National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

MOLECULAR GENETICS OF HANTAVIRUSES:

CLONING, SEQUENCING, EXPRESSION, AND MORPHOGENESIS

A Thesis Submitted to the

School of Graduate Studies

University of Ottawa

In Partial fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology

Faculty of Medicine

By

Dragana Antic

ISBN 0-315-85859-1

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Molecular characterization of the Seoul 80-39 virus, a prototype of the Seoul serotype, was carried out by cloning and sequencing. The large (L) genomic RNA segment is 6530 nucleotides long. The viral complementary-sense RNA contains a single open reading frame (ORF) from the AUG codon at nucleotide positions 37-39 to the UAA stop codon at nucleotide positions 6490-6492. This ORF could encode a polypeptide of 2151 amino acids (246,662 D) which likely corresponds to the L protein detected in purified viral particles (Elliot et al., 1984) and is assumed to be an RNA-dependent RNA polymerase molecule (Schmaljohn and Dalrymple, 1983). The M RNA segment is 3651 nucleotides long. A single ORF was detected in the viral complementary-sense RNA. It starts with the AUG codon at nucleotide positions 47-49 and ends with the UAA codon at nucleotide positions 3446-3448. This ORF could encode a polypeptide of 1133 amino acids which is cotranslationally processed into viral glycoproteins G1 and G2. The S genomic RNA segment was characterized from the 3' end to nucleotide 1332. One long ORF was detected from the AUG codon at nucleotide positions 43-45 to the UAA stop codon at nucleotide positions 1330-1332. This ORF has a capacity to encode a 48 kD protein which corresponds to the viral nucleocapsid (N) protein.

A comparison of coding properties of the tripartite genomes of various hantaviruses allowed construction of the evolutionary tree for the *Hantavirus* genus.

The second part of the study was focused on the maturation of Hantaan 76-118 virus glycoproteins G1 and G2 and virus morphogenesis. Processing of glycoproteins in Hantaan virus infected cells was studied by immunoprecipitation, chemical cross-linking, glycosylation inhibition and sensitivity to endoglycosidase H, F and D; whereas virus morphogenesis was studied by thin-section electron microscopy.

Glycoproteins G1 and G2 form a complex. The basic complex unit consists of one molecule of G1 and one molecule of G2 (heterodimer) which possibly can further assemble into an oligomeric structure. Experiments with the glycosylation inhibitor brefeldin A indicate that complex formation between the two glycoproteins occurs in the ER. Other glycosylation inhibition studies and treatments of glycoproteins with endoglycosidases show that the N-linked oligosaccharide molecules of Hantaan virus glycoproteins are high mannose. Resistance of both G1 and G2 to endoglycosidase D suggests a structure with more than five mannose residues. Therefore, the enzymes of the *mid*- and *trans*-Golgi are not involved in processing of Hantaan virus glycoproteins. However, processing of a high mannose structures ($(Man)_8(GlcNAc)_2$ to $(Man)_7(GlcNAc)_2$ or $(Man)_6(GlcNAc)_2$) may take place in the *cis*-Golgi by the enzyme α-mannosidase I.

Virus morphogenesis was studied by thin-section electron microscopy of mock-infected and Hantaan virus-infected cells with or without brefeldin A treatment. Virus budding was observed only in Hantaan virus-infected cells, in the *trans*-Golgi network. When the Golgi apparatus was destroyed by brefeldin A

treatment, assembly of virus particles was not observed. Therefore, although Golgi apparatus may not be involved in the maturation of viral glycoproteins, the glycoprotein complex is targeted to the Golgi where virus assembly takes place.

The third project was to express nucleocapsid proteins of Seoul 80-39 virus and Hantaan 76-118 virus using baculovirus recombinants. Cloned S genomic segment of Seoul 80-39 virus was inserted into the baculovirus transfer vector, pAcYM1-Hu. *Spodoptera frugiperda* (Sf9) cells were co-transfected with the recombinant transfer vector and wild type AcNPV DNA. Recombinant virus, designated as AcNPV-Hu/Seoul N was isolated by plaque purification. Analysis of recombinant virus-infected Sf9 cell lysates by SDS-PAGE showed that N proteins were expressed in high levels and were identical to virion associated N proteins in size and antigenicity.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. C. Yong Kang. His guidance, encouragement and constructive criticism were invaluable throughout the project. Without his support, this work would not have been possible.

Many thanks to the members of the advisory commitee, Dr. Ken Dimock, Dr. Earl Brown and Dr. Kathryn Wright, who were a constant source of help and useful suggestions and to Dr. C.M. Johnson-Lussenburg who reviewed the thesis.

Dr. Mark Galinski supplied the sequences of the L proteins of the negative-stranded RNA viruses and Dr. Ian Robb performed the electron microscopy of the cell cultures. Dr. Martin Tenniswood's lab provided the Microgenie sequence analysis software and help in using it.

The help received from Dr. Kang's graduate students, both past and present, was invaluable. Of special note is the time and effort given by Drs. Mark Parrington and Erling Rud.

Excellent technical assistance was provided by Bernadette Mah, Nancy Delcellier, and Doris McLean. They, along with the secretaries, staff and other students of the Department of Microbiology, helped make the days more pleasant.

The Selic and Radojicic families were a large and constant source of support. Their contribution to the overall success of the work is immeasureable and heartfully appreciated.

The financial support of the University of Ottawa is greatfully acknowledged.

DEDICATION

To Marko and my parents

Without their endless love, understanding and support

this thesis would not exist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

μCi	microcuries
μg	microgram
μl	microlitre
μmol	micromoles
A	adenine base in a nucleotide sequence
Ab	antibody
AcNPV	*Autographa californica* nuclear polyhedrosis virus
ATCC	American type culture collection
ATP	adenosine triphosphate
b	base
bis	N,N'-Bis-methylene-acrylamide
bp	base pair
BPB	bromophenol blue
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
°C	degrees Celsius
C	Cytosine base in a nucleotide sequence
CAPS	3-[cyclohexylamino]-1-propane-sulfonic acid
cDNA	complementary deoxyribonucleic acid
Ci	Curies
CIAP	calf intestinal alkaline phosphatase

cm	centimetre
CPE	cytopathic effect
cRNA	complementary ribonucleic acid
CTL	cytotoxic T lymphocytes
D	daltons
dATP	deoxyadenosine triphosphate
cCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxyribonucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DEPC	diethyl pyrocarbonate
DSP	dithiobis(succinimidylpropionate)
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid disodium salt

ED	endoglycosidase D
EF	endoglycosidase F
EH	endoglycosidase H
EHF	epidemic hemorrhagic fever
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
rER	rough endoplasmic reticulum
EtBr	ethidium bromide
Fig.	Figure
FCS	fetal calf serum
g	gravity or grams
G	Guanine base in a nucleotide sequence
G1	glycoprotein 1
G2	glycoprotein 2
GAR-AP	goat anti-rabbit alkaline phosphatase
GER	Germiston virus
h	hour
HA	hemagglutination
HI	hemagglutination inhibition
HIFCS	heat inactivated fetal calf serum
HFRS	hemorrhagic fever with renal syndrome
IBI	International Biotechnologies, Inc.

IEC	International Equipment Company
IFA	indirect immunofluorescence assay
Ig	immunoglobulin
Inc.	Incorporated
IPTG	isopropylthiogalactoside
kb	kilo bases
kD	kilo daltons
L	large protein
LAC	LaCrosse virus
LB	Luria broth
LTD	Limited
MAb	monoclonal antibody
MEM	minimal essential medium
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid

NP	nucleocapsid protein
NE	nephropathia epidemica
NEAA	nonessential amino acids
NEN	New England Nuclear
NENS	sodium acetate, EDTA, NaCl and SDS
ng	nanogram
nm	nanometer
NP	nucleocapsid protein
NP40	NonidetP40
NS	nonstructural protein
NT	neutralizing
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PMSF	phenylmethylsulfonyl-fluoride
PPO	2,5-Diphenyloxazole
PRNT	plaque reduction neutralization test
rER	rough endoplasmic reticulum
RIPA	radioimmunoprecipitation assay

RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein core
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	seconds
SSH	Snowshoe Hare virus
SSPE	subacute sclerosing panencephalitis
STE	NaCl-Tris-EDTA
T	Thymine base in a nucleotide sequence
TB	Terrific broth
TBE	Tris-borate, EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
TTBS	TBS plus tween20
U	Uracil base in a nucleotide sequence
UV	ultraviolet
V	volts
Vero E6	African green monkey kidney cells
vRNA	viral ribonucleic acid

WHO	World Health Organization
XGal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YT	yeast extract, tryptone, and NaCl

Abbreviations for amino acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine

T	threonine
V	valine
W	tryptophan
Y	tyrosine

CHAPTER 1 GENERAL INTRODUCTION

1. HANTAVIRUSES: HISTORICAL BACKGROUND

History

Hantaviruses were classified as members of the new, *Hantavirus* genus of the *Bunyaviridae* family in 1987 (Schmaljohn and Dalrymple) after the viral genome was molecularly characterized. This group of viruses is associated with epidemic and endemic human diseases collectively known as hemorrhagic fever with renal syndrome (HFRS). The disease was reported for the first time in 1913, in Russia as hemorrhagic nephroso-nephritis. In the 1930s and 1940s, it was recognized as epidemic hemorrhagic fever (EHF) in China and Japan, as nephropathia epidemica (NE) in Scandinavia and as epidemic nephritis in eastern Europe. Scientists in the Soviet Union and Japan noticed that the disease was acquired through contact with rodents and/or their ectoparasites or excreta, that it can be transmitted from one human to another only by patients sera and that it was not of bacterial origin (Kasahara and Kitano, 1943; Kitano, 1944; Smorodintsev et al., 1959). The major disease outbreak in humans that evoked attention happened during the Korean conflict (1950-1953) when over 3000 United Nations troops, stationed in the demilitarized zone between North and South Korea, developed a disease with capillary hemorrhage and acute renal failure (Smadel, 1953). After many attempts to isolate the etiologic agent of HFRS, H. W. Lee and

P. W. Lee finally succeeded. In 1976, they demonstrated the presence of antigen in the lungs of the striped field mouse, *Apodemus agrarius coreae* using sera from patients convalescing from HFRS. The etiologic agent was passed from infected to uninfected adult *A. agrarius* (Lee H. W. et al., 1978), and was not easily transmitted to laboratory rodents. They were able to infect adult *A. agrarius*, as well as human volunteers, with sera isolated from two acute-phase HFRS patients. The attempt to propagate virus in a cell culture succeeded (French et al., 1981) allowing further characterization of the infectious agent.

Classification of hantaviruses

More than 100 different strains of the virus have been isolated from different natural rodent hosts such as mice (*Apodemus and Mus musculus*), rats (*Rattus*) and voles (*Clethrionomys* and *Microtus*). The serological relationships between different isolates of hantaviruses have been studied by indirect immunofluorescence assays (IFA), plaque reduction neutralization tests (PRNT) and enzyme-linked immunosorbent assay (ELISA). On the basis of the degree of serological cross-reactivity, hantaviruses have been classified into several serotypes (Lee et al., 1978; Niklasson et al., 1984; Schmaljohn et al., 1985; Lee et al., 1985; Sheshberadaran et al., 1988; Arikawa et al., 1989). Hantaan 76-118 virus, Seoul 80-39 virus, Puumala (Hällnäs B1) virus and Prospect Hill virus are prototypes of the four major serotypes (Table 1). These viruses were isolated from different rodent hosts and are the etiologic agents of different forms of the disease. Hantaan virus

TABLE 1

SEROTYPIC CLASSIFICATION OF HANTAVIRUSES

Serotype	Prototype Virus	Host	Severity of Disease
HANTAAN	Hantaan 76-118	mice (*Apodemus*)	severe
SEOUL	Seoul 80-39	rats (*Rattus*)	moderate
PUUMALA	Hällnäs	voles (*Clethryonomys*)	mild
PROSPECT HILL	Prospect Hill	voles (*Microtus*)	no disease

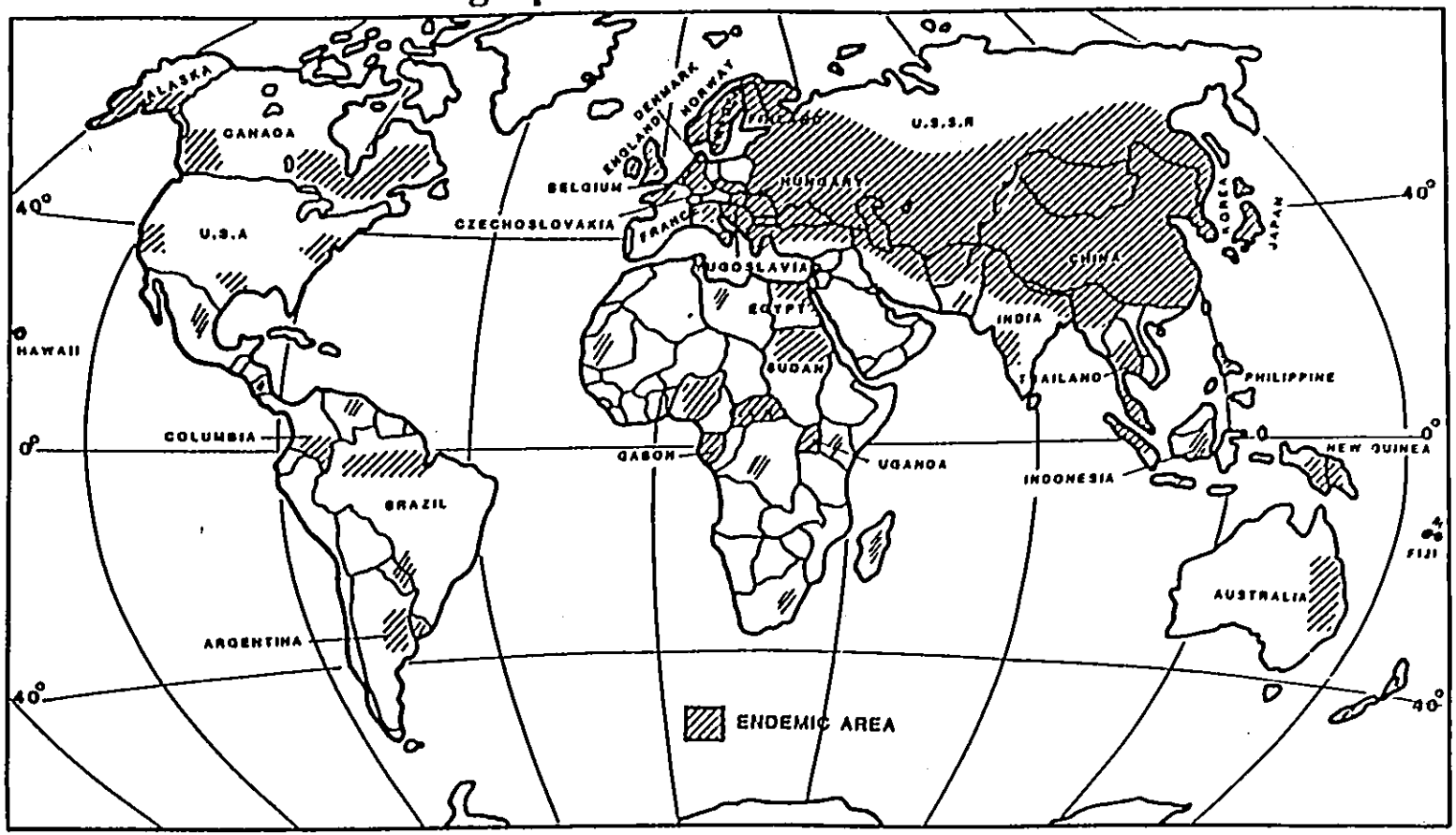
(isolated from the Korean striped field mouse, *Apodemus agrarius coreae*), usually causes a severe disease whereas Seoul virus (isolated from urban rats, *Rattus rattus* in Korea) causes a mild form of hemorrhagic fever. Puumala virus (isolated from bank voles, *Clethrionomys*, Scandinavia) causes nephropathia epidemica. Prospect Hill virus (isolated from meadow voles, *Microtus pennsylvanicus*, in the United States) has not been associated with any form of human disease.

Epidemiology

Hantaviruses are widely distributed. Specific antiviral antibodies have been found in rodents and humans throughout the world (Fig. 1). Human infections develop after a close contact with infected rodents or rodent excreta. An arthropod vector for the virus has not been demonstrated (Lee, 1989a). Farmers, forest workers, military personnel and hunters are the most affected populations and major outbreaks of the disease occur in the late spring and in the fall. This is related to the size of the rodent population (*Apodemus* and *Clethrionomys*) as well as to the activities of people (planting, harvesting and hunting) which peak in these two seasons. However, HFRS cases are reported all year round in the endemic rural areas because rodents usually invade houses during the snow season. Seoul-type viruses were detected in urban rat populations, especially in major seaports. Infection rates can exceed 50% and these rats can serve as a reservoir of human infections (LeDuc et al., 1986). Infections with Seoul viruses occur throughout the year, but tend to be more frequent in fall and winter seasons.

Figure 1. Worldwide distribution of hantaviruses - indicated by the demonstration of antibodies in humans and rodents. Copied from the *Manual of Hemorrhagic Fever with Renal Syndrome*, H.W. Lee (1989).

Geographical Distribution of HFRS



Human disease

Typical severe form of HFRS develops in 20 to 30% of infected individuals. The disease can be divided into five phases: febrile, hypotensive, oliguric, diuretic and convalescent. (Chun et al., 1989). It usually starts with sudden onset of fever with chills lasting 3 to 6 days, conjunctival injection, prostration, anorexia, vomiting, abdominal pain. Hemorrhagic manifestations begin about the third day, proteinuria about the fourth day and hypotension about the fifth. Hypotension or shock develops as a result of decreased effective circulating blood volume due to the capillary leakage of plasma and dilation of capillary and venular beds. Capillary hemorrhages are most visible at the hypotensive phase, but cerebral, conjunctival and gastrointestinal bleeding becomes most prominent during the oliguric phase. Central nervous symptoms and pulmonary edema occur in severe cases and about 50% of the fatal cases occur during the oliguric phase. Renal disorders, varying from mild to acute renal failure, are characteristic of the acute phase and may persist for several weeks. Recovery begins with the diuretic phase and may take 2 to 3 months (Chun et al., 1989). The fatality rate is 5 to 20% and the outcome of the infection depends on the infectious dose and the immune status of the patient. Treatment of patients with ribavirin can significantly improve the clinical picture and decrease mortality rate (Huggins et al., 1991). However, most patients (70% to 80%) infected with hantaviruses develop a mild, influenza-like illness. The disease, caused by Seoul viruses, can be divided in five phases as well, but they are shorter and sometimes it is hard to distinguish between phases. The clinical

manifestations are high fever, strong abdominal pain, myalgia, conjunctivitis, petechial skin rash, hepatomegaly, hepatic dysfunction and mild renal dysfunction. Because of the hepatic involvement, it has been suggested that Seoul virus shouid be considered as one of the etiologic agents of non-A, non-B hepatitis (Lee, 1989a). Nephropathia epidemica is a similar, but significantly milder disease in which the renal manifestation dominates the hemorrhagic features. It usually resolves a few weeks after the onset of symptoms and sequelae are uncommon. Only one fatal case of NE has been reported so far (Linderholm et al., 1991). Quite often, infected individuals may have a subclinical, completely asymptomatic form of the disease.

Animal infection

All rodents develop an asymptomatic persistent infection. Viral antigen is most commonly seen in lungs and kidneys of infected animals. Infectious virus is excreted in saliva, feces and urine and aerosolized excreta are the source of human infection.

Laboratory rodents can be infected with hantaviruses (Lee et al., 1981a, b; Tanishita et al., 1986; Yanagihara et al., 1984). Intraperitoneal or subcutaneous injection of newborn mice with Hantaan virus results in high virus titers in brain, lungs, kidneys and capillary endothelium. Mice develop inflammatory and destructive lesions and die in 2 to 3 weeks (Lee et al., 1981a). Passive immunization effectively protects newborn animals (Zhang et al., 1989). The

resistance to disease increases with age so that 3 week-old mice survive infection. Mice are the most infectious during the first 40 days of their infection. After that period, detectable virus decreases slowly, but remains sufficiently high for transmission of infection for another year. Clearance of the virus is dependent on both humoral and cellular immune responses which develop upon infection (Asada et al., 1987, 1988, 1989; Nakamura et al., 1985a, b; McKee et al., 1985).

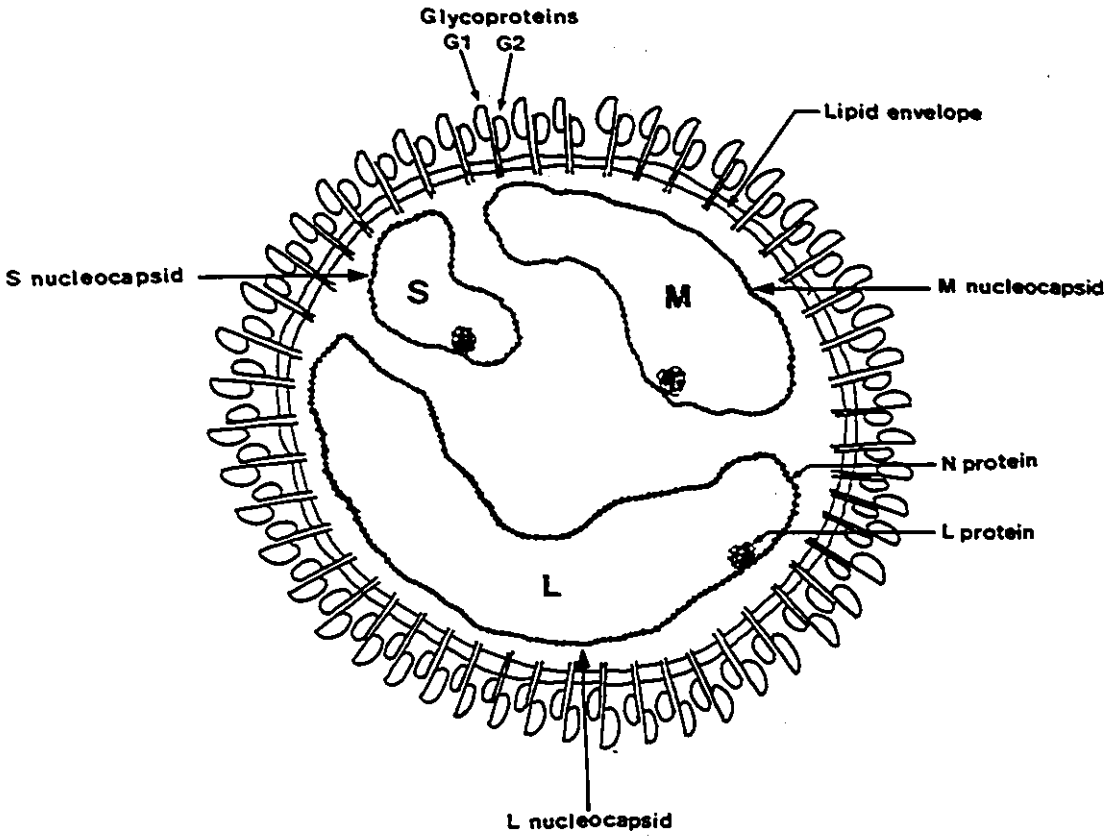
2. VIRION STRUCTURE

Hantaviruses have usually spherical, enveloped virions, 80-120 nm in diameter. The virus particle contains RNA and four structural proteins: two internal proteins, the L (RNA polymerase/transcriptase) and the N (nucleocapsid) proteins and two external glycoproteins, G1 and G2 (Fig 2). Viral glycoproteins are embedded in a lipid bilayer derived from the host cell membrane, and are displayed as surface projections (spikes) or a gridlike pattern on the virion surface. Elongated particles, 110-210 nm in length, are often observed.

Virions contain three single-stranded, negative-sense RNA segments, designated large (L), medium (M) and small (S) (Schmaljohn et al., 1983). The RNA segments are encapsidated with nucleocapsid proteins (Schmaljohn and Dalrymple, 1983) and associated with viral RNA polymerase (Elliot et al., 1984).

All three genome segments have a consensus 3' terminal nucleotide sequence, common to all virus strains examined in the genus (3'-AUCAUCAUCUG), and inversely complementary 5' termini. This sequence is genus specific and

Figure 2. Schematic representation of a hantavirus particle. L indicates a large protein corresponding to the putative RNA polymerase; N represents nucleocapsid proteins; G1 and G2 are viral glycoproteins embedded in a cell derived lipid envelope. L nucleocapsid, M nucleocapsid and S nucleocapsid represent encapsidated L, M and S genomic RNAs.


Glycoproteins
G1 G2
Lipid envelope
S nucleocapsid
S
M
M nucleocapsid
N protein
L protein
L
L nucleocapsid

therefore differentiates hantaviruses from other *Bunyaviridae* genera (Schmaljohn et al., 1985).

The nucleocapsids are packaged inside a lipid envelope during budding at internal cellular membranes, most likely Golgi vesicles (Matsuoka et al., 1991). Assembly of biologically active virions, containing all three segments, must involve interactions between the nucleocapsids and the internal domains of the glycoproteins or with the membrane itself. It has been postulated that the difference in size of virions is directly related to the number of nucleocapsids incorporated into a particular virus particle. Indeed, nonequimolar ratios of L, M and S RNA molecules were extracted from purified virions, suggesting that each virion may contain unequal numbers of three segments, with the S genomic RNA being the most and the L genomic RNA being the least abundant (Yoo and Kang, 1987). The mechanism of assembly is not known.

3. GENOME STRUCTURE

Molecular characterization of the hantavirus genome has been very tedious and slow because of slow virus growth and extremely low virus titer.

When this project began, the coding capacity of the S and M genome segments of Hantaan 76-118 virus was determined by cloning and sequencing (Schmaljohn et al., 1986b, 1987a). It was evident that the S genome segment has a single, long open reading frame (ORF) in the virus-complementary sense RNA with the capacity to encode only one protein with a molecular weight of 48 K

(Schmaljohn et al., 1986b). This size is consistent with the values reported for Hantaan N protein (Schmaljohn et al., 1983; Elliot et al., 1984). Another small ORF, which could potentially code for a 6 kD polypeptide, was identified in the same reading frame immediately following the termination codon of the N gene. However, viral protein of this size was not identified in Hantaan virus-infected cells. This finding made hantaviruses unique among Bunyaviridae genera since the S segments of other Bunyaviruses encode the nucleocapsid (N) protein and a nonstructural (NS_S) protein either from overlapping reading frames (Bunyavirus) or by ambisense coding strategy (Uukuviruses and Phleboviruses).

Like the S segment, the M segment of Hantaan 76-118 virus has only one long ORF in virus-complementary sense RNA, with a potential to encode a polypeptide of 126 kD (Schmaljohn et al., 1987; Yoo and Kang, 1987b). The N-terminal sequencing of the G1 and G2 glycoproteins demonstrated that the M segment gene product is processed into these two glycoproteins most likely by cotranslational processing. A single M segment gene product is common to all Bunyaviruses. However, while the polypeptide of the Hantaan virus, and uukuviruses is processed into two glycoproteins, the M segment encoded polypeptide of bunyaviruses and phleboviruses is processed into two glycoproteins and the nonstructural protein (NS_M). Since the nucleocapsid protein and the two glycoproteins of Hantaan virus were encoded by the S and M segments, the large protein, observed in viral particles, was presumed to be encoded by the L segment.

Until now, the coding capacity of the L segment of Hantaan 76-118 virus, all

three segments of Puumala Hällnäs B1 virus and the S and M segments of Prospect Hill virus has been determined. One of the goals of this study was to molecularly characterize the prototype of the Seoul serotype of hantaviruses, Seoul 80-39 virus. Comparative sequence analysis will provide a better understanding of the evolution and pathogenesis of all four hantavirus serotypes.

4. FUNCTION OF HANTAVIRUS PROTEINS

The large (L) protein of approximately 250 kD has been found in association with purified Hantaan virions (Elliott et al., 1984) and is believed to have RNA-dependent RNA polymerase activity (Schmaljohn and Dalrymple, 1983). However, functions of this protein have not been determined yet and further characterization awaits expression of the L gene or the establishment of hantavirus reconstitution systems.

The G1 and G2 envelope glycoproteins are responsible for *in vitro* hemagglutination (HA) and they both stimulate production of neutralizing (NT) antibodies (Tsai et al., 1984; Okuno et al., 1986). On the basis of neutralization of virus infectivity by antibodies, hantaviruses were grouped into four serotypes. Although monoclonal antibodies (MAbs) to G1 and G2 of Hantaan virus have been made and characterized, the epitopes involved in HA and NT have not been mapped (Arikawa et al., 1989). Only 6 out of 20 MAbs, made to the glycoproteins of Hantaan 76-118 virus, had both hemagglutination inhibition (HI) and neutralization activity (Arikawa et al., 1989). Most of MAbs showing HI activity

only, were MAbs to the glycoprotein G2 and two of them were able to react well with viruses from all four serotypes, Hantaan, Seoul, Prospect Hill and Puumala (Dantas et al., 1986; Arikawa et al., 1989), suggesting the existence of common antigenic sites. Other antibodies were Hantaan-specific or cross-reactive with the Seoul virus. It is evident that both glycoproteins are essential for the induction of a protective, humoral immune response. Indeed, efficient protection of newborn mice from Hantaan virus challenge was demonstrated only upon immunization with recombinant baculovirus or vaccinia virus expressing both glycoproteins (Schmaljohn et al., 1990).

Glycoproteins of hantaviruses mediate cell fusion, at low pH (Arikawa et al., 1985), but it is not known whether both or only one of the two proteins is important for this process.

Like genomes of other negative-stranded RNA viruses, the genomic RNA of hantaviruses is encapsidated with nucleocapsid proteins (NP). The process of assembly has not been elucidated. It is presumed that some sort of specific interaction occurs between the viral genomic RNA and NP. This interaction is believed to be crucial for regulation of transcription and replication, as described in the introduction to Chapter 5.

NP induces production of complement-fixing antibodies most of which are cross-reactive within the genus. Transfer of anti-Puumala immune serum into nude mice decreased titers of Hantaan virus in lungs and spleens. Since only the antibodies against NP cross-react with all HFRS viruses, this protection most likely

reflects NP cross-reactivity. Although the antibodies against NP do not have neutralizing activity, immunization of animals with the recombinant baculovirus expressing NP, protected the animals from Hantaan virus challenge (Schmaljohn et al., 1990). This suggests the importance of a cell mediated immune response to NP in Hantaan virus infections. Indeed, Asada et al. (1987, 1988, 1989) showed that cytotoxic T lymphocytes (CTL) were generated when spleen cells from mice infected with viruses causing HFRS were stimulated *in vitro* with syngeneic cells infected with viruses. CTLs induced by Hantaan 76-118 virus were cross reactive with target cells infected with Seoul virus, Puumala virus or Prospect Hill virus and vice-versa. Because these studies were performed with intact virions it is not possible to determine whether the CTL response was specific to NP.

In order to determine the role of the nucleocapsid protein in virus replication, encapsidation and in the cell-mediated immune response, the expression of the nucleocapsid protein was necessary. Therefore, one of the goals of this study was to express high levels of the nucleocapsid protein using a recombinant baculovirus system.

5. STAGES OF INFECTION

The first event in the virus infection process is attachment of the virus to host cell receptor(s). The attachment is presumably mediated by viral envelope glycoproteins. Since neutralizing and hemagglutination inhibiting sites have been detected on both the G1 and G2 proteins of Hantaan virus, it is most likely that

both proteins are involved in attachment, either directly or in a conformation-dependent manner. Cellular receptor(s) involved in the attachment of Bunyaviruses (including hantaviruses) to the host cell, have not been identified yet. After attachment, viral particles are most likely endocytosed. Low pH in endosomes probably causes fusion of viral membranes with endosomal membranes, resulting in a release of nucleocapsids into the cytoplasm (Schmaljohn and Patterson, 1991).

Transcription and replication of viral genomic RNA segments (vRNAs) occur in the cytoplasm. Virion-associated RNA polymerase starts primary transcription (synthesis of viral-specific mRNAs from the negative-sense vRNA template) most likely by addition of cellular mRNA-derived sequences (approximately 15 nucleotides) to their 5' ends. The 3' base of this short fragment is probably aligned on the 3' base of the template and is used as a primer (Bouloy et al., 1990). The first base incorporated by the polymerase would be position +2. Transcription proceeds, but it never goes to the end of the templates. The transcription of the S and L segments of LaCrosse virus (Patterson and Kolakofsky, 1984; Hacker et al., 1990) and the M segment of Germiston (GER) virus (Bouloy et al., 1990) ends within the template sequence 3' GUUUUU 5'. The transcription of the S segment of the GER virus ends within a similar sequence - 3' GUUUGU 5' (Bouloy et al., 1990) and the M segments of the Snowshoe Hare (SSH) virus (Eshita et al., 1985) and the Rift Valley fever virus (Ihara et al., 1985) end within the 3' ACCCC 5' sequence. The function of these purine rich sequences in transcription

termination is to be determined. The 3' termini of mRNAs of all bunyaviruses are not polyadenylated (Kolakofsky and Hacker, 1991). The 3' terminal sequences of hantavirus mRNAs have not been determined yet.

In contrast to mRNA synthesis, synthesis of antigenomes and progeny genomes initiates with a triphosphate at the position +1. It is hypothesized that nucleocapsid proteins interact immediately with the 5' termini of nascent chains of antigenomes and genomes, allowing synthesis to go right to the end of the template. The hypothetical model explaining the switch between the transcription and replication is explained in greater detail in the introduction to Chapter 5.

Synthesis of bunyavirus NP and probably of the L protein takes place on free polyribosomes, whereas the glycoproteins are synthesized on the membranes of the rough endoplasmic reticulum (rER). Glycoproteins are directed to the Golgi apparatus where they reside (Matsuoka et al., 1991). Encapsidated genomic RNA segments associate with L proteins. It has been shown that phlebovirus RNPs accumulate at the cytoplasmic side of the Golgi membranes at areas in which viral envelope glycoproteins are present at the luminal face (Smith and Pifat, 1982). It is proposed that interaction between the cytoplasmic domain of the glycoproteins and nucleocapsid proteins of RNPs is the first step in the assembly of virions (Garoff and Simons, 1974; Sturman et al., 1980). After budding of virus particles into the Golgi cisternae, virions are further transported through the *trans*-Golgi network and released from the cells by exocytosis (Matsuoka et al., 1991).

Hantaviruses establish noncytolytic, persistent infections in susceptible

mammalian host cells without interfering with host macromolecular synthesis. Most stages in hantavirus infection are similar to those reported for other bunyaviruses. However, published data explaining the processing of hantavirus glycoproteins and virus assembly not only are not clear but are somewhat contradictory. Therefore, another objective of this study was to clarify the maturation of hantavirus glycoproteins and to determine the site of hantavirus morphogenesis.

6. STATEMENT OF THE OBJECTIVES OF THE STUDY

The objectives of the study are the following:

Objective 1.

a). To elucidate the molecular organization of the Seoul 80-39 genome.

b). To compare genome organization, nucleotide and amino acid sequences of the Seoul 80-39 virus with the genomes of other hantaviruses in order to better understand their evolutionary relationships and differences in pathogenicity.

Objective 2.

To investigate the processing of Hantaan virus glycoproteins G1 and G2 and to determine the site of virus morphogenesis.

Objective 3.

To express the nucleocapsid protein of Seoul 80-39 and Hantaan 76-118 viruses as a first step in the characterization of their protein function(s).

CHAPTER 2 MATERIALS AND METHODS

1. PROPAGATION OF HANTAVIRUSES IN CELL CULTURE

Cells, viruses and media

The african green monkey kidney cell line, Vero E6, was obtained from the American Type Culture Collection (ATCC 1008, CRL 1586) and used for the propagation of hantaviruses.

Seoul 80-39 virus and Hantaan 76-118 virus were provided by Dr. Ho Wang Lee (WHO collaborating center for Hantaviruses, Korea University, School of Medicine).

Cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS) and 2 mM L-glutamine (Gibco). Cells were propagated in 100 mm polystyrene dishes (Nunc, Kamstrup, Denmark) at 37°C in a humidified incubator (Shel-Lab, Sheldon Manufacturing Inc. Portland, Oregon) supplemented with 5% CO_2.

Infections

All infectious work with hantaviruses was carried out in the level IV (D) Biohazard Containment Facility of the Faculty of Medicine, University of Ottawa.

