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Short Communication

Recombination between North American strains of porcine reproductive and respiratory syndrome virus

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV), a recently discovered arterivirus swine pathogen, was shown to undergo homologous recombination. Co-infection of MA-104 cells with two culture-adapted North American PRRSV strains resulted in recombinant viral particles containing chimeric ORF 3 and ORF 4 proteins. Nucleotide sequence analysis of cloned recombinant PCR products, encompassing 1182 bases of the 15.4 kb viral genome, revealed six independent recombination events. Recombinant products persisted in culture for at least three passages, indicating continuous formation of recombinant viruses, growth of recombinant viruses in competition with parental viruses, or both. The frequency of recombination was estimated from $<2\%$ up to 10% in the 1182 b fragment analyzed, which is similar to recombination frequencies observed in coronaviruses. An apparent example of natural ORF 5 recombination between naturally occurring wild type viruses was also found, indicating that recombination is likely an important genetic mechanism contributing to PRRSV evolution. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Porcine reproductive and respiratory syndrome virus; Viral recombination; Differential RT-PCR

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae* of non-segmented positive single-stranded RNA viruses, was discovered as the cause of a mystery swine disease in Europe (Lelystad virus, LV; Wensvoort et al., 1991) and in the U.S. (VR-2332; Collins et al., 1992). These two strains (VR-2332 and LV) are extremely diverse in nucleotide sequence (Meulenberg, et al., 1993; Murtaugh et al., 1995; Nelsen, et al., 1999). Substantial sequence variation also occurs among North American PRRSV isolates (Kapur et al., 1996; Andreyev et al., 1997; Murtaugh et al.,

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1998) and such variation could be due to innate error rates of the RNA polymerase (Hubner et al., 1992; Ward et al., 1988) or PRRSV recombination (Kapur et al., 1996). The arterivirus family belongs to the order *Nidovirales*, along with coronaviruses and toroviruses (Cavanagh, 1997). Coronaviruses undergo high frequency viral recombination (Lai et al., 1985; Keck et al., 1988; Masters et al., 1994; Jia et al., 1995; reviewed in Lai, 1996) and toroviruses appear to have undergone non-homologous recombination events (Snijder, et al., 1991). Although putative recombination events among arteriviruses have been reported (Chirnside et al., 1994; Godeny et al., 1998), we provide the first experimental evidence that arteriviruses undergo homologous recombination.

Two vaccine strains of North American PRRSV, RespPRRS® (R, Boehringer Ingelheim Animal Health, St. Joseph, MO) and Prime Pac® PRRS (S, Schering Plough Animal Health, Kenilworth, NJ) were reconstituted from vials (4×10^5 5 plaque forming units (pfu)/ml) and inoculated into separate cultures of MA-104 cells (EMEM/10% FBS) at a multiplicity of infection (moi) of 0.1. The infected cells were incubated at 37°C until the cytopathic effect (CPE) was greater than 80%, and then supernatants were harvested (1.3×10^7 $TCID_{50}$/ml). MA-104 cells were infected with each virus alone or co-infected with R and S at a combined moi of 1.0, strain ratios (R:S) of 1:3, then incubated until CPE was complete (3 days p.i.). Plaque assays of the infected cell supernatants indicated that both plaque size and PFU/ml of the mixed infection viruses appeared similar to vaccine viruses alone (data not shown). Passage 1 media supernatants were used to reinfect MA-104 monolayers at an moi of 1.0 (passage 2) and the infection protocol was repeated twice more (passages 3 and 4). Viral RNA was isolated from each infected cell supernatant.

A comparison of RespPRRS® and of PRIME PAC® PRRS genomic sequence (GenBank submissions AF066183 and AF066384) identified strain specific regions. Differential primers (R9/, S9/, /R13, /S13) were synthesized to amplify a 1182 base region from nucleotides 12886–14067, encoding part of ORF 3 through ORF 5. The forward primers (R9/ and S9/) differed by eight bases and the reverse primers (/R13 and /S13) differed by six bases (Table 1). Total RNA was isolated from 280 μl infected cell supernatant containing infectious progeny virus using the QIAamp Viral RNA Kit (QIAgen Inc.). RNA templates (6 μl each) were each annealed to 2 μl of 50 μM random hexamers and reverse transcribed using 2.5 units/μl Moloney murine leukemia virus (MuLV) reverse transcriptase, 1 unit/μl RNase inhibitor and 1 mM each dNTP in 5 mM $MgCl_2$/50 mM KCl/10 mM Tris–HCl, pH 8.3 at 42°C for 15 min (Perkin-Elmer Cetus). PCR amplification was completed on cDNAs from each infection using each forward primer paired with a homologous or heterologous reverse primer to screen for recombinant sequences. cDNA (5 μl) was added to 20 μl of mastermix such that the final component concentrations were 140 nM each primer, 200 μM each dNTP, 2 mM $MgCl_2$/50 mM KC1/10 mM Tris–HCl, pH 8.3 and 0.025 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Each sample was denatured at 94°C for 4 min, followed by 30 cycles at 68°C for 30 s, 72°C for 45 s and 93°C for 45 s. Amplified fragments were polished at 72°C for 10 min and visualized on an agarose gel. Supernatants from cells infected only with R or S parent virus yielded

