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Generation of an Infectious Clone of VR-2332, a Highly Virulent North American-Type Isolate of Porcine Reproductive and Respiratory Syndrome Virus

H. S. Nielsen,[1,2]† G. Liu,[3] J. Nielsen,[1]† M. B. Oleksiewicz,[1]‡ A. Bøtner,[1] T. Storgaard,[4]* and K. S. Faaberg[3]

Danish Veterinary Institute, Lindholm,[1] Laboratory for Virology and Immunology, Royal Veterinary and Agricultural University, Frederiksberg,[2] and Applied Trinomics, Novo Nordisk A/S, Måløv,[4] Denmark, and Department of Veterinary Pathobiology, University of Minnesota, St. Paul, Minnesota[3]

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A full-length cDNA clone of the prototypical North American porcine reproductive and respiratory syndrome virus (PRRSV) isolate VR-2332 was assembled in the plasmid vector pOK_{12}. To rescue infectious virus, capped RNA was transcribed in vitro from the pOK_{12} clone and transfected into BHK-21C cells. The supernatant from transfected monolayers were serially passaged on Marc-145 cells and porcine pulmonary alveolar macrophages. Infectious PRRSV was recovered on Marc-145 cells as well as porcine pulmonary macrophages; thus, the cloned virus exhibited the same cell tropism as the parental VR-2332 strain. However, the cloned virus was clearly distinguishable from the parental VR-2332 strain by an engineered marker, a *Bst*Z17I restriction site. The full-length cDNA clone had 11 nucleotide changes, 2 of which affected coding, compared to the parental VR-2332 strain. Additionally, the transcribed RNA had an extra G at the 5′ end. To examine whether these changes influenced viral replication, we examined the growth kinetics of the cloned virus in vitro. In Marc-145 cells, the growth kinetics of the cloned virus reflected those of the parental isolate, even though the titers of the cloned virus were consistently slightly lower. In experimentally infected 5.5-week-old pigs, the cloned virus produced blue discoloration of the ears, a classical clinical symptom of PRRSV. Also, the seroconversion kinetics of pigs infected with the cloned virus and VR-2332 were very similar. Hence, virus derived from the full-length cDNA clone appeared to recapitulate the biological properties of the highly virulent parental VR-2332 strain. This is the first report of an infectious cDNA clone based on American-type PRRSV. The availability of this cDNA clone will allow examination of the molecular mechanisms behind PRRSV virulence and attenuation, which might in turn allow the production of second-generation, genetically engineered PRRSV vaccines.

Porcine reproductive and respiratory syndrome is considered one of the most economically important infectious diseases of swine (1, 15). The disease is associated with severe reproductive disorders in sows and gilts and respiratory problems in pigs (11, 13, 16). The causative agent of the disease is porcine reproductive and respiratory syndrome virus (PRRSV), which, together with equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus, are placed in the *Arteriviridae* family, within the order *Nidovirale*s (9).

The PRRSV genome consists of a 5′-capped and 3′-polyadenylated single-stranded positive-sense RNA molecule of 15.1 to 15.5 kb. The viral genome contains, in the 5′ to 3′ direction, a 5′ leader, at least nine open reading frames (ORF 1a, ORF 1b, and ORFs 2 to 7), and a 3′ nontranslated region (14, 23, 32, 39). ORF 1a and ORF 1b are located directly downstream of the 5′ leader and encode a large replicase polyprotein, which is thought to be autoproteolytically cleaved into 13 smaller nonstructural proteins assumed to be involved in virus replication and transcription (4, 35, 38). ORFs 2 to 7 encode the structural proteins associated with the virion. These proteins are expressed from a 3′-coterminal nested set of subgenomic mRNAs (23, 24, 32).

PRRSV emerged almost simultaneously in North America and Europe in the late 1980s and early 1990s, respectively. Even though the same PRRSV-associated disease was observed on both continents, phylogenetic analysis has revealed two distinct genotypes of PRRSV, North American and European, with a sequence homology of only approximately 63% at the nucleotide level (2).

The establishment of infectious full-length cDNA clones has become critical in the study of viruses. The availability of such cDNA clones offers an opportunity for analysis and modification of viral genomes at the molecular level and has greatly aided research on virus replication, pathogenesis, and vaccine development (7). Yet, to date, only one infectious cDNA clone of PRRSV has been established, and this clone is unfortunately not generally available to the scientific community. Furthermore, this cDNA clone is based on the European-type Lelystad virus (25). The large genetic differences observed between the North American and European genotypes of PRRSV make it

* Corresponding author. Mailing address: Applied Trinomics, Preclinical Development, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark. Phone: 45 44 43 61 24. Fax: 45 44 43 45 58. E-mail: TSTS@novonordisk.com.

† Present address: Preclinical Development; Novo Nordisk A/S, Måløv, Denmark.

‡ Present address: Symphogen A/S, Lyngby, Denmark.

TABLE 1. Summary of oligonucleotides used for RT-PCR amplification

Primer[a]	Sequence (5′ → 3′)[b]	Position within VR-2332 genome[c]
5′ terminus (fragment b) primers		
RT416	5′ TGC GAT TGG A 3′	435–445
F20	5′ CTC GAG GGC GCG CC*T AAT ACG ACT CAC TAT AGG* TAT GAC GTA TAG GTG TTG GCT 3′ (*Xho*1, *Asc*I)	1–21
R229	5′ CGT GTG CAG TAT ACT TGG CCC T 3′ (BstZ17I)	249–270
Fragment e primers		
RT4890	5′ AAG GCT TGG A 3′	4910–4919
F251	5′ GGA GGG CCA AGT **A**TA CTG CAC ACG A 3′ (*Bst*Z17I)	247–271
R4774	5′ GTG TCA GGG TCA ACC ACG A 3′	4794–4812
Fragment f primers		
RT7924	5′ TGC ATC AGC A 3′	7944–7953
F4333	5′ ATC TTG GCT GGA GCT TAC GT 3′	4334–4353
R7821	5′ TGG TTG TGC TCA ACC GCG T 3′	7841–7859
Fragment d primers		
RT13019	5′ AGC TGA AGG A 3′	13039–13048
F7406	5′ TCT CAG AGT TGG CGA CCC T 3′	7408–7426
R12887	5′ ATC CTG CAC CAA AGA GAC CT 3′	12907–13026
Fragment c primers		
RT15327	5′ AAT TGA ATA GGT 3′	15347–15358
F12513	5′ TTT CAG CAT CTA GCC GCC A 3′	12515–12533
R15278	5′ AAT CAG TGC C**G**T T**A**A C**C**A CAC ATT CTT CCA 3′ (*Hpa*I)	15298–15327
3′ terminus (fragment a) primers		
RT15392	5′ CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG A AA CGT $T(T)_{38}$3′ (*Xba*I, *Acl*I)	Poly (A) tail
F15301	5′ AGA ATG TGT G**G**T T**A**A C**C**GG CA 3′ (*Hpa*I)	15302–15321
M13	5′ CAG GAA ACA GCT ATG AC 3′	M13 sequence

