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Novel 5' and 3' *cis*-Acting Elements of the Arteriviruses Required for Viral Replication

Yu-Jeong Choi[1†], Sang-Im Yun[1†], Shien-Young Kang[2], and Young-Min Lee[1*]

[1]*Department of Microbiology, College of Medicine and Medical Research Institute, Chungbuk National University Cheongju, Korea,* [2]*Research Institute of Veterinary Medicine, College of Veterinary Medicine, Chungbuk National University*

[†] *These authors contributed equally to this work.*

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in North America in 1987 and shortly thereafter in Europe. It has since become one of the most common and economically significant infectious diseases in the swine industry worldwide. It is characterized by mild to severe reproductive failure in sows and gilts and respiratory problems in piglets. The PRRS virus (PRRSV) was first isolated almost simultaneously in Europe and North America; these strains are designated as Lelystad and VR-2332, respectively. Although these strains induce phenotypically indistinguishable disease symptoms, they are genetically and serologically distinct. They currently constitute the two distinct genotypes of known PRRSV species.

PRRSV belongs to the family *Arteriviridae* in the order *Nidovirales* together with equine arteritis virus (EAV), simian hemorrhagic fever virus, and the lactate dehydrogenase-elevating virus of mice. Like other arteriviruses, PRRSV is a small-enveloped virus with a positive-sense, single-stranded RNA genome of ≈15 kb in length. The genome has a cap structure at its 5' end and a poly (A) tail at its 3' end. The genome contains at least nine open reading frames (ORFs) flanked by 5' and 3' noncoding regions (NCRs). Two overlapping ORFs, ORF1a and 1b, are expressed from the genomic RNA. These two ORFs are predicted to be processed into 13 mature nonstructural proteins that are believed to be involved in viral replication. ORFs 2a, 2b, and 3-7 are translated from the 5' end of a coterminal nested set of subgenomic mRNAs. The small ORF 2b is completely embedded within the larger ORF 2a. These ORFs encode the viral structural proteins.

To analyze positive-sense RNA viruses such as PRRSV at a molecular and genetic level, a number of reverse genetics systems that allow the determination of the functions of the genes and gene products of these RNA viruses through their manipulation and genetic analysis have been developed. With regard to arteriviruses, two "RNA-launched" reverse genetics systems for EAV have been independently

developed by two research groups. Although both functional EAV cDNAs were constructed using the same virus isolate, a different bacteriophage promoter, either the SP6 or T7 RNA polymerase promoter, was placed immediately upstream of the viral genome for run-off transcription *in vitro*. The first infectious EAV cDNA was constructed in a high copy number plasmid, pUC18, and did not show any genetic instability during its construction and propagation in *E. coli*, in contrast to the infectious cDNAs of several other viruses.

For PRRSV, the first "RNA-launched" reverse genetics system was constructed for the European Lelystad strain by assembling its full-length cDNA under the T7 promoter in the low copy number plasmid, pOK12. Identical strategies were also previously adopted in the construction of an infectious cDNA for the North American strain, VR-2332. Recently, two additional T7 promoter-driven "RNA-launched" reverse genetics systems were developed independently for the highly virulent American isolate NVSL #97-7895 and for the highly virulent 'atypical' North American isolate, P129, using the low copy number pBR322 and high copy number pCR2.1 plasmids, respectively. An additional "DNA-launched" system has also been reported for the P129 isolate, and employed full-length cDNA under the human cytomegalovirus immediate early promoter.

We here demonstrated the successful engineering of the RNA genome of porcine reproductive and respiratory syndrome virus (PRRSV) using an infectious cDNA as a bacterial artificial chromosome. Run-off transcription from this cDNA by SP6 polymerase resulted in capped synthetic RNAs bearing authentic 5' and 3' ends of the viral genome that had a specific infectivity of $>5\times10^5$ PFU/μg of RNA. The synthetic viruses recovered from the transfected cells were genotypically and phenotypically indistinguishable from the parental virus. Using our system, a series of genomic RNAs with nucleotide deletions in their 5' end produced viruses with decreased or no infectivity. Various pseudorevertants were isolated and acquisition of novel 5' sequences of various sizes, composed predominantly of A and U bases, restored their infectivity, providing a novel insight into functional elements of the 5' end of the PRRSV genome. In addition, our system was further engineered to generate a panel of self-replicating, self-limiting, luciferase-expressing PRRSV viral replicons bearing various deletions. Analysis of these revealed the presence and location of a 3' *cis*-acting element in the genome that was required for replication. Moreover, we produced EGFP-expressing infectious viruses, which indicates that the PRRSV cDNA/viral replicon/recombinant virus can be developed as a vector for the expression of a variety of heterologous genes. Thus, our PRRSV reverse genetics system not only offers a means of directly investigating the molecular mechanisms of PRRSV replication and pathogenesis, it can also be used to generate new heterologous gene expression vectors and genetically defined antiviral vaccines.