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Uukuniemi virus-like particles: a model system for bunyaviral assembly

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To my wonderful parents

Ge mej kraft att förändra det jag kan
Tålamod att acceptera det jag inte kan förändra
Och vishet att se skillnaden

Carolines klokbok

Skapande består av en massa försök

Populärvetenskaplig sammanfattning

Alla levande organismer vi ser omkring oss är uppbyggda av celler. Det finns i stort sett två olika sorter, eukaryota (t.ex. djur och växtceller) och prokaryota (t.ex. bakterieceller) celler. Virus är inga celler utan små parasiter som lever inuti andra celler, både eukaryota och bakterieceller. Det finns en mängd olika virus som har grupperats in i familjer. Virus inom samma familj delar egenskaper såsom storlek och arvsegenskaper. Olika virus har genom åren specialiserat sig på att infektera och leva i olika celler och organismer. Vissa virus är så specialiserade att de bara kan infektera en speciell art. Poliovirus kan t.ex. endast infektera människor och apor. Man kan då utrota viruset genom att vaccinera hela jordens befolkning. Andra virus såsom Influensavirus kan infektera många olika arter t.ex. människa, fågel och gris. Vissa arter utvecklar ingen sjukdom och sprider bara viruset vidare medan andra orsakar akut sjukdom. Virus sprids ofta med insekter och fästingar som biter och suger blod. Viruset förs då över till värddjuret där de infekterar och förökar sig. Ett sätt att identifiera nya virus är att gå ut i skogen, samla myggor och fästingar och sedan försöka isolera virus. Man kan givetvis också isolera virus från sjuka människor och djur. I början av 60-talet isolerades *Uukuniemiviruset* (UUK virus) från en fästing som plockades i byn Uukuniemi, nära den Ryska gränsen i Finland. Det viruset karaktäriserades under 70-, 80- och 90- talet och har länge varit ett modellvirus för en snabbt växande virus-familj (över 350 medlemmar) som heter *Bunyaviridae.*

UUK viruset är ett ganska okänt virus. Det beror troligtvis på att detta virus inte infekterar människor och orsakar därför inte någon känd sjukdom. Andra närbesläktade virus i samma familj orsakar däremot farliga sjukdomar, såsom blödarfeber. Det är svårt att studera dessa farliga virus därför att man behöver speciella laboratorier med mycket hög säkerhet för att förhindra smitta och spridning.

Min avhandling handlar om UUK virus. Jag har studerat hur de olika virusdelarna sätts ihop inne i en cell för att kunna bilda nya virus. Uukuniemiviruset består av ett RNA-genom (tre olika delar) som innehåller genetisk information för förökning, ett membran som skyddar och kapslar in RNA-genomet och fyra proteiner. Varje virus partikel stjäl en liten bit membran från den infekterade cellen när det bildas, och membranet liknar det membran som omger varje djurcell. Två av de fyra proteinerna kallas glykoproteiner och sitter i membranet. Glykoproteinerna ger stabilitet och är viktiga för virus-cell kontakt och infektion. Ett annat protein binder och skyddar genomet (nukleoprotein), det sista proteinet förökar genomet inne i cellen.

Vi har utvecklat en metod för att studera bildandet av nya viruspartiklar inne i cellen. Med denna metod tillverkar man ”virus-lika partiklar”, dessa partiklar består av olika virusdelar men inte alla. På så sätt kan man studera, ändra och mutera olika virusbeståndsdelar utan att tillverka riktiga virus, som kan infektera och orsaka sjukdom. Med hjälp av denna metod har vi identifierat vilka delar av membranproteinerna som samverkar med genomet och är viktiga för packning av genomet till ett virus. Vi har också studerat hur nya viruspartiklar bildas i cellen, och vilka delar av membran-proteinerna som är involverade i detta.

Genom att utveckla ett system för att tillverka virus-lika partiklar så kan man studera farliga virus från *Bunyaviridae* familjen utan att att bli smittad och sjuk. Det är viktigt att studera basala mekanismer såsom hur virus bildas innuti celler för då lär vi känna dem bättre. Därienom kan nya mediciner utvecklas som fokuserar på att påverka och förhindra just det steget i virusets livscykel, och därigenom lindra eller förhindra sjukdom.

Abstract

Viruses are intracellular parasites that are unable to multiply except when inside a living cell of a host. Outside their host they remain inert. There are many different types of viruses that are classified into different virus families according to their size, nucleic acid composition (RNA or DNA), or the composition of their outer shell (naked or enveloped). Following entry, negative stranded viruses release their genome which is subsequently replicated, transcribed, and the different viral components are assembled into new virus particles, which are released from the host cell and ready to infect new cells. This thesis focuses on how the different viral parts assemble into an infectious particle inside the cell as well as on the identification of parts in the RNA genome important for viral replication.

Uukuniemi virus (UUKV), a prototypic member of the *Bunyaviridae* family, has a segmented RNA genome with negative polarity. The three segments encode four structural proteins, two glycoproteins (G_N and G_C) located in the envelope, one nucleoprotein (N protein), and a RNA-dependent RNA polymerase (L protein). Flanking these open reading frames (ORFs) are non-coding regions (NCRs) containing *cis*-acting signals important for viral transcription, replication, encapsidation and packaging. These NCRs encompass one variable region, and one highly conserved region located in both the 5' and 3' terminal ends of the three segments, which are complementary to each other.

To study the function of the NCRs we used a minigenome system developed for UUKV. In this system the viral protein coding sequence is replaced by sequences encoding a reporter protein. This minigenome is transfected into cells together with the N and L protein necessary for replication and transcription, after which reporter protein expression can be measured. In the first paper we studied the variable region of the NCRs and its effect on promoter strength and packaging efficiency. We performed this by comparing the activity of minigenomes that contained the NCRs derived from all three different segments. We found that the variable region is not only important for the regulation of promoter activity but also for packaging efficiency, since the three different minigenomes all showed different reporter activity.

Next the assembly of the UUKV inside the cell was examined. In order to study packaging and assembly, we first developed a virus-like particle (VLP) system for UUKV. With the addition of the glycoprotein precursor encoding both glycoproteins G_N and G_C to the minigenome system, VLPs containing the minigenome are produced and released into the supernatant. These particles are able to infect new cells where reporter protein expression can be detected. Characterization of these VLPs revealed that they have similar size and surface morphology as *wild-type* UUKV. Moreover we demonstrated that only the two glycoproteins are required for generating VLPs, suggesting that UUKV budding is driven by the two glycoproteins.

We further analyzed the importance of specific amino acids in the cytoplasmic tail of both glycoproteins, G_N and G_C, for packaging of the RNA genome into VLPs and the budding of UUKV. This was done by performing an alanine scan of the G_N cytoplasmic tail (81 amino acids) and the G_C cytoplasmic tail (5 amino acids), and analyzing the effect of these mutations on particle formation in our VLP system. We identified three regions in the G_N tail (amino acids 21-25, 46-50 and 71-81) that are important for minigenome transfer of the VLPs. A more detailed analysis showed, four amino acids in the G_N cytoplasmic tail involved in the packaging interaction with the ribonucleoproteins while two other amino acids are important for the budding into the Golgi compartment. Finally three amino acids in the G_N and two in the G_C cytoplasmic tail are important for the correct localization of the two glycoproteins in the cell.

In conclusion, we show a mechanism of UUKV assembly and demonstrate the usefulness of our experimental system. We also postulate that the VLP system is a useful tool for analyzing packaging, assembly, and budding for other members of the *Bunyavidae* family.

List of original publications

This thesis is based on the following articles, which are referred to by their Roman numerals I to IV

I

Kirsten Flick, [#] Anna Katz, [#] ANNA K ÖVERBY, [#] Heinz Feldmann, Ralf F Pettersson and Ramon Flick. 2004. Functional analysis of the non-coding regions of the Uukuniemi virus (*Bunyaviridae*) RNA segments. Journal of Virology, **78**, 11726-11738
[#] authors contributed equally to this work

II

ANNA K ÖVERBY, Vsevolod Popov, Etienne PA Neve and Ralf F Pettersson. 2006. Generation and analysis of infectious virus-like particles of Uukuniemi virus (*Bunyaviridae*), a useful system for studying bunyaviral packaging and budding.
Journal of Virology, **80**, 10428-10435

III

ANNA K ÖVERBY, Ralf F Pettersson and Etienne PA Neve. 2007. The glycoprotein cytoplasmic tail of Uukuniemi virus (*Bunyaviridae*) interacts with the ribonucleoproteins and is critical for genome packaging. Journal of Virology, **81**, 3198-3205

IV

ANNA K ÖVERBY, Vsevolod Popov, Ralf F Pettersson and Etienne PA Neve. The cytoplasmic tails of Uukuniemi virus (*Bunyaviridae*) G_N and G_C glycoproteins are important for intracellular localization and budding of VLPs. Submitted

Papers not included in this thesis

Laure R Deflubé,[#] ANNA K ÖVERBY,[#] Kerstin Angner, David A Stein, Patrick L Iversen and Ramon Flick. Interference of bunyaviral gene expression *in vitro* with antisense morpholino oligomers. Manuscript
[#] authors contributed equally to this work

Abbreviations

aa	amino acid
BHK cells	baby hamster kidney cells
BiP	IgG heavy chain binding protein
BSL2	bio safety laboratory level 2
BUNV	Bunyamwera virus
CAT	chloramphenicol acetyltransferase
CCHFV	Crimean-Congo Hemorrhagic Fever virus
cRNA	complementary RNA
DI particles	defective interfering particles
ER	endoplasmitic reticulum
ERGIC	ER-Golgi intermediate compartment
GFP	green fluorescent protein
G_C	C terminal glycoprotein
G_N	N terminal glycoprotein
G_N/G_C	glycoproteins
h	hour
HTNV	Hantaan virus
IGR	intragenic region
L protein	RNA dependent RNA polymerase
L segment	large RNA segment
LACV	La Cross virus
LCMV	lymphocytic choriomeningitis virus
M protein	matrix protein
M segment	medium RNA segment
mRNA	messenger RNA
N protein	nucleoprotein
NCRs	non-coding regions
NSm	non-structural protein m
NSs	non-structural protein s
nt	nucleotide
ORF	open reading frame
p110	UUKV glycoprotein precursor
p.i.	post infection
PolI	RNA polymerase I
RNPs	ribonucleoproteins
RVFV	Rift Valley fever virus
S segment	small RNA segment
TM	trans membrane
TSW	Tomato Spotted Wilt virus
UUKV	Uukuniemi virus
VLP	virus-like particle
vRNA	viral RNA
wt	wild type

Content

Introduction

Secretory pathway

Both cellular and viral proteins destined for the plasma membrane travel along the highly conserved route called the secretory pathway. It consists of a complex highly ordered series of membrane bound compartments through which proteins are transported for sequential maturation steps (Rothman and Orci, 1992). The mechanisms by which proteins enter and travel between these compartments have been elucidated by morphological, biochemical and genetic studies. The different compartments include, the endoplasmitic reticulum (ER), ER-Golgi intermediate compartment (ERGIC), and the *cis*- *medial*- and *trans*-compartments of the Golgi network and the endosomes (Fig. 1).

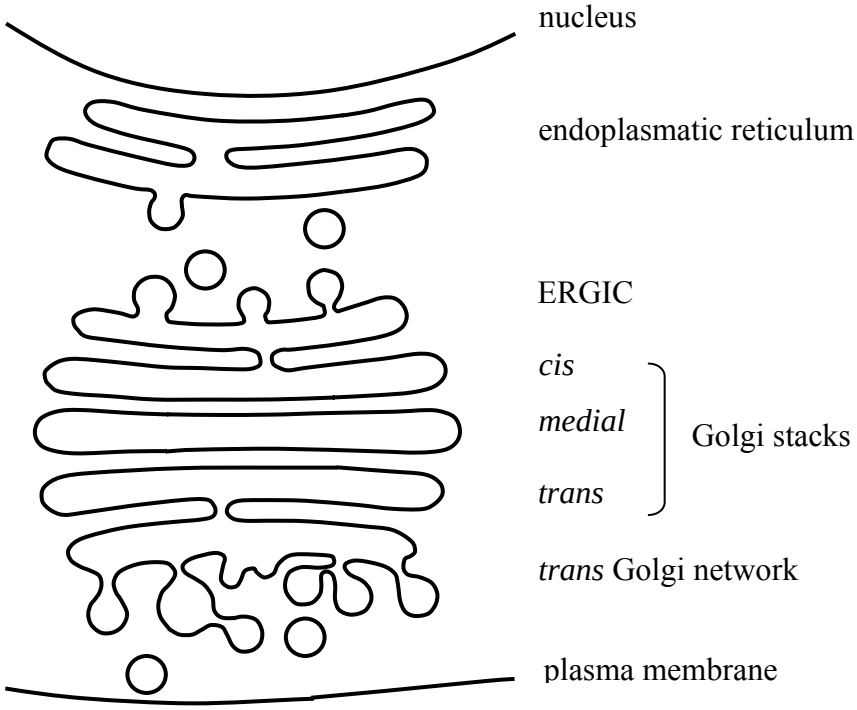

Figure 1. *Schematic overview over the secretory pathway, adopted from (Flint, et al., 2000).*

Endoplasmatic Reticulum

Secreted proteins destined for the extra cellular space and transmembrane proteins destined for the different compartments in the secretory pathway, the plasma membrane, Golgi or ER are translocated into the ER during translation. The precursor protein usually contains a signal sequence at the N-terminus of the protein. This sequence is between 15-30 amino acids long, and contains a hydrophobic core (Hatsuzawa, *et al.*, 1997, von Heijne, 1990). After translation by the ribosome and exposure of the signal sequence, translation is temporarily arrested, due to the binding of the signal recognition particle (SRP) to the

signal sequence and the ribosome. The binding of the SRP to the ribosome-signal sequence directs the complex to the ER membrane (Walter and Johnson, 1994).

