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Molecular Characterization of the 3′ Terminus of the Simian Hemorrhagic Fever Virus Genome

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The 3′ end of the simian hemorrhagic fever virus (SHFV) single-stranded RNA genome was cloned and sequenced. Adjacent to the 3′ poly(A) tract, we identified a 76-nucleotide noncoding region preceded by two overlapping reading frames (ORFs). The ultimate 3′ ORF of the viral genome encodes the capsid protein, and the penultimate ORF encodes the smallest SHFV envelope protein. These two ORFs overlap each other by 26 nucleotides. Northern (RNA) blot hybridization analyses of cytoplasmic RNA extracts from SHFV-infected MA-104 cells with gene-specific probes revealed the presence of full-length genomic RNA as well as six subgenomic SHFV-specific mRNA species. The subgenomic mRNAs are 3′ coterminal. In its virion morphology and size, genome structure and length, and replication strategy, SHFV is most similar to lactate dehydrogenase-elevating virus, equine arteritis virus, and porcine reproductive and respiratory syndrome virus.

Simian hemorrhagic fever virus (SHFV) causes a persistent infection in patas monkeys (*Erythrocebus patas*) with no overt disease symptoms (15, 21). In contrast, this virus induces a fatal hemorrhagic fever in monkeys within the genus *Macaca* (26). Death occurs 1 to 2 weeks after the onset of symptoms in macaque monkeys and is usually due to hypovolemic shock (26). Within an infected primate center colony, mortality rates of up to 100% in susceptible monkeys have been observed (21).

The SHF virion is enveloped and 45 to 50 nm in diameter. The nucleocapsid possesses icosahedral symmetry (35). The positive-sense, single-stranded RNA genome, which was estimated to be 15,000 nucleotides (nt) in length (28), contains a poly(A) tract at its 3′ terminus (29) and a type I cap at its 5′ terminus (30).

On the basis of virion morphology, SHFV was initially placed in the family *Togaviridae* (35). However, because this virus was shown to bud through the endoplasmic reticular membrane (35), as do the flaviviruses, and not through the plasma membrane of the cell, as do the togaviruses, SHFV was subsequently reclassified into the family *Flaviviridae* (39). This virus was later further classified into the hepatitis C virus group within the family *Flaviviridae* (38). In 1993, on the basis of our preliminary results (14), SHFV was reclassified as a member of the genus *Arterivirus* by the International Committee on the Taxonomy of Viruses at the International Congress of Virology in Glasgow, Scotland.

The genus *Arterivirus*, originally classified within the family *Togaviridae* but now free-standing, was created for equine arteritis virus (EAV) when it was discovered that EAV utilized six subgenomic mRNAs during its replication cycle (36, 40). In contrast, other togaviruses produce only a single subgenomic message during their replication cycle. When the EAV genome was sequenced, its genome organization was found to be most similar to that of the coronaviruses and the toroviruses (7). However, EAV differs from the coronaviruses and toroviruses in virion size and morphology as well as in genome length.

Recently, two viruses with RNA genomes closely related to that of EAV have been sequenced: Lelystad virus (LV), a European isolate of porcine reproductive and respiratory syndrome virus (PRRSV) (6, 22), and lactate dehydrogenase-elevating virus (LDV) (12), which causes persistent infections in mice. Both of these viruses are also morphologically similar to EAV and have been included in the genus *Arterivirus*. A study group has been formed by the International Committee on the Taxonomy of Viruses to debate the taxonomical classification of this new group of viruses.

As a first step in determining the molecular characteristics of SHFV, we cloned and sequenced the 3′ end of the SHFV RNA genome and examined the replication strategy of this virus. The evidence reported here demonstrates that SHFV is most similar to EAV, LDV, and LV/PRRSV in its genome organization and replication strategy.

To obtain purified virus as a source of genome RNA, MA-104 cells were infected at a multiplicity of infection of 0.2 with the prototype strain of SHFV, LVR 42-0/M6941. Tissue culture fluid was collected 24 h postinfection, clarified, and layered atop a 10 and 20% discontinuous glycerol gradient made in TNE (10 mM Tris [pH 8.0], 0.1 M NaCl, 1 mM EDTA). Virus was pelleted through the gradient at 24,500 rpm for 16 h. SHFV genomic RNA was purified by sedimentation through a 15 to 40% sodium dodecyl sulfate (SDS)-sucrose gradient after treatment of the virion pellet with SDS, pronase, and vanadyl ribonucleoside complex as previously described (2). Peak RNA fractions were pooled, ethanol precipitated, aliquoted, reprecipitated with ethanol, and stored at −70°C until use.

