Nucleotide Sequence of Classical Swine Fever Virus Strain Alfort/187 and Transcription of Infectious RNA from Stably Cloned Full-Length cDNA

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The complete nucleotide sequence of the genome of classical swine fever virus (CSFV) strain Alfort/187 was determined from three cDNA libraries constructed by cloning of DNA fragments obtained from independent sets of reverse transcription and PCR. The cDNA fragments were then assembled and inserted downstream of a T7 promoter in a P15A-derived plasmid vector to obtain the full-length cDNA clone pA187-1. The first nucleotide of the CSFV genome was positioned at the transcription start site of the T7 promoter. Cleavage at an *SrfI* restriction site introduced at the exact 3' end of the cloned viral cDNA allowed the in vitro synthesis of full-length viral RNA by runoff transcription. This RNA proved to be infectious after transfection into porcine kidney cells. Infectivity was not increased after capping of the synthetic RNA. Virus recovered from transfected cells was titrated in porcine kidney cells by endpoint dilution using indirect immunofluorescence and a CSFV-specific monoclonal antibody. RNA transcripts generated from plasmid DNA isolated from bacteria which had been cultured and cloned 10 times remained infectious, indicating that the full-length clone is stable in bacterial cells. A silent point mutation introduced at position 11842 of the genome was retained in the recombinant virus recovered from transfected cells. An infectious chimeric construct was obtained by replacing a 696-bp fragment in pA187-1 with the corresponding cDNA fragment from the CSFV strain CAP. The stably cloned full-length CSFV cDNA allows site-specific mutagenesis of the viral genome and thus will be useful for detailed molecular characterization of the virus as well as for studies of viral pathogenesis.

Classical swine fever is an economically important, highly contagious disease of pigs caused by the classical swine fever virus (CSFV), formerly referred to as hog cholera virus. Together with bovine viral diarrhea virus (BVDV) and border disease virus, CSFV belongs to the genus *Pestivirus* within the family *Flaviviridae* (48). Its genome is a single positive-stranded RNA of approximately 12.3 kb. The single large open reading frame of pestiviruses codes for a polyprotein of approximately 4,000 amino acids, which is processed co- and posttranslationally by host cell as well as virus-encoded proteases to mature structural and nonstructural proteins (reviewed in reference 29). A large number of CSFV strains ranging from highly virulent to avirulent have been isolated. Although host factors may play a role in the outcome of the disease in pigs, there is strong evidence for viral determinants responsible for the variable virulence observed between different strains (6, 46). In cell culture, most of the CSFV strains and isolates grow without inducing a cytopathic effect. For the CAP strain (24) and for CSFV isolates containing defective interfering particles, cytopathogenicity has been reported (26).

A full-length cDNA clone allowing the recovery of infectious virus would be an invaluable tool to approach a series of open questions, including the functional characterization of viral gene products, the analysis of virus and RNA replication, the determination of virulence factors, and also the elucidation of mechanisms involved in viral pathogenesis. This has been possible for many positive-strand RNA viruses (reviewed in reference 2). At recent scientific meetings, it was reported that infectious RNA was successfully transcribed from a full-length

cDNA copy of the C strain of CSFV (30) and of the NADL strain of BVDV (47).

The complete nucleotide sequence of CSFV has been published for strains Alfort (25), now referred to as Alfort Tübingen (21), Brescia (31), and, more recently, ALD and GPE⁻ (15). The virulent Alfort/187 strain was isolated from a pig that died after experimental infection with the original Alfort strain (1, 6). On the basis of animal experiments performed at our institute and according to the criteria of van Oirschot (46), Alfort/187 must be classified as a moderate-virulent strain (our unpublished data). Data obtained previously from partial sequencing of the Alfort/187 genome (14, 32, 37, 38) indicated that Alfort Tübingen and Alfort/187 were only distantly related strains.

We constructed three independent cDNA libraries of the Alfort/187 genome and determined the complete nucleotide sequence, which was then compared with the corresponding data published for other pestiviruses. We also report the assembly of a full-length cDNA clone in a P15A-derived plasmid vector and demonstrate its stability in bacterial cells. Progeny virus could be recovered from porcine kidney cells transfected with in vitro transcripts derived from the linearized plasmid DNA. Also, a chimeric virus genome was constructed in vitro from cloned cDNA of two distinct virus strains. The virus recovered from cells transfected with the respective RNA had retained the chimeric sequence. This finding shows that the full-length clone that we have established can be used for genetic engineering of the viral genome.

MATERIALS AND METHODS

Cells and viruses. Porcine kidney cell line PK-41 (IFFA-Mérieux, Lyon, France) was grown in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing 5% horse serum (Sebak GmbH, Aidenbach, Germany). The swine kidney cell line SK-6 (17), kindly provided by M. Pensaert, Faculty of

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FIG. 1. Schematic representation of the original cDNA library (fragments J, G, C, M, E, Z9, Z13, P1, P2, PHj, H, Q, R, T, and U) and of the strategy applied for the assembly of the full-length Alfort/187 cDNA clone. Positions relative to the genome map and important restriction sites used in the construction procedure are shown. 5'-NCR and 3'-NCR, 5' and 3' noncoding regions.

Veterinary Medicine, Gent, Belgium, was seeded in minimal essential medium (MEM) containing Hanks salts (Gibco BRL) and 10% (vol/vol) horse serum, and medium was replaced 3 h later by MEM containing Earle's salts (Gibco BRL) and 5% (vol/vol) horse serum.

CSFV strain Alfort/187 (6) batch 73 was kindly provided by B. Liess, Veterinary School, Hannover, Germany. PK-41 cells were infected with Alfort/187, and cells from the second passage were submitted to three cycles of freeze-thawing. The supernatant was clarified by centrifugation at $1,000 \times g$ for 15 min and stored at -70°C . The clarified cell culture extract had a titer of 1.6×10^6 50% tissue culture infective doses (TCID₅₀)/ml on PK-41 cells. The CAP strain of CSFV had been isolated from the persistently infected porcine kidney cell line IB-RS2 (24) and was provided by H. Laude, Joey-en-Josas, France.

Recombinant DNA procedures. DNA cloning was performed by standard procedures (40). Restriction endonucleases were purchased from New England Biolabs, Gibco BRL, Boehringer Mannheim, and Stratagene. T4 DNA ligase was obtained from Promega. DNA fragments were purified from low-melting-temperature agarose gels by using the Wizard PCR Preps DNA purification system (Promega) or from standard agarose gels by electroelution by using a BIOTRAP electroeluter (Schleicher & Schuell, Dassel, Germany). Unless specified, the plasmid constructs were amplified in *Escherichia coli* XL1-Blue cells (Stratagene).

