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Infectious RNA Transcribed from an Engineered Full-Length cDNA Template of the Genome of a Pestivirus

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Infectious RNA was transcribed for the first time from a full-length cDNA template of the plus-strand RNA genome of a pestivirus. The genome of the C strain, which is a vaccine strain of classical swine fever virus, was sequenced and used to synthesize the template. The cDNA sequence of the C strain was found to be 12,311 nucleotides in length and contained one large open reading frame encoding a polyprotein of 3,898 amino acids. Although there were mostly only small differences between the sequence of the C strain and the published sequences of strains Alfort and Brescia, there was one notable insertion of 13 nucleotides, TTTTCTTTTTTTT, in the 3′ noncoding region of the C strain. Furthermore, we showed that the sequences at the 5′ and 3′ termini of the C strain are highly conserved among pestiviruses. We found that the infectivity of the in vitro transcripts of DNA copies pPRKflc-113 and pPRKflc-133 depended on the correctness of the nucleotide sequence. The in vitro transcripts of pPRKflc-133 were infectious, whereas those of pPRKflc-113 were not. In fact, only 5 amino acids among the complete amino acid sequence determined this difference in infectivity. However, virus FLc-133, which was generated from pPRKflc-133, cannot be differentiated from native C-strain virus. Therefore, we exchanged the region encoding the antigenic N-terminal half of envelope protein E2 in pPRKflc-133 with the equivalent region of strain Brescia. The resulting hybrid virus, FLc-h6, could be differentiated from the C strain and from FLc-133 with monoclonal antibodies directed against envelope proteins E^{rns} and E2 of strain Brescia and the C strain. To be suitable for further vaccine development, viruses generated from pPRKflc-133 should grow at least as well as native C-strain virus. In fact, we found that FLc-133, hybrid virus FLc-h6, and the C strain grew equally well. We concluded that pPRKflc-133 is an excellent tool for developing a classical swine fever marker vaccine and may prove valuable for studying the replication, virulence, cell and host tropism, and pathogenesis of classical swine fever virus.

Classical swine fever (CSF) or hog cholera is a highly contagious and often fatal disease of pigs, which is characterized by fever and hemorrhages and can run either an acute or a chronic course (33). Outbreaks of the disease occur intermittently in Europe as well as other parts of the world and can cause large economic losses. The causative agent of the disease is classical swine fever virus (CSFV), a member of the *Pestivirus* genus of the *Flaviviridae* family (7).

The Chinese strain (C strain) is a live attenuated vaccine strain that effectively protects pigs against disease (30). It is considered one of the most effective and safest of the live vaccines. However, pigs that are vaccinated with the C strain cannot be serologically differentiated from pigs that have been infected with a CSFV field strain. This is a major obstacle to the use of this vaccine in western Europe, where the disease is controlled by slaughtering infected and suspected herds and imposing quarantine restrictions. Such measures generally result in large economic losses. One solution may be the use of a so-called marker vaccine, which makes it possible to discriminate vaccinated from infected animals. Such a vaccine could be used during outbreaks of the disease for the vaccination of pigs in affected areas. This would strongly reduce transmission of the field virus, would enable the identification and stamping out of infected herds, and might reduce the quarantine restrictions. Thus, a marker vaccine might significantly reduce the economic losses due to CSF. However, a marker vaccine for CSFV is not yet commercially available.

We previously described two possible CSFV marker vaccines. One is a vector vaccine consisting of an attenuated pseudorabies live virus expressing envelope protein E2 of CSFV (38). This vaccine protects seronegative pigs against both pseudorabies and CSF. However, it is unsuccessful in protecting against CSF when it is administered to pigs that have neutralizing antibodies against pseudorabies virus (21). The other vaccine consists of E2, and it is produced in insect cells by a baculovirus expression vector (10). This vaccine, which is still under investigation, protects pigs against CSF and is safe because it contains only E2. However, since this vaccine consists of a protein, it is expected to be less effective than a live virus vaccine such as the C strain. Moreover, a live virus vaccine will be easier and less costly to produce.

To enable development of a C-strain marker vaccine, its genome must be altered by site-directed mutagenesis. The pestiviruses bovine viral diarrhea virus (BVDV), border disease virus, and CSFV (1, 18, 20) are positive-strand RNA viruses whose genomes vary in length from 12.5 to 16.5 kb (1, 16, 17, 19, 22, 24). Site-directed mutagenesis of RNA is as yet impossible. However, since in vitro transcription of cloned DNA has opened the way to synthesize infectious RNA from a full-length DNA copy of the genomes of positive-strand RNA viruses, the study of these viruses by site-directed mutagenesis of the DNA copy has been greatly enhanced (for a review, see reference 2). Infectious transcripts of full-length cDNAs have been described for the flaviviruses yellow fever virus (25), dengue 4 virus (12), Japanese encephalitis virus (28), and Kunjin

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virus (11). These viruses belong to the genus *Flavivirus* of the *Flaviviridae* family. However, no infectious transcripts have been described for hepatitis C virus and *Pestivirus*, the other two genera of this family.

This report describes for the first time the complete cDNA sequence of the genome of the C strain and the construction of a full-length DNA copy of this sequence, from which infectious RNA was transcribed. Furthermore, by constructing an antigenic variant of the C strain, we demonstrated that the infectious copy can be used to genetically alter the C-strain genome. The infectious copy is an excellent tool for developing a C-strain marker vaccine and may prove valuable for studying the replication, virulence, cell and host tropism, and pathogenesis of CSFV.

MATERIALS AND METHODS

Cells and virus. Swine kidney cells (SK6-M) (29) and bovine turbinate cells were grown in Eagle's basal medium or Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum and antibiotics. Fetal bovine serum was tested for the presence of BVDV and BVDV antibodies as described previously (20). Only sera that contained no BVDV and BVDV antibodies were used.

The C strain of CSFV was adapted to SK6-M cells as described by Terpstra (29). The strain designated Cedipest is noncytopathic and was cloned by three-fold end point dilution. After three amplification steps, a cloned virus stock with a titer of 3.5×10^6 50% tissue culture infective doses ($TCID_{50}$) per ml was produced.

CSFV strains Brescia and Alfort-L, as well as BVDV strains Osloss-L and NADL-L, originated from the collection of pestivirus strains available at the Institute for Animal Science and Health in Lelystad.

