Cytopathogenic and Noncytopathogenic RNA Replicons of Classical Swine Fever Virus

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To determine the minimal requirements for autonomous RNA replication of classical swine fever virus (CSFV), genomes having in-frame deletions within the genes for structural and flanking nonstructural proteins were constructed, based on an infectious cDNA clone of CSFV Alfort/187. RNA was transcribed in vitro from the respective plasmids and transfected into SK-6 swine kidney cells. The replication competence of the RNA was determined by immunostaining transfected cells for CSFV NS3 protein and by analysis of cell extracts for viral RNA, as well as protein synthesis at different times after transfection. The genes encoding Npro*, C, E**, E1, E2, p7, and NS2 proved to be dispensable for RNA replication, but the efficiency of replication varied strongly between individual constructs. RNA replicons containing the complete NS2-NS3 gene persisted in transfected cells and continued to replicate without causing any obvious morphological or functional damage to the cells, whereas genomes lacking the NS2 gene replicated more efficiently and induced a cytopathic effect. These findings suggest that NS2, although it is not essential for pestivirus RNA replication, has a regulatory function therein. Both cytopathogenic and noncytopathogenic replicons were packaged into virus particles provided in trans by a cotransfected full-length helper virus genome.

Classical swine fever virus (CSFV) is the causative agent of a highly contagious disease in pigs. Virulent strains cause characteristic disease symptoms such as fever, neurological disorders, hemorrhages, and high mortality rates, whereas infection with avirulent strains remains clinically apparent but induces a protective immunity. Furthermore, prenatal infection of fetuses can lead to persistently infected animals which shed virus over a long period, as do pigs which exhibit the chronic form of disease (3, 4, 29, 31).

CSFV, bovine viral diarrhea virus (BVDV), and border disease virus form the pestivirus genus within the family Flaviviridae (33). CSFV is a small enveloped virus containing a positive-strand RNA genome of 12.3 kb, which consists of a 5′ untranslated region (5′UTR), a large open reading frame (ORF) encoding a single polyprotein, and a 3′ untranslated region (3′UTR) (16, 29). The 5′UTR contains an internal ribosomal entry site for cap-independent translation initiation of the viral polyprotein which is co- and posttranslationally processed by host cell and viral proteases (reviewed in reference 16). The cleavage sites of the polyprotein have been determined for BVDV, except for p7-NS2 (5, 16, 23, 26). C, E**, E1, and E2 represent the structural proteins found in mature virions. As demonstrated for other flaviviruses (2), RNA replication of pestiviruses is thought to occur on cytoplasmic membranes via the synthesis of a negative-stranded full-length genome (6, 7, 23), while the components of the viral replication complex have not been identified. It has been shown recently that the viral protein NS5B of BVDV has RNA-dependent RNA polymerase activity (37). The NS3 protease is responsible for the cleavage of the viral polyprotein downstream of NS3 and requires NS4A as a cofactor (28, 35).

Furthermore, it possesses both helicase and NTPase activity (27, 32), suggesting a role in RNA replication.

According to their behavior in tissue culture pestiviruses can be divided into two biotypes, noncytopathogenic (NCP) and cytopathogenic (CP). The mechanism responsible for the cytopathic effect (CPE) is poorly understood, but the overexpression of NS3 is a common feature of all CP pestiviruses (16). Furthermore, it has been shown that cells infected with CP BVDV undergo apoptosis (10, 36). CP pestiviruses represent mutants which arise from NCP viruses, presumably by RNA recombination during replication (16). Several mutants have been described for BVDV. Most of them contain rearrangements of viral sequences and/or insertions of host cell sequences (reviewed in reference 16). However, some CP BVDV and all CP CSFV isolates described so far are composed of defective interfering (DI) particles and NCP helper virus. CSFV DI genomes, with the identical deletion of 4,764 nucleotides (nt) corresponding to the genes encoding Npro** through NS2, have been isolated from different sources (12, 14, 17). A genome with a similar deletion of 4,746 nt has been described recently (12). Furthermore, a defective genome was reported which lacks the 4,263 nt encoding C through NS2 but retains the foreign murine ubiquitin gene which had been used to replace Npro** at the 5′ terminus of the ORF in the parental genome (30). After transfection into susceptible cells, such defective CSFV genomes, obtained either by extraction from infected cells or by transcription in vitro from the respective cloned cDNA, replicate and are packaged efficiently in the presence of helper virus RNA, thereby causing a CPE (14, 15).

