THE APPLICATION OF A PRRSV REVERSE GENETIC SYSTEM FOR THE STUDY OF NONSTRUCTURAL PROTEIN (NSP) FUNCTION

by

DAL-YOUNG KIM

D.V.M., Chungbuk National University, 2000

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2007

ABSTRACT

Infectious cDNA clones of PRRSV make it possible to construct marker viruses for the study of virus replication and pathogenesis. The nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus (PRRSV) is the single largest protein produced during virus replication. The cDNA of the pCMV-129 infectious PRRSV clone was modified by creating unique Mlu I and SgrA I restrictions sites at nucleotide (nt) positions 3,219 and 3,614, respectively: both located within the C-terminal region of nsp2. cDNAs coding for oligo- and polypeptide tags, including FLAG, enhanced green fluorescent protein (EGFP) and firefly luciferase were inserted into the newly created restriction sites. The results showed that only the EGFP-containing genomes were properly expressed and produced virus. EGFP fluorescence, but not EGFP immunoreactivity, was lost during passage of recombinant EGFP viruses in culture. Sequencing of a fluorescence-negative EGFP virus showed that the EGFP remained intact, except for the appearance of mutations that may affect chromophore formation. The results show that nsp2 can be a site for the expression of foreign proteins.

Removal of the region between Mlu I and SgrA I sites resulted in a virus that contained a 131 amino acid deletion. The deleted region was replaced with EGFP or an eight amino acid influenza hemagglutanin (HA) tag. Recombinant viruses were used to infect pigs. Gross and micro-histopathology showed reduced pathogenesis when compared to the parent wild-type virus. The 131 amino acid peptide, when expressed as a recombinant protein and coated onto enzyme linked immunosorbent assay (ELISA) plates, was recognized by sera from pigs infected with wild-type virus, but not the deletion mutants. The results from this study show that nsp2 is a potential target for the development of marker vaccines that can differentiate infected from vaccinated animals (DIVA) and for virus attenuation.

THE APPLICATION OF A PRRSV REVERSE GENETIC SYSTEM FOR THE STUDY OF NONSTRUCTURAL PROTEIN (NSP) FUNCTION

by

DAL-YOUNG KIM

D.V.M., Chungbuk National University, 2000

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2007

Approved by:

Major Professor
Raymond Rowland

ABSTRACT

Infectious cDNA clones of PRRSV make it possible to construct marker viruses for the study of virus replication and pathogenesis. The nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus (PRRSV) is the single largest protein produced during virus replication. The cDNA of the pCMV-129 infectious PRRSV clone was modified by creating unique Mlu I and SgrA I restrictions sites at nucleotide (nt) positions 3,219 and 3,614, respectively: both located within the C-terminal region of nsp2. cDNAs coding for oligo- and polypeptide tags, including FLAG, enhanced green fluorescent protein (EGFP) and firefly luciferase were inserted into the newly created restriction sites. The results showed that only the EGFP-containing genomes were properly expressed and produced virus. EGFP fluorescence, but not EGFP immunoreactivity, was lost during passage of recombinant EGFP viruses in culture. Sequencing of a fluorescence-negative EGFP virus showed that the EGFP remained intact, except for the appearance of mutations that may affect chromophore formation. The results show that nsp2 can be a site for the expression of foreign proteins.

Removal of the region between Mlu I and SgrA I sites resulted in a virus that contained a 131 amino acid deletion. The deleted region was replaced with EGFP or an eight amino acid influenza hemagglutanin (HA) tag. Recombinant viruses were used to infect pigs. Gross and micro-histopathology showed reduced pathogenesis when compared to the parent wil-type virus.. The 131 amino acid peptide, when expressed as a recombinant protein and coated onto enzyme linked immunosorbent assay (ELISA) plates, was recognized by sera from pigs infected with wild-type virus, but not the deletion mutants. The results from this study show that nsp2 is a potential target for the development of marker vaccines that can differentiate infected from vaccinated animals (DIVA) and for virus attenuation.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

LIST OF KEY WORDS

PRRSV : Porcine Reproductive and respiratory Syndrome Virus.

Nsp : Nonstructural protein.

EGFP : Enhanced Green Fluorescent Protein.

HA : Hemagglutinin.

ELISA : Enzyme Linked-Immonosorbent Assay.

DIVA : Differentiated Infected Vaccinated Animals

LV : Lelystad Virus

EAV : Equine Arteritis Virus

SHFV : Simian Hemorrhagic Fever Virus

LDV : Lactate Dehydrogenase Virus.

ORF : Open Reading Frame.

GP : Glycoprotein

M : Membrane.

BHK-21 : Baby Hamster Kidney-21.

TRS : Transcription Regulating Sequence.

RFS : Ribosomal Frameshifting Site.

AP : Accessary Protein.

MP : Main Protease.

RdRp : RNA dependent RNA polymerase.

Z : Zinc-binding domain.

HEL : Helicase

N : NendoU

TM : TransMembrane.

SUD : SARS-CoV Unique Domain.

CAT : Chloramphenicol Acetyltransferase.

PAM : Porcine Alveolar Macrophage.

DMVs : Double Membrane Vesicles

RT-PCR : Reverse Transcriptase-Polymerase Chain Reaction.

S/P : Standard/Peak

VI : Virus Isolation.

DEDICATION

This dissertation is dedicated to my parents, brother.

ACKNOWLEDGEMENTS

The deepest appreciation goes to my primary mentor, Dr. Bob Rowland, who has served as a father figure to me over the last five years and has given me the opportunity to achieve one of my scientific goals. Additionally, I would like to offer my profound thanks to the members of my committee, Dr. Rollie J. Clem, Dr. Richard D. Oberst and Dr. Chang**,** Kyeong-Ok, for all their time and helpful guidance.

I leave as is to express my thanks to the members of my lab for all of their time and advice over the years. Specifically, I would like to thank Maureen Karrigan for her help and time in all studies. It has been a great pleasure to interact with the other lab members as well including : Vinita Singh Chauhan, Luna Miraflor Aguirre, John Patton, Kyle Horlen. I also wish to thank my previous mentors, Dr. Kang, Shien-Young for his advice and motivation.

Finally, I express my deepest gratitude to my dear parents, my elder brother, and all my family members. I am eternally grateful for their everlasting support and faith in all my undertakings.

CHAPTER 1

PRRSV; Overview

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) was first observed in 1987 in the United States (20, 23) where it became widespread throughout North America. Initially, the disease was named mystery swine disease because of the elusive nature of its causal agent. Late in 1990, the first European outbreak of disease was reported in Germany, from where it spread rapidly throughout Europe (31). In mid-January 1991 the disease was reported in the Netherlands in breeding herds, and by the end of March it had spread over all provinces with intensive pig farming. In 1994, PRRS was officially recognized in 16 countries on three different continents; America, Asia and Europe (31).

The etiologic agent responsible for the syndrome was first isolated on alveolar macrophage cultures by Wensvoort et al in Europe in 1991 (52) and was designated Lelystad virus (LV). Soon after that, the virus (ATCC VR-2332) was isolated on an MA104 derived cell line, CL 2621, by Collins et al. (10) in the United States in 1992 and identified as the swine infertility and respiratory syndrome (SIRS) virus.

Initially several names were used to refer to the disease: mystery swine disease (23); swine infertility and respiratory syndrome (SIRS; 20) ; porcine epidemic abortion and respiratory syndrome (PEARS; 50); and blue-eared pig disease (53). However, porcine reproductive and respiratory syndrome (PRRS) is the name now commonly used and the one recognized by the International Office of Epizootics (O.I.E), leading to the causative agent being called porcine reproductive and respiratory syndrome virus (PRRSV).

THE VIRUS

PRRSV is a small enveloped RNA virus that is classified as a member of the family

Arteriviridae (33), within the newly created order *Nidovirales* (8). The family *Arteriviridae* also includes equine arteritis virus (EAV), mouse lactate dehydrogenase elevating virus (LDV), and simian hemorrhagic fever virus (SHFV; 32, 43). PRRSV isolates are more closely related to LDV than to EAV (30, 32). Variations in antigenicity have been evidenced with polyclonal sera or monoclonal antibodies between the North American and European isolates (18, 28, 29) and among American isolates (27). Furthermore, the North American and European isolates have been shown to represent two distinct genotypes, now classified as PRRSV Type 1 for viruses of European lineage and PRRSV Type 2 for the North American lineage. Type 1 and Type 2 genotypes share only about 67% identity at the nucleotide level (29, 30).

CLINICAL ASPECTS

PRRS initially emerged worldwide as a devastating clinical disease, characterized by a massive reproductive disorder affecting pregnant sows, perinatal losses and respiratory distress of piglets. PRRS is also seen by reduced growth performance during the grow-finish stage of production. Over time it became clear that the major characteristic of the disease was a high variability of clinical signs, including a smoldering subclinical course of infection. In retrospect, based on the presence of PRRSV antibodies in Canada and East Germany, PRRSV had already been circulating in herds before the report of outbreaks in North America and Europe (7, 40).

In its epidemic phase, PRRS is characterized in sow herds by massive reproductive disorders that last 1-3 months (11, 52). The onset of the infection in sows often starts with inappetence and anorexia, which, if the infection spreads on a large scale in a herd, is marked as a “rolling inappetence”. Occasionally, incidental cases of respiratory distress, fever or death have been

reported in sows. The reproductive disorders include abortion, premature birth, late term birth, increased incidence of fresh or autolytic stillborns, fetal death with or without mummification, and weak-born piglets that die shortly after birth. A less common finding, more often described in Europe, is a transient discoloration (cyanosis) of the ears, abdomen and vulva (53).

The disease in young pigs is characterized by respiratory disorders, increased mortality as a result of secondary infections and retarded growth (11, 52). The severity of clinical is largely age-dependent, i.e. more severe in nursery pigs compared to finishing pigs.

Currently, PRRSV is endemic in most pig-producing countries throughout the world. Field studies have revealed that PRRSV can persist in herds for years (47, 50). Although limited clinical field studies of endemically infected herds are available, it is generally acknowledged that most infections are subclinical and productivity is usually maintained within acceptable parameters. In some infected herds, sporadic respiratory disease in young piglets or periodic reproductive disease in naïve sows have been reported (14). In the USA and the United Kingdom, the recurrent respiratory disease problems in nursery and grower pigs are referred to as porcine respiratory disease complex (PRDC) and post-weaning respiratory syndrome (17, 47, 55). In these herds, a higher than usual prevalence of secondary pathogens often occurs concurrently with PRRSV infections (42).

IN VITRO GROWTH CHARACTERISTICS OF PRRSV

In the pig, PRRSV primarily targets alveolar lung macrophages. It was also recently shown to replicate in testicular germ cells such as spermatids and spermatocytes in infected boars (49). PRRSV can grow in vitro in primary cultures of alveolar lung macrophages and in African green monkey kidney cells or derivatives thereof (CL2621 or MARC-145; 3, 24). PRRSV can replicate

in several cell lines that cannot be infected by virus particles (35). This finding indicates that cell tropism is determined by the presence or absence of an as yet unidentified receptor on the cell surface. Recently monoclonal antibodies were produced that specifically bind to macrophages and prevent these cells from becoming infected by PRRSV (19). These MAbs recognize a 210-kDa membrane protein, that might function as a putative receptor for PRRSV. Kim et al (2006) showed that simian vimentin could play an important role in transforming nonsusceptible cell lines into PRRSV permissive ones. A sialoadhesin and heparin receptor as well as CD163 are known to be a putative receptor of PRRSV.

PRRSV enters the host via the standard endocytotic route. Electron microscopy shows PRRSV particles in small vesicles, which appear to be clathrin-coated pits (26). Between 3-6 hr after infection, double membrane vesicles are formed (44). These are general features of arterivirus replication. For EAV, the double membrane vesicles are derived from the endoplasmic reticulum (ER) and contain the replication complex (41). PRRSV is assembled when preformed nucleocapsids bud into the lumen of the smooth endoplasmic reticulum or Golgi region or both. After budding, virions accumulate in vesicles, which move to the plasma membrane where they fuse to release the virus. In one-step growth experiments, the maximum release of PRRSV particles is between 10-20 h and the maximum titers in cell culture are $10^{6.5}$-$10^{7.5}$ $TCID_{50}$/ml. The cytopathic effect of PRRSV in macrophages and cell lines is characterized by rounding of the cells and detachment from the culture plate surface.

GENOME ORGANIZATION

Genome sequencing of several PRRSV strains (12, 13) shows that PRRSV has a similar organization as other arteriviruses (45). The PRRSV genome is approximately 15 kb in length

and contains nine ORFs (Fig. 1.2). ORFs 1a and 1b comprise about 75% of the genome and encode the nonstructural proteins (nsp's). The ORF1a and ORF1ab encoded polyproteins are processed into smaller protein products by virus-encoded proteases. For PRRSV, the first two N-terminal cleavage products, nsp1α and nsp1β, have been shown to be papain-like cysteine protease (16). Based on studies of equine arteritis virus, cysteine protease (nsp2) and a serine protease (nsp4) are assumed to cleave the ORF1ab product into 12 nonstructural proteins (Fig. 1.4). Still little is known about the function of the individual PRRSV nsps. Predictions relating to the functions of the ORF1b-encoded replicase subunits nsp9 and nsp10 are derived from comparative sequence analysis. A putative RNA-dependent RNA polymerase motif is present in nsp9, and a nucleoside triphosphate-binding/RNA helicase motif and metal binding domain are in nsp10 (2, 56).

The seven smaller ORFs, 2 through 7, are located at the 3' end of the genome and encode structural proteins (13, 34). The nucleocapsid protein, N (ORF7) and the integral membrane protein M (ORF 6) are not glycosylated. In constrast, GP2a (ORF 2a), GP3 (ORF 3), GP4 (ORF 4), and GP5 (ORF5) proteins are all N-glycosylated. Recently, a novel non-glycosylated structural protein, expressed from an ORF embedded in ORF2 and called ORF 2b codes for a small non-glycosylated protein known as 2b in PRRSV and E in EAV (46, 54).

The proteins derived from ORFs 2 to 7 are translated from a 3' nested set of subgenomic mRNAs (Fig.1.2 and 1.3). The subgenomic mRNAs are composed of a leader sequence, derived from the 5' end of the viral genome and fused to the subgenomic mRNA bodies by a discontinuous transcription mechanism (45, Fig. 1.3). The site of fusion, also called the leader-body junction site, is a conserved sequence of six nucleotides : UUAACC.

GENOME TRANSLATION AND REPLICATION

By definition, the genome of a positive-stranded RNA virus fulfills a dual role in both storage and expression of genetic information. Thus, the reproduction of the arterivirus genomic RNA is a combined process of genome *replication* and mRNA *transcription* . For simplicity, I will use the term 'replication' for the process of genome (mRNA1) synthesis, whereas the term 'transcription' will be used to refer to generation of the subgenomic mRNAs.

The PRRSV replication cycle starts with the expression of the replicase gene from the incoming genome. Replicase ORF1a and ORF1b, which is in the -1 reading frame relative to ORF1a, are both expressed from the genomic mRNA. ORF1b translation requires a ribosomal frameshift just before ORF1a translation is terminated (15). The ORF1a/ORF1b overlap region contains two signals which are assumed to promote this event (4) : a so-called 'slippery' sequence, which is the actual frameshift site, and a downstream RNA pseudoknot structure. In EAV, the putative shift site 5' GUUAAAC 3' is followed immediately by the ORF1a termination codon. In PRRSV and LDV, one additional codon is present between these two elements. The predicted stems 1 and 2 of the RNA pseudoknot typically consist of 11-12 and 6-7 base pairs, respectively (15, 22, 33). The functionality of the arterivirus frameshift signal has only been demonstrated for EAV ; using a reporter gene construct, a frameshift efficiency of 15-20% was observed (15).

In constrast to coronarviruses, little was known about the arterivirus RNA sequences required for genome replication. Molenkamp et al (2000a, 2000b) described defective interfering (DI) particle of EAV, which showed the minimum genomic RNA sequence for viral replication and packaging. In the 3' UTR, which displays 33-47% sequence identity among the arteriviruses, a short conserved sequence motif just upstream of the 3' poly(A)-tail was desribed (22). At the 5'

end, it is noteworthy that in three of the four arteriviruses the ORF1a initiation codon is located immediately downstream of the 3' end of the common leader sequence (only in EAV are 12 additional nucleotides present). As a result, the entire genome 5' UTR is present in all subgenomic mRNAs of LDV, PRRSV and SHFV. Four host proteins from MA-104 cells were reported to bind to in vitro-generated transcripts representing the 3' end of the SHFV genomic negative strand (21). This region, which is the complement of the genomic leader sequence, is assumed to be important for the initiation of plus-strand RNA synthesis. The same four proteins, which remain to be identified, interacted with transcripts representing the corresponding region of the LDV and EAV minus strands, suggesting the involvement of a common set of host factors in arterivirus plus-strand RNA synthesis. The RNA sequences required for encapsidation of the arterivirus genome remain to be determined.

SEQUENCE VARIATION BETWEEN EUROPEAN AND NORTH AMERICAN PRRSV STRAINS

Extensive sequence analysis of field isolates of PRRSV has unexpectedly revealed high sequence variation between North American and European PRRSV isolates (1, 38, 48). The GP5 protein is the most variable structural protein, with only 51-55% amino acid identity between North American and European isolates, whereas the M protein is the most conserved structural protein, with 78-81% amino acid identity.Sequence comparison between the ORF1 genes of Lelystad (LV) and two US strains (1) reveal major differences in this part of the genome. The greatest variation is observed in nsp2. The nsp2 protein of US strains is aprroximately 102 amino acids longer than that of LV and only share 32% identity at the amino acid level. Furthermore, differences in the leader-body junction sites that are used by European and US viruses are also

present. Although the leader-body junction sequence (UUAACC) is conserved in both European and North American isolates, the distance between the junction sequence and the downstream ORF is highly variable. Furthermore, additional junction sites that are less frequently used were identified for the North American strains VR2332 and ISU96 but not for LV (38).

PREVENTION AND CONTROL

Despite the fact that PRRSV infection is widespread throughout the world, noninfected herds still exist. While all routes of virus introduction into a naïve herd are not completely understand at this time, the primary source is the infected pig. Therefore, it is critical to routinely isolate and test breeding stock before introducing them to a PRRS-negative herd. Replacement stock to be added to naïve herds should be obtained from known negative sources that carry out a regular schedule of herd monitoring. In addition, there should be clear and open communication between veterinarians responsible for the health of the source and recipient herds, respectively, prior to purchase of replacement stock.

Following the identification of PRRSV and the development of diagnostic tests, practitioners throughout the world have attempted to control the effects of PRRS. The initial attempts were based on the use of strategies known to be effective for controlling other diseases of swine (e.g., segregated early weaning). Recently, a model for the control of PRRS which focuses on the elimination of subpopulations was developed. The components of the model are as follows. (1) Understanding of the pattern of viral spread throughout the system through the application of population-based diagnostic strategies. (2) Proper development of replacement stock prior to introduction into PRRS-infected herds. (3) Prevention of the transmission of the virus from sow

to piglet through breeding-herd stabilization. (4) Control of the spread of virus in the nursery or finisher populations through weaned-pig management. The purpose of such a model is to provide a generally applicable systems approach for the control of PRRS-related disease problems. The individual components of the model described in greater detail as follows.

PRRSV VACCINES

Several studies have established that vaccination against PRRS can result protective immunity (57, 58). Such studies have led to the extensive use, especially in the United States, of commercial vaccines as an aid in reducing the clinical consequences of infection with virulent PRRSV. Although both attenuated and inactivated vaccines have been described, the former are generally believed to be more efficacious. As a result, virus inactivation has been used mostly as a method of preparing autogenous vaccines.

Currently, two attenuated vaccines are commercially available for use as an aid in the prevention and control of PRRS. RespPRRS/Repro® has been marketed in North America since 1994 for administration to pigs between 3 and 18 weeks of age for the prevention of the respiratory component of PRRS, and since 1996 for administration to nonpregnant gilts and sows for the prevention of the reproductive component of PRRS (The same vaccine is marketed in Europe under the name Ingelvac® PRRS MLV). Prime Pac® PRRS has been marketed in the United States since 1996 for administration to nonpregnant gilts and sows for the prevention of the reproductive component of PRRS.

Much of what is now known about vaccination against PRRS is based on controlled laboratory studies with RespPRRS/ Repro® and with three experimental vaccines developed at the National animal Disease Center (NADC) (58). All of these vaccines, as well as Prime Pac®

PRRS, were derived (i.e., attenuated) by repeated passage of virulent, North American field strains of PRRS virus in cell culture. They differ genetically from one another to some extent, but they are more closely related to each other than to Lelystad (LV) virus.

Information from fields use of PRRS vaccine is limited mostly to RespPRRS/ Repro®, which has been used extensively since it was first licensed and marketed in 1994 under the name RespPRRS/ Repro®. Although RespPRRS® was not approved for use in gilts and sows for the prevention of the reproductive component of PRRS until 1996 (When the name was changed to RespPRRS/Repro®).

The following discussion relative to the safety, efficacious, and limitations of PRRS vaccines is based almost entirely on information derived from laboratory studies with RespPRRS/Repro® and NADC vaccines and from field use of RespPRRS/Repro®. The relative merits of the more recently introduced Prime Pac® PRRS remain to be determined.

It is generally believed that, when used judiciously, attenuated vaccines can be of value in preventing and controlling PRRS. However, to achieve greatest success a number of factors need to be considered when designing a vaccination program. (1) Vaccines virus can persist for weeks and perhaps months (59), and its persistence may be similar to that of virulent virus (58,60). (2) Vaccines virus can be transmitted from vaccinated pigs to naïve pigs (61, 62). (3) Vaccines virus can persist in boars and be disseminated through semen (63). (5) Vaccine-induced protective immunity is slow to develop (59). (6) The time required to develop protective immunity is likely to be strain related; that is, immunity may develop more quickly and more forcefully and last longer against the homologous and antigenically similar strains than against more distantly related strains of PRRSV. (7) Some vaccinated pigs, gilts, and sows may fail to seroconvert for PRRSV following vaccination (Mengeling and Lager, unpublished data). (8) Successful

vaccination of adult females (as judged by seroconversion) may not ensure the complete absence of transplacental infection and vertical transmission of virulent virus (59). In addition, evidence has been presented for the transmission of vaccine virus from vaccinated sows to nonvaccinated sows, and from vaccinated herds to nonvaccinated herds, with the subsequent development of vaccine-virus-induced reproductive failure (61).

Although vaccines, especially attenuated vaccines, have played a role in the prevention and control of PRRS and are likely to continue to do so in the foreseeable future, there is still much to be learned about their efficacy and safety and about the minimal interval between vaccination and exposure to virulent virus necessary for protective immunity. And because of the strain differences there may be a need for multistrain vaccines to provide the earliest protection against the widest range of heterologous strains. Also, inactivated vaccines (63), because of their added level of safety, need to be further evaluated for their potential for the control of PRRS, either when used alone or as an adjunct to attenuated vaccines.

INFECTIOUS cDNA CLONES

The development of infectious cDNA clones is a major breakthough in PRRSV research and allows the construction of genetically-engineered mutant PRRS virus in vitro (35, 5, 6, 39, 51, 9). When the transcripts of a cDNA clone are transfected to BHK-21 or MARC-145 cells, progeny virus is produced which can be further propagated in porcine alveolar macrophages or MARC-145 cells. The genomic transcripts are first transfected into BHK-21 cells because these cells are more efficiently transfected than CL2621 cells or other PRRSV propagating cell lines. Although PRRSV cannot enter BHK-21 cells, once the genomic RNA is introduced into these cells, the virus is produced. In the published literature, there are several examples of specific

modifications to the PRRSV genome, including mutations, deletions, substitutions, and foreign gene expression (5, 6, 9). However, only a few mutations are tolerated by the virus, indicating that the major part of the genomic RNA contains essential sequences for virus replication.

REFERENCES

1. **Allende R, Lewis TL, Lu Z, Rock DL, Kutish GF, Ali A, Doster AR, Osorio FA.** North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J Gen Virol. 1999 Feb;80 (Pt 2):307-15.

2. **Bautista EM, Faaberg KS, Mickelson D, McGruder ED.** Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology. 2002 Jul 5;298(2):258-70.

3. **Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D.** Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest. 1992 Apr;4(2):127-33.

4. **Brierley I.** Ribosomal frameshifting viral RNAs. J Gen Virol. 1995 Aug;76 (Pt 8):1885-92.

5. **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 6,500,662.

6. **Calvert, J.G., Sheppard, M.G., Welch, S.K.W.,** 2003. Infectious cDNA clone of North American porcine reproductive and respiratory syndrome (PRRS) virus and uses thereof. US Patent Application 20030157689.

7. **Carman S, Sanford SE and Dea S.** Antibodies to PRRS virus in serum banks of Ontario swine (1978-1982). Proceedings of the 14th swine health association.

8. **Cavanagh D.** Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol. 1997;142(3):629-33.

9. **Choi YJ, Yun SI, Kang SY, Lee YM.** Identification of 5' and 3' cis-acting elements of the porcine reproductive and respiratory syndrome virus: acquisition of novel 5' AU-rich sequences restored replication of a 5'-proximal 7-nucleotide deletion mutant. J Virol. 2006 Jan;80(2):723-36.

10. **Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS.** Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest. 1992 Apr;4(2):117-26.

11. **Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Gorcyca DE, Chladek DW and Morrison RB.** Swine infertility and respiratory syndrome (mystery swine disease). In: Proceedings of Minnesota Swine Conference for Veterinarians, University of Minnesota, MN, 1991.

12. **Dea S, Gagnon CA, Mardassi H, Milane G.** Antigenic variability among North American and European strains of porcine reproductive and respiratory syndrome virus as defined by monoclonal antibodies to the matrix protein. J Clin Microbiol. 1996 Jun;34(6):1488-93.

13. **Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D.** Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch Virol. 2000;145(4):659-88.

14. **Dee SA, Joo HS.** Recurrent reproductive failure associated with porcine reproductive and respiratory syndrome in a swine herd. J Am Vet Med Assoc. 1994 Oct 1;205(7):1017-8.

15. **den Boon JA, Snijder EJ, Chirnside ED, de Vries AA, Horzinek MC, Spaan WJ.** Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. J Virol. 1991 Jun;65(6):2910-20.

16. **den Boon JA, Faaberg KS, Meulenberg JJ, Wassenaar AL, Plagemann PG, Gorbalenya AE, Snijder EJ.** Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases. J Virol. 1995 Jul;69(7):4500-5.

17. **Done SH, Paton DJ.** Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet Rec. 1995 Jan 14;136(2):32-5.

18. **Drew TW, Meulenberg JJ, Sands JJ, Paton DJ.** Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. J Gen Virol. 1995 Jun;76 (Pt 6):1361-9.

19. **Duan X, Nauwynck HJ, Favoreel HW, Pensaert MB.** Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. J Virol. 1998 May;72(5):4520-3.

20. **Hill HT**. Overview and history of mystery swine disease (swine infertility/respiratory syndrome). In: Proceedings of the Mystery Swine Disease Committee Meeting, Livestock Conservation Institute, Madison, 1990; pp. 29-31.

