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Molecular biology of *Bunyavirus*-host interactions

Baldridge, Gerald Don, Ph.D.

The University of Arizona, 1989

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Molecular Biology of Bunyavirus-Host Interactions

by

Gerald Don Baldridge

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1989

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Gerald Don Baldridge

entitled Molecular Biology of Bunyavirus-Host Interactions

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Martinez J. Hewlett	October 5, 1989 Date
Thomas J. Lindell	October 5, 1989 Date
Danny L. Brower	October 5, 1989 Date
Harris Bernstein	October 5, 1989 Date
Charles P. Gerba	October 5, 1989 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director	10-5-89 Date

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SIGNED: Harold Don Baldridge

Acknowledgements

I wish to thank my committee members for the support and advice they gave me throughout my tenure as a student. Special thanks go to Dr. Martinez Hewlett for his support, guidance and patience in his role as director of my dissertation research. I also wish to thank Dr. Barry Beaty of Colorado State University at Fort Collins, Colorado for his guidance and support of my work.

I am grateful for the support, advice and friendship of my fellow graduate students and wish to thank in particular David Bruce, Frank Flomerfelt and Ed Gammill. Drs. Corrie and John Clerx provided much deeply appreciated advice during their tenure as postdoctoral students at the Universisty of Arizona. Gail Hewlett provided invaluable expert technical assistance with figures.

Lastly, I thank my wife and fellow student Dr. Pamela Shubat for her help and patience as we pursued our degrees.

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Abstract

Ribonuclease T1 oligonucleotide fingerprint (ONF) analysis was used to study genomic stability of La Crosse virus (Bunyaviridae) during vertical and horizontal transmission in the laboratory. No RNA genomic changes were detected in vertebrate cell culture-propagated virus isolated (following oral ingestion and replication) from the natural mosquito host, Aedes triseriatus. Genomic changes were not detected during transovarial passage of virus through two generations cf mosquitoes or in genomes of virus isolated from suckling mice infected by bite of second generation transovarially infected mosquitoes. These results demonstrate that, despite the well-documented phenomenon of rapid nucleotide change in RNA viruses under various conditions, the La Crosse virus genome can remain relatively stable during transovarial transmission (TOT) in the insect host and during transfer between insect and vertebrate hosts.

ONF analysis was used to demonstrate TOT of reassortant California serogroup bunyaviruses in Aedes triseriatus. Mosquitoes were simultaneously inoculated

with temperature sensitive mutants of La Crosse and Snowshoe hare viruses able to replicate at 33 C but not at 40 C. Putative reassortants, selected by replication at 40 C, were isolated from progeny mosquitoes and mice infected by these mosquitoes. ONF analysis confirmed that they were reassortant bunyaviruses.

Approximately 75% of the M segment and 25% of the L segment nucleotide sequences of Inkoo virus (Bunyaviridae) were determined by Sanger dideoxynucleotide sequence analysis of cDNA clones. Comparison of the M segment nucleotide and deduced amino acid sequences with those of four other bunyaviruses, representing two serogroups, revealed greater conservation of nucleotide than of amino acid homology between serogroups. The position of cysteine residues is highly conserved in the five amino acid sequences. Areas of the sequences representing nonstructural protein(s) are less conserved than glycoprotein regions.

The L segment nucleotide sequence begins with the known 3' end of the viral L segment and contains an open reading frame encoding the amino terminal 505 amino acids of the viral L protein. The amino acid sequence contains the glycine-aspartate-aspartate motif which is conserved in many RNA-dependent RNA polymerases. Comparison of the L

segment sequences with those in the GEN Bank Data Base revealed no significant similarities with any other sequence.

Introduction

Perspective

On a yearly basis, 10 to 15% of the world's population is afflicted with serious disease caused by arthropod-vectored pathogens. In addition, vast numbers of livestock and poultry are also affected. We face many well-documented problems in controlling these diseases. Among the most serious is the spread of drug and insecticide resistance among pathogen and vector populations. This is an extremely complex phenomenon involving many biochemical mechanisms such as inducible mixed-function oxidase isozyme systems in insects which metabolize insecticides and synthesize and degrade hormones regulating growth and reproduction. Traditional disease control efforts have been greatly compromised by these many-faceted resistance problems at a time when burgeoning human populations face ever greater exposure to pathogens and their vectors.

It is therefore imperative that we gain a better understanding of the ecology, molecular biology and biochemistry of vector-pathogen interactions if we are to have hope of further success in interrupting arthropod-vectored disease transmission cycles. Some progress has

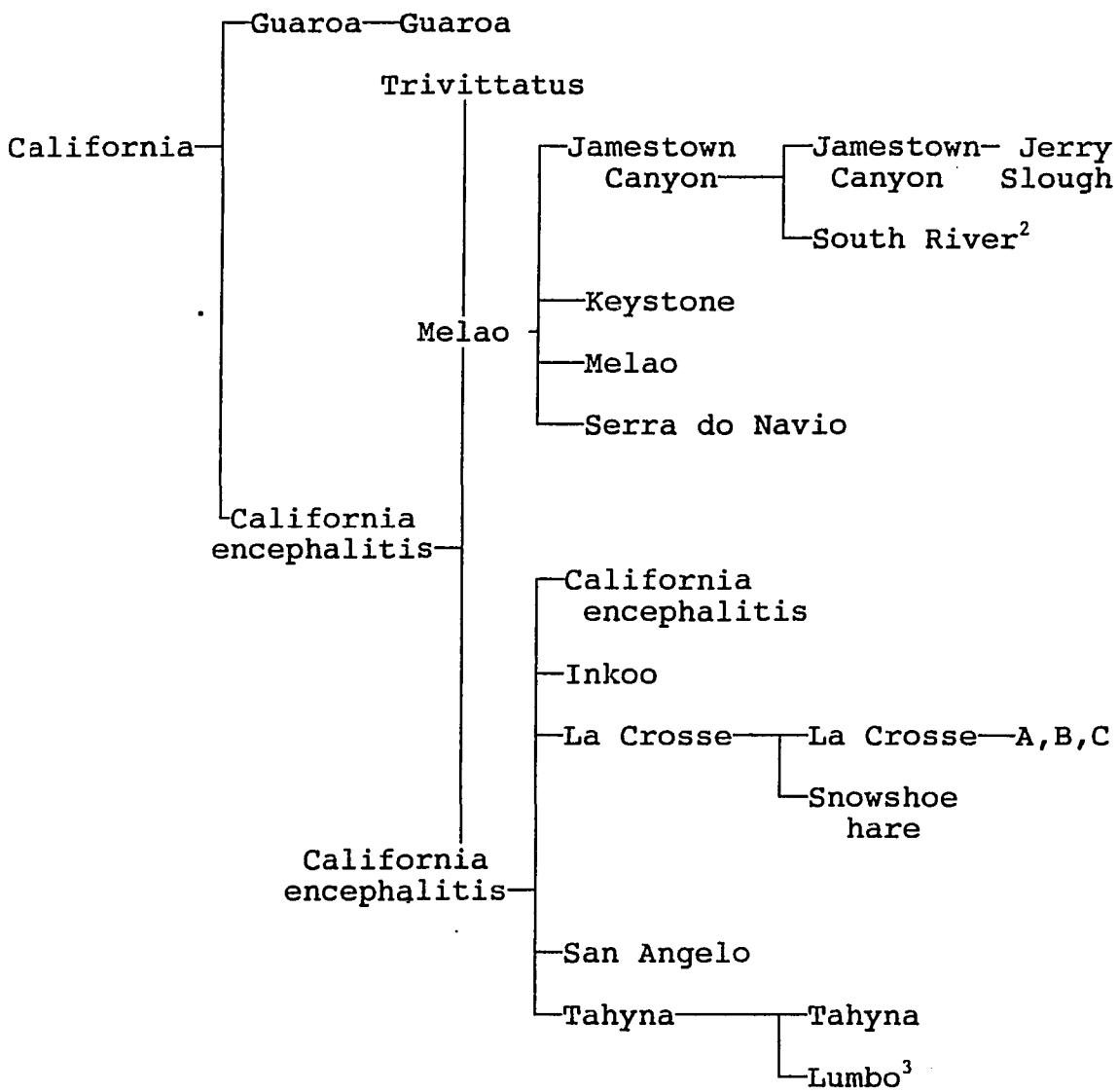
been made in this respect for such major diseases as malaria. But much less is known about molecular aspects of interactions between the more than 500 arthropod-borne animal viruses and their invertebrate hosts.

Taxonomy and Distribution

The Bunyaviridae is the largest arbovirus family and includes over 200 viruses which are distributed worldwide, but have been described primarily from the western hemisphere. The family includes 5 genera defined on the basis of serological and biochemical criteria. The largest genus is designated Bunyavirus and consists of over 100 viruses classified in 15 serogroups. The California serogroup contains 13 viruses including La Crosse (LAC), Snowshoe hare (SSH), and Inkoo (INK) viruses which are the subjects of the research described in this dissertation. Table 1 shows a summary of antigenic relationships among California serogroup viruses. La Crosse virus was originally isolated from brain tissue of a 4 year old girl in La Crosse, Wisconsin (Thompson et al., 1965) and has since been isolated in many other areas of the eastern United States. Snowshoe hare virus occurs in artic and subarctic North America and was originally isolated in

western Montana (Burgdorfer et al., 1961). Antibodies to SSH have recently been detected in blood of residents of Shanghai, China (Gu et al., 1984) and it is likely that SSH has the widest distribution of viruses in the Bunyavirus genus. Inkoo virus has so far been isolated only in Finland (Brummer-Korvenkontio et al., 1973).

Table 1. Antigenic relationships[1] among California serogroup bunyaviruses.



[1] Based on Calisher, (1983). Relationships are primarily determined by complement-fixation for serogroup and complex placement and by neutralization for serotype and subtype.
[2] South River is an unregistered virus believed to be a geographic variety of Jamestown Canyon.
[3] Lumbo is an unregistered variety of Tahyna found in Africa.

Disease Associations

Arboviruses have been recognized as etiologic agents of disease in humans and animals since the historic demonstration at the turn of the century by Walter Reed and colleagues of yellow fever transmission by the mosquito Aedes aegypti to human volunteers. Viruses within 4 of the 5 genera of the Bunyaviridae are responsible for a large number of debilitating and sometimes lethal diseases in humans and livestock.

Bunyaviral diseases display a wide range of clinical manifestations. Examples include Rift Valley fever virus in the genus Phlebotomus which has caused major epizootics throughout this century in Africa. The virus causes an acute febrile illness with a high incidence of death and abortion among ruminant livestock. Occasional epidemics among humans are characterized by acute fever sometimes complicated by encephalitic, ocular and/or fatal hemorrhagic illness. Nairobi sheep disease virus (genus Nairovirus) is the most pathogenic virus disease of sheep in East Africa. It is vectored by ticks and causes acute gastro-enteritis with mortality rates approaching 90%. Hantaan virus, the type virus of the genus Hantavirus, occurs in East Asia where it causes acute hemorrhagic fever

with renal syndrome in humans. In some epidemic outbreaks mortality rates are as high as 20 to 30%. The reservoir of the virus population appears to be rats and there is no known vector. The genus Bunyavirus contains viruses causing disease with clinical manifestations ranging in humans from inapparent viremia with an antibody response to severe encephalitis resulting in death and, in domestic animals, congenital deformities and abortion. The Bunyamwera, Bwamba, Group C, Guama and California serogroups cause disease in humans while members of the Simbu serogroup afflict livestock. A fifth genus, Uukuvirus, contains 11 viruses whose principal hosts are ticks and birds. None of these viruses are known to cause disease in humans or livestock.

California encephalitis virus was the first member of the California serogroup to be implicated as an etiologic agent of human disease (Hammon and Reeves, 1952). Information concerning sources of isolation and vectors of members of this serogroup is summarized in Table 2, while information on distributions and vertebrate hosts is summarized in Table 3. Nationwide surveillance for cases of human encephalitis for the period of 1963 to 1985 indicated 1,657 cases of encephalitis attributable to the California serogroup (Grimstad, 1988). La Crosse virus

appeared to be responsible for most of these cases. With the exception of those years when St. Louis encephalitis virus outbreaks occur, LAC encephalitis "is probably the most important mosquito-borne disease in the United States" (Le Duc, 1979). Onset of cases coincides with the May to September peak in mosquito activity.

Table 2. Sources of isolation and vectors of California serogroup bunyaviruses.

Virus	Source	Primary Vector	Secondary Vector
California encephalitis	Ae. melanimon	Ae. melanimon	Ae. dorsalis
Guaroa	human female	unknown	unknown
Inkoo	Ae. communis	unknown	unknown
Jamestown Canyon	Cs. inornata	Ae. stimulans Ae. communis	Ae. triseriatus An. punctipennis
Jerry Slough	Cs. inornata	unknown	unknown
Keystone	Ae. atlanticus	Ae. atlanticus	Ae. infirmatus
La Crosse	human female	Ae. triseriatus	Ae. canadensis
Melao	Ae. scapularis	unknown	unknown
San Angelo	An. p. pseudo-punctipennis	unknown	unknown
Serra do Navio	Ae. fulvus	unknown	unknown
Snowshoe hare	Lepus americanus	Ae. communis	Cs. inornata
Tahyna	Ae. caspius	Ae. vexans	Ae. cinerus ?
Trivittatus	Ae. trivittatus	Ae. trivittatus	Ae. infirmatus

Ae. = Aedes, An. = Anopheles, Cs. = Culiseta

Table 3. Distributions and vertebrate hosts of California serogroup bunyaviruses.

Virus	Distribution	Principal Hosts	Secondary Hosts
California encephalitis	Western U.S.	California ground squirrel	rabbit
Guaroa	Brazil, Panama	unknown	unknown
Inkoo	Finland	reindeer	moose, cattle
Jamestown Canyon	U.S.	white-tailed deer	elk, mule deer
Jerry Slough	California	unknown	unknown
Keystone	Southern U.S.	rabbit, squirrel	rats, deer
La Crosse	Eastern U.S.	chipmunk, squirrel	fox, rabbit
Melao	Brazil, Panama	unknown	unknown
San Angelo	Western U.S.	unknown	unknown
Serra do Navio	Brazil	unknown	unknown
Snowshoe hare	Canada Northern U.S.	snowshoe hare Citellus squirrels	rodents, deer moose, horses
Tahyna	Europe, U.S.S.R.	European hare and rabbit	hedgehog, swine
Trivittatus	U.S.	cottontail rabbit	domestic rabbits

California serogroup viruses usually produce mild or inapparent infection in humans. Estimates of inapparent to apparent infection ratios for various geographic locations range from 26:1 (Monath et al., 1970) to 1,571:1 (Grimstad et al., 1984). Clinical infection by California serogroup viruses produces 4 distinct generalized syndromes (Grimstad, 1988): (1) Mild to moderate febrile illness with no central nervous system (CNS) involvement (e.g., Guaroa, Inkoo); (2) Moderate to severe febrile illness with respiratory symptoms and occasional CNS complications (e.g., Tahyna); (3) Moderate to severe febrile illness with frequent CNS complications (e.g., California encephalitis, La Crosse, Snowshoe hare, Trivittatus); (4) Febrile illness with frequent CNS complications and frequent respiratory symptoms (e.g., Jamestown Canyon). Clinical infection with other members of the serogroup has not been documented. Clinical infection by neuropathogenic serotypes frequently results in sequelae such as reduced IQ and epileptic siezure. Children are at greater risk than adults of developing clinical infection.

Biological Differences in Virus Infection of Vertebrates and Invertebrates

There are considerable differences in the nature of California serogroup virus infection in invertebrates and vertebrates. When an infected vector bites a susceptible vertebrate host the virus begins replication in tissues at or near the site of primary inoculation. A short-term viremia follows which results in spread of the virus throughout the host reticuloendothelial system. An antibody response occurs and in most instances the infection is terminated. However, in some instances neuropathogenic serotypes are able to cross the blood-brain barrier, apparently as a consequence of replication in endothelial cells of the cerebral capillaries (Johnston, 1983). The virus might also reach the brain by retrograde transmission along neurons following replication at neuromuscular junctions in a manner similar to Rabies virus. Encephalitic disease may then occur.

When a mosquito feeds on a viremic vertebrate host, virus ingested in the bloodmeal must infect and replicate in cells of the midgut before passing through to the hemocoel. Once in the hemocoel, the virus spreads throughout the arthropod and replicates to high titers,

being found in virtually every organ system examined (Beaty and Thompson, 1977). Infection of the arthropod is lifelong. Despite the tremendous numbers of virus particles often found in infected vectors no major negative effects on the arthropod hosts have been reported. Thus, while Bunyavirus genus infection in vertebrate hosts may cause significant morbidity and occasional mortality, particularly among immature hosts outside the normal arbovirus cycle such as children (Parsonson and McPhee, 1985), there appears to be little effect of virus infection on the invertebrate host. This observation extends to in vitro cell culture. Viral replication in vertebrate cells causes cytopathologic effects (CPE) and death while replication in mosquito cells does not cause CPE and persistent infections of long duration can be established (Florkiewicz and Hewlett, 1980; Elliot and Wilkie, 1986).

Virus Life Cycle

The life cycle of LAC virus is the best understood of the California serogroup viruses and the following discussion will pertain to it. The life cycle is diagrammed in Figure 1. The woodland mosquito, Aedes triseriatus, is the principal vector of LAC virus. This

mosquito transmits the virus to the principal vertebrate hosts, chipmunks and tree squirrels. Infection of these hosts serves to amplify and disseminate the virus as they develop viremias sufficient to infect subsequently feeding uninfected mosquitoes. Yuill and co-workers (1983) have also demonstrated a potential role for red foxes in disseminating LAC virus, particularly in areas where trapping pressure is heavy and nonimmune juveniles constitute a high proportion of the fox population. Other woodland mammals such as deer do not develop viremias of sufficient titer to serve as amplifying hosts.

For many years cycling of the virus between the invertebrate and vertebrate hosts was thought to be the mechanism of maintenance of the virus in nature. However, very few adult mosquitoes survive through the winter and of these, only a low proportion would be likely to be infected by virus obtained from an infected vertebrate host. Furthermore, viral infection of the vertebrate hosts is usually self-limiting and transient and could not be expected to serve as a mechanism of winter survival. This apparent "weak-point" in the cycle was explained by the demonstrations of transovarial (Watts et al., 1973) and venereal (Thompson and Beaty, 1977) transmission of the virus within the mosquito population and the ability of the

virus to overwinter in the diapaused eggs of the vector (Watts et al., 1974). These transmission mechanisms greatly diminish the importance of the role of the amplifying vertebrate hosts in maintenance of the virus in nature and it is now widely believed that they are the principal mechanisms of maintaining the virus. Transovarial transmission has also been demonstrated for many other viruses in the families Bunyaviridae and Flaviviridae.

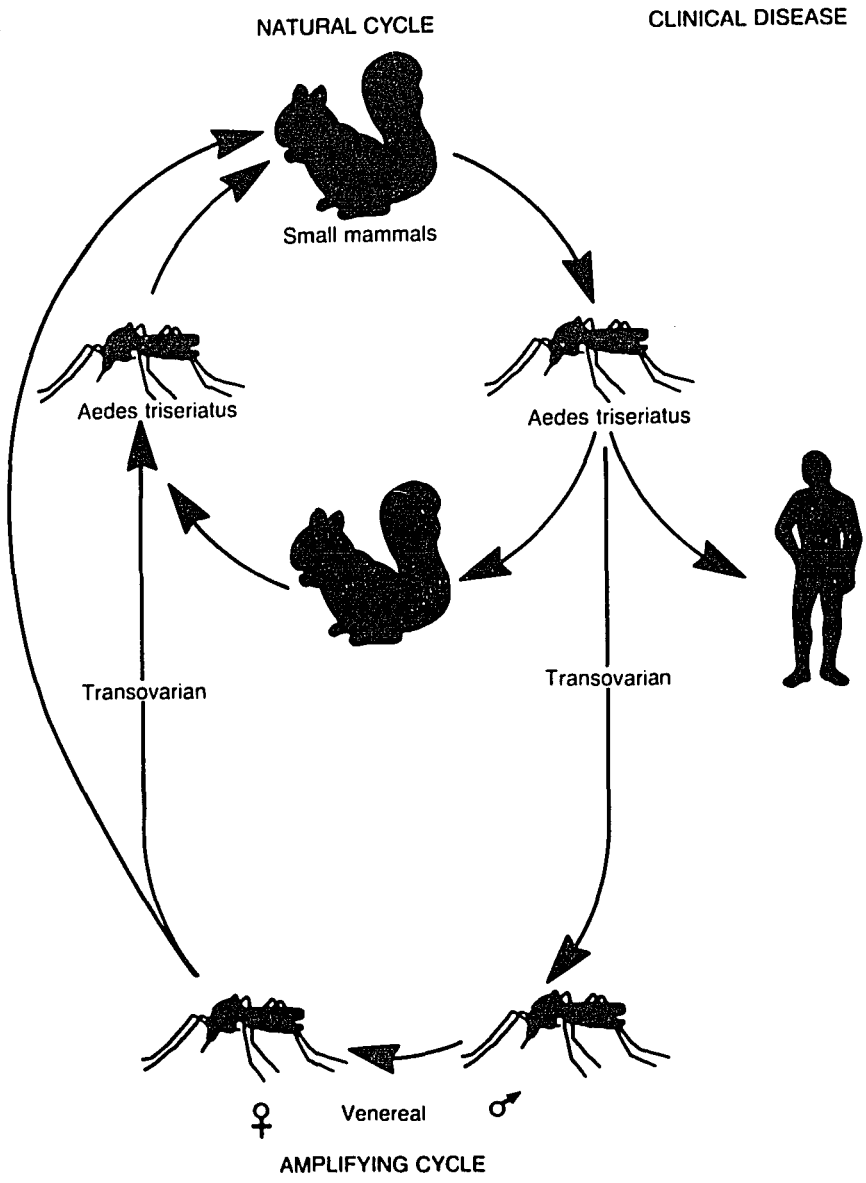


Figure 1. Life cycle of La Crosse virus. The virus is maintained during winter in diapaused eggs infected by transovarial transmission. The virus is amplified by venereal transmission between the transovarially infected male mosquito and uninfected female mosquito and by the inapparent cycle involving small mammals and mosquitoes. Taken from Viral Infections of the Nervous System by Richard T. Johnson.

Molecular Biology

Viruses in the family Bunyaviridae, genus Bunyavirus are lipid-solvent-sensitive and possess a host-derived envelope. The viral particles, as visualized by electron microscopy, are roughly spherical, 90 to 100 nm in diameter, and are coated with surface "spikes" consisting of viral glycoproteins. There are 2 viral glycoproteins designated G1 (110 to 120 kilodaltons) and G2 (30 to 40 kilodaltons) which have covalently associated fatty acid residues, are only lightly glycosylated, and are present in equimolar proportions (Bishop, 1985).

