Studies on the expression strategy and encoded proteins of the bunyavirus S RNA segment

by

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ABSTRACT

A characteristic of viruses in the family Bunyaviridae is that the viral genome consists of three RNA segments of single stranded RNA, designated large (L), middle (M) and small (S). The Bunyaviridae family is sub-divided into five genera, but in all members of the family the S RNA segment encodes the RNA-binding nucleocapsid (N) protein. This study is restricted to viruses in the Bunyavirus genus and concerns the expression strategy of S segment encoded proteins and the nature of the interaction of N protein with viral RNA.

For viruses of the Bunyavirus genus the S segment also encodes a non-structural protein, termed NSs, in an overlapping reading frame downstream of the start of the N open reading frame (ORF). The NSs ORF initiates from a tandem AUG in most bunyaviruses, such as Bunyamwera the prototype virus. The Maguari (MAG) virus belongs to the same serogroup as Bunyamwera virus (BUN), with which it shares high S segment homology, but the MAG S segment in addition to encoding N and NSs proteins, possess a third ORF, potentially encoding a protein of M_r 9.3K, downstream of the N and NSs ORFs. The MAG S segment is 945 bases in length and most of the MAG S segment (bases 22-937) was available on a single cDNA clone, pMAG60. In order to investigate the expression strategy of the MAG S segment fragments of the cDNA, containing either the complete S segment coding region or parts of it, were subcloned downstream of a T7 RNA polymerase promoter sequence in the vector pTZ18U. A series of recombinant pTZ plasmids were used as templates to generate message-sense RNA in T7 RNA polymerase directed run-off transcription reactions. Aliquots of the RNA were translated in a cell free translation system (rabbit reticulocyte lysate and wheat germ extract) and the synthesised proteins, which were labelled with ^{35}S methionine, were analysed by polyacrylamide electrophoresis. Translation of RNA derived from templates containing the entire MAG S coding region produced three major products with electrophoretic mobilities equivalent to the predicted M_r of the encoded products, N, NSs and ORF3 proteins. Translation of transcripts derived from plasmids containing NSs and 9.3K ORFs or 9.3K ORF only produced two major products or one major product repectively, corresponding to NSs and/or the 9.3K protein. These

experiments indicated that the proposed sites of initiation of translation for N, NSs and 9.3K proteins were correct but in addition it demonstrated that all three encoded products were capable of being translated from a single mRNA species .
The NSs tandem initiation codons for NSs in the MAG S cDNA were mutated either singly or together to leave either the first or second or no potential codons at the start of the NSs ORF. Translation of transcripts derived from these templates indicated that NSs translation was initiated at the tandem AUG and that both the first and the second AUG were equally capable of initiating NSs translation. In addition NSs translation was found to be unaffected by the loss of the upstream N initiation codon or coding sequences. However, synthesis of the 9.3K protein was reduced when the upstream N initiation codon was inactivated.
Although the 9.3K protein product could be translated in vitro, its presence could not be demonstrated in radiolabelled MAG virus infected cells due to a high background of labelled host proteins. In an attempt to make a specific antiserum to this protein the 9.3K protein was expressed as a beta-galactosidase (β-gal) fusion protein in bacterial cells. A protein band corresponding to the fusion protein was isolated from preparative polyacrylamide gels and used to inoculate a rabbit at 3-4 week intervals. Test bleeds were used in immunoprecipitation assays of appropriately programmed rabbit reticulocyte lysates, but the serum failed to immunoprecipitate the 9.3K protein. However, the serum was able to recognise the β-gal carrier protein in a seperate immunoblot assay of β-gal protein. It was concluded that the 9.3K protein was poorly immunogenic and no further attempt at raising an antiserum to this protein was made.
The N protein interacts with each RNA genome segment to form internal nucleocapids within the Bunyaviridae particle. Each nucleocapsid consists of a single species of RNA genome segment (L, M or S), multiple copies of N protein and minor amounts of L protein. In virus infected cells N protein has high specificity for full length negative-sense genomic or positive-sense antigenomic RNA, but not viral mRNAs, which have different 5' and 3' ends, and host cell RNA. The nature of this specificity

is unknown and was investigated by studying *in vitro* the RNA binding activity of recombinant expressed N protein with various S segment derived RNA species. Full length S segment cDNAs were assembled for both MAG and BUN viruses. These cDNAs were placed under T7 promoter control to enable full length positive sense RNA transcrips to be obtained. In addition a negative sense BUN S cDNA was made. The BUN S cDNA was further altered to create an abbreviated S segment containing essentially the 5′ and 3′ non-coding regions of the segment. A source of nucleocapsid protein was obtained via the baculovirus expression system. This entailed the placing of the MAG S coding region downstream of the baculovirus polyhedrin promoter in a baculovirus transfer vector, pAcYM1, and recombining the plasmid into the baculovirus genome via homologous recombination. An isolated recombinant baculovirus, AcMAGS, expressed recombinant N protein in infected insect cells, *Spodoptera frugiperda*. A significant fraction of this expressed protein was soluble and was purified to homogeneity in a two stage protocol consisting of N protein enrichment by ammonium sulphate precipitation and ion exchange chromatography (Mono Q FPLC). The MAG N protein was also expressed as a beta-galactosidase fusion protein in *E.coli*. This fusion protein was immunologically recognised by MAG virus specific serum.

The RNA binding activity of recombinant N protein was investigated by three techniques: North-west blot; filter binding; and gel mobility shift assay. Analysis of N-RNA interaction by North-west blot failed to demonstrate any specificity of interaction, for both baculovirus expressed N protein and bacterially expressed beta-galactosidase N fusion protein. Measurement of RNA-N interaction by filter binding assay defined optimum pH, NaCl concentration and temperature with which to obtain maximal levels of RNA-N complex. However, complexes formed under these conditions failed to exhibit specificity of interaction. RNA-N complexes were also analysed by gel mobility shift assay, in which the interaction of the N protein with RNA was visualised on an agarose gel as an alteration of the mobility of radiolabelled RNA when complexed with N protein as opposed to free RNA. Although complexed and free RNA could clearly be distinguished (the conditions employed

to allow interaction to occur were the same as those used in the filter binding assay) specificity of interaction could not be demonstrated. The specificity of interaction that exists in bunyavirus infected cells could not be demonstrated <u>in vitro</u> using the above reagents.

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Abbreviations

aa	amino acid
ATP	adenosine-5'-triphosphate
β-gal	beta-galactosidase
BHK	baby hamster kidney 21, clone 13 cells
BSA	bovine serum albumin
CHAPS	3-cholamidopropyl dimethyl-ammonio-1-propane sulphonate
Ci	Curie
CTP	cytosine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dCTPαS	2'-deoxycytidine-5'-O-(1-thiotriphosphate)
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	dATP, dCTP, dGTP and dTTP
ddATP	2', 3'-dideoxyadenosine-5'-triphosphate
ddCTP	2', 3'-dideoxycytidine-5'-triphosphate
ddGTP	2', 3'-dideoxyguanosine-5'-triphosphate
ddTTP	2', 3'-dideoxythymidine-5'-triphosphate
DI	defective interfering
DNA	deoxyribonucleic acid
ds	double stranded
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	sodium ethylenediamine tetra-acetic acid
g	grams
G	guanine
GTP	guanosine-5'-triphosphate
ITP	inosine-5'-triphosphate
K	kilodalton
kb	kilobase
hr	hour
M	molar
mg	milligram
min	minute
ml	millilitre

mM	millimolar
moi	multiplicity of infection
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NP40	Nonidet P40
N	nucleocapsid protein
NS	nonstructural protein
nt	nucleotides
OD	optical density
ORF	open reading frame
^{32}P	phosphorus-32-radioisotope
PBS	phosphate buffered saline
PEG6000	polyethylene glycol 6000
pfu	plaque forming units
RNA	ribonucleic acid
RNP	ribonucleocapsid
RNase	ribonuclease
rpm	revolutions per minute
^{35}S	sulphur-35-radioisotope
SDS	sodium dodecyl sulphate
Sf	Spodoptera frugiperda
ss	single-strand
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
TTP	thymidine-5-triphosphate
UV	ultraviolet
V	volts
µg	microgram
µl	microlitre
v/v	volume/volume (ratio)
W	Watts
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)
wt	wild type

Amino Acid Symbols:

A-alanine	G-glycine	M-methionine	S-serine
C-cysteine	H-histidine	N-asparagine	T-threonine
D-aspartate	I-isoleucine	P-proline	V-valine
E-glutamate	K-lysine	Q-glutamine	W-tryptophan
F-phenylalanine	L-leucine	R-arginine	Y-tyrosine

Restriction enzymes

Ec	EcoRI
Bm	BamHI
Hd	HindIII
Kp	KpnI
Ps	PstI
Sc	SacI
Sl	SalI
Sm	SmaI
Sp	SphI
Xb	XbaI

Virus Abbreviations:

AcNPV	Autographa california nuclear polyhedrosis virus
BAT	Batai
BUN	Bunyamwera
CCHF	Crimean-Congo Hemorrhagic fever
CMV	Cytomegalovirus
DUG	Dugbe
GER	Germiston
HAZ	Hazara
HIV	human immunodeficiency virus
HSV	herpes simplex virus
HTN	Hantaan
INS	Impatiens necrotic spot
LAC	La Crosse
MAG	Maguari
NE	nephropathia epidemica
PH	Prospect Hill

PT	Punta Toro
PUU	Puumala
RVF	Rift Valley fever
SR	Sapporo rat
SSF	Sicilian sandfly fever
SSH	snowshoe hare
TOS	Toscana
TSW	tomato spotted wilt
VSV	vesicular stomatitis virus
UUK	Uukuniemi

Chapter 1

INTRODUCTION.

1. Introduction and Classification of the Bunyaviridae family

It has been almost fifty years since Bunyamwera virus, the prototype virus of the Bunyaviridae family, was first isolated in the Bunyamwera area of the Semliki forest, Uganda (isolated in 1943, Smithburn et al, 1946). Since then the number of Bunyaviridae isolated has grown dramatically. Today the family comprises of over 300 viruses (Kingsford, 1991) grouped into five genera (Bunyavirus, Hantavirus, Nairovirus, Phlebovirus, and Tospovirus) making it one of the largest groupings of animal viruses. However despite the size, global distribution and presence of many individual virus pathogens within the ranks of the family it is only over the last few years that a greater understanding of the Bunyaviridae has been achieved. This leap in knowledge has been achieved principally through the application of molecular biology techniques to this area of animal virology.

The Bunyaviridae are single stranded RNA viruses of negative sense. Negative stranded RNA viruses are distinguished from positive stranded RNA viruses on the basis that the positive stranded virus particle lacks RNA polymerase activity and the RNA genome acts as mRNA. Once introduced into the cell the RNA is translated by cellular ribosomes into viral proteins. The RNA of negative sense viruses is not infectious and is dependent, once in the cell, upon the virion associated RNA polymerase to transcribe viral genomic RNA into the antigenomic, complementary sense RNA, which constitutes message sense RNA. This mRNA is then translated into viral proteins to enable continuation of the infectious process.

The Bunyaviridae genome is segmented and consists of three RNA segments. Those viruses included in the genera Bunyavirus, Hantavirus and Nairovirus can be regarded as conventional negative stranded RNA viruses such as the the unsegmented filoviruses, paramyxoviruses and rhabdoviruses and the 7 or 8 segmented orthomyxoviruses. In contrast viruses belonging to the Phlebovirus, and Tospovirus genera resemble the unconventional arenaviruses in that part of their segmented genome is both positive and negative sense ie ambisense. All viruses in the Bunyaviridae are however united by a series of common features which are used in the classification of the family.

The criteria for classification as a member of the Bunyaviridae family, as outlined by Bishop et al, 1980, are:

1. The virus particles are spherical (90-100nm in diameter) and enveloped.
2. Each virion contains three unique single stranded segments of RNA. These segments are designated according to size (L, large; M, medium; S, small.)
3. Closely related viruses are able to genetically interact by genome segment reassortment.
4. Each of the single stranded RNA segments in the virion is complexed individually into a helical ribonucleoprotein complex containing nucleocapsid protein and L protein (the presumptive polymerase).
5. The virion envelope contains two viral glycoproteins designated G1 and G2. The glycoproteins exhibit type specific antigenic determinants.
6. The viruses replicate in the cytoplasm of the infected cell and mature by budding into the smooth surface vesicles in or near the Golgi apparatus.

On the basis of serological tests and genetic relatedness, the majority of the Bunyaviridae have been classified into four genera: Bunyavirus, Hantavirus, Nairovirus and Phlebovirus (Francki et al, 1991). Members of each genus are further subdivided into categories of varying degrees of antigenic relatedness from serogroup, complex, serotype to subtype and variety. An example of the serological classification of a single genus is illustrated in table 1. The serological assays employed are complement fixation (CF), neutralisation, haemagglutination inhibition (HI) and more recently ELISA assays (Gonzalez-Scarano et al, 1982; Shope, 1985; Schmaljohn et al, 1988).The neutralisation and HI tests are specific for the antigenic determinants on viral glycoproteins encoded by the M segment (Gentsch et al, 1980). The CF reaction is mainly directed against determinants on the nucleocapsid protein encoded by the S segment. In general the nucleocapsid protein is more conserved than the glycoproteins, therefore as a rule viruses within a genus share CF antibodies and are further differentiated into serogroups on the basis of neutralisation and HI antibodies. Phleboviruses are exceptions in that the N protein is conserved to a lesser extent than the glycoproteins, and so the CF test is type specific and the HI test is cross-reactive, except for the Uukuniemi serogroup where in comparison with other phleboviruses it is the N protein that is more conserved, (Calisher et al, 1981; Shope, 1985; Calisher and Karabatson, 1988; Bishop, 1985; Francki et al, 1991).

2. Natural history of the Bunyaviridae

A. Geographical Distribution.

Individual viruses are named after their site of original geographical isolation and hence the cosmopolitan variety of names lends testament to the worldwide distribution of the Bunyaviridae. Indeed members of the Bunyaviridae family have been isolated from every

continent except Antarctica; despite this near worldwide distribution there appears to be a geographical localisation of viral serotypes to specific continents or geographical regions (Calisher, 1988) eg Bunyamwera virus to Africa and California encephalitis virus to North America (Bishop and Shope, 1979). The reason for such geographical isolation has not been determined although it may possibly be associated with adaption of the virus to susceptible host species within a geographical region and lack of susceptible host species outside that geographical boundary.

B. The Arthropod-borne Genera of the Bunyaviridae Family.

Members of the family are transmitted by biting arthropods except for members of the Hantavirus genus. Virus transmission in nature revolves around the replication of virus in a permissive invertebrate host and virus introduction into a vertebrate host during the course of a blood meal by the invertebrate vector. A wide range of invertebrate and vertebrate hosts, including man, are infected by members of the Bunyaviridae family and many members are important human and or veterinary pathogens.

The Bunyavirus genus is the largest of the five genera with over 160 viruses classified into 16 serogroups (Bishop, 1990). Almost all of these viruses are transmitted by mosquitoes, although members have also been associated with Culicoides midges. Bunyamwera virus, the type virus for the family Bunyaviridae and the Bunyavirus genus was first isolated from Aedes species mosquitoes (Smithburn et al 1946). This virus has subsequently been isolated from Aedes, Mansonia and Culex mosquitoes in the Central Africa region (Berge, 1975).

Named after the type virus, Nairobi sheep disease, the Nairovirus

genus consists of 7 serogroups and all members are transmitted by ticks (Casals and Tignor, 1980; Zeller et al, 1989). Nairobi sheep disease virus is found associated with Rhipicephalus ticks isolated from domestic animals and is localised to Central and Southern Africa (Berge, 1975). Crimean-Congo haemmorrhagic fever virus (CCHF), the causative agent of a potentially fatal human disease, is associated with a wide variety of Hyalomma ticks. CCHF virus is distributed over a wide geographic area including sub-Saharan Africa, eastern Europe and Asia (Gonzalez-Scarano and Nathanson, 1990). The most detailed studies of CCHF virus have been made in South Africa where the virus is highly endemic in cattle and large wild herbivores (Sheperd et al, 1987)

Until relatively recently a single serogroup with 13 members, all of which are transmitted by Ixodes ticks, made up a separate genus known as the Uukuvirus genus (Bishop, 1990). Uukuniemi virus, the prototype of the genus, was originally isolated in Finland in 1960 from a cattle tick and has been repeatedly isolated from sea birds (Gonzalez-Scarano and Nathanson, 1990). It has recently been established that uuku- and phlebo- viruses are genetically closely related and so the uukuviruses have been re-classified as a serogroup within the Phlebovirus genus (Francki et al, 1991).

At least 39 other viruses make up the Phlebovirus genus (Karabatsos, 1985; Tesh et al, 1982; Travassos da Rosa et al, 1983). These viruses are transmitted by mosquitoes and phlebotomine flies (Tesh, 1988). Phleboviruses have been isolated from phlebotomine flies in both hemispheres and all show antigenic relatedness to the type virus, sandfly fever Sicilian (SFS) virus (Gonzalez-Scarano and Nathanson, 1990). SFS virus can cause acute non-fatal influenza like illness, phlebotomous

TABLE 1. *Proposed serologic classification of viruses of family Bunyaviridae. Genus* Bunyavirus[a]

Anopheles A group
Anopheles A
CoAr3624[b]
ColAn57389[b]
Las Maloyas
Lukuni
Trombetas[b]
Tacaiuma
H32580[b]
SpAr2317[b] (Virgin River)
CoAr1071[b] (CoAr3627[b])

Anopheles B group
Anopheles B
Boraceia

Bunyamwera group
Bunyamwera
Batai (Calovo)
Birao
Cache Valley
(Tlacotalpan)
Maguari (CbaAr426[b])
Playas
Xingu[b]

Germiston
Ilesha
Lokern
Northway
Santa Rosa
Shokwe
Tensaw
Kairi
Main Drain
Wyeomyia
Anhembi (Iaco, BeAr328208[b])
Macaua[b]
Sororoca
Taiassui[b]
Bozo[c]
Fort Sherman[b,c]
Mboke[c]
Ngari[c]
Tucunduba[b,c]
AG83-1746[b,c]

Bwamba group
Bwamba
Pongola

C Group
Caraparu
Caraparu (BeH5546,[b] Trinidad[b])
Ossa
Apeu
Vinces
Bruconha[b]
Madrid
Marituba
Murutucu
Restan
Nepuyo (63U11[b])
Gumbo Limbo
Oriboca
Itaqui

California group
California encephalitis
Inkoo
La Crosse (snowshoe hare)
San Angelo
Tahyna (Lumbo[b])
Melao
Keystone
Jamestown Canyon (South River,[b] Jerry Slough)
Serra do Navio
trivittatus
Guaroa
AG83-497[b,c]

Capim group
Capim
Acara
Moriche
Benevides
Bushbush
Benfica
GU71u344[b]
Juan Diaz
Guajara (GU71u350[b])

Gamboa group
Gamboa
Pueblo Viejo (75-2621[b])
Alajuela[b]
San Juan (78V-2441,[b] 75V-2374[b])
Brus Laguna[b,c]

Guama group
Guama
Ananindeua
Moju
Mahogany Hammock
Bertioga
Cananeia
Guaratuba
Itimirim
Mirim
Bimiti
Catu
Timboteua

Koongol group
Koongol
Wongal

Minatitlan group
Minatitlan
Palestina

Olifantsvlei group
Olifantsvlei (Bobia)
Botambi
Dabakala[c]
Oubi[c]

Patois group
Patois
Abras
Babahoyo
Shark River
Zegla
Pahayokee

Simbu group
Simbu
Akabane
Yaba-7[b]
Manzanilla
Ingwavuma
Inini
Mermet
Buttonwillow
Nola
Oropouche
Facey's Paddock[b]
Utinga
Utive[b]
Sabo
Tinaroo
Sathuperi (Douglas)
Shamonda
Sango
Peaton
Shuni
Aino (Kaikalur, Samford[b])
Thimiri

Tete group
Tete
Bahig
Matruh
Tsuruse
Batama

Turlock group
Turlock
Lednice
Umbre
M'Poko
Yaba-1[b]

Ungrouped
Kaeng Khoi
Leanyer[b]

[a] Viruses are classified in three steps indicated by degrees of indentation—complex, virus, and subtype; viruses in parentheses are varieties.
[b] These viruses are not in the published or working *International Catalogue of Arboviruses* as of 1988
[c] Unassigned within the group.

Table taken from Bishop (1990)

fever in humans, but the most important pathogen of the genus is Rift Valley fever virus. This is a particularly virulent pathogen of domestic animals and man. Originally isolated during an investigation in 1930 of an epidemic amongst sheep on a farm in the Rift Valley in East Africa, the virus was shown to be mosquito borne and the causative agent of disease in goats, cattle and humans in the epidemic area (Daubey and Hudson, 1931). The virus can be transmitted by a wide variety of Aedes and Culex mosquito species, which has facilitated the virus spread as far north as Egypt (Gonzalez-Scarano and Nathanson, 1990). The Rift Valley fever virus fluctuates between epizootic periods associated with the rainy season and high mosquito density when the virus is readily isolated, and enzootic periods when isolation is almost impossible (Megan and Shope,1981).

C. Anthropod vector - virus interactions

Infection of insect vectors is asymptomatic and results in a life long persistent infection. Adult insects can acquire the virus in one of two ways;

(a) Oral infection, in which the insect ingests a blood meal from an infected vertebrate host (Berge, 1975).

(b) Venereal transmission between adult insects (Thompson and Beaty, 1978).

It should be noted that the relative epidemiological importance of these two modes of infection under natural conditions has not been evaluated. Baldridge et al (1989), however, suggested that venereal and transovarial transmission (discussed below) are important in maintaining a low rate of viral evolution.

Parental transmission of the virus to offspring is by transovarial infection rather than by the transovum route, as virus can be isolated

from surface sterilised eggs (Beaty and Thompson, 1976). This method of infection has been demonstrated both experimentally (Baldridge et al, 1989) and from a number of field isolates from eggs, larvae and pupae, and is thought to be of particular importance during periods of vector inactivity i.e. overwintering, when infected females deposit eggs in the autumn and in the spring these eggs hatch to yield infected larvae (Watts et al, 1974; Beaty and Bishop, 1988).

D. Hantavirus transmission and infection.

There is no evidence for an arthropod vector associated with members of the Hantavirus genus. Each hantavirus has a single or a few host rodent species in which it can maintain a persistent asymptomatic infection. Viral transmission is thought to be caused by aerosolised rodent excretions (Lee, 1982). On the basis of the degree of serological cross-reactivity, hantaviruses have been classified into several serotypes. Hantaan (strain 76-118), Puumala (strain Hallnas B1), Seoul (strain 80-39), Prospect Hill, Leaky and Four Corners virus are prototypes of the six major serotypes (Baek et al, 1988; Hughes et al, 1993). Hantaan, Puumala and Seoul virus complexes are associated with human diseases collectively known as haemorrhagic fever with renal syndrome (HFRS) (WHO, 1982; Lahdevirta, 1982), whereas the Four Corners virus is associated a relatively recent syndrome described as hantavirus pulmonary syndrome (HPS). Hantaviruses have been detected in indigenous rodents throughout the world both in areas where HFRS occurs and in those where the disease syndrome has not been reported. Epidemic haemorrhagic fever was first recognised among United Nations soldiers in Korea in 1951 and was called Korean haemorrhagic fever. Similar diseases were described in the USSR and China. Wild rodents were suspected of being the source of infection and Hantaan virus, the genus prototype, was first isolated from a field mouse, Apodemus agrarius (Lee et al,

1978). Viral antigens were detected in lung tissue of the mouse by immunofluorescence studies using sera from patients suffering from HFRS. The agent could be passed from infected to uninfected A. agrarius but not to other rodent species. The agent was shown to be a virus belonging to the Bunyaviridae family, and was called Hantaan virus after the Hantaan River in the region where the original cases had been described. The isolation of Hantaan virus quickly led to identification of other hantaviruses such as Puumala virus from Scandinavia, which is associated with a species of vole (Clethrionymys glareolus) (Brummer-Korvenkontio et al, 1980), and Seoul virus associated with rats (Lee et al, 1982).

E. Human pathogenic infections by Bunyaviridae

Human disease has been associated with members of the Bunyavirus, Hantavirus, Nairovirus and Phlebovirus genera. In most cases these infections are incidental and occur only when humans intrude on the natural routes of transmission (Gonzalez-Scarano and Nathanson, 1990). Disease syndromes range from influenza-like illness with transient mild fevers through to encephalitis and severe haemorrhagic fevers (Shope, 1985). It should be noted that a particular disease syndrome is not restricted to any one genus or serogroup and different viruses in the same serogroup or genus may cause different syndromes. Human viral infection in most instances is caused by bites from infected insect vectors, though hantavirus infections are caused by close contact with the natural rodent hosts and the virus conveyed by the same means as rodent-to-rodent infections, i.e. by aerosolised rodent excretia (Lee, 1982). Person to person transmission of hantaviruses via this route has not been reported.

Crimean-Congo haemorrhagic fever nairovirus and Rift Valley fever

phlebovirus, which can be transmitted by bites from ticks or mosquitoes respectively, can also be transmitted to humans by a non-vectored route i.e. exposure to infected animal tissues. Crimean-Congo haemorrhagic fever virus infection can also occur by exposure to infected humans or their body fluids. It is presumed that these non-vectored forms of infection are by aerosol or percutaneous routes (Burney et al, 1980; Sabin and Blumberg, 1947).

F. Tospovirus transmission, infection and classification

The causative agent of the plant disease tomato spotted wilt was first shown to be caused by a virus by Samuel et al,(1930). Tomato-spotted wilt virus (TSWV) has a host range much wider than any other plant virus: at least four hundred plant species from fifty botanical families, both monocots and dicots, are naturally infected by TSWV (Peters et al, 1991). Among the host plants are a number of important crops including tobacco, lettuce, onion and pea (de Haan et al, 1989). The virus also infects ornamentals such as Impatiens, Ageratum, and Chrysanthemum (Goldbach and de Haan, 1993). The virus has a worldwide distribution and is horizontally transmitted by nine species of thrips (de Haan et al, 1989) and is able to replicate in these insect vectors (Wijkamp et al, 1993). In addition the virus can also be vertically transmitted through the seeds of certain plants (Beaty and Calisher, 1991).

Three serologically distinct TSWV groups (I-III) were defined using antibodies directed against their nucleocapsid protein (de Avila et al, 1990, 1993; Law and Moyer, 1990). The serogroup I isolates cross react with antibodies to serogroup II, but not with antibodies to group III. Serogroup III consists of isolates from Impatiens plants and have been

defined as a different virus, Impatiens necrotic spot virus (INVS) (Law et al, 1991). The physicochemical nature and morphogenesis of these viruses are unique amongst plant viruses but similar in nature to the Bunyaviridae (de Haan et al, 1989) and early studies (de Haan et al, 1990) suggested the coding strategy of the TSWV genome is similar to that of phleboviruses. However, the absence of significant sequence homology between TSWV and other members of the Bunyaviridae family suggests that TSWV represents a new genus within the family and the name Tospovirus has been proposed (Peters, 1991).

3. Bunyaviridae Virion Structure.

Electron microscopic studies have demonstrated that virion surface structure varies between members of different genera (Martin et al, 1985). It is presumed that this is because of different arrangements of surface proteins on the virus particle (Martin et al, 1985; Schmaljohn and Patterson, 1990). Bunyaviridae virions are, however, generally spherical, 80-120nm in diameter and have a fringe of surface glycoprotein projections 5-10nm in length which are embedded in a lipid bilayer 4nm thick (Talmon et al, 1987). A schematic diagram of a Bunyaviridae virus particle is provided in Figure 1. Each virion has two species of glycoproteins termed G1 and G2 although Folke et al (1981) detected three species of glycoprotein in Hazara nairovirus. It should be noted that by convention the larger molecular weight glycoprotein is termed G1. The overall chemical composition of an uukuvirus virion was estimated to be 2% RNA, 58% protein, 33% lipid and 7% carbohydrate (Obijeski et al, 1976a; Obijeski and Murphy, 1977).

Bunyaviridae particles possess three internal nucleocapsids. Each nucleocapsid consists of a single species of RNA genome segment (large

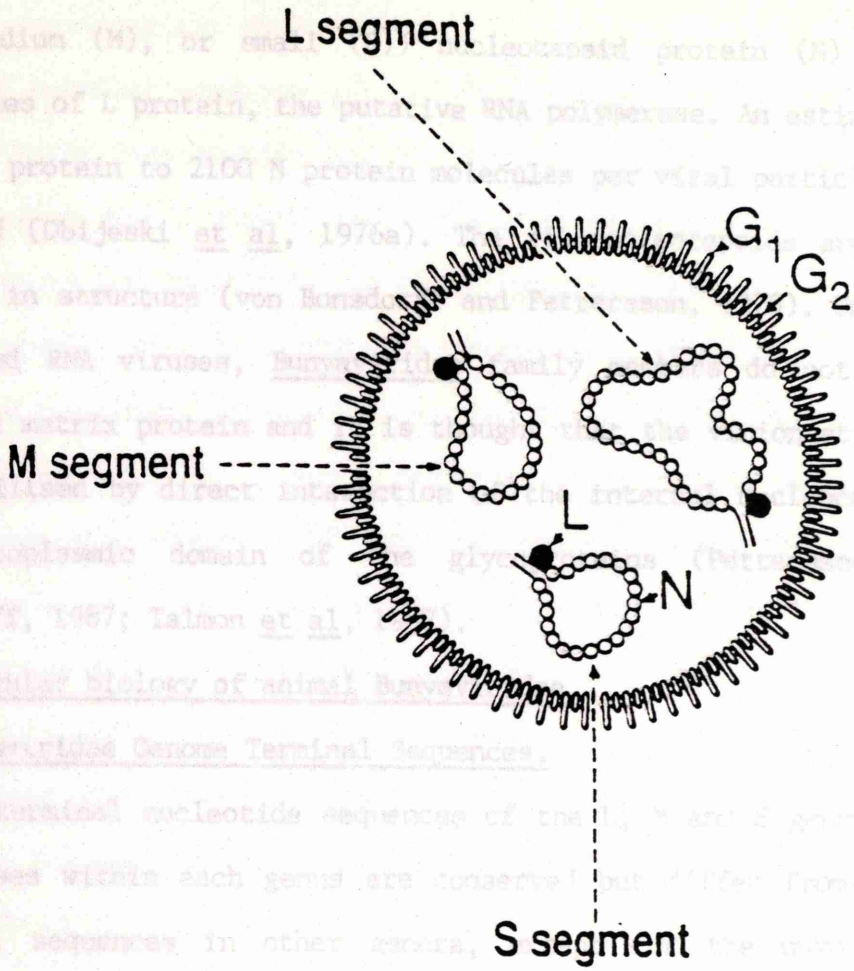


Figure 1.
Schematic representation of a virus particle of the *Bunyaviridae* family. The viral structural proteins L, G1, G2 and N are indicated. The three internal nucleocapsid structures representing each of the three genome RNA segments (L, M and S) individually integrated with N and L proteins are also indicated. Figure adapted from Golbach and de Haan (1993).

(L), medium (M), or small (S)) nucleocapsid protein (N) and minor quantities of L protein, the putative RNA polymerase. An estimated ratio of 25 L protein to 2100 N protein molecules per viral particle has been reported (Obijeski et al, 1976a). The ribonucleocapsids appear to be helical in structure (von Bonsdorff and Pettersson, 1975). Unlike other enveloped RNA viruses, Bunyaviridae family members do not encode an internal matrix protein and it is thought that the virion structure may be stabilised by direct interaction of the internal nucleocapsids with the cytoplasmic domain of the glycoproteins (Pettersson and von Bonsdorff, 1987; Talmon et al, 1987).

4. Molecular biology of animal Bunyaviridae

A. Bunyaviridae Genome Terminal Sequences.

The 3' terminal nucleotide sequences of the L, M and S genome segments of viruses within each genus are conserved but differ from the genome terminal sequences in other genera, except for the uukuviruses and phleboviruses which have identical 3' terminal sequences (Figure 2). The 5' terminal sequences of genome segments have also been determined for a number of representatives from different genera and are complementary to those sequences at the 3' termini (Figure 3).

Under the electron microscope Bunyaviridae nucleocapsids were found to be circular (Obijeski et al, 1976b; Pettersson and von Bonsdorff, 1975; Samso et al, 1975) despite the fact that the RNA segments contain 5' triphosphate and 3' hydroxyl ends (Obijeski et al, 1976). This circularity was postulated to be due to the complementary nature of the 5' and 3' termini which would allow the formation of stable base pairing between segment ends in a panhandle structure. The conserved genus specific terminal nucleotide sequences vary from 8 to 11 bases in length dependent on the genus. The conserved genus specific

terminal sequences are shown in Figure 2.

The terminal sequences of the three genome segments of Bunyamwera and La Crosse bunyaviruses are compared in Figure 3. The terminal 11 bases at both the 3' and 5' ends in all genome segments are conserved, complementary and constitute the genus specific consensus sequences. The complementarity extends beyond the consensus sequence for a further 15 to 20 nucleotides, depending on the segment. Following the genus consensus sequence the S and L segments of both virus genomes exhibit segment specific sequences of four nucleotides whereas the M segment of both viruses exhibit virus specific M segment sequence (Figure 3). Dependent on the genome segment complementarity between the 3' and 5' termini is maintained beyond the consensus sequence such that the longer the segment the longer the panhandle. The position and nature of the loops and bulges does not appear to be conserved.

Interestingly, both viruses possess within the genus consensus sequence an "allowable" U G base pair at position 9 which would constitute an A C mismatch in the antigenome RNA. This would therefore be a potential method of differentiating genomes from antigenomes. However mismatches are not present in the conserved sequences of the other genera. The RNA genome segments of influenza virus also possess complementary 5' 3' termini. Hsu et al (1987) used psoralen cross-linking to demonstrate that the ends of the genome RNAs within intracellular and virion nucleocapsids are base paired. Raju and Kolakofsky (1989a) performed similar psoralen cross-linking experiments on La Crosse virus nucleocapsids in virions and in infected cells. Their results demonstrate that genome segment base pairing does occur, however the level of cross-linking obtained was lower than that for influenza

Figure 2
Consensus 3' and 5' terminal nucleotide sequences of Bunyaviridae genome RNA segments

Bunyavirus	3'	UCAUCACAUGA.....UCGUGUGAUGA	5'
Hantavirus	3'	AUCAUCAUCUG.....AUGAUGAU	5'
Nairovirus	3'	AGAGUUUCU.....AGAAACUCU	5'
Phlebovirus	3'	UGUGUUUC.....GAAACACA	5'
Tospovirus	3'	UCUCGUUAG.....CUAACGAGA	5'

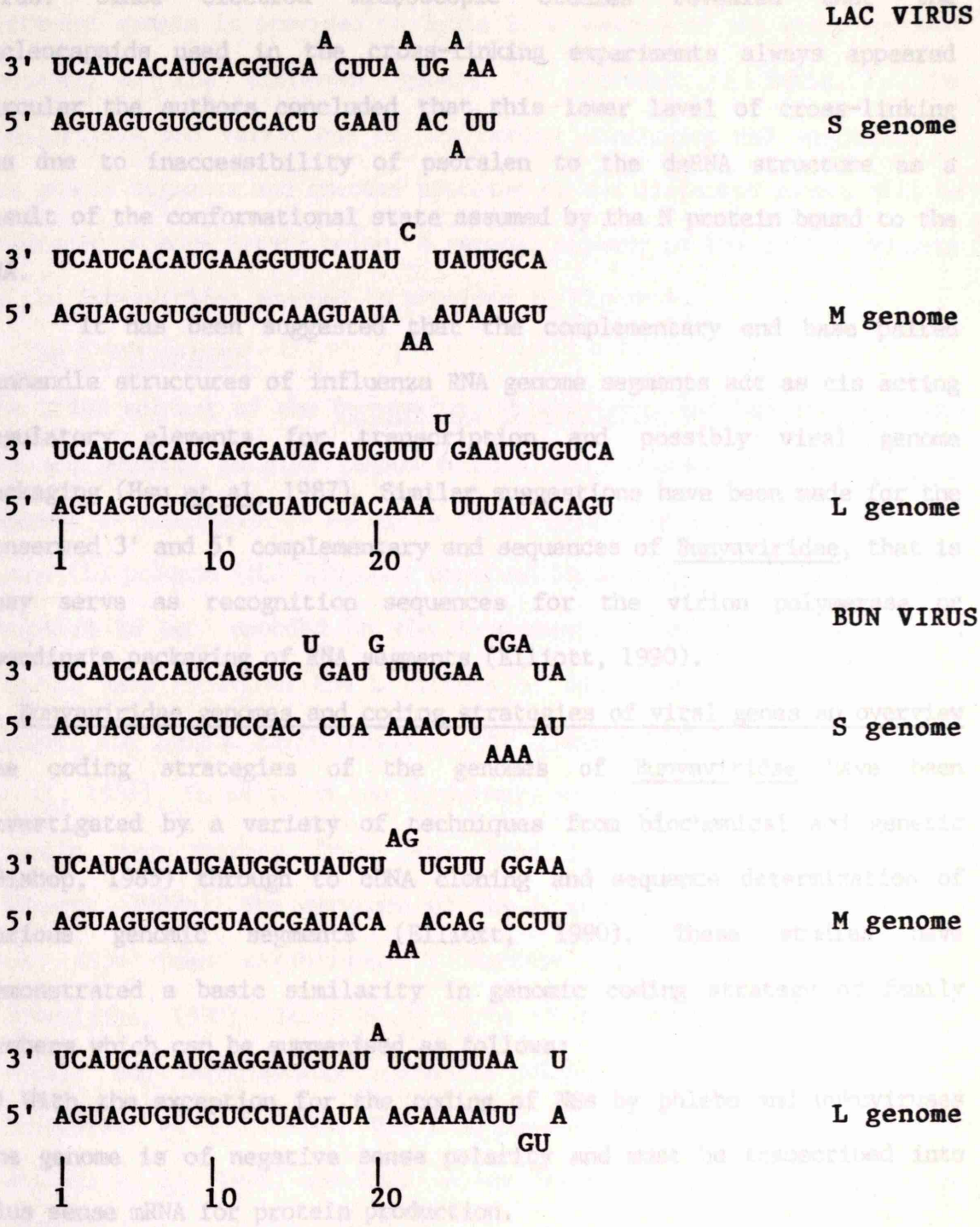


FIGURE 3 A comparison of the complementary sequences and possible base-paired structures of the LAC virus genome segments and the BUN virus genome segments (Elliott, 1990; Hacker et al 1990). Unmatched or mismatched bases are shown above or below the duplex.

virus. Since electron microscopic studies revealed that the nucleocapsids used in the cross-linking experiments always appeared circular the authors concluded that this lower level of cross-linking was due to inaccessibility of psoralen to the dsRNA structure as a result of the conformational state assumed by the N protein bound to the RNA.

It has been suggested that the complementary end base paired panhandle structures of influenza RNA genome segments act as cis acting regulatory elements for transcription and possibly viral genome packaging (Hsu et al, 1987). Similar suggestions have been made for the conserved 3' and 5' complementary end sequences of Bunyaviridae, that is they serve as recognition sequences for the virion polymerase or coordinate packaging of RNA segments (Elliott, 1990).

B. Bunyaviridae genomes and coding strategies of viral genes an overview

The coding strategies of the genomes of Bunyaviridae have been investigated by a variety of techniques from biochemical and genetic (Bishop, 1985) through to cDNA cloning and sequence determination of various genomic segments (Elliott, 1990). These studies have demonstrated a basic similarity in genomic coding strategy of family members which can be summarised as follows:

1) With the exception for the coding of NSs by phlebo and uukuviruses the genome is of negative sense polarity and must be transcribed into plus sense mRNA for protein production.

2) The L RNA encodes the L protein.

3) The M RNA encodes the virion glycoproteins expressed as a polyprotein precursor.

4) The S RNA encodes the nucleocapsid protein.

An overall summary of the sizes of the virus RNA genome segments from different genera is provided in Table 2. A summary of the virus encoded proteins of the different genera is provided in Table 3. The similarities and variations of the coding strategies and sequences of the genome segments and encoded proteins of the different genera will be discussed in more detail below. A general summary of the coding strategy of the Bunyaviridae genomes is provided in Figure 4.

C. The L RNA segment

The L RNA segment of the Bunyavirus, Phlebovirus, and Hantavirus genera are all similar in size (about 6 to 7 Kb), whereas the Nairovirus L segment is estimated to be up to twice this size (Elliott, 1990). The large (L) protein (M_r> 200,000) observed in all Bunyaviridae members is proposed to be encoded on the L segment. Independent re-assortment studies have localised the L protein of Batai, Bunyamwera, La Crosse, Maguari and Tahyna bunyaviruses to the L segment (Elliott 1989b; Endres et al, 1989). In addition the Bunyamwera virus L segment sequence and L protein open reading frame have been determined from cDNA clones (Elliott, 1989b). The sequence of the L segment of three hantaviruses have also been established ; Hantaan virus (Hantaan serogroup) (Schmaljohn, 1990); Seoul 80-39 virus (Seoul serogroup) (Antic et al, 1991a); and Nephropathia epidemica virus, NEV (Puumala serogroup) (Stohwasser et al, 1991). The L segment sequences of Uukuniemi virus (Elliott et al, 1992) and Rift valley fever phlebovirus (Muller et al, 1990) have also been established.

The L segment of Bunyamwera (BUN) virus is 6,875 nucleotides in length and has an open reading frame that encodes a protein of 2,238 amino acids (M_r 259,000) in the viral complementary RNA (Elliott, 1989b)

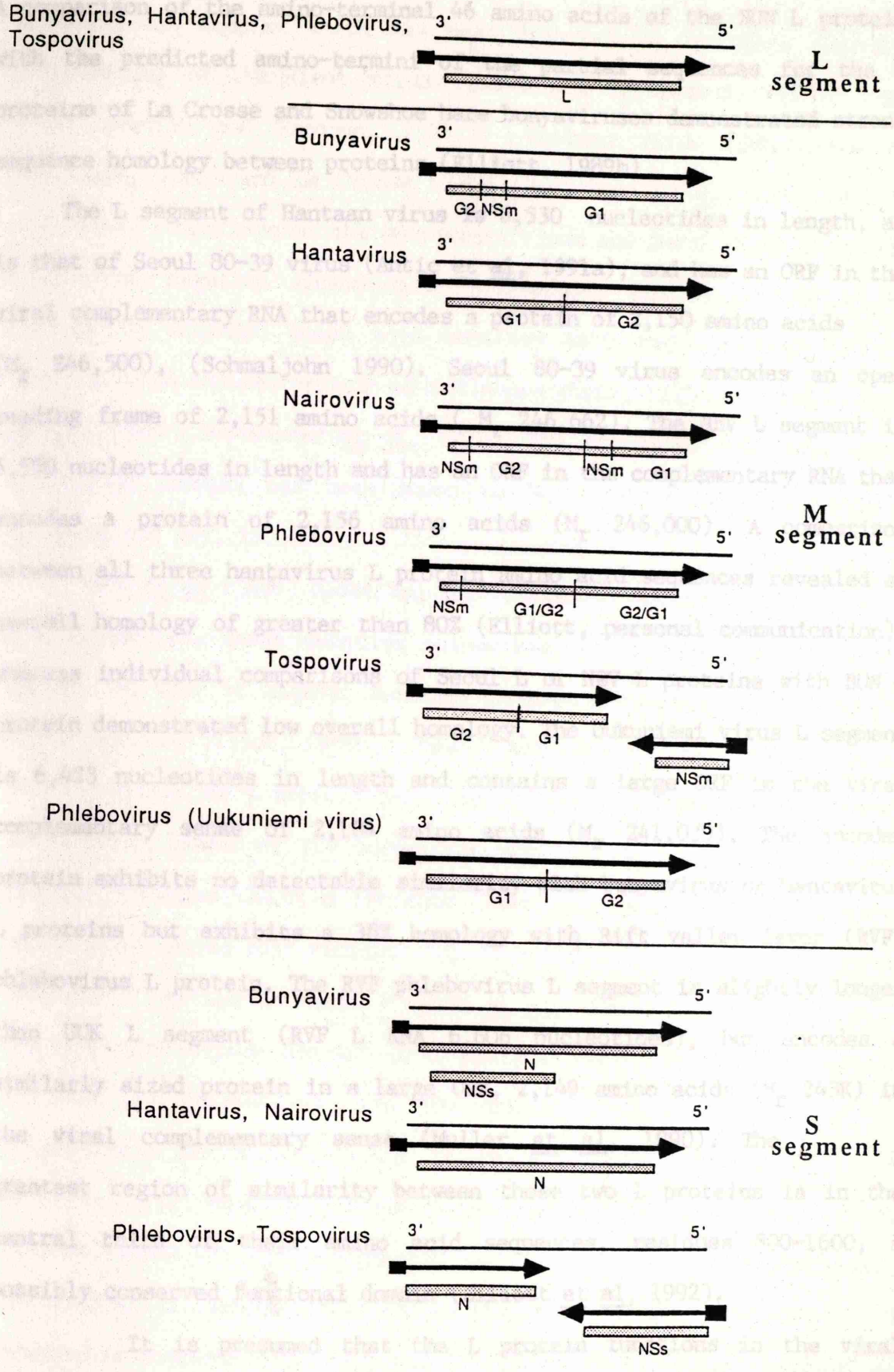
Table 2.
The sizes of the genome segments of the Bunyaviridae. Table adapted from Elliott et al (1991). The sizes are given in nucleotides and are based on nucleotide sequence data from cloned cDNAs, except for those values in brackets which are based on estimates from electrophoresis of RNAs. The Hantavirus L segment values were obtained from Antic et al (1991); Schmaljohn (1990); Stohwasser et al (1991). The Phlebovirus L segment values were obtained Elliott et al (1992) and Muller (1990). The Tospovirus values were obtained from de Haan et al (1990; 1991); Kormelink et al (1992); Law et al (1992). The Nairovirus S segment values were obtained from Marriott and Nutall (1992) and Ward et al (1990).

Bunyaviridae genus	L segment	M segment	S segment
Bunyavirus	6875	4458-4534	850-984
Hantavirus	6530-6530	3616-3682	1696-1785
Nairovirus	(11000-14400)	(4400-6300)	1672-1712
Phlebovirus	6423-6606	3884-4330	1720-1904
Tospovirus	8897	4972	2916

Table 3.
Proteins encoded by the Bunyaviridae (sizes given in kilo daltons). Table adapted from Elliott et al (1991). The Hantavirus L value was obtained from Antic et al (1991); Schmaljohn (1990); Stowwasser et al (1991). The Phlebovirus L values were obtained from Elliott et al (1992) and Muller et al (1990). The Phlebovirus values shown in brackets are for the Uukuniemi virus serogroup, which was recently re-classified as a member of the Phlebovirus genus. The Tospovirus values were obtained from de Avila et al (1993); de Haan et al (1990; 1991); Law et al (1992). The Nairovirus N values were obtained from Marriott and Nutall (1992) and Ward et al (1990).

RNA segment/ protein	Bunyavirus	Hantavirus	Nairovirus	Phlebovirus	Tospovirus
L segment					
L	259	246	>200	243 (231)	331
M segment					
G1	108–120	68–76	72–84	55–70 (70–75)	78
G2	29–41	52–58	30–45	50–60 (65–70)	58
NSm	15–18	None	None	78/14 (None)	34
S segment					
N	19–25	50–54	48–54	24–30 (20–29)	29
NSs	10–13	None	None	29–31 (30)	52

Figure 4.
Coding strategies of Bunyaviridae genomes (not to scale). *Thin lines*, genomic RNAs; *arrows*, mRNAs (■, 5' end; ►, 3' end); *solid boxes*, gene products. This diagram is adapted from Elliott (1990).


Bunyavirus, Hantavirus, Phlebovirus, Tospovirus
3'
5'
L
L segment
Bunyavirus
G2 NSm
G1
Hantavirus
G1
G2
Nairovirus
NSm
G2
NSm
G1
M segment
Phlebovirus
NSm
G1/G2
G2/G1
Tospovirus
G2
G1
NSm
Phlebovirus (Uukuniemi virus)
G1
G2
Bunyavirus
N
NSs
Hantavirus, Nairovirus
N
S segment
Phlebovirus, Tospovirus
N
NSs

A comparison of the amino-terminal 46 amino acids of the BUN L protein with the predicted amino-termini of the partial sequences for the L proteins of La Crosse and Snowshoe hare bunyaviruses demonstrated strong sequence homology between proteins (Elliott, 1989b)

The L segment of Hantaan virus is 6,530 nucleotides in length, as is that of Seoul 80-39 virus (Antic et al, 1991a), and has an ORF in the viral complementary RNA that encodes a protein of 2,150 amino acids (M_r 246,500), (Schmaljohn 1990). Seoul 80-39 virus encodes an open reading frame of 2,151 amino acids (M_r 246,662). The NEV L segment is 6,550 nucleotides in length and has an ORF in the complementary RNA that encodes a protein of 2,156 amino acids (M_r 246,000). A comparison between all three hantavirus L protein amino acid sequences revealed an overall homology of greater than 80% (Elliott, personal communication), whereas individual comparisons of Seoul L or NEV L proteins with BUN L protein demonstrated low overall homology. The Uukuniemi virus L segment is 6,423 nucleotides in length and contains a large ORF in the viral complementary sense of 2,104 amino acids (M_r 241,039). The encoded protein exhibits no detectable similarity with bunyavirus or hantavirus L proteins but exhibits a 38% homology with Rift valley fever (RVF) phlebovirus L protein. The RVF phlebovirus L segment is slightly longer than UUK L segment (RVF L RNA 6,606 nucleotides), but encodes a similarly sized protein in a large ORF, 2,149 amino acids (M_r 243K) in the viral complementary sense (Muller et al, 1990). The greatest region of similarity between these two L proteins is in the central third of their amino acid sequences, residues 800-1600, a possibly conserved functional domain (Elliott et al, 1992).

It is presumed that the L protein functions in the viral

transcriptase complex which has been detected in detergent disrupted preparations of Uukuniemi virus (Ranki and Pettersson, 1975), Lumbo virus (Bouloy and Hannoun, 1976), Hantaan virus (Schmaljohn and Dalrymple, 1983) and La Crosse virus (Patterson et al, 1984). A more widespread comparison of the Bunyamwera virus and Seoul virus L proteins with other negative strand virus polymerases did not result in any regions of extended amino acid homology with the exception of weak homology with the PB1 protein of influenza virus, (Elliott, 1989b; Antic et al, 1991a; Stohwasser et al, 1991). The PB1 protein of influenza A polymerase complex has been shown to be involved in RNA elongation (Romanos and Hay, 1984).

Poch et al (1989) described four conserved motifs in RNA-dependent RNA polymerses. These putative polymerase motifs have been identified within the area of highest homology between influenza PB1 protein and BUN L protein (Elliot, 1989b). These 'Poch' motifs are conserved in all known Bunyaviridae L protein sequences (Jin and Elliott, 1992). Figure 5 shows an alignment of the polymerase motifs from known Bunyaviridae L protein sequences. Each of the four 'Poch' motifs (A,B,C and D) contain an invariant amino acid residue: Asp in motif A, Gly in motif B, Asp-Asp in motif C and Lys in motif D (Poch et al, 1989). In motif C the conserved doublet Asp-Asp (D-D) motif is common to all RNA dependent RNA and DNA polymerases. The SDD triplet seen in motif C of the Bunyaviridae polymerase domain is characteristic of the polymerase proteins of all negative and ambisense segmented genome viruses (Jin and Elliott, 1992). In the unsegmented negative strand viral polymerases a GDN triplet is characteristic and in plus stranded RNA polymerases a GDD triplet is characteristic of motif C (Jin and Elliott, 1992; Poch et al,

1989).

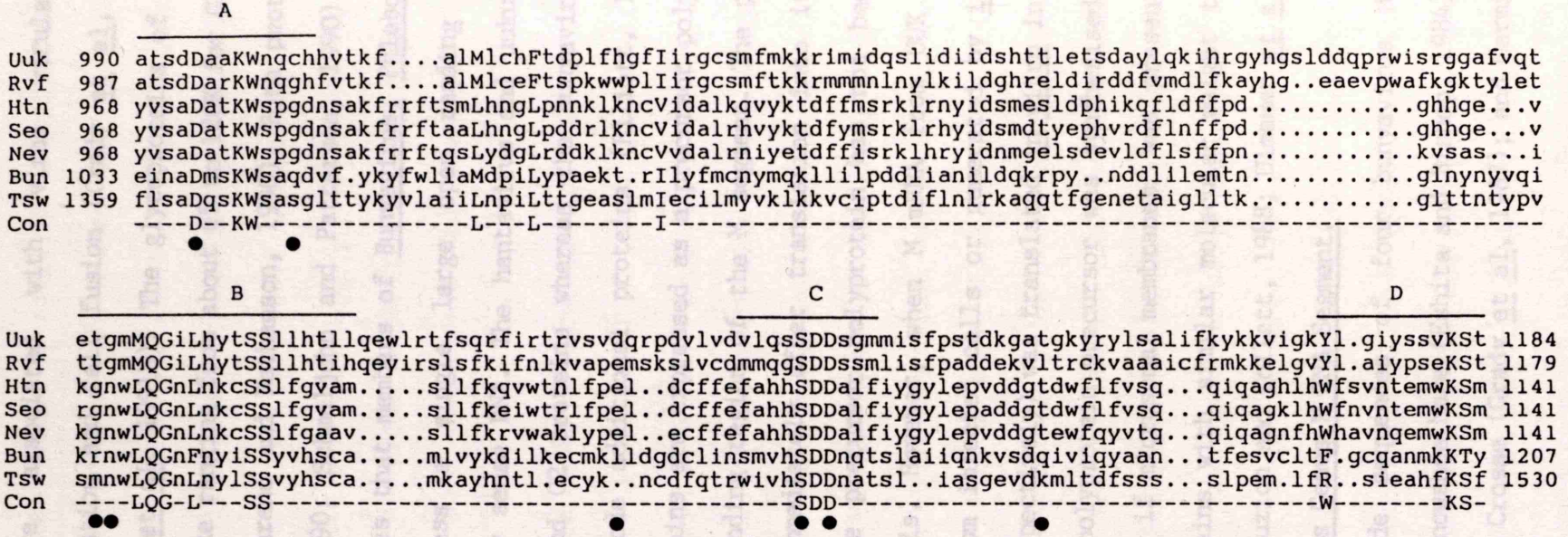
Evidence that the L protein is the viral transcriptase has been reported by Jin and Elliott (1991). By placing cDNA containing the L ORF of Bunyamwera virus in a recombinant vaccinia virus, L protein was expressed in vaccinia infected cells. The expressed protein was found to have the same electrophoretic migration as L protein synthesised in Bunyamwera virus infected cells; to react with L protein monospecific antisera; and lastly, to possess RNA synthesis activity (Jin and Elliott, 1991). More recently Jin and Elliot (1992) carried out specific mutagenesis to the putative polymerase domain of the BUN L protein and the effects these alterations had on the the RNA synthesis activity of transiently expressed L protein was assesed in a nucleocapsid template transfection assay. The residues which were altered are indicated in Figure 5. Jin and Elliott (1992) reported that changes to conserved residues within individual motifs resulted in the loss of L protein activity. Even conversion of the motif C SDD triplet to GDN, found in the non segmented negative strand viral polymerase, resulted in the loss of L polymerase activity. Whereas substitutions made to non conserved motifs, either within individual motifs or outwith a motif, had no effect on L protein activity.

