

Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Québec reference strain*

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Summary. The cDNA sequence of the 3'-terminal genomic region of the Québec IAF-exp91 strain of porcine reproductive and respiratory syndrome virus (PRRSV) was determined and compared to those of other reference strains from Europe (Lelystad virus) and US (ATCC VR2385, MN-1b). The sequence (2834 nucleotides) which encompassed ORFs 3 to 7 revealed extensive genomic variations between the Québec strain and Lelystad virus (LV), resulting from high number of base substitutions, additions and deletions. The ORFs 5, 3, and 7 seemed to be relatively the most variable; the predicted encoding products of the Québec and LV strains displayed only 52%, 54%, and 59% amino acid identities, respectively. Nevertheless, in vitro translation experiments of the structural genes (ORFs 5, 6, and 7) and radio-immunoprecipitation assays with extracellular virions gave results similar to those previously reported for LV. In contrast, close genomic relationships were demonstrated between Québec and US strains. Taking together, these results indicate that, although structurally similar, North American PRRSV strains belong to a genotype distinct from that of the LV, thus supporting previous findings that allowed to divide PRRSV isolates into two antigenic subgroups (U.S. and European).

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small enveloped RNA virus which has been found to be the causative agent of a new pig disease that occurs in North America and Europe [1, 5, 7, 34]. The disease is characterized by severe reproductive failure in sows and respiratory problems affecting pigs of all ages [12]. The European prototype strain of PRRSV, known as the Lelystad virus (LV), shows close similarities with the *Arteriviruses* from morphological,

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biochemical and molecular aspects [21, 33]. This new group of viruses include equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) [26]. The viral genome is a positive single-stranded polyadenylated RNA molecule of about 15 kb in length that contains eight open reading frames (ORFs) similarly organized as EAV [6, 8, 21]. The transcription mechanism of the genomic RNA has similarities with that of *Coronaviruses*, since a 3'-coterminal nested set of six major subgenomic mRNAs has been identified in PRRSV-infected cells [6, 20–22]. The virions contain a nucleocapsid protein of 15 kDa (N), an unglycosylated membrane protein of 18 kDa (M), and a glycosylated membrane protein of 25 kDa (E); it was recently shown that these proteins are encoded by ORFs 7, 6, and 5, respectively [23].

So far, little is known about the origin and evolution of PRRSV. Although the clinical syndromes associated with PRRSV infection are similar in North America and Europe [12], antigenic variations have been reported between US and European isolates and among US isolates [12, 25]. Preliminary sequence analysis of North American isolates revealed high genomic variations with European strains [18, 19]. This is strongly supported by serological findings. To further contribute to the understanding of the genetic basis for antigenic diversity between PRRSV isolates, we determined the cDNA sequence of the 3'-terminal genomic region of the Québec reference strain IAF-exp91. Our prime objective was to compare the genomic sequence of the Québec strain to those of other reference strains from Europe and US. Since important amino acid changes were identified between North American strains and LV, we also analysed the proteins specified by a cell culture-adapted Québec PRRSV isolate (IAF-Klop) and demonstrated that these viruses remain structurally similar.

Materials and methods

Viruses

The origin and propagation in porcine alveolar macrophages (PAM) of the Québec IAF-exp91 and IAF-Klop strains of PRRSV have been previously described [7, 17]. The latter was also adapted to grow in MARC-145 cells, a highly permissive cell clone to PRRSV derived from the MA104 monkey kidney cell line [14]. The cell line was kindly provided to us by J. Kwang (U.S. Meat Animal Research Center, USDA, ARS, Clay Center, Nebraska). The IAF-Klop strain was plaque-purified twice and yielded titers of 10^5 – 10^6 TCID₅₀/ml after five successive passages in MARC-145 cells.

Polyclonal antisera

Hyperimmune sera were prepared against IAF-exp91 and IAF-Klop PRRSV strains in New Zealand Albino rabbits and specific-pathogen-free (SPF) piglets, respectively [7, 17]. The porcine hyperimmune sera had only weak neutralizing activity (VN titers of 1:32) towards IAF-Klop strain, but reached titers of 1:2560–1:5120 by indirect immunofluorescence staining. The monoclonal antibodies (MAbs) SDOW17, VO17 and EP147, directed against the nucleocapsid protein of the ATCC-VR2332 isolate of PRRSV [25] were kindly provided by D.A. Benfield and E. Nelson (South Dakota State University).

cDNA synthesis and cloning

Supernatant fluids of infected cells were clarified and extracellular virions were concentrated by ultracentrifugation, as previously described [18]. Genomic RNA of both Québec PRRSV strains was extracted from 50 μ l aliquots by the guanidinium isothiocyanate-acid phenol method described by Chomczynsky and Sacchi [4]. Cloning of IAF-exp91 3'-end genomic fragments was obtained by setting up a cDNA library, as previously reported [18].