Isolation of cDNA covering the complete genome of the C strain. Cytoplasmic RNA from cells infected with a pestivirus was isolated and amplified by reverse transcription-PCR (RT-PCR) essentially as described previously (20, 35).

The genome of the C strain was amplified and cloned twice. During the first amplification, primers for first-strand cDNA synthesis and PCR were selected on the basis of homology between the sequences of CSFV Strains Brescia (21) and Alfort (15) and BVDV strains Osloss (23) and NADL (5). For the second amplification, new primers were synthesized on the basis of the sequence of cDNA fragments produced in the first amplification.

To obtain cDNA clones containing the 5′ and 3′ termini of pestivirus genomes, we used the 3′-5′ ligation method (13). Cytoplasmic RNA was isolated from cells infected with pestiviruses as described above and was purified through a 5.7 M CsCl cushion (19). Because results of previous experiments suggest that there is no cap structure at the 5′ end of the BVDV genome (3), genomic RNA was ligated without previous treatment with pyrophosphatase. An 8-μg portion of RNA was ligated in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 20 U of rRNasin (Promega), 10 μg of bovine serum albumin per ml, and 1 mM ATP, with 10 U of T4 RNA ligase (New England Biolabs). The mixture was incubated for 4 h at 37°C. RNA was extracted with phenol-choloform, precipitated with ethanol, pelleted, and resuspended in RNase-free water. Portions (2 μg) of RNA were amplified by RT-PCR as described previously (34). Portions (2 μl) of each PCR product were reamplified with a nested set of primers. For RT, a minus-sense primer that hybridizes to the 5′ noncoding region was used. For the two amplification steps, we used plus-sense primers that hybridize to the 3′ noncoding region and minus-sense primers that hybridize to the 5′ noncoding region. After phenol-chloroform extraction and ethanol precipitation, PCR products were ligated into pUC21 (39).

All modification and cloning procedures were carried out essentially as described previously (27). Restriction enzymes and DNA-modifying enzymes were purchased and used as specified by the manufacturer. Plasmids pGEM4Z-blue, pGEM5Zf(+), and pUC21 (Promega) were used as vectors. *Escherichia coli* DH5α (8) was used for propagation of plasmids and cDNA clones.

Sequencing of cDNA. Plasmid DNA used for sequencing was extracted and purified either by alkaline lysis and LiCl precipitation or by CsCl centrifugation (27). The T7 DNA polymerase-based sequencing kit (Pharmacia) was used for direct double-stranded sequencing of plasmid DNA. In addition to the SP6, T7, and universal pUC/M13 forward and reverse primers, we used oligonucleotide primers based on the sequence of CSFV strain Brescia (20). Primers were synthesized with a Cyclone DNA synthesizer (Millipore) or with a 392 DNA/RNA synthesizer (Applied Biosystems). Sequence reaction products were analyzed on a 6% acrylamide gel containing 8 M urea. Sequence data were analyzed with a Compaq 386 computer, using Speedreader hardware and PCgene software (Intelligenetics Inc., Applied Imaging Corp., Geneva, Switzerland), and with an Apple Macintosh computer, using the program MacMolly*tetra.*

Construction of full-length cDNA clones pPRKflc-113, pPRKflc-133, and pPRKflc-h6. Full-length DNA clone pPRKflc-113 was composed from the cDNA clones depicted in Fig. 1B. First, two subclones were constructed in the vector pGEM4z-blue, one (pPRc64) containing the cDNA sequence of the 5′ half of the genome (nucleotides 1 to 5560), and the other (pPRc-111) containing the cDNA sequence of the 3′ half of the genome (nucleotides 5463 to 12311). Plus-sense primer 5′-AGATTGAATTCGTCGACTAATACGACTCACTATAGTATACG AGGTTAGTTCATTCTC-3′ was used for PCR amplification to introduce unique *Eco*RI and *Sal*I sites flanking the T7 RNA polymerase sequence, which is joined directly to the 5′-terminal nucleotide of the C-strain genome, in pPRc64. Minus-sense primer 5′-GAGGGATCCAGATCTAGAGCCCGGGC CGTTAGAAATTACC-3′ was used for PCR amplification to introduce unique *Srf*I and *Xba*I sites flanking the C-strain sequences in pPRc111. When the *Srf*I site was digested, the exact 3′ terminus of the C-strain cDNA sequence was generated. To increase the stability of the plasmids, we recloned inserts of plasmids pPRc64 and pPRc111 in vector pPRK, which resulted in pPRc108 and pPRc123, respectively. The vector pPRK, which is derived from the low-copy-number vector pOK12 (39), was used for all further full-length cloning. The vector still contains unique *Spe*I, *Not*I, *Eag*I, *Bam*HI, *Eco*RV, *Eco*RI, and *Xba*I sites in the multiple-cloning site. Finally, full-length cDNA clone pPRKflc-113 was constructed by joining the inserts of pPRc123 and pPRc108 at the unique *Nco*I site (nucleotide 5532) in the sequence of the C strain.

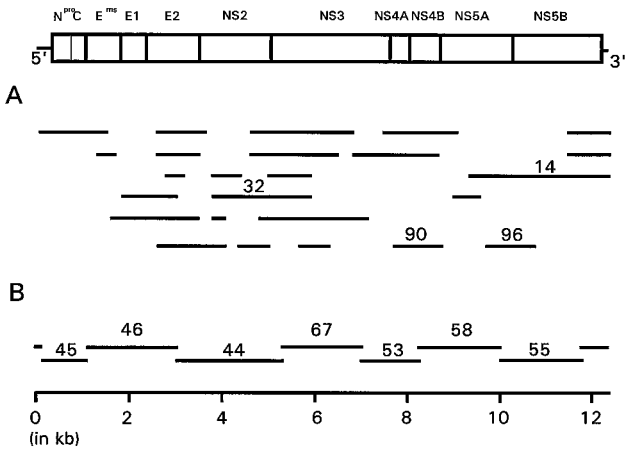

FIG. 1. Schematic representation of the cDNA clones used to determine the nucleotide sequence of the C strain. (A) First-round cDNAs. Clone 14 was the only first-round cDNA used for construction of pPRKflc-113 (see Fig. 4). cDNA clones 32, 90, and 96 were used to change pPRKflc-113 into pPRKflc-133 (see Fig. 4). (B) Second-round cDNAs. The numbered second-round cDNAs were used to construct pPRKflc-113 (see Fig. 4). Positions of the cDNA inserts in the nucleotide sequence of the C strain are indicated by the scale bar (in kilobases) at the bottom of the figure. A schematic representation of the pestivirus genes and their organization in the genome is presented at the top of the figure.

