Rapid Communication

Chimeric porcine reproductive and respiratory syndrome viruses reveal full function of genotype 1 envelope proteins in the backbone of genotype 2

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A R T I C L E   I N F O

Article history:
Received 29 October 2010
Returned to author for revision 1 December 2010
Accepted 27 December 2010
Available online 20 January 2011

Keywords:
Porcine reproductive and respiratory syndrome virus
Two genotypes
Chimeric
Envelope proteins

A B S T R A C T

Porcine reproductive and respiratory syndrome virus (PRRSV) is classified into two genotypes, type 1 and type 2, which share only about 60% genetic identity. Here, we report viable chimeric viruses in which the envelope protein genes from ORF2a to ORF5 of vSHE (type 1) were swapped into the genetic backbone of vAPRRS (type 2). We found that the envelope proteins of genotype 1 were fully functional in genotype 2 PRRSV, and the rescued chimeric progeny viruses showed robust genetic stability and similar replication properties to the parental strains in vitro. To our knowledge, this is the first study to report the substitution of complete ORFs between different genotypes of porcine arterivirus. These findings pave the way to further elucidate the structure-function relationship of PRRSV envelope proteins, and may enable the development of novel marker vaccines that can be used to differentiate vaccinated from infected animals.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes severe economic losses to the swine industry worldwide. PRRSV belongs to the family Arteriviridae, order Nidovirales (Cavanagh, 1997; Snijder and Meulenberg, 1998), which includes the other viruses, equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV). PRRSV is classified into two genotypes, the European genotype (type 1) and the North American genotype (type 2) (Nelsen et al., 1999; Snijder and Meulenberg, 1998). Interestingly, these two genotypes first appeared in the two different continents virtually simultaneously and cause almost identical clinical manifestations, despite sharing only about 60% genetic identity at the nucleotide level over the entire genome (Hanada et al., 2005; Nelsen et al., 1999). Such a paradox raises questions regarding which factors determine a functional TRS for PRRSV transcription.

PRRSV is an enveloped virus containing a single positive-strand RNA genome (Conzelmann et al., 1993). The genome comprises a 5′ terminal untranslated region (5′-UTR), nonstructural protein regions (ORF1a and ORF1b), structural protein regions (ORF2a–ORF7), and a 3′ terminal untranslated region (3′-UTR), followed by a poly(A) tail (Meulenberg, 2000; Meulenberg et al., 1995; Snijder and Meulenberg, 1998; Wu et al., 2001). PRRSV gene expression adopts a discontinuous transcription strategy to synthesize a set of nested subgenomic mRNAs (sg mRNAs) which possess the same 5′-UTR and 3′-UTRs with the genomic RNAs (Pasternak et al., 2006). During the sg mRNAs synthesis, the transcription-regulating sequence (TRS) is believed to play a key role in mediating the discontinuous jumping of the nascent “body” RNA to the leader (Pasternak et al., 2001). The leader TRS (TRS-L), a hexa-nucleotide (UUAACC), is conserved while the “body” TRSs (TRS-Bs) are diversified among different genotypes of PRRSV (Meng et al., 1996; Meulenberg et al., 1993; Nelsen et al., 1999). PRRSV not only synthesizes sg mRNAs that code for the structural proteins using canonical TRS, but it also generates subgenomic RNAs (sg RNAs) by noncanonical TRS (Meng et al., 1996; Yuan et al., 2000; Zheng et al., 2010). This poses question regarding which factors determine a functional TRS for PRRSV transcription.

ORF1 encodes at least 13 proteins, mainly involved in viral genome replication and transcription (Kroese et al., 2008; Meulenberg, 2000). Of the seven structural proteins, GP2a (encoded by ORF2a), GP3 (ORF3) and GP4 (ORF4) are thought to interact with each other to form multimeric complexes, and along with E (ORF2b), they are embedded in the virion envelope and are dubbed as minor envelope proteins (Wieringa et al., 2003; Wissink et al., 2005). The four minor envelope proteins are presumed to be involved in the process of virus entry into target cells because loss-of-function of any of these proteins affects viral infectivity, and it has been found that GP2a and GP4 interact with the cell receptor CD163 (Das et al., 2010; Wissink et al., 2005). The non-glycoprotein E has been reported to possess ion channel-like properties (Lee and Yoo, 2006). The major envelope glycoprotein GP5 (ORF5) forms a disulide-linked heterodimer with the membrane protein M (ORF6), which is critical for arterivirus
particle assembly (Faaberg et al., 1995; Snijder et al., 2003; Wissink et al., 2005).

