JOURNAL OF VIROLOGY, Mar. 2005, p. 3787–3796 Vol. 79, No. 6
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.6.3787–3796.2005

The E2 Glycoprotein of Classical Swine Fever Virus Is a Virulence Determinant in Swine

G. R. Risatti,[1]†‡ M. V. Borca,[1]†* G. F. Kutish,[1]‡ Z. Lu,[1] L. G. Holinka,[1] R. A. French,[2]‡ E. R. Tulman,[1]‡ and D. L. Rock[1]‡

Plum Island Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York,[1] and Department of Pathology and Veterinary Science, The University of Connecticut, Storrs, Connecticut[2]

Received 26 May 2004/Accepted 9 November 2004

To identify genetic determinants of classical swine fever virus (CSFV) virulence and host range, chimeras of the highly pathogenic Brescia strain and the attenuated vaccine strain CS were constructed and evaluated for viral virulence in swine. Upon initial screening, only chimeras 138.8v and 337.14v, the only chimeras containing the E2 glycoprotein of CS, were attenuated in swine despite exhibiting unaltered growth characteristics in primary porcine macrophage cell cultures. Additional viral chimeras were constructed to confirm the role of E2 in virulence. Chimeric virus 319.1v, which contained only the CS E2 glycoprotein in the Brescia background, was markedly attenuated in pigs, exhibiting significantly decreased virus replication in tonsils, a transient viremia, limited generalization of infection, and decreased virus shedding. Chimeras encoding all Brescia structural proteins in a CS genetic background remained attenuated, indicating that additional mutations outside the structural region are important for CS vaccine virus attenuation. These results demonstrate that CS E2 alone is sufficient for attenuating Brescia, indicating a significant role for the CSFV E2 glycoprotein in swine virulence.

Classical swine fever (CSF) is a highly contagious and often fatal hemorrhagic disease of swine and is caused by *Classical swine fever virus* (CSFV), a member of the genus *Pestivirus* of the family *Flaviviridae* (5). Intermittent CSF outbreaks in Europe and other parts of the world result in significant economic losses. While infection with highly virulent CSFV strains can cause acute CSF characterized by high morbidity and mortality, isolates of moderate to low virulence induce a prolonged, chronic disease (35).

CSFV is a small enveloped virus with a single-stranded, 12.5-kb RNA genome of positive polarity. The CSFV genome contains a single open reading frame encoding an approximately 4,000-amino-acid polyprotein which through cellular and viral protease-mediated co- and posttranslational processing gives rise to the following 11 to 12 final cleavage products: NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (23). Protein C and the glycoproteins E^{rns}, E1, and E2 represent structural components of the virion (28). E1 and E2 are anchored to the envelope by their carboxy termini, with E^{rns} loosely associated with the envelope. E^{rns} and E2 are present as homodimers linked by disulfide bridges on the surfaces of CSFV virions, whereas E2 is also found dimerized with E1 (28, 37, 38). The genetic basis of CSFV virulence and host range remains poorly understood (35).

Development of infectious CSFV cDNA clones has enabled genetic approaches for defining mechanisms of viral replication and pathogenesis. Infectious clones (IC) of the attenuated CSFV C-strain and the pathogenic Alfort/Tübingen and Eystrup strains have been constructed, enabling identification of E^{rns} and N^{pro} as virulence factors in swine and of the role of different E^{rns} mutations in virus attenuation (17, 19). Additionally, attempts to mutate and inactivate E^{rns} in the attenuated C-strain resulted in a virus atypically cytopathic for cell cultures (10, 39). Thus, CSFV IC hold great promise for identifying viral proteins or protein domains functioning in viral virulence and host range and for rationally engineering marker live attenuated CSF vaccines (17, 19, 21, 30, 31).

Here we have used chimeras of the highly pathogenic Brescia strain and the attenuated vaccine strain CS to identify genetic determinants of CSFV virulence and host range. Results demonstrate that the CSFV E2 glycoprotein is a major determinant of virulence in swine.

MATERIALS AND METHODS

Viruses and cells. Swine kidney cells (SK6) (27) free of bovine viral diarrhea virus (BVDV) were used throughout this study. SK6 cells were cultured in Dulbecco's minimal essential medium (Gibco, Grand Island, N.Y.) with 10% fetal calf serum (Atlas Biologicals, Fort Collins, Colo.). CSFV strain Brescia (obtained from the Animal and Plant Health Inspection Service, Plum Island Animal Disease Center) was propagated in SK6 cells and used for IC construction. The CS vaccine strain is derived from the LK VNIIVIM parental vaccine strain by serial passages and cloning (40). Titrations of CSFV from clinical samples were conducted by using SK6 cells in 96-well plates (Costar, Cambridge, Mass.), stained via immunoperoxidase assay 4 days postinoculation by using the CSFV monoclonal antibody 303 (4) and the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.) (24), calculated by using the method of Reed and Muench (22), and expressed as 50% tissue culture infective dose ($TCID_{50}$)/milliliter. As performed, test sensitivity was ≥ 1.8 $\log_{10}/TCID_{50}$/ml. Primary swine macrophage cell cultures were prepared as described by Zsak et al. (41).

Construction of CSFV Brescia (*pBIC*), CS (*pCSIC*), and chimeric cDNA IC. Total cellular RNA was Trizol extracted (Gibco) from SK6 cells infected with peripheral blood obtained from pigs infected with the CSFV strain Brescia or vaccine strain CS. cDNAs spanning the entire genome of CSFV Brescia and CS were obtained by reverse transcription (RT) as follows: the reaction mixture (1 μl of Moloney murine leukemia virus RT buffer, 4 mM deoxynucleosidetriphos-

* Corresponding author. Mailing address: Plum Island Animal Disease Center, USDA/ARS/NAA, P.O. Box 848, Greenport, NY 11944-0848. Phone: (631) 323-3019. Fax: (631) 323-3044. E-mail: mborca@piadc.ars.usda.gov.

† These authors contributed equally to the results presented in this report.

‡ Present address: Department of Pathobiology and Veterinary Science and Center of Excellence for Vaccine Research, The University of Connecticut, Storrs, CT 06269-3089.

TABLE 1. Oligonucleotides used for construction of pBIC, pCSIC, and chimeric p 138, p319.1, and p312.1 infectious clones[a]

Primer	Sequence	Function
MCSFBrescia	5′TCGAG***CCATGG***AT***GGCCGCTACGGCC***AT***ATGCAT***AT***ACGCGT***GACTAGTGACGT3′	Multiple cloning
MCSRBrescia	5′CACTAGTC***ACGCGT***AT***ATGCAT***AT***GGCCTAGCGGCC***AT***CCATGG***C	Brescia
R4567	5′CTCCTGCTATTTCATCTA3′	RT 5′ end
Brescia 5′	5′ACTAGT***ACGCGTTAATACGACTCACTATAG***TATACGAGGTTAGTTCA3′	PCR 5′ end
CS5′	5′GTCAGTCGACTT***AATACGACTCACTATAGT***ATACGAGGTTAGTTCA3′	PCR 5′ end
R3668	5′ATCCATTTCTTTATAGGC3′	PCR 5′ end
R8538	5′CCCTATCCTATCATCCGT3′	RT middle
R8127	5′GTGTTCTCTTCTGCT CAC3′	PCR middle
R12292	5′GGGCCGTTAGAAATTACCTTA3′	RT 3′ end
Brescia 3′	5′ATTG***CCATGGCCCGGGC***CGTTAGAAATTACCTTA3′	PCR 3′ end
F8017	5′AAGCGATGGTTTGCTAGG3′	PCR 3′ end
F2379	CCTCATCTGCTTGATAAAAG	p138.8
R6636	CCTTCTCTGGGCTTGTTC	p138.8
F*Sac*II	5′GAACAACTCGCCGCGGGTCTACAGTTAGG3′	SacII site
R*Sac*II	5′CCTAACTGTAGACCCGCGGCGAGTTGTTC3′	SacII site
MCS+	5′CCGGCCGCGTCGACATAGATCTAT***GGCCGTAGCGGCC***3′	Multiple cloning
MCS−	5′TCGAGGCCGCTACGGCCATAGATCTATGTCGACGCGGCCGGACGT3′	site for CS
F2008	5′GATTCTCCATGAGATGGG3′	PCR middle
CS3′	5′ATTGCTCGAGCCCGGGCCGTTAGGAAATTACCTTA3′	PCR 3′ end
F8210	5′GATGAATTAGTCAAGGAG3′	PCR 3′ end
F3237	5′ACACAACTGTCAAGGTGC3′	PCR middle
R3126	5′TCTGTAACCTGTCTCATT3′	PCR 5′ end

