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REPLICATION STRATEGIES AND APPLICATIONS OF SEMLIKI FOREST VIRUS

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LIST OF ORIGINAL PUBLICATIONS

I Vasiljeva, L., Merits, A., Golubtsov, A., **Sizemskaja**, **V**., Kääriäinen, L., Ahola, T. (2003). Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J Biol Chem* 278(43):41636–41645.

II Tamberg, N.*, **Lulla**, **V**.*, Fragkoudis, R., Lulla, A., Fazakerley, J. K., Merits, A. (2007). Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. *J Gen Virol* 88:1225–1230.
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IV **Lulla**, **V**., Sawicki, D. L., Sawicki, S. G., Merits, A., Ahola, T. Molecular defects in Semliki Forest virus temperature sensitive mutants ts10 and ts14 mapping to nsP1. Manuscript.

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Some unpublished data is also presented.

Author's contribution

Paper **I**: Responsible for the cloning of the set of plasmids (deletion constructs); participation in the discussion of the manuscript.

Paper **II**: Equal contribution with N. Tamberg; responsible for the performing of pilot experiments with tested marker viruses, infectious center assay, protein labelling and IP experiments; together with N. Tamberg and/or A. Lulla: virus growth curves, RNA labelling and northern blot analysis, active participation in the discussion and the writing of the manuscript.

Paper **III**: Main person responsible for the paper including planning, experimental part, analysis and active participation in the writing of the manuscript.

Manuscript **IV**: Main person responsible for the paper including planning, experimental part (except RNA labelling experiments), analysis and active participation in the writing of the manuscript.

ABBREVIATIONS

aa	amino acid
AdoHcy	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-methionine
BHK	baby hamster kidney
CP	capsid protein
cDNA	complementary DNA
CPV	cytoplasmic vacuoles type I
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxynucleotide 5'-triphosphate
EEE	eastern equine encephalitis
EGFP	enhanced green fluorescent protein
GFP	green fluorescent protein
GMP	guanosine monophosphate
GT	guanylyltransferase
GTP	guanosine 5'-triphosphate
HCV	hepatitis C virus
ICA	infectious center assay
IF	immunofluorescence
kb	kilobase
kDa	kilodalton
m^7GMP	methyl-^{7}guanosine monophosphate
MCS	multiple cloning site
m.o.i.	multiplicity of infection
MT	methyltransferase
NC	nucleocapsid
NLS	nuclear localization signal
NTP	nucleotide triphosphate
NTPase	nucleosidetriphosphatase
ns	nonstructural
nsP	nonstructural protein
nt	nucleotide(s)
P1234	polyprotein 1234 (the same abbreviation is used for P123, P12 and P34)
PFU	plaque forming unit
p.i.	post infection
RC	replication complex
RC_{MINUS}	replication complex synthesizing minus-strand RNA
RC_{PLUS}	replication complex synthesizing plus-strand RNA
RdRp	RNA-dependent RNA polymerase
RNase	ribonuclease
RT-PCR	reverse transcription – polymerase chain reaction

RTPase	RNA triphosphatase
SARS-CoV	severe acute respiratory syndrome coronavirus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
SG	subgenomic
SIN	Sindbis virus
ts	temperature sensitive
VEE	Venezuelan equine encephalitis
VLP	virus-like particle
wt	wild type

1. INTRODUCTION

Semliki Forest virus (SFV) is one of the best studied members of the genus *Alphavirus* (family *Togaviridae*). The alphavirus genome is a single-stranded positive RNA of approximately 11.5 kb in length. It encodes two large polyprotein precursors which are co- and post-translationally processed first into active processing intermediates and then to mature proteins (Strauss and Strauss, 1994). The structural proteins, encoded by the 3' third of the genome, are translated from a subgenomic (SG) mRNA generated by internal initiation on the complementary minus-strand template. In contrast, the nonstructural (ns) polyprotein, designated P1234, is translated directly from the viral genomic RNA. It is processed into its individual components, the nsP1-nsP4. The nsPs have the multiple enzymatic and non-enzymatic functions required in viral RNA replication (Kääriäinen and Ahola, 2002). The regulation of the replication of alphaviruses has been studied for several decades but many of the exact mechanisms involved in that process remain unknown.

One of the well-known approaches for the characterization and the analysis of SFV replication machinery is based on the use of conditional-lethal mutants. Several groups of SFV temperature sensitive (ts) mutants were isolated in 1970s (Keränen and Kääriäinen, 1974). These mutants have been divided into different phenotypic groups on the basis of their ability to induce viral RNA synthesis at the restrictive temperature. So far these ts phenotypes were not designated specifically to the mutations in the viral genome.

The broad host range of alphaviruses, their highly efficient gene expression and relatively simple genome organisation have facilitated the development of alphavirus-based gene expression systems. Alphavirus-based vectors and replicon systems have been extensively used in different studies and have a potential to be used in medicine. Unfortunately, it has been found that many of these vectors tend to suffer from genomic instability, especially *in vivo*.

Therefore, the aims of the current thesis were to enlighten the molecular mechanism of the regulation of the sequential processing of SFV polyprotein and based on this data to construct the SFV marker virus for studying viral pathogenesis *in vivo*; to map known ts mutations to the SFV genome, then study viral RNA synthesis and polyprotein processing using ts mutants and also test these mutations for a potential application for the construction of temperature-regulated expression vectors.

2. REVIEW OF LITERATURE

2.1. Alphaviruses

Alphaviruses represent a relatively small group of enveloped positive-strand RNA viruses, which belong to the family *Togaviridae*. Alphaviruses infect vertebrate hosts and are generally transmitted by arthropods, mainly mosquitoes. Birds and rodents serve as the natural reservoirs of alphaviruses, while man and bigger mammals are usually dead-end hosts. Alphaviruses are classified into three large groups according to their serological cross-reactivity and sequence homology: the VEE/EEE (Venezuelan equine encephalitis/ eastern equine encephalitis) group, the SFV group and SIN (Sindbis) group. By the diseases caused by alphavirus infection they can be divided into two pathological groups. New World alphaviruses (e.g. VEE and EEE) cause encephalitis, while Old World alphaviruses (e.g. SIN, SFV and Ross River virus) are milder pathogens and typically induce fever, rash and arthritis (Strauss and Strauss, 1994). Historically, the medical impact of alphavirus infection has been relatively low. However, it is rapidly changing since 2005, when a Chikungunya virus outbreak of unprecedented magnitude was detected and is currently ongoing in the Indian Ocean territories (Schuffenecker et al., 2006).

Among all alphaviruses the most studied are the two: SFV and SIN; their laboratory stains are considered non-pathogenic for humans. They have been extensively studied for already more than three decades and have become well-established models not only for alphaviruses, but for related pathogenic viruses as well. In addition, alphaviruses have been used as good models for studying viral replication, the modification and transport of glycoproteins, endocytosis and the process of membrane fusion (Helenius, 1995; Kielian, 1995; Atkins et al., 1999; Helenius and Aebi, 2001; Ellgaard and Helenius, 2003). Alphaviruses also represent one of the best known model systems of macromolecular assembly (reviewed in Garoff et al., 2004).

During the last 15 years, alphavirus-based vectors have been increasingly used as the expression vectors for basic research, for the production of recombinant proteins as well as for the generation of vaccine candidates and systems for gene therapy applications (see “Alphavirus-based expression vectors” section for details).

2.1.1. Virion structure

The structural architecture of alphavirus virions has been studied by cryo-electron microscopy image reconstruction and by X-ray crystallography of structural proteins (Vogel et al., 1986; Choi et al., 1991; Cheng et al., 1995; Mancini et al., 2000). Significant progress was made in the structural studies of alphavirus virions in the last years (Gibbons et al., 2004a; Gibbons et al., 2004b; Mukhopadhyay et al., 2006; Roussel et al., 2006).

SFV virion is a spherical enveloped particle with a diameter of about 65 nm. The inner nucleocapsid (NC) consists of the genomic RNA enclosed in the shell formed by 240 copies of the capsid protein (CP) arranged as hexamers and pentamers. The NC is surrounded by a lipid envelope which is usually derived from the plasma membrane of the host cell. The envelope contains virus-encoded glycoproteins: two integral membrane proteins E1 and E2, which are present in the form of heterodimers, and small peripheral membrane protein E3. Both the NC and the envelope are organized with T=4 icosahedral symmetry (Garoff et al., 1974; Cheng et al., 1995; Fuller et al., 1995).

2.1.2. Genome organization

Many positive-strand RNA viruses have significant similarities in their genome organization and based on this criterion they can be divided into large groups termed superfamilies. Members of the alphavirus-like superfamily include animal viruses from the genera *Alphavirus*, *Rubivirus*, and *Hepevirus*, as well as insect viruses of *Tetraviridae* family, and numerous groups of plant viruses. The replicase module of these viruses contains three conserved protein domains dedicated as methyltransferase (MT), helicase and RNA-dependent RNA polymerase (RdRp) domains. Other similarities shared by the members of alphavirus-like superfamily include membrane-associated replication, presence of cap0 (m^7GpppA) structure at the 5' end of plus-strand RNAs, an extra untemplated G residue at the 3' end of minus-strand of RNA, the asymmetry of replication producing an excess of plus-strand RNA, the shut-off of the minus-strand RNA synthesis in the late infection, and the regulated production of SG mRNA molecules by internal initiation on SG promoter in minus-strand template (Strauss and Strauss, 1994; Buck, 1996; Kääriäinen and Ahola, 2002).

The genomic RNA of alphaviruses (11.5 kb for SFV, 11.7 kb for SIN) is positive-sense single-stranded RNA and often designated as 42S RNA for SFV (Fig. 1). It can be divided into two parts: the 5' two-thirds encode for nsP1, nsP2, nsP3 and nsP4, which are required for RNA replication; and the 3' third of the genome encodes for the structural proteins: CP, E3, E2, 6K and E1, which are expressed exclusively from SG RNA (often designated as 26S RNA for SFV). Both 42S and 26S RNA molecules have a 5'-terminal cap0 and 3'-

terminal poly(A) tail structure similar to eukaryotic mRNA molecules (Sawicki and Gomatos, 1976; Kääriäinen and Söderlund, 1978; Strauss and Strauss, 1994).

2.1.3. Life cycle

The scheme of the alphavirus life cycle is shown in Figure 2. Alphaviruses can infect a variety of host cell types and appear to be able to utilize more than one type of cell surface receptor for entry. The entry pathway of SFV has served as a model for the studies of the entry process of enveloped viruses in general: SFV was the first virus shown to enter cells by receptor-mediated endocytosis via clathrin coated vesicles, followed by membrane fusion after the exposure to low pH in the endosomes (Helenius et al., 1980; Marsh and Helenius, 1989). Following internalization into the cell, the acidic pH of the endosomes promotes the dissociation of the E1-E2 heterodimers and the formation of new E1 homotrimers which represent an active form of the membrane fusion protein. This rearrangement leads to the fusion of the virion envelope with the endosome membrane. As a result, the NC is released into cytoplasm and becomes subsequently disassembled by ribosomes, thus releasing the RNA genome to the cytoplasm (reviewed in Garoff et al., 1994; Kielian, 1995).

Upon delivery to the cytoplasm, the viral RNA serves as mRNA for the translation of nsPs (Fig. 1). In the early phase of infection the 5' two-thirds of the 42S RNA are translated into a precursor polyprotein P1234, which is then autocatalytically cleaved into P123 and nsP4, yielding the early RNA polymerase, responsible for the minus-strand RNA synthesis. After the cleavage of P123 into nsP1, nsP2, and nsP3, in turn the minus-strand is used as template for the subsequent synthesis of new 42S RNA plus-strands, as well as 26S RNAs (reviewed in Kääriäinen and Ahola, 2002).

Alphaviruses alternate between the infection of arthropod and vertebrate hosts. Although the infection of vertebrates is acute and often accompanied by disease, the continuing transmission of the virus in nature requires that the infection of arthropods must be persistent and relatively asymptomatic. Therefore specific mechanisms for the control of viral RNA synthesis have evolved to moderate the pathogenicity of the viruses in their arthropod hosts. It has been shown that nsP2 serves as a master regulator of the host response to the infection regulating the infection outcome, lytic versus persistent infection (Kim et al., 2004; Sawicki et al., 2006). Details of this regulation are discussed in the section 2.1.4.

The translation of 26S RNA leads to the production of structural proteins, which are initially synthesized as a single polypeptide precursor CP-E3-E2-6K-E1 (Fig. 1). The CP is cleaved autocatalytically from the nascent chain to the cytoplasm of the infected cell, where it first associates with the large ribosomal subunit and then is rapidly transferred to the 42S RNA, which contains an

encapsidation signal, to form the NC (Ulmanen et al., 1976; Melancon and Garoff, 1987). After the cleavage of CP, the modifications of viral glycoproteins take place on the membranes of rough endoplasmic reticulum, where E3 and E2 are first released as the precursor protein p62, which is co-translationally glycosylated (Garoff et al., 1978; Pesonen and Kääriäinen, 1982). E1 is preceded by a hydrophobic 6K protein, the N-terminus of which serves as a signal peptide. The cellular signal peptidase cleaves between p62 and 6K, and between 6K and E1. The E1 and p62 form heterodimers and are transported via Golgi complex to the plasma membrane. During this transport the p62 is cleaved into E3 and E2 by furine-like protease from the *trans*-Golgi network (Melancon and Garoff, 1986; Garoff et al., 1990; Liljeström and Garoff, 1991b).

The final stage of virion assembly is initiated by specific interaction between the NC and the cytoplasmic tails of the spike protein E2 at the plasma membrane (Suomalainen et al., 1992). The carboxy terminal tail of the E2 protein contains a conserved Tyrosine-X-Leucine motif, which is important for the interaction of the spike proteins with the NC. This interaction triggers the release of viral particles from cells by budding (Sjöberg and Garoff, 2003). The functions of the hydrophobic 6K protein are not fully understood. It is known that this protein is membrane associated and covalently modified by fatty acid chains. Mutations and deletions in 6K affect virion assembly and budding stages, but are not lethal for SFV infection (Liljeström et al., 1991; Loewy et al., 1995; McInerney et al., 2004).

2.1.4. Replication strategies: how and where?

The replication strategy of SFV relies on the production of replicase proteins in the form of ns polyprotein precursor, which is then co- and post-translationally processed (Fig. 1). The processing of the ns polyprotein is performed by viral ns protease and is temporarily highly regulated. As indicated above, the P1234 is cleaved immediately after or during its synthesis, resulting in the formation of an early replication complex (RC) consisting of the P123 polyprotein and nsP4. This complex is responsible for the viral minus-strand RNA synthesis (RC_{MINUS}). The minus-strand RNAs are synthesized only during the first few hours of infection and their synthesis requires continuous translation (Sawicki and Sawicki, 1980). In contrast, positive-strand RNAs are synthesized throughout the infection and their synthesis continues even after the addition of protein synthesis inhibitors (reviewed in Kääriäinen et al., 1987). This behaviour was explained by differences in the composition of polymerase complexes and switching the template preference. When P123 is cleaved into mature nsP1, nsP2 and nsP3, they, in turn, together with nsP4 form the late positive-strand RNA synthesizing complex (RC_{PLUS}), responsible for the

synthesis of full-length genomic RNA and a SG mRNA (Lemm et al., 1994; Shirako and Strauss, 1994; Lemm et al., 1998; Gorchakov et al., 2004; Kim et al., 2004; McInerney et al., 2005).

The protease activity of nsP2 was mapped to its C-terminus (aa 459-799) which was classified as papain-like cysteine protease domain (Gorbalenya et al., 1991; Strauss et al., 1992; Vasiljeva et al., 2001). All the three junctions between nsPs are cleaved by nsP2 protease (Ding and Schlesinger, 1989; Hardy and Strauss, 1989), whereas the efficiencies of those cleavages are not equal. It was shown, using short recombinant substrates, that the site between nsP3 and nsP4 (the 3/4 site) was processed very readily, the 1/2 site was processed much less efficiently, and the cleavage of the 2/3 site remained extremely poor even with a large excess of enzyme (Vasiljeva et al., 2001). Presumably, the recognition of 2/3 site needs different requirements and plays a special role in switching from the early to the late replicase. The analysis of SFV cleavage sites with shuffled N-terminal and C-terminal half-sites showed that the main determinants of cleavage efficiency are located upstream of the cleavage site. Amino acid residues in positions P4, P3, P2, and P1 of the 3/4 site cannot tolerate much variation, whereas in the P5 position most residues were permitted. The aa in position P1' had a significant effect on cleavage efficiency, and in this regard the protease markedly preferred a glycine residue over the tyrosine natively present in the 3/4 site (Lulla et al., 2006).

The strategies used in RC formation by SIN and SFV differ in several aspects. In contrast to that of SFV, the P123 of SIN is an outcome of an opal termination codon after nsP3 gene present in SIN. Therefore in SIN infection the full-length P1234 is translated only by read-through of the opal termination codon (Strauss et al., 1983). It is also very much likely that the processing of P1234 of SIN may differ from that one of SFV (Golubtsov et al., unpublished).

At the late stages of alphavirus infection, the translation of cellular mRNAs and viral nsPs is suppressed and the processing pattern of the P1234 is altered. Because of the high free nsP2 concentration at the late stage of the infection, P1234 is cleaved prematurely at 2/3 site, yielding aberrant P12 and P34, not capable of active RC formation (Sawicki et al., 2006).

Alphaviruses encode only four nsPs, which are required for the replication of viral genome and for the transcription of the SG RNA. However, the RCs also appear to include cellular proteins and assemble on cellular organelles. The viral RNA synthesis takes place in the cytoplasm of infected cells and is associated with membranes. The analysis of the purified RC revealed all the four nsPs (Ranki and Kääriäinen, 1979; Gomatos et al., 1980). An original approach was used on SIN to determine the composition and location of RCs. The assembly of the in-frame fusion nsP3 with the green fluorescent protein (GFP) into stable complexes was studied at different stages of recombinant SIN infection. It was found that the protein complexes which are associated with nsP3-GFP contain a high concentration of cytoskeleton proteins, chaperones,

elongation factor 1A, heterogeneous nuclear ribonucleoproteins, 14-3-3 proteins, and some of the ribosomal proteins. These proteins were proposed to be essential for RC formation and functioning (Cristea et al., 2006; Frolova et al., 2006). Recently the recombinant SIN, expressing GFP in fusion with nsP2, was constructed and used to isolate the nsP2-specific protein complexes formed in the SIN infected cells. Besides the proteins previously detected in nsP3-GFP complexes, several new RNA-binding proteins were found by this approach (Atasheva et al., 2007). However, it should be mentioned that at the current stage the functional significance and/or the precise function of any of these proteins for alphavirus replication has not been directly demonstrated.

The late viral RNA synthesis in alphavirus-infected cells, which generates viral plus-strand RNAs, takes place at the structures called cytoplasmic vacuoles type I (CPVI, hereafter CPV), which are modified endosomes and lysosomes with a diameter of 0.2-1 μm. The membrane of CPV is covered by regular membrane invaginations or spherules (diameter 50 nm), which are shown to be the sites of RNA synthesis. Cryo-immunoelectron microscopy of infected cells at 5 to 6 hours p.i. has shown that all the four nsPs are associated with spherules together with nascent RNA, indicating that the active synthesis of plus-strand RNAs takes place in these structures (Froshauer et al., 1988; Kujala et al., 2001). The exact structure and composition, as well as the exact pathway of biogenesis of spherules, are not known. It was proposed that the nsP1 of alphaviruses acts as an anchor for the attachment of the RC to the membrane of spherule (Peränen et al., 1995) and that this association is already formed at the polyprotein stage (Salonen et al., 2003; Spuul et al., 2007).

2.2. Functions of the nonstructural proteins

Each of the alphavirus nsPs has been assigned specific roles in the viral replication based on the mapping and the analysis of the properties of ts mutants, the biochemical analyses of recombinant proteins, and primary sequence analysis (Kääriäinen and Ahola, 2002).

2.2.1. NsP1

NsP1 (in SFV 537aa, 64kDa) is a multifunctional protein and several of its functions have been revealed in the last decades.

NsP1 is the main enzyme taking part in the capping of alphavirus 42S and 26S RNAs. The first RNA capping reaction in alphaviruses is catalyzed by nsP2 (RNA triphosphatase, RTPase) whereas the next two are catalyzed by nsP1: methyltransferase (MT) and guanylyltransferase (GT). NsP1 is a guanine-7-methyltransferase, transferring a methyl group from S-adenosyl-methionine

(AdoMet) to guanine nucleotide. Evidence for this was obtained first for SIN, and then this activity was directly demonstrated on recombinant SIN and SFV nsP1 proteins, expressed in *E. coli* and in insect cells (Mi et al., 1989; Mi and Stollar, 1991; Laakkonen et al., 1994). The mechanism of viral nsPs catalyzed capping reaction differs from that used in cellular capping reactions (reviewed in Furuichi and Shatkin, 2000) (Table 1). First, in the MT reaction, nsP1 prefers GTP and dGTP as methyl group accepting substrates, whereas cellular enzymes methylate cap analogs and unmethylated guanosine-capped RNAs (Lampio et al., 1999). Second, in the GT reaction, nsP1 forms a covalent complex exclusively with m^7GMP, whereas cellular enzymes form a GMP-enzyme complex. NsP1 does not seem to have any obvious structural similarity to the cellular GT proteins, more specifically, there are no conserved lysine residues in nsP1. Instead, the histidine residue (H38 in SFV), absolutely conserved among all the alpha-like viruses, is a good candidate for the covalent binding of m^7GMP. This prediction has been supported by findings that mutations of this residue completely abolish covalent nucleotide binding by nsP1 (Ahola and Kääriäinen, 1995; Ahola et al., 1997; Ahola and Ahlquist, 1999; Kääriäinen and Ahola, 2002).

Table 1. Synthesis pathways of the mRNA cap structure in eukaryotes and viruses of alphavirus-like superfamily (modified from Shuman, 2002).

Capping in eukaryotic cells		Capping in alphavirus-like superfamily	
$pppN(pN)_n \rightarrow ppN(pN)_n + P_i$	**(RTPase)**	$pppN(pN)_n \rightarrow ppN(pN)_n + P_i$	**(RTPase, nsP2)**
$GTP + enzyme \rightarrow GMP\text{-}enzyme + PP_i$ $GMP\text{-}enzyme + ppN(pN)_n \rightarrow$ $\rightarrow GpppN(pN)_n + enzyme$	**(GT)**	$GTP + AdoMet \rightarrow$ $\rightarrow m^7GTP + AdoHcy$	**(MT, nsP1)**
$GpppN(pN)_n + AdoMet \rightarrow$ $\rightarrow m^7GpppN(pN)_n + AdoHcy$	**(MT)**	$m^7GTP + \mathbf{nsP1} \rightarrow m^7GMP\text{-}\mathbf{nsP1} + PP_i$ $m^7GMP\text{-}\mathbf{nsP1} + ppN(pN)_n \rightarrow$ $\rightarrow m^7GpppN(pN)_n + \mathbf{nsP1}$	**(GT)**

Thus, alphaviruses have a specific and unique mechanism for the cap synthesis. A similar virus-specific mechanism has been demonstrated for tobacco mosaic virus (Merits et al., 1999), brome mosaic virus (Ahola and Ahlquist, 1999), bamboo mosaic potexvirus (Li et al., 2001) as well as for hepatitis E virus (Magden et al., 2001). Thus, the same capping mechanism is used through the large alphavirus-like superfamily of RNA viruses. This functional similarity, together with aa sequence homology between virus specific MTs, indicates a common evolutionary origin of the superfamily (Kääriäinen and Ahola, 2002).

Tight binding to intracellular membranes is another well-documented function of nsP1. The membrane anchoring of nsP1 occurs when it is expressed alone in mammalian cells, indicating a possible role of nsP1 in anchoring the alphavirus RCs to intracellular membrane structures (Peränen et al., 1995; Ahola et al., 2000). NsP1 is palmitoylated on cysteine residues 418–420 in

yeast, insect and mammalian cells (Laakkonen et al., 1996). The substitution of those cysteines to alanines leads to the weaker membrane binding, but both SFV and SIN, containing this mutation, remain viable and can replicate in the cell culture. However, these mutant viruses have lost their pathogenicity for mice (Ahola et al., 2000) and the palmitoylation-defective mutants of SFV have shown the tendency to acquire compensatory mutations to restore high levels of RNA synthesis. Fluorescence microscopy studies revealed that these mutant nsP1 proteins remained bound to the membranes of infected cells (Ahola et al., 2000; Žusinaite et al., 2007). These facts, together with the viability of these mutant viruses, indicate the existence of palmitoylation-independent membrane-binding site in nsP1, and it was shown that the short peptide localized between aa residues 245–264 of nsP1 of SFV is indeed responsible for nsP1 membrane binding. The sequence comparison of the known nsP1 sequences of alphaviruses revealed that this peptide is well conserved (Ahola et al., 1999). It has been demonstrated that the association with anionic membrane phospholipids is absolutely required for the enzymatic activities of nsP1 (Ahola et al., 1999). It has also become clear that the nsP1 is the sole membrane anchor of the alphavirus RC, since the other replication proteins have no independent membrane affinity in transfected cells (Salonen et al., 2003). Currently the mechanism of the membrane binding of alphavirus RC looks as follows: initially, nsP1 reversibly associates with membranes via amphipathic membrane-binding peptide. This interaction followed by the palmitoylation of the nsP1 and then the palmitoylated form of nsP1 is tightly and irreversibly bound to the membrane (Lampio et al., 2000; Kääriäinen and Ahola, 2002; Spuul et al., 2007).

