Deletions in the hypervariable domain of the nsP3 gene attenuate Semliki Forest virus virulence

Sareen E. Galbraith,1,2† Brian J. Sheahan2 and Gregory J. Atkins1

1Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland
2University College Dublin School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Correspondence
Sareen E. Galbraith sagalbra@utmb.edu

Major virulence determinants of Semliki Forest virus (SFV) lie within the non-structural genes that form the replicase complex proteins. Gene exchange between virulent and avirulent viruses has shown that the nsP3 gene, which has essential 5′ conserved domains and a non-essential hypervariable 3′ domain, is one of the virulence determinants. This protein plays a role in subgenomic 26S and negative-strand RNA synthesis and is thought to function with nsP1 to anchor replication complexes to cell membrane structures. Studies to date have focused on analysing the effect of mutational changes spread over the whole gene on virulence of the virus. The virulent SFV4 virus, derived from an infectious clone, was utilized to analyse the effect on virulence of large deletions in the hypervariable domain of nsP3. Two viruses with different in-frame deletions that spanned this domain showed reduced rates of RNA synthesis and multiplication in cell culture. In adult BALB/c mice, these viruses were avirulent after intramuscular and intraperitoneal inoculation, and brains sampled from infected mice showed minimal or no evidence of pathology. These deleted viruses had greatly reduced virulence when administered by the intranasal route and brains from infected mice showed lesions that were much less severe than those seen in SFV4 infection. Mice surviving infection with the deleted viruses resisted challenge with the virulent L10 strain, indicating induction of protective immunity. This work establishes that deletions in the nsP3 hypervariable domain attenuate virulence after peripheral inoculation and also reduce virulence after intranasal inoculation.

INTRODUCTION

Semliki Forest virus (SFV), a member of the genus Alphavirus in the family Togaviridae, is an enveloped, positive-stranded RNA virus, which usually causes mild febrile disease in humans. The SFV4 strain of SFV is derived from the infectious clone pSP6-SFV4 (Liljestrom et al., 1991) and causes encephalitis in mice of all ages, leading to death a few days after intranasal (i.n.) inoculation. Only a proportion of mice die when inoculated intramuscularly (i.m.) and less die when the virus is inoculated intraperitoneally (i.p.). The L10 strain causes lethal encephalitis, irrespective of the route of infection (Atkins et al., 1999). After peripheral inoculation SFV causes a high titre viraemia that enables virus passage across the blood–brain barrier (BBB) and infection of central nervous system cells occurs, particularly neurons and oligodendrocytes (Pathak & Webb, 1974; Soilu-Hänninen et al., 1994; Fazakerley, 2002, 2004).

The SFV genome encodes four non-structural and five structural proteins. On entering the cell, the positive-strand genomic RNA acts as a messenger for translation of the non-structural polyprotein and as a template for synthesis of negative-strand replicative intermediate RNA. This negative-strand RNA is the template for synthesis of new genomic RNA and the subgenomic 26S RNA, which encodes the structural polyprotein. The non-structural polyprotein is post-translationally cleaved into four proteins (nsP1, nsP2, nsP3 and nsP4) and the structural polyprotein into five proteins [capsid (C), 6K plus E1, E2 and E3 envelope proteins; Strauss & Strauss, 1994].