There are 3 size classes of internal nucleocapsid which are circular or loosely helical in shape. They consist of many copies of a nucleocapsid protein (N, 19 to 26 kilodaltons), a few copies of a large replicase/transcriptase protein (L, 150 to 200 kilodaltons) and 1 of 3 unique species of single-stranded negative-sense genomic RNA (Bishop, 1985). The large or L RNA (3 megadaltons) encodes the replicase/transcriptase protein (Endres et al., 1989). The medium or M RNA (2 megadaltons) encodes the coat glycoproteins and 1 or more nonstructural proteins in the same reading frame. The M segment mRNA is translated as a large polyprotein which is proteolytically

processed to yield mature proteins (Eshita and Bishop, 1984; Fazakerley et al., 1988). The small or S RNA (0.3 to 0.5 megadaltons) encodes the nucleocapsid protein and a nonstructural protein in an overlapping reading frame (Bishop et al., 1983). The function of the nonstructural proteins is unknown.

The 3 genomic RNA species have a common consensus 3' end sequence of UCAUCACAUG and an inverted complementary 5' sequence (Clerx-van Haaster et al., 1982; Bishop, 1985). The sub-genomic plus sense viral messenger RNA species have heterogenous capped 5' end sequences of about 14 nucleotides which are presumably parasitized from host mRNA species as a means of priming plus sense RNA transcription (Bishop et al., 1983; Patterson et al., 1984; Eshita et al., 1985). The subgenomic mRNAs lack sequences from the 5' ends of the genomic RNAs and are not polyadenylated at their 3' ends. Replication occurs in the cytoplasm of infected cells (Goldman et al., 1977). Actinomycin D and alpha-amanitin are not capable of inhibiting viral RNA replication or transcription (Vezza et al., 1979). Viral maturation occurs in the Golgi of infected cells (Murphy et al., 1968; Smith and Pifat, 1982). Many details of viral infection, replication, transcription, translation and assembly remain to be elucidated.

RNA segment reassortment has been demonstrated both *in vitro* and *in vivo* in the arthropod host (Beaty et al., 1981) between members of the California serogroup, or the Bunyamwera serogroup, or the Group C serogroup but not between members of the different serogroups (Bishop, 1982). This phenomenon is analogous to the "genetic shift" seen in influenza viruses and is of great evolutionary and epidemiologic importance.

Purpose of the Dissertation

In the *Bunyavirus* life cycle the genetic information of the virus is replicated and expressed in the distinctly different biological systems of the vertebrate and invertebrate hosts. Extra- and intracellular molecular events must determine the interactions between the viruses and their hosts and must also underly the differences seen in the nature of the virus-vertebrate and virus-invertebrate interactions. Since these events are mediated on a molecular level it follows that the techniques of molecular biology and biochemistry provide an appropriate means of investigation leading to a greater understanding of arboviral host interactions, life cycles and disease. Such an increased understanding may ultimately lead to

increased ability to control arboviral disease as well as greater knowledge of molecular differences between vertebrate and invertebrate cellular processes.

To these ends, I have pursued 3 major avenues of investigation into the molecular biology of Bunyaviruses and their host-interactions. These investigations are as follows:

Genomic Stability of La Crosse Virus During Vertical and Horizontal Transmission

In view of differences in the nature of LAC virus infection in vertebrate and invertebrate hosts and the high mutability and rapid evolutionary potential of RNA viral genomes (for reviews see Reanney, 1982; Steinhauer and Holland, 1987; Strauss and Strauss, 1988), one might expect that environmental differences faced by the virus in the two host types could select for different genotype distributions in the virus population. Such a phenomenon could have important epidemiological implications including changes in transmission potential, virulence, host tropisms, and ability to evade prevalent immunologic barriers in vertebrate hosts (Beaty et al., 1988). In a collaborative effort with Dr. Barry Beaty of Colorado State

University, I have used the technique of RNase T1 oligonucleotide fingerprinting (ONF) to study the possibility that the LAC virus genome may evolve when passaged between (horizontal) and within (transovarial or vertical transmission) invertebrate and vertebrate hosts in the laboratory.

Transmission of Reassortant Bunyaviruses

In a second collaboration with Dr. Beaty, I have again used ONF to study generation and transmission of reassortant California serogroup viruses in mosquitoes and mice. The reassortant phenomenon is of evolutionary significance and, in the case of influenza viruses, of great epidemiologic importance. Bunyaviruses are known to reassort. We have addressed the unanswered question of the potential of Bunyavirus reassortants to be transovarially transmitted.

Sequence Analysis of Inkoo Virus M and L Genomic Segments

I have used Sanger dideoxynucleotide chain termination sequencing technology to obtain partial sequences of the

INK virus M and L genomic RNA segments. I have compared these sequences and their deduced amino acid sequences with published sequences for other viruses in order to obtain new information concerning evolutionary relationships among the viruses and to deduce details of their RNA and protein molecular structures.

Materials and Methods
for
La Crosse Virus Genomic Stability Studies

Virus

La Crosse virus, originally isolated from a fatal human encephalitis case (Thompson et al., 1965) and serially passaged many times in BHK-21 cell culture monolayers, was further passaged 1 time in a mouse and 1 time in BHK-21 cell monolayers. The virus was then plaque-purified 3 times on BHK-21 cell monolayers, followed by a final low multiplicity of infection (MOI) passage in BHK-21 cell monolayers to prepare inoculum stock.

Mosquitoes

Aedes triseriatus mosquitoes were collected in La Crosse, Wisconsin in 1981. Virus and mosquitoes were kindly provided by Dr. Wayne Thompson, University of Wisconsin. The mosquito colony has been maintained at Colorado State University for 7 years under the following conditions: photoperiod 16 hours light/8 hours dark, 60-

80% relative humidity, 23-25 C. Adults were maintained on sugar cubes, raisins, apples and water. Larvae were fed TetraMin™ (Tetra Works, Melle, W. Germany).

Cell Culture

BHK-21 cells were obtained from the American Type Culture Collection and were grown at 37 C, 8% carbon dioxide in DMEM media (Gibco) supplemented with 10% bovine calf serum (Hyclone). Medium for production of ^{32}P-labelled virus was phosphate-free DMEM with 25mm HEPES (Hazleton) supplemented with 2% dialyzed fetal bovine serum (Hyclone) and ^{32}P-orthophosphate (ICN).

Mice and Reagents

Balb/C mice were obtained from Charles River Laboratories, Willington, Ma. Sources of materials for ONF were as follows: Polyacrylamide was from BDH Chemicals, LTD, Poole, England and bisacrylamide was from Kodak; urea and boric acid were from IBI and Tris base was from Research Organics Inc., Cleveland, Ohio; Seaplaque™ low-melting point agarose was from FMC Corp; phenol was from

Boehringer Mannheim Biochemicals; ribonuclease T1 and tRNA were from BRL.

Artificial Bloodmeal Preparation and Infection of Mosquitoes

Uninfected female mosquitoes were allowed to feed on artificial bloodmeals containing plaque-purified LAC virus. The bloodmeals were prepared by growing virus in BHK-21 cell culture monolayers infected at an MOI of 0.01. When cells exhibited 3+ CPE they were scraped into the media and transferred to a centrifuge tube. Following a 10 min, 200x g centrifugation, all but 2 mls of the supernatant was poured off. The cells were resuspended and gently mixed with 2 mls each washed human red blood cells, heat-denatured fetal bovine serum and 10% sucrose. The mixture was warmed to 37 C and was offered to mosquitoes as droplets placed on the cage screening. The virus titre was 7.1 $\log_{10}$ Tissue Culture Infectious $Dose_{50}$/ml. Titers were determined as described in the section, Virus Assays, on page 35. The infection rate of mosquitoes taking bloodmeals was determined by immuno-flourescence examination of a leg (Beaty and Thompson, 1975) removed from each mosquito 14 days after the bloodmeal.

Growth of Virus Isolates and Preparation of Viral RNA for Oligonucleotide Fingerprinting

All mosquitoes and mouse brains stored at -70 C were homogenized in 100 ul DMEM. The homogenates were diluted 100-fold with sterile phosphate-buffered saline and were passed through 0.2 um syringe filters. A 1 ml volume of each filtrate was used to infect a 175 cm^2 tissue culture flask of confluent BHK-21 cells. When the cells exhibited 3+ CPE the culture media was collected and clarified by a 10 min, 1,000x g centrifugation and was stored as a virus stock at -70 C. These stocks were used to infect BHK-21 cell monolayers at MOI values of 0.1-1.0 in order to prepare ^{32}P-labelled viral RNA for ONF. I have chosen BHK-21 cells in preference to a mosquito cell line because of technical difficulties in obtaining sufficient ^{32}P-labelled virus for ONF from mosquito cell cultures. Following infection, the cell cultures were maintained at 33 C for 24-36 hr in phosphate-free DMEM supplemented with 2% dialyzed fetal bovine serum and ^{32}P-orthophosphate at 10 uCi/ml. The cell culture medium was collected and clarified by a 30 min, 10,000 RPM centrifugation in a Sorvall SS-34 rotor at 4 C. The supernatant was loaded onto 7 ml

cushions of 30% w/w glycerol in TE buffer (10 mM Tris/HCL, 1 mM EDTA, pH 8.0 at 4 C,). The virus was pelleted at 4 C for 2 1/2 h at 24,000 RPM or for 15 h at 15,000 RPM in a Beckman SW-28 rotor. The pellets were resuspended for 1 hr on ice in 0.4 ml each of TE buffer and 25 ul of 5M NaCl. Three mls of resuspended virus was loaded onto a linear gradient prepared with 5 ml of 30% w/w glycerol in TE buffer and 4.5 ml of 50% w/w sodium potassium tartrate in TE buffer. The gradients were centrifuged at 4 C for 2-4 hr at 35,000 RPM or 15 hr at 18,000 RPM in a Beckman SW-41 rotor. Visible virus bands (present about halfway down the gradients) were collected and diluted to 10 ml final volume with TE buffer. The diluted virus was loaded onto 2 ml cushions of 30% w/w glycerol in TE buffer and was pelleted for 2 hr at 4 C and 35,000 RPM in the SW-41 rotor. The viral pellets were resuspended in 0.3 ml TE buffer with 2% SDS at room temperature, and were extracted twice with equal volumes of phenol saturated with TE buffer, and once with 24:1 v/v chloroform/isoamyl alcohol. The aqueous phase, containing viral RNA, was ethanol-precipitated overnight at -20 C. The RNA was pelleted, resuspended in 40 ul TE buffer/2% SDS, and the viral L, M, and S RNA segments were separated by electrophoresis in 1% low-melting point agarose gels buffered with TBE at 5V/cm for 6

hr. The position of L, M and S RNA in the gels was determined by autoradiography. Sections of gel containing the L, M and S RNA were excised, melted at 65 C and extracted twice with equal volumes of phenol and once with chloroform/isoamyl alcohol. The aqueous phases were ethanol-precipitated with 10 ug tRNA as carrier.

RNase T1 Oligonucleotide Fingerprinting

The viral RNAs were pelleted, resuspended in 50 ul TE buffer and digested for 1 hr at 37 C with 100 units of RNase T1 (BRL). The digests were phenol and chloroform extracted as above and ethanol precipitated overnight at -20 C. The viral RNA was pelleted, resuspended in 6 ul 6M urea and subjected to two-dimensional polyacrylamide gel electrophoresis essentially as described by DeWachter and Fiers (1972) with the modifications of Lee and Fowlks (1982). Autoradiography, following electrophoresis of the second dimension gels, was performed with Kodak X-OMAT AR or Konica Type A x-ray film at -70 C with intensification screens.

Materials and Methods
for
Transmission of Reassortant Bunyavirus Studies

Virus

Temperature sensitive (ts) mutant LAC and SSH viruses were kindly provided by Dr. David Bishop of the NERC Institute of Virology, Oxford, U. K. These viruses have a ts lesion in the M RNA segment (Group I, SSH-I-3) or the L RNA segment (Group II, LAC-II-5) as described (Gentsch et al., 1977). They replicate at the permissive temperature of 33 C but not at the nonpermissive temperature of 40 C.

Mosquitoes, BHK-21 cells, Balb/C mice and all other reagents were as described above for the LAC virus genomic stability studies.

Virus Assays

Stock virus and experimental samples were titrated and assayed by Dr. Beaty's staff for the ts phenotype by two methods; microtitration or plaque assay. For microtitration, serial 10-fold dilutions of the samples were made in cell culture growth medium (Minimum Essential

Medium - Earle's base, (MEM-E) containing 7% newborn bovine serum (NBS), 10% tryptose phosphate broth (TBP), 100 units/ml penicillin and 100 ug/ml streptomycin). Dilutions were added to wells of a 96-well tissue culture plate (50 ul per well). Four to six replicates were plated for each dilution. After dilutions were placed in the plates, BHK-21 cells in growth medium were added (4,500 cells per well). For each sample, duplicate plates were made and incubated at 33 C and 40 C. After 4 days incubation, plates were examined for CPE. Titers were calculated by the method of Karber (1931). For plaque assays, serial 10-fold dilutions of virus samples were made in growth medium and inoculated onto monolayers of VERO cells in 6-well plates (0.2 ml per well). Virus was allowed to adsorb for 1 hour before media removal. Monolayers were overlaid with 1% agarose (Sea-Plaque, FMC Corp.) in maintenance medium (MEM-E, 4% NBS, 10% TBP, 100U/ml penicillin and 100 ug/ml streptomycin). The plates were incubated for 5 days at 33 C and 40 C. To visualize plaques, monolayers were fixed and stained with crystal violet (0.25% w/v in 20% methanol). Titers were calculated by the method of Karber (1931).

Infection of Mosquitoes

Stock viruses were diluted in phosphate buffered saline and intrathoracically inoculated into female Aedes triseriatus mosquitoes. Mosquitoes received approximately 30 Tissue Culture Infectious $Dose_{50}$ each of LAC-II-5 and SSH-I-3 diluted virus stocks. Control mosquitoes received equivalent doses of LAC-II-5 or SSH-I-3 alone. Following inoculation, mosquitoes were allowed to mate with uninfected male mosquitoes and were offered an adult mouse as a blood source on days 6, 14, and 21 post-inoculation. Engorged females were offered an oviposition dish 3 days after feeding. Egg liners were collected, air dried for 24 hours and stored in a humidified box. On day 28 post-infection all mosquitoes were dissected. Ovaries were removed and triturated and held at -70 C till assay for virus as described above.

Transmission Studies

Eggs of infected mosquitoes were hatched and reared. Progeny mosquitoes were assayed for virus by the immunoflourescence leg-pull test (Beaty and Thompson, 1975). Infected mosquitoes were allowed to feed on

individual suckling mice before trituration and storage at -70 C. Mice were held until ill, brains were removed and triturated, and stored as described for results of the LAC virus genomic stability studies. To determine the genotype of reassortant viruses the ts stock viruses and 3 experimental samples that exhibited the wild type phenotype by plaque assay were examined by ONF. These samples were: (1) ovary of female #12 that was dually infected by intrathoracic inoculation; (2) transovarially infected progeny mosquito #6; and (3) virus isolated from mouse #6 that had been fed upon by progeny mosquito #6. All ONF procedures were as described for the LAC virus genomic stability studies with the exception that the 3 samples exhibiting the wild type or reassortant phenotype were grown at 40 C to suppress simultaneous growth of the ts mutants.

Materials and Methods for Inkoo Virus M and L Segment Sequence Analysis

Reagents

Exonuclease III, S1 nuclease, T4 DNA polymerase, T4 DNA ligase, AMV reverse transcriptase and the Klenow fragment of E. coli DNA polymerase B were from BRL. Restriction enzymes were from BRL or Promega Biotech. Sequenase™ kits were from United States Biochemical. Urea and boric acid were from IBI. Polacrylamide was from BRL or BDH Chemicals, LTD, Poole, England and bisacrylamide was from Kodak. Tris base was from BRL or Research Organics Inc., Cleveland, Ohio. Phenol was from Boehringer Mannheim Biochemicals. Alpha-^{35}S dATP was from New England Nuclear.

Cloning of Inkoo Virus Genome Segments

Preparation of INK virus cDNA was executed in the laboratory of Dr. Martinez Hewlett by Dr. Melanie K. Spriggs as described in her dissertation (Spriggs, 1984). In brief, INK viral nucleocapsids were purified on CsCl

gradients and full-length double-stranded RNA representing both plus and minus strand genomic viral RNA was recovered. The RNA was used as template to synthesize cDNA with reverse transcriptase and the Klenow fragment of E. coli DNA polymerase B. The cDNA was size-fractionated by electrophoresis in 5% polacryamide gels buffered with TBE at pH 8.3. The cDNA greater than 1,000 bp in length was recovered by electroelution, ethanol precipitated, and C-tailed with terminal deoxynucleotidyl transferase. The C-tailed cDNA was annealed to G-tailed plasmid pBR322 opened at the Pst I site and was used to transform E. coli strain mm294. Drug resistant colonies containing cDNA inserts were identified as virus specific by dot blot hybridization analysis. Segment specificity of positive clones was determined by electrophoresing purified viral RNA in agarose gels followed by transfer to nitrocellulose filters. The filters were probed with individual clones that had been ^{32}P radiolabelled by nick translation. A panel of 7 clones containing cDNA inserts of 1,000 bp or greater was selected for further work (Table 4).

Table 4. Estimated insert size and segment assignments of Inkoo virus cDNA clones.

Clone	Insert size[1]	Segment[2]
pINK 130	7,000	L
pINK 140	1,000	M
pINK 210	1,700	M
pINK 220	1,800	L
pINK 350	2,500	M
pINK 360	1,000	M
pINK 430	2,000	L

[1] size in number of base pairs.
[2] L = large RNA segment and M = medium RNA segment.

Subcloning of Inkoo Virus cDNA Plasmids

The cDNA plasmid stocks described in Table 4 were subjected to large scale plasmid purification by standard techniques as described in Maniatis et al., 1982. The cDNA inserts were removed from the plasmids by digestion with Pst I and were purified by electrophoresis in 1% agarose or 5% polyacrylamide gels buffered with TBE at pH 8.3. The inserts were visualized by staining the gels with ethidium bromide and illumination with a hand-held UV lamp. Appropriate bands were excised and the DNA was electroeluted into TBE at pH 8.3, phenol extracted, ethanol precipitated and resuspended in TE buffer, pH 8.0. The resuspended DNA was subjected to digestion with various restriction enzymes and the digestion fragments were gel-purified as described above. The recovered fragments were cloned into the sequencing vectors $M13mp_{18}$ and $M13mp_{19}$ (Messing, 1983). These recombinant plasmids were used to produce template for DNA sequencing.

The cDNA inserts in plasmids pINK220L and pINK430L were also recloned into the plasmid pBSM13 (Vector Cloning Systems, San Diego, CA). These recombinant plasmids were subjected to exonuclease III degradation followed by treatment with S1 nuclease and T4 DNA polymerase before

religation with T4 DNA ligase as described in the company's protocol in order to generate nested sets of deletion plasmids for DNA sequencing.

Sequencing of Inkoo Virus cDNA

Sanger dideoxynucleotide chain termination sequencing (Sanger et al., 1977) was used to determine partial or complete sequences of the inserts in the 7 INK cDNA-containing plasmids described in Table 4. Single-stranded M13 templates were prepared as described by Messing (1983) and were sequenced by standard techniques with Klenow fragment, AMV reverse transcriptase or Sequenase™ (a chemically modified phage T7 DNA polymerase) with ^{35}S dATP as label. Some M13 subclones were used to prepare double-stranded template and were sequenced as described in Lim and Pene (1988) with reagent conditions as described in the Sequenase kit protocol. The pBSM13 nested deletion plasmids were used to prepare single-stranded template as described in the company protocol and were sequenced with Sequenase. Those templates which could not be accurately sequenced with dGTP due to GC compressions were re-sequenced with Sequenase and dITP substituted for dGTP to resolve the compressions.

The products of the sequencing reactions were routinely electrophoresed on 44 cm long, 0.4mm thick, 7.5% polyacrylamide gels made 7 M urea and buffered with TBE at pH 8.3. The gels were run at 1,600 to 2,000 V for 2 h. For those templates over 200 bp, a second 5% gel was run for 4 to 5 h and in some instances a third 5% gel was run for 7 to 9 h. This allowed resolution of bands up to 600 bp from the primer site. The gels were fixed for 20 to 30 min in 5% acetic acid, 5% methanol (v/v), and were mounted on blotting paper to be vacumn dried at 80 C for 1 h. The dried gels were exposed to Kodak X-OMAT AR or Konica Type A x-ray film with intensification screens at -70 C for 12 to 72 h.

Analysis of Sequence Data

Sequence gel autoradiograms were read by hand with use of a lightbox and the data was entered into an Artisoft™ AT personal computer. Overlap analysis of subclones was executed by hand and/or with use of the Dave Mount 5 (DM5) computer programs. This package of programs was released on April 15, 1987 by the Genetics PC-Software Center on behalf of the Arizona Board of Regents. Splicing of subclones, restriction site searches, open reading frame

analysis, codon usage and dinucleotide analysis were executed with the DM5 program. The Nucalign and PrtAlign programs of Walter and Lippman were used to conduct nucleic acid and protein sequence homology alignments. Ktuple values of 2 and 3 were employed for amino acid and nucleotide comparisons respectively. Similarity searches between the INK sequences and all sequences in the GEN Bank database were conducted with the University of Wisconsin Genetics Computer Group (UWGCG) Bestfit program (Devereux et al., 1984). Dot matrix plots of homology alignments were executed with the UWGCG DotPlot program.

Results

Genomic Stability of La Crosse Virus During Vertical and Horizontal Transmission

The passage history of LAC virus isolates subjected to ONF analysis in this study is diagrammed in Figure 2. A group of mosquitoes, infected with plaque-purified virus by artificial bloodmeal, were allowed to feed a second time on uninfected adult mice and oviposit. One of these mosquitoes (Original Female) was triturated and stored at -70 C. Her eggs were hatched and reared, and the adult F1 progeny were tested for virus infection. The transovarially infected females were allowed to feed on uninfected adult mice and oviposit. One of these females F1 Daughter #8) was triturated and stored at -70 C. Her eggs were hatched and reared and the adult F2 progeny were tested for virus infection. The transovarially infected F2 progeny were allowed to bloodfeed independently on individual uninfected suckling mice before the mosquitoes were triturated and stored at -70 C. Mosquitoes F2 Daughter #2 and F2 Daughter #3 were from this group. The suckling mice were held until ill (symptoms included

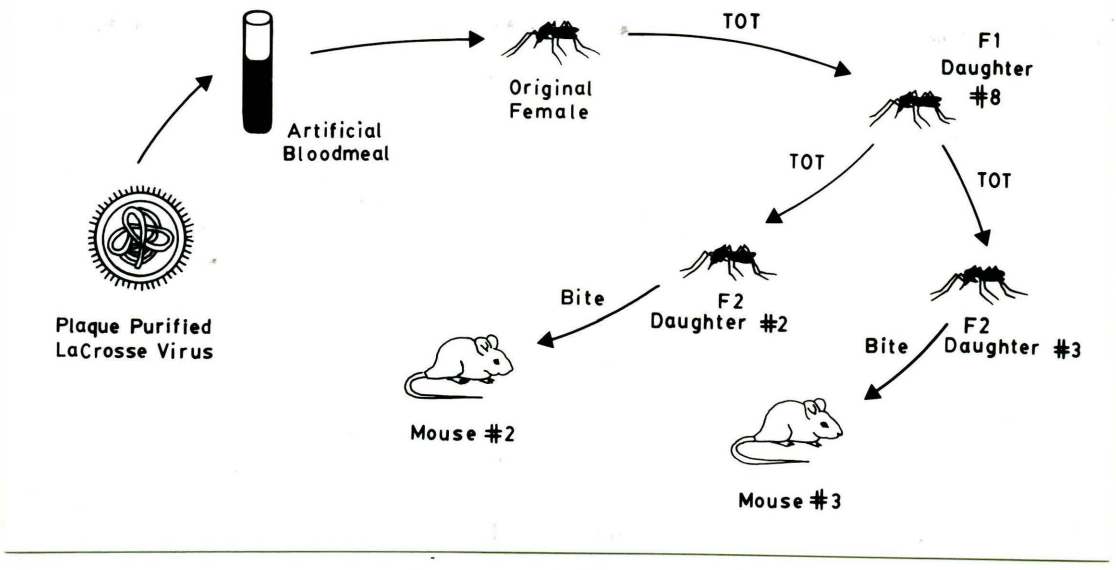


Figure 2. Passage history of La Crosse virus. Plaque-purified virus was passaged through this series of hosts. Virus was isolated from each host and subjected to oligonucleotide fingerprint analysis. TOT = transovarial transmission. Bite = transmission by bite of an infected mosquito.

shaking, inability to remain upright, cessation of nursing, and dehydration) and brains were then removed, homogenized and stored at -70 C. Mouse #2 and Mouse #3 were infected by bite of mosquitoes F2 Daughter #2 and #3 respectively.

