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Reverse genetic manipulation of the overlapping coding regions for structural proteins of the type II porcine reproductive and respiratory syndrome virus

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ABSTRACT

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.

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 (Keffaber, 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; Mounir et al., 1995; 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

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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 sgmRNAs (van Marle et al., 1999). TRS-flanking sequence could also affect nidoviral 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 sgmRNA 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 sgmRNAs (Nelson et al., 1993; Meng et al., 1995; Zheng et al., unpublished data). In addition, the role of TRS-flanking sequence plays for PRRSV sgmRNA 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, Swa 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, Swa 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, Swa I, and Asc I sites inserted between ORF1/2. Meanwhile, an Nde I restriction site was created 3 nucleotides (CAT) such that p5ND7 was constructed for separation of ORF4/5, and pORF7Ua was constructed by inserting ATG between ORF7/3′ UTR in similar manner. pORF56c and pORF673 were constructed by inserting restriction sites (Pac I, Swa 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 pORF7Ua, 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 aliquoted, kept at −80 °C, and designated the initial generation (P_0). An aliquot (200 μl) of 1000 fold diluted P_0 viral liquid was used to infect fresh Marc-145 cells. The supernatant of cell culture was harvested at 5dpi, designated P_1, aliquoted and frozen. Serial passage was conducted to generate passage P_2–P_5.

To prove that the rescued viruses were indeed from the tranfected 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 vORF7Ua (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, P_1 and P_5 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

