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Infectious Transcripts from Cloned Genome-Length cDNA of Porcine Reproductive and Respiratory Syndrome Virus

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The 5′-terminal end of the genomic RNA of the Lelystad virus isolate (LV) of porcine reproductive and respiratory syndrome virus was determined. To construct full-length cDNA clones, the 5′-terminal sequence was ligated to cDNA clones covering the complete genome of LV. When RNA that was transcribed in vitro from these full-length cDNA clones was transfected into BHK-21 cells, infectious LV was produced and secreted. The virus was rescued by passage to porcine alveolar lung macrophages or CL2621 cells. When infectious transcripts were transfected to porcine alveolar lung macrophages or CL2621 cells, no infectious virus was produced due to the poor transfection efficiency of these cells. The growth properties of the viruses produced by BHK-21 cells transfected with infectious transcripts of LV cDNA resembled the growth properties of the parental virus from which the cDNA was derived. Two nucleotide changes leading to a unique *Pac*I restriction site directly downstream of the ORF7 gene were introduced in the genome-length cDNA clone. The virus recovered from this mutated cDNA clone retained the *Pac*I site, which confirmed the de novo generation of infectious LV from cloned cDNA. These results indicate that the infectious clone of LV enables us to mutagenize the viral genome at specific sites and that it will therefore be useful for detailed molecular characterization of the virus, as well as for the development of a safe and effective live vaccine for use in pigs.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the *Arteriviridae* family, which also comprises equine arteritis virus (EAV), lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (SHFV) (20). Recently, the International Committee on the Taxonomy of Viruses has decided to incorporate this family in a new order of viruses, the *Nidovirales*, together with the *Coronaviridae*, and *Toroviridae* (3). The order *Nidovirales* represents enveloped RNA viruses that contain a positive-stranded RNA genome and synthesize a 3′ nested set of subgenomic RNAs during replication. The subgenomic RNAs of coronaviruses and arteriviruses contain a leader sequence which is derived from the 5′ end of the viral genome (27, 32). However, the subgenomic RNAs of toroviruses lack this leader sequence (31). Open reading frames 1a and 1b (ORF1a and ORF1b), which encode the RNA-dependent RNA polymerase, are expressed from the genomic RNA, but the smaller ORFs at the 3′ end of the genomes of *Nidovirales*, which encode the structural proteins, are thought to be expressed from the subgenomic mRNAs. The genomes of arteriviruses (approximately 13 to 15 kb) are much smaller than those of coronaviruses (28 to 30 kb) and toroviruses (26 to 28 kb).

The causative agent of a new disease, now known as porcine reproductive and respiratory syndrome, was first identified in 1991 by Wensvoort et al. (39) and was named Lelystad virus (LV). The main symptoms of the disease are respiratory problems in pigs and abortions in sows. Although major outbreaks, such as those observed at first in the United States in 1987 and in Europe in 1991, have diminished, this virus still causes significant economic losses in herds in the United States, Europe, and Asia. PRRSV preferentially grows in porcine alveolar lung macrophages (PAMs) (39). A few cell lines, such as CL2621 and other cell lines cloned from the monkey kidney cell line MA-104, are also susceptible to the virus (1, 5, 13). The genomic cDNA sequence of LV and other isolates of PRRSV was determined (6, 20, 25). In addition to the RNA-dependent RNA polymerase (ORF1a and ORF1b), the genomic sequence encodes four envelope glycoproteins named GP_2 (ORF2), GP_3 (ORF3), GP_4 (ORF4), and GP_5 (ORF5), as well as a nonglycosylated membrane protein M (ORF6) and the nucleocapsid protein N (ORF7) (18, 21, 22, 35). Immunological characterization and nucleotide sequencing of U.S. strains have indicated that they are antigenically different from European strains (25, 26, 37).

The production of cDNA clones from which infectious RNA can be transcribed in vitro has become an essential step in the molecular genetic analysis of positive-strand RNA viruses. These clones are useful in studies focused on genetic expression, replication, function of viral proteins, and recombination of RNA viruses, as well as for the development of new viral vectors and vaccines (for a review, see reference 2). The technology is applicable to positive-strand RNA viruses, whose RNA genomes function as mRNA and initiate a complete infectious cycle upon introduction into appropriate host cells. Although infectious clones have been described for several other positive-strand RNA viruses, no infectious cDNA clone has been described for PRRSV until now. In this study, we generated for the first time an infectious clone of the LV isolate of PRRSV and used it to produce LV mutants.

MATERIALS AND METHODS

Cells and viruses. The *Ter Huurne* strain of LV was isolated in 1991 (39) and grown in PAMs. Passage 6 of the *Ter Huurne* strain (TH) was used in this study, as well as a derivative of this strain, LV4.2.1, which was adapted for growth on CL2621 cells by serial passage. PAMs were maintained in RPMI 1640 growth medium (Flow), whereas CL2621 cells were maintained in Hanks' minimal essential medium (Gibco-BRL/Life Technologies). Virus titers (expressed as 50% tissue culture infective doses [$TCID_{50}$] per milliliter) were determined on PAMs or CL2621 cells by end point dilution, as described previously (38). BHK-21 cells were maintained in Dulbecco's minimal essential medium. For transfection experiments, the BHK-21 cells were grown in Glasgow minimal

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essential medium (GIBCO-BRL/Life Technologies) by the method of Liljeström and Garoff (17).

Isolation of viral RNAs. At 24 h after infection, intracellular LV RNA was isolated from PAMs or CL2621 cells infected with TH and LV4.2.1, respectively, at a multiplicity of infection of 1 as described previously (20). To isolate virion genomic RNA, virions were purified on sucrose gradients as described by van Nieuwstadt et al. (35) and were resuspended in TNE (10 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM EDTA). A 1-ml volume of proteinase K buffer (100 mM Tris-HCl [pH 7.2], 25 mM EDTA, 300 mM NaCl, 2% [wt/vol] sodium dodecyl sulfate) and 0.4 mg of proteinase K (Boehringer Mannheim) were added to 1 ml of purified LV virions (10^8 $TCID_{50}$). This reaction mixture was incubated at 37°C for 30 min. The RNA was extracted once with phenol-chloroform (1:1) and precipitated with ethanol. The RNA was stored in ethanol at −20°C. One-tenth of this RNA preparation was used in reverse transcription reactions.