The non-structural proteins form the virus replication and transcription complexes (Kääriäinen & Söderlund, 1978). The nsP2 protein cleaves nsP4 from the non-structural polyprotein to give the cleavage intermediate (nsP123). Negative-strand RNA synthesis is carried out by the nsP123 and nsP4 replicase complex and this process is unstable because of the processing of nsP123. The intermediate partially cleaved replicase complex (nsP1, nsP23 and nsP4) can synthesize both negative- and positive-strand RNA. Positive-strand RNA synthesis of full-length and subgenomic 26S RNA is
then carried out by the completely processed replicase complex consisting of nsP1, nsP2, nsP3 and nsP4 (Wang et al., 1994; Shirako & Strauss, 1994; Lemm et al., 1994, 1998; Vasiljeva et al., 2003; Kim et al., 2004). The nsP1 protein caps the viral mRNAs (Wang et al., 1996; Ahola et al., 1999, 2000; Kääriäinen & Ahola, 2002), interacts with nsP4 (Shirako et al., 2000; Fata et al., 2002a, b) and plays a role in negative-strand RNA synthesis (Hahn et al., 1989; Wang et al., 1991). This protein acts with nsP3 to mediate association of the non-structural polypeptide with membranes in the cell and to target the polypeptide to intracellular vesicles (Peränen et al., 1995; Laakkonen et al., 1996; Ahola et al., 1999; Lampio et al., 2000; Kujala et al., 2001; Salonen et al., 2003). It can also modulate the proteinase activity of nsP2 (de Groot et al., 1990). The nsP2 protein is involved in initiation of RNA-capping reactions and therefore in the regulation and synthesis of positive-strand subgenomic 26S and negative-strand RNA (Suopanki et al., 1998; Vasiljeva et al., 2000). This protein is responsible for the proteolytic processing of the non-structural proteins (Strauss & Strauss, 1994; Vasiljeva et al., 2001; Merits et al., 2001). The nsP4 protein is the catalytic subunit of the viral RNA-dependent RNA polymerase and has the GDD motif, which is common to the catalytic subunits of RNA polymerases (Strauss & Strauss, 1994; Kääriäinen & Ahola, 2002).

The nsP3 protein comprises three domains; the first is conserved among alphaviruses, coronaviruses, Hepatitis E virus and Rubella virus (Koorn & Dolja, 1993; Pehrson & Fuji, 1998), the second is conserved among alphaviruses and the third C-terminal domain is hypervariable (Strauss & Strauss, 1994). nsP3 is a phosphoprotein and the phosphorylation sites have been mapped to serine and threonine residues, which are located mainly in the hypervariable domain (Li et al., 1990; Peränen, 1991; Vihinen & Saarinen, 2000; Vihinen et al., 2001). The phosphorylation of this domain could indicate an accessory function in negative-strand RNA synthesis (De et al., 2003). The functions of the nsP3 protein are not well understood, although it is known to play a role in subgenomic 26S and negative-strand RNA synthesis (Hahn et al., 1989; Lemm & Rice, 1993; Wang et al., 1994; LaStarza et al., 1994a, b; Lemm et al., 1994; Shirako & Strauss, 1994). It has been proposed that it acts with nsP1 to mediate association of the replication complex with cytoplasmic membrane structures (Peränen et al., 1988, 1995; Peränen, 1991; Peränen & Kääriäinen, 1991). It is also known to affect the cleavage specificity of the nsP2 proteinase (de Groot et al., 1990; Strauss & Strauss, 1994).

Virulence of SFV is polygenic with determinants of this phenotype distributed through most of the genome (Glasgow et al., 1991, 1994; Santagati et al., 1995, 1998; Rikkonen, 1996; Tarbatt et al., 1997; Ahola et al., 2000; Tuittila et al., 2000; Vihinen et al., 2001; Fazakerley et al., 2002; Tuittila & Hinkkanen, 2003). Two studies established, by analysis of chimeras between the virulent SFV4 strain and avirulent strains, that the nsP3 gene is a virulence determinant (Tarbatt et al., 1997; Tuittila et al., 2000). Tarbatt et al. (1997) found that virulence determinants throughout the genome act additively, although 65 % of the mutational base changes found lie within the non-structural region. Tuittila et al. (2000) showed that virulence determinants lie mainly within non-structural genes and more specifically in the nsP3 gene, although the chimeric viruses generated were only tested by peripheral inoculation and not by the more stringent i.n. route. In a second study, again only examining virulence after peripheral inoculation, Tuittila & Hinkkanen (2003) showed that individual amino acids, mainly in the conserved domain of nsP3, act additively in determining virulence. However, three of the eight amino acid mutations examined fell within the hypervariable domain. In addition, a recent study showed that a virulent virus with a non-phosphorylated nsP3 gene gave reduced virulence in mice after peripheral inoculation (Vihinen et al., 2001) and this phosphorylation is known to occur mainly within the hypervariable domain (Li et al., 1990; Peränen, 1991; Vihinen & Saarinen, 2000; Vihinen et al., 2001).