The role of nsP1 in the negative-strand RNA synthesis was first revealed by the use of SIN ts mutants: it was demonstrated that nsP1 directly participates in the initiation and the elongation of the minus-strand RNA synthesis. One of these mutants, termed ts11, exhibits a rapid and selective cessation of the minus-strand RNA synthesis upon temperature shift. The single point mutation, responsible for this phenotype, has been identified as A348T in nsP1 (Sawicki et al., 1981b; Hahn et al., 1989b). It was also demonstrated that during the minus-strand RNA synthesis the physical interaction between nsP1 and nsP4 takes place (Shirako et al., 2000) and it is logical to assume that this interaction is required for polymerase complex functioning.

2.2.2. NsP2

NsP2 (in SFV 799aa, 86kDa) is another multifunctional protein, which has several important functions in the viral RNA synthesis and polyprotein processing. The intracellular distribution of nsP2 correlates with its multiple activities: alphavirus nsP2 localizes both in the cytoplasm and the nucleus of the

infected cells (Peränen et al., 1990). In the cytoplasm, a part of the SIN nsP2 is associated with the large membranous complexes that contain polymerase activity, while similar amounts of the nsP2 can be found in the soluble fraction, which has almost no cellular organelles or membranes (Frolova et al., 2006).

NTPase activity of nsP2 was directly demonstrated for the recombinant SFV nsP2. It was also demonstrated that the point mutation in the putative NTP-binding motif GVPGSG**K**^{192}S to GVPGSG**N**^{192}S (K192N) leads to the complete inhibition of NTPase activity. The insertion of this mutation to the virus genome resulted in the almost complete inhibition of viral replication during the several days post transfection and only after longer incubation a revertant virus appeared (Rikkonen et al., 1994). Thus, NTPase activity of nsP2 is essential for efficient alphavirus replication.

The analysis of nsP2 sequence revealed typical helicase motifs in the N-terminal part of the protein (Koonin and Dolja, 1993). As RNA-helicase, SFV nsP2 was shown to unwind partially double-stranded RNA *in vitro* in the presence of NTPs or dNTPs. This activity was also completely inhibited by K192N mutation, thus indicating a partial overlap of the helicase and the RTPase functions in this site. RNA helicase activity was demonstrated only for the full-length nsP2, but not for its N-terminal half. The exact role of nsP2-helicase, as well as of other helicases from different plus-stranded RNA viruses, remains unclear. It is believed to function either as a separator of double-stranded RNA replication intermediates or in removing of the secondary structures from template RNAs (Gomez de Cedrón et al., 1999; Kääriäinen and Ahola, 2002).

As mentioned above, nsP1 has both MT and GT functions, required in mRNA capping reactions, but lacks RTPase activity. It was directly demonstrated by using short γ-^{32}P-labelled RNA molecules that nsP2 specifically cleaves the γ,β-triphosphate bond at the 5' end of RNA. The same activity was demonstrated for nsP2 of SIN, as well as for N-terminal fragment of SFV nsP2 (residues 1-470). Similarly to NTPase and helicase activities, RTPase reaction was also completely abolished by K192N mutation (Vasiljeva et al., 2000).

Many animal viruses encode proteinases, required for processing of viral polyproteins. NsP2 was first identified as a candidate for the role of the SFV ns protease, based on its homology with the known proteases (Gorbalenya et al., 1991). The mutation of the putative catalytic cysteine residue of nsP2 (C478A, nsP2^{CA}), when introduced into SFV polyproteins P1234, P123 and P23, completely abolished all proteolytic cleavages. It was also demonstrated that nsP2 is the only proteinase needed for the processing of the SFV P1234 (Merits et al., 2001). The isolated N-terminal fragment of nsP2 (Pro39) also has a protease activity. The activity of recombinant Pro39 can be inhibited by N-ethylmaleimide, Zn^{2+}, and Cu^{2+}, but not EDTA (ethylenediaminetetraacetic acid), PMSF (phenylmethylsulfonyl fluoride), or pepstatine, in accordance with the thiol proteinase nature of nsP2 protease (Vasiljeva et al., 2001). The

regulatory role of nsP2 protease in the replication process is discussed in details in section 2.1.4.

It has been demonstrated by immunofluorescence (IF) microscopy that a significant portion of the nsP2 is transported to the nucleus of the infected cells. The nuclear transport of nsP2 is rapid: ~50% of nsP2 was found in the nuclear fraction of SFV-infected BHK-21 cells after a short pulse followed by 20 min chase (Peränen et al., 1990). At the same time nsP2, if expressed alone, localized almost exclusively in the nucleus of BHK-21 cells. The nuclear localization signal (NLS) of the SFV nsP2 was identified as $R^{648}R^{649}R^{650}$ sequence and mutations changing $R^{648–649}$ to D (RDR, DRR and DDR) resulted in the cytoplasmic location of the nsP2 (Rikkonen et al., 1992). The nuclear localization of nsP2 apparently plays some role in SFV induced neuropathogenicity: R649D mutation in NLS significantly reduces the neuropathogenicity of SFV. Some delay in the P1234 processing in the cells, infected with mutant viruses, was also observed, suggesting that this mutation also affects the proteolytic activity of nsP2 (Fazakerley et al., 2002).

The genetic approach revealed that alphavirus nsP2 is involved in the synthesis of the 26S RNA and that some interaction between nsP2 and 26S RNA probably also occurs (Hahn et al., 1989b; Suopanki et al., 1998). However, it can not be excluded that the effect of mutations in the C-terminus of nsP2 on the 26S RNA synthesis might be partly connected to polyprotein processing since the synthesis from the 26S promoter is activated only by fully cleaved nsPs (Lemm et al., 1994; Shirako and Strauss, 1994). This finding reflects the fact that 26S promoter in the newly synthesized minus-strands is recognized by nsP2 as well (Sawicki et al., 1978; Suopanki et al., 1998). Shut-off of the minus-strand synthesis is also regulated by nsP2 (Sawicki and Sawicki, 1993; De et al., 1996; Suopanki et al., 1998), but curiously the minus-strand synthesis can be reactivated in several SIN ts mutants even in the presence of protein synthesis inhibitors (Sawicki et al., 1981b; Sawicki and Sawicki, 1986b; Sawicki et al., 1990; Sawicki and Sawicki, 1993). Though the mechanisms of this reactivation phenomenon are poorly understood, it is tempting to speculate that some conformational changes in mutant nsP2, resulting from temperature shifts, result in the rearrangement of RC and change the template preference in the case of these mutants. It was recently shown that alphavirus nsP2 functions in the engagement of the host response to infection and activates a switch from the early to the late phase of infection. The loss of this function leads to continuous viral minus-strand synthesis and the production of unstable RC_{PLUS} (Sawicki et al., 2006).

NsP2 is also responsible for the cytopathogenic effect caused by SFV and SIN in infected vertebrate cells. The expression of nsP2 alone is sufficient for inducing transcriptional inhibition in these cells. Mutations mapped to the C-terminal positions of nsP2 both in SIN and SFV led to a significant reduction of the virus induced cytopathic effect, possibly affecting both transcriptional

shutdown and apoptosis, the basic death mechanism in alphavirus infection (Perri et al., 2000). Interestingly, in contrast to SIN and SFV, the New World alphaviruses (VEE and EEE) have developed an alternative mechanism of transcription inhibition that is mainly determined by their CP, but not by the nsP2 (Garmashova et al., 2007).

Finally, the first crystal structure of alphaviral nsP has been solved recently: the structure of the protease domain of the VEE nsP2 was determined at 2.45 Å resolution. The structure of this enzyme was found to be rather unique. The VEE protease consists of two distinct domains: the N-terminal domain of nsP2 protease differs by its protein fold significantly from any known cysteine protease, and even more surprisingly the C-terminal domain of nsP2 protease displays structural similarity to S-adenosyl-L-methionine-dependent RNA methyltransferases. By the combination of these structural data with the data from genetic experiments and biochemical analysis it was suggested that this domain provides essential elements that may contribute to substrate recognition by alphavirus protease (Russo et al., 2006).

2.2.3. NsP3

Relatively little is known about the functions of nsP3 (in SFV 482 aa, 61 kDa), which is, however, somehow involved in the synthesis of viral RNA, since ts mutation in nsP3 of SIN renders the virus unable to produce RNA at nonpermissive temperatures (Hahn et al., 1989b). nsP3 is also suggested to function in the minus-strand and the SG RNA synthesis (LaStarza et al., 1994; Wang et al., 1994). In addition, several independent studies support the essential role of nsP3 in alphavirus virulence (Tuittila et al., 2000; Tuittila and Hinkkanen, 2003; Suthar et al., 2005; Galbraith et al., 2006).

The N-terminal region of nsP3 was defined as macro domain and is highly conserved among alphaviruses (Koonin and Dolja, 1993). The homologous domains were also found in bacteria, archae and eucaryotes (Pehrson and Fuji, 1998). The 3D-structure of macro domain of another positive-stranded RNA virus, the severe acute respiratory syndrome coronavirus (SARS-CoV), has been solved recently. It was shown that the macro domain of SARS-CoV dephosphorylates ADP-ribose-1"-phosphate, a side product of cellular tRNA splicing, to ADP-ribose in a highly specific manner (Saikatendu et al., 2005). However, the targeted mutagenesis of potential active-site residues in SARS-CoV had no apparent effect on the replication of the virus, indicating that coronavirus replicase polyproteins may have evolved to include nonessential functions (Putics et al., 2005). In the case of alphaviruses it was recently shown that the conserved N-terminal domain of nsP3 is capable of binding poly(ADP-ribose) with high affinity (Egloff et al., 2006). Is has also been proposed that the

macro domain is involved in the cell response to alphavirus infection (De et al., 2003), but so far the mechanism(s) of its activities have not been understood.

The C-terminal part of alphavirus nsP3 is highly variable both in length and sequence (Strauss and Strauss, 1994). It is phosphorylated at the cluster of serines and threonines by cellular kinases. The most active phosphorylation sites of SFV nsP3 were mapped to aa residues $S^{320,327,332,335}$ and $T^{344,345}$ (Peränen et al., 1988; Vihinen and Saarinen, 2000; Vihinen et al., 2001). The comparative analysis of SIN nsP3 mutants with changes in the N-terminal region showed that the reduced phosphorylation of nsP3 correlated with the reduced minus-strand RNA synthesis (De et al., 2003). However the elimination of nsP3 phosphorylation or even the deletion of the full C-terminal region of nsP3 did not abolish SFV replication (Vihinen et al., 2001).

IF microscopy of mammalian cells transiently expressing nsP3, revealed that nsP3 is localized in the structures somewhat similar to the CPV observed in virus infected cells. Biochemical analysis also revealed that nsP3 had weak membrane affinity (Peränen et al., 1988; Peränen and Kääriäinen, 1991). Indeed, it has been demonstrated that the formation of CPV structures requires the presence of nsP3 in the ns polyprotein precursors. In contrast to earlier reports it was shown that the structures formed by transiently expressed nsP3 do not have any similarities to real CPV, contain no membranes, have no clear structure and most likely represent the artefacts of high-level transient expression (Salonen et al., 2003). Therefore it is not clear which function the individual nsP3 might have in alphavirus infection.

2.2.4. NsP4

NsP4 (in SFV 614aa, 68kDa) is the catalytic subunit of the SFV RNA polymerase. This was first suggested by the presence of a GDD in the polymerase sequence motif, which is typical for all RdRps (Koonin and Dolja, 1993)**.** This suggestion was strongly supported by the experimental data obtained for various SIN nsP4 ts mutants: these mutants were unable to synthesize any viral RNA at nonpermissive temperature (Keränen and Kääriäinen, 1979; Sawicki et al., 1981a). The best-characterized mutant has been SIN ts6, which failed to synthesize any RNA upon shift to restrictive temperature, and had a specific defect in the elongation step in RNA synthesis (Barton et al., 1988).

As RdRp, mature nsP4 is required for RNA replication. More specifically, nsP4 and uncleaved P123 function in minus-strand RNA synthesis (Lemm et al., 1994; Shirako and Strauss, 1994), whereas individual nsP1, nsP2, nsP3 and nsP4 were capable to produce SG RNAs (Li et al., 2005). The binding of nsP4 to SG and genomic promoters has been extensively studied in SIN. By itself, nsP4 did not bind to the SG promoter. Rather, all the four nsPs were required

for the binding of the RdRp to the promoter; a peptide fragment directly binding to the SG promoter contained a sequence corresponding to aa residues 329–334 of nsP4 (Li and Stollar, 2004). A specific sequence in nsP4 responsible for recognition of genomic promoter was also determined: it corresponds to aa residues 531–538 of nsP4. The differences in nsP4 binding to SG and genomic promoters are strongly supported by the fact that specific aa residue changes in nsP4 (R331A and R332A) prevented the synthesis of SG RNA without affecting the synthesis of genomic RNA (Li and Stollar, 2007). Thus, alphavirus RdRps have different and distinct sites for the recognition of the promoters for the synthesis of SG and genomic RNA.

It was recently shown that the recombinant SIN Δ97nsP4, purified from *E. coli*, possesses terminal adenylyltransferase activity, as it specifically catalyzed the addition of adenine to the 3' end of RNA in the presence of divalent cations. This finding suggests a novel function of the alphavirus RdRp in the maintenance and repair of the poly(A) tail, an element required for the replication of the viral genome (Tomar et al., 2006). In addition to terminal adenylyltransferase activity, the real polymerase activity was shown for individual nsP4 which was purified from SIN infected BHK-21 cells: nsP4 containing the cellular extract was capable of copying SIN plus- and minus-strand template RNAs, but was not able to synthesize SG RNA (Thal et al., 2007).

NsP4 is the most unstable nsP of alphaviruses and its amount in the infected cell is low in comparison to other nsPs. In several alphaviruses like SIN, nsP4 is produced only by the translational read-through of the opal termination codon (UGA) upstream of the nsP4 gene (Strauss et al., 1983). Additionally, in alphavirus infected cells nsP4 is degraded by the N-end rule pathway in proteasomes (de Groot et al., 1991; Takkinen et al., 1991; Merits et al., 2001). However, it was demonstrated that the infected cells contain a pool of highly stable nsP4 molecules, and it was suggested that they may be protected from degradation by other components of the RC (Takkinen et al., 1991).

2.3. Alphavirus-based expression systems

Nowadays alphavirus, particularly SFV, are the most widely used viral expression vectors for transient gene expression (Baldi et al., 2007). The number of features, such as the rapid production of high-titer virus stocks, the broad host range (including a variety of mammalian cell lines and primary cell cultures), the high RNA replication rate in the cytoplasm and extreme transgene expression levels, led to the development of the broad range of vectors from SFV (Liljeström and Garoff, 1991a), SIN (Xiong et al., 1989) and VEE (Davis et al., 1989). Typical disadvantages of the alphavirus-based vectors are their short-term expression mode and cytotoxic effects on host cells by inducing the

shutdown of cellular biosynthesis and the cell death by apoptosis. On the other hand, these properties can be considered as useful under certain conditions, for example, in anti-tumour and anti-microbial immunotherapy (Riezebos-Brilman et al., 2006).

Alphavirus-based vectors represent important tools for several applications. For example, many transfection systems used for the studies of the cells from nervous system result in the significant transfection of glial cells with the minimal transfection of neuronal cells. If the goal of the research is to transfect neuronal cells rather than other cells, then alphaviruses represent almost an ideal delivery system. For example, the injection of SFV-GFP vector into neuronal tissue slice cultures resulted in the high percentage (> 90%) of the GFP-positive neurons (Ehrengruber et al., 1999; Lundstrom, 1999).

To overcome the cytotoxic effects caused by the alphavirus vectors, the less cytopathic vectors capable of persistent replication have been constructed for both SIN and SFV (Agapov et al., 1998; Perri et al., 2000). So far all the mutations resulted in reduced cytotoxicity are located in nsP2 gene. However, it is known that mutations and deletions in nsP3 (Tuittila et al., 2000; Vihinen et al., 2001; Galbraith et al., 2006) and in nsP1 (Ahola et al., 2000) could also be used to create less cytopathogenic vectors.

The development of ts alphavirus vectors is an area of significant interest for future biotechnology and gene therapy applications. A temperature-regulated SIN vector enabled the expression of the highly toxic apoptosis inducing death domain of the receptor interacting protein (RipDD) after induction at 29ºC, with no expression and high cell viability at the nonpermissive temperature of 37ºC (Boorsma et al., 2000). In a similar way, some of SFV ts vectors have been developed (Lundstrom et al., 2001a). The introduction of two additional point mutations in nsP2 to such a SFV vector resulted in a novel vector with both noncytopathogenic and ts phenotype (Lundstrom et al., 2001b).

There are many different ways for the construction of alphavirus-based expression vectors; below two of them will be discussed: the replicon vectors (also called non-replicating expression vectors) and the vectors expressing the foreign protein as a component(s) of the viral polyprotein (also called replication-competent marker viruses). Both of these vectors have a big potential in different applications and vectors of those types have been developed and tested in this study.

2.3.1. Alphavirus replicon vectors

In replicon vectors the region encoding for viral structural proteins (Fig. 1) has been replaced by a multiple cloning site (MCS). As a result, these vectors retain the entire ns region as well as the natural SG promoter. Packaged alphavirus-like particles can be produced by the cotransfection of *in vitro* transcribed replicon-

RNA and a helper-RNA, encoding for structural proteins (Liljeström and Garoff, 1991a; Bredenbeek et al., 1993). Productive replication and a high level of the expression of foreign genes can be initiated either by transfection of the replicon RNA into the cytoplasm of the cell or by the infection of the cells with replicon-RNAs packaged in alphavirus-like particles. The system is self-limiting because the helper-RNAs, which lack the packaging signal, are not encapsidated. Thus, replicons are single-cycle vectors incapable of spreading from the infected to the non-infected cells. The major drawback of this system is the risk, although at low probability, of the recombination between the two vectors, leading to the generation of wild type (wt) genomes (Weiss and Schlesinger, 1991). To reduce the appearance and/or amplification of replication-competent viruses, further manipulations of the helper plasmid have been engineered. In one approach mutation abolishing intracellular cleavage of p62 is introduced into the helper system. As a result, the expressed p62 cannot undergo normal proteolytic processing to activate viral entry functions, but may be activated by cleavage with chymotrypsin *in vitro* (Helper-2 system) (Berglund et al., 1993). In another approach, the structural genes are split between the two separate helper vectors: one expressing the CP, another – viral glycoproteins (Frolov et al., 1997; Smerdou and Liljeström, 1999).

It is also possible to enhance the expression of target genes by the use of the so-called capsid enhancer sequence. In earlier works it was noticed that in BHK-21 cells, infected with SFV based packaged replicons, the yield of recombinant β-galactosidase was approximately 50 μg per 10^6 cells, which was far below the amount of CP produced in virus-infected cells. This difference was found to be due to the presence of an "enhancer" sequence located in native 26S SG RNA at position 25 nt downstream of the start codon for CP. The use of the same sequence in replicon vectors stimulates translation (and foreign protein expression) about 10-fold (Sjöberg et al., 1994; Sjöberg and Garoff, 1996). Several applications of alphavirus replicon vectors have already been described in neurobiological studies, in gene therapy, for vaccine development and in cancer therapy (Lundstrom, 1999).

2.3.2. Replication-competent marker viruses

An alternative strategy to the removal of structural genes is to duplicate SG promoter, insert internal ribosome entry site (IRES) elements or to insert foreign genes into the natural gene expression units of the alphavirus genome. In the first vector of such a type the EGFP marker gene was inserted into the structural region of SIN. The 3' end of the EGFP sequence was linked to the sequence encoding the 2A autoprotease of the foot-and-mouth disease virus and then inserted between the capsid and E3 regions of SIN. This recombinant virus displayed greater marker protein expression stability and was less attenuated in

newborn mice than the corresponding vectors containing the duplicated SG promoter (Thomas et al., 2003). As with the expression from a duplicated SG promoter, this strategy has the potential for a high level of recombinant protein expression but it has also the potential limitation that the expression depends on the activation of the viral SG promoter and occurs at a relatively late stage of infection.

In order to take an advantage of the early expression, SIN genomes with a marker gene inserted into the replicase gene have been produced. First, nsP3-luciferase fusion viruses were constructed and used to study inhibition of alphavirus replication (Bick et al., 2003); nsP3-EGFP fusion viruses were constructed to study the formation of nsP3-specific protein complexes (Frolova et al., 2006). Similarly to nsP3-EGFP viruses, the SIN viruses encoding nsP2-GFP chimeric protein, were developed by the same research group by the use of transposon-based random library construction and selection. They demonstrated various applications of these viruses for the study of the functions of nsP2 in SIN infection. At the same time the attempts to construct similar fusion viruses for nsP1 region did not result in any infectious viruses (Atasheva et al., 2007). This may indicate that there are certain limitations in the construction of alphavirus genomes with marker proteins fused to their own nsPs.

The production of nonstructural-marker fusion proteins represents an important direction in the research of different RNA viruses. This approach has also been successful for poliovirus, hepatitis C virus (HCV), equine arteritis virus and several filamentous plant viruses (Dolja et al., 1993; Mueller and Wimmer, 1998; Moradpour et al., 2004; Rajamaki et al., 2005; van den Born et al., 2005). All these recombinant genomes have been used for multiple studies. As an example, an attractive approach was used on HCV. Different fluorescent proteins were inserted in frame into 3' terminal region of replicase protein NS5A, and simultaneous infection by two resulting replicons gave a perfect model for studying the superinfection exclusion in HCV infection (Schaller et al., 2007).

2.4. Temperature sensitive mutants

Ts mutations represent a specific class of conditional lethal mutations. They are characterised by a specific phenotype: the cells and the viruses harbouring them are able to grow at low temperature (called permissive) but are not able to grow at higher temperature (called nonpermissive or restrictive). Ts mutations often result from the changes at the sites within a protein, which are important for maintaining their molecular conformation: the structure of the mutant-protein is disrupted at higher temperatures, but is normal at lower temperatures. It has been shown a long time ago that the characterization of ts mutants, defective in

viral RNA synthesis at nonpermissive temperatures, allows the identification of possible functions of the wt counterparts (Burge and Pfefferkorn, 1966). Below the properties of ts mutants of SFV and SIN with defects in replication are discussed.

2.4.1. SFV ts mutants

Ts mutants of SFV were isolated from the N-methyl-N'-nitro-N-nitrosoguanidine treated virus culture (Keränen and Kääriäinen, 1974) and were divided into three phenotypic groups on the basis of their ability to induce viral RNA synthesis at the restrictive temperature (Table 2).

Table 2. Properties of the ts mutants of SFV (Keränen and Kääriäinen, 1974; Keränen and Kääriäinen, 1979). Specific references and additional information can be found in the text.

Mutant	Pheno-type	RNA synthesis at 39ºC % of wt	Shift up 28ºC to 39ºC		Molar ratio 42S:26S RNA		Late defect identified
			Accumulation of P123	PFU/cell	After shift to 39ºC	At 28ºC	
ts4	RNA^-	0.9	+	16	0.98	0.14	26S/protease
ts6	RNA^-	1.1	+	83	0.94	0.12	26S/protease
ts9	RNA^-	0.9	–	4	1.17	0.33	NONE
ts11	RNA^-	4.4	–	190	0.82	0.13	NONE
ts14	RNA^-	3.3	–	240	0.53	0.11	NONE
ts10	RNA±	7.2	–	0.003	0.43	0.16	NONE
ts13	RNA±	19	–	0.002	1.16	0.30	NONE
ts1	RNA+	114	P12/P123	0.001	1.10	0.60	26S RNA
wt	RNA+	100	–	140	0.50	0.13	NONE

Seven of SFV ts mutants, which were unable to synthesize virus specific RNA at nonpermissive temperature (39ºC), were classified as RNA^- (RNA-negative) mutants. Two mutants, designated RNA± (RNA plus-minus) mutants, made less virus specific RNA at the restrictive temperature than did the wt virus. Six RNA+ (RNA-positive) mutants synthesized 42S and 26S RNA at 39ºC at almost the same amounts and ratio as the wt virus. However, one of the RNA+ mutants, the ts1, made a higher proportion of 42S RNA, presumably at the expense of 26S RNA, both at 27ºC and at 39ºC. It also should be mentioned that the synthesis of 42S RNA and 26S RNA in the case of the wt SFV infected cells is somewhat temperature dependent on its own. There is an increase in synthesis of 42S RNA compared to the synthesis of the 26S RNA at 39ºC, the situation being reversed at 27ºC (Keränen and Kääriäinen, 1974; Keränen and Kääriäinen, 1975).