Table 1
Primers used in RT-PCR analysis and sequence determination. Forward primers are indicated by a slash (/) following the primer name, reverse primers are denoted by a slash before the primer name

Primer	Sequence
S9/	CAGCGCTACGAACCTGGCAAGGT
R9/	GAGATCTACGAACCCGGTAGGTC
/S13	AACCAGACCAACTGTGTCAAGGAAG
/R13	GACTAAAGCGACTGTGTCAAGGAAA
p55/	CACCTGAGACCATGAGGTGG
/P56	GATCTTACCCAGCATCTGGCAC
416P4/	TATGGTGCCCTCACTACCAG
/05P2	GAAGCAAGTCAACGCAGCC
513P4/	GAATTGTGCTTTCACCTTCGG
/06P1	GCGGCACTTTCAACGTGG
/P34	GCAACTGATGTCTTGGAGG
P41/	GACGGCGGCAATTGGTTTC
761P7/	CCAGCTACGTCCAACATG

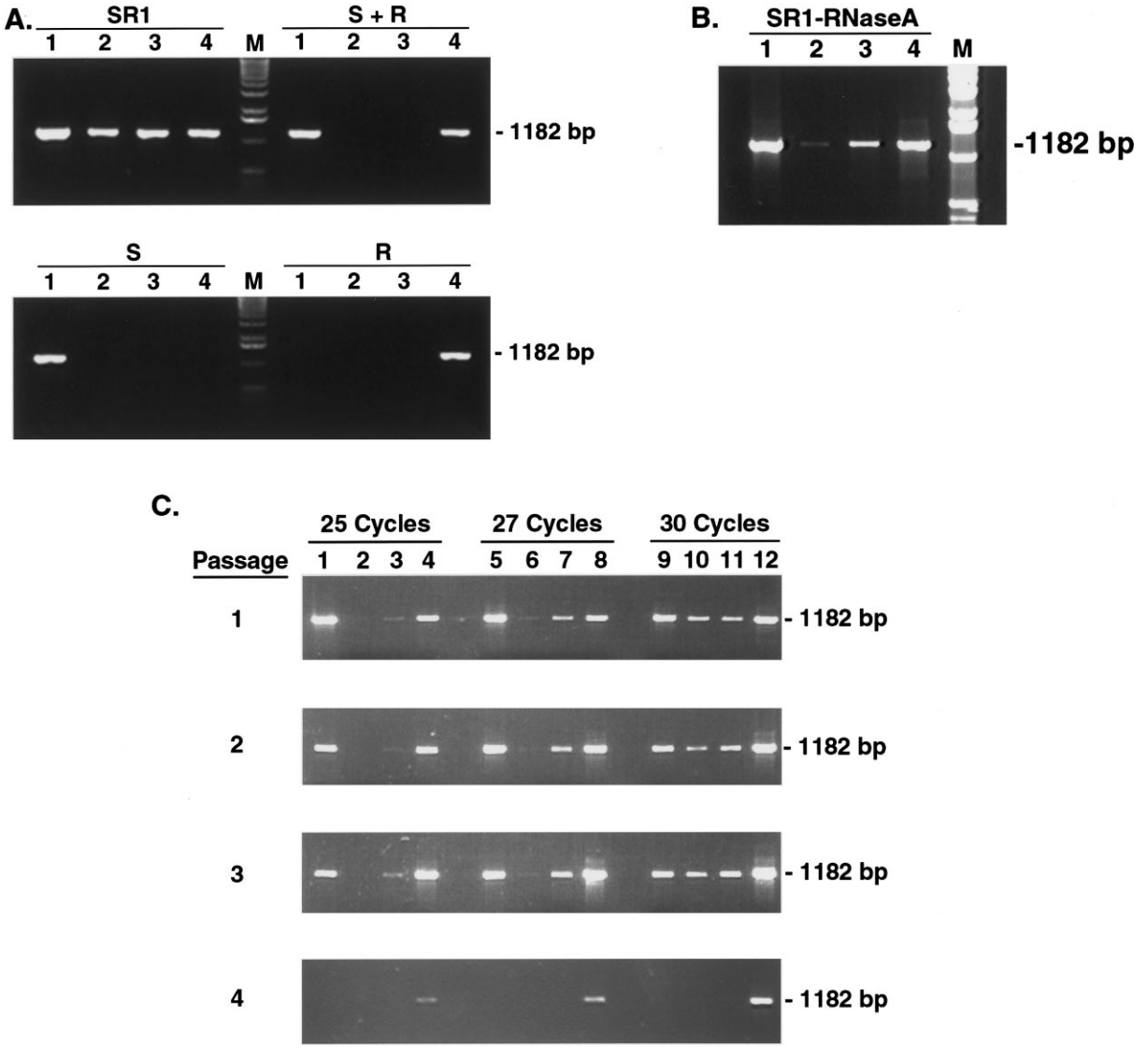

Fig. 1. Reverse transcription and polymerase chain reaction (RT-PCR) analysis of first passage infected cell culture supernatants. (A.) Panel SRI: co-infection of MA-104 cells with strains S and R; Panel S + R: in vitro mixture of RNA generated by separate infection of MA-104 cells with strains S and R; Panel S: infection of MA-104 cells with strain S alone; Panel R: infection of MA-104 cells with strain R alone. Lane 1: primer pair S9/S13; lane 2: primer pair S9/R13; lane 3: primer pair R9/S13; lane 4: primer pair R9/R13. (B.) SRI cell supernatant was treated with 33 μg/ml RNase A for 30 min at 37°C, followed by protease K digestion (300 ng/ml) in the presence of 0.6 units/μl RNase inhibitor and 1 mM dithiothreitol (DTT) for 30 min at 37°C. SRI viral RNA was then isolated, reversed transcribed and amplified as described. (C.) cDNAs generated from passages 1–4 were PCR amplified for 25, 27 or 30 cycles with primer pairs S9/S13 (lanes 1, 5, 9); S9/R13 (lanes 2, 6, 10); R9/S13 (lanes 3, 7, 11), and R9/R13 (lanes 4, 8, 12). A 1 kb DNA ladder standard was used for analyses (M). The agarose gels were digitally processed for publication using Adobe Photoshop® 5.0 on a Power Macintosh 8500/150.

product DNA only from homologous virus primer pairs (Fig. 1A, panels S and R). In contrast, supernatants from co-infected cells yielded DNA products from both heterologous and homologous primer pairs, indicating that the two vaccine viruses had recombined in the genomic region encoding ORF 3-5 (Fig. 1A, panel SRI). In order to exclude the possibility that this result occurred postharvest, due perhaps to enzymatic template switching, the individual vaccine viral RNAs from P1 cell supernatants were pooled and analyzed in the same RT-PCR experiment. In this case, as shown in Fig. 1A (panel S + R, lanes 2 and 3), heterologous primer pairs did not amplify recombinant bands. This result indicated that viral RNA recombination events occurred during co-culture of the parent viruses and were not artificially generated during RT-PCR amplification. To show that recombination resulted in packaged