PACKAGING OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS REPLICON RNA BY A STABLE CELL LINE EXPRESSING ITS NUCLEOCAPSID PROTEIN

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Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the Arteriviridae family, is one of the most common and economically important swine pathogens. Although both live-attenuated and killed-inactivated vaccines against the virus have been available for a decade, PRRSV is still a major problem in the swine industry worldwide. To explore the possibility of producing single-round infectious PRRSV replicon particles as a potential vaccine strategy, we have now generated two necessary components: 1) a stable cell line (BHK/Sinrep19/PRRSV-N) that constitutively expresses the viral nucleocapsid (N) protein localized to the cytoplasm and the nucleocapsid and 2) a PRRSV replicon vector (pBAC/PRRSV/Replicon–ΔN) with a 177-nucleotide deletion, removing the 3’-half portion of ORF7 in the viral genome, from which the self-replicating propagation-defective replicon RNAs were synthesized in vitro by SP6 polymerase run-off transcription. Transfection of this replicon RNA into N protein-expressing BHK-21 cells led to the secretion of infectious particles that packaged the replicon RNA, albeit with a low production efficiency of 0.4×10⁷ to 1.1×10⁸ infectious units/ml; the produced particles had only single-round infectivity with no cell-to-cell spread. This trans-complementation system for PRRSV provides a useful platform for studies to define the packaging signals and motifs present within the viral genome and N protein, respectively, and to develop viral replicon-based antiviral vaccines that will stop the infection and spread of this pathogen.

Keywords: porcine reproductive and respiratory syndrome virus, viral replicon, nucleocapsid, stable cell line, trans-complementation

Porcine reproductive and respiratory syndrome virus (PRRSV) causes an economically important pandemic disease, PRRS, which is characterized by severe reproductive failures in sows and respiratory illness in pigs of all ages (Done and Paton, 1995; Rossow et al., 1995; Botner, 1997; Zimmerman et al., 1997; Rossow, 1998). The disease was first recognized in North America in the late 1980s (Keffaber, 1989) and subsequently appeared in Europe in the early 1990s (Paton et al., 1992; Collins et al., 1993). Two overlapping ORFs, ORF1a and ORF1b, are expressed from the genomic RNA; ORF1a encodes the pp1a polyprotein, and ORF1b is expressed via a ribosomal frame shift, leading to the translation of a large pp1ab polyprotein (Snijder and Meulenberg, 1998; Fang and Snijder, 2010). The pp1a and pp1ab polyproteins are processed by viral proteases into 13 putative nonstructural proteins (NSPs) that are involved in RNA replication and innate immunity: NSP1 to NSP12, numbered according to their arrangement in the viral genome (den...
Generation and characterization of the stable BHK-21 cell line that constitutively expresses PRRSV N protein. (A) Schematic representation of the recombinant plasmids used for this experiment. pSinrep19 is a noncytopathic Sindbis virus-based vector for heterologous gene expression. pSinrep19/PRRSV-N, a derivative of pSinrep19, encodes the PRRSV ORF7 (N) and puromycin N-acetyltransferase (PAC) genes under a separate subgenomic promoter (26S promoter). MCS, multiple cloning sites. (B) Expression and localization of PRRSV N proteins. The BHK-21 cell lines (designated BHK/Sinrep19 and BHK/Sinrep19/PRRSV-N) were established by transfection of naïve BHK-21 cells with each of the RNAs transcribed in vitro from either pSinrep19 or pSinrep19/PRRSV-N, followed by selection with puromycin. Selected cells were fixed with formaldehyde, permeabilized with Triton X-100, and immunostained with a mouse anti-N monoclonal antibody and subsequently with a FITC-conjugated goat anti-mouse IgG (α-PRRSV N, green fluorescence). Cell nuclei were stained with propidium iodide (PI, red fluorescence) in the presence of RNase A. Images were obtained with a Zeiss Axioskop confocal microscope. Merged images are also presented.