[a] Primer names are organized in groups. Prefixes: RT, reverse transcription primer; F, forward PCR primer; R, reverse PCR primer.
[b] The T7 RNA polymerase promoter sequence in primer F20 is shown in italics. Restriction sites introduced by PCR are underlined and specified in parentheses at the end of the sequence. Silent mutations within the viral sequence are shown in boldface. Fragments a through f correspond to the letters in Fig. 1.
[c] The nucleotide positions within the VR-2332 genome are based on GenBank accession numbers PRU87392 and AF094475 (26, 30).

highly relevant to obtain infectious cDNA clones representing both genetic types of PRRSV.

The present article describes the establishment of an infectious full-length cDNA clone of the North American-type isolate VR-2332. VR-2332 was chosen because it is the prototypical North American-type isolate, is well characterized, and has been shown to be highly virulent for sows and piglets (5, 10, 13). In addition, VR-2332 forms the basis of an attenuated live vaccine (3, 22), the complete genome sequences of both VR-2332 and the vaccine virus have been published (3, 26, 41), and both VR-2332 and the vaccine virus are freely available. Given the availability of complete genome sequences for the vaccine and VR-2332 strains, the infectious clone of VR-2332 should be immediately applicable for the identification of genetic attenuation and virulence determinants in the PRRSV genome, with a view to the development of second-generation, genetically engineered vaccines.

MATERIALS AND METHODS

Cells and virus. The virus used in the present study was a third cell culture passage of the highly virulent North American-type isolate VR-2332 (American Type Culture Collection).

Marc-145 cells were grown in Eagle's minimal essential medium (EMEM) with 5% fetal calf serum. BHK-21C cells were grown in EMEM with 10% fetal calf serum. Porcine pulmonary alveolar macrophages (PPAM) were obtained by lung lavage of specific-pathogen-free piglets free of PRRSV, as previously described (6). Prior to use, PPAM were resuspended in EMEM with 5% fetal calf serum. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Marc-145 and PPAM are fully permissive for VR-2332 and were used to propagate and titrate the virus. To rescue virus from the infectious cDNA clone, cells had to be transfected with in vitro-transcribed RNA. Pulmonary macrophages are difficult to transfect, and preliminary experiments showed a slightly better transfection efficiency with BHK-21C cells than Marc-145 cells (not shown). Although BHK-21C cells cannot be infected by VR-2332, infectious virus was generated after transfection with viral RNA, similar to the observation for Lelystad virus (25). Therefore, BHK-21C cells were used to rescue infectious virus by transfection with in vitro-transcribed RNA.

RNA extraction and RT-PCR. Viral RNA was isolated by binding to silica particles in guanidine thiocyanate as previously described (28, 29) and used immediately for cDNA synthesis.

cDNA synthesis was performed with SuperScript II reverse transcriptase (RT) (Invitrogen A/S, Taastrup, Denmark) and specific RT primers (Table 1). A total of six fragments, covering the complete VR-2332 genome (two small fragments representing the viral termini and four large fragments representing the internal part of the genome), were subsequently PCR amplified with *PfuTurbo* DNA polymerase according to the manufacturer's protocol (Stratagene, Aarhus, Denmark). The cycling conditions were 94°C for 1 min, then 30 cycles of 92°C for 20 s, 58°C for 60 s, and 72°C for 6 min, followed by 25 cycles of 92°C for 20 s, 58°C for 60 s, and 72°C for 6 min with the time increased by 10 s per cycle, and finally 72°C for 10 min.

A fragment representing the 5′ end of the viral genome immediately preceded by a T7 RNA polymerase site was made by PCR with the F20 sense and R229 antisense primers (Table 1, fragment b). The F20 primer contained, in the 5′ to 3′ direction, an *Xho*I site, an *Asc*I site, the T7 promoter, and nucleotides 1 to 21 of the VR-2332 sequence (based on GenBank accession number AF094475) (30). The R229 primer introduced a silent mutation at nucleotide 259 (based on GenBank accession numbers PRU87392 and AF094475) (26, 30), leading to the creation of a *Bst*Z17I restriction site (Table 1, fragment b). A fragment representing the 3′ end of the viral genome was constructed by reverse transcription with primer RT15392, containing, in the 5′ to 3′ direction, an M13 primer site, an *Xba*I site, an *Acl*I site, and a T_{38} sequence (Table 1, fragment a). The reverse transcription reaction was followed by PCR with M13 antisense (Invitrogen A/S) and F15301 sense primers (Table 1, fragment a). The F15301 primer contained silent mutations at nucleotides 15314 and 15318, introducing an *Hpa*I site at the 5′ end of the fragment.

