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Generation of cytopathogenic subgenomic RNA of classical swine fever virus in persistently infected porcine cell lines

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Abstract

Two biological clones (A.1 and B.2) of the classical swine fever virus strain Alfort/187 and the recombinant virus vA187-1, derived from a cDNA clone of Alfort/187, were used to establish persistently infected cultures of the swine kidney cell lines SK-6 and PK-41. It was found that 100% of the cells in the passaged cultures were positive for viral antigen throughout the course of the experiment. Additionally, supernatants collected upon passaging of the cells continuously contained high titers of infectious virus. In six separate cultures persistently infected with either the biological clones or the recombinant virus, a cytopathic effect occurred spontaneously between passage 8 and 94. The cytopathogenic agent in the supernatants of these cultures could be passaged repeatedly, suggesting the generation of a mutant virus. Analysis of RNA from such cultures revealed the presence of a subgenomic viral RNA of approximately 8 kilobases (kb). In all six cases, this RNA had an identical internal deletion of 4764 nucleotides, including the region coding for all structural proteins. The subgenomic RNA replicated and was packaged in the presence of wild-type virus. Cells infected with cytopathogenic virus contained increased amounts of the viral protein NS3 thought to be involved in pestivirus cytopathogenicity. © 1997 Elsevier Science B.V.

Keywords: Classical swine fever virus; Cytopathogenicity; Persistence; Pestivirus

1. Introduction

Classical swine fever virus (CSFV), a small enveloped virus, is the causative agent of a highly contagious disease in pigs leading to substantial economical losses. It belongs to the genus Pestivirus within the family Flaviviridae (Wengler et al., 1995), together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV) of sheep. The CSFV genome is a positive-stranded RNA of approximately 12.3 kilobases (kb) containing a single open reading frame (ORF) which codes for a polyprotein of 3898 amino acids that is co- and posttranslationally processed by viral as well as cellular proteases (Thiel et al., 1996).
With respect to their effect on tissue culture cells, two biotypes can be distinguished for all pestiviruses (Thiel et al., 1996). The noncytopathogenic (ncp) biotype replicates without obvious damage to the host cell whereas the cytopathogenic (cp) variant destroys the infected cells, presumably through the apoptosis pathway (Zhang et al., 1996). In the case of BVDV, the cp viruses are considered to represent mutants of the respective ncp form, which are generated by RNA recombination during replication in persistently infected cattle. The genomes of most cp isolates contain duplications or rearrangements of viral sequences, insertions of host cellular sequences with or without duplication of viral sequences or internal deletions (Meyers et al., 1989, 1991, 1992; Qi et al., 1992; Tautz et al., 1993, 1994). In all cases analyzed so far, these mutations lead to production of the nonstructural protein NS3 in addition to unprocessed protein NS2/3 present in cells infected with ncp BVDV. Thus, NS3 is a marker protein for cp BVDV and also a prime candidate for the cp agent in this system. In contrast, cells infected with ncp CSFV or BDV contain both NS3 and NS2/3 (our results and Becher et al., 1994; Thiel et al., 1996), raising doubts about the direct correlation of NS3 expression with cytopathogenicity in cell culture.

Most CSFV strains grow in cell culture without exhibiting a cytopathic effect (CPE) (Moennig and Plagemann, 1992; Thiel et al., 1996) and reports about cytopathogenicity of the CAP strain (Laude, 1978) could not be confirmed in our laboratory. Only recently, three cp CSFV isolates were described and shown to contain defective interfering (DI) particles (Meyers and Thiel, 1995). In contrast, cp BVDV is always isolated together with an antigenically related ncp virus from animals with mucosal disease (Brownlie et al., 1984; Bolin et al., 1985). In most cases the two biotypes can be cloned and propagated as homogeneous populations in cell culture. In contrast, cytopathogenicity of CSFV is caused by a defective virus which can only replicate in the presence of a wild-type helper virus.

