Helsinki University Biomedical Dissertations No. 48

Replication of Tula Hantavirus

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Academic Dissertation

To be publicly discussed, with the permission of the Faculty of Biosciences of the University of Helsinki, in the Small Lecture Hall, Haartman Institute, on April 2nd at 12 o'clock noon.

Helsinki, 2004

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ISSN 1457-8433
ISBN 952-10-1731-7 (paperback)
ISBN 952-10-1732-5 (PDF)
http://ethesis.helsinki.fi
Multiprint
Helsinki, 2004

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their Roman numerals in the text

I **Kukkonen, S. K. J.**, Vaheri, A., and Plyusnin, A. (1998). Completion of the Tula hantavirus genome sequence: properties of the L segment and heterogeneity found in the 3' termini of S and L genome RNAs. *Journal of General Virology* 79, 2615-2622.

II **Kukkonen, S. K. J.**, Vaheri, A., and Plyusnin, A. (2004). Tula hantavirus L protein is a 250 kDa perinuclear membrane-associated protein. *Journal of General Virology*, in press.

III Plyusnin, A., **Kukkonen, S. K.**, Plyusnina, A., Vapalahti, O., and Vaheri, A. (2002). Transfection-mediated generation of functionally competent Tula hantavirus with recombinant S RNA segment. *EMBO Journal* 21:1497-503.

IV Li, X-D.*, **Kukkonen, S.***, Vapalahti, O., Plyusnin, A., Lankinen, H., and Vaheri A. (2003). Tula hantavirus infection of Vero E6 cells induces caspase-mediated apoptosis involving TNF-receptor 1 mediated signal pathway. Submitted

* equal contribution

ABBREVIATIONS

aa	amino acid
Bax	Bcl-2 associated protein X
Bcl-2	B-cell lymphocyte/leukemia-2 protein
BrUTP	5-bromouridine 5'-triphosphate
cRNA	antigenomic ribonucleic acid
EGFP	enhanced green fluorescent protein
GTP	guanosine triphosphate
HFRS	hemorrhagic fever with renal syndrome
HIV-1	Human immunodeficiency virus type-1
HPS	hantavirus pulmonary syndrome
Hsp70	heat shock protein 70
IFA	immunofluorescence assay
IPTG	isopropyl-ß-D-thiogalactopyranoside
L	large genome segment of *Bunyaviridae*
L protein	RNA polymerase of *Bunyaviridae*
M	medium genome segment of *Bunyaviridae*
mRNA	messenger ribonucleic acid
N protein	nucleocapsid protein *Bunyaviridae*
nt	nucleotide
NTP	nucleotide triphosphate
NTR	nontranslated region
ORF	open reading frame
PCR	polymerase chain reaction
p.i.	post infection
RNA	ribonucleic acid
RT-PCR	reverse transcription - polymerase chain reaction
S	small genome segment of *Bunyaviridae*
T7 pol	bacteriophage T7 RNA polymerase
TNF	tumor necrosis factor
UMP	uridine monophosphate
UTP	uridine triphosphate
vRNA	genomic RNA
vRNP	viral ribonucleoprotein complex

1. ABSTRACT

Hantaviruses are carried by rodents and cause hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas. HFRS is characterized by vascular hemorrhage with kidney dysfunction and HPS by vascular hemorrhage with acute pulmonary edema. Search for the causative agent of HFRS lead to the discovery of Hantaan virus in 1976. To date more than 20 hantavirus species have been found, including Tula virus, which was detected in European common voles, *Microtus arvalis*, captured in Tula, Russia.

Hantaviruses are enveloped viruses that have two glycoproteins, G1 and G2 embedded in the lipid bilayer. The virions contain three viral ribonucleoprotein complexes (vRNP) that consist of a negative-strand RNA segment: S (small), M (medium), or L (large), encapsidated by nucleocapsid (N) protein with one L protein associated with each vRNP. The 5' and 3' termini of the viral RNAs contain a consensus sequence found in all hantaviruses. Based on these properties, hantaviruses are assigned into the same genus, *Hantavirus*, of the *Bunyaviridae* family.

In the current work, the sequencing of the Tula virus genome was completed. The L RNA was determined to have 6541 nucleotides (nt) with an antigenomic open reading frame of 6459 nt that encodes the L protein. It contains the regions conserved in all RNA-dependent RNA polymerases. The 3' ends of S and L vRNAs were heterogeneous with deletions in over 40% of the termini. As the termini contain the promoter required for RNA synthesis, the deletions can interfere with RNA synthesis lowering virus production.

The detection of a hantavirus L protein in infected cells had not been accomplished previously. In this study polyclonal antibodies were raised against L protein. The protein was found to be expressed as a perinuclear membrane protein of approximately 250 kDa. The RNA polymerases of all positive-strand RNA viruses are known to be membrane-associated but this is the first such finding for a negative-strand RNA virus.

Sequencing of Tula virus RNA isolated from rodents suggested that homologous recombination might have occurred. In the current work we infected cells with one Tula virus strain and then expressed, via transfection-mediated expression, the S RNA of another Tula strain. Homologous recombination could be observed and recombinant viruses could be isolated. This proves that at least in this experimental setting homologous recombination is possible and that it could be used to introduce genomic material into hantaviruses.

It has been debated whether hantaviruses cause apoptosis. When Tula virus was propagated in Vero E6 cells, it caused cell death. The dying cells had a degraded genome that could be observed as a DNA ladder. The cells were positive in TUNEL staining and the PARP protein was cleaved by a caspase. Tumor necrosis factor α enhanced the observed cell death. Based on these findings we determined the cell death to be apoptotic.

2. REVIEW OF THE LITERATURE

2.1. Discovery and classification of hantaviruses

Hantaviruses were discovered in the search for the etiology of hemorrhagic fever with renal syndrome (HFRS). Rodents were suspected to be carriers of the disease and, in an immunofluorescence assay (IFA), sera of HFRS patients bound to tissue from striped field mice, *Apodemus agrarius*, that had been trapped from the banks of the river Hantaan, South Korea. This demonstrated the presence of the infectious agent in the rodent tissue. The infectious agent could be transferred to striped field mice in a laboratory setting (Lee *et al.*, 1978). The infectious agent was isolated and named Hantaan virus. It later gave the name for the genus *Hantavirus*.

Based on similarities in the clinical picture and epidemiology of HFRS in Korea and nephropathia epidemica in Europe, the causative agents were thought to be related. This was confirmed by positive IFA of *Apodemus agrarius* tissue with serum from a nephropathia epidemica patient (Lee *et al.*, 1979; Svedmyr *et al.*, 1979). Since the causative agent of HFRS was found in rodents, this was thought to be also the case for nephropathia epidemica and, indeed, Puumala virus was isolated from bank voles, *Clethrionomys glareolus*, trapped in Puumala, Finland (Brummer-Korvenkontio *et al.*, 1980; 1982).

As the search for hantaviruses has been extended to more rodent species, new hantaviruses have been found. Tula virus was discovered from the European common vole, *Microtus arvalis* (Plyusnin *et al.*, 1994). Interest in hantavirus research grew when it was discovered that the outbreak of acute respiratory illness with high mortality in the United States was caused by a hantavirus, later named Sin Nombre virus, carried by the deer mouse, *Peromyscus maniculatus* (Nichol *et al.*, 1993). To date, more than 20 hantaviruses have been discovered (Plyusnin, 2002). With the growing number of hantaviruses found from various rodent species, it has become apparent that each hantavirus is typically carried by only one rodent species with closely related rodents having also closely related hantaviruses. This indicates that the viruses have been co-evolving with their rodent hosts (Hjelle & Yates, 2001; Plyusnin & Morzunov, 2001; Plyusnin, 2002).

Hantaviruses are grouped into one genus, *Hantavirus*, due to their genetic similarity, especially in the conserved terminal sequences. Based on similarities in the genome structure and other properties, hantaviruses are further grouped together with the genera *Orthobunya*-, *Nairo*-, *Phlebo*-, and *Tospovirus* to the family *Bunyaviridae* (Elliott *et al.*, 2000). Hantaviruses are rodent-borne, whereas all other viruses of this family are arthropod-borne. All viruses of this family have a genome consisting of three negative-strand RNAs: L (large), M (medium), and S (small). Each genus of the *Bunyaviridae* family has specific 5'-

and 3'-terminal nucleotide sequences that are conserved in all viruses of the genus (Fig. 2.1.1.) (Elliott *et al.*, 1991).

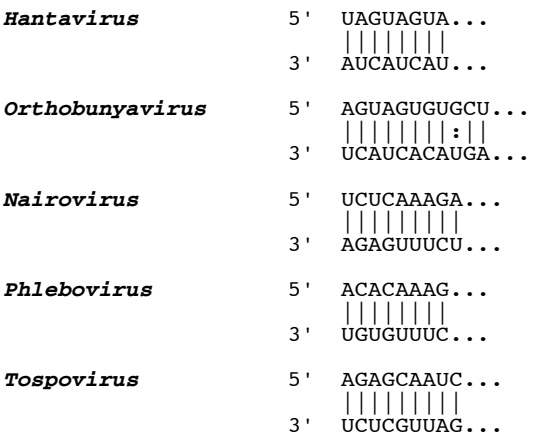

Figure 2.1.1. The genus-specific 5' and 3' termini of *Bunyaviridae* (Elliott *et al.*, 1991). Possible base pairs are indicated by vertical lines and noncanonical base pairs by a colon

2.2. Hantavirus-associated diseases

Hantaviruses are thought to cause persistent infections of their rodent hosts without any apparent disease (Yanagihara *et al.*, 1985; Hutchinson *et al.*, 1998; Botten *et al.*, 2000). As human-to-human transmission of hantaviruses does not normally occur, humans are dead-end hosts for the viruses; therefore, all hantavirus adaptations have occurred to promote spread of the virus in rodents. Humans can be infected by hantaviruses through the respiratory tract when air containing aerosolized viruses shed from rodent feces, urine, or saliva is inhaled (Tsai, 1987). Person-to-person transmission has been reported only for Andes virus (Wells *et al.*, 1997; Padula *et al.*, 1998). Whereas hantavirus infections tend to be asymptomatic in rodents (Tkachenko & Lee, 1991), they are known to cause two types of diseases in humans: HFRS and hantavirus pulmonary syndrome (HPS).

HFRS is a disease characterized by vascular hemorrhage and kidney dysfunction (Cosgriff & Lewis, 1991). The disease is caused by Hantaan, Seoul, Puumala, Dobrava, and Saaremaa hantaviruses which are carried by rodents in Asia and Europe, although the hosts of Seoul virus, the brown rat, *Rattus norvegicus*, and the black rat, *Rattus rattus*, are found worldwide. There are over 100,000 cases worldwide annually with case fatality rate ranging from 0.1 to 10 % depending on which hantavirus has caused the infection (Lee *et al.*, 1990; Brummer-Korvenkontio *et al.*, 1999). In Europe there are annually approximately 3000 cases in Russia (seroprevalence 6%), 1000 in Finland (5%), 300 in Sweden (8% in northern Sweden) and 100 or less in other European countries (Vapalahti *et al.*, 2003).

HPS is characterized by vascular hemorrhage and acute pulmonary edema and has a case fatality rate of 20-40 % in hospitalized cases (Khan & Young, 2001). Typically the disease starts with fever followed by interstitial edema (Duchin *et al.*, 1994; Ketai *et al.*, 1994; Zaki *et al.*, 1995). HPS is caused by Sin Nombre and related viruses in North America, and Andes and related viruses in South America, all of which are carried by rodents of the Sigmodontinae family (Schmaljohn & Hjelle, 1997). Between 1993 (when the disease was first characterized) and 2002 there were 1598 reported cases with 530 in Argentina, 344 in the USA, 271 in Chile, 240 in Brazil, and altogether 213 cases in the other countries of the Americas (Pan American Health Organization, 2003).

Diagnosis of hantavirus infections is based mainly on serological methods (Krüger *et al.*, 2001). Of the four structural proteins of hantaviruses, the N protein is the most commonly used antigen in the serological tests; it is easier to express than the other hantavirus proteins and it is also highly immunogenic (Vapalahti *et al.*, 1995). Furthermore, antibodies against N protein reach high titers in the acute phase of infection whereas antibodies against the two glycoproteins do not reach high titers until the late convalescent phase (Lundkvist *et al.*, 1993; Kallio-Kokko *et al.*, 2001). It is not known whether antibodies against the fourth viral protein, L protein, exist in patients and there are no serological tests using L protein as antigen.

Many hantaviruses have been considered nonpathogenic e.g. of European hantaviruses Tula virus and of North American hantaviruses Prospect Hill virus (Vapalahti *et al.*, 2003). There is a report claiming that Tula virus was associated with a disease in a patient bitten by a rodent. Serological tests showed that the patient had antibodies against hantavirus antigens and the serum of the patient showed specificity to Tula virus in a focus reduction neutralization test (Schultze *et al.*, 2002). Furthermore, the patient had IgM antibodies that decreased with time. However, it was suggested that the described symptoms of this patient are better explained by a bacterial disease, rat-bite fever (Clement *et al.*, 2003) but the symptoms are not typical of this disease (Schultze *et al.*, 2003). In another report it was claimed that a patient having fever, renal syndrome, and pneumonia would have had an illness caused by Tula virus. The patient's serum had antibodies against hantavirus antigens and the strongest reaction was with Tula virus in a focus reduction neutralization test (Klempa *et al.*, 2003). Although the authors showed that Tula virus does circulate in rodents in the area where the patient lives, it is not certain whether the illness was actually caused by a Tula virus infection. In contrast to e.g. Puumala virus, transmission of Tula virus to humans was exceedingly rare even in an area with high Tula virus prevalence in rodents (Vapalahti *et al.*, 1996).

How hantaviruses cause disease in humans is still not well known. Hantaviruses infect endothelial cells causing an increase in vascular permeability. This is probably not

caused by direct damage to the endothelial cells, as hantaviruses do not appear to cause a cytopathic effect in these cells (Yanagihara & Silverman, 1990; Pensiero *et al.*, 1992; Zaki *et al.*, 1995; Sundstrom *et al.*, 2001).

Comparisons of pathogenic and nonpathogenic hantaviruses can help elucidate how hantaviruses cause disease. However, it should be noted that even subtle amino acid changes can cause dramatic changes in the pathogenicity of hantaviruses e.g. a single amino acid substitution in the glycoprotein sequence markedly increased the pathogenicity of Hantaan virus to suckling mice (Isegawa *et al.*, 1994; Ebihara *et al.*, 2000).

Pathogenic hantaviruses use β3 integrins as receptors, whereas nonpathogenic hantaviruses use β1 integrins (Gavrilovskaya *et al.*, 1998; Gavrilovskaya *et al.*, 1999). Furthermore, the pathogenic viruses: Hantaan, Seoul, Puumala, Sin Nombre, and New York virus blocked endothelial cell migration, whereas the nonpathogenic Prospect Hill and Tula virus did not (Gavrilovskaya *et al.*, 2002). This differential effect may explain why the pathogenic hantaviruses impair normal endothelial function increasing vascular permeability resulting in disease.

HFRS-causing Hantaan virus, HPS-causing New York virus, and the nonpathogenic Prospect Hill virus differ also in the effect that they have on gene expression in infected human umbilical vein endothelial cells. A DNA microarray study showed that infection with the nonpathogenic Prospect Hill virus elicited a high-level induction of interferon stimulated genes early on (1 day p.i.) whereas infections with the pathogenic Hantaan and New York viruses elicited the interferon response only after 4 days p.i. This indicates that the pathogenic viruses might be better equipped to evade the antiviral response of the host cell. Of the genes induced by interferon, the greatest induction was detected in the amount of MxA-coding mRNA (Geimonen *et al.*, 2002). MxA inhibits infection by *Bunyaviridae* including hantaviruses as has been shown with Hantaan, Tula, and Puumala viruses (Frese *et al.*, 1996; Kanerva *et al.*, 1996). The mechanism of inhibition has not been studied with hantaviruses but it has been studied with other *Bunyaviridae*. In cells infected with La Crosse and Bunyamwera virus, genus *Orthobunyavirus*, and Rift Valley Fever virus, genus *Phlebovirus*, MxA sequesters N protein into fibrillary structures in the perinuclear region making it unavailable for virus assembly (Kochs *et al.*, 2002).

All hantaviruses that cause HPS have in the cytoplasmic tail of G1 a signaling element called immunoreceptor tyrosine-based activation motif (Geimonen *et al.*, 2003b). This motif directs receptor signaling. Nonpathogenic and HFRS-causing hantaviruses do not have this motif with the exception of Tula virus, which, however, lacks an adjacent cysteine residue which can alter the conformation of the motif. By interfering with the mechanism that controls cellular responses to external stimuli, the signaling element of G1 might alter vascular permeability (Geimonen *et al.*, 2003b).

Animal models have been developed for both HFRS and HPS. Infection of cynomolgus macaques (*Macaca fascicularis*) with Puumala virus caused a disease that resembled nephropathia epidemica, the mild form of HFRS, by all measured parameters (Klingström *et al.*, 2002). Infection of Syrian hamsters (*Mesocricetus auratus*) with Andes virus produced a lethal HPS-like disease that had similar clinical symptoms, disease progression, histopathology of the lungs and spleen, and incubation period as HPS in humans (Hooper *et al.*, 2001). These models could be used in the study of pathogenesis mechanisms and in development of vaccines and treatment of disease.