Little is known about the structure–function relationship of PRRSV structural proteins. In this study, we investigated whether the envelope proteins of the type 1 PRRSV could function in the backbone of the type 2. We constructed a panel of chimeric full-length PRRSV cDNA clones in which the genes encoding the type 2 envelope proteins (ORF2a to ORF5) were replaced by the equivalent genes from the type 1 virus. Viable chimeric progeny viruses were rescued and analysed, based which further functional dissection of these PRRSV structural proteins can be conducted.

Results

Transfer of the type 1 envelope protein genes into a type 2 genetic background

Limited homology is observed between the envelope proteins (GP2a to GP5) of the type 1 and type 2 PRRSV strains (Table 1). The ORFs differ in length, nucleotide identity ranging from 61 to 72%, while the maximum amino acid sequence identity is 76%. To dissect the structure–function relationship of the envelope proteins, we investigated whether the envelope proteins could be functional in the genetic background of a different PRRSV genotype. Utilizing two full-length infectious cDNA clones pAPRRS (derived from type 2 strain) and pSHE (derived from type 1 strain), we constructed a panel of chimeric clones (Fig. 1). For swapping heterologous genes conveniently, an Ascl restriction enzymatic recognition site was inserted between ORF1b and ORF2a of pAPRRS to generate pAPRRSSasc. Such genetic manipulation did not significantly impair virus replication (Yu et al., 2009). Based on pAPRRSasc, the envelope protein genes (ORF2a–ORF5) were replaced with their counterparts from pSHE. Because the coding regions for the minor envelope proteins share long overlapping sequences between adjacent ORFs, we generated chimeric plasmids pAPRRS-SHE234, which contained the entire genomic region from ORF2a to ORF4 of vSHE. To further investigate if the virus viability was affected by the potential interaction between the GP5, M and minor envelope proteins, we also constructed chimeric plasmids pAPRRS-SHE5 and pAPRRS-SHE2345, in which the ORF5 or all of the envelope protein genes, ORF2a–ORF5, were replaced with those of vSHE. Nucleotide sequencing analysis verified the identity of the plasmids.

Cytopathic effect (CPE), such as cells agglomeration and detachment, was observed on days 4 to 6 post-transfection in MARC-145 cells which transfected with in vitro RNA transcripts derived from pAPRRSSasc, pAPRRS-SHE234, pAPRRS-SHE2345 and pAPRRS-SHE5, respectively, but not in mock-transfected control. Indirect immunofluorescence assay (IFA) confirmed that the observed CPE was PRRSV-specific, as the transfected cells reacted positively when stained with monoclonal antibodies (mAbs) specific for the nucleocapsid protein (N) and nonstructural protein 2 (NSP2) of type 2 PRRSV (Fig. 2). To further confirm that the rescued viruses were originated from the transfected RNA, the viral RNA was extracted from the supernatants of passage 1 (P1) viruses for complete genome sequencing. Sequence analysis confirmed that the progeny viruses possessed chimeric genomic structures, in which the target ORFs of vAPRRS had been successfully replaced by their counterparts from vSHE (data not shown). Several nonsynonymous point mutations were detected in other genomic regions compared to parental virus vAPRRS, as summarized in Supplementary Table S1. The rescued viruses were designated vAPRRSSasc, vAPRRS-SHE234, vAPRRS-SHE2345 and vAPRRS-SHE5, respectively. We further assessed the genetic stability of the chimeras in vitro. The chimeric viruses were passaged consecutively to passage 8 (P8) in fresh MARC-145 cells using a 1000-fold diluted inoculum. The P8 viral RNA was extracted, and the whole structural protein encoding genes were analysed by RT-PCR and sequencing. The results demonstrated that the heterologous ORFs were stably maintained in P8 viruses, and no amino acid mutation was detected compared to P1 chimeras (data not shown). This indicated that the chimeric PRRSVs have robust genetic stability at least in vitro.