Confluent monolayers of Vero E6 cells were infected with hantaviruses at

a multiplicity of infection (MOI) of approximately 0.01 plaque forming units per cell (pfu/cell). After addition of the hantavirus inoculum, cells were incubated for 2 hours at 35°C to allow virus adsorption to occur. Fresh DMEM supplemented with 10% FCS was added and infected cells were incubated for 4 days. The medium was changed and incubation was continued for another 4 days. Cell culture medium was harvested 8 days after infection and at the same time fresh medium was added to the cell culture. Incubation was continued for another 4 days. Cell culture fluid collected on days 8 and 12 post-infection was used for virus purification.

Plaque assay

Vero E6 cells were grown in 6 well plates (Multidish 6, Nunclon, Denmark). Monolayers were infected with 0.2 ml of 10 fold serial dilutions of hantaviruses. After virus adsorption, inoculum was removed and cells were overlaid with 2 ml of EMEM-agarose medium (1 x EMEM, 10% FCS, 8mM L-glutamine, 0.1 mM nonessential amino acids (NEAA), 10 units/ml penicillin G, 100 μg/ml streptomycin (Gibco) and 0.6% SeaKem Agarose (Marine Colloids, Inc., Baush and Lomb). Cells infected with Hantaan 76-118 virus were incubated for 8 days and cells infected with Seoul 80-39 virus were incubated for 10 days at 35°C when the second overlay containing 1 X EMEM, 10% FCS, 8 mM L-glutamine, 0.1 mM NEAA, 10 units/ml penicillin G, 100 μg/ml streptomycin and 5% neutral red was added. The incubation was continued for another 2 to 3 days, plaques were counted and virus

titer was determined.

2. RNA ISOLATION AND ANALYSIS

Radiolabeling of viral RNA

To label hantavirus RNA [^{3}H]-uridine (10 μCi/ml, 20-30 Ci/mmol, Amersham, Arlington Heights, IL) was added to the culture medium at day 4 and at day 8 after infection. The radioactivity associated with viral genomic RNA was determined in a liquid scintillation counter (LKB Wallac, 1214 Rackbeta), after RNA isolation.

Isolation of Seoul 80-39 virus genomic RNA

Virus was pelleted by centrifugation of the cell culture fluid at 24,000 rpm for 2.5 hours at 4°C in a SW 28 rotor (L8-70 Beckman ultracentrifuge). The virus pellet was resuspended in STE (10 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA) buffer (50 μl for every 30 ml of medium). Viral particles were disrupted by addition of an equal volume of 2 x NENS buffer (100 mM sodium acetate, pH 5.1; 200 mM NaCl; 20 mM EDTA; 1% SDS) containing 500 μg/ml of proteinase K (Boehringer Mannheim GmbH) and subsequent incubation at 37°C for 30 minutes. The RNA was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), once with chloroform-isoamyl alcohol (24:1) and precipitated with 0.5 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol. All RNA

precipitations were done at -70°C, overnight.

Purification of viral RNA in a CsCl gradient

After chloroform-isoamyl alcohol extraction, RNA was pooled, sodium acetate pH 5.5 was added to a final concentration of 25 mM and the volume was adjusted to 2.7 ml with water. 2.918 g of CsCl was dissolved in this solution and the sample was overlaid on a 1 ml cushion of 5.7 M CsCl, 25 mM sodium acetate (pH 5.5) and centrifuged in a SW 50.1 rotor (L8-70 Beckman ultracentrifuge) for 18 hours at 37,000 rpm and 25°C. The supernatant was aspirated and the pelleted RNA was resuspended in 240 μl of diethyl pyrocarbonate-treated water (DEPC-H_2O) and heated to 68°C for 5 minutes. RNA was then ethanol precipitated as described previously.

Isolation of total cellular RNA

Cells were washed with cold phosphate buffered saline solution (PBS). Fresh PBS was then added, the cells were scraped from plates and pelleted at 2000 rpm for 5 minutes (IEC HN-SII centrifuge, Damon, Needham Hts., MASS). 3 ml of guanidium thiocyanate solution (4M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0 and 100 mM β-mercapto ethanol) was added and the cells were disrupted with 2 passes through an 18 G 1 1/2 Needle. Lysed cells were overlaid on 1.2 ml of 5.7 M CsCl, 25 mM sodium acetate (pH 5.5) and centrifuged for 12 hours at 36,000 rpm and 20°C in a SW50.1 rotor (L8-70 Beckman ultracentrifuge).

Following aspiration of the supernatant, the pelleted RNA was resuspended in DEPC-H_2O and ethanol precipitated.

Glyoxal agarose gel electrophoresis of RNA

RNA was dissolved in 5 μl of DEPC-H_2O and added to 15 μl of sample buffer containing 10 mM sodium phosphate, pH 7.0 (6.1 mM Na_2HPO_4, 3.9 mM NaH_2PO_4); 0.9 M (6%) deionized glyoxal (BDH) and 50% dimethylsulfoxide (DMSO). The RNA sample was heat denatured at 60°C for 20 minutes, loaded on a 1.5% agarose gel, 22 cm long, and run for 3 hours at 100 volts (EC-103 power pack, Mendel Scientific Co.) in 10 mM sodium phosphate buffer, pH 7.0. The gel was fixed in 10% acetic acid, 30% methanol for 1 hour, then in methanol for 1 hour and incubated in 3% PPO (2,5-Diphenyloxazole) in methanol overnight. Finally, the gel was washed in water, dried and exposed to X-ray film (Du Pont) at -70°C.

3. CLONING OF SEOUL 80-39 VIRUS GENOME

First and second strand cDNA synthesis

The virus specific cDNA library was made using CsCl purified viral RNA as a template (minimum 150,000 cpm), random hexanucleotide primers and cDNA Synthesis System Plus (Amersham International plc, Amersham UK). Reactions were performed as suggested by the manufacturer. Double stranded cDNA was

size fractionated through Sepharose 4B columns, large cDNA molecules were ligated into the *Sma* I site of pUC19 and recombinant plasmids were used to transform E. coli JM 101 cells, as described in section 9.

Reverse transcription - polymerase chain reaction (RT-PCR)

First strand cDNA synthesis was performed on the purified viral RNA template or on the total cellular RNA (cDNA Synthesis System Plus, Amersham). The resulting RNA-DNA hybrid was phenol-chloroform extracted, ethanol precipitated and used for the second strand synthesis by PCR (Gene amp kit, Perkin Elmer Cetus Corp., Norwalk, CT). In both reactions viral specific oligonucleotides were used as primers. cDNA products were purified by gel electrophoresis and inserted into pUC19.

4. DNA ISOLATION AND ANALYSIS

Small scale isolation of plasmid DNA from *E. coli* (mini-prep)

The procedure used for isolation of plasmid DNA was adapted and modified from the alkaline extraction method described by Birnboim and Doly (1979). Bacteria were grown overnight in Luria broth (LB, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 100 μg/ml of ampicillin at 37°C. Cells were pelleted and resuspended in 200 μl of solution I (50 mM glucose - 25 mM TRIS-HCl, pH 8.0 - 10 mM EDTA - 5 mg/ml lysozyme). After incubation for 5 minutes at room

temperature, the bacteria were lysed by adding 400 μl of a freshly prepared alkaline solution, solution II (0.2 N NaOH - 1% SDS) and incubated for 5 minutes on ice. 300 μl of 7.5 M ammonium acetate was added, the contents of the tube were mixed and incubation was continued for another 15 minutes. After centrifugation for 15 minutes at 4°C in a microcentrifuge (IEC Micro-MB centrifuge), the supernatant was transferred to another tube, 0.6 volumes of isopropanol were added and the resulting solution incubated at room temperature for 15 minutes. The DNA precipitate was pelleted by centrifugation at 15,000 rpm for 15 minutes. The supernatant was removed by aspiration and the pellet was resuspended in 100 μl of TE buffer. 100 μl of 5 M ammonium acetate was then added and mixed. After incubation on ice for 30 minutes, the suspension was centrifuged for 15 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube and 2.5 volumes of ice cold 100% ethanol was added. Another centrifugation for 10 minutes was performed to pellet plasmid DNA. The supernatant was discarded and the pellet was dried in a Speed-Vac (Savant) for 5 minutes. The pellet was resuspended in 50 μl of H_2O.

Large scale isolation of plasmid DNA (maxi-prep)

The procedure was essentially the same as the small scale procedure with following modifications. Bacteria were grown overnight in 40 ml of Terrific broth (TB; 1.2% bacto tryptone, 2.4% bacto yeast extract, 0.4% glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4) containing 100 μg/ml of ampicillin. Cells were pelleted in

Oak Ridge tubes using a JA-21 rotor (J2-21 Beckman centrifuge) for 5 minutes at 10,000 rpm. Incubation times and solutions used were as described in the mini-prep procedure except 5 ml of solution I, 10 ml of solution II and 7.5 ml of 7.5 M ammonium acetate was used in the individual steps. After isopropanol precipitation, the pelleted DNA was resuspended in 200 μl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) and RNase A (20 μg/ml) was added and the mixture incubated at 37°C for 30 minutes. 200 μl of 5 M ammonium acetate was then added and after further incubation on ice for 30 minutes was centrifuged for 15 minutes. The supernatant was transferred to another microcentrifuge tube and plasmid DNA was precipitated as described in the mini-prep procedure.

Alternatively, after isopropanol precipitation, plasmid DNA was purified on CsCl gradients as described by Sambrook et al. (1989), or Qiagen ">plasmid<" kit was used as described by manufacturer.

DNA agarose gel electrophoresis

A DNA sample (10 μl) was mixed with 1 μl of sample buffer (50% glycerol, 1% SDS, 100 mM EDTA, 0.1% bromphenol blue) and loaded onto 0.7 to 1.5 % agarose gels prepared with Tris-borate buffer (TBE, 89 mM Tris; 89 mM boric acid; 2.5 mM EDTA). A "minnie"™ Submarine gel apparatus, model HE33 (Hoefer Scientific Instruments) or wide mini-Sub™ cell (Bio-Rad) was used and electrophoresis was typically allowed to proceed for 2 hours at 100 V.

Isolation of DNA fragments from agarose gels

A modified "freeze-squeeze" method (Tautz and Renz, 1983) was used to recover DNA from agarose gels. Gels were stained with ethidium bromide (EtBr) and DNA was visualized under long-wave UV light. Bands of interest were cut from the gel and the agarose slices were placed in 0.5 ml microcentrifuge tubes which had been punctured through the cap and the bottom with a 21 G needle. The tubes were placed in 1.5 ml decapped microcentrifuge tubes and centrifuged for 5 minutes. After centrifugation an equal volume of 0.3 M sodium acetate, pH 5.2 was added to the smashed agarose in the 1.5 ml tube. The agarose - sodium acetate mix was transferred with a 1 ml needleless syringe to 0.5 ml tubes which had been punctured (through the cap and the bottom) and plugged with siliconized glass wool. These tubes were then placed in 1.5 ml decapped tubes and frozen at -70 °C for 30 minutes. After thawing, samples were centrifuged for 5 minutes. During centrifugation, the liquid phase containing DNA passed into the 1.5 ml tube and the agarose particles remained behind on the glass wool in the 0.5 ml tube. If necessary, the 0.5 ml tubes were transferred to another 1.5 ml decapped tube and centrifugation was repeated until no liquid was evident in the 0.5 ml tube. The eluted volumes were pooled and the EtBr was removed by extracting three times with H_2O saturated n-butanol. The DNA was then precipitated with 2.5 volumes of ethanol, at -20°C.

Restriction analysis

Restriction endonucleases were purchased from New England Biolabs (NEB, Beverly, MA), Pharmacia (Dorval, Quebec, Canada), Bethesda Research Laboratories (Gaithersburg, MD) or Promega Biotech (Madison, WI). All enzyme reactions were performed in buffers and at temperatures recommended by the supplier. Usually, 2 to 3 units of an enzyme were used per 1 μg of DNA.

5. DNA MODIFICATIONS

Ligation

Typically, 50 to 500 ng of cDNA or a restriction fragment was mixed with 100 to 200 ng of the linearized plasmid or the linearized replicative form of M13. The reaction was carried out at 16°C, overnight in ligation buffer (20 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 10 mM DTT; 1 mM ATP) with 5 units of T4 DNA ligase (Pharmacia) and in a final volume of 20 μl.

Phosphorylation

Up to 1 μg of DNA and 10 units of T4 polynucleotide kinase were incubated in 70 mM Tris-HCl, pH7.6 - 10 mM $MgCl_2$ - 5 mM DTT and 5 mM ATP in a final volume of 20 μl at 37°C for 30 minutes. Subsequently, the enzyme was inactivated by incubation at 65°C for 15 min. The reaction was slowly cooled down to room temperature and the DNA was phenol-chloroform extracted and ethanol

precipitated.

Dephosphorylation

Up to 1 μg of DNA was treated with 0.5 units of calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim) in 50 mM Tris-HCl, pH 9.0 containing 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$ and 1 mM spermidine in a final volume of 50 μl. For dephosphorylation of protruding 5' termini, the reaction was incubated at 37°C for 30 minutes, another 0.1 unit of CIAP was added and incubation was continued for another 30 minutes at 37°C. Recessed 5' termini or blunt ends were dephosphorylated by incubating the reaction at 37°C for 15 minutes, then at 56°C for 15 minutes. Another 0.1 unit of CIAP was added and incubation at both temperatures was repeated. After the dephosphorylation reaction, 40 μl of H_2O, 10 μl of 10 x STE and 5 μl of 10% SDS were added to the sample. The sample was heated to 68°C for 15 minutes and the DNA was phenol-chloroform extracted and ethanol precipitated.

Filling of recessed 3' ends

Approximately 1 μg of DNA in 50 mM Tris-HCl, pH 8.0 containing 10 mM $MgSO_4$; 0.1 mM DTT and 50 μg/ml BSA was incubated with 80 μM of dNTPs and 1-5 units of Klenow DNA polymerase (Pharmacia) at room temperature for 30 minutes in a final volume of 20-50 μl. The DNA was phenol-chloroform extracted and ethanol precipitated.

Conversion of a 3' overhang to a blunt end

This reaction was done either using the 3'-5' exonuclease activity of Klenow DNA polymerase or using the 5'-3' polymerase and 3'-5' exonuclease activity of T4 DNA polymerase. When Klenow DNA polymerase was used, the reaction was set as described in the previous section except dNTPs were not added. When T4 DNA polymerase (NEB) was used, 0.1 mM dNTPs were added and the reaction was allowed to proceed as suggested by the supplier.

6. PREPARATION OF LABELED DNA PROBES AND HYBRIDIZATION

Nick-translation

50 μl reaction mixtures containing 1 μg of DNA, 10 μM dCTP, dGTP, dTTP, 100 μCi of [α-^{32}P]-dATP (800 Ci/mmol, Amersham), approximately 5 x 10^{-4} units of deoxyribonuclease I (DNase I) and 5 units of DNA polymerase I in 50 mM Tris-HCl, pH 7.2 containing 10 mM $MgSO_4$; 0.1 mM DTT and 50 μg/ml BSA were incubated for 1.5 hours at 16°C. Unincorporated nucleotides were removed by passing the labeled DNA through a Sephadex G-50 column. Fractions containing the nick-translated probe were pooled, denatured by boiling for 10 minutes and used for hybridization.

Colony lifting and hybridization

Bacterial colonies were lifted onto Colony/PlaqueScreen Hybridization

Transfer Membrane (NEN Research Products, DuPont), processed and used for hybridization as recommended by the manufacturer.

Northern blotting and hybridization

Immediately after glyoxal gel electrophoresis, RNA was electrophoretically transferred from the gel to NEN's Gene Screen *Plus* membrane (DuPont) using a Trans-Blot™ cell (Bio-Rad Laboratories) for 12 hours at 24 V and 22°C. The membrane was subsequently processed and used for hybridization as recommended by the manufacturer.

7. SEQUENCING

Reactions

Plasmid DNA was denatured prior to sequencing in 0.2 M NaOH - 0.2 mM EDTA for 5 minutes at room temperature. The reaction was neutralized with sodium acetate buffer, pH 5.0 (final concentration 0.3 M) and DNA was precipitated with 2 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried and used for sequencing.

Sequencing was performed with either modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation, Cleveland, OH) or with *Taq* DNA polymerase (TaqTrack Sequencing System, Promega). Protocols supplied by manufacturers were followed. Universal forward and reverse pUC primers and

specific primers were synthesized in an Applied Biosystems 380B DNA synthesizer (Biotechnology Research Institute, University of Ottawa) and used to prime the sequencing reactions.

Gels

6% acrylamide - 0.3% bis-acrylamide or 8% acrylamide - 0.4% bis-acrylamide gels containing 8 M urea in 1 x TBE were used for electrophoresis of sequencing reactions on the IBI sequencing apparatus Model STS-45. The running buffer was 1 x TBE and electrophoresis was carried out at a constant power of 55 watts. When sequencing samples were electrophoresed for 10 hours or longer, 6.5% acrylamide - 0.35% bis-acrylamide gels containing 7.8 M urea and 1.2 x TBE were used. The upper reservoir contained 1 x TBE and bottom reservoir contained 1.5 x TBE as running buffers and electrophoresis was carried out at a constant power of 50 watts. After electrophoresis, gels were transferred from the glass plate onto Whatman (3MM) paper, dried under vacuum (Gel dryer model 583, Bio-Rad) at 80°C for 20 minutes and exposed to X-ray film (DuPont).

8. M13 PHAGE

All cDNA clones were subcloned into the *Kpn*I - *Bam* HI linearized replicative form (RF) of M13mp18 and M13mp19. Transformation of *E. coli* JM101 competent cells with RF of M13, plaque assay, growth of M13, detection of single-

stranded phage DNA in culture supernatants and isolation of single-stranded DNA were performed as described by Messing (1983).

Isolation of single-stranded M13 phage DNA (maxi-prep)

In a 125 ml Erlenmeyer flask, 12 ml of 2 x YT medium (1.6% bacto tryptone, 1% bacto yeast extract and 0.5% NaCl) was combined with 50 µl of an overnight culture of *E. coli* JM101. Bacteria were grown for 1 hour at 37°C, 50 µl of phage stock was added and the bacteria were grown for 6 hours at 37°C. Cells were pelleted by centrifugation. The supernatant was transferred to another tube, 0.67 ml of 40% polyethylene glycol (PEG) and 0.67 ml of 5 M sodium acetate (pH 7.0) were added, the solution was vortexed and incubated overnight at 4°C. Phage was pelleted at 20,000 rpm for 10 minutes in SW41 rotor (L8-M Beckman ultracentrifuge). The supernatant was discarded and the remaining liquid was removed with cotton swabs. The pellet was resuspended in 0.6 ml of 20 mM Tris-HCl (pH 7.2), transferred to a microcentrifuge tube and extracted once with phenol, two times with phenol-chloroform (1:1/v:v) and once with chloroform. The aqueous phase was combined with 0.05 volumes of 2.5 M sodium acetate (pH 5.2) and 2 volumes of ethanol and incubated overnight at -70°C. DNA was pelleted by centrifugation, washed with 70% ethanol, dried in a Speed-Vac and resuspended in 60 µl H_2O. The concentration was determined by reading the A_{260} in the Beckman DU-8B spectrophotometer.

9. *E. COLI* CULTURES

Preparation of JM101 and JM109 competent cells

Competent cells were prepared as described by Hanahan (1983). 25 single colonies were picked from a freshly streaked SOB agar plate (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, $MgSO_4$, 1.5% agar) and dispersed in 5 ml of SOB liquid medium (the same as above, but without agar) by vortexing. The cells were inoculated into 250 ml of SOB and incubated at 37°C with moderate agitation for 2 - 3 hours (until the density reaches 4 - 7 x 10^7 viable cells/ml, A_{650} = 0.45 - 0.55). The culture suspension was dispensed into 50 ml tubes (Falcon 2070 Blue Max™, Becton Dickinson) and chilled on ice for 10 - 15 minutes. The cells were pelleted by centrifugation at 3,000 rpm in a clinical centrifuge (IEC HN-SII) for 12 - 15 minutes at 4°C. The supernatant was discarded and the remaining liquid was aspirated. The cells were resuspended in 1/3 of the initial volume of cold RF1 solution (100 mM RbCl, 50 mM $MnCl_2$ x 4 H_2O, 30 mM KOAc, 10 mM $CaCl_2$ x 2 H_2O, 15% glycerol; pH adjusted to 5.8 with 0.2 M acetic acid), incubated on ice for 15 minutes and pelleted by centrifugation at 3,000 rpm and 4°C for 15 minutes. Pelleted cells were resuspended in 1/12.5 of the original volume of cold RF2 solution (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$ x 2 H_2O, 15% glycerol; pH adjusted to 6.8 with 0.2 M acetic acid) and incubated on ice for 15 minutes. Aliquots of 200 µl were dispensed into chilled screw cap tubes (NuncCryotubes) and frozen in liquid nitrogen.

Transformation of JM101 or JM109 cells

Cells were thawed on ice. As soon as the cell suspension became liquid, aliquots of 200 μl were placed in 10 ml snap cap tubes (Falcon 2057) and 10 μl of DNA was added. The cells were incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds and then chilled by returning the tubes to ice. 800 μl of SOC medium (SOB medium with 20 mM glucose) was added, the cells were incubated at 37°C for 1 hour with moderate agitation and then plated onto the selective medium (LB plates containing 100 μg/ml of ampicillin, 50 μM isopropyl-thio-galactoside (IPTG) and 40 μg/ml of Xgal).

Preparation of RR1 competent cells

A fresh overnight culture of RR1 cells was diluted in LB (1:50) and grown at 37°C until A_{650} reached 0.40 - 0.50. The culture was then incubated on ice for 20 minutes and cells were pelleted at 3000 rpm and 4°C in clinical centrifuge. Cells were resuspended in 1/2 of the initial volume of ice cold 0.1 M $MgCl_2$, repelleted immediately and resuspended in 1/15 of the initial volume of ice cold 0.1 M $CaCl_2$. Cells were spun down, drained and resuspended in 1/10 of the initial volume of ice cold 85 mM $CaCl_2$, 15% glycerol.

Transformation of RR1 cells

For each transformation, 0.3 ml of competent cells was thawed on ice, 1/5 of a ligation reaction was added and the sample was incubated on ice for 30

minutes. Cells were heated for 2 minutes at 42°C and chilled on ice. 0.7 ml of LB was added, the suspension was incubated for 1 hour at 37°C and then plated onto selective medium.

10. PROTEIN ANALYSIS

Radiolabeling of viral proteins

Eight days after infection, Hantaan virus infected cells were rinsed with phosphate buffered saline (PBS) and methionine-free DMEM (supplemented with mM glutamine and 10% FCS) was added. After 2 hours incubation at 35°C, the medium was replaced with fresh DMEM containing 100 μCi/ml of L-[^{35}S]methionine (NEN, DuPont). The proteins were labeled for 4 hours at 35°C. After labeling, the cells were rinsed in ice-cold PBS, scraped with a rubber policeman and pelleted. When purified virions were used for protein analysis, the labeling period was extended to 24 hours. Virus was pelleted from medium collected from twenty 100 mm plates. The pellet was resuspended in 1 ml of lysis buffer (NP-40 or RIPA buffer) and used for three immunoprecipitation reactions with MAbs.

Preparation of cell lysates

NP-40 cell lysates were prepared by addition of 1 ml of NP-40 lysis buffer (20 mM Tris, pH 8.0; 137 mM NaCl; 1 mM $CaCl_2$; 0.5 mM $MgCl_2$; 10% glycerol; 1%

NP-40 and 4 mM phenylmethyl sulfonyl fluoride (PMSF)) to the cells harvested from one T75 flask. RIPA cell lysates were prepared by the addition of 1 ml of RIPA buffer (NP-40 buffer without glycerol and with 1% sodium deoxycholate and 0.1% SDS) to the pelleted cells. The cells were vortexed, incubated for 15 minutes at 4°C and spun at 10,000 rpm for 10 minutes. The supernatant was used for three immunoprecipitations.

Immunoprecipitation

To 300 μl of clarified cell lysate, 2-5 μl of MAb was added and the mixture was incubated for at least 2 hours at 4°C with constant rotation. Protein A Sepharose beads (Pharmacia) were added (50 μl of 50 mg/ml suspension) and incubation was continued for another hour. The protein A Sepharose beads were pelleted and washed four times with cold wash buffer (100 mM Tris; 500 mM LiCl, pH 9.0). Protein-antibody conjugates were dissociated from protein A by boiling for 5 minutes in sample buffer (1% SDS; 25% glycerol; 10 mM Tris, pH 6.9 and 0.02% bromphenol blue, with or without 50 mM dithiotreitol (DTT)). Samples were either loaded onto a SDS-polyacrylamide gel or stored at -70°C.

Endoglycosidase H, endoglycosidase F and endoglycosidase D digestions

Immunoprecipitated samples were first washed and then resuspended in 30 μl of either endoglycosidase H (EH) buffer (50 mM sodium acetate, pH 5.2), endoglycosidase F (EF) buffer (50 mM sodium acetate (pH 7.0); 0.25 mM EDTA;

1% NP-40) or endoglycosidase D (ED) buffer (0.1 M citrate-phosphate buffer, pH 6.5). Digestion was carried out with 2 mU of EH, 0.1 U of EF or 10 mU of ED for 12 hours after which a fresh aliquot of enzyme was added and incubation was continued for another 10 hours. Enzymes were purchased from Boehringer Mannheim.

Glycosylation inhibition

Eight days after infection, cells were treated with glycosylation inhibitors during the prelabeling period (2 hr), after which fresh drug was added for the 4-hr labeling period. The concentrations of inhibitors used were: 10 μg/ml or 2.5 μg/ml of brefeldin A (Epicentre Technologies, Bio/Can Scientific Inc.), 3 mM castanospermine (Boehringer Mannheim), 300 ng/ml of swainsonine (Boehringer Mannheim) and 1 μM or 10 μM monensin (Boehringer Mannheim).

Chemical cross-linking

The cleavable chemical cross-linker dithiobis(succinimidylpropionate) (DSP) was added to NP-40 solubilized cell lysates to a final concentration of 1 mg/ml. The samples were incubated for one hour at 27°C with constant rotation. The reaction was terminated by addition of glycine to a final concentration of 2 mM.

Sucrose gradient sedimentation analysis

The clarified cell lysate was cross-linked and layered onto 5 to 20% (wt/vol)

linear sucrose gradients in 100 mM NaCl; 20 mM Tris, pH 7.5; 30 mM MOPS, pH 7.5; 0.1% NP-40. The gradients were centrifuged in an SW41 rotor at 37,000 rpm for 24 hours at 4°C. Fractions of 0.6 ml were collected by puncturing the bottom of the tube. Hantaan virus glycoproteins were immunoprecipitated from the collected fractions with MAbs.

Polyacrylamide gel electrophoresis

Protein analysis was done by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separating gel consisted of 10% acrylamide, 0.27% bis-acrylamide, 0.1% SDS and 0.75 M Tris, pH 8.8. The stacking gel consisted of 5% acrylamide, 0.13% bis-acrylamide, 0.1% SDS and 66 mM Tris, pH 6.8. Electrophoresis was carried out at 100 V in 25 mM Tris, 192 mM glycine, 0.1% SDS buffer using the Mini-Protean II or Protean II Electrophoresis Cell (Bio-Rad). After electrophoresis, gels were fixed in 30% methanol, 10% acetic acid for 30-60 minutes and prepared for fluorography with Amplify (Amersham) for 1 hour. Gels were dried under vacuum and exposed to X-ray film. Alternatively, gels were stained with Coomasie blue (0.1% coomasie blue, 40% methanol, 10% acetic acid) for 30 minutes and proteins were visualized after several washes in destain solution (30% methanol, 10% acetic acid).

Western blot

Proteins were transferred onto Immobilon PVDF membranes (Milipore).

Membranes were handled as suggested by manufacturer. Prior to electroblotting, gel, membrane and sponges were equilibrated in transfer buffer (10 mM CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid), pH 11; 10% methanol) for 15 min. The transblotting sandwich (Bio-Rad) was assembled and electroblotting was performed at 90 V (300 mA) and room temperature for 30 minutes.

Immuno-blot assay

Immuno-staining of Western blots was performed with the Bio-Rad Immuno-Blot™ Assay Kit as suggested by manufacturer.

11. EXPRESSION OF PROTEINS IN INSECT CELLS

Insect cell culture

Spodoptera frugiperda (Sf9) cells were provided by Dr. M. D. Summers, Texas A & M University, College Station, TX. They were grown in TNM-FH medium (M. Summers and G. Smith, 1987) supplemented with 2 mM glutamine and 10% FCS at 27 °C.

Cotransfection

Sf9 cells were seeded into 35 mm plates at a density of 2.5 X 10^6 cells per plate. Cells were allowed to attach in serum free TNM-FH medium for 30 minutes. Cells were transfected with 2 μg of *Autographa californica* nuclear polyhedrosis

virus (AcNPV) DNA and 2 μg of pAc recombinant plasmid DNA using the calcium phosphate method described in the CellPhect Transfection Kit (Pharmacia). The DNA solution was aspirated, cells were gently rinsed and covered with fresh media and incubated for 3 days at 27°C. Medium was collected for plaque purification of the virus.

Plaque assay

Sf9 cells were plated at a density of 0.5 x 10^6 cells/ml and infected with 10-fold dilutions of the virus in complete cell culture medium. After 1 hour adsorption, the virus was aspirated and the cells were overlaid with 0.5 x TNM-FH - 1.5% low-melting agarose (Bio-Rad). When the overlay hardened, complete TNM-FH was added and infected cells were incubated for 3-4 days at 27°C. Plaques were either counted for determination of virus titer or selected and isolated for purification of the recombinant viruses.

Infection of Sf9 cells with recombinant baculoviruses

For the preparation of a virus stock, 1 x 10^6 Sf9 cells/ml were infected with the recombinant AcNPV using a multiplicity of infection (MOI) of 0.1 to 0.5 pfu/cell.

To achieve a high level of expression of the recombinant protein, 2.5 x 10^6 Sf9 cells/ml were infected with the recombinant virus using a MOI of 5 pfu/cell.

Detection of recombinant proteins

Infected cells were harvested, rinsed with PBS and lysed in protein sample buffer (10% SDS; 10% β-mercapto ethanol; 25% glycerol, 10 mM Tris, pH 6.9 and 0.02% bromphenol blue). Aliquot was loaded onto SDS - polyacrylamide gels, elecrophoresed for 2 hours at 100 V and stained with Coomasie blue, as described in section 10.

The specificity of expressed proteins was determined by Western blot analysis, as described in section 10.

12. ELECTRON MICROSCOPY

Processing of tissue culture cells for thin section electron microscopy

Cells were fixed *in situ* with 2.5% sodium phosphate-buffered glutaraldehyde (pH 7.4) for 2 hours at 4°C, washed three times in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 2% sodium phosphate-buffered osmium tetroxide. After being washed two times in water, cells were stained in water-saturated uranyl acetate for 15 minutes, washed again two times in water and dehydrated in ethanol (15 minutes in 50% ethanol, 15 minutes in 70% ethanol and 3 times for 20 minutes in 100 % ethanol). Embedding and thin sectioning of cells, as well as electron microscopy was performed by Dr. Ian Robb, Division of Pathology, Children's Hospital of Eastern Ontario.

CHAPTER 3 MOLECULAR CHARACTERIZATION OF SEOUL 80-39 VIRUS

INTRODUCTION

Seoul 80-39 virus is the prototype of the Seoul serotype of hantaviruses. All virus isolates antigenically related to the Seoul 80-39 virus have been considered strains of the serotype and are associated with moderate forms of the human disease known as hemorrhagic fever with renal syndrome (HFRS). Similarly, virus isolates which are antigenically related to the Hantaan 76-118 and Hällnäs B1 viruses, respectively, are strains of the Hantaan and Puumala serotypes and are associated with severe form of HFRS or with nephropathia epidemica, respectively. The Prospect Hill isolate is the prototype of the fourth serotype and is not known to cause human disease (Table 1). In order to better understand differences in the pathogenicity of the strains of the four hantavirus serotypes, and the evolutionary relationship between them, molecular characterization of their genomes was necessary. Before this project began, the coding properties of the M and S genomic RNA segments of Hantaan 76-118 virus had been determined and work on Puumala and Prospect Hill viruses was in progress. Therefore, the objective of this part of the project was to determine the genome organization and coding capacity of the Seoul 80-39 virus, a representative of the Seoul serotype.

To determine molecular characteristics of the Seoul 80-39 virus, a cDNA library was made using purified viral RNA as a template and random hexanucleotides as primers. The clones obtained were grouped by cross-

hybridization and their genomic assignment was determined by Northern blot hybridization. The nucleotide sequences of cDNA clones were determined. The remaining parts of the Seoul 80-39 genome were cloned by reverse transcription - polymerase chain reaction using specific primers. The coding capacity of each genomic RNA segment was determined and the amino acid sequence was deduced from the nucleotide sequence information.

Molecular characteristics of the entire L and M segments and of the S segment (from the 5' end of the viral cRNA to the nucleotide position 1332) of Seoul 80-39 virus will be presented, discussed and compared with those of other hantaviruses. A possible evolutionary pathway of hantaviruses will be presented.

RESULTS

1. CONSTRUCTION AND CHARACTERIZATION OF THE SEOUL 80-39 cDNA LIBRARY

Seoul 80-39 virus genomic RNA was isolated from pelleted virions from the culture fluid of infected Vero E6 cells and used as a template for cDNA synthesis with random hexanucleotide primers. The cDNA molecules were size fractionated by chromatography through Sepharose 4B columns and cloned into the *Sma*I site of pUC19 by blunt end ligation. *E. coli* JM101 competent cells were transformed and ampicillin resistant, white colonies were selected for further analysis.

The size of the cDNA clones was determined by digestion of plasmids with restriction endonucleases *Kpn*I and *Bam*HI. cDNA clones were grouped by cross-hybridization using the colony-hybridization method. The specificity of cDNA clones was determined by Northern blot hybridization (not shown). The cross-hybridization and Northern blot hybridization results are shown in Table 2.

Eight groups of clones (L-1 to L-8) were assigned to the L genomic RNA segment. Group L-1 was represented by clone 1 (791 bp) and it did not hybridize to any other group of clones in cross-hybridizations. Northern blot hybridization revealed that this clone was L segment specific. Clone 2 (1424 bp) was a representative of a group of 4 overlapping clones (L-2). This clone did hybridize with the representative of the L-3 group (clone 3, 1803 bp), but it did not hybridize with the representative of the L-4 group (clone 4, 778 bp). Similarly, clone 4

TABLE 2

GENOMIC ASSIGNMENT OF VIRUS SPECIFIC cDNA CLONES

SEGMENT ASSIGNMENT[1]	GROUP[2]	REPRESENTATIVE CLONE	OTHER CROSS-HYBRIDIZING CLONES IN THE GROUP
L	L-1	1	-
	L-2	2	3, 24, 25
	L-3	3	2, 4
	L-4	4	3, 5, 6
	L-5	5	4, 6, 26
	L-6	6	4, 5, 7, 26
	L-7	7	6, 8, 26, 27
	L-8	8	7, 27
M	M-1	9	10, 16, 17
	M-2	10	9, 11, 15, 16, 17, 18, 19
	M-3	11	10, 18, 19
	M-4	12	13, 23
	M-5	13	12, 14, 20, 21, 22, 23
	M-6	14	13, 20, 21, 22, 23

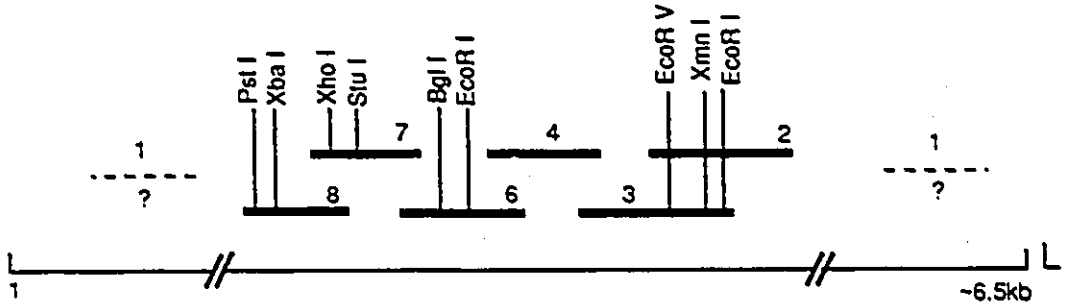
[1] Segment assignment was determined by Northern blot hybridization analysis

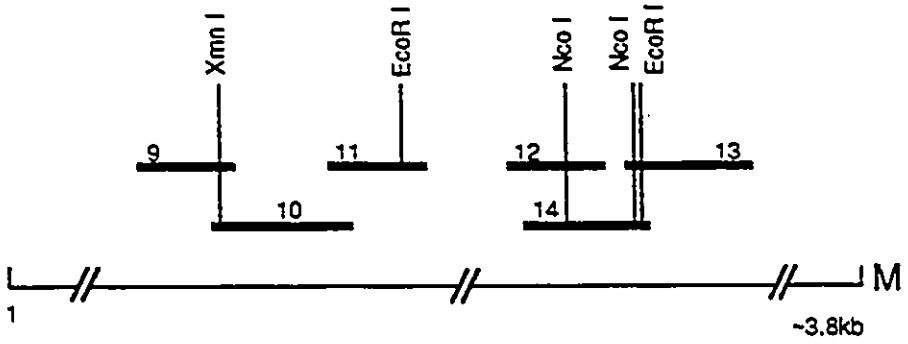
[2] Distinct groups of clones were identified by cross hybridization analysis

hybridized with clones 3, 5 and 6 (representative clones of groups L-3, L-5 and L -6), clone 5 hybridized with clones 4 and 6 and clone 6 (929 bp) hybridized with clones 4, 5 and 7. Therefore, we concluded that group L-5 completely overlaps with groups L-4 and L-6. Clone 7 (representative of group L-7, 877 bp) hybridized with clones 6 and 8 (representative of group L-8, 737 bp). Since clone 3 was the largest clone in cross-hybridizing groups L-2 to L-8, the insert of clone 3 was isolated from an agarose gel, labeled by nick-translation and used for Northern blot hybridization. Clone 3 hybridized with the L genomic RNA segment. Therefore, cDNA groups L-1 to L-8 were L segment specific.