RNA isolation and cDNA synthesis. RNA of CSFV Alfort/187 was obtained by lysis of the clarified extract from infected PK-41 cells in 20 mM Tris-HCl (pH 7.5)–5 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–0.2 mg of proteinase K per ml at 45°C for 30 min and subsequent phenol-chloroform extraction and ethanol precipitation. Total RNA was used for coupled reverse transcription (RT) and PCR for 30 cycles as previously described (38). For each reaction, the amount of RNA used corresponded to 50 μl of the clarified cell culture extract. A set of reverse and forward oligonucleotide primers was designed on the basis of either the consensus sequence of Alfort Tübingen (25) and Brescia (31) or our own sequencing data for Alfort/187. Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Overlapping DNA fragments covering the complete genome (except the 5' and 3' ends) were amplified in at least three independent sets of RT-PCR and ligated either into plasmid p123T (28) or into plasmid pBluescriptIIISK+ (Stratagene), using unique restriction sites introduced into the forward and reverse primers.

Analysis of the 5' and 3' ends. Infected cells were submitted to three cycles of freeze-thawing, and supernatant was clarified twice by centrifugation at $1,000 \times g$ for 15 min. To increase the ratio between viral RNA and cellular RNA, virions were pelleted at $28,000 \times g$ for 3 h at 4°C , and RNA was extracted as described above.

For DNA amplification across joined 5' and 3' ends, RNA concentrated from 1 ml of extract of infected cells was ligated at 37°C for 6 h in a 10- μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM β -mercaptoethanol, 1 mM ATP, 10 μg of bovine serum albumin per ml, 1 mM hexamine cobalt trichloride, 10% (vol/vol) dimethylsulfoxide, and 10 U of T4 RNA ligase

(New England Biolabs). Two microliters of the ligation reaction mixture was then used directly in a 50- μl RT-PCR (38) containing 50 pmol of antisense primer PEST2 (49), which is complementary to nucleotides 383 to 363, and 50 pmol of sense primer JL1 (5' GTT ACT GCA GCC GCC AGT AGG ACC CTA TTG 3'), homologous to nucleotides 12095 to 12114. Ten microliters of the RT-PCR mixture was separated on an agarose-ethidium bromide gel. A second, seminested PCR of 30 cycles was performed after piercing the gel at the expected fragment length of 597 bp and rinsing the tip in PCR buffer containing primers JL1 and GR1 (5' AAA CTG CAG CCC AGT TCG GCC GTC 3'), complementary to nucleotides 95 to 79. Both JL1 and GR1 had additional nucleotides at their respective 5' ends to obtain a *Pst*I restriction site. The 316-bp amplification product was digested with *Pst*I, electroeluted from a 1.5% agarose gel, and ligated into pBluescriptIIISK+ (fragment J; Fig. 1).

The 5' end was amplified by ligation-anchored PCR mainly as described by Trout et al. (45). For this purpose, anchor oligonucleotide RT7G (5' CTA TAG TGA GTC GTA TTA AGA TCT GTC GAC GCG TC 3') and its complementary oligonucleotide LT7G, containing a *Sal*I restriction site in addition to a T7 promoter, were designed. First-strand cDNA was synthesized at 37°C for 60 min in a 25- μl reaction mixture containing RNA derived from 1 ml of extract of infected cells, 40 pmol of antisense primer MR4 (5' CTC GCT GCT CCC TGT CT 3'), complementary to nucleotides 1770 to 1754, 25 mM Tris-HCl (pH 8.4), 75 mM KCl, 2.5 mM MgCl₂, 250 μM each deoxynucleoside triphosphate, 12.5 U of RNase inhibitor (Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). RNA was hydrolyzed by heating at 65°C for 30 min in 200 mM NaOH. The reaction was neutralized with 200 mM HCl and diluted with H₂O to a final volume of 500 μl , and cDNA was purified and concentrated by centrifugation through a Microcon 100 spin column (Amicon). Anchor oligonucleotide RT7G was phosphorylated at the 5' end and blocked at the 3' end with ddATP and terminal transferase (Boehringer Mannheim), and 10 pmol was ligated to the purified first-strand cDNA with T4 RNA ligase under the same conditions as described above.

One microliter of the ligation reaction was added directly to 50 μl of PCR mix containing 50 pmol each of primers LT7G and MR4, and PCR was performed for 30 cycles. Reamplification for 30 cycles with the same primers was performed after piercing the gel at the expected fragment length of 1,805 bp and rinsing the tip in the PCR mix. The 1,805-bp fragment was electroeluted from the agarose gel, cut with *Sal*I and *Aat*II, and ligated in pAMP-2 (see below).

Nucleotide sequencing. Double-stranded plasmid DNA was prepared by using the Wizard Maxipreps DNA purification system (Promega) and sequenced by the dideoxy-mediated chain termination method (41) with incorporation of [³⁵S]dATP, using either a Sequenase version 2.0 DNA Sequencing kit (United States Biochemical) or a Sequitherm Long-Read Cycle Sequencing kit (Epicentre Technologies). The full-length constructs were sequenced on a LI-COR model 4000L automated DNA sequencer (LI-COR, Lincoln, Neb.), using a set of 23 sense and 23 antisense fluorescence-labeled primers (MWG-Biotech GmbH, Ebersberg, Germany) and a Sequitherm Long-Read Cycle Sequencing

kit. Twenty-two sense and 22 antisense oligonucleotides were designed from the Alfort/187 sequence, and two primers were designed from the flanking regions in the backbone vector. Primers between 20 and 22 nucleotides in length were designed and selected for similar annealing temperatures, using the Oligo Primer Analysis version 4.0 software (National Biosciences, Plymouth, Minn.). For further analysis of the nucleotide sequences obtained, the GeneWorks version 2.4 software (IntelliGenetics, Mountain View, Calif.) was used.

Construction of the full-length cDNA clone. For assembly of the full-length cDNA, cloned RT-PCR fragments having the Alfort/187 consensus sequence determined from at least three independent libraries were selected. The two fragments T7G1P and HU3'SrfI (Fig. 1), representing approximately one half of the genome each, were assembled separately in pBluescriptISK+ in T7 and T3 orientations, respectively. The original cDNA fragments, their assembly, and important restriction sites involved in the construction are shown in Fig. 1. In the following description, CSFV-specific restriction endonuclease sites are given with their positions (indicated by superscript numbers) in the viral genome.

