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Identification of virulence determinants of porcine reproductive and respiratory syndrome virus through construction of chimeric clones

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ABSTRACT

In order to determine virulence associated genes in porcine reproductive and respiratory syndrome virus (PRRSV), a series of chimeric viruses were generated where specific genomic regions of a highly virulent PRRSV infectious clone (FL12) were replaced with their counterparts of an attenuated vaccine strain (Prime Pac). Initial genome-wide scanning using a sow reproductive failure model indicated that non-structural (ORF 1a and 1b) and structural (ORF2-7) genomic regions appear to be sites where virulence determinants of PRRSV may reside. These results thus confirm the multigenic character of PRRSV virulence. Additional chimeras containing each individual structural ORFs (2 through 7) of Prime Pac and ORF5 of Neb-1 (parental strain of Prime Pac) within the FL12 backbone were generated and tested individually for further mapping of virulence determinants. Our results allow to conclude that NSP3–8 and ORF5 are the location of major virulence determinants, while other virulence determinants may also be contained in NSP1–3, NSP10–12 and ORF2.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of swine worldwide, characterized by reproductive failure such as late-term abortions in sows, respiratory disease, and increased mortality in young pigs. A recent analysis of the economic impact of PRRS indicates that losses due to this disease are estimated to be greater than $ 560 million each year in the United States alone (Neumann et al., 2005). The causative agent, PRRSV, belongs to the family *Arteriviridae* in the order *Nidovirales* (Cavanagh, 1997; Posthuma et al., 2006) that also includes lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV). The genome of PRRSV consists of a linear, positive-stranded, single molecule of RNA of approximately 15 kb in length, possessing a 5′ cap structure and 3′ poly (A) tail. The genome contains 9 open reading frames (ORFs) flanked by untranslated regions (UTRs) at the 5′- and 3′-termini (Conzelmann et al., 1993; Meulenberg et al., 1993; Snijder and Meulenberg, 2001; Wu et al., 2005). The first 2 ORFs located at the 5′-end of the viral genome (ORFs 1a and ORF1ab) occupy around 80% of the viral genome and encode viral non-structural proteins (NSPs) which are involved in viral polyprotein processing and replication (Bautista et al., 2002; Meulenberg et al., 1993; Snijder and Meulenberg, 1998, 2001). The remaining 7 ORFs (ORF2a, 2b, 3–7) at the 3′-end of the viral genome encode viral structural proteins and are translated from a co-terminal nested set of sub-genomic mRNAs with a common leader sequence at the 5′-end (Dea et al., 2000; Meulenberg et al., 1995a, 1997; Wu et al., 2001, 2005).

PRRSV strains circulating in the field are highly diverse. Some studies have compared the pathogenicity of PRRSV strains and have described a range of degree of virulence among PRRSV strains (Halbur et al., 1995, 1996). However, a typical avirulent or attenuated PRRSV phenotype corresponds to some of the live commercial vaccines that have been developed (Gorcyca et al., 1995). These modified live vaccines are considered to be the only PRRS vaccines with well documented capacity to induce effective protection against PRRSV infections (Osorio et al., 1998; Zuckermann et al., 2007), especially if the challenge and vaccine strains maintain close homology. The Prime Pac (PP) attenuated vaccine has been previously demonstrated to control PRRS effectively in both reproductive and respiratory disease models (Hesse, 1996a,b; Hesse et al., 1997). This PP and other attenuated PRRSV strains used for vaccination were developed by conventional methodology involving multiple serial passages in a continuous cell line, a process that is concomitant with a progressive loss of the *in vivo* virulent phenotype.

Previous studies, based exclusively on sequence comparisons between parental virulent and their avirulent derivative strains have suggested that important determinants of virulence would be scattered throughout the PRRSV genome, and may include: 5′ UTR, ORFs 1a (NSP2), 1b (NSP10), 2, 3, 4, 5 and 6 (Allende et al., 2000; Grebennikova et al., 2004; Madsen et al., 1998; Oleksiewicz et al., 1999; Oleksiewicz et al., 2002; Storgaard et al., 1999; Yang et al., 1998; Yuan et al., 2001). Furthermore, many of the mutations are not necessarily related to *in vivo* virulence but rather to the adaptation of

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the viral strain to grow in the cultured host cell. In spite of these *a priori* inferences, the actual molecular basis for the virulence and attenuation of PRRSV still needs to be determined. Live attenuated vaccines can revert back to virulence, mainly due to the natural properties of RNA viruses to easily mutate under selective pressures. A number of vaccine-related revertants have been previously reported (Botner et al., 1999, 1997; Madsen et al., 1998; Nielsen et al., 2001, 2002; Stadejek et al., 2002; Storgaard, Oleksiewicz, and Botner, 1999); thus the understanding of the molecular basis of attenuation of PRRSV virulence is critical for future generation of novel, more rationally attenuated PRRSV vaccines.