An autonomously replicating defective BVDV genome which lacks the genes encoding C, E**, E1, E2, p7, and NS2 has been described recently by Behrens et al. (1). It demonstrates that none of these proteins is essential for RNA replication. In the same study it was suggested that the 5′ terminal region of the ORF contains a cis signal required for RNA replication, since the Npro** gene could neither be deleted completely nor be replaced by a ubiquitin gene. However, the replacement of the Npro** gene by ubiquitin in the CSFV Alfort/187 genome yielded infectious virus (30).
In this report a series of in vitro-constructed defective CSFV genomes derived from strain Alfort/187 were analyzed with respect to autonomous RNA replication in SK-6 cells. The question of whether these defective genomes can be packaged in the presence of a helper virus and whether they are cytopathogenic was also addressed.

MATERIALS AND METHODS

Cells and viruses. The swine kidney cell line SK-6 (11), kindly provided by M. Persaert (Faculty of Veterinary Medicine, Ghent, Belgium), was propagated in Dulbecco’s modified Eagle medium supplemented with 5% horse serum. CP CSFV vA187-1 used as a control was derived from a persistently infected SK-6 cell culture medium and subjected to RT-PCR with the primers either with biologically cloned strain Alfort/187 (17) or vA187-Ubi (30). To these deletions were spontaneously generated in SK-6 cells persistently infected Apar (-3390 and -390) or vA187-Ubi (30), treated with Klenow polymerase, and religated. To confirm the presence of the predicted in-frame deletion of 60 nt within the protease domain of NS3 was confirmed by DNA sequencing.

In vitro transcription and electroporation. In vitro transcription from Sf9l(1-369), or Nrl1(1-378)-linearized plasmids was performed by using the T7 Megascript kit (Ambion) as described before (20, 25, 30). After Dnase I digestion, transcripts were purified through MicroSpin S-400 HR columns (Pharmacia) and quantified with a GeneQuant II photometer (Pharmacia). SK-6 cells were washed twice and resuspended in ice-cold phosphate-buffered saline (PBS). A total of 2 × 105 cells in a volume of 0.8 ml were mixed with 15 μg of RNA, transferred to a 0.4-cm cuvette and electroporated immediately by using a Gene Pulser (Bio-Rad) set at 450 V and 500 μF. Alternatively, 0.4 ml of cell suspension at a density of 107 cells/ml was mixed with 5 μg of RNA, transferred to a 0.2-cm cuvette and electroporated at 2000 V and 500 μF. After electroporation the cell suspension was kept for 10 min at room temperature, then diluted in Dulbecco’s modified Eagle medium containing 5% horse serum, seeded, and harvested for analysis at different times after electroporation.

Cell staining. After electroporation 4 × 105 cells per well were seeded in 24-well plates and fixed at the indicated time as described before (17). For the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay performed with the in situ cell death detection kit (Boehringer Mannheim) or, for Western blotting as described before (17, 20). Porcine anti-pestivirus NS3 (kindly provided by E. Peterhans, Institute of Veterinary Virology Mannheim) or, for amplification of fragments shorter than 1 kb, were used. For PCR either the Expand long-template PCR kit (Boehringer Mannheim) or, for amplification of fragments shorter than 1 kb, polymerase (Promega) was used.

