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Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype*

R. Allende[1], **G. F. Kutish**[2], **W. Laegreid**[3], **Z. Lu**[2], **T. L. Lewis**[2,†], **D. L. Rock**[2], **J. Friesen**[1], **J. A. Galeota**[1], **A. R. Doster**[1], and **F. A. Osorio**[1]

[1]Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska, U.S.A.
[2]Plum Island Animal Disease Center, USDA/ARS, Greenport, New York, U.S.A.
[3]U.S. Meat Animal Research Center, USDA/ARS, Clay Center, Nebraska, U.S.A.

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Summary. Although live-attenuated vaccines have been used for some time to control clinical symptoms of the porcine reproductive and respiratory syndrome (PRRS), the molecular bases for the attenuated phenotype remain unclear. We had previously determined the genomic sequence of the pathogenic PRRSV 16244B. Limited comparisons of the structural protein coding sequence of an attenuated vaccine strain have shown 98% homology to the pathogenic 16244B. Here we have confirmed the attenuated phenotype and determined the genomic sequence of that attenuated PRRSV vaccine and compared it to its parental VR-2332 and the 16244B strains. The attenuated vaccine sequence was colinear with that of the strain 16244B sequence containing no gaps and 212 substitutions over 15,374 determined nucleotide sequence. We identified nine amino acid changes distributed in Nsp1β, Nsp2, Nsp10, ORF2, ORF3, ORF5 and ORF6. These changes may provide the molecular bases for the observed attenuated phenotype.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), is a devastating viral disease characterized by reproductive failure in sows, pre-weaning mortality and respiratory illness in piglets [1, 24]. The causative agent, PRRSV [30], belongs to the *Arteriviridae* family together with lactate dehydrogenase-elevating virus

*The GenBank accession number for the PRRSV modified live vaccine (complete genome) reported in this paper is AF159149.

†Deceased

(LDV) of mice, equine arteritis virus (EAV) and simian haemorrhagic fever virus (SHFV) [3].

Inactivated and live-attenuated vaccines have been developed to control the clinical symptoms of PRRS [5, 10, 22, 26]. Although attenuated PRRSV vaccines have been in use since 1994, the molecular basis for their reduced pathogenicity remains unclear. Two sequencing studies of the structural protein coding region of the attenuated PRRSV vaccine that has been in use the longest, have been conducted [15, 33]. From these studies, two nucleotides changes in ORF5 and ORF6 respectively were described as possibly influencing the PRRSV virulence [15]. However, no data have been reported on molecular changes which may have occurred elsewhere in the entire genome during the attenuation process and probably involved in loss of PRRSV virulence.

We have previously determined the genomic sequence of the North American pathogenic 16244B PRRSV isolate [2]. Preliminary analysis conducted in our laboratory, of the 3′ end of the 16244B strain and the attenuated PRRSV vaccine strain referenced above revealed a high genetic identity of 98% (unpublished data). Furthermore the sequence comparison of PRRSV 16244B and the parental strain of the vaccine VR-2332 [19] revealed high identity (unpublished data). Taken together, these observations suggest that as previously reported in other PRRSV field isolates the PRRSV 16244B could be one example of the virulent derivatives of the attenuated PRRSV vaccine that have been recently reported to occur in the field [17]. Therefore, the pathogenic potential of 16244B becomes of interest to characterize. Furthermore, in order to determine which changes may actually be responsible and which not for the reduction of PRRSV virulence we confirmed the in vivo phenotype and determined the nearly entire genomic sequence of the attenuated vaccine strain and compared it to that of its parental VR-2332 (a bonafide pathogenic strain [6]) and the closely related PRRSV 16244B field isolate.

This study showed that nine amino acid changes distributed in Nsp1β, Nsp2, Nsp10, ORF2, ORF3, ORF5, and ORF6 might be responsible for the attenuated PRRSV phenotype.