[a] Restriction sites are shown in bold italics and underlined. 5′ to 3′, for MCSF Brescia, MluI, NsiI, SfiI, and NcoI; for Brescia, 5′, MluI; and for Brescia, 3′, overlapping NcoI and SrfI; bold letters indicate the T7 promoter. RT, reverse transcription. 5′ end, middle, and 3′ end are amplicons shown in Fig. 1.

phates, 10 pmol of specific reverse primer R12292, R8538, or R4567 [Table 1], 2 μl of total RNA, and 5.3 μl of water) was incubated at 70°C for 5 min, cooled at room temperature for 20 min, and further incubated for 1 h at 37°C after addition of 2 U of Moloney murine leukemia virus reverse transcriptase (Stratagene, Cedar Creek, Tex.). The entire viral genome of both strains was PCR amplified in three overlapping fragments, using specific primers as described below (Table 1 and Fig. 1A).

A 4.3-kb region comprising the 3′ end of the CSFV genome was amplified by seminested PCR, using the primer combination F8017–Bresia3′ (Brescia virus) or F8017–CS3′ (CS virus), and then reamplified by using the primer combination F8210-Bresia3′ or CS3′. The amplified DNA fragments were excised from a 1% agarose gel and cloned, using SfiI/NcoI sites (Brescia virus) or SfiI/XhoI sites (CS virus), into *pACYC177GR* and *pACYC177AZ* (see below) to obtain plasmids *pBrescia 3′end* or *pCS 3′end*, respectively.

A central 4.8-kb genomic region was amplified by using the primer combination F3237-R8127 (Brescia virus) or F2008-R8127 (CS virus). The amplified fragments were purified as described above and cloned into *pCR4Blunt*, using a Topo cloning kit (Invitrogen, San Diego, Calif.) to create the plasmid *pTOPOB-middle* (Brescia virus) or *pTOPOCSmiddle* (CS virus). *pTOPOBmiddle* was then NsiI-SfiI digested, and the fragment was cloned into *pACYC177GR* to create the plasmid *pBresciamiddle*. Similarly, *pTOPOCSmiddle* was BglII-SfiI digested, and the fragment was cloned into *pACYC177AZ* to generate the plasmid *pCSmiddle*.

A 3.1-kb region comprising the 5′ end of the CSFV genome was amplified by using primers Brescia 5′ and R3126 (Brescia virus) or CS 5′ and R3668 (CS virus). Amplified products were cloned in *pCR4Blunt* as described above to generate plasmid vectors *pTOPOB5′end* and *pTOPOCS5′end*. *pTOPOB5′end* was MluI-NsiI digested and cloned into *pBresciamiddle* to generate the vector *pBresciamiddle-5′end. pBrescia 3′end* was SfiI-SacII digested and cloned into *pBresciamiddle-5′end* to generate a full-length plasmid, *pBIC* (Fig. 1A). Similarly, *pTOPOCS5′end* was SalI-BglII digested and cloned into *pCSmiddle* to generate the vector *pCSmiddle-5′end*. *pCS3′end* was SfiI-XhoI digested and cloned into *pCSmiddle-5′end* to generate a full-length plasmid, *pCSIC* (Fig. 1A).

The low-copy-number plasmids used here, *pACYC177GR* and *pACYC177AZ*, are *pACYC177* derivatives (New England Biolabs, Beverly, Mass.), obtained by removing a 1.2-kb AatII-XhoI fragment from *pACYC177* and replacing it with a synthetic oligonucleotide containing unique restriction sites (Table 1).

Chimeric cDNA ICs p189.9, p182.1, p138.8, p167.1, p113.1, and p337.14 were constructed by replacing, respectively, the restriction fragments ClaI-SrfI, ClaI-NaeI, NaeI-BamHI, BamHI-SfiI, SfiI-SrfI, and ClaI-SacII in *pBIC* with the homologous fragment from the CS strain (Fig. 2). Chimeric cDNA ICs p137.1 and p343.8 were constructed by replacing, respectively, the restriction fragments NaeI-BamHI and ClaI-SacII in *pCS* with the homologous fragment from *pBIC* (Fig. 2).

Chimeric cDNA ICs p319.1 and p312.1 were constructed as follows. The NaeI-BamHI fragments from *pBIC* and *pCSIC* were obtained by PCR by using the primers F2379 and R6636 and cloned by using a TA Topo kit (Invitrogen) to create the plasmids *pTOPOBreN/B* and *pTOPOCSN/B*, respectively. These plasmids were used as targets for site-directed mutagenesis, using the QuikChange XL kit (Stratagene) and the specific oligonucleotide primers FSacII and RsacII, complementary to each strand of the target. Following mutagenesis, the reaction was treated with 10 U of the endonuclease DpnI to eliminate methylated target DNA, and extended products were transformed into XL10 Gold ultracompetent cells (Stratagene). DNA obtained from the transformants was purified (QIAGEN plasmid kits, Valencia, Calif.) and sequenced to verify the presence of the desired mutation. Extension resulted in creation of a SacII site and introduction of a silent mutation at position 3540 to yield the following plasmids: *pTOPOBreN/BSacII* and *pTOPOCSN/BSacII*. NaeI-SacII fragments of each plasmid containing the genomic region E2/p7-NS2-3 were reciprocally inter changed, creating the chimeric plasmids *pTOPOBreCS* and *pTOPOCSBre* (Brescia/CS and CS/Brescia, respectively). Finally, the NaeI-BamHI fragments from *pTOPOBreCS* and *pTOPOCSBre* were cloned in *pBIC* to create cDNA ICs p319.1 and p312.1, respectively (Fig. 2).

Rescue of CSFV BICv, CSICv, and chimeras. Full-length genomic ICs were linearized with SrfI and in vitro transcribed by using the T7 Megascript system (Ambion, Austin, Tex.). RNA products were precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W with a BTX 630 electroporator (BTX, San Diego, Calif.). Cells were plated in 12-well plates and 25-cm^2 flasks and incubated for 4 days at 37°C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above. Stocks of rescued viruses were stored at −70°C.

DNA sequencing and analysis. Full-length ICs, in vitro rescued viruses, and viruses recovered from infected animals were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain termination method (25). Sequencing reactions were prepared with the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Reaction products were sequenced on a PRISM 3700 automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org), with confirmatory assemblies performed by using CAP3 (7). The final DNA consensus sequence represented on average eightfold redundancy at each base position.

Nucleotide and amino acid comparisons and alignments were done by using BLAST (bl2seq) (1) and Clustal (29). Protein characterization and secondary structure predictions (Chou-Fasman and Garnier-Osguthorpe-Robson) were done by using the MacVector (Accelrys, Inc.) and GCG (3) software packages, SAPS (2), memsat (14), and psipredict (13).

