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LIANE VIRU

Development and analysis of novel alphavirus-based multifunctional gene therapy and expression systems

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UNIVERSITY OF TARTU
PRESS
1632

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Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on August 28th, 2013 by the Council of Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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Commencement: Auditorium 121, Nooruse 1, Tartu, at 14.15 on January 24th, 2014

Publication of this thesis is granted by the Institute of Technology, Faculty of Science and Technology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund

ISSN 2228–0855
ISBN 978–9949–32–470–5 (print)
ISBN 978–9949–32–471–2 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I **Ülper L**., Sarand I., Rausalu K., Merits A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of Virological Methods*, 148, 265–270.

II **Viru L**., Heller G., Lehto T., Pärn K., El Andaloussi S., Langel Ü., Merits A. (2011). Novel viral vectors utilizing intron splice-switching to activate genome rescue, expression and replication in targeted cells. *Virology Journal*, 8, 243.

III Ratnik K., **Viru L**., Merits A. (2013). Control of the rescue and replication of Semliki Forest virus recombinants by the insertion of miRNA target sequences. *PLoS One*, 8, e75802.

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Author's contribution:

I Performed infectivity assay, analysis of stability of rescued virus genome and growth curve experiments; analyzed proteins expressed by rescued virus.

II Participated in the construction of full-length SFV DNA/RNA layered vectors; performed all experiments with full-length SFV DNA/RNA layered constructs.

III Performed and designed experiments together with K. Ratnik; performed growth curve experiments.

LIST OF ABBREVIATIONS

aa	amino acid (residue)
ASO	antisense oligonucleotides
C	alphavirus capsid protein
cDNA	complementary DNA
CHIKV	Chikungunya virus
CMV	human cytomegalovirus
CNS	central nervous system
CPV	cytopathic vacuoles (also known as cytoplasmic vacuoles)
DC	dendritic cell
DENV	dengue virus
Gluc	*Gaussia* luciferase
EEEV	Eastern equine encephalitis virus
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
HCV	hepatitis C virus
HSV	herpes simplex virus
ICA	infectious center assay
icDNA	infectious complementary DNA
IRES	internal ribosome entry site
kb	kilobase
Luc	firefly luciferase
miRNA	microRNA
MOI	multiplicity of infection
NC	nucleocapsid
NLS	nuclear localization signal
ns	nonstructural
nsP	nonstructural protein
ORF	open reading frame
RdRp	RNA-dependent RNA polymerase
SFV	Semliki Forest virus
SG	subgenomic
SINV	Sindbis virus
SSO	splice-switching oligonucleotide
TAA	tumor associated antigene
TF	transframe protein
TGF-β	transforming growth factor beta
TK	thymidine kinase
Ts	temperature-sensitive
UTR	untranslated region
VEEV	Venezuela equine encephalitis virus
VRP	virus-replicon particle
WEEV	Western equine encephalitis virus
wt	wild type

INTRODUCTION

Viruses are usually described as non-living objects because they lack independent metabolism and therefore require living cells for procreation. Usually, though not always, viral infection is harmful for cells and can even result in cell death. Although there is no direct correlation between the cytotoxicity of a virus and its *in vivo* pathogenicity, the same also applies at the level of the organism: viral infection disturbs numerous processes within the organism and activates various defense reactions that in combination with other factors, may lead to sickness and death of the organism. For this reason, viruses capable of infecting humans are often medically important pathogens. On the other hand, both killing of cells and the activation of defense reactions of cells and organisms by viruses provide researchers with the opportunity to use viruses and virus-based vectors to treat various genetic disorders and as anti-cancer agents.

Alphaviruses are small positive-strand RNA viruses that make up the genus *Alphavirus* of the family *Togaviridae*. The species of the genus are rather numerous and genetically diverse. The type member of the genus is Sindbis virus (SINV); other well-studied members of the genus include Semliki Forest virus (SFV) (Strauss and Strauss, 1994) and, more recently, Venezuelan equine encephalitis virus (VEEV) and Chikungunya virus (CHIKV). Alphaviruses infect a large variety of cell types within organisms. This property makes it possible to use these viruses to develop replication-competent vectors that may have clinical applications. However, as with many other viruses, the potential for creating clinically useful vectors can only be exploited if virus replication and gene expression are subject to a certain level of control. This is challenging because, on the one hand, wild-type (wt) alphaviruses often cause serious diseases. On the other hand genetically modified viruses are often too severely attenuated to represent potent tools against rapidly growing cancers. Finally, all modified viruses display a tendency to revert to their wt forms.

This thesis is focused on the development of novel types of SFV-based vectors. For this, a novel DNA/RNA layered system capable of producing infectious RNAs inside transfected cells was developed. Subsequently, the corresponding vectors were engineered to contain different control systems. First, a system based on the use of aberrantly spliced introns and the rescue of corresponding defects using splice-switch oligonucleotides (SSOs) was developed. Second, the target sites of host micro RNAs (miRNAs) were used to down-regulate viral infectivity, replication and gene expression. The properties of both types of vectors were characterized using various cell-culture-based assays, which revealed the potential of such vectors but also highlighted problems associated with the use of these control systems.

REVIEW OF LITERATURE

Alphaviruses

The family *Togaviridae*, which includes the genera *Alphavirus* and *Rubivirus*, contains small viruses with enveloped virions and positive-strand non-segmented RNA genomes. The genus *Alphavirus* contains approximately 30 recognized members. The proteins encoded by different alphaviruses are characterized by similarities in the amino acid (aa) sequences: approximately 60% similarity is observed for non-structural (ns) proteins, and at least 45% similarity exists for structural proteins (Strauss and Strauss, 1994; Luers et al., 2005).

Alphaviruses are distributed worldwide. Those that infect birds and mammals can be divided into Old World and New World subgroups. The New World alphaviruses are VEEV and Western (WEEV) and Eastern (EEEV) equine encephalitis virus. The Old World alphavirus subgroup includes SFV, SINV and CHIKV, as well as many others (Strauss and Strauss, 1994). With the exception of species that infect fish, all alphaviruses are spread by arthropod vectors, usually mosquitoes. Infection of the arthropod vector is not associated with visible pathology and is therefore considered asymptomatic. Alphaviruses can also infect vertebrates, including humans. Similar to infections caused by other arboviruses, alphavirus infection in vertebrates is characterized by high-titer viremia, which is essential to ensure vector transmission – an essential phase in the natural life cycle of the virus. In addition, infection in humans is often associated with illnesses such as rash, arthritis or encephalitis (Aguilar et al., 2011). In general, the primary symptoms caused by alphavirus infection are fever and headache. However, some New World alphaviruses, as evident from their names, can cause encephalitis, and mortality rates as high as 50–70% have been observed for EEEV (Strauss and Strauss, 1994). Viruses from the Old World subgroup are more likely to cause subclinical infections; even in cases of acute illness, the mortality rate associated with infection by these viruses is generally low. Therefore, the Old World alphaviruses have until recently been considered to be medically less important than the New World alphaviruses. This situation changed with the recent (2005–2007) outbreak of CHIKV, which caused a variety of medical, social and economic problems (Kelvin et al., 2011). On islands in the Indian Ocean, this epidemic resulted in millions of infected people; the associated mortality rate was 0.1% (Das et al., 2010; Schwartz and Albert, 2010).

To date, the best-studied alphaviruses are SINV and SFV. In contrast to medically more important alphaviruses such as VEEV and CHIKV, attenuated laboratory strains of SFV and SINV are considered non-pathogenic to humans. Consequently, these viruses are used both as objects and as tools of basic research. The extensive knowledge obtained through the study of these viruses has been used to characterize other alphaviruses (although in some cases this has led to erroneous generalizations) and even to improve understanding of other enveloped viruses with RNA genomes (Ellgaard and Helenius, 2003). In addition, alphavirus-based gene expression vectors have been found to possess

numerous useful properties and are therefore thought to have potential as therapeutic and/or anti-cancer agents (Riezebos-Brilman et al., 2006; Johansson et al., 2012). Alphaviruses and vectors based on these viruses have small, simple genomes that can be easily modified. The vectors can be used to infect various cell types, and high levels of foreign gene expression can often be achieved (Strauss and Strauss, 1994). However, like all viral vectors, alphavirus-based expression systems have intrinsic limitations. In general, they are highly cytotoxic, making them unsuitable for long-term gene expression but suitable for brief gene expression and the virus-mediated killing of targeted cells. In addition, like the vast majority of RNA replicases of RNA viruses, the RNA-dependent RNA polymerase (RdRp) of alphaviruses lacks proofreading activity. Therefore, the alphavirus replicase makes 10^{-3}–10^{-5} synthesis errors per nucleotide per replication cycle. Coupled with the very efficient RNA recombination and the general growth advantages of wt viruses, this error rate makes modified RNA genomes of alphavirus-based vectors unstable (Rausalu et al., 2009). As a consequence, alphavirus vectors have an intrinsic tendency to eliminate inserted therapeutic genes of interest and/or to revert pathogenicity-reducing mutations. Therefore, during their propagation, constructed alphavirus-based vectors often revert to wt viruses or pseudorevert to viruses with growth characteristics similar to those of the wt virus (Domingo et al., 1997).

Virion and genome organization

The alphavirus virion has icosahedral symmetry, and its diameter is 65–70 nm. The genome is located inside the virion and consists of a single molecule of single-stranded RNA. The genome is surrounded by 240 copies of the viral capsid protein (C), which form a coat termed the capsid. Capsid together with genome is termed nucleocapsid (NC), has a diameter of 40 nm and in virions is covered with a host-derived lipid bilayer envelope. The alphavirus envelope contains 80 glycoprotein spikes, each of which is formed by three copies of each viral envelope protein (heterodimers of the E1 and E2 transmembrane proteins and, in some alphaviruses, extrinsic E3) (Cheng et al., 1995; Fuller et al., 1995). The outer glycoprotein layer covers most of the surface of the virion membrane. Together with the one-to-one molar ratio of C and membrane proteins, this gives alphavirus virions a highly regular structure.

The RNA genome of alphaviruses is approximately 11.5 kb in length. The genomic RNA, which in the case of SFV is historically also referred to as 42S RNA, is similar in structure to cellular mRNAs: it contains a cap structure at the 5’ end and a poly(A) tail at the 3` end (Figure 1). Unlike most cellular RNAs, however, it contains two large open reading frames (ORFs), of which only the first is translated from the viral genome. For the expression of proteins encoded in the second ORF, an additional RNA molecule, termed ‘subgenomic’ (SG) RNA (also referred as 26S RNA), is produced in infected cells. SG RNA resembles genomic RNA in that it has the same polarity as genomic RNA and contains cap

and poly(A) structures, but it is shorter, being collinear to the 3' one-third of the genomic RNA. Apart from different strategies for their expression, the products of the two different ORFs also have distinctly different functions. The first ORF, which represents nearly two-thirds of the alphavirus genome, encodes a ns-polyprotein (P1234 polyprotein) that is the precursor of the ns-proteins nsP1, nsP2, nsP3 and nsP4, the virus-encoded subunits of the RNA replicase complex. The structural proteins (C, E1, E2, E3, 6K and the recently described TF protein) are transcribed from the second ORF in the form of a structural polyprotein by means of SG mRNA, which is itself generated by internal initiation of transcription from the SG promoter. The SG promoter is located on the third RNA molecule found in alphavirus-infected cells, the complementary negative-strand RNA. This molecule is transcribed from genomic RNA and in turn serves as a template for both types of positive-strand RNAs. Because there are only two ORFs but in total ten mature proteins, both nsPs and structural proteins are expressed as polyprotein precursors. The conversion of these polyproteins to mature proteins occurs as a result of tightly controlled processing by two viral (nsP2, C) and several host cell proteases (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 2007; Firth et al., 2008).

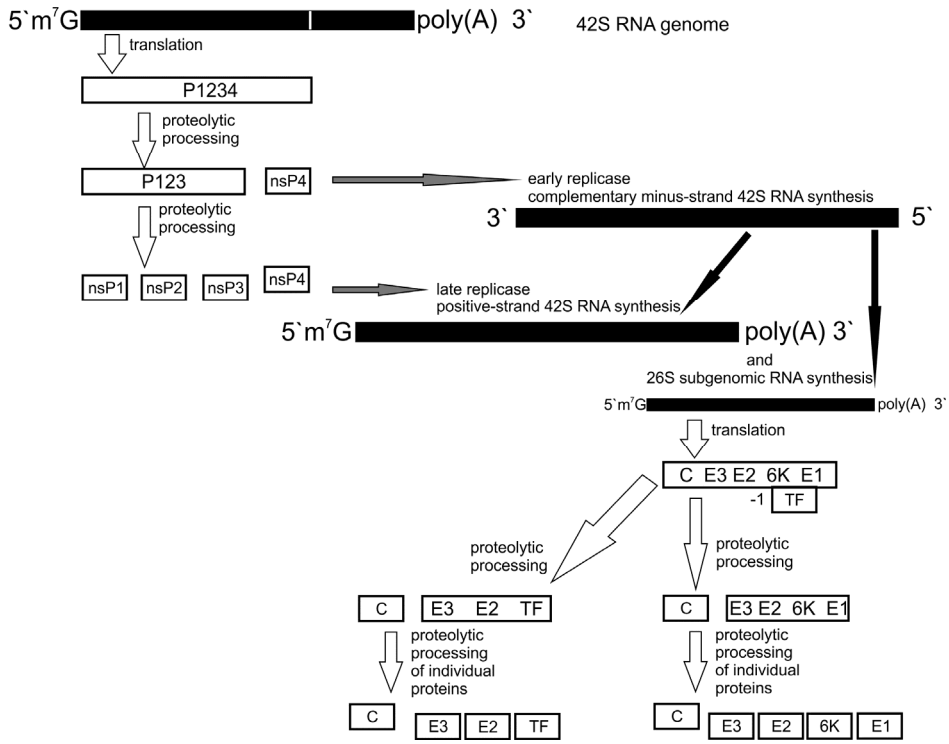

Figure 1. Basic schema of SFV RNA synthesis, translation of genomic and SG RNAs and processing of ns- and structural polyproteins. For ns-polyproteins, only the early processing pathway leading to the formation of replicase complexes is shown.

In addition to its coding regions, the alphavirus genome also contains three relatively short untranslated regions (UTR). Two of these UTRs are located at the 5`and 3`ends of the genome and play important roles in viral RNA replication; the third is located between two ORFs and partially overlaps with sequences important for the synthesis of SG RNA (Schlesinger and Schlesinger, 2007).

Infection cycle

Entry. All alphaviruses use membrane glycoprotein E2 as an antireceptor (Tucker and Griffin, 1991; Smith et al., 1995). In contrast, no cellular receptor common for all alphaviruses is known. Instead, various studies have identified different proteins as receptors for different alphaviruses; the identity of the receptor depends both on the virus species and on the cell type and origin (e.g., the vertebrate host or an insect vector) (Wang et al., 1992; Klimstra et al., 2003; Jose et al., 2009; Rose et al., 2011). There is also some controversy concerning the alphavirus entry mechanism. However, the vast majority of studies have shown that alphaviruses use receptor-mediated endocytosis to enter host cells. After the virion binds to the cell surface, clathrin-mediated endocytosis takes place; subsequently, the internalized virions are localized in early endosomes. As the endosomes mature, the pH within them decreases; after it reaches a critical level (for alphaviruses, pH 6 and below), conformational changes occur in the virion envelope proteins. One outcome of these changes is the formation of E1 homotrimers that act as fusion proteins to cause merging of the virion envelope with the endosomal membrane (Wahlberg et al., 1992; Gibbons et al., 2000). Membrane fusion releases the viral NC to the host cell cytoplasm, where it is unpacked by ribosomes, resulting in release of the viral genome within the cytoplasm (Salminen et al., 1992; Singh and Helenius, 1992; Smith et al., 1995; Vonderheit and Helenius, 2005).

The RNA genome that has been released within the cytoplasm initiates the subsequent steps of infection (Figure 1). Importantly, these steps are similar or even identical regardless of the method of entry and the exact origin of the genomic RNA. This similarity is extremely useful for the experimental manipulation of alphavirus vectors because it allows infection to be initiated using naked RNA molecules prepared using *in vitro* transcription or synthesized in the cell nucleus from delivered complementary DNA (cDNA) of the virus genome.