Our laboratories have previously generated and characterized a highly virulent PRRSV strain (vFL12) derived from a full-length PRRSV infectious cDNA clone (FL12) (Truong et al., 2004), and more recently we constructed an infectious clone of attenuated PP vaccine strain (vPP18) (Kwon et al., 2006). Sequence comparisons indicated that these two strains exhibit approximately 90% homology with a number of sequence differences distributed throughout the genome. Moreover, the biological properties of these infectious clone-derived viruses were substantiated in either a young pig (vFL12) or a pregnant sow models (vFL12 and vPP18) by unequivocally reproducing the highly virulent and attenuated phenotypes of parental viruses respectively (Kwon et al., 2006). In the present study, we describe the generation of a series of chimeric viruses established between two different PRRSV strains of divergent virulence phenotypes and their use for *in vivo* studies (sow reproductive failure model) to determine the molecular basis of the PRRSV virulence and attenuation. Our results indicate that PRRSV virulence is multigenic, with the PRRSV virulence residing in both the non-structural and structural genes. Among the non-structural genes, NSP3–8 regions are the location of major virulence determinants while ORF5, encoding glycoprotein (GP) 5, is the principal structural determinant of virulence.

Results and discussion

Construction of chimeric clones and recovery of viable chimeric viruses

By systemically exchanging genomic regions and generating a series of chimeric viruses spanning the entire genome of PRRSV, we confirmed specific regions that are associated with virulence. Similar approaches were reasonably successful to demonstrate the determinants of virulence, plaque phenotype, pathogenesis and immunogenicity in several other viruses, such as swine vesicular disease virus (Kanno et al., 1999), porcine circovirus type 1 and 2 (Fenaux et al., 2003), classical swine fever virus (Risatti et al., 2005), foot- and mouth disease virus (Beard and Mason, 2000), simian immunodeficiency virus (Haddrick et al., 2001) and vaccinia virus (Kim et al., 2003). Importantly, a recent report validates the development of chimeras between a virulent and a vaccine strain of PRRSV and the study of the biological properties of these chimeras by inoculation of young pigs. The findings suggest that both structural and non-structural regions of PRRSV genome may be important for virulence (Wang et al., 2008).

With the highly virulent FL12 full-length infectious clone serving as the genomic backbone, a series of full-length chimeric cDNA clones containing various genomic regions spanning the entire genome of PP vaccine strain were generated. These clones, shown in Fig. 1, contain: 5′ UTR and NSP1 and part of NSP2 (cP5U.NSP1.2); part of NSP2 and part of NSP3 (cPNSP2.3); part of NSP3 to NSP8 (cPNSP3.8); NSP9 (cPNSP9); NSP10 to NSP12 (cPNSP10.12); ORF3 to 7 and 3′ UTR (cPORF3.3U) genes; and the entire region spanning all the structural genes and 3′ UTR (cPORF2.3U) of the PP vaccine virus genome (Fig. 1). Likewise, additional chimeric viruses containing individual structural genes of PP vaccine within the context of the FL12 genomic backbone were also constructed. In these cases a full-length infectious clone (PP18) derived from PP vaccine virus described previously (Kwon et al., 2006) was used to provide genomic components of PP vaccine virus to the FL12 backbone in order to generate chimeric clones containing individual structural genes of PP vaccine virus or ORF5 of Neb-1, the parental strain of PP as shown in Fig. 2. This approach generated a series of chimeric strains, which represent respectively: ORF2 (cPORF2), ORF3 (cPORF3), ORF4 (cPORF4), ORF5 (cPORF5), ORF6 (cPORF6), and ORF7 gene and 3′ UTR (cPORF7.3U) of PP vaccine virus genome as well as the ORF5 (cNORF5) of Neb-1 (Fig. 2).

All the individual chimeric clones were confirmed by sequencing and a number of nucleotide changes and resulting amino acid alterations including introduced restriction endonuclease (RE) sites were observed throughout the genome as described previously (Kwon et al., 2006) (Table 2). Table 2 summarizes the nucleotide changes

