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Erratum to “Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains”

Shishan Yuan, Daniel Mickelson, Michael P. Murtaugh, Kay S. Faaberg *

Department of Veterinary Pathobiology, 205 Veterinary Science Building, University of Minnesota, 1971 Commonwealth Avenue, St. Paul, MN 55108, USA

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Abstract

Two full-length porcine reproductive and respiratory syndrome virus (PRRSV) genomes, strain VR-2332 and its cell culture passaged descendent RespPRRS vaccine strain, were compared and analyzed in order to identify possible sites of attenuation. Of the 41 nucleotide changes, 12 resulted in conservative changes and 18 produced non-conservative changes. The results suggest that key amino acids in ORF1 may contribute to the phenotype of RespPRRS, which includes increased growth rate on MA-104 cells and decreased virulence in swine. The results provide a genetic basis for future manipulation of a PRRSV reverse genetics system. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first recognized as a ‘mystery swine disease’ in the United States in 1987 (Kef-faber, 1989). Since the virus was identified in Europe (Lelystad virus; Wensvoort et al., 1991) and in the USA (VR2332; Benfield et al., 1992; Collins et al., 1992), PRRSV has become a serious pathogen of swine herds world-wide (Rossow et al., 1999). PRRSV, along with lactate dehydroge-nase-elevating virus (LDV), equine arteritis virus

(EAV) and simian hemorrhagic fever virus (SHFV), is a member of the family Arteriviridae in the order Nidovirales (Cavanagh et al., 1990; Plagemann and Moennig, 1992).

PRRSV causes respiratory disease in young swine and production of mummified, weakborn or aborted piglets in pregnant sows through an unre-solved mechanism. Clues to the genetic basis of pathogenicity can be gleaned from comparison of the nucleotide sequence of a virulent parent viral strain with that of the cell-passaged attenuated variant. One such pair is the prototype North American isolate, strain VR-2332, and Resp-PRRS, its MA-104 cell adapted attenuated de-scendent. In this article, we report the complete

* Corresponding author. Tel.: +1-612-6249746; fax: +1-612-6255203.

E-mail address: kay@lenti.med.umn.edu (K.S. Faaberg).

nucleotide sequence of both strains of PRRSV and have deduced and characterized their nucleotide and protein differences. Several key amino acid changes between parental and vaccine strains were identified using this approach, but many other nucleotide and amino acid changes also occurred in genomic regions of unknown function. The nucleotide sequences were also compared to strain 16244B, which is claimed to be a virulent field revertant of RespPRRS (Allende et al., 2000). A full comparison of all three PRRSV strains shows that the origin of 16244B ('Michelle' strain) is unclear.

2. Materials and methods

2.1. Viruses

PRRSV strains VR-2332 and RespPRRS have been described in previous reports (Collins et al., 1992; Nelsen et al., 1999; Yuan et al., 1999). Strain 16244B (Michelle) was described by Allende et al. (1999). For growth curve analysis, MA-104 cells were infected with each PRRSV strain at an m.o.i. of 0.1. After 1 h of adsorption, the unattached virus was washed off of the cells and 5 ml of fresh cell medium (EMEM/10% FBS or DMEM/2% Swine Serum) was added. The culture was placed at 37°C, 5% CO₂ and 0.5-ml aliquots of virus were removed and replaced with 0.5 ml fresh medium at 4, 8, 12, 16, 24, 36, 48, 60, and 72 h. Viral plaque assays of each time point were completed on MA-104 cells with a 5-ml agar overlay (an equal volume of mixture of 2% low gelling point agarose (FMC SeaPlaque) and 2 × MEM medium). Incubation at 37°C, 5% CO₂ for 96–120 h revealed the presence of plaques, which were quantitated for each time point.

2.2. 5'-Race RT-PCR

PRRSV was harvested from infected MA-104 cells on day 5 post-infection (p.i.). After pelleting of cellular debris at 12000 rev./min, the supernatants were layered onto a 2 ml 0.5 M sucrose cushion in an SW 27 ultracentrifuge tube and

centrifuged at 24000 rev./min for 3 h. The pelleted virions were resuspended in STE buffer (200 µl; 10 mM NaCl/10 mM Tris pH 7.5/1 mM EDTA, 4°C) and transferred to a microcentrifuge tube. Another 200 µl of STE was used to collect any remaining disrupted virions and the two fractions of viral suspension were pooled and stored at –20°C.

