were designed using Oligo6.0 software based on PRRSV genomic sequences (GenBank accession number: AF184212) and synthesized. Five pairs of primers (Table 1) containing restriction sites were designed using pAOPRS as a template and based on PRRSV genomic sequences. Site-specific mutations were created using the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene) according the manufacturer’s recommendation with modifications. Briefly, oligonucleotide primers were designed such that the foreign insertion sequence were incorporated at the 5’ end or in the middle of the primer, leaving at least 10 nucleotides at the 3’ end matching with the template sequence. Using circular plasmid DNA as the template, PCR were run as described in the instruction manual of the QuickChange mutagenesis kit. The plasmid template was eliminated by Dpn I digestion (New England Biolabs, Ipswitch, MA), followed by transformation of Top 10 competent cells (Invitrogen) with the digested PCR mixture. The intermediate plasmid
was screened and verified by restriction enzyme mapping and nucleotide sequencing, and the target region were swapped into the pAPRRS backbone treated with the same restriction enzymes, resulting in the final full-length mutant clone. The purified full-length mutant plasmid was all verified by PCR, restriction enzyme digestion and nucleotide sequencing.

Fig. 5. The mutant viruses utilized the native canonical TRS-B for sgRNA transcription. Leader–Body Junction sequences of v5ND7 RNA 5, vORF56c RNA 6, and vORF673 RNA 7 were RT-PCR amplified using forward primer in leader region and reverse primer in the respective ORF, followed by nucleotide sequencing of the sgRNA-specific PCR product. The upper strand in the alignments represents the TRS-L region of the genomic leader sequence, and the lower strand with trace file is the mRNA sequence, from which the individual TRS-B is indicated. The broken line box denotes the inserted sequence, and arrowheads indicate the jumping direction of Leader–Body. (A) Leader–Body fusion site of mRNA5 from v5ND7; (B) Leader–Body fusion site of mRNA6 from vORF56c; (C) mRNA 6.1 from vORF56c; (D) Leader–Body fusion site of mRNA7.1, and (E) mRNA 7.2 from vORF673.
Table 1
Primers used for PCR

| Namea | Sequence (5′−3′)b | Positionc | Application |
|-------|------------------|------------|-------------|
| ORF12F | TTAATTAATTTAAATGGCGCGCCAATGAAATGGGGTCCATGC | 12182−12223 | PCR to insert Pac I Swa I and Asc I between ORF1 and 2 |
| ORF12R | GGGGGCCGCATTTAATTAATTAATATTACGCTTGAAGTTGG | 12160−12203 | PCR to insert CAT between ORF4 and 5 |
| ND5F | CATTATATGGCTGACAAATTAAAAGGGCCCTACGATGGGGTCGTCCCTAGACGACTTTTG | 14484−14532 | PCR to separate ORF5 and 6 |
| ND5R | GCCTATGACCTAATTAAATGCTTCCTAGACGACTTTTG | 14484−14532 | PCR to separate ORF5 and 7 |
| ORF56F | TTAATTAATTTAAATGGCGCGCCATGAAATGGGGTCCATGC | 12182−12223 | PCR to insert Pac I Swa I and Asc I between ORF1 and 2 |
| ORF56R | GGGGGCCGCATTTAATTAATTAATATTACGCTTGAAGTTGG | 12160−12203 | PCR to insert CAT between ORF4 and 5 |
| NDUR | GCTTACAACATGACGACCTAAAGGGCCCTACGATGGGGTCGTCCCTAGACGACTTTTG | 14484−14532 | PCR to separate ORF5 and 6 |
| ORF67F | TTAATTAATTTAAATGGCGCGCCATGAAATGGGGTCCATGC | 12182−12223 | PCR to insert Pac I Swa I and Asc I between ORF1 and 2 |
| ORF67R | GGGGGCCGCATTTAATTAATTAATATTACGCTTGAAGTTGG | 12160−12203 | PCR to insert CAT between ORF4 and 5 |

a Primer names are organized in groups. Prefixes: RT, reverse transcription primer; SF, forward PCR primer; SR, reverse PCR primer.
b Restriction sites introduced by PCR are underlined.
c The nucleotide positions within the viral sequence are based on GenBank accession number AF184212.

Transfection of cells with RNA transcripts

Mutant plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) followed by identification by electrophoresis, restriction enzyme map identification. The full-length mutant clones were linearized by cleavage with the restriction enzyme BstXI (New England Biolabs, Frankfurt, Germany) and ligated into pCR-Script vector (Stratagene). The resulting plasmids were transformed using the DH5α competent cells (Stratagene) followed by identification by electrophoresis and sequencing.

Indirect immunofluorescence analysis

Marc-145 cells were infected with a low multiplicity of infection by primary passage (P0) of the rescued virus (MOI), incubated for 72 hpi. The cell monolayer was washed twice with PBS, followed by fixation in cold methanol for 10 min at room temperature. The fixed cells were processed by 0.1% BSA at room temperature for 30 min, and then incubated at 37 °C for 2 h with anti-Nsp2 monoclonal antibody of PRRSV (kindly provided by Dr. Ying Fang at South Dakota State University) at 1:800 dilution in phosphate-buffered saline (PBS). After three washes with PBS, cells were incubated at 37 °C for 1 h with fluorescein isothiocyanate-conjugated (FITC) secondary goat anti-mouse antibody. Finally, cells were washed five times with PBS and visualized under Olympus inverted fluorescence microscope fitted with a camera.