RNA$^-$ mutants were not able to synthesize the detectable amount of viral RNA at the nonpermissive temperature and accordingly they showed defects only in early functions. This group includes mutants designated SFV ts4, ts6, ts9, ts11 and ts14. Strictly speaking, two of them, ts11 and ts14, actually showed some RNA synthesis at the nonpermissive temperature, but were included in the RNA$^-$ group since in the shift-up experiment they behaved like RNA$^-$ mutants (Keränen and Kääriäinen, 1974).

The RNA synthesis by SFV ts4 at the restrictive temperature was found to be about 1% of that synthesized by the wt virus, whereas at the permissive temperature both 42S and 26S RNAs of SFV ts4 were synthesized normally. There was a cessation of 26S RNA synthesis after the shift to the restrictive temperature, while the synthesis of 42S RNA continued. The production of 26S RNA resumed when the infected cultures were shifted back to the permissive temperature, indicating that the defect was reversible (Saraste et al., 1977; Kääriäinen et al., 1978). SFV ts4 has been so far the only ts mutation identified and mapped to ns region of SFV. The corresponding lesion was found in the nsP2 region, where M781 has been changed to T. This change was found to be responsible for the inhibition of P1234 processing, for the inhibition of the normal shut-off of the minus-strand synthesis and for the inhibition of 26S synthesis possibly by detaching RC from the SG promoter (Suopanki et al., 1998).

There are two mutants of SFV, ts10 and ts13, which were defined as RNA± mutants. At restrictive temperature they showed somewhat more RNA synthesis than did RNA$^-$ mutants and therefore they had defects both in the early and in the late functions in the replication cycle. In addition, ts10 mutant showed a polymerase defect even at the permissive temperature (Keränen and Kääriäinen, 1974).

SFV ts1 mutant, which was determined as RNA+ mutant, is one of the most intriguing among all SFV ts mutants. At all tested temperatures this mutant synthesized considerably less 26S RNA than did the wt virus. Since the total amount of RNA synthesized by ts1 was not reduced, the defect of 26S RNA synthesis was compensated by the increased synthesis of 42S RNA. SFV ts1 was also found to be defective in structural functions: a single aa change in E3 protein (C58Y) inhibited the intracellular transport of SFV envelope protein complex. It was demonstrated that at 39ºC the envelope protein complex (E1-p62) of SFV ts1 was arrested in the rough endoplasmic reticulum. Only when the infected cultures are shifted to 28ºC, the complex was transported to the cell surface (Syväoja et al., 1990).

Thus, some limited information about SFV ts mutants has been available for some years. However, their full analysis has not been carried out and the potential of their use in SFV research has therefore not yet been exploited. In contrast, as reviewed below, many important investigations have been carried out with the use of the set of ts mutants of SIN.

2.4.2. SIN ts mutants

Ts mutants of SIN (Table 3) have been used in studies of the viral RNA synthesis, polyprotein processing and the intracellular transport of viral proteins, and the maturation of virus particles. These studies have yielded important insights into the different stages of viral RNA replication, such as the regulation of minus-strand and SG RNA syntheses. The RNA^- ts mutants of SIN are best characterized ts mutants of alphaviruses and have been historically assigned to four complementation groups: A, B, F and G. It was demonstrated later that these complementation groups correlate with specific mutations in nsPs (Rice et al., 1987).

Several useful outcomes from the investigation of SIN ts mutants have been already discussed in previous chapters. Therefore only the summary of the most important findings is given below.

Table 3. Properties of ts mutants of SIN (modified from Ahola, 1997).

Mutant	Mutation	Defect[a]
ts11	nsP1 A348T (rev)[b]	Minus-strand initiation/elongation
ts14	nsP2 E163K (rev)	Replication complex formation/conversion
ts16	nsP2 V275I	Replication complex formation/conversion
ts21	nsP2 C304Y (rev)	Replication complex formation/conversion
ts19	nsP2 L416S (rev)	Replication complex formation/conversion
ts118	nsP2 V425A (rev)	Replication complex formation
	nsP4 Q93R	Both mutations are needed for RNA^- phenotype
ts18	nsP2 F509L (rev)	Replication complex formation/conversion, 26S RNA, processing
ts17	nsP2 A517T (rev)	Replication complex formation/conversion, 26S RNA, processing, (–)RNA reactivation
ts7	nsP2 D522N	(–)RNA reactivation (both mutations), initial replication
	nsP3 F312S	complex formation
ts133	nsP2 N700K (rev)	Replication complex formation/conversion, 26S RNA, processing, (–)RNA reactivation
ts24	nsP2 G736S (rev)	Replication complex formation/conversion, 26S RNA, processing
ts138	nsP3 A68G	Replication complex formation/conversion
ts4	nsP3 A268V (rev)	Initial replication complex formation, 26S RNA, (–)RNA
ts6	nsP4 G153E (rev)	Elongation, 26S RNA
ts110	nsP4 G324E (rev)	Elongation, 26S RNA

a) General properties of the mutants have been examined by Keränen and Kääriäinen (1979), and by Sawicki and Sawicki (1985). See text for specific references.
b) rev indicates that a ts mutation has reverted back to the original aa.

First, the properties of some ts mutants, mapped in nsP4 (SIN ts6, ts110 and ts118), strongly supported the hypothesis that nsP4 is the catalytic subunit of alphavirus polymerase (Hahn et al., 1989a). Interestingly, it was found that some of the ts mutations in nsP4 can be complemented with the different mutations in other nsPs. This finding indicates the importance of interactions between the components of the RC for virus viability (Sawicki and Sawicki, 1985; Hahn et al., 1989a; Shirako et al., 2000; Fata et al., 2002b). Second, three mutations, which were mapped to nsP3 (SIN ts138, ts4 and ts7) resulted in defect in the minus-strand RNA synthesis (Hahn et al., 1989b; Wang et al., 1994), indicating that nsP3 is needed for formation of the initial minus-strand RC. Third, four mutations (SIN ts14, ts16, ts21 and ts19) mapped to the helicase domain of nsP2 resulted in defects in early infection: all these mutants stopped the accumulation of viral RNA upon shift to nonpermissive temperature, but previously assembled stable polymerases remained active. Thus the effect of these mutations could be explained by the defects caused in the assembly of initial minus-strand polymerase or in the conversion of this activity to the activity required for the plus-strand synthesis (Hahn et al., 1989b). Fifth, many ts mutations were mapped to the protease domain of nsP2 (SIN ts18, ts17, ts133 and ts24). All the viruses with corresponding mutations were found to be defective at polyprotein processing, which can be best observed in early infection. The majority of these mutants had complicate phenotypes, which in most cases resulted from the fact that these mutants carried more than one mutation (Sawicki et al., 1981a; Sawicki and Sawicki, 1985). Sixth, only one ts mutant, SIN ts11, was mapped to nsP1. It was shown that at restrictive temperatures ts11 causes a specific and rapid cessation of the minus-strand synthesis, while the synthesis of the plus-strand and SG RNA continued normally (Sawicki et al., 1981a), confirming the role of nsP1 in the formation of the early RC.

Thus, the extensive study of SIN ts mutants allowed the identification of various functions of nsPs and properties of RC; it also opened an opportunity for the usage of these mutations in the construction of the SIN based temperature-regulated expression vectors.

3. RESULTS AND DISCUSSION

3.1. Objectives

The first aim of the present study was to analyze the mechanisms involved in the regulation of the proteolytic processing of ns polyprotein P1234 of SFV. Detailed understanding of the molecular mechanism of the regulation of the sequential processing of SFV polyprotein and the analysis of the properties of nsP2 protease were then subsequently used for the design and construction of the new type of stable SFV marker virus.

Some limited information about SFV ts mutants has been available for some years. However, their location in the SFV genome was not known and the full potential of their use in SFV research has not yet been exploited. Therefore the second aim of the current thesis was to map the ts mutations of SFV, to study the viral RNA synthesis, polyprotein processing and other related functions of these ts mutants; and also to test these mutants for a potential application as a material for the construction of temperature-regulated gene expression vectors.

3.2. Mechanisms involved in regulation of processing of P1234

3.2.1. P1234 processing *in vitro* and *in vivo* (I, III)

Ns polyprotein P1234 is processed by nsP2 protease at three junctions: 1/2, 2/3 and 3/4. To understand the mechanism of orchestrating of its proteolysis, we analyzed the proteolytic processing of various *in vitro* translated SFV polyproteins (containing either non-cleavable sites or deletions in the vicinity of the cleavage sites) by different nsP2-containing proteases. Our results demonstrated different efficiency, order and mode (*in cis* or *in trans*) of the processing of different cleavage sites, suggesting the existence of possible control mechanism(s) for SFV P1234 processing.

It was shown previously on *in vitro* translated $P12^{CA}34$ substrate (C478A in nsP2, the mutation which inactivates protease activity) that both full-length nsP2 and its C-terminal part Pro39 (341 aa residues) efficiently cleave 3/4 site, whereas 1/2 site was processed less efficiently by both nsP2 and Pro39 (Vasiljeva et al., 2001). It was also shown that 2/3 site in $P2^{CA}3$ substrate was readily cleaved by nsP2, but very poorly by Pro39 (Fig. 7, **I**). This finding indicates that the cleavage of 2/3 site needs the N-terminal part of nsP2 which is missing in Pro39. At the same time it was found that the polyproteins, containing the non-cleavable 1/2 site (P1^23, P1^234, symbol ^ indicates that the proteins remain obligatorily connected), could not self-process at the 2/3 site

despite the presence of protease activity in their nsP2 region (Fig. 1, **I**). This indicates the inhibitory role of the unprocessed nsP1 part of polyprotein for the cleavage of 2/3 site.

To prove that the presence of unprocessed nsP1 has an inhibitory effect on the 2/3 site processing serial deletions were made in nsP1 part of P1^23. It was found that the largest deletion (64 C-terminal aa residues in nsP1 sequence were removed) did not activate the cleavage. In contrast, shorter internal deletions affecting the last 20 C-terminal aa residues of nsP1 (adjacent to the 1/2 site) caused the partial activation of the 2/3 processing; at the same time deletions extending over the cleavage point of the 1/2 site to the N-terminus of nsP2 eliminated this activation effect. Taken together, these results supported the hypothesis that the intact N-terminus of nsP2 is important for the 2/3 site processing, and only the release from the nsP1 allows the N-terminus of nsP2 to function as activator for 2/3 cleavage (Fig. 6, **I**). According to these and other experiments, cleavages at the 1/2 and 3/4 sites are independent of those taking place at the other sites, whereas proteolysis at the 2/3 site takes place only after the 1/2 cleavage.

To understand whether the cleavage of the 1/2 and 2/3 sites takes place *in cis* or *in trans* the effect of dilution on the processing kinetics of P12 and P23 was examined. It was found that in all of the diluted samples the amount of P12 continued to decrease during the observation period at about the same rate as in the undiluted sample. This indicates that the processing of P12 is an intramolecular reaction, i.e. it takes place *in cis*. This fact also explains why recombinant proteases cleave this site rather poorly *in trans* (Fig. 7D, **I**). In contrast, the dilutions drastically inhibited the processing of the 2/3 site, a finding which strongly supports the hypothesis, that 2/3 site is processed intermolecularly i.e. *in trans* (Fig. 3, **I**). It should be also mentioned that similar experiments carried out on the 3/4 site failed to produce clear results due to the extremely high speed of processing (our unpublished data). Therefore it can not be concluded how the 3/4 site is processed in infected cells: *in cis* or *in trans*. The fact that this site can be efficiently cleaved *in cis in vitro* does not prove that this is also the case in infected cells. It remains possible that in infected cells both ways of processing are used: in the early phase of infection the cleavage can occur *in cis* (yielding P123 + nsP4 complex) while in the later infection it most certainly happens *in trans* (results in processing of P34 into nsP3 and nsP4).

The fact that 2/3 site was cleaved very inefficiently by Pro39 (Vasiljeva et al., 2001), but quite efficiently by full-length nsP2 (Fig. 2B, lanes 10–12, **I**), suggested that nsP2 N-terminal sequence contains a "cofactor" required for this cleavage. Serial deletions in the N-terminal domain of nsP2 revealed that the possible cofactor would be located within the first 120 N-terminal residues of nsP2 (Fig. 5, **I**). The deletion of 5–20 N-terminal aa residues of nsP2 protease (Fig. 6, B and D, **I**) or the addition of extra aa residues to the N-terminus of

nsP2 (Fig. 7A, **I**) prevented the cleavage of 2/3 site, indicating that the intact N-terminus was one of the requirements of 2/3 site cleavage. Hence this cleavage also requires, in addition to 20–120 residues from the helicase domain, a precise N-terminus, which is released when nsP1 is cleaved from the polyprotein. Interestingly, these results and conclusions were confirmed by the recent studies of the nsP2 of SIN (Atasheva et al., 2007). These researchers found that the insertion of GFP sequence at the sites located in the N-terminus of nsP2 inhibited P23 processing. This study provides indirect evidence that the mechanism of 2/3 site processing is similar for SFV and SIN and that the cleavage rules revealed in *in vitro* experiments are also followed in infected cells.

Thus, the combined data from those studies indicate that the different regulation mechanisms are used in the early and the late processing pathways (Fig. 8, **I**). In contrast to the pathway used in early infection (described above), in later infection, when functional RCs are no longer formed, the polyprotein is first cut preferentially at the 2/3 site by free nsP2 protease and P12 and P34 become the dominant processing intermediates (Fig. 1B, **III**). The results obtained from the studies of the ts mutants of SFV also allow us to suggest that nsP2 plays a key-role in the viral replication since 6 out of 9 ts mutations were mapped to nsP2 and showed protease-dependent defects (**III**).

3.2.2. Possible mechanism for regulation of processing (I, III)

The synthesis of the alphavirus positive- and negative-strand RNAs and the transcription of the SG RNA are regulated temporally by the processing of the ns polyproteins (Lemm et al., 1994; Shirako and Strauss, 1994). According to the model, proposed by Lemm et al., nsP4 and uncleaved P123 serve as the minus-strand RNA replicase. The cleavage of P123 results in the cessation of the negative-strand RNA synthesis and the synthesis of both genomic and SG positive-strand RNAs. More recently, Fata et al. have suggested that the switch from the minus-strand synthesis to the synthesis of SG RNA is associated with the cleavage of P23 (Fata et al., 2002a).

Based on the data obtained for SIN and SFV, we proposed a model for the processing pathway of P1234 (Fig. 8A, **I**). The first and the most efficient cleavage of 3/4 site leads to the formation of the RC_{MINUS} (P123+nsP4). In this scheme the 3/4 site in P1234 is processed *in trans* (Fig. 8A, **I**). Scheme is based on the combined data from *in vitro* studies (Merits et al., 2001; Vasiljeva et al., 2001; **I**). However, it should be mentioned, that none of the experiments, described in these studies, rule out the possibility of the *in cis* processing of the 3/4 site in P1234. The direct proof of the existence of *in cis* pathway is still missing, but several of our subsequent studies, where the processing pattern of P1234 was studied in cells, infected with SFV mutants (Lulla et al., 2006; **II**),

indirectly support the hypothesis of the existence of both processing pathways. Therefore it is tempting to speculate that at the early stage of infection the cleavage of 3/4 site takes place *in cis*, i.e. the cleavage of P1234 into P123 and nsP4 is an intramolecular reaction. The next event is *in cis* cleavage of the 1/2 site and the formation of an intermediate unstable complex nsP1+P23+nsP4. In contrast to the cleavage of 3/4 site it takes place in a rather slow manner leaving for P123 enough time to function as part of RC_{MINUS}. The tight regulation of 2/3 site cleavage is preregulated by the cleavage of 1/2 site, so the proteolysis of P23 is the key-step in processing and converts replicase preference from the synthesis of minus-strands to the plus-strands, forming the stable RC_{PLUS}: nsP1+nsP2+nsP3+nsP4. The exact molecular basis of this switch is not known, but it can be speculated that the cleavage of 2/3 site triggers some conformational rearrangements affecting the template specificity of polymerase.

It is well known that the alphavirus nsP1 is a membrane-bound protein (Ahola et al., 1999), therefore the *in cis* cleavage of 1/2 site is almost certainly a membrane-associated process. In contrast, very rapid cleavage of the 3/4 site may happen before membrane binding of polyprotein. Most probably the slow cleavage of 1/2 site also enables the proper folding of the P123 polyprotein so that protein-protein interactions between the replicase components can take place before proteolytic processing (Salonen et al., 2003). Therefore it was proposed that the active membrane-associated RCs can be formed only through this polyprotein pathway. Only thereafter proteolytic processing converts the replicase into the stable polymerase, which synthesizes plus-strand RNAs. Later in infection, when the concentration of free nsP2 is high, co-translational *in trans* cleavage of site 2/3 is favored, as shown by the appearance of short-lived precursors P12 and P34 in infected cells (Fig. 8B, **I**) (Lachmi and Kääriäinen, 1976; Lehtovaara et al., 1980). At this point the possibility of the formation of functional RCs is lost; the P12 is processed *in cis* resulting in individual nsP1, which is targeted to the plasma membrane (Peränen et al., 1995), and nsP2, which is at least partially transported to the nucleus (Peränen et al., 1990). P34 is cleaved *in trans* by free nsP2 resulting in the aggregation of nsP3 in the cytoplasm (Salonen et al., 2003) where it may become rapidly degraded due to the properties of its C-terminal sequence (**II**). Similarly, the nsP4 is rapidly degraded via the ubiquitin pathway (Fig. 8B, **I**) (de Groot et al., 1991; Takkinen et al., 1991).

3.3. Construction and testing of novel marker viruses

3.3.1. Design of the replication-competent marker viruses (II)

First, we investigated whether the EGFP marker could be cloned into the C-terminus of nsP3 to form nsP3-EGFP fusion protein. The corresponding virus, designated SFV(3F)4-EGFP (Fig. 1A, **II**), was found to be viable and it expressed the expected nsP3-EGFP fusion protein. IF analysis revealed that as expected, the EGFP signal produced by this virus co-localized with the viral RC (Fig. 2C, **II**). In this respect the SFV(3F)4-EGFP resembles the recently described SIN construct also expressing nsP3-EGFP fusion protein (Frolova et al., 2006). In contrast to this SIN construct, which is reported to be rather stable, the plaque purification followed by the analysis of the fluorescence produced by individual plaques demonstrated that SFV(3F)4-EGFP was genetically highly unstable and therefore it was not suitable for most applications in the cell culture or for animal-based studies.

One likely explanation for the genetic instability of SFV(3F)4-EGFP is that the larger size of the nsP3-EGFP fusion protein affects replicase function and that this exerts a strong negative selection pressure. To produce a system with minimal impact on the viral replicase, the native C-terminus of nsP3 was restored by the duplication of its last 30 aa residues. An optimized artificial nsP2 protease recognition sequence was then inserted between the end of the nsP3 coding sequence and the start of the EGFP coding sequence thus flanking the EGFP marker protein with nsP2 protease recognition sites (Fig. 1A, **II**). Based on our studies of nsP2 and its substrate requirements as protease, the inserted sequence was originated from the 3/4 site which is cleaved with high efficiency by nsP2. The first aa residue after the inserted cleavage point was also changed from Y to G since the nsP2 protease prefers G in the P1' position and G represents a stabilizing aa (Varshavsky, 1996; Lulla et al., 2006). The P1' G was followed by aa residues 2–7 from the N-terminus of nsP4 to increase the processing efficiency at the recombinant site. The resulting virus was designated as SFV(3H)4-EGFP (Fig. 1A, **II**).

3.3.2. Properties and applications of SFV(3H)4-EGFP (II)

To determine whether genetic modifications used for the construction of SFV(3H)4-EGFP affected virus infectivity, infectious centre assays were performed. Typically $(3.1–4.4)\times10^5$ plaques per μg RNA were obtained with both pSFV(3H)4-EGFP and pSFV4 transcripts. The plaque sizes of these two viruses were also similar. To further characterize the growth of SFV(3H)4-EGFP relative to SFV4, one-step growth curves were compared. Relative to SFV4 virus, the production of the infectious SFV(3H)4-EGFP virus was

delayed by approximately one hour and the final titre was approximately 10-fold lower (Fig. 1B, **II**). However, since SFV(3H)4-EGFP was able to replicate to 10^9 pfu/ml within 10 hours, it can be concluded that the insertion of EGFP between nsP3 and nsP4 did not substantially affect recombinant virus multiplication.

The genetic stability of alphavirus vectors has been previously estimated by assessing marker gene expression in infected animals or in infected cell cultures (Cook and Griffin, 2003; Thomas et al., 2003). However, both of these methods are only able to detect relatively large changes in the virus population. The relatively simple and more sensitive method, used in this study, directly detected and quantified EGFP-negative genomes. This analysis revealed that 100% of plaque purified viruses from the second passage of SFV(3H)4-EGFP and >90% of plaque purified viruses from the fifth passage expressed the EGFP marker. Thus, despite the small duplication of the viral sequence necessitated by the addition of the nsP2 processing site upstream of the EGFP sequence, SFV(3H)4-EGFP demonstrated remarkably improved genetic stability relative to SFV(3F)4-EGFP. This stability was considerably exceeded compared to that observed for any of six different recombinant SFV vectors with a duplicated SG promoter (our unpublished results). However, it should be noted that at late passages some genomes with a deletion in the marker gene region were present in virus samples. Attempts to reduce this by removing 6 duplicated aa residues from the N-terminus of inserted EGFP, or by decreasing the length of the nsP3 C-terminal fragment fused to the C-terminus of EGFP resulted in viable viruses, but did not increase their genetic stability. Thus, our results indicate that the deletions in the marker gene do occur upon passage of the SFV(3H)4-EGFP virus. Whereas the frequency of this process may be low, given their growth advantage (Fig. 3B, **II**), deleted genomes are likely to increase in the viral population following multiple passages. Importantly, following the replication and spread of SFV(3H)4-EGFP in the mouse brain, no deletion variants were observed until the fifth passage demonstrating the utility of this virus for *in vivo* pathogenesis studies. The possible reason for higher *in vivo* stability includes the different cellular environment, differences in virus infection in infected cells as well as smaller viral amounts used for each infection (1,000 PFU for *in vivo* and 500,000 PFU for *in vitro* passage).

In SFV infected BHK-21 cells, nsP3 is known to associate with modified endosomes and lysosomes (Froshauer et al., 1988). In contrast, free EGFP is typically diffusely localized in both the nucleus and the cytoplasm. In SFV(3F)4-EGFP infected cells, EGFP co-localized with nsP3 to punctuate cytoplasmic structures, presumably virus RCs (Fig. 2C, **II**). In contrast, SFV(3H)4-EGFP infected cells showed granular cytoplasmic staining for nsP3 and diffuse predominantly nuclear staining for EGFP (Fig. 2D, **II**). The independent localization of nsP3 and EGFP in the SFV(3H)4-EGFP infected cells indicates that EGFP was released from the RCs.

The expression of the nsPs in SFV(3F)4-EGFP and SFV(3H)4-EGFP infected cells was examined by Western blotting (Fig. 2A, **II**). Individual nsP1, nsP2, nsP3, nsP4 and EGFP were readily observed in SFV(3H)4-EGFP infected cells. No nsP3-EGFP fusion protein was detected but a small amount of unprocessed EGFP-nsP4 was detected by antibodies to nsP4 and, albeit to a lesser extent, to EGFP (Fig. 2A, **II**). In SFV(3F)4-EGFP infected cells, nsP3 was present predominantly as an nsP3-EGFP fusion protein. All these data indicate that the artificial cleavage site between the nsP3 and EGFP was cleaved very efficiently.

To study the dynamics of cleavage at the inserted cleavage site between nsP3 and EGFP, the cells infected with SFV(3H)4-EGFP were metabolically pulse-labelled and the nsP3 and its precursors immunoprecipitated with an anti-nsP3 antibody. By this approach P123-EGFP, P3-EGFP-4 and P3-EGFP processing intermediates were all detected. In chased samples processing intermediates were not detected (or were present in lower quantities) and the amount of fully processed nsP3 was increased. Thus, all the expected processing products, except the very rapidly processed P1234 and P123-EGFP-4, were detected and no apparent disturbance of polyprotein processing was observed for SFV(3H)4-EGFP (Fig. 3). As it is evident from Figure 3, the artificial site between nsP3 and EGFP was processed very rapidly: only traces of nsP3-EGFP fusion protein are visible in the pulse sample and its amount is below the detection limit in the chased sample. Thus, the addition of the fourth cleavage site in the ns polyprotein did not change the processing pattern of the ns polyprotein.

