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Replacement of the heterologous 5' untranslated region allows preservation of the fully functional activities of type 2 porcine reproductive and respiratory syndrome virus

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The 5' untranslated region (UTR) is believed to be vital for the replication of porcine reproductive and respiratory syndrome virus (PRRSV), yet its functional mechanism remains largely unknown. In this study, to define the cis-acting elements for viral replication and infectivity, The 5' UTR swapping chimeric clones pTLV8 and pSHSP5 were constructed based on two different genotypes full-length infectious cDNA clone pAPRRS and pSHE backbones. Between them, vTLV8 could be rescued from pTLV8 and had similar virological properties to vAPRRS, including phenotypic characteristic and RNA synthesis level. However, pSHSP5 exhibited no evidence of infectivity. Taken together, the results presented here demonstrate that only the 5' UTR of type 1 PRRSV did not affect the infectivity and replication of type 2 PRRSV in vitro. The 5' UTR of type 2 PRRSV could be functionally replaced by its counterpart from type 1.

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Introduction

The 5' untranslated region (UTR) of positive-sense, single-stranded RNA viruses has been demonstrated to play a key role in the regulation of viral replication and many biological properties. Research on many positive-sense RNA viruses has shown that their important secondary structure in the 5' UTR protects against degradation by exonucleases (Gallie, 1998). For example, the 5' cloverleaf plays a crucial role in maintaining the stability of poliovirus RNA (Barton et al., 2001) and a hairpin motif located close to the 5' end is believed to be important for the stabilization of the RNA (TR and B, 1989). In Dengue virus, the 5' UTR is also critical for RNA synthesis because a stem–loop RNA structure at the 5' end promotes specific RNA synthesis (Alvarez et al., 2005; Lodeiro et al., 2009) and a binding site for viral RNA-dependent-RNA polymerase (RdRp) at the 5' end of the genome facilitates template recognition at the 3' end of the genome via long-range RNA–RNA interactions (Filomatori et al., 2007). The 5' end of the Aichi virus genome encodes RNA elements important for both negative-strand and positive-strand RNA synthesis (Nagashima et al., 2005b). In addition to involvement in the stability of the genome, the cloverleaf structure at the 5' end of the poliovirus genome has been demonstrated to be essential for both plus- and minus-strand RNA synthesis (Andino et al., 1990; Gamarnik and Andino, 1998; Herold and Andino, 2000). Furthermore, in the case of alphaviruses, flaviviruses and some picornaviruses, the 5' UTR has also been shown to contain distinct core promoter elements for RNA synthesis (Frolov et al., 2001; Frolov et al., 1998; Nagashima et al., 2005a). In addition, the 5' UTR affects translational control. Internal ribosome entry site (Mihalek et al., 1999) function has been found in the 5' UTR of some members of the Flaviviridae (Dumas et al., 2003) and Picornaviridae (Rijnbrand et al., 2000, 1997).

Porcine reproductive and respiratory syndrome (PRRS) emerged in the late 1990s in Europe and North America, resulting in tremendous economic loss to the swine industry. The PRRS viruses (PRRSV) isolated from Europe and North America display huge diversity in genetic and serological performance, although they induce indistinguishable clinical manifestations (Halbur et al., 1995; Nelsen et al., 1999). As a result, PRRSV has been characterized into two distinct genotypes, European type 1 and North American type 2 (Wang et al., 2008). PRRSV is a small, enveloped RNA virus, a member of the genus Arterivirus, family Arteriviridae, order Nidovirales (Cavanagh, 1997; Snijder, 2001). The PRRSV genome consists of at least ten open reading frames (ORFs), which are flanked by the 5’ and 3’ UTRs. The 5’ UTRs of the
Type 1 and type 2 PRRSV are different in length (approximately 220 and 190 nucleotides (nt), respectively) and share approximately 60% nucleotide homology (Forsberg, 2005; Hanada et al., 2005; Lu et al., 2011). Nevertheless, highly conserved stem-loop structures have been identified in the leader of all PRRSV strains, which are believed to play a part in viral replication (Oleksiewicz et al., 1999). PRRSV adopts a unique discontinuous transcription mechanism for sg mRNA transcription. The structural proteins are translated from the 5' ends of a nested set of co-terminal sg (Khromykh et al., 2003) mRNA2 to 7 (Snijder and Meulenberg, 2007).
and chimeric viruses were propagated in MARC-145 cells as described previously (Yuan and Wei, 2008).

