Classical Swine Fever Virus E\textsuperscript{rns} Deletion Mutants: \textit{trans}-Complementation and Potential Use as Nontransmissible, Modified, Live-Attenuated Marker Vaccines

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An SK6 cell line (SK6c26) which constitutively expressed the glycopolypeptide E\textsuperscript{rns} of classical swine fever virus (CSFV) was used to rescue CSFV E\textsuperscript{rns} deletion mutants based on the infectious copy of CSFV strain C. The biochemical properties of E\textsuperscript{rns} from this cell line were indistinguishable from those of CSFV E\textsuperscript{rns}. Two E\textsuperscript{rns} deletion mutants were constructed, virus Flc23 and virus Flc22. Virus Flc23 encoded only the utmost N- and C-terminal amino acids of E\textsuperscript{rns} (deletion of 215 amino acids) to retain the original protease cleavage sites. Virus Flc22 is not recognized by a panel of E\textsuperscript{rns} antibodies, due to a deletion of 66 amino acids in E\textsuperscript{rns}. The E\textsuperscript{rns} deletion mutants Flc22 and Flc23 could be rescued in vitro only on the complementing SK6c26 cells. These rescued viruses could infect and replicate in SK6 cells but did not yield infectious virus. Virus neutralization by E\textsuperscript{rns}-specific antibodies was similar for the wild-type virus and the recombinant viruses, indicating that E\textsuperscript{rns} from SK6c26 cells was incorporated in the viral particles. Pigs vaccinated with Flc22 or Flc23 were protected against a challenge with a lethal dose of CSFV strain Brescia. This is the first demonstration of \textit{trans}-complementation of defective pestivirus RNA with a pestiviral structural protein and opens new ways to develop nontransmissible modified live pestivirus vaccines. In addition, the absence of (the antigenic part of) E\textsuperscript{rns} in the recombinant viral particles can be used to differentiate between infected and vaccinated animals.

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Materials and Methods

Cells and viruses. Swine kidney (SK6) cells were grown in Eagle's basal medium containing 5% fetal bovine serum, glutamine (0.3 mg/ml), and the antibiotics penicillin (200 U/ml), streptomycin (0.2 mg/ml), and mycostatin (100 U/ml). Fetal bovine serum was tested for the absence of BVDV and BDV antibodies as described previously (18). Virus stocks were prepared by passaging the virus 8 to 10 times on SK6c26 cells. The virus titers obtained ranged from \(10^8\) to \(10^9\) TCID_50/ml. Since CSFV tends to be associated with the host cells, lysates were used for reinfection of fresh cells and as vaccine inoculation unless indicated otherwise. These lysates were prepared by freezing and thawing cell culture twice and clarification.

Construction and characterization of an SK6 cell line (SK6c26) expressing Erns. The SK6 cell line constitutively expressing Erns was designated SK6c26. The SK6 cell line expressing Erns was designated SK6c26. The amino acid sequence for the Erns gene was constructed, but the 5' noncoding region was retained for intact protease cleavage sites. Two complementary oligomers, the 5'-noncoding region; 3'-noncoding region.

Construction of recombinant CSFV deletion Erns mutants. A deletion of amino acids 422 to 488 in Erns of strain C was accomplished by PCR amplification of the Erns gene, using the forward primer p974 and the reverse primer p1120 (5'-GAC GGA TTC GGC ATA GGC GCC AAA TTG-3'). The HA epitope, amino acid sequence GAC GTC GAC AGA TCT-3'), and the reverse primer p295 (5'-CAT AAG GCC CAA ACC AGG TTT G-3'), the PCR product was phosphorylated and ligated into the plasmid pPRK16 that was digested with BglII and SalI, resulting in plasmid pPRKc45. After PCR amplification of pPRKc45 with the forward primer p935 (5'-CCG AAA ATA TAA CTC AAT GGT TT-3') and the reverse primer p1120 (5'-GAC GGT CTC TAT CCT GAT TTC-3'), the PCR product was phosphorylated and ligated into the plasmid pPRKc50. Clones in which the mutated Erns gene was inserted in the correct orientation were transfected to SK6 cells and tested for expression of E2 by immunostaining with antibodies against E2-specific Mabs B3 and B4 (35).