For assembly of the 5' half of the genome (fragment T7G1P), fragments M and E released from pAM4 by *Pst*I-*Bsa*I²⁵²⁰ digestion and from pAE19 by *Bsa*I²⁵²⁰-*Bam*HI digestion were ligated in vitro and cut with *Pst*I and *Bam*HI, and the ME fragment was inserted in pBluescriptISK+, resulting in pAME-1. Part of E was obtained from pAE19 by restriction with *Sst*I²⁶⁸⁶-*Apo*I³⁴⁶², and the Z13 fragment was cut out of pAZ13 with *Apo*I³⁴⁶²-*Hind*III. The two fragments were ligated in vitro, recut with *Sst*I-*Hind*III, and inserted into pBluescriptISK+, yielding pAEZ13-2 (EZ13 in Fig. 1). Plasmid pAP2 (P2 in Fig. 1) was digested with *Pst*I and *Nhe*I⁵⁰⁵⁷ and ligated to the *Pst*I⁴¹⁶⁶-*Nhe*I⁵⁰⁵⁷ fragment of pAZ9 (Z9 in Fig. 1), resulting in pAZ9P-3 (Z9P in Fig. 1). Plasmid pAMP-2 carrying the MP fragment was constructed by ligation of the *Nsi*I³²⁴²-*Bgl*I⁴⁶³² fragment of pAEZ13-2 and the *Bgl*I⁴⁶³²-*Bam*HI⁶⁴³⁷ fragment of pAZ9P-3 in pAME-1 cut with *Nsi*I³²⁴² and *Bam*HI⁶⁴³⁷. Plasmid pAMP-2 was then cut with *Sal*I and *Aat*II¹⁵⁴⁰ and ligated with the 1,805-bp *Sal*I-to-*Aat*II¹⁵⁴⁰ fragment obtained by anchor-ligated PCR (see above), resulting in pAT7GP2-1/5 (T7GP2 in Fig. 1). Finally, a new RT-PCR fragment was synthesized from viral RNA by using primer LT7/1-21 (5' GAC GCG TCG ACA GAT CTT AAT ACG ACT CAC TAT AGT ATA CGA GGT TAG TTC ATT C 3'), containing a *Sal*I restriction site and the T7 promoter immediately followed by the first 21 nucleotides of the Alfort/187 genome, and primer CR2 (5' CTA ATC CAC TTT AGG GTT 3'), which is complementary to nucleotides 840 to 823. This PCR product was then used to replace the *Sal*I-to-*Aat*II¹⁵⁴⁰ fragment of pAT7GP2-1/5. The nucleotide sequence between *Sal*I and *Aat*II¹⁵⁴⁰ was analyzed, and the construct carrying T7G1P with the correct sequence was named pAT7G1P-4.

For assembly of the 3' half of the genome (fragment HU3'SrfI), the R fragment released from pAR2 with *Pst*I⁸⁶⁷⁶ and *Hind*III was ligated to pAQ29 (Q in Fig. 1) cleaved with *Pst*I⁸⁶⁷⁶ and *Hind*III, yielding pAQR-7 (QR in Fig. 1). The T clone pAT32 was cut with *Sph*I¹¹¹⁶² and *Kpn*I and ligated to the U fragment obtained from pAU6 by *Sph*I¹¹¹⁶²-*Kpn*I digestion, and the resulting TU construct was named pATU-1. Plasmid pAQR-7 was then partially cut with *Sst*I, and linear DNA that had been cut only at one *Sst*I site was isolated and digested with *Kpn*I. The 5,535-bp *Sst*I⁹⁹⁷⁶-to-*Kpn*I plasmid DNA was then used to construct pAQU-7 (QU in Fig. 1) by ligation to the *Sst*I⁹⁹⁷⁶-*Kpn*I fragment of pATU-1. The H fragment obtained after cleavage of pAH18 with *Bam*HI⁶⁴³⁷ and *Nhe*I⁷³⁷⁹ was subsequently inserted into the large *Nhe*I⁷³⁷⁹-to-*Bam*HI fragment of pAQU-7, yielding pAHU-2 (HU in Fig. 1). To introduce a unique *Srf*I site at the 3' terminus of the genome, a RT-PCR fragment was amplified from the original RNA by using antisense primer 3'URSx (5' TTC CTC GAG CCC GGG CCG TTA G 3'), complementary to the last 10 nucleotides of the genome and containing *Srf*I and *Xho*I restriction sites, and sense primer UL3 (5' TCA ACT ACT TAC TAC TA 3'), homologous to nucleotides 11645 to 11661. The 654-bp U3'SrfI fragment was cut with *Hind*III¹⁷²⁹ and *Xho*I and used to replace the corresponding fragment in pAHU-2. The sequence of the inserted PCR fragment was verified by nucleotide sequencing, and a plasmid which had acquired a silent mutation (T instead of C) at position 11842 was selected and named pAHU(3'SrfI)-24 (HU3'SrfI in Fig. 1).

As a vector to assemble the complete genome, plasmid pACYC177 (4) was modified by replacing the 1,615-bp *Aat*II-to-*Xho*I fragment by the 259-bp *Aat*II-to-*Xho*I polylinker cassette of pSL1180 (Pharmacia). The resulting 2,584-bp plasmid was multiplied in *E. coli* HB101 and termed pACNR1180. Plasmid pAT7G1P-4 was cut with *Sal*I and *Bam*HI⁶⁴³⁷, and the 6,464-bp fragment was ligated between *Sal*I and *Bam*HI of pACNR1180. The resulting plasmid pNRAT7G1P-8 was cut with *Bam*HI⁶⁴³⁷ and *Xho*I and ligated to the 5,866-bp *Bam*HI⁶⁴³⁷-*Xho*I fragment of pAHU(3'SrfI)-24. The selected full-length clone was named pNRA24-12. Plasmid pAKpnBam-1 (AKpnBam in Fig. 1) was constructed by ligation of the 179-bp *Kpn*I⁴⁴⁵³-*Bgl*I⁴⁶³² fragment of pAZ13 and the 425-bp *Bgl*I⁴⁶³²-*Nhe*I⁵⁰⁵⁷ fragment of pAZ9 into pAP2 cut with *Kpn*I and *Nhe*I⁵⁰⁵⁷. The nucleotide sequence between *Kpn*I⁴⁴⁵³ and *Bam*HI⁶⁴³⁷ of pAKpnBam-1 was verified, and the *Kpn*I⁴⁴⁵³-*Bam*HI⁶⁴³⁷ fragment of pNRA24-12 was replaced by the corresponding fragment of pAKpnBam-1. The final full-length clone from which infectious RNA could be transcribed was termed pA187-1.

A chimeric cDNA clone, pA187-C1-14, was constructed by replacing the *Eag*I¹⁸²-*Bsp*DI⁷⁷⁸ fragment of pA187-1 by the corresponding fragment of pCAPG30, a pBluescriptISK(+)-derived cDNA clone of CSFV strain CAP.