21. **Hwang YK, Brinton MA.** Cell proteins bind to a 67 nucleotide sequence within the 3' noncoding region (NCR) of simian hemorrhagic fever virus (SHFV) negative-strand

RNA. Adv Exp Med Biol. 1998;440:235-40.

22. **Godeny EK, Chen L, Kumar SN, Methven SL, Koonin EV, Brinton MA.** Complete genomic sequence and phylogenetic analysis of the lactate dehydrogenase-elevating virus (LDV). Virology. 1993 Jun;194(2):585-96.

23. **Keffaber KK**. Reproductive failure of unknown etiology. American Association of Swine Practitioners Newsletter 1989; 1: 1-19.

24. **Kim HS, Kwang J, Yoon IJ, Joo HS, Frey ML.** Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch Virol. 1993;133(3-4):477-83.

25. **Kim JK, Fahad AM, Shanmukhappa K, Kapil S.** Defining the cellular target(s) of porcine reproductive and respiratory syndrome virus blocking monoclonal antibody 7G10. J Virol. 2006 Jan;80(2):689-96.

26. **Kreutz LC, Ackermann MR.** Porcine reproductive and respiratory syndrome virus enters cells through a low pH-dependent endocytic pathway. Virus Res. 1996 Jun;42(1-2):137-47.

27. **Magar R, Larochelle R, Dea S, Gagnon CA, Nelson EA, Christopher-Hennings J, Benfield DA.** Antigenic comparison of Canadian and US isolates of porcine reproductive and respiratory syndrome virus using monoclonal antibodies to the nucleocapsid protein. Can J Vet Res. 1995 Jul;59(3):232-4.

28. **Magar R, Larochelle R, Nelson EA, Charreyre C.** Differential reactivity of a monoclonal antibody directed to the membrane protein of porcine reproductive and respiratory syndrome virus. Can J Vet Res. 1997 Jan;61(1):69-71.

29. **Mardassi H, Mounir S, Dea S.** Identification of major differences in the

nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. J Gen Virol. 1994 Mar;75 (Pt 3):681-5.

30. **Meng XJ, Paul PS, Halbur PG, Lum MA.** Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch Virol. 1995;140(4):745-55.

31. **Meredith MJ**. Porcine reproductive and respiratory syndrome (PRRS). Pig disease information centre, University of Cambridge, 1995.

32. **Meulenberg JJ, de Meijer EJ, Moormann RJ.** Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology. 1993 Jan;192(1):62-72.

33. **Meulenberg JJ, de Meijer EJ, Moormann RJ.** Subgenomic RNAs of Lelystad virus contain a conserved leader-body junction sequence. J Gen Virol. 1993 Aug;74 (Pt 8):1697-701.

34. **Meulenberg JJ, Petersen-den Besten A, De Kluyver EP, Moormann RJ, Schaaper WM, Wensvoort G.** Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology. 1995 Jan 10;206(1):155-63.

35. **Meulenberg JJ, Bos-de Ruijter JN, van de Graaf R, Wensvoort G, Moormann RJ.** Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. J Virol. 1998 Jan;72(1):380-7.

36. **Molenkamp R, Rozier BC, Greve S, Spaan WJ, Snijder EJ.** Isolation and characterization of an arterivirus defective interfering RNA genome. J Virol. 2000a Apr;74(7):3156-65

37. **Molenkamp R, Greve S, Spaan WJ, Snijder EJ.** Efficient homologous RNA recombination and requirement for an open reading frame during replication of equine arteritis virus defective interfering RNAs. J Virol. 2000b Oct;74(19):9062-70.

38. **Nelsen CJ, Murtaugh MP, Faaberg KS.** Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol. 1999 Jan;73(1):270-80.

39. **Nielsen HS, liu G, Nielsen J, Oleksiewicz MB, Botner A, Storgaard T, Faaberg KS.** Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. J Virol. 2003 Mar;77(6):3702-11.

40. **Ohlinger VF, Pesch S and Bischoff C.** Key factors that influence PRRS-epidemiology in European swine production. In: Proceedings of 3rd International Symposium on PRRS and Aujeszky disease. Ploufragen, France, 1999, pp. 199-201.

41. **Pedersen KW, van der Meer Y, Roos N, Snijder EJ.** Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. J Virol. 1999 Mar;73(3):2016-26.

42. **Pijoan C, Solano G and Segales J.** PRRS virus and secondary disease. In: Proceedings of A.D. Leman Conference, St. Paul, MN, 1994, pp. 225-226.

43. **Plagemann PG, Moennig V.** Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv Virus Res. 1992;41:99-192.

44. **Pol JM, Wagenaar F.** Morphogenesis of Lelystad virus in porcine lung alveolar

macrophages, Am, Assoc. Swine Pract. Newslett. 4 (1992) 29.

45. **Snijder EJ, Meulenberg JJ.** The molecular biology of arteriviruses. J Gen Virol. 1998 May;79 (Pt 5):961-79.

46. **Snijder EJ, van Tol H, Pedersen KW, Raamsman MJ, de Vries AA.** Identification of a novel structural protein of arteriviruses. J Virol. 1999 Aug;73(8):6335-45.

47. **Stevenson GW, Van Alstine WG, Kanitz CL, Keffaber KK.** Endemic porcine reproductive and respiratory syndrome virus infection of nursery pigs in two swine herds without current reproductive failure. J Vet Diagn Invest. 1993 Jul;5(3):432-4.

48. **Suarez P, Zardoya R, Martin MJ, Prieto C, Dopazo J, Solana A, Castro JM.** Phylogenetic relationships of european strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res. 1996 Jun;42(1-2):159-65.

49. **Sur JH, Doster AR, Christian JS, Galeota JA, Wills RW, Zimmerman JJ, Osorio FA.** Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. J Virol. 1997 Dec;71(12):9170-9.

50. **Terpstra C, Wensvoort G, Pol JM.** Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. Vet Q. 1991 Jul;13(3):131-6.

51. **Truong HM, Lu Z, Kutish GF, Galeota J, Osorio FA, Pattnaik AK.** A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology. 2004 Aug 1;325(2):308-19.

52. **Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, Wagenaar F.** Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q. 1991 Jul;13(3):121-30.

53. **White M.** "Blue ear" disease in pigs. Veterinary Record 1991; 128:574.

54. **Wu WH, Fang Y, Farwell R, Steffen-Bien M, Rowland RR, Christopher-Hennings J, Nelson EA.** A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology. 2001 Aug 15;287(1):183-91.

55. **Zeman D, Neiger R, Yaeger M, Nelson E, Benfield D, Leslie-Steen P, Thomson J, Miskimins D, Daly R, Minehart M.** Laboratory investigation of PRRS virus infection in three swine herds. J Vet Diagn Invest. 1993 Oct;5(4):522-8

56. **Ziebuhr J, Snijder EJ, Gorbalenya AE.** Virus-encoded proteinases and proteolytic processing in the Nidovirales. J Gen Virol. 2000 Apr;81(Pt 4):853-79.

57. **Hesse RA, Couture LP, Lau ML, Dimmick SK, and Ellsworth SR.** 1996 Efficacy of Prime Pac PRRS in controlling PRRS reproductive disease: Homologous challenge. In Proc 27th Annu Meet Am Assoc Swine Pract, pp. 103-105.

58. **Mengeling WL, Larger KM, Vorward AC.** 1996. An overview an vaccination for porcine reproductive and respiratory syndrome. In Proc 23d Allen D. Leman Swine Conf, pp. 139-142.

59. **Mengeling WL, Vorwald AC, Lager KM, Brockmeier SL**. Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. Am J Vet Res. 1996 Jun;57(6):834-9.

60. **Wills RW, Zimmerman JJ, Yoon KJ, Swenson SL, McGinley MJ, Hill HT, Platt KB.** 1995. Porcine reproductive and respiratory syndrome virus: Isolation from chronically

infected swine. In Proc 26th Annu Meet Am Assoc Swine Pract, pp. 387-389.

61. **Botner A, Strandbygaard B, Sorensen KJ, Have P, Madsen KG, Madsen ES, Alexandersen S.** Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet Rec. 1997 Nov 8;141(19):497-9.

62. **Mengeling WL, Lager KM, Vorwald AC.** Clinical effects of porcine reproductive and respiratory syndrome virus on pigs during the early postnatal interval. Am J Vet Res. 1998 Jan;59(1):52-5.

63. **Christopher-Hennings J, Nelson EA, Nelson JK, Benfield DA.** Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. Am J Vet Res. 1997 Jan;58(1):40-5.

64. **Swenson SL, Hill HT, Zimmerman JJ, Evans LE, Wills RW, Yoon KJ, Schwartz KJ, Althouse GC, McGinley MJ, Brevik AK.** 1995. Preliminary assessment of an inactivated PRRS virus vaccine on the excretion of virus in semen. Swine Health and Production 3(6): 244-247

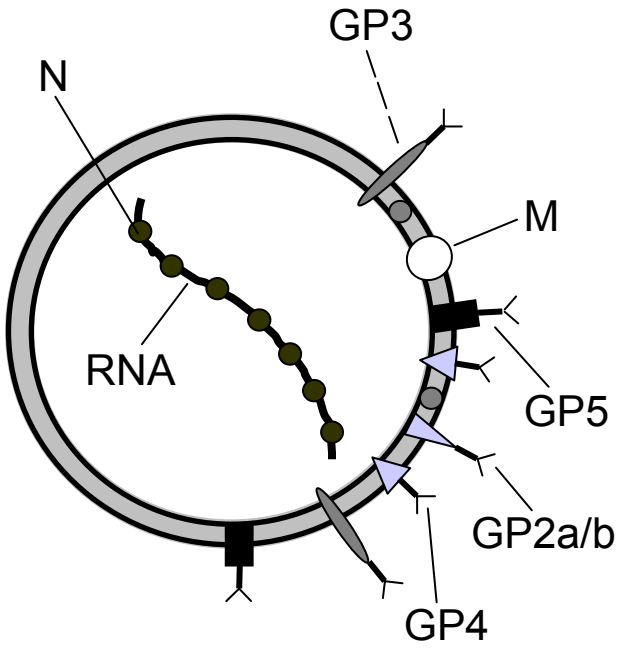


Fig. 1.1. Schematic presentation of the virus particle of PRRSV and the location of the structural proteins GP2-GP5, M and N.

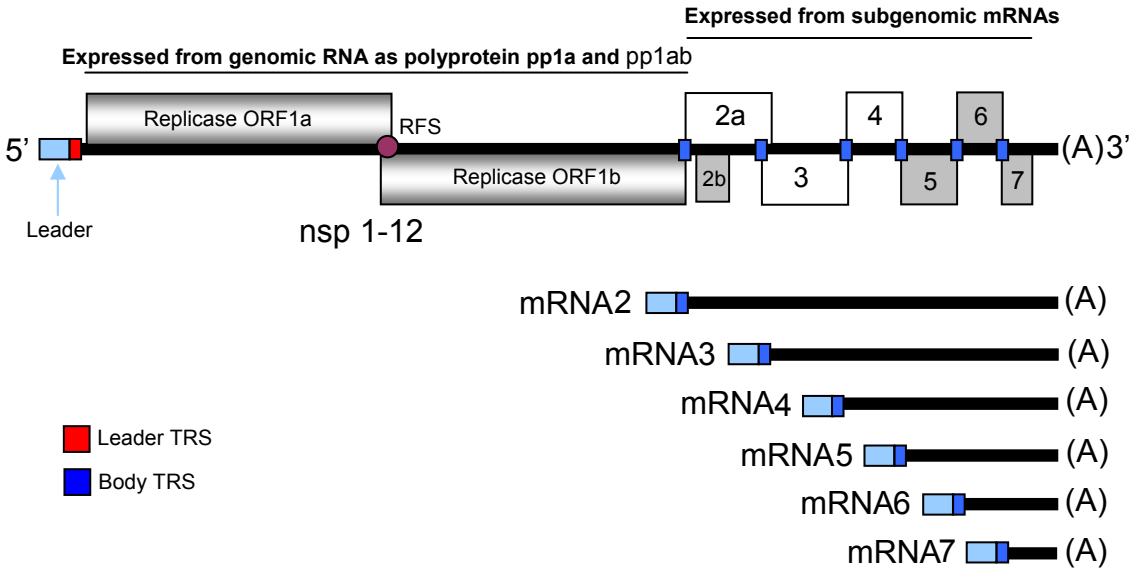


Fig. 1.2. Genome organization of PRRSV. The replicase gene, consisting of the open reading frame (ORFs) 1a and 1b, encodes a polyprotein that is cleaved, forming smaller protein products designated as nonstructural proteins (nsp 1-12). ORF1 is followed by ORFs 2 to 5 encoding glycoproteins GP2 to GP5 ; ORF6 encodes the membrane protein M ; and ORF7 encodes the nucleocapsid protein N. An internal ORF present within ORF2 encodes 2b. 3' nested set of subgenomic mRNAs synthesized during PRRSV replication. The 5' leader (thin blue box), derived from the genomic RNA and fused to the subgenomic RNAs, is shown. Leader TRS (Transcription regulating sequence; thick red box) ; Body TRS (thick blue box) ; RFS (Ribosomal frameshifting ; purple box).

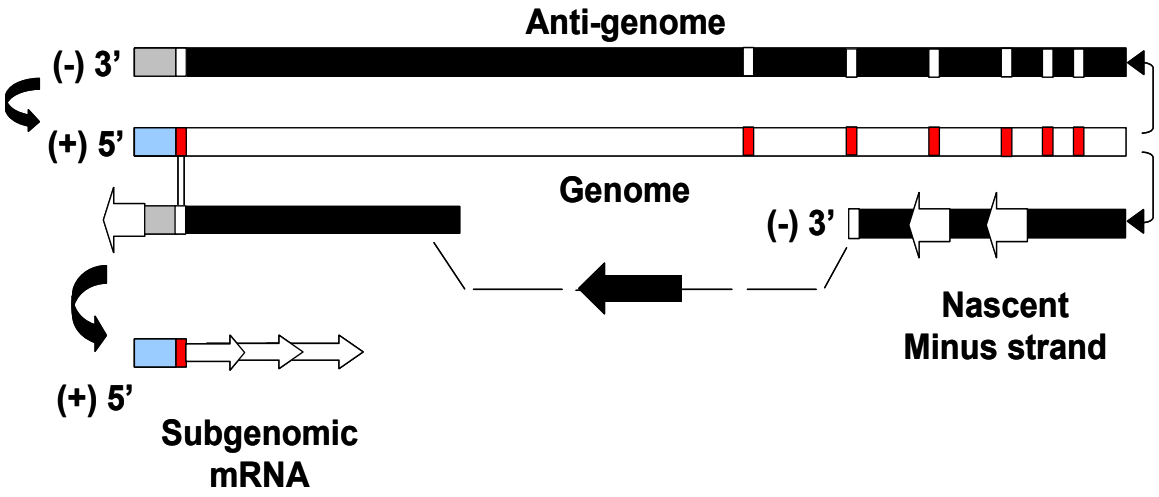


Fig. 1.3. Model for nidovirus subgenomic RNA synthesis by discontinuous extension of minus strands. Whereas, genome replication relies on continuous minus strand synthesis (antigenome), subgenomic minus strands are produced by atteunuation of nascent strand synthesis at a body TRS (red bar), followed by translocation of the nascent strand to the leader TRS in the genomic template. Following base-pairing between the body TRS complement at the 3' end of the minus and the leader TRS, RNA synthesis would resume to complete the subgenomic minus strand that would then serve as template for the transcription of subgenomic mRNAs.

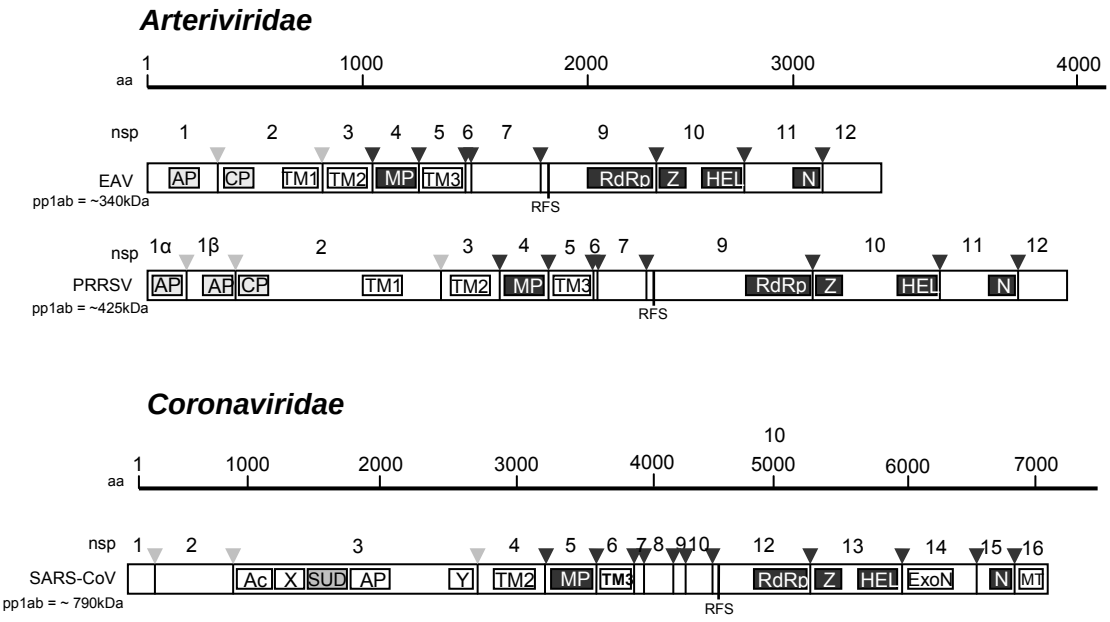


Fig. 1.4. Nidovirus replicase comparison. The replicase gene organization of the arteriviruses EAV and porcine reproductive and respiratory virus (PRRSV) and coronavirus SARS-CoV are depicted in the form of polyprotein pp1ab. The border between ORF1a- and ORF1b-encoded residues is indicated as RFS (ribosomal frameshift). Gray and black arrows represent the sites that are cleaved by accessory papain-like protease (AP) and the main (3C-like) protease (MP), respectively. The proteolytic cleavage products are numbered, and within the cleavage products, the location of the domain that have been identified as structurally or functionally related are indicated in black boxes. These include the four nidovirus-wide conserved domains encoded by ORF1b (RdRp, zinc-binding domain [Z], helicase [HEL], and NendoU [N]), putative transmembrane domain (TM), coronavirus-specific conserved domains (Ac, X, Y, Exo, and MT), and one SARS-CoV specific insertion (SUD). Note that the arterivirus and coronavirus polyprotein are drawn to different scales.

CHAPTER 2

The expression and stability of foreign tags inserted into nsp2 of porcine reproductive and respiratory syndrome virus (PRRSV)

ABSTRACT

The nonstructural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus (PRRSV) is the single largest protein produced during virus replication. The cDNA of the pCMV-129 infectious PRRSV clone was modified by creating unique Mlu I and SgrA I restrictions sites at nucleotide (nt) positions 3,219 and 3,614, respectively: both located within the C-terminal region of nsp2. cDNAs coding for oligo- and polypeptide tags, including FLAG, enhanced green fluorescent protein (EGFP) and firefly luciferase were inserted into the newly created restriction sites. The results showed that only the EGFP-containing genomes were properly expressed and produced virus. EGFP fluorescence, but not EGFP immunoreactivity, was lost during passage of recombinant EGFP viruses in culture. Sequencing of a fluorescence-negative EGFP virus showed that the EGFP remained intact, except for the appearance of mutations that may affect chromophore formation. The results from this study show that nsp2 can be a site for the expression of foreign proteins and creates the possibility of constructing marker viruses for the study of virus replication.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae,* is an economically important disease of swine. Other members of the arterivirus family include lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV : see review 22). *Arteriviridae* along with the *Coronaviridae*, and the recently established *Roniviridae* belong to a relatively new order, *Nidovirales* (8). Arterivirus reverse genetic systems have greatly improved the understanding of arterivirus RNA replication, subgenomic mRNA synthesis and the role of cis-acting elements required for RNA packaging (1, 2, 3, 26, 14, 16, 25). The inclusion of foreign genes also provides the opportunity to develop marker viruses, which can be used in studies of virus replication, gene function and pathogenesis. One approach for creating a marker virus is the insertion of cDNA into one of the structural genes. For example, the chloramphenicol acetyltransferase (CAT) gene was inserted into ORF2 of EAV, but without the production of infectious virus (26). A cDNA fragment encoding the influenza virus hemagglutinin (HA) was ligated into the 5' or 3' end of the nucleocapsid (N) gene, ORF7, of PRRSV (9). However, the synthesis of the resulting HA-N fusion protein resulted in nonviable progeny virus. Another approach is to create a cassette, containing a marker, with an upstream transcription regulating sequence (TRS). The result is the synthesis of an additional subgenomic mRNA encoding the gene of interest. This approach was used to express EGFP in EAV and PRRSV (5, 1, 2, 27, 12). In both cases, the recombinant arterivirus expressed EGFP fluorescence and replicated to the same levels as the parental virus.

The approach incorporated in this study was the insertion of a marker into the 12 kb region occupied by the non-structural proteins (nsp's). The nsp's of PRRSV are produced as the result

of the proteolytic processing of the pp1a and pp1b polyproteins, which are translated from ORF1a and ORF1ab, respectively. The complete processing of pp1a and pp1ab is predicted to yield 12 nsp polypeptides (15, 29, 30). The relatively large nsp2 region of North American PRRSV isolates occupies amino acids 383 to 1263 within pp1a (15). Based on studies of EAV, nsp2 is predicted to contain a cysteine protease domain in the N-terminal end, which is responsible for proteolytic cleavage of the nsp2/3 junction (15, 22, 28). The C-terminal end interacts with nsp3 and together these form a scaffolding complex that supports the formation of double membrane vesicles and replication complexes (21, 22, 23, 28). Genetic sequence comparisons of PRRSV isolates identified a hypervariable region in nsp2 that naturally incorporates both nucleotide insertions and deletions (20, 7). Based on these properties, we reasoned that nsp2 could support the insertion of foreign proteins.

MATERIALS AND METHODS

Cells.

MARC-145 cells and BHK-21 cells were maintained in Eagle's MEM with 7% FBS and supplemented 0.008% Fungizone and 0.01% penicillin-streptomycin. The MARC-145 cell line, a derivative of MA-104 cells (11), was used in those experiments involving PRRSV infection. Porcine alveolar macrophage (PAM) cells were obtained from the lungs of four to six week-old pigs by lung lavage with PBS. Cells were plated in RPMI with 10% FBS with antibiotics. After two days, cells were infected with virus. Virus yield was measured by end-point titration of virus-containing culture media on MARC-145 cells. Serial 10-fold dilutions of virus were placed on 96-well tissue culture plates containing MARC-145 cells. After 3 days, plates were fixed in 80% acetone then stained with SDOW-17. Results were reported as $TCID_{50}$/ml (19).

Introduction of Mlu I and SgrA I restrictions site into nsp2 of pCMV-S-129.

Insertion of unique restriction sites into nsp2 region of pCMV-S-P129 infectious clone was performed using the QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene). Sequences for forward and reverse primers used to insert the Mlu I and SgrA I sites are shown in Table 1. QuikChange II XL reactions were as recommended by the manufacturer and contained 150 picomoles of each primer and 10 ng of plasmid DNA as template. Reaction conditions included an initial denaturation step at 95˚C for 60 seconds followed by 18 cycles of 95˚C (50 sec), 60˚C annealing (50 sec), and 68˚C extension (20 sec). The reaction was concluded with a 7-minute incubation at 68˚C. The insertion of the unique restrictions sites was performed in two steps and shown in Fig. 1. First, the Mlu I site was created at position 3,219 by performing site-directed mutagenesis on the full-length infectious clone. The changing of only two nucleotides allowed the introduction of a Mlu I site without altering the encoded amino acid sequence. The plasmid was named pCMV-S-129-Mlu. To create the SgrA I site, the infectious clone was cut with Mlu I and Pme I and subcloned into a shuttle, pCR-XL-3201-7700. A naturally occurring Pme I site is located at nt position 7,701, within nsp8. Site-directed mutagenesis was performed on the shuttle vector to create the SgrA I site at nt position 3,614. The shuttle vector was cut with Mlu I and Pme I and ligated into the Mlu I and Pme I sites in the full-length cDNA clone to create the plasmid pCMV-S-129-Mlu-SgrAI.

Transfection of MARC-145 and BHK-21 cells on twelve well plates was performed with 2 ug of plasmid DNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations.

Antibody detection of FLAG, EGFP and PRRSV nucleocapsid (N) protein

Cells grown on plastic plates were fixed in 80% acetone for 10 min and then air-dried. All antibodies were diluted in PBS with 5% FBS (PBS-FBS). Except where indicated, all incubations were performed at 37^{o} C for one hr followed by extensive washing with PBS. For detection of the FLAG epitope tag, cells were stained for two hr with anti-FLAG antibody (Molecular Probes) diluted 1:200. Cells were incubated with a 1:500 dilution of AlexaFluor 584-labeled anti-rabbit antibody (Molecular Probes) and visualized under a fluorescence microscope. The immunological detection of the EGFP protein was performed by staining cells for one hr with a 1:200 dilution of goat anti-GFP antibody (Rockland), followed by staining with peroxidase-labeled rabbit anti-goat antibody (Sigma-Aldrich) diluted 1:1,000 in PBS-FBS and incubated for one hr. Peroxidase activity was detected using a DAB substrate system (Amresco). PRRSV nucleocapsid (N) protein antigen was detected using the anti-N protein mAb antibody, SDOW-17 (a kind gift of Eric Nelson, South Dakota State University). Cells were incubated with SDOW-17 for one hour followed by staining with AlexaFluor 594-labled goat anti-mouse IgG antibody. AlexaFluor 594 was detected with an A540 cutoff fluorescence filter.

Confocal microscopy

Glass coverslips of pCMV-Mlu-EGFP-SgrA infected MARC-145 cells were washed with PBS, fixed for 10 minutes with 2% paraformaldehyde in PBS. Cells were counterstained with the nuclear stain, TO-PRO-3. Coverslips were mounted and viewed under a Zeiss LSM 510 confocal microscope.

RT-PCR for the detection of the EGFP insert

The presence of the EGFP insert in nsp2 was detected by reverse transcription-polymerase chain reaction (RT-PCR) amplification of total RNA from media of virus-infected cells. Total RNA was isolated from cells using a TRIzol, (Invitrogen). The primers chosen for RT-PCR amplification flanked the EGFP insert. The sequence of the 2941F and 3421R forward and reverse primers were 5'-AGCCAATTTTTGTGTCTGCACTGCAACACAAATTT-3' and 5'–CCCAAACCTGCCATCTAAGGTGCGAAACGCCTGGT-3', respectively.

Viral RNA was reverse transcribed using SuperScript III (Invitrogen) and the 3421R primer. Amplification was performed using TaKaRa Ex Taq^{TM} according to the manufacture's recommendations. Reaction conditions included an initial denaturation step of 95˚C for 60 seconds followed by 30 cycles of 94˚C (35 sec), 58˚C annealing (35 sec), and 72˚C extension (1 min). The reaction was concluded with a 10-minute incubation at 72˚C. PCR products were separated on an agarose gel and stained with ethidium bromide. PCR products were cloned into pCR2.1 TOPO vector (Invitrogen) according manufacturer's instruction, expressed in E. coli, and sequenced in both directions using M13 forward and reverse primers, which were located in the pCR2.1 vector.

