



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

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

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

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

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

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

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

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

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

MOLECULAR CHARACTERIZATION OF GENOMIC SEGMENTS OF THE
PROSPECT HILL STRAIN OF *HANTAVIRUS* AND EXPRESSION OF
REGULATORY PROTEINS OF HUMAN IMMUNODEFICIENCY VIRUS

A Thesis Submitted to the
School of Graduate Studies
University of Ottawa

In partial fulfillment of the Requirements for the Degree of
Doctor of Philosophy
Department of Microbiology and Immunology
Faculty of Medicine

By

Mark A. Parrington

© Mark A. Parrington, Ottawa, Canada, 1991



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-75070-7

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

PART 1: PROSPECT HILL VIRUS

Hemorrhagic fever with renal syndromes (HFRS) are acute diseases in humans caused by viruses in the *Hantavirus* genus of the Bunyaviridae family. The severity of HFRS, caused by a specific hantavirus is related to its serotype which is related to its rodent host. There are four antigenically distinct serotypes recognized in the *Hantavirus* genus. Each antigenically distinct serotype was isolated from a different rodent species. Serotype 1 includes *Apodemus* derived strains like Hantaan virus strain 76-118 (Hantaan) which is associated with the severe form of HFRS. Serotype 2 includes *Rattus* derived strains like Sapporo rat virus strain SR-11 (SR) that are generally associated with a moderate although potentially still fatal form of HFRS. Serotype 3 includes *Clethrionomys* derived strains like Puumala virus strain Hällnäs B1 (Hällnäs) which is associated with a milder form of disease. Serotype 4 includes *Microtus* derived strains like Prospect Hill virus, strain Prospect Hill-1 (PH) that have not been linked to any disease in man.

Genomic analyses revealed PH virus has three RNA segments large (L), Medium (M) and Small (S), like all Bunyaviridae, with relative molecular masses (M_r) of 2.2×10^6 , 1.3×10^6 and 0.6×10^6 respectively.

Complementary DNA representing the genomic M RNA segment of the PH virus was cloned and its nucleotide sequence determined. The PH virus M RNA segment consists of 3707 nucleotides. As with other Bunyaviridae the 3' and 5' termini are inversely complementary and could possibly allow the M RNA segment to form a circular structure. The PH

virus M RNA segment has a single long open reading frame (ORF) in the viral complementary-sense RNA with a coding capacity of 1142 amino acids with a predicted M_r of approximately 126 K. This protein is the putative glycoprotein precursor that would be cleaved into the two viral envelope glycoproteins G1 and G2. The predicted gene product of the PH virus M segment was compared with the corresponding gene products of Hantaan virus, SR virus and Hällnäs virus. There was 74% and 79% amino acid sequence similarity between the G1 and G2 proteins of PH virus and Hällnäs virus respectively. In contrast, there was only 50% amino acid sequence similarity between the G1 proteins of PH virus and SR virus or Hantaan virus. However, the G2 proteins of SR virus and Hantaan virus were more closely related to the G2 protein of PH virus with amino acid sequence similarity of approximately 62%. The G1 proteins of all four viruses had three potential asparagine-linked glycosylation sites conserved and there was one conserved site in the G2 proteins. Hydrophilicity plots of the four virus glycoproteins were very similar. The region of greatest hydrophilicity was conserved in the Hällnäs virus, SR virus and Hantaan virus, and was located near the C-terminus of the G1 protein. In contrast, the region of greatest hydrophilicity in the PH virus glycoprotein precursor was located closer to the N-terminus of the G1 protein. Our data demonstrate that despite differences in the serotypic profiles and virulence of PH virus and Hällnäs virus, their G1 and G2 proteins are closely related.

Complementary DNA representing the genomic S RNA segment of the PH virus was also cloned and its nucleotide sequence determined. The PH virus S RNA segment consists of 1675 nucleotides and like the PH virus M RNA segment the 3' and 5' termini are inversely complementary.

Analysis of the PH virus S RNA sequence also revealed an energetically stable hairpin structure near the 5' end of the virion RNA which may serve as a transcription termination signal. The PH virus S RNA segment had a long ORF in the viral complementary-sense RNA that could potentially encode a 433 amino acid (49K) nucleocapsid (N) protein. The predicted gene product of the PH virus S segment was compared with the corresponding gene products of Hantaan virus, SR virus and Hällnäs. There was 79.9% amino acid sequence similarity between the N proteins of PH virus and Hällnäs virus. In contrast, there was only approximately 62% amino acid sequence similarity between the N proteins of PH virus and SR virus or Hantaan virus. However, the N proteins of SR virus and Hantaan virus were more closely related to each other with an amino acid sequence similarity of approximately 83%. Comparisons between the amino acid sequences of these four virus N proteins revealed the *Hantavirus* N protein could be divided into two conserved regions and one non-conserved region. The first conserved region includes the first 233 amino acids of the N protein and these four viruses have 57.3% amino acid sequence similarity in this region. The second conserved region is composed of the last 124 amino acids at the C-terminus. The four virus N proteins have 79% amino acid sequence similarity in this region. In between these two conserved regions is a non-conserved region in which the four virus N proteins only have 10.4% similar amino acid sequence. Hydrophilicity plots of the four virus N proteins were very similar. However, the region of greatest hydrophilicity in the PH virus and Hällnäs virus profiles was in the C-terminal half of the N proteins. In contrast, the region of highest hydrophilicity in the SR virus and Hantaan virus profiles was located near the N-terminus of the N proteins.

A second smaller ORF was recognized in the viral complementary-sense S RNA of PH virus, and could potentially encode a 90 amino acid (10.5K) protein. This is very similar in size to the NS_S protein coded for by the S RNA segments of some bunyaviruses. Although this second ORF was not observed in the viral complementary-sense S RNA of SR virus or Hantaan virus it was observed in Hällnäs virus. This second ORF in the viral complementary-sense S RNA of Hällnäs virus was identical in position and size to the one in PH virus and the putative NS_S proteins these ORFs could potentially encode had 50% amino acid sequence similarity. This putative PH virus NS_S gene was expressed using the baculovirus expression system in *Spodoptera frugiperda* 9 (SF9) cells. The putative PH virus NS_S protein was expressed to reasonable levels in this system. The partially purified putative NS_S protein was used to raise specific antibodies in rabbits. Although these antibodies could immunoprecipitate this protein from SF9 cells it was not able to detect this protein in PH virus infected Vero cells. Therefore, at this time there is no evidence that PH virus or Hällnäs virus express a second protein using their S RNAs.

Our data demonstrate that despite differences in the serotypic profiles and virulence of PH virus and Hällnäs virus, their G1, G2 and N proteins are closely related. We conclude that PH virus and Hällnäs virus may have evolved along a separate evolutionary pathway in the *Hantavirus* genus from SR virus and Hantaan virus.

PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 *rev* AND *vif* PROTEINS

Human Immunodeficiency virus type 1 (HIV-1) has been identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS) and related disorders. HIV-1 displays a high degree of genetic complexity. In addition to encoding the *gag*, *pol* and *env* proteins characteristic of all replication-competent retroviruses, HIV-1 encodes at least six other proteins (*vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*). These nine viral proteins are encoded by more than 20 distinct mRNA species that are derived from post-transcriptional processing of the initial full length viral transcript.

Two HIV-1 proteins, *rev* and *vif*, that are normally expressed only at low levels in infected cells were expressed to high levels in insect cells using recombinant baculovirus vectors. Sera from HIV-1 infected individuals, with different symptoms of disease, were tested for the presence of antibodies that would recognize the *rev* or *vif* proteins of HIV-1.

Antibody against *rev* was found in sera from some individuals in all stages of HIV-1 infection, although somewhat fewer ARC and AIDS patients had *rev* antibody in comparison to healthy donors or healthy donors at risk. Since *rev* antibodies were found in HIV-1 infected individuals in all stages of disease, detection of *rev* antibodies is of no prognostic value.

In contrast, antibody against *vif* was found only in sera from healthy donors or healthy donors at risk, but were not present in the sera of ARC or AIDS patients. This result is consistent with the suggestion that production of *vif* antibodies early in the HIV-1 infection may be partially

protective and delay the progression to ARC and AIDS. Another explanation is that *vif* antibodies are produced early during the HIV-1 infection and disappear as the diseases progresses to ARC and AIDS. These results differ with some previous results. However, even if *vif* antibodies are not usually present in ARC or AIDS patients the finding that sera from some HIV-1-seronegative individuals and 20% of *Schistosoma mansoni*-infected HIV-1-seronegative individuals have antibodies that cross-react with the *vif* protein severely limits the prognostic value.

Although the HIV-1 *vif* protein is poorly immunogenic, antibodies that recognize this protein were raised in rabbits. This anti-*vif* serum is presently being used in a study to determine if *vif* is a virion associated protein.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. C. Yong Kang for his encouragement and guidance during this research project. Also, without his generous financial support this study could not have been accomplished.

I would also like to thank Dr. Ken Dimock for his good advice and willingness to answer questions during the course of this research project.

I am also grateful to Erling Rud who in the early years assisted with my work by giving timely technical advice.

Special thanks go to Jamie Waring for his help, advice and comradery.

I would also like to thank Nancy Delcellier for preparing oligonucleotide primers and Bernadette Mah and Doris McLean for operation of the automated DNA sequencer.

Finally, I would like to thank the secretarial staff and support staff who have always been very helpful with me.

DEDICATION

This thesis is dedicated to my wife Linda and my daughter Sharon

TABLE OF CONTENTS

ABSTRACT	ii
PART 1: PROSPECT HILL VIRUS	ii
PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 <i>rev</i> AND <i>vif</i>	
PROTEINS	vi
ACKNOWLEDGEMENTS	viii
DEDICATION	ix
TABLE OF CONTENTS.	x
LIST OF TABLES	xvii
LIST OF FIGURES.	xviii
LIST OF ABBREVIATIONS	xxii
CHAPTER 1: INTRODUCTION	1
PART 1: LITERATURE REVIEW OF HANTAVIRUSES	1
1. HISTORY OF HEMORRHAGIC FEVER WITH RENAL	
SYNDROME	1
2. ETIOLOGY.	3
Virus Isolation	3
Antigenic Relationships between Hantaviruses	7
Virion Morphology	7
Hantavirus Morphogenesis	9
Genome Organization and Viral Proteins	11
3. EPIDEMIOLOGY	15
Distribution	15
Epidemiologic Type	17
Transmission.	19
Reservoirs	19

Vectors	19
4. CLINICAL MANIFESTATIONS, PATHOGENESIS, SERO- DIAGNOSIS, TREATMENT	20
Clinical Manifestations	20
Pathogenesis	22
Serological Testing	23
Treatment	23
Vaccine Development	24
5. STATEMENT OF OBJECTIVES.	24
PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 <i>rev</i> AND <i>vif</i>	
PROTEINS	26
1. GENERAL DESCRIPTION OF HIV-1	26
2. HIV-1 <i>rev</i> PROTEIN	27
Function of the HIV-1 <i>rev</i> Protein	27
3. HIV-1 <i>vif</i> PROTEIN	28
Function of the HIV-1 <i>vif</i> protein	28
4. STATEMENT OF OBJECTIVES.	30
CHAPTER 2: MATERIALS AND METHODS	31
1. PROSPECT HILL VIRUS PRODUCTION	31
Virus, Cells and Media	31
Virus Production and Radiolabelling of Viral RNA	31
Assay for PH Virus Production	32
2. PREPARATION OF RNAs	33
Extraction and Purification of PH Virus RNA	33
Extraction and Purification of Intracellular RNA from PH Virus Infected Cells	34
Agarose Gel Electrophoresis of Viral RNA	34

3. BLOTTING AND HYBRIDIZATION OF NUCLEIC ACIDS	35
Northern Blots	35
Colony Blotting of Bacterial Plasmid DNA	35
Dot Blotting of RNA or DNA	35
Nick Translation	36
Nucleic Acid Hybridization	36
4. PLASMID PREPARATION AND ELECTROPHORESIS OF DNA	37
Small-Scale Isolation of Plasmid DNA (Miniprep)	37
Large-Scale Isolation of Plasmid DNA (Maxiprep)	37
Agarose Gel Electrophoresis of DNA and Purification of DNA from Agarose Gels	38
Preparation of Competent Cells	38
Transformation of Competent Cells	40
5. CLONING OF THE PROSPECT HILL VIRUS GENOME	41
Synthesis of Oligonucleotide Primers for Cloning	41
Construction of a Prospect Hill Virus cDNA Bank	41
Molecular Cloning of the 5' and 3' Termini of the PH virus M RNA Segment	42
Molecular Cloning of the 3' and 5' Termini of the PH virus S RNA Segment	44
6. DETERMINATION OF NUCLEOTIDE SEQUENCES	45
Subcloning into the Bacteriophage M13	45
Preparation of Single-Stranded M13 DNA for Sequencing	46
Subcloning into the DNA Plasmid pUC19	46
Preparation of Double-Stranded pUC19 Plasmid DNA for Sequencing	47
Manual DNA Sequencing	47

Urea-Polyacrylamide Sequencing Gels	49
Automated DNA Sequencing	49
Computer Analysis of Nucleotide and Amino Acid Sequences	49
7. PRODUCTION OF RECOMBINANT BACULOVIRUSES	50
Modification of Genes for Protein Expression	50
Cells, Viruses and Preparation of Virus Stocks	51
Preparation of the Baculovirus Transfer Vector	51
Insertion of the Putative PH Virus NS ₅ Gene into the Baculovirus Transfer Vector	52
Insertion of the Human Immunodeficiency Virus Type 1 (HIV-1) <i>rev</i> Gene into the Baculovirus Transfer Vector	52
Insertion of the HIV-1 <i>vif</i> Gene into the Baculovirus Transfer Vector	52
Transfection of Spodoptera Frugiperda Cells with Baculovirus Transfer Vector Constructions	52
Isolation and Purification of Recombinant Baculoviruses	53
Analysis of Recombinant Baculoviruses by Hybridization	55
8. ANALYSIS OF PROTEINS	55
Radiolabeling of PH Virus Infected Cells	55
Radiolabeling of Recombinant Baculovirus Infected Cells	55
Preparation of Cell Lysates	56
Immunoprecipitations	56
SDS-Polyacrylamide Gel Electrophoresis	57
Elution of Proteins from SDS-Polyacrylamide Gels	58
Production of Polyclonal Antisera	58
Western Blot Immunoassay	59

CHAPTER 3: RESULTS	60
PART 1: PROSPECT HILL VIRUS	60
1. STRUCTURAL CHARACTERIZATION OF PH VIRUS.	60
Characterization of the PH Virus Genome	60
2. ASSAY FOR PH VIRUS PRODUCTION	62
3. MOLECULAR CHARACTERIZATION OF THE PH VIRUS M	
RNA SEGMENT	66
Cloning and Sequence Analysis of the PH Virus M RNA	
Segment	66
Coding Strategy of the PH Virus M RNA	76
Comparison of the PH Virus, Hällnäs Virus, SR Virus and	
Hantaan Virus G1 and G2 Proteins	76
4. MOLECULAR CHARACTERIZATION OF THE PH VIRUS S RNA	
SEGMENT	84
Cloning and Sequence Analysis of the PH Virus S RNA	
Segment	84
Coding Strategy of the PH Virus S RNA Segment	89
Comparison of the PH Virus, Hällnäs virus, SR virus and	
Hantaan Virus N Proteins	93
Comparison of the PH Virus and Hällnäs Virus NS _S Proteins . .	98
5. EXPRESSION OF THE NS_S GENE OF PH VIRUS IN INSECT	
CELLS.	101
Deletion and Modification of the Non-Coding Sequence of	
the PH virus NS _S Gene and Transfer of this Gene into	
the Baculovirus Transfer Vector	101
Construction of the Recombinant Baculovirus Containing the	
Putative NS _S Gene	106

Expression of the Putative NS ₅ Protein of PH virus	106
Testing for the Presence of the NS ₅ Protein in PH virus	
Infected Vero Cells	111
PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 <i>rev</i> AND <i>vif</i>	
PROTEINS	114
1. EXPRESSION OF THE <i>rev</i> AND <i>vif</i> GENES OF HIV-1 IN	
INSECT CELLS	114
Deletion and Modification of the Non-Coding Sequence of	
the HIV-1 <i>rev</i> Gene and Transfer of this Gene into	
the Baculovirus Transfer Vector	114
Construction of the HIV-1 <i>rev</i> Recombinant Baculovirus	118
Deletion and Modification of the Non-Coding Sequence of	
the HIV-1 <i>vif</i> Gene and Transfer of this Gene into	
the Baculovirus Transfer Vector	120
Construction of the HIV-1 <i>vif</i> Recombinant Baculovirus	123
Expression of the HIV-1 <i>rev</i> and <i>vif</i> Proteins by	
Recombinant Baculoviruses	124
Phosphorylation of the HIV-1 <i>rev</i> Protein	127
Reaction of Human Sera from HIV-1 Infected Donors Against	
the <i>rev</i> and <i>vif</i> Proteins Expressed in Insect Cells	128
Production of Rabbit Antibodies Specific for the HIV-1 <i>vif</i>	
Protein	131
CHAPTER 4: DISCUSSION	137
PART 1: PROSPECT HILL VIRUS	137
PH Virus Genome	139
Growth Characteristics of PH Virus	140
Features of the M and S RNA Segments of PH virus	141

Coding Strategy of the PH Virus M RNA Segment	144
Coding Strategy of the PH Virus S RNA Segment	149
Concluding Remarks	155
PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 <i>rev</i> AND <i>vif</i>	
PROTEINS	157
Expression of the HIV-1 <i>rev</i> and <i>vif</i> Proteins	157
Prevalence of Antibodies Against <i>rev</i> and <i>vif</i> in HIV-1	
Infected Individuals	158
Production of <i>vif</i> Antibodies in Rabbits.	160
CHAPTER 5: REFERENCES.	161
APPENDIX 1	190

LIST OF TABLES

1. Genus specific 3' terminal consensus nucleotide sequences of Bunyaviridae vRNA segments	5
2. Antigenically recognized serotypes within the <i>Hantavirus</i> genus	16
3. Comparison of the amino acid sequences of the G1 and G2 proteins of Hällnäs virus, SR virus, and Hantaan virus to the G1 and G2 proteins of PH virus	81
4. Comparison of the amino acid sequences of the N proteins of Hällnäs virus, SR virus, and Hantaan virus to the N protein of PH virus	95
5. Prevalence of antibodies against <i>vif</i> and <i>rev</i> in HIV-1-infected individuals	132

LIST OF FIGURES

1. Fluorograph of the genomic RNA of PH virus.	61
2. Production and release of PH virus into the tissue culture medium after infection of Vero cells	63
3. Release of PH virus into the culture medium each day	65
4. Cloning strategy of the PH virus M RNA genomic segment	68
5. Hybridization of a ³² P-labelled PH virus M specific clone to dot blots of total infected cell RNA	71
6. Analysis of the 3' end of the PH virus M RNA segment by comparing PCR amplified products using different primers	72
7. Nucleotide sequence of the PH virus M RNA segment	74
8. Predicted secondary structure of the complementary 3' and 5' termini of the PH virus M RNA segment	75
9. Translation termination codon locations in all reading frames of the PH virus M RNA segment	77
10a. Comparison of the predicted amino acid sequences of the PH virus, Hällnäs virus, SR virus, and Hantaan virus G1 proteins	78
10b. Comparison of the predicted amino acid sequences of the PH virus, Hällnäs virus, SR virus, and Hantaan virus G2 proteins	79
11. Hopp and Woods hydrophilicity profiles of PH virus, Hällnäs virus, SR virus, and Hantaan virus glycoprotein precursors	83
12. Cloning strategy of the PH virus S RNA genomic segment	85
13. Nucleotide sequence of the PH virus S RNA segment	87
14. Predicted secondary structure of the complementary 3' and 5' termini of the PH virus S RNA segment	88

15. Predicted secondary structure in the PH virus S RNA between bases 1580 and 1620, measuring from the 3' terminus	90
16. Translation termination codon locations in all reading frames of the PH virus S RNA segment	91
17. Comparison of the predicted amino acid sequences of the N proteins of PH virus, Hällnäs virus, SR virus, and Hantaan virus	94
18. Hopp and Woods hydrophilicity profiles of PH virus, Hällnäs virus, SR virus, and Hantaan virus N proteins	97
19. Comparison of the predicted amino acid sequences of the putative PH virus and Hällnäs virus NS _S proteins	99
20. Hopp and Woods hydrophilicity profiles of the putative NS _S proteins of PH virus and Hällnäs virus	100
21. Construction of a baculovirus transfer vector for the PH virus NS _S gene	102
22. Primary selection of recombinants with the PH virus NS _S gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers	104
23. Enrichment of PH virus NS _S recombinant baculoviruses using a limiting dilution assay and dot blot hybridization	107
24. Dot blot analysis of PH virus NS _S recombinant baculoviruses after plaque purification	108
25. Expression of the putative NS _S protein of PH virus in insect cells	110

26. Testing the specificity of the PHV-NS ₅ -Ab rabbit serum by immunoprecipitation of the PH virus NS ₅ protein from insect cell lysates	112
27. Testing for the presence of the putative NS ₅ protein in PH virus infected Vero cells by immunoprecipitation of PH virus infected cell lysates with the PHV-NS ₅ -Ab rabbit serum	113
28. Construction of a baculovirus transfer vector for the HIV-1 <i>rev</i> gene	115
29. Primary selection of recombinants with the HIV-1 <i>rev</i> gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers	117
30. Dot blot analysis of HIV-1 <i>rev</i> recombinant baculoviruses after plaque purification.	119
31. Construction of a baculovirus transfer vector for the HIV-1 <i>vif</i> gene	121
32. Primary selection of recombinants with the HIV-1 <i>vif</i> gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers	122
33. Dot blot analysis of HIV-1 <i>vif</i> recombinant baculoviruses after plaque purification.	125
34. Expression of proteins in cells infected with wild type AcNPV, or the recombinant baculoviruses AcRev or AcVif during a 96 h time-course experiment	126
35. Phosphorylation of the HIV-1 <i>rev</i> protein expressed in insect cells	129

36. Western blot of <i>vif</i> and <i>rev</i>	130
37. Western blot of the HIV-1 <i>vif</i> protein expressed in insect cells or <i>E. coli</i>	133
38. Testing the specificity of the HIV-1- <i>vif</i> -Ab rabbit serum by immunoprecipitation of the HIV-1 <i>vif</i> protein from insect cell lysates	135

LIST OF ABBREVIATIONS

A	Adenine base in a nucleotide sequence
ACS	aqueous counting scintillant
AIDS	Acquired Immunodeficiency Syndrome
AMV	avian myeloblastosis virus
ARC	AIDS-related complexes
ATCC	American type culture collection
Bis	N,N'-Bis-methylene-acrylamide
bp	base pair
BPB	bromophenol blue
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
°C	degrees Celsius
C	Cytosine base in a nucleotide sequence
cDNA	complementary DNA
Ci	Curies
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxyribonucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid disodium salt
ELISA	enzyme-linked immunosorbant assay
EtBr	ethidium bromide
FBS	fetal bovine serum
Fig.	Figure
g	gravity or grams
G	Guanine base in a nucleotide sequence
GAR-AP	goat anti-rabbit alkaline phosphatase
G1	largest envelope glycoprotein in Bunyaviridae
G2	smallest envelope glycoprotein in Bunyaviridae
h	hour
Hantaan	Hantaan virus strain 76-118
Hällnäs	Puumala virus strain Hällnäs B1
HFRS	hemorrhagic fever with renal syndrome
HIV-1	human immunodeficiency virus type 1
IBI	International Biotechnologies, Inc.
IEC	International Equipment Company
Inc.	Incorporated
IPTG	isopropyl-1-thio-beta-D-galactoside
K	kilo (X 1000)
Kb	kilobase
l	liter

L	large RNA segment or <i>Hantavirus</i> polymerase protein
LB	Luria-Bertani medium, tryptone, yeast extract, NaCl
Ltd	Limited
M	medium RNA segment or molar
MEM	minimal essential medium
min	minute
ml	milliliter
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection
MOPS	morpholinopropanesulfonic acid
M _r	relative molecular mass
mRNA	messenger ribonucleic acid
NEN	New England Nuclear
nm	nanometer
N	nucleocapsid protein
NENS	Na-acetate, EDTA, NaCl, and SDS
ng	nanogram
NS _m	nonstructural protein encoded by M RNA
NS _s	nonstructural protein encoded by S RNA
NT	nick translation
OD	optical density
pg	picogram
PH	Prospect Hill virus strain Prospect Hill-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol

pfu	plaque forming unit
PPO	2,5-diphenloxazole
RF	replicative form
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNP	ribonucleoprotein core
S	small RNA segment or Svedberg units
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOB	tryptone, yeast extract, NaCl
SOC	tryptone, yeast extract, NaCl, glucose
SSC	standard saline citrate buffer
SR	Sapporo rat virus strain 11
T	Thymine base in a nucleotide sequence
TAE	Tris-acetate, EDTA buffer
TBE	Tris-borate, EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
TTBS	TBS plus tween 20
UV	ultraviolet
U	Uracil base in a nucleotide sequence
vcRNA	viral complementary sense RNA
vRNA	viral sense RNA
YT	yeast extract, tryptone, and NaCl
μ Ci	microcuries

μg	microgram
μM	micromolar
μmol	micromoles

CHAPTER 1: INTRODUCTION

PART 1: LITERATURE REVIEW OF HANTAVIRUSES

1. HISTORY OF HEMORRHAGIC FEVER WITH RENAL SYNDROME

Hemorrhagic fever with renal syndrome (HFRS) may have existed in Asia for at least 1,000 years, since a description of HFRS was recorded in a Chinese medical book written in approximately 960 AD (Lee, 1982a). In 1932 the Russians observed a new disease similar to HFRS in people living along the lower Amur river basin in the Soviet Far East. Thereafter, there were yearly outbreaks of this disease along the Amur river (Smorodintsev *et al.*, 1959). When the Japanese army invaded Manchuria there were 12,000 cases of this disease among their 1 million troops (Ishii *et al.*, 1942; Nakazawa, 1966). Russian and Japanese researchers studying this disease independently defined its clinical and epidemiological features. They determined that it was infectious and was probably caused by a virus. Both groups found human volunteers could be infected by intravenous or intramuscular injections with blood or urine from infected patients if the samples were taken early during their illness (Kasahara *et al.*, 1944; Smorodintsev, 1944). They were unable however, to establish an infection in experimental animals.

In 1951 United Nations troops fighting near the front in Korea began contracting an acute hemorrhagic fever. The course of this severe and often fatal disease, which became known as Korean Hemorrhagic fever (KHF), was unusual. Patients developed cardiovascular instability, shock and renal failure, and physicians recognized this was a disease previously

unknown in the Western world (Smadel, 1953). During the Korean war over 3,200 cases were confirmed among all United Nations troops with a mortality of 10-15% (Sheedy *et al.*, 1954). American efforts, although more extensive than the Russian or Japanese ones, failed to establish the disease in experimental animals or tissue culture (McClure and Ley, 1954). HFRS became the major infectious disease problem for the Korean army after it took control of the demilitarized zone. After 1961 HFRS began occurring more often in the civilian population and farther south than the endemic area previously recognized during the war (Lee, 1982b; Lee and Lee, 1988).

Hemorrhagic fever diseases very similar to HFRS have been reported throughout Europe and Asia. These include Hemorrhagic nephrosonephritis or HFRS in the Soviet Union (Smorodintsev *et al.*, 1944; Smorodintsev *et al.*, 1959), Songo fever or epidemic hemorrhagic fever (EHF) in China (Ishii *et al.*, 1942; Kasahara *et al.*, 1944; Nakazawa *et al.*, 1966), nephropathia epidemica (NE) in Scandinavia (Myhrham, 1951), epidemic nephritis or EHF in Eastern Europe (Gajdusek, 1962; Gaon *et al.*, 1986) and EHF in Japan (Tamura, 1964). War nephritis, which had symptoms similar to HFRS was reported among British soldiers in World War I and in Northern armies during the American Civil War (Abercrombie, 1916; Bradford, 1916; Lee, 1982b).

In 1982 the World Health Organization (WHO) recommended that all the above diseases with different names be referred to as "hemorrhagic fever with renal syndrome (HFRS)" (World Health Organization, 1983).

2. ETIOLOGY

Virus Isolation

A major breakthrough in the study of HFRS throughout the world was achieved in 1976 with the isolation of the etiologic agent of KHF from the lung tissue of the striped field mouse *Apodemus agrarius* (Lee and Lee, 1977; Lee *et al.*, 1978). This virus is now officially known as Hantaan virus (Karabatos, 1985). The prototype Hantaan virus strain 76-118 (Hantaan) was adapted to Fisher and Wistar rats (Lee *et al.*, 1981c) and subsequently adapted to the continuous cell lines; A549 (human lung carcinoma cells) and the E6 clone of Vero 76 cells (a clone of African green monkey kidney cells) (French *et al.*, 1981; McCormick *et al.*, 1982). Adaptation to cell culture was quickly followed by an immunofluorescent antibody assay (IFA) for serological diagnosis of infection which has identified Hantaan virus or antigenically related viruses throughout the world in several genera of rodents and insectivores (van der Groen *et al.*, 1986).

Hantaan and related viruses were identified as members of the Bunyaviridae family on the basis of their morphological and biochemical characteristics (McCormick *et al.*, 1982; White *et al.*, 1982; Hung *et al.*, 1983a 1983b, 1985; Schmaljohn and Dalrymple, 1983; Schmaljohn *et al.*, 1983, 1985; Elliott *et al.*, 1984). Viruses classified in the Bunyaviridae family have the following characteristics: virus particles are spherical, 90-100 nm in diameter; are enveloped with glycoprotein surface projections; virions contain three unique segments of single-stranded negative-sense RNA in the form of circular ribonucleoprotein complexes and a transcriptase enzyme (Elliott, 1990). The viruses replicate in the

cytoplasm and mature by budding into smooth-surface vesicles in or near the Golgi region (Bishop *et al.*, 1980). The presence of a tripartite single-stranded RNA genome with a negative polarity in Hantaan and related viruses was particularly strong evidence for classification of hantaviruses in the Bunyaviridae family since this is the only animal virus family known to possess an RNA genome with three segments (Schmaljohn *et al.*, 1983). Complement fixation and neutralizing tests using monovalent and polyvalent antisera prepared against 172 bunya and bunya-like viruses revealed that there was no cross-reactivity between Hantaan virus and viruses in the other four Bunyaviridae genera (*Bunyavirus*, *Nairovirus*, *Phlebovirus* and *Uukuvirus*) (Lee and van der Groen, 1989). Viruses in the other four Bunyaviridae genera are transmitted by biting arthropods (mosquitoes, gnats, ticks, etc.) (Bishop and Shope, 1979). In contrast, Hantaan and related viruses are transmitted by several genera of rodents and insectivores (van der Groen *et al.*, 1986). The 3'-terminal nucleotide sequences of the three genomic RNA segments is specific for each genus in the Bunyaviridae family (Table 1) (Clerx-van Haaster and Bishop, 1980; Obijeski *et al.*, 1980; Parker and Hewlett, 1981; Clerx-van Haaster *et al.*, 1982a, 1982b). The 3'-terminal nucleotide sequence of the RNA segments of Hantaan and related viruses differed from the corresponding sequences of the other four Bunyaviridae genera (Schmaljohn and Dalrymple, 1983). Because of these differences between Hantaan and related viruses and viruses in the other four Bunyaviridae genera it was proposed that Hantaan and related viruses be classified in a new Bunyaviridae genus. The genus name *Hantavirus* was proposed and has been accepted by the International Committee on Taxonomy of Viruses (The Executive Committee on Taxonomy of Viruses,

Table 1. Genus specific 3' terminal consensus nucleotide sequences of Bunyaviridae vRNA segments.

Genus	Terminal sequence
<i>Bunyavirus</i>	3'-UCAUCACAUGA
<i>Nairovirus</i>	3'-AGAGAUUCU
<i>Phlebovirus</i>	3'-UGUGUUUC
<i>Uukuvirus</i>	3'-UGUGUUUCU
<i>Hantavirus</i>	3'-AUCAUCAUCUG

1987).

After the first prototype *Hantavirus* was isolated other hantaviruses antigenically similar to the prototype virus were also isolated from patients with KHF in Korea (Lee and Lee, 1977; Lee *et al.*, 1978), and EHF in China (Yu-tu, 1983) and the Far Eastern Soviet Union (Tkachenko *et al.*, 1983, 1984; Dantas *et al.*, 1987).

Between 1981 and 1983, 67 *Hantavirus* strains were isolated from different rodent genera and locations in China (Yen *et al.*, 1984). Several hantaviruses have been isolated from *Apodemus flavicollis*, including the Fojnica and Pletvice strains in Yugoslavia (Gligic *et al.*, 1986). These viruses were isolated from areas where severe forms of HFRS had been observed. The Porogia strain was isolated from a patient with severe HFRS in Greece (Antoniadis *et al.*, 1987).

Puumala virus, the etiologic strain of mild HFRS, NE, was isolated from the bank vole *Clethrionomys glareolus* in Sweden (Yanagihara *et al.*, 1984b, 1984c; Niklasson and LeDuc, 1984), in the European USSR (Tkachenko *et al.*, 1983; Tkachenko *et al.*, 1984; Gavrilovskaya *et al.*, 1983) and in Yugoslavia (Gligic *et al.*, 1986).

Five hantaviruses were isolated in Belgium all from *C. glareolus* (Yanagihara *et al.*, 1984b; van der Groen *et al.*, 1983) in an area with clinical cases of mild HFRS.

In the United States the hantaviruses Prospect Hill and Leaky were isolated from the meadow vole *Microtus pennsylvanicus* and from *Mus musculus* respectively (Lee *et al.*, 1982c; Lee *et al.*, 1985a; Back *et al.*, 1988).

Several hantaviruses have been isolated from *Rattus* species, in Korea (Lee *et al.*, 1982a), in China (Song *et al.*, 1983), in Brazil (LeDuc *et al.*,

1985), in Japan (Sugiyama *et al.*, 1984; Arikawa *et al.*, 1985b) and in the United States (LeDuc *et al.*, 1984; Tsai *et al.*, 1985; Childs *et al.*, 1988) and some have been associated with disease.

Hantaviruses have also been isolated from laboratory rats in Japan (Kitamura *et al.*, 1983) and Korea, and from rat tumors in Japan (Yamanishi *et al.*, 1983) and England (Lloyd and Jones, 1986).

Antigenic Relationships between Hantaviruses

Hantaviruses have presently been divided into four antigenically distinct serotypes by the immunofluorescent antibody, immune adherence hemagglutination, hemagglutination inhibition, and plaque reduction neutralization tests (Lee *et al.*, 1985b; Sugiyama *et al.*, 1987, Arikawa *et al.*, 1989). Each antigenically distinct serotype was isolated from a different rodent genus and the serotype of the rodent isolate was dependent on the reservoir host genus and not the geographical region in which the virus was isolated (Lee *et al.*, 1985b, Arikawa *et al.*, 1989). Serotypes were suggested based on the prototype virus of each major rodent genus. Serotype 1 included *Apodemus*-derived strains, serotype 2 included *Rattus*-derived strains, serotype 3 included *Clethrionomys*-derived strains, and serotype 4 included *Microtus*-derived strains (Lee *et al.*, 1985b; Yanagihara and Gajdusek, 1987).

There is some evidence however, that there may be other serotypes not yet fully characterized (Lee *et al.*, 1985b).

Virion Morphology

All other Bunyaviridae are spherical with a mean diameter of 95 nm varying between 75 and 115 nm (Elliott, 1990). Hantaviruses however, are generally larger, with a mean diameter of 122 nm and also have a greater variation in size (75 nm to 200 nm) (Hung *et al.*, 1983b, 1985).

Hantaviruses are also more polymorphic than other Bunyaviridae in that elongated and rod forms have been observed (Hung *et al.*, 1985).

Like other Bunyaviridae particles the *Hantavirus* particle consists of four structural proteins: two internal proteins, the transcriptase component (L protein) with a relative molecular mass (M_r) of approximately 200 K, and the nucleocapsid protein (N) with a M_r of approximately 50-53 K, and two surface glycoproteins, designated as G1 and G2 with M_r 's of approximately 68-72 K and 56-57 K respectively, that are inserted in the viral membrane (Schmaljohn *et al.*, 1983; Schmaljohn and Dalrymple, 1983, 1984; Elliott *et al.*, 1984; Schmaljohn *et al.*, 1986a; 1986b).

Bunyaviridae have a ribonucleoprotein core composed of three circular nucleocapsids (Bouloy *et al.*, 1973/74; Pettersson and von Bonsdorff, 1975; Samso *et al.*, 1976; Obijeski *et al.*, 1976b; Hewlett *et al.*, 1977; Pardigon *et al.*, 1982). It has been demonstrated that hantaviruses also have three separate nucleocapsids (Schmaljohn *et al.*, 1983) but it has not been determined if they are circular. The Bunyaviridae nucleocapsids are composed of three single-stranded RNA species which form 1-2% of the particle by weight and each of these segments is associated with N protein (2100 molecules/particle) and L protein (25 molecules/particle) (Obijeski *et al.*, 1976a).

The *Hantavirus* ribonucleoprotein core is surrounded by a membrane bilayer approximately 4 nm thick (McCormick *et al.*, 1982; White *et al.*, 1982; Hung *et al.*, 1983a, 1983b). The viral membrane bears two surface glycoproteins which give the virus a knobby appearance (McCormick *et al.*, 1982; White *et al.*, 1982; Hung *et al.*, 1983a, 1983b, 1985). Ultrastructural studies using negative-stain electron microscopy revealed

Hantaan virus had a surface structure composed of a grid-like pattern of morphologic subunits not seen in any other Bunyaviridae or any other animal viruses (Martin *et al.*, 1985). Bunyaviridae do not encode an internal matrix protein so the virion structure is thought to be stabilized by direct interaction between the internal nucleocapsids with the membrane or with the cytoplasmic domain of the inserted glycoproteins (Pettersson and von Bonsdorff, 1987; Talmon *et al.*, 1987).

Hantavirus Morphogenesis

Generally, maturation of Bunyaviridae occurs by budding into smooth vesicles at or near the Golgi complex (Lyons and Heyduk, 1973; Murphy *et al.*, 1973; Kuismanen *et al.*, 1982, 1984; Smith and Pifat, 1982). During maturation the glycoproteins accumulate in the Golgi complex which causes a progressive vacuolization (Kuismanen *et al.*, 1984). This effect is mediated only by the glycoproteins (Gahmberg *et al.*, 1986a). Accumulation of the viral glycoprotein in the Golgi does not block the ability of the Golgi to glycosylate and transport authentic cell glycoproteins (Gahmberg *et al.*, 1986b). Although some G1 protein has been detected on the surface of infected cells, virions bud into the Golgi vesicles where they are transported to the cell surface and released by exocytosis (Elliott, 1990). Expression of Hantaan virus and *Phlebovirus* proteins in vaccinia virus vectors indicates that Golgi targeting is an inherent property of the glycoproteins (Matsuoka *et al.*, 1988; Pensiero *et al.*, 1988). This Golgi targeting signal however, has not been determined. Bunyaviridae maturation, including that of hantaviruses, can be inhibited by the monovalent ionophore monensin (Cash, 1982; Kuismanen *et al.*, 1985; Schmaljohn *et al.*, 1986a) which inhibits intracellular transport of membrane glycoproteins by blocking the release of secretory vesicles from

Golgi membranes and also interferes with fusion of Golgi vesicles with the plasmalemma (Tartakoff and Vassalli, 1978). Morphological studies on hantaviruses employing thin-section and immunoelectron microscopy have indicated that hantaviruses mature in cisternae of the endoplasmic reticulum in contrast to other viruses in the Bunyaviridae (Hung *et al.*, 1983a, 1983b, 1985). It appears that *Hantavirus* morphogenesis is more complex than that of other genera in the Bunyaviridae family, in that little or no association with the Golgi complex was observed, budding through cytoplasmic vesicles was rarely observed and large viral inclusion bodies were observed (Hung *et al.*, 1983b, 1985, 1987). Three types of inclusion bodies were found in infected cells; granular, granulofilamentous, and filamentous (Hung *et al.*, 1987). The granular type were spherical and 500-2,000 nm in diameter. The most common were the granulofilamentous type which were pleomorphic and measured 2,500-7000 nm. The least common were the filamentous type of inclusion body which occasionally spanned the entire length of the infected cell. Inclusion bodies were found only in the cytoplasm of infected cells and were often associated with rough endoplasmic reticulum and Golgi cisternae (Hung *et al.*, 1987). Immuno-staining revealed these inclusion bodies were composed of viral antigen and ultrastructurally it was determined these inclusion bodies were either virus aggregates or accumulations of virus-related precursors (Hung *et al.*, 1987). Another feature of *Hantavirus* infected cells was the presence of a viral antigen layer on infected cells (Hung *et al.*, 1985). This viral antigen layer was approximately 40 nm thick and was found on the inner surface of membranous vesicles and on invaginated cavities of plasma membranes (Hung *et al.*, 1985).

Genome Organization and Viral Proteins

Hantaan virus was shown to have a tripartite single-stranded RNA genome with a negative polarity (Schmaljohn and Dalrymple, 1983; Schmaljohn *et al.*, 1983). The three genomic RNA segments, large (L), medium (M), and small (S) have molecular weights of approximately 2.2, 1.3 and 0.5 X 10⁶, respectively (Yoo and Kang, 1987a). The results of cDNA cloning and nucleotide sequence determination of various genome segments has yielded precise information on their size and coding capacity.

The L RNA segment of two hantaviruses, Hantaan virus and Seoul virus strain 80-39 (Seoul) have been cloned and the nucleotide sequence determined (6,530 bases) (Schmaljohn, 1990; Antic *et al.*, 1991b). The Hantaan virus and Seoul virus L RNA segments can encode, in the viral complementary sense, either 2,150 or 2,151 amino acids respectively (Schmaljohn, 1990; Antic *et al.*, 1991b). These proteins have calculated M_r's of approximately 247 K which is similar in size (>200 K) to the L protein (polymerase) detected in purified Hantaan virus particles (Elliott *et al.*, 1984). The nucleotide sequence of the L segment of only one other virus in the Bunyaviridae family has been reported: Bunyamwera virus, a member of the *Bunyavirus* genus (Elliott, 1989). The L protein potentially encoded by the Bunyamwera L RNA segment was shown to have a small region with amino acid similarity to part of the PB1 polymerase protein of Influenza virus (Elliott, 1989). The Seoul virus polymerase protein was also similar to the Bunyamwera virus polymerase protein and Influenza virus PB1 polymerase protein in a small region (46 amino acids), but not to the polymerase proteins of LCMV, measles virus, Newcastle disease virus, parainfluenza virus, respiratory syncytial virus,

Sendai virus, rabies virus or VSV (Antic *et al.*, 1991b). Work on the *in vitro* transcriptase activity in Hantaan virus revealed that the polymerase (L) protein was an RNA-dependent RNA polymerase absolutely dependent on the presence of manganese divalent cations (Schmaljohn and Dalrymple, 1983).

The M RNA segment of at least one disease causing *Hantavirus* within serotypes 1, 2 and 3 has been cloned and the nucleotide sequence determined. The Hantaan virus (serotype 1), SR virus (serotype 2), Biken 1 virus (B-1) (serotype 2), Seoul virus (serotype 2) and Hällnäs virus (serotype 3) M RNA segments were shown to consist of between 3,616 and 3,682 bases (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Izegawa *et al.*, 1990; Antic *et al.*, 1991a). These virus M RNA segments contain single long open reading frames (ORF), in the complementary sense, which can encode a glycoprotein precursor consisting of 1136-1148 amino acids with a predicted M_r of approximately 126 K (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Izegawa *et al.*, 1990; Antic *et al.*, 1991a). This precursor is cleaved into the G1 and G2 proteins. Amino acid sequencing of the N-termini of the Hantaan virus G1 and G2 proteins established the gene order as 5'-G1-G2-3' (Schmaljohn *et al.*, 1987b). Amino acid sequencing also demonstrated that Hantaan virus and SR virus have signal peptides of 18 and 16 amino acids respectively, preceding the G1 protein (Schmaljohn *et al.*, 1987b; Arikawa *et al.*, 1990). Asparagine-linked sugars are associated with both the G1 and G2 proteins and the majority are of the high mannose type (Elliott *et al.*, 1984; Schmaljohn *et al.*, 1986a). The M_r 's of the unglycosylated G1 and G2 proteins are approximately 62 K and 54 K respectively, whereas the glycosylated G1

and G2 proteins migrate between 68 K to 72 K and 56 K to 57 K respectively (Elliott *et al.*, 1984; Schmaljohn *et al.*, 1986a). The M_r of the unglycosylated G1 protein (62 K), estimated on gels, is smaller than the calculated M_r of this protein (70.3 K) as determined by its amino acid composition. To explain this discrepancy it was suggested that there is a small intergenic region, which is removed by cleavage, between the carboxy terminus of the G1 protein and the amino terminus of the G2 protein (Schmaljohn *et al.*, 1987b). The Hantaan virus envelope glycoproteins were demonstrated to have hemagglutinating activity (Tsai *et al.*, 1984; Okuno *et al.*, 1986) and can induce cell fusion at low pH (Arikawa *et al.*, 1985a). It is not presently known however, whether the fusion property is associated with G1, G2 or both. Work with monoclonal antibodies (MAbs) has identified neutralizing sites in both the G1 and G2 proteins and whereas all neutralizing MAbs inhibit hemagglutination some MAbs to G2 inhibit hemagglutination but do not neutralize virus (Yamanishi *et al.*, 1984; Dantas *et al.*, 1986; Arikawa *et al.*, 1989). Comparison of seven hantaviruses using a radioimmunoprecipitation assay demonstrated that G1 was the least cross-reactive protein and the G2 protein was only weakly cross-reactive (Shesberadaran *et al.*, 1988).

Viruses in the Bunyaviridae family do not all use the same M RNA coding strategy. The M coding strategy of hantaviruses is similar to that of viruses in the *Uukuvirus* genus in that two glycoproteins are generated from the glycoprotein precursor. However, the precursor protein of viruses in the *Bunyavirus* and *Phlebovirus* genera are cleaved to generate a third protein termed NS_m which is non-structural (Gentsch and Bishop, 1979; Fuller and Bishop, 1982; Elliott, 1985; Collett *et al.*, 1985; Ihara *et al.*, 1985; Battles and Dalrymple, 1988; Takehara *et al.*, 1989). The

precursor protein containing these proteins could not be detected in infected cells suggesting cleavage is a cotranslational event (Pennington *et al.*, 1977; Lees *et al.*, 1986). In bunyaviruses the M RNA segment, specifically the G1 protein, has been identified as a major determinant of virulence and infectivity (Beaty *et al.*, 1981, 1982; Shope *et al.*, 1981; Gonzalez-Scarano *et al.*, 1985, 1988; Janssen *et al.*, 1986; Sundin *et al.*, 1987). It has not been determined if this is also true for hantaviruses.

The S RNA segments of at least one disease causing *Hantavirus* within serotypes 1, 2 and 3 has also been cloned and the nucleotide sequence determined (Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990). The Hantaan virus (serotype 1), SR virus (serotype 2), and Hällnäs virus (serotype 3) S RNA segments were shown to consist of between 1,696 and 1,785 bases (Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990). These virus S RNA segments contain a single long ORF, in the complementary sense, which can encode a protein with a predicted M_r of 48 K to 49 K which is almost identical to the M_r of the actual Hantavirus N protein (50-53 K) (Schmaljohn and Dalrymple, 1983; Elliott *et al.*, 1984; Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990). Expression of the Hantaan virus S RNA segment by a recombinant baculovirus demonstrated that if the first AUG initiation codon in the ORF was removed no N protein was synthesized (Schmaljohn *et al.*, 1988b). The *Hantavirus* N protein is the major cross-reactive antigen between serotypes and this presumably reflects areas of sequence conservation on the S RNA segments detected with cross-hybridization studies (Schmaljohn *et al.*, 1987a; Sheshberadaran *et al.*, 1988).

Coding strategy of the S RNA segment varies between genera in the

Bunyaviridae family (Bishop, 1985; Elliott, 1990). Viruses in the *Bunyavirus* and *Phlebovirus* genera encode an N protein and a non-structural (NS_S) protein with their S RNA segments. However, the coding strategies of the S segments differ between these genera. Members of the *Bunyavirus* genus use overlapping ORF's to encode a 19-26 K N protein and a 10-12 K NS_S protein, whereas viruses in the *Phlebovirus* and *Uukuvirus* genera encode a 26-28 K N protein and a 29-32 K NS_S protein using an ambisense coding strategy (Bishop, 1985; Simons *et al.*, 1990). The function of these NS_S proteins is not presently known. The S RNA segments of viruses in the *Hantavirus* and *Nairovirus* genera appear to only encode an N protein with a M_r of approximately 50 K (David-West, 1974; Clerx and Bishop, 1981; Clerx *et al.*, 1981; Foulke *et al.*, 1981; Cash, 1985; Watret and Elliott, 1985; Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990; Elliott, 1990).

3. EPIDEMIOLOGY

Distribution

Seroepidemiological surveys have revealed that hantaviruses are widely distributed throughout the world, as demonstrated by the presence of antibodies against hantaviruses in sera from humans, field rodents, urban rats and laboratory rats (Lee and Antoniadis, 1981; Lee *et al.*, 1981d; Lee, 1982b; Lee *et al.*, 1982b; Lee *et al.*, 1984; LeDuc *et al.*, 1986; Lee, 1986; van der Groen *et al.*, 1986; Yanagihara and Gajdusek, 1987; LeDuc, 1987).

There are at least three forms of HFRS; severe, moderate, and mild, and severity of the disease is at least partially dependent on the serotype of the etiologic agent (Table 2) (Lee and van der Groen, 1989). Serotype

Table 2. Antigenically recognized serotypes within the *Hantavirus* genus.

Serotype	Virus	Host	Disease
1	Hantaan 76-118	<i>Apodemus agrarius</i>	severe HFRS
2	Sapporo rat 11	<i>Rattus norvegicus</i>	moderate HFRS
3	Hällnäs	<i>Clethrionomys glareolus</i>	mild HFRS (NE)
4	Prospect Hill	<i>Microtus pennsylvanicus</i>	non-pathogenic

1 includes *Apodemus*-derived strains, like Hantaan virus strain 76-118 (Hantaan), which are associated with the severe form of HFRS (Lee *et al.*, 1978). Serotype 2 includes *Rattus*-derived strains, like Sapporo rat virus strain 11 (SR), which are associated with a moderate although potentially still fatal form of HFRS (Lee *et al.*, 1982a; Kitamura *et al.*, 1983). The severe and moderate forms of HFRS are common in Asian countries. In the Republic of Korea there are several hundred cases a year in rural and urban areas with a case fatality rate of approximately 5% (Lee, 1988b; Lee and van der Groen, 1989). In China, there are an estimated 50,000 to 100,000 cases a year with mortality rates of 5-20% depending on the province (Jiang, 1983; Song *et al.*, 1984; Lee, 1988a). Serotype 3 includes *Clethrionomys*-derived strains, like Puumala virus strain Hällnäs B1 (Hällnäs), and is associated with the milder form of HFRS (NE) (Brummer-Korvenkontio *et al.*, 1980). The majority of cases caused by Puumala virus in Europe are mild HFRS (NE) with a mortality rate of approximately 0.2% (Settergren *et al.*, 1988a). Serotype 4 includes *Microtus*-derived strains, like Prospect Hill virus strain Prospect Hill I (PH), that have not been linked to any disease in man (Lee *et al.*, 1982c, 1985a; Yanagihara *et al.*, 1984a, 1987).

