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# **Studies on the Hantavirus S Segment Gene Products**

by

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A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY  
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## SUMMARY

Hantaviruses, members of the *Hantavirus* genus, *Bunyaviridae* family, are enveloped, single-stranded, negative-sense RNA viruses, among which are the causative agents of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. The virus genome consists of three segments, designated large (L), medium (M), and small (S), that are packed into helical nucleocapsids. These segments encode the RNA polymerase, glycoproteins G1 and G2, and nucleocapsid (N) protein, respectively.

Studies on the S genome segment products encoded by hantaviruses were undertaken using different molecular approaches. The initial aim of the project was to attempt a development of a reverse genetics system based on the systems established for other negative-strand RNA viruses. As a basis, the system successfully developed for Bunyamwera virus was chosen.

To this end, recombinant Puumala (PUU) virus-like RNA transcripts containing the CAT reporter gene were designed and produced that would be recognised and transcribed by transiently expressed recombinant PUU virus proteins. Two modified cell lines, BHK T7-SIN and Vero T7, that stably expressed bacteriophage T7 polymerase, were transfected with plasmids containing the reporter RNA or PUU protein sequences under control of the T7 promoter.

Several reporter constructs were used. These contained an antisense sequence encoding the open reading frame of the chloramphenicol acetyl transferase (CAT) gene that replaced the coding region in a negative sense PUU virus S or L segment untranslated regions (UTR). The presence of the complete 3' and 5' UTRs was thought to be essential for the transcripts from the reporter construct to act as authentic viral RNAs. The exact 3' and 5' terminal sequences were also considered to be prerequisites important for successful recognition and transcription by the viral polymerase. These were achieved by cloning the reporter construct immediately downstream of different versions of T7 promoter or *cis*-active hammerhead ribozyme sequence to generate the exact 5' termini, and by placing either a *Bbs*I restriction enzyme site or hepatitis delta virus self-cleaving ribozyme immediately downstream of the 3' terminus to generate

the exact terminal nucleotides. However, no evidence for recognition of the reporter construct was obtained in numerous experiments.

As an alternative approach, the recently described polymerase I system was employed. This system utilizes cellular RNA polymerase I to produce transcripts with correct 3' ends thus avoiding the need for expressing run-off transcripts or the use of a self-cleaving hepatitis delta ribozyme and also ensures production of the exact 5' termini. The proteins in this system are expressed from a plasmid containing CMV promoter, the cellular RNA polymerase type II promoter. However, this approach was also unsuccessful.

The mammalian two-hybrid system (M2HS), an *in vivo* assay to study protein-protein interactions, was employed to investigate the ability of the Puumala virus N protein to self-associate and to determine the domains on the protein responsible for the interaction. Not only was it shown that molecules were able to interact with one another but it was also revealed that the interaction occurs through the N- and C-terminal domains, confined to amino acids 1-105 and 385-432. Based on the results obtained via M2HS, a 'head-to-tail' model is proposed in which the association of the N proteins takes place through their terminal regions. The results of the M2HS were further confirmed using a co-immunoprecipitation assay.

Possible interactions between N and different fragments of L protein were also studied using the M2HS, however, no conclusions could be made as to whether or not (and if yes then how) the two proteins interact. This may be because the L protein was expressed as separate fragments and any interaction domains could be disrupted.

Finally, the expression of a putative second gene product (ORF 2) expressed from the S segment of hantaviruses was investigated. The results of immunoprecipitation and Western blot analyses with anti-ORF2 peptide antibodies indicated that the putative ORF2 protein could be detected from the plasmid pTMPUUS, that contains all the S gene sequence, and from pTMPUUORF2 containing only ORF2 coding sequence. The protein was also detected in Puumala virus infected cells by Western blot analysis. Although the results of this work are preliminary, they suggest that the PUU S segment does indeed express a protein in addition to N.

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## Abbreviations

<sup>14</sup> C	carbon-14	DOPE	dioloeoyl L- $\alpha$ -phosphatidyl ethanolamine
<sup>35</sup> S	sulphur-35	dTTP	deoxythymidine triphosphate
A	adenine	dH <sub>2</sub> O	de-ionised water
A <sub>280</sub>	absorbency at 280nm	DI	defective-interfering
AD	activation domain	DMSO	dimethylsulphoxide
BD	binding domain	DNA	deoxyribonucleic acid
BSA	bovine serum albumin	ds	double-stranded
C	cytosine or carboxy	DTT	dithiothreitol
°C	Celsius	ECL	enhanced chemiluminiscence
CaCl <sub>2</sub>	calcium chloride	<i>E.coli</i>	<i>Escherichia coli</i>
CAT	chloramphenicol acetyltransferase	EDTA	ethylenediaminetetra-acetic acid
cDNA	complementary DNA	ER	endoplasmic reticulum
Ci	Curie	G	guanine
cm	centimetre	g	gram or gravity ( <i>g</i> )
cm <sup>2</sup>	square centimetres area	h	hour
CoA	co-enzyme A	HCl	hydrogen chloride
cpm	counts per minute	HFRS	haemorrhagic fever with renal syndrome
cRNA	complementary RNA	HPS	hantavirus pulmonary syndrome
CsCl	caesium chloride	IP	immunoprecipitation
CTP	cytidine triphosphate	IRES	internal ribosome entry site
dATP	deoxyadenosine triphosphate	IFN	interferon
dCTP	deoxycytidine triphosphate	IRF	interferon response factor
DDAB	dimethyldioctadecyl ammonium bromide	kb	kilobase
dGTP	deoxyguanosine triphosphate	KCl	potassium chloride
DMEM	Dulbecco's modified Eagle medium	kD	kilodalton
dNTP	deoxynucleotide	KOAc	potassium acetate

M	Molar	PAGE	polyacrylamide gel electrophoresis
M2H	mammalian two-hybrid	PBS	phosphate-buffered saline
mA	milli Amp	PCR	polymerase chain reaction
μCi	micro Curie	PEG	polyethylene glycol
min	minute	PEI	polyethylene imine
mg	milligram	pmol	picomole
μg	microgram	RBD	RNA binding domain
MgCl <sub>2</sub>	magnesium chloride	RNA	ribonucleic acid
MgSO <sub>4</sub>	magnesium sulphate	RNase	ribonuclease
ml	millilitre	RNasin	placental ribonuclease inhibitor
μl	microlitre	rpm	revolutions per minute
mm	millimetre	RT	room temperature
mmol	millimole	s	second
moi	multiplicity of infection	SL	stem-loop
mRNA	messenger RNA	SDS	sodium dodecyl sulphate
N	nucleocapsid protein or amino	ss	single-stranded
Na	sodium	T	thymine
NaCl	sodium chloride	TAE	Tris-acetate EDTA
NaOH	sodium hydroxide	TBE	Tris-borate EDTA
ng	nanogram	TE	Tris-EDTA
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	ammonium sulphate	T7	T7 RNA polymerase
nm	nanometre	TEMED	NNN'N' tetramethylethylenediamine
NP	nucleoprotein	TLC	thin-layer chromatography
NP40	nonidet P40	TnT	transcription and translation
TEN	Tris-EDTA-NaCl	TSB	transformation and storage buffer
NS	nonstructural	U	uracil or unit
NSV	negative-strand virus	vRNA	viral RNA
UTR	untranslated region	V	volts
nt	nucleotide	w/v	weight by volume
oligo	oligonucleotide		
ORF	open reading frame		

## Abbreviated virus names

AND	Andes virus	PUU	Puumala virus
BAY	Bayou virus	RIOS	Rio Segundo virus
BCC	Black Creek Canal virus	RIOM	Rio Mamore virus
BUN	Bunyamwera virus	RSV	Rous sarcoma virus
DOB	Dobrava virus	RVF	Rift Valley fever virus
DUG	Dugbe virus	SEO	Seoul virus
ELMC	El Moro Canyon virus	SeV	Sendai virus
HIV	human immunodeficiency virus	SN	Sin Nombre virus
HTN	Hantaan virus	SV40	simian virus 40
ISLA	Isla Vista virus	THAI	Thailand virus
KHAB	Khabarovsk virus	TOP	Topografov virus
LAC	La Crosse virus	TPM	Thottapalayam virus
LN	Laguna Negra virus	TSW	tomato spotted wilt virus
MUL	Muleshoe virus	TUL	Tula virus
NY	New York virus	UUK	Uukuniemi virus
PH	Prospect Hill virus	VSV	vesicular stomatitis virus
		vTF7-3	recombinant vaccinia virus expressing T7 RNA polymerase

## Amino acids

A	Ala alanine	N	Asn asparagine
B	Asx asparagine/aspartic acid	P	Pro proline
C	Cys cysteine	Q	Gln glutamine
D	Asp aspartic acid	R	Arg arginine
E	Glu glutamic acid	S	Ser serine
F	Phe phenylalanine	T	Thr threonine
G	Gly glycine	V	Val valine
H	His histidine	W	Trp tryptophan
I	Ile isoleucine	Y	Tyr tyrosine
K	Lys lysine	Z	Glx glutamine or glutamic acid
L	Leu leucine		
M	Met methionine		

## **CHAPTER 1: INTRODUCTION**

### **1.1. The problem of hantaviral fevers**

Hantaviral fevers are infectious diseases distributed throughout the world, the causative agents of which are members of the *Hantavirus* genus, family *Bunyaviridae*. Since the isolation in 1976 of the first hantavirus in South Korea from the striped field mouse, *Apodemus agrarius*, until the present time, in different regions of the world more than 500 hantaviral strains have been isolated (Schmaljohn and Hjelle, 1997). Antigenic, biochemical and molecular-genetic studies allowed assignment of those strains to groups considered as serotypes of hantavirus. At present, the genus comprises 22 serotypes including those that cause hemorrhagic fever with renal syndrome, hantavirus pulmonary syndrome and viruses that are not pathogenic to humans. The natural reservoir and the major source of human infection are wild rodents. Transmission to humans appears mostly via inhalation of aerosolized infected rodent urine, saliva and excreta (Peters *et al.*, 1999; Feldmann, 2000; Schmaljohn and Nichol, 2001).

Hemorrhagic fever with renal syndrome (HFRS) is a severe human disease that comprises a variety of clinically similar illnesses such as Korean hemorrhagic fever, epidemic hemorrhagic fever, and nephropathia epidemica. At least four distinct hantaviruses are known to cause HFRS: Hantaan, Puumala, Seoul, and Dobrava viruses (Lee *et al.*, 1985; Schmaljohn *et al.*, 1985; Dantas *et al.*, 1987; McKee *et al.*, 1991; Xiao *et al.*, 1994; Antoniadis *et al.*, 1996). Approximately 150,000 to 200,000 cases of HFRS involving hospitalization are reported each year throughout the world, with more than half of those in China. Hundreds to thousands of HFRS cases are reported by Russia and Korea, and most remaining cases (hundreds per year) are found in Japan, Finland, Sweden, Bulgaria, Greece, Hungary, and Yugoslavia. Depending on which hantavirus is responsible for the illness, HFRS can appear as a mild, moderate, or severe disease.

Death rates range from less than 0.1% for HFRS caused by Puumala virus to approximately 10% to 15% for HFRS caused by Hantaan virus (Lee, 1996). Clinical manifestations of the disease include hemorrhage, proteinuria, myositis, conjunctival infection, eye pain and myopia. Therapy for HFRS generally consists of supportive care which may include renal or peritoneal dialysis. The drug ribavirin statistically improves HFRS disease outcome but is not always effective (Huggins *et al.*, 1991).

The term 'hantavirus pulmonary syndrome' (HPS) is used for a severe respiratory illness found in North and South America. Sin Nombre virus was identified as the etiologic agent of an outbreak of the disease in the south-western United States in the summer of 1993 (Nichol *et al.*, 1993). At present, at least four more hantaviruses are known to cause HPS: Black Creek Canal, Bayou, New York, and Andes. Unlike HFRS, in HPS capillary leakage is localized exclusively in the lungs, and the kidneys are largely unaffected. As with HFRS, clinical differences can be observed among patients with HPS caused by different hantaviruses. The case fatality rate for this illness was reported to be greater than 50% (Schmaljohn *et al.*, 1995; Hjelle *et al.*, 1996; Khan *et al.*, 1995; CDC, 2002).

The worldwide distribution, high rates of human infection, significant frequency of severe forms of the disease leading to fatal outcome, lack of specific means of treatment and prevention demonstrate the high importance of the problem of hantaviral fevers.

Because of the lack of specific means to prevent HFRS and HPS, the development of a vaccine against hantaviruses is one of the most important tasks connected with their study. However, vaccine development efforts have been hampered by the inability to propagate hantaviruses in many cell lines, the propensity of these viruses to cause persistent infections both in their rodent hosts and in cell culture, and their slow and low-titered replication in cell culture (Schmaljohn *et al.*, 1990). This has hindered the study of the viruses in the laboratory; consequently, many questions remain concerning the potential genetic changes which may influence the ability of hantaviruses to replicate in permissive cell culture systems.

For this reason, it is essential to get a deeper understanding of basic properties of the viral genome, to identify basic viral components reliable for eliciting the host immune

response, and to consider constant spontaneous changes in hantaviral genomes (genome mutations and recombinations) caused by joint circulation in natural foci of the representatives of different rodent families - carriers of different hantaviral serotypes.

## 1.2. Characteristics of the *Bunyaviridae*

The *Bunyaviridae* is a large family that contains more than 350 mainly arthropod-borne viruses. They are distributed worldwide and share certain morphological, serological, biochemical and molecular-genetic characteristics. The family is divided into five genera: *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Tospovirus*, and *Hantavirus* (Elliott, 2001). The viruses are capable of infecting birds, mammals or plants, sometimes causing severe disease. Several members of the family are of human and veterinary importance. The human diseases include: several types of encephalitis (California group of bunyaviruses) which may be rare or endemic depending on the virus; epidemic fevers (Simbu and Oropouche bunyaviruses, Rift Valley fever phlebovirus); endemic fevers (sand fly fever group of phleboviruses) or hemorrhagic fevers (hantaviruses and nairoviruses) (Table 1.1) (Calisher, 1996).

All viruses of the *Bunyaviridae* studied so far are enveloped, spherical particles, about 90 to 120 nm in diameter. A single-stranded, tripartite, negative-sense or ambisense RNA genome is surrounded by the envelope which is composed of host derived lipid with glycoprotein spikes and is approximately 4 nm thick (Bishop, 1996). Based on the structure and its components, the overall chemical composition is calculated to be 1-2% RNA, 58% protein, 33% lipid, and 7% carbohydrate (Obijeski and Murphy, 1977).

The three genomic RNA segments are designated S (small), M (medium) and L (large). Their lengths have been shown to vary considerably among viruses in different genera of the family: the L segment ranges from 6.4kb for Uukuniemi virus to 12.2kb for Dugbe virus, whereas the M segment ranges from 3.2kb for Uukuniemi virus to 4.9kb for Dugbe virus, and the S segment shows a difference in size from 0.9kb for Bunyamwera

Genus	Viruses	Disease	Vector
<i>Orthobunyavirus</i>	Bunyamwera	Human	Mosquitoes
	Cache Valley	Sheep, cattle	Mosquitoes
	California encephalitis	Human	Mosquitoes
	Germiston	Human	Mosquitoes
	La Crosse	Human	Mosquitoes
	Snowshoe hare	Human	Mosquitoes
	Tahyna	Human	Mosquitoes
<i>Nairovirus</i>	Crimean Congo Haemorrhagic Fever	Human	Ticks, culicoid flies
	Dugbe	Human	Ticks
	Nairobi sheep disease	Sheep, Human	Ticks, culicoid flies, mosquitoes
<i>Phlebovirus</i>	Sandfly Fever	Human	Phlebotomine flies (also airborne)
	Rift Valley Fever	Human, cattle	Mosquitoes
	Uukuniemi	Human	Ticks
	Toscana	Human	Phlebotomine flies
	Punta Toro	Human	Phlebotomine flies
<i>Hantavirus</i>	Hantaan	Human	rodents
	Dobrava	Human	rodents
	Puumala	Human	rodents
	Seoul	Human	rodents
	Sin Nombre	Human	rodents
	Black Creek Canal	Human	rodents
<i>Tospovirus</i>	Tomato spotted wilt virus	Over 360 plant species	Thrips

Table 1.1. Some important pathogens in the family *Bunyaviridae*.  
Adapted from Calisher (1996).

virus to 2.9kb for tomato spotted wilt virus (Fig.1.1). However, the pattern of genome segment sizes is conserved within a genus.

All viruses examined to date encode their nucleocapsid protein in the S segment and their envelope glycoproteins in the M segment. The L RNA segment encodes the viral polymerase. Orthobunyaviruses, tospoviruses and some phleboviruses also code for a nonstructural protein, NSm, in their M segment. The NSm of tospoviruses is encoded in ambisense orientation. The S segment of phleboviruses and tospoviruses also encodes a nonstructural protein NSs, however, unlike orthobunyaviruses that also code for NSs, the members of these two genera have been shown to utilize an ambisense coding strategy (Fig.1.1).

The virus L protein (approximately 25 copies per virion) is associated with the N protein (approximately 2100 copies per virion), and with each of the three genomic RNA segments to form ribonucleoprotein (RNP) complexes termed nucleocapsids (Fig.1.2) (Obijeski *et al.*, 1976). The nucleocapsid is the template for mRNA synthesis and genome replication (Kolakofsky and Hacker, 1991).

All three viral RNA species have highly conserved complementary terminal sequences specific for each genus. The mRNA transcripts have heterogeneous, nonviral 5' end sequences (8 to 15 nucleotides in length) that are acquired from host mRNA sequences for the purposes of priming viral mRNA. Another feature of these viral mRNAs is that they are truncated at the 3' end by approximately 50-110 nucleotides compared to the full-length cRNA (Patterson and Kolakofsky, 1984; Bouloy *et al.*, 1990; Jin and Elliott, 1993b). These characteristics are shared with influenza virus mRNAs, however, unlike influenza virus, bunyavirus mRNAs do not appear to be polyadenylated (Krug, 1981).

Viral replication occurs in the cytoplasm of infected cells. Virions mature by budding into smooth surface vesicles in or near the Golgi complex (Elliott, 1990; Schmaljohn and Pettersson, 1990).

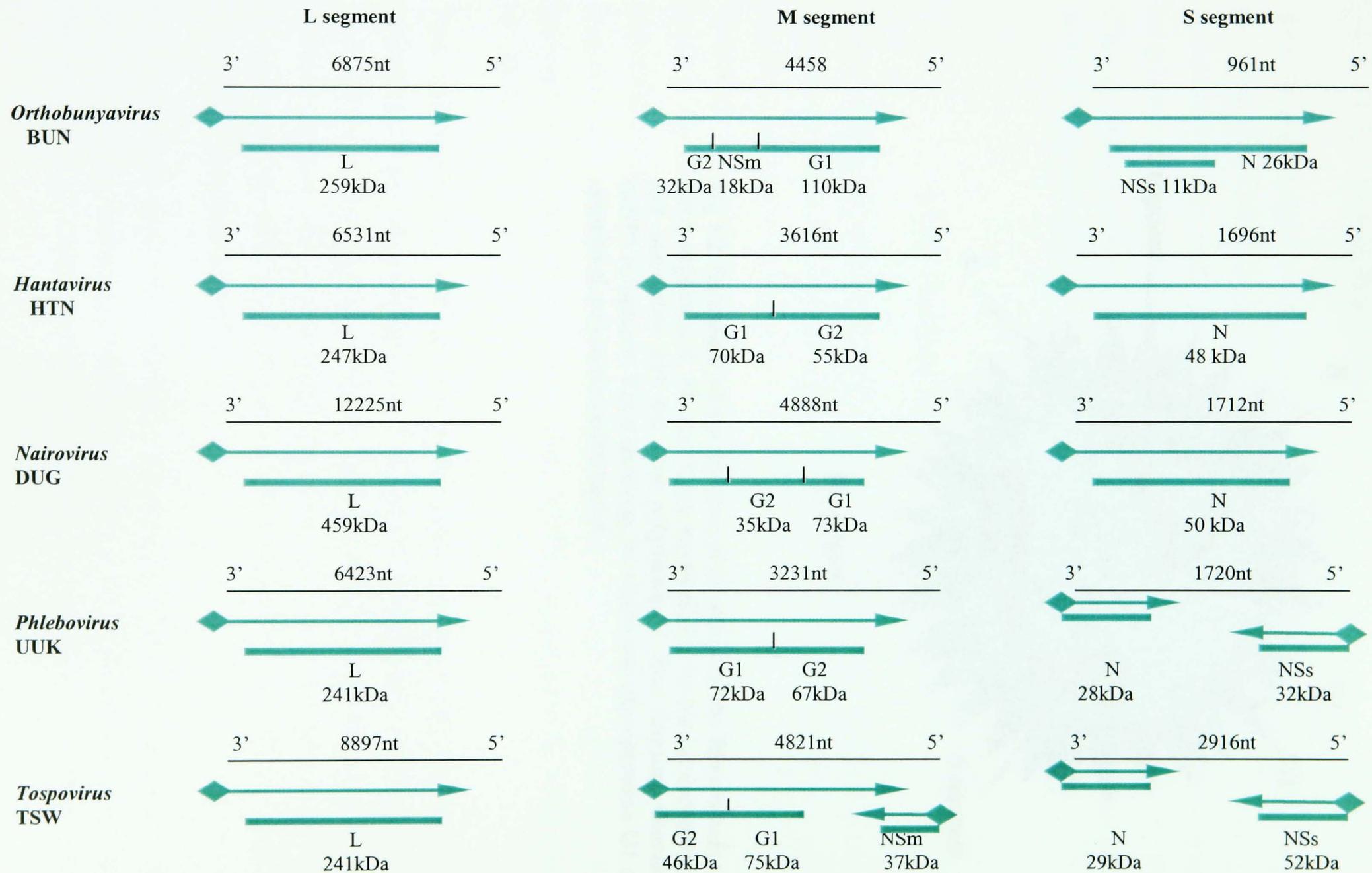


Fig. 1.1. Coding strategies of members of the *Bunyaviridae*. Thin lines represent the viral genome RNA, arrows mRNA, and rectangles polypeptides. The caps on mRNAs are shown as diamonds. From Elliott (1996).

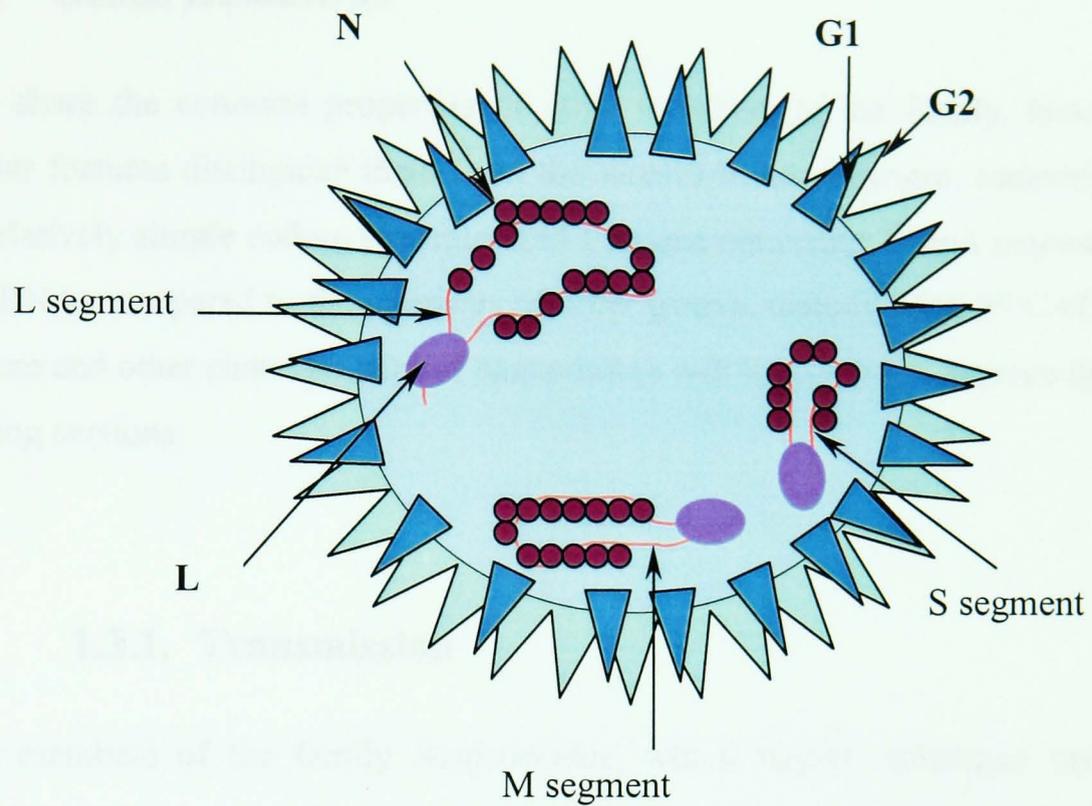


Fig 1.2. Schematic of the structure of members of the *Bunyaviridae*. The three RNA segments S, M, and L are encapsidated by the nucleocapsid protein N and associated with the viral polymerase L thus forming ribonucleoprotein (RNP) complexes. Lipid envelope, in which two glycoproteins G1 and G2 are embedded, surrounds nucleocapsid.

### **1.3. Genus *Hantavirus***

Hantaviruses share the common properties of other members of the family, however, three particular features distinguish them from the viruses in other genera: transmission by rodents, relatively simple coding strategies and a unique consensus 3' end sequence in the genomic RNAs compared to the members of other genera, namely AUCAUCAUC... (Fig.1.3). These and other characteristics of hantaviruses will be discussed in more details in the following sections.

#### **1.3.1. Transmission**

Unlike other members of the family *Bunyaviridae*, which require arthropod vectors, hantaviruses appear to be primarily associated with a specific rodent species, causing a persistent, asymptomatic, life-long infection in that species. They are thought to be transmitted via infectious aerosol generated by contaminated urine and feces, and possibly via saliva during bites. Most of the current data appears consistent with cospeciation of hantaviruses and their rodent hosts being the predominant pattern in the long-term evolution of this group of viruses (Antic *et al.*, 1992b; Morzunov *et al.*, 1995; Morzunov *et al.*, 1996; Plyusnin *et al.*, 1994a, 1994b; Spiropoulou *et al.*, 1994; Xiao *et al.*, 1994).

Well characterized Old World hantaviruses cause diseases collectively known as hemorrhagic fever with renal syndrome. They include Hantaan (HTN), Dobrava (DOB), Seoul (SEO), and Puumala (PUU) viruses that are transmitted by the striped field mouse, *Apodemus agrarius*, the yellow-necked field mouse, *A.flavicolis*, the Norway rat, *Rattus norvegicus*, and the bank vole, *Clethrionomys glareolus*, respectively (Table 1.2) (Hjelle *et al.*, 1995; Plyusnin *et al.*, 1996).

Sin Nombre (SN) virus was identified as the causative agent in an outbreak of severe pulmonary disease, hantavirus pulmonary syndrome, in the southwestern United States in 1993 (Nichol *et al.*, 1993; Chizhikov *et al.*, 1995; Spiropoulou *et al.*, 1994). The deer

<i>Orthobunyavirus</i>	3'-UCAUCACAUGA.....UCGUGUGAUGA-5'
<i>Hantavirus</i>	3'-AUCAUCAUCUG.....AUGAUGAU-5'
<i>Nairovirus</i>	3'-AGAGUUUCU.....AGAAACUCU-5'
<i>Phlebovirus</i>	3'-UGUGUUUC.....GAAACACA-5'
<i>Tospovirus</i>	3'-UCUCGUUAG.....CUAACGAGA-5'

Fig.1.3. Consensus terminal sequences of *Bunyaviridae* genome RNA segments. The terminal sequences are complementary and conserved within each genus.

Species	Disease	Principal Reservoir	Distribution of virus
Hantaan (HTN)	HFRS	<i>Apodemus agrarius</i> (striped field mouse)	China, Russia Korea, Taiwan, China
Dobrava-Belgrade (DOB)	HFRS	<i>Apodemus flavicolis</i> (yellow-neck mouse)	Balkans
Seoul (SEO)	HFRS	<i>Rattus norvegicus</i> (Norway rat)	Worldwide
Puumala (PUU)	HFRS	<i>Clethrionomys glareolus</i> (bank vole)	Europe, Russia, Scandinavia
Thailand (THAI)	Nd	<i>Bandicota indica</i> (bandicoot rat)	Thailand
Prospect Hill (PH)	Nd	<i>Microtus pennsylvanicus</i> (meadow vole)	U.S., Canada
Khabarovsk (KHB)	Nd	<i>Microtus fortis</i> (reed vole)	Russia
Thottapalayam (TPM)	Nd	<i>Suncus murinus</i> (musk shrew)	India
Tula (TUL)	Nd	<i>Microtus arvalis</i> (European common vole)	Europe
Sin Nombre (SN)	HPS	<i>Peromyscus maniculatus</i> (deer mouse)	U.S., Canada Mexico
New York (NY)	HPS	<i>Peromyscus leucopus</i> (white-footed mouse)	U.S.
Black Creek Canal (BCC)	HPS	<i>Sigmodon hispidus</i> (cotton rat)	U.S.

El Moro Canyon (ELMC)	Nd	<i>Reithrodontomys megalotis</i> (western harvest mouse)	U.S., Mexico
Bayou (BAY)	HPS	<i>Oryzomys palustris</i> (rice rat)	U.S.
Topografov (TOP)	Nd	<i>Lemmus sibiricus</i> (Siberian lemming)	Russia
Andes (AND)	HPS	<i>Oligoryzomys longicaudatus</i> (long-tailed pygmy rice rat)	Argentina
Isla Vista (ISLA)	Nd	<i>Microtus californicus</i> (California vole)	U.S.
Laguna Negra (LN)	HPS	<i>Calomys laucha</i> (vesper mouse)	Paraguay
Bloodland Lake (BLL)	Nd	<i>Microtus ochrogaster</i> (prairie vole)	U.S.
Muleshoe (MUL)	Nd	<i>Sigmodon hispidus</i> (cotton rat)	U.S.
Rio Segundo (RIOS)	Nd	<i>Reithrodontomys mexicanus</i> (mexican harvest mouse)	Costa Rica
Rio Mamore (RIOM)	Nd	<i>Oligoryzomys microtis</i> (small-eared pygmy rice rat)	Bolivia

Table 1.2 Members of the genus *Hantavirus*, family *Bunyaviridae*  
HFRS, hemorrhagic fever with renal syndrome, HPS, hantavirus pulmonary syndrome, Nd, not defined

mouse, *Peromyscus maniculatus*, an indigenous North American rodent of the subfamily *Sigmodontinae*, was quickly identified as the primary natural reservoir for SN virus. Subsequently, additional distinct hantaviruses associated with rodents of other *Sigmodontinae* genera were discovered in North and South America. The viruses named Bayou (BAY), Black Creek Canal (BCC), Andes (AND), and Laguna Negra (LN), associated with *Oryzomys palustris*, *Sigmodon hispidus*, *Oligoryzomys longicaudatus*, and *Calomys laucha* rodents, respectively, have already proven to be pathogenic for humans (Khan *et al.*, 1995; Morzunov *et al.*, 1995; Ravkov *et al.*, 1995; Rollin *et al.*, 1995; Torrez-Martinez and Hjelle, 1995; Lopez *et al.*, 1996; Nichol *et al.*, 1996; Williams *et al.*, 1997). El Moro Canyon (ELMC) virus, transmitted by the harvest mouse, *Reithrodontomys megalotis* (Hjelle *et al.*, 1994), and several hantaviruses from the Prospect Hill (PH) virus group (found in several different North American *Microtus* species) (Parrington *et al.*, 1991; Song *et al.*, 1995) have not been associated with human disease.

### **1.3.2. Coding strategies**

The coding strategies of hantaviruses appear to be the simplest among those described for the L, M, and S segments of the members of the other four genera in the family. The genome of hantaviruses consists of three negative-strand RNA segments: large (L) (6.5-6.6 kb), medium (M) (3.6-3.7 kb), and small (S) (1.7-2.1 kb). They were shown to encode in the virus complementary-sense only the structural proteins: the L segment encodes an RNA polymerase (~250 kDa), the M segment two glycoproteins (G1 ~70 kDa and G2~50kDa, without glycans) and the S segment a nucleocapsid protein (~50kDa) (Schmaljohn, 1996).

However, sequence data revealed that some hantaviruses such as Prospect Hill and Sin Nombre viruses may be able to encode a second protein in the S genome segment (Parrington and Kang, 1990; Stohwasser *et al.*, 1990; Spiropoulou *et al.*, 1994). Further, statistical analysis of the third base substitution frequency in the region encoding

potential second ORF protein (ORF2) in these viruses showed greatly reduced level of third position base substitution in comparison to N ORF outside ORF2 (Bowen *et al.*, 1995; Spiropoulou *et al.*, 1994). A lower third base substitution frequency in this region suggests that the second ORF codes for a functional protein. A similar statistical analysis predicted a functional overlapping ORF in the P gene of vesicular stomatitis virus (Bilsel *et al.*, 1990) (discussed in more detail in Chapter 5).

Despite this suggestive finding, the existence of a hantaviral nonstructural protein has not been demonstrated. Moreover, Hantaan virus, the prototype virus of the genus, has not even been shown to possess an ability to encode a protein similar to the one of orthobunyaviruses, phleboviruses, and tospoviruses that code for a nonstructural protein NSs in their S segment (Schmaljohn *et al.*, 1986b).

### **1.3.2.1. N protein**

The hantavirus nucleocapsid protein (N), which is in the range of 428 to 433 amino acids (~50kDa), is larger than those found in most other members of the family by approximately 160 to 200 amino acids, except for nairoviruses, which also have an N protein of approximately the same length (Spiropoulou *et al.*, 1994; Ravkov *et al.*, 1995; Bishop, 1996; Schmaljohn, 1996). N protein localizes at the perinuclear region in infected or N-expressing cells (Ravkov and Compans, 2001) and was shown to bind to filamentous actin similar to influenza virus N protein (Digard *et al.*, 1999).

N is the major antigenic protein, and a strong antibody response can be detected at an early phase of the disease in patient sera. The protein possesses immunodominant, linear, cross-reactive epitopes in the first 100 amino acids of the N terminus (Yamada *et al.*, 1995; Elgh *et al.*, 1996; Gott *et al.*, 1997). In addition, serotype-specific conformational epitopes have been detected between amino acids 155 and 429 of the N using serotype-specific monoclonal antibodies (MAbs) (Fig.1.4) (Ruo *et al.*, 1991; Yoshimatsu *et al.*, 1996).

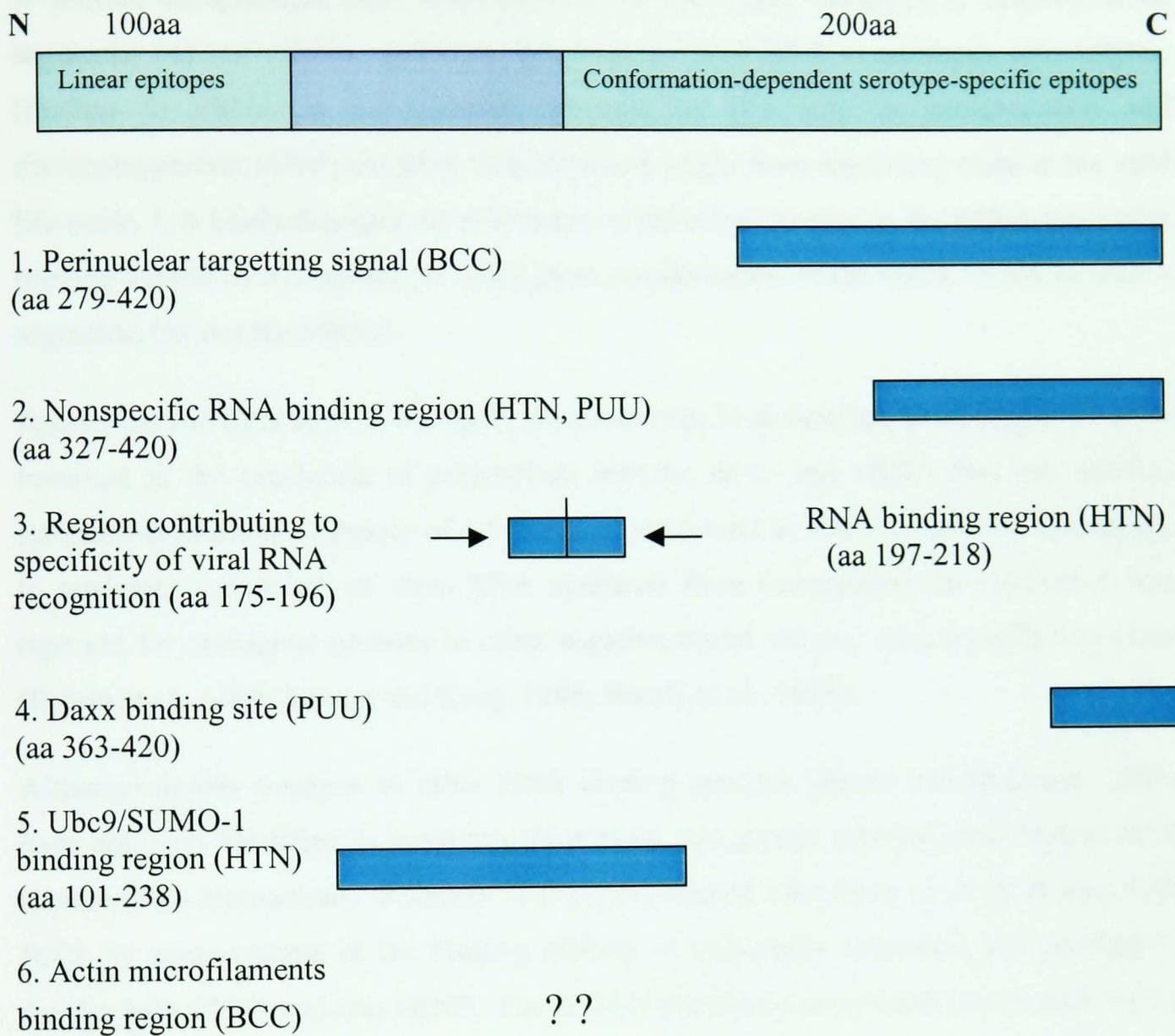


Fig. 1.4. A schematic representation of the functional domains in the N protein of hantaviruses. According to published reports, the functional domains (shown as blue bars) are as follows: 1. The perinuclear targeting signal; 2. The nonspecific RNA binding region; 3. The specific RNA binding region; 4. The Daxx binding site; 5. The Ubc9/SUMO-1 binding region. 6. The actin microfilament binding region. BCC – Black Creek Canal; HTN – Hantaan; PUU- Puumala viruses.

N protein encapsidates both viral genomic (vRNA) and antigenomic (cRNA) RNA segments, but not mRNA, and only the genomic viral RNA is packaged into virions. Binding to vRNA is an essential property to N's role in encapsidation and ribonucleoprotein (RNP) complex formation and might have regulatory roles in the viral life cycle. It is likely that specific sequences or structures present in the vRNA molecules provide a point of nucleation for subsequent encapsidation of the entire vRNA or cRNA segments, but not the mRNA.

Apart from the formation of the RNP, the hantavirus N protein has been suggested to be involved in the regulation of polymerase activity, as c- and vRNA (but not mRNA) synthesis depends on a supply of soluble (i.e., not bound to RNA) N protein. The ability to modulate the switch of virus RNA synthesis from transcription to replication was reported for analogous proteins in other negative-strand viruses, such as influenza virus (Patton *et al.*, 1984; Beaton and Krug, 1986; Honda *et al.*, 1988).

Although motifs common to other RNA binding proteins (Siomi and Dreyfuss, 1997) have not been identified in hantavirus N protein, two groups reported their studies of N protein-RNA interactions. Severson *et al* (1999) studied interaction of the N protein with RNA by measurement of the binding affinity of bacterially expressed and purified N protein with vRNA and non-vRNA. The HTN N protein demonstrated a preference for its full-length vRNA S segment as compared to its binding with an RNA encoding the open reading frame on the S segment. Furthermore, a strong preference was noted for the S segment vRNA as compared to nonspecific RNA. Additional experiments found the 5' end of the S segment vRNA to be necessary and sufficient for the binding reaction (Severson *et al.*, 2001). Preferential binding of the Bunyamwera virus N protein to the 5' end of the S segment vRNA has also been reported (Osborne and Elliott, 2000). In the second report, Xu *et al* (2002) showed that minimal RNA binding domain (RBD) is located between amino acid residues 175 and 217 that may extend into aa 217 to 249 by using a filter binding assay (Fig.1.4). It is also possible that additional regions of the N protein contribute to the interaction with the vRNAs. As Xu *et al.* showed (2002) the region of the N protein corresponding to amino acids Pro-196 through Ser-218 as well as determinants in 175-195 were necessary for a functional RBD. In addition to the

evidence provided by deletion mapping, the amino acids that follow 232 are highly nonconserved among HV sequences (aa 233-309). Studies with other RNA viruses have also reported that the nucleocapsid proteins bind vRNA through a localized, conserved domain, and that nonconserved regions are less likely to contain functional domains. For example, in the influenza virus nucleoprotein, a highly conserved region among A-, B- and C-type viruses was mapped (Albo *et al.*, 1995; Kobayashi *et al.*, 1994). Also, a conserved region in the C terminus of the rabies virus N protein, aa 298 to 353, was shown to bind directly to the vRNA (Kouznetzoff *et al.*, 1998). Similarly, RNA binding domain of the mouse hepatitis virus N protein was mapped to a central, conserved region, comprising amino acids 169 to 308 (Masters, 1992; Nelson and Stohlman, 1993). However, identification of common features of RBDs among the hantaviral nucleocapsid proteins may require elucidation of their three-dimensional structures.

Severson *et al* (2001) proposed a two-step model for encapsidation of the viral genome and antigenome RNAs that entails both specific and nonspecific interactions with the N protein. Initially, a specific interaction occurs between the N protein and the sequences in the single-stranded region of the predicted stem loop (SL) structure comprising amino acids 1 to 39 in the 5' end of the nascent vRNA. Initial binding may be followed by N-N protein interaction, which could drive the nonspecific binding of the remaining vRNA template.

The hantaviral nucleocapsid protein was also shown to interact with cellular proteins. Using a yeast two-hybrid screening system, Li *et al* (2002) found that the PUU N interacts with the Fas-mediated apoptosis enhancer, Daxx, at the C-terminal 57-aa residues in the N (Fig.1.4). They hypothesized that the interaction of the PUU N with Daxx regulated the localization of the N and was involved in the apoptotic process of the infected cells (Li *et al.*, 2002). Maeda *et al* (2003) have recently reported the interaction between the HTN N and SUMO-1. SUMO-1, a small ubiquitin-like modifier-1, conjugating enzyme 9 (Ubc9) conjugates SUMO-1 to target proteins and modulates cellular processes such as signal transduction, transcription regulation, and cell growth regulation. The results of their research showed that the aa 101-238 region in the N of HTN is necessary and sufficient for Ubc9-binding (Fig.1.4). Interestingly, Daxx interacts

with Ubc9 and is covalently conjugated with SUMO-1. This led the authors to suggest that it might be possible that the complex of N-Ubc9-SUMO-1-Daxx regulates the subcellular localization of the HTN N (Maeda *et al.*, 2003).

### **1.3.2.2. G1 and G2 glycoproteins**

The M genome segment of hantaviruses encodes the glycoprotein precursor (GPC) that is posttranslationally cleaved into two integral membrane surface glycoproteins, G1 and G2. The gene order of hantavirus M RNA with respect to messenger-sense RNA is 5'-G1-G2-3' (Schmaljohn *et al.*, 1987). G1 and G2 form a heterodimer in the endoplasmic reticulum (ER) before they are targeted to the Golgi compartment (Antic *et al.*, 1991, 1992a; Pensiero and Hay, 1992; Ruusala *et al.*, 1992). The G1/G2 heterodimers form the spikes on the virus particles which mediate receptor binding and fusion (Arikawa *et al.*, 1985, 1989; Dantas *et al.*, 1986).

Sequence determination revealed that the amino terminus of the mature G1 starts at position 18 (threonine) and with the G2 at 649 (serine) (Schmaljohn *et al.*, 1987). Hantaviruses apparently share the common *Bunyaviridae* property of a leader sequence before the first encoded glycoprotein, as has been observed for the phlebo- and orthobunyaviruses (Collett *et al.*, 1985; Ihara *et al.*, 1985b; Eshita *et al.*, 1984; Lees *et al.*, 1986). The absence of a NSm coding region between the signal sequence and G1 distinguishes hantaviruses from the phleboviruses Punta Toro and Rift Valley fever viruses, which have long (30K and 16K, respectively) stretches of polypeptides prior to the amino-terminal sequences of their first mature glycoprotein (Collett *et al.*, 1985; Ihara *et al.*, 1985b).

Four hydrophobic domains are found on the GPC (Fig.1.5). Domain I extends from amino acid position 1 to 17 and most likely serves as a signal peptide. The hydrophobic domain II (position 441 to 515) is unusually long and the actual membrane spanning portion is unknown. Domain III extends from position 627 to 648 and ends with a highly conserved pentapeptide motif WAASA at the amino terminus of G2. Domains II and IV

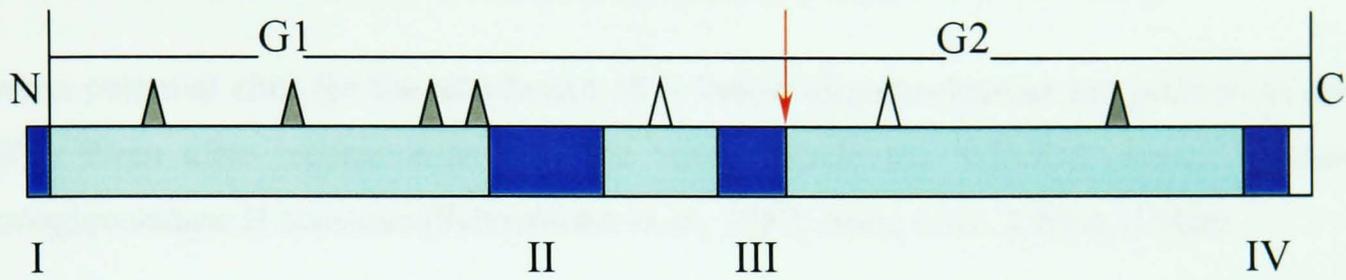


Fig. 1.5. Schematic illustration of the Hantaan virus glycoprotein precursor. The hydrophobic domains I (aa 1-17), II (aa 441-515), III (627-648), and IV (1097-1127) are shown in dark blue. Seven potential glycosylation sites (five in G1 and two in G2) are indicated as triangles, five of which are likely to be glycosylated (shown in gray). The red arrow indicates the potential cleavage site between G1 and G2 (highly conserved pentapeptide motif WAASA at the carboxyl-terminal end of domain III).

(position 1097 to 1127) are the transmembrane domains of G1 and G2, respectively. It was shown that pentapeptide WAASA in the termini of the domain III is found with all molecularly characterized hantaviral glycoproteins (Spiropoulou *et al.*, 1994).

Seven potential sites for the attachment of N-linked oligosaccharides are present on the GPC. Even after incorporation into the virus particle the N-linked glycans remain endoglycosidase H sensitive (Schmaljohn *et al.*, 1987; Antic *et al.*, 1992a, 1992b).

Until recently, the GPC has not been identified following hantavirus infection nor using recombinant expression from a plasmid containing the M segment ORF (Schmaljohn *et al.*, 1987; Pensiero and Hay, 1992; Schmaljohn, 1996). Previous evidence for the expression of such a precursor in the family *Bunyaviridae* had been obtained through *in vitro* translation experiments for Rift Valley fever virus of the genus *Phlebovirus* (Suzich and Collett, 1988). Recently, Lober *et al.* (2001) demonstrated the expression of HTN glycoprotein precursor in mammalian cells. The cleavage of the precursor GPC followed the rules for signal peptides, indicating that a signal peptidase complex is responsible for the processing into the glycoproteins G1 and G2. Detection of the precursor GPC could only be achieved through complete or partial cleavage inhibition. Amino acid sequence comparison of bunyavirus glycoprotein precursors in this particular cleavage region (WAASA sequence in the end of domain III) indicated that similar concept for cleavage is employed not only by hantaviruses but also by many other members of the family *Bunyaviridae* (Lober *et al.*, 2001).

The Golgi complex was thought to be the site of assembly for all hantaviruses (Plyusnin *et al.*, 1996) similar to the other viruses in the family *Bunyaviridae* data on which was obtained by electron microscopy, immunofluorescence analysis, and studies of the expression of viral glycoproteins (Anderson and Smith, 1987; Kuismanen *et al.*, 1982; Matsuoka *et al.*, 1994). For example, the use of indirect immunofluorescence demonstrated that recombinant HTN G1 and G2 could be colocalized with mannosidase II, a Golgi marker protein. Neither G1 nor G2 could be detected at the plasma membrane when analyzed by surface immunofluorescence or by biotinylation (Lober *et al.*, 2001).

Intracellular maturation of the virus particles in the Golgi cisternae has also been visualized by electron microscopy (Hung, 1988).

However, recent studies with viruses designated as New World hantaviruses, challenge the idea that the intracellular mode of virus assembly is the only mechanism utilized by hantaviruses (Goldsmith *et al.*, 1995; Ravkov *et al.*, 1998). Electron microscopy of Vero E6 cells infected with Sin Nombre virus showed accumulation of the virus particles on the cell surface and their absence in the Golgi complex and other intracellular compartments (Goldsmith *et al.*, 1995). Similar findings were obtained in studies with Black Creek Canal virus (BCC), another representative of the New World hantaviruses, in polarized epithelial cells using electron microscopy and immunofluorescence. It was shown that BCC assembly and release occur at the apical cell surface (Ravkov *et al.*, 1998). Therefore, there may be differences in the site of maturation between the New World and Old World hantaviruses.

A number of studies indicate that in bunyaviruses the signal specifying Golgi localization resides in just one of the glycoproteins. For example, for Bunyamwera virus the targeting signal was mapped to the N-terminal glycoprotein of the precursor (G2) (Lappin *et al.*, 1994), for Punta Toro phlebovirus it was mapped to the transmembrane domain and the first 10 amino acids of the cytoplasmic tail of the G1 protein (Matsuoka *et al.*, 1996), and for Uukuniemi virus, it was mapped to residues 10 to 40 of the G1 cytoplasmic tail (Andersson *et al.*, 1997). At the same time, the results of studies on hantaviruses concerning the Golgi targeting of G1 expressed separately from G2 were somewhat conflicting. When expressed individually, G1 and G2 were retained in the ER, whereas when coexpressed from separate plasmids, both proteins localized to the Golgi. Pensiero and Hay (1992) reported that G1 reached the Golgi in the absence of G2, whereas Ruusala *et al.* (1992) showed that G1 alone was unable to leave the ER without coexpression of G2. Similar to the results obtained by Ruusala *et al.* (1992), Shi and Elliott (2002) demonstrated by using double-staining immunofluorescence and confocal microscopy with a series of truncated cDNA constructs that Golgi targeting and retention of the glycoproteins require the coexpression of G1 and G2, and when separately expressed, both G1 and G2 remained predominantly in the ER. The signal for Golgi

localization of the virus glycoproteins may depend on the conformation of the oligomerized G1 and G2 complex rather than a primary amino acid sequence as is the case for other bunyaviruses.

As reported for other members of the *Bunyaviridae*, and other enveloped viruses, hantavirus envelope proteins possess haemagglutinating (HA) activity and induce pH-dependent cell fusion (Tsai *et al.*, 1984; Arikawa *et al.*, 1985; Okuno *et al.*, 1986). These functions are thought to play important roles in virus attachment to susceptible cell surfaces and in the uncoating of virions during the initial stages of infection.

Nine distinct overlapping antigenic sites, two on G1 and seven on G2, were demonstrated for hantaviruses. Analysis of the antigenic sites by HA inhibition and plaque-reduction neutralization tests showed that all of the sites, except one on G1 and two on G2, were related to viral HA. Only one of the G1 antigenic sites and two of the G2 sites were involved in viral neutralization. The nine antigenic sites could be further divided into 13 based upon the serological cross-reactivity of MAbs with viruses representative of each of the four major antigenic groups within the *Hantavirus* genus, i.e. Hantaan, Seoul, Puumala and Prospect Hill viruses (Arikawa *et al.*, 1988). It is now well established that antibodies to G1 and G2 neutralize the virus, distinguish viral serotypes, and protect animals from hantavirus infection (Arikawa *et al.*, 1989; Arikawa *et al.*, 1992; Chu *et al.*, 1995; Dantas *et al.*, 1986; Lundkvist and Niklasson, 1992a, 1992b; Pensiero *et al.*, 1988; Schmaljohn and Hjelle, 1997; Arikawa *et al.*, 1989; Chu *et al.*, 1995; Chu *et al.*, 1994). Differences within G1 and G2 neutralization determinants differentiate hantavirus serotypes and define functional relationships between pathogenic hantavirus strains (Schmaljohn and Hjelle, 1997). Neutralizing antibodies to both G1 and G2 were demonstrated to passively protect animals from infection with HTN virus. Immunizing hamsters with expressed G1 and G2 proteins in combination, but not separately, induced a protective immune response (Schmaljohn *et al.*, 1990). Those results suggest that both G1 and G2 are immunologically relevant.

### 1.3.2.3. L protein

The hantavirus 250 kDa L protein, the viral RNA-dependent RNA polymerase, is encoded by the L segment. The L protein possesses polymerase and endonuclease activities and is thought to be responsible for all steps of viral RNA transcription and replication.

Alignment of amino acid sequences of the RNA-dependent polymerases of different segmented and nonsegmented negative-strand RNA viruses revealed six conserved regions, designated domains I-VI, separated by more variable regions. These domains were proposed to be important for the various enzymatic activities of the polymerase, while the more variable regions between the domains contribute to the overall conformation of the protein (Feldhaus and Lesnaw, 1988; Poch *et al.*, 1990; Sidhu *et al.*, 1993; Smallwood *et al.*, 2002). Domains II and III have been proposed to represent the polymerase module. Domain II contains a region, designated pre-Motif A, with three invariant amino acids and one highly conserved charged residue. Domain III contains four regions designated motifs A, B, C, and D (Poch *et al.*, 1990) that together contain four invariant amino acids and one highly conserved residue when compared to 23 RNA-dependent RNA or DNA polymerase, including enzymes from paramyxoviruses, rhabdoviruses, bunyaviruses, arenaviruses, influenza viruses, a filovirus, and HIV (Muller *et al.*, 1994). Motif A is an acidic motif; motif B is the core motif for nucleotide binding; motif C is the core motif for catalytic function; and motif D is a basic motif (Poch *et al.*, 1990) (Fig.1.6).

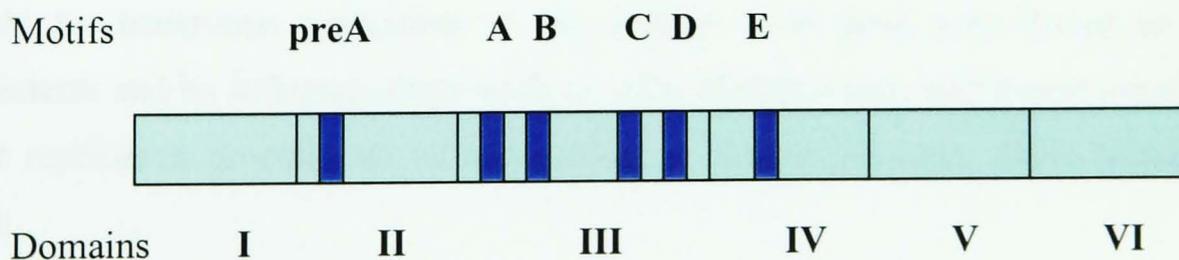


Fig.1.6. Schematic presentation of the domains and motifs on the polymerase protein. Domains II (pre-motif A) and III (motifs A, B, C, and D) have been proposed to represent the polymerase module.

Several of these previously identified motifs and premotifs common to all RNA-dependent polymerases are found in the hantavirus L protein and are shown in Fig. 1.7. (Piiparinen *et al.*, 1997). Premotif A just upstream of motif A (Muller *et al.*, 1994) and motifs A-D (Poch *et al.*, 1990) were found in the most highly conserved amino-terminal half of the hantavirus L protein (from amino acids 860 to 1191) (Chizhikov *et al.*, 1995). Also observed are the two recently identified N-terminal conserved domains preceding premotif A and motif A found previously in bunyavirus and arenavirus L proteins (Muller *et al.*, 1994). Between these two conserved stretches, a region (from amino acids 178-604) variable among the different hantaviruses was found. Motif E (Muller *et al.*, 1994) just downstream of motif D containing the tetrapeptide E(F/Y)XS, also common to segmented negative-stranded RNA viruses, was found. In addition, regions of high conservation among the hantaviruses can also be seen in the carboxy-terminal half of the L protein, including one particularly acidic residue-rich domain close to the carboxy terminus of the protein. A similar acidic domain was previously noted in the L protein of tomato spotted wilt virus (de Haan *et al.*, 1991). The carboxy-terminal part of the L protein was proposed to serve for specific interaction with host cell-encoded *trans*-acting transcriptional cofactors (Sidhu *et al.*, 1993). The high conservation of these domains among hantavirus L proteins implies some important role in the L protein structural conformation or interaction with cellular factors necessary for L polymerase function.