Viral RNA was isolated using the QIAamp viral RNA kit (Qiagen) and stored at –80°C after quantitation by optical density and native RNA agarose gel electrophoresis. Approximately 1 µg of purified viral RNA in 6 µl was added to 2 µl of 2 µM reverse primer /1a-p3668 (5'-GGT-CGTTGACAAGTTGGTCATCTACCGGTTT-ATCCTCGGA), incubated at 67°C for 10 min and placed on ice. First strand cDNA synthesis was then completed as described previously (Yuan et al., 1999). The reverse transcribed product was purified (Microcon 100, Amicon) and the cDNA was eluted in 40 µl of RNase-free water. Multiple adenosine or guanosine residues were added to the 3'-end of purified PRRSV cDNA (~1 µg) using a terminal deoxynucleotide transferase procedure described by the manufacturer (TdT; New England Biolabs), diluted to 200 µl and stored at –20°C. First round PCR was completed as described previously using 2 µM Qt (5'-CCAGT-GAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTTTT) or Qc (5'-CCA-GTGAGCAGAGTGACGAGGACTCGAGCTCAAGCCCCCCCCCCCCCCCCCCC) and 15 µM Qo (5'-CCAGTGAGCAGAGTG-ACG) as the forward primers and 15 µM /1a-p3668 as the reverse primer (Frohman, 1994; Yuan et al., 1999). An identical second round of PCR was then completed using 10 µl first round PCR product and Qi (5'-GAGGACTCGAGCTCAAGC) and /1a-p1457 (5'-CCTTCGGCAGGCGGGGAGTAGTGTGAGGTGCTCAGC; 15 µM each) as the primer pair.

2.3. Sequence analysis

Automated sequencing reactions were completed with Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) using a PE 2400 Thermocycler (Perkin–Elmer) at the Univer-

sity of Minnesota Advanced Genetic Analysis Center. Comparison of the parental and vaccine genomes and deduced ORF amino acid comparison was completed using computer software included in the LASERGENE package (DNASTAR Inc., Madison, WI), Wisconsin Package Version 10.0 and (Genetics Computer Group (GCG), Madison, WI). GenBank accession numbers used for sequence analysis include the complete VR-2332 (U87392, Nelsen et al., 1999), RespPRRS (AF066183; Yuan et al., 1999) and 16244B (AF046869) sequences.

3. Results

3.1. Determination of the 5' terminal sequence of VR-2332 and RespPRRS vRNA

Primer extension experiments had suggested

that 20 nucleotides were unaccounted for in the published sequence of strain VR-2332 (Nelsen et al., 1999). In order to determine the unresolved bases, we derived several clones of PCR products generated from different passages of VR-2332 and RespPRRS (Fig. 1). Initially, several standard 5'-RACE was performed on passage 2 from the original VR-2332 field isolate brain homogenate, passage 31 of strain VR-2332 and passage 4 of RespPRRS vaccine strain (Frohman, 1994). Three different 5'-terminal base patterns were elucidated, with added ambiguity due to the poly T tract added during the 5'-RACE procedure, immediately preceding an apparent 18 base PRRSV sequence GACGUAUAGGUGUUGGC. Interestingly, heterogeneity at the 5'-end was detected early during passaging of the original PRRSV field isolate, yet no heterogeneity was seen after viral plaque purification to produce strain VR-

10	20	30	40	50	60	
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTATGCCTTGGCATTTGTATTGTCAGGAGCTGTGACCATTGGCAC						VR-2332
<i>TTTTTTTT</i> GACGTATAGGTGTTGGC						VR-2a.c1
<i>TTTTTTTT</i>						VR-2b.c29
<i>TTTTTAT</i>						VR-2b.c24
<i>TTTATAT</i>						VR-2b.c25
<i>TTTATAT</i>						VR-2b.c28
<i>TTTTTAT</i>						VR-31.c16
<i>TTTTTAT</i>						VR-31.c2
<i>TTTTTAT</i>						VR-31.c16
<i>TTTTTAT</i>						VR-31.c17
<i>CCCCCAT</i>						VR-24.c11
<i>CCCCCAT</i>						VR-24.c12
<i>CCCCCAT</i>						VR-24.c2
<i>CCCCCAT</i>						VR-24.c7
<i>CCCCCAT</i>						VR-24.c9
<i>TTTTTAT</i>T.						R-4.c1
<i>TTTTTAT</i>T.						R-4.c2
<i>CCCCTAT</i>A.						R-12.c1
<i>CCCCTAT</i>A.						R-12.c2
<i>CCCCTAT</i>A.						R-12.c3
<i>CCCCTAT</i>A.						R-12.c4