Docking of SRP to the ER located SRP-receptor induces a tight binding of the ribosome to the translocon in the ER membrane (Walter and Johnson, 1994), which forms an aqueous pore, spanning the membrane (Crowley*, et al.*, 1994). This induces release of the SRP and the SRP-receptor, and introduction of the signal sequence into the pore and translation is resumed (Johnson and van Waes, 1999). The growing polypeptide is inserted through the translocon into the ER lumen during translation and trans membrane (TM) domains are simultaneously inserted in the correct orientation into the lipid bilayer. The signal sequence is integrated into the ER membrane, it can be either cleaved by a signal peptidase as observed for soluble proteins, or remain attached to the protein as a membrane spanning domain for membrane proteins (Johnson and van Waes, 1999). Membrane proteins with a cleavable signal sequence contain a second hydrophobic sequence which functions as a stop-transfer sequence, a TM domain. Proteins with one TM domain and with a cleavable N-terminal signal sequence are called type I membrane proteins. In type II membrane proteins the signal-anchor in the N-terminus forms the membrane spanning domain. Proteins that cross the membrane several times are referred to as polytopic membrane proteins (Spiess, 1995).

The ER lumen contains many enzymes which are responsible for the folding, modifications and quality control of the emerging proteins. The ER proteins that help with protein folding are called molecular chaperones e.g. IgG heavy chain binding protein (BiP), calnexin and calreticulin (Zhang*, et al.*, 1997). These proteins do not alter the proteins covalently, but assist the unfolded proteins to fold correctly. BiP binds to hydrophobic sequences thereby preventing exposure and aggregation, so that the proteins can assume their correct folding (Helenius*, et al.*, 1992). These proteins also play an important role in the ER quality control, preventing missfolded or aggregated proteins to exit the ER by remaining attached (Zhang*, et al.*, 1997). Thus, only correctly folded and assembled proteins can exit the ER and enter the secretory pathway.

Another post-translational modification of proteins that occurs in the ER is the formation of covalent disulfide bonds between pairs of cysteine residues. These bonds are rarely formed in the cytoplasm due to the reducing environment (Helenius*, et al.*, 1992). The ER lumen on the other hand is more oxidative, and contains protein disulfide isomerase (PDI) which is the protein catalyzing the formation of disulfide bonds (Helenius*, et al.*, 1992). These disulfide bonds are essential for correct folding, and contribute to the stability of the folded protein. Formation of the disulfide bonds is often the rate limiting step in the protein folding.

Many soluble and transmembrane proteins are glycoproteins, where sugars are covalently attached to the protein. These sugars are important for folding, solubility and function. One of the most common modifications is the N-linked glycosylation (Helenius, 1994). An oligosaccharide rich in mannose preassembled on a lipid carrier is added onto an asparagine residue, within the Asn-X-Ser/Thr consensus sequence, by the oligosaccharyl transferase. Some sugar residues are later trimmed off in the ER by glucosidase I and II,

and ER mannosidase in preparation for additional modifications in the Golgi (Helenius, 1994).

Post-translational modification of proteins on the cytoplasmic side

Fatty acid modification of proteins occurs on the cytoplasmic side of the ER and Golgi, and results in the attachment of the protein to cell membranes. S-palmitoylation is the addition of a thioester linkage of palmitate, a C16 saturated fatty acid, to cysteine residues. This modification is reversible and catalyzed by membrane-bound palmitoyl transferases (PATs) (Greaves and Chamberlain, 2007). Many cellular and viral proteins are palmitoylated and this modification has been shown to be important for cellular protein trafficking and sorting (Greaves and Chamberlain, 2007, Resh, 2006).

Vesicular traffic

When proteins in the ER are correctly folded and assembled they are packaged into secretory vesicles. The mechanism to control that the vesicles are loaded with the appropriate cargo is not fully understood. But it is thought that most proteins display a transport signal which is recognized by complementary receptor proteins in the membrane and that these become trapped in the budding vesicles by interacting with the vesicle coat proteins (Rothman and Wieland, 1996). These transport vesicles bud from the ER with the help of proteins that form an external coat on the vesicle, such as the protein complex COPII and small GTPases (Rothman and Wieland, 1996). After the vesicle has been released by pinching off the ER membrane the coat is removed and the vesicle is transported via the ER-Golgi intermediate compartment (ERGIC) also called the vesicular tubular cluster in order to reach the Golgi. The ERGIC is a complex membrane cluster between the ER and Golgi, and it was initially defined following the identification of a lectin marker ERGIC-53 (Appenzeller-Herzog and Hauri, 2006, Saraste and Svensson, 1991). The function of the ERGIC is not well understood but it is thought to contribute to the concentration, folding and quality control of newly synthesized proteins (Appenzeller-Herzog and Hauri, 2006).

Membrane receptors (t-Snare) on the target membrane are proposed to define the specificity with which the transport vesicles deliver their cargo, and only vesicles containing a complimentary Snare are able to interact with a specific target membrane Snare (Rothman, 1994). There are different types of coats for vesicles within the secretory pathway; COPII is responsible for ER-Golgi, COPI for the retrograde transport to the ER (Golgi-ER) and the forward transport from the Golgi-plasma membrane, and Clathrine coated vesicles for transport from the plasma membrane to the endosome (Rothman and Wieland, 1996).

Golgi

The Golgi complex is located near the cell nucleus and consists of a variable number of flattened cisternae with dilated rims, arranged parallel with each other. The Golgi contains different compartments each with different protein modifying activities, as well as stations where specific sorting decisions are made (Blazquez and Shennan, 2000).

Proteins enter the Golgi apparatus from the ER via the ERGIC and *cis*-Golgi network, which is composed of connected tubules and stacks, and proteins leave the Golgi from the *trans*-Golgi network. Between the *cis*- and *trans*-Golgi networks are the *cis*- *medial*- and *trans*-cisternae which together forms the Golgi stacks (Fig. 1) (Blazquez and Shennan, 2000).

The oligosaccharides that were attached in the ER are further processed as the proteins move through the Golgi stacks. The removal of mannose groups occurs in *cis*- and *medial*-cisterna while, the addition of N-Acetylglucosamine is catalyzed in the *medial*-cistern and the addition of galactose and sialic acid occurs in the *trans*-cistern (Hobman, 1993). Proteins targeted to the plasma membrane, secretory granules, lysosomes and endosomes are mostly sorted in the *trans*-Golgi network (Rothman and Wieland, 1996). In addition the *trans*-Golgi network also receives proteins recycled from the plasma membrane and the endosomal compartment through a retrograde pathway.

The Golgi compartment is also the place were O-linked oligosaccharides are synthesized and added onto certain serines or threonines (Rottger*, et al.*, 1998).

Secretion

Exocytosis is the transport from the *trans*-Golgi network to the cell exterior. Fifteen years ago it was though that this secretion occurred by default, however, it has now been established that there are specific sorting signals for basolateral and apical targeting (Rodriguez-Boulan and Musch, 2005), this pathway is also called selective constitutive-like secretory pathway (Blazquez and Shennan, 2000). Secretion is also a highly regulated process, in the regulated secretory pathway the secreted material is concentrated into granules, and stored within the cell for several hours, or days until an extracellular stimulus induces their secretion (Blazquez and Shennan, 2000).

Uukuniemi virus a member of the *Bunyaviridae* family

The *Bunyaviridae* family is one of the largest family of viruses with over 350 members and they are found worldwide. They are divided into five different genera; *Phlebovirus, Nairovirus, Orthobunyavirus, Hantavirus* and *Tospovirus*, (Table 1) (Fauquet*, et al.*, 2005). The first four genera infect animals and the last genus contains plantviruses. Members of the *Bunyaviridae* are mostly arboviruses (arthropod-borne viruses), transmitted between vertebrate hosts by mosquitoes, ticks, and biting flees; however, viruses in the *Hantavirus* genus are not transmitted by arthropods but are transmitted by excreta from persistently infected rodents (Calisher, 1996). Many of the members cause serious disease in humans such as hemorrhagic fever, hanta virus pulmonary syndrome and encephalitis. In addition, some of them are classified as category A pathogens (http://www2.niaid.nih.gov/biodefense/bandc_priority.htm) by the National Institute of Allergy and Infectious Diseases (NIAID), which means that they could potentially be used as bioterrorist weapons. Unfortunately, no commercially available human vaccine is available for any of the

Genus	Viruses
Phlebovirus	Uukuniemi virus (UUKV) Rift Valley fever virus (RVFV)
Nairovirus	Dugbe virus Crimean-Congo Hemorrhagic Fever virus (CCHFV)
Orthobunyavirus	Bunyamwera virus (BUNV) La Cross virus (LACV)
Hantavirus	Hantaan virus (HTNV) Tula virus
Tospovirus	Tomato Spotted Wilt virus (TSWV)

Table 1: *Examples of viruses from the different genera*

bunyaviruses requiring that research on the pathogenic viruses would be performed under high biosafety laboratory conditions e.g. BSL 3 and BSL 4.

Uukuniemi virus (UUKV) a member of the *Phlebovirus* genus was isolated from a tick in Finland 1964 and was named after the small village Uukuniemi close to the Russian border (Oker-Blom*, et al.*, 1964). Neutralizing antibodies against UUKV have been found in seabirds and cattle, however, no antibodies have been found and no disease in humans has been associated with UUKV (Saikku, 1973). UUKV has been used as a model virus for the *Bunyaviridae* family for over 35 years, and many features of the *Bunyaviridae* family were first discovered in UUKV (Pettersson*, et al.*, 1977, Pettersson and von Bonsdorff, 1975).

Structure

UUKV is an enveloped, spherical virus with a diameter of ~90 nm. The virions consist of four structural proteins. Two glycosylated spike proteins G_N and G_C encompass the viral envelope and have been reported to form both heterodimers and homodimers (Persson and Pettersson, 1991, Ronka*, et al.*, 1995, von Bonsdorff and Pettersson, 1975), and the nucleoprotein (N protein) which is associated with the RNA, and the RNA dependent RNA polymerase (L protein). The members of the *Bunyaviridae* do not have a matrix (M) protein which is known to determine the structure and stability of other viruses (Jayakar*, et al.*, 2004, Nayak*, et al.*, 2004, Takimoto and Portner, 2004). It is therefore thought that the integrity, shape, and structure of the UUKV particle is determined by the two glycoproteins. UUKV has been shown to generate a very homogenous population of progeny particles both with regard to virion size and surface morphology (von Bonsdorff and Pettersson, 1975). The two UUKV glycoproteins arrange themselves in clusters forming cylindrical hollow morphological units, with a T=12 and P=3 icosahedral surface

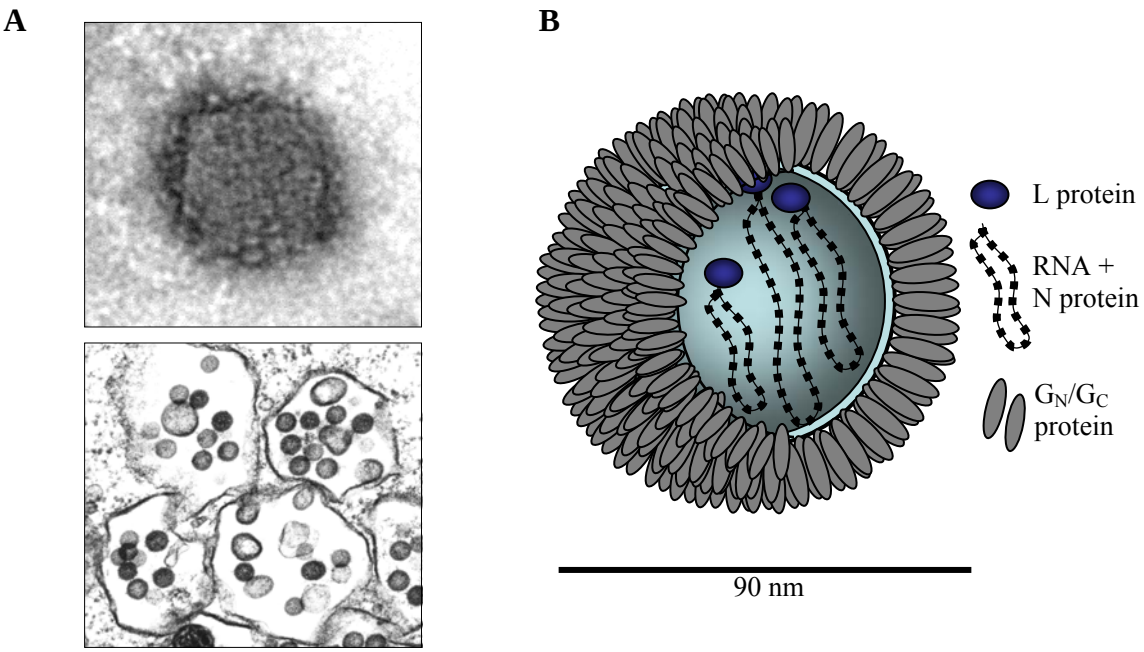

Figure 2: A. *Surface morphology of UUKV, with negative staining electron microscopy upper panel and thin section of UUKV infected BHK cells lower panel.* ***B.*** *Schematic representation of UUKV particle with its structural components.*

lattice (von Bonsdorff and Pettersson, 1975). The surface projections are between 8 to 10 nm long and the hexon-hexon distances are estimated to be 12.5 to 16 nm for stained viral particles (von Bonsdorff and Pettersson, 1975), and a typical staining is shown in Fig. 2A. It is currently not known if these clusters are formed by homo- or heterodimers. Other members of the *Bunyaviridae* family, for example virions in the *Orthobunyavirus* and *Nairovirus* genera (e.g. BUNV, LACV, CCHFV and Dugbe viruses) do not seem to have an ordered surface lattice (Booth*, et al.*, 1991, Martin*, et al.*, 1985, Talmon*, et al.*, 1987).