Cloning of the 5′ and 3′ termini of the LV genome. The 5′ end of the viral genome of LV was cloned by a modified single-strand ligation to single-stranded cDNA procedure (11). Virion RNA, prepared as described above, was used in a reverse transcription reaction with primer 11U113 (5′ TACAGGTGCCTGAT CCAAGA 3′), which is complementary to nucleotides 1242 to 1261 of the genome of LV. The reverse transcription reaction was performed in a final volume of 20 μl, as described previously (19). Then the single-stranded cDNA was ligated to an anchor primer, ALG3 (5′ CACGAATTCACTATCGATTCT GGATCCTTC 3′), as specified in the protocol of the 5′-Amplifinder rapid amplification of cDNA ends kit (Clontech). Primer ALG3 contains *Eco*RI, *Cla*I, and *Bam*HI sites, and its 3′ end is modified with an amino-blocking group to prevent self-ligation. The ligated cDNA was used as template in a PCR with primers LV69 (5′ AGGTCGTCGACGGGCCCCGTGATCGGGTACC 3′) and ALG4 (5′ GAAGGATCCAGAATCGATAG 3′). Primer LV69 is complementary to nucleotides 604 to 625 of the LV genome, whereas ALG4 is complementary to anchor primer ALG3. The PCR conditions were as described by Meulenberg et al. (19), and the product obtained was digested with *Eco*RI and *Sal*I and cloned in pGEM-4Z. A similar strategy was used to clone the 5′ terminus of the LV genome from intracellular LV RNA. For these experiments, 10 μg of total cellular RNA isolated from CL2621 cells infected with LV4.2.1 was used.

A 3′-end cDNA clone containing a long poly(A) tail was constructed by reverse transcription of LV RNA with primer LV76 [5′ TCTAGGAATTCTAG $ACGATCG(T)_{40}3'$], which contains *Eco*RI, *Xba*I, and *Pvu*I sites. The reverse transcription reaction was followed by PCR with primers LV75 (5′ TCTAGGA ATTCTAGACGATCGT 3′), which is identical to LV76 except for the poly(T) stretch, and 39U70R (5′ GGAGTGGTTAACCTCGTCAA 3′), a sense primer corresponding to nucleotides 14576 to 14595 of the LV genome and containing an *Hpa*I site. The resulting PCR products were digested with *Hpa*I and *Eco*RI and cloned in cDNA clone pABV39 restricted with the same enzymes (see Fig. 2A). One cDNA clone containing a poly(A) stretch of 109 A's (pABV392) was used in further experiments.

Sequence analysis. The correct genomic sequence of newly generated cDNA clones was assessed by oligonucleotide sequencing. Oligonucleotide sequences were determined with the PRISM Ready Reaction Dye Deoxy Terminator cycle-sequencing kit and automatic sequencer (Applied Biosystems).

Construction of full-length genomic cDNA clones of LV. cDNA clones generated previously to determine the nucleotide sequence of the genome of LV (20) were ligated at convenient restriction sites in high-copy-number plasmid pGEM-4Z (see Fig. 2A). Plasmid pABV254 was constructed from pABV clones 25, 11, 12, and 100 and had been used in a previous study (8). Standard cloning procedures were carried out as described by Sambrook et al. (29). Plasmid pABV369 is derived from pABV331 but encodes a Leu instead of a Pro at amino acid 1084 in ORF1a and was generated by substituting nucleotides 3413 to 3615 with a newly generated reverse transcription-PCR fragment encoding the Leu residue. To establish overlap between pABV20 and pABV5, two new cDNA fragments were generated by reverse transcription-PCR. The *Xba*I site (incorporated in the PCR primer), the internal *Apo*I site (nucleotide 6016), and the *Bam*HI site (nucleotide 6750) were used to ligate these fragments in pABV20 (see Fig. 2A). Since further ligation of cDNA fragments in pGEM-4Z resulted in unstable clones, the inserts of pABV331/369, pABV384, and pABV368 were ligated to each other and to the 5′ and 3′ cDNA fragments in low-copy-number vector pOK12 (see Fig. 2B) (36). The plasmids were transformed to *Escherichia coli* DH5α and grown at 32°C in the presence of 5 to 15 μg of kanamycin per ml to keep their copy number as low as possible. First, the cDNA fragment of pABV392 [$(A)_{109}$] was excised by digestion with *Eco*RI, modification of this site with Klenow polymerase (Pharmacia) to a blunt end, and digestion with *Bam*HI. This fragment was cloned in pOK12 digested with *Bam*HI and *Fsp*I (the latter site was also modified to a blunt end), resulting in pABV395. A 5′ cDNA clone, which contained the T7 RNA polymerase promoter directly fused to the newly determined 5′ terminus of the LV genome, was amplified by PCR from pABV387 with primers LV83 (5′ GAATTCACTAGTTAATACGACTCACTA TAGATGATGTGTAGGGTATTCC 3′) and LV69. LV83 is composed of, in order from 5′ to 3′, an *Eco*RI site and a *Spe*I site, a T7 RNA polymerase promoter sequence, a single G for initiation of transcription, and nucleotides 1 to 19 of the LV genome. The PCR fragment was cloned in the *Eco*RI and *Sal*I sites of pOK12, resulting in pABV396. Subsequently, the 5′ cDNA fragment of pABV396 and the 3′ cDNA fragment of pABV395 were ligated to the cDNA fragments of pABV331/369, pABV384, and pABV368, using the restriction sites indicated in Fig. 2B. In this way, two genome-length cDNA clones were obtained; they were designated pABV414 and pABV416. These genome-length cDNA clones encode identical viral protein sequences except for one amino acid at position 1084 in ORF1a, which is a Pro in pABV414 and a Leu in pABV416.

To introduce a unique *Pac*I site in the genome-length cDNA clone directly downstream of the ORF7 gene, the T and A at nucleotides 14987 and 14988 were both replaced by an A in a PCR with sense primer LV108 (5′ GGAGTGGTT AACCTCGTCAAGTATGGCCGGTAAAAACCAGAGCC 3′) plus antisense primer LV112 (5′ CCATTCACCTGACTGTTTAATTAACTTGCACCCTGA 3′) and with sense primer LV111 (5′ TCAGGGTGCAAGTTAATTAAACAG TCAGGTGAATGG 3′) plus antisense primer LV75. The PCR fragments were ligated in pABV395 with the created *Pac*I site and flanking *Hpa*I and *Xba*I sites, resulting in pABV427. This fragment was then inserted in pABV414 with the same unique *Hpa*I and *Xba*I sites, resulting in pABV437 (see Fig. 6A). To detect the marker mutation in the virus recovered from transcripts of pABV437, RNA was isolated from the supernatant of infected PAMs. This RNA was used in reverse transcription-PCR to amplify a fragment of approximately 0.6 kb [spanning nucleotide 14576 to the poly(A) tail of variable length] with primers LV76, LV75, and 39U70R. The presence of the genetic marker was detected by digesting the PCR fragments with *Pac*I.

