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Classical Swine Fever Virus: Recovery of Infectious Viruses from cDNA Constructs and Generation of Recombinant Cytopathogenic Defective Interfering Particles

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The 5′- and 3′-terminal sequences of the genomic RNA from classical swine fever virus (CSFV) were determined, and the resulting information was used for construction of full-length CSFV cDNA clones. After transfection of in vitro-transcribed RNA derived from a cDNA construct, infectious CSFV was recovered from porcine cells. To confirm the de novo generation of infectious CSFV from cloned DNA, a genetically tagged CSFV was constructed. In comparison with parental CSFV, the recombinant viruses were retarded in growth by about 1 order of magnitude. Introduction of a deletion by exchange of part of the full-length construct for corresponding cDNA fragments derived from the genomes of cytopathogenic CSFV defective interfering particles (DIs) (G. Meyers and H.-J. Thiel, J. Virol. 69:3683–3689, 1995) resulted in recovery of cytopathogenic DIs from in vitro-transcribed RNA. This finding confirmed the hypothesis that the deletion previously identified in the DI genomes is responsible for their cytopathogenicity. The established system will allow novel approaches to analysis of pestiviral molecular biology and in particular to elucidation of the molecular basis of attenuation and cytopathogenicity of these viruses.

Classical swine fever virus (CSFV) is a member of the genus *Pestivirus*, which also comprises bovine viral diarrhea virus (BVDV) and border disease virus of sheep. Together with the genus *Flavivirus* and the hepatitis C virus group, the genus *Pestivirus* is included in the family *Flaviviridae*. Flaviviruses represent small enveloped viruses with positive-stranded RNA genomes of 10 to 13 kb which contain continuous long open reading frames (ORFs). Translation of the viral genomic RNA results in a polyprotein which is co- and posttranslationally processed by cellular and viral proteases to give rise to the mature viral proteins.

Pestiviruses represent important pathogens of ruminants and pigs and cause major losses in stock farming (21). Recent outbreaks of classical swine fever in the European Community and the appearance of an apparently new, highly pathogenic form of BVDV in Canada and the northern part of the United States (5, 24, 29) have emphasized the economic impact of diseases induced by these viruses.

For all pestiviruses, two biotypes can be distinguished in tissue culture, namely a noncytopathogenic (noncp) form replicating without obvious damage to the host cells and a cytopathogenic (cp) variant provoking lysis of infected cells (21). Molecular analyses revealed that cp viruses represent mutants of the noncp form. The observed alterations in the genomes of cp pestiviruses result from nonhomologous recombination leading to integration of cellular RNA, duplication or deletion of viral RNA (15–20, 27, 36, 37). For CSFV, three independent cp isolates were found to contain defective interfering particles (DIs) which all exhibit identical deletions. Data obtained after RNA transfection and virus dilution experiments showed that the DIs are responsible for the cytopathic effect of these CSFV isolates, while the autonomously replicating helper viruses are noncp (20). Cytopathogenicity of pestiviruses is correlated with the expression of the nonstructural protein NS3 (18–20, 25, 26). Changes with respect to the expression of this protein can be attributed to the alterations identified in the genomes of the cp pestiviruses (20, 36, 37).

Application of modern molecular techniques allowed us to address obvious issues, including the genome organization of pestiviruses, the functions of pestiviral proteins, and the basis for cytopathogenicity of these viruses. However, one of the most powerful means of modern virology, namely studying the effects of defined mutations introduced into the viral genome, was not applicable for these viruses. This "reverse genetics approach" is dependent on an infectious cDNA copy which has not been available for pestiviruses so far. We report here the generation of an infectious CSFV cRNA derived from a full-length cDNA clone and its first application to studies of cytopathogenicity.

MATERIALS AND METHODS

Cells and viruses. PK15 cells were obtained from the American Type Culture Collection (Rockville, Md.). CSFV Alfort/Tübingen was reisolated from organs of an experimentally infected moribund animal (31). CSFV ATCC (7) was obtained from the American Type Culture Collection (ATCC VR-531). The cytopathogenic variant CSFV Alfort/M was isolated after about 230 passages in pig lymphoma cell line 38A1D (20). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and nonessential amino acids. Cells and virus stocks were tested regularly for the absence of mycoplasma contamination.

Infection of cells. Since pestiviruses tend to be associated with the host cells, lysates of infected cells were used for reinfection of culture cells. Lysates were prepared by freezing and thawing cells 48 h postinfection and were stored at −70°C. Unless indicated otherwise in the text, a multiplicity of infection (MOI) of about 0.5 was used for infections.

Virus neutralization test and immunofluorescence assay. Virus-containing samples were serially diluted, and 50 μl was mixed with the same volume of monoclonal antibody (MAb) a18 (42) or control MAb, incubated at 37°C for 2 h, mixed with 100 μl of medium containing 1.5×10^4 PK15 cells, and seeded in 96-well plates. After 72 h, cells were analyzed by immunofluorescence.

For immunofluorescence with a mixture of anti-CSFV MAbs a18 and 24/16 (41, 42), cells were fixed with ice-cold methanol-acetone (1:1) for 15 min, air dried, rehydrated with phosphate-buffered saline (PBS), and then incubated with

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the antibodies. Bound antibodies were detected with a fluorescein isothiocyanate-conjugated goat anti-mouse serum (Dianova, Hamburg, Germany).

cDNA cloning and nucleotide sequencing. Establishment of cDNA libraries in lambda ZAPII (Stratagene, Heidelberg, Germany), library screening, and nucleotide sequence determination were done essentially as described before (18). Probes for library screening were a 0.6-kb *Eco*RI-*Hin*dIII fragment from cDNA clone 4.0 for the 5′ end and a 0.5-kb *Hin*dIII-*Eco*RI fragment from clone 1.9 for the 3′ end (clones described in reference 15). Sequence analysis was done with Genetics Computer Group software (6). The oligonucleotides for priming of first-strand cDNA were random hexanucleotides (Pharmacia, Freiburg, Germany) for the determination of the 5′ end, oligo(dT) for RNA ligated with oligo(A), and ol-18 for RNA ligated with ol-40 (ol-18 and ol-40 are described in reference 4). The sequence of ol-18 is 5′ TTTAGCGACCGTTCGATC 3′. The sequence of ol-40 is 5′ GATCGATCGAATTCATATGCGCGATCGAACG GTCGCTAAA 3′.