Little is also known about the function of the nsP3 protein in other alphaviruses. Ross River virus, O’nyong-nyong virus, Salmon pancreas disease virus, Sleeping disease virus, Venezuelan equine encephalitis virus (VEEV) and Sindbis virus (SINV) all show a conserved 3’ domain that is essential for replication and a hypervariable 5’ domain in their nsP3 genes (Strauss et al., 1988; Meissner et al., 1999; Weston et al., 2002; Saleh et al., 2003). For VEEV, the hypervariable domain is thought to reflect adaptation to growth in different hosts or vectors (Oberste et al., 1996). For SINV, viruses with larger deletions in the hypervariable domain gave decreased virus yield and total viral RNA synthesis early in infection in vitro. The largest deletion mutants were defective at initiating a productive infection in mosquito cells, although once infection was established virus yields were comparable with the wild-type virus (LaStarza et al., 1994b).

In this study, we have investigated the effect of non-revertible deletions in the hypervariable domain of nsP3 on RNA synthesis and multiplication of SFV4 in vitro and on virulence in vivo. Two SFV strains with deletions in the hypervariable domain showed reduced rates of RNA synthesis and multiplication in vitro possibly due to reduced binding of the replicase complexes to cellular membranes. These deletion mutants were avirulent after peripheral inoculation and showed reduced virulence after i.n. inoculation. Thus, we have shown that the hypervariable domain of the nsP3 gene plays an important role in the pathogenicity of SFV.

**METHODS**

**Virus and cells.** The cDNA infectious clone of the SFV4 strain of SFV (pSP6-SFV4, Fig. 1) was obtained from P. Liljestrom (Karolinska Institute, Sweden). Baby hamster kidney cells (BHK-21; ATCC) were cultured in BHK-21 medium supplemented with 5% fetal calf serum, 5% tryptose phosphate broth, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. BALB/3T3 cells

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Fig. 1. Schematic diagram of the construction of nsP3 deletions. (a) Nucleic and amino acid sequence of the wild-type SFV4 virus and nsP3 gene deletion mutants derived from it, which are named according to the restriction sites (underlined in the SFV4 nucleic acid sequence) used to create them. The SN deletion (amino acids in shaded box) stretches from the SacII site to the NaeI site and the TN deletion (amino acids in unshaded box) stretches from the NaeI site to the Tth111I site. The cleavage site at the end of the nsP3 gene is preserved. (b) pSP6-SFV4 plasmid used in deletion construction. AflII and BglII sites were used to transfer the nsP3 gene into the L28 cloning vector. The deletions were constructed in the L28 vector and the same sites used to transfer the fragment back into pSP6-SFV4. (c) Agarose gel electrophoresis of the nsP3 gene PCR products from SFV4, SFV4-SN and SFV4-TN using plasmid DNA (P), cDNA reverse transcribed from infected BHK-21 cell RNA (R) and cDNA reverse transcribed from infected mouse brain RNA (B). The SFV4 band is 920 bp in size, indicating a full-length hypervariable region, whereas the SFV4-SN band is 623 bp in size and the SFV4-TN band is 749 bp in size, indicating presence of the deletions in the hypervariable region.

(ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. To produce infectious virus from the cDNA clone, viral RNA was in vitro transcribed, electroporated into BHK-21 cells as described previously (Liljestrom et al., 1991) and the infectious virus was passaged once in BHK-21 cells. The virulent L10 strain was prepared from a plaque-purified seed stock and grown and titrated in BHK-21 cells (Balluz et al., 1993).

To measure virus growth in BHK-21 and BALB/3T3 cells, triplicate monolayers were mock infected or infected with virus at an m.o.i. of 0.1. After 1 h, the virus inoculum was removed and the monolayers thoroughly washed three times with PBS and 3 ml medium was added. The medium was harvested from these cells at 2 h intervals for 12 h, aliquoted and frozen at −70 °C for plaque assay. This procedure was repeated for cells infected at an m.o.i. of 10.

To measure viral RNA synthesis, triplicate monolayers of BHK-21 cells were infected with virus at an m.o.i. of 100 and incubated at 37 °C for 1 h. Control wells were mock infected with medium alone. The virus inocula were replaced with medium containing 5 μg actinomycin D (Sigma) ml⁻¹. Samples were taken at 2, 4, 6 and 8 h post-infection (p.i.) and the cultures were pulse-labelled with 1 μCi (37 kBq) [5,6-³H]uridine (Amersham) ml⁻¹ for 2 h in the presence of 5 μg
actinomycin D ml⁻¹. After the pulse, the monolayers were lysed in 1% SDS, precipitated with 10% (v/v) trichloroacetic acid and harvested by suction onto filter mats. The radioactivity associated with the dried filter mats was determined by liquid scintillation counting.