RESULTS

Expression properties of oligo- and polypeptide tags in nsp2 of pCMV-S-P129.

Site-directed mutagenesis was used to introduce unique Mlu I and SgrA I restriction enzyme sites at nucleotide positions 3,219 and 3,614 of pCMV-S-129, a DNA-launched PRRSV infectious clone (12). The creation of the sites required changing only two nucleotides for the

introduction of the Mlu I site and only a single nucleotide change for the creation of the SgrA I site (see Table 1). Both sites were created without altering the encoded amino acid sequence. The restriction sites are located within the C-terminal region of nsp2 and are distant from the N-terminal histidine and cysteine residues involved in nsp2 cysteine protease function, and are also distant from the conserved C-terminal end.

Nsp2 possesses considerable nucleotide and peptide sequence hypervariability, including the capacity to support significant insertions and deletions (20, 6, 18, 7). To better understand peptide sequence hypervariability as it related to the location of the Mlu I and SgrA I sites, the nsp2 peptide sequences from 14 PRRSV isolates, obtained from GenBank and our lab, were aligned using CLUSTALX (24). The resulting histogram, presented in Fig. 2.2B, shows the number of substitutions for each 10 amino acids versus the amino acid position in nsp2. The results confirmed that the peptide sequence in C-terminal region of nsp2 is highly variable. However, hypervariability was not uniform across the region but contained two hypervariable regions, we call HV-1 and HV-2, which flank a more conserved region. The site chosen for the insertion of the Mlu I site lies within this island of relatively conserved amino acids. In contrast, the SgrA I site is located within a highly variable domain, we call HV-2. It is interesting to note that the SgrA I site is in the same vicinity as a naturally occurring insertion of 250 amino acids in the PrimePak PRRSV isolate (20).

The investigation into the expression of foreign cDNA tags in nsp2 incorporated the cloning of three different-sized cDNAs into the Mlu I site of pCMV-S-P129-Mlu (Fig. 2.2A). The first insertion was a 24 bp cDNA encoding the 8 amino acid FLAG oligopeptide, DYKDDDDK (pCMV-S-P129-Mlu-FLAG). The second was the insertion of a 798 bp cDNA, which coded for the enhanced green fluorescent protein (EGFP; pCMV-S-P129-Mlu-EGFP). And finally, we

chose a 1650 bp cDNA coding for firefly luciferase (pCMV-S-P129-Mlu-LUC). PCR amplification, incorporating primers with Mlu I restriction sites on the 5' and 3' ends of each cDNA were used to clone each tag into the Mlu I site of the infectious clone. The correct orientation and sequence of each inserted fragment was confirmed by DNA sequencing incorporating primers that flanked the Mlu I site of the infectious clone. Plasmids were transfected into baby hamster kidney (BHK-21) and MARC-145 cells. The use of BHK-21 cells was based on earlier observations showing increased transfection efficiency of BHK cells (14, 16). When checked at 24 and 72 hr after transfection with pCMV-S-P129-Mlu-FLAG, both cell lines were negative for anti-FLAG staining. However, media from cells at 72 hr after transfection contained viable virus, as demonstrated by the presence of positive staining with the anti-nucleocapsid antibody at two days after infection of MARC-145 cells (data not shown).

The transfection of MARC-145 cells with pCMV-S-P129-Mlu-EGFP resulted in the appearance of single cells with green fluorescence, which first became visible at about 12 hr after transfection. By three days, the individual cells were replaced with clusters of fluorescent cells indicating that virus had spread to adjacent cells. An example of a fluorescent focus at four days after transfection of MARC-124 cells is shown in Fig. 3A. The transfection of BHK-21 cells with the pCMV-S-P129-Mlu-EGFP produced single fluorescent cells at 24 hr, which eventually disappeared without the formation of foci. This result is consistent with the non-permissiveness of BHK-21 cell for PRRSV infection. The P129-MLU-EGFP virus obtained at four days after transfection of MARC-145 cells with pCMV-S-P129-Mlu-EGFP was used to infect PAMs. Within 24 hr, EGFP expression was clearly visible (see photomicrograph in Fig. 2.3B), indicating that the P129-MLU-EGFP virus was capable of infecting porcine macrophages. Two additional EGFP-expressing constructs were made. The first incorporated the insertion of EGFP

cDNA into the SgrA I site, to produce the virus P129-SGR-EGFP (see Fig. 2.2A). The second plasmid, pCMV-S-P129-Mlu-EGFP-Sgr, was made by adding Mlu I and SgrA I sites to the 5' and 3' ends of EGFP, respectively, and then ligating the EGFP cDNA into the Mlu I and SgrA I sites of the infectious clone. The resulting virus, P129-MLU-EGFP-SGR contained a replacement of the 395nt Mlu I- SgrA I fragment with the 798 nt EGFP (see Fig. 2.2A). The two new EGFP constructs possessed the same properties as the P129-MLU-EGFP virus, including the formation of fluorescent foci cells and infection of porcine alveolar macrophage (PAM) (data not shown).

During arterivirus replication, nsp2 is predominately perinuclear where it associates with double membrane vesicles (DMVs), which contain the replication complexes (31, 32). MARC-145 cells infected with P129-MLU-EGFP-SGRA were fixed at 24 hr after infection and counterstained with the nuclear stain TO-PRO-3. As shown in Fig. 2.4, EGFP was found in the cytoplasm with an accumulation of fluorescence around the nucleus. This result indicates that the localization of the nsp2-EGFP fusion protein is consistent with nsp2.

Luciferase activity was measured in MARC-145 cells after transfection with pCMV-S-P129-Mlu-LUC. For the purpose of comparison, the plasmids, pISRE-Luc and pCMV-S-P129, were included as positive and negative controls, respectively. The level of luciferase activity at 24 hr after transfection of MARC-145 cells with pCMV-S-P129-Mlu-Luc was equivalent to the level obtained from cells transfected with the positive control (Fig. 2.3C); whereas, cells after transfection with the pCMV-S-P129 remained negative for luciferase activity. Even though the pCMV-S-P129-Mlu-Luc cells were positive for luciferase, PRRSV N protein antigen was not detected, and media obtained at 4 days after transfection was negative for the presence of virus.

These results indicate that luciferase activity was present, but without the production of infectious virus.

Replication properties of recombinant viruses

Yields of the recombinant viruses obtained four days after the transfection of MARC-145 cells were measured using MARC-145 and PAM cells. The results, presented in Fig. 2.5, showed that all recombinant viruses, except for P129-LUC, replicated in MARC-145 cells and PAMs. The yields of recombinant EGFP and FLAG viruses was similar to the P-129 parent virus. Virus yields for the P129-MLU-EGFP and P129-SGR-EGFP viruses were reduced by 1 to 2 logs, but recovered to P129 levels after a single passage on MARC-145 cells. These results demonstrate that the insertion of EGFP into the Mlu I or SgrA I sites does not affect the in vitro replication properties of the P129 virus. The replacement of 132 amino acids in nsp2 with EGFP, in the virus P129-MLU-EGFP-SGR, also had no significant effect on virus replication.

Stability of EGFP expression

To study the stability of EGFP expression by recombinant PRRS viruses, fluorescence was followed during serial passage of P129-MLU-EGFP and P129-MLU-EGFP-SGR viruses on MARC-145 cells. Viral passage was initiated by harvesting media at three days after transfection with plasmid and placing a 1:100 dilution of culture supernatant on a fresh monolayer of MARC-145 cells. The second passage was performed in a similar manner using media from infected cells. After a total of five passages of the P129-MLU-EGFP virus on MARC-145 cells, EGFP fluorescence had disappeared (Fig.2. 6A). The presence of virus was confirmed by positive staining for the N protein (Fig.2. 6B). Even though EGFP fluorescence

was absent, infected cells still expressed the EGFP polypeptide, which was evident by positive staining with a polyclonal anti-GFP antibody (Fig. 2.6C). Since the DAB chromogenic substrate used to detect the peroxidase activity in anti-GFP stained cells destroyed EGFP fluorescence, anti-GFP staining was performed on a duplicate well of infected cells. In contrast to the P129-MLU-EGFP, the P129-MLU-EGFP-SGR virus produced fluorescent foci after five passages (see Fig. 2.6D and E). However, there were several nucleocapsid-positive cells that lacked EGFP fluorescence suggesting that some viruses within the population had become fluorescence negative (see arrow in Fig. 2.6E). As expected, the P129-MLU-EGFP-SGR foci reacted with the anti-GFP antibody (Fig. 2.6F). The results indicate that EGFP fluorescence, but not EGFP immunoreactivity, is lost during serial passage of EGFP viruses. However, the replacement of the 132 amino acid region between Mlu I and SrgA I, with EGFP was able to improve the stability of fluorescence expression (set up the duplication)

RT-PCR products containing the EGFP region were generated by amplifying the region flanking the Mlu I sites in P129-MLU-EGFP. The results, presented in Fig. 2.7A, showed that the predicted 1378 bp product, was obtained for each passage, indicating that the EGFP insert remained intact. To better understand the mechanism for loss of fluorescence, we sequenced the EGFP PCR product from the P5 virus. There were only two point mutations in EGFP: both resulted in a change in amino acid. The first was a change from arginine to cysteine at position 96 and the second, a change from asparagine to tyrosine at position 106 (See Fig. 2.7B). There were no mutations in the region of the cDNA product flanking the EGFP insert.

DISCUSSION

The insertion of foreign oligo- and polypeptide sequences into nsp2 of PRRSV provides the opportunity to develop marker viruses for use in studies of virus replication and nsp2 function. The choice of nsp2 as a site for foreign protein insertion was based on its large size and natural capacity to support the insertion of polypeptides. The incorporation of fluorescent tags, such as EGFP, provides the mean to follow replication in live cells over time. Since the level of fluorescence in a culture well provides a semi-quantitative measurement of virus replication, EGFP-labeled viruses can be incorporated into high throughput screening methods for the identification of antiviral compounds.

This study identified some potential limitations in the types of tags suitable for insertion into the Mlu I and SgrA I restriction sites chosen in this study. One cDNA fragment, encoding the FLAG peptide tag, DYKDDDDK, inserted into the Mlu I site was not detected in transfected cells but resulted in the release of viable virus into the media (Fig. 2.5). To determine if the absence of antibody recognition was unique to the FLAG tag, we constructed a similar plasmid incorporating the HA sequence, YPYDVPDY. Again, viable virus was generated, but with the same negative antibody staining result (data not shown). The absence of detection of small oligopeptide tags could be related to the location of the tag within nsp2. For optimal detection, small oligopeptide tags are typically placed on the N- or C-terminus of a protein, which allows for easy access for binding an anti-tag antibody. In this study, the peptide tags were embedded within nsp2, with large polypeptides regions flanking the insertion site. The folded protein may bury the tag, making it inaccessible to antibody. We also investigated the properties of a relatively large luciferase cDNA inserted into the Mlu I site. Luciferase activity was readily detected after transfection of the pCMV-129-S-Mlu-Luc plasmid (Fig. 2.3C), but without the production of replication competent virus or the synthesis of nucleocapsid protein. Nsp2 is

essential for the synthesis of genome, anti-genome and subgenomic mRNAs. Therefore, the absence of nucleocapsid antigen expression after transfection with the pCMV-S-P129-Luc plasmid suggests that the ORF7 subgenomic mRNA was not being properly synthesized. The likely effect of the insertion of luciferase is the inhibition of nsp2 function. Since PRRSV is a positive stranded RNA virus, the source of the nsp2-luciferase-containing transcript is from the full-length genomic RNA driven by the CMV promoter on the pCMV-S-129-Mlu-Luc plasmid.

BHK-21 and MARC-145 cells transfected with pCMV-S-P129-Mlu- EGFP produced both green fluorescent cells and viral progeny. PAM cells supported the replication of the MARC 145 cell-derived EGFP viruses (Fig. 2.3B). Cells infected with EGFP recombinant viruses expressed EGFP fluorescence to relatively high levels. The distribution of EGFP fluorescence was predominately cytoplasmic and perinuclear (Fig.2. 4), consistent with the localization properties of arterivirus nsp2 (32). After five passages of the P129-MLU-EGFP virus, fluorescence had disappeared. However, viral foci stained positive with an anti-GFP polyclonal antibody, indicating that the EGFP was still immunologcally intact (see Fig. 2.6). Fluorescence was maintained longer during the serial passage of P129-MLU-EGFP-SGR, but fluorescence was eventually lost after further passaging on MARC-145 cells.

RT-PCR, incorporating primers that flanked the EGFP insert of P129-MLU-EGFP, showed that the size of the insert remained intact over eight passages (Fig. 2.7A). DNA sequencing of EGFP and the flanking regions of a virus after five passages showed two mutations in EGFP, an Arg to Cys change at position 96 and a Asn to Tyr change at position 106. The chromophore of EGFP is formed by Thr-65, Tyr-66 and Gly-67, which are embedded within a barrel strucuture formed by 11 anti-parallel beta sheets (4, 17). After translation, the nascent EGFP polypeptide is non-fluorescent. Fluorescence becomes active after the formation of an imidazolidinone ring by

Tyr-66 and Gly-67 followed by the auto-oxidation of Tyr-66. Mutations in the chromophore, nearby amino acid residues, or amino acids residues involved in the formation of the beta-barrel can inactivate fluorescence (13). The tertiary structure of GFP, derived from X-ray crystallographic studies (17), shows that Arg-96 forms a hydrogen bond with the carbonyl group of the imidazolidinone ring. Arg-96 is likely to participate in the formation or stabilization of the Tyr-66/Gly-67 ring structure (17). Therefore, a change from Arg-96 to Cys-106 may prevent fluorescence by blocking the formation of the imidazolidinone ring. Additional support for mutations that affect chromophore formation comes from the nucleotide sequence analysis of three fluorescence-negative isolates from pigs infected with P129-MLU-EGFP-SGR. One isolate possessed a Tyr to Ser change at position 66, within the tri-peptide chromophore. The second isolate possessed a Tyr to His change at position 66 along with the Arg to Cys mutation at position 96. The third possessed a deletion that covered the chromophore region.

Even though it appears that nsp2 can easily tolerate the insertion of EGFP, the accumulation of point mutations in amino residues that affect chromophore formation suggests that the chromophore may affect nsp2 function. EGFP is a commonly used tag in virology and cell biology. The possibility that chromophore formation can affect the function of the fusion partner is a unique observation. The mechanism for how chromophore formation might affect nsp2 function remains to be determined.

REFERENCES

1. **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 6,500,662.
2. **Calvert, J.G., Sheppard, M.G., Welch, S.K.W.,** 2003. Infectious cDNA clone of North American porcine reproductive and respiratory syndrome (PRRS) virus and uses thereof. US Patent Application 20030157689.
3. **Choi, Y.J., Yun, S.I., Kang, S.Y., Lee, Y.M.,** 2006. Identification of 5' and 3' cis-acting elements of the porcine reproductive and respiratory syndrome virus: Acquisition of novel 5' AU-Rich sequences restored replication of a 5'-proximal 7-nucleotide deletion mutant. J. Virol. 80, 723-736.
4. **Cormack, B.P., Valdivia, R., Falkow, S.,** 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene. 173, 33-38.
5. **De Vries, A.A., Glaser, A.L., Raamsman, M.J., Rottier, P.J.,** 2001. Recombinant equine arteritis virus as an expression vector. Virology 284, 259-276.
6. **Fang, Y., Kim, D.Y., Ropp, S., Steen, P., Christopher-Hennings, J., Nelson, E.A., Rowland, R.R.R.,** 2004. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus. Res. 100, 229-235
7. **Fang, Y., Schneider, P., Zhang, W., Faaberg, K.S., Nelson, E.A., Rowland, R.R.R.,** 2007. Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: Analysis of isolates collected between 1999 and 2004. Arch. Virol. (In press)
8. **Gonzalez, J.M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A.E., Enjuanes,**

L.A., 2003. Comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Arch. Virol. 148, 2207-2235.

9. **Groot Bramel-Verheije, M.H., Rottier, P.J., Meulenberg, J.J.,** 2000. Expression of a foreign epitope by porcine reproductive and respiratory syndrome virus. Virology 278, 380-389.
10. **Heim, R., Prasher, D.C., Tsien, R.Y.,** 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. U. S. A. 91, 12501-12504.
11. **Kim, H.S., Kwang, J., Yoon, K.J., Joo, H.S., Frey, M.L.,** 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. 133, 477-483.
12. **Lee, C., Calvert, J.G., Welch, S.K., Yoo, D.,** 2005. A DNA-launched reverse genetics system for porcine reproductive and respiratory syndrome virus reveals that homodimerization of the nucleocapsid protein is essential for virus infectivity. Virology. 331, 47-62.
13. **Li, X., Zhang, G., Ngo, N., Zhao, X., Kain, S.R., Huang, C.C.,** 1997. Deletions of Aequorea victoria green fluorescence protein define the minimal domain required for fluorescence. J. Biol.Chem. 272, 228545–28549.
14. **Meulenberg, J.J., Bos-de Ruijter, J.N., van de Graaf, R., Wensvoort, G., Moormann, R.J.,** 1998. Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. J. Virol. 72, 380-387.
15. **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, 270-280.

16. **Nielsen, H.S., Liu, G., Nielsen, J., Oleksiewicz, M.B., Botner, A., Storgaard, T., Faaberg, K.S.,** 2003. Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. J. Virol. 77, 3702-3711.

17. **Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., Remington, S. J.,** 1996. Crystal structure of the Aequorea victoria green fluorescent protein. Science. 273, 1392–1395

18. **Ropp, S.L., Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg, K.S.,** 2004. Characterization of emerging European-like PRRSV isolates in the United States. J. Virol. 78, 3684-3703

19. **Rowland, R.R.R., Robinson, B., Stefanick, J., Guanghua, L., Lawson, S.R., Benfield, D.A.,** 2001. Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma and recovery of virus replication with 2-aminopurine. Arch. Virol. 146, 539-555.

20. **Shen, S., Kwang, J., Liu, W., Liu, D.X.,** 2000. Determination of the complete nucleotide sequence of a vaccine strain of porcine reproductive and respiratory syndrome virus and identification of the Nsp2 gene with a unique insertion. Arch. Virol. 145, 871-883.

21. **Snijder, E.J., Wassenaar, A.L., Spaan, W.J.,** 1994. Proteolytic processing of the replicase ORF1a protein of equine arteritis virus. J. Virol. 68, 5755-5764.

22. **Snijder, E.J., Meulenberg, J.J.,** 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961-979.

23. **Snijder, E.J., van Tol, H., Roos, N., Pedersen, K.W.,** 2001. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. J. Gen. Virol. 82, 985-994.

24. **Thompson, J. D., T. J. Gibson, T.J., Jeanmoughin, F. Higgins, D.G.,** 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

25. **Truong, H.M., Lu, Z., Kutish, G.F., Galeota, J., Osorio, F.A., Pattnaik, A.K.,** 2004. A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology 325, 308-319.

26. **van Dinten, L.C., den Boon, J.A., Wassenaar, A.L., Spaan, W.J., Snijder, E.J.,** 1996. An infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. Proc. Natl. Acad. Sci. U.S.A. 94, 991-996.

27. **Yoo, D., Welch, S.K., Lee, C., Calvert, J.G.,** 2004. Infectious cDNA clones of porcine reproductive and respiratory syndrome virus and their potential as vaccine vectors, Vet. Immunol. Immunopathol. 102, 143-154.

28. **Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E.,** 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81, 853-879.

29. **Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A.,** 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80, 307-315.

30. **Wootton, S., Yoo, D., Rogan, D.,** 2000. Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. Arch. Virol. 145, 2297-2323.

31**. Molenkamp R, van Tol H, Rozier BC, van der Meer Y, Spaan WJ, Snijder EJ.,** The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription. J. Gen. Virol. 81, 2491-2496.

32. **Pedersen, K.W., van der Meer, Y., Roos, N., Snijder, E.J.,** 1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. J. Virol. 73, 2016-2026.

Table 2.1- Primers used for introducing Mlu I and SgrA I sites in nsp2 of pCMV-S-129*

3219 Mlu I	
Forward	5'CTGTCAAGTGTTAGGATCAC**G**CG**T**CCAAAATACTCAGCTC
Reverse	5' GAGCTGAGTATTTTGG**A**CG**C**GTGATCCTAACACTTGACAG
SgrA I (3597-3637)	
Forward	5'CTCGGGAAAATAGAAAA**C**ACCGGTGAGATGATCAACCAGGG
Reverse	5'CCCTGGTTGATCATCT**C**ACCGGT**G**TTTTCTATTTTCCCGAG

*Restriction sites are underlined. Mutations needed to create the restriction sites are in bold.

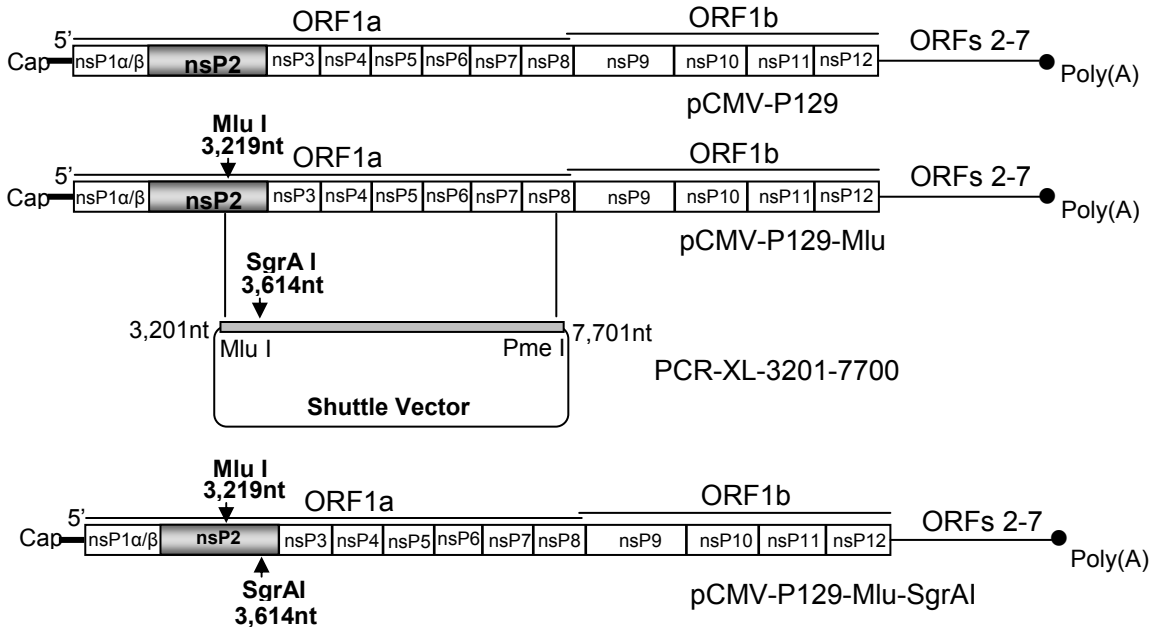


FIG. 2.1. Insertion of unique Mlu I and SgrA I cloning sites into nsp2 of the pCMV-S-129 infectious cDNA clone. The Mlu I site was introduced at genome position 3,219 of the pCMV-S-P129. The SgrA I site was inserted after subcloning the Mlu I-Pme I fragment to produce the shuttle vector pCR-XL-3201-7700 followed by the insertion of the SgrA I site. The Mlu I-Pme I fragment was then ligated into pCMV-S-129 cDNA.

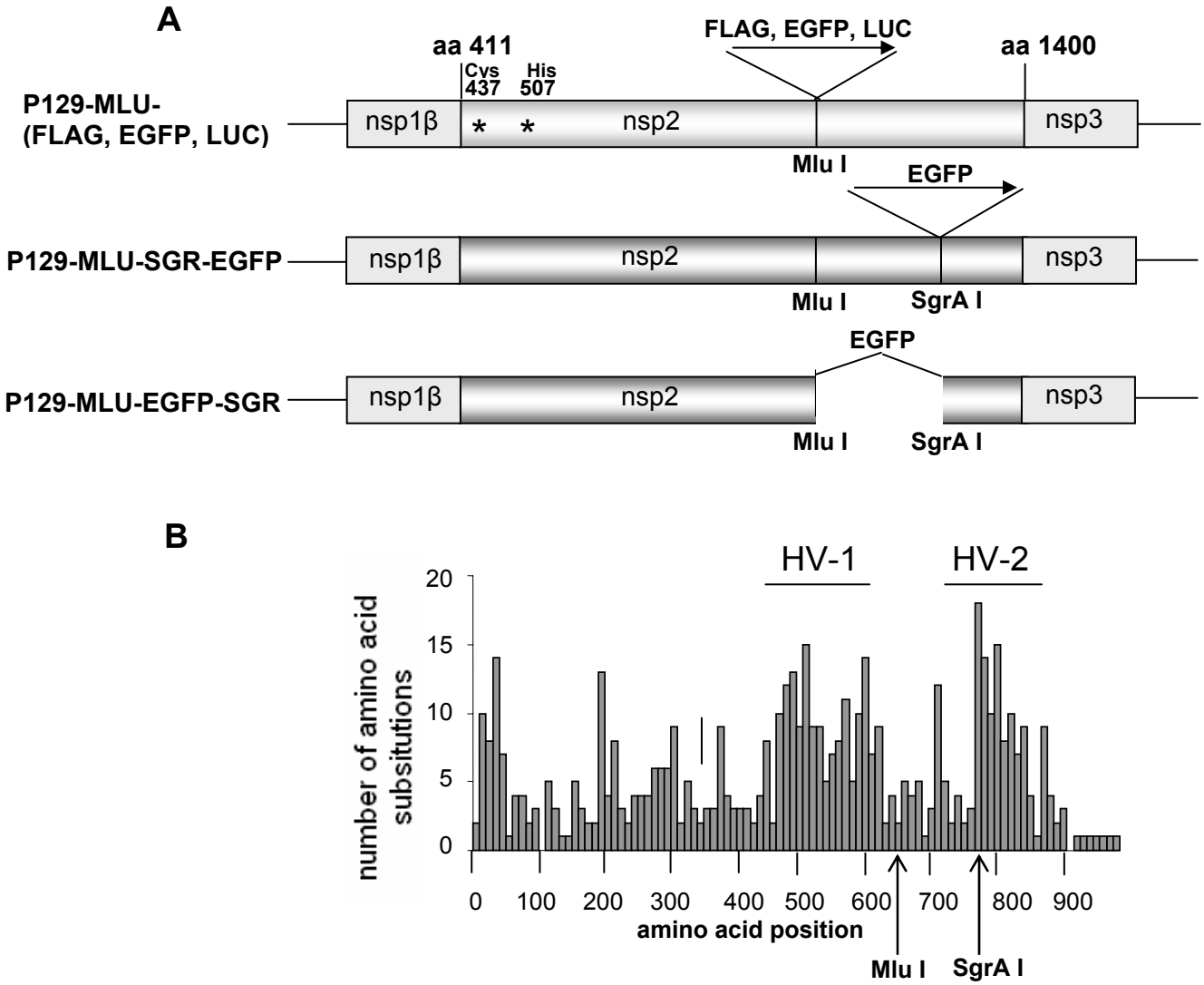


Fig. 2.2 Recombinant viruses used in this study. (A) Location of olig-peptide and polypeptide tags in nsp2. Asterisks identify the location of histidine and cyteine residues which are conserved among the arteriviruses and form part of the protease active site. (B) Location of Mlu I and Sgr A I sites within the conserved and hypervariable regions in nsp2. Nsp2 peptide sequences from 14 PRRSV isolates were aligned using Clustal-X. The bar graph shows the number of amino acid substitutions along the nsp2 peptide sequence. Each bar represents 10 amino acids. Location

chosen for the insertion of the Mlu I and SgrA I sites are identified by arrows. Two hypervariable domains are identified as HV-1 and HV-2. The nsp2 sequences were obtained from GenBank as well as our lab isolate, SD23983. The viruses and GenBank accession numbers used to create the graph were VR-2332(AY150564), SP(AF184212), 01NP1.2(DQ056373), BJ-4(AF331831), PL97-1(AY582141), CH-1a(AY032626), HB-2(sh)/2002(AY262352), JA142(AY424271), NVSL(AY545985), P129(AF494042), HN1(AY457635), PA8(AF176348), RespPRRSV (AF066183).