Six groups of clones (M-1 to M-6) were assigned to the M genomic RNA segment. Clone 9 (489 bp) was a representative of the M-1 group and it hybridized with a representative of the M-2 group (clone 10, 1463 bp) which further hybridized with a representative of the M-3 group (clone 11, 582 bp). A representative clone of the M-4 group (clone 12, 377 bp) hybridized with a representative clone of the M-5 group (clone 13, 821 bp) and clone 13 hybridized with a representative clone of the M-6 group (clone 14, 953 bp). Since clone 10 was the largest clone in cross-hybridizing groups M-1 to M-3 and clone 14 was the largest in cross-hybridizing groups M-4 to M-6, the inserts of these clones were isolated from agarose gels, labeled by nick-translation and used for Northern blot hybridization. These clones hybridized with M genomic RNA segment and therefore groups M-1 to M-6 were M segment specific. A physical map of clones (Fig. 3) was constructed after restriction enzyme analysis with 20 different

Figure 3. Physical map of the L segment- and M segment-specific clones.


Pst I
Xba I
Xho I
Stu I
Bgl I
EcoR I
EcoR V
Xmn I
EcoR I
1
?
7
8
4
6
3
2
1
?
1
L
~6.5kb


Xmn I
EcoR I
Nco I
Nco I
EcoR I
9
10
11
12
14
13
1
M
~3.8kb

endonucleases.

cDNA clones representing the S genomic RNA segment were not obtained.

2. GENOMIC ORGANIZATION AND CODING CAPACITY OF THE L GENOMIC RNA SEGMENT

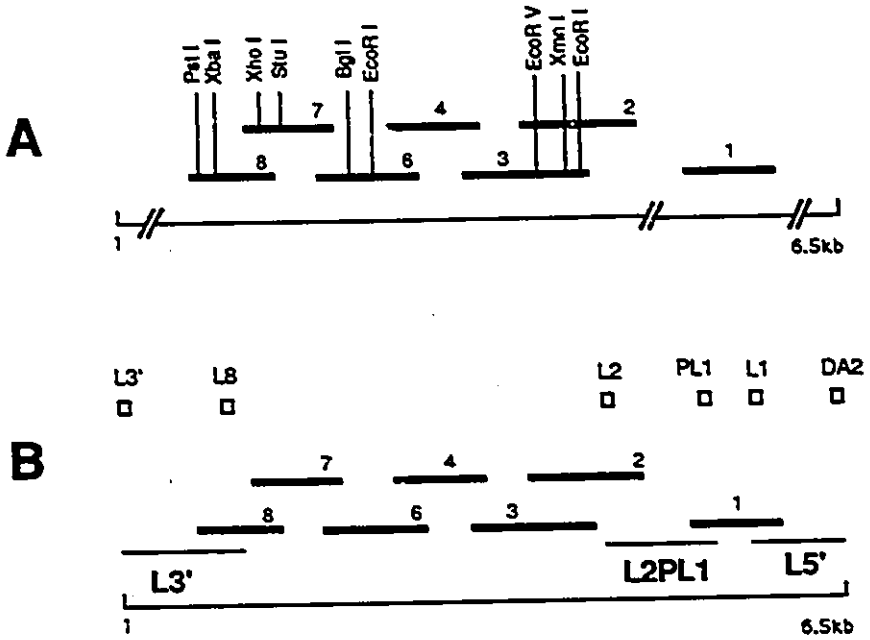
Sequencing strategy

Seven L segment specific clones, 1, 2, 3, 4, 6, 7 and 8 (Fig. 3), were used for sequencing. The cDNA fragments were subcloned into the *Kpn*I - *Bam*HI sites of replicative forms of both M13mp18 and M13mp19 DNA. Single stranded DNA was isolated and sequenced by the dideoxy chain termination method in the presence of the universal M13 forward primer or specific sequencing primers.

Strategy for cloning of the full length L genomic RNA segment

Once the sequence of the L segment specific clones was determined, we were able to design specific primers for reverse transcription - polymerase chain reaction for cloning of the 3' end, the 5' end and the gap between clone 1 and the group of overlapping clones (Fig. 4). The L3' primer has 25 nucleotides complementary to the previously determined 3' end of the Seoul L RNA segment (Schmaljohn et al., 1985), whereas the DA2 primer has 10 bases complementary to the 3' end of cRNA (the sequence was predicted from the inverse complementarity between the 3' and 5' ends of the M and S segment genomic

Figure 4. Strategy for cloning of Seoul 80-39 virus L RNA segment. (A): Position and restriction map of the cDNA clones of the L RNA segment. The 52 bases at the 3' end of the L RNA segment, the region between nucleotides 4989 and 5343 (354 bases) and the region from end of clone 1 to the 5' end of the vRNA were cloned using PCR. (B) and (C): The position and sequences of primers used for cloning of the 3' end, the 5' end and the gap between clone 1 and the group of overlapping clones. All primers had a *Pst*I site at their 5' ends allowing insertion of the clones into the *Pst*I site of pUC19. Oligonucleotides L3', L2 and L1 were used to prime first strand cDNA synthesis. Subsequent amplification reactions were performed in the presence of two primers: L3' and L8 for obtaining the 3' end; L2 and PL1 for the synthesis of the L2PL1 clone and L1 and DA2 for the cloning of the 5' end of the L RNA segment. (D): Amplified DNA sequences representing the 3' end (592 bp), the gap between the clones 2 and 1, L2PL1 (719 bp) and the 5' end (816 bp) of the L segment are shown by arrows.



C

Pst I

L3' CAGCTGCAG TAGTAGTAGACTCCGGAAGAGACAA

L2 CAGCTGCAG TGCAGAGGCAATCTGTGCATACTG

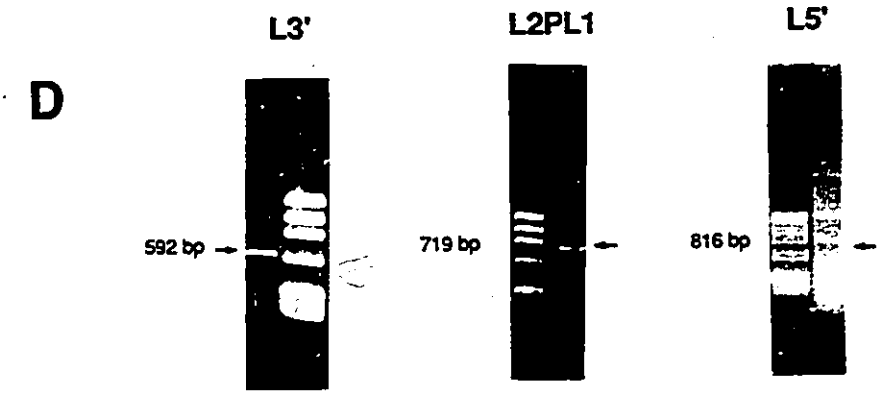
L1 CAGCTGCAG TCATCAGGACTGCTAGAGTACT

Pst I

L8 CAGCTGCAG ATCTCATGTATTGAACGACACCAT

PL1 CAGCTGCAG CTGTCCTCAATGCACAGT

DA2 CAGCTGCAG TAGTAGTAGA



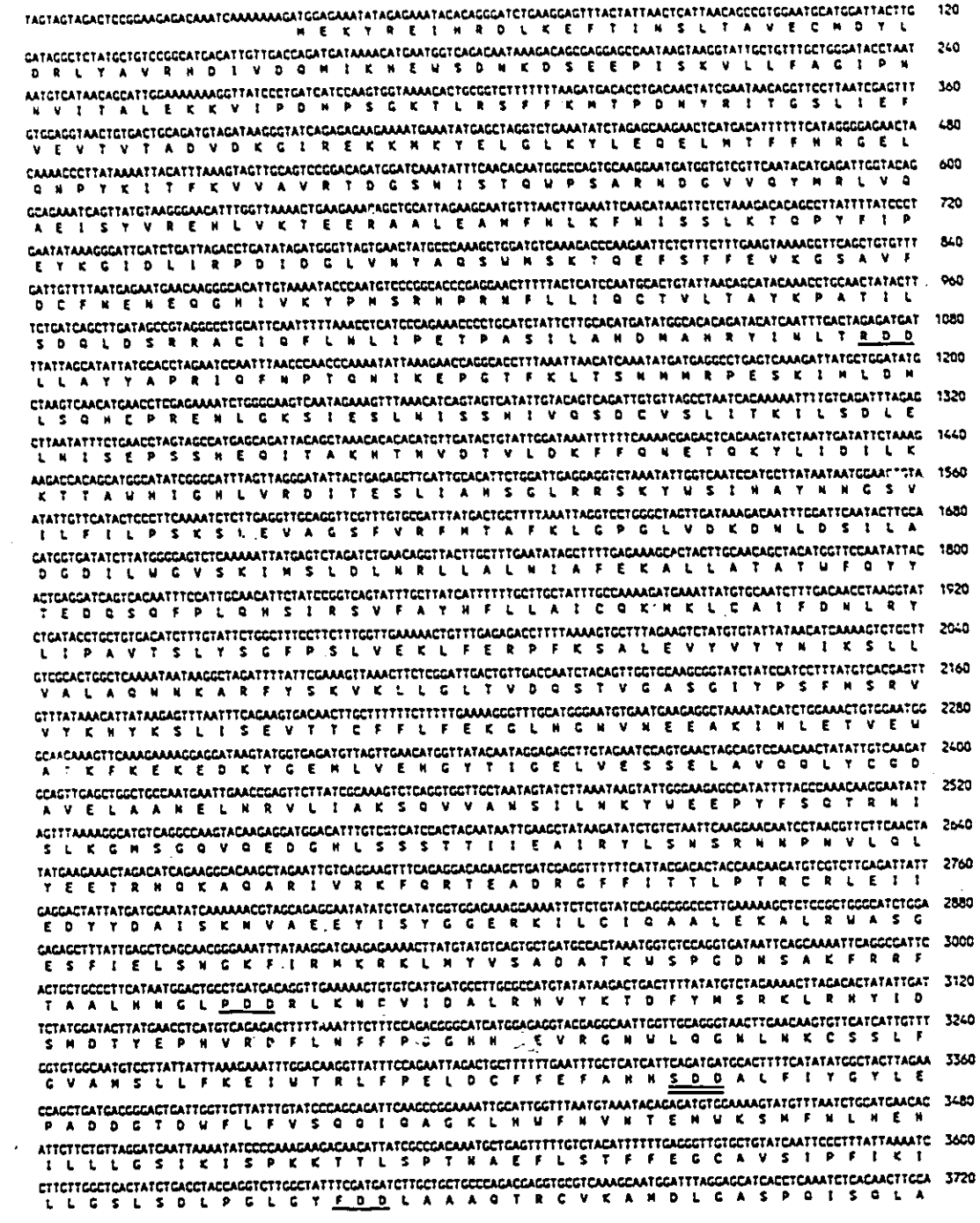
RNAs). Other primers were designed according to the sequences of the clones 8 (L8), 2 (L2) and 1 (L1 and PL1) (Fig. 4).

To clone the 3' end of the L segment, the oligonucleotide L3' was used to prime the first strand cDNA synthesis. Since the position of clone 1 (relative to the group of 6 clones) was not known, amplification reactions were performed in the presence of two primers: L3' and either L8 or PL1. The product was obtained in the reaction with L3' and L8 primers (Fig. 4) showing that clone 1 is positioned downstream from the group of 6 overlapping clones. Therefore, the gap between clone 2 and clone 1 (clone L2PL1) was filled by priming the reverse transcription with the L2 primer and subsequent polymerase chain reaction with primers L2 and PL1. Similarly, the 5' end of the L segment was obtained by reverse transcription with the L1 primer and amplification reaction with primers L1 and DA2 (Fig. 4). All primers had a *Pst*I site at their 5' termini allowing insertion of the clones into the *Pst*I site of pUC19. Clones L3', L2PL1 and L5' were sequenced in both directions by the dideoxy chain termination method using specific as well as universal pUC forward and reverse primers.

Nucleotide sequence and the deduced amino acid sequence of the L segment

The complete nucleotide sequence of the L genomic RNA segment was determined and is presented as complementary, messenger sense DNA in Fig. 5. The L segment is 6530 nucleotides long and has a base composition of 32.6% A, 16.4% C, 21.1 % G and 29.9% U. The 3' and 5' termini are inversely

Figure 5. Nucleotide sequence (presented as cDNA) and the deduced amino acid sequence of the Seoul 80-39 virus L genomic RNA segment. The X-D-D motifs, present in RNA dependent RNA polymerase molecules, are underlined. The SDD motif, conserved in L proteins of Bunyaviruses (hantavirus and bunyavirus) and in the PB1 protein of influenza virus, is double underlined.



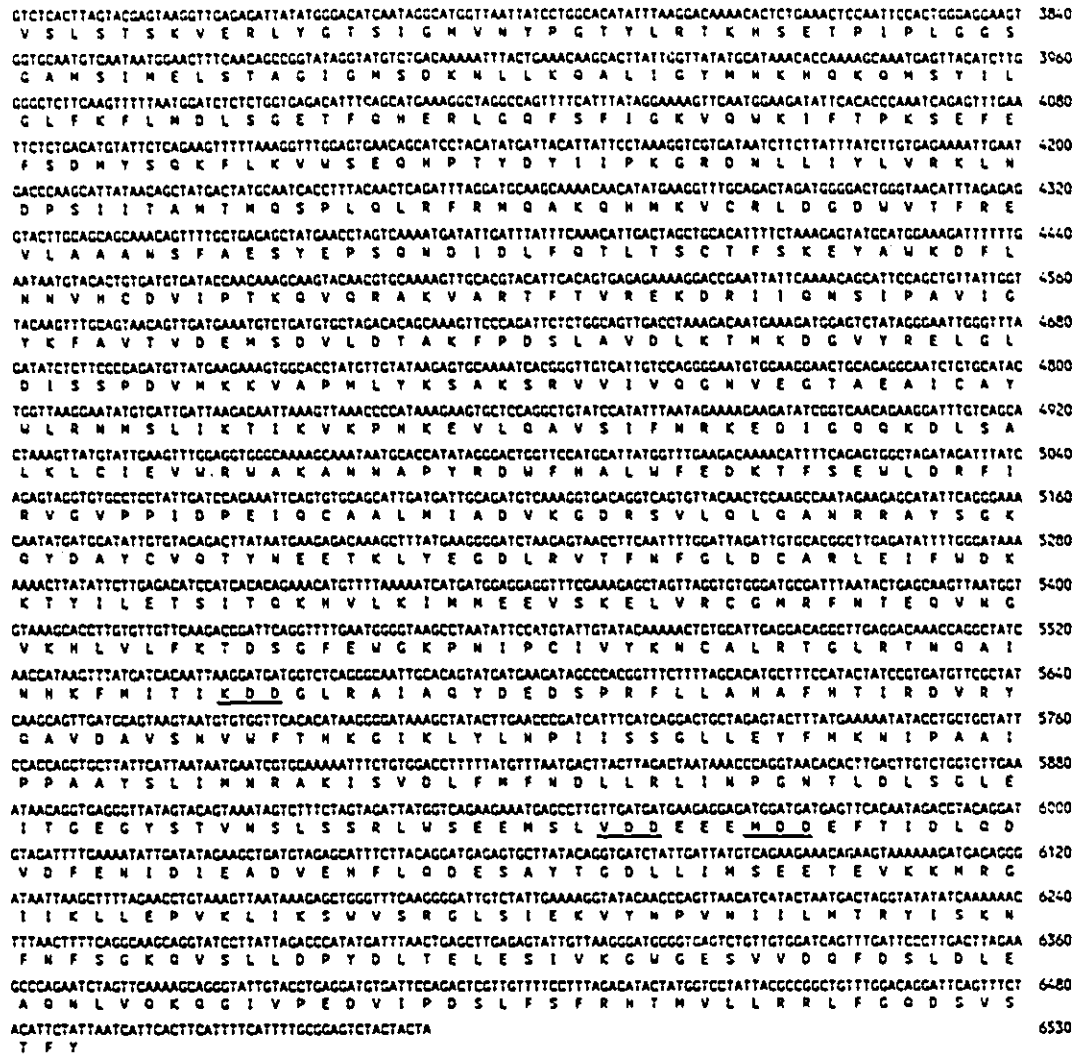
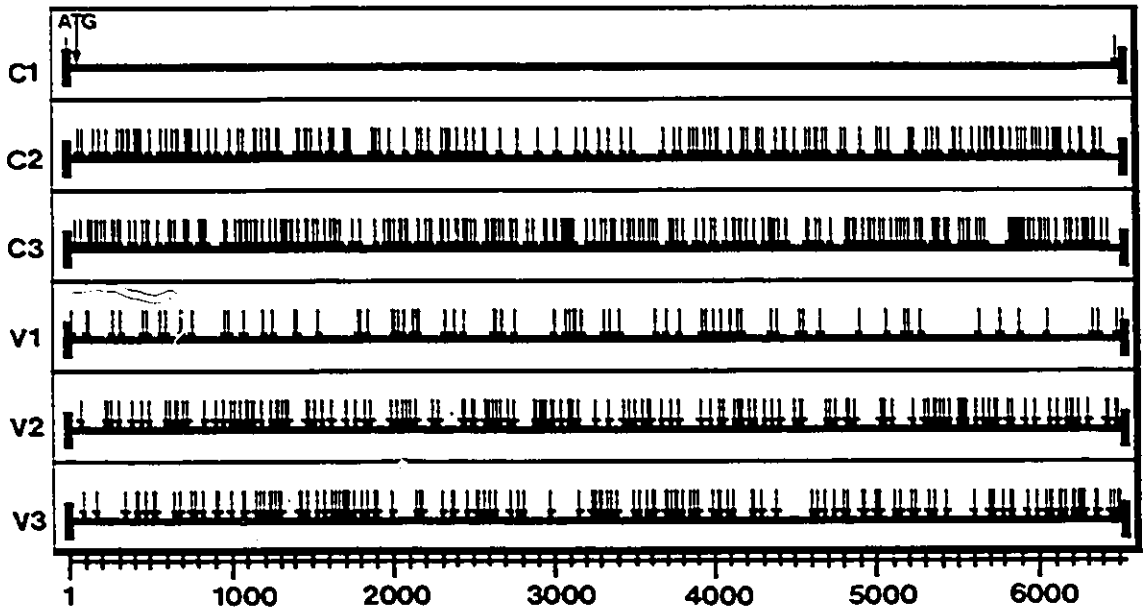


Figure 6. Translation termination codon locations in all reading frames of the Seoul 80-39 virus L RNA segment; three viral complementary (messenger) sense reading frames (C1, C2, C3) and three viral sense reading frames (V1, V2, V3). The first potential translation initiation codon is indicated by an arrow.


ATG
C1
C2
C3
V1
V2
V3
1
1000
2000
3000
4000
5000
6000

complementary for 15 nucleotides and the secondary structure that would result from base pairing (shown in Fig. 13) would have a free energy of -39.3 kcal (calculated for the terminal 50 nucleotides). A single open reading frame (ORF) was detected in the first frame of the viral complementary (+) sense RNA (Fig. 5 and Fig. 6). It starts from the AUG codon at nucleotide position 37-39 and ends with the UAA stop codon at nucleotide position 6490-6492. This ORF could encode a polypeptide of 2151 amino acids (246,662 D) which corresponds to the size of the L protein detected in purified viral particles (Elliot et al., 1984).

3. GENOMIC ORGANIZATION AND CODING CAPACITY OF THE M GENOMIC RNA SEGMENT

Sequencing strategy

Six M segment specific clones, 9, 10, 11, 12, 13 and 14 (Fig. 3), were subcloned into the *Kpn*I - *Bam*HI sites of the replicative form DNA of bacteriophages M13mp18 and M13mp19. Single stranded DNA was isolated and used for sequencing with the universal M13 forward primer or with specific primers.

Strategy for cloning of the full length M genomic RNA segment

In order to obtain clones representing the 3' end, the 5' end and the gap between the two groups of clones, we performed first strand cDNA synthesis on

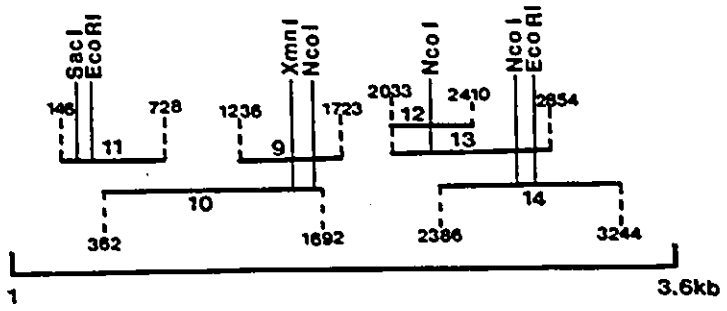
the viral RNA template followed by the polymerase chain reaction using specific primers, as shown in Figure 7. Primer DA1 contains 20 nucleotides complementary to the previously determined 3' end of the Seoul M RNA (Schmaljohn et al., 1985) whereas primer DA2 represents 10 bases identical to the predicted 5' terminus of virion M genomic RNA segment (prediction based on the inverse complementarity between the 3' and 5' termini of Hantaan 76-118 virus M RNA (Schmaljohn et al., 1987a, Yoo and Kang, 1987b). Other primers (DA11, DA9, DA12 and DA14) were designed according to the previously determined sequences of the clones. Oligonucleotide primers DA1, DA9 and DA14 were used to prime first strand cDNA synthesis. Subsequent amplification reactions were performed in the presence of two primers: DA1 and DA11 to obtain the 3' terminus, DA9 and DA12 for the synthesis of the middle (M) section and DA14 and DA2 were used to synthesize the 5'terminus of the M RNA segment. All primers had a *Pst*I site at their 5' termini allowing insertion of resulting clones into the *Pst*I site of pUC19. Clones were sequenced in both directions using universal pUC forward and reverse primers.

Nucleotide sequence and the deduced amino acid sequence of the M genomic RNA segment

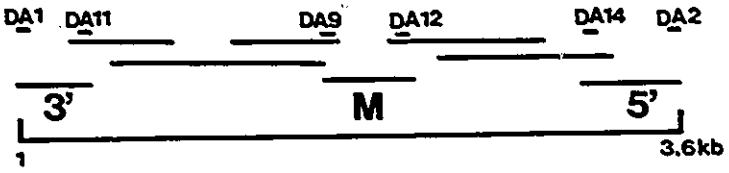
The nucleotide sequence of the M genomic RNA segment of the Seoul 80-39 virus is presented in Figure 8. The virion M genomic RNA segment contains 3651 nucleotides with a base composition of 30.2% A, 18.8% C, 21.7% G and 29.3% U.

Figure 7. Strategy for cloning of Seoul 80-39 virus M genomic RNA segment. (A): Precise position and restriction map of the cDNA clones of the M RNA segment. The 146 bases at the 3' terminus, the region between nucleotides 1723 and 2033 (310 bases) and the region from nucleotide 3244 to the 5' end (407 bases) of the vRNA were cloned using PCR. (B) and (C): The position and sequences of primers used for cloning of the 3' end, the 5' end and the middle section of the Seoul 80-39 virus M genomic RNA segment by the polymerase chain reaction. All primers had a *Pst*I site at their 5' termini allowing insertion of the clones into the *Pst*I site of pUC19. Oligonucleotides DA1, DA9 and DA14 were used to prime first strand cDNA synthesis. Subsequent amplification reactions were performed in the presence of DA1 and DA11 primers to obtain the 3' terminal clone; DA9 and DA12 primers for the synthesis of the middle (M) section and DA14 and DA2 primers for the cloning of the 5' terminus of the M genomic RNA segment. (D): Amplified DNA sequences representing the 3' terminus (235 bp), the middle section (338 bp) and the 5' terminus (565 bp) of the M genomic RNA segment are shown by arrows.

A



B



C

DA1 CAGCTGCAGTAGTAGTAGACTCCGCAAGA (Pst I)

DA11 CAGCTGCAGCATGTTGCATGAGCTCTC (Pst I)

DA9 CAGCTGCAGTGTGAGATCTGTAAGTAC (Pst I)

DA12 CAGCTGCAGTCAAGGTCTGTATGCATA (Pst I)

DA14 CAGCTGCAGAGTGTGACACTCTCACGA (Pst I)

DA2 CAGCTGCAGTAGTAGTAGA (Pst I)

D

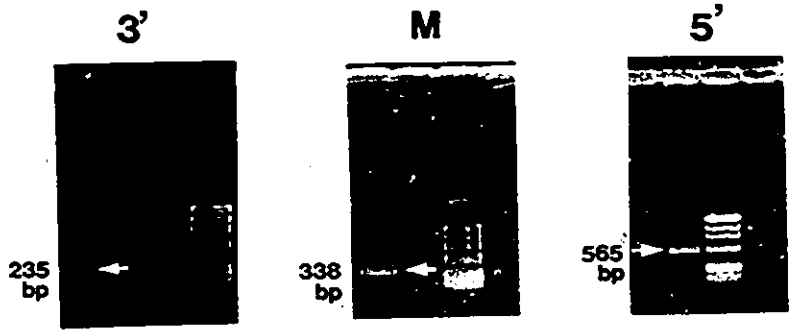


Figure 8. Nucleotide sequence (presented as complementary, messenger sense DNA) and the deduced amino acid sequence of the Seoul 80-39 virus M genomic RNA segment. Potential N-linked glycosylation sites (N-X-S(T) where X can be any amino acid except P or D) are boxed. The four main hydrophobic domains are underlined.

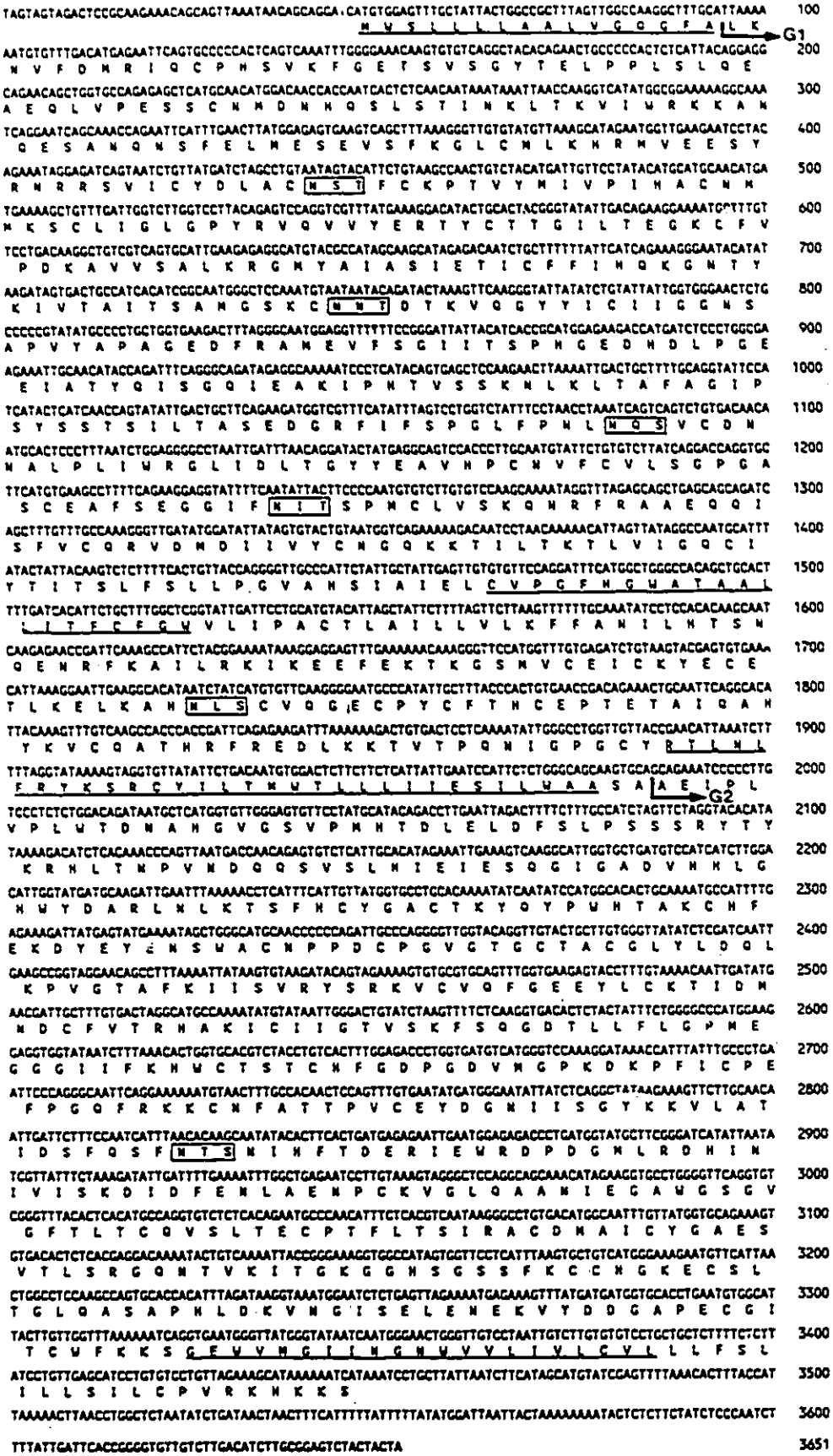
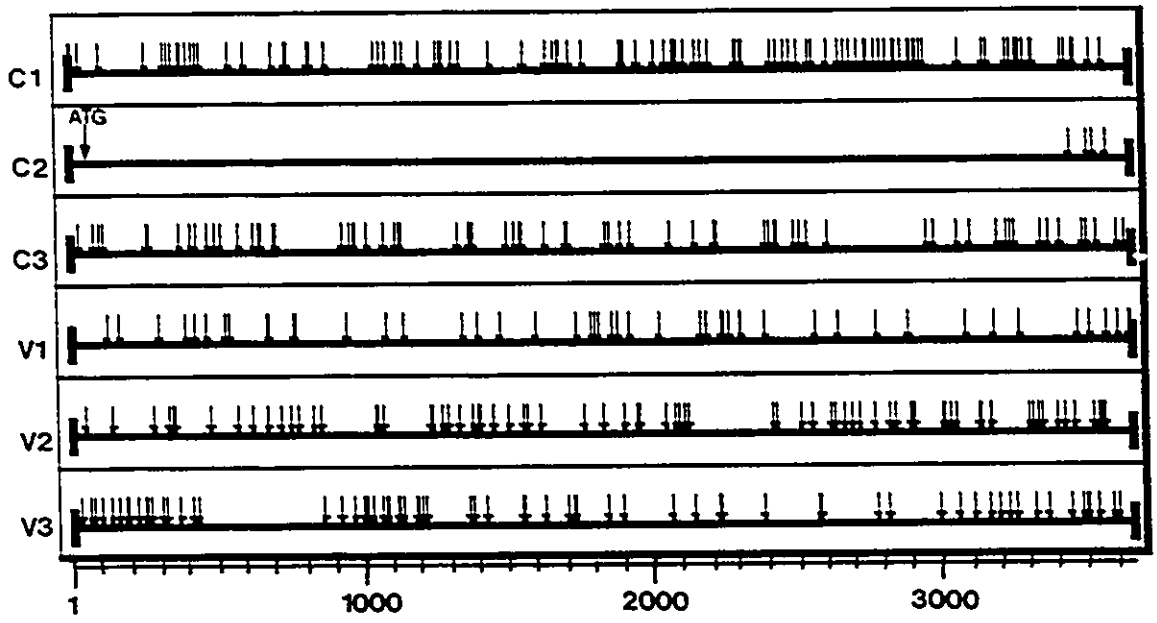


Figure 9. Positions of translation termination codons in all reading frames of the Seoul 80-39 virus M RNA segment; three viral complementary (messenger) sense reading frames (C1, C2, C3) and three viral sense reading frames (V1, V2, V3). The first potential translation initiation codon is indicated by an arrow.


C1
ATG
C2
C3
V1
V2
V3
1
1000
2000
3000

The 3' and the 5' termini of the Seoul 80-39 M genomic RNA segment are inversely complementary for 20 bases and the structure that could result from base pairing (shown in Fig. 17) would have a free energy of -45.0 kcal (calculated for the terminal 50 nucleotides). A single open reading frame was detected in the second frame of the complementary, messenger sense RNA (Fig. 8 and Fig. 9). The ORF starts with the AUG codon at nucleotide position 47-49 and ends with the UAA codon at nucleotide position 3446-3448. The predicted M segment encoded protein has 1133 amino acids (Fig. 8) and represents a glycoprotein precursor. A search for potential asparagine-linked glycosylation sites (Asn-X-Ser/Thr, where X represents any amino acid except Asp or Pro) (Bause, 1983, Kornfeld and Kornfeld, 1985) revealed that the putative G1 and G2 glycoproteins of the Seoul 80-39 virus contain five and one potential glycosylation sites, respectively (Fig. 8). The M segment encoded protein is relatively cysteine rich (5.4%). A hydropathy plot (shown in Fig. 19) of the predicted protein shows four principal regions of hydrophobicity (underlined in Fig. 8). The first domain is located at the N terminus (amino acid positions 1-16) and is the signal peptide for G1. The second domain is located between amino acid position 486 and 506 of G1 and most likely represents a transmembrane region. The third hydrophobic region (amino acids 627-657) represents either another transmembrane region or represents a signal peptide for G2. A highly hydrophilic region is located between the second and the third hydrophobic domain. A fourth hydrophobic domain is located at the C terminus of G2 (amino acid positions 1106-1126) and probably represents a

transmembrane region of the G2. This domain is followed by a short stretch of hydrophilic amino acids which may represent a cytoplasmic tail of G2.

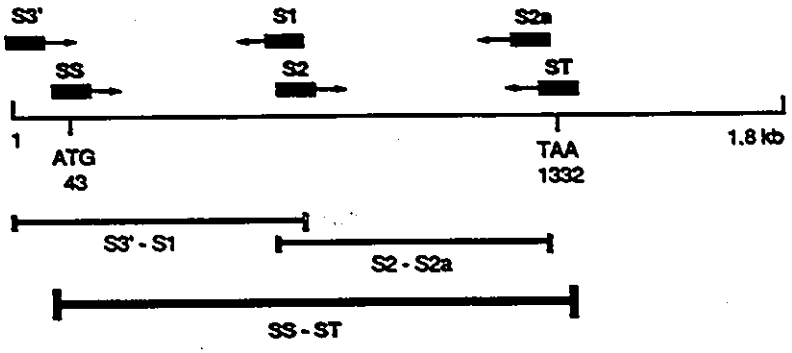
4. GENOMIC ORGANIZATION AND CODING CAPACITY OF THE S GENOMIC RNA SEGMENT

Strategy for cloning of the S genomic RNA segment

The S segment (from the 5' end of the viral cRNA to the stop codon of the ORF) was cloned by reverse transcription - polymerase chain reaction. The published sequence of the S genomic RNA segment of SR-11 virus (Arikawa et al., 1990), which represents another strain of the Seoul serotype, was used to design oligonucleotide primers for cloning the S segment of the Seoul 80-39 virus by PCR. Two clones, S3'-S1 and S2-S2a (Fig. 10), representing 1332 nucleotides of the S genomic RNA segment were obtained by priming first strand cDNA synthesis with primers S3'or S2. Subsequent amplification reactions were primed with sets of two primers: S3' and S1 or S2 and S2a. Primer S3' was designed so that a *Pst*I site was added to the 5' terminus of the oligonucleotide and primers S1, S2 and S2a were representing part of the S segment sequence containing an internal *Pst*I site. The presence of the restriction site allowed cloning of the cDNA fragments into the *Pst*I site of pUC19. Sequence of the clones was determined using the dideoxy chain termination method in the presence of viral specific or pUC universal forward and reverse primers.

