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.

Virus Research 148 (2010) 8–16

Contents lists available at ScienceDirect

Virus Research

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

Recombinant PRRSV expressing porcine circovirus sequence reveals novel aspect of transcriptional control of porcine arterivirus

Haihong Zheng[a,b], Zhi Sun[a], Xing-Quan Zhu[b], Jinxue Long[a], Jiaqi Lu[a], Jian Lv[a], Shishan Yuan[a,*]

[a] Department of Swine Infectious Diseases, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 518 Ziyue Road, Shanghai 200241, China
[b] College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province 510642, China

ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form
15 November 2009
Accepted 25 November 2009
Available online 29 November 2009

Keywords:
PRRSV
Novel subgenomic RNAs
Transcription-regulating sequence
Cryptic TRS
Porcine circovirus

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) expresses its genes via a set of nested subgenomic (sg) mRNAs. Such discontinuous transcription is unique yet poorly understood for arterivirus. The utilization of transcription-regulating sequence (TRS) remains a puzzle, as many TRS-like sequences exist in viral genome, yet only six or seven sg mRNAs were transcribed in arterivirus infected cells. To investigate the transcriptional control of the porcine arterivirus, a recombinant PRRSV infectious cDNA clone pCPV expressing the capsid gene of porcine circovirus 2 (PCV2) between PRRSV ORF1b and ORF2a was developed. The rescued recombinant viruses contained a range of disparate deletions of the inserted PCV2 sequence, yet two stable recombinant viruses containing 41 and 275 nt of foreign sequences were generated upon plaque purification and serial passages. Further analysis of the sg RNA2 profile revealed that an array of novel sg RNA species was generated in cells infected with the recombinant virus. One group was formed by utilizing the inserted PCV2 sequence as TRS; another group was generated from cryptic TRS-like PRRSV sequences located 19, 37 and 97 nt immediately downstream of the PRRSV ORF2 AUG. These results demonstrated that (1) the recombinant virus from direct insertion of foreign sequences was genetically unstable, while two recombinant PRRSVs containing foreign sequence of 41 or 275 nt in length, respectively, became stable upon plaque purification and further serial passages; (2) PRRSV can utilize foreign TRS-like sequence as transcriptional promoter; (3) the insertion of foreign sequence provoked the generation of novel subgenomic RNAs utilizing cryptic TRS-like sequences that remain non-functional in native PRRSV.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the family *Arteriviridae*, order *Nidovirales* (Snijder and Meulenberg, 1998). Similar to most Nidoviruses, PRRSV gene expression adopts a unique discontinuous transcription strategy for synthesis of a nested set of subgenomic (sg) mRNAs (sg mRNAs), sharing the same genomic terminal untranslated regions (UTR) and the poly(A) tail with the viral genome (Pasternak et al., 2006; Snijder and Meulenberg, 1998). During this process, the transcription-regulating sequence (TRS) is believed to play a vital role by mediating the discontinuous jumping of the nascent body RNA to the leader (Makino et al., 1991; Pasternak et al., 2001, 2004; Sola et al., 2005; van Marle et al., 1999; Zuniga et al., 2004), probably via a mechanism of similarity-assisted RNA recombination (Pasternak et al., 2006; Spaan et al., 1983) Several different models for discontinuous transcription have been proposed to illustrate the mechanism of such unique sg RNA synthesis (Pasternak et al., 2006; Sawicki and Sawicki, 1995; Snijder and Meulenberg, 1998). Sawicki and Sawicki (1995) first proposed that the discontinuous jumping happens during the synthesis of the negative-strand RNA. According to this minus-strand RNA extension model, the negative-strand RNA synthesis starts from the 3′ end of the genomic RNA, and attenuates at the TRS body (TRS-B) site, where the transcriptase complex and nascent RNA jump to the leader TRS (TRS-L). More and more experimental data supported the minus-strand extension model, while key questions remain to be answered (den Boon et al., 1996; Hussain et al., 2005; Pasternak et al., 2006, 2001; Sethna et al., 1989; van Marle et al., 1999). For instance, what constitutes the attenuation signals during minus-strand RNA extension? How important a role that TRS-flanking sequences play in the transcriptional process? What is the mechanism of discontinuous jumping of the transcriptase-nascent RNA complex? Equine arterivirus (EAV) has been utilized as a model for molecular dissection of the arteriviral transcription, whereas little is known of porcine arteriviral RNA synthesis.

The development of reverse genetics system paves the way for molecular dissection of the arterivirus transcription. Snijder and coworkers developed the first infectious cDNA clone of arterivirus, and gained significant observations on EAV transcription process

* Corresponding author. Tel.: +86 21 3429 3137; fax: +86 21 5408 1818.
E-mail address: shishanyuan@shvri.ac.cn (S. Yuan).

0168-1702/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2009.11.014

Table 1
Primers used for RT-PCR.

Name	Sequence (5′–3′)	Position	Application
SFPCV	ccTTAATTAAATGACGTATCCAGGGAGGCG	1716–1735	PCR to insert PCV2 ORF2
SRPCV	aaaGGCGCGCCTTAAGGGTTAAGTGGGGGG	1034–1052	between ORF1 and 2
SF11422	GCGTCCCTCCCACATGCCTTC	11,422–11,442	PCR for vCPV genomic
SR12582	GCCTCGCTCACCACCTGTTTC	12,562–12,582	RNA
SF5	CGTATAGGTGTTGGCTCTATGC	5–26	PCR for vCPV1 and
SR12644	TTTACAGGTCTCGGCTTC	12,644–12,661	vCPV2 sg RNA2

Prefixes: SF, forward PCR primer; SR, reverse PCR/RT primer; RT, reverse transcription. Restriction sites introduced by PCR are underlined. The lowercase letters represent protective nucleotides. The nucleotide positions are based on the full-length PRRSV sequence (GQ330474) and PCV2 sequence (DQ104419).

(Pasternak et al., 2000, 2001; van Dinten et al., 1997; van Marle et al., 1999). Utilizing reverse genetic manipulation, these authors found that base-paring between the TRS-L and TRS-B was critical but not sufficient for mediating the discontinuous transcription of EAV subgenomic mRNA, for which local secondary structure seems also to play a significant role (Pasternak, 2003; Pasternak et al., 2001, 2004; van Marle et al., 1999).

As a relatively newly emerging pathogen, PRRSV transcriptional mechanism has remained largely unknown. It is known that the PRRSV TRS-L is a hexa-nuleotide, UUAACC, which is conserved among different PRRSV genotypes (Meng et al., 1996; Meulenberg et al., 1993; Nelsen et al., 1999). However, the TRS-B is diversified among different genotypes, PRRSV isolates, and sgmRNAs code for different proteins of the same virus strain. In addition, several noncanonical TRSs have been identified as leader–body junction site for sg mRNAs (Meng et al., 1996; Nelsen et al., 1999). Among these diversified TRSs, however, it seems that an individual TRS may be preferentially employed for certain subgenomic RNA transcription (Nelsen et al., 1999). In addition, several noncanonical TRS-like sequences exist in the arteriviral genome, implying that arteriviral transcription requires more than just the hexameric TRS sequences. Moreover, novel subgenomic transcripts were detected from cells infected with a recombinant EAV, MHV and TGEV (de Vries et al., 2001; Fischer et al., 1997; Sola et al., 2003), possibly by activation of cryptic nonfunctional TRS sequence generated from inserted heterologous gene containing TRS-like sequence. These phenomena pose questions about what factors determine a functional TRS, and cautions have to be taken during the development of nidovirus as an expression vector for expression of the inserted gene.

