universität wien

DIPLOMARBEIT

“Establishing a trans-packaging system for TBEV replicons to study events of complementation and recombination in the genus *Flavivirus*“

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Wien, Juli 2009

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Studienrichtung /Studienzweig (lt. Studienblatt):	Molekulare Biologie
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Danke…

…an alle die mich beim Entstehen dieser Arbeit fachlich unterstützt haben.

Ich möchte mich herzlich bei Prof. Franz X. Heinz bedanken für die Möglichkeit am Institut für Virologie meine Diplomarbeit durchzuführen und die spannende Welt der Viren näher kennenzulernen. Herzlich danken will ich all meinen Laborkollegen aus der virologischen Forschung, die immer hilfsbereit, interessiert und aufgeschlossen waren. Ein ganz großes Dankeschön geht dabei an Dr. Christian Taucher für eine tolle Betreuung in Praxis und Theorie. Danke auch an Dr. Gabriel O´Riordain, die mir half meiner Arbeit den letzten sprachlichen Schliff zu geben.

…an alle die meine Freuden und Sorgen während meiner Diplomarbeit geteilt haben.

Ich danke meinen Eltern und meiner Schwester Nina, die mich immer unterstützen und an mich glauben. Danke Ida und Cäcilia, die mich mit ihrem Temperament immer auf andere Gedanken bringen. Vielen Dank an Constanze Hauser und Paula Carranza fürs Zuhören und Diskutieren über Viren und viele andere Dinge.

…an meinen Freund Leo, dass du für mich da bist, wann auch immer.

Contents

Summary

Tick-borne encephalitis virus (TBEV) is a member of the family *Flaviviridae*, genus *Flavivirus*. The flavivirus genome consists of a single, positive-strand RNA molecule which contains only a single open reading frame, that is translated into a polyprotein and then further processed by viral and host proteases yielding three structural and seven non-structural proteins. Flavivirus genomes defective in the production of infectious virus particles can be constructed by deletion of one or more structural proteins. This type of genome is called a replicon because it is still capable of initiating RNA replication. Replicons have been used to study a variety of different aspects of flavivirus biology including RNA recombination and packaging. However, the use of replicons is often limited to cell types where efficient transfection methods are available. The main goal of the thesis was to package TBEV replicons into virus particles to allow the infection of different cell types. To achieve this, we utilized the alphavirus expression vector VEEV for the expression of the structural proteins CprME of the TBE virus. Upon co-transfection of RNA of the alphavirus VEEV coding for CprME of TBEV and TBEV replicons in BHK-21 cells, virus particles containing replicon RNA were recovered. These packaged replicons were only capable of infecting a cell once and were called single round infectious particles (SIPs). In contrast, previous studies showed that complementation of replicons allowed multiple rounds of infection when the multiplicity of infection was high enough to allow co-infection. Passages of such complementing replicons were used to study recombination.

The second aim was to use the produced SIPs containing replicon RNA in co-infection experiments to test whether they could be used for studies on recombination. We demonstrated that the infectivity of the produced SIPs strongly varied between the different cell types BHK-21, MEF, HEK 293T, and IRE-18. Furthermore, we showed that trans-complementation only worked well in HEK 293T where the multiplicity of infection was greater than 10 %. However, the number of infected cells was generally too low to allow efficient complementation in the majority of the tested cell types and therefore no passages could be performed to select recombinant viruses.

As a consequence, we tried to optimize the production of SIPs. First, we utilized HEK 293T cells for packaging of TBEV replicons. We succeeded in packaging TBEV replicons into SIPs in HEK 293T cells by co-transfection of TBEV replicons with VEEV-CprME, but failed to augment the amounts of produced SIPs. Secondly, we cloned the CprME

coding sequence into the backbone of the plasmid DNA expression vector, phSV40. The resulting phSV40-CprME construct and TBEV replicons were sequentially transfected into HEK 293T cells. Again, trans-packaging of TBEV replicons into SIPs was successful. However, once more we did not achieve the desired optimization of efficiency of the SIPs production. Overall, we successfully established a trans-packaging system for TBEV replicons into SIPs. Unfortunately we could not produce high enough titers of SIPs to allow investigation of recombination. Nevertheless, the established system may prove very useful in studies that do not depend on high titers.

Zusammenfassung

Das Frühsommer-Meningoenzephalitis (FMSE) Virus gehört zur Familie der *Flaviviridae*, Gattung *Flavivirus*. Das Genom der Flaviviren besteht aus einem einzelsträngigen RNA Molekül mit positiver Polarität, das nur über einen Leserahmen verfügt und in ein Polyprotein translatiert wird, welches durch virale und zelluläre Proteasen prozessiert wird. Dadurch entstehen die drei Strukturproteine und die sieben Replikationsproteine der Flaviviren. Flavivirusgenome, die keine infektiösen Viruspartikel produzieren, können durch die Deletion eines oder mehrerer Strukturproteine generiert werden. Diese Art von Genomen werden als Replikons bezeichnet, da Replikons noch in der Lage sind die Replikation der Virus RNA zu starten. Replikons können zur Untersuchung vieler Aspekte der Flavivirusbiologie, wie zum Beispiel RNA Rekombination und Virusverpackung eingesetzt werden, wobei die Verwendung von Replikons meist auf Zelllinien beschränkt ist für die es effiziente Transfektionsprotokolle gibt. Um die Infektion von verschieden Zelltypen zu ermöglichen, war das primäre Ziel der präsentierten Arbeit die Verpackung von FSME Virus Replikons in Viruspartikel. Um die FMSE Virus eigenen Strukturproteine CprME zu exprimieren, verwendeten wir den Alphavirus Expressionsvektor VEEV. RNA des CprME exprimierenden Alphavirus VEEV wurde zusammen mit FSME Virus Replikons in BHK-21 Zellen transfiziert, und somit konnten Viruspartikel die Replikon RNA enthielten, produziert werden. Diese verpackten Replikons konnten eine Zelle nur einmal infizieren und wurden daher als einfach infektiöse Partikel bezeichnet. Im Gegensatz dazu, konnte in vorangegangenen Studien gezeigt werden, dass Replikons durch Komplementation, Wirtszellen mehrfach infizieren konnten, wenn die Infektionsrate hoch genug war um die gleichzeitige Infektion der Wirtszelle mit mehreren Replikons zu gewährleisten.

Unsere weitere Zielsetzung war die Untersuchung von Rekombination in verschiedenen Zelltypen. Dazu wurden unterschiedliche Zelltypen mit den verschiedenen produzierten einfach infektiösen Viruspartikeln zweifach infiziert und einer Kontrolle auf Komplementation zwischen den verschieden Replikons unterzogen. Wir zeigten, dass die Infektiosität der produzierten einfach infektiösen Viruspartikeln stark zwischen den untersuchten Zelltypen BHK-21, MEF, HEK 293T und IRE-18 variierte. Weiters zeigten wir, dass Komplementation *in trans* nur in HEK 293T Zellen stattfand, wo die Infektionsrate mehr als 10% betrug. Tatsächlich war die Anzahl der infizierten Zellen im Allgemeinen zu gering um eine effiziente Komplementation zwischen den Replikons in

der Wirtszelle in den meisten untersuchten Zelltypen nachzuweisen und daher konnten auch keine Zellpassagen durchgeführt werden um rekombinante Viren zu nachzuweisen.
Daher versuchten wir die Produktionseffizienz der einfach infektiösen Partikel zu optimieren. In diesem Sinn verwendeten wir die Zelllinie HEK 293T Zellen um die FSME Virus Replikons in Viruspartikel zu verpacken. Nach der Transfektion von FSME Virus Replikons zusammen mit VEEV-CprME, konnten einfach infektiöse Viruspartikel produziert werden, gleichzeitig konnte die Effizienz der Produktion nicht verbessert werden. In einem weiteren Versuch das System zur Verpackung von FSME Virus Replikons zu optimieren, wurde die Sequenz die für die Strukturproteine kodierte in das DNA Expressionsplasmid phSV40 eingefügt. Das generierte Konstrukt phSV40-CprME und die FSME Replikons wurden aufeinanderfolgend in HEK 293T Zellen transfiziert. Widerrum, konnten die FSME Virus Replikons in einfach infektiöse Viruspartikel *in trans* verpackt werden, allerdings konnte eine Optimierung der Effizienz der Methode wieder nicht erreicht werden. Letztendlich, konnten wir das Verpackungsystem für FSME Replikons *in trans,* erfolgreich etablieren. Dennoch konnte die Produktion der einfach infektiösen Viruspartikel nicht in einem sehr hohen Maßstab erfolgen und daher war uns die Anwendung zur Untersuchung von Rekombination nicht möglich. Nichtsdestotrotz, kann das etablierte Verpackungssystem in vielen anderen Studien, die keine hohe Verpackungseffizienz verlangen, Anwendung finden.

1. Introduction

1.1. Family *Flaviviridae*, genus *Flavivirus*

The family *Flaviviridae* consists of the three genera *Flavivirus*, *Pestivirus* and *Hepacivirus*. All members of this family are single-stranded RNA viruses with positive polarity and share similarities in genome organization, virion morphology, and replication strategies. The genus *Flavivirus* is the largest genus containing over 70 viruses, most of which have arthropod vectors. Therefore, the genus can be divided into three major groups, mosquito-borne viruses, tick-borne viruses and viruses with no known arthropod vector (Fields, Knipe et al. 2007).

Upon infection of humans, flaviviruses cause diverse diseases including hemorrhagic fever, fever arthralgia rash syndrome, and infections of the central nervous system. The most important human pathogens are found among the mosquito-borne group, such as yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and dengue virus (DENV) and have been considered as potentially emerging disease agents (Mackenzie, Gubler et al. 2004). Furthermore, the tick-borne encephalitis virus (TBEV) is the most important member of the tick-borne group causing severe neurological infections in humans (Mandl 2005). Table 1 gives an overview of medically important flaviviruses, their vectors, and natural hosts.

Table 1. Medically important flaviviruses.

Virus	Main clinical syndromes*	Main vectors	Natural hosts
Mosquito-borne viruses			
Dengue virus (serotypes 1-4)	FAR, HF	*Aedes Aegypti*	Humans, (macaque monkeys in Africa)
Yellow fever virus	Hepatitis, HF	*Aedes* and other species	Primates (monkeys, chimpanzees, baboons), humans
Japanese encephalitis virus	CNS	*Culex triaeniorhynchus*	Waterfowl (egrets, herons), chicken, pigs
West Nile virus	FAR, CNS	*Culex pipiens*	Waterfowl and other birds
St Louis encephalitis	CNS	*Cules pipiens, tarsalis, nigripalpus*	Birds (pigeons, sparrows)
Murray Valley encephalitis virus	CNS	*Culex annulirostris*	Waterfowl, rabbits, marsupials
Kunjin virus	FAR	*Cules annulirostris*	(?Waterfowl, chickens)
Rocio virus	CNS	*Psorophora species*	Wild birds
Tick-borne viruses			
Tick-borne encephalitis virus	CNS	*Ixodes* species°	Forest rodents (mice, hedgehogs)
Louping ill virus	CNS	*Ixodes ricinus*	Sheep, shrews, field mice, grouse
Powassan virus	CNS	*Ixodes* species°°	Rodents (shrews, rats, squirrels), land birds, bats
Omsk haemorrhagic fever virus	HF	*Dermacentor* species	Rodents (muscrats, voles)
Kyasanur forest disease virus	HF	*Haemophysalis* species°	Rodents, birds, bats, monkeys

*FAR=fever arthralgia rash syndrome, HF=haemorrhagic fever, CNS=central nervous system infection.
° Also transmitted via unpasteurized milk; °° also transmitted by direct contact with infected carcasses.

1.1.1. Disease, distribution, and transmission

Most vector-borne flaviviruses are transmitted by two groups of blood-suckling arthropods, mosquitoes and ticks, and circulate between arthropods and wild vertebrate hosts. The transmission to humans occurs by the bite of an infected arthropod, but humans are mostly dead-end hosts. In rare cases direct transmission between vertebrates has also been reported (Kuno and Chang 2005). Once flaviviruses are introduced through the skin, the epidermal Langerhans cells are probably among the first infected cells (Chambers and Diamond 2003). The virus is transferred by these cells to the local lymph nodes and further spreads to the lymphoid compartments. There, virus replication leads to infection of other tissues and viremia. The causative diseases in humans are diverse including hemorrhagic fever, fever arthralgia rash syndrome, and infections of the central nervous system.

1.1.1.1. Yellow fever virus

YFV is the prototype of the genus *Flavivirus* and has been employed as a model to reveal insights into genome structure and replication strategies. YFV is found in West and Central Africa, as well as in South and Central America (fig.1) where it causes yellow fever in humans. Transmission occurs mostly through the bite of *Aedes* species. Severe cases of yellow fever display symptoms such as high fever associated vomiting and hepatitis. There exists a live-attenuated YFV 17D vaccine which is regarded as one of the safest live vaccines available (Roukens and Visser 2008).

1.1.1.2. Tick-borne encephalitis virus

Three subtypes of TBEV are phylogenetically defined: European, Far Eastern, and Siberian (Ecker, Allison et al. 1999). TBEV is endemic in regions of Central and Eastern Europe, Siberia, Northern China, and Japan as shown in figure 1. In Central Europe, the main vector of transmission is the tick species *Ix. ricinus*, while in Eastern Eurasia it is *Ix. persulcatus*. In addition, occasional transmission to humans by drinking unpasteurized milk has been reported (Kaiser 2008). Most infections with TBEV are asymptomatic but in about one third of the cases severe neurological symptoms such as meningitis, meningoencephalitis, or meningoencephalomyelitis occur. Effective prevention against TBEV is provided by a formalin-inactivated whole virus vaccine. Vaccination campaigns

in Austria demonstrated that the incidence of disease could dramatically reduce TBEV-caused neuroinfections (Kunze and Kunze 2003).

1.1.1.3. West Nile virus

WNV is the causative agent of West Nile fever in humans, horses, and birds. WNV is predominately transmitted by the mosquito species *Culex pipiens*. The virus originated in Africa, was transported to Europe and Asia via migratory birds, and emerged world-wide in the 1990s (fig.1). Most infections of humans display mild symptoms; however in 1 of 150 cases, infected people develop meningitis or encephalitis (Petersen, Roehrig et al. 2002). An inactivated whole virus vaccine is in use in horses but chimeric live attenuated vaccine candidates are currently being tested in human clinical trials (Dauphin and Zientara 2007).

1.1.1.4. Japanese encephalitis virus

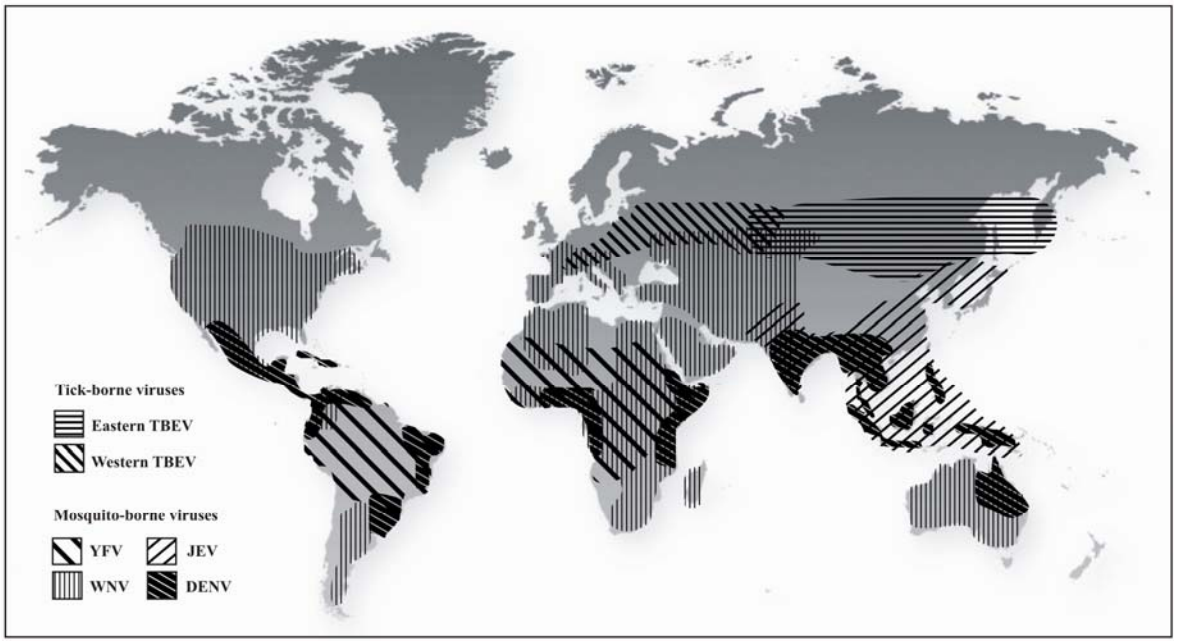
JEV causes viral encephalitis in eastern and southern Asia (fig.1). Most infections of humans are asymptomatic but in one third of the reported cases it can lead to severe illness displaying neurological symptoms (Mackenzie, Gubler et al. 2004). The natural hosts of JEV are pigs and birds from whom the virus is transmitted to the human dead-end host mainly by *Culex tritaeniorrhynchus*. For prevention of disease, a babymouse brain derived vaccine is available. Furthermore, a vaccine candidate derived from Vero cells will soon be on the market (Tauber, Kollaritsch et al. 2007).

1.1.1.5. Dengue viruses

DENVs are transmitted through *Aedes agypti* to humans which are also the virus' natural host. DENV is endemic in tropical Asia, Africa, Australia, and America and is responsible for the highest rates of disease and mortality among all members of the flaviviruses. Four serotypes of DENV are known to cause a spectrum of diseases ranging from mild fever to severe illnesses such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which is primarily caused by cross reactions due to secondary infections with a different serotype. Moreover, sequential infections by multiple serotypes can worsen a DENV infection due to an antibody-dependent enhancement. All four virus serotypes

cause similar illness, but severe and fatal hemorrhagic fever is more often associated with DENV-2 and DENV-3 serotypes. At present, no efficient vaccine against all four serotypes is available (Mackenzie, Gubler et al. 2004).

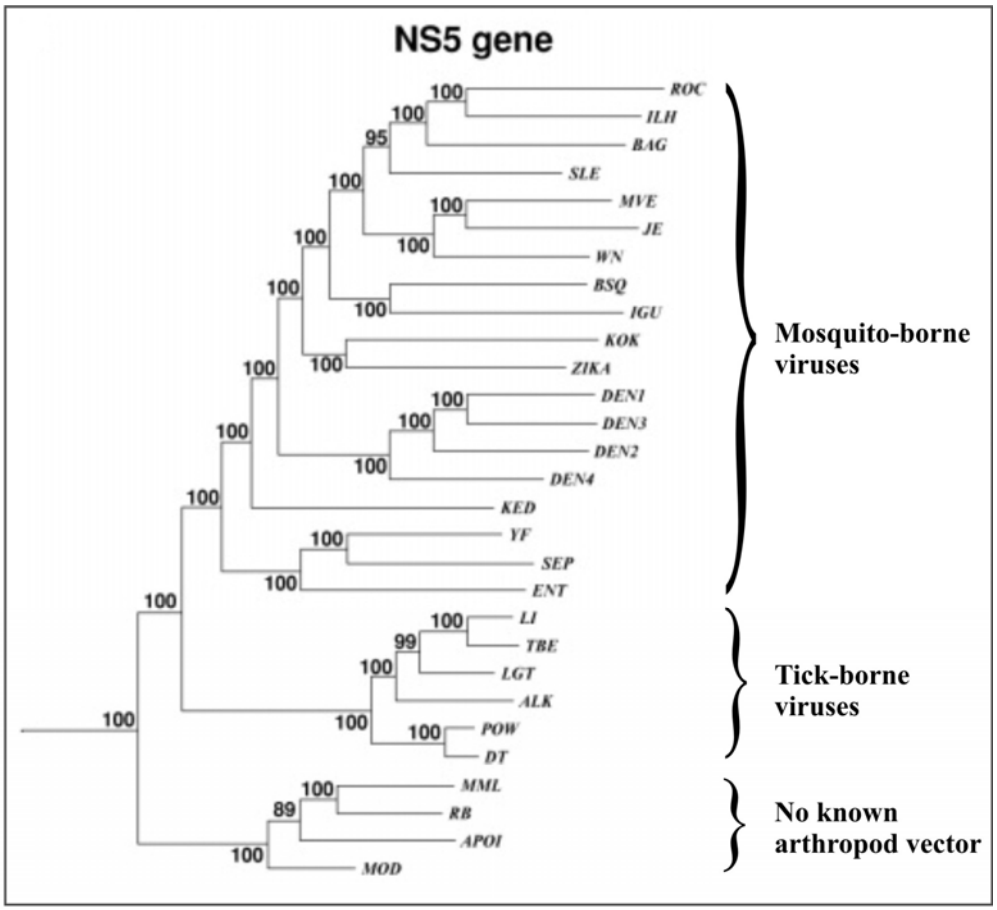
Fig.1. Approximate global distribution of neurotropic flaviviruses; the map was drawn according to references published in 2007 by the Centers for Disease Control and Prevention (CDC); from (Fields, Knipe et al. 2007).



1.1.2. Phylogeny and evolution

As stated above, flaviviruses can be divided into three distinct groups according to their ecological relationship with their known vectors as the mosquito-borne group, the tick-borne group, and viruses with no known arthropod vector. This division largely reflects the selective constraints imposed on the viruses by the vertebrate hosts, the invertebrate vectors, and the associated ecologies. Actually, phylogenetic studies based on the flavivirus genetic sequence, such as of NS5 which is a gene required for flavivirus replication, showed characteristic branching patterns that confirmed these groupings (fig.2). Therefore, the NS5 based phylogram suggested that from a putative ancestor of the genus *Flavivirus*, two major branches emerged, the non-vector and the vector-borne cluster. Further, the tick-borne and the mosquito-borne viruses emerged from the vector-borne cluster (Kuno, Chang et al. 1998).

Fig.2. Phylograms of flaviviruses based onto the NS5 gene sequence. The phylograms' branching patterns confirm the grouping of the genus into mosquito-borne viruses, tick-borne viruses, and viruses with no known arthropod vector; adapted from (Fields, Knipe et al. 2007).



Evolution of RNA viruses is based on different molecular mechanisms such as RNA recombination. Recombination among the genomes of RNA viruses is known to be a relevant factor in viral evolution and can lead to the emergence of new viral species and significantly influence the pathogenicity and fitness of viruses (Worobey and Holmes 1999; Gould, de Lamballerie et al. 2003).