A Clal-NcoI fragment of pPRKc48 and pPRKc50 was isolated and ligated into the Clal-NcoI-digested vector pPRKc2 (previously named pPRKc133) (19), and the resulting full-length cDNA CSFV strain C Erns mutants were named pPRKc22 and pPRKc23, respectively. A schematic representation of these constructs is shown in Fig. 1.

Regeneration of recombinant viruses. Plasmids pPRKc22 and pPRKc23 were purified on columns (Qiagen) and linearized with 

Characterization of recombinant Erns viruses. The growth kinetics of the viruses was determined in SK6c26 cells. Subconfluent monolayers in M24 wells (2 cm²) were infected at a multiplicity of infection of 0.05. Viruses adsorbed for 1.5 h. Before the cells were supplied with fresh medium, the first sample was collected: this was defined as time zero. At 0, 1, 2, 3, 4, 5, 6, and 7 days after infection, the monolayers were frozen-thawed twice and clarified by centrifugation for 10 min at 5,000 × g at 4°C. Virus titers (log TCID₅₀ per milliliter) of total lysates (cell lysates plus supernatant) were determined on SK6c26 cells. The virus neutralization index (log reduction of virus titer [TCID₅₀/milliliter]) was performed as described previously (19). RNA (1 μg) was transfected with Lipofectin (Gibco BRL) to SK6c26 cells grown in 10-cm² tissue culture plates. RNA transfection was performed in duplicate. Four days after transfection, one sample was immunostained with MAb b3 specific for E2. When the E2 immunostaining was negative, the duplicate sample was passaged and split into two samples. One of these samples was used for immunostaining 4 days after passing. From monolayers, which showed E2 expression, supernatant was applied onto fresh SK6c26 or SK6 cells to determine the presence of infectious virus. After 4 days, the monolayers were immunostained as described above.

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The pigs were vaccinated by several routes of inoculation and agarose gel in Tris-acetate-EDTA (TAE), and purified on Costar Spin-X tubes. Four pigs born from conventional sows free of antibodies against pestiviruses were divided into two groups of two pigs; one group was vaccinated with virus Flc2, while the other group was vaccinated with virus Flc22. The pigs were vaccinated by several routes of inoculation and received, via each route, 2 ml of vaccine corresponding to 2 virus Flc22. The pigs were challenged intranasally with 100 LD50 of CSFV strain Brescia. Negative control pigs that were inoculated with the same LD50 derived from this stock solution before and after this pilot study all died within 14 days after infection (1, 27). All pigs were tested by direct immunofluorescence for the presence of viral antigen.

Vaccination and challenge of pigs. Four pigs born from conventional sows free of antibodies against pestiviruses were divided into two groups of two pigs; one group was vaccinated with virus Flc2, while the other group was vaccinated with virus Flc22. The pigs were vaccinated by several routes of inoculation and received, via each route, 2 ml of vaccine corresponding to 2 × 10^6 TCID_{50} in total, each pig received 8 ml of vaccine, corresponding to 8 × 10^6 TCID_{50}. The pigs were sedated, placed on their backs, and inoculated with a virus suspension in phosphate-buffered saline into the nostrils, intravenously, and intradermally. In addition, the vaccines Flc22 and Flc23 were administered intramuscularly with a double-oil-water emulsion, since a recently performed study with an attenuated pseudorabies virus vaccine administered intramuscularly with a water-oil emulsion resulted in a slight decrease of E\textsuperscript{rns}-stained cells to approximately 80%.