Animal infections. Initial screening of chimeric viruses 189.9v, 182.1v, 138.8v, 167.1v, 113.1v, 337.14v, 137.1v, and 343.8v to determine a virulence phenotype was conducted by intranasal inoculation of two 10- to 12-week-old pigs with 10^5

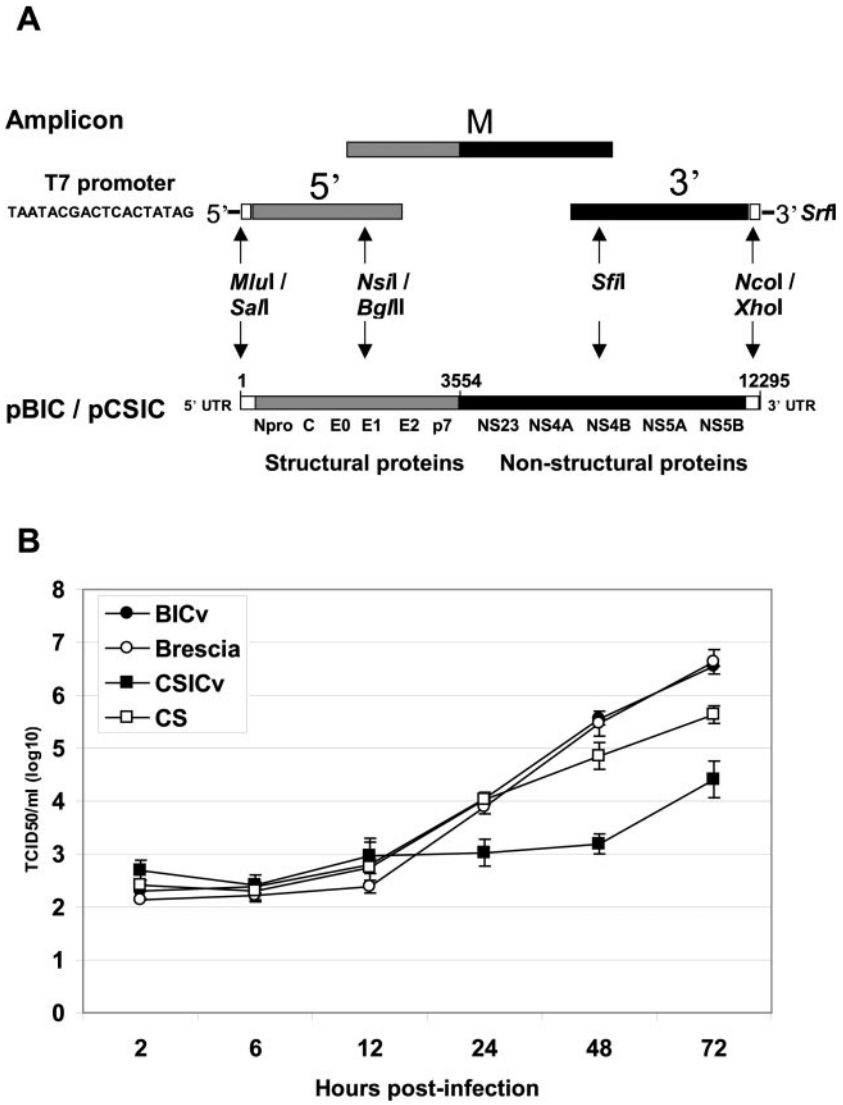


FIG. 1. (A) Construction of CSFV infectious cDNA clones *pBIC* and *pCSIC*, using amplicon fragments 5′, M, and 3′. Corresponding restriction sites and nucleotide numbers are depicted (*pBIC*/*pCSIC*). (B) Growth curve of CSFV Brescia and CS and viruses derived from ICs BIC and CSIC. Primary macrophage cell cultures were infected (MOI = 0.1) and harvested at the indicated time points. Samples were titrated on SK6 cells with virus detected by immunohistochemistry as described in Material and Methods. Data are means and standard errors from three independent experiments.

$TCID_{50}$ of each virus. The presence of clinical symptoms and temperature were observed daily. More-detailed pathogenesis studies with attenuated chimeric viruses 138.8v, 319.1v, and 312.1v were conducted with similar pigs (n = 30) randomly allocated into five groups of six animals each. Pigs were intranasally inoculated with 10^5 $TCID_{50}$ of BICv, CSICv, or chimeric viruses. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea, and cough) were observed daily throughout the experiment and scored as previously described (20). One pig per group was necropsied at 6 and 8 days postinfection (dpi), at which time blood, serum, nasal swabs, and tonsil scraping samples were obtained. Tissue samples (tonsil, mandibular lymph node, and spleen) were snap-frozen in liquid nitrogen for virus titration or fixed in 10% neutral buffered formalin for histopathological studies.

Total and differential white-blood-cell counts. Blood was collected from the anterior vena cava in tubes containing EDTA (Vacutainer). Total white-blood-cell, lymphocyte, and platelet counts were obtained with a Beckman Coulter ACT (Beckman Coulter, Fullerton, Calif.).

Immunohistochemistry. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Tissue sections were allowed to adhere to Superfrost/plus slides (Fisher Scientific, Pittsburg, Pa.), heated for 20 min at 65°C, and then deparaffinized with xylene. Sections were rehydrated through a graded alcohol series and washed with phosphate-buffered saline (PBS) (pH 7.4). Immunohistochemistry was performed as described by Sur et al. (26). Briefly, 4-μm sections were first treated with 3% hydrogen peroxide in PBS for 20 min, followed by washes in PBS and digestion with 0.05% Protease XIV (Sigma Chemical Co., St. Louis, Mo.) for 2 min at 37°C. After several washes with PBS, sections were incubated in blocking solution (5% normal goat serum in PBS) for 30 min at room temperature and then incubated for 2 h at 4°C with CSFV-specific monoclonal antibody directed against the E2 glycoprotein (4) diluted 1:500 in PBS. After PBS washes, slides were incubated with alkaline phosphatase-conjugated, goat antimouse antibody for 20 min at room temperature.

Nucleotide sequence accession numbers. Brescia and CS genomic sequences were deposited in GenBank under accession numbers AY578687 and AY578688, respectively.

RESULTS

Construction of CSFV Brescia (*pBIC*) and CS (*pCSIC*) infectious clones. Infectious RNA was in vitro transcribed from a full-length copy of CSFV strain Brescia or CS (Fig. 1A).

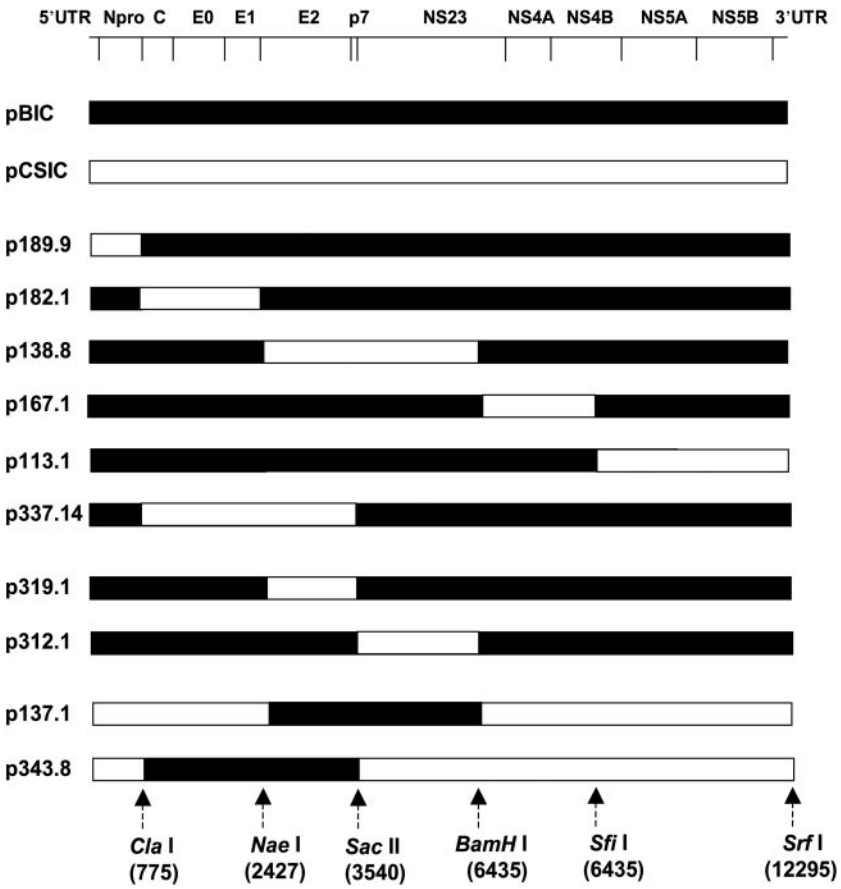


FIG. 2. Construction of CSVF chimeric viruses. Corresponding restriction sites and nucleotide numbers are depicted.

cDNAs amplified by RT-PCR (see Materials and Methods) using specific primers (Table 1) were cloned into the low-copy-number plasmids *pACYC177GR* and *pACYC177AZ* to obtain *pBIC* and *pCSIC*, full-length DNA copies of the Brescia and CS viral genomes, respectively (Fig. 1A). RNA transcripts were obtained from these plasmids and used to transfect SK6 cells. Virus was rescued from transfected cells at day 4 posttransfection. Nucleotide sequences of the rescued virus BICv/CSICv genomes were identical to those of parental viruses Brescia and CS, respectively.