Viral persistence is normally used in terms of chronic or latent viral infections in animals or humans. Examples such as BVDV, human immunodeficiency virus, herpes simplex virus or hepatitis B virus show that viruses can persist in host organisms for very long periods of time. However, the molecular basis of persistence is largely unknown and is additionally complicated by the fact that various mechanisms seem to operate in different cell–virus systems (reviewed in Holland, 1990; Oldstone, 1991; Ahmed et al., 1996). Persistently infected cell cultures represent a valuable tool to study the mechanisms of viral persistence and the molecular evolution of viruses and cells (de la Torre et al., 1985, 1988). The latter aspect is especially interesting in the case of single-stranded RNA viruses which lack proof-reading and postreplicative error correction mechanisms and are believed to have a high mutation rate (Lai, 1992; Domingo et al., 1996). We report here the establishment of cell cultures persistently infected with CSFV and the spontaneous generation of cp subgenomic RNA upon repeated passage of the cells.

2. Materials and methods

2.1. Cells and viruses

Porcine kidney cell line PK-41, obtained from IFFA-Credo (Lyon, France) was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL/Life Technologies) containing 5% (v/v) horse serum (Sebak, Berlin, Germany). The swine kidney cell line SK-6 (Kasza et al., 1972), kindly provided by M. Pensaert (Faculty of Veterinary Medicine, Gent, Belgium) was seeded in minimal essential medium (MEM) containing Hanks salts (Gibco BRL/Life Technologies) and 10% (v/v) horse serum; 3 h later, the medium was replaced by MEM containing Earle’s salts (Gibco BRL/Life Technologies) and 5% (v/v) horse serum.

CSFV strain Alfort/187, originally isolated from a pig that died after experimental infection with the original Alfort strain (Dahle and Liess, 1995), was obtained from B. Liess (Institute for Virology, Hannover Veterinary School, Hanover, Germany). It was passaged twice and subsequently cloned by three rounds of end-point
dilution. Two of the resulting clones termed A.1 and B.2 were expanded by two further passages which were then used as virus stocks. Recombinant CSFV vA187-1 was rescued after transfection of in vitro transcribed RNA from a full-length cDNA clone of Alfort/187 (Ruggli et al., 1996).

2.2. Infection of cells

For the generation of persistently infected cultures, $5 \times 10^6$ cells were seeded in 75-cm$^2$ cell culture flasks as described above and infected 24 h later at a multiplicity of infection (m.o.i.) of 1 or mock-infected. The infected cells were passaged twice weekly and supernatants were collected, clarified by low-speed centrifugation and stored at $-70^\circ$C.

For virus passages, monolayers of cells in 25-cm$^2$ flasks were infected with 0.5 ml supernatant or 0.5 ml virus stock obtained from freeze-thawed infected cells, diluted with 1.5 ml serum-free medium. After 2 h, 3 ml medium containing 5% (v/v) horse serum were added. After 48 h (PK-41: 72 h), the virus was released from the infected cells by two cycles of freezing and thawing. Virus stocks obtained by low-speed centrifugation were stored at $-70^\circ$C.

For titration of virus, 10-fold dilutions of the supernatants or virus stocks in medium containing 2% horse serum were dispensed each into five wells of a 96-well plate seeded with either SK-6 or PK-41 cells. The titer was calculated after monitoring of CSFV-specific infection by using an indirect immunofluorescence or immunoperoxidase assay 48 h postinfection.

For CPE assays, cells in 24-well plates were infected for 2 h with 0.3 ml of a 1:10 dilution of the virus passages followed by the addition of 0.7 ml medium containing 3% (v/v) horse serum, incubated for 48 h at 37°C postinfection and stained with crystal violet as described below.

2.3. Indirect immunofluorescence assay

After removal of the medium, infected cells were fixed with ethanol at $-20^\circ$C for 10 min. Cells were then incubated for 30 min at 37°C in the presence of CSFV-specific monoclonal antibody HC/TC 26 (Dr. W. Bommeli AG, Bern, Switzerland) directed against glycoprotein E2 (Greiser-Wilke et al., 1990). After washing with phosphate-buffered saline (PBS), cells were incubated with FITC-conjugated rabbit anti-mouse immunoglobulins (Dako) for 30 min at 37°C. Finally, cells were washed with PBS and analyzed by fluorescence microscopy.

