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Identification of Antigenic Regions of the E^{rns} Protein for Pig Antibodies Elicited during Classical Swine Fever Virus Infection

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The structural glycoprotein E^{rns} of classical swine fever virus (CSFV) is one of the major antibody targets upon infection of pigs with the virus. Molecular dissection of the structure of E^{rns} would define the minimal immunodominant regions that induce antibody responses after infection and may thus help design an effective diagnostic reagent or vaccine. In this study, deletion analysis was made within amino acids (aa) 297 to 776 of the CSFV Alfort/187 polyprotein containing the large C-terminal portion of the E^{rns} protein (aa 27 to 227), the entire E1 protein (aa 1 to 195), and the N-terminal portion of the E2 protein (aa 1 to 87). Various protein fragments with target deletions from N- or/and C-terminal ends were constructed with pET30, expressed in *Escherichia coli* and probed on Western blots with antisera from pigs infected with CSFV. This has resulted in the identification within E^{rns} of three overlapping antigenic regions: AR1($E^{rns}_{aa\ 65–145}$), AR2 ($E^{rns}_{aa\ 84–160}$) and AR3 ($E^{rns}_{aa\ 109–220}$). N- or C-terminal deletions as small as 3 residues introduced into these regions disrupt their reactivity with antibodies, indicating that they are the minimum requirements for recognition by pig antibodies. The three minimal antigenic regions correlated well with the hydropathy profiles and the 3D structural model of E^{rns}. Each individual region and a protein fragment containing AR1, AR2 and AR3 reacted equally well with pig anti-CSFV sera. Since variable and conserved sequences are present within the three overlapping antigenic regions of E^{rns} of different pestiviruses, specific serological detection of CSFV infection or broad detection of pestivirus infections may be achieved with the use of a single E^{rns} region or a combination of two or three E^{rns} regions.

Key words: antibody, antigenicity, classical swine fever virus, recombinant protein, structural glycoprotein.

Classical swine fever virus (CSFV) is an enveloped positive-stranded RNA virus (*1*) of the genus *Pestivirus* of the *Flaviviridae* family (*2*). Other members classified into this genus are bovine viral diarrhoea virus (BVDV) and border disease virus of sheep (BDV). These viruses are antigenically and genetically closely related. CSFV can cause a highly contagious disease in pigs with substantial economic loss. Like other members of the genus, CSFV contains a genome of approximately 12.5 kb with a single large open reading frame that is translated to a polyprotein precursor of approximately 4,000 amino acids (aa). The polyprotein is cleaved co- and post-translationally by cellular and viral proteases into structural proteins including C, E^{rns}, E1, and E2, and nonstructural proteins including NS2, NS3, NS4A, NS4B, NS5A and NS5B (*3*).

The envelope glycoprotein E^{rns} forms a disulfide-bridged homodimer in the virion (*4*, *5*). E^{rns} is highly modified by *N*-linked glycosylation, which contributes about 50% to the molecular mass of E^{rns} (*6*, *7*). The C-terminal region of E^{rns} can translocate the full-length protein across eukaryotic cell membranes (*8*). E^{rns} can be found both on the surface of pestivirus-infected cells and in the culture medium (*6*). The protein can bind to several cell types and inhibits CSFV and BVDV infection in cell culture, suggesting that E^{rns} is involved in attachment to or entry of the viruses into susceptible cells (*9*). The interaction of E^{rns} with the cell surface glycoaminoglycans (heparan sulfate) contributes in part to the binding of the virus to susceptible cells (*10*, *11*). The E^{rns} protein contains ribonuclease activity (*7*, *12*, *13*), which is a unique feature for a viral surface protein. Homology in two stretches of the E^{rns} sequence to members of the Rh/T2/S RNase superfamily provides a structural basis for the ribonuclease activity (*5*, *12*). The biological function of E^{rns} ribonuclease activity is not yet clear. Mutations in E^{rns} destroying the RNase activity give rise to viruses that are more cytopathic in culture and are attenuated *in vivo* (*14*, *15*). Antibodies that inhibit ribonuclease activity also tend to neutralize virus infectivity (*7*). The CSFV E^{rns} is capable of inducing apoptosis in lymphocytes of several species (*16*); and although no data has been reported to show that the RNase activity of E^{rns} is related to its toxicity, cytotoxicity is a feature of other soluble ribonucleases (*17*).

Antibodies directed against E^{rns} and E2 have been demonstrated in infected animals (*18*–*21*). Monoclonal antibodies to E^{rns} (*22*) or E2 (*23*, *24*) can neutralize virus

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Table 1. **Primers used for molecular cloning.**

Primer[a]	Sequence[b]	Annealing sites (nucleotides)
Forward primers		
P68	5′-GGATCCGCAGAAGCTTGCATGGGATCTG-3′	1251–1272
P133	5′-ACAGGATCCTGGTACAATATAGACCCCTG-3′	1424–1443
P134	5′-ACAGGATCCGACGTCAACGTGGTCACCCA-3′	1535–1554
P135	5′-ACAGGATCCGTGGAGGATATCTTGTATGG-3′	1655–1674
P167	5′-ACAGGATCCGAGTGCGCTGTGACTTGTAG-3′	1499–1518
P170	5′-ACAGGATCCGTGACTTGTAGGTATGATAA-3′	1508–1527
P171	5′-ACAGGATCCAGGTATGATAAAGATGCTGA-3′	1517–1536
P172	5′-ACAGGATCCAAAGATGCTGACGTCAACGT-3′	1526–1545
P179	5′-ACAGGATCCATGAATAGAACCCAAGCAAA-3′	1454–1473
P216	5′-ACAGGATCCGCCACAGACACGGAACTGAA-3′	1310–1329
P217	5′-ACAGGATCCAACTATACGTGCTGTAAGTT-3′	1367–1386
Reverse primers		
P69	5′-CTCGAGGGTCCCGTCGAACAGGAGCTCG-3′	2680–2701
P116	5′-ACACTCGAGAAAGGAACCTGCCACGCA-3′	2582–2599
P117	5′-ACACTCGAGTGATGATATTGCGTACCTGTA-3′	2462–2482
P118	5′-ACACTCGAGGAGGAATGCCGTGGTTGATGC-3′	2342–2362
P119	5′-ACACTCGAGAGCCACCTTGGTTTCATATG-3′	2244–2263
P120	5′-ACACTCGAGTGTGTCACAGCCTTCAGGTTC-3′	2123–2143
P131	5′-ACACTCGAGTACATTACAGTAAGGTGATAG-3′	1856–1876
P132	5′-ACACTCGAGTTGCCTCCCGAGCCAAGATG-3′	1776–1795
P140	5′-ACACTCGAGTATAGTGTTGGTCATTCCAT-3′	1725–1744
P141	5′-ACACTCGAGGAGCAAACTGCCGCACTCAT-3′	1680–1699
P142	5′-ACACTCGAGGGAAACATTGAAATTACATG-3′	1635–1654
P143	5′-ACACTCGAGAGAAAAATTTTTTCCTTTCT-3′	1590–1609
P144	5′-ACACTCGAGGTTTCTGGCCTGGGTGACCA-3′	1545–1564
P145	5′-ACACTCGAGCCTACAAGTCACAGCGCACT-3′	1500–1519
P163	5′-ACACTCGAGTTTTCCTTTCTTGCAGCCGG-3′	1581–1600
P164	5′-ACACTCGAGCTTGCAGCCGGTCAGGGTTG-3′	1572–1591
P165	5′-ACACTCGAGGGTCAGGGTTGTTGGCCTGT-3′	1563–1582
P166	5′-ACACTCGAGGTTGACGTCAGCATCTTTAT-3′	1524–1543
P218	5′-ACACTCGAGTTTGCTTCTACCCTCCAACC-3′	1815–1834

[a]Primers are synthesized based on the sequence of the CSFV strain Alfort/187 genome (*29*) (GenBank accession No. X87939). [b]Restriction enzyme sites (*Bam*HI, *Xho*I, and *Hin*dIII) incorporated at 5′ ends or contained in the genome sequence are underlined.

infectivity, and both proteins can confer protective immunity to pigs (*25–27*). The antigenic sites or regions of the pestivirus E[rns] are not well defined, except that the C-terminal end (37mer peptide) was shown, based on its probable independent folding and good exposure in the modular structure of E[rns], to bind antibodies in sera from infected animals (*18*). In this study, various truncated forms of the CSFV E[rns] protein were expressed in *Escherichia coli* and these recombinant products used to map the binding regions of E[rns] for pig antibodies elicited during infection. This has allowed for the definition of an immunodominant region composed of three overlapping immunogenic regions on E[rns], which may thus have the potential for diagnostic applications.