In vitro and in vivo analysis of CSFV BICv and CSICv. Growth kinetics of BICv and CSICv were compared with their parental viruses, Brescia and CS, in a multistep growth curve. Primary porcine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at times postinfection through 72 h. Replication kinetics and virus yields between the parental Brescia and its IC-derived virus, BICv, were indistinguishable (Fig. 1B). However, titers of CSICv were approximately 10-fold lower than those of parental CS virus (Fig. 1B). To assess pig virulence phenotypes, three pigs were inoculated intranasally with 10^5 $TCID_{50}$ of parental viruses (Brescia and CS) or IC-derived viruses, BICv and CSICv. No significant differences in time to onset of clinical disease, mortality rate (which was 100%), time to death, or viremia titers were observed for Brescia- and BICv-infected animals (data not shown). Animals inoculated with CS or CSICv were free of CSFV-related clinical signs during the experimental period (data not shown). These data indicate that the IC-generated viruses, BICv and CSICv, have parental phenotypes in vitro and in vivo.

CSFV E2 is a virulence determinant. To identify CSFV virulence and host range determinants, we constructed chimeric viruses in which genomic regions of the pathogenic Brescia strain were replaced with homologous regions from the CS vaccine strain (Fig. 2). Chimeric viruses 189.9v, 182.1v, 138.8v, 167.1v, 113.1v, and 337.14v were constructed as described in Material and Methods and found to contain nucleotide sequences identical to those of their corresponding cDNA IC (data not shown). Chimeras were screened for virulence in pigs by intranasal inoculation of 10^5 $TCID_{50}$/animal. Only two chimeras, 138.8v and 337.14v, exhibited the attenuated phenotype of CSICv, while the other four chimeras exhibited the virulence phenotype of parental BICv (Table 2). The CS region common to both 138.8v and 337.14v encoded the E2 gene, suggesting that a significant virulence determinant absent in CS E2 was responsible for attenuation of these chimeras.

To confirm the role of E2 in swine virulence and further map the region responsible for 138.8v attenuation, two additional chimeric viruses, 319.1v and 312.1v, were constructed to contain CS regions encoding E2 only or p7 and the amino-terminal 894-amino-acid residues of NS2-3, respectively (Fig. 2). Chimeric viruses were rescued, and their nucleotide sequences

TABLE 2. Swine survival, clinical scores, and fever response following infection with BICv, CSICv, and chimeric viruses

Virus	No. of survivors/ total no.	Mean time to death (days ± SD)	Fever	
			No. of days to onset (± SD)	Duration, no. of days (± SD)
BICv	0/10	9.1 (3.1)	4.4 (0.8)	4.6 (0.8)
CSICv	4/4			
189.9v	0/4	8.75 (2.6)	3 (0)	5.2 (3.2)
182.1v	0/2	11.5 (6.4)	4 (0.7)	5.5 (4.9)
138.8v	2/2		8.5 (0.5)	1 (0)
167.1v	0/2	12 (4.65)	6.5 (0.7)	9 (2.8)
113.1v	0/2	8 (0)	4 (0)	4 (0)
337.14	2/2			
137.1v	2/2			
343.8v	2/2		8[a]	1

[a] Only one animal showed fever for 1 day.

were found to be identical to those of their corresponding cDNA IC (data not shown).

Growth characteristics of 138.8v, 319.1v, and 312.1v relative to those of BICv and CSICv were examined in a multistep growth curve by infecting primary swine macrophage cell cultures (MOI = 0.1) and determining titers at various times postinfection (Fig. 3A). While growth characteristics of BICv, 138.8v, 312.1v, 319.1v, and 337.14v were similar, CSICv growth was reduced by approximately 1,000-fold. Notably, the plaque size of 138.8v and 319.1v was reduced by approximately 50 to 60% relative to that of BICv and 312.1v. The plaque size of CSICv was reduced by 90% relative to that of BICv (Fig. 3B).

Chimeric viruses 319.1v and 312.1v were evaluated for swine virulence relative to BICv, CSICv, and 138.8v. Parental viruses exhibited characteristic virulence phenotypes (Table 3). Animals infected with chimera 138.8v or 319.1v survived infection and, with the exception of a transient fever (1 to 2 days), remained clinically normal. All animals infected with 312.1v presented with clinical signs of CSF starting at 3 to 7 dpi, with kinetics and severity of disease indistinguishable from those observed for animals infected with BICv (Table 3). White-blood-cell and platelet numbers dropped drastically by 6 dpi in BICv- or 312.1v-infected animals and remained low until death, while a transient and much less dramatic effect was observed for 138.8v- or 319.1v-infected animals (Fig. 4). CSICv infection induced no significant hematological changes.

Viremia in 138.8v- or 319.1v-infected animals was transient (8 to 10 dpi) and significantly reduced by 10^5 to 10^6 $\log_{10}$ from that with BICv or 312.1v infection but was higher than that observed for CSICv-infected animals where no viremia was detected (Fig. 5C). Similar titration patterns were obtained for nasal swab and tonsil scraping samples (Fig. 5A and B).

Nucleotide sequences of viruses recovered from infected animal tonsil scraping samples (6 dpi) were identical to those of 138.8v, 319.1v, and 312.1v stock viruses used for inoculation (data not shown).

For a more-detailed comparison of the pathogenesis in-

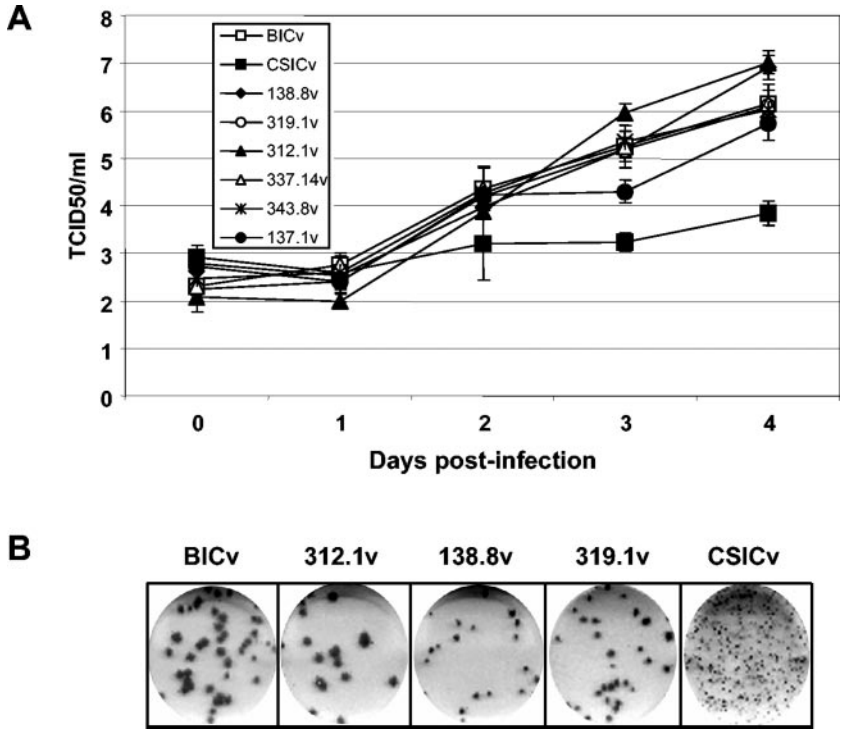


FIG. 3. (A) Growth characteristics of CSFV BICv, CSICv, and chimeric viruses 138.8v, 312.1v, and 319.1v in swine macrophage cell cultures. Primary swine macrophage cell cultures were infected (MOI = 0.1) with CSFV BICv, CSICv, 138.8v, 319.1v, 312.1v, 337.14v, 343.8v, or 137.1v. At times postinfection, samples were collected and titrated for virus yield. Data are means and standard errors from three independent experiments. (B) Plaque formation of CSFV BICv, CSICv, and chimeric viruses on SK6 cell cultures. Cell cultures were infected with 50 to 100 $TCID_{50}$, overlaid with 0.5% agarose, and incubated at 37°C for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry as described in Materials and Methods.

