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Each of the eight simian hemorrhagic fever virus minor structural proteins is functionally important

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ABSTRACT

The simian hemorrhagic fever virus (SHFV) genome differs from those of other members of the family *Arterivirus* in encoding two adjacent sets of four minor structural protein open reading frames (ORFs). A stable, full-length, infectious SHFV-LVR cDNA clone was constructed. Virus produced from this clone had replication characteristics similar to those of the parental virus. A subgenomic mRNA was identified for the SHFV ORF previously identified as 2b. As an initial means of analyzing the functional relevance of each of the SHFV minor structural proteins, a set of mutant infectious clones was generated, each with the start codon of one minor structural protein ORF mutated. Different phenotypes were observed for each ortholog of the pairs of minor glycoproteins and all of the eight minor structural proteins were required for the production of infectious extracellular virus indicating that the duplicated sets of SHFV minor structural proteins are not functionally redundant.

Introduction

Simian hemorrhagic fever virus (SHFV) was first identified in the 1960s when it was found to be the causative agent of fatal hemorrhagic fever outbreaks in captive rhesus macaque colonies in the United States, Russia and Europe (Tauraso et al., 1968). Experimental SHFV infections in several macaque species produced clinical symptoms including fever, anorexia, adipsia, cyanosis, skin petechial and nose bleeds and ultimately, led to death by 7 to 13 days after infection (Allen et al., 1968; London, 1977; Palmer et al., 1968). These symptoms closely resembled those induced by other types of hemorrhagic fever viruses, such as Ebola Zaire and Marburg viruses in rhesus macaques (Bray, 2005). Various species of African monkeys are the natural hosts of SHFV, and in baboons, vervets, patas monkeys and African green monkeys, SHFV infections are typically asymptomatic and often persistent (Gravell et al., 1986). Previous SHFV outbreaks in macaque colonies are thought to have been caused by inadvertent mechanical transfer of SHFV present in the blood of a persistently infected African monkey to a macaque followed by efficient transmission of the virus between macaques (Palmer et al., 1968). Humans exposed to SHFV-infected macaques did not develop disease symptoms or seroconvert (Dalgard et al., 1992; Palmer et al., 1968).

SHFV is a member of the family *Arteriviridae* that also includes equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase elevating virus (LDV) and wobbly possum disease virus (Dunowska et al., 2012; Snijder and Meulenberg, 1998). The *Arteriviridae*, *Coronaviridae* and *Roniviridae* families are classified within the Order *Nidovirales* based on similarities in genome organization and replication strategy (Snijder and Kikkert, 2013). The SHFV genome is a 5′ capped and 3′ polyadenylated, positive-sense, single-stranded RNA of approximately 15.7 kb. The 5′ two-thirds of the genome encodes the ORF1a and ORF1ab polyproteins (Fig. 1A). The nonstructural proteins proteolytically cleaved from these polyproteins are required for the replication and transcription of the viral genomic and subgenomic (sg) RNAs (Beerens et al., 2007; Snijder and Meulenberg, 1998).

The viral structural proteins are encoded at the 3′ end of the genome and are expressed from a 3′ and 5′ coterminal, nested set of sg mRNAs. The 3′ most gene (ORF7) encodes the 15 kDa nucleocapsid (N) protein which forms disulfide-linked homodimers that interact to form the nucleocapsid (Dea et al., 2000; Snijder and Meulenberg, 1998). The interaction of the basic N-terminal domain of the N protein with genomic RNA is thought to facilitate packaging of the viral RNA into the nucleocapsid (Dea et al., 2000). The two other major structural proteins are the 19 kDa non-glycosylated membrane (M) protein and the 26 kDa major glycoprotein (GP5). These two virion envelope-associated proteins interact to form disulfide-linked heterodimers

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that function in virion attachment (Delputte et al., 2002,2007). The M protein is the most conserved arterivirus structural protein and is essential for progeny virion assembly (Delputte et al., 2002; Snijder and Meulenberg, 1998). The macrophage-restricted protein sialoadhesion (CD169) serves as the arterivirus receptor and mediates virion internalization (Van Breedam et al., 2010; Welch and Calvert, 2010), while the macrophage-specific CD163 antigen is required for virion entry/uncoating (Calvert et al., 2007; Van Gorp et al., 2008). Recently, an additional ORF (GP5a) that starts at an alternative AUG within the major glycoprotein GP5 ORF was identified in the genomes of each of the known arteriviruses (Firth et al., 2011). An EAV reverse genetic study indicated that GP5a was not an essential protein but its absence reduced virus yields and GP5a was detected in purified PRRSV particles (Johnson et al., 2011; Sun et al., 2013).

The EAV, PRRSV and LDV genomes also encode four minor structural proteins: E, GP2, GP3 and GP4. The sequences of these proteins are less conserved between different arteriviruses than those of the major structural proteins. The minor structural GPs form complexes located on the surface of virions that are postulated to be involved in receptor binding and virion uncoating (Snijder and Kikkert, 2013). The E protein is translated from bicistronic sg mRNA 2 that also expresses GP2 and it has been proposed that E oligomers function as ion-channels during virion entry (Lee and Yoo, 2006; Snijder et al., 1999). The SHFV genome encodes two adjacent sets of four minor structural protein ORFs (Godeny et al., 1998).