The core of the viral particles consists of three negative stranded RNA segments, designated large (L), medium (M) and small (S) segment (Pettersson*, et al.*, 1977) which are encapsidated with the N protein. They are seen as circular structures in the electron microscope due to complementary base pairing between the ends (Pettersson and von Bonsdorff, 1975).

Genome organization

Viral genome

The three genome segments L, M and S all have non-coding regions (NCRs) flanking the open-reading frames (ORFs), and the terminal sequences at the 3' and 5' end of these NCRs are highly conserved among the segments and within each genus. However the sequences varies between the different genera (Giorgi, 1996). These highly conserved nucleotides (nt) are complementary between the 3' and 5' ends of the segments, and base pairing has been shown by electron microscopy (Pettersson and von Bonsdorff, 1975), in

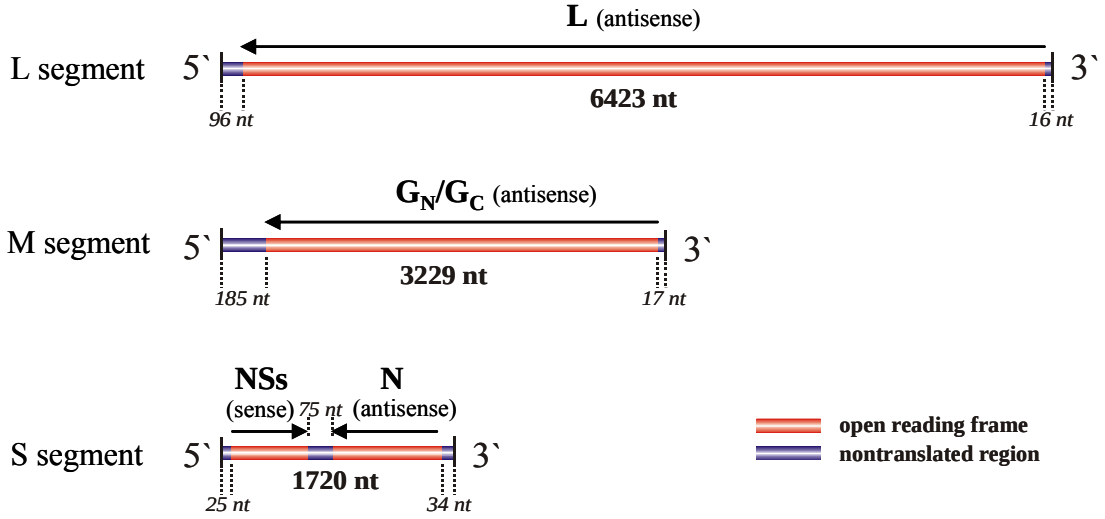

Figure 3: *UUKV genome organization, the three negative stranded RNA segments encode four structural proteins and one non structural protein. The L segment encodes the RNA dependent RNA polymerase (L protein), the M segment encodes the glycoprotein precursor p110 which is post-translationally processed into two glycoproteins G_N and G_C. The S segment has an ambisense coding arrangement encoding the non-structural protein (NSs) in a sense direction and the N protein in the antisense direction. Flanking these open reading frames are non coding regions (blue) containing cis-acting signals important for replication, transcription, encapsidation and packaging.*

which the NCRs form stable panhandle structures and circularizes the RNA segments. The conserved terminal nucleotides of the three segments L, M and S of UUKV are: 3' **UGUGUUUC**UGxxGxU... and 5' **ACACAAAG**xCxCxxC..., the bold nucleotides are conserved within the genus (Giorgi, 1996). The NCRs are also thought to contain regulatory elements important for replication, transcription initiation and termination, encapsidation and packaging (Barr, *et al.*, 2003, Barr, *et al.*, 2006, Kohl, *et al.*, 2006, Osborne and Elliott, 2000, Schmaljohn, 2001). The viral (vRNA) and complimentary RNA (cRNA) are in complex with the nucleoprotein and these complexes together with the L protein constitute the ribonucleoprotein (RNPs) which is the functional template for the RNA dependent RNA polymerase transcription and replication (Lopez, *et al.*, 1995).

L segment

The UUKV L segment is 6423 nt long with a 5' and 3' NCRs of 95 nt and 16 nt respectively. The L segment encodes one single ORF encoding the L protein (Elliott, *et al.*, 1992). The L protein (~ 200 kDa) the largest of the viral proteins is a cytoplasmic protein, which replicates and transcribes the vRNA and cRNA. The L protein of Tula virus (*Hantavirus* genus) has been shown to be membrane associated, localized to the perinuclear region, and partially co-localized with the Golgi marker GM130 (Kukkonen, *et al.*, 2004).

M segment

The M segment of UUKV is 3229 nt long with 5' and 3' NCRs of 185 nt and 17 nt respectively (Ronnholm and Pettersson, 1987). The M segment encodes the p110 precursor which is post-translationally processed into the two glycoproteins G_N (G1) and G_C (G2) in the ER (Ronnholm and Pettersson, 1987). Other viruses in the family *Bunyaviridae* encode an additional protein on the M segment a non-structural m (NSm) protein. The orthobunyaviruses have the NSm ORF inserted between the G_N and G_C, while some members in the genus *Phlebovirus* have the NSm encoding sequence upstream of the G_N encoding region, the tospoviruses have an ambisense coding strategy generating the NSm from a subgenomic RNA. No NSm have been reported among the hantaviruses, nairoviruses or UUKV (Schmaljohn, 2001).

The Bunyamwera virus (BUNV) NSm is a transmembrane protein shown to localize in the Golgi compartment together with the G_N and G_C glycoproteins. NSm interacts with the N protein and the glycoproteins, and the N terminal domain of NSm has been shown to be important for virus assembly (Shi*, et al.*, 2006). Not much is known about NSm for the other members of the *Bunyaviridae* family.

The UUKV glycoproteins are type I trans-membrane proteins, with the N terminus facing the ER lumen and the C terminus facing the cytoplasm. G_N has a cytoplasmic tail of 98 amino acids (aa) in which the C terminal end of 17 aa constitutes the G_C signal sequence, and it is currently not known if this signal sequence stays in the membrane or flips out to generate a 98 aa long cytoplasmic tail (Andersson*, et al.*, 1997b). G_C has a very short lysine rich cytoplasmic tail of 5 aa (Ronnholm and Pettersson, 1987). The G_N and G_C proteins each contain four sites for N-linked glycosylation (Pesonen*, et al.*, 1982), and in mature virions these are endoglycosidase (endo) H resistant in G_N while most of them are endo H sensitive in G_C (Kuismanen, 1984). Both glycoproteins are transiently associated with the folding chaperones BiP (Persson and Pettersson, 1991), calnexin and calreticulin (Veijola and Pettersson, 1999). The G_N and G_C proteins also have different folding kinetics measured by disulfide-bond formation; the G_N is correctly folded in about 10 min, in contrast to G_C which folds much slower, 45-60 min (Persson and Pettersson, 1991). After synthesis, glycosylation and folding, the two glycoproteins form heterodimers (Persson and Pettersson, 1991), and due to the different maturation times of the two proteins, G_N forms a heterodimer with a G_C proteins synthesized 40 min earlier (Persson and Pettersson, 1991).

Both glycoproteins are palmitoylated, although changing the cysteins to alanines did not affect intracellular localization of the two glycoproteins. The functional implications of this modification for the virus are currently not known (Andersson and Pettersson, 1998). Some other viral membrane proteins are also palmitoylated at their cytoplasmic tail, including the Influenza virus hemagglutinin H (Chen*, et al.*, 2005, Jin*, et al.*, 1996, Zurcher*, et al.*, 1994), vesicular stomatitis virus (VSV) G (Whitt and Rose, 1991), Sindbis virus E1 (Smit*, et al.*, 2001), murine coronavirus Spike (Thorp*, et al.*, 2006), and retrovirus Env (Rousso*, et al.*, 2000). The functional implications of these modifications are not precisely known, but palmitoylations may be important for membrane fusion activity (Lambrecht and Schmidt, 1986), virus assembly and budding (Thorp*, et al.*, 2006), or incorporation of glycoproteins in the virions (Rousso*, et al.*, 2000).

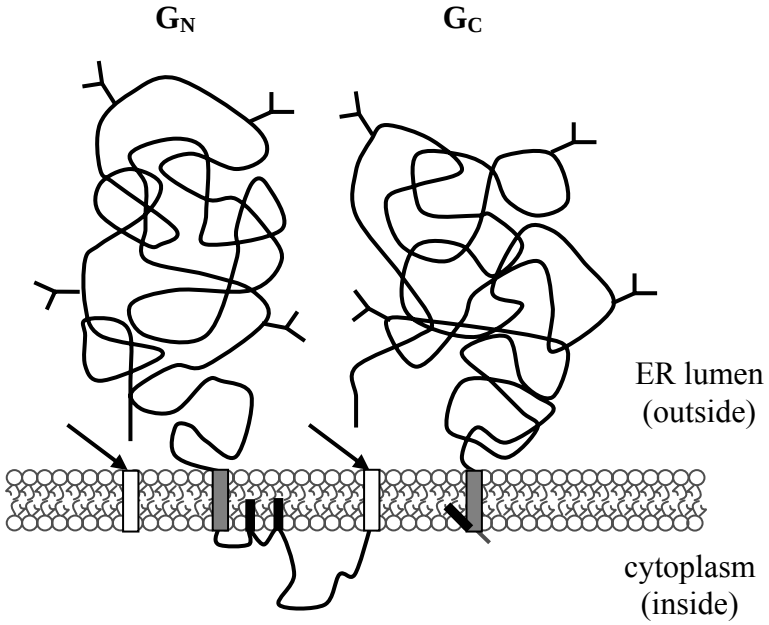

Figure 4: *Schematic representation of UUKV G_N/G_C, white boxes are signal sequences, gray are transmembrane domains, black is palmitoylations, arrows indicate signal sequence cleavage sites and Y represents glycosylations.*

S segment

The UUKV S segment is 1720 nt long with a 25 and 34 nt long 5' and 3' NCR respectively. The S segment contains two non overlapping reading frames (Simons, *et al.*, 1990). The 5' end of the viral sense RNA encodes the nucleoprotein (N protein) and the 5' end of the complementary RNA, encodes the non structural protein (NSs) (Fig. 3) (Simons, *et al.*, 1990), and this ambisense coding strategy is shared with the other members of the genus *Phlebovirus* and *Tospovirus*. Between the two open reading frames is an intragenic region (IGR) of 75 nt, containing a short palindromic sequence which could potentially form a stem loop structure (Simons and Pettersson, 1991). This stem loop has been proposed to be important for termination of transcription (Simons and Pettersson, 1991). The orthobunyaviruses encode a NSs protein from an overlapping reading frame, while the nairoviruses and hantaviruses lack a NSs protein (Schmaljohn, 2001).