cDNA tailing reaction. First-strand cDNA was prepared from 5 μg of RNA isolated from partially purified CSFV (31), phenol extracted, and further purified by passage through a Sephadex G-50 spun column (32) and ethanol precipitation. The RNA-DNA hybrid was resuspended in 40 μl of tailing buffer (140 mM potassium cacodylate, 30 mM Tris-HCl, 1 mM $CoCl_2$, 0.1 mM dithiothreitol, and 0.2 mM deoxynucleoside triphosphate [dGTP for G tailing or dATP for A tailing]). After incubation at 37°C for 30 min with 20 U of terminal deoxynucleotidyltransferase (Boehringer-Mannheim, Mannheim, Germany), again phenol extraction and ethanol precipitation were performed. The tailed product was resuspended in 33 μl of first-strand cDNA buffer (cDNA kit; Pharmacia) containing 1 μg of oligo(dC) (G-tailed cDNA) or oligo(dT) (A-tailed cDNA) and added to a cDNA second-strand mix (cDNA kit; Pharmacia) and was further treated as recommended by the supplier.

Oligonucleotide-RNA ligation. Forty nanograms of oligonucleotide ol-40 or oligo(A) (Pharmacia) was ligated to 4 μg of partially purified CSFV RNA (31) with 150 ng of T4 RNA ligase (Pharmacia) in 30% polyethylene glycol–50 mM Tris-HCl (pH 7.8)–5 mM $MgCl_2$–0.5 mM ATP–10 mM β-mercaptoethanol for 2 h at 20°C in a volume of 10 μl. After phenol extraction and ethanol precipitation, the reaction product served as a template for cDNA synthesis.

Construction of full-length CSFV cDNA clones. The cDNA clones used for construction of the full-length construct were described before (15). Restriction, subcloning, and other standard procedures were done essentially as described previously (32). Restriction and modifying enzymes were purchased from NEB (Schwalbach, Germany), Pharmacia, and Boehringer-Mannheim.

The *Hin*dIII-*Bgl*II fragment of clone 4.0 was ligated together with the *Bgl*II-*Eco*RI fragment from clone 4.2 into pEMBL18, restricted with *Hin*dIII and *Eco*RI, resulting in clone pM5. The clone pM3, covering the 3′ region of the genome, was established in the same vector, with the *Eco*RI-*Kpn*I fragment from clone 4.2 and the 0.5-kb *Kpn*I-*Hin*dIII fragment from clone 1.9 with subsequent insertion of the 4-kb *Kpn*I fragment from clone 5.5. The inserts from pM5 and pM3 were combined in *Hin*dIII-cut pBR322 after restriction with *Eco*RI and *Hin*dIII to yield pGesM. For the 5′ end, oligonucleotides CS5plus and CS5minus were hybridized and inserted together with the *Eag*I-*Hin*dIII fragment from clone 4.0 into pEMBL18/*Eco*RI-*Hin*dIII, resulting in pE5. For the 3′ end, oligonucleotides CS3plus and CS3minus were hybridized and ligated with the *Hin*dIII-*Aat*II fragment from clone 1.9 into pEMBL18/*Hin*dIII-*Bam*HI, resulting in plasmid pE3. The inserts from pE5 and pE3, cut with *Eco*RI-*Hin*dIII and *Hin*dIII-*Bam*HI, respectively, were cloned together in pBR322/*Eco*RI-*Bam*HI, leading to p5/3Fus. To p5/3Fus, digested with *Hin*dIII and dephosphorylated with calf intestine phosphatase, the insert from pGesM, released with *Hin*dIII, was ligated, resulting in pB/CSFV. For construction of pA/CSFV, the 12-kb *Xho*I-*Bam*HI fragment from pB/CSFV was inserted into pACYC177 (NEB) restricted with the same enzymes. The resulting plasmid was cut with *Xho*I, and after end filling with Klenow polymerase, the *Eco*RI (blunt-ended)-*Bgl*II fragment from pB/CSFV was inserted. Subsequently, the 3′-terminal *Bam*HI-*Nsi*I fragment was exchanged for an equivalent fragment resulting from reverse transcription (RT)-PCR with oligonucleotides ol-3′Srf and ol-Nsi/plus.

The sequences of the oligonucleotides used are as follows: CS5plus, 5′ AATT CTAATACGACTCACTATAGTATACGAGGTTAGCTCTTTCTCGTATAC GATATTGGATACACTAAATTTCGATTTGGTCTAGGGCACCCCTCCA GCGAC 3′; CS5minus, 5′ GGCCGTCGCTGGAGGGGTGCCCTAGACCA AATCGAAATTTAGTGTATCCAATATCGTATACGAGAAAGAGCTAAC CTCGTATACTATAGTGAGTCGTATTAG 3′; CS3plus, 5′ CCACAGTTGG ACTAAGGTAATTTCCTAACGGCCCCCATTGGCCG 3′; CS3minus, 5′ GA TCCGGCCAATGGGGGCCGTTAGGAAATTACCTTAGTCCAACTGTG GACGT 3′; ol-3′Srf, 5′ CGGGATCCGCCCGGGCCGTTAGGAAATTACC TT 3′; and ol-Nsi/plus, 5′ CCTACAGACCCCTGGCTTGC 3′.

Site-directed mutagenesis. Mutagenesis according to the method of Kunkel et al. (12) was done with the Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad, München, Germany) essentially as recommended by the manufacturer, with the exception that single strands were produced with the filamentous phage VCSM13 (Stratagene). The presence of the desired mutations was verified by nucleotide sequencing. The oligonucleotide used for the mutagenesis was ol-CSFV13 (5′ GCTGCCACTGGCGCCGTCA 3′).

In vitro transcription. Two micrograms of the respective cDNA construct was linearized with the appropriate restriction enzyme and purified by phenol extraction and ethanol precipitation. Transcription with T7 RNA polymerase (NEB) was carried out in a total volume of 50 μl of transcription mix (40 mM Tris-HCl [pH 7.5]; 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 0.5 mM [each] ATP, GTP, CTP, and UTP; 10 mM dithiothreitol; 100 μg of bovine serum albumin per ml) with 50 U of T7 RNA polymerase in the presence of 15 U of RNAguard (Pharmacia). After incubation at 37°C for 1 h, the reaction mixture was passed through a Sephadex G-50 spun column (32) and further purified by phenol extraction and ethanol precipitation.