Epidemiologic Type

There are three epidemiologic types of HFRS that are determined by the location of the disease outbreak and the reservoir host of the etiologic agent; rural, urban, and animal room (Lee *et al.*, 1982b).

The reservoir in rural endemic areas of Asia are field rodents, particularly *Apodemus* species (Lee *et al.*, 1981a). Although field rodents they will invade homes during the snowy season. There are two seasonal peaks of rural type HFRS, late spring and fall, when numbers of

Hantavirus infected *Apodemus* mice are usually high (Lee, 1982a, 1982b). Victims are usually 20-50 years of age and are farmers or soldiers working or stationed in the field. European HFRS is also of the rural type and the main reservoir in Scandinavia is *Clethrionomys glareolus* (Niklasson *et al.*, 1987; Niklasson and LeDuc, 1987; Settergren *et al.*, 1988b). In the rest of Europe other field rodents are the reservoir hosts (van der Groen *et al.*, 1986; Tkachenko *et al.*, 1983, 1987). Rural types of HFRS are caused primarily by hantaviruses in serotypes 1 and 3.

The source of urban cases of HFRS is house rats (*Rattus*) (Lee *et al.*, 1980; Chumakov and Garvrilovskaya, 1980; Lee *et al.*, 1982a; Song *et al.*, 1984; Sugiyama *et al.*, 1984). Since 1983 there have been approximately 100 cases/year of urban HFRS in metropolitan areas of Seoul and other large Korean cities (Lee, 1989). In Japan there were several cases in the 1960's and 1980's in urban areas of Osaka (Tamura, 1964; Morimoto *et al.*, 1986). In China mild cases of HFRS have occurred in several cities (Lee and van der Groen, 1989). There are cases of urban HFRS throughout the year, but they are most frequent in fall and early winter seasons in temperate zones (Lee and van der Groen, 1989). Urban cases of HFRS are usually caused by hantaviruses in serotype 2, particularly Seoul virus (Tamura, 1964; Lee *et al.*, 1980; Shortridge *et al.*, 1987).

The reservoir for animal room cases is colonized experimental rats. This was demonstrated by the presence of antibody and isolation of a *Hantavirus* from experimental rats (Lee and van der Groen, 1989). By 1985 there had been 16 cases of laboratory infections in Korea and 126 in Japan, with 1 fatality (Lee *et al.*, 1986a; Kawamata *et al.*, 1987). Laboratory infections have also been reported in Korea, Belgium, The Netherlands, France and Great Britain (Umenai *et al.*, 1979; Lee and

Johnson, 1982; Lloyd *et al.*, 1984; Osterhaus *et al.*, 1984; Dournon *et al.*, 1984). Laboratory infections can occur at anytime but are most frequent in winter when the air is dry. Seoul virus (serotype 2) is a common cause of laboratory infections.

Transmission

The infection pattern indicates that hantaviruses are spread by aerosols. Large quantities of virus are excreted from the urine, saliva and feces of infected mice (*Apodemus agrarius*) and bank voles (*Clethrionomys glareolus*) (Lee *et al.*, 1981b; Yanagihara, 1985). Excretion of Hantaan virus from mouse saliva and feces could be detected for one month and in urine for up to a year. Horizontal transmission of Hantaan virus has been demonstrated in *Apodemus* mice and is transmitted primarily via the respiratory tract. There is no evidence of direct human-to-human transmission of the virus.

Reservoirs

The reservoir host for Hantaan virus in rural areas of Korea is *Apodemus agrarius*; in Finland and west of the Ural mountains the reservoir host of Puumala virus is *Clethrionomys glareolus* and field mice; in urban areas of Korea, Japan and China *Rattus* and *Rattus norvegicus* are the reservoirs of Seoul virus. Colonized experimental rats are also a reservoir for Seoul virus. Antigen from hantaviruses has been detected in 16 different rodent species and 4 different insectivore species (Tkachenko *et al.*, 1983; Gavriovskaya *et al.*, 1983; van der Groen *et al.*, 1986).

Vectors

The identification of hantaviruses as a new genus in the Bunyaviridae family initially suggested that it was an arthropod-transmitted virus like all other members of this family. However, the long term viruria and

aerosol transmission suggest that hantaviruses do not require arthropod vectors (Traub *et al.*, 1954; Umenai *et al.*, 1979; Lee *et al.*, 1981a; Bishop *et al.*, 1980). However, it has been hypothesized that Hantaan virus can be transmitted from ectoparasites on *Apodemus* mice (Kasahara *et al.*, 1944). Hantaan virus has been recovered from *Haemolaelaps glasgowi* and *Eulaelaps stabulans* collected from *Apodemus* mice in China. It was also shown that these mites and *Ornithonyssus bacoli* and *Haemolaelaps casalis* could be infected by feeding on infected rodents (Tsai, 1987). Whether these mites can transmit hantaviruses is not known, however, they are not required for transmission since horizontal transfer can occur between clean mice that have not been ectoparasitized. Also hantaviruses have not been isolated from any arthropods or successfully grown in insect tissue culture cell lines.

4. CLINICAL MANIFESTATIONS, PATHOGENESIS, SERO-DIAGNOSIS, TREATMENT AND VACCINE DEVELOPMENT

Clinical Manifestations

Before isolation of the etiologic agent of HFRS which allowed serodiagnosis, this disease could only be identified on clinical grounds. The major manifestations are fever, prostration, vomiting, proteinuria, hemorrhagic phenomena, shock and renal failure (Earle, 1954). The incubation period is usually 14-21 days but can vary between 4 and 42 days. In moderate and mild cases of HFRS the hemorrhagic phenomena and proteinuria are often absent.

The severe form of HFRS can be divided into five distinct phases; febrile, hypotensive, oliguric, diuretic, and convalescent (Smadel, 1953;

Earle, 1954; Gajdusek, 1962). In moderate cases of HFRS the five phases are usually of shorter duration. In mild cases of HFRS diagnosis is very difficult on clinical grounds because renal manifestations dominate the hemorrhagic features (Lähdevirta, 1971, 1982; Settergren *et al.*, 1988a).

The febrile phase lasts 3-7 days and is characterized by a high fever (40°C), general malaise, weakness and generalized myalgia. There is extensive retroperitoneal or peritoneal edema resulting from extravasation of plasma due to increased capillary permeability. Near the end of this phase conjunctival hemorrhage occurs and fine petechiae are observed in folds of the face, neck, soft palate and anterior chest wall.

The hypotensive phase has a sudden onset and can last from hours to 2 days. Classical signs of shock including tachycardia, narrowed pulse pressure, hypotension and cold and clammy skin are observed. In mild cases blood pressure returns to normal in a few hours but in severe forms hypotension manifests as clinical shock and one-third of deaths are associated with irreversible shock in this phase. The pathogenic mechanism of hypotension likely results from plasma loss from the vascular system due to capillary dilation, decreased cell mass and decreased arterial tonicity.

The oliguric phase lasts from 3-7 days. Blood pressure begins to return to normal but about 60% of patients become hypertensive because of their hypervolemic state. Patients suffer severe nausea and vomiting. The bleeding becomes more severe and conjunctival, cerebral and gastrointestinal hemorrhaging can occur. Approximately 50% of HFRS fatalities occur during this phase.

The diuretic phase can last days or weeks and clinical recovery is initiated by diuresis. Diuresis is sometimes delayed by dehydration and

electrolyte imbalance. Urine output will vary between 3-6 liters a day but urine volume, and length of this phase is related to the severity of the disease.

The convalescent phase requires 2-3 months and while mild anemia can persist, recovery is the rule. Glomerular filtration rate, renal blood flow and urinary concentration ability return to about 70% of normal within 6 months.

Pathogenesis

The pathogenesis of a hantavirus infection in humans is not understood. A primate model for this disease does not exist although Yanagihara *et al.* (1988) demonstrated that some cynomolgus monkeys (*Macac fascicularis*) and a chimpanzee (*Pan troglodytes*) showed mild symptoms of acute nephropathy when infected with PH virus. The mechanism(s) by which hantaviruses produce capillary dysfunction is unknown.

Human material obtained from autopsied patients or biopsied specimens demonstrated hantavirus antigen in the pituitary, brain, spleen, kidney and liver. The kidney subcortical medullary vessels were congested in Chinese HFRS patients dying in the febrile or hypotensive phases but no tubular damage was observed. As the disease progressed vascular congestion occurs in the intertubular spaces and was followed by progressive tubular damage (Yanagihara and Gajdusek, 1987).

Several groups have attempted to infect various rodents with hantaviruses. A lethal suckling mouse model for hantavirus infection was developed (Tsai *et al.*, 1982; Yamanouchi *et al.*, 1984; Kurata *et al.*, 1983; Kim and McKee, 1985; McKee *et al.*, 1985) and ribavirin therapy showed limited success in suckling mice (Huggins *et al.*, 1986). It was shown that

both humoral and cellular immunity were required to protect mice against Hantaan virus infection, and T cells possessing L3 TQ4 Lyt 2⁺ markers were especially important in clearing virus *in vivo* (Asada *et al.*, 1987, 1988, 1989; Tamura *et al.*, 1989). Cross-reactive studies with cytotoxic T lymphocytes (CTLs) suggests that CTLs may recognize epitopes common to all hantaviruses whereas epitopes that elicit neutralizing antibodies were shown to be mainly specific to each virus (Asada *et al.*, 1989; Zhang *et al.*, 1989). Using a hamster challenge model it was demonstrated that to induce protective high titer antibody responses against Hantaan virus, an animal required exposure to both the G1 and G2 envelope glycoproteins (Schmaljohn *et al.*, 1990).

Serological Testing

Serological diagnosis of HFRS is the method of choice and can be made by demonstrating a rise in titer of specific immunofluorescent antibodies (IFA) and neutralizing antibodies against hantaviruses. IFA and neutralizing antibodies appear during the first week of disease and peak by the end of the second week. IFA antibodies can be detected with *Apodemus* lung sections (Lee *et al.*, 1978) or A594 and Vero E6 cells (van der Groen and Beelaert, 1985) infected with Hantaan virus. Several methods such as hemagglutination inhibition, complement fixation, immune adherence hemagglutination, enzyme-linked immunosorbent assay (ELISA) and solid-phase radioimmunoassay are also now available (Yanagihara and Gajdusek, 1987). To identify the specific hantaviral agent, plaque reduction neutralization with reference prototype hantaviruses is the recommended technique (Tanishita *et al.*, 1984; Tsai *et al.*, 1984; Schmaljohn *et al.*, 1985; Arikawa *et al.*, 1985; Lee *et al.*, 1985b; Takenaka *et al.*, 1985).

Treatment

The treatment of patients is mainly supportive since there is no specific measure or drug treatment for HFRS. However, HFRS patients in China and Korea were recently treated with ribavirin within seven days after onset of fever and this reduced mortality and shortened the course of the illness (Lee and van der Groen, 1989). Hemodialysis during the oliguric phase has also proved useful (Cohen, 1982; Lee, 1982b).

Vaccine Development

Production of a vaccine for HFRS has been a definite goal, but development of a *Hantavirus* vaccine has been hampered by the slow growth and low-titer replication of hantaviruses in cultured cells, the requirement for a high level containment facility to grow the virus and the lack of an animal model to test the efficacy of a potential vaccine. Presently, inactivated Hantaan virus-infected mouse or rat brain preparations have been tested in North and South Korea (Yan, 1985; Kim and Ryu, 1988; D. J. Suh, J. W. Song and H. W. Lee, Virus Information Exchg. Newsl. 6, 131). Inactivated Hantaan virus grown in tissue culture is under development and testing in Japan and China (Yamanishi *et al.*, 1988; U. -X. Yu and Z. -Y. Zhe, Virus Information Exchg. Newsl. 6, 131).

5. STATEMENT OF OBJECTIVES

All the hantaviruses that have previously been molecularly characterized cause a human disease, whereas PH virus is known to be non-pathogenic (Yanagihara *et al.*, 1984a, 1987). Therefore, a comparison between pathogenic and non-pathogenic viruses may lead us to map

domain(s) responsible for pathogenesis. Furthermore, sequence comparisons of Asian, European and North American isolates of *Hantavirus* could reveal the evolution of this group of viruses, and PH virus represents one of the North American isolates.

- 1) To develop an assay to monitor the progress of a PH virus infection in tissue culture and determine the best times to harvest the virus and extract its RNA.
- 2) To clone the PH virus M RNA segment and determine its nucleotide sequence.
- 3) To compare the putative gene products of the PH virus M RNA segment with the homologous gene products of disease causing hantaviruses from serotypes 1, 2 and 3.
- 4) To clone the PH virus S RNA segment and determine its nucleotide sequence.
- 5) To compare the putative gene product of the PH virus S RNA segment with the homologous gene product of disease causing hantaviruses from serotypes 1, 2 and 3.
- 6) To express the potential gene product of the second overlapping open reading frame on the viral-complementary S RNA of PH virus, and determine if this gene product is expressed in PH virus infected Vero cells.

PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 *rev* AND *vif* PROTEINS

1. GENERAL DESCRIPTION OF HIV-1

Human Immunodeficiency virus type 1 (HIV-1) has been identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS) and related disorders (Barre-Sinoussi *et al.*, 1983; Broder and Gallo, 1984; Gallo *et al.*, 1984; Popovic *et al.*, 1984; Sarngadharan *et al.*, 1984). HIV-1 displays a high degree of genetic complexity. In addition to encoding the *gag*, *pol* and *env* structural proteins characteristic of all replication-competent retroviruses, HIV-1 encodes at least six other proteins. Two of these proteins *tat* and *rev*, are essential *trans* regulators of viral gene expression, while two other proteins *pr* and *vif* are important for the morphogenesis and release of infectious virions (Dayton *et al.*, 1986; Cullen and Greene, 1990). The roles of the proteins *nef* and *vpr* is not clear and both are dispensable for HIV-1 replication in cell culture (Kim *et al.*, 1989; Ogawa *et al.*, 1989; Cohen *et al.*, 1990b). Recent data suggest however, that *vpr* is a virion structural protein whose expression moderately enhances the rate of viral replication (Ogawa *et al.*, 1989; Cohen *et al.*, 1990a, 1990b). These nine viral proteins are encoded by more than 20 distinct mRNA species that are derived from post-transcriptional processing of the initial full length viral transcript (Muesing *et al.*, 1985; Felber *et al.*, 1990; Robert-Guroff *et al.*, 1990; Schwartz *et al.*, 1990a, 1990b).

2. HIV-1 *rev* PROTEIN

The HIV-1 *rev* protein is 116 amino acids in length and is encoded by two exons put together in a double-spliced mRNA approximately 2 Kb in length (Arya *et al.*, 1985; Feinberg *et al.*, 1986; Sodroski *et al.*, 1986). The *rev* protein is required for expression of the *gag*, *pol*, *env*, *vif* and *vpr* proteins which require unspliced or single-spliced mRNA (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986; Knight *et al.*, 1987; Malim *et al.*, 1988; Sadaie *et al.*, 1988; Terwilliger *et al.*, 1988; Hadzopoulou-Cladaras *et al.*, 1989; Garret *et al.*, 1991). In the absence of *rev* the *gag*, *pol* and *env* proteins are not produced, and the virus is replication defective (Sodroski *et al.*, 1986; Terwilliger *et al.*, 1988).

Function of the HIV-1 *rev* Protein

Expression of *rev* protein during HIV-1 replication causes a switch from small multiply-spliced mRNAs, which encode regulatory proteins, to singly-spliced or unspliced mRNAs, which encode structural proteins, by inducing export of the mRNAs from the nucleus to the cytoplasm before complete splicing can occur (Malim *et al.*, 1988; Chang and Sharp, 1989; Emerman *et al.*, 1989; Felber *et al.*, 1989; Hammariskjold *et al.*, 1989). The *rev* protein also increases the half-life of these larger mRNA species (Felber *et al.*, 1989). The mRNA transport function is caused by *rev* reacting with a cis-acting, highly structured 234 nucleotide target sequence, the *rev* responsive element (RRE), located within the envelope gene of HIV-1 (Rosen *et al.*, 1988; Dayton *et al.*, 1989; Emerman *et al.*, 1989; Felber *et al.*, 1989; Hammariskjold *et al.*, 1989; Malim *et al.*, 1989b). The *rev* protein was shown to react with the RRE by direct binding (Dayton *et al.*, 1989; Zapp and Green, 1989; Heaphy *et al.*, 1990; Malim *et*

al., 1990; Olsen *et al.*, 1990). The regulation of mRNA transport from the nucleus induced by the *rev* protein is dependent on the fact that HIV-1 mRNA species use suboptimal splice sites (Chang and Sharp, 1990).

The HIV-1 *rev* protein is localized in the nucleus (Cullen *et al.*, 1988; Felber *et al.*, 1989) and is specifically associated with nucleoli (Cullen *et al.*, 1988; Kubota *et al.*, 1989; Cochrane *et al.*, 1990; Venkatesh *et al.*, 1990). *Rev* is directed to the nucleus by a basic stretch of amino acids (amino acids 25-50) that is rich in arginine residues (Malim *et al.*, 1989a; Perkins *et al.*, 1990).

The *rev* protein is a phosphoprotein (Hauber *et al.*, 1988; Cochrane *et al.*, 1989b) and is phosphorylated on two serine residues by a specific serine kinase localized in the nucleus (Cochrane *et al.*, 1989b). However, the *rev* protein does not require phosphorylation to be functionally active (Hauber *et al.*, 1988; Cochrane *et al.*, 1989a, 1989b; Malim *et al.*, 1989a).

3. HIV-1 *vif* PROTEIN

The HIV-1 *vif* gene is located between the *pol* and *env* genes as a single exon that could encode a 192 amino acid protein (Ratner *et al.*, 1985; Wein-Hobson *et al.*, 1985). The HIV-1 *vif* protein is encoded by a singly-spliced mRNA of approximately 5 Kb and is *rev* dependent (Garret *et al.*, 1991). Because *vif* is *rev* dependent it is a late gene like *gag*, *pol*, and *env*.

Function of the HIV-1 *vif* protein

Using HIV-1 mutants deficient for *vif* expression it was demonstrated that *vif* was required for the efficient transmission of cell-free virus in culture (Fisher *et al.*, 1987; Strebel *et al.*, 1987). These *vif* deficient

proviruses appear to express all other proteins to normal levels and release virus from the cell at normal efficiency. Although the released virions are morphologically identical to virions released from *vif*⁺ HIV-1 infected cells, they were found to be approximately 1,000 times less infectious (Fisher *et al.*, 1987; Strebel *et al.*, 1987). Interestingly, the absence of *vif* does not appear to affect the efficiency of direct cell to cell spread of HIV-1 (Fisher *et al.*, 1987; Strebel *et al.*, 1987). Strebel *et al.* (1987) demonstrated virus of normal infectivity could be generated by transcomplementation. This suggested that *vif* was required for absorption, penetration, endocytosis, or uncoating and suggests *vif* is virion-associated (Strebel *et al.*, 1987). Presently, *vif* has not been demonstrated to be a virion-associated protein.

Recently the HIV-1 *vif* protein was found to have some amino acid sequence similarity with cysteine proteases (Guy *et al.*, 1991). When the *vif* protein and *env* protein (gp160) were co-expressed in mammalian cells the envelope protein gp120 was released into the medium (Guy *et al.*, 1991). Immunofluorescence and immunogold techniques showed that the *vif* protein was localizing in the Golgi apparatus and on the surface of vesicles (Guy *et al.*, 1991). If a cysteine protease inhibitor was added or gp160 was expressed alone, gp120 was found to accumulate in the cell (Guy *et al.*, 1991). Size analysis of the proteins showed the envelope protein gp41 was approximately 1 K smaller when expressed with *vif* (Guy *et al.*, 1991). Using antisera raised against synthetic peptides, Guy *et al.* (1991) showed that the extreme C-terminus of the gp41 protein was cleaved when expressed with the *vif* protein but only in the absence of the cysteine protease inhibitor. The *env* protein recognizes the CD4 molecule at the cell surface and then enters the cell via pH-independent

membrane fusion involving gp41 (Stein *et al.*, 1987). Therefore, it has been postulated that virions possessing gp41 proteins that have not been correctly processed by *vif* may be unable to enter the cell efficiently (Guy *et al.*, 1991).

4. STATEMENT OF OBJECTIVES

The baculovirus expression vector system used to express the putative NSs protein of PH virus, was employed to express two important regulatory proteins, *rev* and *vif*, of HIV-1. Expressed *rev* and *vif* was used to study the prevalence of antibodies against *rev* and/or *vif* in HIV-1 infected individuals in order to determine the efficacy of these proteins as prognostic markers for the progression of AIDS.

- 1) To express the *rev* and *vif* proteins of HIV-1 to high levels in insect cells using recombinant baculovirus vectors.
- 2) To test sera from HIV-1 infected individuals, with different symptoms of disease, for the presence of antibodies that would recognize the *rev* or *vif* proteins of HIV-1.
- 3) To use partially purified HIV-1 *vif* protein to raise specific antibodies in rabbits that could eventually be used to determine if *vif* is a virion-associated protein.

CHAPTER 2: MATERIALS AND METHODS

1. PROSPECT HILL VIRUS PRODUCTION

Virus, Cells and Media

Prospect Hill (PH) virus (strain Prospect Hill-1), provided by Dr. J.M. Dalrymple (USAMRIID, Frederick, MD), was propagated in Vero E6 cells (ATCC 1008, CRL 1586). Cells were grown and maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) (Dulbecco and Freeman, 1959), supplemented with heat-inactivated fetal bovine serum (FBS) (10%) (v/v) and L-glutamine (2mM) (Gibco, Grand Island, NY) (complete DMEM).

Virus Production and Radiolabelling of Viral RNA

To propagate PH virus, Vero cells in 100 mm tissue culture dishes (Nunc/GIBCO/BRL Life Technologies Inc., Burlington, Ontario) were infected with a multiplicity of infection (MOI) between 0.03 and 0.005 pfu/cell and maintained at 35°C in a humidified Shel-lab incubator (John's Scientific Inc., Toronto, Ontario) with 5% CO₂. The medium on the cells was changed 4 days post-infection and PH virus was harvested 8, 12, and 15 days after infection. Labelling of PH virus RNA was accomplished by adding 100 µCi of [5,6-³H]-uridine (1 mCi/ml, 40-60 Ci/mmol, Amersham, Arlington Heights, IL) to the medium of each plate 4, 8, and 12 days after infection. Tissue culture fluid containing the virus was harvested 8, 12 and 15 days after infection. Tissue culture fluid was clarified by centrifugation for 30 min at 3,000 g at room temperature (Damon/IEC, model HN-S, Needham Heights, MA). Virus particles in clarified tissue culture fluid were pelleted by centrifugation for 2 h at 81,000 g at 4°C in a Beckman SW 28 rotor (Beckman, Cedar Grove, NJ).

Assay for PH Virus Production

Vero E6 cells in 100 mm tissue culture plates were infected with PH virus as described above or mock infected. After a 1 h adsorption at 37°C each culture dish was given a final volume of 10 ml of complete DMEM containing 100 μ Ci of [5,6-³H]-uridine. The tissue culture dishes were incubated at 37°C which was constant for the assay. At 1, 2 and 3 days after infection 1 ml of tissue culture fluid was harvested from each dish and replaced with 1 ml of fresh complete DMEM containing 10 μ Ci of [5,6-³H]-uridine. On day 4 post-infection all of the medium was removed and 1 ml was used in the assay. Each plate was given 10 ml of DMEM containing bovine serum albumin (BSA) (2%) and 100 μ Ci of [5,6-³H]-uridine. Medium was removed and replaced on days 5, 6, 7, 9, 10, 11 and 12 as described for days 1, 2 and 3 except the medium contained 2% BSA instead of 10% FBS. Medium was removed and replaced on day 8 as described for day 4. The 1 ml sample removed each day was centrifuged at 14,000 g for 3 min. Some of the supernatant (800 μ l) was removed, diluted in 10 ml of cold PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂) and centrifuged at 165,000 g for 1 h at 4°C in a SW41 rotor (Beckman). The supernatant was discarded and the pellet was washed by resuspending in 10 ml of cold PBS and centrifuged as before. After discarding the supernatant the pellet was resuspended in 200 μ l of STE (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 0.5% Triton X-100 and mixed with 10 ml of aqueous counting scintillant (ACS) (Amersham). The radioactivity was measured in a liquid scintillation counter (LKB, Model 1214 Rackbeta, Sweden). Duplicate plates were set up for both the PH virus infected sample and mock infected controls. The counts per minute/ml (cpm/ml)

from duplicate cultures were averaged and then plotted.

2. PREPARATION OF RNAs

Extraction and Purification of PH Virus RNA

Virus pellets were resuspended in a total of 450 μ l of STE buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA), then mixed with 450 μ l of 2X NENS buffer (200 mM NaCl, 20 mM EDTA, 100 mM sodium acetate, pH 5.1, 1% SDS) containing 500 μ g/ml of proteinase K (Boehringer Mannheim, Laval, Quebec) and incubated at 37°C for 30 min. Virion RNA was extracted with one volume of phenol-chloroform-isoamyl alcohol (25:24:1) and re-extracted with chloroform-isoamyl alcohol (24:1). If the virion RNA was required for gels or Northern blots it was precipitated by adding three volumes of cold ethanol and stored overnight at -20°C or at -70°C for at least 60 min. The RNA was pelleted by centrifugation in a microcentrifuge at 14,000 g for 30 min at 4°C. However, if the PH virus RNA was to be used for cDNA synthesis it was purified after the extraction steps. The purification of PH virus RNA was based on the procedure of Chirgwin et al. (1979). In a typical experiment, a 2.7 ml solution of PH virus RNA was mixed with 2.918 g of CsCl, and layered on a 1 ml cushion of 5.7 M CsCl buffered with 25 mM Na-acetate (pH 5.5). RNA was pelleted by centrifugation for 18 h in a SW 50.1 rotor (Beckman) at 121,000 g at 25°C. Pelleted RNA was resuspended in H₂O by brief heating at 68°C, and then ethanol precipitated.

Alternatively, the virus pellets were dissolved in a total of 3 ml of 4 M guanidinium thiocyanate stock containing 100 mM 2-mercaptoethanol and buffered with 25 mM Na-citrate, pH 7.0. This was layered on a 1.2

ml cushion of 5.7 M CsCl buffered with 25 mM Na-acetate (pH 5.5) (Chirgwin et al. 1979). The PH virus RNA was pelleted by centrifugation for 12 h in a SW 50.1 rotor at 121,000 g at 20°C. Pelleted RNA was resuspended in H₂O by brief heating at 68°C, and then ethanol precipitated.

Extraction and Purification of Intracellular RNA from PH Virus Infected Cells

Vero E6 C1008 cells were infected with PH virus and maintained as described above. At 8 or 9 days post-infection the cell monolayers were washed once with cold PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂), the cells were scraped off the plates, resuspended in PBS and pelleted by centrifugation at 3,000 g at room temperature (Damon/IEC, model HN-S). Pelleted cells were dissolved in 3 ml of a 4 M guanidinium thiocyanate stock containing 100 mM 2-mercaptoethanol and 25 mM Na-citrate, pH 7.0, and layered on a 1.2 ml cushion of 5.7 M CsCl buffered with 25 mM Na-acetate (pH 5.5). The intracellular RNA was pelleted by centrifugation for 12 h in a SW 50.1 rotor at 121,000 g at 20°C. Pelleted RNA was resuspended in H₂O by brief heating at 68°C, and then ethanol precipitated.

Agarose Gel Electrophoresis of Viral RNA

Viral RNA was prepared for agarose gel electrophoresis by denaturing with a buffer containing 0.9 M deionized glyoxal, 50% DMSO and 10 mM NaH₂PO₄, pH 7.0 at 60°C for 20 min. The RNA was electrophoresed in 1.5% agarose gels prepared in 10 mM sodium phosphate buffer, pH 7.0. Gels were electrophoresed for 4 h at 100 volts with continuous recirculation of the buffer (McMaster and Carmichael, 1977).

For fluorography, the gels were fixed for 1 h in 30% methanol and

10% glacial acetic acid. After fixing, gels were given two 30 min washes in 100% methanol and then impregnated with methanol containing 3% PPO for at least 12 h. The PPO was then precipitated in the gels by soaking in H₂O for 1 h. Gels were then dried with the Bio-Rad Model 583 Slab Dryer (Bio-Rad, Mississauga, Ontario) and exposed to Cronex X-ray film (DuPont, Wilmington, DE) at -80°C (Bonner and Laskey, 1974).

3. BLOTTING AND HYBRIDIZATION OF NUCLEIC ACIDS

Northern Blots

After electrophoresis the RNA was electroblotted onto a GeneScreen Plus membrane (New England Nuclear Research products (NEN), Mississauga, Ontario) in TAE blotting buffer (12 mM Tris, 6 mM Na-acetate, 0.3 mM EDTA, pH 7.5) at 10 volts for 1 h then 40 volts for 2 h. The membrane was placed in 50 mM NaOH for 30 sec to reverse the glyoxal reaction. The membrane was then neutralized by placing in a 1X SSC buffer (150 mM NaCl, 15 mM Na-citrate, 200 mM Tris, pH 7.5) for 30 sec.

Colony Blotting of Bacterial Plasmid DNA

Bacterial transformants were inoculated onto Colony/PlaqueScreen membranes (NEN) on LB plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Bacto-agar) containing 100 µg/ml of ampicillin. After an overnight incubation at 37°C the bacterial colonies were lysed on the membrane with alkali using the method of Grunstein and Hogness (1975).

Dot Blotting of RNA or DNA

RNA or DNA to be blotted was first denatured in 0.5 N NaOH and then neutralized with 1/10 volume of 10 M NH₄-acetate. Aliquots of

different dilutions of the nucleic acid were loaded into wells and vacuum blotted onto a GeneScreen Plus membrane pre-soaked in 1 M NH_4 -acetate containing 0.02 N NaOH using a Bio-Dot vacuum blotter (Bio-Rad). Each well was rinsed twice with 1 M NH_4 -acetate containing 0.02 N NaOH and the membrane was dried at room temperature overnight.

Nick Translation

DNA was nick translated using a modified procedure of Rigby *et al.* (1977). Approximately 1 μg of DNA was incubated in Nick Translation (NT) buffer (50 mM Tris, pH 7.2, 10 mM MgSO_4 , 0.1 mM dithiothreitol (DTT), 50 $\mu\text{g}/\text{ml}$ of BSA). To this mixture was added 30 μmol each of dGTP, dATP, dCTP, dTTP, 100 μCi of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (800 Ci/mmol, Amersham), 440 pg of deoxyribonuclease I and 20 units of *E. coli* DNA polymerase I (Pharmacia Biotechnology, Uppsala Sweden). This solution was then incubated at 15°C for 60 min. The labelled DNA was passed through a Sephadex G-50 column to remove unincorporated nucleotide triphosphates.

Nucleic Acid Hybridization

GeneScreen Plus membranes or Colony/PlaqueScreen membranes were prehybridized in 1 M NaCl, 10% dextran sulfate, 1% SDS and 400 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA. During prehybridization membranes were incubated for at least 6 h at 65°C if DNA was bound to the membrane or 60°C if RNA was bound to the membrane. After prehybridization a specific denatured nick translated DNA probe was added to the solution and hybridization continued for 16 h at the same temperatures described above. Membranes were then washed twice (30 min each) in 2X SSC (300 mM NaCl, 30 mM Na-citrate, 400 mM Tris, pH 7.5) and 0.1% SDS and once (30 min) in 0.1X SSC (15 mM NaCl, 1.5 mM Na-citrate, 20 mM Tris,

pH 7.5) and 0.1% SDS, all at room temperature. A final wash was performed for 30 min in 0.1X SSC and 0.1% SDS at 65°C if DNA was bound to the membrane or 60°C if RNA was bound to the membrane. After air drying membranes were exposed to Cronex X-ray film at -80°C.

4. PLASMID PREPARATION AND ELECTROPHORESIS OF DNA

Small-Scale Isolation of Plasmid DNA (Miniprep)

Bacterial cultures were grown for 5-6 h at 37°C in 2 ml of LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) containing 100 µg/ml of ampicillin. Miniprep isolation of plasmid DNA was done using the alkaline lysis method (Birnboim and Doly, 1979) with modifications by Morelle (1989). Pelleted miniprep DNA was resuspended in RNase A (200 µg/ml) to remove RNA.

Large-Scale Isolation of Plasmid DNA (Maxiprep)

A seed stock of bacterial cultures was first grown for 5-6 h at 37°C in 5 ml of LB medium containing 100 µg/ml of ampicillin. This seed stock was used to inoculate 1 liter of LB medium and incubation was continued at 37°C for 12-16 h. Bacteria were lysed and plasmid DNA extracted using the lysis by alkali method (Maniatis *et al.*, 1982). Closed circular plasmid DNA was purified by centrifugation to equilibrium in CsCl and ethidium bromide (EtBr) gradients in a vTi 65 rotor (Beckman) at 325,000 g for 16 h at 20°C. EtBr was removed from the plasmid DNA by several extractions with H₂O saturated n-butanol. Plasmid DNA was precipitated using standard methods and was quantitated by measuring the Optical Density (OD) at wavelengths of 260 nm.

Agarose Gel Electrophoresis of DNA and Purification of DNA from Agarose Gels

DNA was electrophoresed in agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA samples were prepared for electrophoresis by mixing with a 1/10 volume of DNA loading buffer (445 mM Tris pH 8.0, 445 mM boric acid, 10 mM EDTA, 0.4% Bromophenol Blue, 0.4% Xylene Cyanol FF, 50% glycerol). To photograph the DNA, gels were stained with EtBr (1 µg/ml in H₂O) and visualized with a short-wave ultraviolet (UV) light.

DNA fragments were isolated from agarose gels using a modification of the "freeze squeeze" method (Tautz and Renz, 1983). DNA was visualized with a long-wave UV light in the gel after EtBr staining. DNA bands of interest were cut out from the gel and placed in a 500 µl microcentrifuge tube that had a 21 gauge needle hole in the top and bottom. The gel slice was pelleted at 10,000 g into a decapped 1.5 ml microfuge tube. The agarose in the 1.5 ml tube was mixed with 100 µl of 0.3 M Na-acetate (pH 5.2) and placed in a 500 µl microfuge tube with a 21 gauge needle hole in the top and a 21 gauge needle hole in the bottom plugged with silanized glass wool. After placing this tube in a decapped 1.5 ml microfuge tube the sample was frozen at -70°C for 20 min. After freezing the sample was pelleted at 10,000 g through the glass wool for 5 min. Centrifugation was repeated twice more in fresh 1.5 ml tubes for 4 min and 3 min. The eluted samples were pooled, extracted three times with H₂O saturated n-butanol, and the DNA was ethanol precipitated.

Preparation of Competent Cells

The JM101 strain of *E. coli* (Yanisch-Perron *et al.*, 1985), provided by Dr. P. Lau (Biotechnology Institute, National Research Council of Canada,

Montreal, Quebec), was made competent for DNA transformation using the method of Simanis (1985). Briefly, 13 colonies of JM101 cells were picked off freshly streaked SOB plates (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 2% Bacto-agar) and used to inoculate a 125 ml culture of SOB medium. This culture was incubated at 37°C with moderate agitation until the cell density reached $4-7 \times 10^7$ cells/ml (OD at 550 nm of 0.45-0.55). Cells were collected, chilled on ice for 10-15 min and pelleted at 1,000 g for 13 min at 4°C. Pelleted cells were resuspended in 42 ml of ice cold RF1 buffer (100 mM RbCl, 50 mM MnCl₂·4H₂O, 30 mM K-acetate, 10 mM CaCl₂·2H₂O, 15% (w/v) glycerol, pH 5.80) and incubated on ice for 15 min. Cells were pelleted as described above, resuspended in 10 ml of ice cold RF2 buffer (10 mM MOPS, 10 mM RbCl, CaCl₂·2H₂O, 15% (w/v) glycerol, pH 6.8) and incubated on ice for 15 min. Cells were distributed in 0.5 ml aliquots, flash frozen in liquid nitrogen and stored at -70°C. JM101 cells prepared this way routinely gave transformation efficiencies of $2 \times 10^7/\mu\text{g}$ of circular pUC19 (Pharmacia Biotechnology) plasmid DNA.

The RR1 strain of *E. coli* was made competent as follows. A 10 ml culture of 2X YT medium (1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl) was inoculated with RR1 cells and was incubated overnight at 37°C without shaking. The overnight culture was used to inoculate 100 ml of P medium (20 mM KPO₄ pH 7.0, 15 mM (NH₄)₂SO₄, 1.8 μM FeSO₄, 10 mM MgSO₄, 1% casamino acids, 0.25% glucose) which was incubated at 37°C with shaking until the OD at 600 nm was 0.3-0.4. The cells were then cooled on ice and pelleted at 1,300 g for 10 min at 4°C. Pelleted cells were resuspended in 100 ml of ice cold 10 mM NaCl and centrifuged as above. The cell pellet was resuspended in 50 ml of ice cold 50 mM

CaCl₂, placed on ice for 15 min and pelleted as before. The cell pellet was resuspended in 10 ml of freezing buffer (50 mM CaCl₂, 16% glycerol), distributed in 0.5 ml aliquots, quick frozen in an ethanol/dry ice bath and stored at -70°C. RR1 cells prepared this way routinely yielded transformation efficiencies of $5 \times 10^5/\mu\text{g}$ of circular pAcYM1 plasmid DNA.

E. coli strain DH5 α competent cells, library efficiency, were purchased from Bethesda Research Laboratories (BRL) (Gaithersburg, MD). These cells gave transformation efficiencies of $1 \times 10^8/\mu\text{g}$ of circular pUC19 plasmid DNA.

Transformation of Competent Cells

Transformation of competent cells with plasmid or phage DNA was carried out according to standard procedures (Maniatis *et al.*, 1982). A tube of competent cells was thawed on ice and 200 μl of cells was placed in a 15 ml Falcon polypropylene tube (Fisher Scientific, Nepean, Ontario). Less than 10 μl of plasmid DNA was added to the cells and the mixture was placed on ice for 30 min. The cells were "heat shocked" by incubating at 42°C for 45-90 sec. The transformation mixture was placed on ice for 2 min, mixed with 800 μl of LB (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) or SOC medium (SOB, 20 mM glucose) and incubated at 37°C for 50 min. The transformation mixture was then diluted and 100 μl was spread on the appropriate plates (LB or SOB) containing 100 $\mu\text{g/ml}$ of ampicillin.

5. CLONING OF THE PROSPECT HILL VIRUS GENOME

Synthesis of Oligonucleotide Primers for Cloning

Unless otherwise stated, all oligonucleotide primers for cloning were synthesized using an Applied Biosystems Model 380B DNA synthesizer (Applied Biosystems, Mississauga, Ontario) at the University of Ottawa Biotechnology Research Institute.

Construction of a Prospect Hill Virus cDNA Bank

PH virus RNA, purified by pelleting through CsCl, was used as a template for the synthesis of cDNA as described (Gubler and Hoffman, 1983). First strand cDNA synthesis was done in a reverse transcriptase reaction mixture containing 1 mM dTTP, 1 mM dCTP, 1 mM dGTP, 200 μ M dATP, 100 mM Tris (pH 8.4), 130 mM KCl, 10 mM MgCl₂, 1 mM DTT, 20 units of RNasin (Promega Biotec, Madison, WI), 50 μ Ci of [α -³²P] dATP (800 Ci/mmol), and 40 units of AMV reverse transcriptase (Pharmacia Biotechnology). Approximately 10 ng of viral RNA and 200 ng of random primers (Pharmacia Biotechnology) were used and the reaction mixture was incubated for 1 hr at 42°C. The cDNA was converted to double-stranded DNA by adding 1/10 volume of 10X NT buffer (50 mM Tris, pH 7.2, 10 mM MgSO₄, 0.1 mM DTT, 50 μ g/ml of BSA), 2 units of RNase H (BRL) and 30 units of *E. coli* DNA polymerase I. The mixture was incubated for 2 hr at 15°C and then terminated by the addition of EDTA and SDS to final concentrations of 50 mM and 1% respectively. The double-stranded cDNA was size fractionated through a Sepharose 4B column, and fractions containing large cDNA molecules were collected. The double-stranded cDNA was either blunt-end ligated into the *Sma*I site of pUC19 or was first ligated to phosphorylated *Pst*I linkers (New

England Biolabs, Beverly, MD), digested with the restriction endonuclease *Pst*I (Pharmacia Biotechnology), then ligated into the *Pst*I site of pUC19. Ligations were done in ligation buffer (50 mM Tris, pH 7.8, 10 mM MgCl₂, 20 mM DTT, 1 mM ATP, 50 µg/ml BSA) containing 10 units of T₄ DNA ligase and were incubated at 15°C for 12-16 h. This DNA was used to transform competent *E. coli* JM101 cells prepared using the method of Simanis (1985). Ampicillin resistant colourless colonies were picked from LB plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Bacto-agar) containing 100 µg/ml of ampicillin, 100 mM IPTG (BRL) and 2% Bluo-gal (BRL) (Messing, 1983).

Molecular Cloning of the 5' and 3' Termini of the PH virus M RNA Segment

The 5' terminus of the PH virus M RNA segment was cloned using the polymerase chain reaction (PCR) using primers MP62 and MP61. Primer MP62 had 18 nucleotides complementary to viral M RNA (5'-CAGCTGCAGGTGTAGGCACTGGAT, Fig. 4, nucleotide positions 2379-2396, measuring from the 3' end of M genomic RNA) and was used for first strand cDNA synthesis, using PH virus M RNA as template, as described above. Primer MP61 had 16 nucleotides identical to the predicted 5' terminus of virion M RNA (5'-CAGCTGCAGTAGTAGTAGACTCCGC) (Schmaljohn et al., 1985). Prediction of the 5' terminus was based on the usually observed inverse complementarity between the 3' and 5' termini of *Hantavirus* RNA segments (Schmaljohn et al., 1986b, 1987b). The cDNA was amplified by PCR performed with the Geneamp kit and method provided by Perkin-Elmer Cetus Corp. (Norwalk, CT). Briefly PCR was done by mixing first-strand cDNA with 50 ng of MP62 and MP61 in 100 µl of the PCR reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5

mM MgCl₂, 100 µg/ml of gelatin) containing 200 µM of each dNTP and 5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus Corp.). The sample was heated to 94°C for 2 min and then subjected to 30 cycles of PCR; 1 min at 94°C (denaturation), 2 min at 55°C (annealing), and 3 min at 72°C (extension) using a DNA thermal cycler (Perkin-Elmer Cetus Corp.). The 72°C extension step was prolonged for another 7 min at the end of the 30th cycle. Both primers had *Pst*I sites at their 5' ends (sequence shown underlined) so the resultant clone M5' could be inserted into the *Pst*I site of pUC19.

To obtain 3' terminus of the PH virus M RNA, 2 µg of total cellular RNA from PH virus infected Vero E6 cells, extracted 9 days post-infection, was mixed with 500 ng of primer MP63 which had 18 nucleotides complementary to the PH virus M mRNA (5'-CAGCTGCAGAT-TGAAGCATGTGCCITC, Fig. 4, nucleotide positions 608-625, measuring from the 3' end of the PH virus M genomic RNA) and 500 ng of primer MP61 which had 16 bases identical to the 3' end of PH virus M RNA (5'-CAGCTGCAGTAGTAGTAGACTCCGC, Fig. 4) (Schmaljohn et al., 1985) in 100 µl of the PCR reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 100 µg/ml of gelatin) containing 200 µM of each dNTP, 32 units of AMV reverse transcriptase and 5 units of *Taq* DNA polymerase. The sample was incubated at 42°C for 60 min, heated to 94°C for 2 min, and then subjected to 40 cycles of PCR as described previously except the extension time was not prolonged until after the 40th cycle. Both primers were constructed with *Pst*I sites at their 5' ends (sequence shown underlined) for the purpose of cloning. However, the resultant PCR product had an internal *Pst*I site so the DNA fragment M3' was blunt-end ligated into the *Sma*I site of pUC19 instead.

Molecular Cloning of the 3' and 5' Termini of the PH virus S RNA Segment

The 3' terminal clone of the PH virus S RNA was obtained with PCR using two specific oligonucleotide primers MP11 and MP12. Primer MP11 has 21 nucleotides complementary to the 3' terminus of the PH virus S RNA (Fig. 12) (Schmaljohn et al., 1985), and was used for first strand cDNA synthesis as previously described. Primer MP12 has 20 nucleotides complementary to PH virus S cDNA (Fig. 12, nucleotide positions 497-516, measuring from the 3' end of the PH virus S genomic RNA). The cDNA of the S genomic segment was amplified by PCR in 100 μ l of the PCR reaction buffer containing 200 μ M of each dNTP, 100 ng each of primers MP11 and MP12 and 5 units of *Taq* DNA polymerase. The sample was heated to 94°C for 3 min and then subjected to 30 cycles of PCR as described previously for the 5' end of the PH virus M RNA segment except the annealing temperature was 50°C. Both primers had *Pst*I sites at their 5' ends (Fig. 12) so the resultant DNA fragment PS3, could be inserted into the *Pst*I site of pUC19.

The 5' terminal clone of the PH virus S RNA was prepared in essentially the same way as the 3' terminal clone. Two specific oligonucleotide primers MP13 and MP14 were prepared. Primer MP13 had 20 nucleotides complementary to the PH virus S RNA segment (Fig. 12, nucleotide positions 1451-1470, measuring from the 3' end of the PH virus S genomic RNA). Primer MP14 had 8 nucleotides identical to the predicted 5' terminus of PH virus S RNA (Fig. 12). Prediction of the 5' terminus was again based on the usually observed inverse complementarity between the 3' and 5' termini of *Hantavirus* RNA segments (Schmaljohn et al., 1986b, 1987b). Primer MP13 was used to make first strand cDNA as

described above, and this cDNA was amplified by PCR using the conditions previously described for the 3' end of the PH virus S RNA segment using 50 ng each of primers MP13 and MP14. The *Pst*I sites at the 5' ends of these primers (Fig. 12) allowed insertion of the DNA fragment PS5 into the *Pst*I site of pUC19.

6. DETERMINATION OF NUCLEOTIDE SEQUENCES

Subcloning into the Bacteriophage M13

The replicative form (RF) of M13mp18 and M13mp19 (Pharmacia Biotechnology) were digested with *Pst*I or with *Eco*RI (Pharmacia Biotechnology) and *Hind*III (Pharmacia Biotechnology) and then dephosphorylated with alkaline phosphatase. Purified DNA fragments were ligated into the appropriately cleaved M13 RF DNA and used to transform competent *E. coli* JM101 cells. Recombinant phage plaques were identified by their colourless appearance in the presence of 2% Blue-gal and 100 mM IPTG (Messing, 1983). Colourless M13 plaques were used to inoculate 2 ml cultures of 2X YT medium (1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl) and were incubated at 37°C for 7 h with vigorous shaking. Bacterial cells were then removed from the medium by centrifuging at 10,000 g for 5 min. To 18 µl of the supernatant was added 2 µl of 10X loading buffer (0.5% bromophenol blue, 2% EDTA, 50% glycerol, 2% SDS). These samples were then electrophoresed in a 0.8% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) for 5 h at 100 volts. Comparison of the migration of wild type M13 phage DNA with recombinant phage DNA confirmed the presence of DNA inserts (Messing, 1983). These supernatants were the recombinant phage stocks

and were stored at -70°C.

Preparation of Single-Stranded M13 DNA for Sequencing

A single colony of *E. coli* strain JM101, from a M9 plate, was inoculated into 2X YT medium and was grown overnight at 37°C with vigorous shaking. 50 µl of this overnight culture was used to inoculate 12 ml of 2X YT medium and this culture was grown at 37°C with vigorous shaking. After 1 h, these cells were infected with 50 µl of recombinant phage stock and incubated at 37°C with vigorous shaking for 7 h. After incubation samples were centrifuged at 13,000 g for 10 min in a JA-20 rotor (Beckman). The supernatant was mixed with 0.67 ml of 40% PEG-8000, 0.67 ml of 5 M Na-acetate (pH 7.0) and incubated for 1 h at 4°C. Recombinant phage was pelleted at 13,000 g for 15 min in the JA-20 rotor. The supernatant was discarded and the phage pellet was allowed to dry for 1 h. The phage pellet was resuspended in 600 µl of 20 mM Tris-HCl buffer (pH 7.2) and extracted once with one volume of phenol saturated with 100 mM Tris-HCl buffer (pH 8.0), twice with one volume of phenol-chloroform-isoamyl alcohol (25:24:1) and re-extracted twice with chloroform-isoamyl alcohol (24:1). The aqueous phase was combined with 0.5 volumes of 8M NH₄-acetate, 2 volumes of ethanol and precipitated overnight at -20°C. The phage DNA was pelleted at 10,000 g in a microcentrifuge, washed with 70% ethanol and dried in a Speed Vac Concentrator (Savant Instruments/Emerston Instruments Inc., Richmond Hill, Ontario). The DNA was resuspended in H₂O and the yield was determined by measuring the OD at 260 nm. 100-120 µg of recombinant phage DNA was routinely obtained with this method.

Subcloning into the DNA Plasmid pUC19

Circular pUC19 plasmid was digested with the restriction

endonucleases *Pst*I or *Sma*I and then dephosphorylated with alkaline phosphatase. Complementary DNA or agarose gel purified DNA fragments were blunt-end ligated into *Sma*I cleaved pUC19 DNA and used to transform competent JM101 cells. Alternatively cDNA fragments were ligated to phosphorylated *Pst*I linkers, digested with the restriction endonuclease *Pst*I, ligated into the *Pst*I site of pUC19 and used to transform competent JM101 cells. Cells with the recombinant plasmid were identified by their colourless appearance in the presence of 2% Blue-gal and 100 mM IPTG. Colourless colonies were used to inoculate 2 ml cultures of LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Bacto-agar) containing 100 µg/ml of ampicillin. Recombinant plasmid DNA was isolated using the miniprep method. Plasmids of interest were isolated in large amounts using the maxiprep procedure.

Preparation of Double-Stranded pUC19 Plasmid DNA for Sequencing

The double-stranded plasmid DNA, purified by the maxiprep procedure, was prepared for sequencing using the alkaline-denaturation method (Chen and Seeburg, 1985; Haltiner *et al.*, 1985; Hattori and Sakaki, 1986). Briefly, 2-3 µg of double-stranded plasmid DNA was denatured in 0.2 N NaOH for 5 min at room temperature, neutralized by the addition of 0.4 volumes of 5 M NH₄-acetate (pH 7.5) and precipitated by placing at -70 °C for 5 min after adding 4 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried and stored at -20 °C until used.