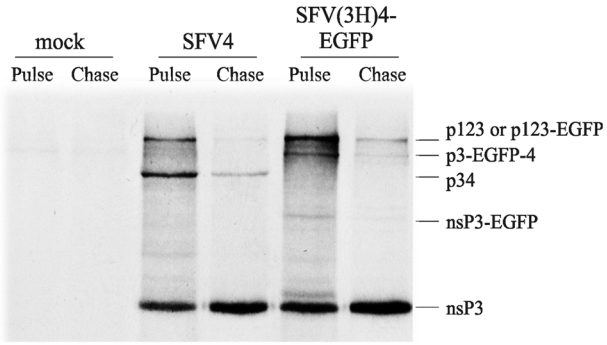

Figure 3. Polyprotein processing in SFV(3H)4-EGFP and SFV4 infected BHK-21 cells. At 3 hours p.i. cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 15 minutes (pulse) followed by chase for 45 minutes with medium containing an excess of unlabelled methionine and cysteine. Cell lysates were immunoprecipitated with an anti-nsP3 antibody and analyzed by SDS-PAGE and visualized by autoradiography.

In BHK-21 cells EGFP is generally quite stable with an estimated half-life of 24h. However, in BHK-21 cells infected with SFV(3H)4-EGFP the EGFP signal detected late in infection by fluorescence microscopy had decreased considerably suggesting low EGFP stability. To assess the stability of the EGFP and nsPs in the infected cells, BHK-21 cells infected with SFV(3H)4-EGFP or SFV4 were metabolically labelled with [^{35}S]methionine/cysteine, chased for 1, 3, 8 or 24 hours and labelled EGFP, nsP1 and nsP3 were immunoprecipitated. For both viruses the nsP1 was stable over the 24 hours chase period and only a small decrease in the amount of nsP3 was detected (Fig. 2B, **II**). In contrast by 24 hours the amount of EGFP was below the detection limit of this analysis (Fig. 2B, **II**). Thus the low amounts of EGFP observed by fluorescent microscopy represent the rapid degradation of EGFP rather than the low levels of expression. EGFP has been repeatedly used as a marker protein in alphavirus vectors and such an effect has never been described previously. Therefore it is logical to assume that the modification in EGFP termini, resulting from the cloning procedure, must be responsible for the protein instability. The processed EGFP has a G residue at its N-terminus (Fig. 1A, **II**) which according to the N-end rule is a stabilizing residue. Therefore the most likely explanation is that the instability results from the duplicated C-terminal sequence of nsP3 (aa residues 453–482; Fig. 1A, **II**) which remains attached to the C-terminus of EGFP. If this is the case, then the effect of the same sequence on its native location (at C-terminus of nsP3) is either different or is suppressed by interaction(s) with other components of SFV RC. This explanation is supported by our observation that the nsP3-EGFP fusion protein, expressed by SFV(3F)4-EGFP, is much more stable than the EGFP expressed by SFV(3H)4-EGFP.

The RNA synthesis by SFV4 and SFV(3H)4-EGFP was compared by [^{3}H]-uridine labelling of total virus RNA and by the Northern blot analysis. Both techniques revealed a delay in virus RNA synthesis in SFV(3H)4-EGFP infected cells relative to SFV4 infected cells (Fig. 3A, **II**); this was most obvious at 3–4 hours p.i. To analyze whether the insertion of the marker genes altered the temporal expression of SFV structural proteins or the shutdown of host cell translation, protein synthesis in BHK-21 cells infected with SFV4 or SFV(3H)4-EGFP was studied by metabolic labelling. As observed with virus growth (Fig. 1A, **II**) and virus RNA synthesis (Fig. 3A, **II**), the production of viral proteins C, E1 and p62 in SFV(3H)4-EGFP infected cells started approximately 1 hour later than in SFV4 infected cells (Fig. 3B, **II**). A longer, approximately 2 hours delay, was observed for the host cell translational shutdown.

The *in vivo* phenotype of the recombinant virus was detected in animal experiments. It was found that after intracerebral infection in mice the recombinant virus was able to replicate and spread efficiently in the mouse brain; furthermore SFV(3H)4-EGFP infected brain cells expressed sufficient amount of EGFP to be observed by fluorescence microscopy (Fig. 1E, **II**).

Taken together, the virus constructed in this study, SFV(3F)4-EGFP, was similar to the SIN constructs described earlier (Bick et al., 2003; Frolova et al., 2006). Although the SFV(3F)4-EGFP virus was viable it was genetically unstable, most probably due to defect(s) in the formation and/or functioning of the virus RCs. This would be consistent with the finding that the position of EGFP insertion into the SIN nsP3 region affects the stability of the construct (Frolova et al., 2006). As with SIN nsP3-fusion constructs (Frolova et al., 2006; Ventoso et al., 2006) SFV(3F)4-EGFP and its analogues may have uses in *in vitro* systems to study replicase gene expression, replicase protein localization and interactions, but its instability precludes application in *in vivo* pathogenesis studies.

The EGFP marker gene was effectively moved to the nsP3/nsP4 junction and flanked on both sides by nsP2 recognition sites in SFV(3H)4-EGFP. In all the measurements of virus replication and growth SFV(3H)4-EGFP was slightly slower than the parental SFV4. The possible reasons for this are numerous but include the effects resulting from the times required for the transcription and the translation of the larger genome or larger ns polyprotein sizes and delays or defects in RC formation. This new system was designed to allow EGFP to be easily substituted with other markers such as *Renilla* luciferase. Our strategy of marker insertion at this point in the replicase gene is also compatible with the introduction of mutations in other structural and nonstructural regions of the SFV genome (our unpublished results). The new constructs described here are likely to have utility in many experimental studies both in the cell culture and in animal model systems.

3.4. Identification and analysis of SFV ts mutants

3.4.1. Mapping of ts mutations (III)

Virus stocks of SFV ts mutants were isolated more than 30 years ago (Keränen and Kääriäinen, 1974) and stored at –70ºC in Helsinki. These viruses were used as the source of the RNA for RT-PCR and sequencing as well as materials for comparison with newly constructed recombinant viruses. We have sequenced the entire ns region of the second passage stocks of SFV ts1, ts6, ts9, ts10, ts11, ts13, and ts14 and published these sequences in the GenBank database (Table 1, **III**). We were particularly interested in ns defects to tie the replication phenotype with the detected mutation in nsPs. However the structural part of two mutants, ts10 and ts13, was also sequenced (GenBank accession numbers DQ189082 and DQ189084, respectively), but the analysis of the identified structural defects of these mutants remains to be performed.

Overall five missense mutations were mapped to nsP2, two mutations were mapped to nsP1 and ts13 was found to have two mutations: one in nsP2 and

another in nsP4 (Table 1, **III**). No mutations were found and therefore no new insights into the poorly understood functions of nsP3 were gained in the present study. Similar to our results, most of the ts mutations of SIN were also found to localize in the nsP2 region indicating its key role in the replicase formation and functioning. The localization of the identified ts mutations of SFV and those published for SIN is summarized in Figure 4.

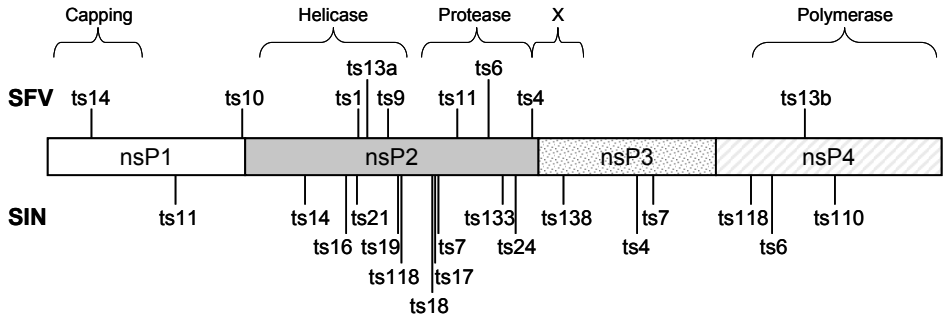

Figure 4. Identified ts mutations of the ns region of SFV (**III**) and SFV ts4 mapped by Soupanki et al. (1998) are given in upper panel. SIN mutations (see section 2.4.2. for references) mapped to the ns region are given in lower panel. Functional domains are indicated above.

To confirm that the identified mutations were indeed responsible for the ts phenotype of the corresponding genome, the revealed nt changes were introduced into a wt full-length infectious cDNA clone of SFV (pSFV4). The resulting recombinant clones and viruses were used to prove that they would recapitulate the original ts phenotype. The recombinant ts viruses with RNA$^-$ phenotype were designated SFots6, SFots9, SFots11 and SFots14. When the recombinant virus had the mutations, originating from the mutants initially classified as RNA+ or RNA± (implying the presence of additional mutations in the structural proteins) introduced in its ns region, recombinant viruses were named SFons1, SFons10, or SFons13. For ts13, which had two aa changes in the nsPs (Table 1, **III**), three separate recombinant viruses were generated: SFons13ab (contained both changes), SFons13a and SFons13b (each one contained one change). The mutation in ts4 was mapped previously (Suopanki et al., 1998), and both ts4 and SFots4 were included in the current research as additional controls. All these recombinant viruses and corresponding controls have been used for the studies of virus replication, the RNA synthesis, polyprotein processing and for the analysis of other properties related to the known functions of nsPs.

3.4.2. Properties of the original and recombinant ts viruses (III)

Several assays were performed to compare the properties of the original and recombinant ts virus strains (Table 2, **III**). It was needed in order to prove that the identified mutations are the ones responsible for ts phenotype. Additionally it was needed because the pSFV4 and original ts mutants have a slightly different genetic background and, finally, because the assay components (chemicals, media, etc) have been changed over 30 years making the results obtained in the 1970s difficult for direct comparison with those from the early 2000s. First, the both original and recombinant virus stocks were titrated in BHK-21 cells at 28°C (permissive temperature) and at 39°C (restrictive temperature) by plaque assay. Second, to measure the leak yield at the restrictive temperature, two parallel sets of BHK-21 cells were infected with each virus stock at m.o.i. of 10, one was incubated at 28°C for 16 hours and another at 39°C for 8 hours. The accumulated virus stocks were collected and titrated by plaque assay at 28°C. Third, the total RNA synthesis at the restrictive temperature and SG RNA synthesis after the shift-up to the restrictive temperature was measured. Forth, the polyprotein processing was studied after shift-up to the restrictive temperature.

As expected, the wt virus used as a control, did not reveal any significant temperature-dependent behaviour. In contrast, both the original ts mutants and the newly constructed recombinant viruses displayed the set of ts phenotypes. As it was already discussed, the RNA+ mutant ts1 has a defect in the intracellular transport of the envelope protein complex due to a mutation in the E3 (Syväoja et al., 1990) and it makes a reduced amount of 26S RNA. This second defect was found to be due to the mutation S308N in the helicase domain of nsP2 (Table 2, **III**): SFons1 grew like a wt virus at 39°C, but produced considerably less 26S RNA (Fig. 1A, **III**). It was demonstrated later that ts1 had a wt-like protease activity both *in vivo* and *in vitro* (Balistreri et al., 2007). Thus the phenotype of ts1 is unlikely due to the defect in ns polyprotein processing, the reverse is true for the most of the rest ts mutants (see below). These findings suggest that there are several mechanisms by which different ts mutants mapped to nsP2 region cause the reduced SG RNA synthesis (for comparison, see the results obtained for ts9 and ts13a below). In this respect ts1 forms a unique class: the ts1 mutation itself is located in helicase motif IV, which is involved in RNA binding (Caruthers and McKay, 2002) and ts1 appears to be unique in overproducing the nsPs (Sawicki and Sawicki, 1986a). Therefore it can be suggested that ts1 could influence SG RNA synthesis either by affecting the RNA binding of replicase or helicase activity of nsP2.

Mutation ts9 is also mapped to the helicase domain of nsP2. The corresponding recombinant virus, SFots9, fully recapitulated the RNA^{-} phenotype of the original virus ts9, demonstrating clear defects in virus titer, leak and 26S RNA synthesis (Tabe 2, **III**). An interesting pattern was observed

in SFots9 ns polyprotein processing after the shift-up to the restrictive temperature: the mutation caused the accumulation of P123 precursor (Fig. 5), whereas other ts mutations in nsP2 caused the accumulation of both P1234 and P123 (Fig. 1B, **III**). In *in vitro* experiment no defect in the cleavage of the Trx34 substrate was observed for the ts9-nsP2, but the enzyme was found to be inactive in cleaving 2/3 site in recombinant substrate (Balistreri et al., 2007).

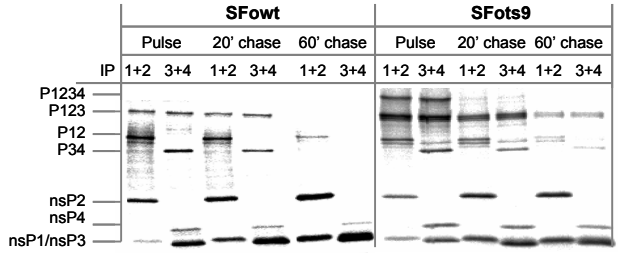

Figure 5. Analysis of ns polyprotein processing of recombinant viruses SFowt and SFots9 after shift-up to the restrictive temperature. For experimental procedure see legend to Fig. 1B (**III**).

Thus, ts9 mutation in the helicase region affects the protease activity not in general, but in a site-specific manner. We have demonstrated that the 2/3 site processing requires the correct N-terminus of nsP2 (**I**). A specific effect of the ts9 mutation on the 2/3 site processing may therefore result from the disturbance of the N-terminus of nsP2. Finally, ts9-nsP2 was almost completely inactive as an NTPase and RTPase when assayed *in vitro* (Balistreri et al., 2007). It is worth to mention that SIN ts mutants, mapped to the helicase domain of nsP2, do not have protease defects (De et al., 1996) despite of the fact that several ts mutations of SIN are located at the same area as SFV ts9 (Fig. 4).

Third ts mutant mapped to helicase domain of nsP2 was ts13. As indicated above, it contained two aa changes in the ns region (Table 1, **III**). Their combination in SFons13ab was required to reproduce the phenotype of ts13, which now scored as RNA^{-} (original ts13 behaved as RNA±, Keränen and Kääriäinen, 1974). The reduced RNA synthesis at the restrictive temperature was observed both in SFons13a and SFons13b, but the mutation in nsP2 caused a stronger defect (Table 2, **III**). It was also found that the recombinant nsP2 with ts13a mutation showed unexpectedly severe protease defect both *in vivo* and *in vitro*. Finally, similarly to that found in ts9, ts13a-nsP2 had also reduced RTPase and NTPase activities *in vitro* (Balistreri et al., 2007). Interestingly, there is also a SIN ts mutant in which mutations both in nsP2 and nsP4 are required for the ts phenotype, and this has been interpreted as evidence supporting the existence of a functional interaction between these proteins (Hahn et al., 1989a).

The original ts6 behaved as RNA^- mutant with defects in ns polyprotein processing and 26S RNA synthesis (Keränen and Kääriäinen, 1979), whereas SFots6 showed lesser defects in the virus titer and RNA production: compared to the wt virus SFots6 had three fold reduction of RNA synthesis at the restrictive temperature, much less than original RNA^- ts6 stock (Table 2, **III**). A possible reason for such behaviour could be that the genome sequences of pSFV4 and the original cloned wt (parental sequence of ts6) have several sequence differences. However, SFots6 was still defective in polyprotein processing and 26S RNA synthesis, showing that these phenotypes were due to the mutation A662T in nsP2 (Fig. 1, **III**). Additionally that mutation resulted in greatly reduced protease activity of the purified nsP2 protease (Balistreri et al., 2007). Similar results have also been obtained for the isolated protease domain, Pro39, containing individual mutations ts4, ts6 and ts11. It was also demonstrated that after a short exposure to 39ºC the cleavage efficiency of Pro39 carrying these ts mutations did not revert to the initial 28ºC level. Therefore it was suggested that ts4, ts6 and ts11 mutations affected rather protein stability than protease catalytic activity itself (Golubtsov et al., 2006).

The mutation in ts11 was mapped to the protease domain of nsP2. SFots11 fully reproduced the phenotype of the original virus ts11, showing clear defects in polyprotein processing and a partial defect in 26S RNA synthesis (Table 2, **III**). Similarly to ts6 and ts4, containing mutation in protease domain of nsP2, ts11-nsP2 had reduced protease activity at higher temperatures *in vitro*, but displayed normal NTPase and RTPase activities (Balistreri et al., 2007). Thus, as it was logical to expect, all the ts mutations located within the C-terminal protease domain of nsP2 caused defects in polyprotein processing. All the mutations resulting in defective 26S RNA synthesis were found to localize within nsP2, and this defect was observed irrespective in which domain (helicase or protease) the mutation was detected. In contrast, as it has been shown previously, SIN ts mutations located within the protease domain often caused an associated defect in SG RNA synthesis, but mutations in SIN helicase domain (with the possible exception of SIN ts21) did not (Sawicki and Sawicki, 1985; Hahn et al., 1989b; De et al., 1996; Suopanki et al., 1998).

As it was revealed on the recently solved 3D-structure of the VEE virus protease, alphavirus protease consists of two subdomains: the N-terminal core protease domain and the C-terminal methyltransferase-like domain. It was proposed that the C-terminal domain also participates in substrate binding (Russo et al., 2006). Concerning the mutations detected and analyzed in this study, the ts11 lesion is localized in the core protease domain while ts4 and ts6 are localized in the methyltransferase-like domain. Since ts6 and ts4 both have clear defects in polyprotein processing, then our findings also support the idea that the C-terminal domain of nsP2 protease is indeed essential for protease activity.

3.4.3. Functional analysis of ts mutations mapped to nsP1 (III, IV)

Two mutants, ts10 and ts14, were found to contain mutations in nsP1 region (Table 1, **III**). The mutant ts10 was classified as RNA± and, accordingly, the ns mutation of SFons10 did not considerably reduce the virus yield. At the same time this mutation resulted in the reduction of the RNA synthesis at the restrictive temperature, and the observed level of the RNA synthesis was typical for RNA± mutants. In contrast, SFots14 exactly reproduced the RNA$^-$ phenotype in the titer, the leak and the RNA synthesis (Table 2, **III**). Neither SFons10 nor SFots14 did have a defect in 26S RNA synthesis (Table 2, **III** and Table 1, **IV**).

Since ts10 was mapped close to the 1/2 site, it was logical to assume that it can cause a defect in the processing of this site. The 1/2 site cleavage plays a central role in regulating the template switch (plus- versus minus-strand) in the RNA synthesis of alphaviruses (Hahn et al., 1989b; Hardy and Strauss, 1989) and the processing defect could explain some ts properties of the mutant virus. However, it was found that in processing of *in vitro* translated P12-ts10 and P12-ts14 the first order kinetic constant did not differ significantly from that of wt-P12, whereas P12-ts4, used as a control, had greatly reduced kinetic constant values at higher temperatures (Fig. 1C, **IV**). Similarly, in SFons10 infected cells, the polyprotein processing occurred exactly as during infection with wt SFV, whereas in SFots14 infected cells the processing of P1234 was only slightly slower (Fig. 1D, **IV**). This defect was very mild compared to the defects in polyprotein processing observed for SFots4, SFots6, SFots9 and SFots11 (Fig. 1B, **III**, Fig. 5). Therefore we conclude that under studied conditions ts10 and ts14 do not have significant defects in polyprotein processing.

Since ts14 was mapped within the capping domain of nsP1, we performed the *in vitro* analysis of the known capping reactions of SFV. We demonstrated that nsP1-ts10, nsP1-ts14 and nsP1-wt showed equal and strong MT activity both at 28°C and 39°C (Fig. 2B, **IV**). Similarly to MT, in GT reaction nsP1-ts10, nsP1-ts14 and nsP1-wt also demonstrated equal and strong activity at both temperatures (Fig. 2C, **IV**). Finally, the flotation analysis of nsP1-ts10 and nsP1-ts14 revealed that both mutant proteins were, similar to nsP1-wt, membrane-associated in this system (Fig. 5, **IV**). The subcellular localization of nsP1-ts10 and nsP1-ts14 was studied in HeLa cells. Both the wt and the two mutant proteins showed the association with the plasma membrane, with the induction of prominent filopodia-like structures at both temperatures (data not shown), as it has been previously described for wt nsP1 (Laakkonen et al., 1996; Spuul et al., 2007). These results strongly suggest that defects in the RNA capping reactions are not the reasons behind ts phenotypes caused by ts10 and ts14 mutations.

The only ts mutation mapping to nsP1 in SIN is ts11. This mutant was found to be specifically defective in the minus-strand synthesis at the restrictive

temperature (Sawicki et al., 1981; Wang et al., 1991). Therefore the synthesis of the minus-strand RNA was also measured for SFots14 and SFons10 and the SIN ts11 was used as control in all these experiments. It was found that when infected cells were shifted to 40°C at 3 hours p.i. or 4 hours p.i., a rapid cessation of the minus-strand synthesis by SFons10 and SFots14, similar to the rapid shut-off by SIN ts11, occurred (Fig. 4, **IV**). This proves that similarly to the ts11 of SIN the SFV nsP1 mutants ts10 and ts14 are specifically defective in minus-strand RNA synthesis. The shift to 40°C and incubation in a medium containing cycloheximide led to a rapid cessation of the minus-strand synthesis by SFons10, SFots14, and SIN ts11 (data not shown). The rate of the loss of activity was similar to that seen for the temperature shift alone. This indicates that the shift to 40°C alone was sufficient to prevent further production of functional nsP1. Thus our results further support the conclusion that the SFV nsP1 mutants have ts defects in the functions required for the minus-strand synthesis, possibly in promoter recognition or the initiation of the synthesis. Therefore, our results support the idea that alphavirus nsP1 is specifically involved in the regulation of the minus-strand RNA production.

The minus-strand synthesis depends on the formation of the functional replicase consisting of several subunits. One possible explanation of the defect in the minus-strand synthesis is a failure of nsP1 to interact with its partners required for replicase formation. To study the possible defects in protein-protein interactions, both nsP1-ts10 and nsP1-ts14 were co-expressed in BHK-21 cells with nsP3. In this assay both mutant proteins showed clear interaction, similar to that shown by nsP1-wt at all used temperatures (Fig. 5B, **IV**). This finding does not, however, rule out the possibility that the interaction of mutant nsP1 with other ns proteins and/or host factors may have ts defects.

3.4.4. Construction and testing of replicon vectors with ts mutations (A. Iofik and V. Lulla, unpublished)

SFV is the most widely used viral expression vector for transient gene expression (Baldi et al., 2007). So far only one ts mutation has been used in the SFV based ts vectors (Lundstrom et al., 2001b), therefore this area of significant interest was clearly underdeveloped. Accordingly it was important to test whether the newly discovered ts mutations (**III**) have also an impact in the context of the temperature mediated regulation of foreign gene expression.

To construct replicons with ts phenotype the pSFV1 vector (Liljeström, 1994) with modified MCS was used as a backbone. Fragments containing ts mutations were transferred from corresponding ts mutations containing plasmids (**III**) using *Sac*I and *Eco*RV restriction enzymes for the transfer of ts10 and ts14 mutations; *Sac*I and *Eco*81I – for the transfer of ts1, ts13a, ts9, ts11, ts6 (Table 1, **III**) and ts4 (Suopanki et al., 1998) mutations; and *Eco*47III

and *Bgl*II for the transfer of ts13b mutation to this vector (Table 1, **III**). Luciferase gene from pGL3 plasmid (Promega) was inserted in ts replicon plasmids by using *Bgl*II and *Xba*I restriction sites. Corresponding replicon plasmids were designated pSFV1-ts1-luc, pSFV1-ts4-luc, pSFV1-ts6-luc, pSFV1-ts9-luc, pSFV1-ts10-luc, pSFV1-ts11-luc, pSFV1-ts13a-luc, pSFV1-ts13b-luc and pSFV1-ts14-luc.

Virus-like particles (VLPs) were produced by the co-electroporation of transcripts from replicon plasmids and pHelper-1 as described earlier (Liljeström and Garoff, 1991a) and designated wt-VLP, ts1-VLP, ts4-VLP, ts6-VLP, ts9-VLP, ts10-VLP, ts11-VLP, ts13a-VLP, ts13b-VLP and ts14-VLP. VLP stocks were titered by IF; obtained titers varied from $1 \cdot 10^7$ to $4 \cdot 10^8$ IU/ml (IU – infectious units). BHK-21 cells were infected with wt-VLPs for different times to find the optimal time for luciferase expression (the reaching of plateau level); it turned out to be the same time as it was detected in wt SFV growth curve experiments: 10 hours at 39ºC or 16 hours at 28ºC (data not shown). The luminescence of luciferase (in relative light units, RLU) was measured for 10 s in samples prepared essentially as described by the manufacturer (Promega, Luciferase Assay System).

In order to analyze whether the ts phenotypes of recombinant viruses are also reproduced in the corresponding replicons, we infected BHK-21 cells with both ts and wt VLPs with m.o.i. of 50, measured luciferase activity at selected time points at 28ºC and 39ºC and compared relative luciferase activities produced per cell at these temperatures (Fig. 6).