Ns-protein expression and virus genome replication. The incoming virus genome serves as a template for the production of ns-polyprotein or polyproteins, depending on the presence or absence of leaky stop codon in the region corresponding to the C-terminus of nsP3. The full-sized P1234 contains over 2400 aa residues and is processed by nsP2 and/or by the corresponding regions of ns-polyproteins into four individual nsPs. Processing does not occur at random; instead, the order and timing of the cleavages are fixed. This is essential because the formation and activation of the viral replicase as well as the coordination of its functions are achieved *via* changes in ns-polyproteins.

Individually expressed nsPs fail to interact normally; cannot form structures characteristic of alphavirus replicase complexes (see below); and are unable to synthesize viral RNAs. The artificial acceleration of P1234 processing is reported to be detrimental for virus replication (Strauss et al., 1992). Similarly, slow down of P1234 processing results in different adverse effects on virus infectivity; the nature of defects depends on which processing sites are affected (Lulla et al., 2006, 2012, 2013; Gorchakov et al., 2008). Thus, the only known pathway for formation of functional alphavirus replicase complexes involves normal expression and processing of ns-polyprotein.

P1234 processing is also associated with the formation of replicase complexes and with changes in their RNA substrate specificity. To form early replicase, P1234 must initially be cleaved between nsP3 and nsP4. This is a rapid reaction, and at the early stage of infection it is also the first cleavage of ns-polyprotein to occur (Merits et al., 2001; Lulla et al., 2006). The early replicase complex binds to the genomic RNA of the virus and uses it as a template; hence, it is responsible for the synthesis of full-length negative-strand RNA. This particular pattern of processing dominates only at the early stage of infection, which lasts for approximately 4 hours (Sawicki and Sawicki, 1980). Nevertheless, this amount of time is sufficient for the generation of approximately 20000 negative stand RNAs (anti-genomes) per cell (the exact number generated seems to depend on the size of the cell) (Wang et al., 1991; Mai et al., 2009; Frolova et al., 2010). The negative-strand RNA is complementary to the viral RNA genome except that it contains one unpaired G nucleotide at the 3`end and has no cap-structure at 5' end (Sawicki and Gomatos, 1976; Wengler et al., 1979). Early studies have shown that the negative strand of alphavirus RNA may also contain a poly(U) sequence that corresponds to a poly(A) tail. However, these findings have been challenged by more recent studies indicating that synthesis of negative-strand RNA is initiated immediately upstream of the poly(A) sequence of the positive-strand template and, accordingly, negative-strand RNA must lack poly(U) (Hardy and Rice, 2005; Hardy, 2006). Indeed, the double-stranded RNAs that form the replication intermediates of alphaviruses have the ability to bind oligo(dT) resin, indicating that they lack a poly(U) element in the negative strand (Nikonov et al., 2013). There is also no indication that the negative-strand RNAs of alphaviruses exist as individual, single-stranded RNA molecules. Instead, negative-strand RNA molecules are always found in duplexes with positive-strand RNA. The only possible explanation for this is that each individual positive-strand RNA serves as a template for the synthesis of only one anti-genome. Consequently, once the negative strand is made, the replicase must be re-organized to carry out the synthesis of positive strands. Indeed, the mechanism by which this switch occurs has been described. Cleavage of the remaining P123 polyprotein leads to the formation of a late replication complex containing nsP1, nsP2, nsP3 and nsP4; within this complex, negative-strand RNA is used as a matrix for the synthesis of both genomic and SG mRNAs (Li and Rice, 1993; Lemm et al., 1994; Griffin, 2001).

Unlike early replicase, the late replication complexes are capable of many rounds of synthesis and, once formed, remain active until the death of the host cell. The late replicase complexes have the appearance of membrane-bound vesicles, appearing as spherules with an average diameter of 50 nm. The spherules are formed at the plasma membrane of infected cells (Frolova et al., 2010; Spuul et al., 2010); later in infection, they become located in structures termed type I cytopathic vacuoles (CPV), which represent modified endosomes and lysosomes (Froshauer et al., 1988; Kujala et al., 2001; Gorchakov et al., 2008). Unless certain mutants, characterized by discontinuous synthesis of negative-strand RNAs, are used the spherules are formed only during the early stages of infection. Their formation ceases approximately 4 hours post-infection due to two mechanisms. First, the excess of free nsP2 protein causes changes in P1234 processing that prevent the formation of early and consequently also late replicase complexes; second, virus-induced shutdown of translation subsequently abolishes the synthesis of ns-polyprotein. Thus, while the early stages of alphavirus infection are characterized by the synthesis of both negative- and positive-strand RNAs, only the latter molecules are produced at late stages of infection.

Similar to the replication of all eukaryotic positive-strand RNA viruses, the replication of alphaviruses occurs in membrane-bound compartments. Of the nsPs of alphaviruses, **nsP1**, which contains 537 aa, is the only membrane-binding protein (Ahola et al., 2000; Spuul et al., 2007) and is essential for the binding of the alphavirus replicase complex to cytoplasmic membranes (Salonen et al., 2003). Equally importantly, nsP1 has methyltransferase and guanylyltransferase activities, both of which are required for the addition of a cap-structure to the alphavirus genomic and SG RNAs (Ahola and Kääriäinen, 1995). nsP1 is also abundantly found outside of virus replication complexes. In infected cells, most of the free nsP1 is located at the inner surface of the plasma membrane, (Ahola et al., 1997) where it is responsible for the disruption and rearrangement of the cellular actin cytoskeleton and the induction of outgrowth of filopodia-like structures (Laakkonen et al., 1998; Zusinaite et al., 2007).

nsP2, which contains 799 aa, is the largest ns-protein of alphaviruses; similarly, it has the largest number of known enzymatic and non-enzymatic activities. The C-terminal portion of nsP2, which is a papaine-like protease, is responsible for the autocatalytic processing of ns-polyprotein (Merits et al., 2001). The N-terminal portion of nsP2 has functions that are important for viral RNA synthesis and modification. It has NTPase activity (Rikkonen et al., 1994) and RNA 5` triphosphatase activity; the latter contributes to the first reaction of synthesis of cap-structures on viral positive-strand RNA (Vasiljeva et al., 2000). The RNA helicase activity of nsP2 has also been thought to be associated with the N-terminal part of the protein; however, recent data from our research group suggest that both the N- and C-terminal regions of nsP2 are required for this function (unpublished data). In addition to its enzymatic activities, nsP2 of Old World alphaviruses is responsible for shutting down host cell RNA and protein synthesis and is capable of inhibition of the antiviral interferon response (Frolov

et al., 1999; Breakwell et al., 2007; Fros et al., 2013). The nsP2 of SFV, but not that of all Old World alphaviruses, has a motif that is similar to the nuclear localization signal (NLS). The presence of this motif appears to be important for the virus because almost half of synthesized nsP2 is localized to the cell nucleus during infection (Rikkonen et al., 1992). Until recently, the nuclear functions of nsP2 were not understood. However, it was recently shown that nsP2 within the nucleus is capable of initiating the degradation of the catalytic subunit of cellular RNA polymerase II and thereby of shutting down cellular transcription. Interestingly, the protease activity of nsP2 seems not to be required for this activity (Akhrymyk et al 2012). Modification of the putative NLS of the nsP2 of SFV results in predominantly cytoplasmic localization of the protein and remarkably reduces the cytotoxicity of the virus as well as its ability to counteract the induction of type I interferons (Breakwell et al., 2007; Tamm et al., 2008; Nikonov et al., 2013). Mutations of this type also reduce the pathogenicity of SFV infection for mice (Fazakerley et al., 2002).

nsP3 (482 aa) has been and remains the least well understood alphavirus replicase protein. In the majority of alphaviruses, the region encoding the C-terminal portion of nsP3 contains an in-frame termination codon. Termination at this codon results in the synthesis of a shorter P123 polyprotein, while its read-through, which occurs at a frequency in the range of 10–20%, results in expression of full-length P1234 (Strauss and Strauss, 1994). Unlike other nsPs, nsP3 lacks any enzymatic activity that can be clearly linked to the viral infection cycle. Nevertheless, it is an essential part of the replication complex and is required for synthesis of negative-strand RNA and SG RNA (Dé et al., 2003). nsP3 interacts with nsP1 and, as part of the P123 and P1234 polyproteins, redirects the localization of replicase complexes from the plasma membrane to intracellular membranes. Thus, nsP3 is clearly important for the formation of CPVs (Salonen et al., 2003). Two globular domains exist within the conserved N-terminal two-thirds of nsP3: a macro domain and a zinc-binding domain (Shin et al., 2012). In contrast, the C-terminal region of nsP3 is not conserved and is apparently intrinsically disorganized. It is very likely used as a “hook” that is essential for interaction with various host proteins. The roles of these interactions have been revealed only for a few nsP3-binding proteins (Panas et al., 2012). Because the C-terminal portion of nsP3 is likely designed to acquire and/or lose various motifs that are required for interactions with host proteins, this region is also very tolerant of mutations (LaStarza et al., 1994). Similarly, the C-terminal region is tolerant of the insertion of large tags, including fluorescent proteins and luciferases (Attarzadeh-Yazdi et al., 2009).

nsP4 (614 aa) is an RdRp and therefore essential for viral replication (Rubach et al., 2009). nsP4 also has terminal adenyl transferase activity, which is needed for the synthesis of poly(A) tails on positive-strand RNAs (Hardy and Rice, 2005; Tomar et al., 2006). The N-terminal region of nsP4 has no homology with known proteins and is likely involved in interactions with other nsPs. It has been observed that the levels of nsP4 in infected cells are much lower than those of other ns-proteins. One reason for this is that on its own nsP4

is very unstable; unless it is located in complexes with other ns-proteins, it is rapidly degraded (de Groot et al., 1991). A second reason is that the full-length P1234 of the majority of alphaviruses is 5- to 10-fold less abundant than P123, which is produced as result of termination at the in-frame termination codon (Strauss and Strauss, 1994). The exact reasons for the observed down-regulation of nsP4 levels are not known; however, the downregulation of expression of RdRp is clearly not unique to alphaviruses because a similar phenomenon is frequently observed in different viruses with RNA genomes.

Synthesis of structural protein; NC and virion assembly. The translation of the alphavirus structural polyprotein C-p62(E3E2)-6K/(TF)-E1 on the SG RNA template takes place in the vicinity of CPVs. In SINV and SFV, the region of SG-RNA that encodes the amino terminus of the C-protein is essential to boost expression of the structural polyprotein and is therefore termed a 'translational enhancer' (Frolov and Schlesinger, 1994). This element is active only in infected cells and is not universally present in all alphaviruses; for example, VEEV and apparently CHIKV lack this element. Like the ns-polyprotein, the structural polyprotein is also processed rapidly. During this processing, C-protein, which possesses a serine protease domain at its carboxy-terminus, cleaves itself from the rest of the polyprotein. This cleavage uncovers a signal at the N-terminal region of the remaining p62-6K-E1 polyprotein that targets it to the endoplasmic reticulum (ER) membrane, where translation continues. The remaining cleavages of the structural polyprotein are catalyzed by cellular enzymes. In ER membranes, the peptide bonds between p62 (the precursor of E3 and E2), 6K and E1 are processed. p62 and E1 form heterodimers that are transported to the plasma membrane. During this transport, p62 is cleaved in the trans-Golgi compartment to form the E2 and E3 proteins (Zhang et al., 2003). Transport in the form of p62:E1 complexes rather than in the form of mature E2:E1 complex is essential to prevent premature conformational change of E1 into the fusogenic form during its transport through cellular compartments with low pH. A ribosomal frameshift signal has been found in the sequence that encodes the 6K protein. In cases in which the frameshift occurs (the frequency of this event is approximately 10%), translation of the structural polyprotein results in the synthesis of TF protein (Figure 1). TF protein is associated with assembled virions (Firth et al., 2011), and its presence is essential for alphavirus-induced *in vivo* pathogenesis (Snyder et al., 2013).

The assembly of alphavirus virions begins with the formation of NC. This is initiated by the interaction of C-protein with genomic RNA. The encapsidation signal, which is essential for this interaction, is localized to the ns-region of RNA genome; thus, only genomic RNAs but not SG RNAs are packed into NCs. It has been shown that C-protein assembles with the encapsidation signal area and that icosahedral NCs are formed. The viral glycoprotein complexes formed as described above are transported to the cell surface, where the spike-structures are inserted into the plasma membrane. The key to the final formation and budding of mature virions is the interaction between the NC and the E1:E2

dimer (Suomalainen et al., 1992; Jose et al., 2009). The released virions are capable of infecting other cells.

Infection of the central nervous system (CNS). The names of New World alphaviruses reflect their ability to infect the CNS. However, some Old World alphaviruses also possess this property. During a recent outbreak, several lethal cases of CHIKV infection were associated with infection of the CNS (Couderc et al., 2008). Although several commonly used laboratory strains of SINV and SFV are pathogenic for mice and rats while other strains are not, all SFV strains can cross the blood-brain barrier. Therefore, these viruses have been used as model systems in attempts to understand the mechanisms of viral neuropathogenesis. It has been found that infection of the CNS by alphaviruses depends not only on the virus strain but also on the age of the animal (or, in case of *in vitro* systems, on the developemental stage of cultivated neurons) (Griffin, 2005). All strains of SFV and SINV can cause lethal disease in neonatal and suckling mice (Atkins et al., 1999; Fazakerley et al., 2002; Griffin, 2005), while only pathogenic strains can kill adult mice. The route of infection is another important factor. Infection with the SFV4 strain is pernicious to 60–70% of Balb/c mice when the virus is administered intraperitoneally (i.p.); when given intranasally (i.n.), the same virus strain killed all mice by causing lethal encephalomyelitis at 5–7 days post-infection. Avirulent strains of SFV, such as the A7(74) strain, do not cause encephalitis in adult mice; instead, they induce nonlethal demylinating disease that generally lasts for up to a month after infection (Glasgow et al., 1991, 1994; Santagati et al., 1995, 1998; Tarbatt et al., 1997; Atkins et al., 1999; Fazakerley, 2004). The genetic basis for the differing neurovirulence of SFV strains is not completely understood. Several older reports suffered from problems with viral icDNA clones, and these topics are currently under re-investigation in our laboratory and in other laboratories.

The mechanism of immune clearance of non-lethal alphaviruses from the CNS has been mostly studied using SINV. Such studies have revealed that viral clearance occurs in three phases. During the first phase (days 3–7 after exposure to the virus), the infectious virus is cleared. This is followed by the clearance of the majority of viral RNA (days 8–60) and finally by a phase in which a very low level of viral RNA is maintained (Metcalf and Griffin, 2011). The immune mechanisms responsible for viral clearance are generally non-cytolytic; both cytokines and antibodies are required for the clearance to occur (Metcalf et al., 2013).

Alphavirus-based vector systems

Any alphavirus can be used to develop a vector system. However, the alphaviruses most frequently used as vectors, are those that are also the most studied. All alphavirus-based vectors are constructed using infectious cDNA (icDNA) clones of the corresponding viruses. The first SFV-based vector was constructed in 1991 when an infectious cDNA clone of SFV was developed (Liljeström et al., 1991).

Several methods can be used to classify alphavirus-based vectors. In this work, the vectors are divided into two major groups, full-length vectors and replicon vectors, based on the presence or absence of the structural region of the viral genome. Both types of vectors can exist as RNA-launched systems and as DNA/RNA-layered vectors (Figure 2). Common elements of all these vector constructs are cDNA of sequences corresponding to the ns-region, 3' and 5' UTR elements and natural or duplicated SG promoters of the virus genome. Unlike replicon vectors, full-length vectors also contain sequences essential for virion formation. The major difference between RNA-launched and DNA/ RNA-layered vectors is the mode of production of infectious RNA molecules. In case of RNA-launched vectors these molecules are produced in a test tube using *in vitro* transcription; accordingly the cDNA of the vector is under control of a bacteriophage (SP6 or T7) RNA polymerase promoter. In the case of DNA/RNA-layered vectors, transcription is carried out in the nucleus of a cell that has been directly transfected with plasmid containing the cDNA of the vector; in these vectors, the cDNA sequences are placed under the control of a promoter for eukaryotic RNA polymerase II (the immediate early promoter of human cytomegalovirus (CMV) is the most frequently used). A more detailed description of each type of vector is given below.