To further define the transcriptional control of porcine arterivirus, and test the insertion size and sequence nature of the gene of interest (GOI) using the ORF1 and 2 intergenic locus, we inserted the capsid protein (ORF2) of the porcine circovirus 2 (PCV2), another major pathogen that often synergistically infects pigs with PRRSV. Utilizing the recombinant PRRSV, we analyzed the involved subgenomic RNA synthesis. The results demonstrate that PRRSV transcription is a sophisticated process, and the recombinant PRRSV is useful for the development of PRRSV marker vaccine.

2. Materials and methods

2.1. Cells and viruses

Marc-145 cells (ATCC, Manassas, VA) were propagated in EMEM medium (Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD), and maintained in EMEM with 2% FBS at 37 °C with 5% CO_2. The viruses rescued from the Type II PRRSV infectious clone pAPRRS (Yuan and Wei, 2008) and pORF12 (Yu et al., 2009), respectively, were used as parental viruses in all experiments.

2.2. Construction of recombinant plasmids

The oligonucleotide primers with incorporated restriction site Pac I and Asc I, respectively, were designed using Oligo6.0 software based on PCV2 genomic DNA sequences (GenBank™ accession number: DQ104419) and synthesized by Tiangen Inc. (Shanghai, China). The PCR primers used for PCV2 ORF2 amplification were listed in Table 1. The PCV2 DNA clone spanning the ORF2 was used for PCV2 ORF2 gene amplification (Zhang et al., 2009). The PCR amplification parameters included 30 cycles of 95 °C denaturation (30 s), 58 °C annealing (30 s) and 72 °C extension (1 min), followed by one round of 10 min incubation at 72 °C. The PCR product and pORF12 were digested with Pac I and Asc I (New England Biolabs, Ipswitch, MA), ligated, and the resulting full-length mutant clone was designated as pCPV.

2.3. In vitro RNA transcription and transfection

The full-length plasmid pCPV was isolated using a QIAprep Spin Miniprep kit (QIAgen, Hilden, Germany), and linearized with the restriction enzyme Xho I that cuts immediately downstream of the poly(A) tail, followed by recovering with a QIAquick PCR purification kit (QIAgen, Hilden, Germany). 1 μg of the purified linearized pCPV was used as template for RNA *in vitro* transcription, using a T7 mMessage Machine Kit (Ambion INC, Austin, TX) according to the manufacturer's recommendations. The RNA was dissolved in nuclease-free water, and identified by native RNA agarose gel electrophoresis as described previously (Yuan and Wei, 2008). Marc-145 cells were propagated in a 6-well plate (2×10^5 cells/35 mm well) and grown for 2 days to approximately 60–80% confluence. The subconfluent Marc-145 cells were transfected with 1 μg RNA per well using the DMRIE-C reagent (Invitrogen, Carlsbad, CA), and incubated at 37 °C with 5% CO_2 and was monitored daily for cytopathic effect (CPE). When usually 80% CPE was observed, the supernatant of the cell culture was harvested, aliquoted, and stored in −70 °C freezer as the primary rescued virus stock, designated as vCPV passage 0 (P0). The 1000-fold diluted P0 was used for inoculating fresh cells, and the supernatant was harvested at 4 dpi, designated as P1. The P2–P5 virus stocks were prepared in the same manner.

2.4. Viral plaque assay

The P0 suspension of the rescued vCPV was 10-fold diluted serially, and 200 μl viral preparation was inoculated in Marc-145 monolayer in a 6-well-plate. After 1 h adsorption at 37 °C, the cell monolayer was overlaid with 2 × EMEM containing 4% FBS, and mixed with an equal volume of 2% low melting agarose (Cambrex, Rockland, ME). After the agarose overlay was solidified at room temperature, the plate was inversely (bottom up) placed in humidified CO_2 incubator at 37 °C for 4–5 days. For viral plaque purification, the agarose plug containing a visible plaque was directly picked using a Gilson pipet tip (P20) into an eppendorf

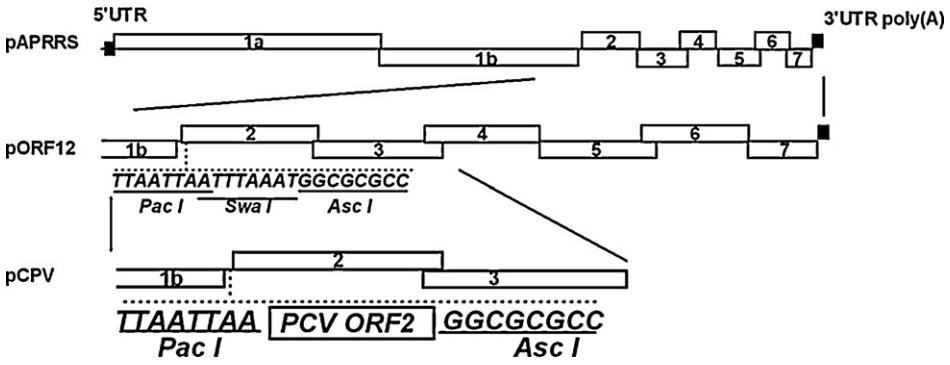

Fig. 1. Construction of recombinant PRRSV expressing PCV2 capsid gene. The boxed numbers 1a, 1b, 2a, 2b, 3–7 represent PRRSV open reading frame (ORF) 1a-ORF7, respectively. For the mutant PRRSV clones, only the region covering the 3′ part of ORF 1b through the 3′ end of the genome is shown. The pORF12 was constructed (Yu et al., 2009) by inserting restriction sites (Pac I, Swa I and Asc I, a total of 23 nt) directly between ORF1b and ORF2 of the wild-type pAPRRS. pCPV was then constructed by inserting Pac I-PCV2 ORF2-Asc I in the same insertion site as pORF12.

tube containing 500 μl of EMEM, followed by pipetting up-and-down, vortexing, and freezing-and-thawing three times to dissolve the agarose plug. After centrifugation at 12,000 × g for 10 min, the supernatant was used directly for inoculation of Marc-145 cells in a 6-well-plate, and the resultant viral plaques were picked and treated as mentioned above. Such plaque-to-plaque purification was carried out for three times. The supernatant of the infected cells by the 3rd round plaque suspension was harvested at 4–5 dpi, when about 80% cells developed CPE, followed by serial passages at 0.01 multiplicity of infection (MOI) up to five passages, and designated as P2–P5, respectively.