**Construction of plasmids**

The subclone pCBSA, which contains the 5′ terminus (nt 1-2573) of the type 2 vAPPRS genome, was used as the shuttle plasmid vector for Quick-Change site-directed PCR mutagenesis during the reverse genetic manipulation (Lu et al., 2011). Two nucleotides, “at”, were inserted between TRS and “AUG” to create the Nde I restrict endonuclease site between the end of the 5′ UTR and the translation initiation codon of ORF1. The mutant fragment was transferred subsequently into the corresponding region of pAPPRS using Spf I in front of the T7 promoter and Ap II at nt 1688 in pAPPRS (Yuan and Wei, 2008). As a result, the mutant pTLNd4 was created. The Pac I restriction enzyme site was also created through the insertion of four nucleotides, “taa”, ahead of TRS to create the mutant pPa2. These plasmids are shown in Fig. 1C. The primer pairs SFLNd/SRLNd and SPF/SPpac for PCR mutagenesis are listed in Table 1. pLVNd1 is a shuttle plasmid that was generated by truncating the genomic sequences in the full-length cDNA clone pSHE between the Not I site ahead of the CMV promoter and the Nde I site behind TRS, and was cloned into pBlueScript SK+ vector. Following this, the 5′ UTR of type 2 clone pTLNd4 was replaced by that of the type 1 strain to construct the chimeric clone pTLv8 using these two restriction enzyme sites. All of the plasmids were verified by restricted enzymatic mapping and nucleotide sequencing (Shanghai Sunny Inc, China).

For the construction of converse chimeras pSHSP5, the pNSHE was used as the backbone in which a Nde I was inserted between the TRS and ORF1 translation initiation codon according to the pTLNd4 construction method. The vNSHE also showed similar virological characteristics to vSHE (Yuan et al., unpublished data), just like vTLNd4 to vAPPRS. Using Not I and Nde I digestion, the CMV promoter region and 5′ UTR of type 2 were substituted with that of type 1 in pNSHE backbone.

Splicing-overlap-extension (Liao et al., 2011) PCR was used to construct the chimeric clone revertants pTLv7823, 8401, 8914, and 9433. The restriction endonucleases used to transfer the mutant fragments into the related region in pTLv8 were Kpn I at nt 5388 bp, Pme I at nt 7800 bp, Avr II at nt 8959 bp and Xba I at nt 14772 bp. Throughout the study, pAS was used as a non-replicative plasmid control, as described previously (Lu et al., 2011).

**Plasmid transfection and virus recovery**

The parental plasmid pAPPRS and mutant plasmids pTLNd4, pPa2, pTLv8, pSHSP5 were isolated using a QIAprep Spin Miniprep kit as instructed (Qiagen, Hilden, Germany and identified further by spectrophotometry and 0.8% agarose gel electrophoresis (AGE)).
Samples of each DNA (3 μg), 15 μL of Lipofectamine™ LTX and 3 μL Plus Reagent (Invitrogen) were transfected directly into 70% confluent BHK-21 cells, according to the manufacturer’s protocol. The cell supernatants were harvested at 24 h post transfection (hpt), diluted BHK-21 cells, according to the manufacturer’s protocol. The Plus Reagent (Invitrogen) were transfected directly into 70% confluent BHK-21 cells. The BHK-21 cells were grown in six-well plates to 70% cell density. Transfected BHK-21 cells were harvested at 24 h post transfection (hpt), diluted 1:600 and stored at –80 °C, as P0. The P0 viral supernatants were used for passage in MARC-145 cells in six-well plates as virus stocks (five passages, P1–P5) for use in further experiments, as described previously (Sun et al., 2010; Yu et al., 2009; Yuan and Wei, 2008; Zheng et al., 2010).

**Indirect immunofluorescence assay (IFA)**

The BHK-21 cells were grown in six-well plates to 70% confluence, and each well was transfected by pAPRRS, pSH, pHE, or mock control to verify the expression of viral nucleocapsid protein (N), as reported previously (Gao et al., 2012; Sun et al., 2010). The cell monolayer was fixed at 24 hpt with ice-cold methanol for 10 min at room temperature and blocked subsequently with 0.1% bovine serum albumin (BSA) for 30 min, followed by incubation with a monoclonal antibody against the N protein of type 1 PRRSV (kindly provided by Ingenas Co., Madrid, Spain) and type 2 PRRSV (kindly provided by Dr. Ying Fang at South Dakota State University) at 1:600 dilution for 2 h at 37 °C. After five washes, the cells were incubated at 37 °C for 1 h with Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) (Invitrogen). The fluorescence signal was visualized using an inverted fluorescence microscope (Olympus IX71), as described previously (Gao et al., 2012). For investigation of the viability of the mutant transfectants on BHK-21 cells, the anti-N IFA was repeated at 48 h post infection (hpi), using P0 viral supernatants from BHK-21 cells on the MARC-145 cell monolayer. The procedure is described in detail above and the cells were incubated at 37 °C for 1 h with Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) (Invitrogen) (Gao et al., 2012).

**5' RACE, RT-PCR and full-length sequencing**

For identification of mutant sites, genomic RNA was extracted from viral supernatants (P1–P5) using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s procedure. Following this, the viral RNAs were used as the templates for RT-PCR. The primary transcription was conducted using avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa, Dalian, China) and primer SR2573 (Table 1). The cDNAs were synthesized with the forward primer pair SFDNLTX and 3'-primer LTX and 3 μL Plus Reagent (Invitrogen), aliquoted and stored at –80 °C, as P0. The P0 viral supernatants were used for passage in MARC-145 cells in six-well plates as virus stocks (five passages, P1–P5) for use in further experiments, as described previously (Sun et al., 2010; Yu et al., 2009; Yuan and Wei, 2008; Zheng et al., 2010).