Treatment of HFRS and HPS is currently mainly supportive. A ribonucleoside analog, ribavirin, can interfere with viral replication and, reportedly, lowered the mortality of HFRS patients (Huggins *et al.*, 1991) but had no significant effect in HPS patients (Chapman *et al.*, 1999). In cells infected with Hantaan virus, ribavirin appears to be incorporated into viral RNAs resulting in loss of sequence information and consequently loss of production of functional viral proteins (Severson *et al.*, 2003).

Several vaccines based on inactivated virus have been developed and have also been used in South Korea and China resulting in a decrease in HFRS cases (Krüger *et al.*, 2001). Recombinant vaccinia viruses expressing hantavirus proteins, recombinant hantavirus glycoproteins, N protein, virus-like particles, and, lately, also nucleic acid vaccines have been studied as potential vaccines though human trials with these have not been reported yet.

2.3. Structure and genome of hantaviruses

All *Bunyaviridae* have a segmented negative-strand genome consisting of three RNA's: L (large), M (medium), and S (small). The reverse complement of L segment encodes L protein which has been shown to be the RNA polymerase of the virus (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Flick & Pettersson, 2001; Blakqori *et al.*, 2003; Flick *et al.*, 2003). The reverse complement of M segment encodes a glycoprotein precursor that is cleaved to yield glycoproteins G1 and G2, and the reverse complement of S segment encodes N protein (Elliott, 1990). Out of hantaviruses with the complete genome sequenced, Seoul virus has the shortest open reading frames for all of the four viral proteins with the following number of amino acids (aa) in the deduced sequences: L 2151 aa, G1 647 aa, G2 486 aa, and N 429 aa; Puumala virus has the longest open reading frames for all four proteins: L 2156 aa, G1 658 aa, G2 490 aa, N 433 aa (Plyusnin, 2002). L protein sequences are the most conserved with a maximum amino acid divergence of 31.4% and the glycoprotein sequences are the most divergent, with divergence of up to 46.9% (Nemirov *et al.*, 2003).

Bunyaviridae virions are spherical and have a diameter of approximately 100 nm (Obijeski *et al.*, 1976a; Lee & Cho, 1981; Martin *et al.*, 1985). The viruses are enveloped and the two glycoproteins, G1 and G2, form spikes on the virions (Fig. 2.3.1). Based on electron microscopy images these spikes protrude approximately 6 nm from the surface of hantavirus virions (Martin *et al.*, 1985). The glycoproteins mediate cell attachment and fusion (Tsai *et al.*, 1984; Arikawa *et al.*, 1985; Okuno *et al.*, 1986). The glycoproteins are glycosylated with the sugar moieties being mainly of the high-mannose type (Schmaljohn *et al.*, 1986).

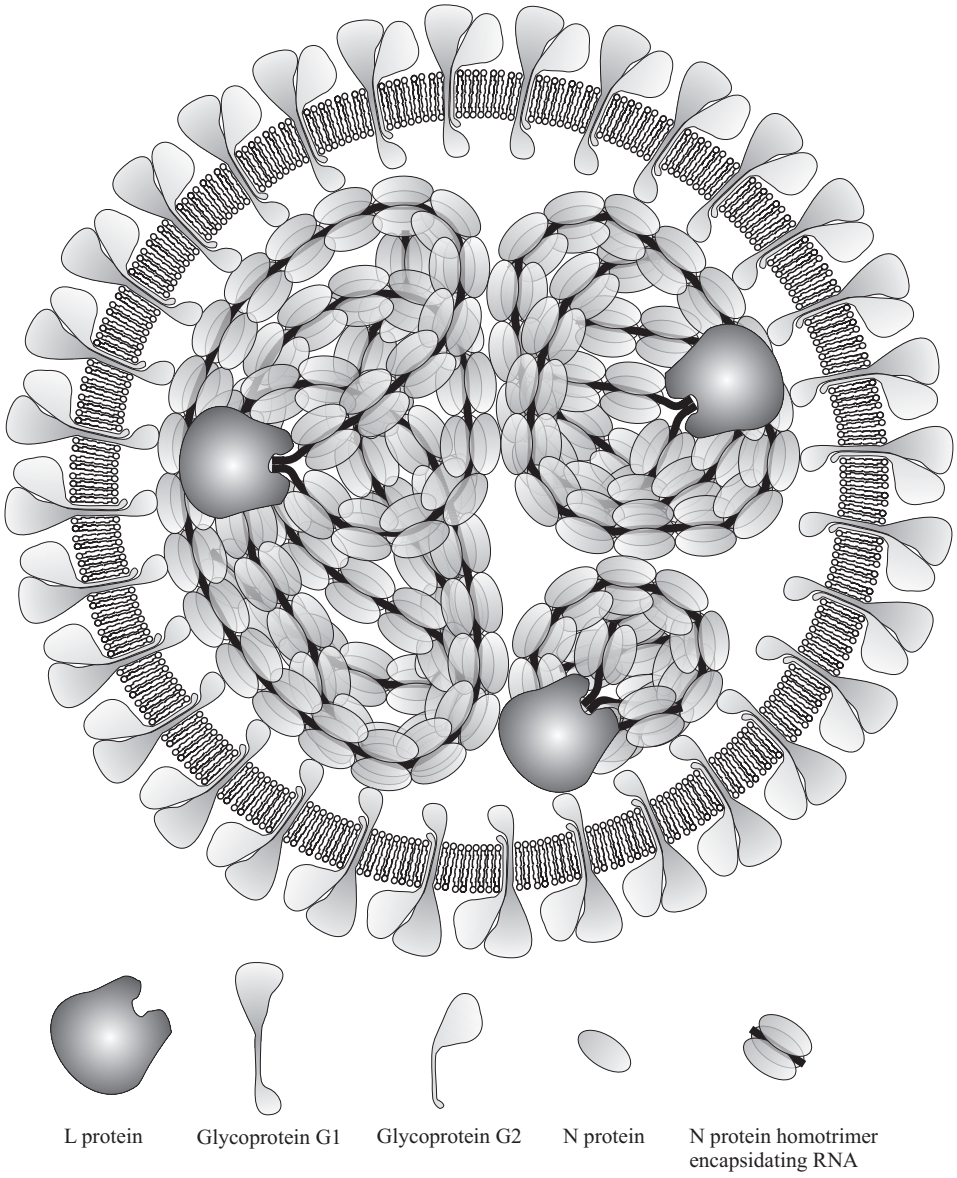

Figure 2.3.1. Hantavirus virion. The virion is enveloped by a phospolipid bilayer and contains three vRNPs: L (large), M (medium), and S (small), with one L protein bound to each.

Inside the virions there are three viral ribonucleoprotein complexes (vRNP), which consist of the genomic S, M, and L vRNAs encapsidated by the N protein (Fig. 2.3.1) (Obijeski *et al.*, 1976b; Elliott *et al.*, 1984; Gott *et al.*, 1993). The virions have been shown to contain equimolar amounts of the three genomic vRNAs (Hutchinson *et al.*, 1996). Small amounts of L protein are also associated with the vRNPs (Obijeski *et al.*, 1976b).

When recombinant N protein and the two glycoproteins are expressed in cells, virus-like particles are formed even in the absence of viral genomic RNA and the L protein (Betenbaugh *et al.*, 1995). The N protein and the two glycoproteins are, thus, the proteins that form the ultrastructure of the virions.

The vRNPs of *Bunyaviridae* appear to be circular (Pettersson & von Bonsdorff, 1975) due to the complementarity and subsequent base pairing of the 5' and 3' termini of the vRNAs (Pardigon *et al.*, 1982). The vRNPs of viruses of the families *Arenaviridae* and *Orthomyxoviridae* are also circular due to the base pairing of the 5' and 3' ends (Palmer *et al.*, 1977; Hsu *et al.*, 1987).

Only the above described viral proteins: the N protein, the two glycoproteins and the L protein, have been detected in *Bunyaviridae* virions (Obijeski *et al.*, 1976a; Elliott *et al.*, 1984; Kormelink *et al.*, 1994). Additional nonstructural proteins are encoded by the S and M segments of orthobunya-, phlebo-, and tospoviruses. The tospovirus nonstructural protein encoded by the M segment is involved in cell-to-cell movement (Kormelink *et al.*, 1994). The nonstructural protein (NSs) encoded by the S RNA of Bunyamwera orthobunyavirus (Bridgen *et al.*, 2001) and Rift Valley fever phlebovirus (Bouloy *et al.*, 2001) inhibits the interferon response in infected cells; the NSs of San Angelo, California encephalitis, and La Crosse orthobunyaviruses, inhibits protein translation and induces apoptosis (Colon-Ramos *et al.*, 2003).

Hantaviruses carried by Arvicolinae and Sigmodontinae rodents, e.g. Tula, Puumala, and Sin Nombre viruses, have an additional small open reading frame (ORF) overlapping the major open reading frame of the S mRNA (Plyusnin, 2002). In Tula virus this ORF could encode a protein of 90 amino acids (Plyusnin *et al.*, 1994) but no hantavirus nonstructural protein has been detected yet. The S mRNAs of the Murinae-carried Hantaan, Seoul, Dobrava, and Saaremaa viruses do not have this additional ORF (Plyusnin, 2002).

2.4. Virus entry into cells

Hantaviruses infect endothelial cells and were thus suspected to use endothelial cell receptors to gain entry into cells. Indeed, an integrin ligand, vitronectin, blocked infection of cells by HFRS-causing hantaviruses: Hantaan, Seoul, and Puumala viruses, and HPS-causing hantaviruses: Sin Nombre and New York viruses. Another integrin ligand, fibronectin, blocked the infection of the nonpathogenic Prospect Hill virus (Gavrilovskaya *et al.*, 1998; Gavrilovskaya *et al.*, 1999). Monoclonal antibodies to β3 and β1 integrins blocked the infection of pathogenic and nonpathogenic hantaviruses, respectively, indicating that these integrins are used as receptors in virus entry. Furthermore, expression of recombinant β3 integrins in CHO cells that normally do not express the protein made it possible for Sin Nombre and New York viruses to infect these cells (Gavrilovskaya *et al.*, 1998). In addition to the integrins, a 30 kDa protein has been reported to be used as a receptor by hantaviruses (Kim *et al.*, 2002). Only the size, not the identity, of the protein was reported.

The entry of hantaviruses into cells was studied with Hantaan virus (Jin *et al.*, 2002). The entry was dynamin-dependent indicating that uptake of the virus occurs by endocytosis. Inhibition of caveolae-mediated endocytosis did not inhibit virus entry, wheras inhibition of clathrin-dependent endocytosis did. Furthermore, clathrin co-localized with viral proteins, confirming that the entry of Hantaan virus occurs by the clathrin-dependent endocytic pathway. Viral proteins co-localized with an early endosome marker 90 min p.i. and with a lysosome marker 4 hours p.i. Raising the pH of the acidic late endosomes and lysosomes inhibited virus entry indicating that the acidic environment is required for the intracellular fusion of the viral membrane and release of the vRNPs into the cytoplasm (Jin *et al.*, 2002).

The N protein of Black Creek Canal virus interacts with actin and disruption of the filaments inhibits virus assembly and/or release (Ravkov *et al.*, 1998). Therefore, it is conceivable that vRNPs would be transported from the cytoplasmic entry site to a site of RNA synthesis utilizing actin filaments. However, disruption of actin filaments by pretreatment of cells for 30 min with cytochalasin D prior to infection and treating the cells for 2 hours during infection, did not lower virus yield significantly (Ravkov *et al.*, 1998). This demonstrates that actin filaments are not required in the early stages of infection. The cytochalasin D treatment lasted only 2 hours during infection and, therefore, it cannot be ruled out that actin filaments may play a role in transport of the vRNPs from the site of virus entry.

2.5. Viral RNA synthesis and the RNA polymerase

2.5.1. Location of RNA synthesis

The site of *Bunyaviridae* RNA synthesis has been studied with La Crosse orthobunyavirus and it was found to be localized in the cytoplasm (Rossier *et al.*, 1986) but there have been no studies to localize the site within the cytoplasm. The N protein of Black Creek Canal, Puumala, and Seoul hantaviruses is localized in the cytoplasm around the nucleus, i.e. the perinuclear region (Ravkov & Compans, 2001; Kariwa *et al.*, 2003). As N protein is known to be required for viral RNA synthesis in *Bunyaviridae* (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Accardi *et al.*, 2001; Flick & Pettersson, 2001; Blakqori *et al.*, 2003; Flick *et al.*, 2003), the perinuclear region is also likely to be the site of RNA synthesis. The N protein of Black Creek Canal hantavirus was found to co-localize with the Golgi marker α-mannosidase II in the perinuclear region (Ravkov & Compans, 2001) but the N protein of Seoul hantavirus did not co-localize with Golgi markers (Kariwa *et al.*, 2003). Of other *Bunyaviridae*, the N protein of the Crimean-Congo hemorrhagic fever nairovirus is of similar size to hantavirus N protein and localizes in the perinuclear region even when the Golgi is disrupted (Andersson *et al.*, 2004). Based on these data, the Golgi complex is not likely to be the site of RNA synthesis. The perinuclear localization of the N protein of Crimean-Congo hemorrhagic fever nairovirus was abolished when actin filaments were disrupted (Andersson *et al.*, 2004). Therefore, it is possible that the RNA synthesis machinery of *Bunyaviridae* would be associated with actin filaments in the perinuclear region.

There have been no studies on the localization of hantavirus L protein, nor have there been reports on the localization of newly synthesized viral RNAs. Of viruses with a segmented negative-strand genome, the localization of RNA polymerase subunits has been studied only for influenza A virus which replicates in the nucleus where the heterotrimer formed by the three polymerase subunits also localizes (Akkina *et al.*, 1987).

The RNA replication of all positive-strand RNA viruses is associated with membranes (Salonen *et al.*, 2004). The RNA synthesis occurs in vesicles or appressed membranes where the replication intermediates are sequestered resembling the RNA synthesis of retroviruses and double-stranded RNA viruses that sequester the double-stranded replication intermediates in protein cores (Schwartz *et al.*, 2002). Schwartz *et al.* (2002) have suggested that the sequestering of replication intermediates might help the viruses avoid the antiviral host defenses triggered by double-stranded RNA. Of the hantavirus proteins required for RNA synthesis, the membrane association of N protein has been studied with Black Creek Canal virus and the protein was found to be membrane-

associated (Ravkov & Compans, 2001). It remains to be seen whether the RNA synthesis of negative-strand RNA viruses, such as *Bunyaviridae*, is also associated with membranes.

The membrane-association of RNA synthesis can also bring about advantages of surface catalysis discussed by Lyle *et al.*, (2002) by: 1) limiting reactant movement to two dimensions instead of three, 2) providing the RNA template with multiple binding sites due to the close proximity of several RNA polymerases; this should increase the affinity of the polymerase to the RNA template, and 3) retention of reaction products which can then rapidly be used as templates in the synthesis of the complementary RNA-strand in RNA replication. Surface catalysis is enhanced by the formation of two-dimensional arrays of RNA polymerase oligomers that has been observed with poliovirus (Lyle *et al.*, 2002). RNA polymerase oligomers have also been observed with a negative-strand virus, Sendai paramyxovirus (Smallwood *et al.*, 2002; Cevik *et al.*, 2003).

2.5.2. Transcription of mRNA

The genomic RNAs of negative-sense viruses cannot function as mRNAs. Therefore, after entry into cells viruses of the family *Bunyaviridae* have to synthesize positive-strand mRNAs using the genomic RNAs as templates. Newly synthesized N protein is required for genome replication (Abraham & Pattnaik, 1983; Pattnaik & Abraham, 1983; Patterson & Kolakofsky, 1984) and therefore mRNA transcription and translation of viral proteins precedes RNA replication.

The L, M, and S genome segments of all *Bunyaviridae* have nontranslated regions (NTR) of varying lengths at the 5’ and 3’ termini of the RNAs. These NTRs contain regions that function as promoters as has been demonstrated in assays where coding regions of reporter genes are flanked by viral NTRs (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Flick & Pettersson, 2001; Blakqori *et al.*, 2003; Flick *et al.*, 2003). The 5' and 3' termini of RNA genome segments of many hantaviruses have been determined (Chizhikov *et al.*, 1995; Meyer & Schmaljohn, 2000; Padula *et al.*, 2002). All negative-strand RNA viruses have complementarity between the 5’ and 3’ termini (Barr & Wertz, 2004) and this also the case for all three hantavirus RNA segments (Fig. 2.5.2.1a). Furthermore, it has been shown for Bunyamwera orthobunyavirus that base pairing between the 5’ and 3’ termini is required for RNA synthesis with perfect complementarity allowing the most active RNA replication (Barr & Wertz, 2004). Like the termini of other *Bunyaviridae*, the terminal sequences of hantaviruses could fold into a corkscrew structure (Fig. 2.5.2.1b) that has been suggested to function as the promoter for mRNA transcription in *Bunyaviridae* (Flick *et al.*, 2002). The corkscrew structure could be a universal promoter structure among segmented negative-strand RNA viruses, as the vRNA termini of influenza A virus, family *Orthomyxoviridae* (Flick *et al.*, 1996; Flick & Hobom, 1999; Pritlove *et al.*, 1999; Leahy *et al.*, 2001a; Leahy

et al., 2001b) and tenuiviruses (Flick *et al.*, 2002) could also fold into this structure. Among *Bunyaviridae* only RNA termini of viruses of the genus *Phlebovirus* cannot form the corkscrew structure (Flick *et al.*, 2002).