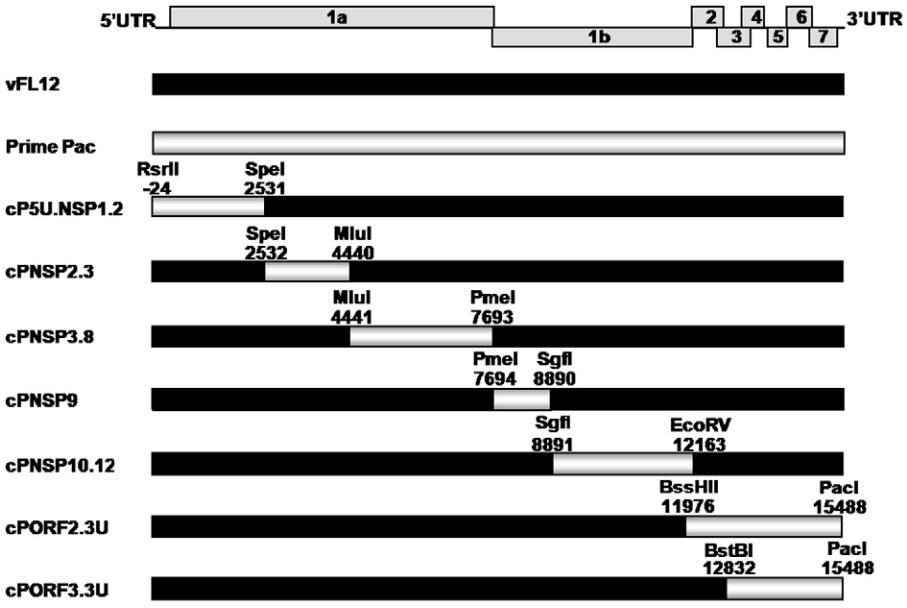

Fig. 1. Construction of chimeric PRRS viruses for genome-wide scanning for genes involved in attenuation. The genome organization of PRRSV is shown at the top of the figure. The colored boxes represent the regions of parental viruses vFL12 (black) and Prime Pac (gray), respectively. Corresponding restriction sites and nucleotide numbers used for cloning are depicted. The designation of each chimeric virus is shown on the left.

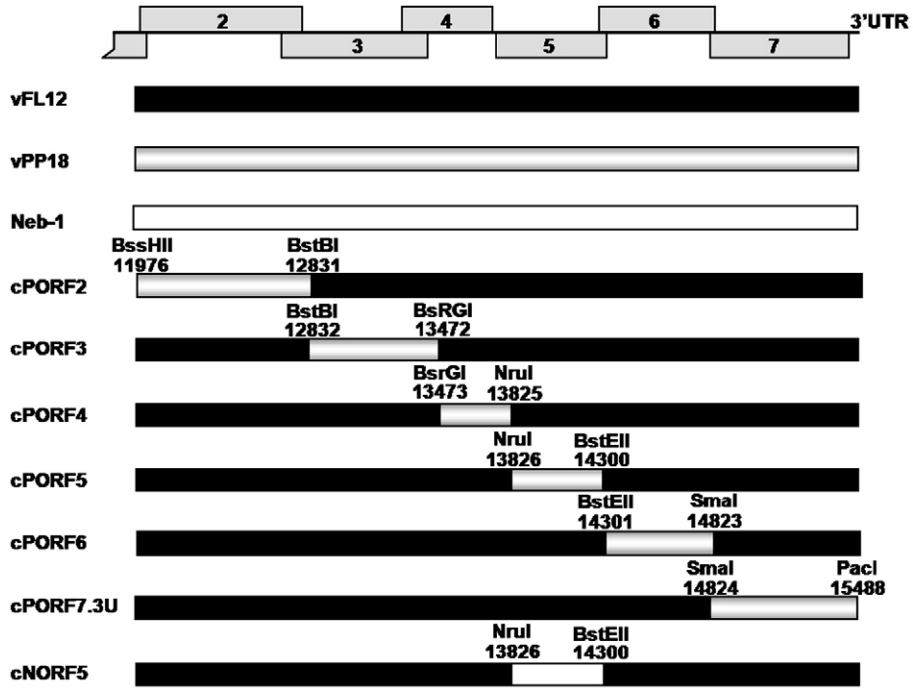

Fig. 2. Construction of single gene chimeric PRRS viruses within structural regions. The genome organization of PRRSV is shown at the tope of the figure. The boxes with different color represent the regions of parental viruses vFL12 (black), vPP18 (gray) and Neb-1 (white) respectively. Corresponding restriction sites and nucleotide numbers used for cloning are depicted. The designation of each chimeric virus is shown on the left.

caused by the incorporation of RE sites to facilitate genome replacement and their predicted amino acids changes. Overall, 14 nucleotide changes resulted in 4 non-conservative (Ala782Val, Ser254Asn, Leu48Thr, Trp256Arg) and 1 semi-conservative (Ser171Gly) amino acid changes within ORF 1a, 2, 3 and 5 regions (Table 2). The full-length chimeric cDNA clones were linearized by digestion with AclI, as described in Materials and methods, and used for *in vitro* transcription followed by electroporation into MARC-145 cells. Viable chimeric viruses were recovered as described previously (Kwon et al., 2006; Truong et al., 2004). By sequence analysis, we confirmed that the genomic sequences of the rescued viruses were derived from their corresponding chimeric clones and maintained the nucleotides changes.