RNA recombination has been demonstrated to occur frequently among the family *Flaviviridae*, particularly in members of the genus *Pestivirus*. In the case of the bovine diarrhea virus, a member of the pestiviruses, RNA recombination is continuously observed in nature and is responsible for changes in viral pathogenicity (Becher, Orlich et al. 2001).

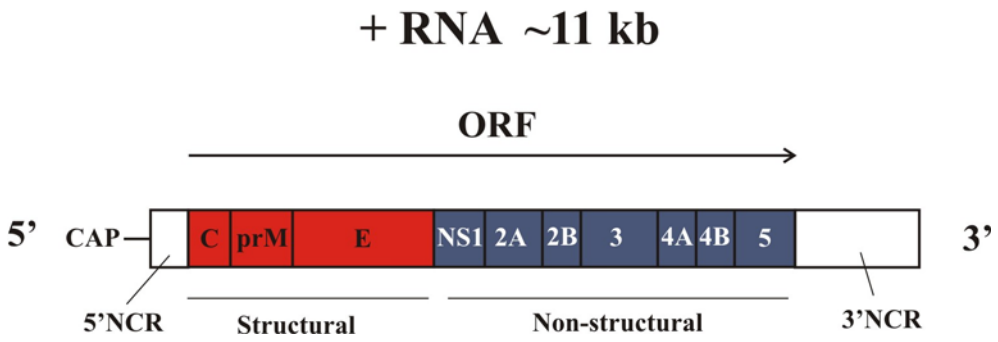
Members of the genus *Flavivirus*, in spite of their relatedness to the pestiviruses, appear to be different with respect to their propensity for RNA recombination. Actually, it is most likely that the members of the genus *Flavivirus* emerged through clonal evolution, where diversity is generated largely by the accumulation of point mutations, rather than through recombination events (Gould, de Lamballerie et al. 2003). Conversely, some sequence-based phylogenetic studies have suggested that homologous recombination has happened among strains of the same viral species. Other studies have identified recombinant flaviviruses by computational sequence analysis of viral isolates from infected patients and have suggested that homologous recombination has occurred between closely related virus strains of all four dengue virus serotypes (Holmes, Worobey et al. 1999; Tolou, Couissinier-Paris et al. 2001), JEV, and St. Louis encephalitis virus (Twiddy and Holmes 2003). However, there is no direct experimental proof of flavivirus recombination e.g. in a cell or host that is double-infected. It is not known whether recombination takes place between different species of flaviviruses nor have inter-species recombination events been fundamentally characterized yet.

1.1.3. Molecular biology of flaviviruses

1.1.3.1. Genome organization

The flavivirus genome is a single, positive-strand, ~11 kb long RNA molecule (fig.3). The 5' terminal end carries a type I CAP structure ($m^7GpppAmpN^2$) followed by a conserved dinucleotide AG. In contrast to cellular mRNA, the 3' terminus of the genome lacks a terminal poly (A) tail and ends with the dinucleotide CU which is complementary to the 5' terminal AG. Genomic RNA contains a single open reading frame (ORF) and serves as the only viral mRNA which is translated into a polyprotein containing three structural (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Fields, Knipe et al. 2007). The ORF is flanked by two non-coding regions (NCR). Depending on the virus species, the 3' NCR consists of 400 to 800 nucleotides, in contrast to the 5' NCR which is only about 100 nucleotides in length (Markoff 2003).

Fig.3. Schematic sketch of the flavivirus genome. The 11 kb long genome encodes a large polyprotein in one ORF. Structural proteins (shown in red) are encoded at the 5' terminus, while non-structural proteins (shown in blue) are located at the 3' terminus. Picture is not drawn to scale.

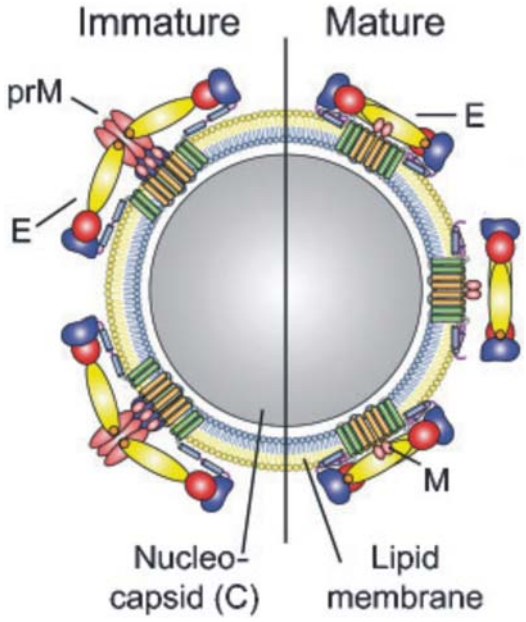


1.1.3.2. Virus particles

Flavivirus virions are small spherical, enveloped particles of about 50 nm in diameter and are composed of three structural proteins (Mukhopadhyay, Kuhn et al. 2005). The surface of viral particles is composed of the two glycoproteins, membrane (M) and envelope (E) which are embedded in a lipid bilayer. The large E glycoprotein mediates attachment and fusion. Protein M is cleaved from the precursor protein prM. This proteolytic cleavage step occurs in the trans-Golgi network of the host cell and is essential for maturation of virions (fig.4). The envelope encompasses the nucleocapsid that is composed of multiple copies of the small basic capsid protein C. Although, electron density is observed below the

membrane envelope, the nucleocapsid appears to have discernible symmetry and lacks a defined form (Fields, Knipe et al. 2007). The viral genomic RNA was shown to interact with the N- and C-terminal part of the capsid protein (Khromykh and Westaway 1996). This interaction was proposed to be similar to cellular histones (Mukhopadhyay, Kuhn et al. 2005).

Fig.4. Schematic representation of the immature and mature flavivirus particles. Nucleocapsid and glycoproteins prM, E, and the lipid membrane are indicated. Cleavage of prM causes formation of M, the release of the pr part from the virion and dimerization of E; adapted from (Stiasny and Heinz 2006).



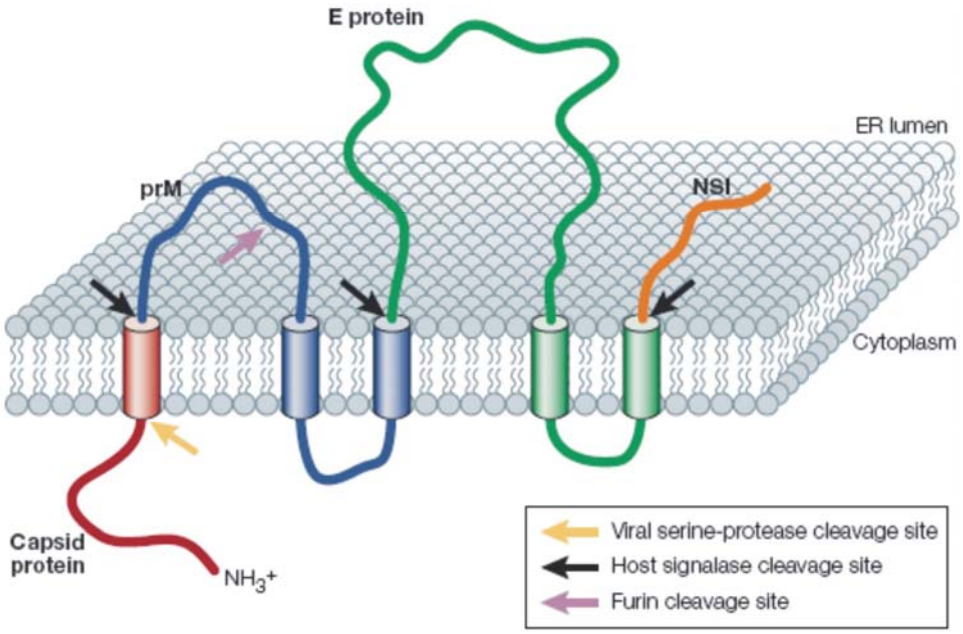
In addition to mature virions, infected cells also release noninfectious subviral particles. These particles are composed of an envelope containing the proteins M and E but lack the nucleocapsid and the RNA genome (Smith, Brandt et al. 1970). Further subviral particles can be produced by co-expressing protein prM and E in cells. Those particles are termed recombinant subviral particles (RSP), are 30 nm in diameter and therefore smaller than mature virions (Schalich, Allison et al. 1996). RSPs have similar functional features as the infectious particles and are therefore used as a model system for studying assembly and entry processes (Lorenz, Kartenbeck et al. 2003).

1.1.3.3. Viral proteins

Translation of the genomic RNA gives rise to a polyprotein of about 3400 amino acids (aa). During translation, this polyprotein is translocated and anchored in the ER membrane

by various signal sequences and membrane anchor domains (fig.5). Co- and post-translational processing by host and viral proteases yields the 10 distinct viral proteins.
The N-terminal section encodes the three structural proteins (C-prM/M-E) followed by the seven non-structural proteins (NS1-2A-2B-3-4A-4B-5) (see also fig.3). The majority of the non-structural flavivirus proteins are multifunctional and all seven proteins are directly or indirectly involved in RNA replication (Fields, Knipe et al. 2007).

Fig.5. Schematic drawing of the topology of the structural proteins in the ER membrane. Transmembrane regions are indicated as cylinders, cleavage sites are indicated by arrows; modified from (Mukhopadhyay, Kuhn et al. 2005)



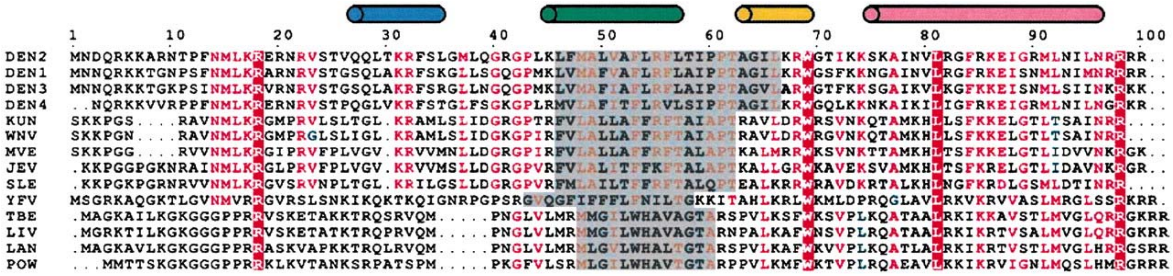
1.1.3.3.1. Capsid protein C

The capsid (C) protein has about 11 kDa molecular weight and represents the sole building block of the nucleocapsid. The C protein has basic residues at the N- and C-termini which interact with the genomic RNA. Furthermore, it was observed that C proteins form dimers that assemble into capsid-like particles when interacting with nucleic acids (Kiermayr, Kofler et al. 2004).
All flavivirus C proteins contain a conserved internal hydrophobic region (fig.6) that plays an important role in virion assembly (Kofler, Heinz et al. 2002; Ma, Jones et al. 2004). Deletion studies with TBEV C protein revealed that deletions of this hydrophobic domain up to 10 aa are tolerated, albeit with increased production of capsidless subviral particles (Kofler, Heinz et al. 2002). At the C-terminus, the mature C protein is followed by a

hydrophobic anchor that serves as an internal signal sequence initiating translocation of the prM protein into the ER lumen (fig.5).

The maturation of the capsid is a timely coordinated process between the cleavages of two proteases. First, cleavage at the C-terminus of the capsid occurs by the viral protease NS2B/3. This liberates the cleavage site of the host protease signalase resulting in the processing of prM. This coordinated process is important for flavivirus assembly because decoupling of the two cleavages resulted in increased production of capsidless subviral particles (Lobigs and Lee 2004).

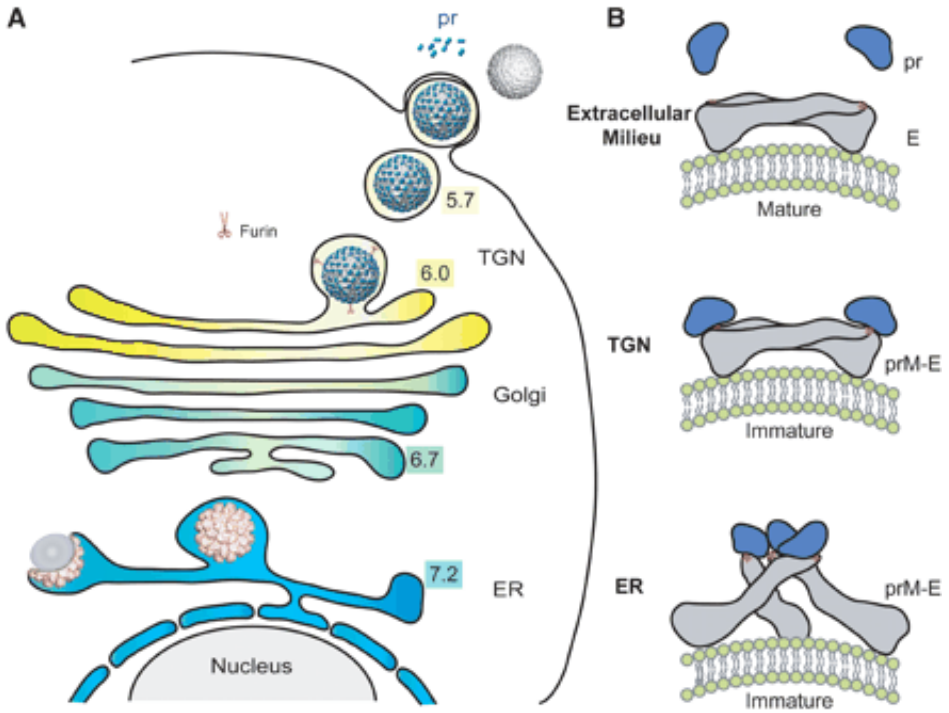
Fig.6. Multiple sequence alignment of flavivirus C proteins. Residues with high similarity (>50%) are red, and the conserved residues are highlighted. The secondary structure is indicated at the top. The conserved internal hydrophobic region of flaviviruses is shaded in gray; modified from (Ma, Jones et al. 2004).



1.1.3.3.2. Membrane glycoprotein prM/M

The 26 kDa glycoprotein prM is the precursor of the membrane protein M. In immature virions, the main function of prM is to prevent envelope protein E from intracellular fusion during transport through the host secretory pathway by shielding the fusion loop of protein E (Heinz, Stiasny et al. 1994). Protein prM folds rapidly after translation and assists protein E in proper folding (Lorenz, Kartenbeck et al. 2003). Upon translocation into the ER membrane, protein prM is anchored by two C-terminal transmembrane domains (fig.5). During the maturation process of the flavivirus, the acidification in the trans-Golgi network (TGN) induces a rearrangement of the glycoproteins, resulting in exposure of the furin cleavage site (fig.7). The prM protein is cleaved by furin in the TGN, but the proteolytic product pr stays associated with the virion to prevent membrane fusion. Upon release to the extracellular milieu, pr dissociates at neutral pH, and the result is a mature virus (Yu, Zhang et al. 2008).

Fig.7. Alterations of glycoprotein prM by furin activity during travel through the host secretory pathway. The immature particles bud into the ER and travel through the Golgi network to the TGN where acidification induces the conformational changes of the glycoproteins and results in the exposure of the furin cleavage site on protein prM. Furin cleavage results in the formation of protein M but the cleavage product pr remains associated with the virion till its release at the cell surface. There, the neutral pH causes the dissociation of the pr parts from the virion surface; the fusion loops are indicated by red stars; adapted from (Yu, Zhang et al. 2008).

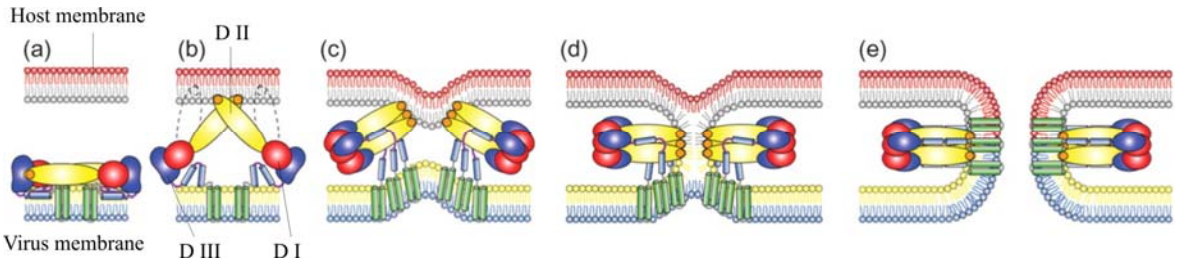


1.1.3.3.3. Envelope glycoprotein E

The envelope glycoprotein E is the largest of the three structural proteins with a molecular weight of about 53 kDa. It mediates attachment as well as membrane fusion. Protein E is arranged in parallel as dimers in head to tail orientation. Each monomer is composed of three domains: the central domain I is involved in dimerization; the immunoglobulin-like domain, domain III, is thought to contain the putative receptor-binding sites (Rey, Heinz et al. 1995), and domain II contains the highly conserved fusion loop that upon exposure to acid pH is able to integrate into target membranes.

Similar to protein M, protein E comprises two C-terminal transmembrane domains that serve to anchor the protein in the ER membrane (fig.5). Furthermore, the trans-membrane domain II functions as the signal sequence for NS1 protein translocation (Chambers, Hahn et al. 1990). The protein is cleaved twice at the N- and the C-terminus respectively. At low pH conditions, E protein dimers dissociate into monomers and finally form a trimer structure. This conformational change triggers virus and host cell membrane fusion and is described in detail in figure 8 (Stiasny and Heinz 2006).

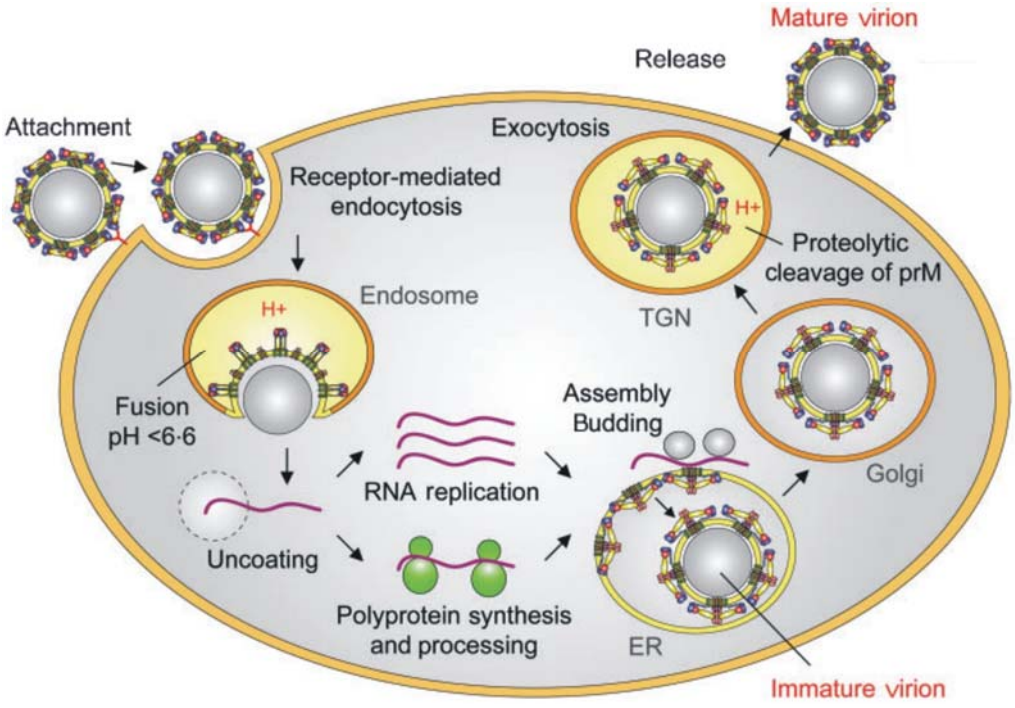
Fig.8. Scheme of the stages of the flavivirus membrane fusion triggered by conformational changes of protein E. The three domains of protein E are indicated in different colors: yellow-DII, red-DI, blue-DIII. (a) Metastable E dimer at the surface of a mature virion. (b) Low pH-induced dissociation of E dimer and interaction of E monomers with the target membrane. Dotted lines indicate flexibility at the junction between DI and DII. (c) Initiation of hairpin formation and E trimerization through the relocation of DIII and zippering of the stem along DII. (d) Hemifusion intermediate in which only the leaflets of the two membranes that face each other (outer leaflets) have mixed. (e) Generation of the final post-fusion structure (E trimer) and opening of the fusion pore; adapted from (Stiasny and Heinz 2006)**.**



1.1.4. Flavivirus life cycle

The binding of flaviviruses to the surface of the host cell is mediated via interaction of the envelope protein E with one or more cellular receptors. Only a few cellular receptors have been characterized so far, e.g. highly sulfated glycosaminoglycans such as heparan sulfate have been suggested to play an important role in the attachment of flaviviruses to the cell surface (Chen, Maguire et al. 1997). Heparan sulfates are highly conserved molecules and it is likely that they play a role during attachment and entry of flaviviruses but the exact mechanism mediated by heparin sulfate is still elusive (Kroschewski, Allison et al. 2003). After attachment, the virion is internalized via receptor-mediated endocytosis followed by transport in clathrin-coated pits to a pre-lysosomal endocytotic compartment (fig.9) (Chu and Ng 2004). There, the fusion of the viral and the endosomal membrane is catalyzed by low pH in the endosome and results in the release of the nucleocapsid into the cytoplasm. After release, the nucleocapsid disassembles and translation and replication starts in association with virally induced cellular membranous structures at the perinuclear region. Genomic RNA is directly translated into a polyprotein precursor that undergoes co- and post-translational processing by viral and host proteases. Subsequently, RNA replication initiates where full-length negative strand copies of the genome are synthesized. Those serve as templates for synthesis of plus-strand RNA.

Fig.9. The flavivirus life cycle. For details see text; modified after (Stiasny and Heinz 2006).



The membrane protein prM and the envelope protein E are retained in the ER via sequences in their C-terminal transmembrane anchors (Op De Beeck, Rouille et al. 2004) and the N-termini are released by host protease cleavage. Packaging of the nucleocapsid containing the viral RNA genomes is most likely ensured by the concerted cleavages of first the capsid protein and then prM (see above), because no accumulation of preformed capsids can be observed and prM and E alone are the driving force for intra-cellular budding in the ER. The immature virions travel through the host secretory pathway to late TGN where proteolytic cleavage of prM occurs by the host protease furin (Stadler, Allison et al. 1997). Finally, the mature virions are released from the cell by fusion of the transport membrane with the plasma membrane.