2.4. Indirect immunoperoxidase assay

After removal of the medium, infected cells were dried for 30 min at 37°C and fixed for 2 h at 80°C. Cells were then incubated for 15 min at 37°C in the presence of CSFV-specific monoclonal antibody HC/TC 26. After washing with PBS, 0.1% Tween 80, cells were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) for 10 min at 37°C. Cells were washed again with PBS, 0.1% Tween 80, and incubated with chromogen solution (4 mg/ml 3-amino-9-ethyl-carbazol in N,N-dimethylformamide, AEC), freshly diluted 1/20 in 50 mM sodium acetate (pH 5.0) and 0.05% H$_2$O$_2$). Finally, cells were washed with deionized water and examined for specific staining by light microscopy.

2.5. Crystal violet staining

Cells were fixed as described for the indirect immunoperoxidase assay before the addition of staining solution containing 0.4 mg/ml crystal violet in PBS, 10% (v/v) formaldehyde, and incubated for 3 min at 25°C. After washing with deionized water, the cells were air-dried.

2.6. RNA extraction

Total RNA was extracted from cells 20 h postinfection using Trizol according to the manufacturer’s protocol (Gibco BRL/Life Technologies). Briefly, cells were lysed by the addition of Trizol and several-fold pipetting up and down. The lysate was chloroform-extracted and the RNA contained in the aqueous phase was precipitated with isopropanol, washed once with ethanol and air-dried.
RNA from supernatants of infected cells was extracted by proteinase K digestion: briefly, 0.45 ml of supernatant was mixed with 45 μl 10 × extraction buffer (200 mM Tris—HCl (pH 7.5), 50 mM EDTA (pH 8.0), 5% (w/v) SDS) before adding 5 μl proteinase K (20 mg/ml). The reactions were incubated for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

2.7. Northern blotting

Northern blots were performed essentially following standard procedures (Sambrook et al., 1989). Briefly, 10 μg of total cellular RNA was separated by electrophoresis on formaldehyde denaturing 1% agarose gels and blotted onto positively charged nylon membranes (Boehringer Mannheim) by vacuum transfer in the presence of 20 × SSC, and crosslinked by irradiation with a 254-nm light source (Stratalinker UV Crosslinker, Stratagene) as recommended by the manufacturer. A 32P-labeled antisense RNA probe hybridizing to the 3' terminus of the viral genome (nt 12095–12298) was prepared by in vitro transcription from a cDNA clone of Alfort/187 (Ruggli, unpublished data) with T7 polymerase in the presence of 0.5 mM each ATP, GTP, CTP, 12.5 μM UTP and 1.5 μL [c-32P]UTP (800 Ci/mmol, Amersham). The membrane was incubated at 60°C for 7 h in prehybridization/hybridization solution (5 × Denhardt's reagent, 5 × SSPE, 1% (w/v) SDS, 100 μg/ml herring sperm DNA, 50 μg/ml yeast RNA, 50% (v/v) formamide), followed by overnight incubation at 60°C in fresh hybridization solution supplemented with the labeled probe. Subsequently, the blot was washed three times for 30 min in 1 × SSPE/0.5% SDS and once in 0.1 × SSPE/0.5% SDS. All washing steps were performed at 65°C. Bands were visualized by electronic autoradiography (Instant Imager 2024, Packard, Meriden, CT) and by exposure to X-ray film.

2.8. Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extracted by proteinase K digestion from 0.2 ml supernatant of CSFV infected cells was reverse-transcribed with the Expand Reverse Transcriptase kit (Boehringer Mannheim) in the presence of 100 pmol reverse primer HR3 (complementary to nts 8207–7191 of the CSFV Alfort/187 genome) for 60 min at 42°C. For PCR, the Expand High Fidelity PCR System kit (Boehringer Mannheim) was used. Reactions were performed with 1/10 of the reverse-transcribed cDNA, 20 pmol primers LT7G1 (corresponding to nts 1–21 of the CSFV Alfort/187 genome) and PR2 (complementary to nts 6440–6410 of the CSFV Alfort/187 genome). The cycling profile was 94°C for 10 s, 62°C for 30 s, and 68°C for 8 min with 20 s extension per cycle aimed at amplifying simultaneously the 1704 bp fragment from the subgenomic RNA as well as the corresponding 6478 bp fragment of the full-length RNA in the same tube.