Materials and methods

Virus, cells and RNA isolation

We used the commercially available modified live PRRSV vaccine RespPRRS /Repro (NOBL Laboratories, Inc.), a cell passaged attenuated strain derived from the pathogenic PRRSV field isolate VR-2332 [4]. The vaccine strain was derived by twenty-five passages in monkey kidney cell line MA-104 at 35 °C−37 °C, followed by twelve passages at 31 °C in the same cell line. The commercial product (Serial #JA-441A-203) obtained from a licensed vaccine dealer, was reconstituted and used to inoculate monolayers of MARC-145 cells, a subclone of the monkey kidney cell line MA-104 [12]. After two cell culture passages, supernatants containing infectious virus were collected, titered in monolayers of MARC-145 cells and stored for further animal inoculations. The total RNA was extracted from the infected cell monolayers using TRIzol (GibcoBRL) following manufacturer's protocol.

The pathogenic PRRSV strain 16244B isolated in Nebraska, U.S.A., in 1997 has already been described [2]. The 16244B virus was replicated in monolayers of MARC-145 cells. After two cell culture passages, the infected monolayers were frozen and thawed and clarified by low speed centrifugation. Viral suspensions were titered in monolayers of MARC-145 cells and stored for further animal inoculation.

Animal inoculations

Two groups of four PRRSV seronegative pregnant sows (Landrace breed) each were inoculated at 90 days of gestation. One group received $10^{4.7}$ $CCID_{50\%}$/2 ml of vaccine virus and the second group received $10^{5.0}$ $CCID_{50\%}$/2 ml of 16244B PRRSV strain. All the animals received one ml inoculum in each nostril. Animals were kept in strict isolation in separate rooms and observed daily. Two markers for viral pathogenicity were observed, 1) the induction of abortion and 2) the rate of piglet survival at 10 days after farrowing. The results of farrowing performance and piglets health were recorded throughout a two week period. The general appearance, signs of respiratory distress, diarrhea and overall body conditions were monitored during this period. Serum samples were collected from sows at 7 days after farrowing for PRRSV antibody detection. Lung and spleen tissue, lymph nodes and serum samples were taken, from piglets born alive and from others, shortly after death, for viral isolation in monolayers of MARC-145 cells. Viral identification was performed in formalin fixed and paraffin-embedded tissues from the same piglets using immuno-histochemistry (IHC) as described by Sur et al. [27].

The pathogenicity of PRRSV VR-2332 in pregnant sows has been previously reported [6].

Table 1. Oligonucleotides used for amplification of PRRSV vaccine strain

Code	Sequence (5′–3′)	Location[a]
RA-1F	TGCCTCGGCATTTGTATTGTCAG	24–46
RA-3R	CTTGCAGCCTCCGCTGTAGGTACT	1100–1123
RA-8R	CGAACGATCGGAATCGCCAG	1907–1926
RA-9F	CTGCCTGCCTTGACTGGCT	1832–1850
RA-12R	ACTGCCGCCGCCGAACTCAATCTT	3003–3026
RA-11F	ACCGCCGTGCTCTCCAAGTTGGAA	2395–2418
RA-16R	CAATGCCAAGCCTAAGCA	4304–4321
RA-17F	CCTGTTCGCAGCCTTGTTGTG	4213–4233
RA-22R	CGGGCAATCAGCTATAG	5813–5830
RA-21F	TCGGAGGCATGGCTCATAGG	5198–5218
RA-26R	AGCGAGGGCACCAGTCAGAGACTC	6784–6807
RA-27F	CTTGTTGGCGATGGAGTGTTCTCT	6685–6708
RA-30R	CATCGATCGGTCTCGCAACCGGGT	7861–7884
RA-31F	CAGCGGCTTGACCCGCTGTGGTCG	7706–7729
RA-37F	TGGTGATCTATGCACAGCATATGG	9085–9108
RA-42R	GGAATCAATAGTGATGGCGTC	10596–10616
RA-43F	CTGTCAGGTATGGGCAGG	10546–10563
RA-48R	GGCAGGGCGCGTACGGAG	12249–12266

[a]Locations are from the sequence of PRRSV 16244B strain (GenBank Accession No. AFO46869)

RT-PCR, PCR and cDNA cloning

Viral cDNAs were synthesized by RT-PCR using random hexanucleotide primers and SuperScript II reverse transcriptase (GibcoBRL) following the supplier's instructions. The resulting cDNA was used as template in the PCR. The PCR was performed with PRRSV-specific primers designed from the sequence of the North American PRRSV 16244B strain [2] (Table 1). PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) following manufacturer's instruction.