Because it had previously been demonstrated that SHFV contains a poly(A) tract at the 3′ terminus of its genome (29), oligodeoxythymidine was used to prime the viral genome for reverse transcription. The resulting cDNA was made double stranded with DNA polymerase by the method of Okayama and Berg (25) and inserted into the pUC18 plasmid vector (Pharmacia Biotec, Inc., Piscataway, N.J.), using *Eco*RI linkers. The plasmid DNA was amplified in *Escherichia coli* DH5α and purified by using a Miniprep Kit Plus (Pharmacia).

Northern (RNA) blot hybridization analysis (31) using purified, full-length SHFV genome RNA and radiolabeled cDNA probes synthesized from the cDNA clones revealed SHFV specificity of the clones (data not shown). Two clones were chosen and then sequenced by the dideoxy-chain termination

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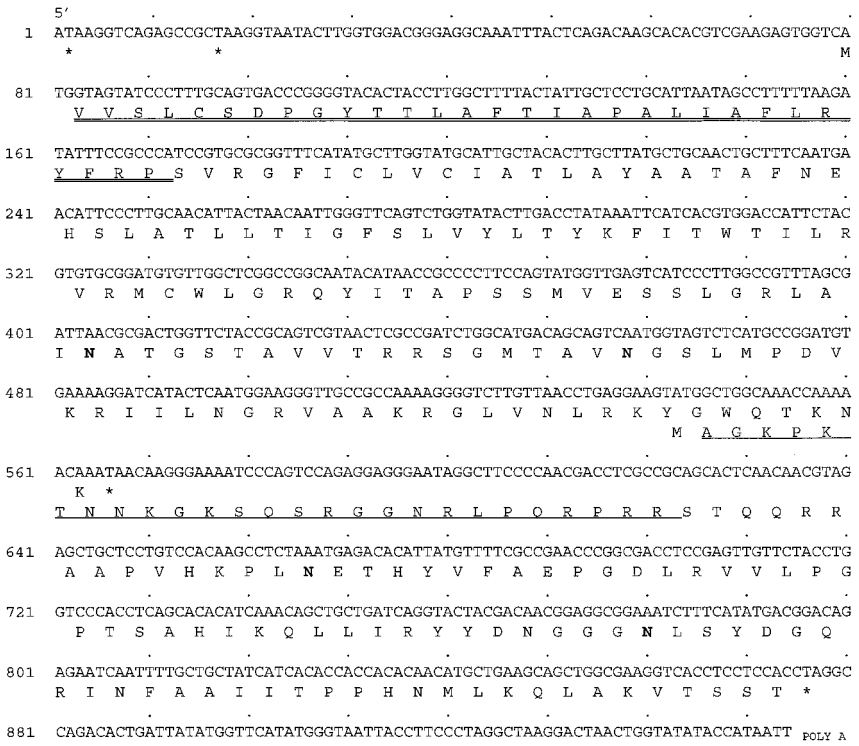

FIG. 1. Nucleotide sequence of the 3′ terminus of the SHFV genome. The amino acids encoded by the ORFs are shown below the nucleotide sequence. An asterisk indicates a termination codon. Amino acids which were also obtained by N-terminal amino acid sequencing of the SHFV p15 (single underline) and p20 (double underline) proteins are shown. Potential N-linked glycosylation sites are indicated in boldface.

method (32), using the Sequenase enzyme (United States Biochemical Corp., Cleveland, Ohio). The sequences obtained from these two clones were identical except that one clone contained an additional adenosine residue in the poly(A) tract relative to the other clone (12 versus 11 nt). Preceding the poly(A) tract, 806 unique 3′-terminal SHFV nucleotides were identified (Fig. 1). An additional 148 nt were obtained by direct sequencing of the SHFV genomic RNA, as previously described (12), using a primer complementary to nt 175 through 197 (Fig. 1). The amino acid sequences of the potential open reading frames (ORFs) encoded by the SHFV 3′ sequence were derived by using the University of Wisconsin Genetics Computer Group (GCG) software (9). No other reasonably sized ORFs were found in this sequence.