Full-length RNA-launched vectors are also called genomic or replication-competent vectors (Figure 2A). In their RNA form, these vectors contain the complete (or nearly complete) viral genome; in addition, they typically contain inserted gene(s) of interest and/or regulatory elements. The most important property of these vectors is that they can not only perform RNA replication, a property that is shared with replicon vectors, but also express structural proteins and produce infectious virions. Such vectors are essentially modified alpha-viruses that can infect cells and spread in cell culture (or in organism); with the exception of their ability to express some foreign genes, they have the same features as wt alphaviruses. The use of full-length RNA-launched vectors offers several advantages. First, these vectors are easy to produce in large amounts in cell culture (no packaging systems or packaging cell lines are required). Second, these vectors are almost always characterized by high-level but short-term expression of a gene of interest, such as a sequence coding for a tumor antigen, and by the ability to induce apoptosis of infected cells. These properties make them promising as tools in anti-cancer therapy (Barry et al., 2010; Quetglas et al., 2010). One vector of this type that was constructed on the basis of SINV has been experimentally shown to have the ability to destroy cancer cells *in vivo* upon being directly injected into the tumor (Tseng et al., 2009). However, because alphaviruses can infect a variety of cell types, full-length vectors also tend to kill other types of cells and, importantly, can spread infection from the injection site to other tissues. Thus, their use could be beneficial (for example, in targeting of tumor metastasis) but also could be dangerous to the organism. Full-length vectors must therefore be modified either to replicate only in targeted cells or, at the very least, modified so that they are restricted from infecting cells, tissues and organs (such as cells of the CNS) in which they

otherwise might cause serious damage resulting in disease. Interestingly, SINV has been reported to possess natural tropism to tumor cells (Gardner et al., 2000; Tseng et al., 2002). However, most alphaviruses seem to lack this useful property; therefore, the vectors derived from these viruses must be controlled by artificially generated means.

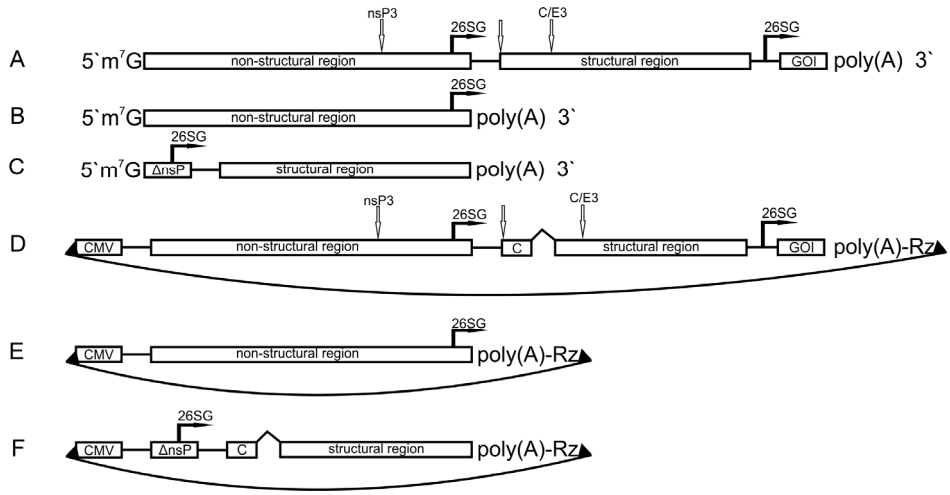

Figure 2. Schematic representation of the main types of alphavirus-based vectors. The constructs shown in panels A-C represent *in vitro* transcripts prepared using test-tube reactions; they are identical to the RNA molecules present in transfected/infected cells. Elements of the vector construct that are present only in the DNA plasmid are not shown.

A. Full-length RNA-launched vector with duplicated SG promoter and inserted gene of interest (GOI). Other commonly used sites for insertion of foreign genetic information are indicated by open arrows.
B. RNA-launched replicon vector. Commonly, the gene of interest is inserted under the control of the native SG promoter; however, the vector may also contain duplicated SG promoters and/or inserted IRES elements.
C. Basic helper vector for RNA-launched replicon vector.
D. Full-length DNA/RNA layered vector in the form of an infectious plasmid. CMV – immediately early promoter of CMV, ^ – inserted intron (essential for some but not all DNA/RNA layered alphavirus vectors), Rz – negative strand ribozyme of hepatitis D virus.
E. DNA/RNA layered replicon vector in the form of a plasmid construct.
F. Helper vector for DNA/RNA layered replicon vector in the form of a plasmid construct. Integration of similar DNA into the genome of cell allows construction of packaging cell lines usable for both types (RNA-launched or DNA/RNA layered) of replicon vectors.

All of the vectors shown in the figure can be further engineered to contain mutations, deletions, insertions and various control elements.

In some cases, alphavirus-based vectors that rely solely on the intrinsic properties of the parental virus (such as cell killing or activation of the interferon response) and require minimal, if any, modification are designed. However, in most cases, it is necessary to design vectors that also express foreign sequences and/or contain other artificially generated elements such as miRNA-encoding sequences or target sites for cellular miRNAs. There are several different ways to insert and express foreign genes using full-length alphavirus-based vectors. The main hurdles are keeping the vector genetically stable and achieving the desired (usually high) level of foreign protein expression. One obvious way of achieving these goals is to express the gene of interest together with the virus' own ns- or structural proteins. Examples of the former are constructs in which fluorescent proteins, luciferases or other marker proteins have been inserted into the carboxy-terminal region of nsP3. This approach provides the opportunity to visualize viral replication in infected cells. In one such vector, a region encoding enhanced green fluorescent protein (EGFP) was placed between the coding regions for nsP3 and nsP4 and flanked by nsP2 protease recognition sites, which were required to achieve release of EGFP from the ns-polyprotein (Tamberg et al., 2007; Fragkoudis et al., 2009). Sequences coding for fluorescent proteins have also been inserted in different locations within the nsP3-coding region in SINV, resulting in viruses that express nsP3-EGFP (Frolova et al., 2006) or nsP2-EGFP (Atasheva et al., 2007) fusion proteins. These viruses were used to visualize the synthesis and location of nsP2 and nsP3 and to identify host proteins that interact with these viral proteins and/or with the viral replicase. Because the expression of ns-proteins by alphaviruses is short-term, usually ending by 6–8 hours post-infection, and occurs at a relatively low level, such constructs are not suitable for applications in which a high level of expression of the gene of interest is essential.

The expression of alphavirus structural proteins occurs at a much higher level (100-fold or more) than expression of ns-proteins and, importantly, continues during the late stages of infection. This has made the structural ORF a promising region for the insertion of foreign sequences. The first such successful strategy developed was the insertion of an EGFP sequence between the C-protein and E3 regions (Thomas et al., 2003) of the SINV vector; the resulting virus was reported to show a high level of marker protein expression and superb genetic stability. However, this was not the case for similar SFV-based vector: the corresponding virus was clearly growth-attenuated and relatively unstable (Fragkoudis et al., 2009; our unpublished data). More recently, strategies to express marker proteins fused to the glycoproteins of alphaviruses have also been described.

An alternative option is to express the gene of interest independently of viral proteins; two approaches can be used. In the first approach, the extra protein can be expressed using an inserted internal ribosome entry site (IRES). For unknown reasons, this approach did not work in the context of full-length SFV vectors (Rausalu et al., 2009), although it did work well in the context of SFV replicon vectors (Kiiver et al., 2008). Nevertheless, the successful construction

and use of full-length constructs obtained using the IRES insertion strategy have been described (Volkova et al., 2008; Guerbois et al., 2013). However, by far the easiest (and hence most commonly used) way to create an independent expression unit in an alphavirus vector is duplication of the SG promoter. For this, two factors are important. The first is the length of the sequence representing the duplicated SG promoter. The use of SG promoters that are too long can generate problems with vector stability; hence, the promoters should be as short as possible and as long as required. In most alphaviruses, the minimal active SG promoter has a length of 24 bases (spanning from position -19 to position +5; coordinates are given with respect to the start site of SG RNA) (Levis et al., 1990) and possesses activity equal to approximately one-fifth that of the native full-length SG promoter. SFV, however, seems to be an exception in that SG promoters nearly twice as long (for example, from position -37 to position +17) are required for effectiveness (Rausalu et al., 2009). Second, the location of the duplicated SG promoter element (and thus also that of the foreign gene) in the vector genome is also important. Again, there are differences between different alphaviruses. It has been shown that the best region in which to place such an insertion in full-length SFV vectors is the region immediately downstream of the structural genes (Rausalu et al., 2009). In contrast, for CHIKV and SINV vectors, insertion between the ns- and structural regions is preferred (Pierro et al., 2003; Tsetsarkin et al., 2006). Again, the reasons for these discrepancies are not understood.

Two factors set a limit how many SG promoters, IRES elements and foreign genes can be inserted into the genome of a full-length alphavirus vector. First, each insertion reduces and/or slows down replication of the recombinant genome either by interfering with natural gene expression (promoter competition can be observed in cases in which duplicate SG promoters are used) or simply by increasing the size of the genome, which then takes a longer time to replicate. Second, because the icosahedral NC has a fixed volume, there is a limit to the size of the RNA genome that can be incorporated into the NC. Although mutations that change the structure of the NC of SINV and increase its volume have been described (Ferreira et al., 2003), in general only 2000 or slightly more extra nucleotides can be incorporated into full-length alphavirus vectors. This number of nucleotides corresponds to a relatively short gene of interest and thus represents a serious limitation. As the limit of the gene length is approached, the genetic instability of the recombinant full-length vector increases rapidly because such vectors have serious growth disadvantages compared to wt viruses or aberrant viruses that have lost most of the inserted sequences. Accordingly, the large genomes will be rapidly outcompeted during vector preparation and propagation. The elimination of the original vector with the inserted sequence from the population can be slowed down somewhat by selecting appropriate conditions for passaging (in general, the lower the multiplicity of infection (MOI) is, the more rapidly inserts are lost) but cannot be completely prevented, making the use of such constructs for medical purposes highly problematic.

RNA-launched replicon vectors (Figure 2B) are basically truncated versions of the alphaviral genome in which the structural region has been replaced by foreign gene(s) that are typically expressed using the native 26S promoter; however, replicon vectors with duplicated SG promoters and/or IRES elements have also been developed (Kiiver et al., 2008). Unlike full-length vectors, replicon vectors represent a single-cycle expression system. Cells transfected with such vector RNAs become infected and, if the vector is based on an Old World wt alphavirus, undergo apoptosis. Apoptosis is triggered both by high-level replication of the replicon RNA and by the cytotoxic properties of wt nsP2; it may be further enhanced (or, in some cases, slowed down) by the properties of the expressed protein of interest. In general, cells transfected with replicon vectors tend to survive slightly longer than cells transfected or infected with the corresponding full-length vectors. The reason for this is the lack of ER stress caused by the expression of alphavirus glycoproteins (Barry et al., 2010). In contrast, replicon vectors based on New World alphaviruses have only moderate or low cytotoxicity. This difference is due to the fact that the main cytotoxic protein of New World alphaviruses is not nsP2 but C-protein (Garmashova et al., 2007a, 2007b), which is not produced from the replicon RNA.

In vitro-transcribed replicon vector RNAs can be used directly for transfection of the cells of interest. However, this is often impractical due to the limited number of cells that can be transfected using transfection reagents or physical methods (electroporation). Furthermore, many types of cells can be infected by alphaviruses but cannot be efficiently transfected with large RNA molecules. To overcome these issues, packing systems for alphavirus replicon vectors have been developed. The most straightforward approach is the use of a so-called helper vector. Helper RNA is capable of replication in the presence of the replicon RNA and, unlike the latter, triggers the expression of structural proteins. The basic design of helper vectors is simple. They also consist of a truncated alphavirus genome that, in this case, lacks most of the ns-region (including the packing signal) but possesses the *cis*-active sequences needed for replication and transcription (5'- and 3'- UTRs, 26S promoter) and the region encoding the viral structural proteins. Co-transfection of cells with replicon RNA and helper RNA leads to the production of virus-replicon particles (VRP). These resemble infectious alphavirus virions but instead of a full-length genome, contain packed replicon RNA lacking structural genes. Such VRPs are "suicide particles" because they are capable of only one round of infection.

The primary safety concern associated with the use of VRPs is the possibility that replicon and helper RNAs will recombine, leading to the formation of replication-competent full-length viral genomes. Several approaches have been developed to minimize the likelihood of such events. One of these is the use of a split helper system. In this system, the regions encoding C-protein and glycoproteins were separated in two independent helper RNAs that should then be co-electroporated with replicon RNA to obtain VRPs (Smerdou and Liljeström, 1999). A recent study showed that the 26S promoter is not strictly required for the production of functional structural proteins from helper RNA

(Kamrud et al., 2010b). This permitted the development of promoterless helpers, further increasing the safety of the replicon systems. Finally, safeguards that render the potential full-length genomes non-viable (or strongly attenuated) in cells infected with VRP stocks can be inserted into helper vectors (Kamrud et al., 2010a).

The greatest advantage of standard alphavirus replicon vectors is their ability to express high levels of foreign protein. It has been shown that SFV-based replicons can express as much as 100 μg β-galactosidase per million infected cells (Liljeström and Garoff, 1991). Experiments with VEEV replicons expressing Lassa virus N-protein showed that the foreign protein constituted approximately 20% of total cellular protein (Pushko et al., 1997). For SFV- or SINV-based replicon vectors, the expression of foreign proteins can be further boosted by the use of translational enhancers: in such vectors, sequence encoding the N-terminal portion of C-protein is fused to the 5' end of the sequence encoding the protein of interest (Sjöberg et al., 1994). This approach was used to boost the vector-induced anti-tumoral response caused by expression of high levels of IL-12. When high doses of a vector expressing IL-12 fused to the capsid enhancer were used in mice model, complete tumor regression was observed in approximately 80% of cases; even at lower doses, vectors with enhancer were more efficient than standard vectors. That study also demonstrated that alphavirus-based vectors can be used as anti-cancer agents (Rodriguez-Madoz et al., 2005).

As indicated above, alphavirus-based vectors can express more than one gene of interest simultaneously. This property can be used for more efficient targeting of the same disease or to simultaneously achieve other goals. For the first option, additional genes of interest are included in the vector using an extra copy or copies of the SG promoter. Although this approach guarantees simultaneous expression of multiple proteins of interest, the expression levels of different proteins are typically not identical even if their expression is mediated by SG promoters of the same sequence and length. A vector of this type expressing the three CMV proteins pp65, IE1 and gB was constructed and used to vaccinate mice against CMV infection. Vaccination with the vector resulted in high numbers of antigen-specific T-cells and high titers of neutralizing antibodies against CMV (Reap et al., 2007a, 2007b). Thus, such an approach is possible, though not necessarily beneficial. A simple and possibly more efficient alternative would be to vaccinate using a combination of alphavirus-based vectors each of which has been engineered to express one antigen. It is certainly easier to manipulate the expression levels of different antigens by changing the composition of the mixture than by attempting to manipulate their expression levels from a more complex vector. An example of a study in which multiple inserted sequences had different goals utilized an SINV replicon vector engineered to express luciferase using an SG promoter as well as a herpes simplex virus (HSV) thymidine kinase (TK) fused to nsP3. In this case, the generated TK activity offered a means of increasing the safety of the vector because its replication and spread could be controlled by the use of the anti-herpesvirus

compound ganciclovir (Tseng et al., 2009). Regardless of the goal, it must also be kept in mind that although alphavirus replicon vectors can accommodate rather large insertions of foreign sequences (up to 6–8 kb), the use of multiple SG promoters and multiple genes of interest can easily reach this limit. Finally because all duplications represent potential recombination points inside the vector sequence, the use of duplications may result in enhanced recombination and deletion of one or several genes of interest during the preparation of VRP stocks.