These preliminary results provide evidence that the central region of the BUN L protein contains the polymerase domain but further work is required to fully identify those residues, conserved between different Bunyaviridae L proteins and within the motifs, that are essential for polymerase activity.

D. The M RNA segment

The M segment encodes two structural glycoproteins, G1 and G2. The

Figure 5.
Alignment of the putative polymerase domains of Bunyaviridae L proteins (taken from Jin and Elliott (1992)). The alignment was generated using the programs PILEUP and PRETTY in the GCG package (Devereux et al, 1984), and the regions corresponding to the four motifs (A to D) identified by Poch et al (1989) are overlined. 'Con' shows the residues conserved in all Bunyaviridae L proteins and the large dots indicate the amino acid residues which were changed in the Bunyamwera virus L protein. The sequences compared were Uuk, Uukuniemi (Elliott et al, 1992); Rvf, Rift Valley fever (Muller et al, 1991); Htn, Hantaan (Schmaljohn, 1990); Seo, Seoul (Antic et al, 1991); Nev, nephropathia epidemica (Stohwasser et al, 1991); Bun, Bunyamwera (Elliott, 1989b); Tsw, tomato spotted wilt (de Haan et al, 1991) viruses.


A
Uuk 990 atsdDaaKWnqchhvtkf....alMlchFtdplfhgfIirgcsmfmkkrimidqslidiidshttletsdaylqkihrgyhgslddqprwisrggafvqt
Rvf 987 atsdDarKWnqghfvtkf....alMlceFtspkwwplIirgcsmftkkrmmmnlnylkildghreldirddfvmdlfkayhg..eaevpwafkgktylet
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Seo 968 yvsaDatKWspgdnsakfrrftaaLhngLpddrlkncVidalrhvyktdfymsrklrhyidsmdtyephvrdflnffpd.....ghhge...v
Nev 968 yvsaDatKWspgdnsakfrrftqsLydgLrddklkncVvdalrniyetdffisrklhryidnmgelsdevldflsffpn.....kvsas...i
Bun 1033 einaDmsKWsaqdvf.ykyfwliaMdpiLypaekt.rIlyfmcnymqkllilpddlianildqkrpy..nddlilemtn.....glnynyvqi
Tsw 1359 flsaDqsKWsasglttykyvlaiiLnpiLttgeaslmIecilmyvklkkvciptdiflnlrkaqqtfgenetaiglltk.....glttntypv
Con ----D--KW---------L---L-------I----------
B
C
D
Uuk etgmMQGiLhytSSllhtllqewlrtfsqrfirtrvsvdqrpdvlvdvlqsSDDsgmmisfpstdkgatgkyrylsalifkykkvigkYl.giyssvKSt 1184
Rvf ttgmMQGiLhytSSllhtihqeyirslsfkifnlkvapemskslvcdmmqgSDDssmlisfpaddekvltrckvaaaicfrmkkelgvYl.aiypseKSt 1179
Htn kgnwLQGnLnkcSSlfgvam.....sllfkqvwtnlfpel..dcffefahhSDDalfiygylepvddgtdwflfvsq...qiqaghlhWfsvntemwKSm 1141
Seo rgnwLQGnLnkcSSlfgvam.....sllfkeiwtrlfpel..dcffefahhSDDalfiygylepaddgtdwflfvsq...qiqagklhWfnvntemwKSm 1141
Nev kgnwLQGnLnkcSSlfgaav.....sllfkrvwaklypel..ecffefahhSDDalfiygylepvddgtewfqyvtq...qiqagnfhWhavnqemwKSm 1141
Bun krnwLQGnFnyiSSyvhsca.....mlvykdilkecmklldgdclinsmvhSDDnqtslaiiqnkvsdqiviqyaan...tfesvcltF.gcqanmkKTy 1207
Tsw smnwLQGnLnylSSvyhsca.....mkayhntl.ecyk..ncdfqtrwivhSDDnatsl..iasgevdkmltdfsss...slpem.lfR..sieahfKSf 1530
Con ----LQG-L---SS----------SDD----------W--------KS-

proteins are associated with virus virulence, neutralisation, haemagglutination and cell fusion (Grady et al, 1987: Arikawa et al, 1989: Antic et al, 1991b). The glycoproteins of different genera vary widely in size ranging from about 65 to 120K for G1 and 34 to 65K for G2 (Gonzalez-Scarano and Nathanson, 1990). Both proteins are glycosylated (Elliott, 1990; Schmaljohn and Patterson, 1990). Nucleotide sequence data indicates that members of Bunyavirus, Phlebovirus, and Hantavirus genera possess a single large open reading frame in the viral complementary sense RNA. The hantavirus and uukuvirus M segments only encode G1 and G2 proteins whereas the bunyavirus and phlebovirus M segments encode additional proteins (Elliott, 1990). All M segment encoded proteins are expressed as a precursor polyprotein equivalent to the entire coding region of the M segment. The polyprotein is cleaved during or immediately after translation into its individual protein products. The precursor polyprotein has not been detected in virus infected cells. However, when M mRNA from UUK or RVF phleboviruses (isolated from infected cells or generated by in vitro transcription from cDNA respectively) was translated in vitro in cell-free translation systems the polyprotein precursor was synthesised. This polyprotein is not observed if microsomal membranes was present during translation. Instead proteins with similar molecular weight to viral glycoproteins were found (Suzich and Collett, 1988; Ulmanen et al, 1981).

(i) Bunyavirus Genus M RNA Segment.

The nucleotide sequences of four bunyavirus M segments have been determined: snowshoe hare (Eshita and Bishop, 1984); Bunyamwera (Lees et al, 1986); La Crosse (Grady et al, 1987); and Germiston (Pardigon et

al,1988). The M segments range in length from 4458 to 4534 bases. The precursor polyprotein ORF encodes the two viral glycoproteins G1 (M_r 108K to 125K) and G2 (M_r 29K to 41K) and a nonstructural protein, NSm, (M_r 11K to 16K) (Gentsch and Bishop, 1987; Fuller and Bishop, 1982; Elliott, 1985). The precursor polyprotein has not been detected in infected cells (Pennington et al, 1977; Lees et al, 1986). In addition, attempts to translate bunyavirus M segment-specific mRNA from infected cells have been unsuccessful (Abraham and Pattnaik, 1983; Elliott, 1985).

The gene order of snowshoe hare (SSH) bunyavirus M segment ORF was determined as 5' G2-NSm-G1 3'. This result was obtained by the use of direct amino acid sequence analysis of G1 and G2 glycoproteins together with specific peptide antibodies to NSm (Fazakerley et al, 1988). A comparison of known bunyavirus M segment protein coding regions has been carried out on the basis of the above derived gene order (Elliott, 1990). The open reading frame begins with a signal sequence varying in length from 13 to 21 amino acids, which is presumed to be cleaved from the mature G2 protein prior to assembly. The G2 proteins of snowshoe hare (SSH) and La Crosse (LAC) virus, members of the California serogroup, demonstrate a 95% homology (Fazakerley et al, 1988: Grady et al, 1987). A comparison of the G2 proteins of SSH virus with Bunyamwera virus of Bunyamwera serogroup showed 60% homology (Lees et al, 1986; Gonzalez-Scarano and Nathanson, 1990). The four bunyavirus G2 sequences exhibit 66% homology (Elliott, 1990). It should be noted that in all cases the positions of two predicted N-linked glycosylation sites (Asn-X-Ser/Thr) are strictly conserved (Elliott, 1990).

The non-structural protein, NSm, which lies between the coding

regions of G2 and G1, has an overall conservation of 50% homology (Elliott, 1990). The G1 proteins of all four viruses display about 40% similarity, with a greater conservation of sequence in the carboxy half of the protein (Elliott, 1990). The positions of the Cys residues are strictly maintained which suggests that the proteins might assume similar folded structure. The G1 proteins of viruses within the same serogroup demonstrate a higher homology than G1 proteins from different serogroups. The G1 proteins of SSH virus and LAC virus exhibit a high degree of homology, 89%,(Gonzalez-Scarano and Nathanson, 1990), whereas SSH virus and Bunyamwera virus G1 proteins share 41% homology (Gonzalez-Scarano and Nathanson, 1990). The G1 protein has a single strictly conserved N-glycosylation site; a second site is conserved within serogroups (Elliott, 1990).

The hydropathy profiles of the four polyproteins were also compared and found to be very similar (Elliott, 1990). It is thought that the G1 protein is anchored in the viral envelope via the hydrophobic sequence close to its carboxy terminus with amino-terminus external to the membrane (Elliott, 1990: Gonzalez-Scarano and Nathanson, 1990). Futher evidence to support this hypothesis comes from the fact that the amino terminal domain contains epitopes accessible to monoclonal antibodies. In addition the amino terminus also possesses a conserved trypsin cleavage site (Fazakerley et al, 1988: Gonzalez-Scarano, 1985). The G2 proteins contain a long hydrophobic domain in the middle of the molecule (Fazakerley et al, 1988), presumed to be a transmembrane region. The orientation of the two termini of the G2 protein has not been resolved due to the lack of G2 monoclonal antibodies. According to amino acid sequence information and hydropathy profiles the NSm protein is

suggested to be a membrane bound protein (Elliott, 1990), however neither the function nor the intracellular location of NSm is known.

(ii) Hantavirus Genus M RNA segment

The Hantavirus genus is represented by five major serotypes and the nucleotide sequences of the viral M segments of five viruses belonging to three of these serotypes have been published: Hantaan virus (76-118) of Hantaan serotype (Schmaljohn et al, 1987; Yoo and Kang, 1987); nephropathia epidemica virus (NEV) (Giebel et al, 1989) of Puumala serotype; and Seoul 80-39 virus (Antic et al, 1991b), Biken 1 virus (B1) (Isegawa, 1990) and Sappora rat virus (SR-11) (Arikawa et al, 1990) of Seoul serotype. The M segment is between 3,616 (Hantaan virus) and 3,682 nucleotides in length (NEV). Hantaviruses encode a polyprotein in the viral complementary sense RNA of between 1,133 to 1,148 amino acids in length. The polyprotein is processed to G1 and G2 envelope glycoproteins with molecular weights of 70K and 55K respectively (Elliot et al, 1984; Schmaljohn et al, 1986; Schmaljohn et al, 1987). The gene order for Hantaan virus, the prototype of the genus, was established as 5' G1-G2 3' (Schmaljohn et al, 1987). The initial precursor protein of 126K is processed into G1 (64K) and G2 (53.7K). Mature G1 starts 18 amino acids beyond the first AUG of the ORF and is preceded by a hydrophobic sequence (Schmaljohn et al, 1987; Arikawa et al, 1989). The G2 protein, which starts at amino acid 649, is preceeded by an intergenic region of 6K, which encodes a hydrophobic amino acid sequence and is thought to be a membrane-spanning region (Schmaljohn et al, 1987). A similar 6K membrane-spanning intergenic region, which is thought to serve as a signal sequence for envelope proteins, has been demonstrated between the two envelope protein coding sequences of the alphaviruses (Garoff et al,

1980; Strauss and Strauss, 1986).

A comparison of the deduced amino acid sequences of the G1 and G2 proteins for SR-11 HTN and NEV revealed 43% homology for G1 and 50% homology for G2 (Arikawa et al, 1990). The degree of homology was found to be higher within serotypes than between: B1 and SR-11 viruses of Seoul serotype exhibited 98% homology for G1 and 99% homology for G2 (Isegawa et al, 1990); whereas Seoul 80-39 and SR-11 viruses exhibited 77% homology for both glycoproteins (Antic et al, 1991b). The hydropathy profiles of the M segment encoded proteins are similar (Antic et al, 1991). Hantaan 76-118 virus encoded glycoproteins possess five potential N-linked glycosylation sites (four in the G1 protein and one in the G2 protein) (Schmaljohn et al, 1987; Antic et al, 1987). A comparison of the encoded amino acid sequences of SR-11, NEV and HTN M segments revealed that three potential sites are conserved within G1 and one is conserved within the protein G2. Both SR-11 and B-1 glycoproteins share six conserved potential glycosylation sites; five on G1 and one on G2 (Isegawa et al,1990; Anwaka et al, 1990). Seoul 80-39 M segment ORF was also found to have five potential sites in G1, three of which are conserved with SR11 and B1 G1 proteins, and one potential conserved site in G2 (Antic et al, 1990).

Expression of Hantaan M and S RNA segment ORFs using recombinant vaccinia viruses revealed that the specificity of virus neutralisation resided on the glycoproteins (Arikawa et al, 1989). Baculovirus and vaccinia virus recombinants expressing the M segment ORF or portions of the segment encoding either G1 or G2 alone produced antigenically authentic Hantaan virus proteins retaining all of the antigenic sites as defined by a panel of monoclonal antibodies to Hantaan virus

glycoproteins (Arikawa et al, 1989; Schmaljohn et al, 1990). In addition, the expressed proteins were able to induce antibodies in test animals which could recognise authentic viral particles in ELISA assays and also neutralise viral infectivity (Schmaljohn et al, 1990). Although baculovirus recombinants which expressed only Hantaan nucleocapsid protein (N) also appeared to protect some animals from Hantaan virus challenge (Schmaljohn et al, 1990), this contrasts the situation in vitro where MAb to G1 and G2 but not N were found to neutralise infectivity (Arikawa et al, 1989; Dantas et al, 1986).

(iii) Phlebovirus Genus M RNA segment

The nucleotide sequence has been determined for two phleboviruses M segments: Rift Valley fever virus (RVFV) (Inhara et al, 1985) and Punta Toro virus (PT) (Collett et al, 1985; Takehara et al, 1989). The RVFV M segment is 3,885 nucleotides in length and has an ORF encoding a polyprotein of 1,197 amino acids on the viral complementary sense RNA. The Punta Toro virus M segment is 4,330 nucleotides in length and encodes a polyprotein of 1,313 amino acids. Amino-terminal sequence analysis of purified G1 and G2 from RVFV revealed a gene order of 5' G2-G1 3' but this is reversed in PT virus ie 5' G1-G2 3'.

Sequence comparison indicates, however, that the G2 of PT is 49% homologous to the G1 protein of RVFV and that G1 of PT has approximately 35% homology with G2 of RVFV (Ihara et al, 1985), hence the apparent difference in coding order reflects only the nomenclature which is based on the relative sizes of the two encoded glycoproteins. Both PT and RVFV possess extra coding potential in a pre-glycoprotein region, or NSm region, between the AUG initiation codon and the start of the mature glycoprotein coding sequences. In the case of RVFV this pre-glycoprotein

region has five in frame AUG codons which precede the mature G2 and potentially encodes a protein of 17K. Punta Toro NSm region has 13 in frame initiation codons before G1 and potentially encodes a protein of 30K (Ihara et al, 1985).

Translation in vitro of a message sense RNA transcript of RVFV M cDNA produced a translation product of 133K (Suzich and Collett, 1988) which could be further processed in the presence of microsomal membranes to yield glycoproteins G1 (Mr 65K) and G2 (Mr 56K) as well as additional polypeptides of 78K and 14K (Suzich and Collett, 1988). Expression experiments using recombinant vaccinia viruses or baculoviruses containing cDNAs of RVFV M segment ORF with different ORF initiation codons such that specific sequence changes removed one or more of the five in frame initiation codons in the NSm region demonstrated that the 78K protein initiated at the first AUG and the 14K protein at the second AUG (Suzich et al, 1990) (Figure 6). The 78K protein was presumed to represent an unprocessed polyprotein of NSm and G2 and the 14K protein only pre-G2 sequence (Suzich and Collett,1988; Kakach et al 1988).

Using pulse chase experiments it was not possible to demonstrate that the 78K proteins served as a precursor to G2, and removal of the first AUG had no negative effect on G2 production (Kakach et al,1988). Indeed, the functional roles of the 78K and 14K proteins remain unclear, as both G2 and G1 glycoproteins could be synthesised and processed in their absence and only the hydrophobic amino acid sequence immediately preceding the mature glycoprotein coding sequences was shown to be required for authentic glycoprotein production (Kakach et al, 1988).

Monoclonal antibodies to either the G1 or G2 glycoproteins of RVFV and PT virus are capable of neutralising infection in cell culture

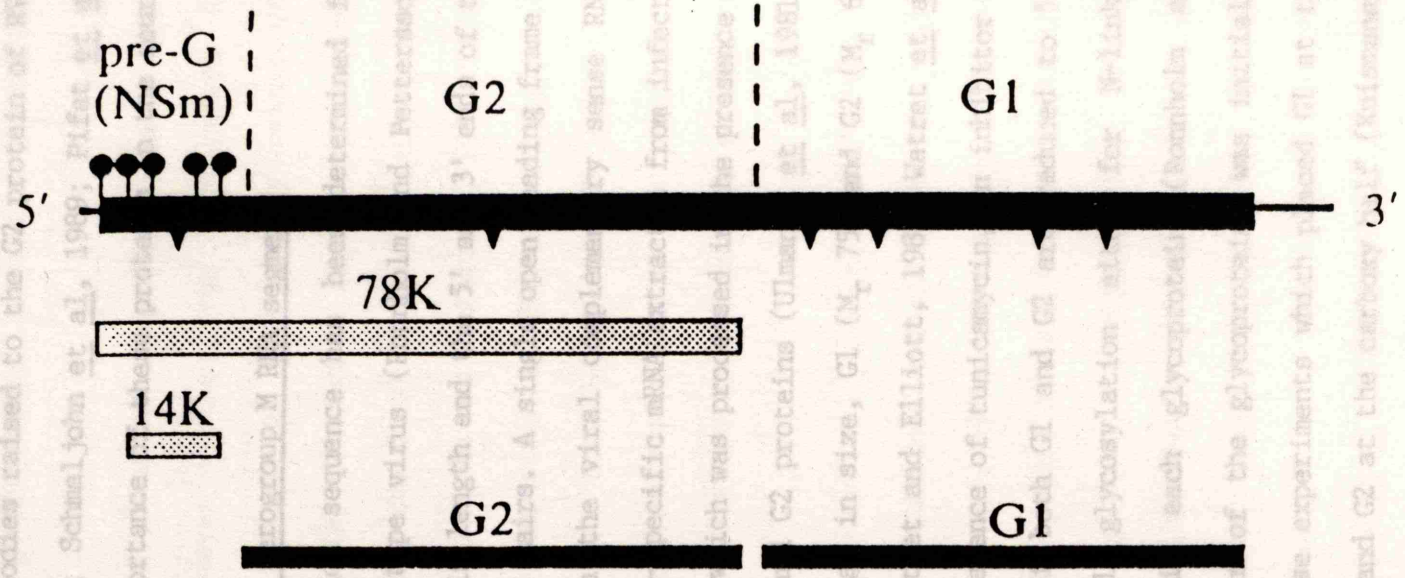

Figure 6.
Expression strategy of the Rift Valley fever virus M RNA segment. The top of the figure shows the M RNA with the coding region for NSm, G2 and G1 indicated. On the M segment AUG codons are shown as ● and N-linked glycosylation sites indicated as ▼. The four protein products detected in infected cells are indicated below as 14K, 78K and G2 and G1. This diagram is taken from Elliott (1990) and is a modification of Collett *et al* (1989).

(Keegan and Collett, 1986; Pifat et al, 1988), however protection in animals is only afforded by antibodies raised to the G2 protein of RVFV and G1 of PT (Collett et al,1987; Schmaljohn et al, 1989; Pifat et al, 1988) which may emphasise the importance of these proteins in the course of animal infections.

(iv) Phlebovirus Genus- Uukuniemi serogroup M RNA segment

The complete M segment nucleotide sequence has been determined for Uukuniemi virus, the serogroup type virus (Ronnholm and Pettersson, 1987). The segment is 3231 bases in length and the 5' and 3' ends of the RNA are complementary for 30 base pairs. A single open reading frame of 1,008 amino acids is encoded in the viral complementary sense RNA. Translation in vitro of M segment-specific mRNA extracted from infected cells produced a protein of 110K which was processed in the presence of microsomal membranes to give G1 and G2 proteins (Ulmanen et al, 1981). The viral glycoproteins are both similar in size, G1 (M_r 75K) and G2 (M_r 62-65K) (Pettersson et al, 1971; Watret and Elliott, 1985; Watret et al, 1985). When synthesised in the presence of tunicamycin, an inhibitor of N-linked glycosylation, the Mr of both G1 and G2 are reduced to 55K (Kuismanen,1984). Four potential glycosylation sites for N-linked glycans have been identified in each glycoprotein (Ronnholm and Pettersson, 1987). The gene order of the glycoproteins was initially determined by in vitro pulse chase experiments which placed G1 at the amino half of the 110K precursor and G2 at the carboxy half (Kuismanen, 1984). This was later confirmed by comparing partial amino-terminal sequences of purified G1 and G2 proteins with the polyprotein sequence (Ronnholm and Pettersson, 1987). The M segment does not possess any additional coding capacity for non-structural proteins, however both

encoded mature glycoproteins are preceded by strongly hydrophobic sequences thought to be Golgi translocational sequences (Ronnholm and Pettersson, 1987).

Serological cross-reactivity has not been demonstrated between uuku- and phlebo- viruses, however viruses from both genera share several genetic features that suggest an evolutionary relationship. Uukuniemi, Punta Toro and Rift Valley fever virus share complementary 3' and 5' nucleotide sequences for the first 10 to 13 residues (Collet et al, 1985; Inhara et al, 1985; Ronnholm and Pettersson, 1987; Takehara et al, 1989). In addition, a comparison of M segment polyproteins demonstrated a low but significant homology between M segment gene products, particularly towards the carboxy end of the precursor. No similarity was detected between uukuvirus and bunyavirus glycoproteins (Ronnholm and Pettersson, 1987).

(v) Nairovirus Genus M RNA segment

Estimation of M segment size for individual viruses range from 4,458 up to 6,300 nucleotides (Clerx and Bishop, 1981; Clerx et al, 1981; Watret and Elliott, 1985). This makes M segments of nairoviruses larger than M segments of viruses from other genera. Two glycoproteins have been detected: G1 (M_r 72-84K) and G2 (M_r 30-45K) (Clerx and Bishop, 1981; Clerx et al, 1981; Cash, 1985; Watret and Elliott, 1985), although 3 glycoproteins of M_r 84K, 45K and 30K have been detected in virion preparations of Hazara virus (Foulke et al, 1981).

The nucleotide sequence of Dugbe (DUG) nairovirus M segment has been established by Marriott et al (1992). The segment is 4,888 nucleotides in length and therefore at the lower end of the estimated nairovirus M segment size range. The segment contains one ORF encoded in the viral

complementary sense. This ORF extends from an AUG start codon at bases 48-50 to a stop codon at bases 4701-4703, with a coding capacity for a polypeptide of 173.3K. The DUG M segment was found not to exhibit any sequence homology with the M segments of other members of the Bunyaviridae or with their predicted polypeptide products (Marriott et al, 1992). Two surface glycoproteins have been reported for DUG virus, G1 (M_r 73K) and G2 (M_r 35K), (El-Ghorr et al, 1990). The G1 protein was mapped to the carboxyl end of the DUG M ORF by amino-terminal sequencing and cross reactivity of anti-G1 serum to carboxyl DUG M fusion protein expressed in E.coli (Marriott et al, 1990).

Unlike other Bunyaviridae glycoproteins the DUG G1 protein does not appear to be processed co-translationally from a polyprotein representing the entire ORF. Instead the 73K G1 protein is cleaved over a period of hours from a 85K precursor, which is able to be detected in virus infected cells (Marrriott et al, 1992). Marriott et al (1992) suggest that this precursor protein is encoded from residue 830 to the carboxyl-terminus of the M segment ORF.

The G2 coding sequence is located upstream of the G1 sequence on the M ORF but immunoprecipitation of pulse labelled protein from infected cell lysate using a G2 specific antibodies indicate that the protein is initially expressed as a non-structural protein with an M_r of 70K. This non-structural protein is encoded between the carboxyl terminus of G2 and the amino-terminal start of the M segment ORF (Marriott et al, 1992).

Although pulse chase experiments of nairovirus infected cells suggest that nairovirus glycoprotein expression is similar to the strategy employed by other Bunyaviridae ie. mature glycoprotein is

obtained from a larger precursor polypeptide (Clerx and Bishop, 1981; Watret and Elliott, 1985). The results obtained by Marriott et al (1992) indicate that processing of the nairovirus M segment primary product(s) is more complex than the M segment protein processing observed in other genera.

E. The S RNA segment

The S RNA segment in all members of the Bunyaviridae encodes a nucleocapsid (N) protein in the viral complementary sense RNA (Elliott, 1990; Simons et al, 1990; Ward et al, 1991). The N protein is the most conserved viral structural protein within each genus. For the hantaviruses and nairoviruses the N protein is approximately twice the size of the N protein of members of other genera. The S segments of hantaviruses and nairoviruses do not encode any additional proteins (Elliott, 1990; Ward, 1991). Members of the Bunyavirus, and Phlebovirus genera encode a non-structural (NSs) protein (M_r 10-30K). There are two possible coding strategies for NSs: the bunyaviruses encode the NSs protein as an overlapping reading frame within the N protein open reading frame (Elliott, 1990); the phleboviruses encode NSs on the viral sense strand of the S segment (Ihara et al, 1984; Marriott et al, 1989; Simons et al, 1990).

(i) Bunyavirus Genus S RNA segment.

The nucleotide sequence of six bunyavirus S segments have been published. Bunyamwera, Germiston and Maguari viruses of the Bunyamwera serogroup (Elliott, 1989a; Gerbaud et al, 1987; Elliott and McGregor, 1989); La Crosse and snowshoe hare viruses are members of the California serogroup (Akashi and Bishop, 1983; Cabradilla et al, 1983;

Bishop et al, 1982) and Aino is a member of the Simbu serogroup (Akashi et al, 1984).

The S segment length ranges from 850 to 984 bases (Elliott,1990) and all encode a nucleocapsid protein (M_r 19K-26K) and a non-structural protein (M_r 10K-12K) in overlapping reading frames. A comparison of the six bunyavirus S RNA segment encoded N and NSs proteins was made by Elliott (1989) and is shown in Figure 7. The encoded N proteins are similar in size (233 to 235 amino acids in length) whereas the NSs proteins are more variable (91 to 109 amino acids in length). Overall the N proteins of the 6 viruses exhibit a 40% homology in their amino acid sequence. Certain regions of the N protein sequence are strictly conserved, particularly between residues 62 to 102, 123 to 169 and the carboxy-terminal 15 residues. It is possible that these conserved sequences are involved in RNA/protein interactions, although there is no obvious sequence homology to other known RNA binding proteins such as heterogeneous nuclear RNPs, nucleolin, mRNA polyadenylate binding protein and small nuclear RNPs (Chan et al, 1989; Query et al, 1989).

The NSs proteins demonstrate about 25% similarity between all 6 viruses but exhibit 70% homology within a serogroup. The region which demonstrates the highest similarity represents the carboxy terminal of the shortest NSs protein. Four out of the six NSs amino acid sequences initiate from a tandem AUG whereas Aino NSs and Germiston NSs initiate from a single AUG. The function of NSs has not yet been determined but it may interact with L in a fashion similar to the non structural protein / L protein interaction in VSV.

(ii) Hantavirus Genus S RNA segment

The nucleotide sequences of four hantavirus S segments have been

(*a*)

```
           1                                                                  60
BUN.N      .MiELeFhDV AantssTFDP EvaYanFkrv httgLsYDhI RIFYikgrei KtsLaKrsEW
MAG.N      .MiELeFnDV AantssTFDP EiaYvnFkri httgLsYDhI RVLYikgrei KtsLtKrsEW
GER.N      .MlELeFeDV pnnigsTFDP EsGYtnFqrn ylpgvtLDqI RIFYikgrei KnsLSKrsEW
LAC.N      .MsDLvFyDV AstgangFDP DaGYmdFcvk naesLnLaaV RIFFlnaaka KaaLSrkpER
SSH.N      .MsDLvFyDV AstgangFDP DaGYmaFcvk yaesvnLaaV RIFFlnaaka KaaLSrkpER
AINO.N     manqFiFqDV pqrnlaTFnP EvGYvaFiak hgaqLnFDtV RfFFlnqkka KmvLSKtaqp
Consensus  -M-EL-F-DV A-----TFDP E-GY--F--- ----L-LD-- RIF------- K--LSK--EW

           61                                                                 120
BUN.N      eVtLnLGGWK ItVyNtnFPG NRNNPVPDdg LTLHRLSGFL ARYlLEk.ml kvsEpekliI
MAG.N      eVtLnLGGWK VaVfNtnFPG NRNsPVPDdg LTLHRLSGFL ARYlLEkilk vsd.pekliI
GER.N      eVtLnLGGWK VpVlNtnFPG NRNNaVPDyg LTFHRiSGYL ARYlLgkYla .etEpeklim
LAC.N      kanpkFGeWq VeViNnhFPG NRNNPIgnnd LTiHRLSGYL ARWvLDqYne nddEsqhelI
SSH.N      kanpkFGeWq VeVvNnhFPG NRNNPInsdd LTiHRLSGYL ARWvLEqYke nedEsrrelI
AINO.N     sVdLtFGGiK ftlvNnhFPq ytaNPVPDta LTLHRLSGYL AkWvaDqC.. ktnqiklaea
Consensus  -V-L--GGWK V-V-N--FPG NRNNPVPD-- LTLHRLSGYL AR--LE-Y-- ---E-----I

           121                                                                180
BUN.N      ksKIINPLAE kNGITWndGe EVYLSFFPGs EMFLgTFrFY PLaIGIYkVq rkeMEPkYLe
MAG.N      ksKIINPLAE kNGITWadGe EVYLSFFPGs EMFLgTFkFY PLaIGIYkVq kkeMEPkYLe
GER.N      rtKIVNPLAE kNGITWesGp EVYLSFFPGa EMFLgTFrFY PLaIGIYkVq rkeMDPkFLe
LAC.N      rttIINPiAE sNGVgWdsGp EIYLSFFPGt EMFLeTFkFY PLtIGIhrVk qgmMDPqYLk
SSH.N      kttIINPiAE sNGVrWdsGa EIYLSFFPGt EMFLeTFkFY PLtIGIYrVk qgmMDPqYLk
AINO.N     meKIVmPLAE vkGcTWteGl tmYLgFaPGa EMFLeTFeFY PLvIdmhrVl kdgMDvnFMr
Consensus  --KIINPLAE -NGITW--G- EVYLSFFPG- EMFL-TF-FY PL-IGIY-V- ---MDP-YL-

           181                                                          237
BUN.N      KtMRQRYmgL eAatWtvsKl teVqsALtvV ssLgWkKtnv SaAARdFLaK FGInM*.
MAG.N      KtMRQRYmgL eAatWtvsKV neVqaALtvV sgLgWkKtnv SaAAReFLaK FGInM*.
GER.N      KtMRQRYlgi dAqtWtttKl geVeaALkvV sgLgWkKtnv SsAAReFLsK FGIrM*.
LAC.N      KaLRQRYgtL tAdkWmsqKV aaIaksLkdV eqLkWgKggl SdtAktFLqK FGIrLp*
SSH.N      KaLRQRYgsL tAdkWmsqKV taIaksLkeV eqLkWgrggl SdtARsFLqK FGIrLp*
AINO.N     KvLRQRYgtL tAeqWmtqKI daVraAFnaV gqLsWaKsgf SpAARaFLaq FGIni*.
Consensus  K--RQRY--L -A--W---KV --V--AL--V --L-W-K--- S-AAR-FL-K FGI-M--
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(*b*)

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           1                                                                  60
BUN.NSS    MMSLltpavl LTqrshtlTL svstplglVM ttYeSStlkd aRlkLvSqke vnGkLhLtLg
MAG.NSS    MMSLltpavl LTqrlhtlTL svstplglVM ttFeSStlkd aRlkLvSqke vsGrLrLtLg
GER.NSS    .MSLitsgvl LTqsqdtlTF svttcqglrL tkFaSStlkd aRlkivSqke vnGkLrLtLg
LAC.NSS    MMShqqvqmd LilmqgiwTs vlkmqnhstL lqLgSSssml qRprLlSrvs qrGrLtLnLe
SSH.NSS    MMShqqvqmd Lilmqgiwhs vlnmqnqsIL lqLgSSssmp qRprLlSrvs qrGrqiLnLe
AINO.NSS   .MfLngislr LTrrsgmwhL llnmgpnsIs ipLdSSssir rRprwySvrr hnqvLiLhLv
Consensus  MMSL------ LT------TL ---------L --L-SS---- -R--L-S--- --G-L-L-L-

           61                                                          111
BUN.NSS    AGRllYiIrI FLaTGTtQFl TmVLPSTasV Dslpgtylrr c*..... .
MAG.NSS    AGRllYlIqI FLaTGTvQFq TmVLPSTdsV Dslpgtylrk f*..... .
GER.NSS    AGRylYsIrI sLeTGTmQcl TtVLPSTvsV Dtlpgtyles tlqrqnqkss *
LAC.NSS    sGRwrlsIiI FLeTGTtQLv TtILPSTdyl gi*..... ..... .
SSH.NSS    sGRwrlsIiI FLeTGTiQLt atILPSTdcq Di*..... ..... .
AINO.NSS   AsslhWlItI F..pnTqQil cqtLPSlsiV sqdi*..... ..... .
Consensus  AGR--Y-I-I FL-TGT-Q-- T-VLPST--V D--------- ---------- -
```

Figure 7.
Six-way alignment of (a) the nucleocapsid (N) proteins and (b) the non-structural (NSs) proteins of Bunyamwera (BUN), Maguari (MAG), Germiston (GER), La Crosse (LAC), snowshoe hare (SSH) and Aino viruses. The alignments were prepared by using the GAP and PRETTY programs of Devereux et al (1984). The 'consensus' sequence is based on a plurality of 4, and is a convenient way of highlighting regions of strong conservation. This figure is taken from Elliott (1989a).

published: HTN (Schmaljohn et al, 1986b); SR-11 virus (Arikawa et al, 1990); NEV (Stohwasser et al, 1990); and Prospect Hill virus (PHV) (Parrington and Kang, 1990). The HTN virus S segment is 1,696 bases in length. SR-11 and NEV S segments are slightly larger, 1,769 and 1,785 bases in length respectively, whereas PHV S segment is 1,675 nucleotides long. The encoded N proteins are similar in size (429 - 433 amino acids in length) and the M_r of 46-48K is approximately twice the value for a bunyavirus N protein. A comparison of the predicted N protein amino acid sequences of all four viruses demonstrated an overall homology of 58% (Arikawa et al, 1990). Individual comparisons of HTN N protein sequence with SR-11 N, NEV N and PHV N protein sequences gave homology values of 82%, 61% and 62% respectively (Arikawa et al, 1990; Stohwasser et al, 1990; Parrington and Kang, 1990). All four N proteins possess stretches of strong amino acid homology and the carboxy terminal third of the N proteins were nearly identical (amino acids 303-429) (Arikawa et al, 1990; Parrington and Kang, 1990). Both HTN and SR-11 possess two in frame AUG initiation codons (at positions 37 and 46 for HTN and 43 and 52 for SR11) at the start of the N open reading frame. Overall the amino and carboxy termini regions of the different hantavirus N protein sequences are more conserved than the middle of the protein sequence (Elliott et al, 1991). A comparison of the distribution of acidic and basic residues between the four N protein sequences demonstrates that there is a strong conservation of the charged residues throughout the N protein sequences (Figure 8). Overall Figure 8 shows that the first hundred residues from the amino terminal of the hantavirus N protein sequence is strongly charged with acidic and basic residues; the second hundred residues contains a cluster of basic

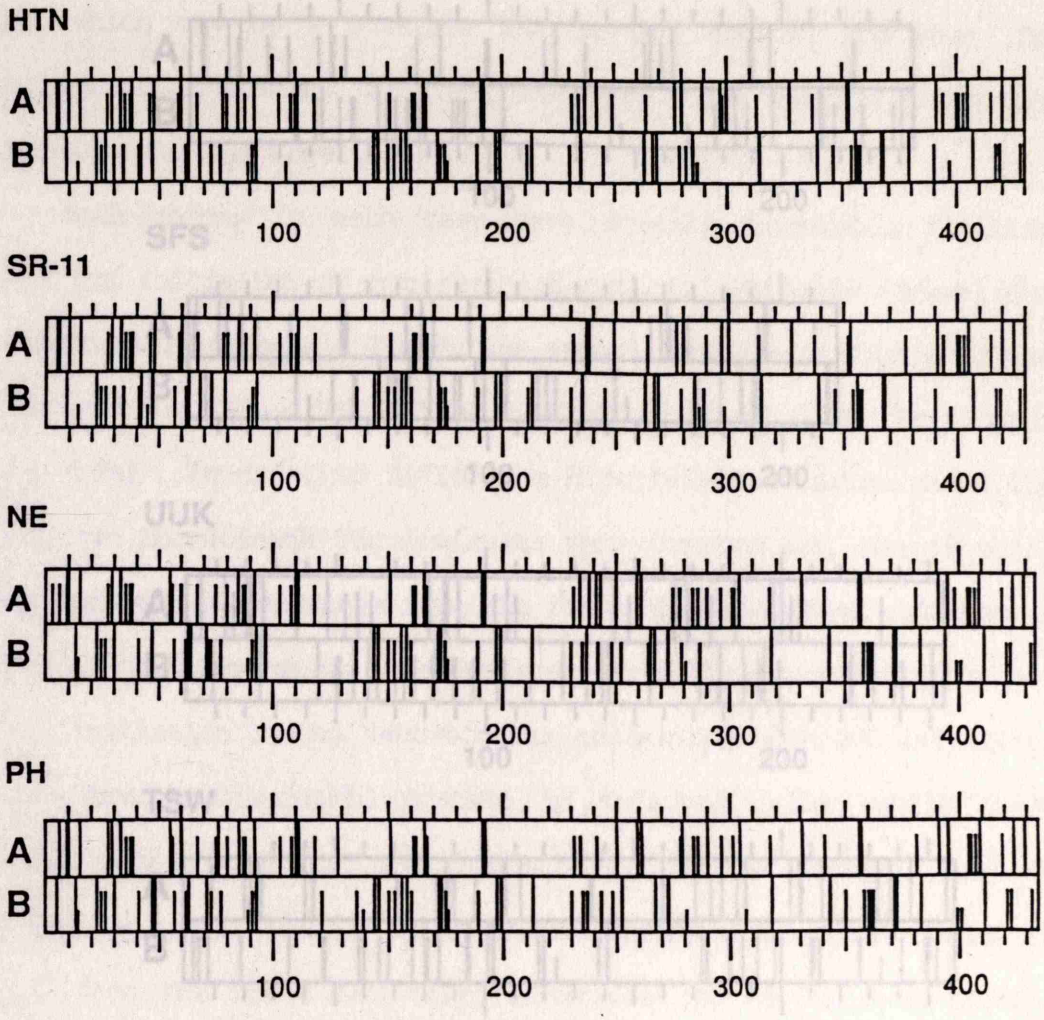


Figure 8. Distribution of acidic and basic residues in the N protein of Hantaan (HTN), Sapporo rat (SR-11), nephropathia epidemica (NE) and Prospect Hill (PH) viruses. Distribution analysis was carried out using the DNA strider sequence analysis program (Marck, 1988). The acidic (A) and basic (B) amino acids are indicated with vertical lines. For A, *full vertical lines* represent glutamic acid residues and *intermediate lines* represent aspartic residues. For B, *full lines* represent arginine residues and *intermediate lines* represent lysine residues and *short lines* represent histidine residues. *Numbers* represent amino acid residue position within the ORF. Figure taken from Elliott et al (1991).

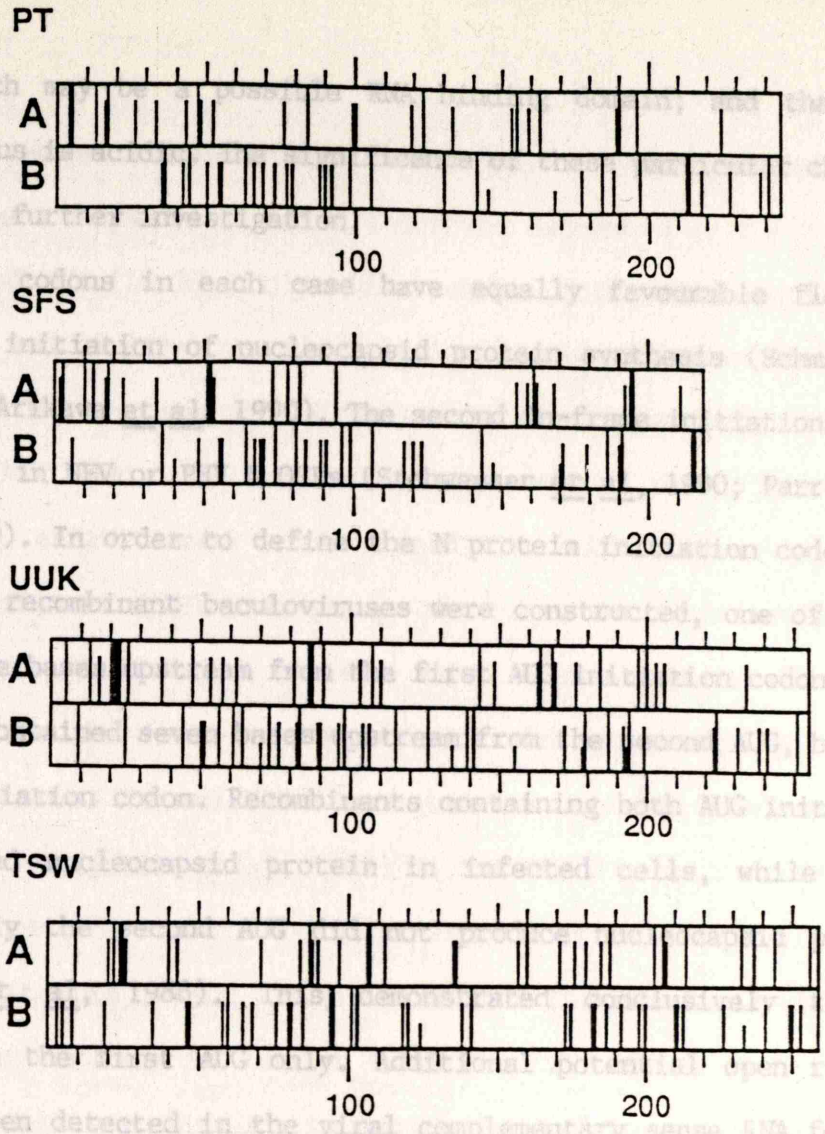


Figure 9.
Analysis of the distribution of the acidic and basic residues in the N protein of Punta Toro (PT), Sicilian sandfly fever (SFS) and Uukuniemi (UUK) phleboviruses and tomato spotted wilt tospovirus (TSW). Analysis of the distribution of the acidic (A) and basic (B) amino acid residues was carried out using the programme of Marck (1988). For A, *full vertical lines* represent aspartic acid residues and *intermediate lines* glutamic acids. For B, *full lines* represent the positions of the arginine residues, *intermediate lines* are lysines and *short lines* histidines. *Numbers* represent amino acid residue position within the ORF. Figure taken from Elliott et al (1991).

residues, which may be a possible RNA binding domain; and that the carboxy terminus is acidic. The significance of these particular charged domains awaits further investigation.

Both codons in each case have equally favourable flanking sequences for initiation of nucleocapsid protein synthesis (Schmaljohn et al, 1986b; Arikawa et al, 1990). The second in-frame initiation codon is not present in NEV or PHV N ORFs (Stohwasser et al, 1990; Parrington and Kang, 1990). In order to define the N protein initiation codon for HTN virus two recombinant baculoviruses were constructed, one of which included twelve bases upstream from the first AUG initiation codon and a second which contained seven bases upstream from the second AUG, but not the first initiation codon. Recombinants containing both AUG initiation codons produced nucleocapsid protein in infected cells, while those containing only the second AUG did not produce nucleocapsid protein (Schmaljohn et al, 1988). This demonstrated conclusively that N initiated from the first AUG only. Additional potential open reading frames have been detected in the viral complementary sense RNA for HTN virus (48 codons) (Schmaljohn et al, 1986), NEV virus (90 codons), and PHV (90 codons), but not for SR-11 (Arikawa et al, 1990; Parrington and Kang, 1990). Expression of these encoded prospective polypeptides could not be demonstrated (Schmaljohn et al, 1986b; Stohwasser et al, 1990; Parrington and Kang, 1990).

(iii) Nairovirus Genus S RNA segment

The nucleotide sequence of the S segment of Crimean-Congo Hemorrhagic fever (CCHF), Hazara (HAZ) and Dugbe (DUG) nairoviruses have been determined (Marriott and Nuttall, 1992; Ward et al, 1990). The S segments range in size from 1,672 to 1,712 nucleotides in length and encode an N

protein in the viral complementary sense RNA. A comparison of the termini of these S segments revealed conserved sequences at the 3' (21 nucleotides) and 5' (18-30 nucleotides) ends. Both CCHF and HAZ N proteins (M_r 54 and 54.2KDa) are slightly larger than DUG N proteins (M_r 49.4). CCHF and HAZ viruses are closely related antigenically (Casals and Tignor, 1980) and are members of the same serogroup (CCHF serogroup), but exhibit weak cross reactivity with members of the Nairobi sheep disease (NSD) serogroup of which HAZ is a member. A comparison of the predicted N protein amino acid sequences of these viruses reinforces the inter-relationship of the two serogroups, with 60% homology between CCHF and HAZ N proteins and only 54% homology with DUG N protein (Marriott et al, 1992). The DUG N protein shows a 40 residue deletion at the carboxy end relative to CCHF and HAZ N proteins. This implies that this region is not essential for function. Although the S RNA coding strategy is similar in both nairo- and hanta- viruses and the N proteins are similar in size no significant homology was observed between genera (Ward et al, 1990).

(iv) Phlebovirus Genus S RNA segment

The nucleotide sequences of four phlebovirus S segments have been determined: Punta Toro (PT) virus (Ihara et al, 1984); sandfly fever Sicilian (SFS) virus (Marriott et al, 1989); Rift Valley fever virus (RVFV) (Giorgi et al, 1991); and Toscana (TOS) virus (Giorgi et al, 1991). The S genome segments range from 1,690 bases (RVFV) to 1,904 bases (PT virus) in length and exhibit conserved inverted complementary 5' and 3' terminal sequences of between 6-14 nucleotides. The S segment of the prototype virus, SFS, is 1,746 nucleotides in length and encodes

an N protein of M_r 25K in the 3' half of the virus S segment in the complementary sense RNA. The viral S segment also encodes a non-structural protein NSs (M_r 30K) in the viral sense RNA on the 5' half of the segment in a non-overlapping ORF. The N protein is translated from mRNA corresponding to the 3' half of the virion RNA whereas the NSs is made from a virion sense mRNA identical to the 5' half of the virion RNA (Ihara et al, 1984, 1985; Marriott et al, 1989). This coding strategy has been termed ambisense and is also found in S and L RNA segments of members of the Arenaviridae family (Auperin et al, 1984). TOS and RVFV S segments encode N (27.7K and 27.4K respectively) and NSs (36.7K and 29.9K respectively) in a similar ambisense strategy (Giorgi et al, 1991). The NSs protein has been detected in SFS, RVFV and Karimabad phlebovirus infected cells (Watret et al,1985; Smith and Pifat, 1982; Struthers and Swanepoel, 1982) and the subgenomic mRNA for NSs was detected in PT virus infected cells (Marriott et al, 1989). In addition, cDNAs of N and NSs ORFs of PT virus were expressed in two separate baculovirus recombinants and mono-specific antisera raised to these proteins immunoprecipitated either N or NSs proteins from PT virus infected cells (Overton et al, 1987).

(v) Phlebovirus Genus- Uukuniemi serogroup S RNA segment

The nucleotide sequence of the S RNA segment of Uukuniemi virus has been determined (Simons et al, 1990). The segment is similar in size (1,720 bases) to phlebovirus S RNA and also possesses an ambisense coding strategy in which N and NSs proteins are encoded in non-overlapping reading frames.

The N protein, 254 amino acids in length (M_r 28.5K), is encoded in the 3' half of the viral S RNA in the complementary sense. The NSs

protein , 277 amino acids in length, (M_r 32K) is encoded in the 5' end of the viral sense RNA. Northern Blot analysis demonstrated that N and NSs proteins are translated from subgenomic mRNA of opposite polarity and about 800 and 850 nucleotides in length respectively. Giorgi et al (1991) carried out a sequence comparison for both N and NSs proteins of UUK, RVFV, PT, TOS and SFS phleboviruses. A comparison with RVFV N protein established a descending order of relatedness of PT>TOS>SFS>UUK. A comparison with RVFV NSs protein produced an order of SFS>PT>TOS>UUK. Overall the N protein was more similar among phleboviruses than the NSs protein (Giorgi et al, 1991). The amino acid sequence of the N protein exhibited a 35% and 33% homology with PT and SFS N proteins, whereas PT and SFS N proteins exhibited a 47% homology (Marriott et al, 1989; Simons et al, 1990). In addition, the extreme 5' and 3' termini of the S RNA segments of PT, SFS, TOS and RVFV demonstrate a high degree of conservation with UUK S segment termini. Thus demonstrating the inter-relationship between these viruses. A comparison of the charge distribution along the amino acid sequence of the ambisense encoded N proteins of PT, SFS, UUK and TSW viruses revealed that they have a common acidic amino terminal region of about 30 residues followed by a basic region (Figure 9). Although these features are less pronounced in TSWV, it does suggest a structural feature that is common to these proteins, which may be important in N protein-RNA interaction.

5. Tospovirus genus virion structure and molecular biology

TSWV virions are similar to other Bunyaviridae virions in that they are spherical enveloped particles 80-110nm in diameter with two membrane glycoproteins, G1 (M_r 78K) and G2 (M_r 58K), located on the surface of the virus particle (Mohamed et al, 1973; Tas et al, 1977). The viral

genome comprises three segments of single stranded RNA: S RNA (2.9Kb), M RNA (4.8Kb) and L RNA (8.9Kb), (de Haan et al, 1989; 1990; 1991; Van den Hark et al, 1977; Kormelink et al, 1992a; Mohamed, 1981). Each segment is individually encapsulated by nucleocapsid (N) protein (M_r 28K) and is associated with minor amounts of L protein (M_r 331K) (de Haan et al; Mohamed, 1973; Tas et al, 1977). The terminal sequences of the S, M and L RNA segments of TSWV exhibit 5' and 3' complementarity as is the case with other Bunyaviridae members (de Haan et al, 1989; 1991; Kormelink et al, 1992a) and the conserved 5' and 3' termini exhibit some similarity with phleboviruses (Figure 2).

The complete nucleotide sequence of the TSWV have been determined. The L RNA segment is 8,897 nucleotides in length and encodes a protein with a predicted M_r of 331K in the viral complementary sense (de Haan et al, 1991). The predicted protein sequence exhibits regions of homology with the L protein and presumptive polymerase of the animal Bunyaviridae, Bunyamwera virus and Hantaan virus, (de Haan et al, 1991). The TSWV L protein also exhibits homology with the core polymerase PB1 protein of influenza A virus (de Haan et al, 1992). The regions of strongest homology encompass the four putative polymerase motifs described by Poch et al (1989). An alignment of the TSWV L protein putative polymerase domain with similar regions of the L proteins of other Bunyaviridae is shown in Figure 5.

The TSWV S segment is 2,916 nucleotides in length and possesses an ambisense coding strategy similar to that of S RNAs of phleboviruses (de Haan et al, 1990). The N protein (M_r 28.8K) is encoded in the viral complementary sense RNA. A non-structural protein (NSs, M_r 52.4K) is encoded in the viral sense RNA (Kormelink et al, 1991). Both ORFs are separated by an intergenic region which has the potential to fold into a long stable hairpin structure that may act as a termination

signal for the two subgenomic mRNAs which terminate in this region (de Haan et al, 1990). The 5' ends of the S mRNAs isolated from TSWV infected cells were found to posses non-viral heterogenous sequences of 12 to 20 nucleotides in length (Kormelink et al, 1992b). This would imply that all tospovirus mRNAs similarly possess such 5' non-viral sequences, a feature common to viral mRNAs of animal-infecting members of the Bunyaviridae. These short heterogenous sequences are generated from capped host mRNA species by a cap snatching mechanism involving the viral transcriptase and is subsequently used to initiate transcription on the viral genome (see viral replicative cycle: primary transcription). This is the first plant virus reported to use such a mechanism.

A partial sequence covering 95% (2.8Kb) of the S RNA from a Bulgarian isolate of TSWV has also been reported (Maiss et al, 1991). The N protein and the NSs protein are encoded in a similar fashion to that reported for the original TSWV isolate. A nucleic acid sequence comparison revealed a 96% similarity and the predicted sequences of N and NSs proteins were 92% similar (Maiss et al, 1991). Whereas a partial sequence of the S segment of the serologically distinct Impatiens necrotic spot tospovirus (INSV) encoding the N protein exhibited only a 62% homology with TSWV N protein sequence, strengthening the serological assay result which placed both viruses in different serogroups (Law et al, 1991). A similar result was obtained when the nucleotide and amino acid sequence of the N gene of seven tospovirus isolates, representing the three serogroups, were compared (de Avila et al, 1993) ie protein sequences were strongly conserved within groups (up to 99% homology), but less conserved between groups, additionally groups I and II shared more homology with each other (75% homology) than with groupIII (approximately 57% homology).

An alignment of the N protein sequences from viruses of the three

serogroups showed regions of conservation spread over the length of the protein but no recognised RNA binding motif (de Avila et al, 1993) and although the tospovirus nucleocapsid protein is similar in size to that of phleboviruses no distict sequence similarity was able to be detected in the N protein sequences of these ambisense S encoded viruses (de Avila et al, 1993; de Haan et al, 1990).

The sequences of TSWV and INSV M RNA segments have been determined (Kormelink et al, 1992a; Law et al, 1992). The TSWV RNA segment is 4821 nucleotides in length and INSV M segment is 4972 nucleotides long both have complementary 5' and 3' ends. Each segment encodes two glycoproteins, G1 and G2 (78K and 58K respectively), in a single ORF in the viral complementary sense. In vitro transcription and tranlation of portions of the INSV M segment cDNA demonstrated that the G2 protein was encoded from the amino terminus and the G1 protein from the carboxyl end of the polypeptide. This is a similar coding strategy to bunyavirus and phlebovirus M segments. An alignment of INSV G2-G1 ORF with that of Puta Toro and Rift Valley fever phleboviruses revealed a 36% and 39% similarity respectively (Law et al, 1992). TSWV G2-G1 ORF was found to exhibit homology with the Bunyamwera G1 protein (Kormelink et al, 1992a).