Stability of the plasmid clone pA187-1. The stability of pA187-1 in bacteria was assessed by several cloning and growth cycles in *E. coli* XL1-Blue cells. Bacteria

from the original colony were grown to late log phase by overnight shaking at 37°C in LB medium supplemented with ampicillin. Bacteria from the saturated culture were then transferred to LB agar plates containing ampicillin. After overnight incubation, a single colony was selected and used to inoculate fresh medium. This procedure was repeated 10 times. After 5 and 10 passages, plasmid DNA was purified and analyzed by restriction digestion. In vitro-transcribed RNA from the linearized plasmid DNA was tested for infectivity as described below.

In vitro transcription. Plasmid containing the full-length viral cDNA was linearized with *Srf*I (Stratagene), treated with proteinase K (Sigma), and purified by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in diethyl pyrocarbonate-treated H₂O to a final concentration of 500 ng/μl. In vitro transcription was performed by using a MEGascript T7 kit (Ambion Inc., Austin, Tex.). As instructed by the supplier, a 20-μl reaction mixture containing 1.5 μg of linearized plasmid DNA, 1× transcription buffer, 7.5 mM each ATP, CTP, GTP, and UTP, and 2 μl of T7 polymerase-RNase inhibitor mix was incubated at 37°C. For synthesis of capped RNA, the GTP concentration was reduced to 1.5 mM and transcription was performed in the presence of 6 mM m⁷G(5')ppp(5')G. For radiolabeling, transcription was carried out in the presence of 20 μCi of [α-³²P]UTP (Amersham). For removal of template DNA, the reaction mixture was incubated for 15 min at 37°C in presence of 2 U of DNase I. Unlabeled runoff transcripts were either used directly for electroporation or stored in aliquots at -70°C. Alternatively, RNA from transcription reactions was purified by phenol-chloroform extraction and precipitation in the presence of 500 mM ammonium acetate, 10 mM EDTA, and 1 volume of isopropanol. RNA was resuspended in diethyl pyrocarbonate-treated H₂O and used for transfection and for further analysis. Photometric quantification of total runoff transcript was carried out in a GeneQuantII photometer (Pharmacia). A transcription reaction mixture extracted and precipitated immediately after addition of the T7 polymerase served for background subtraction. For calculation of the percentage of apparent full-length transcripts, [α-³²P]UTP-labeled transcripts were separated by formaldehyde agarose gel electrophoresis, and the wet gel was sealed in a plastic bag. The numbers of counts of the full-length and the total transcripts were determined by electronic autoradiography using the InstantImager 2024 Electronic Autoradiography system (Packard Instrument Company, Meriden, Conn.).

Analysis of RNA. Northern (RNA) blotting was performed essentially as described by Sambrook et al. (40). Briefly, RNA was separated by electrophoresis on 1% denaturing formaldehyde agarose gels and blotted onto positively charged nylon membranes (Boehringer Mannheim) by vacuum transfer in the presence of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and cross-linked by irradiation with 254-nm light, using a Stratilinker UV cross-linker (Stratagene). A ³²P-labeled antisense RNA probe was prepared by transcription of *Bam*HI-linearized pATU-1 DNA with T7 polymerase in the presence of 0.5 mM each ATP, GTP, and CTP, 15 μM UTP, and 20 μCi of [α-³²P]UTP (800 Ci/mmol). The membrane was incubated at 55°C for 4 h in prehybridization-hybridization solution (5× Denhardt's reagent, 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH 7.7], 1% SDS, 100 μg of herring sperm DNA per ml, 50 μg of yeast RNA per ml, 50% formamide) and then incubated overnight at 55°C in fresh solution supplemented with the labeled probe. The blot was washed three times in 1× SSPE-0.5% SDS and once in 0.1× SSPE-0.5% SDS. All washing steps were performed at 60°C for 30 min. Bands were visualized by electronic autoradiography and by exposure to X-ray film.

Transfection of porcine cells and virus rescue. SK-6 and PK-41 cells were transfected either by lipofection with DOTAP transfection reagent (Boehringer Mannheim) or by electroporation using a Gene Pulser apparatus with Capacitance Extender (Bio-Rad).

For liposome-mediated transfection, 2.5 μg to 5 μg of RNA and 50 μg of DOTAP were incubated in a total volume of 200 μl of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4) for 5 min at room temperature. After dilution with 5 ml of OptiMEM 1 (Gibco BRL), the DOTAP-RNA mixture was added to the SK-6 or PK-41 cell monolayer that had been incubated in OptiMEM 1 for at least 4 h. After 16 h, OptiMEM 1 was replaced by the appropriate cell culture medium supplemented with 5% horse serum.

For electroporation, monolayers of confluent SK-6 or PK-41 cells were trypsinized, washed once with growth medium, and resuspended at a density of 10⁶ cells per ml in Hanks MEM and 10% horse serum or DMEM and 10% horse serum, respectively. Variable amounts of runoff transcripts, either purified or as crude transcription reaction, were added to 0.8 ml of the cell suspension and immediately transferred to 0.4-cm electroporation cuvettes (Bio-Rad). Electroporation was carried out at room temperature by applying two successive pulses at 300 V (750 V/cm) and 960 μF. Cells were allowed to recover for 5 min and then resuspended in 8 ml of Hanks MEM with 10% horse serum for SK-6 cells and DMEM supplemented with 5% horse serum for PK-41 cells. A total of 4 × 10⁶ cells were seeded in a 75-cm² flask for later virus recovery, and 8 × 10⁵ cells were seeded per well in 35-mm wells for indirect immunofluorescence assays. For SK-6 cells, the medium was replaced 16 h later by MEM containing Earle's salts and 5% horse serum.

Sixty-five hours after transfection, cells were either fixed and stained by indirect immunofluorescence (see below) or harvested by resuspension in the growth medium and lysed by three cycles of freeze-thawing, and the supernatant was

FIG. 2. Comparison of the 45 5'-terminal nucleotides of the genome of Alfort/187 with the corresponding sequences of other CSFV and BVDV strains. The highly conserved 5' ends (box I) and their complementary inverted sequences (box II) are shown. Dots represent nucleotides that are identical to those found for Alfort/187, and dashes indicate gaps in the alignment or nonavailable sequence data (5' ends of Alfort Tübingen and Brescia).

clarified by centrifugation at $1,000 \times g$ for 10 min. Virus was passed several times on cell monolayers with RNase A and DNase I treatment between each passage: 20 μ l of cleared supernatant was incubated for 1 h at 37°C in the presence of 2 U of DNase I (Ambion Inc.) and 10 μ g of RNase A (Sigma), diluted in serum-free medium, and used directly to inoculate a fresh monolayer. After 2 h, cells were washed and incubated for 65 h in the appropriate medium containing 2% horse serum.