1.1.5. Flavivirus virion assembly

Flavivirus virions assemble intracellularly by budding into the ER as electron microscopy of infected cells has shown (Lorenz, Kartenbeck et al. 2003). Translocation of the structural proteins in the ER membrane and the assembly of virions are intimately linked mechanisms. During translation of the polyprotein, the structural proteins are translocated in the ER membrane by signal sequences and anchor domains. After appropriate proteolytic cleavage, the C protein and the viral RNA genome are localized in the cytoplasm where protein C interacts with the RNA molecule via its basic residues located at the N- and C-termini (Kiermayr, Kofler et al. 2004). Protein C remains associated with the ER membrane and the precursor of the nucleocapsid (NC) forms from multiple copies of protein C. The exact mechanism of the formation of the NC is not known but biochemical studies suggest NC formation starts with a C protein dimer building block (Kiermayr, Kofler et al. 2004).

On the luminal side of the ER, the prM and E protein form a stable heterodimer. Both proteins are retained in the ER membrane by their C-terminal transmembrane regions. The prM and E containing envelope around the NC is formed by budding of NC into the ER lumen (fig.9). Although NC can be assembled *in vitro* (Kiermayr, Kofler et al. 2004), they are rarely found in infected cells, indicating that virion formation is a coordinated process between the membrane-associated C protein and the prM–E complex on the ER luminal side. Subviral particles are frequently found during flavivirus infection. These capsidless particles consist of structural proteins M and E and the lipid bilayer. Due to the presentation of the major flavivirus antigen, protein E, in a particular form, subviral particles display immunogenic features similar to virions. Recombinant subviral particles (RSPs), containing only M, E proteins, and lipid, can be produced by co-expression of prM and E protein alone. This indicates that virion assembly is driven solely by the action of prM and E.

Studies employing RSPs as a model system observed accumulation of RSPs in the rough and smooth ER, and in downstream compartments of the secretory pathway. Furthermore, experiments with membrane anchor-free E protein suggest that formation of the prM-E complex is the rate limiting step for RSP and native virion assembly (Lorenz, Kartenbeck et al. 2003).

1.1.6. Replicon-based experimental systems

1.1.6.1. Replicons

Replicons are defined as self-replicating, non-infectious RNAs, which are generated by deleting parts or all of the coding regions of the structural proteins C, prM, and E. Thereby, all the non-structural proteins of the viral genome including the flanking non-coding sequences are maintained because they are required *in cis* for RNA replication (Kofler, Aberle et al. 2004). Replicons, upon artificial introduction into the host cell via e.g. electroporation, are capable of autonomous self-replication, but due to the deletions in their structural proteins cannot form infectious mature virions. In former studies of our group replicons have been used to study a variety of different aspects of the flavivirus biology including packaging (Gehrke, Ecker et al. 2003)

1.1.6.2. Reciprocal trans-complementation system

In a recent study of our group, a novel reciprocal trans-complementation system was established which consisted of two replicons replicating independently in the same host cell. Both replicons had the ability to replicate independently their genomes but neither could form infectious progeny by itself due to the lack of at least parts of one structural protein. A combination of replicons which contained deletions of different structural proteins, for example one lacking the sequence coding for the capsid protein and the others lacking the sequence coding for the proteins prM and E, were shown to be able to complement each other. Infectious virions (SIPs) that contained replicons' RNA were produced from co-transfected cells at a multiplicity high enough to allow co-infection and further rounds of passages were possible. Passages at limiting dilutions were used to provide optimal conditions for the selection of possible recombinant full-length viruses that could be generated by recombination between the two complementing replicons. Indeed, recombination was demonstrated between replicons of Japanese encephalitis virus during passages in BHK-21 cells whereas no recombination was detected with replicons of TBEV.

1.1.6.3. Trans-packaging of replicons into SIPs

On the other hand, most experimental systems that have been developed to study packaging of flavivirus RNAs employ a single replicon, which is packaged into single round infectious particles by providing some or all of the structural proteins *in trans* by various heterologous expression systems. Upon transfection of one replicon genome into suitable cells, the structural proteins, which are essential for packaging, are provided by a second source, only the single replicon genome is packaged into the virus particles. The resulting single round infectious particles (SIPs) are capable of establishing one round of infection but do not produce progeny virions as they lack some or all structural genes essential for virion formation. Thus, unlike the reciprocal trans-complementation system, infection with preparations containing one SIP species cannot lead to a second round of production of infectious particles because only a single defective genome is present in the particles.

However, the trans-packaging of replicons into SIPs holds two main advantages over the reciprocal trans-complementation system. First, once SIPs containing different replicon genomes are packaged into a suitable cell line at high efficiency, co-infection experiments with two SIP species can be performed. Thereby, events of trans-complementation or recombination among the replicons' genomes can be studied in various different cell types independently. Secondly, the introduction of the viral genomes into the host cells by infection with SIPs resembles the natural way of a wild-type virus infection better than the artificial introduction via transfection of the cells by electroporation. Furthermore, transfection of many cell types is rather difficult or has not been established so far.

Different studies have established various systems to generate flaviviral SIPs by expressing missing structural proteins *in trans*. These systems include expression plasmids or cell lines that have been stably transfected to continuously express flavivirus structural proteins (Konishi, Fujii et al. 2001; Gehrke, Ecker et al. 2003; Fayzulin, Scholle et al. 2006). Additionally, positive, single-stranded RNA viruses have been successfully employed as vectors for heterologous gene expression. In various studies, viral vectors have been developed from the non-infectious replicons of picornaviruses, flaviviruses, and alphaviruses (Hewson 2000). Among those, the most advanced replicon gene expression systems are derived from the members of the genus *Alphavirus*.

1.2. Family *Togaviridae*, genus *Alphavirus*

The family *Togaviridae* is defined into the two different genera *Alphavirus* and *Rubivirius*. All togaviruses are enveloped, positive, single-stranded RNA viruses with icosahedral symmetry (Fields, Knipe et al. 2007). Unlike the genus *Rubivirus* which comprises only a single member, the Rubella virus, the genus *Alphavirus* consists of 28 members that can be further antigenically classified into at least seven serocomplexes (Powers, Brault et al. 2001). Alphaviruses are mainly transmitted by arthropods and circulate between invertebrate insect vectors and vertebrate reservoir hosts which are generally mammals or birds. Additionally, fish have also been reported as hosts (Weston, Villoing et al. 2002). Alphaviruses cause a plethora of human and animal diseases, involving encephalitis, arthritis, fever, and rash (table 2).

Table 2. Human pathogenic alphaviruses.

Virus	Vertebrate reservoir host	Human disease	Animal disease
Antigenic complex BF			
Barmah Forest virus	Birds	Fever, arthritis, rash	
Antigenic complex SF			
Chikungunya virus	Primates	Fever, arthritis, rash	
Getah virus	Mammals	Fever	Horse
Mayaro virus	Mammals	Fever, arthritis, rash	
O'nyong-nyony virus	?	Fever, arthritis, rash	
Ross River virus	Mammals	Fever, arthritis, rash	
Semliki Forest virus	?	Fever, encephalitis	Horse
Antigenic complex EEE			
Eastern equine encephalitis virus	Birds	Fever, encephalitis	Horse, pheasant, emu, pigeon, turkey
Antigenic complex VEE			
Everglades virus	Mammals	Fever, encephalitis	
Venezuelan equine encephalitis virus	Mammals	Fever, encephalitis	Horse
Antigenic complex WEE			
Sindbis virus	Birds	Fever, arthritis, rash	
Western equine encephalitis virus	Birds, mammals	Fever, encephalitis	Horse, emu

1.2.1. Disease, distribution, and transmission

Similar to flavivirus infection, the virus is introduced into the host via arthropods bites. Most often, the insect vector is one of the mosquito species. First, the local muscle cells or Langerhans cells in the skin are infected. The Langerhans cells carry the virus to the lymph nodes and it spreads from there to the CNS, skin, and joints leading to encephalitis, rash, arthritis, and fever.

Alphaviruses are widely distributed throughout the world. According to distribution and to theories explaining the origin and emergence of viruses by migratory birds, alphaviruses have classically been described as Old World and New World viruses (Weaver, Hagenbaugh et al. 1993). As it is likely that several transoceanic exchanges have occurred (Powers, Brault et al. 2001), here we classify alphaviruses according to pathogenesis rather than to distribution.

Alphaviruses that are primarily associated with encephalitis are Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV). EEEV causes localized outbreaks of equine, pheasant, and human encephalitis in the summer. With a mortality rate of 30 to 40% in humans (Fields, Knipe et al. 2007), EEEV is the most lethal virus among the encephalitis associated alphaviruses. The virus is found in North America along the Atlantic seaboard, in Central America, and along the north and east coast of South America. WEEV has caused epidemics of encephalitis in humans, horses, and emus and is widely distributed in the western plains and valleys of the United States, Canada, and in South America. The case fatality rate of WEEV is 10% in humans. VEEV causes equine and human encephalitis, displaying a low mortality rate of less than 1% in humans. Infections occur in subtropical and tropical areas of America. For prevention, formalin-inactivated vaccines against EEEV, WEEV, and VEEV are available for horses and against EEEV for emus. A live attenuated vaccine against VEEV for humans has been developed but displayed substantial side effects after immunization (Pittman, Makuch et al. 1996).

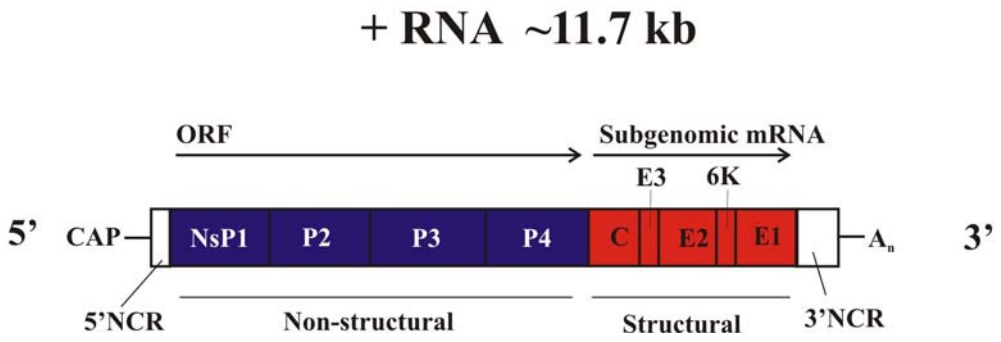
Syndromes of polyarthritis, rash, and fever are mainly associated with the Chikungunya virus (CHIKV), the O'nyong-nyong virus (ONNV), the Ross River virus (RRV), Sindbis virus (SINV), the Barmah Forest virus (BFV), and the Semliki Forest virus (SFV). Those viruses are distributed across Asia, Europe, Africa, and Australia. A live attenuated vaccine against CHIKV is available for humans (Levitt, Ramsburg et al. 1986).

1.2.2. Molecular biology of alphaviruses

1.2.2.1. Genome organization

The togavirus genome consists of a single, positive-strand, ~11.7 kb long RNA molecule that contains a 5' terminal 7-methylguanosine and a 3' terminal poly (A) tail (fig.10). At the 5' end, two-thirds of the genome encode the non-structural proteins (nsP1, nsP2, nsP3, nsP4) while at the 3' term, one-third codes for the structural proteins (C, E3, E2, 6K, E1). The coding region is flanked by non-coding regions (NCR) that are about 60 nucleotides long at the 5' terminus and approximately 320 nucleotides long at the 3' terminal end of the RNA genome. During virus replication, non-structural proteins are directly translated from the genomic RNA, while structural proteins are translated from a subgenomic mRNA. Template for the production of subgenomic mRNA and the whole-length plus-strand RNA genome is a minus-strand RNA intermediate (Fields, Knipe et al. 2007).

Fig.10. Schematic drawing of the alphavirus genome. The 11.7 kb long RNA genome is segregated into two segments with the replication region (shown in blue) at the 5' end and the structural region at the 3' terminus (shown in red). Picture is not drawn to scale.

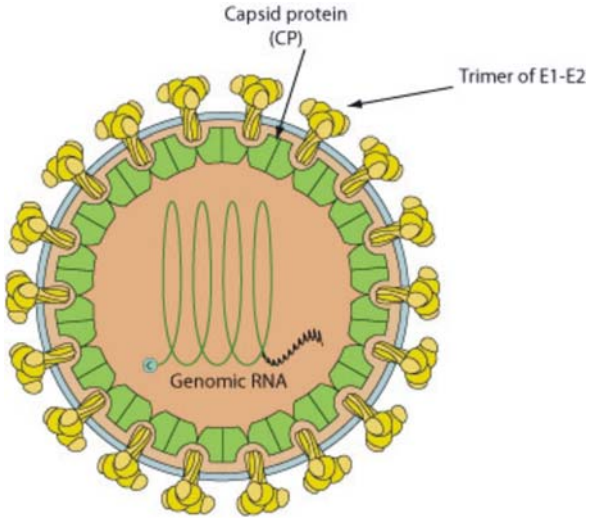


The alphavirus genome comprises 5' terminal and 3' terminal conserved sequence elements (CSE) which display *cis*-acting activity on viral replication and translation and are thought to recruit host factors during viral replication (Pardigon and Strauss 1992; Frolov, Hardy et al. 2001). Furthermore, a subgenomic promoter, preceding the start of mRNA synthesis, controls the production of subgenomic mRNA and is also thought to bind host cellular factors (Wielgosz, Raju et al. 2001).

1.2.2.2. Virion structure

Most knowledge about the alphavirus virion structure comes from studies on SINV and SFV. Alphavirus virions are small, spherical, enveloped particles of about 70 nm in diameter and are composed of four structural proteins: the capsid protein C, the transmembrane glycoproteins E1 and E2, and the small membrane-associated protein 6K. Transmembrane glycoproteins E1 and E2 form spikes that consist of three E1-E2 heterodimers (fig.11). Spikes are anchored in a host-derived lipid bilayer that is enriched in cholesterol and sphingolipid. Furthermore, the small membrane-associated protein, 6K, is also found in the viral particles (Gaedigk-Nitschko and Schlesinger 1990; Lusa, Garoff et al. 1991). The capsid proteins build up the icosahedral nucleocapsid containing the genomic RNA which is finally enclosed by the lipid layer with the inserted transmembrane proteins (Fields, Knipe et al. 2007).

Fig.11. Schematic drawing of the alphavirus virion. Capsid proteins (green) and the three E1-E2 heterodimers (yellow) which form one spike are indicated. Within the nucleocapsid, the genomic RNA is sketched; modified from www.expasy.ch



1.2.2.3. Viral proteins

In the initial step of translation, the 5' two-thirds of the genomic RNA are directly translated to produce a polyprotein of about 2500 amino acids. The polyprotein encodes the four non-structural proteins (nsP1, nsP2, nsP3, nsP4) that are required for RNA replication and transcription and is processed by nsP2 protease activity.

The five structural proteins (C, E3, E2, 6K, E1) are translated from a subgenomic mRNA which is transcribed from a minus-strand RNA intermediate. The translated polyprotein is

about 1240 amino acids long and co- and post-translationally processed by host and viral proteases. Structural proteins comprise the virion components and are dispensable for viral RNA replication in the alphavirus life cycle (Fields, Knipe et al. 2007).

1.2.2.3.1. Non-structural proteins

The non-structural proteins are multifunctional and essential for alphavirus RNA replication. NsP1 has been shown to have methyl- and guanylyltransferase activity therefore it is necessary to cap the RNA genome. Additionally, nsP1 is required for the initiation of minus-strand RNA synthesis (Hahn, Grakoui et al. 1989; Laakkonen, Hyvonen et al. 1994).

NsP2 functions as an RNA helicase, a nucleoside triphosphatase, an RNA triphosphatase, and a protease responsible for processing the non-structural polyprotein (Rikkonen, Peranen et al. 1994; Gomez de Cedron, Ehsani et al. 1999; Vasiljeva, Valmu et al. 2001).

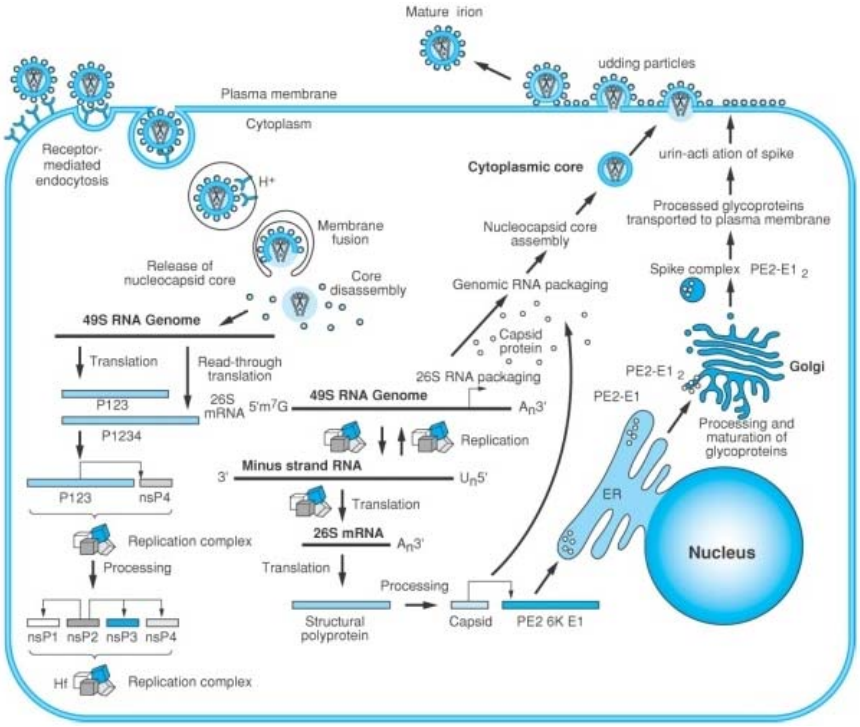
The function of nsP3 is still uncertain but studies indicate an importance in minus-strand RNA synthesis (LaStarza, Lemm et al. 1994).

NsP4 encodes the RNA-dependant RNA polymerase (RdRP) necessary for RNA replication of the viral genome (Hahn, Grakoui et al. 1989).

1.2.3. Alphavirus life cycle

Attachment of the alphavirus to the host cell is mediated by surface glycoprotein E2. A variety of different molecules such as heparan sulfate, laminin receptor, and C-type lectine have been proposed to act as putative receptors for virus binding (Ludwig, Kondig et al. 1996; Klimstra, Nangle et al. 2003; La Linn, Eble et al. 2005). To display such a wide host range, it is assumed that the virus uses a ubiquitous highly conserved receptor or the surface protein E2 contains multiple receptor-binding sites. After entry, the virions proceed via clathrin-dependant pathway to the fusion of cellular and viral membrane catalyzed by acidification of the vesicles (fig.12). Alphavirus fusion occurs through destabilization of the E1-E2 heterodimer followed by insertion of the distal tip of fusion protein E1 in the target membrane. The E1 proteins align and form timers which finally cause pore formation and the release of the nucleocapsid into the cytoplasm (Kielian and Rey 2006).

Figure 12. Alphavirus life cycle; for details see text; adapted from (Fields, Knipe et al. 2007).



Once the nucleocapsid is disassembled, the replication proteins are directly translated from the genomic RNA in a polyprotein. After proteolytic cleavage mediated by viral protease activity, the distinct proteins form a replication complex. Viral replication occurs in the cytoplasm through a minus-strand RNA intermediate that also serves as template for the production of subgenomic mRNA. The polyprotein translated from the subgenomic mRNA

encodes the structural proteins and is processed by cellular and viral protease activity (Strauss and Strauss 1994).

Capsid protein C frees itself from the polyprotein through autocatalytic protease activity. Following autoproteolysis, protein C assembles into a core particle that encapsides a single molecule of genomic RNA. Meanwhile, the nucleocapsid is formed in the cytoplasm, the polyprotein encoding pE2, K6, and E1 is translocated into the ER membrane where it is processed and undergoes post-translational modifications. pE2 and E1 form the heterodimer spike in the ER which is transported through the Golgi network. Prior to arrival at the plasma membrane, pE2 is cleaved by furin releasing E3 and the mature E1-E2 spike forming complex (Fields, Knipe et al. 2007).

In the final stage, nucleocapsid cores and glycoprotein spikes interact to promote virus budding at the cytoplasmic membrane.

1.2.4. Alphavirus-based experimental systems

1.2.4.1. Defective interfering genomes

Defective interfering (DI) genomes replicate and are packaged in the presence of helper virus, and retain all *cis*-acting sequences necessary for RNA replication. Several alphaviral defective genomes have been molecularly characterized showing that the study of DI genomes provided a powerful genetic tool to identify the location and function of required *cis*-acting sequence elements (Levis, Weiss et al. 1986). Investigating DI genomes paved the way for the development of replicons which are capable of RNA replication but cannot infect new cells for they lack the region coding for the structural genes (Schlesinger 2000). However, replicons can be packaged by supplying structural proteins with additional helper RNAs, resulting in infectious viral particles (Bredenbeek, Frolov et al. 1993).

1.2.4.2. Replicon systems

Alphavirus replicons have become a standard gene expression model. The advantages of alphavirus-based expression systems include a broad range of susceptible host cells such as insect, avian and mammalian cells. Furthermore, high levels of heterologous proteins can be expressed by engineering infectious recombinant RNAs that express additional subgenomic RNAs. Another way employs the replacement of the structural genes to produce replicons that can be packaged into infectious particles using defective helper RNAs or packaging cell lines (Frolov, Hoffman et al. 1996).

Replicon systems derived from SINV, SFV, and VEEV have been established so far (Liljestrom and Garoff 1991; Pushko, Parker et al. 1997; Schlesinger 2000). In a wild type virus genome, efficient subgenomic promoters drive the expression of the alphavirus structural proteins. Replacement of the structural genes with heterologous genes yields a non-infectious alphavirus expression vector. Alphavirus expression systems can be employed in basic research to study mechanisms in virology such as replication, packaging, fusion, and recombination. Further, applications in vaccine development or production of diagnostic assays for other viruses are of increasing interest.