The tospovirus M segment also encodes a non-structural (NSm) protein in the viral sense with a predicted M_r of 34K (INSV) or 33.6K (TSWV) (Kormelink et al, 1992a; Law et al, 1992). Although ambisense Bunyaviridae S segments have previously been reported these are the first reports of a Bunyaviridae M segment with an ambisense nature. Subgenomic M segment transcripts of both viral and complementary sense mRNA have been detected in INSV and TSWV infected tissue (Kormelink et al, 1992a; Law et al, 1992) which implies that both ORFs are expressed. In both INSV and TSWV the ORFs are encoded in opposite ends of the segment and are separated by an A-U rich intergenic region. The A-U rich

region has the potential to form a stable stem loop structure and is thought to play a part in mRNA transcription termination. Similar A-U rich intergenic regions are found in the ambisense tospovirus S segment of both TSWV and INSV (de Haan et al, 1990; Law et al, 1991) and in the viral ambisense S segment of Punta Toro phlebovirus (Emery and Bishop, 1987). The tospovirus M segment although sharing features common to other Bunyaviridae M segments is unique because of its ambisense nature. Recently Mushegian and Koonin (1993) identified through conserved amino acid sequence motifs that the tospovirus NSm protein was a member of a superfamily of RNA and DNA plant virus cell to cell movement proteins. Such proteins are essential for the dissemination of virus infection within the infected plant.

Recombinant expression studies of TSWV encoded proteins has so far been limited to the NSs protein. A recombinant baculovirus expressing the TSWV NSs open reading frame was used to raise antisera in rabbits to the 52.4K protein. Immunogold labelling experiments of tissue sections from TSWV infected plants, using the NSs antisera, located the NSs protein either dispersed in the cytoplasm or associated with flexible filamental fibres (Kormelink et al, 1991). Intracellular filaments of NSs have been reported in PT virus infected cells and were shown to associate with virions and nucleocapsids (Overton et al, 1987). However, infection of insect cells with a recombinant baculovirus expressing TSWV NSs at high levels failed to produce fibrous structures and it is therefore unlikely that the NSs protein is solely responsible for these structures (Kormelink et al, 1991). The role of the NSs protein in TSWV infection cycle requires further study.

6. Viral replicative cycle

A. Virus attachment and absorption into the host cell

As with other animal viruses Bunyaviridae initiate infection and entry into host cells by attaching to receptors on the host cell membrane. Attachment of the virus to the cell membrane is via the external viral glycoproteins. The cellular receptor for glycoprotein attachment has not been identified for any Bunyaviridae. The involvement of the viral glycoproteins in the attachment process has been demonstrated by the fact that the proteolytic digestion of the glycoproteins on LAC bunyavirus particles severely affects virus infectivity (Obijeski et al, 1976a; Kingsford and Hill, 1983). The relative importance of each of the encoded glycoproteins in the attachment process would seem to vary between family members and between virus host cells. The G1 and G2 proteins of HTN hantavirus (Arikawa et al, 1989; Dantas et al, 1986) and RVF and PT phleboviruses (Keegan and Collett, 1986; Pifat et al, 1988) have been shown to have neutralising and HI sites which implies that both proteins are involved in attachment. In contrast, neutralisation of LAC virus was obtained by G1 antibodies (Grady et al, 1983; Kingsford et al, 1983) and treatment of LAC virions with pronase, which degrades G1 protein but not G2, was found to produce non-infectious virus (Kingsford and Hill, 1983). Ludwig et al (1989) reported that protease removal of G1 from virus particles increased LAC virus affinity for mosquito cells but did not alter virus infectivity.

The ability of Bunyaviridae viruses to mediate fusion of infected cells at acidic pH has been reported (Gonzalez-Scarano et al, 1982, 1985: and Gonzalez-Scarano, 1985), and is believed to relate to the mechanism of viral penetration and uncoating. This process is similar to the method of entry used by influenza virus and VSV and was first reported for alphaviruses (Marsh and Helenius, 1980). The pathway envolves endocytosis of virus particles and sequestration in endosomes

which subsequently become acidified (Tycko and Maxfield, 1982). This results in the fusion of cell and viral membranes and the release of the viral nucleocapsid into the cell cytoplasm. The presence of virus-filled endosomes has been observed in electron microscope studies of RVFV infected cells (Ellis et al, 1988). Evidence showing the ability of the G1 protein to cause cell fusion at acidic pH has been demonstrated for LAC virus through the use of neutralising monoclonal antibodies (MAb) that also inhibit haemagglutination (HI) activity (Gonzalez-Scarano et al, 1985). Pobjecky et al (1989) however, postulated that it is a hydrophobic domain of G2 rather than the G1 protein that is responsible for fusion activity. The authors found that virus particles lacking G1, through protease degradation, were able to cause liposome fusion, whereas G1 containing liposomes were unable to induce fusion. Ludwig et al (1991) suggest that G1 mediates virus attachment to vertebrate cells and that G2 serves the same purpose in mosquito midgut cells. The authors speculate that the 'protease resistant' G2 protein may have evolved to serve as the virus attachment protein in the midgut under conditions in which G1 is conformationally altered or removed from the virus envelope. These suggestions were based on several experimental findings: purified G1 binds to mammalian and mosquito cloned cell lines in a dose-dependent manner but was incapable of binding to mosquito midgut cells isolated ex vivo, whereas G2 would bind to both mosquito cell lines but not to the mammalian cells. In addition, LAC virus infection of mammalian cells was inhibited by treatment of the cells with purified G1 and infection in mosquito cells was reduced by treatment of the cells with a combination of G1 and G2 proteins (Ludwig et al, 1991). This indicates that G1 is able to interact with mosquito cells and mammalian cells but is unable to interact with mosquito midgut cells. This would imply an important role for G2 protein in initiating mosquito infection in the mosquito midgut.

B. Primary transcription

The initial requirement for replication of a negative sense RNA virus upon entry into the cell and uncoating of the viral genome is for primary transcription of the viral genome by the virion associated polymerase (Bouloy and Hannoun, 1976; Ranki and Pettersson, 1975). As mentioned previously transcription activity has been detected in disrupted preparations of Bunyaviridae viruses (Ranki and Pettersson, 1975; Bouloy and Hannoun, 1976; Schmaljohn and Dalrymple, 1983; Patterson et al, 1984) although the activity was low compared to that of other negative strand viruses. The mechanism by which the Bunyaviridae members initiate transcription of their mRNA has not yet been fully defined, however many similarities have been found with the mechanism adopted by influenza viruses. Patterson et al (1984) showed that La Crosse bunyavirus polymerase in vitro was stimulated by dinucleotides (e.g. ApG), cap analogues (e.g. m^7GpppAm) and natural mRNAs (e.g. alfalfa mosaic virus (ALMV) RNA 4). These results are similar to those obtained for influenza polymerase (Krug, 1981). Krug and his co-workers demonstrated that with influenza virus this stimulation was due to a virus encoded endonuclease that was part of the polymerase complex. The endonuclease generates an oligonucleotide primer containing the 5' capped end of the mRNA together with the following 12 to 14 nucleotides which are then incorporated into the influenza transcripts (Krug, 1981; Plotch et al, 1981; Ulmanen et al, 1983; Ulmanen et al, 1981).

Patterson et al (1984) demonstrated the presence of a methylated cap dependent endonuclease which cut ALMV 4 RNA 13 to 14 nucleotides from its methylated capped 5' terminus. It was presumed that these capped and methylated fragments were the same as those used to prime LAC

primary transcription as these transcripts possessed 5' extensions of 10 to 14 nucleotides (Patterson et al, 1984). Other bunyavirus, phlebovirus and tospovirus RNA transcripts have been shown to possess non-viral coded 5' terminal heterogeneous extensions of 12-15 nucleotides in length (Bishop et al, 1983; Collett, 1986; Kormelink et al, 1992; Patterson et al, 1984; and Bouloy et al, 1990). Individual clones of Germiston virus mRNAs have been sequenced and their 5' termini show partial homology with some host cellular mRNAs (Bouloy et al,1990). More recently it has been shown that the sequences of LAC mRNA from infected cells are capped because they can be specifically selected by anti-cap antibodies (Hacker et al, 1990). Thus the mechanism of RNA transcription of influenza viruses and bunyaviruses would seem to be remarkably similar except that: (a) bunyavirus mRNAs are synthesised in the cytoplasm using a pool of stable cellular mRNA transcripts (Rossier et al, 1986) whereas influenza viruses derive their primers from newly synthesised RNA in the cell nucleus (Ishihama and Nagata, 1988); (b) consequently bunyavirus transcription is not inhibited by actinomycin D or α-amanitin whereas influenza activity is inhibited (Vezza et al, 1979); (c) influenza viruses employ three (P) proteins in a polymerase complex whereas bunyaviruses probably use just one, the L protein (Krug, 1987; Jin and Elliott, 1991).

Primary transcription in other negative sense RNA families such as Orthomyxoviridae, Rhabdoviridae and Paramyxoviridae has been shown to be independent of host-cell protein synthesis by use of inhibitors such as cycloheximide and puromycin (Fields et al, 1990). Reports of the dependency of Bunyaviridae primary transcription for ongoing host-cell protein synthesis are contrary to the relative independence exhibited by

other negative sense RNA viruses. Abraham and Pattnaik (1983) demonstrated that primary transcription of both Bunyamwera and Akabane bunyaviruses in infected cells were sensitive to the presence of cycloheximide. In addition, no or greatly reduced levels of S mRNA could be detected through hybridisation studies of LAC virus infected cells when cycloheximide or puromycin was present (Patterson and Kolakofsky, 1984: Raju and Kolakofsky, 1986: Raju and Kolakofsky, 1987). Similar results were also obtained for Germiston virus mRNA synthesis in infected cells in the presence of anisomycin or cycloheximide (Gerbaud et al, 1987). The requirement for ongoing protein synthesis in infected cells sharply contrasts with the ability of Bunyaviridae particles to carry out RNA transcription in vitro in its absence since in vitro transcription was thought to represent primary transcription in vivo (Bouloy and Hannoun, 1976; Gerbaud et al, 1987; Ranki and Pettersson, 1975; Schmaljohn and Dalrymple, 1983).

A re-examination of the in vitro products of the LAC polymerase revealed all the S mRNAs to be incomplete products which terminated at nucleotide 175 and that drugs such as cycloheximide had no effect on this reaction (Bellocq et al, 1987). Furthermore, full length S mRNAs, which terminated at nucleotide 886 were only synthesised in vitro if active rabbit reticulocyte lysate was added (Bellocq et al, 1987). Addition of cycloheximide into this latter system was found to cause transcription to terminate at nucleotide 175 but did not effect the rate of mRNA initiation (Bellocq et al, 1987). The pre-terminating 175 nucleotide transcript of S mRNA was also shown to be synthesised in LAC virus infected cells in the presence of cycloheximide (Raju et al, 1986). Further in vitro studies revealed that readthrough of the

termination site at nucleotide 175 to the end of the S mRNA, at nucleotide 886, in the absence of on-going protein synthesis was possible by the replacement of guanosine residues on the nascent chain with inosine (Bellocq and Kolakofsky, 1987). In order to explain these results Bellocq and Kolakofsky proposed that premature termination was due to base pairing of the nascent mRNA chain with its template and that concurrent ribosomal translation of the nascent chain was required to break these interactions (Bellocq and Kolakofsky, 1987). Vialant and Bouloy (1992) recently demonstrated that Germiston bunyavirus transcriptase was able to synthesise S mRNA and antigenomic S RNA *in vitro* in the presence of rabbit reticulocyte lysate treated with a protein synthesis inhibitor edeine. Edeine acts by inhibiting the formation of the 40S and 60S ribosomal complexes. However, this inhibitor does not stop the 40S ribosomal subunit binding to and scanning the nascent mRNA template (Kozak and Shatkin, 1978). Vialant and Bouloy (1992) proposed that scanning 40S ribosomal subunits on the nascent mRNA were sufficient to prevent RNA-RNA interactions between the nascent chain and its template thereby preventing premature termination of transcription in accordance with the model proposed by Bellocq and Kolakofsky, 1987).

The requirement of on-going protein synthesis for primary transcription in *Bunyaviridae* infected cells has been shown to be host cell-type dependent. Raju *et al* (1989) demonstrated a translational requirement for S mRNA synthesis in BHK, HeLa and Vero cells infected with LAC virus but not in infected mosquito cells. Moreover the cell type dependent translational requirement could be reproduced *in vitro* by the use of a cytoplasmic extract (Raju *et al*, 1989). The authors

postulated that translational dependency was due to host cell factors which were present in some cell lines, such as BHK, which enhance RNA/RNA interaction. Cell factors which enhance RNA-RNA hybridisation have been found in HeLa cells (Munroe, 1988). The presence or absence of such a factor in cell lines may account for the discrepancies reported in the ability to detect full-length mRNA in infected cells treated with puromycin (Vezza et al, 1979; Eshita et al, 1985).

For both ambisense and non-ambisense segments the 3' ends of the mRNA transcripts never extend to the end of their templates; consequently the ends of the mRNA do not possess complementary 5' and 3' ends and are unable to form circular structures. The bunyavirus S and M segment mRNAs have truncated 3' termini of between 60-120 nucleotides in comparison with their viral template (Bouloy et al, 1984, 1990; Cash et al, 1979; Eshita et al, 1985; Jin and Elliott, 1993; Patterson and Kolakofsky, 1984; Pattnaik and Abraham, 1983) all have truncated 3' termini of about 100 nucleotides in comparison with their viral template. Germiston S mRNA has been mapped to end 110-120 nucleotides from the 5' end of the template within the template sequence 3'...GGGGUUUGUUAG...5', whereas Germiston M mRNA has been mapped to end 80 nucleotides from the 5' end of the template at the sequence 3'...AUGUUUUUGUU...5' (Bouloy et al, 1990) and Bunyamwera S mRNA to terminate approximately 100 nucleotides from the 5' end of the template at the sequence 3'..UUGGGUGUUUU..5' (Jin and Elliott, 1993). Potential transcription termination sites have been proposed for the S mRNAs of LAC and SSH viruses at or near the genomic sequence 3'...G/CUUUUU...5' (Bouloy et al, 1990; Patterson and Kolakofsky, 1984; Eshita et al, 1985). In all cases these termination sites exhibit similarity with the mRNA transcription termination and polyadenylation signal found in influenza virus i.e. 3'...GUUUUU...5' (Gupta and Kingsbury, 1982). However, polyadenylation of Bunyaviridae mRNA probably does not occur as

demonstrated by the inability to recover bunya- or phlebo- virus mRNAs from oligo dT columns (Pattnaik and Abraham, 1983; Suzich and Collett, 1988). It should be noted that most mapped Bunyaviridae mRNAs have the potential to form stem loop structures upstream of their 3' ends, which may substitute for the 3' mRNA stability usually provided by a polyA tail on polyadenylated mRNAs.

The ambisense S segments of phleboviruses (Ihara, 1984; Simon et al, 1990) as well as the S segment of TSWV (de Haan et al, 1990) produce mRNA transcripts for N and NSs of opposite polarity. Both N and NSs subgenomic mRNAs possess heterogeneous 5' terminal sequences similar to S mRNAs of bunyaviruses (Ihara et al, 1985; Collett, 1986). The ORFs for N and NSs proteins, encoded on antigenomic and genomic sense strands respectively, do not overlap and are separated by an intergenic region. In PT, UUK and TSWV viruses, this region is AU rich and capable of forming a stable stem loop structure of 360,74 and 503 nucleotides respectively (Simons et al, 1990: de Haan et al, 1990). Similar structures have been identified in arenaviruses (Romanowski and Bishop, 1985), and are thought to be involved in transcription termination. The transcription termination sites for N and NSs mRNAs have been mapped close to the terminal loop of the stem structure for PT virus S RNA (Emery and Bishop, 1987).

The SFS virus S segment intergenic region is 250 nucleotides in length, C rich in the genomic sense and does not possess any obvious base paired hairpin loop structure (Marriott et al, 1989). Similarly, the intergenic regions of TOS and RVFV phleboviral S segments, intergenic regions of 62 and 82 bases respectively, lack secondary structure and are C rich in the genomic sense RNA (Giorgi et al, 1991).

These phleboviruses may employ an alternative means of transcription termination to a stem loop structure in the template, possibly involving the homopolymeric C or G stretches in the intergenic region.

C. Genome replication

In negative sense RNA viruses a change from primary transcription to replication is a switch from mRNA synthesis to synthesis of full length antigenomes followed by synthesis of full length genomic RNA. It is presumed that viral and or host factors are involved in this switch since genome replication and subsequent secondary transcription are prevented by inhibitors of translation (Abraham and Pattnaik, 1983; Raju and Kolakofsky, 1986). mRNA differ from full length antigenomes in that mRNA 5' ends are capped and the 3' end never extends to the end of their genomic RNA template and preterminate at mRNA transcription termination sites. In order to transcribe full length antigenomic RNA presumably these factors influence directly or indirectly the addition of capped primers to the 5' end of antigenomic sense RNA and override the mRNA transcription termination signal on the virus genome sense RNA template. The processes involved in this transcriptional switch have not yet been determined for the Bunyaviridae. It is possible that the increasing concentration of unassembled N protein could initiate the switch towards replication as is the case of the non-segmented negative strand RNA viruses (Wertz et al, 1987). Another possible candidate is NSs protein, where present, which may bind directly to the polymerase protein and convert the transcriptase to a replicase.

The functional role of the NSs protein in virus replication is unknown. However, for this protein to be able to function in the initial stages of replication requires that the protein is either expressed

early in infection or is a constituent of the virus particle. The latter has never been reported and the ambisense nature of the S RNA segments of phleboviruses dictate that NSs mRNA cannot be transcribed into full length viral complementary sense RNA. However, the full length S viral complementary sense RNA as well as viral sense RNA have been detected in Uukuneimi virions (Simons et al, 1990). This implies that early expression of NSs protein in infected cells is possible by transcription of NSs mRNA from virion derived antigenome S RNA template. Direct evidence of the early expression of virus sense encoded NSs protein comes from time course studies of RVF phlebovirus infection of cells. Parker et al (1984) demonstrated that NSs protein could be detected as early as two hours after infection and that initial detection of NSs protein was concurrent with the initial detection of N protein. In contrast to these results studies of UUK virus infected BHK 21 cells detected N protein as early as 4 hours after infection but were unable to detect NSs until 8 hours post infection (Simons et al, 1992). In addition a RVF phlebovirus mutant with a 600 base deletion in the coding sequence of NSs has been reported. This mutant is able to replicate to wild type level in Vero and mosquito cells but is defective in human lung diploid MRC-5 cells (Simons et al, 1992). These results would imply that NSs protein is not necessary for initiation of replication and is non essential in particular cell lines. Simons et al (1992) have shown that UUK NSs protein is strongly associated with the 40S ribosomal complex and that this association takes place soon after translation. Vialant and Bouloy (1992) have recently reported that Germiston bunyavirus requires active 40S ribosomal subunit for the efficient transcription of message length and full length S RNA in an in vitro

transcription assay. Given the reported affinity of the NSs protein for the 40S ribosomal complex a possible role for the NSs protein in viral transcription can be envisaged; either in maintaining a pool of 40S ribosomal subunits and/or in coupling the 40S scanning activity on the nascent strand with viral transcriptase activity on the template strand. A possible tenuous link can be drawn between the reqirement for on-going protein synthesis and polymerase activity in particular cell types (Raju et al, 1989). The affinity of the NSs protein for the 40S complex of the translational apparatus and the essential requirement of NSs protein in particular cell types (Simons et al, 1992) requires investigation. Investigation of such a possible link would undoubtedly provide a better understanding of the functional significance of the NSs protein.

A better understanding of the detailed events and interactions that occur during Bunyaviridae genome replication in infected cells awaits further study.

D. Virus maturation

Maturation of Bunyaviridae members differs from other negative strand RNA viruses in that the virions are formed by a budding process at the intracellular smooth membranes of the Golgi (Lyons and Heyduk, 1973; Murphy et al, 1973). The positive strand RNA viruses corona- and flaviviruses similarly bud at intracellular membranes and the corresponding viral glycoproteins accumulate intracellularly rather than being transported to the cell surface. Machamer and Rose (1987) showed that three hydrophobic membrane-spanning domains of coronavirus E1 glycoprotein were required for its retention in the Golgi membrane. Recombinant vaccinia viruses expressing glycoproteins G1 and G2 of RVFV, PT, or HTN virus in infected cells have demonstrated that the

glycoproteins are targetted to the Golgi and this targetting is independent of other viral proteins or RNA (Wasmoen et al, 1988; Matsuoka et al, 1988; Pensiero et al, 1988). In addition, the Golgi signal sequence for RVFV glycoproteins was shown to be encoded within the G1 and G2 coding sequence as removal of the NSm, the preglycoprotein region , did not inhibit localisation of the glycoprotein to the Golgi (Wasmoen et al,1988). Indeed, a recombinant vaccinia virus encoding G2 of PT virus with a hydrophobic sequence from the N-terminal NSm region of PT virus M segment ORF produced a G2 in infected cells that was unable to be transported to the Golgi complex, indicating that NSm was not the Golgi signal sequence (Chen et al, 1991). Expression of the full length G2 protein on its own in another recombinant vaccinia virus resulted in the transportation of the G2 protein to the cell surface and not as expected a localisation in the Golgi. This suggested that although G2 protein may possess its own Golgi signal sequence it lacks a Golgi retention sequence, however transportation of G2 to the cell surface was less efficient than that for VSV-G or influenza HA proteins (Rose and Doms, 1988). Co-expression of G1 and G2 proteins resulted in retention of the glycoproteins in the Golgi complex (Chen et al, 1991). The authors speculated that either G1 possessed the Golgi retention signal or that the retention signal was generated as a result of non-covalent interaction between G1 and G2 proteins when they formed hetero-dimers. Hetero-dimerization of G1 and G2 proteins has been reported to occur in the ER of Uukuniemi virus infected cells implying a similar functional interaction (Persson and Pettersson, 1991). Investigation into the Golgi signal sequence of PT G2 by expression of full length protein with a 13 amino acid hydrophobic leader sequence or G2 protein

without a hydrophobic leader sequence indicated that the hydrophobic domain preceding G2 was able to function as a translocational signal peptide. In addition a hydrophobic domain near the carboxy terminus of G2 protein was shown to act as a membrane anchor as a truncated G2 protein lacking this region was secreted from the cell surface into the culture medium (Chen et al, 1991).

Following retention in the Golgi the glycoproteins accumulate in the Golgi in increasing concentrations causing vacuolation (Gahmberg et al, 1986; Kuismanen et al, 1984). Bunyaviridae family members lack an equivalent to the matrix protein present in most other negative sense RNA viruses. The matrix protein functions as a bridge between the nucleocapsid and the glycoproteins (Yoshida et al, 1976). Thus in Bunyaviridae assembly direct interaction between ribonucleoproteins, which accumulate on the cytoplasmic side of the Golgi, and the viral glycoproteins, on the luminal face, during virion assembly is suggested to occur at the site of virus maturation, and is determined by a critical level of glycoproteins. Such interactions have been observed by the electron microscope in SFS phlebovirus infected cells (Smith and Piffat, 1982). Presumptive glycoprotein transmembrane regions have been suggested from predicted hydropathic plots of Bunyaviridae glycoproteins (Schmaljohn and Patterson, 1990; Smith and Piffat, 1982). Glycoprotein interaction with ribonucleoprotein has been reported in Uukuniemi virus infected cells treated with tunicamycin, a protein glycosylation inhibitor, where the glycoproteins were retained in the rough endoplasmic reticulum but still exhibited interaction (Kuismanen et al, 1984).

After the virus particles are assembled by budding into the Golgi

cisternae they are released in small vesicles and transported to the plasma membrane where fusion of the vesicle and plasma membrane result their release (Smith and Piffat, 1982). The only known example of direct budding of the virus from the plasma membrane was reported for RVFV in infected primary rat hepatocytes, however virus budding into the Golgi cisternae was also noted to occur in the same cells (Anderson and Smith, 1987).

7. Genetics of Bunyaviridae

A. Genome segment reassortment.

The segmented genome of Bunyaviridae family members enables one virus to potentially reassort segments of its genome with segments from another virus within the same genus to create reassortant progeny. This can confer beneficial traits on a progeny reassortant virus, such as increased or altered host range. Reassortment has been shown to occur in nature in La Crosse virus (Klimas et al, 1981) and in the Patois group of bunyaviruses (Ushima et al, 1981). Experimentally virus reassortment has been achieved in dually infected mosquitoes (Beaty et al, 1981, 1982, 1985; Shope et al, 1981) and although reassortment in a vertebrate host has not been demonstrated it has been shown to occur for other segmented viruses (Beaty and Bishop, 1988) which implies the possibility of its occurence in Bunyaviridae infected vertebrate cells. Dual infection in tissue culture has demonstrated that reassortment occurs between viruses within the same genera but not between genera. Even within a genus, reassortment can only be shown to occur between viruses within the same serogroup such as within the California encephalitis (CE) or the Bunyamwera serogroups of the Bunyavirus genus (Bishop et al, 1980; Gentsch and Bishop, 1976; Gentsch et al, 1977; Gentsch et al, 1979; Iroegbu and Pringle, 1981; Ozden and Hannoun, 1980), but not between members of different serogroups (Bishop et al, 1984; Elliott et al, 1984; Iroegbu and Pringle, 1981).

Even within the same serogroup, certain viruses appear unable to reassort with other members. This barrier is clearly not due to geographical isolation as demonstrated by the fact that Maguari virus (South American) and Bunyamwera virus (African) are able to freely

reassort whereas Northway and Main Drain, both found in the same area (North America) remain genetically isolated (Elliott et al, 1984). It would seem likely that the specificity for recombination may be due to the virus encoded gene products, given the relatedness of reassortment groups (Pringle et al, 1984).

Temperature sensitive (ts) mutants have been employed in the selection of reassortant viruses. The ts mutants were obtained by mutagenization of wild type virus using 5-fluorouracil, 5-azacytidine or methyl-N-nitro-guanidine. These ts mutants enable selection of progeny virus at the restrictive temperature if the two parental viruses possess mutations in different virus segments. Almost two hundred ts mutants have been isolated belonging to either the CE or Bunyamwera serogroups (Elliott, 1990). In addition, ts mutants have also been obtained for UUK uukuvirus (Gahmberg, 1984). In one case in the Bunyamwera serogroup, a further restriction on viral reassortment through the linkage of viral L and S segments which co-segregate in heterogeneous crosses involving temperature sensitive mutants of Batai Bunyamwera and Maguari bunyaviruses, whereas the M segment would reassort freely. This linkage could be overcome when heterologous reassortants were used as parental viruses (Elliott et al, 1984; Pringle et al, 1984).

Reassortment experiments with ts mutants of CE serogroup viruses resulted in their being placed in two non-overlapping reassortant groups with a ts lesion in either the L segment (group II) or in the M segment (group I) (Gentsch et al, 1977, 1979; Bishop et al, 1984). The inability to obtain bunyavirus mutants with lesions in the S segment has been suggested to be due to the coding strategy of this segment: as the N and NSs proteins are encoded in overlapping reading frames any mutation in

the overlap region would have a greater likelihood of being lethal to the virus rather than being a ts mutation (Murphy and Pringle, 1987). Reassortment experiments with Bunyamwera serogroup ts mutants resulted in three distinct reassortant groups, presumably representing each of the three segments. Only one group, group II, has been clearly assigned to a genome segment i.e. the M segment (Iroegbu and Pringle, 1981).

By reassortment experiments Uukuniemi virus ts mutants were classed into two reassortant groups, although group assignment to individual segments has not been reported (Gahmberg, 1984).

B. Genome variation and Bunyaviridae evolution

Two possible means by which Bunyaviridae evolve are by RNA segment reassortment, discussed in the above section, and by genetic drift. The process of genetic drift involves the accumulation of point mutations, and possibly sequence deletions, duplications and inversions (Beaty and Bishop, 1988). Indeed, the spontaneous mutation rate of SSH virus has been proposed to be as high as 1-2% (Bishop and Shope, 1979), and evidence indicates that this genomic plasticity may also apply to another member of the same serogroup, the LAC virus. Several field isolates of LAC virus were obtained from various geographical locations and these were subjected to genomic analysis by oligonucleotide fingerprinting. Analysis revealed that each of the isolates possessed individually distinguishable genome profiles (El Said et al, 1979; Klimas et al, 1981). In contrast the stabilty of the Bunyaviridae genomes has also been demonstrated. The genomes of two isolates of LAC virus obtained 18 years apart, from brain tissues of fatally infected individuals, possessed very similar oligonucleotide fingerprints (Bishop and Shope, 1979). In addition, no genomic changes were detected during

transovarial passage of LAC virus through two generations of mosquitoes, nor in the LAC virus isolates from suckling mice that had been infected by second generation transovarially infected mosquitoes (Baldridge et al, 1989).

Bilsel et al (1988) reported that Toscana phlebovirus, serially passaged by transovarial transmission over a period of two years and through twelve generations of the natural sandfly host, underwent no detectable genomic change. Conversely, Tesh and Gubler (1975) have demonstrated the presence of small plaque variants in the population of transovarially passaged LAC virus, indicating a heterogeneous virus population. Furthermore, Sundin et al (1987) were able to isolate a LAC virus variant by use of monoclonal antibodies in tissue culture. This mutant exhibited decreased cell fusion ability, loss of mouse neuroinvasiveness and decreased infectivity in mosquitoes, implying that the LAC virus genome is able to respond adaptively to selective pressure. The virus was able to revert back to wild type phenotype following recovery from an infected mosquito and passage in BHK-21 cells. The definitive effects on genome stability and selection of viral variants during transovarial and venereal transmission in invertebrate hosts and viral passage between the invertebrate and vertebrate hosts await further study.

8. Defective interfering particles

As with other animal viruses high multiplicity passage of Bunyaviridae in mammalian cells has been reported to produce defective interfering (DI) particles (Kacscak and Lyons, 1978; Cunningham and Szilagyi, 1987; Verani et al,1984). Based on studies of better defined DI particles such as those of vesicular stomatitis virus, VSV, (Holland, 1990) it is suggested that Bunyaviridae DI particles

contain only a portion of the parental virus genome. The DI particles therefore require co-infection with competent homologous helper virus to replicate in a host cell. In such an infection the helper virus replication is greatly supressed by a process termed autointerference.

In comparison with reports of DI particles of VSV, Sendai and influenza viruses there are few reports of Bunyaviridae DI particles. Kascsak and Lyons (1978) observed a species of Bunyamwera DI particle which co-sedimented with the S RNA segment of the wild type virus; whereas Cunningham and Szilagyi (1987) detected truncated L RNA genome subunits in the cytoplasm of GER virus infected BHK-21 cells. Mammalian cells persistently infected by Dugbe nairovirus (David-West and Porterfield, 1974) or Toscana phlebovirus (Verani et al, 1984) suggest the involvement of DI particles but these have not been directly demonstrated. Persistently infected C6/36 mosquito cells produced auto-interfering particles containing an intact S segment but devoid of L and M RNAs (Elliott and Wilkie, 1986). More recently Scallan and Elliott (1992) reported the presence of subgenomic RNAs derived from the L RNA in BUN virus persistently infected C6/36 cells. These shorter RNAs were unable to be packaged into nucleocapsids. Patel and Elliott (1992) reported the isolation of DI particles from BUN virus infected mouse L cells. These particles were found to contain defective L RNAs, which unlike those reported by Scallan and Elliott (1992), were able to be efficiently packaged into the virion. Analysis of the DI RNAs from the DI particles reported by Patel and Elliott (1992) revealed that they had a single internal deletion of between 72% to 77% of the L segment but retained the 5' and 3' terminal sequences. These results indicate that the

inability of defective L RNAs in persistently infected C6/36 cells, identified by Scallan and Elliott (1992), to be packaged into nucleocapsids may be because these RNAs do not possess the necessary terminal sequence(s). Resende et al (1991) have shown that TSWV supports the replication of DI RNAs formed during the serial passage of the virus at high multiplicity in Nicotiana rustica plants. Further characterisation of DI RNAs from four isolates revealed that they were all derivatives of the L RNA segment and that 5' and 3' termini were retained but approximately 60% to 80% of the L RNA segment was missing in a single internal deletion (Resende et al, 1992), as similarly reported for BUN DI L RNA by Patel and Elliott (1992). Resende et al (1992) proposed that DI RNA generation is accomplished by the viral polymerase 'jumping' across the internal sequence from one secondary structure to another containing the repeated sequences, during the process of replication of the viral complementary strand. This mechanism of detachment and reinitiation of synthesis at a specific signal on the same molecule has been proposed to explain the generation of DI molecules in a number of viral systems (Lazzarini et al, 1981).

The formation of DI particles would seem common to all Bunyaviridae, but the process by which DI particles are formed and the role of Bunyaviridae DI particles in the natural infection cycle are at present unknown and await further investigation.

9. Persistent Bunyaviridae infection

In contrast to infection of mammalian cells, infection of mosquito cell cultures by arthropod-borne members of the Bunyaviridae family causes little cellular cytopathic effect and an asymptomatic persistent infection is readily established (Newton et al, 1981). A number of

persistently infected mosquito cell lines have been established with Bunyamwera, LAC and Marituba bunyaviruses and Toscana phlebovirus (Carvalho et al, 1986; Elliott and Wilkie, 1986; Newton et al, 1981; Nicoletti and Verani, 1985; Rossier et al, 1988). These persistently infected (PI) cells are resistant to superinfection by homologous virus which implies that part or all of the genome is expressed in the PI cell (Newton et al, 1981; Elliott and Wilkie, 1986). Indeed, a small percentage of the PI cells continue to shed virus (Elliott and Wilkie, 1986; Newton et al, 1981). A comparative study of Bunyamwera virus infected mammalian and mosquito cells (incubated at 37°C and 26°C respectively) carried out by Newton et al (1981) revealed a high level of synthesis and accumulation of viral proteins in BHK cells which was sustained until 30 hours post infection (hpi) when the first signs of cytopathic effect were visible. In contrast viral protein synthesis in mosquito cells was low and was reduced to barely detectable levels by 30 hpi. The authors suggested that the outcome of infection in cultured cells is determined by the rate of "synthesis, clearance and accumulation" of virus-coded proteins such that if the rate of synthesis and accumulation is high and clearance low then cytopathic effect will result. In contrast Raju and Kolakofsky (1988) have attributed the cause of cytopathic effect in mammalian cells to a general virus-induced mRNA instability.

Rossier et al (1988) carried out a detailed study of LAC bunyavirus infection of BHK and mosquito cells under identical conditions of cell growth and temperature (33°C). It was found that even under identical conditions virus specific proteins and RNA accumulated more slowly in mosquito cells. Maximum levels of mRNA and full length genomic RNA

synthesis were obtained in BHK cells by 6 hpi and cytopathic effect became visible at 14 hpi. The maximum levels in mosquito cells were not reached until 24 hpi and the concentration of virus specific proteins and RNAs in mosquito cells at this point was equivalent to, and exceeded, that in BHK cells. The mosquito cells then progressed from an acute phase of infection to establishment of persistent infection (post 24 hour) characterised by a reduction in virus N protein synthesis and virus replication. Curiously, the decline of N protein synthesis was not related to a change in the S mRNA level which remained almost unaltered and implied that a form of translational control was in operation (Rossier et al, 1988).

Hacker et al (1989) postulated that beyond a certain threshold level of N protein concentration, the protein interacted with its own (S) mRNA which would inhibit mRNA translation and production of more N protein. This would in turn reduce the level of available N protein and therefore reduce virus replication. Replication of VSV and Sendai virus in BHK cells has been shown to be influenced by the level of their nucleocapsid proteins (Raju and Kolakofsky, 1987). In order to prove this hypothesis Hacker et al (1989) investigated the levels of encapsidated mRNA in infected mosquito cells during infection. At 12 hpi less than 10% viral mRNA was encapsidated, whereas between 24-48 hpi 75% of viral mRNA was assembled with N protein. Encapsidation was found to be specific for virus mRNA, which could not be translated in vitro (Hacker et al, 1989). It is, however, unlikely that the down regulation of virus replication is due simply to the level of unassembled N protein as its pool increases with time and N protein is highly specific for genome and antigenomes, and viral mRNAs are only a

secondary target. Hacker et al (1989) therefore suggested that the down regulation of genome replication may be due to some factor directly affecting the polymerase, since the polymerase is expressed at low levels in mosquito cells and would therefore be a suitable target to control by modification. This factor was suggested to inhibit the polymerase replicase activity, which would impede genome and antigenome synthesis but not viral mRNA synthesis. The transcriptase products would in turn be down regulated by the increasing levels of unassembled N protein, resulting in the reduced levels of viral products seen after 24 hpi which would establish persistent infection. This hypothesis awaits further investigation.

10. Specificity of protein-RNA interactions

This project is partially concerned with the study of interaction between bunyavirus nucleocapsid protein and the S RNA genome segment. Therefore a general review of protein-RNA interactions is presented in this section as an introduction to this area of study.

Nucleic acid binding proteins can be categorised into DNA and RNA binding proteins. The DNA binding proteins have been studied in great detail. In general they all interact with B-form DNA in a sequence specific manner and recognition of specific sites is through small discrete binding domains. On the basis of common primary sequence and proposed three dimensional structures these DNA binding domains can be sub-classified into: helix turn helix motif; zinc finger binding motif; leucine zipper protein dimerisation and binding motif; β-ribbon recognition element; helix-loop-helix structure (Harrison, 1991). These protein domains all fold such that they present a protruding surface/structure that enables sequence specific contact with base pairs

predominantly in the major groove of β-form DNA.

In comparison with DNA binding studies our knowledge of RNA binding is poor. Undoubtedly RNA binding studies are complicated by the structural versatility of RNA molecules which are able to form both single stranded and double stranded structures. The determinants of RNA binding specificity are therefore more likely to include the three dimensional RNA structure as well as the primary RNA sequence. In this respect RNA-protein interaction is more analogous to protein-protein binding than protein-DNA binding. RNA primary and tertiary structures that enable specificity of interaction have been identified in some RNA binding studies. Similarly some protein domains that enable selective RNA binding have been identified. However only in very few situations have both RNA and protein binding structures been fully understood eg TMV capsid structure (Namba et al, 1989)

A. RNA binding protein motifs

(i) The RNA recognition motif

The best characterised RNA binding motif consists of approximately 80 amino acids. Initially called the RNA binding domain (Dreyfuss, et al, 1988) it was later named the RNA recognition motif, RRM (Query et al, 1989). The RRM contains two elements: a highly conserved ribonucleoprotein consensus sequence of 8 amino acids (RNP 1); and a less well conserved sequence of 6 amino acids (RNP2) (Nagai et al, 1990; Query et al, 1989). The RRM was initially identified in yeast poly (A) binding protein which contains four copies of this structural motif (Sachs et al, 1986). The RRM has subsequently been found in several prokaryote and eukaryote proteins that bind single stranded nucleic acid including: nucleolin; heterogenous nuclear RNP proteins; rho protein; E.coli

protein T4 gp 32; Drosophila genes Sxl, Tra and elav; and components of U1 and U2 snRNPs (Query et al, 1989). It has been demonstrated that a polypeptide slightly larger than the RRM motif is sufficient for RNA binding activity (Scherly et al, 1990a), whereas deletion analysis indicated that the entire RRM is required for sequence specific binding (Query et al, 1989).

Although the RRM is recognised as the hallmark of an RNA binding protein it has been difficult to provide a consensus model for RNA binding by this motif especially since it is extremely flexible in that it enables recognition of a variety of RNA structures. Thus while rho protein is thought to bind C-rich unstructured RNA, U1 70K and A proteins of U1 snRNP bind specifically to stem loop structures, and poly (A) binding protein recognises a stretch of adenosines. Secondary and tertiary models for RRM have been proposed and consist of four anti-parallel β strands with two intercalated strands behind (Ghetti et al, 1989). The X-ray crystallographic structure of the RNA binding domain of U1 snRNP A protein revealed a similar structure to the model (Nagai et al, 1990). The conserved consesus sequences RNP1 and RNP2 were found to lie side by side on the middle of the two middle β strands and would be able to intercalate with RNA bases via two aromatic residues (one in each motif). In addition the X-ray crystallographic structure revealed two parallel flexible loops at one end of the β sheets. These loops contained more than half the arginine and lysine residues present in the RRM. Crystallographic studies of protein DNA complexes have shown that lysine and arginine residues are crucial for binding to the phosphate backbone of DNA molecules (Harrison and Aggarwal, 1990). A similar role is suggested for these basic loops in the RRM, such that they act as

basic jaws that enable binding or gripping of the RNA. Additionally site directed mutagenesis has demonstrated that the specificty of interaction with the target RNA bases is obtained by these loops through interaction with serine residues (Nagai et al, 1990). The site of RNA interaction takes place at a stem loop structure of comparable size with the RRM domain (stem loop II of U1 snRNA). Specific recognition of the RNA by protein A occurs on the ten nucleotide loop structure. Alteration of the amino acid sequence emphasised the importance of specifically positioned cytosine residues in order to maintain specificty of the interaction. These cytosines are believed to hydrogen bond with protein A serine residues located on the RNA binding loops (Nagai et al, 1990).

Although polypeptides no larger than the RRM are able to bind specifically to RNA (Query et al, 1989; Scherly et al, 1990a) external influences are able to affect the specificty of binding by this motif. The RRM protein U2B", a component of U2 snRNP, binds specifically to stem loop IV of U2 snRNA. In contrast to U1 A protein however U2B" requires a second protein, U2A', with which it dimerises to enable specific binding to the target RNA (Scherly et al, 1990a). In the absence of U2A', U2B" binds non specifically and displays equal affinity for stem loop structures in U1, U2 and U6 RNA. The U2A' protein does not contain a RRM but specifically interacts with the RRM of U2B" protein causing specific conformational changes that enable U2B" to interact specifically with target RNA (Scherly et al, 1990b).

(ii) The arginine rich RNA binding motif

A separate class of sequence specific RNA binding proteins has been identified. These proteins although lacking a RRM posses an arginine rich motif of 6 to 8 basic amino acids usually flanked by non basic

polar and charged amino acids. This motif was first identified in λ N protein (Lanzinski et al, 1989) and has been identified in a number of other RNA binding proteins including HIV proteins Tat and Rev; ribosomal and snRNP core proteins; and RNA virus coat proteins (Figure 10).

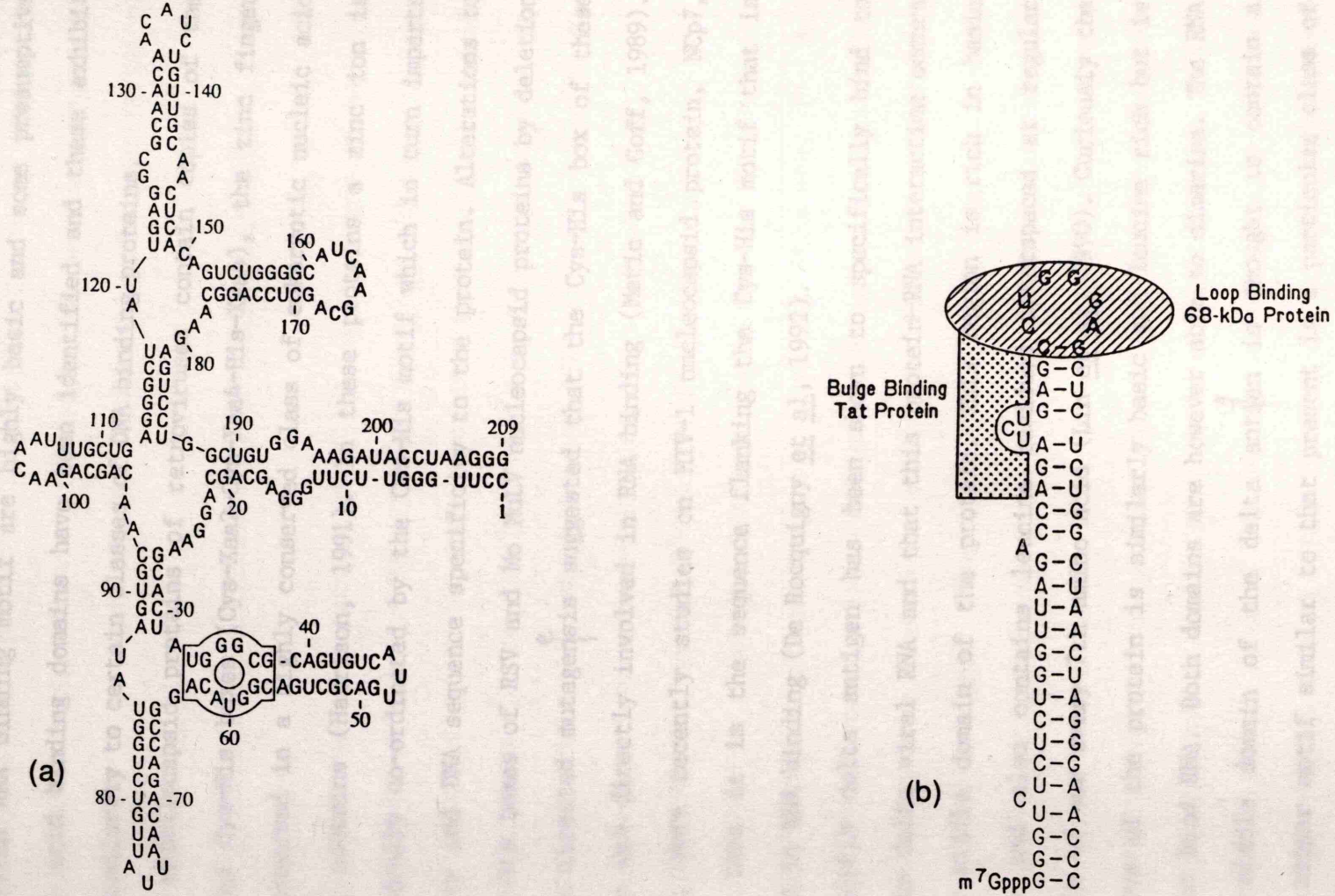
The best studied example of this class of binding protein is Tat. Synthetic peptides of 24 and 12 amino acids in length bearing the Tat arginine rich region were able to bind specifically to Tat RNA (Weeks et al, 1990; Subramanian et al, 1990). However a slight loss in specificty was obtained as peptides would bind to both positive and minus sense target RNA (Heaphy et al, 1991). This implied that other regions outside the arginine-rich motif were implicated in selective RNA binding. Further evidence for this suggestion was obtained when the arginine-rich region of Tat protein was substituted for a similar region in Rev protein. Although both Tat and Rev proteins have distinct target RNAs the hybrid Tat-Rev protein bound to Tat target RNA with normal binding activty (Subramanian et al, 1990). A dependence on domains outside the the arginine-rich motif for complete specificity for the target RNA has also been demonstated for the λ N protein (Lanzinski et al, 1989). Whereas in contrast to Tat the arginine rich motif is sufficient for Rev specific binding (Steffy and Wong-Staal, 1991).

Rev and Tat proteins interact with different target RNA structures. Rev protein binds to a stem bulge stem structure whereas Tat binds to a stem loop structure (Figure 11). In both cases however a distorted sugar phosphate backbone surrounded by double base pair regions is a critical determinant for recognition (Heaphy et al, 1991; Steffy and Wong-Staal, 1991) and may be a common RNA motif for this group of RNA binding proteins.

Figure 10. Conservation of the arg-rich motif in potential RNA binding proteins, taken from Lanzinski et al (1989).
Abbreviations: B, basic; O, nonbasic polar; Z, charged; J,acidic; HIV, human immuno-deficiency virus; Ad 2, adenovirus 2; SSV, simian sarcoma virus; MMSV, mouse mammary sarcoma virus; FeSV, feline sarcoma vius; LCV, lymphocytic choriomeningitis virus; B. stear., Bacillus stearothermophilus; and L. chor., Liverwort chloroplast.

rkvsdrglts	**RDRRRiaRwEK**	riayalkngv	Nun of phage HK022
mdA	**qTRRRERRaEK**	qaqwkaanpl	N of Lambda
ivwkeskgtA	**KSRyKaRRaEl**	iaerrsneal	N of phage 21
ygkstfagnA	**KTRRHERRRKl**	aierdticni	N of phage 22
itkalgisyg	**RkKRRgRRRap**	qgsqthqvsl	Tat of HIV-1
npypqgpgtA	**sQRRnrRRRwK**	qrwrqilala	Rev of HIV-2
ewiyynyyte	**RaKRRDRRRRs**	vchartwfcf	E4 13kd of Ad2
qerekkeaee	**RERRRDRRqEK**	nltril	Gag of SSV
eerrrtedeq	**KEKeRDRRRhR**	emsrll	Gag of MMSV
gprtptaspi	**aSRlRERREnp**	aeesqal	Gag of FeSV
ldfsevsnvq	**RimRKERRDDK**	dlqrlrslnq	Nucleoprotein of LCV
kgqvvkavvv	**RTKRgvRRpDg**	syirfdenac	L14 of B. Stear.
rmktgnkiry	**nSKRRhwRRtK**	lgl	L39 of Rat
vteaspmvkA	**KDeRRERRDDf**	anetaddaea	S6 of E.coli
mn	**KSKRssRRRmp**	pirsgeiidy	S18 of L. chlor.
lakhglyekk	**KTsRKqRkERK**	nrmkkvrgta	S19 of Xenopus
vscaihskvv	**RNRsREaRKDR**	tppprfrpag	S26 of Rat
	BOBRBJRRZZB	[Consensus]	

Figure 11. Target RNA binding sites of (a) rev and (b) tat HIV regulatory proteins. (a) Predicted secondary secondary structure of the rev response element (RRE) of HIV-1 (bases 7786-8010); the primary rev binding site in this region has been identified as a purine rich bubble sequence, indicated as a boxed sequence in (a). Figure taken from Heaphy et al (1991). (b) Predicted structure of the trans activation response element (TAR). The TAR RNA stem loop is present at the 5' end of all HIV mRNA species and tat protein is believed to bind to the bulged loop in the TAR stem. An endogenous 68K RNA binding protein that also binds to TAR is also indicated. Figure taken from Cullen et al (1990).


(a)
(b)
Loop Binding
68-kDa Protein
Bulge Binding
Tat Protein
m7GpppG

(iii) Other RNA recognition motifs

Other RNA binding proteins although not possessing a distinctive RRM or Arg-rich RNA binding motif are highly basic and some presumptive nucleic acid binding domains have been identified and these exhibit great similarity to certain classes of DNA binding proteins.

The metallocapsid proteins of retroviruses contain copies of the conserved Cys-His boxes (Cys-Xaa2-Cys-Xaa4-His-Xaa4), the zinc finger motif observed in a highly conserved class of eukaryotic nucleic acid binding proteins (Harrison, 1991). In these proteins a zinc ion is tetrahedrally co-ordinated by the Cys-His motif which in turn imparts stability and DNA sequence specificity to the protein. Alterations to the Cys-His boxes of RSV and Mo MuLV nucleocapsid proteins by deletion or site directed mutagenesis suggested that the Cys-His box of these proteins was directly involved in RNA binding (Meric and Goff, 1989). However, more recently studies on HIV-1 nucleocapsid protein, NCp7, suggest that it is the sequence flanking the Cys-His motif that is involved in RNA binding (De Rocquigny et al, 1992).

Hepatitis delta antigen has been shown to specifically bind to hepatitis delta viral RNA and that this protein-RNA interaction occurs via the middle domain of the protein. This region is rich in basic residues and also contains leucine residues interspaced at regular intervals of one every 7th amino acid (Lin et al, 1990). Curiously the N-terminus of the protein is similarly basic and leucine rich but is unable to bind RNA. Both domains are however able to dimerise. The RNA binding middle domain of the delta antigen is thought to contain a leucine zipper motif similar to that present in a particular class of DNA binding proteins (Landshulz et al, 1988). In these proteins the leucine rich region is also located next to a basic region. Dimerisation of the molecule through interaction of leucine side chains on individual

molecules causes a conformational change in the protein that enables the basic region of the protein to interact with its target site on the DNA. Hepatitis d antigen however, unlike its DNA binding counterparts would seem not to be dependent upon dimerisation for specificity of interaction (Lin et al, 1990).

B. Specific interaction between capsid proteins and viral RNA in RNA viruses

Selective interaction between viral RNA and coat protein has been demonstrated for a number of RNA viruses. The E.coli RNA bacteriophages were some of the first to be shown to exhibit this specificity and the site of RNA interaction was localised to a bulged hairpin structure in all cases (Witherell and Uhlenbeck, 1989). The specificity of the interaction enables efficient and selective encapsidation of viral genomic RNA and invariably this specificity can be localised to particular RNA sequences which are able to form a three dimensional structure such as a stem loop structure. The coat protein of tobacco mosaic virus (TMV) exhibits high specificity for its genomic RNA and the protein-nucleic acid interactions have been a subject of intense study. The virus particle consists of a single stranded 6.4kb RNA genome coated in a helical array by around 2,100 copies of the coat protein (M_r 17K). Infectious TMV particles can be reconstituted in vitro from purified RNA and coat protein components. Assembly is initiated by a specific interaction between a coat protein disk aggregate and an internal RNA sequence of approximately 75 bases, the assembly origin, located about 1Kb from the 3' end of the genome (Turner et al, 1988; Zimmern and Butler, 1977). This RNA sequence contains a putative stem loop structure and encapsidation by TMV coat

protein of heterologous RNA *in vitro* and *in vivo* has been obtained by the inclusion of this 75 base sequence (Gallie *et al*,1987; Sleat *et al*, 1986; Sorger *et al*, 1986). Selective interaction between coat protein and RNA was later shown to take place at the apex loop and specifically by hydrogen bonding to 2 of 3 regularly repeated G residues (Turner *et al*, 1988; Namba *et al*, 1989). Viral assembly is assumed to take place subsequently by the sequential addition of coat protein aggregates to a growing helical nucleoprotein rod (Namba *et al*, 1989).

Viral RNA sequences containing putative stem loop structures that enable RNA selection by capsid protein have subsequently been identified in turnip crinkle virus (TCV), alfalfa mosaic virus (AMV) and MHV coronavirus (Stohlman *et al*, 1988; Wei *et al*, 1990; Zuidema *et al*, 1983). A selective encapsidation signal has also been reported for Sindbis alphavirus (Weiss *et al*, 1989) although no specific stem loop structure has been suggested. These selective RNA sequences are located near the 5' terminus of some RNA genomes (eg Sindbis alphavirus and MHV coronavirus) and near the 3' terminus in others (eg TMV and AMV). In contrast TCV is reported as having a selective sequence near both 5' and 3' termini.

RNA binding in some cases may require heterogenous protein interactions between capsid protein and other viral protein(s) before sequence specific RNA binding is able to take place. VSV nucleocapsid protein has been shown to specifically bind to the 5' ends of both negative and positive strand leader like VSV RNAs but not to other VSV RNAs (Blumberg *et al*, 1983; Moyer *et al*, 1991). RNA sequences conferring specificity have been localised to 19 nucleotides from the 5' end of the positive strand RNA (Moyer *et al*,1991). The specificity of the interaction is

such that over 75% of the leader RNA in the infected cell is encapsidated by N protein. However, specificity *in vitro* can only be demonstrated in the presence of an additional viral protein, NS, with which it complexes to impede sequence non-specific interactions (Masters and Banerjee, 1988). A similar result is obtained *in vivo*. When N protein is expressed in COS cells in the absence of other VSV viral proteins, the expressed protein behaves as if bound to cellular RNA (Sprague *et al*, 1983). In contrast, in VSV infected cells such binding does not occur, presumably due to the presence of NS protein (Masters and Banerjee, 1988). It should be noted that N protein sequence-specific RNA binding can also be achieved *in vitro* if a cytoplasmic extract from uninfected cells or poly(A) is present (Moyer *et al*, 1991). It is presumed that these factors are in some way able to mimic the action of NS protein, possibly by impeding N self-aggregation (Moyer *et al*, 1991).