As shown in Fig. 1, there is a 76-nt noncoding region (NCR) adjacent to the poly(A) tract of the SHFV genome. The length of the SHFV 3′ NCR is similar to the lengths of the 3′ NCRs of EAV, LDV, and LV/PRRSV, which are 59 (7), 80 (12), and 114 (6, 22) nt, respectively. Using the GCG software GAP program (24) with a gap weight of 5.0 and a gap length weight of 0.3, we determined that the nucleotide identities between the SHFV 3′ NCR and the 3′ NCRs of EAV, LDV, and LV/PRRSV were only 37, 32, and 34%, respectively. The identity between the EAV and LV/PRRSV 3′ NCRs was 44%, while the identity in this region between EAV and LDV was 37%. The 3′ NCRs of LDV and LV/PRRSV were most similar, with a nucleotide identity of 47%.

It had previously been shown that the last 8 nt at the 3′ termini of the 3′ NCRs of SHFV, LDV, and LV/PRRSV were conserved, with a consensus sequence of 5′ CC(A/G)(T/G/A)AATT 3′ (12). The 3′-terminal nucleotides of the EAV genome (5′ CCAGGAACC 3′) were more divergent but did resemble the consensus sequence present in these other viruses. It has been proposed that this conserved 3′ sequence may function as a *cis*-acting signal for viral RNA replication (12).

Adjacent to the SHFV 3′ NCR is an ORF of 336 nt (Fig. 1) which encodes a very basic protein of 111 amino acids with a calculated molecular mass of 12.3 kDa and an estimated pI of 11.7 at neutral pH. Since the capsid protein genes of EAV, LDV, and LV/PRRSV each map to the ultimate 3′ ORFs of their respective genomes and since the capsid proteins of most RNA viruses are basic proteins, it seemed likely that the 3′ ORF of SHFV encodes the capsid protein.

To determine the map position of the capsid protein gene on the SHFV genome, we first identified the SHFV capsid protein among the viral structural proteins and then determined its N-terminal amino acid sequence. Extracellular SHFV that had been metabolically radiolabeled with a ^{14}C-amino acid mixture (DuPont-NEN Research Products, Boston, Mass.) was harvested 48 h after infection at a multiplicity of infection of 0.1 and partially purified by pelleting through a discontinuous glycerol gradient as described above. Pelleted virus was resuspended in Laemmli sample buffer (19), and the viral proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The gel was fixed, stained, and autoradiographed to visualize the viral protein bands. Four prominent SHFV protein bands were observed in the SHFV-containing lane of the gel by this procedure (Fig. 2). These proteins migrated with apparent molecular masses of 15, 20, 38 to 46, and 48 to 60 kDa and were designated p15, p20, p42, and p54, respectively (Fig. 2, lane A). Two additional protein bands with apparent molecular masses of 28 and 35 kDa were observed; however, preliminary Western blot (immunoblot) analyses sug-

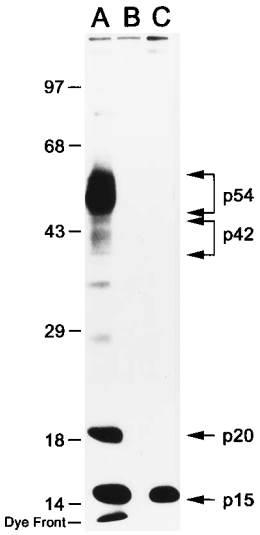

FIG. 2. Autoradiograph of ^{14}C-amino acid-labeled SHFV structural proteins. Proteins from extracellular SHFV particles (lane A), media obtained from mock-infected MA-104 cells (lane B), and viral nucleocapsids (lane C) were pelleted through a glycerol gradient and separated by SDS-PAGE. Lines indicate the positions (kilodaltons) of standard proteins, and arrows indicate possible SHFV structural protein bands.

gest that these two proteins may be cellular contaminants (data not shown and reference 11). The SDS-PAGE pattern for the SHFV proteins is similar to that previously reported for the LDV structural proteins, which have estimated molecular masses of 14, 18, and 24 to 44 kDa (3, 23), and the structural proteins of EAV, with estimated molecular masses of 14, 16, 25, and 30 to 42 kDa (10).