RT reactions were performed by using the Expand RT kit (Boehringer Mannheim), primer HR3, and Michaelis S-400 HR columns for subsequent cDNA purification (17, 20). For PCR either the Expand long-template PCR kit (Boehringer Mannheim) or, for amplification of fragments shorter than 1 kb, Taq DNA polymerase (Promega) was used.

Protein analysis. SK-6 cells were lysed in a hypotonic buffer (20 mM MOPS [morpholinepropanesulfonic acid], 10 mM NaCl, 1.5 mM MgCl2, 1% Triton X-100 [pH 6.5]), and the extracts were used either for chloramphenicol acetyl transferase–enzyme-linked immunosorbent assay (CAT-ELISA) (Boehringer Mannheim) or for Western blotting as described before (17, 20). Porcine anti-pestivirus hyperimmune serum SRT12 (19) and MAb 49DE directed against pestivirus NS3 (kindly provided by E. Peterhans, Institute of Veterinary Virology, University of Bern, Bern, Switzerland) served for the detection of NS3.

TABLE 1. Oligonucleotides used for PCR-based site-directed mutagenesis and RT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position (nt)*</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>CsF-L001</td>
<td>1–21</td>
<td>GTATACGAGGTTAGGTTACCC</td>
</tr>
<tr>
<td>Pest1</td>
<td>310–329</td>
<td>CCTGTAGGGGTGCGAGAG</td>
</tr>
<tr>
<td>NS2-LMFe</td>
<td>3764–3779</td>
<td>ITTCATLGTTGTCAGCAAGAG</td>
</tr>
<tr>
<td>NS2-L</td>
<td>3767–3783</td>
<td>P-GTGGAGATCGAGAGAGAG</td>
</tr>
<tr>
<td>NS3-L</td>
<td>5141–5161</td>
<td>P-GGGCCCCTGTTGGCAAGAA</td>
</tr>
<tr>
<td>HL3</td>
<td>6967–6983</td>
<td>AGTGCAGCTGCTATC</td>
</tr>
<tr>
<td>NTR-R</td>
<td>376–378</td>
<td>CATGTTGAGTACAGCAAGAG</td>
</tr>
<tr>
<td>Npro-R</td>
<td>877–856</td>
<td>GCAACTTGTAACCCCAATAG</td>
</tr>
<tr>
<td>E2-RMFe</td>
<td>3553–3528</td>
<td>GGCAATGTTGCTGTTGAAACATG</td>
</tr>
<tr>
<td>PR5</td>
<td>5596–5577</td>
<td>GGCGGAGTTCTGATGCTG</td>
</tr>
<tr>
<td>HR3</td>
<td>7197–7181</td>
<td>GGCTTCTAGCCTGTG</td>
</tr>
</tbody>
</table>

* Numbers refer to the location on the genome of CSFV Alfort/187.

** Overhangs and mutations are in italics, 5’ phosphorylation of oligonucleotides is indicated by the prefix ‘P-‘, and MfeI restriction sites are underlined.

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cultures. After low-speed centrifugation, 1 ml of undiluted supernatant was used to infect 2 × 10^5 SK-6 cells seeded the day before and the inoculum was replaced after 1 h. An aliquot of the cell culture medium was collected 48 h after infection for RNA extraction and RT-PCR, which was performed as described before (20).

CPE assay. CPE induced by replicon RNA was monitored on SK-6 cells seeded at a density of 1.5 × 10^4 (for subsequent infection) or 4 × 10^4 (after electroporation) per well in 24-well plates. For infection, virus obtained either from the cultures in which the packaging had been performed or from cultures in which the respective packaged replicons had been passaged once or twice was used. Undiluted supernatant obtained after centrifugation of the respective frozen and thawed cultures served as the inoculum, which was replaced with fresh medium 1 h after infection. The cells were examined twice a day by light microscopy for the development of a CPE. After 48 or 72 h the medium was removed, and the cells were fixed and stained with the addition of a crystal violet solution (0.4 mg of crystal violet per ml and 0.37% formaldehyde in PBS) for 1 h to visualize the CPE macroscopically.