The sequence-specific RNA binding of the nucleoprotein (NP) in the ribonucleoprotein (RNP) core of the influenza virion particle may similarly be subject to heterogenous protein interaction before RNA sequence specific interactions can occur. Each RNP consists of a RNA genome segment associated with RNA polymerase and numerous copies of NP. It has been demonstrated using a reporter gene encoding chloramphenicol acetyltransferase (CAT) that the 5' terminal 22 nucleotides and the 3' terminal 26 nucleotides of the viral RNA segments contain the signals necessary for transcription, replication and packaging of influenza virus RNAs *in vivo* (Luytjes *et al*, 1989). However, binding studies carried out *in vitro* between purified NP and virus specific RNA failed to demonstrate specificity of interaction and NP bound non specifically

to any RNA (Yamanaka et al, 1990). Therefore for sequence specific interaction to occur the presence of one or all of the RNA polymerase components (PB1, PB2 and PA) may be required.

Recognition and interaction of viral RNA by viral capsid proteins is dependent upon specific sequences on the RNA molecule that allow the protein to interact specifically with the RNA. Subsequent encapsidation of any downsteam sequence is similarly dependent upon the initial sequence specific protein-RNA interaction. The relative importance that homogenous or heterogenous protein interactions may have on the ability of the capsid protein to recognise RNA signals in different virus systems awaits further investigation.

11. Aims of the project

A. Bunyavirus S segment protein coding strategy and expression

The complete nucleotide sequence of a number of bunyavirus S segments have been published (Elliott, 1990). All encode N and NSs proteins in overlapping reading frames. The NSs ORF in all but one case initiates from a tandem AUG and in two S segments a third overlapping reading frame is present. Despite a detailed knowledge of the coding strategy of the S segment proteins little is known about their expression.

The aims of the project were to investigate N and NSs protein expression in vitro from polycistronic and monocistronic mRNAs derived from S cDNAs under control of a T7 promoter and translated in cell free translation systems. Oligonucleotide site directed mutagenesis was also applied to determine the importance of the NSs tandem initiating AUG. These studies utilised the available Maguari bunyavirus S cDNAs. The S segment of this virus encodes an additional third overlapping reading frame and its expression was similarly investigated. In addition an

attempt was made to raise a monospecific antiserum to this protein to enable study of its expression in virus infected cells.

B. Bunyavirus nucleocapsid assembly

Nucleocapsids isolated from infected cells contain either negative sense genomic RNA or full length plus sense RNA but not bunyavirus mRNAs which have different 5' and 3' termini. The mechanism of this selectivity is unknown. This project studied the interaction of N protein with full length and abbreviated S RNAs in an attempt to define selectivity and the optimum conditions for interaction. These studies were carried out using a recombinant Maguari virus N protein expressed in a baculovirus or bacterial expression system. The N protein was reacted with ^{32}P labelled S RNA specific transcripts generated by T7 run-off transcription reaction of Bunyamwera S cDNA templates under T7 promoter control.

Chapter 2

1.Materials.

1.1 Chemicals

Chemicals were obtained from either BDH Ltd or Sigma Chemical Co.Ltd., except for the following:

Beecham Research Labs Ltd.:ampicillin (Penbritin).

Bio-Rad Labs Ltd.: ammonium persulphate, TEMED, Coomassie Brilliant Blue R250.

Du Pont UK Ltd.: En^{3}hance autoradiography enhancer.

James Burrough (FAD) Ltd.: boric acid, caesium chloride.

May and Baker Ltd.: acetic acid (glacial), chloroform, glycerol, hydrochloric acid.

Pharmacia LKB Ltd.: Ficoll 400, 2'-deoxyribonucleosides 5'-triphosphates, ribonucleoside 5'-triphosphates, 2'-deoxycytidine 5'-O-(1-α thiotriphosphate), T7 promoter and universal sequencing primers, and cap analogue [m7 G(5')ppp(5')G].

Johnson Matthey GMBH Alfa Products: methyl mercury hydroxide.

Gibco BRL Ltd.: Xgal (5 bromo-4-chloro-3-indolyl-B-galactoside), IPTG (isopropyl-B-D thiogalacto-pyranoside).

1.2. Miscellaneous Materials

Dialysis membrane from Medicell International.

Nitrocellulose BA85 and BA83 from Schleicher and Schuell.

λ DNA digested with HindIII as size markers from Gibco BRL Ltd.

RNAsin recombinant RNase inhibitor from Promega.

Bacterial and tissue culture plastics from Gibco BRL Ltd.

Trypsin (0.25% w/v) from Gibco BRL Ltd.

X-Omat S film for autoradiographs from Kodak Ltd.

Gene Clean DNA purification kit from Stratech Scientific Ltd.

RNaid RNA purification kit from Stratech Scientific Ltd.

Western Blot Horseradish peroxidase (HRP) system from Promega.

Wheat Germ Extract translation system from Amersham.

Rabbit reticulocyte lysate translation system from NEN Ltd.

Protein assay reagent from Pierce, Ltd.

Pansorbin cells (a 10% suspension of formalin fixed S. aureus cells) from Calbiochem Corporation.

1.3. Enzymes

Restriction enzymes were obtained from Bethesda Research Labs and New England Biolabs.

Calf intestinal phosphatase was manufactured by Boehringer Mannheim.

T7 DNA polymerase from Pharmacia LKB Ltd.

T4 polynucleotide DNA kinase from New England Biolabs.

T4 polynucleotide DNA ligase from Gibco BRL Ltd.

RQ1 DNase from Promega.

T7 RNA polymerase from Promega.

Phosphorylated Restriction enzyme linkers from New England Biolabs.

1.4 Radiochemicals

Radiolabelled compounds were supplied by Amersham International PLC.

	Specific Activity.
[^{35}S]-L-methionine	>800 Ci/mmol.
5'[α-^{32}P] deoxyadenosine triphosphate	>3000 Ci/mmol.
5'[α-^{32}P] cytidine triphosphate	>400 Ci/mmol.

1.5 Bacterial strains

All strains used were E.coli K 12 derivatives. They were stored on plates at 4°C or as overnight cultures in 40% v/v glycerol at -20°C. A list of bacterial strains used is given in Table 1.

1.6 Bacterial plasmids

A list of bacterial plasmids used is given in Table 2. Plasmid DNA was stored at -20°C.

1.7 Bacteriophage

All bacteriophage were derivatives of M13 phage. A list of bacteriophage used is given in Table 3. Bacteriophage were stored either at 4°C as phage supernatants in 2TY broth or as double-stranded replicative form (RF) DNA at -20°C.

1.8. Media

Luria (L) broth (per litre):	Tryptone	10g.
	Yeast extract	5g.
	NaCl	5g.
	Thymine	3.5mg.
	Glucose	1g.
Luria (L) agar (per litre):	Luria broth with agar added to 15g/l.	
2TY broth (per litre):	Tryptone	16g.
	NaCl	5g.
	Yeast extract	10g.
M9 medium (per litre):	$CaCl_2.2H_2O$	3.7mg.
	$MgSO_4.7H_2O$	62mg.
	$NaHPO_4$	1.5g.

	KH_2PO_4	75g.
	NaCl	1.25g.
	NH_4Cl	27g.
	Glucose	4g.
	Thiamine (B1)	5mg.
M9 agar:	M9 medium with agar added to 12g/l.	
Top agar:	Tryptone	10g.
	Yeast extract	5g.
	NaCl	5g.
	Agar	7g.

Supplementation of broth/agar with antibiotics: if required, broth/agar were supplemented with ampicillin at 50ug/ml or tetracycline at 12ug/ml.

2. Methods

Recombinant DNA Techniques.

2.1 Preparation of plasmid DNA (Maniatis et al, 1982)

A single colony from an agar plate was inoculated into 10ml of Luria broth, supplemented with the appropriate antibiotic to maintain selection for plasmid carrying bacteria. The culture was shaken overnight at 37°C. Five mililitres of this starter culture and the appropriate antibiotic were added to 350 ml of Luria broth and shaken overnight in a 2 litre flask. The culture was poured into centrifuge bottles and centrifuged at 5000 rpm for 20 minutes in a GS 3 rotor. The cells were resuspended in 100ml of STE (0.1M NaCl, 10mM Tris HCl pH 8.0, 1mM EDTA) and re-pelleted (5000rpm/10 minutes).

The washed bacterial pellet was resuspended in 8ml of solution I (50mM glucose, 25mM Tris HCl pH 8.0, 10mM EDTA, 1mg/ml lysozyme). The

Table 1. List of bacterial strains.

Strain	Genotype
NM522	F′ *lacI*^{q}*Δ(lacZ)M15 proA*$^+$*B*$^+$ */supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5* (r_K^- m_K^- McrBC$^-$)
JM101	F′ *traD36 lacI*^{q}*Δ(lacZ)M15 proA*$^+$*B*$^+$ */supE thi Δ(lac-proAB)*
DH5 α	F′*/endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF)U169 deoR* (ϕ80d*lacΔ(lacZ)M15*)
TG1	F′ *traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$*B*$^+$*/supE Δ(hsdM-mcrB)5* (r_K^- m_K^- McrB$^-$) *thi Δ(lac-proAB)*

Table 2.
List of bacterial plasmids

pMAG60
pTZ18U
pTZMAGS
pTZMAGN
pTZMAGS(NSs1)
pTZMAGS(NSs2)
pTZMAGSΔN(NSs1)
pTZMAGSΔN(NSs2)
pTZMAGNSs
pTZMAGNSs1
pTZMAGNSs2
pTZORF3
pUC9
pUC18
pUCT7MAGS5
pUCT7MAGS
pAcYM1
pYM1MAGS
pTF7.5
pTF7.5MAGS
pTF7.5MAGNSs
pUEX1
pEX2
pUEX/ORF3
pEXN
pUCT7BUNS(+)
pUC9T7BUNS
pUCT7BUNS(-)
pUCT7BUNS300
pTZBUNS660
pTZVSVNS

The plasmids listed above were obtained as a direct result of the work reported in this thesis, except for: pTZVSVNS, which was constructed during separate research work (R M Elliott and A McGregor, unpublished work); pAcYM1, obtained from B. Possee (Institute of Virology, Oxford); pTF7.5, obtained from B. Moss (NIH, Bethesda, USA); pMAG60, pEX2 and pUEX1, supplied by R.M. Elliott; pUC9, pUC18 and pTZ18U supplied by Pharmacia.

Table 3. List of M13 constructs.*

Construct
M13mp19MAGS(BB)
M13mp19MAGS(BB)ORF3
M13mp19MAGS(61-945)
M13mp19T7MAGS(10-86)
M13mp19T7MAGS(1-86)
M13mp19T7BUNS
M13mp19BUNS(12-387)
M13mp19BUNS(1-387)
M13mp18T7BUNS
M13mp18T7BUNS(Ec98)
M13mp18T7BUNS(Ec98,768)
M13mp18T7BUNS(-)
M13mp18BUNS(387-944)
M13mp18BUNS(387-961)
M13mp18BUNS300

*Recombinant M13 were derived from either parental M13mp18 or M13mp19 as indicated.

cells were transferred to SS34 tubes and left at room temperature for 10 minutes. The tubes were then transferred to ice and 14ml of solution II (0.2N NaOH, 1% w/v SDS) was added. This was mixed gently and left on ice for a further 10 minutes, after which time 10ml of solution III (60ml 5M KOAc, 11.5ml glacial acetic acid, 28.5ml water per 100ml) was added and mixed gently by inversion. After a further 10 minutes on ice the lysate was clarified by centrifugation at 18,000 rpm in a SS 34 rotor for 20 minutes at 4°C. The supernatant was decanted carefully and 0.6 vol of isopropanol added, mixed and left at room temperature for 20 minutes. The nucleic acid was then recovered by centrifugation at 10,000 rpm for 20 minutes at 15°C. The supernatant was carefully poured off and the pellet and the walls of the tube were washed with 70% v/v ethanol and dried under vacuum. The pellet was resuspended in 10ml of TE to which 10g of caesium chloride (CsCl) and 1ml of stock ethidium bromide (EtBr) solution (10mg/ml) were added. The sample was then transferred into a Beckman Quick Seal tube. A caesium chloride density gradient was obtained by centrifugation at 40,000 rpm in a Ti50 or Ti65 rotor at 14°C for 48 hours.

Chromosomal and plasmid bands were observed under long wavelength UV illumination and the lower plasmid band harvested. This was achieved by puncturing the side of the tube with a syringe and withdrawing the plasmid band. Ethidium bromide in the plasmid preparation was extracted with saturated isopropanol (isopropanol was saturated in aqueous 5M NaCl, 10mM Tris HCl pH 8.0, 1mM EDTA). Caesium chloride was removed from the preparation by dialysis against TE for a period of 12 hours at 4°C and the DNA recovered by the addition of 2½ volumes of ethanol and 1/10th volume of sodium acetate (pH 5.4). Plasmid DNA was resuspended in

sterile distilled water and stored at -20°C. The DNA concentration was determined by A_{260nm} reading assuming A_{260nm} of 1= 50ug/ml DNA.

2.2 Preparation of M13 RF DNA

Single plaques were picked from a bacterial lawn using cocktail sticks and each one inoculated into a universal containing 2ml of YT broth containing a 1/100 dilution of an overnight culture of a suitable bacterial strain (JM101,NM522,TG1) in 2TY broth. The cultures were shaken for 6-7 hours at 37°C then transferred to an Eppendorf tube and centrifuged for 1 minute. The supernatant was then transferred to a fresh Eppendorf tube and if not used immediately, stored as phage stock at 4°C.

Three hundred millilitres of 2TY broth in a 2 litre flask was inoculated with 3ml of a suitable bacterial strain and shaken at 37°C until the optical density at 600_{nm} reached about 0.2 at which point 500ul of phage stock was added. The culture was then shaken overnight at 37°C after which the cells were pelleted and treated as for preparation of plasmid DNA.

2.3 Rapid Bulk Preparation Of DNA

As an alternative method of preparing plasmid and M13 RF DNA bacterial pellets from bulk cultures (section 2.1 or 2.2) were treated with solutions I and II as described in section 2.1 after which 9ml of 3M sodium acetate pH 4.6 was added per tube, mixed thoroughly by inversion and left on ice for 10 minutes. The supernatant was clarified by centrifugation in a SS 34 rotor at 18,000 rpm for 20 minutes at 4°C. To the cleared supernatant 100ul of RNAse (10mg/ml)was added, mixed, and the tube incubated at 37°C for 30 minutes. The sample was extracted twice with phenol:chloroform (1:1), and the DNA was then precipitated by

the addition of 2 vols of ethanol. The pellet was resuspended in 1.6ml of water and 0.4ml of 4M NaCl and 2ml of 13% w/v PEG added, mixed and the sample incubated on ice for at least 2 hours. The DNA was pelleted by centrifugation at 10,000 rpm in an SS34 rotor at 4°C for 20 minutes. The supernatant was removed and the pellet washed with 70% v/v ethanol and dried under vacuum. The DNA was resuspended in water and stored at -20°C until required.

2.4 Bacterial Transformation

The protocol for bacterial transformation was a variation of a protocol of Cohen et al(1972).

A: Preparation of competent cells.

One millilitre of an overnight culture of an E.coli strain was inoculated into 100ml of 2TY broth in a 250ml flask and the culture then shaken at 37°C until the cells had reached an OD_{600nm} of about 0.3. The culture was then pelleted by centrifugation (3000 rpm for 5 minutes) on a bench-top centrifuge. The cell pellet was resuspended in 40ml of ice cold 0.1M $CaCl_2$ and placed on ice for 30 minutes. The cells were then repelleted, resuspended in 3ml of ice cold 0.1M CaCl2 and stored on ice.

B: Transformation.

Plasmid DNA (approximately 100ng) was added to 200ul of competent cells in an Eppendorf tube and incubated on ice for 45-60 minutes. The cells were then subjected to heat shock at 42°C for 3 minutes and immediately returned to the ice for 5 minutes before the addition of 0.4ml L-broth. Two hundred microlitre aliquots of the suspension were plated out on L-agar plates plus appropriate antibiotic. The plates were incubated overnight at 37°C.

If lac α complementation screening system was used to identify

plasmids with inserts 40µl X-gal (20mg/ml in N,N dimethylformamide) and 30µl IPTG (20mg/ml in water) were added immediately before plating out.

C: Transfection.

Two hundred microlitres of competent cells were mixed with M13 RF DNA (or a derivative) and incubated for 1 hour on ice. Meanwhile 1ml of an overnight culture was diluted into 10ml of 2TY broth and shaken at 37°C for 1 hour. After 1 hour the transfection reaction was heat shocked at 42°C for 3-4 minutes and returned to the ice for 5 minutes. The transfection reaction was then added to a 4 X ½ inch glass tube and 1ml of freshly grown bacteria added. The tube was then transferred to the 42°C water bath where 4ml of molten top agar was then added (together with 40ul X-gal and 30µl IPTG if necessary). The tube was mixed and the contents poured on to a L-broth agar plate. The top agar was allowed to set, after which time the plates were incubated overnight at 37°C.

2.5 Small Scale Preparation of DNA

A: Plasmid DNA.

Single colonies were picked and inoculated into 2ml of L-broth with appropriate antibiotic selection and shaken overnight at 37°C. The culture was transferred to an Eppendorf tube and pelleted. The pellet was resuspended in 100ul of Solution I (section 2.1) and incubated for five minutes at room temperature. Two hundred microlitres of solution II (section 2.1) was added, gently mixed and the tubes placed on ice for 5 minutes, then 150ul of solution III (section 2.1) was added, mixed by vortexing and incubated on ice for a further 5 minutes. The sample was centrifuged for 5 minutes, the supernatant poured off and extracted with an equal volume of phenol/chloroform (1:1). The plasmid DNA was ethanol precipitated and washed with 70% v/v ethanol, dried under vacuum and

resuspended in 40µl H_2O. Six microlitre aliquots were subsequently digested with restriction endonucleases in the presence of RNAse A at 20ug/ml and analysed by gel electrophoresis.

B: M13 RF DNA.

A single plaque was picked and inoculated into 2ml of YT broth containing a 1/100 dilution of an overnight culture of a suitable bacterial strain and shaken for 6-7 hours at 37°C. The culture was then removed to an Eppendorf tube and pelleted. The cell pellet was then treated as in section A.

2.6 Preparation of DNAase-Free RNAase A

Pancreatic RNAase (RNAase A) was dissolved at a concentration of 10mg/ml in 10mM Tris HCl pH 7.5, 15mM NaCl, heated to 100°C for 15 minutes and allowed to slowly cool to room temperature. The stock solution was then aliquotted and stored at -20°C.

2.7 Preparation of Dialysis Tubing

Dialysis tubing was cut into lengths of 10-20cm and submerged in a solution of 2% w/v sodium bicarbonate and 1mM EDTA pH 8.0. The tubing was boiled for 10 minutes and then allowed to cool before being rinsed in distilled water. The tubing was boiled for a further 10 minutes in distilled water, allowed to cool slowly and stored in 50% v/v ethanol.

2.8 Preparation of Phenol

Phenol was melted at 68°C and placed in aliquots in universals and these aliquots were stored at -20°C. When required an aliquot of phenol was melted at 68°C and an equal volume of buffer (10mM Tris HCl pH 8.0) was added at room temperature and thoroughly mixed with the phenol. The mixture was allowed to settle out into two phases. The aqueous phase was removed and replaced with fresh 10mM Tris HCl pH 8.0, mixed and allowed

to settle into two phases. The phenol solution was then ready for use and could be stored for up to two months at 4°C. The phenol:chloroform mixture consisted of equilibrated phenol mixed with chloroform and isopropyl alcohol in the proportion of 25:24:1.

2.9 Ligation

DNA fragments were mixed in the ratio of from 3 parts insert to 1 part vector in a total volume of 20ul containing 4µl of 5X ligation buffer (5X ligation buffer consisted of 50mM Tris HCl pH 7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% w/v PEG 8000) and 2 units of T4 DNA ligase. The final DNA concentration for cohesive end ligation was approximately 30µg/ml and for blunt-end ligation 300µg/ml. The ligation reaction was incubated overnight at 16°C and then either used immediately in a transformation reaction (section 2.4) or stored at -20°C until required.

2.10 Ligation of Phosphorylated Linkers To Blunt-ended DNA

In an Eppendorf tube 0.1-0.5µg of blunt ended DNA was mixed with 1-2µg of phosphorylated linkers and water added to a total volume of 16µl. Two units of T4 DNA ligase and 2µl of 10X blunt-end ligation buffer (10X ligation buffer consisted of 0.66M Tris HCl pH 7.6, 50mM $MgCl_2$, 50mM DTT and 5mM Spermidine) were added and the ligation reaction incubated overnight at 16°C. The ligation reaction was then treated as in section 2.9.

2.11 Blunt End Repair

2ug of DNA was restricted with the desired endonuclease then phenol extracted and ethanol precipitated (section 2.12). The DNA was resuspended in 10µl of H_2O and 2µl of 10 X T4 polymerase buffer (0.33M Tris-acetate pH 8.0, 0.66M Potassium acetate, 0.1M Magnesium acetate, 5mM DTT, 1mg/ml BSA (fraction V, Sigma)). One microlitre of 2mM of all

four dNTP's and water to 20µl total volume was added. Two units of T4 polymerase were mixed with the DNA solution and incubated at 37°C for 15 minutes. At the end of that time 1U Klenow was added and the incubation extended for a further 5 minutes before the reaction was halted by phenol extraction and ethanol precipitation.

2.12 Ethanol Precipitation

Ethanol precipitation was carried out by the addition of 1/10th volume 3M sodium acetate pH 5.4, and 3 volumes of ethanol, mixed and incubated for 20 minutes on dry ice or overnight at -20°C. DNA was pelleted by centrifugation for 10 minutes and washed with 70% v/v ethanol and then dried under vacuum. The DNA was resuspended in water and stored at -20°C or -70°C.

2.13 Restriction Endonuclease Digestion of DNA

Restriction endonuclease digests of DNA plasmid or M13 RF DNA samples were carried out according to the manufacturers guidelines using an approximate X 10 concentrate restriction buffers supplied with the restriction enzymes. The following protocol was adhered to. A solution containing the required amount of DNA was mixed with an appropriate volume of X 10 restriction buffer and water, to make the volume up to the required amount. One unit of restriction enzyme was added per ug of DNA, mixed, and the sample incubated at 37°C for at least 2 hours before digestion was halted. In the case of double digestions the restriction enzyme which required the lowest salt concentration was used first. This was then followed by restriction with the second enzyme after adjustment of the salt concentration by use of the appropriate X 10 restriction buffer and heat inactivation of the first enzyme if necessary. The DNA sample was then analysed or purified by agarose gel

electrophoresis or alternatively phenol:chloroform (1:1) extracted, ethanol precipitated and stored at -20°C until required.

Digested vector DNA that was to be used in a ligation reaction was dephosphorylated after restriction to prevent self-ligation. Basically 1U of calf intestinal phosphatase (CIP) was added per µg of DNA, mixed and incubated at 37°C for a further 30 minutes.

2.14 Analytical Agarose Gel Electrophoresis of DNA

Generally, 10µl (0.05-1µg) of DNA sample was mixed with an appropriate amount of 10 X loading buffer (50% v/v glycerol, 0.4% w/v bromophenol blue, 0.4% w/v xylene cyanol, 1mM EDTA) and loaded into slots in an agarose gel (dimensions 10 X 7cm). Gels were made in 1 x TBE buffer (90mM Tris HCl, 90mM boric acid, 1mM EDTA) containing 0.5µg ethidium bromide per ml of gel and electrophoresis was carried out with the gel submerged in 1 X TBE buffer. One to 1.3% gels were used and electrophoresed at 90V until the bromophenol blue in the loading buffer had run at least ¾ of the length of the gel. The gel was transferred to a short wave-length UV transilluminator and were photographed using type 667 Polaroid film.

2.15 Purification of DNA Restriction Fragments

Five to twenty micrograms of plasmid DNA was restricted as required. The whole sample was mixed with loading buffer and loaded on to a preparative agarose gel (1% to 3%) and electrophoresed at 90V. The gel was transferred to a long wavelength UV transilluminator. The required DNA band was cut out of the gel using a sterile scalpel. The agarose slice was then treated in one of the following ways:

A: Electroelution.

The protocol employed was based on Maniatis et al (1982). The gel slice

was placed in dialysis tubing in a minimum volume of 1 X TBE (see section 2.14). The dialysis tubing was then sealed using clips and placed in a gel tank containing sufficient 1 X TBE to submerge the tubing. The gel slice was electroeluted for 2 hours at 200V, then the polarity was reversed for 2 minutes. The power was switched off and the dialysis tubing removed from the tank. The solution surrounding the gel slice was pipetted into an Eppendorf tube, phenol:chloroform (1:1) extracted, ethanol precipitated and resuspended in 10-30µl of water. A fraction of the sample was analysed by gel electrophoresis to determine the DNA recovery and the remainder stored at -20°C until required.

B: Gene Clean.

Alternatively DNA was purified using a commercial kit i.e. Gene Clean™. This kit contains a specifically formulated silica matrix called Glassmilk which binds single and double stranded DNA (Vogelstein and Gillespie, 1979). The protocol followed was as described by the manufacturer. The agarose gel slice was placed in an Eppendorf tube , crushed and dissolved in 3 volumes of saturated NaI (containing TBE modifier solution) at 50°C for 5 minutes. A suspension of glass milk (5µl/5µg DNA) was added to the solution. After incubation at room temperature for 5 minutes the glass milk was pelleted by a 30 second spin in a microcentrifuge. The glass milk was then washed three times (200µl of wash buffer per wash). After the final wash the glass milk was resuspended in 1-2 volumes of water and DNA eluted from the solid matrix by incubation at 50°C for 5 minutes. The glass beads were pelleted and the supernatant removed to another Eppendorf tube. A fraction of the sample was analysed by gel electrophoresis and the remainder stored at -20°C until required.

2.16 Dideoxy Sequencing Of DNA

The method of sequencing used was the chain-termination method of sequencing developed by Sanger et al (1977). In the original procedure primer extension was catalysed by Klenow fragment of E.coli DNA polymerase I. However in this updated method (Kristensen et al, 1988), T7 DNA polymerase is used. A list of solutions used in the sequencing reactions is provided in Table 4.

A: Preparation of single-stranded M13 template DNA.

Single plaques were picked and inoculated into 2ml of 2TY broth containing a 1/100 dilution of an overnight culture of E.coli strains JM101, TG1 or NM522.The culture was shaken vigorously at 37°C for 5-6 hours. The culture was transferred to an Eppendorf tube and clarified by spinning in a microcentrifuge for 1 minute. The supernatant was transferred to a fresh tube and M13 phage precipitated by the addition of 200ul of 20% PEG, 3.5M ammonium acetate, mixed and allowed to stand for 5 minutes at room temperature. The supernatant was spun in a microcentrifuge for 5 minutes. The supernatant was poured off and the tube briefly re-spun for a few seconds. The residual supernatant was completely removed using a Pasteur pipette.

The phage pellet was resuspended in 100µl of TE (10mM Tris HCl, pH 8.0, 1mM EDTA), extracted with an equal volume of phenol (equilibrated in TE) by vigorous vortexing for 15-30 seconds and centrifuged for 5 minutes.The aqueous layer was then extracted with phenol/chloroform (1:1) before being ethanol precipitated. The precipitated DNA was washed with 70% ethanol, dried under vacuum and resuspended in 30µl of water.

B: Annealing of primer to single-stranded template.

Three microlitres of template DNA (roughly 2µg of DNA) and 4ul of water was added to an Eppendorf tube and 2µl of universal primer (0.8pmol/µl) was added. After the addition of 2µl of annealing buffer (5 X concentrate, see Table 4) the tube was vortexed and briefly centrifuged. The sample was incubated at 70-80°C for two minutes and then transferred to a water bath at 37°C for > 30 minutes. The sample was then allowed to stand at room temperature until required.

C: Labelling reaction.

To the side of the annealed template/primer tube the following was added: 1µl 0.1M DTT, 2µl labelling mix, 1µl [α-^{32}P] dATP and 3 units T7 DNA polymerase. The labelling reaction was initiated by the mixing of the contents of the reaction tube by brief centrifugation. The tube was then left at room temperature for 5 minutes. Meanwhile 2µl of termination mixes for G,A,T and C tracks (see Table 4) were added to individual wells in a microtitre plate (one set per template/primer reaction). The plate was sealed and preincubated at 37°C for 1 minute.

D: Termination reaction.

Three and a half microlitre aliquots of the labelling reaction were transferred to each of the four termination mixes on the microtitre plate. The samples were mixed by brief centrifugation and incubated at 37°C for 5 minutes, then 4µl of stop solution (see Table 4) was added and mixed by centrifugation.

E: Annealing of primer to double-stranded template.

In an Eppendorf tube 2µg of plasmid DNA in a total volume of 8µl was denatured by the addition of 2µl 2M NaOH, mixed by vortexing and

incubated at room temperature for 10 minutes. Three microlitres of 3M sodium acetate (pH 4.5) and 7ul of water were then added. The DNA was precipitated by the addition of 60µl of ethanol and incubated on dry ice for 10 minutes before being centrifuged for 10 minutes. The pellet was then washed with 70% ethanol, dried under vacuum and resuspended in 10µl of distilled water. Two microlitres of annealing buffer (see Table 4) and 2ul of primer solution (0.8µM) were added and the sample was incubated at 37°C for at least 30 minutes. Sequencing protocol was then carried out as described in steps (C) and (D).

TABLE 4. Sequencing Solutions

5 X Annealing Buffer:	200mM Tris HCl (pH 7.5) 100mM $MgCl_2$ 250mM NaCl.
Labelling Mix	8μM dGTP, 8μM dCTP, 8μM dTTP.
ddG Termination Mix	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddGTP, 50mM NaCl.
ddA Termination Mix	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddATP, 50mM NaCl.
ddT Termination Mix.	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddTTP, 50mM NaCl.
ddC Termination Mix	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddCTP, 50mM NaCl.
Stop Solution	95% v/v Formamide 20mM EDTA 0.05% w/v Bromophenol Blue 0.05% w/v Xylene Cyanol.

2.17 Sequencing Gels

A: 8% Sequencing stock solution.

Two hundred and eighty eight grams of urea were added to about 400ml of water, and heated until dissolved. Forty five point six grams of Electran grade acrylamide and 2.4g of electran grade N N-methylene bisacrylamide were added and the volume adjusted to 500ml with the addition of water. Twenty grams of Amberlite were added and the solution mixed for 30 minutes on a magnetic stirrer. The Amberlite was then removed by filtration through a Whatman 113^{V} prefolded filter, 60ml of 10 X TBE added and the total volume of the solution adjusted to 600ml by the addition of water. The 8% stock solution was stored in the dark at 4^{o}C until required. Ten times TBE buffer was prepared according to the following recipe per 4 litres; 432g Tris base, 220g boric acid and 37.2g EDTA.

B: Sequencing gel.

Two glass plates (44.5 X 33cm), one notched, were treated with Repelcote from BDH and further treated with ethanol to remove excess Repelcote. Repelcote, which is water repellent, is a 2% solution of dimethyl-dichlorosaline in 1,1,1-trichloroethane and produces a silicone surface on glass. A glass plate sandwich using the two glass plates together with 0.2mm spacers was assembled and the bottom of the plates were sealed with masking tape. Ninety millilitres of 8% sequencing gel solution was mixed with 0.9ml of 10% ammonium persulphate and 90ul of TEMED. The solution was poured between the two plates and a comb inserted. The gel was allowed to set for at least 1 hour before use. The sequencing gel was run on a Gibco BRL kit and powered by a LKB power pack set at between 60-90 watts. The running buffer was 1 X TBE and the

gel was pre-run for about 10 minutes before the samples (2-4µl volumes) were loaded onto the gel. The gel was normally run until the bromophenol dye front reached the bottom of the gel. The gel was then removed from the kit, the two plates separated and the gel transferred onto Whatman paper and dried down under vacuum. The gel was exposed to X-ray film at room temperature.

2.18 Oligonucleotide Synthesis and Purification

Oligonucleotides were synthesised by Dr. J Mc Lauchlan on an 8600 Applied Biosystems machine on a micromole scale. The synthesised oligonucleotide was eluted with ammonium hydroxide and heated for about 5 hours at 55°C. The deprotected oligonucleotide was then transferred to Eppendorf tubes and dried down overnight on a Speedvac evaporator (Savant). The sample was resuspended in 200ul of water and stored at -20°C. Oligonucleotides were further purified by polyacrylamide gel electrophoresis (acrylamide: bisacrylamide ratio 19:1) in the presence of urea (7M). The concentration of acrylamide used was dependent upon the size of oligonucleotide to be purified.

Acrylamide %	Fragment Size
6	35 to 130
8	19 to 75
10	12 to 55

The following gel mixes were prepared:

Acrylamide gel mix	6%	8%	10%
Urea	288.0g	288.0g	288.0g
Acrylamide	34.2	45.6	57.0
Bisacrylamide	1.8	2.4	3.0

Each gel mix was prepared as described for 8% sequencing stock solution (section 2.17).

Two glass plates (16.5 X 26.5 cm) were assembled with 1mm spacers. The bottom and the sides of the cast were sealed with masking tape. Sixty millilitres of a particular gel solution was mixed with 0.6ml of 10% ammonium persulphate and 60ul TEMED before being poured between the plates, and a comb inserted. The gel was allowed to polymerise for at least 40 minutes before use. The comb was then removed and the plates were attached to the gel tank. The bottom and top troughs were filled with 1 X TBE buffer. The sample was diluted in an equal amount of 2 X formamide loading buffer (1 X TBE, 90% w/v deionised formamide, 0.5% w/v bromophenol blue, 0.5% w/v xylene cyanol) and heated to 90°C for 3 minutes prior to loading onto the gel. The gel was run at between 20-40 V/cm constant voltage until the bromophenol blue dye had reached the bottom of the gel. The gel was then taken from the tank and the top plate of the gel removed. The gel was then covered by plastic wrap, inverted, the bottom plate removed and the other exposed gel face covered by the plastic wrap. The gel was placed on a thin layer chromatography (TLC) plate. The TLC plate had a polyester silica gel surface incorporating a fluorescent indicator.

The oligonucleotide bands were visualised by shadowing the gel with a short-wave UV lamp. The desired band was cut out, placed in a 15ml Falcon tube and submerged in a minimum volume of sterile water, then shaken overnight at 37°C. The oligonucleotide solution was then transferred to Eppendorf tubes and dried down under vacuum. The oligonucleotide was then resuspended in approximately 200µl of H_2O and the concentration of oligonucleotide estimated by measuring the OD at

260nm, assuming OD 1 = 20µg/ml of single stranded DNA.

2.19 Oligonucleotide Site-Directed Mutagenesis

Oligonucleotide site-directed substitution, insertion or deletion mutagenesis was performed using the Eckstein method (Taylor et al, 1985) and either employed a kit purchased from Amersham in which case manufacturer's guidelines were followed, or alternatively the protocol given by Sayers et al (1988) was followed. All reactions were carried out in microcentrifuge reaction tubes.

Mutagenesis Procedure:

A: Phosphorylation of oligonucleotide primer.

Two microlitres of the oligonucleotide (at a concentration of 500U/ml) was treated with 2U of T4 polynucleotide kinase in 1 X kinase buffer (1mM ATP, 100mM Tris HCl pH 8.0, 10mM $MgCl_2$, 7mM DTT) in a total volume of 30ul at 37°C for 15 minutes. The reaction was halted by heat inactivation at 70°C for 10 minutes.

B: Annealing reaction.

The annealing reaction was carried out by incubating 10µg of single stranded M13 template DNA and 5µl of phosphorylated oligonucleotide solution in 125mM Tris HCl pH 8.0, 125mM NaCl in a total volume of 40µl. The annealing reaction was heated to 70°C for 5 minutes, placed in a 56°C water bath for 20 minutes and finally incubated at 37°C for 20 minutes before being placed on ice in readiness for the next step.

C: Synthesis and ligation of mutant DNA strand.

The annealing solution was adjusted to 50mM Tris HCl pH 8.0, 50mM NaCl in a total volume of 90ul by the addition of the following: $MgCl_2$ to 8mM, ATP to 800µM, dATP, dGTP and dTTP to 200µM each, dCTP (α-S) to 200µM. Six units of Klenow and six units T4 ligase were then added

simultaneously, the sample mixed and briefly spun. The sample was then incubated at 16°C for between 16-48 hours.

D: Removal of background single stranded DNA template and Nci I RF DNA nicking.

To the reaction sample 170µl of water and 30µl of 5M NaCl were added and thoroughly mixed. The sample was then passed through two 0.45µm nitrocellulose filters to remove single stranded DNA. An additional 100µl of 0.5M NaCl was passed through the filters and the total filtrate was collected in a single Eppendorf to which was added 1/10 volume 3.5M sodium acetate and 2.5 volumes of ethanol. The sample was then precipitated on dry ice for 20 minutes, centrifuged, washed with 1ml of cold ethanol, dried under vacuum and resuspended in 250µl of nicking solution (25mM Tris HCl pH 8.0, 25µM DTT, 50µM $MgCl_2$, 75mM NaCl). Fifty microlitres of the sample was digested with 10U of Nci I for 90 minutes at 37°C. The remainder of the DNA sample was stored at -20°C until required.

E: Removal of wild type DNA template strand.

After 90 minutes of Nci I digestion the salt concentration was adjusted to 100mM and 50U of exonuclease III was added and the sample incubated for a further 30 minutes. The sample was then immediately transferred to a 70°C water bath for 10 minutes to terminate the exonuclease reaction.

F: Repolymerisation.

To the gapped DNA sample ATP was added to a final concentration of 800µM and 4dNTP's to 200µM in a total volume of 80µl. DNA polymerase I (3U) and T4 ligase (2U) were added and the sample was incubated at 16°C overnight. The sample was then stored at -20°C until required.

G: Transfection and recovery of mutants.

Twenty microlitres of the reaction mix (F) were used to transform 300µl of competent TG1 cells prepared as described previously (see 2.4 C). Bacterial plates were incubated overnight at 37°C and the following day individual M13-derived plaques were picked and grown up as described previously (2.2). In order to identify mutant changes either M13 RF DNA or single stranded M13 DNA template was prepared as previously described (2.5B and 2.16A). M13 RF DNA was used when the mutation could be identified by the gain of additional restriction enzyme sites. Alternatively single stranded DNA was sequenced to identify the mutation.

Oligonucleotide site directed mutagenesis ranged in efficiency from 5% to 100%.

3 RNA And Protein Analytical Techniques

3.1 RNA Transcription in vitro

The protocol followed was based on Krieg and Melton (1987) and T7 RNA polymerase (Promega) manufacturer's protocol for *in vitro* transcription reactions. Various cDNAs derived from S genome segments of Maguari (MAG) or Bunyamwera (BUN) bunyaviruses or from VSV NS gene were positioned downstream of a T7 RNA polymerase promoter. This was carried out by either subcloning appropriate restriction digested cDNA fragments into the polycloning site of the T7 promoter containing plasmid pTZ18U (Mead *et al*, 1986) or alternatively oligonucleotide site directed mutagenesis was used to specifically introduce a T7 RNA polymerase promoter sequence upstream of BUN S and MAG S cDNAs.

To create a suitable template for use in run-off RNA transcription reactions the 3' ends of cDNA templates were linearised by restriction

digestion with a suitable enzyme. Hind III, Xba I and Sma I were used routinely. RNA was synthesised in a 50µl reaction which comprised the following:

10µl 5 X transcription buffer (200mM Tris HCl pH 8.0, 40mM $MgCl_2$, 125mM NaCl, 10mM spermidine)

5µl 100mM DTT

5µl 5mM of each rNTP

2µl of DNA template (1µg/µl)

1µl of RNasin

1µl of RNA polymerase (20U)

26µl of H_2O

The reaction was carried out at 37°C for 1 hour 45 minutes. At the end of this time the template DNA was removed by the addition of 2 units of DNase (RQ 1) and incubated at 37°C for 15 minutes. The reaction volume was then increased to 100µl by the addition of H_2O and the sample extracted with an equal volume of phenol/chloroform. The aqueous phase was transferred to a fresh Eppendorf tube and the RNA precipitated by the addition of 1/10 volume 7.5M ammonium acetate and 2½ volumes of ethanol. The solution was mixed, placed on dry ice for 15 minutes followed by centrifugation for 10 minutes, washed with 70% v/v ethanol and dried under vacuum. The RNA pellet was resuspended in 10µl of sterile water and stored at -70°C.

To produce capped RNA transcripts the rGTP level was reduced to 0.1mM and m^7GpppG was included at 0.5mM.

3.2 Production of ^{32}P Labelled High Specific Activity RNAs by in vitro Transcription

In order to produce ^{32}P labelled RNA transcripts the following reaction conditions were used:

4µl	5 X transcription buffer
2µl	100mM DTT
20U	RNasin
4µl	2.5mM each of rATP, rGTP, rUTP.
2.4µl	100µM CTP
1µl	linearised template (1µg)
5µl	[$\alpha^{32}P$] CTP
1µl	T7 RNA polymerase (20-25U) 20µl final volume

The reaction conditions were as described in the Promega "Biological Research Products-Protocols & Applications Manual" and the reaction was carried out for 1 hour at 37°C. One unit of RQ 1 DNAase was then added and the sample incubated for a further 15 minutes. The sample was then phenol/chloroform extracted followed by removal of unincorporated nucleotides by treatment with the RNAid™ kit (see section 3.3).

3.3 Purification of ^{32}P Labelled RNA Transcripts from Unincorporated Nucleotides Using RNaid™ Kit

After DNase treatment of the in vitro transcription reaction the reaction volume was increased to 100µl and RNA transcripts purified using RNaid™ kit (Bio 101). The kit contains a specifically formulated silica matrix which under appropriate conditions binds RNA but not ribonucleotides. The protocol followed was as described by the manufacturer. Three volumes of RNA binding buffer was mixed with the transcription reaction and 1µl of RNA MATRIX™ was then added. After a

brief vortex the sample was incubated at room temperature for 5 minutes.The sample was then centrifuged for 1 minute and the supernatant was removed. The pellet was washed twice with RNA WASH^{TM} before being resuspended in 50µl of water. The RNA was then eluted from the matrix by heating at 50°C for 5 minutes, briefly vortexed, and spun for 1 minute in a microcentrifuge. The supernatant containing the RNA was subjected to another round of purification using the RNaid^{TM} kit before elution of RNA into 200µl of water. The RNA was stored at -70°C until required.

To determine the efficiency of incorporation of label into the transcript, precipitation was carried out on final purified product. One microlitre of purified product was mixed with 100µl of H_2O containing carrier DNA at 100µg/100µl before the addition of 1ml of ice cold 10% w/v TCA. The sample was incubated on ice for 30 minutes and filtered through two glass fibre paper filters (2.1cm GF/C Whatman). The filter was then washed with 1ml of ice cold ethanol, dried and the amount of incorporated label determined by counting in a scintillation counter (Beckman LS 5000CE). This value was compared with that for an equivalent volume of starting material to determine the percentage of incorporation of the label.

3.4 Analysis of RNA in vitro Transcripts

A: Polyacrylamide gel electrophoresis.

^{32}P labelled RNA transcripts were analysed by polyacrylamide gel electrophoresis (19:1 acrylamide to bisacrylamide) under non-denaturing and denaturing conditions.

For non-denaturing conditions a stock 4% acrylamide gel solution was prepared as follows: 22.8g acrylamide and 1.2g of bisacrylamide were dissolved in a total volume of 90ml. Ten millilitres of 10 X TBE was then

added to make the volume up to 100ml. For denaturing conditions a stock 4% acrylamide solution was prepared by dissolving acrylamide and bisacrylamide as above but in addition urea was added at 48g/100ml stock solution. The urea containing solution was treated with Amberlite (5g) for 30 minutes. The Amberlite was removed by filtration through a Whatman 113^{V} prefolded filter and 10ml of 10 X TBE (section 2.17) added to make the volume up to 100ml. In order to pour a gel two plates were coated with repelcote and two spacers were assembled into a glass sandwich. The bottom and sides of the plates were sealed with masking tape. The gel solution was then polymerised by the addition of 700µl of 10% w/v ammonium persulphate and 70µl of TEMED to 70ml of 4% acrylamide gel solution. The gel solution was poured between the plates and a comb inserted. The gel was allowed to set for at least 1 hour before use. The vertical gel was placed in the gel rig and upper and lower reservoirs filled with 1 X TBE. The gel was briefly pre-run at a constant voltage of 300V before the samples were loaded. After loading the gel was electrophoresed until the xylene cyanol band was ¾ down the gel length. The gel was then removed from the rig, the backing plates removed and the gel transferred onto Whatman 3MM paper and dried down. RNA transcripts were then detected by autoradiography.

B: Methyl mercuric hydroxide agarose gel electrophoresis.

Following a protocol from Current Methods in Molecular Biology RNA transcripts were analysed by agarose gel electrophoresis in the presence of methyl mercuric hydroxide. A stock 10 X running buffer was initially prepared which consisted of 0.05M sodium borate, 0.1M sodium sulphate, 0.01M EDTA, 0.15M boric acid. A 1.3% w/v agarose gel was prepared by melting 1.3g of agarose in 100ml of 1 X running buffer. The gel was

cooled to 50°C and 500ul of 1M methyl mercuric hydroxide added. A horizontal gel was poured and a comb was then inserted. The gel was allowed to set for at least 45 minutes before use. Meanwhile RNA samples were prepared in the following manner: 5µl of 2 X sample buffer (20% v/v glycerol, 0.05% w/v Bromophenol Blue, 2 X running buffer, 1µl of 0.1M methyl mercuric hydroxide was added, vortexed and the sample briefly spun. The gel was placed in the gel tank and semi-submerged in 1 X running buffer and the comb was removed. The samples were quickly loaded onto the gel. The gel was then run at a constant voltage between 60-100V. A peristaltic pump was used to recirculate the buffer between the two reservoirs. Once the bromophenol blue had run to the end of the gel the gel was removed and either transferred onto 3MM Whatman paper to be dried down for visualisation of ^{32}P labelled transcripts by autoradiography, or alternatively the gel was transferred to a solution of 0.05M ammonium sulphate to which 10µl of stock ethidium bromide had been added. After 20 minutes the gel was transferred to a transilluminator and RNA transcripts visualised under UV illumination.

3.5 Filter Binding Assay

Purified ^{32}P labelled RNA transcripts were incubated with purified nucleocapsid protein at room temperature for 30 minutes in a final volume of 50µl which contained 10mM Tris HCl pH 7.0, 130mM NaCl, 5mM $MgCl_2$, 0.5mM EDTA, 20% w/v glycerol and 2mM DTT. The mixture was filtered through a 0.45µm nitrocellulose filter and washed with 1 X PB (10mM Tris HCl pH 7.0, 50mM NaCl, 1mM EDTA.) The filters were dried and the amount of ^{32}P RNA retained determined by Cerenkov counting.

3.6 Translation of RNA in vitro

Message sense in vitro synthesised MAG S or BUN S RNA transcripts were

translated *in vitro* using nuclease-treated rabbit reticulocyte lysate or wheat germ extract cell-free translation systems. Proteins synthesised in these systems were identified by incorporation of (^{35}S) methionine. At the end of the translational assay an equal volume of dissociation mix (for recipe see section 3.8) was added to each reaction system. The sample was heated for 3 minutes at >90°C and a fraction run on an SDS-PAGE gel. Proteins were visualised by fluography. A summary of the individual assay conditions used is provided below.

A: NEN Nuclease-treated rabbit reticulocyte lysate.

A solution of translational cocktail, 1M potassium acetate and 50mM magnesium acetate supplied in the translation kit were mixed together with rabbit reticulocyte lysate, ^{35}S methionine and RNA in the following proportions:

^{35}S Methionine	5µl
Translation cocktail	5.5µl
1M Potassium acetate	2µl
50mM Magnesium acetate	0.5µl
lysate	10µl
RNA (or H_2O)	1µl
H_2O	1µl (Total 25µl)

Each reaction was carried out for 90 minutes at 37°C and stopped by the addition of dissociation mix or removal of samples to -20°C.

B: Amersham wheat germ extract.

1M potassium acetate, 1mM methionine-depleted amino acid pool and water were added to wheat germ extract together with ^{35}S methionine and RNA in the following proportions:

^{35}S Methionine	4µl	
1M Potassium acetate	2µl	
1mM (met^-) amino acid pool	2µl	
Wheat germ extract	15µl	
H_2O	6µl	
RNA (or H_2O)	1µl	(Total 30µl)

Each reaction was incubated at 26°C for 90 minutes and the reaction stopped by the addition of an equal volume of dissociation mix or removal of the samples to -20°C.

3.7 Radioimmunoprecipitation

After the in vitro translation assay the reaction volume was increased to 200µl by the addition of RIPA buffer (1% v/v Triton X 100, 1mM PMSF, 1% w/v sodium deoxycholate, 50mM Tris HCl pH 7.5, 150mM NaCl, 0.1% w/v SDS). Bunyamwera antisera (Watret et al, 1985) was added at 1/100 dilution and the sample incubated overnight at 4°C with continuous mixing. Antigen/antibody complexes were precipitated by the addition of 50ul of S.aureus cells (Pansorbin cells) and the sample mixed for 20 minutes. The cells were pelleted by brief centrifugation and the supernatant poured off. The cells were then washed three times with 500ul of wash buffer (0.1M Tris HCl pH 7.5, 0.5M LiCl). After the final wash the cells were resuspended in an equal volume of dissociation mix, boiled for 5 minute and vortexed to release bound protein. The sample was briefly centrifuged to pellet the cells and the supernatant loaded on to an SDS-PAGE gel. The immunoprecipitated proteins were visualised by fluorography.

3.8 Protein Analysis

Polyacrylamide gel recipes based on the SDS (denaturing) discontinuous

buffer system of Laemmli (1970) were used to analyse protein samples by one-dimensional gel electrophoresis. A glass plate sandwich using two 15.5 X 19cm glass plates (one of which was notched) together with 1mm spacers was assembled and the bottom and sides of the plates sealed with Kapton tape.

A resolving gel of 8, 10, 12, 15, 18 or 20% final acrylamide concentration was prepared as described on the next page, and poured between the glass plates. The top of the resolving gel was layered with water and the gel was allowed to polymerise for approximately 15 minutes.

Resolving Gel Final Acrylamide Concentration

	8%	10%	12%	15%	18%	20%
Acrylamide :bis (75:1)	6.88ml	8.60	10.30	12.75	15.50	17.10
1M Tris HCl pH 8.8	12.30ml	12.30	12.30	12.30	12.30	12.30
H_2O	13.42ml	11.70	10.00	7.55	4.80	3.20
10% SDS	0.33ml	0.33	0.33	0.33	0.33	0.33
10% ammonium persulphate	0.33ml	0.33	0.33	0.33	0.33	0.33
TEMED	30µl	30µl	30µl	30µl	30µl	30µl

Meanwhile a stacking gel was prepared according to the following recipe:

Acrylamide:bisacrylamide (75:1)	2ml
1M Tris HCl pH 6.8	1.25ml
H_2O	16ml
10% SDS	0.2ml
10% Ammonium persulphate	0.2ml
TEMED	10µl

The water overlay was poured off and a stacking gel made to the above recipe was poured on top of the polymerised resolving gel. A comb was then inserted and the stacking gel was allowed to set for approximately 60 minutes before use.

After the gel had polymerised the comb and the tape at the bottom of the gel were removed. The gel was placed in a gel tank and the top and bottom troughs filled with running buffer. Stock running buffer 10 X Tris/glycine was made as follows: 576g Glycine and 121.2g Tris were dissolved in a total volume of 4 litres. Running buffer was made by dilution of stock solution 1 part in 9 parts water and the addition of SDS to 0.1%.

Samples were resuspended in an equal volume of dissociation mix (0.125M Tris HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) and heated to 90°C for 5 minutes before loading on to the gel. Gels were run at 4°C, 40mA constant current until the bromophenol band was almost at the bottom of the gel. The gel was then removed from the rig and proteins visualised by staining with Coomassie Brilliant Blue (see section 3.9A) or by silver staining (see section 3.9B). Alternatively, if radioactive samples were to be analysed the gel was fixed in 3 changes of fixing solution,(fixing solution consisted of 50% v/v methanol, 10% v/v acetic acid; H_2O), treated with EN^{3}Hance, dried down and exposed to film at -70°C. Proteins were then detected by fluography.

3.9 SDS-PAGE Staining Techniques

Protein bands on SDS-PAGE gels were stained by one of the following methods

A: Coomassie Blue staining of proteins in gels.

Coomassie Blue stain solution was made up to the following recipe; 50% (v/v) methanol, 0.05% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad), 10% (v/v) acetic acid, 40% (v/v) water. Sufficient staining solution was then added to the SDS-PAGE gel in a plastic container such that the gel floated freely. The gel was shaken for up to 2 hours and then destained by replacing the staining solution with fixing solution (50% v/v methanol, 10% v/v acetic acid, H_2O.) The gel was then destained by shaking, over several changes of the fixing solution.

B: Silver staining of proteins in gels.

The gel was initially fixed in solution I (30% v/v ethanol, 10% v/v acetic acid) for between 30 minutes to overnight. The gel was then transferred to solution II (30% v/v ethanol, 0.5M NaOAc, 0.5% v/v glutaraldehyde, 0.2% w/v $Na_2S_2O_3$) for between 3 minutes to 1 hour. It was then rinsed thoroughly in distilled water for 30 minutes before being soaked in solution III (0.1% w/v $AgNO_3$, 0.02% v/v formaldehyde) for between 15 to 60 minutes. The gel was then developed in solution IV (2.5% w/v Na_2CO_3, 0.01% v/v formaldehyde), and the staining reaction stopped by transfer of the gel to 0.05% w/v EDTA solution.

3.10 Electrotransfer Of Proteins From SDS-PAGE Gels To Nitrocellulose Filters-Western Blot

The method of horizontal electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose filters is based on Towbin et al (1979). The protocol followed was that described by the manufacturer of the electrophoresis transfer kit. SDS-PAGE gels were loaded and run as described previously. At the end of electrophoresis the gel was removed from the tank and separated from the glass plates. Six pieces of Whatman

3MM paper and one piece of nitrocellulose membrane of identical dimensions to the gel were soaked in transfer buffer (39mM glycine, 48mM Tris, 0.0375% w/v SDS, 20%v/v methanol). They were then carefully layered on to the anode electrode plate of the Nova Blot Electrophoresis Transfer Kit (LKB). The nitrocellulose was placed on top of the 3MM paper. The resolving gel was separated from the stacking gel and layered on to the nitrocellulose membrane. A further six pieces of 3MM paper soaked in the transfer buffer were placed on top of the gel. At all times care was taken to avoid bubbles. The cathode was placed on top of the stack and the blot apparatus was connected to a power pack and subjected to a constant current of 0.8mA/cm^2 for 1 hour. At the end of this time the unit was dismantled and the nitrocellulose membrane transferred to a polythene bag, sealed and stored at 4^{o}C until required.