Two weeks after the bloodmeal, 65/83 or 78% of mosquitoes ingesting bloodmeals tested positive for virus infection. This compared favorably with the 95% infection rate for a second group of mosquitoes intrathoracically inoculated with virus. The F1 generation progeny of the Original Female had a transovarially transmitted filial infection rate of 19% and the F2 progeny of F1 Daughter #8 had a rate of 46%.

An autoradiogram of LAC and SSH virus RNAs, prepared as described and electrophoresed in low-melting point agarose gels, is shown in Figure 3. Only 3 major bands are present, corresponding to the viral L, M and S segments. The absence of other bands demonstrates that these viral RNA preparations were relatively free of cellular contaminants and were suitable for ONF.

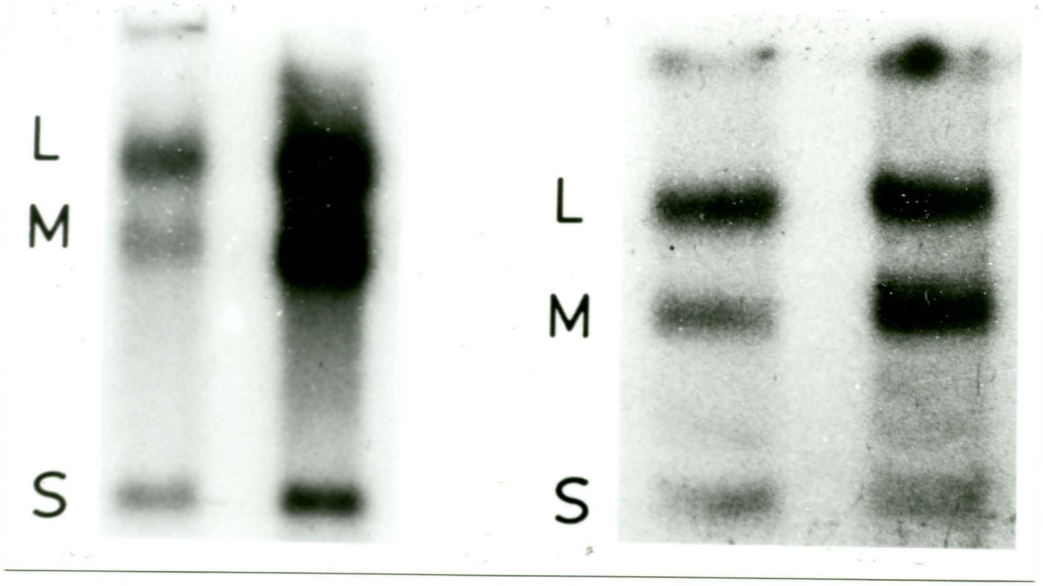


Figure 3. Autoradiogram of four preparations of La Crosse and Snowshoe hare virus RNAs separated in 1% low-melting point agarose gels. From left to right: LAC virus isolated from the infective bloodmeal, original plaque-purified LAC virus, LAC L-II-5 ts mutant virus, and SSH S-I-3 ts mutant virus. L = large RNA. M = medium RNA. S = small RNA.

The L, M and S segment oligonucleotide fingerprints of the original plaque-purified LAC virus and virus isolated from the Original female and Female #8 are shown in Figure 4. The fingerprint patterns of the original plaque-purified LAC virus and virus isolated from F2 Daughter #2 and Mouse #3 are shown in Figure 5. There were no detectable changes in these fingerprint patterns of the three viral RNA segments of LAC virus isolated from any host in the passage history diagrammed in Figure 2. From 1-4 replicate fingerprints were obtained for each RNA segment of virus isolated from any particular host. It must be noted that these virus isolates were passaged twice in BHK-21 cells in order to produce ^{32}P-labelled viral RNA for ONF analysis.

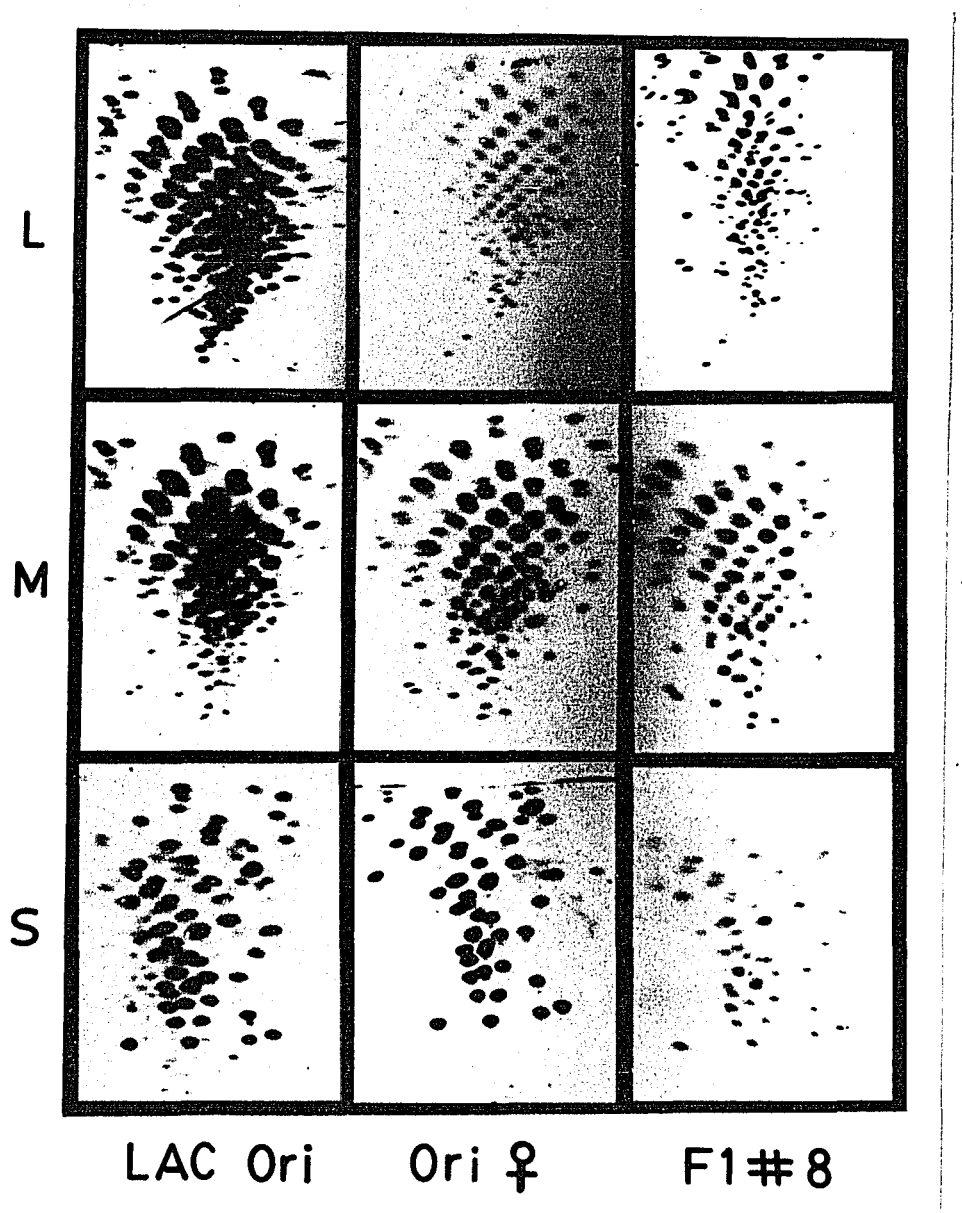


Figure 4. Oligonucleotide fingerprint autoradiograms of original plaque-purified La Crosse virus (left) and of virus isolated from the Original female (middle) and from F1 Daughter #8 (right).
L = large RNA. M = medium RNA. S = small RNA.

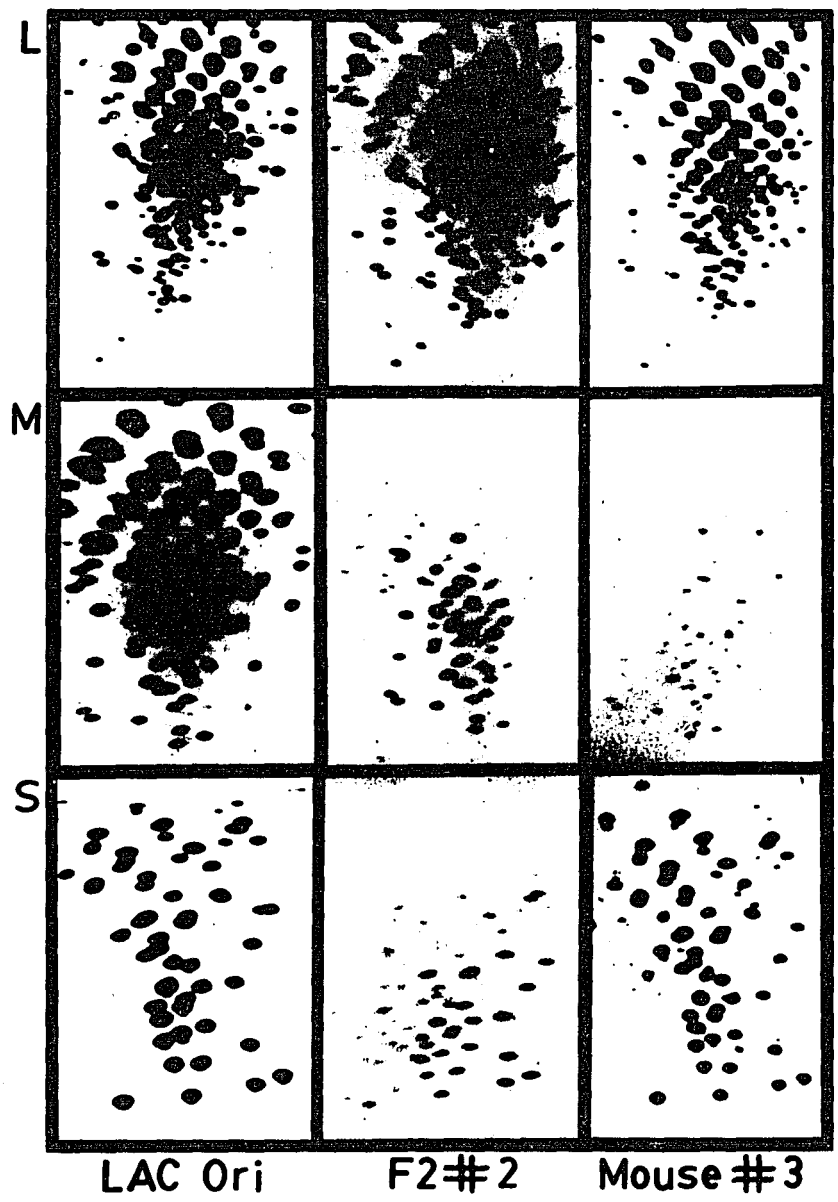


Figure 5. Oligonucleotide fingerprint autoradiograms of original plaque-purified La Crosse virus (left) and of virus isolated from F2 Daughter #2 (middle) and from Mouse #3 (right). L = large RNA. M = medium RNA. S = small RNA.

Transmission of Reassortant Bunyaviruses

Temperature-sensitive (ts) mutant stocks of LAC and SSH viruses were simultaneously inocculated into uninfected adult female Aedes triseriatus mosquitoes in an effort to generate reassortant virus infections in these mosquitoes. Thirty-five of 200 mosquitoes intrathoracically inocculated with the mixture of ts mutant viruses (LAC-II-5 and SSH-I-3) survived to 28 days post-infection. Of these, 22 (63%) contained reassortant virus as determined by ability to replicate and form plaques at the nonpermissive temperature of 40 C. None of the mosquitoes infected with LAC-II-5 or SSH-I-3 alone contained virus able to replicate at the nonpermissive temperature.

A total of 1,027 female progeny mosquitoes were obtained. Of these, 66 (6.4%) contained viral antigen. Fifty-eight of these transovarially infected mosquitoes survived to the time of assay for reassortant virus. Six (10%) of these mosquitoes contained reassortant virus. Transmission of reassortant virus to mice was attempted with these mosquitoes and was successful with 3 (50%).

In order to determine the parental origin of each RNA segment in the reassortant viruses, the ts mutant viruses LAC-II-5 and SSH-I-3 were subjected to oligonucleotide

fingerprinting (ONF). Virus isolated from female #12 that had been dually infected by intrathoracic inocculation and tested positive for reassortant virus was subjected to ONF as was virus obtained from her daughter, progeny mosquito #6, and from mouse #6 infected by bite of progeny mosquito #6. The genotypes of LAC-II-5, SSH-I-3 and the 3 experimental samples are shown in Table 5. Fingerprint autoradiograms of LAC-II-5, SSH-I-3 and virus from mosquito #12, mosquito #6 and mouse #6 are shown in Figure 6. The genotypes, shown in parentheses according to parental origin in the order large, medium and small RNA segments, were LAC-II-5 (LLL), SSH-I-3 (SSS), mosquito #12 (SLL), mosquito #6 (SLS) and mouse #6 (SLS).

Table 5. Genotypes of viruses used in transmission of reassortant bunyaviruses study.

Virus Source	Genotype[1]
LAC-II-5 stock	LAC/LAC/LAC
SSH-I-3 stock	SSH/SSH/SSH
Mosquito #12 ovary	SSH/LAC/LAC
Progeny mosquito #6	SSH/LAC/SSH
Mouse #6	SSH/LAC/SSH

[1] Genotype is expressed as parental source of each virus segment: large/middle/small.

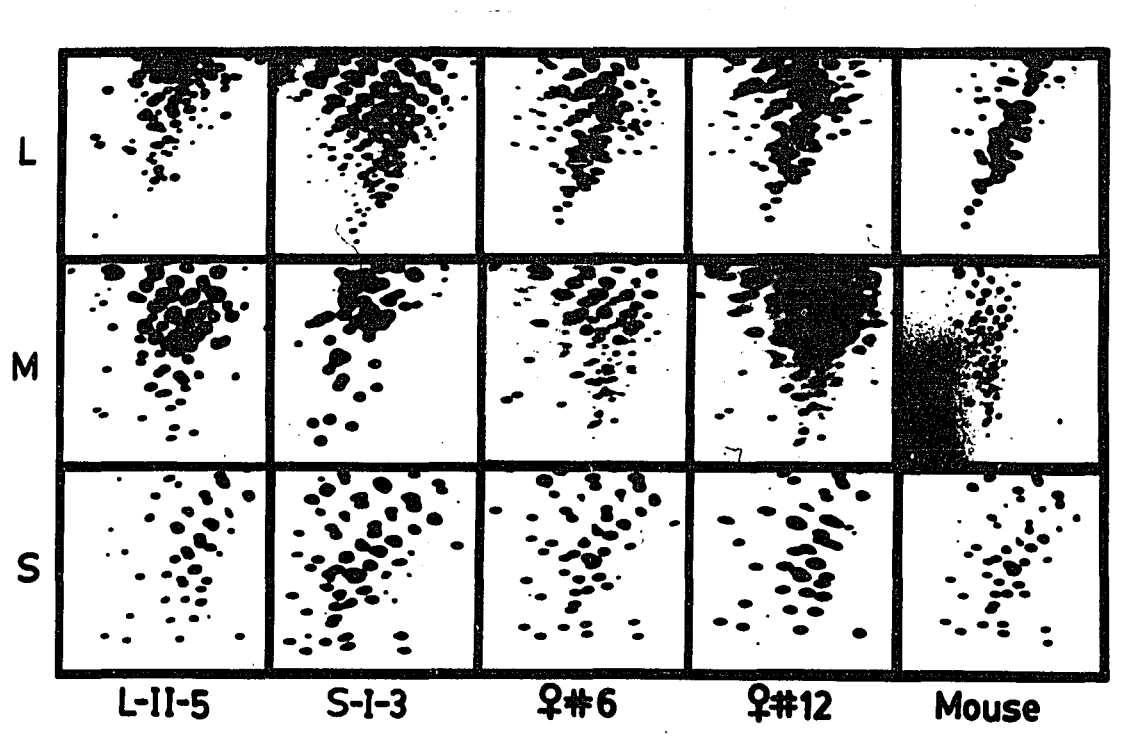


Figure 6. Oligonucleotide fingerprint autoradiograms of La Crosse (L-II-5) and Snowshoe hare (S-I-3) parent viruses and reassortants isolated from mosquitoes #6 and #12 and from mouse #6. L = large RNA. M = medium RNA. S = small RNA.

Sequence Analysis of Inkoo Virus M Segment

The INK virus M segment RNA encodes the viral coat glycoproteins (G1 and G2) and nonstructural proteins (NsM) of unknown function. An M segment subgenomic complementary sense RNA is translated as a polyprotein which is post-translationally processed to yield mature proteins.

The complete sequences of the INK cDNA inserts in plasmids pINK 140M, 210M, 350M and 360M were obtained by sequencing restriction fragments subcloned into the M13 sequencing vectors. Restriction fragments obtained from these cDNA inserts, and the extent to which various restriction fragments were sequenced, are shown in Figure 7. Comparison of the nucleotide sequences of the pINK 140M and 360M cDNA inserts revealed that they were identical to each other and to approximately one-half of the pINK 210M cDNA insert. A region of approximately 30 deoxycytidine residues separated this portion of the pINK 210M cDNA insert (210B) from a second portion of the insert (210A) whose sequence matched an internal region of the large pINK 350M cDNA insert. It is likely that this "composite" structure of the pINK 210M cDNA insert is an artifact of the dC-tailing procedure executed by Dr. Spriggs. Comparison of the sequences of the INK M segment cDNAs with

the published sequence of the closely related SSH virus M segment (Eshita and Bishop, 1984) allowed determination of the plus or negative sense of the cDNA sequences and their relative positions within the INK viral M RNA sequence (Figure 7). Approximately 300 nucleotides at the 3' end of the viral negative-sense RNA and 800 nucleotides beginning approximately 2,750 nucleotides from the 3' end of the viral RNA were not contained in this panel of cDNA clones.

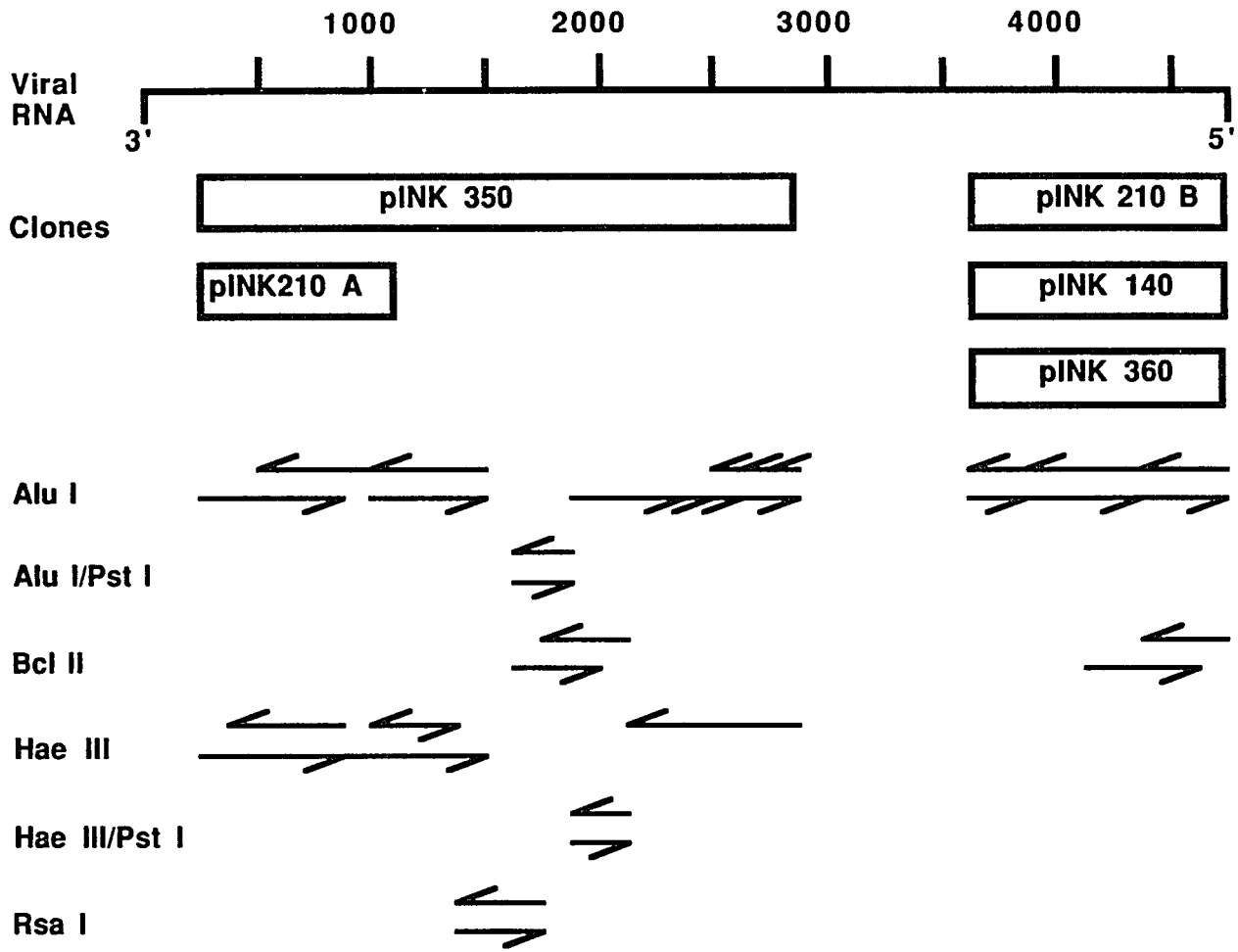


Figure 7. Inkoo virus M segment cDNA clones and sequencing strategy. Subcloned restriction fragments are shown with arrows representing the direction of sequencing. Multiple clones of each fragment were sequenced as described in the text. Numbers above the viral RNA line refer to number of nucleotides from the 3' end of the viral RNA.