**Construction of nsP3 deletions.** The region from BglII (6714 bp) to SfdI (7783 bp) in pSP6-SFV4 (Fig. 1) was replaced with the corresponding region from the A7 virus genome to remove the third Avfl site at position 7114 bp. The plasmid was then digested with Avfl and religated to remove the Avfl fragment (2572–3915 bp; pSP6-SFV4AFL). The unique Avfl and BglII sites were used to transfer the nsP3 gene fragment into the L28 cloning vector. The in-frame deletions in the nsP3 gene were made using the unique restriction sites that were present in the nsP3 gene after ligation into the L28 cloning vector. To make nsP3-SN, the plasmid was digested with SacI (5056 bp) and Nael (581bp) and the 325 bp fragment was removed. The overhanging ends on the SacI restriction site were removed using Klenow polymerase (NEB; according to manufacturer’s instructions) and the plasmid religated. To make nsP3-TN, the plasmid was digested with Nael (5381 bp) and Tth111I (5509 bp) and the 129 bp fragment was removed. The overhanging ends on the Tth111I restriction site were blunt end-filled and the plasmid religated in the same way. The nsP3 gene deletions were religated into the pSP6-SFV4AFL plasmid using the unique Avfl and BglII sites. The deleted Avfl fragment was religated back into the plasmids to form pSP6-SFV4-SN and pSP6-SFV4-TN.

**RT-PCR detection of nsP3 deletions.** To detect the presence of the deletion mutants in cells and mouse tissue, and to check the stability of the deletion mutants, an RT-PCR detection method was used. Total RNA was isolated from virus-infected BHK-21 cells by immediate solubilization of the cell monolayer in RNA isolator (Genosys). Total RNA was isolated from infected mouse brain by homogenizing 100 mg tissue per ml of RNA isolator in a Bio-Pulverizer tube (Anachem). Negative control reaction mixes, using uninfected BHK-21 cells and uninfected mouse brain tissue, were analysed for each RT-PCR reaction set. Oligonucleotide primers were designed to amplify the area around the deletions in the non-structural coding region of SFV. The forward primer was 5’-GCCGAATTCCTCATTTTCCCCTCCCGA-3’ (position 4951) and the reverse primer was 5’-CCCGAATTCCGGGCGTGAATCTTTTCATTTTTCGA-3’ (position 5871). First-strand cDNA was synthesized from 1 µg RNA using the reverse primer and AMV reverse transcriptase in a 20 µl reaction, according to the manufacturer’s instructions (Promega). The reverse transcription reaction was incubated at 42°C for 60 min, at 99°C for 5 min and then at 4°C for 5 min. A 5 µl aliquot of the cDNA product was incorporated in a 100 µl PCR reaction according to the manufacturer’s instructions (Promega). Briefly, the PCR reaction mix consisted of 25 mM MgCl₂, 10 × reaction buffer, PCR nucleotide mix (10 mM each dNTP) and 5 U Taq polymerase. Primers were added at a concentration of 0.005 µg forward and reverse. Samples were incubated for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. A final extension time of 5 min at 72°C was used and the amplified products were analysed on a 1% agarose gel.

**Mouse pathogenicity.** Specific pathogen-free female 40–60-day-old BALB/c mice were inoculated i.n. with 10⁶ p.f.u. virus in 20 µl PBS, placed on the end of the nostril so that the droplet was inhaled (Sammin et al., 1999). Mice were inoculated i.p. with 10⁶ p.f.u. virus in 500 µl PBS. Mice were lightly anaesthetized with halothane before i.m. inoculation with 10⁶ p.f.u. virus in 50 µl PBS in the right tibialis anterior leg muscle. Immunity of surviving mice was tested by i.p. challenge with 10⁶ p.f.u. L10 virus in 500 µl PBS, which is lethal for unprotected mice.