The four fragments covering the main internal part of the genome (ORFs 1 to

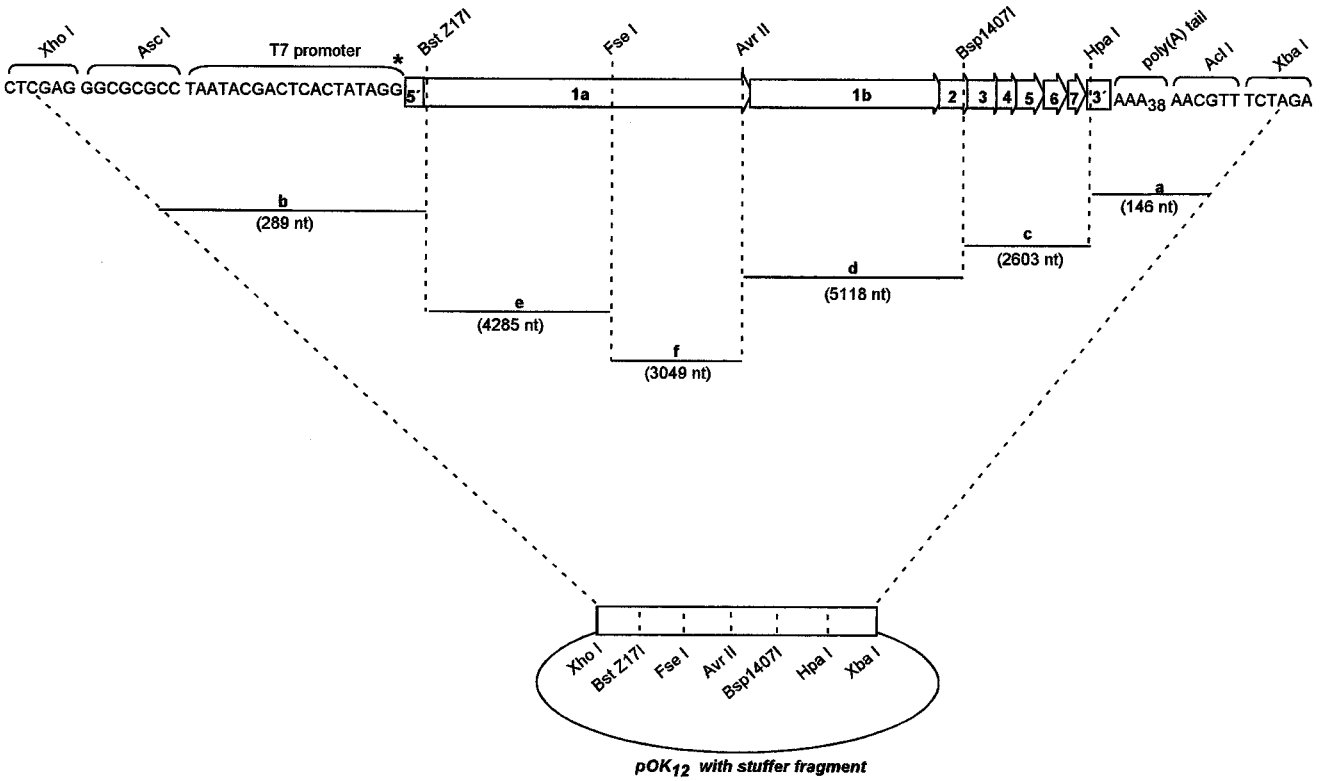

FIG. 1. Multistep strategy used to assemble full-length cDNA clone of VR-2332. In the top cartoon, the organization of the viral genome is shown, as are the positions of the unique restriction sites used for cloning purposes. The numbers 1a, 1b, and 2 through 7 indicate the PRRSV open reading frames. 5′ indicates the 5′ leader. and 3′ indicates the 3′ nontranslated region. At the 5′ end of the genome, *Xho*I and *Asc*I restriction sites and a T7 RNA promoter were fused to the genome. The asterisk indicates the transcription start site of T7 RNA polymerase, resulting in the sequence 5′-m^7G(5′)ppp(5′)G cap analog-TA TGA CGT ATA GGT. . .3′ as the predicted 5′ terminus of RNA transcribed in vitro with the T7 mMessage Machine kit. Downstream of the 3′ nontranslated region, a poly(A) tail of 38 A's and the restriction sites *Acl*I and *Xba*I were inserted. The complete viral genome was divided into six fragments flanked by unique restriction sites, represented by the horizontal lines labeled a through f. The length of each fragment is indicated in parentheses below the horizontal lines (in nucleotides). As shown in the bottom cartoon, these fragments were individually cloned into the pOK_{12} vector in the order indicated by the letters a to f. Prior to viral genome assembly, pOK_{12} was prepared by inserting a stuffer fragment containing all the unique restriction sites shown in the top cartoon in the *Xho*I and *Xba*I sites.

7, divided among fragments c through f; Fig. 1 and Table 1) were designed to allow assembly with unique restriction sites naturally found in the viral sequence. The primers used for RT-PCR amplification of these four internal fragments of the viral genome are described in Table 1 (fragments c through f). Finally, a small stuffer fragment which contained all these unique restriction sites (*Xho*I, *Bst*Z17I, *Fse*I, *Avr*II, *Bsp*1407I, *Hpa*I, and *Xba*I) was made by PCR with two overlapping synthetic oligonucleotides.

All PCR-amplified fragments were gel purified and cloned in the pCR-Blunt II-Topo vector (Invitrogen A/S). Before being used for assembly of the full-length clone (see below), these individual subclones were cycle sequenced with fluorescent BigDye chain terminators (Applied Biosystems, Nærum, Denmark), and the sequences were determined by capillary electrophoresis on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Construction of full-length cDNA clone. A full-length cDNA clone of VR-2332 was assembled by following the multistep strategy illustrated in Fig. 1. First, the pOK_{12} plasmid (37) was prepared by inserting the above-mentioned stuffer fragment in the *Xho*I and *Xba*I sites. Then, each of the viral subclones (Fig. 1, fragments a through f) was excised from pCR-Blunt II-Topo, gel purified, and ligated into the pOK_{12} plasmid after digestion of pOK_{12} with the same restriction enzymes. Following each ligation step, the pOK_{12} construct was transformed into *Escherichia coli* DH5α cells and grown overnight at 37°C in the presence of kanamycin (50 μg/ml).

Thus, in order to assemble the total VR-2332 genome in pOK_{12}, six sequential rounds of cloning were performed in the following order. The fragment covering ORF7 and the 3′ nontranslated region, followed by the 5′ leader, ORFs 3 to 7, ORFs 1b and 2, and finally the two fragments of ORF 1a (Fig. 1; the lettering a through f indicates the order in which the viral subclones were inserted in pOK_{12}). To ensure that no deletions had occurred during the multiple clonings, several cDNA clones were tested by single digestion with the multicutting enzyme *Pvu*II or *Pst*I. Finally, after assembly was complete, the complete VR-2332 sequence in the pOK_{12} plasmid (complete 15.4-kb fragment flanked by *Xho*I and *Xba*I sites in Fig. 1) was resequenced, and the result was submitted to GenBank.