RESULTS

Replication competence of defective genomes. The defective genomes shown in Fig. 1 were transcribed in vitro from the respective plasmids, and 15 μg of each RNA was transfected into 2.5 × 10^5 SK-6 cells. Subsequently, the cells were split in order to perform immunostaining (Fig. 2), to analyze viral RNA (Fig. 3 and 4) and viral protein expression (Fig. 5) at different times after electroporation.

Immunoperoxidase staining of SK-6 cells was performed at 16 and 44 h after electroporation of the individual in vitro transcripts, by using the C16 MAb directed against NS3 (Fig. 2). More than 50% of the cells transfected with A187-ΔApa (Fig. 2C), -Δ4764Ubi (Fig. 2H), or -Δ4764 (Fig. 2I) RNA scored positive at 16 h after electroporation, but the latter two showed a more intense staining than A187-ΔApa. A lower percentage of NS3-positive cells was observed after electroporation of A187-ΔE2 (Fig. 2B), -Δ3390 (Fig. 2D), and -Δ4263 (Fig. 2G), whereas no stained cells were found after electroporation of either A187-Δp7 (Fig. 2A), -ΔAcc (Fig. 2E), -Δ4764ΔBN (Fig. 2K), or of 3′ truncated A187-ΔApa RNA (Fig. 2F). The proportion of NS3-expressing cells remained constant between 16 and 44 h after electroporation in the case of A187-ΔE2 (Fig. 2B), -ΔApa (Fig. 2C), and -Δ3390 (Fig. 2D). In contrast, both the proportion of cells positive for NS3 and the total number of cells were significantly reduced at 44 h after electroporation of A187-Δ263 (Fig. 2G), -Δ4764Ubi (Fig. 2H), and -Δ4764 (Fig. 2I) when compared to 16 h after electroporation. The absence of wild-type CSFV in the electroporated cells was confirmed by immunostaining for CSFV E2 with the MAb HC/TC 26 (data not shown).

Northern blotting was performed with total RNA extracted from electroporated SK-6 cells 1, 20, and 48 h after electroporation (Fig. 3A to D). The blots shown in Fig. 3A to C were hybridized with the negative-stranded RNA probe J1L complementary to the 5′UTR of CSFV. As no detectable amounts of positive-stranded RNA were expected to be synthesized 1 h after transfection, this time point served for analysis of the stability of the different RNAs. Although equal amounts of RNA were electroporated the hybridization signals obtained for viral RNA extracted after 1 h varied considerably for the different transcripts (Fig. 3A). The low concentration of transcripts found for cells transfected with A187-ΔE2 and A187-ΔAcc RNA suggests that these RNAs are rapidly degraded. As expected, no signal was obtained for the transcript derived from NruI-linearized plasmid, since it lacked the 3′UTR to which the riboprobe was complementary. Total RNA extracted 20 h after electroporation (Fig. 3B) contained increased amounts of positive-stranded viral RNA for A187-Δ4764Ubi and A187-Δ4764 RNA, and, less pronounced, for A187-ΔApa RNA. At 48 h after electroporation (Fig. 3C), the signal was further increased in the case of A187-ΔApa RNA, whereas it was reduced for A187-Δ4764 RNA and absent for A187-Δ4764/Δubi RNA. The strong decline in detectable RNA for the latter two constructs was expected due to the almost complete destruction of the respective cultures by these cytopathic replicons. Negative-stranded viral RNA corresponding in size to the respective sense-stranded RNA was detected at 20 h after electroporation of A187-ΔE2, -Δ4764Ubi, and -Δ4764 transcripts, but the amounts were significantly larger for the latter two RNAs (Fig. 3D). The specificity of the riboprobe used for the detection of negative-stranded viral RNA was confirmed by hybridization to in vitro transcripts of pA187-1 of negative and positive polarities (Fig. 3D). For A187-ΔE2 and A187-Δ263 RNA a signal for positive-stranded viral RNA of the predicted size was observed both at 20 and 48 h after electroporation, and for A187-Δ3390 RNA at 48 h but not at 20 h (Fig. 3B and C). However, negative strands of these viral RNAs could not be detected (Fig. 3D). Transcripts from SrfI-linearized pA187-Δp7, -ΔAcc, and -Δ4764ΔBN and from NruI-linearized pA187-ΔApa did not yield detectable amounts of