DNA sequencing and analysis

Sequencing of cDNA clones was performed on both strands by the dideoxynucleotide chain termination method [28] using T7 DNA polymerase (Pharmacia). The nucleotide and amino acid sequences were analysed with the aid of the Geneworks 2.2 (IntelliGenetics) sequence analysis program. All comparisons were performed with a k-tuple length of one, and costs to open and to lengthen a gap of 5 and 25, respectively.

In vitro translation of PRRSV structural genes

Enzymatic amplification of ORFs 5 and 6 of the IAF-Klop strain was achieved by RT-PCR [19]. Oligonucleotide primers were designed according to the 3'-end sequence of the IAF-exp91 strain. Two restriction sites for *Eco*RI (sens primers) and *Bam*HI (antisens primers) were added at the 5' end of each primer for directional cloning. Sequence and position of oligonucleotide primers were the following:

1005PS: 5'GAATTCGAATTCATGTTGGGGAAATGCTTGACC 3' (1210–1230);
1005PR: 5'GGATCCGGATCCGGCAAAGTCATCTAGGG 3' (1807–1824);
1006PS: 5'GAATTCGAATTCAGTTTCAGCGGAACAATGG 3' (1782–1800); and
1006PR: 5'GGATCCGGATCCAGCTGATTGACTGGCTGGC 3' (2357–2372).

The amplified products of ORFs 5 and 6 were agarose-gel purified, digested with *Eco*RI and *Bam*HI, and finally ligated into a similarly treated pBluescript SK+ plasmid vector (Stratagene) [27]. Enzymatic amplification of ORF 7 was achieved using primer pair 1008PS/1009PR [18] to which *Eco*RI restriction sites were added at the 5' end of both primers to allow cloning into pBS SK+ vector. The resulting recombinant plasmids pBS5, pBS6 and pUC7 contained the coding sequences of ORFs 5, 6 and 7, respectively, under control of the T7 RNA polymerase promoter. After being linearized by digestion with the appropriate endonuclease (*Bam*HI or *Eco*RI), recombinant plasmids were transcribed in vitro using T7 RNA polymerase (Ambion Inc). Capping of transcripts was achieved by adding 0.75 mM of m⁷Gppp (Pharmacia LKB) to the reaction mixture. Two μ l of each transcript were in vitro translated using wheat germ extract (Promega) in the presence of 50 μ Ci of trans-labeled [³⁵S]methionine (Sp. act. >1 200 Ci/mM, ICN Biochemical). For post-translation modification studies, in vitro translation experiments were also done using lysates of rabbit reticulocytes (Promega) in the presence of canine microsomal membranes (Promega).

In vivo labeling of PRRSV proteins and immunoprecipitation

Confluent monolayers of MARC-145 cells were infected with IAF-Klop strain at a MOI of 0.1 TCID₅₀/cell. At 36h post-infection, cells were washed with PBS, starved for 2h in methionine-free medium, then reincubated for 6 h in culture medium containing 50 μ Ci/ml of [³⁵S]methionine. Alternatively, the labeling period was extended until maximum cytopathic effect was achieved, and extracellular virus was prepared as previously described [17]. Proteins were immunoprecipitated

from infected cell lysates or extracellular virions, then analyzed by electrophoresis in 12 or 15% SDS-polyacrylamide gels, as previously described [17]. In one experiment, immune complexes were treated with 200 mU of endoglycosidase F/N-glycosidase F (glyco F) (Boehringer Mannheim) as performed by De Vries et al. [9]. In case of the translated products of ORFs 5 to 7, they were either directly analyzed by electrophoresis or after immunoprecipitation.

Results

Comparison of the IAF-exp91 sequence with that of LV

The sequence of the 3'-terminal portion of the genome of Québec reference strain IAF-exp91 of PRRSV was obtained from 8 viral specific clones having in common their 3' ends. Sequence data obtained from these clones were assembled and a sequence consisting of 2834 nucleotides (nt) was derived. For any given region, at least two distinct clones were sequenced on both strands, except for the 5' most 634 nucleotides which were determined from only the longest clone where no base mismatches between the two strands were observed. The nucleotide and deduced amino acid (aa) sequences determined for the Québec IAF-exp91 strain of PRRSV have been submitted to EMBL Data Library and have been assigned accession no. L40898.