2.5. Growth curve of virus

The vCPV1 and vCPV2 suspensions were diluted 10-fold serially, and 200 μl viral suspension was inoculated onto Marc-145 monolayers, and then treated by the procedures mentioned in 2.4. The resulting plaques were stained by crystal violet (5% (w/v) in 20% ethanol). To determine the multi-step growth curve, Marc-145 cells in 6-well-plate were infected with 200 μl (0.01MOI) of P5 viruses. An aliquot of the cell culture supernatant was harvested and replenished with an equal volume of EMEM containing with 2% FBS at the indicated time points (6, 12, 24, 36, 48, 72, 84, 96, 108, and 120 h) post-infection, 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 (Sun et al., 2007).

2.6. RNA extraction and RT-PCR

Viral genomic RNA was isolated using a QIAprep Viral RNA Mini Kit (QIAgen, Hilden, Germany), and total intracellular RNAs were isolated using TRizol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was suspended in RNase-free water, quantified by UV spectrometer, aliquoted and stored at −70 °C. The primers used to detect recombinant PRRSVs are listed in Table 1. For confirming the existence of inserted PCV2 ORF2 in the genomic RNA of the recombinant PRRSVs, RT-PCR amplification was conducted for 30 cycles of 95 °C denaturation (30 s), 60 °C annealing (30 s) and 72 °C extension (1.5 min), followed by incubation at 72 °C for 10 min. The PCR product was purified by QIAquick PCR purification kit as instructed by the supplier (QIAgen, Hilden, Germany), followed by cloning into pGEM-T vector (Promega, Madison, USA). RT-PCR for detecting sg RNA2 species of the recombinant viruses included 40 cycles of 95 °C denaturation (30 s), 58 °C annealing (30 s) followed by incubation at 72 °C for 10 min. The PCR products were gel-purified and sub-cloned into pGEM-T vector (Promega, Madison, USA), and followed by nucleotide sequencing.

2.7. RNA structure analysis

The computer programs Mfold and RNAVIZ were used to analyze the secondary structure of TRS-B used for novel sg RNA2. 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 TRS-B used for novel sg RNA2 including flanking sequence.

3. Results

3.1. Construction of recombinant PRRSV expressing PCV2 sequence

PRRSV adopts a tightly controlled gene expression strategy, and overlapping regions exist between most of the ORFs coding for the structural proteins, posing problems for dissecting the transcriptional process. However, there is one nucleotide separating ORF1 and 2, downstream of the canonical TRS2 for sgmRNA2 synthesis. To facilitate foreign gene cloning in this locus, we previously developed an infectious clone, pORF12, containing a poly-cloning linker consisting of three restriction enzyme sites (Pac I, Swa I, and Asc I) between ORF1b and ORF2a (Yu et al., 2009). In this study, we set out to investigate sg RNA profile of the recombinant virus expressing PCV2 capsid gene (ORF2). As shown in Fig. 1, the PCV2 coding sequence (702 bp) was inserted into the full-length infectious mutant clone, producing the resultant full-length mutant cDNA clone pCPV which contained a Pac I site at the 5′ end of the insertion followed by the intact PCV2 ORF2 and the Asc I site directly connecting with the PRRSV ORF2. The resultant recombinant plasmid pCPV was verified by restriction enzyme mapping and nucleotide sequencing.

In vitro RNA transcripts from the linerized pCPV DNA template were used to transfect Marc-145 cells, which developed CPE at 7 days post-transfection, approximately 48 h delayed than that of the synthetic RNA from the parental pORF12. The supernatant of the transfected cells was harvested, designated passage 0 (P0), and the rescued virus was named as vCPV. The latter was serially passaged five times, and designated as P1-P5, respectively. IFA was conducted with monoclonal antibody against Nsp2 and N protein of PRRSV, respectively (data not shown). These results demonstrated that the rescued virus was PRRSV-specific.

3.2. Recombinant PRRSV contained a range of deletions of the inserted PCV2 gene

Since we failed to detect PCV2-specific antigen from cells infected by the recombinant virus, we hypothesized that the inserted PCV2 gene was altered by either deletion and/or changes of open reading frame. To this end, RT-PCR of the viral genomic RNA was performed with a primer set (Table 1) flanking the poly-cloning site, which produced a calculated PCR product of 1878 bp in length using the recombinant pCPV plasmid as the template. However, a major PCR band of approximately 1450 bp in length was detected in P1 virus preparation, meaning that about 400 bp of the inserted gene was deleted (data not shown). These results indicated that nucleotide sequence deletions occurred in vCPV, while also retained partial inserted PCV2 sequence. To further investigate the sequence nature of the recombinant virus, the PCR products were cloned. Ten plasmid clones were used for analysis of nucleotide sequence. The nucleotide sequencing results showed that two types of PCV2 sequence deletion occurred, with four clones contained 41 nt and the remaining six clones retained 275 nt of foreign sequences (Fig. 5). In both cases, the flanking PRRSV genomic RNA sequence remained unchanged. These results demonstrated that the recombinant virus from direct insertion of the PCV2 sequence was genetically unstable.

3.3. Plaque-purified recombinant PRRSV expressing partial PCV2 sequence can be genetically stable and displayed similar characteristics with those of the parental virus

To further investigate the genetic plasticity of the recombinant virus, we conducted viral plaque purification and molecular characterization of the insertion region. A total of 10 plaques were picked, and each plaque was further purified by three rounds of plaque-to-plaque assay. The afore-mentioned RT-PCR and nucleotide sequencing analysis were performed on the purified genomic RNAs of the first passage of the selected viral plaques. Again, the same two PCV2 sequence deletion patterns were identified, with three plaques retained 41 nt and another seven plaques contained 275 nt of the inserted foreign sequences. To assess the genetic stability of the plaque-purified viruses, two representative plaques, vCPV1 and vCPV2, were further serially passaged by infecting Marc-145 cells at 0.01MOI for five times, and designated as P1–P5, respectively. The same RT-PCR and nucleotide sequencing were carried out on P1, P3, and P5 of vCPV1 and vCPV2 genomic RNAs. At least 10 independent clones for each RT-PCR product of each sample were sequenced. Surprisingly, the nucleotide sequences at the site of insertion remained the same during the serial passage process, and were identical with what were found in the originally rescued vCPV (P0). Specifically, vCPV1 contained 41 nt located at the 3′ end of the foreign sequence, while vCPV2 contained 275 nt sandwich a 443 nt (74–516 nt) deleted middle region of PCV2 ORF2 deleted (Fig. 5), while there is no alteration in the flanking PRRSV genomic RNA sequence in both viruses and their progenies. These results demonstrated that the recombinant virus from direct insertion of foreign sequence without an additional TRS was genetically unstable, however, plaque-purified viruses can be maintained genetically stable at the insertion site for at least five passages.