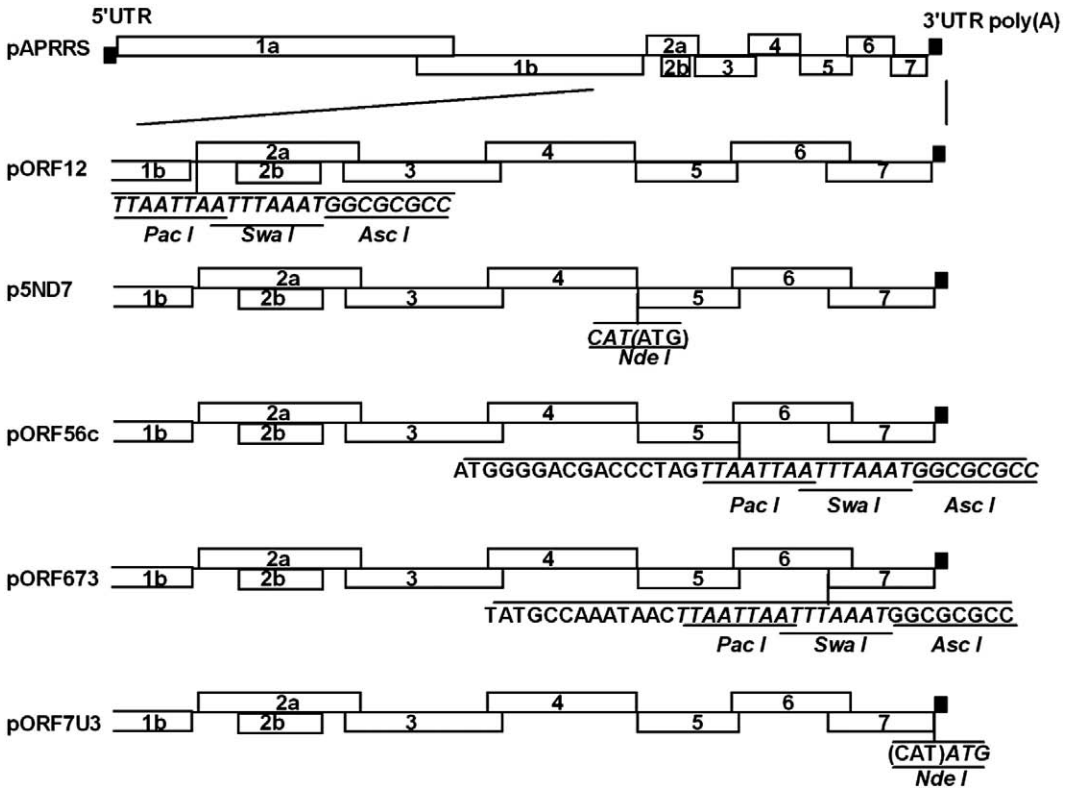


Fig. 1. Genomic structure of the full-length mutant PRRSV cDNA clones. The boxed numbers 1a, 1b, 2a, 2b, and 3 through 7 represented PRRSV open reading frame (ORF) 1a – ORF7. For the mutant PRRSV clones, only the region covering the part of 1b through ORF7 and 3′ UTR is shown. The position and inserted nucleotide sequences are shown. The restriction sites (Pac I, Swa I, Asc I, or Nde I) are underlined and labeled.

results revealed that the foreign insertion sequences were retained in P_1 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, P_1, and P_5 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

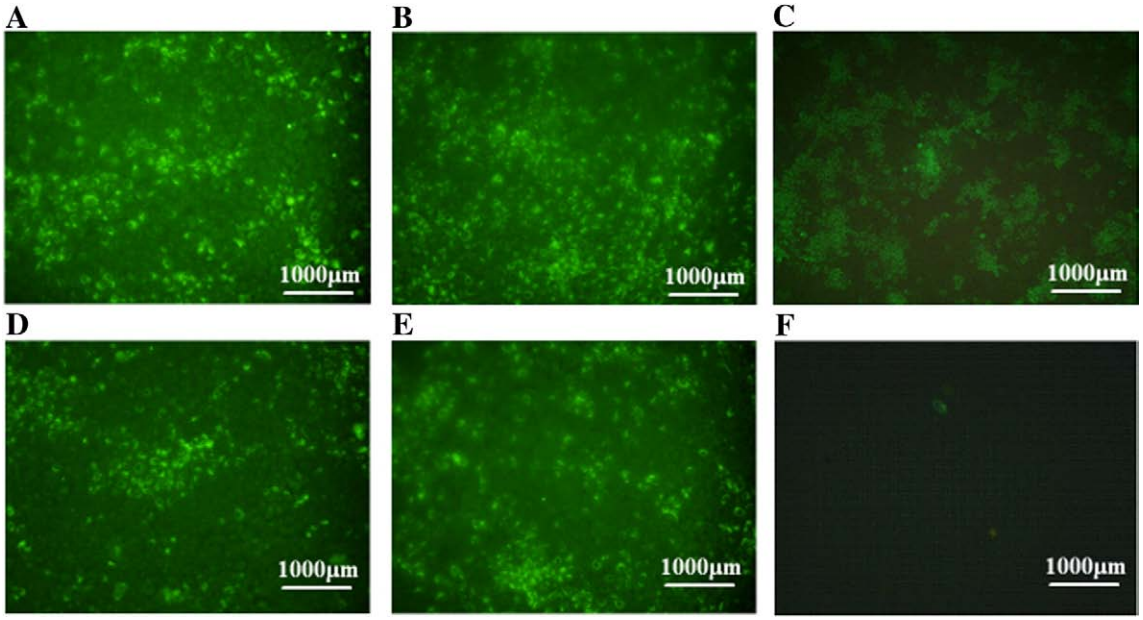


Fig. 2. Indirect immunofluorescence assay showed that the rescued viruses are PRRSV-specific. The supernatant of the transfected cells was collected and aliquoted as virus stock P0, of which 0.01 MOI was inoculated on Marc-145 cells of cell dishes, and incubated at 37 °C for 72 hpi. The infected cells were fixed and then stained 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), followed by incubation with fluorescein isothiocyanate-conjugated (FITC) secondary goat anti-mouse antibody, and visualized under Olympus inverted fluorescence microscopy. Panel (A) vORF12, (B) v5ND7, (C) vORF56c, (D) vORF673, (E) vORF7Ua, and (F) negative control.

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 7.58×10^4 PFU ml^{-1} at 84 h, considerably lower than the1.51×10^6 PFU ml^{-1}at 72 hpi of wild-type virus, indicating that the insertion between ORF 5 and 6 could adversely affects 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.