SHFV particles were next incubated with 1% Nonidet P-40, to remove the viral envelope, and the nucleocapsids were then pelleted. SDS-PAGE analysis of the nucleocapsids revealed a single protein, p15 (Fig. 2, lane C). These data indicated that p15 is the SHFV capsid protein and that p20, p42, and p54 are associated with the viral envelope.

To determine the N-terminal amino acid sequence of p15, the SHFV structural proteins were separated by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.), and stained as previously described (13). The capsid protein, p15, was excised from the membrane, and its amino-terminal sequence was determined by David W. Speicher of the Protein Microchemistry Facility at the Wistar Institute (Philadelphia, Pa.), using an Applied Biosystems model 475A protein sequencer as described previously (34). A 26-amino-acid sequence was obtained for the N terminus of the SHFV capsid protein; this amino acid sequence mapped to the 5′ end of the ultimate 3′ ORF of the SHFV genome (Fig. 1).

Analyzed with the GCG software GAP program with a gap weight of 3.0 and a gap length weight of 0.1, the SHFV capsid protein showed about the same degree of amino acid similarity to the capsid proteins of EAV (39%), LDV (39%), and LV/PRRSV (41%). Amino acid similarity between the capsid proteins of EAV and LDV was 47%, and that between EAV and LV was 42%. The capsid proteins of LDV and LV/PRRSV showed the greatest degree of amino acid similarity (57%).

The amino acid sequence of the SHFV capsid protein contains two potential N-linked glycosylation sites: one site is located in the middle of the capsid protein sequence, while the other site is located toward the C terminus of the protein (Fig. 1). The capsid protein sequence of LV/PRRSV contains one potential N-linked glycosylation site near the N terminus of the protein (6, 22), and the EAV capsid protein sequence contains no potential N-linked glycosylation sites (7). Although the capsid protein sequence of LDV contains three putative N-linked glycosylation sites located near the C terminus of the protein (12, 17), it has been reported that the mature capsid protein of LDV is not glycosylated (3). Recent attempts to metabolically radiolabel the sugar residues on SHF virion particles indicate that the capsid protein in mature virus particles is also not glycosylated (data not shown and reference 11).

Adjacent to the SHFV p15 ORF is an ORF of 489 nt, which overlaps the capsid protein ORF by 26 nt (Fig. 1). Overlapping ORFs are characteristic of the EAV, LDV, and LV/PRRSV genomes but are not found in the coronavirus, torovirus, togavirus, or flavivirus genomes. The second ORF encodes a very hydrophobic protein of 162 amino acids with a calculated mass of 17.8 kDa. A hydropathicity plot of this peptide, by the method of Kyte and Doolittle (18), and an estimation of the secondary structure, by the method of Chou and Fasman (5), suggest that three hydrophobic, potential membrane-spanning regions are present within the first 90 N-terminal amino acids of this sequence (data not shown). Proteins with similar triple membrane-spanning structures have been shown to be encoded by the 3′ penultimate ORFs in the genomes of LDV (12), EAV (7, 10), and LV/PRRSV (22). A similar protein structure has also been described for the M proteins of the coronaviruses (1, 8, 27).

To confirm that the smallest of the SHFV envelope proteins, p20, mapped to the penultimate 3′ ORF of the SHFV genome, the N-terminal sequence of this viral protein was determined by the method described above for the SHFV capsid protein. A 30-amino-acid sequence was obtained, and as shown in Fig. 1, this amino acid sequence mapped to the 5′ end of the penultimate 3′ SHFV ORF.

The amino acid similarities between the SHFV p20 sequence and the sequences of those proteins encoded by the penultimate ORFs of EAV, LDV, and LV/PRRSV were determined to be 48, 54, and 57%, respectively, using the GAP program with the constraints described above for the capsid protein comparisons. The amino acid sequence similarity between the EAV and LDV proteins was 44%, and that between the EAV and LV/PRRSV proteins was 47%. Surprisingly, a 70% similarity was observed between the LDV and LV/PRRSV proteins.

