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Canada
MOLECULAR CHARACTERIZATION AND REPLICATION STRATEGY
OF HANTAVIRUS

A Thesis Submitted to
The School of Graduate Studies and Research
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In Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology
School of Medicine

by

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ABSTRACT

Hantaan virus strain 76-118, a prototype virus causing hemorrhagic fever with renal syndrome (HFRS) previously known as Korean hemorrhagic fever (KHF), and serologically related viruses are widely distributed through the world. Genomic analysis demonstrated that hantaviruses possess three species of RNA in the virions. The large (L) RNA segment of Hantaan virus was estimated to have a molecular weight of $2.2 \times 10^6$, and the molecular weights of the middle (M) and small (S) RNA segments were estimated to be $1.25 \times 10^6$ and $0.6 \times 10^6$, respectively. When genomic RNAs of three other hantavirus isolates, Prospect Hill, USSR cg38-83, and ROK 83-109, were compared, similar but distinct tripartite genomic RNA profiles were observed. The molar ratios of the three genomic RNA segments of hantaviruses were not equal. The molar ratios of L : M : S RNA segments were $1 : 1.6 : 2.2$, $1 : 3.3 : 6.3$, and $1 : 2.2 : 3.6$ for Hantaan virus strain 76-118, Prospect Hill, and USSR cg38-83, respectively. Four structural proteins of Hantaan virus with estimated molecular weights of 180 K, 70 K, 55 K and 44 K, were identified by SDS-PAGE. These four proteins correspond well to the structural proteins of bunyaviruses. These proteins were believed to be the RNA polymerase (L), two glycoproteins (G1 and G2) and nucleocapsid protein (N) of hantavirus, respectively. Structural features; a tripartite RNA genome and four
structural proteins suggest that Hantaviruses belong to the family Bunyaviridae.

The cDNA clone banks were constructed using random primers as well as specific primers representing the 3' terminal sequences of the genomic segments. Clones were grouped by Southern blot-cross hybridization and assigned to each genomic segment by Northern blot hybridization. The 5' terminal clones of the M and S RNAs were constructed by dCTP tailing and oligo (dG) priming using two oligonucleotides deduced from nucleotides 3545–3500 of the M RNA and 1585–1600 of the S RNA. The clones containing the 3' terminal sequence were screened by oligonucleotide hybridization using labeled primers as probes. Physical maps of the cDNA clones representing the M and S RNAs were constructed on the basis of cross hybridization and Northern blot analyses. Complete nucleotide sequences of the M and S RNAs were determined, using cDNA clones representing the genomic RNA segments, by the dideoxy-nucleotide chain termination method. As with other bunyaviruses, inverted complementary sequences of 20 nucleotides and 22 nucleotides in length were present at both termini of the M and S RNA, respectively. Two mismatches were seen in the complementary termini for the M RNA and three mismatches were observed for the S RNA. The M RNA segment of Hantaan virus contains 3616 nucleotides with a single large open reading frame in the antigenomic sense. This large open reading frame was capable of encoding a
polypeptide of 1135 amino acids (126 KDa). This putative gene product is a possible precursor of the G1 and G2 viral envelope glycoproteins. Seven potential asparagine-linked glycosylation sites were found to be scattered throughout the predicted protein sequence. A hydropathy plot suggested that the typical signal sequence were located at both the G1 and G2 proteins. Transmembrane anchor domains were present at carboxy termini of both the G1 and G2 proteins. These features of both the G1 and G2 proteins resemble the properties of a typical glycoprotein. No other open reading frames were found which would produce any functional proteins.

The complete sequence of the S RNA was 1694 nucleotides long. One large open reading frame was found in the viral complementary sequence. This reading frame is capable of encoding a polypeptide of 367 amino acids with a molecular weight of 41 K. This polypeptide is a possible nucleocapsid protein. A region encoding a nonstructural protein, characteristics of certain bunyaviruses, does not seem to exist on the S RNA segment of Hantaan virus.

Two viral specific intracellular RNAs in subgenomic length were identified using strand-specific cDNA probes. The polarities of these two intracellular RNAs were complementary to the M and S genomic RNAs. This observation is consistent with the nucleotide sequence data which revealed a single large open reading frame in the antigenomic sense RNA of both the M and S RNA of Hantaan virus.
A full length cDNA clone specific for the mRNA was constructed and subsequently modified, to remove the non-coding flanking sequences and to contain a trinucleotide ACC/mmediately upstream of the translation-initiating ATG codon of the glycoprotein gene, using oligonucleotide-directed mutagenesis. The modified glycoprotein gene was subcloned into a baculovirus transfer vector pAcYM1. Following cotransfection of Spodoptera frugiperda cells with the recombinant transfer vector and the DNA of Autographa californica nuclear polyhedrosis virus (ACNPV), polyhedrin-negative recombinant baculovirus was isolated. S. frugiperda cells infected with the recombinant baculovirus synthesized the G1 and G2 envelope proteins of Hantaan virus. Furthermore, the glycoprotein precursor was cleaved in S. frugiperda cells.
ACKNOWLEDGEMENTS

All the glory to God.

I wish to express my deepest gratitude to Dr. C. Y. Kang for his guidance and encouragement throughout this research project. Also, without his financial support this study would not have been achieved.

I would like to give my special appreciation to Dr. K. Dimock for his assistance, discussion and fruitful advice.

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Last but not least, I would like to acknowledge the Hankook Yakult Institute for their scholarship support during the course of this study.
DEDICATION

This thesis is dedicated to
HeiRyun and my mother YoungOk Ahn
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GLOSSARY OF ABBREVIATIONS

b  base
beta-gal  beta-galactosidase
bp  base pair
C  degree in Celsius
cDNA  complementary deoxyribonucleic acid
Ci  Curie
cpm  counts per minute
CsCl  cesium chloride
Da  dalton
DEP  diethylopyrocarbonate
DMF  N, N-dimethylformamide
DMSO  dimethylsulfoxide
dNTP  deoxynucleotide triphosphate
ddNTP  dideoxynucleotide triphosphate
ds  double-stranded
DTT  dithiothreitol
EDTA  ethylene diamine tetraacetic acid
EtBr  ethidium bromide
EtOH  ethyl alcohol
Hantaan  strain 76-118 of hantaviruses
HFRS  hemorrhagic fever with renal syndrome
hr  hour
IPTG  isopropyl-1-thio-beta-D-galactoside
K  kilo (X 1000)
L  large RNA segment
M  medium RNA segment
min  minute
moi  multiplicity of infection
mol. wt.  molecular weight
mRNA  messenger ribonucleic acid
NS_M  nonstructural protein encoded by M RNA
NS_S  nonstructural protein encoded by S RNA
NTP  ribonucleotide triphosphate
PBS  phosphate-buffered saline
PEG  polyethylene glycol
pfu  plaque forming unit
PPO  2,5-diphenyloxazole
RF DNA  replicative form of bacteriophage M13 DNA
rRNA  ribosomal ribonucleic acid
S  small RNA segment
SDS  sodium dodecylsulfate
ss  single-stranded
SSC  standard saline citrate
TCA  trichloroacetic acid
ttS  temperature sensitive
vRNA  viral ribonucleic acid
vcrRNA  complementary viral ribonucleic acid
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactoside
CHAPTER 1: INTRODUCTION

1.1 Literature review of the Hantaan Group of Viruses

1.1.1 Historical Background of Hemorrhagic Fever with Renal Syndrome

Hemorrhagic fever is a term that first came into general use during the Korean War (1950-1953) when over 3,000 United Nations troops developed the disease and were hospitalized with capillary hemorrhages and acute renal failure (Smadel 1953). This disease has become known as Korean hemorrhagic fever (KHF). Diseases similar to KHF had been reported as Songo fever or epidemic hemorrhagic fever (EHF) in China (Ishii et al. 1942), as hemorrhagic nephroso-nephritis in the Soviet Union (Smorodintsev et al. 1944), as nephropathia epidemica (NE) in Scandinavian countries (Myhrman 1951), and as epidemic nephritis in Eastern Europe (Gajdusek 1962). In spite of much effort, little was known of the etiologic agent of the disease until, in a major breakthrough, Lee H. et al. (1978) reported the isolation of the long-sought causative agent of Korean hemorrhagic fever from the pulmonary tissues of the striped field mouse, Apodemus agrarius, captured in the KHF endemic area. The agent was named Hantaan virus after the river which runs through the endemic region near the demilitarized zone in Korea. Virus isolation has made it possible to study the nature of the disease, the possible vectors, and its relation to similar clinical syndromes occurring elsewhere in the world. The World Health
Organization (1982) recommended the name hemorrhagic fever with renal syndrome (HFRS) for Korean hemorrhagic fever and closely related diseases with an associated renal syndrome. The prototype virus Hantaan strain 76-118 was adapted to Fisher and Wistar rats (Lee P. et al. 1981c), and was subsequently adapted to growth in two continuous cell lines, A549 human lung carcinoma cells (French et al. 1981) and Vero E6 cells (McCormick et al. 1982). In vitro propagation of Hantaan virus was quickly followed by development of an immunofluorescent antibody assay for serological diagnosis and sero-epidemiological studies.

1.1.2. Epidemiology of Hantaan and Related Viruses

A number of viruses antigenically related to but distinguishable from Hantaan virus strain 76-118 have been isolated from various geographic regions and reservoir hosts (Table 1). Three epidemiological types of hantaviruses (a collective name of Hantaan and serologically related viruses, Desmyter et al. 1984, Schmaljohn et al. 1985) have been reported according to the outbreak and the host. These are rural, urban, and experimental animal types (Lee H. 1982a). Among 9 species of field rodents in the rural endemic area in Korea, only Apodemus mouse species harbor Hantaan virus (Lee H. et al. 1978, 1981a). Puumala and Hallnas strains were isolated from the lungs of bank voles (Clethrionomys glareolus) in Finland and Sweden (Brummen-Korvenkontio et al. 1980, Yanagihara et al. 1984). Discontinued distribution of
<table>
<thead>
<tr>
<th>Virus</th>
<th>Host</th>
<th>Location</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hantaan 76-118 1</td>
<td>Apodemus</td>
<td>Korea</td>
<td>severe KHF</td>
</tr>
<tr>
<td>ROK 83-109 2</td>
<td>Human</td>
<td>Korea</td>
<td>severe KHF</td>
</tr>
<tr>
<td>Urban Rat 80-39 3</td>
<td>Rattus</td>
<td>Korea</td>
<td>mild KHF</td>
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<tr>
<td>Tchoupitoulas 4</td>
<td>Rattus</td>
<td>LA, USA</td>
<td>?</td>
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<tr>
<td>Girard Point 5</td>
<td>Rattus</td>
<td>PA, USA</td>
<td>?</td>
</tr>
<tr>
<td>Prospect Hill 6</td>
<td>Microtus</td>
<td>MD, USA</td>
<td>?</td>
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<tr>
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<td>Fisher Rat</td>
<td>Japan</td>
<td>HFRS</td>
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<tr>
<td>Puumala 8</td>
<td>Clethroneomys</td>
<td>Finland</td>
<td>NE</td>
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<td>Hallnas 9</td>
<td>Clethroneomys</td>
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<td>NE</td>
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<tr>
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</tr>
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</tr>
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</tr>
<tr>
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<td>Fisher rat</td>
<td>Japan</td>
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<tr>
<td>GB-B 14</td>
<td>Wistar Rat</td>
<td>England</td>
<td>HFRS</td>
</tr>
</tbody>
</table>

Table 1. Virus Isolates Associated with Hemorrhagic Fever with Renal Syndrome

Apodemus mice from Asia to Europe suggests the bank vole is a reservoir of nephropathia epidemica (NE) in Scandinavia and the Western USSR (Brummer-Korvenkontio et al. 1980, Yanagihara et al. 1985, Casal et al. 1969, Niklasson and LeDuc 1984). Virus-infected rodents excrete large amounts of virus in saliva, feces, and urine. Particularly in urine, virus is secreted for at least 12 months (Lee H. et al. 1981a). Two seasonal peaks of high incidence seem to occur, one in the late spring and a larger peak in the late fall (Earle 1954, Gauld and Graig 1954, Lee H. 1978, Xu et al. 1985). The seasonality of high transmission of virus to man is linked to increased prevalence of Hantaan virus in Apodemus mice, their reproductive stages, environmental factors, and occupational risk in man (Lee H. 1982a, 1982b). The greatest incidence of nephropathia epidemica is also observed in the late fall in northern Scandinavia with 10,000–20,000 cases annually. In this area, bank vole populations are highly cyclic, with a peak in numbers followed by a dramatic crash, about every 4 years (Johnson 1980, LeDuc 1987). Large outbreak have frequently occurred during military operations with massive field exposure (Brown 1916, Lee H. 1982b, Sutton 1985, LeDuc 1987).

A new HFRS-related virus has been identified in Microtus species captured in Prospect Hill, Maryland (Lee P. et al. 1982a, Childs et al. 1985). Immunofluorescent antibody studies and radioimmunoassay have demonstrated that this new virus is cross-reactive to but
distinguishable from other hantaviruses (Lee P. et al. 1982a, 1985a, Schmaljohn et al. 1985). Although the clinical features of Prospect Hill virus infection are unclear, the virus appears to be widely distributed among indigenous wild rodents in the USA (Childs et al. 1985, Yanagihara et al. 1987).

Many cases of HFRS have been reported in individuals who reside in urban centers (Tamura 1964, Lee H. et al. 1980). It has been concluded that domestic rats (Rattus rattus) are the source of the urban disease (Lee H. et al. 1980, Chumakov and Garvrilovskaya 1980, Lee H. et al. 1982, Song et al. 1984, Sugiyama et al. 1984). When investigations were performed at several port cities in the USA, a large number of captured rats were positive for Hantaan virus antigen and antibody. It is hypothesized that domestic rats infected with a hantavirus may have disseminated through international shipping. By indirect fluorescent antibody techniques (IFA) and plaque reduction neutralization tests (PRNT), the urban rat hantaviruses were different from Hantaan virus (Tsai et al. 1982, LeDuc et al. 1982, Lee H. et al. 1980, Lee P. et al. 1985c).

Laboratory-associated outbreaks of HFRS have been documented among laboratory staff. An outbreak in a Moscow institute involving 82 laboratory personnel was related to introduction of large numbers of voles captured in an endemic area (Casals et al. 1966). Laboratory-acquired infections have been reported in Japan and Korea (Umenai
1979, Lee and Johnson 1982). Antibodies against Hantaan virus strain 76-118 have been demonstrated both in patients and in the rats they handled. Infected laboratory rats are found in Japan not only in scientific institutions but also in commercial distribution. The infection pattern indicates that transmission is due to infectious aerosols. Unlike other bunyaviruses, the rodent vector with long-term viruria and aerosol transmission suggest that hantaviruses have no need for arthropod vectors (Traub et al. 1954, Umenai et al. 1979, Lee H. et al. 1981b, Bishop et al. 1980a). Similar observations involving laboratory outbreaks have been made in Finland, Belgium, France, the United Kingdom, and in the Netherlands (Desmyter et al. 1983, Dournon et al. 1984, Osterhaus et al. 1984, Lloyd et al. 1984).

Seroepidemiological surveys have suggested that the geographical distribution of HFRS is nearly worldwide. The serologically confirmed, severe form of HFRS has been described in Korea, China, Japan, and the Eastern part of the USSR (Lee H. 1978, Mery et al. 1983). Serologically confirmed cases of mild HFRS have been reported as NE in Sweden (Niklasson et al. 1983, Svedmyr et al. 1982), Finland (Brummer-Korvenkontio et al. 1983), Norway (Svedmyr et al. 1979, 1982), as a mild form of epidemic hemorrhagic fever (EHF) in China (Song et al. 1984, Chen et al. 1986), as epidemic nephritis in Yugoslavia, Hungary, Czechoslovakia (Gresikova et al. 1984), Greece (Gajdusek...

1.1.3. Clinical Manifestations, Sero-diagnosis, and Treatment of Hemorrhagic Fever with Renal Syndrome

Until serological diagnosis was developed, KHF was diagnosed only on clinical grounds. Its typical manifestations are fever, prostration, vomiting, proteinuria, hemorrhagic phenomena, shock, and renal failure. The incubation period is generally 2-3 weeks. Mild and moderate forms of the disease occur frequently, but there had been no way to identify these forms of KHF. The severe form of KHF is divided into five phases according to
clinical, laboratory and pathophysiological findings-febrile, hypotensive, oliguric, diuretic, and convalescent phases (Earle 1954, Smadel 1953, Gajdusek 1962). The overall death rate is 6-8%, but in certain outbreaks case-fatality rates have been reported as high as 33% or more (Earle 1954). Nephropathia epidemica, which is a mild form of HFRS found in Europe, has shown clinical features similar to KHF (Chumakov and Garvrilovskaya 1980, Lahdevirta 1982). Clinical symptoms include sudden onset, fever, abdominal or low back pain and evidence of renal dysfunction. Fatalities are rarely seen. Perhaps the most common feature of NE and the Far Eastern form is the renal manifestation (Lahdevirta 1982, Gajdusek 1962). Very severe forms of European HFRS have been recently reported in Balkan countries including Bulgaria and Greece (LeDuc et al. 1986a). This severe form is characterized by more frequent occurrence of shock and hemorrhagic manifestations with mortality rates ranging from 15-35% (LeDuc 1987).

Using Hantaan virus strain 76-118, indirect immunofluorescent antibody (IFA) tests have been applied to diagnose HFRS by demonstration of rising titers against hantavirus. The IFA using Hantaan-infected A 549 cells or Vero E6 cells (French et al. 1981, McCormick et al. 1982, Kitamura et al. 1983) is superior to infected Apodemus lung sections (Lee H. et al. 1978) because of the ease of sample preparation and the reduced likelihood of either contamination with other agents or non-specific
fluorescence (Goldgaber et al. 1982, Song et al. 1983, Lee P. et al. 1982b). However, the specificity of immuno-fluorescent titer is often doubtful. Plaque reduction neutralization tests are more specific for Hantaan and its variants (Lee P. et al. 1985b, Takenaka et al. 1985). But this technique is somewhat cumbersome due to the difficulties in plaque formation and long incubation periods (12-18 days). Apparently, a rapid, sensitive, and more specific diagnostic tool is needed (Tkachencho et al. 1981, Goldgaber et al. 1985).

The treatment of HFRS patients is mainly supportive. Hemodialysis performed during acute renal failure has been beneficial (Cohen 1982, Lee H. 1982a). A lethal suckling mouse model has been very recently developed (McKee et al. 1985, Kim and McKee 1985), and ribavirin therapy has been tried with certain limits in suckling mice (Huggins et al. 1986). No immuno-prophylaxis is available yet. Production of a vaccine for HFRS is a definite goal.

1.2. Literature Review of the Family Bunyaviridae

Recent studies of the physico-chemical and morphological properties of Hantaan virus suggest that Hantaan virus is bunyavirus-like (Lee H. 1982a, White et al. 1982, Hung et al. 1983). Thus, the general characteristics of the Bunyaviridae are reviewed. Among the five families of negative-stranded RNA viruses, the Bunyaviridae includes most of the arthropod-borne viruses
comprising over 200 viruses which infect vertebrates and/or invertebrates (Porterfield et al. 1973/74, 1975/76). Four genera of viruses, Bunyavirus, Nairovirus, Phlebovirus, and Uukuvirus, have been established according to serologic and some limited molecular analyses (Bishop et al. 1980a). In addition, some viruses such as the Bakau serogroup, Kaisodi group, Mapputta group, Thogoto group, Turlock group, and several unassigned viruses have been considered as possible members of the family (Bishop et al. 1980a). In this review, bunyavirus as a general term will be used to represent all the viruses within the family Bunyaviridae.