RNA transfection. Transfection was done with a suspension of 3×10^6 PK15 cells and about 0.5 μg of in vitro-transcribed RNA bound to DEAE-dextran (Pharmacia). For positive controls, usually 5 μg of total RNA from PK15 cells infected with the respective CSFV isolate was used for transfection. The RNA-DEAE-dextran complex was established by mixing RNA dissolved in 100 μl of HBSS buffer (5 g of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, and 1 g of dextrose per liter [pH 7.05]) (39) with 100 μl of DEAE-dextran (1 mg/ml in HBSS) and incubation for 30 min on ice (39). Pelleted cells were washed once with Dulbecco's modified Eagle's medium without fetal calf serum, centrifuged, and then resuspended in the RNA-DEAE-dextran mixture. After 30 min of incubation at 37°C, 20 μl of dimethyl sulfoxide was added, and the mixture was incubated for 2 min at room temperature. After addition of 2 ml of HBSS, cells were pelleted and washed once with HBSS and once with medium without fetal calf serum. Cells were resuspended in Dulbecco's modified Eagle's medium with fetal calf serum and seeded in a 10.0-cm-diameter dish. Forty-eight to 72 h posttransfection, cells were split and seeded as appropriate for subsequent analyses.

Northern (RNA) hybridization. RNA preparation, gel electrophoresis, radioactive labelling of the probe, hybridization and posthybridization washes were done as described before (37). A 2.2-kb *Sal*I fragment from CSFV Alfort/Tübingen cDNA clone 4.2 (15) was used as a probe.

RT-PCR. RT of 2 μg of heat-denatured RNA (2 min at 92°C, 5 min on ice in 11.5 μl of water in the presence of 30 pM reverse primer) was done after addition of 8 μl of RT mix (125 mM Tris-HCl [pH 8.3]; 182.5 mM KCl; 7.5 mM $MgCl_2$, 25 mM dithiothreitol; 1.25 mM [each] dATP, dTTP, dCTP, and dGTP), 15 U of RNAguard (Pharmacia), and 50 U of Superscript (Life Technologies/BRL, Eggenstein, Germany) for 45 min at 37°C. After addition of paraffin (Paraplast; melting point, 55°C) and after 2 min at 80°C, the tubes were placed on ice, and 30 μl of PCR mix (8.3 mM Tris-HCl [pH 8.3]; 33.3 mM KCl; 2.2 mM $MgCl_2$; 0.42 mM [each] dATP, dTTP, dCTP, and dGTP; 0.17% Triton X-100; 0.03% bovine serum albumin; 5 U of *Taq* polymerase [Appligene, Heidelberg, Germany]) was added. Amplification was carried out in 30 cycles (30 s at 94°C, 30 s at 54°C, 60 s at 72°C).

The oligonucleotides used had the following sequences: ol-BVDV32, 5′ AAATCTCTGCTGTACATGGCACATG 3′; and ol-HCV19, 5′ TGAGTTAT GTTCTAGGCTGCTACAGG 3′. The sequences of oligonucleotides ol-Nsi/plus and ol-3′Srf are given in the section "Construction of full-length clone."

RESULTS

Determination of the 5′- and 3′-terminal sequences of the CSFV genome. Even though the infectivity of an in vitro-transcribed RNA with a truncated 5′ end has been reported (11), it is generally agreed that an infectious clone should contain the complete genetic information of the respective virus, including the sequences at the utmost 5′ and 3′ ends. The standard methods of cDNA synthesis and cloning do not allow determination of the 5′- and 3′-terminal sequences of an RNA molecule. Therefore, the 5′-terminal sequence of CSFV Alfort was determined after tailing at the 3′ end of first-strand cDNA. Two separate tailing reactions were run—one with dGTP and one with dATP. In the case of G tailing, oligo(dC) was included as a primer in the cDNA second-strand synthesis, while for the A-tailed DNA, oligo(dT) was added. Eleven independent clones with G tails and 10 with A tails were isolated from the resulting cDNA libraries (Table 1). Downstream of the homopolymeric tail, the sequence 5′ GTATACGAG. . . 3′ or 5′ DGTATACGAG. . . 3′ was identified in these clones (Table 1). The D in the second sequence stands for a G, A, or T residue.

To obtain further information on the 5′ end of the CSFV RNA, primer extension experiments were carried out. The overwhelming amount of the extension products was obviously derived from an RNA with a 5′ end corresponding to the 5′ GTATACGAG. . . 3′ version (data not shown). We therefore chose this sequence as the 5′ end of our full-length cDNA clone.

For analysis of the 3′-terminal sequence of the CSFV ge-

TABLE 1. Tailing at the 3′ end of first-strand cDNA to determine the 5′-terminal sequence of the CSFV Alfort/Tübingen RNA

Sequence	No. of clones with:	
	G tailing	A tailing
(Tail)GTATAC...	6	5
(Tail)GGTATAC...	1	3
(Tail)AGTATAC...	1	2
(Tail)TGTATAC...	3	—[a]
(Tail)CGTATAC...	—	
Total	11	10

[a] —, clones with the respective sequence cannot be distinguished from those containing (tail)GTATAC...

nome, oligonucleotides were ligated to the viral RNA, and first-strand cDNA synthesis was started with primers complementary to the oligonucleotide extensions. Two separate reactions were carried out. In one case, an RNA oligonucleotide [oligo$(A)_{12–18}$] served as the substrate for the RNA ligase, while in the other case, a 40-mer DNA oligonucleotide (ol-40) (4) was used. After cDNA synthesis, cloning, and library screening, clones with inserts derived from the 3′ region of the viral RNA were isolated, and the termini of the inserts were sequenced. Six clones with 3′-terminal oligo(A) and 13 clones containing the ol-40 sequence were identified. In 12 of 19 clones, the synthetic oligonucleotides were preceded by the sequence 5′...TTTCCTAACGGCCC 3′ (sequence given in the orientation of the viral genome), while in the other cases one or two extra C residues were present (Table 2). Since it seems difficult to explain this result by a technical problem, it is likely that the 3′ end of the viral RNA is heterogeneous, displaying three to five C residues.