Figure 10. Strategy for cloning of Seoul 80-39 S genomic RNA segment. (A) and (B): Position and sequences of primers used for cloning of the S segment (from the 5' end to the stop codon of the long ORF of the viral cRNA). Oligonucleotides S3', S2 and SS were used to prime first strand cDNA synthesis and subsequent PCR was carried out in the presence of two primers: S3' and S1 for the synthesis of clone S3' (C); S2 and S2a to obtain clone S2-S2a (C); SS and ST for the cloning of the ORF only (C).

A



B

S3' CAGCTGCAGTAGTAGTAGACTCCCTAAAGAGCTA
Pst I

S1 ACATACTGCAGTGCGGAATCT
Pst I

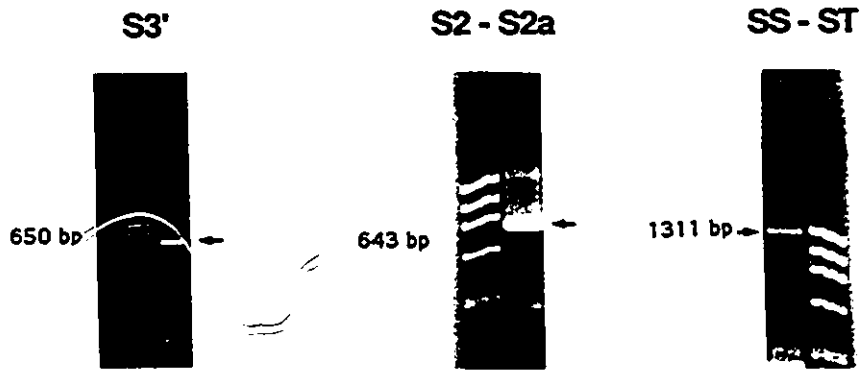
S2 CGCACTGCAGTATGTGGACTATAT
Pst I

S2a CAGCTGCAGAGATCTGAGCCAGGCTAC
Pst I

SS CTCGAGCTCTATAAATATGGAGGAAATCCAGAGAGA
Sac I

ST ACCGGTACCTTATAATTTCATAGGTTCCTG
Kpn I

C

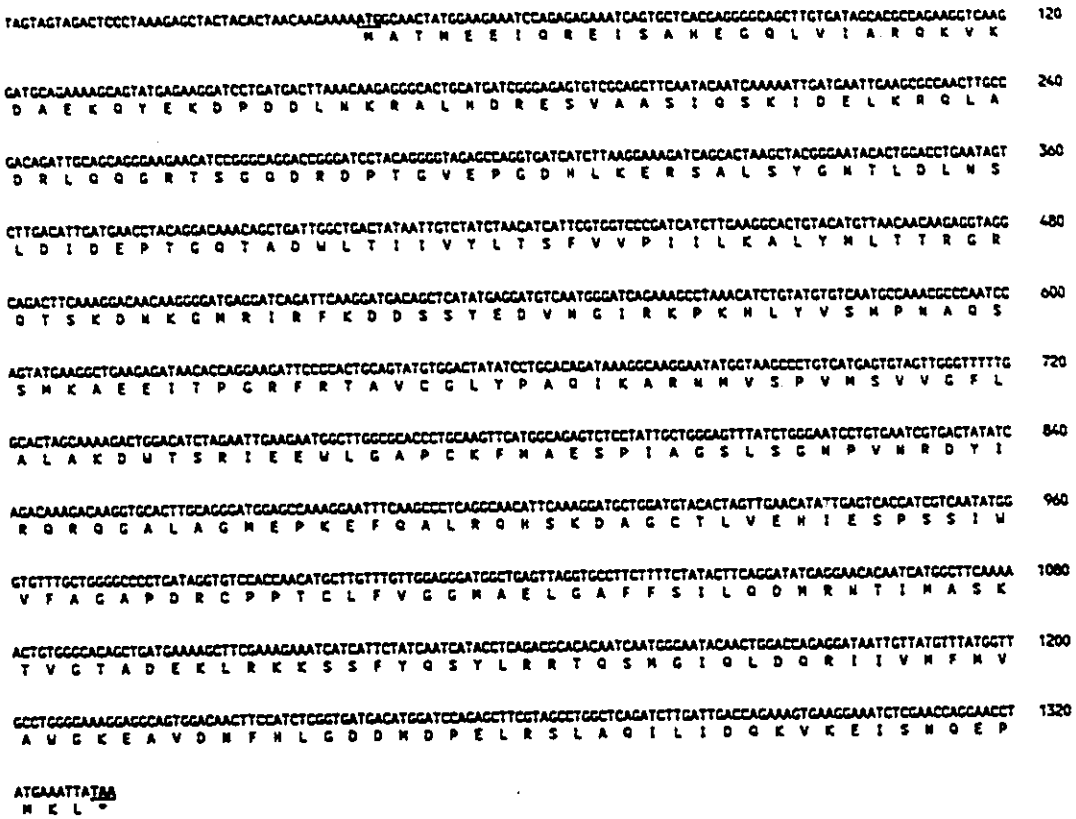


Once the sequence was determined, SS and ST oligonucleotide primers were prepared and used to prime cDNA synthesis (SS) and PCR (both SS and ST). The SS primer was complementary to the part of the S segment RNA containing the translation initiation codon and the ST primer was identical to the S genomic RNA containing the translation termination codon. A *Sac*I restriction site and a TATAAAT sequence which provides the optimal context for translation initiation in insect cells, were added to the 5' terminus of the SS oligonucleotide. A *Kpn*I site was added to the 5' terminus of the ST oligonucleotide. The SS-ST fragment was inserted into the *Sac*I-*Kpn*I sites of pUC19 and sequenced in both directions. Sequencing of this clone confirmed the sequence of the ORF of the S genomic RNA segment.

Nucleotide sequence and the deduced amino acid sequence of the S genomic RNA segment

The nucleotide sequence of the cDNA representing 1332 nucleotides of the S genomic RNA segment was determined and is presented in Figure 11. The coding region starts with the translation initiation (ATG) codon at nucleotide positions 43-45 and ends with the translation termination (TAA) codon at nucleotide positions 1330-1332. Other open reading frames were not detected in these 1332 nucleotides of the S segment. The product of 48 kD encoded by this open reading frame corresponds in size to the nucleocapsid protein detected in virions.

Figure 11. Nucleotide sequence (presented as complementary, messenger sense DNA) and the deduced amino acid sequence of the Seoul 80-39 virus S genomic RNA segment (from the 5' terminus to nucleotide position 1332). Translation initiation and translation termination codons are underlined.


TAGTAGTAGACTCCCTAAAGAGCTACTACACTAACAACAAAAATGGCAACTATGGAAGAAATCCAGAGACAAATCAGTGCTCACCAGGCCCAGCTTGTCATACCACCCCAGAAGGTCAAG 120
M A T M E E I Q R E I S A H E G Q L V I A R Q K V K
GATGCAGAAAAGCACTATGAGAAGGATCCTGATGACTTAAACAAGAGGGCACTGCATGATCGGCAGAGTGTCCCAGCTTCAATACAATCAAAAATTGATGAATTGAAGCCCCAACTTGCC 240
D A E K Q Y E K D P D D L N K R A L H D R E S V A A S I Q S K I D E L K R Q L A
GACAGATTGCAGCAGCGAAGAACATCCGGGCAGGACCCGGCATCCTACAGGGGTAGAGCCAGGTGATCATCTTAAGGAAAGATCAGCACTAAGCTACGGGAATACACTGCACCTGAATAGT 360
D R L Q Q G R T S G Q D R D P T G V E P G D H L K E R S A L S Y G H T L D L N S
CTTGACATTGATGAACCTACAGGACAAACAGCTGATTGGCTGACTATAATTGTCTATCTAACATCATTCGTCGTCCCGATCATCTTCAAGGCACTGTACATGTTAACAACAAGAGGTAGG 480
L D I D E P T G Q T A D W L T I I V Y L T S F V V P I I L K A L Y M L T T R G R
CAGACTTCAAAGGACAACAAGGGGATGAGCATCAGATTCAAGGATGACAGCTCATATGAGGATGTCAATGGGATCAGAAAGCCTAAACATCTGTATGTGTCAATGCCAAACGCCCAATCC 600
Q T S K D N K G M R I R F K D D S S Y E D V N G I R K P K H L Y V S M P N A Q S
AGTATGAAGGCTGAAGAGATAACACCAGGAAGATTCCCCACTGCAGTATGTGGACTATATCCTGCACAGATAAAGGCAAGGAATATGGTAACCCCTGTCATGAGTGTAGTTGGGTTTTTG 720
S M K A E E I T P G R F R T A V C G L Y P A Q I K A R N M V S P V M S V V G F L
GCACTAGCAAAAGACTGGACATCTAGAATTGAAGAATGGCTTGGCGCACCCTGCAAGTTCATGGCAGAGTGTCCTATTGCTGGGAGTTTATCTGGGAATCCTGTGAATCGTGACTATATC 840
A L A K D W T S R I E E W L G A P C K F M A E S P I A G S L S G N P V N R D Y I
AGACAAAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCCTCAGGCAACATTCAAAGGATGCTGGATGTACACTAGTTGAACATATTGAGTCACCATCGTCAATATGG 960
R Q R Q G A L A G M E P K E F Q A L R Q H S K D A G C T L V E H I E S P S S I W
GTGTTTGCTGGGGCCCCTCATAGGTGTCCACCAACATGCTTGTTTGTTGGAGCGATCGCTCAGTTAGGTGCCTTCTTTTCTATACTTCAGGATATCAGGAACACAATCATGGCTTCAAAA 1080
V F A G A P D R C P P T C L F V G G M A E L G A F F S I L Q D M R N T I M A S K
ACTGTGGCCACAGCTGATGAAAAGCTTCGAAAGAAATCATCATTCTATCAATCATACCTCAGACGCACACAATCAATGGCAATACAACTGCACCACAGGATAATTGTTATGTTTATGGTT 1200
T V G T A D E K L R K K S S F Y Q S Y L R R T Q S M G I Q L D Q R I I V M F M V
GCCTGGGAAACGACGCCAGTGGACAACTTCCATCTCGGTGATGACATGGATCCACAGCTTCGTAGCCTGGCTCAGATCTTGATTGACCAGAAAGTGAAGGAAATCTCGAACCAGCAACCT 1320
A W G K E A V D N F H L G D D M D P E L R S L A Q I L I D Q K V K E I S N Q E P
ATGAAATTATAA
M K L *

DISCUSSION

The objective of my initial research was to determine genome organization and coding capacity of the Seoul 80-39 virus. Once the first part of the project was finished, the characteristics of the virus genes were compared with those of other hantaviruses allowing us to better understand the evolutionary relationships within the *Hantavirus* genus and to identify conserved structures that may be crucial for structure and/or function of gene products.

Comparison of coding properties of the L, M and S genome segments of hantaviruses

Strict negative-sense coding strategy was observed in all three genomic RNA segments of all hantaviruses analyzed so far. The L genomic RNA segment encodes the L protein (Antic et al., 1991b); the M genomic RNA segment encodes viral glycoproteins (Antic et al., 1991a) as a precursor polyprotein and the S genomic RNA segment encodes the nucleocapsid (N) protein. None of the hantavirus genomic RNA segments has been shown to encode for a nonstructural protein. Relatively short, noncoding sequences are flanking the coding region of each genomic RNA segment (Table 3). Therefore, approximately 94% of the genome contains coding information.

TABLE 3

CODING PROPERTIES OF THE L, M AND S GENOME SEGMENTS OF HANTAVIRUSES

Genome Segment	Virus (strain)	Nucleotide Sequences			Number of amino acids	Predicted Mr	Gene Products	Reference (EMBL Accesion No.)
		Total	Noncoding					
			5'	3'				
L	Seoul (80 - 39)	6,530	36	38	2,151	246,660	L	Antic et al. 1991 (X56492)
	Hantaan (76 - 118)	6,533	37	40	2,151	246,500	L	Schmaljohn et al. 1991 (X55901)
	Puumala (Hällnäs)	6,550	36	46	2,156	246,000	L	Stohwasser et al. 1991 (M63194)
M	Seoul (80 - 39)	3,651	46	203	1,133	125,500	G1, G2	Antic et al. 1991 (X56493)
	Seoul (SR - 11)	3,651	46	203	1,133	125,500	G1, G2	Arikawa et al. 1990 (M34882)
	Hantaan (76 - 118)	3,616	40	168	1,135	126,300	G1, G2	Schmaljohn et al. 1987 (M14627) Yoo and Kang 1987 (Y00386)
	Puumala (Hällnäs)	3,682	40	195	1,148	126,800	G1, G2	Giebel et al. 1989 (M29979)
	Prospect Hill	3,707	49	231	1,142	125,800	G1, G2	Parrington et al. 1991 (X55129)
S	Seoul (80 - 39)	**	42	**	429	48,100	N	Antic and Kang 1991 (not registered)
	Seoul (SR - 11)	1,769	42	437	429	48,100	N	Arikawa et al. 1990 (M34881)
	Hantaan (76 - 118)	1,696	36	370	429	48,100	N	Schmaljohn et al. 1986 (M14626)
	Puumala (Hällnäs)	1,785	42	441	433	49,400	N	Stohwasser et al. 1990 (M32750)
	Prospect Hill	1,675	42	229	433	49,000	N	Parrington and Kang 1990 (X55128)

Noncoding regions correspond to the 5' and 3' end sequences of the mRNAs.

** Clones representing the 3' end of the S genomic RNA segment of Seoul 80-39 virus were not obtained .

Comparison of the Seoul 80-39 L genome segment with L genome segments of other hantaviruses and L genes of other negative-strand RNA viruses

The nucleotide sequence of the Seoul 80-39 L genomic RNA segment was compared with nucleotide sequences of L genomic RNA segments of Hantaan 76-118 (Schmaljohn, 1991) and Puumala Hällnäs B1 virus (Stohwasser et al., 1991), as well as with nucleotide sequences of L genes of other negative-strand RNA viruses using the Microgenie computer program. Similarity was found only between three hantaviral L genomic RNA segments (Fig. 12).

The 3' and the 5' termini of the Seoul virus, Hantaan virus and Puumala virus are inversely complementary for 15, 9 and 20 bases respectively. If a panhandle is formed by base pairing of the two ends, the structures would have the free energies of -39.3 kcal for Seoul virus, -23.6 kcal for Hantaan virus and -44.9 kcal for Puumala virus (Fig. 13).

The L protein of the Seoul 80-39 virus was compared with L proteins of Hantaan 76-118 virus, Puumala Hällnäs B1 virus and with the polymerase proteins of other negative-stranded RNA viruses (Microgenie computer program). Hantaviral L proteins are highly conserved (Fig. 12) and long stretches of identical amino acids can be found (Fig. 14). However, L genes of Hantaan virus and Seoul virus are more closely related to each other than to the L gene of Puumala virus. Comparison with RNA polymerases of other negative-stranded RNA viruses revealed 44% sequence similarity only with part of the Bunyamwera virus L protein (Elliott, 1989) and very little similarity (17%) with part of the PB1 protein of

Figure 12. (A) and (B): Comparison of hantavirus L segments. Comparison is expressed as percentages of identical nucleotide (nt.) and amino acid (aa.) residues. Numbers in brackets indicate percent of similarity when conservative amino acid changes (R=K; S=T; D=E; Q=N; Y=F; V=L=I=M; A=G; A=V) were counted as identical.

A.

L Segment Similarity	Puumala (Hällnäs)		Hantaan (76 - 118)		Seoul (80 - 39)	
	nt.	aa.	nt.	aa.	nt.	aa.
Seoul (80 - 39)	64	69	74	85	100	100
Hantaan (76 - 118)	64	70	100	100		
Puumala (Hällnäs)	100	100				

B.

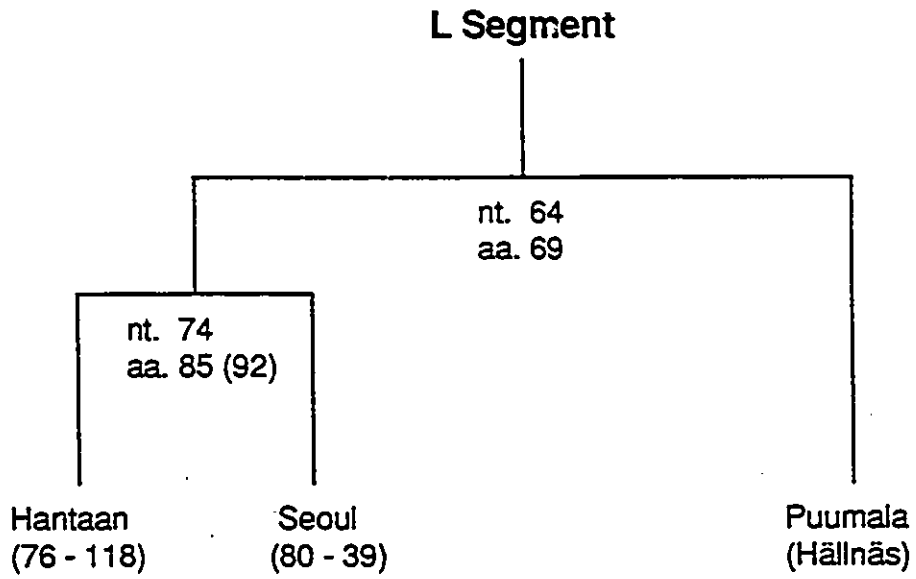
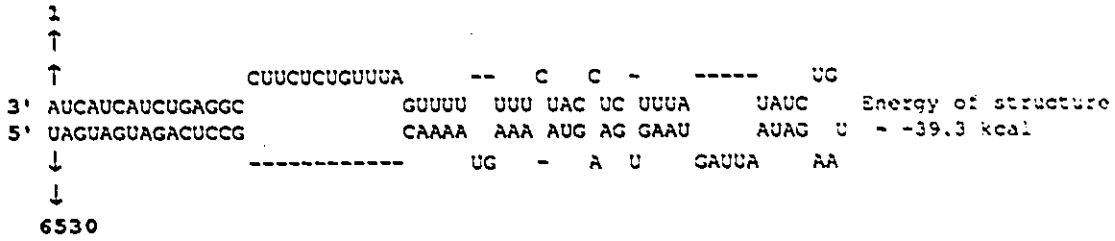
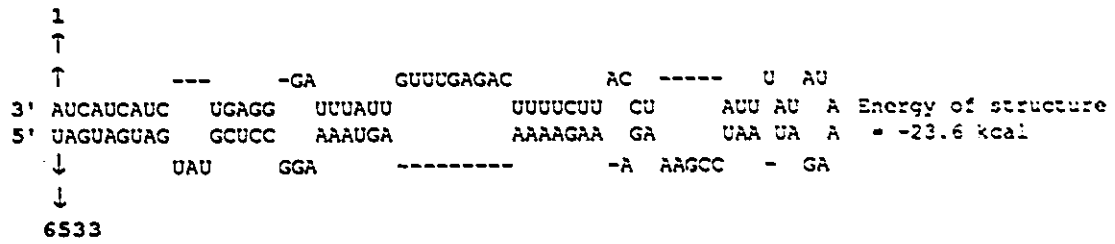


Figure 13. Predicted secondary structure of the complementary 3' and 5' termini of Seoul 80-39 virus; Hantaan 76-118 virus; Hällnäs B1 virus L genomic RNA segments. The base pairing between the 3' and 5' terminal 50 nucleotides is shown. The free energy values were calculated using the RNA folding program of the PC/Gene computer program (version 5.11).

SEOUL



HANTAAN



PUUMALA

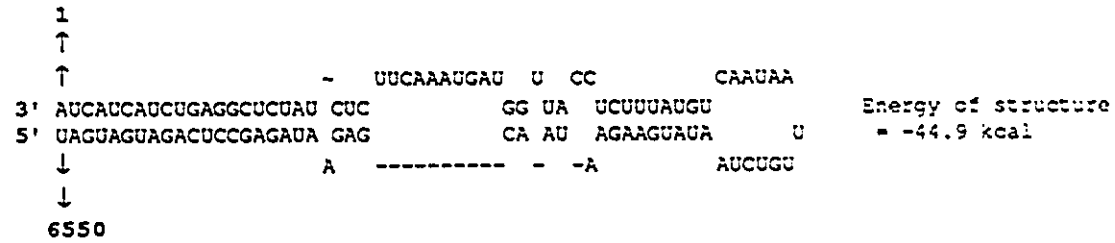
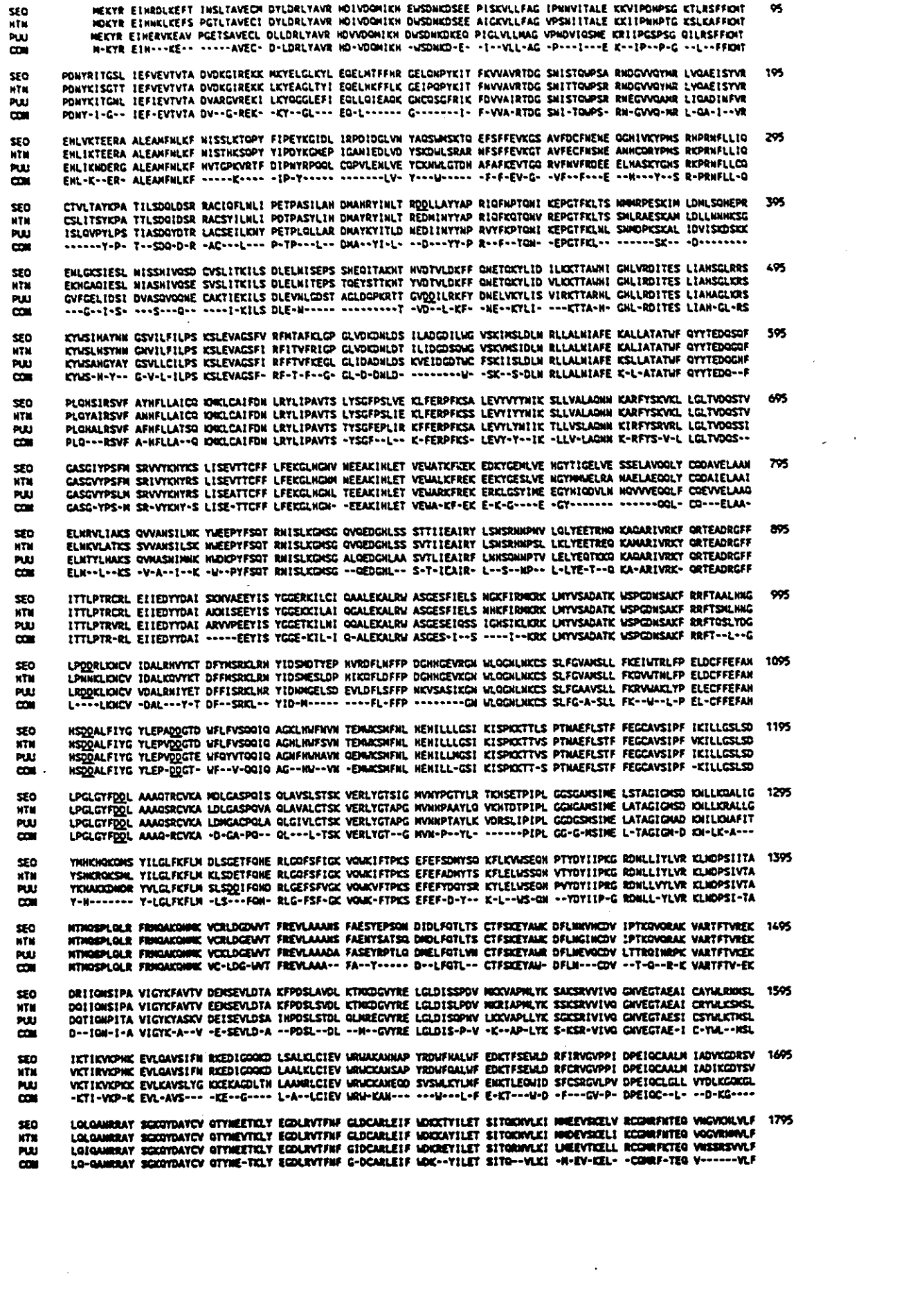


Figure 14. Alignment of the deduced amino acid sequences of Seoul 80-39 virus (SEO), Hantaan 76-118 virus (HTN) and Puumala Hällnäs B1 virus (PUU) L segments was performed by the Pileup program of the University of Wisconsin GCG Sequence Analysis Software. The consensus sequence (CON) shows amino acids which are conserved among all three viruses. D-D sequences are underlined.



```
SEQ     KTDSGFEVGK PNIPCIVYKN CALRTGLRTN QAINNKFMIT IKDDGLRAIA QYDEDSPRFL LAHAFNTIRD VRYQAVDAVS NVWFTNKGIK LYLNPIISSG  1895
NTN     KTESGFEVGK PNIPCIVYKN CVLRTSLRTT QAINNKFMIT IKDDGLRAIA QHDEDSPRFL LAHAFNTIRD IRYQAVDAVS NVWFINKGVK LYLNPIISSG
PUU     KTESGFEVGK PNVPCIVYRN CTLRTGLRVR QPTNKAFSIT IQANGFRAMA QLDEENPRFL LAHAYHNLKD VRYQALQAVG NVWFKNTQNK LFINPIISAG
CON     KT-SGFEVGK PN-PCIVY-N C-LRT-LR-- Q--N--F-IT I---G-RA-A Q-DE--PRFL LAHA-N---D -RYQA--AV- NVWF-----K L--NPIIS-G

SEQ     LLEYFMKNIP AAIPPAAYSL INNRAKISVD LFMFNDLLRL INPGNTLDLS GLEITGEGYS TVNSLSSRLW SEEMSLVDDE EEMDD..... EFTIDLQDVD  1900
NTN     LLENFMKNLP AAIPPAAYSL INNRAKISVD LFMFNDLLKL INPRNTLDLS GLETTGDEFS TVSSMSSRLW SEEKSLVDDD EELDD..... EFTIDLQDVD
PUU     LLENFMKGLP AAIPPAAYSL INNKAKISVD LFMFNELLAL INPGNVLNLD GIEETSEGYT TVSTISSTQW SEEVSLTNDD SDDDGDASGL DYTIDLDDID  1905
CON     LLE-FMK--P AAIPPAAYSL INN-AKISVD LFMFN-LL-L INP-N-L-L- G-E-T----- TV---SS--W SEE-SL--D- ---D------ --TIDL-D-D

SEQ     FENIDIEADV EHFLQDESAY TGDLLIMSEE TEVKDNRGII KLLEPVKLIK SWVSRGLSIE KVYNPVNIIL NTRYISKNFN FSGKQVSLLD PYDLTELESI  2090
NTN     FENIDIEADI EHFLQDESSY TGDLLISTEE TESKDNRGIV KILEPVRLIK SWVSRGLSIE KVYSPVNIIL NSRYISKTFN LSTKQVSLLD PYDLTELESI
PUU     FETIDLKEDI EHFLQDESAY TGDLLIQTEE TEIRKLRGNI KILEPVKLIK SWVSKGLSID KIYNPVNIIL NTRYNSKHYN FNAKQLSLND PYDLTEFESI  2095
CON     FE-ID---D- EHFLQDES-Y TGDLLI--EE TE--K-RG-- K-LEPV-LIK SWVS-GLSI- K-Y-PVNIIL N-RY-SK--N ---KQ-SL-D PYDLTE-ESI

SEQ     VKGWGESVVD QFDSLDLEAQ NLVQKGGIVP EDVIPQSLFS FRNTMVLLRR LFGQDSVSTF Y        2151
NTN     VRGWGECVID QFESLDREAQ NMVVNKGICP EDVIPQSLFS FRNTMVLLRR LFPQDSISSF Y
PUU     VKGWGECVKD RFIELDQEAQ RKVTEERVLP EDVLPQSLFS FRHADILLKR LFPRDSASSF Y        2156
CON     V-GWGE-V-D -F--LD-EAQ --V------P EDV-PQSLFS FRN---LL-R LF--DS-S-F Y
```

Figure 15. Comparison of homologous parts of L proteins of Seoul virus (SEO), Bunyamwera virus (BUN) and influenza virus PB1 protein (PB1). Numbers indicate amino acid positions of proteins. The SDD motif is double underlined.

```
SED 1179 RGNULQGNLNKCSSLFGV AMSLLFKEIVTRLFPELD   CFFEFANNSDD 1225
BUN 1118 R NULQGNFNYISSTVNSCAM LVYKDILKECMKLLDGDCLINSMVNSDD 1165
PBI 405 P GNNNNGNFNNLSTVLGVSIL NLGQKR        YTKTTYNVDGLQSSDD 446
```

influenza virus, as shown in Fig. 15. These relatively conserved regions contain the SDD sequence motif surrounded by predominantly hydrophobic amino acids. Similar sequence motifs (GDD, LDD and MDD) have been found in RNA-polymerase molecules of bacteriophages, retroviruses and other RNA viruses (Argos, 1988) suggesting their evolutionary conservation and functional importance. One MDD, one SDD and six other DD motifs were observed in the L protein of Seoul 80-39 virus. The L protein of Hantaan 76-118 virus has one LDD, one SDD and four other DD motifs and the L protein of Puumala Hällnäs B1 virus has one LDD, one MDD, two SDD and four other DD motifs. The position of the SDD motif is conserved in all three L proteins (Fig. 14). Homology to polymerase proteins of LCMV, measles virus, Newcastle disease virus, parainfluenza virus, respiratory syncytial virus, Sendai virus, rabies and vesicular stomatitis virus (VSV) was not detected.

Comparison of the Seoul 80-39 M genome segment with M genome segments of other hantaviruses

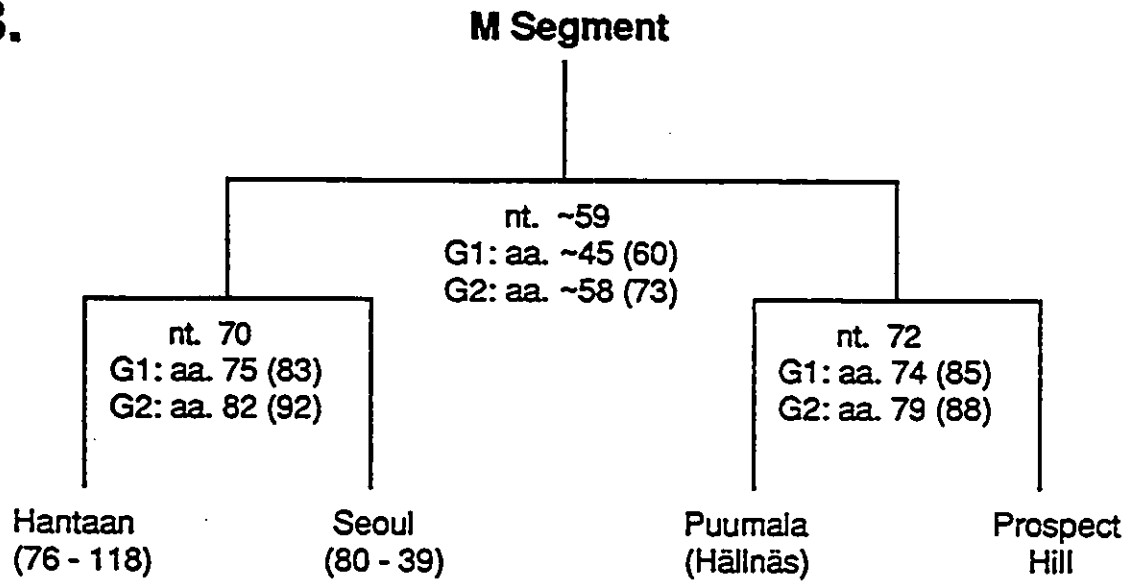
The nucleotide sequence of the Seoul 80-39 M genomic RNA segment was compared with nucleotide sequences of M genomic RNA segments of SR-11 virus (Arikawa et al., 1990), Hantaan 76-118 virus (Schmaljohn et al., 1987a; Yoo and Kang, 1987b), Puumala Hällnäs B1 virus (Giebel et al., 1989) and Prospect Hill virus (Parrington et al., 1991) (Fig. 16). Extremely high conservation of the primary structure was observed between the two viruses of the same serotype i.e.

Figure 16. (A) and (B): Comparison of hantavirus M segments. Comparison is expressed as percentages of identical nucleotide (nt.) and amino acid (aa.) residues. Numbers in brackets indicate percent of similarity when conservative amino acid changes (R=K; S=T; D=E; Q=N; Y=F; V=L=I=M; A=G; A=V) were counted as identical.

A.

M Segment Similarity	Prospect Hill		Puumala (Hällnäs)		Hantaan (76 - 118)		Seoul (SR - 11)		Seoul (80 - 39)	
	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.
Seoul (80 - 39)	62	54	56	53	70	77	96	99	100	100
Seoul (SR - 11)	62	54	58	54	72	77	100	100		
Hantaan (76 - 118)	63	54	59	54	100	100				
Puumala (Hällnäs)	72	76	100	100						
Prospect Hill	100	100								

B.



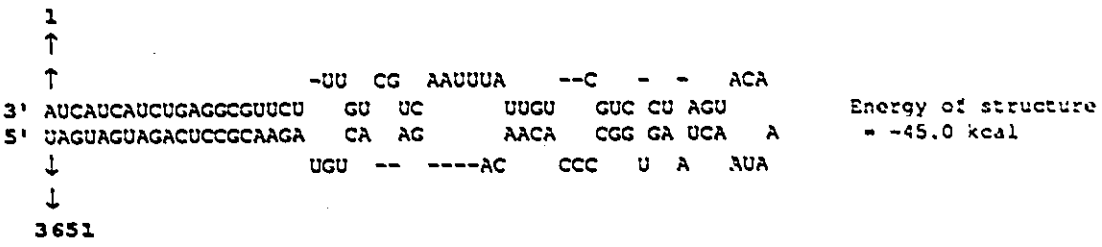
Seoul 80-39 virus and SR-11 virus (Fig. 16A). It is interesting that 81% of these base changes are uridine (U) to cytidine (C) or adenine (A) to guanine (G) transitions and they did not result in a significant change of the protein structure. Representative viruses of different serotypes have diverged more (Fig. 16), but it is evident that Hantaan and Seoul viruses are more closely related to one another than to other hantaviruses and that Puumala and Prospect Hill viruses are more similar to each other than to Seoul or Hantaan viruses (Fig. 16).

The 3' and the 5' termini of M genomic RNA segments of all five viruses are inversely complementary. While the terminal sequences of the M genomic RNA segments of Seoul 80-39 and SR-11 viruses have inversed complementarity for 20 bases, Hantaan 76-118, Hällnäs B1 and Prospect Hill have inversed complementarity for 18, 21 and 16 bases, respectively. The predicted secondary structures resulting from base pairing would have free energies of -45 kcal for Seoul 80-39 virus, -41.8 kcal for Hantaan 76-118 virus, -65.9 kcal for Hällnäs B1 virus and -47.4 kcal for Prospect Hill virus (Fig. 17). The free energy values were calculated for the 3' and 5' terminal 50 nucleotides.

M segments of all hantaviruses have one long open reading frame which encodes envelope glycoproteins G1 and G2. Amino-terminal sequence analysis of the G1 proteins of Hantaan and SR-11 viruses (Schmaljohn et al., 1987a; Arikawa et al., 1990) showed that the terminal residue of both proteins is leucine. Sequences preceding the leucine residues of Hantaan and SR-11 viruses are not similar, but they both have characteristics of typical signal sequences (vonHeijne,

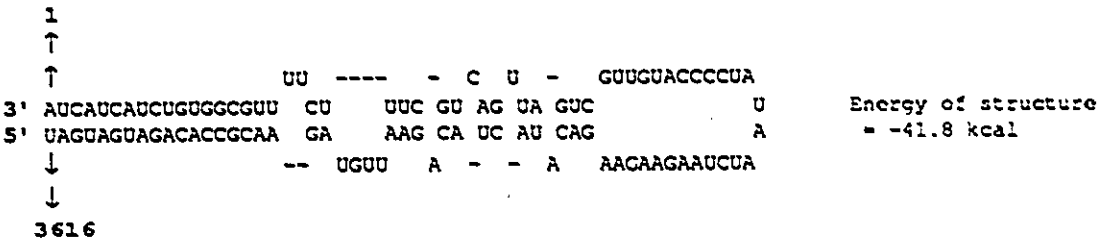
Figure 17. Predicted secondary structure of the complementary 3' and 5' termini of Seoul 80-39 virus; Hantaan 76-118 virus; Hällnäs B1 virus and Prospect Hill M genomic RNA segments. The base pairing between the 3' and 5' terminal 50 nucleotides is shown. The free energy values were calculated using the RNA folding program of the PC/Gene computer program (version 5.11).