1.2.1. Genomic Composition of Bunyaviruses

The genome of bunyaviruses is composed of three unique species of RNA designated the large (L), medium (M), and small (S) segments (Bouloy et al. 1973-74, Pettersson and Kaarianen 1973, Clewley et al. 1977, Gentsch et al. 1977a, Obijeski et al. 1976b, Ushijima et al. 1980, Robeson et al. 1979, Pettersson et al. 1977). Gel electrophoresis of the genomic segments has shown that the L, M, and S RNA species in most bunyaviruses have molecular weights of approximately 3 X 10^6, 1.5-2.0 X 10^6, and 0.5-0.8 X 10^6, respectively (Bishop 1980a). Somewhat lower molecular weights have been reported for Uukuniemi viral RNA species (Pettersson et al. 1977). Until 1977, one question concerning the segmented genome in bunyaviruses was whether each RNA segment was a deletion product of the genome or whether each RNA species represented unique genetic
information. Oligonucleotide fingerprints of La Crosse, snowshoe hare, and uukuniemi virus demonstrated that the L, M, and S RNA segments contained unique nucleotide sequences (Clewley et al. 1977, Pettersson et al. 1977). The 3' terminal nucleotide sequence analysis demonstrates that all three viral RNA species possess a consensus 3' sequence (Parker and Hewlett 1981, Akashi and Bishop 1983, Clerx-van Haaster and Bishop 1980). No polyadenylation tract was found for La Crosse virus, Lumbo virus, snowshoe virus or Uukuniemi virus RNA species (Bouloy et al. 1973/74, Clewley et al. 1977, Pettersson et al. 1977). As shown in Table 2, the 3' terminal RNA sequences of viruses representing all four genera within the family Bunyaviridae have been determined. A common 3' terminal sequence is highly conserved within each genus (Obijeski et al. 1980, Clerx-van Haaster et al. 1982b). Terminal nucleotide analysis of the viral RNA species of snowshoe hare, La Crosse, and Uukuniemi viruses have shown that the 5' terminal nucleotide has the structure 5'pppAp and seems to be neither capped nor methylated (Bishop 1979, Bishop and Shope 1979). Furthermore, accumulated sequence data have suggested that the 3' terminal consensus sequences are inverted and complementary to the 5' termini of the RNA species (Bishop 1985). Electron microscopic examination revealed that the viral RNA species and nucleocapsids were often observed in circular forms (Hewlett et al. 1977, Pettersson and von Bonsdorff 1975, Samso et al. 1975).
However, when the viral RNA was denatured, only linear RNA species were visualized (Dahlberg et al. 1977, Hewlett et al. 1977). The electron microscopic studies together with the nucleotide sequence data suggest that each viral RNA species exists in a non-covalently linked closed circular configuration.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Representative</th>
<th>Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bunyavirus</td>
<td>La Crosse\textsuperscript{a}</td>
<td>3'-UCAUCACAUGA...</td>
</tr>
<tr>
<td>Uukuvirus</td>
<td>Uukuniemi\textsuperscript{b}</td>
<td>3'-UGUGUUUCUGG...</td>
</tr>
<tr>
<td>Phlebovirus</td>
<td>Punta Toro\textsuperscript{c}</td>
<td>3'-UGUGUUUCG...</td>
</tr>
<tr>
<td>Nairovirus</td>
<td>Qalyub\textsuperscript{d}</td>
<td>3'-AGAGAUUCU...</td>
</tr>
</tbody>
</table>


1.2.2. Structural Proteins of Bunyaviruses

Relatively little information is available on the structural components of most bunyaviruses due to difficulties with their adaptation to tissue culture. The chemical composition of one bunyavirus, Uukuniemi virus,
has been reported to be 2% RNA, 58% protein, 33% lipid, and 7% carbohydrate (R. F. Pettersson and O. Renkonen, cited in Obijeski and Murphy 1977). Three major virion polypeptides, two glycoproteins (G1 and G2) and a nucleocapsid protein (N), have been reported in many bunyaviruses (McLerran and Arlinghaus 1973, Obijeski et al. 1976a, 1977, Goldman et al. 1977, Pettersson and von Bonsdorff 1975, Gentsch et al. 1977a, Ushijima et al. 1980, Bishop et al. 1981). For bunyamwera virus, the prototype virus of the family Bunyaviridae, the molecular weights of these major polypeptides are reported as 115 K, 38 K, and 19 K for G1, G2, and N respectively (Gentsch et al. 1977a). Both G1 and G2 glycoproteins can be removed by proteolytic enzyme treatment. In addition, iodine-labelling of the La Crosse virion in vitro labelled only two glycoproteins (Obijeski et al. 1977). Electron microscopy of enzyme treated virions showed intact particles but a lack of spikes. (von Bonsdorff and Pettersson 1975). Thus, the spikes located on the surface of the virus are assumed to be composed of the glycoproteins. An internal ribonucleoprotein complex is released from purified virions treated with non-ionic detergents (Pettersson et al. 1971, Rosato et al. 1974, White 1975, Bouloy and Hannoun 1976b). By equilibrium density gradient centrifugation, three species of RNP molecules were separated. Each of the species of nucleo-capsid consists of a major polypeptide (N) and a single species of single-stranded RNA. In addition to the
three major viral structural polypeptides, a minor 120-180 KDa large protein (L) has been reported to be associated with the purified nucleocapsids (McPhee and Westway 1981). The L protein is believed to be a virion-associated RNA polymerase. At least three bunyaviruses, Lumbo (Bouloy and Hannoun 1976a), snowshoe hare (Vezza et al. 1979), and Uukuniemi (Ranki and Pettersson 1975), have been shown to contain an RNA-directed RNA polymerase activity.

1.2.3. Replication Strategies of Bunyaviruses

Following virus attachment and penetration, the first step in replication of Bunyaviridae is the synthesis of subgenomic complementary mRNA species from the individual genomic segments by the virion-associated transcriptase. The viral RNA replication and secondary transcription follows the translation of mRNA species (Bishop and Shope 1979). Viral RNA replication is not affected by actinomycin D (Bouloy and Hannoun 1973, Vezza et al. 1979), which indicates that cellular transcription is not required for the initiation of bunyavirus infection. Recently primer extension and S1 mapping studies have revealed that the S mRNA species of snowshoe hare virus (Bishop et al. 1983b, Eshita et al. 1985, Ihara et al. 1985b) and La Crosse virus (Patterson and Kolakofsky 1984) have 12-15 nucleotides long, heterogenous 5' non-viral sequences that extended beyond the 3' end of the viral RNA. Similarly, some 12-14 nucleotides of unknown origin were reported to be present at the 5' end of the M mRNA of Rift Valley fever virus.
(Collett 1986). They probably represent host-derived RNA primer sequences used by viral transcriptase (Krug et al. 1979, Lamb and Choppin 1983). The messenger RNAs of bunyaviruses have also been reported to be truncated (Cash et al. 1979, Patterson and Kolakofsky 1984, Bouloy et al. 1984, Eshita et al. 1985, Collett 1986).

The S RNA of snowshoe hare virus (Bishop et al. 1982), La Crosse virus (Cabraddilla et al. 1983), and Aino virus (Akashi et al. 1984) have been cloned and sequenced. Sequence analysis together with genetic studies (Gentsch and Bishop 1978, Bouloy et al. 1984) have shown that the S RNA of the bunyavirus codes for both nucleocapsid protein (N) and a non-structural protein (NS$_g$). These two proteins are encoded by overlapping reading frames in the viral complementary sequence. Only a single mRNA species specific for snowshoe hare S RNA has been demonstrated by Northern blot hybridization and by viral-sense oligonucleotide-directed cloning analysis (Bishop et al. 1983a, Eshita et al. 1985). Although Patterson et al. (1983) reported the probable existence of multiple mRNA species of La Crosse S RNA, it is believed that only one S mRNA serves for the synthesis of both N and NS$_g$ proteins in snowshoe hare virus infected cells (Fuller et al. 1983, Eshita et al. 1985).

More recently, Ihara et al. (1984) have reported that the N protein of Punta Toro Plebovirus is encoded in the complementary sequence of the 3' half of the S RNA. A non-structural protein, NS$_g$ is possibly encoded in the 5' half
of the S RNA although its existence has not been demonstrated in cell extracts. Thus, the S RNA of Punta Toro virus has been termed ambisense. Hybridization analyses has established that subgenomic mRNA species representing the two genes are present in infected cells (Ihara et al. 1984). Evidence has accumulated that lymphocytic choriomeningitis virus (Romanowsky et al. 1985), Pichinde virus (Auperin et al. 1984), and Lassa virus (Auperin et al. 1986) in the family Arenaviridae also have a similar ambisense coding strategies (Bishop 1985, 1986).

Genetic recombination studies and sequence analyses have shown that the two glycoproteins G1 and G2 are encoded by the M RNA segment (Fuller et al. 1983, Gentsch and Bishop 1979, Fuller and Bishop 1982, Collett et al. 1985, Ihara et al. 1985a). The nucleotide sequence of the M RNA also reveals that it codes for a single polypeptide in the viral complementary sequence (Eshita and Bishop 1984, Lees et al. 1986). This presumptive precursor was not found in virus infected cells but was detected by in vitro translation of the glycoprotein mRNA (Ulmanen 1981). Thus, this precursor is cleaved to yield G1 and G2 protein as well as a non-structural protein, NSM (Fuller and Bishop 1982, Kuismanen 1984). Since the 200 KDa L protein has been identified (Obijeski et al. 1976a), by elimination, the L RNA segment is presumed to encode the large, L, protein.

For many animal viruses, such as vesicular stomatitis
virus (Strous and Lodish 1980, Zilberstein et al. 1981), Semliki Forest virus (Garoff et al. 1982), and influenza virus (Klenk and Rott 1980), the viral glycoproteins are synthesized in the endoplasmic reticulum and transported to the plasma membrane via the Golgi complex. Thus, virus maturation takes place at the cell surface. Recently, coronavirus has been shown to mature at the membranes of the smooth or rough endoplasmic reticulum (Sturman and Holmes 1983). By contrast, bunyaviruses appear to mature exclusively at the membrane of the Golgi apparatus (Kuismanen et al. 1982, Smith and Pifat 1982, Murphy et al. 1973, Madoff and Lenard 1982). Both G1 and G2 and the N proteins accumulate in the Golgi region (Kuismanen et al. 1984). Virus maturation is effectively inhibited by the carboxylic ionophore monensin (Cash et al. 1980, Cash 1982, Kuismanen et al. 1985). Monensin has been known to inhibit intracellular transport of membrane glycoproteins by blocking the secretory vesicles from the Golgi complex (Tartakoff and Vassalli 1978). The release of the virus particles from cells is thought to occur through vesicular carriers which fuse with the plasma membrane.

1.2.4. Genetics and Pathogenesis of Bunyaviruses

Genetic studies have mainly been reported for Bunyamwera and California encephalitis serogroup viruses. Genetic recombination by reassortment of genomic subunits has been reported using temperature sensitive (ts) mutants in heterologous infections of Batai virus, Bunyamwera
virus, and Maguari virus, three members of the Bunyamwera serogroup (Iroegbu and Pringle 1981, Ushijima et al. 1981, Pringle et al. 1984). Furthermore, ts mutants of Maguari virus could be classified into three recombination groups corresponding to the three genomic segments (Pringle and Iroegbu 1982). In California serogroup viruses, recombination involving RNA segment reassortment has also been demonstrated employing ts mutants of snowshoe hare, La Crosse, Tahyna, Lumbo, California encephalitis, and Trivittatus viruses (Gentsch and Bishop 1976, Gentsch et al. 1979, Rozhon et al. 1981, Beaty et al. 1981b). For each of these California group viruses, only L and M RNA ts mutants have been identified (Gentsch et al. 1977b, 1979). No genetic recombination has been documented between viruses from different serogroups.

The coding assignments of RNA segments have been deduced by analysing the structural proteins of reassortant viruses (Gentsch and Bishop 1978). Relationships between the gene products and virulence of bunyaviruses have also been investigated using reassortants (Rozhon et al. 1981, Gentsch et al. 1980). By the use of reassortants of virulent La Crosse and avirulent snowshoe hare parents, the M RNA segment has been shown to be the major determinant for dissemination of La Crosse virus (Beaty et al. 1982). Reassortants containing the La Crosse virus M RNA produced disseminated infection and were transmitted by mosquitoes. By contrast, viruses with a snowshoe hare virus M RNA were
as inefficiently transmitted as the parental snowshoe hare virus. Although attenuating mutations in other La Crosse RNA species may affect the properties of the M gene (Rhazon et al. 1981), the principle determinants for pathogenicity of bunyaviruses appear to be the viral glycoproteins (Shope et al. 1981, Gonzalez-Scarano et al. 1982, Beaty et al. 1981a, 1982, Bishop et al. 1980b).

1.3. The Role of Viral Envelope Glycoproteins in Interactions between Viruses and Host Cells

Many enveloped viruses are important pathogens and their envelope components exhibit functional and immunological properties important for the biology of these viruses and for their interaction with host cells. General features of the membrane structure of viruses include the presence of a lipid bilayer, glycosylated proteins, and internal non-glycosylated proteins (Compans and Klenk 1979). The lipids are derived from the host cell membranes during maturation. The carbohydrates are also specified by cellular enzymes (Compans and Klenk 1979). The internal matrix proteins are not found in arenaviruses and bunyaviruses unlike other negative stranded, enveloped RNA viruses (Bishop and Shope 1979, Howard 1987). Viral membrane glycoproteins are present at the surface in the form of spikes. These glycoproteins may be either identical molecules or fragments that derived from a common precursor polypeptide by proteolytic cleavage. Some viruses such as
rhabdoviruses possess one type of spike (Katz et al. 1977) while other viruses such as orthomyxoviruses and bunyaviruses contain two types of spikes, each one formed by a different glycoprotein species (Skehel and Waterfield 1975, Schied et al. 1972, Bishop 1985). The glycoproteins are amphipathic. They consist of an external hydrophilic part and a hydrophobic region including carboxy termini by which the spikes are associated with the lipid bilayer. The glycoprotein synthesis is initiated by the synthesis of a short amino acid sequence at the amino terminus. This signal sequence is responsible for insertion of the nascent viral glycoprotein into the membrane of the rough endoplasmic reticulum (Blobel and Dobberstein 1975, Garoff et al. 1978). The signal sequence is proteolytically cleaved during the insertion process (Lingappa et al. 1978). Glycosylation of viral glycoproteins appears to be initiated by the transfer of preformed oligosaccharide chains to the newly synthesized polypeptide chain. The carbohydrate side chains of viral glycoproteins are generally linked to asparagine residues. Two major types of glycoproteins, a mannose-rich type and a complex type, are found. The carbohydrate transfer process seems to be extremely rapid. It has been recognized that membrane insertion and glycosylation of the protein are synchronous for alphaviruses and rhabdoviruses (Klenk and Rott 1980). Glycosylated proteins seem to be resistant to proteolytic cleavage (Klenk and Rott 1980). Glycoproteins then migrate
to the site of virus assembly (Lafay 1974, Knap et al. 1977, Gahmberg et al. 1986). Bunyaviruses have a unique maturation property with glycoprotein accumulating in Golgi complex whereas glycoproteins are transported to plasma membrane for most other viruses (Kuismanen et al. 1982, Kuismanen et al. 1985, Gahmberg et al. 1986). The viral glycoproteins play important roles in the interaction between viral envelopes and cellular membranes. They are responsible for the first step in the infectious process, adsorption and penetration (Klenk and Rott 1980). Models for adsorption and penetration are hemagglutination and membrane fusion processes respectively. Viral glycoproteins also induce and react with neutralizing antibodies. These neutralization reactions may prevent either the attachment of the virus to the receptor, entry into cells, or uncoating and release of the viral genome (Choppin and Schied 1980). Neutralizing antibodies against several bunyaviruses have also been shown to recognize the sites on the glycoproteins (Grady et al. 1983; Gonzalez-Scarano et al. 1985). Bunyavirus glycoproteins are probably the major determinants of virulence (Genstch et al. 1980, Beaty et al. 1981a, 1982, Kingsford et al. 1982, Sundin et al. 1987) and therefore the most obvious antigens for the induction of virus neutralizing antibodies (Kingsford and Hill 1983, Wiley 1985).
1.4. Statement of Objectives

In spite of the clinical significance of hantaviruses and their world-wide distribution in nature, very little is known about their molecular biology. The major obstacles have been the long lag period of virus production, which varies from 7 to 24 days and the low virus yields, typically $1-5 \times 10^6$ pfu/ml. In order to overcome such difficulties and to understand the strategy of virus replication, one potentially useful approach is the molecular cloning of the virus genome. Another important aspect of hantavirus research is to investigate the possibility of developing a recombinant vaccine for HFRS. Using the prototype virus Hantaan strain 76-118 as a model system for hantaviruses, the objectives of this study have been:

1) to characterize the structural components and to define the proper taxonomy of Hantaan virus

2) to clone the virus genome and to elucidate the genomic structure and organization of Hantaan group viruses

3) to sequence the genomic components and to investigate the coding assignments and replication strategy of Hantaan virus

4) to express the viral gene encoding glycoproteins and, as a long-term goal, to develop a recombinant subunit vaccine against hemorrhagic fever with renal syndrome.
CHAPTER 2: MATERIALS AND METHODS

2.1. The Structure of Hantaan Virus

2.1.1. Cells and Viruses

The ROK 83-109 isolate and the prototype virus, Hantaan 76-118, were provided by Dr. H. W. Lee, WHO Collaborating Center, Korea University, Seoul, Korea. The Prospect Hill isolate was obtained from Dr. P. W. Lee, National Institutes of Health, Bethesda, Maryland. The USSR cg38-83 strain was provided by Dr. G. van der Groen, Institute of Tropical Medicine, Antwerp, Belgium. For virus production, a cloned line of Vero E6 cells (ATCC C1008, CRL 1586) was grown and maintained at 37 C in Dulbecco's Modified Eagle's Medium (DMEM) (Dulbecco and Freeman 1959) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM glutamine (Gibco, Grand Island, NY). Since hantaviruses have been assigned to Biosafety Level D in the Guidelines of the Medical Research Council of Canada, all of the work involving infectious virus was conducted in the D biohazard containment facilities of the University of Ottawa.

2.1.2. Virus Production and Radiolabeling of Viral Nucleic Acids

To produce the virus, cells were infected at a low multiplicity of infection (0.01-0.001 pfu/cell), and maintained at 37 C in a humidified incubator with 5% CO₂. The medium was changed at 4 days post-infection, and the cells were maintained for another 4 days. To label Hantaan
virion RNA, [³H]-uridine (10 uCi/ml, 20-30 Ci/mmol, Amersham, Arlington Heights, IL) was added to the culture medium on day 4 post-infection. Cell culture medium was harvested on day 8 post-infection and clarified by centrifugation for 30 min at 3,000 g at room temperature (Damon/IEC, model HN-S, Needham Heights, MA). The virus was pelleted by ultra-centrifugation in a Beckman SW 28 rotor (Beckman, Palo Alto, CA) at 81,000 g for 2 hr at 4 C. For production of other hantavirus isolates, similar procedures were applied except for an additional fluid change and labelling on day 8 post-infection. Virus was harvested on day 12 post-infection. For further purification, the virus pellet was resuspended in TE buffer, pH 7.4 (10 mM Tris, 1 mM EDTA), layered on a 10%-60% linear sucrose gradient and centrifuged for 16 hrs at 195,000 g in a Beckman rotor SW 41 at 4 C. The gradient fractions were identified by bottom puncture of the tube. The virus band was identified by scintillation counting or by refractive index of 1.3958 (38% sucrose w/w) (Schmaljohn et al. 1983).

2.1.3. Extraction and Gel Electrophoresis of Viral RNA

The virus pellet was dissolved in 100 ul of buffer containing 100 mM NaCl, 10 mM EDTA, 50 mM sodium acetate, pH 5.2, and 0.5% SDS followed by incubation at 37 C for 30 min in the presence of 300 ug/ml of proteinase K. The virion RNA was extracted once with an equal volume of phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1).
Three volumes of cold ethanol were added and stored at -20 C overnight. The RNA was recovered by centrifugation in a microcentrifuge for 30 min at 4 C. For gel electrophoresis, RNA was denatured with 1 M deionized glyoxal and 50% DMSO in 10 mM sodium phosphate buffer, pH 7.0, at 60 C for 15 min. The gel, consisting of 1.4% agarose, was prepared and electrophoresed in 10 mM sodium phosphate buffer, pH 7.0 for 4 hr at 100 volts (McMaster and Carmichael 1977). The gel was fixed in 10% glacial acetic acid and 30% methanol. The gel was then impregnated with 3% PPO in DMSO, soaked in water to precipitate the PPO, dried, and exposed to Cronex X-ray film (Du Pont, Wilmington, DE) at -70 C (Bonner and Laskey 1974).

2.1.4. SDS-Polyacrylamide Gel Electrophoresis of Viral Proteins

The virus suspension, in TE buffer, was diluted with an equal volume of electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.0025% bromophenol blue, and 20% glycerol). Samples were boiled for 5 min prior to gel electrophoresis. Electrophoresis was carried out for 1 hr at 200 volts in a discontinuous polyacrylamide gel (Laemmli 1970) under reducing or non-reducing conditions using a mini-protein gel apparatus (Bio-Rad, Richmond, CA). For reducing conditions, 2-mercaptoethanol was added to the sample to give a final concentration of 5% (v/v). The separating gel consisted of 7.5% acrylamide, 0.02% bisacrylamide, 1.0% (w/v) SDS, and 0.375 M Tris-HCl, pH
8.8, and the stacking gel consisted of 4.0% acrylamide/bis, 1.0% (w/w) SDS, and 0.125 M Tris-HCl, pH 6.8. The protein bands were visualized by staining with 0.1% Coomassie blue R-250 in fixative (40% methanol, 10% acetic acid). For Western blot analysis, the proteins were electrophoretically transferred onto nitrocellulose membrane (Burnette 1981). Electroblotting was carried out in 0.025 M Tris, 0.192 M glycine and 20% (v/v) methanol for 1 hr at 100 V using a Bio-Rad mini transblot apparatus. The procedure for immunoassay is detailed in Section 2.7.2.

2.1.5. Densitometry

Autoradiographs of genomic RNA were scanned with the Beckman Model DU-8B spectrophotometer using a Slab Gel Scanning System. The densitogram was plotted with the aid of a Compuset™ Module microprocessor (Beckman). The area of the peak was calculated in valley to valley mode to subtract the background. Wavelength was calibrated using a holmium oxide filter. Five readings at a wavelength of 600 nm were performed for peak determination and an average was determined. The scanning speed and the chart speed were 2 and 5 cm/min, respectively. The calculated area of the peak corresponding to each genomic segment was used to calculate molar ratios according to the estimated size of each RNA segment.

2.1.6. Measurement of Radioactivity

A small aliquot of labeled sample was added to a 10 ml glass tube. 25 ul of bovine serum albumin (10 mg/ml) and 5
ml of 10% TCA were added. The mixture was kept on ice for 15 min and the precipitate was collected by centrifugation. After being washed two times with 5 ml of 10% TCA, the pellet was resuspended in 100 ul of 2 N NaOH and 200 ul of water was added. The sample was then mixed with aqueous counting scintillant (ACS, Amersham) and radioactivity was measured in a liquid scintillation counter (LKB, Model 1214 Rackbeta, Sweden).