This genomic region contains five ORFs corresponding to ORFs 3 to 7 of the LV and extends to the poly (A) tail. All the ORFs overlapped, except ORFs 4 and 5. These ORFs each encoded a polypeptide with predicted sizes of 29K, 19.6K, 22.4K, 19.1K and 13.6K, respectively (Table 1). Sequence analyses revealed a high degree of genomic variation between Québec IAF-exp91 and LV strains. A high rate of base substitutions, additions and deletions, randomly distributed along the nucleotide sequence was noted.

The percentages of aa identity between predicted encoding products of ORFs 3 to 7 of the IAF-exp91 strain and those of the LV are presented in Table 2. The result indicate that ORFs 5, 3, and 7 are the most variable with only 52%, 54%, and 59% aa identities, respectively, when compared to LV. In contrast, ORFs 4 and 6 of the IAF-exp91 strain were relatively less variable, sharing respectively 68 and 81% aa sequence identities with those of the LV (Table 2). Variations in the length of each

Table 1. IAF-exp91 ORFs 3 to 7 characteristics

ORF	Position	Number of amino acids encoded	Calculated size (kDa)	Potential N-linked glycosylation sites
7	2311–2679	123 (128)	13.6 (13.8)	1 (1)
6	1797–2318	174 (173)	19.1 (18.9)	1 (2)
5	1210–1809	200 (201)	19.0 22.4 (22.4)	2 (2)
4	663–1196	178 (183)	17.4 19.6 (20.0)	4 (4)
3	118–879	254 (265)	26.3 29.0 (30.6)	7 (7)

The corresponding values for LV are indicated within the parenthesis. In case of ORFs 3, 4, and 5, the lower values correspond to the molecular mass of the proteins after removal of the predicted N-terminal signal sequence, determined using the weight matrix of Von Heijne [31]

Table 2. Percentage amino acid identity between predicted IAF-exp91 ORFs 3 to 7 products and those of LV, LDV, and EAV

ORF	LV	LDV	EAV
7	59	49	23
6	81	51	17
5	52	46	18
4	68	35	17
3	54	24	11

ORF was also noted making the products of ORFs 7, 5, 4, and 3 of the Québec IAF-exp91 strain shorter than those of the LV (Table 1), whereas ORF 6 of the Québec strain had an additional aa residue.

Comparison of the aa sequences of the predicted encoding product of each of the ORFs also revealed that most of the variations are located at the amino terminus (Fig. 1). This was particularly evident in case of ORF 5 where 32 aa changes were identified within the 35 most *N*-terminal residues. The same situation was observed at the *N*-terminus of ORF 3 product, where only 29% identity was found within the 35 most *N*-terminal residues. Interestingly, despite these extensive aa changes, the potential *N*-linked glycosylation sites as well as the general hydrophathy profiles of the ORFs products were highly conserved (data not shown). Comparative analyses with LV of the IAF-exp91 ORF 7 and the 3'-terminal non coding region have been previously reported [18]. The ORF 7 product showed 59% aa identity with that of LV (Table 2).

A conserved sequence motif, (U/A)(C/U/A)(A/G)ACC, has been previously identified for LV which might serve as part of the junction site for the leader sequence during transcription of mRNAs [6,22]. Except for ORFs 7 and 6 where the sequence motif AACC was present at nearly the same position upstream the ATG start codon, such consensus sequence was not identified upstream the ORFs 5, 4, and 3 of the IAF-exp91 strain (data not shown).

Sequence comparison of IAF-exp91 with US PRRSV isolates

As shown in Fig. 1, the IAF-exp91 strain appeared to be closely related to the US PRRSV isolate ATCC VR 2385 [20]. Amino acid identities were of 90% for ORF 5 and 96% for both ORFs 6 and 7. No base deletions or additions could be demonstrated between these two North American strains. As is the case for IAF-exp91 and LV, it appears from these comparisons that ORF 5 is relatively highly variable among PRRSV isolates. Interestingly, most aa substitutions identified within ORF5 products of IAF-exp91 and ATCC VR 2385 strains, corresponded to variable sites between LV and IAF-exp91 strains.