In vitro transcription and transfection of RNA. Full-length genomic cDNA clones were linearized with *Pvu*I, which is located directly downstream of the poly(A) stretch. Plasmid pABV296, consisting of Semliki Forest virus (SFV) vector pSFV1 expressing the GP_4 protein encoded by ORF4 of LV (23), served as control for in vitro transcription and transfection experiments and was linearized with *Spe*I. The linearized plasmids were precipitated with ethanol, and 1.5 μg of these plasmids was used for in vitro transcription with T7 RNA polymerase (full-length cDNA clones) or Sp6 RNA polymerase (pABV296) by the methods described for SFV by Liljeström and Garoff (16, 17). The in vitro-transcribed RNA was precipitated with isopropanol, washed with 70% ethanol, and stored at −20°C until use.

BHK-21 cells were seeded in 35-mm wells (approximately 10^6 cells/well) and were transfected with 2.5 μg of in vitro-transcribed RNA or 2.5 μg of intracellular LV RNA mixed with 10 μl of Lipofectin in Optimem as described by Liljeström and Garoff (17). Alternatively, RNA was introduced into BHK-21 cells by electroporation. In this case, 10 μg of in vitro-transcribed RNA or 10 μg of intracellular LV RNA was transfected to approximately 10^7 BHK-21 cells under the electroporation conditions of Liljeström and Garoff (17). The electroporated cells were seeded in four 35-mm wells. The medium was harvested 24 h after transfection and transferred to CL2621 cells or PAMs to rescue infectious virus. Transfected and infected cells were tested for the expression of LV proteins by an immunoperoxidase monolayer assay (IPMA), essentially as described by Wensvoort et al. (38). Monoclonal antibodies (MAbs) 122.13, 122.59, 122.9, and 122.17, directed against the GP_3, GP_4, M, and N proteins, respectively (35), were used for staining in this assay.

RESULTS

Infectivity of LV RNA. Although it has been assumed that genomic RNA of LV is infectious, experiments to prove the infectivity of genomic LV RNA had failed until now, because we were unable to transfect PAMs with nucleic acids. Since cell line CL2621 and other clones derived from the monkey kidney cell line MA-104 are the only cells which were shown to propagate LV and other isolates of PRRSV (1, 5, 13), we first tested these in transfection experiments to demonstrate the infectivity of LV RNA. When intracellular RNA isolated from CL2621 cells infected with LV was transfected to CL2621 cells at different doses by using different transfection reagents, such as Lipofectin, Lipofectamin, DEAE-dextran or electroporation, no cythopathic effect or plaques were observed and no production of structural proteins could be detected in IPMA with LV-specific MAbs. RNA transcribed in vitro from pABV296 (23) was used as control in these experiments. This RNA was transfected most efficiently by electroporation. However, only 0.01% of the CL2621 cells stained with GP_4-specific MAbs in IPMA. In contrast, when BHK-21 cells were electroporated under similar conditions, 90 to 100% of the cells stained. Since these results indicated that BHK-21 cells were much more efficiently transfected than CL2621 cells, we used them to test the infectivity of LV RNA. Therefore, intracellular LV RNA (2.5 μg, which was estimated to contain approximately 1 to 2 ng of LV genomic RNA) was transfected to 10^6 BHK-21 cells with Lipofectin. At 24 h after transfection, approximately 5 to 15 individual cells were stained with LV-specific MAbs, but no