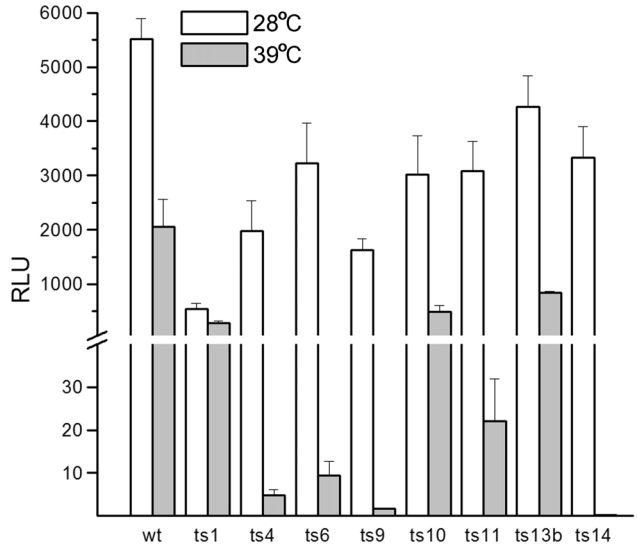

Figure 6. Activity of luciferase per BHK-21 cell infected with VLPs with corresponding ts mutation (in RLU, relative light units). White columns represent activities obtained at 28ºC and grey columns – at 39ºC.

First, the relative luciferase activities at permissive temperatures were compared. It was found that almost all ts replicons produced luciferase at the same level, which was slightly lower than that observed for wt-VLPs (Fig. 6). Second, the productions of luciferase at restrictive temperatures were compared. As it is evident from Figure 6, replicons with mutations ts4, ts6, ts9, ts11 and ts14 (RNA^- group, **III**), showed a drastic reduction in luciferase expression at the restrictive temperature. Therefore it would be logical to assume that these ts replicons fully reproduce the RNA^- phenotypes of corresponding viruses. Based on this assumption, one would expect similar transgene expression levels (at restrictive temperatures) for all these mutants. Our data indicate that this is not exactly the case: different levels of the reduction of luciferase expression at restrictive temperature were obtained for different mutants. In BHK-21 cells ts14 turned out to be the mutation with the biggest impact, greatly reducing the marker protein expression (more than 10^4 fold), whereas others 4 mutations caused 10^2–10^3 fold reduction in luciferase expression under the restrictive temperature (Fig. 6). It is very intriguing that the defect caused by mutation in nsP1 (ts14) is more severe compared to the defects caused by the mutations mapped to nsP2 (ts4, ts6, ts9 and ts11). Therefore it is tempting to speculate that the highest temperature dependent effect, observed among all ts replicons, may be due to some specific role of nsP1 in replication of SFV. Our data indicate that the mutation ts14 blocks the negative-strand RNA synthesis at restrictive temperature (**IV**) and consecutively the synthesis of all viral RNA. It may be speculated that this phenomenon is responsible for the very efficient suppression of the marker protein expression in the case of the corresponding replicon vector as well.

One mutant, namely ts13a (RNA±), was found reproducible to produce very low amounts of VLPs in transfected BHK-21 cells. These VLP yields were too low to be concentrated enough to perform infection by m.o.i. of 50 and therefore this mutant was excluded from the subsequent analysis. At the same time the defect is interesting on its own. This can indicate that ts13a mutation severely affects replication of Helper-RNA and, as a result, the expression of structural proteins from SG promoter in Helper-RNAs.

As it was expected, two RNA± mutations (ts10 and ts13b) as well as RNA+ mutant ts1 did not significantly differ in luciferase expression levels (at restrictive temperature) from wt replicons, thus indicating that mild RNA± phenotype is too weak to be used in replicons with the temperature-regulated expression. The combination of 13a and 13b mutations, as well as many other possible combinations, was not analyzed in the current study but definitely deserve attention and further investigation.

Engineering of the temperature-regulated expression system based on alphaviruses is still a developing field; the possibility of using newly described ts mutations of SFV in replicons and also in replicating SFV vectors is now available. It is possible to combine these ts mutations with non-cytopathic

mutations to obtain the regulation of the transgene expression and reduced cytotoxicity similarly to the vectors which were reported earlier (Lundstrom et al., 2001b). As tools regulated by temperature, ts replicons can be used not only in temperature shift-up experiments for the inactivation of further foreign protein expression from the SG promoter, but also in temperature shift-down approaches to activate the foreign protein expression at the needed time point.

4. CONCLUSIONS

The results presented in this study demonstrate the important issues concerning the regulation of the replication process of SFV. They also demonstrate the possibilities how the mechanisms involved in SFV replication can be used for different applications. The main results of the present thesis can be presented as follows:

1. SFV P1234 polyprotein processing was investigated both *in vivo* and *in vitro*. It was found that the proteolysis of the 2/3 site takes place *in trans* and is triggered only after the cleavage of 1/2 site, which itself is processed only *in cis*. Additionally, the mapping of the regulatory sequences, affecting the 2/3 and 1/2 cleavages was performed; based on these and earlier results a mechanism of the temporal regulation of P1234 processing was proposed.
2. A novel SFV based marker virus, containing EGFP marker gene inserted into the virus replicase between nsP3 and nsP4, flanked by enhanced artificial nsP2 protease recognition sites, was constructed on the basis of the results from previous studies of ns protease of SFV. The infection by this marker virus did not significantly differ from that by wt virus. It also demonstrated good genetic stability and thus this marker virus represents a good tool for the studies of virus infection both in the cell culture and in animal model systems.
3. We have sequenced the panel of SFV ts mutants and constructed the recombinant viruses, which contain the mutations identified by sequencing. Resulting recombinant viruses were shown to reproduce the phenotypes described for the original mutant viruses. All the mutations mapped to the protease domain of nsP2 caused defects in ns polyprotein processing and SG RNA synthesis; all the mutations in the helicase domain of nsP2 affected SG RNA synthesis. These types of defects were not associated with ts mutations found in other nsPs.
4. We found that the enzymatic activities of recombinant nsP1, containing ts10 or ts14 lesions, were not temperature sensitive on their own. These mutant proteins were also membrane-bound, interacted normally with nsP3 and there was no gross defect in the processing of the polyproteins containing these mutations. However, it was found that these two mutant viruses were specifically defective in the minus-strand RNA synthesis at the restrictive temperature.
5. Based on the revealed ts mutations, a set of SFV ts replicons with luciferase marker gene was constructed. Replicons with ts4, ts6, ts9, ts11 or ts14 mutations showed a drastic reduction of luciferase expression in BHK-21 cells at the restrictive temperature. Thus, the characterization of these ts mutations resulted in the construction of a set of temperature-regulated SFV based vectors.

SUMMARY IN ESTONIAN

Semliki Forest viiruse replikatsiooni strateegiad ja rakendused

Semliki Forest viirus (SFV) on perekonda *Alphavirus* kuuluv positiivse polaarsusega RNA genoomne virus. Tänu oma suhteliselt lihtsa ehitusele on SFV olnud ja on siiani oluliseks mudeliks RNA genoomsete viiruste elutsükli uurimisel. Peale selle kujutab SFV endast ühte kõige lootustandvamat vektorit nii geeniteraapia kui ka biotehnoloogia (nt. rekombinantsete valkude ekspressiooni) jaoks.

Käesoleva doktoritöö eesmärkideks olid analüüsida SFV polüproteiini protsessingu mehhanisme, iseloomustada selle viiruse temperatuuritundlikuid mutante ja identifitseerida vastavad mutatsioonid ning, kasutades eelpool nimetatud tööde käigus saadud informatsiooni, konstrueerida uudesed SFV-l põhinevad vektorid ja marker-viirused.

Käesolevas doktoritöö raames läbi viidud uuringud andsid olulist uut informatsiooni SFV replikatsiooniprotsessi regulatsioonis osalevate mehhanismide kohta. Peale selle näitasid saadud tulemused ka alfaviirustel põhinevate biotehnoloogiliste süsteemide uusi kasutamisvõimalusi. Põhilised tulemused ning nende põhjal tehtud järeldused on järgmised:

1. SFV polüproteiini protsessingu uurimine *in vivo* ja *in vitro* näitas, et 2/3 saidi proteolüüs toimub *in trans* ja see teostatakse ainult peale 1/2 saidi lõikamist; 1/2 saidi lõikamine toimub aga *in cis*. Samuti identifitseeriti 2/3 ja 1/2 saitide lõikamist reguleerivad järjestused. Nende ja varasemate andmete põhjal pakuti välja P1234 protsessingu regulatsiooni selgitav mudel.
2. Lähtudes eelnenud SFV mittestruktuurse proteaasi lõikamissaidi uuringutest konstrueeriti uudne SFV-l põhinev marker-viirus. EGFP marker-geen ümbritseti kunstlikke nsP2 proteaasi lõikamissaitidega ja sisestati viiruse genoomi nsP3 ja nsP4 regioonide vahel. Selle tulemusena saadi viirus, mille infektsioon ei erine oluliselt metsiktüüpi SFV infektsioonist. Seega kujutab saadud marker-viirus endast head mudelit SFV infektsiooni uurimiseks nii rakukultuuris kui ka katseloomades.
3. Sekveneeriti seitsme SFV temperatuuritundliku mutandi genoomid ja konstrueeriti vastavaid mutatsioone sisaldavad rekombinantsed viirused. Näidati, et saadud rekombinantsed viirused reprodutseerisid varem kirjeldatud temperatuuritundikud fenotüübid. Kõik mutatsioonid, mis paiknesid nsP2 proteaasses domeenis, põhjustasid mittestruktuurse polüproteiini protsessingu ja subgenoomse RNA sünteesi defekte; kõik mutatsioonid, mis paiknesid nsP2 helikaasses domeenis, mõjutasid ainult subgenoomse RNA sünteesi. Teistes mittestruktuursetes valkudes paiknevad mutatsioonid seda tüüpi defekte ei põhjustanud.
4. Näidati, et nsP1-s asuvate temperatuuritundlikute mutatsioonide ts10 ja ts14 sisseviimine rekombinantsetesse valkudesse ei põhjusta nsP1 peamiste

funktsioonide temperatuurist sõltuvat defekti; ka olid sellised rekombinantsed valgud membraanidega seondunud ja ka mutantsete nsP1 ning nsP3 valkude interaktsioon oli sarnane metsikut tüüpi valkude interaktsioonile. Samuti ei leitud vastavate mutantsete viiruste polüproteiinide protsessingus tõsiseid defekte. Samas näitas läbiviidud analüüs, et restriktiivsel temperatuuril olid need kaks mutanti spetsiifiliselt defektsed RNA negatiivse ahela sünteesi poolest.

5. Lähtudes analüüsitud temperatuuritundlikutest mutatsioonidest valmistati rida lutsiferaasi markergeeni kandvaid SFV replikon-vektoreid. Kui infektsioon vastavate viiruslaadsete partiklitega viidi läbi restriktiivsel temperatuuril, siis näitasid ts4, ts6, ts9, ts11 ja ts14 mutatsioonidega replikonid lutsiferaasi geeni ekspressiooni järsku vähenemist. Seega võimaldab saadud replikonide temperatuuritundlik fenotüüp nende kasutamist temperatuuri abil reguleeritavate SFV-l põhinevate vektoritena.

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REFERENCES

Agapov, E. V., Frolov, I., Lindenbach, B. D., Pragai, B. M., Schlesinger, S. and Rice, C. M. (1998) Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. *Proc Natl Acad Sci U S A*, **95**, 12989–12994.

Ahola, T. and Kääriäinen, L. (1995) Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. *Proc Natl Acad Sci U S A*, **92**, 507–511.

Ahola, T. (1997) Semliki Forest virus RNA capping enzyme. *Academic Dissertation*. Helsinki, ISSN 1239–9469.

Ahola, T., Laakkonen, P., Vihinen, H. and Kääriäinen, L. (1997) Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities. *J Virol*, **71**, 392–397.

Ahola, T. and Ahlquist, P. (1999) Putative RNA capping activities encoded by brome mosaic virus: methylation and covalent binding of guanylate by replicase protein 1a. *J Virol*, **73**, 10061–10069.

Ahola, T., Lampio, A., Auvinen, P. and Kääriäinen, L. (1999) Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. *EMBO J*, **18**, 3164–3172.

Ahola, T., Kujala, P., Tuittila, M., Blom, T., Laakkonen, P., Hinkkanen, A. and Auvinen, P. (2000) Effects of palmitoylation of replicase protein nsP1 on alphavirus infection. *J Virol*, **74**, 6725–6733.

Atasheva, S., Gorchakov, R., English, R., Frolov, I. and Frolova, E. (2007) Development of Sindbis viruses encoding nsP2/GFP chimeric protein and their application for studying nsP2 functioning. *J Virol*, **81**, 5046–5057.

Atkins, G. J., Sheahan, B. J. and Liljeström, P. (1999) The molecular pathogenesis of Semliki Forest virus: a model virus made useful? *J Gen Virol*, **80**, 2287–2297.

Baldi, L., Hacker, D. L., Adam, M. and Wurm, F. M. (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol Lett*, **27**, 677–684.

Balistreri, G., Caldentey, J., Kääriäinen, L. and Ahola, T. (2007) Enzymatic defects of nsP2-proteins of Semliki Forest virus temperature-sensitive mutants. *J Virol*, **81**, 2849–2860.

Barton, D. J., Sawicki, S. G. and Sawicki, D. L. (1988) Demonstration *in vitro* of temperature-sensitive elongation of RNA in Sindbis virus mutant ts6. *J Virol*, **62**, 3597–3602.

Berglund, P., Sjöberg, M., Garoff, H., Atkins, G. J., Sheahan, B. J. and Liljeström, P. (1993) Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *Biotechnology (N Y)*, **11**, 916–920.

Bick, M. J., Carroll, J. W., Gao, G., Goff, S. P., Rice, C. M. and MacDonald, M. R. (2003) Expression of the zinc-finger antiviral protein inhibits alphavirus replication. *J Virol*, **77**, 11555–11562.

Boorsma, M., Nieba, L., Koller, D., Bachmann, M. F., Bailey, J. E. and Renner, W. A. (2000) A temperature-regulated replicon-based DNA expression system. *Nat Biotechnol*, **18**, 429–432.

Bredenbeek, P. J., Frolov, I., Rice, C. M. and Schlesinger, S. (1993) Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. *J Virol*, **67**, 6439–6446.

Buck, K. W. (1996) Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv Virus Res*, **47**, 159–251.

Burge, B. W. and Pfefferkorn, E. R. (1966) Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology*, **30**, 204–213.

Caruthers, J. M. and McKay, D. B. (2002) Helicase structure and mechanism. *Curr Opin Struct Biol*, **12**, 123–133.

Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H. K., Smith, T. J. and Baker, T. S. (1995) Nucleocapsid and glycoprotein organization in an enveloped virus. *Cell*, **80**, 621–630.

Choi, H. K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M. G. and Wengler, G. (1991) Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature*, **354**, 37–43.

Cook, S. H. and Griffin, D. E. (2003) Luciferase imaging of a neurotropic viral infection in intact animals. *J Virol*, **77**, 5333–5338.

Cristea, I. M., Carroll, J. W., Rout, M. P., Rice, C. M., Chait, B. T. and MacDonald, M. R. (2006) Tracking and elucidating alphavirus-host protein interactions. *J Biol Chem*, **281**, 30269–30278.

Davis, N. L., Willis, L. V., Smith, J. F. and Johnston, R. E. (1989) *In vitro* synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. *Virology*, **171**, 189–204.

de Groot, R. J., Rumenapf, T., Kuhn, R. J., Strauss, E. G. and Strauss, J. H. (1991) Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc Natl Acad Sci U S A*, **88**, 8967–8971.

De, I., Sawicki, S. G. and Sawicki, D. L. (1996) Sindbis virus RNA-negative mutants that fail to convert from minus-strand to plus-strand synthesis: role of the nsP2 protein. *J Virol*, **70**, 2706–2719.

De, I., Fata-Hartley, C., Sawicki, S. G. and Sawicki, D. L. (2003) Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. *J Virol*, **77**, 13106–13116.

Ding, M. X. and Schlesinger, M. J. (1989) Evidence that Sindbis virus nsP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology*, **171**, 280–284.

Dolja, V. V., Herndon, K. L., Pirone, T. P. and Carrington, J. C. (1993) Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *J Virol*, **67**, 5968–5975.

Egloff, M. P., Malet, H., Putics, A., Heinonen, M., Dutartre, H., Frangeul, A., Gruez, A., Campanacci, V., Cambillau, C., Ziebuhr, J., Ahola, T. and Canard, B. (2006) Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. *J Virol*, **80**, 8493–8502.

Ehrengruber, M. U., Lundstrom, K., Schweitzer, C., Heuss, C., Schlesinger, S. and Gahwiler, B. H. (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc Natl Acad Sci U S A*, **96**, 7041–7046.

Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol*, **4**, 181–191.

Fata, C. L., Sawicki, S. G. and Sawicki, D. L. (2002a) Alphavirus minus-strand RNA synthesis: identification of a role for Arg183 of the nsP4 polymerase. *J Virol*, **76**, 8632–8640.

Fata, C. L., Sawicki, S. G. and Sawicki, D. L. (2002b) Modification of Asn374 of nsP1 suppresses a Sindbis virus nsP4 minus-strand polymerase mutant. *J Virol*, **76**, 8641–8649.

Fazakerley, J. K., Boyd, A., Mikkola, M. L. and Kääriäinen, L. (2002) A single amino acid change in the nuclear localization sequence of the nsP2 protein affects the neurovirulence of Semliki Forest virus. *J Virol*, **76**, 392–396.

Frolov, I., Frolova, E. and Schlesinger, S. (1997) Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus RNA. *J Virol*, **71**, 2819–2829.

Frolova, E., Gorchakov, R., Garmashova, N., Atasheva, S., Vergara, L. A. and Frolov, I. (2006) Formation of nsP3-specific protein complexes during Sindbis virus replication. *J Virol*, **80**, 4122–4134.

Froshauer, S., Kartenbeck, J. and Helenius, A. (1988) Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J Cell Biol*, **107**, 2075–2086.

Fuller, S. D., Berriman, J. A., Butcher, S. J. and Gowen, B. E. (1995) Low pH induces swiveling of the glycoprotein heterodimers in the Semliki Forest virus spike complex. *Cell*, **81**, 715–725.

Furuichi, Y. and Shatkin, A. J. (2000) Viral and cellular mRNA capping: past and prospects. *Adv Virus Res*, **55**, 135–184.

Galbraith, S. E., Sheahan, B. J. and Atkins, G. J. (2006) Deletions in the hyper-variable domain of the nsP3 gene attenuate Semliki Forest virus virulence. *J Gen Virol*, **87**, 937–947.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E. and Frolov, I. (2007) The old world and new world alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol*, **81**, 2472–2484.

Garoff, H., Simons, K. and Renkonen, O. (1974) Isolation and characterization of the membrane proteins of Semliki Forest virus. *Virology*, **61**, 493–504.

Garoff, H., Simons, K. and Dobberstein, B. (1978) Assembly of the Semliki Forest virus membrane glycoproteins in the membrane of the endoplasmic reticulum *in vitro*. *J Mol Biol*, **124**, 587–600.

Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U. and Liljeström, P. (1990) The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J Cell Biol*, **111**, 867–876.

Garoff, H., Wilschut, J., Liljeström, P., Wahlberg, J. M., Bron, R., Suomalainen, M., Smyth, J., Salminen, A., Barth, B. U., Zhao, H. (1994) Assembly and entry mechanisms of Semliki Forest virus. *Arch Virol Suppl*, **9**, 329–338.

Garoff, H., Sjöberg, M. and Cheng, R. H. (2004) Budding of alphaviruses. *Virus Res*, **106**, 103–116.

Gibbons, D. L., Reilly, B., Ahn, A., Vaney, M. C., Vigouroux, A., Rey, F. A. and Kielian, M. (2004a) Purification and crystallization reveal two types of interactions of the fusion protein homotrimer of Semliki Forest virus. *J Virol*, **78**, 3514–3523.

Gibbons, D. L., Vaney, M. C., Roussel, A., Vigouroux, A., Reilly, B., Lepault, J., Kielian, M. and Rey, F. A. (2004b) Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature*, **427**, 320–325.

Golubtsov, A., Kääriäinen, L. and Caldentey, J. (2006) Characterization of the cysteine protease domain of Semliki Forest virus replicase protein nsP2 by *in vitro* mutagenesis. *FEBS Lett*, **580**, 1502–1508.

Gomatos, P. J., Kääriäinen, L., Keränen, S., Ranki, M. and Sawicki, D. L. (1980) Semliki Forest virus replication complex capable of synthesizing 42S and 26S nascent RNA chains. *J Gen Virol*, **49**, 61–69.

Gomez de Cedrón, M., Ehsani, N., Mikkola, M. L., Garcia, J. A. and Kääriäinen, L. (1999) RNA helicase activity of Semliki Forest virus replicase protein nsP2. *FEBS Lett*, **448**, 19–22.

Gorbalenya, A. E., Koonin, E. V. and Lai, M. M. (1991) Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. *FEBS Lett*, **288**, 201–205.

Gorchakov, R., Frolova, E., Williams, B. R., Rice, C. M. and Frolov, I. (2004) PKR-dependent and -independent mechanisms are involved in translational shutoff during Sindbis virus infection. *J Virol*, **78**, 8455–8467.

Hahn, Y. S., Grakoui, A., Rice, C. M., Strauss, E. G. and Strauss, J. H. (1989a) Mapping of RNA– temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. *J Virol*, **63**, 1194–1202.

Hahn, Y. S., Strauss, E. G. and Strauss, J. H. (1989b) Mapping of RNA– temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J Virol*, **63**, 3142–3150.

Hardy, W. R. and Strauss, J. H. (1989) Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both *in cis* and *in trans*. *J Virol*, **63**, 4653–4664.

Helenius, A., Kartenbeck, J., Simons, K. and Fries, E. (1980) On the entry of Semliki Forest virus into BHK-21 cells. *J Cell Biol*, **84**, 404–420.

Helenius, A. (1995) Alphavirus and flavivirus glycoproteins: structures and functions. *Cell*, **81**, 651–653.

Helenius, A. and Aebi, M. (2001) Intracellular functions of N-linked glycans. *Science*, **291**, 2364–2369.

Kääriäinen, L., Sawicki, D. and Gomatos, P. J. (1978) Cleavage defect in the non-structural polyprotein of Semliki Forest virus has two separate effects on virus RNA synthesis. *J Gen Virol*, **39**, 463–473.

Kääriäinen, L. and Söderlund, H. (1978) Structure and replication of alphaviruses. *Curr Top Microbiol Immunol*, **82**, 15–69.

Kääriäinen, L., Takkinen, K., Keränen, S. and Söderlund, H. (1987) Replication of the genome of alphaviruses. *J Cell Sci Suppl*, **7**, 231–250.

Kääriäinen, L. and Ahola, T. (2002) Functions of alphavirus nonstructural proteins in RNA replication. *Prog Nucleic Acid Res Mol Biol*, **71**, 187–222.

Keränen, S. and Kääriäinen, L. (1974) Isolation and basic characterization of temperature-sensitive mutants from Semliki Forest virus. *Acta Pathol Microbiol Scand [B] Microbiol Immunol*, **82**, 810–820.

Keränen, S. and Kääriäinen, L. (1975) Proteins synthesized by Semliki Forest virus and its 16 temperature-sensitive mutants. *J Virol*, **16**, 388–396.

Keränen, S. and Kääriäinen, L. (1979) Functional defects of RNA-negative temperature-sensitive mutants of Sindbis and Semliki Forest viruses. *J Virol*, **32**, 19–29.

Kielian, M. (1995) Membrane fusion and the alphavirus life cycle. *Adv Virus Res*, **45**, 113–151.

Kim, K. H., Rumenapf, T., Strauss, E. G. and Strauss, J. H. (2004) Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology*, **323**, 153–163.

Koonin, E. V. and Dolja, V. V. (1993) Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit Rev Biochem Mol Biol*, **28**, 375–430.

Kujala, P., Ikäheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P. and Kääriäinen, L. (2001) Biogenesis of the Semliki Forest virus RNA replication complex. *J Virol*, **75**, 3873–3884.

Laakkonen, P., Hyvönen, M., Peränen, J. and Kääriäinen, L. (1994) Expression of Semliki Forest virus nsP1-specific methyltransferase in insect cells and in *Escherichia coli*. *J Virol*, **68**, 7418–7425.

Laakkonen, P., Ahola, T. and Kääriäinen, L. (1996) The effects of palmitoylation on membrane association of Semliki Forest virus RNA capping enzyme. *J Biol Chem*, **271**, 28567–28571.

Lachmi, B. E. and Kääriäinen, L. (1976) Sequential translation of nonstructural proteins in cells infected with a Semliki Forest virus mutant. *Proc Natl Acad Sci U S A*, **73**, 1936–1940.