viral RNA, first passage supernatant from the co-culture was treated with RNase A before RNA isolation. The RNA was protected from RNAse A degradation and resulted in a similar RT-PCR pattern of recombination (Fig. 1B). Purified RNA added to culture supernatant was not similarly protected (data not shown), indicating that the supernatants contained RNases and that recombinant PCR products came only from packaged viral RNAs.

Sequencing of recombinant PCR products in Fig. 1A confirmed that the DNAs were derived from recombinant viral molecules. Potential recombinant PCR products were purified using Microcon-100 microconcentrators (Amicon, Inc.). Nucleotide sequences (1172 bp) of the 1182 bp PCR products were determined by the Advanced Genetic Analysis Center of the University of Minnesota (St. Paul, MN) with primers P41, /P34 and 761P7/ (Table 1) and compared to parental vaccine viruses (S and R). One recombinant sequence, RS (generated by R9/S13), exhibited 100% identity to strain R for 445 bases at the 5′ end but 100% identity to strain S for 627 bases at the 3′ end (Fig. 2). Two DNA sequence ambiguities (denoted N in Fig. 2) were evident in the middle portion of the RS sequence. An SR recombinant (generated by S9/R13) exhibited 100% identity to strain S for 78 bases at the 5′ end but showed 100% identity to strain R for 659 bases at the 3′ end. Eight sequence ambiguities in the middle portion of the SR recombinant sequence were observed (Fig. 2).

RS product DNAs were ligated into the pGEM®T vector (Promega) and used to transform DH5α *E. coli*. Five individual clones (RS131, 132, 135, 136, and 137) were sequenced as above. Sequence analysis and comparison was completed with computer software included in the LASERGENE package (DNASTAR Inc.), Wisconsin Package Version 9.1 (Genetics Computer Group (GCG)), and EUGENE (Molecular Biology Information Resource). Sequence analysis of five independent clones from the RS recombinant DNA product revealed five unique patterns of recombination within the 1182 bp region (Fig. 2). Clones RS136, RS131, RS135 and RS137 each appeared to represent a single and unique cross-over event (Fig. 2). Clone RS132 appeared to be a triple cross-over recombinant, as its sequence was 100% identical to parent strain R for the first 200 bases, 100% identical to parent strain S for the next 562 bases, 100% identical to strain R for 115 bases, then 100% identical to strain S for the remainder of the sequence. Recombination did not disrupt the encoded proteins. Three clones encoded chimeric ORF 3 proteins (RS132, RS135, RS137), three clones encoded chimeric ORF 4 proteins (RS131, RS132, RS136), and none of the clones resulted in a chimeric ORF 5 protein.