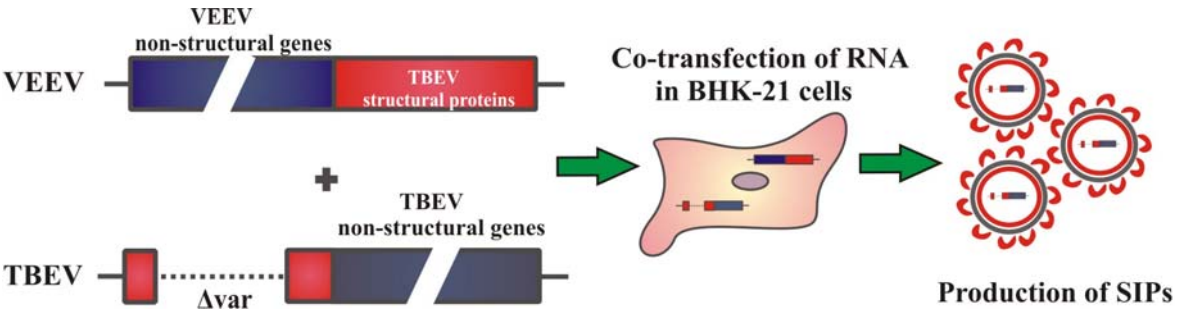
The genomes of alphaviruses provide versatile genetic tools as the alphaviral structural genes can be replaced by heterologous genes resulting in high level expression of heterologous proteins. Through recombinant DNA techniques, alphavirus-based vectors

expressing the flavivirus structural genes can be generated. Upon co-transfection of RNA derived from such an expression vector together with flavivirus replicon RNA in a suitable cell line, SIPs can be produced. The missing structural proteins that are required for virion formation are provided *in trans* by the alphavirus recombinant construct yielding packaged flavivirus replicons. Upon transfection with alphavirus-based vectors, mammalian cells often display shut-down of the host cell protein translation, and cytopathic effects (CPE), finally resulting in cell death within a few days caused by the continuous viral replication. This problem could be solved by introducing a mutation in nsP2 of SINV replicon (Frolov, Agapov et al. 1999). For VEEV replicon system, mutations in the 5' NCR and nsP3 resulted in non-cytopathic replication in mammalian cells (Petrakova, Volkova et al. 2005).

2. Scientific aim

The first goal of the thesis was to establish an efficient trans-packaging system that would allow packaging of TBEV replicons. To achieve this, the structural proteins C, prM, and E should be expressed *in trans* by a non-flaviviral source. First, an alphavirus replicon should be generated to express the flavivirus structural proteins, then co-transfection of BHK-21 cells with this construct and flavivirus replicons should lead to packaging of this otherwise packaging deficient virus genomes (fig.13).

Fig.13. Scheme of the replicon packaging system. The VEEV non-cytotoxic vector encoded VEEV replicative proteins as well as all three structural proteins of TBEV. Different TBEV replicons with variable deletions in CprME were encoded by a second RNA. After *in vitro* transcription, RNA of VEEV and of the TBEV replicons is co-transfected into BHK-21 cells. TBEV replicons are *trans*-packaged by the structural proteins provided by VEEV yielding the production of SIPs.



Furthermore, packaging should be achieved by co-transfection in other cell types or by providing the structural proteins from a different vector such as a plasmid expression vector.

Virions that are produced by providing the structural proteins *in trans* contain a replicon which lacks one or more proteins that are essential for the production of infectious virus particles. This means that after infection no further viral spread occurs, accordingly such virions are designated single round infections particles (SIPs). However, earlier studies have shown that two replicons with reciprocal deletions of different structural proteins can also produce infectious virus particles. In contrast to the production of single-round infectious particles, this reciprocal trans-complementation between two flavivirus replicons was shown to work for several passages if the multiplicity of infection was high enough to allow co-infection of the same cell with virus particles containing different replicons. In previous studies, such passages were used to select possible recombinant full-length viruses between the complementing replicons. However, this approach requires artificial transfection of the replicons prior to passages which complicates recombination studies

due to the lack of efficient transfection methods for many cell types. Therefore, the second goal of this thesis was efficient packaging of replicons lacking different regions of the structural proteins. Then a mixture of different SIPs at high titers should be used in co-infection experiments in different cell types to test if complementation could be observed and passages could be performed. This should provide an important tool for many studies including the determination of recombination frequency of flaviviruses in different cell types.

3. Materials and methods

3.1. Manipulation of nucleic acids

3.1.1. Plasmids and bacteria

Expression plasmids employed in this study were derivates of pBR322 (Invitrogen) or pUC (Stratagene) and contained an Amp^r gene for selection. All plasmids encoded structural proteins derived from the genome of western subtype strain Neudoerfl (GenBank accession number U27495). Plasmid pTNd/5' contained cDNA corresponding to the 5' one-third of the genome (Mandl, Ecker et al. 1997) and was utilized for amplification and expression of structural proteins C, prM, and E in two different vectors.

Plasmid pVEEV (courtesy of Ilya Frolov, University of Texas) consisted of a pBR322 backbone encoding a SP6 promoter followed by nt 1 to 7481 of the VEEV TC-83 genome and a 1074-nt-long sequence of VEEV TC-83 including the 3' NCR followed by a poly(A) sequence and a unique *Mlu*I restriction site (Petrakova, Volkova et al. 2005). Between the two VEEV genomic regions, a Neudoerfl-derived sequence of CprME plus signaling sequence was introduced (fig.14A). The origin of the second expression plasmid pSV40-CrpME was the purchased vector phRL-SV40 from Promega. The sequence coding for the Renilla Luciferase protein was replaced by CprME plus NS1 signaling sequence (fig.14B). Further, the plasmid comprised a T7 promoter and a 420-nt-long SV40 enhancer and promoter sequence.

For subcloning followed by side-directed mutagenesis of CprME, vector pSG5 from Stratagene was employed (fig.15).

The pBR322 derived replicon plasmids ΔC, ΔME, ΔE also contained cDNA corresponding to the genome of western Subtype TBE virus strain Neudoerfl. The replicon plasmids encoded all non-structural proteins and parts of the structural proteins (fig.16). pTBEV-ΔC, a derivative of pTNd/c containing a large deletion (62 amino acids) of the sequence coding for the capsid protein as well as individual mutations in the signal sequence of the capsid protein, was used as the DNA template for *in vitro* synthesis of replicon ΔC. The production of replicon ΔME lacking the complete sequence coding for structural protein E and prM was previously described (Gehrke, Ecker et al. 2003).

Plasmid pTBEV-ΔE, the template for generating TBEV replicon ΔE, was constructed by first performing PCR using the primers 5'-ttttaccggtttacgctgatgttggttgcgctgtgga-3' and 5'-ttccatcgatagtgtgactagcaggccatgagca-3' with pTNd/5' as a template and then cloning this PCR product into pTNd/5' using the restriction enzymes *Age*I and *Cla*I.

All plasmids were amplified in *Escherichia coli* strain HB101.

Fig.14. Vector maps of pVEEV and pSV40 expressing CprME. Two plasmids were generated to provide the TBEV structural proteins *in trans*. (A) pVEEV consisted of a pBR322 backbone encoding a SP6 promoter followed by nt 1 to 7481 of the VEE TC-83 genome, proteins CprME, NS1 signaling sequence, and a 1074-nt-long sequence of VEE TC-83 including the 3' NCR followed by a poly(A) and a *Mlu*I restriction site. (B) pSV40-CprME derived from the purchased vector phRL-SV40 (Promega©) and comprised a T7 promoter followed by CprME plus NS1 signaling sequence and a 420-nt-long SV40 enhancer and promoter sequence. TBEV structural proteins are shown in red, VEEV derived sequences in dark blue, NS1 in light blue, vector backbone structures in black.

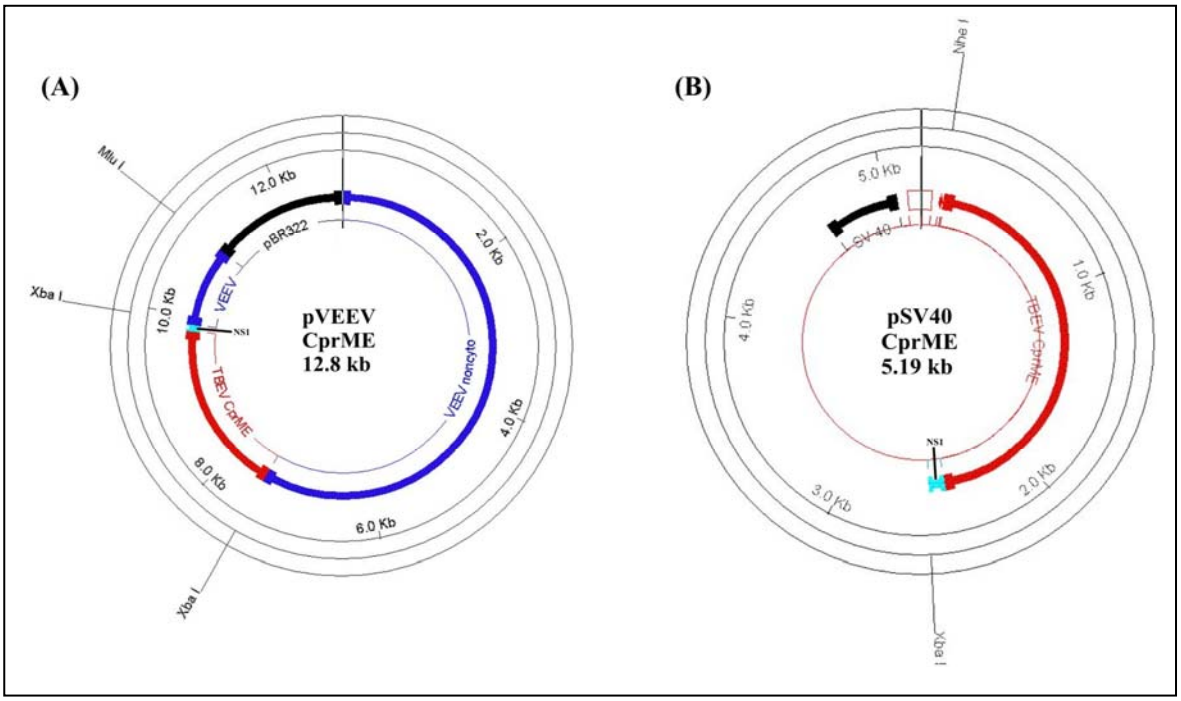


Fig.15. Vector map of pSG5 (Stratagene©). pSG5 was employed for subcloning of CprME followed by side-directed mutagenesis (adapted from www.stratagene.com).

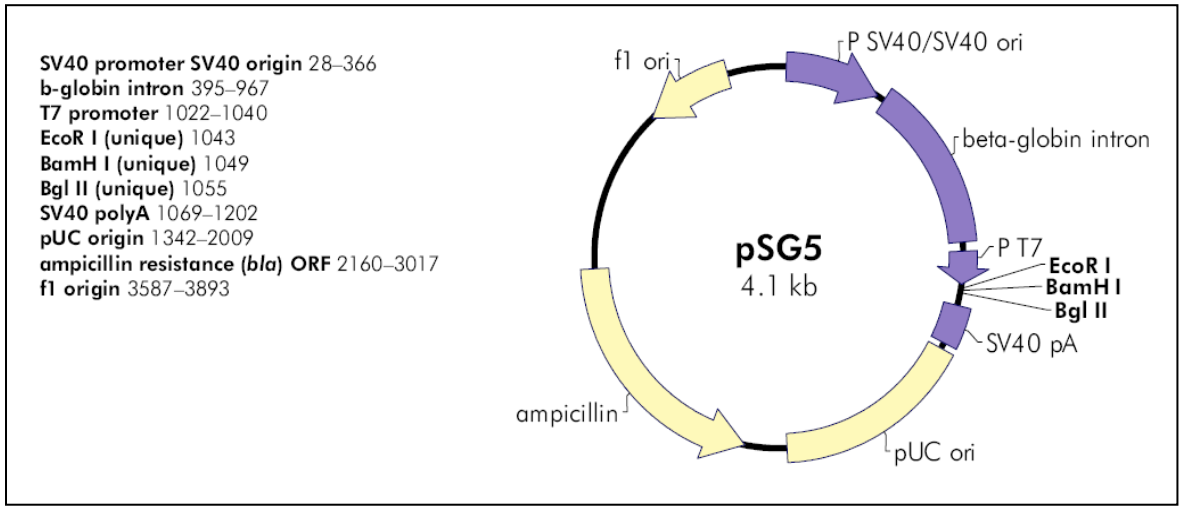
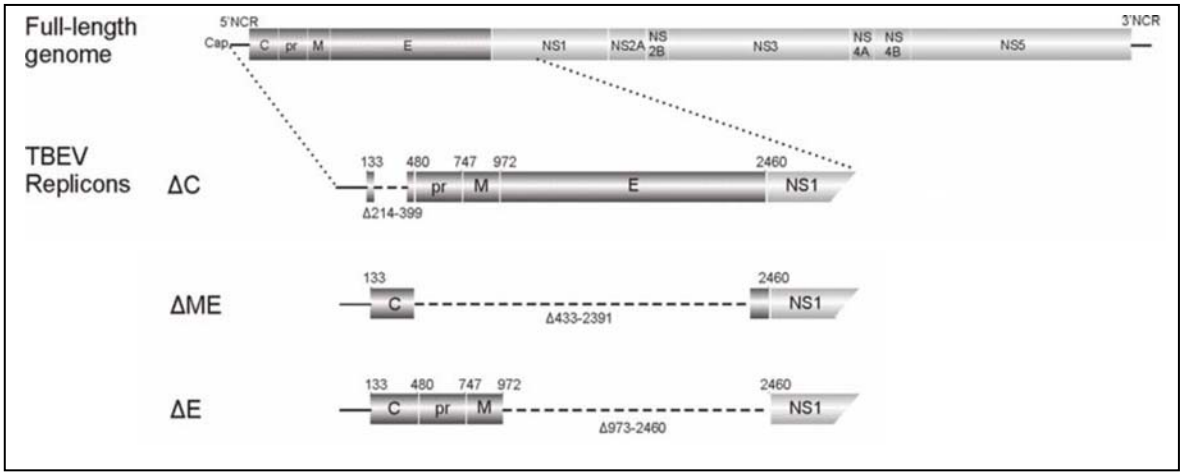


Fig.16. TBEV replicons ΔC, ΔME, and ΔE. pTBEV-ΔC contained a deletion of 62 amino acids in the C protein; pTBEV-ΔME had the complete prM protein and 1419 aa of the protein E deleted; pTBEV-ΔE lacked the whole protein E sequence.



3.1.2. Polymerase chain reaction (PCR)

For cloning of pVEEV, the insert CprME plus NS1 signaling sequence was amplified in a two-step PCR using Clontech Advantage™ HF2 PCR System. Primers comprised an *Xba*I restriction site and matching sequences of the pVEEV vector and the structural proteins and were purchased from Sigma (table 3). 35 µl H_2O_{dd}, 1 µl sense primer (10 pmol), 1 µl antisense primer (10 pmol), 5 µl 10x Advantage PCR Buffer, 5 µl 10x HF2 dNTP Mix and 1 µl 50x Advantage HF2 Pol Mix were added to 2 µl pTNd/5' template (undiluted or diluted in H_2O_{dd}). Reactions were carried out in a Biometra thermal cycler with the following settings:

1’ 94°C → 3x [15’’ 94°C → 1’ 40°C → 5’ 68°C] → 20x [45’’ 94°C → 5’ 68°C] → 4°C.

To create the pSV40-CprME, the insert CprME plus NS1 signaling sequence was amplified in a PCR using Clontech Advantage™ HF2 PCR System. Sense and antisense primers containing *Nhe*I or *Xba*I restriction sites respectively were obtained from VWR-Biotech (table 3). 70 µl H_2O_{dd}, 2 µl sense primer (5 pmol), 2 µl antisense primer (5 pmol), 10 µl 10x Advantage PCR Buffer, 10 µl 10x HF2 dNTP Mix and 2 µl 50x Advantage HF2 Pol Mix were added to 4 µl pTNd/5’ template (undiluted or diluted in H_2O_{dd}). Reactions were carried out in a Perkin Elmer thermal cycler with the following settings:
15’’ 94°C → 35x [15’’ 94°C → 1’ 55°C → 4’ 68°C] → 4°C.

Table.3. Primers used for PCR reactions. Restriction sites are highlighted in red.

Expression Plasmids	Primer sequence	Primer orientation
pVEEV-CprME	AGTCTAGAGCTTGCCGCCACCATGGTCAAGAAGGCCATCCT	sense
	GCTCTAGACTTGGCGGACTAGACTATGTCGTAGTCCATTCAG	antisense
	GTTACGCCCCCACTCCAAGGGTCA	
pSV40-CprME	ATAGGCTAGCCATGGTCAAGAAGGCCA	sense
	GCTCTAGAATTAGTCATACCATTCTGAGAC	antisense

3.1.3. Purification of PCR products

PCR products that were required for further cloning steps, were purified using the Wizard® SV Gel and PCR Clean-Up System from Promega.
An equal volume of membrane binding solution was added to the PCR reaction, mixed, and applied to a provided SV minicolumn. The incubation of 1’ at room temperature was followed by centrifugation in a tabletop microcentrifuge at 16,000 x g for 1’. The flow-through was discarded and column-bound DNA was washed once with 700 µl membrane wash solution (16,000 x g for 1’) and a second time with 500 µl membrane wash solution (16,000 x g for 5’). An additional centrifugation at 16,000 g for 1’ allowed all residual ethanol to evaporate. DNA was eluted by adding 50 µl nuclease free H_2O_{dd} (Ambion) directly onto the membrane followed by incubation at room temperature for 1’ and by centrifugation at 16,000 x g for 1’. Eluted DNA was stored at -20°C.
Purification was performed prior to digestion with restriction enzymes.

3.1.4. DNA digestion and restriction enzymes

Digestion of DNA was performed with restriction enzymes purchased from New England Biolabs or Fermentas according to the protocols of the manufacturer. Different amounts of DNA were digested in an appropriate volume of buffer and enzyme for 60 to 90 minutes at 37°C. *Nhe*I and *Xba*I were from Fermentas; *Mlu*I from NEB; Klenow fragment from NEB.
In the case of a preparation of a vector backbone for cloning, the whole reaction was applied to preparative gel electrophoresis followed by DNA elution from the agarose gel using Wizard® SV Gel and PCR Clean-Up System from Promega. If a PCR-derived insert was digested, the reaction was also purified by employing the same system but treated like a PCR reaction.

3.1.5. Agarose gel electrophoresis of DNA

Gel electrophoresis was executed in electrophoresis cells from BioRad. Agarose was melted in 1x TBE buffer (1% w/v) followed by addition of ethidium bromide to a final concentration of 2.5 µg/ml. The melted agarose gel was poured into a tray with a comb placed for the formation of gel pockets. After polymerization of the gel, the comb was removed and the tray was placed in the electrophoresis cell filled with 1x TBE buffer. DNA samples were mixed with 5x loading buffer and H_2O_{dd} according to the samples initial volume and applied to the gel pockets. *Hind*III digested lambda phage derived DNA was employed as the length marker with fragment sizes of 23130, 9416, 6557, 4361, 2322, 2027, 564, and 125 bp. Separation of DNA fragments occurred at 110V for 30 to 60 minutes depending on their length. Due to intercalation of ethidium bromide into DNA, the samples could be visualized with UV light in a transilluminator at wavelength 302 nm.

3.1.6. Preparative agarose gel and DNA elution

In order to elute DNA from an agarose gel, the separated DNA fragments were visualized and the desired bands were excised under UV light. Cut gel fragments were transferred into 1.5 ml microcentrifuge tubes, weighed, and DNA eluted employing the Wizard® SV Gel and PCR Clean-Up System from Promega.
An equal volume of Membrane Binding Solution was added to the weighed gel fragment, incubated at 60°C for 10', and frequently mixed to dissolve the agarose. Subsequently, the

dissolved mixture was transferred to a SV minicolumn and prepared as described in chapter 3.1.3.

3.1.7. Dephosphorylation of DNA

The 5' phosphates of the linearized and gel-purified vector were removed using the calf alkaline phosphatase (20 U/µl) purchased from Roche. Per 1 µl of DNA, 10 U of phosphatase and 1/20 volume of 10 x dephosphorylation buffer were added to the reaction mix and incubated for 45' at 37°C. The whole reaction was purified as described in chapter 3.1.3.

3.1.8. Ligation of DNA fragments

Ligation of vector and insert was carried out with the T4 DNA Ligase System from Fermentas. To ensure high ligation efficiency, insert-vector ratios of 3:1 up to 10:1, depending on the length of DNA fragments, were used. To an appropriate volume of nuclease free H_2O_{dd}, 10 x T4 ligation buffer, DNA fragments, and 1 µl T4 DNA Ligase (30U/µl) were added. A reaction mix without insert DNA was prepared as a negative control. The reaction mix was incubated for 60' at room temperature followed by phenol/chloroform extraction and ethanol precipitation.

3.1.9. Phenol-chloroform extraction of DNA

After ligation, the reaction mix was adjusted to a volume of 200 µl with nuclease free H_2O_{dd}. An equal volume of H_2O-saturated phenol/chloroform (Applied Biosystems) was added to the aqueous sample and the mixture was gently inverted for approximately 30''. To exact phase separation, the mixture was spun down in a tabletop centrifuge and the upper aqueous phase was carefully transferred into a fresh 1.5 ml tube. This step was repeated once followed by addition of an equal volume of chloroform:isoamylalcohol 24:1 to remove any residual phenol. After mixing, the sample was again centrifuged to allow phase separation. The aqueous upper layer containing the DNA was transferred to a new tube and concentrated by ethanol precipitation.

3.1.10. Ethanol precipitation

Aqueous solutions of DNA were mixed with 3 volumes of absolute ethanol and 1/10 volume of 3M sodium acetate (pH 5.2) and precipitated at -20°C for 20'. After centrifugation (30', 16,000 x g, room temperature), the DNA pellet was washed with 70% ethanol, centrifuged (15', 16,000 x g, room temperature), air-dried, and finally resuspended in 21 µl TE buffer.

3.1.11. Preparation of electrocompentent bacteria

5 ml of LB medium were inoculated with *E. coli* HB101 and grown overnight at 37°C in a shaking incubator at 280 rpm. The next day, overnight cultures were added to 500 ml LB medium and incubated at 37°C shaking at 280 rpm until bacterial growth reached an optical density between 0.6 to 0.8. Optical density was determined in a Hitachi U2001 Spectrophotometer at wavelength of 600 nm. The bacterial culture was chilled for 15' to 30' on ice and was constantly kept on ice during the following procedure. All steps were performed with sterile solutions and containers. The culture was aliquoted in 6 centrifuge tubes and pelleted by centrifugation (30', 4°C, 5,000 rpm, rotor JLA-16.250, Beckman Avanti J-20 centrifuge). Each pellet was resuspended in 165 µl ice-cold, sterile H_2O_{dd} and centrifuged again (15', 4°C, 5,000 rpm). The procedure was repeated three times with 500 ml cold, sterile H_2O_{dd}. Next the pellets were resuspended in 20 ml 10% sterile glycerol followed another centrifugation step. Finally, bacteria resuspended in 2.5 ml 10% glycerol, frozen in aliquots of 50 µl in liquid nitrogen, and stored at -80°C.