In cells infected with UUKV the nucleoprotein (28.5 kDa) is the most abundant viral protein. The N protein is localized in the cytoplasm and binds to both vRNA and cRNA (A. Katz personal communication) to make a functional template for the L protein. For phleboviruses, dimers of the N protein (Le May, *et al.*, 2005) bind to an encapsidation signal located within the NCR of the genome segments (Blakqori, *et al.*, 2003, Flick and Pettersson, 2001). This interaction is followed by an oligomerization event that covers the entire RNA genome. For hantaviruses the N protein forms trimers (Kaukinen, *et al.*, 2004) and these have been shown to bind to the panhandle triggering encapsidation and oligomerization (Mir and Panganiban, 2005). Although, the orthobunyavirus N protein does

not form dimer or trimers, however, it still binds the RNA as a monomer and oligomerizes directly on the RNA in a head-head and tail-tail interaction (Leonard, *et al.*, 2005). Two different regions in the UUKV and hantavirus N proteins are important for oligomerization and both the C- and N-termini seem to be involved (A. Katz personal communication), (Alfadhli, *et al.*, 2001, Lindgren, *et al.*, 2006, Yoshimatsu, *et al.*, 2003).

The UUKV NSs protein (32 kDa) is a cytoplasmic protein but the function remains unknown. The protein has not been found in virions or nucleocapsids isolated from infected cells, however, it has been found associated with the 40S ribosomal subunit (Simons, *et al.*, 1992). The UUKV N protein is synthesized within 4 hours (h) post infection (p.i.) and is stable for several hours while the NSs protein was identified only 8 h p.i. with a half live of about 1.5 h (Simons, *et al.*, 1992). For RVFV, another phlebovirus, NSs was shown to package cRNA of the S segment. This cRNA could after infection serve as a template for NSs mRNA (Ikegami, *et al.*, 2005b), resulting in the expression of the NSs protein early during infection. RVFV NSs has been also been shown to enhance RNA transcription (Ikegami, *et al.*, 2005a). The NSs of RVFV is phosporylated and accumulates as fibrillar structures in the nucleus (Struthers and Swanepoel, 1982), so the function of RVFV NSs and UUKV NSs appear different. RVFV NSs has been shown to be an α/β interferon (IFN) antagonist (Bouloy, *et al.*, 2001). It does not inhibit IFN transcription like other INF antagonist, but binds to components of the TFIIH transcription factor complex, preventing the assembly of the TFIIH subunits, resulting in a dramatic drop in cellular RNA synthesis (Le May, *et al.*, 2004). The NSs of BUNV and LACV are also IFN antagonists (Blakqori, *et al.*, 2007, Bridgen, *et al.*, 2001, Weber, *et al.*, 2002) but the exact mechanism of action is only partly understood for BUNV and not at all for LACV. BUNV NSs has been shown to inhibit phosphorylation of the cellular polymerase II thereby preventing transition between transcription initiation to elongation of the IFN-β gene (Thomas, *et al.*, 2004). The LACV NSs has been reported to induce apoptosis (Blakqori and Weber, 2005), and if overexpressed it suppresses RNA interference in mammalian cells (Soldan, *et al.*, 2005). However, this effect was not seen in mosquito cells (Blakqori, *et al.*, 2007).

Virus life cycle

Attachment and entry

The mechanism by which viruses of the family *Bunyaviridae* enter the cell is not well studied and fully understood, but appears similar to that of other enveloped viruses. The first step is the attachment of the viral G_N and/or G_C protein to a receptor on the cell surface. The presence of neutralizing antibodies towards both glycoproteins indicates that both proteins might be involved in receptor binding for phleboviruses and hantaviruses (Arikawa, *et al.*, 1989, Keegan and Collett, 1986). However, G_C is the major attachment protein for the members of the orthobunyaviruses (Plassmeyer, *et al.*, 2005). The specific receptors that UUKV or most of the other members in the family utilize have not yet been identified. Following attachment the virus most likely enters the cell via endocytosis in coated vesicles similar to RVFV (Ellis, *et al.*, 1988). These vesicles are transported to the

endosome were membrane fusion and release of RNPs occurs. The infection of UUKV requires acidic conditions, since blocking the acidification of the endosomal compartment with ammonium chloride inhibited UUKV infection (Ronka, *et al.*, 1995). The pH drop generates a conformational change in the G_C, leading to dissociation of the homodimer into monomers, but the G_N is not affected by acidification (Ronka, *et al.*, 1995). The morphological changes can also be observed in the electron microscope (Ronka, *et al.*, 1995), and this conformational change might be involved in the fusion step. After fusion of the viral membrane with the endosomal membrane the RNPs and L protein are released into the cytoplasm.

Transcription and replication

In the cytoplasm, primary transcription of the vRNA segments into mRNA occurs by the L protein. Only nucleoprotein associated RNA can function as a template for transcription (Dunn, *et al.*, 1995, Lopez, *et al.*, 1995). The initiation of the viral mRNA synthesis is primed by capped fragments of cellular RNAs, a mechanism called cap-snatching (Raju, *et al.*, 1990). The caps are taken by the L protein from cellular mRNA present in the cytoplasm (Simons and Pettersson, 1991), a similar cap-snatching mechanism is used by Influenza virus, but the Influenza virus removes the caps from the cellular mRNA in the nucleus (Flint, *et al.*, 2000).

The viral mRNA transcripts for bunyaviruses have been found to be shorter at the 3' end compared to the vRNA segments. The transcription termination occur approximately 100 nt from the 5' end of the template RNA (Bouloy, *et al.*, 1984, Collett, 1986, Simons, 1992). The transcription termination signal for the BUNV S segment has been identified to be a 33 nt long RNA segment and located in the 5' NCR (Barr, *et al.*, 2006). A six nt segment appears very important and seems to be conserved in the L segment amongst the other orthobunyaviruses suggesting that the mechanism of bunyavirus transcription termination may be common to other viruses in the same family (Barr, *et al.*, 2006).

The RNA transcripts from the ambisense S segment of UUKV are about half the size of the template (Pettersson, *et al.*, 1985, Ulmanen, *et al.*, 1981), and the hair pin secondary structure predicted in the IGR may be involved in the transcription termination (Simons and Pettersson, 1991). No poly-adenylation has been identified on the mRNAs, despite several attempts (Bouloy, *et al.*, 1984, Pettersson, *et al.*, 1985, Simons, 1992, Ulmanen, *et al.*, 1981).

After primary transcription and protein synthesis a switch from mRNA synthesis to replication and synthesis of full-length cRNA and vRNA must occur. This means that the L protein must change from using capped oligonucleotides as a primer for making uncapsidated mRNA transcripts, to initiate primer independent replication at the 3' end of the template and continue to the very 5' end making an exact complementary copy of the template RNA that can be encapsidated. The exact mechanism behind this switch is not known. The nucleoprotein may however be involved since both vRNA and cRNA are encapsidated by the N protein, and the N protein might serve as an anti termination signal, similar to other negative stranded RNA viruses (Banerjee, 1987, Gubbay, *et al.*, 2001). However, no correlation between the quantity of nucleoprotein in the cell and mRNA

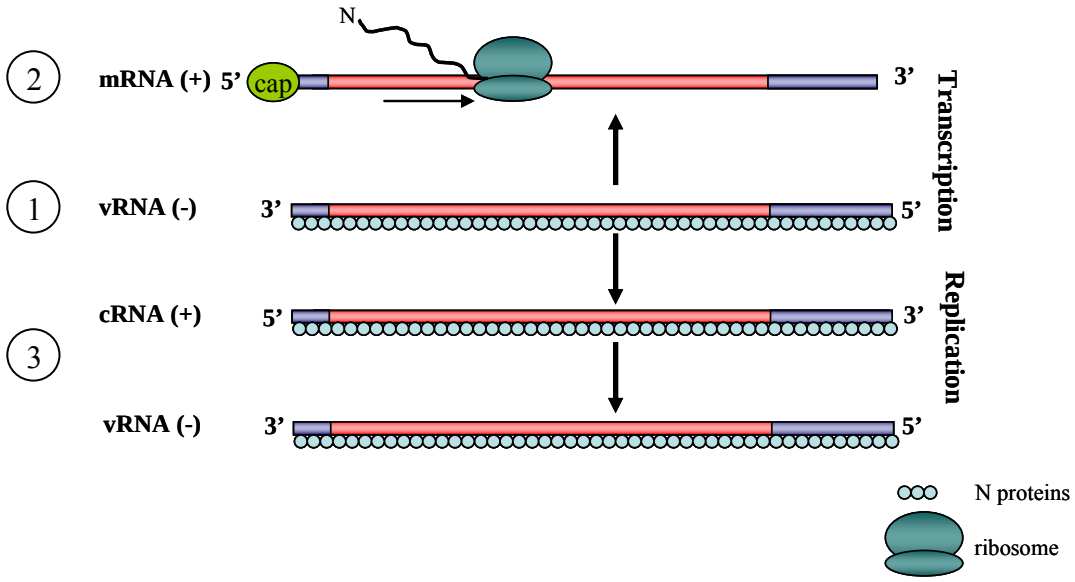

Figure 5: *Schematic Representation of bunyaviral replication.* ***1.*** *Viral infection, the vRNA enters the cytoplasm as RNPs.* ***2.*** *In the cytoplasm, the viral RNA polymerase initiates primary transcription to generate mRNA and viral proteins.* ***3.*** *After transcription the replication of the full length RNA segments occur, generating cRNA templates for producing vRNA, both of these products are encapsidated by nucleoprotein.*

transcripts have been demonstrated in UUKV (Flick and Pettersson, 2001) or the lymphocytic choriomeningitis virus (LCMV), (*Arenaviridae*) (Pinschewer, *et al.*, 2003).

Unlike most other replicating viruses, UUKV does not affect host cell DNA, RNA or protein synthesis during the first 35 h of viral infection (Pettersson, 1974), and only a slight cytopathic effect is observed late in the viral infection (40-90 h post infection).

Assembly and release

After replication and synthesis of the structural components of UUKV, assembly occurs. Unlike the alpha-, arena-, orthomyxo-, paramyxo-, rabdo-, and retroviruses which bud from the plasma membrane to the extracellular space, the members of the bunya-, and corona-, and flavivirus families and Rubella virus bud into intracellular organelles like ER, ERGIC or Golgi. The factors that determine the site of budding, are thought to be the targeting of the viral glycoproteins to the specific intracellular compartment. After synthesis, folding and maturation of the two UUKV glycoproteins they form heterodimers in the ER and enter the secretory pathway. Studies have shown that G_N, when expressed alone is targeted to and retained in the Golgi due to a signal located in the G_N cytoplasmic tail between amino acids 10 and 40 (Andersson, *et al.*, 1997a, Andersson and Pettersson, 1998). The G_C when expressed alone is unable to exit the ER probably due to a lysine based

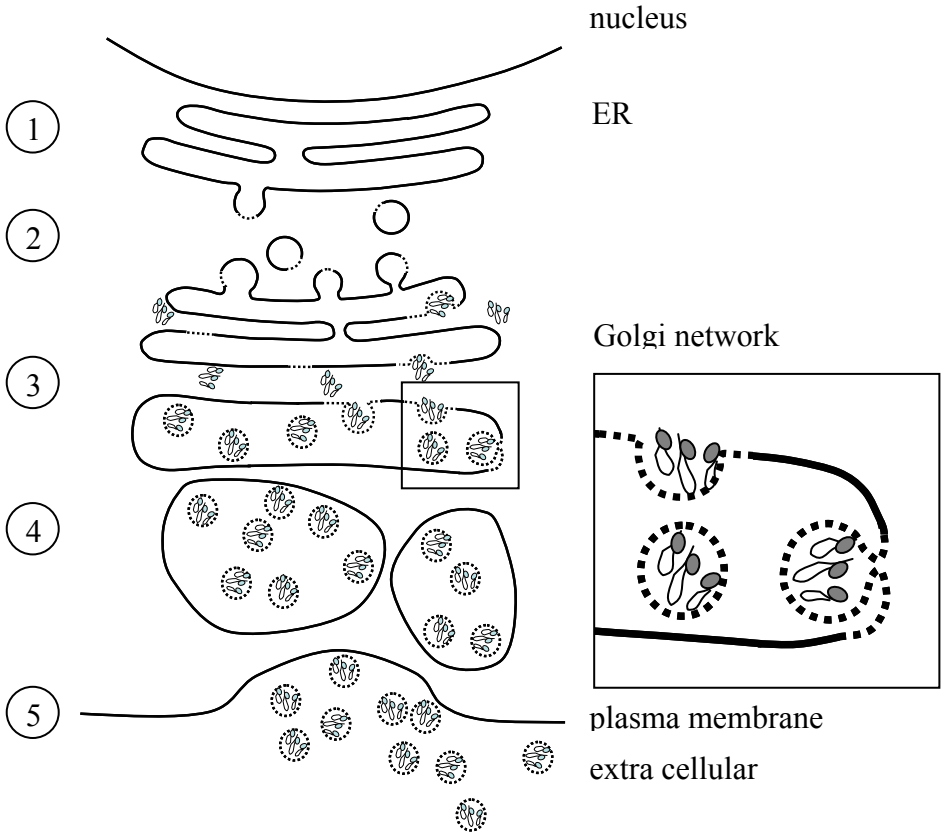

Figure 6: *Overview of the assembly and release pathway for bunyaviruses.* ***1.*** *The glycoproteins are translated and translocated into the ER membrane, they fold and form heterodimers.* ***2.*** *The heterodimers are packaged into cellular cargo vesicles and are transported to the Golgi were they accumulate.* ***3.*** *The replicated RNPs line up on the cytoplasmic side of the Golgi and when a critical concentration of viral glycoprotein is reach in the Golgi they start to bud into Golgi.* ***4.*** *Accumulation of glycoprotein and viral particles in the Golgi results in a morphological change of Golgi, it becomes more vacuolized. The large membrane compartments filled with virus are transported to the plasma membrane.* ***5.*** *At the plasma membrane the vesicle fuses with the membrane and viruses are released.*

ER retention signal in its short G_C cytoplasmic tail (Ronnholm, 1992). It is therefore essential that heterodimerization occurs between the G_N and G_C for the exit of the G_C from the ER. The heterodimers G_N/G_C accumulate in the Golgi membrane inducing a progressive morphological change of the Golgi causing vacuolization and dispersion (Fig. 6) (Gahmberg, *et al.*, 1986a, Gahmberg, *et al.*, 1986b, Kuismanen, *et al.*, 1984, Kuismanen, *et al.*, 1982). Concomitantly with the accumunation of the glycoproteins in the Golgi, the helical RNPs line up on the cytoplasmic side of the Golgi (Kuismanen, *et al.*, 1985).