Next, we investigated the expression profiles of the swapped heterologous genes in chimeric viruses. Virus-infected MARC-145 cells were stained with mAbs (kindly provided by Dr Hsuan Nauwyck at Ghent University) against GP3 (VII2D/5-1D) and GP5 (VII2H/2-4D) of type 1 PRRSV, and a customer-made peptide-specific antibody against GP4 (ARV-SHE-GP4, Shanghai GL Biotech company) of vSHE. As shown in Fig. 2, the type 1 specific proteins encoded by swapped genes were detected in corresponding chimeras infected cells. As no specific antibodies against the GP2a and E proteins were available, we were unable to investigate the expression of these heterologous proteins in vAPRRS-SHE234 and vAPRRS-SHE2345 infected cells. However, we speculate that these two envelope proteins were also expressed, as all of these proteins are believed to be crucial for viral infectivity.

The virological characteristics and transcriptional profiles of the chimeras

To investigate the virological characteristics of chimeric viruses, we performed viral plaque morphology and growth kinetics analysis with passage 3 (P3) viruses. As shown in Fig. 3A, the plaque morphology of the chimeric viruses, vAPRRS-SHE234 and vAPRRS-SHE2345, was similar to the plaques observed with vAPRRS. However, vAPRRS-SHE5 exhibited much smaller plaques, although its peak titer was virtually similar with that of other chimeras. The peak titers of vAPRRS-SHE234, vAPRRS-SHE2345 and vAPRRS-SHE5 were 5.1 × 10^5, 1.0 × 10^6 and 2.0 × 10^5 plaque forming unit per ml (PFU/ml), respectively, which were a little lower than that of the parental strains vAPRRS (7.9 × 10^5 PFU/ml) and vSHE (6.3 × 10^5 PFU/ml). The finding that chimeric viruses had a lower peak titer than the wild-type viruses was not unexpected. However, there was no significant difference in the growth curve between chimeras and parental strains (Fig. 3B), suggesting that the heterologous envelope protein genes did not dramatically affect viral growth, and the envelope proteins of type 1 were fully functional in type 2 PRRSV.

Both type 1 and type 2 PRRSVs utilize the same TRS-L (UAACC), yet their TRS-Bs are diversified. To investigate how the transcription process was affected in the chimeric viruses, sg RNAs were RT-PCR amplified from the total cellular RNA, followed by cloning and nucleotide sequence determination. The leader–body junction sequences and their genomic positions of sg RNAs mediated by canonical TRS-Bs were summarized in Table 2. The sg RNA2 from all of the chimeras, sg RNA5 from vAPRRS-SHE5, the TRS-Bs and their flanking sequences were the same as the parental strain vAPRRS. While sg RNAs transcribed from swapped genes and the genes immediately downstream, the TRS-Bs and their flanking sequences were the same as the donor virus vSHE. In addition, some small sg RNAs were detected from chimeras while not parental viruses, in which the leader–body junction sites were situated downstream of the initial codon of the corresponding ORFs (supplementary Table S2).

Table 1

<table>
<thead>
<tr>
<th>ORF</th>
<th>nucleotide (bp)</th>
<th>nt identity (%)</th>
<th>deduced a.a. length</th>
<th>a.a. identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vSHE</td>
<td>vAPRRS</td>
<td>vSHE</td>
<td>vAPRRS</td>
<td></td>
</tr>
<tr>
<td>ORF2a</td>
<td>750</td>
<td>771</td>
<td>67</td>
<td>249</td>
</tr>
<tr>
<td>ORF2b</td>
<td>213</td>
<td>222</td>
<td>72</td>
<td>70</td>
</tr>
<tr>
<td>ORF3</td>
<td>798</td>
<td>765</td>
<td>65</td>
<td>265</td>
</tr>
<tr>
<td>ORF4</td>
<td>552</td>
<td>537</td>
<td>67</td>
<td>183</td>
</tr>
<tr>
<td>ORF5</td>
<td>606</td>
<td>603</td>
<td>61</td>
<td>201</td>
</tr>
</tbody>
</table>

* The identity were aligned by the clustal W method in DNASTar software.
The M protein (Verheije et al., 2002). In this study, we exchanged the GP5 with that of PRRSV or LDV, and demonstrated that the ectodomain of virologically functional chimeric EAVs, in which the ectodomain of GP5 was replaced which shared about 95% identity. Dobbe and coworkers engineered genes between various PRRSV strains belong to the same genotype, 2008). All of these chimeric viruses were generated by exchanging chimeric arteriviruses have previously been constructed to investigate virus virulence determinants or cell tropism. Using two infectious cDNA clones derived from two distinct virulent type 2 PRRSV strains, Wang and colleagues constructed chimeric viruses in infectious cDNA clones derived from two distinct virulent type 2 PRRSV, the two genotypes of PRRSV, type 1 and type 2, are fully functional in type 2 PRRSV. Unfortunately, the two genotype strains of PRRSV shared the same cellular tropism in vitro. Further development of inter-species chimeric viruses with different cell tropism is therefore required to investigate the viral attachment protein, an enigma so far for arterivirus.