So far, only one ORF 4 sequence data is available from the US PRRSV isolates, namely the MN-1b strain [15]. Surprisingly, as much as 17% aa variations exists between this US isolate and IAF-exp91. In addition, the length of this ORF seemed

IAF-exp91	ORF3	MANSRTFLYILLCCSFLYSFCSTVVADSNATYCFWFP LVRGNFSFELTVNYTVCPCLTRQAAAEIYEPGRALWC	75
LV	ORF3	..HQCARFHFF..GFIC.LVH.ALASN.SS.LC....AH..T.....I...I.MP.STS...RQL.....NM..	75
IAF-exp91	ORF3	RIGHDRCCEDDHDELGLFVPPGLSNEGRLTSVYAWLAFLSFSYTAQFHPFIFGIGNVSKVYVDIKHQFICAVHDG	150
LV	ORF3	K.....E.R.....LMSI.S.YD.L-K.EG.....A.....L.....R.F..KR.....E... 149	
IAF-exp91	ORF3	QNTTLPRHDNISAVFQTYQHQVDGGNWFHLEWLRPFSSWLVLNVSWFLRRSPASHVSVRVFQTSRPIPPQQA	225
LV	ORF3	H.S.VSTGH...LYAA..H..I.....L.....I.....V.P..R.IY..IL..TR..RLPV 224	
IAF-exp91	ORF3	LLSSKTSAV--LGIATRPLRRFA-K.-----L.---AARR	254
LV	ORF3	SW.FR..I.SD.TGSQQRK.K.PESRPNVVKPSVLPSTS.	265
IAF-exp91	ORF4	MAASLLFLLVGFCELLVSQAFACKPCFSSSLSDIKTNTTAAAGFVVLQDISCLR-HSHSPS-ETIR--KIPQCRT	71
MN-1b	ORF4	...AT...M...K.....-----ANHVA.....S.A.....-RN.A.-.A.-.....A 66	
LV	ORF4	...AT..F.A.AQHIM..E.....TH...E.....M.....N.F.P.GV.AAQ.K.SFG.SS...E 75	
IAF-exp91	ORF4	AIGTPVYITVTANVTDENYLHSSDLLMLSSCLFYASEMSEKGFKVFIGNVSGIVAVCVNFTSYVQHVKEFTQRS	146
MN-1b	ORF4I.....E.V.....R.....LL. 141	
LV	ORF4	.V...Q...I.....S..YNA.....A.....V.SA.....D..A..TQH..QHH 150	
IAF-exp91	ORF4	VV-DHVRLHFMTPETMRWATVLAFLAILLAI	178
MN-1b	ORF4	---R.....	171
LV	ORF4	L.I..I.....LTPSA.....TI.....	183
IAF-exp91	ORF5	MLGKCLTAGYCSQLPFLWCIVPFC--FVALVNSANSYSSSHLQLIYNLTICELNGTDWLNKGFDWAVETVIFPVL	73
VR 2385	ORF5Y...L.....S.--.....S..GN.G.N.....L.....AN.....C..... 73	
LV	ORF5	.RCSHKLGRFLTPHSCF.WLFL.L.TGLSWSFADGN.GD..TY.Y.....SSH.G.....LY..A 75	
IAF-exp91	ORF5	THIVSYGALTTSHFLDTVGLITVSTAGYLHGRYVLSIIYAVCALAALICFVIRLTKNCMSWRYSCTRYTNFLD	148
VR 2385	ORF5V.....FV.....M.....A..... 148	
LV	ORF5	...L.LGF.....F.AL..GA.....FVG.....C.V.GA..F..FV....AA....AC..AR..F...IV.D 150	
IAF-exp91	ORF5	KGKLYRWRSPVIEKGGKVEVEGHLIDLKRVVLDGSAATPVTKVSAEQWCRP	200
VR 2385	ORF5	..R.....R.....I.....R.....S.. 200	
LV	ORF5	R.RVH..K..IVV..L..A..D.N.VTI.H...E.VK.Q.L.RT.....-EA 201	
IAF-exp91	ORF6	MVSSLDDEFDCHDSTAPQKVLLAFSITYTPVMYALKVSRGRLGLLHLLIFLNCAFTFGYMTTFHFQSTNRVALTM	75
VR 2385	ORF6	.E.....I.....V.....K..... 75	
LV	ORF6	.-GG.....N.PI.A..LV.....I.....I.....S.....YV.....L 74	
IAF-exp91	ORF6	GAVVALLWGVYSIETWKFITSRCRLCLLGRKYILAPAHHVESAAGFHP IAASDNHAFVVRPSTTVNGTLVPG	150
VR 2385	ORF6N..... 150	
LV	ORF6FT.S.....C..R.....L.S.S..G.R.YA..K..L..... 149	
IAF-exp91	ORF6	LKSLVLGGKKAVKRGVVNLVKYAK	174
VR 2385	ORF6R...Q..... 174	
LV	ORF6	.R.....R.....GR 173	
IAF-exp91	ORF7	MPNNGRQQKKK-----GDGPVNQLCQMLGKIIAQQNQSRGKGPCKKKNKKNPEKPHFPLATEDDVRHHFTPS	70
VR 2385	ORF7	...T.K...R.....H..... 70	
LV	ORF7	.AGK.-QS....STAPM.N.....L..AM.KS.R.---QPR.GQA...K.....A...I...L.QT 75	
IAF-exp91	ORF7	ERQLCLSSIQTAFNQAGTCTLSDSGRISYAVEFSLPTHHTVRLIRVTASPSA-----	123
VR 2385	ORF7T..... 123	
LV	ORF7	..S...Q.....AS..S..KV.FQ...M..VA.....ST-..SQGAS 128	