Sequence analysis

RT-PCR products and cloned cDNA inserts were sequenced by dideoxynucleotide chain-termination method [23]. Sequencing reactions were prepared with the BigDye Terminator Cycle Sequencing kit (Perkin Elmer). Sequences were determined with an ABI PRISM 377 automated DNA sequencer and an ABI PRISM 3700 DNA analyzer. The ABI sequence sequence software version 3.3 was used for lance tracking and trace extraction. Further cDNA sequencing, genome assembling and computer analysis was done as described in Allende et al. [2]. The consensus sequence had at least fourfold redundancy at each nucleotide position in both directions, with an average ten-fold redundancy.

Sequence comparison to PRRSV VR-2332 was done using the GenBank data base Accession No. PRU87392 [19].

Results

Animal inoculations

The phenotypes of both strains (RespPRRS/Repro and 16244B) were different as defined by the two markers of viral pathogenicity (Table 2). Animals incoculated with PRRSV 16244B farrowed an average of six live piglets and the rate of survival at 10 days after farrowing was of 29%. Similar results were reported in sows inoculated with the parent of the vaccine PRRSV VR-2332 [6] (Table 2). In contrast animals inoculated with the vaccine strain had an average of 13 piglets born alive and the rate of survival at 10 days post farrowing was of 94%. The piglets

Table 2. Comparison of the phenotype of the PRRSV vaccine, 16244B and VR-2332 strains by intranasal inoculation of sows at 90 days of gestation

	Vaccine				16244B					VR-2332[a]		
Sow ID:	A	B	C	D	E	F	G	H		7	8	9
Days to farrowing	113	115	115	113	106	114	115	117	$P = 0.55$[b]	112	114	110
Piglet status:												
-born alive	14	13	13	12	5	8	4	7	$P = 0.02$	5	8	5
-dead	1	1	1	1	4	4	2	2	$P = 0.01$	4	3	2
-mummified	1	–	–	–	2	–	3	1	$P = 0.01$	4	3	2
Piglet survival at 10 days:	12	12	12	12	0	1	3	3	$P = 0.02$			

[a]Christianson et al. [6]

[b]Mann-Whitney U test for median comparison

born alive in the group inoculated with PRRSV 16244B looked weak, unthrifty and feverish. The piglets born from the group inoculated with the attenuated vaccine strain appeared normal. Gross pathology and histopathology studies were performed in all the piglets that died shortly after birth. The results indicated that some piglets in the PRRSV 16244B-inoculated group had mild interstitial pneumonia and a mild vasculitis involving branches of the pulmonary artery was noted in one of these piglets. Infectious virus was isolated from serum samples of these piglets which were also PRRSV positive by IHC performed on samples of their lung tissues. In the PRRSV vaccine-inoculated group, perinatal mortality was unrelated to PRRSV infection (i.e. traumatic as a result of being lied on by the dam). The gross pathology examination of these piglets was normal. Lung tissue samples from these piglets were PRRSV negative by IHC. Infectious virus was isolated from a pool of sera taken at 7 days post farrowing from the healthy piglets born from sow A of the vaccinated group. Attempts to recover infectious virus from the other litters in the vaccinated group were negative. Nevertheless, the median time of gestation to farrowing was similar in both groups of sows. All sows in the experiment except for sow D from the vaccine-inoculated group, developed PRRSV antibodies as indicated by the ELISA positive results. The lack of seroconversion upon intranasal inoculation in one animal coincides with our previous observations that indicate that this PRRSV modified live vaccine, when given by the recommended route, can also fail to induce antibodies as detected by ELISA [20].

Genome sequence analysis

The determined consensus sequence of the vaccine genome was 15,374 bases long comprising the entire coding region, 3′ non-coding region (NCR) and 165 nucleotides of the 5′ NCR. The consensus sequence of the vaccine PRRSV strain was more than 99% identical to and colinear with the sequence of the North American PRRSV 16244B [2] with 212 nucleotide substitutions and neither deletions nor insertions. From these nucleotide substitutions 20 were transversions (T>A; A>T; C>G; T>G; G>T or A>C). In 196 out of these 212 positions the vaccine was identical to its parental strain [19]. Analysis of the comparison indicated that the vaccine strain had 29 non-conservative and 46 conservative amino acid changes over the entire coding region. The observed non-conservative changes were located in ORF1a (23 changes), ORF1b (2 changes), ORF2 (1 change), ORF3 (2 changes) and ORF5 (1 change).