SEOUL



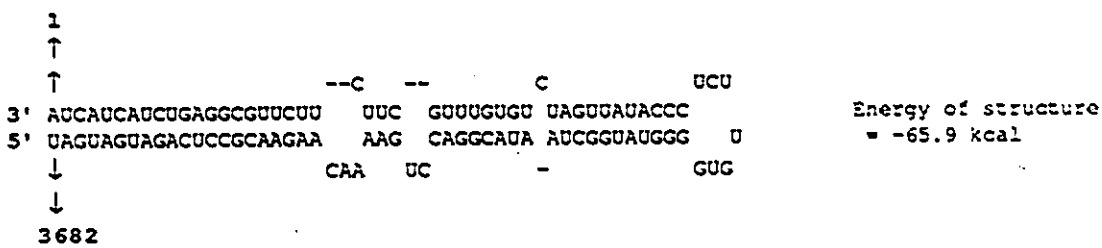
Energy of structure = -45.0 kcal

HANTAAN



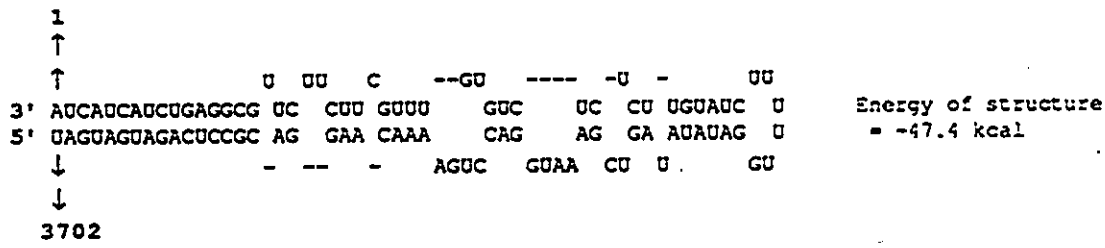
Energy of structure = -41.8 kcal

PUUMALA



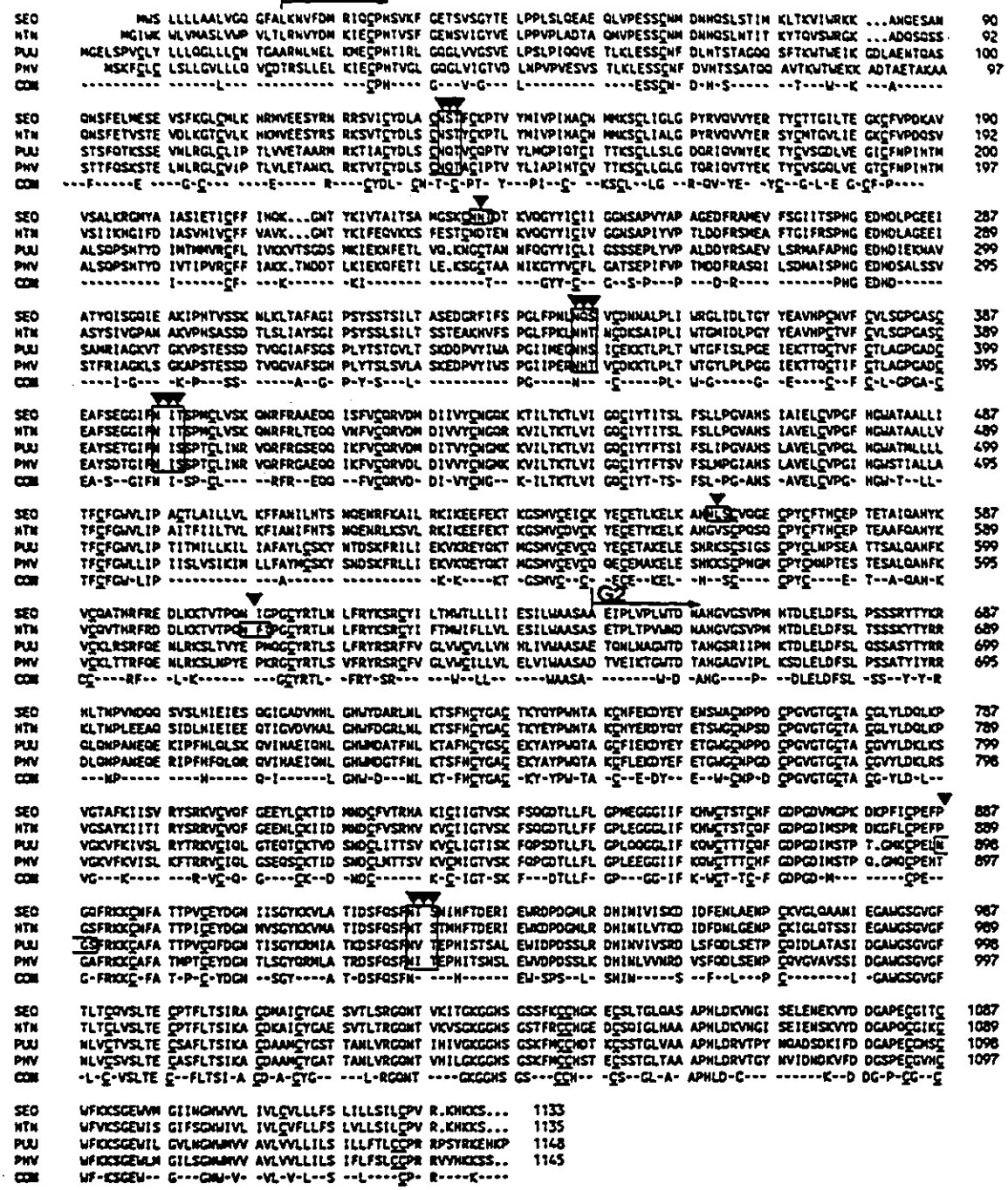
Energy of structure = -65.9 kcal

PROSPECT HILL



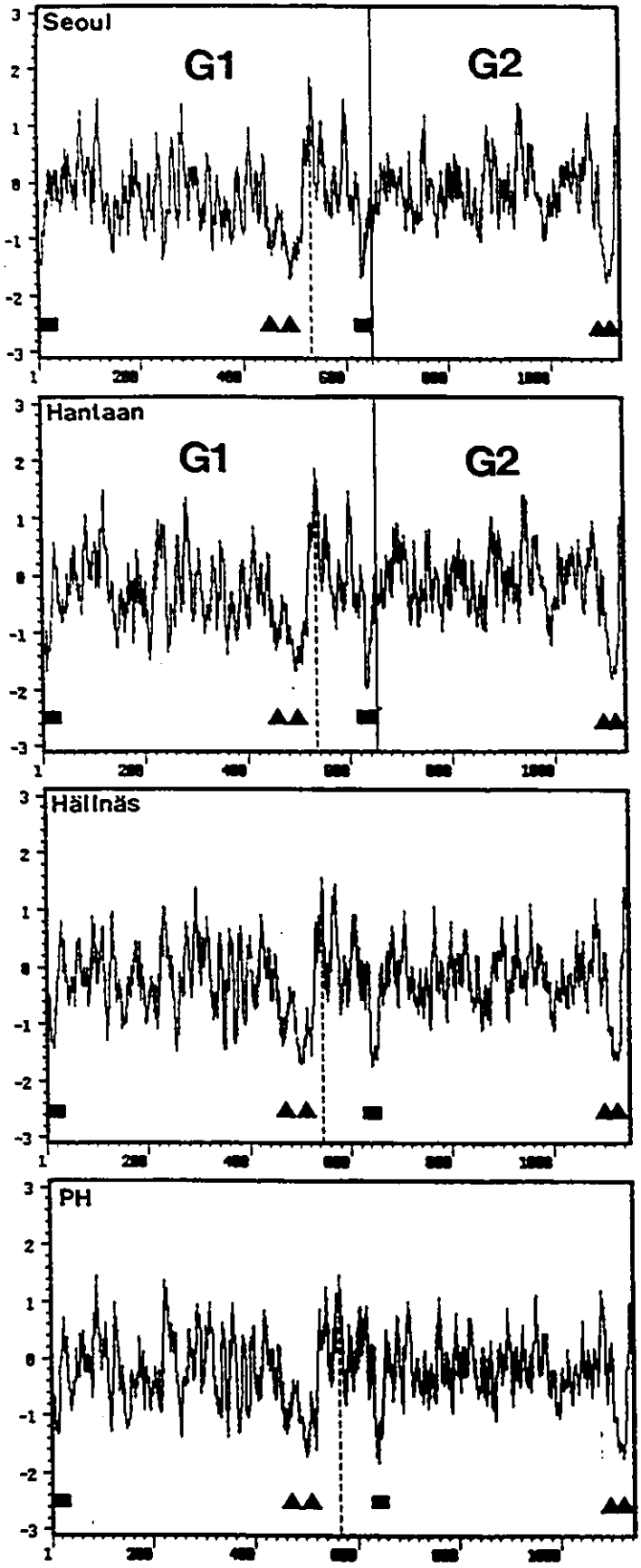
Energy of structure = -47.4 kcal

Figure 18. Alignment of the deduced amino acid sequences of Seoul 80-39 virus (SEO), Hantaan 76-118 virus (HTN), Puumala Hällnäs B1 virus (PUU) and Prospect Hill virus (PHV) M segments was performed by the Pileup program of the University of Wisconsin GCG Sequence Analysis Software. The consensus sequence (CON) shows amino acids which are conserved among all four viruses. The amino termini of the G1 and G2 proteins of Hantaan and SR-11 viruses were determined (Schmaljohn et al., 1987; Arikawa et al., 1990) and are indicated above the sequence. Cysteine residues are underlined. Potential N-linked glycosylation sites are boxed and marked: (▾▾) represents sites conserved in all four viruses and (▾) represents unique sites.


G1
G2

1983). The G2 amino-terminal residues of Hantaan and SR-11 viruses were found to be serine and alanine, respectively (Schmaljohn et al., 1987a; Arikawa et al., 1990). Although amino-terminal sequence data have not been obtained for the G2 protein of Puumala and Prospect Hill viruses, the five amino acids preceding the G2 of Hantaan and SR-11 viruses are conserved in all four viruses. This suggests that the G2 N-terminal residues of the Puumala and Prospect Hill viruses are glutamic and aspartic acids, respectively (Fig. 18). The carboxy-terminal amino acid of the Hantaan virus G2 protein is encoded by the last codon of the M gene (Schmaljohn et al., 1987a). However, the carboxy-terminal amino acid of the Hantaan virus G1 protein has not been determined. Hantaan and Seoul viruses have 641 and 643 codons, respectively, between the N-terminus of the G1 and the N-terminus of the G2. If the G1 protein contains all the amino acids, the molecular weight of the native polypeptide would approximately be 70 K. However, based on the polyacrylamide gel electrophoresis, the glycosylated G1 protein of Hantaan virus is estimated to be 68 kD. Specific immunoprecipitation of the G1 was observed with antisera to peptide 588-614 of the G1, suggesting that the C-terminus of the G1 extends at least to amino acid residues 588-614 (Schmaljohn et al., 1987a). Therefore, a small intergenic region (~6 K) may exist between the carboxy terminus of G1 and the amino terminus of G2. This putative intergenic region contains the third main hydrophobic domain (amino acids 627-657) preceded by a highly hydrophilic region (Fig. 8). A similar 6 K membrane spanning intergenic region has been demonstrated in alphaviruses (Garoff et al., 1980; Strauss and

Figure 19. Hydrophilicity profiles of the Seoul 80-39 virus, Hantaan 76-118 virus, Puumala Hällnäs B1 virus and Prospect Hill (PH) virus M segment encoded proteins generated using the ANTIGEN program of the PC/Gene computer program (version 5.11) with an interval of 9 amino acids. Y axis represents the hydrophillic index of the protein: hydrophillic sequences have positive values and hydrophobic sequences have negative values. X axis represents the amino acid numbers of the protein. Four main hydrophobic regions are marked: (▬) represents possible signal sequences and (▲▲) represents possible transmembrane domains.


Seoul
G1
G2
Hantaan
G1
G2
Hällnäs
PH

Strauss, 1986). It is located between the two envelope proteins, E1 and E2, and was shown to serve as a signal sequence for the E2.

A comparison of the G1 and G2 proteins of all four viruses revealed overall amino acid sequence homology of 43% (58% if conservative changes are counted as identical) for the G1 and 55% (71% with conservative substitutions) for the G2. Despite the relatively low amino acid sequence similarity, hydrophilicity profiles of the M segment encoded proteins are nearly superimposable (Fig. 19). The positions of almost all cysteine residues are conserved among all of the viruses as well (Fig. 18), suggesting conformational similarities of the envelope glycoproteins of all hantaviruses. The positions of potential asparagine-linked glycosylation sites are also quite conserved among the viruses. Four glycosylation sites (three in G1 and one in G2) have been conserved in representative viruses of all four serotypes (Fig. 18).

Comparison of the Seoul 80-39 S genome segment with S genome segments of other hantaviruses

The sequence of 1332 nucleotides of the Seoul 80-39 S genomic RNA segment was determined, the amino acid sequence of the encoded protein (nucleocapsid protein) was deduced and both were compared with the sequences of other hantaviruses.

The sequence of the S genomic RNA segment of the Seoul 80-39 virus was primarily compared with the S segment sequence of the SR-11 virus (Arikawa et al.,

1990). Comparison revealed that 1332 nucleotides of S segments of these two strains of the Seoul serotype differ only by 22 nucleotides. Twenty of these changes are uridine (U) to cytidine (C) or adenine (A) to guanine (G) transitions which is similar to changes observed for the M genomic RNA segments of these two viruses. The changes at the nucleotide sequence level resulted in a single amino acid change at the position 251 (Leu/Pro). The S genomic RNA segment of the Seoul 80-39 virus was compared with S segments of other hantaviruses and data are summarized in Figure 20. As was observed for the L and M segments, the S segments of Hantaan and Seoul viruses are more closely related to one another than they are to those of Puumala and Prospect Hill viruses and vice versa.

Although the 5' end sequence of the S genomic RNA segment of Seoul 80-39 virus was not determined in this study, high conservation of the primary structure between the two Seoul isolates suggests that the 3' and 5' terminal sequences of the S genomic RNA segments are conserved. Therefore, the ends of the S genomic RNA of the Seoul 80-39 virus are expected to be inversely complementary, as was shown for the other two genomic RNA segments (Fig. 21A). Secondary structure of the entire S segment genomic RNA molecule of the SR-11 virus (Fig. 21B) was determined by RNAFOLD program of the University of Wisconsin GCG Sequence Analysis Software. The 3' and 5' termini form a base paired stem in the folded RNA suggesting a functional importance of terminal sequence conservation and complementarity.

Alignment of the amino acid sequences of the nucleocapsid proteins of these

Figure 20. (A) and (B): Comparison of hantavirus S segments. Comparison is expressed as percentages of identical nucleotide (nt.) and amino acid (aa.) residues. Numbers in brackets indicate percent of similarity when conservative amino acid changes (R=K; S=T; D=E; Q=N; Y=F; V=L=I=M; A=G; A=V) were counted as identical.

A.

S Segment Similarity	Prospect Hill		Puumala (Hällnäs)		Hantaan (76 - 118)		Seoul (SR - 11)		Seoul (80 - 39)	
	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.	nt.	aa.
Seoul (80 - 39)	60	63	60	62	72	82	98	99.8	100	100
Seoul (SR - 11)	60	63	60	62	72	82	100	100		
Hantaan (76 - 118)	62	62	60	63	100	100				
Puumala (Hällnäs)	69	80	100	100						
Prospect Hill	100	100								

B.

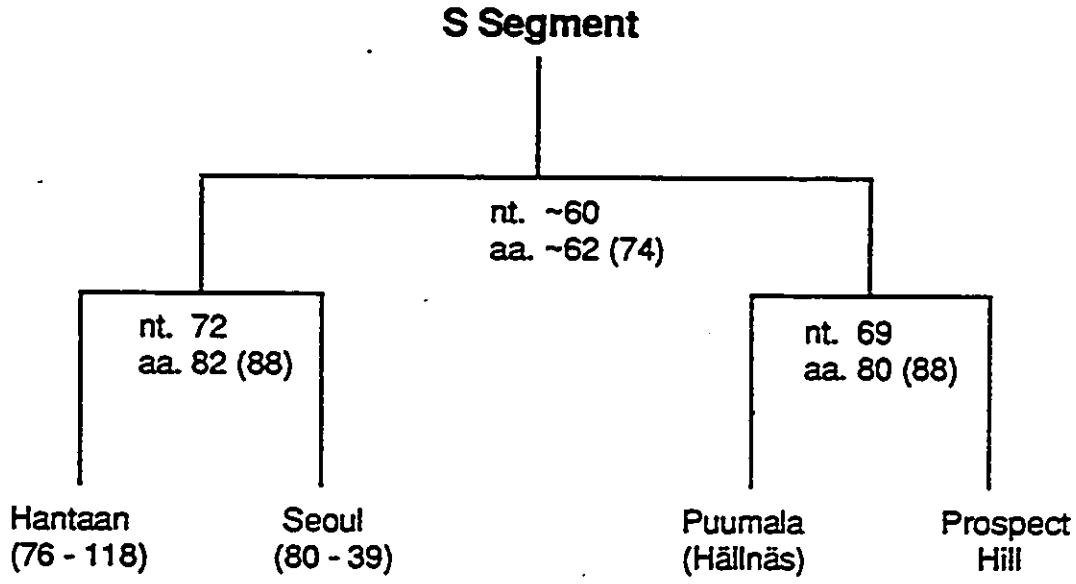
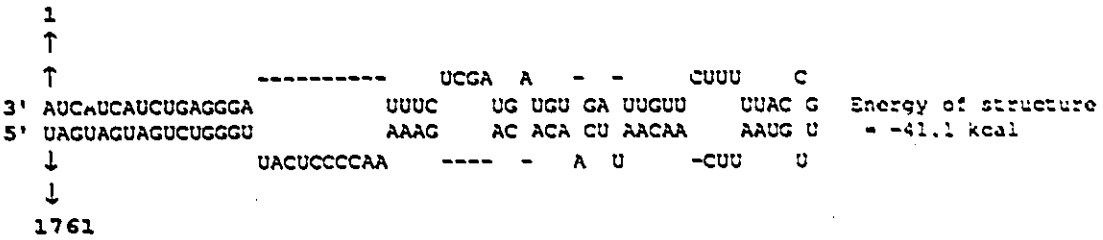


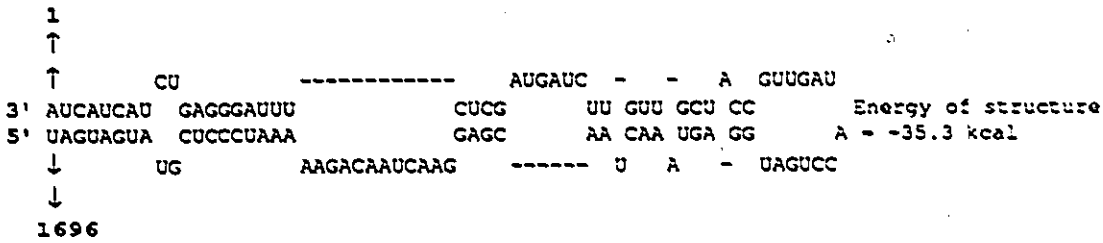
Figure 21. (A): Predicted secondary structure of the complementary 3' and 5' termini of Seoul SR-11 virus (assumed to be identical to Seoul 80-39 virus); Hantaan 76-118 virus; Hällnäs B1 virus and Prospect Hill S genomic RNA segments. The base pairing between the 3' and 5' terminal 50 nucleotides is shown. The free energy values were calculated using the RNA folding program of the PC/Gene computer program (version 5.11). (B): Secondary structure of the S segment genomic RNA molecule of the SR-11 virus as determined by the RNAFOLD program of the University of Wisconsin GCG Sequence Analysis Software.

A.

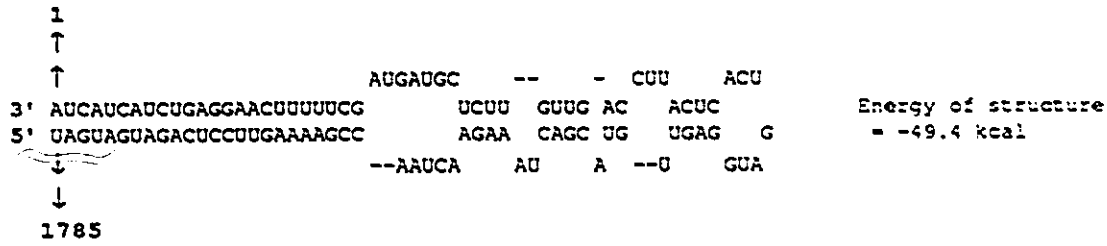
SEOUL



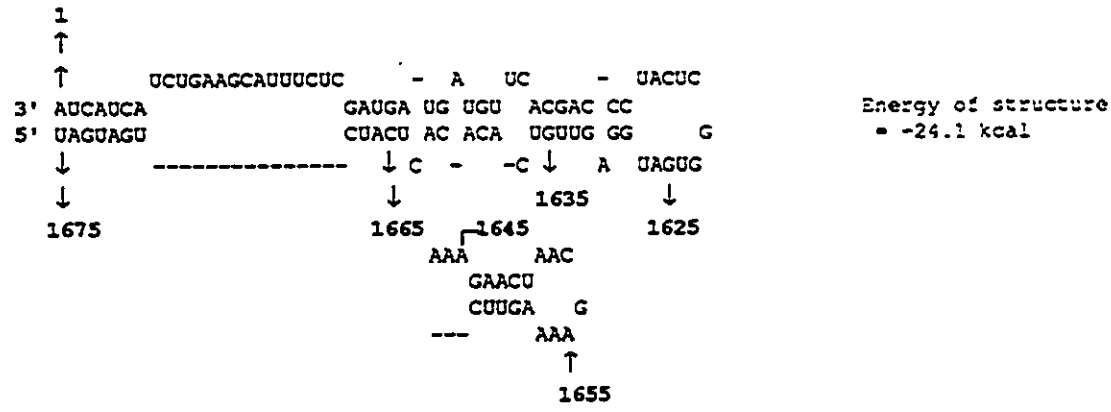
HANTAAN



PUUMALA



PROSPECT HILL



B.

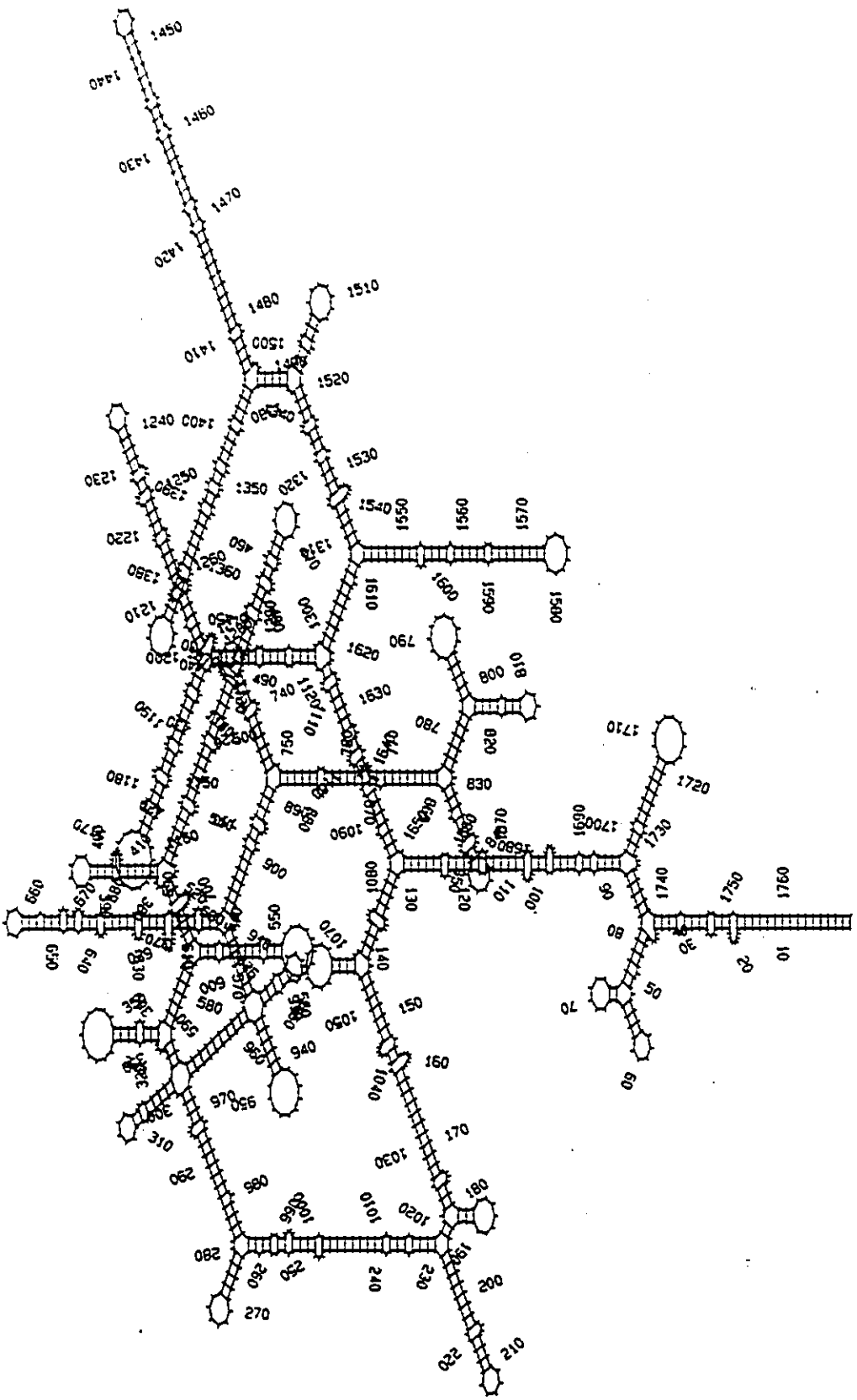
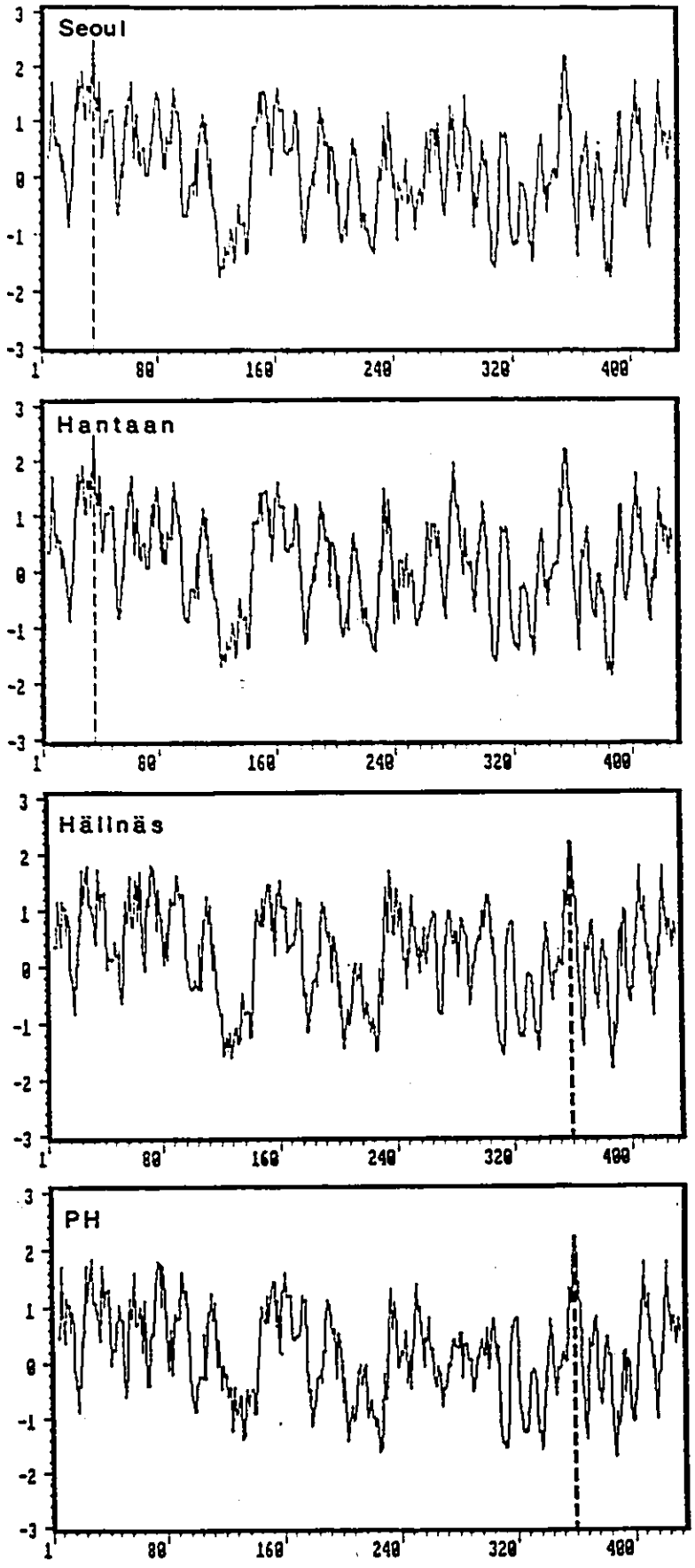


Figure 22. Alignment of the deduced amino acid sequences of Seoul 80-39 virus (SEO), Hantaan 76-118 virus (HTN), Puumala Hällnäs B1 virus (PUU) and Prospect Hill virus (PHV) S segments was performed by the Pileup program of the University of Wisconsin GCG Sequence Analysis Software. The consensus sequence (CON) shows amino acids which are conserved among all five viruses.

```
SEQ     METMEEIQRE ISANEGQLVI ARQKVKDAEK GYEKDPDQLN KRALMDRESV AASIQSKIDE LKRQLADRLQ QGRTSGQDRD PTGVEPGDHL KERSALSYGN  100
NTN     METMEELQRE INANEGQLVI ARQKVRDAEK GYEKDPDELN KRTLTDREGV AVSIQAKIDE LKRQLADRIA TGKNLGKEQD PTGVEPGDHL KERSHLSYGN
PUU     MSDLTDIQEE ITRMEQQLVV ARQKLKDAER AVEVDPDDVN KSTLQARQQT VSALEDKLAD YKRRMADAVS RKKMDTKPTD PTGIEPDDHL KERSSLRYGN
PHV     MSQLRETQEE ITRMEQQLVI ARQKLKEAER TVEVDPDDVN KSTLQSRRSA VSTLEDKLAE FKRQLADVIS RQKMDEKPVD PTGIELDDHL KERSSLQYGN
CON     M------Q-E I--ME-QLV- ARQK---AE- --E-DPD--N K--L--R--- ------K--- -KR--AD--- ---------D PTG-E--DHL KERS-L-YGN

SEQ     TLDLNSLDID EPTGQTADVL TIIVYLTSFV VPIILKALYM LTTRGRQTSK DNKGMRIRFK DQSSYEDVNG IRKPKHLYVS MPNAQSSMKA EEITPGRFRT  200
NTN     VLDLNHLDID EPTGQTADVL SIIVYLTSFV VPILLKALYM LTTRGRQTTK DNKGTRIRFK DQSSFEDVNG IRKPKHLYVS LPNAQSSMKA EEITPGRYRT
PUU     VLDVNAIDIE EPSGQTADWY TIGVYVIGFT IPIILKALYM LSTRGRQTVK ENKGTRIRFK DGTSFEDING IRRPKHLYVS MPTAQSTMKA EELTPGRFRT
PHV     VLDVNSIDIE EPSGQTADVL KIGSYIIEFA LPIILKALNM LSTRGRQTVK ENKGTRIRFK DQSSYEDVNG IRRPKHLYVS MPTAQSTMKA EELTPGRFRT
CON     -LD-N--DI- EP-GQTADW- -I--Y---F- -PI-LKAL-M L-TRGRQT-K -NKG-RIRFK DQ-S-ED-NG IR-PKHLYVS -P-AQS-MKA EE-TPGR-RT

SEQ     AVCGLYPAQI KARMNVSPVM SVVGFLALAC DWTSRIEEWL GAPCKFNAES PIAGSLSGNT V....NRDYI RQRGALAGM EPKEFQALRQ HSKDAGCTLV  296
NTN     AVCGLYPAQI KARGNISPVM SVIGFLALAC DWSDRIEQWL IEPCKLLPQT AAVSLLGGPA T....NRDYL RQRGVALGMM ETKESKAIRQ HAEAAGCSMI
PUU     IVCGLFPTQI GVRNIMSPVM GVIGFSFFVC DWPEKIREFM EKECPFIKPE VKPGTPAQEV EFLKRNRVYF NTRQDVLDKN KVADIDKLID YAASGDPTSP  300
PHV     IVCGLFPAQI NARNIISPVM GVIGFAFFVC DWADKVKAFL DQKCPFLKAE PRPGQPAGEA EFLSSIRAYL NNRGAVLDET HLPQIDALVE LAASGDPTLP
CON     -VCGL-P-QI --R---SPVM -V-GF----K DW-------- ---C------ ---------- --------Y- --RG--L--- ---------- ----------

SEQ     EMIESPSSIW VFAGAPDRCP PTCLFVGGNA ELGAFFSILQ DMRNTIMASK TVGTADEKLR KCSSFYQSYL RRTQSMGIQL DQRIIVNFMV AWQKEAVDNF  396
NTN     EDIESPSSIW VFAGAPDRCP PTCLFIAGIA ELGAFFSILQ DMRNTIMASK TVGTSEEKLR KCSSFYQSYL RRTQSMGIQL GQRIIVLFNV AWQKEAVDNF
PUU     DDIESPNAPW VFACAPDRCP PTCIYVAGNA ELGAFFSILQ DMRNTIMASK TVGTAEEKLK KCSSFYQSYL RRTQSMGIQL DQRIILLTNL EWGREMVDNF  400
PHV     DSLENPHAAW VFACAPDRCP PTCIYIAGNA ELGAFFAILQ DMRNTIMASK TVGTAEEKLK KCSAFYQSYL RRTQSMGIQL DQRIILMYMI EWGNEVVNNF
CON     -----P---W VFA-APDRCP PTC----G-A ELGAFF-ILQ DMRNTIMASK TVGT--EKL- KCS-FYQSYL RRTQSMGIQL -QRII---M- -WG-E-V--F

SEQ     MLGDDMDPEL RSLAQILIDQ KVKEISWQEP MKL  429
NTN     MLGDDMDPEL RTLAQSLIDV KVKEISWQEP LKL
PUU     MLGDDMDPEL RGLAQSLIDQ KVKEISWQEP LKI  433
PHV     MLGDDMDPEL RQLAQALIDQ KVKEISWQEP LKI
CON     MLGDDMDPEL R-LAQ-LID- KVKEISWQEP -K-
```

Figure 23. Hydrophilicity profiles of the Seoul 80-39 virus, Hantaan 76-118 virus, Puumala Hällnäs B1 virus and Prospect Hill (PH) virus S segment encoded proteins generated using the ANTIGEN program of the PC/Gene computer program (version 5.11) with an interval of 6 amino acids. The Y axis represents the hydrophilic index of the protein: hydrophilic sequences have positive values and hydrophobic sequences have negative values. The X axis represents the amino acid numbers of the protein.


Seoul
Hantaan
Hällnäs
PH
3
2
1
0
-1
-2
-3
1
80
160
240
320
400

viruses (Fig. 22) reveals highly conserved regions, particularly at the carboxy terminus, as well as regions with very little similarity. The conserved domains may be important for interaction of nucleocapsid proteins with the viral RNA or with other viral proteins. Positions of acidic and basic amino acids are also conserved among all four N proteins and therefore hydrophilicity profiles (Fig. 23) are very similar, suggesting structural similarities of these proteins. However, the points of highest hydrophilicity which are nearly in or adjacent to an antigenic site, predicted by the program (Hopp and Woods, 1981), are located at the N termini of N proteins of Seoul and Hantaan viruses (amino acid positions 33-38) and at the C termini of N proteins of Hällnäs B1 and Prospect Hill viruses (amino acid positions 357-363). Six out of seven amino acids, representing the highest hydrophilic peak of Puumala and Prospect Hill viruses, are conserved in all the viruses, the only difference being a conservative substitution. Additionally, amino acids adjacent to this epitope (from the position 320 to 380) have been highly conserved as well. Therefore, this region may represent a cross-reacting epitope. Indeed, the sera of nephropathia epidemica patients were able to cross-react with Hantaan nucleocapsid protein (Zöller et al., 1989). However, the highest hydrophilic peak of Hantaan and Seoul viruses is not in the most conserved region of the N protein. This may explain the observation that the sera from HFRS patients were not able to cross-react with Puumala nucleocapsid protein (Zöller et al., 1989). Although these two regions may represent epitopes in nucleocapsid proteins of the viruses, it is unlikely that they are the only ones. Mapping of

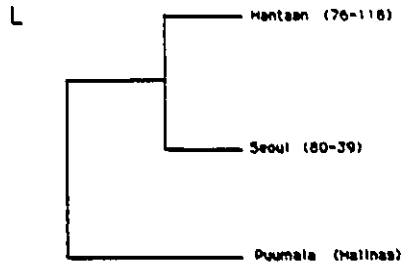
antigenic domains would provide the answers to these questions.