Manual DNA Sequencing

The DNA sequence was determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using the United States Biochemical (Cleveland, OH) Sequenase DNA sequencing kit with [α -³⁵S]dATP as described by the manufacturer. Briefly, 1-2 µg of M13

single-stranded or 2-3 μg of denatured double-stranded pUC19 DNA was annealed with 8 ng of primer (pUC/M13 17-mer forward primer, pUC 21-mer reverse primer or specific oligonucleotide primers, all synthesized as described above, in Sequenase buffer (40 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 50 mM NaCl) in a final volume of 10 μl . The primer was extended and labelled by incubating at room temperature for 10 min after the addition of 1 μl of 0.1 M DTT, 2 μl of diluted dGTP labelling mix (1.5 μM dGTP, 1.5 μM dCTP, 1.5 μM dTTP), 0.5 μl of [α - ^{35}S]dATP (>1000Ci/mmol) and 2 μl of diluted Sequenase (diluted 1:8 in ice-cold TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) prior to use. After labelling, 3.5 μl of primer-template mixture was added to four tubes containing 2.5 μl of the appropriate A, C, G or T deoxy/dideoxy (d/dd)NTP termination mix. The concentration of dNTP's was 80 μM and the concentration of the chain-terminating ddNTP was 8 μM in all four termination mixes. Termination reactions were incubated at 37°C for 5 min, mixed with 4 μl of stop solution (95% Formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) and stored at -20°C.

Alternatively, the DNA sequence of clones was determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using *Taq* DNA polymerase as described by Innis *et al.* (1988). Briefly, 1-2 μg of M13 single-stranded or 2-3 μg of denatured double-stranded pUC19 DNA was annealed with 8 ng of primer in sequencing buffer (10 mM Tris-HCl, pH 8.5, 3 mM MgCl_2) in a final volume of 10 μl . The primer was extended and labelled by incubating at 37°C for 2 min after the addition of 2 μl of dGTP labelling mix (10 μM dGTP, 5 μM dCTP, 5 μM dTTP), 1 μl of [α - ^{35}S]dATP (>1000Ci/mmol, Amersham, Arlington Heights, IL), 5 μl of H_2O and 2 μl of *Taq* DNA polymerase (5 U/ μl). After labelling, 4 μl

of primer-template mixture was added to four tubes containing 2 μ l of the appropriate A, C, G or T d/ddNTP termination mix. The concentration of dNTP's was 30 μ M in all four termination mixes. The concentration of the ddNTP in the specific reactions was 120 μ M for ddGTP, 1 mM for ddATP, 1.5 mM ddTTP and 500 μ M for ddCTP. Termination reactions were incubated at 70°C for 2 min, mixed with 2 μ l of stop solution (95% Formamide, 20 mM EDTA, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol FF) and stored at -20°C.

Urea-Polyacrylamide Sequencing Gels

Sequencing reactions were electrophoresed on 37 cm X 40 cm X 0.04 cm 6% polyacrylamide (5.7% acrylamide, 0.3% N'N'-Bis-methylene-acrylamide (Bis)) 7 M urea gels buffered with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Glass plates were treated with dimethyldichlorosilane (BDH, Toronto, Ontario) before the gel was cast. Electrophoresis was done with an IBI (New Haven, CT) Model STS-45 Thermoplate sequencing apparatus at a constant power of 55 watts. After electrophoresis gels were dried onto Whatmann 3 mm paper using a Bio-Rad Model 583 Slab Dryer and exposed to Cronex X-ray film at -80°C.

Automated DNA Sequencing

Automated DNA sequencing was done by B. Mah or D. McLean using the DuPont Genesis 2000 automated DNA sequencer according to the protocol provided by DuPont.

Computer Analysis of Nucleotide and Amino Acid Sequences

Analysis of secondary structure of RNA, searches for potential open reading frames, translation of DNA sequences, location of signal peptide cleavage sites, hydrophilicity profiles, searches for potential antigenic sites, and location of potential transmembrane helices were completed

using the PC/Gene program (Version 5.11, 1987, Department of Medical Biochemistry, University of Geneva, Switzerland, distributed by Intelligenetics, Inc., Mountainview, CA).

Analysis of secondary structure of RNA was based on Zucker's method (Zucker and Stiegler, 1981) with modifications (Jacobsen et al., 1984).

Hydrophilicity profiles and searches for potential antigenic sites were done using the method of Hopp and Woods (1981).

Signal sequence cleavage sites were determined by the method of von Heijne (1986).

The consensus sequence used to determine potential asparagine-linked glycosylation sites was N-X-S/T-X, where X cannot be proline (P) (Bause, 1983).

Prediction of transmembrane helices was based on the method of Rao and Argos (1986).

7. PRODUCTION OF RECOMBINANT BACULOVIRUSES

Modification of Genes for Protein Expression

The upstream and downstream flanking regions of genes to be expressed in baculovirus were removed and/or modified using site-specific recombination directed by double-stranded crossover linkers (Sung *et al.*, 1986). Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer and double-stranded crossover linkers were prepared by combining equal molar ratios of complementary oligonucleotides, heating the mix to 95°C for 3 min and cooling slowly to room temperature. A 10 fold molar excess of the crossover linker was

ligated to the plasmid, containing the gene of interest, that had been completely digested with one or two restriction endonucleases. The entire ligation mixture was electrophoresed on a TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) agarose gel and the linear plasmid band was purified. This purified material was then used to transform competent JM101 cells on LB plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Bacto-agar) containing 100 µg/ml of ampicillin

Cells, Viruses and Preparation of Virus Stocks

Spodoptera frugiperda (SF9) cells were obtained from Dr. M. D. Summers (Texas A & M University, College Station, TX). *Autographa californica* nuclear polyhedrosis virus (AcNPV) was provided by Dr. D. H. L. Bishop (NERC Institute of Virology, Oxford, U.K.). Wild type and recombinant baculoviruses were propagated in SF9 cells at 27°C in TNM-FH medium (Hink, 1970; Summers and Smith, 1987) supplemented with heat-inactivated FBS (10%) (v/v) and L-glutamine (2mM) and containing antibiotics (penicillin 100 u/ml, streptomycin 100 mg/ml, kanamycin 100 mg/ml) (complete TNM-FH). Stocks of recombinant viruses were prepared by picking a virus plaque and resuspending it in 1 ml of complete TNM-FH medium. This stock was used to infect approximately 3.5×10^6 SF9 cells in a T-25 flask (25 cm², Corning, Corning, NY) at an MOI of approximately 0.01 pfu/cell. Virus was propagated for 4-5 days at 27°C. This virus stock was then used to infect 1.5×10^7 SF9 cells in a T-75 flask (75 cm², Corning) with an MOI of 0.1 pfu/cell. The virus supernatant would again be harvested 4-5 days post-infection.

Preparation of the Baculovirus Transfer Vector

The baculovirus transfer vector pAcYM1 (Matsura *et al.*, 1987) was prepared using the maxiprep method, digested with *Bam*HI (Pharmacia

Biotechnology) and dephosphorylated with alkaline phosphatase.

Insertion of the Putative PH Virus NS_S Gene into the Baculovirus Transfer Vector

A 281-bp *Bgl*II fragment, containing the putative PH virus NS_S gene, recovered from the plasmid pUCPHVNS_S was ligated into the *Bam*HI site of pAcYM1. This plasmid, pAcPHVNS_S, was used to transform competent *E. coli* strain RR1 cells. Orientation of the putative PH virus NS_S gene in pAcYM1 was determined by digesting plasmids with *Ava*I and *Bam*HI.

Insertion of the Human Immunodeficiency Virus Type 1 (HIV-1) *rev* Gene into the Baculovirus Transfer Vector

A 369-bp *Bgl*II fragment, containing the HIV-1 *rev* gene, recovered from the plasmid pUC19-*rev*3 was ligated into the *Bam*HI site of pAcYM1. This plasmid, pAcYM1-*rev*, was used to transform competent *E. coli* strain RR1 cells. Orientation of the *rev* gene in pAcYM1 was determined by digesting plasmids with *Ava*I.

Insertion of the HIV-1 *vif* Gene into the Baculovirus Transfer Vector

A 592-bp *Bam*HI fragment, containing the HIV-1 *vif* gene, recovered from the plasmid pUC19-*vif*3 was ligated into the *Bam*HI site of pAcYM1. This plasmid, pAcYM1-*vif*, was used to transform competent *E. coli* strain RR1 cells. Orientation of the *vif* gene in pAcYM1 was determined by automated sequencing using the DuPont Genesis 2000 automated DNA sequencer.

Transfection of *Spodoptera Frugiperda* Cells with Baculovirus Transfer Vector Constructions

SF9 cells were co-transfected with a baculovirus transfer vector containing either the putative PH virus NS_S gene (pAcPHVNS_S), the HIV-1

rev gene (pAcYM1-*rev*), or the HIV-1 *vif* gene (pAcYM1-*vif*) and purified wild type AcNPV DNA, using the calcium phosphate precipitation technique (Graham and Van Der Eb, 1973) as modified for insect cells (Smith *et al.*, 1983). Briefly, 3 μ g of AcNPV DNA was mixed with 3 μ g or 6 μ g of plasmid DNA in 950 μ l of co-transfection buffer (20 mM HEPES, 1 mM Na₂HPO₄, 5 mM KCl, 125 mM NaCl, 10 mM glucose, pH 7.05). The addition of 50 μ l of 2.5 M CaCl₂ caused the DNA in the solution to form a fine precipitate. This solution was mixed with 1 ml of complete TNM-FH media, overlaid on $1.5\text{--}2.0 \times 10^6$ cells in 35 mm tissue culture plates (Corning) and incubated at 27°C for 3 h. The overlay was removed and replaced with 2.5 ml of complete TNM-FH media and incubated at 27°C for 3 days. The tissue culture fluid was then harvested and titrated on monolayers of SF9 cells in 35 mm or 96-well (flat bottom) tissue culture plates.

Isolation and Purification of Recombinant Baculoviruses

Recombinant baculoviruses were selected using two methods. In the first method, SF9 cells in serum-free TNM-FH medium were seeded into 35 mm plates at a density of 1.2×10^6 cells. During cell attachment serial 10 fold dilutions of virus were prepared. Co-transfection supernatants were diluted from 10^{-3} to 10^{-6} while plaque picked viruses were diluted from 10^{-2} to 10^{-4} . After cell attachment medium was removed and 200 μ l of diluted virus was added to each plate. Virus was allowed to absorb for 1 h at 27°C and then the medium was removed. A 2.5 ml overlay of 1.5% low melting agarose in TNM-FH complete medium was added to each plate. After the agarose solidified, 2 ml of complete TNM-FH medium was added to each plate and plates were incubated at 27°C for 5-7 days in a humid environment (Brown and Faulkner, 1977).

Once plaques were well formed they were visually examined against a dark background with a strong light. Clear plaques were marked and then examined further with an inverted phase contrast microscope at 400X. Those clear plaques showing no evidence of polyhedra were picked and re-titrated on SF9 cells. Three consecutive plaque purifications were usually sufficient to isolate recombinant virus free from wild type virus.

In the second method, a limiting dilution assay was done using a modification of the procedure described by Kafatos *et al.* (1979). The wells in a 96 well flat bottom microtiter plate were seeded with 1.5×10^4 SF9 cells in 100 μ l. A series of 10-fold dilutions was made with the co-transfection culture supernatant from 10^{-1} to 10^{-8} . 100 μ l of a particular dilution was added to each well in a row of 8 wells (A-H). The plate was incubated at 27°C for 7 days. The culture supernatants from each well were then transferred to another 96 well microtiter plate and stored at 4°C. The infected cells in each well of the plate were lysed by adding 200 μ l of 0.5 N NaOH and mixing. The solution was neutralized by adding 20 μ l of 10 M NH_4 -acetate to each well and mixing. The infected cell DNA was blotted onto GeneScreen Plus membrane using a Bio-Dot vacuum blotter. After drying the membrane was incubated in hybridization buffer with a nick translated plasmid containing the gene of interest using the hybridization method described above. The culture supernatant harvested from wells at a high dilution ($>10^{-5}$), where the infected cell DNA yielded a strong hybridization signal, was used in a plaque assay as described in the first method. Clear plaques were selected as described previously and usually only one round of plaque purification is required.

Analysis of Recombinant Baculoviruses by Hybridization

SF9 cells infected with a recombinant baculovirus were harvested 4-5 days after infection. DNA from infected cells was recovered by lysing cells with 0.5 N NaOH. The solution was neutralized by adding a 1/10 volume of 10 M NH_4 acetate. The DNA was dot blotted onto GeneScreen Plus membrane and hybridized as described in section 3.

8. ANALYSIS OF PROTEINS

Radiolabeling of PH Virus Infected Cells

At 10 days post-infection PH virus or mock infected Vero cells, in 100 mm tissue culture plates were starved with 10 ml of methionine-free MEM (Flow Laboratories, Mississauga, Ontario) containing 10% FBS and 2 mM L-glutamine at 35°C for 1 h. The medium was removed and replaced with 1 ml of methionine-free MEM containing 10% FBS, 2 mM L-glutamine and 100 μCi of L-[^{35}S]-methionine (1066 Ci/mmol, Amersham). Cells were labelled for 6 h at 35°C.

Radiolabeling of Recombinant Baculovirus Infected Cells

Monolayers of SF9 cells, in T-25 flasks, were infected with recombinant baculoviruses and incubated at 27°C for 48 h. The cells were starved with 5 ml of Grace's methionine-free or phosphate-free medium for 1 h at 27°C. The medium was removed and replaced with 1 ml of Grace's methionine-free or phosphate-free medium containing 100 μCi of L-[^{35}S]-methionine (1066 Ci/mmol, Amersham) or 400 μCi of [γ - ^{32}P]-orthophosphate (8 mCi/ml, Amersham) and incubation at 28°C was continued for 2 h.

Preparation of Cell Lysates

SF9 cells, in T-25 flasks, were scraped off the flask, using a rubber policeman, resuspended in 10 ml of cold PBS (137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , 0.9 mM CaCl_2 , 0.5 mM MgCl_2) and were pelleted at 1,000 g for 5 min. Cells were washed three times with 10 ml of cold PBS and pelleted as before. SF9 cells were then lysed in 200-250 μl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10 mM Tris pH 7.5, 10% β -mercaptoethanol, 10% SDS, 0.02% bromophenol blue, 25% glycerol), placed in boiling water for 10 min and stored at -20°C .

PH virus or mock infected Vero cells, in 100 mm tissue culture plates, were washed once with 4 ml/plate of cold DMEM. The cells were scraped off the plates, using a rubber policeman, resuspended in 10 ml of cold DMEM and were pelleted at 1,000 g for 5 min. Cells were washed three times with cold PBS and pelleted. Vero cell pellets were lysed in 400 μl /plate of RIPA buffer plus detergents (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) at 4°C . Samples were sonicated to shear the DNA and were then heated to 100°C in boiling water for 10 min. Samples were pelleted at 10,000 g for 10 min at 4°C in a JA-20 rotor (Beckman). The supernatants were pelleted again at 14,000 g for 30 min at 4°C in a microcentrifuge. The supernatants were used immediately in immunoprecipitation reactions.

Immunoprecipitations

Up to 10 μl of a baculovirus infected cell lysate was added to 900 μl of RIPA buffer plus detergents (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) containing 50-100 μl of specific rabbit antiserum and 15 mg of protein A Sepharose CL-4B

beads (Pharmacia Biotechnology). Samples were placed at 4°C overnight with gentle agitation. Beads were washed 4 times in cold RIPA buffer plus detergents. Proteins precipitated by the antibody were removed from the beads by resuspending in SDS-PAGE sample buffer (10 mM Tris pH 7.5, 10% β -mercaptoethanol, 10% SDS, 0.02% bromophenol blue, 25% glycerol) and heating in boiling water for 10 min.

Up to 900 μ l of PH virus or mock infected Vero cell lysate in RIPA buffer plus detergents was mixed with 100 μ l of specific rabbit sera and 15 mg of protein A Sepharose CL-4B beads. Samples were then treated as above.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by SDS-PAGE in slab gels using the discontinuous buffer system (Laemmli, 1970). Stacking gels were composed of 3% acrylamide, 0.08% Bis, 0.1% SDS and 125 mM Tris-HCl, pH 6.8. The resolving gels for the *rev* and *vif* proteins were composed of 15% acrylamide, 0.4% Bis, 0.1% SDS and 380 mM Tris-HCl, pH 8.8. Other samples were electrophoresed through a step gel system with two resolving gels (12% acrylamide, 0.32% Bis, 0.1% SDS and 380 mM Tris-HCl, pH 8.8 and 20% acrylamide, 0.53% Bis, 0.1% SDS and 380 mM Tris-HCl, pH 8.8). The SDS-PAGE electrophoresis buffer consisted of 25 mM Tris, 190 mM glycine and 0.1% SDS. Proteins were denatured in SDS-PAGE sample buffer before electrophoresis in the Protean II Slab Electrophoresis Cell (Bio-Rad) at 70 volts overnight or the Mini Protean II Electrophoresis Cell (Bio-Rad) at 80 volts for 2-3 h.

Visual identification of proteins was made by staining gels for 1 h in 0.25% Coomassie blue, 50% methanol and 10% glacial acetic acid and destaining by diffusion in 10% methanol and 10% glacial acetic acid.

For fluorography, gels were fixed in 25% propanol and 10% glacial acetic acid for 30 min at room temperature with constant shaking. After fixing, gels were soaked in Amplify (Amersham) for 30 min at room temperature with constant shaking. The Amplify was discarded and the gel was dried with the Bio-Rad Model 583 Slab Dryer onto a piece of filter paper and exposed to Cronex X-ray film at -80°C.

Elution of Proteins from SDS-Polyacrylamide Gels

Samples containing proteins of interest were denatured in SDS-PAGE sample buffer (10 mM Tris pH 7.5, 10% β -mercaptoethanol, 10% SDS, 0.02% bromophenol blue, 25% glycerol) before electrophoresis in the Bio-Rad Protean II Slab Electrophoresis Cell and were electrophoresed overnight at 70 volts. Proteins to be eluted were visualized by staining with 0.1% Coomassie blue, 50% methanol and 10% glacial acetic acid for 25 min, and destained for 55 min in 5% methanol and 10% glacial acetic acid. The protein bands of interest were cut out of the gel and soaked in volatile elution buffer (50 mM NH_4HCO_3 , 0.1% SDS) for 30 min. The gel slices were then loaded into a Bio-Rad Model 422 Electro-Eluter and the protein was eluted out of the gel by electrophoresing at 10 mA/tube in volatile elution buffer. The eluted sample was diluted four fold with H_2O , frozen at -70°C and dried overnight in a Speed Vac Concentrator, to remove the volatile buffer. Protein samples were resuspended in H_2O , frozen and dried. The protein sample was resuspended in H_2O and stored at -20°C.

Production of Polyclonal Antisera

Male New Zealand white rabbits were inoculated in the muscle of both hind legs with 40 μg of eluted protein mixed with Freund's complete adjuvant. After two weeks the rabbits were inoculated in the muscle of each hind leg with 40 μg of eluted protein mixed with Freund's

incomplete adjuvant. 28-36 days after injection a small amount of blood was removed and tested for the specific antibody using the Western Blot Immunoassay. If the specific antibody was present in the serum tested, all the blood was removed by cardiac puncture.

Western Blot Immunoassay

Proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) using the Mini Trans-blot Electrophoresis Cell (Bio-Rad) at 100 volts for 1 h in blotting buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3) (Burnette, 1981). Free protein binding sites on protein-bound membranes were blocked by incubating for 1 h in TBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl) containing 3% gelatin at room temperature. Following two 10 min washes in TTBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% tween 20), the membrane was incubated overnight at room temperature in antibody buffer (TTBS, 1% gelatin) containing specific antibodies. Unbound antibody was removed from the membrane with two 10 min washes in TTBS (TBS, 0.05% tween 20). Membranes were then incubated with a 1:3,000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (GAR-AP, Bio-Rad) in antibody buffer (TTBS, 1% gelatin) for 1 h at room temperature. The membrane was given two 10 min washes in TTBS and one 10 min wash in TBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl). Antigens were visualized by incubating membranes in carbonate buffer (100 mM 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 p-toluidine salt) for 10-15 min. The colour development was stopped by placing the membrane in distilled water.

CHAPTER 3: RESULTS

PART 1: PROSPECT HILL VIRUS

1. STRUCTURAL CHARACTERIZATION OF PH VIRUS

Characterization of the PH Virus Genome

To label the PH virus RNA, [^3H]-uridine was added to PH virus infected cells when the tissue culture medium was exchanged for fresh medium on days 4 and 8 post-infection. On day 11 post-infection the RNA was extracted from pelleted virions, denatured with glyoxal and analyzed by agarose gel electrophoresis and fluorography. The three different [^3H]-uridine labelled PH virus RNAs, Large (L), Medium (M) and Small (S) were easily visualized (Fig. 1, lanes 1, 2). Electrophoresis of denatured [^3H]-uridine labelled ribosomal RNA markers in the same agarose gel made estimation of the relative molecular masses (M_r) of the PH virus RNA species possible (Fig. 1, lane 3). The PH virus L, M and S segments had M_r 's of 2.2×10^6 , 1.3×10^6 and 0.6×10^6 respectively. These sizes are the same as reported by Yoo (1988).

The relationship between the intensity of the [^3H]-uridine labelled genomic RNA bands on the fluorographs and their molecular weights was not linear (Fig. 1, lanes 1, 2). This was due to a difference in the molar ratios of the three PH virus RNA segments and was previously reported (Yoo and Kang, 1987a).

Smaller RNA species were also observed on the fluorograph (Fig. 1, lanes 1, 2). These smaller RNAs may well represent cellular RNA species which were packaged into PH virus since these smaller RNAs were co-

Figure 1. Fluorograph of the genomic RNA of PH virus. RNA was labelled with [³H]-uridine and resolved on a 1.5% agarose gel after denaturation with glyoxal. The gel was impregnated with 3% PPO, dried and exposed to X-ray film at -70°C. lanes 1, 2, PH virus RNA; lane 3, Eucaryotic ribosomal RNA.

1 2 M

L - —

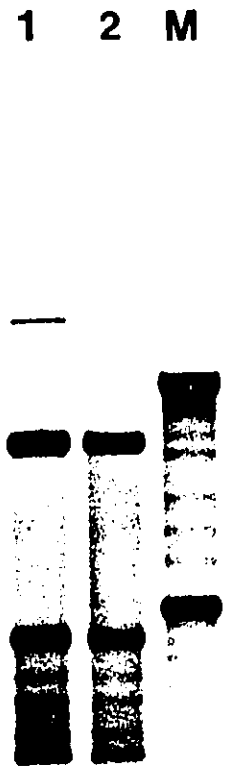
M -

S -

- 28S

- 18S

- 5S

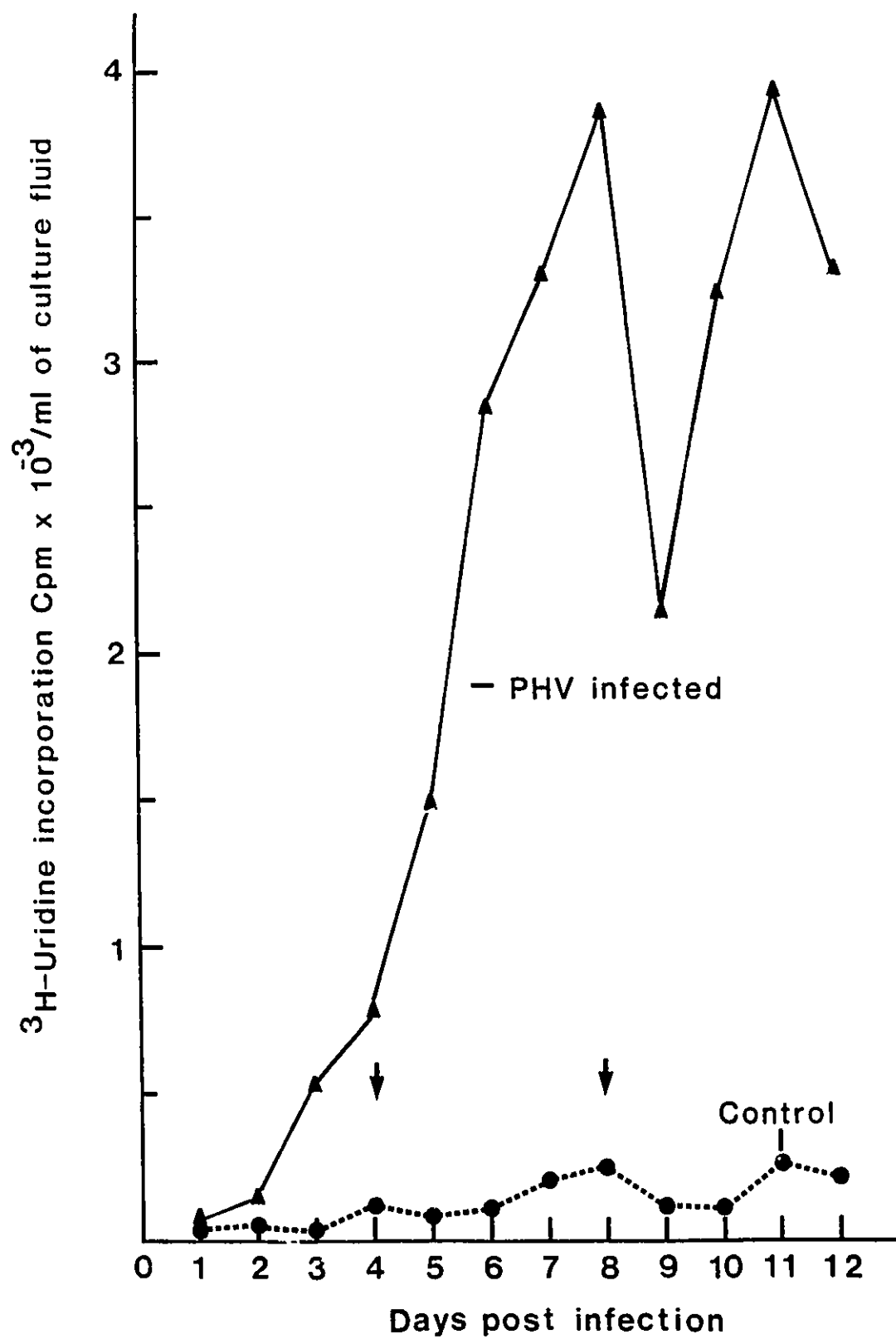


purified with the virus in the gradient. Also, these smaller RNAs did not hybridize with PH virus specific DNA probes (data not shown). Packaging of cellular nucleic acid in enveloped RNA viruses is a well known phenomenon.

2. ASSAY FOR PH VIRUS PRODUCTION

An assay was developed to measure the release of PH virus from infected Vero cells on a daily basis (see Materials and Methods). Infected or mock infected cells were labelled with [^3H]-uridine and the amount of labelled PH virus released into the tissue culture fluid was measured each day. On days 1 and 2 post-infection there was little or no release of PH virus into the medium (Fig. 2). Beginning on day 3 post-infection the amount of uridine labelled material pelleted from the culture supernatants from the PH virus infected cells began to increase in comparison to the mock infected control, indicating that uridine labelled PH virus was being released into the medium. The amount of labelled PH virus accumulating in the medium continued to increase until day 8 post-infection when the amount of labelled PH virus in the medium peaked (Fig. 2). This occurred even though all of the medium was replaced on day 4 post-infection and therefore all virus in the culture medium was removed. Because the medium was replaced on day 8 post-infection the amount of PH virus in the medium showed a decrease on day 9 post-infection (Fig. 2). The amount of PH virus again accumulated in the medium until reaching a second peak on day 11 post-infection (Fig. 2). The amount of labelled PH virus in the medium decreased between days 11 and 12 post-infection possibly because degradation of the extracellular

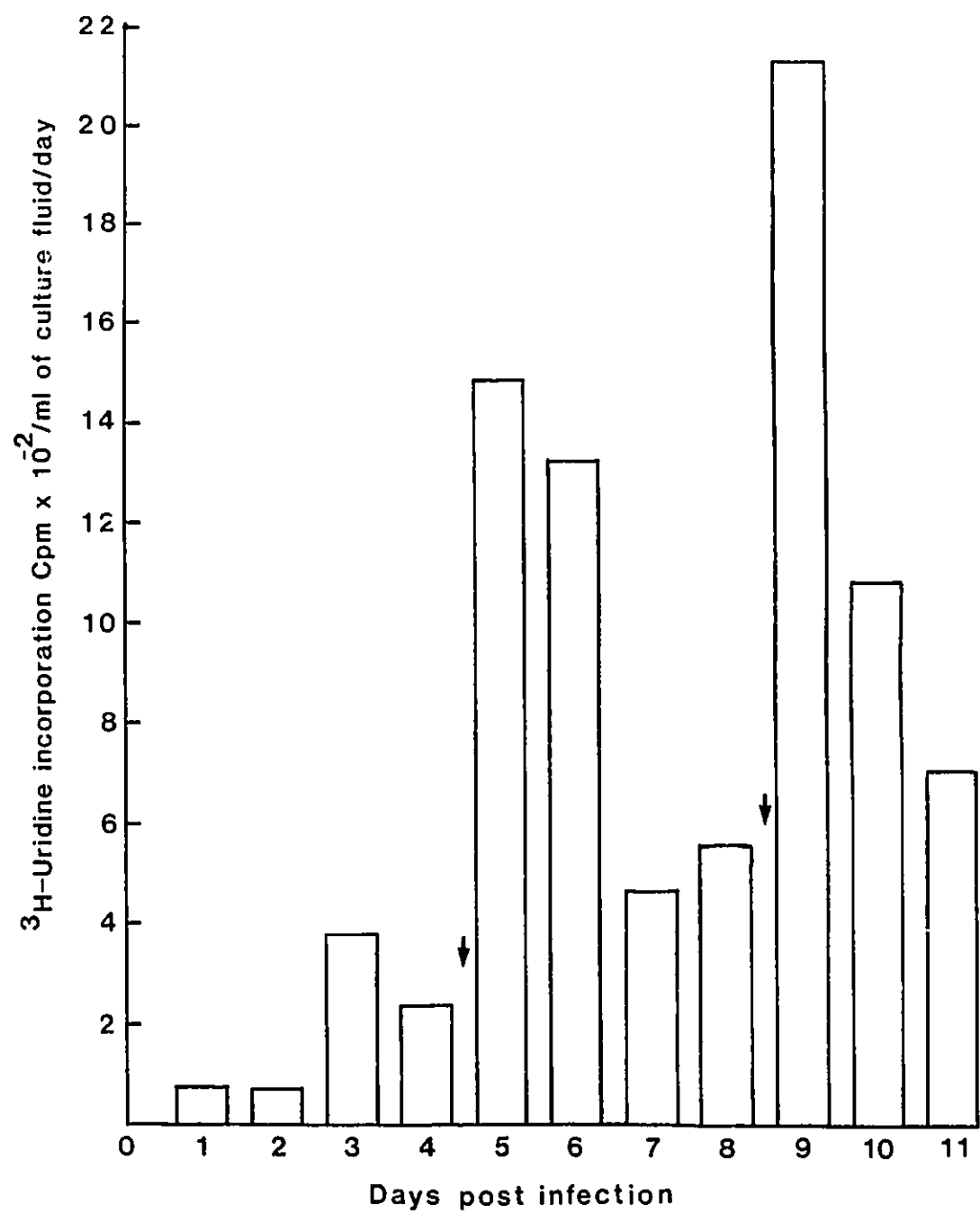
Figure 2. Production of and release of PH virus into the tissue culture medium after infection of Vero cells. Vero E6 cells were infected with PH virus or mock infected. The cells were labelled with [^3H]-uridine and labelled material was pelleted from the medium as described in Materials and Methods. The amount of ^3H cpm pelleted from tissue culture fluid of PH virus or mock infected cells was plotted each day for 12 days. Duplicate samples were obtained each day and the amounts of pelleted ^3H labelled material were averaged before plotting. To accurately indicate the amount of [^3H]-uridine labelled PH virus pelleted each day this value was determined by subtracting the ^3H cpm from the mock infected sample on each day from the ^3H cpm obtained from the PH virus infected sample. Arrows indicate when the culture fluid was removed and replaced with fresh medium.



virus began to exceed the release of new virus into the culture medium (Fig. 2). Assayed medium from mock infected cells showed consistently low pelleted [^3H]-uridine counts over the duration of the assay (Fig. 2). Although the control counts were low we wanted to verify that labelled PH virus was actually being pelleted and quantitated by the assay. Therefore, the RNA was extracted from labelled material recovered during the assay procedure. A fluorograph of this glyoxal denatured RNA after electrophoresis was identical to the fluorograph in Figure 1 (lanes 1, 2), indicating most of the peltable [^3H]-uridine counts represent PH virus (data not shown). The assay was discontinued after day 12 post-infection because of the deteriorating condition of the infected cells.

This assay only measures the labelled virus released from the cells into the medium and does not necessarily reflect virus production in the cells. Also the amount of virus pelleted on day 8 post-infection is the total accumulation of virus released on days 5, 6, 7 and 8 post-infection, minus the virus lost to degradation and the 1 ml samples removed on days 5, 6 and 7 post-infection. Although the rate of virus degradation is unknown, if it is relatively constant then the amount of virus released each day can be approximated by subtracting the amount of labelled virus released on previous days from the amount of labelled virus pelleted on that day. This works for all days but 5 and 9 post-infection when zero is subtracted from the [^3H]-uridine labelled PH virus counts because the medium was completely exchanged on days 4 and 8 post-infection. Figure 3 demonstrates that during the first four days post-infection the amount of labelled PH virus in the medium was relatively low. However, on day 5 post-infection, one day after the medium was exchanged for fresh medium, there was over a 5-fold increase in the amount of labelled PH

Figure 3. Release of PH virus into the culture medium each day. To determine when the largest amounts of PH virus are released into the medium, the amount of [^3H]-uridine labelled PH virus pelleted from the medium each day, shown in Fig. 2, was subtracted from the amount counted on the previous day. The exception to this was on days 5 and 9 when zero was subtracted since the medium had been removed and exchanged for fresh medium on the previous day. Arrows indicate when the culture fluid was removed and replaced with fresh medium.



virus pelleted from the medium. The amount of labelled PH virus released into the medium was almost as high on day 6 post-infection. In contrast the amount of PH virus released on days 7 or 8 post-infection was approximately 1/3 as high as the amount released on either of the two previous days. On day 9 post-infection, again one day after the medium was changed, there was a large increase (over 4-fold) in the amount of labelled PH virus released into the medium. The amount of labelled PH virus released on day 9 post-infection was over 30% more than the amount of PH virus released in any other one day period. The amount of labelled PH virus released day 10 post-infection showed a decrease of 50% from day 9 post-infection and dropped approximately another 35% on day 11 post-infection. By day 12 post-infection the released PH virus was degrading faster than virus production and this resulted in a net decrease in the amount of labelled PH virus pelleted from the medium (data not shown). These results indicate that giving the PH virus infected cells fresh medium caused a "burst" in the amount of virus released into the medium. However, feeding of the cells with fresh medium more often (every three days) had a detrimental effect on PH virus production (data not shown).

3. MOLECULAR CHARACTERIZATION OF THE PH VIRUS M RNA SEGMENT

Cloning and Sequence Analysis of the PH Virus M RNA Segment

A cDNA library of clones representing the PH virus RNA segments was made using random primers as described in Materials and Methods. The specificity of some of these clones (L, M or S) was determined by

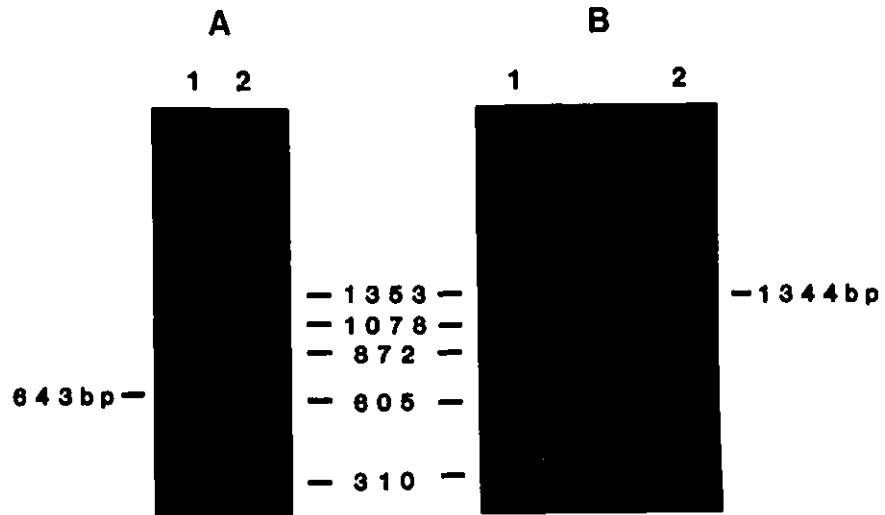
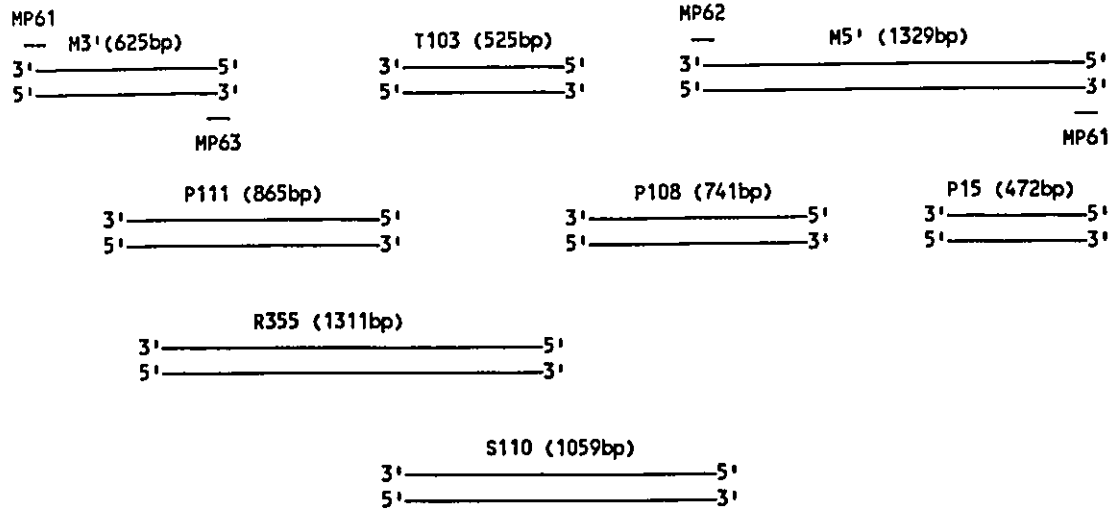
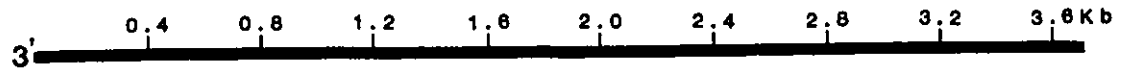
Northern analysis. Clones representing the PH virus M RNA segment were used in cross-hybridization studies to identify other clones representing the PH virus M RNA segment. A combination of the Northern analysis and cross-hybridization studies identified six clones that represented most of the PH virus M RNA segment (Fig. 4). The nucleotide sequences of these six clones, P111, R355, T103, S110, P108 and P15 were determined using the dideoxy chain termination method (Sanger *et al.*, 1977). The nucleotide sequence of these clones were compared with the nucleotide sequence of the Hällnäs virus M RNA segment (Giebel *et al.*, 1989). The comparison revealed the following; clone P111 began approximately 340 bases from the 3' end of the PH virus M RNA segment, there was a gap of approximately 500 bases between clones P108 and P15, and clone P15 ended approximately 20 bases from the 5' end of the PH virus M RNA segment. The 5' and 3' termini of the PH virus M RNA segment were cloned by the polymerase chain reaction (PCR) method using *Taq* DNA polymerase.

The 5' end of the M RNA segment was cloned with PCR by amplifying cDNA made from the genomic RNA segment with primers MP62 and MP61 (see Materials and Methods). Primer MP62 was designed to initiate cDNA synthesis inside of clone P108 so that the gap between clones P108 and P15 could also be cloned. The resultant 1344-bp PCR fragment (Fig. 4B, lane 2) was ligated into the *Pst*I site of pUC19 and designated M5'.

Initially, attempts were made to clone the 3' end of the PH virus M RNA segment using a similar method as for the 5' terminus. First-strand cDNA was synthesized using genomic M RNA as template and a primer MP40 (5'-CAGCTGCAGTAGTAGTAGTACTCCGCTAAGA) that should have 21

Figure 4. Cloning strategy of the PH virus M RNA genomic segment. The PH virus M RNA segment is represented by the solid line at the top of the figure. The size of clones M3', P111, R355, T103, S110, P108, P15, M5', and the portion of the PH virus M RNA they represent is shown below. The clones are represented as double-stranded to illustrate where the primers anneal. The sequence of primers MP61, MP62, and MP63 are shown in Materials and Methods. A; lane 1, 1/8 of the DNA from amplification of the 3' end of the PH virus M RNA segment using primers MP61 and MP63. The 643-bp fragment is M3' with the additional 9-bp *Pst*I site from the 5' end of each primer; lane 2, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; B; lane 1, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; lane 2, 1/10 of the DNA from amplification of the 5' end of the PH virus M RNA segment using primers MP62 and MP61. The 1344-bp fragment is M5' with the additional nucleotides from each of the primers.

Prospect Hill Virus M Genome



nucleotides complementary to the reported 3' terminus of the PH virus M RNA segment (underlined nucleotides) (Schmaljohn *et al.*, 1985). PCR amplification of this cDNA with primers MP40 and MP41 (5'-CAGCTGCA-GTTCTGTACTCTTTGA, nucleotide positions 356-370 measuring from the 3' end of the PH virus M RNA) which is complementary to PH virus M cDNA (underlined nucleotides) generated a fragment of the expected size (approximately 400-bp). However, the nucleotide sequence of this PCR fragment showed that the sequence of primer MP40 appeared three times whereas primer MP41 was not present. This fragment, although not representing the PH virus M RNA segment, showed some sequence similarity with the PH virus L RNA segment (data not shown). When a larger primer, MP60 (5'-CAGCTGCAGTAGTAGTAGACTCCGCTAAGAAAG) complementary to the entire PH virus M RNA 3' terminus (underlined nucleotides) reported by Schmaljohn *et al.* (1985), was used, no cDNA was produced. These results suggested that the PH virus 3' terminal sequence reported by Schmaljohn *et al.* (1985) was incorrect. All *Hantavirus* M RNA segments previously cloned and sequenced are identical for the first 16 bases at their 3' termini (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Antic *et al.*, 1991a). A primer MP61 (see Materials and Methods) that was complementary to this 16 base sequence also failed to generate any cDNA. A complex stable base-paired structure formed by the 3' and 5' termini of *Hantavirus* M RNA segments (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Antic *et al.*, 1991a) may have interfered with cDNA synthesis initiated by a primer this small. Therefore, it was decided to use PH virus M mRNA in total cell RNA isolated from PH virus infected cells as the template for first-strand cDNA synthesis.

To determine when total cellular RNA was rich in PH virus specific RNA, total RNA from PH virus infected cells was isolated 8 or 9 days post-infection. Dilutions of this RNA were blotted and hybridized using a nick translated M clone (S110) as a probe (Fig. 5). The total cell RNA extracted at day 9 post-infection clearly shows more PH virus specific RNA/ μ g of total cell RNA than does RNA extracted from cells on day 8 post-infection (Fig. 5). Presumably the majority of this PH virus specific RNA is mRNA. Cloning of the 3' end of the PH virus M RNA was done with primers MP61 and MP63 using PH virus M mRNA as template, as described in Materials and Methods, and generated a 643-bp PCR fragment (Fig. 4A, lane 1) that was blunt-end ligated into the *Sma*I site of pUC19 and designated M3'. Nucleotide sequence analysis of this clone revealed differences with the putative 3' terminal sequence of the PH virus M RNA previously reported (Schmaljohn *et al.*, 1985). The previously reported 3' terminal M RNA sequence had two additional bases at positions 17 and 23 (3'-AUCAUCAUCUGAGGCG**AUUCUUUC**, underlined and bolded bases) (Schmaljohn *et al.*, 1985) that were not present in our clone (3'-AUCAUCAUCUGAGGCGUUCUUC). To verify that our sequence was correct we repeated this experiment with primer MP63 and other 3' end primers (Fig. 6). Two primers, MP61 and MP67, were identical with the 5' terminal sequence we determined for the PH virus M cDNA and they yielded equal amounts of the 643-bp PCR fragment (Fig. 6, lanes 1, 4). Primer MP68 has a one nucleotide mismatch with the PH virus M cDNA at position 18 (G instead of A) and this primer generated a lower amount of the 643-bp PCR fragment (Fig. 6, lane 5). Primer MP60 has two additional bases at nucleotide positions 17 and 23 which are not present in our PH virus M cDNA and this primer yielded the least amount of the 643-bp PCR

Figure 5. Hybridization of a ^{32}P -labelled PH virus M specific clone to dot blots of total infected cell RNA. Total cell RNA was isolated from PH virus infected Vero cells 8 or 9 days after infection, dot blotted onto GeneScreen Plus and hybridized to nick-translated M clone S110. 1; 10 μg of total cell RNA from PH virus infected cells; 2; 5 μg of total cell RNA from PH virus infected cells; 3; 2.5 μg of total cell RNA from PH virus infected cells; 4; 1.25 μg of total cell RNA from PH virus infected cells; 5; 0.625 μg of total cell RNA from PH virus infected cells. The 8 or 9 above each dot indicates the number of days after infection the total cell RNA was isolated.

1	2	3	4	5
$\frac{8}{9}$	$\frac{8}{9}$	$\frac{8}{9}$	$\frac{8}{9}$	$\frac{8}{9}$
●	●	●		
●	●			
●				

Figure 6. Analysis of the 3' end of the PH virus M RNA segment by comparing PCR amplified products using different primers. The 3' end of the PH virus M RNA segment was amplified by PCR using primer MP63 and primers matching the M cDNA sequence in Fig. 7 or with a number of nucleotide changes causing mis-matches. A; Primers used in the analysis. Base changes are bolded and underlined; B; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; lane 1, Primer MP61; lane 2, Primer MP63; lane 3, Primer MP60; lane 4, primer MP67; lane 5, Primer MP68.

A

PH virus M cDNA: 5'-TAGTAGTAGACTCCGC AAGAA G

MP60: 5'-TAGTAGTAGACTCCGCTAAGAAAG

MP61: 5'-TAGTAGTAGACTCCGC

MP67: 5'-TAGTAGTAGACTCCGC AAGA

MP68: 5'-TAGTAGTAGACTCCGC AGGA

B



fragment (Fig. 6, lane 3) with the exception of the negative control (Fig. 6, lane 2). Furthermore, our sequence was identical to the reported 3' terminal sequence of the Hällnäs virus M RNA segment for the first 35 bases (Giebel *et al.*, 1989). Therefore, clone M3' most likely represents the actual 3' end of the PH virus M RNA segment. The nucleotide sequence of clones M3' and M5' were determined using the dideoxy chain termination method (Sanger *et al.*, 1977). The combined and consensus sequence (3707 bases) of clones M3', P111, R355, T103, S110, P108, P15, and M5' is shown in Figure 7. Comparison of the 3' and 5' termini of the PH virus M RNA segment in the genomic RNA sense revealed an inverse complementary sequence involving 38 of the terminal 45 nucleotides (Fig. 8). The calculated free energy of this structure is -47.1 kcal/mol at 25°C. The stability of this structure is similar to that of the Hantaan virus M segment (-42.4 kcal/mol at 25°C) and SR virus M segment (-39.6 kcal/mol at 25°C), but is much lower than that of the Hällnäs virus M segment (-64.8 kcal/mol at 25°C) (Giebel *et al.*, 1989). Complementarity between the terminal nucleotides of an RNA segment is a Bunyaviridae characteristic (Parker and Hewlett, 1981; Bishop *et al.*, 1982; Cabridilla *et al.*, 1983; Ihara *et al.*, 1984; Eshita and Bishop, 1984; Colett *et al.*, 1985; Schmaljohn *et al.*, 1986b, 1987b; Giebel *et al.*, 1989; Stohwasser *et al.*, 1990; Antic *et al.*, 1991a, 1991b). Therefore, it was concluded that clones M3', P111, R355, T103, S110, P108, P15, and M5' represented the entire PH virus M RNA segment. The base composition of the PH virus M RNA segment is 29.8% A, 18.8% G, 21.7% C, and 29.7% U. These values are similar to those of the Hällnäs virus, SR virus and Hantaan virus M RNA segments (Schmaljohn *et al.*, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990).

Figure 7. Nucleotide sequence of the PH virus M RNA segment. The sequence is presented as the viral complementary DNA (5'-3'). Nucleotides are numbered on the left and right ends of each line. The translation initiation codon (ATG, nucleotide positions 50-52) and translation termination codon (TAA, nucleotide positions 3476-3478) of the putative PH virus glycoprotein precursor are underlined.