A stable, full-length cDNA infectious clone for SHFV-LVR (SHFVic) was constructed. The replication characteristics of the virus produced by MA104 cells transfected with *in vitro*-transcribed SHFVic RNA were similar to those of the parental SHFV-LVR virus. A separate sg mRNA was identified for the SHFV ORF previously identified as 2b. A set of mutant infectious clones, each lacking the expression of one of the duplicated 3′ ORFs, was made and their ability to produce infectious virions was analyzed. The data indicate that all eight of the minor structural proteins are required for the efficient production of infectious virions and suggest that SHFV particles are more complex than those of the other known arteriviruses.

Results

Construction of a full-length cDNA infectious clone of SHFV-LVR

A full-length cDNA clone of the SHFV, strain LVR, genome was constructed using a previously described strategy (Fang et al., 2006b; Nielson et al., 2003; Yount et al., 2000, 2002). Five overlapping cDNA fragments covering the genome were amplified from SHFV RNA by RT-PCR as described in the *Materials and Methods* section (Fig. 1B and C). A few nts in each fragment differed from the Genbank SHFV consensus sequence (Accession number AF180391.1) and initially each of these nts was corrected by site-directed mutagenesis of the appropriate fragment clone. The corrected individual fragment clones were then cut with PflMI, simultaneously ligated and cloned into pACYC184. The complete cDNAs in three clones were sequenced and no unexpected mutations were found. MA104 cells transfected with either 100 or 500 ng of *in vitro* transcribed SHFV RNA were observed daily for the development of cytopathic effect (CPE). At 120 h after transfection, cells were lysed and lysates analyzed for SHFV nucleocapsid and nsp1β proteins by Western blotting. The transfected cells did not show any obvious CPE and viral proteins were not detected in cell lysates through 120 h (data not shown). The harvested culture fluid was serially passaged four times in an attempt to recover virus but neither CPE nor intracellular viral protein was detected after any of these passages (data not shown).

Table 1
Nucleotide differences between GenBank (AF180391.1) and SHFVic sequences.

Position	GeneBank	New sequencing	Amino acid change	Gene
511	C	T	P101L	nsp1α
849	G	T	V214F	nsp1β
1658	G	A	silent	nsp1γ
2503	G	C	G765A	nsp2
3726	C	–	10 aa frame shift	nsp2
3757	–	A	10 aa frame shift	nsp2
3785	A	G	silent	nsp2
4277	T	G	silent	nsp3
4895	A	G	silent	nsp4
5575	G	T	G1889V	nsp5
5695	G	A	R2018H	nsp5
5704	A	T	K2021I	nsp6
5707	C	G	P2022R	nsp6
5727	A	T	I2029F	nsp6
6088	G	C	G1960A	nsp7
8276	T	G	S2879A	nsp9
8340	G	T	W2900L	nsp9
10359	T	C	L3373S	nsp11

Because the Genbank consensus sequence (GenBank Assession number AF180391.1) of SHFV LVR was obtained by older sequencing methods from shot gun clones and it was likely that this sequence contained deleterious errors. Viral RNA extracted from parental SHFV LVR was subjected to 454 sequencing as described in the Materials and Methods section. In addition, each of the fragment clones was reamplified from SHFV LVR RNA by RT-PCR and sequenced. The same 18 nt differences compared with the previous GenBank consensus sequence were detected by both 454 genomic sequencing and individual fragment sequencing (Table 1). The GenBank sequence was updated based on 454 sequencing data (GenBank Assession number AF180391.2). The reamplified fragment clones were then used to construct new full-length clones (SHFVic). The sequences of three independently constructed full length clones were confirmed. Viral RNA was *in vitro* transcribed from linearized plasmid RNA and analyzed on an RNase-free denaturing agarose gel (Fig. 1D). Six RNA bands with sizes of about 15, 13, 12, 7, 4 and 3 kb were consistently detected. The 15 kb band was appropriately sized to represent the full-length viral genome RNA. The shorter products were likely the result of premature termination or aberrant transcription. MA104 cells transfected with either 100 or 500 ng of *in vitro* transcribed SHFVic RNA showed CPE by 72 h after transfection and Western blot analysis detected nsp1β in lysates from cells transfected with either concentration of viral RNA at 72 h after transfection (Fig. 2A). The higher amount of viral protein detected after transfection of 100 ng of RNA suggested the possibility that increased amounts of the shorter RNAs negatively affected the efficiency of viral replication. To confirm that extracellular infectious virus was produced, 100 μl of undiluted culture fluid from the transfected cells was passaged onto fresh MA104 cells. These cells showed CPE starting at 24 h after infection and Western blot analysis detected intracellular nsp1β protein in cell lysates harvested at 72 h (Fig. 2A).

To compare the replication kinetics of the infectious clone virus and the parental virus, MA104 cells were infected with either first passage infectious clone virus or with SHFV-LVR parental virus at an MOI of 1 and viral yields were quantified by plaque assay on MA104 cells at various times after infection (Fig. 2B). The replication kinetics of the parental virus and the infectious clone virus were similar and both cultures produced peak titers of $\sim 10^6$ PFU/ml by 72 h after infection. The kinetics of intracellular viral protein synthesis were assessed by Western blotting of lysates from