## Table 1

Oligonucleotides used for RT-PCR mutagenesis, 5’RACE and Northern blotting analysis.

| Name       | Sequence                  | Position | Application            |
|------------|---------------------------|----------|------------------------|
| SFND       | 5'-ATGACGATATTGTAAGTGTGTCCATATGCGCTTGGCATTGACATTG-3' | 1–37     | RT-PCR                |
| SR2573     | 5’-CTGGCCAGGCCTACTCTGCGCAAGTC-3' | 2548–2573 | RT-PCR                |
| SPLn       | 5’-GGTCCTCTCCACCCCATTTACACGTAAGCTGATCGTCCAGTGACG-3' | 170–219  | PCR mutagenesis        |
| SRnl       | 5’-CAGCTGACGATATCGATTCCCAAGAACLCTGAAGGCTGAC-3' | 172–217  | PCR mutagenesis        |
| SPac       | 5’-CTTCAGTTCCCATCTAATACGTGGTGTTGACTCGGATATGACG-3' | 0        | PCR mutagenesis        |
| SFL1V0     | 5’-AGCCCAAACCTATCTCCTTCTC-3' | 100–121 (LV) | RT-PCR                |
| SR683      | 5’-GGACGGCAGTTGGTAAACAGCTG-3' | 658–683  | RT-PCR                |
| SR345      | 5’-TAGCCAAAGACTCCTCCTCTC-3' | 323–343  | Nucleotide sequencing  |
| SR1224     | 5’-CTTGCAGCCCGCCTGATGATCTGTCG-3' | 1098–1124 | 5’ RACE               |
| SFLVI      | 5’-ATGATTGTAAGGATATCCTTC-3' | 1–19 (LV) | Nucleotide sequencing  |
| SF2480     | 5’-CAGTTGaAACCTAGACC-3' | 2480–2503 | Full-length nucleotide sequencing |
| SR4463     | 5’-CGTTGACACCGACAGAATCG-3' | 4444–4463 | Full-length nucleotide sequencing |
| SF4344     | 5’-GCCCTCGCGTTGCTCACTGTCGGATTTTTTT3'-3' | 4344–4373 | Full-length nucleotide sequencing |
| SR9753     | 5’-GTACCGGCAACTCTGGCTATCCCTCCCTCAT-3' | 9722–9753 | Full-length nucleotide sequencing |
| SR9348     | 5’-TCCATACATTCAATAAC-3' | 9348–9366 | Full-length nucleotide sequencing |
| SR12013    | 5’-CCGTTAACACCCATCAATG-3' | 11992–12013 | Full-length nucleotide sequencing |
| SFL1120    | 5’-TTCATTAAAGCTACATGACC-3' | 11210–11236 | Full-length nucleotide sequencing |

**Notes:**

- a Abbreviations in primer names: SF = forward PCR primer; SR = reverse PCR primer.
- b Italic lowercase represents sequences different from vAPRSS.
- c Numbers in the primer names denotes the nucleotide positions in the parental vAPRSS. LV represents the type 1 strain.

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were reverse transcribed using the primer Qst (Table 1), and the cDNAs were used as templates for amplification of five overlapping genomic fragments using the primers RF1 (SFLV1/SR2573), RF2 (SF2480/SR4463), RF3 (SF4344/SR9573), RF4 (SP9348/SR12013), and RF5 (SF11210/Qst) (Table 1). The PCR products were purified by 1% agarose gel electrophoresis and cloned into the Zero Blunt® II-TOPO® PCR vector (Invitrogen) for sequence analysis. The resultant nucleotide sequences were assembled by SeqMan and aligned using the Lasergene® software package (Lu et al., 2011).

The 5′ end sequences of vTLV8 and vAPRRS (P1–P5) were also detected by 5′ RACE (Yuan et al., 2001). Total viral RNAs were extracted from infected cells using TRIzol® reagent (Invitrogen) and stored at −80 °C. Samples of viral RNAs (100 ng) were used for the transcription using avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa, Dalian, China) and primer SR2573. The reverse-transcribed PCR products were purified using a QIAgen purification kit (QIAgen, Hilden, Germany) according to the manufacturer’s instructions. The cDNAs were eluted in 40 μl of DNase/RNase-free water. Poly(G)-tailing (TaKaRa, Dalian, China) was conducted on the 3′-terminal of purified cDNA using a terminal deoxynucleotide transferase (New England Biolabs, USA). After this, the PCR products were eluted in 60 μl ddH₂O and stored at −30 °C for further use. First round PCR was completed as described previously (Yuan et al., 2001) using 2 pM Qc and 10 pM Qo as the forward primers and 10 pM SR1124 as the reverse primer. The first round PCR was performed using the primer pair Qi and SR683 and initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 60 s. An identical second round of PCR was then performed using 1 μl of the first round PCR product; the parameters were initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 40 s. After electrophoresis in 1% agarose gels, the PCR products were purified using a QIAquick gel- purification kit (QIAgen, Hilden, Germany) according to the manufacturer’s protocol. The purified PCR products were used directly as templates for subcloning and nucleotide sequencing.