MATERIALS AND METHODS

Materials—E. coli strain INVαF′, DH5α, pCR2.1 vector, and *Taq* DNA polymerase were obtained from Invitrogen (Carlsbad, CA) while *E. coli* BL21(DE3)pLysS and pET30 vectors were purchased from Novagen (Madison, WI). The anti–histidine tag monoclonal antibody was obtained from QIAGEN (Santa Clarita, CA). Expand Long Template PCR system (*Taq* and *Pwo* DNA polymerase mixture) was obtained from Roche Diagnostics (Laval, Quebec, Canada), and other molecular biology reagents were purchased from New England Biolabs (Mississauga, Ontario, Canada). The Wizard PCR Preps DNA purification system, the Wizard Plus Minipreps DNA purification system, M-MLV reverse transcriptase, and rRNasin were from Promega (Madison, WI). The GenElute Plasmid Minipreps kit was supplied by Sigma (Oakville, Ontario, Canada). All chemicals were of commercially available analytical grade. Classical swine fever virus (CSFV) antisera were obtained from experimentally infected swine (*28*).

RNA Extraction—Total RNA was extracted from a 75-cm^2 flask of PK15 cells (monolayer), which had been infected with CSFV strain Alfort/187 for 3 days, using a TriPure isolation reagent (Roche Diagnostics) according to the manufacturer's instructions. The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated RNase-free water (50 µl) and stored frozen (–80°C) until use.

Synthesis and Cloning of CSFV cDNA by RT-PCR—cDNA was derived from the total RNA with reverse transcriptase. Briefly, the RNA material (20 µl) was mixed with 10 µl of random hexamer (0.5 mg/ml) and 137.5 µl of H_2O, heated at 70°C for 5 min, and cooled on ice. Then M-

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Construct	Encoded Polypeptide	Reactivity with pig anti-CSFV serum
pET68-69	$E^{rns}_{aa\ 27\text{-}227}E1E2_{aa\ 1\text{-}87}$	ND
pET68-116	$E^{rns}_{aa\ 27\text{-}227}E1E2_{aa\ 1\text{-}53}$	ND
pET68-117	$E^{rns}_{aa\ 27\text{-}227}E1E2_{aa\ 1\text{-}14}$	ND
pET68-118	$E^{rns}_{aa\ 27\text{-}227}E1_{aa\ 1\text{-}169}$	ND
pET68-119	$E^{rns}_{aa\ 27\text{-}227}E1_{aa\ 1\text{-}136}$	+
pET68-120	$E^{rns}_{aa\ 27\text{-}227}E1_{aa\ 1\text{-}96}$	+
pET68-131	$E^{rns}_{aa\ 27\text{-}227}E1_{aa\ 1\text{-}7}$	+
pET68-132	$E^{rns}_{aa\ 27\text{-}207}$	+
pET68-140	$E^{rns}_{aa\ 27\text{-}190}$	+
pET68-141	$E^{rns}_{aa\ 27\text{-}175}$	+
pET68-142	$E^{rns}_{aa\ 27\text{-}160}$	+
pET68-143	$E^{rns}_{aa\ 27\text{-}145}$	+
pET68-163	$E^{rns}_{aa\ 27\text{-}142}$	–
pET68-164	$E^{rns}_{aa\ 27\text{-}139}$	–
pET68-165	$E^{rns}_{aa\ 27\text{-}136}$	–
pET68-144	$E^{rns}_{aa\ 27\text{-}130}$	–
pET68-166	$E^{rns}_{aa\ 27\text{-}123}$	–
PET68-145	$E^{rns}_{aa\ 27\text{-}115}$	–
pET133-131	$E^{rns}_{aa\ 84\text{-}227}E1_{aa\ 1\text{-}7}$	+
pET167-131	$E^{rns}_{aa\ 109\text{-}227}E1_{aa\ 1\text{-}7}$	+
pET170-131	$E^{rns}_{aa\ 112\text{-}227}E1_{aa\ 1\text{-}7}$	–
pET171-131	$E^{rns}_{aa\ 115\text{-}227}E1_{aa\ 1\text{-}7}$	–
pET172-131	$E^{rns}_{aa\ 118\text{-}227}E1_{aa\ 1\text{-}7}$	–
pET134-131	$E^{rns}_{aa\ 121\text{-}227}E1_{aa\ 1\text{-}7}$	–
PET135-131	$E^{rns}_{aa\ 161\text{-}227}E1_{aa\ 1\text{-}7}$	–

Fig. 1. **Schematic representation of deletion constructs and their encoded products used to map the antigenic regions on E^{rns}.** Interactions of the recombinant proteins with the antiserum from an experimentally CSFV-infected pig at 44 dpi are analyzed by Western Blots and the results indicated by + (specific reaction showing bands of similar intensity), – (reaction not detected) and ND (reaction not determined). The consensus region among the antibody-reactive E^{rns} fragments is indicated by a shaded box. Deleted residues are represented by a thin line.

MLV reverse transcriptase reaction buffer (50 µl, 5×), dNTP (12.5 µl, 10 mM), rRNasin (10 µl, 30 U/µl) and M-MLV reverse transcriptase (10 µl, 200 U/µl) were added, and the mixture was incubated at 37°C for 1 h. The reverse transcriptase reaction was terminated by heating at 95°C for 5 min. The cDNA thus synthesized was used as template to amplify a DNA fragment corresponding to nucleotides 1251 to 2701 of the CSFV strain Alfort/187 genome (*29*) (GenBank accession number X87939) by PCR with primers P68 and P69 (Table 1) in a 50-µl volume (1× buffer 1; 1.75 mM Mg^{2+}; 0.4 mM for each of dATP, dCTP, dGTP and dTTP; 0.5 µM of each primer; 12 µl of template; 1 µl of *Taq* and *Pwo* DNA polymerase mixture) using the Expand Long Template PCR System (Roche Diagnostics). Reaction mixtures were incubated in a GeneAmp PCR system 9700 thermocycler (Perkin Elmer, Foster City, CA) for 2 min at 94°C , followed by 40 cycles of amplification at 94°C for 30 s, 56°C for 45 s, and 72°C for 4 min and a final extension for 10 min at 72°C. The PCR product (about 1,450 bp) was purified with the Wizard PCR Preps DNA purification system and ligated into pCR2.1 to create pCR68-69.

Creation of Deletion Constructs—All DNA manipulations were performed according to established procedures (*30*). pET68-69 was generated by subcloning a ~1.45 kb *Bam*HI–*Xho*I fragment of pCR68-69 into the *Bam*HI and *Xho*I sites of pET30c. This expression construct contains the CSFV sequence (nt 1251 to 2701) coding for the C-terminal portion of the E^{rns} protein (aa 27 to 227), the entire E1 protein (aa 1 to 195), and the N-terminal portion of the E2 protein (aa 1 to 87), corresponding to aa 294 to 776 of the CSFV polyprotein. All deletion constructs were created by amplification of a desired CFSV region from pCR68-69 using PCR as described above with oligonucleotide primers listed in Table 1 and cloning into pET30a or pET30c at either the *Bam*HI and *Xho*I sites or the *Hin*dIII and *Xho*I sites. These constructs, named according to the primers used in PCR, are: pET68-116, pET68-117, pET68-118, pET68-119, pET68-120, pET68-131, pET68-132, pET68-140, pET68-141, pET68-142, pET68-143, pET68-144, pET68-145, pET68-163, pET68-164, pET68-165, pET68-166, pET133-131, pET134-131, pET135-131, pET167-131, pET167-142, pET167-143, pET179-143, pET179-142, pET133-142, pET217-142, pET216-142, pET167-141, pET167-140, pET167-132, pET167-218, and pET133-218. The constructs were propagated in *E. coli* strain INVαF′ and introduced into *E. coli* BL21(DE3)pLysS for expression of deletion proteins. All constructs were sequenced using an automatic sequencer to ensure that the inserted gene segments were correct and in frame at the fusion point. Each recombinant protein has an N-terminal fusion of 49–58 residues, including a six-histidine tag, and an additional fusion of eight residues at the C-terminal end.