### **1.3.3. Stages of hantavirus replication**

Models for hantavirus replication at the cellular level have been based on direct experiments and by inference from work on other bunyaviruses, and the principal stages of the replication process can be summarised as follows (Bishop, 1996; Schmaljohn, 1996):

**Attachment of virus** is mediated by G1 and G2 glycoproteins which are thought to take part in the recognition of receptor(s) on host cell surface.



```

PUU/Seq 1540 EGVYRELGLD ISQPNVLKKV APLLYKSGKS RIVIVQGNVE GTAESICBYW LKTMSLVKTI KVKPKKEVLK
PUU/B1820 1540 .....
SN 1541 .....GL.E...RI.....A.R..V...E.....RS.....R.....R
SEQ 1540 D.....B.D.M...M...A...V.....A.A...RN...I...H...O
PUU/Seq 1540 EGVYRELGLD ISQPNVLKKV APLLYKSGKS RIVIVQGNVE GTAESICBYW LKTMSLVKTI KVKPKKEVLK
PUU/B1820 1540 .....
SN 1541 .....GL.E...RI.....A.R..V...E.....RS.....R.....R
SEQ 1540 D.....B.D.M...M...A...V.....A.A...RN...I...H...O
HTN 1540 D.....L.D.M.RI..M...S...V.....A..R...S.....R...H...O

PUU/Seq 1610 AVSLYGGKKEK VGDLTHLAAM RLCIEVWRWC KANEQDSVTW LKYLVFENKT LEQWDLFCS RGVLPIDPEI
PUU/B1820 1610 .....A.....S.....M.....I.S...V....
SN 1611 .....ST..N..L.LODDY..T.....D.NVND..NA.Y..KO..MD..ER.RR..K..V....
SEQ 1610 ...IFNR..D..I.OQKD.S.L.K.....A...NAPYRD..FHA.W..D...FSE.L.R..R.V..P....
HTN 1610 ...IFNR..D..I.OQKD...L.K.....SAPYRD..FQA.W..D...FSE.L.R..R.V..P....

- - - - -Conserved region two- - - - -
PUU/Seq 1680 QCLGLLVYDL KGQKGLLQIQ ANRRAYSQKQ YDAYCVQTYN EETKLYEGDL RVTNFGIDC ARLEIFWDKK
PUU/B1820 1680 .....R
SN 1681 ...IA..L..V..L.Y.SV..M.....R.....L.....
SEQ 1680 ...AA.MIA..V..DRBY..L.....L.....
HTN 1680 ...AA.MIA..I..DYSV..L.....V.....L.....

- - - - -
PUU/Seq 1750 EYILETSITQ RVNLKILMEE YTKELLRCGM RPKTEQVNS RSVLFKTES GFEWGKPNVP CIVYRNCTLR
PUU/B1820 1750 .....
SN 1751 .....H...LM...O.....SHT...L.....FKH.A..
SEQ 1750 T.....KH...M...S...V...N...GV KHL...D...I...K..A..
HTN 1750 A.....KH...M.D..S...IK...N...QVG..HM.....I...K..V..

- - - - -
PUU/Seq 1820 TGLRVRHPTN KAFSITIQAN GFRAMAQLDE ENPRFLAHA YHNLKDVRYQ ALQAVGNVWF KMTQHKLFIN
PUU/B1820 1820 .....O.....
SN 1821 ...TKOA..E.M.NV..D...I..M.M..S...T.R...V...QTA....
SEQ 1820 ...TNOA..HK.M...KDD..L..I..Y..DS...F.TIR...VD..S...THKG...YL.
HTN 1820 ...S..TTOA..HK.M...KDD..L..I..H..DS...F.TIR..I...VD..S...IHKGV..YL.

- - - - -Conserved region three- - - - -
PUU/Seq 1890 PISAGLLEN FMKGLPAAIP PAAYSLIMNK AKISVDLFMF NELLALINPQ NVLNLDGIEE TSEGFTTVST
PUU/B1820 1890 .....Y....
SN 1891 ...S.....V..R.....YS..TS
SEQ 1890 ...S...Y...N.....R.....D..R...G..T.D.S.L.I..G..YS..NS
HTN 1890 ...S.....N.....R.....D..K...R..T.D.S.L.T..GDE.S...S

```

Fig.1.7. continued

**Entry and uncoating** of the virions are thought to occur via endocytosis and subsequent fusion of the viral membrane with the endosomal membrane.

After entry, L protein-mediated **primary transcription** of three viral-complementary mRNAs occurs in the cytoplasm. The process involves cleavage of capped sequences from host cellular mRNAs by the viral polymerase to prime transcription of the S, M, and L mRNAs by L (Fig.1.8).

**Translation** yields N, L, and the glycoproteins. Free cellular ribosomes are utilised for translation of the N and L proteins. In the case of the glycoproteins the ribosomes are bound to the membrane of the endoplasmic reticulum and the glycoproteins are processed in the Golgi apparatus where they are also glycosylated.

Following mRNA translation, transcription shifts from mRNA to a full-length positive-sense **intermediate antigenome** (cRNA) by the viral polymerase, and ribonucleoprotein (RNP) structures are formed (Schmaljohn and Dalrymple, 1983). This is subsequently copied back into the negative-sense genome, also by L protein. What mediates the switch from transcription to replication is unknown.

**Secondary transcription** to generate the viral mRNAs can then take place and more viral proteins are synthesized, while replication is ongoing.

**Morphogenesis:** evidence suggests that RNPs may use microfilaments for transport to virus assembly sites (Ravkov *et al.*, 1998). Nucleocapsids accumulate under Golgi membranes and are packaged into virions by an undefined association with the glycoproteins G1 and G2.

**Virus release:** the virions are transported to the plasma membrane in vesicles which fuse with the membrane and release virions. Virions that assemble at the plasma membrane bud directly into the extracellular space.

A number of the principal stages will be discussed in detail further within the section.

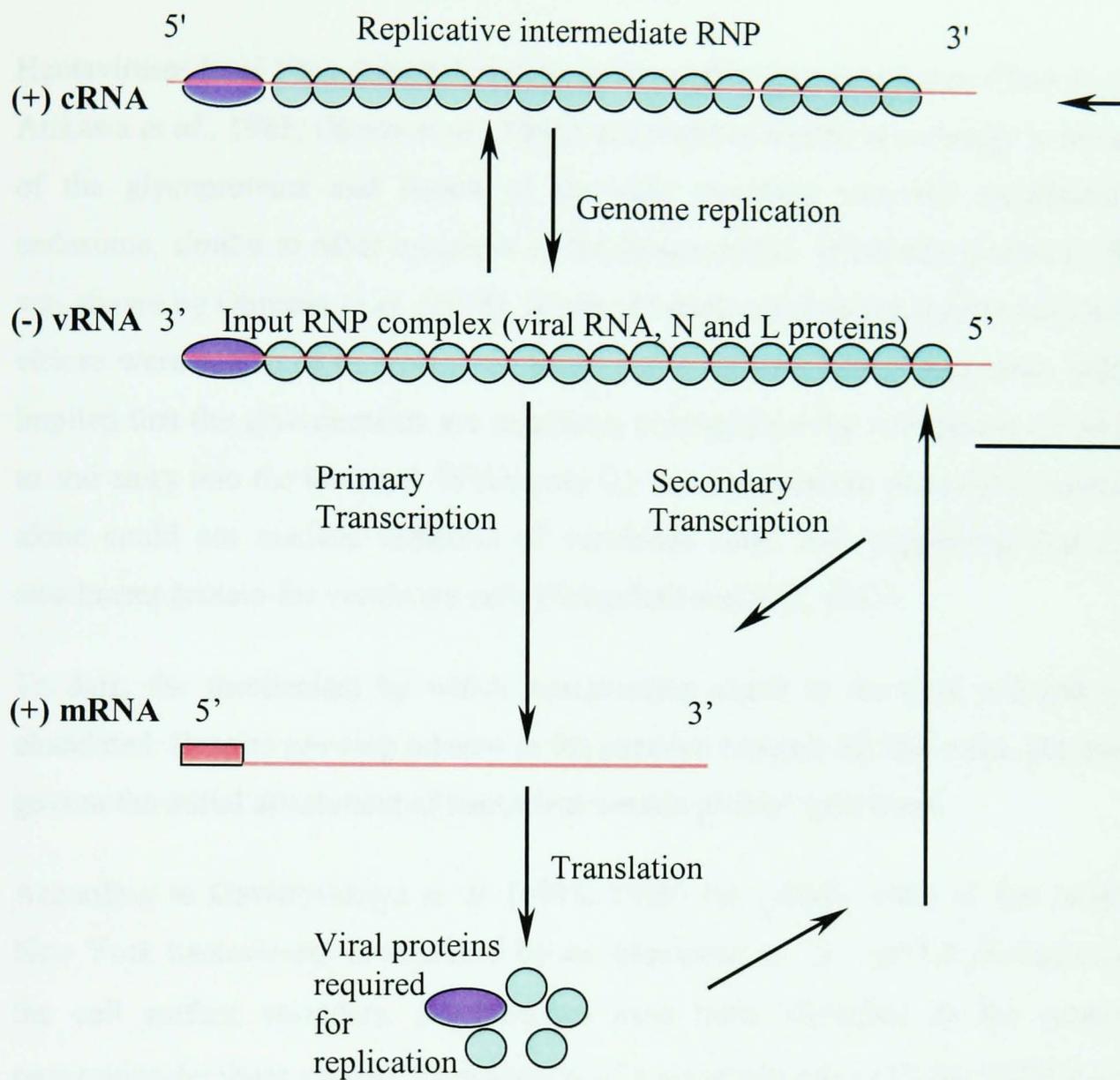


Fig.1.8. Hantavirus replication. The input genome is a ribonucleoprotein (RNP) complex which cannot be translated. RNA dependent RNA polymerase is associated with the input genome and transcribes mRNA molecules as the first step of replication. Translation of primary transcripts produces proteins which together with full-length (+) cRNA form RNP replicative intermediate. This then serves as template for new genome synthesis. The mRNAs are truncated compared to the cRNA and are not encapsidated into RNP. Their 5' ends are capped

### **1.3.3.1. Attachment and entry**

Hantaviruses have been demonstrated to undergo pH-dependent fusion (Tsai *et al.*, 1984; Arikawa *et al.*, 1985; Okuno *et al.*, 1986), presumably related to a change in the structure of the glycoproteins and fusion of the viral envelope with the membrane of the endosome, similar to other members of the *Bunyaviridae* (Gonzalez-Scarano, 1985). As was shown by Obijeski *et al.* (1976), levels of infectivity dropped significantly after LAC virions were subjected to proteolytic treatment to remove their glycoprotein spikes. This implied that the glycoproteins are necessary prerequisites for recognition of, attachment to and entry into the host cell. When only G1 was subjected to proteolytic treatment, G2 alone could not mediate infection of vertebrate cells, thus suggesting that G1 is an attachment protein for vertebrate cells (Kingsford and Hill, 1983).

To date, the mechanism by which hantaviruses attach to the host cell has not been elucidated. Despite growing interest in the putative receptor for this virus, the events that govern the initial attachment of hantavirus remain poorly understood.

According to Gavrilovskaya *et al.* (1998, 1999) the cellular entry of Sin Nombre and New York hantaviruses is mediated by an interaction of G1 and G2 glycoproteins with the cell surface receptors.  $\beta 3$  integrins have been identified as the receptors for recognition by these viruses. Transfection of a non-permissive cell line (CHO) with a  $\beta 3$  integrin expressing plasmid increased infectivity whereas blocking the attachment by the  $\beta 3$  integrin ligand, vitronectin, or antibodies against the receptor reduced infectivity. Although virus infection was not completely inhibited by treatment with antibodies, the percentage of inhibition was within the range described for icosahedral viruses which have been previously shown to enter cells via integrins. No other receptors were found so far to be important for recognition by the other members of the genus, and there is no direct evidence that Hantaan virus, for example, also binds to  $\beta 3$  integrins on the cell surface. It is possible that additional interactions exist between the virus and the host cells. It is currently unclear whether  $\beta 3$  integrin is a virus-binding protein (or site) on cells or whether it is involved in the internalization of virus only.

### **1.3.3.2. Transcription**

The process of mRNA synthesis by hantaviruses is similar to the one described for other members of the *Bunyaviridae* and influenza virus. Upon entry into the cell, hantaviruses begin primary transcription, i.e. synthesis of mRNA which is then translated to generate the viral proteins required for subsequent transcription (Fig.1.8). Initial transcription produces positive-sense mRNA, which is truncated at the 3' termini by approximately 50-110 nt relative to the antigenome RNA and possesses a non-templated capped sequence at the 5' end via an unusual process where capped RNA fragments are cleaved from host cell mRNAs to be used as primers for virus transcription (Patterson and Kolakofsky, 1984; Jin and Elliott, 1993b; Li and Palese, 1994; Pritlove *et al.*, 1998; Elton *et al.*, 1999). As these mechanisms render viral mRNA inexact complements of their templates, they cannot serve in turn as substrates for synthesis of progeny vRNA. Instead, the infecting RNPs are secondarily transcribed via mechanism thought to use unprimed initiation to produce exact complements of the genome segments. The genome is then replicated via an antigenome intermediate prior to secondary transcription taking place, which generates the majority of viral mRNA in the cell (Bishop, 1996). Only encapsidated RNA that forms ribonucleoprotein complexes together with viral N and L proteins can be used as a template for both transcription to generate mRNA and replication. Unlike genome and antigenome RNAs, mRNA is not encapsidated by N protein (Bouloy *et al.*, 1990), and is not polyadenylated as is the case for influenza virus (Lamb and Krug, 1996).

Different kinetics for the hantaviral N, GPC, and L protein mRNA accumulation in Vero E6 cells have been demonstrated by Hutchinson *et al.* (1996). Differential mRNA synthesis was shown in SN virus-infected Vero E6 cells, with the quantity of each mRNA (N mRNA ≥ GPC mRNA ≥ L mRNA) correlating inversely with RNA length. The general pattern of mRNA expression resembles that seen in mosquito cells persistently infected with La Crosse (LAC) virus (Rossier *et al.*, 1988).

### **1.3.3.2.1. Untranslated regions**

The coding regions of each genome segment of hantaviruses do not extend to either terminus. Instead, they are flanked by an untranslated regions (UTR) from both termini. The lengths of the UTRs differ from 20nt to about 730nt, with the 3' UTR always being significantly longer than the corresponding 5' UTR of the genomic-sense RNA.

While the genomic RNAs of hantaviruses show relatively few differences in the length of the L and the M segment, the length of the S segment varies significantly, mainly in its 3' UTR which represents perhaps the most interesting part of the hantavirus genome. Within a certain hantavirus type the length and sometimes even the sequence of this region does not undergo dramatic changes suggesting that it has a functional role. In contrast, between different hantavirus types, the S segment 3' UTR varies widely both in length (from 229nt in PH to 728nt in SN) and in its nucleotide sequence, except for the terminal nucleotides forming the panhandle structures (Antic *et al.*, 1992a, 1992b; Hjelle *et al.*, 1994; Plyusnin *et al.*, 1994a; Spiropoulou *et al.*, 1994; Morzunov *et al.*, 1995; Ravkov *et al.*, 1995).

Most hantaviruses carry within this region motifs that resemble the sequence 3' CCCCACCCAGUCA 5' found at the proposed mRNA termination site in HTN (Dobbs and Kang, 1994) and in the corresponding regions of other bunyaviruses (Dunn *et al.*, 1994; Bowen *et al.*, 1995; Vapalahti *et al.*, 1996). Another motif, 3' GAUGGAGU 5', with a still unclear function, can be found in single or multiple copies in all hantaviruses close to the highly conserved 5' terminus of the S segment (Ravkov *et al.*, 1995).

Also, numerous precise and imprecise repeats with no overall similarity in their structure and pattern may be detected in the 3' UTR. The verified sequences of the 3' termini of all hantavirus S segments contain the second conserved sequence 3' UCGAUGAU5' from positions 21 to 28 and single conserved nucleotides at positions 16 and 18. However, the 3'-terminal sequences of the M segments are less conserved, with conserved nucleotides at positions 15 to 17, 20, 24 to 27, 35, and 38. The hantavirus L segment was found to

possess the conserved sequences at positions 18, 20, 21, 24 to 26, and 33 to 35 (Chizhikov *et al.*, 1995). Supposing that the 3' UTR participates in such steps of viral reproduction as packaging, there could be at least two possible explanations for the above-mentioned differences in its primary structure: first, molecular mechanisms operating at these steps differ from one host to another; second, the secondary rather than the primary structure of the 3' UTR is crucial for its proper activity.

The terminal 14 nucleotides of the S, M, and L genomic segments of each of the members of the different serotypes of the *Hantavirus* genus were found to be highly conserved, containing the sequence 3' AUCAUCAUCUGAGG 5' at the 3' termini and 5' UAGUAGUAU(G)CUCC 3' at the 5' termini. Panhandle structures at least 17 bp long potentially could be formed by complementary regions of the 5' and 3' termini of each segment (Fig.1.9). The complementarity of terminal sequences is also observed in other viruses. For example, with Sendai virus, a paramyxovirus, the terminal complementarity is 12 bases, with human parainfluenza virus type 3 (HPIV3), the extent of terminal complementarity is 17 bases (Hoffmann and Banerjee, 2000), and with vesicular stomatitis virus, the prototypical rhabdovirus, it is only 8 bases (Pattnaik and Li, 1994).

Analysis of some negative-strand RNA viruses has shown that 5'- and 3'-terminal nucleotide sequences, as well as putative panhandle-like structures formed by the 5' and 3' termini of RNA molecules, are involved in the process of initiation and regulation of viral transcription, replication and encapsidation (Pattnaik and Li, 1994; Tiley *et al.*, 1994). For instance, bases 1-12 at the termini of the HPIV3 genome were critical for promoting replication, whereas bases 13-55 of the leader were of moderate importance in promoting replication (Hoffmann and Banerjee, 2000). A similar picture is observed in 3' terminal regions of the vesicular stomatitis virus genome RNA where bases 1 to 12 were demonstrated to be involved in the encapsidation process, whereas bases 13 to 18 were not. In addition, bases 19 to 24 were shown to be involved in replication and virus assembly (Pattnaik and Li, 1994).

Dunn *et al* (1995) developed a reverse genetics system for analysis of the *cis*-acting signals involved in BUN transcription by using a negative-sense chloramphenicol

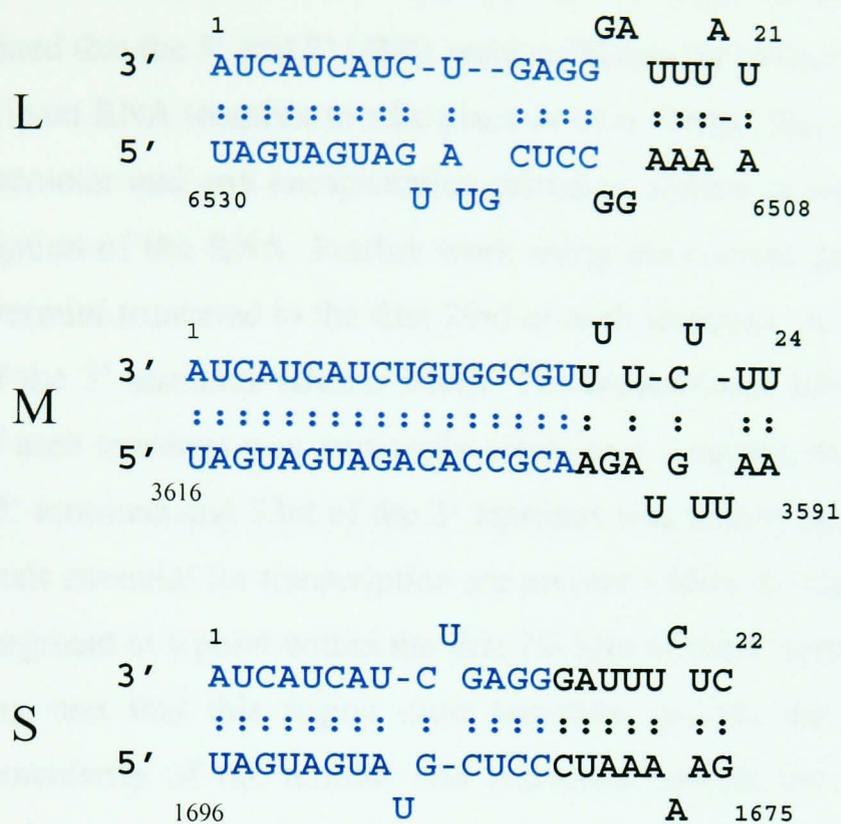


Fig. 1.9. Panhandle-forming 3'- and 5'-terminal sequences of the genomic S, M, and L RNA segments of HTN. The genus-specific terminal sequences are in blue. Numbers indicate nucleotide sequence with respect to the 3' terminus of virus-sense RNA (Schmaljohn, 1996)

acetyltransferase (CAT) RNA flanked by the negative-sense BUN termini. It was determined that the 5' and 3' UTRs were sufficient for transcription and replication of the recombinant RNA template to take place *in vivo*. Hence, they were thought to contain the viral promoter and any encapsidation initiation signals necessary for encapsidation and transcription of the RNA. Further work using the reverse genetics system involved the use of termini truncated to the first 20nt of each terminus, or 32nt of the 5' terminus and 33nt of the 3' terminus (Dunn, 2000). The recombinant RNA containing only the first 20nt of each terminus was minimally active as a template, but the RNA containing 32nt of the 5' terminus and 33nt of the 3' terminus was active. Hence, it was determined that the signals essential for transcription are present within the region of RNA from each end of the segment to a point within the first 20-32nt of the 5' terminus, and 20-33nt of the 3' terminus, and that this region must therefore include the viral promoter. When the complementarity of the termini was increased beyond the first eleven nucleotides to include the first 18nt, activity in the assay was restored. Hence, complementarity would appear to be an important feature in the UTRs for providing transcriptional regulatory signals.

The complementarity of terminal sequences of hantaviruses is not always complete, and a mismatch at position 9 and a noncanonical U-G pair in position 10 have been reported by several groups (Kukkonen *et al.*, 1998; Sun *et al.*, 2001; Piiparinen *et al.*, 1995; Bowen *et al.*, 1995). The exception to this pattern, a single nucleotide difference, was found at position 10 in the S segment 5' termini of Prospect Hill and Bayou viruses, where a G –A change leads to the restoration of complementarity (replacing a U-G pairing). The observation of incomplete complementarity of hantavirus RNA termini is similar to the situation seen in other negative-strand RNA viruses. For instance, it was shown that the mismatch region in the panhandle structure formed by genome segment termini of influenza virus is the virus polymerase binding site, and conversion of the termini to exact complementarity destroys polymerase binding (Tiley *et al.*, 1994).

It is therefore possible to speculate, that the highly conserved bases 1 to 14 found at the 3' termini of hantavirus plus and minus RNA templates may be involved in initiation of encapsidation and /or binding virus RNA polymerase, whereas the nucleotide differences

in positions 20 to 28 between different RNA segments could determine the differential rate of RNA segment transcription or replication. The highly conserved noncomplementary nucleotide pair at position 9 of the hantavirus RNA panhandle structure formed between the 5' and 3' termini of genome RNA may also serve an important role in polymerase binding and regulation of virus RNA transcription and replication (Chizhikov *et al.*, 1995).

#### **1.3.3.2.2. Cap-snatching mechanism for initiation of mRNA synthesis**

Primary transcription of mRNA resembles that described for bunyaviruses and occurs in the cytoplasm by interaction of the virion-associated polymerase and the three genome templates. An endonuclease associated with the polymerase complex cleaves host mRNAs to generate capped fragments which act as primers, and the presence of a methylated 5' cap structure on the host mRNA is required for this cleavage to occur (Bishop *et al.*, 1983; Schmaljohn, 1996; Simons and Pettersson, 1991; Garcin *et al.*, 1995b). The 5' terminal extensions of approximately 10 to 18 nucleotides that are heterogeneous in sequence and are not templated from genome RNA have been also found on the mRNAs of viruses in the *Orthobunyavirus* (Bishop *et al.*, 1983; Bouloy *et al.*, 1990; Eshita *et al.*, 1985; Jin and Elliott, 1993b; Pattersson and Kolakofsky, 1984), *Phlebovirus* (Collett, 1986; Ihara *et al.*, 1985a), *Nairovirus* (Jin and Elliott, 1993b), and *Tospovirus* (Kormelink *et al.*, 1992) genera of the family. This cap-snatching mechanism was first described for influenza virus (Plotch *et al.*, 1981; Krug, 1981; Pattersson and Kolakofsky, 1984; Krug *et al.*, 1989; Kolakofsky and Hacker, 1991), with the important difference that cap snatching occurs in the cytoplasm of *Bunyaviridae*-infected cells, as opposed to the nucleus in influenza virus-infected cells. This is due to the fact that *Bunyaviridae* members replicate exclusively in the cytoplasm, and, therefore, the viral endonuclease uses a pool of mature cellular mRNAs as substrates for primers.

Despite heterologous sequence, the 5' terminal extensions described for different viruses in the family show preferences for specific mono-, di-, and tri-nucleotides at the -1 to -3 positions with respect to the 5' terminus of the mRNA, although no information is

available as to whether these bases are present in the cellular mRNA and specifically chosen for priming, or they are added by the viral polymerase using a slippage mechanism with 3' vRNA termini as a template. For example, it was demonstrated for eight out of eleven NSs mRNAs of Uukuniemi virus which had intact terminal sequence 5'-AC AC--, that all had C at -1, six of these also had A at -2, and five had C again at -3 (Simons and Pettersson, 1991). Similar results were obtained by Jin and Elliott (1993) for Bunyamwera virus S mRNA. In twenty out of twenty one sequenced clones, a U residue was present at position -1 relative to the viral terminus which has the double triplet 5'-AGU AGU--. Ninety percent of the clones had G at position -2 and half had an A residue at -3. These results are in accordance with the findings reported for Germiston (Bouloy *et al.*, 1990) and snowshoe hare viruses (Eshita *et al.*, 1985). Hantaviruses display similar preferences and were shown to initiate mRNA with a G residue at position -1 relative to the viral terminus (Garcin *et al.*, 1995b).

### **1.3.3.2.3. Prime-and-realign model for the initiation of RNA synthesis**

Recent work on hantavirus RNAs (Garcin *et al.*, 1995b) suggests the existence of the so-called prime-and-realign mechanism of chain initiation, in which mRNAs are initiated with a G-terminated host cell primer and genomes with GTP, not at the 3' end of the genome template but internally (opposite the template C at position +3), and after extension by one or a few nucleotides, the nascent sequence repeats, before processive elongation takes place (Fig. 1.10). For genome initiation, an endonuclease, perhaps that involved in cap snatching, is postulated to remove the 5' terminal extensions of the genome, leaving the 5' pU at position +1.

According to observations of Garcin and coworkers (1995b):

- There is a strong preference for the viral endonuclease to cut the host mRNA after a G



- The 3' G of the resulting capped fragment aligns opposite the C at the position +3 of the template
- The primer is elongated for a few nucleotides until G +6 (capped primer –GUAG OH) but elongation by only a single nucleotide is sufficient and perhaps preferable
- Before further elongation proceeds, the nascent chain realigns on the template such that the original 3' G of the primer is at position –1 and the following UAG is opposite positions +1 to +3, thus creating the nearly ubiquitous G at position –1. mRNAs that lack precisely the first UAG repeat could result when the original G of the capped primer first aligns opposite the template C +6 and, after elongation by a few nucleotides, realigns on the template such that this G is opposite position +3 rather than position –1. This is equivalent to placing this G at position –1 and leaving a 3 nt gap (Garcin *et al.*,1995b).

This prime-and-realign mechanism can explain similarity of the sequences at the 3' end of the host primer and the 5' end of the antigenome, as well as the precise deletion of a single trinucleotide repeat in a large fraction of the HTN mRNAs.

The fact that a 5' residue of the Hantaan virus is a monophosphorylated U residue can also be explained by the slippage event by the viral RNA polymerase. It is possible that the 5' pU ends arose by endonuclease action on a longer chain. In contrast to mRNA initiation, the same polymerase-associated endonuclease would cleave the nascent chain in the same way (3' unpaired G), except that cleavage would occur after rather than during initiation, and the requirement for a cap group would be relieved.

Arenaviruses are another family of segmented negative-strand RNA viruses in which a similar situation occurs. For Tacaribe virus (Franze-Fernandez *et al.*, 1987; Garcin and Kolakofsky, 1990, 1992), the genome 3' ends are OH GC GU ..., whereas the 5' ends are pppG CG CA.... When genomes and antigenomes are annealed intermolecularly or their complementary ends are annealed intramolecularly, there is a single-base overhang at each 5' end which can be specifically removed with RNase I in a high salt concentration and which appears to be pppG (Garcin and Kolakofsky, 1990; Raju *et al.*, 1990). Despite

the 3' terminal G of the template, the available evidence suggests that the GTP which started the chain did so opposite the penultimate G (position +2). This pppG is thought to end up as a 5' overhang via a slippage mechanism by being extended to a pppGpC dinucleotide (opposite positions +2 and +3), the pppGpC is proposed to realign upstream on the template by two positions such that its cytidine is opposite the template 3' terminal G, and the 5' pppG overhang is locked in place when the replicase resumes RNA synthesis processively (Garcin and Kolakofsky, 1992).

The feature of the prime-and-realign model, namely, its ability to repair damaged genome ends by restoring small terminal deletions and mutations, is important in maintaining virus infectivity when these ends undergo limited damage. Genomes which lack a few nucleotides at the 3' end can be repaired by simply extending this end on a intact complementary 5' end. However, genomes which lack a few nucleotides at the 5' end would require a different mechanism for repair, as conventional synthesis takes place only in the 5' to 3' direction.

#### **1.3.4. Apoptosis caused by hantaviruses**

Over the past few years, evidence has accumulated to suggest that a growing number of viruses induce cell death by apoptosis, an active and physiologically regulated process of cellular self-destruction (Shen and Shenk, 1995; Razvi and Welsh, 1995). The mechanisms underlying virus-induced cell death are important in understanding of the pathogenesis of viral infection, however, they are still not clear. Cells infected by various viruses may initiate death programs as a part of host defense. Conceivably, the ability of virus-infected cells to develop apoptosis would help hosts to restrict or slow the spread of virus.

Unlike other members of the *Bunyaviridae* family that show acute cytopathic infections in permissive vertebrate cell cultures, hantaviruses routinely establish semipersistent and noncytolytic infections in cultured cells. Although some hantaviruses are highly pathogenic in human hosts, they cause chronic infections in rodents with no apparent

cytopathicity (Lee *et al.*, 1981). *In vitro*, these viruses are fastidious and can be propagated only in certain cell types (French *et al.*, 1981; McCormick *et al.*, 1982). Vero E6 cells, used for growth and production of a cell-free virus in high titer usually remain viable up to 3 weeks of HTN infection with little or no apparent cytopathic consequences (Schmaljohn *et al.*, 1983). Formation of plaques in this cell line, however, suggests a transient and incomplete cytopathicity. Infection by HTN leads to a gradual loss of cell viability and eventually results in cell death.

The experimental data obtained by Kang *et al* (1999) strongly suggests that apoptosis certainly plays a role in the process of cell death by hantavirus infection and that Vero E6 cells infected by HTN undergo apoptosis through a general pathway leading to the degradation of chromosomal DNA. However, one noticeable difference in the HTN-induced apoptosis from the cases reported for other cytolytic viruses was the relatively slow progression of the apoptotic process: less than 30% of the cells died at 7 days postinfection with appearance of fragmented nuclear DNA. Infection by virus of higher titer did not affect the temporal pattern of apoptotic cell death. Adsorption of UV-inactivated virus did not induce apoptosis. The relative resistance of Vero E6 cells against apoptosis early in HTN infection suggested that cellular endonuclease activities required for apoptotic DNA cleavage were perhaps suppressed early in HTN infection and that a full-scale viral replication was permitted before activation of these enzymes.

At present, it is not clear how exactly HTN infection induces apoptosis. It has been suggested, as in poliovirus infection, that the cell death pathway may be activated by shutting off host protein synthesis (Tolskaya *et al.*, 1995). According to this hypothesis, virus-infected cells apparently require ongoing synthesis of protective cellular proteins to avoid activation of the death pathway. One protein, the most frequently proposed for such a protective role, is the 26-kDa membrane-associated proto-oncogenic protein Bcl-2, known to prevent apoptosis induced by multiple agents in a variety of cells. One mechanism responsible for HTN-induced apoptosis could be the suppression of intracellular Bcl-2 protein which was shown to be down-regulated in HTN-infected cells (Zhong *et al.*, 1993).

In addition, interesting results came from yeast two-hybrid screening with a HeLa cDNA library (Li *et al.*, 2002). They demonstrated that PUU N protein interacts with the protein Daxx, well known as a Fas death-domain adaptor protein, which transduces death signals through the Jun N-terminal kinase (JNK) pathway. It is possible therefore, that hantaviruses may interfere with the apoptotic pathway at the post-translational level and could use Daxx as a mediator. It was demonstrated that when HeLa cells were cotransfected with both Daxx and N protein, they co-localized in nucleus. Recently, Ravkov and Compans (2001) showed that PUU N could be found at perinuclear membranes in infected Vero E6. It is possible to speculate that the interaction of PUU N with Daxx may be transient and takes place either in the cytoplasm or in the nuclei prior to nucleocapsid assembly and/or under stimulation of stress factors like apoptotic stimuli, Fas ligand or TNF-alpha.

## **1.4. Reverse genetics systems for negative-sense RNA viruses**

### **1.4.1. Introduction**

Viruses with completely or predominantly negative-sense RNA genomes span seven viral families: the nonsegmented *Bornaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, and *Filoviridae*, and the segmented *Orthomyxoviridae* (6-8 segments), *Bunyaviridae* (3 segments), and *Arenaviridae* (2 segments). These virus families include a number of human and animal pathogens such as influenza A, B, and C viruses, Lassa virus, rabies virus, Ebola virus, Marburg virus, measles virus, canine distemper virus, respiratory syncytial virus, mumps virus, human parainfluenza virus types 1-4, and methods for engineering attenuated variants or recombinant viruses for vaccine purposes were needed.

In addition, many of these viruses serve as important models for basic research on all aspects of viral replication. Thus the recovery of complete negative-stranded RNA viruses from cloned cDNAs was among the most important breakthroughs in RNA virology in the 1990s since it became the basis to directed engineering of the viral genomes and a detailed understanding of the function of the viral genes and their products.

Reverse genetics, a technique used to engineer specific mutations into viral genomes, was first performed for DNA viruses, either by transfecting cells with plasmids encoding the viral genome or by heterologous recombination of plasmids bearing viral sequences with the virus genome (Panicali and Paoletti, 1982; Mackett *et al.*, 1982). They were followed by manipulations of positive-strand RNA genomes. Transfection of plasmids, or RNA transcribed from plasmids, containing the poliovirus genome, into susceptible cells led to recovery of infectious poliovirus (Racaniello and Baltimore, 1981; Kaplan *et al.*, 1985). However, the genomes of negative-strand RNA viruses were less amenable to artificial manipulations in comparison with the DNA and positive-strand RNA viruses.

In contrast to positive-strand RNA viruses, the genome of which is also a functional mRNA, the naked genomic RNA of a negative-strand RNA virus is not able to initiate infection when expressed in or transfected into a permissive cell line. Their genomes are the complement of mRNA and therefore cannot be directly translated to give viral proteins without first being copied into complementary mRNA. The minimal infectious particle of this type of virus is the transcriptionally active ribonucleoprotein (RNP) complex. This complex is composed of the genomic viral RNA (vRNA) complexed with the viral nucleoprotein and the RNA-dependent RNA polymerase protein. The viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome RNA template due to the fact that animal cells do not possess such an enzyme, and this function must be supplied preformed in the input virion. Hence, the deproteinized genomic RNA of negative-strand RNA viruses cannot initiate infection. Moreover, the mRNA is different to the positive complementary RNA intermediate which is used for replication in that it is 3' truncated (Fig.1.11). As a result, the mRNA

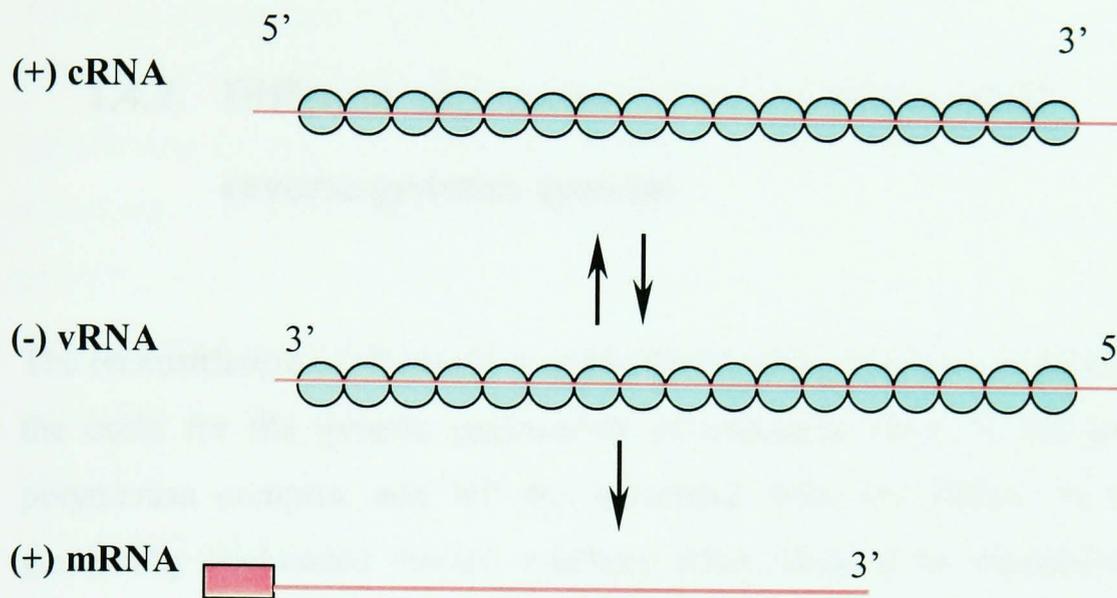


Fig.1.11. Infected cell RNAs of segmented negative strand RNA viruses. Both vRNA and cRNA are exactly complementary and encapsidated by N protein. mRNA contains 5' sequences derived from host cell capped mRNAs as a result of cap-snatching mechanism. mRNA is shorter than vRNA and cRNA as it is 3'truncated.

does not contain all the viral specific information required for production of new genomes and can not serve as template for transcription.

### **1.4.2. Different approaches to the development of reverse genetics systems**

The reconstitution of ribonucleocapsid (RNPs) using synthetic or purified vRNA became the basis for the genetic engineering of influenza virus. In the isolated RNPs, the polymerase complex and NP are associated with the vRNA. In order to generate genetically engineered viruses, synthetic RNA needs to be assembled into replication-competent RNPs. *In vitro* reconstitution of RNPs resulted in the transcription of a synthetic RNA template and, more important, of full-length vRNAs purified from virions (Plotch *et al.*, 1981; Parvin *et al.*, 1989; Honda *et al.*, 1990). Honda *et al.* (1987) isolated RNPs, which catalyzed the synthesis of short RNA transcripts, from detergent-treated virions by glycerol gradient centrifugation. Furthermore, by differential centrifugation of isolated RNP cores through a discontinuous CsCl-glycerol gradient, they separated the RNA polymerase-RNA fraction from NP. The RNA polymerase-RNA complex could initiate RNA synthesis, but it was unable to synthesize full-length RNA unless NP was added to the reaction, indicating that the polymerase complex is sufficient to initiate RNA synthesis, whereas NP is needed for elongation of the RNA transcript. Incubation of influenza virus naked RNA, either transcribed from plasmids *in vitro* or purified by phenol extraction, with free NP/polymerase fractions, resulted in the formation of transcriptionally active complexes. The reconstituted RNP resembled native RNP in *in vitro* transcription reactions with regard to temperature and salt optima and kinetics of label incorporation (Honda *et al.*, 1987).

Reconstitution *in vitro* of RNPs containing a single negative-sense RNA segment (derived by *in vitro* transcription from a DNA clone), the nucleoprotein, and the three polymerase subunits resulted in recovery of the first recombinant influenza virus

incorporating an engineered RNA segment. Luytjez and coworkers (1989) devised the first system to modify influenza viruses by constructing a plasmid that contained the coding region for chloramphenicol acetyltransferase (CAT) in place of the NS gene in the antisense orientation flanked by the 3' and 5' noncoding regions of the vRNA of the influenza virus segment eight (Fig.1.12). This cassette was flanked by a T7 RNA polymerase promoter and a restriction enzyme site that allowed the production of *in vitro* transcripts containing an exact virus-like 3' end. *In vitro* transcription from the T7 promoter resulted in a RNA molecule containing the terminal non-coding sequences, matching those found in influenza virus segment eight, flanking an antisense copy of the CAT gene. This RNA could not be translated to give active CAT protein unless it was first itself used as a template to make positive strand mRNA. Since eukaryotic cells do not possess either RNA-dependent RNA polymerase or CAT activities, the new approach was a sensitive reporter system. RNA transcripts were mixed with purified NP and polymerase proteins to allow the formation of RNP complexes. Prior to or after their transfection with RNPs, the cells were infected with helper influenza virus to provide the viral proteins required for RNA amplification (Fig.1.13). CAT activity in lysates derived from transfected and infected cells indicated the transcription of vRNA-like CAT transcripts to give message-sense RNAs. The recombinant CAT RNA was not only transcribed and replicated, but also packaged into progeny virus particles as demonstrated by the ability of the media from transfection experiments to induce CAT activity in cells after serial passage even after RNase A treatment. These experiments demonstrated that the noncoding regions on influenza virus RNAs contain all the signals required for transcription, replication, and packaging of CAT-vRNA.

This technique was subsequently refined to create influenza virus containing neuraminidase (NA) proteins derived from plasmid cDNAs (Enami *et al.*, 1990). It relied on reconstitution of viral RNPs from *in vitro*-transcribed RNA and purified nucleocapsid proteins. The protein-RNA complex was transfected into cells, followed by infection with a helper influenza virus with a strong counter-selectable phenotype which was well characterized. Influenza virus A/WSN-HK is a reassortant containing seven segments from influenza A/WSN/33 and the neuraminidase gene segment from influenza virus A/HK/8/86. This reassortant virus can only form plaques in MDBK cells when the cell

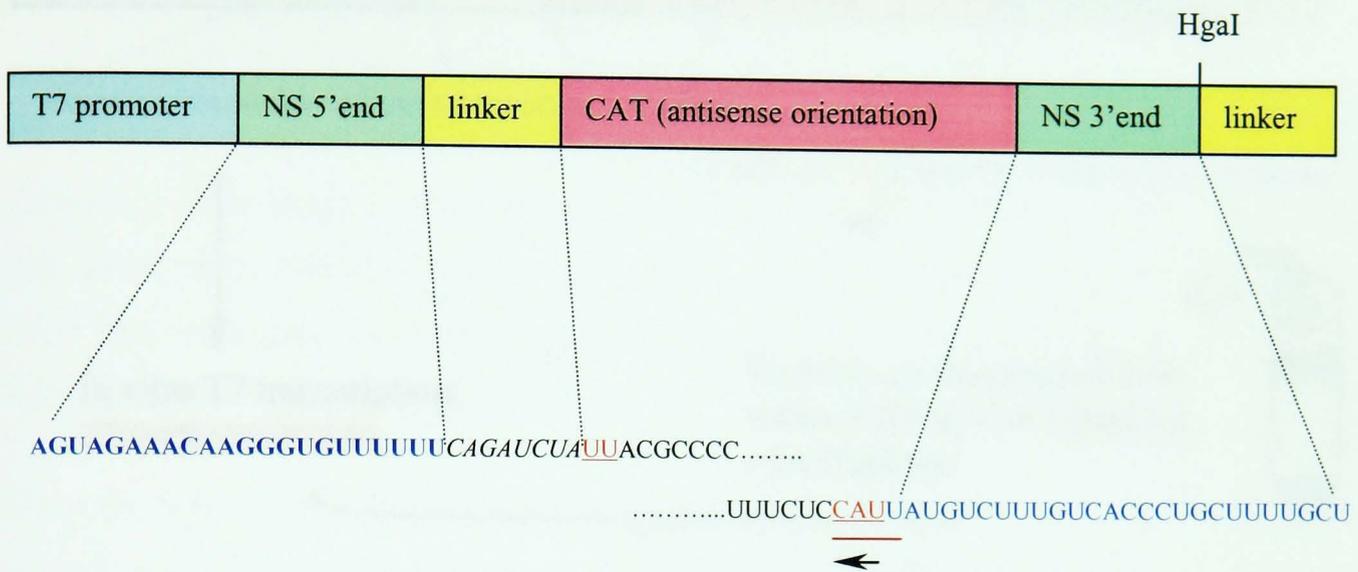


Fig. 1.12. Transcription cassette for the generation of transfectant influenza virus. The coding sequence for CAT (in the antisense orientation) is flanked by short linker sequences (introduced for cloning purposes), the 5' and 3' ends of vRNA, and a restriction site to generate vRNA-like 3' ends. In vitro transcription by T7 polymerase results in a synthetic vRNA that can be packaged into infectious influenza virus. vRNA sequences are shown in blue. The start and stop codons for CAT are doubly and singly underlined, respectively. The arrow indicated the orientation of the CAT reading frame. The linker sequence is shown in italics (Luytjes *et al.*, 1989)

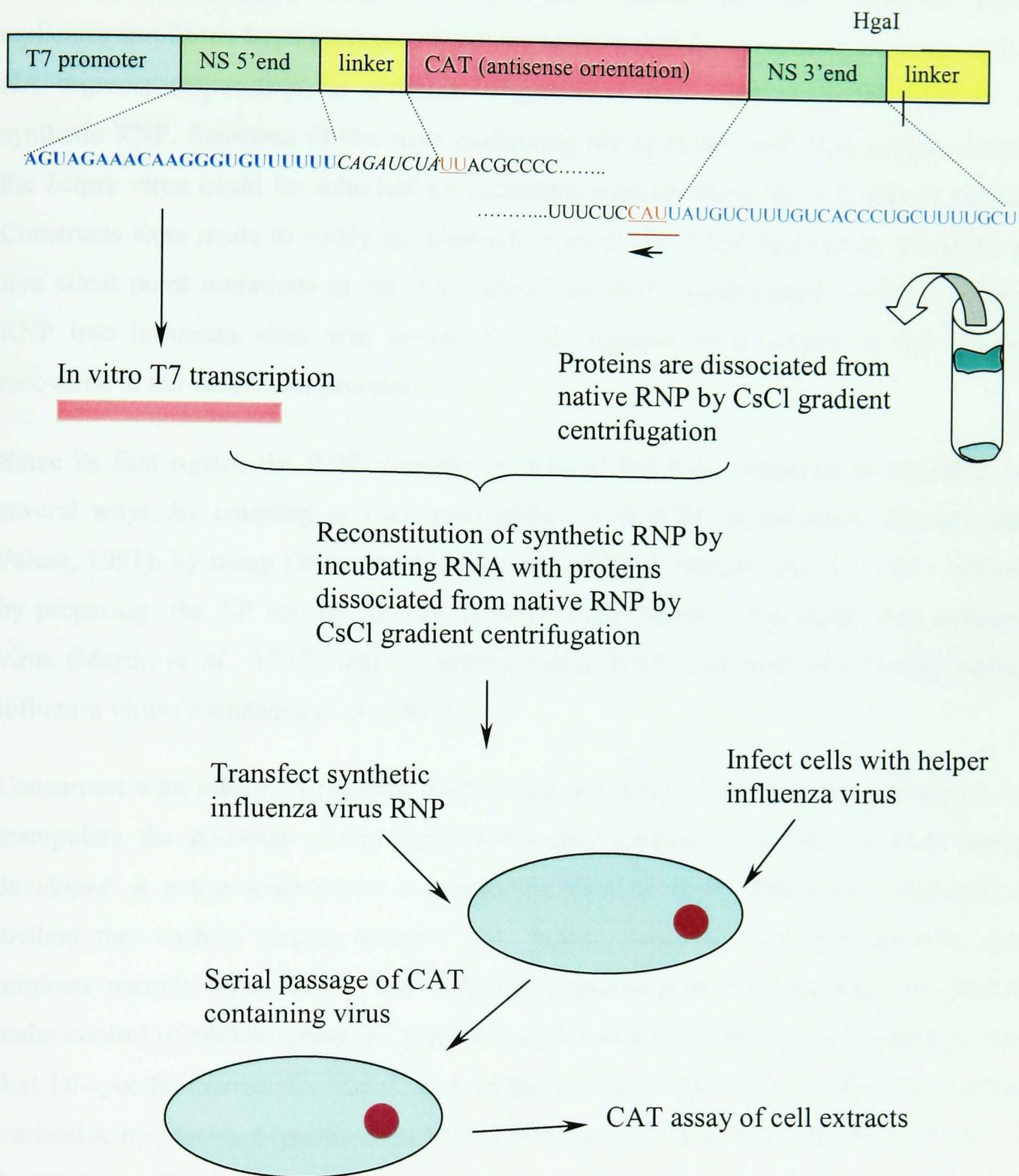


Fig. 1.13. RNP transfection method for the rescue of transfectant influenza virus. Purified NP and polymerase proteins are assembled with *in vitro* synthesized vRNA to form RNP complexes. Following RNP transfection and helper virus infection, the transfectant virus, containing RNA derived from cloned cDNA is selected. The recombinant CAT RNA is not only transcribed and replicated, but also packaged into progeny virus particle as demonstrated by the ability of the media from transfection experiments to induce CAT activity in cells after serial passage (Luytjes *et al.*, 1989).

culture media is supplemented with a protease, whereas the parent WSN/33 strain replicates and forms large plaques without the requirement for exogenous protease. WSN NA segment responsible for protease-independence was supplied in the form of a synthetic RNP. Selection of the virus containing the synthetic WSN/NA segment from the helper virus could be achieved by excluding protease from the cell culture media. Constructs were made to verify the plasmid origin of the WSN segment by introducing five silent point mutations in the NA coding sequence. Incorporation of the synthetic RNP into influenza virus was proved by the presence of mutations in the viruses recovered in the absence of protease.

Since its first report, the RNP transfection method has been improved or modified in several ways: by coupling *in vitro* transcription with RNP reconstitution (Enami and Palese, 1991), by using electroporation instead of DEAE transfection (Li *et al.*, 1995a), by preparing the NP and polymerase proteins from infected cells rather than purified virus (Martin *et al.*, 1992), and by adding native RNP cores instead of using helper influenza virus (Yamanaka *et al.*, 1991).

Concurrent with efforts to perform reverse genetics with influenza virus, techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses were being developed. A major achievement was made by Pattnaik *et al.* (1992) who employed a method that enables rescue, without VSV helper virus, of virus like particles that originate entirely from cDNA. The cDNA corresponding to a DI genome was placed under control of bacteriophage T7 promoter such that transcription would initiate on the first DI-specific nucleotide. The 3' end of the T7 transcript was cleaved at the last DI nucleotide by placing hepatitis delta virus (HDV) antigenomic ribozyme downstream of the DI RNA. The ribozyme was positioned such that autocatalytic cleavage released DI genome RNA with the exact termini required. Plasmid derived RNA was successfully produced *in vivo* by transfecting this construct into cells previously infected with a recombinant vaccinia virus vTF7-3 as a source of T7 polymerase (Fuerst *et al.*, 1986). Co-transfection of further T7 constructs containing VSV genes enabled efficient encapsidation and replication of the DI RNA. In the presence of all VSV genes the DI

RNA could be packaged into VSV DI particles which budded from the cells (Pattnaik *et al.*, 1992).

Similar approach was later used by Schnell and coworkers (Schnell *et al.*, 1994) who successfully recovered recombinant rabies virus. These authors cotransfected vaccinia virus-infected cells with plasmids, encoding the viral nucleocapsid protein (N) and the polymerase proteins (L and P) under the control of T7 promoters, together with a plasmid encoding a full-length antigenomic viral RNA under the control of T7 promoter at the 5' end and a self-cleaving ribozyme at the 3' end. After transcription of RNAs from the T7 promoters and translation of the encoded proteins, nucleocapsid proteins assemble around the antigenomic RNAs, and polymerase proteins then replicate these RNPs to form RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and translation, infectious virus is assembled.

This reverse genetics technique was adapted by laboratories studying other nonsegmented negative-strand RNA viruses, resulting in the rescue of vesicular stomatitis virus (Lawson *et al.*, 1995; Whelan *et al.*, 1995), measles virus (Radecke *et al.*, 1995), respiratory syncytial virus (Collins *et al.*, 1995), Sendai virus (Garcin *et al.*, 1995a; Kato *et al.*, 1996), human parainfluenza virus 3 (Hoffman and Banerjee, 1997; Durbin *et al.*, 1997a), and simian virus 5 (He *et al.*, 1997). Some modifications to the original technique have been made, such as the use of stably transfected cell lines expressing the T7 RNA polymerase protein, or one or more of the viral proteins required for genome replication (Radecke *et al.*, 1995). For example, Kato *et al.* (1996) provided the second report of recovery of Sendai virus from cDNAs. In this report, virus was recovered from both negative and positive sense RNAs transcribed *in vivo* in the vaccinia T7 system, or transfected into the system after being transcribed *in vitro*. The efficiency of recoveries from antigenomic RNA constructs was much higher than reported by Garcin and coworkers (Garcin *et al.*, 1995a). These results were achieved by: i) truncation of the T7 promoter (by removing the guanosine triplet) thus providing a precise 5' end to the viral RNA transcripts; ii) optimization of the NP, P, and L plasmid ratios, and iii) inhibition of vaccinia cytopathic effect by incubating in the presence of both ara C and rifampicin (Kato *et al.*, 1996).

In most of the methods, the positive sense antigenome RNA was used as opposed to the negative sense genome RNA. This is critical because of an antisense problem. If the negative sense genome is used instead, mRNAs encoding viral proteins can hybridize to the naked genomic RNA and prevent the critical assembly of the genome into the RNP, the template for transcription and replication (Roberts and Rose, 1998). As was already mentioned, the negative-strand viruses always keep their genome in RNP form, probably in part to avoid this antisense problem. When one starts with the positive-strand antigenome, this RNA can form RNP without any interference from the mRNAs. Once in RNP form, the positive strand can then be replicated to form full-length minus strand RNPs that are wrapped into RNPs as nascent RNA chains and thus immune to interference from mRNAs.

Using a reverse genetics approach, bunyavirus promoter elements and the viral proteins that are required for transcription and replication were studied. Dunn *et al* (1995) cloned the CAT gene in the negative-sense orientation between the 5' and 3' nontranslated regions of the Bunyamwera bunyavirus S RNA segment. As with influenza virus, the terminal sequences of bunyavirus RNAs are complementary and highly conserved. It has therefore been assumed that these sequence elements define the bunyavirus promoter and are crucial for promoter activity. Cells were transfected with constructs expressing the proteins encoded by the L and S segments followed by transfection with *in vitro* transcribed RNA, which resulted in CAT activity. The bunyavirus S segment encodes two proteins, N and NSs, in overlapping reading frames. To determine whether both of these proteins are required for transcription and replication, constructs expressing only N or NSs were tested. N protein expression, together with L protein expression, resulted in CAT activity from the reporter RNA, whereas no CAT activity was detected with the L and NSs expression constructs. Thus, it was concluded that the L and N proteins are sufficient for transcription and replication of a bunyavirus-like RNA. In addition, it was shown that deletion of five nucleotides at the 3' end of the viral RNA drastically reduced CAT expression. In contrast, addition of two nucleotides at the 5' end, or of 11 or 35 nucleotides at the 3' end, did not abolish CAT expression. Therefore, like the influenza virus polymerase complex, the bunyavirus polymerase protein can apparently start transcription and /or replication internally (Dunn *et al.*, 1995).

A year later, the first recovery of the Bunyamwera virus entirely from cloned DNAs was reported by Bridgen and Elliott (1996). In contrast to the influenza system, which required helper virus, a helper-free system was employed. Each antigenomic RNA construct was expressed from a T7 promoter and had the self-cleaving hepatitis delta virus ribozyme at the 3' end. Each antigenome transcript contained two extra nonviral guanosine residues at the 5' end (Fig.1.14). Three plasmids were transfected expressing the three antigenomic viral segments (L, M, and S) along with three T7-plasmids expressing the viral mRNAs encoding all the viral proteins (N, NSs, G1, G2, NSm, and L) into HeLa T4 cells infected with the recombinant vaccinia virus expressing T7 polymerase. To increase the number of bunyavirus particles relative to the number of vaccinia virus particles, the authors took advantage of the ability of Bunyamwera virus to replicate in mosquito cells and introduced a passage step through *Aedes albopictus* C6/36 cells. Therefore, extracts of the cells harvested after transfection were used to infect C6/36 cells, and after 1 week, supernatants from these cells were assayed for the presence of Bunyamwera virus by plaque formation on BHK cells. The rescue efficiency was about 10-100 plaques per  $10^7$  cells in the original transfection, and the transfectant viruses grew with the same kinetics and to the same titre as authentic Bunyamwera virus (Bridgen and Elliott,1996).

A similar approach to the one used by Dunn *et al* (1995) was subsequently utilized for Rift Valley fever phlebovirus which has an ambisense S RNA segment: the N and NSs proteins are encoded in distinct ORFs, with the NSs ORF being in the vRNA sense. Both proteins are translated from specific subgenomic mRNAs. In the reverse genetics system developed for RVF virus (Lopez *et al.*, 1995; Prehaud *et al.*, 1997), the antisense CAT reporter cDNA was also expressed using the T7-vaccinia virus system, while the L and N proteins were supplied from vaccinia virus recombinants.