Unlike other negative stranded viruses the members of the *Bunyaviridae* family do not have a matrix (M) protein. The M protein is in many cases important for the initiation of budding, determining the morphology of the progeny virus particles and serves as a

bridge between the envelope proteins and the interior nucleocapsid and RNPs (Eichler, *et al.*, 2004, Gomez-Puertas, *et al.*, 2000, Lee, *et al.*, 2002, Schmitt, *et al.*, 2002). Because M is absent in UUKV, a direct interaction between the RNPs and the envelope glycoproteins G_N and G_C has been suggested, and co-immunoprecipitation studies have confirmed an interaction between the N protein and the G_N/G_C proteins (Kuismanen, 1984). Since UUKV G_N has an cytoplasmic tail of 81 amino acids and G_C only 5 amino acids, this interaction is more likely to be between the cytoplasmic tail of G_N and the RNPs, and it is believed that the viral RNPs initiated budding into the Golgi (Betenbaugh, *et al.*, 1995, Kuismanen, *et al.*, 1985). Budding of UUKV can be observed in the ERGIC (Jantti, *et al.*, 1997), however, the main site of budding is the Golgi (Kuismanen, *et al.*, 1984, Kuismanen, *et al.*, 1982). The viruses are subsequently transported in large vesicles along the secretory pathway to the plasma membrane were membrane fusion occurs and viruses are released (Kuismanen, *et al.*, 1985). However, the precise mechanism how bunyaviruses bud into the Golgi, which viral proteins are necessary, and if additional cellular factors are required has not been analyzed.

Budding of other viruses

Since different viruses assemble and bud from different cellular compartments such as the plasma membrane, ER, ERGIC and Golgi, they have different requirements for budding and display a diversity of budding mechanisms.

Coronaviruses are an example of how structural proteins assist each other to localize to the proper budding compartment. Coronaviruses are positive-stranded RNA viruses that bud into the ERGIC. They encode at least three envelope proteins; the spike (S), the membrane and the envelope (E) protein. The membrane protein is the most abundant protein in the viral membrane and is transported to the Golgi when expressed alone, it also interacts with E, S, and the nucleoprotein. The S protein when expressed alone is transported to the plasma membrane. The E protein is only a minor protein in viral particles, and is retained in the ERGIC by an unknown mechanism, and is the key to defining the site of budding. Coronaviruses do not have a matrix protein and the nucleoprotein is not involved in the initiation of budding (Vennema, *et al.*, 1996). Instead, the E protein plays a major role in morphogenesis, and the E protein together with the membrane protein can by themselves form particles (Vennema, *et al.*, 1996). Alpha viruses on the other hand require the nucleocapsid for the initiation of budding, and specific interactions between the membrane proteins and the nucleocapsid are the driving force in the budding (Lopez, *et al.*, 1994, Suomalainen, *et al.*, 1992). The nucleocapsid also plays a central role in type D retroviruses, which can bud in the absence of envelope proteins (Hunter, 1994).

For negative-strand viruses a well known force driving budding is the M protein. These virus families, e.g. arena, filo, paramyxo, rhabdo, orthomyxoviruses are budding from the plasma membrane. The M proteins are able bind to the membrane via hydrophobic domains, and they form a matrix underneath the envelope by homo-oligomeric interactions. The M protein also functions as a bridge between the glycoproteins in the membrane and the viral RNPs (Schmitt and Lamb, 2004). Although these viruses are diverse, most of them

have been shown to contain a late assembly (L) domain in their M protein (Craven, *et al.*, 1999, Harty, *et al.*, 1999, Licata, *et al.*, 2003, Schmitt and Lamb, 2004, Schmitt, *et al.*, 2005, Urata, *et al.*, 2006). The L domain was first identified in the retrovirus Gag proteins and when mutated the viral particles failed to bud off from the host cell. The L domain is characterized by the presence of a distinct core sequence. Multiple types of L domains have now been characterized in different viruses. The L domain seems to function by recruiting of cellular proteins to assist viral budding. Several cellular factors have been identified that interact with the L domain of the different viruses by yeast 2 hybrid screens, and one of these is Tsg101 (Schmitt and Lamb, 2004). Which is involved in the formation of cytoplasm-containing vesicles within the endosome (Bieniasz, 2006).

Packaging of RNPs into virions

Very little is known about the packaging mechanism of bunyaviruses, how the three genome segments assemble and are packaged into virions. The packaging interaction between the RNPs and the glycoproteins has to occur between the cytoplasmic tail of either G_N or G_C and the three components of the RNPs, the RNA, nucleoprotein or the viral polymerase, however, specific interaction sites have not been mapped. For TSWV G_C has been shown to interact and colocalize with the N protein, and it was suggested that this interaction is common for other bunyaviruses (Snippe, *et al.*, 2007). However, for UUKV it has been argued that the G_C cytoplasmic tail functions more as an ER retention signal and not as a packaging motif (Ronnholm, 1992), while the 81 aa long G_N cytoplasmic tail functions as the interacting partner for the RNPs. It has been shown that the NCR of UUKV and BUNV contains signals sufficient for packaging of an artificial genome into progeny particles (Flick and Pettersson, 2001, Kohl, *et al.*, 2006).

Defective interfering (DI) particles are virus particles that have packaged a genome segment containing a major deletion. They are known to be produced when cells are infected with high multiplicities of virus. DI particles in the *Bunyaviridae* family usually contain a major deletion in only one segment, the L segment (Marchi, *et al.*, 1998, Patel and Elliott, 1992, Resende Rde, *et al.*, 1992). The deletion is typically restricted to the ORF of the L segment leaving the NCR intact (Marchi, *et al.*, 1998, Patel and Elliott, 1992, Resende Rde, *et al.*, 1992). Since the *cis*-acting signals located in the NCR are undisturbed, this short segment has a replication advantage compared to the long wild type (wt) segment, resulting in a more efficient replication, encapsidation and packaging of this truncated segment if the trans-acting proteins are present. DI particles can stay in a virus population when cells are infected with undiluted viruses, because the functional polymerase is provided by a wt virus infecting the same cell (Marchi, *et al.*, 1998). The progeny virus population however, will become less infectious for each passage due to accumulation of DI particles.

One feature of segmented viruses is the ability to reassort their genome segments, meaning that the viral particles can package genome segments from different strains of viruses. Reassortment of viruses happens when one single cell is infected with two different segmented viruses from the same serogroup, the progeny virus can then package segments from the different strains generating viruses with a new genetic makeup (Pringle, 1996).

This mechanism is the basis for the dramatic antigenic shifts in Influenza virus leading to global pandemics (Young and Palese, 1979). Studies of different bunyavirus reassortments have shown that the mixing of genome segments does not occur at equal frequency indicating that packaging is not a random process (Pringle, 1996). In some of these reassortment experiments viruses containing two S, M and L segments originating from both parental strains was found (Rizvanov, *et al.*, 2004), showing that one virus particle can not only package the L, M and S in one copy, but several additional segments. Likewise, UUKV and RVFV particles have not been found to contain equimolar ratios of the three segments, but rather a molar ratio of 1:4.6:2, for UUKV L, M and S segments (Pettersson and von Bonsdorff, 1975), and 1:3.9:3.9 for the RVFV L:M:S segments respectively (Gauliard, *et al.*, 2006). The rational for this discrepancy is currently not known, but this suggests that the packaging is not a random process.

Packaging of Influenza virus

Influenza virus is a negative stranded RNA virus with 8 different segments, and there are two different models at the moment explaining the packaging of the 8 segments. The first model suggests a random packaging mechanism, in which the 8 segments are randomly packaged into virions, due to a common structural feature. The second model is the selective packaging model, in which each segment has a unique structural feature "the packaging signal" ensuring the incorporation of all individual segments into each virion (Palese and Shaw, 2007). There is increasing evidence for the second model, since specific packaging regions have been identified located in both the NCR and the ORFs of NA (Fujii, *et al.*, 2003), HA (Watanabe, *et al.*, 2003), NS (Fujii, *et al.*, 2005), PB2, PB1, and PA (Liang, *et al.*, 2005, Muramoto, *et al.*, 2006) segments. The morphology of the interior of the virus also supports the selective packaging model. Noda and coworkers recently showed that the 8 viral segments are clustered together in a very ordered fashion, 7 segments surrounding a centrally located segment (Noda, *et al.*, 2006). The segment-specific packaging probably occurs via specific RNA-RNA or protein-RNA interactions (Palese and Shaw, 2007).

Research tools for negative stranded RNA viruses

Reverse genetics

In classical genetics there is a search for a gene that cause a specific phenotype. Reverse genetics is the opposite, and attempts to find the function of a specific gene by mutating it and analyzing the phenotypic effects. In molecular virology, reverse genetics is the generation of infectious virus from a cloned cDNA. This technology was applied in the early 80ies for Poliovirus a positive-stranded RNA virus. Poliovirus could be rescued from cells transfected with plasmids having a copy of the poliovirus genome (Racaniello and Baltimore, 1981). The major advantage of positive stranded RNA viruses is that they can utilize the host cell machinery to initiate their life cycle. The reverse genetics of negative stranded RNA viruses on the other hand was more of a challenge, since cDNA or synthetic

RNA itself is not infectious without the addition of the viral RNA dependent RNA polymerase. The vRNA or cRNA input would need to be encapsidated in the form of RNPs (Conzelmann, 2004), and precise 3' ends are required for replication and packaging of the viral genome (Conzelmann, 2004, Neumann and Kawaoka, 2004).

There are nevertheless two different approaches to study negative sense RNA viruses with reverse genetics. The first one is by using a "minigenome" or "minireplicon" system, in which a reporter gene e.g. green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT) or luciferase is cloned between the 5' and 3' NCRs. This minigenome can be transcribed and replicated by co-expression with the viral proteins. The second approach is the "rescue" system whereby infectious viruses are recovered from cDNA transfected cells, however, these viruses often contain specific genetic alterations. Most rescue systems were preceeded by the generation of a minigenome system.

Minigenome systems

Several minigenome systems have been developed for the members of the *Bunyaviridae* family, the first one in 1995 for BUNV and RVFV (Dunn, *et al.*, 1995, Lopez, *et al.*, 1995). It was not until 2001 that such a system was developed for UUKV (Flick and Pettersson, 2001). In this system the M segment 5' and 3' NCRs were used to flank the CAT reporter (Fig. 7). There are two main systems for generating RNA segments with exact 5' and 3' termini required for efficient replication, the T7 phage polymerase and the endogenous RNA polymerase I (PolI) used for synthesis of ribosomal RNA. The first systems developed used T7 polymerase which had to be provided to the cells by either a recombinant vaccinia virus or by an expression plasmid. That system was later refined by the use of stable T7 expressing cell lines (Lowen, *et al.*, 2004). The PolI system was first developed for Influenza virus (Neumann, *et al.*, 1994), and was later used in the rescue of Influenza virus (Fodor, *et al.*, 1999, Neumann, *et al.*, 1999). Both T7 and PolI have been successfully used in minigenome systems.

Minigenome systems have also been used to identify *cis*-acting signals in the NCR (Gauliard, *et al.*, 2006, Kohl, *et al.*, 2004, Kohl, *et al.*, 2006) and to optimize the conditions for rescuing complete infectious virus (Dunn, *et al.*, 1995, Lowen, *et al.*, 2004). The UUKV promoter and regulatory regions were mapped to the terminal nucleotides in the conserved region of the NCRs and mere conservation of the basepairing maintaining the panhandle between the 5' and 3' termini was not sufficient for an active promoter (Flick, *et al.*, 2002). The minigenome systems are quite limited when analyzing particle assembly, maturation, RNP packaging and receptor binding. These studies require either a full rescue system or a virus-like particle system.