1	TAGTAGTAGA	CTCCGCAAGA	AGAAGCAAAC	ACAGAGAGAA	CATAGAAAAA	TGAGTAAATT	60
61	TTGTCTGTGT	TTGAGTCTTT	TGGGAGTACT	ACTACTGCAG	GTCTGTGACA	CCAGGAGCCT	120
121	TCTGGAATTG	AAGATTGAAT	GTCCCTATAC	TGTAGGTTTA	GGTCAAGGCC	TTGTAATAGG	180
181	GACTGTGGAT	TTAAACCCTG	TCCCTGTTGA	GTCTGTGAGC	ACATTAAAGT	TAGAAAGCTC	240
241	TTGTAACTTT	GATGTGCATA	CAAGCTCAGC	CACACAGCAG	GCTGTTACAA	AGTGGACATG	300
301	GGAAAAGAAA	GCTGACACAG	CAGAAACAGC	AAAGGCAGCT	TCAACAACAT	TTCAATCAAA	360
361	GAGTACAGAA	TTAAATTTAC	GGGGCTTATG	TGTTATTCCA	ACTTTGGTCC	TTGAGACTGC	420
421	TAATAAGCTC	CGTAAGACTG	TCACATGTTA	TGATCTTTCA	TGTAATCAGA	CAGCATGCAT	480
481	CCCAACAGTT	TACTTAATTG	CTCCCATACA	TACATGTGTG	ACAACAAAGA	GCTGTTTACT	540
541	GGGATTAGGA	ACTCAACGGA	TTCAAGTGAC	TTATGAAAAG	ACATACTGTG	TGTCTGGACA	600
601	ACTGGTAGAA	GGCAGATGCT	TCAATCCGAT	CCACACAATG	GCATTATCTC	AACCAAGCCA	660
661	TACATATGAT	ATTGTCACATA	TACCAGTCAG	GTGTTTTTTC	ATTGCAAAGA	AAACAATGA	720
721	TGATACCCCTT	AAAATAGAGA	AACAGTTTGA	GACAATTCTA	GAGAAATCAG	GGTGTACAGC	780
781	TGCAAAATATA	AAAGGCTATT	ATGTCTGTTT	TTTAGGTGCA	ACATCAGAAAC	CTATATTTGT	840
841	GCCAACAAATG	GATGATTTTA	GAGCATCCCA	GATCTTGTCT	GATATGGCAA	TTTCTCCTCA	900
901	CGGGGAAGAT	CACGACAGTG	CTCTATCATC	TGTGAGTACC	TTTAGAATTG	CAGGGAAATT	960
961	AAGCGGAAAG	GCACCATCCA	CTGAGTCATC	CTGACTGTGC	CAAGGTGTAG	CATTTTCAGG	1020
1021	ACATCCATTG	TATACGTCCC	TTTCCGTGCT	AGCCTCAAAG	GAAGATCCAG	TTTACATTTG	1080
1081	GTCACCTGGA	ATAATCCCGG	AACGGAATCA	CACTGTATGT	GACAAGAAGA	CACTACCATT	1140
1141	GACATGGACA	GGTTATTTGC	CATTGCCTGG	AGGGATCGAA	AAGACCACAC	AGTGCACCAT	1200
1201	ATTTTGTACT	CTTGCTGGGC	CCGGTGCAGA	TTGTGAAGCA	TATTCAGACA	CAGGTATTTT	1260
1261	CAATATCAGT	TCACCCACAT	GTCTCATAAA	TCGAGTTCAG	AGATTTCCGG	GGGCAGAGCA	1320
1321	ACAAATCAAA	TTTGTGTGTC	AGCGGGTAGA	CCTGGATATC	GTTGTGTACT	GTAATGGCAT	1380
1381	GAAGAAGGTA	ATTCTTACTA	AAACCTTGT	TATAGGTCAG	TGCATATATA	CATTCAACAAG	1440
1441	TGTTTTCTCC	CTAATGCCTG	GAATTGCTCA	TTCACTTGCA	GTGGAATTGT	GTGTCCCTGG	1500
1501	CATACACGGG	TGGTCAACAA	TTGCTTTACT	TGCAACATTC	TGTTTTGGCT	GGCTCCTTAT	1560
1561	CCCTATCATA	TCTCTAGTAT	CAATCAAGAT	AATGCTACTT	TTTGCTTATA	TGTGTTCAAA	1620
1621	ATATAGTAAT	GACTCGAAAT	TCCGGTTACT	TATAGAAAAG	GTTAAGCAGG	AATATCAGAA	1680
1681	AACTATGGGT	TCTATGGTCT	GTGAGGTTTG	TGAGCAGGAA	TGTGAAATGG	CAAAAAGATT	1740
1741	GGAGTCTCAC	AAGAAAAGTT	GTCCTAATGG	TATGTGCCCT	TACTGCATGA	ACCCTACAGA	1800
1801	GTCTACTGAG	TCTGCCCTGC	AAGCTCATTT	CAAAGTGTGT	AAACTTACAA	CAAGGTTCCA	1860
1861	GGAGAACCCT	CGCAAGTCAC	TAAATCCATA	TGAGCCATAA	CGAGGTGTCT	ATAGGACACT	1920
1921	CTCTGTGTTT	AGATATCGGA	GTAGGTGCTT	TGTGGGCCTT	GTTTGGTGCA	TCCTACTTGT	1980
1981	ATTAGAACTT	GTCATATGGG	CTGCAAGTGC	GGATACTGTA	GAAATAAAAA	CAGGTTGGAC	2040
2041	TGACACTGCA	CATGGAGCTG	GGGTAATACC	ACTCAAATCA	GATTTAGAGC	TTGATTTTTT	2100
2101	GTTGCCATCA	TCTGCAACAT	ACATATACAG	ACGAGATCTT	CAAAACCCAG	CAAAATGAGCA	2160
2161	AGAAAAGATC	CCTTTTCACT	TCCAGCTACA	CGCTCAGGTG	ATTCATGCAG	AGATTCAAAA	2220
2221	TTTAGGCCAC	TGGATGGATG	GAACATTTAA	TCTGAAAACA	TCATTCCATT	GTTACGGTGC	2280
2281	TTGTGAAAAG	TATGCTTATC	CGTGGAACAC	TGCAAAATGT	TTCTTAGAGA	AAGATTATGA	2340
2341	ATTTGAAACA	GGGTGGGGCT	GTAATCCTGG	TGATTGCCCA	GGTGTAGGCA	CTGGATGTAC	2400
2401	TGCTTGTTGA	GTTTATTTGG	ATAAATTGCG	CTCTGTTGGC	AAGGTATTCA	AAGTGATATC	2460
2461	CCTAAAGTTC	ACAAGACGGG	TTTGTATACA	ATTAGGCAGT	GAACAATCAT	GTAAGACCAT	2520
2521	TGATTGCAAT	GACTGTTTGA	TGACAACATC	TGTGAAGGTT	TGCATGATTG	GGACAGTTTC	2580
2581	AAAATTTTCA	CCCGGTGATA	CCCTTTTATT	TTTAGGCCCT	TTAGAGGAAG	GTGGTATTAT	2640
2641	ATTTAAGCAA	TGGTGTACTA	CTACCTGTCA	CTTTGGAGAC	CCGGGGGACA	TCATGAGTAC	2700
2701	TCCGCAGGGT	ATGCAATGCC	CAGAGCATAC	AGGTGCCTTT	AGAAAGAAAT	GTGCATTTGC	2760
2761	TACAATGCCA	ACATGTGAGT	ATGATGGAAA	CACCTTTTCT	GGTTACCAGC	GGATGCTTGC	2820
2821	AACAAGGGAT	TCTTTCCAGT	CTTTCAACAT	TACAGAACCA	CATATTACCA	GCAATTCACT	2880
2881	TGAATGGGTA	GATCCAGATA	GTTCAATTAA	GGATCATATT	AATTTGGTAG	TTAATCCCGA	2940
2941	TGTATCATTT	CAGGACTTAT	CTGAAAATCC	CTGTCAAGTT	GGTGTGGCCG	TGTCTTCCAT	3000
3001	TGATGGTGCA	TGGGGCTCAG	GTGTGGGTTT	TAATCTGGTA	TGTAGTGTGA	GTTTGACAGA	3060
3061	ATGTGCTAGT	TTTCTAACTT	CAATAAAGGC	ATGTGACGCT	GCAATGTGTT	ACGGTGCAAC	3120
3121	AACAGCTAAT	CTAGTGAGAG	GGCAAAATAC	TGTGCACATT	TTAGGAAAAG	GGGGACACTC	3180
3181	TGGCTCAAAA	TTTATGTGTT	GCCATAGCAC	TGAATGTTCA	TCAACAGGCT	TGACAGCTGC	3240
3241	TGCACCACAT	CTTGATAGAG	TCACTGGCTA	TAATGTTATC	GACAATGATA	AAGTATTTGA	3300
3301	TGACGGCTCT	CCTGAGTGTG	GTGTACACTG	CTGGTTTAAA	AAGTCTGGGG	AATGGTTAAT	3360
3361	GGGGATTTTG	AGTGGTAATT	GGATGGTAGT	GGCAGTTTTA	GTCGTACTGC	TGATCTTATC	3420
3421	AATATTTCTTA	TTCAGCTTAT	GTTGCCCTCG	AAGGGTTGTC	CATAAAAAAT	CCTCGTAACT	3480
3481	TAGATCCATA	ATCATTTTACT	CACAAATGTG	CATGTTGTTA	ACCCTAGAAT	TACACTTATG	3540
3541	TATTACTTCT	ATTATAGCAC	TTTAAACTAA	ACATACTAAT	TTAATAAGTA	ATAAATAGCA	3600
3601	TATATATATT	CACTCTTAAT	CATATATCAA	AAAAAACTAA	AACTTTGGTA	TACTAACCCT	3660
3661	ATATATCAGC	TTTACCTGGA	CTTTTGTTC	TGCGGAGTCT	ACTACTA		3707

Figure 8. Predicted secondary structure of the complementary 3' and 5' termini of the PH virus M RNA segment. The terminal 45 nucleotides of the PH virus M RNA, as deduced from the cDNA sequence, are shown. The free energy value for this structure (-47.1 kcal/mol at 25°C) was calculated using the RNA folding program in the PC/Gene computer program (Version 5.11).

[illegible]

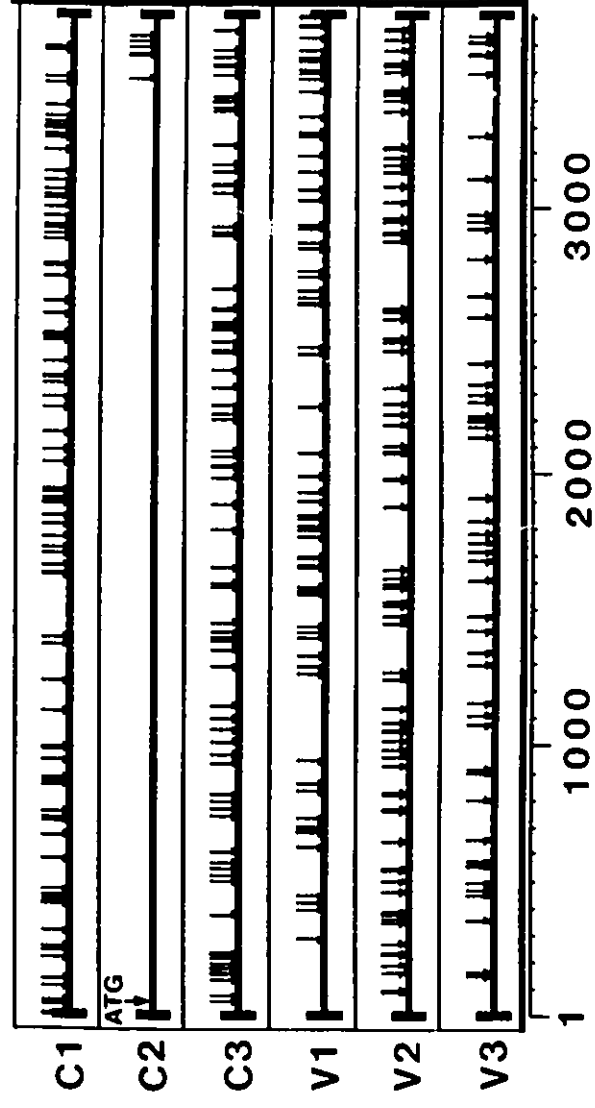
Coding Strategy of the PH Virus M RNA

The PH virus M RNA segment is 3707 bases long (Fig. 7). This is the largest *Hantavirus* M segment reported to date as compared with 3682 bases for Hällnäs virus, 3651 bases for SR virus, Seoul virus and Biken 1 virus, and 3616 bases for Hantaan virus (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Isegawa *et al.*, 1990; Antic *et al.*, 1991a). Positions of translation termination codons were determined for the six potential reading frames of the PH virus M RNA sequence. A large open reading frame (ORF) was observed in frame 2 of the M cRNA (Fig. 9). The first potential in-frame translation initiation codon in the PH virus M ORF is at nucleotides 50-52 near the 5' end of the cDNA (Fig. 7). The flanking sequence for this initiation codon is not considered optimal for translation, since although there is an A at the -3 position there is also an A at the +4 position (Kozak, 1984, 1986b, 1986b) (Fig. 7). This ORF terminates at nucleotides 3476-3478 and has a coding capacity of 1142 amino acids (Fig. 7). This is 8 amino acids larger than the SR virus (1134 amino acids) glycoprotein precursor and 6 amino acids larger than the Hantaan virus (1136 amino acids) glycoprotein precursor (Schmaljohn *et al.*, 1987b; Yoo and Kang, 1987b; Arikawa *et al.*, 1990), but 6 amino acids smaller than the Hällnäs virus glycoprotein precursor (Giebel *et al.*, 1989).

Comparison of the PH Virus, Hällnäs Virus, SR Virus and Hantaan Virus G1 and G2 Proteins

The deduced amino acid sequences of the PH virus, Hällnäs virus, SR virus, and Hantaan virus G1 and G2 proteins were compared (Fig. 10a, and 10b). The cleavage site between the signal peptide and N-terminus of G1 is known for SR virus and Hantaan virus (Schmaljohn *et al.*, 1987b;

Figure 9. Translation termination codon locations in all reading frames of the PH virus M RNA segment. Translation termination codons in the PH virus M RNA are indicated by arrows in the three reading frames of the viral complementary sense (C) and viral sense (V). The first potential ATG initiation codon in the longest open reading frame is also indicated by an arrow.



Nucleotides

Figure 10a. Comparison of the predicted amino acid sequences of the PH virus, Hällnäs virus, SR virus, and Hantaan virus G1 proteins. Amino acids are numbered on the left and right ends of each line. Non-identical amino acids of Hällnäs virus, SR virus, and Hantaan virus G1 proteins are presented below the PHV G1 protein sequence. Identical amino acids are denoted by dots, conservative amino acid changes are underlined, and missing amino acids in any sequence are denoted with a dash. Small boxes with 3 amino acids denote potential N-linked glycosylation sites. The large box denotes the potential transmembrane domain common to all four viruses.

|---- G1 ---->

PH: 1 --MSKFCLCLSLGVLQVCDTRSL-ELKIECPHTVGLGQGLVIGTVDLNPVPVESVS 57
Hallnas: 1 MGELSPVCLYL..QG..CNTGAARN.N...M.....IR.....V.S.E.PSL.IQQ.E 60
SR: 1 -----MWSL..LAA.VGGGFALKNVFDMR.QL..S.NF.ETS.S.Y.EFP.LSLOEAE 53
Hantaan: 1 -----MGIWKW.VMAS.VWPVL.LRNVYDM.....SF.GNS...Y.E.P...LADTA 55

PH: 58 TLKLESSCNFVDHTSSATQQAHTKWTWEKKADTAETAKAASSTTFQSKSTELNLRGLCVIP 117
Hallnas: 61L...T.G..SF.....I.G.L..NTQ.S..S..T..S.V.....L.. 120
SR: 54 Q.VP.....M.N.Q.LS.I---N.L.KVIWRKK.NQES.NQNS.EVVE.SVSFK...MLK 110
Hantaan: 56 QMVP.....M.N.Q.LN.I---Y.QVSWRGK.DQSQS.QNS.ETV...VD.K.T..LK 112

PH: 118 TLVLETANKLRKTVTCTYDLSONQTAACIPTVYLIAPHTCVTTKSCLLGLGTQRIQVTEK 177
Hallnas: 121 ...V...ARM...IA.....V.Q....MG..Q..I.....S..D.....N... 180
SR: 111 HRMV.ESYRN.RS.I....A..B.F.K....M.V...A.NMM....I...PY....V..B 170
Hantaan: 113 HKMV.ESYRS..S.....B.V.K..L.M.V...A.NMM....IA..PY.V..V..B 172

PH: 178 TYCVSGQLVEGTCFNPHTMALSQPSHTYDIVTIPVRCFFIAKK-TNDDTLKIEKFETI 236
Hallnas: 181D....I.....M.MM....LVI..V.SG.SM...N...L 240
SR: 171 ...TT.I.T..K..V.DKAVVSALKRGM.A.AS.ETI...HQ.GNTYKIYTAITSAMGS 230
Hantaan: 173 ...MT.V.I..K..V.DQSVVSIKHGIF..ASVHIV...V.V.GNTYKIFEQV..SFES 232

PH: 237 LEKSGCTAANIKGYVCFLGATSEPIFVPTMDDFRASQILSDMAISPHGEDHDSALSSVS 296
Hallnas: 241 VQ.N....N.FQ...I..LI.SS...LY..AL..Y.SAEV..R..FA.....IEKNA.. 300
SR: 231 KCMN--DTKVQ...I..I.GN.A.VYA.AGE...MEVF.GIIT.....LPGEEIA 288
Hantaan: 233 TCMN--ENKVQ...I..IV.GN.A..Y...L.....MEAFGTGIFR.....L.GEEIA 290

PH: 297 TFRIAGKLSGKAPSTESSDTVQGVAFSGHPLYTSLSVLASKEDPVYIWSPGIIPERMTV 356
Hallnas: 301 AM.....VT..V.....I....S....TG..T..D.....A....M.G..BI 360
SR: 289 .YQ.S.QIEA.I.H.V..KNLKL.T.A.I.S.S.T.I..AS..GRF.F...LF.NL.QB 348
Hantaan: 291 SYS.V.PANA.V.HSA....LSLI.Y..I.S.SL..I.T.STEAKHYF...LF.KL..N 350

PH: 357 CDKKTPLPLTWTGYLPLPGGIEKTTQCTIFCTLAGPGADCEAYSDTGIFNIBSPTCLINRV 416
Hallnas: 361 .E.....FIS...E.....V.....E..... 420
SR: 349 ..NNA...I.R.LID.T.YY.AVHP.NV..V.S....S...F.EG...T..M..VSKQ 408
Hantaan: 351 ...SAI..I...MID...YY.AVHP..V..V.S....S...F.EG...T..M..VSKQ 410

PH: 417 QRFRGAEQQIKFVCQVRDLDIVVYCNMGKKVILTKTLVIGQCIYFTFSVFSLMPGIAHSL 476
Hallnas: 421S.....M..T.....L.....I...I..V.... 480
SR: 409 N...A.....S.....M..I....Q..T.....I..L..L..V...I 468
Hantaan: 411 N...LT...VN.....M.....QB.....I..L..L..V...I 470

PH: 477 AVELCVPGIHGWSTIALLATFCFGWLLIPIISLVSIKIMLLFAYMCSKYSNDBKFRLLIE 536
Hallnas: 481A.ML..L.....V...T.TMILL..LIA...L.....NT.....I... 540
SR: 469 .I.....F...A.A..I.....V...ACT.AILLVLKF..NILHTSNOENR.KAILR 528
Hantaan: 471F...A.A..V.....V...A.TFIILTVLKFI.NIFHTSNOENRLKSVLR 530

PH: 537 KVKQEYQKTMGSMVCEVCQCECEMAKELESHKKSCPNGMCPYCMNPTESTESALQAHFKV 596
Hallnas: 541 ...R.....Y...T.....R...SI.S....L..S.A.T..... 600
SR: 529 .I.E.FE..K.....I.KY...TL..KA.NL.VQ.E...FTHC.P..T.I...Y.. 588
Hantaan: 531 .I.E.FE..K.....D..KY...TY...KA.GV...QSQ...FTHC.P..A.F...Y.. 590

PH: 597 CKLTTRFQENLRKSLNPYEPKRGCYRTLVSFRYRSRCFVGLVWCILLVLELVIWAASA 654
Hallnas: 601 ...RS.....TV...MQ.....L.....F.....V...HH.IV.... 658
SR: 589 .QA.H..R.D.K.TVT.QNIGP.....NL...K...YILTM.TL..II.SIL.... 646
Hantaan: 591 .QV.H..RDD.K.TVT.QNFTP.....NL...K...YIFTM.IF.....SIL.... 648

Figure 10b. Comparison of the predicted amino acid sequences of the PH virus, Hällnäs virus, SR virus, and Hantaan virus G2 proteins. Amino acids are numbered on the left and right ends of each line. Non-identical amino acids of Hällnäs virus, SR virus, and Hantaan virus G2 proteins are presented below the PHV G2 protein sequence. Identical amino acids are denoted by dots, conservative amino acid changes are underlined, and missing amino acids in any sequence are denoted with a dash. Small boxes with 3 amino acids denote potential N-linked glycosylation sites. The large box denotes the potential transmembrane domain common to all four viruses.

PH: 655 DTVEIKTGWTDTAHGAGVIPLKSDLELDFSLPSSATYIYRRDLQNPANEQERIPFHFQLQ 714
 Hallnas: 659 E.QNLNA.....SRI..M.T.....Q...S.T...Q.....K....L..S 718
 SR: 647 AEIPLVPL...N...V.SV.MHT.....SK.T.K.H.T..V.D.QSVSL.IEIE 706
 Hantaan: 649 SETPLTPV.N.N...V.SV.MHT.....T..SK.T...K.T..LE.AQS.DL.IEIE 708

PH: 715 RQVIHAEIQNLGHWMDGTFNLKTSFHCYACEKYAYPWQTAKCFLEKDYEFETGWGCNPG 774
 Hallnas: 719 K.....H.....A.....A.....S.....G..I.....Y.....P 778
 SR: 707 S.G.G.AVHH....Y.ARL.....T..Q...H....HF.....Y.NS.A...P 766
 Hantaan: 709 E.T.GVDVHA....F..RL.....T..E...H....HY..R..QY..S.....S 768

PH: 775 DCPGVGTGCTACGVYLDKLRSVGKVKVISLKFTRRVCIQLGSEQSCKTIDSNDCLMTTS 834
 Hallnas: 779K.....IV...Y..K.....T..T...V.....I... 838
 SR: 767L...Q.KP..TA..I..VRY.S.K..V.F.E.HL....M...FV..RH 826
 Hantaan: 769L...Q.KP..SAY..I..TIRYS....V.F.E.NL..I..M...FVSRH 828

PH: 835 VKVCMIGTVSKFQPGDTLLFLGLEGGIIFKQWCTTTCHFGDPGDIMSTPQ-GMQCPEH 893
 Hallnas: 839L...I.....S.....QQ..L.....Q.....T...K...L 897
 SR: 827 A..I..I.....SQ.....M.G.....H...S.....V.GPKDKPFI...F 886
 Hantaan: 829I.....SQ.....F....G..L...H...S...Q.....PRDK.FL...F 888

PH: 894 TGAFRKKCAFATMPTCEYDGNLTSQYQRMALTRDSFQSFNITEPHITSNSLEWVDPDSSL 953
 Hallnas: 898 N.S.....T.V.QF...I...K..I..K.....V.....STSA...I..... 957
 SR: 887 P.Q.....N...T.V.....II...KKV...I.....TSNI.F.DERI..R...GM. 946
 Hantaan: 889 P.S.....N...T.I.....MV...KKVM..I.....TSTM.F.DERI..K...GM. 948

PH: 954 KDHINLVVNRDVSFQDLSENPCQGVAVSSIDGAWGSGVGFNLVCSVSLTECASFLTSIK 1013
 Hallnas: 958 R....VI.S..L.....T...IDL.TA.....T.....SA..... 1017
 SR: 947 R....I..ISK.ID.EN.A...K..LQAN.E.....T.T.K.....PT..... 1006
 Hantaan: 949 R....IL.TK.ID.DN.G....KI..LOT...E.....T.T.L.....PT..... 1008

PH: 1014 ACDAAMCYGATTANLVRGQNTVHILGKGGHSGSKFMCCHSTECSSGTAAAPHLDRTVG 1073
 Hallnas: 1018S.....I..V.....D.K.....V.....P 1077
 SR: 1007 ...M..I....ESVT.S.....K.T.....S.K...GK.....Q.S.....K.N. 1066
 Hantaan: 1009 ...K..I....ESVT.T.....KVS.....T.R...GED..QI..H.....K.N. 1068

PH: 1074 YNVIDNDKVFDDGSGPCGVHCWFKKSGEWMGILSGNWMVAVLVVLLILSIFLFLCCP 1133
 Hallnas: 1078 ..QA.S...I....A....MS.....IL.V.N.....L..T... 1137
 SR: 1067 ISELE.E..Y...A....IT.....V...IN...V..LI..C...LF.LI.L..IL.. 1126
 Hantaan: 1069 ISE.E.S..Y...A.Q..IK...V....IS..F...I..LI..C.F.LF.LV.L..IL.. 1128

PH: 1134 -RRVVHKKSS-- 1142
 Hallnas: 1138 -.PSYR.EHKP 1148
 SR: 1127 V.K--..... 1133
 Hantaan: 1129 V.K--..... 1135

Arikawa *et al.*, 1990). However, the signal peptide cleavage sites for PH virus and Hällnäs virus were based on computer predictions. Conservation of cysteine residues between PH virus, Hällnäs virus, SR virus, and Hantaan virus is very high (G1 = 78.4%, and G2 = 93.1%). The results of the amino acid comparison of the G1 and G2 proteins of Hällnäs virus, SR virus and Hantaan virus to PH virus are summarized in Table 3. The PH virus G1 and G2 proteins have more amino acid sequence similarity with the Hällnäs virus G1 and G2 proteins than with the G1 and G2 proteins of SR virus and Hantaan virus (Table 3). The G2 proteins of all four viruses have been conserved to a greater extent than the G1 proteins (Fig. 10b; Table 3). A possible variable region was identified in the G1 protein of these four viruses (PH virus amino acid positions 209-282) (Fig. 10a). More amino acid sequence heterogeneity between the four viruses was observed in this region as compared with other regions of the G1 or G2 proteins. Amino acid sequence similarity between the G1 proteins of the four viruses was 38.7% and 52.2% between the G2 proteins of these viruses. If conservative amino acid substitutions are included these numbers increase to 54.6% for the G1 proteins and 67.1% for the G2 proteins.

Three of the four potential asparagine-linked glycosylation sites in the PH virus G1 protein were conserved in the other three viruses. A potential glycosylation site at amino acid positions 527-529 was not conserved in the other three viruses (Fig. 10a). However, SR virus had two other potential sites (amino acid positions 233-235 and 560-562) while Hantaan virus had only one other potential site (amino acid positions 235-237) (Fig. 10a). Interestingly, a possible glycosylation site that is probably not used in Hällnäs virus (N-P-S, amino acid positions 585-587),

Table 3. Comparison of the amino acid sequences of the G1 and G2 proteins of Hällnäs virus, SR virus, and Hantaan virus to the amino acid sequences of the G1 and G2 proteins of PH virus.

VIRUS	G1 PROTEIN		G2 PROTEIN	
	I ^a	(I + C) ^b	I ^a	(I + C) ^b
Hällnäs	74.3%	85.8%	79.0%	88.4%
SR	46.9%	62.6%	62.5%	75.4%
Hantaan	49.4%	66.0%	61.3%	73.4%

^a Identical amino acids.

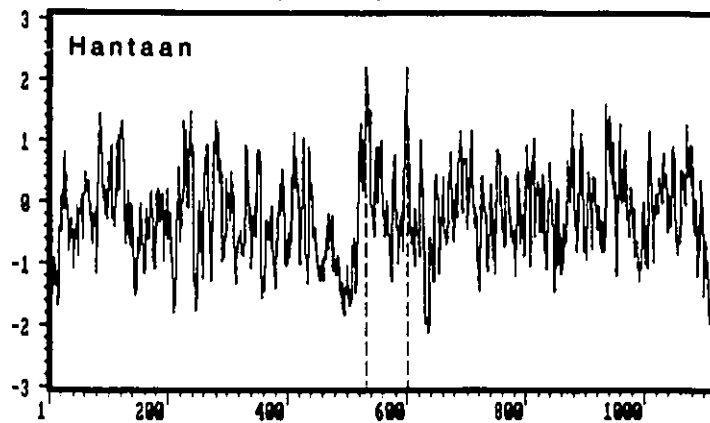
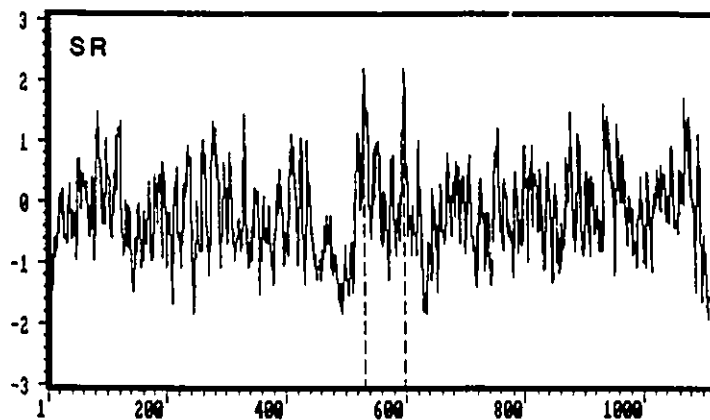
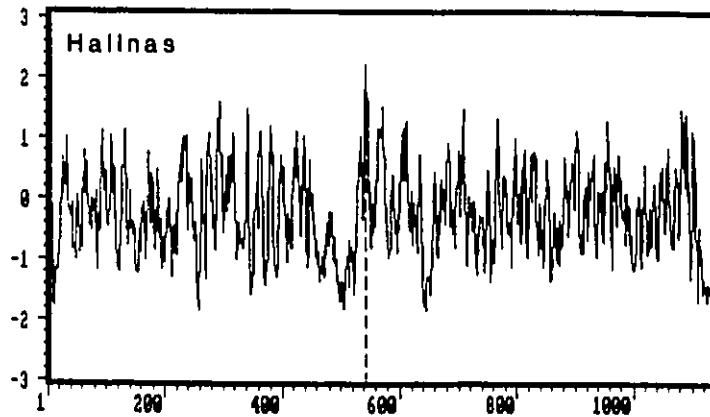
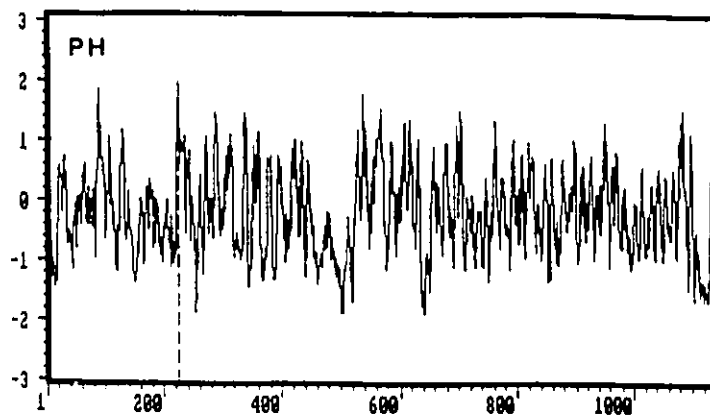
^b Identical amino acids plus conservative amino acid substitutions using the following criteria for conservative substitutions: R=K, S=T, D=E, Q=N, V=L, I=M, A=G, A=V, Y=F.

because of the proline residue (Bause, 1983), is also present in PH virus (N-P-T, amino acid positions 581-583) (Fig. 10a). The one potential glycosylation site in the PH virus G2 protein was conserved in all four viruses (Fig. 10b). A second site in the Hällnäs virus G2 protein (amino acid positions 898-900) is not observed in the PH virus G2 protein (Fig. 10b).

The analysis of potential transmembrane helices (Rao and Argos, 1986) identified only one conserved region in the G1 protein and one conserved region in the G2 protein of all four viruses. The region in the G1 protein ends 132 amino acids from the C-terminus (PH virus amino acid positions 447-522) and was 76 amino acids in length in PH virus and Hällnäs virus, and 71 amino acids in length in SR virus and Hantaan virus (Fig. 10a). The conserved region in the G2 protein is near the C-terminus (PH virus amino acid positions 1102-1132) and is 31 amino acids long in all four viruses (Fig. 10b).

Hydrophilicity profiles (Hopp and Woods, 1981) of the four virus glycoprotein precursors were compared (Fig. 11). There were many similarities between the hydrophilicity profiles of the four viruses. The region of greatest hydrophilicity in the Hällnäs virus profile was conserved in the SR virus and Hantaan virus profiles, and is located near the C-terminus of G1 beginning fourteen amino acids after the potential transmembrane domain (Fig. 11). The SR virus and Hantaan virus profiles also have a second equally hydrophilic region near the C-terminus of G1 (Fig. 11). In contrast, the region of greatest hydrophilicity in the PH virus glycoprotein precursor is closer to the N-terminus (amino acid positions 220-225, Fig. 11). This region is apparently associated with an amino acid deletion in the PH virus G1 protein not observed in the G1

Figure 11. Hopp and Woods hydrophilicity profiles of PH virus, Hällnäs virus, SR virus, and Hantaan virus glycoprotein precursors. Amino acid numbers are shown below each profile. The point of highest hydrophilicity in each profile is indicated by a broken vertical line.



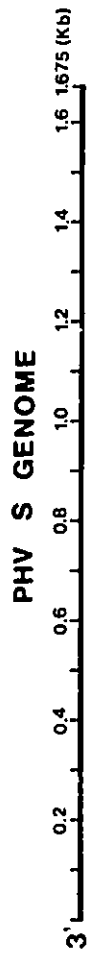
proteins of the other three viruses (Fig. 10a). When hydrophilicity profiles of the G2 proteins were compared, the highest peak of hydrophilicity in Hällnäs virus, SR virus, and Hantaan virus was located at the C-terminus. The region of greatest hydrophilicity in the PH virus G2 protein however, is located twelve amino acids prior to the transmembrane domain (amino acid positions 1084-1089, Fig. 10b).

4. MOLECULAR CHARACTERIZATION OF THE PH VIRUS S RNA SEGMENT

Cloning and Sequence Analysis of the PH Virus S RNA Segment

A cDNA bank of clones representing the PH virus RNA was made using random primers as described in Materials and Methods. PH virus S RNA specific clones were identified by Northern blot analysis. The largest S specific clone (P275) isolated from this bank represented 1176 bases of the PH virus S RNA, and therefore P275 could not represent the entire PH virus S RNA segment. The nucleotide sequence of this clone was determined and compared with the nucleotide sequence of the Hantaan virus S RNA segment (Schmaljohn et al., 1986b). This comparison revealed clone P275 lacked approximately 500 bases from the 3' end of the PH virus S RNA, and approximately 50 bases from the 5' end. We decided to clone the 3' and 5' ends of the PH virus S RNA segment by PCR using the same method used for the 5' end of the PH virus M RNA segment. PCR amplification of the 3' end of the PH virus S RNA was done using primers MP11 and MP12 (Fig. 12) as described in Materials and Methods. Amplification generated a 534-bp fragment (Fig. 12A, lane 2) that was cloned into the *Pst*I site of pUC19 and designated

Figure 12. Cloning strategy of the PH virus S RNA genomic segment. The PH virus S RNA segment is represented by the solid line at the top of the figure. The size of clones PS3, P275, and PS5, and the portion of the PH virus S RNA they represent is shown below. The clones are represented as double-stranded to illustrate where the primers anneal. The sequences of primers MP11, MP12, MP13, and MP14 are shown below the clones. Dark solid lines above the primers indicate sequences homologous with viral RNA. Dark solid lines below the primer indicate viral complementary sequence. Light solid lines indicate the *Pst*I sites on all the primers that allowed insertion of the amplified products into the *Pst*I site of pUC19. A; lane 1, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; lane 2, 1/5 of the DNA from amplification of the 3' end of the PH virus S RNA segment using primers MP11 and MP12. The 534-bp fragment is PS3 with the additional 9-bp from each of the primers; B; lane 1, 1/10 of the DNA from amplification of the 5' end of the PH virus S RNA segment using primers MP13 and MP14. The 242-bp fragment is PS5 with the additional 9bp from each of the primers; lane 2, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers.



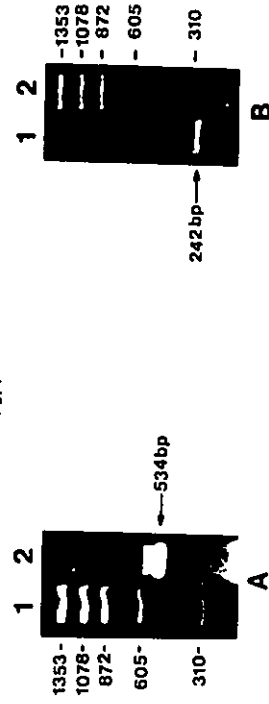
P275 (1176bp)

3' 5'

MP11 PS3 (516bp) 3' 5' MP12 MP13 PS5 (224bp) 3' 5' MP14

MP11: 5'-CAGCTGCAG TAGTAGTAGAC TTCGTAAGA-3' MP13: 5'-CAGCTGCAGCTAATTCACAATTAACATT-3'

MP12: 3'-TATTCGCCCTGTTCCCTAGTCCGACGTCGAC-5' MP14: 3'-ATGATGATGACGTCGAC-5'



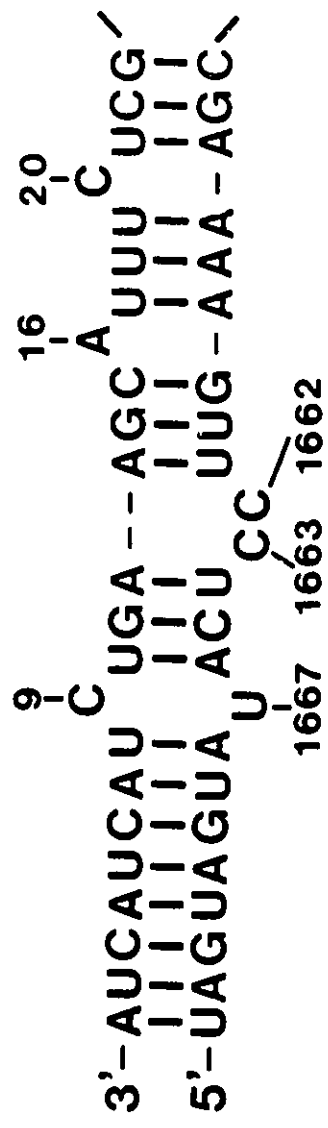
PS3. Nucleotide sequence analysis of PS3 revealed that it contained the reported 3' terminal sequence for the PH virus S RNA segment (3'-AUC-AUCAUCUGAAGCAUUUCUCGAU) (Schmaljohn et al., 1985). Therefore, clone PS3 most likely represented the 3' end of the PH virus S RNA segment. The 5' end of the PH virus S RNA was also cloned using the PCR method (see Materials and Methods) with primers MP13 and MP14 (Fig. 12). The 242-bp amplified product (Fig. 12B, lane 1) was ligated into the *Pst*I site of pUC19, and designated PS5. Nucleotide sequencing of PS5 demonstrated that clone P275 was missing only 20 nucleotides from the 5' terminus of the PH virus S RNA segment. The nucleotide sequence of clones PS3, P275, and PS5 were determined by the dideoxy chain termination method (Sanger et al., 1977).

The combined and consensus sequence (1675 bases) of PS3, P275, and PS5 is shown in Figure 13. Comparison of the 3' and 5' termini of the consensus sequence in the genomic RNA sense revealed a complementary sequence involving 20 of the terminal 23 nucleotides (Fig. 14). The base-paired structure that could result had a calculated free energy of -15.0 kcal/mol at 25°C which is lower than that of the Hällnäs virus S segment (-41.0 kcal/mol at 25°C), SR virus S segment (-29.6 kcal/mol at 25°C) and Hantaan virus S segment (-23.6 kcal/mol at 25°C). Complementarity between the terminal nucleotides of an RNA segment is characteristic of the Bunyaviridae (Parker and Hewlett, 1981; Bishop et al., 1982; Cabridilla et al., 1983; Ihara et al., 1984; Eshita and Bishop, 1984; Colett et al., 1985; Schmaljohn et al., 1986, 1987; Giebel *et al.*, 1989; Stohwasser *et al.*, 1990; Antic *et al.*, 1991a, 1991b). Therefore, we concluded that clones PS3, P275, and PS5 represented the entire PH virus S RNA segment. Base composition of the PH virus S RNA was calculated as 25.9% A, 20.9%

Figure 13. Nucleotide sequence of the PH virus S RNA segment. The sequence is presented as the viral complementary DNA (5'-3'). Nucleotides are numbered on the left and right ends of each line. The translation initiation codon (ATG, nucleotides 43-45) and translation termination codon (TAG, nucleotides 1342-1344) of the putative PH virus N gene are bolded and underlined. The translation initiation codon (ATG, nucleotides 83-85) and translation termination codon (TGA, nucleotides 353-355) of the putative PH virus NS₅ protein are underlined.

1	TAGTAGTAGA	CTTCGTAAAG	AGCTACTACT	ACAAGTGCTG	GGATGAGCCA	50
51	ACTCAGGGAA	ATACAGGAAG	AGATCACTCG	CCATGAGCAG	CAGCTTGTCA	100
101	TTGCCCCGCA	GAAGCTCAAG	GAAGCTGAAC	GGACGGTGGA	GGTGGACCCA	150
151	GATGACGTTA	ACAAAAGTAC	ACTGCAAAGC	AGGCGGTGAG	CAGTGTCAAC	200
201	ATTGGAGGAC	AAATTGGCAG	AGTTCAAGAG	GCAGCTTGCA	GATGTCATCT	250
251	CACGTCAGAA	GATGGATGAG	AAACCTGTGG	ATCCAACCTGG	TATTGAGCTT	300
301	GACGACCATC	TTAAGGAGAG	GTCAAGCCTC	CAATATGGAA	ATGTCCTTGA	350
351	TGTGAATTCC	ATTGATATAG	AAGAACCTAG	TGGCCAGACA	GCTGATTGGC	400
401	TTAAGATTGG	CAGCTACATC	ATAGAATTG	CACTACCTAT	CATCTTGAAA	450
451	GCCTTGcata	TGTTGTCAAC	TAGAGGGAGG	CAAACTGTAA	AAGAGAATAA	500
501	GGGGACAAGG	ATCAGGTTCA	AAGATGATAG	TTCCTATGAA	GATGTGAATG	550
551	GCATTAGACG	CCCAAAGCAC	CTTTATGTGT	CTATGCCAAC	AGCCCAGTCA	600
601	ACAATGAAAG	CTGAGGAATT	AACACCAGGG	AGATTCAGGA	CAATTGTTTG	650
651	TGGACTATTT	CCTGCACAGA	TCATGGCAAG	AAATATCATC	AGTCCTGTAA	700
701	TGGGTGTGAT	CGGATTTGCA	TTTTTTGTAA	AGGATTGGGC	TGACAAAGTA	750
751	AAGGCATTTC	TTGACCAGAA	ATGTCCATTC	CTAAAGGCTG	AGCCACGTCC	800
801	TGGACAGCCT	GCCGGTGAAG	CAGAATTCT	CAGTAGTATT	AGGGCCTACC	850
851	TCATGAACCG	GCAAGCAGTC	CTAGATGAAA	CACATCTGCC	AGACATAGAT	900
901	GCACTAGTTG	AACTTGCTGC	CTCAGGGGAT	CCAACACTGC	CAGACTCACT	950
951	TGAAAATCCA	CATGCAGCTT	GGGTCTTTGC	ATGTGCTCCT	GACCGATGTC	1000
1001	CACCAACATG	CATCTATATT	GCAGGGATGG	CAGAACTTGG	CGCATTTTTT	1050
1051	GCAATCCTAC	AGGATATGCG	AAATACCATC	ATGGCATCTA	AAACCGTAGG	1100
1101	AACAGCTGAA	GAAAAGCTTA	AAAAGAAGTC	TGCATTTTAT	CAGTCGTACC	1150
1151	TACGAAGAAC	ACAATCTATG	GGGATCCAGC	TAGACCAGAG	GATTATCCTC	1200
1201	ATGTACATGA	TTGAGTGGGG	AAATGAGGTC	GTTAACCACT	TCCACCTGGG	1250
1251	TGATGATATG	GATCCCGAGC	TAAGGCAGTT	AGCTCAAGCT	CTCATTGACC	1300
1301	AAAAGGTCAA	AGAGATATCT	AACCAAGAGC	CACTAAAGAT	ATAGTAACCT	1350
1351	GCTTGcatag	CTTCTACTTT	TATAGCCTAT	ATATCCAGCT	GCTTATTATG	1400
1401	ATTTATAATC	ACAATAATTA	TACAAGTTAT	TGTCATGTTA	ATCTCACTTA	1450
1451	CTAATTCACA	ATTAAACATT	GCACTTAACT	AACACTTAGG	CACATAATTG	1500
1501	ATGGAACAAT	ACTGTAAAAC	TGAGCTATCC	CTGTGATTCA	TCTCTGTCTT	1550
1551	CATCATCTCT	AAGGCCACTA	CCTACCTCAA	TACACTTATA	TATATGCACG	1600
1601	TAGCATATAT	ATAAGTGTAT	ATATACTACC	TCAACAGTGT	GTTTCTTTGA	1650
1651	TTGCTTTTCA	AGGAGTATAC	TACTA	1675		

Figure 14. Predicted secondary structure of the complementary 3' and 5' termini of the PH virus S RNA segment. The terminal 23 nucleotides of the PH virus S RNA, as deduced from the cDNA sequence, are shown. The free energy value for this structure (-15.0 kcal/mol at 25°C) was calculated using the RNA folding program in the PC/Gene computer program (Version 5.11).



G, 21.7% C, and 31.5% U. These values are similar to those of the Hällnäs virus, SR virus and Hantaan virus S RNA segments (Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990).

Coding Strategy of the PH Virus S RNA Segment

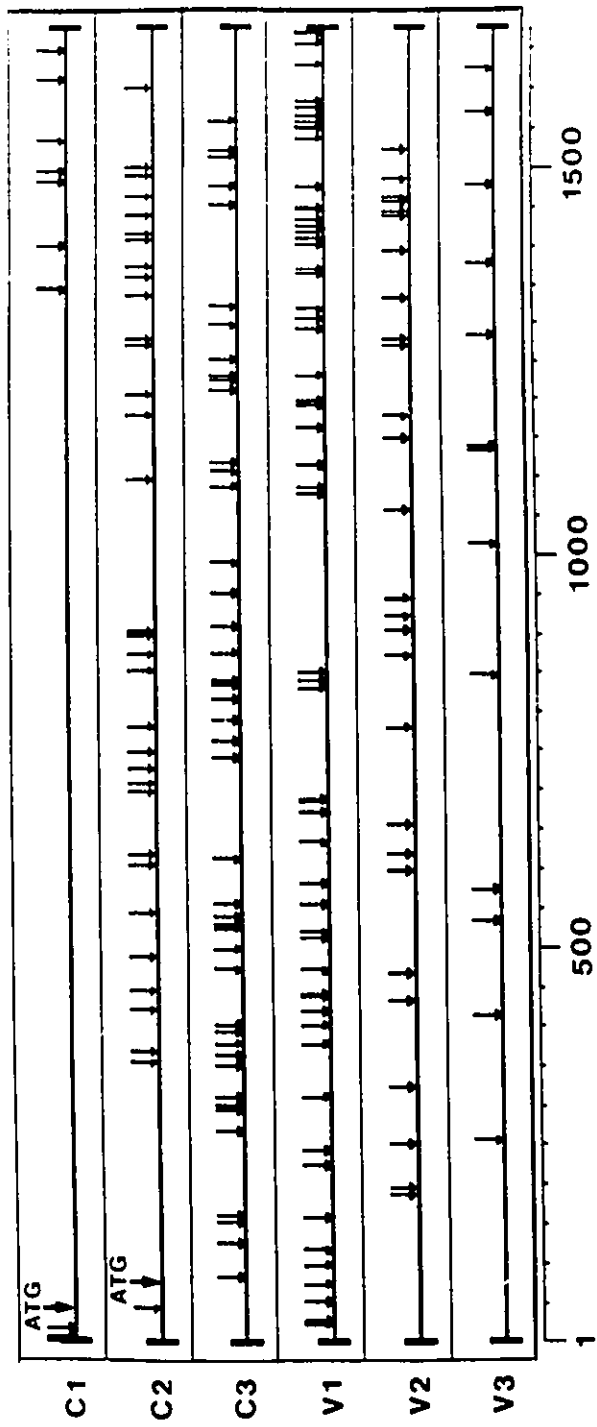
The PH virus S RNA segment is 1675 bases long (Fig. 13). This is the smallest *Hantavirus* S genomic RNA segment reported to date as compared to 1785 bases for Hällnäs virus, 1769 bases for SR virus, and 1696 bases for Hantaan virus (Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990). The PH virus S RNA is potentially capable of forming a large hairpin structure near its 5' end, between nucleotide positions 1580 and 1620 (Fig. 15). This potential hairpin structure has an 18 base-paired stem structure, and is energetically stable with a predicted free energy of -28.6 kcal/mol at 25°C. No similar potential hairpin structure was identified in the S RNA segments of Hällnäs virus, SR virus, or Hantaan virus (Schmaljohn *et al.*, 1986b; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990).

Positions of translation termination codons were determined for the six potential reading frames of the PH virus S RNA sequence. A large open reading frame (ORF) was observed in frame 1 of the S cRNA (Fig. 16). The first potential in-frame translation initiation codon is at nucleotides 43-45 from the 5' end of the PH virus M cDNA (Fig. 13). The flanking sequence for this initiation codon is not considered optimum for translation initiation since there is a G in the -3 position and an A in the +4 position (Kozak, 1984, 1986a, 1986b). However, there are no other in-frame translation initiation codons in this ORF until nucleotide position 261. This ORF extends to a translation termination codon (TAG) at nucleotide position 1342, and could encode a 433 amino acid

Figure 15. Predicted secondary structure in the PF1 virus S RNA between bases 1580 and 1620, measuring from the 3' terminus. This potential hairpin structure was predicted, and its free energy (-28.6 kcal/mol at 25°C) was calculated using the RNA folding program in the PC/Gene computer program (Version 5.11).

		C		
		G		A
		U		U
		G.C		
		C.G		
1595-	A.U	-	1605	
	U.A			
	A.U			
	U.A			
	A.U			
1590-	U.A	-	1610	
	A.U			
	U.A			
	A.U			
	A.U			
1585-	G.C	-	1615	
	U.A			
	G.C			
	U.A			
	A.U			
1580-	U.A	-	1620	
UGGAUGGAGU	/		\	UAUAUGAUGG

Figure 16. Translation termination codon locations in all reading frames of the PH virus S RNA segment. Translation termination codons in the PH virus S RNA are indicated by arrows in the three reading frames of the viral complementary sense (C) and viral sense (V). The first potential ATG initiation codon in each of the two longest open reading frames was also indicated by arrows.



Nucleotides

nucleocapsid (N) protein with a predicted M_r of approximately 49 K. This is identical in size to the N protein of Hällnäs virus (433 amino acids, and 49 K) (Stohwasser *et al.*, 1990) but is 4 amino acids larger than the N proteins of SR virus (429 amino acids, and 48 K) (Arikawa *et al.*, 1990) and Hantaan virus (429 amino acids, and 48 K) (Schmaljohn *et al.*, 1986b).

A second, smaller overlapping ORF was observed in frame 2 of the PH virus S cRNA (Fig. 16), and has a potential in-frame translation initiation codon at nucleotides 83-85 from the 5' end of the PH virus S cDNA (Fig. 13). However, with a G at the -3 position and an A at the +4 position this flanking sequence is also not considered optimal for translation initiation (Kozak, 1984, 1986a, 1986b). This is however, similar to the flanking sequence of the initiation codon of the PH virus N protein. With a translation termination codon (TGA) at nucleotide position 353 (Fig. 13), this ORF could encode a 90 amino acid protein with a predicted M_r of approximately 10.5 K. The PH virus S RNA segment therefore, could possibly encode an NS_s protein on an overlapping ORF as observed in bunyaviruses (Bishop *et al.*, 1982; Akashi and Bishop, 1983; Fuller *et al.*, 1983; Akashi *et al.*, 1984; Bouloy *et al.*, 1984; Elliott, 1985; Gerbaud *et al.*, 1987; Elliott and McGregor, 1989). A second overlapping ORF identical in size and position was observed in frame 2 of the Hällnäs virus S cRNA (90 amino acids, and 11.1 K) (Stohwasser *et al.*, 1990), but not in the SR virus or Hantaan virus S cRNA sequence (Schmaljohn *et al.*, 1986b; Arikawa *et al.*, 1990). No other reading frames in the PH virus S segment had ORF's capable of encoding proteins of more than 50 amino acids (Fig. 16).