3.11 North Western Blot Analysis

Total cell lysate of Spodoptera frugiperda (SF) cells infected with a recombinant baculovirus (AcMAG S) expressing Maguari virus nucleocapsid protein and mock infected SF cells were run side by side on 12% or 15% SDS-PAGE gels and the separated proteins electrotransferred to nitrocellulose membrane as described previously. The membrane was divided into strips which contained both MI and AcMAG S infected SF cell tracks. The strips were probed with ^{32}P-labelled RNA by a modification of a protocol of Bowen et al (1980.) The blots were washed in probe buffer (10mM Tris HCl pH 7.0, 50mM NaCl 1mM EDTA, 1 X Denhardt solution) for 20 minutes and incubated for another 20 minutes in this buffer in the presence of carrier RNA (100-500µg/ml) to inhibit non-specific binding. ^{32}P-labelled RNA probes generated by in vitro transcription reaction were then added to the nitrocellulose strips. The blot was

shaken for a further 1 hour before unbound probe was removed by washing for 10 minutes in probe buffer three times. The strips were then blotted dry and exposed against film at either room temperature or -70°C. The carrier RNA was derived from Saccharomyces cerevisiae, E.coli, or Spodoptera frugiperda cells or BHK cells. E.coli bacterial and eukaryotic RNA were obtained by methods described below (section 3.12). S.cerevisiae RNA was commercially obtained

3.12 Isolation Of RNA From E.coli

A method from "Current Protocols in Molecular Biology" was followed. Cells from a 100ml overnight culture were pelleted and resuspended in 10ml of protoplasting buffer (15mM Tris HCl pH 8.0, 0.45M sucrose, 8mM EDTA) to which 1ml of a 50ug/ml lysozyme solution was added and mixed by inversion. The samples were incubated on ice for 15 minutes before being centrifuged for 10 minutes in an SS34 rotor at 5000rpm at 4°C.

The protoplasts were resuspended in lysing buffer (10mM Tris HCl pH 8.0, 10mM NaCl, 1mM sodium citrate, 1.5% w/v SDS) and 150ul of DEPC. The tube was incubated at 37°C for 5 minutes, chilled on ice and 2.5ml of saturated NaCl added and mixed by inversion. After 10 minutes on ice the sample was centrifuged for 15 minutes at 10,000 rpm in an SS34 rotor at 4°C. The supernatant was removed, mixed with 10ml of ethanol and precipitated on dry ice for 30 minutes. The samples were then centrifuged for 20 minutes at 10,000 rpm at 4°C,washed with 70%w/v ethanol, dried under vacuum and re-dissolved in sterile distilled water. The yield of RNA was determined by UV absorbance at 260nm assuming OD1=40ug/ml. Typically this protocol produced 7mg RNA/100ml culture.

3.13 Isolation Of RNA From Eukaryotic Cells

RNA was isolated from SF cells and BHK cells by acid guanidine

thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987). The following protocol was applied per 1 X 10^6 cells.

Cells were washed in PBS before being resuspended in 100ul of denaturation solution (denaturation solution consisted of the following per 100ml: guanidine thiocyanate 50g, sarcosine 0.5g, antifoam A 0.33ml, 1M Tris pH 7.5 5ml, 0.5M EDTA, H_2O to 99.3ml, filter sterilise; 0.7ml 2-mercaptoethanol added before use). The lysed cells were transferred to a microcentrifuge tube and sequentially 10ul 2M sodium acetate pH 4.0, 100 ul of water-saturated phenol and 20ul of chloroform-isoamyl alcohol mixture (49:1) were added with thorough mixing by inversion after the addition of each reagent. The sample was then vortexed for 10 seconds and placed on ice for 15 minutes before centrifugation for 10 minutes. The aqueous phase was transferred to a fresh tube, mixed with 100ul of isopropanol and then placed at -20°C for at least 1 hour before centrifugation for 10 minutes. The RNA pellet was redissolved in 300ul of denaturation solution, precipitated with 300ul of isopropanol and stored at -20°C for 1 hour. After centrifugation for 10 minutes the pellet was washed with 70% v/v ethanol and dried under vacuum. This protocol yielded approximately 5ug of RNA per 10^6 cells.

3.14 Gel Mobility Shift Assay

RNA-N protein interaction was investigated by gel electrophoresis. Various dilutions of ^{32}P labelled RNA were incubated with a fixed concentration of purified N protein at 30°C for approximately 20 mins under conditions described in the filter binding assay protocol (section 3.5). The sample was then analysed by electrophoresis at room temperature. Electrophoresis was performed on a horizontal 0.9% or 1.75 agarose slab gel (14x11x0.5 cm) containing 10mM sodium phoshate (pH 7).

The gel was run semi-submerged in recirculated 10 mM phoshate running buffer at a power rating of 3-4 V/cm. A blank control lane containing bromophenol blue dye was run on the same gel and electrophoresis was halted when the bromophenol blue dye had run 3/4 the length of the gel. The gel was transferred to 3MM paper, dried down under vaccum and the RNA bands visualised by autoradiography.

3.15 Immuno- Western Blot

A ProtoBlot^{R} Western Blot HRP system kit (Promega) was used to detect specific proteins transferred to a nitrocellulose membrane from a denaturing polyacrylamide gel after electrophoresis (section 3.10). The method is based on the enzyme-linked immunodetection of an antigen-antibody complex between immobilised protein and antigen specific antibodies using anti-IgG secondary antibodies conjugated with horseradish peroxidase.

The protocol followed was according to manufacturers guidelines. The nitrocellulose membrane containing electrotransferred protein was initially treated with a buffer to block non-specific protein binding sites (1% BSA in TBST). TBST comprised of 10mM Tris HCl pH 8.0, 150mM NaCl, 0.05% v/v Tween 20. After 30 minutes the blocking solution was replaced with TBST containing the primary antibody (either rabbit anti-Bunyamwera serum (Watret et al, 1985) diluted 1/100 or rabbit anti B-gal ORF3 fusion protein diluted 1/50 or 1/100) and incubated at room temperature for 30 minutes. The membrane was washed three times with TBST, before being incubated with rabbit anti-IgG horseradish peroxidase conjugate (diluted 1/2500 in TBST). The membrane was then again washed three times with TBST, and the colour development substrate added (per 10ml this consisted of 50ul of stock 4-chloro-1-napthol (100mg/ml

methanol) which was added to 2ml methanol and 8ml TBS (TBST without Tween 20)). Thirty five millilitres of hydrogen peroxide (30% v/v) was also added to the colour development substrate and the reaction allowed to continue until the desired intensity of colour had been obtained. The reaction was then stopped by placing the membrane in water.

4 Cell Culture of BHK Cells and Bunyaviruses

4.1 Medium

Glasgow minimal essential medium 10 X concentrate (Stoker and MacPherson, 1961) was diluted in distilled water to 1 X and supplemented with 100U/ml each of penicillin and streptomycin together with L-glutamine at 5mM and sodium bicarbonate at 2.75g/l. New born calf serum was added to a final concentration of 5% or 10%. The medium was stored at 4°C.

4.2 BHK-21 Clone 13 Cells

BHK-21 cells (MacPherson and Stoker, 1961) were provided by the Cytology unit, Institute of Virology, and grown in the above medium.

4.3 Preparation Of Virus Stocks

Preparation of virus stock was based on Watret et al,(1985). Virus stocks of Bunyamwera and Maguari viruses were prepared by infecting confluent monolayers of cells in large flasks at a multiplicity of infection of 0.01 pfu/cell and incubated at 31°C in a minimal volume of medium. The culture fluid was harvested when the cells displayed extensive cytopathic effect . The culture fluid was transferred to a 50ml Falcon tube and clarified by centrifugation at 3000 rpm for 5 minutes in a bench top centrifuge. The fluid was aliquoted and stored at -70°C.

4.4 Virus Titration

Bunyavirus virus titres were determined by a protocol based on Iroegbu and Pringle (1981). On day 1 a number of 35mm plates were seeded at 1 X 10^6 cells per plate and incubated overnight at 37°C. On day 2 the confluent monolayers of cells were infected with 100ul of serial 10 fold dilutions of viral stock in PBS + 2% calf serum. After 1 hour at 37°C the cells were overlaid with 2ml per plate of overlay solution (overlay solution consisted of 310ml of Eagles A, 60ml Eagles B without phenol red, 125ml 3.6% Difco agar, 7ml calf serum) and incubated at 37°C. After four days at 37°C the cells were fixed in 1% glutaraldehyde and stained with Giemsa stain. Plaques were counted to determine virus titre.

4.5 Preparation Of Virus Nucleocapsids

The following protocol equivalent to Leppert et al, (1979) was followed: Infected cells from four large flasks were scraped into PBS. Cells were pelleted by centrifugation in a bench top centrifuge (10 minutes at 3000 rpm). The cell pellet was then resuspended in 4ml of cold lysis buffer (0.15M NaCl, 0.05M Tris HCl pH 7.5, 0.6% v/v Nonidet NP_{40}) and left on ice for 5 minutes. The cells were then vortexed for 2 minutes, transferred to a 30ml Corex tube and spun at 8K for 5 minutes at 4°C in an SS34 rotor. The supernatant was removed and made 6mM EDTA by the addition of 50ul 0.5M EDTA.

A 6ml 20-40% w/v continuous caesium chloride gradient in 25mm Tris HCl pH 7.5, 2mM EDTA was set up. This was prepared in TST41 tubes and overlaid with 2.5ml 5% w/v sucrose in 50mM NaCl, 25mM Tris HCl pH7.5, 2mM EDTA. The cell extract was gently layered on top of the sucrose and the sample spun overnight at 32K at 12°C on a TST41 rotor. A visible band around the middle of the gradient was harvested by side puncture

and diluted 3 fold in NTE (100mM NaCl, 10mM Tris HCl pH 7.5, 1mM EDTA) and pelleted in a TST41 rotor spun at 40,000 rpm for 2 hours at 4°C. The nucleocapsid material was stored at -70°C until required.

4.6 Radiolabelling Of Intracellular Proteins

Confluent cell monolayers in 35mm Petri dishes were infected at a multiplicity of infection of 5 pfu/cell. At various times after infection the medium was replaced with 1ml PBS containing 30uCi [^{35}S] methionine and incubated for 1 hour at 37°C. The radioactive solution was removed and the cells lysed in 500ul of dissociation mix (see section 3.8). Cell lysates were stored at -20°C and boiled for 3 minutes before being run on an SDS-PAGE gel.

5 Baculovirus Expression System

The baculovirus used in this study was Autographa californica nuclear polyhedrosis virus (AcNPV). Both wild type and recombinant baculovirus (AcMAGS) were propagated in tissue culture on Spodoptera frugiperda (Sf9) clonal insect cell line.

5.1 Insect Cell Line Maintenance

Spodoptera frugiperda cells were maintained as monolayers in large flasks containing TC-100 medium (Gibco-BRL) supplemented with 5% or 10% foetal calf serum (FCS). Cells were incubated at 28°C without CO_2 and routinely passaged once confluency was reached. Confluent monolayers were dislodged by vigorous shaking into 10 ml of medium and were passaged at a 1/10 split (approximately 3x10^6 cells) every 5-7 days. Alternatively cells were maintained in suspension cultures in a 500 ml spinner culture flask. Spinner cultures were seeded at 1-2x10^5 cells/ml and incubated at 28°C with stirring (50 rpm) until a cell density of 2-3x10^6/ml was reached at which point the suspension was subcultured. Cell counts were

obtained by taking samples from the culture and counting the cells under a microscope by use of a Neubauer counting chamber, which enabled the calculation of cells/ml of culture.

5.2 Preparation and Titration of Baculovirus Stocks

Stocks of wild type and recombinant baculovirus were generated by infection of Sf9 cell monolayers in large flasks at a moi of between 0.1 to 1 pfu/cell for 1hr at 28°C with either AcMAGS or AcNPV. Medium was added to the infected flask and cells were incubated for a futher 2-4 days, until cells exhibited obvious cpe. The supernatant was harvested and centrifuged at 2,000 rpm form 10 min at 4°C to remove cells and cellular debris. The supernatant was aliquoted off and stored as virus stock at 4°C.

Recombinant and wild type baculovirus stocks were titrated on Sf9 cell monolayers in 35mm plates. After removal of the medium the plates were inoculated with ten fold serial dilutions of virus and incubated for 1 hr before the inoculum was removed and the cells overlaid with 2 ml of warm overlay. The overlay consisted of a mixture of equal volumes of 3% molten low melting point agarose and TC-100 medium with 10% FCS. The infected cells were incubated at 28°C for 3-4 days and then stained for 24 hr with 0.5 ml of medium containing a 1/20 dilution of 0.4% neutral red. Virus titres varied between 10^7 and 10^{11} pfu/ml. High and low titre stocks were also made from infected suspension cultures. Suspension cultures infected at a moi of 0.1 pfu/cell were harvested approximately 6-7 days post infection. The cells were removed by centrifugation (2000-3000 rpm for 10 min at 4°C). Supernatants at that point were stored at 4°C as low titre stock or transferred to ultracentrifuge tubes and virus particles pelleted at 24000 rpm on a TST41 rotor for 1hr at 4°C. The

supernatant was discarded and the viral pellet resuspended overnight at 4°C in TE buffer. The pellet was loaded on a 10-50% one step sucrose gradient in TST41 tubes and spun at 24000 rpm for 1hr at 4°C. The purified virus band was harvested using a pasteur pipette and repelleted in TE solution to remove the remaining sucrose. The virus pellet was resuspended overnight in TE buffer and stored at 4°C as high titre stock.

6 Bacterial Fusion Proteins

6.1 Analysis And Purification Of Bacterial Fusion Proteins

Analysis and purification of the bacterial fusion proteins was carried out using a protocol by Smith et al, (1990). Overnight cultures of bacteria carrying pEX (Stanley and Luzio, 1984) and pEUX (Bressan and Stanley, 1987) based expression plasmids or recombinant derivatives of these plasmids were diluted 1/50 in LB media and grown in a shaking incubator at 30°C to an optical density at 600nm of about 0.2. Flasks of cells were then transferred to a 42°C water bath for 5 minutes and then placed in a shaking incubator set at 37°C for 2 hours. Cells were recovered from a 100ml culture by centrifugation in an SS34 rotor (5000 rpm for 5 minutes). The cells were washed in STE buffer (see 2.1), resuspended in 4ml of lysozyme solution (0.1 mg/ml in 15% sucrose, 50mM Tris HCl pH 8.0, 50mM EDTA) and incubated on ice for 40 minutes. Cells were then lysed by the addition of 5.6ml Triton X-100 (0.2% v/v in 19mM Tris HCl pH 8.0, 1mM EDTA) and incubation on ice for 5 minutes. The insoluble fraction was recovered by centrifugation of the lysate for 15 minutes on an SS34 rotor at 10,000rpm. The pellet was resuspended in 2.5ml of 8M urea containing 2% v/v 2-mercaptoethanol, an equal volume of dissociation mix added and the sample boiled for 5 minutes. Fusion protein samples were analysed by electrophoresis on a 10% denaturing

SDS-PAGE gel (see section 3.8).

Further purification of fusion proteins was carried out by preparative gel electrophoresis. Two millilitre samples were fractionated on a single gel-width slot on an 8% denaturing SDS-PAGE gel (section 3.8). The fusion protein was located by staining the gel in 0.1% Coomassie Brilliant Blue for 10 minutes. The fusion band was cut out of the gel using a sterile scalpel. The acrylamide slice was crushed, mixed with water and passed through an 18-G syringe needle a number of times. This preparation was then used as an inoculum to raise antifusion protein antibodies in rabbits.

7.2 Preparation Of Anti-fusion Protein Antibodies

Rabbits were injected subcutaneously with approximately 200ug of purified protein in acrylamide slurry and boosted at 10-14 day intervals by the same route with similar doses of protein. Test bleeds were taken at 6 weeks and 8 weeks after the initial injection and used in radioimmunoprecipitation (section 3.7) and Western immunoblot (section 3.14) assays to determine the presence of anti-fusion protein antibodies in the serum.

Chapter 3

Expression of the Small (S) RNA Segment of Maguari Bunyavirus

1 Summary

This chapter describes *in vitro* expression studies on the proteins encoded by the S RNA segment of Maguari bunyavirus. The Maguari virus S RNA segment encodes three proteins in overlapping reading frames: the nucleocapsid (N) protein; the non-structural (NSs); and a third protein designated the ORF3 protein. This chapter also reports attempts to identify the ORF3 protein in virus infected cells and to raise antibodies to the ORF3 protein via its expression as a bacterial fusion protein.

2 Introduction

All bunyavirus S RNA segments for which sequence information is available encode both the N and NSs proteins in overlapping reading frames. Studies on the S RNA in La Crosse virus infected cells led Patterson *et al* (1983) to claim that mRNAs with different 5' ends were responsible for the separate translation of the N and NSs proteins. Subsequently Raju and Kolakofsky (1986) proved these different RNAs to be degradation products. Only one species of S mRNA was isolated from snowshoe hare bunyavirus infected cells (Bishop *et al*, 1983b) and therefore it is probable that both the N and NSs proteins are translated from the same mRNA species, the result of alternative initiation of translation. In order to substantiate this possibility the expression of the Maguari bunyavirus S RNA segment was investigated by *in vitro* transcription/translation.

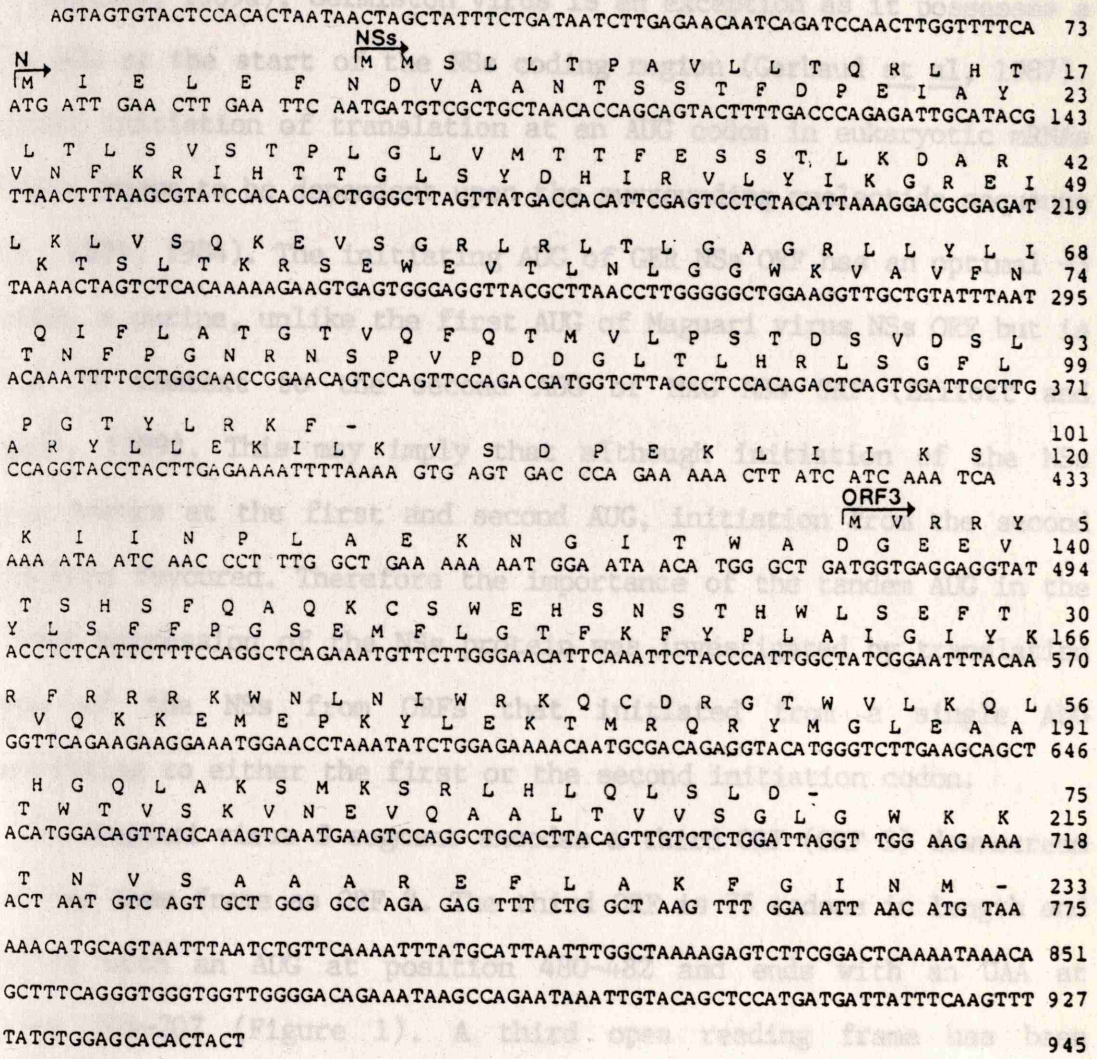
The Maguari virus was chosen for these studies principally because the complete nucleotide sequence of the S RNA segment had been determined by R.M.Elliott (Elliott and McGregor, 1989) and a cDNA clone

of the entire coding region of the segment was therefore available. The nucleotide sequence information revealed that in addition to encoding the nucleocapsid and non-structural proteins, this RNA segment had the potential to encode a third protein in an overlapping reading frame and hence the expression strategy of this protein was also of interest. A similar third overlapping reading frame had been reported for another bunyavirus (Germiston virus) S RNA segment (Gerbaud et al, 1987). An additional reason for carrying out these studies on the Maguari virus S RNA is that Maguari virus is the only bunyavirus for which a ts mutant is available with the lesion on the S segment (Pringle and Iroegbu, 1982). Therefore a better understanding of this virus S segment expression strategy is required.

The majority of the nucleotide sequence of the S RNA segment of Maguari (MAG) virus genome, bases 14-937, was obtained from sequencing the cDNA clone pMAG60 (Elliott and McGregor, 1989). The 5' and 3' terminal sequences were determined by primer extension reactions of infected-cell (+) strand RNA and virion (-) strand RNA templates respectively. The segment is 945 bases in length and as with other bunyaviruses encodes the nucleocapsid (N) and non-structural (NSs) proteins in overlapping reading frames in the complementary, positive RNA sense. Open reading frame (ORF) 1 is 233 codons in length from AUG at position 74-76 to UAA at position 773-775 (Figure 1). The second ORF (ORF 2) is 101 codons in length and extends from a tandem AUG at position 93-98 to UAA at position 396-398. On the basis of predicted size of encoded proteins (ORF1 M_r 26K; ORF2 M_r 11K) and similarity of the predicted amino acid sequence to other bunyavirus N and NSs proteins (Figure 7, chapter 1) ORF 1 was assumed to encode the N protein and

Figure 1.

Nucleotide sequence of the Maguari virus S segment. The sequence is presented as the viral complementary sense and is written as DNA. The three AUG-initiated ORFs (N, NSs and ORF 3) are indicated (taken from Elliott and McGregor, 1989).


AGTAGTGTACTCCACACTAATAACTAGCTATTTCTGATAATCTTGAGAACAATCAGATCCAACTTGGTTTTCA 73
NSs
N
M M S L L T P A V L L T Q R L H T 17
M I E L E F N D V A A N T S S T F D P E I A Y 23
ATG ATT GAA CTT GAA TTC AATGATGTCGCTGCTAACACCAGCAGTACTTTTGACCCAGAGATTGCATACG 143
L T L S V S T P L G L V M T T F E S S T L K D A R 42
V N F K R I H T T G L S Y D H I R V L Y I K G R E I 49
TTAACTTTAAGCGTATCCACACCACTGGGCTTAGTTATGACCACATTCGAGTCCTCTACATTAAAGGACGCGAGAT 219
L K L V S Q K E V S G R L R L T L G A G R L L Y L I 68
K T S L T K R S E W E V T L N L G G W K V A V F N 74
TAAAACTAGTCTCACAAAAAGAAGTGAGTGGGAGGTTACGCTTAACCTTGGGGGCTGGAAGGTTGCTGTATTTAAT 295
Q I F L A T G T V Q F Q T M V L P S T D S V D S L 93
T N F P G N R N S P V P D D G L T L H R L S G F L 99
ACAAATTTTCCTGGCAACCGGAACAGTCCAGTTCCAGACGATGGTCTTACCCTCCACAGACTCAGTGGATTCCTTG 371
P G T Y L R K F - 101
A R Y L L E K I L K V S D P E K L I I K S 120
CCAGGTACCTACTTGAGAAAATTTTAAAA GTG AGT GAC CCA GAA AAA CTT ATC ATC AAA TCA 433
ORF3
M V R R Y 5
K I I N P L A E K N G I T W A D G E E V 140
AAA ATA ATC AAC CCT TTG GCT GAA AAA AAT GGA ATA ACA TGG GCT GATGGTGAGGAGGTAT 494
T S H S F Q A Q K C S W E H S N S T H W L S E F T 30
Y L S F F P G S E M F L G T F K F Y P L A I G I Y K 166
ACCTCTCATTCTTTCCAGGCTCAGAAATGTTCTTGGGAACATTCAAATTCTACCCATTGGCTATCGGAATTTACAA 570
R F R R R K W N L N I W R K Q C D R G T W V L K Q L 56
V Q K K E M E P K Y L E K T M R Q R Y M G L E A A 191
GGTTCAGAAGAAGGAAATGGAACCTAAATATCTGGAGAAAACAATGCGACAGAGGTACATGGGTCTTGAAGCAGCT 646
H G Q L A K S M K S R L H L Q L S L D - 75
T W T V S K V N E V Q A A L T V V S G L G W K K 215
ACATGGACAGTTAGCAAAGTCAATGAAGTCCAGGCTGCACTTACAGTTGTCTCTGGATTAGGT TGG AAG AAA 718
T N V S A A A R E F L A K F G I N M - 233
ACT AAT GTC AGT GCT GCG GCC AGA GAG TTT CTG GCT AAG TTT GGA ATT AAC ATG TAA 775
AAACATGCAGTAATTTAATCTGTTCAAAATTTATGCATTAATTTGGCTAAAAGAGTCTTCGGACTCAAAATAAACA 851
GCTTTCAGGGTGGGTGGTTGGGGACAGAAATAAGCCAGAATAAATTGTACAGCTCCATGATGATTATTTCAAGTTT 927
TATGTGGAGCACACTACT 945

ORF 2 to encode the NSs protein.

Initiation of the NSs ORF with a tandem AUG is a common trend in bunyaviruses (Akashi et al, 1984; Bishop et al, 1982; Cabradilla et al, 1983; Elliott, 1989a). Germiston virus is an exception as it possesses a single AUG at the start of the NSs coding region (Gerbaud et al, 1987). Efficient initiation of translation at an AUG codon in eukaryotic mRNAs has beeen shown to be dependent upon the surrounding nucleotide sequence (Kozak, 1981, 1984). The initiating AUG of GER NSs ORF has an optimal -3 sequence, a purine, unlike the first AUG of Maguari virus NSs ORF but is similar in context to the second AUG of MAG NSs ORF (Elliott and McGregor, 1989). This may imply that although initiation of the NSs protein occurs at the first and second AUG, initiation from the second AUG is more favoured. Therefore the importance of the tandem AUG in the efficient expression of the NSs protein was investigated by translation studies of the NSs from ORFs that initiated from a single AUG corresponding to either the first or the second initiation codon.

The Maguari virus S segment encodes a third ORF (ORF 3) downstream and in the same frame as ORF 2. The third ORF is 75 codons in length and initiates with an AUG at position 480-482 and ends with an UAA at position 705-707 (Figure 1). A third open reading frame has been reported for one other bunyavirus S segment, that of Germiston (GER) virus (Gerbaud et al, 1987). The GER S ORF 3 is also 75 codons in length and in the same frame as NSs protein (Figure 2). Maguari and Germiston viruses are very closely related as they belong to the same serogroup ie Bunyamwera serogroup. A comparison of the amino acid sequences of the three predicted proteins by an homology alignment program (Figure 3) shows that the N and NSs proteins can be readily aligned with the

Figure 2.

The nucleotide sequence of the Germiston virus S segment. The sequence is presented as the complementary sense RNA. The three AUG initiated ORFs are indicated (taken from Gerbaud et al, 1987).

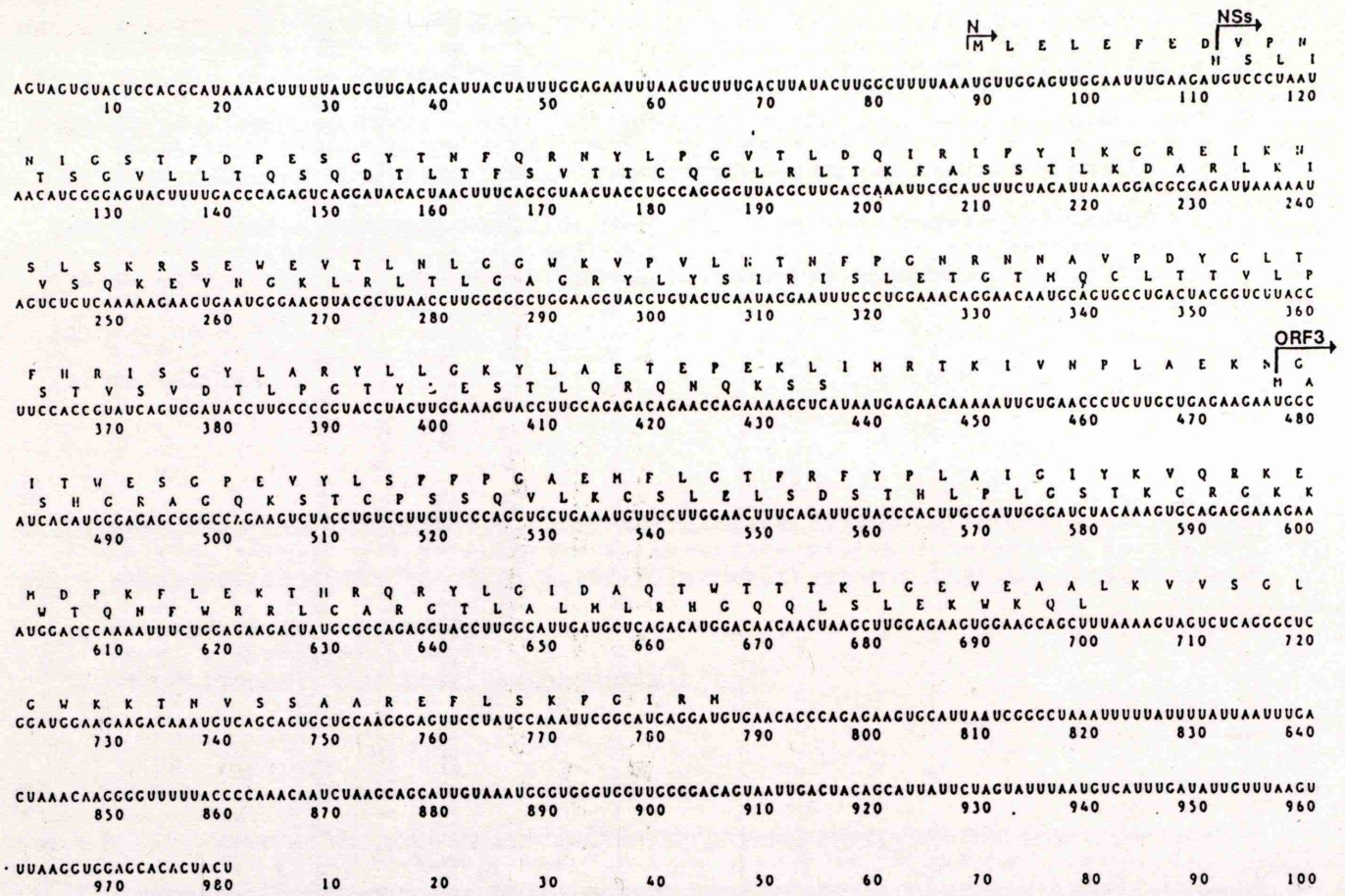
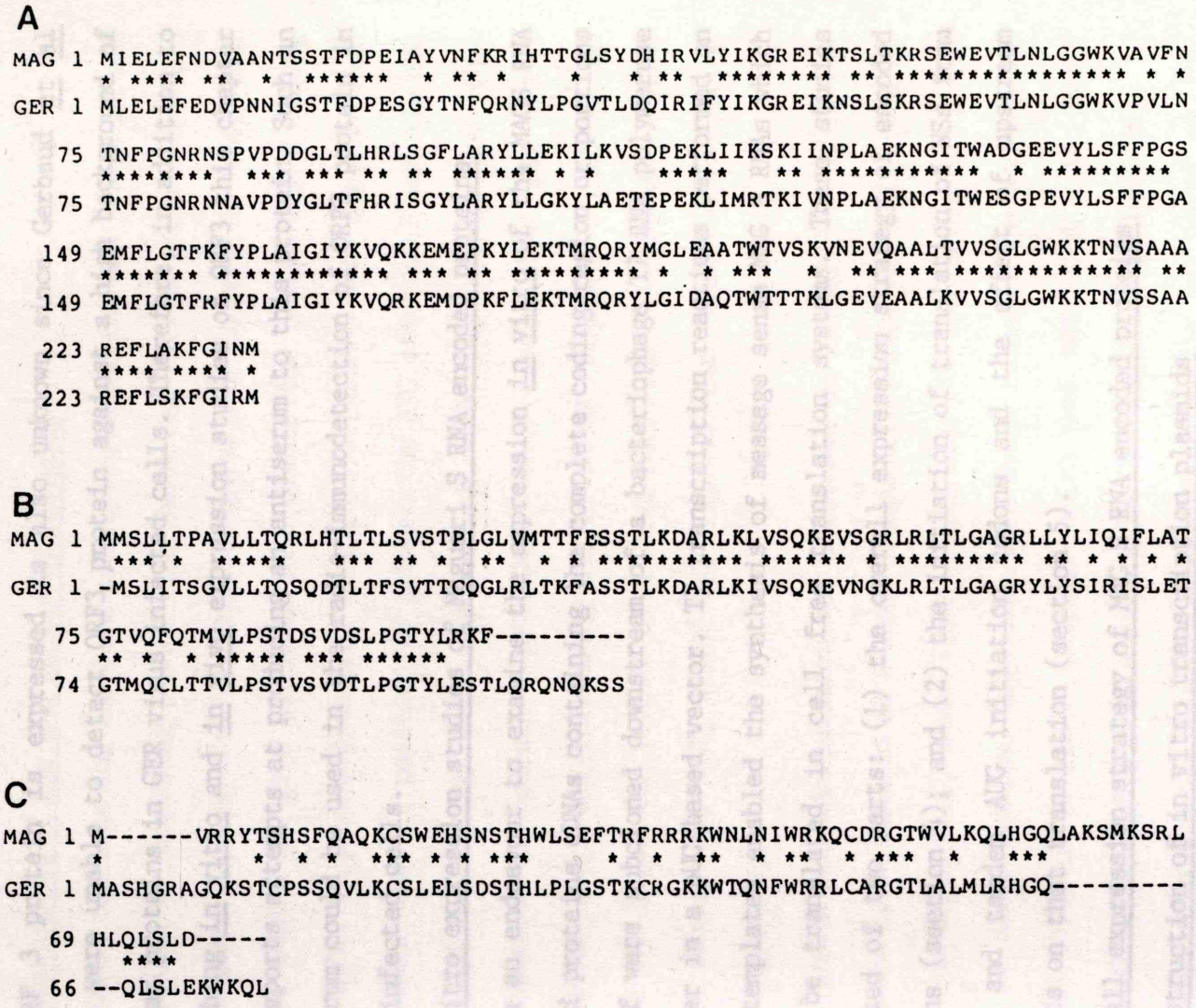

N
NSs
ORF3

Figure 3.

Comparison of the amino acid sequences of (A) the N proteins, (B) the NSs proteins and (C) the predicted protein products of the third ORF of Maguari (MAG) and Germiston (GER) viruses. The alignments were made using the HOMOL program of Taylor (1984)


A
MAG 1 MIELEFNDVAANTSSTFDPEIAYVNFKRIHTTGLSYDHIRVLYIKGREIKTSLTKRSEWEVTLNLGGWKVAVFN
GER 1 MLELEFEDVPNNIGSTFDPESGYTNFQRNYLPGVTLDQIRIFYIKGREIKNSLSKRSEWEVTLNLGGWKVPVLN
75 TNFPGNRNSPVPDDGLTLHRLSGFLARYLLEKILKVSDPEKLIIKSKIINPLAEKNGITWADGEEVYLSFFPGS
75 TNFPGNRNNAVPDYGLTFHRISGYLARYLLGKYLAETEPEKLIMRTKIVNPLAEKNGITWESGPEVYLSFFPGA
149 EMFLGTFKFYPLAIGIYKVQKKEMEPKYLEKTMRQRYMGLEAATWTVSKVNEVQAALTVVSGLGWKKTNVSAAA
149 EMFLGTFRFYPLAIGIYKVQRKEMDPKFLEKTMRQRYLGIDAQTWTTTKLGEVEAALKVVSGLGWKKTNVSSAA
223 REFLAKFGINM
223 REFLSKFGIRM
B
MAG 1 MMSLLTPAVLLTQRLHTLTLSVSTPLGLVMTTFESSTLKDARLKLVSQKEVSGRLRLTLGAGRLLYLIQIFLAT
GER 1 -MSLITSGVLLTQSQDTLTFSVTTCQGLRLTKFASSTLKDARLKIVSQKEVNGKLRLTLGAGRYLYSIRISLET
75 GTVQFQTMVLPSTDSVDSLPGTYLRKF---------
74 GTMQCLTTVLPSTVSVDTLPGTYLESTLQRQNQKSS
C
MAG 1 M------VRRYTSHSFQAQKCSWEHSNSTHWLSEFTRFRRRKWNLNIWRKQCDRGTWVLKQLHGQLAKSMKSRL
GER 1 MASHGRAGQKSTCPSSQVLKCSLELSDSTHLPLGSTKCRGKKWTQNFWRRLCARGTLALMLRHGQ---------
69 HLQLSLD-----
66 --QLSLEKWKQL

minimum of gaps with 73% and 62% amino acid identity respectively. In contrast the alignment of the predicted products of ORF 3 show only 36% identity and requires the insertion of large gaps to maximise homology. Given this contrast the significance of ORF 3 remained unclear. Whether the ORF 3 protein is expressed is also unknown since Gerbaud et al (1987) were unable to detect ORF3 protein against a high background of cellular proteins in GER virus infected cells. Therefore, in addition to describing in vitro and in vivo expression studies of ORF3 this chapter also reports attempts at producing an antiserum to this protein. Such an antiserum could be used in the radio-immunodetection of ORF3 protein in virus infected cells.

3 In vitro expression studies of Maguari S RNA encoded proteins

In an endeavour to examine the expression in vitro of the MAGS RNA encoded proteins cDNAs containing the complete coding region or portions thereof were subcloned downstream of a bacteriophage T7 RNA polymerase promoter in a pUC based vector. T7 transcription reactions performed on these templates enabled the synthesis of message sense MAG S RNAs which could be translated in cell free translation systems. These studies consisted of two parts: (1) the overall expression strategy of encoded proteins (section 4); and (2) the initiation of translation of NSs from single and tandem AUG initiation codons and the effect of upstream elements on that translation (section 5).

4 Overall expression strategy of MAG S RNA encoded proteins

4A Construction of in vitro transcription plasmids

A derivative of pBR322, designated pMAG60, contained a MAG S cDNA insert (bases 14-937) cloned into the PstI site of the vector. Fragments of the pMAG60 insert were subcloned into the T7 promoter-containing

plasmid pTZ18U at the appropiate sites in the polylinker to create: pTZMAGS, pTZ MAGNSs and pTZORF3. In addition MAG S cDNA fragments were subcloned into another T7 transcription vector pTF7.5 at a unique BamHI site to create pTF7.5MAGS and pTF7.5MAGNSs.

(i) Construction of the pTZ series of plasmids

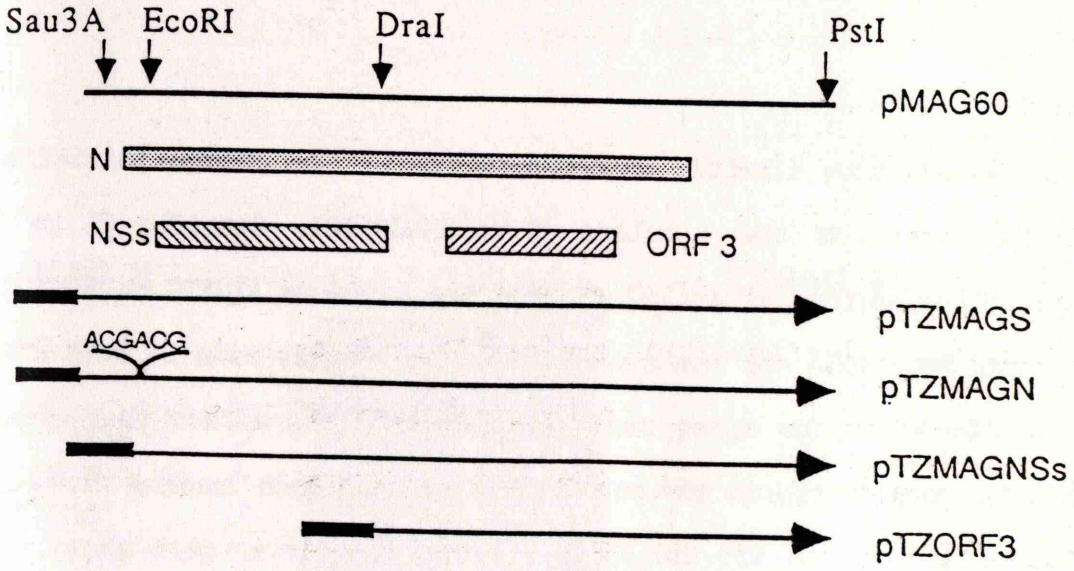
pTZMAGS contains the complete coding region of the MAG S segment and was obtained by digesting pMAG60 with Sau3A and PstI to release a 876bp cDNA fragment (bases 61-937). This fragment was ligated to BamHI-PstI digested pTZ18U to give pTZMAGS; this placed the entire MAG S coding region under T7 promoter control (Figure 4). pTZMAGS was digested with EcoRI (which cuts at position 86 between the ATG start of N and NSs and on the plasmid polylinker upstream of the MAGS cDNA) and then recircularised to produce pTZMAGNSs; this encodes NSs and ORF 3 proteins but lacks the initiating ATG of N ORF (Figure 4). pTZORF3 was obtained by digesting pMAG60 with DraI-PstI which released a 543bp fragment (bases 394-937) which was ligated into SmaI-PstI digested pTZ18U (Figure 4).

An additional manipulation was performed on pTZMAGS to enable the MAGS insert to be handled as a BamHI fragment: pTZMAGS was linearised at the 3' end with PstI, made flush ended with T4 DNA polymerase, and ligated to BamHI linkers. Digestion with BamHI released the MAG S cDNA (bases 61-937) as a BamHI fragment which was subcloned into BamHI digested M13mp19 or pTZ18U to create M13mp19MAGS(BB) and pTZMAGS(BB).

Oligonucleotide site directed mutagenesis was performed on the M13mp19MAGS(BB) template to alter the tandem ATG start of NSs to ACGACG by use of a 20 base long oligonucleotide with the sequence 5'dGAATTCAACGACGTCGCTGC (Figure 5) and mutated DNA referred to as MAGN,

Figure 4.

The coding strategy of the Maguari virus S segment cDNA and construction of subclones in the plasmid vector pTZ18U. pMAG60 is the cDNA copy of the S segment which was cleaved at the appropriate restriction enzyme sites indicated and the fragments were ligated into appropriately cleaved pTZ18U DNA to give the designated plasmids pTZMAGS, pTZMAGN, pTZMAGNSs and pTZORF3. The S segment coding strategy on these plasmids is indicated. In pTZMAGN the tandem ATG which specifies the initiation of the NSs protein was altered by site directed mutagenesis to ACGACG. The T7 promoter is indicated (▬▸) and RNA transcripts were synthesised on PstI linearised plasmid templates using T7 RNA polymerase.


Sau3A
EcoRI
DraI
PstI
pMAG60
N
NSs
ORF 3
pTZMAGS
ACGACG
pTZMAGN
pTZMAGNSs
pTZORF3

was subcloned as a BamHI fragment into BamHI digested pTZ18U to give the construct pTZMAGN (Figure 4).

(ii) Construction of the pTF7.5 series of plasmids

The pTF7.5 plasmid is a pUC-based plasmid that contains a unique BamHI cloning site flanked by T7 promoter and T7 terminator sequences. The plasmid was created by Fuerst et al (1986) to enable transient expression *in vivo* of genes cloned into this plasmid under T7 promoter control: a recombinant plasmid would be transfected into eukaryotic cells infected with a vaccinia virus that expresses T7 polymerase enabling message sense transcripts to be made from the recombinant plasmid template.

Two recombinant pTF7.5 plasmids containing either the complete coding region of the MAG S cDNA (pTF7.5MAGS) or part of the coding region (pTF7.5MAGNSs) were constructed. pTF7.5MAGS was created by isolating the MAGS insert from pTZMAGS(BB) as a BamHI fragment and subcloning it into pTF7.5 linearised with BamHI. The correct orientation of the insert was ascertained by dideoxysequencing using the T7 promoter sequencing primer. pTF7.5MAGNSs was created by digesting pTZMAGS with EcoRI which cut at position 86 between N and NSs initiation codons on the MAGS cDNA. The 5' end was then blunt ended with T4 DNA polymerase and ligated to BamHI linkers. Digestion with BamHI released a 847bp fragment (bases 86-945) which was subcloned into BamHI-digested pTF7.5. The correct orientaion of the insert was verified by dideoxysequencing.

4B In Vitro Transcription and Cell Free Translation

The RNA polymerase specified by bacteriophage T7 is a small subunit enzyme of M_r 100K that is highly specific for its cognate promoter. The core T7 promoter sequence necessary to direct transcription is

5'TAATACGACTCACTATAG3' (Dunn and Studier, 1983). Any DNA sequence can be transcribed into RNA provided it is positioned downstream of this sequence. The enzyme has a rapid rate of transcription (230 nucleotides per second) and is efficiently recycled *in vitro* such that each enzyme can carry out multiple rounds of transcription. Therefore production of multiple RNA copies of a single DNA template molecule is possible within a relatively short time period. The 3' end of the RNA is defined *in vitro* by linearising the DNA with an appropriate restriction enzyme at the 3' end of the template sequence. Run-over transcripts have been reported to appear in addition to the expected transcript when linearised templates have 3' protruding ends, as a result of initiation of transciption on the opposite strand. Therefore only those restriction sites that generate 5' overhangs or blunt ends are suitable for generating linearised templates.

The pTZ plasmids described above were linearised at the HindIII site in the polylinker downstream of the MAG S cDNA insert, whereas the presence of a T7 terminator removed the necessity to linearise the template in the pTF7.5 based plasmids. Messenger sense RNA was synthesised using T7 RNA polymerase (see Materials and Methods) and the total RNA synthesised in a standard reaction was dissolved in 10µl of water and stored in liquid nitrogen until use. Transcripts were translated in rabbit reticulocyte lysate or wheat germ cell free translation systems. The amount of RNA (1µl) used in the cell free systems was assumed to be at saturation level since increasing the amount of RNA added did not increase the level of translation.

(i)Rabbit reticulocyte lysate translation system

Translation of transcripts from pTZ series of plasmids

Translation of pTZMAGS RNA in the rabbit reticulocyte lysate yielded three major polypeptides with electrophoretic mobilities consistent with the predicted molecular weights of ORF1 (N protein, 26K), ORF 2 (NSs protein 11K) and ORF 3 (9.3K protein) products (Figure 6). By normalising with respect to the number of methionine residues in each of the polypeptides it was estimated by densitometeric scanning of the autoradiograph that the three products were synthesised in roughly equimolar amounts.

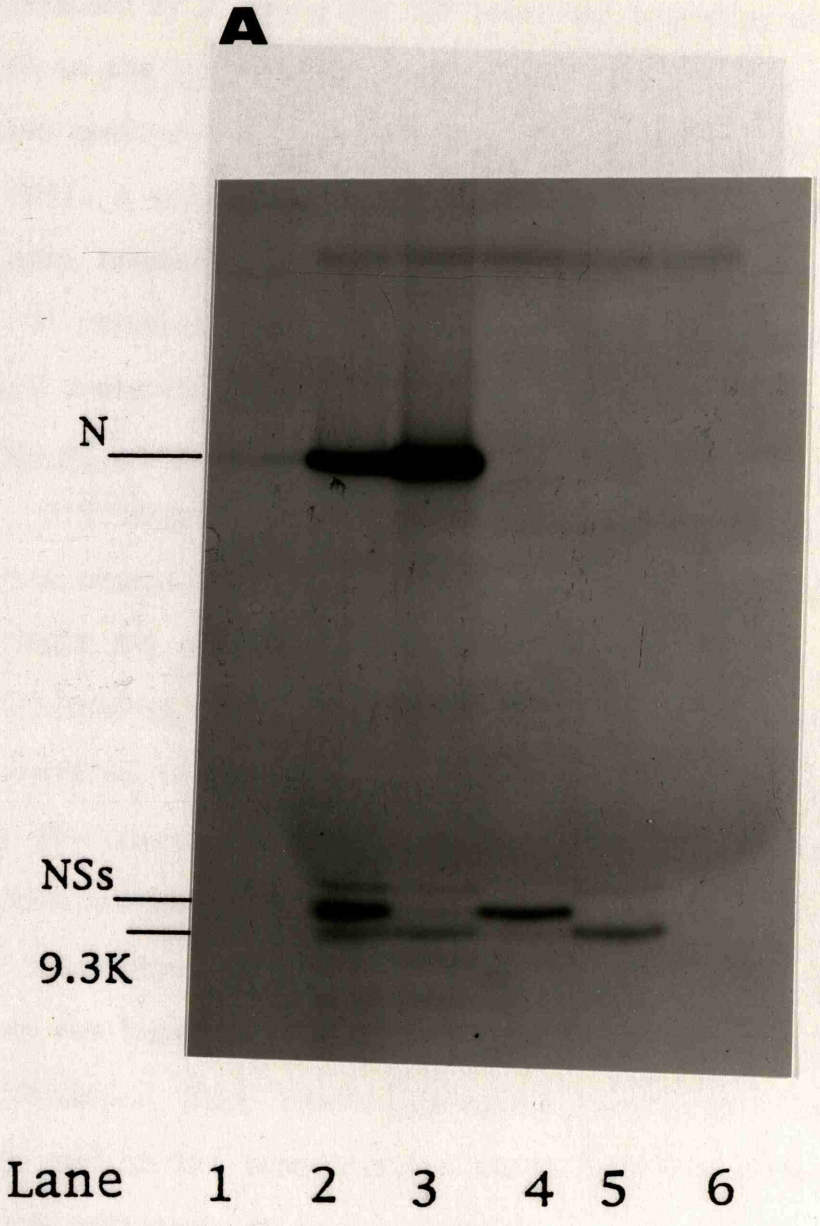
pTZMAGN RNA was translated to give the N and 9.3K proteins and on longer exposures of the autoradiogram (Figure 6B) a small amount of NSs was observed suggesting that a low level of initiation of NSs synthesis occurred at the tandem ACG (Figure 6B). Translation of pTZMAGNSs RNA produced NSs protein at a level equivalent to that synthesised by pTZMAGS RNA, whereas the 9.3K protein was synthesised at about 10% the level obtained by pTZMAGS RNA (Figure 6B). Translation of pTZORF3 RNA yielded only the 9.3K protein at a level similar to that obtained by pTZMAGS RNA. Longer exposure of the autoradiogram (Figure 6B) showed minor amounts of products smaller than the 9.3K protein possibly as a result of product breakdown or internal initiation of translation from AUG or even non AUG codons. A band is also seen above the NSs band which correlates with the appearance of the 9.3K protein, and may result from interaction between the 9.3K protein and a component of the reticulocyte lysate. This band was not observed with the *in vitro* translation of BUN S RNA which does not encode a third ORF (Chapter 4).

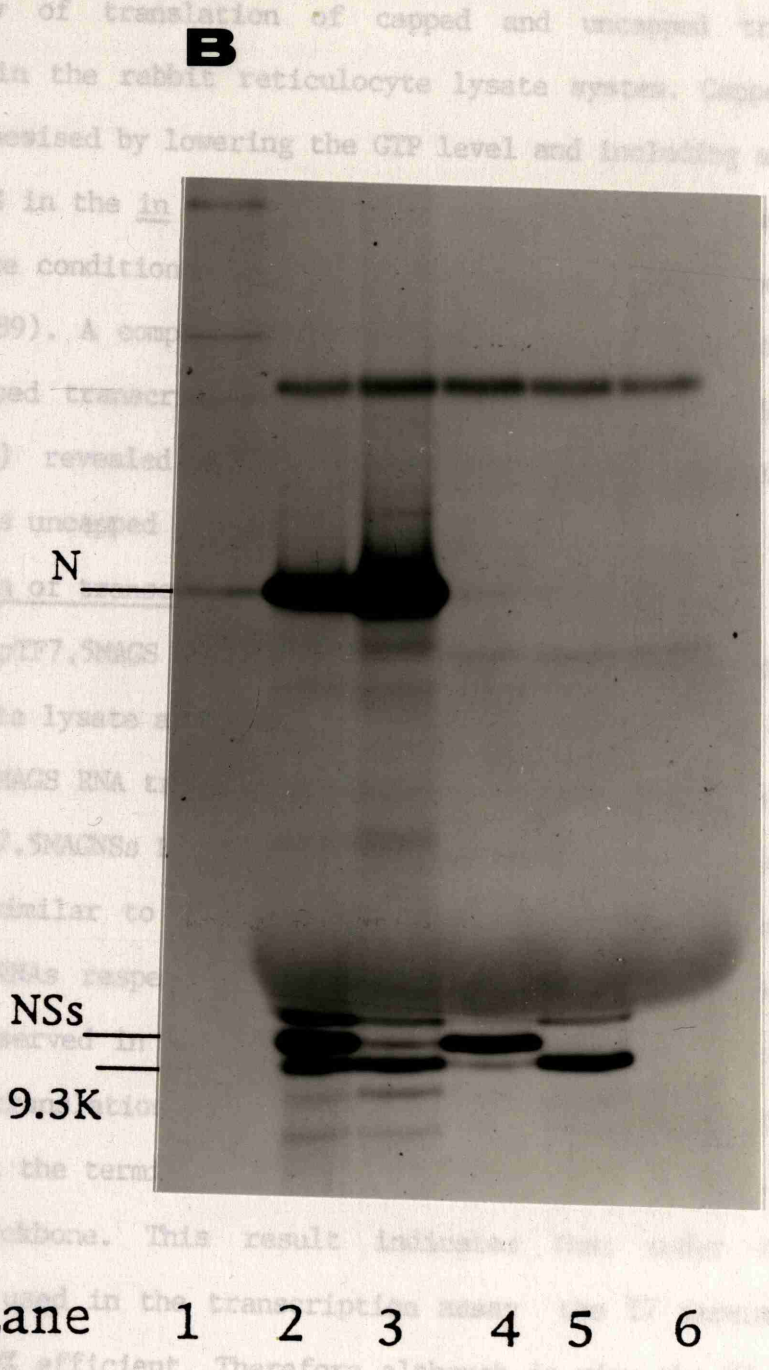
The above experiments were performed with uncapped RNAs. The

Figure 6.