In vitro transcription and transfection. The full-length cDNA clone was linearized by cleavage with *Acl*I, which cuts downstream of the poly(A) tail. Linearized plasmid DNA was used for in vitro transcription of capped RNA [m^7G(5′)ppp(5′)G cap analog] with the mMessage Machine kit (Ambion) according to the manufacturer's instructions and including treatment of the RNA with DNase to remove input plasmid. The RNA was purified by acid phenol-chloroform followed by isopropanol precipitation and redissolved in Tris-EDTA buffer by heating at 70°C. To check the size and quality of the in vitro-transcribed RNA, a sample was denatured in urea-based RNA sample buffer (New England Biolabs, Hellerup, Denmark) and electrophoresed on a 1% native agarose gel in Tris-borate-EDTA buffer with 1 μg of ethidium bromide per ml.

For transfection, BHK-21C cells were seeded in six-well plates (200,000 cells/well in 2 ml of medium) and grown overnight to approximately 80% confluency. Then 5 μg of in vitro-transcribed RNA was mixed with 10 μl of DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol) (Invitrogen A/S) and added directly to the medium. As a negative control, DMRIE-C without RNA was added to BHK-21C cells. As a positive control, BHK-21C cells were transfected with viral RNA from the parental VR-2332 isolate. This control RNA was extracted by acid phenol-chloroform purification followed by binding of the RNA to silica particles in guanidine thiocyanate (29). After 4 h of exposure to DMRIE-C and RNA, the monolayers were washed, and fresh medium was added. Supernatants from cells at 24 h posttransfection were serially passaged first on Marc-145 cells (four passages, each for 5 days) and then on PPAM (one passage, 3 days).

Titration and detection of virus were performed with an immunoperoxidase monolayer assay essentially as described by Bøtner et al., with the monoclonal antibody SDOW17 directed against the PRRSV nucleocapsid protein (27).

Discrimination between the cloned virus and VR-2332. Viral RNA was extracted with the QiaAmp viral RNA minikit (Qiagen, Hilden, Germany) and reverse transcription with Ready-to-go RT beads (Amersham Bioscience, Hørsholm, Denmark) and random hexamers was performed as described previously (29). A 990-bp fragment containing the *Bst*Z17I site engineered in the cloned virus was PCR amplified with the primers 5′-GCA TTT GTA TTG TCA GGA GCT-3′ and 5′-CAA GTC AAA CAA GCT CCA CC-3′. RT-PCR amplicons were digested with *Bst*Z17I and analyzed on a 2.5% agarose gel.

Inoculation in pigs. Twelve 5.5-week-old pigs from a specific-pathogen-free and PRRSV-seronegative herd were divided into three groups, each consisting of four animals. The pigs were Landrace/Yorkshire crossbred. The first group received $10^{4.8}$ 50% tissue culture infectious doses ($TCID_{50}$) of the cloned virus (fourth passage on Marc-145 cells) per ml, the second group received $10^{5.4}$ $TCID_{50}$ of the parental virus isolate VR-2332 (fourth passage on Marc-145 cells) per ml, and the third group was mock inoculated with EMEM. All the animals received 1 ml of inoculum in each nostril. The animals were kept in separate rooms throughout the experiment and observed daily for clinical signs of disease. Blood samples were collected on days 0, 7, 14, and 21 postinfection and tested for PRRSV-specific antibodies by blocking enzyme-linked immunosorbent assay and the immunoperoxidase monolayer assay (6, 33). All pigs were euthanized on day 21 postinfection.

Nucleotide sequence accession number. The complete genomic sequence for the infectious full-length cDNA clone of VR-2332 described in this report has been deposited as GenBank accession number AY150564.

RESULTS

Assembly of full-length cDNA clone of VR-2332. Marc-145 cells were infected with the VR-2332 isolate (third passage on Marc-145 cells) at a multiplicity of infection of 1.3 $TCID_{50}$/cell, and virus was harvested 5 days postinfection. From this material, a full-length cDNA clone covering the entire genome of the pathogenic North American isolate VR-2332 was assembled from overlapping PCR fragments flanked by unique restriction sites (Fig. 1). In order to minimize the number of PCR mutations, the amplifications were performed with a proofreading thermostable DNA polymerase (12). Nevertheless, even a proofreading polymerase would be expected to introduce mutations in a target of this size (Fig. 1; the North American-type PRRSV genome is 15.4 kb long). Therefore, to minimize PCR artifacts, the individual PCR fragments (labeled a through f in Fig. 1) were verified by sequencing before being selected for the final assembly. Finally, to ensure that the infectious clone was as well characterized as possible, the completely assembled VR-2332 sequence in the full-length clone (complete 15.4-kb construct flanked by *Xho*I and *Xba*I sites in pOK_{12}, as shown in Fig. 1) was DNA sequenced.

In total, 11 nucleotide differences were identified (Table 2) when the DNA sequence of the full-length cDNA clone was compared to previously published full-length sequences of North American isolates (GenBank accession numbers AF046869, AF066183, AF159149, AF176348, and PRU87392) (2, 3, 26, 38, 41). Three of these differences were silent mutations that were introduced intentionally to generate the *Bst*Z17I and *Hpa*I restriction sites (Tables 1 and 2). The remaining eight nucleotide differences were either PCR artifacts or the result of genetic variation in the VR-2332 isolate. Only two of the nucleotide mutations resulted in amino acid changes. These were at nucleotides 5520 (Tyr → Ile) and 6854 (Asp → Asn), both located in ORF 1a (Table 2).

Capped RNA was in vitro transcribed from the *Acl*I-linearized full-length cDNA clone with T7 RNA polymerase, and the quality of the RNA was verified by gel electrophoresis (data not shown). At the 5′ end, the in vitro-transcribed RNA had a nonviral G corresponding to the transcription initiation site of the T7 RNA polymerase (Fig. 1).

TABLE 2. Nucleotide differences between the parental VR-2332 isolate and the full-length cDNA clone

Nucleotide position within VR-2332 genome[a]	"Consensus" nucleotide of North American isolates[b]	Nucleotide in cDNA clone	Change
259	G	A	Silent
1075	C	T	Silent
5520	C	T	Y → I
5611	T	A	Silent
6854	G	A	D → N
6967	T	C	Silent
7555	T	C	Silent
10644	T	C	Silent
13788	T	C	Silent
15314	G	T	Silent
15318	T	C	Silent

[a] Nucleotide positions within the VR-2332 genome are based on GenBank accession numbers AF094475 and PRU87392 (26,30).