Comparison of the PH Virus, Hällnäs virus, SR virus and Hantaan Virus N Proteins

A best-fit comparison was made between the predicted amino acid sequence of the N proteins of PH virus, Hällnäs virus, SR virus and Hantaan virus (Fig. 17). Conservation of cysteine residues between the PH virus, Hällnäs virus, SR virus and Hantaan virus N proteins is very high. All four virus N proteins have 5 cysteine residues (Fig. 17). There is 100% cysteine conservation between the N proteins of PH virus and Hällnäs virus and 80% cysteine conservation with the N proteins of SR virus and Hantaan virus (Fig. 17). The results of the amino acid sequence comparison of the N proteins of Hällnäs virus, SR virus and Hantaan virus to PH virus are summarized in Table 4. The PH virus N protein has more amino acid sequence similarity with the Hällnäs virus N protein than with the N proteins of SR virus and Hantaan virus (Table 4). Amino acid sequence similarity between the N proteins of all four viruses was 55.2%. If conservative amino acid substitutions are included this number increases to 70.2%. Based on the comparison of the PH virus, Hällnäs virus, SR virus and Hantaan N proteins it appears that the *Hantavirus* N protein has two conserved regions and one non-conserved region. The first conserved region includes the first 232 amino acids measuring from the N-terminus of each N protein (Fig. 17). In this region, 133 amino acids (57.3%) were identical in type and position between the four virus N proteins. If conservative amino acid substitutions are included, there is 75% amino acid sequence similarity between the four virus N proteins in this region. The second conserved region was very highly conserved and was located in the C-terminal portion of the N proteins of the four viruses (PH virus amino acid

Figure 17. Comparison of the predicted amino acid sequences of the N proteins of PH virus, Hällnäs virus, SR virus, and Hantaan virus. Amino acids are numbered on the left and right ends of each line. Non-identical amino acids of Hällnäs virus, SR virus, and Hantaan virus N proteins are presented below the PH virus N protein sequence. Identical amino acids are denoted by dots, conservative amino acid changes are underlined, and missing amino acids in any sequence are denoted with a dash.

PH: 1 MSQLREIQEEITRHEQQLVIARQKLKEAERTVEVDPDDVNKSTLQSRRSA 50
 Hällnäs: 1 ..D.TD.....V.....D...A.....A.QQT 50
 SR: 1 .ATME...R..SA..G.....V.D..KQY.K...L..RA.HD.E.V 50
 Hantaan: 1 .ATME.L.R..NA..G.....VRD..KQY.K...EL..R..TD.EGV 50

PH: 51 VSTLEDKLAEFKRQLADVISRQKMDEKPVDPGTGIELDDHLKERSSSLQYGN 100
 Hällnäs: 51 ..A.....DY..RM..AV..K...T..T.....P.....R... 100
 SR: 51 AASIQS.ID.L.....R.AAG.NIGQDR....V.PG.....A.S... 100
 Hantaan: 51 AVSIQA.ID.L.....R.ATG.NLG.EQ....V.PG.....M.S... 100

PH: 101 VLDVNSIDIEEPSGQTADWLKIGSYIIEFALPIILKALHMLSTRGRQTVK 150
 Hällnäs: 101A.....YT..V.V.G.TI.....Y..... 150
 SR: 101 T..L..L..D..T.....T.IV.LTS.VV.....Y..T.....S. 150
 Hantaan: 101 ...L.HL..D..T.....S.IV.LTS.VV..L....Y..T.....T. 150

PH: 151 ENKGTRIRFKDDSSYEDVNGIRRPKHLVSMPTAQSTMKAELTPGRFRFT 200
 Hällnäs: 151T.F..I..... 200
 SR: 151 D...M.....K.....N...S.....I..... 200
 Hantaan: 151 D.....F.....K.....L.N...S.....I....Y.. 200

PH: 201 IVCGLFPAQIMARNIISPVMGVIGFAFFVKDWADKVKAFLDQKCPFLKAE 250
 Hällnäs: 201T..QV...M.....S.....PE.IRE.MEKE...I.P. 250
 SR: 201 A....Y....K...MV....S.V..LALA...TSRIEEW.GAP.K.MAES 250
 Hantaan: 201 A....Y....K..QM.....S....LALA...S.RIEQW.IEP.KL.PDT 250

PH: 251 PRPGQPAGEAEFLSSIRAYLMNRQAVLDETHLPDIDALVELAASGPPTLP 300
 Hällnäs: 251 VK..T..Q.V...KRN.V.F.T..D...KN.VA...K.IDY.....S. 300
 SR: 251 LIA.SLS.----NPVN.D.IRQ..GA.AGMEPKFQ..RQHSKDAGC..V 296
 Hantaan: 251 AAVSLLG.----PATN.D..RQ..VA.GNMETKESK.IRCH.EAAGCSMI 296

PH: 301 DSLENPHAAWVFACAPDRCPPTCIYIAGMAELGAFFAILQDMRNTIMASK 350
 Hällnäs: 301 .DI.S.N.P.....V.....S..... 350
 SR: 297 EHI.S.SSI....G.....LFVG.....S..... 346
 Hantaan: 297 EDI.S.SSI....G.....LF...I.....S..... 346

PH: 351 TVGTAEKKLKKKSAFYQSYLRRTQSMGIQLDQRIILMYMIEWGNEVVNHF 400
 Hällnäs: 351S.....L..L...R.M.D.. 400
 SR: 347D...R...S.....V.F.VA..K.A.DN. 396
 Hantaan: 347S....R...S.....G....VLF.VA..K.A.DN. 396

PH: 401 HLGDDMDPELRQLAQALIDQKVKEISNQEPLKI 433
 Hällnäs: 401G...S..... 433
 SR: 397S...I.....M.L 429
 Hantaan: 397T...S...V.....L 429

Table 4. Comparison of the amino acid sequences of the N proteins of Hällnäs virus, SR virus, and Hantaan virus to the N protein of PH virus.

VIRUS	N PROTEIN	
	I ^a	(I + C) ^b
Hällnäs	79.9%	87.5%
SR	63.0%	74.8%
Hantaan	61.7%	74.4%

^a Identical amino acids.

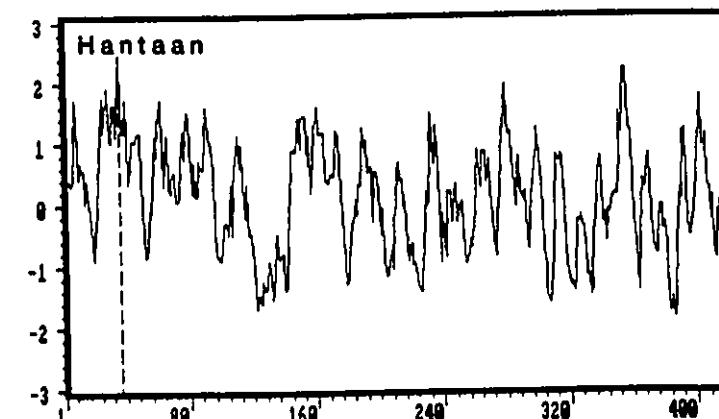
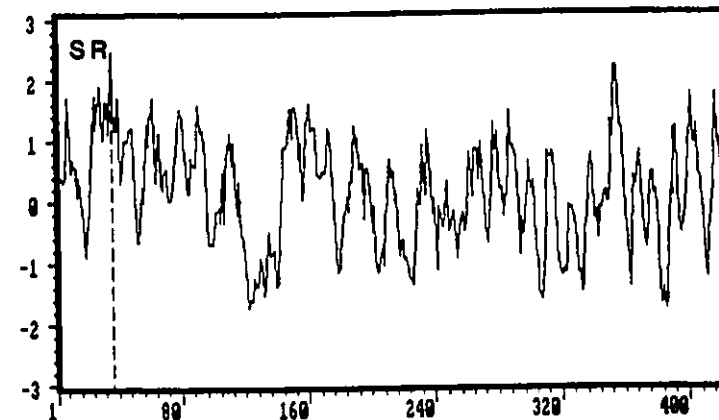
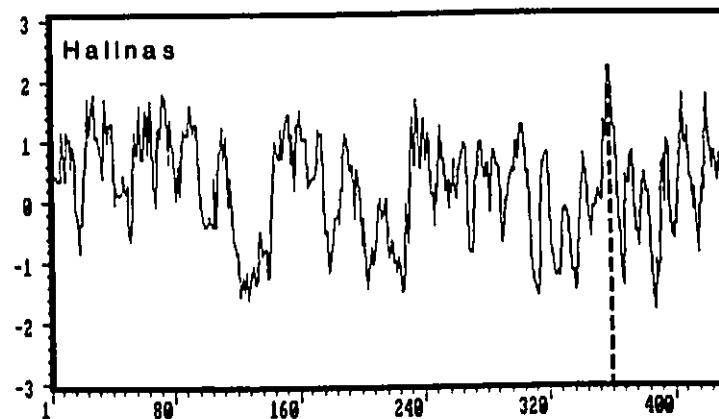
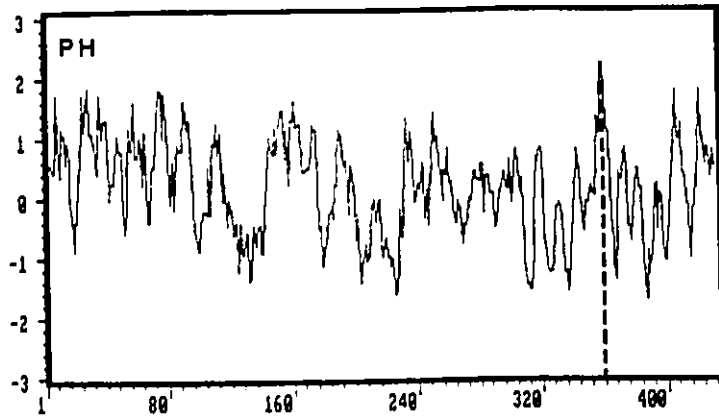
^b Identical amino acids plus conservative amino acid substitutions using the following criteria for conservative substitutions: R=K, S=T, D=E, Q=N, V=L=I=M, A=G, A=V, Y=F.

positions 310-433) (Fig. 17). Within this region, 98 (79%) of the 124 amino acids were identical in type and position between the four viruses. There is 90.3% amino acid sequence similarity in this region between the four viruses if conservative amino acid changes are included. The space between these two conserved regions (PH virus amino acid positions 233-309) was not highly conserved, and is the location of the four additional amino acids in the PH virus and Hällnäs virus N proteins as compared with the SR virus and Hantaan virus N proteins (Fig. 17). Approximately 40% of the amino acid differences between the PH virus N protein and each of the other three virus N proteins are clustered within this region. There is only 10.4% amino acid sequence similarity in this region between the four viruses. Even if conservative amino acid substitutions are included, there is still only 23.4% amino acid sequence similarity.

The calculated isoelectric points of the N proteins of PH virus ($pI = 5.53$) and Hällnäs virus ($pI = 5.59$) (Stohwasser *et al.*, 1990) are similar. In contrast, the calculated isoelectric points of the N proteins of SR virus ($pI = 7.25$) (Arikawa *et al.*, 1990) and Hantaan virus ($pI = 6.7$) (Schmaljohn *et al.*, 1986b) are more neutral than the PH virus and Hällnäs virus N proteins.

Hydrophilicity profiles (Hopp and Woods, 1981) of the N proteins of PH virus, Hällnäs virus, SR virus and Hantaan virus were compared (Fig. 18). Overall, the four virus N protein hydrophilicity profiles were similar (Fig. 18). The region of highest hydrophilicity in the PH virus profile is located in the C-terminal half of the protein (amino acid positions 356-362) and was conserved in the Hällnäs virus profile (amino acid positions 356-362) (Fig. 18). The seven amino acids in this region were identical in the PH virus and Hällnäs virus N proteins (Fig. 17). In contrast, the

Figure 18. Hopp and Woods hydrophilicity profiles of PH virus, Hällnäs virus, SR virus, and Hantaan virus N proteins. Amino acid numbers are shown below each profile. The point of highest hydrophilicity in each profile is indicated by a broken vertical line.



highest region of hydrophilicity in the SR virus and Hantaan virus profiles was located near the N-terminus (amino acid positions 33-38) of the N proteins (Fig. 18). The site in the PH virus N protein predicted by the computer to have a 100% probability as an antigenic site (amino acids 356-362) was similar in the N proteins of Hällnäs virus, SR virus and Hantaan virus (Fig. 17). The Hällnäs virus amino acid sequence was identical at this site (amino acids 356-362) whereas the SR virus (amino acids 352-358) and Hantaan virus (amino acids 352-358) amino acid sequences had two conservative substitutions or one conservative substitution respectively (Fig. 17).

Comparison of the PH Virus and Hällnäs Virus NS_S Proteins

Members of the *Bunyavirus* genus use overlapping ORF's to encode a 10-12 K NS_S protein whose function is not yet known. As mentioned previously the PH virus and Hällnäs virus S cRNA's also contain a second overlapping ORF that could encode 90 amino acid NS_S proteins (Fig. 16). Because of this apparent similarity with viruses in the *Bunyavirus* genus the deduced amino acid sequence of putative PH virus and Hällnäs virus NS_S proteins were compared (Fig. 19). Amino acid sequence similarity between the NS_S proteins of PH virus and Hällnäs virus is 50%. If conservative amino acid substitutions are included the amino acid sequence similarity between these two NS_S proteins increases to 58.9%.

The calculated isoelectric points of the PH virus (pI = 12.72) and Hällnäs virus (pI = 10.92) NS_S proteins were both basic, although the PH virus NS_S protein was more basic.

Hydrophilicity profiles (Hopp and Woods, 1981) of the PH virus and Hällnäs virus NS_S proteins were compared (Fig. 20). The hydrophilicity profiles were similar although the highest peak of hydrophilicity in the

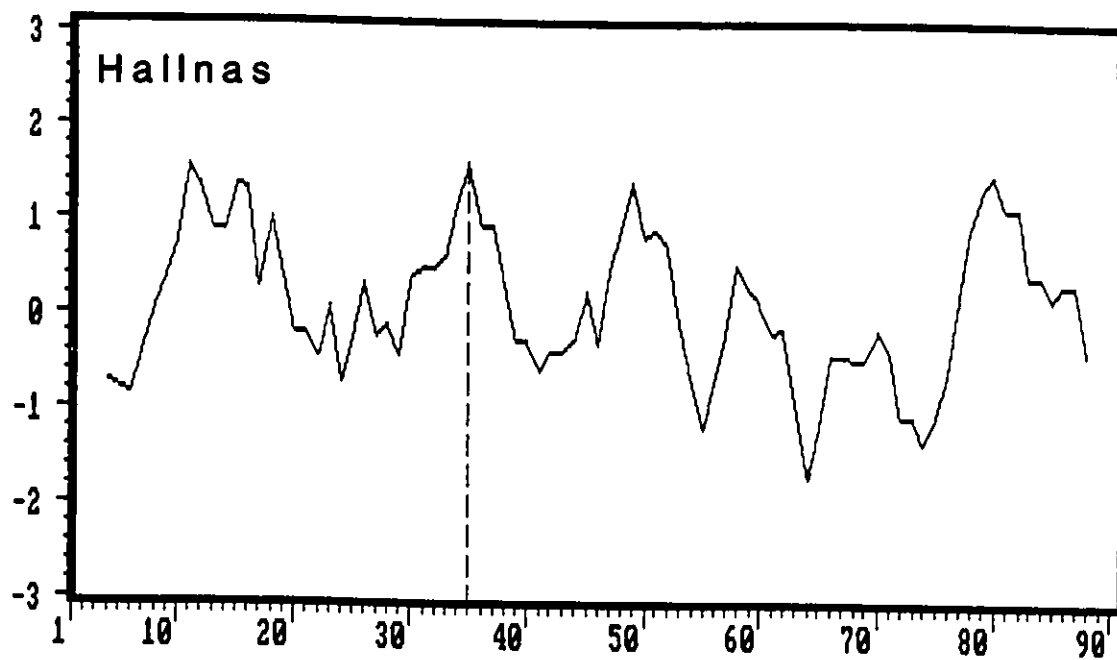
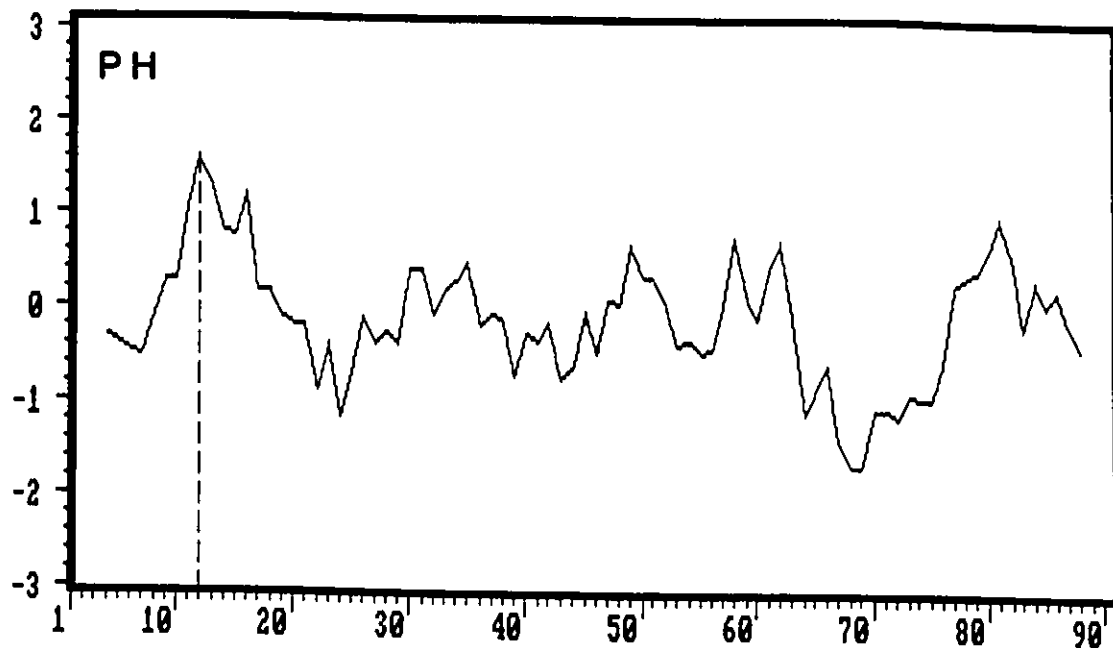
Figure 19. Comparison of the predicted amino acid sequences of the putative PH virus and Hällnäs virus NS₅ proteins. Amino acids are numbered on the left and right ends of each line. Non-identical amino acids of Hällnäs virus NS₅ protein are presented below the PH virus NS₅ protein sequence. Identical amino acids are denoted by dots and conservative amino acid changes are underlined.

PH: 1 MSSSLSLPGR SSRKLNGRWR WTQMTLTKVH CKAGGQQCQH 40
Hällnäs: 1 ..NN.L..DK N..MQREQ.K ..R....RA. Y.QDNK.... 40

PH: 41 WRTNWQSSRG SLQMSSHVRR WMRNLWIQLV LSLTTILRRG 80
Hällnäs: 41 ..I.S..TT.E EW..LCPG.K .IL..LTR.G .N.MI.S..D 80

PH: 81 QASNMEMSLM 90
Hällnäs: 81 ..LD..... 90

Figure 20. Hopp and Woods hydrophilicity profiles of the putative NS₅ proteins of PH virus and Hällnäs virus. Amino acid numbers are shown below each profile. The point of highest hydrophilicity in each profile is indicated by a broken vertical line.



PH virus NS_S protein was near the N-terminus of the protein (amino acid 12) (Fig. 20). In contrast, the highest peak of hydrophilicity in the Hällnäs virus NS_S protein was located closer to the middle of the protein (amino acid 35) (Fig. 20).

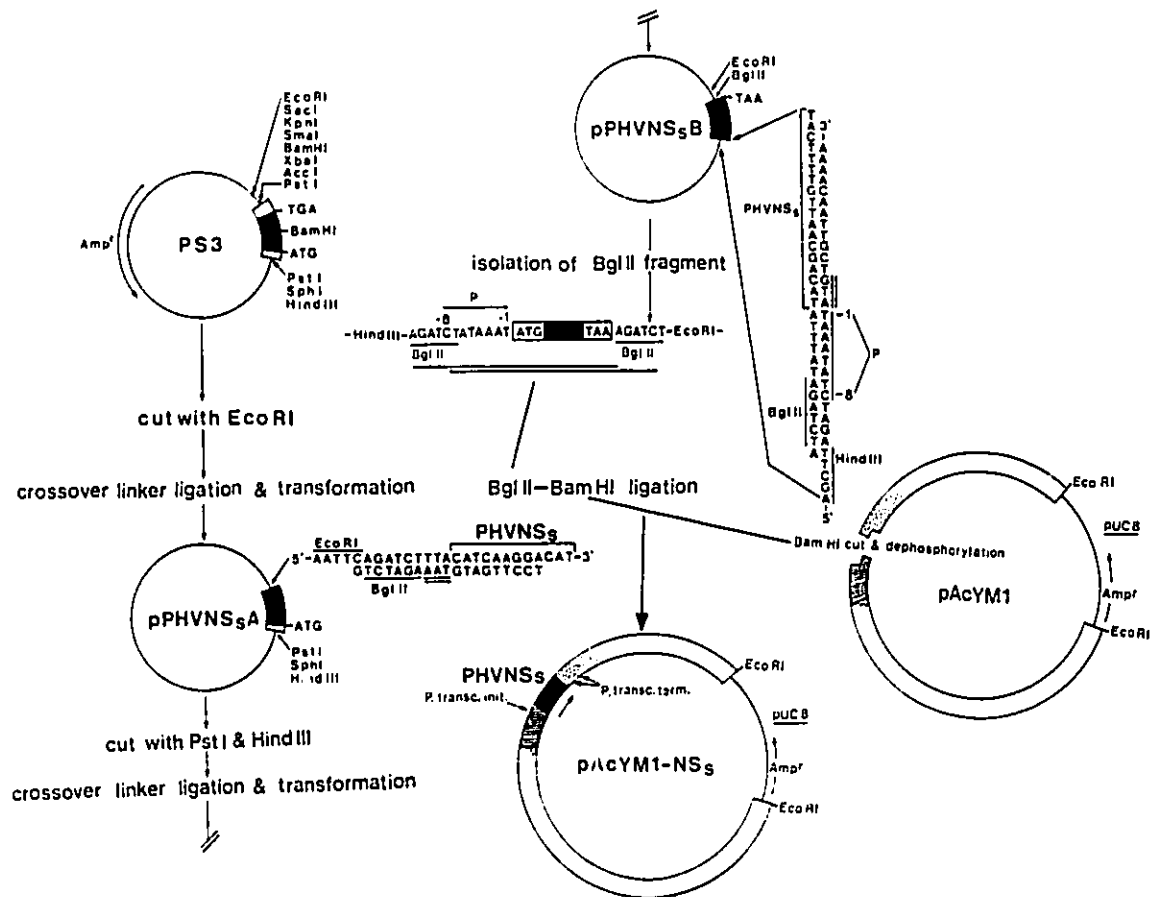
5. EXPRESSION OF THE NS_S GENE OF PH VIRUS IN INSECT CELLS

To determine if an NS_S protein is expressed by the PH virus S RNA segment, this over-lapping ORF was expressed in the baculovirus expression system. Antibodies were made that recognized this protein and were used in an attempt to detect this protein in PH virus infected Vero cells.

Deletion and Modification of the Non-Coding Sequence of the PH virus NS_S Gene and Transfer of this Gene into the Baculovirus Transfer Vector

The entire ORF that could encode a putative PH virus NS_S protein is contained in clone PS3 (Fig. 12 and 13). The 270-bp ORF is located in the 534-bp insert in the *Pst*I site of pUC19. To modify the upstream and downstream untranslated regions of the PH virus NS_S gene, site-specific recombination directed by double-stranded crossover linkers was used (Sung *et al.*, 1986) (Fig. 21). To modify the downstream untranslated region a double-stranded synthetic crossover linker was constructed with an *Eco*RI cohesive end (AATTC), a *Bgl*II recognition site downstream of the termination codon to facilitate insertion into the baculovirus transfer vector, and the NS_S coding sequence (AATGTAGTTCCT) (Fig. 21). This linker was ligated to *Eco*RI digested PS3. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker and NS_S gene following transformation of *E. coli* JM101 cells with the ligation mix (Fig.

Figure 21. Construction of a baculovirus transfer vector for the PH virus NS_S gene. Non-coding untranslated sequence was removed upstream and downstream of the NS_S gene using site-specific recombination directed by double-stranded crossover linkers. This method was also used to add the flanking sequence CTATAAAT (P) immediately upstream of the initiating ATG and to add *Bgl*II sites on both ends of the NS_S gene. The modified PH virus NS_S gene was inserted into the *Bam*HI site of pAcYM1. The resultant vector pAcYM1-NS_S has the PH virus NS_S coding sequence in the correct orientation and under the control of the polyhedrin promoter.

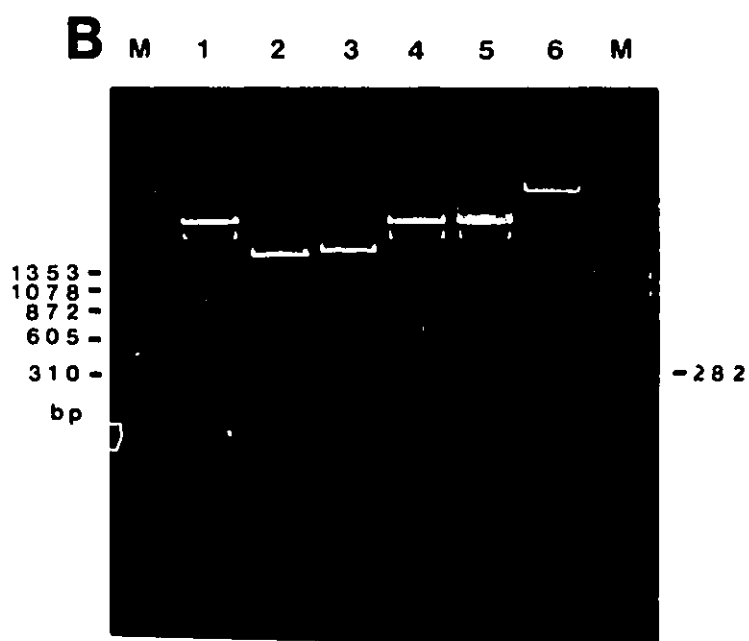
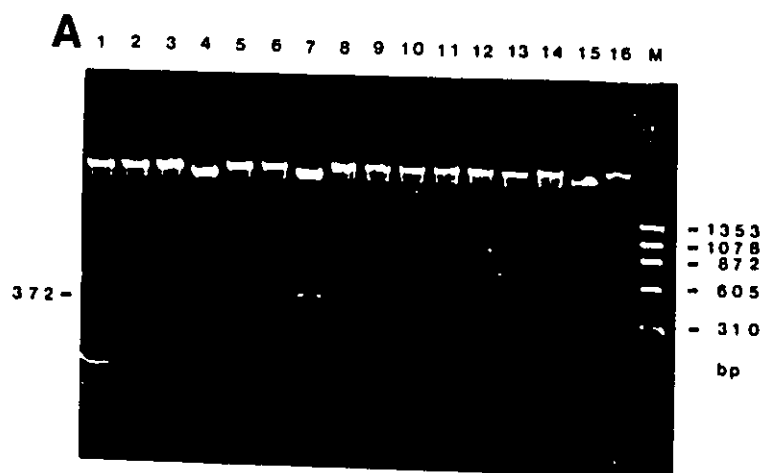


21). The modified plasmid would have the 173-bp downstream untranslated region removed and a *Bgl*II site added. Primary selection of recombinants was done by digesting 16 plasmid clones with *Bgl*II and *Hind*III since this would generate a 372-bp fragment only in modified plasmids (Fig. 22A). Clones 4, 7 and 15 yielded the correct size fragment after the digestion (Fig. 22A, lanes 4, 7, 15). Clone 7 was designated pPHVNS_SA (Fig. 21).

For high levels of protein expression, when using the baculovirus expression system, it is recommended that the upstream untranslated region be as small as possible (Summers and Smith, 1987). It has also been suggested that the flanking sequence ACC upstream of the translation initiation ATG, provides a preferred context for translation initiation in eukaryotes (Kozak 1986a, 1986b). However, work in our laboratory has demonstrated that the flanking sequence CTATAAAAT' upstream of the initiating ATG sometimes gives increased levels of protein expression in the baculovirus system.

To modify the upstream untranslated region of the PH virus NS_S gene, site-specific recombination directed by double-stranded crossover linkers was again used. A double-stranded synthetic crossover linker was constructed with a *Hind*III cohesive end (AGCTT), a *Bgl*II recognition site upstream of the initiation codon to facilitate insertion into the baculovirus transfer vector, the CTATAAAAT' flanking sequence also upstream of the initiation codon, and the putative NS_S coding sequence starting with nucleotide 83 of the PH virus S RNA segment cDNA (ATGACGTTAACAAAA) (Fig. 21). This linker was ligated to plasmid pPHVNS_SA that had been digested with *Pst*I and *Hind*III. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker

Figure 22. Primary selection of recombinants with the PH virus NS_s gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers. A; lanes 1-16, Primary selection of 16 possible recombinant clones was made by digesting their plasmid DNA with *Bgl*II and *Hind*III after crossover linker mutagenesis of the downstream flanking region; lane M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; B; lanes 1-6, Primary selection of 6 possible recombinant clones was made by digesting their plasmid DNA with *Bgl*II after crossover linker mutagenesis of the upstream flanking region; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers.



and NS₅ gene following transformation of JM101 cells with the ligation mix (Fig. 21). Modified plasmids would have the 82-bp upstream untranslated region removed, being replaced with the CTATAAAT flanking sequence and a second *Bgl*II recognition site would have been added (Fig. 21). Therefore, digestion with *Bgl*II should yield a 282-bp fragment from correctly modified plasmids. Screening of six recombinant plasmids, digested with *Bgl*II, indicated clones 1, 4 and 5 were correctly modified (Fig. 22B, lanes, 1, 4, 5). Clone 4 was designated as pPHVNS₅B (Fig. 21).

The modifications to the upstream and downstream untranslated regions were verified in pPHVNS₅B by determining the nucleotide sequence in the junction regions with a DuPont Genesis 2000 automated DNA sequencer (data not shown).

The baculovirus transfer vector that was used, pAcYM1, is a pUC8 derived plasmid that contains the *Eco*RI-I fragments of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome that flank the polyhedrin gene and serves as the site for *in vivo* recombination with wild type AcNPV DNA (Fig. 21). Plasmid pAcYM1 contains all of the upstream polyhedrin gene sequence including the A of the ATG initiation codon and the polyhedrin promoter for transcription initiation (Fig. 21). Most of the polyhedrin coding region was removed and replaced with a *Bam*HI recognition sequence for insertion of foreign genes (Matsuura *et al.*, 1987). To make the baculovirus transfer vector, the 282-bp *Bgl*II fragment from pPHVNS₅B was ligated into the *Bam*HI site of pAcYM1 (Fig. 21). This plasmid pAcYM1-NS₅ contains the CTATAAAT sequence upstream from the PH virus NS₅ coding sequence and is in the correct orientation under the control of the polyhedrin gene promoter (Fig. 21).

Construction of the Recombinant Baculovirus Containing the Putative NS_S Gene

To construct a recombinant baculovirus expressing the putative NS_S gene of PH virus, the plasmid pAcYM1-NS_S DNA was mixed with wild type AcNPV DNA and used to co-transfect SF9 cells. Culture supernatant was harvested 3 days after co-transfection and titrated using a limiting dilution assay in SF9 cells grown in a 96-well plate. After 7 days the culture supernatant from each well was harvested and the DNA from the cells in each well was subjected to dot blot hybridization using nick translated plasmid pUCPHVNS_S as a probe (Fig. 23). The culture supernatant from the well showing a positive signal in the column with the highest dilutions (Fig. 23, well C6), was titrated by plaque assay in SF9 cells. Nine plaques lacking occlusion bodies (polyhedrin) were picked and plaque purified one time before propagation. To further characterize these nine polyhedrin-negative viruses, DNA was prepared from cells infected with these viruses, dot blotted onto a Colony/PlaqueScreen membrane and hybridized using nick translated plasmid pPHVNS_SB as a probe (Fig. 24). While the DNA from wild type infected cells (Fig. 24, dot WT) did not hybridize with the probe, DNA from the cells infected with the nine polyhedrin-negative viruses (Fig. 24, dots 1-9) and the 282-bp *Eg*/II fragment recovered from plasmid pPHVNS_SB (Fig. 24, dot P) clearly hybridized. These results verified that the PH virus NS_S gene was present in the nine recombinant baculoviruses. Recombinant baculovirus 3A1 was designated as AcPHVNS_S and was used in subsequent experiments.

Expression of the Putative NS_S Protein of PH virus

To determine if cells infected with the recombinant baculovirus

Figure 23. Enrichment of PH virus NS₅ recombinant baculoviruses using a limiting dilution assay and dot blot hybridization. All wells in columns 1-8 of a 96 well microtiter plate were seeded with SF9 cells. Three days after co-transfection with plasmid pAcYM1-NS₅ and wild type AcNPV DNA serial 10 fold dilutions were made on the culture supernatant from 10^{-1} - 10^{-8} . Each dilution was used to infect one column of SF9 cells from column 1-8 (eg. 10^{-1} , column 1). After 7 days culture supernatants from each infected well were collected and saved in the corresponding well of another 96 well microtiter plate. Infected cells from each well were lysed, the DNA was dot blotted onto a GeneScreen Plus membrane in the same position as the original well and hybridized to nick-translated NS₅ clone pPHVNS₅B. columns 1, 2, 3, 4, 5, 6, 7, 8 were blotted with the DNA from SF9 cells infected with dilutions of the co-transfection culture supernatant of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} respectively; Positions A9 and H9 were dot blotted with approximately 10 ng of the 282-bp *Bgl*II fragment recovered from plasmid pPHVNS₅B.

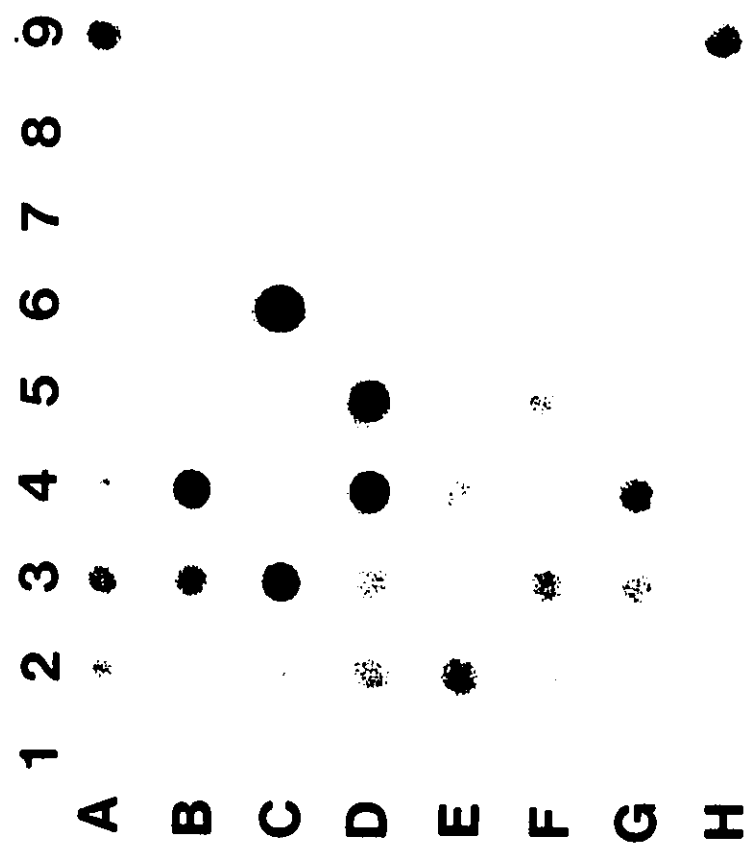
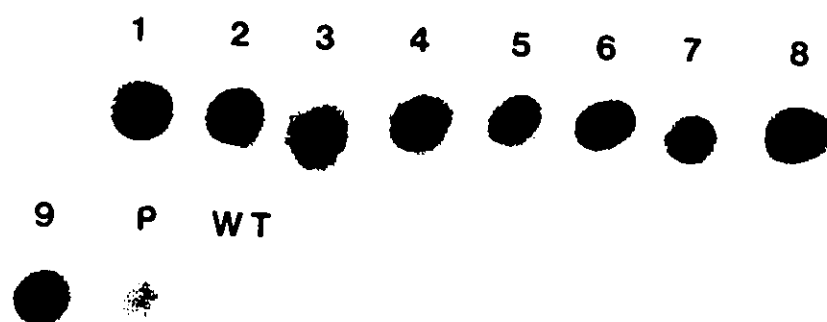


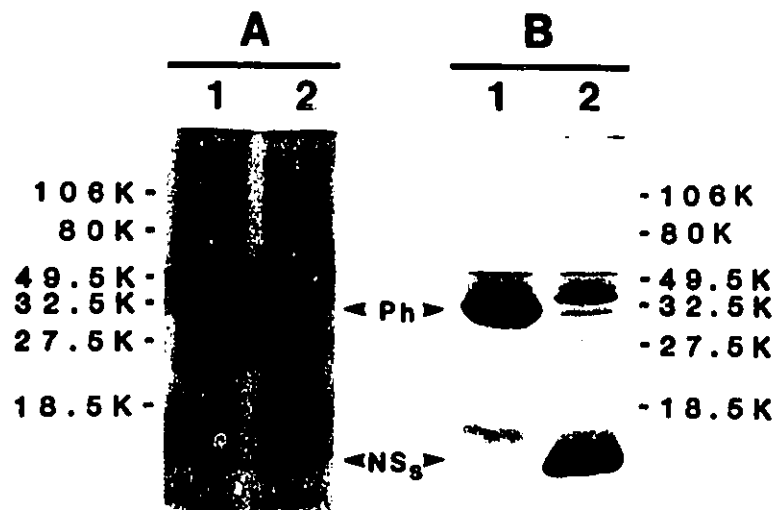
Figure 24. Dot blot analysis of PH virus NS₅ recombinant baculoviruses after plaque purification. Purified polyhedrin-negative plaques or wild type AcNPV was used to infect SF9 cells. Five days after infection 10% of the infected cells were lysed and their DNA was dot blotted onto a Colony/PlaqueScreen membrane. The DNA was hybridized with the nick translated plasmid pPHVNS₅B. dots 1, 2, 3, 4, 5, 6, 7, 8, 9, DNA from cells infected with the PH virus NS₅ recombinant baculoviruses 1A1, 1B1, 2A1, 3A1, 4A1, 5A1, 6A1, 7A1, and 7B1 respectively; dot P, 10 ng of the 282-bp *Bgl*II fragment recovered from plasmid pPHVNS₅B; dot WT, DNA from cells infected with wild type AcNPV.



AcPHVNS_S, synthesized the PH virus NS_S protein, SF9 cells were infected with AcPHVNS_S or the wild type baculovirus AcNPV. Two days after infection the cells were labelled with [³⁵S]-methionine *in vivo*, lysed in SDS-PAGE sample buffer and the cell lysates were analyzed by SDS-PAGE. Proteins were visualized by Coomassie blue staining and by fluorography (Fig. 25). After Coomassie blue staining of the gel no additional protein bands were observed in the AcPHVNS_S infected cell lysates (Fig. 25A, lane 2) as compared with wild type AcNPV infected cells (Fig. 25A, lane 1). However, a protein band with an M_r of 11.8 K stained more intensely in the AcPHVNS_S recombinant virus infected sample (Fig. 25A, lane 2) than in the wild type AcNPV infected sample (Fig. 25A, lane 1). A fluorograph of this gel revealed that the 11.8 K protein band was visible only in the AcPHVNS_S recombinant baculovirus infected cell lysate (Fig. 25B, lane 2). However, the 11.8 K protein band in the wild type AcNPV sample was not visible on the fluorograph (Fig. 25B, lane 1). This 11.8 K protein band is similar in size to the predicted 10.5 K size of the putative NS_S protein of PH virus. Also, since 7 of the 90 amino acids in the NS_S protein are methionine residues it was expected to label well with [³⁵S]-methionine. Therefore, it appears the NS_S protein is being expressed by the recombinant baculovirus AcPHVNS_S and this protein co-migrates with a wild type baculovirus protein with an M_r of 11.8 K.

To produce antibodies against the putative NS_S protein this protein was expressed in SF9 cells infected with AcPHVNS_S, partially purified with SDS-PAGE and used to inoculate rabbits. This rabbit serum prepared against the putative NS_S protein of PH virus was designated as PHV-NS_S-Ab. The specificity of the rabbit serum for the putative NS_S

Figure 25. Expression of the putative NS_s protein of PH virus in insect cells. SF9 cells were infected with the recombinant baculovirus AcPHVNS_s or wild type AcNPV. At 48 h post-infection the cells were harvested after labelling *in vivo* with [³⁵S]-methionine. The cells were lysed in SDS-PAGE sample buffer, heated to 100°C for 10 min and were analyzed by SDS-PAGE in a 12%-20% polyacrylamide gel. A; Coomassie blue stained gel; lane 1, 2% of the wild type AcNPV infected cell lysate; lane 2, 2% of the recombinant baculovirus AcPHVNS_s infected cell lysate; B; Fluorogram of the same gel shown in A. NS_s, PH virus small ORF from the S RNA segment expressed in insect cells; Ph, polyhedrin protein; Protein size determinations were made using prestained low molecular size markers supplied by Bio-Rad: phosphorylase B (106 K), bovine serum albumin (80 K), ovalbumin (49.5 K), carbonic anhydrase (32.5 K), soybean trypsin inhibitor (18.5 K).



protein was demonstrated using radioimmunoprecipitation. AcPHVNS_S or AcNPV infected SF9 cells were labelled with [³⁵S]-methionine *in vivo* and part of the cell lysates were immunoprecipitated with the rabbit serum PHV-NS_S-Ab or pre-immune rabbit serum and analyzed by SDS-PAGE (Fig. 26). Rabbit pre-immune serum did not precipitate any [³⁵S]-methionine labelled proteins from either the AcPHVNS_S or AcNPV infected cell lysates (Fig. 26, lanes 2, 4). The rabbit serum PHV-NS_S-Ab did not precipitate any [³⁵S]-methionine labelled proteins from the wild type AcNPV infected cell lysate (Fig. 26, lane 3). In contrast, one strongly [³⁵S]-methionine labelled protein was immunoprecipitated from the recombinant AcPHVNS_S infected cell lysate (Fig. 26, lane 5). Comparison with the [³⁵S]-methionine labelled AcPHVNS_S infected cell lysate (Fig. 26, lane 1) showed that the ³⁵S labelled protein precipitated with the rabbit serum co-migrated with the putative NS_S protein. Therefore, the PHV-NS_S-Ab rabbit serum is specific for the putative NS_S protein of PH virus expressed in insect cells.

Testing for the Presence of the NS_S Protein in PH virus Infected Vero Cells

PH virus infected or mock infected Vero cells were labelled at 10 days post-infection with [³⁵S]-methionine *in vivo* for 6 h. PH virus infected and mock infected cell lysates were immunoprecipitated with rabbit pre-immune serum or the PHV-NS_S-Ab rabbit serum (Fig. 27). No protein was precipitated from the PH virus or mock infected Vero cells that co-migrated with the NS_S protein (Fig. 27, lanes 1, 2, 3, 4, 5). Therefore, at this time there is no evidence that PH virus expresses a putative NS_S protein using its S mRNA.

Figure 26. Testing the specificity of the PHV-NS_s-Ab rabbit serum by immunoprecipitation of the PH virus NS_s protein from insect cell lysates. Fluorograph of [³⁵S]-methionine labelled immunoprecipitates from SF9 cells infected with the recombinant baculovirus AcPHVNS_s or wild type AcNPV. SF9 cells were infected with AcPHVNS_s or AcNPV. At 48 h post-infection the cells were harvested after labelling *in vivo* with [³⁵S]-methionine. The cells were lysed in SDS-PAGE sample buffer and heated to 100°C for 10 min. The cell lysates were immunoprecipitated with rabbit pre-immune serum or PHV-NS_s-Ab in the presence of protein A Sepharose CL-4B beads. The immunoprecipitated proteins were analyzed by SDS-PAGE, in a 12%-20% polyacrylamide gel, and fluorography. lane 1, 0.5% of the recombinant baculovirus AcPHVNS_s infected cell lysate; lane 2, 5% of the wild type AcNPV infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 3, 5% of the wild type AcNPV infected cell lysate immunoprecipitated with the PHV-NS_s-Ab rabbit serum; lane 4, 5% of the recombinant baculovirus AcPHVNS_s infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 5, 5% of the recombinant baculovirus AcPHVNS_s infected cell lysate immunoprecipitated with the PHV-NS_s-Ab rabbit serum; NS_s, PH virus small ORF from the S RNA segment expressed in insect cells; Protein size determinations were made using prestained low molecular size markers supplied by Bio-Rad: phosphorylase B (106 K), bovine serum albumin (80 K), ovalbumin (49.5 K), carbonic anhydrase (32.5 K), soybean trypsin inhibitor (18.5 K).

1

2 3 4 5

106K -

80K -

49.5K - 

32.5K - 

27.5K - 

18.5K - 

 < NS_s

Figure 27. Testing for the presence of the putative NS_S protein in PH virus infected Vero cells by immunoprecipitation of PH virus infected cell lysates with the PHV-NS_S-Ab rabbit serum. Fluorograph of [³⁵S]-methionine labelled immunoprecipitates from PH virus infected Vero cells. Vero cells were infected with PH virus or mock infected, at 10 days post-infection the cells were harvested after labelling for 6 h *in vivo* with [³⁵S]-methionine. We confirmed infection of the cells by IFA using PH virus specific anti-serum. The cells were lysed in RIPA buffer plus detergents and heated to 100°C for 10 min. The cell lysates were sonicated and then pelleted at 10 K for 20 min at 4°C. The cell lysate supernatants were immunoprecipitated with rabbit pre-immune serum and PHV-NS_S-Ab in the presence of protein A Sepharose CL-4B beads. The immunoprecipitated proteins were analyzed by SDS-PAGE, in a 12%-20% polyacrylamide gel, and fluorography. lane 1, 1,000 cpm of the immunoprecipitated NS_S protein from SF9 cells (Fig. 26, lane 5); lane 2, 50% of the mock infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 3, 50% of the mock infected cell lysate immunoprecipitated with the PHV-NS_S-Ab rabbit serum; lane 4, 50% of the PH virus infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 5, 50% of the PH virus infected cell lysate immunoprecipitated with the PHV-NS_S-Ab rabbit serum; NS_S, PH virus small ORF from the S RNA segment expressed in insect cells; Protein size determinations were made using prestained low molecular size markers supplied by Bio-Rad: phosphorylase B (106 K), bovine serum albumin (80 K), casein (49.5 K), carbonic anhydrase (32.5 K), soybean trypsin inhibitor (18.5 K).

1 2 3 4 5

106K -
80K -

49.5K -
32.5K -
27.5K -

18.5K -

NS_s —

PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 *rev* AND *vif* PROTEINS

The techniques used to modify the putative NS₅ gene of PH virus and express this gene in large quantities in insect cells using a recombinant baculovirus proved to be a simple way to express and characterize foreign proteins. Therefore, we decided to use this approach to express and study the HIV-1 *rev* and *vif* proteins.

1. EXPRESSION OF THE *rev* AND *vif* GENES OF HIV-1 IN INSECT CELLS

Deletion and Modification of the Non-Coding Sequence of the HIV-1 *rev* Gene and Transfer of this Gene into the Baculovirus Transfer Vector

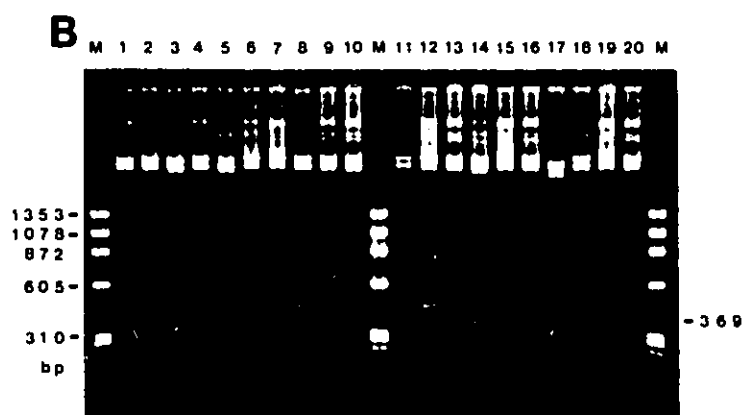
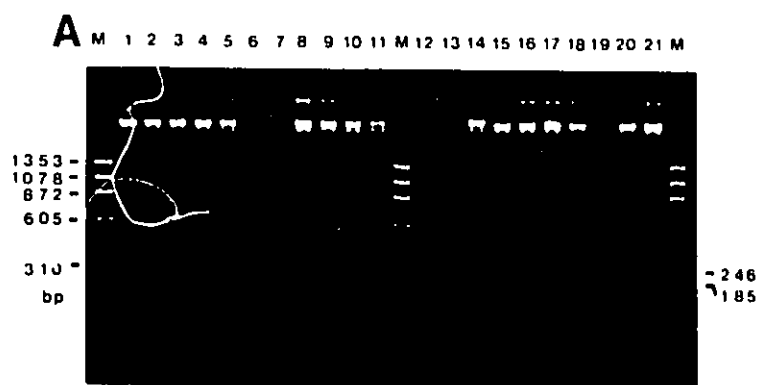
A plasmid, pIBI31-tatrev, containing the cDNA of the 2 Kb HIV-1 *tat* and *rev* mRNA was obtained from Dr. R. Gallo (NIH, Bethesda, MD) (Fig. 28). This clone has both exons comprising the entire 348-bp *rev* gene. Plasmid pIBI31-tatrev was digested with *Rsa*I which generated a 493-bp fragment containing the entire *rev* gene and this fragment was blunt-end ligated into the *Hinc*II site of pUC19 creating the new plasmid pUC19-*rev*1 (Fig. 28). To modify the upstream untranslated region of the HIV-1 *rev* gene, site-specific recombination directed by double-stranded crossover linkers was used. A double-stranded synthetic crossover linker was constructed with a *Xba*I cohesive end (CTAGA), a *Bgl*II recognition site upstream of the translation initiation codon to facilitate insertion into the baculovirus transfer vector, the CTATAAAT flanking sequence also upstream of the translation initiation codon, and the HIV-1 *rev*

Figure 28. Construction of a baculovirus transfer vector for the HIV-1 *rev* gene. Non-coding flanking sequence was removed upstream and downstream of the *rev* gene using site-specific recombination directed by double-stranded crossover linkers. This method was also used to add the flanking sequence CTATAAAT (P) immediately upstream of the initiating ATG and to add *Bgl*II sites on both ends of the *rev* gene. The modified HIV-1 *rev* gene was inserted into the *Bam*HI site of pAcYM1. The resultant vector pAcYM1-*rev* has the HIV-1 *rev* coding sequence in the correct orientation and under the control of the polyhedrin gene promoter.

coding sequence beginning with the *rev* protein start codon (ATGGCAGG-AAGA) (Fig. 28). This linker was kinased and ligated to plasmid pUC19-*rev1* that had been digested with *Xba*I and dephosphorylated with alkaline phosphatase. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker and *rev* gene following transformation of JM101 cells with the ligation mix (Fig. 28). Modified plasmids would have the 73-bp upstream untranslated region removed, being replaced with the CTATAAAT flanking sequence and a *Bgl*II recognition site added (Fig. 28). Therefore, digestion with *Bam*HI should yield a 246-bp fragment from incorrectly modified plasmids and a 185-bp fragment from correctly modified plasmids (Fig. 29A). Screening of 20 recombinant plasmids, digested with *Bam*HI, indicated clones 3, 4, 15, 16 and 17 were correctly modified (Fig. 29A, lanes, 4, 5, 16, 17, 18). Clone 3 was designated as pUC19-*rev2* (Fig. 28).