Analysis of (-) gRNAs and sg mRNAs

Total RNAs were extracted from transfected BHK-21 cells using TRIzol® reagent (Invitrogen) and dissolved in DNase/RNase-free water. Removal of input DNA from RNA preparations was conducted by incubating with DNase I at 37 °C for 30 min according to the protocol of the DNA-free kit (Ambion). The purified RNAs were used as the templates in RT-PCR for detection of (-) gRNAs and sg mRNAs. A house-keeping β-actin mRNA was used as the internal control, as reported previously (Lu et al., 2011). The PCR products were quantified using Quantity One® 1-D analysis software version 4.6.2. The wild type virus vAPRRS was set to be 100%.

The forward primers SF12 and SFLV1 were used for cDNA synthesis from 2 μg of total RNAs using SuperScript TM II Reverse Transcriptase (Invitrogen). The first strand cDNA was treated with 2 μg RNase A (Ambion) at 37 °C for 30 min to eliminate the remnant RNA. The RNase A activity was removed by heating at 95 °C for 10 min. Samples of cDNA (2 μl) and the primer pairs SF12/SR683, SFLV101/SR683, SFLV101/SRLV572 and SF12/SRLV572 were prepared for the first round PCR. The reaction parameters consisted of initial denaturation at 95 °C for 5 s followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The nested PCR was conducted using 2 μl 1000-fold diluted primary PCR product and primer pairs SF12/SR343, SFLV101/SR343, SRLV101/SRLV378 and SF12/SRLV378. The PCR cycle parameters were the same as above.

Subgenomic mRNA7 of the mutant viruses was amplified by the leader–body junction RT-PCR (Lu et al., 2011; Zheng et al., 2010) using TaKaRa AMV and LA Taq (TaKaRa, Dalian, China). The reverse primer Qst was used for the RT reaction, and the four primer pairs SF12/SR15284, SFLV1/SR15284, SFLV1/SRLV14835 and SF12/SRLV14835 were used for detection of sg mRNAs. The reaction parameters consisted of initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 30 s. All PCR products were gel purified using a QIAquick gel- purification kit (QIAgen, Hilden, Germany), and subsequently subcloned and sequenced.

Titration of rescued viruses

The first round rescued viruses from transfection of BHK-21 cells were titrated in MARC-145 cells. The viral titers (TCID₅₀ mL⁻¹) were determined as described previously (Gao et al., 2012). Each of the viral titrations was repeated three times, and the results shown in the figures indicated the mean value.

To determine the growth dynamics, MARC-145 cells in six-well plates were infected with all of the mutant and wild type viruses (P1) at 0.01 MOI, as described previously (Lu et al., 2011; Sun et al., 2010; Yu et al., 2009). After 1 h of adsorption, the cell monolayers were washed. Supernatants (200 μl) were harvested at the indicated time points and frozen at −80 °C until use. Viral titration was conducted by quantitation of viral plaques on MARC-145 cells (Sun et al., 2007; Yu et al., 2009).

Viral plaque assay

MARC-145 cells in six-well plates were infected with 200 μl of 10-fold serially diluted P1 viruses. The plates were incubated at room temperature for 1 h. Following this, cell monolayers were washed with PBS and overlaid with 1% low melting agarose in MEM (Invitrogen) containing 2% FBS. The agarose solidified plates were inverted (placed top down) placed in a 5% CO₂ incubator and incubated at 37 °C. The cell monolayers were stained at 5 days post infection (dpi) with 5% crystal violet in 20% ethanol (Gao et al., 2012).

Viral RNA isolation and Northern blotting

MARC-145 cells were infected with vTLV8, vTLNd4 and vAPRRS (PS) at MOI of 0.01. Total RNA from each sample was isolated at 36 hpi using a QIAgen RNeasy Mini kit (QIAgen, Hilden, Germany). Samples of total RNA (5 μg) were loaded and separated by 1% formaldehyde-denatured agarose gel electrophoresis (AGE). The separated total RNA was transferred to a BrightStar-Plus membrane (Ambion) for 8 h, and cross-linked by UV light for 15 min. Pre-hybridization was performed at 42 °C for 30 min followed by detection using the genotype-specific 3′ UTR or 5′ UTR oligodeoxynucleotide probe P3/PVR5 (Table 1). Blots were hybridized for 12 h and washed with low-stringency and high-stringency buffers, blocking buffer and wash buffer according to the manufacturer’s instructions (Ambion). The filters were incubated with alkaline phosphatase-conjugated streptavidin followed by reaction with the chemiluminescent substrate CDP-STAR (Ambion). The over laid filters were obtained by exposure for 12 h in a dark cassette box in a dark room.
The RNA secondary structures were predicted using the energy minimization program of Mfold server (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/secondary-structure-form1-2.3.cgi) (Zuker, 2003) and edited using the secondary structure drawing and modifying software RNAviz version 2 (De Rijk et al., 2003).