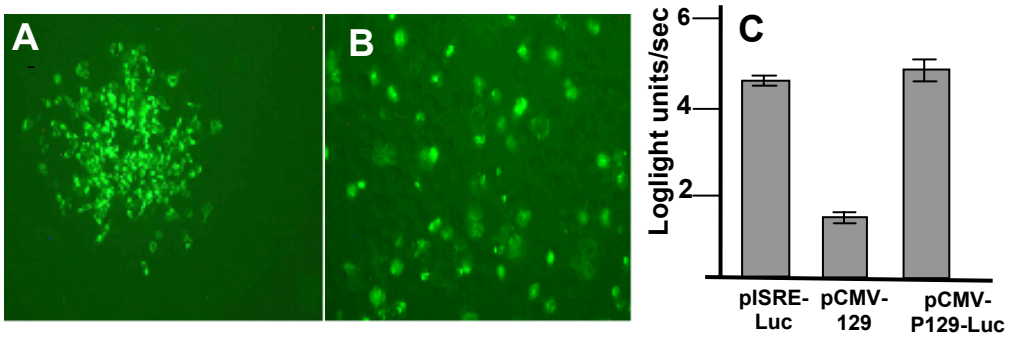


Fig.2.3. Properties of EGFP and luciferase recombinant viruses. (A) The formation of a single focus of EGFP fluorescing cells in a monolayer of MARC-145 cells. The photomicrograph was taken at four days after transfection with pCMV-S-P129-Mlu-EGFP. (B) EGFP fluorescence in PAM cells infected with virus derived from pCMV-S-P129-Mlu-EGFP-transfected MARC-145 cells. Photomicrograph was taken at one day after infection with an m.o.i. of approximately 10 $TCID_{50}$/cell. (C) Luciferase activity in MARC-145 cells transfected with pCMV-S-P129-Mlu-Luc. pISRE-Luc and pCMV-S-P129 were used as positive and negative controls, respectively. Measurements were made at 24 hr after transfection. Cell lysates were analyzed for luciferase activity using the Luciferase Assay System (Promega) and a Lumax luminometer (Molecular Technologies). Values are presented as the mean values of two independent experiments.

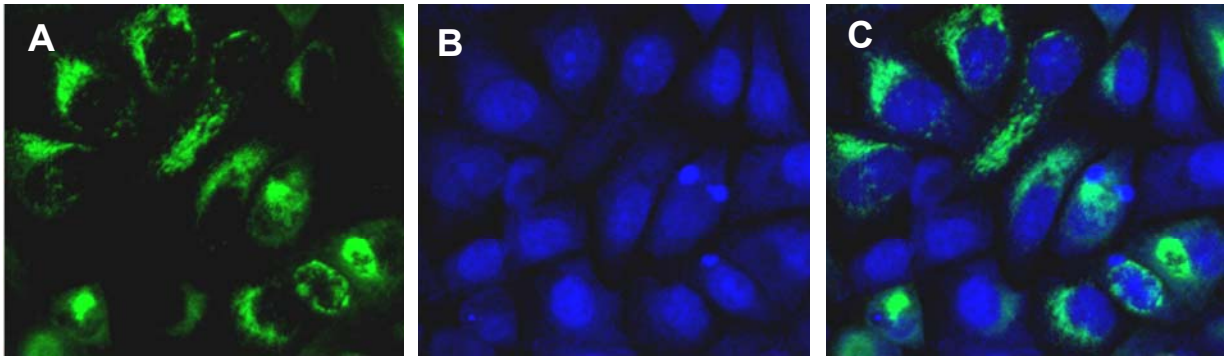


Fig. 2.4. EGFP localization in MARC-145 cells infected P129-MLU-EGFP-SGR. Confocal microscope images showing the localization of EGFP (green) and TO-PRO-3 nuclear staining (blue) in MARC-145 cells at 24 hr after infection. Panel C is the merged image.

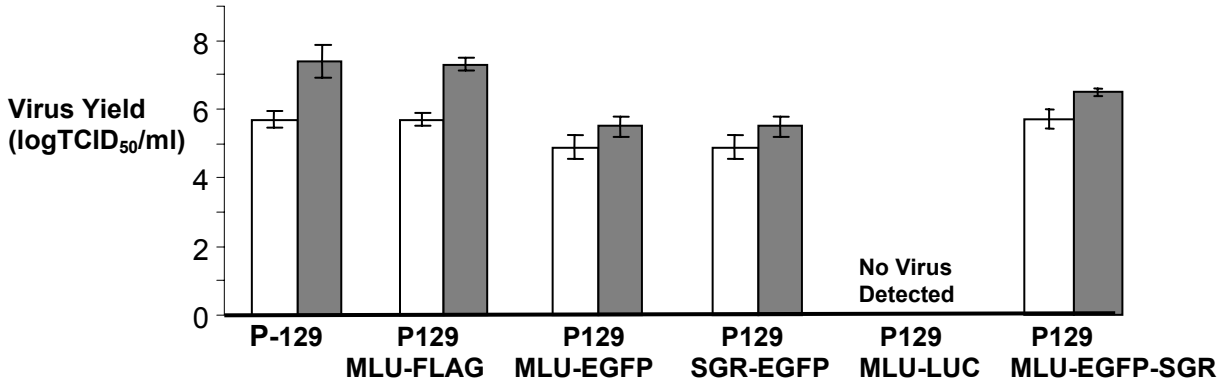


Fig. 2.5. Viral yields of recombinant plasmids. (A) Virus yields from cells transfected with pCMV-S-P129 plasmids. Media was harvested from MARC-145 cells on a 12 well plate at 4 days after transfection with 2 ug of plasmid DNA. The amount of virus recovered was measured by titration on MARC-145 cells (open bars) and on PAMs (closed bars).

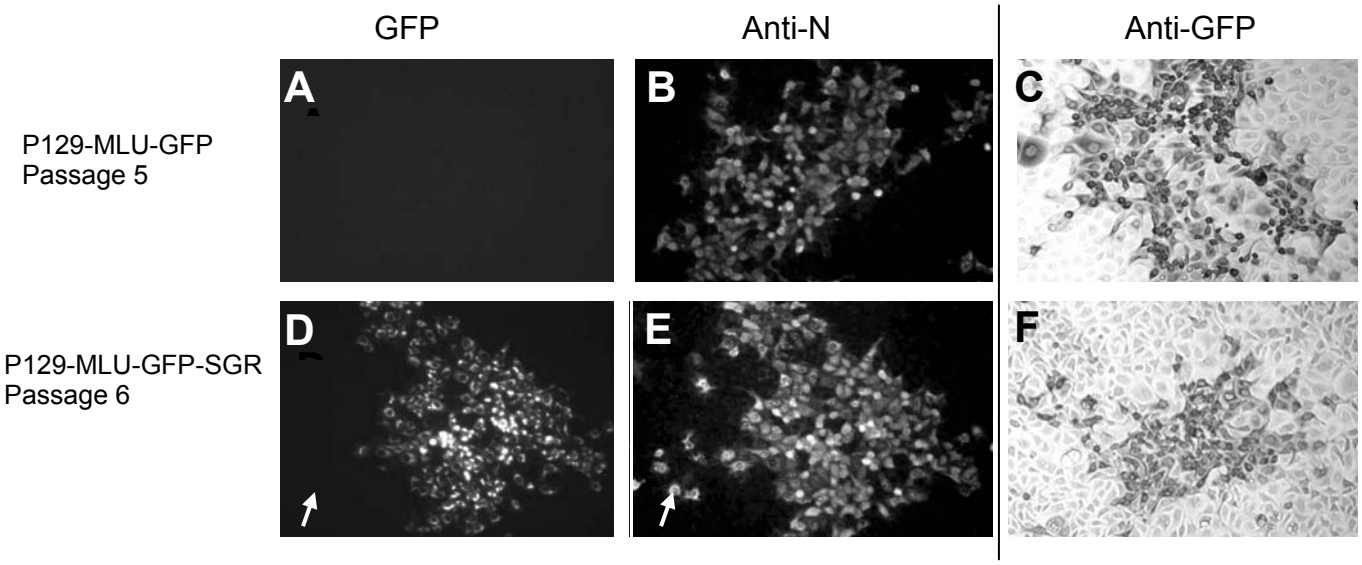
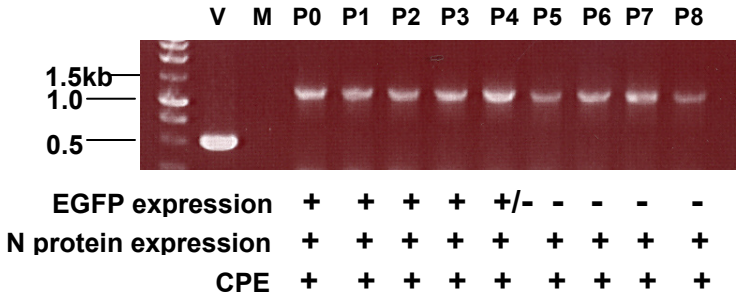


Fig. 2.6. Stability of EGFP fluorescence and immunoreactivity. (A) EGFP fluorescence and (B) anti-nucleocapsid staining in the same viral focus in cells infected with Fig. 3. Intracellular distribution of nsp2-EGFP. Confocal microscopic images showing co-localization of nsp2-EGFP with PRRSV nucleocapsid protein. (A) EGFP expression in MARC-145 cells infected with P129-MLU-EGFP. (B) The same focus stained with Alexifluor-labeled anti-nucleocapsid antibody. (C) A different focus on a duplicate well stained with peroxidase anti-GFP antibody. (D) EGFP fluorescence in MARC-145 cells infected with passage 6 of P129-MLU-EGFP-SGR. (E) The same focus stained with AlexiFluor-labeled anti-nucleocapsid antibody. (F) A different focus on a duplicate well stained with peroxidase anti-GFP antibody. Photomicrographs were obtained at three days after infection with each recombinant virus.

A



B

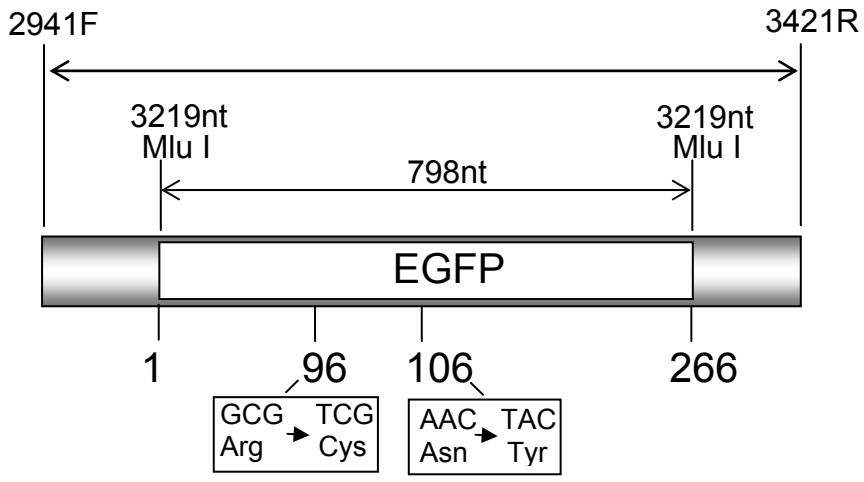


Fig. 2.7. Molecular analysis of EGFP during serial passage of P129-MLU-GFP. EGFP expression was determined by the presence (+) or absence (-) of EGFP fluorescent cells. The presence of infection was determined by staining cells with anti-nucleocapsid mAb. Samples included media from transfected MARC-145 cells (P0) through the eighth passage (P8). The location of primers used for PCR are shown in (B). The location of mutations in EGFP of a virus after five passages in MARC-145 cells is shown in (B).

CHAPTER 3

Nsp2 of porcine reproductive and respiratory syndrome virus; a site for insertion of a vaccine deletion marker and virulence attenuation

ABSTRACT

Vaccines that differentiate infected from vaccinated animals (DIVA) are a new development in PRRS vaccine design. The non-structural protein, nsp2, is the single largest viral product and plays multiple roles in polypeptide processing and the formation of replication complexes. Using reverse genetics and a PRRSV infectious cDNA clone, we constructed several viruses which contained deletions and insertions in nsp2. One construct contained a 131 amino acid deletion within a relatively conserved region of nsp2. The deleted region was replaced with either green fluorescent protein (GFP) or hemagglutinin (HA). The tagged viruses produced normal yields on MARC-145 cells and porcine alveolar macrophages. However, gross and micro-histopathology showed reduced pathogenesis in pigs. The 131 amino acid peptide, when expressed as a recombinant protein and coated onto enzyme linked immnosorbent assay (ELISA) plates, was recognized by sera from pigs infected with wild-type virus, but not the deletion mutants. The results from this study show that nsp2 is an important target for the development of marker vaccines and for virus attenuation.

INTRODUCTION

Deletion marker vaccines for differentiating infected from vaccinated animals (DIVA) provides the means to pursue disease eradication simultaneous with the vaccination of animals with modified live virus (MLV) vaccines. Perhaps the best example in the use of a DIVA-based vaccine is in the eradication of pseudorabies. The deletion of the gI or gX of the psuedorabies MLV provides for the use of a companion ELISA test to detect ant-gI or gX antibodies, elicited in response to infection or exposure to a field virus (28). For small RNA viruses, the creation of a deletion marker can be problematic. A limited number of critical genes, overlapping gene segments and bi-cistronic reading frames in the genome make deletion or modification of an immunogenic region difficult.

Infections caused by porcine reproductive and respiratory syndrome virus (PRRSV) are responsible for approximately $560 million per year in losses to the U.S. swine industry, making it the number one infectious disease in swine. PRRSV belongs to the order *Nidovirales* and along with equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) form a single family, *Arteriviridae*. PRRSV is an enveloped, positive polarity, non-segmented, single-stranded RNA virus possessing a 15 kb genome, which contains at least 9 open reading frames (ORFs). The coding portion is flanked by 5' leader and 3' untranslated regions. The genes for the structural proteins occupy the 3' end of the coding region and comprise ORFs 2 through 7 (1, 15). The major structural proteins, glycoprotein 5 (GP5), matrix (M) and nucleocapsid (N) are derived from ORFs 5, 6, and 7, respectively (10). Whereas, GP2a, 2b, GP3 and GP4, translated from ORFs 2a, 2b, 3 and 4, respectively, are considered minor structural proteins whose functions remain unclear, but are required for infection (31). Based on studies of EAV, GP2b, GP3 and GP4 form a heterotrimer associated with the surface of the virion

(30). The ORF2b protein is an integral viral protein and shares similarities with the small coronavirus E protein (23, 33, 34).

Reverse genetic experiments that produce arterivirus genomes with deletions of single structural protein genes have failed to produce viable virus. Therefore, the essential nature of the structural proteins makes them unsuitable for construction of a deletion marker virus. Other targets for deletion are the nonstructural proteins, which occupy three quarters of the proteome. Arterivirus non-structural proteins (nsp's) are produced by the proteolytic processing of the ORF1a and ORF1ab polyproteins, known as pp1a and pp1ab, respectively. Based primarily on studies of EAV, the estimated 3,960 amino acid pp1ab protein of PRRSV is processed to yield 12 products, labeled nsp1-12 (1, 15, 32). In addition, nsp1 of PRRSV undergoes an autocleavage to yield nsp1α and nsp1β. Both nsp1α and 1β proteins possess papain-like cysteine proteinase (PCP) activities (11, 15). Nonstructural protein 2 (Nsp2) is a multifunctional protein, which, after processing, forms the single largest viral protein. A chymotrypsin-like protease (CP) domain, located at the N-terminal end is responsible for the cleavage of the nsp2/3 junction (21). In addition, nsp2 is a co-factor with nsp3-8 in the cleavage of the nsp4/5 junction (29). In EAV, the C-terminal region of nsp2 forms a strong non-covalent association with nsp3. It has been proposed that the hydrophobic domains in nsp2 and 3 form part of a scaffolding system responsible for anchoring the remaining replicase proteins within double membrane vesicles (DMVs), which house the replication complexes. The co-expression of EAV nsp2 and nsp3 is sufficient to form structures that closely resemble DMV structures (24). The C-terminal end of nsp2 also contains several conserved cysteine residues, which may be involved in binding zinc (35). The main protease, nsp4, contains a 3C-like serine protease (SP;22). The cleavage of

Glu/Gln and Gly/Ser recognition sites by nsp4 yields the remaining non-structural proteins (1, 15, 32).

The RNA-dependent RNA polymerase (RdRp) and nucleoside triphosphate (NTP)-binding / RNA helicase activities are found in the ORF1b proteins, nsp9 and nsp10, respectively (2, 20). Nsp11 possesses a NendoU domain with similarities to a poly(U)-specific endoribonuclease, XendoU, from *Xenopus laevis* (25). Nsp12 is a relatively small protein of unknown function.

Within pp1ab, nsp2 posses two important properties that make it a potential target for the construction of deletion marker virus. First, it is highly immunogenic possessing at least eight B cell epitopes. Secondly, field viruses have been identified that contain naturally occurring nucleotide insertions and deletions within nsp2. In this paper, using a PRRSV infectious cDNA clone, we describe the construction of a virus that contained a 395 nt deletion in nsp2. Replacement of the deleted region with GFP or HA in one of the constructs resulted in a live virus that replicated in MARC-145 cells and primary cultures of porcine macrophages and produced green fluorescence. Nsp2-deleted viruses also replicated in pigs, but possessed an attenuated phenotype compared to the parent virus. A recombinant polypeptide corresponding to the deleted region was recognized by sera from pigs infected with the intact nsp2 region, but not by the sera from pigs infected with the deletion mutants. Therefore, this study identifies deletions in nsp2 which should be useful in the preparation of marker vaccines and for the attenuation of virulent isolates.

MATERIALS AND METHODS

Cells

MARC-145 cells were maintained in MEM with 7% fetal bovine serum (FBS, Amresco) and supplemented with pen/strep and funzizone. Porcine alveolar macrophage (PAM) cells were obtained from the lungs of four to six week-old pigs by lung lavage with PBS. Cells were plated in RPMI with 10% FBS with antibiotics. After two days, cells were infected with virus.

The construction of recombinant PRRSV expressing HA epitope tag.

Detailed procedures of the recombinant viruses (pCMV-P129-Mlu-SgrA & pCMV-P129-Mlu/GFP/SgrA) are described in the Materials and Methods section in Chapter 2.

The HA duplex epitope tag having partial Mlu I and SgrAI sites were resuspended in RNase-Free Duplex Buffer (Integrated DNA Technologies) to 50µM concentration. Heat to 94°C for 2 minutes, and allowed to cool to room temperature. The resulting product was fully resuspended in stable, double-stranded form. The phosphorylated duplex HA epitope tag was ligated into 5,161bp of Mlu I-SgrAI fragment of pCR2.1-3219▪MluI-4875▪AflII. The Mlu I▪HA▪SgrAI clone having intact HA tag was selected by sequence analysis and restriction enzyme digestion. At the last step, the 1,305bp Mlu I▪HA▪AflII fragment was ligated into 17,231bp Mlu I-AflII fragment of pCMV-S-AflII. The resulting product was pCMV-Mlu▪HA▪SgrA-AflII (Primer lists are in Table 1).

Virus preparation and measurement of virus yield

Recombinant viruses were initially harvested from MARC-145 cells on a 12 well plate at four days after transfection with 2µg of plasmid DNA. Viruses were subjected to two more rounds of amplication on MARC-145 cells. Passaging was performed by taking a 1:50 dilution of virus and placing it on a T-25 flask of confluent MARC-145 cells and harvesting virus three days later.

The flask was freeze-thawed prior to harvest. The fourth passage (P4) of the virus was performed by infecting six T-75 flasks. After harvest, the virus was aliquoted into 1ml quantities and stored at -80C. This represented the stock virus for inoculation of pigs and other experiments. Virus yield was measured by end-point titration of cell culture medium on MARC-145 cells. On the day of vaccination, PRRSV stocks were diluted to a concentration of 10^5 $TCID_{50}$/ml in culture medium and were transported on ice to the research facility. A 4 ml challenge dose of diluted stock solution (2 ml per nostril plus a 2 ml intramuscular injection) containing approximately $4X10^5$ $TCID_{50}$/ml was delivered to each pig.

Study design and challenge

Prior to challenge, contact controls assigned to each treatment were removed from the pen and housed separately. Contact controls were returned to the group at 4 hrs after challenge. At approximately 4 weeks of age (Day 0), 10 randomly allotted pigs from treatment groups T02-T04 were challenged intranasally (IN) and intramuscularly (IM) as shown in Table 2. The six animals allotted to the T01 group received a mock challenge IN and IM. All pigs were restrained manually by placing one hand around the snout to limit head movement and the other arm around the midsection of the pig. The pigs were challenged IN with a total challenge volume of 2 ml by instilling 1 ml of challenge material in each nostril. The challenge material was instilled drop wise in each nostril in concurrence with an inspiration movement. Pigs were held in a head-up position for approximately one minute after challenge. Pigs were injected intramuscularly in the neck with 2 ml of the same challenge material.

Antibody detection of viral proteins

Detection of antigens in transfected and infected cells was performed by fixing cells for 10 min in 80% acetone. All antibodies were diluted in PBS with 5% FBS and incubations performed for one hr at room temperature. Cells were extensively washed in PBS between incubation with primary and secondary antibodies. For detection of HA, the anti-HA mAb (Courtesy of Dr. Park Man-Sung, Mount Sinai School of Medicine) was diluted 1:100 in PBS-FBS, followed by incubation with FITC-labeled anti-mouse (Rockland). Viral nucleocapsid antigen was detected using SR-30 mAb (a kind gift of Eric Nelson) diluted 1:100 followed by incubation with AlexaFluor 584 (Rockland). GFP was detected using a 1:100 dilution of rabbit anti-GFP antibody, prepared against the whole molecule followed by the detection of rabbit antibody with peroxidase-labeled goat anti-rabbit. Peroxidase activity was detected using a DAB staining kit (Vector Labs).

Antibody detection of viral antigens in infected pigs

Total antibody was measured using the PRRS HerdCheck ELISA (IDEXX). The results were reported as S/P ratio. Antibodies against the nsp2 protein were detected using an ELISA-based method. Each well of a 96-well ELISA plate was coated with 100 ul of protein at a concentration of 10 ug/ml per well. Plates were washed with phosphate buffered saline (PBS) and blocked with PBS containing 5% goat serum (PBS-GS). Each serum sample was initially diluted 1:50 in PBS-GS and then serially diluted 1:2 serial diluted on the ELISA plate. Plates were incubated at 37 °C for 1hr and then washed 3x with PBS containing 0.01% Tween 20 (PBST). Peroxidase-labeled rabbit anti swine Ig, diluted 1:1,000, was added to each well and incubated at 37 °C for one hour. Wells were washed extensively with PBST and peroxidase activity measured by the addition ABTS peroxidase chromagenic substrate (KPL) and plates read at A_{405}. Antibodies against

EGFP were detected by indirect fluorescence assay (IFA) against cells that expressed EGFP. Since free EGFP leaks out of acetone-fixed cells, Vero cells were transfected with a pEGFP plasmid containing EGFP fused to equine infectious anemia virus Rev protein (EGFP-ERev) followed by incubation with AlexaFluor 584 (Rockland). Transfection into MARC-145 cells was performed on twenty-four well plates using 1 ug of plasmid DNA and Lipofectamine^{TM} 2000 (Invitrogen). Procedures were performed according to the manufacturer's instructions. The next day and after the appearance of EGFP fluorescence, cells were fixed in 80% acetone for 10 min. and then air dried. Pig serum samples, diluted in PBS-GS, were incubated on cells for one hour at 37 °C followed by extensive washing with PBS. Pig antibody was detected with biotin-labeled goat, diluted in PBS-GS, washed extensively and then incubated with anti-pig followed by 1:2,000 dilution of Texas red-labeled streptavidin, diluted in PBS-GS supplemented with 5% pig serum. Incubation was continued for an additional 30 min at 37°C. The cells were extensively washed with PBS and cells viewed under FITC and rhodamine filters. A positive result was identified by the co-localization of EGFP and Texas red staining in the same cells. HA-specific antibody was detected using an ELISA method. ELISA plates were coated with 100 ul/well of 12 ug/ml, wells were blocked with PBS-GS. Samples were initially diluted 1:10 and then subsequent serial 1:2 dilutions. Plates were incubated for one hr at 37°C then extensively washed with PBS. Peroxidase-labeled rabbit anti-swine Ig, diluted 1:1,000, was added to each well and incubated at 37°C for one hr. Wells were washed extensively with PBST and peroxidase activity measured by the addition of ABTS peroxidase chromagenic substrate (KPL).

Expression and purification of nsp2 (628-759) polypeptide

The cDNA fragment corresponding to nucleotides 3219 through 3614 was amplified using the forward and reverse primers, SacII 3219F and BamHI 3614R, respectively. The forward and reverse primers contained Sac II and BamHI, for cloning into the SacII and BamHI sites of the pHUE expression vector. This vector is designed to express a 5xHis-ubiquitin fusion protein in E. coli (6). The resulting plasmid, pUb-nsp2(628-759), was transduced into BL21(Invitrogen) E. coli bacteria. A fresh overnight culture was inoculated into 100 ml of pre-warmed SOB with 50 ug/ml ampicillin. Bacteria were grown to an A_{600} value of 0.4-0.6 and then expression of foreign protein was induced by the addition of 1 ml of 100 mM IPTG. After 4 hr, the bacteria were harvested and the pellet resuspended in 8 ml of binding buffer (10 mM $NaPO_4$, 500 mM NaCl, 5 mM imidazole, pH 7.4). 1 ml of protease inhibitor cocktail (Biocompare) was added followed by 8 mg of lysozyme and the sample placed on ice for 30 min. The sample was sonicated and centrifuged for 20 min at 20,000 x g loaded onto a His GraviTrap Ni column (GE) equilibrated with binding buffer. The column was washed with 12 ml of binding buffer and His-tagged protein eluted with binding buffer containing 500 mM imidazole, pH 7.4. Three ml fractions were collected and protein-containing fractions identified by SDS PAGE on a 12% polyacrylamide gel followed by staining with coomassie blue. Protein-containing fractions were pooled concentrated down to 500 µl on a 20,000 MW cut-off Centricon-Plus filter (Amicon) according the manufacturer's instructions.