The sequence of the cDNA inserts contained in the plasmids pINK 350M and 210M is shown in Figure 8 as the plus or viral antigenome-sense cDNA and is designated M35#10. The sequence of the cDNA inserts contained in the plasmids pINK 140M, 210M and 360M is shown in Figure 9 as the plus or viral antigenome-sense cDNA and is designated M21#8. The base composition of these sequences (as negative-sense viral RNA), and representing approximately 75% of the INK M segment, was 15.3% G, 19.9% C, 31.0% A, 33.8% U for M35#10 and 14% G, 19.7% C, 30.3% A, 36% U for M21#8. These values are similar to those reported for other Bunyaviruses.

Analysis of the INK M segment cDNA sequences for open reading frames (ORF) revealed large numbers of stop codons distributed throughout all 3 possible reading frames of the viral negative-sense RNA sequences. When the complementary plus-sense sequences were analyzed, many stop codons were found throughout frames 1 and 2, but frame 3 was open throughout the 2,420 nucleotide length of M35#10 and the first 857 of the 954 nucleotides in M21#8. M21#8 terminates with a UAG codon. The 806 amino acids encoded by M35#10 are shown in Figure 8 and the 285 amino acids encoded by M21#8 in Figure 9. Their amino acid compositions are shown in Figure 10. The relatively high

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  1 ATA TCA ATG TTA TTG AAG TTG GCG ATG ACT TGT CAC TTC ATA
      Ile Asn Val Ile Glu Val Gly Asp Asp Leu Ser Leu His Thr 14

 43 CGG AAT CAT ATA TTT GCA GTG CTG ACT GTG TCA TAA CGG TTG
      Glu Ser Tyr Ile Cys Ser Ala Asp Cys Val Ile Thr Val Asp 28

 85 ACA AAG AAA CAG CTC AAA TTC GTT TAC AAA CTG ACA ACA CAA
      Lys Glu Thr Ala Gln Ile Arg Leu Gln Thr Asp Asn Thr Asn 42

127 ACC ACT TTG AGG TTG CTG GTA CTA CAG TTA AAT CAG GGT GGT
      His Phe Glu Val Ala Gly Thr Thr Val Lys Ser Gly Trp Phe 56

169 TCA AGA GTA CTA CAT ATA TTA CAC TTG ATC AAA CAT GTG AGC
      Lys Ser Thr Thr Tyr Ile Thr Leu Asp Gln Thr Cys Glu His 70

211 ATC TGA AAG TGT CAT GCG GAC CTA AAT CAA TAC AGT TCC ATG
      Leu Lys Val Ser Cys Gly Pro Lys Ser Ile Gln Phe His Ala 84

253 CTT GCT TTA ATC AAC ATA TGT CAT GTG TGC GAT TTC TGC ATA
      Cys Phe Asn Gln His MET Ser Cys Val Arg Phe Leu His Arg 98

295 GGA CAA TAC TTC CAG GTT CGA TAG CAA ATT CTA TAT GTC AGA
      Thr Ile Leu Pro Gly Ser Ile Ala Asn Ser Ile Cys Gln Asn 112

337 ATA TAG AAA TAA TTA TTC TGG TAA CTT TGT CTT TAG TGA TTT
      Ile Glu Ile Ile Ile Leu Val Thr Leu Ser Leu Val Ile Phe 126

379 TTA TAT TTT TAA CAA TAG TTA GTA AGA CAT ATG TAT GTT ACC
      Ile Phe Leu Thr Ile Val Ser Lys Thr Tyr Val Cys Tyr Leu 140

421 TAT TAA TGC CTA TAT TTA TCC CAA TAG CAT ATA TTT ACG GGT
      Leu MET Pro Ile Phe Ile Pro Ile Ala Tyr Ile Tyr Gly Trp 154
```

Figure 8. Nucleotide and deduced amino acid sequences of M35#10.

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463 GGA TAT ATA ACA AAT CTT GTA AAA AAT GTA AAT TGT GTG GGT
      Ile Tyr Asn Lys Ser Cys Lys Lys Cys Lys Leu Cys Gly Leu 168

505 TAG TTT ACC ACC CAT TTA CCG AGT GTG GGA CAC ATT GTG TTT
      Val Tyr His Pro Phe Thr Glu Cys Gly Thr His Cys Val Cys 182

547 GTG GTG CAA GAT ATG AAA CTT CTG ATA GGA TGA AAT TGC ATA
      Gly Ala Arg Tyr Glu Thr Ser Asp Arg MET Lys Leu His Arg 196

589 GGG CAT CTG GGT TGT GTC CGG GGT ATA AGA GTC TCA GAG CAG
      Ala Ser Gly Leu Cys Pro Gly Tyr Lys Ser Leu Arg Ala Ala 210

631 CCA GAG TTA TGT GCA AAT CAA AAG CGC CAG CAT CAG TTT TAT
      Arg Val MET Cys Lys Ser Lys Ala Pro Ala Ser Val Leu Ser 224

673 CCA GTT ATG AGC CAG CAG TTC TCA TAC TGA CAG CGT TTG TCA
      Ser Tyr Glu Pro Ala Val Leu Ile Leu Thr Ala Phe Val Thr 238

715 CTC CTA CTA GTG CTA TGG TCA TTG GTG AAT CAA GTG AAG TGT
      Pro Thr Ser Ala MET Val Ile Gly Glu Ser Ser Glu Val Tyr 252

757 ACA CAC TAG ATG ACT TGC CTG ATG ATA TGG TAG AAA TGG CAG
      Thr Leu Asp Asp Leu Pro Asp Asp MET Val Glu MET Ala Glu 266

799 AAA GAG TAA ATA TCT ACT ATC AGA TCA TTA TAA TTA ATT ACA
      Arg Val Asn Ile Tyr Tyr Gln Ile Ile Ile Ile Asn Tyr Ser 280

841 GCA TAA CAT GGG TTT TCT TAC TAC TTG GCT TAA TTA TAG CAT
      Ile Thr Trp Val Phe Leu Leu Leu Gly Leu Ile Ile Ala Tyr 294
```

Figure 8 continued.

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883 ATT TCT TTA GGA ATA TTC AAC ATA GAT TTT TAA GCT TTT ATG
      Phe Phe Arg Asn Ile Gln His Arg Phe Leu Ser Phe Tyr Ala 308

925 CAA TGT ATT GTA AAG AAT GTG ACA TGT ATC ATG AAA GAG CTG
      MET Tyr Cys Lys Glu Cys Asp MET Tyr His Glu Arg Ala Gly 322

967 GTT TGA CCT ACT GGG GTG ACT TTA CAA ATA AAT GCA GGC AAT
      Leu Thr Tyr Trp Gly Asp Phe Thr Asn Lys Cys Arg Gln Cys 336

1009 GCA CAT GTG GGC AAT TTG AGG ATG TTA CTG GGT TGA TGG TGC
       Thr Cys Gly Gln Phe Glu Asp Val Thr Gly Leu MET Val His 350

1051 ATA AGA AAT CTT ACA ATT GCA TAA TTA AAT ACA AAG CAA AGT
       Lys Lys Ser Tyr Asn Cys Ile Ile Lys Tyr Lys Ala Lys Trp 364

1093 GGA TAA GAG ATA TGC TAA TCA CAT ATT TAC TTA TCA TGC TAA
       Ile Arg Asp MET Leu Ile Thr Tyr Leu Leu Ile MET Leu Ile 378

1135 TCA AAG ATT CAC TAT TAT TTG TCG GTG CGA CAG GAA CAG ATT
       Lys Asp Ser Leu Leu Phe Val Gly Ala Thr Gly Thr Asp Phe 392

1177 TCG CTG CAT GTA TAG ATG AGA AAA CAG TCA CAT GGA ATT GTA
       Ala Ala Cys Ile Asp Glu Lys Thr Val Thr Trp Asn Cys Thr 406

1219 CTG GAC CAT TTC TAA ATT TAG GCA GGT GCG ATA AAT CCC AGA
       Gly Pro Phe Leu Asn Leu Gly Arg Cys Asp Lys Ser Gln Lys 420

1261 AGA AGA CTG GTT ATA CTG ATA TAG CTG ATC AGT TGA AAG GTT
       Lys Thr Gly Tyr Thr Asp Ile Ala Asp Gln Leu Lys Gly Leu 434

1303 TGG GTG CGA TAT CAG TGC TAG ATA TTC CAA TGA TAA AAA AGA
       Gly Ala Ile Ser Val Leu Asp Ile Pro MET Ile Lys Lys Ile 448
```

Figure 8 continued.

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1345 TCC CAG ATG AGA TAT CTG GAT CAC TGA AGT ATA TAG ATG ATT
       Pro Asp Glu Ile Ser Gly Ser Leu Lys Tyr Ile Asp Asp Leu 462

1387 TAG AGA CAT ATC ATG AAC AAT TGA CTG CTG AAT ATG CCT TCC
       Glu Thr Tyr His Glu Gln Leu Thr Ala Glu Tyr Ala Phe Leu 476

1429 TTA CTA GAT ACT GCG ATT ATT ATA CAC AGT ACA CTG ATA ATT
       Thr Arg Tyr Cys Asp Tyr Tyr Thr Gln Tyr Thr Asp Asn Ser 490

1471 CAG TTT ATA GTC AAA CTG TGT GGA GGA CTT ATT TGC GAT CTC
       Val Tyr Ser Gln Thr Val Trp Arg Thr Tyr Leu Arg Ser His 504

1513 ATG GTT TTC GGT GCA TGT GTC GTT TAT CCA AAT CAA CAT TTC
       Gly Phe Arg Cys MET Cys Arg Leu Ser Lys Ser Thr Phe Leu 518

1555 TGC AGT GTG TAA AAC ATG GTG ATA AGT GCA ATA GTG CTA AGT
       Gln Cys Val Lys His Gly Asp Lys Cys Asn Ser Ala Lys Trp 532

1597 GGG ATT TCG CAA ATG AAA TGA AAA CAT TTT ATA CTG GTC AGA
       Asp Phe Ala Asn Glu MET Lys Thr Phe Tyr Thr Gly Gln Lys 546

1639 AAG CAA AAT TTA ACA AAG ATT TAA ATT TGG CAT TAT TGG TAT
       Ala Lys Phe Asn Lys Asp Leu Asn Leu Ala Leu Leu Val Leu 560

1681 TAC ACA AAG CAT TTA GAG GAA CTG GCA CTT CAT ATA TTG CAG
       His Lys Ala Phe Arg Gly Thr Gly Thr Ser Tyr Ile Ala Glu 574

1723 AAA GTA TGG CCC GAA AAG ATA ACA GGT CTA TGA CAC ATT TTG
       Ser MET Ala Arg Lys Asp Asn Arg Ser MET Thr His Phe Ala 588

1765 CCT TAG AGA TAA AGA AAA AAT TCC CAA CAA ATG CGC TAT TGA
       Leu Glu Ile Lys Lys Lys Phe Pro Thr Asn Ala Leu Leu Thr 602
```

Figure 8 continued.

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1807 CAG CAT TGA TAG ATT ACA TTT CTT ATT TAC AAT CAC TTG CAG
       Ala Leu Ile Asp Tyr Ile Ser Tyr Leu Gln Ser Leu Ala Glu 616

1849 AGA TGT CTA CTT TTG AAC TTG ATG AGG ATT GGG AAG ATT TCA
       MET Ser Thr Phe Glu Leu Asp Glu Asp Trp Glu Asp Phe Lys 630

1891 AAT ACA ACC TGA AGC CAA CAG AAT CGC CTC AAC TTA GAT CAC
       Tyr Asn Leu Lys Pro Thr Glu Ser Pro Gln Leu Arg Ser His 644

1933 ATA GAA CTG ATC AGT ATA ATT TTA AAA ATG CAG ATA GTA ATA
       Arg Thr Asp Gln Tyr Asn Phe Lys Asn Ala Asp Ser Asn Ser 658

1975 GCA ATG TTA AAG TCT GCA AAA ATA TTC AAA AAG TGT CAT GTA
       Asn Val Lys Val Cys Lys Asn Ile Gln Lys Val Ser Cys Ile 672

2017 TTT CAC CTA GGT CTA GAG CTA AAT TTG AAA ATA TAA TAG CAT
        Ser Pro Arg Ser Arg Ala Lys Phe Glu Asn Ile Ile Ala Cys 686

2059 GTG GAG AAC ATG CAA ACC CAT CAG TAT ATA TGA TGC CCA ATG
       Gly Glu His Ala Asn Pro Ser Val Tyr MET MET Pro Asn Val 700

2101 TCA GTA TTT ACC AAT CTA ATT CAG AGA GGA GCC ACT ACT GTA
       Ser Ile Tyr Gln Ser Asn Ser Glu Arg Ser His Tyr Cys Ile 714

2143 TAG CTG ACT CAC ATT GTT TAG AAG AAT ATG AAG TAG TGG AAA
       Ala Asp Ser His Cys Leu Glu Glu Tyr Glu Val Val Glu Thr 728

2185 CAG AAC TCT TAA ATG CAT TGA AAA AAT CTA AAT GTT GGG CTA
       Glu Leu Leu Asn Ala Leu Lys Lys Ser Lys Cys Trp Ala Lys 742

2227 AAG AAA TGG ATG ATA TTG TTC TGC ACA AAC AAG TTG ATG GCT
       Glu MET Asp Asp Ile Val Leu His Lys Gln Val Asp Gly Leu 756
```

Figure 8 continued.

```
2269 TAA GGA GTT GTA GAA TGA AAG ATA CTG GGA ACT GTA ACG TTC
       Arg Ser Cys Arg MET Lys Asp Thr Gly Asn Cys Asn Val Gln 770

2311 AAG GAA ATG ATT GGA CTA TTG TTT TAT GTG AGA ATG GCA ATT
       Gly Asn Asp Trp Thr Ile Val Leu Cys Glu Asn Gly Asn Tyr 784

2353 ATT ATT ATT CAG AAG CTC ACA GAG ACT ATG ATA AAG ATC AAG
       Tyr Tyr Ser Glu Ala His Arg Asp Tyr Asp Lys Asp Gln Asp 798

2395 ATG TAG GTC ACT TTT GTC TCA GCC CT
       Val Gly His Phe Cys Leu Ser Pro 806
```

Figure 8 continued.

```
  1 TAG TGT ACT CTA AAG GAT GTG GGA ATG TTC AAA AAA TAA ATG
      Val Tyr Ser Lys Gly Cys Gly Asn Val Gln Lys Ile Asn Gly 14

 43 GGA CAA ACT ATG GCA ATG GAT TAC CAA AGT TTG ATT ATT TAT
      Thr Asn Tyr Gly Asn Gly Leu Pro Lys Phe Asp Tyr Leu Cys 28

 85 GCC ATT TAG CAA GTA GAA AAG AAG TTA TAC TAA GGA AAT GTT
      His Leu Ala Ser Arg Lys Glu Val Ile Leu Arg Lys Cys Phe 42

127 TCG ATA ACG ATT ACC AAG CTT GCA AAT TTC TAC AAA GTC CTC
      Asp Asn Asp Tyr Gln Ala Cys Lys Phe Leu Gln Ser Pro Pro 56

169 CAA GCT ATC GTT TAG AGG AGC ATC AAG GCA CAG TAA CAG TAA
      Ser Tyr Arg Leu Glu Glu His Gln Gly Thr Val Thr Val Ile 70

211 TAG ATT ATA AAA AAA TTC TAG GCA CTA TAA AGA TGA AGG CTA
      Asp Tyr Lys Lys Ile Leu Gly Thr Ile Lys MET Lys Ala Ile 84

253 TAT TAG GAG ATG TGA AAT ATA AGA ATT ACC AGA GTA ATA TTG
      Leu Gly Asp Val Lys Tyr Lys Asn Tyr Gln Ser Asn Ile Glu 98

295 AAA TCA ATG CTG AAG GAA CAT GTG CTG GAT GTA TAA ATT GTT
      Ile Asn Ala Glu Gly Thr Cys Ala Gly Cys Ile Asn Cys Phe 112

337 TTG AAA ATA TTC ACT GTG AAT TTA CTA TAC ATA CCA CAG TAG
      Glu Asn Ile His Cys Glu Phe Thr Ile His Thr Thr Val Glu 126

379 AAG CAA GTT GCC CTA TAG TGA CAG ACT GTG TTT CAT TCC ATG
      Ala Ser Cys Pro Ile Val Thr Asp Cys Val Ser Phe His Asp 140