**Results**

**Conserved domains exist in the 5’ UTR of type 1 and 2 PRRSVs and their secondary structures have similar feature**

Five type 1 PRRSV strains (GenBank accession Nos. M96262, DQ489311, AY366525, EU076704 and AY588319) and eleven type 2 PRRSV strains (GenBank accession Nos. AY150564, GQ330474, EF488048, EF112445, AF184212, EU109503, EU200962, U87392, EF112447, AY424271, and AF331831) were compared and aligned using the Mfold program, followed by modification using the RNAviz software. The predicted secondary structures of the type 1 and type 2 PRRSV 5’ UTR were analyzed using the Mfold program, followed by modification using the RNAviz software. The predicted secondary structures of the type 1 and type 2 PRRSV 5’ UTR are shown in Fig. 1B (i) and (ii). They were characterized by six major putative helical stem–loops, which we arbitrarily designated as ESL1–5 for type 1 PRRSV and NSL-1–5 for type 2 PRRSV, respectively (Fig. 1B i and 1B ii). The E-SL1 in type 1 PRRSV could be separated into two minor stem–loops, designated as E-SL1a and E-SL1b, which corresponded to N-SL1 in type 2 PRRSV. Similarly, NSL4 in type 2 PRRSV also could be divided into two minor stem–loops, named N-SL4a and N-SL4b, which corresponded to E-SL4 in type 1 PRRSV (Lu et al., 2011). Although the primary nucleotide sequences of these strains showed low sequence homology, their RNA secondary structures were similar, especially the most important leader TRS hairpin (LTH) parts, as shown by the shaded grey box in Fig. 1B, that is, the prominent N/E-SL5.

**Virus rescue. RNA synthesis and gene expression show quite different consequences for the mutants and the WT**

pAPRRS, pSHE and the mutant cDNA clones, pTLNld4, pPa2, pTLV8 and pSHP5 were transfected into BHK-21 cells, and subsequently used to infect MARC-145 cells for identification of viral infectivity. When passaged in MARC-145 cells, pTLNld4 and pAPRRS developed CPE at 3–4dpi. When approximately 80% cytopathic effect (CPE) had developed, viral supernatants from the infected MARC-145 cells were harvested and designated vTLNld4 and vAPRRS (P1), respectively. Both the transfected BHK-21 cell monolayers and infected MARC-145 cell monolayers were examined via IFA using MAb against two genotypes of PRRSV N protein. The results revealed that the similar N protein expression level for pAPRRS and pTLNld4 transfected wells. Moreover, N protein expression of type 1 WT, pSHE, showed comparable level with that of type 2 WT, while only a few isolated spots were found in pTLV8 and pPa2 transfected wells, as shown in Fig. 2C. However, none of the fluorescent signal could be detected in pSHP5 transfected well. When transfec tant supernatants were used to infect MARC-145 cells, pTLV8 showed a subtly different IFA pattern from that of vAPRRS. However, mutant pPa2 and chimeric pSHP5 did not develop CPE even after the fifth passage in MARC-145 cells. The IFA result showed that pPa2 developed a PRRSV-specific fluorescent signal in a few of transfected BHK-21 cells but it did not show any infection of MARC-145 cells. N-specific fluorescent signal could not detected in pSHP5 transfected BHK-21 cells and MARC-145 cells inoculated with supernatant collected from pSHP5 transfected BHK-21 cells, as shown in Fig. 2C. To further confirm whether pSHP5 was infectious, we repeated the transfection experiments using BHK-21 cells three more times and transferred the transfection supernatants to MARC-145 cells for three blind passages. No typical CPE were observed in the MARC-145 cells, and no viral genomic RNA was detected in either the total intracellular RNA or the culture supernatant by RT-PCR. This result suggested that the viral rescue of pPa2 and pSHP5 did not occur in MARC-145 cells. Therefore, the insertion of the four nucleotides “ttaa” in front of TRS caused inactivation of the pPa2, while “at” insertion between TRS and translational initiation codon of ORF1 did not affect the production of infectious progeny virus. The replacement of 5’ UTR of type 2 with 5’ UTR of type 1 in the context of type 2 PRRSV clone did not affect virus viability. Otherwise, the converse replacement in a context of type 1 PRRSV was lethal.

For the negative strand gRNA (−) gRNA RT-PCR analysis, in order to detect whether the possible interferences from the input DNA and the RNA transcript generated by the CMV promoter, the RNAs extracted from pAS and WT transfected cells were used for RT-PCR, as our previously reported (Lu et al., 2011). The results in Fig. 2A showed that DNase I and RNase A digestion, nothing could be detected by the RT-PCR (data not shown). The levels of RNA synthesis levels of rTLNld4 showed almost no difference from those of WT. Otherwise the level of chimeric vTLV8 was reduced compared with vAPRRS. Remarkably, the level of RNA synthesis of the mutant pPa2 showed great similarity to that of pAPRRS, although it did not develop CPE in the tested cell cultures. For pSHP5, (−) gRNA could also be detected, but the synthesis level was largely reduced compared with pSHE, as shown in Fig. 2B. The leader–body junction PCR was performed for identification of sg mRNA7, using four primer pairs as follows, SF12/SR15284, SFLV1/SRLV14835 and SF12/SRLV14835. The sg mRNA7 transcription level of vTLV8 dropped to 50% compared with pAPRRS. Otherwise, the sg mRNA7 could not be detected in pSHP5 transfected cells, indicated that the sg mRNA transcription was dramatically impaired. Taken together, the results indicated that 5’ UTR of type 1 PRRSV substitution did not affect type 2 PRRSV propagation in MARC-145 cells. The Chimera pTLV8 shared almost the similar RNA synthesis level and virological characteristics with the parental type 2 PRRSV strain, vAPRRS. However, the converse chimera pSHP5, in which 5’ UTR of a type 1 PRRSV was substituted with counterpart of the type 2 PRRSV, was lethal. Although the (−) gRNA replication could be detected, the replication level was significantly impaired and the sg mRNA transcription was absolutely abolished. Therefore, the type 1 5’ UTR replacement could allow maintain fully functional activities of type 2 PRRSV.