Fig. 1. 5'-terminal nucleotides of strains VR-2332 and RespPRRS. VR-2332 (VR) or RespPRRS (R) from different passages were subjected to rapid amplification of cDNA ends (RACE) and products were cloned and sequenced as described in Section 2. VR-2332 represents sequence submitted to GenBank prior to elucidation of the 5' terminal nucleotides. Strain VR-2332-infected brain homogenate was passaged two times on two separate occasions (VR-2a, VR-2b), or plaque purified and passaged 24 (VR-24) or 31 (VR-31) times. RespPRRS was passaged four (R-4) or 12 (R-12) times. Bold letters indicate previously unresolved bases and letter in italics refer to the non-viral nucleotides (T or C) arising from the RACE procedure.

2332 (Fig. 1). Further analysis of PCR clones derived from 5'-RACE experiments using poly (G) addition discriminated the remaining 5' terminal bases of strains VR2332 and RespPRRS. Only one additional base, corresponding to an adenine residue, was identified for strain VR-2332 (Fig. 1). Two additional bases, TA, were detected for the 5' terminal bases of four PCR clones derived from vaccine strain RespPRRS. The number of subsequent passages shown in Fig. 1 (2, 4, 12, 24, 31) indicates the relative stability of the cloned virus strains. These results are discordant with a recent report with respect to the first nucleotide of VR2332 and RespPRRS. Oleksiewicz and investigators reported a 5'-terminal U in strain VR-2332 and lack of such in strain RespPRRS (Oleksiewicz et al. 1999), whereas we observed a 5'-terminal U only in the RespPRRS vaccine strain. The complete leader sequence of RespPRRS vaccine strain shares 95.2% identity with another PRRSV vaccine strain, PrimePac PRRS (Shen et al., 2000) (data not shown).

We have determined that the genome size for VR-2332 is 15411 bases (U87392) and that of RespPRRS, with an extra 5'-terminal U, is 15412 bases (AF066183). RespPRRS leader sequence acquired two additional mutations when passaged for another eight passages at high m.o.i. (U → A base 36 and U → C at base 63). From the sequence data, we cannot determine exactly when the 5'-terminal thymidine residue of strain RespPRRS was acquired during passaging. However, the possibility exists that strain RespPRRS has developed individual genomes of variable nucleotide sequence due to strain evolution over the course of more than 70 passages. The RespPRRS sequence which we derived from 3- to 7-fold coverage of the genome (AF066183) is somewhat different from the sequence of RespPRRS recently reported (Allende et al., 2000).

3.2. Nucleotide comparison of the parental and vaccine PRRSV strains

Complete genome analysis of the parental strain VR-2332 and its cell-culture adapted descendant, strain RespPRRS, revealed that 41 nu-

cleotides had changed during viral strain attenuation. The changes appeared throughout the genome (Fig. 2A), except for ORF7 and the 3' untranslated region (UTR), and no single ORF exhibited less than the 99% identity between parental and vaccine strains (Fig. 2B). Of the 41 altered nucleotides, 10 resulted in silent mutations such that the encoded protein was not changed. One of these 10 silent mutations resided in the 5'-leader, 8 resided in ORF 1, and one silent change was detected in ORF3. The rest of the nucleotide changes resulted in amino acid changes in each of the remaining identified ORFs. Detailed analysis of the nucleotide changes, corresponding amino acid changes and potential coding domains are listed in Table 1. For ease of understanding, nucleotide numbering in Table 1 is based on the sequence of VR-2332 (1–15411). The 5'-terminal uracil residue of strain RespPRRS is numbered as zero, and all site designations are one less than the value of the sequence deposited in GenBank.