The cell line SK6c26 was further characterized with respect to the biochemical characteristics of the E\textsuperscript{rns} produced (Table 1). The cell line could be immunostained with the same E\textsuperscript{rns} antibodies (MAb C5, 140.1, and 137.5 and polyclonal serum R716) as could SK6 cells infected with CSFV strain C (Flc2).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Immunostaining with E\textsuperscript{rns}-specific antibody:</th>
<th>Amt of E\textsuperscript{rns} \textsuperscript{a} (ng/10^5 cells) \textsuperscript{b}</th>
<th>RNase activity \textsuperscript{c} (A\textsubscript{260} min^{-1} mg^{-1}E\textsuperscript{rns})</th>
<th>Dimerization \textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK6c26</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK6c22 infected \textsuperscript{e}</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK6</td>
<td>-</td>
<td>-</td>
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</table>

\textsuperscript{a} The amount of E\textsuperscript{rns} in the cell lysates per 10^5 cells was extrapolated from a standard curve prepared from an immunoaffinity-purified preparation of E\textsuperscript{rns} produced in insect cells (5).

\textsuperscript{b} The RNase activity was determined as absorbance at 260 nm (A\textsubscript{260}) units per milligram of E\textsuperscript{rns} per use as described previously (7).

\textsuperscript{c} Detection of dimers of E\textsuperscript{rns} by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown).

\textsuperscript{d} SK6 cells were infected with virus Flc2 with a multiplicity of infection of 0.05 and analyzed 4 days after infection.

With MAb C5 specific for CSFV E\textsuperscript{rns}, positive cells were cloned twice to ensure clonality. The SK6c26 cell line showed detectable expression of E\textsuperscript{rns} in the cytoplasm in more than 95% of the cells, whereas the parental SK6 cells showed no expression (data not shown). Continuous passage of the SK6c26 cells in the presence of histidinol retained persistent expression in more than 95% of the cells for at least 10 months (46 passages). Passage in the absence of histidinol for 10 passages resulted in a slight decrease of E\textsuperscript{rns}-stained cells to approximately 80%.

The cell line SK6c26 was further characterized with respect to the biochemical characteristics of the E\textsuperscript{rns} produced (Table 1). The cell line could be immunostained with the same E\textsuperscript{rns} antibodies (MAb C5, 140.1, and 137.5 and polyclonal serum R716) as could SK6 cells infected with CSFV strain C (Flc2).

The amount of E\textsuperscript{rns} quantified in the SK6c26 cell lysate was 5 ng of E\textsuperscript{rns} per 10^5 cells, as determined by ELISA and extrapolation from a standard curve of immunoaffinity-purified E\textsuperscript{rns} (5). By this quantification method, CSFV-infected SK6 cells contained 15 ng of E\textsuperscript{rns} per 10^5 cells. In all experiments, the RNase activity of the SK6c26 cells and CSFV-infected SK6 cells was determined, and the two systems were shown to have comparable RNase activity. The E\textsuperscript{rns} protein of the stable cell line had a similar mobility to the wild-type E\textsuperscript{rns} as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown) and was efficiently dimerized like the E\textsuperscript{rns} found in virions (28). Thus, in conclusion, the SK6c26 line expresses E\textsuperscript{rns} that is indistinguishable from CSFV-encoded E\textsuperscript{rns}.

**Construction and recovery of C strain CSFV E\textsuperscript{rns} recombinant viruses Flc22 and Flc23.** Two full-length CSFV recombinants were constructed. The first E\textsuperscript{rns} recombinant contained a C-terminal deletion of 66 amino acids from position 422 to 488. This E\textsuperscript{rns} deletion mutant was first constructed with a C-terminal HA tag in the expression vector pEVH1sD12 (pPRK43). The latter HA tag was incorporated as a control for the correct open reading frame. Immunostaining of transient-expression products of this plasmid in SK6 cells was positive only with antibodies against the HA tag and not with antibodies against E\textsuperscript{rns}, including neutralizing MAb C5. Transient expression of plasmid pPRK47, encoding the entire E\textsuperscript{rns} gene with the C-terminal HA tag, was positive with both the E\textsuperscript{rns} and the HA antibodies (data not shown). Thus, the deleted C-terminal amino acids (422 to 488) are essential for recognition of the antigenic site(s) on E\textsuperscript{rns}. The recombinant E\textsuperscript{rns} sequence of pPRK43 without the HA epitope was transferred to the full-length infectious copy of the CSFV strain C pPRKflc2 (19), yielding the full-length clone pPRKflc22 (Fig. 1).