Although the only cleavage product of EAV ORF1ab polyprotein that has been confirmed for PRRSV is Nsp1 (Nsp1α and Nsp1β), here we will adopt the nomenclature proposed for EAV ORF1ab polyprotein processing products and name the putative PRRSV non structural proteins Nsp1 to 12 [24, 28, 29].

5′ NCR

We determined 165 bases from the predicted 189 base length of the 5′ NCR. The 5′ NCR of the vaccine strain had only one nucleotide transition (C>T) at

position 103 when compared to PRRSV 16244B but was identical to the parental strain VR-2332 at this position [19]. The consensus ORF1a start sequence 5′-UAACCAUG-3′ [2, 18, 19] was conserved in the vaccine strain.

Analysis of the ORF1a

From the total of 212 nucleotides substitutions observed over the entire vaccine sequence, 117 were located in ORF1a. However the polyprotein encoded by the vaccine strain was 2503 residues long and 97.9% identical to 16244B isolate [2] All four protease domains, their six putative catalytic residues and cleavage sites were conserved in the vaccine strain sequence. A total of 29 conservative and 23 non-conservative amino acid changes were identified in the ORF1a polyprotein (Table 3).

The predicted non-conservative amino acid changes were concentrated in the cleavage products Nsp1β and Nsp2, evenly distributed on both cleavage products and not associated with any particular catalytic or cleavage site. The vaccine strain's Nsp1β had 5 non-conservative amino acid changes over a total length of

Table 3. Nucleotides and amino acids differences found between vaccine and 16244B strains of PRRSV

Genome region	No. of changes/total for each region (%)		
		amino acid	
	nucleotide	conservative	non-conservative
5′ NCR	1/165(0.6)	–	–
ORF1a:			
Nsp1α	4/498 (0.8)	2/166 (1.2)	0/166 (0)
Nsp1β	16/651 (2.4)	3/217 (1.4)	5/217 (2.3)
Nsp2	61/2, 940 (2.1)	16/980 (1.6)	17/980 (1.7)
Nsp3	16/1, 338 (1.2)	3/446 (0.7)	0/446 (0)
Nsp4	4/612 (0.6)	2/204 (1.0)	0/204 (0)
Nsp5–8	15/1, 335 (1.1)	3/445 (0.7)	1/445 (0.2)
Junction	2/71 (2.8)	–	–
ORF1b:			
Nsp9	25/1, 938 (1.3)	2/646 (0.3)	2/646 (0.3)
Nsp10	16/1, 323 (1.2)	2/441 (0.4)	0/441 (0)
Nsp11	3/672 (0.4)	1/223 (0.4)	0/223 (0)
Nsp12	6/456 (1.3)	2/152 (1.3)	0/152 (0)
ORF2	10/768 (1.3)	1/256 (0.4)	1/256 (0.4)
ORF3	10/762 (0.8)	3/254 (1.2)	2/254 (0.8)
ORF4	3/534 (0.6)	1/178 (0.6)	0/178 (0)
ORF5	15/600 (2.5)	3/200 (1.5)	1/200 (0.5)
ORF6	8/522 (1.5)	2/174 (1.1)	0/174 (0)
ORF7	1/369 (0.3)	0/123 (0)	0/123 (0)
3′ NCR	0/150 (0)	–	–

Table 4. Nucleotides and amino acids differences found between PRRSV vaccine and its parental strain (PRRSV VR-2332)[a]