The roles of these small sg RNAs remained to be determined. Overall, the chimeric viruses transcribed corresponding hybrid sg RNAs, which shared the same 5′-UTR with VAPRRS, and the TRS-Bs from the donor virus remained functional in the chimeric viruses. These results indicated that TRS-Bs were provoked to form novel sg RNAs from swapped genes.

**Discussion**

As a relatively small RNA virus, PRRSV paradoxically encodes six known envelope proteins (GP2, E, GP3, GP4, GP5 and M), all of which are crucial for virus infectivity (Meulenberg et al., 1995; Nelsen et al., 1999; Wu et al., 2001). The structure–function relationship of these proteins remains largely undetermined. Despite causing similar clinical symptoms, the two genotypes of PRRSV, type 1 and type 2, are genetically diversified. Whether the genes and encoded proteins from different genotypes play the same roles and work in concert is an interesting question.

Chimeric arteriviruses have previously been constructed to investigate virus virulence determinants or cell tropism. Using two infectious cDNA clones derived from two distinct virulent type 2 PRRSV strains, Wang and colleagues constructed chimeric viruses with exchanged structural proteins, and showed that such chimeric virus exhibited good immunogenicity and attenuated virulence, suggesting that chimeric viruses could be promising vaccine candidates (Wang et al., 2008). Another group also constructed similar chimeric PRRSVs which contained different envelope-associated structural proteins from different parental viruses (Kim and Yoon, 2008). All of these chimeric viruses were generated by exchanging genes between various PRRSV strains belong to the same genotype, which shared about 95% identity. Dobbe and coworkers engineered viable chimeric EAVs, in which the ectodomain of GP5 was replaced with that of PRRSV or LDV, and demonstrated that the ectodomain of GP5 is not the main determinant of EAV cellular tropism (Dobbe et al., 2001). Using the same approach, similar conclusion was also made for the M protein (Verheije et al., 2002). In this study, we exchanged the complete envelope protein genes (ORF2a–ORF5) between two PRRSV genotypes and generated viable inter-genotypic chimeric viruses. The chimeras exhibited similar virological characteristics to their parental viruses, indicating that the envelope proteins of type 1 virus were fully functional in type 2 PRRSV. Unfortunately, the two genotype strains of PRRSV shared the same cellular tropism in vitro. Further development of inter-species chimeric viruses with different cell tropism is therefore required to investigate the viral attachment protein, an enigma so far for arterivirus.

Little is known about the functional domains of the PRRSV envelope proteins. Amino acid sequences comparison indicate that the envelope proteins between two genotypes does contain conserved domains and putative N-linked glycosylation sites, despite low sequences identity (Fig. 4). It has previously been reported that the GP5 and M proteins are key components for arterivirus assembly, and that the formation of the GP5-M heterodimer in type 1 PRRSV, which is linked by a disulfide bond between cysteine (Cys) residues at positions 50 and 8, respectively, is a prerequisite for virion assembly (Faaberg et al., 1995; Snijder et al., 2003; Wissink et al., 2005). In both vAPRRS-SHE2345 and vAPRRS-SHE5, the GP5 and M proteins originate from different genotype PRRSVs. However, progeny viruses were successfully rescued suggesting that the hybrid GP5-M heterodimers, in which GP5 protein originated from vSHE and M protein from vAPRRS, were still formed. According to the deduced amino acids sequences, the conserved Cys residues were located at position 48 in the GP5 and 9 in the M protein of vAPRRS, respectively (Fig. 4). Overlapping sequences exist between ORF5 and ORF6 in vAPRRS and vSHE. In vAPRRS-SHE2345 and vAPRRS-SHE5, the exact sequences of ORF5 from vSHE were retained while the overlapping sequences of vAPRRS ORF6 with ORF5 were changed to that of vSHE (as shown in Fig. 1), such that the conserved Cys in the M protein remained at position 8 in the chimeras. Accordingly, the Cys residue at position 50 of the GP5 likely interacts with the Cys at position 8 of the M in the two chimeras, and such interaction would lead to the formation of a hybrid heterodimers. Recently, Das et al. (2010) reported that the GP5 protein also strongly interacts with GP4. In the present study, vAPRRS-SHE5 exhibited smaller plaque in MARC-145 cells (Fig. 3A). This may
result from a somewhat impaired interaction between the GP4 (originated from vAPRRS) and GP5 (from vSHE). However, such virological characteristics were not observed in vAPRRS-SHE234, which also harbors a heterologous pair of GP4 (vSHE) and GP5 (vAPRRS). It could be possible that the GP4 of vSHE interacts well with the GP5 of vAPRRS since GP4 is more inter-genotypic conserved (Fig. 4). If this is the case, the exact residues or domains involved in this process would be an interesting subject for future study. PRRSV GP3 contains a hypervariable region at the C terminus, and a truncated sequence in vAPRRS (Fig. 4). Nonetheless, functional complementation of the GP3 protein in vAPRRS with that of vSHE indicated that the C terminus could be functionally nonessential. This finding was consistent with previous reports that an increasing number of GP3 deletion mutants are being isolated in the field (Oleksiewicz et al., 2000; Ropp et al., 2004).