Fig. 1. Comparison of the predicted amino acid sequences of the 3'-terminal genes (ORFs 3 to 7) of Québec PRRSV strain IAF-exp91 with LV and corresponding available sequences from two American isolates (ATCC VR 2385, and MN-1b). The analysis was performed with the aid of the GeneWorks 2.2 (IntelliGenetics) program using default settings. Dots indicate residues identical to those of IAF-exp91, dashes represent gaps introduced into the sequences and amino acid changes are noted. The LV sequences used in this alignment are based on the sequence data presented by Meulenber et al. [21]. Sequence data of the two US strains, ATCC VR 2385 and MN-1b, were taken from previous reports of Meng et al. [20] and Kwang et al. [15], respectively

to vary from one strain to the other (178, 171, and 183 aa for IAF-exp91, MN-1b, and LV, respectively). This finding was unexpected, since ORF 4 was less variable than ORFs 5, 3, and 7, when IAF-exp91 strain was compared to LV (Table 2).

It has been shown that the 3'-terminal non-coding region (151 nt) of the IAF-exp91 strain is 22 nt longer than that of the LV [18]. On the basis of this dissimilarity, a RT-PCR assay was set up that permitted not only detection but also differentiation between Québec and European strains of PRRSV [19]. The homologous non-coding region of the ATCC VR2385 strain has the same length than that of the Québec strain and displays 94% nt identity (Fig. 1).

In vivo and in vitro analysis of the structural genes

The Québec IAF-Klop strain of PRRSV was used for *in vivo* labeling experiments and *in vitro* analysis of structural genes. Contrary to IAF-exp91 strain, IAF-Klop could be serially propagated in the continuous cell line MARC-145, in which labeling experiments could be readily achieved. Sequence analysis showed that IAF-Klop was closely related to IAF-exp91 with amino acid identities higher than 90% for their structural genes (ORF 5: 94%, ORF 6: 98%, ORF 7: 99%). Furthermore, both strains had identical protein profiles as revealed by western immunoblotting (data not shown).

From IAF-Klop infected cell lysates, five major viral-induced proteins with apparent M_r s of 15 K, 19 K, 24.5 K, 29 K, and 42 K could be consistently immunoprecipitated by the homologous porcine hyperimmune serum (Fig. 2A, lane 3). These proteins were not present in mock-infected cultures (Fig. 2A, lane 1). Among these proteins, only the 15 K and 19 K species were efficiently immunoprecipitated by the rabbit hyperimmune serum directed against the IAF-exp91 strain (Fig. 2A, lane 2). Furthermore an additional minor protein species with apparent M_r of 14.5 K was revealed by the rabbit antiserum, but failed to react with the homologous porcine antiserum. Glyco F treatment of immunoprecipitated proteins resulted in loss of the 24.5 K, 29 K and 42 K polypeptide species, thus suggesting their glycosylated nature (data not shown).

To assess the structural nature of the viral proteins identified above, immunoprecipitation experiments were performed using preparations of ^{35}S methionine-labeled and concentrated extracellular virions. Following incubation with homologous porcine hyperimmune serum, only the 15 K, 19 K, and 24.5 K protein species were clearly immunoprecipitated (Fig. 2B, lane 2). Interestingly, glycoF treatment of the immunoprecipitated proteins resulted in loss of the 24.5K protein; instead a new species with estimated M_r of 16.5K was observed (Fig. 2B, lane 3).