DNA extraction and RT-PCR

Viral RNA was isolated using a Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instruction, and suspended in RNase-free water, quantified by UV spectrometer, aliquoted and stored at −70 °C. The resulting plasmids were transfected into 293T cells using a calcium phosphate precipitation technique. The transfected cells were cultured in DMEM supplemented with 10% FBS and G418 for 7 days, then harvested and stored at −80 °C.

Growth curve of virus

Marc-145 cells were infected with a low multiplicity of infection (MOI), and 200 μl of the cell supernatant was harvested at different time points (0 h, 12 h, 24 h, 36 h, 48 h, 72 h, 84 h, 96 h, 108 h, and 120 h) and stored at −70 °C. Viral titration was performed by plaque assay, and a growth curve was determined from the plaque number results as described previously (Gao et al., 2007).
Meng, X.J., Paul, S.S., Halbur, P.G., Morozov, I., 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 76 (Pt 12), 3181–3188.

Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31 (1), 1–21.

Meulenberg, J.J., Bos-de Ruijter, J.N., Wensvoort, G., Moormann, R.J., 1998. An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. Adv. Exp. Med. Biol. 419, 199–206.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moors, A.A., de Vries, A.A., Glaser, A.L., Raamsman, M.J., de Haan, C.A., Sarnataro, S., Godeke, G.J., 2002. Kissing domain in viral targeting. Virology 303 (2), 364–373.

Meng, X.J., Paul, S.S., Halbur, P.G., Morozov, I., 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 76 (Pt 12), 3181–3188.

Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31 (1), 1–21.

Meulenberg, J.J., Bos-de Ruuijer, J.N., Wensvoort, G., Moormann, R.J., 1998. An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. Adv. Exp. Med. Biol. 419, 199–206.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moors, A.A., de Vries, A.A., Glaser, A.L., Raamsman, M.J., de Haan, C.A., Sarnataro, S., Godeke, G.J., 2002. Kissing domain in viral targeting. Virology 303 (2), 364–373.

Meng, X.J., Paul, S.S., Halbur, P.G., Morozov, I., 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 76 (Pt 12), 3181–3188.

Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31 (1), 1–21.

Meulenberg, J.J., Bos-de Ruuijer, J.N., Wensvoort, G., Moormann, R.J., 1998. An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. Adv. Exp. Med. Biol. 419, 199–206.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moors, A.A., de Vries, A.A., Glaser, A.L., Raamsman, M.J., de Haan, C.A., Sarnataro, S., Godeke, G.J., 2002. Kissing domain in viral targeting. Virology 303 (2), 364–373.

Meng, X.J., Paul, S.S., Halbur, P.G., Morozov, I., 1995. Sequence comparison of open reading frames 2 to 5 of low and high virulence United States isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 76 (Pt 12), 3181–3188.

Meulenberg, J.J., 2000. PRRSV, the virus. Vet. Res. 31 (1), 1–21.

Meulenberg, J.J., Bos-de Ruuijer, J.N., Wensvoort, G., Moormann, R.J., 1998. An infectious cDNA clone of porcine reproductive and respiratory syndrome virus. Adv. Exp. Med. Biol. 419, 199–206.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moors, A.A., de Vries, A.A., Glaser, A.L., Raamsman, M.J., de Haan, C.A., Sarnataro, S., Godeke, G.J., 2002. Kissing domain in viral targeting. Virology 303 (2), 364–373.
MN184 using chimeric construction with vaccine sequence. Virology. 371 (2), 418–429.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.

Wootton, S., Yoo, D., Rogan, D., 2000. Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. Arch. Virol. 145 (11), 2297–2323.

Wu, W.H., Fang, Y., Rowland, R.R., Lawson, S.R., Christopher-Hennings, J., Yoon, K.J., Nelson, E.A., 2005. The 2b protein as a minor structural component of PRRSV. Virus Res. 114 (1–2), 177–181.

Yount, B., Roberts, R.S., Lindesmith, L., Baric, R.S., 2006. Rewiring the severe acute respiratory syndrome coronavirus (SARS-CoV) transcription circuit: engineering a recombination-resistant genome. Proc. Natl. Acad. Sci. U. S. A. 103 (33), 12546–12551.

Yuan, S., Murtaugh, M.P., Faaberg, K.S., 2000. Heteroclite subgenomic RNAs are produced in porcine reproductive and respiratory syndrome virus infection. Virology 275 (1), 158–169.

Yuan, S., Wei, Z., 2008. Construction of infectious cDNA clones of PRRSV: separation of coding regions for nonstructural and structural proteins. Sci. China C. Life Sci. 51 (3), 271–279.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31 (13), 3406–3415.