REPLICATION AND EXPRESSION ANALYSIS OF PRRSV DEFECTIVE RNA

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1. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the family *Arteriviridae*, which was recently grouped together with the coronaviruses and the toroviruses in the newly established order of the *Nidovirales.*[1] PRRSV is a spherical, enveloped virus with a diameter of 50 to 60 nm[2] and a positive-stranded RNA genome of 15.0–5.5 kilobases.[3]

Nidovirus defective RNAs have been well documented and studied, especially for coronaviruses[4–6] and equine arterivirus (EAV).[7] Defective RNAs are truncated, and in some cases, rearranged genomes that have usually lost the potential to replicate autonomously due to deletions in the viral replicase gene(s), the replication of which depends on the replicase encoded by the helper virus.[7] Defective RNAs have retained all replication signals and, frequently, also the sequences required for RNA encapsidation. However, the generation of many of nidovirus defective RNAs requires serial undiluted viral passage in cultured cells.[4, 5, 7] We have suggested that PRRSV defective RNAs are different because they are persistently generated during infection, and arise *in vitro* as well as *in vivo* and have termed these RNA species *heteroclites* (*latin:* uncommon forms). PRRSV defective RNAs are heterogeneous in size and sequence and consist of, exclusively, complete 5' and 3' termini joined by short but variable nucleotide repeats. Thus, they contain different lengths of ORF1a and 3'-end sequences and are packaged into virions along with full-length PRRSV genomes.[8,9]

In order to further characterize PRRSV heteroclites, we have sequenced several species that represent varying lengths, and have prepared cDNA clones that correspond to two species (S1 and S7). In this study, we examined basic features of heteroclites S1 and S7 such as maintenance, RNA packaging and the encoding capability.

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2. METHODS

Cells, virus, modified heteroclites and RNA transcription, transfection and analysis: MA-104 cells were grown in DMEM medium containing 10% FBS (Invitrogen) at 37˚C, 5% CO2. PRRSV North American strain VR-2332 infectious clone pVR-V7 was used in the study. In-frame insertion mutagenesis of type 2 porcine circovirus ORF2 gene (PCVORF2) into different positions of PRRSV heteroclite cDNAs of S7 or S1 was achieved by overlapping extension PCR**.** The resultant constructs were confirmed by sequencing and named pS7-PCVORF2/StuI (after leader sequence), pS7-PCVORF2/KpnI (genome position 480), and pS1-PCVORF2/SnaBI (genome position 1338). Linearized PRRSV infectious clone pVR-V7 and PCVORF2 gene modified S1 and S7 derivatives were obtained. RNAs were synthesized using mMESSAGE mMachine (Ambion). VR-V7 RNA was transfected alone or cotransfected with S7-PCVORF2/StuI, S2-PCVORF2/SnaBI or S7-PCVORF2/KpnI RNAs as described.[10] Northern blot analysis on infected cell supernatant was performed as described by Yuan.[8] Heteroclite RNA was translated with Flexi Rabbit Reticulocyte Lysate System (Promega) in the presence of ^{35}S-methionine (Amersham) and immunoprecipitated.[9,11]

Immunizations: Groups of 3–4 weeks PRRSV negative pigs (PCV antibody negative) were immunized by intramuscular injection of 2 ml of each PRRSV modified virus discussed above. Blood samples were collected from all animals on days 0, 3, 7, 14, 21 and 28 and examined for the presence of heteroclites and development of αPCV ORF2 antibodies. Total RNAs from swine serum samples were isolated (QIAamp Viral RNA; Qiagen) followed by nested RT-PCR to amplify the PCV ORF2 gene.

3. RESULTS

3.1. *In Vitro* Transcribed Heteroclites are Replicated and Packaged by PRRSV

After 6 cell passages, the heteroclites were detected by Northern blot analysis using a PCVORF2 gene-specific probe, demonstrating that PCVORF2 S7 and S1 RNAs were efficiently replicated and packaged during PRRSV infection (Fig. 1a). Inoculation of growing swine with passage 2 cell supernatants containing full-length virus and modified heteroclites was completed to assess replication of these heteroclites *in vivo*. Nested RT-PCR analysis on swine serum samples post-infection revealed that PCVORF2 mRNA could still be detected, which suggested that these defective RNA species could be replicated *in vivo*. PCVORF2 modified S7 replicated more efficiently than S1, because pigs inoculated with PCVORF2 modified S7 harbored the ORF2 gene for a longer time period (Fig. 1B). [Due to unknown reasons, control swine (α-PCV antibody negative) harbored PCVORF2 gene, but they were predominantly detected only in early infection.]