Genome region	No. of changes/total for each region (%)		
		amino acid	
	nucleotide	conservative	non-conservative
5′ NCR	1/165(0.6)	–	–
ORF1a:			
Nsp1α	1/498(0.2)	0/166(0)	0/166(0)
Nsp1β	2/651(0.3)	1/217(0.4)	1/217(0.4)
Nsp2	4/2,940(0.13)	2/980(0.2)	1/980(0.1)
NSp3	2/1,338(0.15)	1/446(0.2)	0/446(0)
Nsp4	0/612(0)	0/204(0)	0/204(0)
Nsp5–8	2/1,335(0.15)	0/445(0)	1/445(0.2)
Junction	0/71(0)	–	–
ORF1b:			
Nsp9	0/1,938(0)	0/646(0)	0/646(0)
Nsp10	6/1, 323(0.45)	1/441(0)	2/441(0.4)
Nsp11	5/672(0.4)	3/224(1.3)	1/224(0.4)
Nsp12	2/456(0.4)	0/152(0)	0/152(0)
ORF2	3/768(0.39)	2/256(0.8)	0/256(0)
ORF3	5/762(0.65)	2/254(0.8)	1/254(0.4)
ORF4	1/534(0.18)	1/178(0.6)	0/178(0)
ORF5	2/600(0.3)	1/200(0.5)	1/200(0.5)
ORF6	3/522(0.57)	1/174(0.6)	1/174(0.6)
ORF7	0/369(0)	0/123(0)	0/123(0)
3′ NCR	0/150(0)	–	–

[a]Nelsen et al. [19]; GenBank Accession No. PRU87392

217 residues and the Nsp2 had 17 non-conservative changes over a total length of 980 amino acids. The remaining cleavage products Nsp1α and Nsp3 to Nsp8 were very similar to 16244B with a total of 11 amino acid mutations (Table 3). When compared to its parental VR-2332 strain, the vaccine ORF1a had only 6 amino acid mutations (Table 5). However, the vaccine strain was identical to the 16244B strain at 3 of these positions (Table 5), thus suggesting that only 3 ORF1a residues identified as F_{331}, F_{668} and K_{952} could be considered as putative candidates for attenuation PRRSV phenotype. From them, the F_{331} and F_{668} are non-conservative substitutions which may represent a stronger change in the composition of the putative proteins (Nsp1β and Nsp2).

The ORF1a-ORF1b junction region

There were two nucleotide changes in this region. The G_{7687} A in the stem 1 of the predicted vaccine RNA pseudoknot structure giving rise to a slightly stronger structure than 16244B (−82.5 KJ/mol) and the G_{7724} A in the loop region

Table 5. Amino acids changes between PRRSV vaccine strain and VR-2332 and its comparison to 16244B PRRSV strain

Genome region	Vaccine	VR-2332[a]	16244B
ORF1a:			
Nsp1α	–	–	–
Nsp1β	F_{331}	S_{331}	S_{331}
Nsp2	F_{668}	S_{668}	S_{668}
	N_{951}	D_{951}	E_{951}
	K_{952}	E_{952}	E_{952}
Nsp3	A_{1498}	S_{1498}	A_{1498}
Nsp4	–	–	–
Nsp5–8	L_{2162}	P_{2162}	L_{2162}
ORF1b:			
Nsp9	–	–	–
Nsp10	E_{760}	G_{760}	E_{760}
	H_{952}	Y_{952}	Y_{952}
	R_{1042}	C_{1042}	R_{1042}
Nsp11	T_{1126}	S_{1126}	T_{1126}
	E_{1181}	G_{1181}	E_{1181}
	L_{1184}	V_{1184}	L_{1184}
	A_{1217}	G_{1217}	A_{1217}
Nsp12	–	–	–
ORF2	F_{10}	L_{10}	L_{10}
	S_{122}	A_{122}	S_{122}
ORF3	E_{83}	G_{83}	G_{83}
	V_{94}	I_{94}	V_{94}
	S_{106}	G_{106}	S_{106}
ORF4	G_{43}	S_{43}	G_{43}
ORF5	Q_{13}	R_{13}	R_{13}
	G_{151}	R_{151}	R_{151}
ORF6	E_{16}	Q_{16}	Q_{16}
	G_{121}	R_{121}	G_{121}
ORF7	–	–	–

[a]Nelsen et al. [19]; GenBank Accession No. PRU87392

(Table 3). The vaccine strain was identical to its parental strain at these positions [19] (Table 4).