Lampio, A., Ahola, T., Darzynkiewicz, E., Stepinski, J., Jankowska-Anyszka, M. and Kääriäinen, L. (1999) Guanosine nucleotide analogs as inhibitors of alphavirus mRNA capping enzyme. *Antiviral Res*, **42**, 35–46.

Lampio, A., Kilpelainen, I., Pesonen, S., Karhi, K., Auvinen, P., Somerharju, P. and Kääriäinen, L. (2000) Membrane binding mechanism of an RNA virus-capping enzyme. *J Biol Chem*, **275**, 37853–37859.

LaStarza, M. W., Lemm, J. A. and Rice, C. M. (1994) Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *J Virol*, **68**, 5781–5791.

Lehtovaara, P., Ulmanen, I., Kääriäinen, L., Keränen, S. and Philipson, L. (1980) Synthesis and processing of Semliki Forest virus-specific nonstructural proteins *in vivo* and *in vitro*. *Eur J Biochem*, **112**, 461–468.

Lemm, J. A., Rumenapf, T., Strauss, E. G., Strauss, J. H. and Rice, C. M. (1994) Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J*, **13**, 2925–2934.

Lemm, J. A., Bergqvist, A., Read, C. M. and Rice, C. M. (1998) Template-dependent initiation of Sindbis virus RNA replication *in vitro*. *J Virol*, **72**, 6546–6553.

Li, M. L. and Stollar, V. (2004) Identification of the amino acid sequence in Sindbis virus nsP4 that binds to the promoter for the synthesis of the subgenomic RNA. *Proc Natl Acad Sci U S A*, **101**, 9429–9434.

Li, M. L., Lin, Y. H. and Stollar, V. (2005) A cell-free system for the synthesis of Sindbis virus subgenomic RNA: importance of the concentration of the initiating NTP. *Virology*, **341**, 24–33.

Li, M. L. and Stollar, V. (2007) Distinct sites on the Sindbis virus RNA-dependent RNA polymerase for binding to the promoters for the synthesis of genomic and subgenomic RNA. *J Virol*, **81**, 4371–4373.

Li, Y. I., Chen, Y. J., Hsu, Y. H. and Meng, M. (2001) Characterization of the AdoMet-dependent guanylyltransferase activity that is associated with the N terminus of bamboo mosaic virus replicase. *J Virol*, **75**, 782–788.

Liljeström, P. and Garoff, H. (1991a) A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology (N Y)*, **9**, 1356–1361.

Liljeström, P. and Garoff, H. (1991b) Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J Virol*, **65**, 147–154.

Liljeström, P., Lusa, S., Huylebroeck, D. and Garoff, H. (1991) *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol*, **65**, 4107–4113.

Liljeström, P. (1994) Alphavirus expression systems. *Curr Opin Biotechnol*, **5**, 495–500.

Loewy, A., Smyth, J., von Bonsdorff, C. H., Liljeström, P. and Schlesinger, M. J. (1995) The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. *J Virol*, **69**, 469–475.

Lulla, A., Lulla, V., Tints, K., Ahola, T. and Merits, A. (2006) Molecular determinants of substrate specificity for Semliki Forest virus nonstructural protease. *J Virol*, **80**, 5413–5422.

Lundstrom, K. (1999) Alphaviruses as tools in neurobiology and gene therapy. *J Recept Signal Transduct Res*, **19**, 673–686.

Lundstrom, K., Rotmann, D., Hermann, D., Schneider, E. M. and Ehrengruber, M. U. (2001a) Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem Cell Biol*, **115**, 83–91.

Lundstrom, K., Ziltener, P., Hermann, D., Schweitzer, C., Richards, J. G. and Jenck, F. (2001b) Improved Semliki Forest virus vectors for receptor research and gene therapy. *J Recept Signal Transduct Res*, **21**, 55–70.

Magden, J., Takeda, N., Li, T., Auvinen, P., Ahola, T., Miyamura, T., Merits, A. and Kääriäinen, L. (2001) Virus-specific mRNA capping enzyme encoded by hepatitis E virus. *J Virol*, **75**, 6249–6255.

Mancini, E. J., Clarke, M., Gowen, B. E., Rutten, T. and Fuller, S. D. (2000) Cryo-electron microscopy reveals the functional organization of an enveloped virus, Semliki Forest virus. *Mol Cell*, **5**, 255–266.

Marsh, M. and Helenius, A. (1989) Virus entry into animal cells. *Adv Virus Res*, **36**, 107–151.

McInerney, G. M., Smit, J. M., Liljeström, P. and Wilschut, J. (2004) Semliki Forest virus produced in the absence of the 6K protein has an altered spike structure as revealed by decreased membrane fusion capacity. *Virology*, **325**, 200–206.

McInerney, G. M., Kedersha, N. L., Kaufman, R. J., Anderson, P. and Liljeström, P. (2005) Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol Biol Cell*, **16**, 3753–3763.

Melancon, P. and Garoff, H. (1986) Reinitiation of translocation in the Semliki Forest virus structural polyprotein: identification of the signal for the E1 glycoprotein. *EMBO J*, **5**, 1551–1560.

Melancon, P. and Garoff, H. (1987) Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *J Virol*, **61**, 1301–1309.

Merits, A., Kettunen, R., Mäkinen, K., Lampio, A., Auvinen, P., Kääriäinen, L. and Ahola, T. (1999) Virus-specific capping of tobacco mosaic virus RNA: methylation of GTP prior to formation of covalent complex p126-m7GMP. *FEBS Lett*, **455**, 45–48.

Merits, A., Vasiljeva, L., Ahola, T., Kääriäinen, L. and Auvinen, P. (2001) Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J Gen Virol*, **82**, 765–773.

Mi, S., Durbin, R., Huang, H. V., Rice, C. M. and Stollar, V. (1989) Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology*, **170**, 385–391.

Mi, S. and Stollar, V. (1991) Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli. *Virology*, **184**, 423–427.

Moradpour, D., Evans, M. J., Gosert, R., Yuan, Z., Blum, H. E., Goff, S. P., Lindenbach, B. D. and Rice, C. M. (2004) Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. *J Virol*, **78**, 7400–7409.

Mueller, S. and Wimmer, E. (1998) Expression of foreign proteins by poliovirus polyprotein fusion: analysis of genetic stability reveals rapid deletions and formation of cardioviruslike open reading frames. *J Virol*, **72**, 20–31.

Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J. and Rossmann, M. G. (2006) Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Structure*, **14**, 63–73.

Pehrson, J. R. and Fuji, R. N. (1998) Evolutionary conservation of histone macroH2A subtypes and domains. *Nucleic Acids Res*, **26**, 2837–2842.

Peränen, J., Takkinen, K., Kalkkinen, N. and Kääriäinen, L. (1988) Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J Gen Virol*, **69**, 2165–2178.

Peränen, J., Rikkonen, M., Liljeström, P. and Kääriäinen, L. (1990) Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J Virol*, **64**, 1888–1896.

Peränen, J. and Kääriäinen, L. (1991) Biogenesis of type I cytopathic vacuoles in Semliki Forest virus-infected BHK cells. *J Virol*, **65**, 1623–1627.

Peränen, J., Laakkonen, P., Hyvönen, M. and Kääriäinen, L. (1995) The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. *Virology*, **208**, 610–620.

Perri, S., Driver, D. A., Gardner, J. P., Sherrill, S., Belli, B. A., Dubensky, T. W., Jr. and Polo, J. M. (2000) Replicon vectors derived from Sindbis virus and

Semliki Forest virus that establish persistent replication in host cells. *J Virol*, **74**, 9802–9807.

Pesonen, M. and Kääriäinen, L. (1982) Incomplete complex oligosaccharides in Semliki Forest virus envelope proteins arrested within the cell in the presence of monensin. *J Mol Biol*, **158**, 213–230.

Putics, A., Filipowicz, W., Hall, J., Gorbalenya, A. E. and Ziebuhr, J. (2005) ADP-ribose-1"-monophosphatase: a conserved coronavirus enzyme that is dispensable for viral replication in tissue culture. *J Virol*, **79**, 12721–12731.

Rajamaki, M. L., Kelloniemi, J., Alminaite, A., Kekarainen, T., Rabenstein, F. and Valkonen, J. P. (2005) A novel insertion site inside the potyvirus P1 cistron allows expression of heterologous proteins and suggests some P1 functions. *Virology*, **342**, 88–101.

Ranki, M. and Kääriäinen, L. (1979) Solubilized RNA replication complex from Semliki Forest virus-infected cells. *Virology*, **98**, 298–307.

Rice, C. M., Levis, R., Strauss, J. H. and Huang, H. V. (1987) Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *J Virol*, **61**, 3809–3819.

Riezebos-Brilman, A., de Mare, A., Bungener, L., Huckriede, A., Wilschut, J. and Daemen, T. (2006) Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy. *J Clin Virol*, **35**, 233–243.

Rikkonen, M., Peränen, J. and Käriäinen, L. (1992) Nuclear and nucleolar targeting signals of Semliki Forest virus nonstructural protein nsP2. *Virology*, **189**, 462–473.

Rikkonen, M., Peränen, J. and Kääriäinen, L. (1994) ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J Virol*, **68**, 5804–5810.

Roussel, A., Lescar, J., Vaney, M. C., Wengler, G., Wengler, G. and Rey, F. A. (2006) Structure and interactions at the viral surface of the envelope protein E1 of Semliki Forest virus. *Structure*, **14**, 75–86.

Russo, A. T., White, M. A. and Watowich, S. J. (2006) The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure*, **14**, 1449–1458.

Saikatendu, K. S., Joseph, J. S., Subramanian, V., Clayton, T., Griffith, M., Moy, K., Velasquez, J., Neuman, B. W., Buchmeier, M. J., Stevens, R. C. and Kuhn, P. (2005) Structural basis of severe acute respiratory syndrome coronavirus ADP-ribose-1"-phosphate dephosphorylation by a conserved domain of nsP3. *Structure*, **13**, 1665–1675.

Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E. and Kääriäinen, L. (2003) Properly folded nonstructural polyprotein directs the Semliki Forest virus replication complex to the endosomal compartment. *J Virol*, **77**, 1691–1702.

Saraste, J., Kääiäinen, L., Söderlund, H. and Keränen, S. (1977) RNA synthesis directed by a temperature-sensitive mutant of Semliki Forest virus. *J Gen Virol*, **37**, 399–406.

Sawicki, D., Barkhimer, D. B., Sawicki, S. G., Rice, C. M. and Schlesinger, S. (1990) Temperature sensitive shut-off of alphavirus minus strand RNA synthesis maps to a nonstructural protein, nsP4. *Virology*, **174**, 43–52.

Sawicki, D. L. and Gomatos, P. J. (1976) Replication of Semliki Forest virus: polyadenylate in plus-strand RNA and polyuridylate in minus-strand RNA. *J Virol*, **20**, 446–464.

Sawicki, D. L., Kääriäinen, L., Lambek, C. and Gomatos, P. J. (1978) Mechanism for control of synthesis of Semliki Forest virus 26S and 42S RNA. *J Virol*, **25**, 19–27.

Sawicki, D. L. and Sawicki, S. G. (1980) Short-lived minus-strand polymerase for Semliki Forest virus. *J Virol*, **34**, 108–118.

Sawicki, D. L., Sawicki, S. G., Keränen, S. and Kääriäinen, L. (1981a) Specific Sindbis virus-coded function for minus-strand RNA synthesis. *J Virol*, **39**, 348–358.

Sawicki, D. L. and Sawicki, S. G. (1985) Functional analysis of the A complementation group mutants of Sindbis HR virus. *Virology*, **144**, 20–34.

Sawicki, D. L. and Sawicki, S. G. (1993) A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. *J Virol*, **67**, 3605–3610.

Sawicki, D. L., Perri, S., Polo, J. M. and Sawicki, S. G. (2006) Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. *J Virol*, **80**, 360–371.

Sawicki, S. G., Sawicki, D. L., Kääriäinen, L. and Keränen, S. (1981b) A Sindbis virus mutant temperature-sensitive in the regulation of minus-strand RNA synthesis. *Virology*, **115**, 161–172.

Sawicki, S. G. and Sawicki, D. L. (1986a) The effect of overproduction of nonstructural proteins on alphavirus plus-strand and minus-strand RNA synthesis. *Virology*, **152**, 507–512.

Sawicki, S. G. and Sawicki, D. L. (1986b) The effect of loss of regulation of minus-strand RNA synthesis on Sindbis virus replication. *Virology*, **151**, 339–349.

Schaller, T., Appel, N., Koutsoudakis, G., Kallis, S., Lohmann, V., Pietschmann, T. and Bartenschlager, R. (2007) Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. *J Virol*, **81**, 4591–4603.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M. C., Lavenir, R., Pardigon, N., Reynes, J. M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M. P., Brehin, A. C., Cubito, N., Despres, P., Kunst, F., Rey, F. A., Zeller, H. and Brisse, S. (2006) Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med*, **3**, e263.

Shirako, Y. and Strauss, J. H. (1994) Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J Virol*, **68**, 1874–1885.

Shirako, Y., Strauss, E. G. and Strauss, J. H. (2000) Suppressor mutations that allow Sindbis virus RNA polymerase to function with nonaromatic amino acids at the N-terminus: evidence for interaction between nsP1 and nsP4 in minus-strand RNA synthesis. *Virology*, **276**, 148–160.

Shuman, S. (2002) What messenger RNA capping tells us about eukaryotic evolution. *Nat Rev Mol Cell Biol*, **3**, 619–625.

Sjöberg, E. M., Suomalainen, M. and Garoff, H. (1994) A significantly improved Semliki Forest virus expression system based on translation enhancer segments from the viral capsid gene. *Biotechnology (N Y)*, **12**, 1127–1131.

Sjöberg, E. M. and Garoff, H. (1996) The translation-enhancing region of the Semliki Forest virus subgenome is only functional in the virus-infected cell. *J Gen Virol*, **77**, 1323–1327.

Sjöberg, M. and Garoff, H. (2003) Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. *J Virol*, **77**, 3441–3450.

Smerdou, C. and Liljeström, P. (1999) Two-helper RNA system for production of recombinant Semliki Forest virus particles. *J Virol*, **73**, 1092–1098.

Spuul, P., Salonen, A., Merits, A., Jokitalo, E., Kääriäinen, L. and Ahola, T. (2007) Role of the amphipathic peptide of Semliki Forest virus replicase protein nsP1 in membrane association and virus replication. *J Virol*, **81**, 872–883.

Strauss, E. G., Rice, C. M. and Strauss, J. H. (1983) Sequence coding for the alphavirus nonstructural proteins is interrupted by an opal termination codon. *Proc Natl Acad Sci U S A*, **80**, 5271–5275.

Strauss, E. G., De Groot, R. J., Levinson, R. and Strauss, J. H. (1992) Identification of the active site residues in the nsP2 proteinase of Sindbis virus. *Virology*, **191**, 932–940.

Strauss, J. H. and Strauss, E. G. (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev*, **58**, 491–562.

Suomalainen, M., Liljeström, P. and Garoff, H. (1992) Spike protein-nucleocapsid interactions drive the budding of alphaviruses. *J Virol*, **66**, 4737–4747.

Suopanki, J., Sawicki, D. L., Sawicki, S. G. and Kääriäinen, L. (1998) Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. *J Gen Virol*, **79**, 309–319.

Suthar, M. S., Shabman, R., Madric, K., Lambeth, C. and Heise, M. T. (2005) Identification of adult mouse neurovirulence determinants of the Sindbis virus strain AR86. *J Virol*, **79**, 4219–4228.

Syväoja, P., Peränen, J., Suomalainen, M., Keränen, S. and Kääriäinen, L. (1990) A single amino acid change in E3 of ts1 mutant inhibits the intracellular transport of SFV envelope protein complex. *Virology*, **179**, 658–666.

Takkinen, K., Peränen, J. and Kääriäinen, L. (1991) Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein. *J Gen Virol*, **72**, 1627–1633.

Thal, M. A., Wasik, B. R., Posto, J. and Hardy, R. W. (2007) Template requirements for recognition and copying by Sindbis virus RNA-dependent RNA polymerase. *Virology*, **358**, 221–232.

Thomas, J. M., Klimstra, W. B., Ryman, K. D. and Heidner, H. W. (2003) Sindbis virus vectors designed to express a foreign protein as a cleavable component of the viral structural polyprotein. *J Virol.*, **77**, 5598–5606.

Tomar, S., Hardy, R. W., Smith, J. L. and Kuhn, R. J. (2006) Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity. *J Virol*, **80**, 9962–9969.

Tuittila, M. and Hinkkanen, A. E. (2003) Amino acid mutations in the replicase protein nsP3 of Semliki Forest virus cumulatively affect neurovirulence. *J Gen Virol*, **84**, 1525–1533.

Tuittila, M. T., Santagati, M. G., Roytta, M., Maatta, J. A. and Hinkkanen, A. E. (2000) Replicase complex genes of Semliki Forest virus confer lethal neurovirulence. *J Virol*, **74**, 4579–4589.

Ulmanen, I., Söderlund, H. and Kääriäinen, L. (1976) Semliki Forest virus capsid protein associates with the 60S ribosomal subunit in infected cells. *J Virol*, **20**, 203–210.

van den Born, E., Stein, D. A., Iversen, P. L. and Snijder, E. J. (2005) Antiviral activity of morpholino oligomers designed to block various aspects of Equine arteritis virus amplification in cell culture. *J Gen Virol*, **86**, 3081–3090.

Varshavsky, A. (1996) The N-end rule: functions, mysteries, uses. *Proc Natl Acad Sci U S A*, **93**, 12142–12149.

Vasiljeva, L., Merits, A., Auvinen, P. and Kääriäinen, L. (2000) Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of nsP2. *J Biol Chem*, **275**, 17281–17287.

Vasiljeva, L., Valmu, L., Kääriäinen, L. and Merits, A. (2001) Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. *J Biol Chem*, **276**, 30786–30793.

Ventoso, I., Sanz, M. A., Molina, S., Berlanga, J. J., Carrasco, L. and Esteban, M. (2006) Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes Dev*, **20**, 87–100.

Vihinen, H. and Saarinen, J. (2000) Phosphorylation site analysis of Semliki Forest virus nonstructural protein 3. *J Biol Chem*, **275**, 27775–27783.

Vihinen, H., Ahola, T., Tuittila, M., Merits, A. and Kääriäinen, L. (2001) Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J Biol Chem*, **276**, 5745–5752.

Vogel, R. H., Provencher, S. W., von Bonsdorff, C. H., Adrian, M. and Dubochet, J. (1986) Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. *Nature*, **320**, 533–535.

Wang, Y. F., Sawicki, S. G. and Sawicki, D. L. (1994) Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. *J Virol*, **68**, 6466–6475.

Weiss, B. G. and Schlesinger, S. (1991) Recombination between Sindbis virus RNAs. *J Virol*, **65**, 4017–4025.

Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. and Huang, H. V. (1989) Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science*, **243**, 1188–1191.

Žusinaite, E., Tints, K., Kiiver, K., Spuul, P., Karo-Astover, L., Merits, A. and Sarand, I. (2007) Mutations at the palmitoylation site of non-structural protein nsP1 of Semliki Forest virus attenuate viral replication and cause accumulation of compensatory mutations. *J Gen Virol*, in press.

PUBLICATIONS

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Molecular defects in Semliki Forest virus temperature-sensitive mutants ts10 and ts14 mapping to nsP1

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Alphavirus replicase protein nsP1 has multiple functions during viral RNA synthesis. It catalyzes methyltransferase and guanylyltransferase activities needed in viral mRNA capping, attaches the viral replication complex to cytoplasmic membranes and is required for minus-strand RNA synthesis. Two temperature sensitive mutations in Semliki Forest virus (SFV) were previously identified within nsP1: ts10 (E529D) and ts14 (D119N). Recombinant viruses SFons10 and SFots14 containing these mutations reproduced the features of the original ts strains. We now find that the enzymatic activities of recombinant nsP1, containing ts10 or ts14 lesions, were not temperature sensitive. The mutant proteins also were membrane-bound, mutant nsP1s interacted normally with nsP3, and there was no gross defect in non-structural polyprotein processing in the mutants. The two mutant viruses were specifically defective in minus-strand RNA synthesis at the restrictive temperature.

The alphaviruses are positive-strand RNA viruses, whose genome of approximately 11.5 kb encodes four nonstructural (ns) proteins, nsP1-nsP4, in its 5' end region. The structural protein genes, located at the 3' end of the genome, are translated from a subgenomic mRNA generated by internal initiation on the complementary minus-strand template. In the case of Semliki Forest virus (SFV), the nonstructural proteins are initially produced as a single large polyprotein P1234 of 2,432 amino acid residues, which is processed to the final products in a carefully controlled sequential order (Vasiljeva et al., 2003). The transient processing intermediate P123 together with nsP4 is responsible for negative-strand synthesis, whereas completely processed nsPs in mature, stable replication complexes are active only in genomic and subgenomic positive-strand synthesis (Kim et al., 2004; Lemm et al., 1994; Shirako and Strauss, 1994; Wang et al., 1994).

Many temperature sensitive (ts) strains of Sindbis virus (SIN) and SFV have been isolated and used in studies of RNA synthesis, processing and intracellular transport of viral proteins, and maturation of virus particles (for clarity, SIN ts mutants will be designated with the prefix SIN, whereas SFV ts mutants will be shown without a prefix). The ts strains have yielded several important insights into the different stages of viral RNA synthesis (reviewed in Kääriäinen and Ahola, 2002; Strauss and Strauss, 1994). SIN ts6 causes a rapid cessation of all RNA synthesis, when cells or cell extracts are shifted to the nonpermissive temperature, indicative of a defect in the polymerase (Keränen and Kääriäinen, 1979; Barton et al., 1988). Thus nsP4 was identified as the polymerase subunit genetically a long time ago, but only very recently was the first direct demonstration of nsP4 as the alphavirus RNA-dependent RNA polymerase achieved (Thal et al., 2007). So far, both genetics and biochemistry have struggled in revealing the exact roles of nsP3, although several pieces of the puzzle are emerging. NsP3 participates in the formation of replication complexes and in the synthesis of negative-strand RNA (Wang et al., 1994; Dé et al., 2003). NsP3 is phosphorylated in the carboxy-terminal tail region (Vihinen et al., 2001), and the conserved amino-terminal domain of the protein is capable of binding poly(ADP-ribose) with high affinity (Egloff et al., 2006).

Early on, analysis of RNA synthesis intermediates led to the suggestion that a replicase protein regulates subgenomic RNA synthesis by directly and reversibly binding to the subgenomic promoter (Sawicki et al., 1978). This regulatory factor was later revealed to be nsP2, as many mutants causing defects in subgenomic RNA production are found in nsP2, and particularly in the carboxy-terminal domain of the protein (Suopanki et al., 1998). A curious feature of several of the nsP2 ts mutants is that when shifted to the restrictive temperature late in infection, they can reactivate minus-strand RNA synthesis in the mature replication complexes (Sawicki and Sawicki, 1993). This behaviour has not been demonstrated for the wild type replication complex under any circumstances, but it indicates that there is flexibility in the organisation of the final stable complex and that nsP2 is the central regulator of RNA synthesis. The N-terminal domain of nsP2 carries out the first of the viral RNA capping reactions, RNA triphosphatase (Vasiljeva et al., 2000). The N-terminal domain is also an NTPase, which uses the same active site as triphosphatase (Balistreri et al., 2007). The NTPase activity fuels the RNA helicase activity of nsP2 (Gomez de Cedrón et al., 1999). The carboxy-terminal domain carries out the highly regulated site-specific processing of the ns polyprotein (Hardy and Strauss, 1989; Vasiljeva et al., 2003).

The only SIN ts mutant mapping to nsP1, SIN ts11, is specifically defective in minus-strand synthesis at the restrictive temperature (Sawicki et al., 1981; Wang et al., 1991). This defect is due to a substitution A348T in nsP1 (Hahn et al., 1989), indicating that nsP1 is specifically involved in the regulation of minus-strand production. Biochemical studies with recombinant proteins have shown that nsP1 possesses guanine-7-methyltransferase and guanylyltransferase activities needed in viral RNA capping (Mi and Stollar, 1991; Laakkonen et al., 1994; Ahola and Kääriäinen, 1995). NsP1 has affinity to cytoplasmic membranes, and especially to the inner surface of the plasma membrane; the essential membrane binding activity is primarily mediated by an amphipathic helix that can bind to negatively charged phospholipids (Ahola et al., 1999; Spuul et al., 2007). It should be noted that only a fraction of the nsPs is present in the active replication complexes, which are located on the cytoplasmic surface of endosomes and lysosomes. In addition to this common site, each of the proteins has a specific individual localization pattern, which is independent of the other nsPs: nsP1 on the inner side of the plasma membrane, nsP2 in the nucleus, nsP3 in cytoplasmic aggregates and nsP4 dispersed in the cytoplasm (Kääriäinen and Ahola, 2002; Salonen et al., 2003; Vasiljeva et al., 2003).