Efficiency of both liposome-mediated transfection and electroporation was monitored by using 5 μ g of the β -galactosidase-expressing plasmid pSV- β -galactosidase (Promega). Cells were stained for β -galactosidase expression 24 h after transfection by fixing with 0.05% glutaraldehyde for 10 min at room temperature, threefold washing with phosphate-buffered saline (PBS), and, finally, overlaying with PBS containing 1 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Boehringer Mannheim) per ml, 5 mM potassium ferriicyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$. The percentage of blue cells was estimated by light microscopy.

Infectivity of runoff transcripts was determined by liposome-mediated transfection of SK-6 and PK-41 cells with 10-fold dilutions of *in vitro* transcripts. Each dilution was distributed in 5 wells of a 24-well plate, and indirect immunofluorescence was performed 65 h later. The titer based on total RNA was expressed in TCID₅₀ per microgram of RNA and extrapolated from transfection of 2.5 ng to 2.5 pg of RNA per well.

Titration of virus. At different time points after transfection or infection, cells were resuspended in the growth medium and lysed by three cycles of freeze-thawing, and the supernatant was clarified by centrifugation at $1,000 \times g$ for 10 min. Tenfold dilutions of the clarified supernatant were distributed to each of 5 wells of a 96-well plate seeded with SK-6 cells. The titer was calculated after monitoring of CSFV-specific infection by using an indirect immunofluorescence assay 48 h postinfection.

Indirect immunofluorescence assay. Transfected or infected cells were washed once with PBS and fixed with ethanol at -20°C. Cells were then incubated for 30 min at 37°C in the presence of CSFV-specific monoclonal antibody HC/TC 26, directed against glycoprotein E2 (13, 38) and provided by W. Bommeli AG (Bern, Switzerland). After being washed with PBS, cells were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (DAKO A/S, Glostrup, Denmark) for 30 min at 37°C. Finally, cells were washed and analyzed by fluorescence microscopy.

Nucleotide sequence accession number. The complete Alfort/187 nucleotide sequence has been deposited in GenBank and assigned accession number X87939.

RESULTS

Complete nucleotide sequence of the Alfort/187 genome. The complete nucleotide sequences of two cDNA libraries of Alfort/187 obtained from independent RT-PCR were determined by sequence analysis of both DNA strands. Nineteen single nucleotide differences were found by comparing the data from the two complete libraries. The sequence at these positions was determined from a third library, and the nucleotide that was found to be identical at a particular position in two libraries was regarded as the correct one. When RT-PCR was performed across 5'-to-3'-end-ligated viral RNA, a DNA fragment of the expected length was obtained and cloned. Analysis of independent clones revealed the same sequence for the joined 5' and 3' termini. The exact position of the 5' end was determined by sequence analysis of clones obtained from ligation-anchored PCR, and it was concluded that the 5' and 3' noncoding regions were 373 and 228 bases long, respectively. Hence, the genomic RNA of Alfort/187 is 12,298 nucleotides in

length, contains a single large open reading frame coding for 3,898 amino acids, and has the same size as the genomes of the recently published ALD and GPE⁻ strains (15). When the sequence was compared with the data published for Alfort Tübingen and Brescia, 9 and 12 additional nucleotides, respectively, were found at the 5' end. Nucleotides 1 to 9 of Alfort/187 were conserved in CSFV strains ALD and GPE⁻ and in BVDV strains NADL (5), Osloss (33), and SD-1 (8) (Fig. 2, box I). RNA folding analysis suggested that these nine nucleotides form a stem-loop with nucleotides 29 to 31 (Fig. 2, box II). The overall identity to other published complete CSFV sequences is shown in Table 1.

Construction of the full-length Alfort/187 cDNA clone pA187-1. The original cDNA library and the assembled fragments are shown in Fig. 1. The 5' and 3' half of the Alfort/187 cDNA, defined by a naturally occurring unique *Bam*HI⁶⁴³⁷ site, were assembled separately in pBluescriptIISK⁺. By using RT-PCR, a bacteriophage T7 RNA polymerase promoter was placed immediately upstream of the Alfort/187 cDNA, the first nucleotide of the genome corresponding to the start site of RNA transcription (Fig. 3B). The plasmid carrying the T7 promoter fused to the first 6,437 bp of the Alfort/187 sequence was termed pAT7G1P-4. The four nucleotides GGGC were added at the 3' end of the genome in order to form a unique *Srf*I restriction site (GCCCCGGGC), allowing blunt-end cleavage at the exact 3' terminus of the genomic sequence (Fig. 3C). For this purpose, a new RT-PCR fragment was synthesized and used to replace the corresponding *Hind*III¹¹⁷²⁹-to-*Xho*I fragment of pAHU-2. Sequence analysis of 11 cloned fragments carrying the *Srf*I site and originating from three independent RT-PCR revealed one single clone, pAHU(3'*Srf*I)-24, that had acquired a unique silent mutation (T instead of C at position 11842) considered an error generated by PCR. This mutation served as a marker for later recognition of virus derived from the full-length cDNA clone.

Bacteria containing plasmid pAT7G1P-4 grew to very small colonies on LB agar. In liquid LB medium, bacteria from these colonies grew very slowly, and saturation of the culture was

TABLE 1. Overall nucleotide and amino acid sequence identities between Alfort/187 and published CSFV sequences

Sequence	% identity to Alfort/187			
	Alfort Tübingen ^a	Brescia ^b	ALD ^c	GPE ^{-c}
Nucleotide	86.2	94.8	98.6	99.2
Amino acid	93.6	97.6	99.4	99.2

^a Reference 25.

^b Reference 31.

^c Reference 15.

FIG. 3. Details of the regions flanking the 5' and 3' end of the Alfort/187 cDNA insert in the full-length clone pA187-1. (A) Circular map of pA187-1 showing relative positions of the Alfort/187 cDNA, ampicillin resistance gene, and P15A origin of replication. Locations of relevant unique restriction sites and of the T7 promoter are indicated. (B) Nucleotide sequence flanking the 5' end of the Alfort/187 cDNA. The RNA transcription start site (+1) corresponds to the 5' end of the Alfort/187 cDNA. (C) The *XhoI* and *SrfI* restriction sites and the resulting runoff transcript with the authentic 3' end of the genome.

reached only after shaking for approximately 36 h at 37°C. Colonies containing construct pAHU(3'SrfI)-24 behaved similarly on solid medium but grew to saturation in liquid medium within approximately 24 h.