2.2. Cloning of the Hantaan Virus Genome

2.2.1. Construction of a cDNA Bank

Viral RNA was treated with 10 units of RQI DNase (Promega Biotec, Madison, WI) in 40 mM Tris-HCl, pH 7.6, containing 10mM NaCl and 6 mM MgCl₂ at 37 C for 15 min. The DNase-treated viral RNA was used as a template for the synthesis of cDNA as described (Gubler and Hoffman 1983). First strand cDNA synthesis was carried out in the standard reverse transcriptase reaction mixture: 1 mM dTTP, 1 mM dCTP, 1 mM dGTP, 200 µM dATP, 100 mM Tris-HCl, pH 8.4, 130 mM KCl, 10 mM MgCl₂, 1 mM DTT, 20 units of RNAsin (Promega Biotec), 50 uCi of [α-³²P] dATP (Amersham, 800 Ci/mmol), and 30 units of avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources, Tampa, FL) using approximately 1 µg of viral RNA and an 100 fold molar excess of random primers (Pharmacia Biotechnology, Uppsala, Sweden). After a 30 min incubation at 37 C, the reaction mixture was transferred to ice. One tenth volume
of 10X nick-translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 0.01 M DTT, 500 µg/ml BSA fraction V), 2 units of RNase H (BRL, Gaithersburg, MD) and 23 units of DNA polymerase I (BRL) were immediately added and the sample was incubated for 2 hr at 15°C. The reaction was terminated by addition of EDTA and SDS to final concentrations of 50 mM and 1% respectively. The double-stranded cDNA products were fractionated through a Sepharose 4B column and the fractions containing the large cDNA molecules were collected. Plasmid pUC19 was cut with HindII and treated with calf intestinal alkaline phosphatase before ligation with cDNA. The ligation reaction was carried out in ligation buffer (20mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM DTT, 0.6 mM ATP) using 5 units of T₄ DNA ligase (Boehringer Mannheim, Mannheim, Germany) at 15°C for 16 hr. Competent cells of E. coli JM109 (Yanisch-Perron 1985) were prepared as described previously (Hanahan 1983), and the transformation was carried out according to standard procedures (Maniatis et al. 1982). Ampicillin resistant, colorless colonies were picked from B plates (0.8% NaCl, 1.0% tryptone, 2% agar, 0.001% vitamin B-1) containing 50 µg/ml ampicillin, 100 mM IPTG and 2% X-gal as described previously (Messing 1983). For the construction of 3' terminal clones of the genomic RNA segments, two synthetic oligonucleotides, 19mer yk-1s and 16mer yk-m were obtained from Wistar Institute, Philadelphia, PA. These two specific primers were used for cDNA synthesis as described above.
2.2.2. Preparation of Plasmid DNA

For rapid and mini-scale isolation of plasmid DNA, the bacterial cultures were grown overnight in LB medium (Bacto-tryptone 1%, yeast extract 0.5% and NaCl 1%) containing 50 ug/ml of ampicillin. Recombinant plasmids were prepared by the alkaline lysis extraction method (Birnboim and Doly 1979). The RNA was removed by digestion with 100 ug/ml of pancreatic RNase at 37 C for 30 min. For large-scale plasmid isolation, the culture was grown to an O.D. of 0.4 (600 nm), and plasmid was amplified in the presence of chloramphenicol (170 ug/ml). Closed circular forms of DNA were isolated by centrifugation to equilibrium in CsCl-EtBr gradients in a VTi 65 Beckman rotor at 325,000 g for 16 hr at 20 C. The DNA was then purified and precipitated by standard methods as described elsewhere (Maniatis et al. 1982).

2.2.3. Southern and Northern Blot Hybridization Analyses

Recombinant plasmids were digested with EcoRI and HindIII followed by agarose gel electrophoresis in TBE buffer (0.1 M Tris-borate, 0.1 M boric acid, 2 mM EDTA, pH 8.0). The DNA gels were denatured with 0.2 M NaOH containing 0.5 M NaCl for 30 min and neutralized with 4X TAE blotting buffer (40 mM Tris-HCl, pH 7.8, 20 mM sodium acetate, 2 mM EDTA). The DNA in the gel was electrophoretically blotted onto a transfer membrane (Zeta Probe, Bio-Rad) in TAE blotting buffer overnight at 30 volts using a Bio-Rad Model 250/2.5 power supply. The membranes were
baked at 80°C for 2 hr under vacuum. The membrane was then prehybridized at 42°C for 16 hr in a sealed plastic bag containing prehybridization buffer (0.2 ml/cm² of membrane) of 50% formamide, 5X Denhardt's solution (0.1% each of bovine serum albumin, ficoll and polyvinyl-pyrolidone), 50 mM sodium phosphate, pH 6.5, 5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% SDS and 250 μg/ml of sonication-denatured salmon sperm DNA. Hybridization was carried out at 42°C for 20 hr using fresh hybridization solution containing the radiolabeled probe (1-4 X 10⁶ cpm/ml) as described previously (Southern 1975, Alwine et al. 1979, Thomas 1980). The membranes were washed once with 2X SSC containing 0.1% SDS for 1-2 hr, once with 0.1X SSC containing 0.1% SDS for 30 min at room temperature and once for 30 min at 65°C with 0.1X SSC containing 0.1% SDS with continuous shaking. For Northern blot hybridization, genomic RNA segments were separated on an agarose gel after denaturation with glyoxal as described in Section 2.1.3. The gel was then equilibrated and electroblottedted, and the membranes were used for hybridization as described above.

2.2.4. Screening of 3' Terminal Clones with Synthetic Oligonucleotides

Bacterial transformants were lifted onto hybridization transfer membranes (Colony/Plaque Screen, New England Nuclear, Boston, MA) and lysed by alkali (Grunstein and Hogness 1975). The filters were washed in 3X SSC containing 0.1% SDS at 65°C for 16 hr with continuous shaking. The
filters were then prehybridized at 37 C for 2 hr in a solution of 6X SSC, 1X Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate and 100 μg/ml of denatured calf thymus DNA. Hybridization was carried out at 31 C and 39 C for the 16 mer and 19 mer oligonucleotides respectively, in fresh hybridization buffer containing radiolabeled oligonucleotides as probes. The filters were washed several times at room temperature and once at 35 C for 1 hr with washing solution (6X SSC, 0.05% sodium pyrophosphate). The final washes were performed for 10 min at 42 C and 50 C for the 16mer and 19mer oligonucleotides repectively (Wallace et al. 1981, Woods 1984). The washed filters were wrapped with Saran wrap and exposed to Cronex X-ray film at -70 C.

2.2.5. Cloning of the 5’ Termini of the Genomic RNAs

In order to obtain cDNA clones which represent the 5’ termini of the M and S genomic RNA segments, four segment-specific oligonucleotides (hmpl, hmp2, hsp1, hsp2) were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario. Primers were annealed to the genomic RNAs and extended as described in Section 2.2.1. The first-strand cDNA was tailed with dCTP in tailing buffer (200 mM K-Cacodylate, 25 mM Tris-HCl, pH 6.9, 2 mM DTT, 0.5 mM CoCl₂) using 10 units of terminal deoxynucleotidyl transférase (Pharmacia). The second cDNA strand was primed with poly (dC) (Pharmacia) and synthesized using the large fragment of DNA polymerase I (Amersham). Phosphorylated PstI linker (Boehringer
Manheim) was added and ligated to the double-stranded cDNA in ligation buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 5 mM DTT, 1 mM ATP) at 16°C overnight. After phenol extraction and ethanol precipitation, the cDNA was digested with PstI, inserted into the PstI site of either pUC19 or M13 mp19 RF DNA. E. coli JM109 was then transformed with the recombinant DNA.

2.2.6. In Vitro Labeling of Hybridization Probes

Radiolabeled hybridization probes were prepared by nick-translation (Rigby et al. 1977) using [α-³²P] dATP (3000 Ci/mmol, Amersham). The labeled DNA was passed through a Sephadex G-50 column and the unincorporated nucleotide triphosphates were removed. To prepare oligonucleotide probes, 200 ng of oligonucleotide was phosphorylated using 100 uCi of [γ-³²P] ATP (5000 Ci/mmol, Amersham) in a buffer containing 10 mM MgCl₂, 100 mM Tris-HCl, pH 7.6, and 20 mM DTT with 10 units of T₄ polynucleotide kinase (Maxam and Gilbert 1977). The reaction was carried out at 37°C for 1 hr, and the labeled oligonucleotides were used directly as the hybridization probes.

2.3. Determination of Nucleotide Sequences

2.3.1. Subcloning into M13 Phage

The replicative forms of M13mp18 and mp19 were purchased from New England Biolabs (Beverly, MA). The JM101 and JM109 stains of E. coli were obtained from Dr. P. Lau,
National Research Council of Canada, Ottawa. The host strains were maintained on M9 minimal salt plates. For subcloning into M13 phage DNA, plasmid containing the cDNA fragment was digested with EcoRI and PstI, or with EcoRI and HindIII. The DNA fragments were purified as detailed in Section 2.5.1. M13mp18 and mp19 RF DNA were cleaved with the corresponding restriction enzymes, and ligated to the purified cDNA fragments. The ligation mix was then used for transfection of either E. coli JM101 or JM109. The recombinant phage plaques were identified primarily by the lack of a color reaction in the presence of 2% X-gal and 100 mM IPTG. The colorless plaques were picked and grown in 2X YT (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) for 8 hr with vigorous shaking. After a 5 min spin in a microcentrifuge, the supernatant was mixed with 10X loading buffer (0.5% bromophenol blue, 2.0% EDTA, 50% glycerol, 2.0% SDS), and electrophoresed on a 0.8% agarose gel in TBE buffer (100 mM Tris base, 100 mM boric acid, 3 mM EDTA) for 5 hr at 100 volts. The presence of DNA inserts was confirmed by directly comparing the size of the recombinant phage DNA to the wild type DNA (Messing 1983). Alternatively, the replicative forms of phage DNA were prepared by the alkaline lysis method (Birnboim and Doly 1979) and the inserts were analysed after digestion with appropriate restriction enzymes.

2.3.2. Preparation of M13 Phage DNA for Sequencing

A single bacterial colony was picked from a M9 plate
and grown in 2X YT medium overnight with vigorous shaking. The following morning, a fresh 2X YT was inoculated with 0.1% of the overnight culture. When the O.D. reading at 660nm reached 0.1, 2 ml-aliquots were pipetted into 10 ml snap-cap tubes and infected with 50 ul of M13 phage stock (approx. moi of 100 pfu/cell). Incubation was continued for 5-6 hr at 37 C with vigorous shaking. The supernatant was cleared for 5 min in a microcentrifuge. 1.2 ml of clear supernatant was added to 200 ul of 27% PEG-6000 in 3.3 M NaCl. After standing for 30 min, phage was pelleted for 30 min and resuspended in 100 ul of TE buffer, pH 8.0. The phage DNA was extracted with phenol-chloroform and ethanol-precipitated.

2.3.3. Generation of Sequential Overlapping cDNA Clones

To sequence large DNA fragments, a nested series of overlapping clones were generated by sequentially digesting one end of the cloned insert with exonuclease (Dale et al. 1985). Single-stranded M13 DNA was prepared as described in section 2.3.2. An oligomer RD22 (5'-CGACGGCCAGTGATCCCCTCCCC-3') was purchased from IBI (New Haven, CT). Approximately 1 ug of ssDNA template was annealed with 20 ng of RD22 and digested concurrently for 1 hr at 45 C in 33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT containing 20 units of Eco RI. Following inactivation of EcoRI at 65 C, 6 units of T4 DNA polymerase was added to digest the linearized DNA from the 3' end. Every minute, an aliquot was removed and the reaction in
each aliquote was terminated by heating to 65 C for 10 min. Finally all the digests were combined. A tailing reaction was carried out using 10 mM dGTP and 15 units of terminal deoxynucleotidyl transferase. The reaction was stopped by heating, and RD22 oligomer was again annealed to the DNA. The nicked phage DNA which formed a hybrid with the oligomer was ligated for 1 hr in the presence of 1 mM ATP and 1 unit T4 DNA ligase (BRL). The recircularized single-stranded phage DNA was used to transfect JM109 using standard procedures. Phage DNA was prepared from individual plaques and analysed by agarose electrophoresis. Sequentially deleted overlapping clones were identified by comparing the size of the phage DNA as described in Section 2.3.1.

2.3.4. DNA Sequencing Reactions

DNA sequence was determined by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using 35S-labeled deoxynucleoside triphosphate (Biggin et al. 1983, Ornstein et al. 1985). Deoxy- and dideoxynucleotides were purchased from Pharmacia Biotechnology. Deoxyadenosine 5'-[α-35S] thiotri-phosphate (650 Ci/mmol) and Klenow fragment were purchased from Amersham. The 17mer (5'-GTAAAACGACGCTATTTG-3') and 24mer (5'-CGCCAGGGTTTCCAGTCAGGAC-3') sequencing primers, which begin at -20 and -47 nucleotide from the EcoRI site respectively, were purchased from New England Biolabs. Approximately 1 ug of ssDNA template was annealed with primer in a 1 to 5 molar ratio at 60 C for 1-
2 hr in 10 ul of 10 mM Tris-HCl, pH 8.5, and 10 mM MgCl₂. To the annealed primer/template, 15 uCi of [α-35S] dATP and 1 unit of Klenow fragment were added. This primer/template/label/enzyme mix was split into four separate reactions (2.5 ul each). The primers were then extended by adding 2 ul of the relevant dNTP/ddNTP mixes to each reaction. The compositions of the four dNTP/ddNTP mixes are presented in Appendix 1. After 15 min, the reactions were chased with excess amount of cold dNTPs. The reactions were terminated by adding 4 ul of formamide dye mix (0.03% xylene cyanol, 0.03% bromophenol blue, 20 mM EDTA in deionized formamide).

2.3.5. Urea-Polyacrylamide Sequencing Gel Electrophoresis

The sequencing reactions were electrophoresed on a 37 cm X 40 cm X 0.02 cm polyacrylamide gel. One electrophoresis glass plate was siliconized with 2% dimethyldichlorosilane (Repelcote™, BDH Chemicals, Toronto), and the other was treated with γ-meacryloxy-propyltrimethoxy silane (Silane A-174, BDH). The gel consisted of 7 M urea, 6% acrylamide, 0.3% bis-acrylamide, 100 mM Tris base, 100 mM boric acid and 3 mM EDTA. To catalyze the polymerization of the gel, 200 ul of 10% ammonium persulfate and 30 ul of TEMED were added to 50 ml of the gel mix. The sequencing reactions were heated at 95°C for 3 min and immediately loaded on the gel using a shark tooth comb (IBI). Electrophoresis was carried out using an IBI sequencing apparatus model STS 45 at 50 watts constant
power with a LKB Model 2297 Microdrive 5 power supply (LKB, Sweden). The gel was fixed in 5% acetic acid and 5% methanol for 20 min, dried and exposed to Cronex X-ray film.

2.3.6. Computer Analysis of Nucleotide Sequences

Nucleotide sequences were analysed using Apple IIe and IBM Personal System/2 Model 30 computers. Software used for this study were the University of Minnesota Apple II Sequence Analysis Programs (Version 2.1, 1982, Department of Biochemistry, University of Minnesota, St. Paul, MN) and PC/Gene (version 4.05, 1986, Department of Medical Biochemistry, University of Geneva, Switzerland, distributed by IntelliGenetics, Inc., Mountain View, CA). The Minnesota Program was used to join sequences and to search for restriction enzyme sites. Translation of a DNA sequence, hydropathy plots and searches for potential antigenic sites in protein sequences, and secondary structure analysis of RNA were done using the PC/Gene program. The protein coding regions were analysed by RNY pattern (R:purine, N:any base, Y:pyrimidine) (Shepherd 1981). The hydrophilicity/hydrophobicity of proteins were evaluated by the methods of Kyte and Doolittle (1982). Secondary structure of RNA was based upon Zucker's method (Zucker and Stigler 1981) with modifications (Jacobson et al. 1984). Three letter codes were used for amino acid nomenclature (IUPAC-IUB 1966). Restriction enzymes were abbreviated as described by Roberts (1985).
2.4. Intracellular RNAs of Hantaan Virus-Infected Cells

2.4.1. Extraction and Electrophoresis of Intracellular RNA

Vero E6 C1008 cells were infected at a low moi (0.01-0.001 pfu/cell) and maintained as described in Section 2.1.1. On days 6-7 after infection, the cell monolayers were scraped from plates and resuspended in TMNS buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 25 mM NaCl, 250 mM sucrose) containing 25 ug/ml of polyvinylsulfate and 30 ug/ml of spermine. A solution of 10% triton X-100 (v/v) and 10% sodium deoxycholate (v/v) was added to give a final concentration of 1% for each detergent. The cells were disrupted by Dounce homogenization for 10 strokes on ice. The cytoplasmic fraction was collected by centrifugation at 7,000 g for 30 min at 4°C in a Beckman rotor JA-20. An equal volume of 2X NENS (100 mM sodium acetate, pH 5.2, 200 mM NaCl, 20 mM EDTA, 1% SDS) was added. The sample was then digested with 300 ug/ml of proteinase K for 30 min at 37°C. The cellular RNA was extracted, precipitated, and resuspended by standard methods. The RNA species were denatured with glyoxal and resolved on an agarose gel as outlined in Section 2.1.3. The gel was equilibrated with 1X TAE buffer and electroblotted onto Zeta Probe (Bio-Rad). The membrane was cut into longitudinal strips and used for hybridization (Section 2.2.3).

2.4.2. Preparation of Strand-Specific Hybridization Probes

DNA fragments specific for Hantaan virus genomic segments were subcloned into the multiple cloning sites of
both bacteriophage M13 mp18 and mp19 RF DNA. Single-stranded phage DNA was prepared as described in Section 2.3.2: Hybridization probe primer (5'-CACAATTCCACACAAC-3', New England Biolab) (Ricca et al. 1982) was annealed to single-stranded template DNA in 30-100 fold molar excess in 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 10 mM DTT, 10 mM MgCl₂. 100 uCi of [α-32P] dATP (800 Ci/mmol, New England Nuclear, Boston, MA) was added. The reaction was carried out by adding 5 units of Klenow fragment in the presence of 100 μM dGTP, 100 μM dCTP, 100 μM dTTP and 15 μM dATP. Unincorporated nucleotide triphosphates were separated by a Sephadex G-50 column chromatography. The running buffer contained 100 mM NaCl and 1 mM EDTA so that two strands of the probes would not dissociate. The probes were collected by monitoring radioactivity and used for hybridization directly without denaturation.

2.5. Construction and Modification of a Full Length M Gene
2.5.1. Purification of DNA Fragments, Ligation and Transformation

Gel electrophoresis, DNA ligation, plasmid preparations, and transformation procedures are described in Sections 2.2.1., 2.2.2., and elsewhere (Maniatis et al. 1982). For purification of DNA fragments, the bands of interest were located by examining agarose gels with long wavelength UV light after EtBr staining. The DNA bands were excised and eluted using an IBI electroleluter (Model UEA)
in low salt buffer (20 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.2 mM EDTA) typically for 50 min, depending upon the size of the DNA fragment, at 100 volts. The high salt cushion (10 M ammonium acetate) was recovered and extracted with water-saturated butanol 5 times, and DNA was ethanol-precipitated with 20 μg of glycogen as carrier.

2.5.2. Preparation of Oligonucleotide Linkers

A 26mer oligonucleotide, AB279 (5'-GATCCACCATGGGGAT-ATGGAAGTGG-3'), was prepared by the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ONT. A 22 mer, DW1 (5'-TAAAAATCATAGGATCCTGCA-3'), and a 21mer, DW2 (5'-GGATC-CTATGATTTTTATGC-3'), oligonucleotides were synthesized by the University of Ottawa Biotechnology Research Institute. The oligonucleotides were dissolved in 95% formamide and loaded onto a 8% preparative polyacrylamide gel containing 8 M urea in TBE buffer. The DNA was directly visualized under short wavelength UV light by placing the gel on a silica gel TLC plate containing a fluor (260 nm). The DNA bands were excised and purified as described in Section 2.5.1.

2.5.3. Oligonucleotide-directed Mutagenesis using Crossover Linkers

Synthetic oligodeoxynucleotides DW1, DW2 and AB279 were phosphorylated individually in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 4 mM DTT, 2 mM ATP containing 10 units of T₄ polynucleotide kinase at 37 C for 1 hr. For single-stranded crossover-linker mutagenesis, a 1,000 fold molar excess of
AB279 was ligated to the BamHI site of pDWMUC19 (section 3.6.1.) using standard techniques. To prepare the double-stranded crossover linker, DW1 and DW2 were combined in 10 ul of 10 mM Tris-HCl, pH 8.5, and 10 mM MgCl2 and heated at 70 C for 10 min. After cooling slowly to 42 C, the hybrid was added to a mixture of ligation buffer, 1 unit of T4 DNA ligase, 0.6 mM ATP, and 3 fold molar less of pDWMUC19-AB279 (Section 3.6.2.) which had been completely digested with PstI and dephosphorylated. The ligation mixes were incubated at 15 C for 20 hr and used to transform E. coli strain JM101 on 2X YT plates containing 50 ug/ml of ampicillin.

2.5.4. Determination of the Junction Sequences

The Maxam and Gilbert chemical cleavage method (Maxam and Gilbert 1980) was used to sequence the downstream junction region of the glycoprotein coding sequence of Hantaan virus. The plasmid pDWGUC19 (Section 3.6.2.) was cleaved with HindIII. To label the 3' end of the DNA fragment, the filling-in reaction was carried out in a solution consisting of 2 mM of all four dNTPs, 50 mM Tris-HCl, pH 7.2, 10 mM MgSO4, and 0.1 mM DTT at room temperature by adding 100 uCi of [α-32P] dATP (80 Ci/mmol, Amersham) and 5 units of Klenow fragment. After a 15 min incubation, the reaction was chased with a mixture of 0.1 mM cold dNTPs. The reaction was then terminated with 100 mM EDTA followed by heating at 65 C for 10 min. The sample was phenol-extracted and ethanol-precipitated. The second
enzyme digestion was carried out with EcoRV, and the sample was subjected to agarose gel electrophoresis. The wet gel was wrapped with Saran wrap, and exposed to an X-ray film. The one-end labeled, 800 bp HindIII/EcoRV fragment was located and excised using the exposed X-ray film as a template. The purified fragments were subjected to chemical cleavage procedures. Piperidine and hydrazine were purchased from Aldrich (Milwaukee, WI). Dimethyl sulfate and formic acid were purchased from BDH (Toronto, Ont). Briefly, a 25 ul aliquot of end-labeled DNA fragment (approx. 50,000 cpm) was mixed with 5 ul (5 ug) of sonicated calf thymus DNA. The mixture was dispensed into 4 separate reactions and chemical cleavage reactions were performed (Maxam and Gilbert, 1982). Details of chemical cleavage reactions are described in Appendix 3. To sequence the upstream junction region, the GemSeq K/RT sequencing system (Promega Biotec) was used with modifications. One ug of plasmid pDWGUC19 was denatured with 2 N NaOH and neutralized with 3 M sodium acetate pH 5.2, followed by ethanol precipitation. The DNA was then annealed to M13 forward primers (24mer, New England Biolabs) in 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl by incubating for 1 hr at 37 C. Five units of Klenow fragment and 40 μCi of [α-32P] dATP (400 Ci/mmol, Amersham) were added to the annealing mixture. The label/primer/template/enzyme mix was added to 4 separate reactions each of which contained the appropriate deoxy- and dideoxy-nucleotide mix. Each deoxy-
and dideoxy-nucleotide mix is formulated in Appendix 2.
After a 15 min incubation at room temperature, reactions were chased with a mixture of 2 mM dNTPs and terminated by adding 90% formamide containing 20 mM EDTA, 0.3% bromphenol blue and 0.3% xylene cyanol. Samples were heated at 70°C for 3 min and loaded onto 8 M urea-8% polyacrylamide gel (Section 2.3.5.)