3.3. 5' leader sequence mutations

The resolution of the 5' terminal sequence indicated that the 5' ends of both of these PRRSV strains coded for a 34 aa peptide. However, no conclusive evidence has been generated to demonstrate the presence of this potential leader protein. The leader sequence of RespPRRS suggested some virus heterogeneity. In one case (Table 1, mutation 2a), $U_{36} \rightarrow A_{36}$ (VR-2332 → RespPRRS) coded for a conservative change at amino acid 12 (F12Y). In the other case (Table 1, mutation 2b), the $C_{63} \rightarrow U_{63}$ nucleotide change, located in the third base of the 21st codon in this polypeptide sequence, is silent. RNA folding predictions (Mfold; Zucker, 1989) suggested that each of these nucleotide changes resulted in minor alterations in nucleotide-pairing in stems of secondary structure. The 5'-terminal bases, an A residue in strain VR-2332 and an AT dinucleotide sequence in vaccine strain RespPRRS, are predicted to extend 5' from a long stable hairpin located immediately downstream between nucleotides 2 and 45 (data not shown).

Table 1
Comparison of parental PRRSV strain VR-2332 and vaccine strain RespPRRS^a

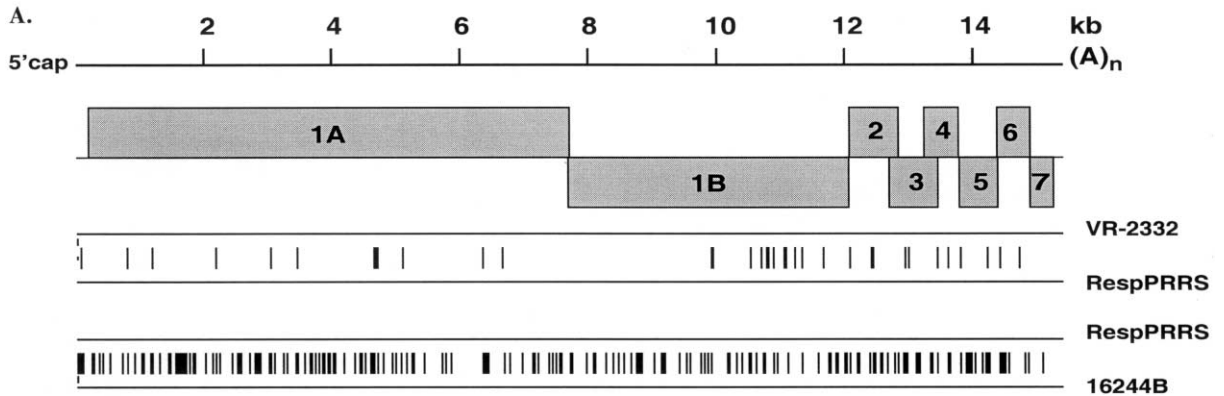
Mutation	ORF	Nt	VR → R	Type of change (codon position)	ORF AA #	VR → R	Type of change	Predicted domain
1	Leader	0	. → U	Addition	–		Nonconservative	Leader RNA
2.1 ^b	Leader	36	U → A	Transition (2)	12	F → Y	Conservative	Leader RNA and possible protein
2.2 ^b	Leader	63	C → U	Transition (3)	21	G → G	Silent	Leader RNA and possible protein
3	1a	784	G → A	Transition (1)	199	V → I	Conservative	NSP1β
4	1a	1181	C → U	Transition (2)	331	S → F	Nonconservative	PCPβ domain NSP1β
5	1a	2192	C → U	Transition (2)	668	S → F	Nonconservative	NSP2
6	1a	3040	G → A	Transition (1)	951	D → N	Semiconservative	NSP2
7	1a	3457	G → A	Transition (2)	1090	D → N	Semiconservative	NSP2
8	1a	4649	A → C	Transversion (2)	1487	N → T	Nonconservative	NSP3
9	1a	4681	U → G	Transversion (1)	1498	A → S	Semiconservative	NSP3
10	1a	4705	G → A	Transition (1)	1506	A → T	Nonconservative	NSP3
11	1a	5097	G → A	Transition (3)	1636	R → R	Silent	NSP3
12	1a	6345	A → U	Transversion (3)	2052	P → P	Silent	NSP5
13	1a	6674	C → U	Transition (2)	2162	P → L	Nonconservative	NSP5
14	1b	9918	U → C	Transition (2)	3244	L → L	Silent	NSP10
15	1b	9958	G → A	Transition (2)	3257	G → E	Nonconservative	NSP10
16	1b	10 533	U → C	Transition (1)	3449	Y → H	Conservative	Helicase domain NSP10
17	1b	10 697	U → C	Transition (3)	3503	A → A	Silent	Helicase domain NSP10
18	1b	10 781	G → A	Transition (3)	3531	T → T	Silent	NSP10
19	1b	10 803	U → C	Transition (1)	3539	C → R	Nonconservative	NSP10
20	1b	10 895	C → U	Transition (3)	3569	D → D	Silent	NSP10
21	1b	11 055	U → A	Transversion (1)	3623	S → T	Semiconservative	NSP11
22	1b	11 081	G → A	Transition (3)	3631	P → P	Silent	NSP11
23	1b	11 221	G → A	Transition (2)	3678	G → E	Nonconservative	NSP11
24	1b	11 229	G → U	Transversion (1)	3681	V → L	Semiconservative	NSP11
25	1b	11 329	G → C	Transversion (2)	3714	G → A	Nonconservative	Coronavirus-like domain; NSP11 (key amino acid)
26	1b	11 666	C → U	Transition (3)	3826	P → P	Silent	NSP12
27	2	12 102	G → U	Transversion (3)	10	L → F	Nonconservative	Signal sequence
28	2b	12 102	G → U	Transversion (1)	9	D → Y	Nonconservative	ND
29	2	12 436	G → U	Transversion (1)	122	A → S	Semiconservative	Extravirion
30	2	12 455	A → G	Transition (2)	128	K → R	Conservative	Extravirion
31	2	12 460	G → A	Transition (1)	130	V → M	Semiconservative	Extravirion