Analysis of ORF1b

Fifty nucleotide substitutions out of the 212 observed over the determined sequence were located in ORF1b. However the ORF1b of the vaccine strain was 99.4% identical to 16244B and only 9 amino acid substitutions over the 1463 residues were present (Table 3). All the Nsp9 to Nsp12 domains and motifs and its cleavage sites [2, 19, 24] were conserved in the vaccine strain.

The comparison of the vaccine strain with the parental VR-2332 strain revealed seven amino acid substitutions (Table 5). Nevertheless the vaccine was identical to the pathogenic strain 16244B at six of this positions. The conservative substitution $Y_{952}H$ was the only one unique to the vaccine strain in ORF1b (Table 5) and was not related to a particular cleavage or catalytic site of the polyprotein.

Analysis of ORF2 to 7

Forty-four nucleotide substitutions were present over the ORF2 to 7 sequence (three of them were located in overlapping regions between two ORFs). The vaccine strain had a total of 14 amino acid mutations in this region being 4 of them non-conservative substitutions. The residue changes at positions $L_{10}F$ (ORF2); $G_{83}E$ (ORF3); $R_{13}Q$ and $R_{151}G$ (ORF5) and ($Q_{16}E$ (ORF6) were unique to the vaccine strain but absent from its parental strain sequence (Table 5). The changes in ORFs 5 and 6 were observed also by Madsen et al. [15]. Although the change G_{83} E (ORF3) was absent from all the North American PRRSV isolates analyzed [11, 16] it was present in three pathogenic field isolates from Denmark from pigs putatively infected with the vaccine strain [15]. Analysis of the sequence of another highly pathogenic PRRSV isolate collected in Iowa in 1997 (unpublished data from this laboratory, strain obtained from NVSL, USDA/APHIS, Ames, IA, USA) indicated similarity to PRRSV 16244B at positions R_{13} and Q_{16} which suggest that changes at these positions may participate in attenuation. The mutation identified in ORF2 was not observed in the vaccine sequence reported by Medsen et al. [15]. There is not much information on the function of PRRSV gp2 but the substitution of a L by a F would change the shape of this region of the putative protein which could be enough to interfere with it function. We did not find any of the amino acid changes described by Yang et al. [33] in the ORF2 to 7 sequence of the vaccine strain.

3′ NCR

The 3′ NCR from the vaccine strain was identical to the 16244B strain [2] and to the VR-2332 strain [33].

Discussion

Here we have confirmed the avirulent phenotype for pregnant female swine and determined the genomic sequence of an attenuated PRRSV vaccine and compared them to those of the parental PRRSV VR-2332 and the pathogenic PRRSV 16244B. Christianson et al. [6] had previously confirmed that the VR-2332 was fully pathogenic when inoculated in sows at 90 days of gestation. Here we obtained similar results with the closely related pathogenic PRRSV 16244B field isolate. Statistically significant differences were observed in the virulence of these strains compared to the attenuated vaccine strain as shown in Table 2. The vaccine strain failed to induce abortions and the rate of piglet survival at 10 days post farrowing was of 94% compared to 29% for isolate 16244B.

The vaccine genome sequence was colinear to the VR-2332 and the16244B strains and there were no insertions or deletions. However, we identified five amino acid mutations in the structural and four amino acid mutations in the non-structural proteins coding region respectively that might be responsible for the attenuated phenotype.