Genetic manipulation of the PRRSV genome could affect viral characteristics. To address this question, we performed viral plaque morphological analysis and growth kinetics of vCPV1 and vCPV2. Virus plaque assay revealed that the recombinant viruses and the parental virus vORF12 virtually had the same plaque morphology (Fig. 2A). Multi-step growth curves demonstrated that vCPV1 and vCPV2 maintained a growth pattern similar to that of vORF12, *albeit* the peak titers of vCPV1 and vCPV2 were a half of a log lower than that of the vORF12 (Fig. 2B). These results indicated that PRRSV can

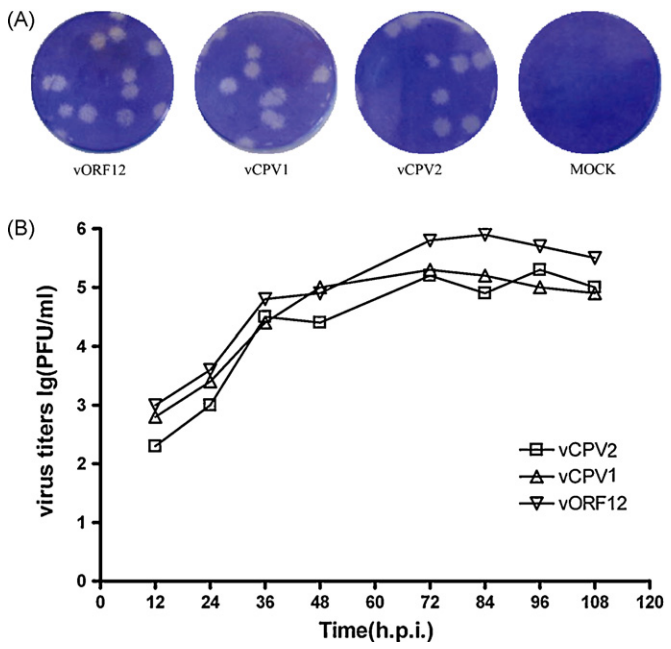

Fig. 2. Mutant viruses retained similar viral properties with parental virus. (A) Viral plaque morphology. Marc-145 cells in 6-well-plate were infected with passage 5 viruses at an MOI of 0.01, or mock-infected with EMEM. The monolayer of infected cells was overlaid with agarose, and stained with crystal violet at 4 dpi. (B) Viral growth kinetics growth analysis. The infection was done as mentioned above. The cell supernatants were harvested at the indicated time points, followed by viral titration by plaque assay on Marc-145.

stably express foreign sequence tag up to 275 nt in length from the intergenic locus of ORF1/2, while maintaining growth properties of the parental virus in cultured cells, which demonstrated that the recombinant PRRSV can be used to produce recombinant marker vaccines to eliminate the infectious disease.

3.4. Novel subgenomic RNAs generated by utilizing foreign sequence as TRS

PRRSV transcriptional control remains largely unknown, and insertion of foreign sequence in intergenic region would disrupt local genomic RNA sequence as well as its secondary structure.

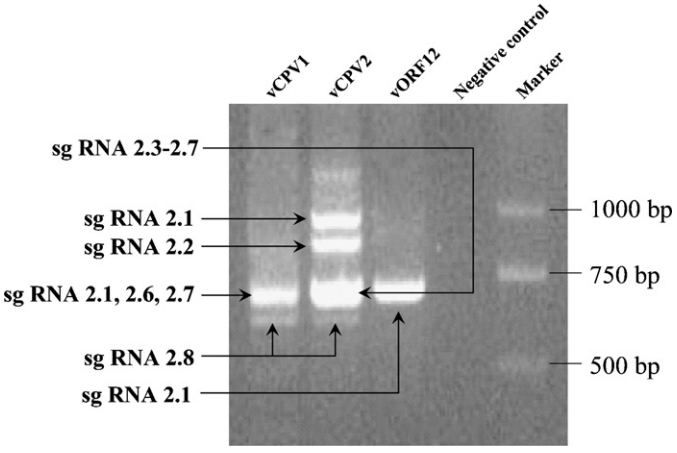

Fig. 3. RT-PCR amplification of leader–body junction specific regions of novel subgenomic RNAs. In order to study the impact of PCV2 sequence on PRRSV transcription, RT-PCR of the novel sg RNA2 s was performed on the total RNA obtained from Marc-145 cells infected by vCPV1 or vCPV2 (P5), using forward primer located in leader region and reverse primer in the ORF2. The bands representing the specific sg RNA2 were shown in the picture.