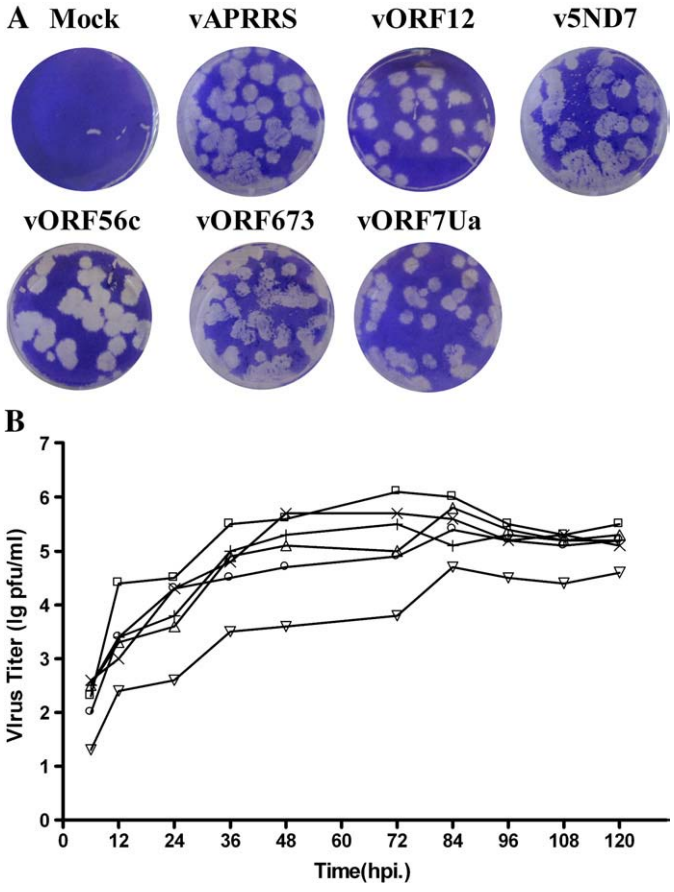


Fig. 3. The mutant viruses retained similar virological properties of parental virus. (A). Viral plaque morphology. Marc-145 cells in 6-well plate were infected with passage 5 viruses at an M.O.I of 0.1, or mock-infected with DMEM. The monolayer of infected cells was overlaid with agarose, and stained with crystal violet at 5 dpi. (B). Viral growth kinetics growth analysis. The infection was done as for viral plaque assay, without agarose overlay. The cell supernatants were harvested at the indicated time points, followed by viral titration by plaque assay on Marc-145. Based on viral titer and timepoints, the growth curves were determined: vAPRRS (□), vORF7Ua (✕), vORF673 (+), vORF56c (▽), v5ND7 (△), vORF12 (○).

The rescued viruses maintain the PRRSV subgenomic RNA profile

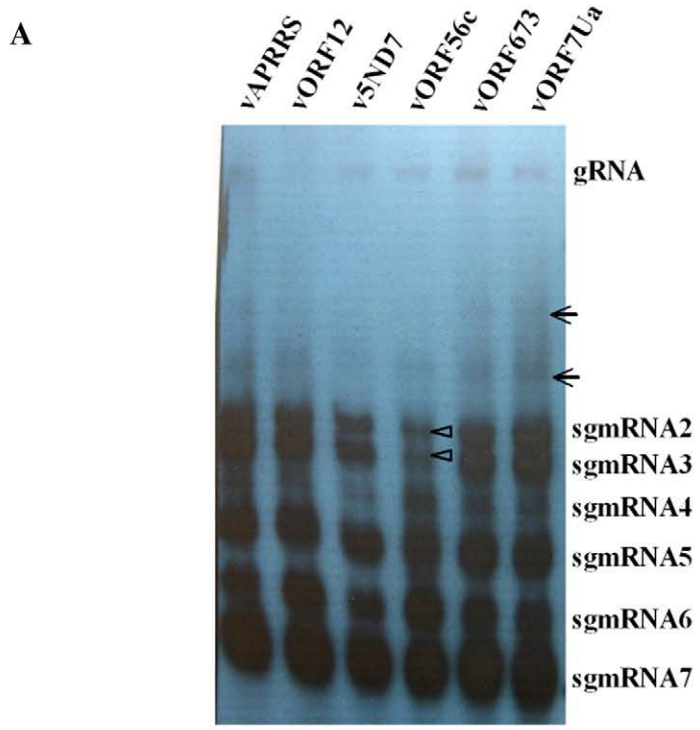
To further define if the mutant viruses retained the viral RNA profiles in infected cells, northern blotting analysis was conducted using a synthetic biotin-labeled probe complementary to the 3′ UTR, shared by all viral RNA species. Consistent with the parental virus vAPRRS, all mutant viruses produced at least seven sgmRNAs in the Passage 5 virus infected cells (sgmRNA2–7, Fig. 4A). Additionally, two subgenomic RNAs (Fig. 4A, black arrow) were also detected between mRNA2 and genomic RNA, which are similar in size to some heteroclite 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 sgmRNA5 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.