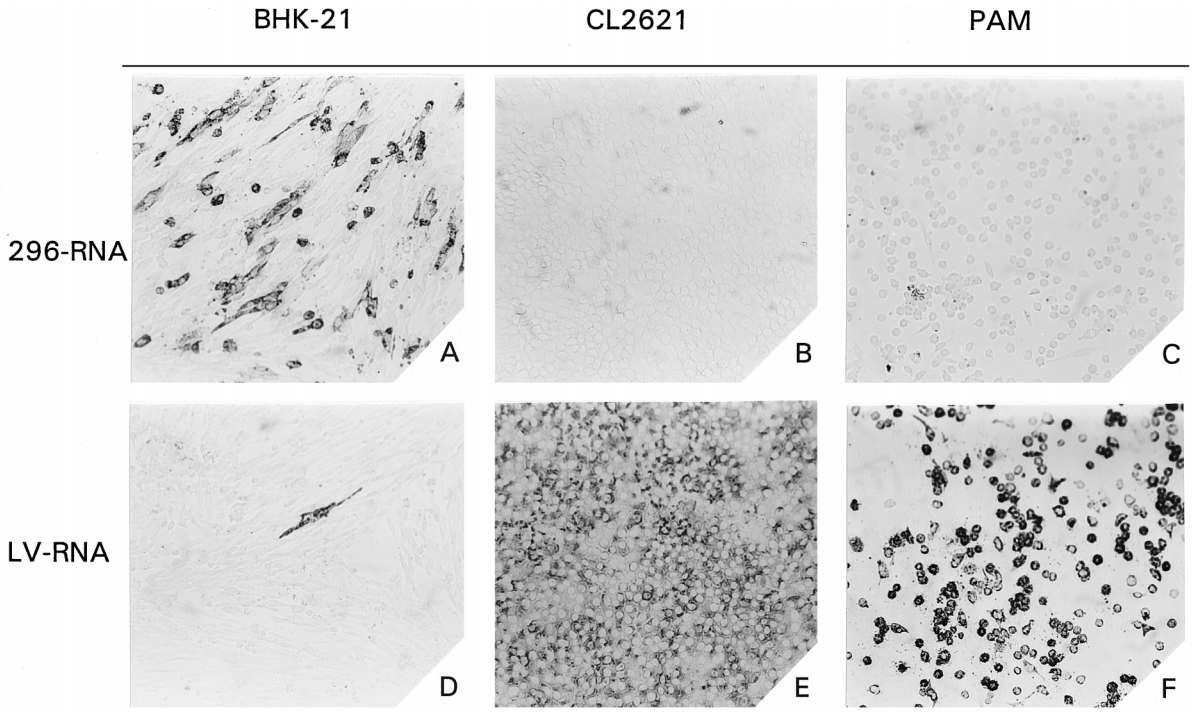

FIG. 1. Infectivity of LV RNA. BHK-21 cells were transfected with pABV296 RNA (expressing GP_4) (A) or LV intracellular RNA (D) by using Lipofectin, and at 24 h posttransfection they were stained in IPMA with GP_4-specific MAb 122.59 (A) or N-specific MAb122.17 (D). The supernatant of BHK-21 cells transfected with pABV296 RNA or LV intracellular RNA was used to infect CL2621 cells (B and E) and PAMs (C and F). These PAMs and CL2621 cells were stained after 2 and 3 days, respectively, with N-specific MAb122.17.

infectious centers or plaques were observed, indicating that the LV did not spread to neighboring cells (Fig. 1D). The number of positive cells increased two- to fourfold when the RNA was transfected to BHK-21 cells by electroporation. Transfection of the control RNA transcribed from pABV296 resulted in 10 to 30% stained BHK-21 cells when Lipofectin was used (Fig. 1A). At 24 h after transfection, the supernatant of the BHK-21 cells transfected with intracellular LV RNA or pABV296 RNA was transferred to PAMs and CL2621 cells. Cythopathic effect was observed in PAM cultures at 2 days and in CL2621 cultures at 3 to 4 days after inoculation with the supernatant from BHK-21 cells transfected with intracellular LV RNA. The infected PAMs and CL2621 cells were positively stained with LV-specific MAbs in IPMA (Fig. 1E and F). Similar results were obtained when RNA isolated from purified virions of LV was transfected to BHK-21 cells (data not shown). No cythopathic effect or staining with LV-specific MAbs directed against the N protein (Fig. 1B and C) or GP_4 (data not shown) was observed in PAMs or CL2621 cells incubated with the supernatant from BHK-21 cells transfected with pABV296 RNA. This was expected since the SFV vector used to construct pABV296 lacks the genes encoding the structural proteins of SFV, which are needed for the assembly of new virus particles. Therefore, these results show that BHK-21 cells can be used to demonstrate the infectivity of LV RNA. Although LV cannot infect BHK-21 cells, probably because they lack the receptor for LV, new infectious virus particles are produced and excreted into the medium once the genomic RNA has been introduced in BHK-21 cells.

Reconstruction of the 5′-terminal sequence of the genomic RNA of LV. It is generally admitted that the entire viral sequence, including the 5′ and 3′ ends, are required to obtain infectious clones. To clone the 5′ end of the LV genome, a modified single-strand ligation to single-stranded cDNA (11) procedure was used. Twelve clones obtained from two independent PCRs on ligated products derived from LV intracellular RNA and 14 clones derived from two independent PCRs on ligated products derived from virion RNA were sequenced. Of these 26 cDNA clones, 22 clones contained an extension of 10 nucleotides (5′ ATGATGTGTA 3′) compared to the cDNA sequence, which was reported in our previous study (20). The other four clones lacked 1 to 3 nucleotides at the 5′ end of this additional sequence (Table 1). This led us to conclude that this 10-nucleotide sequence represents the utmost 5′ end of the LV genome and it was therefore incorporated in the genome-length cDNA clone. Due to the extension of 10 nucleotides, the numbers of nucleotides, the position of primers, and the restriction sites in the genome have been changed and are therefore different from the numbers used in previous papers.

Construction of genome-length cDNA clones of LV. A genome-length cDNA clone of LV was constructed by the strategy depicted in Fig. 2. A T7 RNA polymerase promoter for in vitro transcription was directly linked to the newly determined 5′ terminus of the genome of LV by PCR and inserted in the

TABLE 1. Nucleotide sequences of 5′ end clones of LV

Sequence[a]	No. of clones
ATGATGTGTAGGG	22
TGATGTGTAGGG	1
GATGTGTAGGG	2
ATGTGTAGGG	1

[a] The underlined nucleotides represent additional sequences that were not found in cDNA clones isolated and sequenced previously (20.