Boon et al., 1995; van Dinten et al., 1999; Molenkamp et al., 2000; Ziebuhr et al., 2000; Baptista et al., 2002; van Aken et al., 2006; Nedialkova et al., 2009; Tian et al., 2009; Beura et al., 2010; Chen et al., 2010a, 2010b). ORF2a and ORF3 to ORF7 are translated from a 3′-coterminal nested set of six subgenomic mRNAs (Conzelmann et al., 1993; Meng et al., 1996), whereas ORF2b is embedded within the coding region of ORF2a (Wu et al., 2001). The latter seven ORFs encode the viral structural proteins (Dokland, 2010): GP2a (ORF2a), E (ORF2b), GP3 (ORF3), GP4 (ORF4), GP5 (ORF5), M (ORF6), and N (ORF7) (Meulenberg et al., 1995b; Dea et al., 2000; Wissink et al., 2005; Das et al., 2010; Van Breedam et al., 2010).

One of the seven structural proteins of PRRSV, the ~15 kDa N protein, interacts with the genomic RNA to form the viral nucleocapsid (Spilman et al., 2009). It is the most abundant viral protein in infected cells and is highly immunogenic in infected animals (Meulenberg et al., 1995a; Dea et al., 2000; Murtaugh et al., 2002). The N protein is divided into two domains: an N-terminal RNA-binding domain (RBD) and a C-terminal dimerization domain (DD) (Wootton et al., 2001; Yoo et al., 2003). The RBD contains a large number of basic residues, which are presumably important for its role in RNA binding; within this domain, a hydrophobic α-helix motif is also predicted (Dokland, 2010). A cysteine residue within this α-helix motif is involved in the formation of intermolecular disulfide linkages, thereby forming N-N homodimers, which probably provide stability to the virions (Mardassi et al., 1996; Wootton and Yoo, 2003). The DD forms a well-organized structure that plays a major role in non-covalent dimerization.
of N (Doan and Dokland, 2003). This domain confers self-assembly property on the N protein, providing the basis for viral nucleocapsid formation. Also, the PRRSV N protein is phosphorylated on several serine residues that map to both the RBD and DD domains, suggesting a regulatory role in RNA-binding activity and/or protein-protein interaction (Wootton et al., 2002). Furthermore, the N protein is not only localized to the cytosol of infected cells but is also targeted to the nucleus/nucleolus by nuclear/nucleolar localization signals within the RBD domain (Rowland et al., 1999, 2003; Rowland and Yoo, 2003); however, the functional importance of its nucleolus localization is not yet fully understood.

In our previous study, we developed a reverse genetics system for PRRSV by constructing a genetically stable infectious cDNA, pBAC/PRRSV/FL (Choi et al., 2006). In the present work, we have utilized this system to produce single-round infectious viral replicon particles (VRPs) as a potential vaccine strategy. PRRSV VRPs are generated by a trans-complementation system consisting of two components: 1) a stable BHK-21 cell line constitutively expressing PRRSV N protein encoded by the Sindbis virus-based pSinrep19 vector, and 2) a PRRSV replicon RNA harboring an internal deletion of 177 nucleotides in the N protein-coding region of the viral genome. The PRRSV N proteins produced in the stable cell line were localized throughout the cytoplasm and to the nucleolus. After transfection of the replicon RNA, we found that the N protein-expressing cells were capable of trans-packaging the replicon RNA into single-round infectious VRPs. Our results suggest that this trans-complementation system is a useful tool for dissecting the molecular interactions between the genomic RNA and N protein and between the individual N proteins during oligomerization.

To generate a stable cell line constitutively expressing PRRSV N protein, we first constructed the corresponding gene expression vector, pSinrep19/PRRSV-N, by using the Sindbis virus-based pSinrep19 vector (Agapov et al., 1998). pSinrep19 contains a double subgenomic promoter (26S promoter), one for a foreign gene of interest and the other for the puromycin N-acetyltransferase (PAC) gene that enables selection with the antibiotic puromycin (Fig. 1A, pSinrep19). Thus, the complete ORF7 coding sequence fragment from pBAC/PRRSV/FL (Choi et al., 2006) was amplified by PCR with the following pair of primers: Prorf7F (5'-GATTCTAGAATGCCAAA TAACAAC-3') and Prorf7R (5'-ATCGTTTAAACTTATCAT GCTGAGGGAAGA-3'), where the underlined sequences indicate the XbaI and Pmel restriction endonuclease recognition sequences, respectively, that were used for cloning. The 381 bp XbaI-Pmel portion of these amplicons was then ligated with the 10852 bp XbaI-Pml fragment of pSinrep19, generating pSinrep19/PRRSV-N (Fig. 1A, pSinrep19/PRRSV-N). Both the pSinrep19 and pSinrep19/PRRSV-N plasmids were purified by standard CsCl/ethidium bromide density centrifugation.