We cannot pinpoint the precise cross-over points from the sequences due to significantly long regions of sequence identity between the parent strains. The actual cross-over events presumably occurred within these regions, as was shown with other RNA viruses (Nagy and Simon, 1997). No obvious sequence-dependent, structure-dependent or motif-dependent recombination could be discerned through computer analysis, although potential leader-body junction site motifs (consensus motif UUAACC, Faaberg et al., 1998) were noted near areas of recombination. Leader-body junction motifs have not been shown to influence coronaviral recombination (Lai, 1996).

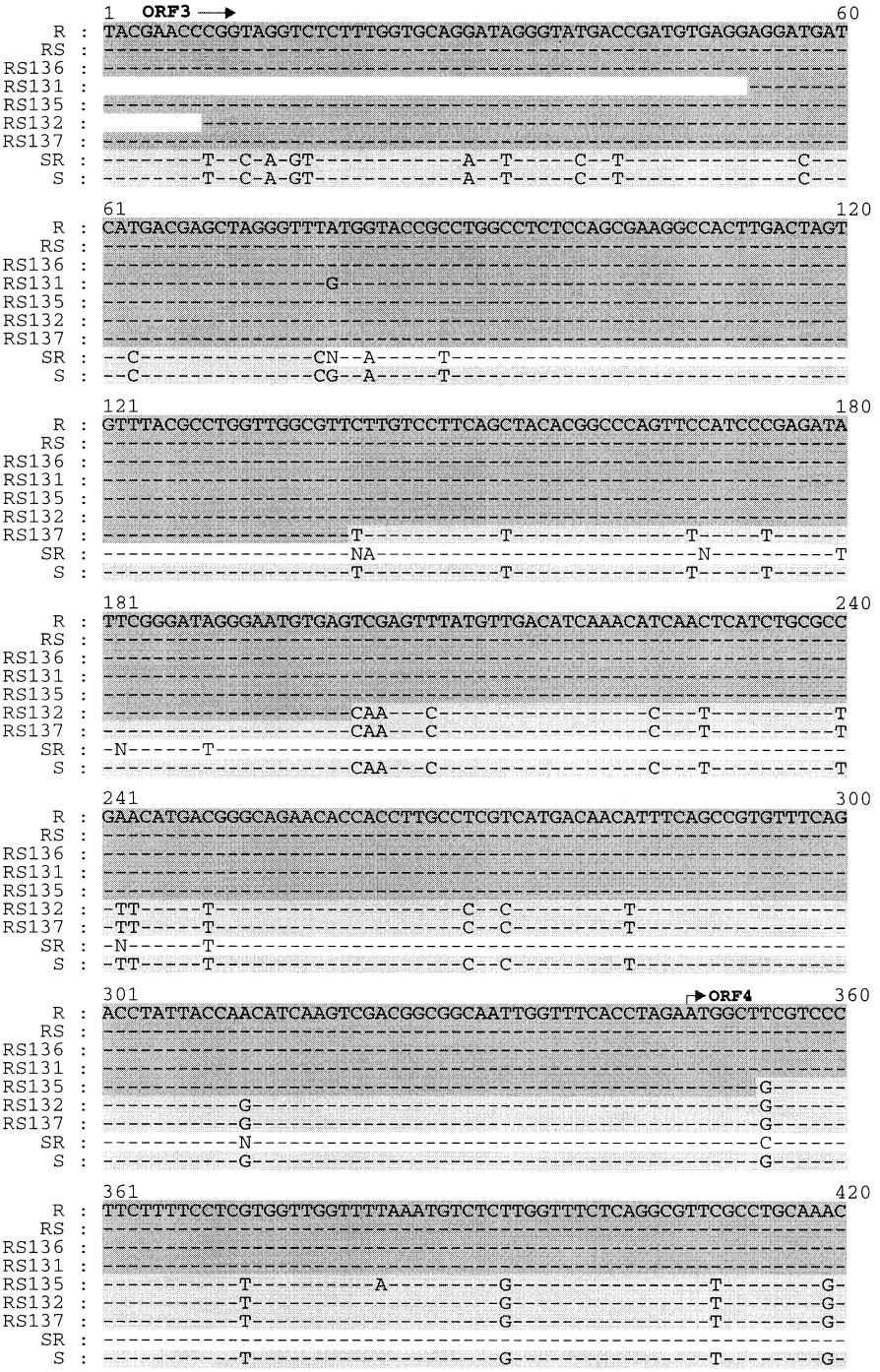

Fig. 2. PCR product and recombinant clone sequences suggest multiple cross-over events. Differential RT-PCR products from the experiment described in Fig. 1, panel SR1 lanes 2 and 3 (SR and RS, respectively) were purified and sequenced. Dashes indicate sequence identity with strain R. Domains representing probable identity with each parental vaccine are shaded (R = darkly shaded nucleotides, S = lightly shaded nucleotides, regions of PCR product sequence ambiguity are unshaded).