FIG. 1. Schematic display of plasmid-encoded full-length and defective genomes of CSFV. (A) Full-length genomes of A187-1 and A187-CAT. The 5′- and 3′UTRs are depicted as black lines, and the open reading frame is shown as a box composed of the individual viral genes encoding structural (hatched) and nonstructural (white) proteins as indicated. Vertical lines indicate the SrfI 12298 and the NruI 12300 sites which were used to linearize the respective plasmids prior to in vitro RNA transcription. The CAT marker gene inserted in A187-CAT at nt 386 of the genome is shown as a separate box (black). (B) Defective genomes. The position and extent of the individual deletions (dashed lines) are given in parentheses for each construct. Numbers refer to the nucleotide sequence of CSFV Alfort/187 (25). The ubiquitin gene is shown as a black box.
input-sized RNA of either polarity 20 and 48 h after electroporation (Fig. 3B to D). One-fifth of the total cellular RNA extracted 48 h after electroporation was digested with RNase-free DNase to remove traces of plasmid DNA prior to performing CSFV-specific RT-PCR with primers HIL3 and HR3. A 241-bp fragment of the NS3 gene present in all deletion mutants was amplified from all samples except for RNA from mock-electroporated cells (Fig. 4). The RT-PCR was also positive for the two RNAs designed not to replicate, namely the 3' truncated A187ΔApa RNA transcribed from SrfI-linearized plasmid, and A187-Δ4764ΔBN RNA which has a deletion within the NS3 protease domain.

To confirm the expression and processing of the viral polyprotein by defective genomes, 5 × 10⁶ electroporated SK-6 cells were lysed 20 h after electroporation, and the extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. MAb 49DE9 and porcine hyperimmune serum N8T12 served for the detection of NS3 protein which comigrated with the respective protein obtained with either A187-Δ4764Ubi or A187-Δ4764 RNA expressed NS3 which was detected by ELISA in hypotonic cell lysates harvested 20 h after electroporation (Fig. 5). A time course experiment confirmed that both A187ΔE2-CAT and A187ΔApa-CAT expressed the marker protein (Fig. 6B). In contrast, electroporation of transcripts from SrfI-linearized pA187ΔAcc-CAT or pA187ΔE2 or from SrfI-linearized pA187ΔApa-CAT did not yield detectable amounts of CAT protein (Fig. 6A and B).

FIG. 2. Expression of NS3 from CSFV replicons. SK-6 cells were stained for NS3 expression 16 and 44 h after electroporation of in vitro transcripts obtained from the respective SrfI-linearized plasmid DNA. A, A187Δp7; B, A187ΔE2; C, A187ΔApa; D, A187Δ3390; E, A187ΔAcc; F, A187ΔApa transcript from SrfI-linearized plasmid; G, A187Δ4263; H, A187Δ4764Ubi; I, A187Δ4764; K, A187Δ4764ΔBN; L, mock.

When the RT-PCR-derived EagIΔ to NcoIΔ fragment of pA187-Δ4764 was sequenced, five point mutations were identified when compared to the authentic sequence of pA187-1. In contrast, no mutations were found in the respective fragments of pA187-Δ4764Ubi and pA187-Δ4764iv (Fig. 1), which was constructed in vitro from a subclone of pA187-1 in analogy to pA187-Δ4263 with primers NS3-L and NTR-R (Table 1). RNA transcribed from pA187-Δ4764 and pA187-Δ4764iv had indistinguishable properties in terms of transfection efficiency, protein expression, and cytopathogenicity (data not shown), indicating that the five mutations in pA187-Δ4764 did not alter its phenotype.