The five nucleotides in pPRKflc-113 that deviate from the predominant sequence (two of three) of the C strain and cause amino acid changes were corrected by exchanging affected DNA fragments with DNA fragments originating from cDNA clones 14, 32, 90, and 96 (Fig. 1), which contain the predominant sequence. The resulting full-length cDNA clone was designated pPRKflc-133.

To demonstrate that antigenically different but viable C-strain mutants can be made from pPRKflc-133, we exchanged the 5′ half of the E2 gene of this construct with the equivalent region of CSFV strain Brescia. The *Nhe*I-*Afl*II fragment of pPRKflc-133 was replaced with the corresponding fragment of pPEh6 (37). The hybrid full-length clone pPRKflc-h6 contains the antigenic region of E2 of CSFV strain Brescia, including a unique *Bgl*II site (34).

RNA transcription and transfection. Plasmid DNA used for the transcription of RNA was purified on columns (Qiagen) as specified by the manufacturer. After plasmid DNA was linearized with *Xba*I or *Srf*I, it was extracted with phenol-chloroform, precipitated with ethanol, vacuum dried, and dissolved in an appropriate volume of RNase-free water. A 1-μg portion of linearized plasmid DNA was used as a template for in vitro transcription. RNA was synthesized at 37°C for 1 h in 100 μl of a reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 100 U of rRNasin (Promega), 0.5 mM each ATP, GTP, CTP, and UTP, and 170 U of T7 RNA polymerase (Pharmacia). Then the template DNA was removed by incubating the reaction mixture with RNase-free DNase I (Pharmacia) for 15 min at 37°C. The reaction mixture was extracted with phenol-chloroform, and the RNA was precipitated with ethanol. The RNA was dissolved in 20 μl of water and quantified by determining the A_{260}.

The RNA transfection mixture was composed by gently mixing 50 μl of lipofectin dilution containing 10 μg of lipofectin (Gibco BRL) with 50 μl of RNA solution containing 1 μg of RNA. The mixture was then incubated at room temperature for 15 min. Subconfluent monolayers of SK6 cells in 35-mm-diam-

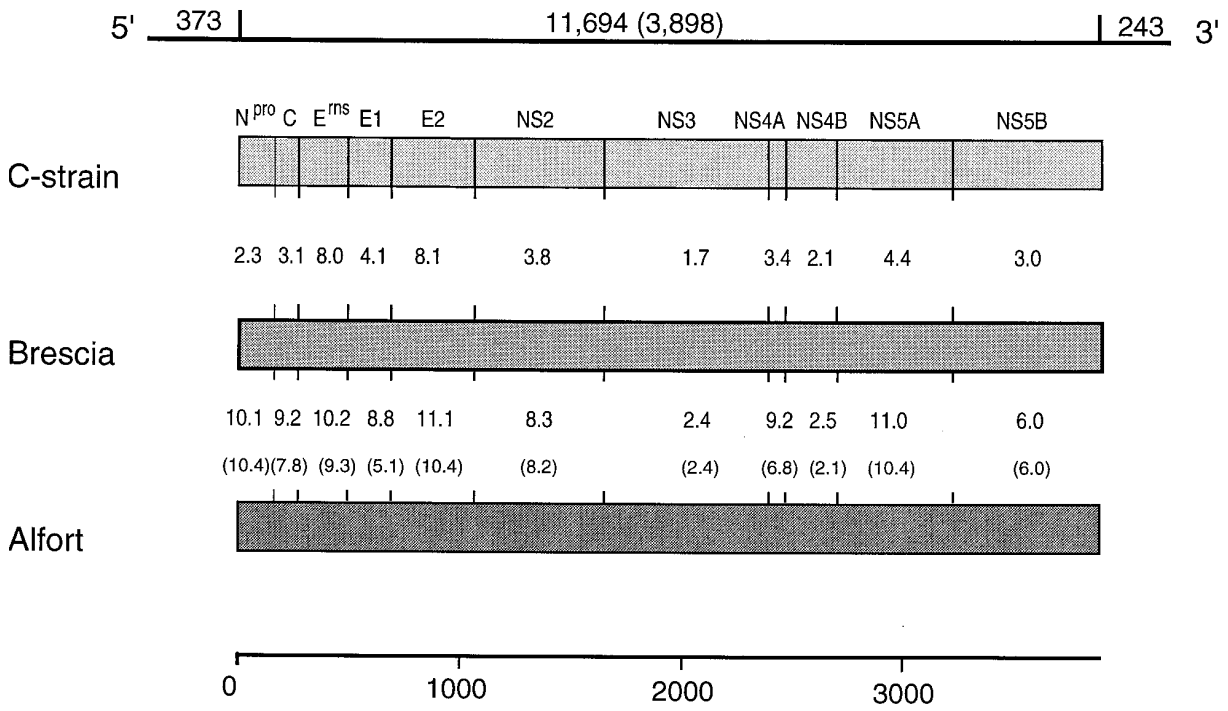

FIG. 2. Schematic representation of the nucleotide sequence of the C strain (top line), indicating the large ORF of 11,694 nucleotides, encoding a polyprotein of 3,898 amino acids, and the 5′ and 3′ noncoding regions of 373 and 243 nucleotides. The middle part shows a comparison of the amino acid sequences encoded by the large ORFs of CSFV Brescia and Alfort and the C strain. The numbers indicate the percentage of amino acids that differ between the proteins of the C strain and strains Brescia or Alfort or the percentage of amino acids that differ between the proteins of strains Brescia and Alfort (numbers in parentheses). The scale bar at the bottom indicates the position of the viral proteins (4, 20, 26, 32) in the polyprotein sequence.

eter wells (Greiner) were transfected with the RNA. The cells were washed twice with DMEM, and 1 ml of DMEM was added, followed by the RNA transfection mixture. After incubation for 16 h at 37°C, the medium was replaced with 2 ml of DMEM supplemented with 5% fetal bovine serum. Incubation was continued for 3 days at 37°C, after which the supernatant was stored. The cells were immunostained with CSFV-specific monoclonal antibodies (MAbs) in an immunoperoxidase monolayer assay as described by Wensvoort et al. (43).