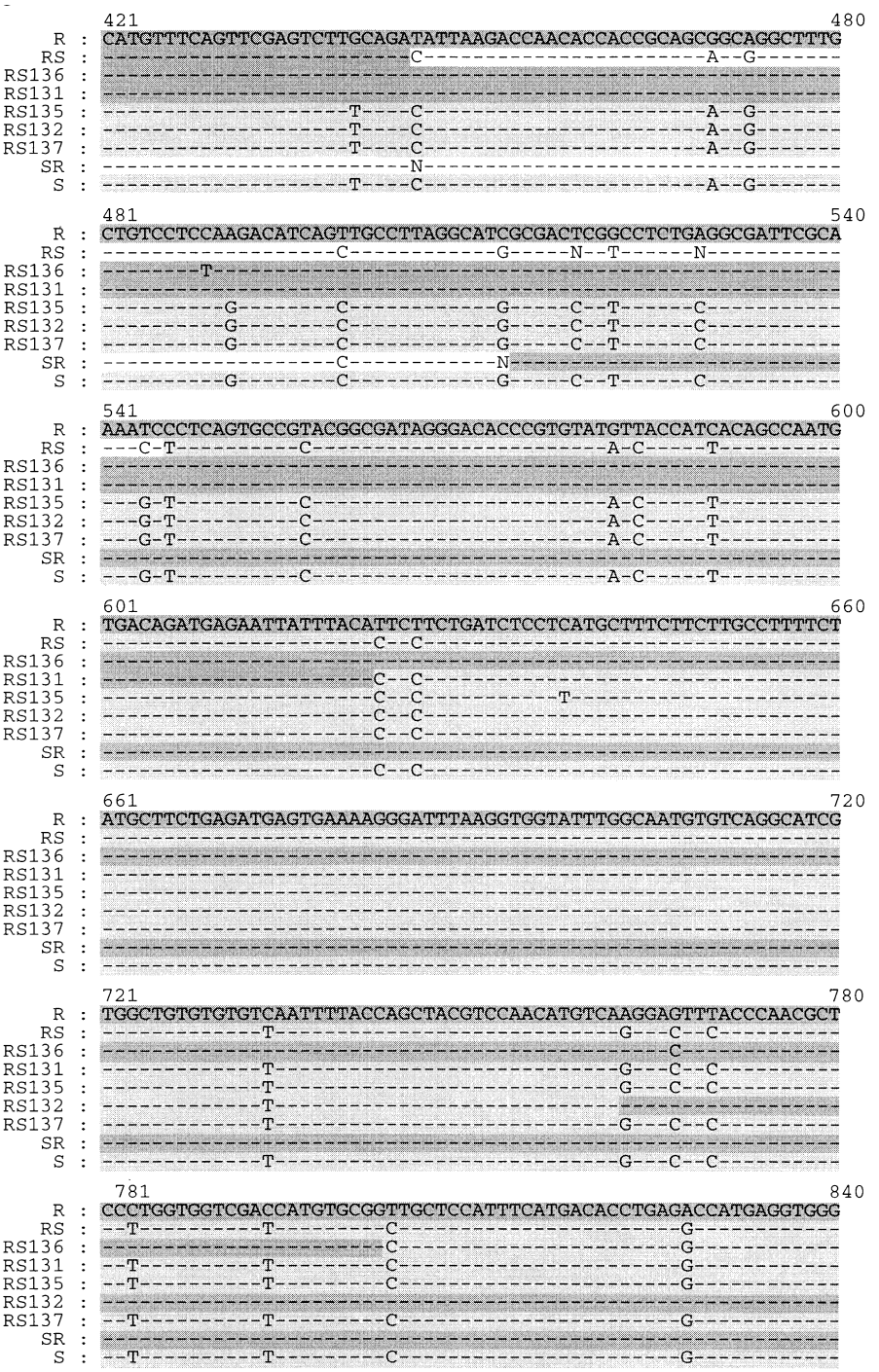

Fig. 2. (*Continued*)