As expected from the aa sequences, *in vitro* translation experiments yielded products with estimated M_r of 15 K and 19 K for ORFs 7 and 6, respectively (Fig. 3A, lanes 3 and 4). Both products were efficiently immunoprecipitated by the anti-IAF Klop porcine hyperimmune serum (Fig. 3B, lanes 3 and 6) and comigrated electrophoretically with the 15 K and 19 K viral proteins. The MAb SDOW 17 reacted toward ORF 7 product (Fig. 3B, lane 5), as it was also the case for Mabs VO17 and EP147 (data not shown). On the other hand, the *in vitro* translated

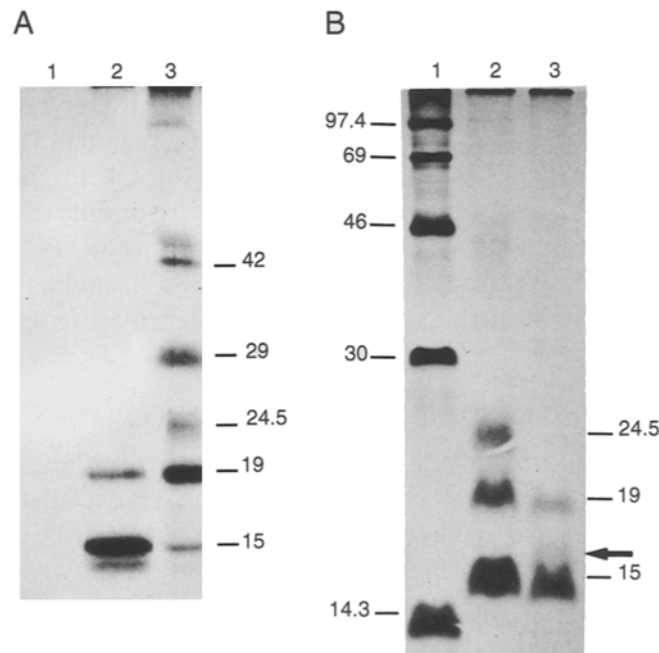


Fig. 2. SDS-PAGE analysis of PRRSV-induced polypeptides. **A** MARC-145 cells were infected with the PRRSV strain IAF-Klop and labeled with [³⁵S]methionine for 6 h. Cell lysates were prepared in RIPA buffer, clarified, and immunoprecipitated with rabbit polyclonal serum raised against the PRRSV strain IAF-exp91 (2) or the porcine homologous hyperimmune serum (3). As negative control, the porcine serum was tested with cell lysates from mock-infected cells (1). The molecular size (in kilodaltons) of the five major viral polypeptides is indicated on the right. **B** RIPA profiles of extracellular virions of the PRRSV strain IAF-Klop collected from supernatants of [³⁵S]methionine-labeled MARC-145 cells after maximum cytopathic effect was achieved. The homologous porcine hyperimmune serum was used for precipitation of viral structural proteins after treatment (3) or no treatment with glyco F (2). Positions of molecular size markers (1) are shown on the left and the viral structural proteins are indicated by their molecular mass (in kilodaltons) on the right. The arrow indicates the possible unglycosylated form of the 24.5 K protein, regularly observed after glyco F treatment. The slight displacement of the treated 15 K and 19 K proteins as compared to their untreated counterparts (3 vs 2) should not be interpreted as resulting from deglycosylation of these proteins but is due to the enzyme incubation buffer as demonstrated in other experiments (data not shown)

product of ORF5 had an apparent M_r of 18.5 K (Fig. 2A, lane 5), which was about 4K less than what was expected from its amino acid sequence (Table 1). This difference could not be due to a defective clone, since all constructions used for in vitro translation experiments were sequenced and no abnormality was detected. Moreover, the ORF 5 product was highly recognized by the anti-IAF Klop porcine hyperimmune serum (Fig 3B, lane 8). Furthermore, when in vitro translation was carried out in the presence of canine microsomal membranes, a larger protein which migrated approximately to the same distance as the 24.5 K viral protein was observed (Fig. 4, lane 3).

