Determinants of Virulence of Classical Swine Fever Virus Strain Brescia

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Two related classical swine fever virus (CSFV) strain Brescia clones were isolated from blood samples from an infected pig. Virus C1.1.1 is a cell-adapted avirulent variant, whereas CoBrB is a virulent variant. Sequence analysis revealed 29 nucleic acid mutations in C1.1.1, resulting in 9 amino acid substitutions compared to the sequence of CoBrB [476]R. Using reverse genetics, parts of the genomes of these viruses, which contain differences that lead to amino acid changes, were exchanged. Animal experiments with chimeric viruses derived from C1.1.1 and CoBrB [476]R showed that a combination of amino acid changes in the structural and nonstructural regions reduced the virulence of CSFV in pigs. Moreover, the presence of a Leu at position 710 in structural envelope protein E2 seemed to be an important factor in the virulence of the virus. Changing the Leu at position 710 in the CoBrB [476]S variant into a His residue did not affect virulence. However, the [710]His in the C1.1.1/CoBrB virus, together with adaptive mutations [276]R, [476]R, and [477]I in E^{rns}, resulted in reduced virulence in pigs. These results indicated that mutations in E^{rns} and E2 alone do not determine virulence in pigs. The results of in vitro experiments suggested that a high affinity for heparan sulfate of C1.1.1 E^{rns} may reduce the spread of the C1.1.1/CoBrB virus in pigs and together with the altered surface structure of E2 caused by the [710]L→H mutation may result in a less efficient infection of specific target cells in pigs. Both these features contributed to the attenuation of the C1.1.1/CoBrB virus in vivo.

Classical swine fever is a highly contagious and sometimes fatal viral disease in pigs. The causative agent, classical swine fever virus (CSFV), is a member of the *Pestivirus* genus within the family *Flaviviridae* (37). The other two members of this genus are bovine viral diarrhea virus (BVDV) and border disease virus. CSFV has been found to be infectious only for pigs. BVDV and border disease virus can infect both ruminants and pigs (3). Pestiviruses are small, enveloped, plus-strand RNA viruses (22). The RNA genome is approximately 12.5 kb (1, 4, 19, 25) and contains a single large open reading frame (ORF) (1, 5, 19, 25), which is flanked by 5′ and 3′ nontranslated regions (NTRs). This ORF is translated into a polyprotein, which is further processed into mature proteins by viral and host cell proteases (27).

The surface structure of pestivirus virions is composed of three glycoproteins, E^{rns}, E1, and E2 (30). E2 is present as a homodimer and as an E2-E1 heterodimer (30, 39). The C terminus of E2 (and probably of E1 as well) functions as a membrane-spanning domain (13) and anchors the E2-E1 and E2-E2 dimers in the viral lipid membrane. The association of E^{rns} homodimers with the virion is not accomplished by a membrane-spanning domain and is tenuous (7). The mechanism of E^{rns} association with virions is currently unknown. All three envelope proteins contain N-linked glycosyl groups (27, 30, 39). E^{rns} is glycosylated to a higher extent than E1 and E2 are. N-linked glycosyl residues account for about half of the mass of an E^{rns} homodimer (27, 44). A considerable portion of the E^{rns} protein produced in infected cells is secreted into the extracellular environment and circulates in the body fluids of infected animals (27). The unexpected finding that E^{rns} possesses RNase activity (9, 28) led to several interesting studies regarding the function of E^{rns} in the life cycle of pestiviruses. The results of in vitro and in vivo studies indicated that E^{rns} (and its enzyme activity) plays a role in the regulation of RNA synthesis in infected cells (10) and in weakening the immune defense of the host early in infection (2, 20).

Recently, it was demonstrated that in vitro cultivation of CSFV in swine kidney cells selects virus variants that attach to the surfaces of cultured cells by interaction with the membrane-associated glycosaminoglycan heparan sulfate (HS) (11). Using reverse genetics, it was shown that replacement of a neutral serine residue by a positively charged arginine residue in the C terminus of E^{rns} (position 476 in the ORF) changes an HS-independent CSFV to a virus that uses HS as an E^{rns} receptor. These findings showed that interaction of virus-bound E^{rns} with HS immobilizes CSFV on the surfaces of cultured swine kidney cells. Animal experiments showed that this Ser-to-Arg change in the C terminus of E^{rns} did not affect virulence (12). In the same study, it was shown that virus reisolated from pigs infected with the HS-independent recombinant virus was able to infect cultured and primary swine kidney cells by an HS-dependent mechanism. These results suggested that the surface properties of CSFV generated in pigs and carrying [476]Ser in the C terminus of E^{rns} are distinct

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from those of genetically identical virus produced in cell culture.

Field isolates and laboratory strains of CSFV vary considerably in their virulence, but until now, no genetic determinants of virulence could be defined despite extensive efforts in sequencing and detailed phylogenetic studies (14, 35). The same holds true for the search for in vitro parameters that would allow grouping of viruses into virulence classes. No significant qualitative or quantitative differences were found between CSFV strains with different degrees of virulence when RNA replication and protein synthesis were investigated by Mittelholzer et al. (21). However, these researchers showed that reduced particle release from SK6 cells was inversely correlated with the virulence of CSFV strains.

Altered virulence properties of viral variants are the basis for their application as live attenuated vaccines. Therefore, it is of particular interest to elucidate the mechanisms causing virus variants to lose the capacity to produce disease in a host and link them to genetic changes in their genomes. Potential mechanisms for the attenuation of viral virulence include the following: (i) changes in viral binding and penetration properties on host membranes, resulting in altered tissue tropisms; (ii) a reduction of viral replication rate in vivo; (iii) a decreased efficiency of virus spread in the host; and (iv) an increased susceptibility of variant viruses to host antiviral responses (reviewed in reference 31). Virulence attenuation has traditionally been achieved by serial passage of virus in cultured cells or laboratory animals (or both), a selection process that can give rise to adaptive changes that result in reduced virulence in the natural or incidental hosts.

We report here on the mechanism for virulence attenuation of laboratory-derived CSFV variants with altered attachment and surface properties in vitro. Therefore, sequences of a virulent Brescia variant (CoBrB ^{476}R [11]) and a cell-adapted avirulent variant C1.1.1 (41), isolated from blood samples from a pig infected with CSFV strain Brescia, were compared. We studied the roles of these adaptive amino acid changes by generating chimeric CSFVs from full-length cDNA constructs. Infection of pigs with these recombinant viruses revealed a possible role of the adaptive Leu→His mutation at position 710 of the polyprotein, but the ^{710}His variant resulted in an avirulent virus only when the variant also contained three adaptive mutations in E^{rns}. Analysis of in vitro- and in vivo-generated viruses suggested that both the high affinity for HS and an altered surface structure of E2 may result in a less efficient infection of specific target cells in pigs. Both these features contribute to the attenuation of CSFV strain Brescia in vivo.

MATERIALS AND METHODS

Cells and viruses. The swine kidney cell line SK6 (15) was maintained as described previously (32). Porcine lung macrophages were isolated from the lungs of 6-week-old specific-pathogen-free piglets as described previously (42). Fetal bovine serum (FBS) and cells were free of BVDV, and the FBS was free of anti-BVDV antibodies. CSFV strain Brescia was isolated from a pig infected with a virulent field isolate (isolate 456610) (40). Brescia virus was grown for 24 passages on PK15 cells and cloned by repeating endpoint dilution (three times). This virus clone (C1.1.1) was passaged three times on PK15 cells to produce a virus stock. This virus stock was adapted to growth on SK6 cells by two extra passages (11). The recently constructed recombinant virus vflC1.1.1 Erns^{R-RI} (11) was derived from the SK6-produced virus stock. Isolation of the HS-inde-

pendent Brescia virus clone Bp2 and its HS-dependent counterpart, Bp3, which was passaged one more time in SK6 cells, have been described recently (11). The constructed recombinant viruses derived from Brescia were named vflCoBrB Erns^{S-ST} (clone Bp2) and vflCoBrB Erns^{S-RT} (clone Bp3) and have been characterized as being as virulent as the pig isolate 456610 from which they were isolated from (11). In this article, viruses vflC1.1.1 Erns^{R-RI}, vflCoBrB Erns^{S-ST}, and vflCoBrB Erns^{S-RT} will be referred to as hereafter as C1.1.1, CoBrB ^{476}R, and CoBrB ^{476}S, respectively.

Sequence comparison of recombinant viruses C1.1.1 and CoBrB ^{476}R. The isolation and sequencing of virus clones C1.1.1 and CoBrB ^{476}R have been described elsewhere (11, 24). The genomic sequences were compared using Auto-Assembler software (Applied Biosystems). Sequence mutations between the two Brescia clones were confirmed by sequencing independent PCR fragments.

Construction, generation, and characterization of chimeric viruses derived from C1.1.1, CoBrB ^{476}R, and CoBrB ^{476}S. Recently, full-length DNA copies of clones C1.1.1 and CoBrB of CSFV Brescia strain have been described (11, 12). These DNA copies were constructed in the same manner as the full-length cDNA of CSFV strain C in low-copy-number plasmid pOK12 (23). The junction between the T7 RNA polymerase promoter sequence and the 5′-terminal nucleotide of C1.1.1 and CoBrB and the junction between the 3′-terminal nucleotide of C1.1.1 and CoBrB and the vector were similar to those described for the full-length copy of strain C in pOK12 (23). Digestion of these full-length cDNAs in pOK12 (named pflC1.1.1 or pflCoBrB) with SrfI generates the exact 3′ terminus of the RNA genome of pflC1.1.1 and pflCoBrB. The viruses derived from these two full-length clones are named C1.1.1 and CoBrB ^{476}R, respectively. Animal experiments showed that C1.1.1 is avirulent (11) and CoBrB ^{476}R is virulent (12). The first chimeric constructs were derived from pflC1.1.1 and pflCoBrB ^{476}R, focusing on the amino acid mutations between pflC1.1.1 and pflCoBrB ^{476}R.