Characterization of recombinant C-strain viruses. Stocks of virus were prepared by mixing 0.1 ml of the supernatant of transfected cells (see previous section) with 2.0 ml of DMEM plus 5% fetal bovine serum and applied to confluent monolayers of SK6 cells in 35-mm wells (Greiner). After incubation for 6 days at 37°C, the cells were trypsinized, diluted 7.5-fold in DMEM plus 5% fetal bovine serum, and grown for another 7 days at 37°C in T75 flasks (Costar). The cells were then freeze-thawed twice, and cell suspensions were clarified by centrifugation at 5,000 × *g* for 10 min at 4°C. Because the clarified supernatants contained only low virus titers ($10^{2.7}$ $TCID_{50}$/ml), they were passaged three more times, resulting in 150 ml of virus stocks with titers of about $10^{5.5}$ $TCID_{50}$/ml.

The viruses FLc-h6 and FLc-133 were characterized by restriction enzyme analysis of viral E2 fragments amplified by RT-PCR. The amplified fragments were then analyzed for the presence of a *Bgl*II site and the absence of a *Hin*cII site (FLc-h6) or vice versa (FLc-133). In addition, viruses were characterized by an immunoperoxidase monolayer assay. For this test, SK6 cells were infected with strains FLc-h6, FLc-133, and Brescia and the C strain. After incubation for 4 days at 37°C, monolayers were immunostained with MAbs directed against conserved (MAb b3) and nonconserved (MAbs b5 and b6) epitopes on E2 of strain Brescia and with MAbs directed against E2 (MAb c2) or E^{rns} (MAb c5) of the C strain (41).

Growth kinetics of the viruses were determined in SK6 cells. Confluent monolayers in T25 flasks (Costar) were infected at a multiplicity of infection of 0.01 with strains Brescia, FLc-133, and FLc-h6 and the C strain. After adsorption of virus for 1.5 h, the medium was removed and fresh medium was added. At 0, 6, 12, 24, 36, 54, 72, 100, and 144 h after infection, duplicate flasks were freeze-thawed twice and clarified by centrifugation at 5,000 × *g* for 10 min at 4°C. The virus titer ($TCID_{50}$ per milliliter) of the supernatants was determined by end point dilution as described previously (43).

Nomenclature. The nomenclature of the pestivirus proteins used in this report will be proposed to the International Committee on Taxonomy of Viruses by the Flaviviridae Study Group. The nomenclature of the pestivirus envelope proteins used by the CSFV research group in Lelystad in previous publications was as follows: E1 (10, 20, 34, 35, 37, 40, 42), renamed E2; E2 (9), renamed E^{rns}; and gp31 (41) or E3 (9), renamed E1.

Nucleotide sequence accession number. The complete nucleotide sequence of the C strain has been submitted to the EMBL/GenBank database and assigned accession number Z46258.

RESULTS

Nucleotide sequences of the C strain of CSFV and the 5′ and 3′ termini of pestivirus genomes. The entire genomic sequence of the C strain was determined by sequencing a minimum of two cDNA fragments, obtained after independent PCRs (Fig. 1). If differences were found between the nucleotide sequences of two clones of a particular region, the consensus nucleotide sequence of that region was determined by sequencing a third independent cDNA clone (Fig. 1A). The nucleotide sequences of cDNA clones that were obtained by the 3′-5′ RNA ligation method (13) and that contained the 5′ and 3′ termini of the C-strain genome (see Fig. 3B) completed the nucleotide sequence (EMBL/GenBank database accession number Z46258).

The sequence contains one large open reading frame (ORF) that is of the same length (11,694 nucleotides) as the large ORFs of CSFV strains Brescia (20) and Alfort-T (15). When the amino acid sequences encoded by these ORFs were compared, the C strain was found to be more closely related to strain Brescia than to strain Alfort-T (Fig. 2). Furthermore, the amino acid sequences of the C strain and strain Alfort-T appeared to diverge as much as did the amino acid sequences of strains Brescia and Alfort-T.

The large ORF is preceded by a 5′ noncoding region of 373 nucleotides. This region is only 1 nucleotide longer than the 5′ noncoding region of the RNAs of strains Brescia and Alfort-T and differs 3.2 and 8.3% from these sequences, respectively (data not shown). In the 5′ noncoding region, sequences of