3.1.12. Transformation of bacteria by electroporation

Electrocompetent *E. coli* HB101 were thawed on ice for not longer than 5', added to 7 µl of purified ligation reaction resuspended in TE buffer, and gently mixed by tapping the reaction tube. The reaction was left on ice for 1', afterwards transferred to a pre-cooled electroporation cuvette (0.1 cm, Biozym) and pulsed in a gene pulser device (Biorad) under following conditions: 1.8 kV, 25 µF, 200 Ω. Immediately after, 1 ml warmed LB medium containing 0.02% glucose was added to the cells. The reaction was transferred into a 14 ml Falcon centrifuge tube and incubated for 60' at 37°C in a shaking incubator at 250 rpm.

Finally, bacteria were spun down, resuspended in about 200 µl of medium, and aliquots of 30 and 70 µl were plated on pre-warmed LB agar plates supplemented with 50 mg/ml ampicillin. The plates were incubated at 37°C overnight.

3.1.13. *Escherichia coli* screening PCR

Screening for correct insertion into the vector was performed using an *Escherichia coli* screening PCR employing reagents from Roche. PCR was performed with primers flanking one ligation site which yielded a PCR product only in the case of correct insertion into the vector.

The clones were picked from LB plates, applied to a 96-well PCR reaction plate and 27.75 µl H_2O_{dd}, 2.5 µl sense Primer (2 pmol), 2.5 µl antisense Primer (2 pmol), 5 µl 10xPCR Buffer II, 8 µl dNTP Mix, 4 µl $MgCl_2$, 0.25 µl AmpiTaqGold™ Polymerase were added to the reaction, which were performed in a Applied Biosystems GeneAmp Thermal Cycler with the following settings:

10’ 94°C → 35x [45’’ 94°C → 30’’ 50°C → 45’’ 72°C] → 7’ 72°C → 4°C.

1/10 of the total PCR reaction volume was subjected to agarose gel electrophoresis to screen for positive clones.

3.1.14. Plasmid preparation

After identification of the positive clones, the corresponding single bacterial colony was picked from the LB plate and transferred in a Falcon tube containing 5 ml LB medium supplemented with 10 mg/ml ampicillin. The culture was incubated overnight at 37°C in a shaking incubator at 250 rpm. The next day, the bacterial cells were pelleted by centrifugation at 12,000 x g for 5’. Isolation of plasmid DNA from the transformed bacterial cells was performed utilizing the Plasmid Mini Kit purchased from Qiagen according to the manufacturer’s protocol.

In order to obtain larger amounts of plasmid DNA, a 5 ml preparatory culture was added to 500 ml LB medium with 10 mg/ml ampicillin and incubated as stated above. Thereafter, bacterial cells were pelleted and plasmid isolation was conducted with the Qiagen Plasmid Mega Kit following the manufacturer’s protocol.

3.1.15. Quantification of DNA

The purity and quantity of isolated plasmid DNA was determined by photometric measurement according to the Christian-Warburg method at the wavelengths of 260 and 280 nm. Samples of DNA diluted 1:100 and 1:50 in TE buffer were subjected to photometry in a UV/Vis quartz glass cuvette. Concentration was calculated by multiplying the absorbance by the dilution factors.
Furthermore, samples of plasmid DNA were subjected to agarose gel electrophoresis to check the DNA quality.

3.1.16. Subcloning

To disrupt an unwanted *Mlu*I restriction site in the CprME sequence, the insert was subcloned in the vector pSG5 obtained from Stratagene.
The insert was cut from the generated pVEEV-CprME vector with the restriction enzyme *Xba*I (Fermentas), purified with the Wizard® System (Promega), and ligated into the prepared vector pSG5. Screening of clones and plasmid isolation was performed according to the described methods in chapters 3.1.13. and 3.1.14.

3.1.17. Site-directed mutagenesis

To achieve the disruption of the unwanted *Mlu*I site in CprME, site-directed mutagenesis was conducted employing the GeneTailor Site-directed Mutagenesis System purchased from Invitrogen.

3.1.17.1. Methylation

Template pSG5-CprME was methylated for 60' at 37°C. The reaction mixture was pipetted to a final total volume of 16 µl as followed: 100 ng plasmid DNA diluted in an appropriate volume of H_2O_{dd}, 1.6 µl methylation buffer, 1.6 µl 10 x SAM, and 1 µl DNA methylase.

3.1.17.2. Mutagenesis

The template was amplified by mutagenesis reaction with two overlapping primers obtained from VWR-Biotech; sequences are stated in table 4. The sense primer contained the altered sequence of a *Mlu*I restriction site where position 3 changes from a guanine to an adenine.

The mutagenesis PCR reaction employed reagents from Clontech Advantage™ HF2 PCR System and was mixed as followed: 2 µl methylated DNA template, 35 µl H_2O_{dd}, 1 µl sense primer (5 pmol), 1 µl antisense primer (5 pmol), 5 µl 10x HF2 PCR buffer, 5 µl 10x HF2 dNTP mix, and 1 µl 50x Advantage HF2 Pol Mix.

Reactions were carried out in a Biometra thermal cycler with the following settings:

15'' 94°C → 20x [15'' 94°C → 30'' 55°C → 7' 68°C] → 5' 68°C → 4°C.

Table 4. Primers used for mutagenesis reaction. The *Mlu*I restriction site is highlighted in red. The introduced point mutation is underlined.

Expression Plasmid	Primer sequence	Primer orientation
pSG5-CprME	GACCGCAACGAAGACACGTCAACCCAGAGTC	sense
	TCTTCGTTGCGGTCTCTTTCGACACTCGTC	antisense

3.1.17.3. Transformation in DH5α™-T1R *E. coli*

The mutagenesis reaction was transfected into the heat shock competent *E. coli* strain DH5α. Therefore, bacteria were thawed on ice for about 5', added to 2 µl mutagenesis reaction, and gently mixed. The mixture was incubated for 10' on ice, further incubated for 30'' at 42°C, and again for 1' on ice. 200 µl pre-warmed SOC medium was added to the reaction followed by incubation for 60' at 37°C in a shaking incubator at 225 rpm. Finally, 125 µl of the transformation reaction were plated onto LB_{Amp} agar plates and incubated at 37°C overnight.

The resulting bacterial colonies were screening by *E. coli* screening PCR, followed by plasmid amplification, isolation, and photometric quantification.

3.1.18. DNA sequencing

To check the outcome of the side-directed mutagenesis reaction, sequencing was performed using fluorescence-labeled dideoxynucleotides. Therefore, the ABI Prism™ Big

Dye Terminator Cycle Sequencing Kit from Applied Biosystems was used according to the manufacturer's instructions. 0.3-0.5 µg DNA resuspended in 13 µl H_2O_{dd}, 3 µl buffer, 3 µl specific sense or antisense primer (2 pmol), and 2 µl Big Dye Ready mix were combined. The sequence reaction was conducted in a Perkin Elmer Thermal Cycler with the following settings:

20'' 96°C → 35x [30'' 96°C → 15'' 50°C → 4' 60°C] → 4°C.

To purify the sequencing reactions, plastic 96-well plates were filled with Sephadex™ G-50 purchased from GE Healthcare and incubated with 300 µl H_2O_{dd}, for three hours at room temperature. Incubated plates were centrifuged (5', 2,500 rpm) and the sequencing reactions were applied with 30 µl H_2O_{dd} to the pre-swollen sephadex wells. In an additional centrifugation step the sequencing reactions were purified and samples were collected. Finally, sequence analysis was performed by an automatic capillary sequencer (Applied Biosystems, GA 310, GA 3100) and the resulting data was examined with the software packages from DNAstar and Corbas Mira Imaging.

3.1.19. Generation of termination points for RNA synthesis and filling in sticky ends

Before viral RNA could be synthesized, pVEEV-CprME was digested with *Mlu*I and TBEV replicons were cut with *Nhe*I in order to create termination points for RNA synthesis. This was carried out under the above stated conditions (see chapter 3.1.4.) followed by purification with the Wizard Purification Kit.

To generate TBEV replicon genomes, the filling in of the sticky ends generated by the restriction cut was performed by employing the Klenow Fragment from Fermentas. 28.5 µl RNAse free H_2O_{dd}, 3 µl CTP, 3 µl TTP, 4 µl NeB2 buffer, and 1.5 µl Klenow Fragment were added to the NheI digested replicons. The mixture was incubated for 15' at 25°C and finally subjected to phenol-chloroform purification and ethanol precipitation (see 3.1.9. and 3.1.10) under RNase free conditions. Samples of 2 µl were checked by agarose gel electrophoresis.

3.1.20. In vitro RNA transcription

In vitro RNA synthesis of VEEV-CprME was performed with SP6-MEGAscript™ Kit purchased from Ambion. To transcribe DNA into RNA, 6 µl template (1 µg DNA) was added to 3.1 µl nuclease-free H_2O_{dd}, 2 µl 10 x buffer, 2 µl 75 mM ATP, 2 µl 75 mM CTP, 2 µl 75 mM UTP, 0.4 µl 75 mM GTP, 0.5 µl 75 mM CAP analog [m7G(5')ppp(5')G], and 2 µl DNA dependent RNA polymerase. The reaction mixture was incubated for 3 hours at 37°C.

To generate TBEV replicon RNA genomes, the Ambion T7-MEGAscript™ Kit was utilized. The reaction was mixed and incubated in the same way as stated above.

3.1.21. DNase digestion

The DNA template was degraded by Turbo DNase from Ambion. 1 µl DNase was added to the RNA synthesis reaction mixture and was incubated for 15' at 37°C.

3.1.22. RNA purification

Synthesized viral RNA was subjected to purification which was performed with RNeasy Kit from Qiagen.

3.1.23. RNA gel electrophoresis

RNA gel electrophoresis was performed in electrophoresis chambers from BioRad. 0.5 g RNase free agarose was melted in 37 ml nuclease-free H_2O_{dd} and 5 ml 10 x MOPS buffer. After cooling, 8 ml formaldehyde (36-38%) were added to the melted agarose, mixed, and poured into a tray with a comb. After gel polymerization, the comb was removed and the tray was placed in an electrophoresis chamber filled with 1 x MOPS buffer.

Samples of 5 µl RNA were mixed with 5 µl H_2O_{dd}, 10 µl RNA loading buffer (Ambion), and 1 µl Radiant Red. The mixture was incubated for 15' at 65°C and immediately after, loaded on the RNA gel. 2 µl of RNA length marker (0.5-9 kbp, Cambrex) were added to 3 µl H_2O_{dd}, 5 µl RNA loading buffer and 0.5 µl Radiant Red and incubated in the same way as the RNA samples.

The settings for the RNA separation were: 35mA constant for 10' followed by 55mA constant for approximately 30' to 50' depending on the length of RNA. Separated RNA bands were viewed in a transilluminator as described in chapter 3.1.5.

3.1.24. Quantification of RNA

The quantity of transcribed RNA was determined in a spectrophotometer according to the Christian-Warburg method at the wavelengths of 260 and 280 nm. Samples of RNA were diluted 1:30 and 1:60 with 10 mM Tris Cl buffer (pH 7.5) and transferred into a UV/Vis quartz glass cuvette. RNA concentration was calculated by multiplying the absorbance with the dilution factor.

3.1.25. Media and buffers

10 x TBE buffer:	108 g Tris
	55 g Boric Acid
	8.3 g EDTA
	Water for a final volume of 500 ml
10 x MOPS buffer:	41.85 g 0.2 M MOPS
	4.1 g 50 mM Sodium actetate
	3.72 g 10 mM EDTA
	Water for a final volume of 1000 ml
	Equilibrated to pH 7.0 with Sodium hydroxide
TE buffer:	5 ml 1M Tris (pH 8.0)
	2 ml 0.25 M EDTA
	493 ml H_2O_{dd}
LB medium:	1 % Bacto-tryptone
	0.5 % Yeast extract
	1 % Sodium chloride
	Equilibrated to pH 7.0 with Sodium hydroxide
	Autoclaved

LB agar medium:	LB medium
	20 g/l Difco agar
	Autoclaved
	Poured in plates under sterile conditions
LB glucose:	LB medium containing 0.02% glucose

3.2. Cell cultures

3.2.1. Cultivation of cells

3.2.1.1. BHK-21

Baby hamster kidney cells (BHK-21) originated from the ATCC (No. CCL-10) and were grown under standard conditions (37°C, 5% CO_2, 95% humidity). These cells were cultivated in 30 ml of growth or minimal medium to dense monolayers in 175 cm^2 tissue culture flasks and were then detached from the surface of the flasks by addition of 5 ml trypsin. Cells were split every 4 days at the ratio 1:10.

Growth medium:	460 ml Eagle´s minimal essential medium (Sigma)
	25 ml foetal calf serum (5%)
	7.5 ml HEPES (1.5%)
	5 ml L-glutamine (1%)
	2.5 ml Neomycin (0.5%)
Minimal medium:	480 ml Eagle´s minimal essential medium (Sigma)
	5 ml foetal calf serum (1%)
	7.5 ml HEPES (1.5%)
	5 ml L-glutamine (1%)
	2.5 ml Neomycin (0.5%)
Trypsin:	20 ml trypsin (0.25%)
	180 ml PBS (pH 7.4)

3.2.1.2. HEK 293T and MEF

Human embryonic kidney cells (HEK 293T) originated from the group of Peter Stäheli (University of Freiburg) and were cultivated under standard conditions (37°C, 5% CO_2, 95% humidity). Cells were grown in 175 cm^2 tissue culture flasks with 30 ml growth or minimal medium till the formation of a dense monolayer. Cells were detached from the

surface by the action of a 1 x trypsin/EDTA mixture and passaged every 3 to 4 days at a split rate of 1:5.
Mouse embryonic fibroblasts (MEF) were kindly provided by the group of Prof. Thomas Decker (University of Vienna). Cell cultivation and passaging was performed in the same way as for HEK 293T cells described above.

Growth medium:	462.5 ml Dulbecco's Modified Eagle Medium (Gibco)
	25 ml foetal calf serum (5%)
	7.5 ml HEPES (1.5%)
	5 ml Penistrep (1%)

Minimal medium:	442.5 ml Dulbecco's Modified Eagle Medium (Gibco)
	5 ml foetal calf serum (5%)
	7.5 ml HEPES (1.5%)
	5 ml Penistrep (1%)

1 x Trypsin:	10 ml 10 x Trypsin/EDTA (Sigma)
	90 ml PBS (pH 7.4)

3.2.1.3. IRE-18

The cell line IRE-18 was derived from the tick species *Ix. ricinus* and were kindly provided by Lesley Bell-Sakyi (Royal Dick School of Veterinary Studies, University of Edinburgh). Tick cells require special conditions of cultivation (28°C, no CO_2, no humidity) in closed 3 cm^2 tubes supplemented with 3 ml growth medium. Cells did not exhibit contact inhibition and therefore grew mostly in three dimensions rather than in monolayers. IRE-18 cells are not strongly adherent and were detached from the tube's surface by pipetting. Cells were splitted weekly at the ratio 1:2.

Growth medium:	17 ml L15 (Invitrogen)
	18 ml 1 x HBSS (Invitrogen)
	2.5 ml tryptose-PO_4-broth (Sigma)
	1.25 ml 10% lactalbumine hydrolysate (Merck)
	10 ml foetal calf serum (Sigma)

0.5 ml L-glutamine (200 mM)
0.5 ml PSA (Invitrogen)

Infection medium: 1 ml HEPES (1 M)
0.3 ml Sodium hydroxide (1 N)
Added to the growth medium

3.2.2. Seeding of cells for infection

Prior to infection experiments, different cell types were seeded in 24-well plates at the following cell counts.

BHK-21	1×10^5
MEF	1×10^5
HEK 293T	5×10^5
IRE-18	5×10^5

Counting of cells was performed with a Casy Cell Counter.

3.2.3. Coating with poly-D-lysine

During cultivation, HEK 293T cells grew semi-adherent. To increase adherence to the surface of plastic and glassware, the containers were coated with poly-D-lysine prior to cell seeding. Therefore, 24-well plates and glass coverslides which were used to perform immunofluorescence were coated with 200 µl of poly-D-lysine solution (0.25mg/ml) diluted in 0.15 M borate buffer and incubated for 2 hours minimum. Afterwards, plastic and glassware was washed three times with sterile PBS and cells were seeded.

0.15 M borate buffer: 57.2 g sodium tetraborate decahydrate
Dissolved in 1000 ml H_2O_{dd}
Adjusted to pH 8.3 with hydrochloride
Sterile filtered

0.025 % poly-D-lysine:	Poly-D-lysine stock (1mg/ml) (Millipore)
	Diluted 1:4 with 0.15 M borate buffer
	Sterile filtered

3.2.4. Transfection protocols

3.2.4.1. Electroporation of BHK-21

Confluent BHK-21 cells were detached from the surface of the cell culture flasks by trypsin activity, collected, and harvested by centrifugation (Sigma, 800 rpm, 14°C, 7'). The pellet was washed twice with ice-cold sterile PBS (pH 8.4). Next, cells were resuspended in an appropriate volume of PBS. 0.8 ml cell suspension was mixed with one, or two, in the case of co-transfection, RNA transcripts and the mixture was transferred into a sterile pre-cooled 0.4 cm gene pulser cuvette. The cells were pulsed twice with 1.8 kV, 25 µF, and 200 Ω by a gene pulser device from BioRad resulting in a time constant of 0.7 ms. After pulsing, the cells were left at room temperature for 10 min followed by dilution 1:10 in growth medium. Finally, cells were seeded at different dilutions in 24-well plates with or without glass coverslides.

Approximately 12 hours post transfection, the medium was replaced with fresh minimal medium. Supernatants were collected from the transfected BHK-21 cells 3 days post transfection, cleared of cell debris (10,000 rpm, 20', 4°C) and stored at -80°C.

3.2.4.2. Electroporation of HEK 293T

Confluent HEK 293T cells were incubated with 1 x trypsin/EDTA solution for 5' to allow detachment from the cell culture flask surface. Cells were pelleted by centrifugation (Sigma, 800 rpm, 14°C, 7') and washed twice with 4°C cold 1 x HeBS. Cells were resuspended in an appropriate volume of electroporation buffer 1 x HeBS and 0.8 ml cell suspension was mixed with the RNA transcripts. The mixture was transferred into a sterile pre-cooled 0.4 cm gene pulser cuvette. The cells were pulsed once with 0.27 kV, 960 µF, and no Ω by a gene pulser device from BioRad resulting in a mean time constant of 14 ms. After pulsing, cells rested for 10' at room temperature. According to incubation time in cell culture, cells were diluted 1:10 when incubated for 2 days and 1:5 when incubated for one day with growth medium and seeded.

After 12 hours of incubation in cell culture, the medium was replaced with minimal medium and supernatants were harvested 2 days after electroporation as described above.

1 x HeBS electroporation buffer:	20 mM HEPES pH 7.05
	137 mM Sodium chloride
	5 mM Potassium chloride
	0.7 mM disodium hydrogen orthophosphate
	6 mM D-Glucose
	Adjusted to pH 7.05 with sodium chloride
	Sterile filtered

3.2.4.3. Transfection of HEK 293T with TransMessenger transfection reagent

5 x 10^5 HEK 293T cells per ml were seeded in poly-D-lysine coated 24-well plates with or without glass coverslides and cultured for 24 hours in growth medium. Transfection was performed according to the manufactures protocol (Qiagen). 2 µl enhancer and 1 µg RNA transcript were diluted in buffer EC-R to a final volume of 100 µl. The mixture was vortexed for 10'' and left for 5' at room temperature. The mixture was spun down, 4 µl TransMessanger transfection reagent were added and again vortexed for 10''. During an incubation period of 10' at room temperature while complex formation took place, cells were washed with twice with PBS using the 1 to 2 fold volume in which cells were seeded. Subsequently, 100 µl medium, without serum or antibiotics supplemented, was added to the transfection mixture and mixed by pipetting up and down twice. The transfection mixture was carefully applied to the cells drop-wise and incubated for 3 hours under standard cell culture conditions. Finally, the complexes were removed, cells washed once with PBS, supplied with minimal medium (supplemented with antibiotics and FCS) and incubated for 24 hours at standard cell culture conditions.

3.2.4.4. Transfection of HEK 283T cells with Lipofectamine

Transfection of DNA expression plasmid phSV40-CprME was performed with Lipofectamin 2000 ™ purchased from Invitrogen. Therefore, 5 x 10^5 HEK 293T cells were seeded in 400 µl DMEM medium supplemented with 5 % FCS but without antibiotics in

24-well plates with or without glass coverslides and cultured for 24 hours. Afterwards, the medium was replaced with DMEM supplemented with 1 % FCS but without antibiotics. In a sterile Eppendorf reaction tube, 3 µg plasmid DNA were diluted in 50 µl Opti-MEM I medium and mixed by gently tapping the reaction tube. In the same way, 6 µl Lipofectamine reagent was mixed with Opti-MEM I medium to a final volume of 50 µl in a separate reaction tube. Both mixtures were incubated for 5' at room temperature before they were combined and complex formation was allowed to proceed during another incubation time of 20' at room temperature. The transfection mixture was gently and applied drop-wise to the 400 µl of medium covering the cells. Finally, incubation for 24 hours at 37°C took place and the medium was replaced with standard minimal medium.

3.2.5. Cell passages and infection of cells

Cells were seeded and cultured in growth medium for 24 hours followed by a single washing step with minimal medium. Cleared and stored supernatants were thawed and immediately placed on ice. 200 µl of supernatant were transferred onto fresh cells, incubated for 2 hours, removed and replaced with fresh minimal medium.
To determine the tier of packaged SIPs in the infectious supernatants, these were serially diluted and applied to fresh cells and incubated in the same way as described above.

3.2.6. Co-infections

Cells of different cell types were seeded at numbers given in chapter 3.2.2. and incubated with 200 µl of thawed preparations of SIPs containing supernatant for 2 hours. After removal of the first preparation, the second preparation was applied to the cells and incubated for a further 2 hours. Finally, the supernatant was removed and cells were washed twice with the respective minimal medium (described above) and cultivated for 3 or 6 days respectively.