The structural glycoprotein gp5 encoded by ORF5 had two amino acid mutations at positions $R_{13}Q$ and $R_{151}G$ that were unique for the vaccine strain. The Q_{13} is a conservative mutation and is located in the putative signal peptide sequence of the gp5, which is removed after translation. This mutation would change the net charge of the signal peptide probably influencing its function and interfering with the transport of gp5 to the membrane of the endoplasmic reticulum. The G_{151} change is located in the hydrophilic portion of the glycoprotein. It has been suggested that G_{151} identifies the vaccine strain and could be one of the many mutations involved in PRRSV attenuation [31]. Since gp5 is probably involved in binding to cellular receptors and contains the major epitopes for inducing both humoral and cellular immune responses [21] is then plausible that the observed change could alter tissue tropism thus contributing to the attenuated phenotype. A single amino acid change in the rabies virus glycoprotein [9] and in the poliovirus Sabin type3 VP3 [32] is sufficient for the acquisition of an attenuated phenotype. The protein encoded by ORF3 (gp3) had one unique mutation $G_{83}E$ which was absent from other North American PRRSV isolates [11, 16]. The change may increase the hydrophilicity of that region of the gp3 possibly affecting the function of the protein that has not yet been determined in PRRSV. However the $G_{83}E$ change was also described in three pathogenic Danish isolates from animals suspected to be infected with the vaccine virus [15]. This data suggest that E_{83} would be not likely involved in attenuation. Madsen et al. [15] reported the conservative amino acid substitution E_{16} Q (ORF6) as possibly influencing virus virulence. We have found the same amino acid substitution in ORF6 of the vaccine and we agree with Madsen et al. [15] that changes at this position could possibly alter the heterodimer (gp5/M) complex formation therefore influencing virus/host or virus/cell interactions. A conservative amino acid substitution was observed in the translation product of PRRSV vaccine ORF2. The function of the PRRSV ORF2 is still unknown. However it has been recently described a novel ORF2a gene in the arterivirus EAV and both ORF2a and ORF2b protein products are required for the production of infectious EAV particles [25]. If PRRSV ORF2 has similar biological functions than EAV ORF2a and ORF2b, then it is plausible to consider that mutations in this ORF could contribute to the PRRSV attenuation phenotype.

Changes in residues of the non-structural proteins encoded by the replicase gene have been reported to attenuate the phenotype of the coronavirus MHV-A59 [13]. Here Nsp1β had a change $S_{331}F$ that was located 8 residues from the catalytic site. The probable conformational change caused by this mutation may be sufficient to alter the efficiency of the proteolytic cleavage, thus resulting in an attenuated phenotype. Two amino acid mutations unique to the vaccine strain were identified in Nsp2. Nsp2 has been suggested to be involved in

species-specific functions [7]. No biological function has yet been identified for this protein in PRRSV but the finding that it contained the majority of the amino acids mutations is consistent with the suggested involvement in host range. It is likely that during the attenuation process through adaptation to MA-104 cell line several mutations took place in the Nsp2 portion of the ORF1a. Thus the attenuated phenotype could be the consequence of the vaccine strain adaptation to MA-104 cell line. However, it has been reported that the EAV Nsp2 acts as a cofactor for the NSP4 protease [29] and its presence was necessary for the major EAV ORF1ab proteolytic pathway [29]. It is then plausible to consider that PRRSV Nsp2 could also be required as a cofactor for PRRSV Nsp4 protease. The observed mutation could then be involved in a mechanism of down regulation of the ORF1ab proteolytic processing. The vaccine ORF1b has a unique mutation $Y_{952}H$ in the Nsp10 region of the polyprotein that contains the metal-binding and helicase domains [18, 24]. Substitution of the aromatic residue Y with a tertiary amine (H) would change the shape and the change of this region of the putative protein. Consequently the efficiency of the helicase function could be compromised, which in turn would interfere with the efficiency of virus replication and then contribute to the acquisition of an attenuation phenotype.

The vaccine RNA pseudoknot stem 1 had a nucleotide change G_{7687} A when compared to the pseudoknot stem 1 from other two pathogenic field isolates (16244B and Iowa). However the vaccine strain was identical to VR-2332 at this position, which suggests that the change may be not responsible for attenuation of PRRSV virulence. We did not determine the complete sequence of the vaccine 5′ NCR, but we identified one nucleotide substitution over the 165 bases sequenced. The function of the 5′ NCR in PRRSV transcription and replication is not known. However, it has been reported that the coronavirus 5′ NCR is involved in regulatory functions in the synthesis of subgenomic mRNAs [14]. Although the vaccine strain was identical to the parental strain at this position we cannot rule out the possibility that changes in the 5′ NCR might alter the efficiency of subgenomic mRNA transcription in PRRSV.

Together these observed molecular differences in ORF2, ORF3, ORF5, ORF6, Nsp1β, Nsp2 and Nsp10 and possible conformational changes in the RNA secondary structure may provide the basis for the attenuated phenotype.

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Authors' address: Dr. F. A. Osorio, Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0905, U.S.A.

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