For the mortality studies, 10 mice were inoculated i.p. with SFV4 and 10 with each of SFV4-SN and SFV4-TN. These mice were observed daily for 14 days and deaths were recorded. Immunity of surviving mice was tested by i.p. challenge with L10 and the mice were observed daily for a further 14 days. This procedure was repeated for the i.m. and i.n. routes.

For the histopathology studies, 12 mice were inoculated i.n., i.p. and i.m. with SFV4, SFV4-SN and SFV4-TN. Three i.p., three i.m. and three i.n. inoculated mice were sampled at 4 and 14 days p.i.; the mice inoculated i.n. with SFV4 were sampled only at 4 days p.i. because all mice died by 6 days p.i. The mice were deeply anaesthetized with halothane and perfused via the left ventricle with 4% formal saline for 5 min. The mice were left overnight in fixative at 4°C and the intact brains and spinal cords removed and processed for paraffin embedding. Four micrometer coronal sections of brain at the level of the olfactory bulbs, frontal cortex, thalamus, optic tracts and pons, and 4 µm coronal and sagittal sections of spinal cord were stained with haematoxylin and eosin (H&E) and examined histologically. Groups of three mice previously inoculated with SFV4, SFV4-SN and SFV4-TN, and surviving challenge with L10, were similarly examined at 4 and 14 days post-challenge.

For the virus titration studies after i.n. inoculation, 15 mice were inoculated with SFV4, SFV4-SN and SFV4-TN and were sampled in triplicate on days 2, 4, 6, 8 and 10 post-inoculation. They were anaesthetized with halothane, ligatured at the neck and the brains were removed. A 10% (w/v) clarified homogenate of the brain was prepared and titrated in BHK-21 cells as described previously (Balluz et al., 1993).

For the virus titration studies after i.m. and i.p. inoculation, 15 mice were inoculated with SFV4, SFV4-SN and SFV4-TN. These mice were sampled in triplicate on days 2, 4, 6, 8 and 10 post-inoculation. For measurement of blood infectivity, mice were anaesthetized with halothane, ligatured at the neck and the thoracic cavity was opened to expose the heart. The heart was incised and a 200 µl sample of blood was removed and diluted 1:10 with medium for plaque assay. Brains from the same mice were also sampled.

**Statistical analysis of survival curves.** Kaplan–Meier survival curves were used to compare SFV4-SN and SFV4-TN with SFV4. The GraphPad Prism 4 program was used for this statistical analysis.

**RESULTS**

**Construction of deletions in the hypervariable domain of nsP3**

Two in-frame deletions in the nsP3 gene were constructed (Fig. 1). A construct having both deletions as one large deletion produced non-viable virus and therefore was not analysed further. However, these two large deletions together span the non-essential hypervariable 3’ domain of the nsP3 gene (Fig. 1b). The SN deletion is 327 nt long, whereas the TN deletion, which is at the extreme 3’ terminus, is 129 nt long (Fig. 1c). The cleavage site at the end of the nsP3 gene (Fig. 1a) was preserved to ensure cleavage of nsP4 from the nsP123 protein.

A PCR detection system was developed to detect these nsP3 gene deletions and the full-length nsP3 gene, to check the stability of the deletions in vitro and in vivo and to ensure the absence of cross-contamination (Fig. 1c). PCR
amplification of the nsP3 gene DNA was performed on plasmid DNA, on cDNA reverse transcribed from BHK-21 cell RNA and on cDNA reverse transcribed from mouse brain RNA. Only deleted viral RNA was detected in the samples from the deletion mutants (Fig. 1c).