2.9. Nucleotide sequencing

DNA obtained by PCR was purified using MicroSpin S-300 columns (Pharmacia). Cycle sequencing was performed using IR41 infrared dye-labeled primers (MWG-Biotech GmbH, Ebersberg, Germany) and the Sequitherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI). Nucleotide sequences were determined on a LI-COR Model 4000L Automated DNA Sequencer (LI-COR, Lincoln, NE). For further analysis of the nucleotide sequences, the GeneWorks Version 2.5 software (IntelliGenetics, Mountain View, CA) was used.

2.10. Protein extraction and SDS–PAGE

Proteins were extracted 17 h after infection with a hypotonic buffer (20 mM MOPS, 10 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, pH 6.5) and the concentration of protein was adjusted to 1 mg/ml. A total of 15 μl of each sample were mixed with an equal volume of 2 × sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, incubated for 5 min at 95°C and separated by SDS–PAGE (Laemmli, 1970).
2.11. Western blotting

Transfer of proteins from SDS–polyacrylamide gels to BioBlot-NC Membranes (Costar), followed by specific immunological detection (Western blotting), was carried out according to the ECL Western blotting protocol (Amersham). After SDS–PAGE, the gels were equilibrated for 60 min in blotting buffer (48 mM Tris–HCl, 39 mM glycine, 20% methanol, pH 9.2) and the proteins transferred for 30 min to BioBlot-NC membranes using a Trans-Blot SD semidry transfer cell (Bio-Rad) at a constant voltage of 15 V. The membranes were then soaked for 2 min in 0.25% Fast Green in 10% acetic acid and destained for 10 min in 100% acetic acid. Non-specific binding sites were blocked by immersing the membranes overnight in 5% (w/v) dried skim milk dissolved in TBS containing 0.1% Tween-20 (TBS-T) at 4°C. All the following steps were carried out at room temperature. Dilutions of serum and secondary antibody were preadsorbed for 30 min in TBS-T supplemented with 3% (w/v) dried skim milk and proteins extracted from 10⁶ non-infected cells using hypotonic buffer (see above) per ml TBS-T. After washing the membranes with TBS-T, they were incubated for 1 h in diluted primary antibody (rabbit polyclonal serum directed against NS3 of CSFV; Bennett, unpublished), washed extensively before incubation for 1 h in 1/100 diluted horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Dako). After an additional five washes, the blot was incubated for 60 s in ECL detection solution and exposed to X-ray film.

3. Results

3.1. Persistently infected cell lines

Two porcine kidney cell lines, SK-6 and PK-41, were infected at an m.o.i. of 1 with one of the two biologically cloned viruses, either A.1 or B.2, derived from the CSFV strain Alfort/187. The infected cell cultures were continuously passaged twice a week for more than 100 passages and examined for the presence of viral antigen. As visualized by immunocytochemistry, 100% of the cells in the infected cultures were positive for viral antigen throughout the course of the experiment (Fig. 1A). The experiment was repeated with an identical set of four cultures. Additionally, we established cultures of PK-41 and SK-6 cells persistently infected with the recombinant virus vA187-1, which were maintained up to passage 80 after infection. Supernatants collected upon passaging the first set of persistently infected cell cultures were assayed for infectious virus and the titer of every fifth passage was determined on the homologous cell line. Titers varied between 10⁵ and 10⁷ tissue culture infectious dose 50% (TCID₅₀) per ml for supernatants of SK-6 cells and 10³.⁷ and 10⁵.⁷ for supernatants of PK-41 cells, and did not decrease with increasing passage number with either cell line used (Fig. 2).