421 ACA GAA TAC TGA TCA CAC CAG ACG AAC ATA AAT ATG CAT TAA
      Arg Ile Leu Ile Thr Pro Asp Glu His Lys Tyr Ala Leu Lys 154
```

Figure 9. Nucleotide and deduced amino acid sequences of M21#8.

463 AAT TGT GTA GTC AAG AAA AAC CTA AAC TGT CTC TAA AAT TTA
Leu Cys Ser Gln Glu Lys Pro Lys Leu Ser Leu Lys Phe Lys 168

505 AGA TCT GTA ACA CAA ATA TTG ATG AGT CTA TGA CTG TTG TGG
Ile Cys Asn Thr Asn Ile Asp Glu Ser MET Thr Val Val Asp 182

547 ATT CCA AGC CGA TCC TAG AGC TTG CAC CAG TGA TCA AAC TAC
Ser Lys Pro Ile Leu Glu Leu Ala Pro Val Ile Lys Leu His 196

589 ATA CAT TAG AGA AAG AGA TGA AAG ATG CAA ATT GGA TGT GCA
Thr Leu Glu Lys Glu MET Lys Asp Ala Asn Trp MET Cys Arg 210

631 GGG TTC GAG ATG AAC CCT TCC AGG TAA TAT TGG AAC CGT TTA
Val Arg Asp Glu Pro Phe Gln Val Ile Leu Glu Pro Phe Lys 224

673 AAA ACC TTT TTG GCT CTT ACA TTG GAA TAT TTT ATA CGG GTA
Asn Leu Phe Gly Ser Tyr Ile Gly Ile Phe Tyr Thr Gly Ile 238

715 TAT TAT GTA TTG TAT GTC TCC TTA TTG TTA TTT ATA TTA TAC
Leu Cys Ile Val Cys Leu Leu Ile Val Ile Tyr Ile Ile Leu 252

757 TGC CTA TTT GTT TCA AGT TGA GGG ACA CTC TAA GGC AAC ATG
Pro Ile Cys Phe Lys Leu Arg Asp Thr Leu Arg Gln His Glu 266

799 AGG ATG CAT ATA AGA AGG AGA TGA AAA TAA GAT GTT CGA ATA
Asp Ala Tyr Lys Lys Glu MET Lys Ile Arg Cys Ser Asn Lys 280

841 AGC ATG GGA TGT CAG CAT AGG TTT AAA TAG TTT TTG GGT TTT
His Gly MET Ser Ala TER Val TER Ile Val Phe Gly Phe Tyr 294

Figure 9 continued.

883 ATG GGG GTG GGG TCA AAC AAA ACT ACT AAA TTT ATT TTA GAA
Gly Gly Gly Val Lys Gln Asn Tyr TER Ile Tyr Phe Arg Ile 308

925 TCA TTT AAA TAT ACT TGG TAG CAC ACT ACT
Ile TER Ile Tyr Leu Val Ala His Tyr 317

Figure 9 continued.

Ala A: 46 (5.7%)	Arg R: 31 (3.8%)	Asn N: 37 (4.6%)	Asp D: 47 (5.8%)
Gln Q: 25 (3.1%)	Glu E: 40 (5.0%)	Gly G: 36 (4.5%)	His H: 24 (3.0%)
Iso I: 59 (7.3%)	Leu L: 70 (8.7%)	Lys K: 57 (7.1%)	Met M: 22 (2.7%)
Phe F: 34 (4.2%)	Pro P: 20 (2.5%)	Ser S: 60 (7.4%)	Thr T: 56 (6.9%)
Cys C: 40 (5.0%)	Trp W: 11 (1.4%)	Tyr Y: 47 (5.8%)	Val V: 44 (5.5%)

Ala A: 11 (3.9%)	Arg R: 9 (3.2%)	Asn N: 15 (5.3%)	Asp D: 14 (4.9%)
Gln Q: 8 (2.8%)	Glu E: 18 (6.3%)	Gly G: 14 (4.9%)	His H: 9 (3.2%)
Iso I: 29 (10.2%)	Leu L: 25 (8.8%)	Lys K: 28 (9.8%)	Met M: 6 (2.1%)
Phe F: 12 (4.2%)	Pro P: 11 (3.9%)	Ser S: 14 (4.9%)	Thr T: 15 (5.3%)
Cys C: 17 (6.0%)	Trp W: 1 (0.4%)	Tyr Y: 13 (4.6%)	Val V: 16 (5.6%)

Ala A: 16 (3.1%)	Arg R: 23 (4.4%)	Asn N: 37 (7.1%)	Asp D: 40 (7.6%)
Gln Q: 19 (3.6%)	Glu E: 37 (7.1%)	Gly G: 16 (3.1%)	His H: 12 (2.3%)
Iso I: 46 (8.8%)	Leu L: 49 (9.4%)	Lys K: 43 (8.2%)	Met M: 17 (3.2%)
Phe F: 31 (5.9%)	Pro P: 17 (3.2%)	Ser S: 38 (7.3%)	Thr T: 28 (5.3%)
Cys C: 7 (1.3%)	Trp W: 8 (1.5%)	Tyr Y: 18 (3.4%)	Val V: 22 (4.2%)

Figure 10. Amino acid compositions of the deduced amino acid sequences of M35#10 (top), M21#8 (middle) and L22#9 (bottom).

cysteine content of 5 to 6% is roughly twice that of an average protein (Klapper, 1977).

Five potential N-linked glycosylation sites are present in the M35#10 amino acid sequence and 2 are present in the M21#8 sequence. A dinucleotide analysis of amino acid codons in the M35#10 and M21#8 sequences was executed and the results are shown in Figures 11 and 12, respectively. Examination of the results reveals strong discrimination against the CG dinucleotide in the codons for all five amino acids (serine, threonine, proline, alanine and arginine) in which it occurs. A comparative summary of various structural features of Bunyavirus genus M segment RNAs and their encoded polyproteins is presented in Table 6. There are no notable differences between INK and the other 4 viruses. The high cysteine content of the polyproteins and the near total conservation of position of these cysteine residues in the polyprotein primary structures are of interest in view of the role played by cysteine in the generation of higher order protein structure.

Codon Usage

TTT-Phe	17(2.1%)	TCT-Ser	5(0.6%)	TAT-Tyr	17(2.1%)	TGT-Cys	14(1.7%)
TTC-Phe	14(1.7%)	TCC-Ser	5(0.6%)	TAC-Tyr	9(1.1%)	TGC-Cys	13(1.6%)
TTA-Leu	24(3.0%)	TCA-Ser	15(1.9%)	TAA-TER	22(2.7%)	TGA-TER	18(2.2%)
TTG-Leu	20(2.5%)	TCG-Ser	3(0.4%)	TAG-TER	22(2.7%)	TGG-Trp	11(1.4%)
CTT-Leu	10(1.2%)	CCT-Pro	3(0.4%)	CAT-His	28(3.5%)	CGT-Arg	1(0.1%)
CTC-Leu	5(0.6%)	CCC-Pro	2(0.2%)	CAC-His	12(1.5%)	CGC-Arg	3(0.4%)
CTA-Leu	19(2.4%)	CCA-Pro	4(0.5%)	CAA-Gln	20(2.5%)	CGA-Arg	3(0.4%)
CTG-Leu	23(2.9%)	CCG-Pro	1(0.1%)	CAG-Gln	26(3.2%)	CGG-Arg	3(0.4%)
ATT-Ile	32(4.0%)	ACT-Thr	14(1.7%)	AAT-Asn	29(3.6%)	AGT-Ser	10(1.2%)
ATC-Ile	9(1.1%)	ACC-Thr	7(0.9%)	AAC-Asn	9(1.1%)	AGC-Ser	3(0.4%)
ATA-Ile	42(5.2%)	ACA-Thr	17(2.1%)	AAA-Lys	24(3.0%)	AGA-Arg	19(2.4%)
ATG-MET	33(4.1%)	ACG-Thr	2(0.2%)	AAG-Lys	25(3.1%)	AGG-Arg	3(0.4%)
GTT-Val	12(1.5%)	GCT-Ala	4(0.5%)	GAT-Asp	7(0.9%)	GGT-Gly	11(1.4%)
GTC-Val	8(1.0%)	GCC-Ala	2(0.2%)	GAC-Asp	2(0.2%)	GGC-Gly	2(0.2%)
GTA-Val	15(1.9%)	GCA-Ala	13(1.6%)	GAA-Glu	6(0.7%)	GGA-Gly	13(1.6%)
GTG-Val	22(2.7%)	GCG-Ala	4(0.5%)	GAG-Glu	9(1.1%)	GGG-Gly	6(0.7%)

Dinucleotide Analysis: Reading 2 bases and skipping 1

AA:	87 (10.8%)	AC:	40 (5.0%)	AG:	35 (4.3%)	AT:	116 (14.4%)
CA:	86 (10.7%)	CC:	10 (1.2%)	CG:	10 (1.2%)	CT:	58 (7.2%)
GA:	24 (3.0%)	GC:	23 (2.9%)	GG:	32 (4.0%)	GT:	57 (7.1%)
TA:	70 (8.7%)	TC:	28 (3.5%)	TG:	56 (6.9%)	TT:	75 (9.3%)

Figure 11. Codon usage and dinucleotide analysis of M35#10 nucleotide sequence.

Codon Usage

TTT-Phe	8(2.8%)	TCT-Ser	4(1.4%)	TAT-Tyr	9(3.2%)	TGT-Cys	13(4.6%)
TTC-Phe	4(1.4%)	TCC-Ser	1(0.4%)	TAC-Tyr	4(1.4%)	TGC-Cys	4(1.4%)
TTA-Leu	8(2.8%)	TCA-Ser	2(0.7%)	TAA-TER	0(0.0%)	TGA-TER	0(0.0%)
TTG-Leu	3(1.1%)	TCG-Ser	1(0.4%)	TAG-TER	0(0.0%)	TGG-Trp	1(0.4%)
CTT-Leu	3(1.1%)	CCT-Pro	4(1.4%)	CAT-His	8(2.8%)	CGT-Arg	1(0.4%)
CTC-Leu	1(0.4%)	CCC-Pro	1(0.4%)	CAC-His	1(0.4%)	CGC-Arg	0(0.0%)
CTA-Leu	7(2.5%)	CCA-Pro	4(1.4%)	CAA-Gln	6(2.1%)	CGA-Arg	1(0.4%)
CTG-Leu	3(1.1%)	CCG-Pro	2(0.7%)	CAG-Gln	2(0.7%)	CGG-Arg	0(0.0%)
ATT-Ile	10(3.5%)	ACT-Thr	4(1.4%)	AAT-Asn	11(3.9%)	AGT-Ser	5(1.8%)
ATC-Ile	5(1.8%)	ACC-Thr	1(0.4%)	AAC-Asn	4(1.4%)	AGC-Ser	1(0.4%)
ATA-Ile	14(4.9%)	ACA-Thr	9(3.2%)	AAA-Lys	18(6.3%)	AGA-Arg	3(1.1%)
ATG-MET	6(2.1%)	ACG-Thr	1(0.4%)	AAG-Lys	10(3.5%)	AGG-Arg	4(1.4%)
GTT-Val	6(2.1%)	GCT-Ala	4(1.4%)	GAT-Asp	10(3.5%)	GGT-Gly	1(0.4%)
GTC-Val	0(0.0%)	GCC-Ala	0(0.0%)	GAC-Asp	4(1.4%)	GGC-Gly	4(1.4%)
GTA-Val	5(1.8%)	GCA-Ala	7(2.5%)	GAA-Glu	10(3.5%)	GGA-Gly	6(2.1%)
GTG-Val	5(1.8%)	GCG-Ala	0(0.0%)	GAG-Glu	8(2.8%)	GGG-Gly	3(1.1%)

Dinucleotide Analysis: Reading 2 bases and skipping 1

AA:	43 (15.1%)	AC:	15 (5.3%)	AG:	13 (4.6%)	AT:	35 (12.3%)
CA:	17 (6.0%)	CC:	11 (3.9%)	CG:	2 (0.7%)	CT:	14 (4.9%)
GA:	32 (11.2%)	GC:	11 (3.9%)	GG:	14 (4.9%)	GT:	16 (5.6%)
TA:	13 (4.6%)	TC:	8 (2.8%)	TG:	18 (6.3%)	TT:	23 (8.1%)

Figure 12. Codon usage and dinucleotide analysis of M21#8 nucleotide sequence.

Table 6. Comparison of the M segment of Inkoo, La Crosse, Snowshoe hare, Germiston and Bunyamwera viruses.

Virus	Size (bases)			Predicted Polyprotein Characteristics			Ratio Conserved/ Nonconserved amino acid sites[3]	
	vRNA	5'NC	3'NC	aa[1]	glycos[2]	%Cys	cys	pro
INK	4500+	?	98	1091+	7+	5.2	----	----
LAC	4526	61	141	1441	5	4.8	51/4	26/4
SSH	4537	61	142	1441	5	4.9	50/5	27/3
BUN	4458	56	103	1433	4	4.9	51/4	13/17
GER	4534	58	164	1437	7	4.9	46/9	11/19

[1] amino acid number
[2] potential glycosylation sites
[3] Inkoo vs other viruses

The M35#10 and M21#8 nucleotide and deduced amino acid sequences were compared for conservation of sequence with the published sequences for 4 other viruses in the genus Bunyavirus. These viruses were LAC (Grady et al., 1987) and SSH (Eshita and Bishop, 1984), members of the California serogroup as is INK, and Bunyamwera (Lees et al., 1986) and Germiston (Pardignon et al., 1988), members of the Bunyamwera serogroup. The results, expressed as total percentage sequence conservation, are shown in Table 7. Examination of the results reveals that LAC and SSH viruses are very closely related and that INK virus is also closely related to them. Bunyamwera (BUN) and Germiston (GER) viruses appear to be less closely related to each other than are the three viruses in the California serogroup. On the basis of amino acid sequence conservation, INK virus appears to be less closely related to BUN and GER viruses than are LAC and SSH viruses. When members of the two serogroups are compared to each other, much greater conservation of nucleotide than of amino acid sequence is evident.

Table 7. Comparison of M segment nucleotide and deduced amino acid sequence conservation[1] among bunyaviruses.

Comparison	Nucleotide	Amino acid
INK v LAC	68	68
INK v SSH	69	69
INK v BUN	59	39
INK v GER	56	36
SSH v LAC	79	89
SSH v BUN	55	43
SSH v GER	--	44
GER v BUN	60	61

[1] Expressed as total percentage sequence identity.
Inkoo = INK, La Crosse = LAC, Snowshoe hare = SSH, Bunyamwera = BUN, Germiston = GER

Dot matrix plots of M segment nucleotide and amino acid sequence comparisons among LAC, SSH, INK and BUN viruses are shown in Figures 13 to 20. The high degree of conservation of nucleotide and amino acid sequence throughout the M segment between LAC and SSH viruses is evident in Figures 13 and 14 respectively. Comparisons of SSH and BUN viruses (Figures 15 and 16), SSH and INK viruses (Figures 17 and 18) and INK and BUN viruses (Figures 19 and 20) reveals that sequence identities are concentrated in the G2 protein region and the carboxy terminal portion of the G1 protein region. Sequence conservation is weak in the amino terminal portion of the G1 protein region and is weakest in the NsM region.

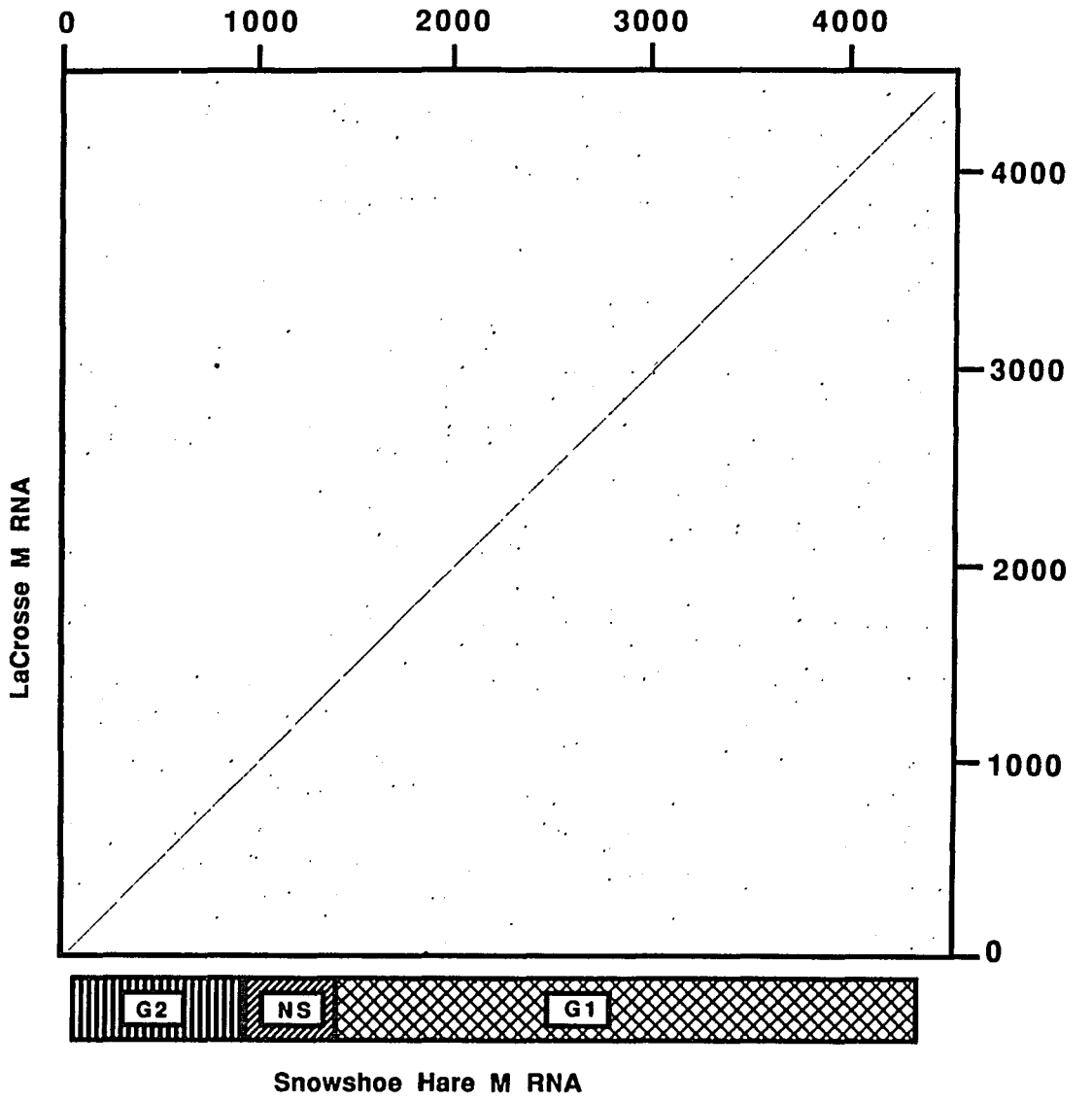

Figure 13. Dot matrix plot of Snowshoe hare and La Crosse virus M segment nucleotide identities. Number of nucleotides from the 5' end of the antigenome RNAs are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom.

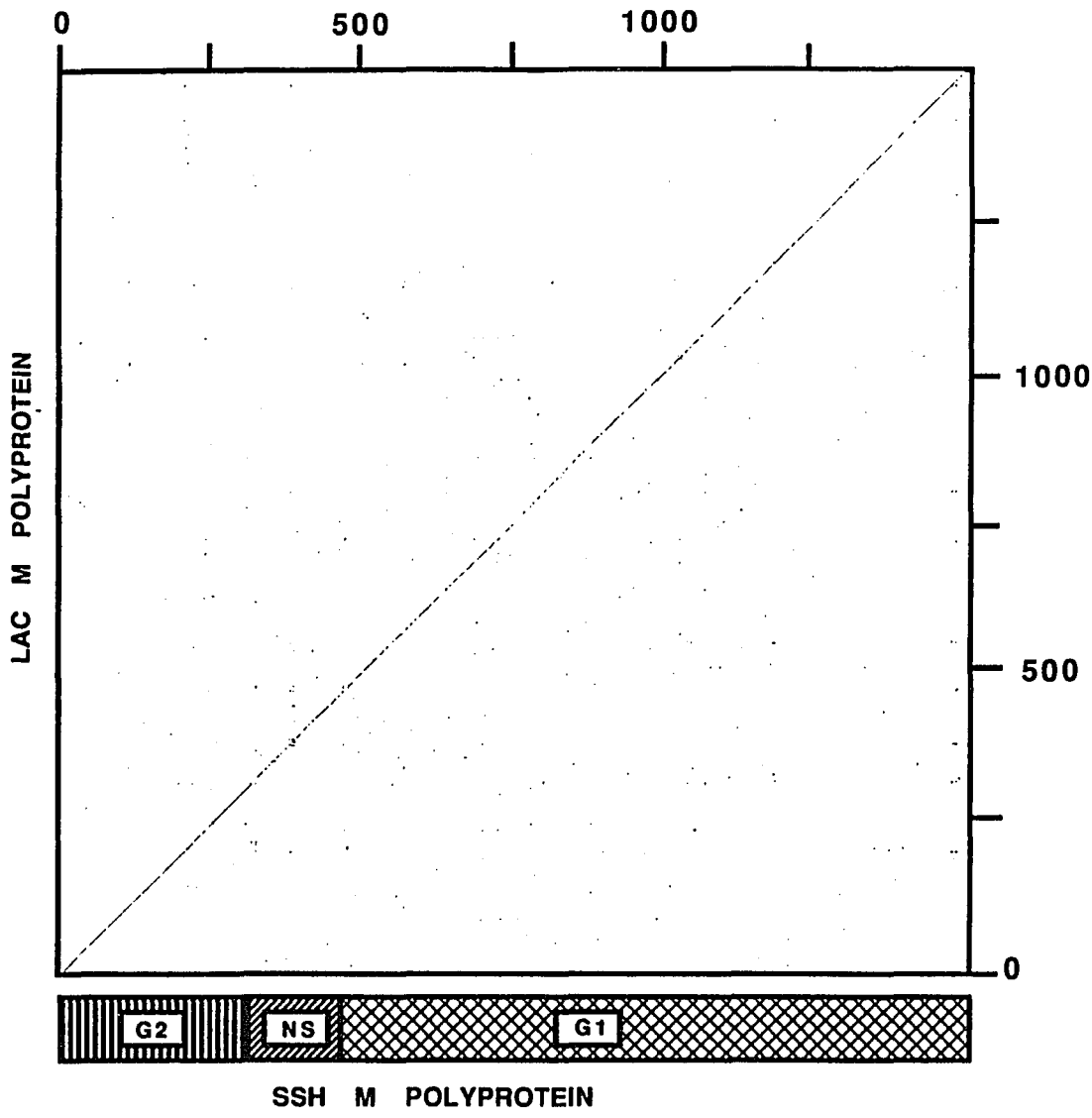

Figure 14. Dot matrix plot of Snowshoe hare and La Crosse virus M segment polyprotein identities. Number of amino acids from the amino termini are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom.

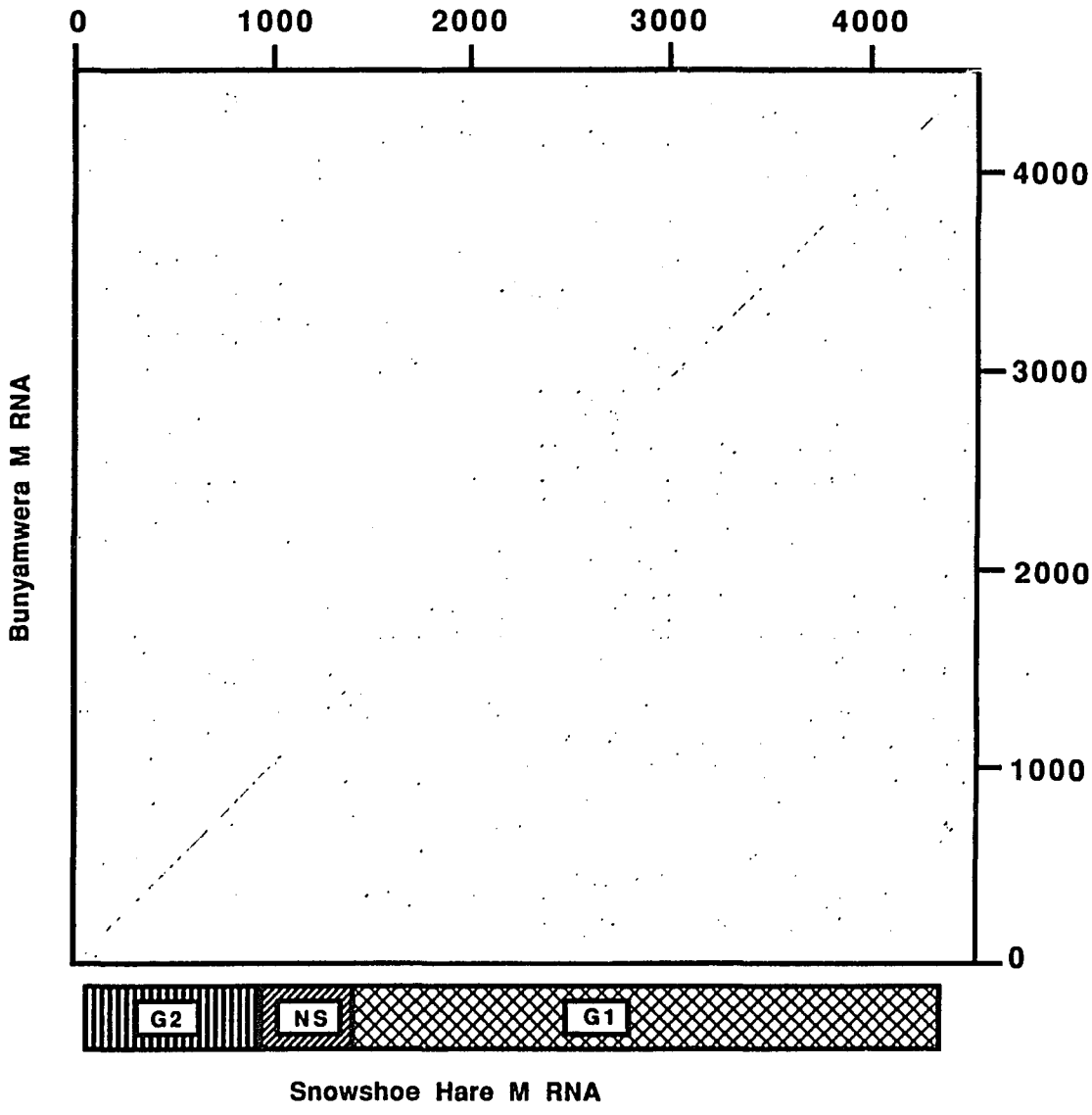

Figure 15. Dot matrix plot of Snowshoe hare and Bunyamwera virus M segment nucleotide identities. Number of nucleotides from the 5' end of the antigenome RNAs are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom.

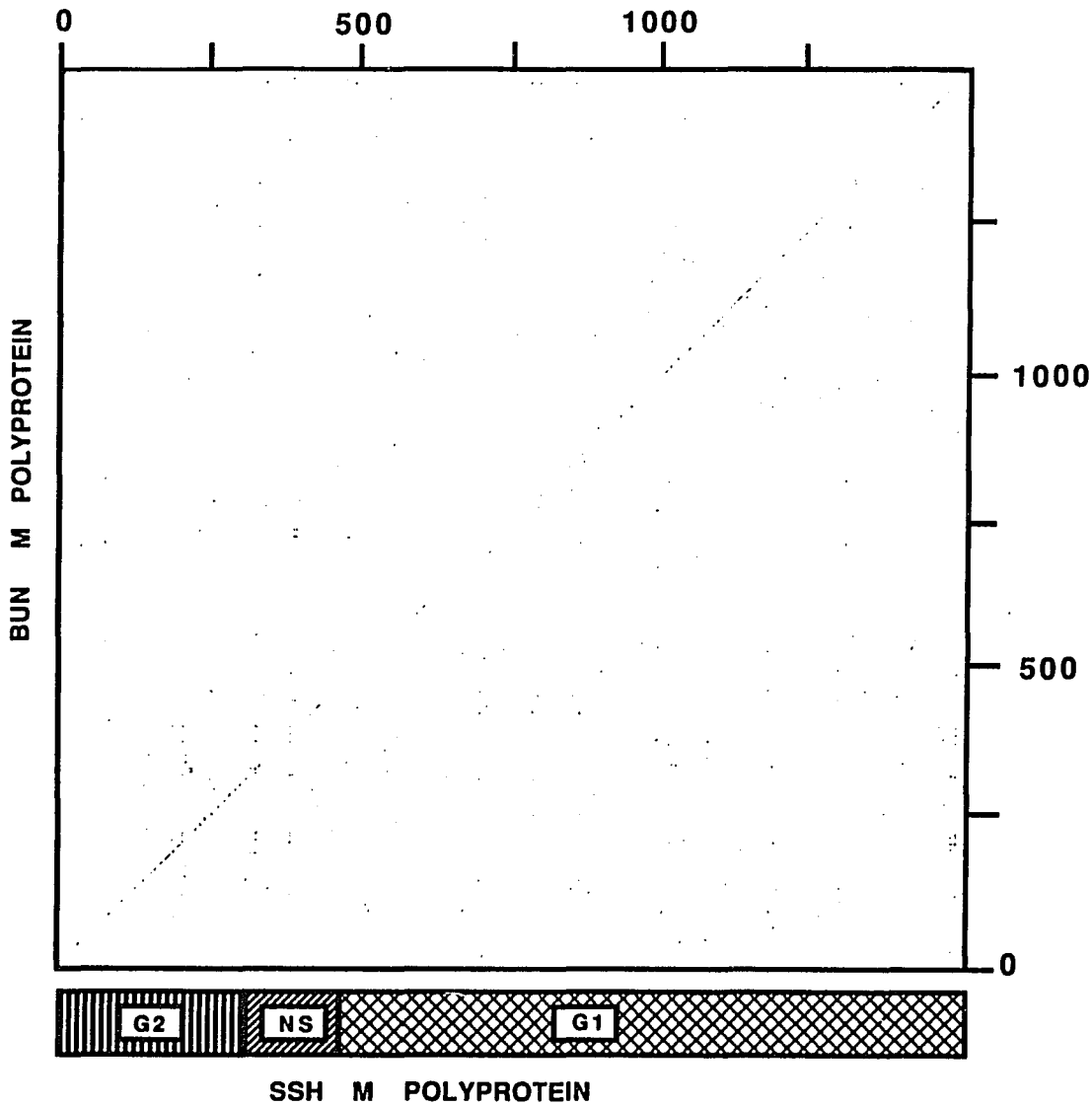

Figure 16. Dot matrix plot of Snowshoe hare and Bunyamwera virus M segment polyprotein identities. Number of amino acids from the amino termini are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom.

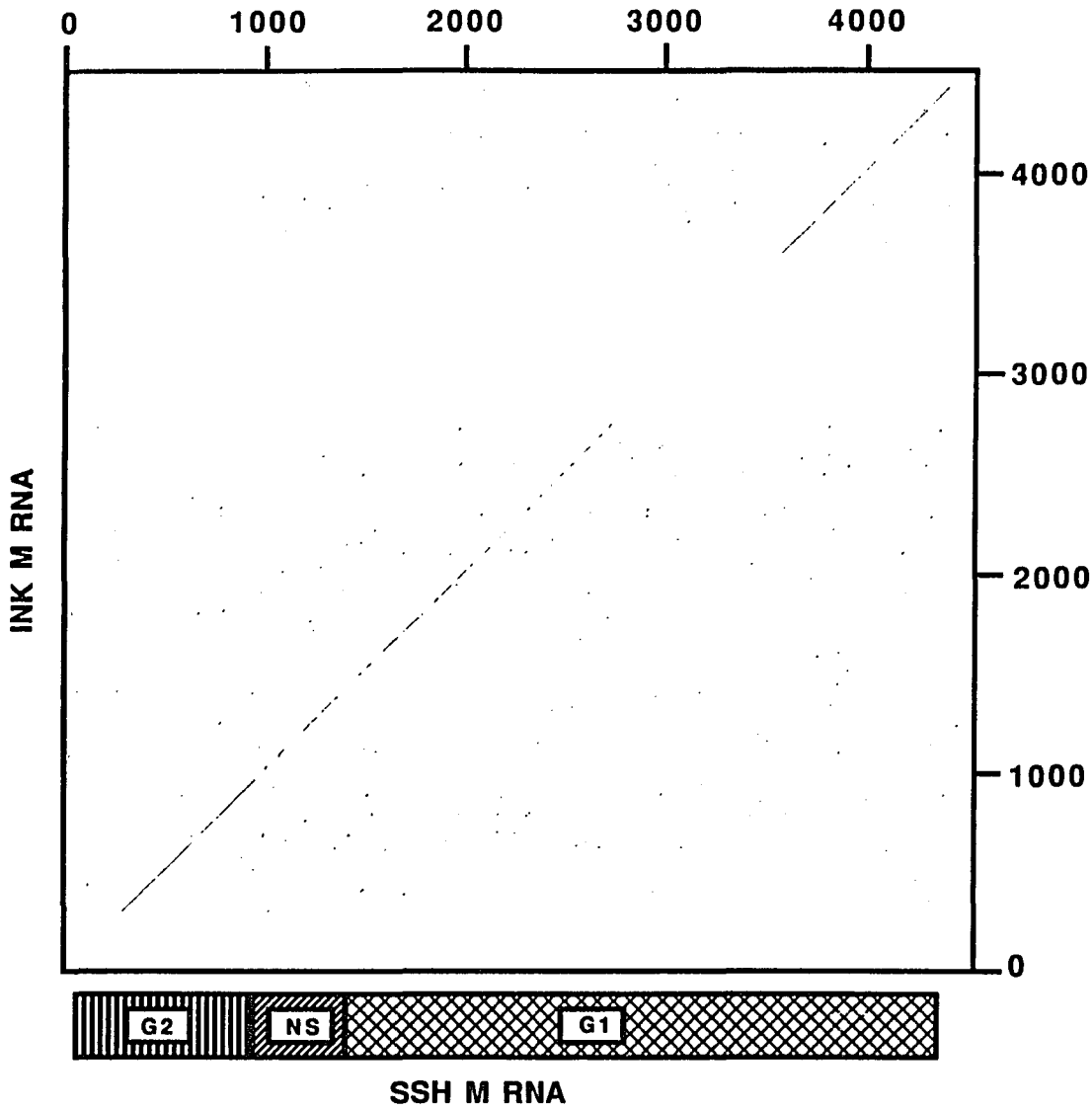

Figure 17. Dot matrix plot of Snowshoe hare and Inkoo virus M segment nucleotide identities. Number of nucleotides from the 5' end of the antigenome RNAs are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom. Approximately 330 nucleotides at the 5' end and 800 nucleotides beginning at position 2,750 of the Inkoo sequence have not yet been obtained.

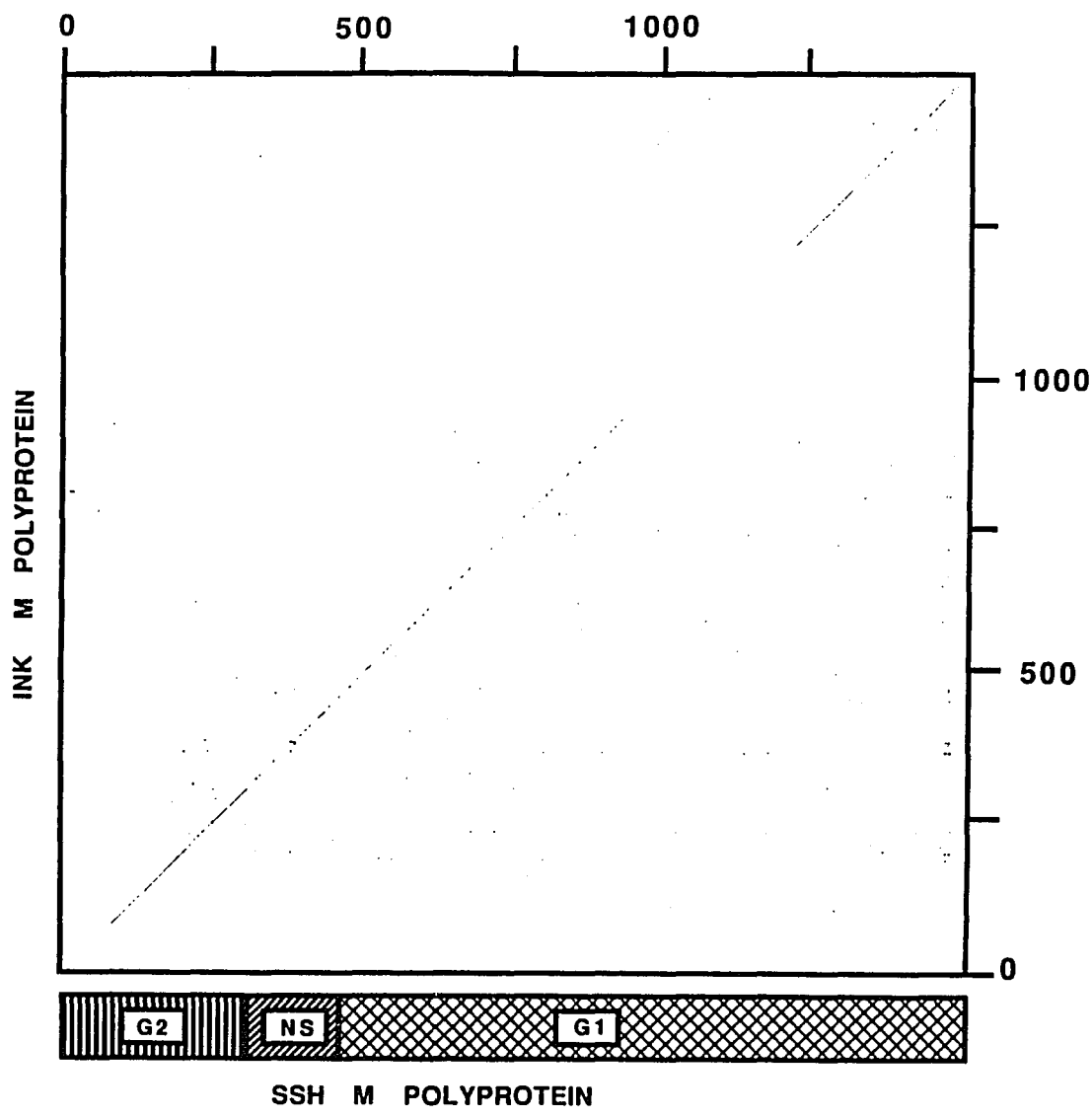

Figure 18. Dot matrix plot of Snowshoe hare and Inkoo virus M segment polyprotein identities. Number of amino acids from the amino termini are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom. Approximately 90 amino acids at the amino terminus and 270 amino acids beginning at position 894 of the Inkoo sequence have not yet been obtained.

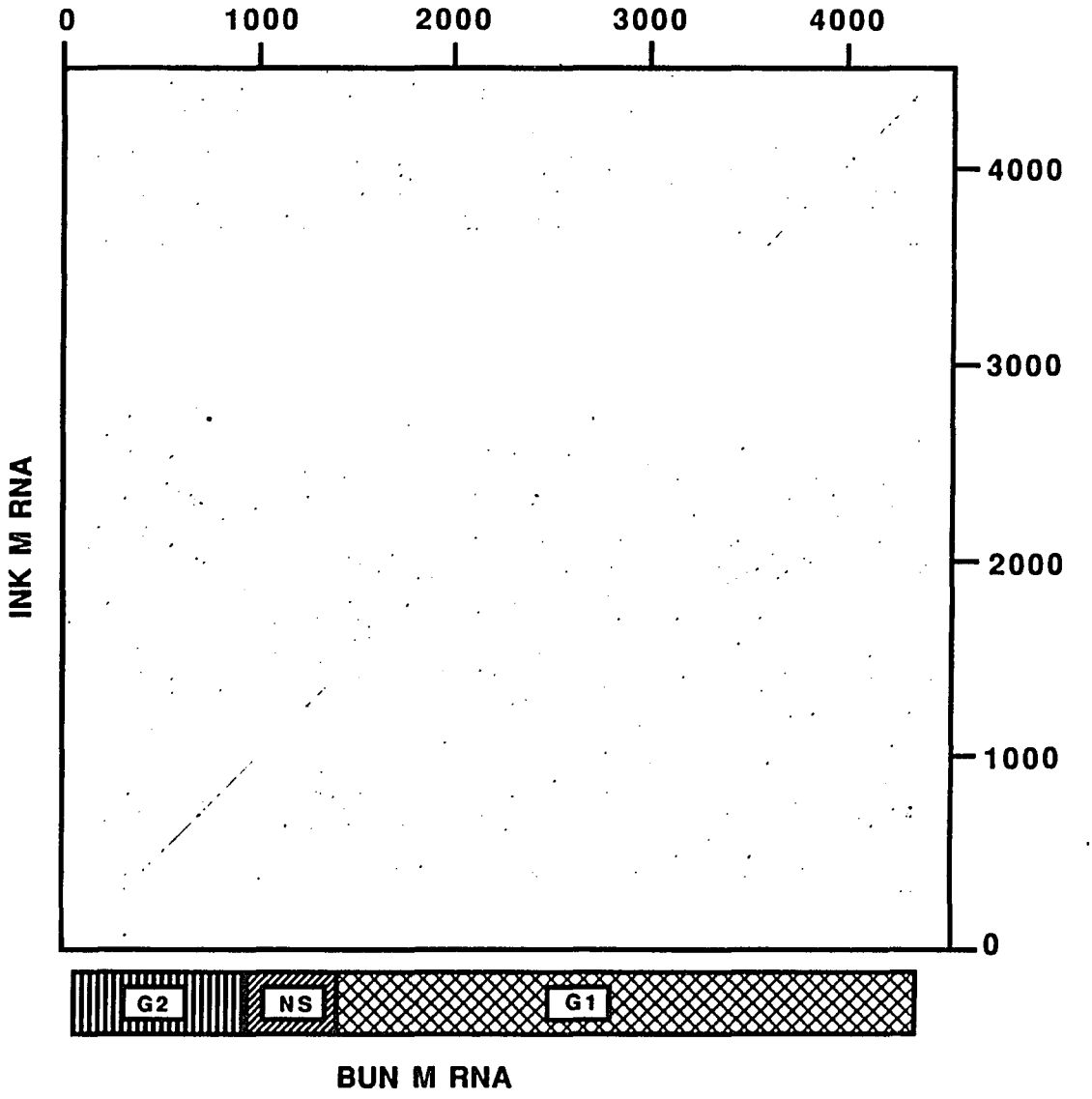

Figure 19. Dot matrix plot of Inkoo and Bunyamwera virus M segment nucleotide identities. Number of nucleotides from the 5' end of the antigenome RNAs are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom (based on known coding strategy of Snowshoe hare virus). Approximately 330 nucleotides at the 5' end and 800 nucleotides beginning at position 2,750 of the Inkoo sequence have not yet been obtained.

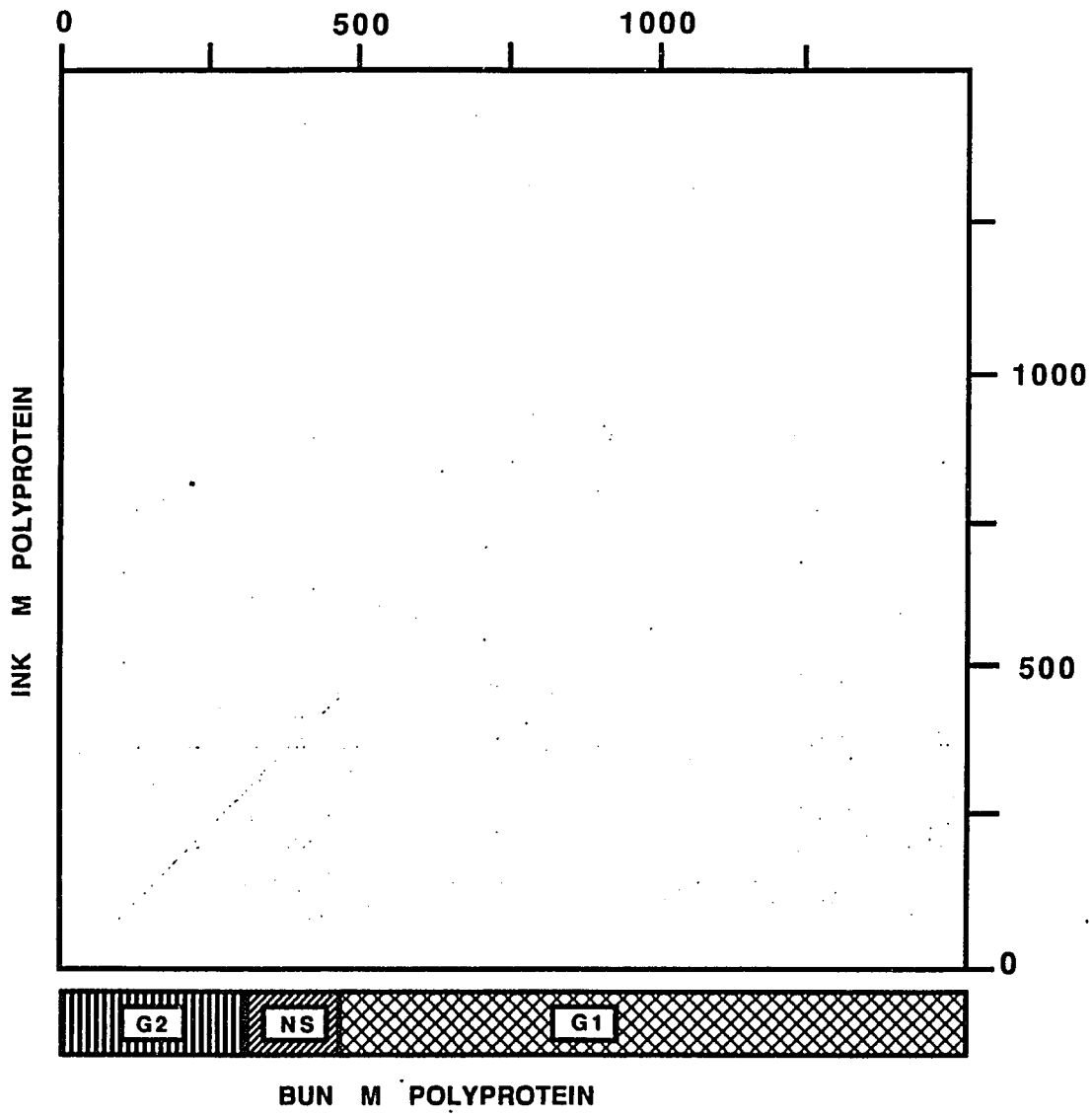

Figure 20. Dot matrix plot of Inkoo and Bunyamwera virus M segment polyprotein identities. Number of amino acids from the amino termini are shown at top and right. Regions encoding the G1 and G2 glycoproteins and the nonstructural or NsM proteins are shown at bottom (based on known coding strategy of Snowshoe hare virus). Approximately 90 amino acids at the amino terminus and 270 amino acids beginning at position 894 of the Inkoo sequence have not yet been obtained.

L Segment

The INK virus L segment RNA encodes the viral RNA-dependent polymerase/replicase protein. The complete sequences of the pINK 220L and 430L cDNA inserts were obtained by sequencing restriction fragments subcloned into the M13 sequencing vectors and by sequencing exonuclease III-generated deletion plasmids of the cDNA inserts cloned into plasmid pBSM13. Restriction fragments obtained from the pINK 220L and 430L cDNA inserts and the extent to which they were sequenced are shown in Figure 21. Efforts to obtain the complete sequence of the pINK 130L cDNA insert were unsuccessful. Overlap analysis of the sequences obtained for the 3 cDNA inserts failed to reveal continuity among them. The nucleotide sequence of the 1,574 base cDNA insert in the pINK 220L plasmid is shown in Figure 22 as the plus or viral complementary-sense cDNA and is designated L22#9. The base composition (as negative-sense viral RNA) is 15.1% G, 18.2% C, 28.6% A, 38.1% U. This cDNA contains an ORF in frame 1 from end to end and encodes 524 amino acids (Figure 22). No ORF greater than 100 amino acids in length was found in any of the 6 possible reading frames of the pINK 430L cDNA insert sequence.

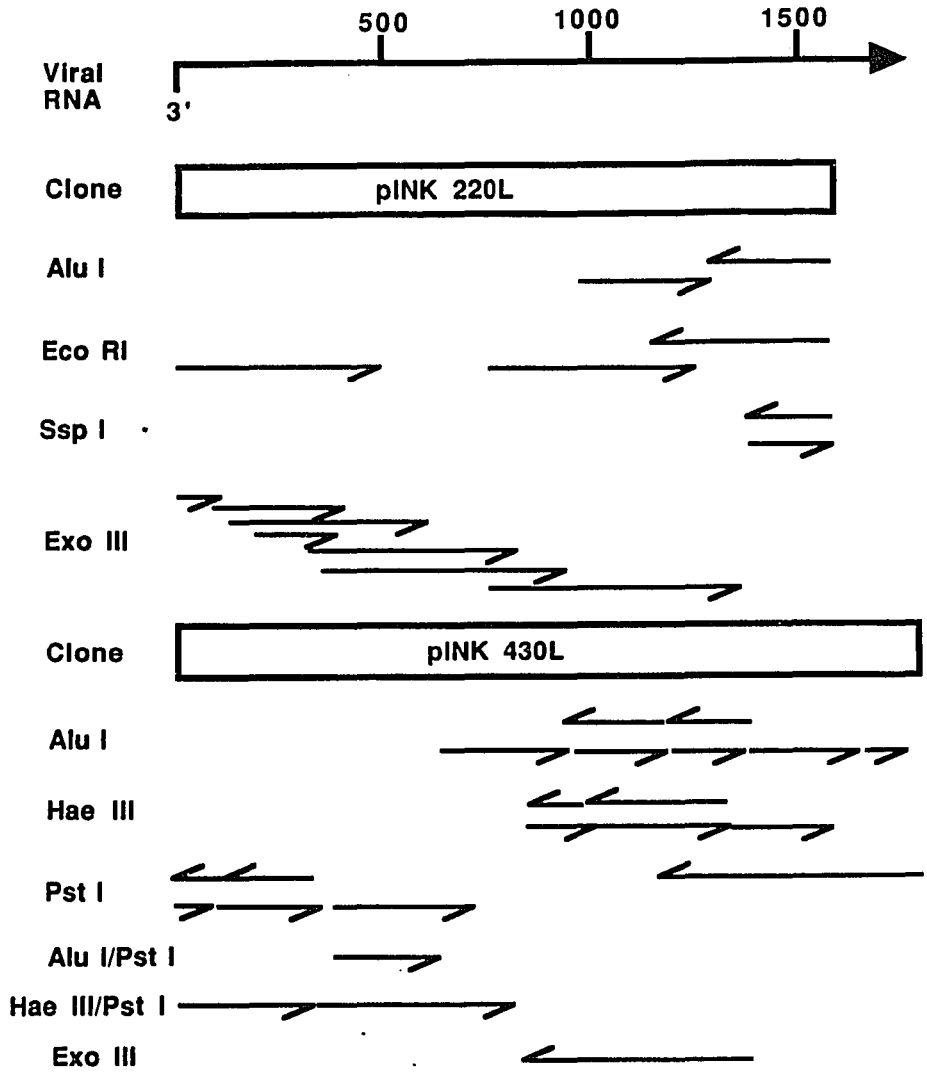

Figure 21. Inkoo virus L segment cDNA clones and sequencing strategy. Subcloned restriction fragments were sequenced as described in the text with arrows indicating direction of sequencing. Multiple clones of each fragment were sequenced. Exo III clones were made by digestion of the cDNA inserts with exonuclease III as described in the text. Numbers above the viral RNA refer to number of nucleotides from the 3' end of the viral RNA. The orientation of the pINK 430 clone with respect to the viral RNA is not known.