To introduce the three adaptive mutations located in the structural region from pflC1.1.1 into a virulent background (pflCoBrB ^{476}R), the ClaI/NcoI fragment (nucleotides [nt] 778 to 5534) from pflC1.1.1 was introduced into ClaI/NcoI-digested pflCoBrB ^{476}R. This resulted in a full-length cDNA pflC1.1.1/CoBrB with adaptive mutations ^{276}S→R and ^{477}T→I in E^{rns} and ^{710}L→H in E2 (see Fig. 3). To introduce six adaptive amino acid mutations located in the nonstructural region from pflC1.1.1 into a virulent background (pflCoBrB ^{476}R), the NcoI/AatII fragment (nt 5534 to 12279) from pflC1.1.1 was introduced into NcoI/AatII-digested pflCoBrB ^{476}R. This resulted in a full-length cDNA pflCoBrB ^{476}R/C1.1.1 with adaptive mutations ^{1915}A→S and ^{1971}S→P in NS3, ^{2638}L→V in NS4b, ^{2846}R→C in NS5a, and ^{3419}N→I and ^{3858}V→A in NS5b (see Fig. 3). A third full-length cDNA, in which CoBrB ^{476}R was introduced into pflC1.1.1, was constructed by introducing the ClaI/NgoMIV fragment (nt 778 to 2439) of pflCoBrB ^{476}R into ClaI/NgoMIV-digested pflC1.1.1. This construct was named pflC1.1.1/CoBrB (N$^{pro'}$-C-E^{rns}-E1) (exchanged proteins shown in parentheses; hybrid proteins resulting from the fusion of C1.1.1 and CoBrB indicated by a prime after the protein) (see Fig. 3).

The second series of chimeric full-length cDNAs were based on pflC1.1.1 and pflCoBrB ^{476}S, focusing on the role of the ^{710}H→L in E2. Therefore, the NgoMIV/NcoI fragment (nt 2439 to 5534) of pflC1.1.1 was introduced into pflCoBrB ^{476}S, resulting in a full-length copy of pflCoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) (see Fig. 3). For the construction of pflCoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1), the NgoMIV/NcoI fragment (nt 2439 to 5534) of pflCoBrB ^{476}S was introduced into pflC1.1.1/CoBrB.

SrfI-linearized DNA (250 ng) of full-length plasmids was transfected into SK6.T7a5 cells as described previously (33). Two days after transfection, the medium was harvested and stored at −70°C, and cells were immunostained with monoclonal antibody (MAb) V3 directed against E2 (38). A portion (100 μl) of the medium collected from wells in which E2 expression was detected (virus passage number 1 [p1]) was used to infect confluent monolayers of SK6 cells grown in 2-cm² tissue culture wells. After 2 days of growth, cells were treated with trypsin, 90% of the cells were transferred to a 25-cm² tissue culture flask, and 10% of the cells were transferred to a 2-cm² tissue culture well. After 3 days of growth, wells were immunostained with MAb V3 and flasks were freeze-thawed twice to prepare virus stock p2. The percent inhibition or reduction of infection of SK6 cells with these recombinant viruses by 225 μg of heparin per ml was determined as described below.

Virus isolation from clinical samples. Leukocytes (white blood cells) were isolated from blood treated with EDTA as described recently (6). Isolated leukocytes were resuspended in the same volume of medium (Eagle basal medium containing 10% FBS and antibiotics) as the volume of blood that was used for isolation. Leukocyte suspensions were stored at −70°C. Tissue suspensions (10% [wt/vol]) in Eagle minimal essential medium with Earle salts (EMEM) containing 5% FBS and antibiotics were prepared as described previously (6).
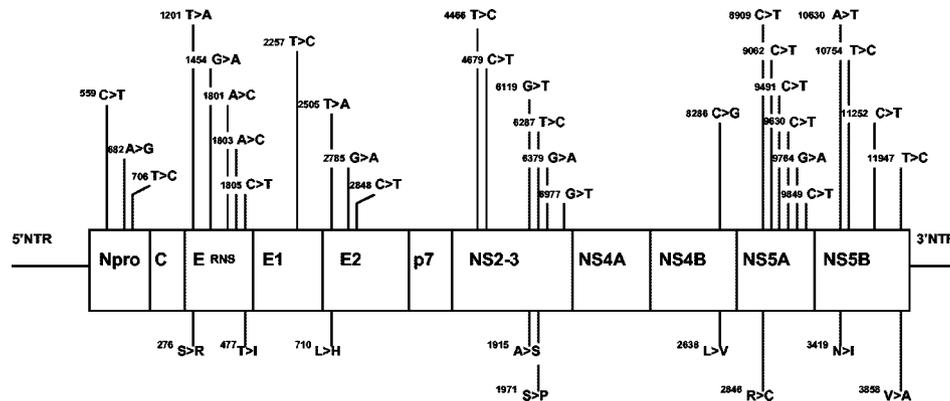
FIG. 1. Comparison of the cDNA sequences of C1.1.1 and CoBrB ^{476}R. Only 29 nucleic acid differences were found (shown above the genome bar); these differences resulted in 9 amino acid substitutions (shown below the genome bar). The sequence differences are shown in the following order: CoBrB ^{476}R > C1.1.1 (original sequence to the adaptive sequence).

These suspensions were stored at −70°C. EDTA-treated blood was frozen at −70°C without further treatment. All leukocyte, tissue, and blood samples, frozen once at −70°C were thawed, divided in aliquots, and stored again at −70°C. In all tests, virus samples that had been freeze-thawed twice were used.

Chemicals, enzymes, and recombinant proteins. Heparin (195 U/mg from porcine intestinal mucosa) and concanavalin A (ConA) were obtained from Sigma, St. Louis, Mo. Lyophilized enzyme was dissolved in storage buffer and stored in aliquots at −70°C as described previously (11). Heparin and ConA were dissolved in EMEM lacking both FBS and antibiotics and stored at 4°C for no longer than 2 weeks.

Inhibition experiments. In the plaque assay, confluent monolayers of SK6 cells, grown in 2-cm^2 tissue culture wells, were washed twice with EMEM lacking both FBS and antibiotics. The cells were preincubated at 37°C for 30 min with 150 μl of EMEM with 300 μg of heparin per ml. Fifty microliters of a dilution of a virus stock in EMEM was added to the wells, mixed, and incubated as described above. The concentration used in the text and figures hereafter corresponds with this diluted concentration (225 μg of heparin per ml). After 30 min, the virus was removed, and the wells were washed twice with 0.5 ml of EMEM and supplied with overlay medium (containing methylcellulose). Cells were grown for 18 h at 37°C, and infectious centers (hereafter referred to as plaques) were detected by immune staining with E2-specific MAb V3. Positive plaques in a well were counted with a microscope. When more than 250 plaques per well were present, a minimum of 100 plaques in a fixed area (at a magnification of ×40) were counted to calculate the total number of plaques in these wells. Percent inhibition was calculated using the formula $100 \times [1 - (e/c)]$, where c is the average number of plaques in duplicate or triplicate wells to which no heparin was added (control well) and e is the average number of plaques in duplicate or triplicate wells to which heparin was added. Percent infection compared to control wells was calculated using the formula $100 \times (e/c)$. For all virus samples, the percentage of inhibition was determined at two different multiplicities of infection (MOIs). For all samples, no seriously different percentages were measured when different amounts of viruses were tested for inhibition by heparin. Therefore, percentages measured at the highest MOI are presented. Relative plaque sizes of viruses were scored in wells to which no heparin was added after 48 h of growth under overlay medium.

Macrophage plaque assay. Infection of alveolar macrophages with CSFV was measured using a modified plaque assay as described by Hulst et al. (12), except no heparin or heparinase I was used in our assay.

ConA inhibition or stimulation assay. Viruses were tested for inhibition or stimulation of infection with different concentrations of ConA as described previously (12).

Ratio of secreted virus to cell-associated virus. SK6 cells grown in 24-well plates were infected with viruses at an MOI of 1. After infection for 30 min, cells were washed two times with complete medium before 1 ml of complete medium was added to each well. At 2 days postinfection (p.i.), the supernatant was collected and stored at −70°C. One milliliter of complete medium was added to the cells in each well before they were freeze-thawed twice at −70°C. Samples were titrated on SK6 cells in 24-well plates in a plaque assay. The ratio of secreted virus to cell-associated virus was determined in a plaque assay and calculated by the following formula: ratio = $\log_{10}$ PFU of cell-associated virus − $\log_{10}$ PFU of secreted virus.

Virus neutralization index. The titers of in vitro- or in vivo-generated viruses CoBrB ^{476}S, CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1), and C1.1.1/CoBrB in the presence or absence of neutralizing antibodies were determined. The virus neutralization index is the log reduction of virus titer, measured in 50% tissue culture infective doses (TCID$_{50}$s), by neutralizing MAb or polyclonal antibodies (PAbs). The virus neutralization index was determined for MAb V4 and V6 and a serum sample (Pab$^{R-RI\ H}$) from blood taken at day 21 p.i. from a C1.1.1/CoBrB-infected pig (animal 5021 [see Table 2]). MAb V4 (4 mg/ml) is directed against a conserved epitope in domain A of the E2 protein of CSFV (38) and was used at a dilution of 1:50. MAb V6 (4 mg/ml) is directed against a nonconserved epitope in domain B of CSFV E2 and was previously characterized as a MAb-resistant mutation for MAb V6 (34) and was diluted at a dilution of 1:50. Pab$^{R-RI\ H}$ serum was used at a dilution of 1:1,000.