Construction of a full-length cDNA. It has been shown for other RNA viruses that transfection of cDNA clones can give rise to infectious virus (8, 9, 23, 28, 33, 34, 43). However, for recovery of recombinant positive-stranded RNA viruses, the strategy of choice is transfection of target cells with genome-like RNA which is generated in vitro from full-length cDNA constructs by runoff transcription with a bacteriophage RNA polymerase (for review, see reference 1). The latter approach was chosen for the CSFV infectious clone.

The first full-length CSFV cDNA construct, which was named pB/CSFV, was established in pBR322 with the insert integrated between the *Eco*RI and *Bam*HI sites of the plasmid (Fig. 1). The utmost 5′ sequence containing the *Eco*RI site, the T7 RNA polymerase promoter, and the 5′ end of the CSFV sequence down to an *Eag*I site at position 80 to 85 was composed of synthetic oligonucleotides.

In analogy to the 5′ end, the 3′-terminal sequences of this clone were produced as oligonucleotides. The rest of the sequence was derived from the cDNA fragments contained in the published clones 4.0, 4.2, 5.5, and 1.9 (15). The different parts of the full-length clone were fused at appropriate restriction sites by standard molecular cloning procedures (32).

TABLE 2. Determination of the 3′-terminal sequence of the CSFV Alfort/Tübingen genome after RNA ligation

Sequence	No. of clones with:	
	Oligo(A) ligation	ol-40 ligation
...GGCCC	4	8
...GGCCCC		2
...GGCCCCC	2	3
Total	6	13

The final version of the full-length cDNA clone, termed pA/CSFV, was constructed in the low-copy plasmid pACYC177 to ensure a higher degree of stability of the inserted sequences. The insert of pA/CSFV is composed of a 10.5-kb *Eco*RI-*Nsi*I fragment derived from pB/CSFV and a 3′-terminal fragment produced by RT-PCR with oligonucleotides ol-Nsi/plus and ol-3′Srf. The downstream primer ol-3′SrfI contains an *Srf*I site overlapping the 3′ end of the CSFV sequence and a *Bam*HI site for cloning (Fig. 1). Restriction with *Srf*I can be used for linearization prior to in vitro transcription. The resulting runoff transcripts terminate with three C residues.

Recovery of infectious virus from RNA transcripts. The pestiviral RNA contains a 5′ noncoding region of about 350 nucleotides which contains up to seven AUG codons preceding the translation initiation codon of the long ORF. It is likely that, similar to hepatitis C virus (10, 38, 40), translation initiation occurs in a cap-independent way. All available data argue in favor of the absence of a 5′ cap structure (2, 14a). Therefore, no cap was introduced when RNA was transcribed in vitro from *Srf*I-linearized pA/CSFV DNA. After several independent transfection experiments, stained foci were detected in the immunofluorescence assay. Since many earlier experiments with noninfectious RNAs capable of directing viral protein synthesis did not result in detectable immunofluorescence, this observation could hardly be imagined to result from translation of input RNA. Thus, replication of the in vitro-transcribed RNA within the transfected cells represents the only plausible explanation for the results obtained. In order to check whether infectious virus was released from the transfected cells, supernatant from the respective cultures was harvested, passed through 0.2-μm-pore-diameter filters, and then used for infection of cells in a virus neutralization assay. In an experiment with a control MAb, positive fluorescence was observed even when the supernatant was diluted 1:128 prior to infection. However, preincubation with CSFV-specific MAb a18 completely inhibited infection already at a 1:2 dilution. This result clearly shows that after transfection of in vitro-transcribed RNA, infectious CSFV was produced and released into the supernatant.

In control experiments with RNA from cells infected with CSFV Alfort/Tübingen, productive transfection of cells could be achieved with less than 10 ng of total cellular RNA. According to our estimations, the amount of viral RNA sufficient to produce a fluorescent focus in these assays was about 10 pg. In the case of in vitro-transcribed RNA, recovery of CSFV was reproducibly observed when 25 ng of RNA was used for transfection. According to estimations based on the number of fluorescent foci, the specific infectivity of the in vitro-transcribed RNA is about 400 to 500 times lower than that of the viral RNA.

To analyze the growth characteristics of the recovered virus, a virus stock was generated after two passages. Cells were infected with aliquots from this recombinant virus stock at an MOI of 0.1, and the number of infectious viruses present in the culture was determined at different time points. While in a parallel experiment parental virus reached a level of 3×10^5/ml after 48 h, the titer of the recovered virus was only about 5×10^4 at the same time point (Fig. 2). However, after five passages in tissue culture, the virus recovered from the in vitro-transcribed RNA exhibited growth characteristics equivalent to those of the wild-type virus (data not shown).

Generation of a genetically tagged virus. In order to provide formal proof for the recovery of infectious virus after transfec-

