Journal of General Virology (2001), **82**, 2607–2614. Printed in Great Britain

Viable porcine arteriviruses with deletions proximal to the 3′ end of the genome

M. H. Verheije,[1] M. V. Kroese,[1] P. J. M. Rottier[2] and J. J. M. Meulenberg[1]†

[1] Department of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health, Lelystad, The Netherlands

[2] Virology Division, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Utrecht, The Netherlands

In order to obtain attenuated live vaccine candidates of porcine reproductive and respiratory syndrome virus (PRRSV), a series of deletions was introduced at the 3′ end of the viral genome using an infectious cDNA clone of the Lelystad virus isolate. RNA transcripts from the full-length cDNA clones were transfected into BHK-21 cells. The culture supernatant of these cells was subsequently used to infect porcine alveolar macrophages to detect the production of progeny virus. It is shown that C-terminal truncation of the nucleocapsid (N) protein, encoded by ORF7, was tolerated for up to six amino acids without blocking the production of infectious virus. Mutants containing larger deletions produced neither virus nor virus-like particles containing viral RNA. Deletion analysis of the 3′ UTR immediately downstream of ORF7 showed that infectious virus was still produced after removal of seven nucleotides behind the stop codon of ORF7. Deletion of 32 nucleotides in this region abolished RNA replication and, consequently, no infectious virus was formed. Serial passage on porcine alveolar macrophages demonstrated that the viable deletion mutants were genetically stable at the site of mutation. In addition, the deletions did not affect the growth properties of the recombinant viruses *in vitro*, while their antigenic profiles were similar to that of wild-type virus. Immunoprecipitation experiments with the six-residue N protein-deletion mutant confirmed that the truncated protein was indeed smaller than the wild-type N protein. The deletion mutants produced in this study are interesting candidate vaccines to prevent PRRS disease in pigs.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-strand RNA virus that belongs to the family *Arteriviridae*, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) and simian haemorrhagic fever virus (SHFV) (Meulenberg *et al.*, 1993*b*). The family *Arteriviridae* has been grouped within the order *Nidovirales* together with the family *Coronaviridae* (Cavanagh, 1997). PRRSV has a single-stranded, polyadenylated RNA genome of about 15 kb that contains at least nine partially overlapping open reading frames (ORFs). ORFs1a and 1b encode the viral replicase genes, which are expressed from the genomic RNA. The other, smaller ORFs are located at the 3′ end of the genome and are expressed from subgenomic (sg) RNAs. These are produced as a 3′-nested set during replication (de Vries *et al.*, 1990; Meulenberg *et al.*, 1993*a*) and encode the structural proteins. ORF7 encodes the nucleocapsid protein, N, ORF6 encodes the membrane protein, M, ORF5 encodes the major envelope glycoprotein, GP_5, and ORFs 2–4 encode the minor envelope glycoproteins, GP_2, GP_3 and GP_4 (Meulenberg *et al.*, 1995). Recently, a novel structural protein, E, was described for EAV (Snijder *et al.*, 1999). This protein is translated from sg mRNA2, which also encodes GP_2.

For vaccine purposes, we aim to generate viable deletion mutants of PRRSV. This raises basic questions about the regions of the viral genome in which deletions are tolerated. In this respect, two considerations are important. Firstly, PRRSV has a concise genome, like other RNA viruses. Since RNA viruses have evolved to optimal fitness, most of the genetic information is expected to be essential. Secondly, the ORFs that encode the structural proteins of the virus are partially

Author for correspondence: Helene Verheije. Present address: Virology Division, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, NL-3584 CL Utrecht, The Netherlands. Fax +31 30 253 6723. e-mail h.verheije@vet.uu.nl

† **Present address:** Amsterdam Molecular Therapeutics, Amsterdam, The Netherlands.

0001-7831

overlapping. Deletions in overlapping regions would therefore result in the mutation of two structural proteins, which would almost inevitably lead to the production of a non-viable virus. Earlier studies showed that deletions in many conserved regions were lethal (M. H. Verheije, unpublished results).

In this study, we focused on the 3′ end of the PRRSV genome, since this region does not contain sequences that overlap other ORFs. Until now, deletions introduced in the N-terminal and middle parts of the coding region of the N protein have not resulted in viable virus (M. H. Verheije, unpublished results). In the present study, we were more successful by focusing on the region around the N gene stop codon. Alignment of the N protein sequence and the 3′ UTR of different PRRSV strains revealed heterogeneity at the C terminus of the N protein and at the 5′ end of the 3′ UTR. A deletion analysis of this region was therefore performed using the available infectious cDNA clone (Meulenberg *et al.*, 1998*a*) of Lelystad virus (LV) in order to determine the limits of the sequences that can be removed while still resulting in the production of a viable virus, but one with reduced pathogenicity. This is the first publication to describe the generation of viable arterivirus mutants that contain a deletion in the viral genome that is maintained stably after multiple passages *in vitro*.