Animal experiments. CSFV recombinant virus stocks (p2) of C1.1.1, CoBrB ^{476}R, C1.1.1/CoBrB (N$^{pro′}$-C-E^{rns}-E1), C1.1.1/CoBrB, and CoBrB ^{476}R/C1.1.1 for animal experiment 1 and virus stocks of CoBrB ^{476}S, CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1), and C1.1.1/CoBrB for animal experiment 2 were diluted in SK6 tissue culture medium to a concentration of 10^5 TCID$_{50}$ per ml (as determined by titration on SK6 cells). Groups of three, 6-week-old, specific-pathogen-free pigs were infected by spraying 1 ml of this virus dilution in each nostril on day 0. In animal experiment 2, one sentinel was placed 24 h p.i. in the stable of each group of three infected pigs, allowing contact exposure with the other pigs. All animals were observed daily for signs of disease, and body temperatures were measured. Fever is defined here as a body temperature of >40°C for at least 2 consecutive days. EDTA-treated blood samples were taken on days 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, and 23. These blood samples were used for isolation of virus from leukocytes (see below) and for monitoring the concentration of thrombocytes and leukocytes. Thrombocytes and leukocytes were counted as described previously (6). Serum samples were taken on days 0, 7, 14, 21, and 23 and tested in an enzyme-linked immunosorbent assay (ELISA) that specifically detects antibodies directed against envelope protein E2 (Ceditest-Ab kit). Pigs were killed when moribund or slaughtered at the end of the trial. At the postmortem examination, tissue samples of the spleen, kidney, tonsil, and ileum were collected, frozen in liquid nitrogen, and stored at −70°C. Duplicate cryostat sections of these tissue samples were tested for viral antigen using a direct immunofluorescence technique (6).

RESULTS

Sequence comparison of viruses C1.1.1 and CoBrB ^{476}R. To find genetic factors that determine the virulence of CSFV Brescia, we cloned and sequenced CoBrB ^{476}R cDNA and compared this sequence to the cDNA sequence of C1.1.1. We found 29 nucleic acid mutations, which resulted in 9 amino acid substitutions (Fig. 1). No sequence differences were found in the 5′ and 3′ NTRs of Brescia C1.1.1 and CoBrB ^{476}R strains, suggesting that these regions of the genome do not determine the virulence of the two viruses. The amino acid

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C1.1.1.      ......>>²⁷²QWNLRDNGT²⁸⁰<<.........>>⁴⁷³RQLRIAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLHGAEG⁷¹⁴<<........
CoBrB ⁴⁷⁶R   ......>>²⁷²QWNLSDNGT²⁸⁰<<.........>>⁴⁷³RQLRTAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLLGAEG⁷¹⁴<<........
Alfort/187   ......>>²⁷²QWNLSDNGT²⁸⁰<<.........>>⁴⁷³RQLRIAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLLGAGG⁷¹⁴<<........
ALD          ......>>²⁷²QWNLSDNGT²⁸⁰<<.........>>⁴⁷³RQLRIAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLLGAGG⁷¹⁴<<........
GPE⁻         ......>>²⁷²QWNLSDNGT²⁸⁰<<.........>>⁴⁷³RQLRIAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLLGAGG⁷¹⁴<<........
C strain     ......>>²⁷²QWNLSDNGT²⁸⁰<<.........>>⁴⁷³RQLRTAGKR⁴⁸¹<<.........>>⁷⁰⁶EIGLLGAGG⁷¹⁴<<........


C1.1.1.      ......>>¹⁹¹¹EYHCSTPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷EDLGPEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHVYGVF²⁶⁴²<<......
CoBrB ⁴⁷⁶R   ......>>¹⁹¹¹EYHCATPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷EDLGSEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHLYGVF²⁶⁴²<<......
Alfort/187   ......>>¹⁹¹¹EYHCATPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷EDLGSEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHLYGVF²⁶⁴²<<......
ALD          ......>>¹⁹¹¹EYHCATPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷EDLGSEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHLYGVF²⁶⁴²<<......
GPE⁻         ......>>¹⁹¹¹EYHCATPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷EDLGSEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHLYGVF²⁶⁴²<<......
C strain     ......>>¹⁹¹¹EYHCATPEQ¹⁹¹⁹<<...>>¹⁹⁶⁷KDLGSEYLD¹⁹⁷⁵<<.......>>²⁶³⁴LVYHLYGVF²⁶⁴²<<......


C1.1.1.      ......>>²⁸⁴²GAGYCGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKIKVEE³⁴²³<<......>>³⁸⁵⁴YEELALARK³⁸⁶²<<......
CoBrB ⁴⁷⁶R   ......>>²⁸⁴²GAGYRGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKNKVEE³⁴²³<<......>>³⁸⁵⁴YEELVLARK³⁸⁶²<<......
Alfort/187   ......>>²⁸⁴²GAGYHGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKNKVEE³⁴²³<<......>>³⁸⁵⁴YEELVLARK³⁸⁶²<<......
ALD          ......>>²⁸⁴²GAGYHGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKNKVEE³⁴²³<<......>>³⁸⁵⁴YEELVLARK³⁸⁶²<<......
GPE⁻         ......>>²⁸⁴²GAGYHGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKNKVEE³⁴²³<<......>>³⁸⁵⁴YEELVLARK³⁸⁶²<<......
C strain     ......>>²⁸⁴²GAGYHGAYL²⁸⁵⁰<<...>>³⁴¹⁵DSEKNKVEE³⁴²³<<......>>³⁸⁵⁴YEELVLARK³⁸⁶²<<......
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FIG. 2. Multiple-sequence alignment of the amino acid sequences of C1.1.1 and CoBrB ⁴⁷⁶R with the sequences of virulent CSFV strains Alfort/187 (19) and ALD (14) and vaccine strains GPE⁻ (14), derived from strains ALD and C (23). The amino acids at the positions where substitutions were found in C1.1.1 and CoBrB ⁴⁷⁶R are shown in bold type.

sequences of C1.1.1 (25) and CoBrB ⁴⁷⁶R were compared with those of CSFV strains Alfort (26), ALD (14), GPE⁻ (14), and C (23). Except for the amino acid at position 2846 in nonstructural protein NS5A, the amino acids of virulent CoBrB ⁴⁷⁶R and other virulent and avirulent CSFV strains are conserved compared to those of the C1.1.1 sequence (Fig. 2).

Construction and characterization of chimeric viruses derived from C1.1.1 and CoBrB ⁴⁷⁶R. To study the role of adaptive amino acid mutations on virulence, three chimeric cDNAs were constructed by exchanging parts of the genomes of C1.1.1 and CoBrB ⁴⁷⁶R. The first full-length cDNA (pflC1.1.1/CoBrB) contains the three adaptive amino acid mutations in the structural region of C1.1.1, ²⁷⁶R and ⁴⁷⁷I in Eʳⁿˢ and ⁷¹⁰H in E2 (Fig. 3) in a virulent background of CoBrB ⁴⁷⁶R. The second full-length cDNA (pflCoBrB ⁴⁷⁶R/C1.1.1) contained the six adaptive amino acid mutations in the nonstructural region originating from C1.1.1, ¹⁹¹⁵S and ¹⁹⁷¹P in NS3, ²⁶³⁸V in NS4b, ²⁸⁴⁶C in NS5a, and ³⁴¹⁹I and ³⁸⁵⁸A in NS5b (Fig. 3). The last of these full-length cDNAs [pflC1.1.1/CoBrB (Nᵖʳᵒ'-C-Eʳⁿˢ-E1)] contains the six adaptive amino acid mutations in the nonstructural region originating from C1.1.1, ¹⁹¹⁵S and ¹⁹⁷¹P in NS3, ²⁶³⁸V in NS4b, ²⁸⁴⁶C in NS5a, and ³⁴¹⁹I and ³⁸⁵⁸A in NS5b, and the adaptive amino acid mutation ⁷¹⁰H in E2 (Fig. 3).

Transfection of SrfI-linearized full-length DNAs into SK6.T7 cells (33) yielded the infectious recombinant viruses C1.1.1/CoBrB, CoBrB ⁴⁷⁶R/C1.1.1, and C1.1.1/CoBrB (Nᵖʳᵒ'-C-Eʳⁿˢ-E1). The transfection medium was used to infect SK6 cells in order to produce p2 virus stocks. The introduced mutations were confirmed by sequencing the amplified DNA of these virus stocks. Virus titers of the p2 stocks of the recombinant viruses were comparable [7.5×10^7 TCID$_{50}$/ml for C1.1.1/CoBrB, 8×10^7 TCID$_{50}$/ml for CoBrB ⁴⁷⁶R/C1.1.1, and 7.3×10^7 TCID$_{50}$/ml for C1.1.1/CoBrB (Nᵖʳᵒ'-C-Eʳⁿˢ-E1)] to those of the parent viruses C1.1.1 (7.3×10^7 TCID$_{50}$/ml) and CoBrB Erns^{S-RT} (7.05×10^7 TCID$_{50}$/ml). No growth differences on porcine alveolar macrophages or SK6 cells could be detected (results not shown).