In vitro growth properties of chimeric viruses

All the chimeric constructs described above and shown in Figs. 1 and 2 resulted in viable viruses. The growth kinetics of the chimeras

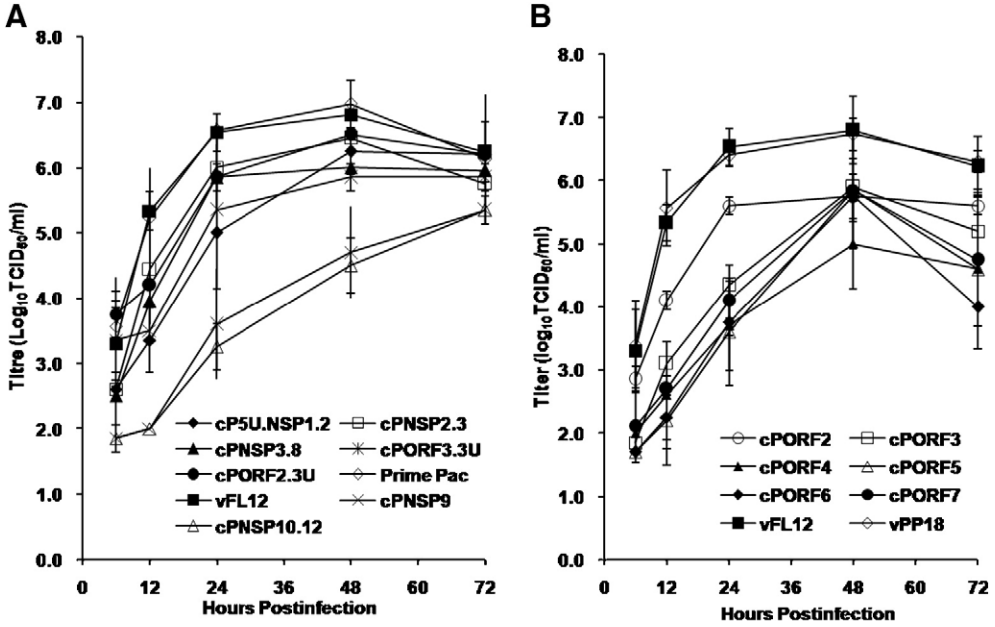

Fig. 3. Growth kinetics of parental and chimeric viruses. The viable chimeric viruses along with the parental viruses were used to analyze their growth kinetics when infecting MARC-145 cells at a MOI of 10. Culture supernatant was collected at 6, 12, 24, 48 and 72 h PI. The virus titers were determined by Reed and Muench calculation of the tissue culture 50% endpoint. Results shown are the mean values of three independent experiments and error bars represent the standard deviation. The viruses depicted in Fig. 1 (Fig. 3A) and Fig. 2 (Fig. 3B) were used for this analysis (except for Neb-1 and cNORF5).

were compared with the parental viruses vFL12, PP vaccine strain and/or vPP18, using MARC-145 cells as previously described (Kwon et al., 2006). The growth kinetics shows that the yields of the chimera cultures were different and that most chimeric viruses had relatively impaired growth compared to parental viruses. The replication pattern and the time to reach peak viral titers were somewhat delayed and in some cases (i.e. cPNSP9, cPNSP10.12) extended to 72 h post infection (PI) (Fig. 3). However the impaired growth in cell culture did not seem to affect viral replication *in vivo*. Based on kinetics of seroconversion by ELISA, the chimeric viruses replicated as efficiently as wild type (wt) upon inoculation of pregnant sows (data not shown).

In vivo virulence phenotype of the chimeric viruses in sow reproductive failure model

Primary genome-wide scanning of PP vaccine strain indicates that major determinants of attenuation would reside within certain non-structural (NSP3-8) and structural (ORF2–3′ UTR) regions

Upon inoculation of sows at 90th day of gestation, the number of viable offsprings at birth and upon weaning at 15 days of age was evaluated. Other parameters, including daily clinical signs and rectal temperatures, total antibody response and viremia in the sows were also evaluated as described previously (Kwon et al., 2006; Osorio et al., 2002). As typical for the swine reproductive failure model when using the FL12 infectious clone or its parental strain (Osorio et al., 2002), no specific clinical signs other than lack of appetite, lethargy and slight fever shortly after infection and prior to reproductive failure, which typically takes place at a later time post inoculation, shortly before or at normal farrowing time were noticed in any group of sows inoculated with all of the different strains throughout the experimental period. The vFL12-inoculated sows confirmed a completely pathogenic phenotype, with 100% mortality of the piglets and the PP vaccine virus-inoculated sows showed normal range of survival rate with 77% (Table 3) in both cases consistent with our previous reports (Kwon et al., 2006). In the primary genome-wide scanning, most important genomic regions contributing to PRRSV virulence were mapped within the NSP3 to 8 and ORF2 to 7 as reflected in the sows inoculated with cPNSP3.8, cPORF2.3U and cPORF3.3U chimeric viruses exhibiting significant piglet survival rates of 75%, 69% and 71%, respectively. While NSP9 (cPNSP9) could be ruled out as an important virulence determinant, it should be noted that most of NSP-encoding regions seemed to play a relative role in virulence, as exhibited by the sows inoculated with cP5U.NSP1.2 (51%), cPNSP2.3 (56%) and cPNSP10.12 (56%) chimeric viruses (Table 3).