```
  1 AGT AGT GTA CTC CTA TTT ACA AAA CTT ACA AAC ACA AAT CGT
    Ser Ser Val Leu Leu Phe Thr Lys Leu Thr Asn Thr Asn Arg 14

 43 TTC CTT GGA ATA ACT ATG GAC AAC ACA GAA TAT CAA CAA TTC
    Phe Leu Gly Ile Thr MET Asp Asn Thr Glu Tyr Gln Gln Phe 28

 85 CTT GCT CGC ATC AAT GCT GCA AGA GAT GCA TGT GTA GCA AAG
    Leu Ala Arg Ile Asn Ala Ala Arg Asp Ala Cys Val Ala Lys 42

127 GAT ATT GAC GTA GAC CTA CTG ATG GCA AGA CAT GAC TAC TTC
    Asp Ile Asp Val Asp Leu Leu MET Ala Arg His Asp Tyr Phe 56

169 GGG AGA GAG TTG TGT AAA TCA TTG AAC ATA GAA TAT AGG AAT
    Gly Arg Glu Leu Cys Lys Ser Leu Asn Ile Glu Tyr Arg Asn 70

211 GAT ATA CCA TTT GTA GAT ATA TTA TTA GAC ATA AAG CCC GAC
    Asp Ile Pro Phe Val Asp Ile Leu Leu Asp Ile Lys Pro Asp 84

253 ATA GAT CCA TTG ACA TTA GAG ATC CCA CAT ATA ACC CCA GAC
    Ile Asp Pro Leu Thr Leu Glu Ile Pro His Ile Thr Pro Asp 98

295 AAC TAC CTA TAT CTG AAT AAT ATC CTA TAT ATC ATA GAT TAT
    Asn Tyr Leu Tyr Leu Asn Asn Ile Leu Tyr Ile Ile Asp Tyr 112

337 AAA GTT TCT GTA TCT AAT GAA AGT AGT GTA ATA ACT ACT AAC
    Lys Val Ser Val Ser Asn Glu Ser Ser Val Ile Thr Thr Asn 126

379 ACA AAG TAT TTT GAA ATG ACA AGA GAC ATA GCA TCT GCG TTG
    Thr Lys Tyr Phe Glu MET Thr Arg Asp Ile Ala Ser Ala Leu 140

421 AAC TTG CAT ATA GAG ATA GTT ATT ATT AGG ATA GAT CCT ATT
    Asn Leu His Ile Glu Ile Val Ile Ile Arg Ile Asp Pro Ile 154
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Figure 22. Nucleotide and deduced amino acid sequences of L22#9.