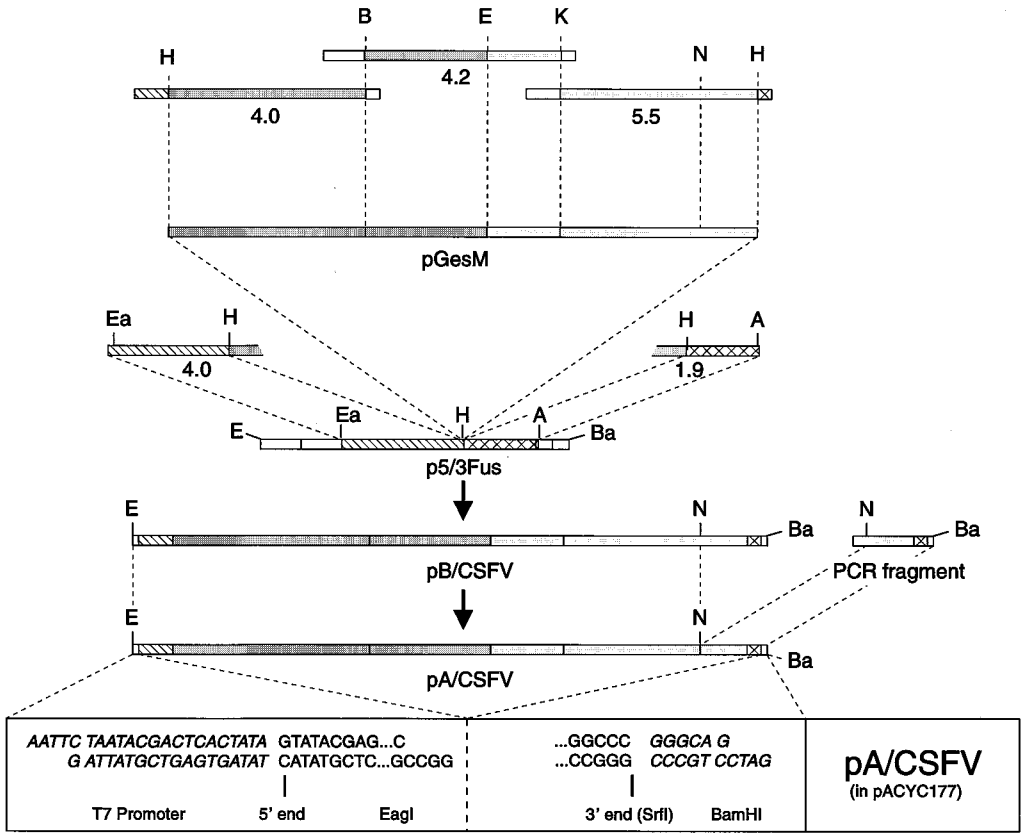

FIG. 1. Construction of CSFV full-length cDNA clone pA/CSFV. The upper part shows the construction of the full-length clone pB/CSFV via the intermediates pGesM (constructed with inserts from cDNA clones 4.0, 4.2, and 5.5 [15]) and p5/3Fus (fusion of restriction fragments obtained from cDNA clones 4.0 and 1.9 [15] with synthetic oligonucleotides corresponding to the 5′ and 3′ ends of the viral genome). Below, the generation of the final construct pA/CSFV by fusion of a PCR fragment with part of pB/CSFV is indicated. The box at the bottom shows the sequences upstream of the 5′ end and downstream of the 3′ end of the viral sequence, which include a T7 RNA polymerase promoter and restriction sites for enzymes used for cloning or linearization of the plasmids. Restriction endonuclease sites: A, *Aat*II; B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RI; Ea, *Eag*I; H, *Hin*dIII; K, *Kpn*I; N, *Nsi*I.

tion of in vitro-transcribed RNA, a mutation was introduced into the cDNA copy. An A-to-C change at position 891 generated an *Nar*I site in the full-length clone. Since the respective position represents the third base of an alanine codon, the exchange is silent. The resulting full-length clone contains a unique *Nar*I site and was termed pA/CSFV/Nar.

After transfection of in vitro-transcribed pA/CSFV/Nar RNA, positive fluorescence was observed. Supernatant from the respective culture dish was harvested, passed through a 0.2-μm-pore-diameter filter, and used for infection of cells. After one passage, total cellular RNA was prepared. As controls, pA/CSFV-derived RNA and RNA isolated from cells infected with parental virus were transfected, and the resulting viruses were treated the same way. The isolated RNAs were first analyzed in a Northern hybridization with a CSFV-specific probe (Fig. 3). As expected, a signal corresponding to CSFV genomic RNA was detected for cells which had been transfected with RNA derived from pA/CSFV, pA/CSFV/Nar, and the positive control (Fig. 3A). No differences were visible between the signals resulting from the parental virus and those from the different recombinant viruses. In contrast, transfection of an in vitro-transcribed control RNA did not result in a detectable signal (Fig. 3B).

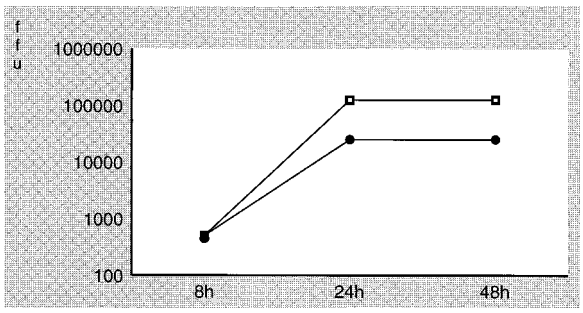

FIG. 2. Growth curve of CSFV Alfort/Tübingen (wild type [□]) and the virus derived from construct pA/CSFV {V(pA/CSFV) [●]}. Monolayers of PK15 cells were infected with the indicated viruses at an MOI of about 0.1 and harvested at the indicated time points, and titers were determined by immunofluorescence. The results are given as focus-forming units (ffu).

For analysis of the genomic region into which the *Nar*I site had been introduced in pA/CSFV/Nar, RT-PCR with the oligonucleotides ol-BVD32 and ol-HCV19 was performed. DNA fragments of the expected size (0.93 kb) were detected for all reaction mixtures containing viral RNA but not for the control (Fig. 4). To verify the presence of the mutated sequence in the genome of the virus derived from pA/CSFV/Nar, aliquots of the amplified products were incubated with *Nar*I. No difference was observed for the fragments amplified from parental virus or V(pA/CSFV), the virus derived from pA/CSFV (Fig. 4). However, the product amplified from V(pA/CSFV/Nar)

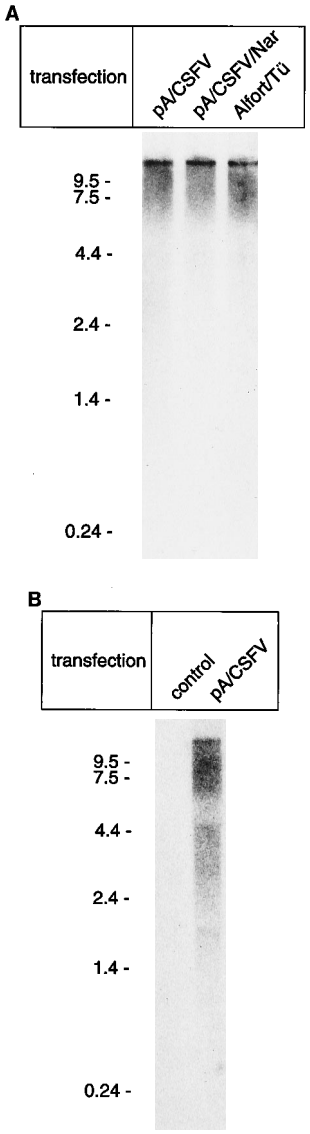

FIG. 3. Northern blot with RNA isolated from cells transfected with RNA transcribed from plasmid pA/CSFV or pA/CSFV/Nar or with total RNA from cells infected with CSFV Alfort/Tübingen (Alfort/Tü). An in vitro-transcribed RNA corresponding to a CSFV genome containing an internal deletion served as a negative control.