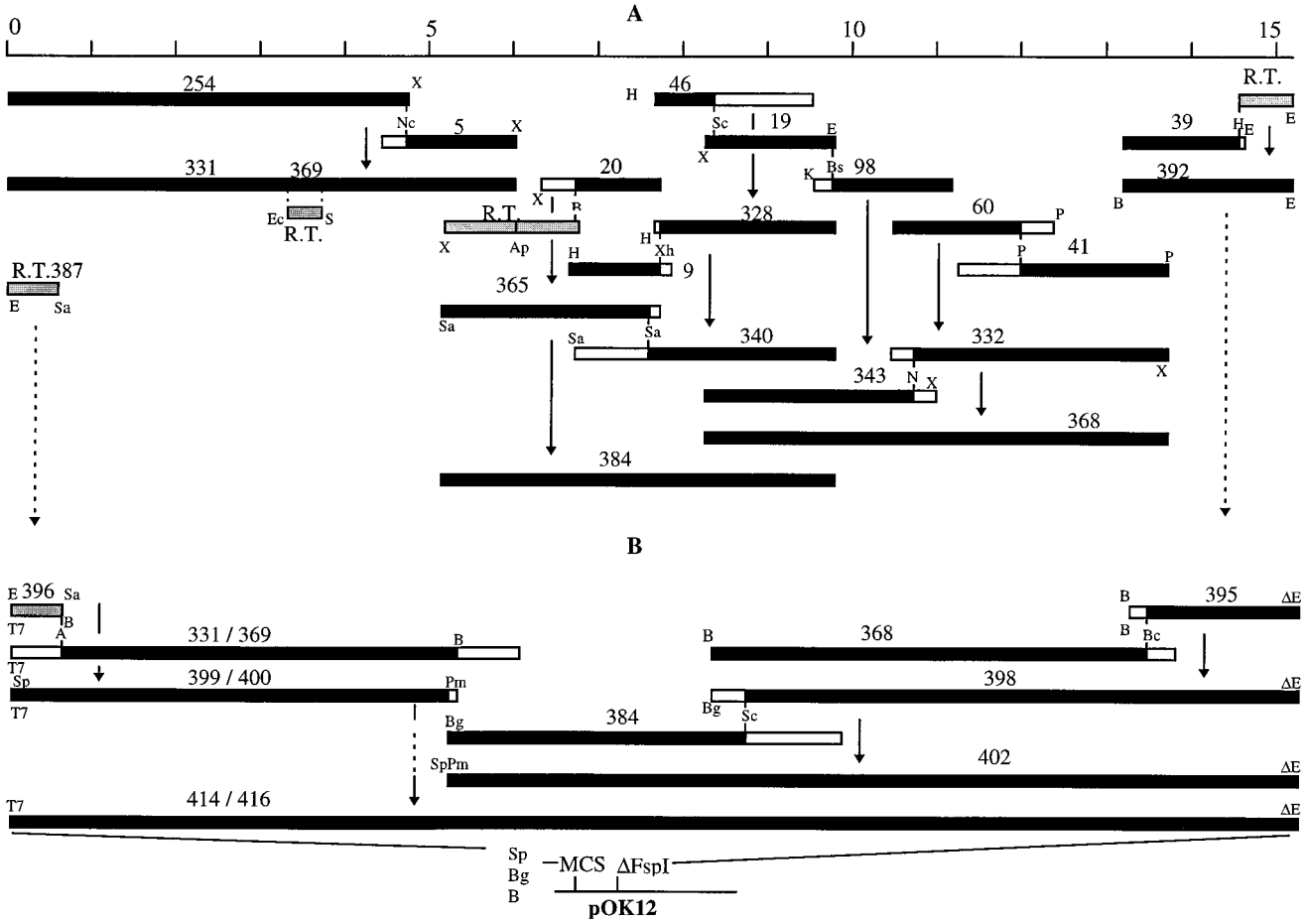

FIG. 2. Construction of genome-length cDNA clones of LV. (A) Ligation of cDNA clones, which were previously sequenced (20), in pGEM-4Z. The pABV numbers of the clones and the restriction sites that were used are indicated. The black boxes represent the parts of the cDNA clones that are fused in the next cloning step. Light grey boxes are cDNA clones newly generated by reverse transcription-PCR (indicated by R.T.) or PCR only. Assembly of the larger cDNA clones pABV331/369, pABV384, and pABV368 with the 5′-end clone pABV396, containing a T7 RNA polymerase promoter, and the 3′-end clone pABV395, containing a poly(A) tail, in low-copy-number vector pOK12. Abbreviations: A, *Apa*I; Ap, *Apo*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bsp*E1; Bc, *Bcl*I; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nar*I; Nc, *Nco*I; S, *Sac*II; Sp, *Spe*I; Sa, *Sal*I; Sc, *Sca*I; P, *Pst*I; Pm, *Pml*I; X, *Xba*I; Xh, *Xho*I; MCS, multiple-cloning site.

genome-length cDNA clone. Resequencing of nucleotides 3420 to 3725 of six newly generated and independent cDNA clones indicated that at nucleotide 3472 a C and T were present at a ratio of 1:1, resulting in a Pro or Leu at amino acid residue 1084 in ORF1a. Since we could not predict the influence of the amino acid substitution at this position on the infectivity of the RNA transcribed from the final genome-length cDNA clone, we constructed two genome-length cDNA clones encoding either a Leu or a Pro at this position. At the 3′ end, a poly(A) stretch of 109 A residues was incorporated in the genome-length cDNA clone.

We tried to ligate the larger cDNA fragments of the pABV331/369, pABV384, and pABV368 clones to the 5′ and 3′ ends in pGEM-4Z, but this resulted in the accumulation of deletions. Therefore, we finally fused these clones to each other in the low-copy-number vector pOK12 (36) and obtained the genome-length cDNA clones pABV414 (Pro) and pABV416 (Leu). These could be stably propagated in *E. coli* under the growth conditions used.

In vitro synthesis of infectious RNA. Transcripts of genome-length cDNA clones pABV414 and pABV416 were transfected to BHK-21 cells to test their infectivity. These transcripts, synthesized in vitro with T7 RNA polymerase, were expected to contain two nonviral nucleotides at the 3′ end (Fig. 3). In addition, they were expected to contain a nonviral G at the 5′ end, which is the transcription start site of T7 RNA polymerase. Approximately 2.5 μg of RNA transcribed in vitro from pABV414 or pABV416 was transfected to BHK-21 cells with Lipofectin, and at 24 h after the transfection, 800 to 2,700 cells stained positive with N-protein-specific MAb122.17 in IPMA (Fig. 4A and D). PAMs that were inoculated with the supernatant derived from BHK-21 cells transfected with transcripts of pABV414 or pABV416 displayed a cythopathic effect after 2 days. Individual plaques were produced after 3 to 4 days in CL2621 cultures that were inoculated with either of the supernatants. The infected PAMs and CL2621 cells stained in the IPMA with MAb122.17 directed against the N protein (Fig. 4B, C, E, and F) and with MAbs directed against the M, GP_4, and GP_3 proteins (data not shown), confirming that these proteins were all properly expressed. No cythopathic effect or staining in IPMA was observed in CL2621 cultures or PAMs that were incubated with the supernatant of BHK-21 cells transfected with pABV296 RNA (negative control). Therefore, these results clearly show that when RNA transcribed from genome-length cDNA clones pABV414 and pABV416 is transfected to BHK-21 cells with Lipofectin, infectious LV