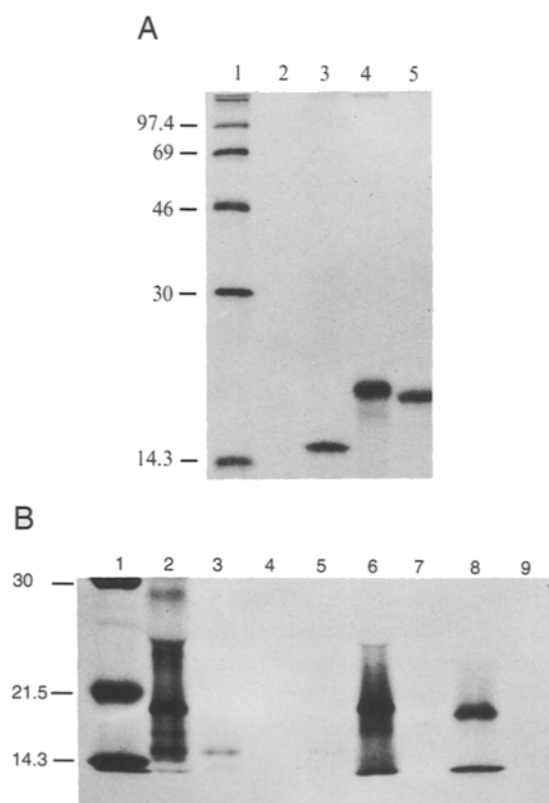


Fig. 3. SDS-PAGE analysis of the in vitro translation products of ORFs 7, 6, and 5. **A** Five μ l of the translation products of ORFs 7 (3), 6 (4), and 5 (5) were solubilized in electrophoresis sample buffer containing 2-mercaptoethanol and analysed on a 15% polyacrylamide gel. The plasmid pBS SK+ was used as negative control (2). Positions of the [14 C] methylated protein markers (1) are shown on the left (in kilodaltons). **B** Radioimmunoprecipitation analysis of the in vitro translation products of ORFs 5, 6, and 7. Infected MARC-145 cell lysates labeled with [35 S]-methionine or translation reaction products of ORF 7, ORF 6, and ORF 5 were incubated with the homologous porcine hyperimmune serum. Antigen-antibody complexes were analysed by SDS-PAGE on a 12% polyacrylamide gel as shown in 2, 3, 6, and 8, respectively. A normal serum collected from a seronegative SPF pig was used as control for precipitation of ORFs 7, 6, and 5 (4, 7, and 9, respectively). The ORF 7 translation product was also immunoprecipitated by monoclonal antibody SDOW17 (5) directed against the 15 K protein of the American reference strain ATCC VR 2332 of PRRSV. Positions of molecular size markers (in kilodaltons) are indicated on the left

Discussion

In the present study, important genomic variations were demonstrated between a Québec reference strain of PRRSV and LV, the European prototype virus. Since these viruses have a distinct geographic origin, and the fact that the Québec strain seemed to have circulated several years before its initial isolation [7], genomic heterogeneity among them was expected. The data obtained from this genomic comparison are in agreement with previous findings by others who demonstrated the existence of antigenic and genomic variations between European and US PRRSV strains, the latter being more heterogeneous [20, 25, 32]. Genomic analyses of the

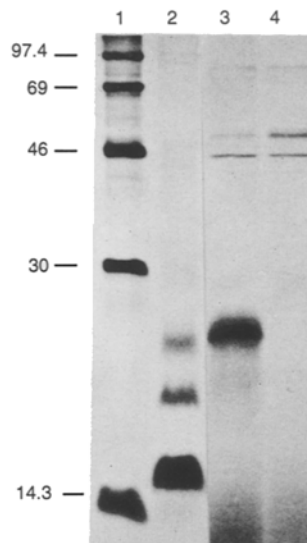


Fig. 4. Glycosylation of the in vitro translated PRRSV ORF 5 product. Linearized pBS 5 and non recombinant pBS SK+ were in vitro transcribed and translated using rabbit reticulocyte lysate in presence of canine pancreatic microsomal membranes. Their products were analysed by SDS-PAGE as shown in 3 and 4, respectively. RIPA profile of the concentrated extracellular virions of the PRRSV IAF-Klop strain, following incubation in the presence of homologous porcine hyper-immune serum, is shown in 2. The [^{14}C] methylated size markers (in kilodaltons) are shown in 1

Québec reference strain further substantiate previous findings suggesting that North American and European PRRSV isolates may have diverged into two distinct genotypes [18, 20]. A common feature in the sequences of European PRRSV isolates is the absence of 22 nucleotides in the first half of the 3'-terminal non-coding region, in comparison with North American isolates studied [18, 20, 30]. It remains to be seen, as more sequence data will be available, whether this property could be considered as a marker trait between European and North American PRRSV isolates.