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463 AGC CGT GAT TTG TAT ATT AGT TCA GAT AGA TTT AAA GAT TTG
    Ser Arg Asp Leu Tyr Ile Ser Ser Asp Arg Phe Lys Asp Leu 168

505 TTC CCA ACA TTA GTG GTT GAT ATT AAT TTT AAC CAA TTT TTT
    Phe Pro Thr Leu Val Val Asp Ile Asn Phe Asn Gln Phe Phe 182

547 GAT TTA AAG CAA GCA TTG TAT GAA AAG TTT GGC GAT GAT GAT
    Asp Leu Lys Gln Ala Leu Tyr Glu Lys Phe Gly Asp Asp Asp 196

589 GAA GAA TTT TTA CTC AAG GTT GCT CAT GGT GAT TTC ACA CTG
    Glu Glu Phe Leu Leu Lys Val Ala His Gly Asp Phe Thr Leu 210

631 ACA GCA CCC TGG TCG AAG ACA GGA TGT CCG CAA GTT TGG CAA
    Thr Ala Pro Trp Ser Lys Thr Gly Cys Pro Gln Val Trp Gln 224

673 CAT CCC ATC TAC AAA GAA TTC AAA ATG AGC ATG CCA ATC CCT
    His Pro Ile Tyr Lys Glu Phe Lys MET Ser MET Pro Ile Pro 238

715 GAG AGG AGA CTT TTT GAA GAA TCT ATG AGA TTT AAT TCA TAT
    Glu Arg Arg Leu Phe Glu Glu Ser MET Arg Phe Asn Ser Tyr 252

757 GAA TCA GAG AGG TGG AAT ACA AAC CTT ATC AGA GTT AGA GAG
    Glu Ser Glu Arg Trp Asn Thr Asn Leu Ile Arg Val Arg Glu 266

799 TAT ACA AAG AAA GAA TAT GAT GAT TTT GTA ACA AAA TCA GCA
    Tyr Thr Lys Lys Glu Tyr Asp Asp Phe Val Thr Lys Ser Ala 280

841 AAG GAA GTT TTC TTA GCT ACA GGG GAT TAC AAA CAG CCA AAT
    Lys Glu Val Phe Leu Ala Thr Gly Asp Tyr Lys Gln Pro Asn 294
```

Figure 22 continued.

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 883 AAA GGC GAA ATT TTG GAA GGC TGG GAG ATG ATG GTA GAG AGA
     Lys Gly Glu Ile Leu Glu Gly Trp Glu MET MET Val Glu Arg 308

 925 GTT CAC CAG CAA AGA GAA GTA AGT AAA TCT ATC CAT GAC CAA
     Val His Gln Gln Arg Glu Val Ser Lys Ser Ile His Asp Gln 322

 967 AAG CCT AGT ATC CAT TTC ATA TGG TCT TCT CAT AAT CCT AAT
     Lys Pro Ser Ile His Phe Ile Trp Ser Ser His Asn Pro Asn 336

1009 AAC AGC AAT AAT GCA ACT TTC AAA TTA ATC TTA TTA TCA AAG
     Asn Ser Asn Asn Ala Thr Phe Lys Leu Ile Leu Leu Ser Lys 350

1051 TCA CTA CAA TCT ATA AAA GGT AAC TCA ACT TAT ACA GAT TCA
     Ser Leu Gln Ser Ile Lys Gly Asn Ser Thr Tyr Thr Asp Ser 364

1093 TTT AGA TCA TTG GGG AAA ATG ATG GAT ATT GGT GAT AAA ATA
     Phe Arg Ser Leu Gly Lys MET MET Asp Ile Gly Asp Lys Ile 378

1135 ACT GAA TAT GAA TCA CAT TGT GAT CAC TTA AAG ACA CAA GCA
     Thr Glu Tyr Glu Ser His Cys Asp His Leu Lys Thr Gln Ala 392

1177 AGG CAA AGC TGG CGA CAA GTT ATG AAC AAA AAG CTA GAG CCA
     Arg Gln Ser Trp Arg Gln Val MET Asn Lys Lys Leu Glu Pro 406

1219 AAA AAG ATA AAT GAT GCT TTA GTC CTT TGG GAA CAA CAG TTC
     Lys Lys Ile Asn Asp Ala Leu Val Leu Trp Glu Gln Gln Phe 420

1261 ATG GTA AAC TCA GAA CTT ATA AAT AAG AAT GAA ATG ATA TTT
     MET Val Asn Ser Glu Leu Ile Asn Lys Asn Glu MET Ile Phe 434

1303 AGA GAT TTC TGT GGG ATA GGT AAG CAC AAG CAA TTT AAA AAC
     Arg Asp Phe Cys Gly Ile Gly Lys His Lys Gln Phe Lys Asn 448
```

Figure 22 continued.

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1345 AAA ATG TTG GAA GAT ATT GAA TTG TCA AAA CCT AAA ATA TTG
     Lys MET Leu Glu Asp Ile Glu Leu Ser Lys Pro Lys Ile Leu 462

1387 GAT TTC AAC GAT GAA AAT ATC TAC TTA TCC AGC CTC ACC ATG
     Asp Phe Asn Asp Glu Asn Ile Tyr Leu Ser Ser Leu Thr MET 476

1429 ATT GAA CAG TGT AAA ATT ATG TTA TCA GAG CAA AAT GGC TTA
     Ile Glu Gln Cys Lys Ile MET Leu Ser Glu Gln Asn Gly Leu 490

1471 AAA AGT AAT AAC TTT ATA CTA GAT GAA TTT GGG AAG AAA ATA
     Lys Ser Asn Asn Phe Ile Leu Asp Glu Phe Gly Lys Lys Ile 504

1513 GGG GAC TGT AAC AAA AAT ACA TTG GAT GTC ATG ACA TCT ATT
     Gly Asp Cys Asn Lys Asn Thr Leu Asp Val MET Thr Ser Ile 518