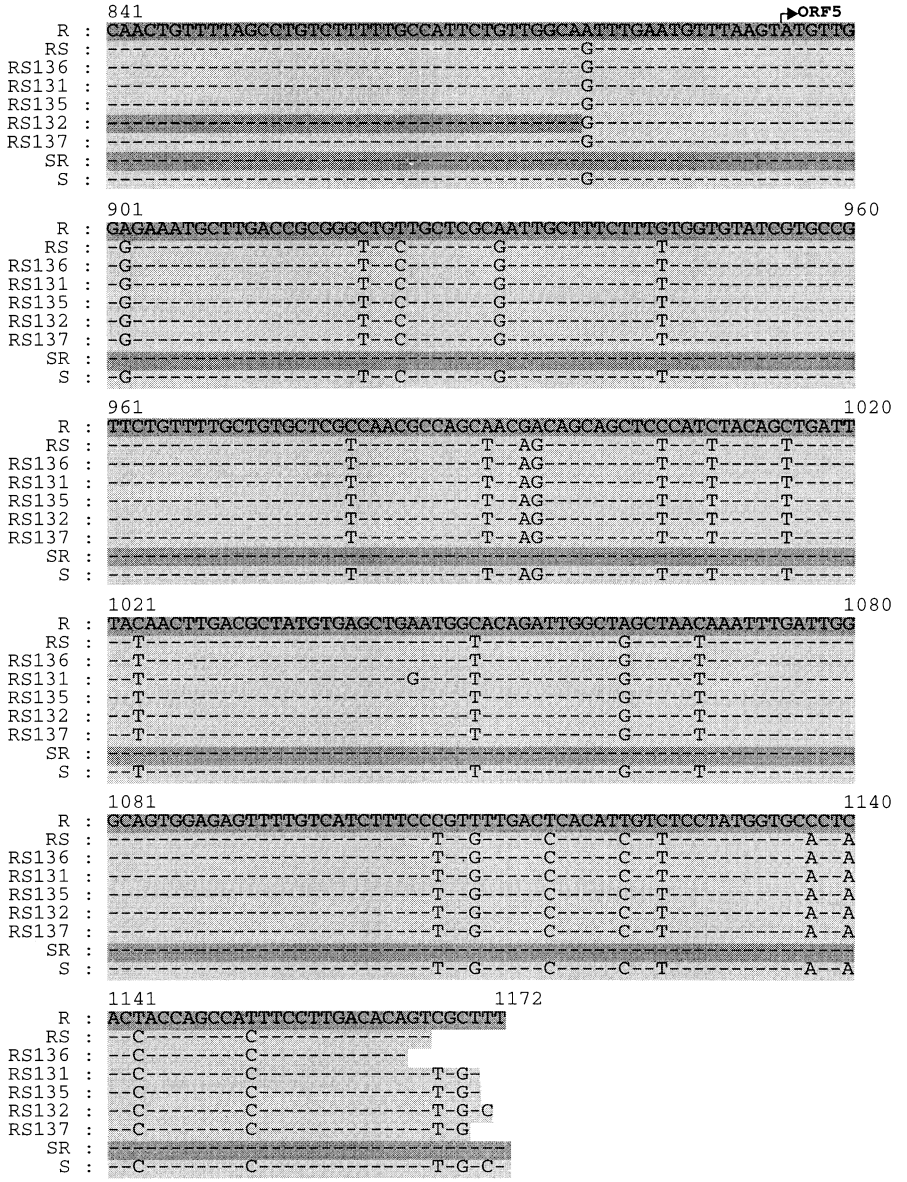

Fig. 2. (*Continued*)

To assess whether or not recombinant viral RNA was maintained during successive passages in culture and to approximate PRRSV recombination frequency, cDNAs from passage 1–4 culture supernatants were amplified for different cycle times with the same homologous and heterologous primer pairs (Fig. 1C). At 25 cycles, the parental RNAs were readily detected in passage 1–3 supernatants, the RS product was barely detected, and almost no SR product was observed. Increasing the cycle number to 27 or 30 revealed that RS products were consistently more

abundant than SR products in passages 1–3. However, at passage 4, only parental vaccine R product was detected in the supernatant. The relative ease with which recombinant RT-PCR bands were detected in passages 1-3 suggests a high frequency event and that recombinant viruses, especially RS recombinants, were continually produced and/or competed for growth with the parental vaccine strains. Passage 4 analysis revealed that strain R was better suited than either strain S or recombinant virus for continuous growth on MA-104 cells. Such a growth disparity also accounts for the inability to observe recombination after 1 passage at an R:S coinfection ratio of 1:1 (data not shown). The relative abundance of RS recombinants in passages 1–3 suggests that genomic sequences upstream of ORF 3 in parental strain R may be more adapted for in vitro growth. Analysis of passage 1 band densities at 25 and 27 cycles allowed an estimation of recombination frequencies. RS recombination occurred at a frequency of up to 10% of parent strain R whereas SR recombination was $< 2\%$, as the input vaccine strains had reached the plateau phase of PCR before recombinant products were visualized. The rates did not change dramatically with two additional passages. This cycle-dependent PCR-yield experiment was repeated two additional times, with no significant difference in the estimated recombination frequency obtained (data not shown). In addition, the frequencies reported may be low due to the fact that recombinations representing multiples of two would not be detected.

For evidence of recombination in naturally occurring PRRSV, 50 field isolate sequences were compared within an 1100 base region encompassing ORFs 5-6. Isolate cDNA was obtained, amplified and analyzed essentially as described previously, except that the annealing temperature for PCR was reduced to 57°C and the primer concentrations increased to 200 nM. Primer pair P55/P56 were used to amplify ORFs 5 and 6 and, to generate ORFs 5 and 6 sequences, primers 416P4/, 513P4/, and /06P1 were used (Table 1). Three isolates of these 50, collected in a 7 week time period from two different farms in close proximity, provided evidence of natural recombination. Isolate 93-15416 appeared to be a recombinant of 93-17957 and 93-22330, as 93-15416 nucleotide identity to the other two isolates shift midsequence (Fig. 3). A 5′ 283 base sequence of isolate 93-15416 was 99.6% identical to isolate 93-17957 but 91.5% identical to isolate 93-22330, while the 3′ 808 bases of the recombinant sequence exhibited 96.5% similarity to 93-17957 but 100% identity to isolate 93-22330 (Fig. 3A). A short intervening sequence of 31 bases in 93-15416 is identical to both of the other isolates, again indicating that short regions of sequence identity appear sufficient to allow PRRSV recombination.