![Fig. 2](image-url) Construction of the PRRSV replicon vector, pBAC/PRRSV/Replicon-ΔN. (A) Schematic representation of the constructs used for this experiment. pBAC/PRRSV/FL is the full-length infectious PRRSV cDNA that we constructed previously (Choi et al., 2006). Highlighted is the locus of PRRSV ORF7 (N), according to the complete genome sequence of the PRRSV P1.97-1/LP1 strain (GenBank accession no. AF612613). pBAC/PRRSV/Replicon-ΔN, a derivative of pBAC/PRRSV/FL, is the PRRSV replicon vector that has an internal in-frame deletion of 177 nucleotides (Ant 15081-15257) corresponding to the C-terminal 59 amino acids of the PRRSV N protein. (B) Expression of PRRSV NSP1α and N proteins in BHK-21 cells transfected with the RNAs derived from pBAC/PRRSV/FL and pBAC/PRRSV/Replicon-ΔN. Naïve BHK-21 cells were mock-transfected (Mock) or transfected with the RNAs synthesized in vitro from pBAC/PRRSV/FL (FL) or from each of the two independent clones of pBAC/PRRSV/Replicon-ΔN (Replicon-ΔN C1 and Replicon-ΔN C2). Transfected cells were directly lysed with 1× sample loading buffer at 24 h post-transfection, transferred to PVDF membranes, and probed with either a rabbit anti-NSP1α antiserum (α-PRRSV NSP1α), a rabbit anti-N antiserum (α-PRRSV N), or a rabbit anti-GAPDH antiserum (α-GAPDH) as a sample loading control. Viral proteins were visualized by staining with an alkaline phosphatase-conjugated goat anti-rabbit IgG and subsequent reaction with BCIP/NBT solution as substrate.
Next, the recombinant pSinrep19 and pSinrep19/PRRSV-N plasmids were each used as templates for in vitro run-off transcription using SP6 RNA polymerase, after linearization with XhoI (Yun et al., 2007, 2009). The 25 μl reaction mixtures contained ~200 ng template DNA, 0.8 mM cap analog (m7G(5′)ppp(5′)G; New England Biolabs [NEB]), 1 mM nTPs (GE Healthcare), 40 U RNaseOUT (Invitrogen), 20 U SP6 RNA polymerase, and the buffer provided by the manufacturer (NEB). The reaction mixtures were incubated for 1 h at 37°C. Following transcription, they were treated with 10 U RNase-free DNase I (Ambion) for 30 min at 37°C to remove the template DNA; the RNA transcripts were then purified by phenol/chloroform extraction followed by ethanol precipitation. The integrity of the RNAs was verified by agarose gel electrophoresis (data not shown). To establish a BHK-21 cell line stably expressing the N protein, 10 μg of the Sinrep19/PRRSV-N RNAs was electroporated into naïve BHK-21 cells, under our optimized conditions (980 V, 99 μs pulse length, and five pulses), with an ECM 830 electroporator (BTX). As a control, the same amount of the Sinrep19 RNA was also used for electroporation in parallel. After electroporation, the transfected cells were seeded for ~24 h, after which the medium was replaced with fresh complete medium containing 10 μg/ml of puromycin (Sigma) for selection of 10-14 days. Thereafter, both the Sinrep19 RNA-selected and Sinrep19/PRRSV-N RNA-selected cells were maintained in the presence of puromycin at 1 μg/ml.

To examine the expression and localization of the N protein, we analyzed each of the two stable cell lines by confocal microscopy as described previously (Choi et al., 2006). In brief, the cells were seeded in a 4 well chamber slide for 24 h, fixed with 0.37% formaldehyde, and permeabilized with 0.2% Triton X-100. They were then immunostained with a mouse anti-N monoclonal antibody (6D7/D2; kindly provided by Shien-Young Kang, Chungbuk National University), followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs). Subsequently, the nuclei of cells harboring Sinrep19/PRRSV-N RNA were localized by staining with propidium iodide (5 μg/ml) in the presence of RNase A (5 μg/ml) for 30 min at 37°C. Fluorescence images were captured with a Zeiss Axioskop confocal microscope using the Bio-Rad MRC1024. In the case of cells harboring Sinrep19/PRRSV-N RNA, we found that the generated N proteins were not only distributed throughout the cytoplasm, microscopically flaring out toward the periphery of the cell, but also targeted to the nucleolus (Fig. 1B, BHK/Sinrep19/PRRSV-N). This result is in agreement with previous reports of an intracellular localization for the N protein in infected cells (Rowland et al., 1999, 2003; Rowland and Yoo, 2003). As expected, no staining was observed in cells harboring Sinrep19 RNA (Fig. 1B, BHK/Sinrep19). Identical results were obtained when BHK-21 cells transfected with Sinrep19/PRRSV-N RNA were passaged up to 10 times (data not shown).