To modify the downstream untranslated region a double-stranded synthetic crossover linker was constructed with a *Hind*III cohesive end (AGCTT), a *Bgl*II recognition site included in the linker immediately downstream from the *rev* gene TAG termination codon to facilitate insertion into the baculovirus transfer vector, and the *rev* gene coding sequence (GATAAGAAATCGAGG) (Fig. 28). This linker was ligated to *Pst*I and *Hind*III digested pUC19-*rev2*. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker and *rev* gene following transformation of *E. coli* JM101 cells with the ligation mix (Fig. 28). The modified plasmid would have the 69-bp downstream untranslated region removed and a *Bgl*II site added. Primary selection of recombinants was done by digesting 20 recombinant clones with *Bgl*II since this would generate a 369-bp fragment from only modified plasmids (Fig. 29B).

Figure 29. Primary selection of recombinants with the HIV-1 *rev* gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers. A; lane 1, pUC19-*rev*1 digested with *Bam*HI; lanes 2-21, Primary selection of 20 possible recombinant clones was made by digesting their plasmid DNA with *Bam*HI after crossover linker mutagenesis of the upstream flanking region; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; B; lanes 1-20, Primary selection of 20 possible recombinant clones was made by digesting their plasmid DNA with *Bgl*II after crossover linker mutagenesis of the downstream flanking region; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers.



Clones 3, 5 and 14 yielded the correct size fragment after the digestion (Fig. 29B, lanes 3, 5, 14). Clone 3 was designated pUC19-rev3 (Fig. 28).

The modifications to the upstream and downstream untranslated regions were verified in pUC19-rev3 by determining the nucleotide sequence in the junction regions with a DuPont Genesis 2000 automated DNA sequencer (data not shown).

To make the baculovirus transfer vector the 369-bp *Bgl*II fragment from pUC19-rev3 was ligated into the *Bam*HI site of pAcYM1 (Fig. 28). This plasmid pAcYM1-rev has the CTATAAAT sequence upstream of the HIV-1 *rev* coding sequence and is in the correct orientation under the control of the polyhedrin gene promoter (Fig. 28).

Construction of the HIV-1 *rev* Recombinant Baculovirus

To construct a recombinant baculovirus expressing the HIV-1 *rev* gene, the plasmid pAcYM1-rev was mixed with wild type AcNPV DNA and used to co-transfect SF9 cells. The culture supernatant was harvested 3 days after co-transfection and was titrated on monolayers of SF9 cells. Plaques lacking occlusion bodies (polyhedrin-negative) were picked and plaque-purified three times before propagation. Two polyhedrin-negative baculoviruses were selected by this method. To verify that these two polyhedrin-negative viruses contained the HIV-1 *rev* gene, DNA was prepared from cells infected with these viruses, dot blotted onto a Colony/PlaqueScreen membrane and hybridized using nick translated plasmid pUC19-rev3 as a probe (Fig. 30). The DNA from mock infected cells (Fig. 30, dot C) did not hybridize with the probe. In contrast, DNA from the cells infected with the two polyhedrin-negative viruses (Fig. 30, dots R1, R2) and the plasmid pAcYM1-rev DNA (Fig. 24, dot RC) clearly hybridized. These results demonstrated that the HIV-1 *rev* gene was

Figure 30. Dot blot analysis of HIV-1 *rev* recombinant baculoviruses after plaque purification. SF9 cells were infected with purified polyhedrin-negative plaques or mock infected. Five days after infection 10% of mock infected or infected cells were lysed and their DNA was dot blotted onto a Colony/PlaqueScreen membrane. The DNA was hybridized with the nick translated plasmid pUC19-*rev*3. dot C, DNA from mock infected SF9 cells; dots R1, R2, DNA from cells infected with the HIV-1 *rev* recombinant baculoviruses 1A and 2A respectively; dot RC, 1.5 μ g of the plasmid pAcYM1-*rev*.

C

R1

R2

RC



present in both recombinant baculoviruses. Recombinant baculovirus 1A was designated as AcRev and was used in subsequent experiments.

Deletion and Modification of the Non-Coding Sequence of the HIV-1 *vif* Gene and Transfer of this Gene into the Baculovirus Transfer Vector

A plasmid, pHXB-2D, containing the entire HIV-1, strain HXB, proviral DNA was obtained from Dr. R. Gallo (NIH, Bethesda, MD) (Fig. 31). This clone has the entire 576-bp HIV-1 *vif* gene. Plasmid pHXB-2D was digested with *EcoRI* which generated a 1095-bp fragment containing the *vif* gene. This fragment was made blunt-ended with Klenow and was ligated into the *HincII* site of pUC19, creating a new plasmid pUC19-*vif*1 (Fig. 31). To modify the upstream untranslated region of the HIV-1 *vif* gene, site-specific recombination directed by double-stranded crossover linkers was used. A double-stranded synthetic crossover linker was constructed with a *Bam*HI cohesive end (GATCC), the CTATAAAAT flanking sequence upstream of the translation initiation codon, and HIV-1 *vif* coding sequence starting with the *vif* protein start codon (ATGGAAAAC-AGATGG) (Fig. 31). This linker was ligated to plasmid pUC19-*vif*1 that had been digested with *Bam*HI and *Xba*I. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker and *vif* gene following transformation of JM101 cells with the ligation mix (Fig. 31). Modified plasmids would have the 394-bp upstream untranslated region removed, being replaced with the CTATAAAAT flanking sequence (Fig. 31). Therefore, digestion with *EcoRI* and *Pst*I should yield a 736-bp fragment from correctly modified plasmids (Fig. 32A). Screening of 12 recombinant plasmids, digested with *EcoRI* and *Pst*I, demonstrated clones 1, 7 and 11 were correctly modified (Fig. 32A, lanes, 2, 8, 12). Clone 1 was designated as pUC19-*vif*2 (Fig. 31).

Figure 31. Construction of a baculovirus transfer vector for the HIV-1 *vif* gene. Non-coding untranslated sequence was removed upstream and downstream of the *vif* gene using site-specific recombination directed by double-stranded crossover linkers. This method was also used to add the flanking sequence CTATAAAT (P) immediately upstream of the translation initiation codon, ATG, and *Bam*HI sites on both ends of the *vif* gene. The modified HIV-1 *vif* gene was inserted into the *Bam*HI site of pAcYM1. The resultant vector pAcYM1-*vif* has the HIV-1 *vif* coding sequence in the correct orientation and under the control of the polyhedrin gene promoter.

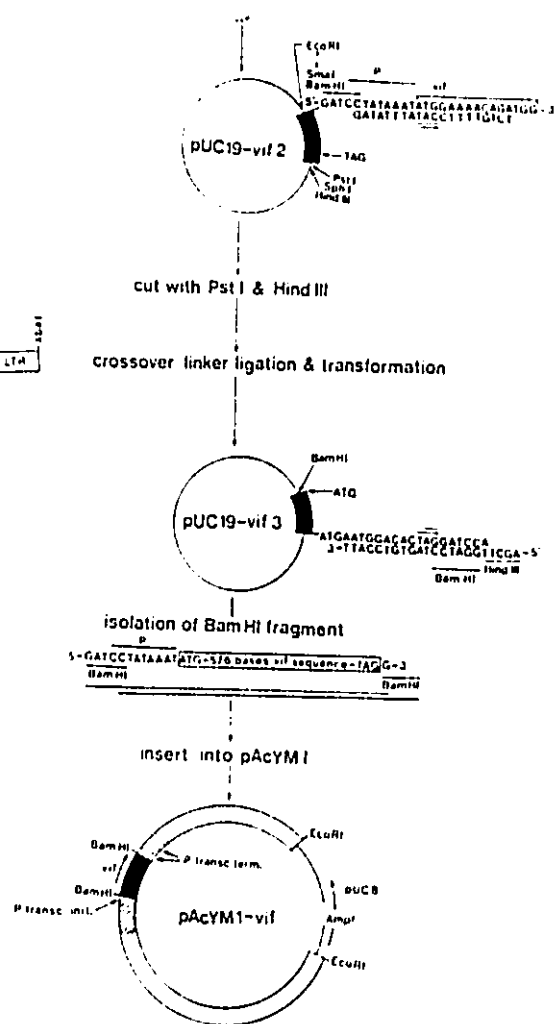
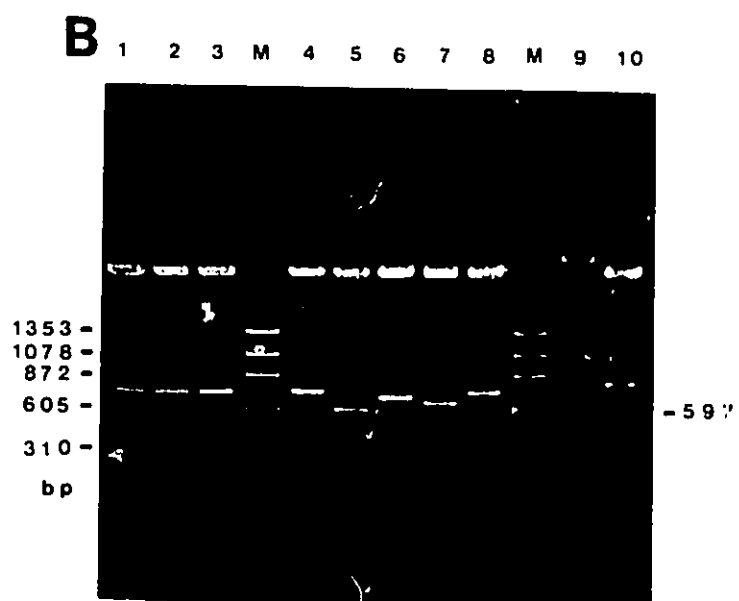
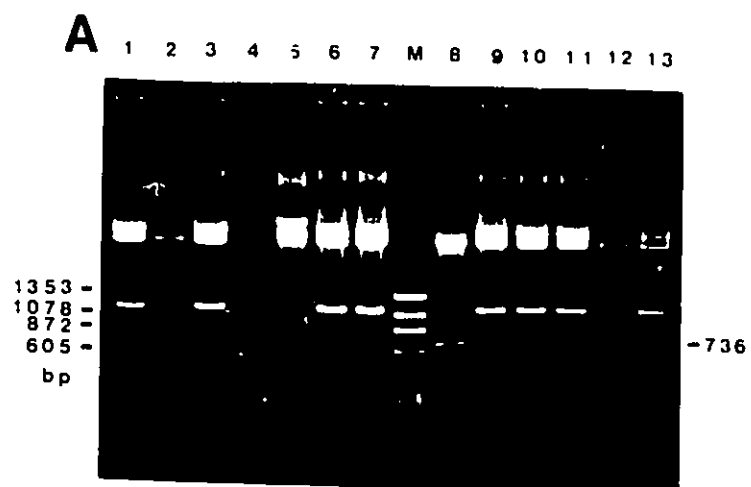


Figure 32. Primary selection of recombinants with the HIV-1 *vif* gene and with the upstream or downstream flanking sequence modified by site-specific recombination directed by double-stranded crossover linkers. A; lane 1, pUC19-*vif*1 digested with *Eco*RI and *Pst*I; lanes 2-13, Primary selection of 12 possible recombinant clones was made by digesting their plasmid DNA with *Eco*RI and *Pst*I after crossover linker mutagenesis of the upstream flanking region; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers; B; lanes 1-10, Primary selection of 10 possible recombinant clones was made by digesting their plasmid DNA with *Bam*HI after crossover linker mutagenesis of the downstream flanking region; lanes M, *Hae*III-digested ϕ X174 phage DNA fragments as molecular weight markers.



To modify the downstream untranslated region a double-stranded synthetic crossover linker was constructed with a *Hind*III cohesive end (AGCTT), a *Bam*HI recognition site included in the linker immediately downstream from the *vif* protein termination codon, TAG, to facilitate insertion into the baculovirus transfer vector, and *vif* gene coding sequence (GATCACAGGTAAGA) (Fig. 31). This linker was ligated to *Pst*I and *Hind*III digested pUC19-*vif*2. Intramolecular recombination occurred *in vivo* between the homologous ends of the linker and *vif* gene following transformation of *E. coli* JM101 cells with the ligation mix (Fig. 31). The modified plasmid would have the 125-bp downstream untranslated region removed and a *Bam*HI site added. Primary selection of recombinants was done by digesting 10 recombinant clones with *Bam*HI since this would generate a 597-bp fragment only from modified plasmids (Fig. 32B). Only clone 5 yielded the correct size fragment after the digestion (Fig. 32B, lane 5). Clone 5 was designated pUC19-*vif*3 (Fig. 31).

The modifications to the upstream and downstream untranslated regions were verified in pUC19-*vif*3 by determining the nucleotide sequence in the junction regions with a DuPont Genesis 2000 automated DNA sequencer (data not shown).

To make the baculovirus transfer vector the 597-bp *Bam*HI fragment from pUC19-*vif*3 was ligated into the *Bam*HI site of pAcYM1 (Fig. 31). This plasmid pAcYM1-*vif* has the CTATAAAT sequence upstream of the HIV-1 *vif* coding sequence and is in the correct orientation under the control of the polyhedrin gene promoter (Fig. 31).

Construction of the HIV-1 *vif* Recombinant Baculovirus

To construct a recombinant baculovirus expressing the HIV-1 *vif* gene, the plasmid pAcYM1-*vif* was used with wild type AcNPV DNA to

co-transfect SF9 cells. The culture supernatant was harvested 3 days after co-transfection and was titrated on monolayers of SF9 cells. Plaques lacking occlusion bodies (polyhedrin-negative) were picked and plaque-purified three consecutive times before propagation. Only one polyhedrin-negative baculovirus was selected by this method. To verify that this polyhedrin-negative baculovirus contained the HIV-1 *vif* gene, DNA was prepared from cells infected with this virus, dot blotted onto a Colony/PlaqueScreen membrane and hybridized with the nick translated *Bam*HI fragment from plasmid pUC19-vif3 (Fig. 33). DNA from mock infected cells (Fig. 33, dot C) did not hybridize with the probe. In contrast, DNA from the cells infected with the polyhedrin-negative virus (Fig. 30, dot V1) and the plasmid pAcYM1-vif DNA (Fig. 33, dot VC) clearly hybridized. These results demonstrated that the HIV-1 *vif* gene was present in this recombinant baculovirus. This recombinant baculovirus was designated as AcVif and was used in subsequent experiments.

Expression of the HIV-1 *rev* and *vif* Proteins by Recombinant Baculoviruses

A time-course experiment was done to analyse expression of the *rev* and *vif* proteins. SF9 cells were mock infected with either of the recombinant baculoviruses AcRev or AcVif, or wild type AcNPV. Each day post-infection for four days infected cells were harvested, lysed in SDS-PAGE sample buffer, heated for 10 min at 100°C, and stored at -20°C. When all the samples were collected they were analyzed by SDS-PAGE (Fig. 34). At 24 hours post-infection (hPi) the protein banding pattern in the baculovirus infected cells (Fig. 34, 24hPi, lanes 2, 3, 4) was similar to the protein banding pattern in mock infected cells (Fig. 34,

Figure 33. Dot blot analysis of HIV-1 *vif* recombinant baculoviruses after plaque purification. SF9 cells were infected with the purified polyhedrin-negative plaque or mock infected. Five days after infection 10% of mock infected or infected cells were lysed and their DNA dot blotted onto a Colony/PlaqueScreen membrane. The DNA was hybridized with the nick translated *Bam*HI fragment from plasmid pUC19-*vif*3. Dot C, DNA from mock infected SF9 cells; dot V1, DNA from cells infected with the HIV-1 *vif* recombinant baculovirus 1A; dot VC, 500 ng of the plasmid pAcYM1-*vif*.

C

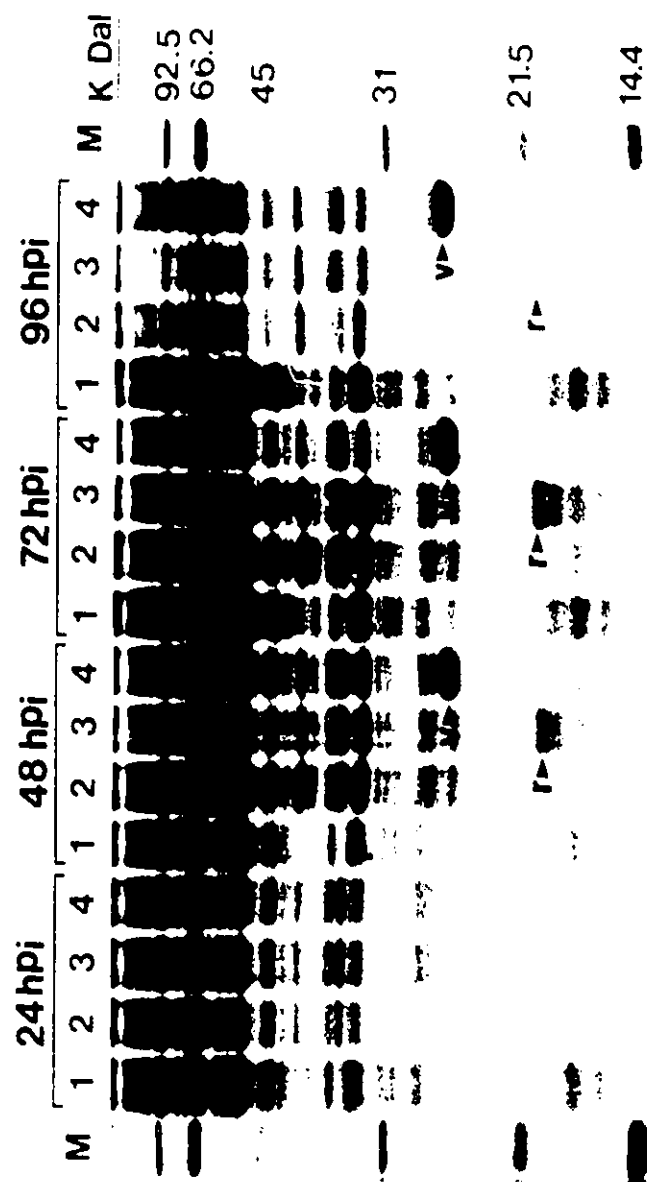
V1



V C



Figure 34. Expression of proteins in cells infected with wild type AcNPV, or the recombinant baculoviruses AcRev or AcVif during a 96 h time-course experiment. SF9 cells were mock infected, infected with either of the recombinant baculoviruses AcRev or AcVif, or the wild type virus AcNPV. Each day post-infection for four days mock infected or baculovirus infected cells were harvested, lysed in SDS-PAGE sample buffer, heated for 10 min at 100°C and analyzed by SDS-PAGE in a 15% polyacrylamide gel. Cells were harvested 24, 48, 72, and 96 hours post-infection (24, 48, 72 and 96hPi). The cells were lysed in SDS-PAGE sample buffer, heated to 100°C for 10 min and were analyzed by SDS-PAGE in a 15% polyacrylamide gel. Protein bands were visualized by staining with Coomassie blue; lanes 1, lysate of mock infected SF9 cells; lanes 2, lysate of wild type AcNPV infected SF9 cells; lanes 3, lysate of recombinant AcRev infected SF9 cells; lanes 4, lysate of recombinant AcVif infected SF9 cells; p, polyhedrin protein; r, HIV-1 *rev* protein; v, HIV-1 *vif* protein; lanes M, low molecular size markers supplied by Bio-Rad: phosphorylase B (92.5 K), bovine serum albumin (66.2 K), ovalbumin (45 K), carbonic anhydrase (31 K), soybean trypsin inhibitor (21.5 K), lysozyme (14.4 K).



24hPi, lane 1). Although some early baculovirus proteins were observed, the polyhedrin protein, HIV-1 *rev* protein and HIV-1 *vif* protein were not observed (Fig. 34, 24hPi, lanes 2, 3, 4). At 48 hPi the baculovirus infected cells had several major protein bands (Fig. 34, 48hPi, lanes 2, 3, 4) not observed in the mock infected cells (Fig. 34, 48hPi, lane 1). Cell lysates infected with each of the baculoviruses had major protein bands not observed in any of the other cell lysates. The cell lysate of wild type AcNPV infected cells had a unique protein with a M_r of 34 K which is the expected size of the polyhedrin protein (Fig. 34, 48hPi, lane 2, p). The cell lysates of the AcRev and AcVif recombinant baculoviruses infected cells had unique proteins with M_r 's of 20 K and 27 K respectively (Fig. 34, 48hPi, lane 3, r; lane 4, v). The size of these proteins is consistent with the expected size of the HIV-1 *rev* and *vif* proteins (Kan *et al.*, 1986; Lee *et al.*, 1986b; Sodroski *et al.*, 1986; Goh *et al.*, 1987; Knight *et al.*, 1987; Cullen *et al.*, 1988; Malim *et al.*, 1988). The amount of *rev* protein continued to increase by 72 hPi but almost completely disappeared from the AcRev infected cells during the next 24 h (Fig. 34, 72hPi, lane 3, r; 96hPi, lane 3, r). The *vif* protein however, continued to accumulate for the remaining 48 h (Fig. 34, 72hPi, lane 4, v; 96hPi, lane 4, v). The HIV-1 *vif* protein in this gel was slightly larger than it has usually been identified (23 K, Kan *et al.*, 1986; Lee *et al.*, 1986b; Sodroski *et al.*, 1986). However, the *vif* protein had a M_r of 23-27 K depending on the molecular weight standards used (data not shown).

Phosphorylation of the HIV-1 *rev* Protein

The HIV-1 *rev* protein was previously found to be a nuclear regulatory phosphoprotein in HIV-1 infected mammalian cells (Hauber *et al.*, 1988; Cullen *et al.*, 1988; Cochrane *et al.*, 1989a, 1989b). To

determine if the HIV-1 *rev* protein is phosphorylated in the insect cells, SF9 cells were infected with wild type AcNPV, the recombinant baculovirus AcRev or the recombinant baculovirus AcPHVNS_S. Two days after infection the cells were labelled with [³²P]-orthophosphate *in vivo*, lysed in SDS-PAGE sample buffer, and the cell lysates were analyzed on an SDS-polyacrylamide gel. Proteins were visualized by fluorography (Fig. 35). Several proteins were labelled with [³²P]-orthophosphate in baculovirus infected cells (Fig. 35, lanes 1, 2, 3). However, a ³²P-labelled protein with a M_r of 20 K was only observed in the AcRev recombinant baculovirus infected cell lysate (Fig. 35, lane 3). This is the size of the HIV-1 *rev* protein and this result demonstrates that the *rev* protein is also post-translationally phosphorylated in the insect cell expression system.

Reaction of Human Sera from HIV-1 Infected Donors Against the *rev* and *vif* Proteins Expressed in Insect Cells

Cell extracts of wild type AcNPV or the AcRev or AcVif recombinant baculovirus infected SF9 cells were prepared and sent to Dr. Devash and co-workers (Medical Products Department, E. I. DuPont Co., Wilmington, DE). They prepared Western blots with these cell extracts and tested the ability of antibodies in sera from HIV-1 infected human donors to recognize the *rev* and *vif* proteins expressed in SF9 cells infected with the recombinant baculoviruses (Fig. 36, from Devash *et al.*, 1990). Some sera from HIV-1 infected human donors were found to contain antibodies that did recognize the baculovirus expressed *rev* or *vif* proteins (Fig. 36, lanes 2, 4). A non-specific protein with a M_r of 24 K was observed in cell extracts from all baculovirus infected SF9 cells (Fig. 36, lanes 1, 2, 3, 4).

Figure 35. Phosphorylation of the HIV-1 *rev* protein expressed in insect cells. Wild type baculovirus (AcNPV) or recombinant baculoviruses carrying either the HIV-1 *rev* gene (AcRev) or the PH virus NS_s gene (AcPHVNS_s) was used to infect SF9 cells. Two days after infection the cells were labelled *in vivo* with [³²P]-orthophosphate. After labelling cells were lysed in SDS-PAGE sample buffer and were analyzed by SDS-PAGE, in a 15% polyacrylamide gel, and fluorography. Lane 1, 10% of the wild type AcNPV infected cell lysate; lane 2, 10% of the recombinant AcPHVNS_s infected cell lysate; lane 3, 10% of the recombinant AcRev infected cell lysate; Ph, polyhedrin protein; rev, HIV-1 *rev* protein; NS_s, PH virus NS_s protein; Protein size determinations were made using prestained molecular size markers supplied by BRL: ovalbumin (47 K), α-chymotrypsin, (33 K), β-lactoglobulin, (24 K), lysozyme (16 K).

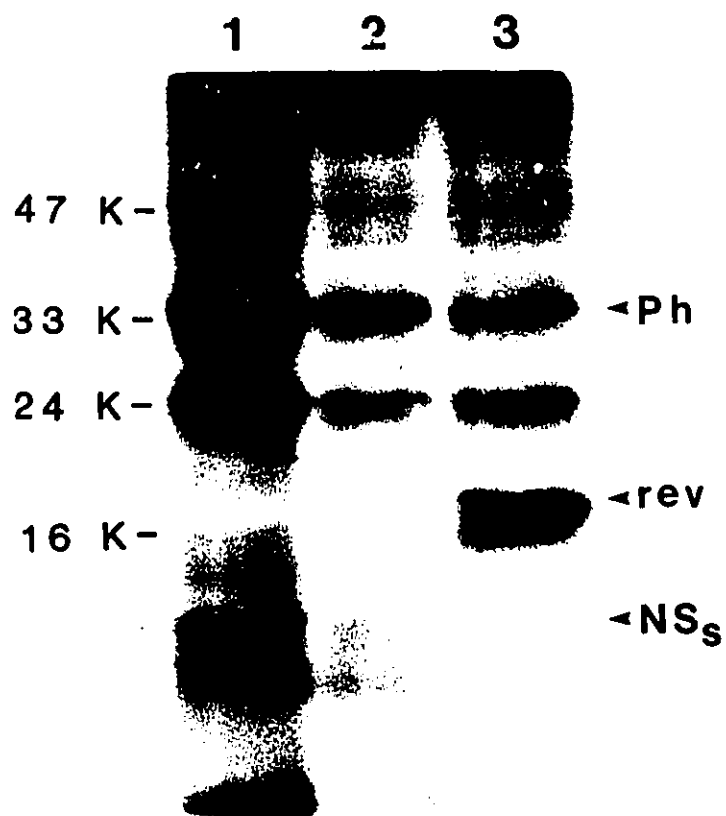


Figure 36. Western blot of *vif* and *rev*. Recombinant baculovirus carrying either the *vif* (AcVif) or *rev* gene (AcRev) was used to infect SF9 cells. The infected cells were collected 72 h post-infection, total cellular protein was subjected to SDS-PAGE, transferred to a nitrocellulose filter and incubated with serum from an HIV-1 infected individual. The reaction was completed after washing and incubations with horseradish peroxidase-conjugated anti-species antibody and precipitable substrate (4-chloro-1-naphthol). Lanes 1 and 3, wild type baculovirus AcNPV infected SF9 cell extracts; lane 2, AcRev infected SF9 cell extracts; lane 4, AcVif infected SF9 cell extracts. The band just below the 24 K marker is a non-specific protein.

1 2 3 4

— 41k —
— 31k —

←-vif

— 24k —

←-rev
— 17k —

The sera from 52 HIV-1 infected healthy donors, 22 HIV-1 infected healthy donors at risk, 18 AIDS-related complex (ARC) patients and 27 Acquired Immune Deficiency Syndrome (AIDS) patients were tested for their ability to react with the *rev* and *vif* proteins expressed in insect cells (Table 5, from Devash *et al.*, 1990). The results showed that approximately 35%-41% of asymptomatic HIV-1 infected individuals have antibodies against the HIV-1 *vif* protein (Devash *et al.*, 1990). However, none of the ARC or AIDS patients tested had antibody against the *vif* protein (Devash *et al.*, 1990). In contrast, HIV-1 infected individuals were found at all stages of infection that had antibodies against the HIV-1 *rev* protein (Devash *et al.*, 1990).

Production of Rabbit Antibodies Specific for the HIV-1 *vif* Protein

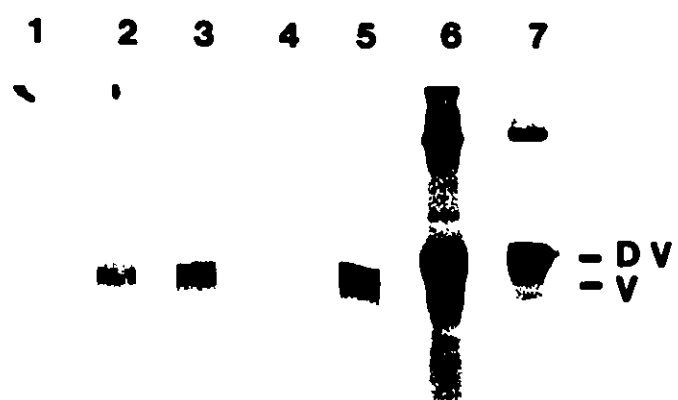
To produce antibodies against the HIV-1 *vif* protein this protein was expressed in SF9 cells infected with AcVif, partially purified with SDS-PAGE and used to inoculate rabbits. The *vif* protein co-migrates with a baculovirus protein during SDS-PAGE so this protein was also unavoidably inoculated into the rabbits. The rabbit serum prepared against the HIV-1 *vif* protein was designated as Vif1-Ab. The specificity of the rabbit serum for the HIV-1 *vif* protein was demonstrated using western blots. Pre-immune rabbit serum did not react to any proteins in the AcVif infected SF9 cell lysate (Fig. 37, lane 1). The rabbit serum Vif1-Ab recognized a single protein band, with the same M_r as the HIV-1 *vif* protein, in both the AcVif and wild type AcNPV infected SF9 cell lysates (Fig. 37, lanes 2, 3). These data indicate that the Vif1-Ab rabbit serum contained antibodies that recognized the wild type baculovirus protein co-purified and inoculated into rabbits with the *vif* protein. From these data it was impossible to determine if the Vif1-Ab rabbit serum was recognizing just

Table 5. Prevalence of antibodies against *vif* and *rev* in HIV-1-infected individuals.

Disease stage	No. of Total <i>vif</i> Ab ⁺ /tested (%)	No. of Total <i>rev</i> Ab ⁺ /tested (%)
Asymptomatic (CDC group I)	17/52 (32.6)	18/52 (34.6)
Asymptomatic (CDC group II)	8/22 (36.3)	9/22 (40.9)
ARC patients (CDC group III)	0/18 (0)	4/18 (22.2)
AIDS patients	0/27 (0)	6/27 (22.2)

HIV-1 positive sera were determined by western blots and gp41-recombinant ELISA. Ab, antibody.

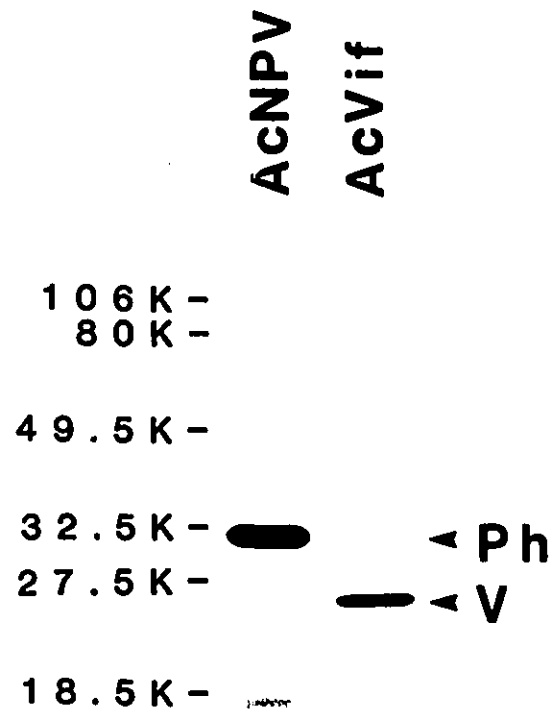
Figure 37. Western blot of the HIV-1 *vif* protein expressed in insect cells or *E. coli*. The recombinant baculovirus AcVif or wild type AcNPV was used to infect SF9 cells. The infected cells were collected 72 h post-infection, total cellular protein was subjected to SDS-PAGE, and transferred to nitrocellulose membranes. Cell lysate of a HIV-1 *vif* fusion protein expressed in *E. coli* was obtained from Dr. Devash (Medical Products Department, E. I. DuPont Co., Wilmington, DE). The nitrocellulose membranes were incubated with either rabbit pre-immune serum, the rabbit anti-*vif* antibody (HIV-1-*vif*-Ab) or an anti-*vif* antibody obtained from Dr. Devash (Medical Products Department, E. I. DuPont Co., Wilmington, DE). The reaction was completed after washing and incubations with horseradish peroxidase-conjugated anti-species antibody and precipitable substrate (4-chloro-1-naphthol). Lane 1, AcVif infected SF9 cell extracts reacted with rabbit pre-immune serum; lane 2, wild type AcNPV reacted with HIV-1-*vif*-Ab; lane 3, AcVif infected SF9 cell extracts reacted with HIV-1-*vif*-Ab; lane 4, wild type AcNPV reacted with the DuPont anti-*vif* antibody; lane 5, AcVif infected SF9 cell extracts reacted with the DuPont anti-*vif* antibody; lane 6, *vif* fusion protein expressed in *E.coli* reacted with the DuPont anti-*vif* antibody; lane 7, *vif* fusion protein expressed in *E.coli* reacted with HIV-1-*vif*-Ab; V, HIV-1 *vif* expressed in insect cells; DV, *vif* fusion protein expressed in *E.coli*.



the wild type baculovirus protein or *vif* protein also. To verify that Vif1-Ab rabbit serum was recognizing the *vif* protein this serum was reacted with a HIV-1 *vif* fusion protein expressed in *E. coli* obtained from Dr. Devash (Medical Products Department, E. I. DuPont Co., Wilmington, DE). The DuPont anti-*vif* antibody and the rabbit Vif1-Ab serum both reacted with the *vif* fusion protein (Fig. 37, lanes 6, 7). These data indicate that the Vif1-Ab rabbit serum does contain antibodies that recognize the HIV-1 *vif* protein. The specificity of the Vif1-Ab rabbit serum for the HIV-1 *vif* protein was further demonstrated using radioimmunoprecipitation. AcVif or AcNPV infected SF9 cells were labelled with [³⁵S]-methionine *in vivo* and part of the cell lysates were used in immunoprecipitation with the rabbit serum Vif1-Ab or pre-immune rabbit serum and precipitated proteins were analyzed by SDS-PAGE. The wild type baculovirus protein that co-migrates with the HIV-1 *vif* protein is only weakly labelled with [³⁵S]-methionine in comparison with the HIV-1 *vif* protein when expressed in insect cells (Fig. 38A, lanes AcNPV, AcVif). Rabbit pre-immune serum did not precipitate any [³⁵S]-methionine labelled proteins from either the AcPHVNS₅ or AcNPV infected cell lysates (Fig. 38B, lanes 2, 4). The rabbit serum Vif1-Ab precipitated a protein weakly labelled with [³⁵S]-methionine from the wild type AcNPV infected cell lysate (Fig. 38B, lane 3). In contrast, one strongly [³⁵S]-methionine labelled protein was immunoprecipitated from the recombinant AcVif infected cell lysate (Fig. 38B, lane 5). Comparison with the [³⁵S]-methionine labelled AcVif infected cell lysate (Fig. 38B, lane 1) showed that the ³⁵S-labelled protein which was precipitated with the Vif1-Ab rabbit serum, co-migrated with the HIV-1 *vif* protein. Therefore, the Vif1-Ab rabbit serum contains specific antibodies that

Figure 38. Testing the specificity of the HIV-1-*vif*-Ab rabbit serum by immunoprecipitation of the HIV-1 *vif* protein from insect cell lysates. Fluorographs of [35 S]-methionine labelled cell lysates or immunoprecipitates from SF9 cells infected with the recombinant baculovirus AcVif or wild type AcNPV. SF9 cells were infected with AcVif or AcNPV, at 48 h post-infection the cells were harvested after labelling *in vivo* with [35 S]-methionine. The cells were lysed in SDS-PAGE sample buffer and heated to 100°C for 10 min. The cell lysates were analyzed by SDS-PAGE, in a 10% polyacrylamide gel, or immunoprecipitated with rabbit pre-immune serum and Vif1-Ab in the presence of protein A Sepharose CL-4B beads. The immunoprecipitated proteins were analyzed by SDS-PAGE, in a 15% polyacrylamide gel, and fluorography. A; lane AcNPV, 0.2% of the wild type AcNPV infected cell lysate; lane AcVif, 0.2% of the recombinant AcVif infected cell lysate; Protein size determinations were made using prestained low molecular size markers supplied by Bio-Rad: phosphorylase B (106 K), bovine serum albumin (80 K), ovalbumin (49.5 K), carbonic anhydrase (32.5 K), soybean trypsin inhibitor (18.5 K); Ph, polyhedrin; V, *vif*; B; lane 1, 0.5% of the recombinant baculovirus AcVif infected cell lysate; lane 2, 5% of the wild type AcNPV infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 3, 5% of the wild type AcNPV infected cell lysate immunoprecipitated with the Vif1-Ab rabbit serum; lane 4, 5% of the recombinant baculovirus AcVif infected cell lysate immunoprecipitated with rabbit pre-immune serum; lane 5, 5% of the recombinant baculovirus AcVif infected cell lysate immunoprecipitated with the Vif1-Ab rabbit serum.

A



B



recognize the HIV-1 *vif* protein expressed in insect cells.

CHAPTER 4: DISCUSSION

PART 1: PROSPECT HILL VIRUS

Hemorrhagic fever with renal syndrome (HFRS) is an acute disease in humans caused by viruses in the *Hantavirus* genus of the Bunyaviridae family. Infection in humans is from exposure to infected rodents or insectivores or their infected excreta. Hantaviruses cause a chronic asymptomatic infection in their rodent reservoir hosts and are apparently only harmful to humans. HFRS is characterized by symptoms of renal dysfunction with varying degrees of severity. In the severest form of HFRS mortality rates can reach 30%. The severity of a HFRS disease, caused by a specific *Hantavirus*, is related to the virus serotype which is related to the rodent host. Hantaviruses are endemic throughout the world and infection appears common, since, based on IFA antibody levels, approximately 17.4 million people have been infected with hantaviruses in Europe alone (Lee and van der Groen, 1989). *Hantavirus* isolates have been divided into four antigenically distinct serotypes using the immunofluorescent antibody, immune adherence hemagglutination, and plaque reduction neutralization tests (Lee *et al.*, 1985b; Sugiyama *et al.*, 1987). During the last few years our understanding of the molecular biology of hantaviruses has been greatly expanded by the cloning and nucleotide sequencing of the M and S genomic segments of pathogenic hantaviruses in serotypes 1, 2 and 3 (Schmaljohn *et al.*, 1986b, 1987b; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Iizegawa *et al.*, 1990; Stohwasser *et al.*, 1990; Antic *et al.*, 1991a), and the L segments of pathogenic hantaviruses in serotypes 1 and 2 (Schmaljohn, 1990; Antic *et*

al., 1991b). The hope has been that by better understanding the molecular biology of these viruses an effective vaccine could eventually be developed.

In this study the growth of PH virus and the coding strategy of the PH virus M and S genomic RNA segments were examined. In addition the putative gene products encoded by the PH virus M and S RNA segments were compared with the homologous gene products of pathogenic hantaviruses in serotypes 1, 2 and 3. Hantaan virus (serotype 1) is the etiologic agent of a severe form of HFRS (Lee *et al.*, 1978), SR virus (serotype 2) is linked to a moderate form of HFRS (Lee *et al.*, 1982a; Kitamura *et al.*, 1983), and Hällnäs virus (serotype 3) is associated with a milder form of HFRS, nephropathia epidemica (NE) (Brummer-Korvenkontio *et al.*, 1980). PH virus was chosen for this study for several reasons. Serological examination demonstrated PH virus was in serotype 4 (Lee *et al.*, 1985b; Sugiyama *et al.*, 1987), and no *Hantavirus* from this serotype had previously been molecularly characterized. Therefore, at the completion of this study the M and S genomic RNA segments of at least one *Hantavirus* in each of the four recognized serotypes would have been molecularly characterized. Also, all of the hantaviruses that have previously been molecularly characterized can cause a human disease, whereas PH virus was demonstrated to be non-pathogenic (Yanagihara *et al.*, 1984a, 1987). Therefore, a comparison between pathogenic and non-pathogenic viruses may lead us to map domain(s) responsible for pathogenesis. Finally, PH virus was isolated in North America, specifically Frederick, MD, USA (Lee *et al.*, 1982c, 1985a). All other presently characterized hantaviruses were isolated in Asia or Europe and by examining PH virus the role, if any, of geography on *Hantavirus*

evolution could be examined.

PH Virus Genome

The PH virus genome was found to be composed of three single-stranded RNA segments Large (L), Medium (M) and Small (S) with M_r values of 2.2×10^6 , 1.3×10^6 and 0.6×10^6 respectively, as previously calculated by Yoo (1988).

Hantaviruses, including PH virus, were found to have unequal molar ratios of their genomic RNA segments (Yoo and Kang, 1987a). Among the viruses examined by Yoo and Kang (1987a), PH virus grows to the lowest titer and also had the greatest imbalance in the molar ratios of its RNA segments, particularly an excess of the S RNA segment (6.3S:1L for PH virus as compared to 2.2S:1L for Hantaan virus, Yoo and Kang, 1987a).

Bunyaviruses were also demonstrated to have unequal molar ratios of their RNA segments with the S RNA segment usually being over represented (Pettersson and Kääriäinen, 1973; Bouloy *et al.*, 1973/74; Obijeski *et al.*, 1976b; Gentsch *et al.*, 1977). Bunyaviruses can readily establish persistent infections in mosquito cell culture. These persistently infected mosquito cells show no differences in their metabolic activities, but continually shed infectious virus (Newton *et al.*, 1981; Nicoletti and Verani, 1985; Carvalho *et al.*, 1986; Elliott and Wilkie, 1986; Rossier *et al.*, 1988). A feature of these persistently infected mosquito cells is that they contain an excess amount of the genomic S RNA segment (Elliott and Wilkie, 1986; Rossier *et al.*, 1988). Defective interfering (DI) particles have been isolated from these cultures that only contain the S RNA segment (Elliott and Wilkie, 1986). *Hantavirus* infected Vero cells have characteristics similar to these persistently infected mosquito cells in that host cell protein synthesis is unaffected, there is no cytopathic

effect and virus is continually shed during the infection. If PH virus was producing DI particles containing only the genomic S RNA segment, similar to those of bunyaviruses, that could interfere with virus replication, this could explain the high molar ratio of genomic S RNA isolated from PH virus and the lower titer of this virus.

Growth Characteristics of PH Virus

A PH virus infection in Vero cells progresses slowly (8-15 days) and grows to a low titer (10^5 pfu/ml). To clone the genomic RNA segments of PH virus it was necessary to develop an assay to determine the best times to harvest the virus during an infection and isolate PH virus RNA. We found that labelling PH virus infected cells with [^3H]-uridine, pelleting the labelled virus from a small sample of medium removed from the culture dishes each day and measuring the amount of [^3H]-uridine labelled virus that could be pelleted gave a good estimate of the amount of virus released from the cells into the medium each day. The growth pattern observed in Figure 2 was not uniform, but varied with the passage history of the PH virus stock. Later passages of virus grew in the cells for 15 days without any cytopathic effect being observed. This allowed PH virus to be harvested three times, 8, 12 and 15 days post-infection. However, one consistent finding in all of the growth assay experiments was the large increase or "burst" in the amount of PH virus released from the cells immediately following the addition of fresh tissue culture medium on the cells. It appears that the addition of fresh medium to the infected cells stimulates PH virus production in the cells, and release of the virus into the tissue culture medium. This finding was supported by the observation that there was an increase in the amount of PH virus specific RNA that could be isolated from PH virus infected cells

24 hours after they were given fresh medium.

Features of the M and S RNA Segments of PH virus

The PH virus M and S RNA segments have the potential to form energetically stable panhandle structures because the 3' and 5' termini of these segments are inversely complementary and can thus base-pair. All other *Hantavirus* RNA segments that have been examined also have inverse complementarity between their 3' and 5' termini and therefore the potential to form a panhandle structure (Schmaljohn *et al.*, 1986b, 1987b, 1990; Yoo and Kang, 1987b; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Izegawa *et al.*, 1990; Stohwasser *et al.*, 1990; Antic *et al.*, 1991a, 1991b). Complementarity between the terminal nucleotides of an RNA segment is also a characteristic of all viruses examined in the Bunyaviridae family (Parker and Hewlett, 1981; Bishop *et al.*, 1982; Cabridilla *et al.*, 1983; Ihara *et al.*, 1984; Eshita and Bishop, 1984; Collett *et al.*, 1985; Elliott, 1990). This panhandle structure is thought to be the reason why *Bunyavirus* and *Uukuvirus* nucleocapsids are circular (Pettersson and von Bonsdorff, 1975; Samso *et al.*, 1975; Obijeski *et al.*, 1976b). Work with bunyaviruses suggests that these conserved terminal sequences are important for encapsidation of the RNA with N protein since usually only genomic RNA and some full length viral complementary RNA can be encapsidated (Raju and Kolakofsky, 1986). In contrast, bunyavirus mRNA, which has a 5' terminal extension and is shorter at the 3' end, is not encapsidated (Raju and Kolakofsky, 1986). These data suggest that the 5' end of viral RNA is important for encapsidation. Raju and Kolakofsky (1989) recently demonstrated that the encapsidated viral RNA definitely forms a panhandle by base-pairing between the 3' and 5' termini.

The unmatched and mismatched base-pairs in the double-stranded RNA

region are thought to be important for protein recognition (Wickens and Dahlberg, 1987). Also the base-pairing of viral RNA and full length viral complementary RNA would be somewhat different since G to U is an allowed base-pairing in RNA but the complementary pair C to A is not. G to U base-pairing is predicted in both the PH virus M and S RNA panhandle structures. It has been suggested that if proteins could distinguish between the panhandles of genome and anti-genome RNA this could be important for transcription and packaging of genomic RNA into virions (Elliott, 1990).

Influenza A viruses are a group of viruses possessing a genome consisting of eight segments of single-stranded RNA with negative polarity. The RNA segments of these viruses were also demonstrated to form circular panhandles held together by base-pairing between the 3' and 5' termini (Hsu *et al.*, 1987; Honda *et al.*, 1988). Since the influenza virus polymerase was shown to be bound to the double-stranded region of RNA it was suggested that the terminal base-paired structure functioned as the promoter for transcription (Honda *et al.*, 1988). However, Parvin *et al.* (1989) demonstrated that virion RNA lacking the complementary sequence at its 5' end was incapable of forming a panhandle, and yet was still transcribed normally. Therefore, the base-paired panhandles that form in Bunyaviridae RNA, possibly including hantaviruses, is likely only important for packaging of the genomic RNA segments.

Nucleotide sequence analysis of the PH virus S RNA segment revealed a possible, energetically stable, hairpin structure. Hairpins are known to serve as transcription termination signals in the S RNA segments of the Punta Toro phlebovirus (Emery and Bishop, 1987), and the Pichinde and lymphocytic choriomeningitis (LCM) arenaviruses (Romanowski and Bishop,

1985). The PH virus S RNA segment hairpin is similar in size and structure to the hairpin observed in the LCM virus S RNA segment. If PH virus uses this hairpin structure as a transcription termination signal, subgenomic mRNAs would be produced. Although we do not know if PH virus produces subgenomic mRNAs, they have been observed in bunyavirus infected cells (Cash et al. 1979; Bishop et al., 1983; Patterson and Kolakofsky, 1984). *Bunyavirus* mRNAs have heterologous non-viral sequences at their 5' ends (Bishop et al., 1983; Patterson and Kolakofsky, 1984; Eshita et al., 1985; Collett, 1986) and the 3' end does not appear to be polyadenylated (Abraham and Pattnaik, 1983; Bouloy et al., 1984; Elliott, 1985). The *Bunyavirus* mRNAs are 60 to 100 nucleotides shorter than full-length transcripts (Elliott, 1990). There is no transcription termination sequence common to the *Bunyavirus* RNA segments (Elliott, 1990). The nature of the 5' end of the *Hantavirus* mRNAs is not presently known but the 3' end was shown not to be polyadenylated (Yoo, 1988). When hairpins function as transcription termination signals transcription usually terminates near the top of the hairpin (Emery and Bishop, 1987). If the hairpin in the PH virus S RNA segment is acting as a transcription termination signal the subgenomic mRNA produced would be approximately 75 nucleotides shorter than a full-length transcript. This would be consistent with what was observed in *Bunyavirus* mRNAs. However, the only way to verify that the hairpin in the PH virus S RNA segment is acting as a transcription termination signal would be to determine the nucleotide sequence at the 3' end of the PH virus S mRNA. Based on the nucleotide sequence data, Hällnäs virus, SR virus, and Hantaan virus S RNA segments are not capable of forming a similar type of hairpin structure.

Coding Strategy of the PH Virus M RNA Segment

A previous comparison of the G1 proteins of Hantaan virus (serotype 1) and SR virus (serotype 2) found amino acid similarities of 75% or 83% if conservative changes were included (Arikawa *et al.*, 1990). The G2 proteins of these viruses were even more highly conserved with amino acid similarities of 82% or 92% if conservative amino acid substitutions were included (Arikawa *et al.*, 1990). These figures were similar to the ones we found when comparing the PH virus (serotype 4) and Hällnäs virus (serotype 3) G1 and G2 proteins (G1-74.3% or 85.8% with conservative differences; G2-79% or 88.4% with conservative differences). When the PH virus (serotype 4) G1 and G2 proteins were compared with the G1 and G2 proteins of Hantaan virus (serotype 1) and SR virus (serotype 2) the amino acid similarity was much lower. These data support earlier work suggesting that serotypes 3 and 4 are antigenically closer to each other than to serotypes 1 or 2 (Sugiyama *et al.*, 1987).