The second E\textsuperscript{rns} recombinant full-length clone, pPRKflc23, lacked the whole E\textsuperscript{rns} gene but retained only the N- and C-terminal utmost amino acids of E\textsuperscript{rns} in order to retain the
original sequences around the cleavage sites (24). This clone contains a deletion of a.a. 273 to 488 (Fig. 1).

Linearized full-length cDNAs of pPRKflc22 and pPRKflc23 were transfected into an SK6 cell line constitutively expressing the bacteriophage T7 RNA polymerase (30). Transient expression of E2 was detected by immunostaining; however, no infectious recombinant virus was recovered, even after repeated passaging of transfected cells (data not shown). Apparently, Erns is required for the formation of such a virus. To investigate whether Erns can complement in trans, in vitro-transcribed RNAs of the plasmids pPRKflc22 and pPRKflc23 were transfected in the cell line SK6c26, which constitutively expressed Erns. At 4 days after transfection, immunostaining of the monolayers with an E2-specific MAb, b3, was negative. The amount of E2 protein of the recombinant viruses might be too small for immunostaining. Therefore, the transfected cells were passaged to obtain higher titers of viruses. E2 could be detected from the wild-type construct (pPRKflc2) two passages after transfection, while virus derived from pPRKflc22 and pPRKflc23 could be detected after four passages. Three to five additional passages were required to obtain a virus titer of approximately 10⁵.5 TCID₅₀/ml, and this stock was used for further characterization of the viruses, which were named Flc22 and Flc23 for clones pPRKflc22 and pPRKflc23, respectively.

Supernatants from SK6c26 cells infected with Flc22 and Flc23 were used for infection of SK6c26 and SK6 cells. Four days after infection, for both viruses approximately 30% of the SK6c26 cells were positive by E2 immunoperoxidase staining (Fig. 2a and b) whereas infection of SK6 cells resulted in only single cells stained or pairs of single cells being stained (Fig. 2d and e). This indicates that the supernatants from SK6c26 cells contain infectious viruses that can infect and replicate in SK6 cells. However, there is no cell-to-cell spread or secondary infection of the mutated viruses in these cells, taking into account that cells infected with CSFV divide normally (once in 24 h). SK6 cells infected with the viruses Flc22 and Flc23 could be immunostained with MAbs directed against E2 but not with Erns-specific antibodies (Table 2). As a control, infection of SK6 cells with wild-type Flc2 grown on SK6c26 cells resulted in a positive immunostaining for both E2 and Erns (Table 2), and secondary infection was observed (Fig. 2). The numbers of wild-type-virus-infected SK6 and SK6c26 cells were comparable, although infected SK6 cells showed a weaker immunostaining (Fig. 2c and f). With supernatant or cell lysates of SK6 cells infected with Flc22 and Flc23, neither SK6 nor SK6c26 cells could be infected. Thus, to obtain infectious virus, the recombinant viral genomes of viruses Flc22 and Flc23

![Image](http://jvi.asm.org/)
require complementation of full-length E\textsuperscript{rms}, which can be delivered in trans by the E\textsuperscript{rms}-expressing SK6c26 cells.

**Characterization of recombinant CSFV viruses Flc22 and Flc23.** Growth kinetics of Flc22 and Flc23 and wild-type Flc2 in the complementing cell line SK6c26 were determined. As shown in Fig. 3, the multistep growth curves of the recombinant viruses Flc22 and Flc23 were very similar but showed a slower growth compared to the parent virus, Flc2. Titers between 10\textsuperscript{5.0} and 10\textsuperscript{5.8} TCID\textsubscript{50}/ml were found for the recombinant viruses after 6 days, whereas the parent strain Flc2 reached this titer within 3 days.