Amongst the PRRSV structural genes, the ORF5 contributes primarily to PRRSV virulence

As a result of this initial genome-wide scanning for PRRSV virulence markers, we concluded that the NSP3-8 segment and the structural protein-coding area are the genomic regions where possible major virulence determinants seem to be located. For practical reasons, we focused on generating individual structural gene chimeras based on the availability of our intermediate plasmid encompassing the majority of structural ORFs and entire 3′ UTR derived from the FL12 (Ansari et al., 2006). By analyzing the virulence phenotypes of the single structural gene chimeras using pregnant sows reproductive failure model, it turned out that ORF5 (cPORF5), reaching 44% survival, principally contributes to virulence within structural regions. This result strongly suggests that the GP5 of PP contains a major virulence determinant which leads to significant increase in survival rate observed with cPORF5-inoculated sows.

To further substantiate this conclusion we also generated a chimera which includes the ORF5 of wt virulent parental strain of PP (Neb-1) within the background of FL12 (Fig. 2). The *in vivo* testing of this construct (cNORF5, Table 4) allowed us to confirm that this chimera, which replaces the ORF5 of PP by its respective wt version, retains virulence at a level equivalent to that of wt FL12. This observation rules out the possibility that the attenuation resulting after gene swapping for the construction of cPORF5 (Table 4) was possibly caused by the mere incompatibility or imperfect matching of viral proteins encoded by genes derived from the two different non-homologous strains composing the chimera (FL12 and PP) rather than being caused by the true attenuation on a virulence determinant. The cNORF5 construct demonstrated its *in vivo* pathogenic properties in the sow reproductive failure model, which clearly supports the contribution of wt ORF5 in virulence. The scores exhibited by vFL12 (0%) and cNORF5 (7%) demonstrate in both cases that the ORF5 gene should be exclusively responsible for the significant differences in reproductive performance between both FL12 and cNORF5 (each of definitely virulent phenotype) and the partially attenuated phenotype of cPORF5 (Table 4). From all of the above it can be concluded that, through the process of attenuation *in vitro* that gave origin to the PP vaccine strain, one of the main factors that led to attenuation of PRRSV came from sequence alteration of certain virulence determinant contained in GP5.

Interestingly only 2 amino acid differences (Phe17Leu and Ser171Gly) were found in the entire coding sequences of GP5 between PP vaccine and Neb-1 wt virulent strains (data not shown). We introduced an RE site at position 171 of PP which led to generation of a mutation from Ser to Gly as shown in Table 2, thus only one amino acid change at position 17 in cNORF5 seems to potentially be involved in virulence. Future single point mutation studies should then examine if indeed this single Phe17Leu is responsible for the ORF5 contribution to PRRSV virulence.

The ORF5 of the PRRSV genome encodes the major viral envelope protein GP5, which is a glycosylated transmembrane protein of approximately 25 kDa (Dea et al., 2000; Gagnon et al., 2003; Meulenberg et al., 1995b). Like EAV and LDV, the PRRSV GP5 and matrix (M) protein interact and form heterodimers, which may play a critical role in virus assembly but for which the mode of interaction has not been demonstrated yet (Delputte et al., 2002; Mardassi, Massie, and Dea, 1996). It has been proposed that the GP5 is involved in the entry of virus into the host cells, presumably by interacting with the host cell receptor sialoadhesin, especially for macrophages (Delputte and Nauwynck, 2004). The GP5 is considered to be important in the infection process because the presence of a major neutralization epitope in the N-terminal ectodomain that might be involved in receptor recognition (Ostrowski et al., 2002). Moreover, recent studies from our laboratories (Ansari et al., 2006) provide evidence that glycosylation of GP5 of PRRSV play an important role in escaping or minimizing virus-neutralizing antibody response by the glycan-shielding mechanism (Johnson et al., 2003; Wei et al., 2003). This glycan shielding mechanism is also observed in LDV (Chen, Li, and Plagemann, 2000), hepatitis B virus (HBV) (Lee et al., 2003), simian immune deficiency virus (SIV) (Reitter, Means, and Desrosiers, 1998), influenza virus (Skehel et al., 1984), and human immune deficiency virus (HIV) (Wei et al., 2003).

With the possible exception of ORF2, the rest of PRRSV structural genes or sequences (ORFs 3, 4, 6, 7 and 3′ UTR) did not contribute significantly to virulence, with all of them reaching negligible survival rates (Table 4). It is interesting to observe that the score attained by cPORF2, which reached a 24% survival, may indicate a partial role of this gene in PRRSV virulence although not to a level as significant as ORF5. Future studies may be necessary to elucidate this possible role of ORF2 in PRRSV virulence.

The use of our highly virulent infectious clone (FL12) as a backbone to construct chimeric viruses has several advantages. First, the highly pathogenic properties of vFL12 in either young pigs or sows reproductive failure model are easily reproducible and distinguishable from the attenuated phenotype. Secondly, vFL12 grows well *in vitro* in both MARC-145 and PAM cells. The FL12 clone can be directly

transfected to susceptible cell line (MARC-145), which allows us to consistently rescue chimeric viruses with relatively high titer. Lastly, because the attenuated PP vaccine infectious clone PP18 was constructed by using the intermediate chimeric clones within the FL12 backbone (Kwon et al., 2006), a number of cloning sites of PP18 are shared with FL12, thus both clones being easily interchangeable.