DNA/RNA layered alphavirus-based vectors. The first SINV-based DNA/RNA layered replicon vector was constructed in 1996; a few years later, similar SFV vectors were described (Dubensky et al., 1996; Berglund et al., 1998). DNA/RNA layered vectors differ from RNA-launched vectors in the design of the plasmid-based part of the vector construct (Figure 2D, 2E, 2F); the vectors themselves (in the RNA form) can be identical. Plasmid constructs of DNA/RNA layered vectors must contain eukaryotic transcription elements, including a promoter for RNA polymerase II (the CMV immediately early promoter is often used, but other promoters with fixed transcription start sites can also be used) upstream of the vector's cDNA and a eukaryotic termination element (poly(A) signal) downstream of it (Atkins et al., 2008). DNA/RNA layered vectors were initially created to avoid expensive RNA production and circumvent the problems associated with the instability of large naked RNA molecules *in vivo*. It should also be noted that there are more (and usually more efficient) available transfection methods for the delivery of DNA plasmids than for the delivery of large RNA transcripts. DNA vectors can also be integrated into the host cell genome, while this is completely impossible with RNA constructs. Finally, the use of DNA permits some types of regulation (the use of promoters with different strengths, the use of inducible promoters) that cannot be used in RNA-launched systems. DNA/RNA layered constructs can also be engineered to contain introns that help stabilize the vector plasmids and improve viral RNA synthesis and export from the nucleus to cytoplasm. DNA/RNA layered vectors have the same advantages as other alphavirus-based vectors: low biohazard, ability of the vector to induce a high-level and high-quality immune response against expressed antigens, and the ability to infect and destroy specifically targeted cells such as cancer cells due either to the cytotoxic properties of the vector or to activation of an anti-cancer immune response.

Alphavirus-based long-term expression systems. Short-term expression of the genes of interest represents an intrinsic limitation of standard alphavirus-based vectors. Therefore, attempts have been made to construct systems that can be used for long-term or even permanent expression of proteins of interest.

Although full-length alphaviruses exert their cytotoxic effects through several different mechanisms, vectors derived from these viruses as well as natural alphaviruses can be adapted to non-cytotoxic growth in a variety of mammalian cells (Atasheva et al., 2010). Furthermore, alphaviruses and vectors derived from them are naturally able to cause persistent infection of insect cells. Unfortunately, these infections are characterized by low-level production of

virus and viral products and are therefore hardly suitable for biotechnological applications. For these reasons, most researchers' attention has been dedicated to the development of alphavirus replicons that can be used to create stably expressing, established cell lines. One approach to this problem is represented by the example of a stably transfected cell line in which a DNA/RNA layered vector of SINV is integrated into the cellular genome; in this system, activation of the replicon and expression of the protein of interest was induced by a change in temperature (Boorsma et al., 2003). However, once induced, the expression may not be permanent because most temperature-sensitive (ts) mutants of alphaviruses do not have non-cytotoxic phenotypes. Therefore, truly non-cytotoxic alphavirus replicons were developed. Because New World alphavirus-based replicons lack structural proteins, which are the major determinants of cytotoxicity in these viruses, minimal effort was needed to develop corresponding non-cytotoxic vectors (Petrakova et al., 2005). A single mutation in nsP2 of SINV was found to be sufficient to render the corresponding replicon vectors capable of permanent replication (Frolov et al., 1999). With SFV replicons, however, the situation proved to be somewhat more complicated. Mutations similar to those used in SINV vectors reduced but did not block the cytotoxicity of SFV replicons (Lundstrom et al., 2001; Tamm et al., 2008). It was later found that two different mutations in nsP2 are needed to attenuate the SFV replicon to a level that allows permanent growth in cell lines (Casales et al., 2008). This requirement may be general for other alphaviruses similar to SFV because two recent studies dedicated to the analysis of the cytotoxicity of CHIKV showed that a similar combination of mutations is required to suppress the cytotoxicity of the CHIKV replicon (Pohjala et al., 2011; Fros et al., 2013). The cell lines generated using non-cytotoxic alphavirus replicons have found various uses ranging from the screening of potential antiviral compounds (Pohjala et al., 2011) to the production of high levels of human therapeutic proteins (Casales et al., 2010).

Potential medical applications of alphavirus-based vectors

The main potential applications of alphavirus-based vectors in medicine lie in the areas of vaccine development and anti-cancer therapies (often the latter also includes elements of anti-cancer vaccination).

Alphavirus-based vectors as components of vaccine candidates. Alphavirus-based vectors can represent components of genetic or recombinant vaccine candidates and, subsequently, vaccines. One of the primary arguments in favor of their use in vaccines is that commonly used alphavirus vectors are not pathogenic to humans. Furthermore, some alphaviruses, such as SFV, which is being used for vaccine development, are not associated with known human diseases. This, however, also creates a specific problem in that there are no existing human vaccines against any alphaviruses; hence, the potential effects (especially the long-term effects and potential adverse side effects) of the use of such vaccines are simply not known. In this regard, alphaviruses have a

disadvantage as vaccine components compared with viruses such as yellow fever virus and vaccinia virus, which have long-documented histories of use as vaccines.

There are a number of obvious advantages associated with the use of alphavirus-based vectors as components of vaccines. They include the broad host range of the viruses and their ability to infect different types of cells, including neuronal and primary cells, their natural tropism to dendritic cells (Nishimoto et al., 2007), the simplicity of production of high-titer virus stocks and the possibility of using VRPs and/or DNA/RNA layered vectors as extremely safe vaccines. Furthermore, because alphaviruses have RNA genomes and replicate in the cytoplasm, there is no risk of integration of viral genetic material into the host cell genome. Alphavirus-based vectors that carry and express many different foreign genes can be constructed (Reap et al., 2007b); alternatively, different constructs can be mixed to obtain multivalent vaccines (White et al., 2013). Like any virus-based system, alphavirus vectors cannot serve as a universal vaccine platform. Some properties of alphaviruses confer benefits for certain applications, while restrictions are implied by others. Thus, although the small genomes of alphaviruses make genetic modification relatively simple, they also preclude the use of large inserts containing foreign genetic information. Furthermore, high-level expression of genes of interest is balanced by the short-term nature of the expression. Alphavirus vectors also activate innate immune responses, both humoral and cellular, ensuring a high-quality immune response against the target; however, anti-vector immunity is also efficiently generated (Lundstrom et al., 2003; Atkins et al., 2008).

Many alphavirus-based vectors have passed preclinical evaluation, and some have been tested at early clinical stages. So far, these vaccine candidates have been based on replicon vectors, which have been used because of their greater safety. VEEV-based vectors are more frequently used than others because in earlier studies it was shown that replicon vaccines derived from VEEV were significantly more immunogenic then replicon vaccines derived from SINV (Perri et al., 2003). However, other alphaviruses such as SFV may also have their benefits; SFV vectors have been shown to induce superior T-cell responses (Knudsen et al., 2012), and they can also be developed into more sophisticated hybrid vectors that induce the production of superior levels of neutralizing antibodies (Rose et al., 2008). The examples provided below describe several vaccine candidates that are based on VEEV replicon vectors:

– The company AlphaVax carried out several studies with the aim of developing vaccine candidates against CMV (with the aim to prevent CMV-induced congenital disease). In one study, VEEV vectors containing single-, double-, and triple SG promoters were used to express the individual CMV proteins gB, pp65 and IE1 or a pp65/IE1 fusion protein. In infected cell cultures, CMV proteins were expressed at high levels, and induction of robust antibody responses to gB and cellular immune responses to pp65 and IE1 was observed in immunized mice (Reap et al., 2007a). This study was followed by a phase 1 clinical trial in which the vaccine candidate was

tested in seronegative adult volunteers. In this trial, two-component replicon vectors expressing gB or a pp65/IE1 fusion protein were used. The vectors were found to be safe and to induce high levels of neutralizing antibodies as well as polyfunctional antigen-specific CD4+ and CD8+ T-cell responses (Bernstein et al., 2009).

- In a study carried out by Global Vaccines Inc., VEEV replicons were used in an attempt to develop a tetravalent vaccine against dengue virus (DENV). The tetravalent vaccine represented a mixture of VRPs, each of which encoded a specific glycoprotein (in the final setup, the E85 configuration of the glycoprotein) of one DENV serotype. This vaccine produced a balanced immune response and fully protected macaques against all four serotypes of DENV. Importantly, no evidence of interference between the various components of the vaccine was observed, and the anti-VEEV immunity was found not to hamper the booster immunization (White et al., 2013).
- Last but not least, AlphaVax tested an alphavirus-based anti-influenza vaccine candidate. The VRPs expressing the NA and HA proteins of influenza virus were found to induce high-titer antibody responses in all of the experimental animals used (mice, rabbits and rhesus macaques), and robust cellular immune responses were observed in mice and rhesus macaques. Toxicology studies performed in rabbits revealed no adverse effects of the vaccine candidate (Hubby et al., 2007).

Use of alphavirus-based vectors in anti-cancer vaccination and cancer treatment. Over the years, alphavirus vectors have been used in numerous attempts to treat various types of cancers including melanoma, prostate cancer, breast cancer and cervical carcinoma. Similarly, many different approaches have been tested, including the use of DNA vaccines, VRPs or even cells transduced with VRPs. Constructed alphavirus vectors have been engineered to express proteins that boost the immune response, tumor-associated antigens (TAA), members of various kinase receptor families, and other functional proteins. Although to date the majority of these studies have been preclinical and have enjoyed varying degrees of success, they have provided important knowledge on how to improve and use alphavirus-based anti-cancer vectors. These studies also revealed for the first time that alphavirus-based DNA vaccines can activate the innate immune response. It was also observed that the quality of host immune response does not depend only on the amount of expressed antigens. Several examples of such studies are provided below.

- Two studies by a single research group describe vaccination of mice against melanoma using DNA/RNA layered SINV replicon vectors. In the first study, vectors expressing tyrosinase-related protein 1 (TRP-1) were used. It was found that the alphavirus-based vector was capable of breaking the host's immune tolerance to the tumor. Furthermore, this effect was not observed in mice lacking RNaseL, indicating that it was dependent on the synthesis of double-stranded RNAs (Leitner et al., 2003). In the second study, the same vector was used in combination with a vector expressing

the anti-apoptotic Bcl-X(L) protein. It was observed that the presence of the Bcl-X(L) expression vector increased the lifespan of transfected cells, boosted the production of tumor antigen and increased the production of antibodies against this antigen. However, animals vaccinated with combination of these two vectors nevertheless displayed decreased protection against aggressive tumors. Thus, a correlation between the ability of the alphavirus-based vector to induce apoptosis of transfected cells and *in vivo* protection against challenge with melanoma was observed. These data indicate that alphavirus-induced apoptosis is essential for protective anti-cancer immunity (Leitner et al., 2004).

– Another strategy is to use antigen-specific dendritic cells (DC) as vaccine against established tumors. In one such study, a tumor overexpressing Neu oncoprotein (Neu is overexpressed in approximate a quarter of human breast cancers) was targeted. First, murine DCs were infected with VRPs of VEEV expressing truncated Neu oncoprotein. Delivery of these DCs to wt mice with established tumors resulted in high levels of Neu-specific CD8(+) T cell and anti-Neu IgG responses; furthermore, these responses were able to induce the regression of the tumors and enhance the survival of the mice (Moran et al., 2007).

Only a few alphavirus-based anti-cancer vaccines have been tested in phase I/II clinical trials. The first such study was conducted by AlphaVax with the aim to evaluate the induction of an immune response against colon cancer using VRPs of the VEEV expressing carcinoembrionic antigen (CEA). Like the pre-clinical studies, this trial showed that the vaccine candidate was safe and that it was capable of breaking immune tolerance to the self-antigen. In another study, it was shown that anti-vector neutralizing antibodies do not necessarily block the therapeutic use of VRPs. A phase I trial conducted using VRPs of VEEV expressing CEA revealed that repeated administration of VRPs was also capable of overcoming the negative effect of elevated levels of suppressive cell populations (Treg). Clinically relevant CEA-specific antibody and T-cell responses were observed in treated cancer patients. Most importantly, the CEA-specific antibodies mediated antibody-dependent cellular cytotoxicity against tumor cells from human colorectal cancer metastases, and patients with CEA-specific T-cell responses exhibited longer overall survival (Morse et al., 2010).
Another clinical trial, this time targeting melanoma, is expected to begin in 2014. The vaccine to be tested is again based on VRPs of VEEV; in this case, the vector encodes the melanoma differentiation antigen TRP-2. It was previously shown that immunological tolerance to the tumors can be broken in mice immunized with VRPs of VEEV or SINV DNA/RNA layered vectors expressing the related TRP-1 antigen (Leitner et al., 2003; Goldberg et al., 2005). Newer data also show that vectors encoding TRP-2 induce the activation of TRP-2-specific humoral (IgG) and effector CD8+ T-cell responses and cause accumulation of CD8+ T-cells within tumors (Avogadri et al., 2010).

Despite these promising results, it is increasingly clear that it is impossible to cure a broad range of tumors by vaccinating only against TAAs. It is frequently not known which TAAs are present in specific types of cancer cells; furthermore, cancers contain different cells, some of which can escape the induced anti-TAA immune response. Therefore, it is important to develop different approaches and frame various strategies. One promising strategy is based on the ability of alphaviruses to kill infected cells. The use of this approach depends on the ability to specifically target tumor cells with viruses. SINV is known to use the laminin receptor (LAMR) to enter mammalian cells (Wang et al., 1992), and the expression of this receptor is upregulated in many cancers (accordingly, there is an excess of free LAMR not bound to laminin on the surface of cancer cells). This results in a natural affinity of SINV for tumor cells, which can likely to be enhanced by modifying the vector. Together with the alternative options that are described briefly in the next section, this type of modification can be used to generate efficient alphavirus-based vectors that not only induce anti-cancer immune responses but also directly kill cancer cells.

Approaches to the control of alphavirus-based vectors

Virus-based replication-competent vectors tend to be more potent tools for foreign protein expression than vectors with impaired replication and/or spread. However, they also carry significant biological danger. Therefore, one of the central tasks in the development of such vectors is to achieve control over their replication and spread. For any virus-based vector, this is no easy task, but it is especially challenging for viruses with RNA genomes. Indeed, viruses with DNA genomes can be manipulated by the use of tissue-specific regulatory elements (promoters, enhancers, etc.) originating from different sources (other viruses, hosts). This approach is not possible with RNA viruses because their replication and gene expression are not controlled by such sequences. Although RNA viruses do display tissue specificity, the reasons for this (aside from the presence of receptors) are generally poorly and often not at all understood. Furthermore, a lack of proofreading function and a tendency to recombine cause instability of recombinant RNA virus-based vectors, which tend to revert, pseudorevert and/or compensate for introduced changes. However, because RNA-virus-based vectors have undeniable potential in numerous applications (see the examples provided above for alphaviruses), numerous approaches to control these vectors have been envisioned and tested. Most of the approaches that have been used are strictly virus-specific and are therefore not applicable to other viruses. The following descriptions give an overview of novel approaches that are applicable to many unrelated viruses and viral vectors.

Regulation of viral vectors using miRNAs. MiRNAs are a class of site-specific post-transcriptional regulators. They are small, approximately 21–25 nt long, non-coding RNA molecules that bind to complementary sequences typically located in the 3’ UTR regions of mRNAs (Lee et al., 1993). MiRNAs are produced in all cells of the body except those without nuclei. Many

miRNAs are tissue-specific. Furthermore, the expression of certain miRNAs is up- or downregulated in cancer cells (Chen, 2005). The varying abundance of different miRNAs (in extreme cases, the complete absence of some miRNAs) in particular cell types provides an opportunity to use the existing miRNA repertoire to control the replication and gene expression of viral vectors. In fact, this strategy is used by viruses themselves; the best known example is hepatitis C virus (HCV), which requires miR-122 as a cofactor for replication (Jangra et al., 2010). Alternatively, viruses can be engineered to express miRNAs. Again, this is a natural property of many DNA viruses that either encode miRNAs or affect the expression of cellular miRNAs (Grey et al., 2008). More recently, it has been reported that RNA viruses can also encode miRNA-like molecules (Hussain et al., 2012).

It is challenging to construct viral vectors whose replication is strictly dependent on the presence of a particular cellular miRNA (that is, to create positive regulation by miRNAs as naturally occurs in the case of HCV). In contrast, negative regulation by existing miRNAs is relatively easy to achieve, requiring only the insertion of miRNA target sites into the genome and/or transcripts of the virus. Negative regulation by existing miRNAs represents an approach that is applicable to all types of viruses. For example, it has been shown that the introduction of target sites of mir-143 or mir-145 into transcripts of the ICP4 gene of an HSV-based vector restricted the vector's replication to human prostate cancer cells, in which the expression of these miRNAs is down-regulated. In a mouse model, the recombinant HSV reduced the growth of human prostate cancers by 80%; importantly, the recombinant virus displayed much lower virulence than the non-targeted virus in normal tissues (Lee et al., 2009). This strategy, in which miRNAs allow the viral vector to replicate only in selected cells, can be designated the "keeping in" approach. The introduction of target sites of the neuron-specific miRNAs mir-124 or Let7 into the polio-virus genome was shown to result in significant reduction of viral neurotoxicity in mice (Barnes et al., 2008). This shows that miRNA-based regulation is applicable to RNA viruses as well. However, in contrast to the previous case, this represents an example of the "keeping out" strategy, in which recombinant virus is restricted from replication in certain cell types. It has also been shown that miRNAs can be used to improve the safety of viral vectors. The intro-duction of target sites of common and abundant miRNAs into helper RNAs of VEEV replicon vectors resulted in drastic down-regulation of the replication and spread of corresponding full-length virus (representing an unwanted product of recombination between helper and replicon RNAs) in cell culture as well as *in vivo* (Kamrud et al., 2010b). Basically, the replication of such RNAs becomes dependent on artificially added miRNA inhibitors; therefore, such genomes can be considered to harbor a conditionally lethal defect.