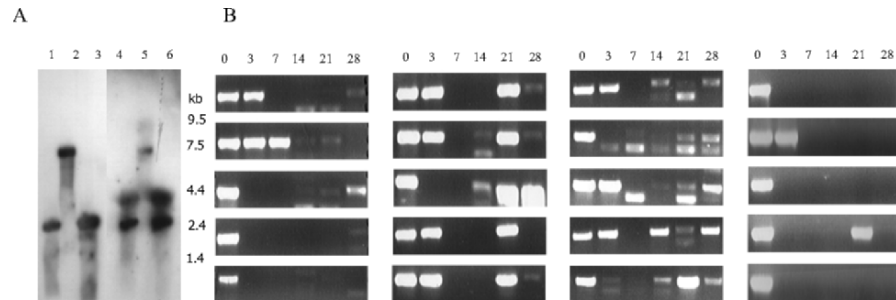

Figure 1. Evidence of PCVORF2 persistence *in vitro* and *in vivo*. A. Northern blot analysis with PCVORF2 probe.[12] Lanes 1–3: *in vitro* transcripts of S7-PCVORF2/StuI, S1-PCVORF2/SnaBI, S7-PCVORF2/KpnI, respectively. Lanes 4–6: S7-PCVORF2/StuI, S2-PCVORF2/SnaBI, S7-PCVORF2-/KpnI modified PRRSV infection supernatants. The appearance of higher molecular weight PCV-specific RNA may be due to a technique anomaly or possibly viral recombination. B. Nested RT-PCR analysis revealed PCVORF2 mRNA in swine serum samples after replication *in vivo.* Column 1: S1-PCVORF2/SnaBI; Column 2: S7-PCVORF2/StuI; Column 3: S7-PCVORF2/KpnI; Column 4: negative control pigs injected with saline. Each panel represents a single pig analyzed at each time point (days post-infection indicated above the individual lane). The 352 bp PCVORF2 DNA product was detected in several serum samples.

3.2. Translation of PRRSV S1 and S7

We next examined the encoding capacity of S1 and S7. Native S1 and S7 as well as PCV or green fluorescent protein (GFP) modified derivatives were used for *in vitro* translation. The results demonstrated native and modified defective RNA species can be easily translated into proteins (Figure 2A). Immunoprecepitation revealed that these proteins could be recognized by specific antibodies against PCV or GFP (Figure 2B). However, we could not detect antibodies in heteroclite and PRRSV inoculated swine (data not shown).

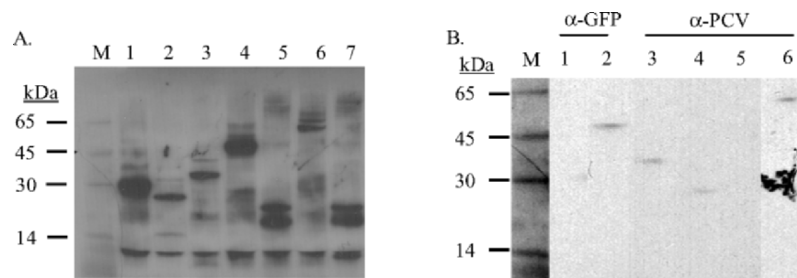

Figure 2. *In vitro* translated native and modified heteroclite species were recognized by specific antibodies. A. *In vitro* translation assay. Lanes 1: S7; 2: S7-PCVORF2/StuI; 3: S7-PCVORF2/KpnI; 4: S7-GFP/BX; 5: S2; 6: S2-PCVORF2/SnaBI, 7: VR-V7. B. Immunoprecipitation of *in vitro* translated proteins with immune serum against PCV and GFP. Lanes 1: S7; 2: S7-GFP; 3: S7-PCVORF2/KpnI; 4: S7-PCVORF2/StuI; 5: S7; 6: S7-PCVORF2/SnaBI.

4. CONCLUSIONS

Defective RNAs serve as an important tool for studying the molecular mechanisms of a virus replication cycle. Previous work has shown that PRRSV heteroclite RNAs, but not subgenomic RNAs, could be packaged and since both heteroclite and subgenomic RNAs have complete 5' and 3' termini, we postulated that the PRRSV packaging signal may lie in the ORF1a region.[8] This notion was derived because the shortest heteroclite, S-9a (junction site nt 476/14344), was found to be in viral particles.[9] In this study, modified defective RNA S7 was packaged efficiently, either with PCVORF2 insertion right after the leader sequence (nt 190) or after the putative packaging signal (nt 476). This suggested that the packaging signal might indeed lie within this 286-bp region, perhaps located somewhere distant from the ORF1a fragment termini, as the insertions did not abort viral packaging. Additional work needs to be done to further define the packaging signal.

S1 and S2 and their derivatives could be easily translated in *in vitro*, but we could not detect any protein products either in virus-infected cells or evidence of protein production, by production of α-PCV antibodies, in inoculated swine. Also, a cell line expressing S7 modified with GFP did not reveal any protein expression (data not shown). Perhaps the *in vitro* system does not reflect the situation in cultured cells or *in vivo*. Unknown factors from PRRSV or the host may inhibit translation or the expression level is not at detectable levels.

5. REFERENCES

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