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 coronaviruses in the same order *Nidovirales*, PRRSV 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


A
vAPRRS
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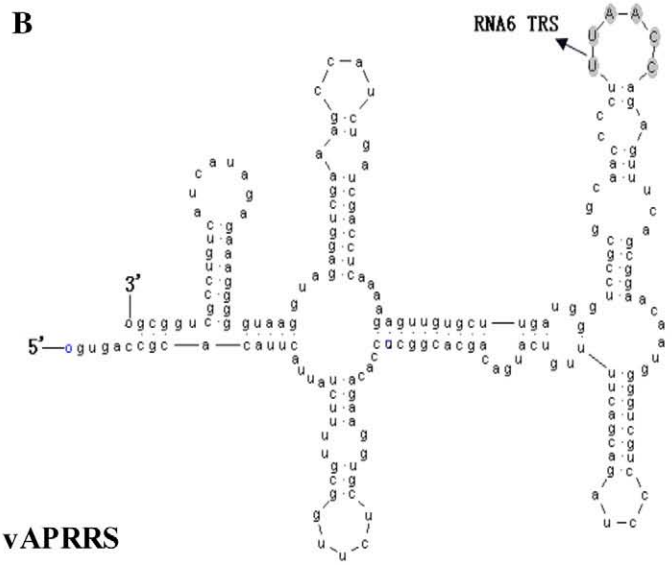
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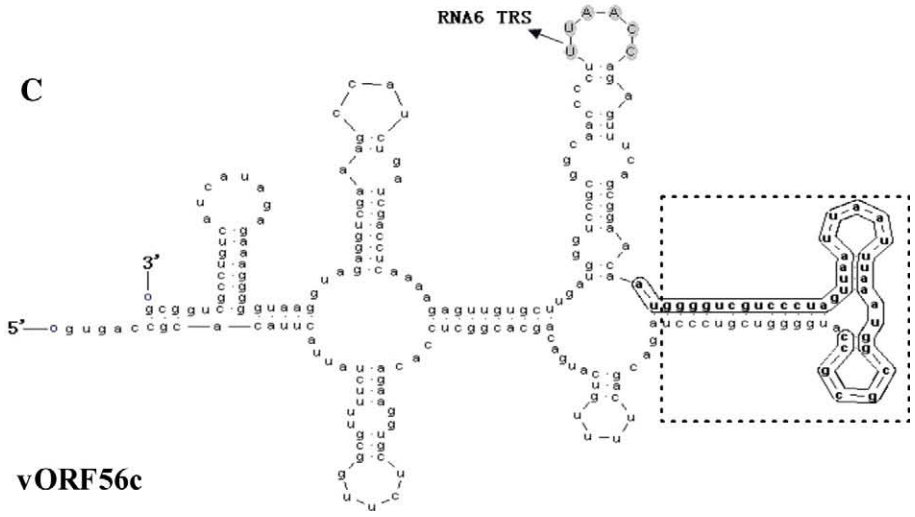
C
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vORF56c