TABLE 3. Swine survival, clinical scores, and fever response following infection with BICv, CSICv, or chimeric viruses

Virus	No. of survivors/ total no.	Mean time to death days (± SD)	Clinical scores, range (5–10 dpi)	Fever		
				No. of days to onset (± SD)	Duration, no. of days (± SD)	Max temp, °F (± SD)
BICv	0/6	12.2 (3.1)	11–20	7 (0)	5.2 (0.5)	106.5 (1)
CSICv	4/4		1–1			103.4 (0.3)
138.8v	6/6		1–1	9.5 (1.1)	1 (0.5)	103.4 (0.2)
319.1v	6/6		1–1.5	9.3 (1.2)	1.25 (1.7)	103.7 (0.7)
312.1v	0/6	10.6 (2.1)	10–20	5.0 (0.0)	8 (0.7)	106.8 (0.5)

duced by 138.8v, 319.1v, and 312.1v, randomly selected animals were euthanized at 6 and 8 dpi (one animal/time point/group), and tissue samples (tonsil, submandibular lymph node, and spleen) were collected for virus titration and histopathological and immunohistochemical analysis. Titers of virus in tissue are shown in Table 4. Replication of CSICv, 138.8v, and 319.1v was transient in tonsils, with tissue titers of virus significantly reduced (approximately 10 to 10^6 $\log_{10}$ depending on virus and time postinfection) relative to those of BICv and 312.1v. CSICv and 138.8v were not detected in regional draining mandibular lymph node or spleen tissues, and 319.1v was detected in these tissues only transiently (6 dpi) and at significantly lower titers (10^2 $\log_{10}$) than those observed for BICv and 312.1v.

Histological lesions in pigs inoculated with BICv were al-

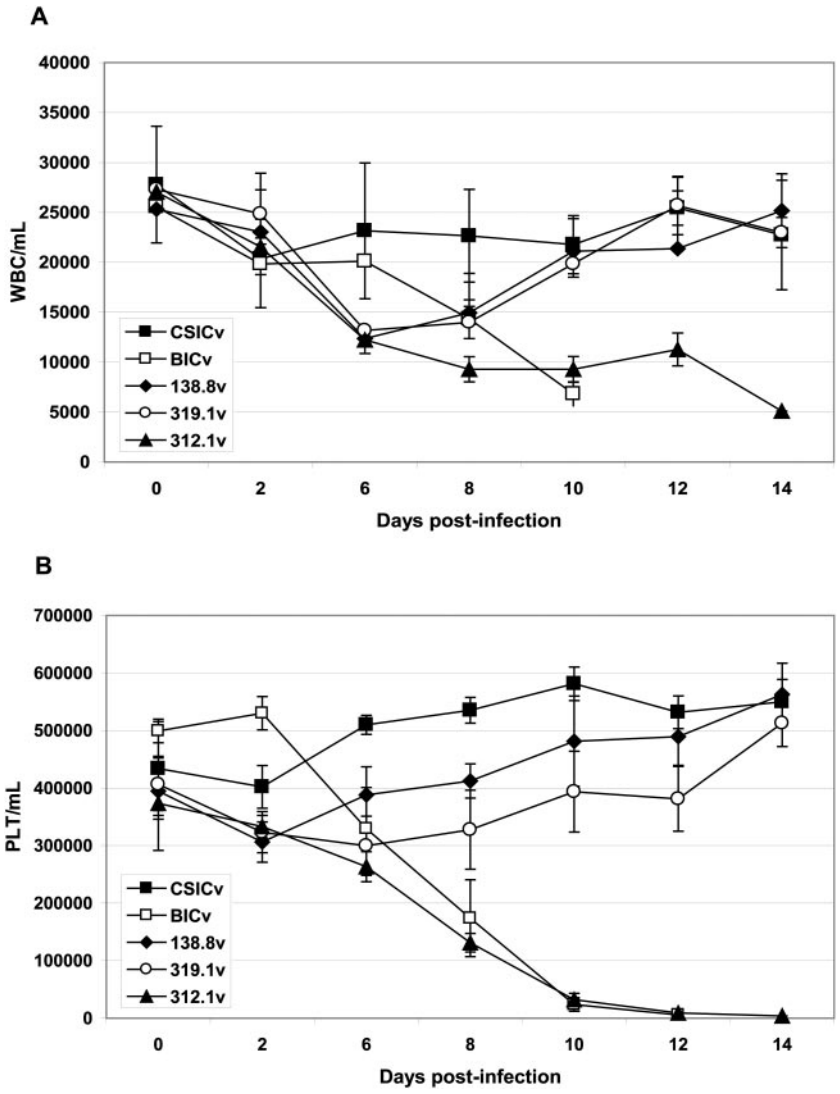


FIG. 4. Cell counts of peripheral white blood cells (A) and platelets (B) for pigs infected with BICv, CSICv, or 319.1v, 138.8v, or 312.1v. Counts were determined as described in Material and Methods. Each point represents the mean and standard errors for four or more animals.

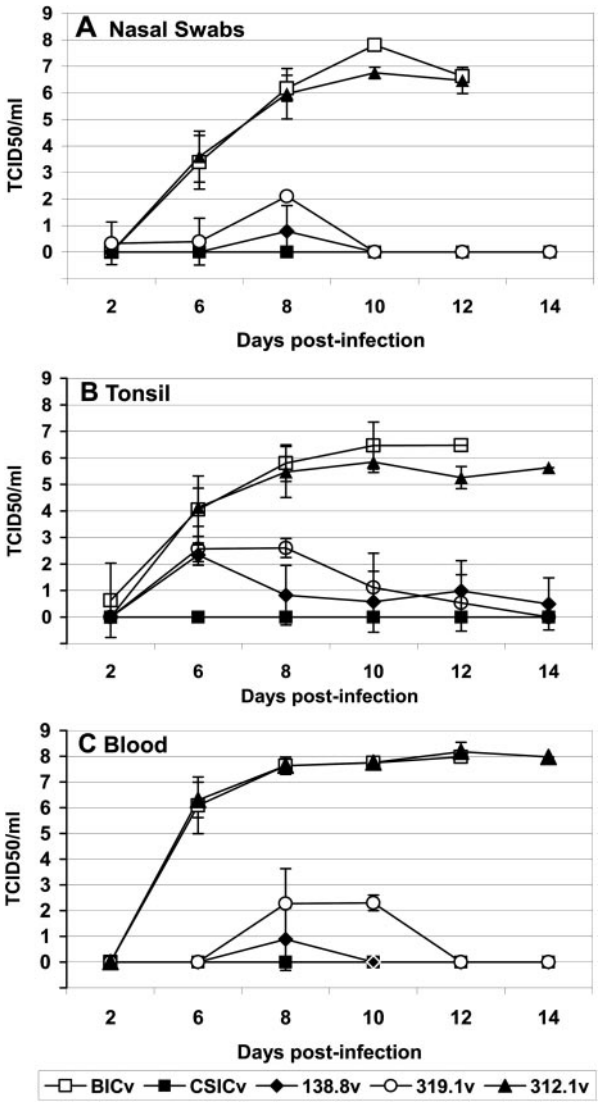


FIG. 5. Titers of virus from (A) nasal swabs, (B) tonsil scrapings, or (C) blood from pigs infected with BICv, CSICv, or chimeric viruses 319.1v, 138.8v, or 312.1v. Each point represents the mean and standard errors for four or more animals.