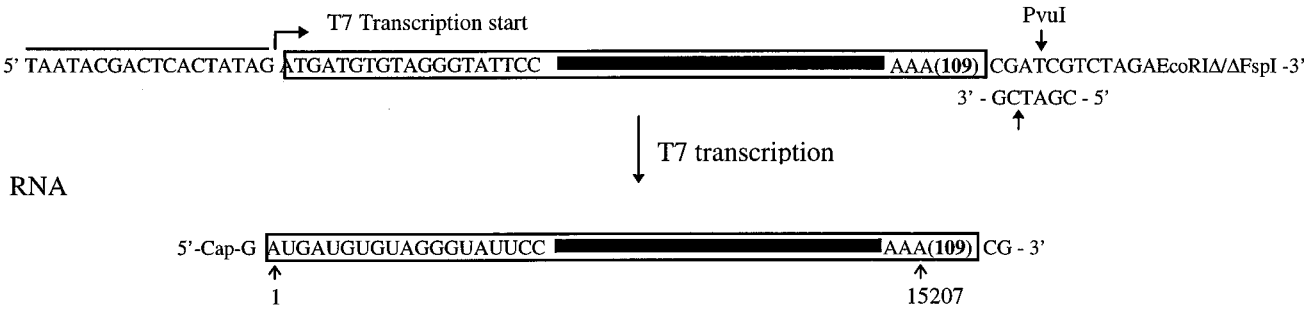

FIG. 3. Terminal sequences of cloned genome-length cDNA of LV and infectious RNA transcribed from this cDNA. Genome-length cDNA clones pABV414 and pABV416 were linearized with *Pvu*I and were transcribed in the presence of the synthetic cap analog $m^7G(5')ppp(5')G$ with T7 RNA polymerase. The resulting RNA should contain one extra nucleotide (G) at the 5′ end and two extra nucleotides (CG) at the 3′ end. The arrows in the RNA indicate the 5′- and 3′-terminal nucleotides corresponding to the authentic LV RNA sequence. The cDNA fragments of pABV414 and pABV416 encode identical viral protein sequences except for one amino acid at position 1084 in ORF1a, which is a Pro in pABV414 and a Leu in pABV416.

is produced and excreted. Moreover, when transcripts of pABV414 or pABV416 were transfected to BHK-21 cells by electroporation instead of by Lipofectin complexes, a two- to fourfold increase in the number of cells staining positive with LV-specific MAbs was obtained. The titer of the recombinant viruses in the supernatant of these electroporated BHK-21 cells was approximately 10^5 $TCID_{50}$/ml.

Growth characteristics of rescued virus. The initial transfection and infection experiments suggested that the rescued recombinant viruses, designated vABV414 and vABV416, infect and grow equally well in PAMs but grow more slowly on CL2621 cells than does the virus rescued from BHK-21 cells transfected with intracellular LV RNA (compare Fig. 1E with Fig. 4B and E, and compare Fig. 1F with Fig. 4C and F). This intracellular LV RNA was isolated from CL2621 cells infected with LV4.2.1, which has been adapted for growth on CL2621 cells. To study the growth properties of vABV414 and vABV416 more thoroughly, growth curves were determined and were compared with those of wild-type LV that has been passed only on PAMs (TH) and with those of LV4.2.1 grown on CL2621 cells. CL2621 cells and PAMs were infected at a multiplicity of infection of 0.05 with these viruses. The culture media were harvested at various intervals, and virus titers were determined by end point dilution on macrophages. The growth rates of the two recombinant viruses did not differ in PAMs; the viruses grew equally well regardless of whether they were derived directly from BHK-21 or further passaged in PAMs (Fig. 5A). The titers of vABV416 derived from BHK-21 were higher than those of vABV416 derived from PAMs, when grown in CL2621 cells (Fig. 5B). Since a similar difference was not observed for vABV414, its biological significance remains unclear. The titers of the recombinant viruses (7.1 to 7.9

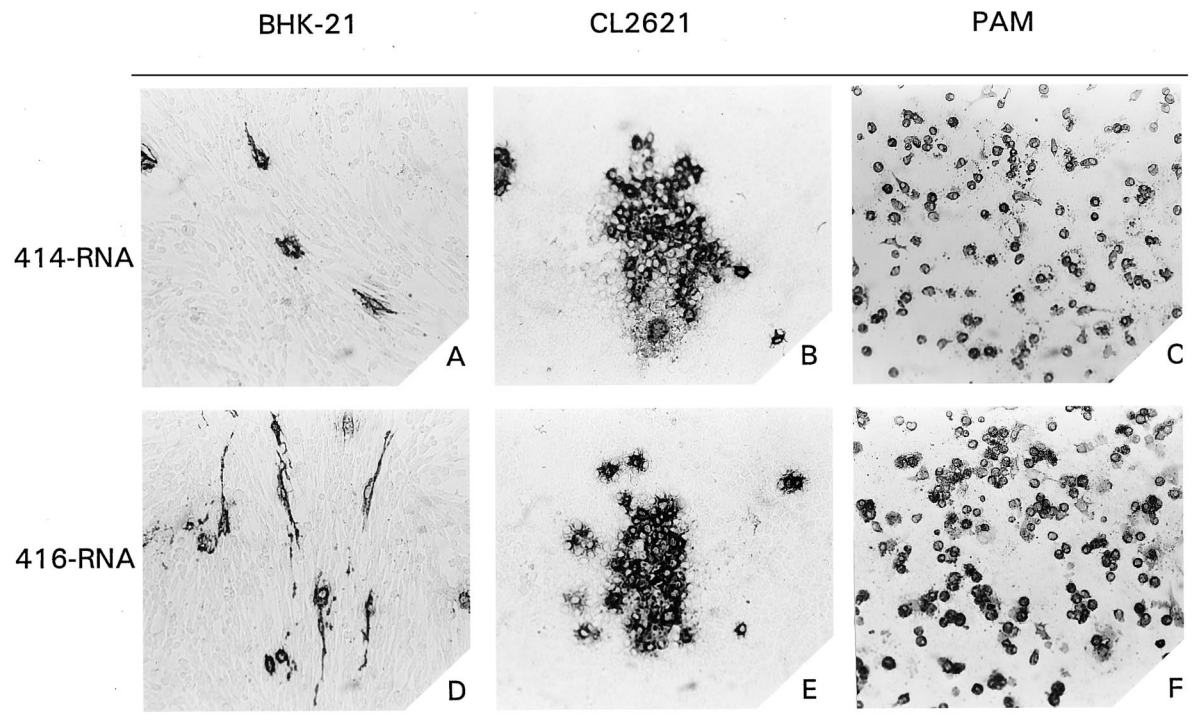

FIG. 4. Infectivity of transcripts from genome-length cDNA clones pABV414 and pABV416. BHK-21 cells were transfected with transcripts from pABV414 (A) or pABV416 (D) with lipofectin and were stained at 24 h posttransfection with N-specific MAb122.17 in IPMA. The supernatant of these transfected BHK-21 cells was used to infect CL2621 cells (B and E) and PAMs (C and F). These PAMs and CL2621 cells were stained after 2 and 3 days, respectively, with N-specific MAb122.17.