fused a 9 amino acids hemagglutinin (HA) tag of the avian influenza fused with N- or C-terminus of the nucleocapsid protein, only to found that HA gene loss was apparent by the second generation, and was completely deleted 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 viability. This is the first report on physical separation of structural protein coding regions of a North American type PRRSV, and provided a platform for further dissection 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 sgmRNA2 and 3. Paradoxically, the sgmRNA4 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 sgmRNA levels and thus virus production. These results implying that the insertion 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 sgmRNA (Pasternak et al., 2006; van Marle et al., 1999), these are not the only factors affecting nidoviral transcription. Indeed, the flanking sequences of TRS influence sgmRNA 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 sgmRNA5.2 from a recombinant EAV containing foreign sequence between ORF5 and ORF6, indicating the landscape for certain sgmRNA 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 sgmRNA2/3 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-unknown 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 RnaViz 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 an expression vector for gene of interest. Because of a series of sgmRNAs 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 rigid icosahedral 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% CO_2. The Type II PPRSV 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 pAPRRS 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 recommendations 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 descried 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

Fig. 4. Subgenomic RNA transcription remains largely unchanged. (A) Northern blot: Marc-145 in 60 mm dishes were infected with passage 5 (1.0 MOI) of each mutant virus and parental virus. The total intracellular RNAs were extracted at 24 hpi by RNAwiz. Ten μg of RNAs for each sample were separated on denaturing agarose gel electrophoresis, and northern blot was conducted by using NorthernMax kit (Ambion) with the biotin-labeled oligo probe complementary to 3′ UTR. The viral genomic RNA (gRNA) and subgenomic mRNAs 2 to 7 (sgmRNA2-7) produced by vAPRRS, vORF12, v5ND7, vORF56c, vORF673, vORF7Ua are shown. Arrows point to the heteroclite-like subgenomic RNAs; Arrow heads indicated the sgmRNA2 and 3 produced by vORF56c. Predicted secondary structure of the TRS-B6 region by MFOLD program: (B) native TRS-B6 region; C vORF56c TRS-B6 plus 39 nt insertion, boxed are the induced extra branched stem-loop structure.