ready present at 6 dpi (Fig. 6). This included tonsillar, splenic, and submandibular lymphoid necrosis and cell depletion along with a moderate to marked (6 and 8 dpi, respectively) histiocytic hyperplasia and reticuloendothelial cell proliferation. Additionally, the spleen was characterized by moderate to severe lymphoid follicular depletion, necrosis, and a mild to moderate histiocytosis. Animals infected with viruses CSICv and 319.1v showed histopathological changes in tonsils and the regional submandibular lymph node similar to those described for the BICv-infected pigs, but the degree of severity was markedly reduced: there was minimal to mild lymphoid necrosis and depletion and a mild histiocytosis. Notably, the pathology already had started to resolve, with evidence of lymphoid hyperplasia, at 6 dpi. In addition, spleens of animals infected with CSICv or 319.1v showed near-negligible changes, with minimal lymphoid necrosis and depletion (Fig. 6).

The presence of CSFV antigen in tissues of infected animals was consistent with the degree and distribution of lesions and titers of virus in tissues. In BICv-infected pigs, there was antigen in superficial and cryptic tonsillar epithelial cells and tonsillar endothelium by 6 dpi. At 6 and 8 dpi, antigen was observed in tonsillar macrophages and lymphoid follicular and parafollicular dendritic cells with moderate to marked immunoreactivity (Fig. 6). Similar immunoreactivity was observed in the spleen, where macrophages and endothelium were affected. Conversely, animals infected with CSICv or 319.1v showed CSFV antigen concentrated in the tonsil, with a significant relative reduction in immunoreactivity in the regional submandibular lymph node and spleen compared to the BICv-infected animals.

These data indicate that glycoprotein E2 markedly affects pig virulence. 319.1v infection is characterized by mild and transient clinical disease, decreased viral replication in tonsils, limited generalization of infection, and a significant reduction in virus shedding.

Brescia structural protein E2 is not sufficient to restore CS virus virulence. To test the ability of Brescia virus E2 to restore a virulent phenotype to the attenuated CS vaccine strain, two chimeric viruses which contained the genomic region encoding all Brescia virus structural proteins (343.8v) or the Brescia E2/p7 protein and the amino-terminal 894 amino acid residues of the NS2-3 protein (137.1v) inserted into the CS genetic background were constructed and evaluated for virulence in swine (Fig. 2; Table 2). Both chimeric viruses, although demonstrating normal growth in swine macrophages, demonstrated an attenuated phenotype in swine, indicating that these Brescia genomic regions alone are not sufficient to restore a virulent phenotype to CS virus.

DISCUSSION

Here we have shown that the CSFV E2 glycoprotein markedly affects viral virulence in swine. The replacement of the E2 gene in the highly pathogenic strain Brescia with the E2 gene from the vaccine strain CS resulted in significant in vivo attenuation. Unlike the acute fatal disease induced by Brescia and its IC BICv, 319.1v infection was subclinical, characterized by decreased viral replication in tonsils, limited generalization of infection, and reduced virus shedding. The 319.1v attenuation phenotype was independent of its ability to replicate in primary

TABLE 4. Titers of virus in tissues after infection with BICv, CSICv, or chimeric viruses

Virus	No. of dpi	$Log_{10}/TCID_{50}/g$ in:		
		Tonsils	Mandibular lymph node	Spleen
BICv	6	4.97	5.13	5.97
	8	5.47	5.97	5.80
CSICv	6	Neg[a]	Neg	Neg
	8	2.13	Neg	Neg
319.1v	6	3.63	3.47	3.63
	8	3.13	Neg	Neg
138.8v	6	2.80	Neg	Neg
	8	Neg	Neg	Neg
312.1v	6	5.80	5.97	5.97
	8	6.30	7.30	7.13

[a] Neg, less than 1.8 $TCID_{50}$ (log_{10}).

macrophage cell cultures. It is notable that no other CS genome region was able to attenuate the virulent Brescia phenotype, indicating the significance of CS E2 in attenuation.

The genetic basis and the molecular mechanisms underlying pestivirus virulence are largely unknown. In the case of CSFV, there are two reports implicating a specific viral protein or genomic region in virulence. A single- or double-codon mutation abrogating E^{rns} RNase activity of the CSFV Alfort/Tübingen strain attenuated the virus for pigs (19). Notably, a similar result was obtained by mutating the same E^{rns} domain of BVDV (18). More recently, N^{pro} has also been shown to be involved in CSFV virulence, since deletion of N^{pro} in the virulent Eystrup strain abrogated its virulence for swine (17). Here a third CSFV virulence determinant associated with the E2 glycoprotein has been identified. Although E2 is a highly immunogenic CSFV glycoprotein, inducing protective immune responses in swine (8, 15, 36, 37), its role in virus virulence has not been previously described.

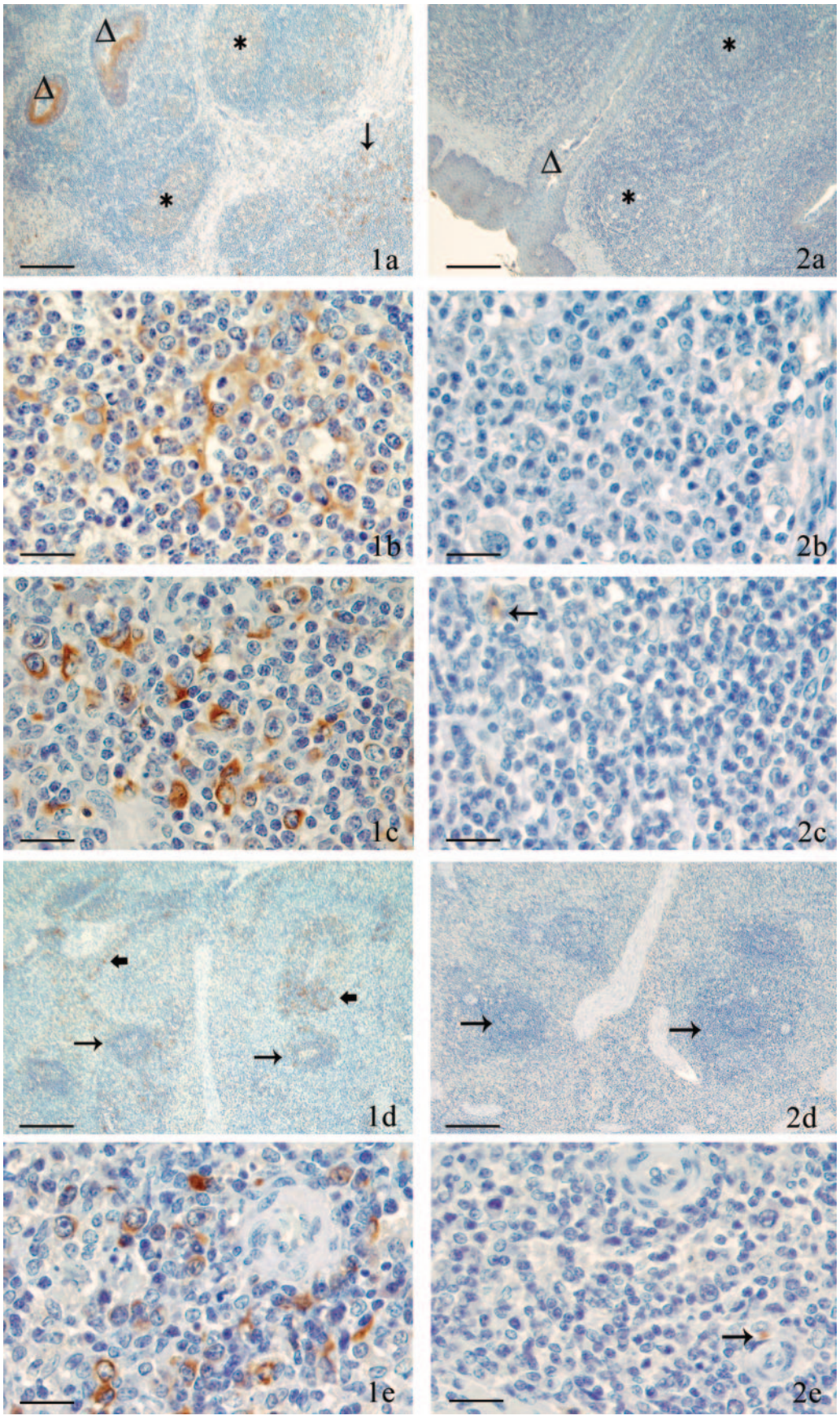
Specific features responsible for the functional difference of CS E2 are unknown. Comparative sequence analysis of Brescia and CS E2 regions revealed no insertions or deletions and 6% nucleotide divergence over 1,119 nucleotides, including 40 synonymous and 22 nonsynonymous substitutions. The 22 predicted amino acid substitutions in CS relative to Brescia are distributed along E2, with only two adjacent positions (238 and 239) affected. Substitutions are generally conservative or neutral, as only six substitutions (T56R, T197M, P200L, S238L, R290S, and L370P) are relatively nonconservative ($\leq$0.3 in a normalized PAM250 substitution matrix [3]). Highly conservative I137V and I342V substitutions were present in a previously predicted fusion peptide region (6) and near the strongly predicted carboxyl-terminal transmembrane domain (position 342 to 366) of CS E2, respectively. Few substitutions were found in regions consistently altered in predicted secondary structure, but they included K45R, a residue previously affecting domain C antigenicity when experimentally mutated in Brescia (34), G99E, and notably T197M and P200L, which locally affect hydrophobicity and predicted B-sheet formation in CS E2. Also notable is L370P, a mutation which resulted in loss of a predicted carboxyl-terminal alpha-helical structure in CS E2 and in the reversed transmembrane topology predicted for CS E2. How these specific changes contribute to the attenuated phenotype observed for CSICv E2 remains to be determined.