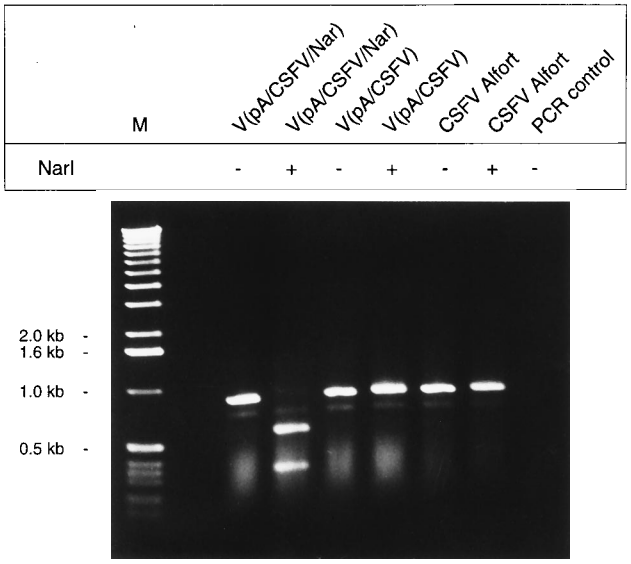

FIG. 4. Ethidium bromide-stained agarose gel with products of RT-PCR derived from RNA of cells infected with the indicated viruses, which were obtained after RNA transfection. The amplified fragments were either loaded without further treatment or after restriction with *Nar*I. M, molecular size markers.

was cut into two fragments of the expected size. Nucleotide sequencing of the amplified fragments further confirmed the absence of the mutation in the parental and the V(pA/CSFV) genomes and its presence in the V(pA/CSFV/Nar) RNA (data not shown). Thus, a genetically tagged virus had been generated from the cDNA construct. The growth characteristics of V(pA/CSFV/Nar) were comparable to those of V(pA/CSFV).

In vitro generation of a cp CSFV DI. Analysis of the three cp CSFV isolates Alfort/M, ATCC, and Steiermark had revealed that all of them are composed of a cp DI and a noncp helper virus. Partial cDNA cloning and sequencing of the three DI genomes revealed identical internal deletions, starting with the second codon of the long ORF and ending with codon 1589 (20). To check whether the deletion was sufficient to produce a cp DI, two cDNA clones corresponding to DI genomes were constructed. Clone pA/CSFV/M contains the deletion and flanking sequences from CSFV Alfort/M, while plasmid pA/CSFV/A contains an equivalent fragment from CSFV ATCC. To generate pA/CSFV/M, a 0.7-kb *Xho*I-*Eco*RI fragment was excised from cDNA clone Alfort/M K1 (20) and inserted into pA/CSFV DNA, from which a 5.3-kb fragment was deleted by restriction with the same enzymes. For construction of pA/CSFV/A, the *Xho*I-*Eco*RI fragment was taken from cDNA clone ATCC K5 (20).

The constructs pA/CSFV/M and pA/CSFV/A were used as templates for in vitro transcription after linearization with *Srf*I. For pA/CSFV/M, a second transcription reaction was conducted with an *Ase*I-restricted template. Since the *Ase*I cleavage site is located about 250 residues upstream of the genomic 3′ end, transcription gives rise to a 3′-terminally truncated RNA. The RNA obtained from the *Ase*I-linearized template can be translated to give rise to all of the DI-encoded proteins, but most likely, replication of this RNA is impossible because of the missing 3′ end. PK15 cells infected with the noncp CSFV Alfort/Tübingen as helper virus were transfected with the different RNAs transcribed from the DI full-length clones and from pA/CSFV. As already shown before (20), transfection of RNA from CSFV Alfort/M-infected cells led to a cytopathic effect and complete lysis of the cells within about 5 days (Fig. 5A). In addition, transfection with the RNA transcribed from the *Srf*I-linearized plasmids pA/CSFV/M and pA/CSFV/A had a cytopathic effect (Fig. 5A). However, RNA derived from the *Ase*I-linearized DI clone was not able to induce cell lysis (Fig. 5A). The same applied to RNA derived from pA/CSFV or pA/CSFV/A restricted with *Srf*I or *Ase*I, respectively, and for RNA derived from cells infected with CSFV Alfort/Tübingen (data not shown and reference 20, respectively). Thus, the exchange of the *Xho*I-*Eco*RI fragment of pA/CSFV for the respective fragment from the DI cDNA clones was sufficient to generate a cp DI. Since transfection of the 3′-terminally truncated RNAs did not lead to cell lysis, it can be speculated that replication of recombinant DI RNA is a prerequisite for induction of a cytopathic effect. Control experiments in which noninfected PK15 cells served as targets for transfection did