Table 1 (Continued)

Mutation	ORF	Nt	VR → R	Type of change (codon position)	ORF AA #	VR → R	Type of change	Predicted domain
32	3	12 943	G → A	Transition (2)	83	G → E	Nonconservative	Extravirion
33	3	12 950	C → U	Transition (3)	85	D → D	Silent	Extravirion
34	3	13 446	G → A	Transition (1)	251	A → T	Nonconservative	Extravirion
35	4	13 446	G → A	Transition (2)	69	C → Y	Nonconservative	Extravirion
36	4	13 614	U → C	Transition (2)	135	V → A	Nonconservative	Extravirion
37	5	13 825	G → A	Transition (2)	13	R → Q	Semiconservative	Signal sequence
38	5	14 238	A → G	Transition (2)	151	R → G	Nonconservative	Intravirion
39	6	14 420	C → G	Transversion (1)	16	Q → E	Conservative	Extravirion
40	6	14 735	C → G	Transversion (1)	121	R → G	Nonconservative	Intravirion
41	6	14 737	G → C	Transversion (3)	121	R → G	Nonconservative	Intravirion

^a Analysis of complete genome sequences and classification of amino acid changes were completed using GCG computational biology computer programs. Predicted domains of viral proteins was based on Hopp–Woods analysis of peptide structure (Jameson and Wolf, 1988) were also completed using GCG. Predicted ORF1 non structural proteins (NSP) were derived from genome comparison of strain VR-2332 with PRRSV strain 16244B (Allende *et al.*, 1999) and with equine arteritis virus (Snijder *et al.*, 1999; van Dinten *et al.*, 1999).

^b Represent two separate sequence analyses of RespPRRS leader (Fig. 1).

**B.**

Genomic Region	Number of Nucleotide Changes	Percent Identity
5'-UTR	2	99.5
1a	11	99.9
1b	13	99.7
2	4	99.5
3	4	99.1
4	2	99.6
5	2	99.7
6	3	99.4
7	0	100
3'-UTR	0	100

Fig. 2. Nucleotide differences between strain VR-2332 and RespPRRS. (A) Full-genome schematic the nucleotide differences between VR-2332 and RespPRRS reveals several changes occurred in ORF1a, a cluster of changes were seen in the 3'-terminus of ORF1b, and discrete changes were seen in all envelope glycoproteins (ORFs 2–5) and in the membrane protein (ORF6). When RespPRRS is similarly compared to strain 16244B, many nucleotide changes are seen throughout the genome. (B) Each region of the PRRSV genome was analyzed for the number of nucleotide changes and the corresponding ORF percent identity between VR-2332 and RespPRRS.