2.6. Production of Recombinant Baculoviruses
2.6.1. Cells, Viruses and Preparation of Stock Viruses

_Spodoptera frugiperda_ (SF21) cells and _Autographa californica_ nuclear polyhedrosis virus (AcNPV) were obtained from Dr. D. H. L. Bishop, NERC Institute of Virology, Oxford, U.K. _Spodoptera frugiperda_ (SF9) cells were provided by Dr. M. D. Summers, Texas A & M University, College Station, TX. Wild type and recombinant viruses were propagated either in SF9 cells in TNM-FH medium (Hinrikus 1970, Summers and Smith 1987) or in SF21 cells in TC 100 medium (Vaughn et al. 1977) containing antibiotics (penicillin 100 u/ml, streptomycin 100 mg/ml, kanamycin 100 mg/ml) either as suspension or as monolayer cultures. The media were supplemented with 10% fetal bovine serum and 2 mM glutamine (Gibco). For the preparation of stock virus, a plaque was picked and resuspended in 1 ml of TC 100 medium. Approximately, 3 X 10^6 SF 21 cells, in a T-25 flask (25 cm², Corning Co., Corning, NY), were infected with the plaque-suspension at a moi of 0.01 pfu/cell and maintained
for 4 days at 28°C. This 4 day-culture fluid was used to infect 1.5 × 10^7 cells in a T-75 flask at a moi of 0.1. The virus supernatant was harvested 5 days post-infection and stored at 4°C. The titer of the virus stock was 3-5 × 10^8 pfu/ml.

2.6.2. Insertion of the Hantaan Virus Glycoprotein Gene into a Baculovirus Transfer Vector

The transfer vector, pAcYM1 (Matsuura et al. 1987), was digested with BamHI and dephosphorylated. A 3.4 kbp BamHI fragment recovered from plasmid pDGUC19 was ligated to the BamHI site of pAcYM1. Following transformation of E. coli strain RR1 with the ligation mix, plasmids were prepared (Sections 2.2.1., 2.2.2., 2.5.1.). The orientation of the glycoprotein gene insert was characterized by digesting the plasmids with EcoRI and EcoRV.

2.6.3. Transfection of Spodoptera frugiperda Cells

Spodoptera frugiperda 21 cells were cotransfected with a mixture of purified wild type Autographa californica viral DNA and pAcYM1-DWG (Section 3.6.3.) by a modification of the procedure described by Smith et al. (1983b). 2 ug of AcNPV DNA was mixed with various concentrations of plasmid DNA (2 to 20 ug) and adjusted to 950 ul with Hepes-buffered saline (20 mM Hepes, 1 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 10 mM glucose, pH 7.05). A fine precipitate was formed by adding 50 ul of 2.5 M CaCl_2, and the DNA mix was inoculated onto a monolayer of 1.2 × 10^6 SF 21 cells in 35 mm tissue culture dishes (Corning) and incubated at room temperature.
After 1 hr, the DNA was removed and 1.5 ml of TC 100 medium was added. The transfected cells were incubated for 4 days at 28 C. The supernatant was harvested and titrated on monolayers of SF21 cells.

2.6.4. Selection and Purification of Recombinant Baculovirus

*S. frugiperda* cells were plated into 35 mm tissue culture dishes (Corning) in TC 100 medium at a density of 1.2'X 10^6 viable cells. Serial 10 fold dilutions of virus inoculum were prepared. Typically, transfection supernatants were diluted 10^-2 to 10^-4, and viruses picked from a plaque were diluted 10^-1 to 10^-3. After medium was removed, 100 ul of diluted virus was inoculated into each dish. After 1 hr incubation for virus adsorption, 2 ml of overlay (1.5% low melting agarose in TC 100 medium) was added. When the overlay was solidified, 1 ml of medium was added and plates were incubated at 28 C for 5-6 days in a humid environment (Brown and Faulkner 1977). The plaques were visually examined by looking against a black background with a fluorescent light source. Clear plaques were differentiated from opaque plaques. The clear plaques were further examined by a light microscope, and those exhibiting no evidence of occlusion bodies were picked and titrated on SF21 cells to obtain pure polyhedrin-negative recombinant viruses.
2.6.5. Preparation of AcNPV DNA and Southern Blot

Hybridization Analysis

To purify AcNPV DNA from extracellular virions, culture fluids were clarified by centrifugation for 30 min at 3,000 g at room temperature (Damon/IEC) and then centrifuged in a Beckman SW27 rotor for 90 min at 81,000 g at 4 C. The virus pellet was resuspended in TE buffer and banded in a 10-50% (w/v) sucrose step gradient in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA for 90 min at 120,000 g using a SW41 rotor at 4 C. The viral band was collected and pelleted. The viruses were disrupted with 1% sodium N-lauryl sarcosinate and 200 ug/ml proteinase K. The DNA was banded in 54% CsCl (w/v) gradients using a SW 50Ti rotor at 195,000 g overnight at 25 C. The DNA band was extracted with water-saturated butanol, dialized in TE buffer and precipitated with ethanol. For partially purified viral DNA preparations, virus-infected cells were lysed with 1% Nonidet P-40 in 30 mM Tris-HCl, pH 7.5 and 10 mM Mg acetate. The nuclei were pelleted in a microcentrifuge (Eppendorf) and digested with proteinase K in 1% sodium N-lauryl sarcosinate at 50 C for 2 hr. The DNA was then phenol-extracted and ethanol-precipitated. For Southern blot analysis, preparations of viral DNA were digested with BamHI and the cleavage products were separated by electrophoresis in a 0.8% agarose gel. The gel was denatured with NaOH and electroblotted to GeneScreen Plus (New England Nuclear) (Section 2.2.3.). The membrane was
incubated in hybridization buffer (Section 2.2.3.) with a nick-translated BamHI fragment (3.4 Kbp) from pDWGUC19.

2.7. Analyses of Gene Products Expressed in Insect Cells

2.7.1. Polyclonal and Monoclonal Antibodies

Polyclonal rabbit antisera and mouse antisera raised against Hantaan virus strain 76-118 were obtained from Dr. J. Dalrymple, U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, MD. and from Dr. P. W. Lee, NIH, Bethesda, MD., respectively. Two monoclonal antibodies, HC02-BC10 and GD05-BC02, as mouse ascitic fluids, were gifts of Dr. J. Dalrymple, USAMRIID, and were originally produced in Dr. J. B. McCormick's laboratory at the Centers for Disease Control, Atlanta, GA.

2.7.2. Protein Gels and Western Blot Immunoassay

Monolayers of SF9 cells were infected with recombinant viruses at a moi of 5-10 and incubated for 48 hr at 28 °C. Cells were harvested and lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.1% SDS). Aliquots of the cell lysates were mixed with equal volumes of 2X dissociation buffer (10% β-mercaptoethanol, 10% SDS, 25% glycerol, 10 mM Tris-HCl, pH 6.8, 0.02% bromophenol blue), boiled for 10 min, and electrophoresed in a 7.5% polyacrylamide gel. Proteins on the gel were transferred onto nitrocellulose membrane (Section 2.1.4.). Antigen-bound membranes were incubated in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 3%
gelatin for 1 hr at room temperature. The membranes were transferred to TTBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) containing Hantaan-specific antibodies and incubated overnight. The membranes were then incubated with goat anti-mouse IgG or with goat anti-rabbit IgG conjugated with alkaline phosphatase (GAP-AP or GAM-AP, Bio-Rad, Richmond, CA). Between each step, membranes were washed in TTBS twice for 10 min each. The viral antigens were detected by immersing the membranes in carbonate buffer (0.1 M NaHCO₃, 1 mM MgCl₂, pH 9.8) containing 0.3 mg/ml of NBT (p-nitro blue tetrazolium chloride) and 0.15 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt) for color development. All the reactions were performed at room temperature.

2.7.3. Radiolabeling and Immunoprecipitation

SF9 cells were infected with virus at a moi of 10 pfu/cell, and maintained at 28 °C for 48 hr. The medium was replaced with methionine-free Grace's medium containing dialyzed fetal calf serum (10%), and the cells were deprived for 1.5 hr. The cells were then labeled with [³⁵S] methionine (1130 Ci/mmol, Amersham) at 150 uCi/ml for 4 hr. After labeling, the cells were washed three times with cold PBS and lysed in RIPA buffer. Cellular extracts were incubated with antibody at 4 °C overnight in the presence of 10 mg of protein A-Sepharose CL-4B (Pharmacia). The beads were washed five times with RIPA buffer, and immune complexes were released by boiling for 5 min in 1X
dissociation buffer. The supernatant were subjected to SDS
gel electrophoresis containing 10% polyacrylamide. After
electrophoresis, the gel was immersed in Amplify (Amersham)
for 30 min, dried and and exposed at -70 C to X-ray film.
CHAPTER 3: RESULTS

3.1. Structural Characterization of Hantaan Virus Strain 76-118 and other Hantavirus Isolates

3.1.1. Characterization of the Genome of Hantaan Virus Strain 76-118

To characterize the viral genome, Hantaan virus infected cells were labeled with $[^3H]$-uridine between 4 and 8 days post-infection. Nucleic acids were extracted from the virions, and analysed by agarose gel electrophoresis and fluorography. Three species of RNA, labeled with uridine, were clearly visualized in both glyoxal denatured or native RNA samples (Figure 1). When the RNA preparations were incubated with pancreatic RNase (100 ug/ml), the three bands disappeared (data not shown). Since uridine is utilized exclusively for the synthesis of RNA molecules in vivo, these results strongly indicate that Hantaan virus possesses three species of genomic RNA. The migration patterns of the native and glyoxal-denatured RNAs were significantly different. Each genomic band appeared to be more diffuse and migrated faster than for the glyoxal-treated preparation. The denatured genomic segments showed sharper bands which allowed the size of each genomic segment to be determined using messenger RNA of vesicular stomatitis virus (Indiana serotype) and ribosomal RNA as markers. The large (L) RNA segment of Hantaan virus was estimated to have a molecular weight of $2.2 \times 10^6$, and the
Figure 1. Autoradiograph of the genomic RNA of Hantaan virus strain 76-118. The genomic RNA was labeled with $[^3\text{H}]$ uridine, and resolved on a 1.4% agarose gel in the native form or after denaturation with glyoxal. The gel was impregnated with 3% PPO and exposed to X-ray film at -70°C. Eucaryotic ribosomal RNA and messenger RNA species of vesicular stomatitis virus were used as molecular weight markers. Numbers in parentheses indicate the number of nucleotides in each of the vesicular stomatitis virus messenger RNAs.
HANTAAN 76/118 Native

HANTAAN 76/118 Denatured

VSV<sub>ind</sub> mRNA

rRNA

18S mRNA (1700)
28S mRNA (6400)

mRNA (1350)
mRNA (850)
molecular weights of the medium (M) and small (S) RNA segments were estimated to be $1.25 \times 10^6$ and $0.6 \times 10^6$, respectively. The electrophoretic profiles of these three RNA genomic bands were similar to those reported for other viruses of the family Bunyaviridae (Obijesky and Murphy, 1977).

3.1.2. Structural Proteins of Hantaan Virus Strain 76-118

To identify the structural proteins of Hantaan virus, pelleted virions were further purified by centrifugation through a sucrose gradient. The purified virions were analysed by SDS-PAGE. Four proteins, with estimated molecular weights of 180,000 (180 K), 70 K, 55 K and 44 K, were clearly visualized by Coomassie blue staining, following electrophoresis under both reducing (Figure 2, lane 1) and non-reducing conditions (Figure 2, lane 2). These four proteins corresponded very well to the structural proteins reported for other viruses of the family Bunyaviridae (Obijesky and Murphy 1977). Recently, Elliot et al. (1984) reported similar observations for Hantaan virion proteins, and designated these proteins L, G1, G2, and N respectively. The G1 and G2 proteins were reported as glycoproteins and the N protein was found to be associated with the nucleocapsid (Elliot et al. 1984). Migration profiles of G1 and G2 appeared to be identical under either reducing or non-reducing conditions. This suggests that the G1 and G2 protein molecules are not linked by disulfide bonds. Western blot analysis using
Figure 2. SDS-polyacrylamide gel electrophoresis of structural proteins of Hantaan virus strain 76-118. The virus was grown in Vero E6 cells, harvested on day 8 post-infection and purified by centrifugation through a sucrose gradient. The virion proteins were separated in an 8% polyacrylamide gel. A: Coomassie-blue stained Hantaan virion proteins; lane 1, proteins were prepared in sample buffer containing mercaptoethanol (reducing conditions); lane 2, without mercaptoethanol in sample buffer (non-reducing conditions); lanes 3, 4, 7, molecular weight markers supplied by Bio-Rad: myosin H-chain (200 K), phosphorylase B (100 K), bovine serum albumin (68 K), ovalbumin (42 K); B, C, D: Western blot immunoassay of Hantaan virion proteins; lane 5, polyclonal mouse antisera raised against Hantaan 76-118 virions; lane 6, polyclonal rabbit antisera; lane 8, monoclonal antibody GD05-HC02 specific for both G1 and G2 glycoproteins (see Section 2.7.1. for the source of antibodies).
antibodies raised against Hantaan virus (Section 2.7.1.) confirmed that the 70 KDa and 55 KDa proteins were the G1 and G2 proteins (Figure 2, lane 8), and the 44 K protein was the nucleocapsid protein (Figure 2, lane 5, 6) (Elliot et al. 1984, Schmaljohn et al. 1983, Yamanishi et al. 1984). Both the mouse and rabbit polyclonal antisera reacted well with the 44 KDa N protein and very weakly with the 55 KDa G2 protein (Figure 2, lane 5, 6). However, neither polyclonal antibody reacted with the 70 KDa G1 or 180 KDa L proteins under these conditions (Figure 2, lane 5, 6). The structural features of Hantaan virus, a tripartite RNA genome and four structural proteins, strongly suggest that Hantaan virus belongs to the family Bunyaviridae.

3.1.3. Comparisons of the Genomic RNA of Different Hantaviruses

Although sero-epidemiological surveys suggest that Hantaan virus strain 76-118 is serologically related to other viruses causing similar diseases (HFRS) in different geographical locations, more direct evidence has not been provided. To verify the relationships among hantavirus isolates, a genomic RNA characterization was conducted for three other strains of hantaviruses isolated from different species of reservoir hosts from various regions of the world (Table 1). Prospect Hill virus was isolated from the wild rodent Microtus pennsylvanicus in Frederick County, MD, USA (Lee P. et al. 1982a), and strain USSR cg38-83
Figure 3. Comparison of genomic/RNAs of Hantaviruses. Vero E6 cells were infected with a low moi (0.01-0.001 pfu/cell) and maintained for 12 days. The [\(^3\text{H}\)] uridine-labeling was performed for 4 days between 8 and 12 days post-infection (10 uCi/ml). For Hantaan virus strain 76-118, cells were maintained for 8 days and labeling was carried out between 4 and 8 days post-infection. The viral RNAs were extracted and denatured with glyoxal before gel electrophoresis. The gel was prepared for fluorography and exposed to X-ray film for 4 days at -70 C. Ribosomal RNA was used as a marker.
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Segment</th>
<th>Molecular weights</th>
<th>Approx. number of nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hantaaan</td>
<td>L</td>
<td>$2.2 \times 10^6$</td>
<td>6,500</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$1.2 \times 10^6$</td>
<td>2,600</td>
</tr>
<tr>
<td>76-118</td>
<td>S</td>
<td>$0.6 \times 10^6$</td>
<td>1,650</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>4.0 \times 10^6</strong></td>
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<tr>
<td>Prospect</td>
<td>L</td>
<td>$2.2 \times 10^6$</td>
<td>6,500</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$1.3 \times 10^6$</td>
<td>3,800</td>
</tr>
<tr>
<td>Hill</td>
<td>S</td>
<td>$0.6 \times 10^6$</td>
<td>1,600</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>4.1 \times 10^6</strong></td>
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<tr>
<td>USSR</td>
<td>L</td>
<td>$2.2 \times 10^6$</td>
<td>6,500</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$1.2 \times 10^6$</td>
<td>3,600</td>
</tr>
<tr>
<td>eg38-83</td>
<td>S</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td>M</td>
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<td>3,800</td>
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<tr>
<td>82-109</td>
<td>S</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>4.2 \times 10^6</strong></td>
<td><strong>12,100 bases</strong></td>
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</tbody>
</table>

Table 3. Estimation of molecular weights and approximate number of nucleotides of the genomic RNA of different hantavirus isolates.
strain was isolated from the vole Clethrionomys glareolus captured in the Bashkirda area in the Western part of the USSR (personal communication, G. van der Groen, Institute of Tropical Medicine, Belgium). Strain ROK 83-109 was isolated from an human HFRS patient in Korea. As with Hantaan virus strain 76-118, all three isolates labeled with uridine showed three distinct species of genomic RNA (Figure 3). The results indicate that all the isolates possessed tripartite RNA genomes. In order to differentiate these isolates from Hantaan virus strain 76-118 and from each other, the labeled genomic RNA preparations from all four isolates were analysed on one gel and the migration profiles were compared (Figure 3). The L RNA segments of all four isolates appeared to be the same size. However, the M RNAs of Prospect Hill and ROK 83-109 isolates were larger than those of Hantaan 76-118 or USSR cg38-83. In contrast, the S RNAs of Prospect Hill, USSR cg38-83 and Hantaan 76-118 were similar in size, but smaller than that of ROK 83-109. The approximate molecular weights and the number of nucleotides of the genomic segments in each isolate are presented (Table 3). The RNA profiles and similarities in the size of the genomic segments of different hantavirus strains clearly demonstrate that all four hantavirus isolates examined are distinguishable but probably belong in the same group of viruses.
3.1.4. Molar Ratios of Genomic RNA Segments

Since the intensity of the $[^{3}H]$-uridine labeled genomic RNA bands on the autoradiograms were not linear with respect to the relative sizes of the RNA segments, the molar ratios of each genomic segment were examined by densitometry (Figure 4). The relative molar ratios were then calculated considering the estimated size of each RNA segment. Another approach was to count the radioactivity of the gel slices corresponding to each genomic band, and to convert these numbers to molar ratios. The latter approach would eliminate the possibility of non-linear correlations between the density of a band and the extent of uridine incorporation due to over-exposure of the X-ray film. As shown in Table 4, the molar ratios of $L: M : S$ RNA segments in Hantaan virus strain 76-118 were estimated to be 1 : 1.6 : 2.2. The differences in the molar ratios of the genomic segments of Prospect Hill and strain USSR cg38-83 were estimated to be 1 : 3.3 : 6.3 and 1 : 2.2 : 3.6, respectively. Since the RNA was labeled with uridine in these experiments, one might speculate that the $S$ RNA segment was possibly uridine-rich as compared to the $L$ or $M$ segments. This possibility was ruled out to some extent by labeling with $[^{3}H]$-guanine. Similar results were obtained (data not shown, see also nucleotide sequence analysis data of the $M$ and $S$ RNA of Hantaan Virus strain 76-118 in Table 5). These results suggest that each virion contains a variable number of RNA molecules rather than equivalent
Figure 4. Densitometric tracing of the genomic RNA profiles of various hantavirus isolates. [\( ^{3}H \)] uridine-labeled viral RNAs were denatured and resolved by agarose gel electrophoresis. Gels were prepared for fluorography and exposed to X-ray film. The exposed film was scanned by densitometer as described in Section 2.1.5.
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Large (L)</th>
<th>Medium (M)</th>
<th>Small (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hantaan 76-118</td>
<td>1</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Prospect Hill</td>
<td>1</td>
<td>3.3</td>
<td>6.3</td>
</tr>
<tr>
<td>USSR cg38-83</td>
<td>1</td>
<td>2.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 4. Molar Ratios of the genomic RNA segments of various hantavirus isolates. The values corresponding to the area of the peak in the densitograms, or the radioactive counts in gel slices, corresponding to the individual RNA segments were converted to the relative number of molecules of each genomic segment by the following equation:

\[
\left( \frac{\text{total number of nucleotides of all three segments}}{\text{number of nucleotides of the particular segment}} \right) \times (\text{area of the peak or radioactive counts})
\]

Molar ratios were normalized using the value for the L segment as 1.
numbers of each RNA segment as is seen in some tripartite plant viruses (see discussion).