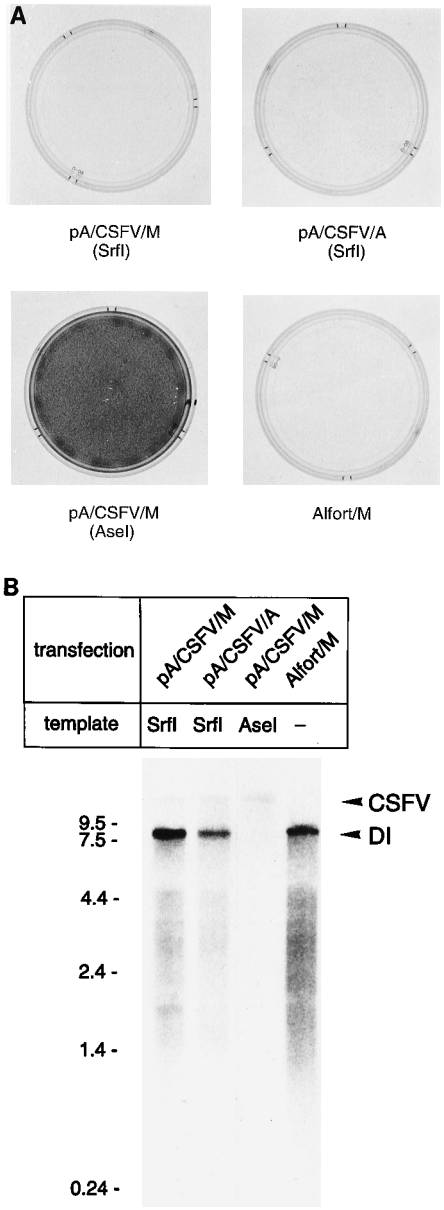

FIG. 5. Results of RNA transfection experiments with PK15 cells infected with CSFV Alfort. RNAs were transcribed from templates pA/CSFV/M or pA/CSFV/A linearized with either *Srf*I or *Ase*I. RNA from cells infected with CSFV Alfort/M served as a control. (A) Crystal violet staining of tissue culture cells which had been seeded after RNA transfection. Cells were washed once with PBS, fixed for 10 min with 5% formaldehyde, washed with water, and stained for 5 min with 1% (wt/vol) crystal violet (in 50% ethanol). (B) Northern blot with RNA derived from the cells 72 h posttransfection hybridized to a CSFV-specific probe. After longer exposure, CSFV genomic RNA was also visible in the lane designated Alfort/M.

not lead to a cytopathic effect, demonstrating that the recovered cp agents indeed are defective and thus are dependent on a helper virus (not shown). RNA was isolated from cells infected with material obtained after the transfection experiments. A Northern blot analysis showed that the presence of DI genomes correlates with detection of a cytopathic effect. For the viruses originating from the transcribed RNA, no obvious difference with respect to CSFV Alfort/M could be detected in this analysis (Fig. 5B). Importantly, the amount of DI RNA was considerably higher than that of the helper virus genome in all three cases. The chimeric character of the artificial DI V(pA/CSFV/A) with the *Xho*I-*Eco*RI fragment derived from CSFV ATCC and the flanking sequences obtained from CSFV Alfort/Tübingen was proved by sequencing of an appropriate RT-PCR product (not shown).

To analyze the growth characteristics of the recovered DIs, infection experiments were carried out with virus stocks produced after one passage. The number of PFU present at different time points postinfection was determined for V(pA/CSFV/M) and V(pA/CSFV/A) and compared with the values observed for CSFV Alfort/M. The titrations were carried out with cells infected with CSFV Alfort/Tübingen 24 h before addition of the DI-containing samples in order to provide enough helper virus even at high dilutions. Similar to the situation described above for V(pA/CSFV), growth retardation with respect to the parental DI was observed for the two recovered DIs. Again, the titers of the recovered DIs determined 48 h postinfection were less than 1 order of magnitude lower than that of the CSFV Alfort/M DI. The growth characteristics of the DIs are comparable with those observed for V(pA/CSFV) and CSFV Alfort/Tübingen, respectively.

DISCUSSION

Application of so-called reverse genetics represents one powerful tool of modern virology. In the case of RNA viruses, reverse genetics usually requires the establishment of a system allowing the generation of infectious virus entirely from cloned sequences. In most instances, a prerequisite for construction of infectious clones is the detailed knowledge of the sequences at the termini of the respective viral genome which are probably crucial for replication of the RNA. Determination of the 3′-terminal sequences of viral genomes without the poly(A) tail can only be achieved after elongation of the 3′ end. Different approaches have been used which basically rely on two enzymatic processes. Elongation of the 3′ end with *Escherichia coli* poly(A) polymerase results in RNAs which can serve as substrates for standard oligo(dT)-primed cDNA synthesis. This approach was used for analysis of the CSFV Brescia RNA (22). The major drawback of the method is that it cannot be decided whether A residues following the last G, T, or C still represent viral sequence or are already part of the enzymatically added tail. Therefore, a precise determination of the genomic 3′ end is not possible when this method is used without accompanying analyses. All other common approaches for the determination of 3′-terminal RNA sequences are based on ligation of additional sequences by T4 RNA ligase. One method which has been used (i.e., for flaviviruses and BVDV) is based on circularization of the genomic RNA (2, 14). In principle, this approach allows simultaneous analysis of both the 3′ end and the 5′ end. However, also in this case, accompanying analyses are necessary to define the border between the 5′ and 3′ ends. In our case, either ribo(A) or a synthetic DNA oligonucleotide was successfully used as the ligation partner. While the data obtained after ligation of the DNA oligonucleotide provide all of the necessary information, there is again the need for accompanying experiments when the oligo(A) reaction mixture is employed.

Interestingly, our analyses did not lead to determination of a definite sequence: instead, variants of a conserved motif were found, which differed in the presence of either three, four, or five terminal C residues. Publications on other pestiviruses report the presence of three C residues for CSFV Brescia (22) and five C residues for BVDV NADL, CP72, and SD1 (2). Even though experimental artifacts cannot be ruled out com-

pletely, it seems likely that pestiviral RNAs indeed are heterogeneous with respect to their 3′ end. The observed heterogeneity could be a result of degradation or of specific features of pestiviral replication. Future analyses have to show whether this property of the viral RNA is functionally important. The experiments described above show that three terminal C residues are sufficient for an RNA to initiate an infectious cycle.

Concerning the 5′ end, again heterogeneity was observed. In about 50% of the clones analyzed, an additional G, A, or T residue was detected. The most likely explanation for this finding is that the nucleotide binding site of the terminal deoxynucleotidyltransferase in these cases was occupied by a previously bound nucleotide which then served as a substrate for the first elongation step. The hypothesis that the variable residue at the 5′ end represents an artifact is supported by the results of primer extension studies and data obtained for BVDV. The 5′-terminal nine residues determined for BVDV strains NADL, CP72, and SD1 after 5′-to-3′-self ligation are 5′ GTATACGAG. . . 3′ (2) and thus are identical to those at the 5′ end of the CSFV Alfort/Tübingen genome without the variable residue. Again, the transfection experiments showed that an RNA starting with the sequence 5′ GTATAC. . . 3′ is infectious.