**nsP3 deletion mutants show reduced rates of multiplication in vitro**

The nsP3 deletion mutants produced similar cytopathic effect (CPE) to the wild-type SFV4 virus in cell culture. Development of this CPE in BHK-21 cells was slower (2–4 h longer) than SFV4 (not shown), which indicated that multiplication of the deletion mutants could be slower than SFV4. To investigate this further, BHK-21 and BALB/3T3 cells were infected at low and high m.o.i. Fig. 2 shows infectious virus release from BHK-21 cells infected at an m.o.i. of 0·1 (more than one round of multiplication; Fig. 2a) and 10 (one round of multiplication; Fig. 2b) and from BALB/3T3 cells infected at an m.o.i. of 0·1 (Fig. 2c) and 10 (Fig. 2d). Infectious virus released from BHK-21 cells infected with the nsP3 deletion mutants at an m.o.i. of 0·1 peaked at 12 h p.i. reaching levels of $5 \times 10^9$ p.f.u. ml$^{-1}$. The level of virus released from BALB/3T3 cells infected with the nsP3 deletion mutants at an m.o.i. of 0·1 peaked at 24 h.p.i. reaching levels of $7 \times 10^8$ p.f.u. ml$^{-1}$ for SFV4-SN and $2 \times 10^8$ p.f.u. ml$^{-1}$ for SFV4-TN, whereas SFV4 virus levels reached $1 \times 10^{10}$ p.f.u. ml$^{-1}$. The level of virus released from BALB/3T3 cells infected with the nsP3 deletion mutants at a m.o.i. of 10 also peaked at 24 h.p.i. reaching levels of $3 \times 10^8$ p.f.u. ml$^{-1}$ for SFV4-SN and $4 \times 10^8$ p.f.u. ml$^{-1}$ for SFV4-TN, whereas SFV4 virus levels reached $1 \times 10^{10}$ p.f.u. ml$^{-1}$. The drop in titre between 2 and 4 h.p.i. in both cell types is the result of residual virus inoculum remaining after the adsorption procedure. The viruses with the nsP3 deletions consistently showed lower titres than SFV4 at both m.o.i. values in both cell types and mutant replication did not reach SFV4 level in the BALB/3T3 cells. Thus, deletions in the hypervariable domain of nsP3 reduce the rate of virus multiplication in vitro.

**nsP3 deletion mutants show reduced rates of RNA synthesis**

To investigate the lower levels of virus released from BHK-21 and BALB/3T3 cells, BHK-21 cells were infected with virus, and viral RNA synthesis was measured by the incorporation of $[5,6^3]$H]uridine. Fig. 3 shows total viral RNA synthesized in BHK-21 cells infected at an m.o.i. of 100. The nsP3 deletion mutants produced significantly less RNA than SFV4 over the 8 h period. The differences in total RNA

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**Fig. 2.** Virus multiplication in BHK-21 and BALB/3T3 cells. BHK-21 cells were infected with virus at an m.o.i. of 0·1 (a) and 10 (b) and BALB/3T3 cells were infected at an m.o.i. of 0·1 (c) and 10 (d). The virus released into the growth medium was quantified by plaque assay and each point on the graph is mean value ± standard error using three replicates at each time point.
synthesis were most pronounced at early times, with SFV4-TN and SFV4-SN producing significantly lower amounts of RNA than the SFV4 virus at 2 and 4 h p.i. These results show that deletions in the hypervariable domain of nsP3 reduce the rate of virus RNA synthesis.

**nsP3 deletion mutants show reduced virulence after i.n. inoculation**

Mice inoculated with SFV4 died by 6 days p.i. Survival rates for mice similarly inoculated with SFV4-SN and SFV4-TN were 20 and 80 %, respectively (Fig. 4a). Virus detected in the brain at 12 h p.i. was due to virus in the blood contaminating the homogenized brain sample (Fig. 4b). Multiplication of the nsP3 deletion mutants in the brain was lower than SFV4. The SFV4-SN mutant showed significantly lower levels of virus than SFV4 at 2 days p.i. and there was no evidence of SFV4-TN infection in the brain at this time point. By 4 days p.i., SFV4-TN showed lower levels of virus in the brain than SFV4, whereas SFV4-SN showed similar levels of virus. Levels of virus in the brain of SFV4-SN-infected mice peaked at 6 days p.i. and dropped off by 8 days p.i., leaving no evidence of virus in the brain at 10 days p.i. Levels of virus in the brain of SFV4-TN-infected mice also peaked at 6 days p.i., but this virus was still present in the brain up to 10 days p.i. Three of these mice were tested at 14 days p.i. and they showed no evidence of virus in the brain (results not shown). Mice inoculated with SFV4-TN and SFV4-SN surviving to 14 days p.i. resisted challenge with L10 (results not shown). For all i.n.-inoculated mice examined at 4 and 14 days p.i., brain lesions were localized primarily in limbic areas, mostly the olfactory bulbs, olfactory cortex, pyriform cortex, hippocampus and thalamus. All mice inoculated i.n. with SFV4 (n = 3) and examined at 4 days p.i. showed extensive laminar and focal areas of malacia accompanied by spongiform degeneration and low-grade perivascular lymphocytic infiltration (Fig. 5a and b). Brain lesions in mice inoculated i.n. with SFV4-SN were less severe and comprised localized areas of malacia, gliosis and low-grade perivascular lymphocytic infiltration in four of six mice at 4 days p.i. (Fig. 5c). Focal areas of malacia, mineralization, demyelination, gliosis and perivascular lymphocytic infiltration were present in all SFV4-SN-inoculated mice (n = 3) at 14 days p.i. In mice inoculated i.n. with SFV4-TN, lesions were less severe than in mice inoculated i.n. with SFV4-SN. Necrotic neurons were an occasional finding and localized areas of gliosis, myelin vacuolation and perivascular lymphocytic infiltration were present in the olfactory cortex in three of five mice at 4 days.
p.i. (Fig. 5d). Focal areas of malacia, mineralization, gliosis, demyelination and perivascular lymphocytic infiltration were present in four of seven SFV4-TN-inoculated mice at 14 days p.i.