Because miRNA inhibitors are typically modified antisense oligonucleotides (ASO), the use of these molecules as essential cofactors for conditionally lethal viral vectors may seem impractical because of the high cost. However, this is not necessarily so. This class of compounds is well-represented in the pipelines

of many pharmaceutical companies, mostly as anti-cancer drug candidates. One such candidate is the ASO AP12009, an inhibitor of growth factor beta (TGF-β, a regulatory molecule that is commonly overexpressed in malignant tumors and is associated with cancer progression). AP12009 has reached phase I/II clinical trials in high-grade glioma, pancreatic carcinoma and melanoma patients and represents a promising candidate drug for the treatment of malignant tumors (Schlingensiepen et al., 2006). Another common target of ASOs is Bcl-2 anti-apoptotic oncoprotein. Considering how difficult it is to achieve complete suppression of cancer growth, one can envision that ASOs targeting genes such as TGF-β or Bcl-2 might be co-delivered with ASOs that enable the replication of miRNA-controlled virus in the targeted cancer.

As indicated above, all RNA viruses are genetically flexible. As a consequence, unfavorable changes to the viral genome are rapidly reverted or compensated for. As expected, this was found to be the case for miRNA targets inserted into RNA genomes (Barnes et al., 2008). The selective pressure toward elimination of the insert can, however, be alleviated by the use of miRNA inhibitors, which should be used at the stage of preparation of VRP or recombinant virus stocks. However, under *in vivo* conditions, viruses would still revert. This is a valid concern, although not necessarily a problem: reversions to wt are also common for several live attenuated vaccines. Thus, initial inhibition/ restriction of virus replication may be sufficient to block unwanted effects. Nevertheless, it is obvious that viral vectors with positive miRNA regulation would be more stable than vectors using negative regulation. This is illustrated by the recent phase II clinical trial of an inhibitor of liver-specific miR-122 which, as indicated above, is an essential co-factor in HCV replication. The drug candidate miravirsen, a locked nucleic-acid-based modified DNA phosphorotrioate ASO, caused long-term reduction in HCV titers, and, importantly, no viral escape mutants were detected (Janssen et al., 2013). Thus, even under clinical conditions, a virus controlled by miRNA in a positive manner was unable to escape from dependence on a particular miRNA. Unfortunately, the artificial construction of vectors with this type of miRNA dependence is difficult, and to my knowledge, no example of such a vector has been described.

Defective introns as tools to control the rescue of recombinant viral genomes from cDNA-containing constructs. Splicing is a biological process that regulates gene expression in eukaryotes by generating mRNAs coding for multiple protein isoforms with diverse functions from single pre-mRNA transcripts (Goemans et al., 2011; Mendell et al., 2012). Because positive-strand RNA viruses of eukaryotes replicate exclusively in the cytoplasm of infected cells, they do not encounter the host cell RNA splicing system. As a consequence, by random chance, their genomes contain numerous sequences that could be recognized by the cellular RNA splicing machinery as cryptic introns. For the use of RNA-launched vectors, this does not represent any problem or benefit. However, the presence of cryptic introns could affect the properties of DNA/RNA layered vectors because in this case, the synthesis of RNA occurs in

the nucleus and mature RNA, acting as the genome of the vector, must be transported to the cytoplasm. Compared to the majority of cellular mRNAs, the alphavirus genome is relatively large (>11 kb); it is rather unusual for an RNA of this size to be copied from a gene containing a single exon. This leads to two problems. First, in the absence of true introns, the cellular splicing machinery will use the cryptic introns that are always present in alphavirus genomes. This will lead to the production of defective RNAs lacking part of the viral genome but having most (or all) of the *cis*-elements required for viral replication. In the presence of a full-length virus (or vector) genome, such RNAs can function as defective interfering RNAs. Second, because the splicing machinery is connected to the systems responsible for transport of mRNA from the nucleus to the cytoplasm, the non-spliced large viral RNAs may accumulate in the nucleus or be transported to compartments of the cytoplasm that are not optimal for translation and the subsequent initiation of replication. Because similar considerations are applicable to all positive-strand RNA viruses, it is hardly surprising that various research groups have begun to incorporate introns into DNA/RNA layered vectors of plant and animal viruses (González et al., 2002; Marillonnet et al., 2005). An additional benefit of this strategy is that it also helps prevent the cryptic expression of viral genes in *E. coli*; as described in the Results and Discussion section, such cryptic expression may cause instability of plasmids containing the cloned cDNA of the virus.

Intron-insertion technology also offers other possibilities. Specifically, not all introns facilitate RNA maturation or are spliced away correctly. It is well known that for many pre-mRNAs different schemes of splicing are used; in addition, defective introns that result in aberrant splicing of preRNAs are also known to occur. The latter, occur during the maturation of β-globin mRNA, results in β-thalassemia, a disease caused by reduced synthesis of the hemoglobin beta subunit. β-thalassemia is a very common illness: approximately 200 different mutations in the β-globin gene (combined, these affect nearly 20% of the human population) are associated with this disease and related disorders (Olivieri, 1999). Several of the mutations associated with the disease are located in the first or second intron of the β-globin gene, where they create incorrect (but efficient) splicing sites and thus cause aberrant splicing of β-globin pre-mRNA (Figure 3). In such RNAs, introns are not completely removed and the reading frame of β-globin is interrupted. In particular, aberrant splicing of the second intron of the β-globin gene is caused by two different and independent (not occurring together in nature) mutations: the change of a T nucleotide to G at position 705 and the change of a C nucleotide to T at position 654. Both of these changes create new 5` aberrant donor sites and activate a common 3`cryptic acceptor site (Figure 3A). Because β-thalassemia is a common disease, numerous studies have attempted to develop compounds capable of blocking this aberrant splicing and thus restoring synthesis of normal β-globin. Some such compounds, called splice-switching oligonucleotides (SSOs), have indeed been developed. Briefly, SSOs are ASOs that bind to the mutant splicing region and prevent its access by the splicosome and other splicing factors

(Figure 3B). This results in a blockade of incorrect splicing and, importantly, restoration of the correct splicing pattern (Dominski and Kole, 1993; Bauman et al., 2009; Svasti et al., 2009).

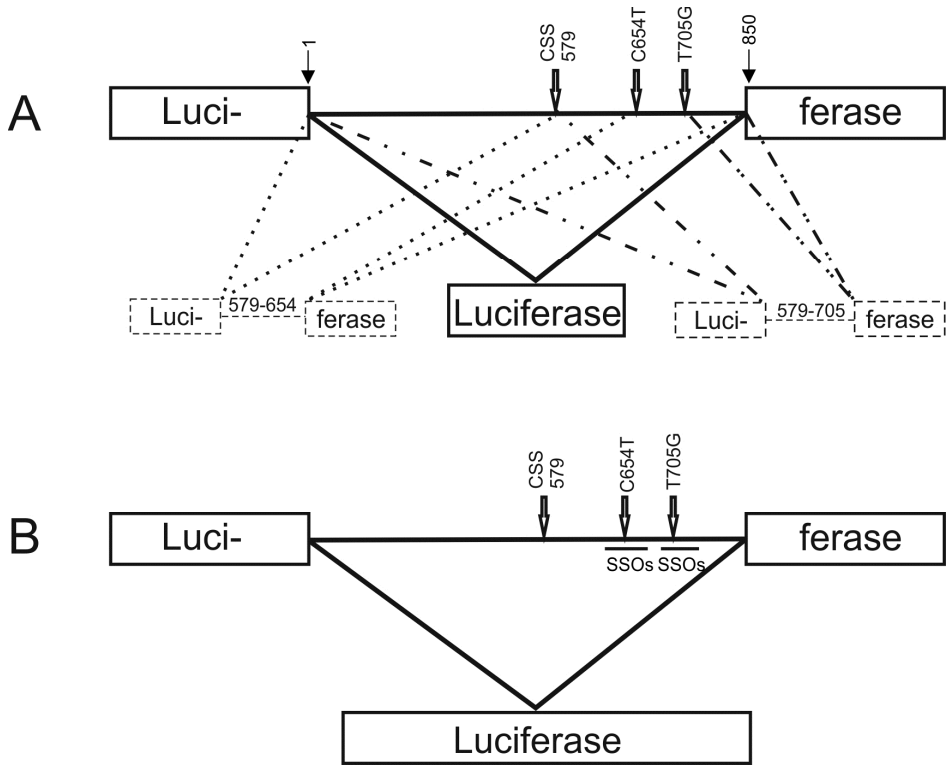

Figure 3. Schematic representation of aberrantly spliced intron (second intron of human β-globin gene). For simplicity, the intron is shown inserted into the coding sequence of the luciferase reporter gene. The beginning and end of the intron are indicated by arrows; the positions of cryptic splicing sites created by mutations associated with β-thalassemia and the common cryptic splice site (CSS) that is activated by these mutations are indicated by open arrows.
A. Aberrant and correct splicing possibilities of introns with mutations associated with β- thalassemia. mRNAs resulting from different aberrant splicing pathways contain parts of an intron that has not been removed and therefore encode defective proteins.
B. Principle of action of splice-switch oligonucleotides (SSO). SSOs bind to mutated sequence and block aberrant splicing; therefore, the intron is completely removed and the resulting mRNA encodes a functional luciferase reporter.

The effects caused by aberrantly spliced introns are also reproduced in the heterologous context. If an incorrectly spliced intron is inserted into a coding region of another gene (including genes of viral vectors), it causes a splicing defect that is reversible by SSOs. Surely, no intron can be used to regulate the replication of the positive-strand RNA genome itself; however, defective introns and SSOs can be used to control the release of infectious RNA from DNA/RNA layered vectors. Again, as in the case of miRNA inhibitors, the main hurdle is likely to be that the approach demands the co-delivery of viral constructs and SSOs. How will SSOs validated for clinical use for this purpose been developed? Does obtaining such SSOs drastically increase the cost of technology? Such risks certainly exist, and it would hinder the development of clinically validated SSOs for such use.

The diseases curable by splicing corrections include Duchenne muscular dystrophy, an X-chromosome linked recessive muscle-wasting disease caused by nonsense mutations in the dystrophin gene. Although there is currently no treatment for Duchenne muscular dystrophy, promising approaches such as exon-skipping therapy could be applied to this disease. This approach is similar to the use of splice correction technology: ASOs are used to block splicing sites flanking exons which contain premature stop codons and thus eliminate such exons from the mRNA coding for dystrophin protein. Like splice-switch technology, this approach can also be adapted for use with DNA/RNA layered vectors of RNA viruses. The possible benefits of the method are obvious: two exon-skipping oligonucleotides are already in late stages of clinical trials, one (Eteplirsen) in phase II and one (Drisapersen) in phase III; at least one additional compound, PRO044, is in phase I/IIa. Thus, it can be envisioned that therapeutic compounds developed to treat genetic disorders that have nothing in common with viruses may one day be used to regulate the rescue of RNA viruses from DNA/RNA layered vectors and/or in other virotherapies. Although this is not likely to happen soon, it does not hurt to think big.

RESULTS AND DISCUSSION

Objectives of the present study

This thesis is based on three logically connected research projects (I, II, III), which were undertaken with following general aims:

1. To identify the reason for the instability of plasmid containing cDNA of RNA-launched full-length SFV4-based vector in *E. coli* and, based on this, to develop a highly stable DNA/RNA layered plasmid construct usable as a platform for the subsequent development of different SFV4-based full-length vectors.
2. To develop full-length SFV4-based DNA/RNA layered vectors in which the rescue of infectious RNAs is controlled by the use of splice-switching technology and to characterize the obtained vectors in cell culture assays.
3. To develop full-length SFV4-based DNA/RNA layered vectors in which the rescue of infectious RNAs and the subsequent spread of rescued virus is controlled by cellular miRNAs and to evaluate different strategies of miRNA target site construction and insertion in the vector genome using cell culture assays.

Each general aim encompassed a number of specific aims; these are highlighted in the subsequent sections.

General rationale of the studies

The RdRps of RNA viruses lack proofreading activity and are prone to switch between homologous RNA templates (copy-choice recombination). For vectors based on such viruses, this inevitably results in the production of heterogenous progeny, competition between different genotypes and subsequent evolution. Through these processes, changes that reduce the fitness of the genome are eliminated sooner or later (Rausalu et al., 2009). Unfortunately, most if not all changes made during the construction of RNA-virus-based vectors fall within the category of "unfavorable". Duplication of SG promoters interferes with natural gene expression, while insertion of IRES elements or genes of interest increases the size of the vector genome, slows replication and (if inserts are too large) hampers the packaging of recombinant genomes into icosahedral NC. The expression of a gene of interest (with the exception of some selective markers, such as antibiotic resistance genes) is at best neutral for the virus; often, the proteins of interest are toxic to the cell or otherwise harmful to virus replication. In addition, the inserted sequences may contain RNA structures that by some (typically unknown) mechanism interfere with crucial viral functions. Mutations in vector genome can appear at any point in time; if they substantially increase the replication capacity of the vector, the mutant genomes can become dominant within one viral generation (Zusinaite et al., 2007). Hence, the stability of such vectors is an intrinsic problem. It can be alleviated

but never eliminated by appropriate design of the vector. Thus, inserts can be constructed so that they have minimal effect on the virus and/or can be inserted in a way that makes their elimination more difficult (Tamberg et al., 2007). It is also helpful to know the primary reasons for loss of any specific marker because this allows the researcher to minimize the damage by using appropriate conditions for vector production and propagation. When these approaches are combined with each other, and with a certain amount of luck, one may obtain an RNA-virus-based vector referred to as "genetically stable" (although, strictly speaking this is not exactly true).

Although these very important aspects of vector design and application were not a primary focus of this study, their existence has had a certain impact on the research described here (studies I-III) and, in some cases, has promoted parallel projects (Rausalu et al., 2009) that are not presented as part of this thesis. Instead, the starting point of my work was a stability problem of a different type. The infectious cDNA clone of SFV4, the plasmid pSP6-SFV4, is very difficult to propagate. Furthermore, when the cDNA of this virus is engineered to produce an RNA-launched vector, the problems are further increased: transformed bacteria refuse to grow, and even if they do, the obtained plasmid more often represents a mixture of different aberrant forms than high-quality homogeneous material. At best, researchers are losing time and money on repeated purification attempts. At worst, they face a complete inability to perform relatively easy tasks such as construction of SFV4-based libraries Thus, in our studies, we were losing the stability of the plasmid vector prior to the rescue of replicating RNA and facing stability problems even before they "had a right" to appear. What was needed was a "foolproof" system that would, at the same time, be inexpensive, fast and convenient to use. When this research was initiated, there was no certainty regarding what would we end up with; as it happened, we obtained the DNA/RNA layered system for SFV that is intricately described in study I. Because this appeared very promising, it seemed appropriate to use it for something more elaborate; thus, largely by itself, the existence of this system promoted studies dedicated to the development of new approaches for the control and regulation of alphavirus-based vectors (studies II and III).

Construction of an SFV-based plasmid without stability problems (I)

The problems with stability and resulting low yields of the pSP6-SFV4 plasmid are known to everyone who has ever worked with this plasmid; in fact, they were immediately observed by the researchers who constructed it in 1991. Nevertheless, for a period of 15 years many research groups used this plasmid as a tool without attempting to discover the reasons for its disturbing lack of stability. However, a few observations that yielded useful information were made by our research group:

– The pSP6-SFV1 plasmid, which contains cDNA of the corresponding SFV replicon vector, has no stability problems.
– The pSP6-Helper1 plasmid, which contains cDNA of the corresponding SFV helper-RNA, has no stability problems
– When pSP6-SFV4 was recreated by combining fragments from pSP6-SFV1 and pSP6-Helper1 (which became standard protocol in our lab), instability always appeared.