[b] After final assembly in pOK_{12}, the infectious clone (15.4-kb fragment flanked by *Xho*I and *Xba*I sites in pOK_{12}; Fig. 1) was sequenced in total (GenBank accession number AY150564). This sequence was compared to a "consensus" sequence of previously published full-length sequences of North American isolates (GenBank accession numbers PRU87392, AF066183, AF176348, AF046869, and AF159149) (2,3,26,38,41). In total, 11 nucleotide differences were observed, as shown in the table. The mutations at nucleotide positions 259, 15314, and 15318 were introduced intentionally to create *Bst*Z17I and *Hpa*I restriction sites, respectively. The sites were used for cloning purposes (see Fig. 1). Furthermore, the *Bst*Z17I site was used as a genetic marker for the cloned virus.

To recover infectious virus from the full-length cDNA clone, BHK-21C cells were transfected with the capped RNA with the transfection reagent DMRIE-C. Supernatants from the transfected BHK-21C cells obtained 24 h posttransfection were serially passaged four times on Marc-145 cells. Positive staining for nucleocapsid protein was detected in Marc-145 cells inoculated with supernatant from passage 1 (Fig. 2). As nucleocapsid production is a late event in PRRSV replication and requires the production of subgenomic mRNA, nucleocapsid staining strongly indicated that the infectious clone was not grossly impeded in any step of intracellular replication (Fig. 2). Similar results were observed after transfection with viral RNA from the VR-2332 isolate. However, no obvious cytopathic effect was detected earlier than passage 3 after transfection with the in vitro-transcribed RNA. This observation deviated from the results obtained when BHK-21C cells were transfected with viral RNA from the parental VR-2332 strain. In this case, cytopathic effect was observed as early as passage 1 on Marc-145 cells.

Finally, the supernatant from the fourth Marc-145 cell passage was passaged once on PPAM. Positive antinucleocapsid staining in the immunoperoxidase monolayer assay confirmed the presence of virus replication in PPAM. These results indicated that the cloned virus possessed the ability to replicate not only in Marc-145 cells but also in PPAM, as observed for the parental VR-2332 virus isolate.

Discrimination between cloned virus and VR-2332. To exclude that the results obtained from the cloned virus were artifactual, for example, due to laboratory contamination with the parental VR-2332 isolate, we assayed the cloned virus

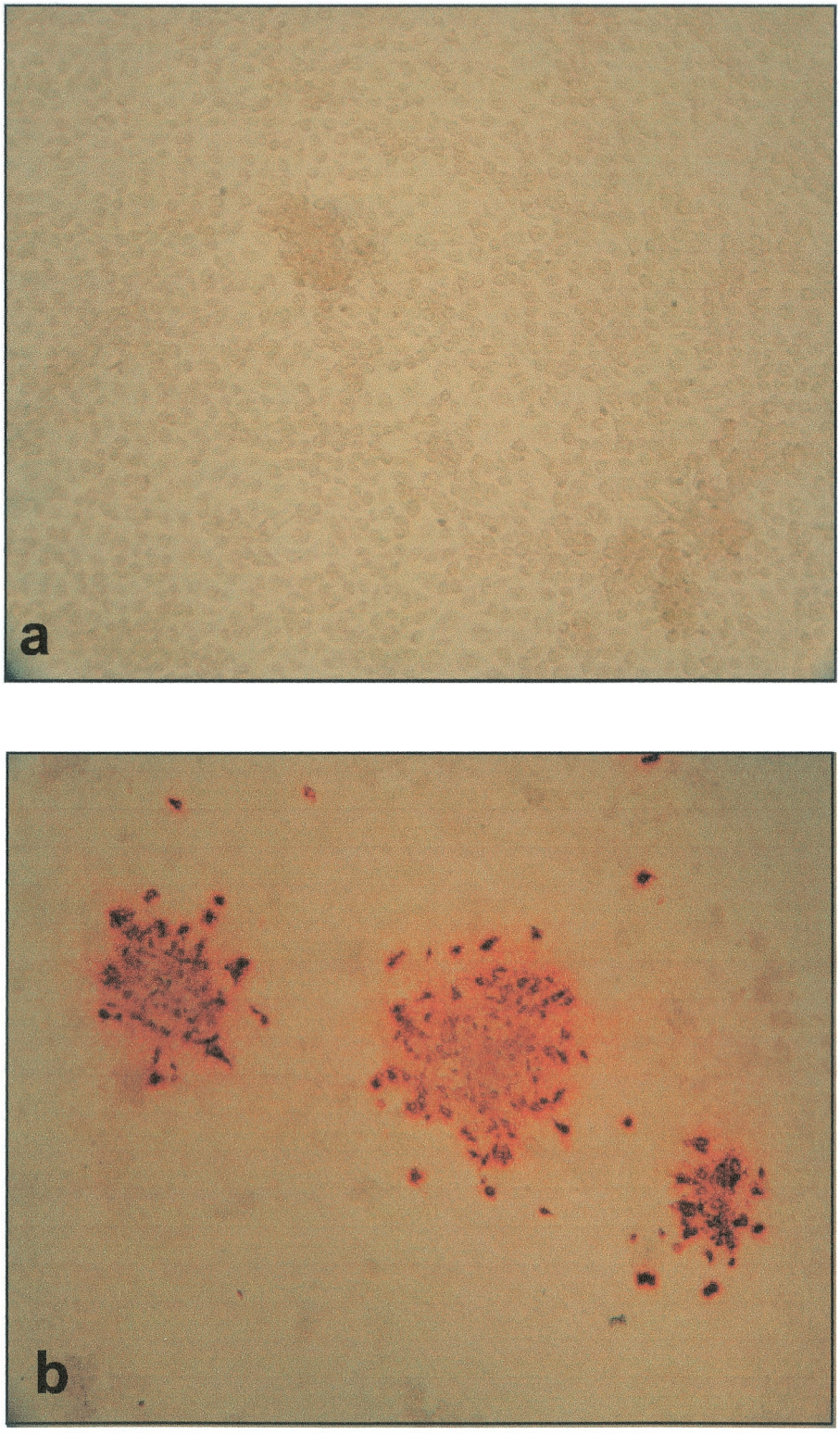

FIG. 2. Detection of cloned virus replication in Marc-145 cells. Supernatants from BHK-21C cells transfected with either (a) transfection reagent DMRIE-C without RNA as a negative control reaction or (b) RNA transcribed in vitro from the full-length cDNA clone were used to infect Marc-145 cultures. At day 3 after infection, the Marc-145 cultures were ethanol fixed and stained with monoclonal antibody SDOW17, directed against PRRSV nucleocapsid protein (a late marker of viral replication), and a horseradish peroxidase-conjugated secondary antibody (immunoperoxidase monolayer assay).