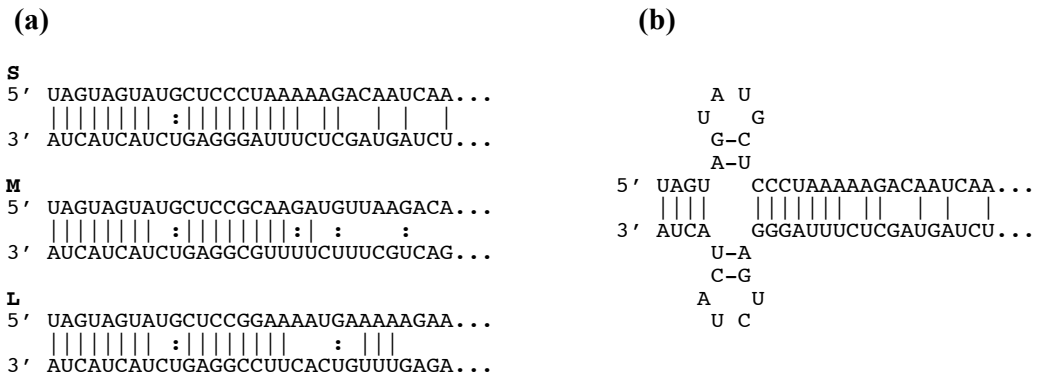

Figure 2.5.2.1. Thirty 5’ and 3’ terminal nucleotides of Hantaan virus (Chizhikov *et al.*, 1995). (a) Possible base pairs are shown with vertical lines and noncanonical base pairs with colons. (b) The corkscrew model of Flick *et al*. (2002) is shown for S vRNA.

N protein encapsidates vRNAs that are the templates for mRNA synthesis (Patterson & Kolakofsky, 1984). Considering the physical constraints in polymerase domains, which will be discussed in following chapters, an encapsidated template would not fit into the putative reaction site. Therefore, N proteins must dissociate from the vRNA as mRNA is being elongated. In influenza A virus, the 5’ and 3’ termini are not encapsidated, as the termini interact with the RNA polymerase and not the NP protein (Klumpp *et al.*, 1997). Initiation of mRNA transcription can, therefore, occur before dissociation of N protein at least for influenza virus.

In eukaryotic cells the 5’ termini of mRNAs contain a cap structure. RNAs lacking a cap are not translated efficiently. The cap structures function as binding sites for eukaryotic initiation factor 4E (eIF4E) which is a subunit of eIF4F. Following binding of eIF4E to the cap, the initiation factors eIF4F and eIF4B, disrupt any secondary structures that might be present in the 5’ terminus of the mRNA and which could interfere with translation. The ribosome can then enter and begin translation. Translation initiation is reviewed e.g. by Merrick & Hershey (1996).

Sequencing of the 5’ termini of mRNAs of viruses with a segmented negative-strand RNA genome, revealed that the 5’ termini of mRNAs had nonviral heterogeneous extensions. These extensions have been found in all the genera of *Bunyaviridae*, as well as *Orthomyxoviridae*, *Arenaviridae*, and tenuiviruses. In Hantaan virus, the type species of the genus *Hantavirus*, the length of the extensions varied from 8 to 17 nucleotides (Garcin *et*

al., 1995). There were few sequence similarities in the sequences of the extension except that there was a strong preference for G at position -1.

Like the 5' termini of eukaryotic mRNAs, the extensions contain cap structures, which has been verified with anti-cap antibodies in experiments with the mRNAs of La Crosse (Hacker *et al.*, 1990) and Germiston orthobunyaviruses (Vialat & Bouloy, 1992). Furthermore, some of the non-viral extensions were found to have identical sequences to the 5’ termini of globin mRNAs of the host cells (Bouloy *et al.*, 1978; Vialat & Bouloy, 1992).

It was deduced that capped RNAs are cleaved from host cell mRNAs by a viral endonuclease and these short capped RNAs are then used as primers in transcription of viral mRNA; this was first discovered with influenza virus (Bouloy *et al.*, 1978; Krug *et al.*, 1979; Plotch *et al.*, 1981). This strategy for obtaining mRNA caps has been dubbed "cap snatching". For influenza A virus it has been demonstrated that it is the viral RNA polymerase which catalyzes the cleavage of the capped primers from host mRNAs in an endonuclease reaction (Li *et al.*, 2001). The use of cap snatching negates the need to encode for capping enzymes such as guanylyl- and methyltransferases.

A 5’-terminal cap makes it possible to distinguish mRNA from antigenomic cRNAs that are produced in genome replication. *Bunyaviridae* cRNAs are encapsidated by N protein (Patterson & Kolakofsky, 1984); this is probably required for cRNA to function as a template in vRNA synthesis. On the other hand, encapsidation of mRNA would interfere with protein translation (Hacker *et al.*, 1989), and as the host-derived capped primer is the only difference to be found in the 5’ termini of mRNAs and cRNAs, it also seems to have the function of preventing encapsidation of viral mRNAs (Kolakofsky & Hacker, 1991).

The last nucleotides in the non-viral extensions of *Bunyaviridae* mRNAs are often the same as first viral nucleotides (Table 2.5.2.1). This is also the case for influenza virus (Beaton & Krug, 1981). One possible explanation for this is that the viral RNA polymerases would cleave the host cell mRNAs preferentially after these nucleotides. However, in an experiment with Germiston orthobunyavirus four out of five mRNA transcripts primed with β-globin mRNA had a U residue and one had GU residues between the globin mRNA and the first viral nucleotides. These nucleotides were in the non-viral extensions but were not part of the globin RNAs (Vialat & Bouloy, 1992).

Genus	Species	3' termini of non-viral extensions	-1 1	viral sequences following non-viral extensions
Orthobunyavirus	Bunyamwera virus	(10/21)	...A G U	A G U A G U...
	Germiston virus	(9/19)	...G U	A G U A G U...
Nairovirus	Dugbe virus	(11/23)	...C U C	U C U C...
		(14/23)	...U C	U C U C...
		(23/24)	...C	U C U C...
Phlebovirus	Uukuniemi virus	(6/11)	...A C	A C A C...

Table 2.5.2.1. Similarity of the nucleotides in the 3' termini of non-viral extensions and the first viral nucleotides. The numbers of mRNA transcripts having the indicated nucleotides out of all sequenced mRNA transcripts are in parentheses. References: Bunyamwera virus (Jin & Elliott, 1993a), Germiston virus (Bouloy *et al.*, 1990), Dugbe virus (Jin & Elliott, 1993b), and Uukuniemi virus (Simons & Pettersson, 1991).

A more likely explanation is a slippage mechanism (Jin & Elliott, 1993b) that has also been proposed for arenaviruses (Garcin & Kolakofsky, 1990; Garcin & Kolakofsky, 1992). In this model the cap-snatched primer would align with the viral RNA template a few nucleotides upstream of the 3' terminus of the viral template. Following elongation of a few nucleotides, the extended primer would be realigned with the viral template so that the 3'-terminal nucleotide of the host-derived primer would be at position -1 (Fig. 2.5.2.2). This proposed mechanism has been dubbed "prime-and-realign" (Garcin *et al.*, 1995).

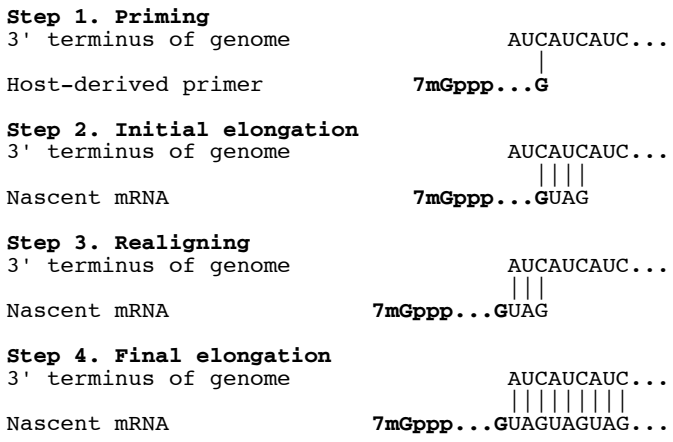

Figure 2.5.2.2. Prime-and-realign model of mRNA transcription of hantaviruses (Garcin *et al.*, 1995). Base pairing is shown by vertical lines.

Bunyaviridae have nucleotide repeats in the termini of their RNAs (Fig. 2.1.1). Without these repeats, the extended primer could not be realigned. These repeats and the prime-and-realign model could explain why the last nucleotides of the non-viral extensions tend to be the same as the first viral nucleotides. If the host-derived primer would be extended to the penultimate repeat and then realigned with the ultimate repeat, this would result in the last nucleotides of the non-viral extensions being templated by the viral RNA. The model also explains why some Hantaan virus mRNAs do not have one of the UAG repeats. The ultimate UAG would be deleted if initial priming and realigning occurred on the penultimate AUC repeat instead of the ultimate repeat (Garcin *et al.*, 1995).

In the case of influenza A virus, the 5' terminus of a vRNA needs to bind to the RNA polymerase before the endonuclease is activated (Cianci *et al.*, 1995; Li *et al.*, 1998). Binding of base paired 5' and 3' termini is most effective in promoting endonuclease activity (Lee *et al.*, 2003). After the host-derived primer is cleaved, mRNA synthesis can begin. The NP protein of influenza virus is not needed for initiation of transcription but it is required for elongation (Hagen *et al.*, 1994; Lee *et al.*, 2002). Also for *Bunyaviridae*, N protein is known to be required for mRNA synthesis (Dunn *et al.*, 1995; Accardi *et al.*, 2001; Flick & Pettersson, 2001; Blakqori *et al.*, 2003; Flick *et al.*, 2003) but whether it is needed only for elongation remains to be seen.

In the elongation phase N protein could be needed to prevent intra-strand base pairing within the vRNA template. The ribosomal subunit 40S has been suggested to play a similar role in preventing intra-strand base pairing within the nascent mRNA or interstrand base pairing between the nascent mRNA and the vRNA template (Bellocq & Kolakofsky, 1987).

Transcription of full-length S mRNAs of orthobunyaviruses have been reported to require ongoing translation in mammalian cells (Abraham & Pattnaik, 1983). Translation inhibitors anisomycin, cycloheximide, and puromycin cause premature termination of S mRNA transcription (Vialat & Bouloy, 1992). However, the transcription of S mRNA is not prematurely terminated in cells treated with another translation inhibitor, edeine, (Vialat & Bouloy, 1992), which does not prevent ribosomal subunit 40S from migrating on mRNA, unlike the three above mentioned inhibitors (Kozak & Shatkin, 1978). The translation inhibition-related premature termination can also be abolished by replacing guanosines with inosines, which prevents G-C base pairing in the nascent mRNA (Bellocq & Kolakofsky, 1987). These findings support the hypothesis of Bellocq and Kolakofsky (1987) that ribosomal subunit 40S prevents base pairing which could lead to premature chain termination. Therefore, ongoing translation would not be required for transcription, but migration of ribosomal subunit 40S on the nascent mRNA would.

When RNA polymerases of detergent-disrupted *Bunyaviridae* virions were used to synthesize RNA *in vitro*, only short RNA transcripts were detected (Ranki & Pettersson, 1975; Bouloy & Hannoun, 1976; Patterson *et al.*, 1984; Adkins *et al.*, 1995). In these experiments RNA synthesis has taken place without ribosomal subunit 40S. The premature chain termination provides further evidence for the requirement of migration of ribosomal subunit 40S on the nascent mRNA.

The mRNAs of *Bunyaviridae* do not extend to the 5' end of the vRNA template (Haaster & Bishop, 1980; Patterson & Kolakofsky, 1984; Ihara *et al.*, 1985; Collett, 1986; Bouloy *et al.*, 1990; Hacker *et al.*, 1990). Consensus sequences for mRNA termination that would be conserved in all *Bunyaviridae* have not been found (Kolakofsky & Hacker, 1991). For hantaviruses, transcription of Hantaan virus S mRNA terminates over 200 nt before the 5’ end of the vRNA template at the sequence 3' ...CCCCACCCAGUCA... 5' (nt 224-236 of the 1696 nt long S segment) (Dobbs & Kang, 1994). Also Sin Nombre hantavirus S mRNA synthesis terminates just downstream of the CCACCC-motif which is found in nearly all hantavirus S vRNAs and is suggested to provide a termination signal (Hutchinson *et al.*, 1996). This motif was found in all hantaviruses carried by Sigmodontinae and Murinae rodents but of Arvicolinae-carried hantaviruses only in Tula virus (Hutchinson *et al.*, 1996). Sin Nombre M mRNAs were polyadenylated unlike S and L mRNAs. M mRNA synthesis terminated at a $(U)_8$ polyadenlyation-transcripition termination signal conserved in hantavirus M vRNAs. L mRNA synthesis extended to the end of the L vRNA template (Hutchinson *et al.*, 1996).

When the ribosome reaches the stop codon of the mRNA, release factors cause the dissociation of the ribosome from the mRNA being translated. The dissociation of the ribosomal subunit 40S would allow intra-strand base pairing within the nascent mRNA, which could lead to termination of the transcription (Vialat & Bouloy, 1992). The inhibitor of protein synthesis, edeine, which allows 40 S ribosomal subunit migration on nascent mRNA but prevents normal ribosome function including release of the ribosome, prevented mRNA chain termination at the normal termination site causing the production of abnormally long mRNAs (Vialat & Bouloy, 1992). This gives further support for the role of ribosomal subunit 40S and intra-strand base pairing in the transcription termination. In influenza A virus intra-chain base pairing forming a hairpin loop at the 5’ end vRNAs is required for polyadenylation of the mRNAs (Pritlove *et al.*, 1999).

The S and L mRNAs of hantaviruses (Hutchinson *et al.*, 1996) and mRNAs of other *Bunyaviridae* do not appear to be polyadenylated (Abraham & Pattnaik, 1983). Algorithms for RNA folding predict that sequences just upstream of the 3' termini of many *Bunyaviridae* mRNAs are capable of folding into stem-loop structures, which could play a

role in transcription termination and stabilization of the mRNAs in the absence of 3' polyadenylation (Hacker *et al.*, 1990; Kolakofsky & Hacker, 1991; Hutchinson *et al.*, 1996).

The amounts of L, M, and S mRNAs differ in Sin Nombre hantavirus-infected Vero E6 cells (African green monkey kidney cells) with plateau values of 3.2 x 10^8, 6.15 x 10^8, and 1.24 x 10^9 copies, respectively (Hutchinson *et al.*, 1996). These give a L:M:S mRNA ratio of 1 : 1.9 : 3.9. The inverse of these numbers would be 1 : 0.52 : 0.26 which is very close to the L:M:S size ratio of the genome segments 1 : 0.56 : 0.31. So, it seems that the mRNAs are produced in amounts that correlate inversely with the sizes of the templates used for mRNA transcription. This would point to elongation as the rate-limiting step in mRNA transcription. For Bunyamwera orthobunyavirus the promoter strength follows the order M > L > S (Barr *et al.*, 2003). However, the comparative efficiency of the L, M, and S vRNA promoters has not yet been determined for hantaviruses. With the newly developed reverse-genetics system (Flick *et al.*, 2003), it should be possible to determine this.

2.5.3. Replication of genomic RNA

In replication of genomic RNAs, antigenomic RNA segments, L, M, and S cRNAs, are synthesized first. These are the full-length exact complements of the genomic vRNAs. The cRNAs are then used as templates in the synthesis of new vRNAs. Synthesis of cRNAs and vRNAs are thought to occur in a similar manner (Kolakofsky & Hacker, 1991; Garcin *et al.*, 1995).

As the full-length cRNAs are not used as mRNAs, they do not require 5'-terminal caps. Indeed, no 5'-terminal nonviral extensions have been found in the 5' termini of *Bunyaviridae* antigenome and genome segments nor are the 5' termini of cRNAs and vRNAs capped (Obijeski *et al.*, 1980; Bishop *et al.*, 1983; Eshita *et al.*, 1985; Raju & Kolakofsky, 1987; Garcin *et al.*, 1995). Therefore, as host-derived primers are not used to prime the synthesis of cRNA and vRNA, the initiation occurs by a different mechanism.

The 5' termini of Hantaan virus genome segments from purified virions were found to contain uridine monophosphates (UMP) instead of triphosphates (UTP) (Garcin *et al.*, 1995). A similar finding was made with the 5'-terminal nucleotides of Sin Nombre virus (Chizhikov *et al.*, 1995). Garcin *et al*. (1995) suggested two possible explanations for the 5'-terminal monophosphates.

(1) Genome synthesis could be initiated with UTP, which has a pyrimidine as the base. An unusual phosphatase, which would remove β- and γ-phosphates leaving the α-phosphate, could then produce the terminal UMP.

(2) The prime-and-realign mechanism suggested for mRNA transcription initiation could also be used to initiate cRNA and vRNA synthesis (Fig. 2.5.3.1). Genome synthesis would begin with GTP being aligned with C_3, elongation of a few nucleotides would follow

and then the nascent RNA would be realigned with the template, so that the 5'-terminal G would be placed at position -1. The GTP would then be cleaved by the endonuclease function of the viral RNA polymerase.