**nsP3 deletion mutants are attenuated after peripheral inoculation**

By 9 days p.i., 70 % of mice inoculated i.m. with SFV4 died. All mice survived i.m. inoculation with the deletion mutants and showed no clinical signs of disease (Fig. 6a). The viraemia induced by SFV4-SN was lower than SFV4, and no detectable viraemia was induced by SFV4-TN (Fig. 6c). SFV4 and SFV4-SN virus detected in the brain at 12 h p.i. was due to virus in the blood contaminating the homogenized brain sample (Fig. 6b). Thus, the deletion mutants did not multiply in the brain, with the exception of a low level of SFV4-SN detected in a single mouse. In mice examined histologically at 4 and 14 days p.i., no lesions were detected at 4 or 14 days p.i. for SFV4-TN, indicating that this deletion mutant was avirulent for the mice when given by this route. For SFV4-SN, no lesions were seen in two of three mice at 4 days p.i. One of three mice examined at this interval showed small foci of gliosis with individual cell necrosis and perivascular lymphocytic infiltrates randomly distributed in the neuropil. No lesions were detected at 14 days p.i.

All mice inoculated i.m. with the deletion mutants survived lethal challenge with L10 virus at 14 days p.i., whereas control uninfected mice died, indicating that protective immunity had been induced in the mice infected with deletion mutants. For SFV4-TN-inoculated mice, no lesions were seen at 4 and 14 days post-challenge and for SFV4-SN-inoculated mice, no lesions were seen in two of three mice at 4 days post-challenge and in three of three mice at 14 days post-challenge. One of three SFV4-SN-inoculated mice examined at 4 days post-challenge showed mature lesions resembling those seen at 4 days p.i. in one SFV4-SN-inoculated mouse, which was unchallenged.

After i.p. inoculation, all the mice receiving the deletion mutants survived and showed no clinical signs of disease, while 20 % of mice receiving SFV4 virus died by 10 days p.i. (Fig. 6d). The viraemia induced by SFV4-SN was lower than that produced by SFV4 and was totally absent from the

**Fig. 5.** Examples of lesions induced in the pyriform cortex and lateral olfactory tract of BALB/c mice at 4 days p.i. (a) in normal brain. (b) SFV4, laminar neuronal necrosis. (c) SFV4-SN, focal neuronal necrosis. (d) SFV-TN, lymphoid perivascular infiltrates and gliosis; neurons appear normal. H&E staining, × 200.
blood by 48 h p.i. The viraemia induced by SFV4-TN was lower than SFV4 at 12 and 24 h p.i., by 48 h p.i. it had reached the levels of SFV4 and by 96 h p.i. the virus was completely absent from the blood (Fig. 6f). The SFV4-TN deletion mutant did not multiply in the brain and the SFV4-SN mutant was present at a low level up to 24 h p.i. (Fig. 6e). In mice examined histologically at 4 and 14 days p.i., no lesions were detected for SFV4-SN and SFV4-TN, showing that the deletion mutants were avirulent for mice when given by the i.p. route. All mice inoculated i.p. with the deletion mutants survived lethal challenge with L10 at 14 days p.i. and no lesions were seen at 4 and 14 days post-challenge.