Biological and molecular similarities between LV, LDV, and EAV, have been well documented [2, 6, 10, 21, 26], with LV and LDV being much more related to each other than to EAV. Despite its high genomic divergence with LV, the Québec PRRSV strain also appeared to be more closely related to LDV than to EAV. The relatedness between PRRSV and LDV in several aspects is intriguing. Indeed, since the latter has been identified many years ago, one can easily speculate that PRRSV may have been derived from LDV. So far, there is no data which suggests an eventual replication of LDV in pigs or PRRSV in mice [13]. Phylogenetic analyses of the polymerase genes of coronaviruses, toroviruses, EAV, LDV, and LV, indicated that both LV and LDV may have derived from an EAV-like progenitor, while coronaviruses hypothetically arose from a torovirus progenitor [10]. Genetic recombinations between positive-stranded RNA viruses have been well demonstrated [11, 29]. A copy-choice mechanism, due to a polymerase "jump" from one negative-sense RNA template to another, has been proposed for the generation of

recombinant coronaviruses [16]. Whether such a mechanism has accounted in the emergence of PRRSV from LDV or from an arterivirus common ancestor, remains questionable. Recently, genomic variations have been also identified within the N and the M genes of several geographically distinct EAV isolates [3, 24]. Despite the distinction of three genomic variants among various EAV strains, their M and N proteins appeared to remain relatively unchanged, which is in agreement with the fact that no distinct antigenic variants of EAV has been identified so far. This contrasts with PRRSV, since the genomic differences between European and North American strains are considerable, and thus, tends to be more heterogeneous.

Recently three structural proteins, N (15 kDa), M (18 kDa), and E (25 kDa), have been identified for LV [23], in agreement with previous findings with IAF-exp91 and ATCC-2332 strains [17, 25]. The viral structural proteins were shown to be encoded by ORFs 7, 6, and 5, respectively [23]. A comparable protein profile was obtained for the cell culture-adapted Québec IAF-Klop strain, suggesting that although genomically highly divergent, PRRSV isolates are structurally related. Indeed, despite high degrees of aa variations, *in vitro* translation experiments demonstrated that the primary structure of the ORFs 5 to 7 of the Québec strain remains comparable to that of LV [23]. However, the difference observed in the electrophoretic mobility of the ORF 5 translated product of the Québec strain (18.5 K) with that predicted from the aa sequence (22.4 K) is causing some concerns. Moreover, a 16.5 K protein species was obtained following glycoF treatment of viral proteins, rather than the 19 K protein predicted from the ORF 5 aa sequence after removal of the putative signal peptide. Studies using monospecific antisera should permit to elucidate whether the 16.5 K protein species could represent the primary unglycosylated form of the 24.5 K protein species. Such discrepancy in the size of the ORF 5 product predicted from the aa sequence, and the M_r value estimated from the electrophoretic mobility of the its *in vitro* translation product, has also been reported in case of LV [23]. A 16.5 K unglycosylated protein was also obtained after glycoF treatment of viral proteins precipitated from lysates of infected cells where the duration of the labeling period was only six hours (data not shown). Therefore, protein degradation could not be considered as an explanation for the discrepancies observed between the *in vivo* and *in vitro* molecular weights of the ORF 5 product. An interesting finding that has never been reported before, is the identification of two additional viral specific proteins in lysates of IAF-Klop infected MARC-145 cells. Two bands corresponding to proteins with apparent M_r s of 29 K and 42 K were constantly immunoprecipitated by the homologous porcine hyperimmune serum. Since these proteins were not detected with concentrated preparations of extracellular virions, they were considered as non-structural. In contrast, in case of LV, two proteins with apparently identical M_r s have been reported by western immunoblotting analysis using purified virion preparations, but both proteins could not be immunoprecipitated from lysates of LV-infected cells [23]. Based on the reactivity of anti-peptide sera directed against LV ORFs 5 and 7, it was speculated that the 42 K protein might represent a homo- or heterodimer of the E protein (25 kDa), whereas the 28 K species a dimeric form of the N protein (15 kDa) [23]. It remains to be demonstrated whether these products are distinct in cases of the LV

and IAF-Klop strains, or whether the antiserum directed against the Québec strain has a distinct specificity pattern from that used for LV.

In conclusion, the results obtained in the present study are in agreement with previous findings on the existence of antigenic diversity between North American and European strains of PRRSV. High degrees of amino acid variations were identified within the structural genes of the Québec and European prototype strains, which might explain the lack of reactivity of the anti-IAF Klop hyperimmune serum when tested against LV in virus neutralization tests (Dea et al., pers. comm.). Nonetheless, it remains to be elucidated whether the products of ORFs 3 and 4, which were also found to be highly variable, also contribute to the antigenic diversity of PRRSV isolates. Although antipeptide sera raised against ORFs 3 and 4 reacted positively with LV-infected PAM cells [23], the exact nature and localization of their products are still unknown.

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