Virulence of chimeric viruses derived from C1.1.1 and CoBrB ⁴⁷⁶R. To analyze the effects of the adaptive amino acid mutations on virulence in pigs, animal experiments were conducted. Pigs were infected intranasally according to the regimen described in Materials and Methods, and results are shown in Table 1.

Pigs infected with the full-length clone of cell-adapted avirulent Brescia clone C1.1.1 (group A) showed no signs of CSF (Table 1). All animals developed a mild leukopenia between days 2 and 7. Analysis of serum samples showed that all animals developed antibodies against E2 in serum samples. No viral antigen could be detected in tonsil, spleen, kidney, and ileum tissue samples after slaughter. Only one pig (pig 4052) developed a mild fever for a short period (3 days) and showed thrombocytopenia. Virus was isolated from blood from this pig at day 7.

In contrast, pigs infected with the virulent CoBrB ⁴⁷⁶R strain (group B) rapidly developed fever (≥40°C) for a period of more than 10 days and symptoms of acute CSF (Table 1). All pigs became seriously ill, as indicated by the lack of appetite, vomiting, dullness, blue ears, and paresis. All animals developed leukopenia and thrombocytopenia. Pig 4062 (group B) developed the chronic form of CSF. No antibodies against E2 were detected in serum samples of this pig, and the pig remained viremic until the end of the trial. After slaughter, viral antigen could also be detected in tissue samples of all pigs. These results clearly showed the avirulent phenotype of C1.1.1 and the virulent phenotype of CoBrB ⁴⁷⁶R.

Only virus CoBrB ⁴⁷⁶R/C1.1.1 (group C) exhibited a level of virulence (death, fever, and clinical signs) comparable to that of CoBrB ⁴⁷⁶R (group B) (Table 1). One pig in group C (pig 4058), however, recovered after having only mild signs of CSF. The presence of antibodies against E2 in serum samples of this pig confirmed infection with CSFV. Virus C1.1.1/CoBrB (group D) caused a mild form of CSF (Table 1). This virus caused fever for several days, and typical signs of CSF were observed in pigs. The pigs of group D recovered rapidly, except
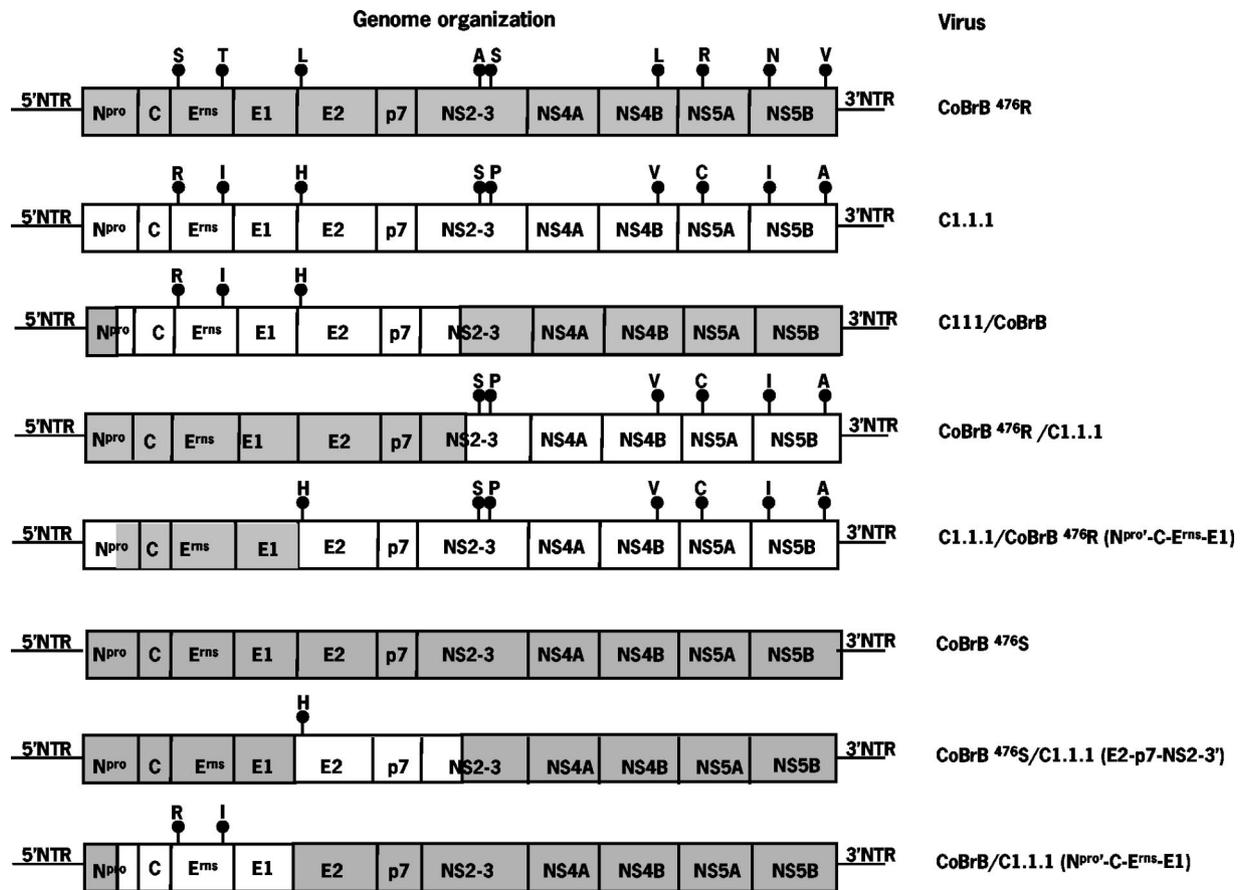
FIG. 3. Schematic representation of full-length cDNA constructs of parent CSFV strains CoBrB (shaded bars) and C1.1.1 (white bars), and chimeric viruses derived from these strains. The genome organization and the name of the virus (exchanged proteins in parentheses) are shown. The original amino acids in CoBrB and the adaptive amino acid mutations in C1.1.1 and chimeric viruses are shown, and localization (lollypops) is indicated. When a fusion of C1.1.1 and CoBrB resulted in a hybrid protein, this was indicated by a prime after the name of the protein.

for one pig (pig 4056), which died of CSF (day 14) before developing antibodies against E2. Another pig (pig 4054) in this group, which had recovered from CSF, died before the end of the trial for unknown reasons. Virus C1.1.1/CoBrB (N$^{pro'}$-C-E^{rns}-E1) (group E), carrying only the 2 amino acids in E^{rns} that originate from the virulent CoBrB ^{476}R, did not cause CSF and is avirulent in pigs (Table 1).

Genetic analysis of in vivo-generated virus. Blood samples taken from pigs were used to infect SK6 cells, and after 2 days, RNA was extracted from these cells and used to determine the sequence of the regions containing mutations. For all viruses, the sequence determined matched the original genotype of the virus administered to the pig. These results showed that replication of these recombinant viruses did not induce genetic changes (such as reversion) in the sequenced regions. However, as in a previous study (12), a minority of revertants were found in blood from a pig infected with CoBrB ^{476}R. The reversion consists of an amino acid substitution in E^{rns} at residue position 476 from an Arg to a Ser, which reflects the phenotypical change from an HS-dependent virus to an HS-independent virus in vitro. This change had no effect on virulence in pigs (12). The amino acid ^{476}S is the predominant variant found in blood from a pig infected with Brescia, pig 456610. Therefore, in further experiments, the CoBrB ^{476}S

variant was used as the parental virulent virus instead of CoBrB ^{476}R virus.

Construction and characterization of chimeric viruses derived from C1.1.1 and CoBrB ^{476}S with adaptive mutations in E^{rns} and E2. The only difference between CoBrB ^{476}R/C1.1.1 and C1.1.1/CoBrB (N$^{pro'}$-C-E^{rns}-E1) is a single amino acid substitution at position 710 (L→H). Because virus CoBrB ^{476}R/C1.1.1 caused CSF (in two of three pigs) and virus C1.1.1/CoBrB (N$^{pro'}$-C-E^{rns}-E1) was avirulent, the results from animal experiment 1 indicate that a single amino acid substitution at position 710 (L→H) may be involved in the virulence of strain Brescia. Therefore, the possible role of the adaptive amino acid mutation ^{710}L→H in virulence was investigated further.

Therefore, two new full-length cDNAs were constructed. The first full-length cDNA [pflCoBrB ^{476}S/C1.1.1(E2-p7-pNS2-3′)] contained the adaptive amino acid mutation at position 710 in the virulent background of CoBrB ^{476}S (Fig. 3). The second full-length cDNA [pflCoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1)] contained three adaptive amino acid mutations in E^{rns} (^{276}R, ^{476}R, and ^{477}I) compared to the CoBrB ^{476}S variant. Virus stocks (p2) of CoBrB ^{476}S and CoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1) were produced as described above. The introduced mutations were confirmed by sequence analysis of the E^{rns} and E2 genomic regions of these recombinant viruses (p2 stocks).