A distinguishing feature of some of the hantaviruses in serotypes 1, 2, and 3 is that they can cause disease in humans. Hantaan virus (serotype 1) is the etiologic agent of a severe form of HFRS (Lee *et al.*, 1978), SR virus (serotype 2) is linked to a moderate although potentially still fatal form of HFRS (Lee *et al.*, 1982a; Kitamura *et al.*, 1983), and Hällnäs virus (serotype 3) is associated with a milder form of HFRS, NE (Brummer-Korvenkontio *et al.*, 1980). In contrast, PH virus (serotype 4) has not been linked to any disease in humans (Yanagihara *et al.*, 1984a, 1987). In bunyaviruses the M RNA segment, specifically the G1 protein, has been identified as the determinant of virulence and infectivity (Beaty *et al.*, 1981, 1982; Shope *et al.*, 1981; Gonzalez-Scarano *et al.*, 1985, 1988; Janssen *et al.*, 1986; Sundin *et al.*, 1987). If this is true for hantaviruses

as well, the large number of amino acid differences observed in the Hällnäs virus G1 and G2 proteins as compared with SR virus and Hantaan virus may explain why this virus only causes the milder form of the disease. The similarity between the G1 and G2 proteins of PH virus and Hällnäs virus suggests that these viruses may have undergone amino acid changes that attenuated virulence in these viruses as compared with SR virus and Hantaan virus. Alternatively, the G1 and G2 proteins of PH virus and Hällnäs virus may have undergone changes that enhanced virulence giving rise to hantaviruses like Hantaan virus and SR virus. There was less conservation observed in the G1 proteins of these viruses as compared with their G2 proteins and this could mean that *Hantavirus* virulence is also associated with the G1 protein. The only major difference observed between the PH virus G1 and G2 proteins and those of the other three viruses was in the hydrophilicity profiles. The highest peak of hydrophilicity in the PH virus glycoprotein precursor is apparently associated with an amino acid deletion in the PH virus G1 protein as compared with the G1 proteins of the other three viruses. Whether any of these differences are involved in the lack of virulence of PH virus is not presently known. However, changes as small as a single amino acid substitution in the hemagglutinin protein were shown to alter the virulence of Influenza A viruses (Kawaoka *et al.*, 1984; Philpott *et al.*, 1990). In these examples however, the Influenza A viruses being compared were mutated forms of the same virus or were closely related, and therefore there were only a small number of amino acid changes to examine. By comparing the G1 and G2 proteins of PH virus with the G1 and G2 proteins of other hantaviruses in different serotypes there were too many differences in the amino acid sequence, probably related to the

different hosts and the antigenic distinctness, to be able to identify changes that could affect virulence. A *Hantavirus*, strain 5302, was isolated in the far Eastern Soviet Union from the vole *Microtus fortis*. Polyclonal and monoclonal antisera identified 5302 virus as a serotype 4 virus antigenically almost identical to PH virus (Sugiyama *et al.*, 1987). The 5302 virus however, is the etiologic agent of an HFRS disease (Tkachenko *et al.*, 1984). An amino acid sequence comparison between the G1 and G2 proteins of PH virus and the G1 and G2 proteins of 5302 virus would give a better chance of determining regions important for virulence in either of these proteins.

Only one potential transmembrane region was conserved in the G1 proteins and one region in the G2 proteins of all four viruses. This suggests that these regions are possibly membrane spanning domains that anchor the G1 and G2 proteins to the surface of the viral envelope. The prediction of potential transmembrane helices using the method of Rao and Argos (1986) sets limits on the length of the predicted helices of between 16 and 35 amino acids to attain one helix that can span a typical membrane. Therefore, it was unexpected that the potential transmembrane region in the G1 protein, predicted by the computer, that is common to all four viruses would be over twice the maximum length allowed in this method (Rao and Argos, 1986). We are unable to determine why such a large region was predicted. The entire 71-76 amino acid region is probably not the membrane anchor domain unless it spans the envelope more than one time. However, this region does not have some of the features of membrane spanning domains that span a membrane more than once. When the method of Eisenberg *et al.* (1984) was used to predict membrane associated helices in the four virus G1

proteins two hydrophobic regions were identified within the large region predicted using the method of Rao and Argos (1986). However, the locations of these two regions, and the number of amino acids between the two regions was not uniform between the four viruses. Therefore, it is difficult to draw any further conclusions about the location and nature of the membrane anchor region in the *Hantavirus* G1 protein.

The method of Rao and Argos (1986) predicted another common transmembrane region in PH virus, Hällnäs virus and Hantaan virus consisting of the last 19-22 amino acids at the C-terminus of their G1 proteins. However, instead of being a possible membrane domain this region is thought to function as a signal sequence for the G2 protein (Schmaljohn *et al.*, 1987b). Schmaljohn *et al.* (1986a) demonstrated that the Hantaan virus non-glycosylated G1 protein had an M_r value of approximately 61 K which is much smaller than the 70.3 K size predicted for a protein with this amino acid composition. To account for this difference, it was postulated that a stretch of amino acids are removed from the C-terminus of the Hantaan virus G1 protein during processing (Schmaljohn *et al.*, 1987b). Using antisera raised against synthetic peptides it was determined that the Hantaan virus G1 protein extended to at least amino acid 614 (Schmaljohn *et al.*, 1987b). This leaves slightly less than 6 K of the Hantaan virus G1 protein C-terminus, including the potential transmembrane region, unaccounted for. There is a similar 6 K region between the two envelope proteins of alphaviruses that contains a membrane spanning domain that is thought to act as a signal sequence for the second protein (Garoff *et al.*, 1980; Strauss and Strauss, 1986). When using the method of von Heijne (1986) to predict signal sequences this potential membrane spanning domain was identified as a signal sequence.

The potential transmembrane domain predicted to anchor the G2 proteins of these hantaviruses in the envelope is within the predicted length (31 amino acids) and, as expected for a membrane anchor domain, is followed by charged amino acids (Rao and Argos, 1986). Therefore, this is probably the membrane anchor domain of the *Hantavirus* G2 protein.

In the results the locations of potential N-glycosylation sites in the G1 and G2 proteins of the four viruses are shown. Three N-glycosylation sites were conserved in the G1 proteins of the four viruses. One of the potential sites in the PH virus and SR virus G1 proteins could be on the cytoplasmic side of the transmembrane domain and therefore would probably not be glycosylated. Also there is some doubt that the amino acid sequence N-D-S/T is recognized for glycosylation (Kornfeld and Kornfeld, 1985). This would eliminate the fourth potential N-glycosylation site in PH virus and one potential site in Hantaan virus. There was only one conserved N-glycosylation site in the G2 proteins of these four viruses.

The sequence reported here for the 24 bases at the 3' end of the PH virus M RNA differs from the sequence reported previously, which was determined by direct RNA sequencing of the PH virus M RNA segment (Schmaljohn *et al.*, 1985). Two bases, a T at position 17 and an A at position 23 were not present in our sequence. We cloned the 3' end using PCR with Taq DNA polymerase. Saiki *et al.* (1988) reported the error frequency of this enzyme during a 30 cycle amplification was 0.25%. However, others in our laboratory used this technique to clone a human parainfluenza virus 3 gene of known sequence and found an error frequency of only 0.03% over a 30 cycle amplification (Murphy *et al.*,

1990). In both of these studies all of the errors were base substitutions, no additions or deletions were observed. A primer, MP60 (24-mer), complementary to the sequence reported by Schmaljohn *et al.* (1985) was unable to generate any cDNA when using PH viral RNA as the template. This primer, MP60, was able to initiate second-strand synthesis in a PCR reaction, but did not work as well as primers identical to the sequence reported in this thesis. If a shorter primer, MP40 (21-mer), complementary to the sequence reported by Schmaljohn *et al.* (1985) was used, cDNA was produced, but it represented the PH virus L RNA segment. Therefore, the sequence reported in this thesis most likely is the correct 3' terminus of the PH virus M RNA segment. However, the differences with the earlier results cannot be explained.

Coding Strategy of the PH Virus S RNA Segment

A previous comparison of the N proteins of Hantaan virus (serotype 1) and SR virus (serotype 2) found amino acid similarities of 82% or 88% if conservative differences were included (Arikawa *et al.*, 1990). These figures are almost identical to the ones found when comparing the PH virus (serotype 4) and Hällnäs virus (serotype 3) N proteins (79.9% or 87.5% with conservative changes). When the PH virus (serotype 4) N protein was compared with the N proteins of Hantaan virus (serotype 1) and SR virus (serotype 2) the amino acid similarity was much lower. These data again support earlier work suggesting that serotypes 3 and 4 are antigenically closer to each other than to serotypes 1 or 2 (Sugiyama *et al.*, 1987). From these data it appears the N protein is the most highly conserved protein between the four viruses, which may explain why the N protein is the major cross-reactive group specific antigen between serotypes (Sheshberadaran *et al.*, 1988).

The comparison of these four *Hantavirus* N proteins demonstrated that the N protein had two conserved regions and one non-conserved region. The non-conserved region is near the middle of the N proteins (PH virus amino acids 233-309). The first conserved region consists of the first 232 amino acids in each N protein and is only moderately conserved (57.3%) between the four viruses. The second conserved region consists of the last 124 amino acids at the C-terminus of each N protein and is highly conserved (79%). The function of these conserved regions remains to be determined. However, since the N protein encapsidates the hantavirus genomic RNA molecules it would be expected to have an RNA binding site. There is a stretch of 37 amino acids in the first conserved region (amino acids 124-161) that has some amino acid sequence similarity with some known RNA binding domains (Chan *et al.*, 1989; Query *et al.*, 1989). This stretch of amino acids has 73% amino acid sequence similarity, 83.8% if conservative substitutions are included, between the four virus N proteins and could represent the putative N protein RNA binding site. The extreme conservation of the 124 amino acids at the C-terminus may indicate this region also contains a functionally important region of the N protein.

A major difference observed between the four virus N proteins was in the hydrophilicity profiles. The highest peak of hydrophilicity in the PH virus and Hällnäs virus N proteins was located near the C-terminus whereas in the SR virus and Hantaan virus N proteins the highest peak of hydrophilicity was closer to the N-terminus.

A feature of the PH virus S RNA sequence warrants further discussion. Bunyaviruses use overlapping ORFs on the S RNA segment to encode their N and NS_s proteins (Bishop, 1985). Hantaan virus and SR

virus however, appear to only encode the N protein with their S RNA segments (Schmaljohn et al., 1986b; Arikawa *et al.*, 1990). Therefore, the presence of a second, shorter overlapping open reading frame in the viral complementary sense of the PH virus S RNA was unexpected. The predicted size of protein encoded by this open reading frame was 90 amino acids (10.5 K) which is very similar in size to the NS_S proteins of the La Crosse, snowshoe hare (92 amino acids, 10.4 K), and Aino (91 amino acids, 10.5 K) bunyaviruses (Bishop et al., 1982; Akashi and Bishop, 1983; Akashi et al., 1984). The fact that Hällnäs virus also has this second overlapping ORF that could potentially encode a similar sized protein (90 amino acids, 11.1 K) with 50% amino acid sequence similarity to the PH virus protein suggested that viruses in serotypes 3 (Hällnäs virus) and 4 (PH virus) may encode a second protein (NS_S) with their S RNA segments.

A key factor in the utilization of a second overlapping ORF in a mRNA is the flanking sequence of the 5'-proximal AUG initiation codon (Kozak, 1986a, 1986b). The leaky scanning model of ribosome initiation postulates that if the 5'-proximal AUG codon is in a suboptimal context then initiation can occur at an AUG codon further downstream (Kozak, 1986a, 1986b). Several of the nucleotides surrounding an AUG codon are known to affect the efficiency of ribosome initiation but the -3 and +4 positions are the most important (Kozak, 1986a, 1986b). The most preferred context has an A at the -3 position and a G at the +4 position, and the occurrence of leaky scanning can be predicted by determining the nucleotides in these two positions (Kozak, 1986a, 1986b). The nucleotides flanking the 5'-proximal AUG codon do not have to be in a weak context just less than optimal, so some of the 40 S ribosomal subunits bypass the

first AUG codon and initiate at the second AUG codon (Kozak, 1986a, 1986b). The second AUG codon is often in a better context for initiation, but this is not a consistent finding (Kozak, 1986a, 1986b). Both Hantaan virus and SR virus have optimal bases at the -3 and +4 positions flanking the N protein AUG codon. In contrast, the AUG codons of the PH virus and Hällnäs virus N proteins have a G at the -3 position and an A at the +4 position (Stohwasser *et al.*, 1990). Although these AUG codons are not in a weak context they are considered suboptimal, and this fits the requirements for an overlapping ORF strategy. Attempts were made to determine if PH virus encodes a NS_S protein. To do this, the putative NS_S gene was expressed using the baculovirus expression system, the expressed NS_S protein was partially purified and used to raise antiserum in rabbits. Once this antiserum was shown to recognize the baculovirus expressed PH virus NS_S protein it was used in attempts to detect this protein in PH virus infected Vero cells. So far attempts to detect this protein in PH virus infected Vero cells have been unsuccessful. There are several possible explanations for this. One possibility is that PH virus does not express a second protein using this second ORF in its S mRNA. However, there are several facts that do not support this explanation. PH virus and Hällnäs virus were isolated from different rodent genera on different continents yet they both have the same size second overlapping ORF. Also, the proteins both of these viruses could encode with this second ORF, initiate and terminate at the same nucleotides, have the same number of amino acids and have 50% amino acid sequence similarity. Furthermore, the putative NS_S proteins these viruses could encode are almost identical in size to the NS_S proteins of some bunyaviruses which are in the same family of viruses as

hantaviruses. The chances of an ORF that is not being used being preserved in two distinct viruses that have been evolving independently would seem remote. There were many technical problems in trying to identify an NS_S protein in PH virus infected cells and these seem to be a more plausible explanation for the present inability to detect this protein in PH virus infected Vero cells. In work with snowshoe hare *Bunyavirus* the NS_S protein was easily identified with SDS-PAGE by labelling infected cells with [³H]-leucine (Fuller and Bishop, 1982). Cells were infected with a high MOI (5-10 pfu/cell) of snowshoe hare virus and by the time labelling was begun (13.5 hours post-infection) host cell protein synthesis was almost completely inhibited (Fuller and Bishop, 1982). As a consequence the proteins primarily being labelled with the [³H]-leucine were of viral origin. Due to the low titers obtained with PH virus, the high MOI infection done with snowshoe hare virus could not be duplicated. This means in a PH virus infection all of the cells are not infected simultaneously. Therefore, if NS_S protein expression is cyclic rather than continuous only a small percentage of cells may be expressing this protein at any given labelling period. In contrast to *Bunyavirus* infection of mammalian cells, *Hantavirus* infection does not result in the shut off of cellular protein synthesis. Under these conditions PH virus encoded proteins had to compete with cellular proteins for the [³⁵S]-methionine label. When [³⁵S]-methionine labelled cell lysates of PH virus infected or mock infected Vero cells were compared using SDS-PAGE, the protein banding patterns were indistinguishable. This finding indicates that cellular proteins are taking up most of the labelled amino acid and PH virus proteins are receiving very little. The second ORF in an overlapping ORF coding strategy is usually expressed at a low level in

comparison to the first ORF gene product (Kozak, 1986b). If PH virus is expressing an NS_S protein it may be taking up very little of the [³⁵S]-methionine over the duration of the labelling period (6 hours) even in comparison to other PH virus proteins. This may explain the present failure to detect this protein in PH virus infected cells. By labelling with [³⁵S]-methionine for longer periods, possibly several days, it might be possible to saturate the cells and enhance labelling of PH virus proteins, including the putative NS_S protein.

La Crosse virus is a *Bunyavirus*, serologically very similar to snowshoe hare virus, and the NS_S protein of La Crosse virus has 87% amino acid sequence similarity with the NS_S protein of snowshoe hare virus (Akashi and Bishop, 1983). The flanking sequence surrounding the La Crosse virus N protein and NS_S protein initiation codons is identical to the homologous sequence of snowshoe hare virus (Akashi and Bishop, 1983). However, an experiment identical to the one that demonstrated the snowshoe hare virus NS_S protein, failed to detect any production of a NS_S protein by La Crosse virus (Fuller and Bishop, 1982). This data may indicate that the La Crosse virus NS_S protein is more labile or that other factors, possibly cellular, are required for expression of some NS_S proteins. If in the case of bunyaviruses these NS_S proteins are important when the virus is in its insect vector then perhaps La Crosse virus only produces this protein in the insect and requires some insect cell factors for expression.

If PH virus does encode an NS_S protein, intriguing questions would be raised about its function and why Hantaan virus and SR virus do not require a similar protein. It might also reveal an evolutionary link between hantaviruses and bunyaviruses.

Concluding Remarks

Although the G1, G2 and N proteins of only four hantaviruses were compared in this study, these viruses are representative of the four major serotypes recognized to date. Comparisons of the M RNA segments and the gene products they could encode between hantaviruses in the same serotype demonstrated nucleotide sequences (> 96%) and amino acid sequences (> 98%) are highly conserved within serotypes (Schmaljohn *et al.*, 1988a; Izegawa *et al.*, 1990; Antic *et al.*, 1991a). The four viruses compared in this study can be considered the prototypes of their serotypes and therefore an overall comparison between each serotype, not just these four viruses, was made.

Amino acid sequence similarity between their G1, G2 and N proteins and the potential to encode a second protein from their S RNA segments clearly demonstrated that PH virus and Hällnäs virus are more closely related to each other than to SR virus and Hantaan virus. Likewise the amino acid sequence similarity between the G1, G2 and N proteins of SR virus and Hantaan virus clearly demonstrated that these viruses are more closely related to each other than to PH virus and Hällnäs virus. These data suggest that the *Hantavirus* genus diverged along at least two evolutionary paths, one composed of the viruses in serotypes 1 and 2, and another of viruses in serotypes 3 and 4. Other hantaviruses not belonging to these four serotypes may belong to one of these two pathways or may reveal further branches in the *Hantavirus* genus. This divergence may have been caused by virus adaptation to the host, since rodent species, not geography, determines the serotypic profile (Lee *et al.*, 1985b; Sugiyama *et al.*, 1987). Our data show that antigenically distinct hantaviruses are more closely related than serotypic studies

originally indicated. It also demonstrates the possibility that many antigenically distinct hantaviruses may have diverged along only two evolutionary pathways.

The finding that a North American isolate such as PH virus and a European isolate like Hällnäs virus have very similar amino acid sequences was unexpected. The similarity between these two viruses may indicate that they were recently separated and have not been evolving independently for long.

PART 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 *rev* AND *vif* PROTEINS

Human Immunodeficiency virus (HIV) has been identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS) and related disorders (Barre-Sinoussi *et al.*, 1983; Broder and Gallo, 1984; Gallo *et al.*, 1984; Popovic *et al.*, 1984; Sarngadharan *et al.*, 1984). HIV-1 displays a high degree of genetic complexity. In addition to encoding the *gag*, *pol* and *env* proteins characteristic of all replication-competent retroviruses, HIV-1 encodes at least six other proteins (*vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*). These nine viral proteins are encoded by more than 20 distinct mRNA species that are derived from post-transcriptional processing of the initial full length viral transcript (Muesing *et al.*, 1985; Felber *et al.*, 1990; Robert-Guroff *et al.*, 1990; Schwartz *et al.*, 1990a, 1990b).

In this study we expressed the *rev* and *vif* proteins of HIV-1 to high levels in insect cells using recombinant baculovirus vectors. Sera from HIV-1 infected individuals, with different symptoms of disease, were tested for the presence of antibodies that would recognize the *rev* or *vif* proteins of HIV-1. The HIV-1 *vif* protein was also used to raise specific antibodies in rabbits with the long term goal of using antiserum as a tool to further examine the role of *vif* in an HIV-1 infection.

Expression of the HIV-1 *rev* and *vif* Proteins

High levels of *rev* and *vif* were obtained in insect cells infected with recombinant baculovirus vectors. Both proteins were easily visualized in SDS-polyacrylamide gels after staining with Coomassie blue. Therefore, insect cell lysates contained plenty of *rev* or *vif* antigen that could be used to test for the presence of specific antibodies to these proteins in

sera from HIV-1 infected individuals.

Prevalence of Antibodies Against *rev* and *vif* in HIV-1 Infected Individuals

Antibody against *rev* was found in sera from some individuals in all stages of HIV-1 infection. Although somewhat fewer ARC and AIDS patients had *rev* antibody in comparison to healthy donors or healthy donors at risk.

These results differed from those of Chandra *et al.* (1988) who found *rev* antibody predominantly in the sera of ARC patients and postulated that *rev* could be a useful prognostic marker for AIDS development. Chandra *et al.* (1988) used bacteria to express *rev* antigen. We found that some other proteins expressed in bacteria showed a high degree of cross-reactivity whereas *rev* expressed in insect cells gave a clean background on western blots.

Antibody against *vif* was only found in sera from healthy donors or healthy donors at risk and were not present in the sera of ARC or AIDS patients. This result is consistent with the suggestion that production of *vif* antibodies early in the HIV-1 infection may be partially protective and delay the progression to ARC and AIDS. Another explanation is that *vif* antibodies are produced early during the HIV-1 infection and disappear as the disease progresses to ARC and AIDS. A way to distinguish between these possibilities would be to examine the sera from the same HIV-1 infected individuals for *vif* antibodies from the time they seroconvert until they develop full-blown AIDS.

These results were similar to those of Ranki *et al.* (1987) who found *vif* antibodies early in HIV-1 infection and Arya (1987) who found, in a limited number of sera, *vif* antibodies less frequently in AIDS patients than in healthy HIV-1 carriers. These results however, contrast strongly

with those of other researchers. Kan *et al.* (1986) found *vif* antibodies in 30% to 50% of HIV-1 infected patients regardless of their stage of disease. Franchini *et al.* (1987) reported similar results, but found an increase in the number of individuals with *vif* antibodies with progression to AIDS. Wieland *et al.* (1990) found *vif* antibodies in only 2.7% of healthy HIV-1 carriers but found the prevalence increased to 36% in ARC patients and 72% in AIDS patients. This divergence in results may be partially explained by the fact that the *vif* protein genes in these studies were not all from the same HIV-1 strain. Some of the differences could also be related to the source of *vif* protein. In studies where results contrasted with ours *vif* was expressed in bacteria (Kan *et al.*, 1986; Franchini *et al.*, 1987; Wieland *et al.*, 1990). We found that *vif* expressed in bacteria showed a high degree of non-specific cross-reactivity whereas *vif* expressed in insect cells gave a cleaner background on western blots.

These conflicting results appear to make *vif* a poor choice for monitoring HIV-1 infection in individuals. Further confusing this issue was the finding, in some studies, that HIV-1 negative, non-risk individuals had antibodies against the HIV-1 *vif* protein (Kan *et al.*, 1986; Arya and Gallo, 1986; Franchini *et al.*, 1987). This finding suggested that some cellular proteins or other infectious agents may be antigenically similar to the HIV-1 *vif* protein. In Africa HIV affects people living in areas where chronic parasitic infections are common. Khalife *et al.* (1990) demonstrated that *vif* antibodies recognize a 170 K protein on the surface of the African parasite *Schistosoma mansoni* and that 20% of the sera from *Schistosoma mansoni*-infected HIV-seronegative individuals can recognize a *vif* peptide in an ELISA test. However, others have not detected any *vif* antibodies in HIV-1 negative, non-risk individuals (Lee *et*

et al., 1986b; Wieland *et al.*, 1990).

Another possible explanation for the differences between the results of studies designed to detect *rev* and/or *vif* antibodies in the sera of HIV-1 infected patients at different stages of disease is the sample size. None of these studies tested more than 200 HIV-1 infected individuals. It may be that the sample sizes in these studies have not been statistically large enough to make an accurate prediction about the prevalence of antibodies to the HIV-1 *rev* or *vif* proteins.

Production of *vif* Antibodies in Rabbits

The HIV-1 *vif* protein expressed in insect cells was partially purified and used to raise *vif* antibodies in rabbits. The *vif* protein was found to be poorly immunogenic in comparison with other HIV-1 antigens expressed in our laboratory. This poor immunogenicity of *vif* may be related to its structure. The predicted amino acid sequence of the *vif* protein does not have a cluster of amino acids that would give this protein a hydrophilic structure with β -turns (Arya and Gallo, 1986). These are two parameters generally thought to be required for strong immunogenicity (Chou and Fasman, 1974; Kyte and Doolittle, 1982). The rabbit *vif* antiserum is presently being used in a study to determine if *vif* is a virion associated protein.

CHAPTER 5: REFERENCES

- ABERCROMBIE, R. G. (1916). Observations on the acute phase of five hundred cases of War nephritis. *J. R. Army Med. Corps.* 27, 10-157.
- ABRAHAM, G. and PATTNAIK, A. K. (1983). Early RNA synthesis in Bunyamwera virus-infected cell. *J. Gen. Virol.* 64, 1277-1290.
- AKASHI, H., and BISHOP, D. H. L. (1983). Comparison of the sequences and coding of La Crosse and snowshoe hare bunyavirus S RNA species. *J. Virol.* 45, 51-63.
- AKASHI, H., GAY, M., IHARA, T., and BISHOP, D. H. L. (1984). Localized conserved regions of the S RNA gene products of bunyaviruses are revealed by sequence analyses of the Simbu serogroup Aino virus. *Virus Res.* 1, 51-63.
- ANTIC, D., LIM, B. -U., and KANG, C. Y. (1991a). Molecular characterization of the M genomic segment of the Seoul 80-39 virus; nucleotide and amino acid sequence comparisons with other hantaviruses reveal the evolutionary pathway. *Virus Res.* 17, 47-58.
- ANTIC, D., LIM, B. -U., and KANG, C. Y. (1991b). Nucleotide sequence and coding capacity of the large (L) genomic RNA segment of Seoul 80-39 virus, a member of the hantavirus genus. *Virus Res.* 17, 59-66.
- ANTONIADIS, A., GREKAS, D., ROSSI, C. A. and LeDUC, J. W. (1987). Isolation of a hantavirus from a severely ill patient with hemorrhagic fever with renal syndrome in Greece. *J. Infect. Dis.* 156, 1010-3.
- ARIKAWA, J., TAKASHIMA, I., HASHIMOTO, N. (1985a). Cell fusion by haemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. *Arch. Virol.* 86, 303-313.
- ARIKAWA, J., TAKASHIMA, I., HASHIMOTO, N., MORITA, C., SUGIYAMA, K., MATSUURA, Y., SHIGA, S., and KITAMURA, T. (1985b). Epidemiological studies of hemorrhagic fever with renal syndrome related virus infection among urban rats in two islands in Tokyo Bay, Japan. *Acta. Virol.* 29, 66-72.
- ARIKAWA, J., SCHMALJOHN, A. L., DALRYMPLE, J. M., and SCHMALJOHN, C. S. (1989). Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. *J. Gen. Virol.* 70, 615-624.
- ARIKAWA, J., LAPENOTIERE, H. F., IACONO-CONNORS, L., WANG, M., and SCHMALJOHN, C. S. (1990). Coding properties of the S and the M genome segments of Sapporo rat virus: Comparison to other causative agents of hemorrhagic fever with renal syndrome. *Virology* 176, 114-125.

- ARYA, S. K. (1987). 3'-orf and sor genes of human immunodeficiency virus: *In vitro* transcription-translation and immunoreactive domains. *Proc. Natl. Acad. Sci. USA* **84**, 5429-5433.
- ARYA, S. K. and GALLO, R. C. (1986). Three novel genes of human T-lymphotropic virus type III: Immune reactivity of their products with sera from acquired immune deficiency syndrome patients. *Proc. Natl. Acad. Sci. USA* **83**, 2209-2213.
- ARYA, S. K., GUO, C., JOSEPHS, S. F., and WONG-STAAAL. (1985). Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* **229**, 69-73.
- ASADA, H., TAMURA, M., KONDO, K., OKUNO, Y., TAKAHASHI, Y., DOHI, Y., NAGAI, T., KURATA, T., and YAMANISHI, K. (1987). Role of T lymphocyte subsets in protection and recovery from Hantaan virus infection in mice. *J. Gen. Virol.* **68**, 1961-1969.
- ASADA, H., TAMURA, M., KONDO, K., DOHI, Y., and YAMANISHI, K. (1988). Cell-mediated immunity to virus causing haemorrhagic fever with renal syndrome: Generation of cytotoxic T lymphocytes. *J. Gen. Virol.* **69**, 2179-2188.
- ASADA, H., BALACHANDRA, K., TAMURA, M., KONDO, K., and YAMANISHI, K. (1989). Cross-reactive immunity among different serotypes of virus causing haemorrhagic fever with renal syndrome. *J. Gen. Virol.* **70**, 819-825.
- BAEK, L. J., YANAGIHARA, R., GIBBS, C. J., Jr., MIYAZAKI, M., and GAJDUSEK, D. C. (1988). Leakey virus: a new hantavirus isolated from *Mus musculus* in the United States. *J. Gen. Virol.* **69**, 3129-3132.
- BARRE-SINOUSSE, F., CHERMAN, J. C., REY, R., NUGAYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLER-BLIN, C., VAZINET-BRUN, F., ROUZIOUX, C., ROZEN BAUM, W., and MONTAGNIER, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* **220**, 868-871.
- BATTLES, J. and DALRYMPLE, J. M. (1988). Genetic variation among geographic isolates of Rift Valley fever virus. *Am. J. Trop. Hyg.* **39**, 623-637.
- BAUSE, E. (1983). Structural requirements of N-glycosylation of proteins. *Biochem. J.* **209**, 331-336.
- BEATY, B. J., ROZHON, E. J., GENSEMER, P. and BISHOP, D. H. L. (1981). Formation of reassortant bunyaviruses in dually infected mosquitoes. *Virology* **111**, 662-6651.

- BEATY, B. J., MILLER, B. R., SHOPE, R. E., ROZHON, E. J., and BISHOP, D. H. L. (1982). Molecular basis of bunyavirus *per os* infection of mosquitoes: role of the middle-sized RNA segment. *Proc. Natl. Acad. Sci. USA* **79**, 1295-1297.
- BIRNBOIM, H. C., and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513.
- BISHOP, D. H. L. (1985). The genetic basis for describing viruses as species. *Intervirology* **24**, 79-93.
- BISHOP, D. H. L. and SHOPE, R. E. (1979). Bunyaviridae. In *Comprehensive Virology*. Edited by H. Fraenkel-Conrat and R. R. Wagner. vol. 14. pp. 1-36. Plenum Press. New York.
- BISHOP, D. H. L., CALISHER, C., CASALS, J., CHUMAKOV, M. P., GAIDAMOVICH, S. Y., HANNOUN, C., LVOV, D. K., MARSHALL, I. D., OKER-BLOM, N. M., PETTERSSON, R. F., PORTERFIELD, J. S., RUSSEL, P. K., SHOPE, R. E., and WESTAWAY, E. G. (1980). Bunyaviridae. *Intervirology* **14**, 1-36.
- BISHOP, D. H. L., GOULD, K. G., AKASHI, H., and CLERX-VAN HAASTER, C. M. (1982). The complete nucleotide sequence and coding content of snowshoe hare bunyavirus small (S) viral RNA species. *Nucleic Acids Res.* **10**, 3703-3713.
- BISHOP, D. H. L., GAY, M. E., and MATSUOKO, Y. (1983). Non-viral heterogenous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA. *Nucleic Acids Res.* **11**, 6409-6418.
- BONNER, W. M., and LASKEY, R. A. (1974). A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83-88.
- BOULOY, M., KRAMS-OZDEN, S., HORODNICEANU, F., and HANNOUN, C. (1973/74). Three segment RNA genome of Lumbo virus (bunyavirus). *Intervirology* **2**, 173-180.
- BOULOY, M., VIALAT, P., GIRARD, M., and PARDIGON, N. (1984). A transcript from the S segment of the Germiston bunyavirus is uncapped and codes for the nucleoprotein and a non-structural protein. *J. Virol.* **49**, 717-723.
- BRADFORD, J. R. (1916). Nephritis in British troops in Flanders. *Q. J. Med.* **9**, 445-459.
- BRODER, S. M. and GALLO, R. C. (1984). A pathogenic retrovirus (HTLV-III) linked to AIDS. *New Engl. J. Med.* **311**, 1292-1297.
- BROWN, M. and FAULKNER, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *J. Gen. Virol.* **36**, 361-364.

- BRUMMER-KORVENKONTIO, M., VAHERI, A., HOVI, T., von BONSDORFF, C. -H., VUORIMIES, J., MANNI, T., PETTINEN, K., OKER-BLOM, N., and LAHDEVIRATA, J. (1980). Nephropathia epidemica: Detection of antigen in bank voles and serological diagnosis of human infection. *J. Infect. Dis.* **141**, 131-134.
- BURNETTE, W. N. (1981). "Western Blotting" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195-203.
- CABRIDILLA, C. D., Jr., HOLLOWAY, B. D., and OBIJESKI, J. F. (1983). Molecular cloning and sequencing of the La Crosse virus S RNA. *Virology* **128**, 463-468.
- CARVALHO, M. G. C., FRUGULHETTI, I. C., and REBELLO, M. A. (1986). Marituba (Bunyaviridae) virus replication in cultured *Aedes albopictus* cells and in L-A9 cells. *Arch. Virol.* **90**, 325-335.
- CASH, P. (1982). Inhibition of La Crosse virus replication by monensin, a monovalent ionophore. *J. Gen. Virol.* **59**, 193-196.
- CASH, P. (1985). Polypeptide synthesis of Dugbe virus, a member of the Nairovirus genus of the Bunyaviridae. *J. Gen. Virol.* **66**, 141-148.
- CASH, P., VEZZA, A. C., GENTSCH, J. R., and BISHOP, D. H. L. (1979). Genome complexities of the three mRNA species of snowshoe hare bunyavirus and *in vitro* translation of S mRNA to viral N polypeptide. *J. Virol.* **31**, 685-694.
- CHAN, E. K. L., SULLIVAN, K. F., and TAN, E. M. (1989). Ribonucleoprotein SS-B/La belongs to a protein family with consensus sequences for RNA-binding. *Nucleic Acids Res.* **17**, 2233-2244.
- CHANDA, P. K. and GHAYEB, J., and WONG-STAAAL, F. (1988). Nonrandom distribution of antibodies to the TRS protein of human immunodeficiency virus in infected people with different clinical status. *AIDS Res. Hum. Retroviruses* **4**, 11-16.
- CHANG, D. D. and SHARP, P. A. (1989). Regulation by HIV depends upon recognition of splice sites. *Cell* **59**, 789-795.
- CHANG, D. D. and SHARP, P. A. (1990). Messenger RNA transport and HIV *rev* regulation. *Science* **249**, 614-615.
- CHEN, E. Y. and SEEBURG, P. H. (1985). Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* **4**, 165-170.
- CHILDS, J. E., GLASS, G. E., KORCH, G. W., ARTHUR, R. R., SHAH, K. V., GLASSER, D., ROSSI, C., and LeDUC, J. W. (1988). Evidence of human infection with a rat-associated Hantavirus in Baltimore, Maryland. *Am. J. Epidemiol.* **127**, 875-8.

- CHIRGWIN, J. M., PRZYBYLA, A. E., MAC DONALD, R. J., and RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.
- CHOU, P. Y. and FASMAN, G. D. (1974). Prediction of protein conformation. *Biochemistry* **13**, 222-228.
- CHUMAKOV, M. P. and GARVRILOVSKAYA, I. N. (1980). Are there two types of the agent of hemorrhagic fever with renal syndrome? *Lancet* **ii**, 690-691.
- CLERX, J. P. M. and BISHOP, D. H. L. (1981). Qulyub virus, a member of the newly proposed *Nairovirus* genus (Bunyaviridae). *Virology* **108**, 361-372.
- CLERX, J. P. M., CASALS, J., and BISHOP, D. H. L. (1981). Structural characteristics of nairoviruses (genus *Nairovirus*, Bunyaviridae). *J. Gen. Virol.* **55**, 165-168.
- CLERX-VAN HAASTER, C. M. and BISHOP, D. H. L. (1980). Analyses of the 3'-terminal sequences of snowshoe hare and La Crosse bunyaviruses. *Virology* **105**, 564-574.
- CLERX-VAN HAASTER, C. M., AKASHI, H., AUPERIN, D. D., and BISHOP, D. H. L. (1982a). Nucleotide sequence analyses and predicted coding of bunyavirus genome RNA species. *J. Virol.* **41**, 119-128.
- CLERX-VAN HAASTER, C. M., CLERX, J. P., USHIJIMA, H., AKASHI, H., FULLER, F., and BISHOP, D. H. L. (1982b). The 3' terminal RNA sequences of bunyaviruses and nairoviruses (Bunyaviridae). Evidence of end sequence generic differences within the virus family. *J. Gen. Virol.* **61**, 289-292.
- COCHRANE, A. GOULB, A., VOLSKY, D., RUBEN, S., and ROSEN, C. A. (1989a). Functional significance of phosphorylation to the human immunodeficiency virus *rev* protein. *J. Virol.* **63**, 4438-4440.
- COCHRANE, A. KRAMER, R., RUBEN, S., LEVINE, J., and ROSEN, C. A. (1989b). The human immunodeficiency virus *rev* protein is a nuclear phosphoprotein. *Virology* **171**, 264-266.
- COCHRANE, A. W., PERKINS, A., and ROSEN, C. A. (1990). Identification of sequences important in the nuclear localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. *J. Virol.* **64**, 881-885.
- COHEN, M.S. (1982). Epidemic hemorrhagic fever revisited. *Rev. Infect. Dis.* **4**, 992-997.
- COHEN, E. A., DEHNI, G., SODROSKI, J. G., and HASELTINE, W. A. (1990a). Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. *J. Virol.* **64**, 3097-3099.

- COHEN, E. A., TERWILLIGER, E. F., JALINOOS, Y., PROULX, J., SODROSKI, J. G., and HASELTINE, W. A. (1990b). Identification of HIV-1 *vpr* product and function. *J. Acquired Immune Defic. Syndr.* **3**, 11-18.
- COLLETTI, M. S. (1986). Messenger RNA of the M RNA of Rift Valley fever virus. *Virology* **151**, 151-156.
- COLLETTI, M. S., PURCHIO, A. F., KEEGAN, K., FRAZIER, S., HAYS, W., ANDERSON, D. K., PARKER, M. D., SCHMALJOHN, C., SCHMIDT, J., and DALRYMPLE, J. M. (1985). Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144**, 228-245.
- CULLEN, B. R., HAUBER, J., CAMPBELL, K., SODROSKI, J. G., HASELTINE, W. A., and ROSEN, C. A. (1988). Subcellular localization of the human immunodeficiency virus trans-acting *art* gene product. *J. Virol.* **62**, 2498-2501.
- CULLEN, B. R. and GREENE, W. C. (1990). Functions of the auxiliary gene products of the human immunodeficiency virus type 1. *Virology* **178**, 1-5.
- DANTAS, J. R. Jr., OKUNO, Y., ASADA, H., TAMURA, M., TAKAHASHI, M., TANISHITA, O., TAKAHASHI, Y., KURATA, T., and YAMANISHI, K. (1986). Characterization of glycoproteins of viruses causing hemorrhagic fever with renal syndrome (HFRS) using monoclonal antibodies. *Virology* **151**, 379-384.
- DANTAS, J. R. Jr., OKUNO, Y., TANISHITA, O., TAKAHASHI, Y., TAKAHASHI, M., KURATA, T., LEE, H. W., and YAMANISHI, K. (1987). Viruses of hemorrhagic fever with renal syndrome (HFRS) grouped by immunoprecipitation and hemagglutination inhibition. *Intervirology* **27**, 161-165.
- DAVID-WEST, T. S. (1974). Polyacrylamide gel electrophoresis of Dugbe virus infected cells. *Microbios* **11**, 21-23.
- DAYTON, A. I., SODROSKI, J. G., ROSEN, C. A., GOH, W. C., and HASELTINE, W. A. (1986). The trans-activator gene of the human T-cell lymphotropic virus type III is required for replication. *Cell* **44**, 941-947.
- DAYTON, E. T., POWELL, D. M., and DAYTON, A. I. (1989). Functional analysis of CAR, the target sequence for the rev protein of HIV-1. *Science* **246**, 1625-1629.
- DEVASH, Y., REAGAN, K., WOOD, D., TURNER, J., PARRINGTON, M., and KANG, C. Y. (1990). Antibodies against AIDS proteins. *Nature (London)* **345**, 581.
- DOURNON, E., MORINIERE, B., MATHERON, S., GIRARD, P. M., GONZALEZ, J. -P., HIRSCH, F., and McCORMICK, J.B. (1984). HFRS after a wild rodent bite in the Haute-Savoie and risk of exposure to Hantaan-like virus in a Paris laboratory. *Lancet* **i**, 676-677.

- DULBECCO, R., and FREEMAN, G. (1959). Plaque production by the polyoma virus. *Virology* **8**, 396-397.
- EARLE, D. P. (1954). Symposium on epidemic hemorrhagic fever. *Am. J. Med.* **16**, 617-709.
- EISENBERG, D., SCHWARZ, E., KOMARONY, M., and WALL, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **179**, 125-142.
- ELLIOTT, L. H., KILEY, M. P., and McCORMICK, J.B. (1984). Hantaan virus: Identification of virion proteins. *J. Gen. Virol.* **65**, 1285-1293.
- ELLIOTT, R. M. (1985). Identification of non-structural proteins encoded by the Bunyamwera serogroup (family Bunyaviridae). *Virology* **143**, 119-126.
- ELLIOTT, R. M. (1989). Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *Virology* **173**, 332-337.
- ELLIOTT, R. M. (1990). Molecular biology of the Bunyaviridae. *J. Gen. Virol.* **71**, 501-522.
- ELLIOTT, R. M. and MCGREGOR, A. (1989). Nucleotide sequence and expression of the small (S) RNA segment of Maguari bunyavirus. *Virology* **171**, 516-524.
- ELLIOTT, R. M. and WILKIE, M. L. (1986). Persistent infection of *Aedes albopictus* C6/36 cells of Bunyamwera virus. *Virology* **150**, 21-32.
- EMERMAN, M., VAZEUX, R., and PEDEN, K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. *Cell* **57**, 1155-1165.
- EMERY, V. C., and BISHOP, D. H. L. (1987). Characterization of Punta Toro S mRNA species and identification of an inverted complementary sequence in the intergenic region of Punta Toro phlebovirus ambisensed S RNA that is involved in mRNA transcription termination. *Virology* **156**, 1-11.
- ESHITA, Y., and BISHOP, D. H. L. (1984). The complete sequence of the M RNA of snowshoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoprotein. *Virology* **137**, 227-240.
- ESHITA, Y., ERICSON, B., ROMANOWSKI, V., and BISHOP, D. H. L. (1985). Analyses of the M RNA transcription processes of snowshoe hare bunyavirus S and M RNA species. *J. Virol.* **55**, 681-689.
- The Executive Committee of The International Committee on Taxonomy of Viruses. (1987). Minutes of the 17th Meeting, pp. 1-14.

- FEINBERG, M. B., JARRET, R. F., ALDOVINI, A., GALLO, R. C., and WONG-STAAAL, F. (1986). HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**, 807-817.
- FELBER, B. K., HADZOPOULOU-CLADARAS, M., CLADARAS, C., COPELAND, T., and PAVLAKIS, G. N. (1989). *rev* protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Natl. Acad. Sci. USA* **86**, 1495-1499.
- FELBER, B. K., DRYSDALE, C. M., and PAVLAKIS, G. N. (1990). Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *J. Virol.* **64**, 3734-3741.
- FISHER, A. G., ENSOLI, B., IVANOFF, L., CHAMBERLAIN, M., PETTEWAY, S., RATNER, L., GALLO, R. C., and WONG-STAAAL, F. (1987). The *src* gene of HIV-1 is required for efficient virus transmission *in vitro*. *Science* **237**, 888-893.
- FOULKE, R. S., ROSATO, R. R., and FRENCH, G. R. (1981). Structural polypeptides of Hantaan virus. *J. Gen. Virol.* **53**, 169-172.
- FRANCHINI, G., ROBERT-GUROFF, M., ALDOVINI, A., KAN, N. C., and WONG-STAAAL, F. (1987). Spectrum of natural antibodies against five HTLV-III antigens in infected individuals: Correlation of antibody prevalence with clinical status. *Blood* **69**, 437-441.
- FRENCH, G. R., FOULKE, R. S., BRAND, O. A., EDDY, G. A., LEE, H. W., and LEE, P. W. (1981). Korean hemorrhagic fever: Propagation of the etiologic agent in a cell line of human origin. *Science* **211**, 1046-1048.
- FULLER, F. and BISHOP, D. H. L. (1982). Identification of virus-coded non-structural polypeptides in bunyavirus-infected cells. *J. Virol.* **41**, 643-648.
- FULLER, F., BHOWEN, A. S., and BISHOP, D. H. L. (1983). Bunyavirus nucleoprotein, N, and a non-structural protein NS_s, are coded by overlapping reading frames in the S RNA. *J. Gen. Virol.* **64**, 1705-1714.
- GAHMBERG, N., KUISMANEN, E., KERÄNEN, S., and PETTERSSON, R. F. (1986a). Uukemi virus glycoproteins accumulate in and cause morphological changes of the Golgi complex in the absence of virus maturation. *J. Virol.* **57**, 899-906.
- GAHMBERG, N., PETTERSSON, R. F., and KÄÄRIÄINEN, L. (1986b). Efficient transport of Semliki forest virus glycoproteins through a Golgi complex morphologically altered by Uukemi virus glycoproteins. *EMBO* **5**, 3111-3118.
- GAJDUSEK, D. C. (1962). Virus hemorrhagic fever. *J. Pediatr.* **60**, 851-857.

- GALLO, R. C., SALAHUDDIN, S. Y., POPOVIC, M., SHEARER, G. M., KAPLAN, M., HAYER, B. F., PALKER, T. J., REDFIELD, R., OLESKE, J., SAFAI, B., WHITE, G., FOSTER, P., and MARKHAM, P. D. (1984). Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**, 500-503.
- GAON, J., KARLOVAC, M., GRESIKOVA, M., *et al.* (1986). Epidemiological features of hemorrhagic fever. *Folia. Med. Fac. Med. Univ. Saraev.* **3**, 24-41.
- GAROFF, H., FRISCHAUF, A. M., SIMONS, K., LEHRACH, H., and DELIUS, H. (1980). Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. *Nature (London)* **288**, 236-241.
- GARRETT, E. D., TILEY, L. S., and CULLEN, B. R. (1991). Rev activates expression of the human immunodeficiency virus type 1 *vif* and *vpr* gene products. *J. Virol.* **65**, 1653-1657.
- GAVRILOVSKAYA, I. N., APEKINA, N. S., MIASNIKOV, Y. A., BERNSHTEIN, A. D., RYLTSEVA, E. V., GORBACHKOVA, E. A., and CHUMAKOV, M. P. (1983). Features of circulation of hemorrhagic fever with renal syndrome (HFRS) virus among small mammals in the European USSR. *Arch. Virol.* **75**, 313-316.
- GENTSCH, J. R. and BISHOP, D. H. L. (1979). M viral RNA segment of bunyaviruses codes for two glycoproteins. G1 and G2. *J. Virol.* **30**, 767-770.
- GERBAUD, S., VIALAT, P., PARDIGON, N., WYCHOWSKI, C., GIRARD, M., and BOULOY, M. (1987). The S segment of Germiston virus RNA genome can code for three proteins. *Virus Res.* **8**, 1-13.
- GIEBEL, L. B., STOHWASSER, R., ZOLLER, L., BAUTZ, E. K. F., and DARAI, G. (1989). Determination of the coding capacity of the M genome segment of nephropatia epidemica strain Hallnas B1 by molecular cloning and nucleotide sequence analysis. *Virology* **172**, 498-505.
- GLIGIC, A., OBRADOVIC, M., STOJANOVIC, R., *et al.* (1986). Hemorrhagic fever with renal syndrome (HFRS) in Yugoslavia. Isolation of Hantaviruses from *Apodemus flavicollis* and *Clethrionomys glareolus*. Abstr. XIth Int. Congr. Infectious and Parasitic Diseases, Munich, p. 810.
- GOH, W. C., SODROSKI, J. G., ROSEN, C. A., and HASELTINE, W. A. (1987). Expression of the *art* gene protein of Human T-Lymphotropic Virus Type III (HTLV-III/LAV) in bacteria. *J. Virol.* **61**, 633-637.
- GONZALEZ-SCARANO, F., JANSSEN, R., NAJJAR, J. A., POBJECKY, N., and NATHANSON, N. (1985). An avirulent G1 glycoprotein variant of La Crosse bunyavirus with defective fusion function. *J. Virol.* **54**, 757-763.

- GONZALEZ-SCARANO, F., BEATY, B. J., SUNDIN, D., JANSSEN, R., ENDRES, M. J., and NATHANSON, N. (1988). Genetic determinants of the virulence and infectivity of La Crosse virus. *Microbial Pathogenesis* **4**, 1-7.
- GRAHAM, F. L., and VAN DER EB, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456-467.
- GRUNSTEIN, M., and HOGNESS, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **73**, 3961-3965.
- GUBLER, U., and HOFFMAN, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 269-273.
- GUY, B., GEIST, M., DOTT, K., SPEHNER, D., KIENY, M. -P., and LECOCQ, J. -P. (1991). A specific inhibitor of cysteine proteases impairs a vif-dependent modification of human immunodeficiency virus type 1 env protein. *J. Virol.* **65**, 1325-1331.
- HADZOPOULOU-CLADARAS, M., FELBER, B. K., CLADARAS, C., ATHANASSOPOULOS, A., TSE, A., and PAVLAKIS, G. N. (1989). The *rev* (*trs/art*) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. *J. Virol.* **63**, 1265-1274.
- HALTINER, M., KEMPE, T., and TJIAN, R. (1985). A novel strategy for constructing clustered point mutations. *Nucleic Acids Res.* **13**, 1015-1026.
- HAMMARSKJOLD, M. L., HEIMER, J., HAMMARSKJOLD, B., SANGWAN, I., ALBERT, L., and REKOSH, D. (1989). Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. *J. Virol.* **63**, 1959-1966.
- HANAHAAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557-580.
- HAUBER, J., BOUVIER, M., MALIM, M. H., and CULLEN, B. R. (1988). Phosphorylation of the *rev* gene product of human immunodeficiency virus type *art/trs* gene product is enhanced by activation of protein kinase C. *J. Virol.* **62**, 4801-4804.
- HATTORI, M. and SAKAKI, Y. (1986). Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* **152**, 232-238.
- HEAPHY, S., DINGWALL, C., ERNBERG, I., GAIT, M. J., GREEN, S. M., KARN, J., LOWE, A. D., SINGH, M., and SKINNER, M. A. (1990). HIV-1 regulator of virion expression (*rev*) protein binds to an RNA stem-loop structure located within the *rev* responsive element region. *Cell* **60**, 685-693.