As the backbone vector for the assembly of the full-length construct, plasmid pACYC177 was modified by replacing most of the kanamycin resistance gene with a polylinker cassette derived from pSL1180. Thus, the resulting cloning vector, pACNR1180, was 2,584 bp in length and contained the P15A origin of replication, the ampicillin resistance gene, and the polylinker. The *SalI*-to-*BamHI* fragment of pAT7G1P-4 carrying the T7 promoter followed by the 5' half of the genomic cDNA was subcloned into the polylinker sequence of pACNR1180 in both orientations, either between *SalI* and *BamHI* or between *XhoI* and *BamHI*. Clones with the insert in the *SalI*-to-*BamHI* orientation were stable, whereas constructs in the opposite orientation had acquired major deletions or insertions which were not further characterized. Finally, the *BamHI*-to-*XhoI* fragment of pAHU(3'SrfI)-24 containing the 3' half of the genome was ligated into the first of the two latter constructs to obtain plasmids carrying a complete copy of the viral cDNA. Six of the seven plasmids tested which had the T7 promoter fused to the full-length Alfort/187 cDNA between *SalI* and *XhoI* sites of pACNR1180 apparently had neither deletions nor insertions when analyzed by multiple restriction digestion (not shown). Furthermore, all colonies grew normally on solid medium, and the respective cultures reached saturation in liquid medium within 16 h. One full-length plasmid clone, pNRA24-12, was selected for nucleotide sequence analysis, and the complete sequence was determined from both strands by using automated sequencing. A 4-bp duplication was found at the *NheI*⁵⁰⁵⁷ restriction site that had been used at an earlier stage of the construction procedure for ligation of cDNA fragments Z9 and P2 (Fig. 1). This duplication was present in all six constructs analyzed. No other mutation, except the silent marker mutation at position 11842, was found in pNRA24-12. To remove the duplication, the *KpnI*⁴⁴⁵³-to-*BamHI*⁶⁴³⁷ fragment of pNRA24-12 was replaced by the corresponding insert of pAKpnBam-1. Bacteria containing plasmids with the corrected sequence did not show altered growth characteristics, and the expected multiple restriction pattern was retained. The full-length Alfort/187 cDNA clone selected

for further experiments was termed pA187-1 and is shown in Fig. 3A.

In vitro transcription. In vitro runoff transcripts of pA187-1 linearized with *SrfI* were separated on denaturing formaldehyde agarose gels after DNase I digestion and blotted on nylon membranes. Viral RNA extracted from cleared supernatant of infected cells served as size marker. RNA was detected by hybridization with a ³²P-labeled Alfort/187-specific antisense RNA obtained by in vitro transcription from plasmid pATU-1. Runoff transcripts and viral RNA were indistinguishable in length (Fig. 4), suggesting that the in vitro-transcribed RNA corresponded to genomic viral RNA. The absolute amount of full-length wild-type (wt) viral RNA in lane 1 of Fig. 4 was 25 ng when extrapolated from the amount of in vitro transcripts in lanes 2 and 3, considering that 26 and 22%, respectively of the

FIG. 4. Northern blot analysis of wt Alfort/187 RNA (lane 1) and of pA187-1 in vitro transcripts (lanes 2 to 7). Plasmid pA187-1 linearized with *SrfI* was transcribed in vitro for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4), 4 h (lane 5), 6 h (lane 6), and 8 h (lane 7). Lanes 8 and 9, control reactions to which either no T7 polymerase (lane 8) or no pA187-1 DNA (lane 9) was added. After DNase I digestion, the RNA was separated on a denaturing 1% formaldehyde agarose gel and blotted onto a positively charged nylon membrane. CSFV-specific RNA was detected with an antisense ³²P-labeled probe transcribed from pATU-1. The percentage of apparent full-length transcripts is indicated for each time point.

transcripts were apparently full-length RNA. The time required to obtain maximal amounts of full-length RNA was determined by transcribing 1.5 μ g of linearized template for 1 to 8 h. The highest yield of full-length RNA was obtained for an incubation time of 3 h, with 16% apparent full-length RNA (Fig. 4, lane 4). Therefore, all following transcriptions were performed for 3 h at 37°C and yielded approximately 50 μ g of RNA from a 20- μ l reaction mixture supplied with 1.5 μ g of linearized template DNA.

Transfection of porcine kidney cells with in vitro transcripts and characterization of the rescued virus. Liposome-mediated transfection yielded 15 to 20% positive SK-6 cells and approximately 0.1 to 0.5% positive PK-41 cells when quantified by using the pSV- β -Galactosidase vector. The same vector served to optimize the electroporation conditions. For both cell lines, electroporation at 750 V/cm and 960 μ F in complete medium containing 10% horse serum gave the best results, with approximately 10 to 15% of SK-6 cells and 0.1 to 0.5% of PK-41 cells transfected. Using either of the two protocols, SK-6 and PK-41 cells were transfected with runoff transcripts of pA187-1. Supernatant of transfected cells was titrated in SK-6 cells at different time points after transfection. Low-titer (2.0×10^2 TCID₅₀/ml) infectious CSFV was recovered 22 h after transfection. At 65 h after transfection, the mean titer of CSFV rescued from SK-6 cells was 6.3×10^7 TCID₅₀/ml. The recombinant virus was named vA187-1. Indirect immunofluorescence performed on SK-6 cells 65 h after electroporation is shown in Fig. 5A.

The RNA infectivity titer was determined after liposome-mediated transfection and was found to be significantly higher in SK-6 cells than in PK-41 cells (Table 2). This correlates with the difference in transfection efficiency observed for the two cell lines. Photometric quantification of total in vitro transcripts was performed after extraction and precipitation of the RNA. Reference reactions which were extracted before incubation gave reproducibly negligible absorption, indicating that unincorporated nucleotides were efficiently removed during RNA purification. The results from three independent sets of in vitro transcription and RNA titration experiments are shown in Table 2. The mean infectivity of pA187-1 runoff transcripts was 5.6×10^4 TCID₅₀/ μ g of RNA in SK-6 cells. Infectivity was approximately 1 log₁₀ TCID₅₀/ μ g of RNA lower when transcripts were synthesized in the presence of m⁷G(5')ppp(5')G capping agent. Wild-type viral RNA quantified in lane 1 of Fig. 4 had an infectivity titer of 5.0×10^4 TCID₅₀/ μ g of apparent full-length RNA (Table 2).

RNA was extracted from virus that had been passaged three times on SK-6 cells, with treatment of the virus with DNase I and RNase A before each inoculation of fresh cells. The genome region containing the silent marker mutation was amplified by RT-PCR. By direct sequence analysis of the amplified DNA, the presence of the expected silent mutation in the genome of vA187-1 was confirmed (Fig. 6). No corresponding RT-PCR product was obtained from either of two controls in which (i) RNA transcribed from linearized pNRA24-12 (frameshift) and (ii) linearized plasmid DNA pA187-1 was transfected.