TABLE 1. Serological and clinical findings after inoculation of pigs with chimeric viruses derived from
C1.1.1 and CoBrB [476]R in animal experiment 1

Group[a]	Virus	Animal no.	Fever[b]	L/T[c]	IFT (T K S I)[d]	Death (d.p.i.)[e]	0	7	14	21	23	0	2	4	7	9	11	14	16	18	21	23
							Serological findings (% inhibition) by E2 ELISA[f] on d.p.i.:					Viremia[g] on d.p.i.:										
A	C1.1.1	4051	–	+/+	– – – –	–	0	0	90	102	102	–	–	–	–	–	–	–	–	–	–	–
		4052	3 (5)	+/–	– – – –	–	0	0	91	101	102	–	–	–	+	–	–	–	–	–	–	–
		4053	–	+/–	– – – –	–	0	0	96	102	102	–	–	–	–	–	–	–	–	–	–	–
B	CoBrB [476]R	4060	11 (3)	+/+	+ + + +	+ (22)	0	0	0	0		–	–	+	+	+	+	+	+	+	+	
		4061	11 (3)	+/+	+ + + +	+ (14)	0	0	0			–	–	–	+	+	+	+	+			
		4062	12 (4)	+/+	+ + + +	–	0	0	0	0	0	–	–	–	+	+	+	+	+	+	+	
C	CoBrB [476]R/C1.1.1	4057	8 (4)	+/+	+ + + +	+ (14)	0	0				–	–	–	+	+	+					
		4058	–	+/–	– – – –	–	0	0	30	92	99	–	–	–	+	+	–	–	+	–	–	
		4059	9 (7)	+/+	+ + + +	+ (17)	0	0	0			–	–	–	+	+	+					
D	C1.1.1/CoBrB	4054	4 (4)	+/+	– – – –	+ (18)	0	0	97			–	–	+	+	–	–	–	–	–		
		4055	7 (4)	+/+	– – – –	–	0	0	101	102	102	–	–	–	+	–	–	–	–	–	–	
		4056	8 (4)	+/+	+ – – –+[h]	+ (14)	0	0				–	–	–	+	+	+					
E	C1.1.1/CoBrB (N[pro′]-C-E[rns]-E1)	4066	–	+/–	– – – –	–	0	0	51	100	101	–	–	–	–	–	–	–	–	–	–	–
		4067	1 (4)	+/–	– – – –	–	0	0	33	95	100	–	–	–	–	–	–	–	–	–	–	–
		4068	–	+/–	– – – –	–	0	0	18	89	97	–	–	–	–	–	–	–	–	–	–	–

[a] Three pigs in each group were inoculated intranasally once with 10^5 TCID$_{50}$ of each virus.
[b] Fever defined as a body temperature of $\geq 40°C$. The number of days with fever is shown. The day the fever began is shown in parentheses. –, no fever.
[c] Leukopenia (L) and thrombocytopenia (T) are defined as cell/platelet counts below 8×10^6 cells/ml and 200×10^6 cells/ml, respectively.
[d] Immunofluorescence technique (IFT) for detection of viral antigen in cryostat sections from tonsils (T), kidney (K), spleen (S), and ileum (I) at death.
[e] Pigs were either found dead or killed when moribund (+). The time of death (in days p.i. [d.p.i.]) is shown in parentheses. –, animal did not die.
[f] The E2 ELISA specifically detects antibodies against CSFV.
[g] Isolated leukocyte samples were incubated on SK6 cells for detection of virus by using an immunoperoxidase monolayer assay.
[h] A few crypts in tonsils were found positive.

The Ser→Arg mutation at position 476 in E[rns] reflects the phenotypical change from an HS-independent virus to an HS-dependent virus. Therefore, virus stocks (p2) of CoBrB [476]S, CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′), C1.1.1/CoBrB, and CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) were tested for inhibition of infection by heparin. Infection of SK6 cells with C1.1.1/CoBrB and CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) was inhibited by 100% by 225 μg of heparin per ml. In contrast, infection of SK6 cells with CoBrB [476]S and CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) was not significantly affected by heparin (Table 2). When SK6 cells were grown under a methylcellulose overlay medium, the diameters of plaques of both CoBrB [476]S variants (S-ST) were two to three times larger than those of the plaques from both C1.1.1/CoBrB and CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) (R-RI). Furthermore, the titers of p2 virus stocks measured on SK6 cells were about 100-fold lower for CoBrB [476]S and CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) than for CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) and C1.1.1/CoBrB (4.5 and 4.0 log$_{10}$ PFU/ml for the S-ST variants versus 6.7 and 6.3 log$_{10}$ PFU/ml for the R-RI variants, respectively). These results clearly showed that infection of SK6 cells with the R-RI variants occurred by an HS-dependent mechanism and that infection with S-ST variants occurred by an HS-independent mechanism. The [710]L→H mutation in the S-ST and R-RI variants reflects the phenotypical change from nonbinding ([710]L) to binding ([710]H) to MAb V6. After immunostaining, expression of CSFV E2 could be detected with MAb V6 in SK6 cells infected with CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) and C1.1.1/CoBrB. In contrast, no E2 was detected with MAb V6 after staining of SK6 cells infected with the virus variants that carried a Leu at position 710,

TABLE 2. Characterization of clinical samples

Virus or animal no.[a]	Sample[b]	Titer (SK6/Macr)[c]	% Inhibition by heparin[d]	Plaque size[e]
CoBrB [476]S 5012	SK6	4.5/6.8	0	Large
	Tonsil	6.0/7.1	40	Large
	Kidney	5.7/6.9	40	Large
	Blood	6.3/6.6	65	Large
CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) 5016	SK6	4.0/6.3	5	Large
	Tonsil	6.4/7.3	55	Large
	Kidney	5.0/6.6	55	Large
	Blood	4.9/5.7	80	Large
C1.1.1/CoBrB 5020	SK6	6.7/5.9	100	Small
	Tonsil	ND[f]	ND	ND
	Kidney	ND	ND	ND
	Blood	1.0/2.3	100	Small
CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) 5024	SK6	6.3/6.2	100	Small
	Tonsil	7.5/6.9	95	Small
	Kidney	5.5/4.8	100	Small
	Blood	4.3/4.4	100	Small/large[g]

[a] In vitro (SK6)- or in vivo-generated (animal number) virus.
[b] Tonsil and kidney samples were prepared postmortem. EDTA-treated blood, taken at day 14 p.i., was tested.
[c] Titers (log$_{10}$ PFU per milliliter) were measured in a plaque assay using SK6 cells or lung macrophages (Macr).
[d] Percent inhibition of infection of SK6 cells by 225 μg of heparin per ml.
[e] Relative plaque size observed after 2 days of growth in a plaque assay using SK6 cells. Cells were not treated with heparin.
[f] ND, not determined.
[g] In the blood from this animal, approximately 1:100 plaques was a large-plaque variant.

TABLE 3. Serological and clinical findings after inoculation of pigs with chimeric viruses derived from
C1.1.1 and CoBrB [476]S in animal experiment 2

Group[a]	Virus	Animal no.	Clinical findings Fever[b]	L/T[c]	IFT (T K S I)[d]	Death (d.p.i.)[e]	E2 ELISA[f] 0	7	14	21	Viremia[g] 0	2	4	7	9	11	14	16	18	21	23
A	CoBrB [476]S	5012c	13 (5)	+/+	++++	+ (16)	0	0	0		−	−	−	+	+	+	+	+			
		5013	13 (2)	+/+	++++	+ (14)	0	0	11		−	−	+	+	+	+	+				
		5014	13 (3)	+/+	++++	+ (14)	0	0	43		−	−	+	+	+	+	+				
		5015	14 (2)	+/+	++++	+ (14)	0	0	0		−	−	+	+	+	+	+				
B	CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′)	5016c	10 (7)	+/+	++++	+ (16)	0	0	0		−	−	−	+	+	+	+	+			
		5017	10 (2)	+/+	++++	+ (11)	0	0			−	−	+	+	+	+					
		5018	6 (3)	+/+	++++	+ (9)	0	0			−	−	+	+							
		5019	10 (3)	+/+	+−++[h]	+ (16)	0	0	82		−	−	+	+	−	+	−	−	−		
C	C1.1.1/CoBrB	5020c	3 (11)	−/−	+−−−[h]	−	0	0	0	97	−	−	−	−	−	−	+	−	−	−	−
		5021	−	+/−	−−−−[i]	−	0	0	98	101	−	−	−	+	−	−	−	−	−	−	−
		5022	1 (7)	+/−	−−−+[h,i]	−	0	0	100	100	−	−	−	+	−	−	−	−	−	−	−
		5023	−	−/+	+−−−[i]	−	0	0	98	101	−	−	−	+	−	−	−	−	−	−	−
D	CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1)	5024c	7 (12)	+/+	++++	+ (18)	0	0	0		−	−	−	−	−	+	+	+			
		5025	10 (5)	+/+	++++	+ (15)	0	0			−	−	+	+	+	+	+				
		5026	7 (5)	+/+	++++	+ (11)	0	0			−	−	+	+	+	+					
		5027	9 (5)	+/+	++++	+ (18)	0	0	0		−	−	−	+	+	+	+	+	+		

[a] Three pigs in each group were inoculated intranasally once with 10^5 TCID$_{50}$ of each virus. The sentinal (contact exposure) animals (c) were placed in each group 24 h after inoculation of the other animals.
[b] Fever defined as a body temperature of ≥40°C. The number of days with fever is shown. The day the fever began is shown in parentheses. −, no fever.
[c] Leukopenia (L) and thrombocytopenia (T) are defined as cell/platelet counts below 8×10^6 cells/ml and 200×10^6 cells/ml, respectively.
[d] Immunofluorescence technique (IFT) for detection of viral antigen in cryostat sections from tonsils (T), kidney (K), spleen (S), and ileum (I) at death.
[e] Pigs were either found dead or killed when moribund (+). The time of death (in days p.i. [d.p.i.]) is shown in parentheses. −, animal did not die.
[f] The E2 ELISA specifically detects antibodies against CSFV.
[g] Isolated leukocyte samples were incubated on SK6 cells for detection of virus by using an immunoperoxidase monolayer assay.
[h] A few crypts in tonsils were found positive.
[i] In some organ samples taken from the ileum, autolysis was found.

despite the detection of E2 expression in duplicate monolayers after staining with MAb V3 (conserved among pestiviruses).