3.2. Molecular Cloning of the Genomic RNA of Hantaan Virus

3.2.1. Construction of Genomic cDNA Libraries

Viral RNA preparations were often contaminated with significant amounts of cellular DNA. To remove the DNA, the viral RNA preparation was treated with RNase-free DNase I prior to cDNA synthesis. The cloning strategy is illustrated in Figure 5. DNase-treated viral RNA, as a mixture of the three genomic segments, was used as template for the construction of a cDNA clone bank by the RNase H method (Gubler and Hoffman 1983) using random primers. The double-stranded cDNA was size-fractionated by chromatography through a Sepharose 4B column. The approximate sizes of the cDNA from each fraction were estimated by agarose gel electrophoresis. Relatively larger fragments were inserted into the unique HindIII site of the cloning vector pUC19 (Yanish-Peron 1985). Smaller cDNA was used as a probe in Northern blot hybridization to confirm that the synthesized cDNA preparation represented all three viral RNA segments. For the primary selection of recombinant clones, ampicillin-resistant and colorless colonies were selected in the presence of the lac operon inducer IPTG and the chromogenic substance X-gal. This approach takes advantage of the β-lactamase gene and insertional inactivation of the β-galactosidase gene of pUC19 (Jarvin 1982).
Figure 5. Strategy for cloning the genome of Hantaan virus strain 76-118.
Cloning Strategy of Hantaan Virus Genome

Genomic RNAs

ds DNA

Larger size ds DNA

Sepharose 4B

Random primer DNA pol I

RTase RNase H

Multiple cloning sites

Amp' pUC19 2.7 kb

LacZ

Hind II Alkaline phosphatase

EcoRI Hind II

Hind II Hind III

E. coli JM 109 ampicillin resistant and white colonies

Isolation of plasmid DNA

Grouping by Southern blot cross hybridization

Genomic assignments of cDNA clones by Northern blot hybridization
3.2.2. Grouping and Segment Assignments of Genomic Clones

Plasmid DNA which was isolated from each recombinant clone was digested with EcoRI and HindIII, and the fragments were separated on an agarose gel. 80 clones were selected with cDNA inserts of more than 400 bp. The clones were grouped by Southern blot cross hybridization. The clones with large inserts were used for the hybridization probes. One example of cross hybridizations is shown in Figure 6. Clone 41 was hybridized to clone 111 and 146 (Figure 6, A, D). Clone 135 was identified as a member of another group of clones which did not share sequence homology with the clone 41 group (Figure 6, A, C). In some cases, a limited degree of overlap was suggested by the intensity of a hybridization signal. For example, Clone 41 has an internal HindIII site which results in cleavage of the insert into two fragments. Only the smaller fragment of clone 41 hybridized with clone 67 (data not shown) indicating that the clone 67 has sequences which overlap with clone 41. Clones lacking direct overlapping sequences could also be grouped. For example, Clones 29 and 72 did not hybridize to each other; however clone 138 hybridized to clone 29, clone 4 hybridized to clone 72, and clone 138 hybridized to clone 4. These results suggest that clone 29, 138, 4 and 72 have been derived from the same RNA segment (see Figure 9). Northern blot analyses were carried out in order to determine the viral specificity of cDNA clones and to correlate each group of clones with individual viral
Figure 6. Grouping of recombinant cDNA clones by Southern blot cross hybridization. Recombinant plasmids were prepared and digested with EcoRI and HindIII. The DNA digests were electrophoresed on a 1% agarose gel in TBE buffer. (A); The inserts were visualized by staining with ethidium bromide. For Southern blot analysis, the DNA fragments were transferred to hybridization membrane (Zeta probe). The membranes were probed with nick-translated clone 17 (B); with clone 135 (C); or with clone 41 (D). The numbers in panel A indicate clone number.
Figure 7. Assignment of cDNA clones to genomic RNA segments of Hantaan virus. (A) The $[^3H]$-uridine labeled viral RNA and ribosomal RNA were electrophoresed on the same gel and transferred to a membrane. The membrane was sprayed with EN$^3$HANCE (New England Nuclear) and exposed to an X-ray film. This film was used as a marker. (B) The genomic segments were separated by 1.4% agarose gel electrophoresis and transferred to hybridization membrane. The membrane was cut into strips and each strip was hybridized with a $[^32P]$ labeled cDNA probe representing an individual group of clones. Numbers in the panel B indicate recombinant clones.
genomic RNA segments. Following agarose gel electro-
phoresis, viral RNA segments were immobilized on membrane
filters by electrotransfer. The membrane was cut into
several strips, and each strip was hybridized with a nick-
translated cDNA probe representing an individual group of
clones. As shown in Figure 7, clones 135, 144 and 11
clearly hybridized to only the S RNA segment and clone 72
hybridized to only the L RNA segment. Clones 110, 90 and 41
hybridized specifically to the M RNA segment. These results
demonstrate that each group of cDNA clones are viral
specific and represent individual segments of the genomic
RNA.

3.2.3. Identification of cDNA Clones Representing the 3'
Termini of the M and S RNA Segments

Since random primers were used to synthesize the
first-strand of cDNA, the 3' end sequence of the genomic
RNAs would be cloned rarely. Schmaljohn et al. (1985)
determined 3' terminal sequences of the genomic RNAs of
Hantaan virus by direct chemical cleavage of the RNA. It is
noteworthy that the first 19 nucleotides from the 3'
terminus of the S RNA segment are identical to those of the
L RNA segment in Hantaan virus strain 76-118. Using the
sequence information from this report, two oligodeoxy-
nucleotides were synthesized and employed as primers for
construction of two additional clone banks. A complementary
16mer yk-m (5'-TAGTAGTAGACTCCGC-3') was deduced from the 3'
end sequence of the M RNA segment. For the L and S RNA
Figure 8. Oligonucleotide screening for clones containing 3' terminal sequences of the genomic RNAs of Hantaan virus. Bacterial transformants were lifted onto a membrane and lysed. The membrane was washed thoroughly before hybridization, and probed with $^{32}$P labeled yk-m at 31°C overnight. The membrane was washed at 35°C for 1 hr and subsequently at 42°C for 10 min (See 2.2.4.) and exposed to X-ray film overnight. Arrows indicate recombinant clones specific for the probe yk-m.
segment, a 19mer yk-1s (5'-'TAGTAGTAGACTCCCTAAA-3') was used as a common primer. Approximately 400 and 500 colorless transformants were obtained after transformation of E. coli with cDNA synthesized using yk-m and yk-1s primers, respectively. Although first strand synthesis was initiated with the specific synthetic primers, it was possible that the DNA polymerase might not copy the 5' end of the first-strand during second-strand synthesis. Consequently, not all clones would have the 3' terminal sequences of the genomic segments. Thus, to identify the clones which retained 3' terminal sequences of the genomic templates, the 16mer yk-m and 19mer yk-1s oligonucleotides were used as hybridization probes. With low hybridization temperatures and non-stringent washing first cDNA strand during second strand synthesis. The clones selected by oligonucleotide screening were subsequently grouped by cross hybridization with previously characterized clones and assigned to genomic segments by Northern-blot analyses.

3.2.4. Physical Mapping of Genomic Clones

In order to define the orientation and to determine physical maps of the genomic clones, extensive hybridizations were carried out between clones. Clone sp28, which had been characterized as a 3' terminal clone of the M RNA, was used to orient the M clones. For example, clone 67 hybridized with sp28 but clone 90 did not. Similarly, clones 135 and 11 hybridized to clone sp19 which represented the 3' terminus of the S RNA segment. As a
Figure 9. Physical maps of the genomic clones of Hantaan virus. The construction of the map was based upon cross hybridization and Northern blot analyses. The maps were oriented using clones sp28 and sp19 as 3' terminal clones of the M and S segments, respectively. Selected restriction sites are also illustrated.
result, clone 11 was mapped as adjacent to sp13. 3'-L clones were not identified following oligonucleotide screening, therefore clones representing approximately 3 kb of the L sequence were tentatively mapped with respect to each other without knowing their orientation. By cross hybridization and Northern blot analyses, all of the cDNA clones were assigned to specific segments of the genomic RNA. Physical maps of the cloned sequences were constructed using representative clones from each group (Figure 9).

3.3 The Genomic Organization and Coding Strategy of M RNA Segment of Hantaan Virus

3.3.1. Determination of the Nucleotide Sequence of the M RNA

Five clones, sp28, 67, 41, 12 and 90 (Figure 9), were initially selected to determine a nucleotide sequence of the M RNA segment of Hantaan virus. The cDNA fragments were subcloned into the multiple cloning sites of the replicative forms of both M13mp18 and M13mp19 DNA using EcoRI and HindIII. For clone 41, PstI and EcoRI sites were used for subcloning since clone 41 has an internal HindIII site. The sequencing strategy is illustrated in Figure 10. Nucleotide sequences were determined in both directions by the dideoxynucleotide chain termination method (Sanger et al. 1977). To sequence clones 41 and 12, sequential overlapping clones were generated (Dale et al. 1985). Although yk-m, of which sequence was derived from M RNA
Figure 10. Strategy for sequencing the mRNA segment of Hantaan virus. The dideoxy chain termination sequencing method was employed after subcloning cDNA fragments into the single-stranded bacteriophage M13. The bars with the numbers indicate the cDNA clones selected for the sequence determination and the approximate length of each cloned sequence. The arrows indicate sequencing direction and the length of each region sequenced. The entire map corresponds to the viral complementary sequence in the 5' to 3' orientation.
segment, was used as a primer in the cloning the 3' terminus of the M segment, clone sp28 was found to contain the yk-1s primer sequence at its terminus. Sp28 has been confirmed as M specific by hybridization to genomic RNA (data not shown). Two other 3' clones, sp42 and sp37, were also sequenced to further confirm the 3' sequence of the M RNA. Clone m4 (Figure 10) was obtained using a second primer hmp2 (20 mer, 5'-GAGGGGGGCTGGGTTCTGG-3') representing nucleotides 3060-3079 of the M RNA segment as determined by sequencing of clone 90. Clone m4 extended to nucleotide 3565. To determine the 5' terminal sequence of the M RNA segment, a third primer hmp3 (16mer, 5'-GTAGCTTTCTAACCAC-3') was used to prime cDNA synthesis. The hmp3 was complementary to nucleotide 3545-3560 of the M RNA. In clone mc8, which was obtained following hmp3 primed cDNA synthesis, the sequence 5'-GGGGGGTTAGTAGTAGACACC-GCAAGA-3' was apparent. Since first strand of cDNA was tailed with dC and oligo (dG) was used to prime the second strand synthesis, the sequence adjacent to the homopolymeric tail of dG's was predicted to be the 5' terminal sequence of the M RNA. Moreover, this sequence was perfectly complementary to the 3' terminal sequence of the viral M RNA. Considering the general features of the genomic RNAs of bunyaviruses that the sequences at both termini of the RNA segments are inverted complements (Clerx van Haaster et al. 1982b, Bishop et al. 1982, Cabradilla et al. 1983, Ihara et al. 1984, Eshita and Bishop 1984), it
was concluded that clone mc8 contained a complete copy of
the 5' terminal sequence of the M segment.

3.3.2. Sequence Analyses and Features of the M RNA

The complete nucleotide sequence of the M RNA segment
of Hantaan virus strain 76-118 is presented in Figure 11 in
the viral complementary sense beginning with the first 3'
terminal nucleotide of the M RNA segment. The M RNA segment
of Hantaan virus contains 3616 nucleotides. As with other
bunyaviruses, inverted complementary sequences of 20
nucleotides in length are present at the termini of the M
RNA. Two mismatches are seen in the complementary termini
at nucleotides 12 and 3605 and at nucleotides 19 and 3598
(Figure 12). The stability of the stem structure predicted
for the terminal complementary sequence of the M RNA was
assessed by evaluating its free energy. The free energy was
calculated to be -27.9 Kcal/mole. When the nucleotide
sequence was compared to published data (Schmaljohn et al.
1987), as shown in Figure 13, four nucleotides at positions
12, 150, 231 and 558 were found to be different. The T at
nucleotide 12 in the data of Schmaljohn et al. (1987)
suggested there is only one mismatch in the predicted stem
structure. Since a synthetic oligonucleotide deduced from
the 3' sequence of the M RNA has been used to prime cDNA
synthesis in our experiments, the reasons for the sequence
differences are unclear. Three additional nucleotide
differences found in the open reading frame. These three
nucleotide differences alter corresponding amino acids from
Figure 11. Nucleotide sequence of the M RNA segment of Hantaan virus. The sequence is presented as a viral complementary DNA sequence in the 5' to 3' orientation. Nucleotides are numbered. Predicted amino-acids from the first methionine codon are also numbered and illustrated below the corresponding codon in the major open reading frame. Asterisks represent the translation termination codon. A hydrophobic stretch at the amino terminus of the predicted protein is underlined (-). Boxes indicate potential asparagine-linked glycosylation sites.
TAGTAGGACTCCGCCAAAAGAAGACACGTCAATCAGCAAC ATG GGG ATA TGG AAG MET GLY ILE TRP LYS 55

TGG CTA GTG ATG GCC AGT TTA GTA TGG CCT GGT TGG ACA CTG AGA TRP LEU VAL MET ALA SER LEU VAL TRP PRO VAL LEU THR LEU ARG 100

AAT GTG TAT GAC ATG AAA ATT GAG TGC CCC CAT ACA GGA AGT TTT ASN VAL TYR ASP MET LYS ILE GLU CY5 PRO HIS THR VAL SER PHE 145

GGG GGA AAG AGT GTG ATA GGT TAT GTA GAA TTA CCC CCC GTG CCA GLY GLY ASN SER VAL ILE GLY TYR VAL GLU LEU PRO GLU PRO VAL PRO 50

TTG GCC GAC ACA GCA CAG ATG GTG CCT GAG AGT TCT TGT AGC ATG LEU ALA ASP THR ALA GLN MET VAL PRO GLU SER SER CYS SER MET 235

GAT AAT CAC CAA TCG TTG AAT ACA ATA ACA AAA TAT ACC CAA GTA ASP ASN HIS GLN SER LEU ASN THR ILE THR LYS TYR THR GLN VAL 280

AGT TGG AGA GGA AAG GCT GAT CAG TCA CAG TCT AGT CAA AAT TCA SER TRP ARG GLY LYS ALA ASP GLN SER GLN SER SER GLN ASN SER 325

TTT GAG ACA GTG TCC ACT GAA GTT GAC TTT AAA GGA ACA TGT GTT PHE GLU THR VAL SER THR GLU VAL ASP LEU LYS GLY THR CYS VAL 370

CTA AAA CAC AAA ATG GTG GAA GAA TCA TAC CGT AGT AGG AAA TCA LEU LYS HIS LYS MET VAL MET ALA GLU CY5 SER TYR ARG SER ARG LYS SER 415

GTA ACC TGT TAC GAC CTG TCT TGC AAT AGC ACT AAT ACC ACT VAL THR CYS TYR ASP LEU SER CYS ASN SER THR TYR CYS AAG CCA 460

ACA CTA TAC ATG ATT GTA CCA ATT CAT GCA TGC AMT ATG ATG AAA 505

THR LEU TYR MET ILE VAL PRO ILE HIS ALA CYS ASN MET MET LYS 155

AGC TGT TGG ATT GCA TGG GGA CCA TAC AGA GGA TCA CAG GTG GTT TAT SER CYS LEU ILE ALA GLU PRO TYR ARG VAL GLN VAL VAL TYR 550

GAG AGA ACT TAC TGT ATG ACA GGA GTG CTG ATT GAA GGA AAA TGC 595

GLU ARG THR TYR CYS MET THR GLY VAL LEU ILE GLU LYS CYS 185

TTT GTG CCA GAT CAA AGT GTG GTC AGT ATT ATC AAG CAT GGG ATC 640

PHE VAL PRO ASP GLN SER VAL VAL SER ILE LYS HIS GLY ILE 200

TTC GAT ATT GCA AGT GGT CAT ATT GTA TGT TCC TTT GTT GCA GTT PHE ASP ILE ALA SER VAL HIS ILE VAL CYS PHE PHE VAL ALA VAL 685

AAA GGG AAT ACT TAT AAA ATT TTT GAA CAG GGT AAG AAA TCC TTT LYS GLY ASN THR TYR LYS ILE PHE GLU GLN VAL LYS LYS SER PHE 730

GAA TCA ACA TGC AAT GAT ACA GAG AAT AAA ATG CAA GGA TAT TAT GLU SER THR CYS ASN ASP THR GLU ASN LYS VAL GLN GLY TYR TYR 775

ATT TGT ATT GTA GGG GGA AAC TCT GCA CCA ATA TAT GTC CCA ACA ILE CYS ILE VAL GLY GLY ASN SER ALA PRO ILE TYR VAL VAL TYR 820

260
AAG ATA AAG GAA GAG TTT GAA AAA ACA AAA GGC TCA ATG GTA TGT 1675
LYS ILE LYS GLU GLU PHE GLU LYS THR LYS GLY SER MET VAL CYS 545
GAT GTG TGC AAG TAT GAG TGT GAA ACC TAT AAA GAA TTA AAG GCA 1720
ASP VAL CYS LYS TYR GLU CYL GLU THR LYS TYR LYS LEU LYS ALA 560
CAC GGG GTA TCA TGC CCC CAA TCT CAA TGT CCT TAC TGT TTT ACT 1765
HIS GLY VAL SER CYS PRO GLN SER GLN CYL PRO TYR CYS PHE THR 575
CAT TGT GAA CCC ACA GAA GCA GCA TTC CAA GCT CAT TAC AAG GTA 1810
HIS CYS GLU PRO THR GLU ALA ALA PHE GLN ALA HIS TYR LYS VAL 590
TGC CAA GTT ACT CAT AGA TTG AGG GAT GAT CTA AAG AAA ACT GTT 1855
CYS GLN VAL THR HIS ARG PHE ARG ASP ASP LEU LYS LYS THR VAL 605
ACT CCT CAA AAT TTT ACA CCA GGA TGT TAC CGG ACA CTA AAT TTA 1900
THR PRO GLN ASN PHE THR PRO GLY CYS TYR ARG THR LEU ASN LEU 620
TTT AGA TAC AAA AGC AGG TGC TAC ATC TTT ACA ATG TGG ATA TTT 1945
PHE ARG TYR LYS SER ARG CYS TYR ILE PHE THR MET TRP ILE PHE 635
CTT CTT GTC TTA GAA TCC ATA CTG TGG GCT GCA AGT GCA TCA GAG 1990
LEU LEU VAL LEU GLU SER ILE LEU TRP ALA ALA SER ALA SER GLU 650
ACA CCA TTA ACT CCT GTC TGG AAT GAC AAT GCC CAT GGG GTA GGT 2035
THR PRO LEU THR PRO VAL TRP ASP ASP ASN ALA HIS GLY VAL GLY 665
TCT GTT CCT ATG CAT ACA GAT TTA GAG CTT GAT TTC TCT TTA ACA 2080
SER VAL PRO MET HIS THR ASP LEU GLU LEU ASP PHE SER LEU THR 680
TCC AGT TCC AAG TAT ACA TAC CGT AGG AAG TTA ACA AAC CCA CTT 2125
SER SER SER LYS TYR THR ARG LYS LEU THR ASN PRO LEU 695
GAG GAA GCA CAA TCC ATT GAC CTA CAT ATT GAA ATA GAA GAA CAG 2170
GLU GLU ALA GLN SER ILE ASP LEU HIS ILE GLU ILE GLU GLN 710
ACA ATT GGT GTT GAT GTG CAT GCT CTA GGA CAC TGG TTT GAT GGT 2215
THR ILE GLY VAL ASP VAL HIS ALA LEU GLY HIS TRP PHE ASP GLY 725
CGT CTT AAC CTT AAA ACA TCC TTT CAC TGT TAT GGT GCT TGT ACA 2260
ARG LEU ASN LEU THR PHE HIS PHE GLY GLY ALA CYS THR 740
AAG TAT GAA TAC CCT TGG CAT ACT GCA AAG TGC TAT GAA AGA 2305
LYS TYR GLU TYR PRO TRP HIS THR ALA LYS CYS HIS TYR GLU ARG 755
GAT TAC CAA TAT GAG AGG AGC TGG GGT GGT [AAT CCA TCA GAT GGT 2350
ASP TYR GLN TYR GLU THR SER TRP GLY CYS ASN PRO SER ASP CYS 770
CCT GGG GTG GGC ACA GGC TGT ACA GCA TGT GGT TTA TAC CTA GAT 2395
PRO GLY VAL GLY THR GLY CYL THR ALA CYS GLY LEU TYR LEU ASP 785
CAA CTG AAA CCA GTT GTT AGT GCT TAT AAA ATT ATC ACA ATA AGG 2440
GLN LEU LYS PRO VAL GLY SER ALA TYR LYS ILE ILE THR ILE ARG 800
GAG ATA GAA AAT AGT AAA GTA TAT GAT GAT GGG GCA CCG CAA TGT 3295
GLU ILE GLU ASN SER LYS VAL TYR ASP ASP GLY ALA PRO GLN CYS 1085

GGG ATA AAA TGT TGG TTT GTT AAA TCA GGG GAA TGG ATT TCA GGG 3340
GLY ILE LYS CYS TRP PHE VAL LYS SER GLY GLU TRP ILE SER GLY 1100

ATA TTC AGT GGT AAT TGG ATT GTM CTC ATT GTC CTC TGT GTA TTT 3385
ILE PHE SER GLY ASN TRP ILE VAL LEU ILE VAL LEU CYS VAL PHE 1115

CTA TTG TTC TCC TTG GTT TTA CTA AGC ATT CTC TGT CCC GTA AGG 3430
LEU LEU PHE SER LEU VAL LEU LEU SER ILE LEU CYS PRO VAL ARG 1130

AAG CAT AAA AAA TCA TAG CTAAATTCTGTGACTATCTCTCGTTATGTATAGC 3483
LYS HIS LYS LYS SER *** 1135

TTAAACATATACTAATTATTTTTATTATTTTGTAATCTACTATTTAATACACACTAAAAAAA 3442
TAGTAGCTTTCTAACCACAAACTTAGTCTTCCTGCATGATGCTCTAAACATCTTG 3601

CGGGTCTACTACTA 3616
Figure 12. Possible base-paired stem structure of the inverted complementary terminal sequences of the M RNA of Hantaan virus. The RNA sequence was deduced from the complementary DNA sequence. The mismatched nucleotides are numbered.
glycine, serine and threonine to glutamine, asparagine and serine, respectively. These results demonstrate remarkable stability of the M RNA segment of Hantaan virus.