Methods

■ **Cells and viruses.** BHK-21 cells were grown in BHK-21 medium (Gibco BRL) supplemented with 5% FBS, 10% tryptose phosphate broth (Gibco BRL), 20 mM HEPES–NaOH (Gibco BRL), pH 7·4, 200 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 20 μg/ml kanamycin, 5 μg/ml polymyxin B and 0·2 μg/ml fungizone. Porcine alveolar lung macrophages (PAMs) were maintained in MCA-RPMI-1640 medium containing 10% FBS, 100 μg/ml kanamycin, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml polymyxin B and 1 μg/ml fungizone. Serial passage of the recombinant PRRS viruses was performed by inoculation of 500 μl of the culture supernatant of transfected BHK-21 cells onto 1×10^7 PAMs. The inoculum was removed after 1 h and 5 ml fresh medium was added. The culture supernatant containing the produced virus was harvested when the first signs of cytopathic effect were observed, generally around 48 h after infection. The virus was further passaged by repeatedly inoculating 500 μl of the harvested culture medium of the previous passage onto 1×10^7 PAMs and again harvesting the culture supernatant after 48 h. Virus titres (expressed as $TCID_{50}$/ml) were determined on PAMs by end-point dilution (Wensvoort *et al.*, 1986).

■ **Construction of full-length genomic cDNA clones of LV.** PCR mutagenesis was used to introduce sequences into the *Pac*I mutant of the genome-length cDNA clone of LV (pABV437) (Meulenberg *et al.*, 1998*a*). The primers used for PCR mutagenesis are listed in Table 1. PCR fragments generated to introduce deletions into ORF7 were digested with *Hpa*I and *Pac*I and ligated into these sites of pABV437. PCR fragments generated to introduce deletions into the 3′ UTR were digested with *Pac*I and *Xba*I and ligated into these sites of pABV437. Standard cloning procedures were performed essentially as described previously (Sambrook *et al.*, 1989). Transformation conditions were maintained as described previously (Meulenberg *et al.*, 1998*a*). Sequence analysis was performed to confirm the introduced mutations. The constructs are drawn schematically in Fig. 2.

■ **Sequence analysis.** The regions of the full-length cDNA clones that originated from the PCR products were analysed by nucleotide sequencing. Sequences were determined with the PRISM Ready Dye Deoxy Terminator cycle sequencing kit and the ABI PRISM 310 Genetic Analyser (Perkin Elmer).

■ ***In vitro* transcription and transfection of BHK-21 cells.** Full-length genomic cDNA clones were transcribed *in vitro* and the resulting RNA was transfected into BHK-21 cells either using Lipofectin (Gibco BRL) or by electroporation (Meulenberg *et al.*, 1998*a*).

Table 1. Primers used to introduce deletions by PCR and to sequence the introduced mutations

The orientation of the primers (+ and − for sense and antisense) and the location of each primer with respect to the nucleotide sequence of LV (GenBank accession no. M96262) are indicated. Restriction sites are underlined.

Primer	Sequence (5′–3′)	Orientation	Purpose*	Location
119R218R	ATGACATCCGGCACCACC	+	Sequencing	14782
LV20	CCTGATTAAAAGCTTGACCCC	−	Sequencing	15066
LV75	TCTAGGAATTCTAGACGATCG	−	*Xba*I site	15088
LV155	ACGTGCGTTAACCTCGTCAAGTATGGCCGGTAAAAACCAGAGCCAGA	+	*Hpa*I site	14582
LV204	ACGTGCTTAATTAACCTTGACTGGCGGATGTAGA	−	pABV639	14974
LV213	TGCAAGTTAATTAAGGTGAATGGCCGCGA	+	pABV693	14996
LV214	GACTGTTTAATTAACTGGCGGATGTA	−	pABV694	14958
LV215	GACTGTTTAATTAAGTCACGCGAATC	−	pABV695	14942
LV239	TGCAAGTTAATTAAGCCTCTGAGTCA	+	pABV729	15021
LV263	GACTGTTTAATTAAGCGGATGTAGA	−	pABV745	14954
LV264	GACTGTTTAATTAAGATGTAGAAGTC	−	pABV746	14951
LV265	GACTGTTTAATTAAGTAGAAGTCACG	−	pABV747	14948
LV266	GACTGTTTAATTAAGAAGTCACGCGA	−	pABV748	14945

* Use of a primer in plasmid construction is indicated by the plasmid name.

■ **Infection of PAMs.** To rescue infectious virus, the culture supernatant of BHK-21 cells was harvested 24 h after transfection and 200 μl of this culture supernatant was used to inoculate PAMs. After 1 h, the inoculum was removed and fresh culture medium was added. Approximately 15 h after infection, the culture supernatant was harvested and PAMs were washed with PBS, dried and stored at −20 °C until the immunoperoxidase monolayer assay was performed.

■ **Immunoperoxidase monolayer assay (IPMA).** Immunostaining of BHK-21 cells and PAMs was performed by methods described previously (Wensvoort *et al.*, 1986). MAbs against GP_3 (122.14), GP_4 (122.1) and the M protein (126.3) (van Nieuwstadt *et al.*, 1996) and against the different antigenic domains of the N protein [138.22 (domain A), 126.9 (domain B), 126.15 (domain C) and 122.17 (domain D); Meulenberg *et al.*, 1998*b*] were used to detect expression of PRRSV proteins.

■ **Genetic analysis of genomic RNA of recombinant viruses.** In order to analyse the viral RNA in the culture supernatant of PAMs and in the fractions of the sucrose gradient, 200 μl of the culture supernatant or of the fraction was diluted with an equal volume of proteinase K buffer (100 mM Tris–HCl, pH 7·2, 25 mM EDTA, 300 mM NaCl, 2% w/v SDS) and 0·08 mg proteinase K was added. After incubation for 30 min at 37 °C, the RNA was extracted with phenol–chloroform and precipitated with ethanol. The RNA was reverse-transcribed with primer LV76 and PCR was performed with primers 119R218R and LV20, which flank the region of the viral genome that contained the deletions. Amplified fragments were analysed in 2% agarose gels and the PCR fragments were excised from the gel and purified with SpinX columns (Costar). Sequence analysis of the fragments was performed using the antisense primer of the PCR.