Development of a new approach for reverse genetics of influenza virus was reported by Neumann and colleagues (Neumann and Hobom, 1995; Neumann *et al.*, 1994; Zobel *et al.*, 1993) who pioneered the use of an RNA polymerase I (pol I)-based system. This approach eliminated the need for protein purification, *in vitro* transcription, and *in vitro* RNP reconstitution by taking advantage of the characteristics of RNA polymerase I

.....GAATTCTAATACGACTCACTATAGGCCTGGTACCCGGGTCGGGCAT

T7 promoter      *StuI*      *SmaI*      Hep  $\delta$

Transcription

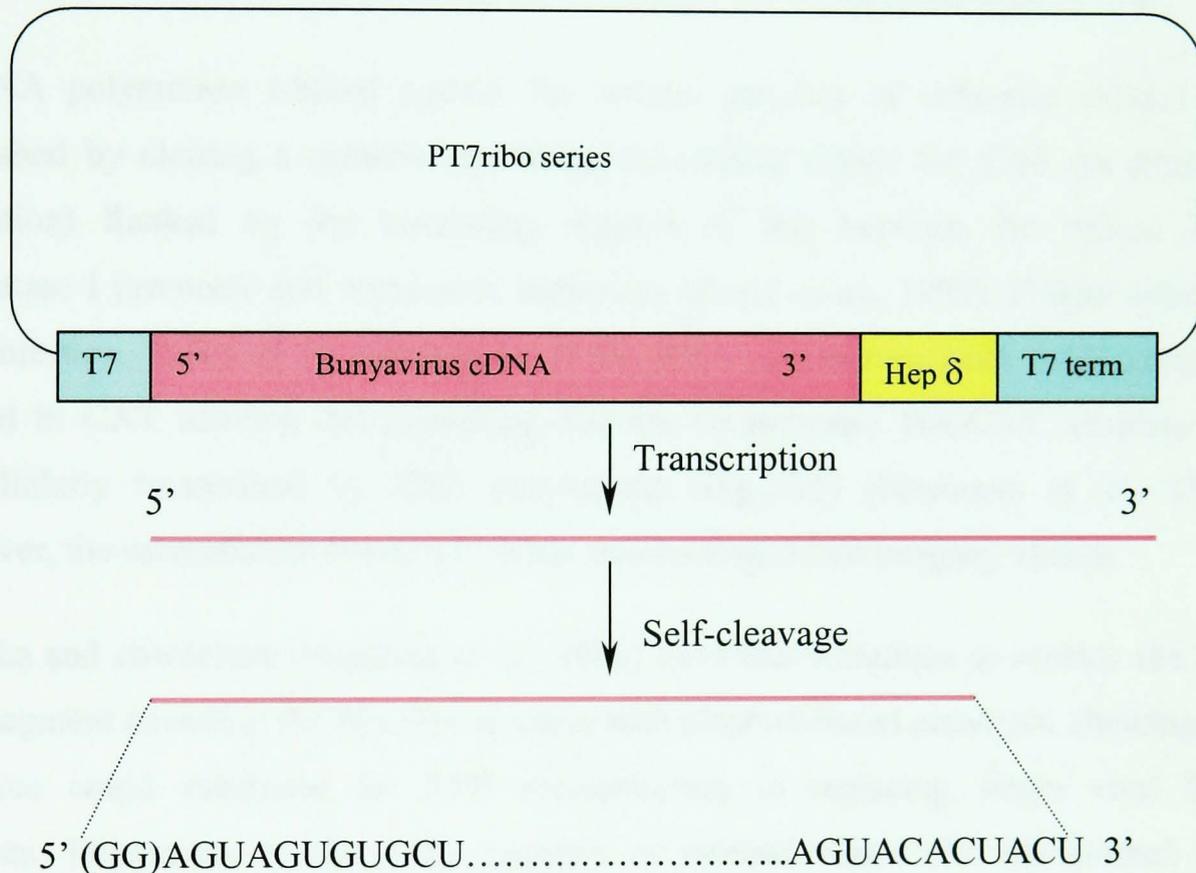


Fig.1.14. Plasmid map of the pT7ribo series. The upper part of the figure shows the sequence around the *StuI* and *SmaI* restriction sites that were used to insert blunt-ended DNA fragments. RNA transcripts produced by bacteriophage T7 RNA polymerase would contain two G residues, derived from the cloning site, before the authentic bunyavirus 5' terminal sequence. The exact 3' end of the RNA is specified by self-cleavage of the nascent RNA by the hepatitis delta virus (Hep  $\delta$ ) antigenome ribozyme. The conserved 11 terminal bases of all three Bunyamwera virus genome segments are shown. T7, T7 promoter; T7term, T7 transcription termination sequence (Bridgen and Elliott, 1996).

transcription. Unlike the mRNA transcripts produced by polymerase II (pol II), RNA polymerase I catalyzes the synthesis of ribosomal RNA (rRNA), which lacks 5' cap and 3' poly(A) structures. Therefore, artificial influenza virus RNA segments with precise 5' and 3' ends could be produced using pol-I driven transcription (Zobel *et al.*, 1993). RNA polymerase I localizes to the nucleus, where influenza virus transcription and replication occur. Hence RNA polymerase I transcription systems can be used to generate vRNA-like transcripts intracellularly (Neumann and Hobom, 1995; Neumann *et al.*, 1994).

An RNA polymerase I-based system for reverse genetics of influenza viruses was established by cloning a cassette containing the coding region for CAT (in antisense orientation) flanked by the noncoding regions of HA between the mouse RNA polymerase I promoter and terminator sequences (Zobel *et al.*, 1993). Helper influenza virus infection, followed by transfection of the RNA polymerase I-HA-CAT construct, resulted in CAT activity, demonstrating that the recombinant HA-CAT template was intracellularly transcribed by RNA polymerase (Fig.1.15) (Neumann *et al.*, 1994). Moreover, the recombinant HA-CAT vRNA was packaged into progeny virions.

Pleschka and coworkers (Pleschka *et al.*, 1996) used this technique to replace the viral RNA segment encoding the NA glycoprotein with plasmid-based construct, showing that technique could substitute for RNP reconstitution in replacing single viral RNA segments. Expression of the RNA segment of interest was under the control of a truncated pol I promoter at the 5' end and pol I terminator at the 3' end. The RNP protein components (PB1, PB2, PA and NP), shown previously to be the minimal proteins required to reconstitute influenza virus polymerase activity (Huang *et al.*, 1990), were encoded on plasmids under the control of pol I promoters. These plasmids were transfected into cells and the RNPs assembled intracellularly.

The system described by Neumann and coworkers (Neumann *et al.*, 1999) represents the conclusion of this work which resulted in the ability to manipulate every gene in the influenza virus genome. They have developed a system that utilizes the host cell for making the equivalent of newly released RNPs by cotransfecting eight plasmids encoding each of the influenza virus genomic RNA segments under control of the RNA

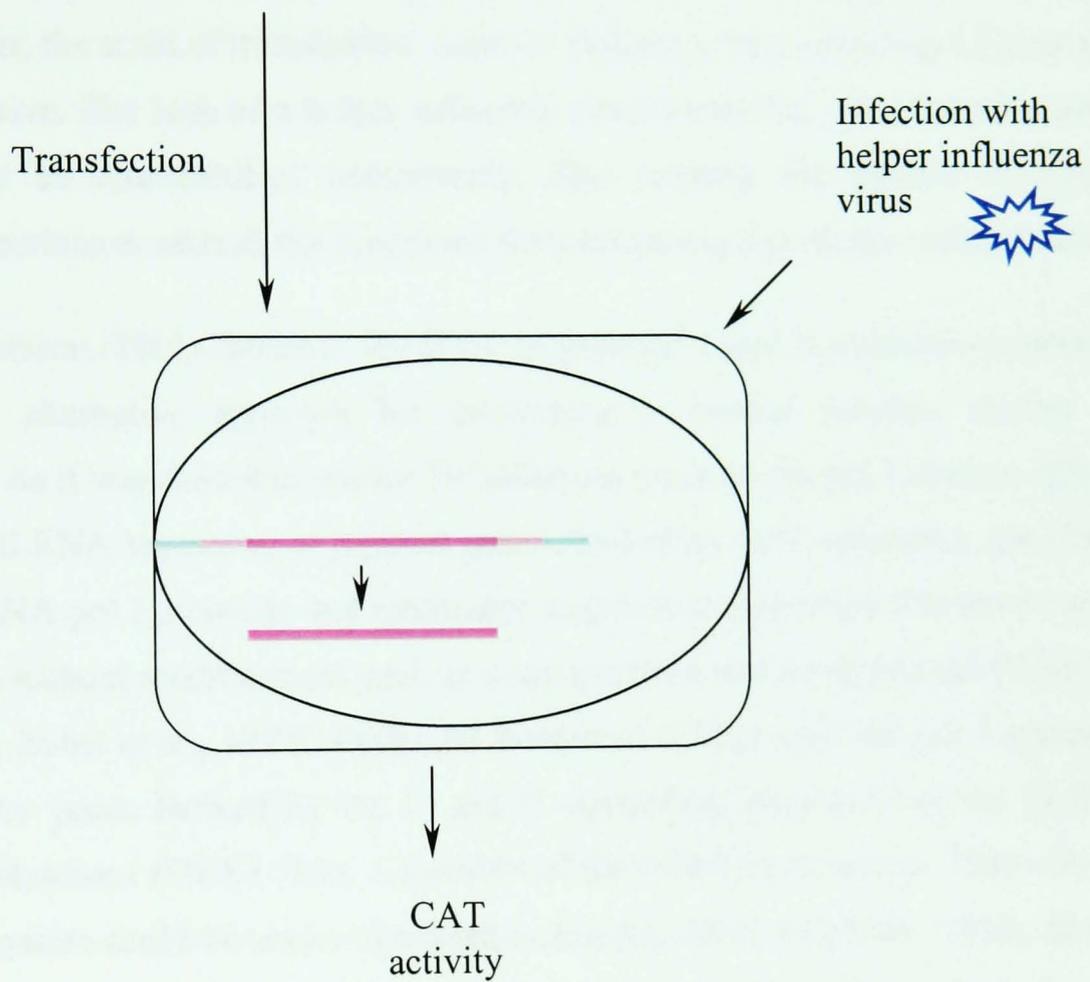


Fig.1.15. RNA polymerase I reverse genetics system. A plasmid containing the coding region for CAT (in antisense orientation) flanked by the noncoding regions of HA between the mouse RNA polymerase I promoter and terminator sequences is transfected into eukaryotic cells. The cells are also infected with helper influenza virus. Intercellular transcription by RNA polymerase I results in expression of CAT gene and CAT activity can be measured (Neumann *et al.*, 1994). Pol I P, polymerase I promoter; Pol I term, polymerase I terminator

pol I promoter and transcription terminator along with four plasmids encoding the polymerase complex proteins and nucleoprotein (NP) cDNAs under control of an RNA pol II promoter. The concept of cotransfecting multiple plasmids to reconstitute a biochemical activity was first used for studying herpes virus DNA replication (Challberg, 1986). However, the scale of transfection used for influenza virus, utilizing 12 plasmids, is very impressive. The lack of a helper influenza virus allows the virus from the initial transfection to be characterized immediately, thus limiting the chance of viruses containing reversions or second-site mutations from becoming significant contaminants.

Flick and Pettersson (2001) turned to the RNA polymerase I (pol I) expression system to use it as an alternative approach for developing a reverse genetics system for *Bunyaviridae*. As it was described earlier for influenza virus, in the pol I system, cDNAs coding for viral RNA segments, or reporter genes flanked by viral sequences, are cloned between the RNA pol I promoter and terminator to generate transcripts that have correct 5' and 3' ends without modifications such as a cap structure and a poly(A) tail (Flick and Hobom, 1999; Zobel *et al.*, 1993). Flick and Pettersson (2001) used the pol I system to express reporter genes flanked by the 5' and 3' noncoding sequences of the M RNA segment of Uukuniemi (UUK) virus, a member of the *Phlebovirus* genus. They showed that the pol I system could be used to synthesize chimeric RNA templates, which, despite lacking a cap structure and poly(A) tail, are transported to the cytoplasm, where they are amplified and transcribed by the UUK virus replicase components supplied either by superinfection with UUK virus or by expression of viral proteins from separate plasmids. The L and N proteins were found to be necessary and sufficient for transcription and replication. One important question is whether the pol I transcript is amplified by replication. As was shown by Flick and Pettersson for UUK virus, although not directly quantifying RNA synthesis, they observed high expression level of CAT and GFP, that could not have been achieved unless replication had occurred. Based on their previous experience, the overall level of CAT activity was much higher than that obtained in the influenza virus up-regulation mutant. Finally, the fact that extracellular medium from transfected and UUK virus-superinfected cells could be used to serially passage CAT activity strongly suggests that the pol I transcript must have been amplified and packaged.

The pol I system offers some advantages over the vaccinia virus-based reverse genetics systems. Vaccinia virus (VV) has been used either to direct the synthesis of the T7 RNA polymerase (Fuerst *et al.*, 1986), which then drives the expression of the reporter construct and the viral helper proteins (Baron and Barrett, 1997; Collins *et al.*, 1995; He *et al.*, 1997; Hoffmann and Banerjee, 1997; Lawson *et al.*, 1995; Lee *et al.*, 2000) or to express the viral helper proteins directly (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Prehaud *et al.*, 1997). VV introduces into the cell a number of unwanted enzymatic activities, which are avoided by using the pol I system. In addition, there is no need to remove the VV, by physical or biochemical means (Lawson *et al.*, 1995; Schnell *et al.*, 1994; Whelan *et al.*, 1995), by passaging the virus through cells not permissive to VV or by using a variant VV (MVA-T7) which does not replicate in mammalian cells (Sutter *et al.*, 1995). However, these problems can be avoided by using cell lines that stably express bacteriophage T7 polymerase. The pol I system also has the advantage of generating the exact 5' and 3' ends of the RNA transcripts, thus avoiding the need for expressing runoff transcripts from restriction enzyme-cleaved plasmids or the use of a ribozyme to produce the correct 3' end. However, unlike influenza virus, transcription of RNA by most negative strand viruses appears in cytoplasm whereas polymerase I produces RNA in nucleus.

### **1.4.3. Application of reverse genetics**

RNP reconstitution *in vivo* and *in vitro* has allowed detailed analysis of the viral promoter and other *cis*-acting signals important for the regulation of transcription and replication (e.g., polyadenylation signals, *cis*-acting signals within the noncoding regions). Reverse genetics has also made it possible to study the functional importance of viral proteins during infection, structure-function relationships of viral gene products and molecular aspects of viral pathogenicity. Transfectant viruses generated by this technique can be used to address issues regarding virus-host cell interactions in transport and assembly processes of viral components.

The recent development of reverse genetics systems for the rhabdoviruses, paramyxoviruses and orthomyxoviruses has provided new tools to investigate in more detail the roles of the *cis*-acting RNA elements involved in replication and transcription. Applying the new approach, Hoffman and Banerjee (2000) demonstrated that nucleotides 1-12 (from the terminus) of the leader promoter formed a domain critical for human parainfluenza virus type 3 replication. In addition, they showed that no mutations in these regions caused defects in transcription, however, mutations in the intergenic sequence and the gene start found at the leader/N gene junction did disrupt transcription.

The functional analysis of the influenza viral RNA promoter via reverse genetics has led to the proposed 'corkscrew' model for the 5' and 3' vRNA terminal sections in their coordinate binding to viral RNA polymerase (Flick *et al.*, 1996; Flick and Hobom, 1999). Although it was believed that the double-stranded panhandle formed by the termini of all influenza virus gene segments might be involved in polymerase recognition, application of this new approach allowed the conclusion that 3' terminal sequences alone could optimally function as a promoter (Parvin *et al.*, 1989). In addition, it was shown that various base-pair exchanges according to that model have not only restored promoter function, but resulted in increased levels of promoter activity, in particular when positions 3 and 8 in the 3' branch or 3 and 8 in the 5' branch of the vRNA promoter structure were involved in such complementary double exchange. Construction of cRNA promoter variants through RNA polymerase I reverse genetics allowed determination of the RNA polymerase-associated, base-paired conformation in a reporter gene read-out system. It turned out to adhere to the 'corkscrew' model, similar, but slightly different in its binding interactions from the corresponding vRNA conformation. The observation of two transcription reactions, initiated in either direction from influenza vRNA and cRNA template molecules, allowed construction of bicistronic, ambisense RNA molecules for simultaneous expression of two proteins from a single segment of viral RNA (Azzeh *et al.*, 2001).

Two other studies used the RNA polymerase I system to determine the function of influenza virus proteins. Neumann *et al* (2000) generated virus-like particles that entirely lacked or possessed mutations in the NS2 gene and examined the effect of these

modifications on vRNP nuclear export. This study confirmed a previous finding by O'Neill *et al.* (1998) that NS2 is critical for vRNP nuclear export, mediated by a nuclear export signal in the N-terminal region of NS2. Watanabe and coworkers (2001) studied the role of the M2 ion-channel protein. Viruses were generated that lacked or contained mutations in the M2 transmembrane domain, indicating that influenza A viruses can undergo multiple cycles of replication without M2 ion-channel activity in cell culture. However, viruses defective in M2 ion-channel activity did not efficiently replicate in mice, demonstrating that this activity is critical for the viral life cycle. Similar approaches can be employed to determine the functions of other influenza virus proteins or cellular events involving specific viral proteins (Watanabe *et al.*, 2001).

Viral attenuation as a result of reverse genetics through specific mutations has practical significance in vaccine development. Such attenuating mutations include those eliminating gene products that are nonessential for replication in tissue culture, those rearranging gene order, and those deleting the cytoplasmic tails of viral glycoproteins (Jin *et al.*, 1996; Kato *et al.*, 1997; Kurotani *et al.*, 1998; Roberts *et al.*, 1998; Wertz *et al.*, 1998). Deletion mutants generally cannot revert, thus permanent attenuation should be possible in such recombinants.

Using reverse genetics approach it is possible, for example, to produce a master strain of influenza virus with multiple attenuating mutations in the genes encoding internal proteins. It can be used to produce a high-yield reassortant virus that possesses the HA and NA from a currently circulating strain. It could be exploited in the production of inactivated vaccines, which currently are generated by conventional genetic reassortment procedures. It can serve as a potentially useful vector for gene transfer into mammalian cells. Studies with helper virus-dependent reverse genetics systems have demonstrated that influenza virus can accommodate additional genetic material. For several short polypeptides, including the V3 loop of HIV-1 gp 120 protein (Li *et al.*, 1993), a highly conserved epitope from the ectodomain of HIV-1 gp41 (Muster *et al.*, 1994), and a B-cell epitope from the outer membrane protein F of *Pseudomonas aeruginosa* (Gilleland *et al.*, 1997), insertion in the antigenic sites of HA resulted in immune responses against the foreign epitope (reviewed in Neumann and Kawaoka, 2001).

The applications of the reverse genetics system can also be well demonstrated using vesicular stomatitis virus (VSV) as an example. It was shown that genes encoding foreign membrane glycoproteins can either be incorporated as extra genes in VSV or can be incorporated in place of the VSV G gene (Schnell *et al.*, 1996, 1997). Therefore, it is possible to obtain viruses containing the foreign proteins in their envelopes by swapping the endogenous VSV G gene for genes encoding foreign glycoproteins. These viruses lack the normally broad tropism conferred by VSV G and can be targeted to specific cells. For example, VSV recombinants expressing the HIV receptor and a coreceptor in place of G incorporate both foreign proteins and are targeted specifically to cells infected with HIV-1 which display the HIV-1 envelope proteins on their surface (Schnell *et al.*, 1997).

Recombinant VSVs expressing foreign antigens have a high potential in vaccine application and have been shown recently to elicit protective immunity in experimental animals. Vaccination of mice with a single dose of recombinant VSV expressing the influenza hemagglutinin provided complete protection from influenza challenge (Roberts *et al.*, 1998). Other examples include expression and incorporation of the influenza A virus neuraminidase (NA) proteins shown by Kretzschmar *et al* (1997), the HIV-1 envelope protein (gp 160) with a VSV-G cytoplasmic tail by Johnson *et al* (1997), the MV fusion (F) and hemagglutinin (HA) proteins by Schnell *et al* (1996), the RSV glycoprotein (G) and fusion (F) protein and the cellular proteins CD4, CXCR4, and CCR5 by Schnell *et al* (1997).

To summarize, reverse genetics approaches have now been described for representatives of most groups of negative-strand RNA viruses and gave an opportunity to study different aspects of viral replication, pathogenesis, interaction with vectors, and to develop genetically engineered vaccines.

## **1.5. Objectives of the project**

The initial aim of the project was to establish a reverse genetics system for hantavirus based on the systems developed for other negative-strand RNA viruses, including that of Bunyamwera virus. This would allow full application of recombinant DNA techniques to manipulate the genome of hantavirus with the particular goal of investigating the initiation of hantavirus antigenome RNA synthesis using expressed viral proteins and a recombinant RNA template.

However, as all the attempts to develop the reverse genetics system for hantavirus failed, the investigation of the interactions of the hantavirus nucleocapsid protein was undertaken by means of the mammalian two-hybrid system. The major objective was to identify whether hantavirus N protein is capable of self-interaction, and if so, which domains on the protein are responsible for interaction. In addition, the possibility of interaction between N and L proteins was examined.

Although hantaviruses have never been shown to possess a nonstructural protein, there were some implications for the potential of an NSs-analogue in certain members of the genus. Therefore, as the last objective, it seemed interesting to determine whether the S segment of Puumala virus encoded a second protein, ORF2 product.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1. Materials**

#### **2.1.1. Enzymes**

Restriction endonuclease enzymes, T4 polynucleotide kinase and T4 DNA polymerase were purchased from New England Biolabs; T7 RNA polymerase and recombinant ribonuclease inhibitor rRNasin were purchased from Promega; calf intestinal phosphatase from Boehringer Mannheim; ribonuclease A from Sigma; *Taq* DNA polymerase was purchased from Qiagen and *Pfu* Turbo polymerase from Stratagene; T4 DNA ligase was purchased from Gibco BRL.

All enzymatic reactions were carried out according to the manufacturer's instructions.

#### **2.1.2. Radiochemicals**

Radiochemicals were supplied by Amersham at the following specific activities:

<sup>35</sup> S-L-methionine	800 Ci/mmol (15 μCi/μl)
<sup>14</sup> C chloramphenicol	58.5 mCi/mmol (0.1 μCi/μl)

### 2.1.3. Synthetic oligonucleotides

Synthetic oligonucleotides were purchased from MWG Biotech.

The sequences of generated oligonucleotides (with restriction sites contained in the oligonucleotides underlined) are as follows:

PuuL <i>Sma</i> I 43+	5'-GGCCCCCGGGGAGAAATACAGAGAGATC ( <i>Sma</i> I)
PuuL <i>Sma</i> I 1200+	5'-GGCCCCCGGGGATATCCAAAGATTCAAAGAA ( <i>Sma</i> I)
PuuL <i>Sma</i> I 2400+	5'-GGCCCCCGGGGTGGTTGAACTTGCTGCTCAA ( <i>Sma</i> I)
PuuL <i>Sma</i> I 3600+	5'-GGCCCCCGGGGTATTAGGTTCTTTATCAGA ( <i>Sma</i> I)
PuuL <i>Sma</i> I 4800+	5'-GGCCCCCGGGGTGGCTAAAGACAATGTCCCT ( <i>Sma</i> I)
PuuL <i>Sma</i> I 5800+	5'-GGCCCCCGGGGATTATTTCATGTTTAACGA ( <i>Sma</i> I)
PuuL <i>Sal</i> I 1260-	5'-GGCCGTCGACGTGCTACATCTATACTATC ( <i>Sal</i> I)
PuuL <i>Sal</i> I 2412-	5'-GGCCGTCGACGAAGTTCAACCACCTCCTGGCA ( <i>Sal</i> I)
PuuL <i>Sal</i> I 3660-	5'-GGCCGTCGACGTACTCTGGGCTGCAGCAAG ( <i>Sal</i> I)
PuuL <i>Sal</i> I 4860-	5'-GGCCGTCGACGTGCAGCTAAATGTGTTAG ( <i>Sal</i> I)
PuuL <i>Sal</i> I 6505-	5'-GGCCGTCGACGTCTGTTAATAAAAAGAA ( <i>Sal</i> I)
PuuL <i>Eco</i> RI 5'	5'-GGCCGAATTCGAGAAATACAGAGAGATC ( <i>Eco</i> RI)
PuuL <i>Eco</i> RI 3'	5'-CGGCGAATTCTAACCCCTCCTTGATACTT ( <i>Eco</i> RI)
SeoNORF <i>Pst</i> I-	5'-GCGCTGCAGTTATAATTTTCATAGGTTCCCTG ( <i>Pst</i> I)
SeoNORF <i>Nco</i> I+	5'-GCGGAATTCGCCATGGCAACTATGGAAGAAATCCAG ( <i>Nco</i> I)
HtnNORF <i>Nco</i> I+	5'-GCGGAATTCGCCATGGCAACTATGGAGGAATTACAG ( <i>Nco</i> I)
HtnNP <i>Pst</i> I-	5'-GCGCTGCAGTTATAGTTTTAAAGGCTCTTGTTGGA ( <i>Pst</i> I)
PuuORF2 <i>Nco</i> I+	5'- GCGGAATTCGCCATGGGCAACAACCTTGTTGTTGCCAGAC ( <i>Nco</i> I)

PuuORF2*Pst*IFLAG-

5'-GCGCTGCAGTCACTTGTCATCGTCGTCCTTGTAGTC-  
CATCAAGGACATTTCCAT (*Pst* I, FLAG)

PuuORF2*Pst*I-

5'- GCGCTGCAGCATCAAGGACATTTCCAT (*Pst* I)

PuuN *Pst*I pktag5

5'- GCGCTGCAGTCACGTAGAATCTAACCGCAACAGGGGATTGGGAAT-  
-GGGTTTACCTGATATCTTTAAGGGTTCTTGGTTTGA (*Pst* I, pktag)

PuuN *Pst*IFLAG-

5'- GCGCTGCAGTCACTTGTCATCGTCGTCCTTGTAGTCTATCTTTAA-  
GGGTTCTTGGTTTGA (*Pst*I, FLAG)

PuuN*Nco*I 43+

5'-GCGGAATTCATGGACTTGACAGACATC (*Nco* I)

PuuS *Eco*RI 5'

5'-GGCCGAATTCGGTGACTTGACAGACATC (*Eco*RI)

PuuS *Bam*HI 3'

5'-CGGCGGATCCATGTCATAACCAGATG (*Bam*HI)

PuuS *Eco*RI 43+

5'-GGCCGAATTCGGTGACTTGACAGACATCC (*Eco* RI)

PuuS *Eco*RI 301+

5'-GGCCGAATTCGATGATCATCTTCTCAAGGA (*Eco* RI)

PuuS *Eco*RI 601+

5'-GGCCGAATTCACCATGAAAGCTGAAGA (*Eco* RI)

PuuS *Eco*RI 906+

5'-GGCCGAATTCATTGACTATGCTGCCTC (*Eco* RI)

PuuS *Eco*RI 1201+

5'-GGCCGAATTCCTGTACATGTTGGAATG (*Eco* RI)

PuuS *Pst*I 360-

5'-GGCCCTGCAGAGCATTACATCAAGGACA (*Pst* I)

PuuS *Pst*I 660-

5'-GGCCCTGCAGGAATAAGCCACATACTA (*Pst* I)

PuuS *Pst*I 960-

5'-GGCCCTGCAGGATTCGATGTCATCAGGCG (*Pst* I)

PuuS *Pst*1270-

5'-GGCCCTGCAGGCTCAGGATCCATGTCA (*Pst* I)

PuuS*Pst*I1342-

5'-GGCCCTGCAGTATCTTTAAGGGTTCTTGGTTTGTATATCTCTTTTACC (*Pst* I)

PuuS *Pst*1560-

5'-GGCCCTGCAGAATTCCTGTATTAATC (*Pst* I)

PuuS *Pst*1780-

5'-GGCCCTGCAGTAGTAGTAGACTCCTTGA (*Pst* I)

#### 2.1.4. Expression vectors and plasmids.

The plasmid pTM1 containing bacteriophage T7 promoter and internal ribosome entry site (IRES) (Moss *et al.*, 1990) was originally supplied by B Moss, NIH, USA

pEGFP-N1 expression vector that contains the immediate early promoter of CMV and the EGFP coding sequences was purchased from Clontech.

Plasmids for the mammalian two-hybrid system, pM1, pVP16 and pG5CAT (Mammalian Matchmaker Two-Hybrid Assay kit, Clontech) were supplied by A Easton, University of Warwick. pG5CAT is a reporter plasmid that contains the CAT gene downstream of 5 consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. pM contains the GAL4 DNA binding domain (BD). pVP16 contains an activation domain (AD) derived from the VP16 protein of herpes simplex virus. Plasmid pM3VP16, a positive control, consists of the AD fused to the BD.

#### 2.1.5. Bacterial strains

The usual host for the propagation of recombinant plasmids was *E. coli* strain DH5 $\alpha$ :  $\Phi$ 80d *lacZ*  $\Delta$ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r K-, mK+), *supE44*, *relA1*, *deoR*,  $\Delta$  (*lacZYA-argF*) Ua69.

#### 2.1.6. Bacterial culture media

The following bacterial culture media were used:

LB-Broth (LB)	10g NaCl, 10g Bactopectone, 15g yeast extract per litre.
LB-agar:	LB-Broth plus 1.5% (w/v) agar
2 $\times$ YT broth:	5g NaCl, 16g Bactopectone, 10g yeast extract per litre.

Ampicillin was added at 75 µg/ml to the medium where appropriate.

Kanamycin was added at 50 µg/ml to the medium where appropriate

### **2.1.7. Cells and tissue culture media.**

BHK-21 cells, clone C-13, a continuous cell line derived from baby hamster kidneys (MacPherson and Stoker, 1962), were grown in Glasgow modified Eagle's medium supplemented with 10% new born calf serum (10% GMEM, Stoker and MacPherson, 1961), 5mM L-glutamine, 14ml/400ml of 7.5% sodium bicarbonate, and 10% tryptose phosphate broth.

293 cells, a human cell line transformed by DNA from human adenovirus type 5 (Graham *et al.*, 1977), were grown in Dulbecco's modified medium containing 10% foetal calf serum and 5mM L-glutamine.

VeroE6 cells, a monkey kidney cell line, were grown in Dulbecco's modified Eagles medium supplemented with 10% foetal calf serum and 5mM L-glutamine.

HeLa cells, a human cell line, were grown in Dulbecco's modified Eagles medium supplemented with 10% foetal calf serum and 5mM L-glutamine.

Cells were grown at 37°C under 5% CO<sub>2</sub> in a humidified incubator. Routine maintenance and passage of cells was done every 3 days.

### **2.1.8. Viruses**

Puumala virus strain cg18-20 was obtained from X Shi of this Institute.

vTF7-3, a recombinant vaccinia virus which expresses T7 polymerase (Fuerst *et al.*, 1986) was originally supplied by B Moss, NIH, USA

### **2.1.9. Commonly used reagents, chemicals and solutions**

All reagents and chemicals were purchased from BDH Chemicals Ltd or Sigma Chemicals Co except as noted.

Acrylamide/bis-acrylamide stock solution:	30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, final ratio 37.5:1. Purchased from Roche Diagnostics and Scotlab.
Agarose gel loading buffer:	0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol.
Ammonium persulphate:	from Biorad.
Blocking buffer (Western blotting):	0.1% Tween-20 in TE buffer with 10% (w/v) non-fat milk
Gel fix:	50% (v/v) methanol, 10% (v/v) acetic acid, 40% dH <sub>2</sub> O
IP buffer:	150mM NaCl, 10mM Tris, pH 7.4, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1mM PSF
Lysis solution:	0.2M NaOH, 1% SDS
Neutralisation solution:	1.32M KOAc, pH 4.8
<i>Pfu</i> PCR buffer (10×):	200mM Tris-HCl, pH 8.8, 20mM MgSO <sub>4</sub> , 100mM KCl, 100mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 1% Triton X-100, 1mg/ml nuclease-free BSA. From Stratagene
PBS:	170mM NaCl, 3.4mM KCl, 10mM HPO <sub>4</sub> , 1.8mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.2, 6.8 mM CaCl <sub>2</sub> , 4.9mM MgCl <sub>2</sub>
Protein dissociation mix:	100mM Tris-HCl, pH 6.8, 4% SDS, 200mM β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue.

Protein sample buffer:	15% (w/v) SDS, 1.5% (w/v) bromophenol blue, 50% (v/v) glycerol
Psi-broth:	250 ml LB medium, 4mM MgSO <sub>4</sub> , 10mM KCl
RIPA:	10mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 1mM PMSF
SDS-PAGE running buffer:	25mM Tris-base, 192mM glycine, 0.1% SDS
STET lysis solution:	8% (w/v) sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris-HCl, pH 8.
TAE:	40mM Tris-acetate, pH 8.0, 1mM EDTA
TBE:	90mM Tris-HCl, 90mM boric acid, 1mM EDTA, pH 8.
TFB1:	100mM RbCl, 50mM MnCl <sub>2</sub> , 30mM CH <sub>3</sub> COOK, 10mM CaCl <sub>2</sub> ×2H <sub>2</sub> O, 15% (w/v) glycerol, pH adjusted to 5.8
TFB2:	10mM MOPS, 10mM RbCl, 75mM CaCl <sub>2</sub> , 15% (w/v) glycerol, pH adjusted to 8.0
TE:	10mM Tris-HCl, pH 8, 1mM EDTA.
TEMED:	from Bio-Rad
TEN:	150mM NaCl, 40mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0.
Transfer buffer:	15mM Tris, 120mM glycine, 20% methanol.
Trypsin solution:	0.25% (w/v) Difco trypsin dissolved in Tris-saline solution plus 0.005% (w/v) phenol red
TSB:	10% PEG, 5% DMSO, 10mM MgCl <sub>2</sub> , 10mM MgSO <sub>4</sub> in 2×YT or LB broth.
TSB-glucose:	TSB supplied with 3.6mg/ml glucose
Versene solution:	PBS supplemented with 0.6mM EDTA and 0.0015% (w/v) phenol red

Visualisation solution for ECL:

from Amersham Life Sciences

### **2.1.10. Miscellaneous materials.**

3MM blotting paper (Whatman)

Disposable plastic reaction vials and pipette tips (Scotlab)

Gene Clean DNA purification kit (Bio 101)

Horizontal gel electrophoresis apparatus Horizon 58 (5.7×8.3×0.3cm) and H5 (14×11×0.5cm, Gibco BRL)

Hybond N nylon blotting membrane (Amersham)

*In vitro* transcription kit (Promega)

Lipofectamine transfection reagent (Gibco BRL)

Nescofilm (Nipon, Bando Chemical Ind)

Opti-MEM media (Gibco BRL)

Pansorbin cells (Calbiochem-Novabiochem)

Plasmid maxi/midiprep kit (Qiagen)

Plasmid miniprep kit (Qiagen)

Polygram SIL G (0.25mm) TLC plates (Camlab)

Protein A sepharose beads (Sigma)

Qiaex DNA purification kit (Qiagen)

Qiafilter maxiprep kit (Qiagen)

Quick-spin columns (Ambion)

SDS-PAGE standards broad range (Bio-Rad)

Semi-dry electrophoresis apparatus (LKB Bromma)

Tissue culture disposable plasticware (Nunc)

*TnT* Coupled Transcription/Translation system (Promega)

Wizard maxiprep kit (Promega)

X-omat S film for autoradiography (Kodak Ltd.)

## **2.2. Methods**

### **2.2.1. DNA manipulation and cloning procedures.**

#### **2.2.1.1. Plasmid preparation**

##### **2.2.1.1.1. Small-scale plasmid preparation: boil-lysis method**

This method was used for diagnostic restriction digestion. Single colonies were picked from agar plates, inoculated into 3 ml aliquots of 2×YT broth, containing the appropriate antibiotic, and incubated overnight at 37°C in a shaking incubator. One and a half ml of bacterial culture was centrifuged at 13,000 rpm in a benchtop centrifuge for 30 seconds, the resultant pellet broken up by vortexing and incubated on ice for 5 minutes in 450 µl STET lysis solution containing 200 µg lysozyme. The solution was boiled for 40 seconds, centrifuged at 13,000 rpm for 20 minutes and the pelleted debris removed with a sterile toothpick. Four hundred and fifty µl isopropanol was added to the supernatant, incubated at -20°C for 30 minutes and centrifuged at 13,000 rpm for 10 minutes. The pellet was washed with 70% ethanol, air-dried and resuspended in 40 µl dH<sub>2</sub>O.

##### **2.2.1.1.2. Small-scale plasmid preparation: alkaline lysis method.**

Single colonies were picked from agar plates, inoculated into 3 ml of 2×YT broth, containing the appropriate antibiotic, and incubated overnight at 37°C in a shaking incubator. One and a half ml of bacterial culture containing the desired plasmid was centrifuged at 13,000 rpm in a benchtop centrifuge for 30 seconds and the resultant pellet resuspended in 100 µl cell resuspension solution (Promega). Two hundred µl fresh lysis solution was added to the bacteria, mixed by inversion and incubated on ice for 3 minutes. One hundred and fifty µl cold neutralization solution was added, mixed by inversion and incubated on ice for 5 minutes. The debris was pelleted at 13,000 rpm for 5

minutes and DNA precipitated from the supernatant by ethanol precipitation. The pellet was washed with 70% ethanol, air-dried and resuspended in 40  $\mu$ l dH<sub>2</sub>O.

#### **2.2.1.1.3. Small-scale plasmid preparation: Qiagen plasmid miniprep kit**

The method was used to produce transfection-quality DNA. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica-gel-membrane in the presence of high salt.

One and a half ml of bacterial culture grown overnight was pelleted at 13,000 rpm for 20 seconds in a benchtop centrifuge. Pelleted bacterial cells were resuspended in 250  $\mu$ l buffer P1 followed by lysis in 250  $\mu$ l buffer P2 by inverting the tube 6 times. The reaction was neutralized by adding 350  $\mu$ l buffer N3 and inverting the tube 6 times. Lysates were cleared by centrifugation at 13,000 rpm in a benchtop centrifuge for 10 minutes and supernatants centrifuged for 30-60 seconds through the column to allow the DNA to adsorb onto membrane. This was then followed by two wash steps in 500  $\mu$ l buffer PB and 750  $\mu$ l buffer PE by centrifugation at 13,000 rpm for 30-60 seconds. To remove residual wash buffer, columns were centrifuged for an additional 1 minute. Forty  $\mu$ l pre-warmed dH<sub>2</sub>O was added onto silica-gel membrane and incubated for 3 minutes at a room temperature. The DNA was eluted from the column into a new tube by centrifugation at 13,000 rpm for 1 minute and stored at -20°C.

#### **2.2.1.1.4. Large scale preparation and purification of plasmid DNA: Promega ‘Wizard’ kit**

Promega ‘Wizard’ kit was used for bulk preparations of DNA according to the manufacturer’s instructions.

Single colonies of *E.coli* strain DH5 $\alpha$  were picked from agar plates, inoculated into 10 ml of 2 $\times$  YT broth, containing the appropriate antibiotic, and incubated overnight at 37°C

in a shaking incubator. One ml overnight culture was inoculated into 300ml pre-warmed LB-broth in a 2 litre flask (containing 75 µg/ml of ampicillin or other antibiotics where appropriate) and shaken for 20 hours at 37°C. Following centrifugation of the cultures at 3,000 rpm (Sorval GS3 rotor) for 10 minutes at 4°C, the bacterial pellet was resuspended in 15ml resuspension buffer. Cells were lysed in 15 ml lysis solution by inversion until the solution was clear. The reaction was neutralized by addition of neutralization solution to the mixture and mixing by inversion. The cell debris was pelleted by centrifugation at 3000 rpm for 10 minutes. The DNA was precipitated with the equal volume of isopropanol at -20°C for 10 minutes, pelleted by centrifugation at 2500 rpm in a swing-bucket rotor for 10 minutes and resuspended in 2 ml dH<sub>2</sub>O. Precipitated DNA was then mixed with 10 ml DNA purification resin and passed through a column under vacuum. The column was washed with 25 ml column wash solution and 5 ml 80% ethanol and dried by centrifugation at 2500 rpm in a swing-bucket rotor for 5 minutes followed by application of vacuum for 5 minutes. One and a half ml dH<sub>2</sub>O was added to the column and incubated at 65°C. The DNA was eluted by centrifugation at 2500 rpm in a swing-bucket rotor for 5 minutes and stored at -20°C.

#### **2.2.1.1.5. Large-scale plasmid preparation: Qiagen ‘Qiafilter’**

The procedure is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to resin under appropriate low-salt and pH conditions, and allows production of transfection-quality DNA.

Single colonies of *E.coli* strain DH5α were picked from agar plates, inoculated into 10 ml aliquotes of 2× YT broth, containing the appropriate antibiotic, and incubated for 8 hrs at 37°C in a shaking incubator. One ml of this culture was inoculated into 100 (250) ml 2× YT broth and incubated overnight. A 100 ml (high-copy) or 250 ml (low-copy) bacterial culture containing the desired plasmid was centrifuged at 3000 rpm for 15 minutes in a swing-bucket rotor at 4°C and the bacterial pellet was resuspended in 10 ml buffer P1. Ten ml buffer P2 was added, mixed thoroughly and the solution was incubated for 5 minutes at a room temperature to allow cell lysis. The solution was neutralised by

adding 10 ml chilled buffer P3, inverting the tube 6 times and incubating on ice for 20 minutes. A tip was equilibrated by applying 10 ml buffer QBT and the column emptied by gravity flow. The bacterial lysate was cleared by being passed through the filter of the syringe into the tip and entered the resin by gravity flow. The tip was washed twice with 30 ml buffer QC and the DNA eluted with 15 ml buffer QF, then precipitated with 10 ml isopropanol and pelleted by centrifugation at 2500 rpm for 3 hours in a swing-bucket rotor at 4°C. The pellet was washed with 70% ethanol, air-dried, resuspended in 1 ml dH<sub>2</sub>O and stored at -20°C.

#### **2.2.1.2. Phenol:chloroform extraction and ethanol precipitation**

An equal volume of 50% phenol, 49% chloroform and 1% isoamyl alcohol was added to 100 or 150 µl of the solution, mixed carefully by vortexing and centrifuged at 13,000 rpm in a benchtop centrifuge for 5 minutes. The upper phase was retained, mixed with an equal volume of chloroform and centrifuged as before. The upper phase was retained, mixed with an equal volume of chloroform and centrifuged as before. The upper phase was retained and 1/10 of the volume of 3M sodium acetate, pH 5.2, and 3 volumes of 100% ethanol were added. The solution was incubated either on dry ice for 20 minutes or at -20°C for 1 hour. The DNA was pelleted by centrifugation at 13,000 rpm for 20 minutes. The pellet was washed with 50 µl 70% ethanol, air-dried and resuspended in dH<sub>2</sub>O.

#### **2.2.1.3. Restriction enzyme digestion of DNA**

Appropriate restriction enzymes were used at 1 unit per µg DNA in buffers supplied by the manufacturers. Restriction digest analysis of small-scale DNA preparations used 2 µl of DNA, digested in a 20 µl reaction volume.

Typical digestion reaction contained:

DNA	2 $\mu$ l
Restriction enzyme 10 $\times$ buffer	2 $\mu$ l
Restriction enzyme	2U
BSA, acetylated (1mg/ml)	2 $\mu$ l
dH <sub>2</sub> O	<u>13 <math>\mu</math>l</u>
	20 $\mu$ l

Digestions were for 1-4 hours at an appropriate temperature specific for different enzymes. DNA fragments were separated by electrophoresis on agarose gels.

For the preparation of DNA to be used in subcloning, 5 to 10  $\mu$ g DNA were digested in a 20 to 50  $\mu$ l reaction volume as above, with 2 units restriction endonuclease per  $\mu$ g DNA, and incubated 2 to 4 hours.

#### **2.2.1.4. End-repair of DNA with the Klenow fragment of *E.coli* polymerase I**

Cohesive ends of plasmid DNA were blunt-ended using the Klenow fragment of *E.coli* polymerase I, which lacks the 3' to 5' exonuclease activity of *E.coli* polymerase I. Reaction consisted of 1  $\mu$ l each of 0.5M dATP, dGTP, dCTP, dTTP and 1-5U Klenow fragment added to a 20 $\mu$ l restriction digestion mixture. The reaction was incubated at 37°C for 15 minutes and the DNA purified by phenol:chloroform extraction.

#### **2.2.1.5. Agarose gel electrophoresis of DNA.**

Electrophoresis of DNA was performed on horizontal slab gels (5.7 $\times$ 8.3 $\times$ 0.3 cm, BRL gel electrophoresis apparatus Horizon 58). 1% (w/v) agarose in 1 $\times$ TBE or 1 $\times$ TAE

containing 0.5 µg/ml ethidium bromide was used. DNA samples were loaded on the gel in a loading dye. Electrophoresis was carried out at 50-100V in 1×TBE or 1×TAE buffers, and the DNA bands were visualized using short or long wave UV illumination.

### **2.2.1.6. Polymerase chain reaction (PCR) amplification of DNA.**

PCR primers were typically 15-30 nucleotides long. 10-50 picomoles of each primer were used in reaction volumes ranging from 10 µl to 50 µl. When plasmid DNA was used as template, triplicate reactions were set up containing 10, 1 and 0.1ng of template. Further replicates allowed reactions to be halted after different numbers of cycles to minimize the number of copying steps and so reduce the possibility of accumulating point mutations. PCR was performed using either *Taq* DNA polymerase or *Pfu* Turbo DNA polymerase. In each case the reaction was carried out in the buffer supplied and under the conditions recommended by the manufacturer. Reactions were performed in a thin-walled 0.5 ml reaction tube using a Techne thermal cycler that does not require the reaction to be overlaid with oil.

Standard reaction using *Taq* DNA polymerase contained:

10× <i>Taq</i> buffer	3µl
25mM MgCl <sub>2</sub>	1.2µl
25mM dNTPs	0.24µl
template DNA, 100ng/µl	1µl
primer 1, 16pmol/µl	0.75µl
primer 2, 16pmol/µl	0.75µl
<i>Taq</i> DNA polymerase, 5 U/µl	0.2µl
dH <sub>2</sub> O	<u>22.86µl</u>
	30µl

Standard reaction using *Pfu* Turbo polymerase:

10× <i>Pfu</i> buffer	5µl
25mM dNTPs	0.4µl
template DNA, 100ng/µl	1µl
primer 1, 16pmol/µl	1.25µl
primer 2, 16pmol/µl	1.25µl
<i>Pfu</i> Turbo DNA polymerase, 2.5 U/µl	0.5µl
dH <sub>2</sub> O	<u>40.6µl</u>
	50µl

Thermal cycling parameters:

segment 1 (duplex melting)	95°C	30 seconds,
segment 2 (primer annealing)	55°C	1 minute
segment 3 (primer elongation)	72°C	3 minutes

These conditions were altered empirically where improvement was required.

Reactions were cycled for 30 PCR rounds, including a final extension step at 72°C for 8 minutes. PCR products were run on 1×TAE agarose gel and purified.

#### **2.2.1.7. Purification of amplification products from PCR using the *High Pure* PCR Product Purification kit (Boehringer Mannheim)**

A gel slice containing the desired DNA was excised from the gel. It was then melted at 55°C for about 5-10 minutes in 900 µl binding buffer. Four hundred and fifty µl isopropanol was added, mixed thoroughly and the mixture was divided into 2 spin columns followed by centrifugation at 13,000 rpm in a benchtop centrifuge for 30 seconds. The column was washed twice with 200 µl wash buffer by centrifuging as described before. The column was centrifuged for an additional 1 minute to ensure optimal purity and the complete removal of residual wash buffer from the glass fibre

fleece. The DNA was then eluted with 40  $\mu$ l pre-warmed dH<sub>2</sub>O by centrifuging at 13,000 rpm for 30 seconds

#### **2.2.1.8. DNA purification from agarose gels by silica matrix adsorption.**

DNA fragments produced by restriction enzyme digestion were separated by electrophoresis through 1% agarose TAE gels containing 0.5 $\mu$ g/ml ethidium bromide. Long wave UV illumination allowed visualization of the DNA bands. A commercial kit, GeneClean (BIO 101 Inc, La Jolla, CA), containing a silica matrix that binds DNA in the presence of high concentrations of sodium iodide, was used for retrieval of DNA from agarose blocks excised from the gels (Vogelstein and Gillespie, 1979). Gel slices containing the required DNA fragments were excised from 1 $\times$ TAE agarose gels and mixed with 3 volumes of saturated NaI solution and incubated for 10 minutes at 55°C until the agarose had completely melted. Silica matrix ('glassmilk') was added (5  $\mu$ l for up to 5 $\mu$ g of DNA) and the mixture incubated for 5 minutes on ice to allow the DNA to bind to the matrix. Following a 5 second centrifugation at 13,000 rpm, the pellet (containing DNA bound to silica particles) was washed with three times with 'NEW' wash (a Tris-buffered mixture of NaCl, ethanol and water) by repeated resuspension/centrifugation cycles. The DNA was eluted from the silica matrix by resuspending the pellet in 20  $\mu$ l dH<sub>2</sub>O, incubating for 5 minutes at 55°C and centrifugation for 5 seconds at 13,000 rpm. The DNA was stored at -20°C.

#### **2.2.1.9. DNA ligation.**

DNA fragments (100-500ng) and vector DNA (100ng), which had been digested previously with the appropriate restriction enzyme(s) and purified, were ligated together in a 20 $\mu$ l mixture. Ligation reactions were set at three molar ratios (1:1, 1:3 and 1:5) of linearized vector to insert DNA.

The mixture contained:

Vector DNA	100 ng
Insert DNA	100-500 ng
T4 DNA ligase	2U
5×ligation buffer	4 µl
dH <sub>2</sub> O	to a total volume 20 µl

Ligation reaction was incubated for 16 to 24 hours at room temperature.

## **2.2.2. Protein analysis**

### **2.2.2.1. SDS polyacrylamide gel electrophoresis (SDS PAGE) of proteins**

Denatured proteins were fractionated by electrophoresis through medium-sized polyacrylamide gels containing SDS, using a discontinuous buffer system (Laemmli 1970). The resolving gel contained 10-20% polyacrylamide in which the acrylamide was crosslinked with N,N'-methylene bisacrylamide in a ratio of 37.5:1(w/w) in resolving gel buffer.

Composition of the resolving gel was as follows:

	12%	15%	20%
Acrylamide	14.4	18.0	24.0 ml
1M Tris, pH8.8	16.8	16.8	16.8ml
dH <sub>2</sub> O	12.9	9.3	3.3ml
10%SDS	0.45	0.45	0.45ml
10%APS	0.45	0.45	0.45ml
TEMED	50µl	50µl	50µl

Ammonium persulphate and TEMED were added immediately prior to pouring the gel into glass plates assembled according to the manufacturer's instructions. The gel was overlaid with isopropanol to leave a smooth interface after polymerisation. The isopropanol was removed when the gel had polymerised by pouring it off. The stacking gel (3.8% polyacrylamide, 62.5mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate and TEMED) was layered on top of the resolving gel and the required comb inserted to form wells. The stacking gel was allowed to polymerise for about 30 minutes before assembling the gel apparatus with glass plates and running buffer placed in the reservoirs (53mM Tris, 53mM glycine, 0.1%SDS). Samples in protein dissociation mix were heated at 100°C for 5 minutes prior to loading to the gel. Electrophoresis was carried out at 40 mA until the bromophenol blue reached the bottom of the resolving gel. The gel was carefully removed from the glass plates after electrophoresis, fixed in gel fix solution for 1 hour to overnight, enhanced for 30 minutes, washed with water three times for 15 minutes each wash, dried under vacuum for 1.5 hour at 80°C and placed in contact with Kodak X-Omat S film for autoradiography.

#### **2.2.2.2. *In vitro* Transcription/Translation of proteins**

*TnT* Coupled Transcription/Translation kit (Promega) was used for *in vitro* translation of the proteins and was performed according to the manufacturer's conditions.

The reaction mixture was composed of:

Rabbit reticulocyte lysate	25µl
Reaction buffer	2µl
T7 RNA polymerase	1 µl
Aminoacid mixture minus methionine, 1mM	1µl
<sup>35</sup> S-methionine (1,000Ci/mmol) at 10mCi/ml	4µl
Rnasin ribonuclease inhibitor, 40U/µl	1 µl
DNA template	1µg
Nuclease-free H <sub>2</sub> O	to final volume 50µl

The reaction mixture was incubated at 30°C for 1.5 hours, 50µl protein dissociation buffer was added and the mixture stored at -20°C.

### **2.2.2.3. Metabolic labelling of intracellular proteins**

Monolayers of cells in 35 mm dishes were radiolabelled with <sup>35</sup>S-methionine in 1 ml of methionine free MEM (30µCi/ml) for 2 hours at 37°C. The radioactive solution was then removed, the cell monolayers washed with 0.5ml PBS and the cells lysed in 200 µl of protein dissociation mix. Cell lysates were heated at 100°C for 5 minutes before loading onto polyacrylamide gel containing SDS.

### **2.2.2.4. Western blotting**

Proteins to be subjected to Western blotting were run on SDS-PAGE as described above. Hybond N nitrocellulose membrane, 4 sheets of 3MM paper and the gel were equilibrated in transfer buffer for 10 minutes. The surface of the blotting apparatus was wet with transfer buffer, and a gel stack was assembled containing (from the bottom) 2 sheets of pre-wet 3MM paper, the nitrocellulose membrane, the gel, and two more sheets 3MM paper. The gel stack was then subjected to a current of 40mA for minigel for 1 hour after which the nitrocellulose membrane was incubated in blocking buffer for 16 hours. The membrane was then incubated with primary antibody in blocking buffer for 1 hour with agitation and washed three times with PBS(A)/0.1% Tween-20. The membrane was then incubated with secondary antibody and washed as before, then subjected to enhanced chemiluminescence by incubating it in freshly-mixed visualisation solution for 3 minutes. Membrane, wrapped in mellotex film, was exposed to an autoradiograph film for 1 second to 10 minutes.

### **2.2.2.5. Immunoprecipitation (IP) of radiolabelled proteins.**

Cell monolayers were radiolabelled with  $^{35}\text{S}$ -methionine (30 $\mu\text{Ci/ml}$ ) in 1 ml methionine free minimal essential medium for 2 to 5 hours. The cells were washed with 0.5ml PBS and 300 $\mu\text{l}$  RIPA buffer containing protease inhibitor was added to each dish to lyse the cells. The dishes were incubated on ice for 30 minutes and the solution transferred to microcentrifuge tubes. Nuclei and cellular debris were removed by centrifugation for 5 minutes at 13,000 rpm and the cleared supernatant was then reacted with 50  $\mu\text{l}$  *Staphylococcus aureus* (Pansorbin, Calbiochem-Novabiochem) for 2 hours at 4°C on a rotating wheel to preadsorb proteins. Pansorbin was pelleted by centrifugation for 30 seconds at 13,000 rpm, 10 $\mu\text{l}$  antibody added to the supernatant and the mixture incubated overnight at 4°C on a rotating wheel. Sixty  $\mu\text{l}$  beads suspension was added to each IP reaction and the mixture rotated for 1 hour at 4°C. Centrifugation at 13,000 rpm for 30 seconds allowed the beads to be pelleted followed by 3 washes with 300 $\mu\text{l}$  RIPA buffer. Beads were resuspended in 60 $\mu\text{l}$  protein dissociation mix, stored at -20°C and boiled for 5 minutes prior to electrophoresis on SDS-polyacrylamide gels.

### **2.2.3. Transfection of mammalian cells**

#### **2.2.3.1. Preparation of cationic liposomes**

The method is based on the one described by Rose *et al* (1991). Dimethyldioctadecyl ammonium bromide (DDAB) was diluted to 4 mg/ml in chloroform and 1 ml was mixed with 1 ml dioleoyl L- $\alpha$ -phosphatidyl ethanolamine (DOPE, 10 mg/ml in chloroform) and the chloroform evaporated with a stream of nitrogen for approximately 5 minutes. The remaining mixture was lyophilised overnight in a freeze drier and the dried lipids were resuspended in 10 ml sterile distilled water by sonication in an ultrasonic bath followed by a sonication probe on ice (constant duty cycle, power 5, 2-3 minutes intervals) until

almost clear. The final suspension had a 1:2.5 ratio by weight of DDAB to DOPE and could be stored at 4°C for up to one month.

### **2.2.3.2. Liposome mediated transfection with ‘in-house’-made liposomes**

Confluent monolayers of cells were washed once with OptiMEM (Gibco) low serum medium prior to application of the transfection mix. The DNA to be transfected (0.5-5 µg) was diluted in 250µl of OptiMEM and combined with separately diluted liposomes (15µl in 250µl OptiMEM). The mixture was incubated at room temperature for 15 minutes before addition to the cell monolayers. After 3 hours incubation at 37°C in a humidified incubator, the transfection mix was removed, cells were washed twice with 0.5ml OptiMEM and 2ml DMEM containing 5% serum was added. Incubation continued for a further 20-48 hours before harvesting the cells for analysis.

### **2.2.3.3. Liposome-mediated transfection with Lipofectamine**

Confluent monolayers of cells were washed once with 1ml OptiMEM (Gibco) low serum medium prior to application of the transfection mix. The DNA to be transfected (0.5-5 µg) was diluted in 100µl OptiMEM and combined with separately diluted Lipofectamine (5µl in 100µl OptiMEM). The mixture was incubated at room temperature for 45 minutes, mixed with 800µl OptiMEM and added to the cell monolayers washed with OptiMEM (0.5 ml per 35mm dish). After 3 to 5 hours of incubation at 37°C in a humidified incubator, the cells were washed twice with OptiMEM and 2ml DMEM was added. Incubation continued for a further 20-48 hours.

### **2.2.4. Chloramphenicol acetyl transferase (CAT) assays.**

CAT assays were adapted from Gorman *et al* (1982). Cell monolayers were harvested by scraping the monolayer off the dish with thin-wall tubing (1 mm bore) and pelleted by

centrifugation at 13,000 rpm for 1 minute in a benchtop centrifuge. The cell pellet was resuspended in 400  $\mu$ l TEN buffer and centrifuged as before. The supernatant was removed by aspirating and the cells were resuspended in 75  $\mu$ l 250mM Tris, pH 7.5, by vortexing. Three freeze-thaw cycles were performed (dry ice/ethanol bath- 37°C water bath) to disrupt the cells. The cell debris was removed by centrifugation at 13,000 rpm for 5 minutes and the supernatant, containing the CAT enzyme, was then heated to 60°C for 10 minutes to inactivate any deacetylase enzymes present. Extracts were then stored at -20°C or assayed immediately.

The CAT assays were performed for between 2 to 20 hours at 37°C in a total volume of up to 75 $\mu$ l depending on the amount of cell extract used. The reaction contained up to 50 $\mu$ l cell extract (equivalent to approximately half a cell monolayer from a 35mm dish), 2  $\mu$ l of 50mM acetyl coenzyme A and 1 $\mu$ l of <sup>14</sup>C chloramphenicol (0.1  $\mu$ Ci/ $\mu$ l; 58.5 mCi/mmol) in 250mM Tris-HCl, pH 7.5.