To determine whether E\textsuperscript{rms} was incorporated into the viral envelope, virus stocks of Flc2, Flc22, and Flc23 were subjected to titer determination in the presence of CSFV neutralizing antibodies (Table 3). All recombinant viruses were neutralized to the same extent as the parent virus Flc2 with both the E\textsuperscript{rms}-specific and E2-specific neutralizing polyclonal antibodies. For Flc22, E\textsuperscript{rms} on the viral envelope could be derived from the SK6c26 complementing cell line as well as from the recombinant E\textsuperscript{rms} protein encoded by the viral genome, but the latter recombinant E\textsuperscript{rms} is not recognized by the polyclonal serum R716 used for neutralizing the viruses. Thus, it remains unclear whether this recombinant E\textsuperscript{rms} is incorporated into the viral membrane. The similar neutralization index obtained with this polyclonal serum suggests that the amount of E\textsuperscript{rms} derived from the complementing cell line in the viral envelopes of Flc22 was comparable to that of Flc2 and Flc23.

After viruses Flc22 and Flc23 were passaged on the complementing SK6c26 cells 10 times, cellular RNA from infected SK6c26 cells was analyzed by RT-PCR with CSFV-specific primers flanking either the E\textsuperscript{rms} or E2 gene. The PCR fragments with primers flanking the E\textsuperscript{rms} gene were of the expected sizes of 857, 401, and 1,055 nucleotides, respectively, for Flc22, Flc23, and Flc2, whereas the E2 gene products were of the expected size of 974 bp for all viruses (Fig. 4). The amplification products of the E\textsuperscript{rms} gene were sequenced, and the obtained sequences were unchanged, indicating that no mutations, no reversion to the wild type, and no recombination with the E\textsuperscript{rms} gene in the cell line could be detected.

**Immunization and challenge of pigs.** To establish the vaccine properties of the E\textsuperscript{rms} deletion mutant viruses, a pilot immunization and challenge experiment was performed. Two pigs in one group were vaccinated with strain Flc22, and two pigs in another group were vaccinated with strain Flc23. Each pig was vaccinated via several routes of inoculation (intramuscularly, intravenously, intradermally, and intratraeheally), since we anticipated that the viruses are nontransmissible and the route of application might be important for efficacy. The pigs were challenged intranasally with 100 LD\textsubscript{50} of CSFV strain Brescia 456610 (27) 4 weeks after vaccination.

After immunization, none of the animals developed clinical signs or fever. After challenge, all the pigs developed a mild fever (40 to 41°C) for 3 days. None of the vaccinated pigs in either group developed leukopenia or thrombocytopenia, although 6 days after challenge a slight drop in the thrombocyte and leukocyte counts was observed for three of the four pigs. After challenge, no virus could be isolated from the leukocytes throughout the experiment. Moreover, the organs of all pigs were IFT negative at the end of the experiment, indicating the clearance of CSFV.

After vaccination of the pigs with viruses Flc22 and Flc23, no CSFV-specific antibodies were detected by the E2 ELISA and the E\textsuperscript{rms} ELISA until the time of challenge (Table 4). This finding was consistent with the NPLA results: all vaccinated pigs remained negative for neutralizing antibodies against CSFV up to the day of challenge 4 weeks after vaccination. After challenge, maximum inhibition percentages were observed in the E2 ELISA in all inoculated pigs, indicating a strong booster effect. Also, all four pigs seroconverted in the

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**TABLE 3. Neutralization of CSF viruses by antibodies**

<table>
<thead>
<tr>
<th>Virus\textsuperscript{a}</th>
<th>Virus neutralizing reduction (log TCID\textsubscript{50}/ml) with serum\textsuperscript{b}</th>
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<tbody>
<tr>
<td></td>
<td>716 (directed against E\textsuperscript{rms})</td>
</tr>
<tr>
<td>Flc2 (wild type)</td>
<td>3.0</td>
</tr>
<tr>
<td>Flc22 (Δ422–488)</td>
<td>3.0</td>
</tr>
<tr>
<td>Flc23 (Δ273–488)</td>
<td>3.25</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Amino acid numbering of CSFV strain C (19).