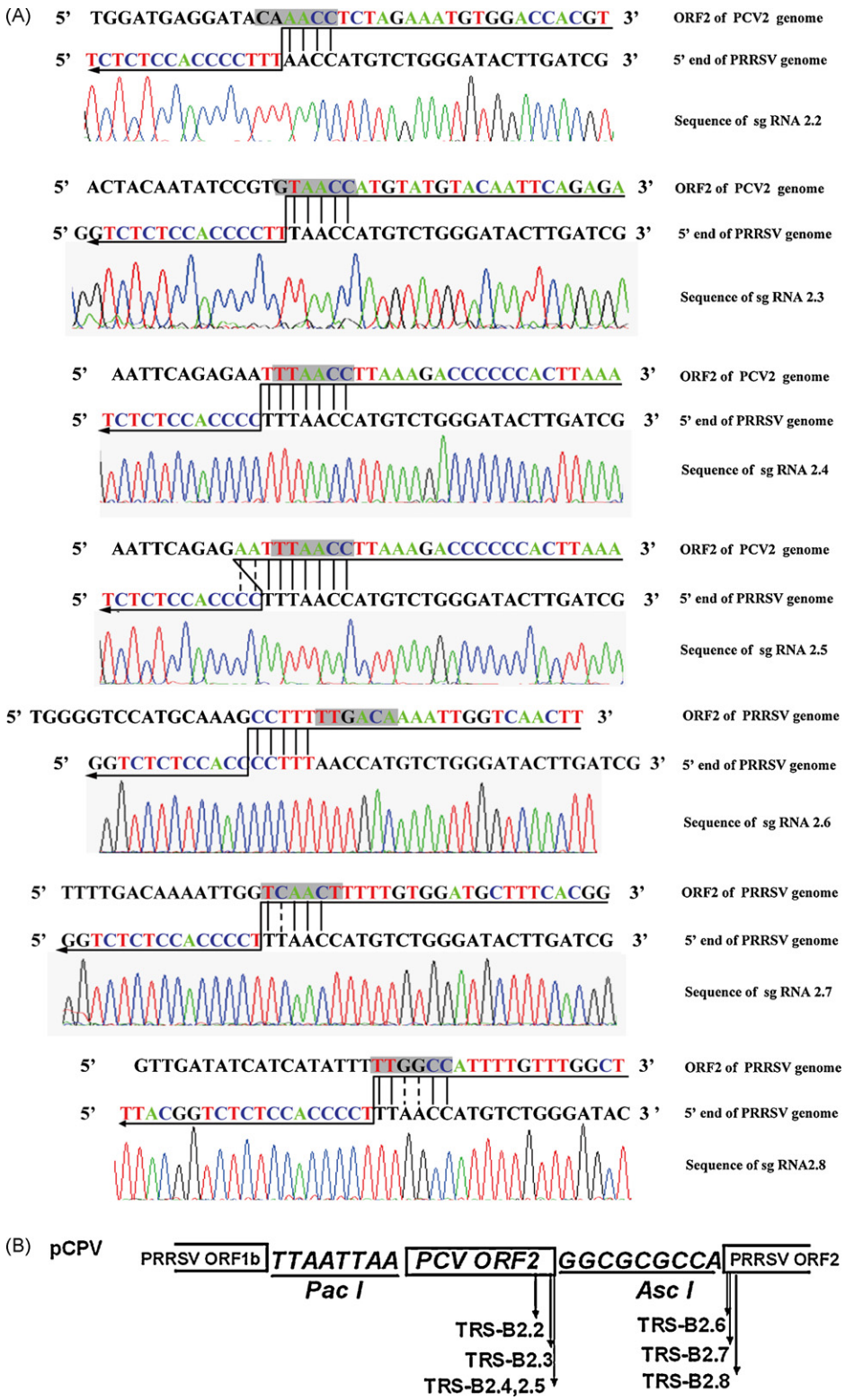

Fig. 4. Identification of the leader–body junction sequence and site for purified vCPV novel sg RNA2. (A) Identification of the leader–body junction sequence for novel subgenomic RNAs. The PCR products were gel-purified and cloned into pGEM-T vector and at least 10 independent clones for each band were sequenced. Sequence analysis reveals that novel subgenomic mRNAs were joined at aberrant junction sites of heterogeneous nucleotides. Sg RNA2.2, sg RNA2.3, sg RNA2.4 and sg RNA2.5, utilized PCV2 ORF2 sequence as TRS. Sg RNA2.6, sg RNA2.7 and sg RNA2.8 utilized a TRS-like sequence located at 19, 37 and 97 nt immediately downstream of the PRRSV ORF2 start codon, respectively. Note that, Sg RNA 2.4 and sg RNA2.5 were formed by utilizing the same TRS sequence, while the leader–body fusion sites were different from each other. The variant fusion site is downsream or upstream of the AAT sequence motif, which is preceding the body TRS (TTAACC) for sg RNA 2.4 and sg RNA 2.5. The upper strand in the alignments represented the TRS-B region of PCV2 ORF2 or PRRSV ORF2 genomic sequence, and the lower strand represents PRRSV genomic leader sequence. Arrowheads indicated the jumping direction of leader–body. The nucleotides in colored letter were derived directly from the sequence profiles. The leader–body junction sequences were indicated by straight and dash line. The sequences of TRS-B were shaded. (B) Identification of the Leader-Body junction sites for novel subgenomic RNAs. For the mutant pCPV, only the region covering the part of 1b and ORF2 of PRRSV is shown. Arrowheads indicate the location in pCPV of TRS-B for novel subgenomic RNAs.

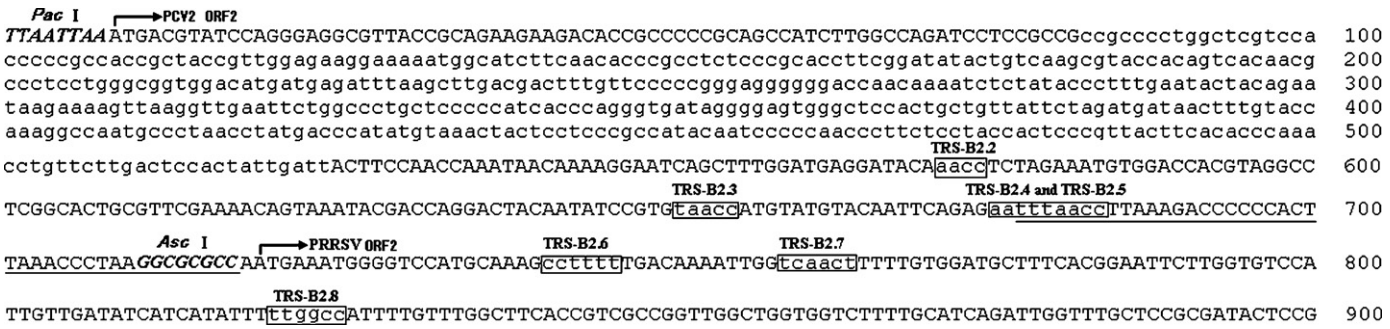

Fig. 5. The genomic RNA sequence of purified vCPV and the Leader-Body junction sequence for novel sg RNAs with their predicted ORFs. The sequence of PCV2 ORF2 was partly deleted. By plaque-to-plaque purification, there were two stable deletion types, which were designated as vCPV1 and vCPV2. In vCPV2 deletion type, the deleted sequence of PCV2 ORF2 was signified by lowercase letter, and in vCPV1 type, the only remained PCV2 ORF2 sequence was showed underlined. The possible Leader-Body junction sequences of all the novel sg RNAs were signified in the rectangle for vCPV2, however, only novel sg RNA2.6–2.8 are synthesized in vCPV1.

Analysis of such genomic RNA alteration would shed light on the mechanism of sgmRNA synthesis and/or translation. In order to study the impact of PCV2 sequence on PRRSV transcription of sgmRNA2, we determined the origin of the leader–body junction sequences of all possible sg RNA2 species in cells infected by P5 viruses of both vCPV1 and vCPV2, by sg RNA2-specific RT-PCR. In comparison with the parental vORF12 producing just one sgmRNA2 (Yu et al., 2009), vCPV1 and vCPV2 produced one and three extra PCR bands, respectively (Fig. 3), implying that novel subgenomic RNA species besides the canonical sg RNA2.1 were generated in the cells infected by the recombinant virus. The purified PCR bands were gel-purified, cloned, and nucleotide sequences were determined. At least 20 independent clones of each PCR products were sequenced. The leader–body junction site for sg RNA2.1 was the same as that of the vORF2 canonical sgmRNA2, while PCV2 sequence in sg RNA2.1 was partly deleted, and the deleted sequence was the same as in vCPV1 and vCPV2 genomic RNA, respectively (data not shown). The only difference between sg RNA2.1 of vCPV1 and vCPV2 was the size of the inserted PCV2 sequence. For vCPV2, sg RNA2.2, sg RNA2.3, sg RNA2.4 and sg RNA2.5 were formed by utilizing PCV2 sequence as TRS. Surprisingly, in the sequence for sgRNA2.3, there was clearly a 5 C residue (CCCCC) at positions 11–15, but this was written out as 4 C and a T residues (CCCCT) in the Leader template (Fig. 4A). The sequences downstream of the leader–body fusion sites of sgRNA 2.3 (CCCCt) have one nucleotide difference (lowercase character) from the Leader template (CCCCC), but the fusion site sequences (TAACC) are identical to the core sequence of TRS-B. The sg RNA 2.4 and sg RNA2.5 were formed by utilizing the same TRS sequence, while the leader–body fusion sites were different from each other. The result indicated that the variant fusion site was downstream or upstream of the AAT sequence motif preceding the body TRS (TTAACC) for sg RNA 2.4 and sg RNA 2.5 (Fig. 4A). We summarized the leader–body junction sites for these novel sg RNA2 located in pCPV (Fig. 4B), and concluded that PRRSV can utilize TRS-like sequence from the inserted foreign gene for generation of novel subgenomic RNA species, and the leader–body junction sequences of all the novel sg RNA2 are from TRS-B.