Cell free translation of *in vitro* transcribed RNAs.

Aliquots of RNA transcripts derived from the pTZ series of plasmids described in Figure 4 were translated in separate rabbit reticulocyte lysate extracts in the presence of ^{35}S methionine and the translation products were analysed by 20% PAGE in the presence of SDS. Proteins were detected by fluorography. The positions of the N, NSs and 9.3K proteins are indicated. Lane (2) pTZMAGS, (3) pTZMAGN, (4) pTZMAGNSs, (5) pTZORF3 and (6) no RNA. Lane (1) Maguari virus infected cell extract. Gel exposure times: A, 6hr and B, 48hr.


A
N
NSs
9.3K
Lane 1 2 3 4 5 6


B
N
NSs
9.3K
Lane 1 2 3 4 5 6

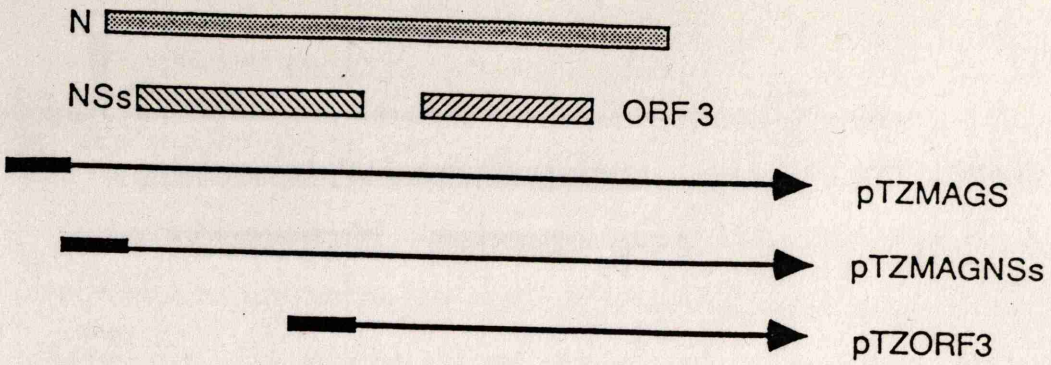
efficiency of translation of capped and uncapped transcripts was compared in the rabbit reticulocyte lysate system. Capped transcripts were synthesised by lowering the GTP level and including an excess level of m^7GpppG in the *in vitro* transcription reactions. It was assumed that under these conditions the majority of transcripts will be capped (Joel *et al*, 1989). A comparison of the efficiency of translation of capped and uncapped transcripts derived from pTZMAGS, pTZMAGNSs and pTZORF3 (Figure 7) revealed no significant differences and in all future experiments uncapped transcripts were used.

Translation of transcripts from pTF7.5 series of plasmids

Both pTF7.5MAGS and pTF7.5MAGNSs RNAs were translated in rabbit reticulocyte lysate and protein products analysed by PAGE (Figure 8). In the pTF7.5MAGS RNA track the N, NSs and ORF3 proteins were seen whereas in the pTF7.5MAGNSs RNA track only the NSs and ORF3 proteins were seen, a result similar to that obtained for the translation of pTZMAGS and pTZMAGNSs RNAs respectively. However a number of additional bands can also be observed in both pTF RNA tracks. These bands are presumably a result of translation of run through transcripts ie the polymerase does not stop at the termination signal and continues to transcribe into the plasmid backbone. This result indicates that under the *in vitro* conditions used in the transcription assay the T7 terminator sequence was not 100% efficient. Therefore although *in vivo* the T7 transcription terminator sequence has been shown to work efficiently (Fuerst and Moss, 1989), for *in vitro* transcription reactions the preferred method of defining the 3' end of the DNA template is by linearising the template with a suitable restriction enzyme.

Figure 7.

Cell free translation of capped and uncapped RNA transcripts. Linearised plasmid templates pTZMAGS, pTZMAGNSs and pTZORF3 were used to generate RNA transcripts with or without a cap analogue at the 5' end of the RNA. The upper part indicates the encoded ORFs on the pTZMAGS, pTZMAGNSs, pTZMAGN and pTZORF3 plasmids. Lower part: the translation efficiency of cap+ and cap- RNAs was compared in rabbit reticulocyte lysate. Protein products labelled with ^{35}S methionine were analysed by SDS-PAGE 18% gel.Lane (1) no RNA, (2) cap+ pTZMAGS, (3) cap- pTZMAGS, (4) cap+ pTZMAGNSs, (5) cap- pTZMAGNSs, (6) cap+ pTZORF3, (7) cap- pTZORF3.


N
NSs
ORF 3
pTZMAGS
pTZMAGNSs
pTZORF3

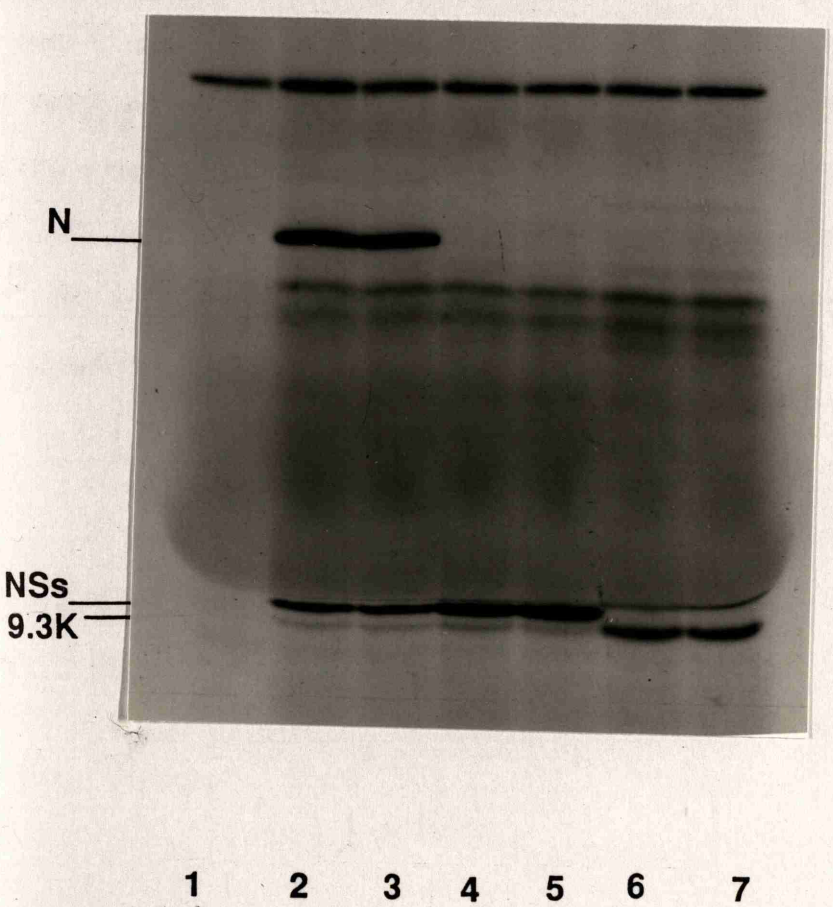
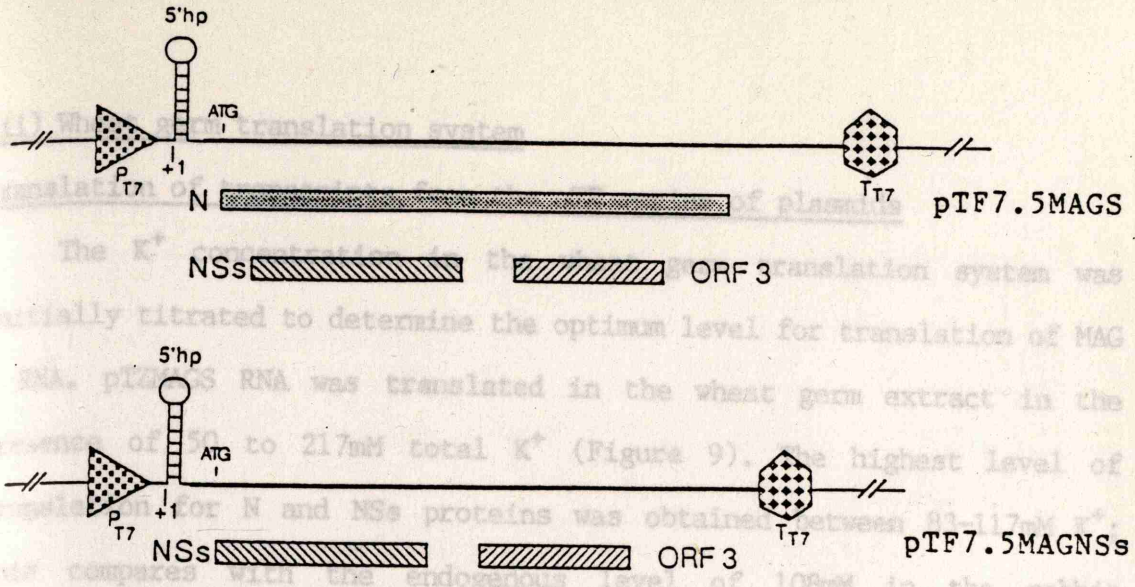
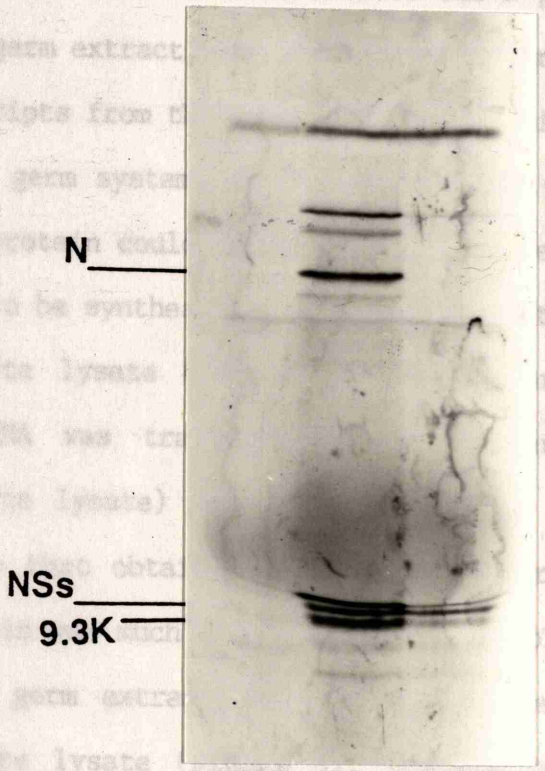

N
NSs
9.3K
1
2
3
4
5
6
7

Figure 8.

Cell free translation of RNA transcripts derived from supercoiled plasmid templates pTF7.5MAGS and pTF7.5MAGNSs. Upper part shows the essential features of the plasmids: MAGS cDNAs are flanked by an upstream T7 promoter sequence (P_{T7}) and a downstream T7 transcription termination site (T_{T7}). In addition a 5' hairpin loop structure, between the T7 promoter and the MAGS cDNA, is incorporated into the 5' end of all transcripts. The N initiation codon in pTF7.5MAGS and the first ATG of NSs ORF in pTF7.5MAGNSs are indicated. Lower part: Aliquots of RNA transcripts were translated in rabbit reticulocyte lysate and labelled translation products analysed by SDS-PAGE 20% gel. Lane (1) no RNA, (2) pTF7MAGS and (3) pTF7MAGNSs. The N, NSs and 9.3K protein products are indicated.


5'hp
ATG
+1
N
NSs
ORF 3
pTF7.5MAGS
5'hp
ATG
+1
NSs
ORF 3
pTF7.5MAGNSs


N
NSs
9.3K

1 2 3

(ii) Wheat germ translation system

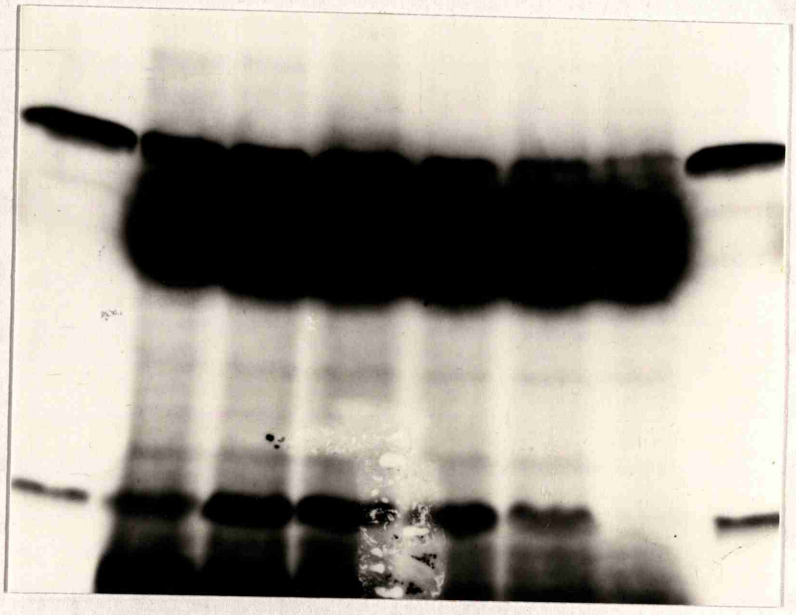
Translation of transcripts from the pTZ series of plasmids

The K^+ concentration in the wheat germ translation system was initially titrated to determine the optimum level for translation of MAG S RNA. pTZMAGS RNA was translated in the wheat germ extract in the presence of 50 to 217mM total K^+ (Figure 9). The highest level of translation for N and NSs proteins was obtained between 83-117mM K^+; this compares with the endogenous level of 108mM in the rabbit reticulocyte lysate (according to manufacturers specifications). Under conditions of 217mM K^+ it is interesting to note that although translation of both proteins is affected; translation of the NSs protein is much more repressed than that of the N protein. Translational assays in wheat germ extract were thereafter routinely performed at 117mM K^+.

Transcripts from the entire pTZ series of plasmids were translated in the wheat germ system. In the pTZMAGS RNA translation track the N, NSs and 9.3K protein could be identified (Figure 10) although less N protein appeared to be synthesised in comparison with the levels obtained in the reticulocyte lysate (Figure 6). No NSs protein was synthesised when pTZMAGN RNA was translated in wheat germ extract (unlike in the reticulocyte lysate) and the 9.3K protein was synthesised to a level similar to that obtained in pTZMAGS RNA translation. Synthesis of the 9.3K protein was much less efficient when pTZORF3 RNA was translated in the wheat germ extract compared to the level obtained in the rabbit reticulocyte lysate (Figure 6). Translation of pTZMAGS and pTZMAGNSs RNAs produced similar levels of NSs in both systems. Synthesis of the 9.3K protein was markedly reduced in pTZMAGNSs RNA programmed systems as compared to pTZMAGS RNA programmed systems.

Figure 9.

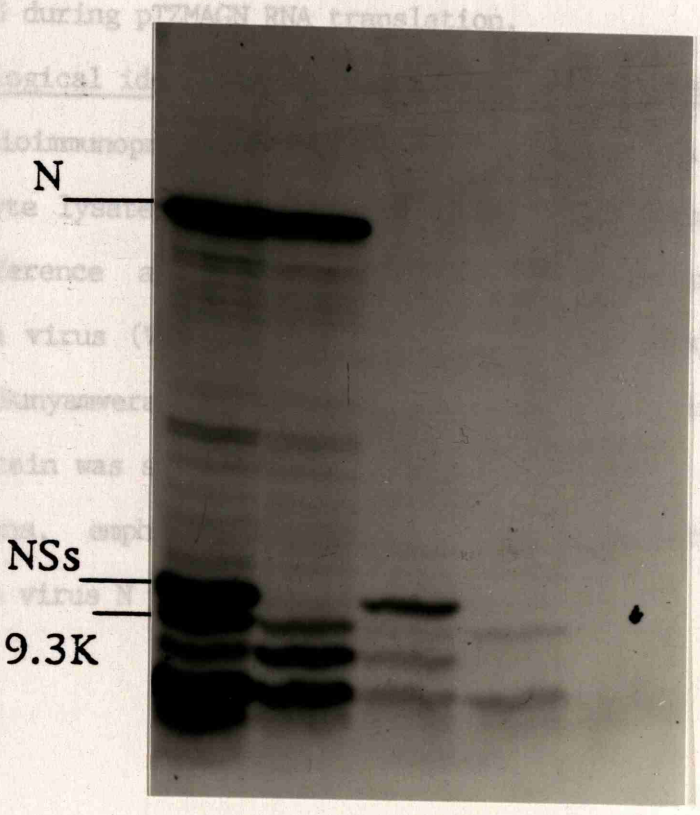
Translation of pTZMAGS derived RNA transcripts in a wheat germ translation system at different concentrations of K^+. Equal aliquots of pTZMAG S RNA were translated in wheat germ extract, under increasing concentrations of K^+, or in rabbit reticulocyte lysate, under standard conditions. Proteins labelled with ^{35}S methionine were analysed 15% SDS-PAGE gel. Lanes (1) and (8) pTZMAGS RNA translated in rabbit reticulocyte lysate. Lanes (2) to (7) pTZMAGS RNA translated in wheat germ extract in increasing concentration of K^+ (50, 83, 117, 150, 183 and 217mM respectively).



Lane	1	2	3	4	5	6	7	8
[K^+] mM	108	50	83	117	150	183	217	108

Figure 10.

Translation of run-off RNA transcripts in wheat germ extract. The upper part shows the MAG S ORFs encoded on the pTZ series of plasmids. Aliquots of RNA transcribed from linearised plasmid templates pTZMAGS, pTZMAGN, pTZMAGNSs and pTZORF3 were used to programme wheat germ extract. Translated proteins labelled with ^{35}S methionine were analysed by SDS-PAGE (20% gel). Lane (1) pTZMAGS, (2) pTZMAGN, (3) pTZMAGNSs, (4) pTZORF3 and (5) no RNA. The N NSs and 9.3K protein products are indicated. Exposure time 48hrs.



Lane 1 2 3 4 5

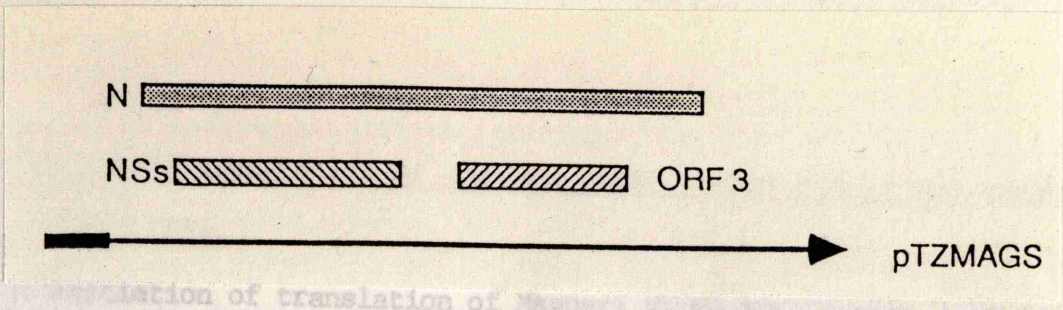
Interestingly there was a greater abundance of small translation products in the wheat germ system compared to the rabbit reticulocyte lysate. The wheat germ system may have a higher protease activity and hence these small products may have arisen from degradation of the primary translation products. Alternatively they may be a result of internal initiation from AUG or non AUG codons. The latter possibility would however seem less likely because NSs did not initiate at the tandem ACG during pTZMAGN RNA translation.

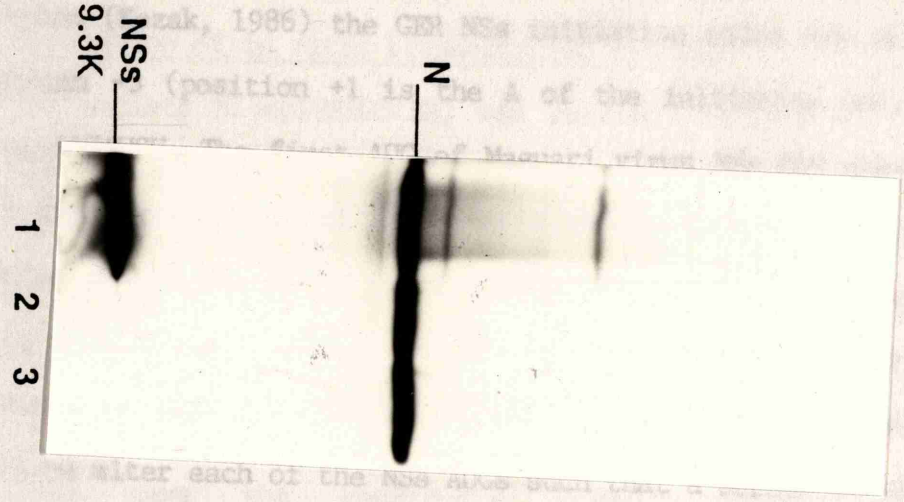
4C Immunological identification of in vitro synthesised N protein

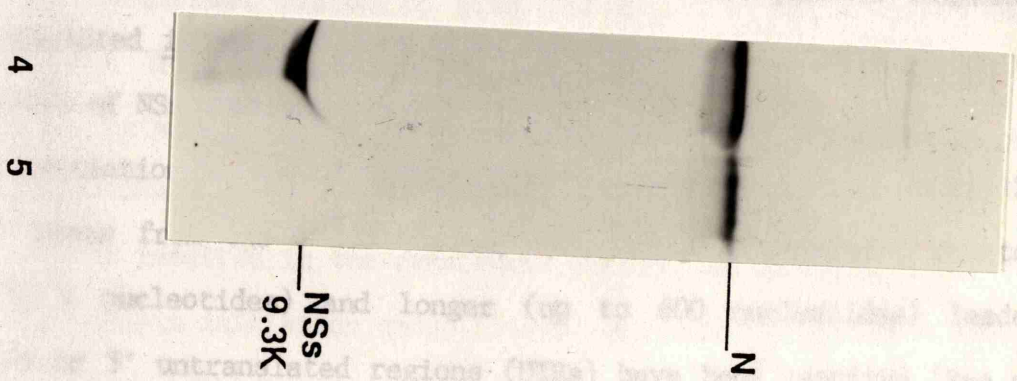
A radioimmunoprecipitation assay of pTZMAGS RNA programmed rabbit reticulocyte lysate (Figure 11) was carried out using either a Maguari virus reference antiserum, a polyclonal antiserum raised against Bunyamwera virus (Watret et al, 1985), or monoclonal antibody ascitic fluid to Bunyamwera virus N protein. The 26K protein corresponding to the N protein was specifically immunoprecipitated by all these antibody preparations, emphasising the high homology between Maguari and Bunyamwera virus N proteins.

Figure 11.

Confirmation of the identity of in vitro translated N protein by radio-immunoprecipitation assays. The upper part indicates the MAG S ORFs encoded on pTZMAGS. Lower part: radiolabelled proteins synthesised in rabbit reticulocyte lysate programmed with pTZMAG S RNA were immunoprecipated by : Maguari virus reference serum (lane 2); ascites fluid of monoclonal antibody (186) to Bunyamwera virus nucleocapsid protein (Elliott, unpublished work) (lane 3); polyclonal antiserum to purified Bunyamwera virus (lane 5). Lanes 1 and 4 are total extracts of pTZMAG S RNA programmed lysate. Protein samples were analysed by SDS-PAGE: lanes 1-3, 15% gel; lanes 4-5, 18% gel.


N
NSs
ORF 3
pTZMAGS


NSs
9.3K
N
1
2
3


4
5
NSs
9.3K
N

5 Initiation of translation of the NSs protein from single and tandem AUG initiated ORFs.

The initiation of translation of Maguari virus NSs protein is from a tandem AUG. According to the Kozak model for the initiation of translation (Kozak, 1986) the GER NSs initiation codon has an optimal A at position -3 (position +1 is the A of the initiation codon) in the sequence AAGAUGU. The first AUG of Maguari virus NSs ORF does not have such an optimal sequence (UCAAUGA) whereas the second initiation codon is similar in context (AUGAUGU) to the GER NSs solo AUG initiation codon (Elliott and McGregor, 1989; Gerbaud *et al*, 1987). To investigate NSs translation initiation oligonucleotide site directed mutagenesis was employed to alter each of the NSs AUGs such that a series of pTZMAGS and pTZNSs vectors was created in which NSs was initiated by either the first or second AUG. Transcripts derived from these plasmid templates were translated *in vitro* to determine the efficiency of initiation of translation of NSs from each AUG.

The initiation signal on most eukaryotic mRNAs is located between 20 and 100 bases from the 5'end (Kozak, 1987). Both appreciably shorter (down to 3 nucleotides) and longer (up to 600 nucleotides) leader sequences or 5' untranslated regions (UTRs) have been reported (Rao *et al*, 1986; Kozak, 1987; Cigan and Donahue, 1987). However, studies *in vitro* of mammalian mRNAs have indicated that a certain minimal length of leader sequence before the initiation codon is required for optimal translation (Spena *et al*, 1985; Strubin *et al*, 1986; Chevrier *et al*, 1988; Pelletier *et al*, 1985). The NSs initiation codon in RNA derived from pTZNSs is between 10-13 bases from the 5' end of the message. Therefore in a separate round of mutagenesis the upstream AUG of N ORF

was altered to abolish initiation from this site and provide a template with an extended 5' UTR before the NSs initiation codon. Transcripts derived from this template were translated in vitro to determine if extension of the length of the 5'UTR has any affect on the level of translation of NSs.

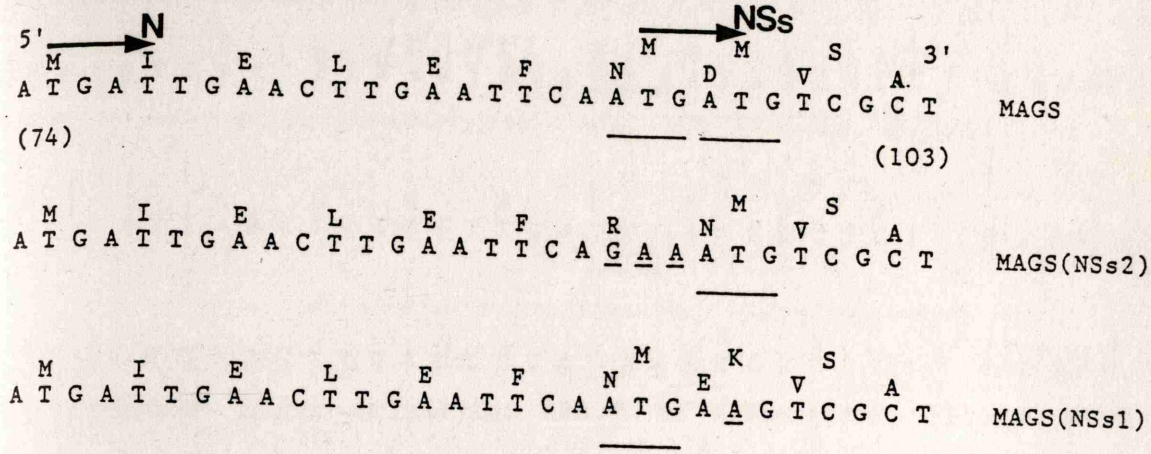
5(A) Construction of in vitro transcription plasmids

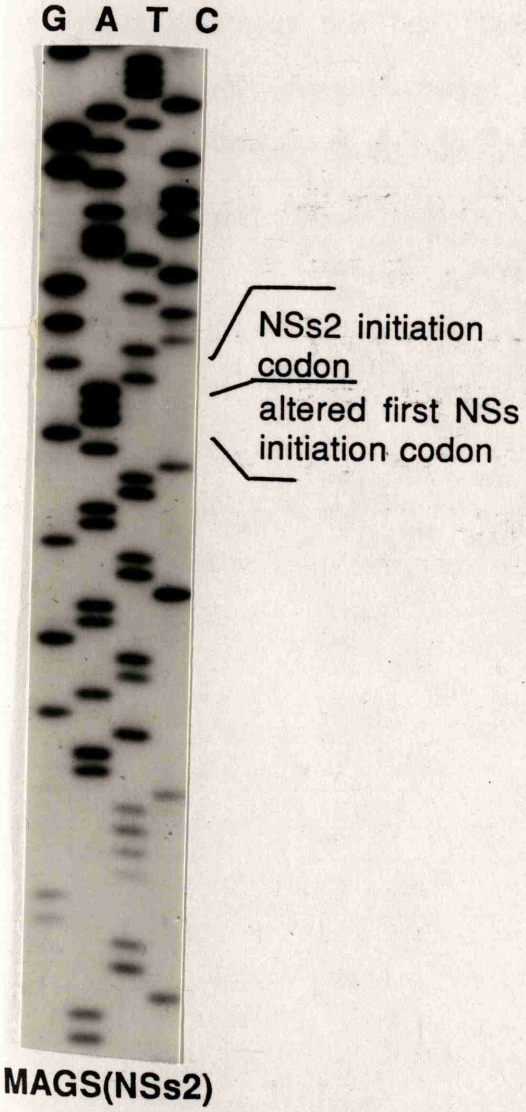
The template M13mp19MAGS(BB) was mutated to convert the tandem initiating AUG (93-98) of NSs to a single AUG either at position 93-95 (NSs1), in which case the second AUG was altered to AAG by use of the oligonucleotide 5'dGAACTTGAATTCAATGAAGTCGCTGCTAAC, or at position 96-98 (NSs2), in which case the first AUG was converted to GAA by use of the oligonucleotide 5'dGAACTTGAATTCAGAAAGTCGCTGCTAAC (Figure 12). These alterations gave the constructs M13mp19MAGS(NSs1) or (NSs2) respectively, which were digested with BamHI and the inserts subcloned into BamHI linearised pTZ18U to give the plasmids pTZMAGS(NSs1) and pTZMAGS(NSs2). These plasmids were digested with EcoRI which cuts at position 86 between the AUG start codons for N and NSs and in the plasmid polylinker at the 5' end of the MAG S cDNA. Recircularization of these plasmids resulted in the constructs pTZNSs1 and pTZNSs2.

The N protein initiation codon (74-76) was also altered in a second round of oligonucleotide site directed mutagenesis to convert the AUG to GAA by the use of the oligonucleotide 5'dGGGATCCAACTTGGTTTTCAGAAATTGAACTTGAATTC on templates M13mp19MAGS(NSs1) and (NSs2) (Figure 13). The mutated DNA inserts were subcloned as BamHI fragments into pTZ18U to give the plasmids pTZMAGSΔNNSs1 and pTZMAGSΔ NNSs2.

Figure 12.

Site specific alterations to the NSs ORF initiation codons. Upper part: Oligonucleotide site directed mutagenesis was performed on the template M13mp19MAGS(BB). The DNA nucleotide sequence of the first ATG initiation codon (bases 93-95) was altered to GAA and this cDNA was designated MAGS(NSs2). The nucleotide sequence of the second ATG (bases 96-98) was altered on another MAGS cDNA from ATG to AAG and the cDNA was designated MAGS(NSs1). Lower part: Verification by dideoxy sequencing of the mutations (A) MAGS(NSs2) and (B) MAGS(NSs1).


5'
N
NSs
3'
MAGS
(74)
(103)
MAGS(NSs2)
MAGS(NSs1)


G A T C
NSs2 initiation codon
altered first NSs initiation codon
MAGS(NSs2)

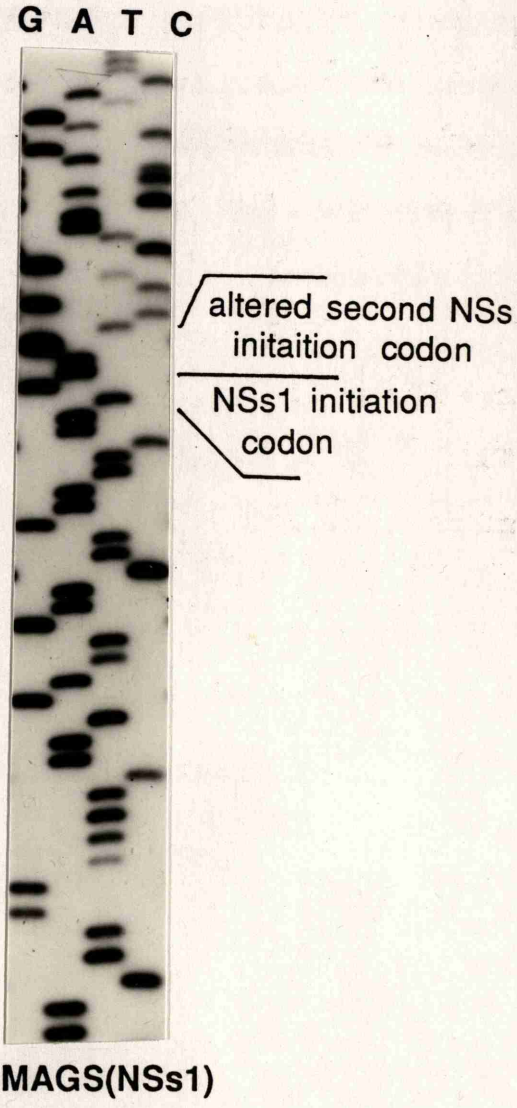
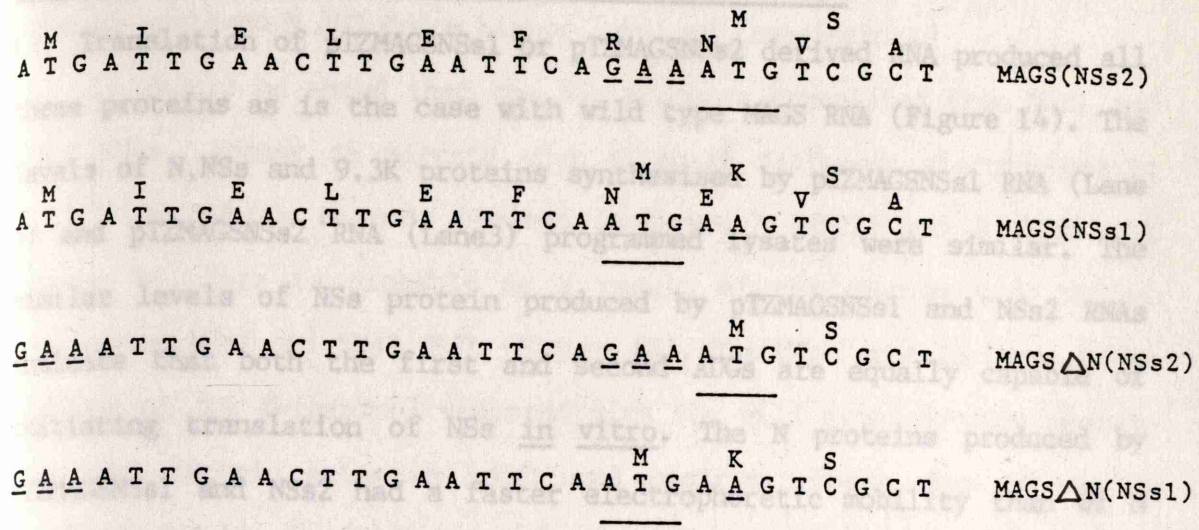
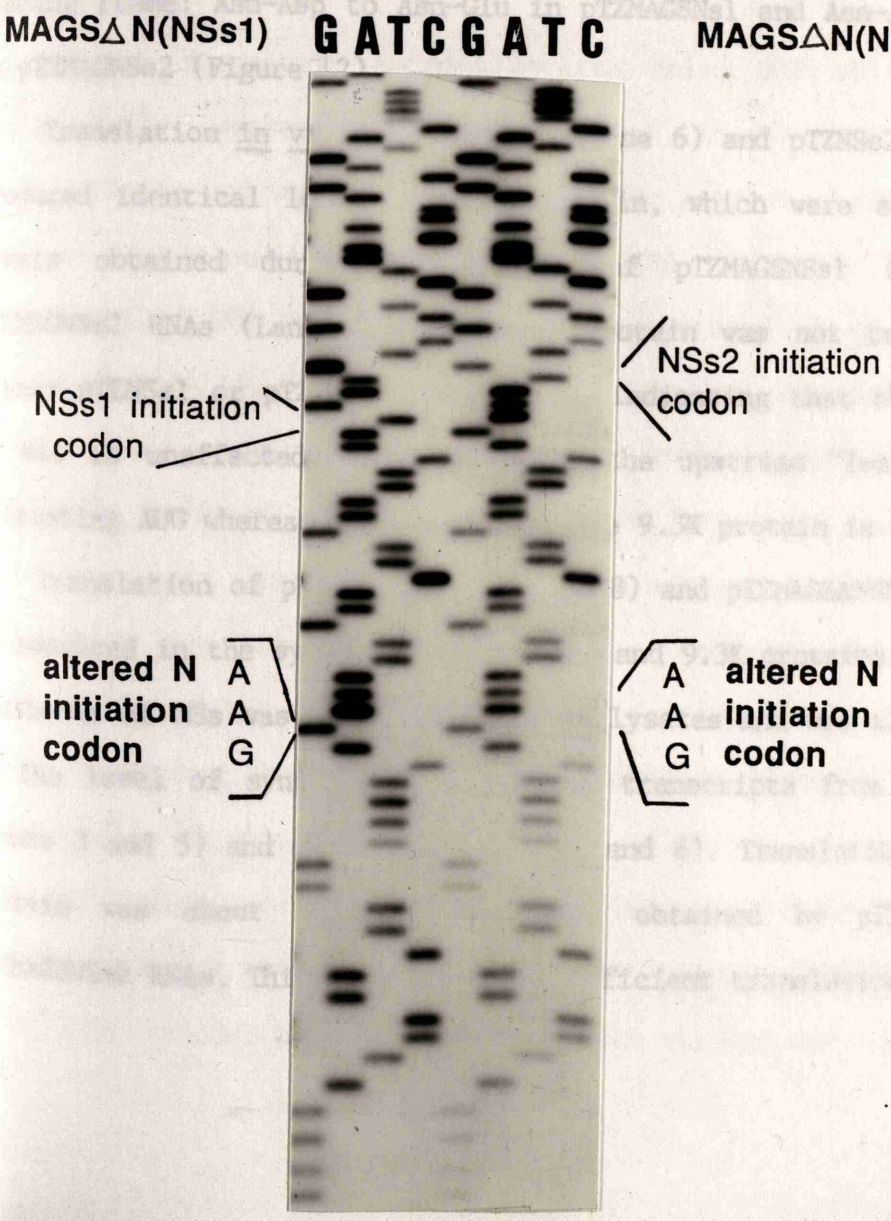

G A T C
altered second NSs initaition codon
NSs1 initiation codon
MAGS(NSs1)

Figure 13.

Site specific mutagenesis of N ORF initiation codon. MAGS(NSs1) and MAGS(NSs2) DNAs were altered by changing the nucleotide sequence of the N ORF initiation codon from ATG to GAA. The altered cDNAs were designated MAGSΔN(NSs1) and MAGSΔN(NSs2) and the specific differences between cDNAs are illustrated in the upper diagram. The changes were verified by dideoxy sequencing shown in the lower part; (A) ATG to GAA on MAGSΔN(NSs1) and (B) ATG to GAA on MAGSΔN(NSs2). The NSs1 and NSs2 initiation codons are indicated.


M I E L E F R N V A
ATGATTGAACTTGAATTCAGAATGTCGCT MAGS(NSs2)
M K S
M I E L E F N E V A
ATGATTGAACTTGAATTCAATGAAGTCGCT MAGS(NSs1)
M S
GAAATTGAACTTGAATTCAGAATGTCGCT MAGSΔN(NSs2)
M K S
GAAATTGAACTTGAATTCAATGAAGTCGCT MAGSΔN(NSs1)


MAGSΔN(NSs1)
GATCGATC
MAGSΔN(NSs2)
NSs1 initiation codon
NSs2 initiation codon
altered N initiation codon
A
A
G
A
A
G
altered N initiation codon

5(B) In vitro transcription and cell free translation

Translation of pTZMAGSNSs1 or pTZMAGSNSs2 derived RNA produced all three proteins as is the case with wild type MAGS RNA (Figure 14). The levels of N,NSs and 9.3K proteins synthesised by pTZMAGSNSs1 RNA (Lane 5) and pTZMAGSNSs2 RNA (Lane3) programmed lysates were similar. The similar levels of NSs protein produced by pTZMAGSNSs1 and NSs2 RNAs indicate that both the first and second AUGs are equally capable of initiating translation of NSs in vitro. The N proteins produced by pTZMAGSNSs1 and NSs2 had a faster electrophoretic mobility than wt N protein, most probably because the alterations made to the tandem AUG start of NSs also affected the amino acids in the overlapping N protein reading frame: Asn-Asp to Asn-Glu in pTZMAGSNs1 and Asn-Asp to Arg-Asn in pTZMAGNSs2 (Figure 12).

Translation in vitro of pTZNSs1 (Lane 6) and pTZNSs2 RNA (Lane 4) produced identical levels of NSs protein, which were similar to the levels obtained during translation of pTZMAGSNSs1 (Lane 5) and pTZMAGNSs2 RNAs (Lane 3). The 9.3K protein was not translated from either pTZNSs1 or pTZNSs2 transcripts, indicating that the translation of NSs is unaffected by the loss of the upstream "leaky" N protein initiating AUG whereas translation of the 9.3K protein is affected.

Translation of pTZMAGSΔNNSs1 (Lane 8) and pTZMAGSΔNNSs2 RNAs (Lane 7) resulted in the synthesis of the NSs and 9.3K proteins. The level of synthesis of NSs was the same for both lysates and was also equivalent to the level of synthesis obtained by transcripts from pTZMAGSNSs1/2 (Lanes 3 and 5) and pTZNSs1/2 (Lanes 4 and 6). Translation of the 9.3K protein was about 30% of the level obtained by pTZMAGSNSs1 and PTZMAGSNSs2 RNAs. This indicates that efficient translation is dependent

upon an upstream N AUG for a higher level of translation.

It should be noted that the NSs proteins translated from NSs1 and NSs2 RNAs appear to migrate with slightly different electrophoretic mobilities, which is most obvious in lanes 7 (NSs2) and 8 (NSs1) of Figure 14. This is probably as a result of the changes made whilst generating NSs1 and NSs2 ORFs (Figure 12). The NSs2 protein loses a Met residue at the N-terminal compared with the wild type sequence whereas the NSs1 protein gains an additional positive charge when at the N-terminal end the second Met is substituted for a Lys residue.

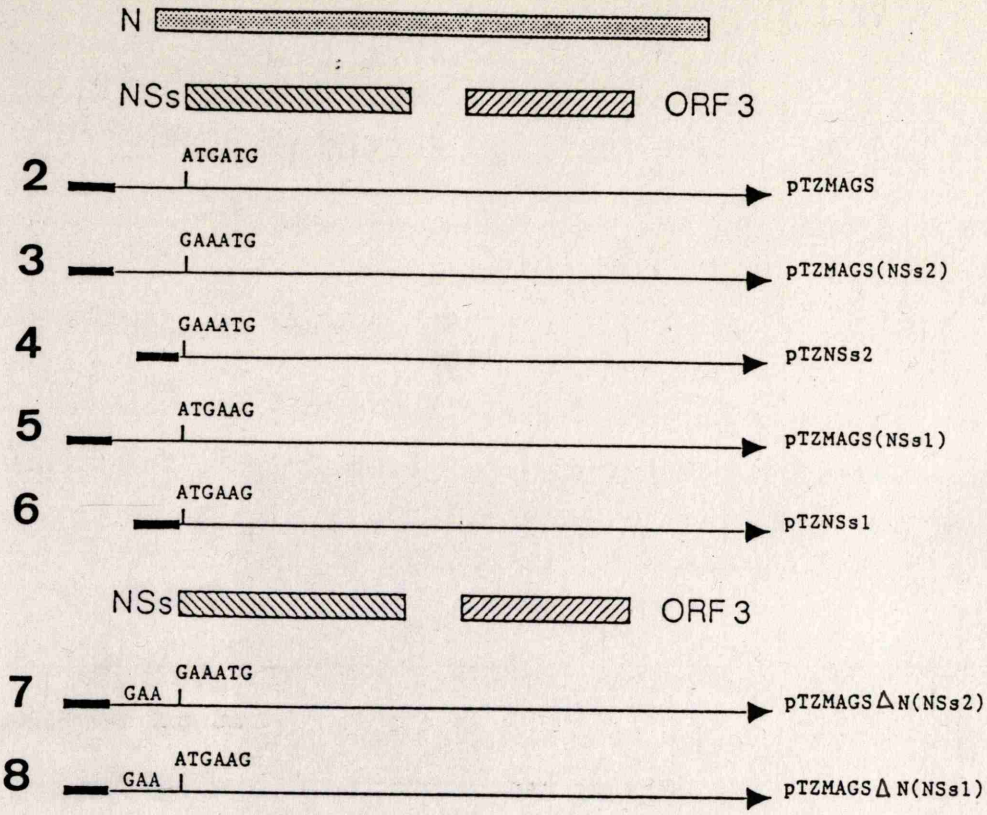
6 The expression of Maguari virus S RNA ORF3 protein in virus infected cells

The 9.3K protein encoded by the third ORF of MAG S RNA was successfully expressed in cell free translation systems from polycistronic and monocistronic mRNAs. The product of the similar third ORF in Germiston (GER) bunyavirus S RNA could not be detected in radiolabelled GER virus infected cells (Gerbaud et al, 1987). In an effort to identify ORF3 protein in radiolabelled Maguari virus infected cell lysates a comparative time course study of protein synthesis in BHK cells infected by Maguari virus and Bunyamwera virus was carried out. Bunyamwera virus was used in this study because it is very closely related to the Maguari virus but does not encode a third overlapping open reading frame on the S RNA genome segment. At 8, 12, 18 and 24hr after infection the cells were radiolabelled with ^{35}S methionine as described in Materials and Methods, and cell lysates of infected or mock infected cells were analysed by PAGE (Figure 15). The N protein was easily detected in both BUN virus and MAG virus infected tracks at 12hr post infection but the NSs protein of both viruses was less distinctive

Figure 14.

Cell-free translation of RNA transcripts containing single NSs initiation codons. A. RNA transcripts derived from Hind III linearised plasmid templates shown diagramatically. The MAG S ORFs encoded on these plasmids are indicated. B. Proteins labelled with ^{35}S methionine were analysed by 20% PAGE in the presence of SDS. Lanes (2)-(8) correspond to lysates programmed with plasmid transcripts from plasmids designated (2)-(8) in part A. Lane (1) is lysate without added RNA.

A


N
NSs
ORF 3
2
ATGATG
pTZMAGS
3
GAAATG
pTZMAGS(NSs2)
4
GAAATG
pTZNSs2
5
ATGAAG
pTZMAGS(NSs1)
6
ATGAAG
pTZNSs1
NSs
ORF 3
7
GAA
GAAATG
pTZMAGSΔN(NSs2)
8
GAA
ATGAAG
pTZMAGSΔN(NSs1)

B

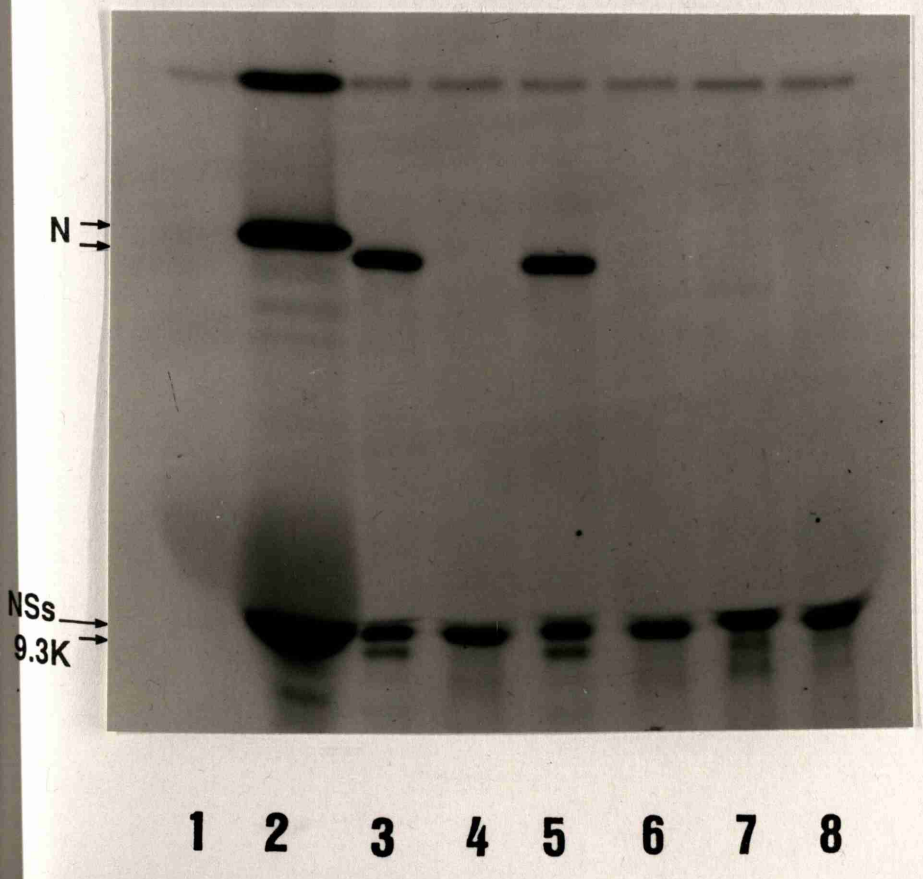

N
NSs
9.3K
1 2 3 4 5 6 7 8

Figure 15.

Time course of protein synthesis in Maguari virus and Bunyamwera virus infected BHK cells. Infected cells were pulse labelled for 1hr with ^{35}S methionine at 8, 12, 18 and 24hrs after infection, cells lysed with dissociation mix and equivalent volumes of cell lysate were analysed by SDS-PAGE 20%. (M), Maguari virus and (B) Bunyamwera virus. Two control tracks are also present: MI, mock infected BHK cells; ORF3, pTZORF3 RNA programmed rabbit reticulocyte lysate. The N and 9.3K ORF3 proteins are indicated.

9.3K
MI
ORF3
MAG (8)
BUN (8)
MAG (12)
BUN (12)
MAG (18)
BUN (18)
MAG (24)
BUN (24)
NSs
MAG N
BUN N

and was best observed at the 24hr time point. However the high cellular background made the detection of the ORF3 protein impossible and both BUN and MAG infected cell lysates resembled each other around the region where the ORF3 protein was expected.

7 Expression of Maguari virus S RNA 9.3K protein as a bacterial fusion polypeptide and attempt to raise an antiserum to this fusion protein

The ORF3 protein could not be identified in radiolabelled Maguari virus infected cells because of a high background of radiolabelled cellular proteins of similar M_r. Therefore an attempt was made to raise a monospecific fusion-protein antibody to the ORF protein which would enable immunoprecipitation of the ORF 3 protein in infected cells.

The fusion protein expression system chosen was that originally developed by Stanley and Luzio (1984), the pEX vector series, and subsequently updated by Bressan and Stanley (1987), the pUEX vector series. The system involves the insertion of foreign sequences into the lac Z gene at the C-terminus of the beta-galactosidase (β-gal) ORF. The lac Z gene is itself fused to the promoter, operator and the cro gene of bacteriophage λ. Expression of the fusion protein is under the control of the temperature sensitive repressor (C_I); and expression may be induced by temperature shift from 30°C to 42°C. The pEX and pUEX plasmids carry a number of unique restriction sites to facilitate cloning of target sequences in all three possible reading frames. The pUEX vectors, unlike the pEX predecessors, are host strain independent as they encode a repressor protein on the plasmid; the pEX vectors do not and therefore are dependent upon specific host cells that express ts C_I to control expression.

7A Construction of the recombinant pUEX expression vector

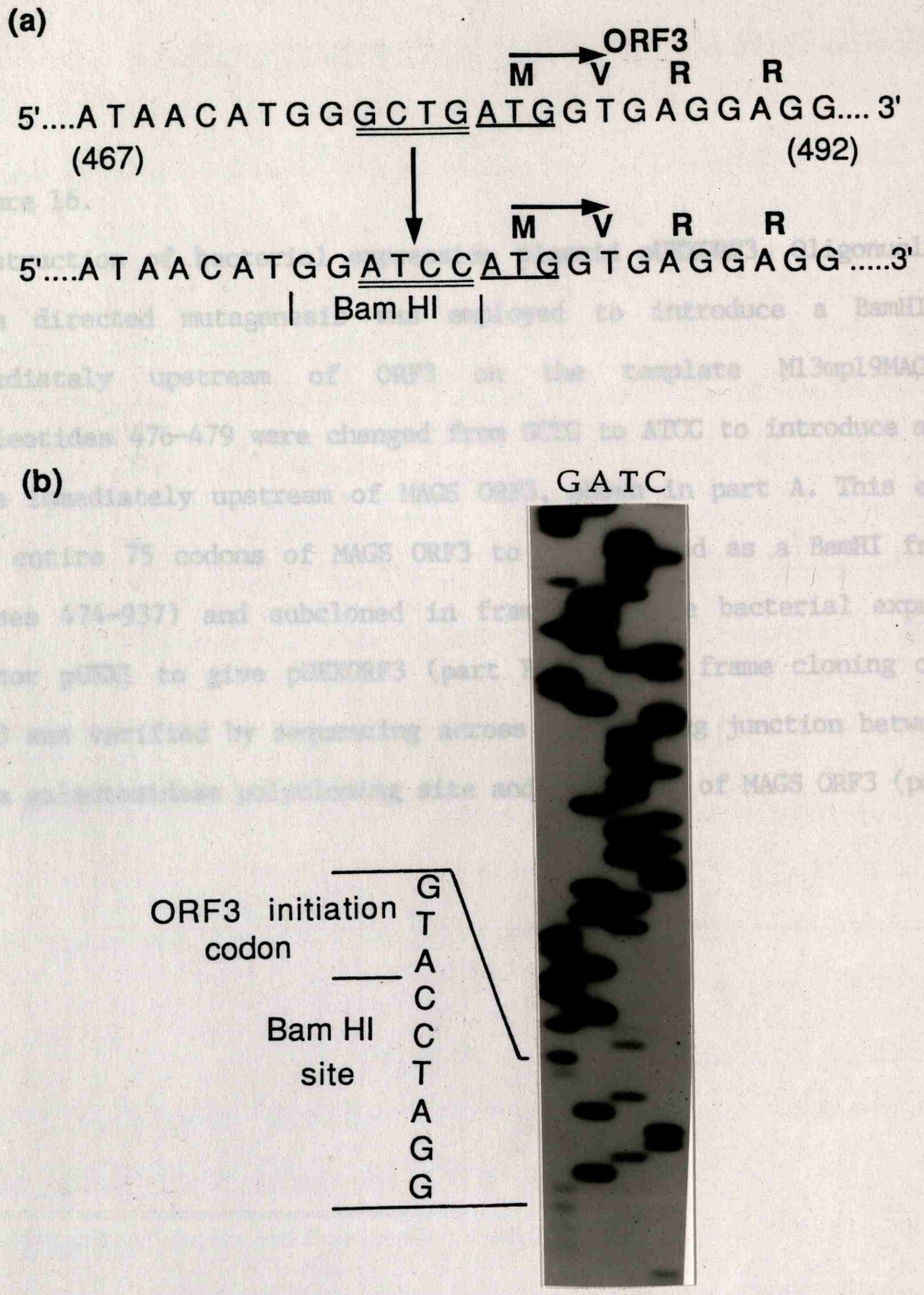
Oligonucleotide site directed mutagenesis was employed to introduce a BamHI site immediately upstream of the MAGS ORF3 AUG initiation codon on the single stranded DNA template M13mp19MAGS(BB) using a 43mer oligonucleotide 5'dGAAAAAAATGGAATAACATGGATCCATGGTGAGGAGGTATACC (Figure 16). RF DNA minipreps of mutants were screened by restriction digestion for the introduction of an additional BamHI site which released the MAGS cDNA insert as two fragments of approximately 400 and 500bp respectively. The entire MAGS ORF3 (amino acids 1-75) was contained on the 500bp BamHI fragment. This fragment was ligated to BamHI-digested pUEXI and competent E.coli (strain DH5α) were transformed with aliquots of the ligation reaction under standard conditions, except a heat shock at 30°C was used to prevent possible expression induction. Colonies carrying recombinant plasmid pUEXORF 3 were initially identified by BamHI digestion, and then sequenced to check the orientation of the BamHI insert and to verify that the reading frame was maintained at the β-gal/ORF 3 junction (Figure 16).