Packaging of replicons in virus particles. To show the packaging of viral RNA, SK-6 cells were coelectroporated with helper A187-CAT RNA and the respective defective RNAs. At 48 h after transfection the cell-free supernatants were collected and used to infect SK-6 cells. The medium of these cultures was collected 48 h postinfection. The RNA was extracted and used to perform RT-PCR with the CSFV-specific primers Pest1 and PR5. A 5.8-kb product representing the A187-CAT
helper genome was amplified from all samples except for those containing either A187-D4764Ubi or A187-D4764 RNA, which both had caused an extensive CPE. Shorter PCR products corresponding in length to the input replicon RNAs were detected for A187-D4263, -D4764Ubi, and -D4764 (Fig. 7A). An additional PCR specific for the marker gene of the helper virus confirmed the presence of vA187-CAT in all samples derived from coelectroporations with the respective in vitro transcript (Fig. 7B).

**Cytopathogenicity of replicons.** Cell morphology and immunostaining after electroporation suggested that the replicons A187-D4263, -D4764Ubi, and -D4764 were cytopathogenic (Fig. 2G, H, and I). To further characterize the cytopathogenic nature of replicons, SK-6 cells were electroporated with the respective RNAs and examined 20 h later by TUNEL assay for fragmentation of genomic DNA as a marker for cell death as well as for the expression of NS3. A high percentage of the cells scored positive in the TUNEL assay in all cultures including mock-electroporated cells (Table 2). This was likely due to the electroporation procedure, which causes extensive damage of the cells. Thus, the defective genomes were packaged into virions by coelectroporation of SK-6 cells with helper A187-CAT RNA as described above. As expected, CPE was observed in cells coelectroporated with helper RNA and, more dramatically, after passage of the supernatants for the transcripts A187-D4263, -D4764Ubi, and -D4764 but not for any of the other defective RNAs or for helper RNA alone (Table 2).

The supernatants of the coelectroporated cells containing the respective packaged replicons together with helper vA187-CAT were diluted 1:50 and used to infect SK-6 cells. Cells were fixed 48 h after infection, before the TUNEL assay in combination with immunostaining for NS3 was performed. Supernatants containing helper virus and particles carrying either A187-D4263, -D4764Ubi, or -D4764 RNA induced CPE as observed by microscopy (Table 2). Foci of rounded cells typical for CSFV-induced CPE contained a high proportion of TUNEL-positive cells (Table 2). These cells were also intensively stained for NS3. However, not all cells positive for NS3 were also positive in the TUNEL assay. A separate immunostaining with MAb HC/TC26 directed against E2 revealed that 100% of the cells were infected with helper virus (data not shown).

**DISCUSSION**

In order to define the genes required for RNA replication of CSFV, a series of defective genomes carrying deletions within the 5' half of the ORF was constructed, based on the infectious full-length clones pA187-1 and pA187-CAT. The genes encoding N through NS2 proved to be dispensable for RNA replication, since A187-D4764 RNA replicated autonomously. As replicon RNA with partially or completely deleted structural genes is not expected to spread from cell to cell, a highly efficient transfection procedure was required to detect low levels of RNA replication. In fact, for the defective genomes
A187-DAp-4764Ubi, and -4764, more than 50% of the cells stained positive for NS3 16 h after electroporation (Fig. 2C, H, and I), and direct evidence for replication was obtained by the detection of negative-stranded RNA of these genomes (Fig. 3D). In contrast, no NS3-positive cells were observed after electroporation of a 39 truncation transcript from pA187-D Ap. Lower proportions of cells staining positive for NS3 were observed for A187-D E2, -D3390, and -D4263 RNA. We conclude that these mutant genomes are replicons as well and that negative-stranded RNA could not be detected due to a less efficient transfection and/or replication of these genomes. Furthermore, in cells transfected with A187-D E2-CAT or A187-D Apa-CAT the marker protein CAT was expressed but not after transfection of the respective 39 truncated RNAs or A187-D Acc-CAT (Fig. 6). This confirms the result obtained with immunostaining for NS3 and shows that translation from input RNA does not yield detectable amounts of protein. The concentration of CAT for the replicons A187-D E2-CAT and