3.5. Foreign sequence insertion provoked cryptic TRS and novel subgenomic RNAs

Using sg RNA-specific RT-PCR, extra more novel sg RNA species were detected from cells infected with the recombinant vCPV than the parental virus (Fig. 3), with the larger ones formed by utilizing the canonical TRS-B2.1 and the inserted PCV2 sequence. We then determined the leader–body junction sites of the two smaller PCR bands. Surprisingly, three distinct leader–body junction sites were located 19, 37 and 97 nt, respectively, immediately downstream of the PRRSV ORF2 start codon for both vCPV1 and vCPV2 (Fig. 4A). Further sequence comparison showed that both TRS-B2.7 (UcAACu) and TRS-B2.8 (UUggCC) are indeed TRS-B (UUAACC)-like. Intriguingly, TRS-B2.6 utilized a noncanonical TRS (ccuuu), and the template switching took place at the sequence motif (ccuuu) including 2 nt of the 5′ end of the canonical TRS-L and 3 nt upstream from the canonical TRS-L (UUAACC), and just preceded the TRS-B-like (UUgACa), which showed that the fusion site of sgRNA2.8 was not just the site of TRS-B, but upstream from it. These results demonstrated that foreign sequence insertion may provoke the generation of novel subgenomic RNAs utilizing cryptic TRS-like and other diversified sequences.

4. Discussions

As a relatively small RNA virus, arterivirus adopts 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 mechanism with other nido- or arterivirus. In the present study, we investigated the nature of porcine arterivirus transcription by inserting a foreign sequence. We found that recombinant PRRSV displayed an array of novel subgenomic RNA species formed by utilizing TRS-like sequence in the inserted foreign sequence and by the cryptic TRSs located 19, 37 and 97 nt immediately downstream of the PRRSV ORF2 start codon provoked when foreign sequences were inserted. Although the novel TRSs were diversified, most of them were TRS-like (UUAACC).

In this study, for sg RNA2.3, contained additional, nontemplated nucleotides. The sequences downstream of the leader–body fusion sites of sg RNA 2.3 (CCCCu) have one nucleotide difference (lowercase character) from the Leader template (CCCCC). PRRSV is RNA virus, and the nucleotide mutation phenomenon usually happens, for RNA-dependent RNA polymerase (RdRp) can permit some nucleotide mismatch when PRRSV replicates and transcripts. So the one nucleotide difference may be caused by nucleotide mismatch during discontinuous transcription. Like SARS-CoV (Hussain et al., 2005), for sgRNA2.5, the RNA polymerase can slide three nucleotides back on the leader template. And for sgRNA2.6, did use noncanonical transcriptional signals, and the CCUUU was used directly as a transcription-regulating signal, the leader–body junction site took place in the CCUUU region. Our results demonstrated that PRRSV can utilize foreign TRS-like sequences as TRS mediating the generation of the novel sg RNA species. de Vries et al. (2001) reported that EAV could utilize TRS-like foreign sequence in the inserted green fluorescent protein (GFP) as TRS to form the novel sg RNAs, which might be related to a sequence specificity of the GFP

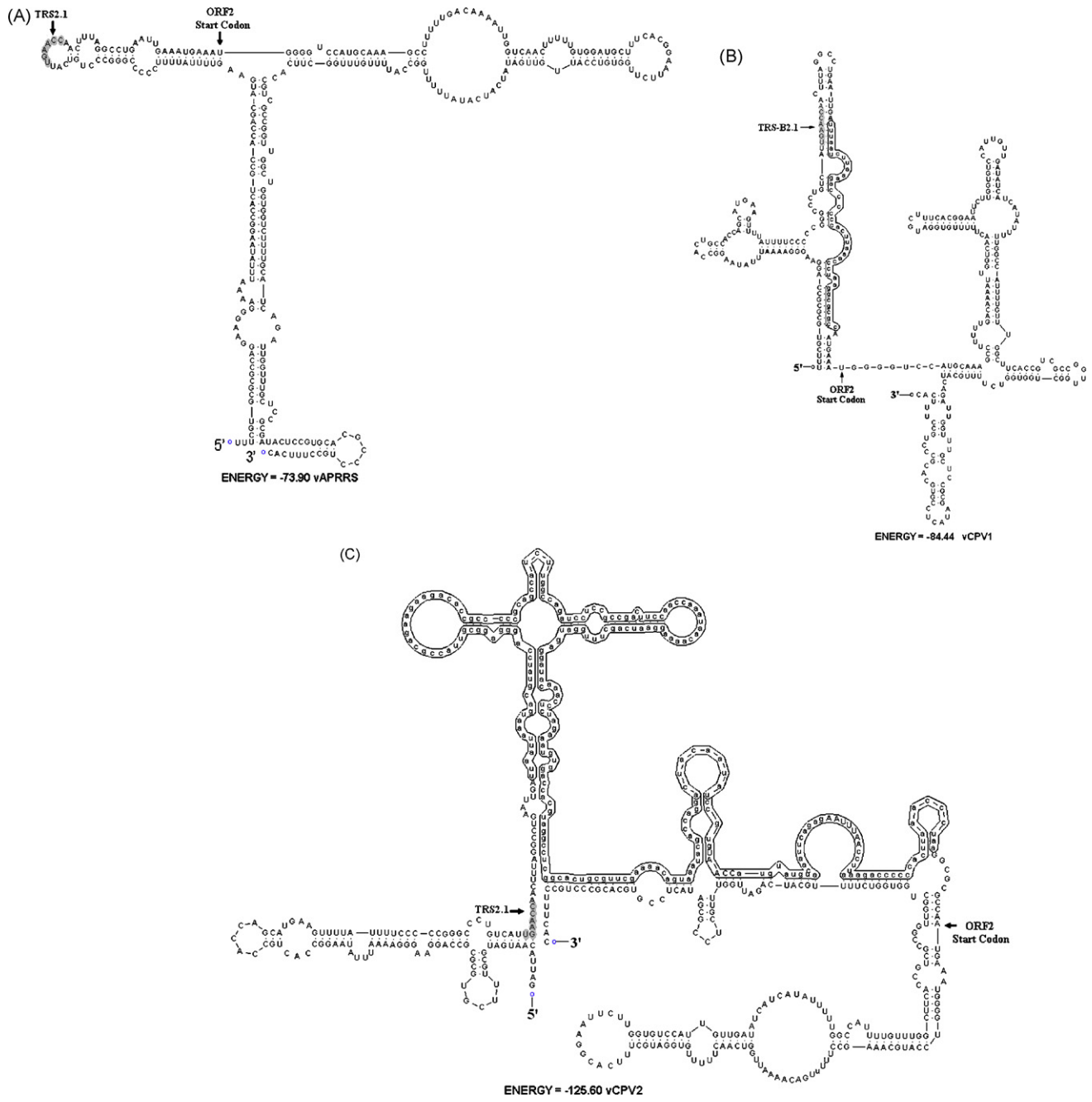

Fig. 6. RNA secondary structure prediction for TRS-B used for novel sg RNA2. The computer programs Mfold and RNAVIZ were used to analyze the secondary structure of TRS-B used for novel sg RNA2. The remnant foreign sequences were boxed. The TRS-B was shaded. The structure indicated the TRS-B for sg RNA2.1 of vAPRRS was located on the loop in the majority of cases, while TRS-Bs for sg RNA2.1 of vCPV1 and vCPV2 were completely on the stem. (A) vAPRRS native TRS-B region; (B) vCPV1TRS-B region; (C) vCPV2 TRS-B region.