Expression of Truncated CSFV Proteins, SDS-PAGE and Western Blotting—*E. coli* strain BL21(DE3)pLysS cells harboring the expression constructs were induced to express recombinant proteins with 1 mM IPTG as described (*19*).Whole cell proteins from induced cells were analysed by SDS-PAGE and Western blotting. Briefly, SDS-PAGE was performed by the method described by Laemmli (*31*), using a 4% stacking gel and a

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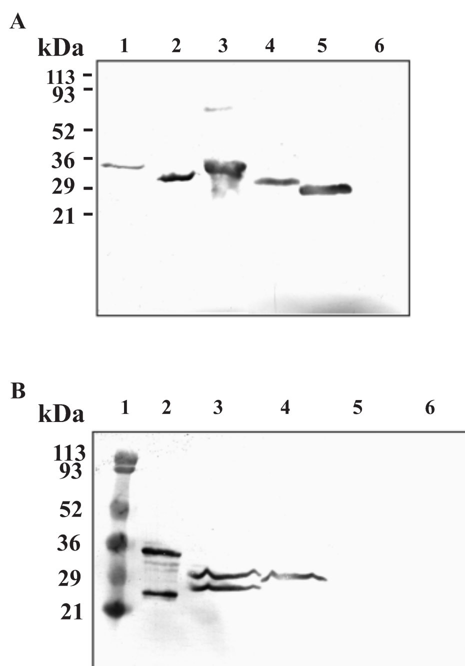

Fig. 2. **Western blot analysis of the products expressed from several selected C-terminal deletion constructs (A) and N-terminal deletion constructs (B).** The total proteins from the cells of each *E. coli* clone equivalent to 1 ml of culture with an A_{590} of 0.2 were analyzed by SDS-PAGE followed by Western blotting probed with an anti-CSFV serum (1:250 dilution) from an experimentally infected pig at 44 dpi. Panel A: Lane 1, pET68-131, expressing $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$; Lane 2, pET68-132, expressing $E^{rns}_{aa\,27-207}$; Lane 3, pET68-140, expressing $E^{rns}_{aa\,27-190}$; Lane 4, pET68-142, expressing $E^{rns}_{aa\,27-160}$; Lane 5, pET68-143, expressing $E^{rns}_{aa\,27-145}$; Lane 6, pET68-144, expressing $E^{rns}_{aa\,27-130}$. Panel B: Lane 1, molecular weight standards; Lane 2, pET68-131, expressing $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$; Lane 3, pET133-131, expressing $E^{rns}_{aa\,84-227}E1_{aa\,1-7}$; Lane 4, pET167-131, expressing $E^{rns}_{aa\,109-227}E1_{aa\,1-7}$; Lane 5, pET170-131, expressing $E^{rns}_{aa\,112-227}E1_{aa\,1-7}$; Lane 6, pET134-131, expressing $E^{rns}_{aa\,121-227}E1_{aa\,1-7}$. The numbers on the left side of the blots indicate the molecular mass of the standards in kilodaltons.

12% resolving gel in a Bio-Rad minigel apparatus (Bio-Rad, Mississauga, Ontario, Canada). The proteins were analyzed either through staining with Coomassie Blue or by use of Western blotting. For Western blots, the proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot SD semi-dry transfer cell. The blots were probed with mouse anti-histidine tag mAb or CSFV antisera from experimentally infected pigs (*19*). Bound antibodies were detected by using horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or HRP-conjugated rabbit anti–swine IgG antibodies (ICN Pharmaceuticals Inc., Aurora, OH) and a 4-chloro-1-naphthol-H_2O_2 substrate kit (Bio-Rad) according to the manufacturer's instructions.

RESULTS

Mapping of Antibody Binding Sites on E^{rns} by C-terminal deletions—The CSFV protein-coding sequence in pCR68-69 was used as a PCR template to generate a number of expression constructs with defined deletions from the C-terminal end (Fig. 1). The focus of this work is to define linear antigenic regions on E^{rns}. Western blots probed with anti-histidine monoclonal antibody (anti-His mAb) failed to detect the expression of protein fragments encoded by pET68-116, pET68-117 and pET68-118. All other constructs were found to produce recombinant proteins detected by anti-His mAb. Further deletions from the C-terminal end resulted in an increase in the level of protein expression for the protein fragments $E^{rns}_{aa\,27-227}E1_{aa\,1-136}$, $E^{rns}_{aa\,27-227}E1_{aa\,1-96}$, $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$, $E^{rns}_{aa\,27-207}$, $E^{rns}_{aa\,27-190}$, $E^{rns}_{aa\,27-175}$, $E^{rns}_{aa\,27-160}$, and $E^{rns}_{aa\,27-145}$. These fragments showed consistently strong reactions with the antiserum from a pig infected with CSFV. The reaction with the CSFV antiserum was essentially lost with a further deletion from the C-terminal end of $E^{rns}_{aa\,27-145}$. Fragments that did not react with the CSFV antiserum include $E^{rns}_{aa\,27-130}$, $E^{rns}_{aa\,27-123}$ and $E^{rns}_{aa\,27-115}$. Figure 2A presents the Western blot results summarizing the reaction of several C-terminally truncated proteins with the CSFV antiserum. The region of 15 aa residues deleted from $E^{rns}_{aa\,27-145}$ to $E^{rns}_{aa\,27-130}$ was investigated more closely through serial deletions of 3 aa residues at a time by constructing $E^{rns}_{aa\,27-142}$, $E^{rns}_{aa\,27-139}$ and $E^{rns}_{aa\,27-136}$. These three constructs expressed the protein fragments that were recognized by anti-His mAb and had an apparent molecular weight close to the predicted size, but none were detected by the CSFV antiserum on Western blots. Thus, the truncated E^{rns} retains the reactivity with the CSFV antiserum until its C-terminal end is deleted beyond Ser^{145}. The whole-cell protein samples provided convenient internal negative controls for Western blots, as the non-specific binding of the antisera or monoclonal antibodies to irrelevant proteins (*i.e.*, *E. coli* proteins) was seen to be negligible compared to the binding to recombinant protein products.

Mapping of Antibody Binding Sites on E^{rns} by N-Terminal Deletion—As C-terminally deleted proteins such as $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$ appeared to be expressed more efficiently in *E. coli* cells than the larger fragments, N-terminal deletions were performed to generate various protein fragments containing the same C-terminal end as $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$ (Fig. 1). This C-terminal end was chosen because the antibody-reactive region had been shown to be located upstream; thus the amino acid sequences downstream could be deleted with confidence that an intact antigenic region is maintained. Sequences of 12 to 47 residues were serially deleted from the N-terminus, resulting in four protein fragments, $E^{rns}_{aa\,84-227}E1_{aa\,1-7}$, $E^{rns}_{aa\,109-227}E1_{aa\,1-7}$, $E^{rns}_{aa\,121-227}E1_{aa\,1-7}$ and $E^{rns}_{aa\,161-227}E1_{aa\,1-7}$. The reactivity with the CSFV antiserum was retained in the former two fragments and clearly lost in the latter two. The area between the N-terminal ends of $E^{rns}_{aa\,109-227}E1_{aa\,1-7}$ and $E^{rns}_{aa\,121-227}E1_{aa\,1-7}$ was then more closely examined through serial deletions of 3 aa residues at a time to produce three protein fragments, $E^{rns}_{aa\,112-227}E1_{aa\,1-7}$, $E^{rns}_{aa\,115-227}E1_{aa\,1-7}$ and $E^{rns}_{aa\,118-227}E1_{aa\,1-7}$. None of these proteins was found to react significantly with the CSFV antiserum. Figure 2B shows an example of Western blotting results for the reactivity of several N-terminally truncated proteins with the CSFV antiserum. It was noted that the $E^{rns}_{aa\,27-227}E1_{aa\,1-7}$ and $E^{rns}_{aa\,84-227}E1_{aa\,1-7}$ preparations contained additional smaller immunoreactive protein bands (Fig. 2B), which may have resulted

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from proteolytic degradation. These deletion experiments indicate that a N-terminally truncated E^{rns} retains its reactivity with the CSFV antiserum until a deletion exceeds Glu^{109}.