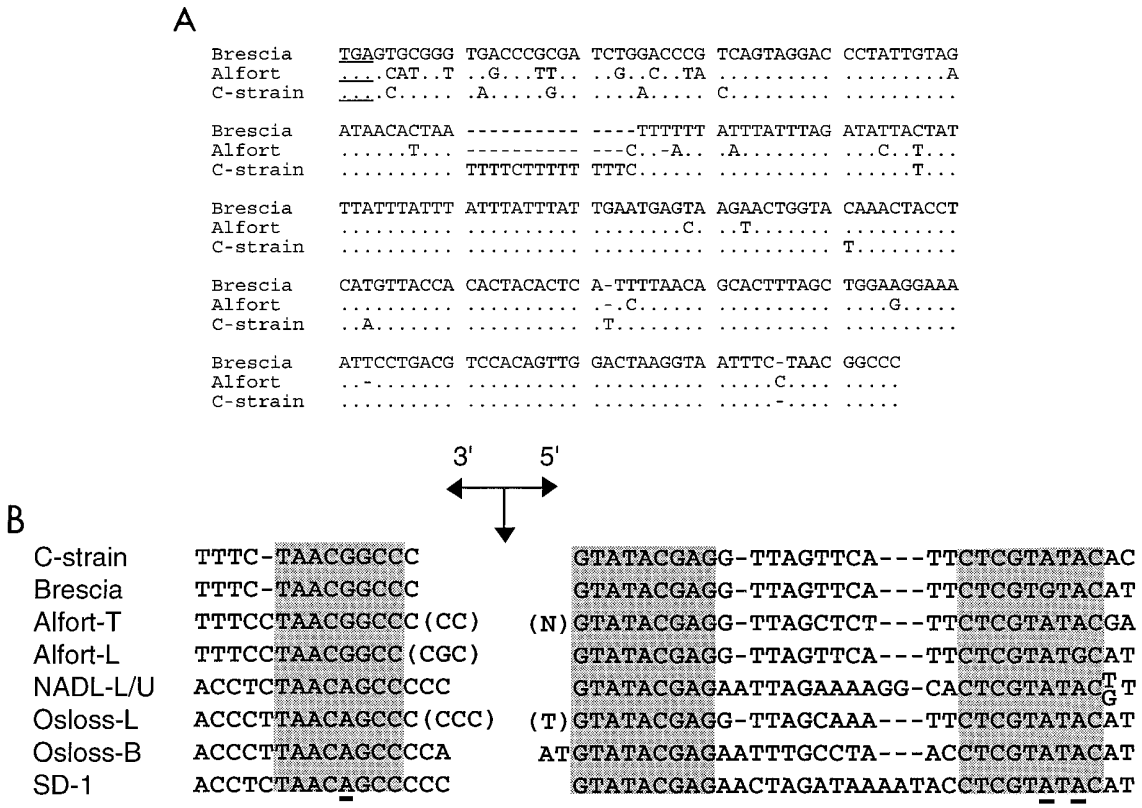

FIG. 3. (A) Alignment of the nucleotide sequences of the 3′ noncoding regions of CSFV strains Brescia and Alfort and the C strain. The TGA stop codons are underlined. (B) Alignment of nucleotide sequences of cDNA originating from the 3′ and 5′ ends of the genomic RNAs of CSFV strains Brescia, Alfort-T, and Alfort-L and the C strain and BVDV strains NADL-L, NADL-U, Osloss-L, Osloss-B, and SD-1. Sequences of strains Brescia, Alfort-L, NADL-L, and Osloss-L were determined essentially as described for the C strain, from batches of virus strains which were biologically cloned and amplified at our institute. All sequences, including that of the C strain, were determined with viral plus-strand RNA. We also determined the 5′ and 3′ termini of strain Brescia from minus-strand RNA. The sequences of strains Alfort-T (14), NADL-U (3), Osloss-B (23), and SD-1 (6) were taken from published data. Shaded boxes show highly conserved regions. Incompletely conserved nucleotides are underlined. Sequences in parentheses were found in a minority of the cDNAs.

strains Brescia and Alfort-T have diverged 8.3%. Following the large ORF, the sequence of the C strain has a 3′ noncoding region of 243 nucleotides, which is longer than the equivalent region in strains Alfort-T and Brescia (229 nucleotides). This difference is caused mainly by the sequence TTTTCTTTTTT TT, which is unique in the RNA of the C strain (Fig. 3A). Except for this inserted sequence, the sequences of the 3′ noncoding regions of strain Brescia and the C strain are less divergent (4.3%) than those of strain Alfort-T and the C strain (12.2%). The sequence divergence in this region between strains Brescia and Alfort-T is 10.9%.

The sequences at the termini of the genomic RNAs of strains Alfort-L, NADL-L, Osloss-L, and the C strain were established by the 3′-5′ RNA ligation method (13) and on the basis of homology with the sequences at the termini of the RNAs of several other BVDV and CSFV strains (Fig. 3B; cDNA sequences are shown). Although the 3′-terminal sequence of the RNA of strain Brescia has been described previously (20), this is the first time the 12 nucleotides at the 5′ terminus have been determined.

At both the 5′ and 3′ termini, the sequences of pestivirus genomic RNAs show heterogeneity and are conserved (Fig. 3B). At the 3′ termini, a nucleotide stretch varying in length from 2 to 6 C's is generally observed. However, in the genomic RNA of strain Alfort-L, a G is occasionally part of this stretch, whereas the RNA of strain Osloss-B terminates with an A. Partly overlapping this stretch of C's is a conserved sequence of 8 nucleotides, TAAC(A/G)GCC. Remarkably, at the one position where this sequence is not completely conserved, a G is found in all the CSFV strains and an A is found in all the BVDV strains. At the 5′ termini, the genomic RNAs of pestiviruses start predominantly with the fully conserved sequence GTATACGAG. Preceding this sequence, one nucleotide is occasionally observed in the genomes of strains Alfort-T and Osloss-L, and an AT was reported at the 5′ terminus of the RNA of strain Osloss-B. After 11 to 15 nucleotides, a second stretch of conserved nucleotides, CTCGTATAC, follows. In this stretch, the first A is replaced by a G in strain Brescia. In strain Alfort-L, the second A is replaced by a G.

Generation of infectious transcripts of a full-length DNA copy of the C-strain genome. Initially, the full-length cDNA clone of the genome of the C strain was constructed in pGEM4z-blue, a high-copy-number plasmid. However, this approach failed because a full-length insert could not be recovered with this vector. To increase the stability of the inserts, a full-length cDNA was constructed in the low-copy-number plasmid pOK12 (39), which was modified by deleting most of the restriction sites of the multiple-cloning site and the T7 promoter sequence. The resulting vector, pPRK, was used for all further full-length cloning. Full-length DNA constructs in pPRK appeared much more stable than in pGEM4z-blue but nonetheless started showing rearrangements and slowly lost inserts after two or three rounds of propagation of transformed *E. coli* DH5α. Therefore, *E. coli* DH5α that was transformed with

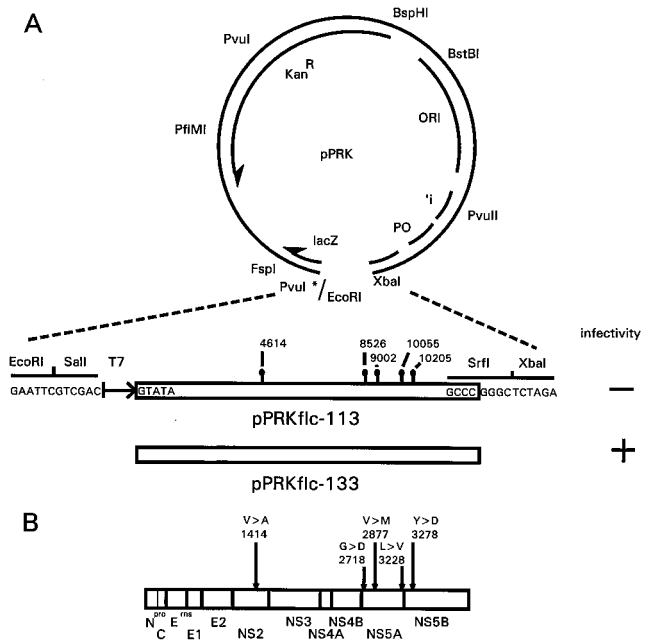

FIG. 4. (A) Schematic representation of the vector and full-length cDNA inserts in clones pPRKflc-113 and pPRKflc-133. Vector pPRK was derived from pOK12 (39). Transcripts initiate from the T7 promoter (T7) and start with GUAU. Linearizing the constructs at the unique *Srf*I site generates transcripts terminating with GCCC. The circles on stalks and numbers in pPRKflc-113 correspond to the nucleotides of the five codons that were changed, resulting in pPRKflc-133. (B) The arrows show positions of the amino acids differing between pPRKflc-113 and pPRKflc-133 in the polyprotein encoded by the large ORF of CSFV. Amino acid residues before and after the arrow are encoded by pPRKflc-113 and pPRKflc-133, respectively. Four of five differences are located in NS5A and NS5B, presumably specifying the viral replicase proteins. The fifth difference is located in the strongly hydrophobic protein, NS2, whose function is unknown.

full-length constructs was never propagated more than once, and amplification of DNA was performed with newly transformed bacteria.