gene. The authors also suggested that the anomalous formation of subgenomic mRNAs is unnecessarily a general consequence of the insertion of foreign genetic information into the EAV genome. In our study, we identified a range of novel sg RNA formed by utilizing TRS-like sequence of PCV2 ORF2, implying that it is common property for coronavirus and arterivirus transcriptional machinery to utilize TRS-like foreign sequences for generation of novel sg RNA species. Like MHV, EAV and TGEV, the location of the functional TRS-like sequences in PCV2 ORF2 for PRRSV is uniformly located at the 3′ end of the inserted foreign sequences (de Vries et al., 2001; Fischer et al., 1997; Sola et al., 2003). This phenomenon may be related to transcription control scheme, in other word, the location of the novel TRSs from foreign sequences may be controlled by the length of transcription. Fischer et al. (1997) proposed that discontinuous transcription could sometimes depend solely on the structure of the viral genome which in turn would be determined by long-range RNA-RNA and/or RNA-protein interactions, and the primary sequence of the foreign sequence as TRS is usually TRS-like. So, for foreign sequence as TRS, its location, the second structure, and the primary sequence are the most important definitive factors.

Our results suggested that alteration of the local RNA structure in recombinant PRRSV provoked the cryptic TRSs in coding region. Several lines of evidences showed that nidovirus utilizes noncanonical TRS when the canonical TRS was altered (Pasternak et al., 2004; Yount et al., 2006; Zhang and Lai, 1994). It is believed that the canonical TRS is located in the loop region of the local RNA sec-

ondary structure (de Vries et al., 2001; van Marle et al., 1999). We analyzed the local RNA second structure of TRS-B2 and its flanking region by Mfold program (Zuker, 2003). As shown in Fig. 6A, the authentic canonical TRS-B2.1 was located in the loop region in the parental virus, whereas was located in the stem in the recombinant vCPV (Fig. 6B and C). The alteration induced by insertion of foreign sequence may affect the functionality of PRRSV canonical TRS-B2.1, as a consequence, the cryptic TRS-B2.6–2.8 were activated.

Further development of PRRSV as an expression vector is of great interest. As the most genetically variable region in PRRSV genome, nsp2 coding sequence has been attempted as site for expressing gene of interest (GOI), but most of such recombinant viruses were proved to be unstable (Fang et al., 2008; Han et al., 2007), or lost the biological activity of the GOI when the recombinant virus was genetically stable (Kim et al., 2007). Calvert et al. (2002) inserted the GFP gene into the P129 infectious clone with an extra TRS element between ORF1b and ORF2a. Such "green" virus was proved to be genetically and phenotypically stable for at least 37 passages. Using the same strategy, Pei et al. (2009) inserted the PCV2 capsid gene with a TRS sequence for the downstream PRRSV ORF2, the recombinant PRRSV-PCV2 virus was stable in both *in vitro* and *in vivo* host. Thus, a synthetic TRS with flanking sequences was introduced to GP2 and E protein normally expressed, and the recombinant viruses are stable (Calvert et al., 2002; Pei et al., 2009). In our study, we directly inserted of PCV2 capsid gene into intergenic region between ORF1 and 2 without an additional TRS. de Vries et al. (2001) reported that EAV could utilize TRS-like foreign sequence in the green fluorescent protein (GFP) inserted to form a novel sg RNA, and these novel sg RNA could code for extended M protein. Our results showed that multiple functional leader-to-body junction sites were located within the PCV2 ORF2 gene, such as TRS-B2.2–2.5. It is believed that arteriviral mRNA translation utilizes a leaky scanning scheme, during which the most 5′ proximal AUG downstream of the leader is probably utilized as the initiation codon of the mRNA. The arteriviral sgmRNA2-coded GP2 and E proteins are vital for virus viability (Snijder et al., 1999; Lee and Yoo, 2006). We obtained viable recombinant PRRSV containing foreign sequence insertion up to 275 nt, indicating that the GP2 and E proteins were expressed from one or more of the sg RNA2 molecules. The sg RNA2.1, utilizing the canonical TRS-B2.1, contained multiple start and stop codons upstream of the authentic initiator AUGs for both ORF2a and ORF2b, making it as unlikely mRNAs coding for the GP2 and E. For sg RNA2.2, the most 5′ proximal AUG is outframe for PRRSV GP2. For sg RNA2.3, the first AUG is in-frame for GP2, however, if sg RNA2.3 can code for an extended GP2, the ribosomes have to read through the termination signals of PCV2 capsid gene as the recombinant EAV coded for extended M protein (de Vries et al., 2001). For sg RNA2.4 and sg RNA2.5, the first AUG is just the start code of GP2. So sg RNA2.4 and sg RNA2.5 may code for the intact GP2 and E protein. So, in our study, it is possible for PRRSV GP2 and E protein to be normally expressed. To be disappointed, the recombinant PRRSV were unstable, in which a chunk of inserted gene was deleted. The instability of the recombinant PRRSV could be caused by disruption of PRRSV ORF2 transcription. Upon insertion of PCV2 ORF2 to the region, TRS2.1 drove transcription of the PCV2 ORF2 gene instead of ORFs 2a and 2b. E protein has been shown to be essential for EAV (Snijder et al., 1999) and PRRSV (Lee and Yoo, 2006). Accordingly, the recombinant PRRSV must make TRS-like sequence active to form the novel sg RNAs for expressing GP2 and E protein by deleting the foreign sequences. However, for EAV, although an expression cassette consisting of the GFP gene flanking EAV-specific transcription-regulating sequences at its 3′ end was constructed, the recombinant EAV was not genetically stable (de Vries et al., 2001). Besides the reason referred to in the paper, we consider that the stability of recombinant virus is related to the location of foreign sequence inserted into genome and the background of genome (Calvert et al., 2002; Pei et al., 2009).

Acknowledgements

S. Yuan was supported by the key program of the China Natural Science Foundation (#30530580), the National Basic Research Program (#2005CB523202), the China national science and technology special program "Prevention and control of highly pathogenic PRRSV" (2007BAD86B06-3), the Ministry of Human Resources of China (#2006-Z3), the National non-profit institute funding program MOST (#2005DIB4J051), the MOST national institute funding program (Shanghai Veterinary Research Institute, CAAS) and the State Key Laboratory of Veterinary Etiological Biology (Lanzhou Veterinary Research Institute, CAAS)(SKLVEB2009KFKT021).