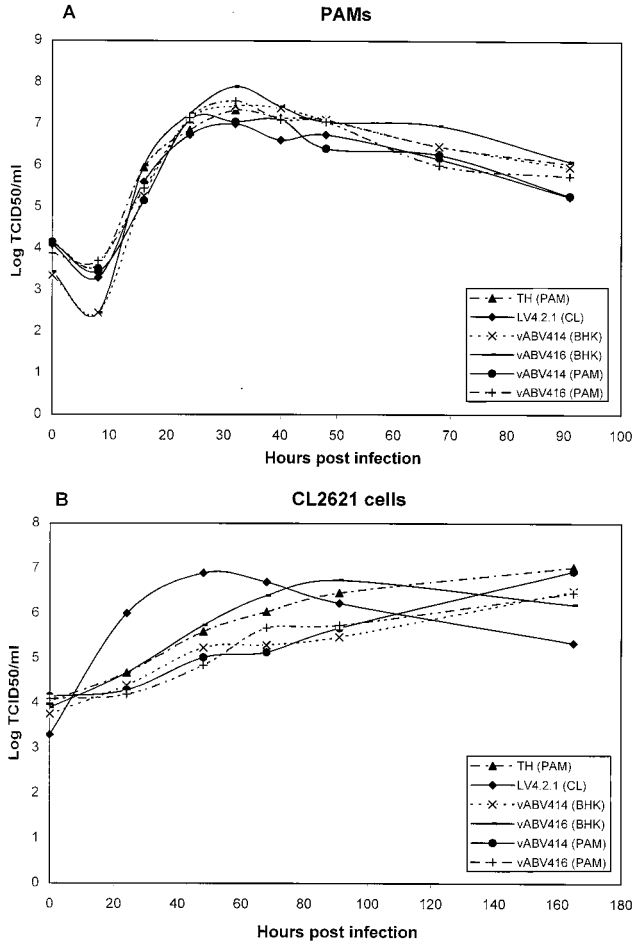

FIG. 5. Growth curves of LV wild-type virus TH, LV4.2.1, and recombinant viruses vABV414 and vABV416 in PAMs (A) and CL2621 cells (B). The recombinant viruses vABV414 and vABV416 produced in BHK-21 cells were either used directly (BHK) or used after multiplication in PAMs (PAM). The TH virus was prepared in PAMs (PAM), whereas LV4.2.1 was prepared in CL2621 cells (CL). The cell cultures were infected in duplicate with the indicated viruses at a multiplicity of infection of 0.05 and harvested at the indicated time points. Virus titers were determined on PAMs by end point dilution, as described previously (38). The mean titers of two independent experiments are shown for each time point. The pooled standard deviation was ±0.2 log $TCID_{50}$/ml.

$TCID_{50}$/ml) in PAMs peaked around 32 h postinfection, whereas the titers in CL2621 were lower and had not yet peaked even at 96 h postinfection. TH virus had similar growth characteristics to the recombinants. In contrast, the CL2621-adapted virus LV4.2.1 grew faster on CL2621 cells than did the viruses vABV414, vABV416, and TH (Fig. 5B). This confirmed the larger plaque size of this virus than that of vABV414, vABV416, and TH observed in CL2621 cells. In summary, these results demonstrate that the growth properties of the recombinant viruses are similar to those of the TH virus. This was expected, since the cDNA sequence used to construct the infectious clones was derived from the parental "nonadapted" TH virus.

Introduction of a genetic marker in the infectious clone of LV. To demonstrate that the genome-length cDNA clone can be used to generate mutant LV strains, a unique *Pac*I site was introduced directly downstream of the ORF7 gene by PCR-directed mutagenesis (Fig. 6A). When RNA transcribed from the genome-length cDNA clone pABV437 containing the *Pac*I site was transfected to BHK-21 cells and the supernatant was transferred to PAMs and CL2621 cells 24 h after transfection, infectious virus was produced. The rescued virus, vABV437, had similar growth properties in PAMs and CL2621 cells to the parental virus, vABV414 (data not shown). A specific region of approximately 0.6 kb [nucleotide 14576 to the poly(A) tail] was amplified by reverse transcription-PCR of viral RNA isolated from the supernatant of PAMs infected with vABV414 and vABV416. Digestion with *Pac*I showed that this restriction site was indeed present in the fragment derived from vABV437 but was absent from the fragment derived from vABV414 (Fig. 6B). Therefore, we were able to exclude the possibility of contamination with wild-type virus and hence we confirmed the identity of vABV437.

DISCUSSION

In this study, we generated for the first time an infectious clone of the LV isolate of PRRSV. In most instances, a pre-

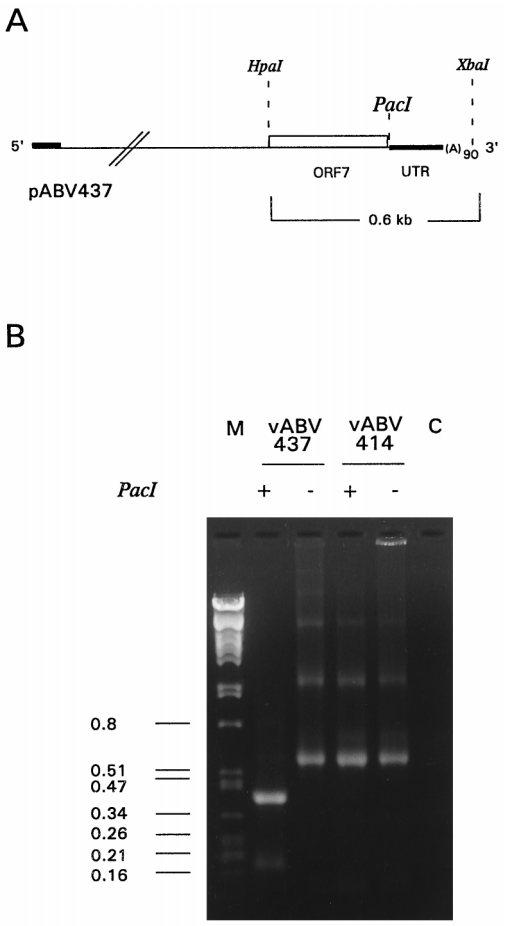

FIG. 6. (A) Introduction of a unique *Pac*I site in the infectious cDNA clone of LV. The *Pac*I site was created by PCR-directed mutagenesis, as described in detail in Materials and Methods. The cDNA fragment containing the *Pac*I site was exchanged in pABV414 by using its unique *Hpa*I and *Xba*I sites, which are indicated. This resulted in pABV437. UTR, untranslated region (B) Identification of the *Pac*I marker in recombinant virus vABV437. In vitro transcripts synthesized from pABV437 were transfected to BHK-21 cells. The supernatant containing the recombinant virus vABV437 was harvested 24 h after transfection. A fragment of approximately 0.6 kb was amplified by reverse transcription-PCR from RNA extracted from recombinant virus vABV437 and its parent vABV414. The PCR fragments were digested with *Pac*I and analyzed together with undigested fragments on a 1.5% agarose gel. Lane C represents a control PCR lacking template DNA; lane M shows the size markers in kilobases.