■ **Radioimmunoprecipitation (RIP).** Metabolic labelling and immunoprecipitation of proteins expressed in PAMs were performed essentially as described previously (Meulenberg & Petersen-den Besten, 1996). MAb 122.17 was used to immunoprecipitate the N protein. PAMs were infected with passage 5 of the viruses at an m.o.i. of 1 and were labelled for 4 h with Tran[35-S] label (ICN) at 15 h post-infection. Samples were analysed by SDS–PAGE using a 14% acrylamide gel.

■ **Virus concentration and purification.** In order to analyse the production of (non-infectious) virus particles, BHK-21 cells were electroporated with RNA transcripts from pABV747 and pABV437 and, 15 h after transfection, the cells were labelled metabolically with 75 μl 10·5 mCi/ml Tran[35-S] label (ICN) for 24 h (Meulenberg & Petersen-den Besten, 1996). The particles in the supernatant were concentrated by centrifuging the supernatant through a 0·5 M sucrose cushion at 26000 r.p.m. for 5 h at 4 °C (Meulenberg & Petersen-den Besten, 1996). The pellet was resuspended in TNE buffer (0·01 M Tris–HCl, pH 7·2, 0·1 M NaCl and 1 mM EDTA, pH 8·0) and layered onto a 20–50% sucrose gradient (van Berlo *et al.*, 1982). The sucrose gradient was centrifuged at 32000 r.p.m. for 19 h at 4 °C. Fractions of 0·5 ml were collected from bottom to top and 5 μl aliquots of the fractions were analysed by SDS–PAGE using a 14% acrylamide gel.

Results

Sequence comparison of the N proteins and 3′ UTRs of PRRSV strains LV and VR2332

Since PRRSV is an RNA virus with a very concise genome, most of its genetic information is expected to be essential. Therefore, genomic cDNA clones containing deletions, especially in the conserved regions, generally do not produce infectious transcripts (M. H. Verheije, unpublished results). In order to identify regions of heterogeneity, where deletions might be tolerated, sequence comparisons were performed. The ORF7 gene at the 3′ end of the LV genome was selected because this ORF does not overlap with other ORFs. Amino acid alignments of the N protein sequence encoded by ORF7 of the prototype European strain (Lelystad virus, LV) and the prototype North American strain (VR2332; Murtaugh *et al.*, 1995) showed 60% overall identity (recently reviewed by Dea *et al.*, 2000). At the C terminus of the N protein, the amino acid sequence is highly conserved up to residue 119 of LV. Downstream of this conserved region, a short stretch without amino acid conservation occurs. In addition, the C-terminal part of the N protein of LV is four amino acids longer than that of VR2332 (Fig. 1A). It was therefore anticipated that deletions in the heterogeneous C terminus of the N protein of LV might be tolerated, and this region was selected as a target to introduce deletions.

Further nucleotide sequence comparison of the 3′ UTR downstream of ORF7 also revealed interesting differences (Allende *et al.*, 1999). The 11 5′-most nucleotides of the 3′ UTR of LV showed no identity to the first nucleotides of VR2332 (Fig. 1B). Directly downstream of these nucleotides, a stretch of 38 nucleotides is present in VR2332 that has no counterpart in LV. In contrast, high sequence conservation was observed further downstream. In view of this heterogeneity, the region directly downstream of the stop codon of ORF7 was also selected as a target site for deletion studies.

LV accepts C-terminal truncations of up to six amino acids of the N protein

cDNA clones with deletions in the sequence encoding the two (pABV639), four (pABV694) and nine (pABV695) C-terminal amino acids of the N protein were constructed by PCR mutagenesis and cloning of the PCR fragments into an infectious cDNA clone of LV containing a *Pac*I site at the stop codon of ORF7 (Meulenberg *et al.*, 1998*a*) (Fig. 2). RNA transcripts of these constructs were transfected into BHK-21 cells and tested for their ability to replicate by analysing the expression of the structural proteins in IPMA (Fig. 2). All transcripts expressed the viral proteins GP_3, GP_4 and M. In order to analyse the expression of the N protein, and in particular its antigenic domains (Meulenberg *et al.*, 1998*b*), we used MAbs 138.22, against antigenic domain A, 126.9, against domain B, 126.15, against domain C, and 122.17, against domain D of the protein in IPMA. For all constructs, we found that the transfected cells could be stained with each of the MAbs. These results indicated that LV genomes containing deletions at the C terminus of the N protein still replicated and that the structural proteins were properly translated. In addition, these deletions did not disturb the antigenic domains of the N protein.