References

Calvert, J.G., Sheppard, M.G., Welch, S.-K.W., 2002. Infectious cDNA clone of North American porcine reproductive and respiratory syndrome (PRRS) virus and uses thereof. US Patent.
De Rijk, P., Wuyts, J., De Wachter, R., 2003. RnaViz 2: an improved representation of RNA secondary structure. Bioinformatics 19 (2), 299–300.
de Vries, A.A., Glaser, A.L., Raamsman, M.J., Rottier, P.J., 2001. Recombinant equine arteritis virus as an expression vector. Virology 284 (2), 259–276.
den Boon, J.A., Kleijnen, M.F., Spaan, W.J., Snijder, E.J., 1996. Equine arteritis virus subgenomic mRNA synthesis: analysis of leader–body junctions and replicative-form RNAs. J. Virol. 70 (7), 4291–4298.
Fang, Y., Christopher-Hennings, J., Brown, E., Liu, H., Chen, Z., Lawson, S.R., Breen, R., Clement, T., Gao, X., Bao, J., Knudsen, D., Daly, R., Nelson, E., 2008. Development of genetic markers in the non-structural protein 2 region of a US type 1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. J. Gen. Virol. 89 (Pt 12), 3086–3096.
Fischer, F., Stegen, C.F., Koetzner, C.A., Masters, P.S., 1997. Analysis of a recombinant mouse hepatitis virus expressing a foreign gene reveals a novel aspect of coronavirus transcription. J. Virol. 71 (7), 5148–5160.
Han, J., Liu, G., Wang, Y., Faaberg, K.S., 2007. Identification of nonessential regions of the nsp2 replicase protein of porcine reproductive and respiratory syndrome virus strain VR-2332 for replication in cell culture. J. Virol. 81 (18), 9878–9890.
Hussain, S., Pan, J., Chen, Y., Yang, Y., Xu, J., Peng, Y., Wu, Y., Li, Z., Zhu, Y., Tien, P., Guo, D., 2005. Identification of novel subgenomic RNAs and noncanonical transcription initiation signals of severe acute respiratory syndrome coronavirus. J. Virol. 79 (9), 5288–5295.
Kim, D.Y., Calvert, J.G., Chang, K.O., Horlen, K., Kerrigan, M., Rowland, R.R., 2007. Expression and stability of foreign tags inserted into nsp2 of porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 128 (1–2), 106–114.
Lee, C., Yoo, D., 2006. The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. Virology 355 (1), 30–43.
Makino, S., Joo, M., Makino, J.K., 1991. A system for study of coronavirus mRNA synthesis: a regulated, expressed subgenomic defective interfering RNA results from intergenic site insertion. J. Virol. 65 (11), 6031–6041.
Meng, X.J., Paul, P.S., Morozov, I., Halbur, P.G., 1996. A nested set of six or seven subgenomic mRNAs is formed in cells infected with different isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 77 (Pt 6), 1265–1270.
Meulenberg, J.J., de Meijer, E.J., Moormann, R.J., 1993. Subgenomic RNAs of Lelystad virus contain a conserved leader–body junction sequence. J. Gen. Virol. 74 (Pt 8), 1697–1701.
Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73 (1), 270–280.
Pasternak, A.O., 2003. Nidovirus transcription-regulating sequences. PhD thesis, Leiden University.
Pasternak, A.O., Gultyaev, A.P., Spaan, W.J., Snijder, E.J., 2000. Genetic manipulation of arterivirus alternative mRNA leader–body junction sites reveals tight regulation of structural protein expression. J. Virol. 74 (24), 11642–11653.
Pasternak, A.O., Spaan, W.J., Snijder, E.J., 2004. Regulation of relative abundance of arterivirus subgenomic mRNAs. J. Virol. 78 (15), 8102–8113.
Pasternak, A.O., Spaan, W.J., Snijder, E.J., 2006. Nidovirus transcription: how to make sense? J. Gen. Virol. 87 (Pt 6), 1403–1421.
Pasternak, A.O., van den Born, E., Spaan, W.J., Snijder, E.J., 2001. Sequence requirements for RNA strand transfer during nidovirus discontinuous subgenomic RNA synthesis. EMBO J. 20 (24), 7220–7228.
Pei, Y., Hodgins, D.C., Wu, J., Welch, S.K., Calvert, J.G., Li, G., Du, Y., Song, C., Yoo, D., 2009. Porcine reproductive and respiratory syndrome virus as a vector: immunogenicity of green fluorescent protein and porcine circovirus type 2 capsid expressed from dedicated subgenomic RNAs. Virology 389 (1–2), 91–99.
Sawicki, S.G., Sawicki, D.L., 1995. Coronaviruses use discontinuous extension for synthesis of subgenome-length negative strands. Adv. Exp. Med. Biol. 380, 499–506.

Sethna, P.B., Hung, S.L., Brian, D.A., 1989. Coronavirus subgenomic minus-strand RNAs and the potential for mRNA replicons. Proc. Natl. Acad. Sci. U.S.A. 86 (14), 5626–5630.
Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79 (Pt 5), 961–979.
Snijder, E.J., van Tol, H., Pedersen, K.W., Raamsman, M.J., de Vries, A.A., 1999. Identification of a novel structural protein of arteriviruses. J. Virol. 73 (8), 6335–6345.
Sola, I., Alonso, S., Zuniga, S., Balasch, M., Plana-Duran, J., Enjuanes, L., 2003. Engineering the transmissible gastroenteritis virus genome as an expression vector inducing lactogenic immunity. J. Virol. 77 (7), 4357–4369.
Sola, I., Moreno, J.L., Zuniga, S., Alonso, S., Enjuanes, L., 2005. Role of nucleotides immediately flanking the transcription-regulating sequence core in coronavirus subgenomic mRNA synthesis. J. Virol. 79 (4), 2506–2516.
Spaan, W., Delius, H., Skinner, M., Armstrong, J., Rottier, P., Smeekens, S., van der Zeijst, B.A., Siddell, S.G., 1983. Coronavirus mRNA synthesis involves fusion of non-contiguous sequences. EMBO J. 2 (10), 1839–1844.
Sun, Z., Wang, J.Y., Zhang, J.W., Qin, A.J., Yuan, S.S., 2007. Identification of porcine reproductive and respiratory syndrome virus regulation sequence in 3′-untranslated region. Wei Sheng Wu Xue Bao 47 (5), 774–778.
van Dinten, L.C., den Boon, J.A., Wassenaar, A.L., Spaan, W.J., Snijder, E.J., 1997. An infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. Proc. Natl. Acad. Sci. U.S.A. 94 (3), 991–996.
van Marle, G., Dobbe, J.C., Gultyaev, A.P., Luytjes, W., Spaan, W.J., Snijder, E.J., 1999. Arterivirus discontinuous mRNA transcription is guided by base pairing between sense and antisense transcription-regulating sequences. Proc. Natl. Acad. Sci. U.S.A. 96 (21), 12056–12061.
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.
Yu, D., Lv, J., Sun, Z., Zheng, H., Lu, J., Yuan, S., 2009. Reverse genetic manipulation of the overlapping coding regions for structural proteins of the type II porcine reproductive and respiratory syndrome virus. Virology 383 (1), 22–31.
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.
Zhang, J.W., Zhuang, J.S., Liu, C.L., Wang, X.M., Yuan, S.S., 2009. Molecular epidemiology analysis on porcine circovirus type 2 in some regions of china in 2007. Sci. Agric. Sin. 42 (8.).
Zhang, X., Lai, M.M., 1994. Unusual heterogeneity of leader-mRNA fusion in a murine coronavirus: implications for the mechanism of RNA transcription and recombination. J. Virol. 68 (10), 6626–6633.
Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31 (13), 3406–3415.
Zuniga, S., Sola, I., Alonso, S., Enjuanes, L., 2004. Sequence motifs involved in the regulation of discontinuous coronavirus subgenomic RNA synthesis. J. Virol. 78 (2), 980–994.