After incubation, the reactions were mixed with 250 $\mu$ l ethyl acetate, vortexed for 15 seconds and incubated for 2 minutes at a room temperature. Following centrifugation at 13,000 rpm for 5 minutes in a microfuge, the upper organic phase was removed and dried under vacuum on a Speed-Vac centrifuge to ensure the complete removal of traces of ethyl acetate. The residue of chloramphenicol was redissolved in 25 $\mu$ l ethyl acetate and applied as a spot near the base of a thin layer chromatography plate. Ascending chromatography was performed using 95% chloroform/5% methanol until the solvent front had reached the top of the plate. The chromatographs were then removed from the tank, dried in a fume hood for 5 minutes and placed directly against Kodak X-omat S film for exposure (12 hours to 5 days).

### **2.2.5. Preparation of competent bacterial cells and transformation.**

A colony of *E.coli* strain DH5 $\alpha$  was picked from the plate, inoculated in 10 ml 2 $\times$ YT broth and incubated in a shaker at 37°C overnight. One ml of an overnight bacterial culture was diluted into 100ml pre-warmed 2 $\times$ YT broth in a 250ml flask and shaken for

approximately 2 hours at 37°C. The culture was cooled on ice for 5 minutes and the bacterial cells were harvested by centrifugation at 3,000 rpm for 10 minutes at 4°C, the pellet was gently resuspended in 30 ml cold TFB1 buffer and placed on ice for an additional 90 minutes. The suspension was centrifuged at 3,000 rpm for 5 minutes and the pellet resuspended in 4 ml ice cold TFB2 buffer. The resulting competent bacterial cells were either used directly for transformation or quick frozen in dry-ice/ethanol and stored at -70°C.

One hundred µl of competent cells were incubated with the ligation reaction (20µl) for 20 minutes on ice, followed by a 90 second heat shock at 42°C to allow uptake of the ligated DNA by the *E.coli* cells. The reaction tube was returned to the ice, 600 µl Psi-broth added and the cells incubated for a further 90 minutes at 37°C in a shaker. Fifty µl of cells was then plated onto LB agar plate, containing appropriate antibiotics, and incubated overnight at 37°C to allow single colonies to form.

#### **2.2.6. Growth and titration of vaccinia virus.**

Large flasks (150 cm<sup>2</sup>) containing almost confluent monolayers of Vero E6 cells were infected with vTF7-3 virus stock at 0.05 pfu/cell in DMEM-10%FCS and the cells were incubated at 37°C for 1 hour with gentle agitation every 15 minutes. After virus adsorption for 1 hour, DMEM containing 5% foetal calf serum was added and the flasks incubated at 37°C for 48 hours. The infected cells were then scraped into the tissue culture medium and pelleted by centrifugation at 3,000 rpm for 5 minutes (Sorval SS34 rotor). The pellet was resuspended in 10mM Tris-HCl, pH 9, at a rate of 2ml per large flask. The cell associated virus was released by three cycles of freeze/thawing and the nuclei removed by centrifugation at 1,000 rpm for 5 minutes. The supernatants were titrated by plaque assay on Vero E6 cell monolayers and stored at -20°C for use as working stocks of vaccinia virus.

### **2.2.7. Infection with vaccinia virus vTF7-3**

Recombinant vaccinia virus vTF7-3 (Fuerst *et al.*,1986) was used as a source of T7 polymerase in cells. A confluent cell monolayer was washed with OptiMEM and vaccinia virus added as  $5 \times 10^6$  pfu/35mm dish in 500  $\mu$ l OptiMEM. The cells were incubated at 37°C for 1 hour, washed once with OptiMEM and transfected as described above.

## **CHAPTER 3. ATTEMPTS TO DEVELOP A REVERSE GENETICS SYSTEM FOR HANTAVIRUSES**

### **3.1. Introduction**

The procedures developed during the 1990s to genetically manipulate the genomes of negative-strand RNA viruses, commonly referred to as reverse genetics, have enhanced the potential of investigation of viral gene expression and the dissection of *cis*-acting regulatory sequences important for replication and transcription. These new methods have facilitated studies of viruses that are present only in low titers in infected cells or whose isolation is problematic. They have provided important information in the study of the genetic expression and replication of RNA viruses by the use of mutagenesis, deletions, insertions and by complementation experiments, but also in the study of natural or induced RNA recombination, and mechanisms generating defective-interfering RNAs.

Although reverse genetics systems for nonsegmented negative-strand RNA viruses such as vesicular stomatitis virus (Pattnaik *et al.*, 1990), Sendai virus (Park *et al.*, 1991) and respiratory syncytial virus (Collins *et al.*, 1991) have long been developed, the development of similar protocols for manipulating the genomes of segmented negative-strand RNA viruses have appeared to be more difficult. Despite this, over the past years, a number of minigenome systems to analyze the transcription and replication processes of segmented negative-strand RNA viruses has been described for the members of the families *Orthomyxoviridae* (Parvin *et al.*, 1989; Luytjes *et al.*, 1989; Zobel *et al.*, 1993; Neumann *et al.*, 1994; Enami *et al.*, 1990; Hoffmann *et al.*, 2000), *Bunyaviridae* (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Prehaud *et al.*, 1997; Flick and Pettersson, 2001) and *Arenaviridae* (Lee *et al.*, 2000).

Minigenome systems for negative-strand RNA viruses are based on intracytoplasmic reconstitution of the RNP complex, which represents the template for the viral polymerase, and is the prerequisite needed to start an infectious cycle. A common feature

of these various systems is that the template RNA is derived from a cDNA clone containing authentic viral terminal sequences. However, there are certain variations in the developed approaches based on the delivery of the template, the source of viral proteins, the type of promoter to drive the transcription and expression, and the sequences present in the template. Transcription of the template RNA requires viral proteins that can be supplied either by recombinant sources (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Collins *et al.*, 1995; He *et al.*, 1997; Hoffmann and Banerjee, 1997) or by helper virus infection (Luytjes *et al.*, 1989; Park *et al.*, 1991). The template can be transcribed within the cell (Flick and Pettersson, 2001) or delivered into the cell either as a transfected naked RNA (Dunn *et al.*, 1995; Park *et al.*, 1991; Collins *et al.*, 1995) or ribonucleoprotein complex (Luytjes *et al.*, 1989). To direct transcription of the template and expression of viral proteins, various types of promoters have been utilized. These included the bacteriophage T7 promoter (Dunn *et al.*, 1995; Park *et al.*, 1991; Collins *et al.*, 1991; Luytjes *et al.*, 1989; Lee *et al.*, 2000; Lopez *et al.*, 1995), RNA polymerase type I (pol I) promoters (Flick and Pettersson, 2001; Neumann *et al.*, 1994) and RNA polymerase type II (pol II) promoters (Flick and Pettersson, 2001; Neumann *et al.*, 1997). The sequences present in the template also may differ and contain either authentic viral transcripts or defective-interfering RNA sequences or reporter genes or mutated RNAs (Collins *et al.*, 1991; Park *et al.*, 1991; Dunn *et al.*, 1995; Pattnaik *et al.*, 1992; Lopez *et al.*, 1995).

To date, methods to study the role of *cis*-acting sequences at the 5' and 3' termini of viral RNA (vRNA) segments of members of the family *Bunyaviridae* have been developed for Bunyamwera (BUN) virus (*Orthobunyavirus* genus) (Dunn *et al.*, 1995), Rift Valley fever (RVF) virus (Lopez *et al.*, 1995; Prehaud *et al.*, 1997) and Uukuniemi virus (UUK) (both *Phlebovirus* genus) (Flick and Pettersson, 2001). The methods for Bunyamwera and Rift Valley fever viruses were based on the now classical T7-vaccinia virus system (Fuerst *et al.*, 1986) to express a chloramphenicol acetyltransferase (CAT) reporter cDNA flanked by 5' and 3' vRNA untranslated regions (UTRs). The reverse genetics system for UUK virus was developed using RNA pol I-mediated transcription of the chimeric viral RNAs also containing CAT reporter gene. In contrast to the vaccinia virus system, the chimeric cDNA (pol I expression cassette) was placed between the murine pol I promoter and terminator.

In this chapter, different strategies that were investigated in an attempt to develop the reverse genetics system for Puumala virus will be described. They were initially based on the system for Bunyamwera virus successfully developed in our laboratory by Dunn *et al.* (1995). Certain modifications had to be introduced to address some special features of hantaviruses, e.g., slow replication, inhibition of hantavirus replication by coinfecting vaccinia virus, and differences in the virus terminal sequences from those of Bunyamwera virus.

Unfortunately, none of the approaches was successful though the results presented may help in designing new strategies in the future.

### **3.2. Reverse genetics system for Bunyamwera virus**

An important step towards development of the reverse genetics system for Bunyamwera virus (BUN), a member of the *Orthobunyavirus* genus, was made by Dunn *et al.* (1995). Initially, a methodology similar to the one described for influenza virus (Luytjes *et al.*, 1989) was adopted to study transcription of a BUN-like RNA containing a reporter gene.

First, they chose to use hybrid vaccinia virus- T7 RNA polymerase system that was developed earlier for the expression of cloned foreign genes in mammalian cells. In its simplest, most versatile and widely used format, cells are infected with recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase, noted for its high transcriptase activity, stringent promoter specificity, and single subunit structure, and then transfected with one or multiple plasmids that contain the target gene of interest preceded by a T7 promoter.

Second, they had to consider sequences at the 5'- and 3'- termini of viral RNAs. Bunyamwera virus is a segmented virus that contains RNA molecules with negative-sense polarity. During the replication cycle, recognition of the 5'- and 3'-structures of the

three vRNA segments by the viral RNA-dependent RNA polymerase results in the replication and transcription of the BUN virus genes. The fact that the terminal sequence elements are highly conserved and complementary indicated that these sequences are important for recognition by the viral polymerase and encapsidation by N protein (Kolakofsky and Hacker, 1991). The development of influenza virus (Luytjes *et al.*, 1989), respiratory syncytial virus (Collins *et al.*, 1991) and vesicular stomatitis virus (Moyer *et al.*, 1991; Smallwood and Moyer, 1993) reverse genetics systems suggested that only the terminal regions of the viral RNA are required for nucleocapsid formation. Therefore, a transcribed artificial RNA should have sequences that are the same as those of the 5' and 3' ends.

In the minigenome system, the RNA template was transcribed from a plasmid comprising a negative-sense reporter gene (chloramphenicol acetyltransferase or CAT) flanked by the exact 5' and 3' untranslated regions of the BUN S RNA segment. A unique downstream restriction enzyme site BbsI allowed run-off transcripts to be made with defined 3' ends. The promoter was truncated (lacked three terminal guanosine residues) so that transcription by polymerase would initiate on the first viral nucleotide (A) of the BUN sequence (Fig. 3.1). Cells infected with recombinant vaccinia virus vTF7-3, which synthesises T7 RNA polymerase, were transfected with chimeric RNA and plasmids expressing BUN L and N proteins under control of a T7 promoter. The transfection of all plasmids into cells resulted in the expression of CAT protein, as measured by its enzymatic activity. This indicated that a negative-sense RNA synthesized from the CAT reporter plasmid was reconstituted intracellularly into functional RNPs. These RNPs were then transcribed by the viral RNA polymerase into mRNA, which was translated into CAT protein (Fig. 3.2).

Dunn and coworkers also showed that transcripts containing 11 (cleaved at the XbaI site) or 35 (cleaved at the HindIII site) extra nucleotides at the 3' end (Fig.3.1) were still recognised by the viral polymerase. However, deletion of the terminal 5 nucleotides at the 3' end, using the HgaI site within the BUN sequence, reduced CAT activity by at least 99.5%.

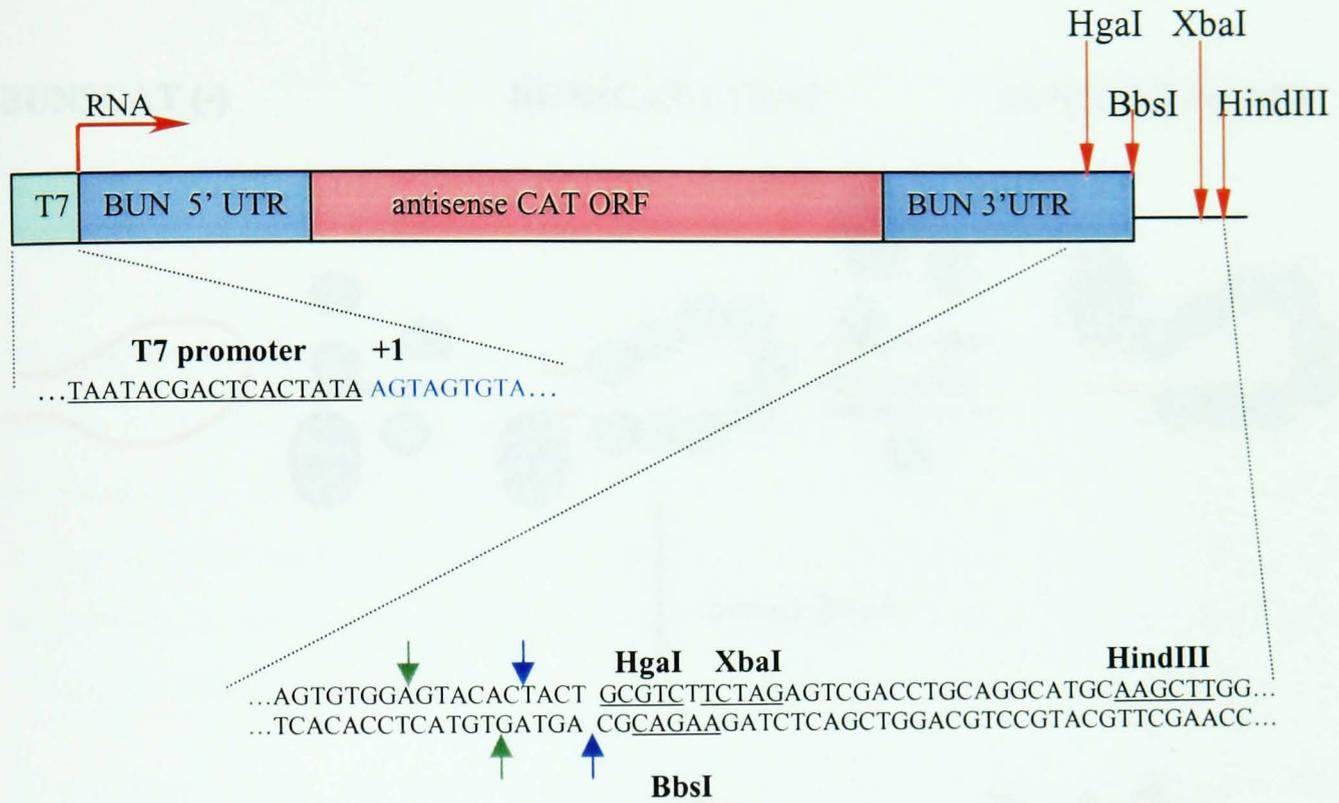


Fig.3.1. Plasmid map of pBUNSCAT (Dunn *et al.*, 1995). The truncated T7 promoter initiates transcription on the first base (A) of the BUN sequence. Runoff transcripts are produced by linearizing the DNA at the indicated restriction sites. The recognition sequences are underlined, and the digestion sites for BbsI are shown by blue arrows and those for HgaI by green arrows. T7, bacteriophage T7 promoter.

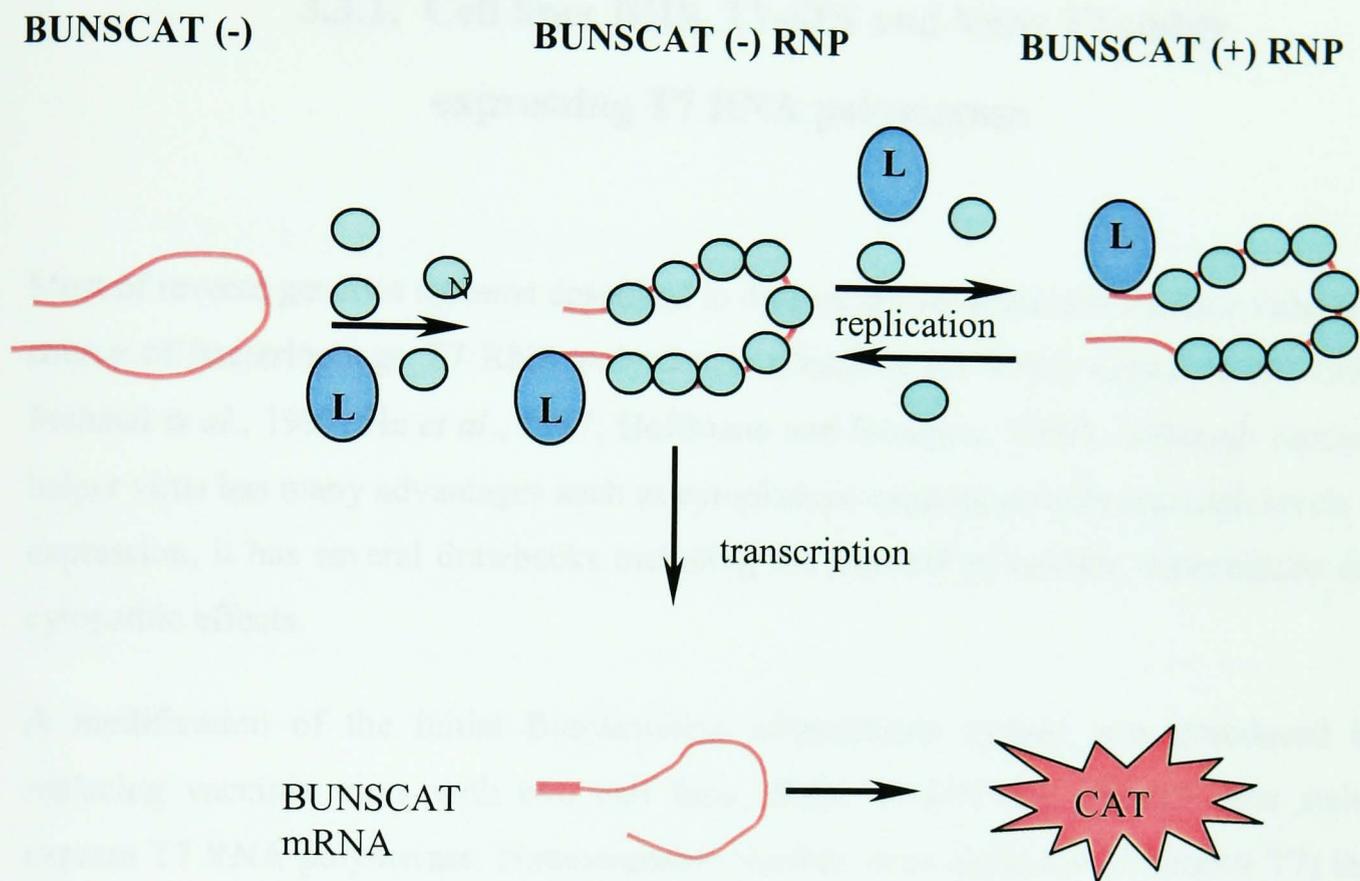


Fig. 3.2. Recognition of the bunyavirus template.

The transfection of all plasmids into cells results in the expression of CAT protein, as measured by its enzymatic activity. A negative-sense RNA is synthesized from the CAT reporter plasmid. It is then reconstituted intracellularly with N protein (supplied by expression plasmid) into functional RNPs. These RNPs are then transcribed by the viral RNA polymerase (L protein also supplied by expression plasmid) into mRNA which is translated into CAT protein. Expression of CAT protein is measured by its enzymatic activity.

### **3.3. T7 RNA polymerase system for Puumala virus**

#### **3.3.1. Cell lines BHK T7-SIN and Vero T7 stably expressing T7 RNA polymerase**

Most of reverse genetics systems described to date utilize recombinant vaccinia virus as a source of bacteriophage T7 RNA polymerase (Dunn *et al.*, 1995; Lopez *et al.*, 1995; Prehaud *et al.*, 1997; He *et al.*, 1997; Hoffmann and Banerjee, 1997). Although vaccinia helper virus has many advantages such as cytoplasmic capping activity and high levels of expression, it has several drawbacks including the shut-off of nuclear transcription and cytopathic effects.

A modification of the initial Bunyamwera minireplicon system was introduced by replacing vaccinia virus with two cell lines, BHK T7-SIN and VeroT7, that stably express T7 RNA polymerase. Noncytopathic Sindbis virus replicons (SINrep19 T7) that allow long-term expression of T7 RNA polymerase in BHK-21 cells have been developed by Agapov *et al* (1998) and kindly provided by Charles M. Rice, Washington University School of Medicine, St.Louis, MO. These replicons contain the gene for puromycin resistance under control of the Sindbis subgenomic RNA promoter. Transfection of *in vitro* transcribed RNA of the SINrep19 T7 into BHK-21 cells produces a population of puromycin-resistant cells due to constitutive replication and transcription of the noncytopathic replicon. Long-term expression of an additional foreign gene has been achieved in cells transformed with replicons that contain a second subgenomic RNA promoter. Cells that carry a replicon that expresses T7 RNA polymerase can transcribe genes under the control of the T7 promoter following transfection of that DNA. The cell line BHK T7-SIN established based on this approach was used in the following experiments.

Another cell line, Vero T7, which stably expresses the T7 RNA polymerase, was supplied by Dr. X. Shi. It was established by transfecting VeroE6 cells, that support hantavirus replication, with the pAM8-1 vector (Zhang *et al.*, 1999), which contains chicken beta-

actin promoter followed by T7 RNA polymerase gene and Rous sarcoma virus promoter followed by the puromycin resistance gene in two reversely arranged expression units (Fig. 3.3).

### **3.3.2. Protein-expressing plasmids**

Two protein-expressing plasmids, pTMPUUS and pTMPUUL, were supplied by Dr. X. Shi.

**pTMPUUS plasmid for expression of Puumala virus N protein.** Puumala virus S segment cDNA (nt 43-1340) was cloned under control of a T7 promoter into an internal ribosome entry site (IRES)-containing cloning vector pTM1 allowing translation in a cap-independent manner (Moss *et al.*, 1990) between NcoI and PstI sites.

**pTMPUUL plasmid for expression of Puumala virus L protein.** The consensus Puumala L segment cDNA (6550nt) was assembled from different individual L clones and cloned between NcoI and XhoI sites of pTM1 cloning vector under control of T7 promoter and IRES.

#### **3.3.2.1. Expression of the N protein from pTMPUUS**

Metabolic labelling of Vero E6 cells infected with recombinant vaccinia virus vTF7-3 and transfected with pTMPUUS plasmid before (Fig. 3.4, A) and after (Fig. 3.4, B) immunoprecipitation with anti-PUU N antibody was performed to determine if the nucleocapsid protein of the correct size could be expressed from pTMPUUS.

The T7 vaccinia virus system was chosen as a simple approach to test the expression of the protein from T7 promoter-containing plasmid as it is known to give high levels of protein expression. In this system, the recombinant vaccinia virus vTF7-3 expresses T7 RNA polymerase. Infection with the virus prior to plasmid transfection results in

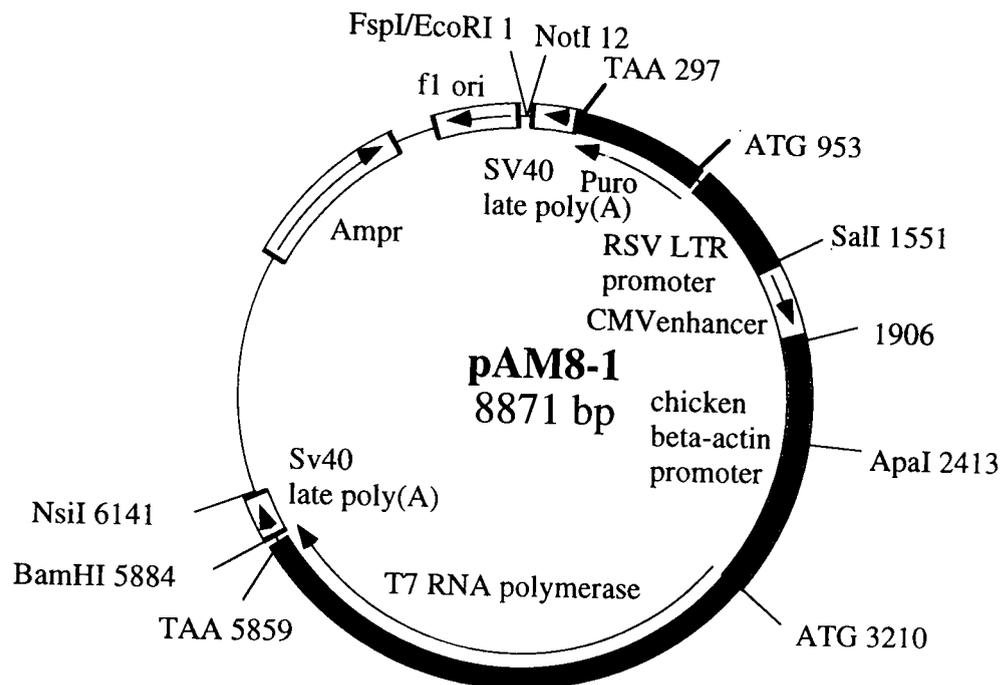


Fig.3.3. Plasmid map of a pAM8-1 vector (Zhang *et al.*, 1999) used to produce Vero T7 cells that stably express bacteriophage T7 polymerase. The plasmid contains chicken beta-actin promoter followed by T7 RNA polymerase gene and Raus sarcoma virus promoter followed by the puromycin resistance gene in two reversely arranged expression units. Transfection of the vector into Vero E6 cells and selection with puromycin produces a population of puromycin-resistant cells that stably express bacteriophage T7 polymerase

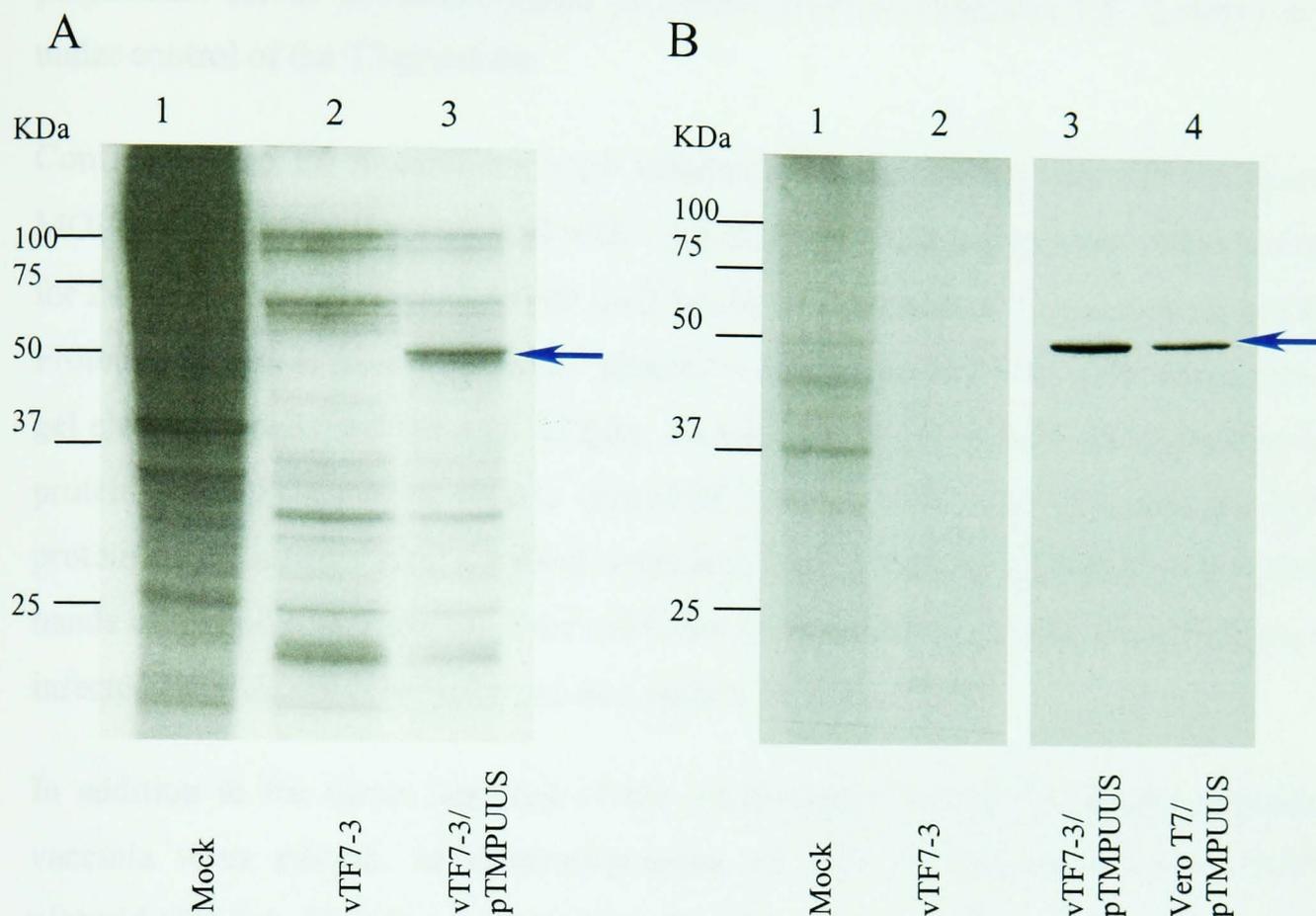


Fig. 3.4. Expression of PUU N protein from pTMPUUS.

**A.** Vero E6 cells were infected with vTF7-3 at 5 pfu/cell and transfected with 1  $\mu$ g pTMPUUS (lane 3). 24 hrs postinfection, the cells were labelled with 50 $\mu$ Ci  $^{35}$ S methionine for 2 hours. Proteins were analysed by 16% SDS-PAGE and autoradiography. The band corresponding to the protein of 50kDa in size is clearly seen on the gel (lane 3) which demonstrates that PUU N protein of a correct size is indeed produced from pTMPUUS expression plasmid. No band corresponding to N protein is seen in lysates from vTF7-3 infected (lane 2) or mock infected cells (lanes 1).

**B.** Immunoprecipitation of PUU N protein with anti-PUU N antibody. N protein was expressed from pTMPUUS either in Vero E6 cells infected with vTF7-3 as a source of T7 RNA polymerase (lane 3) or in VeroT7 cells stably expressing T7 polymerase (lane 4). Bands of the appropriate size corresponding to N protein are marked with a blue arrow. Cell lysates from mock (lane 1) and vTF7-3 (lane 2) infected were immunoprecipitated with anti-N antibody to demonstrate the specificity of the IP procedure. No bands corresponding to N protein were observed. Prestained molecular weight standards were run on the gels and their positions indicated at the left.

expression of T7 RNA polymerase in the cytoplasm of infected cells. The T7 RNA polymerase drives the transcription of mRNA from the plasmid that contains S gene under control of the T7 promoter.

Confluent Vero E6 monolayers were infected with the recombinant vTF7-3 virus at a MOI of 5 pfu/cell and transfected with 1 µg of pTMPUUS 1 hour later. After incubation for 24 hours, the cells were labelled for 2 hrs in the presence of <sup>35</sup>S methionine and lysed. Protein expression from transfected plasmid was analyzed by 16% SDS-polyacrylamide gel electrophoresis and autoradiography. As seen on the gel, a band corresponding to the protein of 50 kDa can be clearly identified (lane 3) which confirms that the PUU N protein of the correct size is indeed expressed from pTMPUUS plasmid. No equivalent bands of a similar size can be observed either in mock-infected (lane 1) or vaccinia virus infected (lane 2) cells that were run as negative controls.

In addition to the direct labelling of the nucleocapsid protein transiently expressed in vaccinia virus system, immunoprecipitation of PUU N expressed from pTMPUUS plasmid (Fig.3.4, B) either in Vero E6 cells infected with vTF7-3 (lane 3) or in Vero T7 cells stably expressing T7 RNA polymerase (lane 4) was performed. Proteins radiolabelled as described before were immunoprecipitated with anti-PUU N antibody (see Methods) and the immunoprecipitates were analysed by 16% SDS-PAGE.

Bands corresponding to the protein of 50 kDa were clearly detected (lane 3 and 4) indicating that PUU N protein of the correct size was successfully expressed both in vaccinia virus system and in Vero T7 cells expressing T7 polymerase. As negative controls, lysates from mock (lane 1) and vaccinia virus infected cells (lane 2) were immune-precipitated using anti-PUU N antibodies. No bands of the corresponding to the N protein size were detected.

This experiment demonstrated that Puumala virus N protein of the correct size (50 kDa) is indeed expressed from pTMPUUS construct using both T7 vaccinia virus system and also Vero T7 cells as alternative sources of the bacteriophage T7 polymerase.

### **3.3.3. 'Transcription' plasmids pPUUSCAT and pT7riboPUUSCAT**

In the first step toward the establishment of a reverse genetics system, a reporter gene is usually used to demonstrate functionality of the system. High conservation and complementarity of the terminal sequences of Puumala virus genome segments indicated that they are essential for recognition by the viral polymerase (see Chapter 1). Similar requirements were demonstrated by minigenome systems developed for Bunyamwera virus (Dunn *et al.*, 1995), influenza virus (Luytjes *et al.*, 1989), respiratory syncytial virus (Collins *et al.*, 1991) and Rift Valley fever virus (Lopez *et al.*, 1995).

To apply the reporter system to Puumala virus, assuming that the transcription and translation signals lie in the non-coding regions (untranslated regions, UTRs) of viral segments, it was decided to base two reporter constructs, pPUUSCAT and pT7riboPUUSCAT, on the most highly expressed segment (the S segment) to increase the levels of reporter gene expression. In hantavirus infected cells, the quantity of each mRNA transcripts correlates inversely with the length of the transcripts. This is also the case for bunyaviruses. However, the results of the reverse genetics system for Bunyamwera virus (Dunn, 2000) have shown that the efficiency of expression of the reporter gene in the context of each of the BUN segments, was in the order of L (taken as 100%)>M (60-70%)>>>S (3-10%), a complete inversion of the ratios of mRNA transcripts found in BUN virus infected cells. This suggests that the conditions of the reporter system differ from infected cells and additional factors must influence the transcript levels in the context of the infected cells. Therefore, it was decided to base one construct (pT7riboPUULCAT) on the L segment 3' and 5' UTRs. These reporter gene containing constructs, kindly supplied by Dr. X.Shi, employed T7 RNA-polymerase promoter and contained CAT gene in antisense orientation flanked by viral 5' and 3' UTRs. It was expected that transcripts from the reporter constructs would behave as authentic viral RNAs.

The terminal sequences are strictly conserved among different hantaviruses. Therefore, in developing a reverse genetics system for Puumala virus, the precise 3' and 5' terminal sequences were assumed to be essential for recognition by the viral polymerase and transcription. To achieve the exact 5' terminus, the viral 5' UTR was placed immediately downstream of the truncated T7 RNA promoter. In bacteriophage T7, RNA polymerase usually initiates on a guanosine residue immediately downstream of the conserved 17bp promoter (Fig.3.5, A). The next two transcribed nucleotides are also guanosines. In the systems developed for Bunyamwera virus (Dunn *et al.*, 1995) and influenza virus (Luytjes *et al.*, 1989), a truncated version of the T7 promoter lacking the last three guanosine (G) residues was used. The required sequences were therefore expected to be obtainable by replacing the usual G triplet with viral sequence.

Influenza virus and bunyavirus cRNA transcripts were successfully generated demonstrating that T7 RNA polymerase was able to initiate transcription starting with an adenine (Fig. 3.5, B). It was decided to use a similar approach for construction of reporter constructs for Puumala virus: the 5' UTR was cloned immediately downstream of a truncated T7 promoter lacking three G residues.

To ensure that the transcribed RNA would have 3' termini authentic to the viral sequence, the constructs contained either BbsI restriction site immediately downstream of PUU 3' UTR to produce run-off transcripts (plasmid pPUUSCAT) or self-cleaving hepatitis delta virus ribozyme sequence, HDV (plasmid pT7riboPUUSCAT) (Fig.3.6).

The plasmid pPUUSCAT directs the synthesis of a PUU virus-like RNA containing the coding region of the chloramphenicol acetyltransferase (CAT) gene. The antisense CAT coding sequence was cloned in place of the PUU S segment coding region in a negative-sense cDNA under control of a T7 promoter. A BbsI restriction site was engineered downstream of the PUU sequence to allow run-off transcription to terminate exactly at the 3' nucleotide of the PUU sequence therefore giving the precise 3' end. The complete untranslated regions of the PUU S segment were included since these were presumed to contain all the transcriptional control signals. Thus T7 transcripts derived from pPUUSCAT would have the authentic termini of PUU S segment RNA and would

A

ϕ T7 genes     ....TAATACGACTCACTATA <sup>+1</sup> **GGG** 

T7 promoter

B

	DNA			RNA
		+1		
BUN	....TAATACGACTCACTATA	<b>AGTAG</b>	TGT...	<b>A</b> GUAGUGU...
INFL	.... TAATACGACTCACTATA	<b>AGTAG</b>	AAA...	<b>A</b> GUAGAAA...
RVF	....TAATACGACTCACTATA	<b>ACACAAAG</b> ...		<b>A</b> CACAAAG...
HTV	....TAATACGACTCACTATA	<b>TAGTAGTAG</b> ...		<b>U</b> AGUAGUAG...

Fig. 3.5. T7 promoter initiation and requirements for RNA transcription by Bunyamwera virus, Rift Valley fever virus, influenza virus and hantavirus. **A.** The 17nt core promoter is written 5'-3' top strand. The optimum initiation nucleotides – three G residues, as found in bacteriophage T7 (Hamm *et al.*, 1990). **B.** DNA sequences transcribed into RNA are shown in bold with the first transcript base shown labelled +1. The alternative bases required for transcription of bunyavirus (BUN), influenza virus (INFL), Rift Valley fever virus (RVF) or hantavirus (HTV)-like transcripts. Except for hantavirus, the three viruses initiate transcription with an A residue (shown in red) and their transcripts were successfully generated. However, in the case of hantavirus, T7 polymerase would have to start transcription with a U residue (shown in blue).

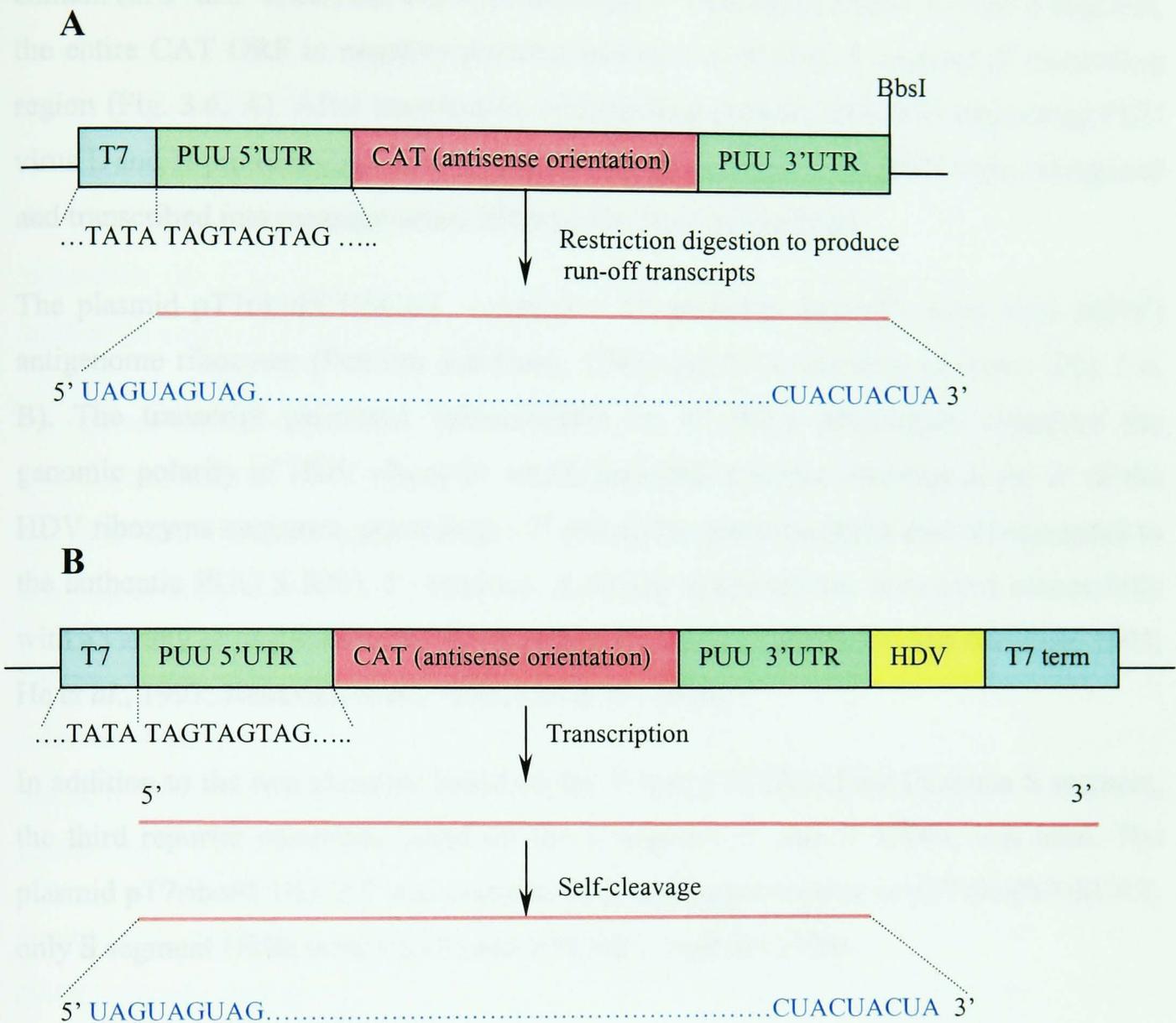


Fig. 3.6. Plasmid maps of reporter constructs pPUUSCAT (A) and pT7riboPUUSCAT (B). In both constructs, the coding sequence for CAT gene (in the antisense orientation) is flanked by the 5' and 3' UTRs of PUU virus S segment. Truncated T7 promoter (without three terminal G residues) defines exact viral 5' terminal sequence. The authentic 3' end of the RNA is specified by restriction digestion with BbsI (A) or by self-cleavage of the nascent RNA by the hepatitis delta virus (HDV) antigenome ribozyme (B). The conserved 9 terminal bases of Puumala virus S genome segment are shown in blue. T7, T7 promoter; T7term, T7 transcription termination sequence; HDV, hepatitis delta virus ribozyme sequence.

contain (in 5' to 3' order) the 442-nucleotide (nt) 5' noncoding region of PUU S segment, the entire CAT ORF in negative polarity, and the 43-nt PUU S segment 3' noncoding region (Fig. 3.6, A). After transfection of linearized plasmid into cells expressing PUU virus L and N proteins CAT activity would only be detected if the RNA were recognized and transcribed into message-sense RNA by the virus polymerase.

The plasmid pT7riboPUUSCAT contains a T7 promoter, hepatitis delta virus (HDV) antigenome ribozyme (Perrotta and Been, 1991) and T7 terminator sequence (Fig. 3.6, B). The transcript generated intracellularly by T7 RNA polymerase contained the genomic polarity of HDV ribozyme which provided autolytic cleavage at the 5' of the HDV ribozyme sequence, generating a 3' end of the upstream RNA that corresponded to the authentic PUU S RNA 3' terminus. A similar approach has been used successfully with a variety of negative-strand RNA viruses (Dunn *et al.*, 1995; De and Banerjee, 1993; He *et al.*, 1997; Neumann *et al.*, 1999; Lee *et al.*, 2000).

In addition to the two plasmids based on the 3' and 5' UTRs of the Puumala S segment, the third reporter construct, based on the L segment 3' and 5' UTRs, was used. The plasmid pT7riboPUULCAT was constructed in analogous manner as pT7riboPUUSCAT, only S segment UTRs were substituted with the L segment UTRs.

### **3.3.4. Transfection and optimization of conditions**

As was shown for Bunyamwera virus (BUN), transcription of the BUN-like RNA containing an antisense CAT gene was dependent on expression of the gene products of the BUN L and S segments and the level of CAT activity produced following transfection of the chimeric RNA was also dependent on the amount and ratio of these proteins synthesized in the cells (Dunn *et al.*, 1995).

The ability of Puumala virus L and N proteins expressed by transfected plasmids to transcribe the chimeric RNA template was investigated by transfection method using 'home-made' liposomes and then measuring CAT activity (see Methods).

Vero T7 and BHK T7-SIN cells were transfected with the mixture comprising two protein-expressing plasmids pTMPUUS, pTMPUUL and one of the reporter constructs: either pT7riboPUUSCAT or T7riboPUULCAT or pPUUSCAT. When plasmid pPUUSCAT was used, it was linearized by digestion with BbsI restriction enzyme and the cells were transfected with the linearized plasmid (see Methods for details). After incubation for 3 to 5 hours at 37°C, growth medium was added (10% DMEM) and incubation continued for 16 to 96 hours.

To optimize the conditions, different ratios and amounts of reporter construct and expression plasmids were used. The amount of pTMPUUL was in the range from 1 to 10 ng, 10 to 100 ng, 100 ng to 1 µg, and 1 to 5 µg, while reporter plasmid and pTMPUUS were taken in equal or different amounts in the range from 0.5 to 2.5 µg.

Different times of incubation were also tested and continued for up to 96 hours. When the cells were incubated for more than 48 hours, the transfection mix together with growth medium were replaced with maintenance medium (2% DMEM) and incubation continued.

Positive and negative controls were included in each experiment. Reporter construct alone was used as a negative control (Fig.3.7, lane 1). Without plasmids expressing PUU proteins no CAT activity would be possible. It would only be detected if the RNA were recognized and transcribed into message-sense RNA by the Puumala virus L and N proteins. The positive control was plasmid pMXCAT (lane 2) that contained CAT gene under control of T7 promoter. Upon transfection into the cells, it would be transcribed by T7 polymerase expressed intracellularly. Expression of CAT gene would result in measurable CAT activity. Each experiment was repeated at least twice, however none of the experiments gave positive results.

To make sure that the negative results were due to specific Puumala virus requirements and not to the requirements of the system, e.g. inactive liposomes, contamination, the reverse genetics system developed in our laboratory for Bunyamwera virus was tested. The original reconstituted system developed by Dunn *et al* (1995) and based on vaccinia

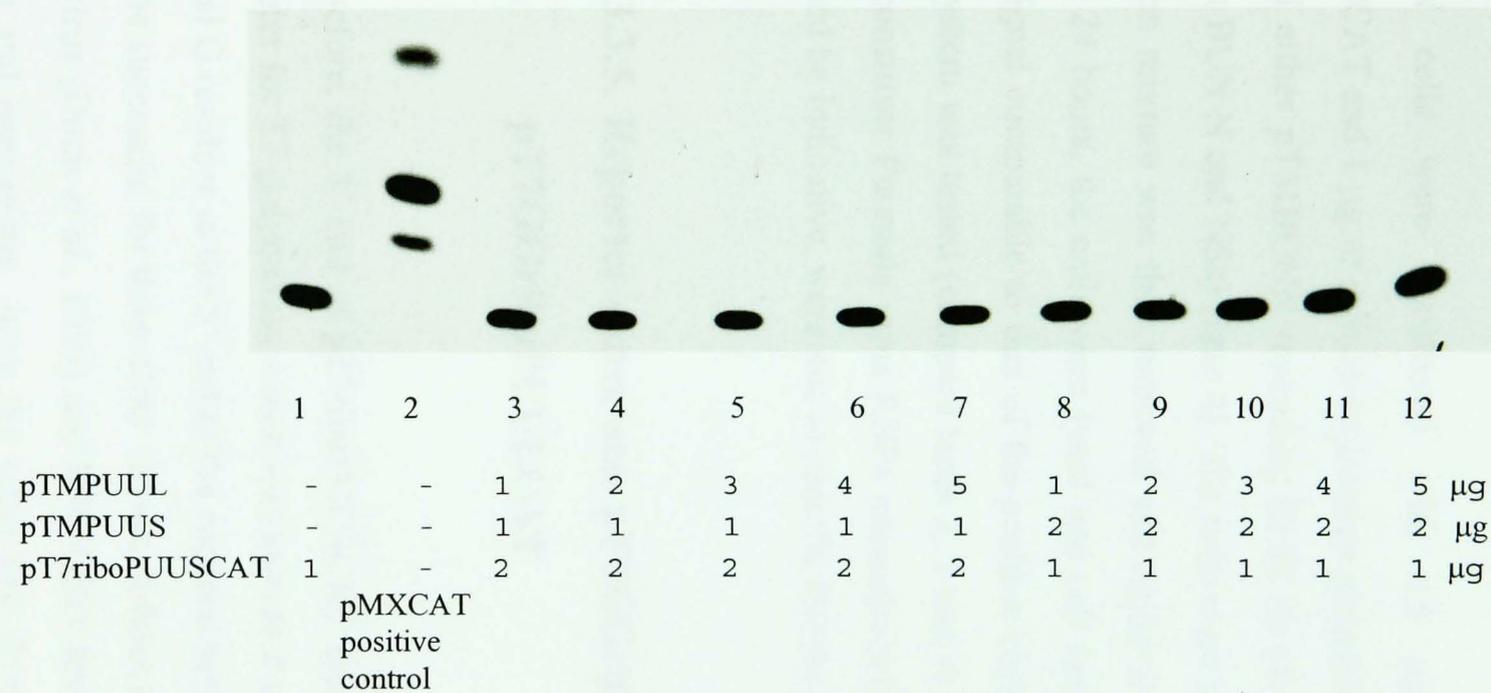


Fig. 3.7. Reporter gene expression from pT7riboPUUSCAT by recombinant Puumala virus proteins. Vero T7 cells (expressing bacteriophage T7 polymerase) were transfected with pTMPUUL (PUU L gene under control of T7 promoter), pTMPUUS (PUU N gene under control of T7 promoter) and a reporter construct pT7riboPUUSCAT (lanes 3-12). Cell extracts were prepared 72 hours posttransfection and assayed for CAT activity. Varying amounts of plasmids are indicated above. Lane 1, negative control: pT7riboPUUSCAT; lane 2, positive control: pMXCAT (CAT reporter gene under T7 promoter control)

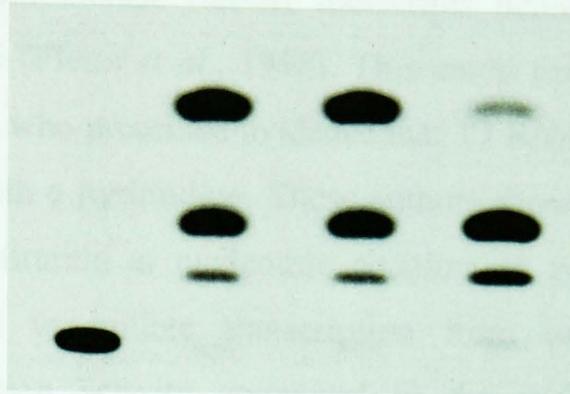
helper virus, vTF7-3, was subsequently modified to express a BUN minireplicon in T7-expressing cells (Weber *et al.*, 2001).

BHK T7-SIN cells were transfected with 0.5 µg of a reporter construct pT7riboBUNSCAT and 1 µg of protein-expression plasmids pTMBUNL (encoding BUN L protein) and either pTMBUNN (encoding BUN N) (Fig.3.8, lane 3) or pTMBUNS (encoding both BUN N and NSs) (lane 4). The cells were incubated at 37°C for 3 hours. The transfection mixture was then removed and replaced with growth medium. After incubation for 24 hours, the cells were lysed and cell lysates subjected to CAT assay. Strong CAT signal comparable to that of the positive control was obtained when BUN minireplicon system was tested (compare lanes 2, 3 and 4). This indicated that the failed attempts to reconstitute Puumala virus RNPs intracellularly, of which expression of the CAT gene would be indicative, were due to specific Puumala virus requirements.

### **3.3.5. Reporter constructs pT7GGriboPUUSCAT and pT7GGriboPUULCAT**

As described before, the 5' end of pT7riboPUUSCAT minigenome was defined by the adjacent promoter for T7 polymerase which was truncated so as not to have an extension of three nonviral G residues to the 5' end of the encoded minigenome. A similar approach was shown to be successful for three other viruses, influenza virus (Luytjes *et al.*, 1989), Bunyamwera virus (Dunn *et al.*, 1995) and Rift Valley fever virus (Lopez *et al.*, 1995). Influenza virus and bunyavirus have the same first five 5' nucleotides: the terminal nucleotide is an adenine residue which is followed by guanosine and then uridine (Fig. 3.5). The cRNA transcripts of these viruses were successfully generated demonstrating that T7 RNA polymerase was able to initiate transcription starting with an adenine residue. However, the hantavirus 5' sequence, unlike genome segments of these viruses, starts with a uridine followed by adenine and then guanosine.

Dunn and Studier (1983) have shown that T7 RNA polymerase can initiate transcription of its own viral genes with a protein-coding sequence in place of the flag-coding nucleotide. It has also been reported that, depending on the sequence downstream of the T7 promoter, the T7 RNA polymerase may initiate transcription from a region of heterogeneity of the 5' terminus (Chapman and Burgess 1987). We have been able to initiate transcription with a promoter that contains a G to T substitution in the T7 promoter (therefore expected to initiate approximately 50% transcription).



	1	2	3	4	
pTMBUNL	-	-	1	1	μg
pTMBUNN	-	-	1	-	μg
pTMBUNS	-	-	-	1	μg
pT7riboBUNSCAT	0.5	-	0.5	0.5	μg
		pMXCAT			

Another attempt to clarify this matter was made in the literature by making a reporter construct pBUNSCAT 5'(-), that contained a deletion of the first two BUN 5' nucleotides (AG) (Dunn, 2000). Initiation of transcription by T7 polymerase would therefore be expected to start from a G. However, the results were not conclusive. It was apparent that transcription from the cleavage site was initiated in the absence of BUN N protein.

**Fig. 3.8. Reporter gene expression from pT7riboBUNSCAT.** BHK T7-SIN cells (expressing bacteriophage T7 polymerase) were transfected with 0.5 μg reporter construct pT7riboBUNSCAT, 1 μg pTMBUNL (encoding BUN polymerase) and either 1 μg pTMBUNN (encoding BUN N protein) (lane 3) or pTMBUNS (encoding both BUN N and NSs proteins) (lane 4). Cell extracts were prepared 24 hours posttransfection and assayed for CAT activity. Lane 1, negative control: pT7riboBUNSCAT; lane 2, positive control: pMXCAT (CAT reporter gene under control of a T7 promoter)

still involved in the process. A second possibility was a weakly active T7 polymerase to give transcription that starts with either G or T. This would explain the previous experiments that suggested transcription from a G. We have now tested the immediate downstream of a T7 promoter by using a reporter construct pT7riboBUNSCAT and pT7riboBUNSCAT(-) that contain an A). A)

Dunn and Studier (1983) have shown that T7 RNA polymerase can initiate transcription of its own viral genes with a purine, either a guanosine or adenine, as the first encoded nucleotide. It has also been reported that, depending on the sequence downstream of the T7 promoter, the T7 RNA polymerase run-off transcripts demonstrate a degree of heterogeneity of the 5' termini (Pleiss *et al.*, 1998). This could explain the findings by Chapman and Burgess (1987) who presented evidence that T7 RNA polymerase may be able to initiate transcription with a pyrimidine. These authors showed that the construct that contained a G to T substitution at nucleotide position +1 within full length T7 promoter (therefore expected to initiate transcription from uridine at +1) gave approximately 80% transcription activity compared to the authentic T7 promoter sequence. However, this was a quantitative study of the transcripts produced rather than qualitative and no attempt was made to determine the actual viral 5' nucleotides produced. It is therefore possible that transcription from the position +1 was in fact initiated from the purine (guanosine) at position +2 as described by Pleiss *et al* (1998). Another attempt to clarify this matter was made in our laboratory by making a reporter construct pBUNSCAT 5'(-2) that contained deletion of the first two BUN 5' nucleotides (AG) (Dunn, 2000). Initiation of transcription by T7 polymerase would therefore be expected to start from a U. However, the results were not conclusive. It was assumed that transcription from this clone could be initiated with a purine (A) at position +2 and not a pyrimidine (U) at position +1 with respect to the T7 RNA promoter. An attempt was also made to determine the nature of the nucleotides at the 5' terminus of transcripts by using RNA ligation of the resulting run-off transcripts followed by reverse transcription and PCR with appropriate primer, however, it was unsuccessful.

Taking into account the fact that the question of transcription initiation with a uridine is still unresolved in the literature, it seemed possible that it would be more difficult for T7 polymerase to give transcription that starts with uridine. This could explain the failure of the previous experiments that employed constructs that had the viral sequence placed immediately downstream of a T7 promoter lacking G residues. Therefore, in the new reporter constructs, pT7GGribo PUUSCAT and pT7GGriboPUULCAT, it was decided to use a truncated version of the T7 promoter sequence containing two G residues (Fig. 3.9, A).