3.3.7. Indirect immunofluorescence

The expression of viral proteins was detected by indirect immunofluorescence. After removal of supernatants, the cells grown on glass coverslides were fixed and permeabilized by adding 500 µl acetone/methanol (1:1) and incubated for 10' at -20°C. Then, the glass

coverslides were air-dried and incubated with 25 µl polyclonal rabbit anti-protein E serum (1:50) for 45' in a humid chamber at 37°C. After rinsing the glass coverslides twice with PBS, the plates were incubated again for 45' with 25 µl fluorescence labeled FITC anti-rabbit (1:10). Finally, the coverslides were washed twice with PBS, air-dried and fixed with DePex (Serva) on microscope slides. Visual inspection was performed with the Epi fluorescence microscope Microphot from Nikon.

4. Results

4.1. Cloning of pVEEV-CprME

In order to provide flavivirus structural proteins C, prM and E *in trans* for the packaging of TBEV replicons, an alphavirus was used as an expression vector. pVEEV-CprME was cloned by introduction of the sequences coding for the proteins C, prM and E of TBEV into the non-cytopathic alphavirus replicon pVEEV which was kindly provided by Ilya Frolov (University of Texas) (fig.17).

The first step to clone pVEEV-CprME was the amplification of the structural genes CprME of TBEV by PCR with primers aligning to the flanking region of TBEV CprME with an additional *Xba*I restriction site (fig.17A). As template for the PCR reaction, the TBE strain Neudoerfl derived plasmid pTNd/5' was used. The PCR reaction produced a 2418 base pairs-long fragment which was subjected to purification and checked by agarose gel electrophoresis. As shown in figure 18A, a PCR product of approximately 2400 base pairs in length was obtained corresponding in size with the length of CprME. The cloning procedure continued with the digestion of the PCR product with restriction enzyme *Xba*I. As backbone pVEEV was digested with *Xba*I and the resulting larger fragment was ligated with the digested CprME fragment (fig.17B). The ligation product was transfected into electrocompetent bacteria. Prior to plasmid isolation, single bacterial colonies were picked and screened for correct insertion of CprME into pVEEV using an *Escherichia coli* screening PCR with primers that flanked one ligation site. If the orientation of CprME in the vector was correct, the screening PCR produced a product of 546 base pairs. To select the positive clones, the screening PCR was subjected to gel electrophoresis. As shown in figure 18B, 7 clones yielded a DNA fragment of the expected length. After identification of the positive clones, the corresponding single bacterial colonies were cultivated and plasmid DNA was isolated large scale.

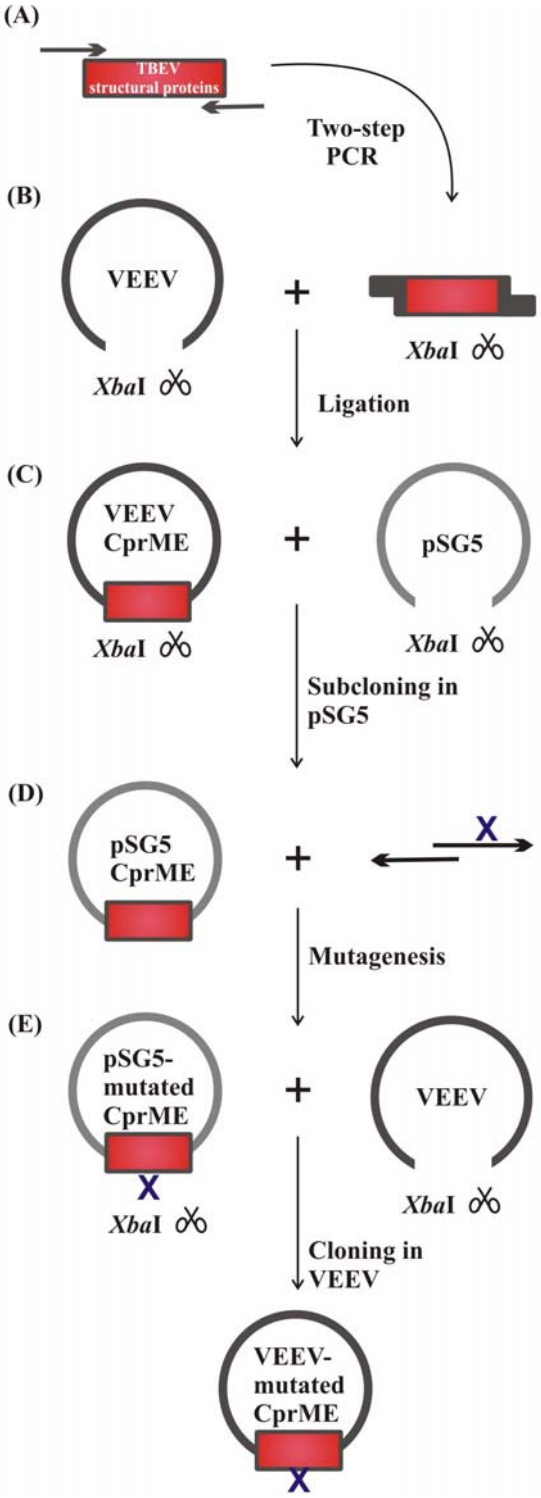


Fig.17. Scheme of the cloning strategy for pVEEV-CprME. Cloning of pVEEV coding for TBEV CprME involved a two-step PCR reaction (A) followed by preparation and cloning of the PCR product into the pVEEV vector (B). The subcloning of the insert into the high-copy plasmid pSG5 (C) was followed by side-directed mutagenesis of the *Mlu*I site in CprME (D). Finally, CprME was cloned back into pVEEV (E). The structural proteins are sketched in red; the blue X indicates the altered *Mlu*I sequence.

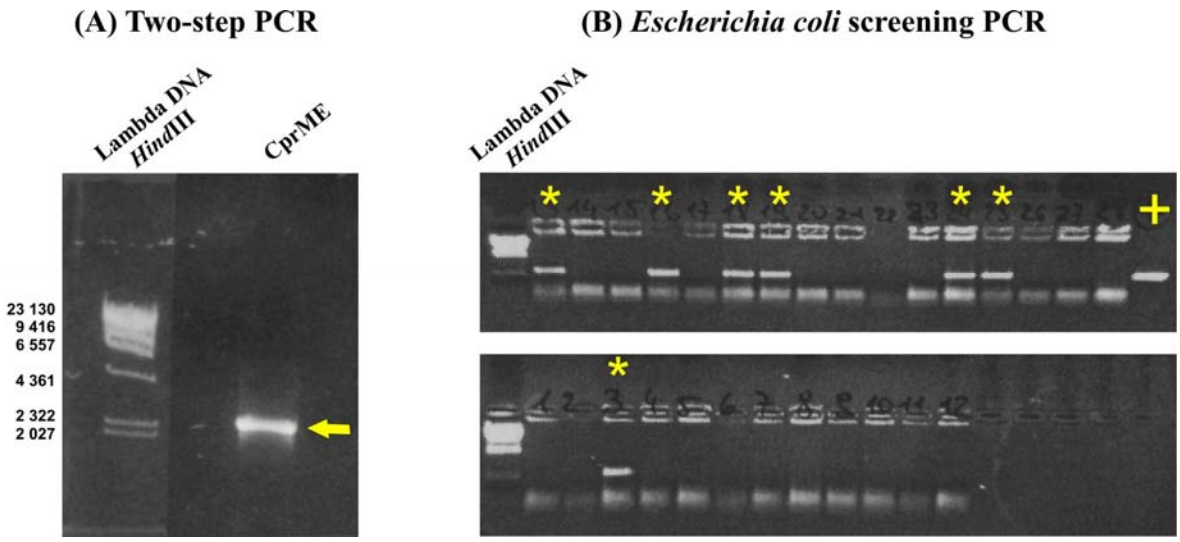


Fig.18. Control of the cloning steps of pVEEV-CprME on 1% agarose gels. (A) The two-step PCR of CprME yielded a 2418 bp product which is indicated by an arrow. Subsequently to *Xba*I digest, ligation, and transformation of bacterial cells, clones were tested for the correct orientation of CprME in the vector by *E. coli* screening PCR **(B).** Clones with correctly orientated CprME displayed PCR products of 546 bp. Lanes with positive clones are indicated by asterisks, + marks the positive control. Lambda DNA marker is given in bp.

Unfortunately, the introduced sequences of TBEV CprME contained an additional *Mlu*I restriction site, which was originally used at the 3' terminus for linearization of the plasmid prior to RNA synthesis. Therefore, the *Mlu*I restriction site in the sequence coding for CprME had to be mutated to allow *in vitro* RNA synthesis from this plasmid. Therefore, a subclone of pVEEV-CprME was generated. pVEEV-CprME was digested with *Xba*I and the resulting smaller of the two fragments containing the sequences coding for CprME cloned into the vector pSG5 (fig.17C). The resulting plasmid pSG5-CprME was subjected to site-directed mutagenesis reaction to disrupt the undesired *Mlu*I restriction (fig.17D). Briefly, pSG5-CprME was methylated, then mutagensis PCR was carried out with primers as described in material and methods (fig.19). The product of this PCR was transfected into DH5α-Max Efficiency cells (Invitrogen) which degraded methylated DNA. As a consequence, methylated and unmutated template DNA pSG5-CprME was degraded in these bacteria yielding only colonies with unmethylated and mutated DNA of the mutagenesis reaction. This allowed selection of pSG5-CprME with a mutated *Mlu*I restriction site. Finally, the altered insert was re-introduced into pVEEV using the XbaI restriction site yielding the desired expression plasmid pVEEV-CprME (fig. 17E).

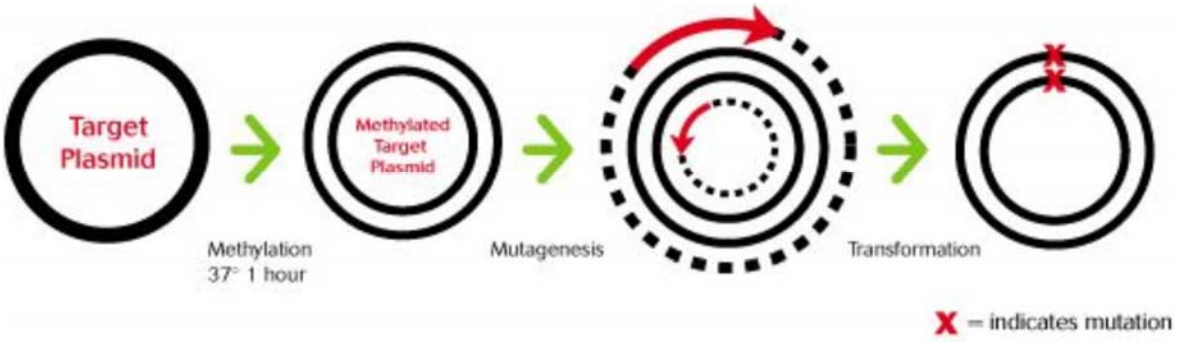


Fig.19. The GeneTailor™ Side-Directed Mutagenesis System process. The mutagenesis reaction involved methylation followed by a PCR reaction with an overlapping primer pair that introduced the mutation. (Adapted from www.invitrogen.com)

4.2. Synthesis of full-length viral RNAs

Full-length viral RNA was synthesized by *in vitro* RNA synthesis. Therefore, pVEEV-CprME or TBEV replicon plasmids were linearized by *Mlu*I or *Nhe*I digestion to create termination points for RNA synthesis (see material and methods). In addition, the generation of TBEV RNA genomes required the action of the Klenow fragment to fill in the sticky ends of the restriction cut creating an exact 3' terminus of the flavivirus genome which is essential for viral RNA replication. *In vitro* RNA transcription of pVEEV-CprME was performed under standard conditions with SP6-MEGAscript™ Kit purchased from Ambion. The quality of the transcribed RNA was checked by RNA gel electrophoresis.

As shown in figure 20A, the *in vitro* transcription of pVEEV-CprME transcript yielded two distinct RNA species. The upper band corresponded to the expected 9892 base pairs long full-length RNA of VEEV-CprME. Moreover, a second shorter RNA fragment of 1000 to 1500 base pairs in length was visible. This additional by-product is also produced from the original VEEV replicon and probably indicated the presence of a cryptic promoter for SP6 polymerase present in the sequence coding for VEEV-CprME. Nevertheless, full-length RNA of VEEV-CprME of high quality could be transcribed.

In figure 20B, two samples of pVEEV-CprME transcripts are shown after treatment with DNase and after purification with RNeasy Mini Kit. The intensity of the RNA bands was observed to decrease after purification. Consequently, the subsequent transfection of cells was performed with non-purified RNA to avoid the significant reduction of RNA amounts caused by the RNA purification step.

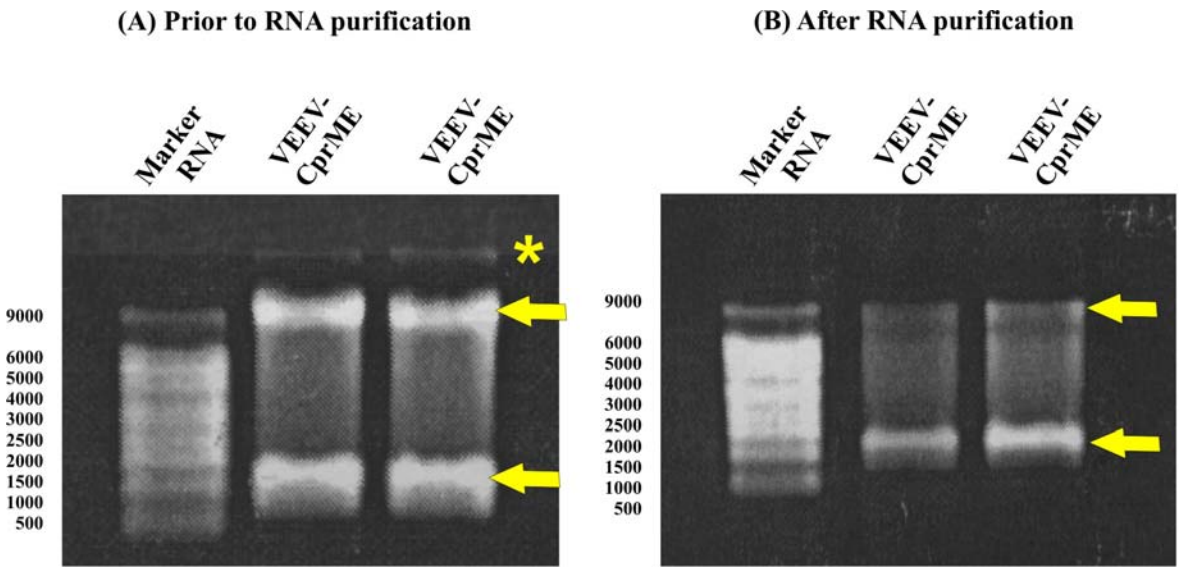


Fig.20. Control of *in vitro* transcription by RNA gel electrophoresis. (A) Directly after transcription, two samples of pVEEV-CprME derived RNAs were subjected to gel electrophoresis. The arrows indicate two RNA species of which the upper one is the expected full-length RNA. The lower band resembles a shortened RNA fragment of 1 to 1.5 kb. The asterisk marks the linearized DNA template. Marker RNA is given in bp. **(B)** Samples of purified RNA were loaded in the middle and the right lane. The weaker bands indicated a considerable loss of RNA due to the purification step.

RNA *in vitro* transcription of TBEV replicons was performed by employing Ambion T7-MEGAscript™ Kit. RNA of the three different TBEV replicons ΔC, ΔME, and ΔE was transcribed and samples subjected to RNA gel electrophoresis which is shown in figure 21. The obtained replicon RNA was of high-quality and co-transfection of RNA could be initiated.

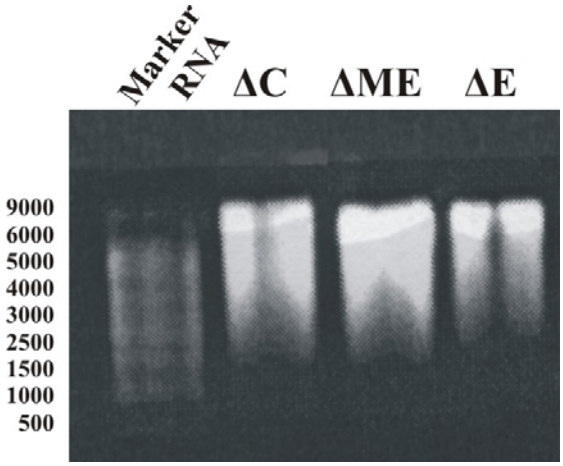


Fig.21. RNA gel electrophoresis of TBEV replicon RNA. From three different TBEV replicons, RNA was produced. To monitor RNA quality, a denaturing RNA gel was run. Marker RNA is given in bp.

In addition, samples of all transcribed RNAs were quantified by photometric measurement and considered as RNA of high concentration and excellent purity.

4.3. TBEV replicons can be packaged by VEEV-CprME

To test whether VEEV-CprME can provide the structural proteins of flaviviruses for the packaging of a TBEV replicon, we made use of a previously described replicon (Gehrke, Ecker et al. 2003). Replicon ΔME lacked the entire region encoding the envelope protein prM and most of the envelope protein E and therefore could not produce infectious virus particles. Transfection of this replicon into BHK-21 cells resulted in cells expressing TBEV proteins, as monitored by immunofluorescence staining 24 hours post transfection with polyclonal TBEV serum (fig.22A). Transfection efficiency was as high as 80-90% of cells. In contrast, transfection of VEEV-CprME RNA yielded only about 40% of positive cells after 24 hours post transfection (fig.22A). However, the presence of cells staining positive with TBEV serum confirmed that TBEV proteins could be expressed by this construct. To monitor the production of trans-packaged TBE virus particles containing replicon ΔME, replicon ΔME and VEEV-CprME were co-transfected into BHK-21 cells (fig.22A). Supernatant was transferred onto fresh cells three days post transfection and 24 hours later immunofluorescence staining was carried out again. As shown in figure 22B, 40% of infected cells exhibited expression of TBEV proteins. This demonstrated that trans-packaging of TBEV replicon ΔME by VEEV-CprME was successful. Interestingly, repeated transfer of supernatant from infected cells resulted in no cells expressing TBEV proteins (data not shown) which indicated that the produced virus particles were indeed only capable of a single round of infection.

To determine the titer of the produced single round infectious particles, supernatants from co-transfected cells where collected, serially diluted and used to infect fresh cells. After 24 hours, cells expressing TBE virus proteins were stained using polyclonal TBEV serum. 8 positive cells were found up to a dilution of 10^{-3}.This showed that 10^3 infectious units of TBEV ΔME SIPs could be produced.

For further trans-complementation studies, two additional TBEV replicons were packaged into single round infectious particles by co-transfection with VEEV-CprME. Replicon ΔC contained a large deletion (62aa) in the region coding for the capsid protein (fig.16) and replicon ΔE had all E gene deleted (fig.16). Both replicons were co-transfected with VEEV-CprME and supernatant was collected three days post transfection. Again, the titer of the produced SIPs was determined by serial dilution of supernatant, infection of fresh BHK-21 cells and immunofluorescence staining against TBEV proteins. In the case of

replicon ΔC two positive cells were found up to a dilution of 10^{-4}, whereas in the case of replicon ΔE only a single positive cell was found up to a dilution of 10^{-4}. Thus, both replicons could be packaged up to a titer of 10^4.

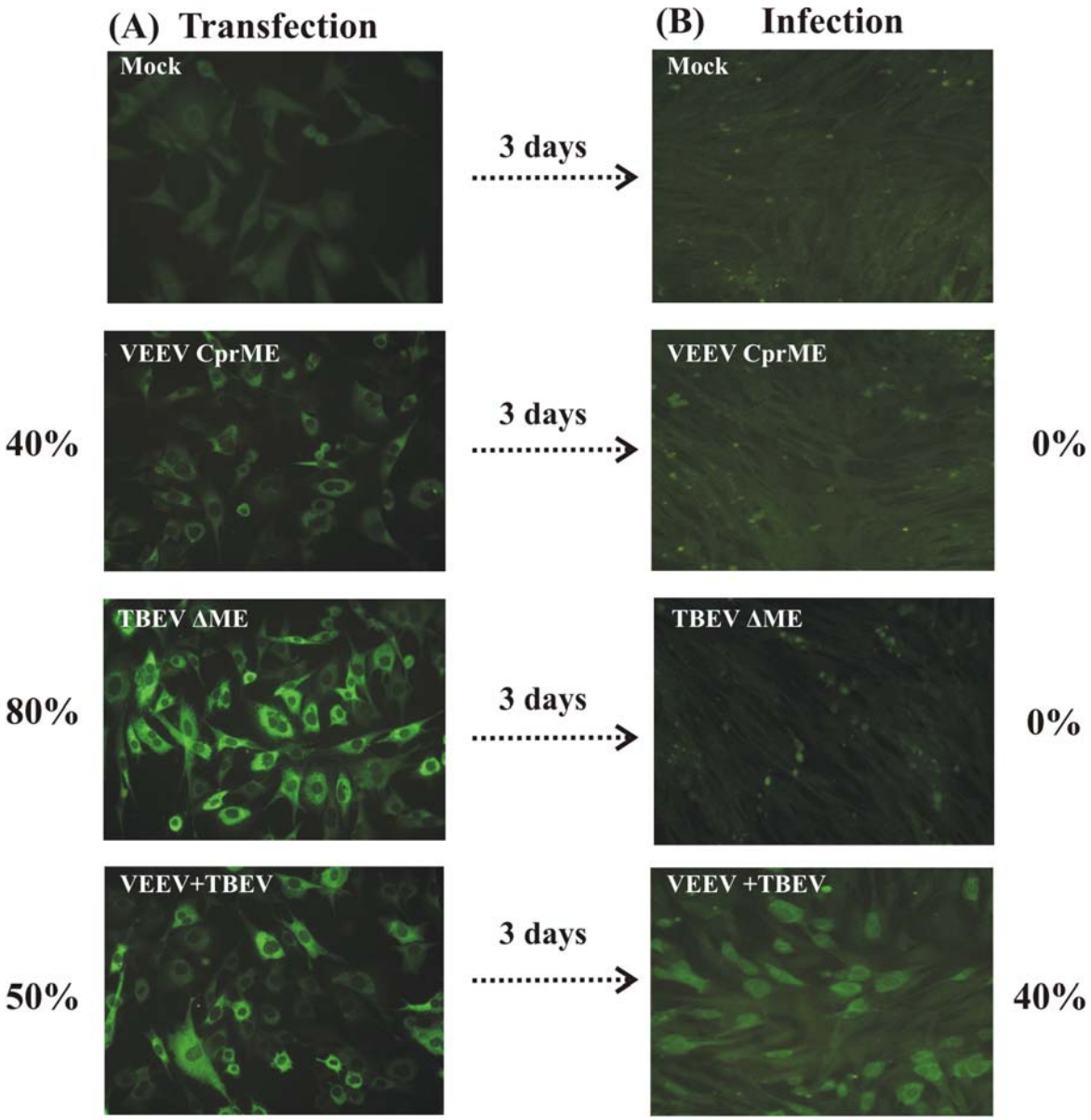


Fig.22. Indirect immunofluorescence of co-transfection and infection experiments with BHK-21 cells. **(A)** Co-transfection of BHK-21 cells with VEEV and TBEV replicon ΔME or each RNA species separately is shown in the left column. Staining was performed 24 hours after transfection. The transfected cell count is given as a percentage next to the picture. **(B)** Infection of fresh BHK-21 cells with supernatant taken three days post transfection is shown in the right column. Immunofluorescence staining was conducted 24 hours after infection with polyclonal TBEV serum.