3.3.3. Coding Strategy and Properties of the Predicted Gene Product of the M RNA

The six possible reading frames of the genomic and anti-genomic sense RNAs were examined. A single large open reading frame was identified in the second frame of anti-genomic sense RNA (Figure 14). The first ATG codon occurs at nucleotides 41-43, and the termination codon (TAG) appears at nucleotides 3346-3348. The nucleotides in positions -4 (i.e., 4 nucleotides upstream from the first ATG), -3, -1 and +4 are C, A, C and G, respectively. These sequences flanking the first ATG codon are a favorable context for translation initiation by eukaryotic ribosomes (Kozak 1986a, 1986b). The predicted amino acid sequence deduced from the nucleotide sequence of the open reading frame is presented in Figure 11. This large open reading frame is capable of encoding a polypeptide of 1135 amino acids with a molecular weight of 126,174. The length of the predicted polypeptide is sufficient to include both G1 and G2 proteins which have been estimated to have molecular weights of 70 K and 55 K, respectively. Seven potential asparagine-linked glycosylation sites were found to be scattered throughout the predicted protein sequence. These are at amino acid residues 134-136 (Asn-Ser-Thr), 235-237 (Asn-Asp-Thr), 347-349 (Asn-His-Thr), 399-401 (Asn-Ile-
Figure 13. Comparison of the nucleotide sequence of the M segment of Hantaan virus with the previously published sequence. The numbers indicate the position of nucleotide differences. Amino-acid differences which result from the nucleotide differences are also indicated below the corresponding codon. (A) our sequence; (B) the sequence reported independently by another laboratory (Schmaljohn et al. 1987).
5'   12   150   231   558   3'  (A).  
Gly  Ser  Thr  

5'   12   150   231   558   3'  (B)  
Glu  Asn  Ser  

M
Figure 14. Potential coding regions in the mRNA of Hantaan virus. The arrows indicate open reading frames initiating with a methionine codon and ending at the first in frame termination codon. The number above each arrow displays the number of amino acids encoded in the potential open reading frame. Small numbers above the central line represent nucleotide position (10^-2).
Thr), 609-611 (Asn-Phe-Thr), 766-768 (Asn-Pro-Ser) and 928-930 (Asn-Thr-Ser). Whether these are in fact glycosylated in the mature viral glycoproteins is not known. Amino-acid composition reveals that the predicted protein is rich in cysteine residues (5.3%) compared to the S RNA gene product (1.3%). The putative M gene product is a possible precursor of both G1 and G2 envelope glycoproteins of Hantaan virus. A hydropathy plot shows that a hydrophobic stretch of 19 amino acids including the first methionine is located at the amino terminus of the potential precursor protein (Figures 11, 15). This feature resembles the typical signal sequence of a glycoprotein (Klenk and Rott 1980). The amino acids adjacent to the carboxy terminus (1106-1127) of the predicted polypeptide were also found to be extremely hydrophobic followed by a stretch of hydrophilic residues (1130-1135) (Figure 31). These features suggest that membrane anchor region and cytoplasmic tail are present on this protein. Two additional hydrophobic regions were identified at amino acid residues 482-509 and 627-648 (Figure 31). They are likely to be an internal membrane anchor domain of the G1 protein and an internal signal sequence of the G2 protein as observed in Semliki Forest virus (Melancon and Garoff 1986). The four most hydrophilic regions which may represent for antigenic determinants (Hopp and Woods 1981) were found at amino acid residues 530-535 (Arg-Lys-Ile-Lys-Glu-Glu), 536-603 (Arg-Asp-Asp-Leu-Lys-Lys), 877-882 (Ser-Pro-Arg-Asp-Lys-Gly) and 942-946
Figure 15. Hydropathy plot of the predicted glycoprotein precursor encoded by the M RNA of Hantaan virus. The darkened areas represent hydrophilic regions and open areas indicate hydrophobic regions. Arrows indicate the position of potential N-linked glycosylation sites. The numbers at the bottom of the figure represent amino acid position starting with the amino terminus of the polypeptide.
(Lys-Asp-Pro-Asp-Gly). Nine other short open reading frames (two from anti-genomic sense and seven from genomic sense) were found in addition to the major open reading frame. Each of these are capable of encoding more than 50 amino acids; however, it remains to be determined if they encode functional proteins.

3.4. Molecular Characterization of the S RNA Segment of Hantaan Virus

3.4.1. Nucleotide Sequencing of cDNA Clones Specific for S RNA

The strategy used to determine the complete nucleotide sequence of the S RNA segment is shown in Figure 16. Three overlapping cDNA clones, sp19, 11 and 135, were initially sequenced in both directions by the chain termination sequencing method. All cDNA fragments were subcloned into PstI and EcoRI sites of M13mp18 and mp19 RF DNA with the exception of clone 135, which required subcloning into the EcoRI and HindIII sites. Sequential overlapping clones were produced in order to sequence clones 11 and 135. Clone sp19 was found to be 466 nucleotides long and to contain the yk-1s primer sequence except for the three terminal nucleotides (TAG). Sp19 was confirmed as a clone encompassing the 3' end of the viral S RNA by sequencing two additional clones sp4 and sp32, which had sequences identical to clone sp19, and retained the yk-1s primer sequence but missing one (T) and two (TA) nucleotides,
Figure 16. Strategy for sequencing the S RNA segment of the Hantaan virus genome. The dideoxy chain termination sequencing method was employed after subcloning cDNA fragment into the single-stranded bacteriophage M13. The bars with numbers indicate the approximate size and position of cDNA clones selected for the sequence determination. The arrows indicate sequencing direction and the length of the region sequenced. The map corresponds to the viral complementary sequence in the 5' to 3' orientation.
respectively. Since clone 135 was the most 5' clone (Figure 16) obtained from the cDNA bank synthesized using random primers, another cDNA library was constructed. Oligonucleotide hsp2 (20mer, 5'-GGGGATGATATGGATCCTGA-3'), derived from nucleotides 1229-1248 of the S RNA segment was used to prime cDNA synthesis. Clone s44 was obtained and enabled the nucleotide sequence analysis to be extended to nucleotide 1605. To extend the 5' sequence of the S RNA even further, oligonucleotide hsp3 (16mer, 5'-CTGGTTTCTCTGCTTA-3'), deduced from nucleotides 1585-1600, was used to prime cDNA synthesis. The first cDNA strand was tailed with dCTP using terminal transferase, and second strand synthesis with the Klenow fragment was primed with oligo (dG). Clone sc5 was obtained from the hsp3 clone bank. When sc5 was sequenced, the heteropolymeric sequence immediately following the dG tail (5'-GGGGGGGGGTAGTAGTAGATGCTCCC-3'), was predicted to represent the 5' terminal sequence of the S RNA segment.

3.4.2. Sequence Analyses and Features of the S RNA Segment of Hantaan Virus

The complete nucleotide sequence of the S genomic RNA of Hantaan virus is presented as a DNA sequence complementary to the viral RNA in the 5' to 3' orientation (Figure 17). The S segment is 1694 nucleotides in length. Examination of the nucleotide sequence reveals that the 3' and 5' termini of the S RNA molecule are complementary with only three mismatches (Figure 18). The stability of this
Figure 17. Nucleotide sequence of the S RNA segment of Hantaan virus. The sequence is presented as a viral complementary DNA sequence in the 5' to 3' orientation. Nucleotides are numbered. The predicted amino acid sequence beginning with the first methionine codon in the major open reading frame are also numbered and illustrated below the corresponding codon. Asterisks represent the translation termination codon.
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GLU ILE GLU ASN SER LYS VAL TYR ASP ASP GLY ALA PRO GLN CYS 1085
GGG ATA AAA TGT TGG TTT GTT AAA TCA GGG GAA TGG ATT TCA GGG 3340
GLY ILE LYS CYS TRP PHE VAL LYS SER GLY GLU TRP ILE SER GLY 1100
ATA TTC AGT GGT AAT TGG ATT GTA CTC ATT GTC CTC TGT GTA TTT 3385
ILE PHE SER GLY ASN TRP ILE VAL LEU ILE VAL LEU CYS VAL PHE 1115
CTA TTG TTC TCC TTG GTT TTA CTA AGC ATT CTC TGT CCC GTA AGG 3430
LEU LEU PHE SER LEU VAL LEU LEU SER ILE LEU CYS PRO VAL ARG 1130
AAG CAT AAA AAA TCA TAG CTAAATTCTGTGACTATCTCTGTTCTTATGATAGC 3483
LYS HIS LYS LYS SER *** 1135
TTTACATATATACATATTTTTTATATTTCCAGTATACATCTATCTAAACACACTAAAAAAA 3442
TAGTAGCTTTCTAACACAAAACCTTAGATTCTTCTTCTGTATGATCTTAAACATCTTG 3601
CGGTGTCTACTACTA 3616
Figure 18. Possible base-paired stem structure of the inverted complementary terminal sequences of the S RNA of Hantaan virus. The RNA sequence was deduced from the complementary DNA sequence. The mismatched nucleotides are numbered.
stem structure was predicted to have a free energy value of -25 Kcal/mole. Such complementarity is consistent with the non-covalently closed circular nature of the genomic RNA species of bunyaviruses (Pettersson and von Bonsdorff 1975, Hewlett et al. 1977). When the nucleotide sequence of the S RNA of Hantaan virus was compared to the previously published report, some important features were found to be different (Figure 19). Schmaljohn et al. (1986b) reported that two additional guanine (G) residues were present in nucleotide positions 83 and 182 within the sequence (Figure 19, B). Furthermore, the nucleotide at position 224 in the data of Schmaljohn et al. (1986b) was a G residue (Figure 19, B) whereas we detected a T residue (Figure 19, A). These three nucleotide changes cause a frameshift and create a new translation initiation codon (see Discussion). In addition to the three major differences between two sequences, three other nucleotide differences were identified at positions 162 and 171 in the upstream untranslated region and at position 1164 which results in an amino acid difference (aspartic acid versus glycine) (Figure 19).

3.4.3. Coding Capacity and the Predicted Gene Product of the S RNA Segment

The coding potential of the S RNA segment of Hantaan virus was examined in the six potential reading frames of genomic and anti-genomic sense RNA. One large open reading frame was found in the second frame of viral complementary
Figure 19. Comparison of the nucleotide sequence of the S segment of Hantaan virus with the previously published sequence. The numbers indicate the nucleotide positions found to be different. Amino-acid differences which result from the nucleotide differences are also indicated below the corresponding codon. The triple dots display the potential translation initiation codon in the major open reading frame. (A), author's sequence; (B) the sequence reported independently by Schmaljohn et al. (1986b).
sequences (Figure 20). The open reading frame begins with an ATG triplet at nucleotide 221-223 and extends to a TAA termination codon at 1322-1324 (Figure 17). This reading frame is capable of encoding a polypeptide of 367 amino acids with a molecular weight of 40,930. The predicted amino acid sequence is presented in Figure 17. The coding capacity of this open reading frame is similar to that of the estimated size of the nucleocapsid protein of Hantaan virus (Figure 2). A second short open reading frame is present in the same frame initiating at nucleotides 1328-1330, two codons beyond the termination codon of the major open reading frame (Figures 17, 20). This frame extends to a TAA codon at 1472-1474; however, while this second open reading frame can code only 48 amino acids (mol. wt. 5,930), it is not known whether the second open reading frame is translated. In viral genomic RNA sense, three short open reading frames with capacities of encoding 65, 72 and 78 amino acids were identified. No other open reading frames larger than these were observed in either the genomic or anti-genomic RNAs (Figure 20). A hydropathy plot suggests that this putative nucleocapsid protein is a relatively hydrophilic polypeptide (Figure 21).

3.5. Base Composition of the M and S RNA Segments of Hantaan Virus Strain 76-118

The molar ratios of the genomic RNAs suggested the possibility that the S RNA segment might be unusually rich
Figure 20. Potential coding regions in the S RNA of Hantaan virus. The arrows indicate open reading frames initiating with methionine codons and ending at the first in frame termination codon. The number above each arrow displays the number of amino acids encoded in the potential open reading frame. Small numbers above the central line represent nucleotide position ($10^{-2}$).
Anti-viral RNA Sense

367 amino acids

Viral RNA Sense

72

78
Figure 21. Hydropathy plot of the predicted nucleocapsid protein encoded by the S RNA of Hantaan virus. The darkened areas represent hydrophilic regions and the open areas indicate hydrophobic regions. The numbers at the bottom of the figure represent amino acid position starting with the amino terminus of the polypeptide.
in uridine and guanine residues (see Section 3.1.3.) Therefore the base composition of the S RNA was compared to that of the M RNA. As shown in Table 5, the amount of guanine residue in the two RNA segments were very similar (18% in M and 19% in S RNA). Furthermore, the relative amount of uridine was nearly identical (30%). Although the base composition of the L segment has not yet been determined, these observations clearly demonstrate that the possibility of differential labeling of genomic RNA segments due to unequal base compositions is unlikely, and suggested that the ratios of the genomic segments in Hantaan virus is not 1:1:1 (Table 4).

3.6. Identification of Viral Specific Intracellular RNA Species

To identify viral-specific RNA species in Hantaan virus-infected cells, total cellular RNA was extracted, electrophoresed on an agarose gel after glyoxal denaturation and transferred to a hybridization membrane. Strand-specific DNA probes were prepared using M13 hybridization probe primers. Because individual probes represented only partial sequences of each genomic segment, the labeled probes were combined in equimolar amounts to include the complete sequence of each segment. The genomic sense, M segment-specific probe hybridized to a single species of intracellular RNA (Figure 22, lane 4) whereas the complementary genomic sense, M segment-specific probe
<table>
<thead>
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<tr>
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<td>C</td>
<td>26.80</td>
</tr>
</tbody>
</table>

Table 5. Base composition of the M and S RNAs of Hantaan virus strain 76-118. The number of nucleotides were determined from the cDNA sequence of the M and S RNA (T, 774; C, 649; G, 1083; A, 1110 bases in M cDNA sequence: T, 400; C, 324; G, 454; A, 516 bases in S cDNA sequence).
did not hybridize to any RNA species (Figure 22, lane 3). Similarly, the genomic sense, S segment-specific probe hybridized to a single RNA species (Figure 22, lane 6) while no other RNA species was identified with the complementary genomic sense, S segment-specific probe (Figure 22, lane 5). To identify L specific intracellular RNA species, L clones were simply nick-translated and used as hybridization probes. This approach failed to detect any RNA species (data not shown). Since the L probes represent more than half of the entire L segment, this is probably due to low abundance of L specific intracellular RNA. The hybridization studies using strand-specific probes indicate that the polarity of the major intracellular RNAs specific for either the M or S segment is complementary to viral genomic RNA. These hybridizable intracellular RNA species seem to be neither the viral genomic RNA nor replicative intermediates. They are probably messenger RNAs (see Discussion). These observations are in agreement with the nucleotide sequence data which reveals a single large open reading frames in the anti-genomic sense of both the M and S segments of Hantaan virus (Figures 14, 20).

3.7. Expression of the Envelope Glycoprotein Gene (M Segment) of Hantaan Virus Strain 76-118 in Insect Cells

Previous studies suggested that the M RNA segment codes for two viral membrane proteins (Section 3.3.). These viral envelope glycoproteins are believed to be responsible
Figure 22. Identification of intracellular RNA species specific for Hantaan virus. Total cellular RNA was prepared from virus-infected Vero E6 cells on day 7 post-infection. The RNA was electrophoresed on an 1.4% agarose gel and immobilized on Zeta probe membrane. The membrane strips were hybridized to strand-specific M13 probes. [$^3$H]-uridine labeled ribosomal RNA (lane 1) and genomic RNA of Hantaan virus (lane 2) were electrophoresed on the same agarose gel and this part of the gel was prepared for fluorography. The exposed X-ray film was used as a marker; (A) M specific probes; lane 3, anti-genomic sense M probe; lane 4, genomic sense M probe; (B) S specific probes; lane 5, anti-genomic sense S probe, lane 6, genomic sense S probe;
for protective immunity (Murphy and Chanock 1985). Since no antiviral agents for HFRS are available, expression of the viral envelope gene would greatly contribute to the development of a recombinant vaccine against diseases associated with hantaviruses. Although foreign gene expression has been successful in many systems, the high efficiency of the viral promoter, the relatively easy construction of recombinants, and the likelihood of post-translational modification of recombinant products have led us to choose baculovirus as an expression system. Thus, this part of the thesis describes the expression of the glycoprotein gene of Hantaan virus in insect cells using a baculovirus expression system.

3.7.1. Construction of a Full Length Glycoprotein Gene

To construct a complete DNA copy of the gene encoding envelope proteins of Hantaan virus strain 76-118, six clones were chosen. The strategy for construction of the envelope protein gene is illustrated in Figure 23. Because two SphI sites were present at nucleotide positions 488 and 2188, clones sp28 and 67 were first ligated together at the SphI site (nucleotide 488). Clones 41 and 12 were joined separately at the downstream SphI site (nucleotide 2188). Clones 90 and m4 were subsequently used to construct a clone 41-12-90-m4 using the BstEII and DraII sites. To construct the final copy, a 900 bp BamHI and HindIII fragment was isolated from clone sp28-67 partially digested with XhoII. A 830 bp BamHI-XhoII fragment was isolated and
Figure 23. Construction of a full-length copy of the glycoprotein-coding sequence of Hantaan virus. The 5' end of the glycoprotein gene was blunt-end ligated to the HindIII site of pUC19. The 3' end was joined at the PstI site of pUC19. The arrows indicate specific restriction sites used for joining fragments. Numbers represent the nucleotide position from the 5' end of the complementary mRNA.
ligated to the 2.8 kbp fragment obtained by digesting the clone 41-12-90-m4 with XhoII and PstI. The ligated fragment was inserted into the BamHI and PstI sites of pUC19. The resulting cDNA clone pDWMUC19 contains the entire sequence, nucleotides 5-3565 numbered from the 3' terminus of the M RNA of Hantaan virus.

3.7.2. Deletion and Modification of Non-coding Sequences of the Glycoprotein Gene

For high levels of gene expression in the baculovirus system, Summers and Smith (1987) suggested that the length of 5' untranslated flanking sequences of foreign genes be as short as possible. It has also been reported that the flanking sequence ACC upstream of the initiating ATG provides a preferred context for translation initiation in eukaryotes (Kozak 1986a, 1986b). Thus, to modify the 5' non-coding sequence of the glycoprotein gene of Hantaan virus, oligonucleotide-directed mutagenesis was carried out using crossover linkers (Sung et al. 1986). A single-stranded, synthetic oligonucleotide AB279 was designed to contain a BamHI cohesive end (GATCC) and a trinucleotide (ACC) followed by glycoprotein coding sequences starting with nucleotide 42 of the M segment of Hantaan virus (ATGGGGATATGGAAG-TGG). The AB279 linker was ligated to the BamHI site of pDWMUC19. Intramolecular recombination occurred in vivo following transformation of E. coli JM101 with the ligation mix (Figure 24). Primary selection of recombinants was done by digesting the resultant plasmids
with XbaI, because unmodified pDWMUC19 would retain a XbaI site in the polylinker region of pDWMUC19. Gene modification was then confirmed by determining the junction sequence of the glyco-protein gene by dideoxy double-stranded DNA sequencing. The sequence 5'-TACCGGGATCCACC-ATGGGG-3' was identified (data not shown). This result indicates that the upstream 40 nucleotides of the glycoprotein gene was removed and the trinucleotide ACC was introduced (Figure 24). The new plasmid was termed pDWMUC19-279.

To remove 3' untranslated sequence, plasmid pDWMUC19-279 was digested with PstI. Because the PstI digestion produced 5' recessed ends, double-stranded crossover linkers were employed. To facilitate the subsequent steps for construction of a baculovirus transfer vector, the BamHI recognition sequence was also introduced immediately downstream of the translation termination codon of the glycoprotein coding sequence (Figure 25). The modified plasmid pDWGUC19 was identified by BamHI digestion, which would release a 3.4 kbp fragment. The 3' junction sequence of the glycoprotein gene was determined by the chemical cleavage method (Maxam and Gilbert 1980), and confirmed to be 5'-AAATCATAGGATCCTGAGG-3' (data not shown). This sequence data demonstrated that 166 nucleotides extending from the first nucleotide following the translation termination codon to the terminal nucleotide of the M segment were removed, and that a BamHI recognition sequence
Figure 24. Modification of 5' non-coding sequences of the glycoprotein gene of Hantaan virus. A single-stranded crossover linker was prepared and ligated to the BamHI site of plasmid pDWTMC19. Intra-molecular recombination occurred in vivo following transformation of *E. coli*. The junction sequence of the modified region was confirmed using the GemSeq/K (Promega Biotec) sequencing system. The triangle indicates the translation initiation codon.
Figure 25. Deletion of 3' non-coding sequences of the glycoprotein gene of Hantaan virus. A double-stranded crossover linker was prepared and used for deletion of the downstream untranslated sequence of the M segment (3449-3616). A BamHI recognition sequence was introduced immediately downstream of the translation termination codon (3446-3448).
pDWMUC19 | Pst I cut

Hantaan M seq.

5' GGTTCATGCTGCA
3' CGAACGTACGG

5' GGTCTAGTAGTTTATTATA
3' ATCGATACTAATAAT

Ligase

5' GGTTCATGCTGCA
3' CGAACGTACGG

ds crossover linker

BamHI

In Vivo | Recombination

pUC19 seq. | Hantaan M seq.