In order to investigate whether the LV mutants with a C-terminally truncated N protein produced infectious virus, we inoculated PAMs with culture supernatants of the transfected

A

```
LV     : MAGKNQSQKKKKSTAPMGNGQPVNQLCQLLGAMIKSQRQ---QPRGGQAKKKKPEKPHFPLAAEDDIRHH :  67
VR2332 : MPNNNGKQQKRK----KGDGQPVNQLCQMLGKIIAQQNQSRGKGPGKKNKKKNPEKPHFPLATEDDVRHH :  66
         *   *  * * *     * ********* **  *  * *      *   *** ********* *** ***


LV     : LTQTERSLCLQSIQTAFNQGAGTASLSSSGKVSFQVEFMLPVAHTVRLIRVTSTSASQGAS : 128
VR2332 : FTPSERQLCLSSIQTAFNQGAGTCTLSDSGRISYTVEFSLPTHHTVRLIRVTASPSA---- : 123
          *  ** *** ************  ** **  *  *** **  *********
```

B

```
LV     : TTAAACAGTCA-------------------------------------GGTGAATGGCCGCGATTGGCG :  32
VR2332 : TGGGCTGGCATTCTTGAGGCATCTCAGTGTTTGAATTGGAAGAATGTGTGGTGAATGGCACTGATTGACA :  70
         *  *   *                                         **********   ***** *


LV     : TGTGGCCTCTGAGTCACCTATTCAATTAGGGCGATCACATGGGGGTCATACTTAATCAGGCAGGAACCAT : 102
VR2332 : TTGTGCCTCTAAGTCACCTATTCAATTAGGGCGACCGTGTGGGGGTGAGATTTAATT-GGCGAGAACCAT : 139
         *   ****** ********************** *   ******* * * *****  ***  *******


LV     : GTGACCGAAATTAAAAAAAA: 122
VR2332 : GCGGCCGAAATTAAAAAAAA: 159
         * * ****************
```

Fig. 1. Alignments of the N protein amino acid sequences (A) and the 3′ UTR nucleotide sequences (B) of PRRSV LV and VR2332. LV (GenBank accession no. M96262) and VR2332 (U87392) are the prototype European and North American PRRSV strains. Conserved residues are indicated by asterisks beneath the alignments. In (B), part of the *Pac*I site present in the 3′ UTR is underlined.

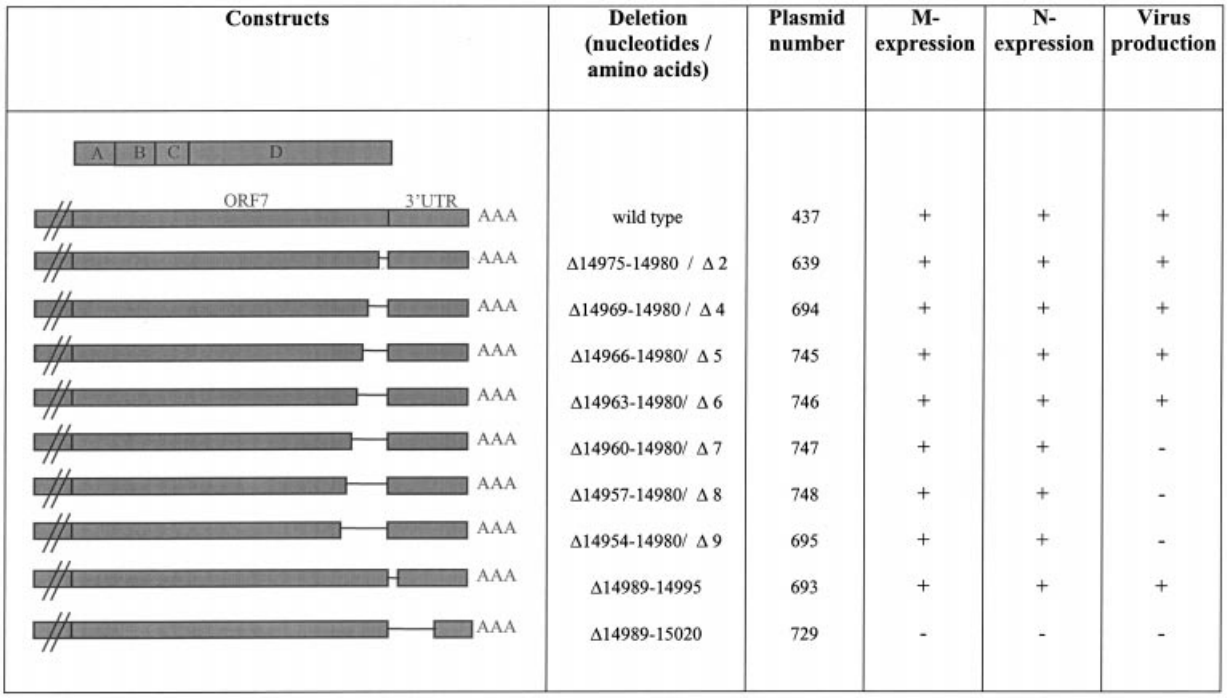

Fig. 2. Design and analysis of the deletion mutants of recombinant cDNA clones of PRRSV. Deletions were introduced by PCR mutagenesis and cloned into the full-length cDNA clone pABV437 (Meulenberg *et al.*, 1998*a*). The constructs, deleted nucleotides, plasmid numbers, observed expression of M and N protein and the production of viable virus are indicated. Boxes indicate regions that were present; lines indicate deleted regions. Expression of viral proteins M and N was analysed by IPMA 24 h after transfection of BHK-21 cells using MAbs 126.3 and 122.17, respectively. Positive staining is indicated by +; no staining is indicated by −. The bar above the constructs indicates the antigenic domains of the N protein.