\textsuperscript{b} log TCID\textsubscript{50} per milliliter reduction of CSFV titers due to the presence of serum.

\textsuperscript{c} Rabbit serum prepared against E\textsuperscript{rms} of CSFV strain C.

\textsuperscript{d} Pig serum specifically directed against E2 of CSFV strain Brescia.
E\textsuperscript{rms} ELISA. In the NPLAs, all inoculated pigs showed high titers against CSFV. These results clearly show that both Flc22 and Flc23 protect pigs against a lethal challenge with the virulent strain Brescia. Moreover, these data indicate that the use of these mutant viruses enables us to discriminate between infected and vaccinated animals based on a CSFV-specific E\textsuperscript{rms} ELISA.

**TABLE 4. Serological response after immunization and challenge of pigs**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Virus</th>
<th>Pig</th>
<th>Response on day postchallenge:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-28</td>
</tr>
<tr>
<td>Ceditest ELISA anti-E2\textsuperscript{b}</td>
<td>Flc23</td>
<td>469</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>476</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Flc22</td>
<td>477</td>
<td>-</td>
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<tr>
<td></td>
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<td>478</td>
<td>-</td>
</tr>
<tr>
<td>Ceditest ELISA anti-E\textsuperscript{rms}</td>
<td>Flc23</td>
<td>469</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>476</td>
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<tr>
<td></td>
<td>Flc22</td>
<td>477</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>478</td>
<td>-</td>
</tr>
<tr>
<td>NPLA for CSFV Brescia-specific neutralizing antibodies\textsuperscript{c}</td>
<td>Flc23</td>
<td>469</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>476</td>
<td>&lt;10</td>
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<tr>
<td></td>
<td>Flc22</td>
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<td>&lt;10</td>
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<tr>
<td></td>
<td></td>
<td>478</td>
<td>&lt;10</td>
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\textsuperscript{a} Pigs were immunized on day -28 with 8 \times 10\textsuperscript{8} TCID\textsubscript{50} of virus. On day 0, the pigs were challenged intranasally with 100 LD\textsubscript{50} of CSFV strain Brescia 46610.

\textsuperscript{b} The Ceditest E2 ELISA specifically detects antibodies against envelope protein E2 of CSFV. Test results are expressed as the percent inhibition of a standard signal; <30\% is negative (-), 30 to 50\% inhibition is doubtful (+), and >50\% inhibition is positive (+).

\textsuperscript{c} The Ceditest E\textsuperscript{rms} ELISA specifically detects antibodies against envelope protein E\textsuperscript{rms} of CSFV. Test results are expressed as the percent inhibition of a standard signal; <50\% is negative (-), ≥50\% is positive (+).

\textsuperscript{d} The range of observed NPLA titers of sera are expressed as the reciprocal of the serum dilution neutralizing 100 TCID\textsubscript{50} of CSFV strain Brescia.

**DISCUSSION**

An SK6 cell line (SK6c26) expressing E\textsuperscript{rms} was established, enabling complementation of CSFV E\textsuperscript{rms} deletion mutants. The biochemical properties of E\textsuperscript{rms} from this cell line were indistinguishable those of from CSFV E\textsuperscript{rms}, which is identified as an RNase (5, 25). It is striking that this cell line expresses active domains of the Erns RNase are located in the N-terminal half of the protein (5, 25).

Defective interfering particles of BVDV and CSFV lacking the N\textsuperscript{pro}, the four structural proteins, and NS2 are strictly dependent on complementation by a helper virus for packaging (14, 15). Thus, all proteins encoded by the structural region can be complemented in trans. trans-complementation of a single protein in the nonstructural regions in the NS1 or NS5 gene has been reported for the flaviviruses yellow fever virus and Kunjin virus, which also belong to the Flaviviridae family (9, 13). This is the first study showing that the glycoprotein E\textsuperscript{rms} is indispensable to obtain infectious virus and that it can be delivered in trans by a complementing cell line. Infectious virus was obtained only when RNAs derived from pPRKflc22 and pPRKflc23 were complemented in trans by the SK6c26 cells. The complemented recombinant viruses could infect and replicate in SK6 cells, but no infectious virus was produced in these cells, since no spread of virus was observed.