4.4. Trans-complementation of TBEV SIPs in different cell types

The major goal of this thesis was to produce SIPs containing different replicons that could be used for co-infection experiments. To test whether the above produced SIPs that contained replicons ΔC, ΔE or ΔME, could complement each other, undiluted supernatants from co-transfected cells containing SIPs of replicon ΔC and ΔE as well as replicon ΔC and ΔME were used to infect different cell types successively according to the scheme shown in figure 23. 24 hours post infection, immunofluorescence staining against TBEV proteins was performed. Infection of approximately 10^5 BHK-21 cells with a mixture of either ΔC and ΔME or ΔC and ΔE containing SIPs yielded approximately 10% positive cells (fig.24A). This was in good agreement with the titer of 10^4 of the produced SIPs which has been determined on BHK cells. In contrast, infection with equal amounts of SIPs resulted in less infected cells of mouse embryonic fibroblasts (MEF) or the tick cell line IRE-18 (fig.24A). This indicated that the infectivity of the produced SIPs strongly varied between different cell types. Interestingly, infection of HEK 293T cells resulted in a higher multiplicity of infection than for BHK-21 cells (fig.24A). In summary, the number of infected cells was low with exception of HEK293T cells. To test if complementation between the replicons had occurred after infection, a cell passage was performed. Undiluted supernatant was transferred from infected cells on fresh cells of each respective cell type. Due to the fact that tick cells have a very slow turnover, supernatant was collected after six days post infection whereas supernatants from all other cells were collected after three days. After 24 hours post infection, immunofluorescence staining was performed against TBEV proteins. No infected cells could be observed from BHK-21 cells or MEF cells indicating that no complementation to infectious virus had occurred in these cells (fig.24B). Interestingly, very few positive cells, three in the case of infection with ΔME and ΔC and one in the case of co-infection with ΔC and ΔE SIPs, were observed after infection with supernatant collected from tick cells (fig.24B). This demonstrated that infectious virus particles were produced from co-infected tick cells. Again and in contrast to all other cell types, a high number of infected cells were observed after infection of HEK-293T cells (fig.24B). This showed that HEK 293T cells not only supported infection of TBEV SIPs very efficiently but also that trans-complementation worked well in this cell type. In summary, passages of complementing replicons were only possible in HEK-293T

cells. However, the efficiency of SIP production seemed to be too low to allow trans-complementation and further passages in most cell types. Therefore, we decided to optimize the production of SIPs.

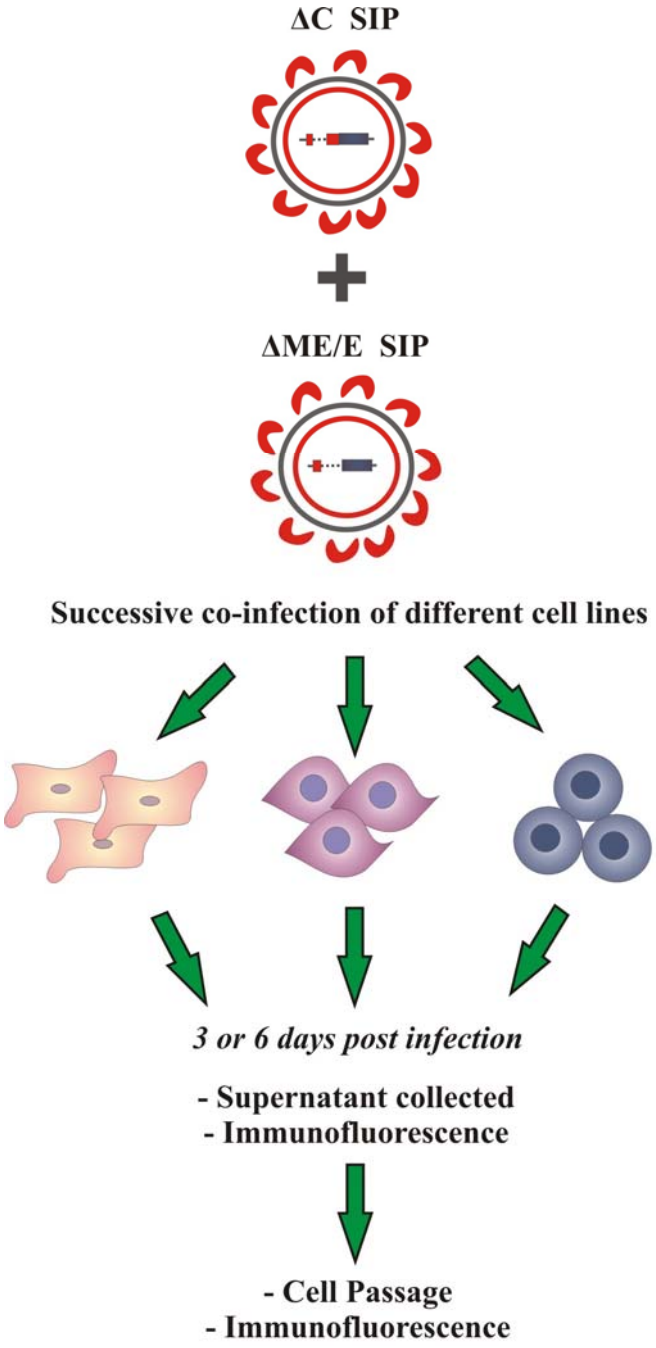


Fig.23. Scheme of successive co-infection with different TBEV SIPs. Various cell lines are co-infected with a SIP pair that could complement deletions of structural genes. Three or six days after infection, supernatants are collected to conduct cell passage and immunofluorescence staining.

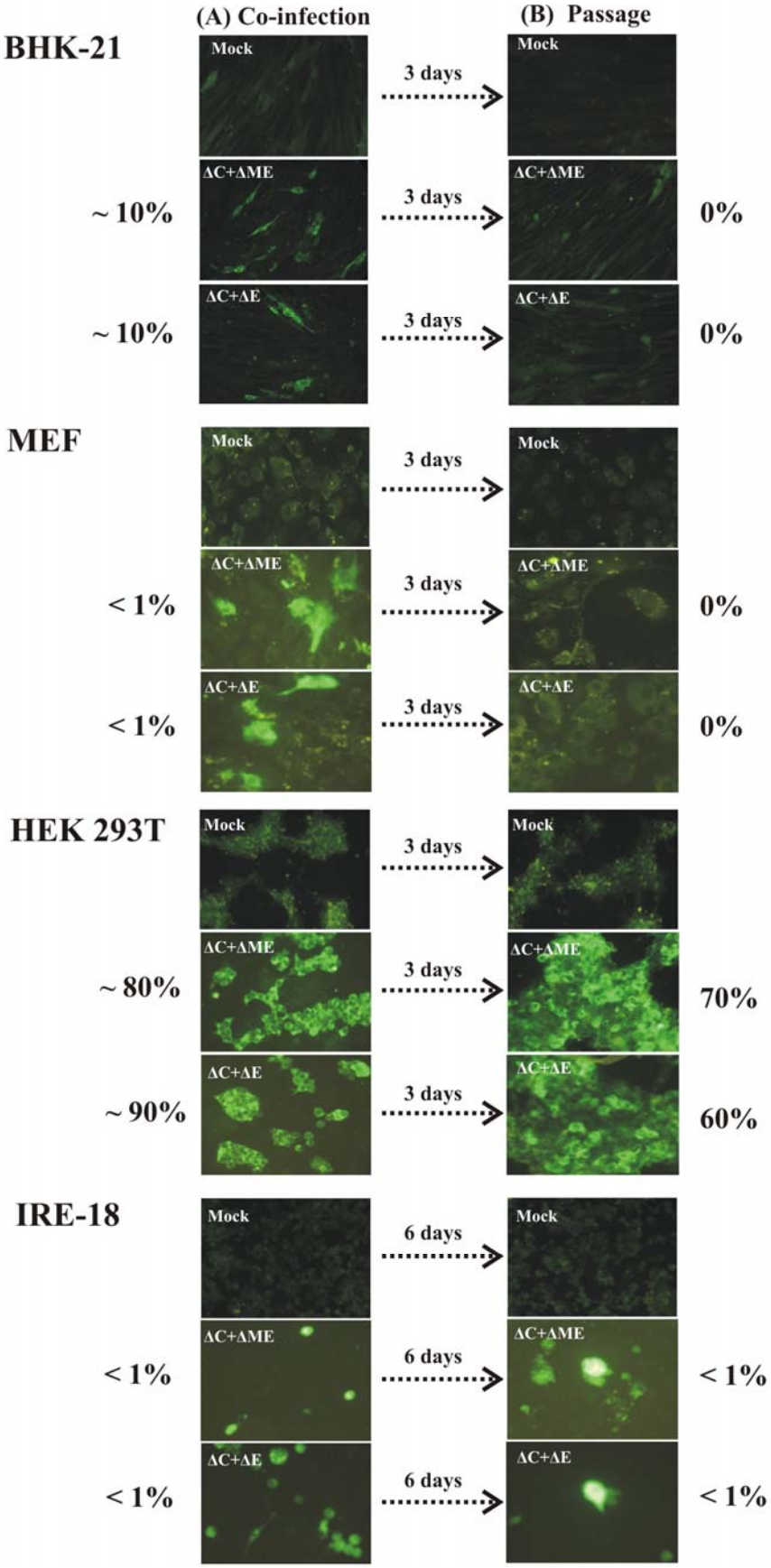


Fig.24. Immunofluorescence of co-infection and passage experiments. (A) Various cell types were co-infected with a mixture of TBEV ΔC and ΔME or ΔE SIPs. Infected cell count was detected by immunofluorescence 24 hours after double infection and is given as a percentage next to the picture. (B) Passage experiments were performed with supernatants collected three or six days after co-infection. Staining was performed six days post infection with polyclonal TBEV serum.

4.5. Optimization of the TBEV replicon packaging system

4.5.1. Transfection of HEK 293T cells.

The co-infection experiments with HEK 293T cells showed that pairs of SIP containing different replicons can efficiently complement each other. Because trans-complementation worked well in HEK 293T cells, we decided to use these cells for the production of TBEV SIPs. For this purpose, VEEV-CprME and TBEV replicons were transfected into HEK-293T cells (fig.25).

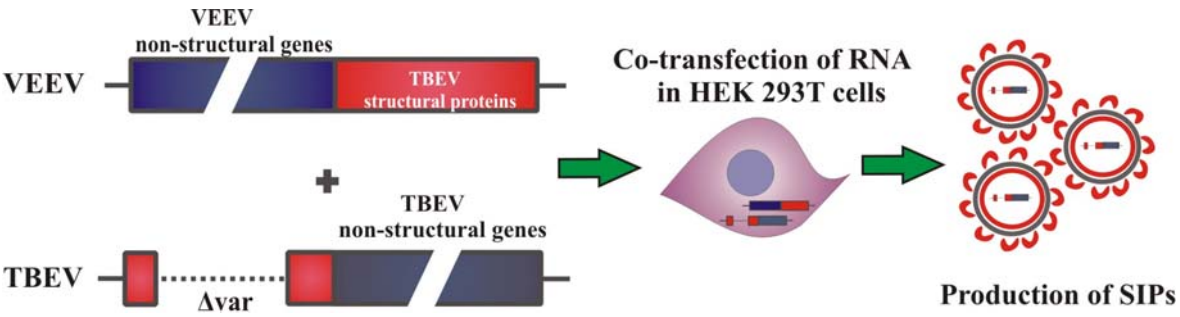


Fig.25. Scheme of the optimized replicon packaging system. VEEV and TBEV replicon RNA were co-transfected in HEK 293T cells. TBEV replicons were trans-packaged by the structural proteins provided by VEEV.

Generally, transfection of HEK 293T cells is performed using standard transfection reagents containing liposomes. We decided to use the TransMessenger Transfection Reagent from Qiagen. Transfection of cells was done according to the manufacturer's instructions. To test SIP production with this transfection method, equal amounts of VEEV-CprME and TBEV ΔME replicon RNA were transfected alone and in combination. 24 hours after Transfection, cells were stained with polyclonal TBEV serum.

The percentage of transfected cells was less than 1% when replicon ΔME or VEEV-CprME RNA was used (fig.26). In addition, co-transfection with both constructs yielded only few positive cells. Thus, the efficiency of RNA transfection by employing TransMessenger Transfection Reagent was not very high and we decided to try an alternative transfection method to increase the transfection efficiency of HEK-293T cells.

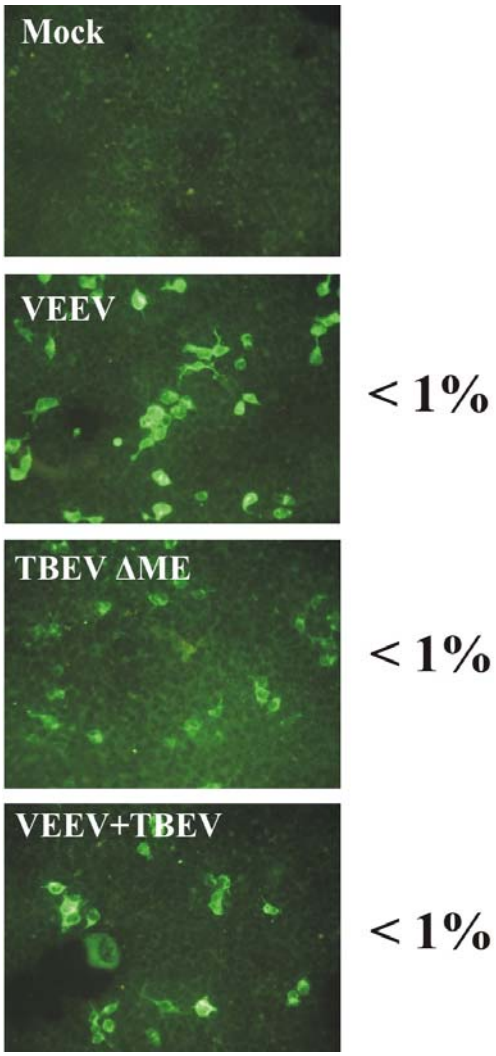


Fig.26. Immunofluorescence of TransMessenger transfected HEK 293T cells. Indirect immunofluorescence took place 24 hours after transfection. All transfections were performed with 1µg of RNA each. The percentage of transfected cells is shown next to the picture.

In a next step, transfection of HEK 293T cells could be achieved by electroporation. To establish a protocol for the electroporation of HEK 293T cells, different setups of electroporation were chosen and 5µg of replicon RNA were transfected under the different conditions. To monitor the efficiency of electroporation, immunofluorescence staining was performed 24 hours after transfection. All electroporation experiments were performed with $1x10^7$ cells in sterile 1 x PBS as electroporation buffer and all showed cell debris. The first experimental setup was conducted under the following conditions: 0.245 kV, 960 µF, one pulse. Less than 1% of the cells were transfected as is demonstrated in figure 27A. Pulsing cells twice with 0.245 kV and 960 µF yielded no transfected cells at all (fig.27B). In the third attempt, cells were pulsed once with 0.260 kV, 550 µF which resulted in less than 1% of transfected cells (fig.27C). Best results were obtained with conditions: 0.260 kV, 960 µF, one pulse. As shown in figure 27D 10% of cells were transfected.

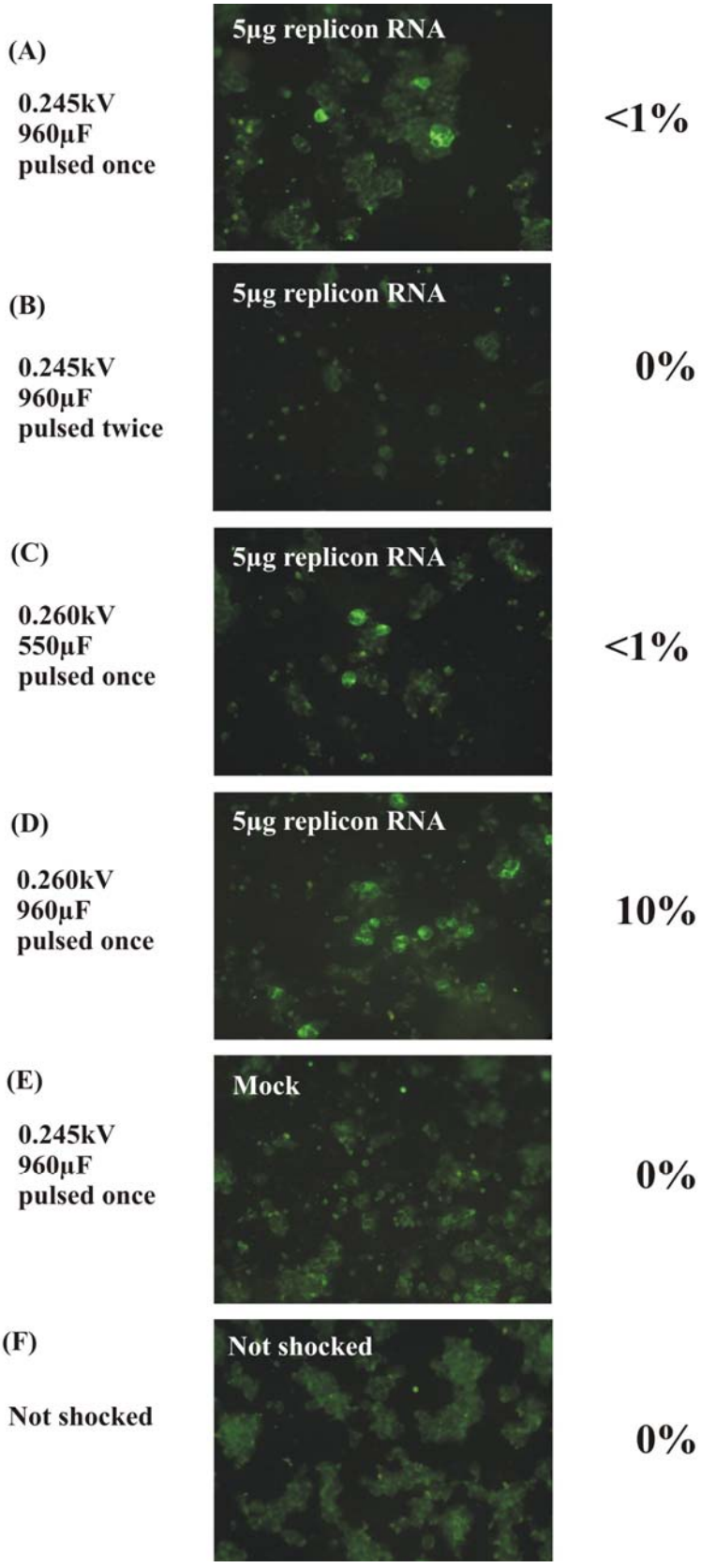


Fig.27. Immunofluorescence after electroporation setup trail for HEK 293T cells. Various electroporation setups were investigated all employing 1xPBS as electroporation buffer. Best results and an electroporation efficiency of 10% were obtained with conditions stated in picture (D).

To further increase electroporation efficiency, PBS was replaced with HeBS electroporation buffer (Chu, Hayakawa et al. 1987). In addition, the RNA was purified after *in vitro* RNA synthesis. Then, HEK 293T cells were transfected with 1xHeBS under electroporation conditions: 0.260 kV, 960 µF, one pulse. As shown in figure 28, the efficiency of electroporation was still less than 10% if non-purified RNA was used. In contrast, HEK 293T transfected with purified RNA yielded 75% positive cells. Therefore, all further electroporation experiments were done with purified RNA in HeBS buffer under the following conditions: 0.260 kV, 960 µF, one pulse.

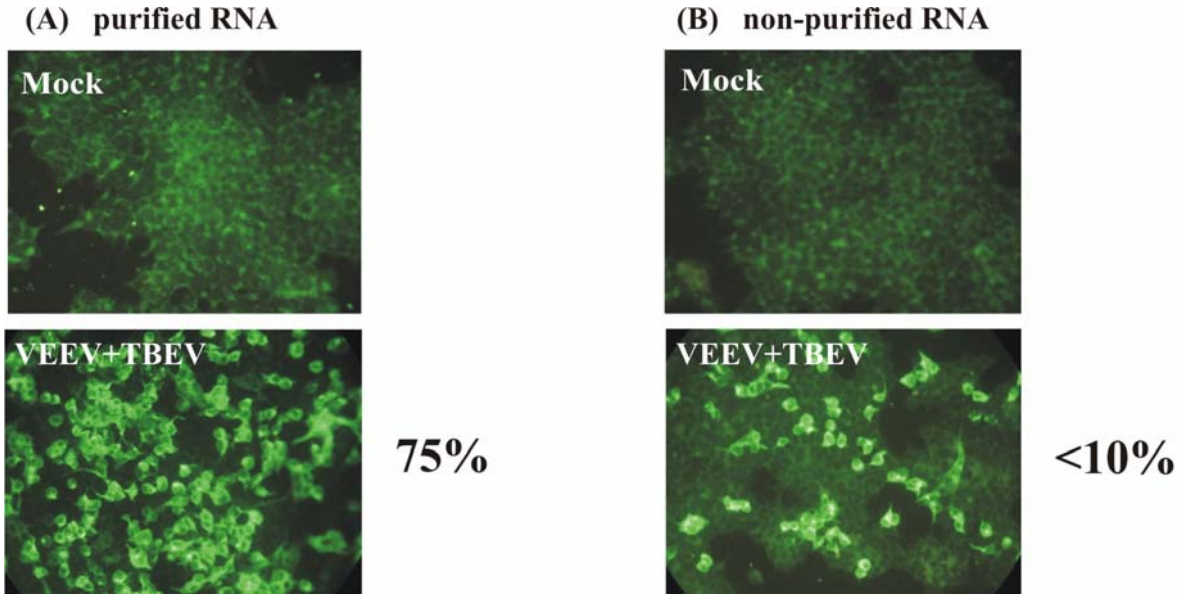


Fig.28. Optimization of electroporation setup for HEK 293T cells. $1x10^7$ cells in 1xHeBS were co-electroporated with (A) purified TBEV ΔME and VEEV RNA and (B) non-purified RNAs. Indirect immunofluorescence was performed 24 hours after co-transfection. The percentage of transfected cells is given next to the picture.