BHK-21 cells, as PRRSV cannot infect BHK-21 cells. Twenty-four h later, the cells were fixed and stained with PRRSV-specific MAbs. PAMs inoculated with the supernatant of BHK-21 cells transfected with transcripts of pABV437, pABV639 or pABV694 stained positive. In contrast, no staining of PAMs was observed after inoculation with supernatant from BHK-21 cells transfected with RNA transcripts from pABV695. In conclusion, LV mutants producing an N protein with a C-terminal deletion of up to four amino acids produced infectious virus, whereas mutants producing an N protein with a C-terminal deletion of nine amino acids did not produce infectious virus at all.

In order to define further the acceptable limits of truncation of the N protein, we made stepwise deletions in the region encoding the five to eight most C-terminal amino acids. The fragments generated by PCR mutagenesis were again intro-

duced into pABV437, resulting in pABV745, 746, 747 and 748, encoding N proteins lacking five, six, seven and eight C-terminal amino acids (Fig. 2). Transfection of their RNA transcripts into BHK-21 cells resulted in the expression of the structural proteins for all constructs, as detected by IPMA. After infection of PAMs with the culture supernatant of the transfected BHK-21 cells, we only detected expression of the structural proteins for vABV745 and vABV746. For mutants lacking the region encoding the C-terminal seven amino acids or more, no staining was observed in IPMA. These results indicate that the maximum region that can be deleted at the 3′ end of ORF7 without abolishing the production of infectious virus comprises 18 nucleotides, encoding the six C-terminal residues of the N protein. The virus produced by this deletion mutant (vABV746) was found to express the set of N protein epitopes, as demonstrated by using our panel of MAbs (data not shown).

Deletion of seven but not of 32 nucleotides at the 5′ end of the 3′ UTR of LV is tolerated

In view of the observed nucleotide sequence variation in the 3′ UTR of the PRRSV genome downstream of ORF7 (Fig. 1B), we also investigated how deletion of these nucleotides would affect the infection process. Deletions were again introduced by PCR mutagenesis and the PCR fragments were introduced into pABV437, directly behind the *Pac*I site at the stop codon of ORF7. The first four nucleotides of the 3′ UTR were left intact, as they are part of this *Pac*I site. This resulted in the plasmids pABV693, which has a deletion of seven nucleotides, and pABV729, in which a deletion of 32 nucleotides occurs at the 5′ end of the 3′ UTR. BHK-21 cells transfected with transcripts of pABV693 expressed the structural proteins. However, BHK-21 cells transfected with transcripts of pABV729 did not express these structural proteins to levels detectable by IPMA, suggesting that RNA replication and/or transcription did not occur. Subsequent infection of PAMs with the culture supernatant of the BHK-21 cells that had been transfected with pABV693 showed expression of the structural proteins in IPMA 24 h after infection. These results demonstrated that at least seven nucleotides at the 5′ end of the 3′ UTR are dispensable for the virus to remain infectious.

Analysis of the stability and growth characteristics of vABV746 and vABV693 *in vitro*

In order to investigate whether the deletions in the viruses generated from pABV746 and pABV693 were maintained stably *in vitro*, these viruses were serially passaged on PAMs. After five passages, viral RNA was isolated from the culture supernatant and studied by genetic analysis. The RNA was reverse-transcribed and the region flanking the introduced deletions was amplified by PCR. Sequence analysis of the fragments showed that the introduced deletion was still present in both cases (data not shown) and that no additional mutations had been introduced in the flanking regions. These results indicated that the deletions had been maintained stably during *in vitro* passaging on PAMs.

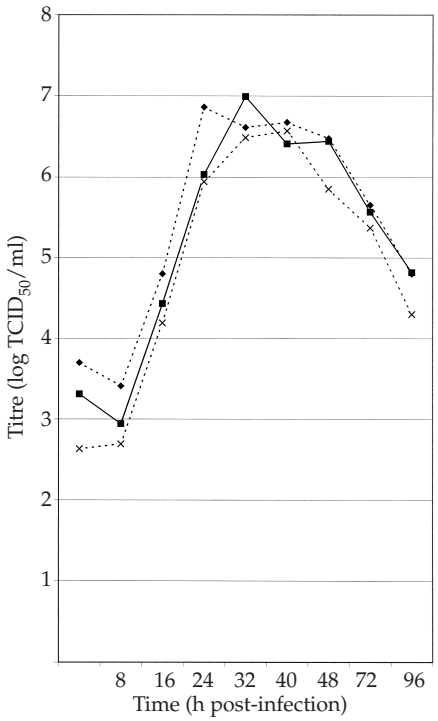

Fig. 3. Growth curves in PAMs of wild-type virus vABV437 (■) and of mutant viruses vABV693 (◆) and vABV746 (×). PAMs were infected in duplicate with passage 5 of the indicated viruses at an m.o.i. of 0·05 and virus was harvested at the indicated time-points. Virus titres were determined by end-point dilution on PAMs (Wensvoort *et al.*, 1986).

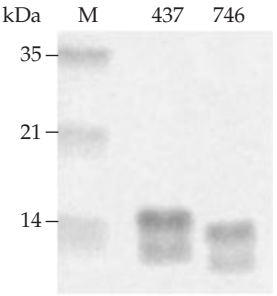

Fig. 4. Analysis of the N proteins expressed by the wild-type virus vABV437 and by the six-amino-acid N protein-deletion mutant vABV746. Proteins were immunoprecipitated from lysates of PAMs infected with passage 5 of vABV437 and vABV746. Labelling was performed for 4 h starting at 15 h after infection. The immunoprecipitated proteins were analysed by SDS–PAGE in a 14% acrylamide gel. Molecular masses of the marker proteins are indicated on the left.