5' AAGCTTGCATGCTGCA
3' TCGAACGTACGG

Pst I

BamHI

Translation termination
was introduced (Figure 25):

3.7.3. Construction of Recombinant Baculovirus

The baculovirus transfer vector pAcYM1 is a pUC8-based plasmid. The pAcYM1 contains the EcoR1-I fragments of the Autographa californica nuclear polyhedrosis virus (AcNPV) genome surrounding the polyhedrin gene which serve as regions for in vivo recombination with wild type AcNPV DNA. The pAcYM1 contains all the upstream sequences of the polyhedrin gene including the A of the initiating ATG codon, but lacks the rest of the polyhedrin coding sequence plus 13 nucleotides downstream from the translation termination codon of the polyhedrin gene. This deleted region was replaced with the BamHI sequence for insertion (Matsuura et al., 1987). In order to construct recombinant baculoviruses, the 3.4 kbp BamHI fragment obtained from pDWSGUC19 was inserted into the unique BamHI site of pAcYM1 to produce pAcYM1-DWG. Therefore, pAcYM1-DWG contains the sequence ACC and the entire coding sequence for the glycoprotein of Hantaan virus, in the correct orientation, downstream of the polyhedrin gene promoter (Figure 26). The plasmid pAcYM1-DWG was used to co-transfect Spodoptera frugiperda cells along with the DNA of wild type Autographa californica virus. The culture supernatant was collected 4 days after co-transfection and titrated in monolayers of S. frugiperda cells. Plaques lacking occlusion bodies (Figure 27, panels B, D) were picked and subsequently plaque-purified three consecutive times before propagation.
Figure 26. Construction of a baculovirus expression vector for Hantaan virus glycoprotein synthesis. The full-length glycoprotein gene (HTN-GPC) was inserted into the BamHI site of pAcYM1. This vector has a deletion between +2 and +749 nucleotides (A of the polyhedrin translation initiation codon as +1). The resultant vector pAcYM1-DWG contains the Hantaan glycoprotein- coding sequence under the control of the polyhedrin promoter.
Figure 27. Microscopic appearance of polyhedrin-positive and polyhedrin-negative plaques. Monolayer cultures of *S. frugiperda* cells were infected with wild type AcNPV or with recombinant baculovirus and incubated for 4 days at 28 C under an agarose overlay. Plaques were examined by phase contrast light microscopy at 400 X magnification (Leitz, Diavert); center (A) and edge (C) of a plaque formed by wild type AcNPV; center (B) and edge (D) of a plaque formed by recombinant virus pAcYM1-DWG-8D. (B) and (D) represent polyhedrin negative in infected cells whereas the (A) and (C) display the appearance of polyhedrin.
To further characterize a selected polyhedrin-negative virus, DNA was prepared from cells infected with the recombinant baculovirus pAcYM1-DWG-8D. The DNA preparation was subjected to Southern blot hybridization using a nick-translated 3.4 kbp BamHI fragment from pDWGUC19 as a probe (Figure 28). While the DNA from uninfected cells (Figure 28, lanes 2, 6) and from wild type AcNPV-infected cells (Figure 28, lanes 3, 7) did not hybridize with the probe, undigested viral DNA (Figure 28, lanes 4, 5) and BamHI digested viral DNA (Figure 28, lanes 8, 9) from pAcYM1-DWG-8D infected cells clearly hybridized. As expected the size of the BamHI digested pAcYM1-DWG-8D fragments were 3.4 kbp (Figure 28, lanes 8, 9). These results verified that the glycoprotein gene of Hantaan virus was present in the recombinant baculovirus genome.

3.7.4. Expression of the Hantaan Virus Glycoprotein Gene by Recombinant Baculovirus

To determine whether cells infected with the recombinant baculovirus pAcYM1-DWG-8D synthesized the Hantaan virus envelope proteins, infected S. frugiperda cells were lysed and the lysates were electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and reacted with monoclonal antibodies specific for the both G1 and G2 envelope glycoproteins of Hantaan virus. As controls, proteins in mock-infected cells and in wild type AcNPV-infected cells were also analysed.
Figure 28. Southern blot analyses of recombinant baculovirus, pAcYM1-DWG-8D. Partially purified viral DNA was prepared from virus-infected *S. frugiperda* cells and electrophoresed on an 1.0% agarose gel prior to (A) or after (B) digestion with BamHI. The DNA products were immobilized on a Zeta Probe membrane and hybridized with a nick-translated 3.4 Kbp BamHI fragment recovered from plasmid pDWGUC19. lane 1, HindIII-digested lambda phage DNA fragments as molecular weight markers; lanes 2, 6, DNA from uninfected cells; lanes 3, 7, DNA from wild type AcNPV infected cells; lanes 4, 5, 8, 9, DNA from the cells infected with the recombinant baculovirus pAcYM1-DWG-8D;
The specificity of the antibody GD05-BC02 for the G1 and G2 was demonstrated by Western blot immunoassay with purified Hantaan virus 76-118 although it is unclear why the monoclonal antibody GD05-BC02 reacted to both G1 and G2 proteins (Figure 2, lane 8). A protein with an approximate molecular weight of 53 K was immunoreactive with monoclonal antibody GD05-BC02 (Figure 29, lane 3). This 53 K protein was not synthesized in mock-infected (Figure 29, lane 5) or in wild type AcNPV-infected cells (Figure 29, lane 4). The molecular weight of the immunoreactive protein produced in recombinant virus pAcYM1-DWG-8D infected cells appeared to be slightly smaller than that of viral glycoprotein G2 (55 K) (Figure 29, lanes 2, 3). This observation may represent the differences in glycosylation. Since the recombinant 53 KDa protein was far smaller than the coding capacity of the gene introduced (predicted molecular weight of 126 K), and appeared to be the G2 protein, we assumed that a large glycoprotein precursor was cleaved and G1 protein should also be produced. However, G1 was not detected due to the presence of a non-specific antibody reactive protein which migrated to the position expected for G1 (Figure 29). To determine if the G1 protein was also synthesized in recombinant baculovirus-infected cells, radioimmuno-precipitation was carried out as follows. Virus-infected S. frugiperda cells were labeled with \[^{35}\text{S} \]methionine in vivo, and the cell lysates were immunoprecipitated with monoclonal antibody GD05-BC02 and analysed by SDS-PAGE.
Figure 29. Synthesis of the G1 and G2 proteins of Hantaan virus in insect cells. *S. frugiperda* cells (SF9) were infected with recombinant baculovirus pAcYM1-DWG-8D or wild type AcNPV. The cells were harvested 48 hr post-infection and analysed by SDS-PAGE. The proteins was transferred to nitrocellulose membrane and incubated with monoclonal antibody, GD05-BC02, specific for both the G1 and G2 proteins of Hantaan virus (see Fig. 2, lane 8). The membrane was washed and incubated with a second antibody, goat anti-mouse IgG conjugated with alkaline phosphatase. Brown color was developed and visualized as described in section 2.7.2. lane 1, prestained molecular weight markers (Bio-Rad): myosin H-chain (200 K), phosphorylase B (100 K), bovine serum albumin (68 K), ovalbumin (42 K); lane 2, Hantaan virion proteins; lane 3, cell lysates from recombinant pAcYM1-DWG-8D infected cells. Arrow indicates the putative G2 recombinant protein; lane 4, cell lysates from wild type AcNPV infected cells; lane 5, uninfected cell lysates.
Figure 30. Immunoprecipitation of G1 and G2 proteins of Hantaan virus. *S. frugiperda* (SF9) cells were infected with recombinant virus pAcYM1-DWG-8D or with wild type AcNPV and incubated for 48 hr. The medium was replaced with methionine-free Grace's medium containing 10% dialyzed fetal calf serum. After 1.5 hr of starvation, the cells were labeled for 4 hr by adding \(^{35}\text{S}\) methionine (100 uCi/ml). The cellular proteins were incubated with monoclonal antibody GD05-BC02 in the presence of protein A Sepharose CL-4B beads. The immunoprecipitated proteins were analysed by SDS-PAGE and fluorography. (A) protein marker stained with Coomassie blue. lane 1, prestained protein markers supplied by Bio-Rad (see legend to Figure 29); lane 2, Hantaan virion proteins, L (180 K), G1 (72 K), G2 (55 K), N (44 K); (B) Autoradiograph of \(^{35}\text{S}\) methionine-labeled immunoprecipitates from *S. frugiperda* cells; lane 3, recombinant virus pAcYM1-DWG-8D infected cells; lane 4, wild type AcNPV infected cells; lane 5, uninfected cells.
Two proteins with approximate molecular weights of 70 K and 53 K were identified (Figure 30, lane 3). The molecular weights of these two proteins were similar to those of G1 and G2 glycoproteins of Hantaan virus strain 76-118 (Figure 30, lane 2). Although some non-specific proteins including polyhedrin were precipitated (Figure 30, lane 4), no bands corresponding to the putative G1 and G2 were detected in mock-infected (Figure 30, lane 5) or wild type AcNPV-infected cells (Figure 30, lane 4). These results were confirmed with anti-glycoprotein monospecific antibody HC02-BC10 that also reacted with both the G1 and G2 proteins of Hantaan virus (data not shown). These results clearly demonstrate that the G1 and G2 Hantaan virus glycoproteins were synthesized in S. frugiperda cells during the recombinant baculovirus pAcYM1-DWG-8D infection. Furthermore, identification of two different protein species with appropriate molecular weights also indicated that the glycoprotein precursor was cleaved and processed in S. frugiperda cells.
CHAPTER 4: DISCUSSION AND CONCLUSIONS

Hemorrhagic fever with renal syndrome (HFRS) in humans is defined as an acute infectious rodent-borne disease caused by a group of viruses closely related to Hantaan virus strain 76-118, the causative agent of Korean hemorrhagic fever (KHF). HFRS gives rise to generalized symptoms of renal dysfunction with the kidney as the main target organ. The mortality rate reaches as high as 30%. Neither effective diagnostic tools, therapeutic agents nor immunoprophylaxis are yet available. An understanding of the molecular biology of Hantaan virus strain 76-118 will provide insight into the nature and replication strategies of the agents of HFRS. Furthermore, this information will eventually lead to the development of a vaccine or antiviral agents for the control of HFRS. In this study, the structural composition and coding strategy of Hantaan virus strain 76-118 were investigated. In addition, the viral gene encoding the two envelope glycoproteins was identified and expressed in insect cells using a recombinant baculovirus vector.

4.1. Structural Components, Genomic Organization and Taxonomy of Hantaan Virus

The identification of four structural proteins in Hantaan virions (Figure 2) is in agreement with other reports. Schmaljohn et al. (1983) separated three species of nucleocapsids from virions treated with a non-ionic
detergent by rate-zonal sedimentation. They observed that a polypeptide was associated with each of the nucleocapsids. Elliott et al. (1984) also reported, following detergent treatment, that a 44 KDa protein was the virus nucleoprotein (N). The nucleoprotein is directly associated with the RNA molecules in bunyaviruses (Bishop 1985). The size of the N protein of Hantaan virus is substantially larger than those of other viruses of the family Bunyaviridae. The molecular weights of the N proteins of La Crosse virus, Rift Valley fever virus, Uukuniemi virus, and Anhembi virus were reported to be approximately 25 K (Rice et al. 1980, Obijeski and Murphy 1977) whereas that of Congo virus (genus Nairovirus) was 50-60 K (Bishop et al. 1980a). 70 KDa and 55 KDa proteins of Hantaan virus were labeled with $[^{3}H]$-glucosamine (Elliott et al. 1984) or with $[^{3}H]$-mannose (Yamanishi et al. 1984). By immunofluorescent and neutralizing antibody studies and by bromelain treatment of virions, these two polypeptides were localized in the envelope of Hantaan virions (Elliott et al. 1984, Yamanishi et al. 1984, Dantas et al. 1986). Thus, the 70 KDa (G1) and 55 KDa (G2) proteins are membrane glycoproteins of Hantaan virus. Similar migration profiles of the G1 and G2 proteins under either reducing or non-reducing conditions suggested that G1 and G2 proteins were not linked by disulfide bonds. (Figure 2, lanes 1, 2). Anti-Hantaan polyclonal rabbit or mouse sera reacted very well with the N protein. These sera also reacted with G2 to
a lesser extent, but not with G1 nor with the large protein (L; 180 KDa). Similar observations were made by Yamanishi et al. (1984) and Schmaljohn et al. (1986b). The very hydrophilic nature of the nucleocapsid protein (Figure 21) probably contributes to its reactivity with the immune system. Although the function of the L protein in bunyaviruses has not been firmly established, demonstration of virion-associated transcriptase activity suggests that the L protein is the most probable candidate for the virion-associated RNA dependent RNA polymerase (Bouloy and Hannoun 1976a, Vezza et al. 1978, Ranki and Pettersson 1975, Patterson et al. 1984). In Hantaan virus, a transcriptase activity was also reported to be associated with the virions although the activity was difficult to detect. (Schmaljohn and Dalrymple 1983). Therefore, the 180 KDa protein associated with Hantaan virions may represent the RNA polymerase.

The genome of Hantaan virus was composed of three species of single-stranded RNA with a total molecular weight of 4.0 X 10^6 (approximately 11,750 nucleotides) (Figures 1, 3, Table 3). Hantaan virus strain 76-118 had no documented serological relationship to most known viruses including other bunyaviruses (McCormick et al. 1982, Tsai et al. 1982, LeDuc et al. 1982, Tsai et al. 1985), but showed cross-reactivity with a number of viruses recently isolated in regions where HFRS was endemic as well as in non-endemic areas (Table 2). The comparison of the
electrophoretic profiles of the genomic RNA of Hantaan virus with the RNAs from serologically related virus isolates from diverse hosts and geographic regions, Prospect Hill, ROK 83-109, USSR cg38-83, demonstrated that these virus isolates had similar but distinct genomic RNA profiles (Figure 3). The comparison of oligonucleotide fingerprints of the genomes of different hantaviruses demonstrated that each isolate possessed unique tripartite genomic segments (Schmaljohn et al. 1985). The only family of animal viruses known to contain tripartite genomic RNA is the Bunyaviridae (Bishop et al. 1980a). The four virion-associated proteins, which correspond to those of the Bunyaviridae, as well as the tripartite genomic RNA suggest that Hantaan 76-118 and related viruses should be classified as members of the family Bunyaviridae.

The molar ratios of the genomic segments of the hantaviruses appeared to be unequal (Tables 4). Non-equimolar amounts of the genomic RNA segments have been observed in bunyaviruses (Bouloy et al. 1973/74, Gentsch et al. 1977a, Obijeski et al. 1976b, Pettersson and Kaariainen 1973). Usually the S RNA segments were found to be over represented as shown in this study. It is possible that each virion contains a variable number of RNA molecules rather than stoichiometric amounts of RNA segments. In plant viruses, Bromoviruses and Cucumoviruses have tripartite RNA genomes and each virion contains only a single RNA segment. Infectivity studies of these viruses
have shown that all three RNA components are required to produce one infectious center (van Vloten-Doting and Jaspers, 1977). A similar situation may exist in hantaviruses which may explain why hantaviruses have such a low infectivity. The infectivity-dilution curve by plaque assay should not deviate from a curve for single hit kinetics. The constitution of the viral genome cannot be deduced from the dilution curve alone, however, as suggested by Peden and Symon (1973), because one of the three essential RNA species may be limiting in its contribution to infectivity relative to the other two components (Fulton 1967, Lane and Kaesberg 1971). Another possible explanation for the non-equimolar ratio of RNA segments may be the presence of defective virus particles. Although the presence of defective virus particles has not been investigated, in any detail, in bunyaviruses, it has been reported that high multiplicity infection with La Crosse virus causes reduced virus yields as compared to lower multiplicity infection (Obijeski et al. 1976b). As suggested by Kasczasak and Lyons (1977), whether interference involves the production of defective virus with lesser or greater than normal quantities of particular RNA species remains to be determined. It is also possible that differential replication of particular viral RNA species may lead to non-equimolar packaging of genomic segments. Another alternative explanation could be differential ribonuclease sensitivity of the RNA molecules, since larger
molecules would be better targets for ribonucleases. If this were the reason, one might expect to find variable results in the molar ratios of the RNA segments from experiment to experiment. However, the data were quite consistent from RNA preparation to preparation. Furthermore, ribonuclease-mediated RNA degradation would produce a random-size distribution. Therefore, the latter explanation for non-equimolar ratios of hantavirus genomic segments is unlikely.

The sequence of the 3' terminus of the L, M and S RNAs of Hantaan virus strain 76-118, 3'-AUCAUCAUCUGA-5', is identical and is also conserved among seven other hantavirus isolates (Schmaljohn et al. 1985). However, this 3'-terminal sequence is different from the consensus sequences reported for other genera of the Bunyaviridae (Table 2). Sequence analyses of the M and the S RNA segments of Hantaan virus revealed that the sequences in both termini of each genomic segment were inverted complements of 20 bp and 22 bp, respectively (Figures 12, 18). The sequence complementarity between the 3' and 5' ends of Hantaan virus genomic RNA is also characteristic of bunyaviruses (Parker and Hewlett 1981, Bishop et al. 1982, Cabradilla et al. 1983, Ibara et al. 1984, Eshita and Bishop 1984). The stable, base-paired stem structure of Hantaan virus RNA is probably involved in the formation of non-covalently closed circular nucleocapsid structures that have been seen in preparation of bunyavirus nucleocapsids.
The sequence conservation and potential circular configuration of genomic RNA segments suggest that this structure may be important in viral morphogenesis, for example, nucleocapsid packaging into virion, and/or in RNA polymerase recognition. The mRNAs specific to the M and S segments of bunyaviruses have been shown to be smaller than the corresponding genomic RNA (Bishop et al. 1983b, Patterson and Kolakofsky 1984, Cash et al. 1979). Thus, the mRNA may lack complementary terminal sequences and, consequently, be unable to form the stem structure predicted for the viral genomic RNA. Therefore, this structural feature may allow RNA polymerase to distinguish viral genomic RNA or replicative intermediates from viral complementary messenger RNA.

It is of interest to note that most members, if not all, of hantaviruses have been recovered directly from rodent species (Lee P. et al. 1978a, Lee H. et al. 1982a, Tsai et al. 1985). Most other bunyaviruses are transmitted by ticks or mosquitoes (Bishop et al. 1980a). No identified arthropod vectors appear to be involved in hantavirus transmission. It is of particular interest that the morphology of hantaviruses is different from that of other bunyaviruses. Although early electron microscopic examination suggested that hantavirus particles morphologically resembled members of the family Bunyaviridae (McCormick et al. 1982, White et al. 1982, Hung et al. 1983), more recent studies have shown that
hantaviruses have a unique surface structure composed of a grid-like pattern of morphologic subunits not previously described for animal viruses (Martin et al. 1985). Aberrant tailed particles and elongated particles were also observed (Hung et al. 1985, Martin et al. 1985). Morphogenesis of hantaviruses appears to be more complex than that of other members of the family Bunyaviridae (Hung et al. 1985, 1987). The unique consensus terminal sequences, the different mode of transmission, the absence of detectable serologic relationship to other bunyaviruses, and the peculiar morphology etc. suggest that hantaviruses constitute a separate genus of the family Bunyaviridae.

4.2. Coding Strategy of the mRNA Segment and Characteristics of the Predicted Glycoprotein Precursor of Hantaan Virus

Analysis of the nucleotide sequence of the M RNA segment revealed that only one open reading frame is present in the viral complementary sense. All other open reading frames in either the genomic or anti-genomic sense were very short (Figure 14). Thus, the 126 KDa (P126) protein seems to be the only polypeptide encoded by the M RNA. Encoding a single gene product on the M RNA is a common property of other bunyaviruses and has been observed for Punta Toro virus (IHara et al. 1985a), Bunyamwera virus (Lees et al. 1986), Rift Valley fever virus (Collett et al. 1985) and snowshoe hare virus (Eshita and Bishop 1984). The first translation initiation codon in the Hantaan virus M
segment occurs 20 nucleotides after the last nucleotide of the consensus sequence. The untranslated region between the consensus sequence and the coding sequence is also seen in La Crosse virus and snowshoe hare virus (Eshita and Bishop 1984, Clerx-van Haaster et al. 1982a). This sequence is not conserved and its function is not known. Translation termination of the major open reading frame occurs 166 nucleotides upstream from the 3' terminus in the viral complementary sequence of the M RNA of Hantaan virus. Thus, the 5' noncoding region is shorter in length than the 3' noncoding region (Figure 11). This has been observed in the M RNA of La Crosse virus and snowshoe hare virus (Eshita and Bishop 1984, Clerx van Haaster 1982a). It is not known whether the length of the 5' non-coding sequence has any significance for the virus replication.

Another important feature of the M RNA of Hantaan virus is the absence of a region encoding a nonstructural polypeptide (NSM). In most bunyaviruses, long amino-acid stretches have been observed between the signal sequence and the amino terminal sequence of mature glycoproteins (G1 or G2). The predicted molecular weights of these putative polypeptides (NSM) deduced from the nucleotide sequence are 12 K for snowshoe hare virus (Fuller and Bishop 1982), 11 K for La Crosse virus (Fuller and Bishop 1982), 36 K for Punta Toro virus (Ihara et al. 1985a), 16 K for Bunyamwera virus (Lees et al. 1986) and 17 K for Rift Valley fever virus (Collett et al. 1985). Moreover, the presence of
nonstructural polypeptides in virus-infected cells have been demonstrated for La Cross virus, snowshoe hare virus, Punta Toro virus (Fuller and Bishop 1982, Overton et al. 1987) and Bunyamwera serogroup viruses (Elliott 1985). Recently, the absence of a region encoding a nonstructural polypeptide, as seen for Hantaan virus, was reported in the M RNA of Uukuniemi virus despite an indication of its evolutionary relationship with Phleboviruses (Ronnholm and Pettersson 1987). Functions of the nonstructural proteins in bunyaviruses are not known.