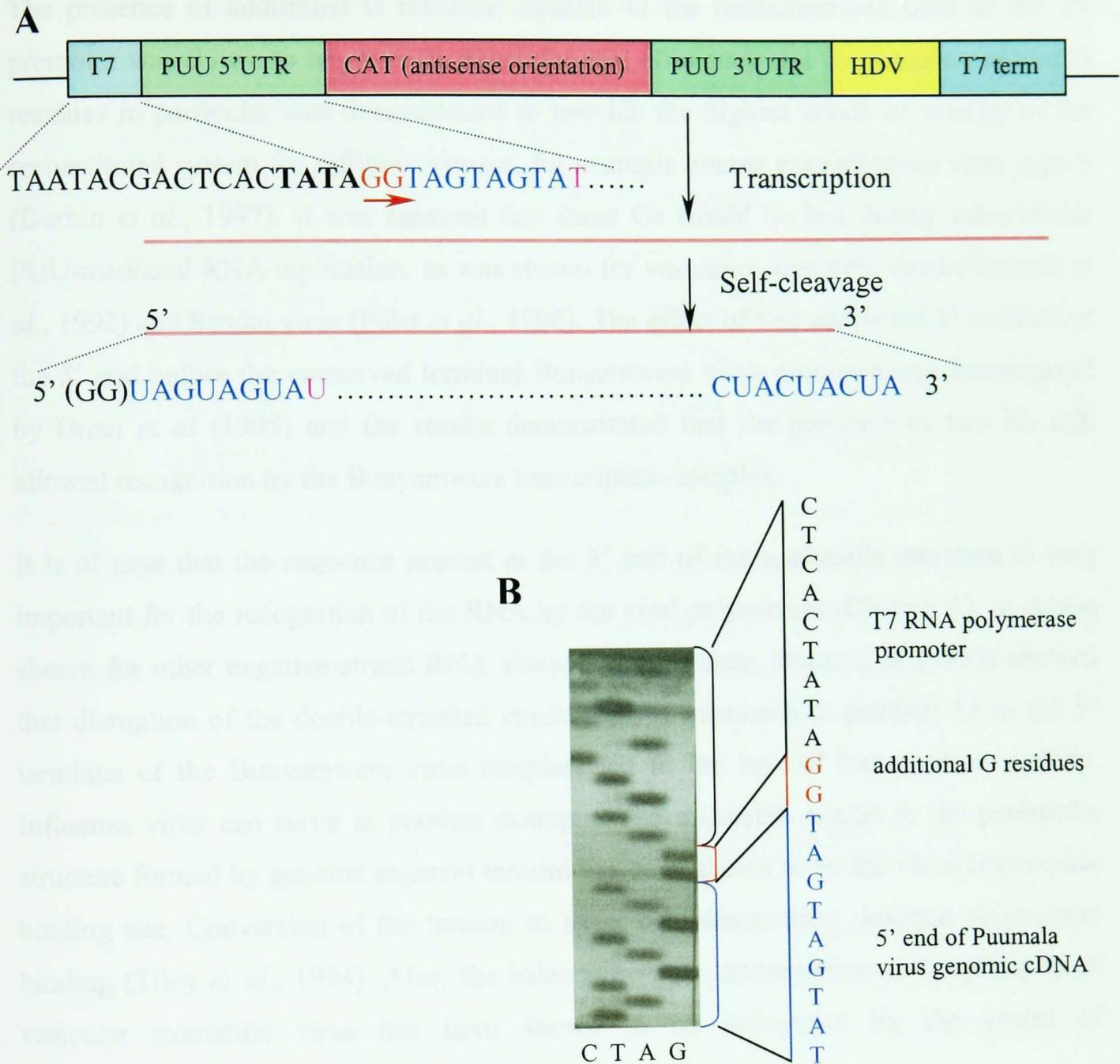


Fig.3.9. Plasmid map of the pT7GGriboPUUSCAT reporter construct. **(A)**. The coding sequence for CAT gene (in the antisense orientation) is flanked by the 5' and 3' UTRs of Puumala virus S segment. RNA transcripts produced by bacteriophage T7 RNA polymerase would contain two G residues, derived from T7 promoter, before the authentic Puumala virus 5' terminal sequence. The exact 3' end of the RNA is specified by self-cleavage of the nascent RNA by the hepatitis delta virus (HDV) antigenome ribozyme. The conserved 9 terminal bases of Puumala virus S genome segment (shown in blue) contain a mismatch in position 9 (shown in pink). T7, T7 promoter; T7term, T7 transcription termination sequence. **(B)**. 5'-end sequence analysis using pT7GGriboPUUSCAT as a template and T7 promoter primer. Partial sequence of T7 promoter is shown in black, two additional G residues in red, and 9 viral 5' terminal nucleotides with a mismatch in position 9 (T instead of G) are shown in blue.

The presence of additional G residues adjacent to the nontranscribed core of the T7 promoter was shown to improve its transcriptional efficiency and the presence of two G residues in particular was demonstrated to provide the highest levels of activity in the reconstituted system for different viruses, for example human parainfluenza virus type 3 (Durbin *et al.*, 1997). It was assumed that these Gs would be lost during intracellular PUU-mediated RNA replication, as was shown for vesicular stomatitis virus (Pattnaik *et al.*, 1992) and Sendai virus (Pelet *et al.*, 1996). The effect of two additional G residues at the 5' end before the conserved terminal Bunyamwera virus sequence was investigated by Dunn *et al.* (1995) and the results demonstrated that the presence of two Gs still allowed recognition by the Bunyamwera transcriptase complex.

It is of note that the sequence present at the 5' end of the panhandle structure is very important for the recognition of the RNA by the viral polymerase (Chapter 1), as it was shown for other negative-strand RNA viruses. For instance, Dunn *et al.* (1995) showed that disruption of the double-stranded structure by a mismatch at position 12 in the 5' terminus of the Bunyamwera virus template led to the loss of transcription activity. Influenza virus can serve as another example. The mismatch region in the panhandle structure formed by genome segment termini has been shown to be the virus polymerase binding site. Conversion of the termini to exact complementarity destroys polymerase binding (Tiley *et al.*, 1994). Also, the balance between transcription and replication of vesicular stomatitis virus has been shown to be influenced by the extent of complementarity of virus RNA termini (Wertz *et al.*, 1994).

However, the data on precise 5' terminal hantaviral sequences is somewhat inconsistent: some 5' and 3' termini are reported to be entirely complementary (Yao *et al.*, 2000; Sun *et al.*, 2000; Plyusnin *et al.*, 1994b; Plyusnin *et al.*, 1995; Kariwa *et al.*, 1999) while others have a mismatch in position 9 (Kukkonen *et al.*, 1998; Sun *et al.*, 2001; Piiparinen *et al.*, 1995; Huang *et al.*, 1996; Bowen *et al.*, 1995). If the authentic hantaviral sequence indeed has a mismatch at position 9 then restoration of complementarity could drastically affect recognition by the viral polymerase. That is why in the new version of the reporter constructs, in addition to two nonviral G residues at the 5' termini, it was decided to use a

sequence that would produce a mismatch in position 9, e.g., being TAGTAGTAT instead of TAGTAGTAG (Fig. 3.9, A, B).

Using new reporter constructs pT7GGriboPUUSCAT and pT7GGriboPUULCAT, a number of experiments were performed based on transfection conditions and different amounts and ratios of plasmids similar to the ones described before. Briefly, Vero T7 and BHK T7-SIN cells were transfected with mixture comprising two protein-expression plasmids pTMPUUS and pTMPUUL, and either pT7GGriboPUUSCAT or pT7GGriboPUULCAT reporter construct. Reporter plasmid and pTMPUUS were taken in equal or different amounts in the range from 0.5 to 2.5 µg. The amount of L protein expressing plasmid varied from 1 to 10 ng, 10 to 100 ng, 100 to 1 µg, and 1 to 5 µg. The cells were incubated for 3 hours at 37°C after which the transfection mixture was replaced by growth medium and incubation continued for additional 48 hours. Each experiment was repeated twice, however, none resulted in CAT expression.

### **3.3.6. Reporter construct pHH&HDVriboPUULCAT**

In another attempt to produce precise 5' viral termini, a new construct was used. In this minigenome reporter construct, a cDNA copy of a *cis*-active hammerhead ribozyme sequence (Birikh *et al.*, 1997) was cloned between the T7 RNA polymerase promoter and the L segment 5' UTR in pT7riboPUULCAT reporter construct (kindly supplied by Dr. X.Shi). Therefore, the exact 5' end would be produced by self-cleavage by a hammerhead ribozyme, while authentic 3' viral termini would be produced by self-cleavage by hepatitis delta virus ribozyme sequence (Fig. 3.10).

The same transfection conditions were applied as before and sets of experiments carried out. Briefly, Vero T7 and BHK T7-SIN cells were transfected with a mixture comprising protein-expression plasmids, pTMPUUS and pTMPUUL, and a reporter construct pHH&HDVriboPUULCAT. The reporter construct and N protein expressing plasmid were taken in the range from 0.5 to 2.5 µg, while the amount of pTMPUUL varied in the range from 100 ng to 5 µg. The cells were incubated for 3 hours and the transfection

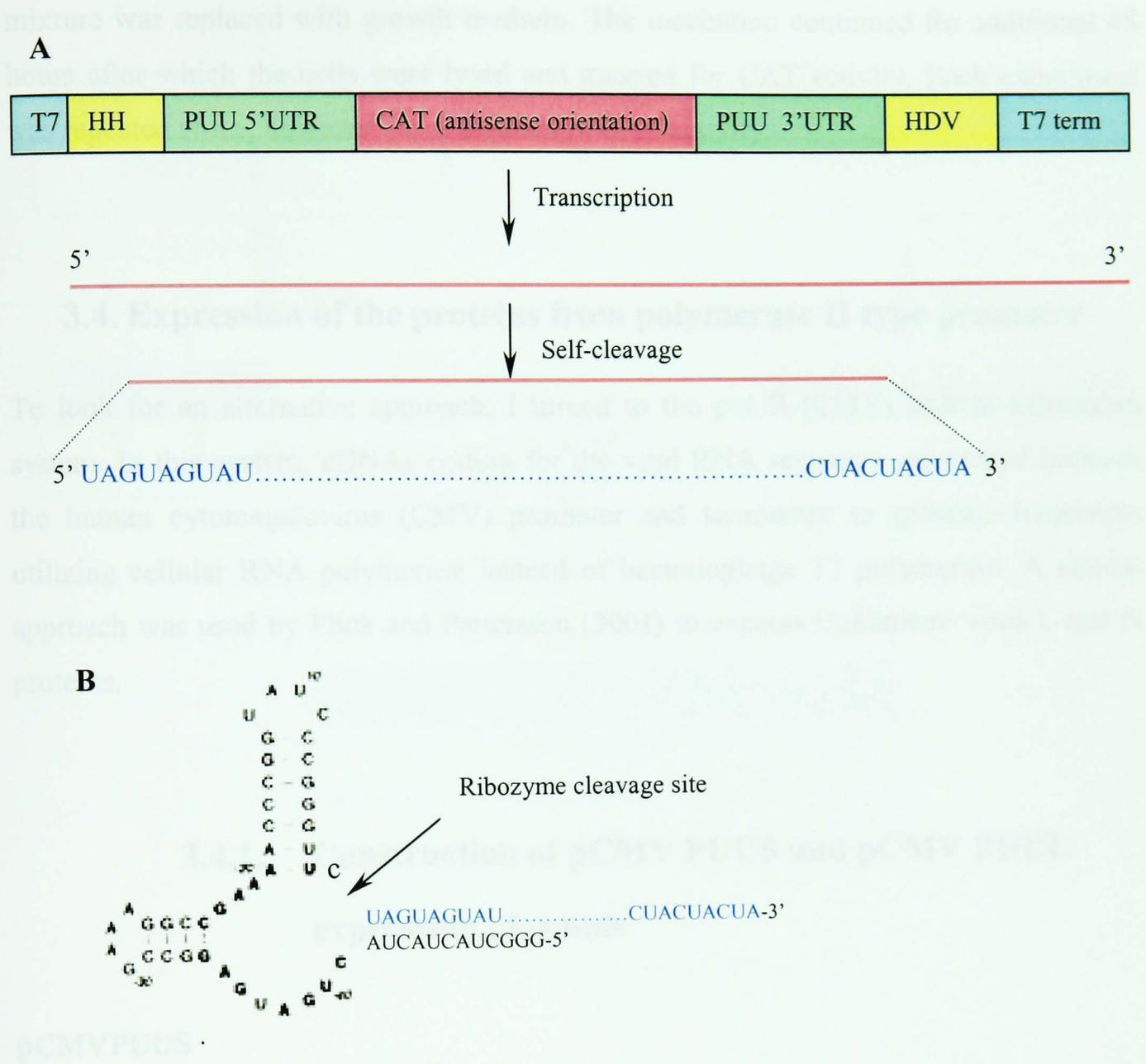


Fig. 3.10. Plasmid map of the reporter construct pT7HH&HDVriboPUULCAT. (A) The coding sequence for CAT gene (in the antisense orientation) is flanked by the 5' and 3' UTRs of PUU virus L segment. Exact viral 5' terminal sequence is defined by hammerhead ribozyme attached to the 5' UTR of Puumala L segment. The authentic 3' end of the RNA is specified by self-cleavage of the nascent RNA by the hepatitis delta virus (HDV) antigenome ribozyme. The conserved 9 terminal bases of Puumala virus S genome segment are shown in blue. (B). Secondary structure of the hammerhead ribozyme attached to the 5' end of the Puumala virus L segment. Core residues of the ribozyme are shown in bold; Puumala virus sequence is in blue. Numbering refers to the first Puumala virus nucleotide as 1. T7, T7 promoter; T7term, T7 transcription termination sequence; HH, hammerhead ribozyme; HDV, hepatitis delta virus ribozyme sequence.

mixture was replaced with growth medium. The incubation continued for additional 48 hours after which the cells were lysed and assayed for CAT activity. Each experiment was repeated twice, however, none resulted in CAT activity.

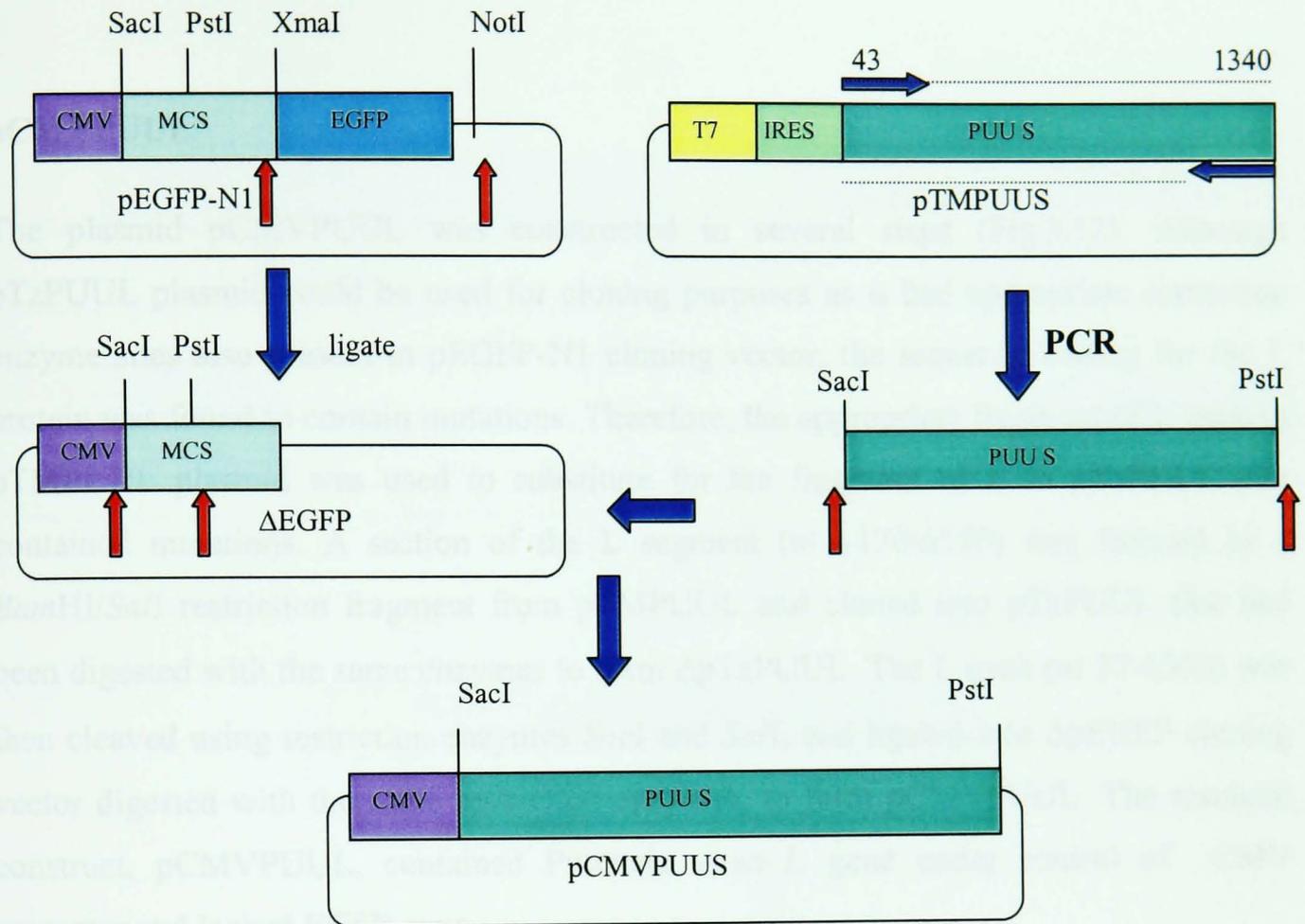
### **3.4. Expression of the proteins from polymerase II type promoter**

To look for an alternative approach, I turned to the pol II (CMV) protein expression system. In this system, cDNAs coding for the viral RNA segments are cloned between the human cytomegalovirus (CMV) promoter and terminator to generate transcripts utilizing cellular RNA polymerase instead of bacteriophage T7 polymerase. A similar approach was used by Flick and Pettersson (2001) to express Uukuniemi virus L and N proteins.

#### **3.4.1. Construction of pCMV PUUS and pCMV PUUL expression plasmids**

##### **pCMVPUUS**

pEGFP-N1 cloning vector, containing human cytomegalovirus (CMV) promoter and a gene coding for EGFP protein was cleaved with *XmaI* / *NotI* restriction enzymes to cut out EGFP gene to form  $\Delta$ EGFP vector (Fig.3.11). Puumala S segment (nt 43-1340) was PCR amplified using pTMPUUS as a template with primers incorporating *SacI* and *PstI* restriction enzyme sites. It was then digested with the same enzymes and cloned into  $\Delta$ EGFP that was cleaved with the same enzymes. The resultant construct pCMVPUUS contained S gene coding for N protein under control of CMV promoter



### 3.4.1.1. Expression of the N protein from pCMVPUUS

To verify that the Puumala virus N protein could be expressed from pCMVPUUS plasmid utilizing cellular RNA polymerase II, 293 cells transfected with the expression efficiency, were transfected with both of the plasmids. The cells were harvested

Fig. 3.11. Construction of pCMVPUUS plasmid expressing Puumala virus N protein under control of CMV promoter. pEGFP-N1 cloning vector, containing human cytomegalovirus (CMV) promoter and a gene coding for EGFP protein was cleaved with XmaI / NotI restriction enzymes to cut out EGFP gene to form ΔEGFP vector. Complete Puumala S segment was PCR amplified using pTMPUUS as a template with primers incorporating SacI and PstI restriction enzyme sites. It was then digested with the same enzymes and cloned into ΔEGFP that was cleaved with the same enzymes. The resultant construct pCMVPUUS contained S segment under control of CMV promoter.

immunoprecipitated using anti-Puumala N antibodies. The bands of the immunoprecipitate

## **pCMVPUUL**

The plasmid pCMVPUUL was constructed in several steps (Fig.3.12). Although pTzPUUL plasmid could be used for cloning purposes as it had appropriate restriction enzyme sites also present in pEGFP-N1 cloning vector, the sequence coding for the L protein was found to contain mutations. Therefore, the appropriate fragment of L gene in pTMPUUL plasmid was used to substitute for the fragment of L in pTzPUUL that contained mutations. A section of the L segment (nt 1170-6550) was isolated as a *Bam*HI/*Sal*I restriction fragment from pTMPUUL and cloned into pTzPUUL that had been digested with the same enzymes to form  $\Delta$ pTzPUUL. The L gene (nt 37-6550) was then cleaved using restriction enzymes *Sac*I and *Sal*I, and ligated into  $\Delta$ pEGFP cloning vector digested with the same restriction enzymes, to form pCMVPUUL. The resultant construct, pCMVPUUL, contained Puumala virus L gene under control of CMV promoter and lacked EGFP gene.

### **3.4.1.1. Expression of the N protein from pCMVPUUS**

To verify that the Puumala virus N protein could be expressed from pCMVPUUS plasmid utilizing cellular RNA polymerase II, 293 cells, known for their high transfection efficiency, were transfected with 1  $\mu$ g of this plasmid. The cells were incubated for 3 hours at 37°C after which the transfection mixture was replaced with growth medium and incubation continued for additional 24 hours. The cells were then labelled with 50  $\mu$ Ci per dish of  $^{35}$ S methionine for 2 hours and the protein immunoprecipitated with an anti-Puumala N antibody. The results of immunoprecipitation are presented in Fig.3.13. As seen on the gel, the band corresponding to the protein of 50 kDa in size can be clearly identified (lane 2). As negative control, lysate from mock cells (lane 1) was immunoprecipitated using anti-PUU N antibodies. No bands of the corresponding to the

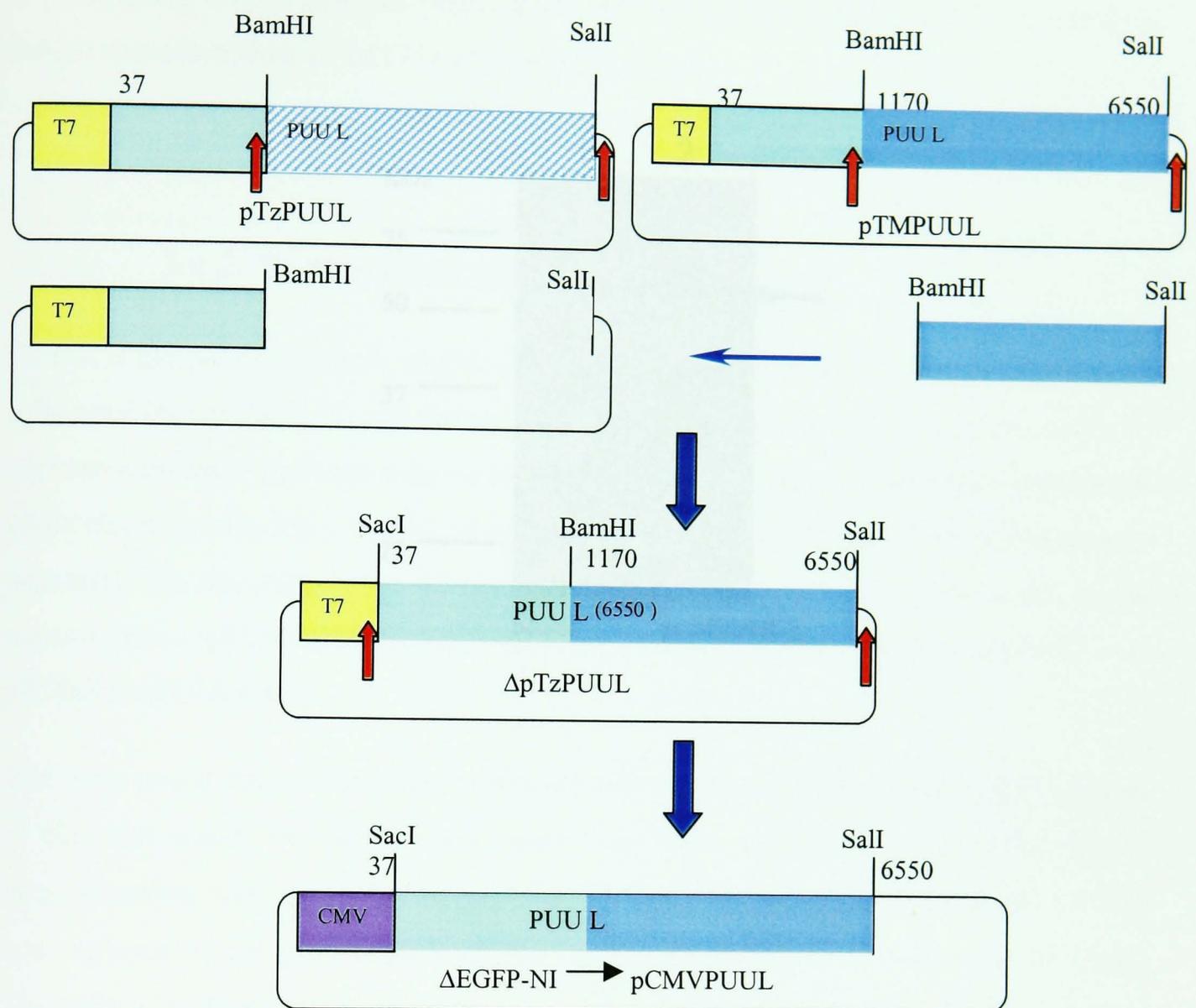


Fig. 3.12. Construction of pCMVPUUL plasmid expressing Puumala virus L protein under control of CMV promoter. A section of L segment (nt1170-6550) was isolated as a BamHI/SalI restriction fragment from pTMPUUL and ligated into the same sites of pTzPUUL to substitute for the same fragment of L containing mutations. The L segment was then cleaved from  $\Delta$ pTzPUUL using restriction enzymes SacI and SalI, and ligated into  $\Delta$ EGFP NI cloning vector that was digested with the same restriction enzymes to form pCMV PUUL.

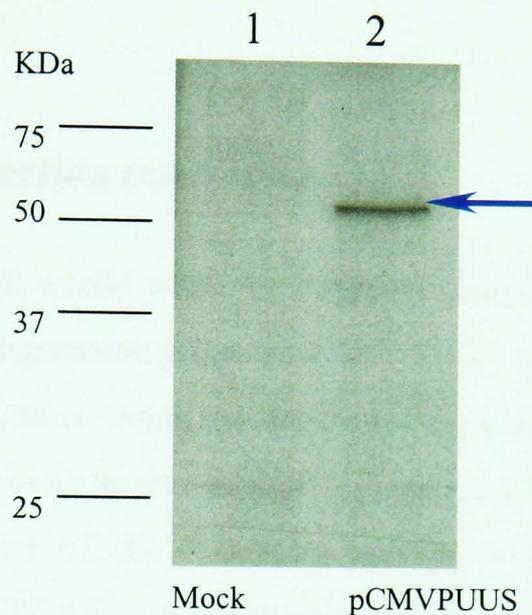


Fig.3.13. Expression of Puumala N protein from pCMVPUUS plasmid. 293 cells were transfected with pCMV PUUS plasmid (lane 2) and PUU N protein was expected to be expressed by the cellular polymerase II. The cells were labelled with 50  $\mu$ Ci  $^{35}$ S methionine for 2 hours. N protein was immunoprecipitated using anti-PUU N antibodies and analysed by 16% SDS-PAGE and autoradiography. Band of the appropriate size corresponding to N protein is marked with blue arrow. No band corresponding to N protein was observed in mock cell lysates used to demonstrate the specificity of the IP procedure (lane 1).

N protein size can be detected. This experiment demonstrates that Puumala N protein is indeed expressed from pCMVPUUS construct.

### **3.4.2. Transfection conditions**

To test if the pol II approach would work for Puumala virus, BHK T7-SIN and VeroT7 cells were transfected with expression plasmids pCMVPUUL and pCMVPUUS, and a T7 reporter construct. Both proteins would be expressed by the cellular RNA polymerase while the chimeric RNA would be transcribed by bacteriophage T7 RNA polymerase expressed by the cells. One of the following reporter constructs was used in the experiments: pT7riboPUUSCAT, pT7riboPUULCAT, pT7GGriboPUUSCAT or pT7GGriboPUULCAT.

The same conditions as ones described before were used and different ratios and amounts of plasmids tested. Briefly, the cells were transfected with the mixture containing all three plasmids, were incubated at 37°C for 3 hours after which the transfection mixture was replaced by the growth medium and incubation continued for additional 48 hours. The cells were lysed and assayed for CAT activity. The amount of pCMVPUUL was in the range from 10 ng to 5 µg, while pCMVPUUS and a reporter construct were in the range from 0.5 to 2.5µg. Each experiment was repeated twice, however, none gave positive results.

### **3.5. Polymerase I system**

To look for an alternative approach for developing a reverse genetics system for hantaviruses, it was decided to use the RNA polymerase I (pol I) expression system. This system, originally developed by Hobom and coworkers (Zobel *et al.*, 1993; Neumann *et al.*, 1994) has been used to study *cis*-acting sequences important for transcription and replication of influenza virus (Flick *et al.*, 1996). In the pol I system, cDNA coding for

reporter gene flanked by viral sequences, is cloned between the RNA pol I promoter and terminator. The chimeric RNA is produced by using cellular RNA polymerase I, which is among the most abundantly expressed enzymes in growing cells. Based on the results from the influenza system, pol I-driven transcription of the reporter RNAs will initiate and terminate exactly at the 5' and 3' ends of the inserted cDNAs, thus giving rise to transcripts with the correct vRNA or cRNA ends (Flick and Hobom, 1999; Zobel *et al.*, 1993). In the case of influenza virus, these pol I transcripts are then replicated and transcribed in the nucleus by the necessary viral proteins.

RNA pol I-catalyzed expression of chimeric viral RNAs was also successfully used for Uukuniemi virus (UUK), a member of the *Phlebovirus* genus, family *Bunyaviridae* (Flick and Pettersson, 2001). In this system, the chimeric cDNA (that contains the CAT gene flanked by the 5'- and 3'-terminal untranslated regions of the UUK virus sense or complementary RNA derived from the medium RNA segment) was cloned between the murine pol I promoter and terminator. After transfection into different eukaryotic cell lines, the resulting construct was transcribed by RNA pol I generating transcripts without any additional nucleotides or with modification at the 5' or 3' end (e.g., cap structure or poly (A) tail).

The possibility of application of the pol I system for Puumala virus was also investigated. The use of the human pol I promoter and highly transfectable human embryonic kidney cells (293T) (Hoffmann *et al.*, 2000; Neumann *et al.*, 1999; Neumann *et al.*, 2000) as opposed to the murine pol I promoter employed for expression of UUK chimeric RNA by Flick and Pettersson (2001) was expected to result in high efficiency of the reporter expression.

To generate the RNA polymerase I construct, the CAT reporter gene flanked by the 3' and 5' UTRs of the L segment was cloned into pHH21 cloning vector (Neumann *et al.*, 1999) between the human RNA polymerase I promoter and mouse RNA polymerase I terminator (kindly supplied by Dr. X.Shi) (Fig.3.14). In analogy to influenza virus protocol, the chimeric RNA was expressed from the pol I promoter while the necessary proteins were expressed from plasmids using the CMV promoter (pCMVPUUL and

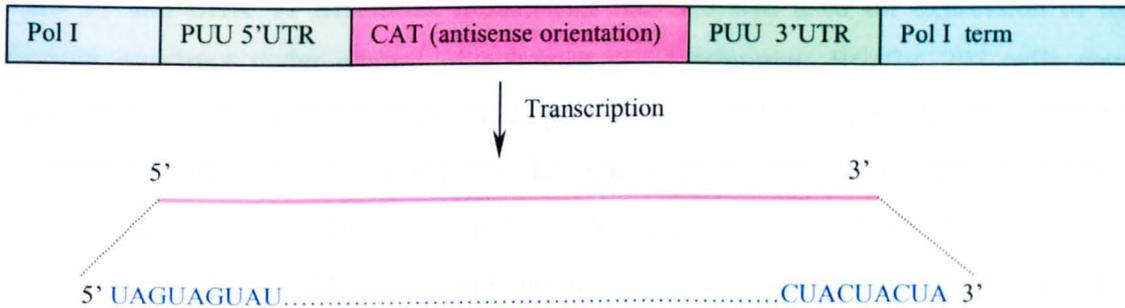


Fig. 3.14. Plasmid map of reporter construct pHH21PUULCAT (supplied by Dr. Shi). The cassette that contains coding sequence for CAT gene (in the antisense orientation) flanked by the 5' and 3' UTRs of PUU virus L segment was placed between human pol I promoter and mouse pol I terminator of the pHH21 cloning vector (Neumann *et al.*, 1999). Transcription by the ribosomal RNA polymerase results in production of transcripts that have exact viral 5' and 3' terminal sequence. The conserved 9 terminal bases of Puumala virus S genome segment are shown in blue. Pol I, human pol I promoter; pol I term, mouse pol I transcription terminator.

pCMVPUUS). Following transport to the cytoplasm, these pol I transcripts would be transcribed and replicated by the necessary viral proteins expressed from plasmids encoding L and N proteins.

Similar transfection conditions as described before were applied, however, instead of Vero T7 and BHK T7 SIN cells, human cell line 293 was used for expression of the reporter construct under control of a human pol I promoter. Briefly, 293 cells were transfected with two expression plasmids pCMVPUUS and pCMVPUUL and a reporter construct pHH21PUULCAT. All plasmids were taken in equal or different amounts in the range from 0.5 to 2.5µg. After incubation for 3 hours at 37°C, the transfection mixture was replaced with growth medium and incubation continued for 48 hours. The cells were lysed and assayed for CAT activity. Each experiment was repeated twice, however, none resulted in measurable CAT activity showing that this approach too failed.

### **3.6. Discussion**

Reverse genetics systems for negative-strand RNA viruses are based on intracytoplasmic reconstitution of the RNP complex, which represents the template for the viral polymerase, and is the prerequisite needed to start an infectious cycle. The expression systems most widely used depend on infection of cells with recombinant vaccinia virus (vTF7-3 or MVA) (reviewed by Palese *et al.*, 1996; Conzelmann, 1998), providing T7 RNA polymerase needed for expression of RNA and proteins from transfected plasmids. Reverse genetics systems for segmented negative-strand RNA viruses based on the T7 vaccinia virus expression system were developed for Bunyamwera virus (Dunn *et al.*, 1995), Rift Valley fever virus (Lopez *et al.*, 1995; Prehaud *et al.*, 1997) (both members of the *Bunyaviridae* family) and for lymphocytic choriomeningitis virus (a member of the *Arenaviridae* family) (Lee *et al.*, 2000). However, vaccinia virus introduces into the cell a

number of unwanted enzymatic activities. Therefore, a modification to an initial BUN minireplicon system was made by using cell lines BHK T7-SIN and Vero T7, stably expressing T7 RNA polymerase, which has proven to be suited for recombinant bovine respiratory syncytial virus mutants (Buchholz *et al.*, 1999) and was also used in a modified reconstituted BUN system (Weber *et al.*, 2001).

Minigenome systems developed for influenza virus (Luytjes *et al.*, 1989), Bunyamwera virus (Dunn *et al.*, 1995) and Rift Valley fever virus (Lopez *et al.*, 1995) used the antisense CAT reporter cDNA that is expressed by the T7-vaccinia virus system, while the L and N proteins are supplied either from vaccinia virus recombinants (BUN) or by T7 vaccinia expression system (RVF and also BUN) or *in vitro* reconstituted RNP (influenza virus). In the bacteriophage T7 promoter, a 17bp core is followed by three guanosine residues. Therefore, T7 RNA polymerase would normally start transcription from the first G residue. However, for these viruses it was important not to have any additional nonviral nucleotides as it would effect recognition and initiation of transcription by the viral polymerase. Therefore, a truncated version of the T7 promoter that lacked last three guanosine residues was employed. Transcription by T7 RNA polymerase would start from the first viral nucleotide. It is important to note, that the 5' terminal sequences of all three viruses start with an adenine residue. This did not seem to present a problem and RNA transcripts were successfully produced which indicated that T7 RNA polymerase was able to start transcription with an adenine. However, in the case of hantavirus it is a uridine residue, and it was thought to be unlikely that T7 RNA polymerase would give transcripts which start with uridine, as was described earlier in the chapter. Still, two different sets of constructs were used in experiments: those that contained a truncated T7 promoter with no G residues (pPUUSCAT, pT7riboPUUSCAT, pT7riboPUULCAT) and those containing two G residues preceding the viral sequence (pT7GGriboPUUSCAT and pT7GGriboPUULCAT). The rationale for the latter approach was that transcripts would start with a G, and not U corresponding to the viral sequence, and these Gs would be 'lost' after transcription thus giving authentic viral terminal sequence. In these constructs, the 3' viral termini was defined either by a *Bbs*I restriction site to give run-off transcripts or by a self-cleaving hepatitis delta virus ribozyme placed immediately downstream of the viral 3' UTR.

Another reporter construct containing hammerhead ribozyme sequence upstream of the 5' UTR was used in order to overcome the complications we might have had with previous constructs. Self-cleavage by the hammerhead ribozyme would produce the exact viral 5' termini. This approach was demonstrated to increase efficiency of poliovirus rescue (Herold and Andino, 2000).

Vero T7 and BHK T7-SIN cells stably expressing T7 polymerase were transfected with one of the reporter constructs and two expression plasmids, pTMPUUS and pTMPUUL that contain genes coding for Puumala N and L proteins, respectively, under the control of a T7 RNA polymerase. Different conditions were explored in an attempt to find the optimum ratios and amounts of reporter construct and expression plasmids. Various times of incubation during and after transfection were also tested. The positive outcome would be determined based on CAT enzyme expression. However, none of the approaches gave detectable CAT activity and thus was negative.

To look for an alternative approach, it was decided to express the proteins using polymerase type II (pol II) promoter as opposed to T7 driven expression. The protein-expressing plasmids were constructed by cloning Puumala S and L genome segments into pEGFP-N1 cloning vector under control of a human cytomegalovirus promoter (CMV), a polymerase II type promoter, in which case the viral proteins would be transcribed by the cellular polymerase instead of bacteriophage T7 polymerase. However, transfection of these expression plasmids together with one of the reporter constructs used before, did not result in measurable CAT activity.

The pol I reverse genetics system was applied as yet another alternative. It has the advantage of generating the exact 5' and 3' ends of the RNA transcripts, thus avoiding the need for expressing run-off transcripts from restriction enzyme-cleaved plasmids or the use of a hepatitis delta ribozyme to produce the correct 3' end. The use of a pol I promoter to generate transcriptionally competent influenza virus model RNAs has been previously reported by Neumann and co-workers (Neumann *et al.*, 1994; Neumann and Hobom, 1995). They used mouse pol I promoter and terminator sequences to ensure the correct formation of the 5' and 3' ends of model RNAs (Zobel *et al.*, 1993). Similar

results were achieved by using human pol I promoter and hepatitis delta virus genomic ribozyme sequences (Pleschka *et al.*, 1996). However, the main difference between the two pol I promoter-based systems was the source of the N and P proteins required for the replication and transcription of the vRNA. The use of pol II promoter-based plasmids instead of infectious influenza viruses to drive the expression of the N and P proteins was shown to result in approximately 100-times-higher levels of CAT expression.

The fact that pol I reporter transcripts are noncapped and nonpolyadenylated raised the concern that these RNAs would not be efficiently transported out of the nucleus. In the case of influenza virus, the pol I transcripts do not have to exit the nucleus, since transcription and replication of vRNAs take place in the nucleus. In contrast, hantaviruses replicate in the cytoplasm and the pol I transcripts therefore have to be exported from the nucleus.

However, as it was shown later by the results of Flick and Pettersson (2001) who applied similar approach for Uukuniemi (UUK) virus, a member of the *Bunyaviridae*, these concerns were unfounded. They suggested that newly synthesized nuclear RNA species rapidly associate with a set of proteins to form RNP structures. Some of these proteins contain an export signal and serve as export factors that guide the RNPs to and through the nuclear pore complex (Nakielny and Dreyfuss, 1999). They speculated that such an export factor(s) binds to the chimeric reporter RNA and facilitates its export to the cytoplasm (Flick and Pettersson, 2001).

To apply the pol I system for Puumala virus, the cells were transfected with reporter construct under control of a human pol I promoter and protein-expressing plasmids under control of CMV promoter. Again, different transfection conditions were explored and similar to the previous experiments they did not give any positive results.

Several different approaches were tested and although similar expression systems have been used for Bunyamwera virus, Rift Valley fever virus, Uukuniemi virus and negative-strand RNA viruses of other families such as Sendai virus, rabies virus, vesicular stomatitis virus, human parainfluenza virus and influenza virus, none of them was

efficient for Puumala virus. The possible explanation for this could be that Puumala L protein was not functional.

Successful development of the minigenome system depends on having biologically functional cDNAs of each gene. It was not possible to detect expression of the L protein either after *in vitro* transcription-translation in a reticulocyte lysate or after transfection of the plasmid pTMPUUL into cells either infected with the recombinant vaccinia virus expressing T7 RNA polymerase or cell lines stably expressing T7 RNA polymerase, because of the naturally low level of expression of this viral protein and the lack of specific antibodies.

The only information available was the identity of nucleotide sequence of the L segment cDNA to the published sequences. However, these sequences were never proven to reflect the authentic functional hantavirus RNA polymerase protein.

This presented a problem, as it was reported that completely conserved residues are essential for the full biological activity of polymerase proteins of such viruses as Bunyamwera virus (Jin and Elliott, 1992; Dunn *et al.*, 1995), Sendai virus (Chandrika *et al.*, 1995) and vesicular stomatitis virus (Sleat and Banerjee, 1993). For example, it was shown that even single mutation in the conserved sequence can drastically affect recognition of the template by Bunyamwera virus proteins. It was demonstrated that certain mutations in the putative polymerase motifs, which are conserved among all RNA-dependent RNA polymerases abolished the RNA synthesis capability of L protein, whereas mutations in unconserved sites still gave rise to functional L protein. Mutation of the conserved Asp at position 1037 in motif A, Asn at position 1119 in motif B, or Asp at position 1165 in motif C resulted in nonfunctional L protein (Poch *et al.*, 1989, Jin and Elliott, 1992; Dunn *et al.*, 1995). Similar to the results obtained for bunyavirus L protein, Chandrika *et al* (1995) showed that completely conserved residues are essential for the full biological activity of L protein of Sendai virus. In two other studies mutational analysis of conserved domain III of the L protein showed this region was important for viral RNA synthesis. Sleat and Banerjee (1993) found overall similar results by mutation of the GDNQ sequence in the vesicular stomatitis virus L protein in domain III, where

changes in conserved amino acids usually yielded proteins inactive in transcription *in vitro*, while substitutions in the three amino acids downstream of this site retained some activity.

To date, it still remains unknown whether Puumala virus L protein was functional as one of the ways to demonstrate this would be to intracellularly reconstitute viral RNPs containing reporter CAT gene, expression of which would result in measurable CAT activity.

Another possibility to check the authenticity of the recombinant L protein would be to assay it for the RNA-dependent RNA polymerase activity. Jin and Elliott (1991, 1993) have described a method to assay the polymerase activity of Bunyamwera virus recombinant L protein. The method relies on the fact that viral RNPs purified on CsCl gradients are inactive for transcriptase activity, probably because the L protein is dissociated or inactivated by high salt concentrations. These authors demonstrated that transcription was restored when these RNPs were transfected into cells expressing recombinant L protein.

It is also possible to test different Puumala virus L clones in the minigenome system employing one of the approaches described in the chapter. Assuming that the unsuccessful attempts to develop the reverse genetics system for Puumala virus were due to L protein not being functional, by testing different L clones it would be possible to identify the one which would work.

## **CHAPTER 4: ANALYSIS OF PUUMALA VIRUS PROTEIN INTERACTIONS**

### **4.1. Interaction between Puumala virus N proteins**

#### **4.1.1. Introduction**

Hantavirus N protein seems to be expressed in excess in infected cells and has been reported to form large granular to filamentous inclusion bodies in the cytoplasm (Hung, 1988; Vapalahti *et al.*, 1995; Ravkov *et al.*, 1998; Ravkov and Compans, 2001). N protein coding regions have been sequenced from a number of hantavirus strains and are well conserved. N is a major antigenic protein. The N proteins of Puumala virus strains vary by up to 5% at the N amino acid level with few effects on their antigenic properties (Plyusnin *et al.*, 1994; Plyusnin *et al.*, 1995).

Hantavirus N proteins are central to the process of virus assembly. Each genomic vRNA associates with the N protein and viral polymerase to create three distinct ribonucleoprotein complexes (RNPs) (Schmaljohn *et al.*, 1983). The N protein has been shown to bind viral RNAs (vRNA) *in vitro* - this function is essential to its role in encapsidation and RNP complex formation, and might have a regulatory role in the viral life cycle (Gott *et al.*, 1993; Severson *et al.*, 1999; Severson *et al.*, 2001). The protein is also likely candidate to bind to the viral glycoproteins during virus assembly or budding. Apart from the role it plays in the formation of the RNP, the hantavirus N protein has been suggested to play a functional role in replication and transcription. In particular, the N protein may modulate the switch of virus RNA synthesis from transcription to replication, as reported for analogous proteins in other negative-strand viruses (Patton *et al.*, 1984; Beaton and Krug, 1986; Honda *et al.*, 1988). It was also shown to interact with Daxx, a protein identified as a Fas-mediated apoptosis enhancer (Li *et al.*, 2002).

To summarize, hantavirus nucleocapsid protein is not only a structural RNA-binding protein that encapsidates the virus genome for the purposes of RNA transcription, replication and packaging, but it also functions as a key adapter molecule between virus and host cell processes through its ability to interact with a wide variety of viral and cellular macromolecules, including RNA, itself, L protein, and cellular polypeptides including actin.

However, the main feature of the protein remains its ability to interact with molecules of one another as homotypic interaction and multimerization are necessary prerequisites of N for packaging and protecting the viral genome. The fact that N aggregates in the cytoplasm of infected cells, forming filamentous structures, also suggests that it is able to self-interact.

To obtain direct evidence of homologous interaction between Puumala virus nucleocapsid proteins and to map the domains of the protein involved in interaction, the mammalian two-hybrid assay system (M2HS) was used (Fields and Sternglanz, 1994). The M2HS is based on the yeast two-hybrid system (Y2HS) developed earlier (Fields and Song, 1989), which provided a genetic approach to identify proteins that interact physically *in vivo*. In addition, the system helps to define contacts among the subunits of multiprotein complexes, as well as to map specific domains within proteins that are responsible for interaction. However, the Y2HS has significant limitations. The host yeast, although a eukaryote, is far removed from human, other mammalian, or higher eukaryotic organisms. Due to the fact that mammalian proteins are likely to retain their native conformation in a mammalian host, and the results would probably represent biologically significant interactions, the use of the M2HS to study protein-protein interactions is therefore more appropriate than the use of the yeast system (Dang *et al.*, 1991).

The mammalian system is similar to the one developed in yeast. Variations for use in mammalian cells were introduced by Vasavada *et al.* (1991) and Takacs *et al.* (1993). It exploits the modular nature of a transcriptional activator that contains two domains, a DNA-binding domain (BD) and a transcription-activation domain (AD). Neither domain alone can activate transcription, and only their reconstruction *in trans* restores activity. This objective is achieved by making two fusion proteins. The first

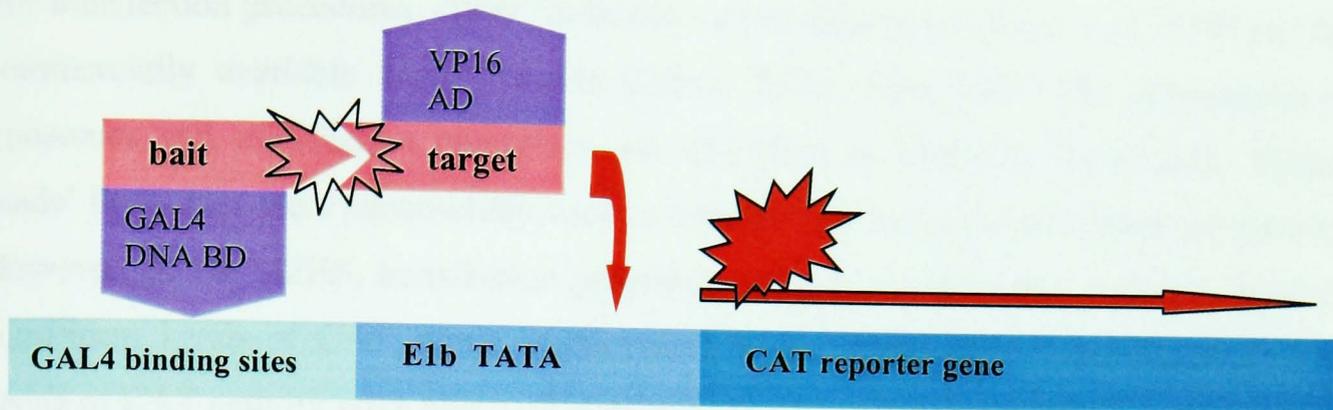
fusion is between the BD and a bait protein, and the second fusion is between AD and a prey or target protein. Both fusions are expressed in the presence of a reporter gene. The fusion proteins are transported to the nucleus where the DNA-BD binds to a specific promoter sequence, GAL4 binding sites, upstream of a reporter gene on a reporter plasmid, and the AD directs the RNA Polymerase II complex to transcribe the downstream reporter gene. The interaction of these two fused proteins restores activation of the transcriptional activator, and turning on the reporter gene allows the cells to be identified (Fig 4.1). The reporter gene used is the chloramphenicol acetyltransferase (CAT) which is detected by the ability of its product to acetylate chloramphenicol in the presence of acetyl CoA.

I used a commercial system, the Mammalian Matchmaker Two-Hybrid assay kit (Clontech). The reporter plasmid, pG5CAT, contains the CAT gene downstream of 5 consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. The minimal E1b promoter does not drive expression of significant levels of the CAT gene, so background should be low in the absence of activation from the GAL4 sites. The pM cloning vector is used to generate fusions of the bait protein to the GAL4 DNA-BD. Similarly, pVP16 is used to construct fusions of the target protein to an AD derived from the VP16 protein of herpes simplex virus. Plasmid pM3VP16, consisting of the AD fused to the BD and thus being a transactivator by itself, is used as a positive control.

#### **4.1.2. Optimisation of conditions**

A number of different cell lines and two DNA transfection techniques were used to optimise the M2HS. Two positive controls were used to test the system. The first one was the Mammalian Matchmaker Two-Hybrid Assay kit plasmid pM3VP16 (Clontech), consisting of the AD fused to the BD and thus being a transactivator by itself. The second positive control included bait and target proteins known to interact and give a positive signal in the M2HS. These included plasmids pAASN and pSGN encoding Bunyamwera virus (BUN) N protein (Osborne, 2001). Three different cell lines, HeLa, 293, and BHK-

A



B



Fig. 4.1 The mammalian two-hybrid system. The DNA-binding domain (BD) binds to a specific promoter sequence, Gal4 binding sites, upstream of a reporter gene on a reporter plasmid pG5CAT, and the AD directs the RNA Polymerase II complex to transcribe the downstream CAT reporter gene. This is possible only when two proteins, one fused as the bait to BD and the other as the target fused to AD, interact, which restores activity of the transcriptional activator resulting in CAT expression (A). If the proteins do not interact the CAT gene remains inactive (B).

21, were tested for their ability to support the M2HS and optimize the results (Fig 4.2). For transfection procedures, either 'in-house'- made liposomes (Rose *et al.*, 1991) or the commercially available Lipofectamine (Gibco BRL) were used. The preparation of liposomes and transfection conditions are described in Methods. Previously, 'home-made' liposomes were successfully used to transfect various cell lines (data not shown). However, in the M2HS, transfection procedure using these liposomes failed to produce significant levels of CAT signal as compared to the commercial Lipofectamine. Low levels of CAT activity were observed in BHK cells (Fig.4.2, A) using both liposomes and Lipofectamine when only the commercial positive control pM3VP16 was used (lanes 1 and 3), but not for BUN N positive control (lanes 2 and 4). Since BHK cells did not yield reproducible and reliable results, they were not used further. Similar results were achieved when the 293 cell line, known to be highly efficient in transfections, was used (B). In this case, transfection using both techniques resulted in strong CAT signal for transactivation with pM3VP16 (lanes 1 and 3). No interaction was observed between positive control BUN N proteins (lanes 2 and 4). HeLa cells (C) showed strong CAT activity not only for commercial positive control, plasmid pM3VP16 (lanes 1 and 3), but also for BUN N proteins (lanes 2 and 4), when the cells were transfected at 30-50% confluency. Therefore, it was decided to carry out the following experiments on identification of protein-protein interaction in hantavirus using HeLa cells and the commercial transfection reagent Lipofectamine. 1 µg of each plasmid was used unless otherwise stated as this amount was sufficient to produce a strong CAT signal.

Subsequent experiments were done in duplicate, and three independent transfections were performed. To eliminate false positive interactions negative controls were included in each experiment. A plasmid encoding the protein fused to AD was cotransfected with an empty pM vector to ensure that the target protein did not function autonomously as a DNA-BD or bind directly to the DNA-BD encoded by pM vector. A plasmid encoding the protein fused to BD was cotransfected with an empty pVP16 vector to determine whether or not the bait protein functions autonomously as a transactivator. In addition, all the constructs used in work were tested for their ability to activate CAT expression by interaction with an 'empty' BD or AD vector.

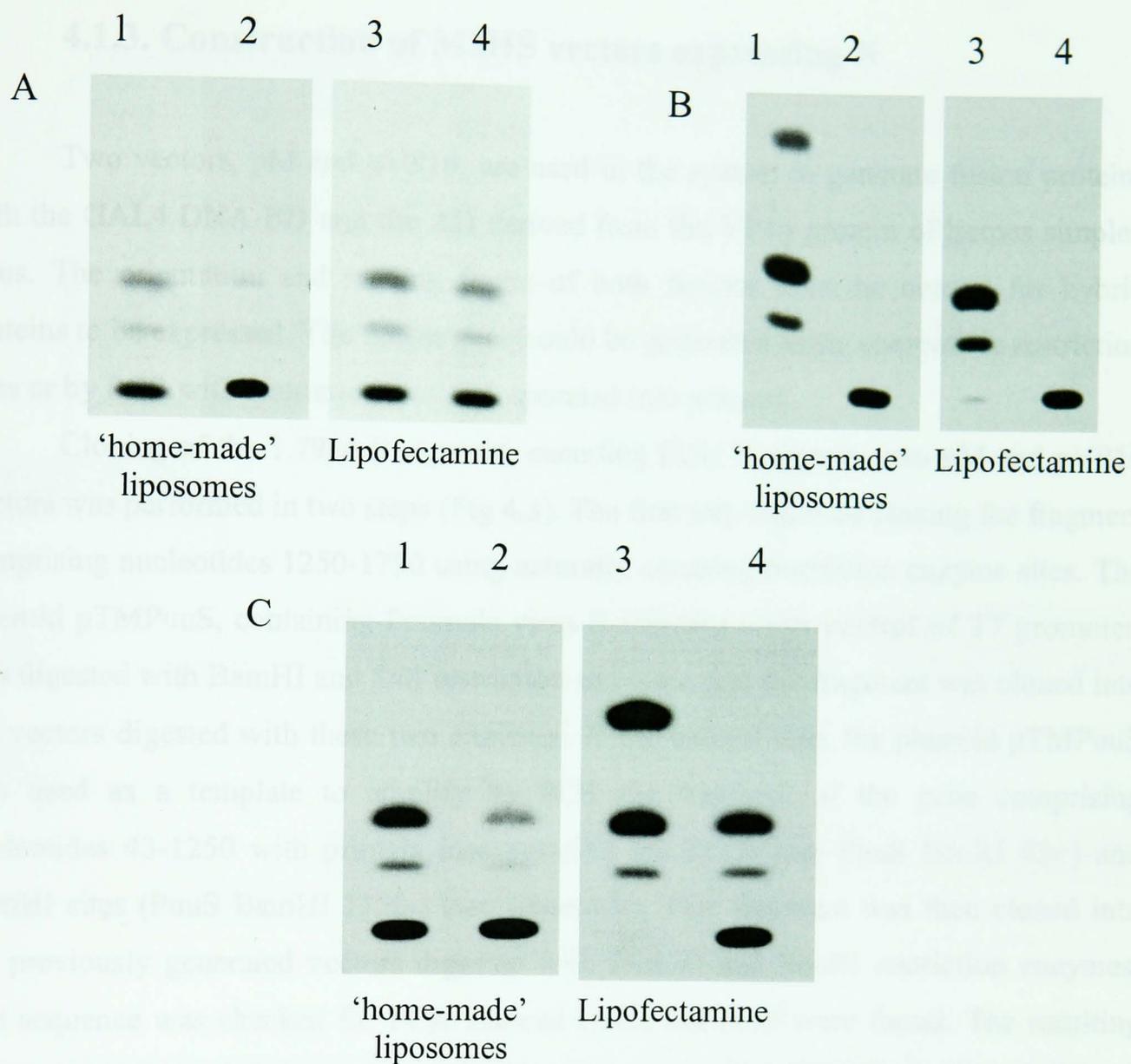


Fig. 4.2. Optimisation of the mammalian two-hybrid system. The cell lines HeLa, 293, and BHK-21 were tested for their ability to support the M2HS using pM3VP16 (lanes 1 and 3) and pAASN and pSGN (lanes 2 and 4) as positive controls. Transfection with 'home-made' liposomes and Lipofectamine were used in each case. A poor signal was obtained with BHK cells using both methods (A). 293 cells (B) gave only strong signal for commercial positive control but not for proteins known to interact. At a low confluency (50%) HeLa cells provided strong signal for both (C). 1  $\mu$ g of each plasmid was used for transfections.

### **4.1.3. Construction of M2HS vectors expressing N**

Two vectors, pM and pVP16, are used in the system to generate fusion proteins with the GAL4 DNA-BD and the AD derived from the VP16 protein of herpes simplex virus. The orientation and reading frame of both fusions must be correct for hybrid proteins to be expressed. The fusion gene could be generated using compatible restriction sites or by PCR with restriction sites incorporated into primers.

Cloning of the 1.79kb S segment, encoding PUU N protein, into pM and pVP16 vectors was performed in two steps (Fig 4.3). The first step involved cloning the fragment comprising nucleotides 1250-1790 using naturally occurring restriction enzyme sites. The plasmid pTMPuuS, containing Puumala virus S segment under control of T7 promoter, was digested with BamHI and SalI restriction enzymes, and the fragment was cloned into the vectors digested with these two enzymes. In the second step, the plasmid pTMPuuS was used as a template to amplify by PCR the fragment of the gene comprising nucleotides 43-1250 with primers incorporating EcoRI (primer PuuS EcoRI 43+) and BamHI sites (PuuS BamHI 1250-) (see Materials). This fragment was then cloned into the previously generated vectors digested with BamHI and EcoRI restriction enzymes. The sequence was checked for PCR-induced errors but none were found. The resulting constructs contained the sequence encoding N protein with a BD (designated pMPuuN) or AD (designated pVPPuuN) fusion tag at the N-terminus.