HEK 293T cells displayed a high metabolic rate and rapid growth. During cell cultivation, the medium was acidified soon after the seeding of cells. Acidification plays an important role in the maturation of TBEV virions as well as in the fusion process for the entry of virions. In the fusion process, TBEV surface proteins undergo conformational changes activated by protonation by an acidic pH (Fritz, Stiasny et al. 2008). This structural rearrangement starts at pH 6.8 and is irreversible. In cell culture media, acidification can induce this irreversible step but leads to the inactivation of virions because they are no longer fusiogenic. To monitor the acidification of cell culture medium by HEK-293T cells, the pH was measured after transfection. HEK 293T cells were co-transfected with TBEV replicon and VEEV RNA according to the established electroporation protocol described above. Next, three different cell counts ($1x10^5$, $2x10^5$, $4x10^5$) of transfected cells were

seeded followed by standard cell cultivation. Changes in pH were followed by a time course measurement and are given in figure 29. 24 hours after Transfection, the pH of all media was 8.0. The first significant decrease in pH from 8 to 7.4 was detected 48 hours after transfection and with a seeding of $4x10^5$ cells. In the case of seeding $2x10^5$ and $4x10^5$ cells, the pH dropped to 7.5 after 72 hours. Subsequently, the pH value of all media declined continuously till reaching a value of about 6.0 to 6.4 at day 7.

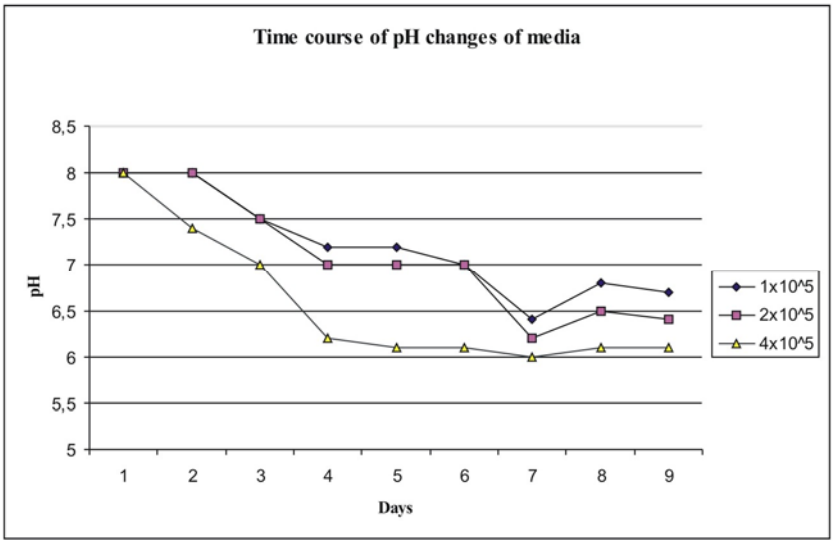


Fig.29. Time course of pH change of media during HEK 293T cell cultivation. Acidification of the medium was monitored by daily pH measurement. The first significant drop of pH value was recognized two days after transfection and seeding of cells.

Considering that the conformational change of protein E starts at pH 6.8, collecting the supernatants prior to the decline of the pH to below 6.8 was necessary. In respect of the observations of pH changes presented in figure 29, $1x10^7$ cells were co-transfected, supernatants were taken 48 hours after transfection, and the pH value was controlled. Thus, supernatants were collected two days after co-transfection. Again, replicon ΔC, ΔME, ΔE were co-transfected with VEEV-CprME according to the above described experimental procedure. To determine the titer of SIPs, both dilution series and infection of fresh HEK 293T cells were performed. Again, immunofluorescence staining was used to detect infected cells. Packaging of replicon ΔC in HEK 293T cells yielded 10^3 single-round infectious particles and a titer of 10^4 was achieved with replicons ΔME and ΔE.

This demonstrated that packaging TBEV replicons in HEK 293T cells was successful. However, there was no increase in titers of packaged replicons in BHK-21 cells to HEK 293T cells.

4.5.2. Cloning of phSV40-CprME

In a final attempt to improve the packaging of TBEV replicons, we used a DNA expression vector phSV40 for the expression of TBEV CprME. First, the region coding for CprME including the signal sequences of NS-1 at the end of protein E were amplified from template pTNd/5' by a PCR reaction with primers containing the appropriate flanking region and additional *Nhe*I and *Xba*I restriction sites (fig.30A). In the second step, pHRSV40 was prepared by a digest with *Nhe*I and *Xba*I restriction enzymes. As backbone for the final expression vector, the larger fragment of this digest was purified from an agarose gel. Then the PCR product was subjected to restriction digest with *Nhe*I and *Xba*I. The two purified DNA fragments were ligated; electroporated into bacteria and the desired pHS40 containing colonies were picked and subjected to large scale plasmid purification. The final phSV40-CprME expression plasmid had the originally encoded Renilla gene replaced with the sequence coding for CprME from TBEV (fig.30B)

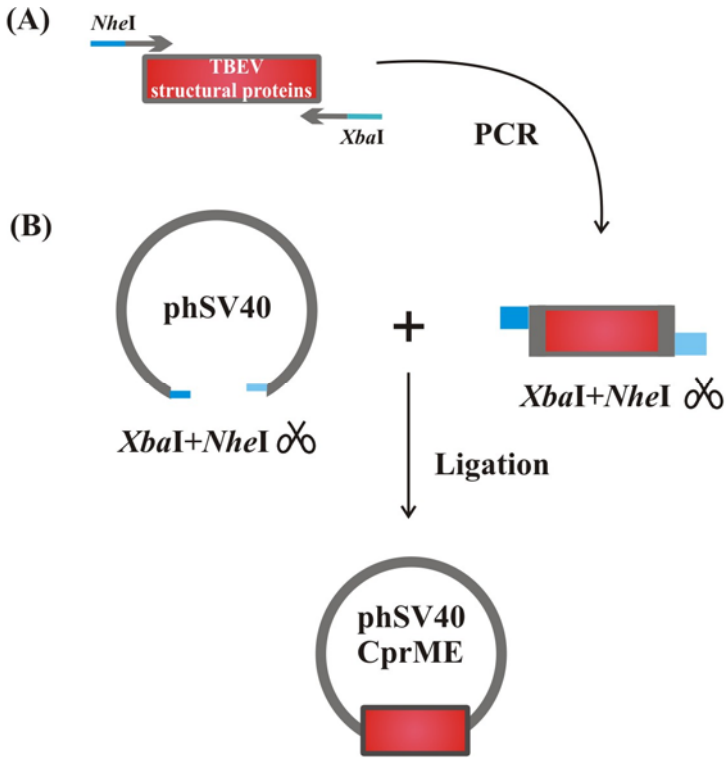


Fig.30. Scheme of the cloning strategy for phSV40-CprME. Cloning of phSV40 coding of TBEV CprME involved (A) PCR reaction with primers with *Xba*I and *Nhe*I restriction sides and (B) cloning of the PCR product into the digested vector.

4.5.3. TBEV replicons are packaged by co-transfection with pHSV40-CprME.

To test whether TBEV replicons could be efficiently packaged by co-transfection with pHSV40-CprME, TBEV replicon RNA was transfected into HEK 293T cells by electroporation with the following conditions: 0.260 kV, 960 µF, one pulse. Since using electroporation to transfect the cells with DNA was inefficient, the DNA plasmid phSV40-CpreME was transfected 24 hours after electroporation with Lipofectamine 2000 reagent purchased from Invitrogen (fig.31).

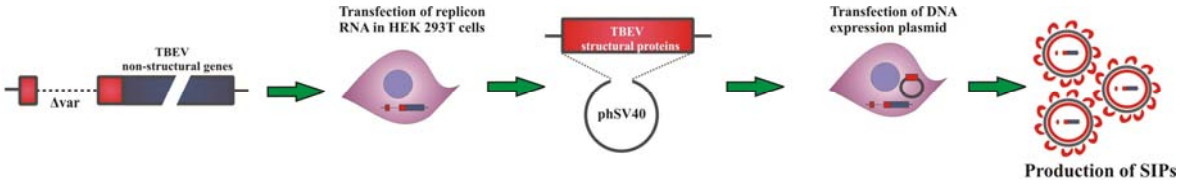


Fig.31. Scheme of the alternative replicon packaging system. First, TBEV replicon RNA were transfected in HEK 293T cells via electroporation. Secondly, DNA expression plasmid encoding a SV40 promoter and all structural genes of TBEV was transfected by employing Lipofectamine reagent. Missing structural proteins of replicons were provided *in trans* by phSV40 resulting in the production of TBEV SIPs.

Transfection of TBEV replicon ΔME yielded 50% positive cells (fig.32A). Transfection of DNA plasmid phSV40-CprME with Lipofectamine showed 70% of cells to be positive. Immunofluorescence after successive transfection of RNA and DNA revealed that up to 90% of cells expressed TBEV proteins. This indicated that the two consecutive transfection method yielded a high transfection efficiency. 24 hours after transfection of phSV40-CprME, supernatants were harvested and transferred onto fresh HEK 293T cells to test whether the production of SIPs was successful. Again, indirect immunofluorescence staining was used to detect infected cells. As expected, the transfection of pHSV40-CprME did not result in the production of SIPs and therefore no infected cells were detected (fig.32B). Similar results were obtained when supernatant was transferred from cells transfected with replicon ΔME which is in good agreement with previous results. However, infection with supernatant from cells co-transfected with pHSV40-CprME and replicon ΔME resulted in 70% infected cells.

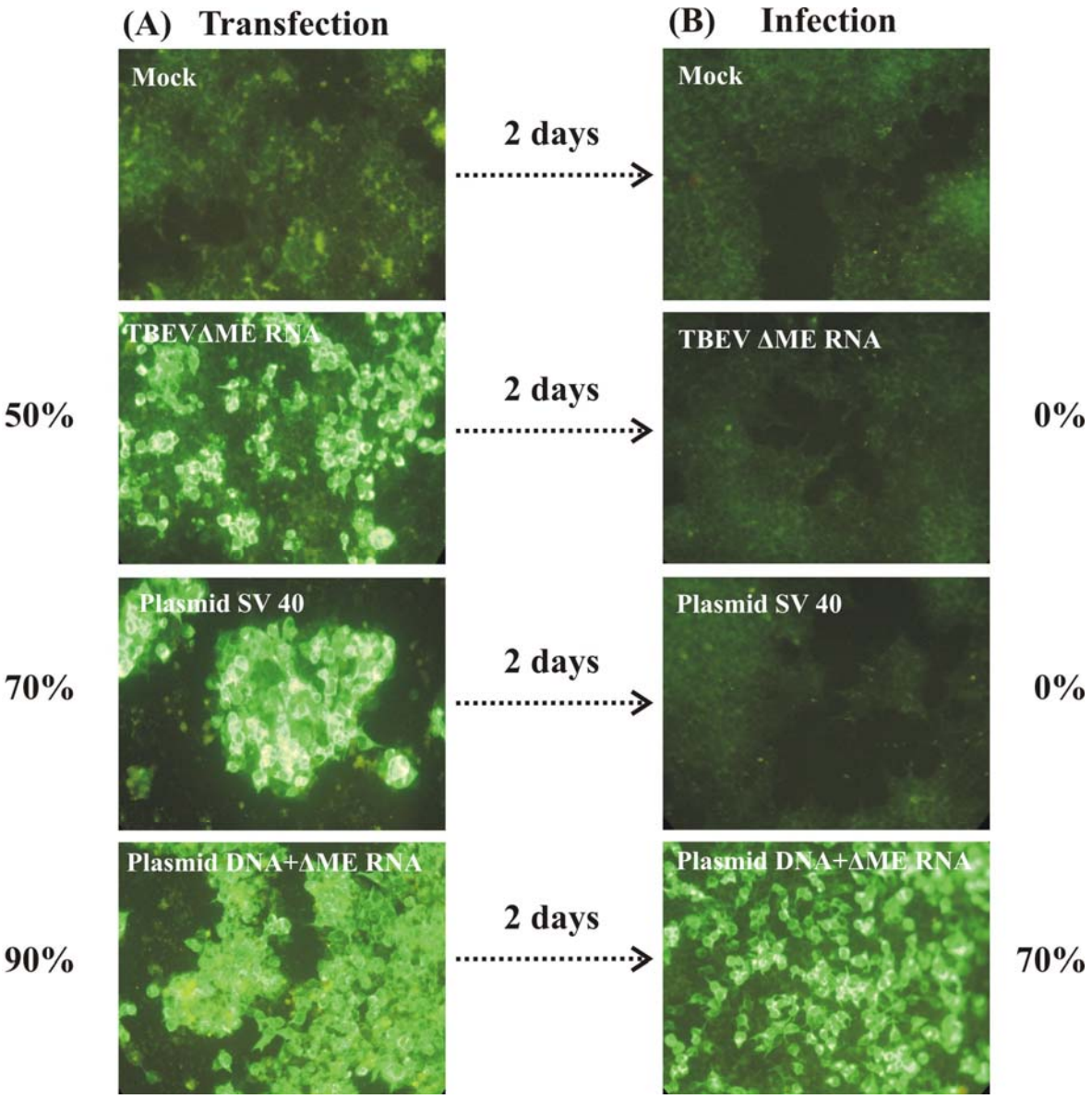


Fig.32. Trans-packaging TBEV replicons with DNA expression plasmid in HEK 293T cells. (A) Transfection of the two components of the packaging system occurred differently. Replicon RNA was transfected via electroporation while phSV40 DNA was transfected using Lipofectamine reagent. The efficiency of transfection was monitored by immunofluorescence 24 hours after. (B) Infection of fresh cells with supernatant taken two days after first transfection is shown. Percentage of positive cells is given next to the picture.

To quantify the amount of packaged TBEV replicons, the titer of SIPs was determined as before. Supernatants were harvested two days after transfection with TBEV replicon RNA. Dilution series and infection of fresh HEK 293T cells were conducted as described in part 4.3.

All three packaged replicons ΔC, ΔME, and ΔE reached a titer of 10^4 single-round infectious particles. Thus, packaging of TBEV replicons with the help of the DNA expression plasmid phSV40 encoding the structural proteins replicon resulted in a similar SIP production efficiency as described with the other systems.

In summary, a trans-packaging system for TBEV replicons could be established. However, the produced amounts of packaged replicons were not sufficient for application to a recombination trap to study recombination in flaviviruses. Two different approaches to optimize the system were performed which did not succeed in increasing the packaging efficiency.

5. Discussion

Positive, single-stranded RNA viruses have been successfully employed as vectors for heterologous gene expression. In various studies, viral vectors have been developed from non-infectious replicons of picornaviruses, flaviviruses, and alphaviruses (reviewed in (Hewson 2000)). Among those, the most advanced replicon gene expression systems are derived from the members of the genus *Alphavirus*. Replicon systems derived from SINV, SFV, and VEEV have been established so far (Liljestrom and Garoff 1991; Pushko, Parker et al. 1997; Schlesinger 2000). In a wild type virus genome, efficient subgenomic promoters drive the expression of the alphavirus structural proteins. Replacement of the structural genes with the desired heterologous genes yields a non-infectious alphavirus expression vector.

In this study, we utilized the non-cytopathic VEEV replicon vector which originated from the group of Ilya Frolov (University of Texas) to provide the structural proteins CprME of the flavivirus TBE *in trans* to establish a trans-packaging system for packaging of TBEV replicons into virus particles. Virions that contain a non-infectious virus genome are only capable of infecting a cell once because no virus progeny can be produced by the packaging defective replicon genome. Therefore, virus particles produced by providing the structural proteins *in trans* are called single round infectious particles (SIPs).

The initial step was the cloning procedure of pVEEV-CprME which was complicated by the fact that the TBEV sequence of CprME comprised a second *Mlu*I restriction site. Digestion of pVEEV-CprME with *Mlu*I was necessary for linearization of the plasmid in order to create a termination point for RNA synthesis. Therefore, the second *Mlu*I restriction site in the CprME sequence had to be mutated to allow the synthesis of the full-length viral RNA from this plasmid. This problem was solved by subcloning the sequence into the backbone of the vector pSG5 followed by side-directed mutagenesis of the *Mlu*I restriction side.

The viral full-length RNA was generated by *in vitro* RNA synthesis from pVEEV-CpreME. Interestingly, *in vitro* transcription of pVEEV-CprME did not only yield the expected full-length RNA but also a second shortened RNA fragment. This additional RNA fragment was also present when RNA was transcribed from the original VEEV replicon and the group of Ilya Frolov observed this byproduct as well (personal communication). One possible explanation for the generation of this small RNA fragment was the presence of a cryptic promoter of SP6 polymerase in the sequence coding for the

non-structural proteins of VEEV. Estimation from the RNA gel indicated that the short RNA fragment was synthesised to greater amounts than the full-length RNA. In addition, the amount of full-length RNA further decreased after a RNA purification step. This led to the synthesis of very little full-length RNA and consequently fewer cells were transfected with VEEV-CprME than after transfection with a TBEV replicon. Although VEEV-CprME was transfected less efficiently, we could demonstrate that packaging of TBEV replicons by cotransfection with VEEV-CprME was successful in BHK-21 cells. However, packaging of replicons ΔME, ΔC, and ΔE reached only titers of 10^4 infectious units (IU) per ml 72 hours after co-transfection. In a previous study, WNV replicons were successfully packaged upon transfection into BHK-21 cells which stably expressed a VEEV replicon encoding West Nile virus CprME (Fayzulin 2006). Due to this, WNV replicon genomes were trans-packaged in virus-like particles to titers of 10^5 to 10^6 infectious units per ml 75 hours after transfection. Although, the titers which were obtained by the system presented in our work could not be directly compared to titers of packaged replicons in other studies, we assumed that our TBEV replicon packaging system did not achieve the highest possible efficiency of trans-packaging replicons. This was probably caused by the low efficiency of transfection of VEEV-CprME.

Nevertheless, we applied our produced SIPs containing different replicons to co-infection experiments in order to investigate whether TBEV replicons ΔC and ΔME or ΔC and ΔE could complement each other in the production of infectious virus particles. By co-infecting different cell types with SIPs of replicon ΔME and ΔC as well as ΔE and ΔC, we showed that (i) complementation only worked when the multiplicity of infection was greater than 10% (ii) the infectivity of the produced SIPs strongly varied between the different cell types and that (iii) the numbers of infected cells were generally too low to allow efficient complementation with the exception of the HEK 293T cells.

Flaviviruses seem to exhibit a very low rate of recombination (unpublished data). Therefore, studies on recombination depend on the observation of recombination in multiple rounds of infection. However, since co-infection experiments revealed that the infectivity of the produced SIPs was too low to allow trans-complementation and cell passages, the selection of full-length viruses in limiting dilution passages was not possible in the desired cell types (e.g. tick cells). As a consequence, we decided to optimize the replicon packaging system. Infectivity and trans-complementation was very low in the cell types used except for HEK 293T cells so we decided to exploit these cells for packaging of TBEV replicons. In order to transfect HEK 293T cells with our replicon constructs, we

first utilized a liposome based transfection reagent which did not prove satisfactory in terms of efficiency of transfection. Therefore, transfection was performed by electroporation with a newly established protocol yielding efficient transfection rates of up to 75%. Yet another problem arose when packaging TBEV replicons in HEK 293T cells. Due their high turnover, HEK 293T cells quickly acidified the medium during cell cultivation (observed in cell culture) which was critical for the production of SIPs. Flavivirus fusion is induced at low pH by a conformational change of protein E in the endosome of the host cell. This is an irreversible step and exposure of virions to low pH in the medium can inactivate the virus particles. To avoid this, pH changes were controlled during SIP production and supernatants were harvested prior to the critical change in the pH value. However, the efficiency of SIP production was not improved and packaging TBEV replicons in HEK 293T cells also yielded only titers of 10^4 IU per ml for replicons ΔC, ΔME, and ΔE. Switching the cell type employed for packaging from BHK-21 to HEK 293T cells did not improve the efficiency of the SIP production at any level. This indicated that upon co-transfection with replicons and VEEV-CprME, the process of replicon packaging is independent of the cell type in which packaging occurred. It was stated before that the *in vitro* transcription of VEEV-CprME yielded low amounts of full-length viral RNA. Therefore, upon transfection this might have caused a limitation of SIP production due to CprME being present in the cells only at very low levels. Hence, it must be concluded that the employed VEEV based expression system did not provide the most efficient conditions for packaging TBEV replicons and the problem of packaging efficiency must be approached by changing the expression system that provided CprME.

In a final attempt to optimize the SIP production, we altered the VEEV based expression system which provided CprME *in trans* to the DNA expression plasmid phSV40. After successful cloning of CprME into the phSV40 backbone, the TBEV replicon RNA and the phSV40-CprME DNA expression plasmid were sequentially transfected into HEK 293T cells. TBEV replicon RNA was transfected first by electroporation followed by transfection of the phSV40-CprME DNA construct with Lipofectamine reagent. This sequential transfection of the cells resulted in an excellent transfection efficiency of 90%. Upon infection with supernatants from the transfected cells, all three packaged replicons ΔC, ΔME, and ΔE reached a titer of 10^4 SIPs per ml. This demonstrated that SIPs were successfully produced. Unfortunately the SIP production was still not higher than with previously used systems. This means that despite our extensive efforts we could not produce TBEV SIPs to satisfying titers. One possible explanation for the low efficiency of

SIP production could lie in the nature of the TBEV replicons used. The efficiency of trans-packaging systems depends on the way the structural proteins are provided. In a wild type virus genome, assembly of virions involves concerted processing of all three structural proteins. Although all of these proteins were provided as a unit in our expression system, packaging might have been inhibited by structural proteins that were encoded on TBEV replicon genomes. Therefore, future experiments to optimize this packaging approach will include the construction of TBEV replicons that lack all three structural proteins. In summary, our attempts to produce TBEV SIPS were successful and may prove useful to studies that do not rely on high titers.

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