Like other arterivirus, PRRSV adopts a sophisticated system of sg mRNA synthesis, but the detailed mechanism is still poorly understood (Pasternak et al., 2006). In the present study, inter-genotypic hybrid sg RNAs was detected, which consisted of the 5′-UTR from vAPRRS and the swapped ORFs from vSHE. The fact that the canonical TRS-Bs in vSHE were still functional indicated that TRS-Bs from both genotypes of PRRSV share common features. In particular, the flanking sequences are probably essential for TRS-Bs function. In addition, we also detected a series of small sg RNAs from chimeras, in which the leader-body junction sites were within the corresponding ORFs. These so-called noncanonical TRS-B-mediated sg RNAs have also been reported by others (Meulenberg et al., 1993; Zheng et al., 2010). We speculate that the activation of noncanonical TRS-Bs was caused by the attenuation of canonical TRS-Bs.

As a RNA virus, PRRSV exhibits a high mutation rate, and genetic recombination has been reported in both genotypes (Li et al., 2009; van Vugt et al., 2001; Yuan et al., 1999). The co-existence of both genotype strains in some countries creates the opportunity for inter-genotypic recombination, although this has not been detected in the field to date (Dewey et al., 2000). The viable inter-genotypic chimeras described here suggest that RNA recombination between different genotypes of PRRSV could be possible. The two genotypes of PRRSV are originated and prevalent in different continents, but this geographical separation has been compromised by the international swine trade and widespread use of a type 2 vaccine, Ingelvac MLV, in Europe for a period of years (Dewey et al., 2000; Ropp et al., 2004). The concern is that if inter-genotypic recombinants were generated in the field, current vaccines and diagnostic techniques may become invalid.

In conclusion, we found that the envelope protein genes (ORF2a–5) of type 2 PRRSV could be replaced by these of type 1, and the heterologous envelope proteins were fully functional for virus replication although
very low homology existed between the two genotype PRRSVs. The chimeric viruses generated here paved the way for further structure–function relationship analysis, and development of novel marker vaccines that can be used to differentiate vaccinated from infected animals.

Materials and methods

Cells, viruses and antibodies

The MARC-145 cell line (ATCC, Manassas, VA, USA) was cultured in Eagle’s minimal essential medium (EMEM) with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD, USA) and maintained in EMEM with 2% FBS at 37 °C with 5% CO2. The type 2 PRRSV strain, vAPRRS, was derived from the infectious clone pAPRRS (GenBank accession: GQ330474; Yuan and Wei, 2008). The type 1 PRRSV strain, vSHE, was rescued from the infectious clone pSHE (GQ461593; Yuan et al., unpublished data), based on the attenuated vaccine strain AMERVAC-PRRS/A3 (Hipra Laboratory). The vSHE shares 95% identity with LV strain (phenotype strain of type 1 PRRSV) in genome level. Monoclonal antibodies (mAbs) specific for N protein (N-McAb) and nonstructural protein 2 (Nsp2-McAb) of type 2 PRRSV were kindly provided by Dr. Ying Fang (South Dakota State University, USA). mAbs against GP3 (VII2D/5-1D) and GP5 (VII2H/2-4D) of type 1 PRRSV were provided by Dr. Hans J. Nauwynck (Ghent University, Belgium). Rabbit

Table 2
The leader-body junction sequences of sg RNAs mediated by canonical TRS-Bs in chimeras, and comparison of the leader-body junction positions between chimeras and parental strains.