The P126 polypeptide is believed to be the precursor for the two envelope glycoproteins, G1 and G2, of Hantaan virus. Such a precursor protein has been identified by in vitro translation of the M mRNA of Uukuniemi virus (Ulmanen et al. 1981). Partial sequence analysis of the amino termini of the G1 and the G2 proteins from Hantaan virions indicated that G1 started at nucleotides 95-97 (Leu), and G2 at nucleotides 1985-1987 (Ser) in the complementary sequence of the M RNA (Schmaljohn et al. 1987). Therefore, the polyprotein is believed to be processed either cotranslationally or post-translationally. Whether the precursor protein actually exists in Hantaan virus-infected cells remains to be demonstrated. Immunoprecipitation studies using antipeptide antisera showed that the carboxy terminus of the G2 protein corresponds to the 3' nucleotide sequences of the major open reading frame (Schmaljohn et al. 1987). Because the position of the carboxy terminus of
the G1 protein has not been determined, the molecular weight of the G1 protein was estimated from the region between the amino termini of the G1 and G2 proteins to be 70 K. However, this value is certainly larger than the 61 K previously reported for the non-glycosylated form of the G1 protein (Schmaljohn et al. 1986a). This finding suggests that a small intergenic peptide exists between the carboxy terminus of the G1 protein and the amino terminus of the G2 protein. Similar situations have been observed in Rift Valley fever virus (Collett et al. 1985) and Uukuniemi virus (Ronnholm and Pettersson 1987). The exact length of the G1 protein is not known at present. Hydropathicity analysis has revealed that four major hydrophobic regions are present in the P126 polypeptide. Figure 31 illustrates the structural characteristics of the P126 polypeptide on the basis of the amino-acid sequence deduced from the nucleotide sequence of the mRNA segment. Two hydrophobic domains, 18 and 22 amino-acid residues in length, precede the mature G1 and G2 glycoproteins, respectively. They are probably the signal sequences for translocation of the proteins into the membrane of the rough endoplasmic reticulum (Klenk and Rott 1980, von Heijne 1983). The internal signal sequence of 22 residues preceding the G2 glycoprotein and the predicted intergenic region between the G1 and G2 proteins are analogous to the 6K protein located between the P62 and E1 glycoprotein of Semliki Forest virus (Garoff et al. 1980, 1982) and Sindbis virus
(Rice and Strauss 1981). Two hydrophobic regions were additionally found to be present at carboxy terminal regions of the G1 and G2 proteins (28 and 22 amino-acid residues in length, respectively). These regions probably represent membrane anchor domains which are associated with the lipid bilayer (Klein et al. 1985). These domains have been previously shown for the HN of influenza virus (Skehel and Waterfield 1975), the G protein of Vesicular stomatitis virus (Katz et al. 1977), the glycoproteins of paramyxoviruses (Schied et al. 1978) and E1 and E2 proteins of Semliki Forest virus (Garoff and Soderlund 1978, Garoff et al. 1982). A 118 amino-acid residue is located between the putative transmembrane anchor domain of the G1 protein and the signal sequence of the G2 protein. By computer analysis, two highly hydrophilic domains are found in this region at amino acids 530-535 and 598-603. Although the cleavage sites in the P126 are not known, it is predictable that one of these hydrophilic domains within the 118 amino-acid residue intergenic region may represent a cytoplasmic tail for the G1 protein. Similarly, an extremely hydrophilic region immediately following the hydrophobic region at the carboxy terminus of the G2 protein probably represents a cytoplasmic tail for the G2 protein. The maturation process of the P126 of Hantaan virus is modeled according to the characteristics of predicted precursor protein. Translocation of the G1 protein would be initiated by the signal sequence, located at amino acid positions
Figure 31. Schematic diagram of the structural characteristics of the glycoprotein precursor of Hantaan virus. Darkened areas indicate hydrophobic domains. Arrows indicate predicted cleavage sites. Numbers represent amino acid positions starting from the amino terminus of the putative precursor protein. S, signal sequence; TM, transmembrane anchor sequence; Y, asparagine-linked potential glycosylation sites.
1-18, in SRP-DP (signal recognition particle-docking protein)-dependent fashion. Insertion would continue until the membrane anchor region of the G1 protein (second hydrophobic region at amino acids 482-509) interacted with the membrane of endoplasmic reticulum (ER). When synthesis of the G1 protein was completed, cytoplasmic tail of the G1 protein would be clipped off and the internal signal sequence (third hydrophobic region at amino acids 627-648) would direct the translocation-reinitiation of the G2 protein across the ER membrane. Similarly, when the G1 protein was completely synthesized, the fourth hydrophobic region at amino acids 1106-1127 would be associated with the membrane. Thus, the G1 protein and G2 proteins of Hantaan virus seemed to be processed co-translationally from a single precursor. It would be of interest to determine whether a short intergenic peptide, similar to the 6K protein of Semliki Forest virus, was generated by cleavages between the carboxy terminus of the G1 protein and internal signal sequence of the G2 protein. If so, it could be a nonstructural protein encoded by the M RNA segment.

Four of seven potential asparagine-linked glycosylation sites are found in the G1 protein and two are in the G2 protein. It is of interest that one potential site for asparagine-linked glycosylation (amino acid position 609) is found in the intergenic region just before the putative signal sequence of the G2 protein. However, it is unlikely to be glycosylated because it is probably not translocated.
into the lumen of the endoplasmic reticulum. A similar situation has been observed in the glycoprotein precursor of Uukuniemi virus (Ronnholm and Pettersson 1987).

4.3. Molecular Characterization of the S RNA Segment of Hantaan Virus

Nucleotide sequence analysis of the S RNA of Hantaan virus revealed that the functional organization of the S RNA is similar to that seen for other bunyaviruses. One major open reading frame is found in the viral complementary S RNA sequence. Beginning with the first AUG codon, the major open reading frame encodes the 41 KDa nucleocapsid protein of Hantaan virus (Figure 17). In the complementary S RNA sequence, the 5' non-coding region is shorter than the 3' non-coding region as observed for the M segment (Figure 17, 220 nucleotides versus 373 nucleotides). This feature has been reported in the S RNA of La Crosse virus (Cabrardilla et al. 1983), snowshoe hare virus (Bishop et al. 1982) and Aino virus (Akashi et al. 1984). The viral complementary 3' terminal 140 nucleotides were highly conserved in La Crosse virus and snowshoe hare virus (Bishop et al. 1982). Although this sequence is not conserved in the S RNA of Hantaan virus or Aino virus, it may have important functions because a non-coding region is found in both the M and S RNAs of all bunyaviruses so far examined. A sequence UGGGUGGG is repeated at 114 nucleotides downstream (nucleotide positions 1439-1446) and 157 nucleotides downstream (nucleotide positions 1482-
from the translation termination codon of the major open reading frame. The tetramer UGGG is also present twice at nucleotides 1427-1430 and 1463-1466. A tandem repeat (CUAAAUU) similar to this has been reported in the S RNA of La Crosse virus (Cabradilla et al. 1983). It may serve as a transcription termination signal for the viral transcriptase or in morphogenesis as a signal for RNA packaging into nucleocapsids or nucleocapsid packaging into virions. Such a repeat, however, is not found in the 3′ non-coding region of the complementary M RNA of Hantaan virus. Therefore, the function of the repeated sequence remains unclear. Another feature of the S RNA of Hantaan virus is that the 3′ non-coding region of viral complementary sequence is A-T, and particularly, T rich. A eucaryotic polyadenylation signal (AATAAA) is not found. Uridylate tracts of 5-6 residues in the 3′ non-coding region have been reported for snowshoe hare virus and La Crosse virus. No such tract is found in the S sequence of Hantaan virus. It is noteworthy that the mRNA species of bunyaviruses are generally thought to contain little or no 3′ terminal polyadenylate sequences (Bouloy et al. 1984). It is not known if Hantaan virus mRNA contains 3′ terminal polyadenylate sequences or where mRNA transcription termination occurs.

Two different S RNA coding strategies have been reported in bunyaviruses. Overlapping reading frames are utilized by most of the bunyaviruses to encode a
nucleocapsid protein (N) and a non-structural protein (NSg). However, ambisense coding strategies for N and NSg have been recognized for Punta Toro virus (Ihara et al. 1984) and Rift Valley fever virus (M. D. Parker, J. F. Smith and J. M. Dalrymple, cited in Collett 1986). In contrast, the S RNA of Hantaan virus does not seem to follow either of these strategies. Although a second short open reading frame is found in Hantaan virus S complementary RNA, in the same reading frame, two codons beyond the termination of the major open reading frame, the polypeptide predicted for this second reading frame is far smaller (6 KDa) than the non-structural proteins reported for the S RNA of other bunyaviruses (Gentsch and Bishop 1978, Bishop et al. 1982, Clerx-van Haaster et al. 1982a, Fuller et al. 1982, Cabradilla et al. 1983, Akashi and Bishop 1983, Fuller et al. 1983, Gerbaud et al. 1987). Analysis of the viral sense RNA revealed three short open reading frames each of which can potentially code for a 7-8 KDa polypeptide (Figure 20). However, these short open reading frames are located within the sequence which encodes the nucleocapsid protein. Thus, this is somewhat different from the ambisense coding strategy in which half of the S RNA encodes nucleocapsid protein and the complementary sequence of the other half encodes a non-structural protein. It is not known whether these short open reading frames are in fact utilized for the synthesis of functional polypeptides.
During the course of our studies, another laboratory has determined the nucleotide sequence of the S RNA segment of Hantaan virus strain 76-118 (Schmaljohn et al. 1986b). We compared the nucleotide sequence of the S RNA of Hantaan virus to the published report by Schmaljohn et al. (1986b). The first potential initiation ATG codon is found at nucleotides 77-79 in the potential open reading frame in our sequence. This codon however will not yield a large product because of two in-frame termination codons present at nucleotides 131-133 and 146-148 (Figure 19). These data suggest that translation of the S mRNA initiates at the second ATG codon at nucleotides 221-223. However, Schmaljohn et al. (1986b) reported that the major open reading frame initiates at the first potential initiation codon. Because Schmaljohn et al. found two additional guanosine (G) residues at positions 83 and 183, translation was predicted to initiate at nucleotides 37-39 and continue uninterrupted until positions 1322-1324. Although the first ATG at nucleotides 37-39 is also present in our sequence, it does not initiate a major open reading frame because of the absence of the two G residues mentioned above. The absence of these two G's results in a frame shift which places eight termination codons in frame before the second ATG at positions 221-223. Moreover, the nucleotide at position 224 in the data of Schmaljohn et al. (1986b) is a G residue whereas we repeatedly detect a T residue. A G residue in this position eliminates a methionine codon.
Consequently, the coding region reported by Schmaljohn et al. (1986b) is 186 nucleotides longer than that predicted on data, which results in a polypeptide 429 amino acids in length (48 KDa) rather than 367 amino acids in length (41 KDa). This finding leads to a discrepancy in the predicted size of the nucleocapsid protein of Hantaan virus. The estimated size as determined by SDS-PAGE is 44 KDa. It is not known whether or not the nucleocapsid protein of Hantaan virus is phosphorylated. We attempted to sequence the N-terminus of the N protein of Hantaan virus. However, due to the low level of virus production and N-terminal blockage of the N protein, we were unable to determine the amino-acid sequence.

According to the sequence analysis, it is quite clear that the coding strategies of the M and S RNAs of Hantaan virus are different from those of other bunyaviruses. Absence of the non-structural protein coding regions in both the M and S RNAs suggests that either non-structural proteins are not synthesized or that a different coding strategy from those of other bunyaviruses is used to encode and synthesize such proteins. It is possible that the viral genes induces host cells to synthesize polypeptides that function like non-structural proteins. Since the nucleotide sequence of the L RNA segment has not been determined, it is not known whether the L RNA contains additional coding capacity for nonstructural proteins. Another possibility for generation of nonstructural proteins is a readthrough
of the stop codons of the major open reading frame as well as the minor reading frames. This implies that a translation termination signal would be suppressed at the end of the major open reading frame, and synthesis of additional polypeptide sequences would be continued until the next stop codon is reached as seen in alphaviruses and retroviruses (Yoshinaka et al. 1985, Collins et al. 1982, Lopez et al. 1985, Kozak 1986c). Alternatively, ribosomes can initiate translation at an internal AUG codon of the major open reading frame. The internal initiation of translation has been observed to occur in influenza virus (Shaw et al. 1983), vesicular stomatitis virus (Herman 1986), and Sendai virus (Curran et al. 1986). Since the roles of non-structural proteins are not known for bunyaviruses, the importance of the presence or absence of such a protein for virus replication is also not known.

4.4. Transcription Strategy of Hantaan Virus

Although coding assignments of the genomic segments have been made in bunyaviruses, little is known about transcription events because of two major obstacles. All bunyaviruses are thought to contain a RNA dependent RNA polymerase. However, the polymerase activity has only been demonstrated for certain viruses in vitro, and even in these cases the activity has been too weak to be practical for transcription studies. Therefore only intracellular viral transcripts are available. However, these intracellular viral transcripts seem to be non-
polyadenylated or have very short polyadenylate sequences. Thus, it is difficult to prepare the transcripts free from genomic RNA or anti-genomic replicative intermediates which differ slightly in length. No direct data are available concerning transcription of the L RNA although viral complementary RNA of similar size to the L segment has been reported (Pettersson et al. 1985). A single M mRNA species has been identified for both Rift Valley fever virus and snowshoe hare virus. For the S mRNA, it is not clear yet whether S RNA-coded polypeptides (N and NS₅) are synthesized from single or multiple mRNA species. While only one S mRNA species was reported in snowshoe hare virus (Fuller et al. 1983, Eshita et al. 1985), claims were made that multiple species of leader RNAs and S mRNA existed in La Crosse virus infected cells (Patterson et al. 1983). It has been suggested that leader RNAs of La Crosse virus allow access to both overlapping reading frames by ribosomes without the need to splice the mRNA (Patterson et al. 1983). Since Hantaan virus does not appear to follow the strategy of overlapping reading frames, as revealed by nucleotide sequence analysis, this situation may not exist in Hantaan virus infected cells. Hybridization studies using strand-specific probes identified a single species of intracellular RNA specific for the M or S segment of Hantaan virus (Figure 22). These intracellular RNA species were complementary to the genomic RNA. In vitro cell-free translation failed to synthesize demonstrable protein
products of the viral genomic RNA (Schmaljohn et al. 1986b). Therefore, these anti-genomic, viral specific intracellular RNA species are either segment specific mRNA species or replicative intermediates. Any intracellular RNAs specific for the L segment have not been identified (probably due to low abundance). These major intracellular RNAs identified are more likely to be mRNAs. These observations are in agreement with the nucleotide sequence data which reveals a single open reading frame in the anti-genomic sense of both the M and S segments of Hantaan virus (Figures 14, 20). Hybridization studies, nucleotide sequence analysis, polymerase activity associated with virions and the inability of genomic RNA to be translated in vitro (Schmaljohn et al. 1983) indicate that the M and S RNAs of Hantaan virus are of negative polarity. Analyses of transcription termination sites, in vitro translation and structure of the 5' ends of mRNA species of Hantaan virus remain to be carried out.

4.5. Expression of the Envelope Glycoprotein Gene of Hantaan Virus in Insect Cells by Recombinant Baculovirus

A helper-independent baculovirus transfer vector has been used for the expression of a variety of foreign genes (Smith et al. 1983a, Luckow and Summers 1988). This vector utilizes the highly efficient polyhedrin promoter of Autographa californica nuclear polyhedrosis virus (AcNPV). The major advantage of the baculovirus expression vector is the abundant expression of recombinant protein. In
addition, baculoviruses are not pathogenic to vertebrates and do not employ transforming elements (Doerfler 1986). Furthermore, the baculovirus expression system has been recognized to utilize insect protein modification and processing systems which may be important for biological function of a recombinant protein (Doerfler 1986, Luckow and Summers 1988). It was of interest to determine whether this insect virus vector system could efficiently express the glycoprotein gene of Hantaan virus. The genome of AcNPV consists of double-stranded circular DNA 128 Kbp in length. Two forms of viral progeny, extracellular virus particles and occluded virus particles are produced during AcNPV infection. Occluded virus particles are embedded in polyhedrin protein (29 KDa) which is the major virus-encoded structural component of the viral occlusions. Polyhedrin protein is detected within 12 hr post-infection and continues to accumulate for 4-5 days until the infected cells lyse (Doerfler 1986). Most baculovirus transfer vectors constructed to date contain sequences of AcNPV, including the polyhedrin gene promoter and various lengths of 5' and 3' sequences flanking the polyhedrin gene, cloned into a bacterial plasmid. The foreign gene is inserted downstream of the polyhedrin promoter and transferred to wild-type AcNPV by homologous recombination, within a cell transfected with both the recombinant plasmid and wild-type viral DNA. Insertion of a foreign gene by in vivo recombination results in the deletion of a major portion of
the polyhedrin gene so that the foreign protein is produced instead of polyhedrin. Recent studies have suggested that the level of foreign gene expression seems to be related to the upstream region of the polyhedrin gene rather than to the polyhedrin-coding or 3' non-coding sequences (Matsuura et al. 1987, Horiuchi et al. 1987, Kang 1988, Luckow and Summers 1988). A new transfer vector pACYM1, containing the A of the initiating ATG codon and all of the upstream sequence of the polyhedrin gene, but lacking the rest of the polyhedrin coding sequences has been developed (Matsuura et al. 1987). It retains the transcription termination signal and intact polyhedrin polyadenylation signal. Thus, the protein produced using pACYM1 will be a non-fused form. For these reasons, pACYM1 was chosen for expression of the glycoprotein gene of Hantaan virus. The glycoprotein gene of Hantaan virus was modified to delete both the 5' and 3' untranslated region and to contain the trinucleotide ACC immediately upstream of the translation initiation codon to provide a preferable translation context (Figures 24, 25). A recombinant baculovirus containing the entire glycoprotein gene of Hantaan virus was constructed and used for expression in Spodoptera frugiperda cells (Figure 26). Western blot and immunoprecipitation demonstrated that two species of proteins were synthesized in insect cells infected with the recombinant baculovirus (Figures 29, 30). These proteins were similar to the G1 and G2 proteins of Hantaan virions.
both in immunoreactivity and in size. The presumptive precursor polypeptide was cleaved to produce two different molecules, G1 and G2 (Figures 29, 30). This indicates that the cleavage of the precursor polypeptide can occur in the absence of virus replication or any other structural proteins of the virus. Proteolytic cleavage of recombinant proteins produced in \textit{S. frugiperda} cells have been documented previously (Smith et al. 1983b, 1985, Maeda et al. 1985, Matsuura et al. 1986, Possee et al. 1986, Kuroda et al. 1986, Madisen et al. 1987, Hu et al. 1987). It is of interest to determine whether the signal peptides of both the G1 and the G2 proteins are also cleaved as observed for human interferon and interleukin 2 expressed in \textit{S. frugiperda} cells (Smith et al. 1983b, 1985, Maeda et al. 1985, Horiuchi et al. 1987).

The putative G2 protein synthesized in \textit{S. frugiperda} cells migrated faster on SDS-Polyacrylamide gel than the G2 protein prepared from the Hantaan virions (Figure 29). This suggests a difference in glycosylation or proteolytic cleavage. N-linked glycosylation of foreign proteins produced in insect cells has been observed in many cases (Smith et al. 1983, Kuroda et al. 1986, Matsuura et al. 1986, 1987, Rusche et al. 1987, Coelingh et al. 1987, Estes et al. 1987, Overton et al. 1987). In these studies, glycosylation was examined by incorporation of radiolabeled sugars, by sensitivity to endoglycosidases or by the use of glycosylation inhibitors. In \textit{Aedes} mosquito cell lines, N-
linked oligosaccharides are found to be deficient in sialic acid, galactose and fucose (Butters and Hughes 1981). Thus, glycosylation of the complex type as seen in mammalian cells does not seem to occur in insect cells, although it remains to be determined for S. frugiperda cells. If only the high mannose type of polysaccharide contributes to the glycosylation of the G1 and G2 proteins produced in S. frugiperda cells, it would result in electrophoretic mobility differences because glycosylation of the G1 and G2 proteins of Hantaan virus in mammalian cells seems to be of a mixture of the high mannose type and the complex type (Schmaljohn et al. 1986b). However, it remains to be determined whether the G1 and G2 proteins synthesized in S. frugiperda cells are partially glycosylated or not glycosylated.

Despite the modification of the glycoprotein gene and the use of an efficient transfer vector (pACYCM1), the level of G1 and G2 proteins synthesized in S. frugiperda cells does not appear to be high. The low level of expression may be due to intrinsic properties of these glycoproteins. It may also be related to the pathway through which the protein is processed in the insect cells. It is not known whether the relatively large size of the inserted gene (3.4 Kbp) has any effect on the level of expression. Whether the low level of expression is due to lower rates of transcription, translation or due to stability of the mRNA species is also not known. It has been suggested that good
levels of expression have been obtained using genes containing their own polyadenylation signal in addition to that of polyhedrin gene (Summers and Smith 1987, Luckow and Summers 1988). Because the mRNA segment of Hantaan virus does not seem to possess a polyadenylation signal in the nucleotide sequence (Figure 11), retention of 3' non-coding sequences of the glycoprotein gene may not affect the level of expression. However, the length of the mRNA of the glycoprotein gene of Hantaan virus will rely solely on the transcription termination signal and polyadenylation signal of the polyhedrin gene, which will likely result in the synthesis of larger than normal mRNA transcripts (approximately 4.0 Kb). It is not known whether such large transcripts are not stable or if there is any premature termination of the transcription or translation of mRNA transcripts.

4.6. Concluding Remarks

Since hantaviruses possess molecular characteristics similar to but unique from other bunyaviruses, they are suggested as new members of the family Bunyaviridae.

Because Hantaan virus represents a significant biohazard, expression of the glycoprotein gene in heterologous system provides an excellent opportunity to study role of the viral glycophotins with safe and easy manipulation. It is of interest to study the sequences necessary for translational or post-translational modification, post- or co-translational cleavage,
translocation reinitiation, cellular transport and immunogenicity of the glycoproteins of Hantaan virus. Since bunyavirus maturation appears to take place in the Golgi apparatus, it will be of particular interest to investigate the sites and mechanisms of Hantaan virus maturation. Furthermore, since the production of any hantaviruses for use as a potential source of viral antigen is difficult, the expression of the surface glycoprotein gene can also be considered as an important first step towards the development of a subunit vaccine against hemorrhagic fever with renal syndrome.
CHAPTER 5: BIBLIOGRAPHY


APPENDIX I: COMPOSITION OF NUCLEOTIDE MIX USED IN M13 SINGLE-STRANDED DNA SEQUENCING REACTIONS

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Chase reaction: 0.5 mM of each four dNTPs
**APPENDIX 2: COMPOSITION OF NUCLEOTIDE MIX FOR dUC19 DOUBLE-STRANDED DNA SEQUENCING REACTIONS**

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APPENDIX 3: BASE-SPECIFIC REACTIONS FOR MAXAM-GILBERT SEQUENCING OF DNA

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(Four reaction were proceeded as follow)

Dry ice/MeOH bath for 10 min
Microcentrifuge for 10 min/4 C
Pellet
Resuspend in 200ul of 0.3M NaOAc pH 5.2, 1mM EDTA
600 ul EtOH
Centrifuge for 10min/4 C
Pellet
70% ethanol rince
Vacuum dry
50 ul of 10% piperidine
Heat at 90 C/15 min
100 ul water
Freeze in dry ice/EtOH
Lyophilize for 100 min
20 ul water
Lyophilize for 30 min
10 ul water
Lyophilize for 30 min
10 ul formamide-dye mix
Heat at 90 C/3 min
Quick chill
Load on a gel in order of G, A, T, C
List of Publications by D. W. Y. Related to This Thesis


Manuscripts in preparation


List of Abstracts and Presentations by D. W. Y. Related to This Thesis