We have recently mapped those SFV ts mutants that display a significant overall phenotype in RNA synthesis to the individual ns proteins (V. Lulla et al., 2006). Similar to SIN, many of the mutations mapped to the largest protein nsP2, and the first biochemical studies carried out with purified proteins containing ts mutations allowed the examination of the connections between the multiple functions of nsP2 (Balistreri et al., 2007). Two individual mutations in SFV nsP1, D119N and E529D were found to be responsible for the ts phenotype of ts14 and ts10, respectively (V. Lulla et al., 2006). Here we have characterized these two mutants in temperature shift-up experiments in cell culture. We have also characterized the enzymatic properties, localization and protein-protein interactions of nsP1 proteins containing these substitutions. We found that both mutants were specifically defective in minus-strand RNA synthesis, whereas the RNA capping activities, membrane binding and interaction with nsP3 were not affected by the mutations.

MATERIALS AND METHODS

Recombinant clones and plasmids. nsP1 genes with ts10 and ts14 mutations from the infectious cDNA clones pSP6-SFV4-ts10 and pSP6-SFV-ts14 (V. Lulla *et al*, 2006) were PCR amplified using specific primers with *Nco*I and *Hind*III adaptors, treated with these enzymes and ligated into the bacterial expression vector pBAT4-nsP1 (Laakkonen et al., 1994) digested with the same enzymes, resulting in plasmids pBAT4-nsP1ts10 and pBAT4-nsP1ts14, respectively. For *in vitro* translation, the two genes were cloned under the T7 promoter in the vector pTSF1, a derivative of pGEM3, giving vectors pTSF1-ts10 and pTSF1-ts14. pBAT-nsP1-ts10 and pBAT-nsP1-ts14 were digested with *Nco*I and *Hind*III and fragments ligated into pcDNA4/TO (Invitrogen), treated with *Afl*II, Klenow fragment and *Hind*III, resulting in mammalian expression plasmids pcDNA4/TO-nsP1-ts10 and pcDNA4/TO-nsP1-ts14. pcDNA4/TO-nsP3 for expression of nsP3 was constructed after PCR amplification of nsP3 with suitable primers. The ts10 and ts14 mutations were transferred from the infectious clones to pTSF-P12 vector for *in vitro* translation of P12 (Merits *et al*, 2001) as *Stu*I-*Sac*I and *Eco*RV-*Dra*III fragments, respectively. The ts4 mutation (M781T in nsP2, Suopanki et al., 1998) containing region was PCR amplified and transferred to pTSF-P12 as *Kpn*I-*Bgl*II fragment.

***In vitro* translation.** *In vitro* translation of nsP1 and P12 proteins was carried out with the T7 TNT rabbit reticulocyte lysate system (Promega) according to the manufacturer's protocol. Reaction mixtures (10 µl) containing 10 µCi of [^{35}S]methionine (GE Healthcare) and 0.5 µg of plasmid DNA were incubated at 30°C for 30 min, after which the translation was stopped by adding cycloheximide to a final concentration of 1 mM. Samples were subjected to SDS-PAGE, and phosphorimaging, and quantification was done using Tina 2.09c software. Calculations of kinetic constants were done using the first order reaction equation.

Expression and flotation of nsP1. To produce active recombinant nsP1, the expression plasmids were transformed into *E. coli* BL21(DE3) strain (Novagen). Cells were grown in the presence of ampicillin (100 µg/ml) in LB media at 37°C until the optical density (OD_{600}) of the culture reached 0.6. The culture was then transferred to 15°C, and protein expression was induced by addition of IPTG, final concentration 500 µM. After 20 h incubation, the cells were collected by centrifugation, washed in buffer containing 15mM Tris, pH 8 and 140 mM NaCl and resuspended in 1/30th of the original volume in lysis buffer (50mM Tris at pH 7.5, 50mM NaCl, 10% glycerol, 1mM DTT, 1mM PMSF). The cell suspension was passed twice through French press at the cell pressure of 10,000 psi. The lysate was centrifuged at 15,000 × g at 4°C for 15 min and the resulting supernatant (S15) was used for in further experiments. S15 fraction of the lysate was used for the flotation assay essentially as described by Laakkonen et al. (1996). Briefly, the sucrose solutions were prepared in a buffer containing 50 mM Tris, pH 7.5 and 100 mM NaCl. The samples were mixed with 67% (w/w) sucrose to give a final sucrose concentration of 60%. Discontinuous flotation gradients were prepared in SW50.1 ultracentrifuge tubes by layering first 0.5 ml of 67% (w/w) sucrose, then 0.5 ml of the sample in 60% sucrose followed by 3 ml of 50% (w/w) sucrose and 1 ml of 10% (w/w) sucrose. These gradients were centrifuged for 16 h at 30,000 rpm in a SW50.1 rotor at 4°C. During the centrifugation, membrane-associated proteins floated to the top of the 50% sucrose layer, while soluble remained in the bottom layers of the gradient. The proteins in gradient fractions were analyzed by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250.

Methyltransferase and guanylyltransferase assays. Guanine-7-methyltransferse activity of the recombinant proteins in S15 was assayed in a buffer containing 50 mM Tris, pH 6.95, 4 mM $MgCl_2$, 2 mM DTT, 10 µM AdoMet, 1 µCi of S-adenosyl-[methyl-^{3}H]methionine and 10 mM GTP in 25 µl volume for 30 min. The reactions were stopped on ice by adding an equal volume of 0.2% SDS in 20 mM EDTA, pH 7.5. The labelled reaction products were isolated in small DEAE sepharose columns prepared in Pasteur pipets, and quantitated by liquid scintillation (Laakkonen et al., 1994). Covalent guanylate complex formation was assayed in a buffer containing 50 mM Tris, pH 7.5, 2 mM $MgCl_2$, 5 mM DTT, 10 mM KCl, 100 µM AdoMet, 5 µCi of α-^{32}P-GTP in 20 µl volume for 20 min at 28°C or 39°C. The reactions were stopped by adding SDS to a final concentration 2% and boiling for 2 min. Samples were subjected to SDS-PAGE, and phosphorimaging, and quantification was done using Tina 2.09c software.

Cells, transfection and immunofluorescence. HeLa and BHK-21 cells were cultivated as described by Salonen et al. (2003). Lipofectine (Gibco) or ExGen (Fermentas) were used as transfection reagents according to manufacturer's instructions. For immunofluorescence, HeLa cells were grown on coverslips in 35 mm dishes. Cells were transfected with pcDNA4/TO constructs using ExGen, followed by incubation for 16 h at 39°C or 28°C. Cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100 for 1 min. The aldehyde groups were quenched with 50 mM NH_4Cl. Cells were treated with rabbit anti-nsP1 antiserum (Kujala et al, 2001), followed by treatment with secondary antibodies conjugated with rhodamine.

Interactions between nsP1 and nsP3. 10^6 of BHK cells were co-electroporated with 3 µg of each of the two plasmids (pcDNA4/TO-nsP1 and pcDNA4/TO-nsP3) and incubated for 24 h at 28°C or 39°C. Cells were lysed in 1 ml buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA and protease inhibitor cocktail (Roche). Clarified lysate was collected after 15 min centrifugation at 15,000 × g. Half of the lysate was incubated with anti-nsP3 antiserum (guinea pig, Kujala et al., 2001), followed by incubation with protein A sepharose beads equilibrated in PBS containing 0.2% BSA. The beads were washed 4 times with lysis buffer, the samples were denatured by SDS, boiled for 5 min and 1/5th of the lysate was applied to SDS-PAGE. Western blotting of the resulting proteins was performed using rabbit anti-nsP1 antiserum.

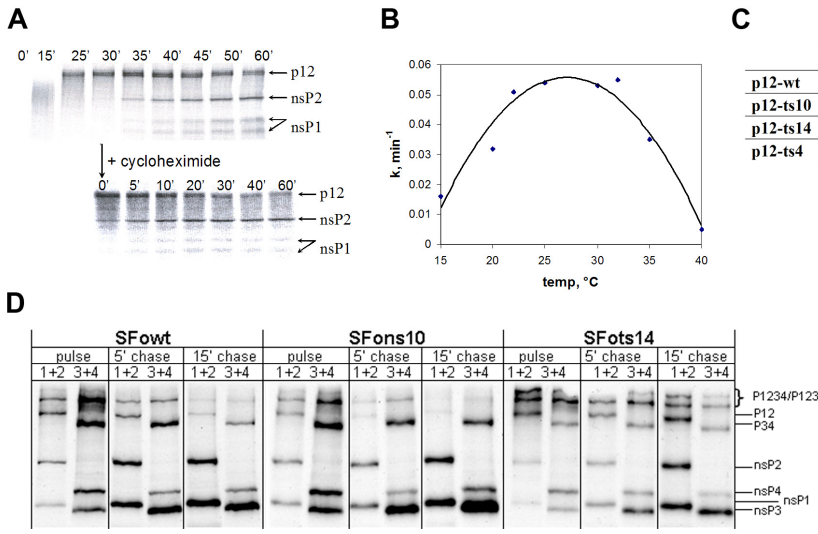
Protein labelling and immunoprecipitation. BHK cells in 35 mm dishes (approximately 10^6 cells/well) were infected at 28°C with 100 plaque forming units (PFU) of each virus (V. Lulla et al., 2006) per cell and incubated for 5.5 h at 28°C, then transferred to 39°C. Cells were washed once with pre-warmed PBS, supplied with methionine-free medium, incubated for 30 min at 39°C and pulsed with 50 µCi/plate of [^{35}S]methionine. Chase periods were done with cold methionine and cysteine (final concentrations 10 mM and 5 mM, respectively). Cells were lysed in 1% SDS followed by boiling. 1/10th of each lysate was used for immunoprecipitation with polyclonal rabbit antisera as described (Kujala et al., 2001). The immunoprecipitates were analyzed by SDS-PAGE and phosphorimaging.

RNA labelling and quantitation of minus-strands. BHK cells in 35 mm dishes (for overall RNA synthesis) or in 60 mm dishes (for negative-strand synthesis) were infected with 25 PFU/cell, then incubated in complete medium containing 20 mM Hepes, pH 7.4 at 30°C until the time of shift up to 40°C or harvest. At the times indicated for each experiment, cells on a 35 mm dish were labelled for a 1 h period with 1 ml [^{3}H]uridine (50 µCi/ml [^{3}H]uridine and 20 µg/ml Actinomycin D), and 60 mm dish with 200 µCi/ml and then harvested. The medium was removed, and 5% LiDS in LET buffer (0.1 M LiCl, 1 mM EDTA, 10 mM Tris, pH 8.0) containing 200 µg/ml proteinase K was added. The cells were harvested approximately at $2.5 \cdot 10^6$ cells/ml (0.7 ml per 35 mm dish). The lysate was passed 4 times through a 27-gauge needle to shear DNA. Duplicate or triplicate samples equivalent to 50,000 cells were precipitated with trichloroacetic acid to measure total incorporation.

To analyze negative-strand synthesis, the lysates were extracted 2-3 times with phenol at pH 4.3, and then 2 times with chloroform-isoamyl alcohol; the aqueous phase was adjusted to 0.2 M LiCl or 0.3 M sodium acetate, and 2 volumes of ethanol were used to precipitate the nucleic acids at –20°C overnight. The precipitate was collected by centrifugation, washed with 70% ethanol, dried and resuspended in 0.2 ml of STE (0.1 M NaCl, 1 mM EDTA and 10 mM Tris, pH 7.4). The samples were subjected to digestion with limited RNase A and chromatography on CF-11 columns, as described (Sawicki et al., 1993). Briefly, the NaCl concentration was adjusted to 0.3 M, and 25 µl of 1 µg/ml RNase A was added for a 15 min incubation at room temperature. Immediately thereafter, the solution was adjusted back to STE by addition of Tris-EDTA and to 35% ethanol. The samples were applied to washed CF-11 columns, tubes were rinsed with 1 ml of 35% ethanol-STE and the wash applied to the column, which was then allowed to drain. Then columns were washed with 15 ml of 35%-STE, followed by 15 ml of 15% EtOH-STE. The replicative forms (RFs) were eluted with 9 ml/column of STE directly into siliconized tubes. 0.5 ml of 4 M LiCl, 100 µg of purified yeast tRNA at 20 mg/ml, and 20 ml of absolute ethanol were added per tube. After through mixing, precipitation was carried out at –20°C overnight, followed by centrifugation at 10,000 rpm for 1 h at 4°C. The pellets were dried and resuspended in 200 µl of nuclease free water. Minus-strand RNA was measured by nuclease protection assay, which determines the amount of heat-denatured labelled RF RNA that was protected from RNase digestion (5 µg RNase A per ml) by hybridization to an about 100-fold excess of unlabelled positive-strand RNA.

RESULTS

It was shown previously that ts10 (E529D) and ts14 (D119N) are mutations in nsP1, which cause a temperature sensitive phenotype in SFV RNA replication. Ts10 was demonstrated to have an RNA± phenotype with 6% RNA synthesis remaining at 39°C, whereas ts14 showed a clear RNA-negative phenotype with very low RNA synthesis at the restrictive temperature. Shift to 39°C after 5 h incubation at 28°C didn't cause any reduction in 26S or 42S RNA synthesis,



	22ºC	32ºC	36ºC
p12-wt	0.050	0.045	0.019
p12-ts10	0.039	0.042	0.021
p12-ts14	0.040	0.040	0.019
p12-ts4	0.045	0.005	0.009

FIG. 1. Processing of wild type and mutant P12 polyproteins. (**A**) Polyproteins P12 were synthesized in a cell-free translation system in the presence of [^{35}S]methionine at 30ºC. Aliquots were taken at the indicated times and analyzed by SDS-PAGE and autoradiography. Arrows indicate the positions of nsPs and their precursor P12. P12 synthesis was stopped after 30 min with 1 mM cycloheximide, and the processed samples were collected at the indicated time points and analyzed by SDS-PAGE and autoradiography. (**B**) Processing kinetics of wild type P12 *in vitro* at different temperatures. Y-axis represents the first order kinetic constant in min^{-1}. (**C**) Comparison of the first order kinetic constants for the processing of wild type and mutant P12 proteins at different temperatures. (**D**) Polyprotein processing in cells infected with SFowt, SFons10 and SFots14. Proteins were labelled with [^{35}S]methionine, the sample from one parallel plate was extracted immediately after the pulse, while other plates were chased with the excess of cold methionine for 5 min or 15 min before extraction. The samples were immunoprecipitated with indicated combinations of antibodies against the ns proteins and analyzed by SDS-PAGE and fluorography. The virus strain, sample (pulse, 5 min or 15 min chases), and antibody combinations used for immunoprecipitation (IP, numbers denote antibodies against the ns proteins) are indicated at the top, the positions of proteins and precursors are marked on the right.

indicating that once synthesized at the permissive temperature, the mature replicase complex is capable of functioning normally at the restrictive temperature (V. Lulla *et al*, 2006). Since several functions are known for nsP1, characterization the specific defect(s) in the mutants was expected to illuminate the roles of nsP1 in RNA replication.

P12 polyprotein processing. As one of the mutations (ts10) mapped very close to the nsP1/nsP2 border (position -9 relative to cleavage site), we considered the possibility of a cleavage defect at this site. The 1/2 site is cleaved relatively slowly to permit the existence of the negative-strand synthesizing replication complex, and the cleavage takes place *in cis* (Vasiljeva et al., 2003). We therefore examined the cleavage efficiency of P12 translated *in vitro*. The wild type P12 polyprotein was translated for 30 min and the synthesis was stopped by cycloheximide at this point, when reasonable amounts of P12 have accumulated, but most of the protein remains uncleaved. The time course of cleavage was monitored by gel electrophoresis and autoradiography (Fig. 1A). First order reaction kinetics was measured both for the decomposition of P12 and for the formation of nsP2 from quantification of the [^{35}S]methionine labelled protein bands. Cleavage experiments at different temperatures showed that there is a very inefficient cleavage of P12 at 39°C (Fig. 1B). This is also the case when purified recombinant protease is used in cleavage studies, showing that the protease is more sensitive to temperature *in vitro* than it is in infected cells (Balistreri et al., 2007). Therefore we used 22°C as permissive temperature, 32°C and 36°C as restrictive temperatures, and compared the processing efficiencies of mutant polyproteins with the wild type P12 processing efficiency *in vitro*. A well-characterized ts protease activity mutant located within the protease domain of nsP2, ts4 (Suopanki *et al*, 1998; Balistreri et al., 2007), was used as a control. As expected, P12-ts4 had greatly reduced kinetic constant values at 32°C and 36°C when compared with the wild type P12 (Fig. 1C). In contrast, for P12-ts10 and P12-ts14, the first order kinetic constant didn't differ significantly from the wild type P12 data (Fig. 1C).

Polyprotein processing in virus-infected cells was studied in pulse-chase experiments with [^{35}S]methionine, as described in Materials and Methods. Recombinant virus containing the ts4 mutation was again taken as a known protease-defective control. The wild type virus SFowt showed almost complete processing of the labelled polyprotein after 60 min chase, whereas in SFots4 infected cells polyproteins P1234, P123, P12 and P34 precursors accumulated, and out the individual proteins only nsP4 could be detected even after 60 min chase (data not shown). Using the shorter chase times revealed that the cleavage of the polyprotein in SFons10 infected cells occurred exactly as during SFowt infection, whereas in SFots14 infected cells the processing of P1234 was slightly slower (Fig. 1D): the precursors were more strongly visible than for SFowt (Fig. 1D). Nevertheless all of the individual nonstructural proteins could also be clearly detected even in pulsed samples. Thus the processing defect of SFots14 is very mild compared to the nsP2 mutants previously examined (Balistreri et al., 2007), and its significance is not understood.

Enzymatic activities of nsP1. To study enzymatic activities of the nsP1 needed in RNA capping, the genes encoding for nsP1-ts10 and nsP1-ts14 were cloned into pBAT4 expression vector and expressed in *E. coli* as recombinant

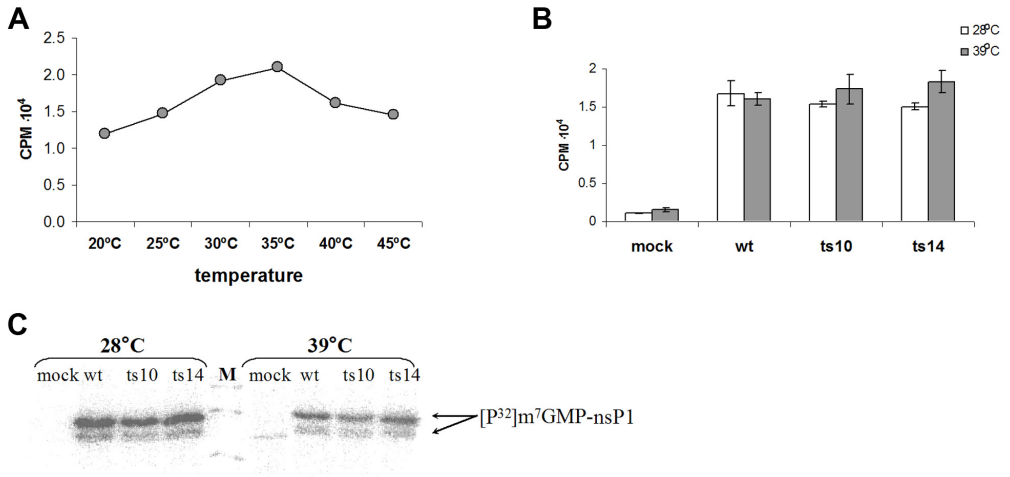


FIG. 2. Enzymatic activities of nsP1 assayed at different temperatures. (**A**) Guanine-7-methyltransferase activity of nsP1-wt. Incorporation of methyl-^{3}H (in counts per minute, CPM) from AdoMet at different temperatures is shown. (**B**) Guanine-7-methyltransferase activities of nsP1-wt, nsP1-ts10 and nsP1-ts14 at 28°C and 39°C. Reactions were done in 3 parallels; error bar indicates standard deviation. (**C**) Formation of enzyme-guanylate complex. S15 fractions of *E. coli* lysates were incubated with [α-^{32}P]GTP at 28°C and 39°C (see Materials and Methods). Proteins were separated by SDS-PAGE followed by phosphoimaging. NsP1 expressed in *E. coli* always yields a major product, which represents the full-length protein, and a minor, shorter product, which most likely arises due to premature translation termination. Both products are enzymatically active (Ahola et al., 1997).

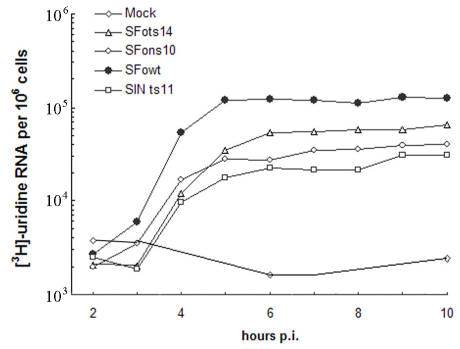


FIG. 3. Positive strand synthesis by wild type SFV and the nsP1 ts mutants. Cells infected with the indicated viruses were labelled with [^{3}H]-uridine in the presence of actinomycin D for 1 h. The labelling was terminated at the indicated time points by cell lysis and RNA extraction. Incorporated label was measured by scintillation counting. The total RNA synthesis is taken to represent positive-strand RNA synthesis, since >90% of total viral RNA synthesis at all time points is positive-strand RNA synthesis.

proteins. *E. coli* transformed with the empty pBAT4 plasmid was used as a control in all the experiments, and exactly the same procedures were performed with the 4 samples, designated as mock, nsP1-wt, nsP1-ts10 and nsP1-ts14, respectively.

Guanine-7-methyltransferase activity was measured in bacterial lysates clarified by a short centrifugation (Ahola et al., 1997). The temperature-dependence of the wild type nsP1 methyltransferase was not as marked as that of the protease (Fig. 2A). Although optimal activity was found around 35°C, the permissive and restrictive temperatures of virus growth, 28°C and 39°C, could easily be used in the assays. All the three proteins nsP1-ts10, nsP1-ts14 and nsP1-wt showed equal and strong methyltransferase activity both at 28°C and 39°C (Fig. 2B).

TABLE 1. Ratio of the synthesis of genome and 26S RNA at 30ºC and after shift to 40ºC

Virus	Molar ratio genome:26S RNA		40ºC/30ºC ratio
	30ºC	shift to 40ºC	
SFowt	0.33	1.3	3.9
SFots10	0.21	0.76	3.6
SFots14	0.38	1.41	3.7
SIN ts11	0.43	0.9	2.1

The other RNA capping reaction catalyzed by nsP1 is guanylyltransferase, which can be measured by covalent complex formation between nsP1 and 7-methyl-GMP (Ahola and Kääriäinen, 1995). Again in this reaction, nsP1-ts10, nsP1-ts14 and nsP1-wt showed equal and strong activity, which was somewhat higher at 28°C than at 39°C for all three proteins (Fig. 2C). Western blotting with anti-nsP1 antibodies was used in these experiments to verify the equal expression levels of the proteins (data not shown). These results strongly suggest that defects in the RNA capping reactions are not involved in the temperature sensitive phenotypes of the ts10 and ts14 mutations.

Positive-strand RNA synthesis. We next turned our attention directly to RNA synthesis in virus-infected cells. We first studied RNA synthesis at the permissive temperature (30°C was used in these experiments) to compare the mutant and wild type viruses. Positive-strand synthesis (measured as cpm incorporated to viral RNA per hour of labelling) increased early and then reached a constant, maximum rate, in a pattern that is typical for alphavirus infection. The SFV nsP1 mutants resembled SIN ts11, another nsP1 mutant, in showing a maximum rate that was ~30-50% of the wild type SFV rate (Fig. 3). There were no ts defects in genome or 26S mRNA synthesis at either 30°C or after shift to 40°C (Table 1), indicating that the mutants are not defective in 26S RNA synthesis. At the higher temperature, genomic RNA synthesis was increased relative to subgenomic RNA synthesis for the

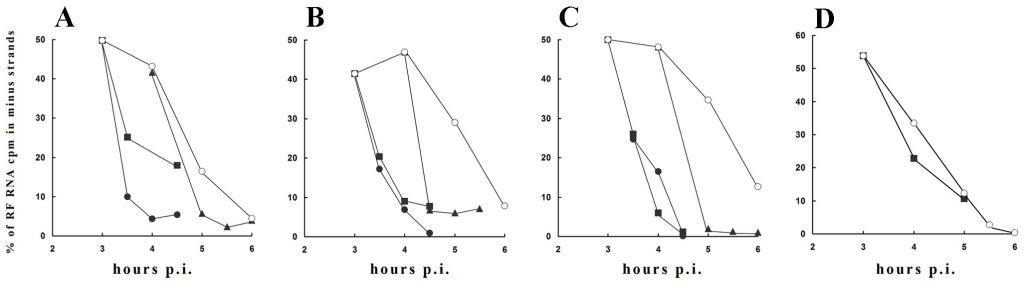


FIG. 4. Negative strand synthesis by the nsP1 ts mutants and wild type SFV. Cells were labelled with [^{3}H]uridine as described in Materials and Methods, and the replicative forms (RF) were isolated. The percentage of labelled negative strand RNA was determined by an RNAse protection assay (see Materials and Methods). The percent of RF RNA cpm in minus-strands was determined for SFons10 (**A**), SFots14 (**B**), SIN ts11 (**C**) and SFowt (**D**); incubations at 30ºC (○), then shift after 3 h to 40ºC (■), shift after 4 h to 40ºC (▲) and shift after 3 h to 40°C in medium containing cycloheximide (●) were done.