The logical continuation of our study on the contribution of the ORF5 gene to PRRSV virulence should consist of uncovering the precise role of single residue(s) for essential virulence within this gene. This will be pursued by site-directed mutagenesis followed by *in vitro* and *in vivo* characterization. More importantly, other virulence determinants should be identified within NSP3 to 8 regions, another cluster of important virulence maker candidates, whose individual characterization will certainly elucidate the reconstitution of full *in vivo* virulence of attenuated strains of PRRSV.

Materials and methods

Cells and viruses

The MARC-145 cells, a sub-clone of MA-104 cells (Kim et al., 1993) were propagated and maintained as described previously (Ansari et al., 2006; Truong et al., 2004). These cells were used for RNA electroporation, virus titration and viral growth kinetics. Second passage (P2) of vFL12 (Truong et al., 2004) and PP vaccine virus (obtained from a commercial batch of Prime Pac vaccine marketed in 1997, Schering-Plough Co.) and first passage (P1) of vPP18 were used for growth kinetics and animal inoculation. For the construction of recombinant chimeric viruses, stocks of comparable titers derived from passages 1 and 3 were only used. The PRRSV Neb-1 strain (Hesse, 1996a), parental virus from which PP virus was derived, was obtained by courtesy of Dr K. Lager (NADC,USDA/ARS, Ames, Iowa).

Preparation of single genomic fragments of PP strain to develop chimeras

The MARC-145 cells were infected with PP vaccine virus (P2) at a multiplicity of infection (MOI) of 0.1 and culture supernatant was collected at 48 h post infection (PI). The culture supernatant was briefly clarified by centrifugation at 3000 ×*g* for 10 min and the virus was pelleted by ultracentrifugation at 100,000 ×*g* on a 15% sucrose cushion for 2 h. The virus pellet was resuspended in PBS and viral RNA was extracted with TRIzol LS (Invitrogen), as per manufacturer's recommendations. The viral RNA was resuspended in nuclease-free water and either directly used for RT-PCR or stored at −80 °C in small aliquots. The cDNA was synthesized by Superscript III (Invitrogen) using specific reverse primers and subsequently used to generate various sizes of individual PCR fragments as shown in Figs. 1 and 2 using the primer sets (Table 1). The PCR was carried out using a mixture of Taq (NEB) and PFU (Stratagene) polymerases to enhance the fidelity during the DNA synthesis.

Construction of full-length chimeric cDNA clones

Full-length FL12 (Truong et al., 2004) and PP18 (Kwon et al., 2006) clones were utilized as either genomic backbones (FL12) or gene donor (PP18) to construct chimeric clones. First, to make chimeric clones covering multiple genes derived from the PP vaccine virus strain, a series of PCR products, which contain naturally present or deliberately introduced RE sites (Table 1), RsrII, SpeI, MluI, PmeI, SgfI, EcoRV, BssHII, BstBI and PacI, were used to replace the corresponding regions of FL12 clone as described previously (Kwon et al., 2006) (Fig. 1). In addition, the construction of single gene chimeric clones comprising individual structural protein encoding genes from either the PP vaccine virus, vFL12 or the ORF5 of Neb-1 strain, additional RE sites (Tables 1 and 2), BsrGI and BstEII were incorporated or NruI was utilized to facilitate the fragment exchange in a similar manner (Fig. 2). The target genes were either directly cloned into full-length FL12 or into an intermediate plasmid encompassing the majority of ORF2, complete ORFs 3 to 7, and the entire 3′ UTR derived from the FL12 as described previously (Ansari et al., 2006). It should be noted that, due to the overlapping character of the PRRSV structural genes, and in order to avoid adding additional non-viral sequences to our constructs, these single gene chimeric constructs included most but not all the corresponding full sequence for each gene. The percentage of authentic gene represented in each case ranges from 57% to 100% (ORF2: 98%, ORF3: 82%, ORF4: 57% (most impacted by the overlapping character of the PRRSV structural genes), ORF5: 79%, ORF6: 85%, and ORF7: 100%). All generated chimeric cDNA clones were confirmed by sequence analysis using the appropriate primers in each case for both FL12 and PP18.