This apparent natural recombination event resulted in a chimeric ORF 5 protein (the putative viral attachment protein) that encodes 90 amino acids, representing a signal sequence, potential viral neutralization site and the first transmembrane domain (Faaberg and Plagemann, 1995), of isolate 93-17957 combined with downstream ORF 5 (Fig. 3B) and ORF 6 (membrane protein; data not shown) amino acids from isolate 93-22330. The ORF 5 and ORF 6 proteins form disulfide bonded heterodimers in the virion (Mardassi, et al., 1996). Therefore, the putative recombinant ORF 5 and 6 heterodimer in isolate 93-15416 demonstrates that recombination events in this important region for PRRSV infection may be tolerated.

This report describes the first experimental data that arteriviruses produce recombinant viral sequences at a relatively high frequency and in the absence of selection. Coronaviruses undergo high frequency homologous recombination under selective pressure, at a rate of about 1% in 1300 nucleotides (Makino et al., 1986; Baric et al., 1990). The coronavirus frequency rate approximates the rate of recombination estimated for PRRSV in this report. We also obtained additional evidence that recombination is a biological mechanism to increase genetic diversity in PRRSV. The evidence of natural recombination between PRRSV field isolates and statistical data obtained previously (Kapur et al., 1996) lend support to the idea that recombination may contribute to the evolution of new PRRSV strains. Recombination in three (ORFs 3-5) of the four PRRSV glycoproteins indicate that sequence ex-