3.2. Cytopathic effect

At variable timepoints between passage 8 and 94, a CPE was observed in six of the 10 persistently infected cultures, irrespective of the virus used for the initial inoculation. Cells became vacuolarized, rounded up and detached from the culture flasks (Fig. 1C). The timepoint of CPE observed in three of the four cultures for which virus titers were determined is indicated in Fig. 2. Inoculation of uninfected cells with clarified supernatants from these cells resulted in rapid induction of a CPE, demonstrating that the cp agent could be passaged. In SK-6 cultures, the CPE became apparent at 20 h after infection and was complete after 72 h, whereas in PK-41 cultures progression of the CPE was somewhat slower, starting at about 30 h postinoculation. In both cell lines, however, a few cells survived and could be further propagated. These cultures continued to produce infectious CSFV (Fig. 2). Infection of either SK-6 or PK-41 cells seeded in 96-well plates with serial dilutions of the cp supernatants showed that a detectable CPE was only induced at a virus dose of at least 10⁴ TCID₅₀ per well. The appearance of focal cell lysis (Fig. 1C) ruled out the possibility that a soluble cytotoxic agent in the supernatants unrelated to the virus was responsible for the CPE. The slower progres-
Fig. 1. (A) Indirect immunofluorescence of PK-41 cells persistently infected with CSFV strain A1. Cells from passage 102 after infection were stained using the monoclonal antibody HC/TC26: 100% of the cells stained positive for viral antigen. (B) Mock-infected cells of the same passage as in (A). (C) Light microscopy: vacuolization of cells infected with cp virus stocks and the focal origin of the CPE.

pression of the CPE observed with supernatants from persistently infected PK-41 cells may reflect the lower titers that were reached with this cell line and thus the lower amounts of the cp agent present in the respective supernatants.

3.3. RNA analysis

Northern blot hybridization was performed using total cellular RNA extracted from persistently infected cells and from cells infected with superna-
tants of persistently infected cells. To avoid detection of RNA molecules resulting from premature termination of RNA replication, a CSFV-specific probe complementary to the 3' terminal 204 nts of the genome was used (Fig. 3A). In all lanes containing RNA from cells infected with cp virus stocks (Fig. 4, lanes 2, 4–6, 8 and 9), a virus-specific band of about 8 kb was detected in addition to the 12.3-kb band representing wild-type (wt) genomic RNA. No such subgenomic RNA was detected in RNA extracts from cells infected with the parental viruses (Fig. 4, lanes 1, 3 and 7), persistently infected cells which did not exhibit a CPE or from uninfected cells inoculated with supernatants of these cells (data not shown), even after very long exposure of the blots. To determine the timepoint of generation of the cp agent, supernatants of SK-6 cells persistently infected with A.1 showing maximal CPE at passage 52 (Fig. 2) as well as supernatants of the respective culture collected three and six passages earlier were passaged on SK-6 cells to obtain high titer stocks for further studies. The CPE appeared within one to three virus passages (ViPa1 to ViPa3) except when virus passages from six cell passages before the initial CPE were used (Fig. 5A). These data indicate that the event leading to the generation of the cp agent occurred between six and three cell passages before the CPE was maximal. Furthermore, the fact that cells infected with the recombinant virus vA187-1 which had been frozen nine passages before maximal CPE occurred could be thawed and passaged for more than 35 passages without showing a CPE indicate that the generation of cp virus is a random event. Northern blot analysis of cells infected with the first virus passage (ViPa1) showed that similar amounts of full-length genomic RNA were detected irrespective of the presence of sg RNA (Fig. 5B). Hence, the production of high amounts of sg RNA did not interfere with the synthesis of wt genomic RNA.

3.4. Sequence analysis

The results of the RNA analyses were reminiscent of the subgenomic RNA of CSFV defective interfering particles recently described by Meyers and Thiel (1995). Similarly, we detected two virus-specific RNAs of 12.3 and 8 kb in cells which showed a CPE. Therefore, we designed an RT-PCR aimed at the amplification of the sequences flanking the putative deletion found in the described subgenomic RNAs (Fig. 3A and B). RT-PCR of RNA extracted from supernatants of infected cells using oligonucleotides LT7G1 and PR2 indeed resulted in the amplification of a subgenomic RNA-specific DNA fragment of the expected size of 1.7 kb. PCR products obtained from two separate amplifications from each cp virus stock were directly sequenced across the deletion in both directions. Alignment of the obtained sequences revealed exactly the same dele-
Fig. 3. Schematic representation of the genome organization of wild-type (A) and subgenomic RNA (B). Location of PCR primers LT7G1 and PR2 and the riboprobe JL1 is shown. Numbers of the nucleotides flanking the deletion are indicated. (C) Partial sequence analysis of the subgenomic RNA found in the CPE-inducing virus stocks (termed 'cp'). The nucleotide sequences flanking the deletion and their deduced translation products are shown. A.1/N and B.2/N originated from a second set of persistently infected cell cultures. The T/A mutation at position 320 already present in the parent virus B.2 allowed to differentiate B.2 from A.1 or vA187-1.

that each subgenomic RNA was derived independently from the respective parent viruses A.1, B.2 and vA187-1.