### **4.1.4. Homologous N-N interactions in the M2HS**

Once plasmids pMPuuN and pVPPuuN were constructed, they were used to investigate possible homologous interaction between PUU N proteins. The relative amounts of each plasmid were titrated against one another to maximise CAT signal (Fig.4.4). The level of CAT activity was not affected by the increase in the amount of plasmid pMPuuN against constant amount of pVPPuuN (lanes 5-8, and 9-12). However, when both plasmids were taken in equal increasing amounts, the level of CAT activity

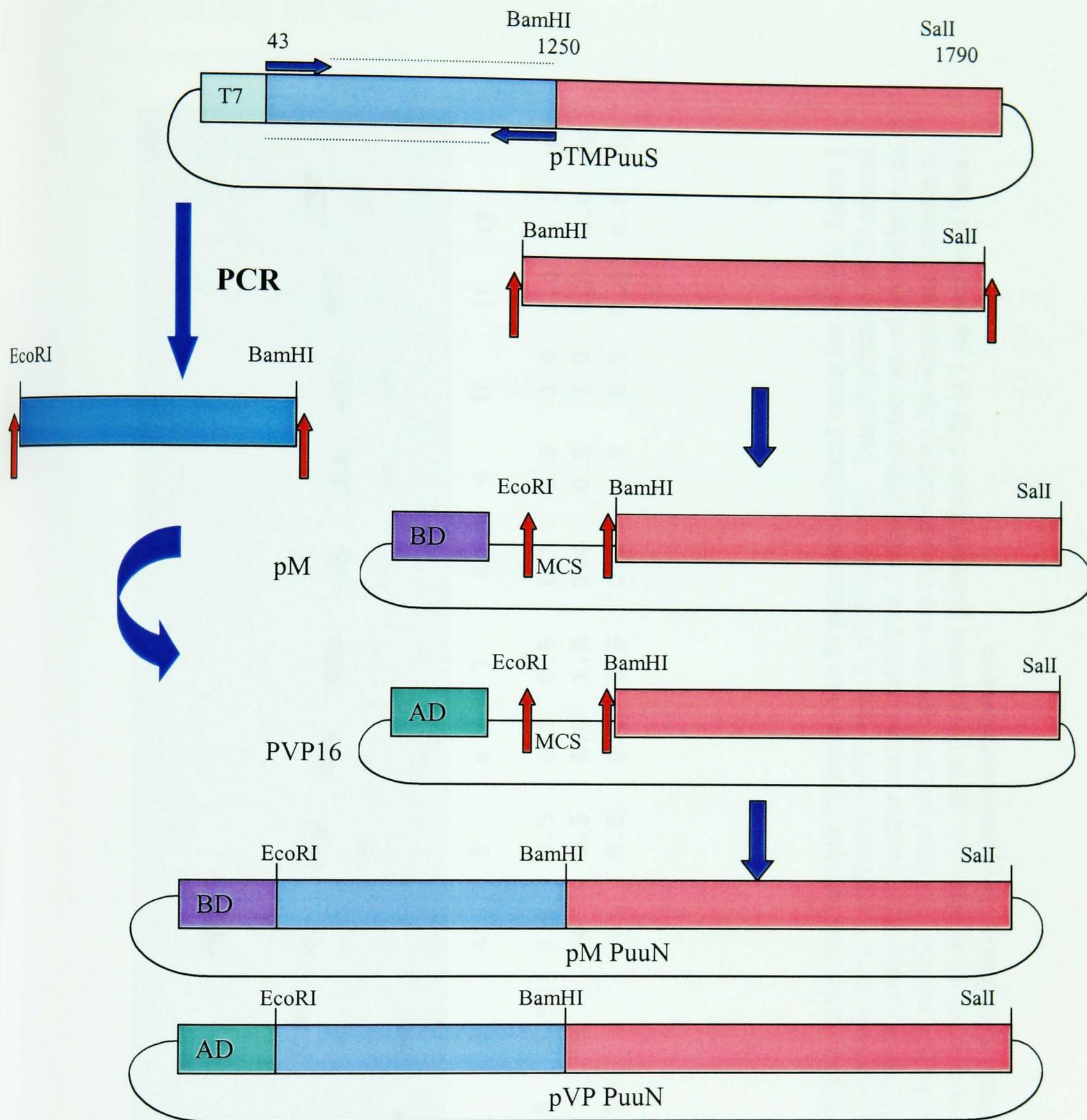
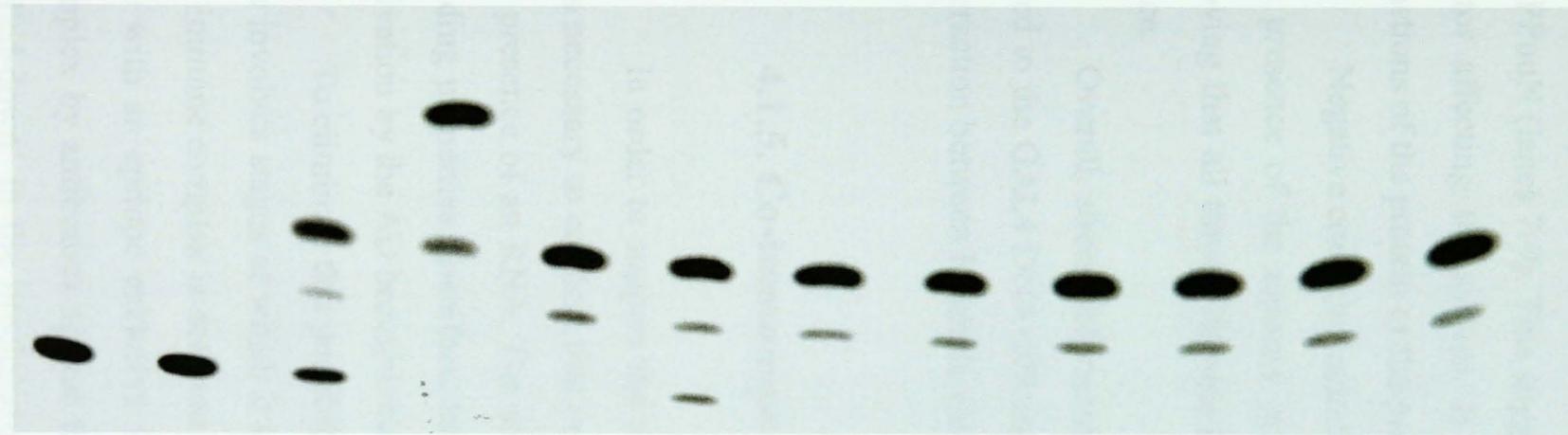


Fig. 4.3. Construction of M2HS vectors expressing Puumala virus N protein. Plasmids pVPPuuN and pM PuuN, containing PUU N sequence were generated in two steps. The first step was to clone a fragment of the gene of 1.2 kb into pM/pVP16 vectors after restriction enzyme digestion of pTMPuuS plasmid with SalI and BamHI. The second step was to PCR-amplify the fragment of the gene encoding the N-terminal part of the protein with primers incorporating EcoRI and BamHI sites using pTMPuuS plasmid as a template. The amplified fragment was subcloned into the vectors generated in the first step.



	1	2	3	4	5	6	7	8	9	10	11	12	
pG5CAT	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	μg
pMPuuN	0.5	0	0	0	<b>0.5</b>	<b>1.0</b>	<b>1.5</b>	<b>2.0</b>	<b>0.5</b>	<b>1.0</b>	<b>1.5</b>	<b>2.0</b>	μg
pVPPuuN	0	0.5	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	μg

Fig. 4.4. Homologous interaction between Puumala virus N proteins monitored using the M2HS. Lanes 1 and 2 - negative controls: lane 1, pMPuuN +pVP; lane 2, pVPPuuN+pM. Lane 3, positive BUN control pSGN +pAASN, lane 4, commercial positive control pM3VP16. Strong interaction was observed between N proteins fused to AD and BD. The level of CAT activity was not affected by the increasing amounts of pMPuuN (shown in bold) when either 0.5 μg of the reporter plasmid (lanes 5-8) or 1 μg was used (lanes 9-12). Each plasmid was taken in the amounts stated above.

decreased (Fig. 4.5, lanes 4-6). This effect was further investigated by titration of increasing amounts of pVPPuuN against constant amount of pMPuuN, and as can be seen from results, the level of CAT activity was decreasing with the increase of the amount of pVPPuuN (lanes 7-9). This suggested that pVPPuuN or its product could be the limiting factor affecting the system. It could be that the AD fusion tag interfered with the functions of the protein or the protein was poorly expressed.

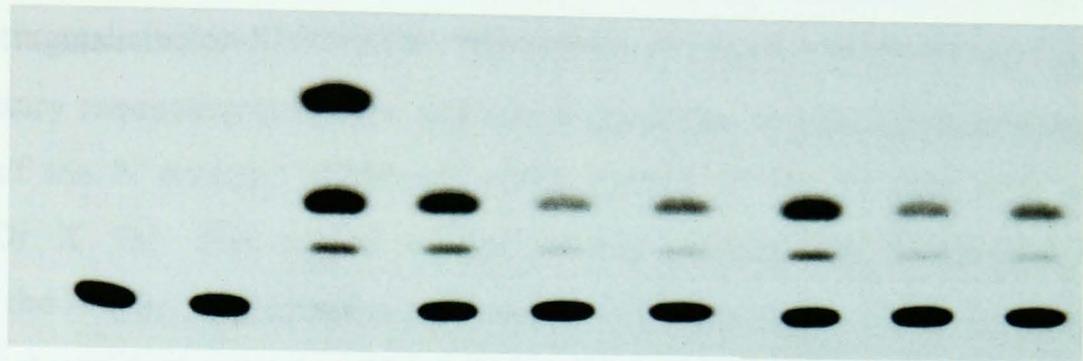
Negative controls, including those testing each plasmid against an empty vector in the presence of the reporter plasmid (lanes 1 and 2), showed no CAT activity, thus proving that all three plasmids had to be present for a reporter gene expression to take place.

Overall, strong interaction was observed between the full-length PUU N protein fused to the GAL4 DNA-BD and PUU N fused to the GAL4 AD, suggesting homotypic interaction between Puumala virus nucleocapsid proteins.

#### **4.1.5. Co-immunoprecipitation assays**

In order to support the results obtained in the mammalian two-hybrid system, it was necessary to confirm that positive interaction between N proteins was not caused by the presence of an RNA. The reason for this is that nucleocapsid proteins possess RNA binding properties, therefore, they may bind RNA which may result in a reporter gene activation by the AD brought into close proximity by this same RNA acting as a bridge.

To eliminate this possibility, a co-immunoprecipitation assay (co-IP) was used, as this involves stages at which it is possible to remove RNA by nuclease digestion before the immune complex is detected. Co-IP involves co-expression of the proteins of interest, one with an epitope exclusive to that protein, and detecting formation of the immune complex by antibodies against this epitope. If two proteins interact, their molecules are 'pulled-down' in the precipitation, and both epitope-tagged and native protein can be detected. Two independent experiments were performed – co-IP of the proteins expressed *in vivo* using recombinant vaccinia virus vTF7-3 as a source of T7 polymerase, and co-IP



	1	2	3	4	5	6	7	8	9
pG5CAT	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pMPuuN	0.5	0	0	0.5	1.5	3.0	0.5	0.5	0.5
pVPPuuN	0	0.5	0	0.5	1.5	3.0	0.5	1.5	3.0

Fig.4.5. Homologous interaction between Puumala virus N proteins. Titration of pVPPuuN. Lanes 1 and 2 – negative control: lane 1, pMPuuN+pVP; lane 2, pVPPuuN+pM. Lane 3, positive control pM3VP16; lanes 4 to 9, pVPPuuN + pMPuuN taken in amounts stated above. The level of CAT activity decreases with an increase in the amount of plasmids (lanes 4-6). Increase in the amount of plasmid pVPPuuN against constant amount of pMPuuN causes decrease in CAT expression (lanes 7-9).

of the proteins expressed *in vitro* using Coupled Transcription/Translation assay 'TnT' (Promega).

For this purpose, it was necessary to generate an N protein with an epitope tag which could be recognized by a specific antibody and was big enough for the tagged protein to be distinguishable on SDS-PAGE. The epitope of choice was the FLAG-tag as it met the necessary requirements of size and immunogenicity. A plasmid expressing a native version of the N protein, pTMPuuS, under control of the T7 promoter, was supplied by Dr X Shi. The tagged version of the protein was constructed by amplification of the N ORF by PCR from start codon 43 to stop codon 432 with primers incorporating *NcoI* and *PstI* sites (PuuNN*NcoI* 43+ and PuuNP*PstI*FLAG) (see Materials). Expression of N protein thus starts with the initial N-terminal amino acid as the *NcoI* site of the vector incorporates the ATG translation start signal. The FLAG epitope was encoded in the reverse primer. The amplified N ORF was cloned into *NcoI-PstI* digested pTM1. The resultant construct was checked for PCR-induced errors but none was found. The new construct containing the N ORF and FLAG tag was designated pTMPuuNFLAG.

pTMPuuS and pTMPuuNFLAG were co-transfected into subconfluent HeLa cells infected one hour previously with recombinant vaccinia virus vTF7-3 (moi 5) as a source of T7 RNA polymerase. The cells were incubated for 20 hours and then labelled with <sup>35</sup>S-methionine for 2 hours followed by immediate lysis. Anti-FLAG antibody was used for co-IP of the lysate. The antibody was precipitated with protein A Sepharose beads and washed with 0.5ml LiCl. Before separation on SDS-PAGE, proteins were dissociated by boiling in protein dissociation buffer. Two distinguishable bands were seen on the gel – one of the native N protein, and the other, slower migrating band, of the FLAG-tagged N protein (Fig.4.6, lane 4). The presence of the two bands on the gel indicated interaction between Puumala virus N proteins detected by anti-FLAG antibodies. FLAG-tagged N was also detected by anti-FLAG antibody when expressed on its own (lane 2) while no band was observed on the gel when the native N was immunoprecipitated with the same antibody (lane 3).

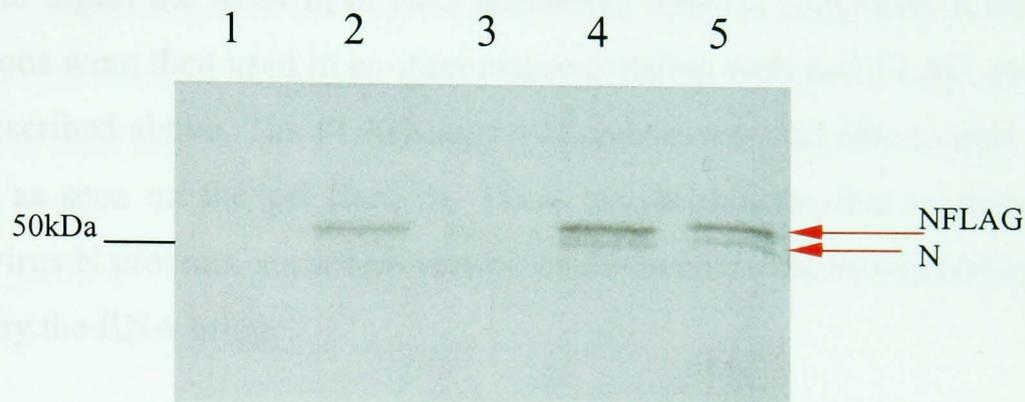


Fig.4.6. Co-immunoprecipitation of N with FLAG-tagged N using anti-FLAG peptide antibody. N and FLAG-tagged were co-expressed in HeLa cells and radiolabelled cells lysates were immunoprecipitated using the anti-FLAG antibody and protein A-Sepharose beads. The products were separated on a 16% SDS-PAGE. Lane 1, mock; lane 2, FLAG-tagged N; lane 3, native N; lane 4, native N and FLAG-tagged N 'pulled down' together as a result of Co-IP; lane 5, native N and FLAG-tagged N expressed in a TnT reaction, incubated with 5mg/ml RNase A and immunoprecipitated with anti-FLAG antibody. 1  $\mu$ g of each plasmid was used in each reaction

To further confirm these results and show that RNA does not affect interaction between N proteins, N and NFLAG proteins were produced by the coupled transcription/translation reaction (TnT) that allows *in vitro* expression of the proteins under control of T7 polymerase supplied in reaction. The proteins were treated with RNase A immediately after incubation of the reaction. To ensure complete digestion of RNA, RNase A was used in 5000-fold excess (5mg/ml) over the amount of RNase sufficient to digest the RNA in *in vitro* assembled RNA-N complexes (Osborne, 2001). The reactions were then used in co-immunoprecipitation with anti-FLAG antibody in the manner described above. The FLAG-tagged N protein was still able to pull down native N protein as seen on the gel (lane 5). These results indicate that interaction between Puumala virus N proteins, shown previously by the mammalian two-hybrid system, is not mediated by the RNA bridge.

## **4.2. Mapping Puumala virus N interaction domains**

After interaction between the full-lengths proteins was shown, the next step was to investigate which domains on the nucleocapsid protein are responsible for the interaction. This was performed by testing possible interactions between full-length N protein and various fragments of N.

Alignment of the deduced amino acid sequences of the nucleocapsid protein of different hantaviruses reveals both regions of high homology and regions of little homology. Two highly conserved regions are located in the N-terminal 150aa and the C-terminal half of the protein, with a highly variable region located between aa 210-310 (Antic *et al.*, 1992). The presence of the regions of high homology among different hantaviruses is indicative of their importance and suggests that protein molecules should possess at least two domains responsible for interaction.

As a first step, interaction between the full-length N protein and its different fragments was investigated. Then, the ability of different fragments of N to interact with each other was tested in order to identify the domains that are involved in interaction between Puumala virus nucleocapsid proteins.

### **4.2.1. Generation of constructs expressing truncated N proteins**

Fragments encoding different regions of N protein were generated using PCR with the appropriate 5' and 3' primers incorporating EcoRI and PstI restriction sites (see Materials). Amplified products were then digested with the restriction enzymes EcoRI and PstI and subcloned into BD and AD vectors to yield a set of plasmids for use in the M2HS. The presence of an insert was verified by restriction enzyme digestion with these two enzymes, and sequences of the constructs were checked for PCR-induced errors but none were found.

As a result, twenty constructs encoding various fragments of N protein fused to either AD or BD were generated (Fig 4.7).

### **4.2.2. Interaction between N-terminal and C-terminal truncated N proteins**

Different truncated proteins were tested for their ability to interact with the full-length N. Very strong interaction was observed between the full-length N and its C-terminal fragments N385-432 and N288-432 (Fig.4.8, lanes 4 and 5, respectively) and to a lesser extent with N185-432 (lane 6) and N85-432 (lane 7). The strength of the signal decreased as the size of the fragment increased (compare lanes 4 to 7). This could indicate that this domain of the protein is buried by additional sequences that 'hide' the interaction domain. Besides, dividing the protein into fragments changes its native conformation that also can affect the results. A weaker interaction compared to N385-432 was observed between the full-length N and the N-terminal 105 aa (lane 8). No interaction was observed between the full-length N and its internal fragments consisting of amino acids 85-205, 185-305, and 288-389 (lanes 9,10 and 11) thus indicating that the internal region of the molecule is not important for interaction. Deletion of 43 aa at the C terminus of the N protein completely abolished the interaction with the full-length protein (lane 12), showing that interaction between two proteins indeed requires C-terminal

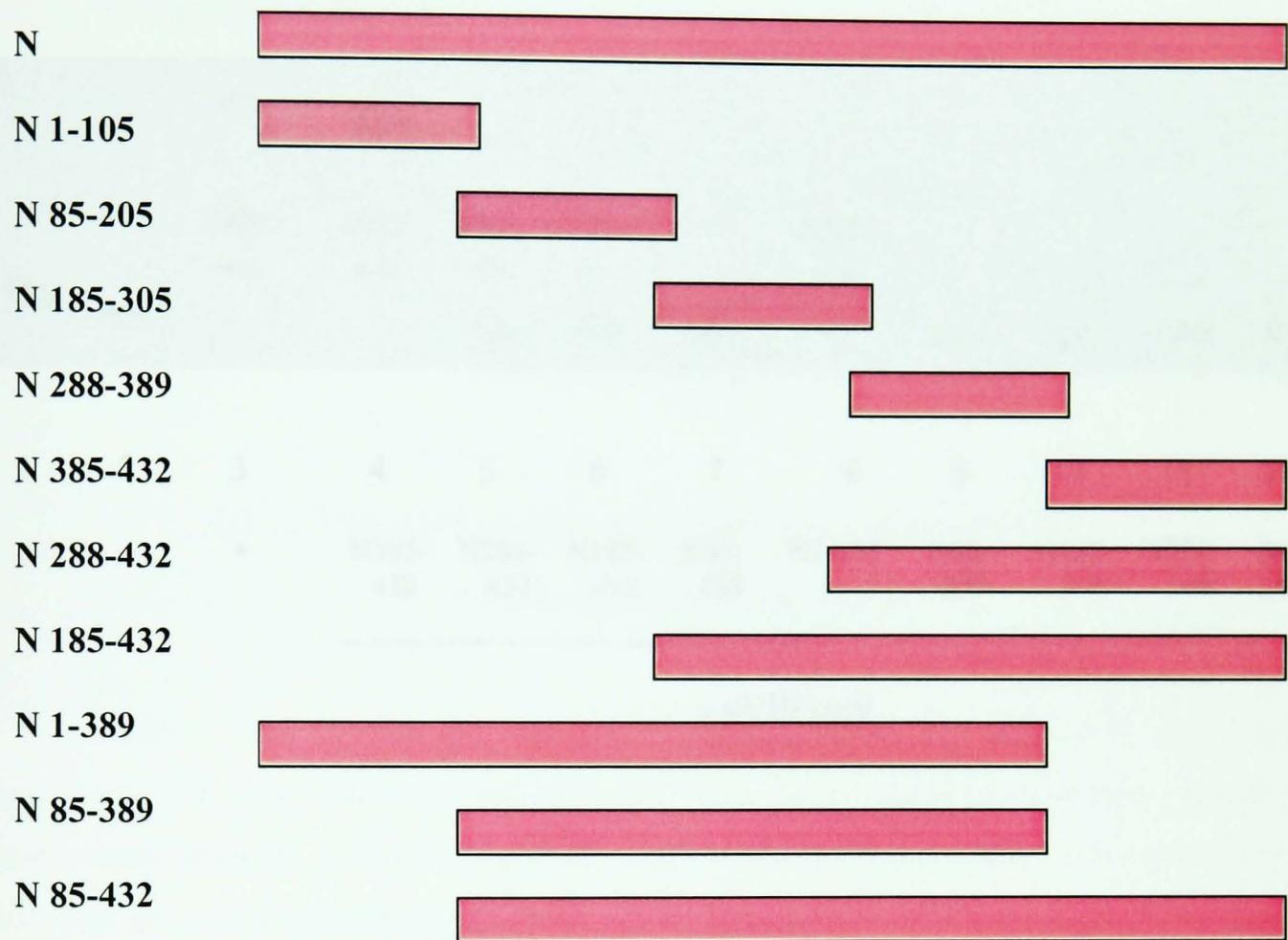


Fig.4.7. Constructs expressing regions of PUU N protein generated for the M2HS to investigate N protein interactions. Constructs were named based on the amino acids of the Puumala virus N protein that were contained in the clone. For example, N 1-105 contains amino acids 1 through 105. Numbers of amino acid residues are indicated

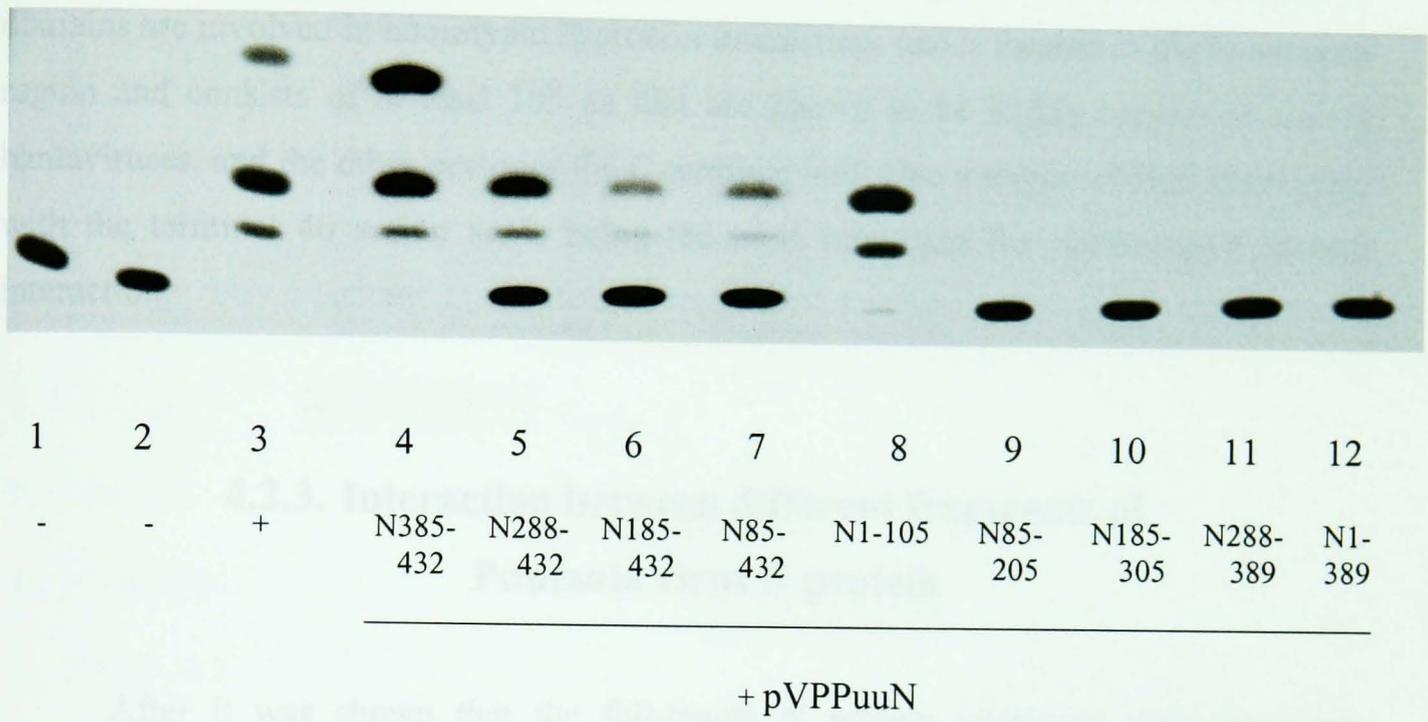


Fig.4.8. Interaction between full-length N protein and various fragments of N. Lanes 1 and 2 - negative control: lane 1, pVPPuuN + pM; lane 2, pMPuuN + pVP. Lane 3, positive control pM3VP16. Strong interaction was observed between full-length N protein and N 385-432 (lane 4), N 288-432 (lane 5), and N1-105 (lane 8), and to a lesser extent with N 185-432 (lane 6) and N 85-432 (lane 7). No interaction was observed between full-length N and its internal fragments N 85-205 (lanes 9), N 185-305 (lane 10) and N 288-389 (lane 11). Deletion of 40aa at the C terminus completely abolished interaction (lane 12). 1 µg of each plasmid was used for transfection.

region. On the other hand, deletion of N-terminal 85 aa did not abolish the interaction with the N protein (lane 7).

These results are summarised in Fig.4.9 and suggest that two independent domains are involved in homotypic N protein interaction: one is located in the N-terminal region and consists of at least 105 aa that are known to be highly conserved among hantaviruses, and the other occupies the C-terminal half, also a region of high homology, with the terminal 46 amino acids being the most important for nucleocapsid protein interaction.

### **4.2.3. Interaction between different fragments of Puumala virus N protein**

After it was shown that the full-length N protein interacted with fragments containing the two terminal domains and not with internal fragments, possible interactions between various fragments of N protein were tested. Knowing which domains are involved in interaction would be the basis for analysis of how precisely interaction involving these two domains occurs. For this purpose, all possible interactions between the fragments of the protein were tested. It was observed that interaction of N-terminal fragments resulted in weak CAT activity (Fig.4.10, lane 4). The strength of the signal for C-terminal fragments increased as the size of the fragment decreased analogous to the results obtained for interaction with the full-length N protein (lanes 5, 6 and 7). The strongest CAT signal was observed when two fragments of N protein comprising the N- and C-terminal domains were tested against one another (lane 8), thus indicating that this type of interaction is the most important for assembly of N protein multimers. No interaction was observed between homologous internal fragments N 85-205 (lane 9), N 185-305 (lane 10) and N 288-389 (lane 11). These results are summarized schematically in Fig.4.11.

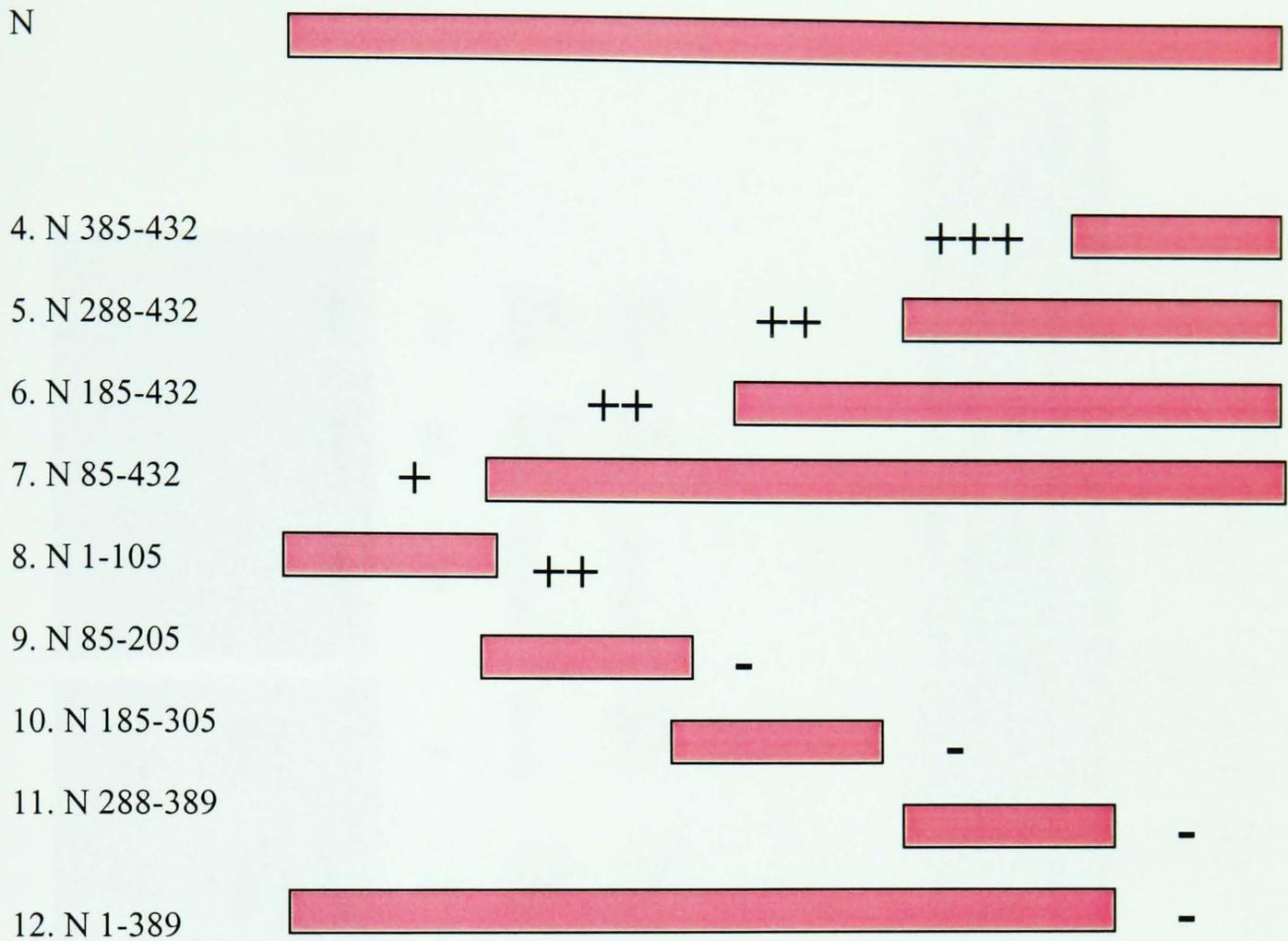


Fig 4.9. Diagram summarizing interaction between full-length N protein and various fragments of N. Numbers of amino acid residues are indicated. Numbers of fragments correspond to the lanes in Fig.4.8. - and + indicate the strength of CAT signal as a result of interaction.

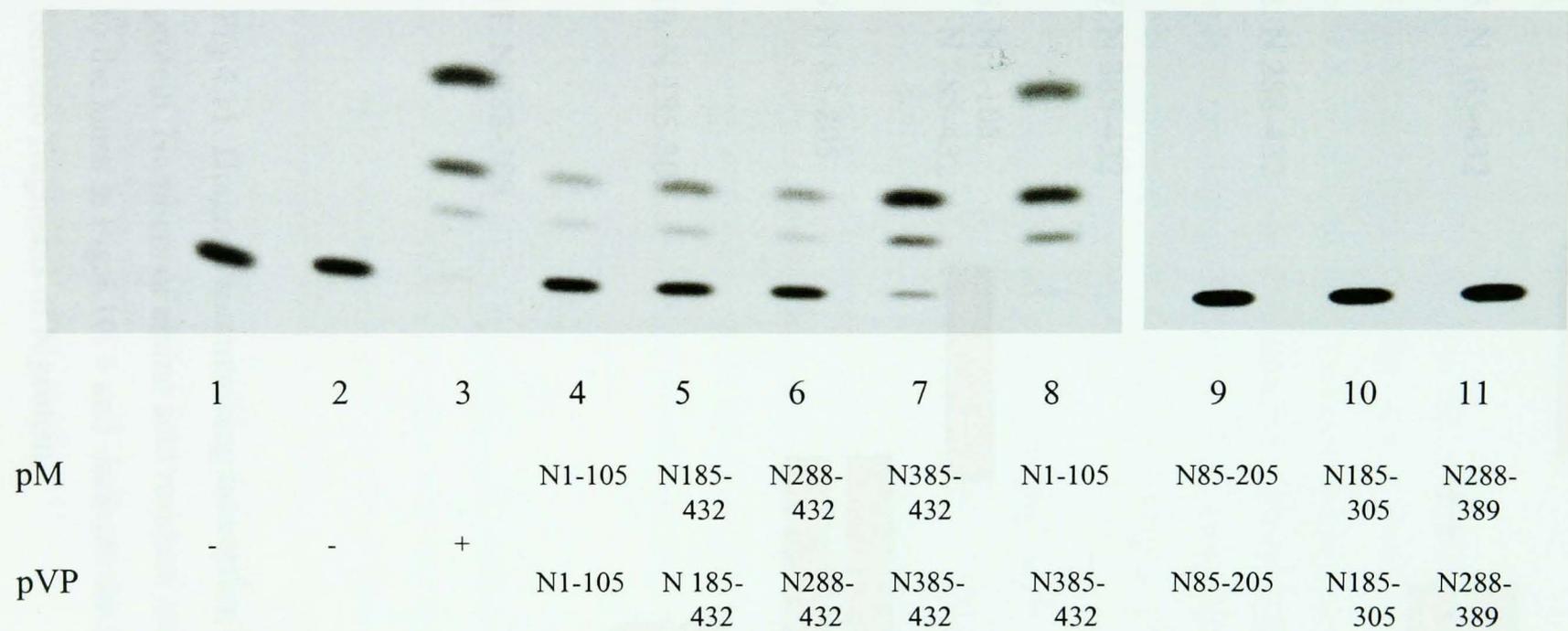


Fig. 4.10. Interaction between various fragments of Puumala virus N protein. Lanes 1 and 2 – negative control: lane 1, pVPPuuN + pM; lane 2, pMPuuN + pVP. Lane 3, positive control pM3VP16. Interaction was observed between homologous terminal fragments N 1-105 (lane 4), N 185-432 (lane 5), N 288-432 (lane 6) and N 385-432 (lane 7). Interaction between N-terminal 105 aa (N 1-105) and C-terminal 46 aa (N 385-432) resulted in strongest CAT signal (lane 8). No interaction was observed between internal fragments of the N protein (lanes 9-11). 1  $\mu$ g of each plasmid was used for each transfection.

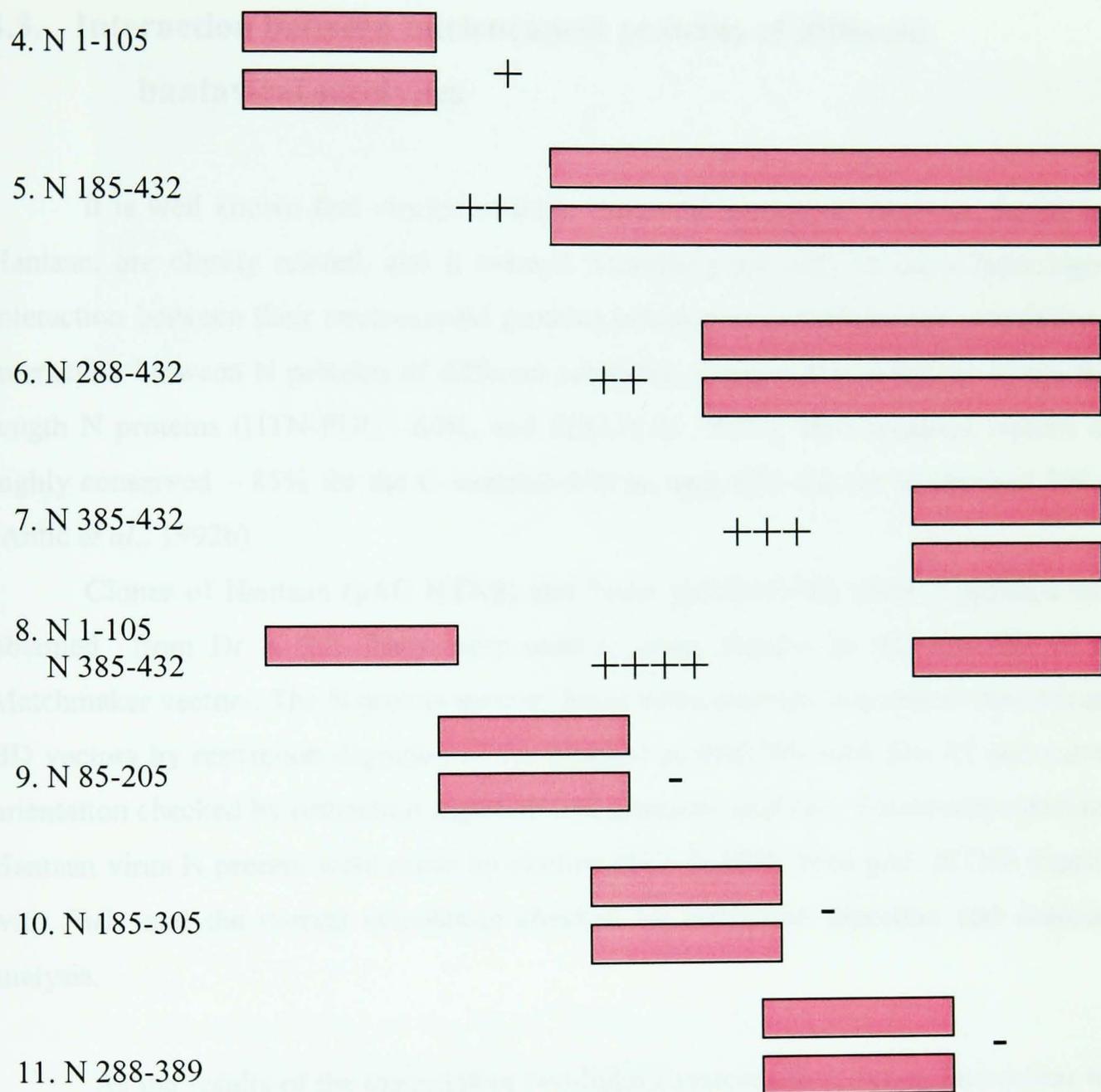


Fig 4.11. Diagram summarizing interaction between various fragments of Puumala virus N protein. Numbers of amino acid residues are indicated. Numbers of fragments correspond to the lanes in Fig.4.10. + and - indicate the strength of CAT signal as a result of interaction between fragments of N protein.

### 4.3. Interaction between nucleocapsid proteins of different hantaviral serotypes

It is well known that viruses of three hantaviral serotypes, Puumala, Seoul, and Hantaan, are closely related, and it seemed interesting not only to show homologous interaction between their nucleocapsid proteins but also to investigate the possibility of interaction between N proteins of different serotypes. Despite low similarity of the full-length N proteins (HTN-PUU –60%, and SEO-PUU –62%), their terminal regions are highly conserved – 85% for the C-terminal 100 aa, and 82% for the N-terminal 200 aa (Antic *et al.*, 1992b).

Clones of Hantaan (pAC HTNS) and Seoul (pCRIIL99S) virus N proteins were obtained from Dr X Shi. They were used to make fusions to AD and BD of the Matchmaker vectors. The N protein gene of Seoul virus serotype was cloned into AD and BD vectors by restriction digestion of the plasmid pCRIIL99S with *Eco* RI and correct orientation checked by restriction digestion and sequence analysis. Constructs containing Hantaan virus N protein were made by cloning HTN N ORF from pAC HTNS digested with *Pst*I and the correct orientation checked by restriction digestion and sequence analysis.

As the results of the mammalian two-hybrid system show, strong interaction was observed between N proteins of each serotype (Fig.4.12, lanes 4, 7, 8) further supporting the idea that hantavirus nucleocapsid proteins are able to interact. Interaction was also observed between N proteins of different serotypes: strong between SEO and PUU (lane 9), and to a lesser extent between HTN and PUU (lane 5), and HTN and SEO (lane 6). An interaction between full-length N protein of Seoul virus and N- and C-terminal fragments of Puumala virus N protein was also tested. It resulted in strong CAT signal indicating interaction (Fig.4.13, lanes 8-10).

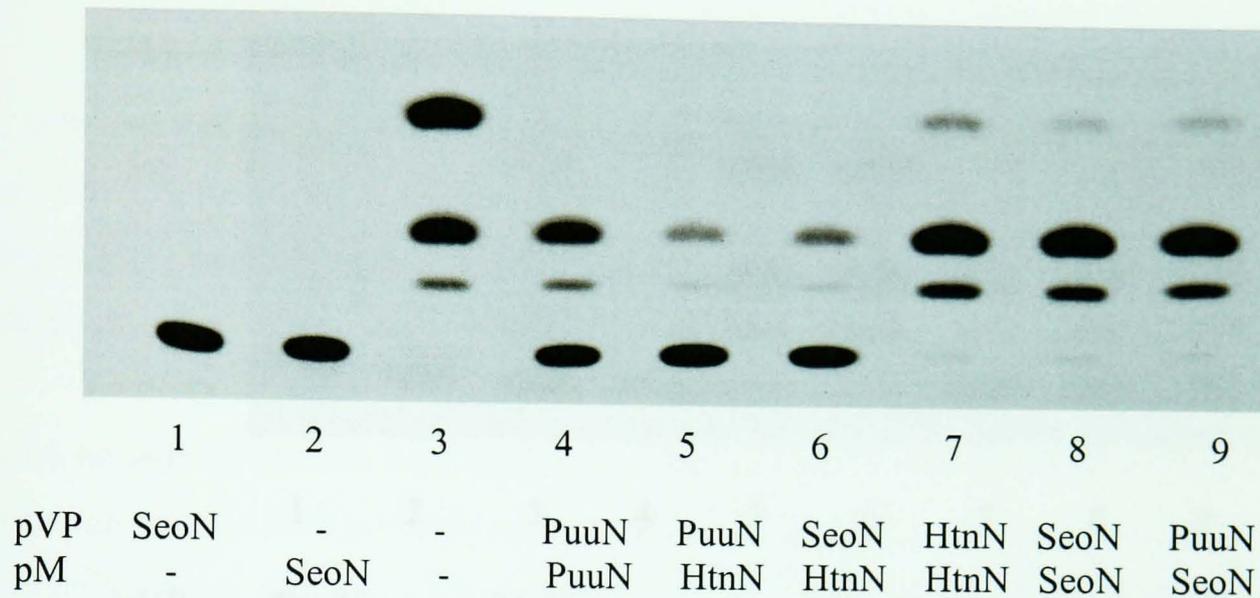


Fig 4.12. Interaction between N proteins of serotypes Seoul, Puumala and Hantaan and between N proteins of different serotypes. Lanes 1 and 2 represent negative controls with empty AD and BD vectors: lane 1, pVPSeoN + pM, lane 2, pMPuuN + pVP; lane 3 – positive control pM3VP16. Strong interaction was observed between N proteins of serotypes Hantaan (lane 7) and Seoul (lane 8). Interaction between N proteins of PUU and HTN (lane 5), SEO and HTN (lane 6) and PUU and SEO (lane 9) also resulted in CAT activity. Plasmids used in the M2HS are indicated above. 1  $\mu$ g of each plasmid was taken for each transfection.

#### 4.4. Interaction between Seoul and Puumala virus N proteins

##### Fragments of *L. grassii*

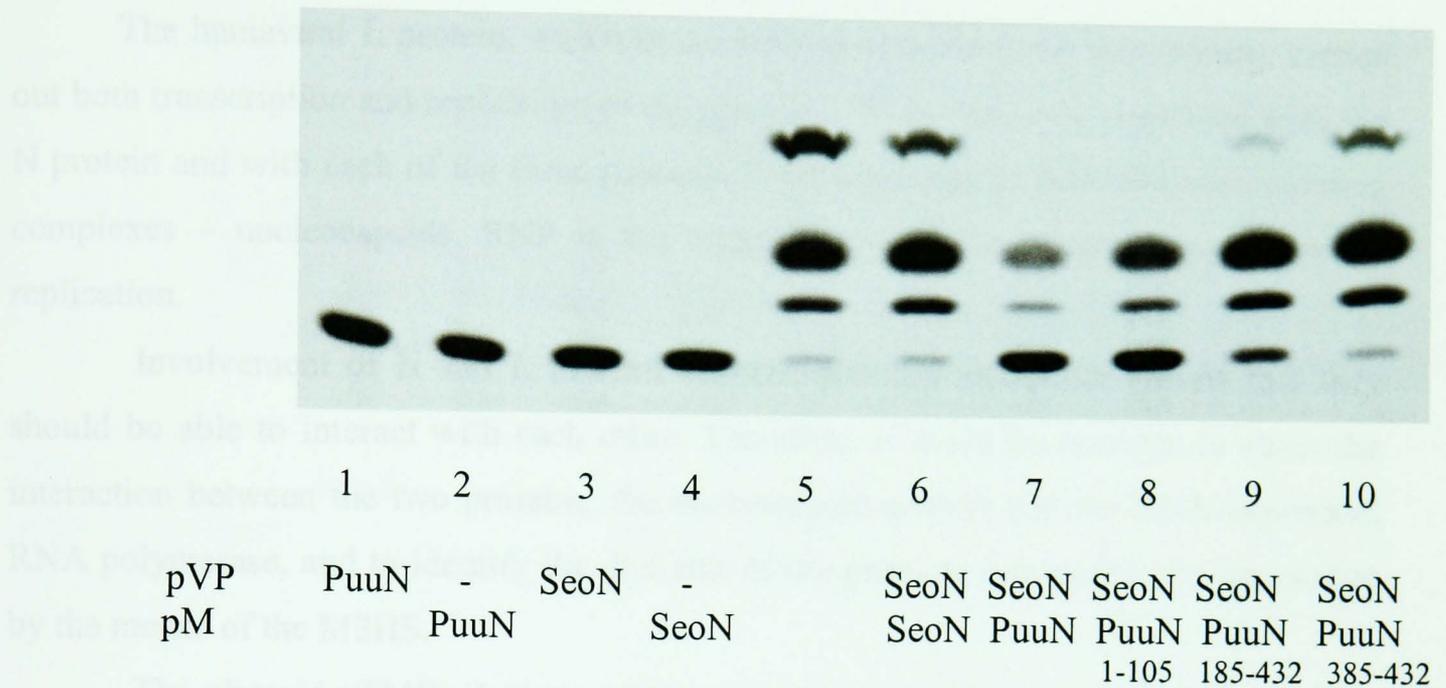


Fig.4.13. Interaction between Seoul and Puumala virus N proteins, and full-length SEO N protein and N- and C-terminal fragments of PUU N. Lanes 1, 2, 3, 4 – negative controls with empty BD and AD vectors; lane 5, positive control pM3VP16. Strong CAT signal (lanes 8, 9, 10) indicates interaction between full-length SEO N and terminal fragments of PUU N. 1  $\mu$ g of each plasmid was used for each transfection.

#### **4.4. Interaction between Puumala virus N protein and different fragments of L protein**

The hantaviral L protein, which is the RNA-dependent RNA polymerase, carries out both transcription and replication of the genome. The L protein is associated with the N protein and with each of the three genomic RNA segments to form ribonucleoprotein complexes – nucleocapsids. RNP is the template for mRNA synthesis and genome replication.

Involvement of N and L in viral transcription and replication means that they should be able to interact with each other. Therefore, it could be possible to show the interaction between the two proteins, the nucleocapsid protein and the RNA-dependent RNA polymerase, and to identify the domains of the proteins responsible for interaction by the means of the M2HS.

The plasmid pTMPuuL that contains sequence encoding L protein under control of the T7 promoter was obtained from Dr X Shi. It was used as a template for amplification of different fragments of the protein as it would not have been possible to use the full-length L protein in the M2HS due to its large size. Several fusions were amplified by PCR with primers incorporating SmaI and SalI sites (see Materials) and subsequently cloned into pVP16 vector digested with these two enzymes. The sequences were checked for PCR-induced errors but none were found. As a result, five constructs were used in the M2HS: pVPPuu L 1-400, pVPPuuL 400-800, pVPPuuL 800-1200, pVPPuuL 1200-1600, and pVPPuuL 1600-2000.

However, no interaction could be detected between full-length N protein and different fragments of L protein in the M2HS (Fig.4.14, lanes 5-9).



## 4.5. DISCUSSION

### 4.5.1. Interaction between hantavirus proteins studied in the M2HS

The N protein of hantaviruses constitutes the main part of the nucleocapsid, the infectious subviral particle, which contains the viral genomic RNA and a few copies of an RNA-dependent RNA polymerase, L protein. Mainly from data on capsid proteins of other enveloped viruses, an important role in the structural organization of the viral life cycle can be ascribed to the Puumala virus N protein. The primary function of viral core proteins, based on their RNA-binding capability, is assumed to provide protection for the viral genome inside. A nonspecific ssRNA binding capacity of the PUU N protein has been demonstrated recently (Severson *et al.*, 2001). Furthermore, a direct interaction with the viral RNA polymerase can be postulated to facilitate its association to the nucleocapsid and possibly mediate transcription and replication. The nucleocapsid protein could also possess a regulatory function during the early events of hantavirus transcription and replication. In a similar context Dunn *et al* (1995) have demonstrated that the N protein of Bunyamwera virus is indispensable for the activity of the viral RNA-dependent RNA polymerase. Similar results have been achieved by Lopez *et al* (1995) for Rift Valley fever virus, a phlebovirus within the *Bunyaviridae*. A necessary prerequisite to fulfil these functions is a capability of the N protein to take part in multiple protein-protein interactions.

In this chapter, evidence for homotypic interaction between N proteins of hantaviruses is presented. The study of Puumala virus N protein interactions was initiated by using the mammalian two-hybrid system in which self-association of Puumala virus N protein was observed. This was additionally supported by the results of co-immunoprecipitation of the proteins expressed both *in vitro* and *in vivo*.

The observation agrees with the data on dimerization and oligomerization capacity of nucleocapsid proteins of two other serotypes within the genus *Hantavirus*, Sin Nombre and Tula viruses (Alfadhli *et al.*, 2001; Kaukinen *et al.*, 2001). It is further supported by the studies on nucleocapsid proteins of a wide range of negative-sense

viruses that include bovine respiratory syncytial virus (BRSV) (Krishnamurthy and Samal, 1998), Marburg virus (Becker *et al.*, 1998) and Sendai virus (Horikami *et al.*, 1992; Myers *et al.*, 1997).

The homomultimerization at the molecular level was dissected by analyzing fragments of the protein. The studies on interaction between the full-length N protein and various fragments of N make it possible to suggest that Puumala virus N protein possesses two independent domains involved in homologous interaction of the protein. These results were obtained in the M2HS where the full-length N protein was shown to interact with its N-terminal 105 aa and minimum 46 aa at the C-termini, but not with internal fragments. Although interactions between homologous N- and C-terminal fragments resulted in some CAT activity, experiments showed that interaction between N- and C-terminal fragments, rather than the homologous interaction between fragments, yielded the strongest CAT signal. The results described above suggest that sequences involved in self-interaction of the N protein are confined to its N-terminal 105 aa and C-terminal 46 aa and that this type of interaction is the most important for assembly of N protein multimers. The fact that deletion of these two domains abolishes the ability to form homodimers in the mammalian two-hybrid system suggests that there are no other interacting domains.

From these results, a 'head-to-tail' organization of the homotypic interaction of the hantavirus N protein can be postulated, involving an N-terminal region (amino acids 1-105) and a C-terminal region (amino acids 385-432) of the protein. This fits the general idea that multimerization requires at least two distinct binding regions and happens in a manner similar to that described for tospovirus N protein (Richmond *et al.*, 1998).

The strength of the interaction between the fragments and the wild-type N protein as shown by CAT signal was apparently stronger than the homotypic interaction of the wild-type N protein. Since it is not likely that both the C-terminal and the N-terminal regions of the N protein perturb the interaction, these results might indicate a limitation of the M2HS in monitoring homotypic interactions. An interaction can be impaired when a fusion protein is folded improperly or inherently unstable, when its expression is poor or when the fused BD or AD partly occludes the site of interaction. It is also possible that optimization of amount of plasmids used in the M2HS could resolve this problem.

Furthermore, an attempt was made to show the ability of nucleocapsid proteins of different hantaviral serotypes to interact with each other. Not only was it shown that N proteins of Seoul and Hantaan virus serotypes interact with each other, but also that Puumala virus N protein interacted with Seoul and Hantaan virus N proteins in the M2HS. Interaction between PUU and HTN N proteins was somewhat surprising since HTN is more distant from PUU than is SEO. These results suggest that interactions between the hantavirus N protein molecules might occur via either highly conserved stretches of amino acid residues or conserved domains of secondary or tertiary structure, despite low homology of the complete sequence. This observation is supported by the data on sequence similarity of the proteins. Despite low similarity of the whole sequence (HTN-PUU – 60%, and SEO-PUU – 62%), alignment of the deduced amino acid sequences of the nucleocapsid proteins of these viruses reveals also regions of high homology: their C terminal 100 aa are 85% identical, and N-terminal 200 aa are 82% identical (Antic *et al.*, 1992b). The N-terminal 100 aa are mainly hydrophilic and highly antigenic. The middle part of the protein, residues 210-310, is a mainly hydrophilic region that is highly variable between different hantaviruses (Jenison *et al.*, 1994; Vapalahti *et al.*, 1995; Elgh *et al.*, 1996; Lundkvist *et al.*, 1995). The C terminus of the protein is highly conserved and has been shown to bind RNA, preferentially to double-stranded RNA (Gott *et al.*, 1993).

To demonstrate that interaction between N proteins of different hantavirus serotypes indeed occurs via highly conserved regions of amino acids at the N- and C-termini, further experiments were carried out. The results indicate that the full-length N protein of Seoul serotype is indeed capable of interacting with N- and C-terminal domains of the Puumala virus N protein.

During the course of this work, two studies on hantavirus N protein interactions were published. Alfadhli *et al.* (2001) used the yeast two-hybrid system, sucrose gradient centrifugation and chemical cross-linking technique to study N protein assembly of Sin Nombre virus (SN). By using the yeast two-hybrid system the SN N protein homotypic interacting domains were mapped to the N-terminal 40aa and to the C-terminal half of the N protein. Furthermore, the SN N protein was found to associate as dimers, trimers and large multimers. Kaukinen *et al.* (2001) investigated the capacity of Tula virus

nucleoprotein molecules to interact with each other and showed the formation of dimers, trimers and further higher molecular mass products, with trimers being the preferential assembly intermediates. Similar to the previous study, this was achieved using the yeast two-hybrid system and confirmed by chemical cross-linking and immunoblotting. These data are in agreement with my findings about Puumala virus N protein interaction obtained via the mammalian two-hybrid system, suggesting that the interacting domains of Puumala virus N protein are located within the first 105 aa of the N protein and at least 46 aa of the C-terminal part.

Owing the fact that nothing is known about the mechanism of how the Puumala virus N protein assembles into nucleocapsid structure, the experimental proof of N protein self-interaction might provide a basis for a better understanding of nucleocapsid formation and the role of the N protein in transcription and replication, since the latter is thought to be regulated by the monomeric/multimeric state of the N protein. One could further speculate that the multimeric state of PUU N protein may be of significance for the specific recognition and correct binding of the viral RNA. This in turn may be obligatory to mediate the accessibility of the RNA for the RNA-dependent RNA polymerase.

As it was shown in this chapter, no interaction was found between Puumala virus N protein and various fragments of the L protein. This is somewhat surprising since it is known that the L protein is associated with the N protein and with each of the three genomic RNA segments to form nucleocapsids. Both L protein and N protein carry out transcription and replication of the genome. The N protein was also suggested to modulate the switch of virus RNA synthesis from transcription to replication, as reported for analogous proteins in other negative-strand viruses. The fact that these two viral proteins are involved in the viral transcription and replication suggests that they should be able to interact with each other.

The negative results on L-N protein interaction are perhaps attributable to the binding sites having been destroyed by expressing L as separate segments. Hence, the intermolecular interactions might take place in infected cells and could be identified in the future by other methods, for example, co-immunoprecipitation assay. Besides, false negative results from two-hybrid system are not without precedent, as failure to identify

other known protein-protein interactions has been reported (Fields and Sternglanz, 1994; Golemis *et al.*, 1997; Cuconati *et al.*, 1998; Van Aelst *et al.*, 1993).