The growth characteristics of viruses vABV746 and vABV693 were investigated by determining their growth curves and comparing them with that of wild-type vABV437. PAMs were infected with viruses from passage 5 at an m.o.i. of 0·05 and samples were taken from the culture media at various time-points. Virus titres were determined by end-point dilution on macrophages. As is clear from Fig. 3, no significant

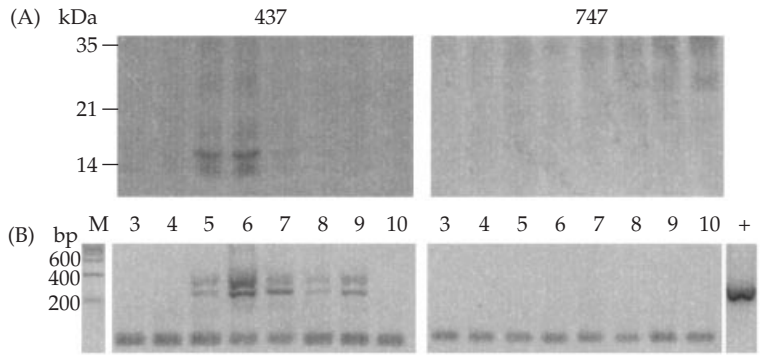

Fig. 5. Analysis of the supernatant of BHK-21 cells transfected with pABV747 and pABV437. Fifteen h after transfection, the cells were labelled for 24 h using 75 µl 10·5 mCi/ml Tran[35-S] label. Particles in the supernatant were concentrated and fractionated as described in Methods. (A) Proteins in the fractions were analysed by 14% SDS–PAGE. (B) RNA was isolated from the fractions and analysed by RT–PCR, for which the primers flanked the region in which the deletion was introduced. The sizes of the markers are indicated on the left. Lane +, positive PCR control.

differences in growth rates could be observed between recombinant viruses and wild-type virus.

Analysis of the truncated N protein of vABV746

In order to confirm the effect of the deletion at the protein level, the size of the N protein expressed by the recombinant virus vABV746 was analysed by immunoprecipitation. PAMs were infected and labelled metabolically with ^{35}S-amino acids and cell lysates were prepared. The N protein in the lysates was precipitated with MAb 122.17, which is directed against the D domain of the protein, and analysed by SDS–PAGE. As expected, an N protein of wild-type size (15 kDa) was immunoprecipitated from lysates of cells transfected with vABV437 (Fig. 4), whereas a protein with an estimated size of 14 kDa was immunoprecipitated from vABV746-infected cell lysates (Fig. 4). The smaller N protein expressed by vABV746 is consistent with the six-residue truncation compared with wild-type vABV437.

Analysis of particle assembly after N protein truncation

The abrupt transition in viability upon deletion of more than six C-terminal amino acids may occur for various reasons. We analysed whether the life cycle of the virus was disturbed at the stage of virus assembly. To this end, BHK-21 cells were transfected with RNA transcripts from pABV747 and proteins were labelled for 24 h starting at 15 h after transfection. Virus particles released into the culture supernatant were concentrated by centrifugation through a sucrose cushion and purified further by equilibrium centrifugation in a sucrose gradient and fractions of this gradient were analysed by SDS-PAGE. Structural proteins of the appropriate size were detected for the positive control pABV437 in fractions 5, 6 and 7. These structural proteins were, however, not detected in any of the fractions of pABV747 (Fig. 5A). In order to confirm the absence of packaged RNA in these fractions, we isolated the viral RNA from each fraction and performed RT–PCR. In none of the fractions was a PRRSV-specific PCR fragment detected, in contrast to the gradient run with pABV437-derived material (Fig. 5B). In each of these fractions, two PCR fragments were observed, in contrast to the lane in which we used cDNA of pABV437 as a template. The second band derived from the fractions could not be identified, however. It might have been derived from deleted, packaged RNAs, resulting in the production of heteroclite sg mRNAs (Yuan *et al.*, 2000). The absence of virus particles or virus-like particles produced by transcripts of pABV747 suggests that virus assembly is disturbed in this mutant.

Discussion

With the eventual aim of obtaining live attenuated PRRSV vaccine candidates, this study describes the construction and analysis of several virus deletion mutants. In view of the genetic variability at the 3′ end of the PRRSV genome, we have tested the effect of deletions in this variable region. We report that constructs lacking the coding sequence for up to six C-terminal amino acids of the LV N protein still yielded infectious virus after transfection of their transcripts into BHK-21 cells. In contrast, further deletions were fully detrimental: the removal of just one additional residue abolished the production of viable virus completely. Furthermore, deletions were also tolerated directly downstream of the stop codon of ORF7. At least seven nucleotides in this region were dispensable for virus production; removal of 32 nucleotides was, however, fatal. Both the virus with a six amino acid truncation of the N protein and the virus with the seven nucleotide deletion in the 3′ UTR had *in vitro* growth characteristics and antigenic profiles similar to those of wild-type virus. Moreover, these viruses were both genetically stable.