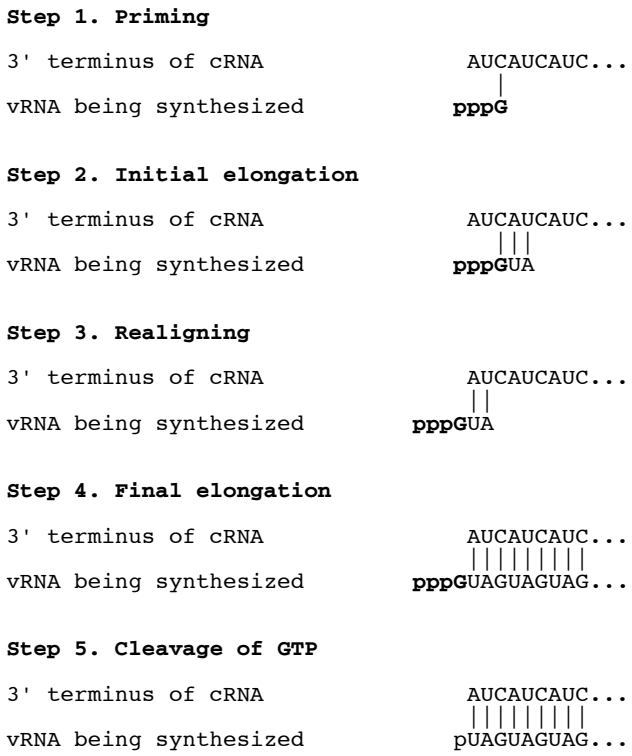

Figure 2.5.3.1. Prime-and-realign mechanism for hantavirus cRNA and vRNA synthesis (Kolakofsky & Hacker, 1991; Garcin *et al.*, 1995).

RNA replication requires protein synthesis in all viruses with a negative-stranded genome, including *Bunyaviridae* (Abraham & Pattnaik, 1983; Patterson & Kolakofsky, 1984; Eshita *et al.*, 1985; Kolakofsky & Hacker, 1991). It has been suggested that the encapsidation of genome and antigenome segments, as they are being synthesized, is required for the synthesis to proceed (Kolakofsky & Hacker, 1991). This would require the synthesis of N proteins before RNA replication could take place.

Unlike mRNAs, both cRNAs and vRNAs are encapsidated by N protein (Patterson & Kolakofsky, 1984). The encapsidation could play a similar role as the ribosomal subunit 40S plays in mRNA transcription, namely, the prevention of intra-strand base pairing within the nascent RNA being synthesized. This base pairing could lead to premature chain termination as in mRNA transcription. If this is the case, encapsidation should proceed on the nascent RNA as it is being synthesized. Indeed, an encapsidation signal with a possible

stem-loop structure was found in the first 39 nucleotides of the 5' terminus of Hantaan virus S vRNA (Severson *et al.*, 2001).

Whereas S and M mRNA synthesis terminates before the 5' end of the vRNA template (Hutchinson *et al.*, 1996), the synthesis of all three cRNAs proceeds to the end of the vRNAs yielding antigenomic RNAs that are exactly complementary to the vRNA templates (Kolakofsky & Hacker, 1991).

In BHK cells infected with La Crosse orthobunyavirus the vRNAs S, M, and L are nearly equimolar (Rossier *et al.*, 1988). For hantaviruses the concentrations of the vRNAs and cRNAs have not been measured, only the concentrations of mRNAs in infected cells have been determined (Hutchinson *et al.*, 1996).

What directs the switch from mRNA synthesis to genome replication is not known. Due to the difference in initiation of mRNA transcription and genome replication, the "decision" whether to produce mRNA or cRNA is made at the start of RNA synthesis. If, during cRNA and vRNA synthesis, N protein is required to prevent base pairing within the nascent RNA or between nascent RNA and the template, then genome replication can proceed only after enough N protein has been synthesized to encapsidate the nascent RNA. Any cRNA synthesis initiated before N proteins were available, would end in premature termination.

It is conceivable that there would be no switch mechanism directing RNA polymerase to start producing cRNAs instead of mRNAs. Since both host-derived primers and GTPs can initiate RNA synthesis, there could be a competition between these two. Binding of host mRNA to the RNA polymerase and subsequent cap snatching could initiate the synthesis of mRNAs. Binding of GTPs could initiate cRNA synthesis which would terminate prematurely until there would be enough newly synthesized N proteins to encapsidate the nascent cRNA.

2.5.4. Generation of genetic variation

Genetic diversity is generated in viruses by accumulation of mutations such as base substitutions, insertions, and deletions and by reassortment and recombination (Worobey & Holmes, 1999). In hantaviruses, genetic variation appears to be caused mostly by accumulation of mutations driven by errors made by the viral RNA polymerase (Plyusnin *et al.*, 1995a; 1995b; Asikainen *et al.*, 2000). The rate of evolution has been estimated for Puumala hantavirus and was found to be slow, 0.7×10^{-7} to 2.2×10^{-6} substitutions per nucleotide per year (Sironen *et al.*, 2001). The slow mutation rate indicates that there is strong selective pressure limiting the accumulation of mutations.

Viruses with a segmented genome can undergo genetic reassortment. This occurs when a host cell is co-infected by two or more viruses and gene segments packaged into

newly assembled viruses originate from more than one progenitor virus. Reassortment has been shown to occur in cell cultures co-infected with two hantavirus strains (Ebihara *et al.*, 2000) and even two distinct hantaviruses (Rodriguez *et al.*, 1998); it has also been suggested to have occurred in nature (Henderson *et al.*, 1995; Li *et al.*, 1995; Plyusnin *et al.*, 1997). Reassortant *Bunyaviridae* among the other genera have been found in nature (Akashi *et al.*, 1997; Akashi *et al.*, 1997; Sall *et al.*, 1999; Bowen *et al.*, 2001; Saeed *et al.*, 2001; Matsumori *et al.*, 2002).

In homologous recombination genetic exchange occurs between homologous RNAs at precisely matched regions. In nonhomologous recombination genetic exchange occurs between two unrelated RNAs at non-corresponding sites and there is no requirement for sequence homology between the RNAs. Both homologous and nonhomologous recombination have long been known to occur in positive-sense RNA viruses (see Worobey and Holmes (1999) and references therein). Homologous recombination has also been detected in the double-stranded RNA virus, rotavirus (Suzuki *et al.*, 1998) but in negative-strand RNA viruses it is an uncommon event (Chare *et al.*, 2003). Nevertheless, homologous recombination has been suggested to have occurred in nature in hantaviruses (Sibold *et al.*, 1999; Sironen *et al.*, 2001; Chare *et al.*, 2003). Homologous recombination in RNA viruses is thought to occur mostly by a copy-choice mechanism in which the RNA polymerase switches from one template RNA to another while nascent RNA is being synthesized (Worobey & Holmes, 1999). This produces a nascent RNA that has been copied from two or more RNA templates. Viral proteins must be properly folded to function and this places restrictions on recombination.

Recombination can be beneficial to the virus by correcting deleterious genomic changes and by accelerating the spread of advantageous mutations. Recombination, thus, provides a means of correcting the high error rate inherent in the RNA replication of RNA viruses. Recombination can also be harmful to viruses by creating defective interfering RNAs, which have terminal sequences required in RNA replication but lack the sequence encoding a proper viral protein. Defective interfering RNAs have been looked for in hantaviruses in a long-term infection, 139 days, of Vero E6 cells by Seoul virus but none could be detected at any time point (Meyer & Schmaljohn, 2000).

2.5.5. Polymerase structures

The L RNA segments, and also the L ORFs, of fully sequenced hantaviruses are nearly identical in size (6530 – 6562 nt, 2151 – 2156 aa) (Plyusnin, 2002). The L segments and the encoded RNA polymerases of other *Bunyaviridae* are of similar size to those of hantaviruses with the exceptions of the L segments of the genera *Tospovirus* and *Nairovirus*, which are significantly larger.

Comparison of amino acid sequences of polymerases has revealed conserved regions. As amino acids critical for polymerase function are more likely to be conserved than other amino acids, sequence comparisons of polymerases of distantly related organisms can reveal functionally crucial amino acids. Conserved amino acid regions have been found in the polymerases of positive-strand RNA viruses (Kamer & Argos, 1984), negative-strand RNA viruses (Tordo *et al.*, 1988), and retroelements (Toh *et al.*, 1985).

All polymerases that use RNAs as template, i.e. RNA-dependent polymerases, have five conserved regions. The first four to be discovered have been named motifs A, B, C, and D (Fig. 2.5.5.1) (Poch *et al.*, 1989). Motif A is centered around a strictly conserved aspartate, motif B around a strictly conserved glycine, motif C around two strictly conserved aspartates, and motif D around a conserved lysine (Fig. 5.1.2, page 35). Single-strand RNA viruses have amino acids in motif C specific for each genome type: negative or positive and segmented or non-segmented RNA genome (Poch *et al.*, 1989). The fifth conserved region, which precedes motif A (Fig. 2.5.5.1), has a conserved lysine and two conserved arginines (Fig. 5.1.2, page 35), and has been named premotif A (Müller *et al.*, 1994). The amino acids centered around the first conserved arginine of premotif A has also been called motif F (Lesburg *et al.*, 1999).

The motifs are in the same order and have similar distances separating them in all RNA-dependent polymerases (Poch *et al.*, 1989; Müller *et al.*, 1994). They are conserved in plus-, minus-, and double-stranded RNA viruses and RNA-dependent DNA polymerases of retroviruses and non-viral retroelements (Poch *et al.*, 1989; Xiong & Eickbush, 1990; Müller *et al.*, 1994). The aspartates of motifs A and C are conserved in all classes of polymerases including DNA-dependent DNA and RNA polymerases in addition to the RNA-dependent polymerases mentioned above (Delarue *et al.*, 1990).

X-ray crystallography has been used to determine the three-dimensional structure of the RNA-dependent RNA polymerase of several viruses including poliovirus (Hansen *et al.*, 1997), hepatitis C virus (Lesburg *et al.*, 1999), bacteriophage Φ6 (Butcher *et al.*, 2001), reovirus (Tao *et al.*, 2002), rabbit hemorrhagic disease virus (Ng *et al.*, 2002), and Norwalk virus (Ng *et al.*, 2004). All of these contain the above-mentioned five motifs, which allows the localization of the conserved amino acids in the polymerase domain. The three-dimensional structures will be discussed below.

RNA polymerases of viruses with a segmented negative-strand RNA genome, (*Arenaviridae*, *Bunyaviridae*, and *Orthomyxoviridae*) have additional conserved amino acids: motif E containing the tetrapeptide E(F/Y)XS following motif D and two residues, glutamate and lysine (Fig. 5.1.2, page 35), between premotif A and motif A (Fig. 2.5.5.1) (Müller *et al.*, 1994). The RNA polymerases of *Arenaviridae* and *Bunyaviridae* have two conserved regions preceding premotif A (Fig. 2.5.5.1) (Müller *et al.*, 1994). In the first

region there are two strictly conserved amino acids, proline and aspartate, and in the second also two strictly conserved amino acids, arginine and tyrosine (Fig. 5.1.3, page 36). Surrounding these are other less conserved amino acids. RNA polymerases of *Bunyaviridae* have, after motif E (Fig. 2.5.5.1), a conserved region centered around four strictly conserved amino acids: a glycine, tyrosine, and two more glycines (Fig. 5.1.4, page 37) (Aquino *et al.*, 2003).

Figure 2.5.5.1. Location of the conserved domains in Hantaan virus L protein. Abbreviations: first N-terminal region (N-1), second N-terminal region (N-2), premotif A (p), two conserved residues (2), motifs A – E, four conserved amino acids (4), and the acidic C-terminal domain (Ac).

In addition to the conserved regions described above, the RNA polymerase sequences of tomato spotted wilt virus, genus *Tospovirus*, and Puumala, Sin Nombre, Dobrava, and Saaremaa viruses, genus *Hantavirus*, all have acidic C-terminal domains (Fig. 2.5.5.1) (de Haan *et al.*, 1991; Stohwasser *et al.*, 1991; Chizhikov *et al.*, 1995; Nemirov *et al.*, 2003).

Three-dimensional structures have been determined to high resolution of all four classes of polymerases: DNA-dependent DNA and RNA polymerases and RNA-dependent DNA RNA polymerases. The core structures, the polymerase domains, of all these polymerases share common features (Sawaya *et al.*, 1994; Joyce & Steitz, 1995; Kim *et al.*, 1995; Butcher *et al.*, 2001). The polymerase domain is made up of subdomains denoted palm, finger, and thumb (Kohlstaedt *et al.*, 1992) that form an open U-shaped cleft in all the polymerases except in RNA polymerases of hepatitis C virus and bacteriophage Φ6 in which the fingers and thumb are connected forming a tunnel (Lesburg *et al.*, 1999; Butcher *et al.*, 2001). The polymerase reaction occurs in the cleft. Of the subdomains, the palm is the most conserved between the polymerases (Lesburg *et al.*, 1999; Butcher *et al.*, 2001).

Motif A, which is folded in a β-strand, and motif C of all the polymerases are located in the palm subdomain with the carboxylate groups of the aspartate side chains facing the cleft (Lesburg *et al.*, 1999). Of the motifs found only in RNA-dependent polymerases (Fig. 5.1.2, page 35), premotif A and an α-helix formed by motif B are located in the fingers subdomain, and an α-helix formed by motif D in the palm subdomain (Lesburg *et al.*, 1999). Motif E is located between the palm and thumb subdomains in an antiparallel β-sheet structure (Lesburg *et al.*, 1999).

Point mutations made to the conserved amino acids have confirmed their importance for the polymerase reaction. Mutating the aspartates of the motifs A and C abolished polymerase activity in the RNA polymerase of Bunyamwera orthobunyavirus (Jin & Elliott,

1992). Similar results have been obtained with other viral polymerases and nonviral polymerases.

Based on three dimensional structures and mutagenesis experiments, it has been possible to determine what role the conserved amino acids play in the polymerase reaction (Bruenn, 2003). The carboxylates of the first aspartates of motifs A and C (Fig. 5.1.2, page 35), which are conserved in all polymerases, bind divalent metal cations. These cations, in turn, activate the 3'-OH of the nascent RNA for nucleophilic attack and bind the phosphates of the incoming NTP. The first arginine of premotif A has been proposedt to increase polymerse fidelity by enhancing proper base pair formation. Motif B might participate in the ribonucleotide triphosphate selection. Motif E interacts with the nascent RNA. As of yet, no function has been assigned to the two N-terminal regions, the lysine and second arginine of premotif A, and motif D.

2.6. Effect of the infection on the host cell

Hantaviruses can infect several cell lines but isolation of hantaviruses has been done in Vero E6 cells, an African green monkey kidney epithelial cell line (Schmaljohn *et al.*, 1985). All tested kidney, spleen, and lung primary cells as well as most established cell lines of these tissues could be infected with Puumala virus, although the virus replicated poorly in many of the cells (Temonen *et al.*, 1993). There was no apparent cytopathic effect and that has usually not been observed with other hantaviruses either (Yanagihara & Silverman, 1990; Pensiero *et al.*, 1992; Zaki *et al.*, 1995; Sundstrom *et al.*, 2001). However, lowering of the pH leads to fusing of infected cells leading to syncytial formation and death. The fusion is probably due to the low pH causing a conformational change in the glycoproteins that then mediate the cell-cell fusion preciding death of the cells (Arikawa *et al.*, 1985; McCaughey *et al.*, 1999).

It has also been reported that apoptosis would occur in Hantaan virus-infected Vero E6 cells (Kang *et al.*, 1999), though apoptosis has not been reported in the studies referred to above. The observed process of cell death was slow with less than 30% of the cells having undergone apoptosis 7 days post infection. When cells of the human embryonic kidney cell line HEK293 were infected with Hantaan, Seoul, Andes, Sin Nombre, and Laguna Negra virus, all of these hantaviruses, interestingly, caused apoptosis in the uninfected bystander cells while cells expressing hantavirus antigen did not undergo apoptotic cell death (Markotic *et al.*, 2003).

Apoptosis is used against viruses as a host defense mechanism: when infected cells die the virus will be deprived of a chance to replicate. Various molecular mechanisms have evolved in viruses to inhibit apoptosis. Viruses also use apoptosis to their advantage by first preventing apoptosis during their replication in the cell and then stimulating apoptosis when

progeny viruses have been assembled. Unlike necrotic cell death, apoptosis does not cause an inflammatory response and, thus, when the virus induces apoptosis, the progeny viruses will be released from the infected cells without having to face a heightened immune response brought about by inflammation (see O'Brien (1998) and references therein).

Hantavirus infection of cells induces cytokine release. In infection of dendritic cells with Hantaan virus, immature cells were activated and the infection induced the release of tumor necrosis factor α and interferon α (Raftery *et al.*, 2002). In infection of both dendritic cells and endothelial cells, Hantaan virus caused increased expression of major histocompatibility complex molecules and intercellular adhesion molecule type I (Raftery *et al.*, 2002). Infection of human lung microvascular endothelial cells with Hantaan and Sin Nombre viruses lead to increase in the expression of the chemokines RANTES and IP-10 (Sundstrom *et al.*, 2001). DNA-array analysis of mRNAs showed that infection of human umbilical vein endothelial cells with New York, Hantaan, and Prospect Hill viruses, lead to activation of the interferon response (Geimonen *et al.*, 2002).

Very little is still known of interactions between hantaviral proteins and cellular components. However, recent studies have revealed some interactions of the glycoproteins and N protein with cellular proteins. HPS-causing hantaviruses have immunoreceptor tyrosine-based activation motifs in the cytoplasmic tail of the G1 glycoprotein. These motifs interact with the host cell kinases: LYn, Syk, and Zap-70, and could alter cell signaling (Geimonen *et al.*, 2003b). These motifs might also downregulate glycoprotein expression by directing ubiquitination and degradation of the glycoproteins (Geimonen *et al.*, 2003a).