The virological properties and sg mRNA transcription level of vTLV8 are indistinguishable from those of the parental virus

The chimeric virus vTLV8 and the other mutants were compared with the parental virus vAPRRS with regard to their titers, plaque morphology and growth profiles in MARC-145 cells. After 48 hpt in BHK-21 cells, the supernatants were harvested and used to infect MARC-145 cells for measurement of their titers. The
results shown in Fig. 3A illustrate that the viral titer of the parental virus vAPRRS was $1 \times 10^{3.67}$ TCID$_{50}$/mL while the chimeric virus vTLV8 did not develop CPE in the first passage in MARC-145. The viral titer of the mutant vTLNd4 was $1 \times 10^{2.23}$ TCID$_{50}$/mL. In addition, the mutant pPa2 did not develop CPE.

There were no differences in viral plaque morphology between vTLV8, vTLNd4, and vAPRRS, as shown in Fig. 3C. Although vTLV8 did not develop CPE in the first passage, the analysis of growth kinetics showed that vTLV8 had almost the same propagation state as vTLNd4 and vAPRRS (Fig. 3D).

In order to analyze the sg mRNA profiles of the MARC-145 cells infected by the mutant viruses, total intracellular RNAs were extracted from the P5 viral supernatants for Northern blotting analysis. The specific oligonucleotide biotin-labeled probes against type 2 PRRSV 3' UTR (PR3) and type 1 PRRSV 5' UTR (PLVR5) were designed to hybridize with the viral RNA. The sequences of the probes are displayed in Table 1. The results demonstrated that the sg mRNAs of chimeric vTLV8 were recognized by both probes, and the sg mRNA7 was most abundant during the viral transcription process, as shown in Fig. 3B. The parental vAPRRS and mutant vTLNd4 were reacted with the type 2-specific probe PR3, not the type 1-specific probe PLVR5.

**Replacement of complete type 2 5’ UTR retains fully functional activities and genetically stable**

To confirm that the mutant viruses contained the specific mutations in the 5’ UTR, mutant sites were detected via RT-PCR and the 5’-proximal sequences were identified by 5’ RACE. The results showed that the Nde I site was present in the mutant viruses, and the authentic type 1 5’ UTR sequence was maintained in vTLV8 throughout five serial passages (P1–P5). The mutants were genetically stable, as shown in Fig. 4.

The effect of Nde I insertion on sg mRNA transcription for vTLNd4 and vTLV8 was also investigated. sg mRNAs 7.1 and 7.2 for vTLNd4 were detected by leader–body junction RT-PCR (Lu et al., 2011; Zheng et al., 2010). nucleotide sequence analysis of each RT-PCR product revealed that the sg mRNA7 level of vTLNd4 was identical to that of the parental vAPRRS, which indicates that the insertion of Nde I had no impact on the viral mRNA transcription of the mutant (data not shown). The sg mRNA-specific RT-PCR for vTLV8 detected the sg mRNAs 2, 3, 4.1, 5.1, 6.1, 7.1 and 7.2. Comparison of the nucleotide sequences illustrated that the leader–body junction sites of vTLV8 were conserved with their counterparts in the parental vAPRRS (data not shown). There was no dramatic difference in the Northern blotting profiles between the WT, vTLNd4 and vTLV8, as shown in Fig. 3B. Taken together, these results show that the mutations did not produce an evident effect on viral sg mRNA transcription.

**Type 1 5’ UTR chimeric mutant possesses nucleotides mutations in the nsp9 region**

The chimeric vTLV8 and vAPRRS genomic sequences were subjected to full-length sequencing analysis. When compared with the parental vAPRRS, there were no other nucleotide
mutations except for five nucleotide substitutions that occurred in the RdRp coding region (ns9) in vTLV8, which caused four amino acid mutations: T7823C caused the amino acid change Leu to Ser, A8401T caused Thr to change to Ser, A8914T caused Ile to change to Phe, and A9433T caused Ile to change to Phe.