- HEWLETT, M. J., PETTERSSON, R. F., and BALTIMORE, D. (1977). Circular forms of Uukuniemi virion RNA: an electron microscopic study. *J. Virol.* **21**, 1085-1093.
- HINK, W. F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature (London)*, **226**, 466-467.
- HONDA, A., UEDA, K., NAGATA, K., and ISHIHAMA, A. (1988). RNA polymerase of influenza virus: role of NP on RNA chain elongation. *J. Biochem.* **104**, 1021-1026.
- HOPP, T. P., and WOODS, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**, 3824-3828.
- HSU, M. -T., PARVIN, J. D., GUPTA, S., KRYSTAL, M., and PALESE, P. (1987). Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proc. Natl. Acad. Sci. USA* **84**, 8140-8144.
- HUGGINS J. W., KIM, G. R., BRAND, O. M., and McKEE, Jr. K. T. (1986). Ribavirin therapy for hantaan virus infection in suckling mice. *J. Infect. Dis.* **153**, 489-497.
- HUNG, T., XIA, S. -M., SONG, G., LIAO, H. -X., CHAO, T. -X., CHOU, Z. -Y., and HANG, C. -S. (1983a). Viruses of classical and mild forms of haemorrhagic fever with renal syndrome isolated in China have similar bunyavirus-like morphology. *Lancet* **i**, 589-591.
- HUNG, T., XIA, S. -M., ZHAO, T. X., ZHOU, J. Y., SONG, G., LIAO, G. X. H., YE, W. W., CHU, Y. L., and HANG, C. S. (1983b). Morphogenic evidence for identifying the viruses of hemorrhagic fever with renal syndrome as candidate members of the Bunyaviridae family. *Arch. Virol.* **78**, 137-144.
- HUNG, T., CHOU, Z., ZHAO, T., XIA, S., and HANG, C. (1985). Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome (HFRS). I. Some peculiar aspects of the morphogenesis of various strains of HFRS viruses. *Intervirology* **23**, 97-108.
- HUNG, T., SEMAO, X., ZINYI, C., GAN, S., and YANAGIHARA, R. (1987). Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome. II. Inclusion bodies-ultrastructural markers of hantavirus-infected cells. *Intervirology* **27**, 45-52.
- IHARA, T., AKASHI, H., and BISHOP, D. H. L. (1984). Novel coding strategy of ambisensed genomic RNA revealed by sequence analysis of Punta Toro phlebovirus S RNA. *Virology* **136**, 293-306.
- IHARA, T., SMITH, J., DALRYMPLE, J. M., and BISHOP, D. H. L. (1985). Complete sequences of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. *Virology* **144**, 246-259.

- INNIS, M. A., MYAMBO, K. B., GELFAND, D. H., and BROW, M. A. D. (1988). DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. USA* **85**, 9436-9440.
- ISEGAWA, Y., FUJIWARA, Y., OHSHIMA, A., FUKUNAGA, R., MURAKAMI, H., YAMANISHI, K., and SOKAWA, Y. (1990). Nucleotide sequence of the M genome segment of hemorrhagic fever with renal syndrome virus strain B-1. *Nucleic Acids Res.* **18**, 4936.
- ISHII, S., ANDO, K., WATANABE, N., MURAKAMI, R., NAGAYAMA, T., and ISHIKAWA, T. (1942). Studies on Songo fever. *Jap. Army Med. J.* **355**, 1755-1758.
- JACOBSON, A. B., GOOD, L., SIMONETTI, J., and ZUCKER, M. (1984). Some simple computational methods to improve the folding of large RNAs. *Nucleic Acids Res.* **12**, 45-66.
- JANSSEN, R., NATHANSON, N., ENDRES, M. J., and GONZALEZ-SCARANO, F. (1986). Virulence of La Crosse virus is under polgenic control. *J. Virol.* **59**, 1-7.
- JIANG, Y. -T. (1983). A preliminary report on hemorrhagic fever with renal syndrome in China. *Chinese Med. J.* **96**, 265-268.
- KAFATOS, F. C., JONES, C. W., and EFSTRATIADIS, A. (1979). Determination of nucleic acid sequences homologies and relative concentrations by dot hybridization procedures. *Nucleic Acids Res.* **7**, 1541-1552.
- KAN, N. C., FRANCHINI, G., WONG-STAAAL, F., DuBOIS, G. C., ROBEY, W. G., LAUTENBERGER, J. A., and PAPAS, T. S. (1986). Identification of HTLV-III/LAV *src* gene product and detection of antibodies in human sera. *Science* **231**, 1553-1555.
- KARABOTOS, N. (ed). (1985). International catalogue of arthropod-borne viruses including certain other viruses of vertebrates. *Am. Soc. Trop. Med. Hyg.*, 445-446.
- KASAHARA, S., KITANO, M., KIKUCHI, H., *et al.* (1944). Studies on pathogen of epidemic hemorrhagic fever. *J. Jap. Pathol.* **34**, 3-5.
- KAWAMATA, J., YAMANOUCHI, T., DOHHMAE, K., MIYAMOTO, H., TAKAHASHI, M., YAMANISHI, K., KURATA, T., LEE, H. W. (1987). Control of laboratory acquired hemorrhagic fever with renal syndrome (HFRS) in Japan. *Lab. Anim. Sci.* **137**, 431-436.
- KAWAOKA, Y., NAEVE, C. W., and WEBSTER, R. G. (1984). Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? *Virology* **139**, 303-316.

- KHALIFE, J., GRZYCH, J. -M., PIERCE, R., AMEISEN, J. -C., SCHACHT, A. -M., GRAS-MASSÉ, H., TARTAR, A., LECOCQ, J. -P., and CAPRON, A. (1990). Immunological crossreactivity between the Human Immunodeficiency virus type 1 virion infectivity factor and a 170-kD surface antigen of *Schistosoma mansoni*. *J. Exp. Med.* 172, 1001-1004.
- KIM, G. R. and McKEE, K. T. Jr. (1985). Pathogenesis of Hantaan virus infection in suckling mice; clinical, virologic, and serologic observations. *Am. J. Trop. Med. Hyg.* 34, 388-395.
- KIM, R. J. and RYU, C. (1985). Study on inactivated vaccine of HFRS. *Proc. Acad. Med. Sci. D.P.R. Korea* 10, 1-9.
- KIM, S., IKEUCHI, K., BYRN, R., GROOPMAN, J., and BALTIMORE, D. (1989). Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* 86, 9544-9548.
- KITAMURA, T., MORITA, C., KOMATSU, T., SUGIYAMA, K., ARIKAWA, J., SHIGA, S., TAKED, H., AKAO, Y., IMAIZUMI, K., OYA, A., HASHIMOTO, N., and URASAWA, S. (1983). Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. *Jpn. J. Med. Sci. Biol.* 36, 17-25.
- KNIGHT, D. M., FLOMERFELT, F. A., and GHAYEB, J. (1987). Expression of the *art/trs* protein of HIV and study of its role in viral envelope synthesis. *Science* 236, 837-840.
- KORNFELD, R. and KORNFELD, S. (1985). Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 54, 631-664.
- KOZAK, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin *in vivo*. *Nature (London)* 308, 241-246.
- KOZAK, M. (1986a). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283-292.
- KOZAK, M. (1986b). Regulation of protein synthesis in virus-infected animal cells. *Adv. Virus Res.* 31, 229-292.
- KUBOTA, S., SIOMI, H., SATOH, T., ENDO, S., MAKI, M., and HATANAKA, M. (1989). Functional similarity of HIV-I rev and HTLV-I rex proteins: identification of a new nucleolar-targeting signal in rev protein. *Biochem. Biophys. Res. Commun.* 162, 963-970.
- KUISMANEN, E., HEDMAN, K., SARASTE, J., and PETTERSSON, R. F. (1982). Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. *Mol. Cell. Biol.* 2, 1444-1458.

- KUISMANEN, E., BANG, B., HURME, M., and PETTERSSON, R. F. (1984). Uukuniemi virus maturation: Immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* **51**, 137-146.
- KUISMANEN, E., SARASTE, J., and PETTERSSON, R. F. (1985). Effect of monensin on the assembly of Uukuniemi virus in the Golgi complex. *J. Virol.* **55**, 813-822.
- KURATA, T., TSAI, T. F., BAUER, S. P. and McCORMICK, J. B. (1983). Immunofluorescence studies of disseminated Hantaan virus infection of suckling mice. *Infect. Immun.* **41**, 391-398.
- KYTE, J., and DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132.
- LÄHDEVIRTA, J. (1971). Nephropathia epidemica in Finland. A clinical, histological and epidemiological study. *Ann. Clin. Res.* **3** (suppl 8), 1-154.
- LÄHDEVIRTA, J. (1982). Clinical features of HFRS in Scandinavia as compared with east Asia. *Scand. J. Infect. Dis. (suppl)* **36**, 93-95.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685.
- LeDUC, J. W. (1987). Epidemiology of Hantaan and related viruses. *Lab. Anim. Sci.* **37**, 413-418.
- LeDUC, J. W., SMITH, G. A., and JOHNSON, K. M. (1984). Hantaan-like viruses from domestic rats captured in the United States. *Am. J. Trop. Med. Hyg.* **33**, 927-928.
- LeDUC, J. W., SMITH, G. A., PINHEIRO, F. P., VASCONCELOS, P. F., ROSA, E. S., and MAIZTEGUI, J. I. (1985). Isolation of a Hantaan-related virus from Brazilian rats and serological evidence of its widespread distribution in South America. *Am. J. Trop. Med. Hyg.* **34**, 810-815.
- LeDUC, J. W., SMITH, G. A., CHILDS, J. E., PINHEIRO, F. P., MAIZTEGUI, J. I., NIKLASSON, B., ANTONIADES, A., ROBINSON, D. M., KHIN, M., SHORTRIDGE, K.F., WOOSTER, M. T., ELWELL, M. R., ILBERY, P. L. T., KOECH, D., ROSA, E. S. T., and ROSEN, L. (1986). Global survey of antibody to Hantaan-related viruses among peridomestic rodents. *Bull. WHO* **64**, 139-44.
- LEE, H. W. (1982a). Hemorrhagic fever with renal syndrome. History of Hantaan virus and epidemiologic features. *Scand. J. Infect. Dis. (suppl)* **36**, 82-85.
- LEE, H. W. (1982b). Korean hemorrhagic fever. *Prog. Med. Virol.* **28**, 96-113.

- LEE, H. W. (1986). Global distribution and molecular biological characteristics of Hantaviruses. *J. Korean Soc. Virol.* 16, 1-5.
- LEE, H. W. (1988a). Hantavirus infection in Asia. In *Nephrology Proceedings Xth International Congress of Nephrology*. Edited by A. M. Davison. vol. 2, pp. 816-839. Bailliere Tindall. London.
- LEE, H. W. (1988b). Seroepidemiologic studies of acute hemorrhagic diseases in Korea from 1985 to 1987 (hemorrhagic fever with renal syndrome, leptospirosis and scrub typhus). *J. Korean Med. Ass.* 31, 581-593.
- LEE, H. W. (1989). Hemorrhagic fever with renal syndrome in Korea. *Rev. Infect. Dis.* 11 (suppl 4), S864-S876.
- LEE, H. W. and ANTONIADIS, A. (1981). Serologic evidence for Korean hemorrhagic fever in Greece. *Lancet* i, 932.
- LEE, H. W. and LEE, P. W. (1977). Korean hemorrhagic fever. II. Isolation of the etiologic agent. *J. Korean Soc. Virol.* 7, 1-9.
- LEE, H. W. and LEE, W. R. (1988). New epidemiologic findings of hemorrhagic fever with renal syndrome in Korea. *J. Korean Soc. Virol.* 18, 67-75.
- LEE, H. W. and JOHNSON, K. M. (1982). Laboratory-acquired infections with Hantaan virus, the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* 146, 645-651.
- LEE, H. W. and van der GROEN, G. (1989). Hemorrhagic fever with renal syndrome. *Prog. Med. Virol.* 36, 62-102.
- LEE, H. W., LEE, P. W., and JOHNSON, K. M. (1978). Isolation of the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* 137, 298-308.
- LEE, H. W., BARK, L. J., BAEK, L. J., CHOI, K. S., WANG, Y. N., and WOO, M. S. (1980). Korean hemorrhagic fever patients in urban areas of Seoul. *Korean J. Virol.* 10, 1-6.
- LEE, H. W., FRENCH, G. R., LEE, P. W., BAEK, L. J. TSUCHIYA, K., and FOULKE, R. S. (1981a). Observation on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever. *Am. J. Trop. Med. Hyg.* 30, 477-482.
- LEE, H. W., LEE, P. W., BAEK, L. J., SONG, C. K., and SEONG, I. W. (1981b). Intra-specific transmission of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, in the rodent *Apodemus agrarius*. *Am. J. Trop. Med. Hyg.* 30, 1106-1112.
- LEE, H. W., BAEK, L. J., and JOHNSON, K. M. (1982a). Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. *J. Infect. Dis.* 146, 638-644.

- LEE, H. W., SEONG, I. W., BAEK, L. J., McLEOD, D. A., SEO, J. S., and KANG, C. Y. (1984). Positive serological evidence that Hantaan virus, the etiologic agent of hemorrhagic fever with renal syndrome, is endemic in Canada. *Can. J. Microbiol.* **30**, 1137-1140.
- LEE, H. W., BAEK, L. J., and KIM, H. D. (1986a). Studies on laboratory rat infection with Hantavirus in animal rooms of institutes in Seoul. *J. Korean Soc. Virol.* **16**, 113-120.
- LEE, P. W., AMYX, H. L., GIBBS, C. J. Jr., GAJDUSEK, D. C., and LEE, H. W. (1981c). Propagation of Korean hemorrhagic fever virus in laboratory rats. *Infect. Immun.* **31**, 334-338.
- LEE, P. W., GIBBS, C. J. Jr., GAJDUSEK, D. C., and SVEDMYR, A. (1981d). Antibody to Korean haemorrhagic fever virus in man in parts of the world where haemorrhagic fever with renal syndrome is not known. *Lancet* **ii**, 256.
- LEE, P. W., GOLDGABER, D., and GIBBS, C. J., Jr., GAJDUSEK, D. C., YANAGIHARA, R. T., SVEDMYR, A., HLACA, C., VESENJAK-HIRJAN, J., and GLIGIC, A. (1982b). Other serotypes of haemorrhagic fever with renal syndrome viruses in Europe. *Lancet* **i**, 1405-1406.
- LEE, P. W., AMYX, H. L., GAJDUSEK, D. C., YANAGIHARA, R. T., GOLDGABER, D., and GIBBS, C. J., Jr. (1982c). New hemorrhagic fever with renal syndrome-related virus in indigenous wild rodents in the United States. *Lancet* **ii**, 1405.
- LEE, P. W., AMYX, H. L., YANAGIHARA, R., GAJDUSEK, D. C., GOLDGABER, D., and GIBBS, C. J., Jr. (1985a). Partial characterization of Prospect Hill virus isolated from meadow voles in the United States. *J. Infect. Dis.* **152**, 826-829.
- LEE, P. W., GIBBS, C. J. Jr., GAJDUSEK, D. C., YANAGIHARA, R. (1985b). Serotype classification of hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. *J. Clin. Microbiol.* **22**, 940-944.
- LEE, T.-H., COLIGAN, J. E., ALLAN, J. S., McLANE, M. F., GROOPMAN, J. E., and ESSEX, M. (1986b). A new HTLV-III/LAV protein encoded by a gene found in cytopathic retroviruses. *Science* **231**, 1546-1549.
- LEES, J. F., PRINGLE, C. R., and ELLIOTT, R. M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: conservation of structural features in the Bunyavirus glycoprotein gene product. *Virology* **148**, 1-14.
- LLOYD, G., and JONES, N. (1986). Infection of laboratory workers with hantavirus acquired from immunocytomas propagated in laboratory rats. *J. Infect.* **12**, 117-125.
- LLOYD, G., BOWEN, E. T. W., and JONES, N. (1984). HFRS outbreak associated with laboratory rats in UK. *Lancet* **i**, 1175-1176.

- LYONS, M. J. and HEYDUK, J. (1973). Aspects of the developmental morphology of California encephalitis virus in cultured vertebrate and arthropod cells and in mouse brain. *Virology* **54**, 37-52.
- MALIM, M. H., HAUBER, J., FENRICK, R., and CULLEN, B. R. (1988). Immunodeficiency virus *rev* trans-activator modulates the expression of the viral regulatory genes. *Nature (London)* **335**, 181-183.
- MALIM, M. H., BOHNLEIN, S., HAUBER, and CULLEN, B. R. (1989a). Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function. *Cell* **58**, 205-214.
- MALIM, M. H., HAUBER, J., LE, S., MAIZEL, V., and CULLEN, B. R. (1989b). The HIV-1 rev transactivator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* **338**, 254-257.
- MALIM, M. H., TILEY, L. S., McCARN, D. F., RUSCHE, J. R., HAUBER, J., and CULLEN, B. R. (1990). HIV-1 structural gene expression requires binding of the rev *trans*-activator to its RNA target sequence. *Cell* **60**, 675-683.
- MANIATIS, T., FRITSCH, E. F., and SAMBROOK, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York, USA.
- MARTIN, M. L., LINDSEY-REGNERY, H., SASSO, D. R., McCORMICK, J. B., and PALMER, E. (1985). Distinction between Bunyaviridae genera by surface structure and comparison with Hantaan virus using negative stain electron microscopy. *Arch. Virol.* **86**, 17-28.
- MATSUOKA, Y., IHARA, T., BISHOP, D. H. L., and COMPANS, R. W. (1988). Intracellular accumulation of Punta Toro virus glycoproteins expressed from cloned cDNA. *Virology* **167**, 251-260.
- MATSUURA, Y., POSSEE, R. D., OVERTON, H. A., and BISHOP, D. H. L. (1987). Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* **68**, 1233-1250.
- McCLURE, W. W. and LEY, H. L. (1954). Epidemiologic studies, fall 1953, commission of Hemorrhagic fever, Armed Forces Epidemiological Board, 2nd Annual Report 1953-1954.
- McCORMICK, J.B., PALMER, E. L., SASSO, D. R., and KILEY, M. P. (1982). Morphological identification of the agent of Korean haemorrhagic fever (Hantaan virus) as a member of the Bunyaviridae. *Lancet* **i**, 765-768.
- McKEE, K. T. Jr., KIM, G. R., GREEN, D. E., and PETERS, C. J. (1985). Hantaan virus infection in suckling mice: virologic and pathogenic correlates. *J. Med. Virol.* **17**, 107-117.

- McMASTER, G. K., and CARMICHAEL, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**, 4835-4838.
- MESSING, J. (1983). New M13 vectors for cloning. In *Methods in Enzymology*. Edited by R. Wu, L. Grossman, and K. Moldave. vol. 101, p. 20-78. Academic Press. Orlando, FL.
- MORELLE, G. (1989). A plasmid extraction procedure on a miniprep scale. *Focus* **11.1**, 7-8.
- MORIMOTO, Y., KISHIMOTO, S., YAMANOUCHI, T., YAMANISHI, K., TAKAHASHI, M., KAWAMATA, J., TAMURA, T., and LEE, H. W. (1986). Clinical features of hemorrhagic fever with renal syndrome in Japan. A clinical and laboratory study on 27 cases in Osaka in the 1960s and 1980s. *Jap. J. Infect. Dis.* **59**, 439-458.
- MUESING, M. A., SMITH, D. H., CABRADILLA, C. D., BENTON, C. V., IASKY, L. A., and CAPON, D. J. (1985). Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature (London)* **313**, 450-458.
- MURPHY, D. G., DIMOCK, K., and KANG, C. Y. (1990). Viral RNA and protein synthesis in two LLC-MK₂ cell lines persistently infected with human parainfluenza virus 3. *Virus Res.* **16**, 1-16.
- MURPHY, F. A., HARRISON, A. K., and WHITFIELD, S. G. (1973). Bunyaviridae: morphologic and morphogenetic similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. *Intervirology* **1**, 297-316.
- MYHRHAM, G. (1951). Nephropathia epidemica, a new infectious disease in Northern Scandinavia. *Acta. Med. Scand.* **140**, 52-56.
- NAKAZAWA, E. (1966). Epidemic hemorrhagic fever in Manchuria. *Jap. Izishinbo* **2280**, 61-65.
- NEWTON, S. E., SHORT, N. J., and DALGARNO, L. (1981). Bunyamwera virus replication in cultured *Aedes albopictus* (mosquito) cells: establishment of a persistent viral infection. *J. Virol.* **38**, 1015-1024.
- NICOLETTI, L. and VERANI, P. (1985). Growth of *Phlebovirus* Toscana in a mosquito (*Aedes pseudoscutellaris*) cell line (AP-61): establishment of a persistent infection. *Arch. Virol.* **85**, 35-45.
- NIKLASSON, B. and LeDUC, J. W. (1984). Isolation of the nephropathia epidemica agent in Sweden. *Lancet* **i**, 1012-1013.
- NIKLASSON, B. and LeDUC, J. W. (1987). Epidemiology of nephropathia epidemica in Sweden. *J. Infect. Dis.* **155**, 269-276.

- NIKlassON, B., LeDUC, J. W., and NYSTROM, K. (1987). Nephropathia epidemica: incidence of clinical cases and antibody prevalence in an endemic area of Sweden. *Epidemiol. Infect.* **99**, 559-562.
- OBIJESKI, J. F., BISHOP, D. H. L., MURPHY, F. A., and PALMER, E. L. (1976a). Structural proteins of LaCrosse virus. *J. Virol.* **19**, 985-987.
- OBIJESKI, J. F., BISHOP, D. H. L., PALMER, E. L., and MURPHY, F. A. (1976b). Segmented genome and nucleocapsid of La Crosse virus. *J. Virol.* **20**, 664-675.
- OBIJESKI, J. F., McCAULEY, J., and SKEHEL, J. J. (1980). Nucleotide sequences at the termini of La Crosse virus RNAs. *Nucleic Acids Res.* **8**, 2431-2438.
- OGAWA, K., SHIBATA, R., KIYOMASU, T., HIGUCHI, I., KISHIDA, Y., ISHIMOTO, A., and ADACHI, A. (1989). Mutational analysis of the human immunodeficiency virus *upr* open reading frame. *J. Virol.* **63**, 4110-4114.
- OKUNO, Y., YAMANISHI, K., TAKAHASHI, Y., TANISHITA, O., NAGAI, T., DANTAS, J. R. Jr., OKAMOTO, Y., TADANO, M., and TAKAHASHI, M. (1986). Haemagglutination-inhibition test for haemorrhagic fever with renal syndrome using viral antigen prepared from infected tissue culture fluid. *J. Gen. Virol.* **67**, 149-156.
- OLSEN, H. S., NELBOCK, P., COCHRANE, A. W., and ROSEN, C. A. (1990). Secondary structure is the major determinant for interaction of HIV *rev* protein with RNA. *Science* **247**, 845-848.
- OSTERHAUS, A., SPIJKERS, J., van STEENIS, B., van der GROEN, G. (1984). Hantavirus infection in Nederland. *Ned. Tijdschr Geneesk.* **128**, 2461-2462.
- PARDIGON, N., VIALAT, P., GIRARD, M., and BOULOY, M. (1982). Panhandles and hairpin structures at the termini of Germiston virus RNAs (Bunyavirus). *Virology* **122**, 191-197.
- PARKER, M. D., and HEWLETT, M. J. (1981). The 3' terminal nucleotide sequences of Uukuniemi and Inkoo virus RNA segments. In *"Replication of Negative Strand Viruses"*, pp. 125-145. Elsevier. New York.
- PARVIN, J. D., PALESE, P., HONDA, A., ISHIHAMA, A., and KRYSTAL, M. (1989). Promoter analysis of influenza virus RNA polymerase. *J. Virol.* **63**, 5142-5152.
- PATTERSON, J. L., and KOLAKOFSKY, D. (1984). Characterization of La Crosse virus small genome transcripts. *J. Virol.* **49**, 680-685.
- PENNINGTON, T. H., PRINGLE, C. R. and McCRAE, M. A. (1977). Bunyamwera virus-induced polypeptide synthesis. *J. Virol.* **24**, 397-400.

- PENSIERO, M. N., JENNINGS, G. B., SCHMALJOHN, C. S., and HAY, J. (1988). Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. *J. Virol.* **62**, 696-702.
- PERKINS, A., COCHRANE, A., RUBEN, A., and ROSEN, C. (1989). Structural and functional characterization of the human immunodeficiency virus *rev* protein. *J. AIDS* **2**, 256-263.
- PETTERSSON, R. F. and KÄÄRIÄINEN, L. (1973). The ribonucleic acids of Uukuniemi virus, a non-cubical tick-borne arbovirus. *Virology* **56**, 608-619.
- PETTERSSON, R. F., and von BONSDORFF, C. -H. (1975). Ribonucleoproteins of Uukuniemi virus are circular. *J. Virol.* **15**, 386-392.
- PETTERSSON, R. F., and von BONSDORFF, C. -H. (1987). Bunyaviridae. In *Animal Virus Structure*. Edited by M. V. Nermut and A. C. Stevens. pp. 147-157. Elsevier. Amsterdam.
- PHILPOTT, M., HIOE, C., SHEERAR, M., and HINSHAW, V. S. (1990). Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian Influenza A virus. *J. Virol.* **64**, 2941-2947.
- POPOVIC, M., SARNGADHARAN, M. G., READ, E., and GALLO, R. C. (1984). Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**, 497-500.
- QUERY, C. C., BENTLEY, R. C., and KEENE, J. D. (1989). A common RNA recognition motif identified within a defined U1 RNA binding domain of the 70K U1 snRNP protein. *Cell* **57**, 89-101.
- RAJU, R. and KOIAKOFISKY, D. (1986). Translational requirements of La Crosse virus S-mRNA synthesis: *in vivo* studies. *J. Virol.* **61**, 96-103.
- RAJU, R. and KOIAKOFISKY, D. (1989). The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired. *J. Virol.* **63**, 122-128.
- RANKI, A., KROHN, M., ALLAIN, J. -P., FRANCHINI, G., VALLE, S. -L., ANTONEN, J., LEUTHER, M., and KROHN, K. (1987). Long latency precedes overt seroconversion in sexually transmitted Human-Immunodeficiency-virus infection. *Lancet* **ii**, 589-593.
- RAO, J. K. M., and ARGOS, P. (1986). A conformational preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta* **869**, 197-214.

- RATNER, L., HASELTINE, W., PATARCA, R., LIVAK, K. J., STARCICH, B., JOSEPHS, S. F., DORAN, E. R., RAFALSKI, J. A., WHITEHORN, E. A., BAUMEISTER, K., IVANOFF, L., PETTEWAY, S. R. Jr., PEARSON, M. L., LAUTENBERGER, J. A., PAPAS, T. S., GHRAYEB, J., CHANG, N. T., GALLO, R. C., and WONG-STAAI, F. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**, 277-284.
- RIGBY, P. W., DIECKMAN, M., RHODES, C., and BERG, P. (1977). Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237-251.
- ROBERT-GUROFF, M., POPOVIC, M., GARTNER, S., MARKHAM, P., GALLO, R. C., and REITZ, M. S. (1990). Structure and expression of *tat*-, *rev*-, and *nef*-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J. Virol.* **64**, 3391-3398.
- ROMANOWSKI, V., and BISHOP, D. H. L. (1985). Conserved sequences and coding of the two strains of lymphocytic choriomeningitis virus (WE and ARM) and Pichinde arenaviruses. *Virus Res.* **2**, 35-51.
- ROSEN, C. A., TERWILLIGER, E., DAYTON, A., SODROSKI, J. G., and HASELTINE, W. A. (1988). Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **85**, 2071-2075.
- ROSSIER, C., RAJU, R., and KOLAKOFSKY, D. (1988). La Crosse virus gene expression in mammalian and mosquito cells. *Virology* **165**, 539-548.
- SADAIE, M. R., BENTER, T., and WONG-STAAI, F. (1988). Site-directed mutagenesis of two trans-regulatory genes (*tat-III*, *trs*) of HIV-1. *Science* **239**, 910-914.
- SAIKI, R. K., GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B., and ERLICH, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.
- SAMSO, A., BOULOY, M., and HANNOUN, C. (1976). Mise en evidence de molécules d'acide ribonucleique circulaire dans le virus lumbo (Bunyavirus). *Compte rendue de l'Academie des sciences D282*, 1653-1655.
- SANGER, F., NICKLEN, S., and COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- SARNGADHARAN, M. G., POPOVIC, M., BRUCH, L., SCHUPBACH, J., and GALLO, R. C. (1984). Antibodies reactive with human T-lymphotrophic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224**, 506-508.

- SCHMALJOHN, C. S. (1990). Nucleotide sequence of the L genome segment of Hantaan virus. *Nucleic Acids Res.* **18**, 6728.
- SCHMALJOHN, C. S., and DALRYMPLE, J. M. (1983). Analysis of Hantaan virus RNA: Evidence for a new genus of Bunyaviridae. *Virology* **131**, 482-491.
- SCHMALJOHN, C. S., and DALRYMPLE, J. M. (1984). Biochemical characterization of Hantaan virus. In *Segmented Negative Strand Viruses*. Edited by R. W. Compans and D. H. L. Bishop. pp. 117-124. Elsevier. New York.
- SCHMALJOHN, C. S., HASTY, S. E., HARRISON, S. E., and DALRYMPLE, J. M. (1983). Characterization of Hantaan virions, the prototype virus of hemorrhagic fever with renal. *J. Infect. Dis.* **148**, 1005-1012.
- SCHMALJOHN, C. S., HASTY, S. E., DALRYMPLE, J. M., LEDUC, J. W., LEE, H. W., von BONSDORFF, C. H., BRUMMER-KORVENKONTIO, M., VAHERI, A., TSAI, T. F., REGNER, H. L., GOLDBERGER, D., and LEE, P. W. (1985). Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science* **227**, 1041-1044.
- SCHMALJOHN, C. S., HASTY, S. E., RASMUSSEN, L., and DALRYMPLE, J. M. (1986a). Hantaan virus replication: Effects of monensin, tunicamycin and endoglycosidases on the structural glycoproteins. *J. Gen. Virol.* **67**, 707-717.
- SCHMALJOHN, C. S., JENNINGS, G. B., HAY, J., and DALRYMPLE, J. M. (1986b). Coding strategy of the S genome segment of Hantaan virus. *Virology* **155**, 633-643.
- SCHMALJOHN, C. S., LEE, H. W., and DALRYMPLE, J. M. (1987a). Detection of hantaviruses with RNA probes generated from recombinant DNA. *Arch. Virol.* **95**, 291-301.
- SCHMALJOHN, C. S., SCHMALJOHN, A. L., and DALRYMPLE, J. M. (1987b). Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. *Virology* **157**, 31-39.
- SCHMALJOHN, C. S., ARIKAWA, J., HASTY, S. E., RASMUSSEN, L., LEE, H. W., LEE, P. W., and DALRYMPLE, J. M. (1988a). Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. *J. Gen. Virol.* **69**, 1949-1955.
- SCHMALJOHN, C. S., SUGIYAMA, K., SCHMALJOHN, A. L., and BISHOP, D. H. L. (1988b). Baculovirus expression of the small genome segment of Hantaan virus and potential use of the expressed nucleocapsid protein as a diagnostic antigen. *J. Gen. Virol.* **69**, 777-786.
- SCHMALJOHN, C. S., CHU, Y.-K., SCHMALJOHN, A. L., and DALRYMPLE, J. M. (1990). Antigenic subunits of Hantaan virus expressed by Baculovirus and Vaccinia virus recombinants. *J. Virol.* **64**, 3162-3170.

- SCHWARTZ, S. B., FELBER, B. K., BENKO, D. M., FENYO, E. -M., and PAVLAKIS, G. N. (1990a). Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J. Virol.* **64**, 2519-2529.
- SCHWARTZ, S. B., FELBER, B. K., FENYO, E. -M., and PAVLAKIS, G. N. (1990b). Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. *J. Virol.* **64**, 5448-5456.
- SETTERGREN, B., JUTO, P., TROLLFORS, B., WADELL, G., and NORRBY, S. R. (1988a). Hemorrhagic complications and other findings in nephropathia epidemica in Sweden. A study of 355 serologically verified cases. *J. Infect. Dis.* **157**, 380-382.
- SETTERGREN, B., JUTO, P., WADELL, G., TROLLFORS, B., and NORRBY, S. R. (1988b). Incidence and geographic distribution of serologically verified cases of nephropathia epidemica in Sweden. *Am. J. Epidemiol.* **127**, 801-807.
- SHEEDY, J. A., FROEB, H. F., BATSON, H. A., CONLEY, C. C., MURPHY, J. P., HUNTER, R. B., CUGELL, D. W., GILES, R. B., BERSHADSKY, S. C., VESTER, J. W., and YOE, R. H. (1954). The clinical course of epidemic hemorrhagic fever. *Am. J. Med.* **16**, 619-628.
- SHESHBERADARAN, H., NIKLASSON, B., and TKACHENKO, E. A. (1988). Antigenic relationship between hantaviruses analysed by immunoprecipitation. *J. Gen. Virol.* **69**, 2645-2651.
- SHOPE, R. E., ROZHON, E. J., and BISHOP, D. H. L. (1981). Role of the middle-sized bunyavirus RNA segment in mouse virulence. *Virology* **114**, 273-276.
- SHORTRIDGE, K. F., LEE, H. W., LeDUC, J. W., WONG, T. W., CHAU, G. W., and ROSEN, L. (1987). Serological evidence of Hantaan-related viruses in Hong Kong. *Trans. R. Soc. Trop. Med. Hyg.* **81**, 400-402.
- SIMANIS, V. (1985). Preparation of competent cells. In *DNA cloning: a practical approach*. Edited by D. M. Glover. vol. 1, pp. 121-127. IRL Press Ltd. Oxford.
- SIMONS, J. F., HELLMAN, U., and PETTERSSON, R. F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, homology to members of the genus *Phlebovirus*. *J. Virol.* **64**, 247-255.
- SMADEL, J. E. (1953). Epidemic hemorrhagic fever. *Am. J. Publ. Hlth.* **43**, 1327-1330.
- SMITH, G. E., SUMMERS, M. D., and FRAZER, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3**, 2156-2165.

- SMITH, J. F. and PIFAT, D. Y. (1982). Morphogenesis of sandfly fever virus (Bunyaviridae family). *Virology* **121**, 61-81.
- SMORODINTSEV, A. A., AL'TCHULER, I. S., DUNAEVSKII, M. I., KAKHREIDZE, K. A., NEUSTROEV, V. D., and CHURILOV, A. V. (1944). Etiology and clinical observations of hemorrhagic nephrosonephritis. *Medgiz Moscow*. 26-47.
- SMORODINTSEV, A. A., CHUDAKOV, V. G., and CHURILOV, A. V. (1959). In *Hemorrhagic nephrosonephritis*. pp. 125. Pergamon Press. London.
- SODROSKI, J., GOH, W. C., ROSEN, C., TARTAR, A., PORTETELLE, D., BERNY, A., and HASELTINE, W. (1986). Replicative and cytopathic potential of HTLV-III/LAV with *sor* gene deletions. *Science* **231**, 1549-1553.
- SONG, G., HANG, C., QUI, X. Z., NI, D., LIAO, H., GAO, G., DU, Y., XU, J., WU, Y., ZHAO, J., KONG, B., WANG, Z., ZHANG, Z., SHEN, H., and ZHOU, N. (1983). Etiologic studies of epidemic hemorrhagic fever (hemorrhagic fever with renal syndrome). *J. Infect. Dis.* **147**, 654-659.
- SONG, G., HANG, C., LIAO, H., FU, J., GAO, G., QUI, H., and ZHANG, Q. (1984). Antigenic differences between viral strains causing classical and mild types of hemorrhagic fever with renal syndrome in China. *J. Infect. Dis.* **150**, 889-894.
- STEIN, B. S., GOWDA, S. D., LIFSON, J. D., PENHALLOW, R. C., BENSCH, K. G., and ENGLEMAN, E. G. (1987). pH-independent HIV entry into CD4-positive cells via virus envelope fusion to the plasma membrane. *Cell* **49**, 659-668.
- STOHWASSER, R., GIEBEL, L. B., ZOLLER, L., BAUTZ, E. K. F., and DARAI, G. (1990). Molecular characterization of the RNA S segment of nephropathia epidemica virus strain Hallnas B1. *Virology* **174**, 79-86.
- STREBEL, K., DAUGHERTY, D., CLOUSE, K., COHEN, D., FOLKS, T., and MARTIN, M. A. (1987). The HIV 'A' (*sor*) gene product is essential for virus infectivity. *Nature (London)* **328**, 728-730.
- STRAUSS, E. G. and STRAUSS, J. H. (1986). Structure and replication of the alphavirus genome. In *The Togaviridae and Flaviviridae*. Edited by S. Schlesinger and M. J. Schlesinger. pp. 35-90. Plenum. New York.
- SUGIYAMA, K., MORIKAWA, S., MATSUURA, Y., SHIGA, S., KOMATSU, T., MORIKAWA, S., KITAMURA, T. (1984). Isolation of a virus related to hemorrhagic fever with renal syndrome from urban rats in non-endemic area. *J. Infect. Dis.* **149**, 472.

- SUGIYAMA, K., MORIKAWA, S., MATSUURA, Y., TKACHENKO, E. A., MORITA, C., KOMATSU, T., AKAO, Y., and KITAMURA, T. (1987). Four serotypes of haemorrhagic fever with renal syndrome viruses identified by polyclonal and monoclonal antibodies. *J. Gen. Virol.* **68**, 979-987.
- SUMMERS, M. D., and SMITH, G. E. (1987). A manual of methods for baculovirus vector and insect cell culture procedures. *Texas Agricultural Experiment Station. Bulletin No. 1555*.
- SUNDIN, D. R., BEATY, B. J., NATHANSON, N., and GONZALEZ-SCARANO, F. (1987). A G1 glycoprotein epitope of La Crosse virus: a determinant of infection of *Aedes triseriatus*. *Science* **235**, 591-593.
- SUNG, W. L., ZAHAB, D. M., MacDONALD, C. A., and TAM, C. S. (1986). Synthesis of synthetic parathyroid hormone genes via site-specific recombination directed crossover linkers. *Gene* **47**, 261-267.
- TAKEHARA, K., MIN, M. -K., BATTLES, J. K., SUGIYAMA, K., EMERY, V. C., DALRYMPLE, J. M., and BISHOP, D. H. L. (1989). Identification of mutations in the M RNA of a candidate vaccine strain of Rift Valley fever virus. *Virology* **169**, 452-457.
- TAKENAKA, A., GIBBS, C. J., Jr., and GAJDUSEK, D. C. (1985). Antiviral neutralizing antibody to Hantaan virus as determined by plaque reduction technique. *Arch. Virol.* **84**, 197-206.
- TALMON, Y., PRASAD, B. V. V., CLERX, J. P. M., WANG, G. -J., CHIU, W., and HEWLETT, M. J. (1987). Electron microscopy of vitrified-hydrated La Crosse virus. *J. Virol.* **61**, 2319-2321.
- TAMURA, M. (1964). Occurrence of epidemic hemorrhagic fever in Osaka city: first cases found in Japan with characteristic features of marked proteinuria. *Biken. J.* **7**, 79-94.
- TAMURA, M., ASADA, H., KONDO, K., TANISHITA, O., KURATA, T., YAMANISHI, K. (1989). Pathogenesis of Hantaan virus in mice. *J. Gen. Virol.* **70**, 2897-2906.
- TANISHITA, O., TAKAHASHI, Y., OKUNO, Y., YAMANISHI, K., and TAKAHASHI, M. (1984). Evaluation of focus reduction neutralization test with peroxidase-antiperoxidase staining technique for hemorrhagic fever with renal syndrome virus. *J. Clin. Microbiol.* **20**, 1213-1215.
- TARTAKOFF, A. and VASSALLI, P. (1978). Comparative studies of intracellular transport of secretory proteins. *J. Cell. Biol.* **79**, 694-707.
- TAUTZ, D., and RENZ, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Anal. Biochem.* **132**, 14-19.

- TERWILLIGER, E., BURGHOFF, R., SIA, R., SODROSKI, J., HASELTINE, W., and ROSEN, C. (1988). The *art* gene product of human immunodeficiency virus is required for replication. *J. Virol.* **62**, 655-658.
- TKACHENKO, E. A., IVANOV, A. P., DONETS, M. A., MIASNIKOV, Y. A., RYLTSEVA, E. V., GAPONOVA, L. K., BASHKIRTSEV, V. N., OKULOVA, N. M., DROZDOV, S. G., SLOVNOVA, R. A., *et al.* (1983). Potential reservoir and vectors of hemorrhagic fever with renal syndrome (HFRS) in the USSR. *Ann. Soc. Belg. Med. Trop.* **63**, 267-269.
- TKACHENKO, E. A., BASHKIRTSEV, V. N., van der GROEN, G., DZAGUROVA, T. K., IVANOV, A. P., and RYLTSEVA, E. V. (1984). Isolation in Vero-E6 cells of hantavirus from *Clethrionomys glareolus* captured in the Bashkiria area of the USSR. *Ann. Soc. Belg. Med. Trop.* **64**, 425-426.
- TKACHENKO, E. A., RYLTSEVA, E. V., MYASNIKOV, A., IVANOV, A. P., and REZAPKIN, G. V. (1987). Study of circulation of hemorrhagic fever with renal syndrome virus among small mammals in the USSR. *Probl. Virol.* **6**, 709-715.
- TRAUB, R. M., HERTIG, M., LAWRENCE, W. H., and HARRIES, T. T. (1954). Potential vectors and reservoirs of hemorrhagic fever in Korea. *Am. J. Trop. Med. Hyg.* **59**, 291-305.
- TSAI, T. F. (1987). Hemorrhagic fever with renal syndrome mode of transmission to humans. *Lab. Animal Sci.* **37**, 428-430.
- TSAI, T. F., BANER, S., McCORMICK, J. B., and KURATA, T. (1982). Intracerebral inoculation of suckling mice with Hantaan virus. *Lancet* *ii*, 503-504.
- TSAI, T. F., TANG, Y. W., HU, S. L., YE, K. L., CHEN, G. L., and XU, Z. Y. (1984). Hemagglutination-inhibiting antibody in hemorrhagic fever with renal syndrome. *J. Infect. Dis.* **150**, 895-898.
- TSAI, T. F., BAUER, S. P., SASSO, D. R., WHITFIELD, S. G., McCORMICK, J. B., BRADFORD, H., CARAWAY, C. T., McFARLAND, L. M., BRADFORD, H., and KURATA, T. (1985). Serological and virological evidence of a Hantaan virus-related enzootic in the United States. *J. Infect. Dis.* **152**, 126-136.
- UMENAI, T., LEE, H. W., LEE, P. W., SAITO, TOYODA, T., HONGO, K., YOSHINAKA, K., NOBUNAGA, T., HORIUCHI, T., and ISHIDA, N. (1979). Korean hemorrhagic fever in staffs in an animal laboratory. *Lancet* *i*, 1314-1316.
- van der GROEN, L. H. and BEELAERT, G. (1985). Immunoperoxidase assay for the detection of specific IgG antibodies to Hantaan virus. *J. Virol. Methods* **10**, 53-58.

- van der GROEN, L. H., LEIRS, H., and VERHAGEN, R. (1986). Polyhostal nature of Hantaan virus. Proc. Second Symp. Recent Advances in Rodent Control, Kuwait, pp. 197-207.
- van der GROEN, G., TKACHENKO, E. A., IVANOV, A. P., and VERHAGEN, R. (1983). Haemorrhagic fever with renal syndrome related virus in indigenous wild rodents in Belgium. *Lancet* **ii**, 110-111.
- VENKATESH, L. K., MOHAMMED, S., and CHINNADURAI, G. (1990). Functional domains of the HIV-1 rev gene required for trans-regulation and subcellular localization. *Virology* **176**, 39-47.
- von HEIJNE, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.
- WAIN-HOBSON, S., SONIGO, P., DANOS, O., COLE, S., and ALIZON, M. (1985). Nucleotide sequence of the AIDS virus, LAV. *Cell* **40**, 9-17.
- WATRET, G. E. and ELLIOTT, R. M. (1985). The proteins and RNA's specified by Clo Mor virus, a scottish Nairovirus. *J. Gen. Virol.* **66**, 2513-2516.
- WHITE, J. D., SHIREY, F. G., FRENCH, G. R., HUGGINS, J. W., BRAND, O. M., and LEE, H. W. (1982). Hantaan virus, aetiological agent of Korean haemorrhagic fever, has Bunyaviridae-like morphology. *Lancet* **i**, 768-771.
- WICKENS, M. P. and DAHLBERG, J. E. (1987). RNA-protein interactions. *Cell* **51**, 339-342.
- WIELAND, U., KÜHN, J. E., JASSOY, C., RÜBSAMEN-WAIGMANN, H., WOLBER, V., and BRAUN, R. W. (1990). Antibodies to recombinant HIV-1 vif, tat and nef proteins in human sera. *Med. Microbiol. Immunol.* **179**, 1-11.
- WORLD HEALTH ORGANIZATION. (1983). Haemorrhagic fever with renal syndrome. Memorandum from a WHO meeting. *Bull WHO* **61**, 269-275.
- YAMANISHI, K., DANTAS, J. R. Jr, TAKAHASHI, M., YAMANOUCHI, T., DOMAE, K., KAWAMATA, J., and KURATA, T. (1983). Isolation of hemorrhagic fever with renal syndrome (HFRS) virus from a tumor specimen in a rat. *Biken J.* **26**, 155-160.
- YAMANISHI, K., DANTAS, J. R. Jr, TAKAHASHI, M., YAMANOUCHI, T., DOMAE, K., TAKAHASHI, Y., and TANISHITA, O. (1984). Antigenic differences between two viruses, isolated in Japan and Korea, that cause hemorrhagic fever with renal syndrome. *J. Virol.* **52**, 231-237.
- YAMANISHI, K., TANISHITA, O., TAMURA, M., ASADA, H., KONDO, K., TAKAGI, M., YOSHIDA, I., KONOBE, T., and FUKAI, K. (1988). Development of inactivated vaccine against virus causing haemorrhagic fever with renal syndrome. *Vaccine* **6**, 278-282.

- YAMANOUCHI, T., DOMAE, K., TANISHITA, O., YAMANISHI, K., TAKAHASHI, M., and KURATA, T. (1984). Experimental infection in newborn mice and rats by hemorrhagic fever with renal syndrome (HFRS) virus. *Microbiol. Immunol.* **28**, 1345-1353.
- YAN, Y. C. (1985). Observation on the safety and effectiveness of EHF inactivated vaccine. *Chinese J. Epidemiol.* **2**, 6.
- YANAGIHARA, R. and GAJDUSEK, D. C. (1987). Hemorrhagic fever with renal syndrome Global epidemiology and ecology of hantavirus infections. In *Medical Virology, vol VI*. Edited by L. M. De la Maza and E. M. Peterson. pp. 171-214.
- YANAGIHARA, R., GAJDUSEK, D. C., GIBBS, C. J., Jr., and TRAUB, R. (1984a). Prospect Hill virus: serological evidence for infection in mammologists. *N. Engl. J. Med.* **310**, 1325-1326.
- YANAGIHARA, R., GOLDGABER, D., LEE, P. W., AMYX, H. L., GAJDUSEK, D. C., GIBBS, C. J. Jr., and SVEDMYR, A. (1984b). Propagation of nephropathia epidemica virus in cell culture. *Lancet* **i**, 1013.
- YANAGIHARA, R., SVEDMYR, A., AMYX, H. L., LEE, P. W., GOLDGABER, D., GAJDUSEK, D. C., GIBBS, C. J., Jr., and NYSTROM, K. (1984c). Isolation and propagation of nephropathia epidemica virus in bank voles. *Scand. J. Infect. Dis.* **16**, 225-228.
- YANAGIHARA, R., AMYX, H. L., and GAJDUSEK, D. C. (1985). Experimental infection with Puumala virus, the etiologic agent of nephropathia epidemica, in bank voles (*Clethrionomys glareolus*). *J. Virol.* **55**, 34-38.
- YANAGIHARA, R., DAUM, C. A., LEE, P. W., BAEK, L. J., AMYX, H. L., GAJDUSEK, D. C., and GIBBS, C. J., Jr. (1987). Serological survey of Prospect Hill virus infection in indigenous wild rodents in the USA. *Trans. R. Soc. Trop. Med. Hyg.* **81**, 42-45.
- YANAGIHARA, R., AMYX, H. L., LEE, P. W., ASHER, D. M., GIBBS, C. J., Jr., GAJDUSEK, D. C. (1988). Experimental hantavirus infection in nonhuman primates. *Arch. Virol.* **101**, 125-130.
- YANISCH-PERRON, C., VIERIRA, J., and MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.
- YEN, YU-CHEN, QIU, FU-XI, *et al.* (1984). Etiologic studies on hemorrhagic fever with renal syndrome (HFRS). Abstr. XIth Int. Congr. Tropical Medicine and Malaria, Calgary, p. 225.
- YOO, D., and KANG, C. Y. (1987a). Genomic comparisons among members of Hantavirus group. In *The Biology of Negative Strand Viruses*. Edited by B. W. J. Mahy, and D. Kolakofsky. pp. 424-431. Elsevier. New York.

- YOO, D., and KANG, C. Y. (1987b). Nucleotide sequence of the M segment of the genomic RNA of Hantaan virus 76-118. *Nucleic Acids Res.* **15**, 6299-6300.
- YOO, D. (1988). Molecular characterization and replication strategy of Hantavirus. Ph.D. Thesis, Department of Microbiology and Immunology, University of Ottawa.
- YU-TU, J. (1983). A preliminary report on hemorrhagic fever with renal syndrome in China. *Chin Med. J.* **96**, 265-268.
- ZAPP, M. L. and GREEN, M. R. (1989). Sequence-specific RNA binding by the HIV-1 rev protein. *Nature (London)* **342**, 714-716.
- ZHANG, X. -K., TAKASHIMA, I., and HASHIMOTO, N. (1989). Characteristics of passive immunity against hantavirus infection in rats. *Arch. Virol.* **105**, 235-246.
- ZUCKER, M., and STIEGLER, P. (1981). Optimal computer folding of large RNA sequences using thermodynamics and auxillary information. *Nucleic Acids Res.* **9**, 133-148.

APPENDIX 1

PUBLICATIONS

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

Devash, Y., Reagan, K., Wood, D., Turner, J., Parrington, M. and Kang, C. Y. (1990). Antibodies against AIDS proteins. *Nature (London)* 345, 581.

Parrington, M. A., Lee, P. -W., and Kang, C. Y. (1991). Molecular Characterization of the Prospect Hill Virus M RNA Segment: a Comparison with the M RNA Segments of other Hantaviruses. *J. Gen. Virol. In Press*.

ABSTRACTS

Ewasyshyn, M., Sabina, L. R., Parrington, M., and Mastrodicassa, A. (1982). Protease profiles of influenza virus infected embryonated eggs and cell culture systems. Can. Soc. Microbiol. Abstracts, p. 106.

Parrington M. A., and Sabina, L. R. (1985). Analysis of Influenza A virus polypeptides by limited proteolysis in SDS-polyacrylamide gels. Can. Soc. Microbiol. Abstracts, p. 97.

Kang C. Y., Parrington, M.A., and Yoo, D. W. (1989). Coding strategies of S genomic segments of different members of Hantavirus genus. The 1st International Conference on Hemorrhagic Fever with Renal Syndrome, p. 42.

Parrington, M., and Kang, C. Y. (1989). Coding strategy of the Prospect Hill virus S genomic segment and its comparison with Hantaan virus. Second Symposium on Arboviruses in the Mediterranean Countries (Including an International Symposium on TBE, Hanta- and CCHF Viruses), p. 29.

Devash, Y., Wood, D., Turner, J., Reagan, K., Parrington, M., and Kang, Y. (1990). Rev and Vif as diagnostic and prognostic indicators. *AIDS Res. Human Retroviruses* 6, 94.