3.5. Protein analysis

To analyze the expression of NS2/3 and NS3 in cells infected with the cp virus stocks or with their respective parent viruses, Western blotting experiments were performed. In protein extracts from SK-6 cells infected with the parental viruses vA187-1, A.1 and B.2, more NS2/3 than NS3 could be detected with an NS3-specific rabbit
antiserum (Fig. 6, lanes 1, 3 and 5), whereas NS2/3 was hardly detectable in extracts from cells infected with cp virus stocks (Fig. 6, lanes 2 and 4). However, high amounts of NS3 were found in the latter samples (Fig. 6, lanes 2, 4 and 6). Comparison of the recombinant virus vA187-1 and its cp virus stock revealed a slighter decrease of NS2/3 but a similar increase in NS3 (Fig. 6, lanes 5 and 6) when compared to A.1 and B.2. This was possibly due to the lower titer of the defective particles in the inoculum used in this experiment which was in agreement with a less pronounced CPE in these cells (not shown).

4. Discussion

The continuous secretion of infectious virus into the supernatant of infected cells for more than 100 cell passages in the present study demonstrates the successful establishment of persistent CSFV infection in cell culture. The virus titers varied within a constant range (Fig. 2) but no cycling as described, for instance, for bovine coronavirus (Hofmann et al., 1990) was observed. Additionally, no decrease of titers over time was detected, irrespective of the cell line used, indicating that only minor selection forces were acting from the host cell side. This was further supported by sequencing data obtained from virus produced by persistently infected cells of high passages (data not shown). They indicate that the virus does not undergo extensive mutations but rather remains genetically stable in this system, at least in the region of the genome which has been sequenced (5' NTR, 5' part of NS3, 3' NTR). More extensive sequence analysis of viral genomes obtained from different passages and of regions of the viral genomes which are known to be more variable will allow a more detailed determination of mutation rates in persistently infected cells.

Before a cytopathic effect occurred, neither the viability nor the growth rates of persistently infected cells underwent significant changes, suggesting that the wild-type virus exerted no negative effects on the cells. The CPE observed in five SK-6 cultures and one PK-41 culture did correlate with the appearance of a subgenomic RNA (Fig. 4) which gave rise to defective particles. Experiments aimed at determining the time-

Fig. 4. Northern blot analysis of cells infected with cp CSFV stocks and their parental viruses. Total RNA was separated in a denaturing formaldehyde agarose gel and virus-specific RNA of positive polarity was detected with the riboprobe JL1. wt, wild-type genomic RNA; sg, subgenomic RNA. 28S marks the position of the 28S ribosomal RNA.
Fig. 5. (A) CPE assay of virus collected from SK-6 cells persistently infected with strain A.1. Supernatant of the cell passage showing maximal CPE (0) as well as supernatants of the respective culture collected three and six cell passages earlier (−3 and −6) were passaged four times. SK-6 cells were inoculated with virus of all four passages (ViPa1 to ViPa4) and stained with crystal violet 48 h after infection. Development of a CPE with virus passages from passage −3 but not from passage −6 shows that the event leading to the generation of the cp agent occurred six to three passages before maximal CPE. (B) Correlation between occurrence of CPE and amount of subgenomic RNA. Crystal violet staining and Northern blot analysis of cells infected with ViPa1 of the same cell passages as in Fig. 5A. Similar amounts of full-length genomic RNA could be detected irrespective of the presence of high amounts of subgenomic RNA. wt, wild-type genomic RNA; sgRNA, subgenomic RNA.

point of generation of the subgenomic RNA (Fig. 5A) indicated that the formation of cp CSFV was a random event and that the cp agent was not present in the original virus stocks. In addition, the generation of a cp CSFV in a cell culture infected with virus rescued from the cDNA clone pA187-1 (Ruggli et al., 1996) proved that the cp agent was generated de novo. These data show that passaging of persistently infected cell cultures can be used to generate cp CSFV. Interestingly, cells which survived the CPE showed altered properties when compared to persistently infected cells which had not experienced a CPE (manuscript in preparation).