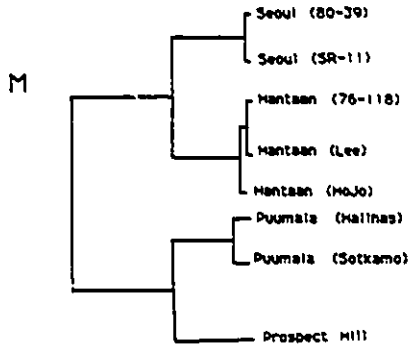
Evolutionary relationships between hantaviruses

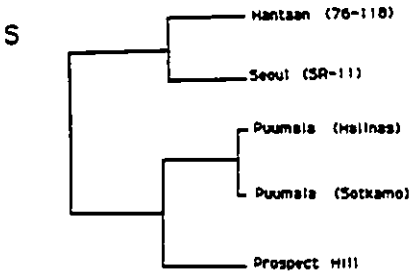
Definition of the serological relationships among hantaviruses helped to elucidate the extent of variation within the genus. However, molecular studies were necessary to determine the evolutionary relationship of different hantavirus serotypes and the basis for differences in their pathogenicity.

Currently, nucleotide sequence information has been reported for some or all of the genome segments of several viruses of the Hantaan serotype (76-118, HoJo, Lee) (Schmaljohn et al., 1986,b; 1987,a; Yoo and Kang, 1987,b; Schmaljohn et al., 1988), of several viruses of the Seoul serotype (80-39, SR-11, R22) (Antic et al., 1991a and b; Arikawa et al., 1990; Xu et al., 1991), of two viruses of the Puumala serotype (Hällnäs B1 and Sotkamo) (Giebel et al., 1989; Stohwasser et al., 1989 and 1991; Vapalahti et al., 1992) and of the Prospect Hill virus (Parrington and Kang, 1990; Parrington et al., 1991). Comparison of nucleotide and amino acid sequences of viruses of the same serotype revealed extremely high conservation (95-99%) of their primary structures (Antic et al., 1992a). However, sequence comparison of viruses of four different serotypes showed that Hantaan and Seoul viruses are more similar to each other than they are to Puumala and Prospect Hill viruses, and that Puumala and Prospect Hill viruses are more closely related to one another than to Hantaan and Seoul viruses (Fig. 24). Therefore, comparative analysis indicates that hantaviruses have evolved from a common

Figure 24. Relative differences among the large (L), medium (M) or small (S) segments of hantaviruses. Dendrograms were generated from multiple sequence alignments by the Pileup program of the University of Wisconsin GCG Sequence Analysis Software. Distance along the horizontal axis is proportional to sequence differences.


L
Hantaan (76-118)
Seoul (80-39)
Puumala (Hallnas)

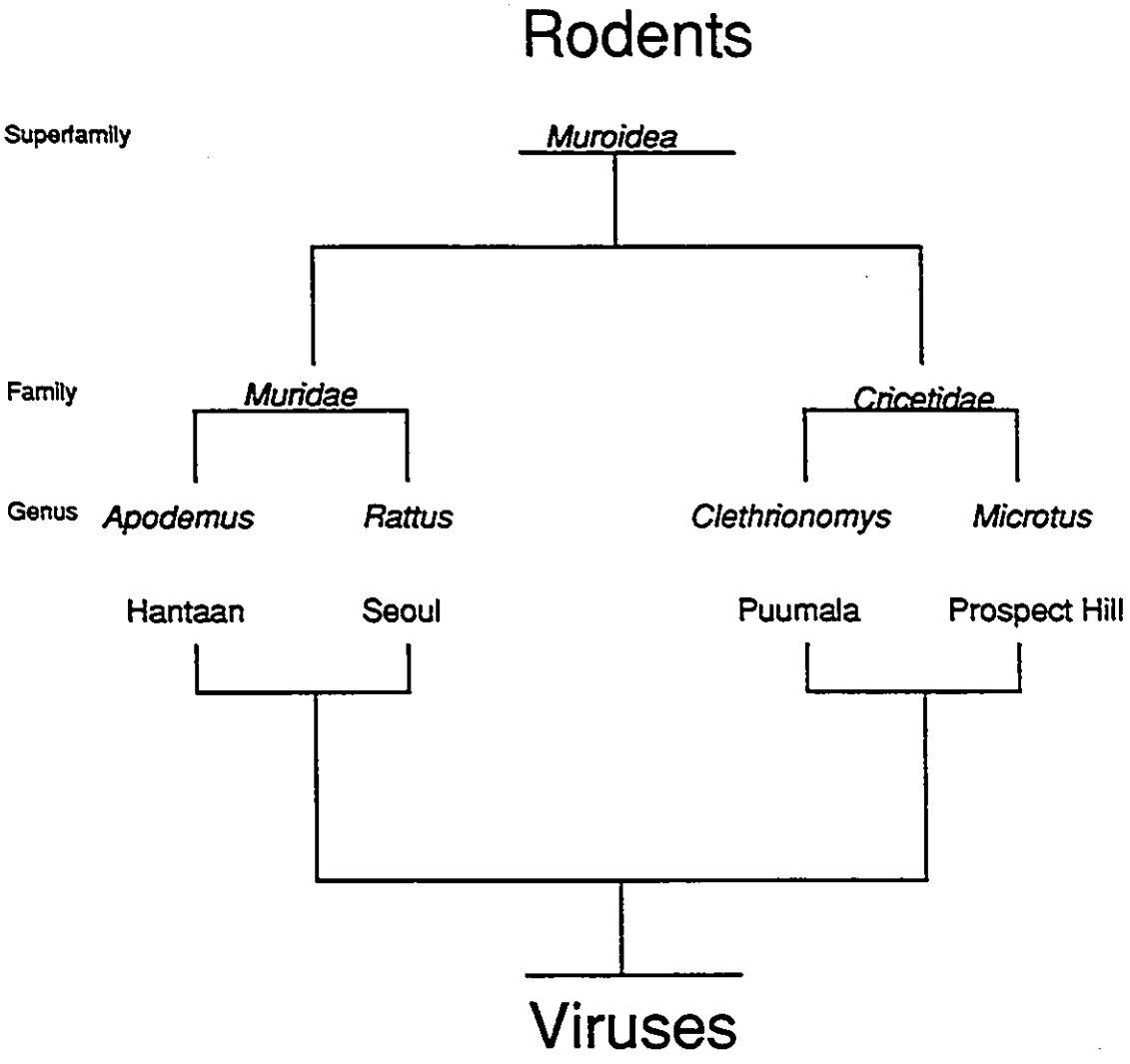

M
Seoul (80-39)
Seoul (SR-11)
Hantaan (76-118)
Hantaan (Lee)
Hantaan (HoJo)
Puumala (Hallnas)
Puumala (Sotkamo)
Prospect Hill


S
Hantaan (76-118)
Seoul (SR-11)
Puumala (Hallnas)
Puumala (Sotkamo)
Prospect Hill

ancestor into at least four host-specific lineages: mouse (Hantaan-type), rat (Seoul-type), bank vole (Puumala-type) and meadow vole (Prospect Hill) isolates (Fig. 25). Furthermore, these viruses can be grouped into two categories. One is represented by Seoul and Hantaan viruses and the other is represented by Puumala and Prospect Hill viruses (Fig. 25). The categorization of hantaviruses correlates well with the categorization of their rodent hosts. All rodent hosts belong to the superfamily *Muroidea* which is divided into families. The family *Muridae* is represented by two genera, *Apodemus* and *Rattus*, which are natural reservoirs for the Hantaan and Seoul viruses, respectively. The other family, *Cricetidae*, is represented by two genera, *Clethrionomys* and *Microtus*, which are hosts for Puumala and Prospect Hill viruses, respectively. Therefore, it seems that the viruses and rodents have co-evolved from common ancestors into two main groups (Fig. 25). The first group represents viruses isolated from rodents of the family *Muridae* and associated with the severe or moderate forms of HFRS (Hantaan and Seoul viruses). The second group represents viruses isolated from rodents of the family *Cricetidae* and associated with nephropathia epidemica (Puumala) or no disease (Prospect Hill).

In all rodents, viral infection may persist for the life of the animal but no virus-induced pathology has been observed. It is evident that the two viruses isolated from the same rodent host do not differ significantly e.g. Seoul 80-39 and SR-11 viruses. However, 81% (for the M genomic RNA segment) and 90% (for the S genomic RNA segment) of all the changes between the two Seoul isolates, at the

Figure 25. Evolutionary trees of hantaviruses and their rodent hosts.


Rodents
Superfamily
Muroidea
Family
Muridae
Cricetidae
Genus
Apodemus
Rattus
Clethrionomys
Microtus
Hantaan
Seoul
Puumala
Prospect Hill
Viruses

nucleotide sequence level were uridine (U) to cytidine (C) or adenine (A) to guanine (G) transitions. The same type of changes have been observed in measles virus isolated from patients with chronic CNS infections (subacute sclerosing panencephalitis) as well as in human parainfluenza virus isolated from persistently infected cells (Wong et al., 1989; Cattaneo et al., 1988; Cattaneo et al., 1989; Murphy et al., 1991). These changes are called biased hypermutation and are explained by the activity of a cellular enzyme which unwinds and modifies double-stranded RNA in eucaryotic cells (Bass and Weintraub, 1987; Wagner and Nishikura, 1988; Bass and Weintraub, 1988). The same or a similar cellular enzyme might cause modifications of viral RNA (Bass et al., 1989), although the existence of a double-stranded viral RNA intermediate has not been observed. These observations suggest that this type of mutation might be a general phenomenon occurring both in the infected organism and cultured cells but only when the virus replicates in an aberrant mode. Therefore, the evolution of RNA viruses could be determined by random viral polymerase errors, by genome reassortment, genetic recombination and by cellular mutational activity.

CHAPTER 4 PROCESSING OF HANTAAN VIRUS GLYCOPROTEINS AND VIRUS MORPHOGENESIS

INTRODUCTION

It has been shown that the bunyaviruses (Madoff and Lenard, 1982), uukuviruses (Kuismanen et al., 1982, Kuismanen, 1984) and phleboviruses (Anderson and Smith, 1987) mature intracellularly by budding through the Golgi area. However, similar data reported for hantaviruses were not clear and were somewhat contradictory. Thin section and immunoelectron microscopy studies have shown that the assembly of immature hantavirus particles occurs at the level of the endoplasmic reticulum (Tao et al., 1983, Tao et al., 1985). However, glycoproteins G1 and G2 were reported to be resistant to endoglycosidase H digestion and sensitive to endoglycosidase F digestion suggesting that the oligosaccharides are of the complex type (Schmaljohn et al., 1986b) and that the proteins have to be processed in the Golgi apparatus. In another study, G1 and G2 expressed by a recombinant vaccinia virus were associated with the Golgi apparatus (Pensiero et al., 1988). Since the mode of glycoprotein maturation correlates well with the site of virus morphogenesis, one would expect that the assembly of viral particles occurs in the Golgi apparatus. Furthermore, a study by Ruusala et al (1990 and 1992) showed that Hantaan virus glycoproteins G1 and G2, expressed from a vaccinia virus vector, were transported to the Golgi region only when G1 and G2 were co-expressed, but they remained endoglycosidase H

sensitive. The expression of these proteins was performed in HeLa or BHK cells which normally do not support hantavirus growth.

The objective of my study was to clarify the maturation pathway of the glycoproteins, G1 and G2, of Hantaan virus and to determine the importance of the Golgi apparatus in virus morphogenesis.

To study processing of Hantaan virus glycoproteins, I performed immunoprecipitations of G1 and G2 from Hantaan virus infected cell lysates prepared with ionic or nonionic detergents using monoclonal antibodies (MAb) specific for glycoprotein G1 or G2. I also treated G1 and G2 with endoglycosidases H, F and D and I used inhibitors of oligosaccharide processing. Morphogenesis of the virus was studied by thin section electron microscopy: Hantaan virus infected cells were compared with mock infected cells and Hantaan virus infected, brefeldin A treated cells.

The importance of the endoplasmic reticulum and the Golgi apparatus for processing of Hantaan virus glycoproteins and virus morphogenesis will be presented and discussed.

RESULTS

1. MATURATION OF HANTAAN VIRUS GLYCOPROTEINS G1 AND G2

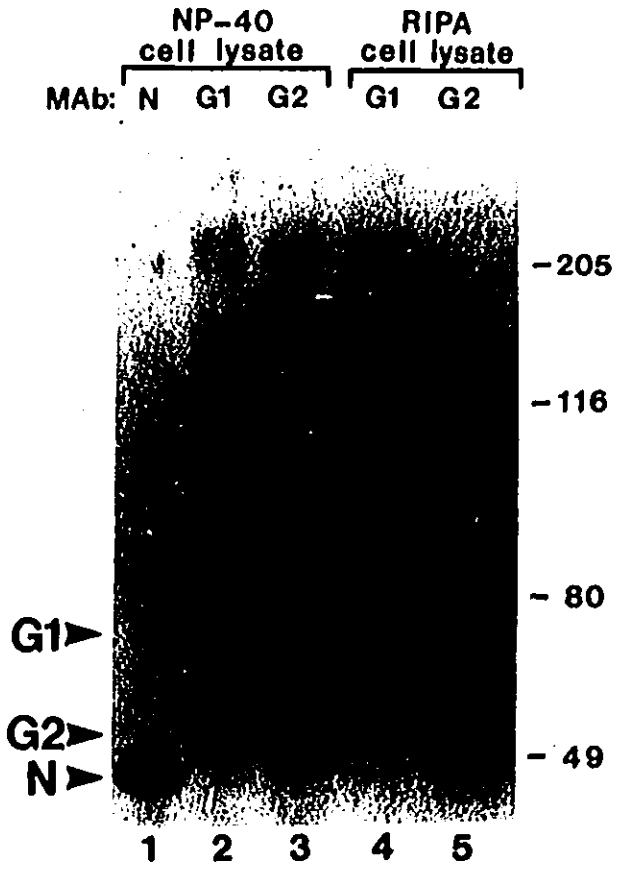
Immunoprecipitation analysis of G1 and G2

Hantaan virus infected Vero E6 cell lysates were used for immunoprecipitation with monoclonal antibodies against glycoprotein G1 (MAbG1) or G2 (MAbG2). When cell lysates were prepared with buffer containing nonionic detergent (NP-40 buffer), both G1 and G2 glycoproteins were precipitated with either MAbG1 or MAbG2 and were detected by reducing (Fig. 26A) as well as nonreducing (not shown) SDS-PAGE. In contrast, when cell lysates were prepared with a buffer containing ionic detergents (RIPA buffer) MAbG1 precipitated only glycoprotein G1 and MAbG2 precipitated only glycoprotein G2 (Fig. 26A). These data suggest that the glycoproteins form a complex that is dissociated by 0.1% SDS.

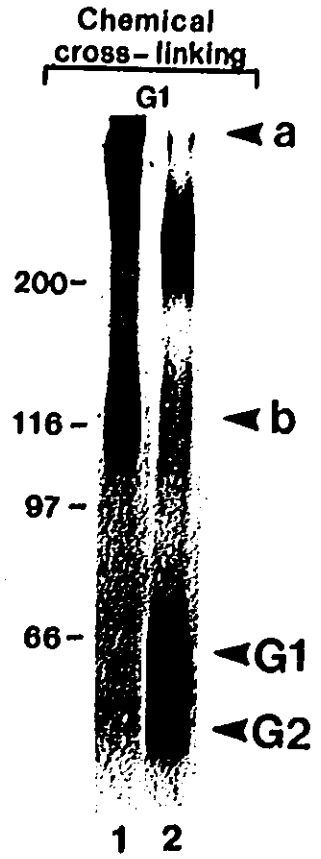
Chemical cross-linking of G1 and G2

Although the immunoprecipitation results suggested that the glycoproteins G1 and G2 form a complex, we were not able to detect an intact complex by SDS-PAGE. To provide direct evidence for the existence of a G1-G2 complex, we have performed chemical cross-linking using dithiobis(succinimidylpropionate) - DSP. Complexes obtained after DSP cross-linking can be dissociated by the addition of DTT.

Figure 26. Immunoprecipitation of Hantaan virus glycoproteins G1 and G2. (A): G1 and G2 were immunoprecipitated from cell lysates prepared with nonionic detergent (lanes 1-3) or from cell lysates prepared with ionic detergent (lanes 4 and 5). Samples were boiled before electrophoresis under reducing conditions. The nucleocapsid protein was immunoprecipitated with a specific MAb as a control (lane 1). MAbs did not precipitate molecules of a similar size from mock infected cells (not shown). (B): G1 and G2 were cross-linked with DSP and immunoprecipitated with MAbG1. One half of the cross-linked, immunoprecipitated sample was electrophoresed under nonreducing conditions (lane 1); the other half was treated with 50 mM DTT prior to electrophoresis (lane 2). a and b represent high-molecular weight complex forms of glycoproteins G1 and G2. Numbers on the side of each gel indicate M_r markers in kilodaltons.


NP-40
cell lysate
RIPA
cell lysate
MAb: N G1 G2 G1 G2
-205
-116
- 80
- 49
G1
G2
N
1 2 3 4 5

A

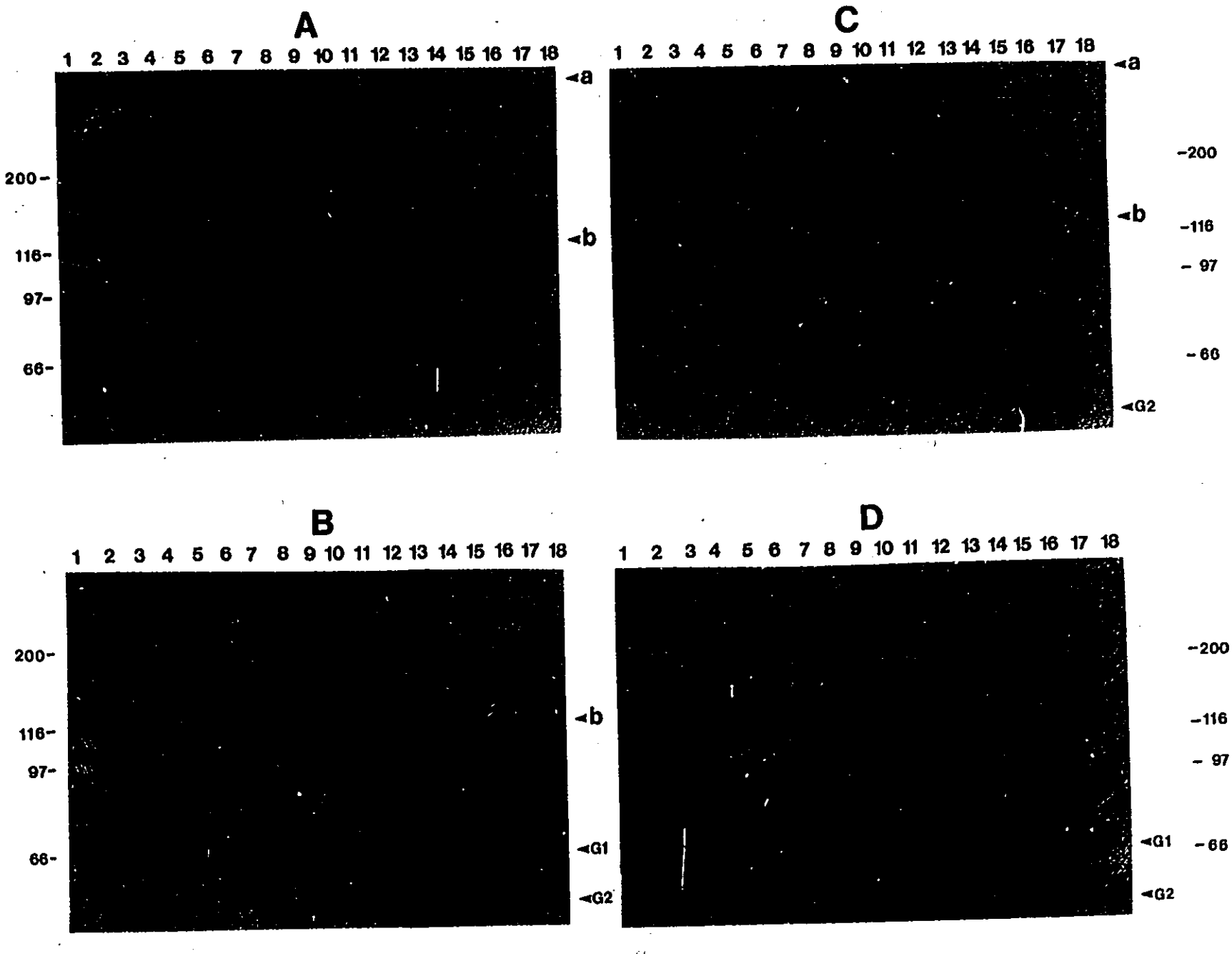

Chemical
cross-linking
G1
a
200-
116-
b
97-
66-
G1
G2
1 2

B

DSP was added to NP-40 solubilized infected cell lysates prior to immunoprecipitation with MAbs against G1 (Fig. 26B) or G2 (not shown). The cross-linked, immunoprecipitated sample, electrophoresed under nonreducing conditions (Fig. 26B, lane 1) contained two high-molecular-weight species (a and b). These two high-molecular-weight bands most likely represent oligomeric (a) and heterodimeric (b) forms of G1 and G2. When DTT was added to the sample, both glycoproteins G1 and G2 were detected (Fig. 26B, lane 2). However, it is evident that cross-linking was not completely reversed by thiol reduction, since oligomeric forms (heterodimers and possibly trimers) were still detected.

To further examine the composition of obtained complexes, cross-linked cell lysates were loaded on sucrose gradients. Fractions collected from one tube were used for immunoprecipitation with MAbG1 (Figs. 27A and 27B) and fractions collected from a second tube were used for immunoprecipitation with MAbG2 (Figs. 27C and 27D). In the first 11 cross-linked, nonreduced fractions, high-molecular-weight forms (a) were precipitated with MAbG1 (Fig. 27A). The same molecule was precipitated with MAbG2 from the first 9 fractions (Fig. 27C). Molecules with a molecular weight of ~120K (b) were precipitated with MAbG1 from fractions 13 to 18 (Fig. 27A). The same 120K band was precipitated with MAbG2 from fractions 11 to 15 (Fig. 27C). However, fractions 16 and 17, used for immunoprecipitation with MAbG2, contained G2 monomers only (Fig. 27C). When DTT was added to the samples after immunoprecipitation (Figs. 27B and 27D), complexes were dissociated to both G1 and G2.

Figure 27. Identification of G1-G2 complex forms by sucrose gradient centrifugation. Eighteen fractions of 0.6 ml were collected by puncturing the bottom of each tube (fraction 1 represents the bottom of the gradient and fraction 18 is the top of the gradient). Hantaan virus glycoproteins were immunoprecipitated from each fraction with either MAbG1 (A and B) or with MAbG2 (C and D). Samples were boiled and electrophoresed under nonreducing (A and C) or reducing (B and D) conditions. a and b represent high-molecular-weight complex forms of glycoproteins G1 and G2.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
200-
116-
97-
66-
◄a
◄b
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
200-
116-
97-
66-
◄b
◄G1
◄G2
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
◄a
◄b
◄G2
-200
-116
- 97
- 66
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
◄G1
◄G2
-200
-116
- 97
- 66

Effect of Brefeldin A on complex formation

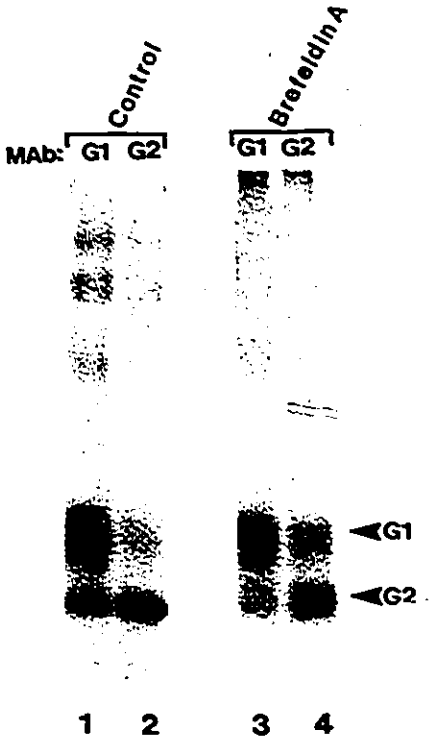
To determine where in the cell the G1-G2 complex was formed, I treated infected cells with brefeldin A (10 μg/ml) and prepared NP-40 cell lysates for immunoprecipitation with MAbs. Brefeldin A causes rapid redistribution of the Golgi region into the endoplasmic reticulum (ER) blocking any transport from the ER to other cellular compartments (Orci et al., 1991, Cheung et al., 1991, Whealy et al., 1991). If the complexes are formed in the ER, as is the case for many other viral glycoproteins (Hurtley and Helenius, 1989), brefeldin A treatment should not prevent complex formation. When infected cells were treated with brefeldin A, G1-G2 complexes were formed since MAbs to either G1 or G2 precipitated both glycoproteins from NP-40 lysates (Fig. 28, lanes 3 and 4) as was observed in untreated cells (Fig. 28, lanes 1 and 2). This experiment demonstrates that a G1-G2 complex is formed in the ER.

Glycosylation inhibition and endoglycosidase digestion

In order to determine the extent of processing of N-linked oligosaccharides, I have used various inhibitors of oligosaccharide trimming and transport. Vero E6 cells infected with Hantaan virus were treated with either brefeldin A, castanospermine, swainsonine or monensin prior to and during labeling. Cell lysates were prepared with RIPA buffer and used for immunoprecipitation of glycoproteins with MAbs to both G1 and G2 (Fig. 29A).

In the presence of 10 μg/ml (Fig. 29A, lane 5) or 2.5 μg/ml (Fig. 29A, lane

Figure 28. Complex formation in the presence of brefeldin A. Using NP-40 buffer, lysates were prepared from untreated infected cells (lanes 1 and 2) or infected cells treated with 10 μg/ml brefeldin A (lanes 3 and 4) prior to immunoprecipitation with either MAbG1 (lanes 1 and 3) or MAbG2 (lanes 2 and 4) and electrophoresis.


Control
Brefeldin A
MAb: G1 G2 G1 G2
G1
G2
1 2 3 4

8) of brefeldin A, immunoprecipitated viral glycoproteins G1 and G2 migrated identically to viral glycoproteins labeled in the absence of the drug (Fig. 29A, lanes 1 and 4), suggesting that trimming or addition of terminal sugars does not occur in the Golgi apparatus. However, trimming of glucose residues from the asparagine-linked precursor is required, as castanospermine, which inhibits α-glucosidase I in the rough ER, caused slower migration of Hantaan G1 and G2 (Fig. 29A, lane 11).

Swainsonine also inhibits a Golgi function, that of the trimming enzyme α-mannosidase II. This enzyme acts late in the processing of carbohydrates from high mannose to complex type sugars. Treatment of infected cells with this inhibitor again did not change the migration of the viral glycoproteins (Fig. 29A, lane 14), confirming that the N-linked oligosaccharides of Hantaan virus glycoproteins G1 and G2 are not complex.

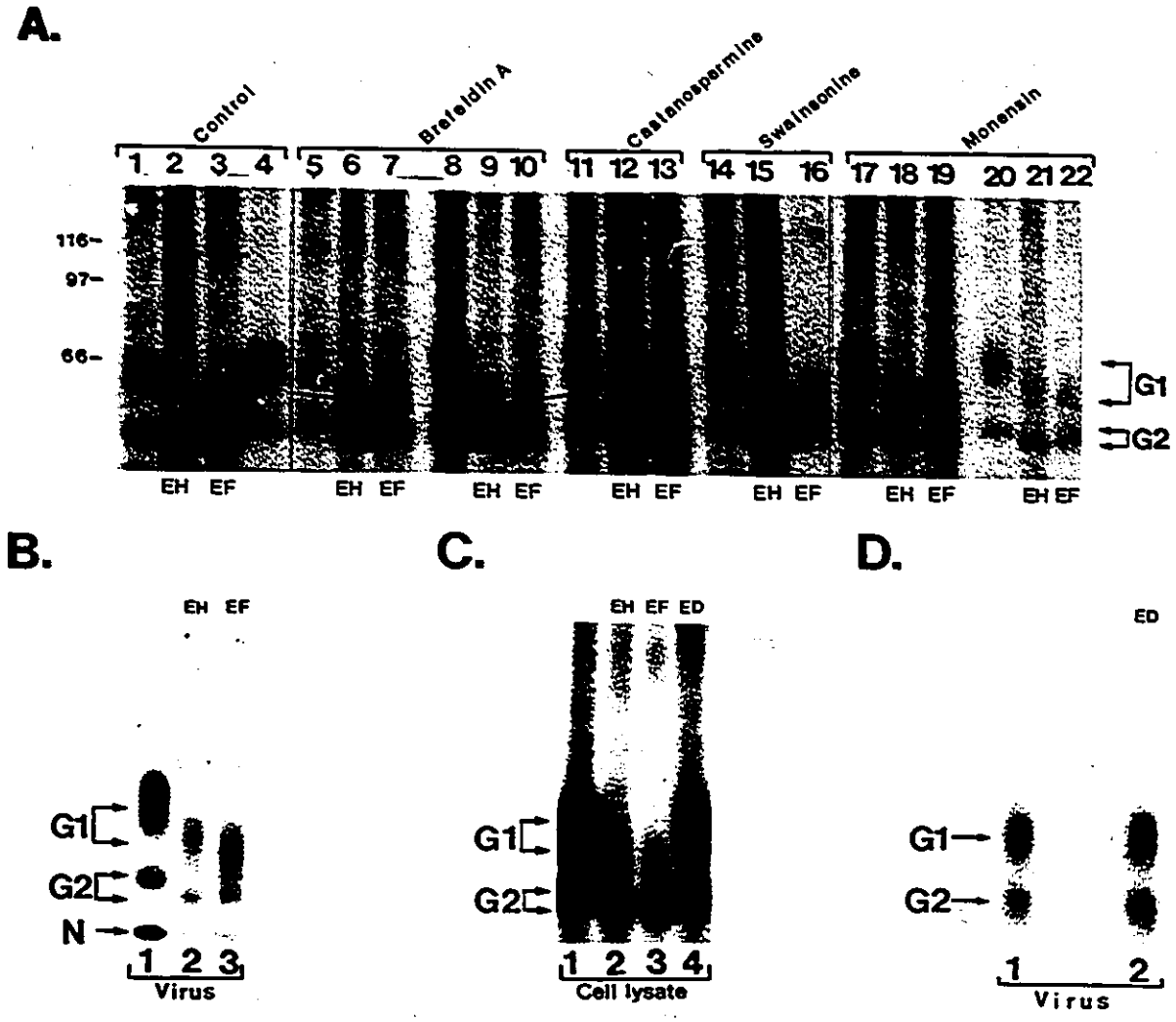
The ionophore monensin slows or arrests intra-Golgi transport and inhibits or prevents late Golgi functions such as terminal N-glycosylation, sulfation of proteoglycans and proprotein processing (Farquar, 1985). Treatment of infected cells with monensin had no effect on the migration (Fig. 29A, lanes 17 and 20) of G1 and G2.

Processing of glycoproteins can be studied by endoglycosidase sensitivity assays. Oligosaccharide chains containing mannose residues only can be trimmed from a glycoprotein both by endoglycosidase H (EH) and endoglycosidase F (EF). However, oliosaccharide chains containing complex sugars can be trimmed from

a glycoprotein by EF only. Therefore, incorporation of complex sugar residues in the *mid*- and *trans*-Golgi can be observed by shift from the EH sensitive to the EH resistant form of a glycoprotein. In both untreated samples (Fig. 29A, lanes 2 and 3) and samples treated with glycosylation inhibitors (Fig. 29A, lanes 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21 and 22), both glycoproteins were sensitive to digestion with endoglycosidase H as well as endoglycosidase F. These results indicate that the mature N-linked oligosaccharides of both G1 and G2 are of the high mannose type. This conclusion was confirmed in a further experiment showing that the glycoproteins immunoprecipitated from virions were also sensitive to endoglycosidase H (Fig. 29 B).

Although these results suggest that events occurring in the *mid*- and *trans*-Golgi are not necessary for processing of G1 and G2, the trimming of the high mannose carbohydrates may still take place in the *cis*-Golgi , by the enzyme α-mannosidase I. This enzyme trims the structure $(Man)_8(GlcNAc)_2$, formed in the ER, to $(Man)_5(GlcNAc)_2$, which is sensitive to endoglycosidase D. To test whether Hantaan virus glycoproteins are trimmed by this enzyme, G1 and G2 were immunoprecipitated from infected cell lysates and treated with endoglycosidase D (Fig. 29C, lane 4). The fact that both G1 and G2 were resistant to endoglycosidase D, but sensitive to endoglycosidase H, suggests a structure with more than five mannose residues. The same result was obtained with G1 and G2 immunoprecipitated from purified virions (Fig. 29D, lane 2). Therefore, trimming of oligosaccharides involves the trimming of glucose residues from the asparagine-

Figure 29. Endoglycosidase sensitivity and inhibition of processing of Hantaan virus glycoproteins G1 and G2. (A): Infected cells were treated with: brefeldin A (10 μg/ml, lanes 5-7 or 2.5 μg/ml, lanes 8-10), castanospermine (lanes 11-13), swainsonine (lanes 14-16) or monensin (1 μM, lanes 17-19 or 10 μM, lanes 20-22) during the prelabeling period (2 hr), after which fresh drug was added for the 4-hr labeling period. Control cultures were infected but were not treated with any inhibitors (lanes 1-4). Lysates were immunoprecipitated with a mixture of MAbG1 and MAbG2. Immunoprecipitated samples were treated with endoglycosidase H (EH) or endoglycosidase F (EF) or were untreated. Lanes 1-4 were exposed for 2 days, lanes 5-16 were exposed for 6 days and lanes 17-22 were exposed for 14 days. (B): EH and EF digestion of G1 and G2 from virions: Glycoproteins and nucleocapsid protein were immunoprecipitated from purified virions (lane 1) and treated with EH (lane 2) or EF (lane 3). (C): Glycoproteins immunoprecipitated from infected cell lysates (lane 1), treated with EH (lane 2), EF (lane 3) and endoglycosidase D (ED) (lane 4). (D): Glycoproteins immunoprecipitated from purified virions (lane 1) and treated with ED (lane 2).


A.
Control
Brefeldin A
Castanospermine
Swainsonine
Monensin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
116–
97–
66–
G1
G2
EH EF
B.
EH EF
G1
G2
N
1 2 3
Virus
C.
EH EF ED
G1
G2
1 2 3 4
Cell lysate
D.
ED
G1
G2
1 2
Virus

linked precursor by α-glucosidase I of the rough ER and possibly the trimming of mannose residues from the structure $(Man)_9(GlcNAc)_2$ to the structure $(Man)_8(GlcNAc)_2$ by α1,2-mannosidase of the rough ER and further to structures $(Man)_7(GlcNAc)_2$ or $(Man)_6(GlcNAc)_2$ by α-mannosidase I of the *cis*-Golgi.