Virulence of chimeric viruses derived from C1.1.1 and CoBrB [476]S with adaptive mutations in E[rns] and E2. To analyze the effect of the adaptive [710]L→H amino acid mutation on virulence in pigs, animal experiments were conducted. Pigs were infected intranasally according to the regimen described in Materials and Methods, and the results are shown in Table 3.

All pigs infected with CoBrB [476]S (group A) and CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) (group B), in which the Leu at position 710 was replaced by His (adaptive amino acid mutation), rapidly developed fever (≥40°C) at 2 or 3 days p.i. that lasted for more than 10 days, and showed symptoms of acute CSF (Table 3). The uninfected sentinels that were placed in the stable of the infected animals of groups A and B at 24 h p.i. to allow contact exposure developed fever for a period of more than 10 days and also became seriously ill. All animals developed leukopenia and thrombocytopenia and were viremic from day 4 p.i. (infected pigs) or from day 7 p.i. (sentinels) onwards. Pigs were killed when moribund. After slaughter, viral antigen was detected in tonsil, spleen, kidney, and ileum tissue samples. With the exception of one pig, no E2 antibodies were detected in the serum samples collected from pigs during the trial and when they were slaughtered. Pig 5019, infected with CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) (group B), developed antibodies against E2, and the virus levels in blood samples disappeared after 11 days. After slaughter, viral antigen was de-

tected only in a few crypts of the tonsils and in spleen and ileum tissue samples.

The pigs infected with C1.1.1/CoBrB (group C), in which the adaptive amino acid mutations in E[rns] ([276]R, [476]R, and [477]I) and E2 ([710]H) were introduced into CoBrB [476]R, showed no signs of CSF (Table 3). Only two pigs developed fever for a short period (≤3 days). Virus was detected in blood samples of all pigs in this group only at day 7 p.i. (infected pigs) and at day 14 p.i. (sentinel pig). All pigs developed antibodies against E2, and after slaughter, viral antigen was detected only in a few crypts in the tonsils of pigs 5020c and 5023 and the spleen of pig 5022.

Virus CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) (group D), in which the adaptive amino acid mutations in E[rns] ([276]R, [476]R, and [477]I) were introduced into virulent CoBrB [476]S, exhibits the same level of virulence as viruses CoBrB [476]S (group A) and CoBrB [476]S/C1.1.1 (E2-p7-pNS2-3′) (group B), except for a delayed onset of fever and spreading to the sentinel pig (Table 3). In conclusion, the [710]L (wild type) or [710]H (adaptive mutation in C1.1.1) in CoBrB [476]S variants does not seem to affect virulence in pigs, whereas the CoBrB/C1.1.1 (N[pro′]-C-E[rns]-E1) variant with [710]L is virulent but with a delayed onset of disease. Interestingly, the C1.1.1/CoBrB (group C) variant with [710]H does not seem to be virulent; however, very mild symptoms in a few pigs were observed (mild fever and viremia).

Genetic analysis of in vivo-generated viruses. The virus titers in blood samples, taken from pigs, were determined in a plaque assay to determine the plaque size after 2 days of

growth under a methylcellulose overlay medium. In wells containing blood samples, taken short before death (14 days p.i.), from pigs infected with CoBrB ^{476}S or CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), all plaques were large and similar in size to plaques formed by CoBrB ^{476}S or CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) produced in SK6 cells (Table 2). In wells containing blood samples taken from pigs infected with C1.1.1/CoBrB at 7 days p.i. (pig 5021) and 14 days p.i (pig 5020), all plaques were small and similar in size to plaques formed by C1.1.1/CoBrB produced in SK6 cells.

The blood samples from pig 5012 (CoBrB ^{476}S), pig 5016 (CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) and pig 5020 (C1.1.1/CoBrB), which were analyzed above for plaque phenotype, were used to infect SK6 cells. After 2 days of growth under a methylcellulose overlay medium, RNA was extracted from these cells and used to determine the sequences of the E^{rns}, E1, and E2 genes. For all these reisolated viruses, the E^{rns}, E1, and E2 sequences were identical to the sequences of the viruses administered to the pigs. These results showed that replication of CoBrB ^{476}S, CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), and C1.1.1/CoBrB did not induce genetic changes in the envelope proteins. However, when the virus titers in blood samples from pig 5026 and 5024 infected with CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1), taken on day 11 p.i. and day 18 p.i, respectively, were determined in a plaque assay (with 225 μg of heparin per ml), large plaques were detected (about one large plaque found for every 20 small plaques), whereas CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) virus produced in SK6 cells formed only small plaques. Large plaques were also detected in blood samples from the two other pigs from this group. However, no large plaques were detected when virus titers of tissue samples, collected from pigs 5024 and 5026 or from the other two pigs, were determined in the presence of heparin. For further analysis, virus isolated from a blood sample (18 days p.i.) from pig 5024 was cloned through endpoint dilution.

To favor infection by virus variants with a large-plaque phenotype, endpoint dilution was performed in medium containing 225 μg of heparin per ml. Of 23 cloned viruses analyzed, one had a large-plaque phenotype. The sequences of the E^{rns}, E1, and E2 genes of this cloned virus, isolated from a blood sample from pig 5024 (virus 5024$^{#18rev}$), were analyzed, together with a small-plaque-phenotype virus isolated from the same blood sample (virus 5024$^{#17wt}$). Sequence analysis of the large-plaque-phenotype virus 5024$^{#18rev}$ showed that the Arg at position 476 in the polyprotein was changed to a His. Therefore, in a blood sample from pig 5024, which was infected through contact exposure with pigs infected with CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1), a minority of a large-plaque-phenotype virus variant [CoBrB/C1.1.1 ^{476}H (N$^{pro′}$-C-E^{rns}-E1)] was found. Sequence analysis of the cloned small-plaque-phenotyped virus 5024$^{#17wt}$ showed that this virus was identical to the virus administered to the pig. As observed for the HS-independent CoBrB ^{476}S virus, additional passages in SK6 cells converted this large-plaque-phenotype CoBrB Erns^{R-HI} E2^L variant to a small-plaque variant (results not shown).

Surface properties of in vivo- and in vitro-generated viruses. Virus titers in organ suspensions and blood samples were determined in a plaque assay with and without 225 μg of heparin per ml. In Table 2, the results of these experiments are presented for pigs 5012 (CoBrB ^{476}S), 5016 [CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′)], 5020 (C1.1.1/CoBrB), and 5024 [CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1)]. Test results obtained with other samples taken from the pigs of the different groups were not significantly different from those presented here. All clinical samples from pigs 5012 and 5016 (CoBrB ^{476}S viruses) formed only large plaques on SK6 cells, which were similar in size to those for in vitro-produced viruses. Only one blood sample (14 days p.i.) from pig 5020 (C1.1.1/CoBrB) was analyzed, because no virus was isolated from organ samples from this pig and the other pigs in this group. Virus from this blood sample formed only small plaques on SK6 cells. Blood samples, taken from pig 5024, formed small and large plaques. When the virus titer for blood from this pig was determined in the absence of heparin, 1 of 100 plaques was large, representing the revertant of CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1). Virus from the other organ samples of this pig and the SK6 cells infected with CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) tested formed only small plaques.

Infection of SK6 cells with virus samples taken from pigs 5020 (C1.1.1/CoBrB) and 5024 [CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1)] was almost completely inhibited (95 to 100%) by 225 μg of heparin per ml. Infection of SK6 cells with virus samples from pigs 5012 (CoBrB ^{476}S) and 5016 [CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′)] showed 40 to 65% inhibition for CoBrB ^{476}S and 55 to 80% inhibition for CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), whereas infection with both viruses produced in SK6 cells was not inhibited.

From the in vitro-produced virus and reisolated viruses from clinical samples, virus titers were determined in a plaque assay after growth for 1 day under a methylcellulose overlay medium. Except for virus isolated from the tonsil of pig 5024, lower virus titers were detected in organ and blood samples for both R-RI variants than in organs and blood samples isolated from pigs infected with both S-ST variants, when samples were analyzed in a plaque assay using lung macrophages. Moreover, for C1.1.1/CoBrB, virus was isolated from the blood of pig 5020 only on one day (14 days p.i.). Note that the virus titers of organ samples and for in vitro-produced CoBrB ^{476}S variants are about 10-fold lower in assays using SK6 cells than the titers in assays using macrophages.