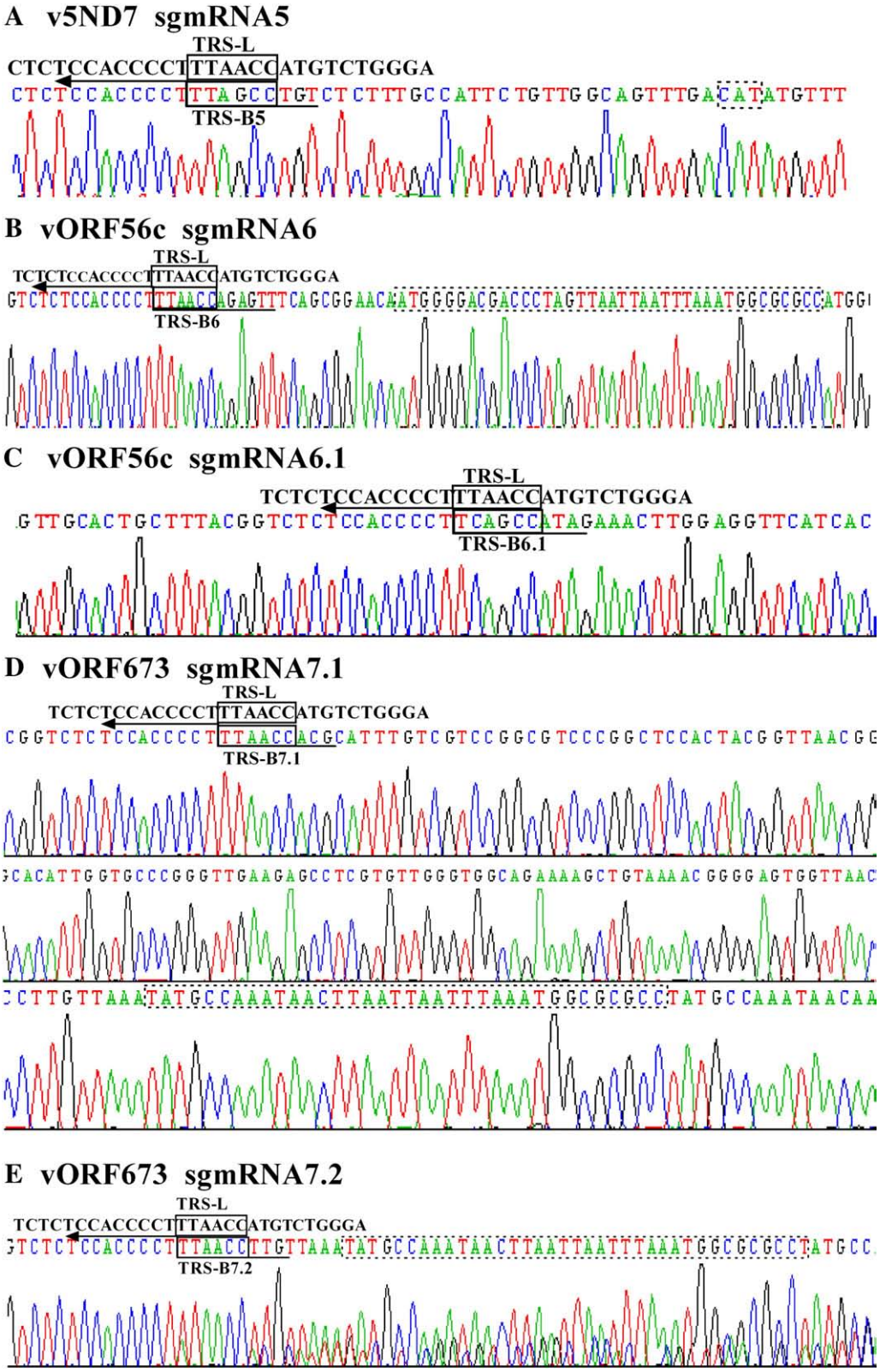

Fig. 5. The mutant viruses utilized the native canonical TRS-B for sgmRNA transcription. Leader–Body Junction sequences of v5ND7 RNA 5, vORF56c RNA 6, and vORF673 RNA 7 were RT-PCR amplified using forward primer in leader region and reverse primer in the respective ORF, followed by nucleotide sequencing of the sgmRNA-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 vORF56c, and (C) mRNA 6.1 from vORF56c; (D) Leader–Body fusion site of mRNA7.1, and (E) mRNA 7.2 from vORF673.

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.

Table 1
Primers used for PCR

Name[a]	Sequence (5′–3′)[b]	Position[c]	Application
ORF12F	TTAATTAATTTAAATGGCGCGCCAATGAAATGGGGTCCATGC	12182–12223	PCR to insert Pac I Swa I and Asc I between ORF1 and 2
ORF12R	GGCGCGCCATTTAAATTAATTAATCAATTCAGGCCTAAAGTTGG	12160–12203	
NDE5F	CTGTTGGCAGTTTGACATATGTTTAAGTATG	13872–13902	PCR to insert CAT between OPF4 and 5
NDE5R	CATACTTAAACATATGTCAAACTGCCAACAG	13872–13902	
ORF56F	TTAATTAATTTAAATGGCGCGCCATGGGGTCGTCCCTAGACGACTTTTG	14484–14532	PCR to separate ORF5 and 6
ORF56R	GGCGCGCCATTTAAATTAATTAAGTTATTTGGCATATTTAACT	14484–14532	
ORF67F	TTAATTAATTTAAATGGCGCGCCTATGCCAAATAACAACGGC	14997–15015	PCR to separate ORF6 and 7
ORF67R	GCCGTTGTTATTTGGCATAGGCGCGCCATTTAAATTAATTAA	14997–15015	
NDUF	AGCATGATGGGCTGGCATATGTCTTGAGGC	15363–15389	PCR to insert ATG between OPF7 and 3′ UTR
NDUR	GCCTCAAGACATATGCCAGCCCATCATGCT	15363–15389	
SF10989	ATTTAGGGCCACAGACAAGCG	10989–11009	Analytical RT-PCR for vORF12
SR12709	CCCCGTCATGCGCAGGTTGTGTAG	12709–12686	
SF12670	CTCTCGGCTACCCATGCTACAC	12670–12691	Analytical RT-PCR for v5ND7
SR14365	ACCGCCAACGATAGAGTCTGCCCTTAGTGTCC	14365–14397	
SF14413	CTGATCGACCTCAAAAGAGTTGTGCTTG	14413–14440	Analytical RT-PCR for vORF56c, vORF673, vORF7Ua and vAPRRS
SR15497	CAATTAAATCTTACCCCCACACGGTCG	15497–15524	
SFSGM	GTATAGGTGTTGGCTCTATGC	6–26	PCR for the sgmRNA
SR14234	GCGTAGATGCTACTCAGGACATACC	14210–14234	PCR for sgmRNA5
SR14880	ATGCGTGGTTATCATTTGCCGTAATC	14855–14880	PCR for sgmRNA6
SR15306	CTCCACAGTGTAACTTATCCTCC	15284–15306	PCR for sgmRNA7