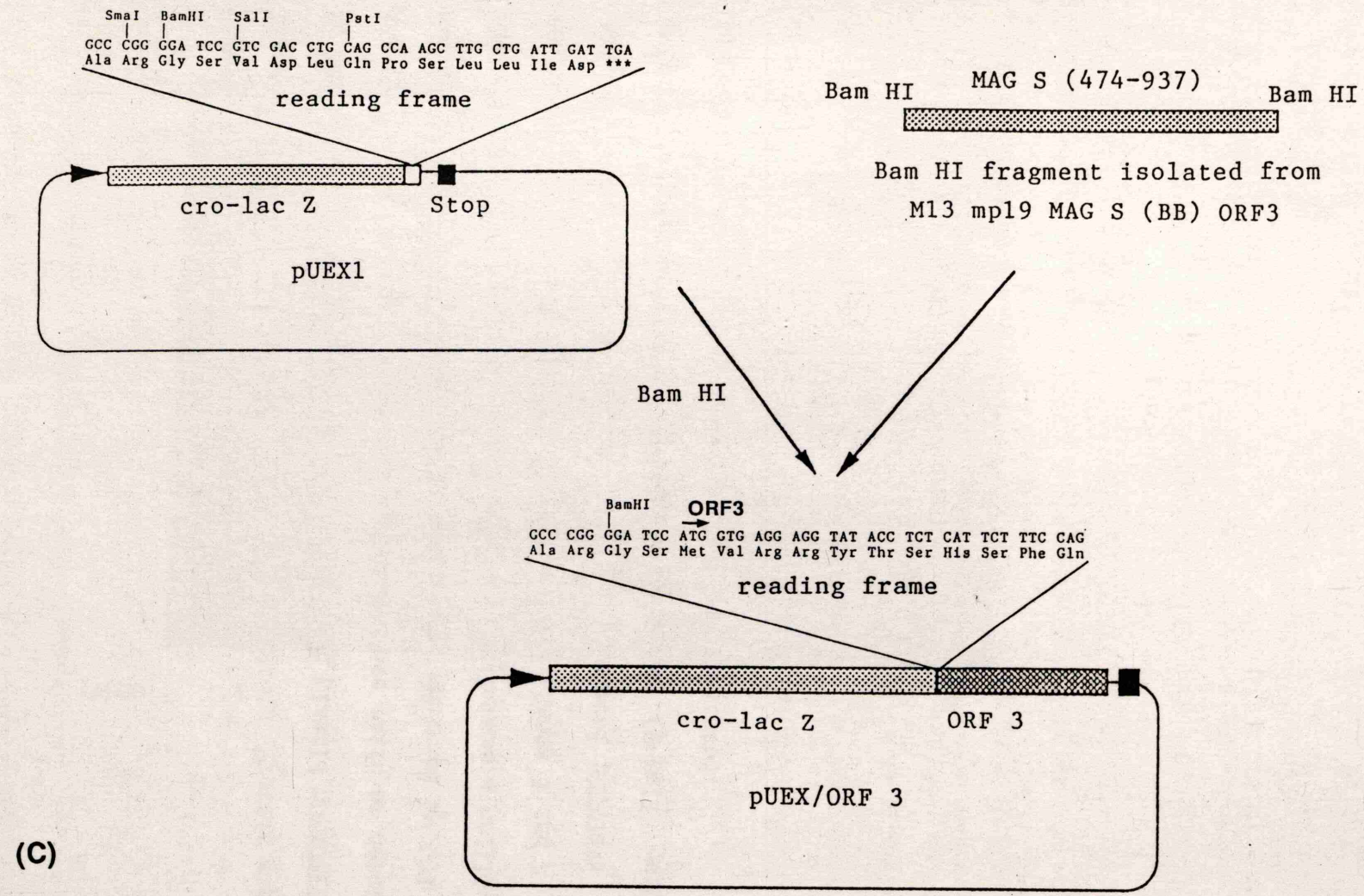
7B Expression of β-gal/ORF 3 Fusion Protein

Expresssion in bacterial cultures carrying recombinant plasmid pUEXORF 3 or wild type plasmid pUEX1 was induced and processed as described in Materials and Methods. Control uninduced cultures were similarly processed. In pUEX1 carrying cells induced expression resulted in the production of a major large M_r protein, presumably β-gal (Figure 17, Lane 1). In pUEXORF 3 cells induced expression resulted in the production of a protein with similar electrophoretic mobility as that produced in induced pUEX1 cells, and a second induced protein which migrated more slowly than β-gal, presumably β-gal/ORF 3 (Lane 3, 5). The

Figure 16.

Construction of bacterial expression plasmid pUEXORF3. Oligonucleotide site directed mutagenesis was employed to introduce a BamHI site immediately upstream of ORF3 on the template M13mp19MAGS(BB). Nucleotides 476-479 were changed from GCTG to ATCC to introduce a BamHI site immediately upstream of MAGS ORF3, shown in part A. This enabled the entire 75 codons of MAGS ORF3 to be isolated as a BamHI fragment (bases 474-937) and subcloned in frame into the bacterial expression vector pUEX1 to give pUEXORF3 (part B). The in frame cloning of MAGS ORF3 was verified by sequencing across the cloning junction between the beta galactosidase polycloning site and the start of MAGS ORF3 (part C).

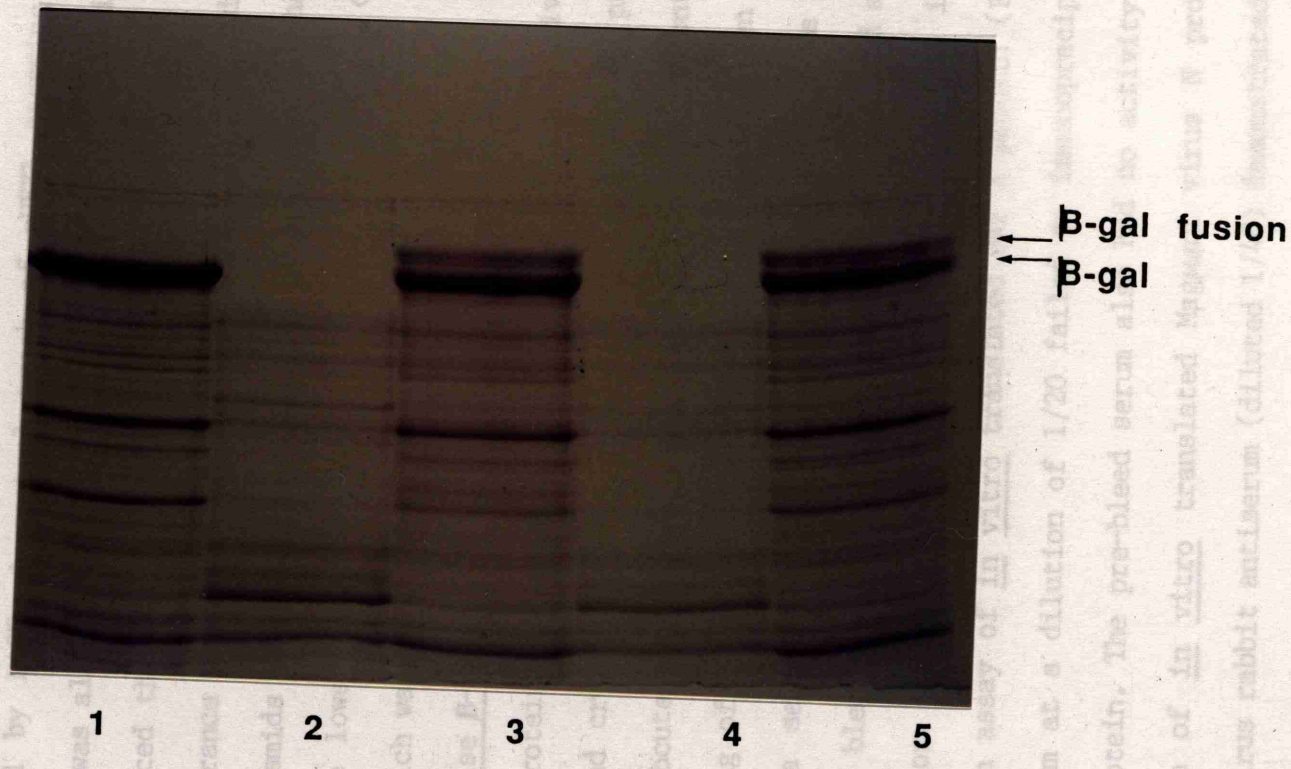

(a)
ORF3
M V R R
5'....A T A A C A T G G G C T G A T G G T G A G G A G G.... 3'
(467)
(492)
M V R R
5'....A T A A C A T G G A T C C A T G G T G A G G A G G .....3'
Bam HI
(b)
GATC
ORF3 initiation codon
Bam HI site
G T A C C T A G G


SmaI
BamHI
SalI
PstI
GCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG CTG ATT GAT TGA
Ala Arg Gly Ser Val Asp Leu Gln Pro Ser Leu Leu Ile Asp ***
reading frame
cro-lac Z
Stop
pUEX1
Bam HI
MAG S (474-937)
Bam HI
Bam HI fragment isolated from
M13 mp19 MAG S (BB) ORF3
Bam HI
BamHI
ORF3
GCC CGG GGA TCC ATG GTG AGG AGG TAT ACC TCT CAT TCT TTC CAG
Ala Arg Gly Ser Met Val Arg Arg Tyr Thr Ser His Ser Phe Gln
reading frame
cro-lac Z
ORF 3
pUEX/ORF 3

(C)

Figure 17.

Expression of beta-galactosidase/ORF3 fusion protein. Protein expression in bacterial cultures, carrying the recombinant plasmid pUEXORF3 or the control plasmid pUEX1, was induced by temperature shift as described in Materials and Methods. Bacterial proteins were analysed by 10% PAGE in the presence of SDS and the bands resolved by Coomassie brilliant blue staining. Lane 1, induced pUEX1 carrying cells. Lanes 3 and 5, induced pUEXORF3 carrying cells. Lanes 2 and 4, uninduced pUEXORF3 carrying cells. Induced proteins beta-galactosidase (β-gal) and beta-galactosidase fusion protein (β-gal/ORF3) are indicated.


β-gal fusion
β-gal
Lane 1 2 3 4 5

unexpected result of both proteins being produced in the same culture suggested that both pUEX1 and pUEXORF 3 were present in the same culture. To investigate this possibility a number of individual recombinant pUEXORF3 colonies were grown up, induced, and expressed proteins analysed by PAGE. A control track of pUEX1 carrying cells expressing β-gal was also analysed on the gel (Figure 18). Each induced recombinant produced the same two high molecular weight protein bands. Since the appearance of the two protein bands was not due to contaminating plasmids in the induced culture it was assumed that the appearance of the lower band was a result of the instability of the fusion protein which was degraded in the bacterial cell.

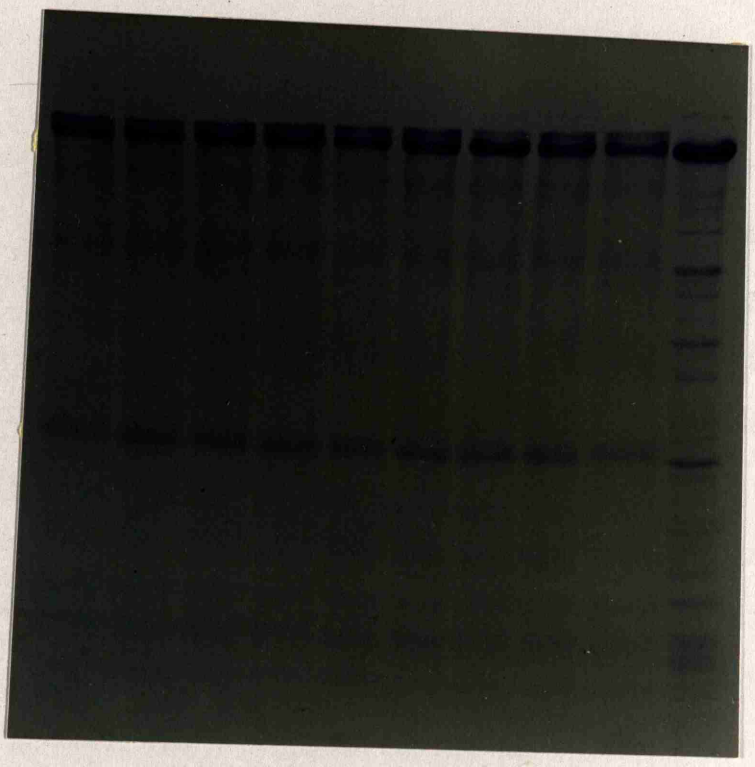
7C Attempts to raise β-gal/ORF 3 fusion protein antiserum

The fusion protein (upper band) was purified by preparative gel electrophoresis and crushed gel slices containing the isolated protein were inoculated subcutaneously into a rabbit. Each injection contained approximately 200μg of protein and booster injections were given every 10-14 days over a seven week period. Fifteen days after the final inoculation a test bleed was made. Serum from the test bleed and a pre-bleed, taken prior to the first inoculation, were used in an immunoprecipitation assay of *in vitro* translated ORF 3 protein (Figure 19). Test antiserum at a dilution of 1/20 failed to immunoprecipitate the 9.3K ORF 3 protein. The pre-bleed serum also had no activity. The immunoprecipitation of *in vitro* translated Maguari virus N protein, using Bunyamwera virus rabbit antiserum (diluted 1/40) demonstrated that the protocol had been successfully carried out.

The inability of the ORF3 fusion antiserum to recognise ORF 3 protein raised the question whether the serum contained activity to any part of

Figure 18.

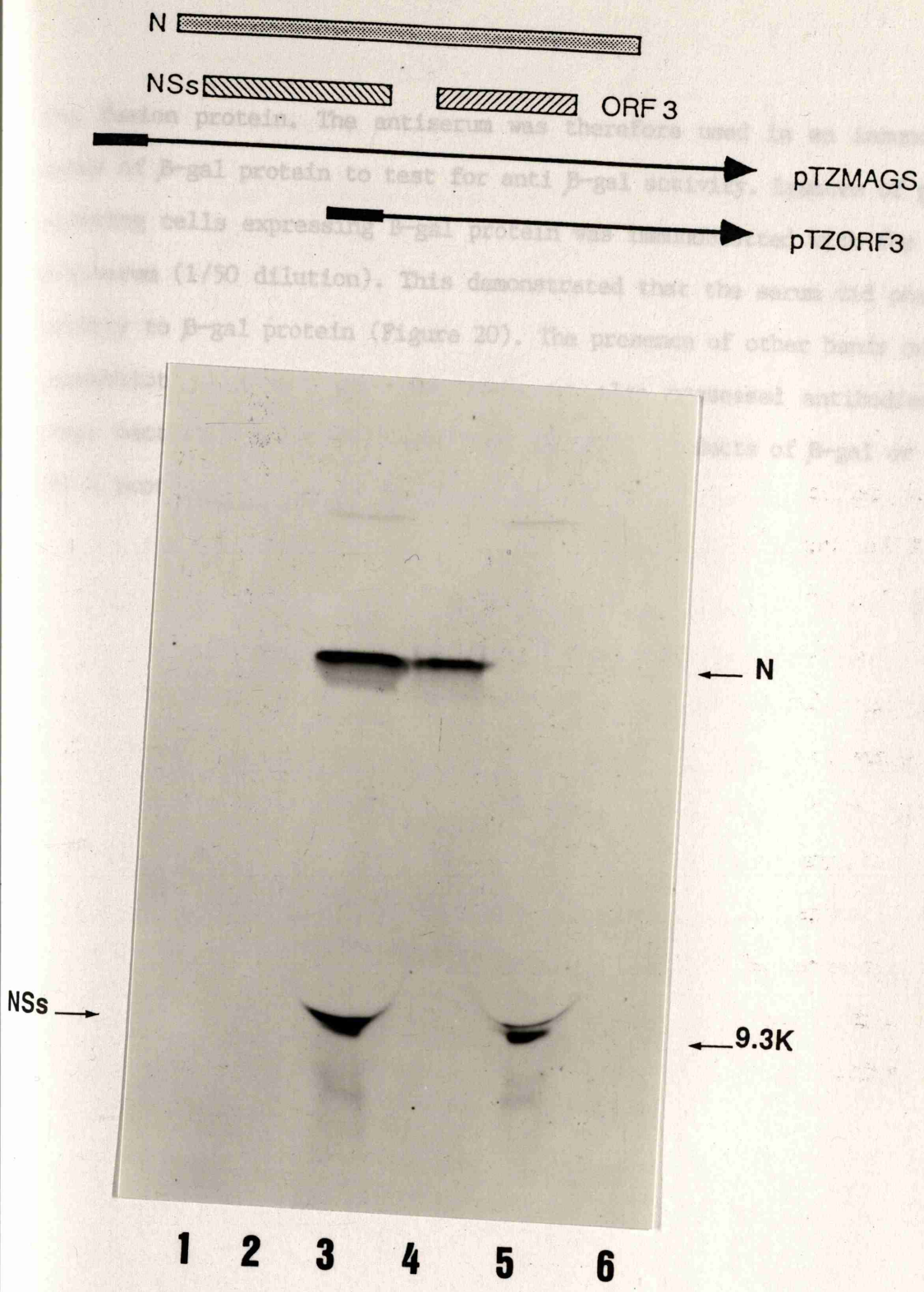
Expression of beta-galactosidase/ORF3 fusion protein in individual clones. Discrete colonies from pUEX ORF3 transformation were picked, grown up and protein expression induced as described in Materials and Methods. Proteins were analysed by SDS-PAGE 10% and bands resolved by Coomassie brilliant blue staining. Lanes 1 to 9; inducd clones carrying pUEXORF3. lane 10; induced pUEX1 carrying cells. Induced proteins beta-galactosidase (β-gal) and beta-galactosidase fusion protein (β-gal/ORF3) are indicated.



Lane 1 2 3 4 5 6 7 8 9 10

Figure 19.

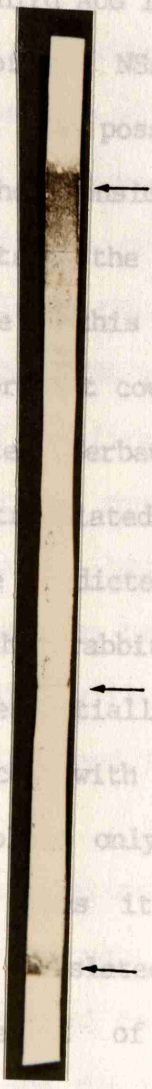
Immunoprecipitation of MAG N and ORF3 proteins. The upper part indicates the ORFs encoded 1n pTZMAGS and pTZORF3. RNA transcripts derived from pTZMAGS and pTZORF3 were translated in rabbit reticulocyte lysate. Lower part: radiolabelled protein products were immunoprecipated using the following: lane 2, lysate without RNA added reacted with polyclonal antiserum to Bunyamwera virus; lane 4, pTZMAG S RNA programmed lysate reacted with polyclonal antiserum to Bunyamwera virus; lane 6, pTZORF3 RNA programmed lysate reacted with beta-galactosidase/ORF3 fusion protein antiserum. Control lanes consist of total rabbit reticulocyte lysate programmed with: lane 3, pTZMAGS RNA; and lane 5, pTZORF3 RNA. Lane 1 is total lysate without RNA added. Proteins labelled with ^{35}S methionine were analysed by 20% PAGE in the presence of SDS.


N
NSs
ORF 3
pTZMAGS
pTZORF3
N
NSs
9.3K
1
2
3
4
5
6

the fusion protein. The antiserum was therefore used in an immunoblot assay of β-gal protein to test for anti β-gal activity. Lysates of pUEX1 carrying cells expressing B-gal protein was immunoblotted with the test antiserum (1/50 dilution). This demonstrated that the serum did possess activty to β-gal protein (Figure 20). The presence of other bands on the immunoblot indicated that the antiserum also possessed antibodies to other bacterial proteins or possibly breakdown products of β-gal or ORF 3 proteins.

Figure 20.

Immunoblot of beta-galactosidase using monospecific beta-galactosidase/ORF3 fusion protein antiserum. Bacterial cells carrying pUEX1 were induced by temperature shift as described in Materials and Methods. Proteins in the bacterial cell lysate were separated by electrophoresis (10% SDS-PAGE) and electroblotted onto a nitrocellulose membrane. The blot was incubated with the rabbit antiserum to beta-galactosidase/ORF3 fusion protein at a dilution of 1/50. Antibody protein reaction was detected by the use of secondary antibody conjugated with horseradish peroxidase as described in Materials and Methods. The location of the beta-galactosidase band on the blot is indicated (β-gal). Undetermined antibody protein interactions are also indicated by arrows.


β-gal

Discussion

An overview of translation of encoded MAG S proteins

The arrangement of overlapping reading frames for the N and NSs proteins in the Maguari virus S RNA is similar to other bunyaviruses (Elliott, 1990). In addition a third AUG initiated ORF is present in the same frame as, and downstream of, the NSs ORF. Another member of the Bunyamwera serogroup, GER virus, also possess this third ORF. The N and NSs proteins of these viruses show considerable amino acid homology in contrast to the predicted products of the third ORFs (Figure 3). On this basis the functional significance of this third protein is questionable. Additionally, the ORF3 protein product could not be detected in Maguari or GER virus infected cell lysates (Gerbaud, 1987). The Maguari virus S segment third ORF was however translated in cell free systems into a 9.3K protein. Indeed, all three predicted MAG S RNA encoded proteins were efficiently produced in either rabbit reticulocyte lysate or wheat germ extract programmed with an essentially complete Maguari virus S RNA transcript. This result is in accord with an earlier report by Bishop et al (1983) who were able to isolate only one species of S mRNA from snowshoe hare infected cells. Thus it would seem that the same bunyavirus mRNA species can be translated to give both the N and NSs proteins presumably as a result of alternative initiation of translation.

Efficient initiation of translation at an AUG codon in eukaryotic mRNAs has been shown to be dependent upon the surrounding nucleotide sequence. An optimal consensus sequence for efficient initiation of translation has been determined by Kozak (1981, 1984) to be 5'A/GCCAUGG with a purine (preferably A) at position -3 and a G residue at +4 (the A

of the AUG being designated +1). The -3 and +4 positions synergistically affect initiation of translation and substitution of a pyrimidine at one or both of these positions greatly affects initiation of translation (Kozak, 1986). A recent survey by Cavener and Ray (1991) of mRNA sequences reemphasised the importance of the -3 purine and to a lesser degree the +4 requirement of a G residue but demoted the importance of the consensus sequence CC at position -1 and -2.

Both the proximity to the 5' terminus and the flanking sequences, dictate which AUG codons initiate translation. The scanning model proposed by Kozak (1986) postulates that a 40S ribosomal subunit binds initially at the 5' end of the mRNA and migrates linearly until it reaches the first AUG codon. If the first AUG is in optimal context the 40S subunit migrates no further and couples with the 60S subunit and protein synthesis initiates. In the leaky scanning model, proposed to deal with bicistronic mRNAs, when the context of the first AUG codon is less favourable some 40S subunits will stop and initiate there while others will bypass the first site and initiate at the next AUG codon downstream (Kozak, 1986).

The sequence context of the initiation codons of N, NSs and ORF 3 are compared to the Kozak consensus sequence in Figure 21. The N and first AUG of NSs are in a similar suboptimal context whereas the second AUG of NSs has an optimal -3 position and the AUG of ORF 3 has an optimal +4 sequence. These suboptimal sequences tend to support the leaky scanning model of initiation of translation being applied to MAG S mRNA. It should be noted that for genes that produce two proteins from the one mRNA by leaky scanning, in general the AUG of the first reading frame is in suboptimal context and only three cases have been described in which

Figure 21.

Sequence contexts of the AUG initiation codons of the Maguari virus S segment products. The optimal sequence is that proposed by Kozak (1986). The NSs protein initiates from a tandem AUG, therefore the context of each AUG is noted.

	-3		+4
Optimal	5' A/GCC	AUG	G
N	UCA	AUG	A
NSs1	UCA	AUG	A
NSs2	AUG	AUG	U
ORF3	CUG	AUG	G

initiation at the first and second initiation codons occurs despite a favourable context around the first AUG: influenza B virus, where proximity of the second AUG codon to the first AUG allows leaky scanning (Williams and Lamb,1989); barley stripe mosaic virus (Petty and Jackson, 1990) and cowpea mosaic virus RNA-M (Holness et al, 1989), where the absence of secondary structure downstream from the first AUG enables leaky scanning.

An examination of the initiation codons for the N protein of other bunyaviruses reveals that they all lie in a suboptimal context. In terms of the Kozak scanning model they are therefore leaky codons which enable some scanning 40S initiation complexes to bypass N AUG and initiate translation downstream at NSs initiation codons (Figure 22). The leakiness of the bunyavirus N initiation codon compares dramatically with the N initiation codons of hanta-, nairo- and uuku- viruses which all possess optimal -3 and +4 sequences (Figure 22). These viruses do not encode a NSs protein in an overlapping ORF, and thus the consensus sequence is optimal to maximise intiation of translation of N protein. On the basis of these observations it would be interesting to progressively manipulate the -3 and +4 sequences surrounding MAG N initiation codon to determine the effect it would have on the synthesis of N and NSs proteins.

An interesting observation is the initiation of NSs translation at a tandem ACG on pTZMAGN RNA. The ATGATG to ACGACG change at the start of the NSs ORF was chosen as it did not change the amino acid coding sequence for N protein in the overlapping reading frame. It was assumed that initiation would not occur at the ACG because of the suboptimal sequence context. Translation of NSs did not occur in the wheat germ

Figure 22.

(a) Sequence context of the AUG initiation codon of the N protein of various bunyaviruses: Maguari (MAG), Elliott and McGregor, 1989; Bunyamwera (BUN), Elliott, 1989a; Batai (BAT), Elliott and McGregor, unpublished results; La Crosse (LAC), Akashi and Bishop, 1983; snowshoe hare (SSH), Bishop et al, 1982.

(b) Sequence context of the AUG initiation codon of the N protein of viruses from different genera: MAG and LAC bunyaviruses (Elliott and McGregor, 1989; Akashi and Bishop, 1983); Hantaan (HTN) hantavirus (Schmaljohn et al, 1986b); Dugbe (DUG) nairovirus (Ward et al, 1990); Rift Valley fever (RVF) phlebovirus (Giorgi et al, 1991); and Uukuniemi (UUK) plebovirus (Simons et al, 1990).

(a)

	-3		+4
Optimal	5' A/GCC	AUG	G
MAG N	UCA	AUG	A
BUN N	UUA	AUG	A
BAT N	CCG	AUG	A
LAC N	GUG	AUG	U
SSH N	GUG	AUG	U

(b)

	-3		+4
Optimal	5' A/GCC	AUG	G
MAG N	UCA	AUG	A
LAC N	GUG	AUG	U
HTN N	ACG	AUG	G
DUG N	AAG	AUG	G
RVF N	AUA	AUG	G
UUK N	AUC	AUG	G

extract but did occur in rabbit reticulocyte lysate at a low level. Naturally occurring ACG initiation codons are rare in eukaryotes but have been reported for adenovirus associated virus coat protein (Becerra et al, 1985) and the Sendai virus C' protein (Curan and Kolakofsky, 1988). In addition, expression in cell free systems of mouse dihydrofolate reductase mRNA (Peabody, 1987) and T7 mRNA (Anderson and Buzash-Pollert, 1985) in which AUG codons were altered to ACG codons have been described. In all these cases however the ACG codon was in a near optimal context unlike NSs initiation codons. It should be noted that the second ACG does have an optimal A nucleotide at -3 which may be sufficient for low level of initiation of translation from an ACG codon in rabbit reticulocyte lysate.

Nucleotide sequences capable of forming a hairpin loop downstream of an unfavourable AUG or non AUG initiation codon have been reported to enhance the the recognition of that codon (Kozak, 1989, 1990). Such structures are optimally placed approximately 14 nucleotides downstream of the initiation codon, the approximate distance between the leading edge of the ribosome and its AUG recognition motif. It is thought that the structure works by presenting a barrier to the scanning ribosome, slowing down the scanning process and thereby enabling the recognition of the unfavourable initiation codon (Kozak, 1990). Analysis of the nucleotide sequence failed to detect such hairpin structure downstream of NSs initiation codons and would in any case fail to account for the discrepancy between ACG initiation in the reticulocyte lysate and the inability for that initiation to occur in the wheat germ extract. The most logical conclusion would be that ACG codons are able to initiate translation even in suboptimal context in rabbit reticulocyte lysate

although at a much reduced level than AUG in a suboptimal context or ACG in an optimal context.

The T7 transcription system, when used in conjunction with a cell-free translation provides a very flexible technique with which to study protein translation of S segment encoded bunyavirus proteins. However these results may not truly reflect the situation in virus infected cells (Kozak, 1989) and so a transient expression system in intact cells would also be desirable to allow the expression of the virus encoded proteins in vivo. To this end pTZMAGS and pTZMAGNSs inserts have been subcloned into transient expression vectors to enable analysis of expression of MAG S encoded proteins in future experiments. Two expression systems have been employed: the p91023 expression vector in COS cells (Wong et al, 1985); and the pTF7.5 plasmid in conjunction with vaccinia virus expressing T7 polymerase in BHK cells (Fuerst et al, 1986).

The p91023 COS cell expression vector contains regulatory elements from SV40 and adenovirus (Wong et al, 1985). The recombinant plasmids p91023MAGS and p91023MAGNSs (containing the MAGS cDNA inserts from pTZMAGS and pTZMAGNSs respectively) were transfected into COS cells and radiolabelled cell lysates were prepared 48hr after transfection and protein samples analysed by SDS-PAGE. Only expression of the N protein was clearly identified in p91023MAGS infected cells whereas the NSs protein could not be identified in either p91023MAGS or p91023MAGNSs transfected cells (Elliott et al, 1989). Similar results were obtained in the transient expression of pTF7.5MAGS and pTF7.5MAGNSs recombinant plasmids in BHK cells (Page, Elliott and McGregor, unpublished results). In both transient expression systems the N protein was identified by

immunoprecipitation using MAG reference antibody. It has been demonstrated in this chapter that this antibody is unable to identify either the NSs or the ORF3 proteins expressed *in vitro* and a similar result is undoubtedly obtained *in vivo*. Therefore in order to monitor the expression of these proteins *in vivo* requires the availability of antisera to these proteins. It should be noted that the NSs and the ORF3 proteins could be translated *in vitro* from transcripts derived from pTF7.5MAGS (Figure 8) and pTF7.5MAGNS and therefore similar expression should be obtained *in vivo*.

Translation of the NSs from single AUG initiating ORFs

Alterations were made to the tandem AUG of the NSs ORF to allow initiation of translation of the NSs protein at either the first (NSs1) or second (NSs2) AUG. These changes did not affect the original context at the -3 and +4 positions: NSs1 AUG was completely unaltered and remained in suboptimal context; NSs2 AUG maintained an optimal purine at -3, but the original A nucleotide was substituted for a G residue. The experimental translation results demonstrate that NSs protein could be initiated from either the NSs1 or the NSs2 AUG codon.

The initiation signal on most eukaryotic mRNA is located between 20 and 100 bases from the 5' end (Kozak, 1987). Initiation codons which have been artificially positioned to lie within 20 bases from the 5' end of the mRNA have been shown to reduce the efficiency of translation (Sedman *et al*, 1990; van den Heuzel *et al*, 1989). The effect of the length of the 5' UTR on the translation of NSs from initiation codons NSs1 or NSs2 was investigated. Alteration of the upstream AUG of the N protein to GAA inhibited N protein expression and effectively created a

pTZNSs type RNA with an extended 5' UTR ranging from 10-13 bases in pTZNSs1 or pTZNSs2 to 61 or 64 bases in pTZMAGSΔNNSs1 or pTZMAGSΔNNSs2 respectively. The level of NSs protein translated from these RNA transcripts was equivalent to that obtained by pTZMAGSNSs1/2 and pTZNSs1/2. Therefore removal of the upstream N AUG or extension of the 5' UTR does not seem to affect initiation of NSs translation at either AUG. This contrasts with the translation of the 9.3K protein which would appear to be influenced by the presence of long 5'UTRs preceding a leaky AUG initiation codon (especially N AUG) both of which serve to maximise ORF 3 translation.

In terms of the scanning model proposed by Kozak the presence of a long 5' UTR allows the accumulation of 40S initiation complexes (Kozak, 1991), one of the essential requirements to maximise downstream translation of ORF 3. Accumulated complexes would in succession scan downstream until an AUG initiation codon was found. If that first AUG was suboptimal then only some of the complexes would initiate translation while others would progress further downstream, where the cycle was repeated. In the case of pTZMAGSNSs1 and pTZMAGSNSs2 RNAs the ORF 3 AUG is preceded by suboptimal N and NSs AUG codons whereas in pTZMAGSΔNNSs1/2 the ORF 3 AUG is preceeded by the suboptimal NSs AUG codon. Since the RNAs have similar 5'UTR it would be expected that the level of 9.3K protein translated would be higher from pTZMAGSΔNNSs1/2 RNAs than from pTZMAGSNSs1/2 RNAs simply because the complex has to progress through the NSs initiation site as opposed to both N and NSs initiation sites, as in pTZMAGSNSs1/2 RNA. Therefore proportionally the number of complexes reaching ORF3 should be greater. However, in reality

it would appear that the reverse occurs such that greater translation of ORF 3 occurs with pTZMAGSNSs1/2 RNA. The presence of an active N initiation codon must allow some initiation complexes to bypass N AUG at a higher proportion than would occur if N AUG is not present. Given the close proximity of the N and NSs initiation codons an intermittent exclusion of initiation of translation from NSs AUG codons may occur. Since the leading edge of a scanning ribosome to the internal AUG recognition covers approximately 19 nucleotides, the leading edge of a N AUG bound scanning ribosome would extend to the NSs initiation codons. Therefore a downstream scanning ribosome that bypassed the N AUG would be unable to initiate at NSs initiation codons if another scanning complex from the 5'UTR was to initiate at N AUG, thereby forcing the complex to initiate further downstream ie at ORF 3. Hence the same number of initiation complexes would get through to ORF 3 even if NSs initiation codons were active (as in pTZMAGS RNA) or inactive (as in PTZMAGN RNA) provided that the long 5'UTR and N AUG were present.

Failure to raise an antiserum to the 9.3K protein of MAG S ORF 3

The failure to detect Maguari virus S segment ORF 3 protein in infected cells was due to a high background of radiolabelled cellular proteins. Thus in order to be able to correctly identify this putative protein in infected cell lysate it was necessary to make an antiserum to this protein. The introduction of a BamHI site immediately upstream of ORF 3 on the MAGS cDNA template M13mp19MAGS(BB) allowed the subcloning of the entire MAGS ORF 3 region into the C-terminus of the β-gal gene at the BamHI site on plasmid pUEX1 and avoided the introduction of any bridging amino acids between the two open reading frames. When protein expression was induced by temperature shift two major products were synthesised: a protein which had a molecular weight of the β-gal protein and a protein of higher molecular weight than the β-gal protein. At first it was assumed that this result was due to a mixed population of bacterial cells carrying either pUEX1 (synthesising the lower band) or pUEXORF 3 (synthesising the upper band). However sequencing of the plasmid DNA did not show signs of a mixed population and analysis of expressed proteins under induced conditions from individual pUEXORF 3 colonies confirmed that pUEXORF 3 carrying cells were solely responsible for the production of the two bands.

The β-gal fusion protein expression system was designed to produce insoluble protein in the bacterial cell and thereby protect it from proteolytic attack. In this instance however some of the fusion protein was degraded back to the carrier protein either as a result of unstable folding of the ORF 3 part of the protein or the presence of a proteolytic cleavage site on the ORF 3 protein. Despite this handicap sufficient quantities of β-gal/ORF 3 protein were isolated from induced

bacteria for use as an immunogen. It would be interesting in a future experiment to examine β-gal/ORF 3 protein expression in protease deficient bacterial cells to determine if a higher yield of fusion protein could be obtained.

The test antiserum possessed activity to β-gal protein but could not immunoprecipitate ORF 3 protein synthesised in a cell free translation system. The failure in obtaining anti-ORF 3 activity may be because ORF 3 protein is poorly immunogenic as a result of its hydrophobic nature. The NSs protein of bunyaviruses is similarly hydrophobic in nature and an attempt at raising antiserum to Maguari virus NSs protein through its expression as a β-gal fusion protein was also unsuccessful (Elliott, RM and Page, K, unpublished results). The fusion protein technique has however been successfully applied in raising antisera to Bunyamwera virus L protein (Jin and Elliott, 1992). The size of the L protein prohibited the expression of the complete protein and therefore only fragments of the L protein were expressed. Those fragments expressed were more hydrophilic in nature than the amino acid sequences of Maguari virus NSs or ORF 3 proteins and consequently the likely reason for their immunogenicity. It should be noted neither the L nor NSs β-gal fusion proteins were unstable when expressed ie the induced band of equivalent M_r to the fusion protein was the only induced band detected. Thus instability cannot be a basis for predicting the success of the fusion protein in raising an effective antiserum.

Since any future investigations of expression of the MAG S ORF 3 protein in virus infected cells or the transient expression of the protein via recombinant eukaryotic expression vectors requires the availability of an effective anti-ORF 3 protein serum it may well be

worth exploring other strategies to obtain this antiserum. Incorporation of the ORF 3 cDNA into a recombinant baculovirus and raising antiserum to virus infected cells expressing ORF 3 protein is a possibility and the availability of the ORF as a BamHI fragment would enable its straightforward subcloning into the baculovirus shuttle vector pAcYM1 (Matsuura et al, 1987). The baculovirus expression system has been successfully used in raising antisera to the N and NSs proteins of Punta Toro phlebovirus (Overton et al, 1987) and the NSs protein of TSWV (Kormelink et al, 1991). The major disadvantage with this technique was that the isolation of the recombinant baculovirus was up until relatively often a slow and laborious process. However, with improved selection techniques (Kitts et al, 1990; Peakman et al, 1992; Vialard et al, 1990) this is no longer the case. Alternatively one could use synthetic peptides corresponding to possible epitopes predicted through a hydrophobicity plot of the protein, as immunogens. These strategies are worth pursuing.

Chapter 4

Construction of full length cDNAs of Maguari virus and Bunyamwera virus S segments under T7 promoter control.

1 Summary.

In order to study effectively the interaction of bunyavirus nucleocapsid protein with S segment derived RNA, a source from which specifically defined full length or abbreviated forms of S RNA could be produced had to be established. This chapter describes the assembly of full length cDNAs of the S segments of Bunyamwera (BUN) and Maguari (MAG) viruses under T7 promoter control which enables run-off T7 transcripts to be generated from these templates. In addition the creation of subgenomic BUN S cDNA templates under T7 promoter control is described.

2 Introduction.

Bunyamwera virus and Maguari virus are two closely related bunyaviruses and the complete nucleotide sequence of the S segment from each virus has been determined (Elliott, 1989a). The Bunyamwera virus S segment, like that of other bunyaviruses, encodes the N and NSs proteins in two overlapping reading frames. The N ORF is 233 codons in length and extends from an AUG at position 86-88 to an UAA at position 785-787. The NSs ORF is 101 codons in length and extends from a tandem AUG at position 105-110 to a UAA at position 408-410 (Figure 1). As with the Maguari S segment it is only the second AUG initiation codon of the NSs ORF that has a favourable context for translation initiation. An alignment of the predicted amino acid sequence of both Maguari and Bunyamwera nucleocapsid proteins demonstrates a 90% conservation of sequence (Figure 2). Amino acid mismatches are spread throughout the protein sequences and approximately half of these mismatches are

Figure 1.

The nucleotide sequence of the Bunyamwera virus S segment (Elliott, 1989a). The sequence is presented in the complementary sense and in the DNA form. The initiation codons for the N and NSs proteins are marked.

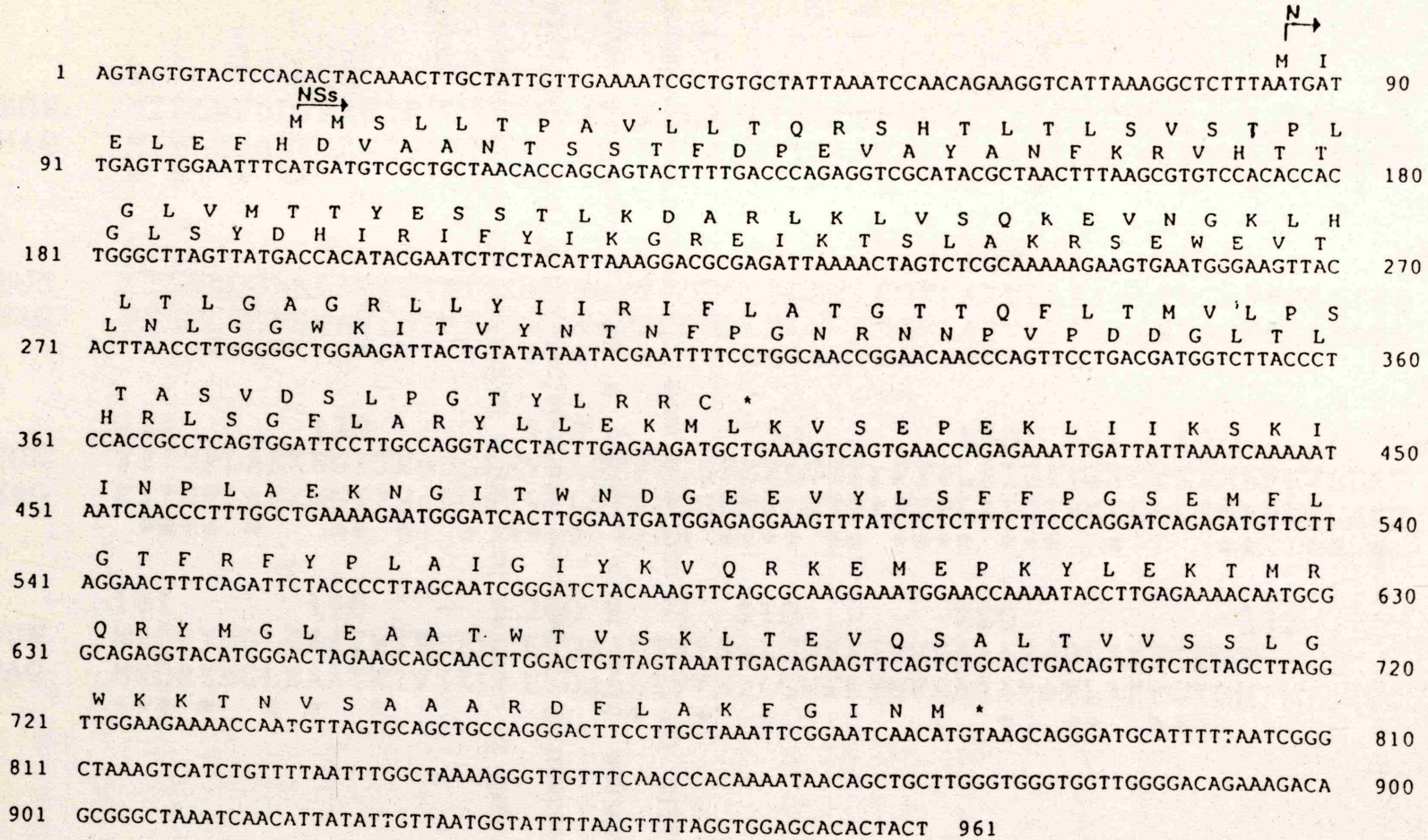

N
M I
1 AGTAGTGTACTCCACACTACAAACTTGCTATTGTTGAAAATCGCTGTGCTATTAAATCCAACAGAAGGTCATTAAAGGCTCTTTAATGAT 90
NSs
M M S L L T P A V L L T Q R S H T L T L S V S T P L
E L E F H D V A A N T S S T F D P E V A Y A N F K R V H T T
91 TGAGTTGGAATTTCATGATGTCGCTGCTAACACCAGCAGTACTTTTGACCCAGAGGTCGCATACGCTAACTTTAAGCGTGTCCACACCAC 180
G L V M T T Y E S S T L K D A R L K L V S Q K E V N G K L H
G L S Y D H I R I F Y I K G R E I K T S L A K R S E W E V T
181 TGGGCTTAGTTATGACCACATACGAATCTTCTACATTAAAGGACGCGAGATTAAAACTAGTCTCGCAAAAAGAAGTGAATGGGAAGTTAC 270
L T L G A G R L L Y I I R I F L A T G T T Q F L T M V L P S
L N L G G W K I T V Y N T N F P G N R N N P V P D D G L T L
271 ACTTAACCTTGGGGGCTGGAAGATTACTGTATATAATACGAATTTTCCTGGCAACCGGAACAACCCAGTTCCTGACGATGGTCTTACCCT 360
T A S V D S L P G T Y L R R C *
H R L S G F L A R Y L L E K M L K V S E P E K L I I K S K I
361 CCACCGCCTCAGTGGATTCCTTGCCAGGTACCTACTTGAGAAGATGCTGAAAGTCAGTGAACCAGAGAAATTGATTATTAAATCAAAAAT 450
I N P L A E K N G I T W N D G E E V Y L S F F P G S E M F L
451 AATCAACCCTTTGGCTGAAAAGAATGGGATCACTTGGAATGATGGAGAGGAAGTTTATCTCTCTTTCTTCCCAGGATCAGAGATGTTCTT 540
G T F R F Y P L A I G I Y K V Q R K E M E P K Y L E K T M R
541 AGGAACTTTCAGATTCTACCCCTTAGCAATCGGGATCTACAAAGTTCAGCGCAAGGAAATGGAACCAAAATACCTTGAGAAAACAATGCG 630
Q R Y M G L E A A T W T V S K L T E V Q S A L T V V S S L G
631 GCAGAGGTACATGGGACTAGAAGCAGCAACTTGGACTGTTAGTAAATTGACAGAAGTTCAGTCTGCACTGACAGTTGTCTCTAGCTTAGG 720
W K K T N V S A A A R D F L A K F G I N M *
721 TTGGAAGAAAACCAATGTTAGTGCAGCTGCCAGGGACTTCCTTGCTAAATTCGGAATCAACATGTAAGCAGGGATGCATTTTTAATCGGG 810
811 CTAAAGTCATCTGTTTTAATTTGGCTAAAAGGGTTGTTTCAACCCACAAAATAACAGCTGCTTGGGTGGGTGGTTGGGGACAGAAAGACA 900
901 GCGGGCTAAATCAACATTATATTGTTAATGGTATTTTAAGTTTTAGGTGGAGCACACTACT 961

Figure 2.

A comparison of the predicted amino acid sequence of Maguari (MAG) and Bunyamwera (BUN) virus nucleocapsid proteins (* indicates those amino acids conserved in the N protein amino acid sequence of other bunyaviruses, information taken from Dunn et al, 1993; Elliott, 1989a).

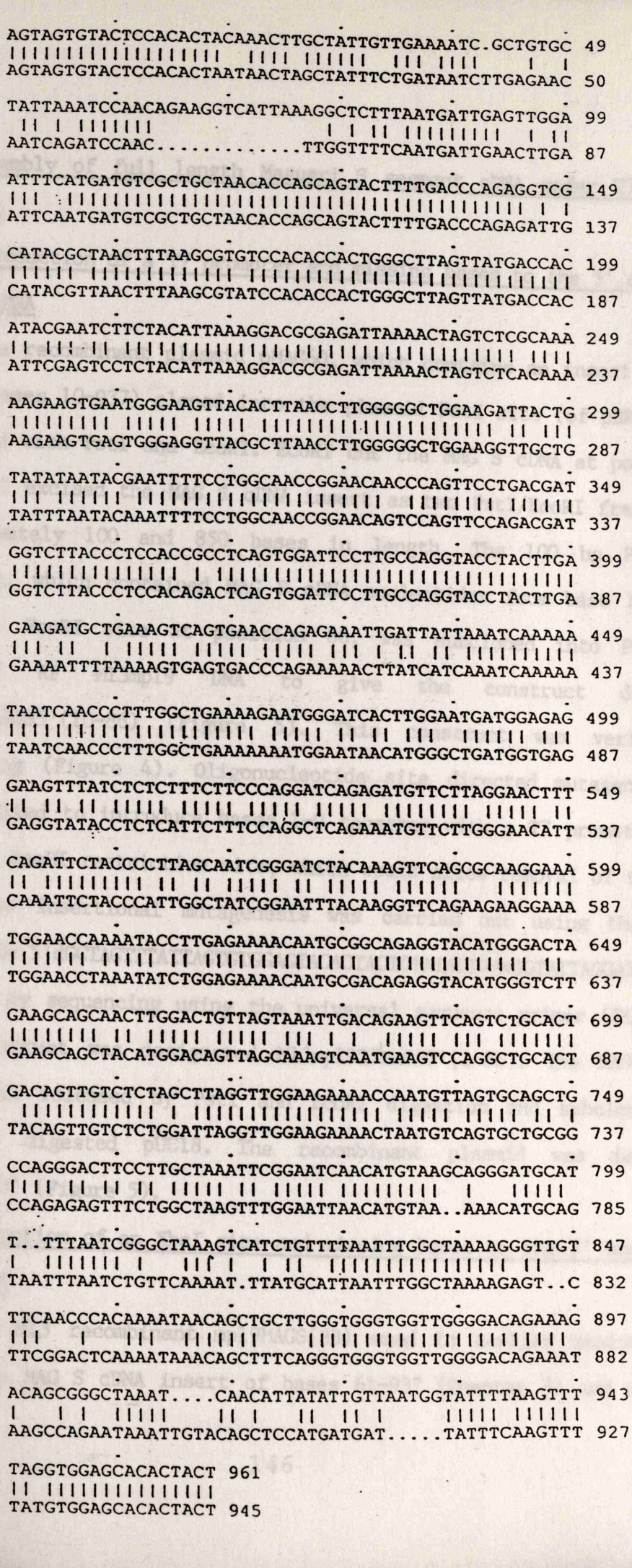
```
          1         10        20        30        40        50        60
BUN   MIELEFhDVAANTSSTFDPEvAYaNFKRvHTTGLSYDHIRifYIKGREIKTSLaKRSEWE
MAG   MIELEFnDVAANTSSTFDPEiAYvNFKRiHTTGLSYDHIRv1YIKGREIKTSLtKRSEWE
      * *   * **         * **  *  *           *  **  *       *  *    *

          61        70        80        90        100       110       120
BUN   VTLNLGGWKitVyNTNFPGNRNnPVPDDGLTLHRLSGFLARYLLEKmLKVSePEKLIIKS
MAG   VTLNLGGWKvaVfNTNFPGNRNsPVPDDGLTLHRLSGFLARYLLEKiLKVSdPEKLIIKS
          ** *   *     ** ***  *     ** ** ** **** **

          121       130       140       150       160       170       180
BUN   KIINPLAEKNGITWnDGEEVYLSFFPGSEMFLGTFrFYPLAIGIYKVQrKEMEPKYLEKT
MAG   KIINPLAEKNGITWaDGEEVYLSFFPGSEMFLGTFkFYPLAIGIYKVQkKEMEPKYLEKT
       **** *   ** *  * **** **** **** ** **** ***  *     **  ** *

          181       190       200       210       220       233
BUN   MRQRYMGLEAATWTVSKLtEVQsALTVVSsLGWKKTNVSAAARdFLAKFGINM
MAG   MRQRYMGLEAATWTVSKLnEVQaALTVVSgLGWKKTNVSAAAReFLAKFGINM
      *** *  * *  *         *   *  *  * *      *  *  **  ***
```

conservative changes. Unlike the Maguari virus S segment the Bunyamwera S segment does not encode a third ORF. Although the BUN S segment is slightly longer than the MAG S segment (961 versus 945 bases respectively) a comparison of the S segment sequences of both viruses reveals a 90% level of homology (Figure 3). It was assumed that because of the high degree of homology between the S segment sequences and also between the encoded N proteins both S segments would be compatible in proposed S RNA segment-nucleocapsid protein interaction studies.

Full length cDNAs to either Bunyamwera or Maguari S segments were unavailable. The longest available cDNAs (MAG S, bases 14-937; BUN S , bases 11-894) were flanked at the 5' and 3' ends by G/C tails as a result of the original cloning procedure of the cDNAs into pBR322. Thus it was necessary to remove these G/C tails and insert the missing nucleotides to convert these cDNAs to full length. For these full length cDNAs to serve as templates for S RNA synthesis a T7 RNA polymerase promoter was introduced upstream of the S cDNA sequence and an XbaI restriction site was introduced immediately downstream of the S cDNA sequence to define the 3' end of DNA template for T7 run-off S transcripts to be generated. The RNA transcripts would be equivalent to full-length anti-genomic (positive sense) RNA. The full-length BUN S cDNA was further manipulated to provide other T7 transcription templates.

Figure 3.