A187-D Apa-CAT was only slightly above the detection limit of the ELISA and about 100-fold lower than for infection of SK-6 cells with vA187-CAT (19). Nevertheless, this shows that CAT is a useful marker for the detection and quantification of replication of mutant CSFV genomes.

The RNAs A187-D p7, -D Acc, and -D 4764BN did not replicate at detectable levels (Fig. 2). This was expected for the latter construct, since the serine protease domain is essential for replication (13, 35). In contrast, the constructs A187-D p7 and A187-D Acc contain all genes required for replication (Fig. 1). We speculate that these RNAs might have an altered secondary structure which affects their ability to serve as a template for RNA replication or translation, since sequencing the template plasmids revealed no mutations which could account for a failure to replicate. Alternatively, correct processing of the viral polyprotein might be affected by the deletions in the E2-p7-NS2 region, yielding nonfunctional proteins. Although the amount of input RNA was standardized, the transfection efficiency as reflected by the proportion of NS3-positive cells varied from below 1% to nearly 100%, depending on the construct (Fig. 2). Furthermore, the amount of RNA detected in cells 1 h after transfection varied strongly, indicating that the stability of the respective transcripts varies. We exclude the possibility that the RNA detected at this time by Northern blot analysis represents newly synthesized RNA. Even after infection of SK-6 cells with wild-type CSFV at a multiplicity of infection of 10 and by using the same detection procedure, we have never observed viral RNA earlier than 6 h after infection (18). Differences in RNA stability might also be responsible for the strong variation in the transfection efficiency between individual constructs (Fig. 2). In addition, it has to be considered that in vitro transcripts of different constructs do not necessarily yield equal proportions of RNA molecules.
TABLE 2. CPE of replicons

<table>
<thead>
<tr>
<th>Assay</th>
<th>Experiment</th>
<th>CPE on cells transfected with:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Δp7</td>
</tr>
<tr>
<td>CPE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Without helper RNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>With helper RNA</td>
<td></td>
</tr>
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<td></td>
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<td></td>
<td>P2</td>
<td></td>
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<tr>
<td>TUNEL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Without helper RNA</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td></td>
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</table>

<sup>a</sup> Defective genomes alone (without helper RNA) or together with full-length helper virus RNA A187-CAT (with helper RNA) were transfected into SK-6 cells. Supernatants of double-transfected cells were passaged twice (P1 and P2).

<sup>b</sup> CPE was examined by light microscopy and macroscopically by crystal violet staining 48 to 72 h after electroporation or infection and scored as follows: confluent monolayer with foci of round cells (+), partially disrupted monolayer (++), less than 10% adherent cells (+++), or no CPE (−).

<sup>c</sup> TUNEL assay was performed either 20 h after electroporation or 48 h after infection.

with a correct, replication-competent secondary structure. Thus, we conclude that the number of input RNA molecules required to start replication in a single cell varies according to the nature of the particular construct.