#### **4.5.2. Proposed model for RNP formation**

In order to form the helical nucleocapsid characteristic of many negative strand RNA viruses like influenza virus (Portela and Digard, 2002) and vesicular stomatitis virus (Green *et al.*, 2000), individual molecules of the N protein have to interact. Cross-linking studies by Alfadhli *et al* (2001) demonstrated that the N protein in mature nucleocapsids, isolated from viruses, existed as dimers and trimers. In the light of the results described in this chapter and studies on Tula and Sin Nombre virus nucleoprotein oligomerization capacity, it seems possible to propose a model for Puumala virus RNP formation. In this model, the association of the N proteins appears through their C and N termini, a so-called 'head-to-tail' mechanism, similar to the one described for tospovirus (Richmond *et al.*, 1998) and Tula virus (Kaukinen *et al.*, 2001). The hypothesis is that two molecules of the N protein come together forming a dimer which then associates with the third molecule available in a monomeric form. The trimer then attaches to the RNA. These trimers then form longer multimers gradually assembling around the RNA (Fig.4.15). Interactions with the viral RNA might assist in correctly orienting the N protein molecules. Divalent cation(s) induce proper folding of the N protein molecules thus facilitating their interactions. When 3 complexes become associated with the RNA, a disc-like oligomer, which is consistent with one turn of the RNP helix is formed – a mechanism similar to the one described for vesicular stomatitis virus and Sendai virus RNP formation (Egelman *et al.*, 1989; Green *et al.*, 2000). A key component in this assembly process should be the RNA which plays a major role in stability of the N protein oligomer. Upon finishing the first turn of the RNP helix, monomer 1 and monomer 10 of the oligomer come into contact to form the disk, a state similar to that seen for tobacco mosaic virus (Durham *et al*, 1971). However, unlike the tobacco mosaic virus disk, the Puumala virus disk is bound to RNA. Additional N-N and C-C-terminal

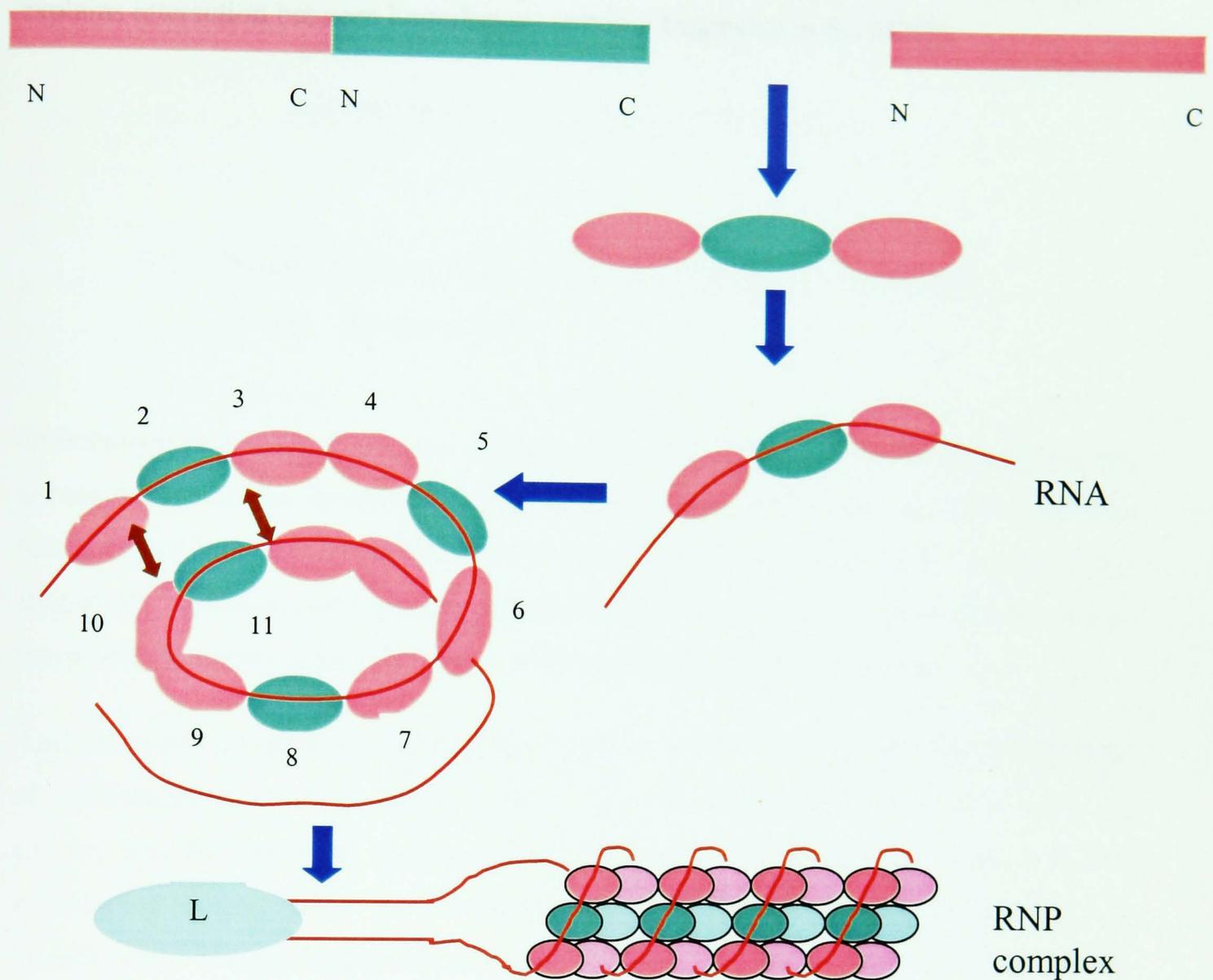


Fig. 4.15. Proposed model of hantavirus RNP formation. The association of the N proteins appears through their C and N termini, a so-called 'head-to-tail' mechanism. Two molecules of the N protein come together forming a dimer which then associates with the third molecule available in a monomeric form. Trimer then attaches to the RNA. These trimers then gradually assemble around RNA forming longer multimers. Interactions with the viral RNA might assist in correctly orienting the N protein molecules. When 3 complexes become associated with the RNA, a disc-like oligomer, which is consistent with one turn of the RNP helix is formed. Monomer 1 and monomer 10 of the oligomer come into contact to form the disk where additional N-N, and C-C terminal interactions may take place to stabilize the structure.

interactions may take place in order to stabilize the helical structure of the complex that explains interaction between homologous terminal fragments in the M2HS.

## CHAPTER 5. POTENTIAL SECOND ORF (ORF2) IN THE S SEGMENT THAT MAY ENCODE AN NSs NONSTRUCTURAL PROTEIN

### 5.1. Nonstructural NSs proteins encoded by members of the *Bunyaviridae*

Orthobunyaviruses, phleboviruses, and tospoviruses code for two nonstructural proteins, termed NSs and NSm, in their S and M segments, respectively. Little is known about the NSs proteins encoded by different members of the *Bunyaviridae*. Not only does the strategy of expression vary between genera but also the primary amino acid sequence of this protein is poorly conserved among different members within a genus.

Orthobunyaviruses possess a nonstructural protein which is smaller than the NSs protein of phleboviruses and tospoviruses. The S segment RNAs of the snowshoe hare, La Crosse, and Bunyamwera viruses are approximately 900 nucleotides long with two overlapping reading frames in the messenger-sense RNA and encode the N protein (19 to 26 kDa) and a NSs polypeptide of 10 kDa to 11 kDa. For phleboviruses and tospoviruses, the NSs proteins which are expressed in an ambisense orientation, are larger than those of orthobunyaviruses. Phleboviruses like Punta Toro and sandfly fever Sicilian viruses have 1.7- and 1.9-kb S segment RNAs, respectively, encoding 25- and 27-kDa N polypeptides at the 5' ends of the messenger-sense RNAs and 29- and 30-kDa NSs proteins at the 5' ends of the genomic sense RNAs. Another member of the *Phlebovirus* genus, Uukuniemi virus, has a nonstructural protein of 32 kDa. An even bigger nonstructural protein of 52 kDa in size is encoded on the S segment of tomato-spotted wilt virus, a member of *Tospovirus* genus (Elliott, 1985; Elliott, 1990; Ihara *et al.*, 1984; Parker *et al.*, 1984; Marriott *et al.*, 1989; Bishop, 1996; Bouloy, 1991).

In addition to the variety in coding strategies and size, it is possible that there may be no functional equivalence between the NSs proteins in different genera and the proteins have

adopted different or only partially overlapping functions with respect to the viral replication cycle. For example, within the *Phlebovirus* genus, NSs appears to vary with different representatives: in Punta Toro virus-infected cells, it localizes in the cytoplasm and is present in minute amounts in purified particles (Overton *et al.*, 1987), in Uukuniemi virus-infected cells, it is associated with the 40S ribosomal subunit (Simons *et al.*, 1992), in Karimabad virus-infected cells, it was found exclusively in the cytoplasm (Smith and Pifat, 1982), and for Rift Valley fever virus, NSs has been shown to be phosphorylated and to form filamentous structures in the infected cell nuclei (Struthers and Swanepoel, 1982; Struthers *et al.*, 1984; Kohl *et al.*, 1999). Further, it was shown that the carboxy-terminal acidic domain of Rift Valley fever virus NSs protein is essential for the formation of filamentous structures but not for the nuclear localization of the protein (Yadani *et al.*, 1999). Studies on a naturally occurring mutant of RVF virus which had a large internal in-frame deletion in the NSs gene, have shown that it replicates normally in some cell lines while establishing abortive infections in others, and is avirulent in mice and hamsters (Muller *et al.*, 1995). It was shown using an *in vitro* transcription-replication system, that NSs of RVF virus had neither a stimulatory nor an inhibitory effect on transcription (Lopez *et al.*, 1995; Prehaud *et al.*, 1997) and also plays a role in antagonism of the interferon (IFN) response (Haller *et al.*, 2000).

The function of the NSs protein of the Bunyamwera (BUN) virus, a member of the *Orthobunyavirus* genus, has been studied more extensively. Weber *et al.* (2001) showed that BUN NSs protein is mainly confined to the cytoplasm but can also enter the nucleus. Using a reverse genetics approach it was demonstrated that transcription and replication of the RNA required only the N and L proteins (Dunn *et al.*, 1995), though the possibility of a regulatory role for NSs was not discounted. Recent results by Weber *et al.* (2001) demonstrated that, unlike the NSs protein of the RVF virus, BUN NSs down-regulates the viral polymerase in a minireplicon system that reconstitutes nucleocapsids from transfected cDNAs. The generation of viruses lacking the NSs gene (Bridgen *et al.*, 2001) showed that although not essential for replication in either tissue culture or in mice, the NSs protein of Bunyamwera virus has several functions in the virus life cycle that contribute to viral pathogenesis. The lack of NSs caused impaired capacity to shut off host cell protein synthesis. It also played an important role in controlling IFN induction

after infection by the virus (Bridgen *et al.*, 2001). BUN NSs is in this sense similar to the NSs protein of the Rift Valley fever virus that is also an antagonist of the interferon response (Haller *et al.*, 2000) and the multifunctional NS1 gene product of influenza A virus, which enables the virus to inhibit host cell gene expression and stimulate its own protein synthesis (Enami *et al.*, 1994; Fortes *et al.*, 1994; Lu *et al.*, 1994), but it also is an IFN antagonist (Garcia-Sastre *et al.*, 1998).

## **5.2. Potential ORF2 in Hantaviruses**

Unlike members of the *Phlebovirus*, *Tospovirus* and *Orthobunyavirus* genera, hantaviruses have not been shown to possess any nonstructural proteins. The S segment RNA of Hantaan virus (HTN), the prototype virus of the genus, though similar in size to those of the phleboviruses, apparently encodes exclusively the 50-kDa nucleocapsid protein. A small ORF, which could code for a 6 kDa polypeptide, is present in the same reading frame on the S segment as N ORF, immediately following the termination codon of HTN N, but a protein of this size has not been detected in HTN virus-infected cells (Schmaljohn *et al.*, 1986b). Moreover, this ORF is not conserved in the S genome segments of the viruses of other serotypes such as Seoul (SEO) (Arikawa *et al.*, 1990), Puumala (PUU) (Xiao *et al.*, 1993), Prospect Hill (PH) (Parrington and Kang, 1990), or Sin Nombre (SN) (Spiropoulou *et al.*, 1994). For these viruses, the sequence data revealed the presence of an overlapping reading frame with a potential to encode for a protein of 6 to 12 kDa in size. However, the existence of nonstructural proteins in these hantaviruses has not been demonstrated by analysis of viral polypeptides in infected cells nor by *in vitro* translation of S segment-specific RNA generated by SP6 transcription of S segment cDNA clones with subsequent immune precipitation (Schmaljohn *et al.*, 1986b; Parrington and Kang, 1990; Stohwasser *et al.*, 1990; Spiropoulou *et al.*, 1994; Stohwasser *et al.*, 1989; Arikawa *et al.*, 1990). Hence, it remained to be determined if these ORFs have any significance.

### **5.2.1. Comparison of S segment sequences of different hantaviruses**

As a first step, the FRAMES programme of the GCG software was used to analyse sequences of S segments of different hantaviruses from the database to look at possible second ORFs. Then, alignments of the potential ORF2 sequences were performed using BESTFIT, GAP, PILEUP, LINEUP and PRETTY programmes of the GCG software packages.

S segment sequences of the following hantaviruses from the database (GenBank accession numbers are shown in brackets) were used for comparison and later divided into three groups according to their capacity to encode a second protein based on the results of the FRAMES programme:

1. Puumala (M32750, X61035, AF324902, AF442613, AJ277030, AJ277033, AJ277034, AJ277075, AJ277076, U14137, Z30708, AB010730, AB010731), Prospect Hill (M34011), Khabarovsk (U35255), and Isla Vista (U31535)
2. Sin Nombre (L25784, L37904), Rio Mammore (U52136), Bayou (L36929), Lechiguan (AF482714), Pergamio (AF482717), Marciel (AF482716), Muleshoe (U54575)
3. Seoul (AF288655, AF288295, M34881), Hantaan (AF329390), Dobrava (AJ410619)

Results of the FRAMES programme are shown in Fig.5.1. One representative of each group, Puumala virus (Fig.5.1, A), Sin Nombre virus (B) and Seoul virus (C), is used as an example.

An ORF for a potential second protein (6-12 kDa) was found in all hantaviruses except for Hantaan (HTN), Seoul (SEO) and Dobrava (DOB) viruses as shown in Fig. 5.2, a schematic summary of the results. A similar potential overlapping second ORF was shown to be present in the S segment of Puumala (PUU), Isla Vista (IV), Prospect Hill

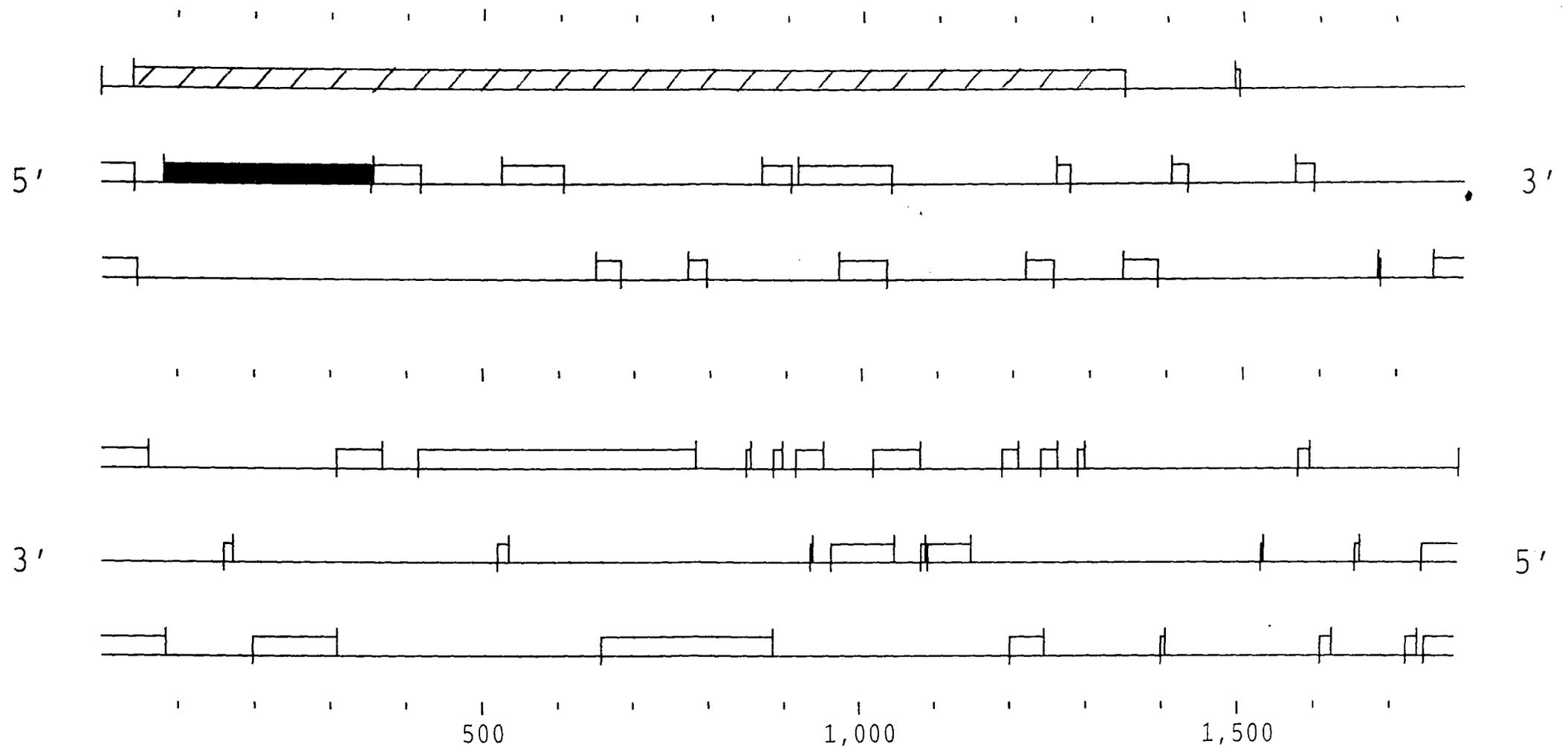


Fig.5.1. Potential open reading frames in the S segment of hantaviruses analysed by the FRAMES programme of the GCG software. Six potential reading frames are presented. Reading frames one to three (from the top) are virus complementary-sense RNA and frames four to six are virus-sense RNA. Vertical lines indicate positions of potential start codons. Striped bar indicates the nucleocapsid protein ORF. Solid bar indicates reading frame of the putative ORF2 protein. A.Puumala virus, B.Sin Nombre virus, C.Seoul virus

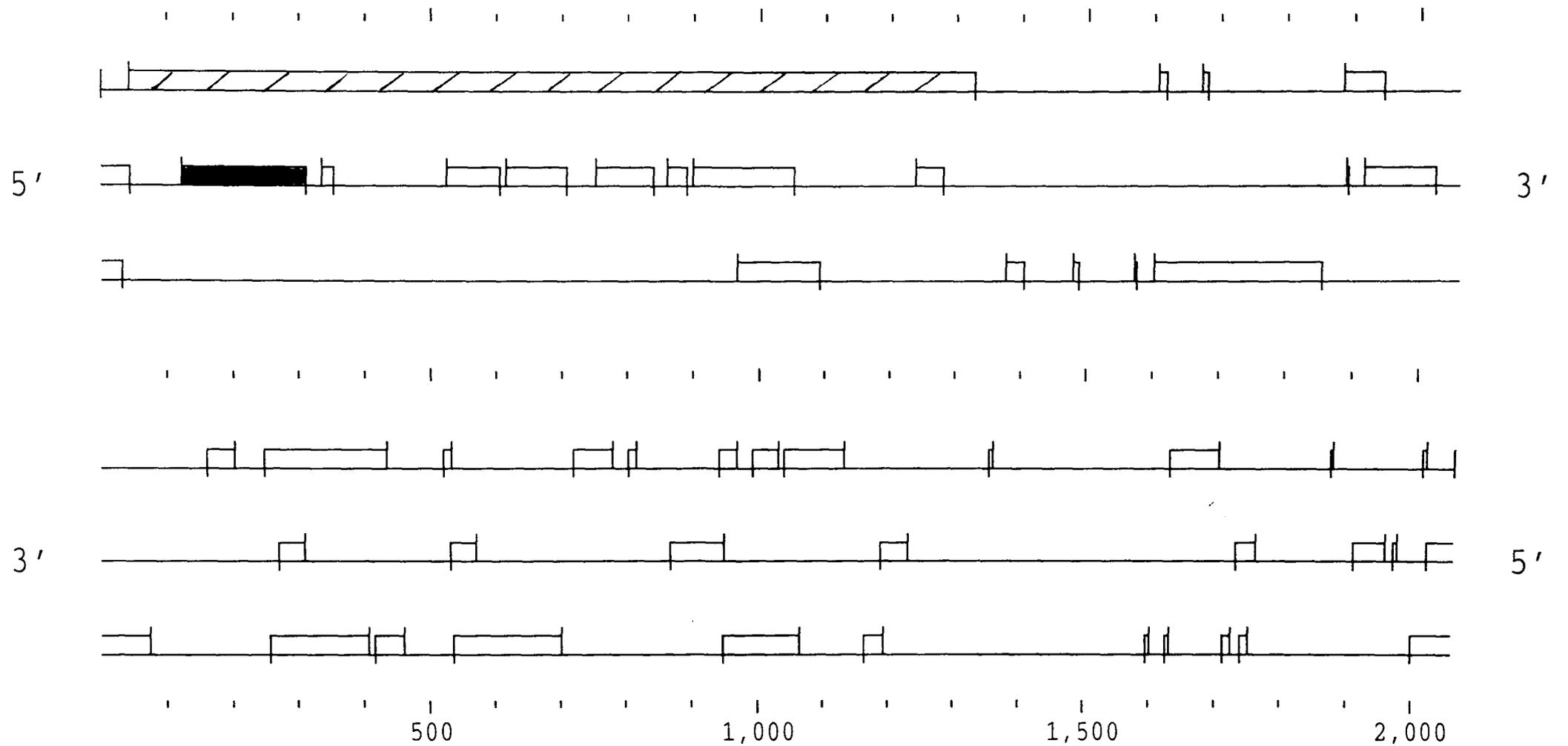


Fig.5.1. **B.** Potential open reading frames in the S segment of Sin Nombre hantavirus analysed by the FRAMES programme of the GCG software. Six potential reading frames are presented. Reading frames one to three (from the top) are virus complementary-sense RNA and frames four to six are virus-sense RNA. Vertical lines indicate positions of potential start codons. Striped bar indicates the nucleocapsid protein ORF. Solid bar indicates reading frame of the putative ORF2 protein.

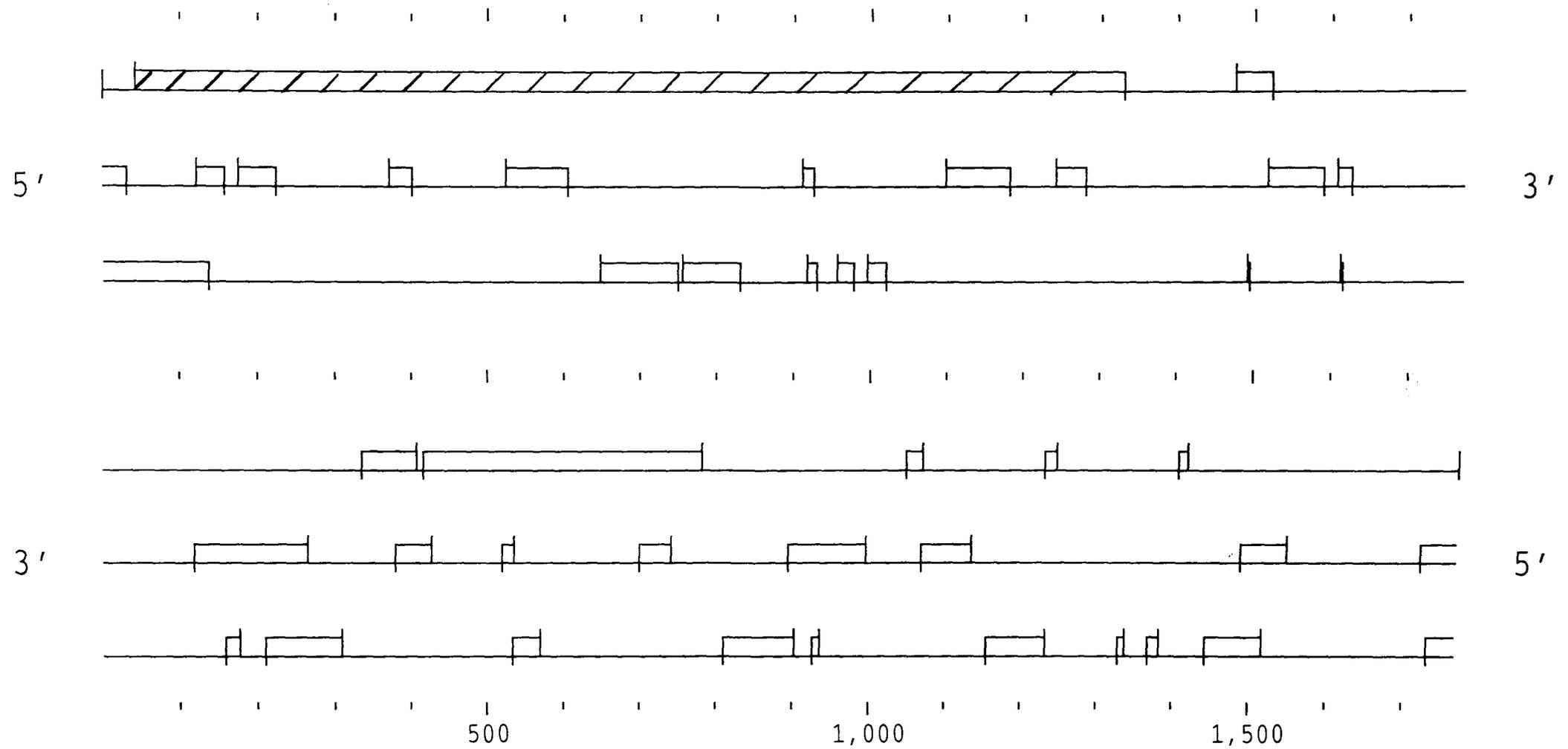
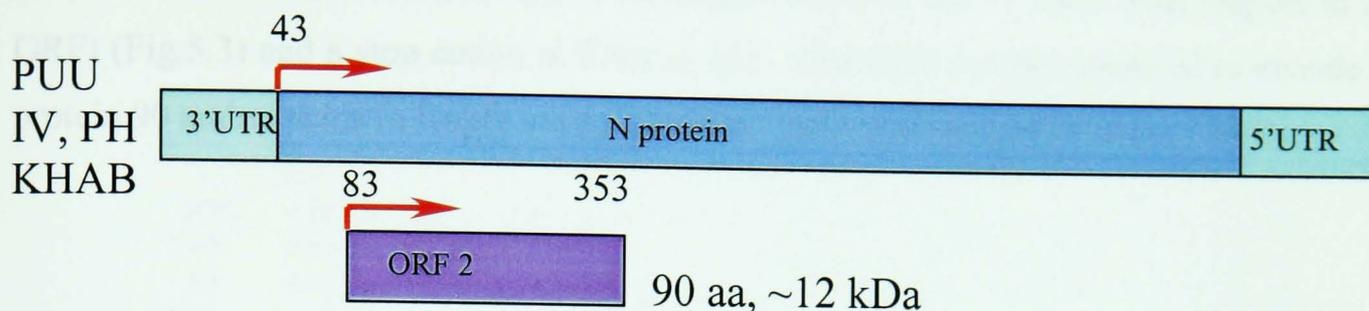
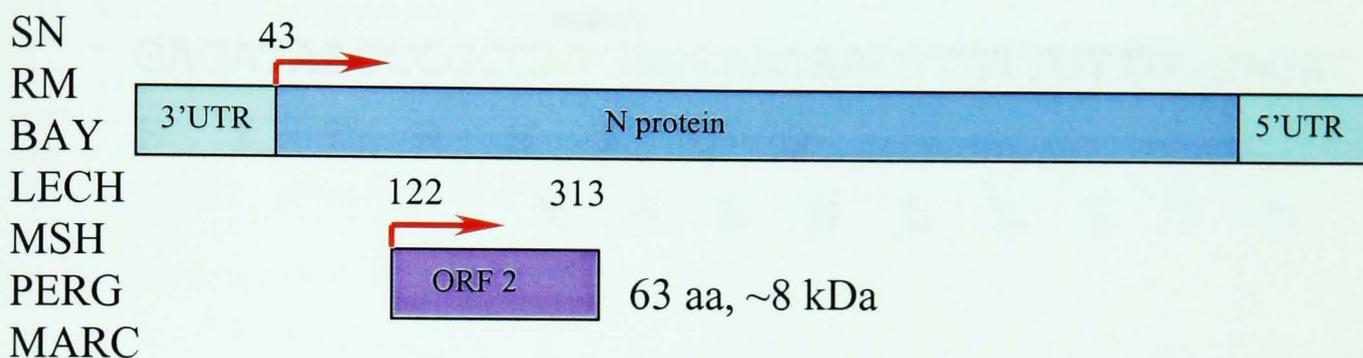


Fig.5.1.C. Potential open reading frames in the S segment of Seoul hantavirus analysed by the FRAMES programme of the GCG software. Six potential reading frames are presented. Reading frames one to three (from the top) are virus complementary-sense RNA and frames four to six are virus-sense RNA. Vertical lines indicate positions of potential start codons. Striped bar indicates the nucleocapsid protein ORF.

A.



B.



C.

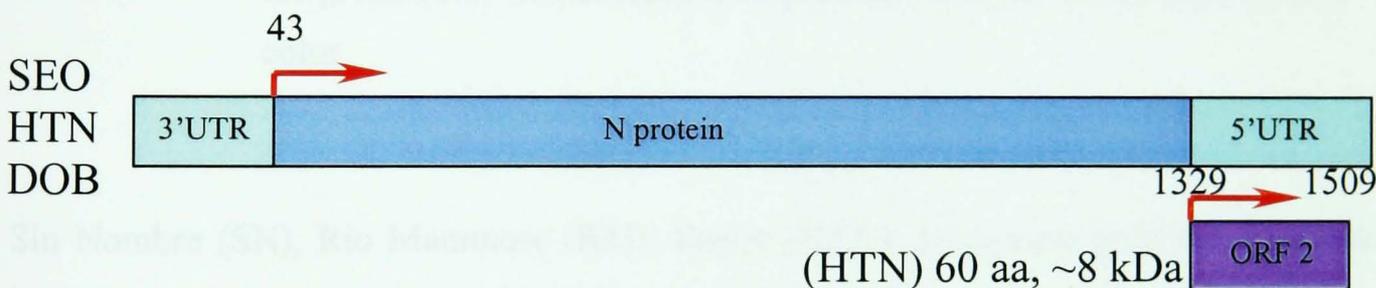


Fig. 5.2. Coding strategy of the S segment of different hantaviruses. Sequences corresponding to the S segment of hantaviruses were analyzed by the FRAMES programme of the GCG software and the results are presented schematically. **(A)**. Puumala (PUU), Isla Vista (IV), Khabarovsk (KHAB) and Prospect Hill (PH) viruses contain second ORF, overlapping that of N protein, with start codon at position 83 and stop codon at position 353 with a potential to encode a protein of 90 amino acids in length. **(B)**. Sin Nombre (SN), Rio Mammore (RM), Bayou (BAY), Lechiguan (LECH), Pergamio (PERG) and Marciel (MARC) viruses contain second ORF with start codon at position 122 and stop codon at position 313 with a potential to encode a protein of 63 amino acids. **(C)**. No second ORF similar to the one encoded by the above hantaviruses was found on the S segment of Seoul (SEO), Hantaan (HTN) and Dobrava (DOB) viruses. However, a small ORF, which could code for a 6 kD polypeptide, is present in the same reading frame on the S segment as N ORF, immediately following the termination codon of HTN N.



	83				→	132
RM	atgaacagca	gcttgtagct	gctcgtcaaa	aacttaagga	tgccgagaag	
BAY	acgaacaaca	actcgtgact	gccaggcaga	agctcaaaga	tcagaaaaga	
SN	acgaacaaca	actcgtgact	gccaggcaga	agctcaaaga	tcagaaaaga	
PERG	acgaacaaca	tctcgtgact	gccaggcaga	aacttaaaga	tgccgagaag	
MARC	acgaacaaca	actcgtgact	gccaggcaga	agctgaaaga	tcagaaaaa	
MSH	atgagcagca	gctggtgaca	gctaaacaaa	agctcaaaga	tgccgaacga	
PUU AB010730	atgaacaaca	acttgttggt	gcccgcaaaa	agcttagaga	tcagagaaa	
PUU AB010731	atgaacaaca	acttgttggt	gcccgcaaaa	agcttagaga	tcagagaaa	
PUU AJ277030	atgaacaaca	acttgttggt	gccagacaga	aactcaagga	tcagaaaag	
PUU AJ277033	atgaacaaca	acttgttggt	gccagacaga	aactcaagga	tcagaaaag	
PUU AJ277034	atgaacaaca	acttgttggt	gccagacaga	aactcaagga	tcagaaaag	
PUU AJ277075	atgaacaaca	acttgttggt	gccagacaga	aactcaagga	tcagaaaag	
PUU AJ277076	atgaacaaca	acttgttggt	gccagacaga	aactcaagga	tcagaaaag	

Fig.5.4. Alignment of partial nucleotide sequences of different hantaviruses using PRETTY programme of the GCG software. Rio Mammore (RM), Sin Nombre (SN), Pergamio (PERG), Marciel (MARC), Muleshoe (MSH) and Bayou (BAY) hantaviruses have a start codon for the putative ORF2 protein at position 122 (shown in blue and arrow). At position 83, a start codon for hantaviruses Puumala, Isla Vista and Prospect Hill, the viruses RM and MSH also contain an ATG and viruses SN, PERG, MARC and BAY have an ACG (shown in blue) indicating that an ATG could be lost over time. However, there is also a stop codon in all of these hantaviruses at position 98 (shown in red).

Hantaan, Seoul and Dobrava hantaviruses were not shown to possess a coding capacity for a protein similar to the ORF2 protein of other hantaviruses of the genus (Fig.5.1, C and 5.2, C). Sequence analysis of viruses Seoul and Hantaan presented in Fig. 5.5, A and B, respectively, reveals the presence of several stop codons in the 83-355 region.

Analogous or similar ORFs were described for PUU strains CG 18-20 and Sotkamo (Stohwasser *et al.*, 1990; Vapalahti *et al.*, 1992), and PH (Parrington and Kang, 1990), Tula (Plyusnin *et al.*, 1994a) and Sin Nombre hantaviruses (Spiropoulou *et al.*, 1994) but not for HTN or SEO viruses (Arikawa *et al.*, 1990; Giebel *et al.*, 1991; Schmaljohn *et al.*, 1986b).

The sequences corresponding to the second open reading frame of different hantaviruses can be readily aligned using BESTFIT programme with a percent identity from 77 to 100. As an example, the results of sequence alignment of two viruses encoding 90aa protein (Prospect Hill and Puumala) and 63aa protein (Pergamio and Rio Mammore) are presented in Fig. 5.6, A and B, respectively.

Additional evidence that the ORF2 encodes a protein is the presence of conserved motifs amongst the putative ORF2 proteins of the studied hantaviruses. Within all putative hantavirus ORF2 proteins, whether they are the 90aa form found in PUU, PH, IV, KHAB viruses or the 63 aa version found in SN, BAY, RM, LECH, MSH, PERG, MARC S RNA sequences, there are 8 conserved residues and 4 conserved positions that contain either lysine or arginine. Ten additional conserved amino acids plus two lysine/arginine positions are present when only the 90aa proteins are examined. The results of amino acid sequence comparison of several hantaviruses, performed using PileUp programme, are presented in Fig. 5.7.

### **5.2.2. Implications for the functionality of the ORF2 protein**

Spiropoulou *et al.* (1994) and Bowen *et al.* (1995) examined the nucleotide substitution frequency distribution within the N ORF of different hantaviruses. If the second ORF

**A**

1 tagtagtaga ctcctaag agctactaca ctaacaagaa aaatggcaac tatggaagaa 60

61 atccagagag aatcagtg<sup>c</sup> <sup>83</sup>→ tcacgagggg cagcttg<sup>tga</sup> tagcacgcca gaaagtcaag 120

121→ gatgcagaaa agcagtatga aaaggatcct gatgact<sup>taa</sup> acaagagggc actgcatgat 180

181 cgggagagtg tcgcagcttc aatacaatca aaaattgatg aat<sup>tga</sup>agcg ccaacttgcc 240

241 gacaggattg cagcagggaa gaatattggg caagaccggg atcctacagg ggtagagccg 300

**B**

1 tagtagtaga ctcctaag agctactata gcaacgatgg caactatgga ggaattacag 60

61 agggaaatca atgccc<sup>atga</sup> <sup>83</sup>→ gggccagctg gtgatagcca ggcagaaggt gagggatgca 120

121→ gaaaaacagt atgaaaagga tccagatgaa ctgaacaaaa gaacatt<sup>aac</sup> agacagagaa 180

181 ggggttgcag catctatcca ggctaagatt gatgaa<sup>tga</sup> aaagacagtt ggcagatagg 240

241 attgcaactg gaaagaacct tggaaaggaa caggacccaa ctgggggttga acctggagac 300

Fig. 5.5. Partial sequences of the S segment of hantaviruses Seoul (strain r22, GenBank Acc N AF288295) (A) and Hantaan (strain A9, GenBank Acc N AF329390) (B). Potential start codons are shown in blue and stop codons in red. Start codon for N protein is shown in bold and underlined. Arrows indicate positions of start codons for the ORF2 proteins encoded by other hantaviruses (either 83 or 122).

**A**

```

83 atgagcagcagcttgtcattgcccgccagaaagctcaaggaagctgaacgg 132
   ||||| || || ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
83 atgaacaacaacttggtggtgccagacagaaactcaaggatgcagaaaag 132

133 acggtggaggtggacccagatgacgttaacaaaagtacactgcaaagcag 182
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
133 gcggtggagatggacccagatgacgttaacaaaacacattgcaagcaag 182

183 gcggtcagcagtgtaacattggaggacaaattggcagagttcaagaggc 232
   || ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
183 gcaacagacagtgtaacactggaggacaaactgcagacttcaagcgac 232

233 agcttgcagatgtcatctcacgtcagaagatggatgagaaacctgtggat 282
   || ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
233 agatggcagatgctgtgtccaggaaaaaatggataactaacctactgac 282

283 ccaactggtattgagcttgacgaccatcttaaggagaggtcaagcctcca 332
   || ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
283 ccgactgggattgagcctgatgaccatctcaaggagcgatcaagcctcag 332

333 atatggaaatgtccttgatgtga 355
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
333 atatgggaatgtccttgatgtaa 355

```

**B**

```

122 atgccgagaagaccgtggaagtggacccagatgaagtcaacaagagcaca 171
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
122 atgccgagaaggcagtggaagtggacccagatgacgttaacaagagcaca 171

172 ttacaaagtagacggggcggctgtgtctacattggagaccaaacttggaga 221
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
172 ttacaaagcagacgggcagctgtgtctacattggagaataaaactcgcaga 221

222 gctcaagaggcagcttgagatttgggtggcagctcaaaagctggctgcaa 271
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
222 actcaagagacagctggcagatttgggtggcagctcaaaaactggcttcaa 271

272 agccagttgatccaacagggttgagcctgatgaccatctga 313
   ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
272 aaccagttgatccaacagggttgagcctgatgaccatttaa 313

```

Fig. 5.6. Sequence comparison of hantaviruses encoding ORF2 proteins of 90 amino acids (A) and 63 amino acids (B) performed using BESTFIT programme of the GCG software. **A.** Sequence comparison of Prospect Hill (GenBank Acc. N M34011) and Puumala (GenBank Acc. N AJ277034) viruses. Percent identity 76.923. **B.** Sequence comparison of Pergamio (GeneBank Acc. N AF482717) and Rio Mammone (GenBank Acc. N U52136) viruses. Percent identity 90.625.

PUU M32750	~~~~~MSNNLL	LPDKNSRMQR	EQWKWTRMTL	TRAHYKQDNK	QCQHWRIINSQ
PUU AF442613	~~~~~MSNNLL	LPDKNSRMQR	EQWKWTRMTL	TRTHYKQGNK	QCQHWRIINSQ
PUU AJ277033	~~~~~MNNNLL	LPDRNSRMQK	RRWRWTQMTL	TKTHCKQGNR	QCQHWRTNLQ
PUU AJ277034	~~~~~MNNNLL	LPDRNSRMQK	RRWRWTQMTL	TKTHCKQGNR	QCQHWRTNLQ
PUU AJ277075	~~~~~MNNNLL	LPDRNSRMQK	RRWRWTQMTL	TKTHCKQGNR	QCQHWRTNLQ
PUU AJ277076	~~~~~MNNNLL	LPDRNSRMQK	RRWRWTQMTL	TKTHCKQGNR	QCQHWRTNLQ
PUU AB010730	~~~~~MNNNLL	LPGKSLEMQR	KQWKWTQMTL	TRTHCKHGNK	QCQHWRIINLP
MSH U54575	~~~~~MSSSSW	QLNKSSKMPN	EQWKWTPMML	TKAHYRADGQ	LCLHWRPNLV
PH M34011	~~~~~MSSSLS	LPGRSSRKLN	GRWRWTQMTL	TKVHCKAGGQ	QCQHWRTNWQ
IV U31535	~~~~~MSSSWL	LQGKNLKMFK	KQWRWTRMMS	TRVHSKADGR	QCQHWRIINLP
SN L25784	~~~~~	~~~~~MQK	ERWNWTPMML	TKAHYRADGQ	LCLHWRPNSE
SN L37904	~~~~~	~~~~~MQK	ERWNWTPMML	TKAHYRADGQ	LCLHWRPNSE
RM U52136	~~~~~	~~~~~MPR	RQWKWTQMTL	TRAHYKADGQ	LCLHWRINSQ
LECH AF482714	~~~~~	~~~~~MPK	RRWRWTRMRL	TRAHYKVDGQ	LCLHWRPNSE
MARC AF482716	~~~~~	~~~~~MQK	KPWTWTQML	TRVHYKVDGQ	LCQHWRTNLE
KHAB U35255	MRSLVTSSSSS	LLDRNSGILR	RRWRRTPMML	TKIHYRQDGR	LCQHWRTNLQ
PUU M32750	TTREEWQMLC	PGRKWILNLL	TRLGLNLMII	SRRDQALDME	MSLM*~
PUU AF442613	TTREEWQMLC	PGRKWILNLL	TRLGLNLMII	SRRDQALDME	MSLM*~
PUU AJ277033	TSSDRWQMLC	PGKKWILNLL	TRLGLSLMTI	SRSDQASDMG	MSLM*~
PUU AJ277034	TSSDRWQMLC	PGKKWILNLL	TRLGLSLMTI	SRSDQASDMG	MSLM*~
PUU AJ277075	TSSDKWQMLC	PGKKWILNLL	TRLGLSLMTI	SRSDQASDME	MSLM*~
PUU AJ277076	TSSDKWQMLC	PGKKWILNLL	TRLGLSLMTI	SRSDRASDME	MSLM*~
PUU AB010730	TSSEEWQMLC	PGKRWILSLL	TRLGLNLMIT	SRRDLASDMG	MSLM*~
MSH U54575	NSRDNWQILL	QLRNWLQNLL	IQQGLSLMTI	*~~~~~	~~~~~
PH M34011	SSRGSQMSS	HVRRWMRNLW	IQLVLSLTTI	LRRDQASNME	MSLM *
IV U31535	TLRDNWQMLY	RARRWMISLL	TLGWNQMTT	SKRDQASGME	MSLM *
SN L25784	NLSGNWLILL	QLRNWLQNLL	IQQGLNLMIT	*~~~~~	~~~~~
SN L37904	NLSGNWLILL	QLRNWLQNLL	IQQGLNLMIT	*~~~~~	~~~~~
RM U52136	NSRDSWQIWW	QLKNWLQNQL	IQQGLSLMTI	*~~~~~	~~~~~
LECH AF482714	NSRDNLQIWW	QLKNWLQNQL	IQQGLSLMII	*~~~~~	~~~~~
MARC AF482716	SSRDSLTTWW	QLKNWLQNQL	IQQGLSLMTI	*~~~~~	~~~~~
KHAB U35255	TSRGSSRIMF	LVRWRMRGLL	IRLGLSQMII	SRRDQASGME	MSLM *

Fig. 5.7 Comparison of amino acid sequences encoding putative ORF2 protein by different hantaviruses. Amino acid sequences of hantaviruses Puumala (PUU M32750, AF442613, AJ277033, AJ277034, AJ277075, AJ277030), Muleshoe (MSH U54575), Prospect Hill (PH M34011), Isla Vista (IV U31535), Sin Nombre (SN L25784 and SN L37904), Rio Mammore (RM U52136), Lechiguan (LECH AF482714), Marciel (MARC AF482716) and Khabarovsk (KHAB U35255) were aligned using PileUp programme of the GCG software. Amino acids (aa) shown in blue are conserved among 90aa ORF2 proteins, amino acids shown in violet are conserved among 63aa version of ORF2 protein, and amino acids shown in red are conserved among all putative hantavirus ORF2 proteins.

were functional then the evolutionary constraint to maintain amino acid sequence in both frames would be expected to lower the third base substitution frequency in this region. Therefore, determination of whether the third base substitution frequency in the region of the N ORF that overlaps the ORF 2 is constrained would suggest that the ORF2 was functional. According to the results of statistical analysis, the third position base substitutions were found to predominate in the N ORF, and the average third position base substitution rate for the region of the N ORF outside ORF2 was higher than the rate for the entire N ORF. Within the overlap region of the N ORF and ORF 2, however, the third position base substitution rate was greatly reduced, dropping to less than 0.5 substitution per position from approximately 1.0 substitution per position in the region of the N ORF that lies outside the ORF 2. This reduction in third base substitution frequency in the overlapping region was statistically significant when compared with either the third base substitution frequency in the non-overlapping region of the N ORF ( $P < 0.00001$ ,  $\chi^2 = 203.42$ ,  $df = 1$ ) or the entire N ORF ( $P < 0.00001$ ,  $\chi^2 = 96.4$ ,  $df = 1$ ), thus suggesting that the putative ORF2 is functional (Spiropoulou *et al.*, 1994).

As seen with previously analysed viruses, a lower third-base substitution frequency in this region provides theoretical evidence that this second ORF codes for a functional protein in hantaviruses. A similar statistical analysis was employed in the case of vesicular stomatitis virus and did successfully predict a functional overlapping ORF in the P gene (Bilsel *et al.*, 1990). The NSs proteins of other members of the *Bunyaviridae* exhibit similar constraints on third base substitution frequency in the region of N ORF that overlaps the NSs ORF.

If this NSs protein prediction is correct, then only the Hantaan, Seoul and Dobrava hantaviruses and probably members of the *Nairovirus* genus are lacking such a protein predicted on the S RNA segment of other members of the *Bunyaviridae* family in either an overlapping ORF or in an ambisense orientation.

### **5.3. Attempts to identify ORF2 protein in Puumala virus infected cells**

So far, the ORF2 protein has not been reported in virus infected cells (Schmaljohn *et al.*, 1986b; Parrington and Kang, 1990; Stohwasser *et al.*, 1990; Spiropoulou *et al.*, 1994; Stohwasser *et al.*, 1989; Arikawa *et al.*, 1990). Therefore, an attempt was made to identify the putative ORF2 protein in Vero E6 cells infected with Puumala virus.

Briefly, subconfluent monolayers of Vero E6 cells were infected with Puumala virus at a MOI 10 pfu/cell. The cells were incubated for 24 hours at 37°C, then the growth media was substituted by maintenance media (2% DMEM) and cells incubated for additional 96 hours. Before labelling, the cells were starved in methionine-free media for 1 hour and then labelled with <sup>35</sup>S-methionine at 50µCi per dish for 4 hours. Cells were harvested and lysed. The cell extracts were analysed by 20% SDS/PAGE as described in Methods followed by autoradiography. Protein profiles of mock- and Puumala virus infected cells are shown in Fig.5.8, lanes 1 and 2, respectively. No distinctive band corresponding to the protein of the expected size of 12 kDa was observed in virus-infected cells compared to mock-infected cells.

Unlike orthobunyaviruses, hantaviruses do not cause host cell synthesis shut off. Therefore, identification of the viral proteins on the gel is problematic. This could explain why no distinctive band of close to ORF2 size was identified by autoradiography of radiolabelled Puumala infected VeroE6 cell extracts.

### **5.4. Construction of pTMPUUORF2, pTMPUUORF2FLAG, pTMHTNN and pTMSEON**

Four plasmids were constructed for the following work. Open reading frames for Hantaan and Seoul virus N proteins were cloned into pTM1 cloning vector that contains a bacteriophage T7 promoter and an internal ribosome entry site (IRES). This would allow

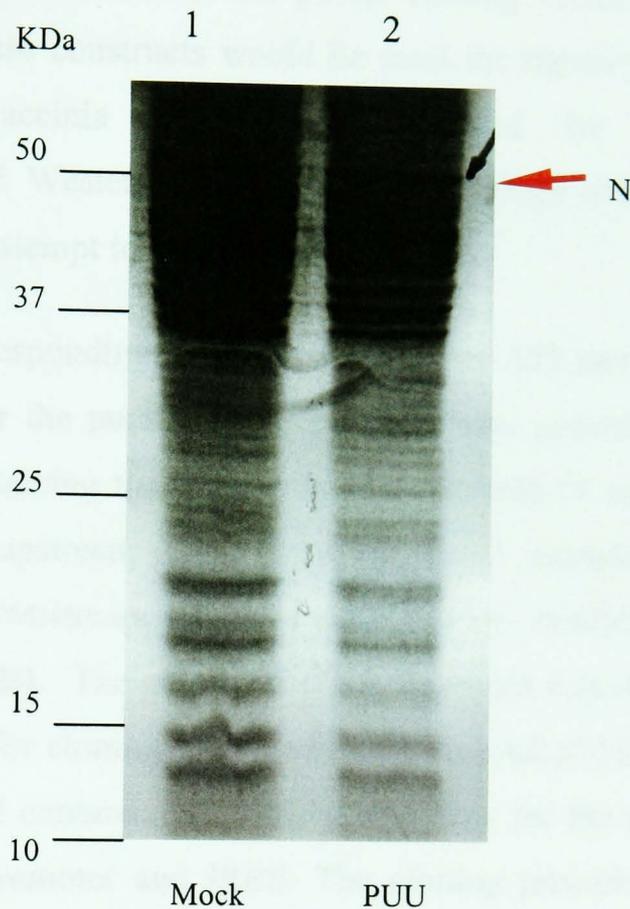


Fig. 5.8. Infection of VeroE6 cells with Puumala virus. Subconfluent monolayers of Vero E6 cells were infected with Puumala virus at MOI 10 pfu/cell. The cells were incubated for 96 hours and then labelled with  $50\mu\text{Ci}$   $^{35}\text{S}$  methionine for 4 hours. Cells were harvested and lysed. Cell lysates were analysed by 20% SDS PAGE followed by autoradiography. Hantaviruses do not cause host cells synthesis shut off. No distinctive band corresponding to the protein of the expected size of about 12 kDa was observed in virus-infected cells. Lane 1, mock infected; lane 2, Puumala infected.

expression of the protein by T7 polymerase using coupled transcription/translation system in rabbit reticulocyte lysates (TnT) and would be used for comparison with Puumala virus. Also, sequences encoding either PUU ORF2 protein or a FLAG-tagged version of ORF2 protein were cloned into pTM1 cloning vector under control of a T7 promoter and IRES. These constructs would be used for transient expression of ORF2 protein using the vaccinia T7 system followed by metabolic labelling, immunoprecipitation and Western blot analysis with either anti-FLAG or anti-ORF2 peptide antibodies in an attempt to identify ORF2 protein.

The DNA fragment corresponding to amino acids 83 to 353 and representing the open reading frame coding for the putative ORF2 protein was generated by PCR (Fig.5.9). Plasmid pTMPUUS containing the S gene coding for both N and ORF2 proteins was used as a template. The upstream primer PuuORF2*NcoI*<sup>+</sup> contained an *NcoI* restriction enzyme site and the downstream primer PuuORF2*PstI*<sup>-</sup> contained a *PstI* restriction enzyme site (see Materials). The amplified DNA fragment was digested with *NcoI* and *PstI* restriction enzymes for cloning into *NcoI/PstI* – cleaved pTM1 vector. The resultant construct pTMPUUORF2 contained the coding sequence for the putative ORF2 protein under control of a T7 promoter and IRES. The cloning procedure was confirmed by restriction enzyme analysis and nucleotide sequencing.

Plasmid pTMPUUORF2FLAG was constructed in a similar manner to pTMPUUORF2, except the primer PuuORF2*PstI*FLAG<sup>-</sup>, incorporating *PstI* restriction enzyme site, also contained a sequence encoding the FLAG peptide (see Materials). The resultant construct contained the sequence encoding ORF2 protein with FLAG peptide epitope (Hopp, 1988) fused to the C terminus under control of a bacteriophage T7 promoter and IRES.

For cloning the N ORF of Hantaan virus (HTN) into pTM1 cloning vector, plasmid pAHTNS supplied by Dr X Shi was used as a template. The DNA fragment representing the open reading frame coding for the N protein was generated by PCR using primers incorporating restriction enzymes *NcoI* (primer HtnNORF*NcoI*<sup>+</sup>) and *PstI* (primer HtnNORF*PstI*<sup>-</sup>) (see Materials). The amplified fragment was then digested with these enzymes and cloned into a *NcoI/PstI* –cleaved pTM1 cloning vector (Fig. 5.10, A).

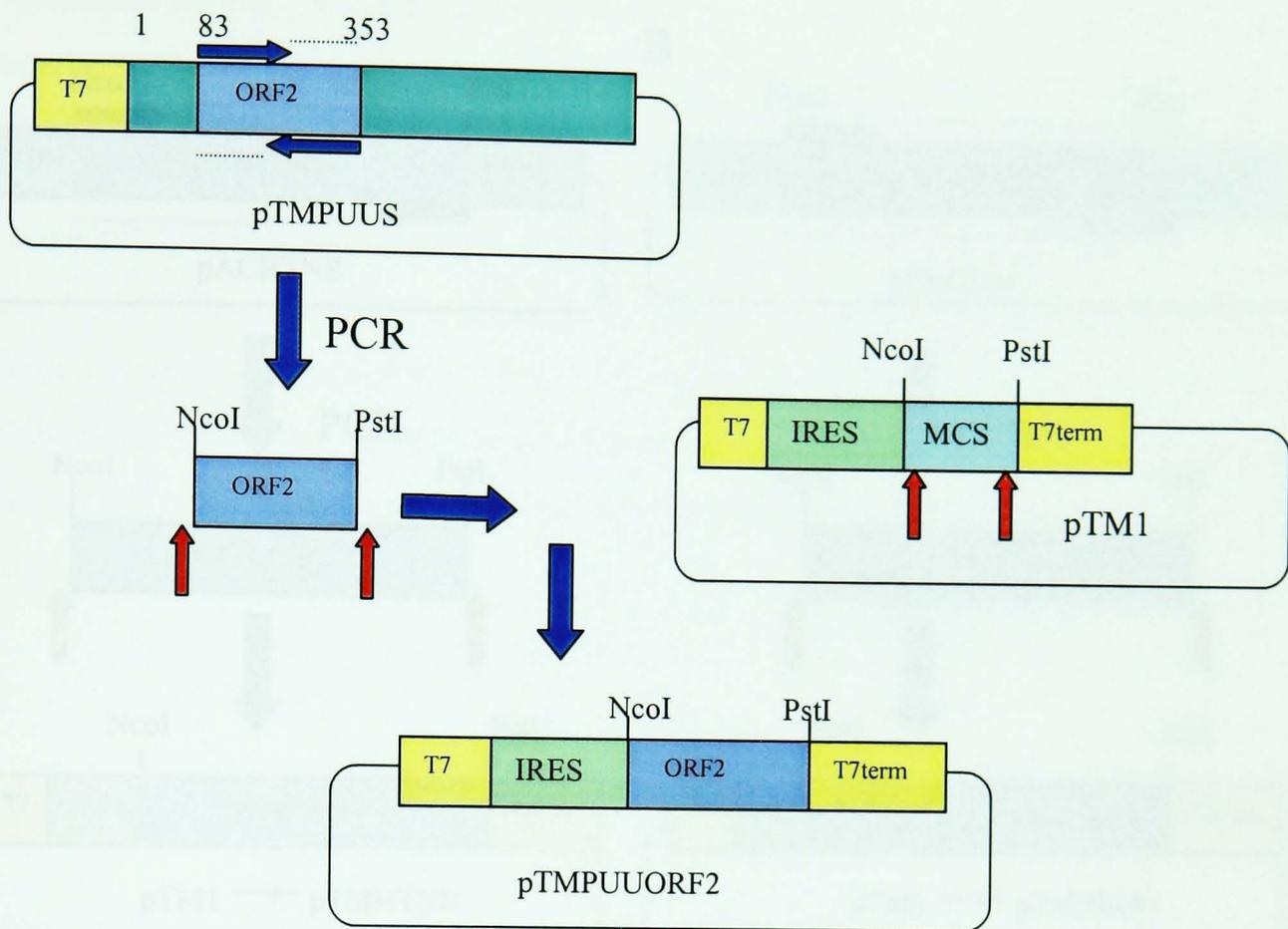


Fig. 5.9. Construction of pTMPUUORF2 expressing putative ORF2 protein. pTMPUUS plasmid was used as a template to PCR amplify second ORF on PUU S segment encoding a putative ORF2 protein with initiation codon at position 83 and stop codon at position 353. The primers contained restriction enzyme sites NcoI and PstI. After restriction digestion with these enzymes, the PCR product was cloned into pTM1 cloning vector cleaved with NcoI and PstI restriction enzymes. The resultant construct pTMPUUORF2 contained ORF2 under control of bacteriophage T7 promoter and IRES

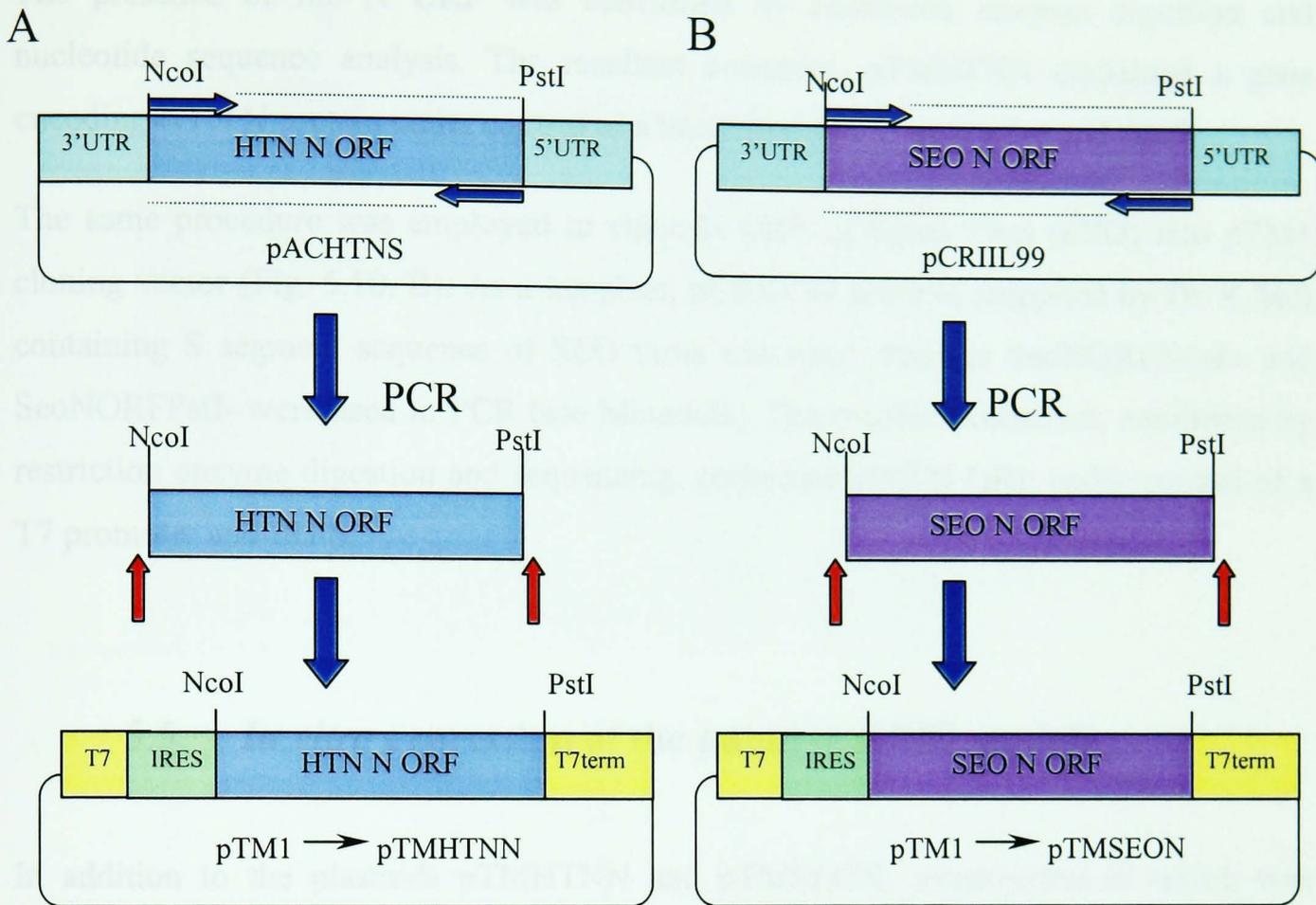


Fig. 5.10. Construction of pTMHTNN and pTMSEON plasmids containing N ORF of Hantaan (HTN) and Seoul (SEO) viruses under control of the bacteriophage T7 promoter.