These observations indicated that the problem was created by the presence of both cDNAs of ns- and structural regions in the same plasmid. It was further observed that all attempts to substitute the SG promoter sequence in pSP6-SFV4 or even in pSP6-Helper1 for the sequence corresponding to IRES of EMCV failed while the same manipulation could be easily done in pSP6-SFV1. The cDNA of EMCV IRES is known to contain sequences that function as promoters for bacterial RNA polymerase. Thus, the following hypothesis was proposed: the cDNA corresponding to the ns-region of the SFV genome (more precisely, the region of cDNA that is absent in the pSP6-Helper1 plasmid) contains sequences that function as weak promoters in bacterial cells. This results in the production of cryptic transcripts that are translated by bacterial ribosomes to viral proteins, one or more of which are toxic to *E. coli*. It was also obvious that the sequence containing the promoter-like regions must be among the sequences coding for the structural proteins of SFV4 as pSP6-SFV1, which lacks corresponding regions also lacks stability problems.

Initial attempts to detect expression of SFV structural proteins in *E. coli* transformed using pSP6-SFV4 failed; the only antibody available to us was an antibody against C-protein that because it was raised against *E. coli*-expressed C-protein, gave a background signal that was too high. The only useful piece of information obtained from this experiment was that the expression of viral structural proteins, if it takes place, must occur at a low level. Therefore, another approach based on the interruption of the structural ORF by insertion of nonsense mutations at various positions was used (I, Fig. 1). This approach provided clear-cut results: all such mutations drastically reduced or completely eliminated the instability of the plasmid in *E. coli*. Analysis of the growth of transformed bacterial cultures revealed that toxicity was associated with cryptic expression of viral glycoproteins, especially E1. The reason for cryptic expression was obviously the presence of one or more weak bacterial promoters in cDNA corresponding to the ns-region of SFV4. Interestingly, cDNA of another SFV isolate, SFVA7(74), apparently lacks such elements; the plasmid corresponding to that isolate is extremely stable in bacteria. This provided a relatively easy means of mapping the region responsible for the effect and could allow us to eliminate the promoter(s) and the problems they caused by using silent mutagenesis. However, although such work can teach us something about bacterial transcription, this work would contribute nothing to our understanding of the molecular biology of alphaviruses. Furthermore, it had the potential to hamper the use of the resulting clones in basic studies. In the case of small RNA viruses, the secondary structure of the genome, which will inevitably be

changed by silent mutagenesis, may contain information that is essential for the viral infection cycle. Loss of or changes in this information may not be harmful in the context of the design and use of SFV-based vectors, but it is not acceptable for studies of SFV molecular biology and pathogenesis. These considerations argued against the use of this approach to solve the instability problem.

An alternative approach to the problem involves blocking the expression of structural proteins in bacterial cells. There are various ways to accomplish this. For example, regulatory elements, such as sequences that bind to the bacteriophage MS2 capsid protein, can be added to the construct. Binding to the capsid protein of MS2 would certainly suppress translation from the cryptic mRNAs. However, because it results in the modification of viral RNA, the addition of regulatory elements could again be used in the context of vector development but not in constructs designed for basic studies of SFV replication. To make the plasmid vector suitable for both applications, an approach that does not involve modification of the viral RNA is needed. One such approach, which is actually well known and is often used in the development of RNA virus-based DNA/RNA layered systems, is interruption of the viral reading frame(s) by insertion of intron(s). Because introns are completely removed by splicing, this approach will produce no alternation in the replicating viral RNA. Thus, it was decided to re-engineer the unstable pSP6-SFV4 plasmid into a DNA/RNA layered plasmid in which the expression of the viral structural proteins could be blocked by intron insertion (I, Fig. 2). To trigger the synthesis of SFV RNA in mammalian cells, the constructed cDNA plasmid contained a CMV promoter; simian virus 40 polyadenylation signal and hepatitis D virus negative strand ribozyme were used to terminate RNA synthesis and to trim the transcript so that it ends in a poly(A) sequence without any extra 3' nucleotides. Most importantly, an intron from rabbit β-globin gene was inserted within the cDNA region encoding C-protein. This location was chosen because it allowed blocking of expression not only of E1 but of all glycoproteins of SFV. It was also chosen so that the eukaryotic transcripts, unless spliced correctly, would have defects that were as severe as possible to avoid the expression of large amounts of truncated glycoproteins in the transfected cells. Nevertheless, the selection of the insertion site involved a fair amount of guessing, and the possibility that the construct needed to be re-designed, possibly more than once, was considered. However, such drastic measures were unnecessary because interruption of the reading frame in the capsid region resulted in complete elimination of instability problems with this vector in bacteria; the modified plasmid vector was able to multiply to a high copy number. Furthermore, problems associated with the rescue of the virus in cell culture were not observed in this study or by other research groups who have been using this construct for nearly five years.

The first critical issue to be determined for new virus-based infectious constructs is the efficiency of rescue of infectious virus from the vector (hereafter, the term "infectivity" is used to designate this efficiency). In the case

of rescue of wt virus, it does not really matter how many cells are actually infected by delivery of the DNA/RNA layered plasmid; the rescued virus spreads, and ultimately all cells will be infected. However, the situation is very different for studies involving the analysis of mutant viruses. Many mutations that severely reduce the infectivity of SFV RNA have been described. In general, mutations that later are reverted, pseudoreverted or compensated tend to reduce the infectivity of RNA-launched constructs as much as 10,000-fold. Thus, unless the infectivity of the original construct exceeds 10^4 plaques/μg of plasmid, such mutations will inevitably (and mistakenly) be classified as lethal mutations (Zusinaite et al., 2007; Lulla et al., 2012). Similarly, if one's aim is the construction of virus-based libraries, their representativity (the number of different virus variants in the library) will be directly proportional to the infectivity of the vector plasmid used. Hence, the availability of a construct with high infectivity is crucial for both of these applications. Using the infectious center assay (ICA), it was found that the infectivity of the layered vector for BHK-21 cells (which, to our knowledge, are the most susceptible host cells for SFV) was $1x10^5$ plaques/μg of plasmid DNA. Compared with transcripts derived from the RNA-launched vector, the infectivity of the new DNA plasmid (now designated pCMV-SFV4) was approximately 5-fold lower; however, its infectivity exceeded that of the SINV-based DNA/RNA layered vector (Dubensky et al., 1996) by almost 10-fold. In addition to its slightly lower infectivity (compared to RNA transcripts), it was also found that infection launched using the pCMV-SFV4 vector was delayed by approximately 1 hour compared to RNA-launched infection (I, Fig. 2). This lag time can be explained by the fact that, unlike infectious RNA transcripts, a DNA vector must enter the nucleus of the host cell and initiate the synthesis of future RNA genomes and the viral RNAs must be transported back to the cytoplasm, processes that require additional time.

The second critical issue associated with the use of DNA/RNA layered vectors with inserted introns is the possibility of formation of defective genomes. In the case of pCMV-SFV4, the intron is inserted into the sequence corresponding to the structural region of SFV. Irrespective of splicing events, the transcripts of this DNA/RNA layered vector will be capable of producing virus replicase proteins, and viral RNA replication will occur. Furthermore, such RNAs can also be packed into virions if the cell also contains wt RNA genomes that can be used to produce structural proteins. Because the cDNA of SFV4 contains numerous cryptic splicing sites, it is possible that incorrect splicing could also occur; for example, one correct splicing site (from the inserted intron) and one incorrect splicing site (a cryptic site from the cDNA) might be used. Again, this could result in the production of defective but replication-competent (at least in the presence of correct transcripts) RNAs. Both of these unwanted events can be minimized by elimination of cryptic splicing sites from the cDNA of SFV by means of silent mutagenesis. However, this situation again involves the use of RNA modifications that are not acceptable in studies of SFV molecular biology and/or pathogenesis. Thus,

there was a real possibility that cells transfected with pCMV-SFV4 would contain two different types of vector-derived RNAs: correctly spliced products and incorrectly spliced RNAs that co-replicate, both of which are packed into virions. If so, the presence of defective genomes could represent a significant problem. For a single virus or vector, serial plaque purification or low-MOI passages can be used to eliminate such defective genomes; however, these methods cannot be used when producing virus-based libraries or to propagate vectors with a tendency to instability (loss of inserts). Hence, it was important to determine whether formation of defectively spliced modified genomes represents a problem for pCMV-SFV4. To clarify the matter, RT-PCR and western blot analysis were performed. RT-PCR analysis revealed that virus stocks rescued from pCMV-SFV4 did not contain detectable levels of contaminating un-spliced or incorrectly spliced RNA. Western blot analysis revealed that all ns-proteins of the virus rescued from pCMV-SFV4 were of the correct size, and no aberrant forms of these proteins were observed. The only defect observed following the use of this method was that in addition to recognition of a prominent C-protein of the correct size, the corresponding antibody also recognized a smaller product that was not observed in cells infected with wt SFV4 (I, Fig. 3). The origin of this product is unclear; we could not detect defective RNAs that could serve as templates for its synthesis. It was therefore concluded that the removal of the inserted intron is correct and virtually complete. These results show that un-spliced or incorrectly spliced RNAs are either absent from this system or present at levels below the level of detection and that they do not represent an obstacle to the use of pCMV-SFV4 or other DNA/RNA layered vectors that are based on this construct.

Thus, the aims of the first study were fully met; in all analyzed aspects, pCMV-SFV4 performed even better than was originally hoped. This success also provided a very efficient tool for our research group and for many other research groups. We have provided the plasmid to more than ten laboratories all over the world, who are now using it instead of pSP6-SFV4 plasmid. Of note, our laboratory has continued to use the older plasmid in addition to the novel one, especially in applications that do not require insertion of additional genes but require initial infectivity to be as high as possible. We have also never managed to replace RNA-launched SFV replicon vectors with DNA/RNA layered vectors: the yields of VRPs achieved using RNA-launched constructs remain superior to those obtained through the use of DNA/RNA layered vectors. Thus, there is clearly room to improve the technology, both with respect to vectors and with respect to their delivery. Regarding the latter, special studies have been performed in collaboration with a research group working on the development of novel transfection reagents (Pärn et al., 2013). Studies II and III of this thesis are also based on the use of pCMV-SFV4. However, these studies took another turn: the focus was shifted to the problem of whether and how the rescue and/or replication of SFV-based constructs can be regulated.

Use of splice-switch technology to regulate rescue of replicating RNA from full-length DNA/RNA layered SFV vectors (II)

The previous study revealed that introduction of a single intron into full-length infectious cDNA of SFV4 carried a number of important consequences: the vector plasmid became more stable, its infectivity became superior to that of other described DNA/RNA layered vectors of alphaviruses, and no adverse effects on the rescue of replicating RNA were observed. An obvious extension of this study was to attempt to introduce additional introns into the pCMV-SFV4 plasmid. This could be accomplished, but no further increase in infectivity was observed. This led us to consider whether the introns can be used for the opposite purpose – to negatively regulate the infectivity of the DNA/RNA layered vector in controlled manner. In parallel studies, it was shown that defective introns, specifically the second intron of the human β-globin gene, which in some individuals carries mutations associated with β-thalassemia, can be used to suppress the expression of firefly luciferase (Luc) if the corresponding gene is integrated into the genome of the cell or delivered into the cell using recombinant adenovirus vectors. Importantly, in both cases, it was shown that this effect could be reversed using SSOs that target the aberrant splicing sites within the intron. Thus, the idea and also a tool-set that includes the wt second intron of the human β-globin gene (control), three aberrantly spliced introns associated with the β-thalassemia that contain point mutation at positions 654 or 705 and an artificially constructed intron containing both of these mutations, as well as SSOs capable of rescuing defects caused by these introns, were available for use. It was also realized that to be efficient aberrantly spliced introns must be inserted into the region corresponding to the ns-region of SFV because only then will their aberrant splicing result in the production of RNAs that cannot be used to produce functional replicase. Furthermore, it was realized that this approach can be used only to control the rescue of RNA genome of the vector. Thus, unlike the case of adenovirus vectors, there is no reversal of the virus infection. It is also obvious that the effects of inserted introns and SSOs on the DNA/RNA layered vector of SFV will be qualitative because one cannot obtain "partially infected" cells. Hence, it was assumed that to achieve efficient control over such vectors, the insertion of more than one aberrantly spliced intron may be required. Therefore, a set from 11 different full-length DNA/RNA layered SFV vectors was constructed. All of these vectors contained a Luc-encoding sequence inserted into the nsP3 region of SFV. The sites used for the insertion of wt or aberrantly spliced introns were selected in the region encoding Luc (the same sites were previously used in adenovirus vectors) and in the region encoding nsP4 (II, Fig. 1, Table 1). Hence, aberrant splicing of any of such introns will prevent expression of functional nsP4 and thus prevent replication of the produced RNA. However, aberrant splicing of the intron within nsP4 would have no effect on the expression of Luc from primary transcripts made using the CMV promoter.

This set of vectors was first tested for efficiency of rescue of replication-competent RNA using the ICA method. It was confirmed that insertion of one or two wt introns into the full length DNA/RNA layered vector of SFV4 did not have a negative effect on the infectivity of the vector in BHK-21 or HeLa cell lines; no increase in infectivity was observed either (data not shown). Thus, the insertion of additional wt introns did not affect the infectivity of the vector. When the same analysis was performed using vectors with aberrantly spliced introns and BHK-21 cells, various levels of inhibition of infectivity were observed (II, Table 2). First, it was found that insertion of an intron with a mutation at position 705 into the Luc region reduced infectivity approximately two-fold. Insertion of an artificial intron harboring two mutations resulted in a five-fold reduction in recombinant virus rescue. The insertion of two aberrantly spliced introns with similar or different point mutations was even more efficient; in this case, the infectivity of the vector was reduced ten-fold. The largest, nearly fifty-fold, reduction in infectivity was achieved with the use of two artificial introns. The ICA performed with HeLa cells yielded less information because no plaques were obtained for any construct containing an aberrant intron or combination of introns. The detection limit of standard ICA is, however, in the range of 10 plaques/μg DNA, and reliable quantification of results requires efficiency to be at least several-fold higher than this. Because vectors containing aberrantly spliced introns failed to reach such efficiencies, we took advantage of a more sensitive assay based on the measurement of vector-mediated Luc expression. This analysis revealed tendencies similar to those found for BHK-21 cells using ICA (II, Fig. 4).

Thus, it could be concluded that aberrantly spliced introns can be used to block rescue of replication-competent RNA from the DNA/RNA layered SFV vectors. While insertion of a single intron had a modest effect, two such introns acting in a synergistic manner increased the overall effect. Though it was not experimentally verified, this observation leads to the suggestion that further increases in the number of aberrantly spliced introns will further increase the effect. Thus, insertion of introns with β-thalassemia-associated mutations into DNA/RNA layered vectors can reduce and very likely ultimately block the rescue of infectious RNA genomes from the full-length DNA/RNA layered vector of SFV.

Thus far, the results shown in this section demonstrate that we have developed a novel and sophisticated way to “kill” the viral vector, but the real challenge is to regulate it. Therefore, the most important part of this project was to test whether it is possible to restore the infectivity of constructed splice defective vectors to some essential level.

It was known that the aberrant splicing pattern caused by a single point mutation in the β-globin intron (either the T705G or the C654T mutation) can be reverted to normal by use of the corresponding SSOs (Svasti et al., 2009). Indeed, it was observed that the infectivity of DNA/RNA layered vectors containing just one mutant intron of such type was efficiently restored by the corresponding SSO. These data, however, are not especially useful because

such vectors also had relatively high infectivity in the absence of SSOs and thus present little practical value. Hence, the truly important point was to discover whether a combination of SSOs can be used to rescue the infectivity of vectors containing several aberrantly spliced introns. Such experiments were therefore performed. The obtained data convincingly demonstrated that the infectivity of vectors carrying two defective introns of natural origin or even of those carrying two artificially created defective introns was efficiently restored. Thus, the greater the inhibitory effect caused by the presence of aberrantly spliced introns, the larger was the rescue efficiency achieved through the use of a combination of SSOs (the largest increase documented was nearly 100-fold; II, Fig. 6). It was also observed that in all experiments the SSO binding to the region near position 654 had higher rescue efficiency than the SSO targeting the sequence near position 705. Furthermore, SSO 654 could also increase the infectivity of vectors containing aberrantly spliced introns with point mutations only at position 705; the opposite effect, rescue of the defect caused by mutation in position 654 by SSO 705, was never observed. Therefore, there are strong reasons to believe that SSO 654 has at least two different activities: first, it can block aberrant splicing site 654 and act as a standard SSO; second, its binding to its target *per se* enhances correct splicing of the mutant intron. This type of effect has not been previously described for this compound; however, splicing-enhancing activity has been described, for example, for ASO binding region near position 632 (Resina et al., 2007; Svasti et al., 2009). It could therefore be assumed that the very high rescue efficiencies observed in this study (II, Fig. 6) resulted from the splice-switch activities of both SSOs and were enhanced by the splicing-enhancing properties of SSO 654.