3.4. Changes in identified ORF 1 domains

Several PRRSV ORF 1 replicase protein domains have been identified (Fig. 3; Snijder and Meulenber, 1998; Allende et al., 1999; Nelsen et al., 1999). From the analysis of both VR-2332 and RespPRRS genomes, it was deduced that only four of the 15 ORF 1 specific nucleotide changes

occurred in these identified domains. One mutation, a C₁₁₈₁ → U₁₁₈₁ transition, which would alter amino acid 331 of ORF 1 from a serine residue in strain VR-2332 to a phenylalanine in the attenuated strain RespPRRS (S331F), resulted in a non-conservative change located in the putative ORF1 cleavage product, nsp2 (den Boon et al., 1995; Allende et al., 1999, 2000; Nelsen et al., 1999). A

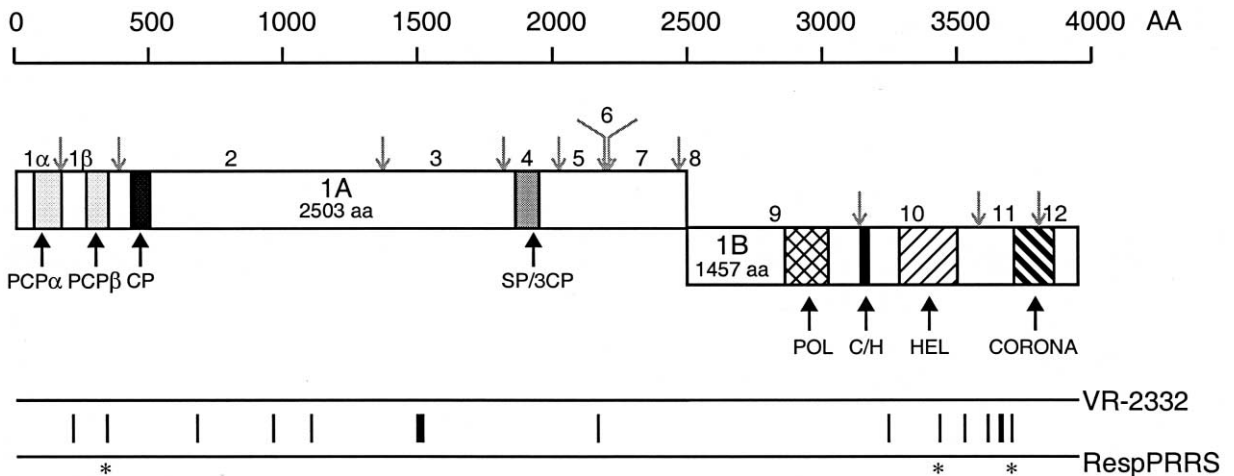


Fig. 3. ORF1 protein amino acid changes between VR-2332 and RespPRRS. ORF1 protein schematic shows identified domains: papain-like cysteine proteases α and β (PCP α and PCP β), Cysteine protease (CP), serine protease/3c-like protease (SP/3CP), polymerase (POL), cysteine/histidine-rich domain (C/H), helicase domain (HEL) and coronavirus-like domain (CORONA). The schematic also shows possible nonstructural proteins (1–12) produced during cleavage by ORF1-encoded proteases at proposed cleavage sites (grey arrows). Seventeen amino acid changes occurred during *in vitro* passaging of strain VR-2332 to produce attenuated strain RespPRRS. Interesting mutations mentioned in the text are indicated by an asterisk.

second recognizable mutation, a $U_{10533} \rightarrow C_{10533}$ transition, resulted in a change from tyrosine to histidine within the helicase domain. The final ORF1 recognizable mutation was located in the coronavirus-like domain. In this instance, the transversion from $G_{11329} \rightarrow C_{11329}$ resulted in amino acid 3714 undergoing a non-conservative change from glycine to alanine. A glycine residue at this position, except for strain RespPRRS, has been shown to be conserved in all nidoviruses analyzed to date (Nelsen et al., 1999).

All other amino acid changes located within ORF1 are in regions of unknown function. However, the cluster of amino acid mutations located near the carboxyl terminal end suggests that the replicase region was altered during passage to result in a more fit virus for replication in cell culture, as evidenced by the *in vitro* one-step growth curve comparison shown in Fig. 4. The growth curve comparison appears to be the reverse of virus strain replication in swine (M. Roof, unpublished data).