requisite for the construction of infectious clones is the identification of the sequences at the termini of the respective viral genome, which are usually crucial for replication of viral RNA (2, 7, 14, 30). In a previous report, it was shown that LV contains a poly(A) tail at the 3′ end (20). In the present study, the exact 5′ end of the LV genome was determined by ligation of an oligonucleotide with a specified sequence to a first-strand primer extension product and amplification by PCR. An extension of 10 nucleotides (ATGATGTGTA) with respect to the published sequence was found in several independent clones and was therefore assumed to represent the utmost 5′ end of the viral genome. Addition of this 10-nucleotide sequence to the 5′ cDNA sequences determined previously (20) formed a complete leader sequence of 221 nucleotides, which is similar in length to the leader of EAV (207 nucleotides) (9) and simian hemorrhagic fever virus (208 nucleotides) (40) but longer than the leader of lactate dehydrogenase-elevating virus (LDV) (155 nucleotides) (4). However, no significant identity exists between the leader sequences of these closely related arteriviruses.

The newly determined 5′-terminal sequence ligated to cDNA fragments covering the entire LV genome resulted in a genome-length cDNA clone of 15.2 kb, from which infectious transcripts could be produced. The infectious clone, described here, is to our knowledge the longest infectious clone of a positive-strand RNA virus thus far developed. Recently, an infectious clone of another arterivirus, EAV, has been reported which is 12.7 kb in length (34). A major problem with the generation of infectious clones is the instability of the virus sequences when cloned in high-copy-number plasmids in bacteria (15, 24, 28, 33). Although initial attempts to assemble a genome-length cDNA clone of LV in high-copy-number plasmid pGEM-4Z failed, the genomic cDNA fragment of 15,207 nucleotides remained stable in low-copy-number plasmid pOK12. However, in subsequent cloning experiments to introduce mutations in the genome-length cDNA clone, we often observed deletions and a decrease in the length of the poly(A) stretch. Our results showed that an extra nonviral G at the 5′ end as well as a nonviral CG at the 3′ end of transcripts of the genome-length cDNA clones of LV did not keep them from being infectious. However, transcripts of full-length cDNA of LV lacking a cap structure were not infectious (data not shown). This indicated that the cap structure is most probably essential for translation of the genomic RNA. No significant difference in the stability of the RNAs transcribed in vitro in the presence or absence of the cap was observed. The infectivity of genomic RNA or transcripts of infectious cDNA clones of other positive-strand RNA viruses has always been tested in cell lines that are susceptible to the virus. This was not possible for LV, due to the poor transfection efficiency of CL2621 cells and PAMs. However, transfection of transcripts from full-length cDNA clones, intracellular LV RNA, and virion RNA to BHK-21, a cell line which is not susceptible to infection with LV, resulted in the production and release of infectious virus, which could be rescued in CL2621 cells and PAMs. The specific infectivity of these transcripts by using Lipofectin was roughly 400 to 1,500 positive cells per μg of RNA. This specific infectivity was two- to fourfold higher in transfections performed by electroporation. It should be mentioned that on the basis of our experiments, the specific infectivity of the infectious transcripts cannot be compared exactly with that of authentic LV RNA, since (i) the intracellular LV RNA used for transfections contained only a very small fraction (roughly estimated to be 0.05 to 0.1%) of genomic LV RNA; (ii) the amount of genomic RNA isolated from virions, which was used for transfections was too small to allow accurate quantification; and (iii) the infectivity of RNA in BHK-21 cells was measured by detecting the expression of structural proteins in IPMA with LV-specific MAbs, which does not necessarily correlate with the production of infectious virus. However, it was clear that the specific infectivity of transcripts from infectious cDNA clones of LV was much lower than that of SFV cDNAs, which were used for comparison. Since infectious virus is produced when LV RNA is transfected into BHK-21 cells but not when these cells are inoculated with LV particles, we hypothesize that it is impossible for LV to enter BHK-21 cells because these cells lack the receptor of LV. A similar difference in the infectivity of LDV and its virion RNA in various cell types has been observed (12). The growth properties of the two recombinant viruses vABV414 and vABV416, which differ only at one amino acid at position 1084 in ORF1a (Pro versus Leu), were similar to those of the parental TH strain, from which the cDNA was originally derived. The wild-type nature of these viruses still has to be confirmed by experimental infection of pigs.

The unique *Pac*I site, which was introduced in the infectious clone downstream of the ORF7 gene at the 5′ end of the 3′ untranslated region of LV, will facilitate the insertion of mutations in the ORFs encoding structural proteins. The virus that could be recovered from this mutated full-length clone had the same growth properties as its parent. This demonstrates that the infectious clone of LV is an excellent tool for site-directed mutagenesis and is an important finding for future projects whose aim is to construct new live vaccines against PRRSV. It might be helpful to develop a so-called marker vaccine by mutagenesis of the genome, so that vaccinated pigs can be distinguished from field virus-infected pigs on the basis of differences in serum antibodies. In addition, the infectious clone of LV might open new opportunities for studies directed at the pathogenesis, host tropism, replication, and transcription of this virus. Arteriviruses and coronaviruses share a discontinuous transcription mechanism, which involves the generation of a nested set of subgenomic RNAs containing a common 5′ leader (27, 32). This specific transcription mechanism is a complex process, which is not yet fully understood. Studies by coronavirus virologists to elucidate the underlying mechanism of leader-primed transcription are restricted to analyses and site-directed mutagenesis of cDNAs of defective interfering RNAs, since the large size of the genome (28 to 30 kb) has impeded the construction of an infectious clone. The infectious clone of the LV isolate of PRRSV might provide a model system to study and unravel the intriguing mechanism of transcription and replication of arteriviruses and coronaviruses.

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