Rescue system

Rescuing infectious virus from cDNA has now been achieved for three different bunyaviruses. The first one was BUNV (Bridgen and Elliott, 1996), however, the yield was inefficient, and that system was optimized some years later (Lowen, *et al.*, 2004). After that both LACV and RVFV have been rescued (Blakqori and Weber, 2005, Ikegami, *et al.*, 2006). In these systems the cRNA of the three segments were cloned into plasmids under

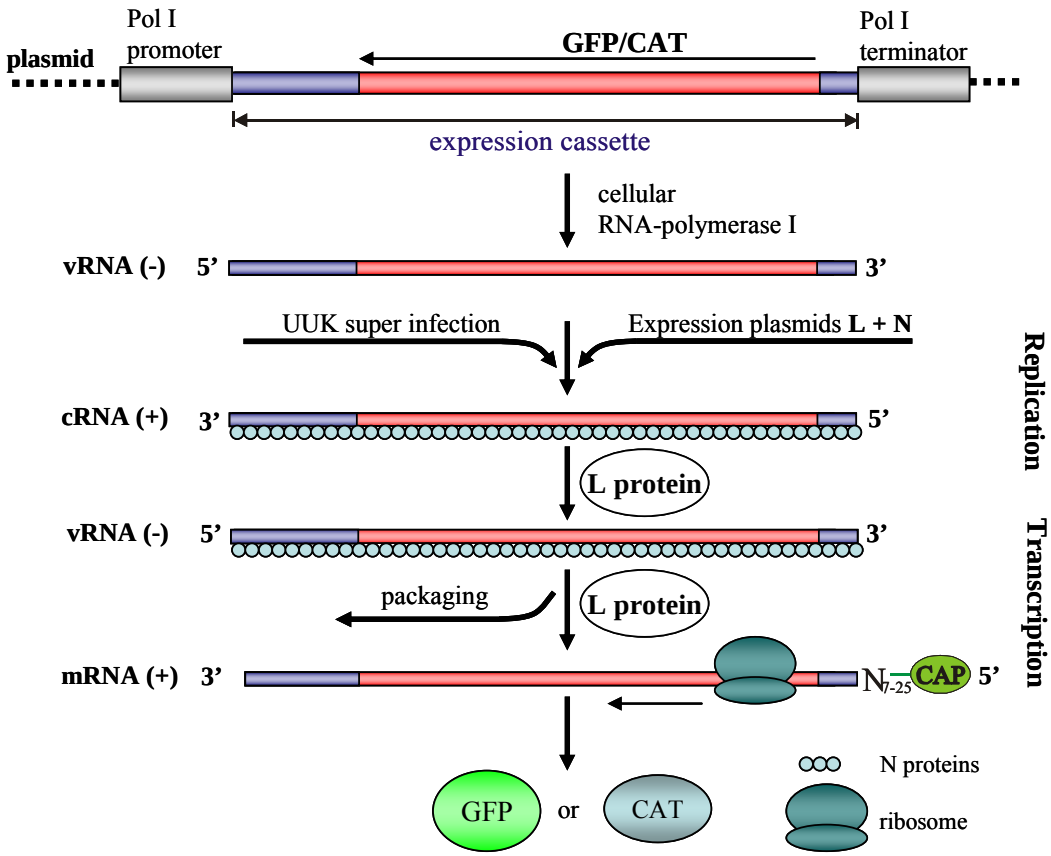

Figure 7: *UUKV minigenome system. The transcription cassette containing the reporter gene (CAT or GFP shown in red) in negative orientation is flanked by the M segment NCRs (blue). The promoter and terminator from the RNA polymerase I (PolI) are flanking the expression cassette and mediate RNA transcription. This plasmid is transfected into BHK-21 cells, and RNA transcription occurs in the nucleus by the RNA PolI. The RNA transcripts of the expression cassettes are transported to the cytoplasm, and in order to replicate the minigenome the viral L and N proteins have to be provided, either by super infection with UUKV or by transfection of the L and the N protein expression plasmids. After replication and transcription of the minigenome the reporter proteins can be monitored by CAT assay or GFP expression. The minigenome can also be packaged into progeny virus particles if the BHK-21 cells were super infected with UUKV.*

the T7 promoter, and transfected into BHK or BSR cell lines stably expressing the T7 polymerase. Support plasmids expressing the L, N and the G_N/G_C proteins were also transfected and found to be necessary for efficient RVFV rescue but not for BUNV or LACV virus rescue (Blakqori and Weber, 2005, Ikegami, *et al.*, 2006, Lowen, *et al.*, 2004). The rescue systems for these three *Bunyaviridae* members have been proven very useful in attempts to elucidate the function of the NSs protein (Blakqori, *et al.*, 2007, Blakqori and Weber, 2005, Ikegami, *et al.*, 2006, Kohl, *et al.*, 2003, Weber, *et al.*, 2002).

These three systems will be very useful in the future to analyze the different components of the virus. For example, the ability of genetically modifying Influenza virus

with the rescue system has provided both knowledge about the virus and a means to generate novel vaccines.

Virus-like particles

Virus-like particles (VLPs) are composed of viral structural proteins, which are embedded or associated with a lipid bilayer for enveloped viruses. These particles resemble the virus from which the structural proteins are derived. However, they lack some or all the nucleic acids, so that they do not permit the generation of infectious progeny viruses. This is a major advantage when working with pathogenic viruses as the work can be performed in a bio safety laboratory level 2 (BSL2) instead of a BSL4. VLPs have been generated for many viruses (Noad and Roy, 2003), and they have been used as vaccines since they resemble the wt virus in morphology but they are not infectious and can not cause disease (Warfield*, et al.*, 2005). VLPs are very useful since they show similar cellular uptake, intracellular traffic, budding and release as wt virus. They have also been used to determine the structural proteins required for assembly, budding and release of viruses and packaging of nucleic acids (Ako-Adjei*, et al.*, 2005, Hsieh*, et al.*, 2005, Lee*, et al.*, 2002, Li*, et al.*, 2003, Licata*, et al.*, 2004, Schmitt*, et al.*, 2002).

Cells can generate and release VLPs after transfection of plasmids encoding the viral structural proteins. By expressing different viral proteins the minimal requirement of budding can be determined, e.g. it was shown that the matrix protein of filovirus and rabdovirus was the only requirement for VLP generation (Hartlieb and Weissenhorn, 2006, Jayakar*, et al.*, 2004), others have shown that the nucleoprotein is not required for coronavirus budding (Vennema*, et al.*, 1996).

Aims

The *Bunyaviridae* family is a large family of viruses, of which many members cause serious disease in humans. Since research tools have only recently been developed for several of the members, little is known about the molecular mechanisms involved in entry, replication and assembly of these viruses. We chose UUKV as a model virus for this family because it does not cause disease in humans, and the molecular biology of the virus has been extensively studied in the past.

The three segments contain NCRs with both a variable and a highly conserved region. These NCRs are proposed to contain all *cis*-acting signals required for replication transcription, encapsidation and packaging. However, it is not known if these NCRs act alone or in the form of a panhandle, or if the three segments are regulated differently.

Bunyaviruses assemble in the Golgi, however, the viral proteins required, and if additional cellular factors are involved is not known. Up till now, glycoprotein localization in the Golgi and the retention signal located in the G_N cytoplasmic tail have been extensively studied. The surface morphology of UUKV particles and the assembly of the viral components have also been studied with electron microscopy. However, the tools for studying what viral components are required for UUKV assembly have up till now been lacking.

- The first aim of this thesis was to analyze the difference between the three segments with respect to promoter strengths and packaging efficiency, to gain further insight in the *cis*-acting signals located in the NCR of the three segments.

- Our second aim was to develop the necessary tools to analyze assembly of UUKV.

- The third aim was to identify regions in the two glycoproteins important for packaging of RNPs and budding of viral particles.

Results and discussion

Characterization and comparison of the cis-acting signals located in the NCR of the three UUKV genome segments (Paper I)

The 5' and 3' NCRs of UUKV contain a highly conserved nt region that can be found in all three segments, and a variable region that varies in length between the different segments. The aim of this study was to analyze the role of the variable portion of the NCR of the three segments in transcription, replication, and packaging. In order to compare the tree different genome segments, minigenomes for the L and the S segment were generated, in addition to the M segment which was published previously (Flick and Pettersson, 2001). Both GFP and CAT protein coding sequences were introduced in between the 5' and 3' NCRs of all three segments. Due to the ambisense coding arrangement of the S segment and the presence of the IGR between the two ORFs, four different S segment based minigenomes were generated. The functions of the IGR were analyzed by introducing it after the reporter gene in one set of minigenomes (sNCR-CAT-IGR-sNCR, and sNCR-IGR-TAC-sNCR) and comparing it with the activity of a minigenome without (sNCR-CAT-sNCR and sNCR-TAC-sNCR). We showed that the IGR in S segment-based minigenomes is important for efficient reporter protein expression. Similar results were obtained for LCMV, (*Arenaviridae*). Members of the *Arenaviridae* family are bi-segmented viruses using an ambisense coding strategy for both segments. Separating the two ORFs is an IGR that is able to form a stem-loop RNA. The LCMV minigenomes lacking the IGRs replicated to the same extent as IGR containing minigenomes, however, no mRNAs were detected, suggesting that the IGR is important for transcription termination but not replication (Pinschewer*, et al.*, 2005). As the UUKV IGR can form a stem-loop similar to that proposed for LCMV it is therefore possible that the UUKV IGR is also involved in viral transcription termination (Simons and Pettersson, 1991).

Comparison of promoters

Next the three genome segment-based minigenomes were compared, and we showed that the NCR of all three segments contain *cis*-acting signals required for efficient transcription, encapsidation and replication of the minigenomes. Although the main promoter has been mapped to the conserved nucleotides in the M segment 5' and the 3' NCRs (Flick*, et al.*, 2002), the variable region of the NCR is also important for regulating transcription and replication. The NCRs of the three segments showed different promoter strengths. The M segment being the strongest and the S segment the weakest. A possible basis for this could be that long genome segments M and L might require a stronger promoter to compensate for their larger size. The protein levels might be regulated at the transcriptional level by using different promoter strengths. The ratio of the RNPs in progeny UUKV particles is 1:4.6:2 for the L:M:S segments (Pettersson and Kaariainen, 1973). It is therefore likely that the RNPs are present in the cell in that specific ratio, and that the different promoter strength are important for maintaining this. A similar order of

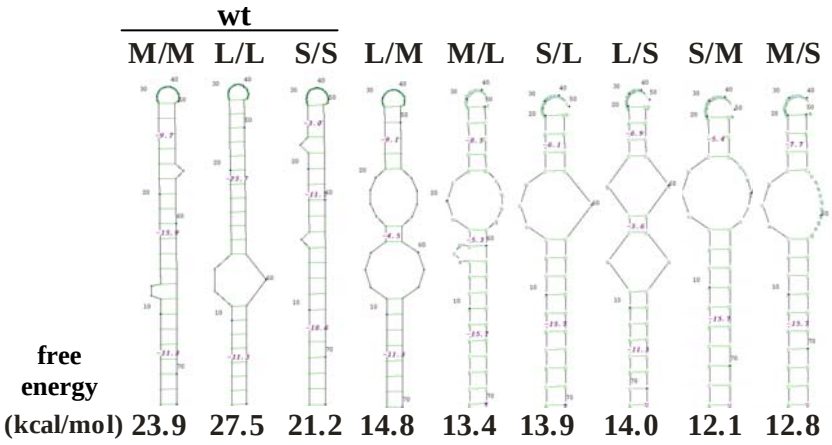

Figure 8: *Predicted secondary structure of the 20 terminal nucleotides from the 5' and 3' wt and chimeric NCRs (GeneBee).*

promoter strength for the viral segments have also been shown for BUNV (M>L>S) (Barr, *et al.*, 2003). The differences in L, M and S segment vRNA levels in the cell also increased when cells were transfected with three minigenomes at the same time (Barr, *et al.*, 2003). An M segment specific region of 12 nt was identified in the NCR to be important for the high replication efficiency (Barr, *et al.*, 2003).