3.5. Changes resulting in altered structural proteins

PRRSV ORFs 2–7 have been shown to code for four glycoproteins (GP2–5), the membrane protein (M) and the nucleocapsid protein (N), respectively. Sequence analysis of strains VR-2332 and RespPRRS indicated that there were 15 nucleotide changes in this region, and all but one of which resulted in amino acid alterations. There were four amino acid mutations within GP2. One amino acid change (aa10, L \rightarrow F) was located within the predicted signal sequence of GP2, but the nucleotide mutation ($G_{12102} \rightarrow U_{12102}$) also altered amino acid 9 from an aspartic acid to a tyrosine of a putative ORF2a protein (Wu, Christopher-Hennings, and Nelson, unpublished data). The remaining three GP2 changes (aa 122, A \rightarrow S; aa 128, K \rightarrow R; aa 130, V \rightarrow M) were clustered in an eight amino acid region in the middle of the protein sequence, predicted to be located on the exterior of the virion. The three amino acid

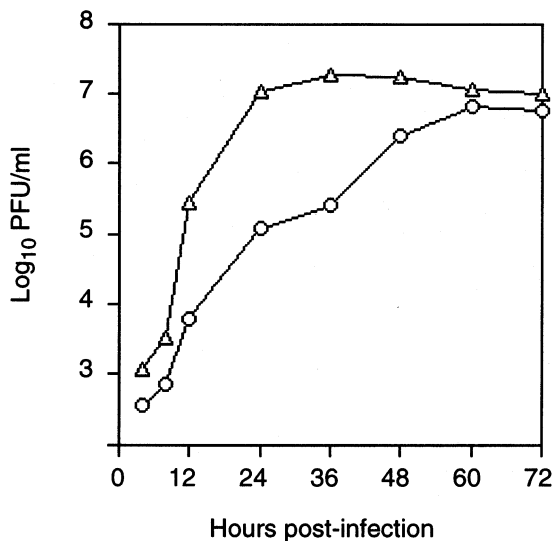


Fig. 4. Viral growth curves of PRRSV strains VR-2332 (○) and RespPRRS (△) reveal that RespPRRS has enhanced growth kinetics *in vitro*. Results are representative of three separate experiments. The number of plaques were determined in triplicate for two separate viral dilutions and were found to deviate by less than 5% in each individual experiment.

modifications that occurred in GP3 (aa 83, G → E; aa 116, G → S; aa 251, A → T) and the two in GP4 (aa 69, C → Y; aa 135, V → A) also are predicted to lie on the exterior of the virion. All of these mutations but one are semi- or non-conservative changes. In the putative viral attachment protein GP5, two amino acids were altered during virus attenuation. One GP5 change occurred within the proposed signal sequence (aa 13, R → Q) and the other change (aa 151, R → G) is predicted to be located in the virion interior (Faaberg and Plagemann, 1995). GP5 has been shown to be disulfide linked to the M protein (Faaberg et al., 1995; Mardassi et al., 1996) and studies with a similar virus have revealed that this disulfide bond is critical to infectivity (Faaberg et al., 1995). While no mutations occurred in the extravirion region of GP5, one mutation did occur within the M protein (aa 16, Q → E) predicted to be located on the outer surface of the virus. Although this M protein mutation was conservative, mutations in this critical region of the PRRSV viral attachment heterodimer could be important. However, analysis of the sixth *in vivo* backpassage of RespPRRS re-

vealed that this mutation had reverted back to a VR-2332-like sequence while maintaining all other ORF 5 and 6 mutations and the avirulent viral phenotype (data not shown). The other M protein mutation was shown to be the result of two nucleotide changes within the codon for amino acid 121, altering the sequence from an arginine to a glycine (R → G). Curiously, both carboxyl terminal GP5 and M mutations resulted in a R → G change.

3.6. Comparison of strains VR-2332 and RespPRRS to PRRSV 16244B field strain

In 1997, PRRSV strain 16244B was isolated from a Nebraska herd and has since been shown to be virulent (Allende et al., 1999, 2000). Full-length genome sequencing revealed that this strain was 98% identical to VR-2332 and RespPRRS (Allende et al., 2000). Whether the isolate is a naturally occurring PRRSV field variant, or directly related to the original VR-2332 field isolate (isolated in 1989) or the RespPRRS vaccine strain (released in 1994) is not known. To address this question, a full-length genome comparison of strain RespPRRS to strain 16244B was completed, as it has been suggested that strain 16244B is a field revertant of RespPRRS (Allende et al., 2000) (Fig. 2A). As can be seen in Fig. 2A, strain 16244B differs from RespPRRS at many nucleic acid residues.