Expression and Antigenicity of Recombinant Proteins Containing the Consensus Sequence Defined by Antibody-Reactive E^{rns} Fragments—N- and C-terminal deletion experiments suggest that a 37 aa common sequence present in all the immunoreactive fragments (Fig. 1) between Glu^{109} and Ser^{145} of E^{rns} constitutes a major antigenic region. The common sequence, embedded in the protein fragment $E^{rns}_{aa\ 109–145}$ and in a slightly larger fragment $E^{rns}_{aa\ 109–160,}$ was expressed from pET167–143 and pET167–142 respectively and tested for reactivity with the CSFV antiserum. Both polypeptides were expressed in abundance, as evidenced by the strong reaction to anti-His mAb; surprisingly, neither protein reacted with the CSFV antiserum (Fig. 3, A and B). When both $E^{rns}_{aa\ 109–145}$ and $E^{rns}_{aa\ 109–160}$ were further analyzed by Western blots probed with 16 different CSFV antisera, a similar result was noted. An example of a Western blot probed with one of the 16 different CSFV antisera is shown in Fig. 3C; the antiserum reacted with a larger E^{rns} fragment, $E^{rns}_{aa\ 27–207}$, and a truncated E2 (*19*) but not with $E^{rns}_{aa\ 109–160}$.

Localization of Overlapping Antigenic Regions on E^{rns}—The findings that $E^{rns}_{aa\ 109–145}$ did not react with the CSFV antiserum while $E^{rns}_{aa\ 109–227}E1_{aa\ 1–7}$, a larger protein fragment containing the same N-terminus as the former (Fig. 1), reacted well with a number of different CSFV pig antisera (data not shown) indicate that the addition of a 88-aa CSFV sequence to the C-terminus of $E^{rns}_{aa\ 109–145}$ can restore the reactivity of the protein with the CSFV antisera. To determine the minimum C-terminal addition required for the restoration of antigenicity, the C-terminal end was extended from $E^{rns}_{aa\ 109–145}$ by 13–17 residues at a time. Five such protein fragments, $E^{rns}_{aa\ 109–160}$, $E^{rns}_{aa\ 109–175,}$ $E^{rns}_{aa\ 109–190}$, $E^{rns}_{aa\ 109–207}$ and $E^{rns}_{aa\ 109–220}$, were expressed from pET167-142, pET167-141, pET167-140, pET167-132 and pET167-218 (Fig. 4), respectively, and tested for reactivity with a CSFV antiserum on Western blots. The antigenic property was restored only with $E^{rns}_{aa\ 109–220}$, which reacted with a similar intensity to the larger fragment $E^{rns}_{aa\ 109–227}E1_{aa\ 1–7}$ (Fig. 5A). Another finding that $E^{rns}_{aa\ 27–145}$, with the same C-terminus as $E^{rns}_{aa\ 109–145}$, reacted well with the CSFV antiserum (Fig. 1) indicates that the antigenicity of $E^{rns}_{aa\ 109–145}$ can be restored by adding a 82-aa CSFV sequence at its N-terminal end. Four proteins, $E^{rns}_{aa\ 94–145}$, $E^{rns}_{aa\ 84–145}$, $E^{rns}_{aa\ 65–145}$, and $E^{rns}_{aa\ 46–145}$, were expressed from pET179-143, pET133-143, pET217-143, and pET216-143 (Fig. 4), respectively, and analyzed with a pig CSFV antiserum to determine the minimum N-terminal addition required for the restoration of antigenicity. The immunological reactivity was achieved with $E^{rns}_{aa\ 65–145}$, and $E^{rns}_{aa\ 46–145}$ (Fig. 5B). Similar N-terminal addition experiments were conducted with $E^{rns}_{aa\ 109–160}$. Four protein fragments, $E^{rns}_{aa\ 94–160}$, $E^{rns}_{aa\ 84–160}$, $E^{rns}_{aa\ 65–160}$, and $E^{rns}_{aa\ 46–160}$, were expressed from pET179-142, pET133-142, pET217-142, and pET216-142 (Fig. 4), respectively; all the proteins but $E^{rns}_{aa\ 94–160}$ reacted with the anti-CSFV serum (Fig. 5C). These experiments have identified three overlapping antigenic regions in E^{rns} which span 156 residues: antigenic region 1 (AR1) covering Asn^{65}–Ser^{145}, AR2 encompassing Trp^{84}–Ser^{160}, and AR3 spanning Glu^{109}–Lys^{220} (Fig. 4). Individually, the three regions react similarly with CSFV antisera (Fig. 5). In contrast, the fragment $E^{rns}_{aa\ 94–175}$, which is located within this 156-aa region yet does not contain the complete sequence of any region, showed no reaction with the CSFV antiserum (Fig. 4).

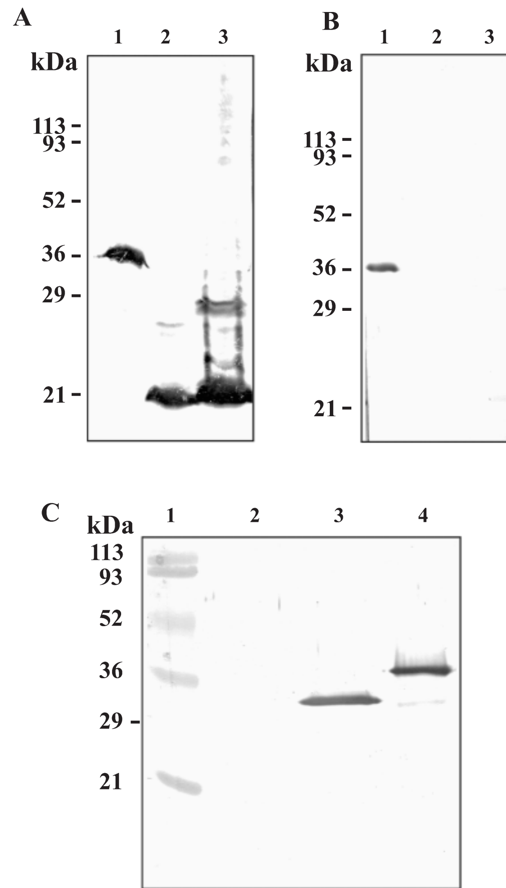

Fig. 3. **Western blot analysis of the two recombinant proteins containing the consensus region defined by immunoreactive E^{rns} fragments.** The total proteins from the cells of each *E. coli* clone (pET167-143 and pET167-142) equivalent to 1 ml of culture with an A_{590} of 0.2 were analyzed by SDS-PAGE followed by Western blotting probed with anti-His mAb at 0.05 μg/ml (A) and with an anti-CSFV serum (B) at 1:250 dilution from an experimentally infected pig at 44 dpi. The $E^{rns}_{aa\ 109–160}$ expressed from pET167-142 was also analyzed with 16 different antisera from experimentally CSFV-infected pigs. An example of a Western blot probed with one of the 16 different CSFV antisera (C) is shown, i.e. the CSFV antiserum from an experimentally infected pig at 166 dpi. Panels A and B: Lane 1, purified truncated E2 (0.5 μg), expressed from pETE2AB as a positive control (*19*); Lane 2, $E^{rns}_{aa\ 109–145}$, expressed from pET167-143; Lane 3, $E^{rns}_{aa\ 109–160}$, expressed from pET167-142. Panel C: Lane 1, molecular weight standards; Lane 2, $E^{rns}_{aa\ 109–160}$, expressed from pET167-142; Lane 3, $E^{rns}_{aa\ 27–207}$, expressed from pET68-132; Lane 4, purified truncated E2 (0.5 μg), expressed from pETE2AB as a positive control (*19*). The numbers on the left side of the blots indicate the molecular mass of the standards in kilodaltons.