**DISCUSSION**

In this study, we analysed the effect of deletions spanning the hypervariable domain of nsP3 on RNA synthesis and multiplication of the virus in vitro, and on virulence in vivo; a deletion spanning the entire hypervariable region produced non-viable virus. The deleted viruses multiplied slower than SFV4 in BHK-21 cells and in mouse BALB/3T3
cells. This was reflected in the lower viral RNA synthesis found in BHK-21 cells. These results are consistent with a previous study showing that an SFV4 virus defective in nsP3 phosphorylation, which occurs mainly in the hypervariable region, gives lower levels of RNA synthesis (Vihinen et al., 2001). LaStarza et al. (1994b) showed that large deletions in the SINV hypervariable domain also decreased viral RNA synthesis and gave lower virus yields earlier in infection. In addition, another study using temperature-sensitive mutants of SINV in a region of the hypervariable domain (nt 4280–5262) that overlaps the SFV4-SN deletion (nt 5056–5381), shows that mutations in this area affect synthesis of RNA and in particular synthesis of the negative-strand replicative intermediate early in infection (Wang et al., 1994).

After i.n. inoculation, none of the mice infected with SFV4 survived, as expected (Atkins et al., 1999), but survival occurred in 20% of mice infected with SFV4-SN and in 80% of mice infected with SFV4-TN. Statistical analysis of the survival curves showed very significant differences between the nsP3 deletion mutants and SFV4 after i.n. inoculation. Consistent with these results, the nsP3 deleted viruses produced lower levels of virus in the brain. For all i.n.-inoculated mice, brain lesions were localized primarily in limbic areas. The extent of the lesions in the limbic system of the brain can be related to the decreased ability of the deletion mutants to replicate in olfactory neurons.

After peripheral (i.m. and i.p.) inoculation, all mice infected with SFV4-SN and SFV4-TN survived and in general there was little evidence of virus replication in the brain. Viraemia in the blood was at lower levels than those of SFV4. Statistical analysis of the survival curves showed very significant differences between the nsP3 deletion mutants and SFV4 after i.m. inoculation. Brain lesions, if present, were low grade and much less severe than those induced by SFV4, and were not detected in SFV4-TN-infected mice or in any of the mice that received the deletion mutants by the i.p. route. All surviving mice resisted challenge with virulent virus. Tuittila & Hinkkanen (2003) showed previously that three amino acid mutations, at positions 435, 442 and 469, within this hypervariable domain are important in determining virulence. Another study showed that a virus defective in phosphorylation of the hypervariable domain was less virulent after i.p. inoculation (Vihinen et al., 2001). However, all of these other studies have only used the i.p. route of inoculation and not the more stringent i.n. route. Thus, deletions in the hypervariable domain of nsP3 render the SFV4 virus avirulent for mice after peripheral inoculation but still allow stimulation of protective immunity. After inoculation, SFV replicates well in the periphery and causes a high titre viraemia, which enables virus passage across the BBB and the induction of brain lesions (Pathak & Webb, 1974; Soili-Hänninen et al., 1994; Fazakerley, 2002, 2004). Thus, replication of these nsP3 deletion mutants in peripheral tissues did not induce a sufficient viraemia to allow passage across the BBB and induction of brain lesions.

Recent work on SFV vectors comparing the use of suicide particles with replication competent SFV4 virus to treat K-BALB tumours induced in the flank of BALB/c mice shows that SFV4 virus treated groups had greater inhibition of tumour growth (Smyth et al., 2005). Thus, replication competent SFV could be more useful in some therapeutic situations since there would be infection of a greater number of cells and therefore greater tissue penetration. The incorporation of nsP3 deletions into a replicating SFV4 virus would attenuate the virus and allow development of a replicating vector based on this strain; this possibility is currently being investigated.

Thus, it can be concluded that deletions in the hypervariable domain of nsP3 reduce rates of virus RNA synthesis and multiplication, probably due to a reduction in formation of the replicase complexes on intracellular membranes, and thereby attenuate the pathogenicity of SFV4 in a non-reversible manner.

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