Attenuation of 319.1v in pigs could conceivably involve some aspect of virus attachment and/or efficiency of entry into critical target cells in vivo. Given that macrophages are targeted in vivo, it is notable that 319.1v exhibited normal growth in macrophage cell cultures in vitro. E^{rns}, E1, and E2 are structural glycoproteins in the CSFV virion envelope, and available evidence indicates that the E2 gene is an essential gene (32). Anchored to the envelope, E2 appears as both homo- and heterodimers linked by disulfide bridges (28, 37, 38) and, along with E^{rns}, is known to be critical for virus reception (9). Notably, pestiviruses containing chimeric E2 proteins have an altered host range. Partial replacement of the amino terminus of CSFV E2 with the homologous sequence from BVDV resulted in a virus that, although differing from BVDV in its capacity to infect fetal bovine epithelial cells, resembled BVDV with a 10-fold decrease in its ability to replicate in SK6 cells (31). Additionally, a chimeric BVDV containing the complete E2 gene of border disease virus (BDV), similarly to BDV, is unable to replicate in MDBK cells, a cell line permissive for BVDV (16).

Consistent with a possible direct or indirect effect for altered E2 on virus attachment and/or spread, 319.1v exhibited a small-plaque phenotype in SK6 cells compared with parental BICv. Plaque size was reduced approximately 50%. Interestingly, CSICv also has a reduced plaque size compared with BICv, although in this case reduction was 90%. Thus, additional genetic determinants in the CSICv genome are likely involved in the small-plaque phenotype. A correlation between plaque size in vitro and pathogenesis in vivo has yet to be established. Hulst et al. (11, 12) serially passaged CSFV Brescia in SK6 cells to generate heparan sulfate binding-dependent small-plaque variants that contained mutations in the E^{rns} protein; however, these varients had an unaltered virulence phenotype in pigs (11, 12).

Despite the data here implicating E2 in swine virulence, the presence of additional CSFV virulence determinants outside the E2 region is indicated. Notably, when inserted into the CS genetic background, all Brescia structural proteins, including E2, were unable to restore a virulent phenotype to CS. This result suggests that CS contains additional mutations affecting virulence in untranslated and nonstructural protein-encoding regions. Given the history of CS virus, a vaccine virus safely used for more than 30 years (40), it is not surprising that CS may contain additional attenuating mutations which affect multiple proteins. In fact, CS demonstrated 531 nucleotide differences with Brescia across the genome, resulting in 105 amino acid substitutions affecting both 5′ and 3′ untranslated regions and all viral proteins except NS4A (data not shown). Additional support for other contributing virulence determinants is suggested by the somewhat more attenuated phenotype for 138.8v (E2/p7/NS2-3 substitution) than for 319.1v (E2 substitution only) observed as reduced blood and tissue titers and the absence of generalized infection (Table 4; Fig. 5). Most significantly, any additional CS mutations affecting virulence were unable to attenuate BICv in the context of the chimeras

FIG. 6. Detection of CSFV antigens by immunohistochemistry at 6 dpi in lymphoid organs of pigs infected with BICv or 319.1v. Note (1a) the abundance of CSFV antigen in tonsillar crypt epithelium (Δ = crypt), lymphoid follicles (*), and parafollicular regions (arrow) of the BICv-infected animal. Both in the follicular (1b) and parafollicular (1c) regions, immunoreactive cells are morphologically characterized as dendritic cells. In the 319.1v-infected animal (2a), there is minimal CSFV antigen in tonsillar crypt epithelium (Δ = crypt) and rare CSFV antigen in lymphoid follicles (* and 2b) and parafollicular (2c) region. In spleen of BICv-infected animal (1d), note lymphoid depletion with poorly defined periarteriolar lymphoid sheaths (large arrows) and abundant CSFV antigen in periarteriolar regions (small arrow) and follicles with associated lymphoid depletion (1e). In the 319.1v-infected animal, there are prominent periarteriolar lymphoid sheaths (2d and e: arrows) with follicles, with minimal to no expression of CSFV antigen in periarteriolar regions and follicles. Lymphoid follicles are well populated with lymphoid proliferation (2e). Bar in (1a), (2a), (1d), and (2d) = 160 μm; bar in (1b), (1c), (2b), (2c), (1e), and (2e) = 30 μm.


1a
2a
1b
2b
1c
2c
1d
2d
1e
2e

generated in this study, further highlighting the significance of E2 in swine virulence.

In summary, a novel CSFV virulence determinant associated with the E2 glycoprotein has been identified. An improved understanding of the genetic basis of CSFV virulence and host range will permit rational design of live attenuated CSF vaccines of enhanced safety, efficacy, and utility.

ACKNOWLEDGMENTS

We thank Aniko Zsak, Adriene Lakowitz, and the PIADC animal care staff for excellent technical assistance.

ADDENDUM

Since completion of the work presented here, additional studies have identified the CSFV E2 protein as contributing to the virulence of strain Brescia; however, these E2 mutants also required mutations in the E^{rns} protein to yield an attenuated phenotype (33).