RT-PCR detection of EGFP insert

The presence of the EGFP insert was detected by reverse transcription-polymerase chain reaction (RT-PCR) amplification using total RNA from sera infected with P129-GFP. Total RNA was isolated from cells using TRIzol, (Invitrogen). The primers chosen for RT-PCR

amplification flanked the EGFP insert. The sequence of the 2941F and 3816R forward and reverse primers were 5'-AGCCAATTTTTGTGTCTGCACTGCAACACAAATTT-3' and 5'–GCAACGGCCCCCCACCGGCCGCATCTACGCCGTCTG-3', respectively.

Viral RNA was reverse transcribed using SuperScript III (Invitrogen) and the reverse primer. Amplification was performed using TaKaRa Ex Taq™ according to the manufacture's recommendations. Reaction conditions included an initial denaturation step of 95˚C for 60 seconds followed by 30 cycles of 94˚C (35 sec), 58˚C annealing (35 sec), and 72˚C extension (1 min). The reaction was concluded with a 10-minute incubation at 72˚C. PCR products were separated on an agarose gel and stained with ethidium bromide. For sequencing products were cloned into pCR2.1 (Invitrogen) and amplified in Top10 E. Coli cells. Minipreps of overnigh cultures were prepared using Wizard DNA purification kit (promega). Sequencing was performed using by ACGT.Inc, Chicago

RESULTS

In vitro properties of nsp2 deletion viruses

Site-directed mutagenesis was used to introduce unique Mlu I and SgrAI restriction enzyme sites at nucleotide positions 3,219 and 3,614, respectively, of ORF1a in the pCMV-S-129 cDNA infectious clone. This clone does not require the in vitro transcription step of RNA prior to transfection, but incorporates a cytomegalovirus (CMV) promoter for the transcription of full-length viral RNA by cellular RNA pol II The restriction sites were created without changing the peptide sequence. Furthermore, the region covered by the Mlu I and SgrAI is a relatively conserved region that covers a portion of nsp2 between hypervariable regions HV-1 and HV-2 (Fig. 3.1). The Mlu-SgrA region is well-removed from the N- and C-terminal domains essential

for nsp2 function. The deleted region, located between the Mlu I and SgrAI sites, was replaced with either a 798 bp EGFP cDNA or the much smaller 24 bp HA tag,YPYDVPDY. Viruses derived from transfected MARC-145 cells were further amplified by performing four additional passages on MARC-145 cells.

EGFP fluorescence appeared within 8 hr after infection with the P129-EGFP virus. Fluorescence was predominantly perinuclear and co-localized with the N protein, two localization properties consistent with the intracellular distribution of nsp2 (referred to in Chapter 4). The HA peptide was not detected in cells after infection with the P129-HA virus or after transfection with the P129-HA plasmid. Similar results were obtained from viruses and infectious viral cDNAs that expressed the FLAG peptide. The transfected cells supported virus replication as demonstrated by the presence of positive staining with antibodies to PRRSV N. The reason for the absence of peptide tag antibody recognition is unclear, but is a likely related to the location of the tag within nsp2. The conformational folding of the protein may embed the peptide tag within the native protein, making it inaccessible to antibody.

Recombinant viruses were tested for replication on MARC-145 and porcine alveolar macrophage (PAM) cells. PAMs are primary cells derived from lung lavage of young pigs and are one of the naturally occurring cell populations that support virus replication in the pig. The P129-GFP virus and the parent wild-type P129 virus, containing the Mlu-SrgA restriction sites, replicated to similar levels in MARC-145 cells; consistently achieving yields of 7 log $TCID_{50}$/ml of media, which are similar to yields obtained for wild-type viruses isolated from infected pigs. In contrast, the P129-HA virus produced an approximate 2 log reduction in yield. However, the continued passage of P129-HA for an additional three times increased the yield to that of the P129-GFP and P129 viruses.

Experimental infection of pigs

P129 (passage five), P129-GFP (passage five) and P129-HA (passage eight) viruses were used to infect pigs. The P129 inoculum was derived from the infectious clone that contained the Mlu I and SgrA I restriction enzyme sites. Groups of 10 PRRSV-negative four week old pigs were infected with 5 log$TCID_{50}$ of each virus using a combination of intranasal and intramuscular administration. Besides the three virus-infected groups, an additional group contained six mock-infected pigs, which received only tissue culture medium. By four days after infection, pigs in the P129 group showed outward signs of PRRS, including respiratory distress and lethargy. The other groups exhibited no change in behavior. At ten days after infection, half the pigs from each group were sacrificed and lungs examined for gross and microscopic changes. Compared to the mock-infected pigs, the lungs from the five P129 pigs appeared heavy with a wet appearance, anatomical changes characteristic of acute PRRS. Representative photomicrographs of lungs from infected and control pigs are shown in Figure 3.2. At the microscopic level, the lungs from the P129 pigs also exhibited features characteristic of acute PRRS, including collapsed alveoli with infiltration of macrophages and accumulation of immature lymphocytes in the interstitium. The P129-HA pigs showed few gross or microhistological changes and appeared similar to the lungs from the mock-infected group. At the microscopic level, the lungs from the P129-EGFP pigs possessed only a few areas of patchy interstitial pneumonia.

Serology and virus isolation were used to determine if the PRRSV pigs were productively infected. We used a commercially available ELISA test to determine the antibody responses in virus and mock-infected pigs. When used for the measurement of antibody in field samples, a 0.4 S/P ratio value is used as the cutoff value for identifying a pig as having a 95% probability of being seropositive for PRRSV. Because of the low background values for the mock-infected

pigs, we increased this cutoff value to an S/P ratio of 1.0. The results showed that all pigs produced antibody to PRRSV, whereas the mock-infected pigsremained seronegative throughout the study. Antibody responses of pigs retained for the full 28 days of the experiment are shown in Fig. 3.3. Typical of the immunological response following PRRSV infection, antibody levels showed a wide degree of variation. Because of this variation, it was not possible to make accurate quantitative comparisons of antibody responses between the groups of infected pigs. However, there appeared a qualitative difference between the P129 and P129-HA groups. At 10 days after infection, all five P129 pigs possessed PRRSV antibodies compared to no pigs in the P129-HA group. These data indicate that the antibody response to the P129-HA virus was delayed.

At four hours after challenge, two naïve, PRRSV-negative control pigs, were placed in each virus group and allowed to intermingle with the other challenge pigs during the remainder of the experiment. These contact control pigs were used as a means to detect the presence of virus shed by infected pigs. Sources of shedding include contact with the innoculum used for challenge or from virus shed after the onset of viremia in the challenge pigs. Pigs typically become viremic two to three days after challenge. All contact control pigs seroconverted demonstrating that virus was being shed. In the P129 group, seroconversion was evident at day 10, which was three days after the appearance of antibody in the challenge pigs. Therefore, infection of the contact pigs was the likely result of virus shed from viremic pen mates. Seroconversion of the contact control pigs in the P129-HA and P129-GFP groups followed a similar pattern, indicating that infection was the result of shedding from viremic pigs.

Virus replication was also assessed by virus isolation (VI) from serum. The results presented in Fig. 3.4A show that all 10 pigs in the P129 group were VI positive by 10 days after infection.

Virus was recovered from 8 of 10 P129-GFP and 8 of 10 P129-HA pigs. Virus was recovered from sera of all contact control pigs. The last VI-positive serum sample in the P129 group was obtained at 14 days after infection. In contrast two of five pigs in the P129-HA group were still VI positive at 21 days. The contact control pigs became VI-positive after the challenge pigs, consistent with the shedding of virus by the acutely infected challenge pigs.

The amount of virus in each serum sample was assessed by measuring the $TCID_{50}$/ml of virus. Mean log$TCID_{50}$/ml values for all virus challenge pigs are shown in Fig. 3.4B. Similar to the antibody results, there was a wide variation between pigs within each group. Generally, the P129 pigs achieved higher levels of virus in the blood with the peak levels of 3.5 log$TCID_{50}$/ml, at Day 7 after infection. This was compared to mean values of approximately 1.0 log$TCID_{50}$/ml for the P129-GFP and P129-HA groups. Even though the amount of virus recovered from sera was lower, the P129-HA and P129-GFP groups appeared to sustain detectable levels of virus for a longer period compared to the P129 group. Collectively, these data indicated that the P129 pigs attained a higher level of viremia. Even though the peak level of viremia in the P129-HA group was lower, viremia was extended for a longer period of time.

Antibody recognition of nsp2 (628-759), HA and GFP tags

The peptide sequence corresponding to amino acids 628-759 of P129 nsp2 is shown in Fig. 3.5A. For the purpose of comparison, we included the peptide sequence from VR-2332, a different isolate and widely regarded as the prototypic example of North American Type 2 PRRSV isolates. There are 19 amino acid differences (86% identity) between the two viruses. Analysis for the presence of B cell epitopes predicted to be immunogenic was performed using the web-based computer program, Antigenic (EMBOS). The results showed seven oligopeptide regions

which can potentially function as B cell epitopes. Recently, Lima et al., conducted an antibody pepscan analysis of the PRRSV proteome. The approach was to screen a set of oligopeptides prepared from PRRSV isolate NVSL 97-7895 (GenBank accession no. AY545985) against antibodies from pigs infected with the 97-7895 virus as well as pigs infected with other isolates. The results showed several linear epitopes within nsp2, including the region covered by amino acids 756-770 in nsp2 of isolate 97-7895 or amino acids 749-763 in P129 (see peptide sequence in Fig. 3.5A surrounded by a dashed line box). The cDNA corresponding to the nsp2 (628-759) peptide of P129 was cloned into pHUE (6) to create the construct pHUE-nsp2(629-759). Four hr after induction with IPTG, the bacteria expressed the peptide corresponding to the nsp2-ubiquitin fusion protein (see Fig. 3.5B). The 5xHis-ubiquitin-nsp2 fusion protein was extracted using native conditions, affinity isolated on a Ni-sepharose column, concentrated and coated onto wells of 96-well ELISA plates. Serum from PRRSV infected pigs was incubated with the immobilized peptide and bound antibodies detected using a peroxidase-labeled anti-pig secondary antibody. Sera from pigs infected with VR-2332 recognized the P129 nsp2(628-759) peptide (data not shown). The P129 nsp2 peptide was incubated with sera from pigs at 28 days after infection with P129, P129-HA and P129-GFP viruses. When compared to mock-infected pigs, four of the five pigs in the P129 group showed elevated A_{405} values. These values were also elevated compared to the other virus-infected pigs. We then measured reactivity of the 28 day serum samples against HA and EGFP antigens. Measurement of anti-HA antibodies was performed using a modified ELISA. There was no evidence of reactivity against HA using sera from P129-HA (data not shown). We also determined if pigs infected with P129-EGFP made antibodies against EGFP. The experimental approach was to incubate dilutions of sera with cells at 24 hr after transfection with a construct that expressed an EGFP-protein fusion protein. Since free EGFP rapidly leaks

out of cells, we incorporated a construct that expressed EGFP fused to an EIAV Rev protein. EGFP-reactive antibody was detected using AlexaFluor 584-labeled anti-pig antibody. A positive result was the localization of red and green fluorescence in the same cells. At dilutions of less than 1:100 there was no reactivity of mock-infected or P129-infected pigs against the fusion protein (data not shown). End point titrations for sera from P129-EGFP pigs ranged between 1:100 and 1:800. These data show that pigs infected with P129 made antibodies against the nsp2(628-759) peptide and peptide-specific antibody was absent in pigs infected with viruses that lacked the peptide region. Furthermore pigs infected with P129-EGFP made antibodies that recognized EGFP. The absence of anti-HA antibodies in P129-HA pigs is consistent with the inability of anti-HA antibody to detect HA in cells after transfection with the infectious clone and after infection of cells with P129-HA. We assume that the small HA epitope is hidden within nsp2 and inaccessible to antibody.

Stability of EGFP expression during infection

Viruses isolated from the sera of the P129-EGFP-infected pigs were propagated on MARC-145 cells. The isolated viruses were no longer fluorescent. However, immunocytochemical staining using anti-GFP antibody indicated that at least a portion of the EGFP protein was still being expressed. An example of a P129-EGFP virus showing a negative fluourescence but positive for anti-GFP staining is shown in Fig. 3.6A. The retention of EGFP immunoreactivity is consistent with the stimulation of an anti-GFP response in pigs. Forward and reverse nsp2-specific PCR primers were chosen that flanked the 5' and 3' ends of the EGFP insert. Of the four isolates, two produced PCR products. The absence of products could be due to loss of the region containing the primer sequence. The PCR-negative viruses were not subjected to further

investigation. The RT-PCR amplification of the other two viruses produced three products. The results showed that product 3 and product 4B possessed an intact EGFP insert, but there were four mutations in product 4B and one mutation in product 3, respectively (Fig.3.6C and D). Product 3 had a Tyr to Ser change at position 66 and product 4B had a Met to Val change at position 89, a Arg to Cys at position 96, a Val to ala at position 151 as well as a mutation at position 66. Product 4A possessed a deletion in EGFP between amino acids 32 to 266 . There were no mutations in the region of the cDNA product flanking the EGFP insert.

DISCUSSION

A major shortcoming of current PRRSV vaccines is the inability to discriminate vaccinated pigs from pigs infected with field isolates. Another limitation in the use of modified live vaccine (MLV) is the potential for reversion into a virulent form, which has been reported to occur in the field (26). The effort to find marker PRRSV viruses is hampered due to the compact arterivirus genomic structure, such as overlapping and bicistronic ORFs. The development of PRRSV reverse genetic system makes it possible to introduce mutations to delineate essential and nonessential domains. Nsp2, the largest single processed protein produced during infection is predicted to be approximately 980 amino acids. This size may be smaller or larger based on which cleavage sites are used, the presence of insertions and deletions and the genotype of the PRRSV isolate. Genetic sequence comparisons of nsp2 peptide from several PRRSV isolates identify at least two hypervariable regions, which naturally incorporate nucleotide insertions and deletions (Fig.3.1A). Mlu I and SgrAI restriction enzyme sites were introduced at nt position 3219 an 3614 of nsp2, in the pCMV-S-P129 cDNA infectious clone by site-directed mutagenesis. The 395nt deleted region, located between the two enzyme sites, was replaced with

798bp EGFP or a much smaller 24 bp HA tag (Fig.3.1A). Virus yields measured just before infection had 5.5 log $TCID_{50}$ in all three viruses and specially, P129-HA virus had three additional passages for cell adaptation and enhancing titration. After infection with recombinant viruses, the P129-HA virus, which had virus yield similar to that of P129 in vitro, showed few gross or microhistological changes and appeared similar to the lungs from mock infected pigs. The deletion of the relatively conserved 395bp domain located between 3219 and 3614 in nsp2 of ORF1a may play a pivotal role in virulence and contributed to the attenuation in vivo. Other approaches were performed to make several different marker viruses, which included an extensive deletion of hypervariable regions of nsp2, but only HA tagged virus was recovered. Logically, HV domains incorporating deletions and insertions should be dispensable for viral replication but it is postulated that they have critical motifs necessary for viral functionality. For example, HV-2, which is connected with transmembrane region, acts as a scaffolding protein for replication complex formation and the disruption of that domain would have a lethal effect on viral replication.

Virus replication was measured by virus isolation (VI) from serum. In Fig.3.4, the P129 group all had VI positive sera, other two groups had 8 of 10 VI positive, but VI positive samples of P129-HA group could be detected by 21 days infection. All contact controls were also positive and these data strongly support the conclusion that acutely infected pigs were shedding virus, and is consistent with the result of seroconversion of naïve contact controls.

Quantitative measurement of VI positive samples showed that P129 had the peak levels of 3.5 logTCID50/ml but other two groups showed two fold below peak, but virus yield of P129-HA could be detected and sustained its viremic condition for a longer period of time. This data indicated, naturally occurring deletion of nsp2 of PRRSV could support viral replication, but

affected an attenuation and maintained a persistent infection in pigs (3, 19). Type 1 North American PRRSV strain with a broad nsp2 variability had a relatively low pathogenesis in experimental condition (12, 13), suffice it to say that the reason of low pathogenesis of HA virus could be associated with the deletion of nsp2 region.

The cDNA corresponding to the nsp2(628-759) peptide of P129 was expressed as 5X his-ubiquitin fusion protein four hr after induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) (6, Fig.3.5B) and it had seven putative B cell epitopes and another linear epitope site which was derived from NVSL 97-7895 PRRSV isolate (GenBank accession no.AY545985, Lima) (Fig.3.5A). With 131 amino acid nsp2 peptide ELISA, antibody reactivity was measured and when compared to mock-infected pigs, four of the five pigs in the P129 group showed elevated A405 values (Fig.3.3). The result of reactivity of the 28 day serum samples against HA and EGFP showed low A405 values compared to P129 (Fig.3.3). This data showed that pigs infected with P129 made antibodies against the nsp2 (628-759) and antibody was absent in pigs infected with viruses that lacked the peptide region. It can open a way for a possibility of PRRSV DIVA vaccine, in order for the accurate evaluation, more field samples should be tested and the standard cut off value be determined.

Another experimental approach was made if P129-EGFP can produce antibodies against EGFP. Serum samples from 28 days after infection in P129-EGFP group showed an immunoreactivity against EGFP fused to an EIAV Rev protein (Fig.3.3). But, the detection of anti-HA antibodies using sera from P129-HA group was failed. This is consistent with the inability of anti-HA antibody to detect HA in cells after transfection with infectious clone and after infection of cells with P129-HA. The peptide tags embedded within nsp2 prevent antibody from recognizing them due to folded native condition.

Viruses isolated from sera of the P129-GFP was grown in the MARC145 cell, it had no fluorescence but supported viral replication and retained immunoreactivity against EGFP (Fig.3.6A). To characterize which motifs could affect negative GFP fluorescence, PCR analysis was performed with two primers flanking the 5' and 3' ends of the EGFP insert. The GFP non-fluorescent clones showed the mutations in Tyr-66 located in chromophore and Arg-96 known to participate in the formation of the Tyr-66/Gly-67 ring structure (Fig.3.6D) (8, 17). An Arg-96 mutation of product 4B were also found at P129-MLU-GFP passage 5 in vitro study (refer to chapter II Fig.2.7B and Fig.3.6D). It was the similar result both in vitro and in vivo and the first report of finding mutations that make EGFP non-fluorescent during in vivo study (refer to chapter II discusstion). In product 4B, a Met to Val change at position 89 and Val to Ala change at position 151 were also discovered but, whether they had a negative effect on EGFP fluorescence, remains to be determined.

With the advent of PRRSV reverse genetics system, the development of attenuated marker vaccine armed with function of a DIVA was not an impossible dream. In this study, we opened a tangible road in PRRSV research field for the first time. Together with the instability of huge nsp2 domain, newly emerging PRRSV quasispecies could be a big barrier for setting up an effective DIVA system. But, relatively conserved and immunogenic nsp2 395bp region will not be easily subject to attack of negative pressure willing to modify its genotype. For the future study, a challenge study against highly virulent PRRSV isolate should be accompanied and except nsp2, the effort of finding conserved but nonessential domains which could be a different selection marker is to be made.

REFERENCES

1. Allende R, Lewis TL, Lu Z, Rock DL, Kutish GF, Ali A, Doster AR, Osorio FA. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J Gen Virol. 1999 Feb;80 (Pt 2):307-15.

2. Bautista, E.M., Faaberg, K.S., Mickelson, D., McGruder, E.D., 2002. Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology. 298, 258-270.

3. Batista L, Dee SA, Rossow KD, Deen J, Pijoan C. Assessing the duration of persistence and shedding of porcine reproductive and respiratory syndrome virus in a large population of breeding-age gilts. Can J Vet Res. 2002 Jul;66(3):196-200.

4. 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 6,500,662.

5. Calvert, J.G., Sheppard, M.G., Welch, S.K.W., 2003. Infectious cDNA clone of North American porcine reproductive and respiratory syndrome (PRRS) virus and uses thereof. US Patent Application 20030157689.

6. Catanzariti AM, Soboleva TA, Jans DA, Board PG, Baker RT. An efficient system for high-level expression and easy purification of authentic recombinant proteins. Protein Sci. 2004 May;13(5):1331-1339.

7. Choi YJ, Yun SI, Kang SY, Lee YM. Identification of 5' and 3' cis-acting elements of the porcine reproductive and respiratory syndrome virus: acquisition of novel 5' AU-rich sequences restored replication of a 5'-proximal 7-nucleotide deletion mutant. J Virol. 2006 Jan;80(2):723-36.

8. Cormack, B.P., Valdivia, R., Falkow, S., 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173, 33-38.

9. de Lima M, Pattnaik AK, Flores EF, Osorio FA. Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus.
Virology. 2006 Sep 30;353(2):410-21.

10. Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch Virol. 2000;145(4):659-88. Review.

11. Den Boon, J.A., Faaberg, K.S., Meulenberg, J.J., Wassenaar, A.L., Plagemann, P.G., Gorbalenya, A.E., Snijder, E.J., 1995. Processing and evolution of the N-terminal region of the arterivirus replicase ORF1a protein: identification of two papainlike cysteine proteases. J. Virol. 69, 4500-4505.

12. Fang Y, Kim DY, Ropp S, Steen P, Christopher-Hennings J, Nelson EA, Rowland RR. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. Virus Res. 2004 Mar 15;100(2):229-35.

13. Fang Y, Rowland RR, Roof M, Lunney JK, Christopher-Hennings J, Nelson EA. A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region.
J Virol. 2006 Dec;80(23):11447-55.

14. Meulenberg JJ, Bos-de Ruijter JN, van de Graaf R, Wensvoort G, Moormann RJ. Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. J Virol. 1998 Jan;72(1):380-7.

15. Nelsen CJ, Murtaugh MP, Faaberg KS. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents.
J Virol. 1999 Jan;73(1):270-80.

16. Nielsen HS, liu G, Nielsen J, Oleksiewicz MB, Botner A, Storgaard T, Faaberg KS. Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. J Virol. 2003 Mar;77(6):3702-11.

17. Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. & Remington, S. J. 1996. Crystal structure of the Aequorea victoria green fluorescent protein. Science 273**,** 1392–1395.

18. Rowland RR, Robinson B, Stefanick J, Kim TS, Guanghua L, Lawson SR, Benfield DA. Inhibition of porcine reproductive and respiratory syndrome virus by interferon gamma and recovery of virus replication with 2-aminopurine. Arch Virol. 2001;146(3):539-55.

19. Rowland RR, Lawson S, Rossow K, Benfield DA. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet Microbiol. 2003 Oct 30;96(3):219-35.

20. Seybert, A., van Dinten, L.C., Snijder, E.J., Ziebuhr, J., 2000. The biochemical characterization of the equine arteritis virus helicase suggests a close functional relationship between arterivirus and coronavirus helicases. J. Virol. 74, 9586–9593.

21. Snijder, E.J., Wassenaar, A.L., Spaan, W.J., Gorbalenya, A.E., 1995. The arterivirus Nsp2 protease. An unusual cysteine protease with primary structure similarities to both papain-like and chymotrypsin-like proteases. J. Biol. Chem. 270, 16671-16676.

22. Snijder, E.J., Wassenaar, A.L., van Dinten, L.C., Spaan, W.J., Gorbalenya, A.E., 1996. The arterivirus nsp4 protease is the prototype of a novel group of chymotrypsin like enzymes, the 3C-like serine proteases. J. Biol. Chem. 271, 4864-4871.

23. Snijder EJ, van Tol H, Pedersen KW, Raamsman MJ, de Vries AA. Identification of a novel structural protein of arteriviruses. J Virol. 1999 Aug;73(8):6335-45.

24. Snijder, E.J., van Tol, H., Roos, N., Pedersen, K.W., 2001. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. J. Gen. Virol. 82, 985-994.

25. Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L., Guan, Y., Rozanov, M., Spaan, W.J., Gorbalenya, A.E., 2003. Unique and conserved Features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. 331, 991-1004.

26. Storgaard T, Oleksiewicz M, Botner A. Examination of the selective pressures on a live PRRS vaccine virus. Arch Virol. 1999;144(12):2389-401.

27. Truong HM, Lu Z, Kutish GF, Galeota J, Osorio FA, Pattnaik AK. A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology. 2004 Aug 1;325(2):308-19.

28. van Oirschot JT, Gielkens AL, Moormann RJ, Berns AJ. 1990. Marker vaccines, virus protein-specific antibody assays and the control of Aujeszky's disease. Vet Microbiol. Jun;23(1-4):85-101.

29. Wassenaar, A.L., Spaan, W.J., Gorbalenya, A.E., Snijder, E.J., 1997. Alternative proteolytic processing of the arterivirus replicase ORF1a polyprotein: evidence that NSP2

acts as a cofactor for the NSP4 serine protease. J. Virol. 71, 9313-9322.

30. Wieringa R, De Vries AA, Post SM, Rottier PJ. Intra- and intermolecular disulfide bonds of the GP2b glycoprotein of equine arteritis virus: relevance for virus assembly and infectivity. J Virol. 2003 Dec;77(24):12996-3004.

31. Wissink EH, Kroese MV, van Wijk HA, Rijsewijk FA, Meulenberg JJ, Rottier PJ. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J Virol. 2005 Oct;79(19):12495-506.

32. Wootton, S., Yoo, D., Rogan, D., 2000. Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. Arch. Virol. 145, 2297- 2323.

33. Wu, W.H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R.R.R., 2001. A 10 kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology. 287, 183-191.

34. Wu, W.H., Fang, Y., Rowland, R.R., Lawson, S.R., Christopher-Hennings, J., Yoon, K.J., Nelson, E.A., 2005. The 2b protein as a minor structural component of PRRSV. Virus. Res. 114, 177-181

35. Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81, 853-879.