To summarize, the obtained results demonstrated that aberrantly spliced introns and SSOs can be used to regulate virus-based vectors. This technology was more efficient in cases of suppression and rescue of adenovirus-mediated gene expression (study II). It is reasonable to assume that the same type of regulation could also be achieved for other DNA viruses replicating in the nucleus, such as herpesviruses. Clearly, for these viruses the technology is not limited to the control of expression of a single gene; aberrantly spliced introns can be used to manipulate the expression of key regulatory proteins such as S13 E1A of adenovirus or ICP4 of HSV, and therefore entire infection cycle of the virus can be controlled. Furthermore, DNA viruses are genetically more stable and less prone to eliminate inserted sequences than viruses with RNA genomes. Therefore, the technology could have excellent potential in the field of DNA virus-based vectors. The extension of this approach to the DNA/RNA layered vectors of alphaviruses has an obvious limitation: only rescue of infectious RNA genomes, but not their subsequent replication and spread, can be controlled. Nevertheless, this approach offers important benefits that alternative approaches do not. For example, unlike regulation based on the use of ts-mutants (good technology on its own, but largely restricted to cell culture), it can also be used in *in vivo* applications. Another benefit of the method is that unlike other mutations introduced into the RNA genome, the aberrantly spliced

introns are only part of the DNA/RNA layered vector and not of the replicating RNA genome; hence, they do not affect RNA replication.

The major limitation of this technology is that it requires a target (a virus with a DNA genome or a DNA/RNA layered vector) and its co-factor (SSO) to be co-delivered. While infection with DNA virus is relatively straightforward, efficient co-delivery of DNA/RNA layered vectors and SSOs is not, especially under *in vivo* conditions. Thus, the applicability of this approach depends on the development of specific (to organs, cell types, cancers) and highly efficient delivery technologies. Our attempts to develop one such approach are presented in an independent publication (Pärn et al., 2013) that is not included as part of this thesis. Some promising results were obtained; however, there certainly remain a long way to go and a number of problems that must be solved. Partially due to these reasons, another study addressing an alternative option for the regulation of viral vectors was initiated. Because the main hurdles revealed in study II are the delivery of SSOs and the lack of post-rescue control over viral RNA replication, a natural question was: do cells naturally (thus, in general, no delivery is required) contain any molecules that could be used to control the rescue of viral RNA genomes from DNA/RNA layered vectors and, ideally, also to control their subsequent replication and spread? One such possibility was exploited and analyzed in study III.

Rescue of recombinant virus from DNA/RNA layered vectors and subsequent spread of infection can be controlled using miRNAs (III)

As described above, different methods of controlling virus replication have been developed. These methods are usually applicable only to a specific virus and/or have significant limitations. For example, ts-mutations are specific for each virus and have limited (if any) use *in vivo*. Insertion of conditionally toxic genes such as TK can increase the safety of different viral vectors, including alphavirus-based vectors (Tseng et al., 2009); however, the use of this approach is hampered by vector instability, which would greatly increase if selective pressure (in this case ganciclovir) were to be applied. Changes in the natural tropism of a virus by specific modification of its antireceptor(s) represent, at least in theory, an excellent approach. However, in practice its use is limited by the fact that viral proteins, including antireceptors, have multiple functions, many of which are not known. Therefore, modification of antireceptors is typically unfavorable for the virus, and introduced changes are therefore often rapidly reverted. In the case of DNA viruses, advantage can be derived from the available tools used to regulate or modify gene expression; for RNA viruses, such options are extremely limited. Thus, there is a clear need to develop approaches that do not suffer from the limitations described above. Therefore, it is not surprising that the potential of using cellular miRNAs has attracted the attention of a number of researchers.

The possibility of using miRNAs is based on their diversity. Some miRNAs are ubiquitously expressed, but there are also miRNAs with remarkable tissue specificity of expression. Furthermore, the panels of miRNAs expressed in normal and cancer cells are different; the expression of miRNAs involved in regulation (repression) of the cell cycle is suppressed in tumors (Chen, 2005). As discussed above, the construction of viral vectors positively regulated by miRNA is complicated. That leaves the possibility of using miRNAs as negative regulators by adding their target sites to virus genomes (RNA viruses) or transcripts. The diversity of miRNAs offers relatively straightforward possibilities for construction of viral vectors that are restricted from some cell types. This type of approach had been used in the development of lentivirus, HSV (Brown et al., 2006; Lee et al., 2009) and RNA virus-based vectors (Barnes et al., 2008; Kelly et al., 2010), including a few vectors based on alphaviruses (Kamrud et al., 2010b; Ylösmäki et al., 2013). The designs used in the majority of these studies have been relatively similar: multiple targets of one or different miRNAs have been introduced as a cluster, and the target sequences have been presented in a form that allows perfect binding of miRNA. The obvious consequence of this is that the targeted RNA becomes cleaved by RNA induced silencing complex; hence the strongest repression is obtained. However, is the strongest response always the best? Multiple studies by different research groups, including ours, have demonstrated that the strongest selection pressure also results in the most rapid and efficient counter-response by the virus. Is the cleavage of viral RNA essential or not? If they have not completely escaped the attention of researchers, these questions have at least escaped discussion in published studies. Because the more subtle regulation of viral vectors may offer possibilities that robust suppression of its replication does not, these questions become central for study III of this thesis.

A standard approach was used for the basic design of the cassette containing miRNA target sites: two copies of three different miRNA target sequences, separated from each other by short spacers, were included in each cassette. The cassettes differed from each other by the selection of target sequences (cl1: let-7, miR-17 and miR-19; cl2: miR-214, miR-143d and miR-218) as well as by their configuration (III, Fig. 1). The rationale behind the selection of miRNAs was that miR-cl1 cassettes are targeted by miRNAs expressed at high levels in almost all cell types; furthermore, their ability to repress alphavirus replication was already known (Kamrud et al., 2010b). The miR-cl2 cassette, on the other hand, consists of targets of miRNAs that are absent or underexpressed in cervical cancer cells and was designed with the aim of studying the possibility of restricting virus replication in non-tumor cells. In addition to the standard configuration of miRNA targets, which is referred to as “Perfect” due to the formation of perfect duplex between miRNA and this type of target, two alternatives were also tested. In the configuration referred to as “Native”, naturally occurring miRNA target sites were used. In the configuration referred to as “Sponge”, a segment of the target sequence essential for cleavage was mutated. It was expected that both Native and Sponge targets would allow

binding of the respective miRNAs but that cleavage of the targeted RNA would not occur; instead, it was assumed that the translation of targeted RNA would be silenced. With one exception, the target cassette was always inserted into the 3`-UTR region of the targeted RNA because this is the region in which most natural targets of miRNAs are located.

Gene expression mediated by DNA/RNA layered alphavirus vectors results from a combination of two different processes. At the first step, transcription of replication- competent RNA from the DNA plasmid occurs. At the second step, cytoplasmic RNA replication and transcription is initiated. Accordingly, the effect of miRNAs on these processes should differ as well. At the first step the production of new RNA transcripts from the DNA template is not affected by miRNAs but the fate of the products of this reaction is. At the second step, binding of miRNA to the RNA genome of the virus could result in its destruction or in translational silencing; thus, both the production of new RNAs and the RNA products could be affected by miRNAs. At the same time one should keep in mind that alphavirus RNA replication is very efficient and is associated with shut-down of cellular transcription and translation. Thus, the production of components essential for functioning of the miRNA system becomes inhibited. Finally, because viral replication is highly error-prone, mutations that provide resistance to miRNA-mediated repression will inevitably occur, and the percentage of such mutants in the population will rapidly increase due to their selective advantage. Therefore, to obtain a better idea of exactly which processes are affected by miRNAs, two types of vectors were constructed and tested: DNA/RNA layered full-length SFV vectors and standard plasmid-based transient expression vectors. To make these two systems as similar to each other as possible, both were engineered to produce mRNAs with the same non-coding regions (corresponding to the UTR-s of SFV4) and containing the same marker, *Gaussia* luciferase (Gluc). The insertion sites of the miRNA target cassettes were also identical; they were placed between the Gluc encoding sequence and the 3' UTR of the virus (III, Fig. 2A).

It was found that in the context of the non-viral vector, all used strategies resulted in reduction of Gluc expression. The extent of the effect depended on the type of inserted cassette. In all tests, miR-cl1 had a much greater effect than miR-cl2, further indicating that the abundance and/or effectiveness of miRNAs targeting miR-cl2 was much lower than that of miRNAs targeting miR-cl1 cassettes. It was also observed that repression of Gluc expression depended on the configuration of the target sequences used; perfect cassettes always caused more prominent repression. The insertion of the most efficient miR-cl1 perfect cassette resulted in nearly 200–fold reduction in Gluc expression compared to the non-targeted vectors, and its effect was very similar in BHK-21 and HeLa cells. The effects of using miR-cl1 cassettes of sponge and native design were less prominent and depended on the cell type: up to 10-fold reduction of Gluc expression was observed in BHK-21 cells, while several-fold more efficient repression was observed in HeLa cells. The miR-cl2 cassettes were always much less efficient; for these sequences, the difference between cassettes of

perfect design and the other two design types was also much less pronounced. Similarly, the repression efficiency was less dependent on the cell type (the effect in HeLa cells was only slightly greater) (III, Fig. 2). These data confirmed that repression based on silencing of mRNA translation is less efficient than repression based on cleavage of targeted mRNA. The different levels of repression observed in different cell types may result from different abundances of miRNAs in different cells and/or from different efficiencies of miRNA-based systems in these cell types. Unexpectedly, the miR-cl2 cassette was no more cell-type-specific than the miR-cl1 cassette. This finding indicated that miRNAs, target sites of which were introduced in this cassette, have low abundance/efficiency in both cell types.

To our knowledge, the efficiencies of identical miRNA target sites inserted into standard expression constructs and these inserted into DNA/RNA layered full-length vectors have never been directly compared. Due to the spread of virus infection, comparison of levels of marker protein activities is valid only at early times post-transfection (as was performed in study II) and is therefore not a method of choice. Therefore, the efficiencies of miRNA targets in the context of SFV DNA/RNA layered vectors were analyzed using ICA. It was found that all the constructed cassettes reduced infectivity of the vectors, although in many cases the effects were small and not statistically significant. In BHK-21 cells, miR-cl1 cassettes of sponge and native design provided modest (2-fold) effects, while the cassette of perfect design caused a massive (approximately 2500-fold) reduction in infectivity. In HeLa cells, the miR-cl1 cassette of perfect design completely blocked rescue of infectious virus; no plaques were detected using ICA, and no Gluc expression or infectious virus release was detected in transfected cell cultures. Surprisingly, however, plaques were rarely detected in HeLa cells transfected with vectors containing miR-cl1 cassettes of native and sponge design, although for these constructs the rescue of infectious virus, which was shown by both Gluc expression and the release of infectious virions, was always observed. At a rough estimate, these cassettes caused approximately 200-fold reduction of infectivity of recombinant vectors in HeLa cells; thus, the extent of repression was approximately 100-fold greater in HeLa than in BHK-21 cells. This difference cannot be solely attributed to the different abundance/efficiency of corresponding miRNAs in these cell lines because differences observed using expression- plasmid-based vectors were only two-to-three fold. However, the situation was strikingly similar to that observed for vectors containing single copies of aberrantly spliced introns (study II). In that case, the insertion of aberrantly spliced introns also reduced plaque formation in BHK-21 cells (ICA) by approximately two-fold but completely blocked it in HeLa cells. Thus, it is reasonable to assume that in BHK-21 cells, which are extremely permissive for alphavirus replication, the effects caused by various repressors are much smaller than those observed in less permissive cells. One can also assume that the most sensitive step in the process of rescue of virus from DNA/RNA layered vector is likely the establishment of replication, as this is the only thing in common between vectors containing aberrantly spliced introns and

these with inserted miRNA target sites. However, it cannot be ruled out that differences in sensitivity also exist at the subsequent stages of alphavirus infection. Therefore, BHK-21 cells may not represent an ideal test system for alphavirus-based vectors because with these cells there is a possibility of critical underestimation of the potency of repressors of alphavirus replication. Notably, however, BHK-21 cells are the only cell line that can be used in some cases. Thus, here we also attempted to compare the effects caused by miR-cl1 (perfect) cassettes inserted in the 3'-UTR region to those caused by insertion of similar cassettes into the ns-region of the viral vector. The vectors used for the latter strategy were engineered to contain an additional marker (III, Fig. 3A) and, presumably due to their increased size, had infectivities that were approximately 200-fold lower than those of vectors lacking the second marker. These levels of infectivity were too low to be reliably measured in any cells other than BHK-21. Using these cells, it was found that insertion of the miR-cl1 perfect target resulted in an additional 7-fold reduction in infectivity; the effect was significant but much smaller than that achieved by insertion of essentially the same target into the 3' UTR region. Thus, based on the results of this study and that of Kamrud and colleagues (2010), it can be concluded that the 3' UTR of alphaviruses is the most favorable position for miRNA target insertion, at least when the highest repression efficiency is desired. The finding that the effects of miR-cl2 target insertion at the very same position were relatively mild in both cell types tested (at best, approximately 4-fold repression was achieved using an miR-cl2 cassette of perfect design in HeLa cells) confirms that the efficiencies of the corresponding miRNAs in both cell lines must indeed be very low.

Similar to the case with vectors containing aberrantly spliced introns, achieving the strongest miRNA mediated repression is not the most important goal. What is needed is efficient and, importantly, stable control over the vector. It was expected that vectors with miR-cl1 of perfect design may well represent an example of strong but unstable repression and that such vectors would most likely require the presence of miRNA inhibitors to replicate to high titer. This was experimentally verified using BHK-21 cells transfected with miRNA inhibitors or irrelevant oligonucleotides (SSO 705, used in study II, was used for this purpose; the choice was based on the finding that this oligonucleotide has no effect on the rescue and replication of SFV). It was observed that, compared to the non-targeted control vector, the rescue of targeted RNA genomes occurred after a delay; however, the corresponding virus replicated to high titer regardless of the presence of miRNA inhibitors (III, Fig. 4). The ability to express a Gluc-marker did, however, clearly depend on the presence of these inhibitors, leading to the conclusion that in the absence of the inhibitors deletions covering the region encoding Gluc (and most likely also the miR-cl1 target) must have occurred. Subsequent analysis performed using northern blotting (III, Fig. 5) and RT-PCR followed by sequencing clearly revealed that this was indeed the case. Somewhat surprisingly, it was observed that similar deletions also occurred in the genomes of virus propagated in the presence of

miRNA inhibitors. This finding indicates that selection pressure against miR-cl1 perfect cassette was clearly too great to allow the corresponding construct to remain genetically intact even in the presence of the miRNA inhibitors. Thus, very prominent repression of infectivity clearly had a downside, and the corresponding constructs used in this part of the study, although of academic interest, may have very little practical value. The same is likely the case for all constructs targeted by the ubiquitous miRNAs present in the majority of cells.
When planning the experiments described in this part of the thesis, more hope was invested in experiments with constructs containing miR-cl2 targets (in fact, these were the first constructs designed and tested; miR-cl1 was constructed and used later). The high infectivity of these constructs for the cervical cancer-derived HeLa cell line further boosted these hopes. Disappointingly, however, it also turned out that the virus with a perfect miR-cl2 site propagated efficiently in essentially every cultivated human cell line tested (data not shown). This was not totally unexpected because, under conditions of continuous growth in cell culture, many miRNAs associated with repression of the cell cycle are obviously downregulated. Lacking access to a reliable supply of primary human cells, we intended to construct cell lines over-expressing miRNA targets that were included in the miR-cl2 cassette. Perhaps not surprisingly, we failed in two cases out of three; only HeLa cells overexpressing miR-214 were obtained. Most likely, the failure with the remainder of these miRNAs was due to the fact that their overexpression was incompatible with continuous growth of the cell culture; thus, transiently transfected cells should most likely have been used in these experiments. Of note, overexpression of miR-214 had an effect on the cells as well, including an unexpected effect on SFV; for unknown reasons, these cells supported SFV replication to a lesser extent (evidenced by the slower replication of control virus) than normal HeLa cells. Nevertheless, with all of its problems, the miR-214-expressing HeLa cell line allowed us to confirm that the growth of targeted virus was indeed specifically reduced (III, Fig. 7), although the effect was not as striking as that observed for viruses containing the miR-cl1 target. This finding led to the prediction that viruses containing the miR-cl2 target may not suffer from instability as did those containing the miR-cl1 target. Indeed, after several passages of the virus in HeLa cells, all plaque-purified isolates were still able to express Gluc and therefore were presumed to contain the miRNA target as well. The use of more sensitive methods (RT-PCR followed by sequencing), however, revealed that all propagated stocks of the recombinant virus and even the initial stock harvested directly from transfected cells contained some viruses with genomes that lacked part of the miR-cl2 region (interestingly, no deletions in the Gluc-encoding regions were detected in the case of this virus). Thus, the existence of even a small selective pressure against the miR-cl2 target was sufficient to cause overgrowth by and accumulation of mutants with growth advantages.