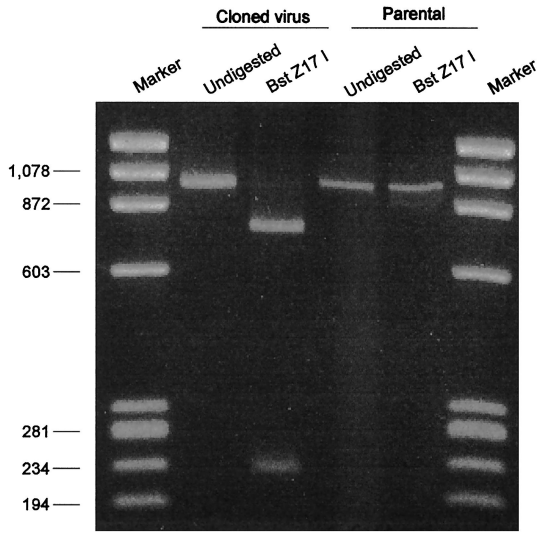

FIG. 3. Differentiation between cloned virus and parental VR-2332 strain. A *Bst*Z17I restriction site was introduced in the full-length cDNA clone of VR-2332 to allow discrimination between cloned virus (tagged with the *Bst*Z17I site) and parental virus (lacks a *Bst*Z17I site). RNA was extracted from lysates of cells infected with either the cloned virus or the parental VR-2332 isolate, and a 990-bp fragment was amplified by RT-PCR as described in Materials and Methods. The amplicons were digested with *Bst*Z17I and analyzed on a 2.5% agarose gel. The presence of a *Bst*Z17I restriction site resulted in fragments of 762 bp and 228 bp. As expected, the restriction site was found in the cloned virus but not in the parental VR-2332 virus isolate.

(second passage on Marc-145 cells) for the presence of a *Bst*Z17I restriction site. As expected, the RT-PCR fragment derived from the cloned virus was cleaved by *Bst*Z17I, generating two fragments of 228 bp and 762 bp (Fig. 3). In contrast, the PCR fragment derived from the parental isolate was not cleaved by *Bst*Z17I (Fig. 3).

Growth kinetics. To determine the growth curve of the cloned virus and to compare it to the growth curve of the parental VR-2332 strain, Marc-145 cells were infected with each of the viruses (fourth passage on Marc-145 cells) at a multiplicity of infection of 0.002 $TCID_{50}$/cell. After 2 h of incubation, the cells were washed twice, and fresh medium was added (time 0). The virus titers of the supernatants were determined by immunoperoxidase monolayer assay on Marc-145 cells at 0, 3, 16, 20, 24, 48, and 72 h postinfection.

The growth curves for the cloned virus revealed a first peak of replication at 16 h postinfection and a second peak at 48 h postinfection (Fig. 4). The same biphasic growth curve was obtained for the parental VR-2332 isolate (Fig. 4). The biphasic kinetics may represent a first and a second replication cycle of PRRSV in Marc-145 cells. Thus, the cloned virus displayed growth kinetics very similar to those of the parental VR-2332 isolate.

The titers of the parental virus were consistently slightly higher than the titers obtained for the cloned virus. This might indicate that the extra G at the 5′ end (Fig. 1) and the other nucleotide changes incurred during cloning (see above, Table 2) affected the replication of the cloned virus. However, the differences in titer between the cloned virus and VR-2332 were quite small at all times.

Inoculation in pigs. In order to investigate the infectivity of the cloned virus in vivo and to compare it to the infectivity of the parental VR-2332 isolate, we performed an inoculation experiment in young pigs. As VR-2332 is a highly virulent isolate, we could gauge infectivity by clinical signs. Additionally, the seroconversion kinetics were monitored.

A blue discoloration of the ears was detected at day 9 postinfection in two of four pigs inoculated with the cloned virus (Fig. 5). Also at day 9, four of four pigs inoculated with the parental VR-2332 isolate exhibited a blue discoloration of the ears (Fig. 5). This discoloration was transient, lasting approximately 6 days. All four pigs in the VR-2332 group exhibited inappetence and appeared lethargic on days 7, 8, and 9 postinfection. Furthermore, two of four pigs in the VR-2332 group exhibited lameness from day 10 postinfection. Postmortem results revealed hemopurulent infection of the knee joint in one pig and the elbow joint in the other. These findings indicated that the lameness was most likely caused by a bacterial infection, in agreement with the known predisposition of PRRSV-infected animals to secondary bacterial infections. Both these pigs appeared lethargic throughout the experiment. The four negative control pigs remained vigorous, had good appetites, and did not at any time show clinical signs of disease.

All pigs were seronegative prior to infection (day 0). In the group infected with the cloned virus, three of four pigs had seroconverted on day 7 postinfection and four of four pigs on day 14 postinfection (Table 3). In the VR-2332-infected group, all four pigs had seroconverted on day 7 postinfection (Table 3). PRRSV-specific antibodies were not detected in pigs from the negative control group (Table 3).

DISCUSSION

In the current work, we established an infectious cDNA clone of the highly virulent, prototypical North American PRRSV isolate VR-2332 (5, 10, 13). While the construction of infectious viral clones is becoming commonplace, the technical

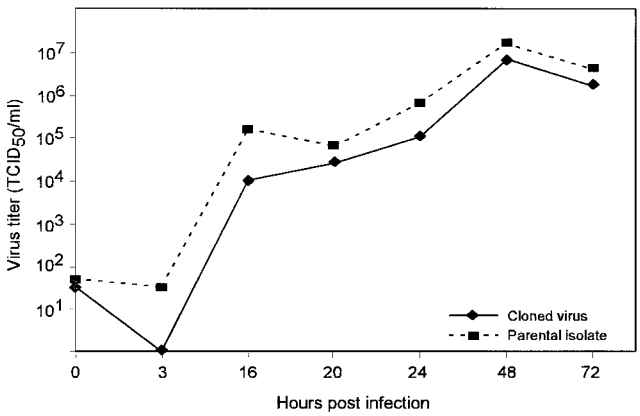

FIG. 4. Growth curves of cloned virus and parental VR-2332 isolate. Parallel cultures of Marc-145 cells were infected at a multiplicity of infection of 0.002 $TCID_{50}$/cell with virus recovered from the full-length cDNA clone and the parental VR-2332 isolate. After 2 h of incubation at 37°C, the cells were washed twice and fresh medium was added (time 0), and the cells were incubated at 37°C. At 0, 3, 16, 20, 24, 48, and 72 h postinfection, samples of the supernatants were taken, and virus titers were determined as described in Materials and Methods.