Table 3.1a- Primers used for introducing AflII site in nsp3 of pCMV-S-129

AflII (4856-4900)	
Forward	5’ CTTTGCCCAGCTGAATGGACTTAAGATCAGGCAAATTTCCAAGCC
Reverse	5’ GAAACGGGTCGACTTACCTGAATTCTAGTCCGTTTAAAGGTTCGG

(Bold and underlined nucleotide is mutated one, Original one is A. Box indicates AflII)

Table 3.1b- Primer lists used for site-directed mutagenesis

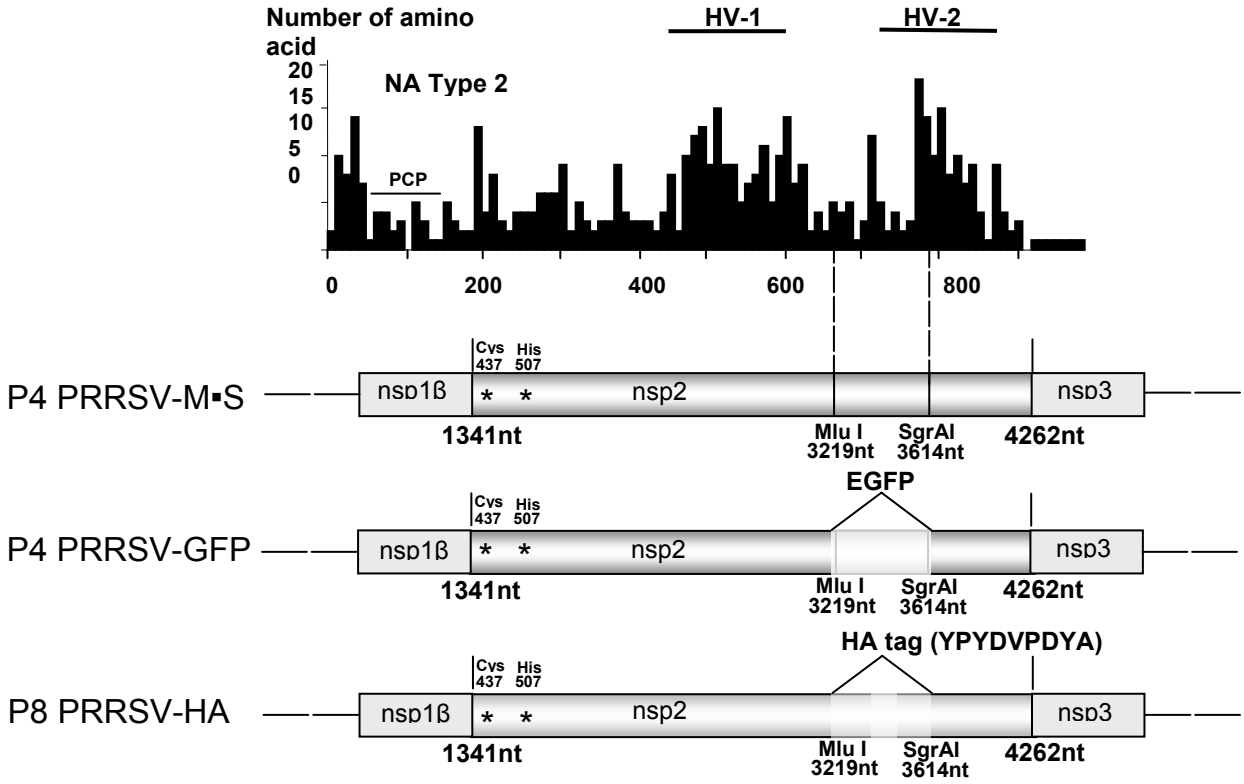
3219 MluI F	5’ ACGCGTCCAAAATACTCAGCTCAA
3614 SgrAI R	5’ CACCGGTGTTTTCTATTTTCCCGAG
3614 SgrAI F	5’ CACCGGTGAGATGATCAACCAGGGA
4875 AflII R	5’ CTTAAGTCCATTCAGCTGGGCAAA
	(Underlined nucleotide is indicated restriction enzyme sites)
HA tag F	5’ **C**GCGTATACCCATACGACGTCCCAGACTACGCA
HA tag R	5’ **C**CGGTGCGTAGTCTGGGACGTCGTATGGGTAT
(Bold and underlined nucleotide was phosphorylated to increase ligation efficiency)	

Table 3.2.

TX	Virus	Passage	Challenge Route	# of pigs
NT1#	NA	NA	NA	6
NT2#	NA	NA	NA	6
T01	MOCK	NA	IN/IM	6
T02	P129-HA	8	IN/IM	10
T03	P129-GFP	5	IN/IM	10
T04	P129-Mlu-SgrA	5	IN/IM	10

#NT1 pigs serve as sentinel animals to monitor the health status of source pigs. NT2 pigs will serve as contact controls for challenge groups, two per room for groups T02 thru T04. IN = intranasal, IM = intramuscular

A.


Number of amino acid
HV-1
HV-2
NA Type 2
20
15
10
5
0
PCP
0
200
400
600
800
Cys 437 His 507
P4 PRRSV-M▪S
nsp1ß
nsp2
nsp3
1341nt
Mlu I 3219nt
SgrAI 3614nt
4262nt
EGFP
P4 PRRSV-GFP
HA tag (YPYDVPDYA)
P8 PRRSV-HA

B.

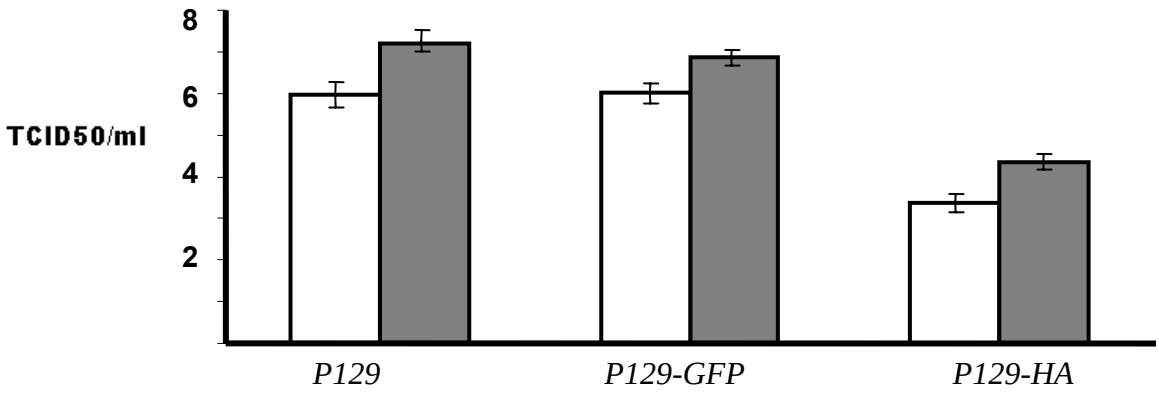

TCID50/ml
8
6
4
2
P129
P129-GFP
P129-HA

Fig.3.1 (A) Hypervariable regions in nsp2 and the construction of recombinant PRRS viruses. Hypervariability regions, HV-1 and HV-2, were identified by comparing the peptide sequences of 14 North American isolates, which were obtained from GenBank. Nsp2 peptide sequences were aligned using Clustal-X. The histogram shows the number of amino acid substitutions along the nsp2 peptide sequence. Each bar covers 10 amino acids. The bottom figure shows the recombinant viruses that were used for pathogenesis study. The last construct contains a 27 nt insert containing the HA peptide. (B) Viral yields of recombinant viruses. Virus yields from cells transfected with three recombinant plasmids. Media was harvested from MARC-145 cells on a 12 well plate at 4 days after transfection with 2 ug of plasmid DNA. The amount of virus recovered was measured by titration on MARC-145 cells (open bars) and on PAMs (closed bars).

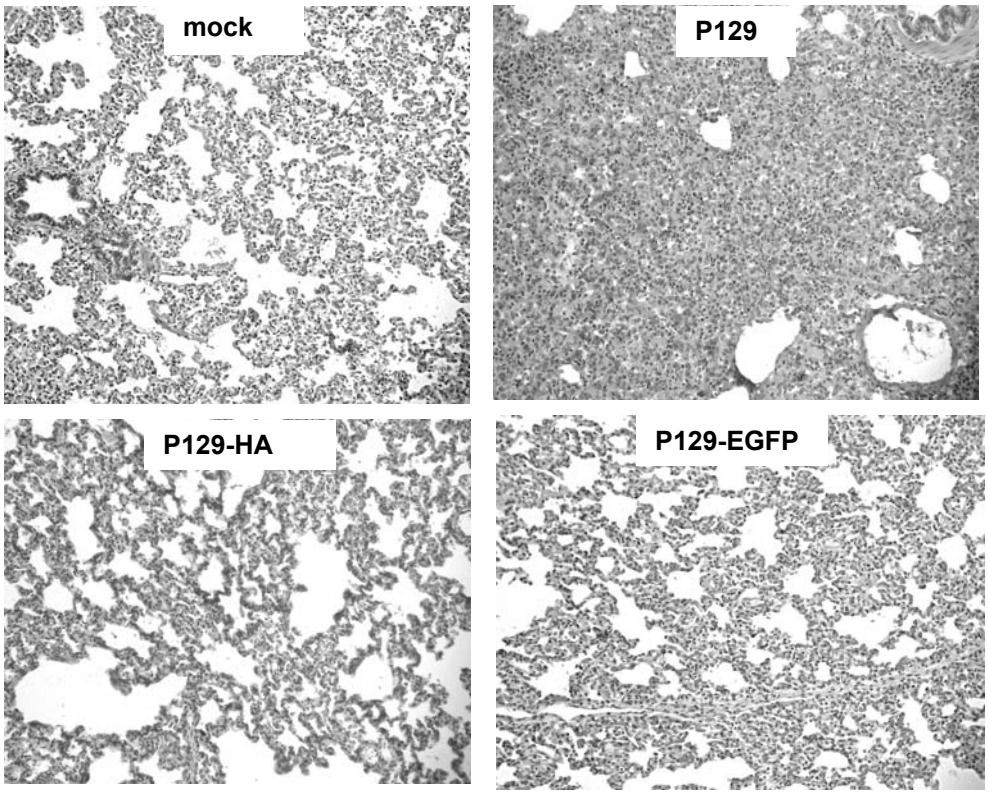


Fig.3.2 Photomicrographs of lungs from infected and control pigs. 10 days postinfection, 5 pigs from three groups infected with recombinant viruses (P129-MluI-SgrAI, P129-HA, P129-EGFP) were examined for histopathology.

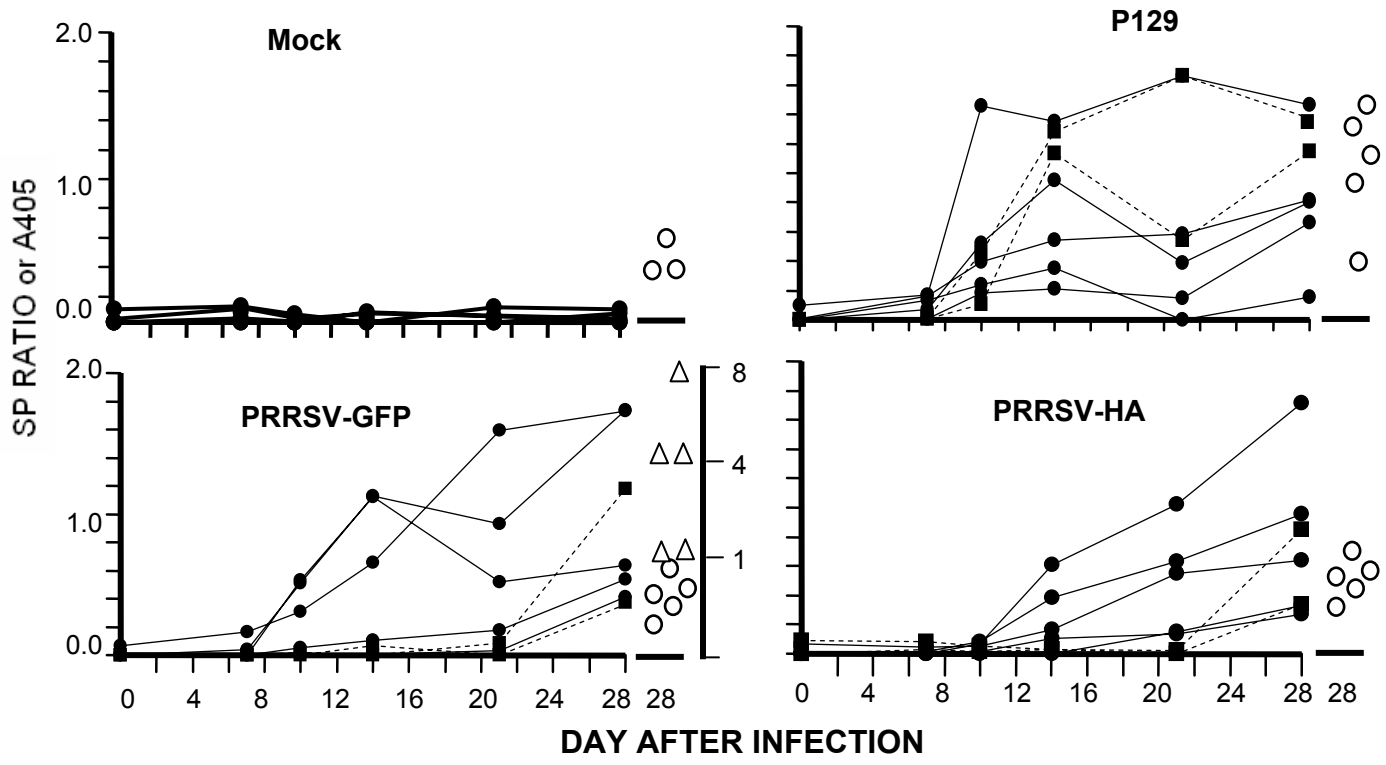
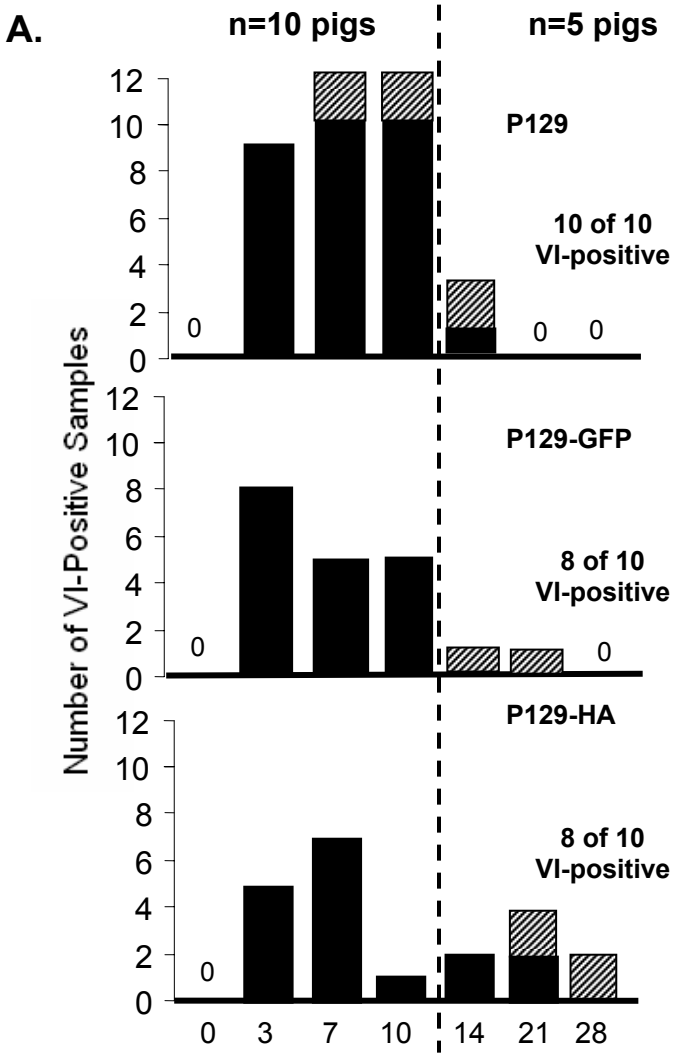


Fig.3.3 Antibody detection of viral antigens in infected pigs. Total antibody was measured using the PRRS HerdCheck ELISA (IDEXX). The results were reported as S/P ratio. Antibodies against the nsp2 protein were detected using an ELISA-based method. Closed circle is antibody response using herdcheck ELISA kit and open circle indicate A_{405} value using the nsp2 (628-759) peptide ELISA. Closed rectanglar are the antibody response of contact control placed in each virus group and allowed to intermingle with the other challenge pigs. In PRRSV-GFP, open triangle shows GFP reactivity against cells transfected with EGFP-ERev.


A.
n=10 pigs
n=5 pigs
P129
10 of 10
VI-positive
P129-GFP
8 of 10
VI-positive
P129-HA
8 of 10
VI-positive
Number of VI-Positive Samples
0 3 7 10 14 21 28

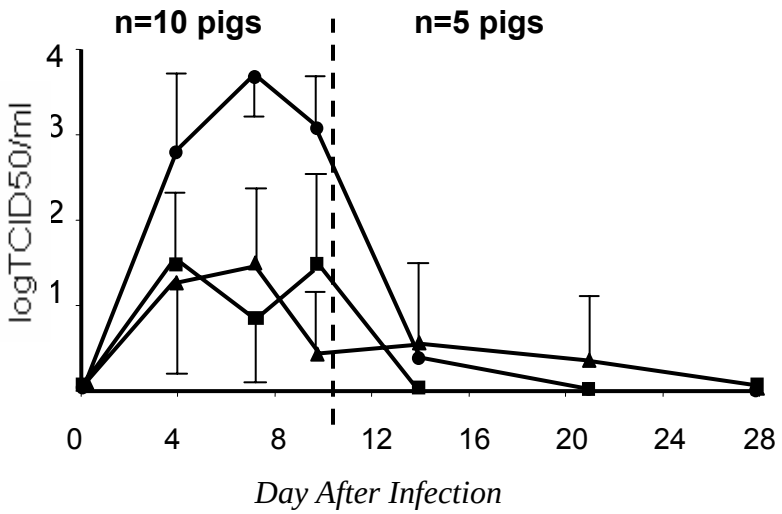

B.
n=10 pigs
n=5 pigs
logTCID50/ml
Day After Infection

Fig.3.4 (A) Virus isolation from infected pigs sera. Black bars represent the number of VI positive pigs and shaded bars are contact control pigs placed four hour after infection. (B) The amount of virus in each serum sample. The virus yield was measured by log$TCID_{50}$/ml. Closed circles are the amount of virus from sera infected with P129, closed rectangles are from sera infected with P129-GFP, and closed triangles are from sera infected with P129-HA.

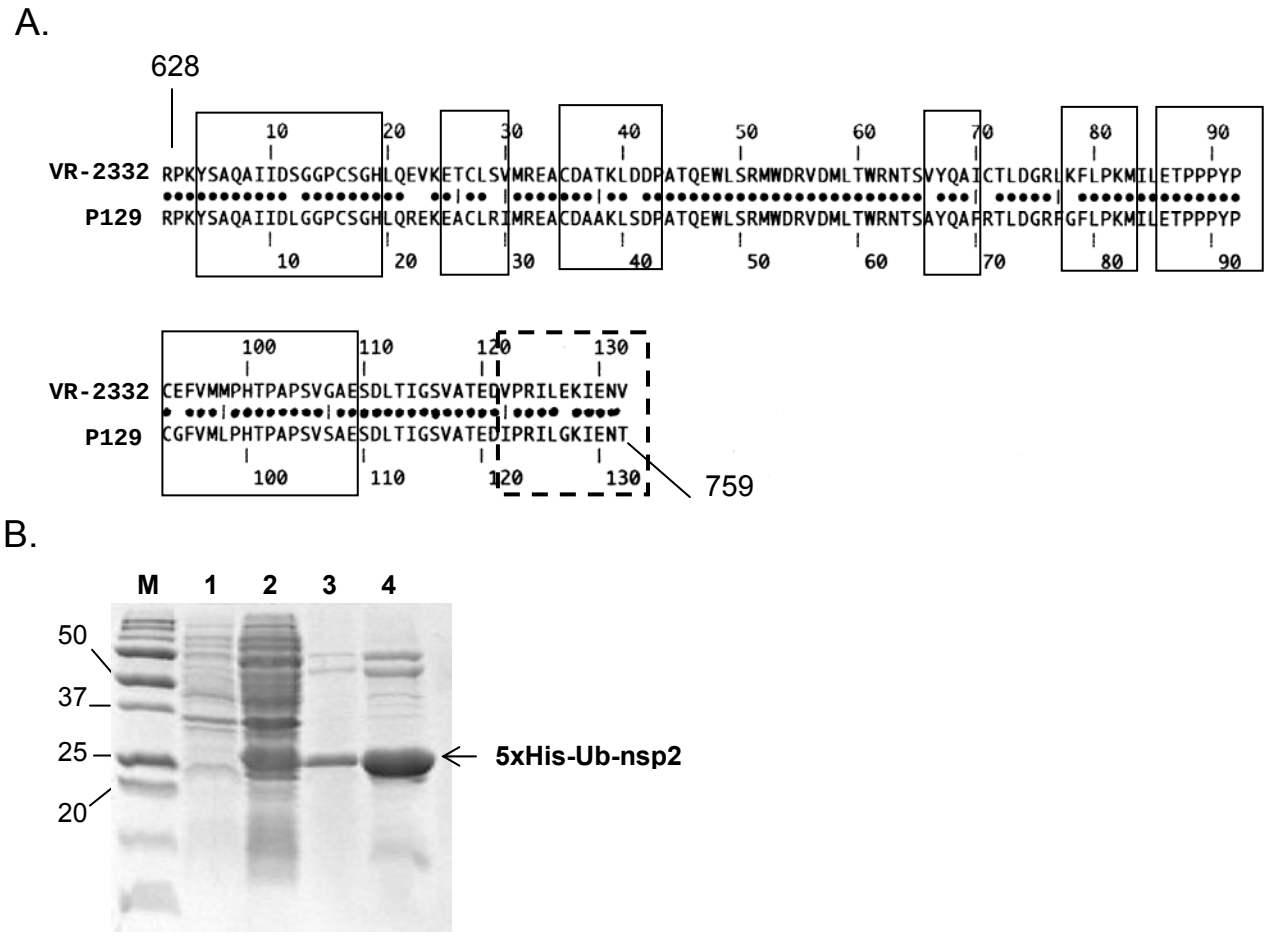


Fig.3.5 Expression and purification of nsp2(628-759) peptide. (A) The above peptide sequence shows the region deleted in the cloned construct PRRSV-▲-GFP, which was derived from the pCMV-S-P129 infectious clone. (B) The cDNA corresponding to this region was cloned into pHUE and expressed as a His-tagged ubiquitin fusion protein (6). Four hr after induction with IPTG, the bacteria expressed the peptide corresponding to the nsp2-ubiquitin fusion protein.

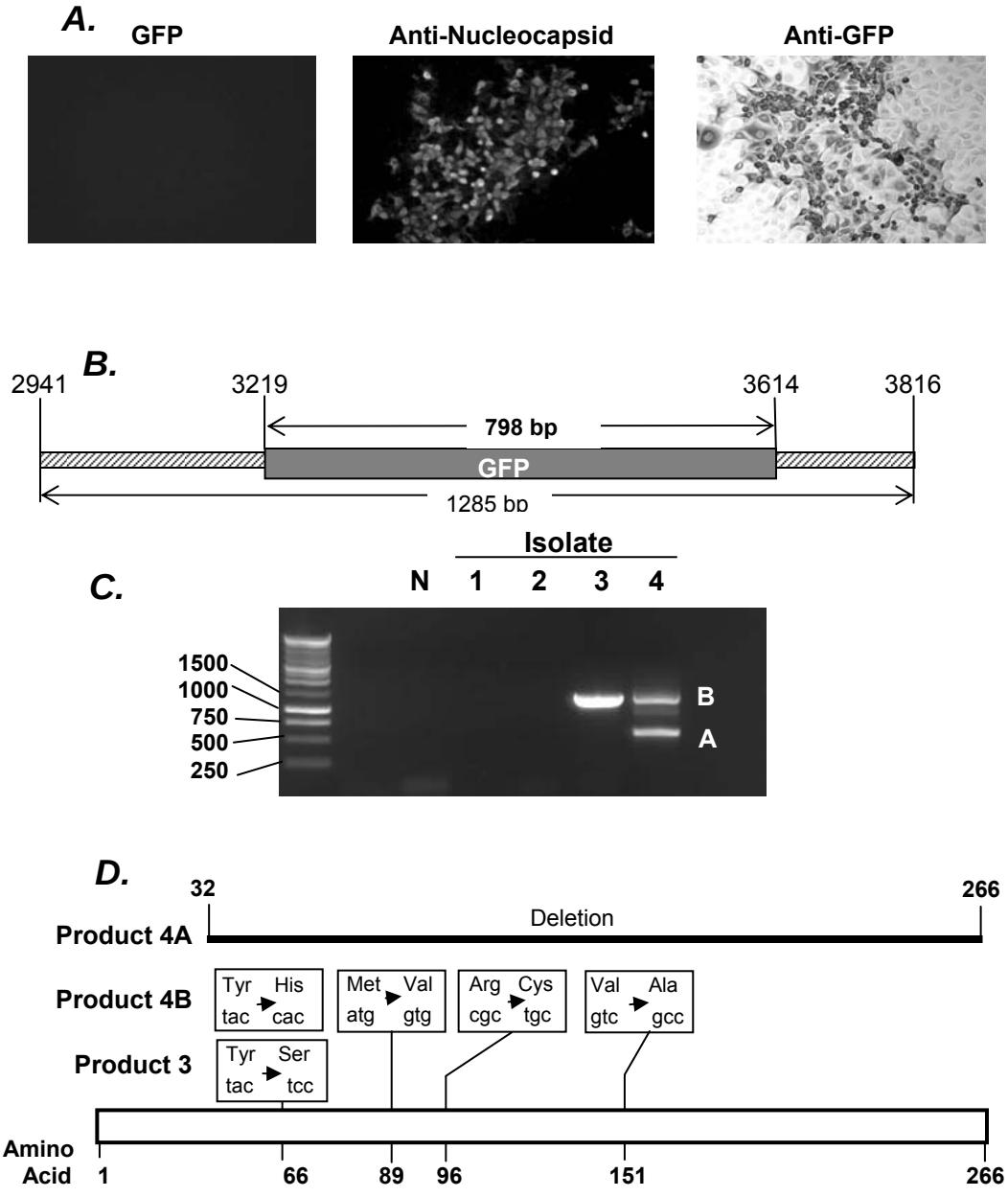


Fig.3.6 (A) Stability of EGFP fluorescence and immunoreactivity. EGFP fluorescence and anti-nucleocapsid staining in the same viral focus in cells infected with GFP-negative sera. (B and C) Molecular analysis of EGFP negative and VI positive sera. 2941F and 3816R primer sets flanking EGFP were used for RT-PCR analysis of four isolates. (D) The amino acids change and deletion of EGFP from isolate number 3 and 4.

CHAPTER 4

Subcellular localization properties of porcine reproductive and respiratory syndrome virus non-structural proteins.

ABSTRACT

Infection of cells with the prototype arterivirus, equine arteritis virus (EAV), involves the formation of replication complexes in association with ER membranes in the perinuclear region of the cell. However, there are examples of arterivirus proteins that localize to other intracellular compartments, such as the nucleus. The purpose of this study was to characterize the intracellular localization properties of the non-structural proteins (nsp's) of porcine reproductive and respiratory syndrome virus (PRRSV). Confocal microscopy of individually expressed enhanced green fluorescent protein (EGFP)-labeled fusion proteins identified nsp 2, 3, 9 and12 as predominately cytoplasmic. Nuclear accumulation was observed for nsp 1, 4, 7, 10 and 11. The main replication complex scaffolding proteins, nsp2 and 3, did not co-localize with anti-Golgi or anti-calnexin (endoplasmic reticulum marker) antibodies. Nsp2, when expressed as an EGFP-tagged protein by a recombinant PRRS virus, also failed to co-localize with endoplasmic reticulum (ER), Golgi and lysozomal associated membrane protein-1 (LAMP-1). Even though this study did not identify the membrane compartment associated with PRRSV nsp localization, these results suggest that there may be some flexibility among the arteriviruses in the source of the membranes recruited in the formation of replication complexes.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for reproductive failure and respiratory disease in neonatal pigs. PRRSV is a member of the family *Arteriviridae,* which also includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (1). The *Arteriviridae*, *Coronaviridae* and *Roniviridae* belong to a single order, *Nidovirales* (2). The common feature within the nidovirus group is the formation of a 3' co-terminal nested set of subgenomic mRNAs, which possess a common leader and a poly-A-tail (3).