In an attempt to address the function of these amino acid mutations, we introduced four nucleotide mutations (T7823C, A8401T, A8914T, and A9433T) into the backbone of pTLV8 in order to obtain the revertants pTLV7823, 8401, 8914, and pTLV9433. In contrast to pTLV8, the four revertant viruses could be rescued from the first passage in MARC-145 cells using the transfection supernatants in BHK-21 cells. Subsequently, we examined genomic RNA replication and sg mRNA7 transcription levels, together with N protein expression, in transfected BHK-21 cells. The results are shown in Fig. 5. Similar levels of sg mRNA7 transcription were found in these four revertants to those in the parental vAPRRS, but not in vTLV8. If the negative strand genomic RNA replication level of vAPRRS was set at 100%, the relative percentages for vTLV7823, vTLV8401, vTLV8914, and vTLV9433 were 60%, 83%, 71%, and 79%. The level of expression of the PRRSV N-protein of these four revertants showed subtle differences from WT, as shown in Fig. 5C. To test the infectivity of these revertant viruses, the supernatants of the transfected BHK-21 cells at 48 hpt were harvested for first cycle viral titration in MARC-145 cells. The viral titer for WT was $1 \times 10^{3.67}$ TCID$_{50}$ mL$^{-1}$, while those of the revertants vTLV8401, vTLV8914, and vTLV9433 were $1 \times 10^{1.23}$ TCID$_{50}$ mL$^{-1}$, $1 \times 10^{1.5}$ TCID$_{50}$ mL$^{-1}$, and $1 \times 10^{1.33}$ TCID$_{50}$ mL$^{-1}$, as shown in Fig. 6A. Although the growth properties of the four mutant viruses were reduced in comparison with the WT, vAPRRS, they could be rescued during the first passage process (P1). Otherwise vTLV8 could not be recovered in P1.

The plaque morphology of these viruses was also assessed, and the results showed that the plaques of the mutant viruses were distinctly different from those of WT in both morphology and numbers when all using 0.01 MOI. The plaque number of vTLV8 was the lowest, and the plaque size was moderate. The viral titer of vTLV8914 manifested by the plaque assay was consistent with that of the first cycle viral titration. The plaque size was the largest and relatively uniform, while vTLV7823 had a very uneven plaque size. The plaque number of vTLV8914 was relatively low, as shown in Fig. 6B.
PRRSV has evolved into a distinct European genotype 1 and a North American genotype 2. Types 1 and type 2 PRRSV share only 60% identity of their overall nucleotide sequences; they are two very distinct genotypes (Murtaugh et al., 1995; Nelsen et al., 1999). However, the two types retain similar genomic organization (Meng et al., 1994; Meulenberg et al., 1993a; Murtaugh et al., 1995; Nelsen et al., 1999). PRRSV continues to evolve rapidly by random genetic mutation and intragenic RNA recombination. Genetic divergence exists between geographically and temporally separate isolates (Matanin et al., 2008).

The 5' UTR of many positive-stranded RNA viruses, including PRRSV, has been demonstrated to contain cis-acting elements required for viral replication and sg mRNA transcription (Liu et al., 2009). In equine arthritis virus (EAV) sg mRNA transcription...
quite different. The TRS of pTLV8 was still located in the loop structure (Fig. 7B). pTLV8 and pSHSP5, the two chimeric viruses were compared with each other and the two structures displayed in Fig. 1B. The TRS location of the two chimeras were drastically different, showing that the RNA structural model predicted by the Mfold website server and in silico analysis, their predicted secondary structure reveals a certain degree of similarity, especially in the top loop of the striking hairpin structure in which TRS is located. The counterpart of the EAV LTH. Compared with the structural models for the PRRSV and EAV 5'-terminal regions, we found that there were more stem–loop (SL) structural elements in the EAV 5'-terminus than that of PRRSV. The LTH of PRRSV was more striking than its counterpart in EAV. The single strand region of the 5'-proximal region of EAV is longer than that of PRRSV. The top loop of the EAV LTH, which was considered to make the leader TRS accessible as an acceptor during template switching, is larger than the top loop of PRRSV LTH. Despite the lack of identity at the level of primary sequence, the two 5'-UTRs are high-ordered and both contained LTHs, which might be considered to be an independent RNA signal in sg mRNA synthesis. Given that thermodynamic algorithms do not show 100% accuracy in the prediction of RNA secondary structure, phylogenetic analysis remains a useful tool for the establishment of structure models. In a study of the 5'-proximal sequence of EAV, van den Born el al. used chemical and enzymatic probing to gain experimental evidence. The combined results of the chemical and enzymatic probing were in good agreement with the structure mode (Van Den Born et al., 2004).

In this study, we entered the 260-bp sequence of pTLV8 and 260-bp sequence of pSHSP5 into the Mfold software, and the RNA secondary structures obtained showed that, although the secondary structure of chimeric pTLV8 and pSHSP5 had changed dramatically compared with each other and the two structures displayed in Fig. 1B, the TRS location of the two chimeras were quite different. The TRS of pTLV8 was still located in the prominent top loop of LTH structure, as shown in Fig. 7A, but the TRS of pSHSP5 was no longer located in the prominent top loop structure (Fig. 7B). pTLV8 and pSHSP5, the two chimeric mutant viruses rescue showed different results. pTLV8 was viable, while pSHSP5 rescue was lethal. The results indicated that the secondary structure of TRS and its adjacent sequences would be very important for viral replication and infectivity. The viral properties of pTLNd4 and pPa2, which contain the TRS, showed only 60% genetic identity. However, according to the secondary structure model predicted by the Mfold website server and in silico analysis, their predicted secondary structure reveals a certain degree of similarity, especially in the top loop of the striking hairpin structure in which TRS is located; the counterpart in EAV. The viral titers of four revertant viruses were higher than those of the parental chimeric virus and the plaque size differed from that of the chimeric vTLV8. The results of the semi-quantitative RT-PCR showed that the level of RNA synthesis in the four mutant viruses was higher than for vTLV8. Taken together, the results of the semi-quantitative RT-PCR showed that the level of RNA synthesis in the four mutant viruses was higher than for vTLV8. Taken together, the results showed that the regulatory function of the 5'-UTR and downstream UTR of the two PRRSV genotypes were essential for viral replication function (data not shown). Therefore, we propose that the key element in the LTH is the top loop in which TRS is located.