Yeast two-hybrid screening has revealed interactions between hantaviral N protein and cellular proteins. Interaction with small ubiquitin-like modifier-1 (SUMO-1) was discovered with both the N protein of Tula virus (Kaukinen *et al.*, 2003b) and Hantaan virus (Maeda *et al.*, 2003). The N protein of Hantaan virus also interacts with SUMO-1 conjugating enzyme 9 (Ubc9) which conjugates SUMO-1 to target proteins and modulates signal transduction, transcription regulation, and regulation of cell growth (Maeda *et al.*, 2003). The N protein of Puumala virus was found to interact with Daxx, an enhancer of apoptosis (Li *et al.*, 2002). Further studies are needed to find out what effect these interactions have on the host cell.

2.7. Virus assembly and release

Hantavirus glycoproteins are synthesized in the endoplasmic reticulum (ER) as a polyprotein precursor protein that is cleaved at a conserved site having the aminoacid sequence WAASA (Löber *et al.*, 2001)yielding the glycoproteins G1 (68 kDa for TULV) and G2 (53 kDa). Sugar groups are added to the proteins in the ER and heterodimers formed by G1 and G2 are then transported to the Golgi (Ruusala *et al.*, 1992; Shi & Elliott, 2002) where the glycosylation is finished (Schmaljohn *et al.*, 1986). The conformation of G1/G2 heterodimers appears to constitute a Golgi retention signal that retains the glycoproteins in the Golgi for virus budding (Shi & Elliott, 2002).

The L, M, and S vRNPs are formed by L and N protein binding to the three vRNAs. A central conserved region of Hantaan virus N protein binds RNA (Xu *et al.*, 2002). Earlier reports found N protein to bind RNA with little sequence specificity (Gott *et al.*, 1993; Severson *et al.*, 1999) but more recently a slight preference for the 5' ends of the vRNAs has been found with Hantaan virus (Severson *et al.*, 2001). Similar preference has been found with Bunyamwera orthobunyavirus N protein and it was suggested that the 5' end of vRNAs might contain the initiation signal for encapsidation (Osborne & Elliott, 2000). However, it is possible that the initation of encapsidation *in vivo* requires that the L protein first be bound to the vRNA and then N protein would bind to the L protein/vRNA complex and encapsidation would proceed. This model would explain why little sequence specificity has been found with N protein binding to RNA alone. With influenza virus it has been shown that it is the RNA polymerase that binds the very termini of the vRNAs not nucleoprotein (Klumpp *et al.*, 1997) and the influenza virus nucleoprotein has been shown to bind the RNA polymerase (Biswas *et al.*, 1998; Medcalf *et al.*, 1999). There are, however, no published studies on interaction between *Bunyaviridae* L and N proteins.

The N protein multimerizes by first forming homotrimers that then interact with each other resulting in a chain (Alfadhli *et al.*, 2001; Kaukinen *et al.*, 2001; Alfadhli *et al.*, 2002; Yoshimatsu *et al.*, 2003). Mapping of the homotypic interactions sites for Tula virus revealed that C-terminal amino acids 393 to 398 are required for the interaction and amino acids 1-43 are also involved (Kaukinen *et al.*, 2003a). The multimerization of N protein allows RNA encapsidation to proceed rapidly once the process has been initiated.

The vRNP assembly is likely to take place in the perinuclear region where N protein of Black Creek Canal, Puumala, and Seoul hantaviruses (Ravkov & Compans, 2001; Kariwa *et al.*, 2003) and Crimean-Congo hemorrhagic fever nairovirus (Andersson *et al.*, 2004) are localized. Once formed the vRNPs need to be transported to the virus assembly site at the Golgi. The N protein of Black Creek Canal virus (Ravkov *et al.*, 1998) and Crimean-Congo hemorrhagic fever virus (Andersson *et al.*, 2004) have been found to interact with actin. Monitoring of virus production indicated that disruption of actin filaments inhibited the

assembly of both viruses. This has led to the suggestion that the vRNPs would be transported via actin microfilaments to the virus assembly site.

At the virus assembly site the N protein of the vRNPs interacts with the cytoplasmic tail of G1 (Koistinen, personal communication) bringing together all of the virion components: glycoproteins G1 and G2, N protein, L protein, and the three vRNAs: S, M, and L. Although in an infected cell all virion components must co-localize at the assembly site, it appears that the glycoproteins and N proteins are responsible for the virion assembly, as virus-like particles can be created by expressing these proteins in cells without the other virion components (Betenbaugh *et al.*, 1995). Furthermore, the study showed that the expression of both glycoproteins and N protein is required for virus budding suggesting that binding of N protein to glycoprotein triggers the budding process.

The packaging signal for hantaviral RNA is not yet known; vRNPs are packaged while the antigenome cRNPs are not. Although in Uukuniemi phlebovirus virions 10% of the RNPs had cRNA instead of vRNA, indicating that specificity for vRNPs is not absolute (Simons *et al.*, 1990). For Uukuniemi phlebovirus (Flick & Pettersson, 2001), La Crosse orthobunyavirus (Blakqori *et al.*, 2003), and Crimean-Congo hemorrhagic fever nairovirus (Flick *et al.*, 2003) the packaging signal appears to reside in the 5'- and/or 3'-terminal NTRs as minireplicons, in which the only viral sequences are derived from the NTRs, are packaged into progeny virions.

Based on electron microscopy studies of Sin Nombre (Goldsmith *et al.*, 1995) and Black Creek Canal viruses (Ravkov *et al.*, 1997), it has been suggested that New World hantaviruses carried by rodents of the subfamily Sigmodontinae would assemble and bud at the plasma membrane in contrast to the Old World hantaviruses that bud into the Golgi. IFA studies demonstrated that the glycoproteins of Sin Nombre virus accumulate predominantly in the Golgi in infected cells, although the proteins could be also detected on the cell surface at late time points (Spiropoulou *et al.*, 2003). In studies with Black Creek Canal virus it was found that both entry and release of the virus occurs preferentially in the apical side of the infected cells (Ravkov *et al.*, 1997). Similar studies have not been reported with Old World hantaviruses.

3. AIMS OF THE STUDY

Tula virus was discovered a few years prior to the beginning of this thesis work. The L RNA and the 5' and 3' termini of all three RNAs, shown to form the promoter for RNA synthesis in other *Bunyaviridae*, had not been sequenced. The N protein and glycoproteins of many hantaviruses, including Tula virus, had been studied but very little was known of the L protein and RNA replication. There were no antibodies against the L protein of any hantavirus and the protein had not been detected in infected cells. Sequencing of Tula virus strains had indicated that homologous recombination might have occurred in nature but there were no studies demonstrating that this type of recombination is possible in negative-strand viruses. There were conflicting reports on the effect of hantavirus infection on the host cells with most studies showing no cytopathic effect but in others infected cells were killed under certain conditions and there was even a report claiming apoptosis to occur.

The specific aims of this thesis work were:

1. Completion of the Tula virus genome by sequencing the L segment and the 5' and 3' termini of all three genome segments.

2. Development of antibodies against L protein, detection of the protein in virions, infected cells, and in vitro translation products and determining the localization of L protein in cells.

3. Study of transfection-mediated homologous recombination in Tula virus infected cells.

4. Study of the effect of Tula virus replication on infected cells.

4. METHODS

The methods are described in detail in the articles referred to by their roman numerals.

Method	**Described in**
Cell culture	I, II, III, IV
Virus infection	I, II, III, IV
Concentration of viruses	I, IV
Virus titration	IV
RNA isolation	I, III
Reverse transcription	I, II, III
PCR	I, II, III
DNA cloning	I, II, III
DNA sequencing	I, II, III
Sequence analysis	I, II, III
Transfection	II, III
Protein expression and purification	II
Immunoblotting	II, III, IV
Affinity purification of antibodies	II
In vitro synthesis of proteins	II
Immunoprecipitation	II
Immunofluorescence assay	II, III, IV
Purification of microsomal membranes	II
Membrane flotation	II
DNA laddering	IV
Flow cytometry	IV

5. RESULTS AND DISCUSSION

5.1. Completion of Tula virus genome sequencing

Before the start of the current work, Tula virus had been isolated and adapted to Vero E6 cell culture (Vapalahti *et al.*, 1996). Antigen-positive lung samples were collected of *Microtus arvalis* captured near Tvrdonice, Moravia, Czech Republic (Plyusnin *et al.*, 1995a). The lung samples were used to infect laboratory-colonized, hantavirus-free *M. arvalis*, which were sacrificed after infection; lung suspensions were prepared and these were used to infect Vero E6 cells (Vapalahti *et al.*, 1996). The Tula virus isolate adapted to the cell culture was designated Tula/Moravia/Ma5302V/94.

The Tula virus S and M gene sequences had been recovered from naturally infected *M. arvalis* (Plyusnin *et al.*, 1994; 1995a; 1996). The same primers were used to determine the S and M RNA sequences of the Vero E6-adapted virus (Vapalahti *et al.*, 1996). We used RNA from the virus adapted to Vero E6 cells to obtain the L RNA sequence of Tula virus. The primers for RT-PCR of the L segment were first designed based on the sequences of the L RNA of Hantaan, Seoul, and Puumala hantaviruses. Later, we used the obtained Tula L RNA sequences to design primers with perfect complementarity with the L sequence. We cloned and sequenced the L segment in five parts (Fig. 5.1.1). Sequencing of the M and L genes and the termini of all three genes of the Tula virus isolate (Vapalahti *et al.*, 1996), **I**) made Tula virus the fifth hantavirus, following Hantaan, Seoul, Puumala and Sin Nombre virus, of which the complete genome was sequenced. Of hantaviruses thought to be non-pathogenic, it is the first and, so far, the only one.

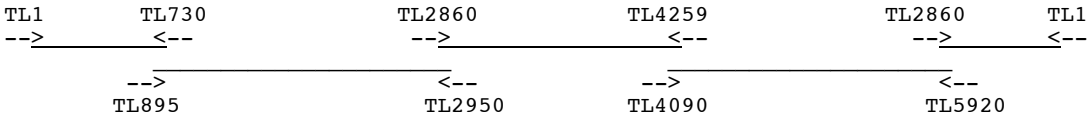

Figure 5.1.1. The L segment was cloned and sequenced in five parts. Primer TL1 annealed at both the 5' and 3' terminus, TL730 annealed around position 950, and TL2860 had two annealing positions around 2860 and 5890.

The length of the L RNA, 6541 nt, was close to the sizes of the previously determined L RNAs of Hantaan (6530 nt), Seoul (6562 nt), Puumala (6550 nt), and Sin Nombre virus (6562 nt). As is the case for the other hantavirus L RNAs, only one large ORF was found and it was in the reverse-complement of the genomic RNA as is also the case for M and S RNAs. The ORF was 6459 nt and could encode a protein of 2153 aa with a deduced molecular mass of 246.8 kDa. The large ORF contained motifs A to D (Fig. 5.1.2) conserved in all RNA-dependent RNA polymerases, premotif A and motif E (Fig. 5.1.2) conserved in the RNA-polymerases of viruses with a segmented negative-stranded RNA

genome, and the glutamate and lysine residues (Fig. 5.1.2) between premotif A and motif A conserved in *Bunyaviridae*, *Arenaviridae*, and *Orthomyxoviridae*.

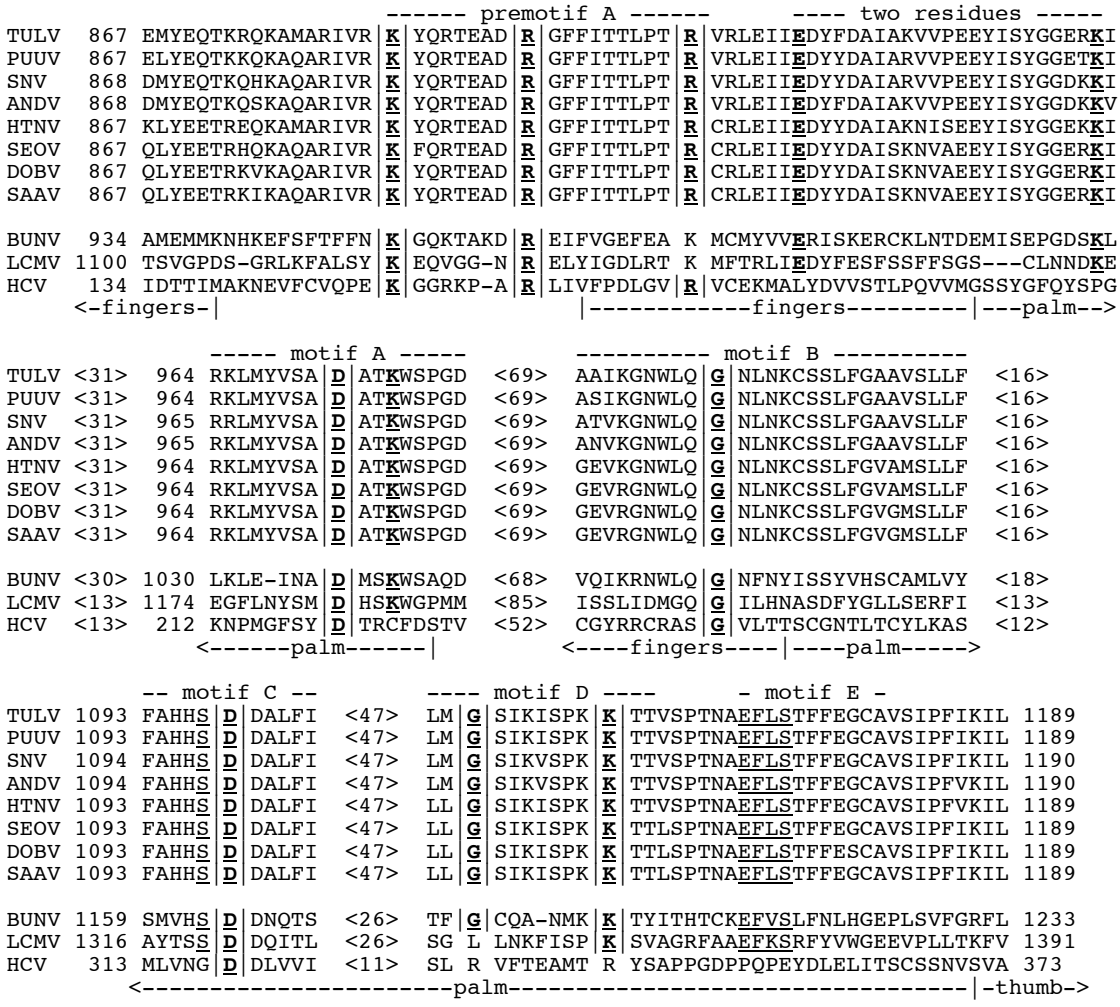

Figure 5.1.2. Hantavirus RNA polymerase sequences from premotif A to motif E. The RNA polymerase of Bunayamwera virus, genus *Orthobunyavirus*, and lymphocytic choriomeningitis virus, family *Arenaviridae*, are included to emphasize amino acids conserved in distantly related polymerases. Hepatitis C virus, family *Flaviviridae*, is included to show location of motifs (Lohmann *et al.*, 1997) in the fingers, palm, and thumb subdomains of the three dimensional structure of the polymerase domain (Lesburg *et al.*, 1999). Underlined amino acids are conserved only in polymerases of segmented negative-strand RNA viruses. Amino acids also in bold are conserved in the polymerases of negative-stranded RNA viruses. Boxed amino acids are generally conserved in the polymerases of all viruses having an RNA genome. Abbreviations and references: Tula (TULV) (Kukkonen *et al.*, 1998), Puumala (PUUV) (Stohwasser *et al.*, 1991), Sin Nombre (SNV) (Chizhikov *et al.*, 1995), Andes (ANDV) (Meissner *et al.*, 2002), Hantaan (HTNV) (Schmaljohn, 1990), Seoul (SEOV) (Antic *et al.*, 1991), Dobrava (DOBV) and Saaremaa (SAAV) (Nemirov *et al.*, 2003), Bunyamwera (BUNV) (Elliott, 1989), lymphocytic choriomeningitis virus (LCMV) (Salvato *et al.*, 1989), and hepatitis C virus (HCV) (Takamizawa *et al.*, 1991).