The localization properties of arterivirus proteins are associated with functions related to virus replication, virion assembly/budding, and escape from host cell defenses. During EAV replication, all nsp's localize to the perinuclear region and are associated with DMVs, which are derived from ER membranes. The staining of EAV-infected cells with antibodies against the ER-associated protein, PDI, shows co-localization with antibodies prepared against the individual nsp's (8). In addition, nsp1 is also found in the nucleus (9). The N proteins of EAV, PRRSV and several coronaviruses also localize to the nucleus during infection and when expressed as GFP-labeled proteins (10, 11, 12). Presumably, nuclear localization of structural and non-structural proteins is involved in the control of nuclear processes during virus replication (9, 10, 12).

One important limitation in the study of the subcellular localization of nsp's in live virus systems is that antisera prepared against an individual nsp also cross-reacts with the much larger parental polyproteins and/or with complexes incorporating other structural and non-structural proteins. The purpose of this study was to follow the localization properties of the PRRSV nsp

products expressed as individual nsp's tagged with enhanced green fluorescent protein (EGFP) and EGFP-nsp2 fusion protein expressed by recombinant PRRSV.

MATERIALS AND METHODS

Virus and Cells

Vero and MARC-145 cell lines were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 8% fetal bovine serum, 0.008% Fungizone and 0.01% penicillin-streptomycin. The MARC-145 cell line, a derivative of MA-104 cells [32], was used in those experiments involving PRRSV-infected cells. Because of relatively poor transfection efficiencies in MARC-145 cells, experiments involving nsp cDNA expression incorporated the use of Vero cells.

nsp plasmid construction

The forward and reverse primers for PCR amplification and cloning of the individual nsp2 regions, shown in Fig. 4.1, are listed in Table 4.1. PCR and cloning was done by standard recombinant DNA techniques. Restriction sites were added to the 5′ ends of the respective sense and antisense PCR primers. For cloning the individual nsp products, total RNA was isolated from MARC-145 cells at 48 hr after infection with North American PRRSV isolate SD2393 using a QIAamp viral RNA kit (Qiagen). cDNA for PCR was prepared by reverse transcription using Superscript II (Invitrogen) and random hexamer oligonucleotide primers and according to manufacturer's recommendations. PCR amplification was performed using *pfu* polymerase (Stratagene) in a reaction volume of 100 µl containing Mg-free 1x PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 1µM of each primer and 2.5 units of polymerase and 2 µl of template.

PCR conditions for all reactions consisted of 1 cycle of 95 ^{0}C for 2 min, 35 cycles at 94 ^{0}C for 30 seconds, 55-60 ^{0}C for 30 seconds, 68 ^{0}C for 1 min per kb pair. A final extension was performed at 70 ^{0}C for 10 min. PCR products were double-digested with appropriate restriction enzymes and ligated in-frame into pEGFP-C3 (Clontech Inc). Plasmid constructs were amplified by transformation into E. coli TOP 10F cells (Invitrogen). Plasmid DNA for transfection was prepared using a Wizard miniprep kit (Promega). All constructs were sequenced prior to use.

The transfection of plasmid DNA into Vero cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Even though MARC-145 cells support PRRSV infection, Vero cells were transfected with a higher efficiency. Approximately 24 hours prior to transfection, 2 X 10^5 Vero cells were seeded into each well of a 12-well plate. Cells were transfected with 2 μg of plasmid DNA. One day after transfection, the cells were viewed under a fluorescence microscope and EGFP expression detected using a FITC excitation filter.

Immunoblot detection of EGFP-labeled proteins and immunofluorescence microscopy

For immunoblot analysis of expressed proteins, transfected cells on a 12-well plate were lysed with 100ul of 2X Laemmli sample buffer, boiled for five minutes, then separated using SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred onto a nylon membrane. Membranes were blocked at room temperature for 1 hr in 5% nonfat dry milk in PBS with 0.2% Tween-20 (PBST). After three washes with PBST, affinity-purified anti-GFP antibody (Rockland) was diluted 1:1,000 in blocking buffer and incubated with the membrane for one hr at room temperature (RT). Membranes were washed three times for 15 minutes at RT with Tris-buffered saline containing 0.2% Tween-20 (TBST). Rabbit anti-goat horseradish

peroxidase-conjugated antibody (Rockland) was diluted 1:1,000 in blocking buffer and incubated with membranes for 1 hr at RT followed by three 15 min washes with TBST. Peroxidase activity was detected using Supersignal West Pico chemiluminescent substrate (Pierce) and membranes were exposed to Kodak autoradiographic film.

For immunofluorescence confocal microscopy, coverslips of transfected cells were washed with PBS, fixed for 10 minutes with 2% paraformaldehyde in PBS, and then stained with antibodies against ER-localized protein (calnexin; mouse mAb AF18 from Abcam, PDI; mouse mAb 1D3 from MBL), Golgi localized protein (mAb 371-4 from Abcam) or LAMP-1 (CD107a; mouse mAb H4A3 from BD Pharmingen), or PRRSV N protein (mAb SDOW-17; courtesy of Eric Nelson, South Dakota State University). Mouse antibodies were detected with goat anti-mouse IgG conjugated with AlexaFluor 594 (Molecular Probes). All antibodies were diluted 1:500 in blocking buffer (PBS containing 3% normal goat sera and 0.2% saponin) and incubations performed for 1 hr at RT followed by extensive washing in PBS. In some experiments, cells were counterstained with the nuclear stain TO-PRO-3, diluted in blocking buffer. Coverslips were mounted and viewed using a Zeiss LSM 510 confocal microscope.

Construction of nsp2-EGFP expressing PRRS virus

A unique Mlu I site was inserted into nucleotide position 3,219 nt in ORF1a of the infectious cDNA clone pCMV-S-P129 (21) using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The EGFP gene of pEGFP-C3, minus the stop codon, was PCR amplified using primers with Mlu I sites on both ends and cloned into pCR2.1. (Invitrogen). The EGFP fragment was removed by cutting with Mlu I and then ligated into the Mlu I site of pCMV-S-P129 to yield the plasmid pCMV-S-P129-nsp2-EGFP. The correct insertion of EGFP was determined by DNA

sequencing. The plasmid was transfected into MARC-145 cells to produce the virus P129-nsp2-EGFP.

RESULTS

Immunoblot detection of pp1ab EGFP fusion proteins expressed in Vero cells

The cloned nsp products, based on the cleavage sites shown in Fig. 4.1 for SD-23983, a typical North American Type 2 PRRSV isolate, included nsp1 (383 amino acids), nsp2 (980 amino acids), nsp3 (446 amino acids), nsp4 (204 amino acids), nsp5 (170 amino acids), nsp7 (259 amino acids), nsp8 (45 amino acids), nsp9 (640 amino acids), nsp10 (441 amino acids), nsp11 (224 amino acids) and nsp12 (152 amino acids). The only construct not made was nsp6, which was predicted to be only 16 amino acids in length. In all constructs, EGFP was fused to the N-terminal end of the nsp. Because antibody reagents were not available for the detection of the individual PRRSV nsp products in Western blots, the fusion proteins were detected using a polyclonal anti-GFP antibody. Nearly all proteins were detected on immunoblots (Fig. 4.2). The only exceptions were nsp2, nsp5 and nsp8. The EGFP-nsp2 fusion protein was easily detected by fluorescence after transfection of Vero cells with the pEGFP-nsp2 plasmid, but was not detected when lysate proteins were transferred to nylon membranes. The negative immunoblot result may have been due to inefficient transfer of the large fusion protein to be efficiently transferred to the nylon membrane. The removal of 280 amino acids from the C-terminal end of nsp2 resulted in the truncated protein, EGFP-nsp2*, which was easily detected in Westerns (Fig. 2). The localization properties of EGFP-nsp2* in transfected Vero cells were the same as the full length GFP-nsp2 construct. The anti-GFP antibody frequently identified minor bands that were smaller than EGFP-nsp2* (see arrow in Fig. 4.2). These smaller products may represent cleavage

products that result from the action of cellular proteases. EGFP-nsp5 and 8 proteins were not detected in Westerns or by fluorescence microscopy in transfected cells. We assumed that these constructs were either unstable or lethal when expressed in Vero cells. Except for nsp1, the remaining EGFP-nsp fusion proteins migrated at their predicted sizes (Fig. 4.2). The intact EGFP-nsp1 product was predicted to migrate at approximately 71 kDa. The appearance of a smaller product on Western blots was consistent with the cleavage of nsp1 into 1α and 1β products (13). The size of the EGFP-labeled nsp1 protein, which migrated at approximately 45 kDa, was consistent with the successful autoproteolytic cleavage between residues C_{126} -R_{127}. Because the EGFP tag is located on the N-terminal end of nsp1, we assumed that only the nsp1α product was being detected in Westerns. This result indicates that the attachment of EGFP does not interfere with the autoproteolytic activity of nsp1.

Subcellular localization of EGFP- nsp's

The localization properties of arterivirus nsp's are largely based on studies of Vero and other cell lines during infection with EAV, the prototypic member of the arterivirus group. During EAV infection, both ORF1a and 1b products have been shown to localize predominately to replication complexes in the cytoplasm. In this study, we utilized confocal microscopy and EGFP labeling to determine the subcellular localization properties of the individual PRRSV nsp's in the absence of infection. The localization of the PRRSV ORF1a products, nsp 1, 2 and 3 were similar to observations reported for the corresponding EAV proteins (9, 14). For instance, PRRSV EGFP-nsp1 accumulated in both the cytoplasm and the nucleus; whereas, nsp2 and 3 were retained in the cytoplasm (Fig. 4.3B, C and D). The accumulation of EGFP-nsp1 in the nucleus was evident by the co-localization of EGFP with the nuclear stain, TO-PRO-3 (Fig.

4.3B). EGFP-labeled nsp4 and 7 localized to the cytoplasm and the nucleus. Since both proteins were expressed as intact fusion proteins (Fig. 4.2), it is unlikely that fluorescence in the nucleus was the result of the removal and diffusion of free EGFP from the cytoplasm through the nuclear pore complex (NPC) and into the cytoplasm. These observations are in contrast to Snijder et al. (14) who reported anti-EAV nsp4 and anti-nsp7 staining as predominately cytoplasmic. It should be noted that during infection, antibodies specific for EAV nsp4 or 7 would also recognize the much larger nsp3-8 polyprotein.

The mechanism for the accumulation of GFP-nsp1 (45 kDa), GFP-nsp4 (47 kDa) and GFP-nsp7 (50 kDa) in the nucleus is unclear. The molecular sizes for these proteins are near the 50 kDa cutoff attributed to proteins that can enter the nucleus by passive diffusion through the NPC [15]. The intracellular distribution of all three proteins resembled the distribution of the 33 kDa EGFP (Fig. 4.3A), suggesting that nuclear accumulation is the result of passive diffusion through the NPC. Another possibility is active transport, which is initiated through an interaction between a peptide nuclear localization signal (NLS) on the cargo and an importin receptor protein (15). A search for classical NLS motifs using the web-based computer program, PSORT (16), showed that only nsp2 and nsp7 possessed potential NLS motifs. In nsp7, the NLS motif, KKKRRR, is located at amino acid 163 of nsp7 or amino acid 2362 in pp1a. A classical NLS could participate in the shuttling of nsp7 into the nucleus. Based on peptide sequence analysis, nsp2 contained a cluster of seven classical NLS motifs located between amino acids 821 and 930 of pp1a. However, since nsp2 is clearly cytoplasmic, the NLS domains are either sterically blocked or superceded by cytoplasmic retention signals.

ORF1b codes for four proteins, a RNA polymerase protein (RdRp) nsp9; a metal-binding protein with helicase activity (M/Hel) nsp10; a NendoU domain-containing protein, nsp11; and a

hydrophobic protein of unknown function, nsp12 (5, 6, 7). All ORF1b proteins are processed via the action of SP (nsp4). The complete proteolytic processing of the ORF1b polyprotein is essential for EAV replication (17). When tagged with EGFP, PRRSV nsp9 was predominately cytoplasmic, with only a small amount of EGFP in the nucleus (Fig. 4.4A). Both EGFP-labeled nsp10 and 11 localized to both the cytoplasm and the nucleus (Fig. 4.4B and C). The localization pattern for PRRSV EGFP-nsp11 was similar to the diffuse distribution pattern described for nsp11 of EAV (18). Since Westerns showed that EGFP-labeled nsp10 and nsp11 were expressed as intact fusion proteins, it is unlikely that the accumulation of fluorescence in the nucleus was a consequence of the diffusion of free EGFP into the nucleus. As shown in the Westerns in Fig. 4.2, EGFP-labeled nsp10 and nsp11 proteins migrated at approximately 75 kDa and 65 kDa, respectively. Both fusion proteins are larger than the size of proteins that enter the nucleoplasm by passive diffusion through the NPC. A search of peptide sequences failed to identify classical NLS motifs. The smallest ORF1b product, nsp12, which was only 53 amino acids or about 40 kDa when fused to EGFP, was retained in the cytoplasm.

The absence of co-localization of nsp2, 3, 9 with ER-specific antibodies

During the replication of EAV in Vero cells, pp1ab-derived proteins assemble into replication complexes that co-localize with PDI, an ER resident protein (19). It is the re-organization and recruitment of ER membranes by viral scaffolding proteins, such as nsp2 and 3, that form the Double membrane vesicles (DMVs) (4, 8). We studied the co-localization of EGFP-labeled PRRSV nsp2 and 3 with antibodies specific for calnexin. Calnexin is an ER-resident protein that participates in calcium release and functions as a chaperone in the folding of N-glycosylated proteins (20). Confocal microscopy was used to study the distribution of the EGFP tagged nsp's

and AlexaFluor 594-labeled antibodies in the same cells. As shown in representative images in Fig. 4.5, there was no co-localization between the cytoplasmic proteins, PRRSV nsp2 and 3, and calnexin antibody, which was evident by the absence of yellow color in the merged images in Fig. 4.5. All images represented relatively thin, 4.74 μM thick sections through each cell. There was also no detectable co-localization between the organelle-specific antibody and the ORF1b protein, nsp9 (Fig. 4.5C).

Localization properties of nsp2 -EGFP expressed by a recombinant PRRS virus

Using a PRRSV cDNA infectious clone, pCMV-S-P129 (21), we attempted to construct recombinant viruses that expressed nsp-GFP fusion proteins. We prepared constructs designed to express nsp2-EGFP, nsp3-EGFP and nsp4-EGFP. Only the nsp2-EGFP virus was viable and produced fluorescent cells. Furthermore, when compared to the parent P129 virus, the EGFP-nsp2 virus replicated to the same levels in MARC-145 cells and in porcine alveolar macrophages. EGFP expression was localized in the cytoplasm; often accumulating within the perinuclear region of the cell. The localization properties of nsp2-EGFP, when expressed alone or by the EGFP virus were similar. The results of co-localization studies using antibodies against the ER or Golgi or autophagic markers showed no co-localization with EGFP in cells infected with the P129-nsp2-EGFP virus (Fig. 4.6A and B, C and D).

Along with the non-structural proteins, structural proteins, such as the N protein of EAV, also localize to replication complexes (22). Furthermore, confocal microscopy shows co-localization between anti-N and anti-nsp2 antibodies in EAV-infected Vero cells. However, the participation of N in the function of the replication complex is unclear, since the expression of N not essential for genome replication or the synthesis of viral subgenomic mRNAs (22). MARC-

145 cells infected with the recombinant PRRSV isolate, P129-nsp2-EGFP, were fixed and stained with AlexaFluor 594-labeled anti-nucleocapsid mAb, SDOW-17. The distribution of the EGFP label was primarily perinuclear and sometimes polarized to one side of the cell, a pattern observed for the localization of EAV ORF1a and 1b products (14, 18). The results presented in Fig. 4.6E show an example of PRRSV N protein distribution, with localization of N in both the nucleus and the cytoplasm. Nuclear staining localized to a region of the nucleus corresponding to the nucleolus (11, 12). The merging of EGFP and N protein images in the same cell showed regions of co-localization, which were evident by areas of yellow staining in the merged images. These data indicate that similar to EAV, the PRRSV N protein localizes with nsp2 in replication complexes.

DISCUSSION

How a viral protein functions in the cell during virus replication is related to its location within a particular subcellular compartment. In the case of the nidoviruses, both structural and non-structural proteins have been shown to localize to the cytoplasm, the site of replication and virion assembly and to the nucleus, a mechanism to modify host cell processes. In this study, we followed the intracellular distribution properties of nine PRRSV nonstructural proteins tagged with EGFP. Even though the proteolytically processed pp1ab gene products of arteriviruses assemble as part of a replication complex in the cytoplasm, at least five PRRSV non-structural proteins, nsp1α, 4, 7, 10 and 11, showed some accumulation in the nucleus. Those nsp's possessing transmembrane segments, such as nsp2 and 3, were retained in the cytoplasm.

Previous studies describing the distribution of EAV ORF1a and 1b products identified only nsp1 as being nuclear (9). The nsp's of EAV localize to replication complexes in the perinuclear

region of the cell. For the smaller PRRSV nsp products, such as EGFP-labeled nsp1α, 4 and 7, the presence of the EGFP-labeled protein in the nucleus can be easily explained by passive diffusion from the cytoplasm, through the NPC and then into the nucleoplasm. Only one PRRSV nuclear protein nsp7, possessed a classical NLS motif; however, whether this peptide domain functions as a legitimate NLS remains unknown. The two PRRSV ORF1b proteins that localized to the nucleus were nsp10 and 11. Unlike the ORF1a products that localized to the nucleus, nsp10 and 11, when tagged with EGFP, exceeded the 50 kDa cutoff size typically associated with proteins that can move into the nucleus by passive diffusion. Both proteins lack a classical NLS. Accumulation in the nucleus for these larger proteins could be the result of the use of a non-classical NLS or by co-transport with a cellular protein.

The role that localization of PRRSV nsp's to the nucleus might play during virus replication is not known. In the case of EAV, nsp1 is essential for subgenomic mRNA synthesis, but is dispensable for genome replication (23). Using a yeast two-hybrid system, (23) showed an association between EAV nsp1 and a cellular protein, p100, a transcription factor. It was postulated that EAV nsp1 could be involved in the recruitment of p100 into the cytoplasmic replication complexes. Another possibility is that the association between nsp1 and p100 in the nucleus is involved in regulating host cell transcription (23). It remains to be determined if PRRSV nsp1 associates with p100 or other transcription-related proteins. In EAV, nsp10 and 11 are RNA binding proteins involved in viral transcription and genome replication. nsp10 possesses NTPase and RNA/DNA duplex-unwinding activities (5, 6). nsp11 contains a NendoU domain, which is conserved in all nidoviruses and shares similarities to the XendoU endoribonuclease of the *Xenopus laevis.* XendoU is a RNA binding protein that participates in the processing of nucleolar RNA (7). Mutagenesis of conserved amino acid residues in the

NendoU of EAV affect both genomic and subgenomic RNA synthesis, including mutations that were lethal. Interestingly, mutations that inhibited endonuclease activity are non-lethal, but result in a reduced plaque size (24). For EAV, the ORF1b products are perinuclear (18). However, antisera against a peptide in nsp11 of EAV recognizes the appropriate 26 kDa product, and immunofluorescence staining of infected cells reveals a nonspecific distribution pattern, with staining throughout the cell. It is tempting to speculate that PRRSV transcription regulatory proteins, such nsp10 and 11, could be transiently shuttled into the nucleus and influence the transcription and processing of host cell RNAs.

Positive polarity, single-stranded RNA viruses typically incorporate cell membranes derived from organelles into the formation of replication complexes. The replication complexes are the factories that produce viral nucleic acids, including viral mRNAs, genomes and anti-genomes. For the nidoviruses, including the coronaviruses and arteriviruses, replication complexes are formed in association with double membrane vesicles (DMVs). In the case of the arteriviruses, the scaffolding proteins nsp2 and 3 are sufficient to form DMV-like structures (4). Scaffolding proteins also anchor and position the other the nsp components, including the RdRP and helicase within the replication complex. At least one arterivirus structural protein, N, is also associated with the replication complex; however, N is not required for viral RNA synthesis (22). Replication culminates in the assembly of virions and internal budding from the ER/Golgi region. For RNA viruses in general, there are several sources of membranes that are recruited into the replication complexes, including the ER, lysosomes, endosomes, Golgi, autophagosomes and mitochondria (25, 26, 27, 28, 29, 30). In the case of the nidoviruses, immunofluorescence staining with nsp-specific antibodies shows that replicase subunits localize to the perinuclear region of the cell. For coronaviruses, such as MHV, the DMVs are associated with membranes

derived from autophagic vacuoles (28). In contrast, for the arterivirus, EAV, the DMVs are derived from the ER (8). Recently, Snijder et al showed that the DMV structures formed during SARS-CoV infection of Vero cells are not associated with autophagic vacuolar proteins, but with membranes derived from the ER. Therefore, within the nidoviruses, there appears to some flexibility in the types of membranes that can be incorporated into replication complexes. The results from this study showed that none of the EGFP-nsp products studied colocalized with either Golgi and ER or autophagic markers. One possibility is that the EGFP-labeled proteins behave differently from nsp's produced during virus infection. However, the incorporation of EGFP-nsp2 into an infections clone showed that GFP fluorescence was able to co-localize with the N protein, but still failed to co-localize with the ER marker, calnexin and PDI, the Golgi and autophagic marker, LAMP-1. Again, the possibility exists that EGFP-labeled nsp2 may behave differently from the wild type nsp2. Another possibility is that similar to the coronaviruses, there maybe differences within the arteriviruses in the types of membranes incorporated into DMVs. It remains to be determined the source of the membranes utilized by the PRRSV replication complex.

REFERENCES

1. **Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A.,** 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80, 307-315.
2. **Bautista, E.M., Faaberg, K.S., Mickelson, D., McGruder, E.D.,** 2002. Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology. 298, 258-270.
3. **Dea, S., Gagnon, C.A., Mardassi, H., Pirzadeh, B., Rogan, D.,** 2000. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145, 659-688.
4. **Pasternak, A.O., Spaan, W.J., Snijder, E.J.,** 2006. Nidovirus transcription: how to make sense...? J. Gen. Virol. 87, 403-421.
5. **Prentice, E., Jerome, W.G., Yoshimori, T., Mizushima, N., Denison, M.R.,** 2004. Coronavirus replication complex formation utilizes components of cellular autophagy. J. Biol. Chem. 279, 10136-10141.
6. **Restrepo-Hartwig, M., Ahlquist, P.,** 1999. Brome mosaic virus RNA replication proteins 1a and 2a colocalize and 1a independently localizes on the yeast endoplasmic reticulum. J. Virol. 73, 10303-10309.
7. **Rowland, R.R., Kervin, R., Kuckleburg, C., Sperlich, A., Benfield, D.A.,** 1999. The localization of porcine reproductive and respiratory syndrome virus nucleocapsid protein to

the nucleolus of infected cells and identification of a potential nucleolar localization signal sequence. Virus. Res. 64, 1-12.

8. **Seybert, A., van Dinten, L.C., Snijder, E.J., Ziebuhr, J.,** 2000. The biochemical characterization of the equine arteritis virus helicase suggests a close functional relationship between arterivirus and coronavirus helicases. J. Virol. 74, 9586–9593.
9. **Snijder, E.J., Wassenaar, A.L., Spaan, W.J.,** 1992. The 5' end of the equine arteritis virus replicase gene encodes a papainlike cysteine protease. J. Virol. 66, 7040-7048.
10. **Snijder, E.J., Wassenaar, A.L., Spaan, W.J., Gorbalenya, A.E.,** 1995. The arterivirus Nsp2 protease. An unusual cysteine protease with primary structure similarities to both papain-like and chymotrypsin-like proteases. J. Biol. Chem. 270, 16671-16676.
11. **Snijder, E.J., Wassenaar, A.L., van Dinten, L.C., Spaan, W.J., Gorbalenya, A.E.,** 1996. The arterivirus nsp4 protease is the prototype of a novel group of chymotrypsin-like enzymes, the 3C-like serine proteases. J. Biol. Chem. 271, 4864-4871.
12. **Snijder, E.J., Meulenberg, J.J.,** 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961-979.
13. **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, 6335-6345.
14. **Snijder, E.J., van Tol, H., Roos, N., Pedersen, K.W.,** 2001. Non-structural proteins 2 and 3 interact to modify host cell membranes during the formation of the arterivirus replication complex. J. Gen. Virol. 82, 985-994.
15. **Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L., Guan, Y., Rozanov, M., Spaan, W.J., Gorbalenya, A.E.,** 2003. Unique and conserved features of

genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. 331, 991-1004.

16. **Snijder, E.J., van der Meer, Y., Zevenhoven-Dobbe, J., Onderwater, J.J., van der Meulen, J., Koerten, H.K., Mommaas, A.M.,** 2006. Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. J. Virol. 80, 5927-5940.
17. **Tijms, M.A., van der Meer, Y., Snijder, E.J.,** 2002. Nuclear localization of non-structural protein 1 and nucleocapsid protein of equine arteritis virus. J. Gen. Virol. 83, 795-800.
18. **Tijms, M.A., Snijder, E.J.,** 2003. Equine arteritis virus non-structural protein 1, an essential factor for viral subgenomic mRNA synthesis, interacts with the cellular transcription co-factor p100. J. Gen. Virol. 84, 2317-2322.
19. **Tu, B.P., Weissman, J.S.,** 2004. Oxidative protein folding in eukaryotes: mechanisms and consequences. J. Cell. Biol. 164, 341-346.
20. **Van der Meer, Y., van Tol, H., Locker, J.K., Snijder, E.J.,** 1998. ORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. J. Virol. 72, 6689–6698.
21. **Van der Meer, Y., Snijder, E.J., Dobbe, J.C., Schleich, S., Denison, M.R., Spaan, W.J., Locker, J.K.,** 1999. Localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. J. Virol. 73, 7641-7657.
22. **Van Dinten, L.C., Wassenaar, A.L.M., Gorbalenya, A.E.,** 1996. Processing of the equine arteritis virus replicase ORF1b protein: Identification of cleavage products containing the putative viral polymerase and helicase domains. J. Virol. 70, 6625–6633.

23. **Van Dinten, L.C., Rensen, S., Gorbalenya, A.E., Snijder, E.J.,** 1999. Proteolytic processing of the open reading frame 1b-encoded part of arterivirus replicase is mediated by nsp4 serine protease and is essential for virus replication. J. Virol. 73, 2027-2037.