Immunological Characterization of the E^{rns} Fragments Containing Overlapping Antigenic Regions—The protein $E^{rns}_{aa\ 65–160}$, expressed from pET217-142, reacts consist-

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Construct	Encoded Polypeptide	Reactivity with pig anti-CSFV serum
pET68-142	$E^{rns}_{aa27-160}$	+
pET216-142	$E^{rns}_{aa46-160}$	+
pET217-142	$E^{rns}_{aa65-160}$	+
pET133-142	$E^{rns}_{aa84-160}$	+
pET179-142	$E^{rns}_{aa94-160}$	–
pET167-142	$E^{rns}_{aa109-160}$	–
pET68-143	$E^{rns}_{aa27-145}$	+
pET216-143	$E^{rns}_{aa46-145}$	+
pET217-143	$E^{rns}_{aa65-145}$	+
pET133-143	$E^{rns}_{aa84-145}$	–
pET179-143	$E^{rns}_{aa94-145}$	–
pET167-143	$E^{rns}_{aa109-145}$	–
pET167-141	$E^{rns}_{aa109-175}$	–
pET167-140	$E^{rns}_{aa109-190}$	–
pET167-132	$E^{rns}_{aa109-207}$	–
pET167-218	$E^{rns}_{aa109-220}$	+
pET167-131	$E^{rns}_{aa109-227}E1_{aa1-7}$	+
pET179-141	$E^{rns}_{aa94-175}$	–

Fig. 4. **Fine mapping of overlapping antigenic regions on E^{rns} with deletion constructs.** The encoded protein products were analyzed by Western blots probed with the antiserum from an experimentally CSFV-infected pig at 44 dpi, and the results are indicated by + (specific reaction showing bands of similar intensity) and – (reaction not detected). The three overlapping antigenic regions are boxed and the consensus region shaded. Deleted residues are indicated by a thin line.

ently with various CSFV antisera (Figs. 5C and 6). As this polypeptide spans the complete sequences of AR1 and 2, it was of interest to determine how the other combinations of regions would react with various anti-CSFV sera. The protein fragment $E^{rns}_{aa\ 84–220}$ was produced from the construct pET133-218 to cover AR2 and 3, and was found to react in a consistent manner with 14 out of 16 different pig anti-CSFV sera, representing a range of animals and days post-infection. One such Western blot is presented in Fig. 6. Similarly, the protein $E^{rns}_{aa\ 65–220}$, expressed from pET217-218 and containing the complete sequences of AR1, 2 and 3, reacted strongly with various CSFV antisera (Fig. 6).

DISCUSSION

This study reports the use of established recombinant DNA techniques to dissect the CSFV structural protein E^{rns} in order to define the protein antigenic architecture responsible for eliciting antibody responses during the virus infection. A strategy that had successfully been used for the definition of a minimal antigenic region on the CSFV E2 protein (*19*) was applied to the construction of various deletions of the CSFV E^{rns}. Based on the reactivity of various E^{rns} deletion mutants (Fig. 1) with a pig anti-CSFV antiserum, a consensus sequence of 37 residues (Glu^{109}–Ser^{145}) on E^{rns} was identified. Surprisingly, an E^{rns} fragment consisting of the consensus sequence showed no reaction with the antiserum. Possible explanations for this are that the consensus sequence may contain only a partial antigenic region or that it may be an antigenic region containing conformational epitopes. A proposed 3D model of E^{rns} (*5*) predicts this cysteine-rich consensus region to be on a large surface-exposed loop between helix 6 and helix 7. The likelihood of two disulfide bonds existing in this region when the protein is in its native form favors the probability of this sequence containing a conformational epitope. By adding a minimum sequence of E^{rns} to either the N- or C-terminal end of the consensus sequence, the reactivity of E^{rns} fragments with the CSFV antiserum was successfully restored. This has led to the first identification of three overlapping linear antigenic regions on E^{rns}: AR1 (Asn^{65}–Ser^{145}), AR2 (Trp^{84}–Ser^{160}) and AR3 (Glu^{109}–Lys^{220}). These are the minimal antigenic regions required for binding pig anti-CSFV antibodies, since small N- or C-terminal deletions introduced into the regions disrupt their reactivity with the pig anti-CSFV antiserum (Figs. 1 and Fig. 4). Such deletions include ^{109}ECA^{111} and ^{143}NFS^{145}. Reactivity of various E^{rns} fragments including AR1, AR2 and AR3 with a number of sera serially collected from CSFV-infected pigs was also demonstrated by enzyme-linked immunosorbent assay (ELISA) and further confirmed the significance of the current findings (*32*). Recently Langedijk *et al.* (*18*), on the basis of probable independent folding and good exposure in the modular structure of E^{rns},

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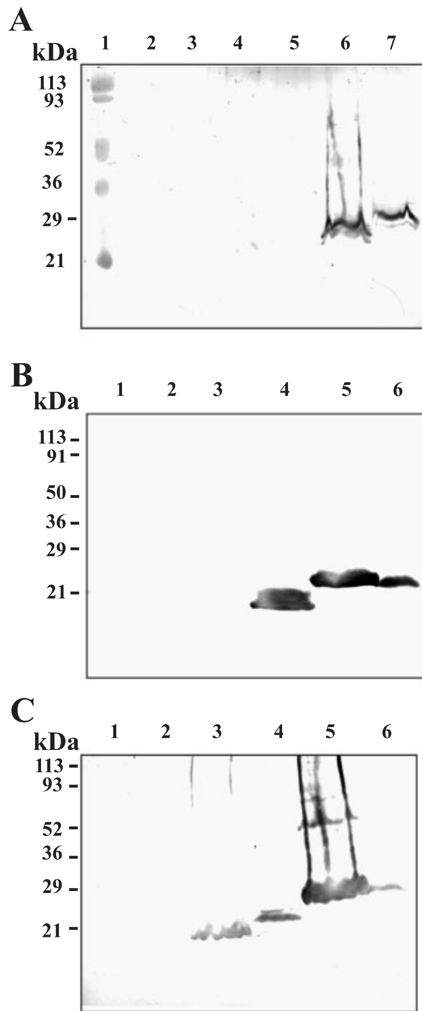

Fig. 5. **Restoration of the immunological reactivity of $E^{rns}_{aa\ 109–145}$ by C-terminal expansion (A) and by N-terminal expansion (B), and $E^{rns}_{aa\ 109–160}$ by N-terminal expansion (C).** The total proteins from the cells of each *E. coli* clone equivalent to 1 ml of culture with an A_{590} of 0.2 were analyzed by SDS-PAGE followed by Western blotting probed with an anti-CSFV serum (1:250 dilution) from an experimentally infected pig at 44 dpi. Panel A: Lane 1, molecular weight standards; Lane 2, $E^{rns}_{aa\ 109–160}$, expressed from pET167-142; Lane 3, $E^{rns}_{aa\ 109–175,}$ expressed from pET167-141; Lane 4, $E^{rns}_{aa\ 109–190}$, expressed from pET167-140; Lane 5, $E^{rns}_{aa\ 109–207}$, expressed from pET167-132; Lane 6, $E^{rns}_{aa\ 109–220}$, expressed from pET167-218, Lane 7, $E^{rns}_{aa\ 109–227}E1_{aa1–7}$, expressed from pET167-131. Panel B: Lane 1, $E^{rns}_{aa\ 109–145}$, expressed from pET167-143; Lane 2, $E^{rns}_{aa\ 94–145}$, expressed from pET179-143; Lane 3, $E^{rns}_{aa\ 84–145}$, expressed from pET133-143; Lane 4, $E^{rns}_{aa\ 65–145}$, expressed from pET217-143; Lane 5, $E^{rns}_{aa\ 46–145}$, expressed from pET216-143; Lane 6, $E^{rns}_{aa\ 27–145}$, expressed from pET68-143. Panel C: Lane 1, $E^{rns}_{aa\ 109–160}$, expressed from pET167-142; Lane 2, $E^{rns}_{aa\ 94–160}$, expressed from pET179-142; Lane 3, $E^{rns}_{aa\ 84–160}$, expressed from pET133–142; Lane 4, $E^{rns}_{aa\ 65–160}$, expressed from pET217–142; Lane 5, $E^{rns}_{aa\ 46–160}$, expressed from pET216-142; Lane 6, $E^{rns}_{aa\ 27–160}$, expressed from pET68-142. The numbers on the left side of the blots indicate the molecular mass of the standards in kilodaltons.