2. VIRUS MORPHOGENESIS

Thin section electron microscopy

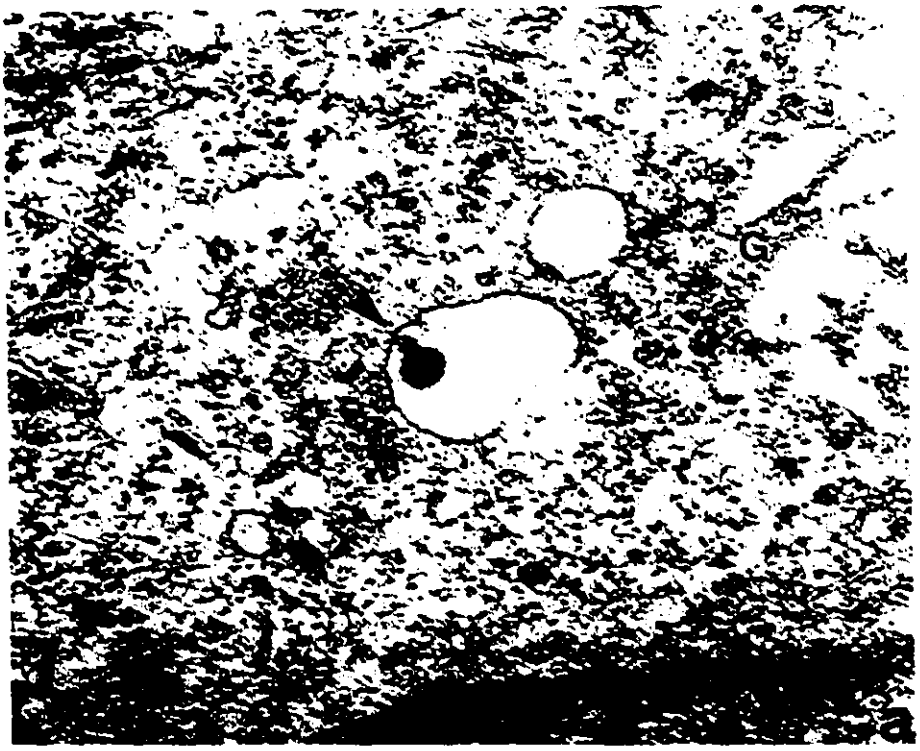
Thin sections of mock infected, Hantaan virus infected and brefeldin A treated, Hantaan virus infected Vero E6 cells were prepared 8 days after infection. Virus-like particles were observed in the region of the *trans*-Golgi network but only in infected, brefeldin A untreated cells (Fig. 30 and 31). The absence of maturing virions in infected, brefeldin A treated cells (Fig. 32) indicates that the Golgi apparatus is an essential cellular compartment for virus morphogenesis, although it may not be important for processing of viral glycoproteins. Virus-like structures were not observed in mock-infected cells (Fig. 33). Although electron micrographs of infected, brefeldin A treated cells (Fig. 32) and mock infected cells (Fig. 33) were taken at magnifications of ca. 14,000 X and 33,000 X, virus-like particles were not observed in these samples under magnification of ca. 100,000 (not shown).

Figure 30. Thin section electron microscopy of Hantaan virus infected cells, 8 days after infection (a and b). Arrows indicate virions in the Golgi apparatus (G). rER indicates rough endoplasmic reticulum. Magnification: (a) ca. 150,000 X, (b) ca. 100,000


G
a

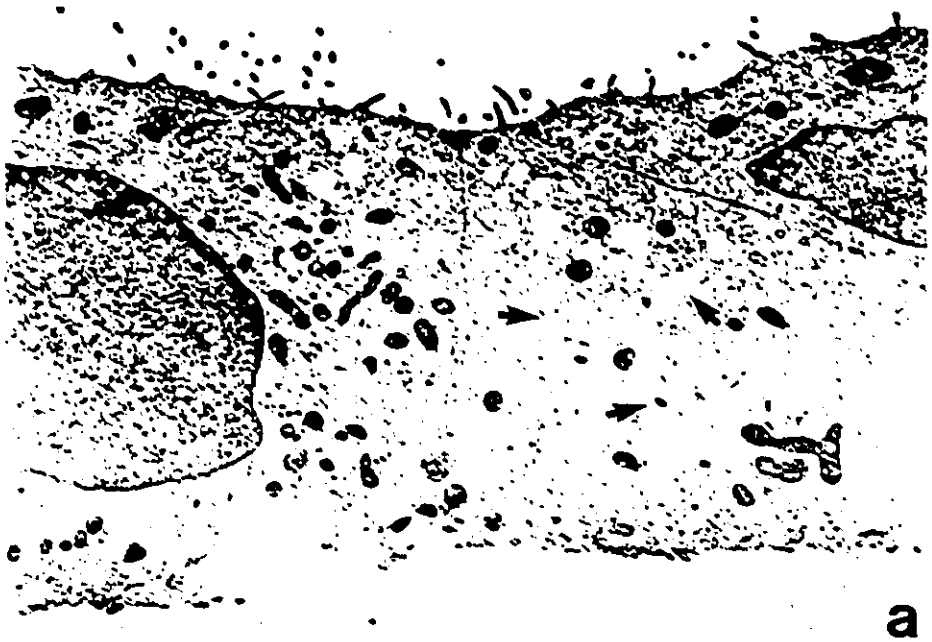

ER
G

Figure 31. Thin section electron microscopy of Hantaan virus infected cells, 8 days after infection (a and b). Arrows indicate budding virions and G indicates Golgi apparatus. Magnification: (a): ca. 100,000 X, (b): ca. 150,000 X.


a


b

Figure 32. Thin section electron microscopy of brefeldin A treated, Hantaan virus infected cells, 8 days after infection (a and b). Absence of Golgi apparatus and dilated endoplasmic reticulum (indicated by arrows) is evident on both electron micrographs. Magnification: (a): ca. 14,000 X, (b): ca. 33,000 X



a

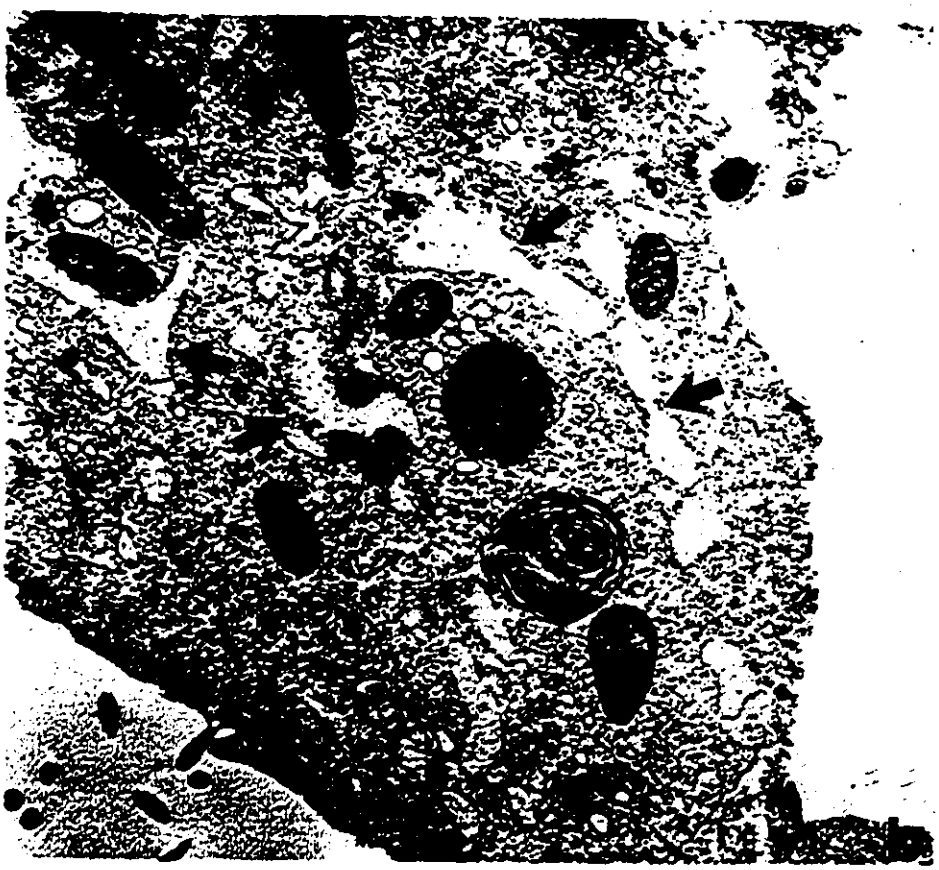
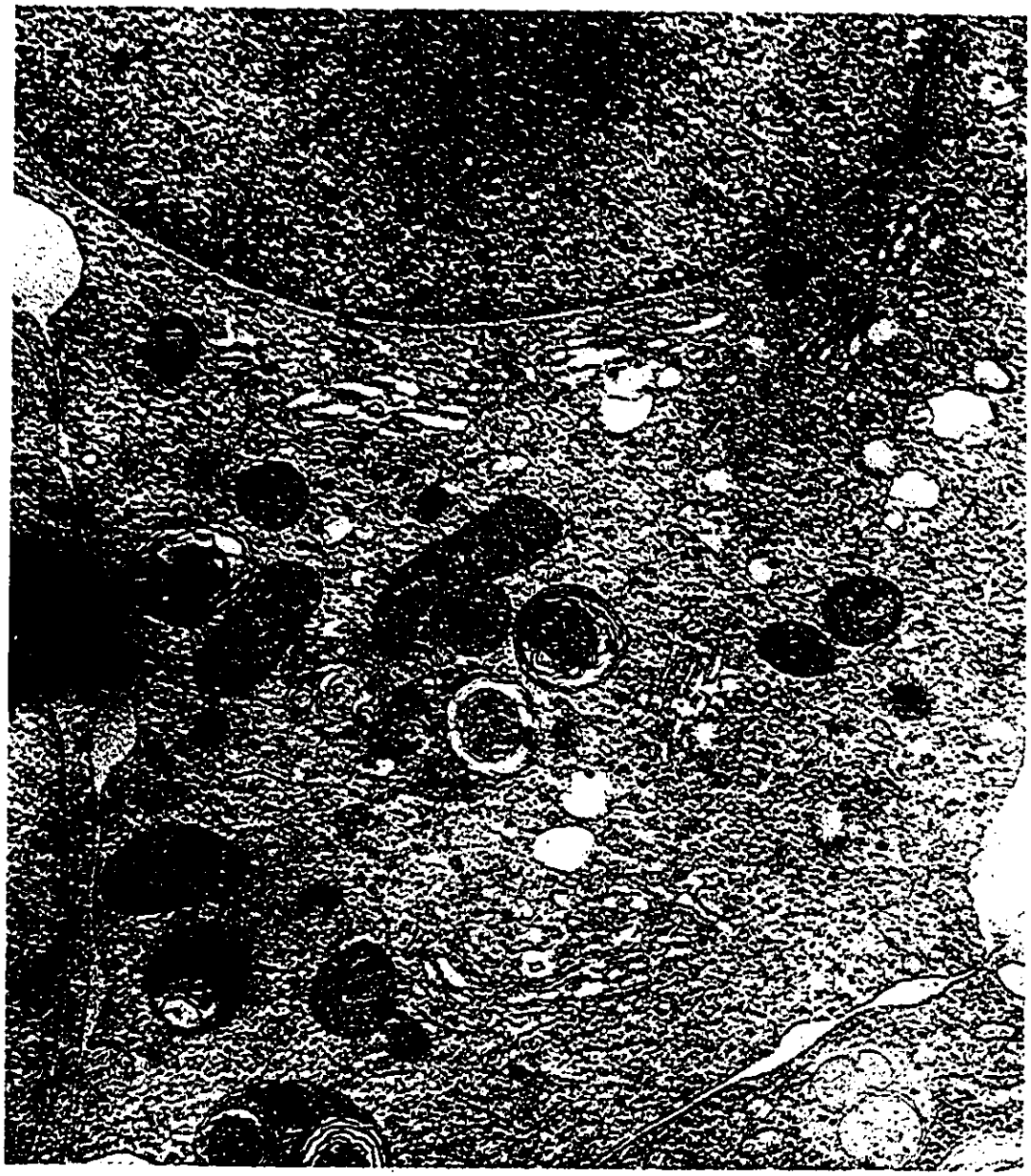


Figure 33. Thin section electron microscopy of mock infected cells. Magnification: ca. 33,000.



DISCUSSION

Virus assembly requires the synthesis and transport of all structural components to the site where the virus particles are formed. Studies on processing of viral envelope glycoproteins have suggested their key role in determining the site of virus maturation. The mRNAs encoding viral glycoproteins are translated on the membranes of the rough endoplasmic reticulum (rER). The proteins are cotranslationally glycosylated in the lumen of the ER. These glycoproteins can be retained in the ER, e.g. Rotavirus glycoproteins VP7 and NS28 (Estes, 1991), or can be transported to the Golgi apparatus where modification of oligosaccharide chains (trimming of mannose residues and addition of complex sugars) occurs. After processing, glycoproteins can be transported to the cell membrane, e.g. alphaviruses (Schlesinger and Schlesinger), rhabdoviruses (Wagner, 1991), paramyxoviruses (Kingsbury, 1991), orthomyxoviruses (Kingsbury, 1991), arenaviruses (Bishop, 1991), retroviruses (Coffin, 1991); or they can be retained in the Golgi apparatus as was shown for coronaviruses (Holmes, 1991) and bunyaviruses (Matsuoka et al., 1991). Other structural components (nucleocapsids) of the virus are directed towards the site of glycoprotein accumulation, where the assembly and budding of virus particles occurs. Rotaviruses mature by budding through the membrane of the ER (Estes, 1991); assembly and budding of alphaviruses (Schlesinger and Schlesinger, 1991), rhabdoviruses (Wagner, 1991), paramyxo- and orthomyxoviruses (Kingsbury, 1991), arenaviruses (Bishop, 1991) and retroviruses (Coffin, 1991) occurs at the

cell membrane. Coronaviruses (Holmes, 1991) and bunyaviruses (Schmaljohn and Patterson) bud through the cisternae of the Golgi apparatus.

Specific targeting mechanisms must exist for selective transport and localization of proteins in various cellular compartments. It has been demonstrated that resident ER proteins can be distinguished from non-resident proteins by the presence of specific signals within the protein sequence. Several soluble ER proteins (Pelham, 1988 and 1989) were found to contain the tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL) which is responsible for their retention in the ER. However, for the efficient transport of proteins from the ER to the Golgi apparatus, the correct folding, assembly and/or oligomerization of proteins was shown to be essential (Rose and Doms, 1988; Copeland et al., 1986; Doms et al., 1988; Gething et al., 1986; Hurtley and Helenius, 1989; Kreis and Lodish, 1986). Glycoproteins of bunyaviruses and coronaviruses seem to behave like Golgi-resident proteins. Machamer and Rose, 1987, have shown that the first transmembrane domain of the E1 glycoprotein of a coronavirus contains a Golgi retention signal. However, studies with glycoproteins of bunyaviruses (PuntaToro virus (Chen et al., 1991; Matsuoka et al., 1991) and Hantaan virus (Ruusala et al., 1992)) expressed in vaccinia virus system demonstrated that the G1 and G2 were targeted to the Golgi complex only when they were co-expressed. Expression of only G1 or G2 resulted in retention of the glycoprotein in the ER. Our results show that glycoproteins G1 and G2 of Hantaan virus form a heterodimer in the endoplasmic reticulum. Therefore, heterodimerization seems to be a necessary

requirement for the transport of Hantaan virus glycoproteins from the ER to the Golgi region. However, we can not conclude that the dimerization is the only signal necessary for Golgi targeting and retention. We can speculate that one (or more) transmembrane regions of the G1 and/or G2 contain a Golgi retention signal, as was shown for the E1 glycoprotein of a coronavirus. Characterization of signals which are important for Golgi retention awaits construction and expression of mutated or chimeric glycoproteins.

In comparison to glycoproteins of other bunyaviruses, our findings show that Hantaan virus glycoproteins G1 and G2 are unique, both in the composition of their oligosaccharide chains and their localization. While glycoproteins of other bunyaviruses have complex sugars and therefore require the Golgi apparatus for processing and glycosylation, glycoproteins of hantaviruses seem to be processed in the ER, but are still transported to the Golgi. Unless the enzyme α-mannosidase I of the *cis*-Golgi is necessary for trimming of mannose residues from the structure $(Man)_8(GlcNAc)_2$ to structures $(Man)_7(GlcNAc)_2$ or $(Man)_6(GlcNAc)_2$, enzymes of the Golgi apparatus do not seem to be involved in processing of Hantaan virus glycoproteins. Therefore, it is possible that both processing of the glycoproteins and complex formation occur in the ER and that the transport and retention of the glycoproteins in the Golgi is necessary only for virus assembly and budding.

CHAPTER 5 EXPRESSION OF THE NUCLEOCAPSID PROTEIN OF HANTAAN 76-118 VIRUS AND SEOUL 80-39 VIRUS

INTRODUCTION

Hantavirus RNA segments are encapsidated with nucleocapsid proteins and associated with small amounts of L (polymerase/transcriptase) protein. It is not clear how the RNA and the N protein interact with each other to form a helical nucleocapsid, but the assembly of RNPs seems to be an important process for regulation of viral transcription and replication. There is not very much data explaining the encapsidation process and its significance in the control of switching between transcription and replication in Bunyaviruses.

Assembly of RNPs is thought to be controlled at the level of initiation of RNA synthesis. The N protein would interact with a specific sequence on its target RNA. Specificity of this reaction would be controlled by sequences conserved at the 5' termini of both genome and antigenome chains, i.e. the terminal 11 nucleotides. However, viral mRNAs also contain this sequence, but not at the precise 5' ends. In the latter case, the affinity of the interaction between the mRNA and the N protein would be much lower. It is proposed that after the first N protein interacts with the conserved RNA sequence, additional N molecules interact with both the RNA and the preexisting N in the complex, allowing encapsidation of sequences further downstream (Kolakofsky and Hacker, 1991). Therefore, if the

concentration of N is relatively low, only genomes and antigenomes would be assembled. mRNAs would be translated and newly synthesized proteins would be used for replication, transcription and virus morphogenesis. As the viral infection progresses, the pool of unassembled N increases and N would begin to associate with viral mRNAs. These assembled mRNAs could not be translated *in vitro* (Hacker et al., 1989) and therefore, N would directly down regulate the synthesis of viral proteins and RNA. Expression of cellular mRNAs presumably would not be affected because the unassembled N protein would have significantly lower affinity for the cellular RNA molecules (Hacker et al., 1989). All this leads to the hypothesis which explains the switch between replication and transcription: as soon as the synthesis of the antigenome chain starts, N protein would recognize and bind to the 5' terminal sequence with high affinity. Other N proteins would then interact with the synthesizing RNA molecule and prevent interactions between the nascent antigenome chain and the template thus preventing premature termination. Because N protein does not have a high affinity for the 5' terminus of the mRNA, the nascent mRNA chains would not be encapsidated. Thus, they would be able to interact with the template and to terminate after the stop codon and before the end of the template (Kolakofsky and Hacker, 1991).

To obtain conclusive information on virus encapsidation, transcription and replication, it is necessary to dissect the viral genome and viral proteins into functional units and perform *in vitro* and *in vivo* reconstitution experiments. One of the steps towards this goal was to express the nucleocapsid protein.

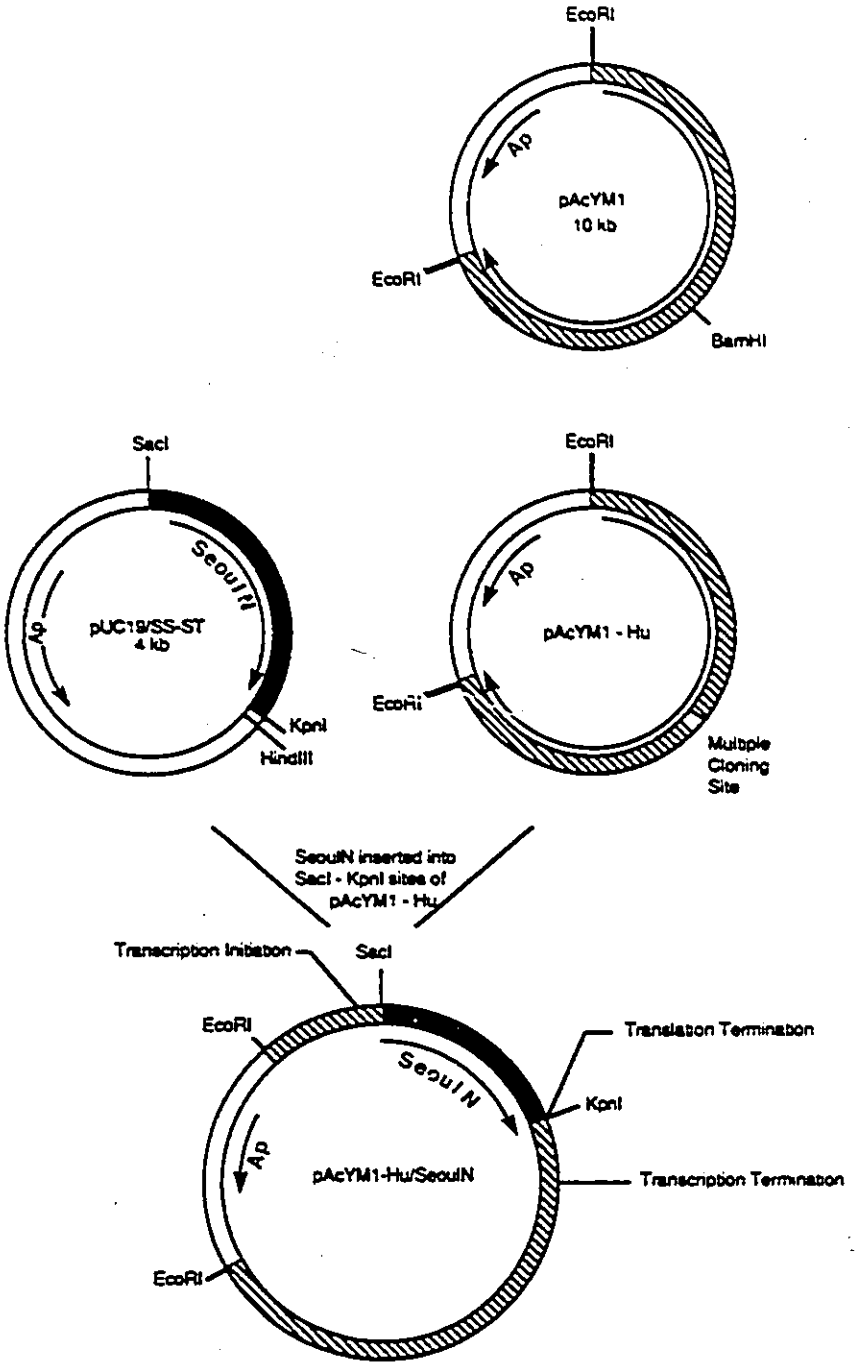
Nucleocapsid proteins of Hantaan 76-118 and Seoul 80-39 virus were expressed using a baculovirus expression system. The polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is not essential for virus infection or replication in the cell culture. Expression of this gene is regulated by a strong promoter and therefore polyhedrin protein accumulates to very high levels in cells, in the form of viral occlusions (polyhedra). Replacement or inactivation of the polyhedrin gene by a foreign gene results in the production of occlusion negative viruses (Occ$^-$) which are easy to select and which express high levels of a recombinant protein (Summers and Smith, 1987).

RESULTS

1. CONSTRUCTION OF A RECOMBINANT BACULOVIRUS

Recombinant baculovirus expressing Hantaan virus nucleocapsid protein (AcNPV/Hantaan N) was obtained from C. S. Schmaljohn, USAMRIID, and recombinant virus expressing the N protein of the Seoul virus was constructed. The full length N gene of Seoul virus, without non-coding sequences, was represented by clone SS-ST obtained by the reverse transcription - polymerase chain reaction (as described in Chapter 3). It was subcloned into the baculovirus transfer vector pAcYM1-Hu (Fig. 34). This vector contains an *Eco*RI fragment of the AcNPV genome inserted into a pUC8-based plasmid. This fragment contains all the upstream sequences of the polyhedrin gene followed by the inserted multiple

Figure 34. Subcloning of the Seoul 80-39 virus nucleocapsid (N) protein gene into the baculovirus transfer vector pAcYM1-Hu. pAcYM1-Hu vector was constructed by inserting the multiple cloning site (*Pst*I-*Sac*I-*Kpn*I-*Sma*I-*Bam*HI) into the *Bam*HI site of pAcYM1 vector (Hu, Y.W.). The Seoul N gene was represented by clone SS-ST (black areas) and was subcloned as a *Sac*I-*Kpn*I insert into pAcYM1-Hu. Shaded areas represent baculovirus derived sequences and clear areas represent pUC8 derived sequences.


EcoRI
Ap
pAcYM1
10 kb
EcoRI
BamHI
SacI
SeoulII
Ap
pUC19/SS-ST
4 kb
KpnI
HindIII
EcoRI
Ap
pAcYM1 - Hu
EcoRi
Multiple
Cloning
Site
SeoulN inserted into
SacI - KpnI sites of
pAcYM1 - Hu
Transcription Initiation
SacI
EcoRI
Translation Termination
SeoulN
KpnI
Ap
pAcYM1-Hu/SeoulN
Transcription Termination
EcoRI

cloning site *Pst*I-*Sac*I-*Kpn*I-SmaI-*Bam*HI (Hu, Y. W., personal communication). It lacks the polyhedrin coding region and the 13 nucleotides downstream from the translation termination codon, but contains following downstream sequences. These sequences would allow in vivo recombination between the pAcYM1-Hu and the wild type AcMNPV DNA (Fig. 35). The SS-ST clone was flanked by *Sac*I and *Kpn*I sequences and therefore was directionally inserted into pAcYM1-Hu, downstream of the polyhedrin gene promoter. *E. coli* RR1 competent cells were transformed with the plasmid designated as pAcYM1-Hu/SeoulN and sterile plasmid DNA was prepared for transfection. *Spodoptera frugiperda* cells (Sf9) were co-transfected with pAcYM1-Hu/SeoulN DNA and with wild type AcMNPV DNA (Fig. 35). The culture supernatant was collected 4 days later and titrated in monolayers of Sf9 cells. Plaques lacking occlusion bodies were picked and subsequently plaque-purified 3 times. Purified recombinant virus designated as AcMNPV-Hu/SeoulN was isolated and used for expression of Seoul N protein.

2. EXPRESSION OF HANTAAN AND SEOUL VIRUS NUCLEOCAPSID PROTEINS

The expression of nucleocapsid proteins in Sf9 cells was determined by electrophoresis of infected cell lysates on SDS-polyacrylamide gels. Gels were stained with Coomasie-blue stain and proteins were visualized after destaining. Proteins expressed in recombinant virus infected cells were compared with proteins expressed in mock- and wild type AcMNPV infected cells (Fig. 36A). To determine the specificity of expressed proteins, mock infected, wild type

Figure 35. Strategy for the production of recombinant baculovirus. Black areas represent inserted foreign gene (Seoul N) to be expressed and shaded areas represent baculovirus derived sequences. Approximate positions of transcription initiation, transcription termination and translation termination is marked.

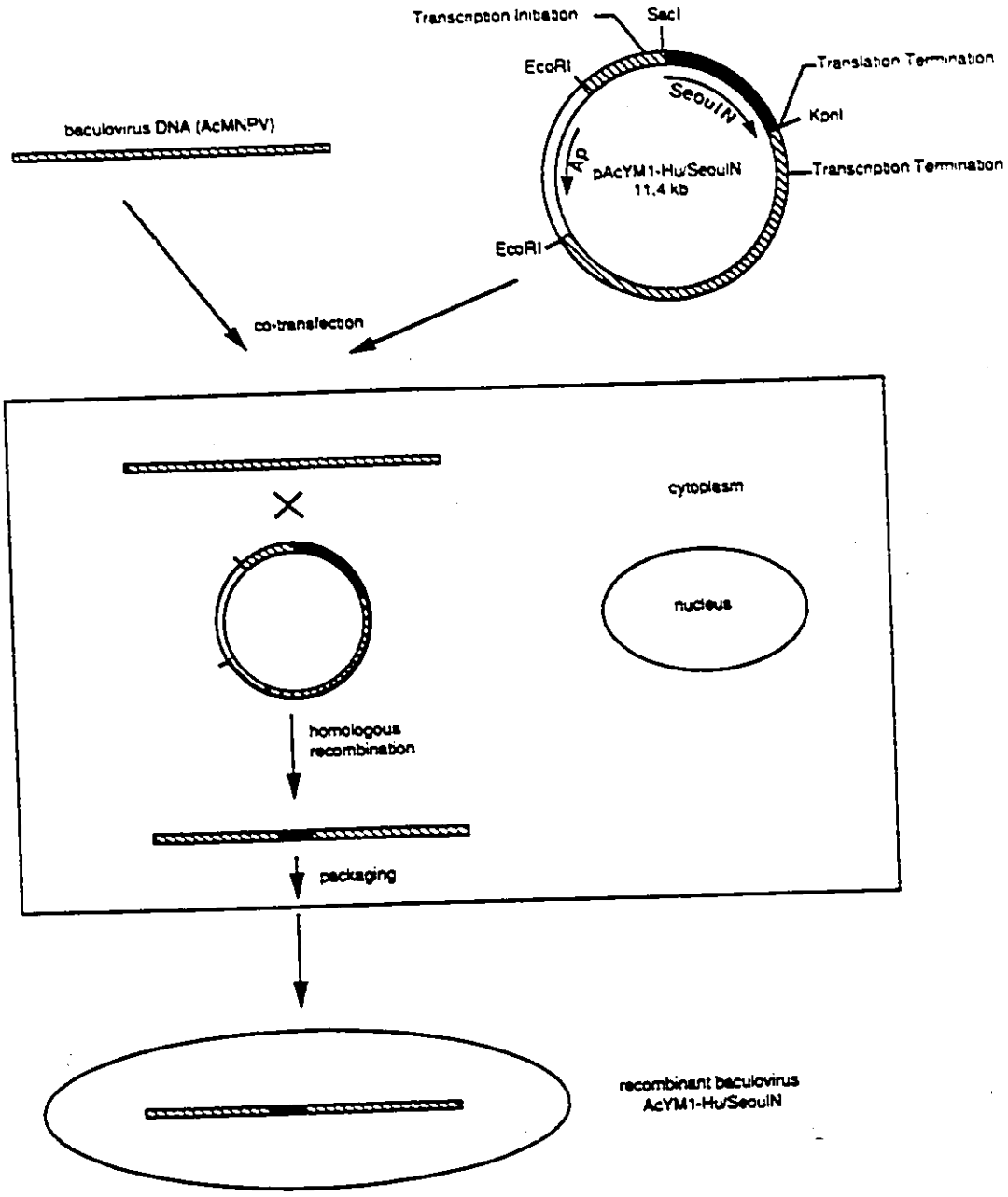
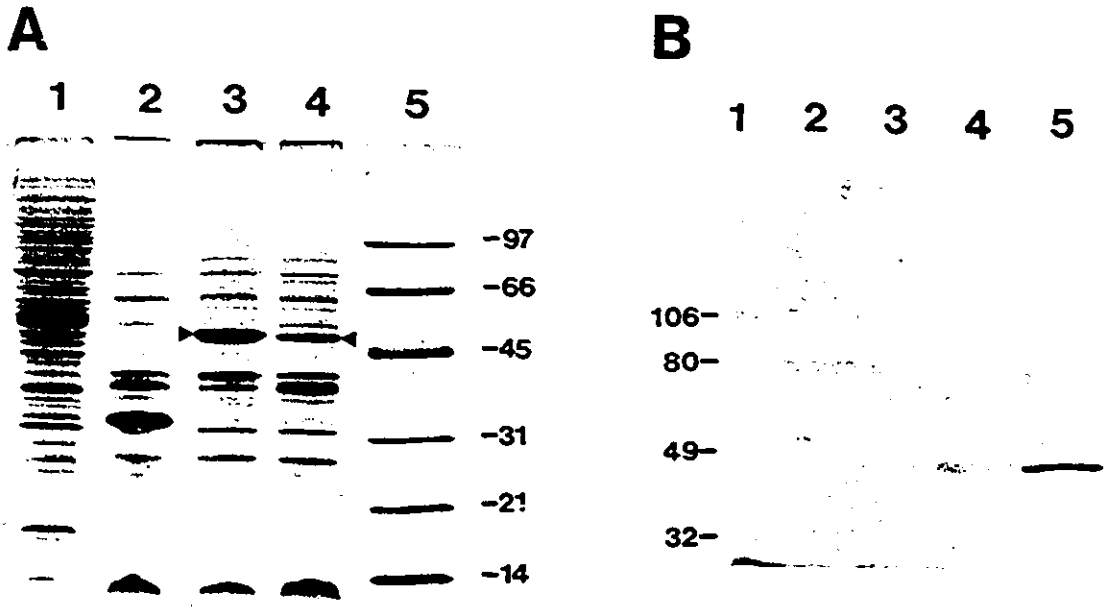

Transcription initiation
SacI
EcoRI
Translation Termination
SeoulN
KpnI
Ap
pAcYM1-Hu/SeoulN
11.4 kb
Transcription Termination
EcoRI
baculovirus DNA (AcMNPV)
co-transfection
cytoplasm
nucleus
homologous
recombination
packaging
recombinant baculovirus
AcYM1-Hu/SeoulN

Figure 36. Analysis of proteins expressed in Sf9 cells. (A): Proteins expressed in mock infected Sf9 cells (lane 1), wild-type AcMNPV infected Sf9 cells (lane 2), recombinant AcMNPV/HantaanN virus infected Sf9 cells (lane 3) and recombinant AcMNPV-Hu/SeoulN virus infected Sf9 cells (lane 4). Low-molecular weight markers (Bio-Rad) were run in lane 5. (B): Immuno-blot staining of mock infected Sf9 cells (lane 2), wild-type AcMNPV infected Sf9 cells (lane 3), recombinant AcMNPV/HantaanN virus infected Sf9 cells (lane 4) and recombinant AcMNPV-Hu/SeoulN virus infected Sf9 cells (lane 5) with the serum of a Seoul virus infected patient. Pre-stained low-molecular weight markers (Bio-Rad) are shown in lane 1. Molecular mass of each marker (in kilo daltons) is shown on the right side of the gel and the left side of the blot.


A
1
2
3
4
5
-97
-66
-45
-31
-21
-14
B
1
2
3
4
5
106-
80-
49-
32-

infected and recombinant virus infected cell lysates were electrophoresed, electroblotted onto nylon membrane and immuno-stained with the convalescent serum of a patient infected with Seoul virus. Recombinant viruses expressed a large amount of products (Fig. 36A, lanes 3 and 4) that had the same characteristics as the native viral N proteins.

DISCUSSION

The objective of this study was to express nucleocapsid proteins of Hantaan 76-118 and Seoul 80-39 virus. To obtain high levels of expression, the baculovirus expression system was employed.

Proteins of approximately 48 kD were expressed in Sf9 cells infected with recombinant baculoviruses (Fig. 36A, lanes 3 and 4). Large quantities of proteins were obtained and their specificity was confirmed by Western blot analysis (Fig. 36B, lanes 4 and 5). Approximately 80 μg of Hantaan N protein and 55 μg of Seoul N protein were obtained from $6x10^6$ cells. The observed difference in the level of expression of these two proteins can be attributed either to the small difference in nature of these two proteins or to the fact that their translation initiation codons were not positioned at the same distance from the promoter. Since the Seoul N gene was inserted into the multiple cloning site of pAcYM1-Hu, it had 12 extra nucleotides in between the translation initiation codon and the promoter, compared to the Hantaan N gene which was inserted into a unique *Bam*HI site of pAcYM1. However, regardless of the observed difference, the level

of expression for both proteins is sufficiently high for use in future purification and functional studies.

CHAPTER 6 CONCLUSIONS

The first objective of this study was to elucidate the genome organization of the Seoul 80-39 virus. A comparison of the molecular characteristics of the Seoul 80-39 virus with other members of the *Hantavirus* genus will provide a better understanding of the evolutionary relationships within this new genus of the *Bunyaviridae* family.

The strategy for determining molecular characteristics of Seoul 80-39 virus was to generate a cDNA library from viral genomic RNA. Fourteen groups of clones were identified by cross-hybridization and their segment assignments were determined by Northern blot hybridization. The remaining parts of the genome were cloned by the reverse transcription-polymerase chain reaction. Sequencing of virus-specific cDNA clones revealed the coding capacity of all three genomic RNA segments (L, M and S) of the Seoul 80-39 virus. All three segments have a single open reading frame in virus-complementary RNA sense. The L genomic RNA segment encodes a large (L) protein which is an RNA-dependent RNA polymerase molecule. The M genomic RNA segment encodes a polypeptide which is cotranslationally processed into two viral glycoproteins G1 and G2 and the S genomic RNA segment encodes a nucleocapsid (N) protein. Out of 11950 nucleotides in the Seoul virus genome, only 802 nucleotides are noncoding. The same coding strategy has been observed for all members of the *Hantavirus* genus.