To obtain C-strain transcripts with the correct 5′ and 3′ termini, we fused the 5′-terminal nucleotide of the C-strain cDNA to the 3′-terminal nucleotide of the T7 promoter sequence (Fig. 4A). Runoff transcripts ending with three consecutive C's were generated by introducing a unique *Srf*I site at the 3′ terminus of the full-length constructs.

Transcripts generated from the first full-length cDNA clone, pPRKflc-113, which was constructed from cDNA obtained after the second round of cloning (numbered cDNAs in Fig. 1B), were noninfectious. After the predominant sequence had been determined, five nucleotide differences were found between pPRKflc-113 and this sequence. These differences resulted in amino acid changes at positions 1414 (V → A), 2877 (V → M), and 3228 (L → V), 2718 (G → D), and 3278 (Y → D) in the polyprotein sequence (Fig. 4B). All amino acid differences were located in the nonstructural protein region of the genome. Four of these differences were located in the predicted viral replicase proteins (4). Replacement of the sequence encoding these five amino acids in pPRKflc-113 by sequences encoding the predominant residues resulted in full-length cDNA clone pPRKflc-133 (Fig. 4A). In contrast to the in vitro transcripts generated from pPRKflc-113, which were noninfectious, those generated from pPRKflc-133 were infectious. As expected, C-strain-specific MAbs reacted identically with this new virus, designated FLc-133, and native C-strain virus (see below). Because of the apparent instability of pPRK flc-133, we analyzed the infectivity of transcripts of five other independent full-length constructs originating from the same transformation as pPRKflc-133. The transcripts of these

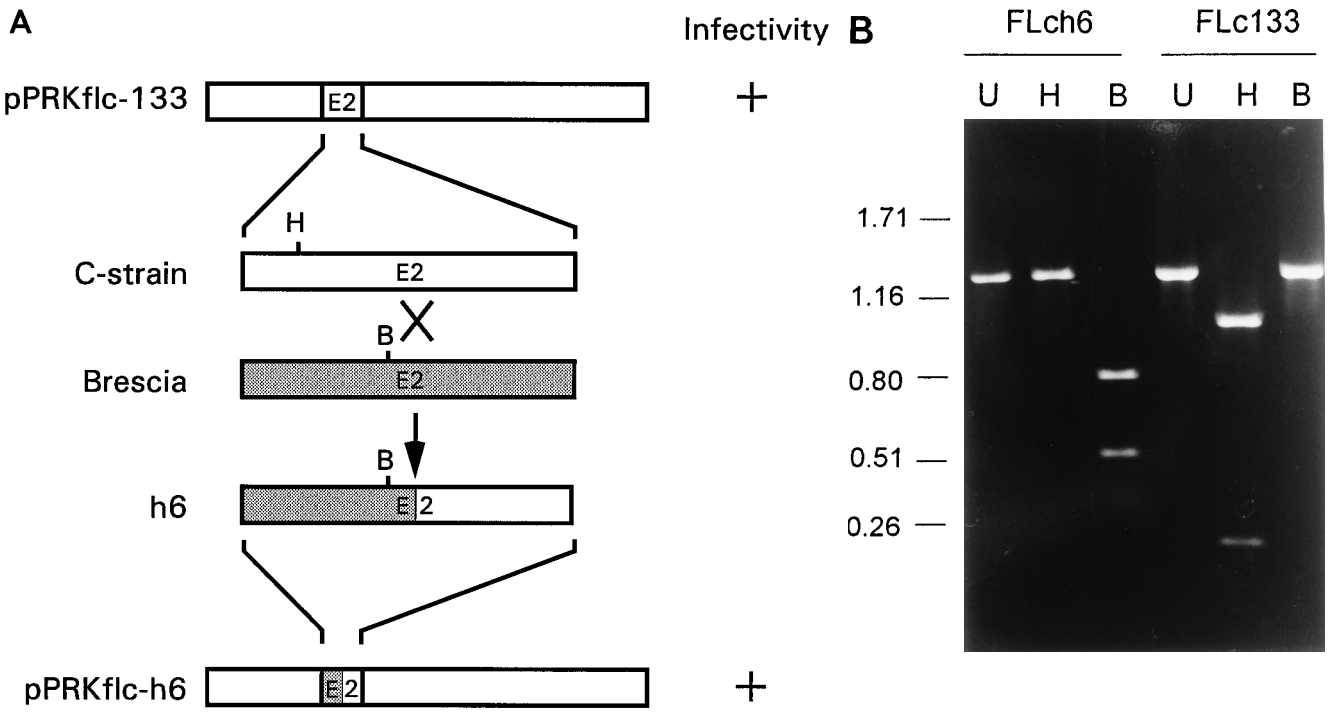

FIG. 5. (A) Construction of full-length clone pPRKflc-h6. The antigenic N-terminal part of E2 in pPRKflc-133, encoded by codons 692 to 877 of the large ORF, was replaced with the equivalent region of E2 of strain Brescia by exchanging the relevant *Nhe*I-*Afl*II fragment of pPRKflc-133 with that of pPEh6 (37). The replacement resulted in the omission of a *Hin*cII site (H) from E2 of pPRKflc-133 and the introduction of a *Bgl*II site (B) into E2 of pPRKflc-h6. (B) Analysis of cDNA fragments covering the E2 genes of FLc-h6 and FLc-133 indicated that both viruses have the expected restriction enzyme pattern. U, undigested; H, digested with *Hin*cII, B, digested with *Bgl*II. Size markers are shown in kilobases.