In vitro transcription, RNA electroporation, and virus recovery

The full-length chimeric cDNA clones were digested with AclI (FL12-based clone) or PacI (PP18-based clone) to linearize and the

Table 1
Primers used for the development of chimeric constructs

Chimeric Constructs	Primer	Sequence[a]	Viral genome Position[b]
cP5U.NSP1.2	T7P+1F	5′ ***ATATAAGCTTCGGACCGTAATACGACTCACTATAGG***ATGACGTATAGGTGTTGGCTCTATGCCATGACATTTGTATTG 3′	1–42
cP5U.NSP1.2	P2546R	5′ CTGGGCGTTGACTAGTTTCAGCA 3′	2524–2546
cPNSP2.3	P2524F	5′ TGCTGAAACTAGTCAACGCCCAG 3′	2524–2546
cPNSP2.3	P4457R	5′ CGACCTGGTCGCACGCGTAAAAGG 3′	4434–4457
cPNSP3.8	P4434F	5′ CCTTTTACGCGTGCGACCAGGTCG 3′	4434–4457
cPNSP3.8	P7749R	5′ GTAACAACCAACCCGCCGCGACCAC 3′	7725–7749
cPNSP9	P7635F	5′ GACAAACTCCAGGACCTGACTAAG 3′	7659–7682
cPNSP9	P8898R	5′ GTGGAGCGATCGCAGGATGCAAG 3′	8876–8898
cPNSP10.12	P8880F	5′ CATCCTGCGATCGCTCCACACCTGC 3′	8880–8904
cPNSP10.12	P12257R	5′ GCTCAGGGTGAAAGGTAGGGCG 3′	12260–12281
cPORF2/cPORF2.3U	P11691F	5′ GCAACAGAAGAGTTGTCGGGTCC 3′	11715–11737
cPORF2	P12842R	5′ CCGTGAGTTCGAAGGAAAAATTGCCCC 3′	12816–12842
cPORF3/cPORF3.3U	P12816F	5′ GGGGCAATTTTTCCTTCGAACTCACGG 3′	12816–12842
cPORF2.3U/cPORF3.3U	A70R	5′ CACACTTAATTAACGT$_{(70)}$AATTTCGGCCGCATG 3′	15399–15419
cPORF4/cPORF5/cNORF5	P13464F	5′ GACACCCGTGTACATCACCA 3′	13464–13483
cPORF3	P13481R	5′ GTGATGTACACGGGTGTCCCTAT 3′	13459–13481
cPORF4	P13969R	5′ GACAAAAGTCTCCACTGCCCAATC 3′	13970–13993
cPORF6	P14291F	5′ GAGGTCGAAGGTCACCTGATCGA 3′	14291–14313
cPORF5/cNORF5	P14313R	5′ TCGATCAGGTGACCTTCGACCT 3′	14292–14313
cPORF6	P14971R	5′ CTGGTTCTGCTGGGCGATGATC 3′	14974–14995

[a] T7 RNA polymerase promoter sequences are shown in bold italics. Underlined sequences represent restriction endonuclease recognition sites utilized in exchanges of amplicons and full-length cDNA assembly. Nonviral sequences are in bold-face type.

[b] Nucleotide position within the NVSL 97-7895 PRRSV isolate (parental strain of FL12 infectious clone) (Truong et al., 2004). GenBank accession number is genbank:AY545985.

Table 2
Nucleotide changes incorporated within the genome of PP vaccine (or PP18) and Neb-1 for the fragment exchange into FL12 and their predicted amino acid changes

Nucleotide position[a]	ORF	Genome of changes incorporated	Nucleotide change[b]	Amino acid position (Protein name)	Amino acid Change
2534	1a	PP	G→A	781 (NSP2)	Silent
2536	1a	PP	C→T	782 (NSP2)	A→V
4442	1a	PP	A→G	1453 (NSP3)	Silent
8893	1b	PP	A→C	404 (NSP9)	Silent
8896	1b	PP	T→C	405 (NSP9)	Silent
12835	2	PP	G→A	254 (GP2)	S→N
12839	3	PP	T→A	48 (GP3)	L→T
12840	2	PP	T→C	256 (GP2)	W→R
13476	4	PP	T→C	78 (GP4)	Silent
14300	5	PP	A→G	171 (GP5)	S→G
14302	5	PP	C→T	171 (GP5)	Silent
14305	5	PP	T→C	172 (GP5)	Silent
14302	5	Neb-1	C→T	171 (GP5)	Silent
14305	5	Neb-1	T→C	172 (GP5)	Silent

[a] Nucleotide positions are based on the genome of NVSL 97-7895 PRRSV isolate (Truong et al., 2004) as shown in Table 1.
[b] The GenBank accession numbers of original nucleotide sequences of PP vaccine, PP18, and Neb-1 are genbank:AF184212, genbank:DQ779791, and genbank:EU755263, respectively.