A comparison of nucleotide and amino acid sequences of hantaviruses

revealed two distinct evolutionary pathways which split into four branches. One pathway includes the Hantaan virus branch and Seoul virus branch, i.e. *Muridae*- (*Appodemus* and *Rattus*) borne viruses. The second pathway includes the Puumala virus and Prospect Hill virus branches, i.e. *Cricetidae*- (*Clethrionomys* and *Microtus*) borne viruses. The evolution of these viruses was probably influenced by many factors, but the most significant factor appears to be the relationship of the virus with its rodent host. That is, viruses isolated from a particular animal species (e.g. *Rattus*) are more similar to other viruses isolated from that same species than to those isolated from a different species (e.g. *Appodemus*, *Clethrionomys* or *Microtus*). At the same time, viruses isolated from one rodent family (e.g. *Muridae*) are more similar to viruses isolated from the same rodent family than to the viruses isolated from the other rodent family (e.g. *Cricetidae*). Geographic factors do not appear to have played a large role in the evolution of these viruses since Hantaan-, Puumala- and Seoul-like viruses have been isolated from rodents all over the world.

Comparisons of the structural proteins of hantaviruses have further indicated sequences which are conserved among all viruses in the genus. The degree of both nucleotide and amino acid sequence similarity identified here for cognate genes, coincides with serologically defined relationships of these viruses. By using this and similar information, it may eventually be possible to localize the antigenic sites on the viral proteins which are responsible for cross-reactive protection to hantaviral infection, or to identify gene regions important for viral

replication.

The second objective of the study was to investigate the processing of Hantaan virus glycoproteins G1 and G2 and Hantaan virus morphogenesis. Hantaan virus-infected cell lysates were used for immunoprecipitation with MAbs against glycoprotein G1 (MAbG1) or G2 (MAbG2). When cell lysates were prepared with buffer containing nonionic detergent, both G1 and G2 glycoproteins were precipitated with either MAbG1 or MAbG2. In contrast when cell lysates were prepared with a buffer containing ionic detergents, MAbG1 precipitated only glycoprotein G1 and MAbG2 precipitated only glycoprotein G2. Heterodimers and possibly higher oligomeric forms of the glycoproteins were detected on nonreducing SDS-polyacrylamide gels only after chemical cross-linking and immunoprecipitation with either MAbG1 or MAbG2. In order to determine the sites of Hantaan virus glycoproteins maturation and the G1-G2 complex formation, infected cells were treated with inhibitors that prevent specific steps of oligosaccharide processing. Furthermore, glycoproteins G1 and G2 immunoprecipitated from infected cell lysates or from isolated virus particles were tested for sensitivity to endoglycosidase H, endoglycosidase F and endoglycosidase D. The results of these experiments show that the G1 and G2 form a complex and that the complex formation occurs in the endoplasmic reticulum. Glycosylation of the G1 and G2 takes place in the endoplasmic reticulum, as well. The N-linked oligosaccharide molecules of Hantaan glycoproteins are high mannose. Therefore, unless the trimming of mannose residues from oligosaccharide chains requires the

activity of α-mannosidase I of the *cis*-Golgi, maturation of Hantaan virus glycoproteins G1 and G2 takes place in the ER. However, the glycoprotein complex is targeted to the Golgi apparatus and thin section electron microscopy studies indicated that the virus morphogenesis takes place in the *trans*-Golgi network.

The last objective was to express the nucleocapsid (N) protein in levels high enough to permit purification and characterization of protein functions. A baculovirus expression system was chosen for expression of the Hantaan and Seoul virus nucleocapsid proteins. The recombinant viruses expressed large amount of products that had the same characteristics as the native viral N proteins.

In conclusion, the main contributions of these studies have been to further the understanding of hantavirus evolution, genetic organization and morphogenesis. This work has opened possibilities and forms the basis for future functional studies of virus structures and biological activities.

LIST OF REFERENCES

Anderson, G.W.Jr., Smith, J.F. (1987) Immunoelectron microscopy of Rift Valley fever viral morphoenesis in primary rat hepatocytes. *Virology* 161, 91-100.

Antic, D., Lim, B.U., Kang, C.Y. (1991a) Molecular characterization of the M genomic segment of the Seoul 80-39 virus; nucleotide and amino acid sequence comparisons with other hantaviruses reveal the evolutionary pathway. *Virus Res.* 19, 47-58.

Antic, D., Lim, B.Y., Kang, C.Y. (1991b) Nucleotide sequence and coding capacity of the large (L) genomic RNA segment of Seoul 80-39 virus, a member of the hantavirus genus. *Virus Res.* 19, 59-66.

Antic, D., Kang, C.Y., Spik, K., Schmaljohn, C.S., Vapalahti, O., Vaheri, A. (1992a) Comparison of the deduced gene products of the L, M and S genome segments of hantaviruses. *Virus Res.* 24, 35-46.

Antic, D., Wright, K.E., Kang, C.Y. (1992b) Maturation of Hantaan virus glycoproteins G1 and G2. *Virology* 189, 324-328.

Argos, P. (1988) A sequence motif in many polymerases. *Nucleic Acids Res.* 16, 9909-9916.

Asada, H., Tamura, M., Kondo, K., Okuno, Y., Takahashi, Y., Dohi, Y., Nagai, T., Kurata, T., Yamanishi, K. (1987) Role of T lymphocyte subsets in protection and recovery from Hantaan virus infection in mice. *J. Gen. Virol.* 68, 1961-1969.

Asada, H., Tamura, M., Kondo, K., Dohi, Y., Yamanishi, K. (1988) Cell-mediated immunity to virus causing haemorrhagic fever with renal syndrome: generation of cytotoxic T lymphocytes. *J. Gen. Virol.* 69, 2179-2188.

Asada, H., Balachandra, K., Tamura, M., Kondo, K., Yamanishi, K. (1989) Cross-reactive immunity among different serotypes of virus causing haemorrhagic fever with renal syndrome. *J. Gen. Virol.* 70, 819-825.

Arikawa, J., Takashima, I., Hashimoto, N. (1985) Cell fusion by haemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. *Arch. Virol.* 86, 303-313.

Arikawa, J., Schmaljohn, A.L., Dalrymple, J.M., Schmaljohn, C.S. (1989) Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. *J. Gen. Virol.* 70, 615-624.

Arikawa, J., Lapenotiere, H.F., Iacono-Connors, L., Wang, M., Schmaljohn, C.S. (1990) Coding properties of the S and the M genome segments of Sapporo Rat Virus: comparison to other causative agents of hemorrhagic fever with renal syndrome. *Virology* 176, 114-125.

Barik, S., Rud, E.W., Luk, D., Banerjee, A.K., Kang, C.Y. (1990) Nucleotide sequence analysis of the L gene of Vesicular Stomatitis Virus (New Jersey serotype): identification of conserved domains in L proteins of nonsegmented negative-strand RNA viruses. *Virology* 175, 332-337.

Bass, B.L. and Weintraub, H. (1987) A developmentally regulated activity that unwinds RNA duplexes. *Cell* 48, 607-613.

Bass, B.L. and Weintraub, H. (1988) An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* 55, 1089-1098.

Bass, B.L., Weintraub, H., Cattaneo, R., Billeter, M.A. (1989) Biased hypermutation of viral RNA genomes could be due to unwinding/modification of double-stranded RNA. *Cell* 56, 331.

Bause, E. (1983) Structural requirements of N-glycosylation of proteins. *Biochem. J.* 209, 331-336.

Baek, L.J., Yanagihara, R., Gibbs, C.J., JR, Miyazaki, M., Gajdusek, D.C. (1988) Leaky virus: a new hantavirus isolated from Mus musculus in the United States. *J. Gen. Virol.* 69, 3129-3132.

Bishop, D.H.L. (1991) Arenaviridae and their replication. p. 567-582. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Blumberg, B.M., Crowley, J.C., Silverman, J.I., Menonna, J., Cook, S.D.,Dowling, P.C. (1988) Measles virus L protein evidences elements of an ancestral RNA polymerase. *Virology* 164, 487-497.

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S., Girard, M. (1990) Characterization of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (*Bunyavirus*). *Virology* 175, 50-58.

Cattaneo, R., Schmid, A., Exchle, D., Baczko, K., ter Meulen, V., Billeter, M.A. (1988) Biased hypermutation and other genetic changes in defective Measles viruses in human brain infections. *Cell* 55, 225-265.

Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Bacyko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Udem, S.A., Billeter, M.A. (1989) Mutated and hypermutated genes of persistent Measles viruses which caused lethal human brain diseases. *Virology* 173, 415-425.

Chen, L., Yang, W. (1990) Abnormalities of T cell immunoregulation in hemorrhagic fever with renal syndrome. *J. Infect. Dis.* 161, 1016-1019.

Chen, S.Y., Matsuoka, Y., Compans, R.W. (1991) Assembly and polarized release of Punta Toro virus and effects of brefeldin A. *J. Virol.* 65, 1427-1439.

Cheung, P., Banfield, B.W., Tufaro, F. (1991) Brefeldin A arrests the maturation and egress of Herpes Simplex virus particles during infection. *J. Virol.* 65, 1893-1904.

Chun, C.H., Laehdevirta, J., Lee, H.W. (1989) Clinical manifestations of HFRS. In: *Manual of Hemorrhagic Fever with Renal Syndrome* p. 19-38. Edited by H.W. Lee and J.M Dalrymple, WHO Collaborating Center for Virus Reference and Research.

Coffin, J.M. (1991) Retroviridae and their replication. p. 645-708. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Copeland, C.S., Doms, R.W., Bolzau, E.M., Webster, R.G., Helenius, A. (1986) Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell. Biol.* 103, 1179-1191.

Dantas, J.R. Okuno, Y., Asada, H., Tamura, M., Takahashi, M., Tanishita, O., Takahashi, Y., Kurata, T., Yamanishi, K. (1986) Characterization of glycoproteins of viruses causing hemorrhagic fever with renal syndrome (HFRS) with monoclonal antibodies. *Virology* 151, 379-384.

Doms, R.W., Ruusala, A., Machamer, C., Helenius, A., Rose, J.K. (1988) Differential effects of mutations in three domains on folding, quaternary structure and intracellular transport of vesicular stomatitis virus G protein. *J. Cell. Biol.* 107, 89-99.

Doms, R.W., Russ, G., Yewdell, J.W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell. Biol* 109, 61-72.

Earle, D.P. (1954) Symposium on epidemic hemorrhagic fever. *Am. J. Med.* 16, 619-709.

Elliott, L.H., Kiley, M.P., McCormick, J.B. (1984) Hantaan virus: identification of virion proteins. *J. Gen. Virol.* 65, 1285-1293.

Elliot, R.M. (1989) Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *Virology* 173, 426-436.

Elliot, R.M. (1990) Molecular biology of the Bunyaviridae. *J. Gen. Virol.* 71, 501-522.

Eshita, Y., Ericson, B., Romanowsky, V., Bishop, D.H.L. (1985) Analysis of the M RNA transcription processes of snowshoe hare bunyavirus S and M RNA species. *J. Virol.* 55, 681-689.

Estes, M.K. (1991) Rotaviruses and their replication. p. 619-644. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Farquar, M.G. (1985) Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1, 447-488.

Feldhaus, A.L. and Lesnaw, J.A. (1988) Nucleotide sequence of the L gene of vesicular stomatitis virus (New Jersey): Identification of conserved domains in the New Jersey and Indianna L proteins. *Virology* 163, 359-368.

Fields, S. and Winter, G. (1982) Nucleotide sequences of influenza virus segments 1 and 3 reveal mosaic structure of a small viral RNA segment. *Cell* 28, 303-313.

French, G.R., Foulke, R.S., Brand, O.A., Eddy, G.A., Lee, H.W., Lee, P.W. (1981) Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin. *Science* 211, 1046-1048.

Galinski, M.S., Mink, M.A., Pons, M.W. (1988) Molecular cloning and sequence analysis of the human parainfluenza 3 virus gene encoding the L protein. *Virology* 165, 499-510.

Garof, H., Simons, K. (1974) Location of the spike glycoproteins in the Semiliki Forest virus membrane. *Proc. Natl. Acad. Sci. USA* 71, 3988-3992.

Garof, H., Fischauf, A.M., Simons, K., Lehrach, H., Delius, H. (1980) Nucleotide sequence of cDNA coding for Semiliki Forest virus membrane glycoproteins. *Nature* 288, 236-241.

Gething, M-J., McCammon, K., Sambrook, J. (1986) Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* 46, 939-950.

Giebel, L.B., Stohwasser, R., Zöller, L., Bautz, E.K.F., Darai, G. (1989) Determination of the coding capacity of the M genome segment of Nephropathia Epidemica virus strain Hallnas B1 by molecular cloning and nucleotide sequence analysis. *Virology* 172, 498-505.

Gonzales-Scarano, F., Endres, M.J., Nathanson, N. (1991) Pathogenesis. *Curr. Top. Microbiol. Immunol.* 169, 217-249.

Gött, P., Zöller, L., Yang, S. Stohwasser, R., Bautz, E.K.F., Darai, G. (1991) Antigenicity of hantavirus nucleocapsid proteins expressed in *E. coli*. *Virus Res.* 19, 1-16.

Hacker, D., Raju, R., Kolakofsky, D. (1989) LaCrosse virus nucleocapsid protein controls its own synthesis in mosquito cells by encapsidating its mRNA. *J. Virol.* 63, 5166-5174.

Hacker, D., Rochat, S., Kolakofsky, D. (1990) Anti-mRNAs in LaCrosse Bunyavirus infected cells. *J. Virol.* 64, 5051-5057.

Holmes, K.V. (1991) Coronaviridae and their replication. p. 471-488. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Hopp, T.P. and Woods, K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* 78, 3824-3828.

Hubbard, S.C., Ivatt, R.J. (1981) Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50, 55-83.

Huggins, J.W., Hsiang, C.M., Cosgriff, T.M., Guang, M.Y., Smith, J.I., Wu, Y.O., LeDuc, J.W., Zheng, Z.M., Meegan, M.M., Wang, Q.N., Oland, D.D., Gui, X.E., Gibbs, P.H., Yuan, G.H., Zhang, T.M. (1991) Prospective, double-blind, concurrent, placebo-controlled clinical trial of intravenous ribavirin therapy of hemorrhagic fever with renal syndrome. *J. Infect. Dis.* 164, 1119-1127.

Hurtley, S.M., Helenius, A. (1989) Control of protein exit from the endoplasmic reticulum. *Ann. Rev. Cell. Biol.* 5, 277-307.

Ihara, T., Matsuura, Y., Bishop, D.H.L. (1985) Analysis of the mRNA transcription processes of Punta Toro phlebovirus (Bunyaviridae). *Virology*, 147, 317-325.

Kasahara, S., Kitano, M. (1943) Studies on pathogen of epidemic hemorrhagic fever. *Japanese Journal of Pathology* 33, 476-483.

Kim, G.R., McKee, K.T. (1985) Pathogenesis of Hantaan virus infection in suckling mice: clinical, virologic and serologic observations. *Am. J. Trop. Med. Hyg.* 34, 388-395.

Kingsbury, D.W. (1991) Paramyxoviridae and their replication. p. 507-526. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Kingsbury, D.W. (1991) Orthomyxoviridae and their replication. p. 527-544. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Kitano, M. (1944) A study of epidemic hemorrhagic fever. *Japanese Army Medical Journal* 370, 269-282.

Klausner, R.D. (1989) Sorting and traffic in the central vacuolar system. *Cell* 57, 703-706.

Klenk, H.D. and Rott, R. (1980) Cotranslational and posttranslational processing of viral glycoproteins. *Curr. Top. Microbiol. Immunol.* 90, 19-48.

Kolakofsky, D., Bellocq, C., Raju, R. (1987) The translational requirement of La Crosse virus S-mRNA synthesis. *Cold Spring Harbor Symp. Quant. Biol.* 52, 373-379.

Kolakofsky, D. and Hacker, D. (1991) Bunyavirus RNA synthesis: genome transcription and replication. *Curr. Top. Microbiol. Immunol.* 169, 143-159.

Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 54, 631-664.

Kreis, T.E., Lodish, H.F. (1986) Oligomerization is essential for transport of vesicular stomatitis virus viral glycoprotein on the cell surface. *Cell* 46, 929-937.

Kuismanen, E., Hedman, K., Saraste, J., Pettersson, R.F. (1982) Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. *Mol. Cell. Biol.* 2, 1444-1458.

Kuismanen, E. (1984) Posttranslational processing of Uukuniemi virus glycoproteins G1 and G2. *J. Virol.* 51, 806-812.

Kuismanen, E., Bang, G., Hurme, M., Pettersson, R.F. (1984) Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* 51, 137-146.

Kuismanen, E., saraste, J., Pettersson, R.F. (1985) Effect of monensin on the assembly of Uukuniemi virus in the Golgi complex. *J. Virol.* 55, 813-822.

LeDuc, J.W., Smith, G.A., Childs, J.E. (1986) Global survey of antibody to Hantaan-related viruses among peridomestic rodents. *Bull. WHO* 64, 139-144.

Lee, H.W., Lee, P.W. (1976) Korean hemorrhagic fever. I. Demonstration of causative antigen and antibodies. *Korean Journal of International Medicine* 19, 371-383.

Lee, H.W., Lee, P.W., Johnson, K.M. (1978) Isolation of the Etiologic Agent of Korean Hemorrhagic Fever. *J. Infect. Dis.* 137, 298-308.

Lee, H.W., French, G.R., Lee, P.W., Baek, L.J., Tsuchiya, K., Foulke, R.S. (1981a) Observations on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever. *Am. J. Trop. Med. Hyg.* 30, 477-482.

Lee, H.W., Lee, P.W., Baek, L.J., Song, C.K., Seong, I.W. (1981b) Intraspecific transmission of Hantaan virus, the etiologic agent of Korean hemorrhagic fever in the rodent *Apodemus agrarius*. *Am. J. Trop. Med. Hyg.* 30, 1106-1112.

Lee, H.W., Johnson, K.M. (1982) Laboratory-acquired infections with Hantaan virus, the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* 146, 645-651.

Lee, P.W., Gibbs, C.J., Jr., Gajdusek, D.C., Yanagihara, R. (1985) Serotypic classification of hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. *J. Clin. Microbiol.* 22, 940-944.

Lee, H.W. (1989) Epidemiology. In: *Manual of Hemorrhagic Fever with Renal Syndrome* p. 39-48. Edited by H.W. Lee and J.M Dalrymple, WHO Collaborating Center for Virus Reference and Research

Lee, H.W. (1989) Hemorrhagic fever with renal syndrome. *Prog. Med. Virol.* 36, 62-102.

Linderholm, M., Settergren, B., Ahlm, C., Burman, L-A., Träff, S., Bäcklund, U., Juto, P. (1991) A swedish fatal case of nephropathia epidemica. *Scand. J. Infect. Dis.* 23, 501-502.

Maass, D.R. and Atkinson, P.H. (1990) Rotavirus proteins VP7, NS28 and VP4 form oligomeric structures. *J. Virol.* 64, 2632-2641.

Machamer, C.E., Rose, J.K. (1987) A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. *J. Cell. Biol.* 105, 1205-1214.

Madoff, D.H. and Lenard, J. (1982) A membrane glycoprotein that accumulates intracellularly: cellular processing of the large glycoprotein of LaCrosse virus. *Cell* 28, 821-829.

Matsuoka, Y., Chen, S.Y., Compans, R.W. (1991) Bunyavirus protein transport and assembly. *Curr. Top. Microbiol. Immunol.* 169, 161-179.

McKee, K.T. Lim, G.R., Green, D.E., Peters, C.J. (1985) Hantaan virus infection in suckling mice: virologic and pathologic correlates. *J. Med. Virol.* 17, 107-117.

Messing, J. (1983) New M13 vectors for cloning. In: *Methods in Enzymology* 101, p. 20-78. Edited by R. Wu, L. Grossman and K. Moldave. Academic Press, New York.

Morgan, E.M. and Rakestraw, K.M. (1986) Sequence of the Sendai L gene: Open reading frames upstream of the main coding region suggest that the gene may be polycistronic. *Virology* 154, 31-40.

Murphy, D.G., Dimock, K., Kang, C.Y. (1990) Viral RNA and protein synthesis in two LLC-MK2 cell lines persistently infected with human parainfluenza virus 3. *Virus Res.* 16, 1-16.

Nakamura, T., Yanagihara, R., Gibbs, C.J., Amyx, H.L., Gajdusek, D.C. (1985a) Differential susceptibility and resistance of immunocompetent and immunodeficient mice to fatal Hantaan virus infection. *Arch. Virol.* 86, 109-120.

Nakamura, T., Yanagihara, R., Gibbs, C.J., Gajdusek, D.C. (1985b) Immune spleen cell-mediated protection against fatal Hantaan virus infection in infant mice. *J. Infect. Dis.* 151, 691-697.

Niklasson, B. and Le Duc, J.M. (1984) Isolation of the nephropathia epidemica agent in Sweden. *Lancet i*, 1012-1013.

Okuno, Y., Yamanishi, K., Takahashi, Y., Tanishita, O., Nagai, T., Dantas, J.R.Jr., Okamoto, Y., Tadano, M., Takahashi, M. (1986) Haemaglutination-inhibition test for haemorrhagic fever with renal syndrome using viral antigen prepared from infected tissue culture fluid. *J. Gen. Virol.* 67, 149-156.

Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J.G., Lippincott-Schwartz, J., Klausner, R.D., Rothman, J. (1991) Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* 64, 1183-1195.

Parrington, M.A., Kang, C.Y. (1990) Nucleotide sequence analysis of the S genomic segment of Prospect Hill virus: Comparison with the prototype hantavirus. *Virology* 175, 167-175.

Parrington, M.A., Lee, P.W., Kang, C.Y. (1991) Molecular characterization of the Prospect Hill virus M RNA segment: a comparison with the M RNA segments of other hantaviruses. *J. Gen. Virol.* 72, 1845-1854.

Patterson, J.L., Kolakofsky, D. (1984) Characterization of La Crosse virus small-genome transcripts. *J. Virol.* 49, 680-685.

Pelham, H.R.B. (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* 7, 913-918.

Pelham, H.R.B. (1989) Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell. Biol.* 5, 1-23.

Pensiero, M.N., Jennings, G.B., Schmaljohn, C.S., Hay., J. (1988) Expression of the Hantaan virus M genome segment by using a Vaccinia virus recombinant. *J. Virol.* 62, 696-702.

Persson, R., Pettersson, R.F. (1991) Formation and intracellular transport of a heterodimeric viral spike protein complex. *J. Cell. Biol.* 112, 257-266.

Pettersson, R.F. (1991) Protein localization and virus assembly at intracellular membranes. *Curr. Top. Microbiol. Immunol.* 170,

Raju, R., Kolakofsky, D. (1989) The ends of La Crosse virus genome and antigenome RNAs within the nucleocapsids are base paired. *J. Virol.* 63, 122-128.

Rose, J.K., Doms, R.W. (1988) Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell. Biol.* 4, 257-288.

Ruusala, A., Schmaljohn, C.S., Pettersson, R.F. (1990) Intracellular transport of Hantaan virus glycoproteins G1 and G2 expressed from Vaccinia virus vectors. *VIIIth International Congress of Virology*, Abstract P44-010, p. 363.

Ruusala, A., Persson, R., Schmaljohn, C.S., Pettersson, R.F. (1992) Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. *Virology* 186, 53-64.

Saiki, R.K., Gelfand, D.H., Stoffer, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491.

Salvato, M., Shimomaye, E., Oldstone, M.B.A. (1989) The primary structure of the lymphocytic choriomeningitis virus L gene encodes a putative RNA polymerase. V *Virology* 169, 377-384.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning* a laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S., Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, 74, 5463-5467.

Schmaljohn, C.S., Hasty, S.E., Harrison, S.A., Dalrymple, J.M. (1983a) Characterization of hantaan virions, the prototype virus of hemorrhagic fever with renal syndrome. *J. Infect. Dis.* 148 (6), 1005-1011.

Schmaljohn, C.S., Dalrymple, J.M. (1983b) Analysis of Hantaan virus RNA: evidence for a new genus of Bunyaviridae. *Virology* 131, 482-491.

Schmaljohn, C.S., Hasty, S.E., Dalrymple, J.M., LeDuc, J.W. (1985) Antigenic and ggenetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science* 227, 1041-1044.

Schmaljohn, C.S., Hasty, S.E., Rasmussen, L., Dalrymple, J.M. (1986a) Hantaan virus replication: effects of monensin, tunicamycin and endoglycosidases on the structural glycoproteins. *J. Gen. Virol.* 67, 707-717.

Schmaljohn, C.S., Jennings, G.B., Hay, J., Dalrymple, J.M. (1986b) Coding strategy of the S genome segment of Hantaan virus. *Virology* 155, 633-643.

Schmaljohn, C.S., Schmaljohn, A.L., Dalrymple, J.M. (1987a) Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order. *Virology* 157, 31-39.

Schmaljohn, C.S., Lee, H.W., Darlymple, J.M. (1987b) Detection of hantaviruses with RNA probes generated from recombinant DNA. *Arch. Virol.* 95, 291-301.

Schmaljohn, C.S., Arikawa, J., Hasty, S.E., rasmussen, L., Lee, H.W., Lee, P.W., Dalrymple, J.M. (1988) Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. *J. Gen. Virol.* 69, 1949-1955.

Schmaljohn, C.S., Arikawa, J., Dalrymple, J.M., Schmaljohn, A.L. (1989) Expression of the envelope glycoproteins of Hantaan virus with vaccinia and baculovirus recombinants. (1989). In: *Genetics and Pathogenicity of Negative Strand Viruses.* Elsevier Science Publishers BV. 58-66.

Schmaljohn, C.S., Chu, Y-K., Schmaljohn, A.L., Dalrymple, J.M. (1990) Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J. Virol.* 64, 3162-3170.

Schmaljohn, C.S. (1990) Nucleotide sequence of the L genome segment of Hantaan virus. *Nucl. Acids Res.* 18, 6728.

Schmaljohn, C.S. and Patterson, J.L. (1991) Bunyaviridae and their replication. p. 545-566. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe et al. Raven Press, New York.

Schlesinger, S. and Schlesinger M.J. (1991) Replication of Togaviridae and Flaviviridae. p. 453-470. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Schubert, M., Harmison, G.G., Meier, E. (1984) Primary structure of the vesicular stomatitis virus polymerase (L) gene: evidence for high frequency of mutations. *J. Virol.* 51, 505-514.

Sheshberadaran, H., Niklasson, B., Tkachenko, E. (1988) Antigenic relationship between Hantaviruses analysed by immunoprecipitation. *J. Gen. Virol.* 69, 2645-2651.

Smadel, J.E. (1953) Epidemic hemorrhagic fever. *Am. J. Public Health* 43, 1327-1330.

Smith, J.F., Pifat, D.Y. (1982) Morphogenesis of sandfly fever viruses (Bunyaviridae family). *Virology* 121, 61-81.

Smorodintsev A.A., Chumakov, V.G., Churilov, A.V. (1959) In: *Haemorrhagic nephrosonephritis.* New York, Pergamon Press, p 120.

Stohwasser, R., Giebel, L.B., Zöller, L., Bautz, E.K.F., Darai, G. (1989) Molecular characterization of the RNA S segment of Nephropathia Epidemica virus strain Hallnas B1. *Virology* 174, 79-86.

Stohwasser, R., Raab, K., Darai, G., Bautz, E.K. (1991) Primary structure of the large (L) RNA segment of Nephropathia Epidemica virus strain Hallnas B1 coding for the viral RNA polymerase. *Virology* 183, 386-391.

Strauss, E.G., Strauss, J.H. (1986) Structure and replication of the alphavirus genome. In: *The togaviridae and flaviviridae*, p35-90. Eds. Schlesinger, S., Schlesinger, M.J. Plenum Press, New York.

Sturman, L.S., Holmes, K.V., Behnke, J. (1980) Isolation of coronavirus envelope glycoprotein with the viral nucleocapsid. *J. Virol.* 33, 449-462.

Summers, M.D., Smith, G.E. (1987) *A manual of methods for baculovirus vectors and insect cell culture procedures.* College Station: Texas Agricultural Experiment Station.

Tamura, M., Asada, H., Kondo, K., Tanishita, O., Kurata, T., Yamanishi, K. (1989) Pathogenesis of Hantaan virus in mice. *J. Gen. Virol.* 70, 2897-2906.

Tanishita, O., Tsakahashi, Y., Okuno, Y. (1986) Persistent infection of rats with hemorrhagic fever with renal syndrome virus and their antibody responses. *J. Gen. Virol.* 67, 2819-2824.

Tao, H., Xia, S.-M., Song, G., Liao, H.-X., Chao, T.-X., Chou, Z.-Y., Hang, C.-S. (1983a) Viruses of classical and mild forms of haemorrhagic fever with renal syndrome isolated in China have similar Bunyavirus-like morphology. *Lancet* 589-591.

Tao, H., Xia, S.-M., Zhao, T.X., Zhou, J.Y., Song, G., Liao, G.X.H., Ye, W.W., Chu, Y.L., Hang, C.S. (1983b) Morphological evidence for identifying the viruses of hemorrhagic fever with renal syndrome as candidate members of the Bunyaviridae family. *Arch. Virol.* 78, 137-144.

Tao, H., Chou, Z.-Y., Zhao, T.X., Semao, X., Hang, C.S. (1985) Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome (HFRS). *Intervirol.* 23, 97-108.

Tao, H., Semao X., Chou, Z.-Y., Gan, S., Yanagihara, R. (1987) Morphology and morphogenesis of viruses of hemorrhagic gever with renal syndrome. *Intervirol.* 27, 45-52.

Tautz, D., and Renz, M. (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Anal. Biochem.* 132, 14-19.

Tordo, N., Poch, O., Ermine, A., Keith, G., Rougeon, F. (1988) Completion of the rabies virus genome sequence determination: Highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. *Virology* 165, 565-576.

Tsai, T.F., Tang, Y.W., Hu, S.L., Ye, K.L., Chen, .L., Xu, Z.Y. (1984) Hemagglutination-inhibiting antibody in hemorrhaic fever with renal syndrome. *J. Infect. Dis.* 150, 895-898.

von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* 133, 17-21.

Wagner, R.W. and Nishikura, K. (1988) Cell cycle expression of RNA duplex unwindase activity in mammalian cells. *Mol. Cell. Biol.* 8, 770-777.

Wagner, R.W. (1991) Rhabdoviridae and their replication. p. 489-506. In: *Fundamental Virology*. 2nd ed. Edited by: B.N. Fields and D.M. Knipe. Raven Press, New York.

Whealy, M.E., Card, J.P., Meade, R.P., Robbins, A.K., Enquist, L.W. (1991) Effect of brefeldin A on Alphaherpesvirus membrane protein glycosylation and virus egress. *J. Virol.* 65, 1066-1081.

Winter, G. and Fields, S. (1982) Nucleotide sequence of human influenza A/PR/8/34 segment 2. *Nucl. Acids Res.* 10, 2135-2143.

Wold, F. (1981) In vivo chemical modification of proteins. *Ann. Rev. Biochem.* 50, 783-814.

Wong, R.C., Ayata, M., Hirano, A., Yoshikawa, Y., Tsuruoka, H., Yamanouchi, K. (1989) Generalized and localized biased hypermutation affecting the matrix gene of a Measles virus strain that causes subacute sclerosing panencephalitis. *J. Virol.* 63, 5464-5468.

Yanagihara, R., Svedmyr, A., Amyx, H.L. (1984) Isolation and propagation of nephropathia epidemica virus in bank voles. *Scand. J. Infect. Dis.* 16, 225-228.

Yanagihara, R., Daum, C.A., Lee, P.W. (1987) Serological survey of Prospect Hill virus infection in indigenous wild rodents in the USA. *Trans. R. Soc. Trop. Med. Hyg.* 81, 42-45.

Yanagihara, R., Amyx, H.L., Lee, P.W., Asher, D.M., Gibbs, C.J., Gajdusek, D.C. (1988) Experimental hantavirus infection in nonhuman primates. *Arch. Virol.* **10**, 125-130.

Yoo, D.W. and Kang, C.Y. (1987a) Genomic comparisons among members of hantavirus group. In: *The Biology of Negative Strand Viruses* 424-431. Edited by B. W. J. Mahy and D. Kolakofsky. Elsevier. New York.

Yoo, D.W. and Kang, C.Y. (1987b) Nucleotide sequence of the M segment of the genomic RNA of Hantaan virus 76-118. *Nucl. Acids Res.* **15**, 6299-6300.

Yusoff, K., Millar, N.S., Chambers, P., Emmerson, P.T. (1987) Nucleotide sequence analysis of the L gene of Newcastle disease virus: Homologies with Sendai and vesicular stomatitis viruses. *Nucl. Acids Res.* **15**, 3961-3976.

Zhang, X.K., Takashima, I., Hashimoto, N. (1989) Characteristics of passive immunity against hantavirus infections in rats. *Arch. Virol.* **105**, 235-246.

Zöller, L., Scholz, J., Stohwasser, R., Giebel, L.B., Sethi, K. K., Bautz, E.K.F., Darai, G. (1989) Immunoblot analysis of the serological response in hantavirus infections. *J. Med. Virol.* **27**, 231-237.

APPENDIX OLIGONUCLEOTIDE SEQUENCES

SEQUENCING PRIMERS

S segment sequencing primers

SF1	5'- GAG AGT GTC GCA GCT - 3'
SR1	5'- ATG ATG TTA GAT AGA CA - 3'

M segment sequencing primers

W-1	5'- CTG AGT ATT GAT TCC TG - 3'
W-2	5'- GAG ACT TGT AAT AGT AT - 3'
W-3	5'- GAG AGT GAA GTC AGC T - 3'
W-4	5'- CAC CAA TGC CTT GAC T - 3'
W-5	5'- ATA GTA GAG TGT CAC CT - 3'
W-6	5'- CAC GTC TAC CTG TCA CT - 3'
W-10	5'- CTG ACC ATT ACA GTA - 3'
W-11	5'- GAG CTC ACT GTA TGA G - 3'
W-12	5'- GAC TGC CTC ATA GTA TC - 3'

L segment sequencing primers

V1	5'- CAT ACT ATC GTG ATG T - 3'
V2	5'- TAG GAG CAT CAC CTC A - 3'
V3	5'- TGC TAA TAG TAT CTT A - 3'

V4	5'- ATC TAC AGT TGG TGC A - 3'
V5	5'- ATA TTG GTC AAT CCA TG - 3'
V6	5'- GCA CAT TCT GGA TTG A - 3'
V7	5'- TGT GAT TAG GCT AAC AC - 3'
V8	5'- AGA CAG CGA GGA GCC AAT - 3'
V3a	5'- TGC TCA TCA TTC AGA TGA - 3'
V3b	5'- CTG TCA TCA GGC AGT C - 3'
U1	5'- AGA CTA TTT ACT GTA CTA - 3'
U2	5'- GCA CTC TTA TAC AAC ATA - 3'
U3	5'- GTC AGA CAT ACC TAT A - 3'
.U4	5'- TCT ACA AGC TCT CCT AT - 3'
U5	5'- CAG TGC GAC ACG CAT GAC - 3'
U6	5'- TAT AAT ACA CAT AGA CT - 3'
U7	5'- TAC TCA TCC AAT GCA CTG TAT - 3'
U8	5'- TGA ACG ACA CCA TCA T - 3'
U3a	5'- ATG TGT TCA TGC AGA T - 3'
U2a	5'- TCA TAG CTC TCA GCA - 3'

REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION PRIMERS

S segment

S3'	5'- CAG CTG CAG TAG TAG TAG ACT CCC TAA AGA GCT A -3'
S2	5'- CGC ACT GCA GTA TGT GGA CTA TAT - 3'
SS	5'- CTC GAG CTC TAT AAA TAT GGA GGA AAT CCA GAG AGA - 3'
S1	5'- ACA TAC TGC AGT GCG GAA TCT - 3'
S2a	5'- CAG CTG CAG AGA TCT GAG CCA GGC TAC - 3'
ST	5'- ACC GGT ACC TTA TAA TTT CAT AGG TTC CTG - 3'

M segment

DA1	5'- CAG CTG CAG TAG TAG TAG ACT CCG CAA GA - 3'
DA9	5'- CAG CTG CAG TGT GAG ATC TGT AAG TAC - 3'
DA14	5'- CAG CTG CAG AGT GTG ACA CTC TCA CGA - 3'
DA11	5'- CAG CTG CAG CAT GTT GCA TGA GCT CTC - 3'
DA12	5'- CAG CTG CAG TCA AGG TCT GTA TGC ATA - 3'
DA2	5'- CAG CTG CAG TAG TAG TAG A - 3'

L segment

L3'	5'- CAG CTG CAG TAG TAG TAG ACT CCG GAA GAG ACA A - 3'
L2	5'- CAG CTG CAG TGC AGA GGC AAT CTG TGC ATA CTG - 3'
L1	5'- CAG CTG CAG TCA TCA GGA CTG CTA GAG TAC T - 3'

L8	5'- CAG CTG CAG ATC TCA TGT ATT GAA CGA CAC CAT - 3'
PL1	5'- CAG CTG CAG CTG TCC TCA ATG CAC AGT - 3'
DA2	5'- CAG CTG CAG TAG TAG TAG A - 3'