Taken together, the results of this study confirm several findings that have already been documented in studies using picornavirus- and flavivirus-based vectors. In the context of vectors based on viruses with RNA genomes, the

primary issue is clearly the instability of recombinant genomes targeted by miRNAs. There is, however, reasonable hope that the impact of this instability is less prominent *in vivo* than it is *in vitro;* at least, this was the case with the stability of marker protein-encoding sequences inserted into the alphavirus genome (Tamberg et al., 2007). It also appears that viruses that are restricted from replication in the CNS performed better than the viruses used in this study; they were less prone to lose inserted sequences, at least while replicating in non-neuronal cells. However, even when combined, these considerations are unlikely sufficient to warrant the clinical use of such vectors; therefore, strategies of recombinant genome construction must be improved. One possible direction for such improvement, the use of native target sites for miRNAs of interest, is provided by this study. The benefit of this approach is that seed regions of miRNAs (and therefore their targets in the genomes of modified viruses) are only 7 nucleotides in length, over three-fold shorter than full miRNAs. These sequences can therefore be engineered in large numbers into coding region(s) of the viral genome without changing the sequence of encoded proteins. Certainly, some of these elements, which are scattered over the viral genome, appear to be in sub-optimal (or in some cases in non-usable) locations, but given the possibility of generating large numbers of targets, comined they may still produce significant effect. Most importantly, these elements cannot be removed by single deletions, which was found to be the most important way in which miR-cl1 and miR-cl2 targets are eliminated, because such removal would also result in the loss of sequences coding for essential viral proteins. Of course, the possible loss of these sequences by reversion still remains. However, given the large number of possible targets, complete reversion should represent a significant challenge even for viruses as genetically flexible as alphaviruses.

Some further perspectives

All of the studies described above were designed and performed with far-reaching aims in mind. Several possibilities concerning how these studies could be extended and the lessons that were learned have been provided in the foregoing sections and are therefore not repeated here. However, it is still interesting to note that the basic design of pCMV-SFV4 was extended to infectious clones of other SFV isolates in subsequent studies: SFV A7(74) and recently also SFV L10 (unpublished data). For SFV A7(74), this was performed for completely different reasons. The SFV A7(74) icDNA clone under the SP6 RNA polymerase promoter is completely stable in bacteria but suffers from another unexplained flaw: it is notoriously difficult to transcribe *in vitro*, as its RNA yield is very poor and inconsistent. Thus, the intron insertion approach used to solve the instability problem with the pSP6-SFV4 plasmid was successfully applied to the problem of a plasmid with poor transcription efficiency. This worked out well, and the obtained pCMV-SFVA7(74) plasmid is as infectious as pCMV-SFV4. In the case of both SFV4 and SFVA7(74), the

improvement was so impressive that once the sequence of SFV L10 isolate became available we did not even make an icDNA clone under the control of SP6 RNA polymerase promoter.

There are certainly many possibilities for the improvement and use of the miRNA-mediated control techniques described in this thesis. Some of these options are listed above. Here, I would like to mention just a few more applications. The strong suppression of virus rescue and replication achieved by use of miRNA targets such as miR-cl1 (perfect) actually has some practical applications. One of these is the development of safer replicon vectors (Kamrud et al., 2010b), while another could be the restriction of alphavirus vectors from one of its hosts. This is possible because vertebrate and insect hosts of alphaviruses have very different sets of miRNAs. Thus, it is very likely that SFV4 containing an miR-cl1 perfect target would replicate normally in cells of insect (including mosquito) origin and that there will be little selection pressure for removal of the miRNA target under these conditions. However, this type of virus represents no real value and therefore this property was never experimentally tested. The opposite combination makes much more sense: a virus carrying a cluster of targets of mosquito-specific miRNAs would be restricted from replication in mosquitoes and would not spread by insect vectors. In the context of the development of live attenuated vaccines against medically important alphaviruses (CHIKV, VEEV, etc.), this offers a clear advantage. In addition, miRNA targeting can also be used, at least to some extent, to improve the immunogenicity of alphavirus vectors. It has been reported that a flavivirus the genome of which was engineered to contain targets for miR-124a induced higher levels of neutralizing antibodies than did a standard attenuated virus strain (Heiss et al., 2011). Without a doubt, there are also many other ways to interfere with the complicated interplay that occurs between host and virus, and these can be utilized to obtain certain advantages or, at the very least, new knowledge.

SUMMARY

This thesis is based on three studies dedicated to the development and analysis of infectious DNA/RNA layered clone of SFV4 and a panel of vector constructs based on this clone. These projects originate from a study performed with the aim of identifying and subsequently eliminating the reasons for the instability of pSP6-SFV4, a standard RNA-launched icDNA clone of SFV, in bacterial cells. It was found that the instability of this plasmid was caused by the toxic effects of SFV envelope proteins that were cryptically expressed in bacterial cells. The problem was eliminated by interrupting the corresponding reading frame with an intron; this manipulation proved to have no adverse effect on the SFV4 rescued from the obtained DNA/RNA layered vector. Thus, this study provided a tool for several further studies, two of which are included in this thesis.

A central problem hampering the medical use of alphavirus vectors is concern over their safety; potential safety issues associated with the use of these viral vectors originate from the fact that alphavirus-based vectors are very difficult to regulate and control, similar to vectors based on other RNA viruses. Two new approaches to control these vectors were tested. First, DNA/RNA layered vectors, in which the rescue of replication-competent RNA genome of SFV was controlled by aberrantly spliced introns and splice-switch oligonucleotides, were designed, constructed and tested. It was shown that rescue of RNA genome can be efficiently inhibited and most likely even blocked by incorporation of increasing numbers of defective introns into the non-structural portions of the vector constructs. Furthermore, the delivery of splice-switch oligonucleotides completely or almost completely restored the infectivity of the vector. This novel principle is also applicable to DNA/RNA layered vectors of other RNA viruses and may be especially useful in controlling and regulating gene expression mediated by vectors constructed from viruses of DNA genomes that replicate in the nucleus of the cell.

Using cellular miRNAs to target the genomes of recombinant alphavirus may also represent a viable method to control rescue as well as replication and spread of the vector. This possibility was tested using a number of viral constructs. It was found that repression of virus replication can be achieved by the insertion of different types of miRNA targets, including those naturally occurring in cellular mRNAs. It was also found that strong inhibition of recombinant virus rescue is relatively easy to achieve and that the main hurdle for development of miRNA controlled alphavirus vector is actually the creation of a vector in which the inserted targets are not lost. Based on the obtained results, construction of recombinant genomes containing natural targets of the miRNAs scattered over the coding region of the alphavirus genome is proposed as the best prospective approach. Such vectors, which may also be engineered to contain other control systems, may find be applied in the development of attenuated virus-based vaccines, recombinant vaccines or anti-cancer agents.

SUMMARY IN ESTONIAN

Alfaviirustel põhinevate multifunktsionaalsete geeniekspressiooni ja -teraapia süsteemide väljatöötamine ja funktsionaalne analüüs

Alfaviirused on positiivse polaarsusega RNA genoomiga viirused sugukonnast *Togaviridea*. Looduses levivad alfaviirused lülijalgsete vektorite abil. Selgroogsetel peremeestel põhjustavad nad erinevaid haigusi; Uue Maailma alfaviirustele on iseloomulik entsefaliidi, Vana Maailma alfaviirustele aga artriidi tekitamine. Enim uuritud alfaviiruste, milliste hulka kuulub ka Semliki Forest viirus (SFV), laboratoorsed tüved on inimesele mitte patogeensed. Kuna nende viiruste genoome on lihtne manipuleerida ja nende kasutamisel vaktsineerimisel või vähivastases teraapias (siiani loom-mudelitel) on täheldatud mitmeid kasulikke efekte (kaasasündinud immuunvastuse aktiveerimine, kasvaja suhtes olemasoleva immuun-tolerantsi murdmine) on need viirused kujunenud perspektiivseteks tööriistadeks geeni- ja kasvajavastaste vaktsiinide väljatöötamisel.

Käesolev väitekiri põhineb kolmel artiklitena avaldatud uurimisetööl mis kajastavad SFV genoomi cDNA-l põhineva infektsioonilise DNA/RNA kihtvektori ning selle alusel valmistatud rekombinantsete vektorite kavandamist, valmistamist ja iseloomustamist. Nende projektide alguspunktiks oli uurimus, mille eesmärgiks oli välja selgitada põhjused, mis muudavad pSP6-SFV4, standardse RNA kaudu käivitatava infektsioonilise klooni, bakterirakkudes geneetiliselt ebastabiilseks. Tehti kindlaks, et seda ebastabiilsust põhjustab SFV membraanivalkude soovimatust ekspressioonist tulenev toksiline mõju bakterile. See probleem lahendati liites SFV lugemisraami introni, mis takistas toksiliste valkude tootmist bakteris kuid ei mõjutanud negatiivselt loodud DNA/RNA kihtvektorist vabanenud viiruse omadusi. Seega loodi antud uurimistöö käigus töövahend, mida kasutati mitmetes järgnevates uurimistes.

Kesksek probleemiks, mis on siiani takistanud alfaviirustel põhinevate vektorite kasutamist meditsiinis, on olnud kahtlused selliste viirustel-põhinevate vektorite ohutuses. See probleem tuleneb omakorda asjaolust, et sarnaselt teistel RNA-genoomsetel viirustel põhinevatele vektoritele on nende vektorite käitumist organismis väga raske kontrollida. Selles väitekirjas katsetati kahte uut lähenemist, mille ühiseks eesmärgiks oli seesuguse kontrolli tekitamine. Uue ja originaalse lähenemisena kavandati ja konstrueeriti SFV põhised DNA/RNA kihtvektorid milles rekombinantse SFV genoomi vabanemist takistavad viiruse kodeerivasse alasse sisse viidud defektsed (valesti splaisitavad) intronid. Selliste valesti splaisitavate intronite poolt põhjustatud defekti on võimalik kompenseerida splaisingu mustrit muutvaid oligonukleotiide kasutades. Läbiviidud uurimistöös näidati, et mitme valesti splaisitava introni lisamine vektori genoomi surub efektiivselt maha (ja eeldatavasti võib ka täielikult blokeerida) viiruse infektsioonilise RNA vabanemist; splaisingu mustrit muutvate oligonukleotiidide lisamine aga vähendab oluliselt või kõrvaldab selliste intronite

põhjustatud negatiivse mõju viirus-vektorile. Nendest tulemustest lähtudes võib väita, et antud meetod sobib ka teiste RNA-viirustel põhinevate kihtvektorite kontrollimiseks ja võib olla eriti efektiivne vektorite, mis põhinevad rakutuumas replitseeruvatel DNA-genoomsetel viirustel, kontrollimiseks.

Raku micro-RNAde (miRNAde) kasutamine alfaviirustel põhinevate vektorite mõjutamiseks kujutab endast lähenemist mille abil võib viiruse paljunemist kontrollida ka peale tema infektsioonilise genoomi vabanemist DNA/RNA kihtvektorist. Vastava uurimuse jaoks valmistatud mutantsete vektorite uurimine näitas, et miRNA vahendatud kontroll viiruse vabanemise ja järgneva leviku üle on suhteliselt lihtsasti saavutatav; selleks on vaja viiruse genoomi liita raku miRNAde sihtmärk-järjestusi. Reaalseks ja palju keerulisemaks probleemiks osutus selliste vektorite geneetilise stabiilsuse tagamine. Saadud tulemuste põhjal võib järeldada, et selle eesmärgi saavutamiseks on vaja kasutada looduslikult esinevaid miRNAde sihtmärke mida saab suurel hulgal sisse viia viirusele olulisi valke kodeerivatesse aladesse. Sellised viirused, millele võib olla lisatud ka muid kontroll-süsteeme, võivad leida kasutust nõrgestatud elusvaktsiinide, rekombinantsete vaktsiinide ja ka kasvaja-vastaste geeniravimitena.

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ACKNOWLEDGEMENTS

I would like to thank all of my lab members, who helped me during the years of my studies. I am grateful to my supervisor, Prof. Andres Merits, for his patience and guidance. I thank my co-authors and former colleagues. Last, but not least, I am extremely grateful to my friends and my family who tolerate my endless complaining.

PUBLICATIONS

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List of publications:

1. **Ülper, L**., Sarand, I., Rausalu, K., Merits, A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of Virological Methods*, 148, 265–270.
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Patents/patent applications:

1. Andres Merits, Mart Ustav, Andres Männik, Rein Sikut, Kaja Kiiver, Valeria Lulla, Aleksei Lulla, **Liane Viru**, Tarmo Mölder, Urve Toots. Transreplicase Constructs; Owner: FIT Biotech Oyj Plc. Priority date: 02.04.2007

2. Kai Rausalu, Valeria Lulla, Liis Karo-Astover, Kristi Luberg, **Liane Viru**, Inga Sarand, Andres Merits, Anna Iofik. A method for creating a viral genomic library, a viral genomic library and a kit for creating the same. Priority number: P200700047; Priority date: 31.08.2007
3. Andres Merits, **Liane Viru**, Ülo Langel, Samir El-Andaloussi, Gregory Ducom Heller, Taavi Lehto. A method and composition for creating conditional lethality for virus mutants and for eliminating the viability of an eukaryotic cell; Priority number: P200900077; Priority date: 07.10.2009

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1. **Ülper, L**., Sarand, I., Rausalu, K., Merits, A. (2008). Construction, properties, and potential application of infectious plasmids containing Semliki Forest virus full-length cDNA with an inserted intron. *Journal of Virological Methods*, 148, 265–270.
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6. Ratnik K., **Viru L**., Merits A. (2013). Control of the rescue and replication of Semliki Forest virus recombinants by the insertion of miRNA target sequences. *PLoS One*, 8, e75802.

Patendid ja patenditaotlused:

1. Andres Merits, Mart Ustav, Andres Männik, Rein Sikut, Kaja Kiiver, Valeria Lulla, Aleksei Lulla, **Liane Viru**, Tarmo Mölder, Urve Toots. Transreplicase Constructs; Omanik: FIT Biotech Oyj Plc. Prioriteedi kuupäev: 02.04.2007

2. Kai Rausalu, Valeria Lulla, Liis Karo-Astover, Kristi Luberg, **Liane Viru**, Inga Sarand, Andres Merits, Anna Iofik. A method for creating a viral genomic library, a viral genomic library and a kit for creating the same. Prioriteedi number: P200700047; Prioriteedi kuupäev: 31.08.2007
3. Andres Merits, **Liane Viru**, Ülo Langel, Samir El-Andaloussi, Gregory Ducom Heller, Taavi Lehto. A method and composition for creating conditional lethality for virus mutants and for eliminating the viability of an eukaryotic cell; Prioriteedi number: P200900077; Prioriteedi kuupäev: 07.10.2009

DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS

1. **Imre Mäger.** Characterization of cell-penetrating peptides: Assessment of cellular internalization kinetics, mechanisms and bioactivity. Tartu 2011, 132 p.
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