DNA was used as templates to generate capped RNA transcripts using the mMESSAGE mMACHINE Ultra T7 kit as per manufacturer's recommendations (Ambion) and as described previously (Truong et al., 2004). The reaction mixture was treated with DNaseI to digest the DNA template and extracted with phenol and chloroform and precipitated with isopropanol. The integrity of the *in vitro* transcripts was analyzed by glyoxal agarose gel electrophoresis followed by ethidium bromide staining. Sub-confluent MARC-145 cells seeded 2 days prior to use were trypsinized and collected for electroporation. About 2×10^6 cells in 400 μl of Dulbecco's modified Eagles medium (DMEM) containing 1.25% DMSO were electroporated with approximately 5.0 μg of *in vitro* transcripts along with 5.0 μg of total RNA isolated from MARC-145 cells by pulsing once using Bio-Rad Gene Pulser Xcell at 225–250 V, 925–950 μF in a 4.0 mm cuvette. The electroporated cells were transferred in a DMEM containing 10% FBS and 1.25% DMSO in a 60-mm cell culture plate for virus recovery and in a separate plate for immunofluorescence staining. Upon initial development of cytopathic effect (CPE), the supernatant from the electroporated cells in 60-mm plate was collected, clarified and passed onto naïve MARC-145 cells in 100 mm plates to prepare stock virus. The culture supernatant was recovered when complete CPE was observed, centrifuged briefly to remove cell debris, and frozen at −80 °C in small aliquots for further studies.

Viral growth properties and kinetics

Growth properties of parental (vFL12, PP vaccine and vPP18) and chimeric viruses were compared in MARC-145. For the growth kinetics, MARC-145 cells were infected with the above-mentioned viruses at an MOI of 10 and incubated at 37 °C. At various time points PI, the culture supernatant from infected cells was collected and virus titer was determined and expressed as tissue culture infectious dose 50 per ml ($TCID_{50}$/ml).

Animal experiments

Pregnant sows were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. Their negative PRRSV infection status was confirmed upon arrival by a commercial ELISA serology test (IDEXX Labs, Portland, ME). Pregnant sows were inoculated at 90 days of gestation and the viability scores of offsprings at birth and weaning (at 15 days of age) were evaluated (Osorio et al., 2002). All animals were tested for anti-PRRSV antibodies by ELISA (IDEXX Labs, Portland, ME). Two sows per group in each separate experiment were infected with parental and chimeric viruses (Tables 3 and 4). In all the cases, a total of 2 ml ($10^{5.2}$ $TCID_{50}$) of virus was administered intra-nasally with 1 ml delivered into each nostril. The rectal temperature and clinical signs of the inoculated animals were monitored daily from 3 days pre-inoculation through farrowing and to weaning. Viremia was always measured by regular titration on MARC-145 cells and in some cases by nested RT-PCR as described previously (Osorio et al., 2002), using the sera collected at 0–7–14–21 days PI. The copy numbers of viral RNA from the sera of sows were measured using a PRRSV real-time quantitative PCR kit (Tetracore, USA).

Table 3
Viability scores of offsprings born from sows infected with parental or chimeric viruses — primary genome-wide scanning[a]

Viruses	Genetic background	Sow no.[a]	Viability at			Survival[b] (%)
			Birth		15 days of age	
			Dead	Live	Live	
vFL12	FL12	1	12	3	0	0
		2	13	1	0	
Prime Pac	Prime Pac	1	0	11	9	77*
		2	0	14	10	
cP5U.NSP1.2	FL12	1	1	12	9	51*
		2	6	6	4	
cPNSP2.3	FL12	1	4	12	9	56*
		2	1	15	9	
cPNSP3.8	FL12	1	0	15	12	75*
		2	3	10	9	
cPNSP9	FL12	1	13	3	0	3
		2	9	11	1	
cPNSP10.12	FL12	1	5	11	10	56*
		2	5	5	5	
cPORF2.3U	FL12	1	0	15	9	69*
		2	2	7	7	
cPORF3.3U	FL12	1	0	10	10	71*
		2	3	9	5	

[a] Two sows each per group. Arbitrary numbers.
[b] Least squares means of each group. The asterisk indicates that the means are significantly different ($P \leq 0.05$) than the mean of vFL12 group.

Table 4
Viability scores of offsprings born from sows infected with chimeric viruses representing single structural genes[a]

Viruses	Genetic background	Sow no.[a]	Viability at			Survival[b] (%)
			Birth		15 days of age	
			Dead	Live	Live	
vFL12	FL12	1	12	3	0	0
		2	13	1	0	
cPORF2	FL12	1	9	6	4	24
		2	7	2	2	
cPORF3	FL12	1	15	0	0	7
		2	9	5	2	
cPORF4	FL12	1	9	1	0	3
		2	12	3	1	
cPORF5	FL12	1	7	7	4	44*
		2	3	7	6	
cPORF6	FL12	1	8	4	0	7
		2	11	4	2	
cPORF7.3U	FL12	1	13	4	1	6
		2	13	2	1	
cNORF5	FL12	1	10	4	2	7
		2	10	5	0	

[a] Two sows each per group. Arbitrary numbers.
[b] Least squares means of each group. The asterisk indicates that the means are significantly different ($P \leq 0.05$) than the mean of vFL12 group.

Statistical analysis

Viral growth properties in MARC-145 cells between parental and chimeric viruses were compared by Dunnett's *t* test and viability scores of offsprings born from sows infected with parental and chimeric viruses were analyzed by Duncan's multiple range test using SAS (version 9.1 for windows; SAS Institute Inc., Cary, NC, USA) program. A *P*-value of less than 0.05 was considered significant.

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