In order to utilize the BHK-21 cell line stably expressing PRRSV N protein to produce PRRSV VRPs, we sought to construct a self-replicating PRRSV replicon containing a large internal deletion in the N protein-coding region of the viral genome. Using the full-length infectious PRRSV cDNA, pBAC/PRRSV/FL (Choi et al., 2006), we initially engineered a set of three viral replicon vectors by introducing an in-frame deletion of various sizes into the N protein-coding region (ORF7). In our preliminary analyses on the replicability of the viral replicon RNAs, we found that removal of any sequences just downstream of the translation initiation codon (AUG) of ORF7 had a deleterious effect on RNA replication (data not shown). This finding prompted us to engineer a viral replicon vector, pBAC/PRRSV/Replicon-ΔN, that contained an internal in-frame deletion of 177 nucleotides (Ant 15081-15257) corresponding to the C-terminal 59 amino acids of the PRRSV N protein (Fig. 2A).

For the pBAC/PRRSV/Replicon-ΔN construct, we employed standard overlap extension PCR with pBAC/PRRSV/FL as the template. Initially, two overlapping fragments flanking a region of deletion intended were amplified by a first round of PCR with two pairs of primers: 1) a 5′ 928 bp fragment with PR222F (5′-TGCAAAGAATTCATGTTGC GCG-3′) and PR228R (5′-AATGCAAGCCCATACGTGACATCATC TTC-3′) and 2) a 3′ 256 bp fragment with PR225F (5′-TGAAGG CTTACCTTGGTA-3′) and PR223R (5′-CACGGATTTCG GACCCATCCTGCA-3′). The two amplicons were gel-purified and used both for a second round of PCR with a pair of the 5′ and 3′ outer primers, PR222F and PR223R. Finally, the 1,109 bp MluI-NotI fragment of the fused PCR products was cloned into the corresponding region in pBAC/PRRSV/FL by a three-piece ligation, by ligating to the 17584 bp NotI-RsrII and 4348 bp RsrII-MluI fragments of pBAC/PRRSV/FL, thereby creating the pBAC/PRRSV/Replicon-ΔN vector (Fig. 2A).

We first examined the replication competency of viral replicon RNAs transcribed from the pBAC/PRRSV/Replicon-ΔN vector. To this end, the pBAC/PRRSV/Replicon-ΔN or pBAC/PRRSV/FL cDNA (as a positive control) was digested with AciI, modified with mung bean nuclease for linearization, and purified by phenol/chloroform extraction and ethanol precipitation (Choi et al., 2006). RNA transcripts were synthesized in vitro using SP6 RNA polymerase, as described earlier for the synthesis of Sinrep19/PRRSV-N RNA, except that the cap analog m7G(5′)ppp(5′)A was added instead of m7G(5′)ppp(5′)G. BHK-21 cells were mock-transfected or transfected with each of the two synthetic RNAs, PRRSV/Replicon-ΔN and PRRSV/FL RNAs. At 24 h after transfection, we analyzed the expression of NSP1α, as an indication of RNA replication, by immunoblotting. A ~18 kDa NSP1α protein was clearly detected in lysates of the cells transfected with PRRSV/Replicon-ΔN RNA, with a level of accumulation similar to or slightly lower than that produced from PRRSV/FL RNA (Fig. 2B, α-PRRSV NSP1α). Also, an additional minor band of ~15 kDa was detected in both the PRRSV/Replicon-ΔN RNA-transfected and PRRSV/FL RNA-transfected cells; the biological importance of this finding requires further investigation. As expected, N protein was expressed in the cells transfected with PRRSV/FL RNA but not with PRRSV/Replicon-ΔN RNA (Fig. 2B, α-PRRSV N). No difference was noted when the BHK/Sinrep19 cell line was used for RNA transfection (data not shown), indicating that Sinrep19 RNA replication does not significantly interfere with PRRSV RNA replication.