<table>
<thead>
<tr>
<th>sg RNA</th>
<th>Chimeras</th>
<th>Leader-body junction sequences of chimeras</th>
<th>Leader-body junction positiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leader</td>
<td>vAPRRS</td>
</tr>
<tr>
<td>2</td>
<td>vAPRRS-SHE234, vAPRRS-SHE2345, vAPRRS-SHE5</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>19 (7)</td>
</tr>
<tr>
<td>3</td>
<td>vAPRRS-SHE234, vAPRRS-SHE2345</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>11 (5)</td>
</tr>
<tr>
<td></td>
<td>vAPRRS-SHE5</td>
<td></td>
<td>83 (4)</td>
</tr>
<tr>
<td>4</td>
<td>vAPRRS-SHE234, vAPRRS-SHE2345</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>83 (5)</td>
</tr>
<tr>
<td></td>
<td>vAPRRS-SHE5</td>
<td></td>
<td>4 (4)</td>
</tr>
<tr>
<td>5</td>
<td>vAPRRS-SHE234, vAPRRS-SHE2345</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>32 (6)</td>
</tr>
<tr>
<td></td>
<td>vAPRRS-SHE5</td>
<td></td>
<td>40 (5)</td>
</tr>
<tr>
<td>6</td>
<td>vAPRRS-SHE2345, vAPRRS-SHE5</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>24 (7)</td>
</tr>
<tr>
<td></td>
<td>vAPRRS-SHE234</td>
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<td>17 (4)</td>
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<tr>
<td>7</td>
<td>vAPRRS-SHE234, vAPRRS-SHE2345, vAPRRS-SHE5</td>
<td>genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic genomic subgenomic</td>
<td>123 (4)</td>
</tr>
<tr>
<td></td>
<td>vAPRRS-SHE234</td>
<td></td>
<td>9 (3)</td>
</tr>
</tbody>
</table>

a The letters underlined indicate the TRS-L in 5’-UTR, TRS-Bs in genome and leader-body junction motif in every sg RNA.

b Distance (in nucleotides) between the leader-body junction motif and the initiation AUG of individual sg RNA.

c The isolated clone number containing the indicated TRS-B.
peptide-specific antibody directed against GP4 of vSHE (ARV-SHE-GP4, recognizes the peptide: PHGVSTAQENIPFGKPSQC) was obtained from GL Biochem (Shanghai, China). The secondary antibodies Alexa fluor 568-labeled goat anti-mouse IgG and Alexa fluor 555-labeled goat anti-rabbit IgG were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

**Construction of chimeric full-length cDNA clones**

Site-specific mutation PCR was conducted to introduce an AscI restriction enzyme recognition site immediately upstream of the ORF2 start codon in the infectious cDNA clone, pAPRRS, as previously described (Yu et al., 2009). This construct was designated pAPRRSasc (Fig. 1). To construct the chimeric full-length cDNA clones, the hybrid DNA fragments which consisted of target ORFs from pSHE and nucleotide sequences from pAPRRS were amplified through splicing overlap extension (SOE) PCR, according to a previous report (Warrens et al., 1997). Briefly, using the construction of pAPRRS-SHE234 as an example, fragment SHE234 (ORF2a–ORF4 of vSHE) was amplified from pSHE using the primer pair FS234asc and RS4-A5, and fragment APRRS56 (ORF5–ORF6 of vAPRRS) was amplified from pAPRRS using the primer pair FS4-A5 and RA14780. Then using SHE234 and APRRS56 as templates, the hybrid fragment SHE234–APRRS56 were amplified via SOE PCR using the primer pair FS234asc and RA14780. All of the oligonucleotide primer sequences were listed in Table 3. The generated hybrid fragment was cloned into the pCR-blunt II-TOPO vector (Invitrogen) following the manufacturer’s recommendations to construct an intermediate plasmid. Then, the hybrid fragment was subcloned into pAPRRSasc, via enzyme digestion with AscI and XbaI (New England Biolabs, Ipswitch, MA, USA), to yield the chimeric full-length cDNA clone pAPRRS-SHE234. The other chimeric plasmids, pAPRRS-SHE2345 and pAPRRS-SHE5, were generated in the same manner (Fig. 1). In this two constructs, the first 16 nucleotide acids of APRRS ORF6 were changed into the last 13 nucleotide acids of SHE ORF5, because overlapping sequences exist between ORF5 and ORF6 in both PRRSVs. All the constructs were verified by sequencing.