TABLE 1. Characterization of recombinant C-strain viruses

Strain	Reaction of CSFV-specific MAbs			
	Directed against E2[a]			Directed against E^{rns}[b]
	Conserved epitope	Brescia-specific epitopes	C-strain-specific epitope	C-strain-specific epitope
C strain	+	−	+	+
Brescia	+	+	−	−
FLc-133	+	−	+	+
FLc-h6	+	+	−	+

[a] MAb b3 recognizes an epitope in domain A of E2 that is conserved among CSFV strains (40). It represents the conserved epitope. MAbs b5 and b6 specifically recognize epitopes in domain B/C of E2 of strain Brescia (40). They represent the Brescia-specific epitopes. MAb c2 (41), which specifically recognizes an epitope in domain B/C of E2 of the C strain (37), represents the C-strain-specific epitope on E2.

[b] MAb c5 (41), which specifically recognizes E^{rns} of the C strain, represents the C-strain-specific epitope on E^{rns}.

cDNAs appeared to be as infectious as those of pPRKflc-133. Moreover, the viruses generated from these transcripts were antigenically indistinguishable from FLc-133 and could be grown to the same titers (results not shown).

Construction and characterization of hybrid full-length clone pPRKflc-h6. To evaluate whether pPRKflc-133 could be used to alter the antigenic properties of the C strain, we constructed a hybrid virus that could be discriminated from strains Brescia and FLc-133 and the C strain with a combination of MAbs specific for strain Brescia and the C strain.

Two distinct antigenic units have been found in the N-terminal half of E2 of CSFV (35). The unit located closest to the N terminus comprises nonconserved antigenic domains B and C, whereas the second unit, which is located C terminally of domain C, contains conserved antigenic domain A (35, 40). We replaced the 5′ half of the E2 gene of pPRKflc-133 with the equivalent region of strain Brescia (which introduced the Brescia-specific epitopes of domains B and C into pPRKflc-133), resulting in hybrid full-length clone pPRKflc-h6 (Fig. 5A).

When RNA transcribed from pPRKflc-h6 was transfected in SK6 cells, an infectious virus, designated FLc-h6, was produced. Because of the replacement, a *Hin*cII site located in E2 of pPRKflc-133 is absent in pPRKflc-h6. Instead, a *Bgl*II site originating from the E2 gene of strain Brescia and absent in the E2 gene of pPRKflc-133 is present in the E2 gene of pPRKflc-h6. The PCR-amplified cDNA of the genomic RNA of FLc-h6 was also found to contain a *Bgl*II site and lacked the *Hin*cII site, indicating that FLc-h6 contained the hybrid E2 gene (Fig. 5B).

The chimeric nature and changed antigenic properties of FLc-h6 were confirmed upon analysis of the epitopes on the viral envelope proteins E^{rns} and E2 of strains Brescia, FLc-133, and FLc-h6 and the C strain (Table 1). As expected, MAb b3, which recognizes a conserved epitope of domain A on E2 (40), reacted with all four strains tested. MAbs b5 and b6, specific for E2 of strain Brescia, reacted only with strains Brescia and FLc-h6. In contrast, MAb c2, which is specific for E2 of the C strain, reacted only with the C strain and FLc-133. Thus, strains Brescia and FLc-h6 could not be differentiated with MAbs directed against E2. However, the use of MAb c5, which recognizes E^{rns} of the C strain but not of strain Brescia (40), enabled us to clearly differentiate these two strains.

Growth kinetics of FLc-133 and FLc-h6. The growth kinetics of FLc-133 and FLc-h6 were established and compared with those of strain Brescia and the C strain. After RNA transfection, virus stocks of FLc-133 and FLc-h6 with titers of $10^{5.7}$ and $10^{5.6}$ $TCID_{50}$/ml, respectively, were prepared. Since these titers did not enable us to analyze the growth kinetics of these viruses under single-step infection conditions, a multistep growth curve was determined (Fig. 6). Cells were infected at a multiplicity of infection of 0.01 $TCID_{50}$ per cell, and virus was adsorbed for 1.5 h (time zero). Although the C strain, FLc-133, and FLc-h6 had comparable growth rates, strain Brescia grew significantly faster. At time zero, titers of the C strain, FLc-133, and FLc-h6 were still at a background level whereas a titer of $10^{2.3}$ $TCID_{50}$/ml was measured for strain Brescia. This finding is probably explained by differences in adsorbance of the viruses or differences in infectivity.

At 144 h after infection, titers of the C strain and strains Brescia, FLc-133, and FLc-h6 reached $10^{5.4}$, $10^{7.8}$, $10^{5.8}$, and $10^{5.6}$ $TCID_{50}$/ml, respectively. These titers agreed with titers of the virus stocks. We concluded that FLc-133 and FLc-h6 grew as well in vitro as the C strain from which they were derived.

DISCUSSION

This report describes for the first time the complete cDNA sequence of the C strain, which is a vaccine strain of CSFV, and the construction of a full-length DNA copy of this sequence from which infectious RNA was transcribed. In addition, the infectious copy was used to construct a hybrid virus in which the 5′ half of the E2 gene was replaced by the equivalent region from CSFV strain Brescia.

Because the C strain originates from China and was attenuated by repeated passage in rabbits and cell lines (31), it was expected that its sequence would differ substantially from those of strains Brescia and Alfort-T, which are two virulent CSFV strains originating from Europe. However, the sequence of the C strain was in fact closely related to the sequences of strains Brescia and Alfort-T. Furthermore, it appeared that strains Brescia and Alfort-T had diverged from each other almost as much as strain Alfort-T had diverged from the C strain. However, there was one notable difference between the sequences of the three CSFV strains, which was that the C strain con-

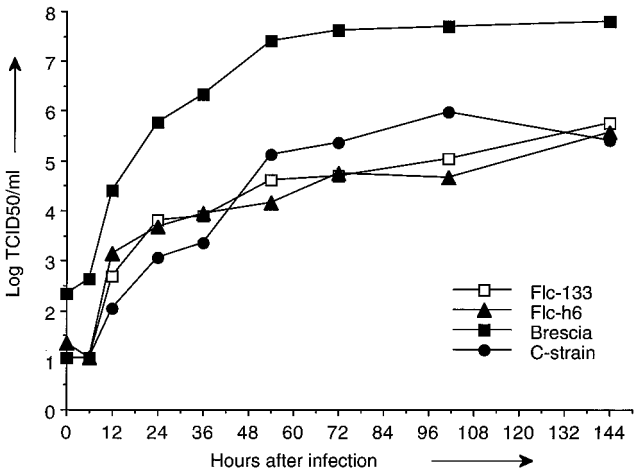

FIG. 6. Multistep growth curves of CSFV strains Brescia, FLc-133, and FLc-h6 and the C strain. Confluent monolayers of SK6 cells were infected at a multiplicity of infection of 0.01 with strains Brescia, FLc-133, and FLc-h6, and the C strain. Virus was adsorbed to cells for 1.5 h, after which cells were supplied with fresh medium. This is time zero. Virus titers ($TCID_{50}$ per milliliter) were established 0, 6, 12, 24, 36, 54, 72, 100, and 144 h after infection from duplicate flasks.

tained an insertion of 13 nucleotides in the 3′ noncoding region.