The Tula L ORF also contained the amino acid regions conserved only in *Bunyaviridae* and *Arenaviridae*, consisting of the two strictly conserved amino acids, proline and aspartate, and the two strictly conserved amino acids, arginine and tyrosine (Fig. 5.1.3).

```
                              50                          75                          99
TULV 29 DRFYAVRHDVVDQMIKHDWSDNKDKEQPIGHVLLMAGVPNEVIQGMEKKIIPG-SPSGQILRSFFKMTPDNYK
PUUV 29 DRLYAVRHDVVDQMIKHDWSDNKDKEQPIGLVLLMAGVPNDVIQSMEKRIIPG-SPSGQILRSFFKMTPDNYK
SNV  29 DRLYAVRHDVVDQMIKHDWSDNKDMERPIGQVLLMAGVPNDVIQGMEKKVIPT-SPSGQILKSFFRMTPDNYK
ANDV 29 DRLYAVRHDLVDQMIKHDWSDNKDVERPIGQVLLMAGIPNDIIQGMEKKIIPN-SPSGQVLKSFFRMTPDNYK
HTNV 29 DRLYAVRHDIVDQMIKHDWSDNKDSEEAIGKVLLFAGVPSNIITALEKKIIPN-HPTGKSLKAFFKMTPDNYK
SEOV 29 DRLYAVRHDIVDQMIKHEWSDNKDSEEPISKVLLFAGIPNNVITALEKKVIPD-HPSGKTLRSFFKMTPDNYR
DOBV 29 DRLYAIRHDIVDQMIKHDWSDNKDSEESIGKVLLFAGVPNNVITAMEKKIIPD-HPSGKTLRSFFKMTPDNYK
SAAV 29 DRLYAIRHDIVDQMIKHDWSDNKDSEESIGKVLLFAGIPNNVITAMEKKIIPD-HPSGKTLRSFFKMTPDNYK

BUNV 27 ADILEARHDYFGRELCNSLGIEYKNNVLLDEIILDVVP-----------------GVNLLNYNIPNVTPDNYI
LCMV 13 LNYIEQDERLSRQKLNFLGQREPRMVLIEGLKLLSRCIEIDS <13> SVETILVESGIVCPGLPLIIPDGYK

     101                         125                         150
TULV ITGSLIEFIEVTVTADVA-RGTREKILKYQAGLEYIEQLLHQESERGNLPGGYRIKFDVVAVRTD 164 <449>
PUUV ITGNLIEFIEVTVTADVA-RGVREKILKYQGGLEFIEQLLQIEAQKGNCQSGFRIKFDVVAIRTD 164 <449>
SNV  ITGALIEFIEVTVTADVA-KGIREKKLKYESGLQFVESLLSQEHKKGNINQAYKITFDVVAVKTD 164 <450>
ANDV ITGNLIEFIEVTVTADVS-RGIREKKIKYEGGLQFVEHLLETESRKGNIPQPYKITFSVVAVKTD 164 <450>
HTNV ISGTTIEFVEVTVTADVD-KGIREKKLKYEAGLTYIEQELHKFFLKGEIPQPYKITFNVVAVRTD 164 <449>
SEOV ITGSLIEFVEVTVTADVD-KGIREKKMKYELGLKYLEQELMTFFHRGELQNPYKITFKVVAVRTD 164 <449>
DOBV ITGSTIEFVEVTVTVDVD-KGIREKRLKYEAGLKYIEQELHNHFLRGDIPQPYKITFQVVSVRTD 164 <449>
SAAV ITGSTIEFVEVTVTVDVD-KGIREKKLKYEAGLKYIEQELHNHFLKGDIPQPYKLTFQVVSVRTD 164 <449>

BUNV WDGHFLIILDYKVSVGNDSSEITYKKYTSLI---------------LPVMSELGIDTEIAIIRAN 132 <509>
LCMV LIDNSLILLECFVRS <11> DTNKLACIREDLAVA----------------GVTLVPIVDGRCD 146 <474>

                     625                         650                         675
TULV 614 TQKMKLCAIFDNLRYLIPAVTSLYSGY-KPLIVKFFERPFKSALDVYLYTIIKTLLVSLAQNNKIRFYSKVR
PUUV 614 SQKMKLCAIFDNLRYLIPAVTSTYSGF-EPLIRKFFERPFKSALEVYLYNIIKTLLVSLAQNNKIRFYSRVR
SNV  615 SQKMKICAIFDNLRYLIPAVTSLYSGY-ELLIEKFFERPFKSALEVYLYNIIKALLISLAQNNKVRFYSKVR
ANDV 615 SQKMKLCAIFDNLRYLIPSVTSLYSGY-ELLIEKFFERPFKSSLDVYLYSIIKSLLISLAQNNKVRFYSRVR
HTNV 614 CQKMKLCAIFDNLRYLIPAVTSLYSGF-PSLIEKLFERPFKSSLEVYIYYYNIKSLLVALAQNNKARFYSKVK
SEOV 614 CQKMKLCAIFDNLRYLIPAVTSLYSGF-PSLVEKLFERPFKSALEVYVYYYNIKSLLVALAQNNKARFYSKVK
DOBV 614 CQKMKLCAIFDNLRYLIPAVTSLYSGF-PSLINKLFERPFKSALEVYVYYYNIKSLLVALAQNNKARFYSKVK
SAAV 614 CQKMKLCAIFDNFRYLIPAVTSLYSGF-PSLISKLFERPFKSALEVYVYYYNIKSLLVALAQNNKARFYSKVK

BUNV 642 ITK-SMLSLTEPSRYMIMNSLAISSHVRDYIAEKFSPYT-KTLFSVYMVNLIKRGCASANEQSS--KIQLRN
LCMV 621 PTK-RNQKQVQSVRYLVMAIVSDFSST--SLMDKLREDLITPAEKVVYKLLRFLIKTIFGTG----------

                     700                         725                         750
TULV LLGLTVDQSTIGASGV--YPSLMSRVVYKHYKSLISEATTCFFLFEKGLHGNLTEEAKIHLETVEWARKFS 753
PUUV LLGLTVDQSSIGASGV--YPSLMSRVVYKHYRSLISEATTCFFLFEKGLHGNLTEEAKIHLETVEWARKFR 753
SNV  LLGLTVDHSTVGASGV--YPSLMSRVVYKHYRSLISEATTCFFLFEKGLHGNLNEEAKIHLETVEWARKFE 754
ANDV LLGLTVDHSTVGASGV--YPSLMSRVVYKHYRSLISEATTCFFLFEKGLHGNLPEEAKIHLETIEWARKFQ 754
HTNV LLGLTVDQSTVGASGV--YPSFMSRIVYKHYRSLISEVTTCFFLFEKGLHGNMNEEAKIHLETVEWALKFR 753
SEOV LLGLTVDQSTVGASGI--YPSFMSRVVYKHYKSLISEVTTCFFLFEKGLHGNVNEEAKIHLETVEWATKFK 753
DOBV LLGLTVDQSTVGASGI--YPSFMSRVIYKHYRSLISEVTTCFFLFEKGLHGNMNEEAKIHLETVEWALKFR 753
SAAV LLGLTVDQSTVGASGI--YPSFMSRVIYKHYRSLISEVTTCFFLFEKGLHGNMNEEAKIHLETVEWALKFR 753

BUNV IYLSDYDITQKGVNDGRNLDSIWFPGKVNLKE-YINQIYLPFYFNAKGLHEKHHVMIDLAKTVLEIEMNQR 779
LCMV ----------------------EKVLLSAKFKFMLNVSYLCHLI-TKETPDRLTDQIKCFEKFFEPKSQFG 727
```

Figure 5.1.3. Hantavirus RNA polymerase sequences preceding premotif A. The RNA polymerases of BUN virus and LCMV have been included to emphasize the conserved amino acids. Amino acids conserved in at least six of the eight RNA polymerases of *Arenaviridae* and *Bunyaviridae* compared by Müller *et al*. (1994) are underlined and in bold. Abbreviations and references are the same as in Figure 5.1.2.

Finally, the Tula ORF also had the region following motif E that is conserved in *Bunyaviridae* and that is centered around four strictly conserved amino acids: a glycine, tyrosine, and two more glycines (Fig. 5.1.4).

```
                  1175                        1200                        1225
TULV 1169  AEFLSTFFEGCAVSIPFIKILLGSLSDLPGLGYFDDLAAAQSRCVKALDM|G|ACPQLAQLGIVLCT
PUUV 1169  AEFLSTFFEGCAVSIPFIKILLGSLSDLPGLGYFDDLAAAQSRCVKALDM|G|ACPQLAQLGIVLCT
SNV  1170  AEFLSTFFEGCAVSIPFIKILLGSLSDLPGLGFFDDLAAAQSRCVKAMDL|G|ASPQLAQLAVVICT
ANDV 1170  AEFLSTFFEGCAVSIPFVKILLGSLSDLPGLGFFDDLAAAQSRCVKSLDL|G|ACPQLAQLAIVLCT
HTNV 1169  AEFLSTFFEGCAVSIPFVKILLGSLSDLPGLGYFDDLAAAQSRCVKALDL|G|ASPQVAQLAVALCT
SEOV 1169  AEFLSTFFEGCAVSIPFIKILLGSLSDLPGLGYFDDLAAAQTRCVKAMDL|G|ASPQISQLAVSLST
DOBV 1169  AEFLSTFFESCAVSIPFIKILLGSLSDLPGLGYFDDLAAAQSRCVKAMDL|G|ASPQVAQLAVALCT
SAAV 1169  AEFLSTFFEGCAVSIPFIKILLGSLSDLPGLGYFDDLAAAQSRCVKAMDL|G|ASPQVAQLAVALCT

BUNV 1214  EFVSLFNLHGEPLSV-FGRFLLPSVGDCAYIGPYEDLASRLSAAQQSLKH|G|CPPSLVWLAISCSH

                        1250                        1275
TULV SKVERL|Y|GTATGMVNNPTSFLKVE-RSSIPIPLG|G|DGSMSIMELATA|G|IGMADKNVLKNAYISFK  1297
PUUV SKVERL|Y|GTAPGMVNNPTAYLKVD-RSLIPIPLG|G|DGSMSIMELATA|G|IGMADKNILKNAFITYK  1297
SNV  SKVERL|Y|GTADGMVNSPVAFLKVT-KAHVPIPLG|G|DGSMSIMELATA|G|IGMADKNILKQAFYSYK  1298
ANDV SKVERL|Y|GTADGMVNSPTAFLKVN-KAHVPVPLG|G|DGSMSIMELATA|G|FGMADKNILKNAFISYK  1298
HTNV SKVERL|Y|GTAPGMVNHPAAYLQVK-HTDTPIPLG|G|NGAMSIMELATA|G|IGMSDKNLLKRALLGYS  1297
SEOV SKVERL|Y|GTSIGMVNYPGTYLRTK-HSETPIPLG|G|SGAMSIMELSTA|G|IGMSDKNLLKQALIGYM  1297
DOBV NKVERL|Y|GTAVGMIKHPSTYLQVK-HGDTPIPLG|G|SGAMSIMELATA|G|IGMSDKNLLKRALLGYI  1297
SAAV NKVERL|Y|GTAVGMINHPSTYLQVK-HSDTPIPLG|G|SGAMSIMELATA|G|IGMSDKNLLKRALLGYI  1297

BUNV WITFFT|Y|NMLDDQINAPQQHLPFNNRKEIPVELN|G|YLNAPLYLIALV|G|LEAGNLWFLINILKKLV  1342
```

Figure 5.1.4. Hantavirus RNA polymerase sequences after motif E. The RNA polymerase of BUN virus has been included to emphasize the conserved amino acids. Amino acids conserved in most *Bunyaviridae* are underlined and in bold. Residues conserved in all *Bunyaviridae* are also boxed. Abbreviations and references are the same as in Figure 5.1.2.

The L RNA had the highest sequence homology with the L RNA of Puumala virus (75% nt identity), followed by Sin Nombre (71%), Seoul (66%), and Hantaan virus (65%) (**I**: Table 1.). The L RNAs of Dobrava, Saaremaa, and Andes viruses were sequenced later and the sequence homologies were 67%, 66%, and 72%, respectively. The amino acid sequence encoded by the large ORF was, expectedly, more conserved, with amino acid identities varying from 85% with Puumala virus L ORF to 68% with that of Hantaan virus (**I**: Table 1.). In a phylogenetic tree, the coding region of the Tula virus L segment is placed together with the L segments of other Arvicolinae-carried hantaviruses (Fig. 5.1.5).

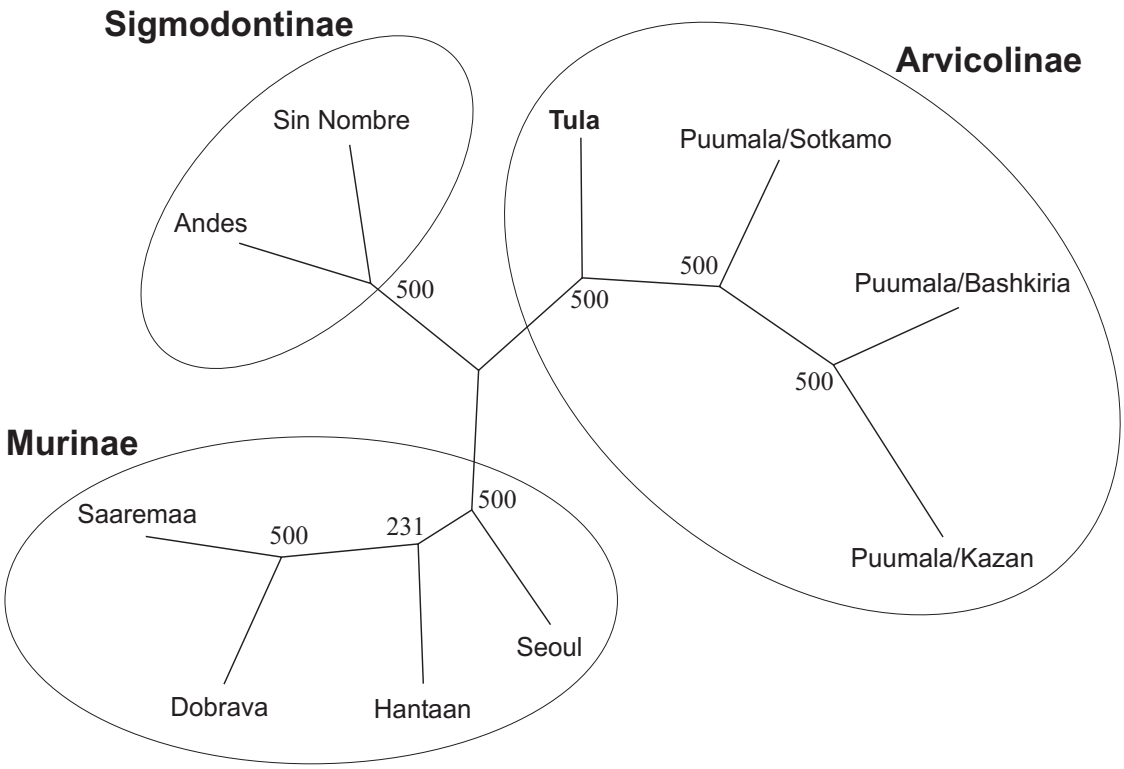

Figure 5.1.5. Consensus phylogenetic tree of the coding regions of hantavirus L segments (courtesy of Kirill Nemirov). Fitch-Margoliash tree fitting algorithm was used in the construction of the tree with 500 bootstrap replicates. The number of replicates supporting each branching is indicated as is the rodent subfamily that the carrier of each virus belongs to.

5.2. Heterogeneity of the 3' ends of S and L vRNAs

The 5’- and 3’-terminal sequences were determined for all three genomic RNAs (vRNAs) both from infected cells and virions. First the ends were ligated by RNA ligation and then the RNAs were amplified by RT-PCR for sequencing. vRNAs isolated from virions were sequenced first. The 5’ termini had the expected conserved consensus sequence found in the 5’ termini of all three vRNAs of all known hantaviruses. Also the 3’ termini of M vRNAs were as expected with 94 % (17 out of 18 clones) having the consensus sequence. In contrast, the 3’ termini of L and S vRNAs were heterogeneous. 47 % (9 out of 19 clones) of the sequenced L 3’ termini were truncated, 37 % (7/19) had extensions and only 16 % (13/19) had the consensus sequence. Of the S 3’ termini 45 % (9/20) were truncated, 20 % (4/20) had extensions and 35 % (7/20) had the expected sequence (**I**: Fig. 2).

There are several possible explanations why the 5' termini are more conserved:

1. There can be more 3'-5' RNA exonuclease than 5’-3’ exonuclease activity in cells.
2. Binding of L protein to vRNAs might protect the 5' ends more than the 3’ ends.

3. It is possible that template requirements are more stringent on the 3' termini, in which case cRNAs with 3'-terminal deletions would not be used as templates in the synthesis of vRNAs as readily as cRNAs with 5'-terminal deletions.

Meyer and Schmaljohn (2000) found deletions in some of the 5’ termini in Vero E6 cells infected with Seoul hantavirus. This led them to propose that the hantavirus RNA polymerase could itself cleave the 5’ termini of vRNAs and that truncated 5’ termini would then be copied into truncated 3’ termini of cRNA. The RNA polymerase is expected to have endonuclease activity which is required in cap snatching, the process in which the 5’ ends of cellular mRNAs are cleaved to be used as primers in synthesis of viral mRNAs (Garcin *et al.*, 1995). However, there is a preponderance of truncated 3’ termini, so cleaving of 5’ termini by the viral RNA polymerase seems an unlikely explanation for the appearance of the deletions in the termini.

For Tula virus, no differences in the heterogeneity of the termini could be detected in vRNAs collected 3 days p.i. to 12 days p.i. However, the 3' termini of vRNAs were more intact in virions than in cells (**I**: Fig. 2) indicating that the 3'-terminal sequences probably play a role in packaging of vRNAs into virions. The packaging signals in hantavirus vRNAs remain yet to be determined.

Experiments with *Bunyaviridae* have shown terminal sequences to be crucial for template function. Deletions in the 3’ termini are not well-tolerated. Meyer and Schmaljohn (2000) found that the appearance of 3’-terminal deletions preceded decreases in the amounts of RNAs and the amount of virions produced. The deletions appear to have similar consequences for virus replication as ribavirin, which is incorporated into viral RNA causing error catastrophe, which leads to viral RNAs losing the capability to function as templates in RNA synthesis (Severson *et al.*, 2003).