**Rescue of the chimeric viruses**

The full-length chimeric plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol, followed by restriction enzyme mapping and nucleotide sequence verification. The plasmids were linearized with XhoI (New England Biolabs), that cuts immediately downstream of the poly(A) tail, and purified with a QIAquick PCR purification kit (Qiagen). Then, 1 μg of the purified DNA template was used for in vitro RNA transcription, using a T7 mMessage Machine Kit (Ambion INC, Austin, TX, USA), according to the manufacturer’s recommendations. The RNA integrity was verified using native RNA agarose gel (1%) electrophoresis, and the RNA was quantified using a spectrometer at
RT-PCR and nucleotide sequencing

Viral genomic RNA was isolated from the supernatant of the cultured cells using a QIAprep Viral RNA Mini Kit (Qiagen), as instructed by the manufacturer. To verify the chimeric genomic RNA in the rescued viruses, RT-PCR was performed using avian myeloblastosis virus reverse transcriptase (TaKaRa) and pfuUltra II Fusion HS DNA polymerase (Stratagene, USA). The complete genomes were amplified in 7 segments, followed by nucleotide sequence determination as previously described (Yu et al., 2009). Total intracellular RNA was isolated using Trizol Reagent (Invitrogen), according to the manufacturer’s instructions. The sg RNAs were amplified by RT-PCR with specific primer pairs. The forward primers and reverse primers were designed to bind the upstream of 5′-LTR of vAPRRS and vSHE, respectively. The reverse primers located in the individual ORFs of the structural proteins (ORF2–ORF7), such that the specific leader-body junctions of the sg RNAs could be determined (Table 3). The RT-PCR products were cloned into the pGEM-T vector (Promega, Madison, USA) and subjected to nucleotide sequencing.

Indirect immunofluorescence analysis

The indirect immunofluorescence assay (IFA) was performed as described previously, with minor modifications (Yu et al., 2009). Briefly, After 48 hours post-inoculation at a low multiplicity of infection (MOI, 0.01), or transfection with RNA transcripts, the MARC-145 cells in six-well plates were fixed in 1% BSA at room temperature. The fixed cells were incubated with specific antibodies against the corresponding target proteins at 37 °C for 2 hours. After extensive washing with phosphate-buffered saline (PBS), the cells were incubated with anti-mouse Alexa-568-labeled or anti-rabbit Alexa-555-labeled secondary antibodies for 1 hour. Finally, the fluorescence was visualized under an Olympus inverted fluorescence microscope fitted with a camera.

Viral plaque and growth kinetics assay

To examine the plaque morphology of the chimeric viruses, tenfold serially diluted virus suspensions were incubated with MARC-145 cells in six-well plates. After a 1-hour adsorption, the cell monolayers were washed off and then overlaid with a mixture of EMEM medium containing 2% FBS and 1% low melting agarose (Cambrex, Rockland, ME, USA). When the agarose overlay solidified, the plate was inverted (bottom up) in a humidified CO2 incubator at 37 °C for four days. The resulting plaques were stained with crystal violet (5% w/v in 20% ethanol).

Multiple-step growth curve analysis was conducted as previously described (Yu et al., 2009). Briefly, MARC-145 cells were infected with the chimeric and parental strains at low MOI (0.01). After a 1-hour adsorption, the cells were washed off with PBS twice and cultured with EMEM (2%FBS). At the indicated time points (12, 24, 36, 48, 60, 72 hours), MARC-145 cells were infected with a low multiplicity of 0.01, and 0.001. After 24 hours post-inoculation, the supernatants of the cell cultures were harvested and the titers were determined by RT-PCR amplification for the sg RNA2–7 of APRRS sequence. The chimeric and parental MARC-145 cells were infected with a MOI of 0.01. After 24 hours post-inoculation, the supernatants of the cell cultures were harvested and the titers were determined by RT-PCR amplification for the sg RNA2–7 of APRRS sequence. The chimeric and parental MARC-145 cells were infected with a MOI of 0.01. After 24 hours post-inoculation, the supernatants of the cell cultures were harvested and the titers were determined by RT-PCR amplification for the sg RNA2–7 of APRRS sequence.
Supplementary data to this article can be found online at doi:10.1016/j.virol.2010.12.048.

References