It has been suggested by Khromykh et al. (11) that sequences in the 5′ and 3′ noncoding regions of flaviviruses are involved in the recognition of the RNA template by the viral replicase and thus are involved in virus replication. As we showed in this study, the native C strain and the recombinant viruses FLc-133 and FLc-h6, which were derived from it, grow to significantly lower titers in vitro than does strain Brescia (Fig. 6). Furthermore, we found a larger amount of intracellular viral RNA in cells infected with strain Brescia than in cells infected with the other three CSFV viruses (results not shown). This suggests that the difference in growth between strain Brescia and the C strain, or its derivatives, is determined at the level of viral RNA synthesis. It is still unclear whether the insertion of 13 nucleotides in the 3′ noncoding region of the C strain is responsible for this difference. However, now that an infectious copy has been made, we can study how this insertion is related, if at all, to replication and whether it is involved in virulence.

The 5′- and 3′-terminal nucleotides of the viral RNA of the C strain have not actually been established. However, we found that the nucleotide sequences of cDNA fragments overlapping the 5′ and 3′ termini of the C strain and strain Brescia were identical. Therefore, we assumed that the termini of the RNA of the C strain are identical to those of strain Brescia. Also, we have not yet established whether the predicted 5′- and 3′-terminal nucleotides of the C strain are the first and last nucleotides of transcripts derived from pPRKflc-133, which was linearized with *Srf*I. However, transcripts generated from plasmids that were linearized at the *Xba*I site and had a 3′ extension of 5 nucleotides were as infectious as transcripts derived from plasmids linearized at the *Srf*I site, which should end at the 3′-terminal C. This finding supports earlier observations that short 3′-terminal extensions do not interfere with infectivity of in vitro transcripts whereas 5′ extensions sometimes do and sometimes do not (see reference 2 for a review).

Lai et al. (12) were able to convert a noninfectious full-length DNA copy of dengue 4 virus into an infectious one by exchanging cDNA fragments suspected of containing deleterious mutations. They made six combinations of cDNA constructs composed from independent cDNAs covering the 5′ and 3′ halves of the viral genome, and they obtained one full-length copy that generated infectious transcripts. In contrast to Lai et al., however, we specifically corrected five amino acid mutations in noninfectious clone pPRKflc-113 and produced the infectious clone pPRKflc-133. Nevertheless, we cannot exclude the possibility that during amplification in *E. coli*, clone pPRKflc-113 or subclones that were used to construct it underwent additional mutations. Further experiments should be done to correct the five mutations in the DNA fragments originating from pPRKflc-113. Only then can it be concluded whether each of the five mutations or a combination of two or more mutations interfere with the infectivity of the in vitro transcripts derived from pPRKflc-113.

It was difficult to quantitatively compare the infectivity of the transcripts of pPRKflc-133 with the infectivity of viral RNA of the C strain, because the in vitro transcripts appeared as only a smear in an agarose gel with a band of low intensity at full-length size. Furthermore, the amount of C-strain RNA isolated from infected cells was so small that it could be visualized only on Northern (RNA) blots (not shown). Nevertheless, we estimate that the full-length in vitro transcripts generated at least 100 to 1,000 times fewer infectious loci than did native C-strain RNA. This finding agrees with observations by other workers (2, 12, 25, 28). The reduced infectivity of our in vitro transcripts is presumably caused mainly by the T7 polymerase, which probably generates a large number of nonviable errors in the full-length RNA. The poor yield of full-length in vitro transcripts is probably caused by premature termination of transcription by the T7 polymerase, as a result of secondary structures in the CSFV sequence. In control experiments in which the T7 polymerase was used to transcribe smaller CSFV-specific DNA sequences or DNA sequences of other origin, we obtained homogeneous full-length transcripts. Furthermore, the plasmid preparations contained high percentages of supercoiled forms (>80%), excluding the possibility that nicked templates caused the size heterogeneity of the transcripts. However, despite the low infectivity of the in vitro RNA, infectious transcripts of at least five independent full-length cDNAs had the same antigenic and growth properties as FLc-133. Moreover, FLc-133 is indistinguishable from the C strain. For these reasons, we believe that pPRKflc-133 is an excellent tool for studying pestiviruses and for developing a CSFV marker vaccine.

A CSFV marker vaccine which must induce antibodies different from those induced by field virus could be developed by mutating domain A of envelope protein E2. Because domain A is conserved among CSFV strains, all field viruses induce antibodies against it (40). Thus, a diagnostic test that could specifically detect antibodies directed against this domain could detect all CSFV field virus infections. However, E2 also induces a protective antibody response against CSFV (10, 38), which suggests that it is important for virus infection. As we showed in this report, hybrid virus FLc-h6 (in which the antigenic N-terminal half of E2 of the C strain was exchanged with the equivalent region of strain Brescia) was viable and had the same growth kinetics as its parent virus FLc-133 (Fig. 6). However, when the same region of E2 of FLc-133 was exchanged with the equivalent region of E2 of BVDV strain NADL, a virus with strongly impaired growth properties in both porcine and bovine cells was obtained (36). Thus, caution should be used in modifying E2 in order not to interfere with its viral functions.

In developing a C-strain marker vaccine, infectious clone pPRKflc-133 can also be used to modify antigenic viral proteins other than E2 or to add a heterologous gene encoding a unique antigenic protein to the genome of the C strain. Moreover, this clone may also prove to be an excellent tool for studying the replication, virulence, cell and host tropism, and pathogenesis of CSFV.

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