Recently, Hulst et al. (12) found that ConA binding to N-linked glycans exposed on the surfaces of virions is responsible for the observed stimulation or inhibition of infection of CSFV strain Brescia. ConA is a lectin that specifically binds with high affinity to terminal mannose (and with less affinity to glucosamine) residues of N-linked glycosyl groups. Most likely, ConA masks negatively or positively charged domains on the surfaces of CSFV particles, leading to either a more (stimulation) or less (inhibition) effective binding of virions to the highly negatively charged sulfate-rich clusters of HS. Therefore, we used ConA binding to demonstrate possible differences in the surface structure of in vitro-generated viruses. Infection on SK6 cells was inhibited for both CoBrB R-RI variants in a dose-dependent manner (Fig. 4). Virus CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) was inhibited 4-fold by a concentration of 50 μg of ConA, whereas virus C1.1.1/CoBrB was inhibited 15-fold by the same concentration of ConA. Infection on SK6 cells was stimulated for both CoBrB ^{476}S variants. Virus CoBrB ^{476}S was stimulated 1,000-fold by a concentration of 12.5 μg of ConA per ml, whereas virus CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) was stimulated 35-fold by a concentration of
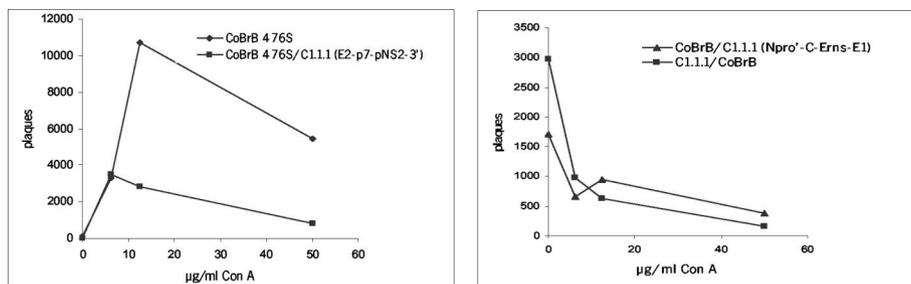
FIG. 4. Stimulation or inhibition of infection by ConA. Virus variants CoBrB ^{476}S, CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), C1.1.1/CoBrB, and CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) (produced in SK6 cells) were preincubated with different concentrations of ConA for 30 min at 37°C. SK6 cells in 2-cm^2 tissue culture wells were preincubated with EMEM with 300 μg of heparin per ml for 30 min at 37°C and then infected with virus. After the virus was removed and the cells were washed, the cells were incubated with 100 mM methyl-α-D-mannopyranoside for 30 min at 37°C. Subsequently, they were washed and supplied with overlay medium and stained after 24 h. Each value is the mean number of plaques calculated from two independent observations.

6.25 μg of ConA per ml. The significantly higher stimulation of infection observed for CoBrB ^{476}S compared to that for CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) indicated that these CoBrB ^{476}S genotypes have different surface properties, although the only difference is the L→H mutation at position 710. In the same way, but to a lesser extent, a slightly higher inhibition of infection was observed for CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) than for C1.1.1/CoBrB.

Ratio of secreted virus to cell-associated virus. We determined the ratio of secreted virus to cell-associated virus on SK6 cells for all CoBrB variants to ascertain whether the virulence of the variants tested correlated with the particle release from SK6 cells. For CoBrB ^{476}S and CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), approximately 1.5 times more virus particles were present in the culture medium than in the cell fraction (Fig. 5). For CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) and C1.1.1/CoBrB, about two times more virus was present in the cellular fraction than in the culture medium.

Virus neutralization index. Appropriate dilutions of MAb V4 (directed against the conserved A domain of E2), MAb V6 (directed against domain B of E2), and a serum sample from a

C1.1.1/CoBrB-infected pig (Pab$^{R-RI H}$) were incubated with in vitro- and in vivo-generated viruses to determine the reduction of the virus titers on SK6 cells through neutralization with MAbs or PAbs (Table 4). MAb V4 neutralized all in vitro-generated viruses by 3.5 log units or more. As expected, MAb V6 neutralized CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) and C1.1.1/CoBrB by 2.6 and 3.0 log units, respectively, whereas CoBrB ^{476}S and CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) were neutralized only by 0.75 and 0.25 log unit, respectively. Interestingly, serum Pab$^{R-RI H}$, which is a PAb serum with antibodies against all viral proteins, showed lower neutralizing indices for the heterologous viruses CoBrB ^{476}S (2.0 log units) and CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) (1.5 log units) than for CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) (3.4 log units) and C1.1.1/CoBrB (3.25 log units), indicating that the ^{710}H→L mutation is also important for recognition in the whole spectrum of antibodies.

To a certain extent, the same results were obtained for the in vivo-generated viruses. Except for C1.1.1/CoBrB, which could not be tested accurately because of low virus titers in the blood samples, all variants were efficiently neutralized by MAb V4 (reduced by 5.0 log units). MAb V6 neutralized CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′) by 2.5 log units, whereas CoBrB/
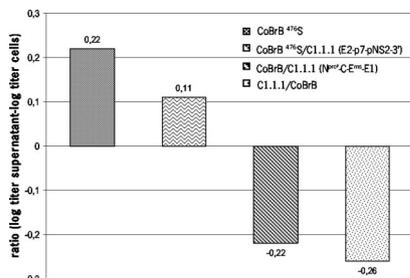
FIG. 5. Ratio of cell-associated virus to secreted virus. SK6 cells grown in 24-well plates were infected with virus variants CoBrB ^{476}S, CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′), C1.1.1/CoBrB, and CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1) (produced on SK6 cells) at an MOI of 1. After infection for 30 min at 37°C, virus was removed, and cells were washed. At 2 days p.i., 1 ml of supernatant was collected and stored at −70°C. One milliliter of culture medium was added to the cells before freeze-thawing twice at −70°C. Samples were titrated on SK6 cells in 24-well plates. The ratio of secreted virus to cell-associated virus was determined in a plaque assay and calculated by the following formula: ratio = log$_{10}$ PFU of cell-associated virus − log$_{10}$ PFU of secreted virus. An average ratio value for all tested viruses was determined from two experiments.

TABLE 4. Virus neutralization of in vitro- and in vivo-generated chimeric viruses derived from C1.1.1 and CoBrB ^{476}S

Virus	Neutralization of SK6-generated virus[a]			Neutralization of in vivo-generated virus[a]		
	V4	V6	Pab^{R-RI}	V4	V6	Pab$^{R-RI H}$
CoBrB ^{476}S	4.0	0.8	2.0	5.0	0.0	1.5
CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′)	3.5	2.6	3.4	4.8	2.5	5.7
CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1)	4.8	0.3	1.5	4.8	0.3	0.8
C1.1.1/CoBrB	5.7	3.0	3.3	ND[b]	ND	ND

[a] Neutralization of in vitro (produced on SK6)- or in vivo-generated chimeric viruses derived from C1.1.1 and CoBrB ^{476}S. Blood samples from animals 5012 (CoBrB ^{476}S), 5016 [CoBrB ^{476}S/C1.1.1 (E2-p7-pNS2-3′)], and 5024 [CoBrB/C1.1.1 (N$^{pro′}$-C-E^{rns}-E1)] were collected, and the titers of virus from the blood samples were determined on SK6 cells with or without neutralizing MAbs or PAbs. The values represent the log TCID$_{50}$ per milliliter reduction of CSFV titers due to the presence of MAbs or PAbs. Neutralizing MAbs and PAbs are as follows: MAb V4 (directed against the conserved A domain of E2), MAb V6 (directed against domain B of E2), and a serum sample from a CoBrB Ems^{R-RI} E2^H-infected pig (Pab$^{R-RI H}$).

[b] ND, not done because the virus titers in blood samples from pigs infected with this virus were too low.

C1.1.1 ($N^{pro\prime}$-C-E^{rns}-E1) was neutralized by 0.3 log unit and CoBrB ^{476}S was not neutralized at all. Regarding the ^{710}H→L mutation, even clearer results were obtained when the serum Pab$^{R\text{-}RI\ H}$ was used to determine neutralizing indices for in vivo-generated viruses. Serum from a C1.1.1/CoBr-infected pig was able to neutralize virus in blood from pig 5016 [CoBrB ^{476}S/ C1.1.1 (E2-p7-pNS2-3′)] by 5.7 log units, whereas low neutralizing indices were obtained for pig 5012 (CoBrB ^{476}S) and pig 5020 [CoBrB/C1.1.1 ($N^{pro\prime}$-C-E^{rns}-E1)]. These results suggest that besides the importance of ^{710}H as an epitope for neutralizing antibodies, this amino acid residue is also crucial for the structure of E2.

DISCUSSION

This study reports on the mechanism of attenuation of laboratory-derived CSFV by host cell adaptation and defines molecular determinants for the loss of the capacity of CSFV strain Brescia variants to produce disease in pigs. Recently, it was demonstrated that limited cultivation of CSFV on porcine kidney cells selects variants that attach to the surfaces of cells by interaction with the membrane-associated HS. A Ser-to-Arg change in the C terminus of E^{rns} at position 476 in the ORF of CSFV strain Brescia is responsible for selection of these HS-binding variants (11). However, this Ser-to-Arg change (CoBrB ^{476}S and CoBrB ^{476}R) did not reduce virulence in pigs (12). The selection of CSFV variants with an altered virulence phenotype by serial passage in porcine kidney cells had been demonstrated earlier for strain Brescia (41). They showed that an extensively passaged (31 times) and cloned virus C1.1.1 was avirulent in pigs. Therefore, we determined the consensus sequences of the genetically closely related strains, virulent CoBrB ^{476}R and avirulent C1.1.1, which were both isolated from the same blood sample of a pig infected with Brescia. We found nine adaptive amino acid changes, besides 20 silent mutations, in C1.1.1. Of the nine adaptive amino acid changes, three were located in the structural region, and the other six were located in the nonstructural region.