The dramatic effect of truncation at the seventh residue of the LV N protein was quite surprising and was not predicted by the sequence. The sequence of the nine C-terminal residues of the LV N protein is very different from that of the VR2332 isolate except for its high content of hydroxy amino acids. In the LV and VR2332 N proteins, six of ten and three of six residues, respectively, at the very C terminus are serines or threonines. The functions of this domain and of these particular residues are unknown. Two other arteriviruses, LDV and

SHFV, also contain hydroxy amino acids at the extreme C terminus of their N proteins, namely 3/10 and 4/10 amino acids, respectively. In contrast, hydroxy amino acids are completely absent from the last ten amino acids of the EAV N protein. While coronavirus N proteins do generally have a relatively high serine content (7–11%; Masters & Sturman, 1990), the proportion of serines and threonines at their C termini is quite insignificant; in these viruses, this region is markedly acidic. Obviously, these variable characteristics do not allow predictions about the role of the C terminus of the N protein in the virus life cycle. The truncated N protein had the same antigenic profile as that of the wild-type N protein, since it reacted with all MAbs directed against antigenic domains of the N protein. This is consistent with observations by Meulenberg *et al*. (1998*b*), who identified that domain D, the most C-terminal domain of N, is a conformation-dependent or discontinuous epitope that involves amino acids 51–67 and 80–90.

Virus particle production appeared to be blocked after truncation of the LV N protein by seven amino acids. This indicates strongly a defect at the level of virus assembly. For a Canadian PRRSV isolate, it has been demonstrated that non-covalent interactions between the C-terminal regions of N proteins are critical for formation of the isometric capsid protein (Wootton & Yoo, 1999). In a system expressing only the N protein, they showed that the last 11 amino acids were involved in these interactions. This might indicate that the C terminus of PRRSV is essential for nucleocapsid formation. Our study supports this idea. Other effects of C-terminal truncation of the N protein can, however, not be excluded, as the N protein has been implicated in various other processes, such as interaction with the viral RNA [Dea *et al*., 2000; for mouse hepatitis virus (MHV), see Cologna & Hogue, 1998; Molenkamp & Spaan, 1997] and interaction with other viral proteins (for MHV, see Narayanan *et al*., 2000). Since it has been described for MHV, the best-studied coronavirus, that a 29 amino acid deletion in the putative spacer region preceding the C-terminal domain of the N protein resulted in temperature-sensitive and thermolabile viruses (Peng *et al*., 1995), we investigated whether our deletion mutants had similar characteristics; they appeared not to have these characteristics. Moreover, infectious virus was still not produced from the deletion mutants expressing truncated N proteins lacking seven amino acids or more after the incubation temperature was lowered to 30 °C. In an earlier study, we demonstrated that extension of the C terminus of the N protein by a nine amino acid sequence of the influenza virus HA protein significantly impaired virus growth (Groot Bramel-Verheije *et al*., 2000). We could not establish whether this was caused by disturbance of virus assembly or disassembly, however. Again, these observations are consistent with the C-terminal region of the LV N protein being involved in N–N interactions essential for the production of nucleocapsids during virus assembly.

RNA viruses have at their termini non-coding sequences that play essential roles in RNA replication and sg mRNA transcription. Mutations in these domains are likely to affect the virus life cycle. Consistently, when we introduced deletions in the 5′-terminal region of the LV 3′ UTR, we found that removal of a small seven-nucleotide variable sequence was accepted, while removal of a somewhat larger, 32-nucleotide stretch was not. From the inability of the RNA transcripts to express the M and N proteins, we conclude that the defect probably resides in an effect on RNA replication or sg mRNA transcription. This suggests that this region of the 3′ UTR contains an essential RNA signal. Our results are in accordance with studies on coronaviruses, which showed that the 5′ terminus of the 3′ UTR is essential in the initial processes of the virus life cycle (Hsue *et al*., 2000). No host or virus protein was found to bind this region of the viral RNA specifically. However, the exact function of this region remains to be elucidated. Since deletion of the 3′-most 27 nucleotides of the N gene did not abolish RNA replication, this region apparently does not contain a replication signal similar to the one observed within the 3′-terminal region of the EAV N gene (Molenkamp *et al*., 2000).

In this study, we aimed to generate viable PRRSV mutants with maximal deletions at the target site. The viruses obtained were characterized *in vitro* and fulfilled the most important requirements; good growth and genetic stability. Because their growth characteristics *in vitro* on PAMs were identical to those of wild-type virus, virus production for *in vivo* studies can be accomplished easily. The growth characteristics *in vitro* do not necessarily correlate with or predict the behaviour of the virus *in vivo*. Thus, many vaccines used currently are attenuated *in vivo*, but show no differences in propagation *in vitro* (Yang *et al*., 1998). Therefore, only animal experiments will tell how these viruses behave *in vivo*, whether they are sufficiently attenuated and whether they induce immune responses that will protect against infection with virulent PRRSV.

We thank E. van Wijk for technical assistance and P. van Rijn for critical reading of the manuscript. This work was supported by Boehringer Ingelheim, Germany.

References

Allende, R., Lewis, T. L., Lu, Z., Rock, D. L., Kutish, G. F., Ali, A., Doster, A. R. & Osorio, F. A. (1999). North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *Journal of General Virology* **80**, 307–315.

Cavanagh, D. (1997). Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Archives of Virology* **142**, 629–633.

Cologna, R. & Hogue, B. G. (1998). Coronavirus nucleocapsid protein–RNA interactions. *Advances in Experimental Medicine and Biology* **440**, 355–359.

Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B. & Rogan, D. (2000). Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. *Archives of Virology* **145**, 659–688.

de Vries, A. A., Chirnside, E. D., Bredenbeek, P. J., Gravestein, L. A., Horzinek, M. C. & Spaan, W. J. (1990). All subgenomic mRNAs of equine arteritis virus contain a common leader sequence. *Nucleic Acids Research* **18**, 3241–3247.

Groot Bramel-Verheije, M. H., Rottier, P. J. M. & Meulenberg, J. J. M. (2000). Expression of a foreign epitope by porcine reproductive and respiratory syndrome virus. *Virology* **278**, 380–389.

Hsue, B., Hartshorne, T. & Masters, P. S. (2000). Characterization of an essential RNA secondary structure in the 3′ untranslated region of the murine coronavirus genome. *Journal of Virology* **74**, 6911–6921.

Masters, P. S. & Sturman, L. S. (1990). Functions of the coronavirus nucleocapsid protein. In *Coronaviruses and their Diseases,* pp. 235–238. Edited by D. Cavanagh & T. D. K. Brown. New York: Plenum Press.

Meulenberg, J. J. & Petersen-den Besten, A. (1996). Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. *Virology* **225**, 44–51.

Meulenberg, J. J. M., de Meijer, E. J. & Moormann, R. J. M. (1993*a*). Subgenomic RNAs of Lelystad virus contain a conserved leader–body junction sequence. *Journal of General Virology* **74**, 1697–1701.

Meulenberg, J. J., Hulst, M. M., de Meijer, E. J., Moonen, P. L., den Besten, A., de Kluyver, E. P., Wensvoort, G. & Moormann, R. J. (1993*b*). Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology* **192**, 62–72.

Meulenberg, J. J. M., Petersen-den Besten, A., De Kluyver, E. P., Moormann, R. J. M., Schaaper, W. M. M. & Wensvoort, G. (1995). Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology* **206**, 155–163.

Meulenberg, J. J., Bos-de Ruijter, J. N., van de Graaf, R., Wensvoort, G. & Moormann, R. J. (1998*a*). Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. *Journal of Virology* **72**, 380–387.

Meulenberg, J. J., van Nieuwstadt, A. P., van Essen-Zandbergen, A., Bos-de Ruijter, J. N., Langeveld, J. P. & Meloen, R. H. (1998*b*). Localization and fine mapping of antigenic sites on the nucleocapsid protein N of porcine reproductive and respiratory syndrome virus with monoclonal antibodies. *Virology* **252**, 106–114.

Molenkamp, R. & Spaan, W. J. (1997). Identification of a specific interaction between the coronavirus mouse hepatitis virus A59 nucleocapsid protein and packaging signal. *Virology* **239**, 78–86.

Molenkamp, R., van Tol, H., Rozier, B. C. D., van der Meer, Y., Spaan, W. J. M. & Snijder, E. J. (2000). The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription. *Journal of General Virology* **81**, 2491–2496.

Murtaugh, M. P., Elam, M. R. & Kakach, L. T. (1995). Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Archives of Virology* **140**, 1451–1460.

Narayanan, K., Maeda, A., Maeda, J. & Makino, S. (2000). Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. *Journal of Virology* **74**, 8127–8134.

Peng, D., Koetzner, C. A. & Masters, P. S. (1995). Analysis of second-site revertants of a murine coronavirus nucleocapsid protein deletion mutant and construction of nucleocapsid protein mutants by targeted RNA recombination. *Journal of Virology* **69**, 3449–3457.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual,* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Snijder, E. J., van Tol, H., Pedersen, K. W., Raamsman, M. J. & de Vries, A. A. (1999). Identification of a novel structural protein of arteriviruses. *Journal of Virology* **73**, 6335–6345.

van Berlo, M. F., Horzinek, M. C. & van der Zeijst, B. A. (1982). Equine arteritis virus-infected cells contain six polyadenylated virus-specific RNAs. *Virology* **118**, 345–352.

van Nieuwstadt, A. P., Meulenberg, J. J., van Essen-Zanbergen, A., Petersen-den Besten, A., Bende, R. J., Moormann, R. J. & Wensvoort, G. (1996). Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. *Journal of Virology* **70**, 4767–4772.

Wensvoort, G., Terpstra, C., Boonstra, J., Bloemraad, M. & Van Zaane, D. (1986). Production of monoclonal antibodies against swine fever virus and their use in laboratory diagnosis. *Veterinary Microbiology* **12**, 101–108.

Wootton, S. K. & Yoo, D. (1999). Structure–function of the ORF7 protein of porcine reproductive and respiratory syndrome virus in the viral capsid assembly. In *Proceedings of the International Symposium on PRRS and Aujeszky's Disease,* pp. 37–38. 21–22 June 1999, Ploufragan, France.

Yang, S. X., Kwang, J. & Laegreid, W. (1998). Comparative sequence analysis of open reading frames 2 to 7 of the modified live vaccine virus and other North American isolates of the porcine reproductive and respiratory syndrome virus. *Archives of Virology* **143**, 601–612.

Yuan, S., Murtaugh, M. P. & Faaberg, K. S. (2000). Heteroclite subgenomic RNAs are produced in porcine reproductive and respiratory syndrome virus infection. *Virology* **275**, 158–169.

Received 2 May 2001; Accepted 7 August 2001