The protein sequence encoded by this second ORF contains two potential N-linked glycosylation sites (Fig. 1). Similarly, the peptide sequence encoded by this ORF in LV/PRRSV (6, 22) contains two putative N-linked glycosylation sites, whereas the corresponding peptide sequences encoded by EAV (7) and LDV (12) contain no potential N-linked glycosylation sites. Preliminary evidence indicates that the mature SHFV p20 protein is not glycosylated (data not shown and reference 11).

EAV, LDV, and LV/PRRSV express their 3′ genes from a 3′-coterminal set of subgenomic mRNAs during replication (17, 22, 36). To investigate the possibility that SHFV generates a similar set of subgenomic mRNAs, Northern blot hybridization analyses were performed on cytoplasmic RNA extracts obtained from SHFV-infected MA-104 cells. Cells were infected with SHFV at a multiplicity of infection of 10. After 7 h, cytoplasmic RNA was extracted as described by Sawicki et al. (33), denatured with glyoxal, separated on a 1% agarose gel, and transferred to a Magna nylon transfer membrane (Micron Separations, Inc., Westborough, Mass.) by standard techniques (31). The nylon membrane was baked at 80°C for 2 h and prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M

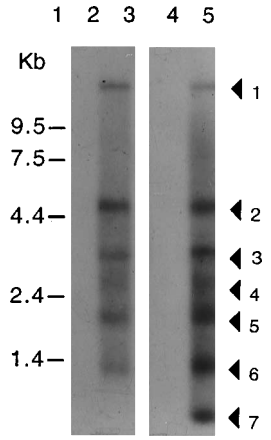

FIG. 3. Northern blot hybridization analyses of total cytoplasmic RNA from mock-infected (lanes 2 and 4) and SHFV-infected (lanes 3 and 5) MA-104 cells. The nylon membrane was incubated with a p20 probe (lanes 2 and 3) or with a 3′-terminus probe (lanes 4 and 5). Lane 1 shows the migration pattern of the RNA standards (Gibco-BRL, Gaithersburg, Md.), which were stained with ethidium bromide.

sodium citrate)–2 mM EDTA–0.01% SDS–200 μg of salmon sperm DNA per ml–50% formamide for 2 h. The RNA probe was added, and incubation continued at 65°C for 16 h. The membrane was washed three times with 0.1× SSC–0.1% SDS for 20 min at 65°C and then autoradiographed at −70°C.

The RNA probe was produced from a cloned PCR fragment template. A forward antisense primer complementary to two residues of the poly(A) tract and the last 18 nt in the 3′ NCR of the SHFV genome and a reverse genomic sense primer starting 47 nt from the 5′ end of the p15 ORF (Fig. 1) were used to generate a 367-nt PCR product from the purified virion RNA template. The PCR product was cloned into the pCRII vector (Invitrogen Corp., San Diego, Calif.) and transfected into *E. coli* DH5α. The recombinant 3′-pCRII plasmid DNA template was linearized with *Bam*HI, and RNA was transcribed in vitro with T7 RNA polymerase in the presence of 50 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The DNA template was digested with DNase after transcription, and the RNA was precipitated with ethanol. This antisense 3′ probe detected full-length viral RNA (15 kb) as well as six subgenomic mRNAs with estimated lengths of 4.7, 3.3, 2.7, 2.0, 1.2, and 0.65 kb in cytoplasmic extracts from SHFV-infected MA-104 cells (Fig. 3, lane 5). No SHFV-specific RNA was detected in uninfected cell extracts (Fig. 3, lane 4). The lengths of the SHFV subgenomic mRNAs are very similar to those of EAV (7), LDV (4), and LV/PRRSV (6, 22), except that the largest SHFV subgenomic mRNA is approximately 1.4 kb longer than the comparable subgenomic mRNAs produced by the other viruses. The detection of six SHFV subgenomic RNAs with the 3′ probe indicates that these mRNAs are 3′ coterminal.