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Brendel, V., P. Bucher, I. R. Nourbakhsh, B. E. Blaisdell, and S. Karlin.** 1992. Methods and algorithms for statistical analysis of protein sequences. Proc. Natl. Acad. Sci. USA **89:**2002–2006.
3. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
4. **Edwards, S., V. Moennig, and G. Wensvoort.** 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet. Microbiol. **29:**101–108.
5. **Francki, R. I. B., D. L. Fauquet, D. L. Knudson, and F. Brown. (ed.).** 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on the Taxonomy of Viruses. Arch. Virol. Suppl. **2:**223–233.
6. **Garry, R. F., and S. Dash.** 2003. Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. Virology **307:**255–265.
7. **Huang, X., and A. Madan.** 1999. CAP3: A DNA sequence assembly program. Genome Res. **9:**868–877.
8. **Hulst, M. M., D. F. Westra, G. Wensvoort, and R. J. M. Moormann.** 1993. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. J. Virol. **67:**5435–5442.
9. **Hulst, M. M., and R. J. M. Moormann.** 1997. Inhibition of pestivirus infection in cell culture by envelope proteins E(rns) and E2 of classical swine fever virus: E(rns) and E2 interact with different receptors. J. Gen. Virol. **78:**2779–2787.
10. **Hulst, M. M., F. E. Panoto, A. Hoekman, H. G. P. van Gennip, and R. J. M. Moormann.** 1998. Inactivation of the RNase activity of glycoprotein E(rns) of classical swine fever virus results in a cytopathogenic virus. J. Virol. **72:**151–157.
11. **Hulst, M. M., H. G. P. van Gennip, and R. J. M. Moormann.** 2000. Passage of classical swine fever virus in cultured swine kidney cells selects virus variants that bind to heparan sulfate due to a single amino acid change in envelope protein E(rns). J. Virol. **74:**9553–9561.
12. **Hulst, M. M., H. G. P. van Gennip, A. C. Vlot, E. Schooten, A. J. de Smit, and R. J. M. Moormann.** 2001. Interaction of classical swine fever virus with membrane-associated heparan sulfate: role for virus replication in vivo and virulence. J. Virol. **75:**9585–9595.
13. **Jones, D. T.** 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. **292:**195–202.
14. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1994. A model recognition approach to the prediction of all-helical membrane protein structure and topology. Biochemistry **33:**3038–3049.
15. **König, M., T. Lengsfeld, T. Pauly, R. Stark, and H.-J. Thiel.** 1995. Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. J. Virol. **69:**6479–6486.
16. **Liang, D., I. Fernandez Sainz, I. H. Ansari, L. H. V. Gil, V. Vassilev, and R. O. Donis.** 2003. The envelope glycoprotein E2 is a determinant of cell culture tropism in ruminant pestiviruses. J. Gen. Virol. **84:**1269–1274.
17. **Mayer, D., M. A. Hofmann, and J. D. Tratschin.** 2004. Attenuation of classical swine fever virus by deletion of the viral N(pro) gene. Vaccine **22:**317–328.
18. **Meyer, C., M. Von Freyburg, K. Elbers, and G. Meyers.** 2002. Recovery of virulent and RNase-negative attenuated type 2 bovine viral diarrhea viruses from infectious cDNA clones. J. Virol. **76:**8494–8503.
19. **Meyers, G., A. Saalmüller, and M. Büttner.** 1999. Mutations abrogating the RNase activity in glycoprotein E^{rns} of the pestivirus classical swine fever virus lead to virus attenuation. J. Virol. **73:**10224–10235.
20. **Mittelholzer, C., C. Moser, J. D. Tratschin, and M. A. Hofmann.** 2000. Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. Vet. Microbiol. **74:**293–308.
21. **Moser, C., A. Bosshart, J. D. Tratschin, and M. A. Hofmann.** 2001. A recombinant classical swine fever virus with a marker insertion in the internal ribosome entry site. Virus Genes **23:**63–68.
22. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
23. **Rice, C. M.** 1996. Flaviviridae: the viruses and their replication, p. 931–959. *In* D. M. Knipe, B. N. Fields, and P. Howley (ed.), Fundamental virology, 3rd ed. Lippincott Raven, Philadelphia, Pa.
24. **Risatti, G. R., J. D. Callahan, W. M. Nelson, and M. V. Borca.** 2003. Rapid detection of classical swine fever virus by a portable real-time reverse transcriptase PCR assay. J. Clin. Microbiol. **41:**500–505.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Sur, J. H., V. L. Cooper, J. A. Galeota, R. A. Hesse, A. R. Doster, and F. A. Osorio.** 1996. In vivo detection of porcine reproductive and respiratory syndrome virus RNA by in situ hybridization at different times postinfection. J. Clin. Microbiol. **34:**2280–2286.
27. **Terpstra, C., R. Woortmeyer, and S. J. Barteling.** 1990. Development and properties of a cell culture produced vaccine for hog cholera based on the Chinese strain. Dtsch. Tierarztl. Wochenschr. **97:**77–79.
28. **Thiel, H.-J., R. Stark, E. Weiland, T. Rümenapf, and G. Meyers.** 1991. Hog cholera virus: molecular composition of virions from a pestivirus. J. Virol. **65:**4705–4712.
29. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgings.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24:**4876–4882.
30. **Tratschin, J. D., C. Moser, N. Ruggli, and M. A. Hofmann.** 1998. Classical swine fever virus leader proteinase Npro is not required for viral replication in cell culture. J. Virol. **72:**7681–7684.
31. **van Gennip, H. G. P., P. A. van Rijn, M. N. Widjojoatmodjo, A. J. de Smit, and R. J. M. Moormann.** 2000. Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. Vaccine **19:**447–459.
32. **van Gennip, H. G. P., A. Bouma, P. A. van Rijn, M. N. Widjojoatmodjo, and R. J. M. Moormann.** 2002. Experimental non-transmissible marker vaccines for classical swine fever (CSF) by *trans*-complementation of E^{rns} or E2 of CSFV. Vaccine **20:**1544–1556.
33. **van Gennip, H. G. P., A. C. Vlot, M. M. Hulst, A. J. de Smit, and R. J. M. Moormann.** 2004. Determinants of virulence of classical swine fever virus strain Brescia. J. Virol. **78:**8812–8823.
34. **van Rijn, P. A., G. K. Miedema, G. Wensvoort, H. G. van Gennip, and R. J. Moormann.** 1994. Antigenic structure of envelope glycoprotein E1 of hog cholera virus. J. Virol. **68:**3934–3942.
35. **van Oirschot, J. T.** 1986. Hog cholera, p. 289. *In* B. S. A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases Of swine, 6th ed. Iowa State University Press, Ames, Iowa.
36. **van Zijl, M., G. Wensvoort, E. de Kluyver, M. Hulst, H. van der Gulden, A. Gielkens, A. Berns, and R. Moormann.** 1991. Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. J. Virol. **65:**2761–2765.
37. **Weiland, E., R. Stark, B. Haas, T. Rümenapf, G. Meyers, and H.-J. Thiel.** 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J. Virol. **64:**3563–3569.
38. **Weiland, F., E. Weiland, G. Unger, A. Saalmüller, and H.-J. Thiel.** 1999. Localization of pestiviral envelope proteins E(rns) and E2 at the cell surface and on isolated particles. J. Gen. Virol. **80:**1157–1165.
39. **Widjojoatmodjo, M. N., H. G. P. van Gennip, A. Bouma, P. A. van Rijn, and R. J. M. Moormann.** 2000. Classical swine fever virus E(rns) deletion mutants: *trans*-complementation and potential use as nontransmissible, modified, live-attenuated marker vaccines. J. Virol. **74:**2973–2980.
40. **Zaberezhny, A. D., T. V. Grebennikova, V. V. Kurinnov, S. G. Tsybanov, I. F. Vishnyakov, S. F. Biketov, T. I. Aliper, and E. A. Nepoklonov.** 1999. Differentiation between vaccine strain and field isolates of classical swine fever virus using polymerase chain reaction and restriction test. Dtsch. Tierarztl. Wochenschr. **106:**394–397.
41. **Zsak, L., Z. Lu, G. F. Kutish, J. G. Neilan, and D. L. Rock.** 1996. An African swine fever virus virulence-associated gene NL-S with similarity to the herpes simplex virus ICP34.5 gene. J. Virol. **70:**8865–8871.