Since the three segments have different promoter strengths, further analysis were required. We wanted to analyze if the 3' or 5' NCRs influence the promoter strength of the three segments, and if the NCRs can act as an independent promoter. In addition, we determine if the base pairing between the 5' and 3' NCRs is important for a functional promoter. Chimeric minigenomes combining the NCR from different genome segments were therefore generated. These chimeric minigenomes were not replication competent, demonstrating that the viral NCRs do not act independently as promoters. Similar results were obtained by two different groups working with BUNV (Barr, *et al.*, 2004, Kohl, *et al.*, 2004). Analysis of the base pairing potential of the terminal 20 nucleotides in the 3' and 5' NCRs was performed, and wt constructs were compared with the chimeras (Fig. 8). The potential for base pairing was severely compromised in the chimeric minigenomes compared to the wt, and the calculated free energy of the chimeras was also much lower compared to the wt. By introducing point mutations in the NCR to enhance base pairing potential, we could restore functional promoter activity, revealing that the complimentary base pairing within the variable part of the 3' and the 5' NCRs is important for promoter functionality. The base pairing between the NCRs seen as circular RNPs was first observed in 1975 with electron microscopy (Pettersson and von Bonsdorff, 1975), however the functional implications was at that time not known.

Analysis of minigenome packaging

The NCRs of the M segment were shown to contain signals required for the packaging of a minigenome into viral particles (Flick and Pettersson, 2001). We analyzed if the other segments contained similar motifs in their 5' and 3' NCRs. Cells transfected with

the UUKV minigenome system were super infected with UUKV. Progeny virus was passaged several times and infected cells were analyzed for CAT expression. Minigenomes for all three genome segments could be packaged into UUK virions and passaged to new cells, verifying that packaging signals are located in the NCRs. The M and the S segment-based minigenomes could be passaged three times before the minigenome was lost. Surprisingly, the L segment-based minigenome was packaged very efficiently and could be passaged four times to new cells without loss of activity. The same order of packaging efficiency was also observed for BUNV (L>M>S) (Kohl*, et al.*, 2006). For Influenza virus the ORFs in all eight segments were shown to contain additional packaging signals (Palese and Shaw, 2007). However, addition of BUNV S segment ORF did not increase the packaging efficiency of the S segment-based minigenome (Kohl*, et al.*, 2006), indicating that the packaging signal for the BUNV S segment is located in the NCRs. For bunyaviral packaging one major question has been whether the packaging mechanism is random or selective. Our results for UUKV together with the results for BUNV suggest that the packaging mechanism indeed is selective, since the L segment based minigenome has the strongest packaging efficiency despite the fact that the M segment is the most abundant segment in the cell (Barr*, et al.*, 2003). Kohl *et al.* also showed that the three segments do not compete with each other for packaging into virions (Kohl*, et al.*, 2006), an observation that also would suggest a selective packaging mechanism.

In this study I used the minigenome system developed for UUKV to study aspects of viral transcription/replication and packaging, and we conclude that the different segments are highly regulated with respect to promoter strengths and packaging efficiencies.

Analysis of UUKV assembly (Papers II, III and IV)

The aim of the studies described in papers II, III and IV was to analyze UUKV assembly. In the absence of a rescue system for UUKV, a VLP system was first developed. In the past VLP systems have been used for many viruses as a tool to study budding, packaging, receptor binding and morphology. In paper II the generation and characterization of the UUK VLP system is described, and in papers III and IV this system is used to identify specific motifs in the glycoproteins important for UUKV RNP packaging and budding.

Generation of an infectious virus-like particle system for UUKV (Paper II)

Considering the lack of an infectious rescue system I needed to generate an infectious VLP system for UUK virus, to facilitate the study on viral assembly and packaging. The initial analysis was aimed to generate and characterize infectious VLPs. Prior to my study only one study of bunyavirus VLPs had been reported, Hantaan virus (HTNV) VLPs were generated by a vaccinia or baculovirus driven expression system (Betenbaugh*, et al.*, 1995).

UUKV, like most other bunyaviruses only has four structural proteins, G_N, G_C, N and L, two of which are already present in the minigenome system (N and L protein). The

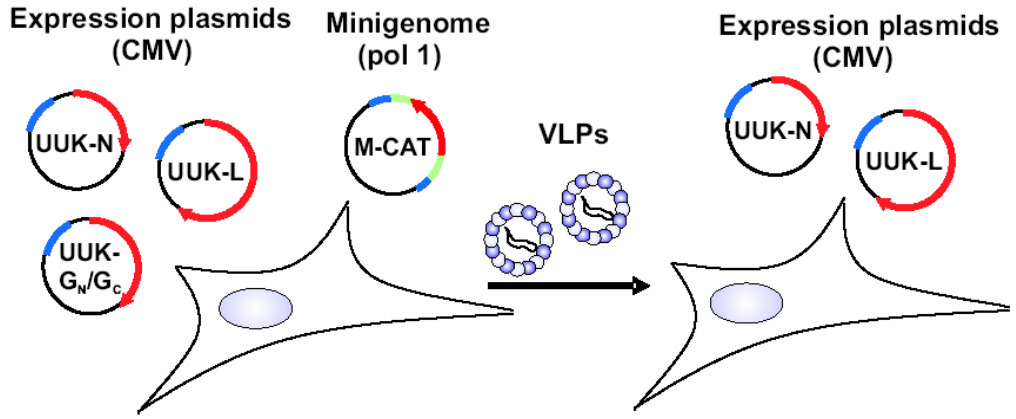

Figure 9: *UUK VLP system. BHK-21 cells are transfected with the minigenome (M-CAT), and the L, N and G_N/G_C expression plasmids. The glycoproteins are expressed and accumulate in the Golgi together with the replicated minigenome-RNPs, and VLPs are generated by budding into Golgi. The VLPs are then transported to the plasma membrane were they are released into the supernatant. The medium containing the VLPs is collected and used for infection of new cells where VLP infection can be measured by minigenome reporter protein expression. Transacting viral proteins required for minigenome replication (L and N) have to be provided by transfection prior to VLP infection. Red arrows indicate ORFs, blue boxes cytomegalovirus (CMV) or PolI promoters and green sections NCR.*

glycoprotein precursor p110 (G_N/G_C) was added to the minigenome system in order to generate VLPs (Fig. 9). The UUK VLPs were characterized with respect to kinetics, morphology, infectivity, and minimal protein composition. We found that the UUK VLPs could package all three minigenomes to the same extent and transfer minigenome activity by VLP infection of new cells. UUK VLPs were also shown to have the same surface morphology and size as wt UUKV. They are generated and released into the media 12 h post transfection, and could be neutralized with UUKV specific immune sera.

The two glycoproteins are necessary and sufficient to generate and release UUK VLPs, and no additional viral proteins or RNA are required for particle formation. HTNV VLPs, were not generated without the presence of both the glycoprotein and the nucleoprotein (Betenbaugh, *et al.*, 1995). This in contrast to the UUKV, were the N protein is not required for VLP formation. The reason for the different requirements between the two VLP systems is not clear but could be due to the different expression system used or to the fact that HTNV (*Hantavirus*) and UUKV (*Phlebovirus*) originates from different genera. This newly developed infectious VLP system for UUKV is an important tool for studying the assembly, packaging and budding of the UUKV in the absence of a full infectious rescue system. We are also one step closer to rescuing the virus.

Identification of amino acid residues in the glycoproteins critical for genome packaging (Paper III)

In the third and the fourth study I describe the identification of functional motifs in the cytoplasmic tail of G_N and G_C important for UUKV assembly. Initially we performed an alanine scan exchanging 5 amino acids at the time in the G_N cytoplasmic tail. Theses glycoprotein mutants were analyzed in the VLP system for their ability to transfer UUKV minigenomes to new cells. Four different mutants with mutations in three regions, corresponding to amino acids 21-25, 46-50 and 71-81, were unable to transfer CAT activity. Analysis of the protein composition of the VLPs released in the supernatant, reveled that regions 21-25 and 46-50 are important for particle generation and release, because no viral proteins could be detected in the supernatant from cells transfected with these two mutants, and will be discussed in paper IV. On the other hand, region 71-81 was shown to be important for packaging of RNPs into VLPs. Equal amounts of glycoprotein (wt and mutated) were released into the supernatant indicating that VLPs are generated to the same extent, however, VLPs containing mutated glycoproteins were completely devoid of N protein. A more detailed mutational analysis of region 71-81 revealed that four amino acids (M76, L79, T80 and R81) are important for packaging of RNPs into VLPs. Interestingly these mutant glycoproteins were also deficient in nucleoprotein binding, as determined by co-immunoprecipitation studies. In paper I, I showed that the three segments have different packaging efficiencies with the L segment the strongest and the S the weakest. To determine if this difference in packaging efficiency was due to the binding of the three segments to different parts of the G_N cytoplasmic tail, all three segment-based minigenomes were tested with the glycoprotein mutants. No segment specific interactions could be observed with any of the mutants, suggesting that the RNPs are packaged into VLPs through an interaction via the N protein and not through a specific RNA interaction.

Budding of UUKV is dependent on two amino acid residues in the G_N cytoplasmic tail and the intracellular localization of G_N/G_C proteins (Paper IV)

The focus of this study was to analyze and identify regions important for budding of VLPs. The two different regions previously identified in the G_N cytoplasmic tail, namely residues 21-25 and 46-50, were further analyzed, as well as the 5 amino acid in the G_C cytoplasmic tail. An alanine scan of these regions was performed and specific amino acids important for generation and release of VLPs were identified. Mutating residues 21-25 in G_N cytoplasmic tail to alanines did not affect the intracellular localization of the G_N/G_C heterodimer, while mutation of residues 46-50 resulted in the retention of both glycoproteins in the ER. Further mutational analysis was performed, and G_NL23 and G_NL24 were identified to be important for the initiation of budding into Golgi, visualized by the absence of VLPs by electron microscopy. Three amino acids (G_NL46, G_NE47 and G_NL50) were shown to be important for the exit from the ER and transport to the Golgi. When these residues were changed to alanines both G_N and G_C were retained in the ER, and as a result these mutants were deficient in budding. These results underline the importance of the intracellular localization of the two glycoproteins in VLP formation and budding.

The short cytoplasmic tail of G_C was also analyzed for functional motifs. The five amino acid long G_C cytoplasmic tail (K V K K S) has been proposed to be an ER retention signal (Ronnholm, 1992). First an alignment the G_C cytoplasmic tail was performed with other members of the *Bunyaviridae* family. One single residue, lysine third position from the end (G_CK3), was found to be highly conserved among the members of the *Orthobunyavirus*, *Phlebovirus* and *Hantavirus* genera, but not the *Nairovirus* or *Tospovirus*. An alanine mutational screen was performed in which the role of single amino acids was analyzed. The mutant glycoproteins were analyzed with the VLP system, and their intracellular localization was determined by immunofluorescence. I identified two amino acids in the G_C tail which resulted in reduced VLP formation compared to wt, and both had a different intracellular localization compared to the wt G_N/G_C. Mutating G_CK3 resulted in exit from the ER but the G_N/G_C heterodimer was not effectively retained in the Golgi, resulting in an accumulation of the glycoproteins at the plasma membrane. Moreover, the C-terminal residue in the G_C protein was shown to be important for the exit out of the ER. Two major conclusions can be drawn from this study; initiation of VLP budding into the Golgi is dependent on two amino acids in the G_N cytoplasmic tail, and the correct intracellular localization of the two glycoproteins is very important for particle formation.

Summary of the functional motifs in the G_N and G_C cytoplasmic tails, implications in UUKV assembly.

I have shown in papers III and IV that the cytoplasmic tail of G_N and G_C are involved in the different steps of UUKV assembly. From the correct targeting to, and retention in the Golgi of the two glycoproteins, to the assembly and budding of virus particles (the specific motifs are marked in Fig. 10). The G_N cytoplasmic tail contains a Golgi targeting and retention signal located to amino acids 10-40 (Andersson and Pettersson, 1998), and when G_N is expressed alone it is retained in the Golgi. However, the targeting of the heterodimer to the Golgi is not only dependent on the 30 amino acids previously identified in the G_N cytoplasmic tail. Four different amino acids, three in the G_N cytoplasmic tail (L46, E47 and L50) and one in G_C cytoplasmic tail (S5) are important for the exit of the glycoproteins from the ER. Since mutating any of these resulted in the retention of both glycoproteins in the ER. There are several examples of mutations that cause missfolding of proteins, which are then unable to exit the ER, due to the quality control mechanisms operating in the ER (Zhang, *et al.*, 1997). However, our mutations are in the cytoplasmic domain, and not in the lumenal domain of the proteins accessible for the recognition by molecular chaperones of the ER quality control (Zhang, *et al.*, 1997). Specific interactions, or lack of these interactions between the two cytoplasmic tails may therefore be responsible for the observed retention. Interactions have been shown to occur both between the G_N-G_N and G_C-G_N cytoplasmic tails by co-immunoprecipitation studies (Fig. 11), and these interactions are probably essential for intracellular trafficking of the heterodimer. The heterodimer formation seems to be strong and independent of the cellular localization of the mutant glycoproteins, as it was also observed for our ER retained mutants. Our data suggest that the heterodimer interactions are not only mediated by the

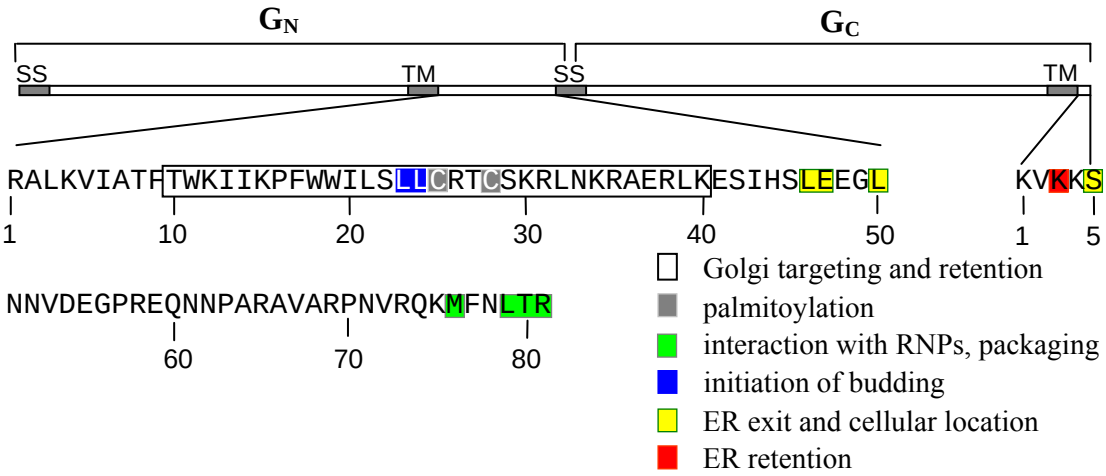

Figure 10: *Summary of identified motifs in the G_N and G_C cytoplasmic tails*

cytoplasmic tails of G_N and G_C, but also by the transmembrane domains of G_N and G_C, since wt G_N could be co-immunoprecipitated with a G_C with a truncated ectodomain (Fig. 11B).