[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 Xho I that cuts downstream of the poly (A) tail, followed by recovering with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany). RNA was synthesized by a T7 mMessage Machine Kit (Ambion INC, Austin, TX) using purified linearized-plasmid as template according to manufacturer's protocol, and including treatment of the RNA with DNase to remove input plasmid. The RNA was dissolved in nuclease-free water. 2×10^5 cell well^{-1} Marc-145 cells were seeded into a 6-well plate and grown for 2 days to approximately 60–80% confluence before transfection. The subconfluent cells were transfected with the RNA transcribed *in vitro* using the DMRIE-C reagent (Invitrogen, Carlsbad, CA), and incubated at 37 °C under 5% CO_2. PRRSV-specific cytopathic effect (CPE) was monitored daily. When the percentage of cytopathic cells exceeded 90%, the supernatant was harvested and stored at −70 °C. The post-transfection cells were serially passaged 5 times as virus stock for use in further growth curves and plaque assays as described previously (Yuan and Wei, 2008).

Plaque assay

The viral suspension was serially 10-fold diluted, and 200 μl viruses were inoculated onto Marc-145 monolayers. After 1 h incubation at 37 °C, the cell monolayers were then overlaid with Modified Eagle Medium (MEM) (2×) containing 2% low melting agarose (Cambrex, Rockland, ME) and 4% fetal bovine serum and incubated for 5 days at 37 °C with 5% CO_2. The resulting plaques were stained by crystal violet (5% w v^{-1} in 20% ethanol).

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 (6 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).

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 by 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.

RNA 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. One-step RT-PCR was performed using a commercial kit (Takara, Dalian, China) according to the protocol in the accompanying manual. The primers and PCR conditions used are listed in Table 1 to detect PRRSV overlapping mutant virus. The PCR products were identified by electrophoresis and sequencing.

sgmRNA5–7 of v5ND7, vORF56c, and vORF673 were amplified by RT-PCR using RevertAidTM M-MuLV Reverse Transcript kit (MBI Fermentas, St.Leon-Rot, Germany) and LA Taq (Takara). The reserve transcription primers and PCR primers used are listed in Table 1. The PCR products were gel purified, subcloned, and followed by nucleotide sequencing.

Northern blot analysis

Monolayers of Marc-145 cells were infected with 1.0 MOI of P5 virus stock of each mutant virus. Total intracellular RNAs were isolated from infected cells harvested at 24 hpi using RNAWiz (Ambion, Austin, TX) according to the manufactures instruction, and 10 μg of total RNAs for each sample were separated on denaturing agarose gels using Agarose-IE (Ambion, Austin, TX) as described previously (Yuan and

Wei, 2008). Northern Blot was performed with the NorthernMax kit (Ambion) according to manufacturer's protocol. Briefly, the gel-separated total cellular RNAs were transferred to a BrightStar-Plus membrane (Ambion, Austin, TX) for 10 h, cross-linked by UV light, prehybridized, and probed with a 3′ UTR-specific oligonucleotide (VR3: 5′ AATTTCGGCCGCATGGTTTTCGCCAATTAAATCTTACCCCCA-CACGGTCGC 3′). The hybridization was incubated at 42 °C overnight, followed by washing with low-stringency buffers (Ambion, Austin, TX). The filters were incubated with alkaline phosphatase-conjugated streptavidin, and incubated with the chemiluminescent substrate CDP-STAR (Ambion, Austin, TX), overlaid with Kodak film.

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