Northern blot hybridization analysis was also performed with a probe specific for the p20 gene. The cloning strategy used to construct the DNA template for this probe and the method used for in vitro synthesis of this RNA probe were similar to those described above for the 3′ probe. The p20 RNA probe was 200 nt in length and started with the first 5′ nucleotide of the p20 ORF (Fig. 1). As shown in Fig. 3, lane 3, this probe hybridized to full-length SHFV genome RNA and to the five largest subgenomic mRNAs; the smallest subgenomic mRNA was not detected with this probe. The p20 probe also did not detect RNA in uninfected cell extracts (Fig. 3, lane 2). It has previously been shown that the smallest EAV subgenomic mRNA contains only the capsid protein ORF and that the EAV capsid protein is translated from that mRNA species (37). The evidence reported here indicates that the smallest SHFV subgenomic RNA (0.65 kb) also contains only the capsid protein ORF; therefore, p15 is likely to be translated from this subgenomic mRNA. It has been proposed for EAV (37) and the coronaviruses (20) that only the 5′ ORF on each subgenomic mRNA is translated. Accordingly, it is expected that p20 is translated from the 1.2-kb SHFV subgenomic RNA.

We report here molecular evidence which supports the recent reclassification of SHFV with EAV, LDV, and LV/PRRSV. First, the genome organization of SHFV is similar to that of EAV, LDV, and LV/PRRSV; the ultimate 3′-terminal ORF of the SHFV genome encodes the capsid protein, and the smallest envelope protein maps to the penultimate 3′ ORF. Second, the SHFV genome consists of multiple overlapping ORFs which, among the positive-sense RNA viruses with icosahedral nucleocapsids, is a characteristic unique to EAV, LDV, and LV/PRRSV. Third, SHFV produces genome-length RNA as well as six subgenomic mRNAs during replication. These subgenomic SHFV mRNAs are nested at the 3′ end of the genome. EAV (36) and LV/PRRSV (6, 22) each produce six subgenomic mRNAs during replication, while LDV produces seven subgenomic mRNAs (4).

EAV, LDV, and LV/PRRSV produce 5′ leader sequences which are derived from the 5′ termini of their respective genome RNAs. These leader sequences are joined to the mRNA bodies at conserved junction sequences, but the mechanism utilized to accomplish this is not well understood. The consensus junction sequences of LDV and LV/PRRSV are similar: 5′ U(A/G)(U/A)AACC 3′ for LDV (12, 16) and 5′ GNUNAACC 3′ for LV/PRRSV (6, 22). The consensus junction sequence of EAV, 5′ UCAAC 3′ (7), shows only some similarity to those of LDV and LV/PRRSV. In the smallest subgenomic mRNA species, the junction sequences of LDV, LV/PRRSV, and EAV are located 14 (12, 16), 17 (6, 22), and 55 (7) nt, respectively, upstream of the capsid protein AUG initiation codon. A sequence, 5′ UUAACC 3′, similar to the consensus junction sequences of LDV and LV/PRRSV is located 15 nt upstream of the p15 ORF of SHFV and functions as the junction sequence for the smallest SHFV subgenomic mRNA (41). Although the junction sequences for the penultimate 3′ ORFs of LDV, LV/PRRSV, and EAV are located 21 (12, 16), 32 (6, 22), and 31 (7) nt, respectively, upstream of the AUG initiation codon for these ORFs, the junction sequence, 5′ UCAACC 3′, preceding the SHFV p20 ORF is located 133 nt upstream of the initiation codon (41). This indicates that the 5′ NCR of the second-smallest SHFV subgenomic mRNA is longer than those of the corresponding subgenomic mRNAs produced by the other three viruses.

Phylogenetic analysis of conserved amino acid domains within the EAV, LDV, and LV/PRRSV RNA-dependent RNA polymerases and helicases, encoded within ORF 1b located in the central portion of their respective genomes, suggest that LV/PRRSV and LDV are more closely related to each other than either is to EAV (12). As presented in this study, amino acid sequence comparisons of both the capsid proteins and the smallest envelope proteins among these three viruses support these relationships. Although the percent amino acid similarities between the capsid protein of SHFV and that of either LV/PRRSV, LDV, and EAV do not differ significantly, amino acid sequence comparisons of the SHFV p20 with the corresponding protein sequences of these three viruses indicated a greater degree of similarity between SHFV

and LV/PRRSV or LDV than between SHFV and EAV. However, because p15 and p20 are structural proteins whose evolution may be influenced by immunologic selection as well as host species variation, the sequences of the conserved domains in the ORF 1b region of the SHFV genome must be determined before a meaningful phylogenetic analysis can be carried out.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U20522.

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