1555 TTT GAA ACT AGA TTT TGG CA    1574
     Phe Glu Thr Arg Phe Trp  524
```

Figure 22 continued.

The nucleotide sequence of the pINK 220L cDNA insert begins with the known 3' end terminal consensus sequence of bunyaviruses (Clerx-van Haaster et al., 1982). A methionine codon is located in the open reading frame at nucleotide 58 and has flanking nucleotide sequences most favorable for initiation of translation (Kozak, 1978; 1984). Assuming translation initiates at this codon, then the L22#9 sequence encodes the 505 amino terminal amino acids of the INK L segment protein. Amino acid composition analysis of the sequence revealed a cysteine content of only 1.3% (Figure 10) and a relatively high content of acidic residues (7.1% Glu, 7.6% Asp). Dinucleotide analysis of the codons revealed strong discrimination against the CG dinucleotide (Figure 23).

Codon Usage

TTT-Phe	19(3.6%)	TCT-Ser	9(1.7%)	TAT-Tyr	13(2.5%)	TGT-Cys	7(1.3%)
TTC-Phe	12(2.3%)	TCC-Ser	1(0.2%)	TAC-Tyr	5(1.0%)	TGC-Cys	0(0.0%)
TTA-Leu	15(2.9%)	TCA-Ser	14(2.7%)	TAA-TER	0(0.0%)	TGA-TER	0(0.0%)
TTG-Leu	14(2.7%)	TCG-Ser	1(0.2%)	TAG-TER	0(0.0%)	TGG-Trp	8(1.5%)
CTT-Leu	7(1.3%)	CCT-Pro	5(1.0%)	CAT-His	9(1.7%)	CGT-Arg	2(0.4%)
CTC-Leu	3(0.6%)	CCC-Pro	3(0.6%)	CAC-His	3(0.6%)	CGC-Arg	1(0.2%)
CTA-Leu	7(1.3%)	CCA-Pro	8(1.5%)	CAA-Gln	15(2.9%)	CGA-Arg	1(0.2%)
CTG-Leu	3(0.6%)	CCG-Pro	1(0.2%)	CAG-Gln	4(0.8%)	CGG-Arg	0(0.0%)
ATT-Ile	12(2.3%)	ACT-Thr	7(1.3%)	AAT-Asn	21(4.0%)	AGT-Ser	8(1.5%)
ATC-Ile	11(2.1%)	ACC-Thr	2(0.4%)	AAC-Asn	16(3.1%)	AGC-Ser	5(1.0%)
ATA-Ile	23(4.4%)	ACA-Thr	19(3.6%)	AAA-Lys	25(4.8%)	AGA-Arg	14(2.7%)
ATG-MET	17(3.2%)	ACG-Thr	0(0.0%)	AAG-Lys	18(3.4%)	AGG-Arg	5(1.0%)
GTT-Val	9(1.7%)	GCT-Ala	5(1.0%)	GAT-Asp	30(5.7%)	GGT-Gly	4(0.8%)
GTC-Val	2(0.4%)	GCC-Ala	0(0.0%)	GAC-Asp	10(1.9%)	GGC-Gly	4(0.8%)
GTA-Val	10(1.9%)	GCA-Ala	10(1.9%)	GAA-Glu	27(5.2%)	GGA-Gly	2(0.4%)
GTG-Val	1(0.2%)	GCG-Ala	1(0.2%)	GAG-Glu	10(1.9%)	GGG-Gly	6(1.1%)

Dinucleotide Analysis: Reading 2 bases and skipping 1

AA:	80 (15.2%)	AC:	28 (5.3%)	AG:	32 (6.1%)	AT:	63 (12.0%)
CA:	32 (6.1%)	CC:	17 (3.2%)	CG:	4 (0.8%)	CT:	20 (3.8%)
GA:	77 (14.7%)	GC:	16 (3.0%)	GG:	16 (3.0%)	GT:	22 (4.2%)
TA:	18 (3.4%)	TC:	25 (4.8%)	TG:	15 (2.9%)	TT:	60 (11.4%)

Figure 23. Codon usage and dinucleotide analysis of L22#9 nucleotide sequence.

Discussion

Genomic Stability of La Crosse Virus During Vertical and Horizontal Transmission

The L, M and S segment RNA oligonucleotide fingerprint patterns of LAC virus isolated from the original female mosquito (infected by artificial bloodmeal), from her F1 and F2 transovarially infected progeny, or from mice infected by bite of the F2 progeny were identical to those of the original plaque-purified LAC virus in the artificial bloodmeal. These results allow me to conclude that no detectable nucleotide changes occurred in the LAC virus genome upon transfer of the virus from vertebrate cell culture to the natural mosquito host in the laboratory by a means closely approximating the natural infection mechanism. Furthermore, no detectable nucleotide changes in the viral genome occurred during 2 generations of TOT in the mosquito host, or upon return of the virus to a vertebrate host by bite of a second generation transovarially infected mosquito.

A small number of nucleotide changes may have occurred and escaped detection due to the fact that ONF

directly samples only 10 to 15% of a large RNA molecule since the greater portion of the RNA is digested into monomers or small oligomers which cannot be resolved by two-dimensional electrophoresis. However, I have probably directly sampled about 25 to 30% of the LAC virus genome by independently analyzing the L, M and S RNA segments.

It is also possible that failure to detect changes in nucleotide sequence is due to use of long-passaged virus, and/or BHK-21 cells rather than mosquito cells to prepare viral RNA for fingerprinting. BHK-21 cells were chosen in preference to available mosquito cell lines because of the mosquito cell's much slower growth rates and the typically low quantities of virus they produce. These characteristics of the mosquito cell lines in conjunction with the cytotoxic effects of ^{32}P-orthophosphate make it practically impossible to obatain sufficient ^{32}P-labelled virus from mosquito cell cultures to conduct ONF analysis. Growth of the virus during many serial passages in BHK-21 cells may have selected a virus strain specifically adapted to these cells. Following isolation of virus from mosquitoes, preparation of labelled virus by growth in ^{32}P-orthophosphate supplemented BHK-21 cell culture might have biologically "filtered" viral variants selected in the mosquitoes. Despite the facts that these are standard

methods mandated by the technical considerations described above, and that they are in common use for ONF analysis of viral genomes, an alternative means of examining genomic stability of arboviruses is needed which avoids potential experimental artifacts due to cell culture propagation of virus. Isolation, cDNA cloning and sequence analysis of virus grown only in mosquito cell culture is one possibilty but would be cumbersome and expensive. However, the recent development of the Taq I polymerase chain reaction technique might be applicable to this problem. Specific targeted areas of the viral genome could be cloned from virus within mosquito tissues and then could be directly sequenced. This approach would eliminate any experimental artifact due to cell culture and would also allow examination of areas of the viral genome inaccesible to ONF analysis.

There is growing interest in the concept of RNA virus populations in existence as heterogenous mixtures of variant genomes or as a "quasispecies" (Domingo et al., 1985) with extremely rapid evolutionary potential exceeding that of their eucaryotic hosts by as much as a million-fold (Holland et al., 1982; Reanney, 1982; Reanney, 1984; Steinhauer and Holland, 1987; Strauss and Strauss, 1988). The quasispecies concept was originally advanced to

describe variable but closely related sets of self-replicating nucleic acid populations during the primeval evolution of life (Eigen, 1971; Eigen and Schuster, 1979). Domingo et al., (1978) were the first to demonstrate that an RNA virus population has a quasispecies distribution. By use of ONF, they showed that an E. coli RNA bacteriophage (QB) stock always yielded an unchanged fingerprint over 50 passages, but individual clones isolated from the stock yielded changed fingerprint patterns. Competition experiments showed that variant clones were always outgrown by the parent stock. The authors concluded that "the genome of QB phage can not be described as a defined structure, but rather as a weighted average of a large number of individual sequences". On the basis of mathematical analysis of their results, they predicted that any particular member of the population differed from the average wild type genome at 1-4 nucleotide sites. Several subsequent studies have demonstrated that the genomes of a wide range of RNA virus species are extremely heterogenous (Fields and Winter, 1981; Schubert et al., 1984; Arias et al., 1986; Catteno et al., 1986). The major source of variation is believed to be the inherent inaccuracy of genome replication by RNA-dependent RNA polymerases (Steinhauer and Holland, 1986),

although other sources include inversions, recombination and, in segmented viruses, reassortment.

Studies of laboratory Vesicular stomatitis virus stocks which were serially passaged at low MOI values in vertebrate cell culture have demonstrated genomic stability (Clewley et al., 1977) while passage at high MOI values generated rapid genomic change (Younger et al., 1981; Spindler et al., 1982). The rates of nucleotide divergence found in cell culture studies are similar to those found for various RNA viruses over the course of disease outbreaks (divergence rates of 0.03-2.0% per year). Examples of rapid and continuous evolution of animal RNA viruses in nature include influenza (Young et al., 1979; Webster et al., 1982), poliovirus (Kew et al., 1981; Nottay et al., 1981), enterovirus 70 (Takeda et al., 1984) and HIV (Hahn et al., 1986). These are all non-arthropod-borne viruses.

This body of evidence underlies the modern concept of an RNA virus quasispecies population which exists in a stable equilibrium dominated by the most genetically fit variants (Steinhauer and Holland, 1987). When environmental conditions change, such as in transfer to a new host, population disequilibrium and rapid continuous evolution of the viral genome may occur.

Evidence indicates that this genomic plasticity may apply to LAC virus. Some 30 field isolates of LAC virus obtained from several geographic locations at the same or at different times were subjected to ONF analysis (El Said et al., 1979; Klimas et al., 1981). None of the isolates yielded identical fingerprints, although some were obviously closely related. Fingerprint analysis of 5 different laboratory stocks of LAC virus in this laboratory by Dr. John Clerx has revealed minor differences among them, as has his analysis of 3 laboratory strains of the Montana isolate of SSH virus. In contrast, 2 SSH virus isolates from mosquitoes in the Yukon Territory, Canada have major differences. These results indicate genetic variation among various geographically isolated populations of LAC virus and it's close relative, SSH virus. The LAC virus genome can respond to changes in selective pressure, as shown by the fact that a monoclonal antibody-selected variant exhibiting decreased cell fusion ability, loss of neuroinvasiveness in mice, and decreased dissemination in mosquitoes reverted following recovery from an infected mosquito and serial passage in BHK-21 cells (Gonzalez-Scarano et al., 1987; Sundin et al., 1987). Tesh and Gubler (1975) have demonstrated the presence of small plaque variants in populations of transovarially passaged

LAC virus, indicating a heterogenous virus population. Furthermore, the spontaneous mutation rate of SSH virus is 1-2% (Bishop and Shope, 1979).

Despite these potential indications of genomic plasticity in LAC virus, I have demonstrated that the virus does not appear to undergo rapid evolution during TOT in the mosquito host or during horizontal transmission from mosquito host to vertebrate host under laboratory conditions. Bilsel et al., (1988) have recently reported that Toscana virus (Phlebovirus; Bunyaviridae), serially passaged by TOT over a period of 2 years through 12 generations of the natural sandfly host, underwent no detectable genomic change as determined by ONF. My extension of these results to LAC virus in mosquitoes allows me to speculate that genomic stability of the viral genome during TOT may be a general phenomenon among Bunyaviridae and perhaps among other arboviruses.

The rate of evolution of Ross River virus (a mosquito-borne positive-sense RNA virus in the family Alphaviridae) has recently been examined by Burness et al., (1988) during the course of a 10-month outbreak of epidemic polyarthritis in a non-immune island population of humans. With use of direct genomic sequencing of 2 areas of the virus genome totaling 1.6 KB, and previously shown to be

variable among different wild isolates of the virus, they demonstrated that only 1 nucleotide substitution occurred in the virus isolates examined over the course of the epidemic. This was particularly surprising because the human host and mosquito vectors were not the normal ones and thus could be expected to have exerted greater selective pressures on the virus than the usual hosts.

Three different arboviruses LAC, Toscana and Ross River, have now been shown to undergo no or very low rates of evolution under conditions in which they might reasonably be expected to evolve at higher rates, and despite the fact that wild isolates of the viruses do exhibit genetic diversity. Furthermore, ONF analysis of various Alphaviruses isolated over a period of 25 years and over wide geographic ranges have demonstrated remarkable similarity among isolates of any particular virus (for review, see Beaty et al., 1988). Taken together, these results may be interpreted to indicate low rates of evolution among arboviruses relative to those of non-arthropod-borne RNA viruses.

The apparent low rate of evolution among arboviruses thus requires explanation. It is unlikely that it is due to inherent genetic stability of arboviruses since almost all RNA viruses show similar rates of spontaneous mutation.

Selective pressures unique to the arbovirus cycle must therefore underlie the low rate of evolution. The fact that arboviruses must be able to infect and then replicate and express their genomes in both vertebrates and invertebrates may itself be a factor in their apparent genetic stability. It has been suggested that transmission between invertebrate and vertebrate hosts may create population disequilibrium in arboviruses leading to generation of new virus populations with different spectrums of variants (Holland, 1984). However, it is also possible that molecular constraints placed on the virus by such disparate hosts may be a powerful selective force for genomic stability by limiting the spectrum of viable and competitive variants in a cycling arbovirus population. The recent demonstration that a translational requirement for complete LAC virus S segment mRNA synthesis is operative in vertebrate cell culture, but not in mosquito cell culture (Raju and Kolokofsky, personal communication), underscores the probable involvement of intra-cellular molecular events in the biological differences seen in arbovirus infections of vertebrate and invertebrate hosts.

During TOT many rounds of virus replication must occur in successive related generations of mosquitoes. If the laboratory observations of LAC and Toscana virus

genomic stability during TOT are true of the situation in nature, then it is possible that this mechanism could confer a low rate of evolution on the virus, either by selection exerted on viral variants or by selection for increased replicative fidelity. The original conclusions of Domingo et al. (1978), that members of an RNA phage population differed on average at 1 to 4 nucleotide sites per genome, have since been extended to field isolates and multiple laboratory clones of foot-and-mouth disease virus (Domingo et al., 1980; Subrino et al., 1983). Steinhauer and Holland (1986), working with Vesicular stomatitis virus, have provided direct sequence evidence that this variability is due to high RNA-directed RNA polymerase error frequencies. Estimates of error frequencies for polymerases of RNA viruses and retroviruses are consistently in the range of 10^{-3} to 10^{-4} per nucleotide site. The available evidence therefore provides little reason to believe that selective pressures for enhanced replicative fidelity might explain instances of apparent genomic stability among RNA viruses. In fact, replicative infidelity is believed to provide RNA viruses with adaptive advantages (Reanney, 1982). The preservation of a genomic consensus sequence within an RNA virus population is therefore most likely due to strong selective pressures for

the most genetically fit and competitive variants within the population. It seems likely that TOT of LAC virus within the mosquito population could exert such selective pressure on LAC variants and that this might be responsible for the apparent genomic stability of the virus rather than selection for replicative fidelity. If one accepts the premise that certain arboviruses, including LAC, may be principally maintained in nature within the invertebrate host by TOT and venereal transmission (Tesh and Shroyer, 1980; Turell, 1988) then a role for these transmission mechanisms in a low rate of viral evolution becomes more plausible.

The genetic variability seen among wild isolates of LAC virus might be partly explained by the existence of virus populations maintained by TOT in reproductively isolated mosquito populations. When such a virus population is transmitted between invertebrate and vertebrate hosts within the arbovirus cycle, dominant varaints are likely to be conserved by physical and biological limitations imposed by small bloodmeal and injected saliva volumes and low virus titers (Yuill, 1983; Burness et al., 1988). However, it must be noted that vertebrates possess immune systems with the demonstrated

ability to select RNA virus variants during persistent infection (Clements and Narayan, 1984).

Transmission of Reassortant Bunyaviruses

The plaque-assay data obtained by Dr. Beaty and colleagues (Chandler et al., submitted) indicated that simultaneous inoculation of temperature sensitive mutant stocks of LAC and SSH viruses into uninfected adult female Aedes triseriatus mosquitoes led to generation of reassortant bunyaviruses in these mosquitoes. The reassortant viruses could be transovarially transmitted to progeny mosquitoes which could then transmit the reassortants by bite to uninfected mice. The ONF data which I have obtained confirms these results. The reassortant genotypes are those expected on a genetic basis due to presence of lesions in the M segment of SSH-I-3 and in the L segment of LAC-II-5 parent viruses. The reassortants have SSH L segments, LAC M segments and LAC or SSH S segments (Table 5). The following conclusions can be made: (1) Heterologous reassortant viruses were generated at relatively high frequency in the vector mosquito, Aedes triseriatus; (2) These reassortant viruses were transmitted by TOT; (3) The transovarially infected

progeny mosquitoes were able to transmit the reassortant viruses to a susceptible vertebrate host, a requirement for epidemiologic significance.

Beaty and colleagues (1981; 1985) have previously demonstrated high frequency generation of Bunyavirus reassortants in Aedes triseriatus following dual inoculation or dual oral ingestion, and that the reassortants can be transmitted to mice by bite of infected mosquitoes. However, the data described in this dissertation is the first demonstration that such reassortants can be transmitted within the vector mosquito by TOT. Since TOT is thought to be a principal mechanism of viral maintenance in nature (Tesh and Shroyer, 1980; Turell, 1988), it is now possible to propose that it might serve as a mechanism leading to fixation of newly generated reassortant viruses within the vector population.

La Crosse and SSH viruses circulate in nature in sympatric cycles as do other viruses in the California serogroup. Furthermore, some species of mosquitoes, including Aedes triseriatus, feed readily on a variety of hosts, may take multiple bloodmeals over the course of their lifespan (DeFoliart, 1983), and quickly seek another host if host defensive behavior leads to interrupted feeding. Studies have also indicated that host-seeking

mosquitoes have a preference for and greater success in taking bloodmeals from viremic hosts. Consequently, there would appear to be ample opportunity for exposure of mosquitoes to different viruses and subsequent reassortant virus generation in nature. Evidence indicates that this does occur among Bunyaviruses (Klimas et al., 1981; Ushijima et al., 1981). However, dual infection by bloodmeal ingestion and reassortant virus generation is limited by the fact that mosquitoes appear to be refractory to superinfection by a second virus within 2 to 7 days after infection by a first virus (Beaty et al., 1985) and by the limited duration of vertebrate viremias. Preliminary evidence indicates that female Aedes triseriatus, transovarially infected with ts mutants of LAC virus, are susceptible to oral superinfection with wild type LAC virus (Sundin, D. and Beaty, B., personal communication). Thus, available evidence and biological characteristics of the Bunyavirus life cycle allow the conclusion that generation of reassortants occurs in nature. How frequently this occurs and how epidemiologically significant it is, remain to be determined.

RNA segment reassortment in nature plays a role in viral evolution in general and the epidemiology of

influenza viral disease in particular (Sugiyama et al., 1981; Laver et al., 1984; Bishop et al., 1987). It is possible that newly evolved reassortant Bunyaviruses might result in rapid changes in vertebrate host range, vector specificity and pathogenicity. How this might have serious epidemiologic consequences is illustrated by the following hypothetical possibility. The Aedes triseriatus-squirrel-LAC virus and the Aedes trivittatus-cottontail rabbit-Trivittatus virus cycles overlap in the forest-grassland ecotone (LeDuc, 1979). These circumstances allow the possibility that a LAC virus reassortant might evolve that could be efficiently vectored by Aedes trivittatus mosquitoes and become fixed in the mosquito population, perhaps by TOT. These mosquitoes are not restricted to forested areas, as are Aedes triseriatus, and are much more likely to encounter and feed upon humans. This hypothetical sequence of events could lead to a significant increase in the incidence of LAC encephalitis.

Studies of LAC and SSH viruses and LAC/SSH reassortants have revealed that the LAC M RNA segment is essential for efficient oral infection of Aedes triseriatus mosquitoes and subsequent transmission to mice (Beaty et al., 1981b; 1982). This serves to demonstrate that not all possible reassortant virus types which could arise under

any given set of circumstances would be likely to result in increased disease problems.

Sequence Analysis of Inkoo Virus M Segment

Bunyavirus M segment RNAs encode the coat glycoproteins G1 and G2 and nonstructural proteins of unknown function which are designated NsM. A subgenomic complementary sense M segment RNA is translated as a large polyprotein which is post-translationally processed to yield the mature proteins. Several features of the INK virus M segment sequence and it's encoded polyprotein are very similar to those reported for LAC, SSH, BUN and GER viruses (see results section for citations). The estimated lengths in nucleotides and encoded amino acids of the INK M segment are approximately the same as those reported for the other 4 viruses. The 3' noncoding portions of the 5 antigenome-sense M RNAs appear to be considerably more variable in length than the 5' noncoding portions, with the INK 3' noncoding sequence being the shortest of the group. The 3' noncoding portion of the M segment sequence is presumably important in initiation and perhaps regulation of negative-sense RNA replication. The significance, if any, of the variability in length and sequence of this

region among the 5 bunyaviruses remains to be determined. The relatively low number of potential N-linked glycosylation sites correlates well with the reported low levels of glycosylation of bunyaviral glycoproteins (Vorndam and Trent, 1979; Lees et al., 1986).

An interesting feature of the predicted M segment polyprotein sequences is their relatively high cysteine content (5 to 6%) and the near total conservation of position of the cysteine residues in the primary structures of the polyproteins (see Table 6). Cysteine contents of 5 to 6% have been reported for the glycoproteins of 3 other genera in the Bunyaviridae (Collet et al., 1985; Ronnholm and Pettersson, 1987; Schmaljon et al., 1987). The conservation of position of cysteine residues in the M segment polyprotein sequences of the Bunyavirus genus extends across serogroup boundaries while that of proline declines greatly. Both cysteine and proline are important in the generation of higher order structure in proteins. Cysteine, through it's ability to form covalent disulfide linkages with other cysteine residues, can play a role in generation of tertiary and even quaternary structure. Cis-Trans isomerization about X-Proline peptide bonds is believed to play an important role in the kinetic mechanism of protein folding, leading to higher order structure.

These phenomena probably underly the high degree of conservation of position of cysteine and proline residues seen in the Bunyavirus M segment polyprotein sequences. It is probable that the envelope glycoproteins of these viruses have a similar three-dimensional structure (a supposition supported by their very similar hydropathy profiles) which has been conserved during the evolution and divergence of the genus. Viral coat glycoproteins are commonly multifunctional and are anchored in the viral envelope as dimers or trimers with highly ordered structures involving cystine linkages (Schlesinger and Schlesinger, 1987). Architectural constraints, perhaps imposed by the necessities of precise intractions of cellular receptors and viral glycoproteins and/or fusion of the viral particle with the cell membrane, have probably resulted in conservation of cysteine-dependent structural features in the glycoproteins during the evolution and divergence of the Bunyaviruses. However, conservation of primary sequence cysteine residue positions is not unique to Bunyaviral glycoproteins. Rather, it is a general phenomenon which has been observed in glycoproteins of other virus families as well as such functionally diverse proteins as polypeptide hormones, cysteine proteases and ferredoxins.

The paucity of codons containing the CG dinucleotide in the INK M segment nucleotide sequence is true of the other viruses in the Bunyaviridae and has also been noted for Flaviviridae (Rice et al., 1985; Dalgarno et al., 1986; Trent et al., 1987). Bulk vertebrate DNA exhibits a deficiency of CG doublets (Bird, 1980) and between 60 and 90% of cytosine residues in these doublets are methylated at the 5 position on the cytosine rings. The rarity of CG doublets is believed to be due to failure of DNA repair mechanisms to recognize deamination of 5-methylcytosine to thymine and this may explain the low abundance of CG doublets in Bunyaviral genes. Unmethylated CG doublets are abundant in areas of vetebrate genomes which seem to be associated with constituitively active genes, perhaps as a consequence of the protective effect of bound regulatory proteins (Bird, 1986). Transcriptionally active sequences in invertebrates also appear to reside in unmethylated areas of their genomes (Bird, 1980). It is possible that the lack of codons containing the CG dinucleotide in Bunyaviral genes is an adaptation leading to the efficient translation of viral proteins in both vertebrate and invertebrate cells (Dalgarno et al., 1983). However, Alphaviruses have a nearly random distribution of CG dinucleotide-containing codons (Rice and Strauss, 1981;

Kinney et al., 1986) resembling the dinucleotide distribution found in mosquitoes and are also capable of efficient replication in both invertebrate and vertebrate hosts. Further work on the details of viral replication, transcription, and translation in invertebrate and vertebrate hosts may lead to an explanation of the functional significance of differences in dinucleotide frequencies among arboviruses.

At present, it is possible to speculate that the Bunyaviridae may be evolutionarily closer to the Flaviridae than to the Alphaviridae. This hypothesis is further strengthened by the fact that Flaviviruses possess a short conserved sequence of nucleotides at the 3' end of plus and minus strand full-length RNAs, a situation similar to the negative-stranded Bunyaviridae (Rice et al., 1986). It is possible that Bunyaviruses may have vertebrate origins and have evolved the ability to replicate in invertebrate vectors. The fact that the Hantavirus genus of the Bunyaviridae appear to be solely mammalian viruses without invertebrate vectors is noteworthy in this respect.

The nucleotide and amino acid sequence comparisons among Bunyaviruses allow the conclusion that the 3 California serogroup viruses (LAC, SSH, INK) are very closely related. When the sequences of these 3 viruses

were compared to those of viruses of 3 other genera in the Bunyaviridae, no significant similarities were revealed whereas comparison with the sequences of BUN and GER viruses, members of another serogroup within the genus Bunyavirus, revealed significant sequence conservation. These results indicate a relatively recent evolutionary divergence of serogroups within the Bunyavirus genus and a much more distant origin of genera within the Bunyaviridae. Lacking fossil evidence or a reliable measure of the rates of nucleotide and amino acid substitutions among RNA viruses, it is impossible to accurately estimate the time scale of these events. However, if we assume that nucleotide substitution rates in RNA viral genomes lie in the range of 0.1 to 2% per year (Reanney, 1982) then much of the divergence within the Bunyavirus genus has probably occurred within the last few hundred to few thousand years and divergence at the family level may have occurred within the last 10 to 100 thousand years. Such a rapid rate of genetic change might be expected to result in epidemiologic consequences within the scale of a human life-time and is a factor which should be considered in devising long-term strategy for dealing with Bunyaviral disease.

The gene order along the M segment nucleotide sequence of SSH virus has recently been determined

(Fazakerley et al., 1988). The G2 protein occupies the amino terminal end of the polyprotein and extends from amino acids 14 to 299. It is preceded by a 13 amino acid signal sequence. The G1 protein occupies the carboxy terminal end of the polyprotein, extending from amino acid 474 to the end of the open reading frame at amino acid 1,441. Lying between G2 and G1, is a stretch of 174 amino acids which the authors demonstrated by immunologic methods to be the source of 10,11 and 19 kilodalton nonstructural proteins (NsM) in infected cell lysates. This coding strategy and the presence of multiple protein species originating from the NsM region are also true of LAC virus (F. Gonzalez-Scarano, personal communication). It is probable that this coding strategy extends to other members of the Bunyavirus genus and it therefore becomes useful to examine homology comparisons among members of the genus in greater detail. Such examination reveals that some areas of the nucleotide and amino acid sequences are more conserved than others. Both nucleotide and amino acid sequences are most strongly conserved in the central and carboxy terminal portions of the G2 protein, and to a lesser extent in the carboxy terminal one third portion of the G1 protein. These conserved areas of the glycoproteins may be important in cell receptor binding and envelope

fusion functions. The conserved areas of the G1 protein may contain immunogenic determinants (Kingsford and Ishizawa, 1984) forming the basis of Bunyavirus serogroup classification.

The NsM region of the nucleotide and amino acid sequences are the least conserved. This is most apparent in the amino acid sequence comparison of INK and BUN viruses. There are many mismatches, semi-conservative substitutions and several gaps necessary to attain alignment in the NsM region. Interestingly, there is total conservation of the 7 cysteine residues in the NsM region in any possible pair-wise comparison among the 5 viruses. The NsM proteins do not form part of the mature virion and it is improbable that they are expressed on the surface of infected cells at a level of abundance comparable to the G1 and G2. If the NsM proteins do appear on the vertebrate cell surface, they would probably occur in conjunction with major histocompatibility antigens and could therfore be of immunogenic significance. However, it is well established that the major host immune response is against the G1 and, to a lesser extent, the G2 proteins. It is unlikely that the NsM proteins are of significance in the neutralizing immune response of vertebrate hosts and they would therefore largely escape immune selective pressure. This

may, at least in part, explain their relative lack of conservation compared to the G1 and G2 proteins.

The conservation of cysteine residues in the NsM region of the polyprotein, and the presence of 10, 11 and 19 kilodalton NsM proteins in SSH virus-infected cell lysates, can be interpreted as evidence that this region of the polyprotein functions to maintain correct conformation during proteolytic cleavage events leading to formation of mature G1 and G2 proteins. This interpretation does not rule out the possibilty that the NsM proteins may play some other role in viral maturation and it must be emphasized that their true function(s) remain unknown.

The greater conservation of amino acid sequence (89%) than of nucleotide sequence (79%) between LAC and SSH viruses is the "normal" finding when making nucleotide and amino acid similarity comparisons. It is surprising that there is 10 to 20% greater conservation of nucleotide than of amino acid sequence when comparing the California serogroup virus sequences to those of the Bunyamwera serogroup. There is no apparent great increase in nucleotide sequence conservation at the 5' and 3' ends of the M segments, areas presumably important in initiation and perhaps regulation of replication and transcription, which could account for the greater nucleotide sequence

conservation. The reason(s) for greater nucleotide than amino acid sequence conservation among the 2 serogroups is enigmatic and one can only speculate on it's significance. It is possible that RNA secondary and tertiary structure is under strong selective pressure, perhaps related to replication and transcription or to packaging in nucleocapsids, which has resulted in conservation of nucleotide sequence. These intra-cellular events must be very similar in invertebrate and vertebrate hosts whereas infective events, mediating host trophisms and ranges, are extra-cellular, presumably more variable in nature, and would be mediated at the protein level. Thus, stringent selective pressure on RNA structure may have resulted in great conservation of nucleotide sequence during divergence of the Bunyavirus genus. Another possibility is selective pressure against nucleotide sequences capable of forming inter- or intra-strand double helical regions. Double helical regions longer than 15 to 20 base pairs in length would be inert to replicase/transcriptase activity and to packaging into nucleocapsids. The M segments of 2 members of the Hantavirus genus, Nephropathica epidemica virus strain Hallnas B1 and Hantaan virus, also share 10% greater nucleotide than amino acid sequence conservation (L. Giebel, personal communication).

Sequence Anlysis of the Inkoo Virus L Segment

Failure to detect continuity among the 3 plasmids containing putative INK L segment cDNA despite the fact that the inserts totaled almost 10,000 bp, while the L segment is believed to be approximately 8,000 nucleotides in length, invites suspicion that at least some portion of the cDNA inserts represents cloned material which is not part of the INK L segment sequence. The pINK 130L plasmid is particularly suspect since cDNA inserts of this size (7,000 bp) commonly contain inversions, repeats, or inserted cloning vector or bacterial sequences (Kolokofsky, D., personal communication).

However, several facts lead me to conclude that the cDNA insert sequence obtained for the pINK 220L plasmid is a portion of the INK L segment sequence. Firstly, the sequence begins with the known 3' end consensus sequence of viruses in the genus Bunyavirus (Clerx-van Haaster et al., 1982). Secondly, dinucleotide analysis of codons in the frame 1 ORF of this sequence shows strong discrimination against the CG dinucleotide, as is true of the INK M segment. Thirdly, the base composition is very close to that of the INK M segment. Fourthly, all Bunyavirus M and

S segments sequenced to date encode ORF with methionine initiation codons at approximately nucleotide 60. The first methionine codon in the pINK 220L cDNA insert sequence is at nucleotide 58 in a context favorable for initiation of translation and the second is at nucleotide 148 in an unfavorable context. It is therefore likely that the 1,574 bp cDNA insert in pINK 220L is a clone of the 3' end portion of the INK L segment and represents about 25% of the total length of the L segment.

This nucleotide sequence and it's deduced amino acid sequence were screened for similarity against all sequences in the GEN BANK database with the University of Wisconsin Genetics Computer Group BestFit program. The glycine-aspartate-aspartate motif, which is conserved in many RNA-dependent RNA polymerases of plant, animal and bacteria viruses and in reverse transcriptases of retroviruses (Kamer and Argos, 1984) appears in the L22#9 deduced amino acid sequence at position 193. No other significant similarities were found. This is not surprising in view of the facts that the INK sequence is incomplete and that the known RNA-dependent RNA polymerase sequences have only very limited similarities with the following exception. The polymerases of Rabies and Vesicular stomatitis viruses (Rhabdoviridae) have 33% overall amino acid homology which

is concentrated in several areas along the sequences (Tordo et al., 1988). Within these particular areas, 10 to 20 % similarities are apparent when the sequences are aligned with those of 2 Paramyxoviridae members, Sendai and Newcastle disease viruses. These 4 viruses are single-stranded negative-sense RNA viruses of animals and employ monocistronic subgenomic mRNAs in their expression strategies which is radically different from the expression strategy of the Bunyaviridae. It is interesting that the glycine-aspartate-aspartate motif does not occur in their polymerase sequences. It is possible that these observations indicate a lack of an evolutionary relationship between the single-stranded negative-sense RNA viruses of animals and the segmented Bunyaviridae.

Conclusion

Arthropod-vectored disease is a problem of mammoth proportions faced by humanity throughout the world. The direct consequences of clinical disease in humans and livestock are augmented by serious socio-economic complications that result in overwhelming burdens for many developing countries in the tropics (Pant, 1987). Many of these countries cannot afford the increasingly expensive chemical control strategies required to contain arthropod-vectored disease in the face of widespread insecticide resistance problems (Curtis and Lines, 1986). This traditional means of controlling arthropod-vectored disease is fraught with other problems, such as toxicant damage to the health of humans and livestock and environmental degradation. Development of alternative means of controlling arthropod-vectored disease is therefore necessary. This can only be done by increasing our knowledge of the ecology, molecular biology and biochemistry of host-pathogen interactions. I believe the research described in this dissertation contributes to this need for new knowledge of host-arbovirus interactions.

The demonstration that reassortant Bunyaviruses can be transovarially transmitted within the vector and that

the inherited reassortants can be transmitted to a vertebrate host is an important contribution to our knowledge of an aspect of Bunyavirus biology which plays a major role in viral evolution and potentially in epidemiology of Bunyaviral disease. This greater understanding of evolutionary mechanisms at work within the virus and vector populations is critical to gaining a predictive capability concerning the potentials and probabilities of future viral disease outbreaks. The spread of human populations into formerly unexploited forested regions of developing countries places increasing numbers of people at risk for hitherto unknown arboviral diseases, while simultaneously increasing the possibilities for alterations in host ranges and distributions of arboviruses through large-scale changes in the environment. The apparent global warming trend can also be expected to cause alterations in the distributions of arboviruses in the developed countries, which are concentrated in temperate regions of the globe. In this country, the recent establishment of Aedes albopictus in gulf coast states is particularly worrisome. This mosquito is an aggressive catholic feeder with the ability to efficiently vector a wide range of arboviruses. It breeds in small pools of water found in treeholes and abandoned containers.

If it is able to spread inland and displace less dangerous mosquitoes, such as Aedes triseriatus which is also a container-breeder, then we can expect an increase in arboviral disease. Predictive capabilities, based on detailed knowledge of arbovirus cycles, could greatly enhance our ability to deal with such potential problems.

If the premise that arboviruses evolve at a low rate in nature, as outlined in this dissertation, proves correct, then the phenomenon of transovarial transmission may provide a means to introduce and maintain genetically engineered virus strains into vector populations. Genetic studies, based on use of reassortants, have led to the discovery that many aspects of Bunyavirus biology such as host range, infectivity and virulence are mediated by the M segment (Beaty and Bishop, 1988; Gonzalez-Scarano et al., 1988). It might be possible to generate reassortants with reduced vertebrate pathogenicity which could be introduced into vector populations in order to displace natural more pathogenic strains.

Knowledge gained from sequence analysis of viral genes coupled with studies of viral expression in the invertebrate host could provide another means of manipulating arbovirus cycles through the use of the phenomenon of pathogen-derived resistance (Sanford and

Johnson, 1985). In this approach, nucleotide sequences obtained from a pathogen are used to genetically modify a host in such a manner that it becomes resistant to the pathogen. The phenomenon of resistance to superinfection among Bunyaviruses provides hope that this approach might be applicable. Pathogen-derived resistance has been demonstrated to be effective in studies with the bacteriophage QB and it's host, E. coli (Grumet et al., 1987).

While these methods of interdicting arboviral life cycles and disease may be somewhat futuristic, practical advances in the development of vaccines are possible now. Sequence analysis of of arboviral nucleotide and amino acid sequences followed by comparative study of the sequences for several members of a family has identified conserved regions of their genomes and proteins, as described for the Bunyaviruses in this dissertation. Identification of shared immunogenic determinants in these conserved regions could lead to genetically engineered serotype or even genus-specific vaccines. Use of cloning vehicles such as the vaccinia virus expression system might ultimately allow production of vaccines against whole families or even several families of viruses. Such a vaccine would be particularly useful in developing tropical countries where

arboviruses and poor medical care are everyday facts of life.

Information derived from sequence analysis of Bunyavirus genomes can also be used to construct synthetic nucleotide and peptide reagents for use in studies designed to unravel details of viral replication, transcription, translation and assembly. Progress in this area has been retarded by such problems as the lack of poly A tails on Bunyavirus plus-sense RNAs, making isolation and quantitative study difficult. Synthetic peptide reagents, constructed on the basis of knowledge gained from comparative analysis of Bunyavirus glycoprotein sequences, may be useful in study of secretory protein maturation and transport. The development of an efficient transfection procedure for mosquito cells (Fallon, 1986) and vectors for expression of transfected genes (A.M. Fallon, personal communication) will allow study of expression of recombinant Bunyaviridae glycoproteins in mosquito cells. Use of such reagents may eventually provide information on differences in intracellular metabolic events in vertebrates and invertebrates.

The demonstrations, as described in this dissertation, that reassortant Bunyaviruses can be transovarially transmitted within the vector, that

Bunyaviruses do not evolve during transovarial transmission in a laboratory mosquito population or when transferred from mosquito to vertebrate host, and the information obtained from sequence analysis of the Inkoo viral genome and it's comparison to the sequences of other Bunyaviruses constitute fundamental contributions to our knowledge of Bunyaviridae. These contributions may ultimately be of practical value in control of Bunyaviral disease.

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