In the first animal experiment, we focused on these nine adaptive amino acid mutations by making chimeric C1.1.1/ CoBrB ^{476}R virus variants, in which either the structural or nonstructural region was exchanged. A minor role for the adaptive amino acid mutations in the nonstructural region on virulence was observed, although some interesting amino acid changes are present. Amino acid ^{1971}P of C1.1.1 is not located in any of the known protease/ATPase/helicase motifs of NS3 (8), but adaptive amino acid mutation ^{1915}S is located 2 amino acids downstream of the DEYH sequence (also known as the Walker motif or motif II [36]), one of the seven conserved motifs that are characteristic for RNA helicases of the DExH type. Grassmann et al. (8) showed that mutations of the third position in the DEYH motif permitted replication, although at a lower level than that of wild-type RNA. In vitro studies with the purified mutant NS3 proteins revealed a significantly impaired helicase activity for the motif II substitutions. Although the adaptive amino acid mutation ^{1915}S in not found in other virulent and avirulent CSFV strains, further in vitro studies should be performed to determine the influence of this mutation in the vicinity of the Walker motif on helicase or nucleotide triphophatase activity. The same holds true for the adap-

tive amino acid mutations ^{3419}I and ^{3858}A in NS5b, which are not located in any of the known RNA-dependent RNA polymerase motifs (17). These amino acids and ^{2638}V in NS4b and ^{2846}C in NS5a may influence virulence by affecting the stability of NS2-3, NS4a, NS4b, NS5a, and NS5b in the replication complex. Mittelholzer et al. (21) found no correlation between RNA levels in vitro and virulence in a study of CSFV strains with different degrees of virulence. Their results suggest that mutations located in the nonstructural region that are involved in replication (such as NS3 and NS5b) are not determinants for virulence in the CSFV strains they tested.

The first animal experiment revealed a possible role for the His residue at position 710 in the ORF of CSFV, since virus CoBrB ^{476}R/C1.1.1 was moderately virulent and C1.1.1/CoBrB ($N^{pro\prime}$-C-E^{rns}-E1) was not virulent. Further studies showed that the ^{710}H by itself was not enough to reduce virulence in pigs, but together with adaptive amino acid changes in E^{rns} (^{276}R, ^{476}R, and ^{477}I), it did reduce virulence. The ^{710}L→H mutation has already been characterized as a MAb-resistant mutation for MAb V6 (MAb V6 epitope located in the nonconserved B domain of E2) (34). Besides an important neutralization site, it also reflects a structural change in E2 that is probably crucial for the function(s) of E2 in vivo.

The most dramatic change in E^{rns} is the ^{476}S→R mutation, which reflects the adaptation to SK6 cells from an HS-independent virus to an HS-dependent virus (11). Binding of baculovirus-expressed E^{rns} recombinant proteins (S-ST, S-RT, R-RI, S-RI, R-RT, and R-ST, representing the amino acid combinations at positions 276, 476, and 477 in E^{rns}) to immobilized heparin-HS-type polysaccharide chains by heparin-Sepharose chromatography showed that an Arg at position 476 increased the affinity of heparin significantly. Furthermore, ^{276}Arg in the N terminus of E^{rns} and ^{477}Ile, which is involved in the recognition of antigenic sites (43), adjacent to ^{467}Arg in E^{rns} in R-RI further increased the affinity of binding of E^{rns} to heparin (M. Hulst, personal communication). Amino acid 276 (S for CoBrB and R for C1.1.1) is located in the N terminus of E^{rns}, upstream of the first RNase domain of E^{rns} (9). The role of this adaptive amino acid residue ^{276}R in E^{rns}, which is conserved among virulent and avirulent CSFV strains and which eliminates a potential N-linked glycosylation site (NXS) (11, 16), was not investigated in vivo. However, studies with baculovirus-expressed E^{rns} recombinant proteins (S-ST, S-RT, R-RI, S-RI, R-RT, and R-ST) revealed that this potential glycosylation site was not glycosylated in insect cells (M. Hulst, personal communication).

For many viruses (reference 29 and references therein), HS-mediated entry is the first step in the infection process, followed by interaction of viral proteins with other cellular mediators (secondary receptors) for adhesion and penetration. Moreover, for foot-and-mouth disease virus, simian virus, Murray Valley encephalitis virus, and Japanese encephalitis virus, it was shown that HS-dependent virus variants, selected in cell culture, are less virulent than their HS-independent counterparts. It was speculated that HS binding in vivo is a disadvantage for the virus, at least for those that spread through the circulation system and do not spread by cell-to-cell contact. Culture-adapted virus variants are rapidly cleared by tissue HS, which is sulfated to a much higher extent than endothelial HS is (29). However, it was shown for CSFV that the Ser-to-Arg

change, from an HS-independent virus to an HS-dependent virus, did not reduce virulence in vivo (12). Our results with the R-RI variants, with a relatively high affinity for heparin compared to that of CoBrB ^{476}S, indicate that spreading of these viruses through the circulation system was reduced. A delayed onset of disease was observed for the animals infected with the CoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1) virus, and the spread to a contact-exposed animal was also delayed compared to the CoBrB ^{476}S virus. This would indeed favor the hypothesis that HS binding in vivo is a disadvantage for the virus. However, for CSFV, it was shown that an HS-independent virus generated in vivo is still able to use HS as an attachment site for primary swine kidney cells (12), probably due to heterogeneity in surface properties of virus particles. Maybe the CoBrB ^{476}S variant uses specific HS motifs displayed on specific host cells or more specific receptors are involved, leading to selection or infection of specific host cells. Therefore, even if HS does not serve as an entrance gate, this may direct the viral pathogen to certain sites where the preferential HS features are displaced, and this can make the difference between easy or more difficult access.

Growth of the HS-dependent variant and CoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1) in pigs yielded revertants with a characteristic large-plaque phenotype. However, the ratio of revertant to nonrevertant phenotypes remained low (<1% on the basis of plaque size) from virus from blood samples, indicating that the pathogenesis in these animals was due to growth of the HS-dependent virus rather than that of the revertant virus. Moreover, nonrevertant viruses were also found predominantly in blood samples from the sentinel pig (ratio of revertant to nonrevertant phenotypes of <1% on the basis of plaque phenotype), indicating that growth of this virus was responsible for the pathogenesis in this pig. Revertants with an amino acid change R→H at position 476 in E^{rns} were isolated from blood samples from infected pigs, whereas no revertants were isolated from tonsil, spleen, kidney, and ileum tissue samples. This reversion reflected the change from an HS-dependent virus to an HS-independent virus. This phenomenon was already observed in a previous study (12), in which a low level of revertants with a large-plaque phenotype was found in blood samples from one of three pigs infected with CoBrB ^{476}R. Analysis of this revertant virus revealed ^{467}Ser instead of ^{467}Arg. This change had no effect on the virulence of this virus in pigs.

The results presented here suggest that the attenuation of cell-adapted CSFV strain Brescia is correlated with the acquisition of positively charged amino acid residues in glycoproteins E^{rns} and E2. The ratio of secreted virus particles (cell-free) to cell-associated virus particles in SK6 cells correlated with the overall (or local) charge of the virions (from positive to negative, R-RI H [0.55], R-RI L [0.6], S-ST H [1.3] and S-ST L [1.7] [ratios shown in brackets]). In contrast to the results of Mittelholzer et al. (21), these results suggested that reduced particle release is not always correlated with the virulence of CSFV strains. According to our standards, CoBrB/C1.1.1 (N$^{pro'}$-C-E^{rns}-E1) could be classified as virulent with delayed onset of disease compared to highly virulent CoBrB ^{476}S, whereas a reduced particle release on SK6 cells was observed. Inhibition or stimulation of infection of CSFV variants on SK6 cells with ConA also correlated with the overall (or local)

charge of the virions (from positive to negative, R-RI H, R-RI L, S-ST H, and S-ST L). The observed differences in neutralizing indices caused by the adaptive amino acid change (L→H) at position 710 in E2 suggest that this mutation plays a crucial role in the structure of E2. Structural changes and/or the acquisition of positively charged amino acids of cell-adapted CSFV variants might have an synergistic effect with the adaptive mutations in E^{rns} and E2, because of the recently shown interaction of E^{rns} and E2 in the virion through a noncovalent interaction (18).

The virulence of CSFV seems to be determined in a rather complex manner. First, the acquisition of positively charged amino acids in E^{rns}, which lead to an HS-independent virus, is not enough to reduce virulence in pigs. HS binding in vivo is considered to be a disadvantage for the virus, but earlier results indicated that this is not completely true for CSFV (12). However, the disease spread less in pigs infected with CoBrB R-RI variants (HS$^+$) than in pigs infected with CoBrB S-ST variants (HS$^-$). This suggests that HS binding in vivo does have an effect on virulence. Second, the ^{710}L→H mutation in E2 leads to reduced virulence only in combination with the adaptive mutations in E^{rns}. The mechanism by which this mutation affects virulence is not clear. As discussed before, the overall (or local) positive charge of the virion seems to be correlated with attenuation of CSFV strain Brescia in vivo. Thus, the mutation in E2 might contribute to the positive charge of the virion and reduced spread in vivo. Another mechanism by which virulence might be affected is reduced spread of virus in vivo in combination with changes in binding and/or penetration of virus in host membranes leading to tissue tropism. Both E^{rns} and E2 are involved in the infection process of CSFV. E^{rns} is involved in HS-mediated entry, which is the first step in the infection process, followed by a possible interaction of E2 with other secondary receptors for adhesion and penetration. Both processes could be disturbed by the adaptive amino acid mutations in E^{rns} and E2 leading to tissue tropism.

Detailed studies of processing and surface structure are needed to obtain insight into how CSFV binds to and associates with host cells. In addition, more-detailed studies on the interaction or replication of CSFV, either cell adapted or in vivo generated, with specific target host cells might elucidate more genetic determinants of virulence for CSFV.

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