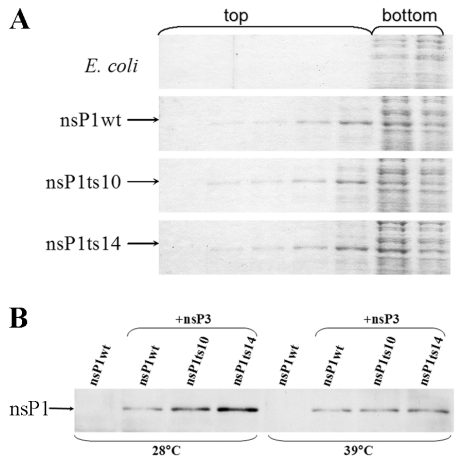


FIG. 5. Interactions of wt and mutant nsP1 of SFV with cellular and viral components. (**A**) Membrane association of nsP1 proteins analyzed by flotation. NsP1 was expressed in *E. coli* and S15 supernatant fraction of the bacterial lysates was subjected to flotation in sucrose gradient. The gradient fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Top and bottom fractions are indicated above the panels, the positions of the analyzed proteins are marked on the left. (**B**) Interactions of wt and mutant nsP1 with nsP3 of SFV at different temperatures. BHK-21 cells were co-electroporated with the plasmids expressing nsP1 and nsP3 at 28°C or 39°C. The cell lysates were incubated with guinea pig anti-nsP3 antiserum, followed by incubation with protein A sepharose and immunoprecipitation. Precipitated samples were denatured and analyzed by SDS-PAGE followed by western blotting using rabbit anti-nsP1 antiserum and visualized by ECL. The analyzed mutant and wt proteins are indicated at the top, the position of nsP1 is marked on the left and temperatures used in the experiments are shown on the bottom.

wild type and for the mutants, as reported previously for both SFV and SIN.

Negative-strand RNA synthesis. Minus-strand synthesis at 30°C was efficient early in infection: ~40% of [^{3}H]-uridine labelled RF RNA was in minus-strands each hour, indicating that ~80% or more of the minus-strands present were newly made during the previous hour. Both wild type SFV (Fig. 4D) and the nsP1 ts mutants (Fig. 4A-C) showed a cessation of minus-strand synthesis by 6 h p.i. (Fig. 4A-C, open circles). When cells were shifted to 40°C at 3 h p.i. or 4 h p.i., this resulted in a rapid cessation of minus-strand synthesis by SFV ts10 and ts14 that was similar to the rapid shutoff by SIN ts11 (Fig. 4A-C, closed triangles and rectangles). Wild type SFV did not show a rapid shutoff when shifted to 40°C, but instead the rate of its normal minus-strand cessation was slightly increased at the higher temperature (Fig. 4D). This argues that the nsP1 mutants SFV ts10 and ts14 are specifically ts for minus-strand synthesis.

Shift to 40°C at 3 h p.i. and incubation in a medium containing cycloheximide (100 µg/ml) led to a rapid cessation of minus-strand synthesis by SFV ts10 and ts14, and SIN ts11 (Fig. 4A-C, closed circles). The rate of loss was similar to that seen for temperature shift alone. This indicates that shift to 40°C prevented any further production of functional nsP1 proteins since it was equivalent to conditions that prevented actual synthesis of nascent nsP1 proteins. The results further support a conclusion that the SFV nsP1 mutants have ts defects in functions required for minus-strand synthesis that would include promoter recognition or initiation.

Protein localization and protein-protein interactions. We were also interested in examining whether the ts mutations would affect nsP1 localization or protein-protein interactions in a temperature-dependent manner. First, the localization of the nsP1 was studied in HeLa cells transiently transfected with derivatives of the pcDNA4/TO vector containing the CMV promoter. Both the wild type and the two mutant proteins showed association with the plasma membrane, with induction of prominent filopodia-like structures, as described before for nsP1 (Laakkonen et al., 1996; Spuul et al., 2007). No temperature dependent (28°C versus 39°C) difference in localization of nsP1 or in induction of filopodia-like structures was apparent for any of the nsP1 derivatives used in this assay (data not shown). Second, the membrane association of recombinant proteins was also studied after expression in *E. coli* by flotation of cell lysates through in a discontinuous sucrose gradient. SDS-PAGE analysis of floated material, visualised by Coomassie Blue staining (Fig. 5A), revealed that both nsP1-ts10 and nsP1-ts14 were, similar to nsP1-wt, membrane-associated also in this system. This data is fully consistent with findings that nsP1-ts10 and nsP1-ts14 were found to be enzymatically active, since these activities are known to depend on membrane association of nsP1 (Ahola et al., 1999). Third, we studied the interactions between nsP1 and nsP3 proteins by co-expression of these proteins in BHK21 cells at 28°C or 39°C. It was found that both nsP1-ts10 and nsP1-ts14, co-expressed in BHK cells, showed clear interaction with nsP3, similar to nsP1-wt (Fig. 5B) indicating that at least this interaction, which has been shown to be crucial for correct replicase complex formation (Salonen et al., 2003), is not temperature dependent in case of ts10 and ts14.

DISCUSSION

We have previously identified and characterized the temperature sensitive mutations of SFV (V. Lulla et al., 2006). Six of them mapped to nsP2 were subsequently characterized in terms of nsP2-related functions (Balistreri et al., 2007). The combination of biochemical assays on the one hand and the studies of the properties of recombinant viruses with ts mutations on the other have proved to be a valuable approach for many occasions. In the case of SFV such an approach resulted in new insights to the functions and properties of nsP2. Here we presented the results of the detailed analysis of two ts mutations mapped to nsP1; both direct functions of the proteins by the use of the purified proteins and indirect effects of the mutations were analyzed.

Subcellular localization, membrane binding and capping activities. In contrast to the situation with SFV nsP2, where ts mutations mapped to nsP2 specifically affected the enzymatic functions of the protein (Balistreri et al., 2007), it was found that direct functions, typical for individual nsP1, such as the subcellular localization, the membrane binding, the guanine-7-methyltransferase and the guanylyltransferase activities were not affected by ts-mutations in nsP1 (Fig 2, 5). Most of these assays were carried out by the use of the recombinant proteins, expressed in *E. coli* and were not pursued in infected cells and extracts, as recombinant protein results were so convincingly negative. Therefore it can be possible that since the expression of the proteins was carried out at low temperature, the ts defects, which would only have an effect during protein synthesis and/or folding, were not noticed in this study. However, we consider this rather unlikely since the ts defects of all the mutations mapped to nsP2 were well reproduced at the recombinant proteins expressed and purified by similar procedure (Balistreri et al., 2007).

The known direct functions of nsP1 are linked with each other: the membrane binding of the nsP1 both determines its subcellular location (Spuul et al., 2007) and is the pre-requisite for its enzymatic functions (Ahola et al., 1999); those in turn are essential for viral RNA replication (Wang et al., 1996). Some limited information about nsP1 structural determinants, responsible for these functions, is already known and, as it is evident from data presented in Fig. 6, neither of the identified ts mutation affects the critical aa residues responsible for these functions. Nevertheless mutations of other residues within the functional domains can also cause functional changes, including ts defects. However, this was found not to be the case for ts14 and ts10. Despite the fact that ts14 lies within the capping domain of nsP1, our results revealed no defects in the RNA capping reactions catalyzed by nsP1 carrying ts14 mutation.

Why no ts mutation analyzed in this study had a clear effect on any direct function of nsP1 remains unsolved. It may simply reflect the fact that the number of ts mutations, mapped to nsP1 of alphaviruses, is rather small compared to the number of the ts mutations mapped to nsP2. As a consequence, the ts mutations affecting the nsP1 mediated capping reaction may have been not found just by chance. While this remains a plausible option, the sharp contrast between the effects of ts mutations in nsP1 and nsP2 is intriguing. In this context it is interesting to note that the deletion of cysteine residues 418-420 (e.g. palmitoylation sequence) from nsP1 of SFV also resulted in the virus with a clear ts phenotype (our unpublished data). Similar to the situation with ts10 and ts14, it was found that this deletion did not affect the enzymatic activities of the nsP1 nor did it prevent the membrane binding of the protein. Since the palmitoylation was found to be unessential for SFV replication (Žusinaite et al., 2007), it can be concluded that the ts

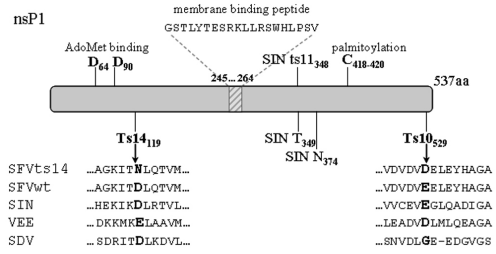


FIG. 6. Schematic representation of nsP1. The amino acid (aa) sequences and positions indicated are according to the sequence of the nsP1 of SFV, positions corresponding to the nsP1 of SIN are indicated with prefix SIN. Locations of conserved and functionally important aa residues are shown. Aa sequence alignments for the regions, corresponding to the positions of ts10 and ts14, for representatives of the principal groups of alphaviruses are provided. VEE indicates Venezuelan equine encephalitis virus and SDV indicates sleeping disease virus.

phenotype of this virus, similar to the ts10 and ts14, most likely represents a consequence of defects in other functions of nsP1.

Polyprotein processing *in vitro* and *in vivo*. Polyprotein processing is a direct function of nsP2, not nsP1. Only a short sequence at the C-terminus of nsP1 – the upstream region of 1/2 cleavage site – is directly involved in this reaction (A. Lulla et al., 2006). Therefore it is not surprising that almost all the ts mutants with ns polyprotein processing defects, analyzed so far, always had lesions in nsP2. As an exception, in the Sindbis-group alphavirus S.A.AR86 a mutation in nsP1 (T538I), which lies within the 1/2 cleavage site, is known to result in the accelerated growth of the virus, processing of nsPs and the synthesis of viral RNAs. These effects are due to the earlier expression from the subgenomic promoter without affecting viral genomic minus- or plus-strand synthesis (Heise et al., 2003). Since ts10 was mapped rather close to the nsP1/nsP2 site, it was logical to assume that it can cause a temperature dependent defect in processing of this site. However, it was not found to be the case neither *in vitro* nor *in vivo*. This finding is in agreement with the fact that ts10 already lies outside the cleavage substrate recognition area (A. Lulla et al., 2006), whereas T538I in S.A.AR86 most probably represents part of the protease recognition sequence itself. Surprisingly, the slight processing defect of ts14 was observed *in vivo*, but it is rather insignificant compared to the defects caused by ts mutations in nsP2 (V. Lulla et al., 2006) and might indicate a slight general conformational disturbance of the whole polyprotein. It is difficult to predict, which kind of effect (if any) this defect may have on the replication of the corresponding virus. On the one hand the 1/2 site cleavage plays a central role in the alphavirus replication regulating the template switch (plus- versus minus-strand) in the RNA synthesis (Hahn et al., 1989; Hardy and Strauss, 1989). On the other hand, one should expect that the slow-down of the polyprotein processing should result in stabilization of the early replicase complex (P123 + nsP4). This stabilization due to the processing defects should either increase or, most likely, not affect the minus-strand synthesis; in reality an opposite effect was observed in temperature shift-up experiments (Fig.4). Therefore the functional significance of this phenomenon remains unknown.

Mediation of the minus-strand synthesis and replicase formation. Similarly to SIN ts11, SFV ts10 and ts14 were found to be normal for the positive-strand RNA synthesis; at the same time all these mutants had specific defect in minus-strand synthesis. It is known that the minus-strand synthesis depends on the formation of the functional early replicase complex consisting of P123 and nsP4 subunits. Therefore one possible

explanation of the defect in the minus-strand synthesis is a failure of nsP1 to interact with its partners required for replicase formation. It has been demonstrated that nsP1 and nsP3 parts of P123 polyprotein are needed for the formation and correct location of the replication complex like structures in transfected cells (Salonen et al., 2003); in addition we have demonstrated that in infected cells at least ts14 causes some delay of P1234 processing at the restrictive temperature most likely by affecting its structure (Fig. 1D). Our experiment, however, failed to reveal any significant temperature dependent defect in interactions between nsP1 and nsP3 (Fig. 5B). There are several possible explanations for this finding. First, it is possible that the defects caused by ts mutation inside nsP1 do not affect the interaction of mature nsP1 and nsP3 and are only detectable if these proteins are expressed in the form of polyprotein precursor. Second, this data may indicate that the ts defects caused by these mutations do not affect interactions within P123 but the interaction of P123 with nsP4. Additional experiments are needed to find out which (if any) of these possibilities constitute the true cause of the ts defect and thus alterations in the interaction of nsP1 with other nsPs, viral RNAs or even with host factors merit further study.

In conclusion, the performed analysis of ts mutations inside nsP1 proved the role of the nsP1 in the alphavirus minus-strand RNA synthesis. Surprisingly, in contrast to the ts mutations mapped to nsP2, these mutations did not influence any of the known enzymatic activities of nsP1. These findings indicate that the mechanism of the action of ts mutations in nsP1 is different from that of the mutations mapped in nsP2. It can be proposed that most likely both ts10 and ts14 influence the minus-strand synthesis by affecting replicase complex formation and/or its interaction with the promoter sequence in the genomic RNA of SFV.

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REFERENCES

1. **Ahola, T., and L. Kääriäinen.** 1995. Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. Proc. Natl. Acad. Sci. USA **92:**507-511.
2. **Ahola, T., P. Laakkonen, H. Vihinen, and L. Kääriäinen.** 1997. Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities. J. Virol. **71:**392-397.
3. **Ahola, T., A. Lampio, P. Auvinen, and L. Kääriäinen.** 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J. **18:**3164-3172.
4. **Balistreri, G., J. Caldentey, L. Kääriäinen, and T. Ahola.** 2007. Enzymatic defects of the nsP2-proteins of Semliki Forest virus temperature-sensitive mutants. J. Virol. **81:**2849-2860.
5. **Barton, D. J., S. G. Sawicki, and D. L. Sawicki.** 1988. Demonstration in vitro of temperature-sensitive elongation of RNA in Sindbis virus mutant ts6. J. Virol. **62:**3597-3602.
6. **Dé, I., C. Fata-Hartley, S. G. Sawicki, and D. L. Sawicki.** 2003. Functional analysis of nsP3 phosphoprotein mutants of Sindbis virus. J. Virol. **77:**13106-13116.
7. **Egloff, M. P., Malet, H., Putics, A., Heinonen, M., Dutartre, H., Frangeul, A., Gruez, A., Campanacci, V., Cambillau, C., Ziebuhr, J., Ahola, T. and Canard, B.** 2006. Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. J. Virol. **80**:8493-8502.
8. **Gomez de Cedrón, M., N. Ehsani, M. L. Mikkola, J. A. García, and L. Kääriäinen.** 1999. RNA helicase activity of Semliki Forest virus replicase protein NSP2. FEBS Lett. **448:**19–22.
9. **Hahn, Y. S., E. G. Strauss, and J. H. Strauss.** 1989. Mapping of RNA$^-$ temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. J. Virol. **63:**3142-3150.
10. **Hardy, W. R., and J. H. Strauss.** 1989. Processing the nonstructural polyproteins of Sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. J. Virol. **63:**4653-4664.
11. **Heise, M. T., J. W. Laura, D. A. Simpson, C. L. Kristen, A. Bernard, R. B. Meeker, and R. E. Johnston.** 2003. An Attenuating Mutation in nsP1 of the Sindbis-group virus S.A.AR86 accelerates nonstructural protein processing and up-regulates viral 26S RNA synthesis. J. Virol. **77**: 1149-1156.
12. **Kääriäinen, L., and T. Ahola.** 2002. Functions of alphavirus nonstructural proteins in RNA replication. Prog. Nucleic Acid Res. Mol. Biol. **71:**187-222.
13. **Keränen, S., and L. Kääriäinen.** 1979. Functional defects of RNA-negative temperature-sensitive mutants of Sindbis and Semliki Forest viruses. J. Virol. **32:**19-29.
14. **Kim, K. H., T. Rumenapf, E. G. Strauss, and J. H. Strauss.** 2004. Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. Virology. **323:**153-163.
15. **Kujala, P., A. Ikäheimonen, N. Ehsani, H. Vihinen, P. Auvinen, and L. Kääriäinen.** 2001. Biogenesis of the Semliki Forest virus RNA replication complex. J. Virol. **75:**3873-3884.
16. **Laakkonen, P., T. Ahola, and L. Kääriäinen.** 1996. The effects of palmitoylation on membrane association of Semliki Forest virus RNA capping enzyme. J. Biol. Chem. **271:**28567-28571.
17. **Laakkonen, P., M. Hyvönen, J. Peränen, and L. Kääriäinen.** 1994. Expression of Semliki Forest virus nsP1-specific methyltransferase in insect cells and in *Escherichia coli*. J. Virol. **68:**7418-7425.
18. **Lemm, J. A., T. Rümenapf, E. G. Strauss, J. H. Strauss, and C. M. Rice.** 1994. Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. EMBO J. **13:**2925-2934.
19. **Lulla, A., V. Lulla, K. Tints, T. Ahola, and A. Merits.** 2006. Molecular determinants of substrate specificity for Semliki Forest virus nonstructural protease. J. Virol. **80:**5413-5422.
20. **Lulla, V., A. Merits, P. Sarin, L. Kääriäinen, S. Keränen, and T. Ahola.** 2006. Identification of mutations causing temperature-sensitive defects in Semliki Forest virus RNA synthesis. J. Virol. **80:**3108-3111.
21. **Mi, S., and V. Stollar.** 1991. Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli. Virology **184:**423-427.
22. **Salonen, A., L. Vasiljeva, A. Merits, J. Magden, E. Jokitalo, and L. Kääriäinen.** 2003. Properly folded nonstructural polyprotein directs the Semliki Forest virus replication complex to the endosomal compartment. J. Virol. **77:**1691-1702.
23. **Sawicki, D. L., L. Kääriäinen, C. Lambek, and P. J. Gomatos.** 1978. Mechanism for control of synthesis of Semliki Forest virus 26S and 42S RNA. J. Virol. **25:**19-27.
24. **Sawicki, D. L., and S. G. Sawicki.** 1993. A second nonstructural protein functions in the regulation of alphavirus negative-strand RNA synthesis. J. Virol. **67:**3605-3610.
25. **Sawicki, D. L., S. G. Sawicki, S. Keränen, and L. Kääriäinen.** 1981. Specific Sindbis virus-coded function for minus-strand RNA synthesis. J. Virol. **39:**348-358.
26. **Shirako, Y., and J. H. Strauss.** 1994. Regulation of Sindbis virus RNA replication: Uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. J. Virol. **68:**1874-1885.
27. **Spuul, P., A. Salonen, A. Merits, E. Jokitalo, L. Kääriäinen, and T. Ahola.** 2007. Role of the amphipathic membrane binding peptide of Semliki Forest virus replicase protein nsP1 in membrane association and virus replication. J. Virol. **81:**872-883.
28. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58:**491-562.
29. **Suopanki, J., D. L. Sawicki, S. G. Sawicki, and L. Kääriäinen.** 1998. Regulation of alphavirus 26S mRNA transcription by replicase component nsP2. J. Gen Virol. **79:**309-319.
30. **Thal, M. A., B. R. Wasik, J. Posto, and R. W. Hardy.** 2007. Template requirements for recognition and copying by Sindbis virus RNA-dependent RNA polymerase. Virology **358:**221-232.
31. **Vasiljeva, L., A. Merits, P. Auvinen, and L. Kääriäinen.** 2000. Identification of a novel function of the alphavirus capping apparatus: RNA 5'-triphosphatase activity of nsP2. J. Biol. Chem. **275:**17281-17287.
32. **Vasiljeva, L., A. Merits, A. Golubtsov, V. Sizemskaja, L. Kääriäinen, and T. Ahola.** 2003. Regulation of the sequential processing of Semliki Forest virus nonstructural polyprotein. J. Biol. Chem. **278:**41636-41645.
33. **Vihinen, H., T. Ahola, M. Tuittila, A. Merits, and L. Kääriäinen.** 2001. Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. J. Biol. Chem. **276:**5745-5752.
34. **Wang, H.-L., J. O'Rear, and V. Stollar.** 1996. Mutagenesis of the Sindbis virus nsP1 protein: effects on methyltransferase activity and viral infectivity. Virology **217:**527-531.
35. **Wang, Y.-F., S. G. Sawicki, and D. L. Sawicki.** 1991. Sindbis virus nsP1 functions in negative-strand RNA synthesis. J. Virol. **65:**985-988.
36. **Wang, Y.-F., S. G. Sawicki, and D. L. Sawicki.** 1994. Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA J. Virol. **68:**6466-6475.
37. **Žusinaite, E., Tints, K., Kiiver, K., Spuul, P., Karo-Astover, L., Merits, A. and Sarand, I.** 2007. Mutations at the palmitoylation site of non-structural protein nsP1 of Semliki Forest virus attenuate viral replication and cause accumulation of compensatory mutations. J. Gen. Virol. in press.

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1998–2001	*B.Sc* in bioorganic chemistry, University of Tartu, Insitute of Bioorganic Chemistry, *cum laude*
2001–2003	*M.Sc* in molecular biology, University of Tartu, Insitute of Molecular and Cell Biology
2003–	*Ph.D* student in molecular biology, University of Tartu, Insitute of Molecular and Cell Biology
2003–	research scientist in Estonian Biocentre, Tartu

Scientific work

Since 2001 I have been working in the group of Prof. Andres Merits. My main research topic has been the replication of Semliki Forest virus, its temperature sensitive mutants and replicon vectors. I have characterized several aspects of the SFV replication, identified and described ts mutants, constructed and tested several novel SFV based vectors. I have also taken part in the characterization of the nsP2 protease of SFV as well as in the development of the method for generation of the SFV based expression libraries.

List of Publications

1. Vasiljeva L, Merits A, Golubtsov A, **Sizemskaja V**, Kääriäinen L, Ahola T. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J Biol Chem.* 2003, 278(43):41636–41645.
3. **Lulla V**, Merits A, Sarin P, Kääriäinen L, Keränen S, Ahola T. Identification of mutations causing temperature-sensitive defects in Semliki Forest virus RNA synthesis. *J Virol.* 2006, 80(6):3108–3111.
3. Lulla A, **Lulla V**, Tints K, Ahola T, Merits A. Molecular determinants of substrate specificity for Semliki Forest virus protease. *J Virol.* 2006, 80(11):5413–22.
4. Tamberg N, **Lulla V**, Fragkoudis R, Lulla A, Fazakerley JK, Merits A. Insertion of EGFP into the replicase gene of Semliki Forest virus results in a novel, genetically stable marker virus. *J Gen Virol.* 2007, 88:1225–1230.

ELULOOKIRJELDUS

Valeria Lulla (Sizemskaja)

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Haridus ja erialane teenistuskäik

1993–1998	Narva Pähklimäe Gümnaasium, *lõpetasin hõbemedaliga*
1998–2001	Bakalaureuseõpe Tartu Ülikoolis, *B.Sc* bioograanilise keemia eralal, *cum laude*
2001–2003	Magistriõpe Tartu Ülikoolis, M.Sc molekulaar- ja rakubioloogia erialal
2003–	Doktorant Tartu Ülikooli Molekulaar- ja Rakubioloogia Instutuudis
2003–	Erakorraline teadur Eesti Biokeskuses, Tartu

Teadustegevus

Alates 2001 aastast olen töötanud Prof. Andres Merits grupis. Minu põhiliseks uurimisteemaks on olnud Semliki Forest viiruse replikatsiooni uurimine, selle viiruse temperatuuri tundlikud mutandid ning nende põhinevad replikon-vektorid. Olen kirjeldanud SFV replikatsioonilisi omadusi, identifitseerinud ja iseloomustanud temperatuuri tundlike mutante, konstrueerunud ja iseloomustanud uudseid SFV-l põhinevaid vektoreid. Olen samuti osalenud nsP2 proteaasi iseloomustamises ja SFV-l põhinevate raamatukogude konstrueerimise meetodi välja töötamises.

Publikatsioonide nimekiri

1. Vasiljeva L, Merits A, Golubtsov A, **Sizemskaja V**, Kääriäinen L, Ahola T. Regulation of the sequential processing of Semliki Forest virus replicase polyprotein. *J Biol Chem.* 2003, 278(43):41636–41645.
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