Infectious RNA (Table 2) could also be transcribed from the chimeric full-length cDNA clone pA187-C1-14 obtained by replacement of most of the 5' noncoding region and of the N^{pro} gene with the corresponding genome region of the CAP strain. Indirect immunofluorescence of SK-6 cells transfected with runoff transcripts of pA187-C1-14 is shown in Fig. 5B. After three passages on SK-6 cells, including extensive DNase I and RNase A treatment, the recovered virus (vA187-C1-14)

FIG. 5. Indirect immunofluorescence of transfected SK-6 cells, using CSFV-specific monoclonal antibody HC/TC 26. Indirect immunofluorescence was performed 65 h after electroporation of SK-6 cells with 1 μ g of runoff transcripts of pA187-1 (after 10 passages in bacteria) (A) or pA187-C1-14 (B) or with 1 μ g of *SrfI*-linearized pA187-1 DNA (C).

TABLE 2. Infectivity of CSFV RNA on SK-6 and PK-41 cells

Sample	Infectivity (TCID ₅₀ /μg of RNA) ^a		
	Expt 1, SK-6	Expt 2, SK-6	Expt 3, PK-41
CSFV Alfort/187 RNA ^b	5.0×10^4	ND	ND
pA187-1 runoff transcripts ^c	6.3×10^4	5.0×10^4	6.3×10^2
pA187-1 runoff transcripts, ^c capped ^d	5.0×10^3	6.3×10^3	ND
pA187-1 cloned 10 times in <i>E. coli</i> , runoff transcripts ^c	2.0×10^4	ND	ND
pA187-C1-14 runoff transcripts ^c	4.0×10^5	5.0×10^4	6.3×10^2
pNRA24-12 runoff transcripts ^c	NI	ND	NI
pA187-1 DNA, linearized with <i>SrfI</i>	NI	NI	NI
Transfection buffer	NI	NI	NI

^a Extrapolated from transfections using DOTAP reagent and 2.5 ng to 2.5 pg of total RNA per well. Results of three independent sets of in vitro transcription and RNA titration experiments are shown. ND, not determined; NI, no detectable infectivity.

^b The infectivity is based on the amount of apparent full-length viral RNA. See text for extraction and quantification.

^c Plasmid DNA was linearized with *SrfI* prior to transcription.

^d Ratio of m⁷G(5')ppp(5')G to GTP in the transcription reaction was 4:1.

had retained both the silent mutation and the CAP-specific sequence on its genome (not shown).

The kinetics of virus replication of wt Alfort/187 and of the recombinants vA187-1 and vA187-C1-14 were compared on SK-6 cells. Cells were infected at a multiplicity of infection of either 0.1 or 10, and virus was extracted and titrated at different time points postinfection. No significant difference was observed for the three viruses (Fig. 7).

Stability of pA187-1 in bacteria. To assess the stability of the full-length plasmid construct in bacteria, pA187-1 was cloned 10 times (10 passages) in *E. coli* XL1-Blue, resulting in a total of 20 multiplication cycles in bacteria, alternating from liquid to solid medium. Multiple restriction digestion of the plasmid purified after 5 and 10 passages generated the expected DNA pattern. Virus could be rescued from SK-6 and PK-41 cells transfected with runoff transcripts of plasmid DNA purified after passages 5 and 10 (indirect immunofluorescence is shown

in Fig. 5A). The infectivity of the RNA after 10 passages remained unchanged (Table 2).

DISCUSSION

Taking advantage of previously published nucleotide sequences of two CSFV strains (25, 31), we used RT and PCR to establish three independent complete cDNA libraries of CSFV strain Alfort/187. To detect errors generated by the reverse transcriptase or by the *Taq* DNA polymerase, we analyzed the complete nucleotide sequences of two libraries. To obtain an authentic sequence at nucleotide positions where differences occurred between the first two libraries, we determined the respective sequence from the third library. This approach has been successfully used for BVDV strain SD-1 by Deng and Brock (8), who calculated an error rate of 4×10^{-5} per nucleotide per cycle. In the present work, single nucleotide dif-

FIG. 6. (A) Identification of the marker mutation at nucleotide position 11842 (arrow) of the recombinant virus vA187-1. The region surrounding nucleotide 11842 was amplified by RT-PCR from extracted RNA of either wt Alfort/187 or recombinant vA187-1 which had been passaged three times in SK-6 cells, with extensive DNase I and RNase A treatment between each passage. The PCR fragments were analyzed by direct sequencing on a LI-COR model 4000L automated DNA sequencer. (B) Nucleotide sequences and the silent mutation (arrow). The encoded amino acids are designated by the single-letter code.

FIG. 7. Growth curves of wt Alfort/187 and recombinants vA187-1 and vA187-C1-14. SK-6 cells seeded in six-well plates (10^6 cells per well) were infected at a multiplicity of infection of 0.1 (A) or 10 (B) either with wt Alfort/187 or with recombinant vA187-1 or vA187-C1-14 rescued from clarified transfection supernatant. After 1 h at 37°C, cells were washed and incubated in fresh medium for up to 32 h. At different time points postinfection, virus was harvested by freeze-thawing of the cultures and titrated on SK-6 cells.

ferences were found at a total of 19 positions in the two completely sequenced cDNA libraries, corresponding to a mutation rate of 2.3×10^{-5} per nucleotide per cycle. Although this result reflects approximately the error rates for *Taq* DNA polymerase of between 2×10^{-4} and 10^{-5} errors per nucleotide per cycle reported by other authors (11, 39), it cannot be excluded that some of the differences found between the clones analyzed were due to sequence heterogeneity in the original virus stock. Previous partial sequencing of the Alfort/187 genome in at least two laboratories had suggested that the nucleotide sequence published for Alfort Tübingen (25) was only distantly related to the sequence of Alfort/187 (14, 32, 37, 38), a clone of the original Alfort strain (1, 6). This is clearly confirmed by the sequence data presented in this work, which revealed an overall nucleotide sequence identity of 86.2% between the Alfort Tübingen sequence (25) and the Alfort/187

sequence. Surprisingly, nucleotide identities were 98.6 and 99.2% when the sequence of Alfort/187 was aligned to the sequences of ALD and of GPE⁻ (15), respectively. The sequence that we have obtained for Alfort/187 also confirms that the first nine nucleotides at the 5' end of the genome are highly conserved between CSFV and BVDV and can form a stem-loop with downstream complementary nucleotides as recently shown by Katayama et al. (18). This secondary structure is likely to be of functional importance.