All three strains were analyzed in detail. We compared identical and non-identical nucleic acid residues between strains VR-2332 and RespPRRS, strains VR-2332 and 16244B, between RespPRRS and 16244B, and between all three strains. We observed 15157 instances in which all three viral genomes contained the identical base, 215 instances in which strains VR-2332 and RespPRRS were identical but different from strain 16244B, 17 cases in which strains VR-2332 and 16244B were identical but different from strain RespPRRS, and 22 instances in which strains RespPRRS and 16244B were identical but different from VR-2332. Importantly, no instances were observed in which the three strains were all different from one another. If strain 16244B were in fact derived from a field reversion of RespPRRS, the data would imply that for 237 nucleotide mutations 16244B reverted back to the exact VR-2332 nucleotide sequence and in no

instance would it have mutated to either of the two possible remaining bases. The likelihood of such a probability is exceedingly low. We also considered the possibility that strain 16244B resulted from a viral recombination event between strain RespPRRS and an unidentified but closely related field isolate. However, no region of considerable length was clearly identified as being derived from strain RespPRRS, which one would expect if viral recombination had taken place. Therefore, the origin of 16244B remains unclear.

4. Discussion

An attenuated PRRSV strain, RespPRRS, was found to differ in sequence from the parental strain, VR-2332, by only 41 nucleotides. This represents a 0.28% variation during 70 passages in MA-104 cells. This rate of variation is comparable to other RNA viruses (Steinhauer and Holland, 1987) and suggests that PRRSV is not susceptible to inherently high spontaneous mutation rates in the absence of immunological and environmental pressures. This may explain the relative stability of strain VR-2332 when passaged alone in cell culture, as this report delineates. However, PRRSV has been shown to undergo high frequency recombination in the presence of two or more virus strains (Yuan et al., 1999) and appears to evolve rapidly in the presence of biological pressure.

Attenuation can result from changes in many areas of viral genomes and the 41 nucleotide mutations described include alterations in several key PRRSV regions. Thus, no definitive site of PRRSV attenuation can be identified simply from sequence analysis. Twelve of the 41 nucleotide changes in strain RespPRRS were silent. However, one or more of these silent mutations could change the secondary or tertiary structure of PRRSV RNA and thus alter the stability of the genome. In other viruses, such as picornaviruses, viral RNA folding may play a critical role in host protein association (Meerovitch et al., 1989) and in neurovirulence (Pilipenko et al., 1989a,b; Westrop et al., 1989).

The 5' and 3' ends of viral sequences have been correlated with attenuation in other viruses, such as poliovirus (Westrop et al., 1989), and shown to be important in viral replication and transcription for coronaviruses, another member of the Nidovirus order (Williams et al., 1999). Only two mutations occurred in the 5'-end of PRRSV during attenuation of strain VR-2332 to RespPRRS, and their relative roles in attenuation must be further investigated. No change occurred in the 3'-end of the PRRSV genome. Viruses may also acquire attenuated phenotypes as a result of mutations in viral proteases (Ni et al., 1995), protease cleavage sites (Tozser et al., 1999), within the polymerase gene (Pelosi et al., 1998; Skiadopoulos et al., 1999), by altering viral proteins to decrease virion stability (Bailly et al., 2000) or to interfere with the virus/host interaction (McCright et al., 1999), as well as by other mechanisms. The comparison of VR-2332 to its vaccine correlate, RespPRRS, revealed that the possibility exists for one or more of these mechanisms to be involved in attenuation.

A recent publication suggested that nine specific amino acid changes may be important in attenuation of RespPRRS (Allende et al., 2000), possibly based on the assumption that PRRSV attenuation may be localized by amino acid comparison of a non-parental genome (16244B) with a parental (VR-2332) and its vaccine offspring (RespPRRS). We have determined that our sequence of the RespPRRS strain is somewhat different from what has been previously reported (Allende et al., 2000), which brings additional complexity to the derivation of potential PRRSV attenuation sites. In addition, the 5'-terminal nucleotides for both VR-2332 and RespPRRS are markedly different from those reported for strain 16244B. In our view, PRRSV attenuation sites may be genotype specific and variable, possibly resulting from a complicated interplay of genomic regions and viral and host specific factors. Postulation of attenuation sites can lead to interesting hypothesis-based experiments. However, a reverse genetics system for PRRSV must be produced and genetically altered at specific nucleotides in order to discern sites of attenuation.

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