identified a 37 aa C-terminal region (aa 191–227), which was recognized by antibodies elicited by pestivirus infections in pigs. An ELISA, based on a peptide containing the C-terminal 37 residues, was developed for the serological diagnosis of CSFV infection. The three antigenic

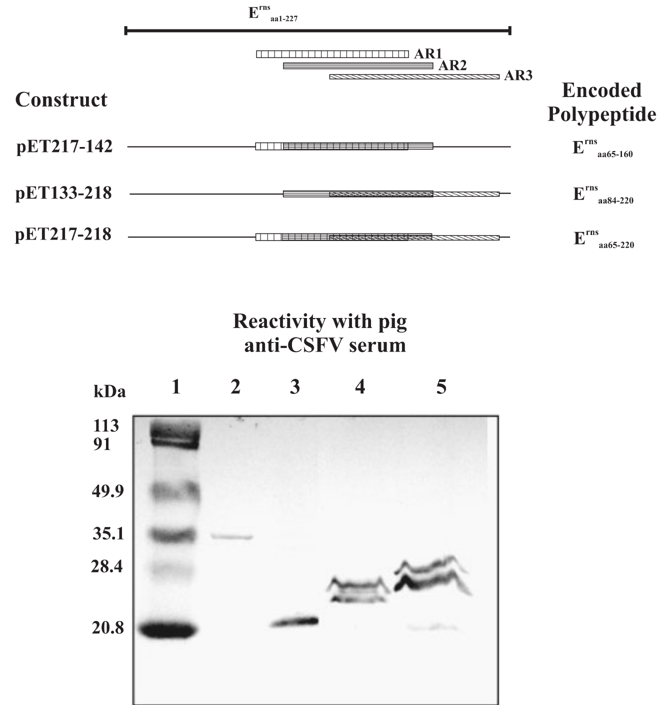

Fig. 6. **Detection of the $E^{rns}_{aa\ 65–160}$ fragment containing AR1 and AR2, the $E^{rns}_{aa\ 84–220}$ fragment containing AR2 and AR3, and the $E^{rns}_{aa\ 65–220}$ fragment containing AR1, AR2 and AR3 with antisera from experimentally CSFV-infected pigs.** The total proteins from the cells of each *E. coli* clone equivalent to 1 ml of culture with an A_{590} of 0.2 were analyzed by SDS-PAGE followed by Western blotting probed with various CSFV antisera. Here is shown one example of a Western blot probed with CSFV antiserum from an experimentally infected pig at 56 dpi. Lane 1, molecular weight standards; Lane 2, purified truncated E2 (0.5 µg), expressed from pETE2AB (*19*) as a positive control; Lane 3, $E^{rns}_{aa\ 65–160}$, expressed from pET217-142; Lane 4, $E^{rns}_{aa\ 84–220}$, expressed from pET133-218; Lane 5, $E^{rns}_{aa\ 65–220}$, expressed from pET217-218. The location of AR1, AR2 and AR3 and the expression constructs are shown above the Western blot.

regions identified here are at least twofold larger than the C-terminal regions of 37 residues reported by Langedijk *et al.* (*18*). These regions are unique in that AR1 and AR2 are located in the central region of E^{rns}, completely separated from the C-terminal antigenic region (aa 191–227). AR3 is located in the C-terminal half of the protein and overlaps with the C-terminal antigenic region (aa 191–227) by 30 residues. Preliminary data (not shown here) demonstrated that the $E^{rns}_{aa\ 27–190}$ fragment containing two overlapping antigenic regions and excluding the C-terminal region (aa 191–227) of Langedijk *et al.* (*18*) reacted on Western blots with 24 of 31 different pig anti-CSFV sera, representing a range of animals and days post infection. This indicates that at least two of the antigenic regions identified are novel and distinct from previously published B-cell epitopes. Interestingly, the consensus region of the three antigenic regions contained one complete T-cell epitope and one partial T-cell epitope sequence described previously (*33*). The overlap of B- and T-cell epitopes in the same protein region has been demonstrated for other viral antigens (*34*) and the CSFV E2 protein (*33*, *35*).

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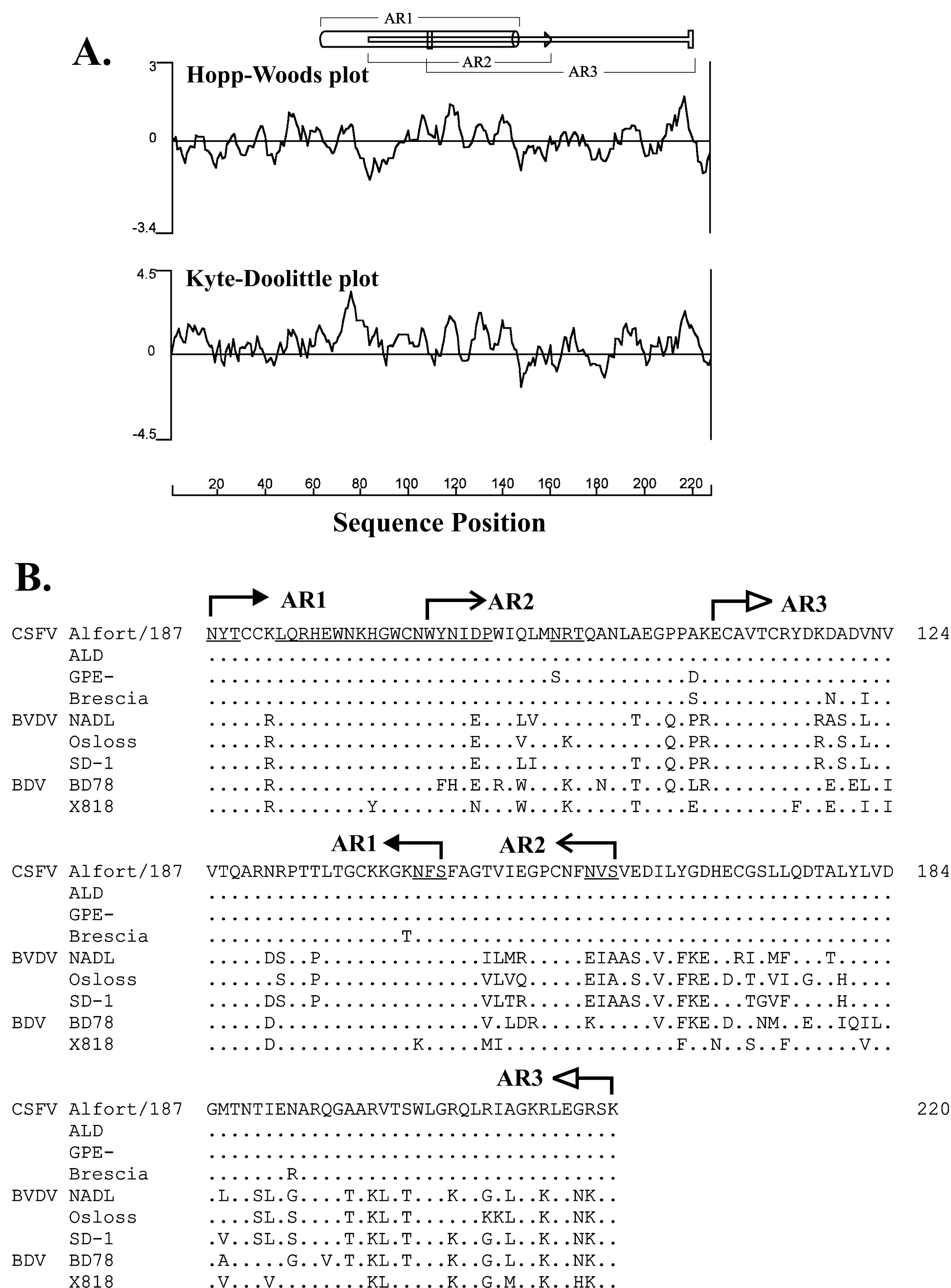