We cannot exclude that in the case of constructs with low transfection efficiency, such as A187-Δ3390, the few cells which stain positive for NS3 (Fig. 2D) contain RNA with an altered sequence compared to its parental template plasmid. In this case, the replicating RNA might have acquired mutations either during in vitro transcription by T7 RNA polymerase or during initial low-level replication in cells. To obtain sufficient material for sequencing and identification of such mutations, RT-PCR amplification of these genomes would be required. However, virus-specific RT-PCR yielded strong signals 48 h during in vitro transcription by T7 RNA polymerase or during initial low-level replication in cells. To obtain sufficient material for sequencing and identification of such mutations, RT-PCR amplification of these genomes would be required. However, virus-specific RT-PCR yielded strong signals 48 h after electroporation (Fig. 4), even for genomes unable to replicate such as A187-Δ4263, A187-Δ4764Ubi, and A187-Δ3390. This shows that input RNA remains detectable over a long time and, therefore, that sequencing of RT-PCR products would be biased.

Interestingly, two types of RNA replicons were found with respect to their replication kinetics and their effect on host cells. The defective genomes A187-ΔE2, ΔApa, and Δ3390 represent the noncytopathogenic replicon type, whereas A187-Δ4263, Δ4764Ubi, and Δ4764 are cytopathogenic replicons. NCP replicons encode NS2-NS3, replicate at low levels, and persist in transfected cells for at least 20 cell passages (data not shown). NS3 expressed by NCP A187-ΔApa was detectable by immunostaining in the majority of the electroporated cells and barely on Western blots, indicating that the expression level is significantly lower than that for CP replicons or virus infection (Fig. 5). On the other hand, CP replicons which do not contain the NS2 gene replicate very efficiently and express large amounts of NS3 (Fig. 2 and 5), the latter being the common feature of CP pestiviruses (16). No functions of pestivirus NS2 have been identified so far (16), but in the closely related hepatitis C virus it was suggested to be involved in the processing of NS2-NS3 (34). Our findings indicate that CSFV NS2 has a regulatory function in RNA replication; nonetheless, it is not essential. Possibly, NS2 prevents an uncontrolled accumulation of viral RNA and proteins leading to host cell death. It is not clear whether mature NS2, a precursor protein, e.g., NS2-NS3, or both could account for this function. Alternatively, the NS2 gene might act at the RNA level, either by altering the secondary structure or by binding modulating factors.

The CP replicons described here correspond in their overall genome structure to the naturally occurring defective CSFV RNAs (12, 14, 17, 30), which carry a large deletion comprising most of or the complete Npro gene, and the coding sequences for all structural proteins, p7, and NS2. We report an additional CP CSFV RNA (A187-Δ4263), which by analogy to the BVDV replicon CP9 (1, 16), contains the complete Npro gene. Remarkably, three different genes with no sequence homology, namely, Npro, murine ubiquitin, and NS3, are located directly downstream of the translation start signal of the respective CP replicons A187-Δ4263, A187-Δ4764Ubi, and A187-Δ4764. This supports our earlier suggestion that the internal ribosome entry site of CSFV Alfort/187 does not overlap with the ORF (30) but is in contrast to what has been reported for CSFV strain C (24) and for BVDV strains NADL (22) and CP9 (1). Upon coelectroporation with a full-length marker genome, the CP replicons were packaged efficiently by the structural proteins provided in trans from a helper genome, giving rise to CP CSFV (Table 2). Analysis of the NCP replicons revealed detectable amounts of packaged RNA for A187-ΔApa but not for A187-ΔE2 and A187-Δ3390. This is not surprising since the transfection efficiencies of the latter two RNAs are very low when compared to A187-ΔApa (Fig. 2). The RT-PCR data (Fig. 7) further indicate that the replicons propagated in the presence of helper virus retained their overall genome structure upon passage, although minor mutations cannot be ruled out. We consider that the transfection efficiency rather than a specific signal on the RNA is critical for the packaging of the replicons described here.

Future studies will aim at a more detailed characterization of NS2 and its role in viral replication. Also, CSFV replicons packaged in virions provided in trans from a heterologous expression system will allow helper virus-free experiments. Finally, CSFV replicons may serve as a model for the investigation of hepatitis C virus replication in cell culture.

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