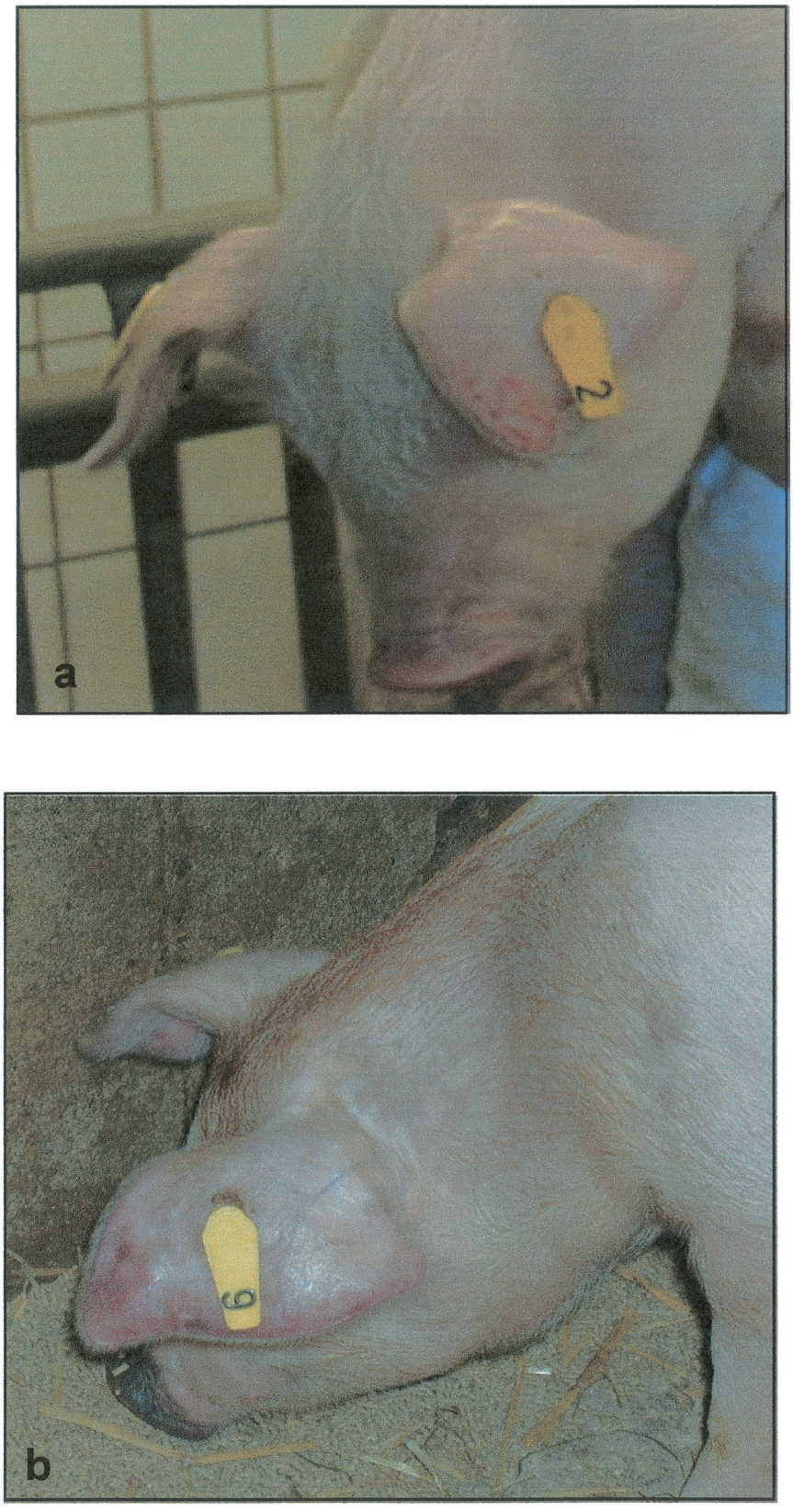

FIG. 5. Development of blue ears in pigs inoculated with cloned virus. A classical clinical sign of PRRSV infection, blue discoloration of the ears, was detected at day 9 postinfection in pigs experimentally infected with (a) the cloned virus or (b) the parental VR-2332 strain.

TABLE 3. Detection of PRRSV-specific antibodies in experimentally infected pigs[a]

Infection	Pig no.	Seroconversion (ELISA/IPMA result) on day postinfection:			
		0	7	14	21
Cloned virus	1	−/0	−/0	+/1,250	+/6,250
	2	−/0	+/50	+/1,250	+/6,250
	3	−/0	+/50	+/250	+/6,250
	4	−/0	+/0	+/6,250	+/6,250
Parental virus	5	−/0	+/250	+/6,250	+/6,250
	6	−/0	+/0	+/1,250	+/6,250
	7	−/0	+/0	+/1,250	+/6,250
	8	−/0	+/0	+/1,250	+/6,250
None (negative control)	9	−/0	−/0	−/0	−/0
	10	−/0	−/0	−/0	−/0
	11	−/0	−/0	−/0	−/0
	12	−/0	−/0	−/0	−/0

[a] Seroconversion was assayed by blocking ELISA and immunoperoxidase monolayer assay (IPMA), two tests routinely used for large-scale examination of field serum samples at the Danish Veterinary Institute. ELISA results: −, no anti-PRRSV antibodies detected; +, anti-PRRSV antibodies detected. Immunoperoxidase assay: sera were tested at 1:50, 1:250, 1:1,250, and 1:6,250 dilutions; 0, negative (anti-PRRSV antibodies not detected); positive results are indicated as the reciprocal of the highest serum dilution at which anti-PRRSV antibodies could be detected.

challenge involved in cloning the genomes of mammalian viruses in bacterial plasmids remains significant (7, 18, 19, 40). In particular, it is impossible to predict in advance how stable a given infectious clone will be during propagation in *E. coli*. Plasmid toxicity is not completely understood but may result from vector-specific as well as insert-specific causes (7, 18, 19, 40). Thus, a key parameter in infectious clone construction is the plasmid backbone. We used the low-copy pOK_{12} plasmid (37), which has been used previously for the assembly of an infectious clone of European-type PRRSV (25), and found that pOK_{12} also provided an appropriate backbone for an infectious clone of American-type PRRSV. Given the relatively large level of genetic differences between European-type and American-type PRRSV (2, 21, 26), we consider this a nontrivial technical achievement which paves the way for further infectious clones of North American-type as well as European-type PRRSV, for example, based on isolates differing in virulence.