5.3. Detection of L protein in infected cells and virions

The lack of antibodies has hindered the study of hantaviral L protein. In our first attempt to raise antibodies we cloned into the vector pGEX-2T the regions 397-685 (289 aa), 944-1174 (231 aa), 1359-1635 (277 aa), and 1655-1936 (282 aa) of the 2153 aa Tula L ORF. This vector has an IPTG-inducible *tac* promoter and the proteins are expressed as glutathione S-transferase (GST) fusion proteins with the GST-tag in the N-terminus. The proteins were expressed in *E. coli*, purified with binding to glutathione-sepharose beads, and used to immunize rabbits and mice. Unfortunately, the anti-sera could not recognize even the antigens themselves. It seems that these regions of the L protein have low immunogenicity.

In our next attempt we tried to express the L ORF in three parts: aa residues 1-718, 719-1436, and 1437-2153. No expression of the N- and C-terminal parts could be observed

by Coomassie Blue staining of protein gels. This could be due to toxicity of these proteins to *E. coli*. Fortunately, the middle part could be expressed. This part contains the motifs conserved in RNA-dependent RNA polymerases, namely, premotif A and motifs A to E. These amino acids form the polymerase domain; therefore, antibodies against this part of the protein are more likely to inhibit polymerase activity, which could increase the potential uses of the antibodies.

As the middle part could not be expressed as a soluble protein, we decided to use purified inclusion bodies to immunize rabbits. Even though inclusion bodies are likely to contain mostly denatured protein, they can also include properly folded epitopes that can stimulate the expression of antibodies against native epitopes. Two rabbits were immunized and both of the two produced antibodies that recognized the antigen itself, though there was a great difference in the binding efficiency; in subsequent experiments immune serum from rabbit II was used.

With the antibodies we were able, for the first time, to detect a hantavirus L protein from both infected cells and virions. When we did immunoblotting of concentrated Tula virus virions (**II**: Fig. 1a) and Tula virus-infected cells (**II**: Fig. 1b) with our new anti-L antibodies, we determined the size of the L protein to be 250 kDa. The antibodies allowed us to demonstrate that we could express L protein in transiently transfected cells (**II**: Fig. 3, Fig. 4b) and also that complete L protein could be expressed *in vitro* by coupled RNA transcription and protein translation (data not shown). Both of these expression methods can now be used to study L protein. With the antiserum we were also able to detect the L protein of Puumala virus from concentrated virions and infected Vero E6 cells (data not shown). Detection of other hantavirus L proteins has not been attempted. Previously a hantavirus L protein had been detected only in virions. Elliott *et al*. (1984) propagated Hantaan virus in the presence of [^{35}S]-methionine, purified virions and detected the virion proteins in a 12.5% polyacrylamide gel (Elliott *et al.*, 1984). They estimated the size of L protein to be 200 kDa but a 12.5% polyacrylamide gel is not ideal for separating proteins this large, which could make their estimate inaccurate.

Hantavirus replication is inefficient in cell culture. An African green monkey kidney cell line, Vero E6, is the most susceptible cell line to hantavirus infections. Even in these cells hantaviruses grow to low titers, approximately 0.01 FFU/cell in our cell cultures. The reason for the low virus production rate is not known. One possibility was that the amount of L protein in cells would be so low that few virions could be assembled. The antiserum we developed against L protein enabled us to determine the amount of the protein in cells. We found approximately 10^4 copies of L protein and 10^7 copies of N protein in cells (**II**) indicating that the low amount of L protein cannot be the main reason for the low virus titers. Immunofluorescence and Northern hybridization assays indicate that the other virion

components: the glycoproteins G1 and G2 and the L, M, and S vRNAs are also abundant in cells. Thus, the assembly of the virion components seems to be very inefficient and finding out the reason for this requires further research.

There is approximately a 5000-fold molar excess of N protein to L protein in infected Vero E6 cells. Even if there are only three L proteins in virions i.e. one L protein bound to each RNA segment, and if L and N proteins were produced in the same proportion in cells as they exist in virions, there would be approximately 15000 copies of the 49 kDa N protein in each virion. This number is improbably high, as the copy number of the 23 kDa N protein of La Crosse virus was determined to be 2100 copies per virion (Obijeski *et al.*, 1976a). It appears that N protein is produced in excessive quantity in cells. It is possible that N protein is required for controlling the host cell response to the infection. As mentioned, hantavirus N protein has been found to interact with SUMO-1 conjugating enzyme 9 (Kaukinen *et al.*, 2003b) and an enhancer of apoptosis, Daxx (Li *et al.*, 2002).

The amounts of the viral proteins: L, N, and G1, were followed in Tula virus-infected Vero E6 cells 1 to 8 days post infection together with the amount of actin as a control. The viral proteins had a similar expression pattern with the amount of each protein increasing until reaching a plateau 4-6 days p.i. (**II**: Fig. 2). Actin amounts remained stable as expected.

5.4. Membrane association of L protein

The RNA replication of positive-strand viruses is associated with membranes for all viruses that have been studied (den Boon *et al.*, 2001). Two methods were used to study the possible membrane-association of the L protein of Tula virus. The first method was purification of microsomal membranes by ultracentrifugation from lysates of Tula virus-infected cells. The pelleted microsomal membranes and associated proteins were immunoblotted as were cytosolic proteins of the supernatant. The second method was membrane flotation assay in which cell lysates were applied to the bottom of a discontinuous sucrose or OptiPrep™-gradient, the gradient was ultracentrifuged, fractions were collected from top to bottom, and the proteins in the fractions were immunoblotted. In this procedure, membranes and associated proteins float to the less dense sucrose or OptiPrep™ at the top, whereas cytosolic proteins remain at the bottom.

We used BAP31, an integral membrane protein of the endoplasmic reticulum (Ng *et al.*, 1997), as a marker for membrane proteins and β-tubulin as a marker for cytosolic proteins (Taverna *et al.*, 2002). When we purified microsomal membranes by ultracentrifugation, the purity of the pelleted membranes and supernatants was checked by detecting BAP-31 and β-tubulin, which were found exclusively in the pelleted membrane

fraction and the supernatant, respectively (**II**, Fig. 3). In Tula virus-infected cells, L protein was found exclusively in the membrane fraction, as was N protein (**II**, Fig. 3). The N protein of the Black Creek Canal hantavirus had been previously found to be membrane-associated (Ravkov & Compans, 2001). Therefore, the membrane association of the N protein of Tula virus was somewhat expected but the membrane association of L protein had not been previously studied.

To check whether L protein expressed alone, without other viral proteins, would also pellet with membranes, we expressed recombinant L protein in HeLa cells using a vaccinia virus-driven T7 RNA polymerase expression system. The cells were first infected with a recombinant vaccinia virus (vTF7-3) that expresses T7 RNA polymerase (T7 pol) and then transfected with a plasmid that had the coding region of L RNA under a T7 promoter. This type of vaccinia virus-driven T7 expression system has been used extensively for the study of *Bunyaviridae* in localization of glycoproteins (Ruusala *et al.*, 1992; Shi & Elliott, 2002), creation of virus-like particles (Betenbaugh *et al.*, 1995), expression of functional L protein of Bunyamwera virus, genus *Orthobunyavirus* (Dunn *et al.*, 1995), and rescue of infectious Bunyamwera virus (Bridgen & Elliott, 1996).

When recombinant L protein was expressed with the system described above, again, all of the protein was found associated with membranes (**II**: Fig. 3.). We expressed a fusion protein of L with enhanced green fluorescent protein (EGFP) with the same system and this fusion protein, which we had made for localization studies discussed below, also was detected only in the pelleted microsomal membrane fraction (**II**: Fig. 3.). On the other hand, EGFP itself was detected in the supernatant containing cytosolic proteins (**II**: Fig. 3.). What these experiments with recombinant L proteins constructs showed was that the membrane association of L protein did not require the presence of other viral proteins. Thus, L protein must itself have a domain that interacts with a membrane component.

The membrane association of L protein and also the other viral proteins: N, G1, and G2, was further studied with membrane flotation assay. Tula virus-infected Vero E6 cells were Dounce-homogenized five days p.i. and cell lysates were applied to discontinuous sucrose or OptiPrep™ gradients. Both types of gradients gave the same results. Again, we used BAP-31 as a marker for membrane proteins and β-tubulin as a marker for cytosolic proteins (Fig. 5.4.1a). L and N proteins were found in membrane-associated fractions and in fractions containing cytosolic proteins (Fig. 5.4.1b). These experiments confirmed that L and N proteins are membrane-associated as are the glycoproteins 1 (data not shown) and 2 (Fig. 5.4.1b). Partial flotation with cytosolic proteins could be due to interactions with the cytoskeleton. The N protein of the Black Greek Canal hantavirus interacts with actin microfilaments (Ravkov *et al.*, 1998). Proteins that interact with the cytoskeleton do not float in membrane flotation assays (Simons & Toomre, 2000), and therefore, interaction

with the cytoskeleton could cause a portion of the N protein and also L protein to fractionate with the cytosolic proteins.

To study the nature of the membrane association of L protein, we treated the cell lysates with 1 M NaCl and 15 mM $NaCO_3$, pH 11, prior to running the discontinuous sucrose or OptiPrep™ gradient. These treatments dissociate peripheral membrane proteins from membranes. Both treatments made the detection of all proteins more difficult but we were able to detect L protein and found the protein only in the cytosolic fractions following treatment (Fig. 5.4.1c), indicating that L protein is a peripheral membrane protein. This is not surprising since no clear membrane-spanning domains were found when we analyzed the amino acid sequence of the L ORF.

The association of viral proteins with lipid rafts has received interest in recent years (Briggs *et al.*, 2003). To study whether any of the Tula virus proteins were associated with lipid rafts, we treated the Tula virus-infected cell lysates with non-ionic detergents, 1% Triton X-100 and 1% Nonidet® P40. The results were the same with both detergents. The membranes, which the control protein BAP-31 as well as Tula virus L and N proteins were associated with, were solubilized by detergent treatment (Fig. 5.4.1d). In contrast, the glycoproteins 1 and 2 fractioned to the top of the gradients, as shown for glycoprotein 2 (Fig. 5.4.1d), indicating that the membranes, which glycoproteins are associated with, are detergent-resistant. Octyl glucoside is known to dissociate lipid rafts and can be used to demonstrate that fractioning in the top fractions in membrane flotation is not due to protein multimers (Simons & Toomre, 2000). Octyl glucoside caused the glycoproteins to fractionate together with cytosolic proteins (Fig. 5.4.1e). As glycoproteins determine the assembly site, these results suggest that the assembly of Tula virus might occur on lipid rafts. As L and N proteins appear not to associate with detergent-resistant membranes, it seems that the site of RNA replication, which is determined by L and N protein localization, occurs on membranes that are distinct from the lipid rafts on which virus assembly might take place.

The membrane association of the polymerases of other negative-strand viruses has not been studied. It could be that the RNA polymerases of all these viruses are either directly or indirectly associated with membranes as is the case for all positive-strand viruses. In future studies, the membrane component that L protein interacts with and the overall arrangement of the hantavirus RNA synthesis machinery could be determined. Deletion and substitution mutations could be used to determine which part of L protein interacts with membranes.

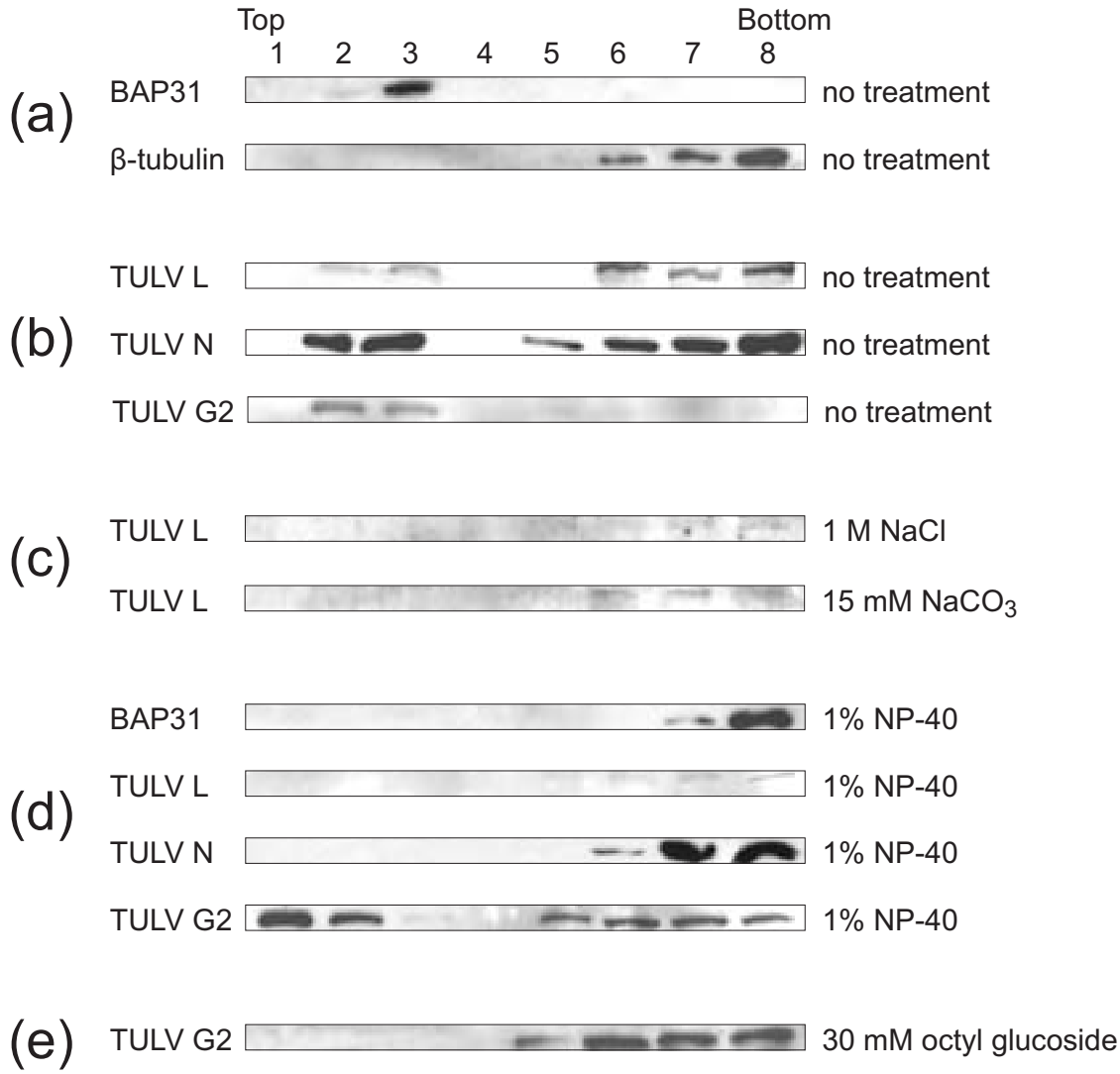

Figure 5.4.1. Membrane flotation. Tula virus-infected cells were lysed 6 days p.i. and the cell lysate ultracentrifuged in a discontinuous sucrose gradient. (a) The control for membrane proteins, BAP31, was found in the top fractions 2 and 3, which floated with membranes, and the control for cytosolic proteins, β-tubulin, remained in the bottom fractions 6-8. (b) Tula virus L and N proteins were found both in the membrane fractions 2-3 and the cytosolic fractions 6-8 and 5-8, whereas, G2 was detected only in the membrane fractions 2 and 3. (c) L protein was dissociated from membranes with NaCl and $NaCO_3$ and detected only in fractions 6-8. (d) Nonidet® P40 treatment caused BAP31, L and N proteins to fractionate with cytosolic proteins in the bottom fractions, whereas most of G2 still floated in the gradient. (e) Octyl glucoside solubilized the membranes G2 associates with, and G2 was in bottom fractions 5-8.

5.5. Localization of L protein in infected cells

We tried various approaches to detect Tula virus L protein in cells by indirect IFA using the anti-L antiserum that we raised. We tried numerous fixation protocols, denaturing the proteins with guanidine hydrochloride before antibody treatment, and addition of a signal amplification step by using biotinylated secondary antibodies detected by streptavidin-conjugated tertiary antibodies. Despite these efforts, we were not able to detect L protein by IFA.

In order to detect L protein in cells, we decided to make fusion protein constructs in which L protein is fused to EGFP. EGFP fusion proteins have the advantage that no antibodies are needed in the detection of the proteins in cells. This allows the detection of the protein even without cell fixation. EGFP fusion proteins have often been used to study viral RNA polymerases and with measles virus it has even been possible to incorporate EGFP coding DNA within the ORF of the RNA polymerase (Duprex *et al.*, 2002). We made two fusion protein constructs in which the EGFP was either in the C-terminus (L-EGFP) or the N-terminus (EGFP-L) of the fusion protein, with the coding region under a T7 promoter (**II**: Fig. 4a). We expressed L-EGFP by transfecting Vero E6 cells with the fusion protein construct and an autogene pCMV/T7-T7pol that has T7 RNA polymerase coding region under both CMV and T7 promoters (Brisson *et al.*, 1999). The CMV promoter drives transcription of T7 pol mRNA and as T7 pol is translated it can bind to the T7 promoter of the autogene and transcribe more mRNA. Thus, there is a cycle leading to a large amount of T7 pol being synthesized in cells. The T7 pol can then transcribe the mRNA of the fusion protein. The fusion protein construct with the N-terminal EGFP (EGFP-L) was expressed in HeLa cells using the vaccinia virus-driven T7 expression system described above. We checked the expression levels of the fusion protein by immunoblotting using both anti-GFP and anti-L antibodies. The amount of protein expressed in cells was much higher with the vaccinia virus-driven system (**II**: Fig. 4b).