Fig. 7. **Hydropathy profiles for the CSFV Alfort/187 E^{rns} protein (A) and comparison of the CSFV Alfort/187 E^{rns} sequence containing the three overlapping antigenic regions with the corresponding regions from other strains of pestiviruses (B).** Both Hopp-Woods and Kyte-Doolittle plots were generated using a scanning window size of 8 amino acids. Bars above the profiles mark the location of three identified overlapping antigenic regions AR1 ($E^{rns}_{aa\ 65–145}$), AR2 ($E^{rns}_{aa\ 84–160}$) and AR3 ($E^{rns}_{aa\ 109–220}$). The E^{rns} sequences are from CSFV strains Alfort/187 (*29*) (GenBank accession No. X87939), ALD (*39*) (GenBank accession No. D49532), GPE- (*39*) (GenBank accession No. D49533), and Brescia (*40*) (GenBank accession No. M31768); BVDV strains NADL (*41*), Osloss (*42*) (GenBank accession No. M96687), and SD-1 (*43*) (GenBank accession No. M96751); and BDV strains BD78 (*44*) (GenBank accession No. U18330), and X818 (*45*) (GenBank accession No. AF037405). Amino acid residues that match exactly those of the CSFV Alfort/187 E^{rns} are represented as dots. The three identified overlapping antigenic regions AR1 ($E^{rns}_{aa\ 65–145}$), AR2 ($E^{rns}_{aa\ 84–160}$) and AR3 ($E^{rns}_{aa\ 109–220}$) are indicated by arrows. Potential *N*-linked glycosylation sites N-X-S/T and one of the two E^{rns} RNase active-sites are underlined.

It is interesting to note that several protein fragments ($E^{rns}_{aa\ 112–227}E1_{aa\ 1–7}$, $E^{rns}_{aa\ 115–227}E1_{aa\ 1–7}$, $E^{rns}_{aa\ 118–227}E1_{aa\ 1–7}$, $E^{rns}_{aa\ 121–227}E1_{aa\ 1–7}$, and $E^{rns}_{aa\ 161–227}E1_{aa\ 1–7}$) containing the C-terminal antigenic region (37 residues) were incapable of reacting with the pig anti-CSFV serum (Fig. 1). This result contrasts with the finding of Langedijk *et al.* (*18*). One possible explanation for this discrepancy is the presence of conformational epitope(s) in the C-terminal antigenic region (37 residues), which is destroyed under the denaturing conditions of SDS-PAGE used in the present

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study. To further investigate this possibility, $E^{rns}{}_{aa\ 121–227}$ $E1_{aa\ 1–7}$, a protein fragment containing the C-terminal antigenic region (37 residues), was purified by eluting from a Ni-agarose column under non-denaturing conditions as described (*19*) and used in ELISA to detect serum antibodies from CSFV-infected pigs. The protein fragment immobilized on an ELISA plate is capable of reacting with anti-CSFV antisera (*32*). This result provides experimental evidence for the presence of conformational epitope(s) in the C-terminal antigenic region (37 residues).

Antigenic sites are typically located in the areas of greatest local hydrophilicity. The hydropathy profiles of E^{rns} were constructed by the methods of Kyte-Doolittle (*36*) and by the approach of Hopp-Woods (*37*) to inspect how well the three identified antigenic regions correlate with the local hydrophilicity of the protein (Fig. 7A). Both methods predict a consensus hydrophilic area in the central part of the protein where two of the three regions are located. The third region has its two ends situated in highly hydrophilic areas with the central part in a less hydrophilic area. Thus, the three antigenic regions identified by the experimental approaches are consistent with the E^{rns} hydropathy profiles. The recent 3D model of E^{rns} (*5*), built based on disulfide bond connectivity and homology modeling, reveals some surface-exposed structures or areas within the three identified overlapping antigenic regions, which are consistent with the present experimental data and may represent the interface of antibody binding.

Examination of the three overlapping antigenic regions reveals the presence of two to three potential *N*-linked glycosylation sites in each antigenic region (Fig. 7B). This implies that the three overlapping regions are important immunogenic sites. However, the experimental data has demonstrated that the binding of expressed regions to pig anti-CSFV antibodies appears to be independent of glycosylation, as *E. coli* does not have glycosylation capability. Also the C-terminal antigenic region (37 residues) contains no glycosylation sites but reacts with CSFV antisera (*18*). Thus, the *E. coli* expression system is appropriate for defining E^{rns} antigenic regions that will help design small peptide antigens necessary for the development of diagnostic tests such as fluorescence polarization assay (*38*). The biological function of E^{rns} ribonuclease activity is not yet understood. Mutations in E^{rns} eliminating the RNase activity give rise to viruses that are more cytopathic in culture and are attenuated *in vivo* (*14*, *15*). Antibodies that inhibit ribonuclease activity also tend to neutralize virus infectivity (*7*). AR1 contains one complete RNase active site (*12*), which partially overlaps with AR2. It would be interesting to see if antibodies directed against this active site are neutralizing, as this would provide further evidence at the structural level of a correlation between the antibody inhibitory effect on E^{rns} RNase activity and the antibody neutralization of virus infectivity.

The study has successfully identified three unique overlapping regions on E^{rns}, which are targeted by antibodies during CSFV infection. These regions may thus have the potential for use in diagnostic applications as shown in a recent study (*32*). Comparison of the sequences accommodating the three overlapping antigenic regions from different strains of pestiviruses (*29*, *39–45*) (Fig. 7B) reveals that (i) these antigentic regions are highly conserved among CSFV strains; (ii) AR1 is more conserved in both terminal regions among the strains of pestiviruses with a variable central region; (iii) AR2 is variable in both terminal regions with a conserved stretch near the C-terminal end among the strains of pestiviruses; and (iv) AR3 is variable in the C-terminal region among the strains of petstiviruses with a relatively conserved N-terminal end. This suggests that while AR1 is less specific and may be capable of detecting a pestivirus infection, AR2 and AR3 may prove to be more specific for the detection of CSFV infection.