On the one hand, the finding that the recombinant viruses Flc22 and Flc23 and the wild-type virus Flc2 have comparable neutralization indices with a serum specific for E\textsuperscript{rms} indicates that the amounts of E\textsuperscript{rms} on the viral envelope of these viruses are comparable. Also, similar maximum virus titers on SK6c26 cells (10\textsuperscript{9.2} to 10\textsuperscript{9.8} TCID\textsubscript{50}/ml) were obtained for these three viruses. However, on the other hand, the wild-type virus Flc2 reached these titers within 3 days whereas the recombinant viruses Flc22 and Flc23 required almost 6 days. Presumably, in-cis interactions between the structural proteins are more effective for virion assembly, although trans-complementation of E\textsuperscript{rms} by the SK6c26 cells still appears to be quite efficient. Another explanation for the retarded growth of the recombinant viruses might be that only one of the cleavage sites at the capsid protein/Erns site or at the Erns/E1 site is cleaved.

Ten-fold-lower titers were obtained for CSFV strain C on SK6c26 cells (10\textsuperscript{-5.8} TCID\textsubscript{50}/ml) compared to the parental SK6 cells (10\textsuperscript{-5.3} TCID\textsubscript{50}/ml). The stable incorporation of the foreign gene has been reported for the flaviviruses yellow fever virus and Kunjin virus, which also belong to the Flaviviridae family (9, 13). This is the first study showing that the glycoprotein E\textsuperscript{rms} is indispensable to obtain infectious virus and that it can be delivered in trans by a complementing cell line. Infectious virus was obtained only when RNAs derived from pPRKflc22 and pPRKflc23 were complemented in trans by the SK6c26 cells. The complemented recombinant viruses could infect and replicate in SK6 cells, but no infectious virus was produced in these cells, since no spread of virus was observed.

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The glycoprotein E\textsuperscript{rms} possesses the unique feature of RNase activity, and inactivation of this activity by a single-amino-acid substitution of the catalytic residues in CSFV strain C resulted in vitro in a cytopathogenic virus (7). In this study, no cytopathogenic effects or apoptosis were observed with cells in-
fected with the E\textsuperscript{rns} deletion mutant Flc22 or Flc23. The titers of the E\textsuperscript{rns} mutants obtained in this study might be too low to induce apoptosis. Otherwise, deletion of the E\textsuperscript{rns} gene in Flc23 might have a different effect on virus-host interaction from that of inactivation of the RNase activity by a single-amino-acid substitution. Furthermore, we do not know whether the C-terminal deletion in E\textsuperscript{rns} of Flc22 inactivates the RNase activity of this protein. This question is now under investigation as part of a study to delineate the functional properties of E\textsuperscript{rns} in relation to the virus-host interaction.

Homologous RNA recombination yielding wild-type virus has been described for other plus-strand RNA viruses like alphaviruses (12) and may carry a potential risk for the use of nonspraying live vaccines. However, no recombination occurred between the E\textsuperscript{rns} genes of the complementing cell line and the deletion mutant genomes after 10 serial passages of defective virus, as analyzed by RT-PCR and sequencing. This is in agreement with complementation studies of nonstructural proteins of flaviviruses YF1 and Kunjin virus, where no recombination was detected after three serial passages of these viruses (9, 13). However, RNA recombination has been described for BVDV strains, including insertions of cellular sequences (e.g., ubiquitin) and genetic rearrangements like deletions, duplications, or even point mutations (14). These recombination events result in cytopathogenic viruses. However, the incidence of these recombination events is very low and is observed in animals that are persistently infected with BVDV and are highly viremic. While cytopathogenic isolates are frequently observed for BVDV, cytopathogenic CSFV isolates are quite rare in vivo and in vitro (11, 15, 17, 29) and no recombination between cellular sequences and the CSFV genome have been described. Nevertheless, although no recombination was observed after 10 serial passages of the mutated CSF viruses, stocks of virus vaccines should always be examined carefully on this point.