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It had been demonstrated that SL2 in the 5'-UTR of type 2 PRRSV could be replaced with its counterpart from a type 1 PRRSV (Lu et al., 2011). We found, further that the complete 5'-UTR of type 2 PRRSV could be replaced with its counterpart from type 1 PRRSV as the promoter for virus replication in the type 2 virus backbone. Therefore, we postulate that the high-ordered structure is more important than the primary sequence in maintaining viral function. Nevertheless, the RNA structural model obtained from in silico analysis must be examined in experimental studies.

RNA-dependent RNA polymerase (RdRp) is an enzyme that catalyzes the replication of RNA from an RNA template. It is an
essential protein encoded in the genomes of all RNA-containing viruses with no DNA stage that have sense-negative RNA, it catalyzes the synthesis of the RNA strand complementary to a given RNA template. The RdRp region of PRRSV is located in Nsp9 (Fang and Snijder, 2010), which is encoded in ORF1b. RdRp and RNA helicase (Hel; Nsp10) are important elements of the viral replication and transcription complex (RTC); the RTC mediates both genome replication and the synthesis of a discontinuous nested set of sg mRNAs. Present research has revealed that viral RdRp plays a function in the viral life cycle, tightly associated with the 5' UTR and 3' UTR. Stem–loop A (SLA) located in the Dengue virus (DENV) 5' UTR provides the binding site for RdRp. Viral genomic synthesis is initiated from the viral 3' end of the viral genome through a process of cyclization, using 5' binding RdRp (Filomatori et al., 2006). It was reported an important cooperative interaction among chimeric hepatitis C/GB virus B NSSA (RdRp), 5' UTR, and 3' UTR in viral replication (Warter et al., 2009). Such an interaction is crucial for viral RNA replication, packaging, and virion assembly in the viral life cycle. In this study, on the based of reverse genetic manipulation of heterogeneous UTR swapping in an infectious cDNA clone of type 2 PRRSV, it was found that substitution of type 1 5' UTR caused several revertant mutation sites to occur in the RdRp region. The chimeric pTLV8 could be recovered during the second passage process (P2), but the revertants pTLV7823, 8401, 8914, 9433 could be rescued only in P1. The results of the analysis of N protein translation level, semi-quantitative RT-PCR and Northern blotting analysis showed that the level of RNA synthesis of the four mutant viruses were higher than that of vTLV8. Viral plaque assay and titration indicated that the vitality of the revertant viruses was greater than that of vTLV8. Taken together, the results show that the regulatory function is closely related to RdRp, and the heterologous 5' UTR with revertant RdRp played a joint role in the process of virus rescue.

The functional studies reported here provide additional data that increase our understanding of the role of the 5' UTR in the regulation of PRRSV RNA replication, and sg mRNA transcription and translation. For the prevention of PRRSV infection, many regulatory mechanisms in the viral life cycle need to be elucidated.

The research on heterogeneous UTR substitution is scarce, especially for PRRSV. Kang et al. (2006) found that the three SL structures in severe acute respiratory syndrome coronavirus (SARS-CoV) 5' UTR could be replaced by their counterparts from mouse hepatitis virus (MHV). However, the entire UTR could not be functionally substituted because of the location of the TRS (Kang et al., 2006). The current study is the first to show that the type 1 5' UTR can have a regulatory function in the type 2 genomic backbone and remain fully functional. The chimeric virus vTLV6 displayed similar virologic characteristics to the WT, which implies that the substantial differences in biological properties between type 1 and type 2 PRRSV may not be due to the 5' UTR. In addition, we performed related research to explore the origin of the 5' UTR 5' terminal exogenous sequences, and the results showed that the insertion sequence which was used for repair was template independent (Gao et al., 2012). We found that SL1 and SL2 of the PRRSV 5' UTR were important cis-acting elements in viral replication and infectivity (Gao et al., 2012; Lu et al., 2011). This study suggests that LTH, especially the top loop in which the TRS located is another cis-acting element in the life cycle of PRRSV. Taken together, the results of this study are consistent with the conclusion of our previous work (Gao et al., 2012; Lu et al., 2011). The secondary structure elements rather than the authentic 5'-proximal primary sequence, are probably indispensable for PRRSV infectivity. This study provides a useful platform for further study on the structural and functional analysis of the PRRSV 5' UTR.

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