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REFERENCES

1. Moennig, V. (1988) Characteristics of the virus in *Classical Swine Fever and Related Viral Infections* (Liess, B., ed.), pp. 55–58, Martinus Nijhoff Publishing, Boston, MA
2. Wengler, G. (1991) Classification and nomenclature of viruses. Fifth report of the international committee on taxonomy of viruses. *Arch. Virol. Suppl.* **2**, 223–233
3. Rice, M.C. and Lindenbach, B.D. (2001) Flaviviridae: the viruses and their replication in *Fields Virology* (Knipe, D.M. and Howley, P.M., eds.) pp. 991–1041, Lippincott Williams & Wilkins, Philadelphia, PA
4. Thiel, H.J., Stark, R., Weiland, E., Rumenapf, T., and Meyers, G. (1991) Hog cholera virus: molecular composition of virions from a pestivirus. *J. Virol.* **65**, 4705–4712
5. Langedijk, J.P., van Veelen, P.A., Schaaper, W.M., de Ru, A.H., Meloen, R.H., and Hulst, M.M. (2002) A structural model of pestivirus E^{rns} based on disulfide bond connectivity and homology modeling reveals an extremely rare vicinal disulfide. *J. Virol.* **76**, 10383–10392
6. Rumenapf, T., Unger, G., Strauss, J.H., and Thiel, H.J. (1993) Processing of the envelope glycoproteins of pestiviruses. *J. Virol.* **67**, 3288–3294
7. Windisch, J.M., Schneider, R., Stark, R., Weiland, E., Meyers, G., and Thiel, H.J. (1996) RNase of classical swine fever virus: biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. *J. Virol.* **70**, 352–358
8. Langedijk, J.P. (2002) Translocation activity of C-terminal domain of pestivirus Erns and ribotoxin L3 loop. *J. Biol. Chem.* **277**, 5308–5314
9. Hulst, M.M. and Moormann, R.J. (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., van Gennip, H.G., and Moormann, R.J. (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
11. Hulst, M.M., van Gennip, H.G., Vlot, A.C., Schooten, E., de Smit, A.J., and Moormann, R.J. (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
12. Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E., and Thiel, H.J. (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* **261**, 1169–1171

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13. Hulst, M.M., Himes, G., Newbigin, E., and Moormann, R.J. (1994) Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. *Virology* **200**, 558–565
14. Hulst, M.M., Panoto, F.E., Hoekman, A., van Gennip, H.G., and Moormann, R.J. (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
15. Meyers, G., Saalmuller, A., and Buttner, M. (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
16. Bruschke, C.J., Hulst, M.M., Moormann, R.J., van Rijn, P.A., and van Oirschot, J.T. (1997) Glycoprotein E^{rns} of pestiviruses induces apoptosis in lymphocytes of several species. *J. Virol.* **71**, 6692–6696
17. Schein , C.H. (1997) From housekeeper to microsurgeon: the diagnostic and theraputic potential of ribonucleases. *Nat. Biotechnol.* **15**, 529–536
18. Langedijk, J.P., Middel, W.G., Meloen, R.H., Kramps, J.A., and de Smit, J.A. (2001) Enzyme-linked immunosorbent assay using a virus type-specific peptide based on a subdomain of envelope protein E^{rns} for serologic diagnosis of pestivirus infections in swine. *J. Clin. Microbiol.* **39**, 906–912.
19. Lin, M., Lin, F., Mallory, M., and Clavijo, A. (2000) Deletions of structural glycoprotein E2 of classical swine fever virus strain alfort/187 resolve a linear epitope of monoclonal antibody WH303 and the minimal N-terminal domain essential for binding immunoglobulin G antibodies of a pig hyperimmune serum. *J. Virol.* **74**, 11619–11625
20. Moormann, R.J., Bouma, A., Kramps, J.A., Terpstra, C., and De Smit, H.J. (2000) Development of a classical swine fever subunit marker vaccine and companion diagnostic test. *Vet. Microbiol.* **73**, 209–219
21. Moser, C., Ruggli, N., Tratschin, J.D., and Hofmann, M.A. (1996) Detection of antibodies against classical swine fever virus in swine sera by indirect ELISA using recombinant envelope glycoprotein E2. *Vet. Microbiol.* **51**, 41–53
22. Weiland, E., Ahl, R., Stark, R., Weiland, F., and Thiel, H.J. (1992) A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. *J. Virol.* **66**, 3677–3682.
23. Weiland, E., Stark, R., Haas, B., Rumenapf, T., Meyers, G., and Thiel, H.J. (1990) Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. *J. Virol.* **64**, 3563–3569
24. Wensvoort, G. (1989) Topographical and functional mapping of epitopes on hog cholera virus with monoclonal antibodies. *J. Gen. Virol.* **70**, 2865–2876
25. Hulst, M.M., Westra, D.F., Wensvoort, G., and Moormann, R.J. (1993) Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. *J. Virol.* **67**, 5435–5442
26. Konig, M., Lengsfeld, T., Pauly, T., Stark, R., and Thiel, H.J. (1995) Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. *J. Virol.* **69**, 6479–6486
27. van Rijn, P.A., Bossers, A., Wensvoort, G., and Moormann, R.J. (1996) Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. *J. Gen. Virol.* **77**, 2737–2745
28. Clavijo, A., Zhou, E.M., Vydelingum, S., and Heckert, R. (1998) Development and evaluation of a novel antigen capture assay for the detection of classical swine fever virus antigens. *Vet. Microbiol.* **60**, 155–168
29. Ruggli, N., Moser, C., Mitchell, D., Hofmann, M., and Tratschin, J.D. (1995) Baculovirus expression and affinity purification of protein E2 of classical swine fever virus strain Alfort/187. *Virus Genes* **10**, 115–126
30. Sambrook, J. and Russel, D.W. (2000) *Molecular Cloning*. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
32. Lin, M., Trottier, E., and Pasick, J. (2005) Antibody responses of pigs to defined E^{rns} fragments after infection with classical swine fever virus . *Clin. Diagn. Lab. Immunol.* **12**, (in press)
33. Armengol, E., Wiesmuller, K.H., Wienhold, D., Buttner, M., Pfaff, E., Jung, G., and Saalmuller, A. (2002) Identification of T-cell epitopes in the structural and non-structural proteins of classical swine fever virus. *J. Gen. Virol.* **83**, 551–560
34. Rodriguez, A., Saiz, J.C., Novella, I.S., Andreu, D., and Sobrino, F. (1994) Antigenic specificity of porcine T cell response against foot-and-mouth disease virus structural proteins: identification of T helper epitopes in VP1. *Virology* **205**, 24–33
35. Yu, M., Wang, L.F., Shiell, B.J., Morrissy, C.J., and Westbury, H.A. (1996) Fine mapping of a C-terminal linear epitope highly conserved among the major envelope glycoprotein E2 (gp51 to gp54) of different pestiviruses. *Virology* **222**, 289–292
36. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132
37. Hopp, T.P. and Woods, K.R. (1983) A computer program for predicting protein antigenic determinants. *Mol. Immunol.* **20**, 483–489
38. Lin, M., Sugden, E.A., Jolley, M.E., and Stilwell, K. (1996) Modification of the Mycobacterium bovis extracellular protein MPB70 with fluorescein for rapid detection of specific serum antibodies by fluorescence polarization. *Clin. Diagn. Lab. Immunol.* **3**, 438–443
39. Ishikawa, K., Nagai, H., Katayama, K., Tsutsui, M., Tanabayashi, K., Takeuchi, K., Hishiyama, M., Saitoh, A., Takagi, M., Gotoh, K. *et al.* (1995) Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE- and the wild-type parental strain ALD. *Arch. Virol.* **140**, 1385–1391
40. Moormann, R.J., Warmerdam, P.A., van der Meer, B., and Hulst, M.M. (1990) Nucleotide sequence of hog cholera virus RNA: properties of the polyprotein encoded by the open reading frame spanning the viral genomic RNA. *Vet. Microbiol.* **23**, 185–191
41. Colett, M.S., Larson, R., Gold, C., Strick, D., Anderson, D.K., and Purchio, A.F. (1988) Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. *Virology* **165**, 191–199
42. De Moerlooze, L., Lecomte, C., Brown-Shimmer, S., Schmetz, D., Guiot, C., Vandenbergh, D., Allaer, D., Rossius, M., Chappuis, G., Dina, D. *et al.* (1993) Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in the 5′ untranslated region. *J. Gen. Virol.* **74**, 1433–1438
43. Deng, R. and Brock, K.V. (1992) Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. *Virology* **191**, 867–869
44. Sullivan, D.G., Chang, G.J., Trent, D.W., and Akkina, R.K. (1994) Nucleotide sequence analysis of the structural gene coding region of the pestivirus border disease virus. *Virus Res.* **33**, 219–228
45. Becher, P., Shannon, A.D., Tautz, N., and Thiel, H.J. (1994) Molecular characterization of border disease virus, a pestivirus from sheep. *Virology* **198**, 542–551

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