The main design feature of our infectious clone is the division of the viral genome into six cassettes flanked by unique restriction sites, making future manipulation of the cloned virus very easy (Fig. 1, cassettes labeled a through f). Furthermore, the clone was completely sequenced (GenBank accession number AY150564), i.e., it has been fully genetically characterized. Finally, the clone is based on a highly virulent PRRSV strain which produces pronounced clinical and histopathological changes that are reproducible and easy to monitor during experimental infection of pigs. We expect that these features will make the infectious clone useful for the rapid identification of attenuation and virulence determinants in the PRRSV genome.

To rescue infectious virus from the pOK_{12} clone, full-length PRRSV genomic RNA was generated by in vitro transcription and transfected into BHK-21C cells. Since previous results indicated that a cap structure enhances the specific activity of transfected PRRSV genomic RNA (25), the cap analog $m^7G(5')ppp(5')G$ was included during in vitro transcription. By passaging supernatants from transfected BHK-21C cells on Marc-145 cells, stocks of infectious virus at a reasonable titer ($10^{4.8}$ $TCID_{50}$/ml, sufficient for experimental infections) could be obtained. Identification of a marker *Bst*Z17I site introduced in the cDNA clone confirmed that the recovered virus did not represent contamination by the parental VR-2332 strain.

An infectious clone should ideally be genetically completely identical to the parental virus. In practice, this is difficult to achieve. First, the sequence for the parental virus, especially when dealing with RNA viruses, may be ambiguous due to the heterogeneity (sometimes referred to as quasispecies structure) often seen in RNA viruses (20, 31). Second, during clone construction, some mutations will invariably occur during RT-PCR amplification (8, 12), other mutations have to be introduced for cloning purposes, and viral 5′ termini are sometimes difficult to reproduce faithfully with the T7 RNA polymerase system. Thus, comparing the biological properties of an infectious clone-derived virus and its parental strain is important. We found that, compared to the parental VR-2332 strain, the cloned virus exhibited the same cell tropism (replicated in Marc-145 cells as well as PPAM), exhibited essentially the same growth kinetics in Marc-145 cells (Fig. 4), and induced seroconversion in experimentally infected pigs (Table 3). Most importantly, experimental infection with the cloned virus induced a classical clinical sign of PRRSV infection, blue discoloration of the ears, which was also observed following infection with the parental VR-2332 strain. Thus, the cloned virus was qualitatively very similar to the parental VR-2332 strain. However, some quantitative differences were observed. First, the cloned virus replicated to slightly lower titers in Marc-145 cells (Fig. 4). Second, the seroconversion kinetics in pigs were marginally slower for the cloned virus (Table 3).

The quantitative biological differences between the cloned virus and VR-2332 mentioned above were minor and have as yet not been confirmed in repeated experiments. Thus, the slightly lower titers in Marc-145 cells (Fig. 4) might be due to experimental variability, and differences in the virus dose used for the animal experiments could account for the marginally quicker seroconversion kinetics (Table 3) and more pronounced clinical changes (see results) in the VR-2332-infected group. Alternatively, the quantitative biological differences observed between the cloned virus and VR-2332 might be real and reflect mutations and genetic defects in the cloned virus, which potentially include (i) 11 nucleotide mutations (Table 2), (ii) an extra 5′ nonviral G, derived from the T7 promoter (Fig. 1 and Table 1), and (iii) a relatively short poly(A) tail (Fig. 1 and Table 1).

The 11 mutations in the cloned virus might potentially influence replication. Only two of these mutations affected coding (Table 2). Both amino acid changes are conservative (17). However, the function of the ORF 1a protein segment, where both these coding mutations are located, is currently unknown, and it cannot be ruled out that they affect viral replication. In a similar vein, noncoding mutations might affect replication. For example, to obtain the fragment covering the 3′ end of the viral genome (Fig. 1, fragment a), an *Hpa*I restriction site was created by introduction of two silent mutations in the 3′non-

translated region (Table 1, primer F15301). With the infectious cDNA clone of the European strain Lelystad, Verheije et al. (37) showed that a highly conserved region in the 3′ nontranslated region folds into a stem-loop, which is engaged in a seven-nucleotide kissing interaction with a similar domain in ORF 7. These domains, which are highly conserved among PRRSV isolates, may play a critical role in virus replication. The mutations introduced by primer F15301 (Table 1) are both situated in this 3′ nontranslated region stem-loop (36). Even though none of the mutations are situated within the seven-nucleotide sequence essential for the kissing interaction, mutations anywhere in this domain might weaken the interaction, thereby reducing viral replication. Experiments are planned to evaluate whether the mutations introduced by primer F15301 (Table 1) interfere with virus replication.

Finally, it should be mentioned here that extension of the 5′ terminus is considered to have a substantial effect on virus replication (7). However, the presence of an additional 5′ nonviral G in the infectious cDNA clone of Lelystad virus did not seem to impair its growth (25). Furthermore, 5′ rapid amplification of cDNA ends of the European-type PRRSV isolate 111/92 revealed a further extension of two nucleotides at the 5′ end compared to the published Lelystad virus sequence (23, 30). Thus, it is possible that there is some natural heterogeneity in the 5′ end of the PRRSV genome without this having a major effect on viral replication.

As mentioned, there are substantial genetic differences between American-type and European-type PRRSVs. Additionally, the two virus types are antigenically very different, and there is only limited cross protection against reinfection with the heterologous type (20). That is, a genetically engineered vaccine based on, for example, an infectious clone of Lelystad virus would not be expected to offer full protection against infection with American-type PRRSV. Thus, the infectious clone of American-type PRRSV generated in the present study represents a significant new opportunity for recombinant vaccine development. Furthermore, previous studies on PRRSV have mainly focused on the functional role of the structural proteins. Regarding the nonstructural proteins, important information concerning their individual roles remains to be addressed. The availability of an infectious clone of VR-2332, supplementing the infectious clones of Lelystad virus (25) and the related equine arteritis virus (34), may broaden the questions that can be examined experimentally in the field of *Arterivirus* replication and pathogenesis.

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