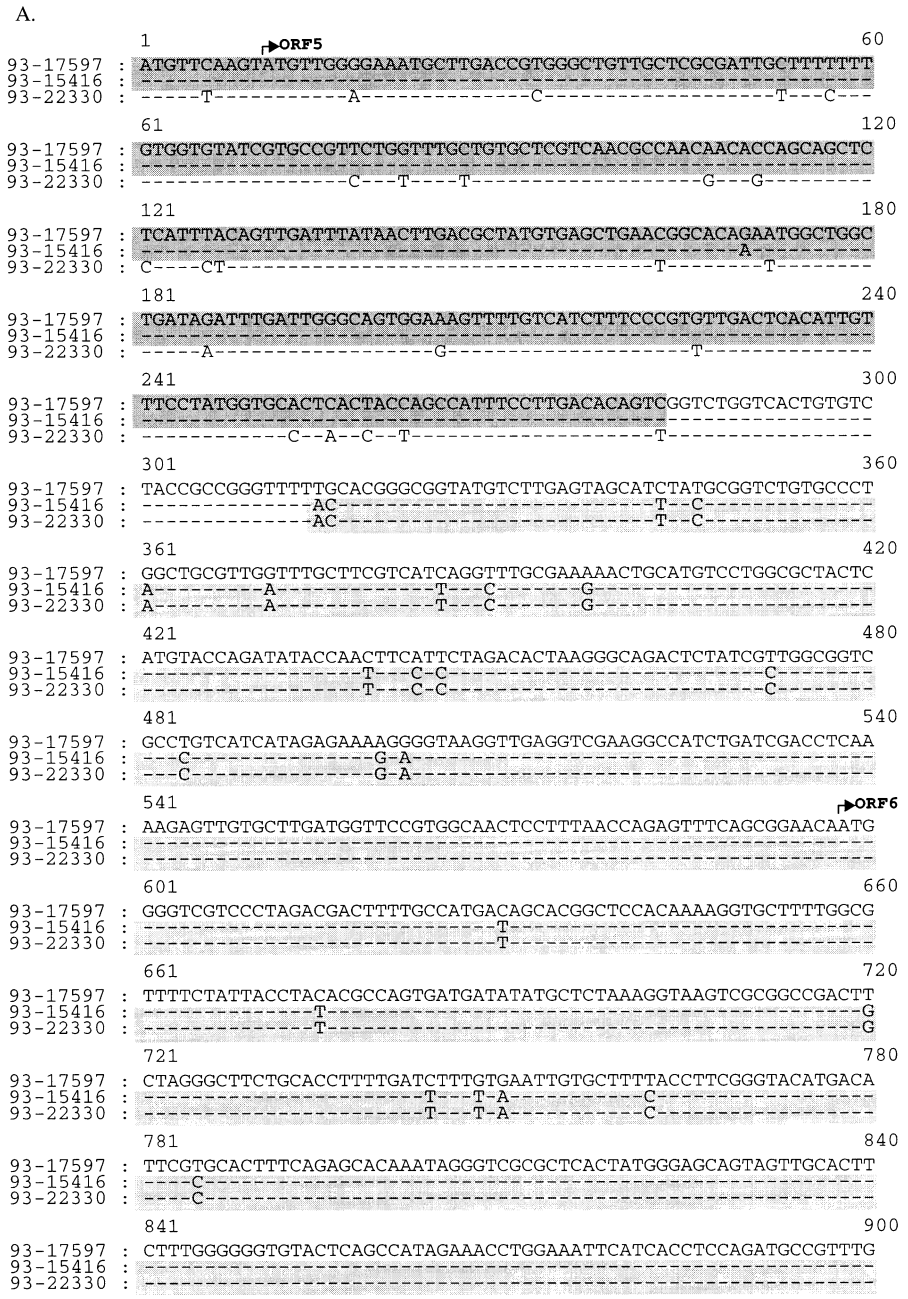

Fig. 3. PRRSV field isolates suggest a natural recombination event. (A.) Nucleotide sequences of three field isolates. (B.) Field isolate predicted amino acid sequences suggest the recombination event resulted in a change in ORF 5 protein composition.

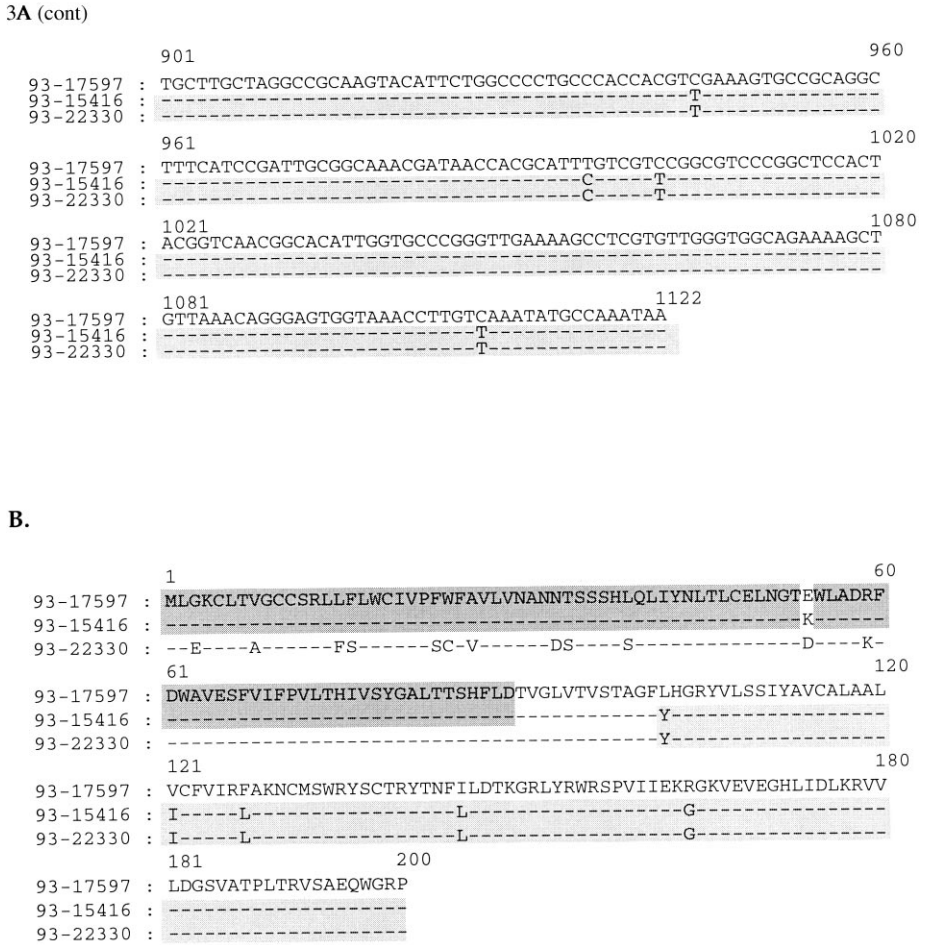

Fig. 3. *(Continued)*

change is permitted within proteins and that recombination between two co-infecting strains may yield unique new virus variants. The frequency with which new recombinant forms become established in the environment may be low, since recombinants obtained in cell culture were unable to compete with parental cell culture-adapted strains over time and the isolation of plaque purified recombinant viruses has not been accomplished to date. Nevertheless, the emergence of new strains due to recombination clearly occurs in the avian coronavirus IBV (Cavanagh, et al., 1990; Wang et al., 1993, 1994; Jia, et al., 1995).

PRRSV recombination reflects precise class I recombination (Nagy and Simon, 1997). Viral recombination between PRRSV strains and the sequences of five recombinant virus clones showed no insertions, deletions, or mismatch mutations close to the crossover region. In addition, the recombination events occurred in regions of extensive nucleotide identity between the viruses. Since recombination appears to be a frequent event in the *Nidovirales*, nidovirus polymerase template switching might also occur between more disparate strains of PRRSV or *Nidovirales* species which share homologous regions of nucleotide sequence identity. Thus, recombination may play an important role in arteriviral evolution,

given that PRRSV emerged recently as a newly described agent infecting swine.

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