The cDNA fragments carrying the consensus sequence were assembled into a full-length cDNA clone. To enable in vitro transcription of viral RNA having the exact genomic 5' end, the CSFV cDNA was placed immediately downstream of a modified bacteriophage T7 RNA polymerase promoter, the 5'-terminal nucleotide of the genome, a guanine, corresponding to position +1 relative to the transcription start site of the promoter. According to Dunn and Studier (10), all 17 authentic promoters for T7 RNA polymerase occurring in the bacteriophage T7 genome have an uninterrupted string of at least four purines downstream of the transcription start site. Changes in positions +1 and +2 were reported to substantially reduce the efficiency of transcription in vitro, whereas the sequence beyond +2 had little effect on the yield of RNA in the transcription reaction (27). In the present work, the T7 RNA polymerase efficiently transcribed RNA that was expected to start with the authentic 5' terminus of the genome (Fig. 3B and Fig. 4), although the nucleotides at positions +2 and +4 were pyrimidines. The flexibility in the T7 promoter sequence has been used previously to synthesize infectious viral RNA without additional nonviral nucleotides at the 5' end (42, 43).

Attempts to assemble the full-length cDNA in the pUC-derived pSL1180 vector failed. Also, cloning of either the 5' or the 3' half of the genomic cDNA in pBluescriptIIISK+ caused strongly reduced growth rates of the transformed bacteria. A similar phenomenon has been reported for the construction of a full-length Kunjin virus cDNA clone (19). In this case, the problem was solved by selecting a pBR322-derived backbone vector. In contrast, pUC-derived plasmids have frequently been used for cloning of full-length cDNA of viral RNA which was shorter than 10 kb and allowed generation of infectious transcripts (9, 20, 36, 42, 44, 50). For cDNA inserts larger than 10 kbp, pBR322-derived vectors were successfully used (7, 19, 23, 35). However, in some cases full-length constructs cloned in pBR322 were not infectious because of instability in bacteria, but infectious RNA was obtained after transcription from in vitro ligated fragments (16, 34, 43). We chose another vector type and constructed plasmid pACNR1180, which is derived from pACYC177 (4), by replacing most of the nonessential sequences with a multiple cloning site. The P15A origin of replication was expected to produce a low number of plasmid copies in bacteria (4) and thus to be advantageous for the stability of a large plasmid construct. The two genome halves were joined in pACNR1180. Interestingly, the 5' half of the genomic cDNA was stable only when it was inserted into pACNR1180 in the opposite orientation with regard to the ampicillin resistance gene. Therefore, this orientation was selected for the assembly of the full-length genomic cDNA. Verification of the complete nucleotide sequence of the full-length construct pNRA24-12 revealed the expected sequence except for a shift in the open reading frame of the genome as a result of a 4-bp duplication. After repair of pNRA24-12, we obtained the cDNA clone pA187-1, from which infectious RNA could be transcribed. Thus, we conclude that the nucleotide sequence that we have initially determined for the Alfort/187 genome does indeed represent the genomic sequence of an

infectious CSFV. Stability of the cDNA clone in bacteria is a prerequisite for experiments involving manipulation of the genome sequence. The conserved DNA restriction pattern and, more importantly, RNA infectivity after serial cloning of pA187-1 and after construction of the chimeric cDNA clone pA187-C1-14 (Table 2) emphasize the stability of the full-length cDNA clone. Nevertheless, introduction of single point mutations at the low error frequency of *E. coli* DNA polymerase cannot be excluded. This is very unlikely, since no point mutation was observed after the numerous cloning steps of the construction procedure, except for the duplication that had occurred at the *NheI*⁵⁰⁵⁷ ligation site. Formal proof that virus recovered after transfection of in vitro-transcribed RNA originated from cDNA was provided by the identification of the chimeric virus vA187-C1-14 and by the retention of the silent mutation at position 11842 of the genome in both vA187-1 (Fig. 6) and vA187-C1-14. The growth characteristics of the recombinant viruses in SK-6 cells were not significantly different from those of the wt virus (Fig. 7). However, other in vitro properties as well as the virulence in pigs have not been examined yet.

We used the control plasmid DNA pSV- β -Galactosidase as a stable eukaryotic expression vector system to monitor the transfection efficiency of the porcine kidney cells. Regardless of the transfection protocol, SK-6 cells were transfected more efficiently than PK-41 cells and therefore were selected for most of the experiments. The difference observed in DNA transfection efficiency between the two cell lines correlated with the difference observed in RNA infectivity, suggesting that, at least for liposome-mediated transfection, the conditions were dependent on the cell line rather than on the nature of the nucleic acid. The infectivity of the in vitro transcripts (Table 2) was calculated on the basis of the total amount of transcribed RNA. The percentage of apparent full-length transcripts calculated after Northern blot analysis in Fig. 4 corresponded to the values obtained after incorporation of [α -³²P]UTP (not shown). Taking in account that the apparent full-length RNA represented 16% of the total RNA when transcription was performed for 3 h, the infectivity of uncapped in vitro-transcribed RNA was apparently higher than the infectivity of extracted wt viral RNA. This difference might reflect the difference in quality of the in vitro transcripts and of the extracted viral RNA containing high amounts of cellular RNA. Furthermore, after capping, the infectivity of in vitro-transcribed RNA was significantly less than that of uncapped RNA (Table 2). This is in contrast to findings for other viruses (12, 22, 34, 35, 42), in which capping of in vitro-transcribed RNA considerably increased its infectivity. This observation correlates well with results obtained for BVDV (3), suggesting that the genomic RNA of pestiviruses does not possess a cap structure. The value of 4.0×10^5 TCID₅₀/μg of RNA (Table 2, experiment 1) for pA187-C1-14 runoff transcripts was the highest infectivity that we obtained in a titration experiment in SK-6 cells. This value might not be representative, since infectivities of runoff transcripts from pA187-C1-14 and pA187-1 were identical in experiments 2 and 3 (Table 2).

We conclude from these data that the RNA produced in vitro by transcription of *SrfI*-linearized pA187-1 is identical in structure and properties to the parental wt viral RNA. The stably cloned full-length cDNA copy of the Alfort/187 genome from which infectious RNA can be transcribed opens new perspectives in the characterization of the virus at the molecular level, of the virus-host interactions, and of viral pathogenesis. Little is known about the functions of virus-encoded proteins and about the role of host proteins in virus replication and morphogenesis. These questions can now be addressed.

CSFV strains and field isolates from different outbreaks have been classified as high virulent, moderate virulent, low virulent, and avirulent (46). Future work will also focus on the identification of the viral and host factors and of the mechanisms responsible for the observed differences in virulence.

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ADDENDUM IN PROOF

After submission of this article, two similar reports were published on the establishment of CSFV cDNA clones from which infectious RNA could be transcribed (R. J. M. Moormann, H. G. F. van Gennip, G. K. W. Miedema, M. M. Hulst, and P. A. van Rijn, *J. Virol.* **70**:763–770, 1996; G. Meyers, H.-J. Thiel, and T. Rumenapf, *J. Virol.* **70**:1588–1595).

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