In the case of viruses with a positive-stranded RNA genome, the strategy of choice for recovery of infectious virus from cDNA relies on construction of full-length clones in which the viral sequences are combined with sequences allowing in vitro runoff transcription (1, 3, 11, 13, 30, 35). The resulting RNA is then used for transfection of appropriate target cells. The possibility of generating RNAs with precisely defined termini and the circumvention of unwanted splicing of the recombinant RNA after expression in the nucleus represent the main advantages of this strategy in comparison with transfection of cDNA constructs. Our attempts to obtain infectious virus from cloned sequences were therefore also based on transfection of in vitro-transcribed RNA. Major problems with this approach concern the generation of the correct 5′ and 3′ ends as well as the stability of the virus sequences when cloned in bacteria. In our case, efficient transcription of an RNA with the optimal 5′ sequence was possible since the virus genome starts with a G residue. However, the production of the desired 3′ end containing five terminal C residues could not be achieved by runoff transcription because of the lack of an appropriate restriction recognition sequence. In contrast to full-length clones of different flaviviruses (13, 30, 35), problems concerning the stability of the CSFV sequences cloned in pBR322 or pACYC177 were not observed during construction of the full-length clones. However, first attempts to establish full-length constructs in plasmids of the pUC, pEMBL, or pBluescript series failed. Moreover, bacteria harboring plasmids with different CSFV-derived inserts in these vectors or full-length constructs in pBR322 or pACYC177 exhibited variable colony sizes, and plasmid yields were very low. These findings indicated toxic effects of the cloned sequences, which in consequence should raise the chance for recovery of unwanted mutants. Without complete nucleotide sequencing, the acquisition of mutations within cloned sequences can never be ruled out and represents one probable reason for a long history of unsuccessful attempts to establish an infectious CSFV clone in our laboratory.

The search for the molecular basis of cytopathogenicity of pestiviruses has led to highly interesting findings during the past 5 years. Most cp pestiviruses analyzed so far have arisen from noncp ancestors by recombination. Integration of cellular sequences, sometimes accompanied by duplication of viral sequences, and different rearrangements of viral sequences, including the generation of cp DIs, have been identified for cp pestiviruses (16–20, 27, 36, 37). The three cp CSFV isolates analyzed so far were found to be composed of cp DIs and noncp helper viruses (20). Partial sequencing of the DI genomes revealed that all three contain exactly the same deletion starting with the second codon of the long ORF and ending with codon 1589 just upstream of the proposed 5′ end of nonstructural protein NS3 (20, 36, 37). Thus, the first protein generated by translation of the DI genomes is NS3. According to our hypothesis, expression of NS3, or in the case of CSFV, its overexpression, represents the reason for the cp phenotype of these viruses. A very interesting open question is whether, in addition to the deletion, other mutations are necessary for cytopathogenicity and/or the ability of the DIs to interfere with the replication of the helper viruses. The data presented above clearly show that the introduction of the respective deletion by exchange of a short cDNA fragment is sufficient for establishment of the cp phenotype. In the case of V(pA/CSFV/M), apart from the deletion, the genomic sequence is changed at only two positions with respect to V(pA/CSFV). One of these nucleotide exchanges is silent, while the other alters residue 1699 from proline to arginine. Since the respective position in the polyproteins of the CSFV DIs ATCC and Steiermark is occupied by a proline, it is very likely that this exchange is not relevant for cytopathogenicity. Even in the case of V(pACSFV/A), the inserted fragment codes for a polypeptide with only four amino acid exchanges with respect to the CSFV Alfort/Tübingen sequence. Therefore, the experiments described above provide strong evidence for the hypothesis that the deletion is the only reason for the cp phenotype of the CSFV DIs. Concerning the ability to interfere with helper virus replication, the available data reveal similar features for the recovered DIs and the CSFV Alfort/M DI. Starting from a limited number of transfected cells, the in vitro-transcribed RNA is able to propagate itself in a way such that after 48 h, more DI RNA than helper virus genome is present, although the cells were infected with CSFV at an MOI of about 0.5 24 h prior to transfection. According to estimations based on different independent transfection experiments, the ratios between DI RNA and the helper virus genome are comparable with those observed for CSFV Alfort/M.

Construction of the chimeric virus V(pA/CSFV/A) demonstrates that sequences from other CSFV strains can replace the parental RNA. Even though the inserted fragment exhibits only a 10% difference with respect to the nucleotide sequence, this finding is important for future projects aiming at construction of new live vaccines against CSFV and the use of pestiviruses as vector systems. In the context of the latter project, it is important to mention that the genomes of cp BVDV isolates contain up to 4 kb of duplicated viral sequences (18, 19). Thus, the use of pestiviruses as vectors carrying large insertions of foreign sequences seems promising.

Both the recovered autonomously replicating viruses and the DIs do not grow as fast as the respective parental viruses. The most likely explanation for this finding is that some kind of mutation is present in the cDNA constructs which influences the growth characteristics of the recombinant viruses. Further experiments with V(pA/CSFV) indicated that the observed defect is not temperature sensitive and is lost after five passages in tissue culture (data not shown). The putative mutation could either be present in *cis*-acting sequences of the genomic RNA or could concern the protein coding region and influence virus propagation through suboptimal function of one or more viral proteins. The available data argue in favor of the first possibility, since in the case of the synthetic DIs, the helper virus should in principle be able to complement for a protein defect in *trans*. One known difference between the viral ge-

nome and the in vitro-generated RNA is that the latter ends with only three C's, while part of the genomic CSFV RNA contains five terminal C residues. Since the 3′ end is most likely involved in initiation of RNA negative-strand synthesis, it is possible that an RNA which is 3′-terminally truncated by two residues is replicated more slowly than the parental RNA. However, the finding that the amounts of genomic RNA were comparable for the recovered and the parental viruses when analyzed 48 h postinfection argues against a defect in RNA replication. Further analyses, including exchange of defined parts of pA/CSFV or pA/CSFV/M with corresponding fragments from independent cDNA clones and changes at the 3′ ends of the full-length constructs are necessary to identify the reason for the growth retardation of the recovered viruses.

The data presented show that it is possible to generate infectious CSFV entirely from cloned cDNA. This opens up new opportunities in pestivirus research and will help to answer a lot of interesting questions.

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