The pilot vaccination and challenge experiment showed that the E\textsuperscript{rns} deletion mutants could protect pigs against a lethal challenge of strain Brescia. Since the recombinant viruses could infect host cells only once, the route of vaccination might be important for protection. Therefore, vaccination was performed simultaneously via multiple inoculation routes. Further research will first focus on the appropriate route of vaccination. A remarkable finding is that vaccinated pigs were negative for CSFV neutralizing antibodies and that no E2 and E\textsuperscript{rns} antibodies could be detected by ELISA until challenge, 28 days postvaccination. The absence of detectable amounts of antibodies can be explained by the low titers of induced antibodies that occur because the complemented viruses can infect target cells only once. König et al. also showed that pigs immunized with E\textsuperscript{rns} were protected even though neutralizing antibodies were not detected (10). In contrast, Terpstra and Wensvoort (27) reported that pigs with a neutralization antibody titer of >32 induced by the live C-strain vaccine were protected against clinical signs of CSF and Bouma et al. (1) showed that pigs with neutralization antibody titers of >50 at 21 days post-vaccination induced by an E2 subunit vaccine were considered to be protected. Our data suggest that pigs can be protected against a lethal challenge even though no antibodies against CSFV are detected.

No differences were found between virus Flc23, lacking the whole E\textsuperscript{rns} gene, and Flc22, lacking the antigenic part of E\textsuperscript{rns} in their in vitro properties, such as growth and neutralization by E\textsuperscript{rns}-specific antibodies, and in their in vivo properties, such as induction of protection against a lethal challenge. Thus, the presence of the N-terminal part of E\textsuperscript{rns} in the mutated genome confers no additional advantage over that of the deletion mutant lacking the whole E\textsuperscript{rns} gene. We focused on the constructing of E\textsuperscript{rns} deletion mutants because E2 is the most powerful protein in the induction of neutralizing antibodies (1, 10, 31).

The vaccination and challenge experiments indicated that the E\textsuperscript{rns} deletion mutants could be useful for further studies on the development of a CSF marker vaccine. However, the strength of a marker vaccine is dependent not only on its immunogenic properties but also on the performance of a diagnostic test that can differentiate between infected and vaccinated animals. Therefore, we have developed an ELISA specific for CSFV E\textsuperscript{rns} (A. J. de Smit, personal communication). However, repeated vaccination of an animal with an E\textsuperscript{rns} deletion mutant virus is needed to demonstrate the suitability of such a test.

Recently we have constructed two CSF marker vaccines based on chimeric CSFV viruses, in which the E2 or E\textsuperscript{rns} gene of the C strain was replaced by that of BVDV type II strain 5250 (H. G. P. van Gennip, submitted for publication). These chimeric viruses protected against a lethal challenge of CSFV strain Brescia and could be serologically differentiated from the wild-type C strain. However, a live genetically engineered chimeric virus, although attenuated, could face problems regarding acceptance as vaccine. The described CSFV deletion mutants described here are safer as vaccines than are the chimeric viruses, since these deletion mutants are nontransmissible.

Although live vaccines are generally considered to have the best immunological properties, the use of a conventional live vaccine, besides the lack of marker properties, can have the disadvantage that the vaccine virus can spread through an animal and is eventually transmitted to a second contact animal. Our results represent a significant advance in the area of the development of a live-attenuated nontransmissible CSF marker vaccine. The possibility of trans-complementing the CSFV glycoprotein E\textsuperscript{rns} also opens up new opportunities for the development of safe, nontransmissible marker vaccines for other pestiviruses, such as BVDV, based on deletion of an antigenic structural protein.

REFERENCES


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