(A) Hantaan virus N ORF was PCR amplified using pAHTNS plasmid as a template, with primers incorporating NcoI and Pst I restriction sites. PCR product was digested with these enzymes and cloned into NcoI/PstI-cleaved pTM1 cloning vector, that contains the bacteriophage T7 promoter and terminator.

(B) Seoul virus N ORF was amplified by PCR with primers incorporating NcoI and PstI restriction sites. Plasmid pCRIIL99 was used as a template. After digestion with NcoI and PstI restriction enzymes, SEO N ORF was cloned into pTM1 cleaved with these enzymes. The resultant construct pTMSEON contained SEO N ORF under control of bacteriophage T7 promoter and EMCV IRES.

T7, T7 promoter; T7term, T7 terminator.

The presence of the N ORF was confirmed by restriction enzyme digestion and nucleotide sequence analysis. The resultant construct, pTMHTNN contained a gene encoding HTN N protein under control of a bacteriophage T7 promoter and IRES.

The same procedure was employed to clone N ORF of Seoul virus (SEO) into pTM1 cloning vector (Fig. 5.10, B). As a template, pCRIL99 plasmid (supplied by Dr X Shi) containing S segment sequence of SEO virus was used. Primers SeoNORFNcoI+ and SeoNORFPstI- were used in PCR (see Materials). The resultant construct, confirmed by restriction enzyme digestion and sequencing, contained SEO N ORF under control of a T7 promoter and IRES.

### **5.5. *In vitro* expression of the putative ORF2 protein**

In addition to the plasmids pTMHTNN and pTMSEON, construction of which was described previously, two other plasmids, pTzPUUS and pGEMSotkamoS (provided by Dr X Shi), containing S segment of Puumala virus strains Vranica and Sotkamo, respectively, under control of a T7 promoter, were used in *in vitro* coupled transcription/translation system (TnT kit, Promega) for expression of the ORF2 protein and comparison to pTMPUUS (containing S segment of Puumala virus strain cg1820).

As expected, no equivalents of ORF2 protein were observed following *in vitro* transcription/translation of cDNA clones of the Hantaan (plasmid pTMHTNN) and Seoul (plasmid pTMSEON) virus S segment. These were previously demonstrated by sequence analysis to lack second ORF. As seen on the gel, only N proteins, that migrate slower than Puumala virus N protein, were expressed (Fig.5.11, lanes 1 and 2).

*In vitro* translation of pTMPUUS, containing S segment sequence (therefore coding for both N and presumably ORF2 proteins) under control of a bacteriophage T7 promoter and IRES, in the rabbit reticulocyte lysate using TnT kit yielded three major polypeptides (lane 5). Electrophoretic mobilities of two of them were consistent with the predicted molecular weights of the N protein (50kDa) and ORF2 protein (12.5 kDa).

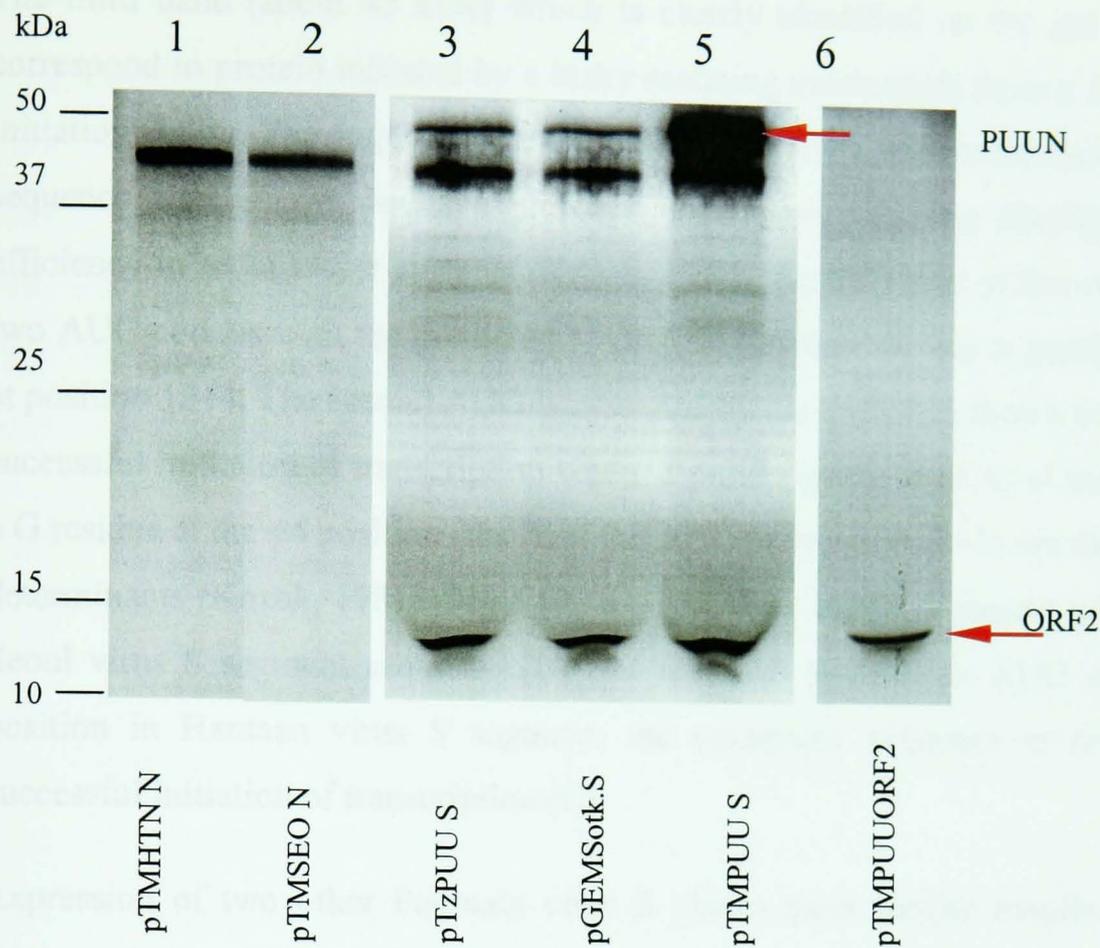


Fig. 5.11. *In vitro* transcription/translation in rabbit reticulocyte lysates using TnT kit (Promega). Proteins encoded on the S segment of Hantaan and Seoul viruses, and three Puumala virus S clones, were expressed *in vitro* using TnT kit and analyzed by autoradiography on a 20% SDS-PAGE. Expression of pTMHTNN and pTMSEON plasmids that contain sequences coding for Hantaan and Seoul virus N proteins, respectively, and lacking the capacity to encode a nonstructural protein gave only bands corresponding to N protein (lanes 1 and 2, respectively). Translation of three Puumala virus S clones from plasmids containing sequences coding for N and ORF2 proteins, pTzPUUS (lane 3), pGEMSotkamoS (lane 4) and pTMPUUS (lane 5) resulted in production of three proteins: N (50 kDa), ORF2 (12 kDa) and a third protein (~45 kDa) generated by a leaky scanning mechanism from a downstream AUG codon. The third protein was similar in size to the one expressed from pTMPUUORF2 plasmid (lane 6). Markers (in kilodaltons) are shown on the left.

The third band (about 45 kDa) which is clearly identified on the gel was assumed to correspond to protein initiated by a leaky scanning mechanism from a downstream AUG initiation codon. The sequence context around the AUG start or secondary AUGs in the sequence were shown to have profound effects on initiation fidelity and translation efficiency in reticulocyte systems (Kozak, 1990). Examination of the sequence revealed two AUG codons with the predicted N ORF, at position 43 and at position 235, to UGA at position 1344. The second AUG is preceded by the sequence shown to be important for successful initiation of transcription where a purine (preferably A) at the -3 position and a G residue at the +4 position (the A of the AUG is designated +1) are the most important determinants (Kozak, 1981) (Fig.5.12, A). No AUG codon is present downstream of the Seoul virus S segment sequence (B) and although there is an AUG codon at the 319 position in Hantaan virus S segment, the consensus sequence is not optimal for a successful initiation of transcription (C).

Expression of two other Puumala virus S clones gave similar results. The translation products in the rabbit reticulocyte lysate from plasmids pTzPUUS and pGEMSoTKamoS are shown in lanes 3 and 4 (Fig.5.11), respectively. The N and ORF2 can be identified as bands corresponding to the protein sizes of 50 and 12.5 kDa, as can protein of a smaller than N size generated by a leaky scanning mechanism from the second AUG codon.

To determine if the PUU ORF2 protein could be expressed from pTMPUUORF2 plasmid, *in vitro* coupled transcription/translation system (TnT kit, Promega) in a rabbit reticulocyte lysate was used. Translation of pTMPUUORF2 gave a protein of 12.5 kDa (Fig. 5.11, lane 6) which was similar in size to the ORF2 protein expressed from pTMPuuS (compare lanes 5 and 6, respectively).

The results of the *in vitro* transcription/translation system using rabbit reticulocyte lysate demonstrate that ORF2 protein, shown by sequence analysis to be encoded on Puumala virus S segment in an open reading frame overlapping that of the N protein, can be expressed from pTMPUUORF2 containing only the sequence coding for ORF2 protein and pTMPUUS containing S gene coding for both N and ORF2 proteins.

## A PUU

1 TAGTAGTAGA CTCCTTGAAA AGCTACTACG AGAACAAC<sup>-3</sup> **GAATG**<sup>+1</sup> GTGA ← 50kDa N  
 51 CTTGACAGAC ATCCAAGAGG AGATAACCC<sup>-3</sup> **CCATG**<sup>+1</sup> GCAA CAACTTGTG ← 12kDa ORF2  
 101 TTGCCAGACA AAAACTCAAG GATGCAGAGA GAGCAGTGGG AGTGGACCCG  
 151 GATGACGTTA ACAAGAGCAC ATTACAAGCA AGACAACAAA CAGTGTCAGC  
 201 ACTGGAGGAT AA<sup>-3</sup>ACTCGCAG ACTACAAGAG A<sup>+1</sup>**GAATG**<sup>+4</sup>CA GATGCTGTG ← 45kDa ΔN  
 251 CCCGGAAGAA AATGGATACT AAACCTACTG ACCCGACTGG GATTGAACCT

## B SEO

1 TAGTAGTAGA CTCCCTAAAG AGCTACTACA CTAACAAGA<sup>-3</sup> **AAATG**<sup>+1</sup> CAAC ← SEO N  
 51 TATGGAAGAA ATCCAGAGAG AAATCAGTGC TCACGAGGGG CAGCTTGTGA  
 101 TAGCACGCCA GAAAGTCAAG GATGCAGAAA AGCAGTATGA AAAGGATCCT  
 151 GATGACTTAA ACAAGAGGGC ACTGCATGAT CGGGAGAGTG TCGCAGCTTC  
 201 AATACAATCA AAAATTGATG AATTGAAGCG CCAACTTGCC GACAGGATTG  
 251 CAGCAGGGAA GAATATTGGG CAAGACCGGG ATCCTACAGG GGTAGAGCCG  
 301 GGTGATCATC TCAAAGAGAG ATCAGCACTA AGCTATGGGA ATACACTGGA

## C HTN

1 TAGTAGTAGA CTCCCTAAAG AGCTACTATA GCA<sup>-3</sup> **ACGATG**<sup>+1</sup> CAACTATGGA ← HTN N  
 51 GGAATTACAG AGGGAAATCA ATGCCCATGA GGGCCAGCTG GTGATAGCCA  
 101 GGCAGAAGGT GAGGGATGCA GAAAAACAGT ATGAAAAGGA TCCAGATGAA  
 151 CTGAACAAAA GAACATTAAC AGACAGAGAA GGGGTTGCAG CATCTATCCA  
 201 GGCTAAGATT GATGAATTGA AAAGACAGTT GGCAGATAGG ATTGCAACTG  
 251 GAAAGAACCT TGGAAGGAA CAGGACCCAA CTGGGGTTGA ACCTGGAGAC  
 301 CATCTTAAGG AGAGATCAAT<sup>-3</sup> **GCTTAGCTAT**<sup>+1</sup> GGCAATGTGC TGGATTTAAA  
 351 CCATCTGGAT ATTGATGAGC CTACAGGGCA GACAGCAGAC TGGCTAAGTA

Fig.5.12. Partial sequences of S segments of viruses Puumala (A), Seoul (B) and Hantaan (C). Potential start codons are shown in blue. The optimal consensus is 5' A/G CCAUGG with a purine (preferably A) at the -3 position and a G residue at the +4 position (the A of the AUG is designated +1) being important determinants (Kozak, 1981) Three major AUG-initiated ORFs are present in the S segment of Puumala virus with third AUG being in optimal sequence context. The residues that are very important for efficient initiation are shown in red, the residues that substitute them are shown in pink.

## 5.6. Metabolic labelling of transiently expressed proteins

Metabolic labelling was performed to further investigate the expression of the ORF2 protein from the plasmid pTMPUUS containing S segment sequence coding for both N and ORF2 proteins. This would demonstrate that the putative ORF2 protein could be expressed *in vivo* as well as *in vitro*.

Recombinant vaccinia virus (vTF7-3) system was used to transiently express protein from transfected plasmid as this vaccinia helper virus is known to express high levels of T7 polymerase. Briefly, Vero E6 cells were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection 5 pfu/cell. The cells were transfected with protein-expression plasmid pTMPUUS, and 24 hrs posttransfection the cells were labelled for 2 hours with 50  $\mu$ Ci of  $^{35}$ S methionine. Cells were harvested and lysed. The cell extracts were analyzed by 20% SDS/PAGE as described in Methods followed by autoradiography (Fig. 5.13).

Protein profiles of mock- and vaccinia virus-infected cells are shown in lanes 1 and 2. N protein, expressed from pTMPUUS, can be clearly identified (lane 3), however, no distinctive band, corresponding to the putative ORF2 protein, can be seen. When compared to ORF2 protein expressed *in vitro* by TnT and used as a positive control (lane 5), it is noted that vaccinia virus protein, similar in size, could be comigrating with the putative ORF2 protein (compare lanes 2, 3 and 5). At the same time, the FLAG-tagged version of the ORF2, expressed from pTMPUUORF2FLAG, can be easily identified on the gel (lane 4).

It is also of note that only a single form of N protein of 50 kDa expressed from pTMPUUS *in vivo* can be detected on the gel (lane 3), therefore, a protein of a smaller size generated by a leaky scanning mechanism from secondary downstream AUG codon was indeed an artifact generated by *in vitro* reticulocyte lysate system.

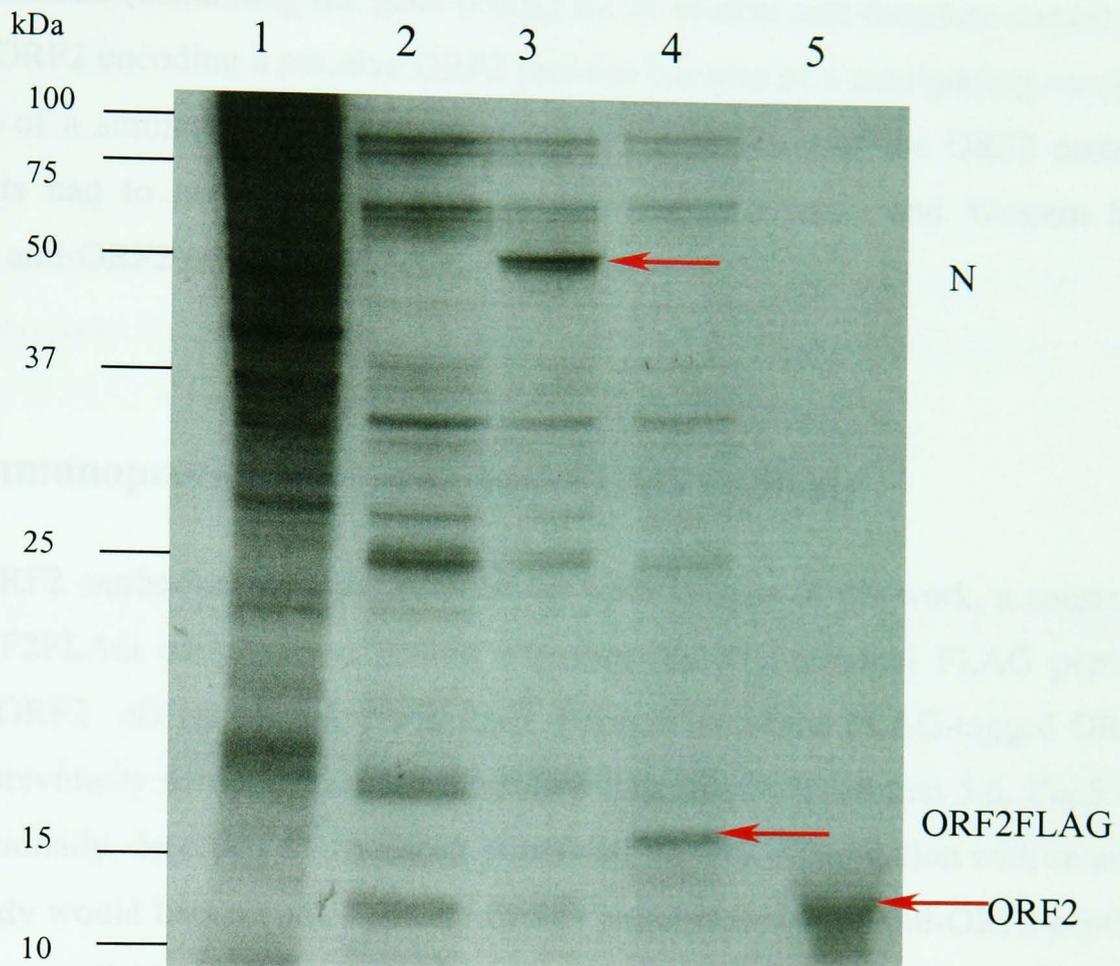


Fig. 5.13. Metabolic labelling of transiently expressed N and ORF2 proteins in vaccinia virus infected Vero E6 cells. Confluent monolayers of Vero E6 cells were infected with recombinant vaccinia virus vvTF7-3 at MOI 5 pfu/cell (lane 2) or mock infected (lane 1) and transfected with pTMPUUS (lane 3) and pTMPUUORF2FLAG (lane 4) plasmids. At 24 hours postinfection, the cells were labelled with 50  $\mu$ Ci of  $^{35}$ S methionine per dish for 2 hrs. The proteins were analyzed by electrophoresis in 20% SDS-PAGE and autoradiography. The positions of the N and ORF2FLAG proteins and the molecular mass markers (in kilodaltons) are shown on the left and right, respectively. Lane 1 – mock infected, lane 2, vvTF7-3 infected, lane 3, pTMPUUS, lane 4, pTMPUUORF2FLAG, lane 5, *in vitro* transcribed ORF2 protein from pTMPUUORF2

It was difficult to conclude whether or not the ORF2 protein was expressed *in vivo* from pTMPUUS plasmid (containing the gene coding for N protein and therefore containing overlapping ORF2 encoding a putative ORF2 protein) because of a comigrating vaccinia virus protein of a similar size. Therefore, to prove the presence of the ORF2 protein, other methods had to be employed, such as immunoprecipitation and Western blot analysis with anti-ORF2 peptide antibodies.

### **5.7. Immunoprecipitation with anti-FLAG antibody**

As no anti-ORF2 antibodies were available at the initial stages of the work, a construct pTMPUUORF2FLAG containing a coding sequence for a C-terminal FLAG peptide fused to the ORF2 cDNA sequence was used. Expression of the FLAG-tagged ORF2 protein was previously demonstrated by direct labelling approach (section 5.6, Fig.5.13, lane 4). Additionally, detection of the ORF2 protein by immunoprecipitation with an anti-FLAG antibody would become the basis for further experiments once anti-ORF2 peptide antibodies were available.

The FLAG-tagged ORF2 protein was transiently expressed by using a T7 vaccinia virus system (described in section 5.6), radiolabelled with 50 $\mu$ Ci of <sup>35</sup>S methionine, immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were analysed by 16% SDS-PAGE (see Methods).

Initial experiments (data not shown) demonstrated that a FLAG-tagged ORF2 protein was seen as a smeared band of a bigger than expected size. It was assumed that the apparent 'smearing' of the band could be the result of the protein degradation by cellular protease as is the case with the nonstructural protein of the Rift Valley fever (RVF) virus, which was shown to be susceptible to the action of cellular protease.

To overcome the degradation of the protein, the cells were treated with protease inhibitor before immunoprecipitation in all of the following experiments. Briefly, cells were labelled as described previously and washed with PBS. 300  $\mu$ l RIPA buffer containing 12

$\mu$ l protease inhibitor (1:25 dilution of protease inhibitor was used per dish) was added onto the cells. The cells were incubated on ice for 30 minutes and immunoprecipitation continued as described in Methods. In contrast to the previous experiment, treatment with protease inhibitor resulted in FLAG-tagged ORF2 protein appearing as a band of about 15 kDa in size (Fig.5.14). Mock (lane 1) and vaccinia virus infected cells (lane 2) were used as negative control. In addition, Puumala N protein was expressed from pTMPUUS plasmid and immunoprecipitated with anti-PUU N antibody. As expected, only one band corresponding to PUU N protein could be detected on the gel (lane 3).

### **5.8. Immunoprecipitation with five anti-ORF2 peptide antibodies**

During the late stages of my work, antibodies were obtained from Dr O Vapalahti, University of Helsinki. Rabbit antibodies were raised against five different PUU ORF2 sequence peptides which are shown in Fig. 5.15.

Expression of the ORF2 protein was analyzed after infection of confluent Vero E6 monolayers with the recombinant vTF7-3 virus at a MOI of 5 PFU per cell. Mock-infected and vaccinia virus infected cells were run as controls (Fig. 5.16, lanes 1 and 2, respectively). After incubation for 24 hours, the cells were labelled for 2 hrs in the presence of  $^{35}$ S methionine and lysed. The proteins were analyzed by 20% SDS-polyacrylamide gel electrophoresis and autoradiography after immunoprecipitation with anti-ORF2 polypeptide antibodies.

The recombinant ORF2 protein expressed from pTMPUUORF2 plasmid was detected by all five antibodies (lanes 4, 6, 8, 10, 12 ). The antibodies also precipitated PUU N protein expressed from pTMPUUS, however, no protein of a similar to the ORF2 size was detected by any of the antibodies (lanes 3, 5, 7, 9, 11). Although similar amounts of the protein were expressed and used for immunoprecipitation with each antibody, different intensities were observed on the gel. Moreover, the intensity of the ORF2 expression was



Fig. 5.14. Immunoprecipitation of transiently expressed ORF2FLAG with anti-FLAG peptide antibody. Confluent monolayers of Vero E6 cells were infected with recombinant vaccinia virus vvTF7-3 at MOI 5 pfu/cell (lane 2) or mock infected (lane 1) and transfected with plasmids pTMPUUS (containing the gene encoding for N and ORF2 proteins) and pTMPUUORF2FLAG (containing C-terminal FLAG sequence fused to the gene encoding ORF2 protein). At 24 hours postinfection, the cells were labelled with 50  $\mu$ Ci of  $^{35}$ S methionine per dish for 2 hrs. N protein was immunoprecipitated with anti-N antibody (lane 3), ORF2 FLAG-tagged protein – with anti-FLAG peptide antibody after treatment with protease inhibitor (lane 4). The immune complexes were analyzed by electrophoresis on 16% SDS-PAGE and autoradiography. The positions of the N and ORF2 proteins and the molecular mass markers (in kilodaltons) are shown on the right and left, respectively. Lane 1, mock infected, lane 2, vvTF7-3, lane 3, pTMPUUS, lane 4, pTMPUUORF2FLAG

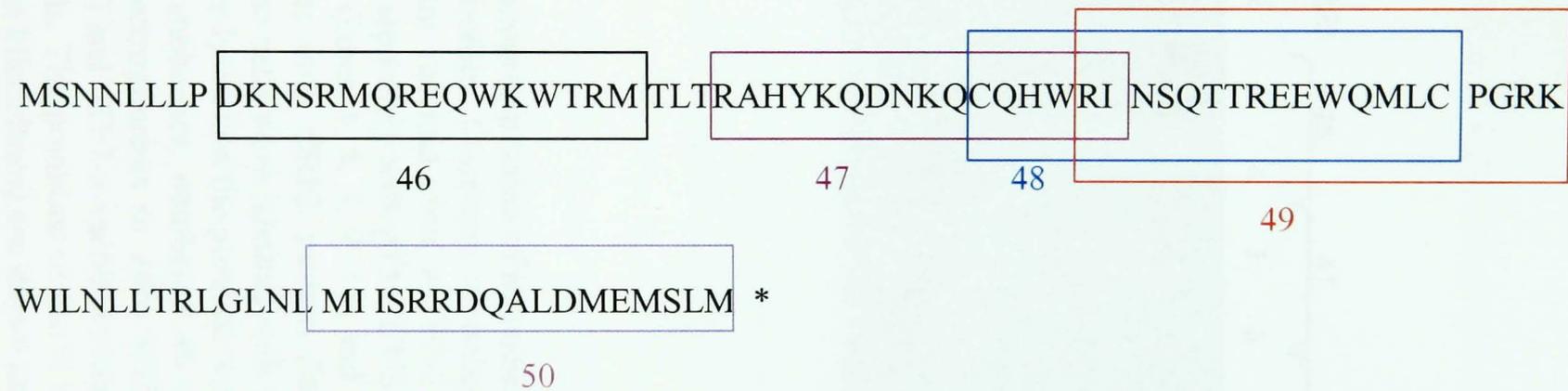


Fig. 5.15. Five anti-ORF2 peptide antibodies used in immunoprecipitation and Western blot analysis. The antibodies were numbered 46 to 50. The regions of the ORF2 peptide used to raise each of the five antibodies are shown in different colors

in inverse proportion to N expression as detected by antibodies N46 (lanes 3 and 4) and N47 (lanes 5 and 6).

### 5.9. Western blot analysis with five anti-ORF2 peptide antibodies

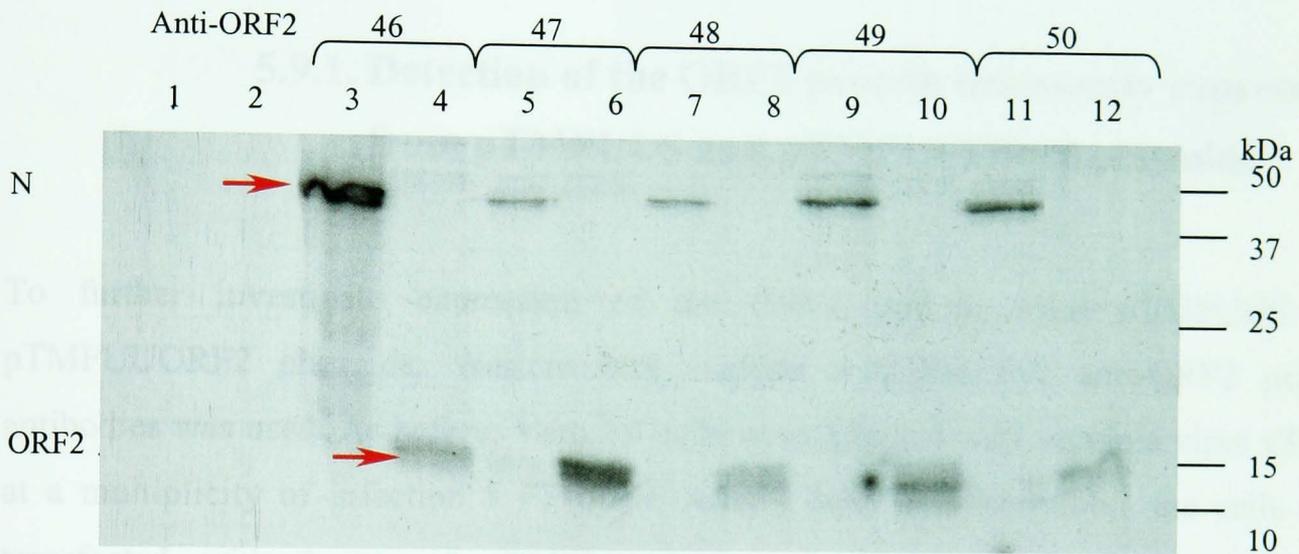


Fig. 5.16. Immunoprecipitation of transiently expressed ORF2 protein with five anti-ORF2 antibodies. Confluent monolayers of Vero E6 cells were infected with recombinant vaccinia virus vvTF7-3 at MOI 5 pfu/cell or mock infected and transfected separately with pTMPUUS containing the gene coding for N and ORF2 proteins (lanes 3, 5, 7, 9, 11) and pTMPUUORF2 plasmid containing gene coding for only ORF2 protein (lanes 4, 6, 8, 10, 12). At 24 hours postinfection, the cells were labelled with 50  $\mu$ Ci of  $^{35}$ S methionine per dish for 2 hrs. Cells were lysed and the proteins were immunoprecipitated with five anti-ORF2 peptide antibodies, numbered 46 to 50. The immune complexes were analyzed by electrophoresis in 20% SDS-PAGE and autoradiography. Mock infected (lane 1) and vvTF7-3 vaccinia virus infected cells (lanes 2) were used as negative controls. The positions of the N and ORF2 proteins and the molecular mass markers (in kilodaltons) are shown on the left and right, respectively.

detected by antibodies N46 (lanes 3, 4), and N47 (lanes 5, 6).

in inverse proportion to N expression as detected by antibodies N46 (lanes 3 and 4) and N47 (lanes 5 and 6).

## **5.9. Western blot analysis with five anti-ORF2 peptide antibodies**

### **5.9.1. Detection of the ORF2 protein transiently expressed from pTMPUUS and pTMPUORF2 plasmids**

To further investigate expression of the ORF2 protein from pTMPUUS and pTMPUORF2 plasmids, Western blot analysis with the five anti-ORF2 peptide antibodies was used. As before, Vero E6 cells were infected with vaccinia virus vTF7-3 at a multiplicity of infection 5 PFU/cell. After 1 hour of adsorption, the cells were transfected with 1  $\mu$ g of pTMPUORF2 and pTMPUUS plasmids. 24 hours posttransfection the cells were lysed and cell extracts subjected to a 20% SDS-polyacrylamide gel electrophoresis and analysed by Western blot (see Methods) with five antibodies. Fig.5.17, A shows whole blot as an example. ORF2 protein expressed from pTMPUORF2 (Fig.5.17, B) was detected by two antibodies, N46 (lane 3) and 49 (lane 6). Mock infected (lanes 1 and 4) and vaccinia infected (lanes 2 and 5) cells were used as negative controls. As no protein was detected by antibodies N 47, 48, and 50, the corresponding blots are not shown.

Results of expression of the ORF2 protein from pTMPUUS are presented in Fig. 5.17, C. Similar to ORF2 protein expressed from pTMPUORF2, the ORF2 expressed from pTMPUUS was detected by antibodies N46 (C, lane 1) and 49 (lane 4). No protein was detected by antibodies N 47 (lane 2), 48 (lane 3), and 50 (lane 5).

To summarize, ORF2 protein expressed from both pTMPUORF2 and pTMPUUS was detected by antibodies N 46 (B, lane 3, C, lane 1) and 49 (B, lane 6, C, lane 4).

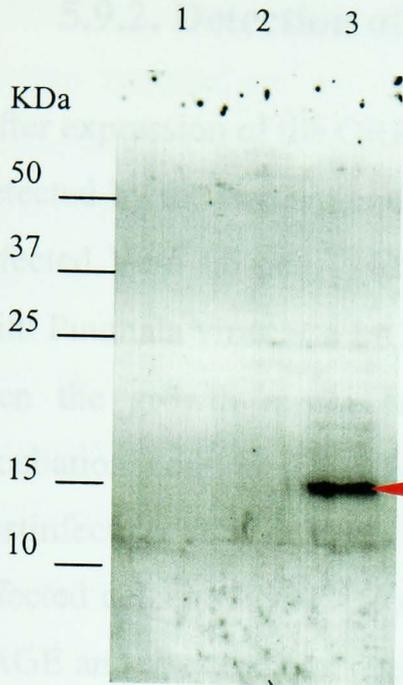
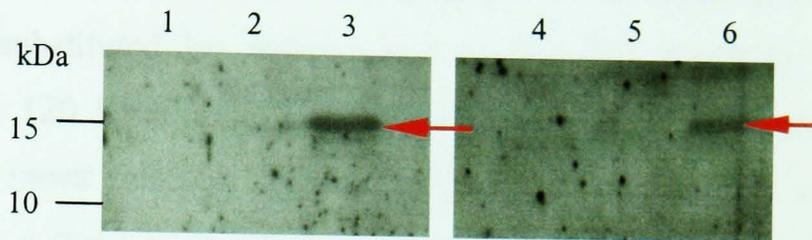
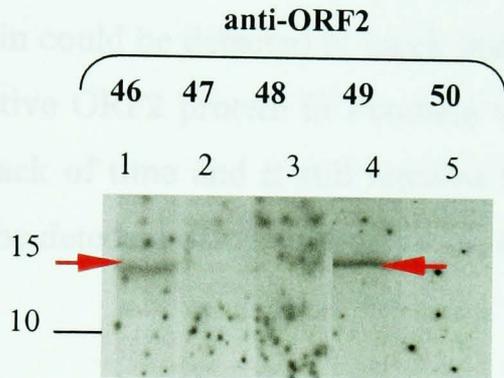
**A****B****C****D**

Fig. 5.17. Western blot analysis using five anti-ORF2 antibodies. Vero E6 cells, infected with vTF7-3 vaccinia virus, were transfected with pTMPUUORF2 plasmid (**B**) containing gene coding for putative ORF2 protein and pTMPUUS (**C**) containing S gene coding for both N and ORF2 proteins. Cell extracts were separated by 20% SDS-PAGE and subjected to Western blot analysis using anti-ORF2 peptide antibodies (numbered 46 to 50). Mock infected (**A**, lane 1; **B**, lanes 1, 4) and vTF7-3 infected (**A**, lane 2; **B**, lanes 2, 5) cells were used as negative controls. ORF2 protein expressed from pTMPUUORF2 plasmid was detected by antibodies N 46 (**B**, lane 3) and 49 (**B**, lane 6). As no protein was detected by antibodies 47, 48, and 50, the corresponding blots are not shown. ORF2 protein expressed from pTMPUUS plasmid was detected by antibodies 46 (**C**, lane 1) and 49 (**C**, lane 4). Antibodies used are shown in bold.

(**D**). Western blot analysis of Puumala virus infected cells. Vero E6 cells were infected with Puumala virus and cell extracts from day 2 (lane 2), 4 (lane 3), 6 (lane 4) postinfection or mock infected (lane 1) were separated by 20% SDS-PAGE and analyzed by Western blot with N 49 anti-ORF2 peptide antibody.

Whole blot (**A**) (fragment of which is presented in section B) is shown as an example

### **5.9.2. Detection of the ORF2 protein in Puumala virus infected cells**

After expression of the ORF2 protein from pTMPUUS and pTMPUUORF2 plasmids was detected by the two antibodies, a further attempt to identify the protein in Puumala virus infected Vero E6 cells was made. Subconfluent VeroE6 cell monolayers were infected with Puumala virus at a MOI 10 pfu/cell. The cells were incubated for 24 hours at 37°C, then the growth media was substituted by maintenance media (2% DMEM) and incubation continued for 24 to 120 hours. The cells were lysed at days 2, 4 and 6 postinfection. The extracts of mock infected (Fig.5.17, D, lane 1) or Puumala virus infected cells at days 2 (lane 2), 4 (lane 3), and 6 (lane 4) were subjected to 20% SDS-PAGE and Western blot analysis (see Methods) with antibody N 49. As the results show, the ORF2 protein was detected by antibody N49 at days 2 to 6. No protein similar to the ORF2 protein could be detected in mock infected cells. No other antibodies were used to detect putative ORF2 protein in Puumala virus infected cells by Western blot analysis due to the lack of time and it still remains to be investigated whether or not the ORF2 protein can be detected using antibodies 46, 47, 48 and 50.

## **5.10. DISCUSSION**

Examination of the S segment sequences of different hantavirus serotypes from the database, including those of several Puumala virus strains and also Khabarovsk, Isla Vista, Sin Nombre, Prospect Hill, Rio Mammore, Bayou, Lechiguan, Pergamio, Marciel, Muleshoe, Seoul, Hantaan, and Dobrava viruses revealed that all viruses, except for Hantaan, Seoul and Dobrava viruses, possessed a second open reading frame (ORF2) overlapping that of the N protein. This ORF2 varies in size and codes for proteins of 6 to 12 kDa in different viruses. The arrangement of the N and putative ORF2 proteins in the Puumala virus S RNA, encoded in overlapping reading frames, is similar to other orthobunyavirus S RNAs. Sequence alignment demonstrated that the sequences could be readily aligned and contained several conserved residues (Fig.5.4, 5.6, 5.7).

Using *in vitro* transcription/translation system in rabbit reticulocyte lysates (TnT kit), the protein encoded on the second ORF was expressed from two plasmids, pTMPUUS, containing S gene (and therefore coding for both N and ORF2 proteins), and pTMPUUORF2, containing only sequence coding for a putative ORF2 protein, to yield the protein of about 12.5 kDa in size.

The fact that the two proteins were efficiently produced in reticulocyte lysates demonstrates that with regard to the N and ORF2 proteins the same mRNA species can be translated to give these products and thus the synthesis of N and ORF2 is most likely the result of alternative translation initiation. The nucleotide sequence context of an AUG codon in eukaryotic mRNAs is important for efficient initiation. The optimal consensus is 5' A/G CCAUGG with a purine (preferably A) at the -3 position and a G residue at the +4 position (the A of the AUG is designated +1) being the most important determinants (Kozak, 1981). The leaky scanning model (Kozak, 1986) has been proposed to deal with the situation of bicistronic mRNAs, whereby some ribosome preinitiation complexes bypass the 5' proximal AUG which is in a suboptimal sequence context and initiate at a downstream AUG. This could also explain why, in addition to N and ORF2 proteins, the third product of about 45 kDa was expressed when the *in vitro* reticulocyte system was used (Fig.5.12). As no such protein was expressed *in vivo* from pTMPUUS using vaccinia virus as a source of T7 polymerase, it therefore remains to be an artifact of the *in vitro* system as the sequence context around the AUG start or secondary AUGs in the sequence were shown to have profound effects on initiation fidelity and translation efficiency in reticulocyte systems (Kozak, 1990).

Despite the successful translation of the ORF2 protein *in vitro*, I failed to detect the protein of a similar size in either Puumala virus-infected cells or expressed from pTMPUUS plasmid in vaccinia virus system using a direct labelling approach. The inability to identify the nonstructural protein *in vivo* in Puumala virus infected cells may be accounted for by the fact that hantaviruses, unlike orthobunyaviruses, do not cause shut-off of the host cell synthesis and a host protein of a similar size could be comigrating with the putative ORF2 protein. Although vaccinia virus shuts off the host cell synthesis,

the inability to detect ORF2 protein expressed from pTMPUUS using vTF7-3 system can be explained by comigrating vaccinia virus protein of a similar to ORF2 protein size.

Five specific anti-ORF2 peptide antibodies were used in attempt to identify the putative ORF2 protein transiently expressed in recombinant vaccinia virus system from both pTMPUUS and pTMPUUORF2 plasmids using immunoprecipitation and Western blot analysis. The results of immunoprecipitation with five anti-ORF2 antibodies revealed that the protein expressed from pTMPUUORF2 plasmid could form immune complexes with all five antibodies. The ORF2 protein could also be detected by antibodies N 46 and 49 using Western blot analysis. Although no antibodies could detect ORF2 protein by immunoprecipitation when pTMPUUS plasmid (that contains S gene encoding both N and ORF2 proteins) was used, two antibodies, N 46 and 49, detected the ORF2 protein expressed from this same plasmid when Western blot analysis was used. When antibody N 49 was used to detect the ORF2 protein in Puumala virus infected cells, the results of the Western blot analysis show its presence on days 2, 4, and 6 postinfection.

Although the results of the experiments described above show that the ORF2 protein can be detected from both plasmids expressed in vaccinia virus system and also in Puumala infected cells using immunoprecipitation and Western blot analysis, additional experiments are needed to be carried out to make the final conclusion of the existence of the ORF2 protein. Further identification of the ORF2 protein in Puumala virus infected cells at different days postinfection using both immunoprecipitation and Western blot analysis with all five antibodies needs to be explored. Immunofluorescence analysis of the ORF2 protein with different antibodies would reveal in which cellular compartments it can be found. It would also be interesting to explore the possibility of phosphorylation of the ORF2 protein as is the case with the Rift Valley fever virus NSs protein. Interestingly, the Puumala ORF2 protein has 12 potential phosphorylation sites, five of which are serine residues, and it was shown for the Rift Valley Fever virus that two serine residues 252 and 256 were the major phosphorylation sites (Kohl *et al.*, 1999). It would also be interesting to perform a differential labelling with different amino acid precursors based on amino acid composition of the Puumala virus ORF2 protein.

## CHAPTER 6. CONCLUSIONS

### 6.1. Attempts to develop reverse genetics system for Puumala virus

A first objective of the project was to attempt to develop a reverse genetics system for Puumala virus (PUU) based on a reporter construct and two viral proteins, N and L, necessary for reconstitution of a transcriptionally active RNP structure.

In this system, it was planned that a recombinant PUU virus-like RNA transcript containing a reporter gene would be recognised and transcribed by transiently expressed recombinant PUU virus proteins.

Two viral proteins, nucleocapsid (N) and viral RNA-dependent RNA polymerase (L), were supplied by transient expression from two plasmids, pTMPUUS and pTMPUUL, using bacteriophage T7 RNA polymerase stably expressed by BHK T7-SIN and Vero T7 cell lines. The reporter plasmid contained an antisense sequence encoding the open reading frame of the chloramphenicol acetyl transferase (CAT) gene. The antisense CAT gene replaced the coding region in a negative sense PUU virus S or L segment cDNA, so that it was flanked by the complete 3' and 5' PUU S or L segment untranslated regions (UTRs). It was expected that transcripts from the reporter construct would behave as authentic viral RNAs since the complete 3' and 5' UTRs of the Puumala virus genome segments are thought to contain the necessary signals for encapsidation, transcription and replication of the RNA transcript.

Differential mRNA synthesis was shown in hantavirus-infected cells, similar to Bunyamwera virus infection, with the quantity of each mRNA (N mRNA  $\geq$  GPC mRNA  $\geq$  L mRNA) correlating inversely with RNA length (Rossier *et al.*, 1988). Therefore, some of the reporter constructs were based on the most expressed viral S segment. However, the results of the reverse genetics system for Bunyamwera virus (Dunn, 2000) have shown that the efficiency of expression of the reporter gene in the context of each of

the BUN segments, was in the order of L (taken as 100%)>M (60-70%)>>>S (3-10%), a complete inversion of the ratios of mRNA transcripts found in BUN virus infected cells. This suggests that the conditions of the reporter system differ from infected cells and additional factors must influence the transcript levels in the context of the infected cells. It is possible that the full length of each genomic segment and coding sequence itself may play a role in control of replication and transcription of the specific segment. For example, the small size of the S segment in comparison with two other segments may suggest that it would be replicated, transcribed and encapsidated by viral proteins at a more efficient rate than L segment. In addition, the N ORF itself could also influence the increased levels of expression.

Therefore, in addition to constructs based on the S segment, which is most expressed in virus infected cells, constructs based on the L segment, most expressed in reporter system, were also used.

It was thought to be important that the reporter RNA transcripts should have the exact 3' and 5' termini of the PUU genome segments since these sequences are highly conserved among different hantaviruses. In order to achieve the exact 3' terminus, the constructs contained either *Bbs*I restriction enzyme site placed immediately downstream of the 3' terminal viral sequence (pPUUSCAT) or hepatitis delta virus ribozyme (pT7riboPUUSCAT, pT7riboPUULCAT). The exact termini was expected to be generated either by run-off transcription from *Bbs*I-digested plasmid or by self-cleavage by the ribozyme.

To achieve the exact 5' termini, the reporter construct was cloned immediately downstream of a truncated T7 RNA promoter so that transcription would initiate at the 5' nucleotide of the PUU sequence (reporter constructs pPUUSCAT, pT7riboPUUSCAT, pT7riboPUULCAT). However, T7 polymerase would have to initiate transcription with a uridine in position +1 and this could be problematic (discussed in section 3.2.5). In order to overcome the possible problem, another set of reporter constructs contained a viral 5' UTR cloned downstream of a truncated T7 promoter that contains two G residues (constructs pT7GGriboPUUSCAT, pT7GGriboPUULCAT). In this case, the T7

polymerase would start transcription with Gs followed by the viral nucleotides, and additional G residues were expected to be lost after transcription giving authentic viral 5' end. As yet another alternative, construct pT7HH&HDVriboPUULCAT was also used in which a cDNA copy of a *cis*-active hammerhead ribozyme sequence (Birikh *et al.*, 1997) was cloned between the T7 RNA polymerase promoter and the L segment 5' UTR. Self-cleavage by the hammerhead ribozyme would give the exact 5' viral termini.

However, none of these approaches was successful, and it was decided to employ pol I system successfully used for influenza virus (Neumann *et al.*, 1994) and Uukuniemi virus (Flick and Pettersson, 2001). This method utilizes cellular RNA polymerase I, which is among the most abundantly expressed enzymes in growing cells, to produce the chimeric RNA transcript. It avoids the need for expressing run-off transcripts from restriction enzyme-cleaved plasmids or the use of a hepatitis delta ribozyme to produce the correct 3' end, and also ensures generation of the exact 5' ends of the RNA transcripts independently of nucleotides present at the viral 5' termini (purines or pyrimidines), as was the case with bacteriophage T7 polymerase. The proteins required for reconstitution of the transcriptionally active RNP complex are expressed from pol II promoter plasmids (in the case of Puumala virus, human cytomegalovirus promoter, CMV), thus using cellular RNA polymerase II. However, this system too failed to produce positive results.

The results described in Chapter 3 may help in designing new strategies in the future. One of the possible explanations for the problems with development of the reverse genetics system could be that viral polymerase was not functional. Different L clones could be tested using one of the reporter systems described. Once the functional clone is identified, it would be possible to develop reverse genetics system based on one of the many approaches used.

Development of the reporter system would allow investigation of Puumala virus RNA synthesis and the proteins involved in transcription and replication. For example, the effect of additional or deleted nucleotides from the extreme ends of the RNA termini could be studied. The importance and function of the conserved sequences found at the PUU 3' and 5' termini could also be determined.

## 6.2. Analysis of Puumala virus protein interactions

A second objective was to identify the ability of the Puumala virus N protein to self-associate, and if so, to determine the domains responsible for interaction.

The study of Puumala virus N protein interactions was undertaken by using the mammalian two-hybrid system (M2HS) in which self-association of the N was observed. The evidence for homotypic interaction between N proteins was additionally supported by the results of co-immunoprecipitation of the proteins expressed both *in vitro* and *in vivo*.

Analysis of different fragments of the N allowed dissection of the homomultimerization at the molecular level. The results of the M2HS suggest that sequences involved in self-interaction of the N protein are confined to its N-terminal 105 amino acids and C-terminal 46 amino acids and that this type of interaction is the most important for assembly of N protein multimers. This became apparent after it was demonstrated that the full-length N protein could interact with both N- and C-terminal fragments, but not with the internal fragments. Moreover, only interaction between heterologous N- and C-terminal fragments and not homologous terminal fragments (C-C or N-N) or terminal fragments with internal fragments, resulted in the strongest CAT signal.

Based on these results, a 'head-to-tail' model is proposed in which the association of the N proteins appears through their C-terminal region comprising amino acids 385-432 and N terminal region comprising amino acids 1-105. A similar mechanism was described for tospovirus (Richmond *et al.*, 1998) and Tula virus (Kaukinen *et al.*, 2001). It is possible to hypothesize that the two molecules of the N protein come together forming a dimer. Association with the third molecule available in a monomeric form results in formation of a trimer.

The results are in agreement with the data on dimerization and oligomerization capacity of nucleocapsid proteins of Sin Nombre and Tula hantaviruses. Using yeast two-hybrid system and a chemical cross-linking studies, it was demonstrated that Sin Nombre virus (Alfadhli *et al.*, 2001) and Tula virus (Kaukinen *et al.*, 2001) N proteins were capable of oligomerization and existed as dimers, trimers and higher molecular mass products, with dimers being a preferential state. Further, the interaction domains of Sin Nombre virus were mapped to the N-terminal 40 amino acids and to the C-terminal half of the proteins.

The ability of Bunyamwera virus N protein to self-associate was also shown using the M2HS and co-immunoprecipitation assays (Osborne, 2001). However, neither half of BUN N was found to be capable of association independently and there was no evidence that the interaction in BUN was mediated by head-to-tail binding as observed with Puumala virus and tospovirus N (Richmond *et al.*, 1998). Therefore, the mechanism of BUN N multimerization differs from that of Puumala, Sin Nombre, Tula viruses and possibly tospoviruses.

Furthermore, the ability for interaction of nucleocapsid proteins of different hantaviral serotypes was also investigated. For this purpose, N proteins of serotypes Puumala, Seoul and Hantaan were tested against each other in the M2HS. Not only was the homologous interaction between N proteins of viruses Hantaan and Seoul shown, but also that proteins of different serotypes could interact as well. The fact that Puumala and Hantaan virus N proteins were able to associate suggests that interactions between molecules of N protein occur via highly conserved stretches of amino acid residues. Despite low similarity of the entire sequence (60%), the proteins share regions of high homology, with their C terminal 100 amino acids being 85% identical, and N-terminal 200 amino acids being 82% identical.

Interaction between N and different fragments of L protein was also studied using the M2HS. However, no interaction was demonstrated using the approach taken. The negative results are perhaps attributable to the fact that L protein was expressed as separate fragments, therefore, the binding sites could be destroyed.

The experimental proof of N protein self-association may provide a basis for a better understanding of nucleocapsid formation. The analysis of the molecular basis of the homotypic interaction and of other interactions with N protein will be the starting point for elucidation the mechanisms of transcription, replication, and assembly of Puumala virus. Proteins of such viruses as vesicular stomatitis virus, HIV, Sendai virus, and some other viruses were shown to possess overlapping binding domains involved in multimerization, RNA binding, or contact to other viral or host proteins (Takacs *et al.*, 1993; Lutzke and Plasterik, 1998; Myers *et al.*, 1997). Hence, there is reason to assume that the mutual influences or interdependence of different or overlapping binding regions may be a fundamental means to regulate various steps of the viral life cycle. Recent findings that there are multiple RNA-binding domains (Gott *et al.*, 1993; Severson *et al.*, 1999; Severson *et al.*, 2001), which overlap the two domains responsible for homotypic interaction, support the idea of an interdependence of these two properties of the N protein. Further investigation of the structural basis for both, the RNA binding and the various protein-protein interactions of the Puumala virus N protein, is needed to better understand the mechanisms of nucleocapsid formation, assembly, and regulation of transcription and replication of Puumala virus.

### **6.3. Potential second ORF (ORF2) in the S segment that may encode an NSs nonstructural protein**

Examination of the S segment sequences of different hantavirus serotypes revealed that viruses Puumala, Khabarovsk, Isla Vista, Sin Nombre, Prospect Hill, Rio Mammore, Bayou, Lechiguan, Pergamio, Marciel, Muleshoe, Seoul, Hantaan, and Dobrava possess a second open reading frame (ORF2) overlapping that of the N protein. This ORF varies in size and potentially codes for proteins of 6 to 12 kDa.

*In vitro* transcription/translation using reticulocyte lysate (TnT kit, Promega) programmed with either pTMPUUORF2 plasmid (containing the gene coding for ORF2 protein only) or pTMPUUS (containing S gene coding for both N and ORF2 proteins) produced protein of about 12.5 kDa in size. It demonstrates that the synthesis of N and ORF2 is most likely the result of alternative translation initiation and the same mRNA species can be translated to give these products.

It was not possible to identify directly the ORF2 protein either expressed in T7 vaccinia virus system or in Puumala virus infected cells. As hantaviruses do not cause host cell synthesis shut off, the host protein could be comigrating with the putative ORF2 protein. This was also the case with vaccinia virus infection where no protein could be identified following transfection of the plasmid, however, the band corresponding to the vaccinia virus protein of a size similar to ORF2 protein was clearly observed.

The ORF2 protein could be detected, however, from both plasmids expressed in vaccinia virus system and also in Puumala infected cells using immunoprecipitation and Western blot analysis with anti-ORF2 peptide antibodies. The protein expressed from pTMPUUORF2 plasmid was detected by all five antibodies in immunoprecipitation assay. Two out of the five antibodies were able to detect the ORF2 protein expressed from pTMPUUORF2 and pTMPUUS as demonstrated by the results of Western blot analysis. The ORF2 protein was also detected in Puumala virus infected cells using Western blot analysis.

Although the results are preliminary, they serve as a starting point for the studies on the ORF2 protein encoded on the S segment of Puumala virus. Additional experiments will help to elucidate the functions of the protein. It would be interesting to investigate the ability of the ORF2 protein to interact with itself in order to form multimers and with other viral proteins and cellular structures as these interactions might be the key in understanding the role of the ORF2 protein. For this purpose, two vectors, pMPUUORF2 and pVPPUUORF2, expressing the ORF2 protein have already been constructed for the use in the mammalian two-hybrid system. As the M2HS constructs expressing N and various fragments of L protein are already available, it would also be possible to

investigate N-ORF2 and L-ORF2 interactions if they exist. In addition, localisation of the protein in cellular compartments could be identified by means of immunofluorescence analysis with different anti-ORF2 peptide antibodies. Two-dimensional gel electrophoresis and mass spectroscopy could help identify the exact molecular mass of the protein. It would also be interesting to perform a differential labelling with different amino acid precursors based on amino acid composition of the proteins, e.g. the Puumala ORF2 protein is rich in leucine, serine, agrinine and glutamine, and poor in cysteine, glycine and proline. The possibility of phosphorylation of the ORF2 protein, similar to the one described for RVF virus (Kohl *et al.*, 1999), could also be explored as the protein has 12 potential phosphorylation sites, five of which are serine residues. Phosphorylation is known to regulate several protein-protein interactions and the degree of phosphorylation may regulate transcription and replication of the genome and also the intracellular localization of the protein.

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