We expressed in cells EGFP alone as a control and then the EGFP fusion protein constructs. Using IFA, we checked co-localization with markers for various cellular organelles. EGFP alone had a diffuse expression pattern, as is characteristic for this protein (**II**: Fig. 5a). In contrast, both fusion protein constructs had punctuate expression patterns characteristic of membrane proteins. Both EGFP/L fusion proteins were localized in the cytoplasm in the perinuclear region (**II**: Fig. 5b,c). There was also some co-localization with both the *cis*-Golgi protein GM130 (**II**: Fig. 5b,c) and N protein (**II**: Fig. 5d). Since both hantavirus L and N protein localize in the perinuclear region in close proximity to the Golgi, this is likely to be the site of RNA synthesis. Assembly of hantaviruses occurs in the Golgi. Having the site of RNA synthesis close to the site of virus assembly may facilitate the combination of these processes. We tried to confirm the localization of the site of viral RNA

synthesis with bromouridine incorporation into nascent RNA followed by indirect IFA with anti-bromouridine antibodies. We infected cells with Tula virus and then transfected them with BrUTP or incubated the infected cells in bromouridine-containing growth medium. However, bromouridine-containing RNA could not be detected by IFA. Hantavirus replication is inefficient in cell culture and the low level of viral RNA being synthesized might cause bromouridine-containing viral RNA to be undetectable.

5.6. Homologous recombination of the S gene

Homologous recombination, in which two or more copies of the same gene are used as templates in the synthesis of new genetic material, is known to commonly occur in positive-strand viruses. There were indications that homologous recombination could have taken place in nature in hantaviruses (Sibold *et al.*, 1999; Sironen *et al.*, 2001), however, homologous recombination had not been strictly proven to occur. Our group had previously cloned Tula virus S segments originating from rodents trapped in Tula, Russia, (Plyusnin *et al.*, 1994) and Moravia, the Czech Republic, (Plyusnin *et al.*, 1995a). These sequences were sufficiently divergent to allow detection of possible recombinants of the two RNAs.

We infected Vero E6 cells with the Tula virus isolate strain from Moravia, Tula/Moravia/Ma5302V/94 and then expressed a truncated S cRNA, Tula23S, of the Russian Tula virus strain, Tula/Tula/Ma23/87, in the same cells (**III**: Fig. 1). The Tula23S was truncated to prevent reassortment and to allow Tula23S sequences to be propagated only through recombination. The Tula23S was expressed by transfecting the Tula-infected cells with two plasmids: 1) the pCMV/T7-T7pol autogene, which has T7 pol gene under both CMV and T7 promoters, and 2) pT7ribo/Tula23S. The latter plasmid has the positive-strand of Tula23S cDNA, with a 5' deletion of 34 nucleotides, under the T7 promoter and the Tula23S gene is followed by Δ7 ribozyme which cleaves the RNA product at the exact 3' end of the S RNA (**III**: Fig. 1).

RNA was extracted from the cells, reverse-transcribed, amplified by PCR, cloned, and sequenced. The S RNA sequence differences between the two Tula virus strains (**III**: Fig. 4) allowed the design of primers that could selectively amplify possible recombinants (**III**: Fig. 2). In the first set of experiments 39 clones were obtained and of these 30 clones had only sequences of the Moravian virus and 6 clones had only Russian sequences, but 3 were recombinants having Moravian sequences in the 5’ part and Russian in the 3’ part, with the break-point being in the nucleotide region 332-368 (**III**: Fig. 2). In the second set of experiments, with an improved PCR protocol, all 20 sequenced clones were recombinants with 12 having the break-point in the nucleotide region 332-368 and 8 at other regions (**III**: Fig. 2). Sequential passaging of the virus in Vero E6 cells led to a gradual disappearance of

the recombinants from the virus pool (**III**: Fig. 3a) indicating that the recombinants were not able to compete with the original viral sequences.

Recombinant viruses could be isolated by using diluted, virus-containing supernatant, for passaging (**III**: Fig. 5). The N protein of the recombinant virus had the same expression pattern in infected cells as that of the cell culture-adapted Tula virus strain (**III**: Fig. 6a). It was also of the expected size and recognized by polyclonal anti-N antibodies (**IV**: Fig. 6b).

These experiments confirmed that homologous recombination can occur in hantaviruses and a hotspot of recombination (nt 332-368) was found. This is the first case where homologous recombination was confirmed to have occurred in a negative-strand RNA virus. This was also the first case in which genomic material expressed from plasmid DNA could be incorporated into hantavirus virions. Rescue of infectious hantavirus from plasmids has not yet been accomplished but the recombination system described here could be used to incorporate genetic material into hantaviruses.

5.7. Reverse genetics

In genetics the effects of pre-existing mutations are studied; often the starting point is a certain phenotype and there is a search for the set of mutations that have caused the phenotype. In contrast, in reverse genetics viral genes are first mutated and then the effects of the introduced mutations are studied. One common reverse genetics system is the minireplicon system. A minireplicon consists of the reverse-complement of the coding region of a reporter gene, such as chloramphenicol acetyl transferase or luciferase, flanked by viral 5'- and 3'-terminal NTRs. The minireplicon is expressed in cells that also express viral proteins required for RNA synthesis. Transfection-mediated transient expression or viral infection can be used to express the viral proteins. The minireplicon is used by the viral RNA polymerase as a template to synthesize reporter gene mRNA. Measurements of the activity of the reporter gene will tell how good a template the minireplicon is for viral mRNA synthesis. Mutations can be introduced to the 5'- and 3'-terminal NTRs to study what is required of the viral promoter for mRNA transcription.

If infection is used to express the viral proteins and the minireplicon is packaged into progeny virions, the packaging signal can be studied by finding out which mutations in the viral NTRs prevent minireplicon packaging. When the viral proteins required for RNA synthesis are expressed in cells by transfection-mediated expression, the minireplicon system can be used to study all the components of the RNA synthesis machinery: L protein, N protein, and the RNA template. Mutations can be introduced to the components to study their effects on RNA synthesis.

Minireplicon systems have now been developed for four of the five *Bunyaviridae* genera: Bunyamwera and La Crosse orthobunyaviruses (Dunn *et al.*, 1995; Blakqori *et al.*, 2003), Rift Valley fever, Toscana, and Uukuniemi phleboviruses (Lopez *et al.*, 1995; Accardi *et al.*, 2001; Flick & Pettersson, 2001), Crimean-Congo hemorrhagic fever nairovirus (Flick *et al.*, 2003), and Hantaan hantavirus (Flick *et al.*, 2003). There is, as of yet, no reverse genetics system for tospoviruses.

By expressing all Bunyamwera orthobunyavirus proteins and the three RNAs: S, M, and L, Bridgen and Elliott (1996) managed even to rescue infectious virus. With a virus rescue system, mutations can be introduced, not only in the components of the RNA synthesis machinery, but also to glycoproteins. The system allows the study of all aspects of the virus life cycle.

We tried to establish a reverse-genetics system for Tula virus. L protein and N protein were expressed by transfecting cells with plasmids that had the proteins under T7 promoter, and T7 pol was supplied using the above-mentioned expression systems: the recombinant vaccinia virus system or the autogene pCMV/T7-T7pol system. L and N protein were also expressed with plasmids that contained the genes under CMV promoter. The minireplicons had S or L NTRs, and the T7 promoter and human and murine RNA pol I promoters were used to express the minireplicon constructs. As reporter gene, both chloramphenicol acetyl transferase and luciferase were tried. Several cell lines were used in the experiments and the reporter gene constructs were also expressed in Tula virus-infected cells. Of the dozens of experiments, we had one positive result, which was, unfortunately, impossible to repeat. Other hantavirus groups were equally unsuccessful, until very recently Flick *et al*. (2003) managed to establish a reverse genetics system for Hantaan virus. They expressed a minireplicon in a mixture of BHK-21 and Vero E6 cells that were infected with Hantaan virus, and in 293-T cells that were transfected with L and N protein expressing plasmids. The minireplicon had, under RNA pol I promoter, the reverse complement of the reporter gene chloramphenicol acetyl transferase flanked by the L NTRs (Flick *et al.*, 2003).

5.8. Apoptosis in Tula virus-infected cells

We observed that when we subcultivated Vero E6 cells 2-4 days after being infected with the Vero E6 cell-adapted Tula virus (strain Moravia/Ma5302v) (Vapalahti *et al.*, 1996) nearly all cells died two to three days after subcultivation. Cell death was also observed without subcultivation 4-6 days p.i. if the multiplicity of infection (m.o.i.) was 0.1 or higher. With lower m.o.i. values, the cell death was delayed. The Tula virus-infected cells were tested for the presence of mycoplasma with a highly sensitive detection assay and the cell culture was found to be mycoplasma-free. Kang *et al*. (2000) had reported that Hantaan

virus infection caused apoptosis in Vero E6 cells but other research groups have not seen Hantaan virus-caused apoptosis of infected cells (Hardestam *et al.*, 2003). We wanted to see whether the cell death we were observing was caused by apoptosis.

Apoptosis triggers a protease cascade executed by cysteine-containing aspartate-specific proteases (caspases), which are activated by cleaving the prodomain of inactive procaspases (reviewed by Hengartner, 2000). Caspase 3 cleaves several proteins including an inhibitor of an endonuclease, which is, thereby, activated and cleaves genomic DNA producing a DNA ladder (Enari *et al.*, 1998; Sakahira *et al.*, 1998), which is characteristic of apoptosis (Wyllie, 1980). We observed the appearance of a DNA ladder in the Tula virus-infected cells from 3 days p.i. onwards (**IV**: Fig. 2a). When TUNEL-staining was done to detect the DNA strand breaks, the Tula virus infected-cells were positive whereas mock-infected were not (**IV**: Fig. 4b). Breaks in DNA strands induce the activation of the 116 kDa poly-[ADP-ribose] polymerase (PARP). In apoptotic cells the activation is inhibited by cleavage of the enzyme by caspase 7 producing a 85 kDa inactive form of the protein (Germain *et al.*, 1999). Cleavage of PARP can, therefore, be used as a marker for apoptosis. We could observe cleavage of the protein in Tula virus-infected cells 5 days p.i., one day after the cleavage of procaspase 3 to its active form (**IV**: Fig. 3a). The cleavage of PARP required Tula virus replication, as cells infected with UV-inactivated virus did not contain the cleaved form of the protein (**IV**: Fig. 3b). Treatment of cells with a broad-spectrum caspase inhibitor, z-VAD-fmk, inhibited both PARP-cleavage (**IV**: Fig. 4a) and DNA strand breaks detected by TUNEL-staining (**IV**: Fig. 4b). These experiments verified that Tula virus replication does, indeed, induce apoptosis in Vero E6 cells.

The progression of apoptosis in Vero E6 cell culture was followed by flow cytometry analysis of propidium iodide stained cells. A sub-G1 peak of apoptotic cell bodies started increasing 3 days p.i. (**IV**: Fig. 2b) and the ratio of apoptotic cells increased from 1% 1 day p.i. to over 40% 8 days p.i. (**IV**: Fig. 2b). Interestingly the G2/G1 ratio was altered in Tula virus-infected cells indicating that apoptosis might be linked to cell cycle (**IV**: Fig. 2b). In Vero E6 cells infected with Hantaan virus, apoptotic cell death became apparent from day 5 onwards after infection (Kang *et al.*, 1999). This slow progression of cells to apoptosis could be due to apoptosis being associated with cell cycle. In a confluent Vero E6 monolayer the majority of cells are senescent due to contact inhibition. The small portion of cells proceeding to cell cycle could be the ones undergoing apoptosis, which would reach detectable levels slowly after enough cells had progressed to cell cycle. Subcultivation of cells eliminates contact inhibition, thus causing the simultaneous progression of nearly all cells to cell cycle. This could lead to the massive apoptotic cell death observed when cells infected with Tula virus are subcultivated.

Members of the B-cell lymphocyte/leukemia-2 (Bcl-2) protein family are critical components of apoptosis having both pro- and anti-apoptotic functions (Rutledge *et al.*, 2002). Kang *et al*., (1999) found that in Hantaan virus-infected Vero E6 cells the amount of Bcl-2 mRNA was decreased, whereas Bcl-2 associated protein X (Bax) and heat shock protein 70 (Hsp70) mRNA amounts were unaffected. In Tula virus-infected Vero E6 cells Bcl-2β and Baxα protein amounts were decreased, whereas Bcl-2α, Baxβ and Bcl-X_L remained unchanged (**IV**: Fig. 5). In the extrinsic pathway of apoptosis cytokines such as inferferons and tumor necrosis factor (TNF) often play a role (Hay & Kannourakis, 2002). In Tula virus-infected Vero E6 cells interferon α did not play a significant role (**IV**: Fig. 6c), whereas the expression of TNF-receptor 1 was elevated (**IV**: Fig. 7a) and treatment of the infected cells with TNF-α lead to increased cleavage of PARP (**IV**: Fig. 7b), indicating that TNF-α amplified the apoptotic response to Tula virus infection in Vero E6 cells. This is interesting, as TNF-α has been elevated in many HFRS patients (Krakauer *et al.*, 1995; Linderholm *et al.*, 1996; Temonen *et al.*, 1996).

6. CONCLUDING REMARKS

The first two decades of hantavirus research has seen a rapid increase in the number of hantaviruses discovered as the search for hantaviruses has been expanded to more regions and rodent species. The effect of the viruses on humans has naturally been the major focus of research as seen in the development of diagnostic methods, vaccines, and, to a lesser extent, treatment of the illnesses caused by hantaviruses. In comparison, research on the molecular biology of hantaviruses has received less attention but has been progressing making use of information that has been acquired with related viruses. Hantavirus research has also made inroads into cell biology, as the interplay between viral proteins and components of the host cell has received more attention.

In the years since its discovery in 1994, Tula virus has become one of the most extensively studied hantaviruses and an apathogenic model virus. It was the fifth hantavirus to have its complete genome sequenced; this was accomplished in the current study (I), and that group of hantaviruses has increased by only three species since. Tula virus is the only virus that has had all four of its structural proteins detected in infected cells which was accomplished with the detection of the L protein with the antibodies that we were able to raise (II). The membrane association and localization of a hantavirus L protein were first studied with Tula virus (II). The current study also showed for Tula virus that homologous recombination can occur in hantaviruses and that can be used to incorporate homologous genetic material to the genome of the virions (III). This study also described the effect that virus infection has on cells with Tula virus, which is a strong inducer of apoptosis (IV).

In the near future it will be possible to rescue infectious hantaviruses by transfecting cells with plasmids expressing the viral RNAs and proteins. This technique, which has in recent years become feasible with influenza viruses and Bunyamwera orthobunyavirus of the *Bunyaviridae* family, will give an important boost to the study of the molecular biology of hantaviruses and the effect the viruses have on cellular functions. However, there are aspects of the virus that will remain difficult to study such as the mechanism of disease in humans. With proper animal models the recombinant hantaviruses can probably help in forming hypotheses concerning this aspect as well.

7. ACKNOWLEDGEMENTS

This study was carried out in the Department of Virology, Haartman Institute, University of Helsinki. I would like to thank Antti Vaheri, who was the professor of the Department of Virology and the head of the Haartman Institute, for providing excellent conditions for research. The head of the former Department of Biochemistry, Professor Carl G. Gahmberg, is thanked for providing an excellent atmosphere for graduate and post graduate studies.

I warmly thank my supervisors, Docent Alexander Plyusnin and Professor Emeritus Antti Vaheri, for their guidance through these years. I was encouraged to try out even the more far-fetched ideas of mine.

I thank Professor Tapani Hovi and Docent Tero Ahola for reviewing the manuscript of this thesis and their helpful suggestions how to improve it. I would like to thank Timo Hyypiä and Maria Söderlund-Venermo for help they provided as members of my thesis committee.

I thank the former, present, and honorary members of the Viral Zoonosis group: Alex, Angelina, Anna, Antti, Anu, Eili, Eliisa, Hannele, Hanni, Heikki, Hilkka, Jie, Juha, Jussi, Kikka, Kirill, Kirsi, Leena, Liina, Mari, Nathalie, Niina, Olli, Ossi, Pasi, Paula, Petteri, Pirjo, Päivi, Raija, Satu, Taija, Tarja, Tomas, Toni, Tuomas, Tytti, Vesa, Virpi, Xiao-Dong, Xiuqi, and Ying. Because of you, it has been nice to come to work even when the work itself has not been progressing as it should. Anu, despite pestering me with her neverending problems with her eMac, has been a very nice neighbor indeed. She, Kirill, and Pasi are also thanked for nice times during our varied free time activities. Leena, Tytti, Raija, Anja, and Pirjo are thanked for excellent technical assistance. Leena has been especially helpful not just towards me but others as well. I've seen people come to her with all kinds of problems and she has always offered assistance. All the other people at the Haartman Institute are thanked as well.

I would like to thank my parents Eevi-Riitta and Juhani, my brother Mika and his fiancée Meri, for support. I would also like to thank my friends Anna-Maija, Matti, Tuula, ToTi, and all others who I've spent time with outside the lab.

The Academy of Finland, Helsinki Graduate School in Biotechnology and Molecular Biology, VITUS, Finnish Cultural Foundation, and Orion Pharma are thanked for financial support.

Helsinki, March 2004

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