To determine whether the replication-competent PRRSV/Replicon-ΔN RNA can be encapsidated into single-round infectious particles by PRRSV N provided in trans, we mock-
transfected or transfected the newly generated BHK/Sinrep19/PRRSV-N cells with either the PRRSV/Replicon-ΔN RNA or with the PRRSV/FL RNA as a positive control. At 48 h after transfection, the culture supernatants were harvested and used to infect naïve Marc-145 cells, and we examined the expression of viral proteins from the packaged replicon RNA by confocal microscopy (Fig. 3A). Immunofluorescence analysis with both a PRRSV-positive pig serum and a rabbit anti-NSP2 antiserum consistently showed an accumulation of viral proteins predominantly around the perinuclear membranes in the cytoplasm of Marc-145 cells inoculated with the medium from PRRSV/Replicon-ΔN RNA-transfected cells or from PRRSV/FL RNA-transfected cells (Fig. 3B). As expected, no immunofluorescent staining was detected in Marc-145 cells inoculated with the supernatant from mock-transfected cells (Fig. 3B). Our results suggest that the PRRSV/Replicon-ΔN RNA was encapsidated into infectious particles, which were then secreted from the BHK/Sinrep19/PRRSV-N cells. Based on immunofluorescence analysis using the rabbit anti-NSP2 antiserum, the yield of PRRSV VRPs was estimated to be inefficient, ranging from $0.4 \times 10^2$ to $1.1 \times 10^2$ infectious units/ml, under our experimental conditions. We verified that there was no evidence of the occurrence of RNA recombination that would potentially produce propagation-competent infectious particles, as demonstrated by the reinfection of naïve Marc-145 cells with the culture supernatant harvested from VRP-infected Marc-145 cells (data not shown).

Over the past two decades, a number of plus-strand RNA viruses have been exploited for development as vectors for foreign gene expression and delivery (Conzelmann and Meyers, 1996; Hewson, 2000; Khromykh, 2000). Although the genome structure and replication mechanism of a particular virus is a major determinant of the strategy to be used to modify its genome, viral vectors are commonly engineered in two ways: as infectious forms and non-infectious replicon forms. In the first approach, a foreign gene of interest is inserted into the viral genome in such a way that all the viral genes that are essential for productive viral infection are functionally intact (Dufresne et al., 2002; Jia et al., 2002; Yun et al., 2003). In contrast, the second approach aims to generate a subgenomic replicon that contains only a limited number of the viral genes required for RNA replication and lacks part or all of the struc-
tural protein-coding and accessory protein-coding regions of the viral genome (Khromykh, 2000; Schlesinger, 2000; Enjuanes et al., 2005). In addition, when the missing viral proteins are supplied in trans, this replicon-based approach can also be further expanded to generate infectious VRPs that are capable of establishing only a single round of infection (Khromykh, 2000; Lundstrom, 2001; Schlesinger, 2001). The most advanced viral vectors are derived from alphaviruses such as Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus (Schlesinger, 2001; Lundstrom, 2005; Atkins et al., 2008) and from flaviviruses such as Kunjin virus and yellow fever virus (Galler et al., 1997; Pijlman et al., 2006). These viruses have a number of characteristics in common that are desirable for the development of a vector to express foreign genes: 1) a wide range of hosts that are susceptible to infection, 2) a large number of cell types that are permissive for replication, 3) a relatively small size of viral genome, 4) a rapid cycle of RNA replication, and 5) cytoplasmic RNA amplification.

In the present study, we have successfully generated a BHK-21 cell line that stably expresses the PRRSV N protein, by employing the Sindbis virus-based foreign gene expression vector, pSinrep19 (Agapov et al., 1998). In this cell line, the ectopically expressed N protein was localized not only to the cytoplasm but also targeted to the nucleus, as seen in PRRSV-infected cells (Rowland et al., 1999, 2003; Rowland and Yoo, 2003); however, it was expressed at a level significantly lower than that produced in PRRSV-infected cells (data not shown). We also created a PRRSV replicon RNA that is replication-competent but propagation-deficient as a result of an internal in-frame deletion of 177 nucleotides, resulting in an AU-rich negative effect on RNA-binding activity of the wild-type N proteins provided in trans. Further investigation will be required to improve the feasibility of our trans-complementation system as a novel vaccine strategy and a new gene delivery system.

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