Meyers and Thiel (1995), who were the first to describe defective particles in CSFV, reported interference of these particles with helper virus replication. We have not detected any interference on RNA replication caused by the subgenomic cp RNA (Fig. 5B). Furthermore, our attempts to reproduce the experiment carried out by Meyers and Thiel (1995) to prove interference by infection of cells with serial dilutions of cp virus gave contradictory results. When the RNA was examined at 16 h postinfection, corresponding to one complete replication cycle, no interference was observed, whereas later in infection when the cytopathic effect appeared, results similar to those described by Meyers and Thiel (1995) were obtained. These data further support the absence of interference at the level of RNA replication (manuscript in preparation). Experiments aimed at investigating interference at other levels, such as protein synthesis or packaging, are currently under way.

All cp CSFV characterized so far, including the ones obtained in the present study, were shown to contain both wild-type virus and defective particles. Meyers et al. (1996) confirmed by the generation of recombinant cytopathogenic defective interfering particles from in vitro transcribed subgenomic RNA that this particular RNA is both necessary and sufficient for CSFV cytopathogenicity. Remarkably, the subgenomic cp RNA in all cases analyzed so far have exactly the same genomic structure, characterized by a deletion of 4764 nucleotides. This suggests a common mechanism of generation such as nonhomologous recombination or template switching of the viral RNA polymerase (reviewed in Lai, 1992). Whether this particular subgenomic RNA is generated exclusively by a site-specific mechanism or
Fig. 6. Western blot analysis of proteins extracted from SK-6 cells infected with the indicated virus stocks. After cell lysis, total protein concentration was determined and equal amounts (16 μg/lane) were subjected to Western blotting. NS2/3 and NS3 were detected with an NS3-specific rabbit antiserum.

whether it is the only RNA from a population of randomly mutated molecules which is replicated and can be packaged is an open question. Also, by performing Northern blot analyses of extracts from various passages of the persistently infected cultures, we have never detected any subgenomic CSFV RNA different from this particular 8 kb species.

The subgenomic cp RNA lacks the genes coding for the amino-terminal autoprotease N\(^{\text{pro}}\), the four structural proteins, p7 and NS2 (Fig. 3), but allows the expression of the nonstructural proteins NS3 to NS5B (Fig. 6 and our unpublished observations). This is particularly interesting as cytopathogenicity of cp BVDV is attributed to the expression of NS3 protein, which is not processed from NS2/3 in the case of ncp BVDV (Thiel et al., 1996). Yet the situation in CSFV and BDV seems more complex. The mere presence of NS3 in infected cells apparently is not sufficient to cause CPE as NS2/3 is at least partially processed into NS2 and NS3 in cells infected with ncp CSFV or BDV (Fig. 5 and Becher et al., 1994) without causing obvious damage to the cells. Meyers and Thiel (1995) suggested that a certain concentration of NS3 has to be reached within a cell to exhibit the CPE or alternatively, that the ratio of NS3 to NS2/3 plays an important role in this process. Our protein analyses of cells infected with ncp and cp CSFV support these hypotheses. High amounts of NS3 were present in cells infected with cp CSFV (Fig. 6) and correlated with the occurrence of the CPE. In addition, expression of NS2/3 (which is coded solely by the helper virus) was decreased, reflecting either interference at the translational level or more efficient processing of NS2/3. Alternatively, the CPE caused by cp CSFV could be due to an NS3 protein which has different properties when compared to NS3 synthesized by ncp pestiviruses. Notably, the cleavage site between NS2 and NS3 and therefore the amino terminus of NS3 is not known for ncp CSFV and BDV.

The persistently infected cell lines described here are useful for the study of long-term interactions between CSFV and host cells but also to further analyze the phenomenon of cp CSFV which correlates with the occurrence of a particular subgenomic RNA and an increased expression of NS3. Studies designed to determine the struc-
tural requirements of subgenomic RNA for replication, packaging and cytopathogenicity will also be carried out by using in vitro transcribed RNA which can be obtained by mutagenesis of the full-length CSFV clone vA187-1. These analyses may lead to better understanding of pestivirus replication and pathogenesis.

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