24. **Vassilakos, A., Cohen-Doyle, M.F., Peterson, P.A., Jackson, M.R., Williams, D.B.,** 1996. The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules. EMBO. J. 15, 1495-1506.

25. **Wassenaar, A.L., Spaan, W.J., Gorbalenya, A.E., Snijder, E.J.,** 1997. Alternative proteolytic processing of the arterivirus replicase ORF1a polyprotein: evidence that NSP2 acts as a cofactor for the NSP4 serine protease. J. Virol. 71, 9313-9322.

26. **Wensvoort,G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemrad, M., deKluyer, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T.H., Verheijden, J.H.M., Braamskamp, J.,** 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet. Quarterly. 13, 121-130.

27. **Wieringa, R., de Vries, A.A., Rottier, P.J.,** 2003. Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. J. Virol. 77, 6216-6226.

28. **Wissink, E.H., Kroese, M.V., van Wijk, H.A., Rijsewijk, F.A., Meulenberg, J.J., Rottier, P.J.,** 2005. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J. Virol. 79, 12495-12506.

29. **Wootton, S., Yoo, D., Rogan, D.,** 2000. Full-length sequence of a Canadian porcine

reproductive and respiratory syndrome virus (PRRSV) isolate. Arch. Virol. 145, 2297-2323.

30. **Wu, W.H., Fang, Y., Farwell, R., Steffen-Bien, M., Rowland, R.R.R.,** 2001. A 10 kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology. 287, 183-191.

31. **Wu, W.H., Fang, Y., Rowland, R.R., Lawson, S.R., Christopher-Hennings, J., Yoon, K.J., Nelson, E.A.,** 2005. The 2b protein as a minor structural component of PRRSV. Virus. Res. 114, 177-181

32. **Yoo, D., Wootton, S.K., Li, G., Song, C., Rowland, R.R.,** 2003. Colocalization and interaction of the porcine arterivirus nucleocapsid protein with the small nucleolar RNA-associated protein fibrillarin. J. Virol. 77, 12173-12183.

33. **Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E.,** 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J. Gen. Virol. 81, 853-879.

Table 4.1. Sequence of primers used to amplify nonstructural proteins of PRRSV

Primer	Sequence[1]	Primer	Sequence[1]
Nsp1		nsp7	
For	5'-*CTCGAG*ATGTCTGGGATACTTGATCGG	For	5'-*CTCGAG*TCTCTGACTGGTGCCCTCGC
Rev	5'-*GAATTC*TAAGCCGTACCACTTGTGACTGCCAA	Rev	5'-*GGATCC*GCTTCCCATTGGACTCTTCC
Nsp2		nsp8	
For	5'-*GAGCTC*GCTGGAAAGAGAGCAAGAAAA	For	5'-*CTCGAGG*CAAAGCTTTCTGTGGAGC
Rev	5'-*GGATCC*GCCCAGTAACCTGCCAAGAATGG	Rev	5'-*GGATCC*TAGCAGTTTAAACACTGCTC
Nsp2*[2]		nsp9	
For	5'-*GAGCTC*ATGGCTGGGAGGAGAGCAAGG	For	5'-*CTCGAG*ATGGGAGCAGTGTTTAAACTGC
Rev	5'-*GGATCC*TAAGGTCCCACATGCGGGAAAGCC	Rev	5'-*GGATCC*TAACTCATAATTGGATCTGAGTTT
Nsp3		nsp10	
For	5'-*CTCGAG*GGGGCACGTTGCATCTGGCA	For	5'-*CTCGAG*ATGGGGAAGAAGTCGAGAGTGT
Rev	5'-*GGATCC*CTCAAGAAGAGACCCAAGCTG	Rev	5'-*GGATCC*TAATTCTAGATCAGCACAAATGGC
Nsp4		nsp11	
For	5'-*CTCGAG*ATGGGTGCTTTCAGAACTCGAAAG	For	5'-*CTCGAG*ATGGGTACGAGCTCTCCGCTCC
Rev	5'-*GGATCC*TAATTCCAGTTCAGGTTTGGCAGCAA	Rev	5'-*GGATCC*TAATTCAAGTTGGAAATAGGCTGT
nsp5		nsp12	
For	5' *CTCGAG*GGGGGCCTCTCCACCGTCCA	For	5'-*CTCGAG*GGTCGCTATTTCACCTGGTA
Rev	5' *GGATCC*CCCTCGGCAAAGTATCGCAAGA	Rev	*5-'GGATCC*TCAATTCAGGCCTAAAGTTG

[1]Restriction enzyme recognition sites added to the primers used for cDNA synthesis are underlined. Primer sequence are based on the SD23983 PRRSV strain of North American PRRSV. The restrictions used for cloning were *Sac* 1 (GAGCTC), *Xho* 1(CTCGAG), *Eco* R1 (GAATTC) and *Bam* H1 (GGATCC).
[2]Truncated nsp2.

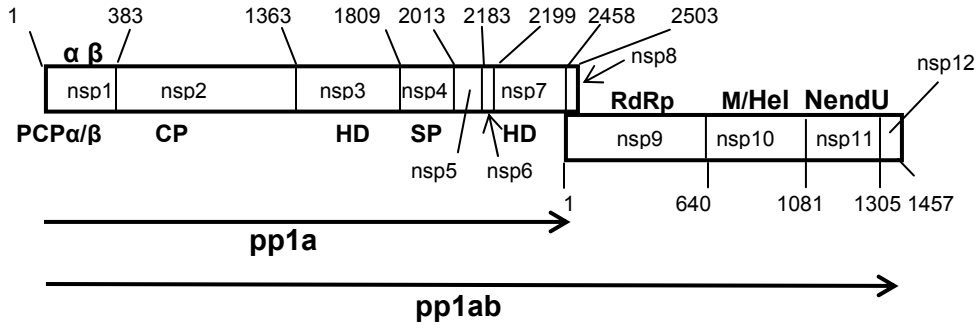


Fig. 4.1. Processing of pp1a and pp1ab proteins of PRRSV. The cleavage sites and products are identified based on the analysis of EAV and PRRSV processing of ORF1a and 1b gene products. PCB, papain-like cysteine protease; CP, chymotrypsin-like protease; HD, hydrophobic domain; SP, serine protease, RdRp, RNA-dependent RNA polymerase; M/Hel, metal binding/helicase; NendoU, NendoU domain.

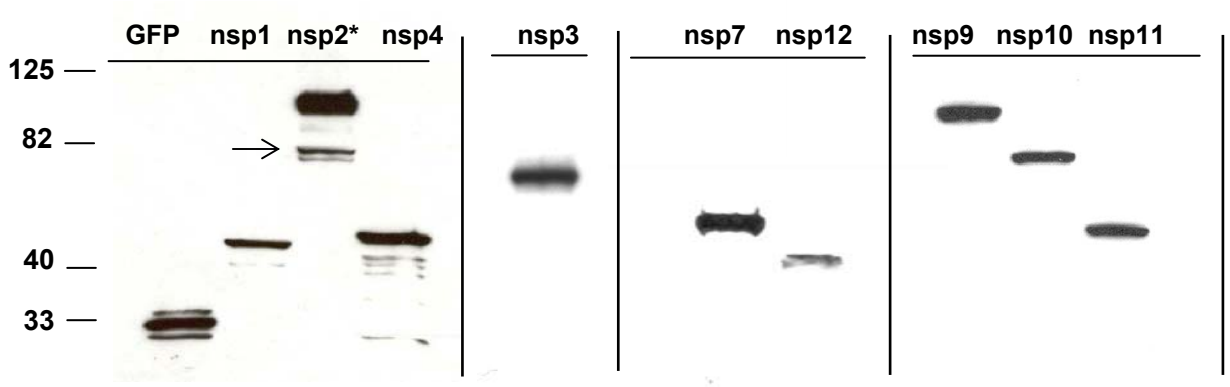


Fig. 4.2. Western blot of PRRSV EGFP-nsp fusion products. Plasmid constructs were prepared that expressed EGFP fused to the N-terminal end of each nsp. Fusion constructs were expressed in Vero cells and detected in immunoblots using a polyclonal anti-GFP antibody. nsp2* is a truncated fusion protein with 280 amino acids removed from the C-terminal end of nsp2. The arrow identifies a smaller product from cells expressing EGFP-nsp2*.

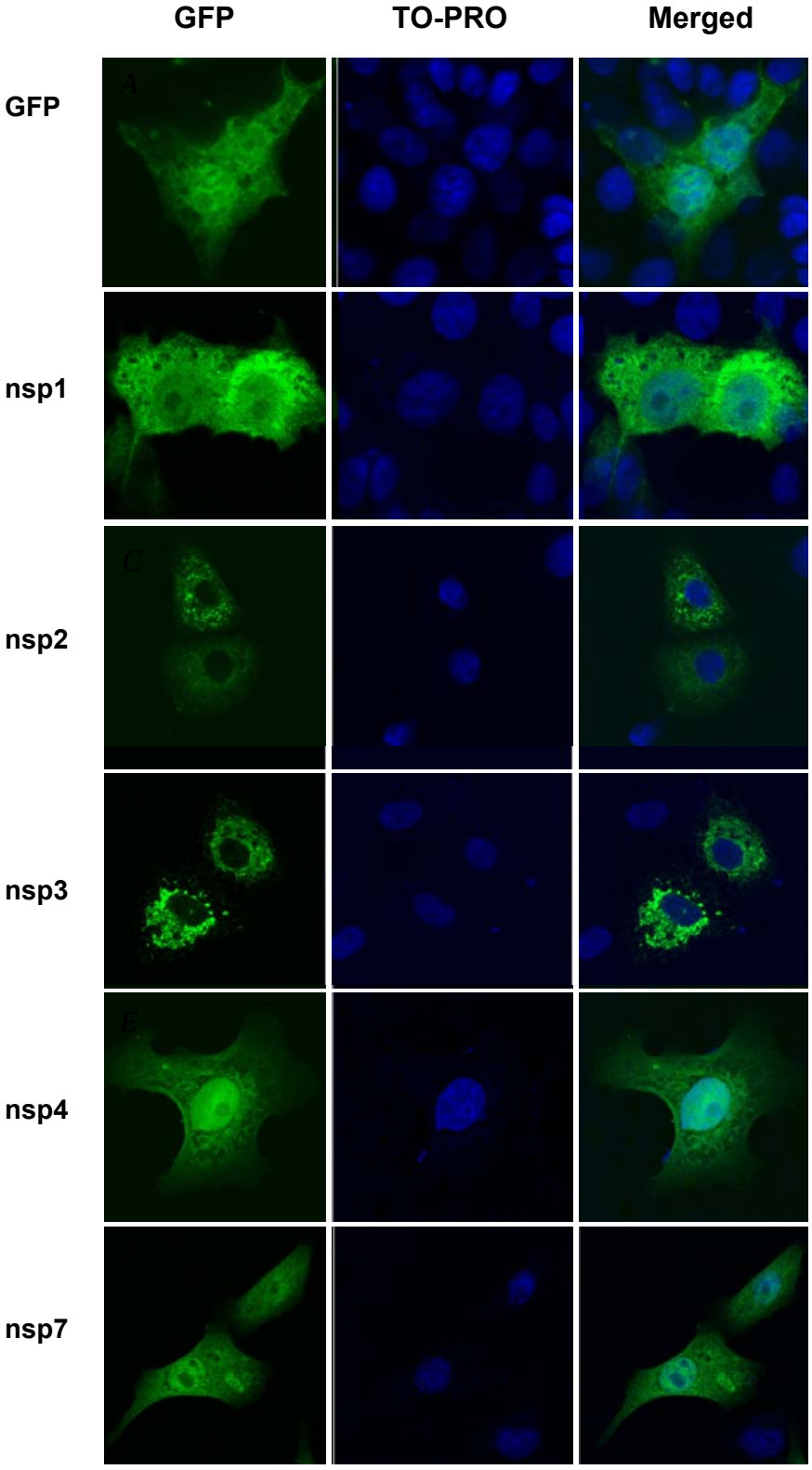

GFP
TO-PRO
Merged
GFP
nsp1
nsp2
nsp3
nsp4
nsp7

Fig. 4.3. Confocal microscopy of ORF1a EGFP-nsp products expressed in Vero cells. Cells were transfected with plasmid constructs expressing ORF1a nsp cDNA products and viewed the next day. Cells were counterstained with the nuclear stain, TO-PRO-3 (blue). Each image represents a 4.74 μM thick slice. In order from right to left: EGFP expression, TO-PRO-3 staining and merged image.

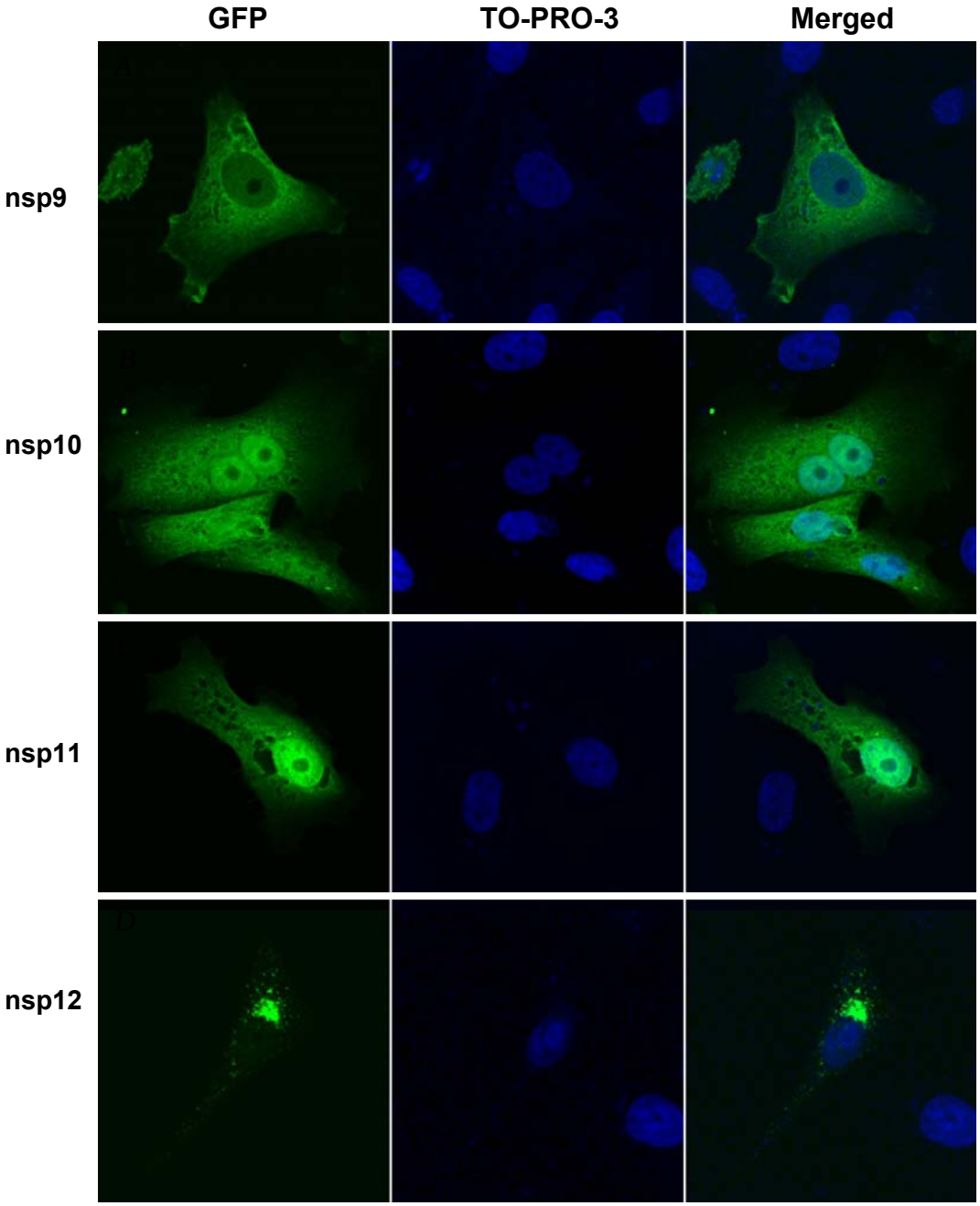

GFP
TO-PRO-3
Merged
nsp9
nsp10
nsp11
nsp12

Fig. 4.4. Confocal microscopy of ORF1b EGFP-nsp products expressed in Vero cells. Cells were transfected with plasmid constructs expressing ORF1b nsp cDNA products and viewed the next day. Cells were counterstained with the nuclear stain, TO-PRO-3 (blue). Each image represents a 4.74 μM thick slice. In order from left to right: EGFP expression, TO-PRO-3 staining and merged image.

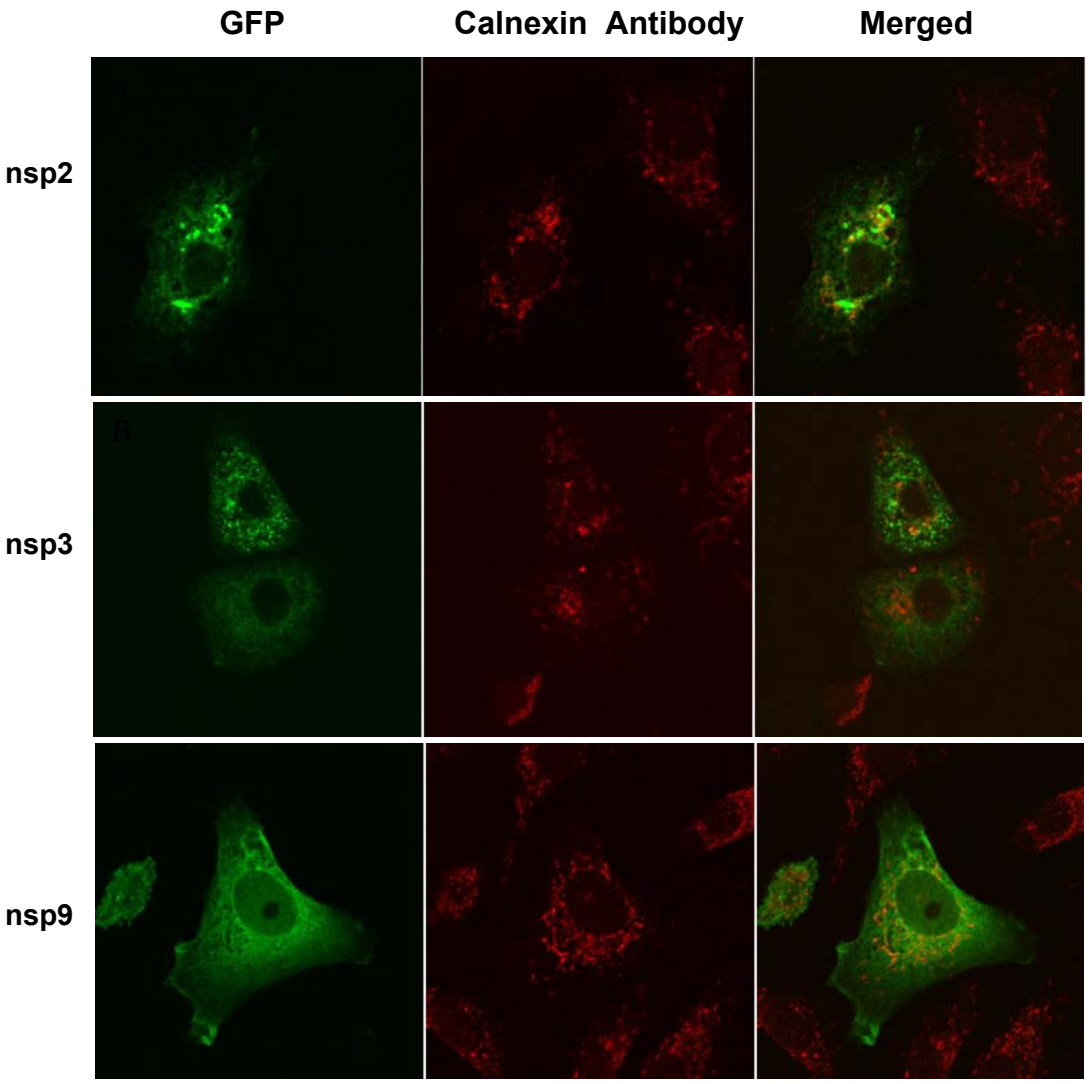


Fig. 4.5. Colocalization of anti-calnexin and GFP. Confocal microscopic images of cells transfected with EGFP-nsp plasmids and stained with the anti-calnexin antibody, mAb AF18. The mouse mAb was detected with AlexaFluor 594-labeled anti-mouse (red color). Each image represents a 4.74 μM thick slice. In order from left to right: EGFP expression, AlexaFluor staining and the merged image of the same cells.

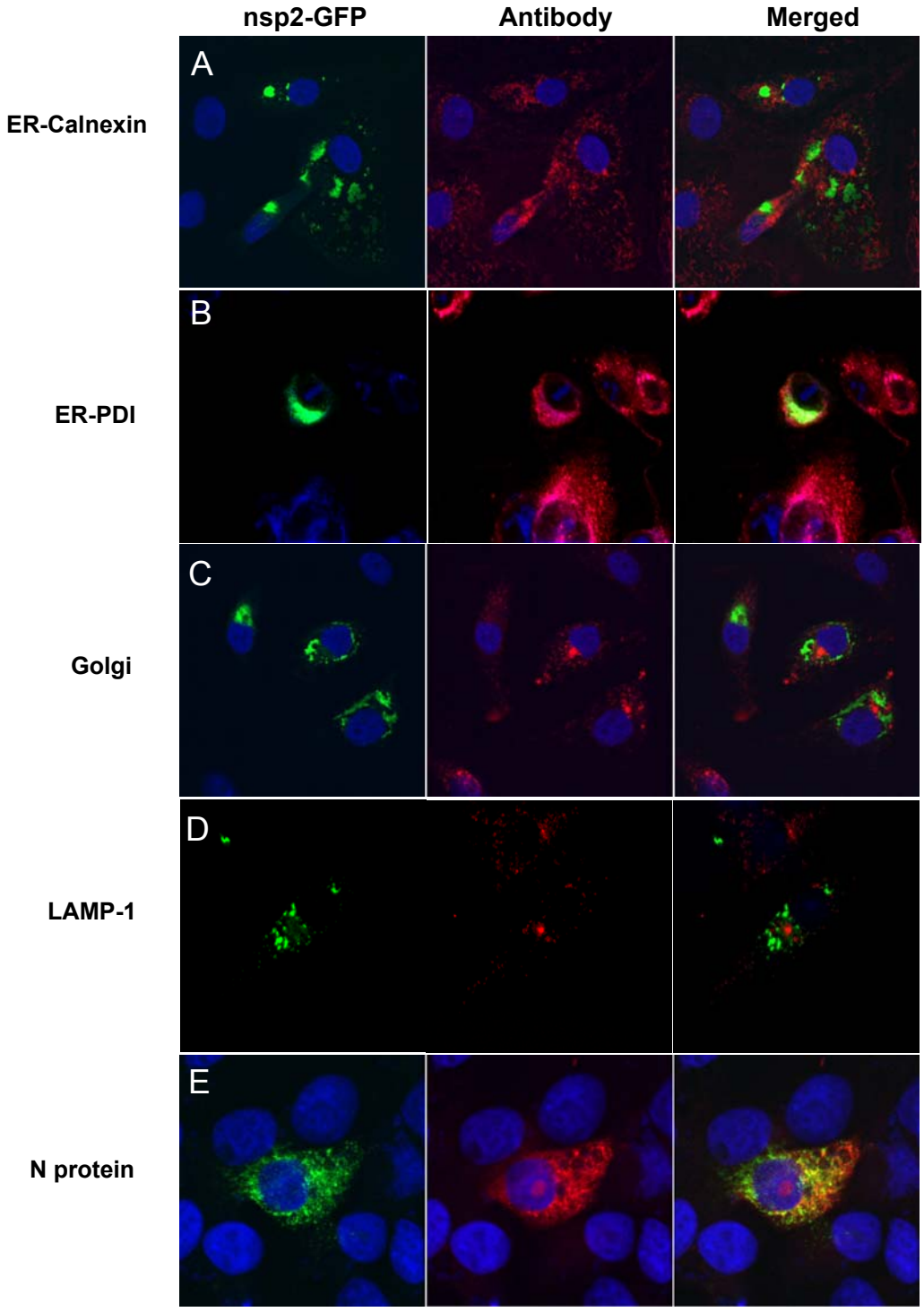

nsp2-GFP
Antibody
Merged
A
ER-Calnexin
B
ER-PDI
C
Golgi
D
LAMP-1
E
N protein

Fig. 4.6. Distribution of anti-calnexin, anti-PDI, anti-Golgi, anti-autophagic marker (LAMP-1), and anti-PRRSV N protein staining in MARC-145 cells infected with P129-nsp2-EGFP. Confocal microscopic images showing co-localization of nsp2-EGFP with anti-calnexin (mAb AF18), anti-PDI (mAb 1D3), anti-Golgi (mAb 371-4) and anti-LAMP-1 (mAb H4A3), anti-nucleocapsid (mAb). Cells were infected with PRRSV isolate, P129-nsp2-EGFP, fixed approximately 48 hr later and stained with antibody. Mouse antibodies were detected with AlexaFluor 594-labeled anti-mouse (red color). Each image represents a 4.74 μM thick slice. In order from left to right: EGFP expression, AlexaFluor staining and the merged image of the same cells.

CONCLUSION AND FUTURE PROSPECTS

Despite the tremendous efforts invested in controlling PRRSV infections, the virus continues to plague the swine industry and damage pig production worldwide. PRRSV infects immune cells and modifies the normal host immune function. The host response to infection is poor and the virus persists in infected pigs for prolonged periods of time. Genetic and antigenic variants rapidly arise, and an effective and safe vaccine is still unavailable. The recent development of reverse genetics systems for PRRSV allows us to engineer the viral genome and to create genetically defined mutant viruses. This study focused on analysis of the multifunctional nonstructural protein (nsp2) that could be a site of foreign gene expression. The usefulness as a viral vector can enlighten an effective gene therapy road against harmful animal viral diseases. Also, our marker PRRSV virus without relatively conserved 131 amino acid region can pave the way for DIVA vaccine for the first time in the PRRSV research field. For better vaccine strategy, the possibility of converting live vaccine into virulent form and recombination among vaccine strains and field isolates can't be excluded.

The next major questions selected, coupled with the capacity to isolate large mutants in each of the PRRSV nonstructural proteins (nsps), could include. (1) Are any nsps beside nsp2 nonessential? (2) Is the gene order of nsps critical? (3) Are nsps interchangeable between Type 1 and 2 PRRSV? (4) How do the replication complexes form on membranes? (5) What nsp complexes regulate discontinuous transcription and synthesis of genome-length and subgenomic-length mRNAs and negative-strand RNAs? (6) What are the structure-function relationships within and between various nsps? (7) What are the functions of each of the nsps, and how do they influence the pathogenesis of PRRSV?

Using the available infectious PRRSV cDNA clones, a specific solution against fundamental questions of PRRSV genetics will be suggested sooner or later. This information will undoubtedly enable a second generation of safe and effective PRRSV vaccine.