I found that G_CK3 the C-terminus in the G_C cytoplasmic tail that is vital for the correct targeting and retention of the heterodimer in the Golgi complex. Mutations of this amino acid resulted in the expression of both glycoproteins at the plasma membrane. I speculate that this lysine is important for the postulated G_C ER retention motif, as well as proper interaction with the G_N Golgi retention signal, since both G_N and G_C move through the Golgi without efficient retention and are recovered at the plasma membrane when mutated. Analysis of the TSWV (*Tospovirus* genus) G_C cytoplasmic tail has shown that G_C interacts with the RNPs (Snippe*, et al.*, 2007). They suggested that this interaction was probably common for all members of the *Bunyaviridae* family. However, in the light of our results this is not likely, as mutations in the UUKV G_C cytoplasmic tail lead to a reduction in VLP formation, but not in RNP packaging (see below). It is more likely that the UUKV G_C is important for the intracellular localization. I could also see that G_CK3 is conserved among the hantaviruses, pheboviruses and orthobunyaviruses but not in the tospoviruses or nairoviruses, suggesting a common mechanism for the members of the hantaviruses, phleboviruses and orthobunyaviruses but not for tospoviruses and nairoviruses and this remains to be investigated.

After the accumulation of the glycoproteins in the Golgi, the RNPs have to gather at the site of budding for virus assembly. I have shown that four amino acids (M76, L79, T80 and R81) in the G_N cytoplasmic tail are important for this interaction. When mutated to alanine empty VLPs were released into the supernatant, demonstrating not only that these residues are important for RNP interaction and packaging but also that the glycoproteins by themselves can initiate budding. It appears therefore important for the virus that the RNPs are present close to the Golgi membrane to prevent formation of empty virus particles. Many viruses create so called “virus factories” in the cell, which is the recruitment of cell

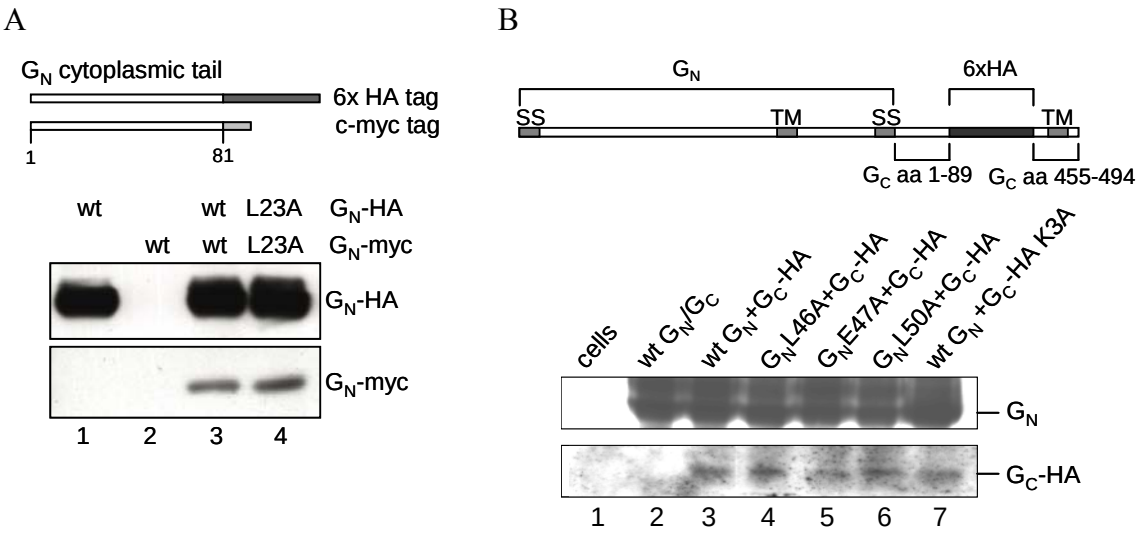

Figure 11: A. *Co-immunoprecipitation of wt or mutant G_N cytoplasmic tail constructs tagged with an HA or c-myc in their C-terminal end. The homodimer complex was immunoprecipitated with a monoclonal antibody recognizing the HA tag, and immunoprecipitated proteins were detected by Western blot using an antibody recognizing the G_N cytoplasmic tail.* ***B.*** *Co-immunoprecipitation of G_N and G_C with a truncated ectodomain. The G_N protein was immunoprecipitated with monoclonal antibody specifically recognizing the G_N protein and immunoprecipitated proteins were detected by Western blot using a polyclonal antibody recognizing the G_C and the G_N proteins.*

organelles and accumulation of viral proteins to enhance replication and assembly of the viruses (Novoa, *et al.*, 2005). Virus factories have been detected around the Golgi in BUNV infected cells (Salanueva, *et al.*, 2003). In UUKV infected cells the N protein was found to accumulate around the Golgi (Kuismanen, *et al.*, 1982), and it is therefore likely that both replication and packaging occurs at this assembly site.

One question which remains unanswered is: whether the packaging mechanism for bunyaviruses is random or selective? Our data in paper I and previous data (DI particles, (Marchi, *et al.*, 1998, Patel and Elliott, 1992, Resende Rde, *et al.*, 1992)) suggested that the viral L segment contains specific motifs in the 5' and 3' NCR important for packaging. However, no specific packaging interactions between the NCRs of the L segment and the cytoplasmic tails of G_N/G_C could be detected (paper III). These results indicate that the specific packaging motif in the L segment might interact with something else, either the polymerase protein or the other viral segments. It was recently reported that in Influenza virus, the 8 segments bind each other in a very ordered symmetry (Noda, *et al.*, 2006). Bunyaviruses may utilize a similar approach. The L segment might bind to the other segments in an ordered manner forming a RNP complex which is incorporated into progeny particles via specific interactions with the G_N cytoplasmic tail. It is known that bunyaviral segments do not reassort at random between members of the same serogroup (Pringle, 1996). Preferential binding of the L segment to another segment in the RNP complex might result in a non random reassortment of two virus strains. Our packaging model suggests a

random packaging interaction between the G_N cytoplasmic tail (paper III) and the RNP complex. The formation of the RNP complex, however, is probably not a random process, since UUKV particles contain the three segments in a specific ratio of L:M:S of 1:4.6:2 (Pettersson and von Bonsdorff, 1975), although we showed that the L segment has the strongest packaging signal (paper III). It is difficult to visualize the organization of the RNPs because of this uneven distribution of the viral segments. One possibility could be that the L protein binds specifically to the NCRs of the different segments, thereby contributing to the ratio of the three segments in progeny viral particles.

The final step in bunyaviral assembly is the budding into the Golgi and formation of virus particles. I have identified two amino acids in the G_N cytoplasmic tail that are involved in the initiation of budding into the Golgi. However, the precise mechanism and if other cellular factors are involved in this process still remains unknown. I did show that the RNPs are not involved in the initiation of budding since glycoproteins alone can form particles. One could postulate that the G_N cytoplasmic tail alone or together with the G_C cytoplasmic tail forms a matrix underneath the membrane, which could initiate the budding. However, the homo-dimerization of the G_N-G_N cytoplasmic tails is not disrupted by mutating the two amino acids L23 and L24 to alanines (Fig. 11A and data not shown). Another alternative is that mutating L23 and L24 disrupts the binding of some unidentified cellular factor important for the initiation of budding. However, this question remains to be answered.

Summary and future perspectives

In this thesis I describe our studies regarding two aspects of the UUKV lifecycle. The role of the NCRs with respect to transcription/replication and packaging was analyzed, however, the major focus of the work has been targeted towards the assembly of UUKV.

Using the UUKV minigenome system we discovered that the variable regions of the NCRs are important regulating transcription/replication and packaging for the three viral segments. In addition, we identified that the 5' and 3' NCRs act together to function as a promoter. The IGR of the S segment was also shown to be important for efficient UUKV minigenome expression, which is in line with its proposed role in transcription termination.

Although the minigenome system developed for UUKV is very useful to study transcription and replication it is of limited use for studying UUKV assembly. In order to study UUKV assembly in the absence of a rescue system I developed an infectious virus-like particle system for UUKV. These VLPs were characterized and the system was optimized. The VLP system is a good complement to the minigenome system already developed for UUKV, especially for studying UUKV assembly in the absence of a rescue system. We used our VLP system two identify specific motifs in the G_N and G_C cytoplasmic tails important for UUKV assembly. The intracellular localization of the glycoproteins, packaging and budding were analyzed.

The next step would be to further analyze the mechanism how, and what cellular factors are involved in RNP packaging and budding. It would also be interesting to analyze the assembly of other bunyaviruses to identify common mechanisms of packaging and budding for this family. Hopefully, the detailed knowledge about bunyaviral assembly will contribute to the development of antiviral targets for the more harmful members of this virus family.

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Doing a PhD takes a number of years and does not always turn out the way you planned in the beginning, at least not for me. But I don't regret anything, even tough the journey traveling between different countries was not always easy and pleasurable. The person you meet and see today is not at all the same as the one that moved to Stockholm and started a PhD four years ago, and I'm truly happy for that. During these years I meet some really wonderful people that I like to thank with all of my heart.

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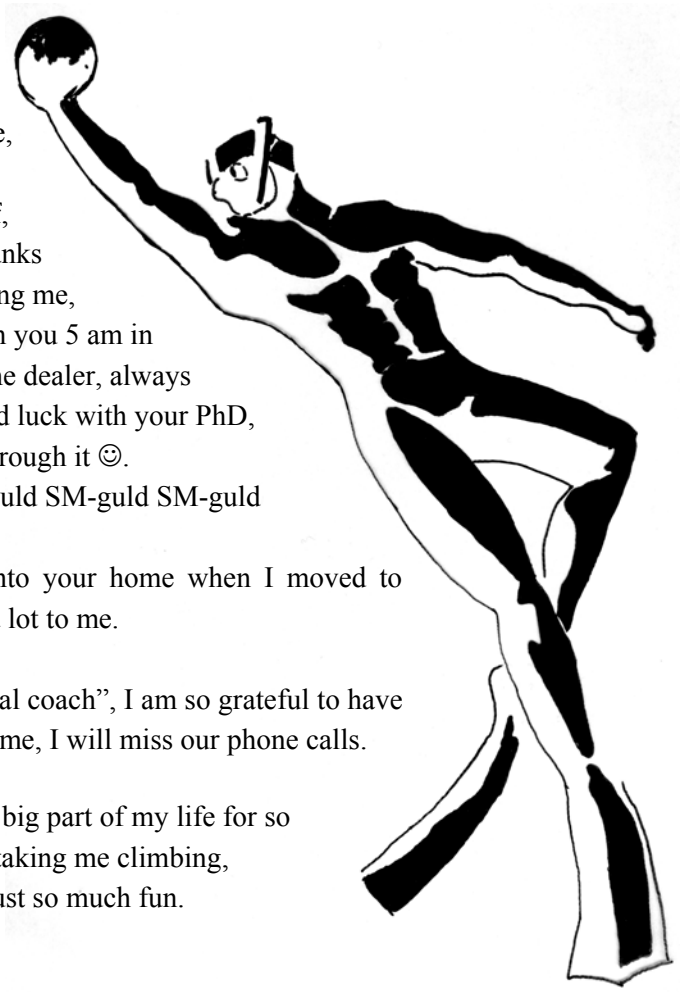

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