Comparison of the nucleotide sequences of Bunyamwera (BUN) and Maguari (MAG) virus S segments. The sequences (BUN S top; MAG S bottom) are presented in the complementary sense and in DNA form. Comparison was carried out using the GAP programme (Devereux et al, 1984)



3. Assembly of full length Maguari S segment cDNA under T7 promoter control

3A Introduction of a T7 promoter and missing bases to the 5' end of the MAG S cDNA

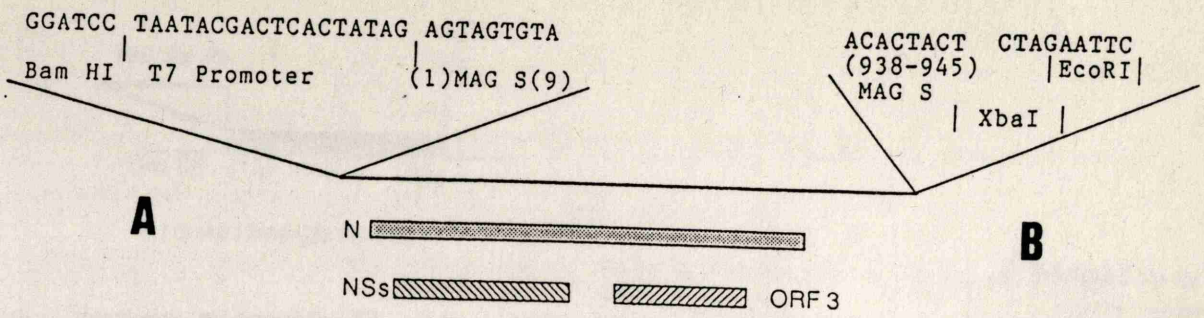
The recombinant plasmid pMAG60, which contains an insert of MAG S cDNA (bases 10-937) cloned into the plasmid PstI site (of pBR322), was digested with PstI and EcoRI. EcoRI cut the MAG S cDNA at position 86 and PstI excised the MAG S cDNA insert as two PstI/EcoRI fragments of approximately 100 and 850 bases in length. The 100 bp PstI/EcoRI fragment, which contained MAG S cDNA nucleotides from base 10 to the internal EcoRI site at position 86, was subcloned into PstI/EcoRI digested RF M13mp19 DNA to give the construct designated M13mp19MAGS5'(10-86) (Figure 5). This construct was verified by sequencing (Figure 4). Oligonucleotide site directed mutagenesis was then applied to introduce bases 1-9 together with a T7 promoter and a flanking BamHI restriction site, which would allow removal of the 5'G/C tail. The insertional mutagenesis was carried out using the oligonucleotide 5'dGCTAGTTTATTAGTGTGGAGTACACTACTCTATAGTGAGCGTATTAGGAT$(C)_8$ and verified by sequencing using the universal sequence primer (Figure 4). This construct was designated M13mp19T7MAGS5'(1-86). The MAG S cDNA insert was released by PstI and EcoRI digestion and subcloned into similarly digested pUC18. The recombinant plasmid was designated pUCT7MAGS5' (Figure 5).

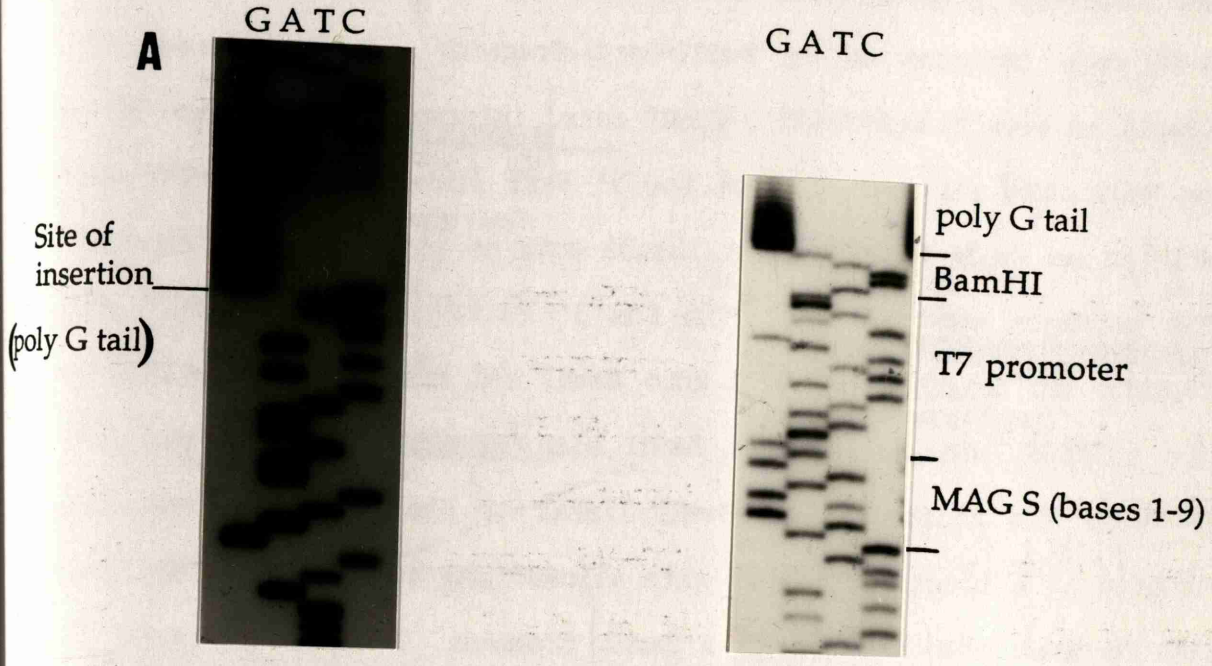
3B Introduction of an XbaI site and missing bases to the 3' end of the MAG S cDNA

The M13 recombinant mp19MAGS(BB) used in earlier manipulations contains a MAG S cDNA insert of bases 61-937 (Chapter 3) and was used

Figure 4.

Construction of full length MAG S cDNA under T7 promoter control. Top part of the figure shows sequences introduced at the 5' and 3' ends of MAG S cDNA clone pMAG60. (A) Missing MAG S bases 1-9 together with a T7 promoter and a BamHI site were introduced at the 5' end of the MAG S cDNA. (B) Missing MAG S bases 938-945 together with XbaI and EcoRI restriction sites were introduced at the 3' end of the MAGS cDNA. Insertion of the above sequences by site directed mutagenesis was verified by dideoxy sequencing as shown in lower figures. (A) 5' insertion (B) 3' insertion.


GGATCC TAATACGACTCACTATAG AGTAGTGTA
Bam HI
T7 Promoter
(1)MAG S(9)
ACACTACT CTAGAATTC
(938-945)
MAG S
EcoRI
XbaI
A
B
N
NSs
ORF 3


A
GATC
Site of insertion
(poly G tail)
GATC
poly G tail
BamHI
T7 promoter
MAG S (bases 1-9)

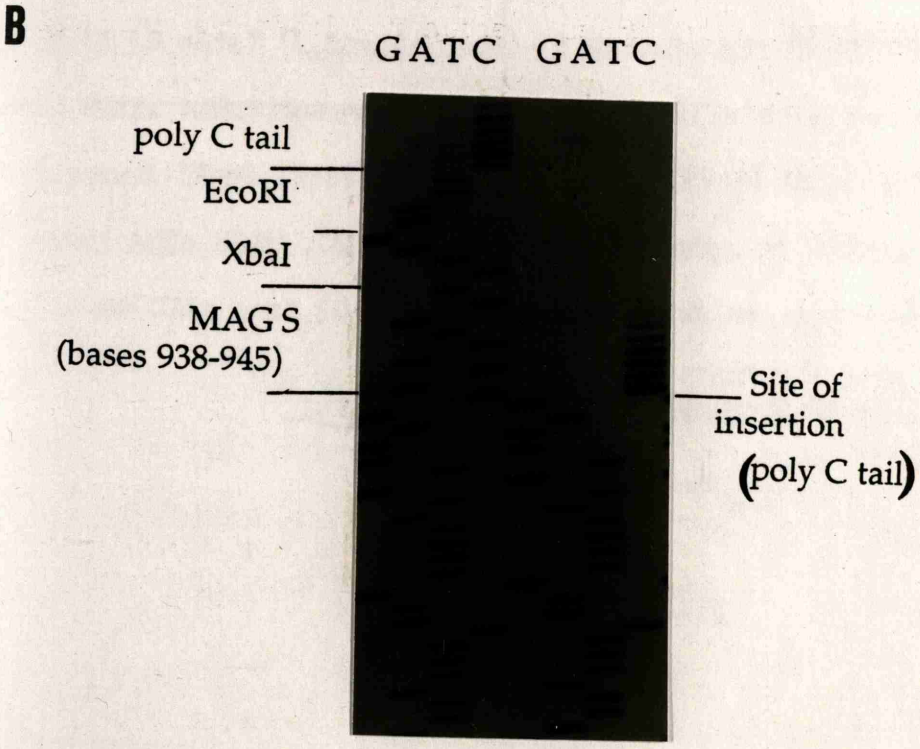
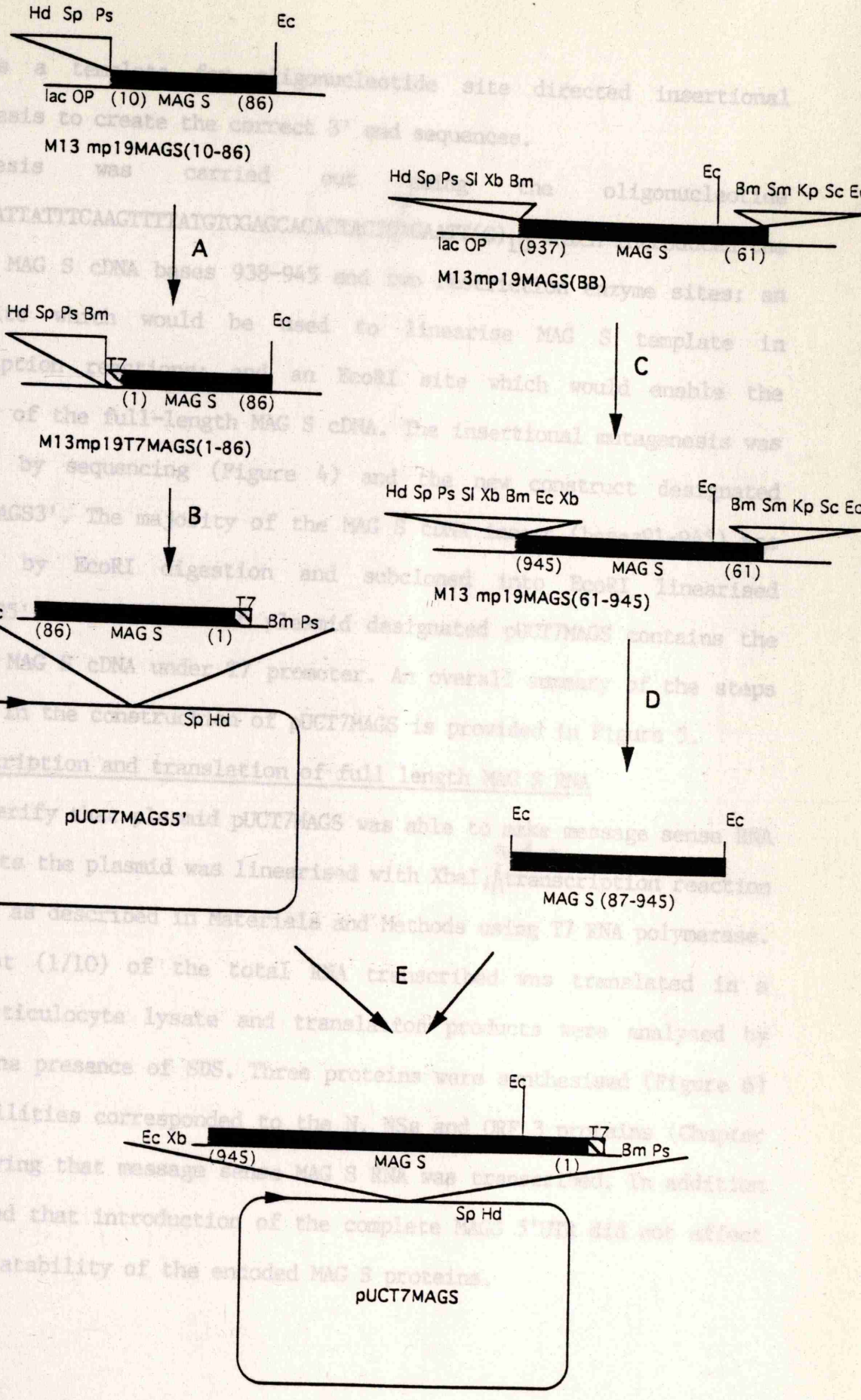

B
GATC GATC
poly C tail
EcoRI
XbaI
MAG S
(bases 938-945)
Site of insertion
(poly C tail)

Figure 5.

Construction of full length MAGS cDNA under T7 promoter control. MAGS cDNA fragments were isolated from pMAG60 and subcloned into M13. Bases 10-86 were isolated as an EcoRI-PstI fragment and subcloned into M13mp19 to give M13mp19MAGS5'. EcoRI cuts internally at position 86 on the MAGS cDNA and PstI cuts in the 5' PstI linker. Bases 61-937 were isolated as Sau3A-PstI fragment. Sau3A cuts at position 61 on the MAGS cDNA sequence and PstI cuts in the 3' PstI linker. This MAGS cDNA fragment was originally cloned into BamHI and PstI digested pTZ18U to give pTZMAGS, which created a BamHI site at the 5' end of the cDNA sequence. The addition of a BamHI linker to the 3' end of the cDNA sequence at a blunt ended PstI site allowed the subcloning of the MAGS cDNA sequence into M13mp19 as a BamHI fragment. The M13 construct was designated as M13mp19MAGS(BB). Oligonucleotide site directed insertional mutagenesis introduced the missing 5' (step A) and 3' (step C) MAGS cDNA sequences together with a T7 promoter and two restriction sites (BamHI and XbaI). MAGS5' cDNA (1-86) was isolated as a PstI, EcoRI fragment and subcloned into pUC18 to give pUCT7MAGS5' (step B). MAGS cDNA bases 87-945 were subcloned as an EcoRI fragment (step D) into pUCT7MAGS5' cut with EcoRI to give pUCT7MAGS (step E).


Hd Sp Ps
Ec
lac OP (10) MAG S (86)
M13 mp19MAGS(10-86)
A
Hd Sp Ps Bm
Ec
T7
(1) MAG S (86)
M13mp19T7MAGS(1-86)
B
Ec
T7
(86) MAG S (1)
Bm Ps
Sp Hd
pUCT7MAGS5'
Hd Sp Ps Sl Xb Bm
Ec
Bm Sm Kp Sc Ec
lac OP (937) MAG S (61)
M13mp19MAGS(BB)
C
Hd Sp Ps Sl Xb Bm Ec Xb
Ec
Bm Sm Kp Sc Ec
(945) MAG S (61)
M13 mp19MAGS(61-945)
D
Ec
Ec
MAG S (87-945)
E
Ec
Ec Xb
T7
(945) MAG S (1)
Bm Ps
Sp Hd
pUCT7MAGS

here as a template for oligonucleotide site directed insertional mutagenesis to create the correct 3' end sequences.

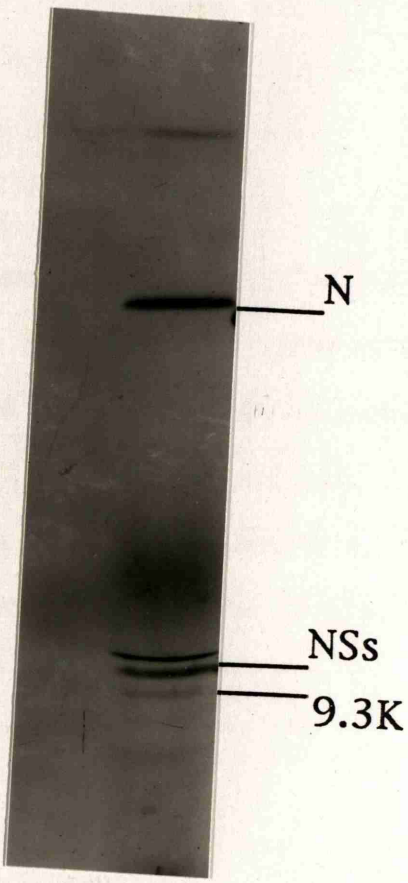
Mutagenesis was carried out using the oligonucleotide 5'dGATGATTATTTCAAGTTTTATGTGGAGCACACTACTCAGAATT$(C)_{10}$ which introduced the missing MAG S cDNA bases 938-945 and two restriction enzyme sites: an XbaI site which would be used to linearise MAG S template in transcription reactions; and an EcoRI site which would enable the assembly of the full-length MAG S cDNA. The insertional mutagenesis was verified by sequencing (Figure 4) and the new construct designated M13mp19MAGS3'. The majority of the MAG S cDNA insert (bases91-945) was released by EcoRI digestion and subcloned into EcoRI linearised pUCT7MAGS5'. The recombinant plasmid designated pUCT7MAGS contains the complete MAG S cDNA under T7 promoter. An overall summary of the steps involved in the construction of pUCT7MAGS is provided in Figure 5.

3C Transcription and translation of full length MAG S RNA

To verify that plasmid pUCT7MAGS was able to make message sense RNA transcripts the plasmid was linearised with XbaI, and a transcription reaction performed as described in Materials and Methods using T7 RNA polymerase. An aliquot (1/10) of the total RNA transcribed was translated in a rabbit reticulocyte lysate and translation products were analysed by PAGE in the presence of SDS. Three proteins were synthesised (Figure 6) whose mobilities corresponded to the N, NSs and ORF 3 proteins (Chapter 3), verifying that message sense MAG S RNA was transcribed. In addition this showed that introduction of the complete MAGS 5'UTR did not affect the translatability of the encoded MAG S proteins.

Figure 6.

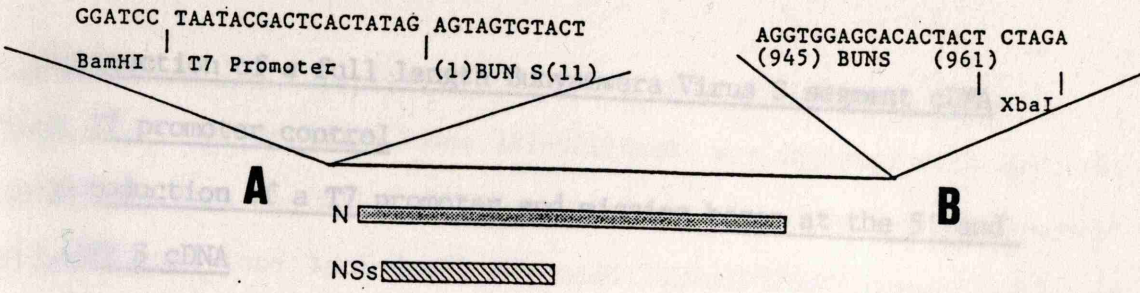
Cell-free translation of pUCT7MAGS RNA. An aliquot of the RNA transcrbed from XbaI linearised pUCT7MAGS was translated in a rabbit reticulocyte lysate. Translated proteins, labelled with ^{35}S methionine, were analysed by SDS-PAGE 18% gel. Lane (2) pUCT7MAGS RNA programmed lysate and lane (1) control lysate without RNA added.

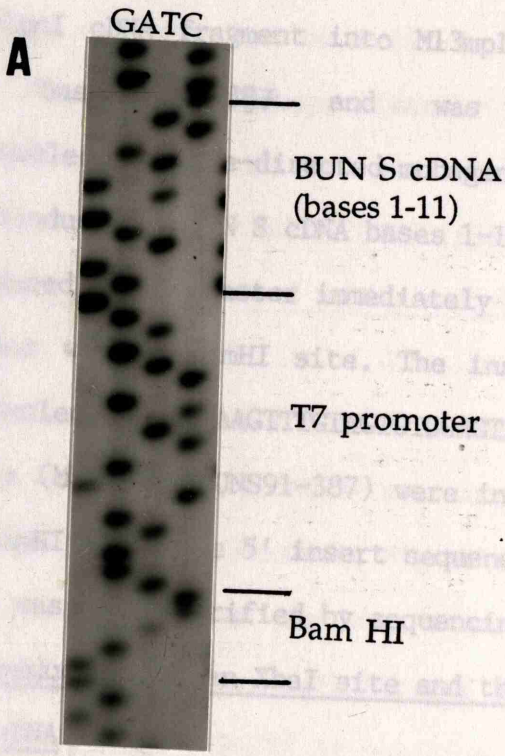


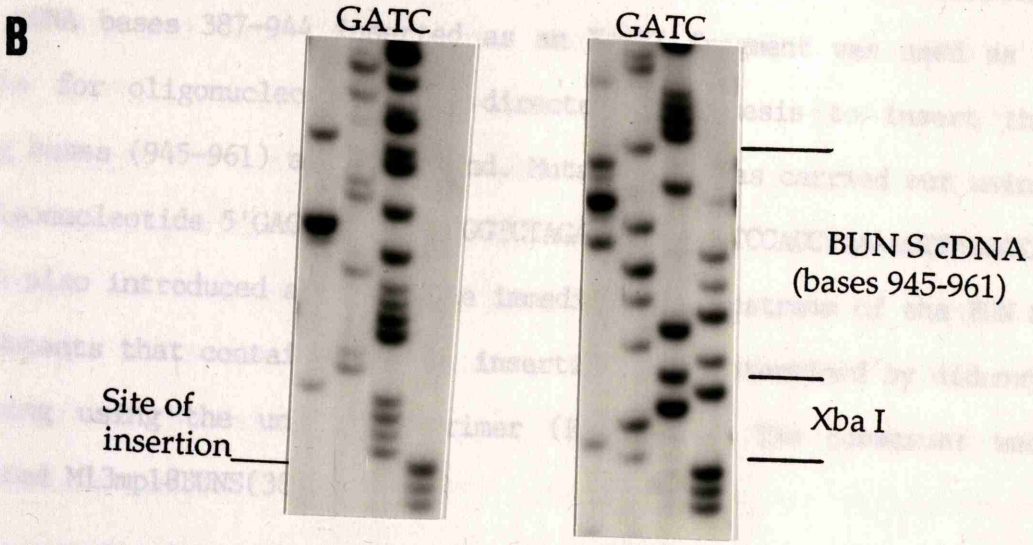
Lane 1 2

Figure 7.

(Top part) Oligonucleotide site directed insertional mutagenesis at the 5' and 3' ends of BUNS cDNA. (A) Insertion of missing 5' bases (1-11) together with a T7 promoter sequence and a BamHI site at the 5' end of the BUN S cDNA. (B) Insertion of missing 3' bases (945-961) together with an XbaI site at the 3' end of the BUN S cDNA. (Lower part) Dideoxy sequencing used to verify 5' and 3' insertions of BUNS cDNA. (A) 5' end (B) 3' end.


GGATCC TAATACGACTCACTATAG AGTAGTGTACT
BamHI T7 Promoter (1)BUN S(11)
AGGTGGAGCACACTACT CTAGA
(945) BUNS (961)
XbaI
A
B
N
NSs


A
GATC
BUN S cDNA
(bases 1-11)
T7 promoter
Bam HI


B
GATC
GATC
BUN S cDNA
(bases 945-961)
Site of
insertion
Xba I

4 Construction of a full length Bunyamwera Virus S segment cDNA under T7 promoter control

4A Introduction of a T7 promoter and missing bases at the 5' end of a BUN S cDNA

Part of a BUN S clone, pBUNS14 (Elliott, 1989a) was subcloned as a PstI-KpnI cDNA fragment into M13mp19. This construct contained BUN S cDNA bases 12-387 and was designated M13mp19BUNS(12-387). Oligonucleotide site-directed mutagenesis was performed on this template to introduce the BUN S cDNA bases 1-11 at the 5' end. The insertion also introduced a T7 promoter immediately upstream of the BUN S cDNA sequence together with a BamHI site. The insertion was carried out using the oligonucleotide 5'dAAGTTTGTAGTGTGGAGTACACTACTCTATAGTGAGTCGTATTAGGAT$(C)_8$. Mutants (M13mp19T7BUNS91-387) were initially identified by the presence of a BamHI site. The 5' insert sequence on the assembled full length BUN S cDNA was later verified by sequencing (Figure 7).

4B Introduction of an XbaI site and the missing bases at the 3' end of a BUN S cDNA

An M13 construct designated M13mp18BUNS309(EcoRI), which contained BUN S cDNA bases 387-944 inserted as an EcoRI fragment was used as a template for oligonucleotide site directed mutagenesis to insert the missing bases (945-961) at the 3' end. Mutagenesis was carried out using the oligonucleotide 5'GAGCTCGAATTCGGGTCTAGAGTAGTGTGCTCCACCTAAAACTTAAAATA , which also introduced an XbaI site immediately downstream of the BUN S cDNA. Mutants that contained the 3' insertion were determined by dideoxy sequencing using the universal primer (Figure 7). The construct was designated M13mp18BUNS(387-961).

4C Assembly of the full length BUN S cDNA under T7 promoter control

RF DNA of M13mp18BUNS(bases 387-961)EcoRI was digested with KpnI and EcoRI to release the BUN S cDNA insert. The isolated KpnI-EcoRI fragment was subcloned into KpnI-EcoRI digested M13mp19T7BUNS(bases 1-387) to give the full length BUN S cDNA construct mp19T7BUNS (Figure 8). The construct was verified by EcoRI-BamHI double digests of RF DNA which released an insert of approximately 1kb. The BUN S cDNA was removed as an EcoRI-BamHI fragment and was subcloned into similarly digested pUC9 to give the construct pUC9T7BUNS (Figure 8).

4D Alteration of BUN S cDNA sequence to consensus virus wild type sequence

Differences between the sequences of cDNA clones pBUNS14 and clones pBUN308, pBUN309 and pBUN3/59 were reported at positions 378 and 379 (Elliott, 1989a). As shown in Figure 9 these differences alter the amino acid coding sequences for both N and NSs proteins. These differences were resolved by primer extension of an oligonucleotide complementary to virion RNA (bases 269-288 of positive sense RNA) to obtain the consensus sequence of the BUN S RNA population. The sequence obtained was the same as that obtained from cDNA clones pBUN308, pBUN309 and pBUN3/59 (Elliott, 1989a). The pBUNS14 cDNA sequence, which was used to construct the full length BUN S cDNA, was therefore assumed not to be consistent with the majority wild type viral population. Thus it was necessary to change these nucleotides on the full length BUN S cDNA to the consensus sequence. The BUN S cDNA was initially subcloned as a XbaI-PstI fragment from pUC9T7BUNS into similarly digested RF M13mp18 to give the construct M13mp18T7BUNS(XbaI,PstI) (Figure 10). This template was subjected to oligonucleotide site directed mutagenesis using the oligonucleotide

Figure 8.

Strategy to construct full length BUNS cDNA under T7 promoter control. Insertional mutagenesis of 5' and 3' ends of the BUNS cDNA were carried out on seperate templates, M13mp19BUNS(12-387) and M13mp18BUNS(387-944) respectively. A full length BUNS cDNA was assembled by subcloning a KpnI-EcoRI cDNA fragment, containing BUNS bases 387-961, into KpnI and EcoRI digested M13mp19BUNS(1-387). KpnI cuts the BUNS cDNA at position 387. The full length BUNS cDNA was subcloned into pUC9 as a BamHI-EcoRI fragment to give pUC9T7BUNS.

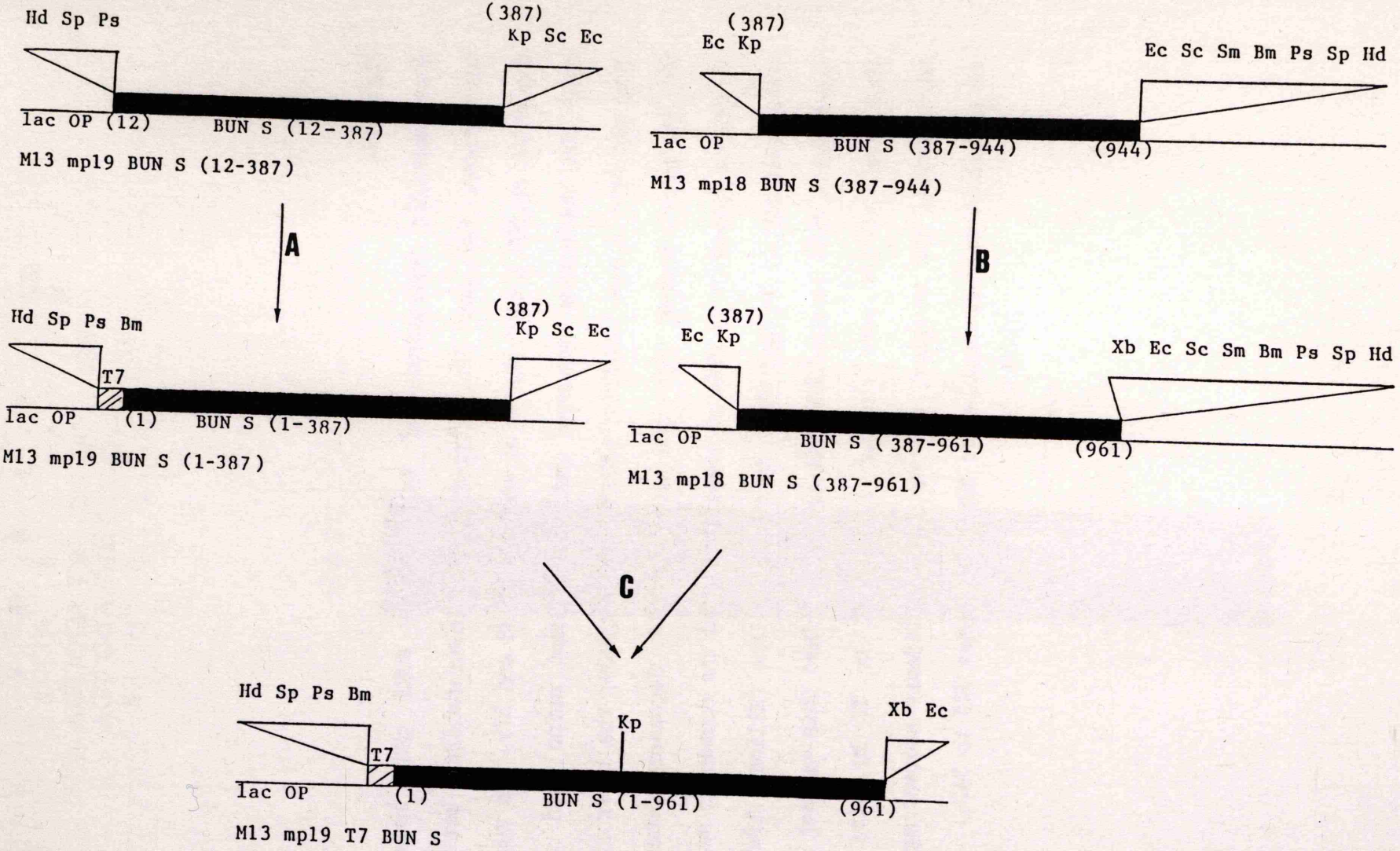

Hd Sp Ps
(387)
Kp Sc Ec
lac OP (12)
BUN S (12-387)
M13 mp19 BUN S (12-387)
(387)
Ec Kp
Ec Sc Sm Bm Ps Sp Hd
lac OP
BUN S (387-944)
(944)
M13 mp18 BUN S (387-944)
A
B
Hd Sp Ps Bm
(387)
Kp Sc Ec
T7
lac OP
(1)
BUN S (1-387)
M13 mp19 BUN S (1-387)
(387)
Ec Kp
Xb Ec Sc Sm Bm Ps Sp Hd
lac OP
BUN S (387-961)
(961)
M13 mp18 BUN S (387-961)
C
Hd Sp Ps Bm
Kp
Xb Ec
T7
lac OP
(1)
BUN S (1-961)
(961)
M13 mp19 T7 BUN S

Figure 9.

Site specific mutagenesis of full length BUNS cDNA sequence at nucleotides 378 and 379. (Upper part) Discrepancies between the sequences of BUN S cDNA clones at positions 378 and 379. The upper lines show the nucleotide sequence and the deduced amino acid sequences obtained from clones pBUN3/59, pBUN308 and pBUN309; the lower lines show the sequence obtained from cDNA clone pBUNS14. Seqeuence determination by primer extension on vRNA as template showed the consensus sequence in the population to be that shown in the upper line (Elliott, 1989a). Lower part: full length BUNS cDNA clone contained this variant sequence. Positions 378/379 were changed from a GT to TC by site directed mutagenesis. The mutation was verified by dideoxy sequencing using an oligonucleotide that annealed to BUNS cDNA bases 325 to 342.

```
          V    D    S    L    P          NSs
          S    G    F    L    A          N
(371)  AGT GGA TTC CTT GCC  (385)
       AGT GGA TGT CTT GCC
          S    G    C    L    A          N
          V    D    V    L    P          NSs
```

GATC

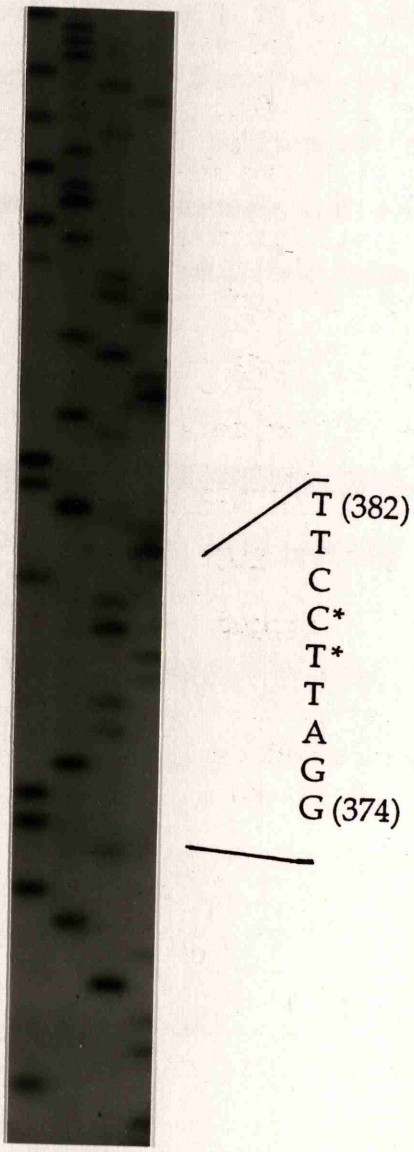
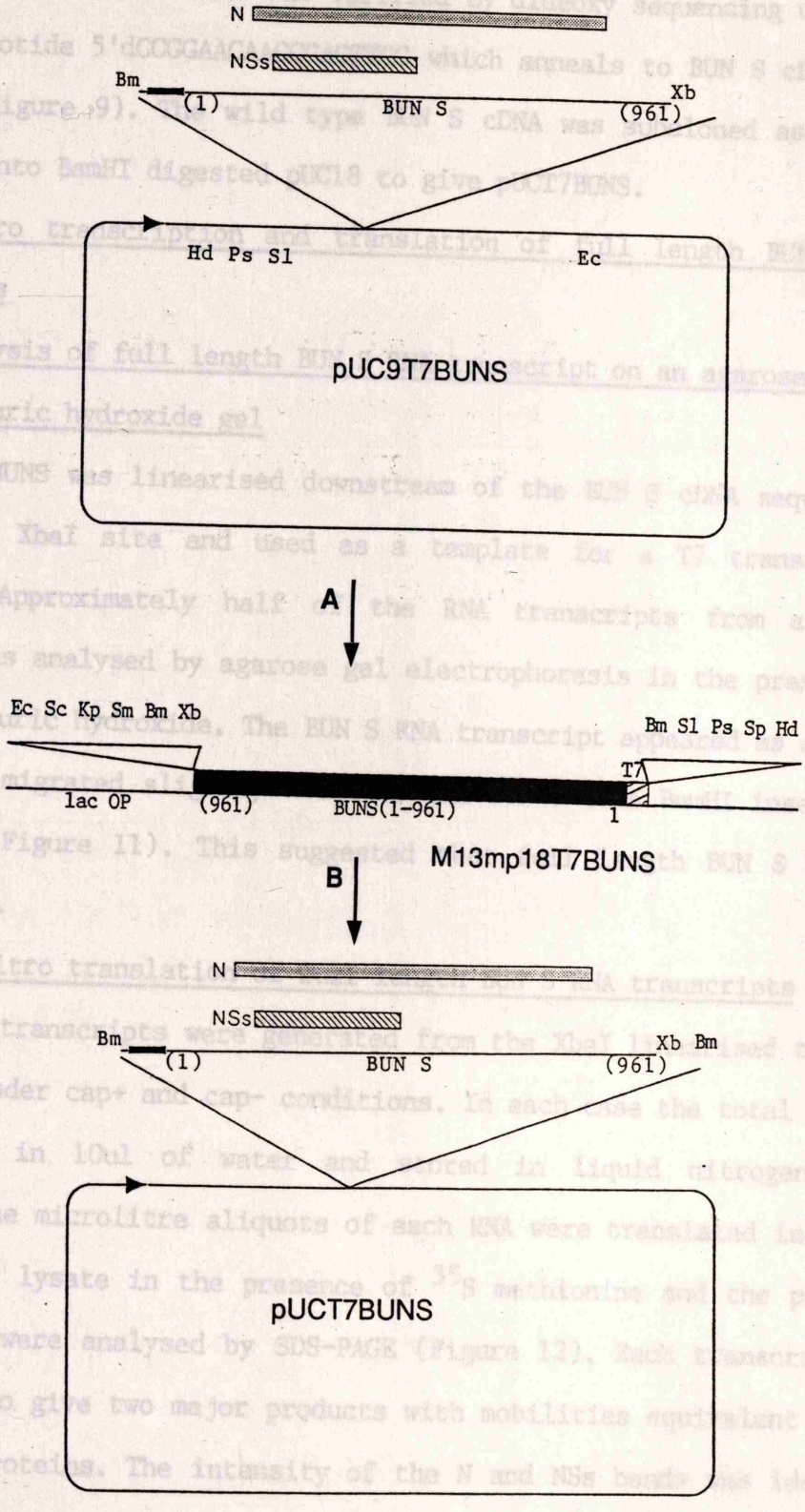


Figure 10.

Subcloning of full length BUN S cDNA into M13mp18 and pUC18. (A) The BUN S cDNA was isolated as a XbaI-PstI fragment from pUC9T7BUNS and subcloned into similarly cut RF M13mp18 to give M13mp18T7BUNS. (B) Site directed mutagenesis was performed on this template to introduce the consensus sequence at nucleotides 378 and 379 on the BUN S cDNA (as described in Figure 9). The altered BUN S cDNA was then isolated as a BamHI fragment and subcloned into pUC18, cut with BamHI, to give pUCT7BUNS.


N
NSs
Bm
(1)
BUN S
(961)
Xb
Hd Ps Sl
Ec
pUC9T7BUNS
A
Ec Sc Kp Sm Bm Xb
Bm Sl Ps Sp Hd
T7
lac OP
(961)
BUNS(1-961)
1
M13mp18T7BUNS
B
N
NSs
Bm
(1)
BUN S
(961)
Xb Bm
pUCT7BUNS

5'dCCGCCTCAGTGGATTCCTTGCCAGGTACC to change nucleotides at positions 378/79 from GT to TC. This was verified by dideoxy sequencing using the oligonucleotide 5'dCCGGAACAACCCAGTTCC which anneals to BUN S cDNA bases 325-342 (Figure 9). The wild type BUN S cDNA was subcloned as a BamHI fragment into BamHI digested pUC18 to give pUCT7BUNS.

4E In vitro transcription and translation of full length BUN S cDNA transcripts

4E(i) Analysis of full length BUN S RNA transcript on an agarose methylmercuric hydroxide gel

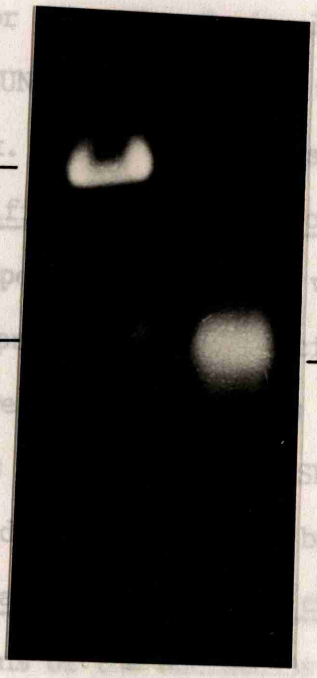
pUCT7BUNS was linearised downstream of the BUN S cDNA sequence at the unique XbaI site and used as a template for a T7 transcription reaction. Approximately half of the RNA transcripts from a single reaction was analysed by agarose gel electrophoresis in the presence of methyl mercuric hydroxide. The BUN S RNA transcript appeared as a single band which migrated slightly ahead of the BUN S cDNA BamHI insert from pUCT7BUNS (Figure 11). This suggested that full length BUN S RNA was synthesised.

4E(ii) In vitro translation of full length BUN S RNA transcripts

T7 RNA transcripts were generated from the XbaI linearised template pUCT7BUNS under cap+ and cap- conditions. In each case the total RNA was resuspended in 10ul of water and stored in liquid nitrogen until required. One microlitre aliquots of each RNA were translated in rabbit reticulocyte lysate in the presence of ^{35}S methionine and the proteins synthesised were analysed by SDS-PAGE (Figure 12). Each transcript was translated to give two major products with mobilities equivalent to the N and NSs proteins. The intensity of the N and NSs bands was identical for the translation of both cap+ and cap- transcripts. It was therefore

Figure 11.

Analysis of full length BUN S RNA transcript by agarose gel electrophoresis. RNA transcripts generated from the XbaI linearised template pUCT7BUNS(+) were analysed on a 1.2% agarose gel containing methyl mecuric hydroxide as described in Materials and Methods. Nucleic acid bands were identified by ethidium bromide staining. Lane 1; pUCT7BUNS(+) digested with BamHI to release the BUNS cDNA insert. Lane 2; BUNS cDNA transcript.


pUC vector
BUNS cDNA insert
BUN S RNA
1
2

assumed that the presence or absence of the synthetic cap structure at the 5' end of the BUN S RNA had no effect on translation in vitro and in subsequent experiments transcripts were synthesised uncapped. Additional bands lower than NSs protein or between that of N and NSs proteins were observed in both BUN S RNA translation tracks but not in the control track. These bands are presumably a result of internal initiation of translation within the N or NSs ORFs. Curiously one band X (Figure 12) appears in both the cap+ BUN S RNA track and the control track but not in the cap- BUN S RNA track. The origin of this band is unknown.

4E(iii)Immunological identification of in vitro expressed N protein

An aliquot of uncapped pUCT7BUNS RNA was translated in rabbit reticulocyte lysate and proteins immunoprecipitated with polyclonal antiserum raised to Bunyamwera virus particles (Watret et al, 1985). The immunoprecipitated proteins were analysed by SDS-PAGE (Figure 13). Only the N protein was recognised and precipitated by the antiserum.

4F Additional alterations carried out on BUNS cDNA

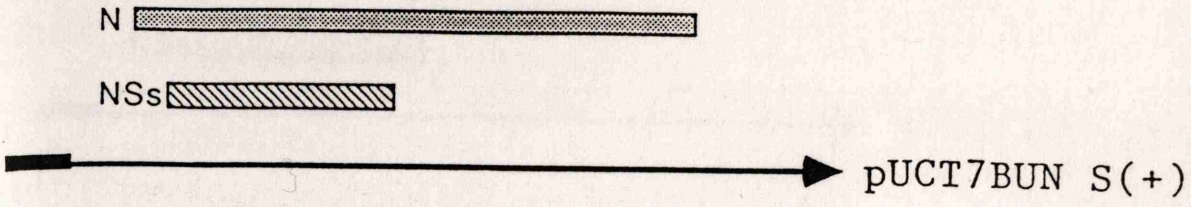
To study the interactions of the nucleocapsid protein with S segment RNA a further series of BUNS cDNA constructs under T7 promoter control were created; pUCT7BUNS300, pTZBUNS 660 and pUCT7BUNS(-). As a consequence of alterations made to the BUNS cDNA sequence during construction of these plasmids the NSs ORF could be isolated and placed under T7 promoter control in a plasmid designated pTZBUNNSs.

4F(i) Construction of pUCT7BUNS 300, pTZBUNS 660 and pTZBUNNSs

pUCT7BUNS 300 contains essentially the non-coding regions of the BUN S cDNA (bases 1-98 and 769-961) under T7 promoter control. M13mp18T7BUNS was mutated using the oligonucleotide 5'dGATTGAGTTGGAATTCCATGATGTCGCTG to introduce an EcoRI site immediately before NSs ORF at nucleotide 98

Figure 12.

Cell free translation of pUCT7BUNS(+) RNA. Aliquots of RNA transcripts derived from XbaI linearised plasmid template pUCT7BUNS(+) under cap+ or cap- conditions were translated in rabbit reticulocyte lysate. The upper part indicates the encoded BUN S ORFs in pUCT7BUNS(+). Lower part: proteins labelled with ^{35}S methionine were analysed by SDS-PAGE 20% gel. Lane: (1) control lysate without added RNA; (2) cap+ pUCT7BUNS(+) RNA; (3) cap- pUCT7BUNS(+) RNA; (4) BUN virus infected BHK cell lysate. The N and NSs proteins are marked together with Y protein which is synthesised by endogenous mRNA. Band X is of unknown origin.


N
NSs
pUCT7BUN S(+)

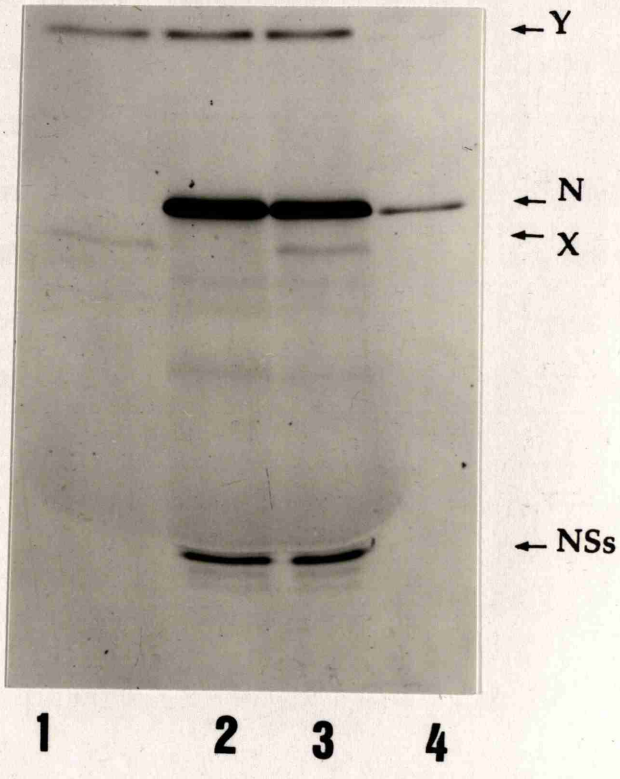
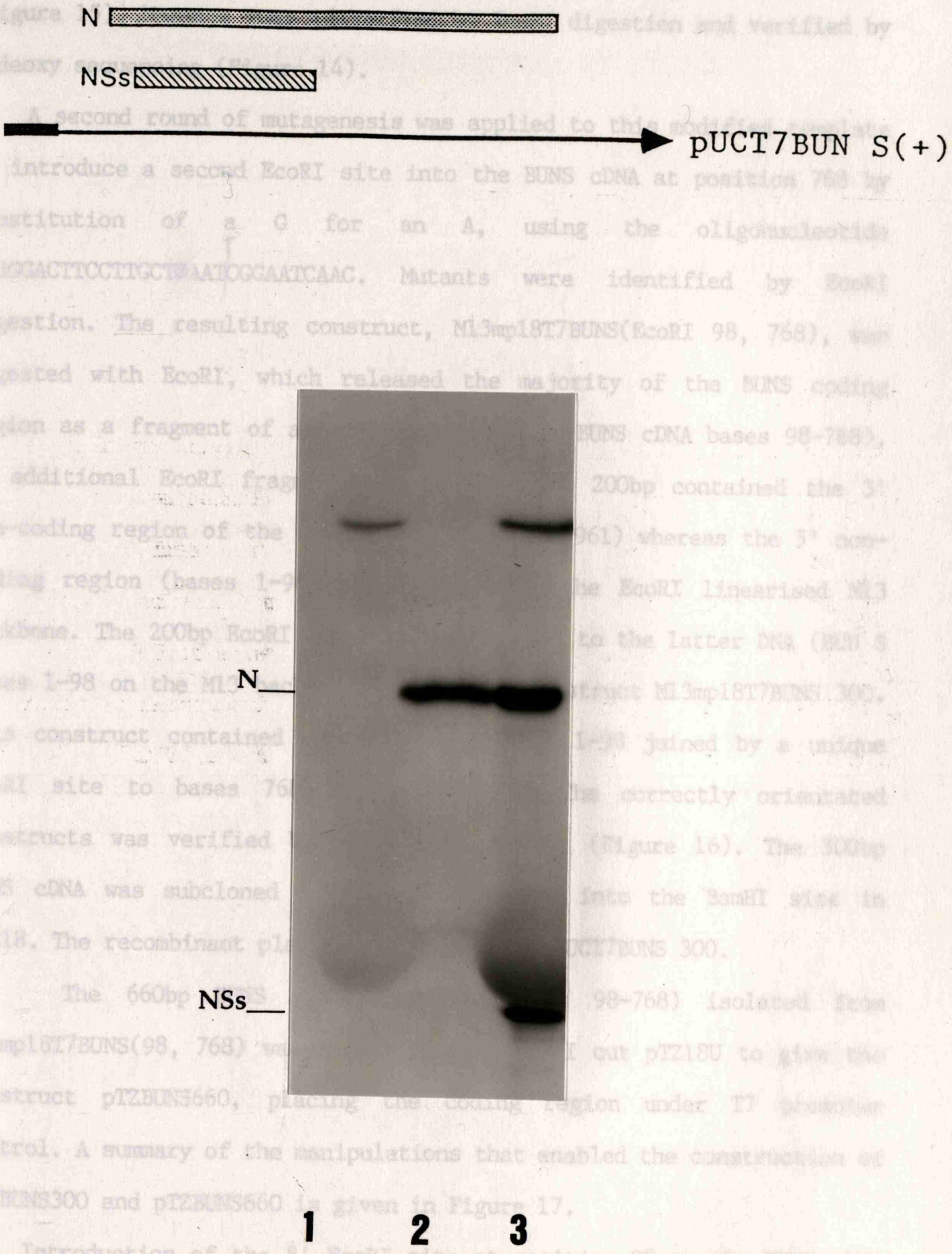

Y
N
X
NSs
1
2
3
4

Figure 13.

Immunoprecipitation of pUCT7BUNS RNA programmed lysate. An aliquot of pUCT7BUNS RNA derived from the template pUCT7BUNS linearised by XbaI was translated in rabbit reticulocyte lysate. Immunoprecipitation was carried out using rabbit polyclonal antiserum to BUN virus (Watret et al, 1985), and analysed by 18% PAGE in the presence of SDS. Lane: (3) proteins translated from pUCT7BUNS RNA; (2) immunoprecipitation of proteins in lane (3); (1) complete lysate without added RNA.


N
NSs
pUCT7BUN S(+)
N
NSs
1
2
3

(Figure 15). Mutants were identified by EcoRI digestion and verified by dideoxy sequencing (Figure 14).

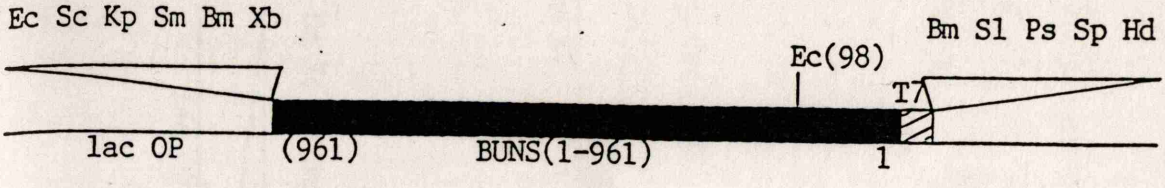
A second round of mutagenesis was applied to this modified template to introduce a second EcoRI site into the BUNS cDNA at position 768 by substitution of a G for an A, using the oligonucleotide 5'dGGACTTCCTTGCTGAATCGGAATCAAC. Mutants were identified by EcoRI digestion. The resulting construct, M13mp18T7BUNS(EcoRI 98, 768), was digested with EcoRI, which released the majority of the BUNS coding region as a fragment of approximately 660bp (BUNS cDNA bases 98-768). An additional EcoRI fragment of approximately 200bp contained the 3' non-coding region of the BUNS cDNA (bases 769-961) whereas the 5' non-coding region (bases 1-98) was retained in the EcoRI linearised M13 backbone. The 200bp EcoRI fragment was ligated to the latter DNA (BUN S bases 1-98 on the M13 backbone)to give the construct M13mp18T7BUNS 300. This construct contained the BUNS cDNA bases 1-98 joined by a unique EcoRI site to bases 768-961 (Figure 15). The correctly orientated constructs was verified by dideoxy sequencing (Figure 16). The 300bp BUNS cDNA was subcloned as a BamHI fragment into the BamHI site in pUC18. The recombinant plasmid was designated pUCT7BUNS 300.

The 660bp BUNS cDNA fragment (bases 98-768) isolated from M13mp18T7BUNS(98, 768) was subcloned into EcoRI cut pTZ18U to give the construct pTZBUNS660, placing the coding region under T7 promoter control. A summary of the manipulations that enabled the construction of pT7BUNS300 and pTZBUNS660 is given in Figure 17.

Introduction of the 5' EcoRI site at position 98 on the BUNS cDNA enabled the isolation of an EcoRI BUNS cDNA fragment (bases 98-961) containing the BUN NSs ORF without the upstream N initiation codon. The

Figure 14.

Introduction of an EcoRI site into BUNS cDNA at nucleotide position 98 by oligonucleotide site directed mutagenesis. A change in the BUNS cDNA sequence at position 103 from T to C introduced an EcoRI site in the BUNS cDNA template M13mp18T7BUNS. The alteration is shown in diagramatic form in the upper part and was verified by dideoxy sequencing (lower part); (A) BUNS wild type sequence and (B) BUNS 5' EcoRI sequence (position 98).



M13mp18T7BUNS(Ec98)

A TGAGTTGGAATTTCATGATG
(91) (110)

B TGAGTTGGAATTCCATGATG
EcoRI

GATCGATC

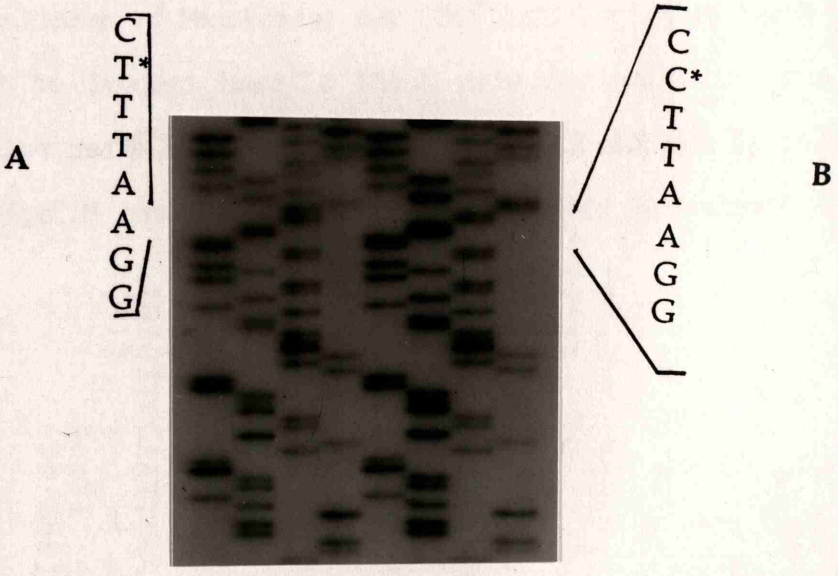
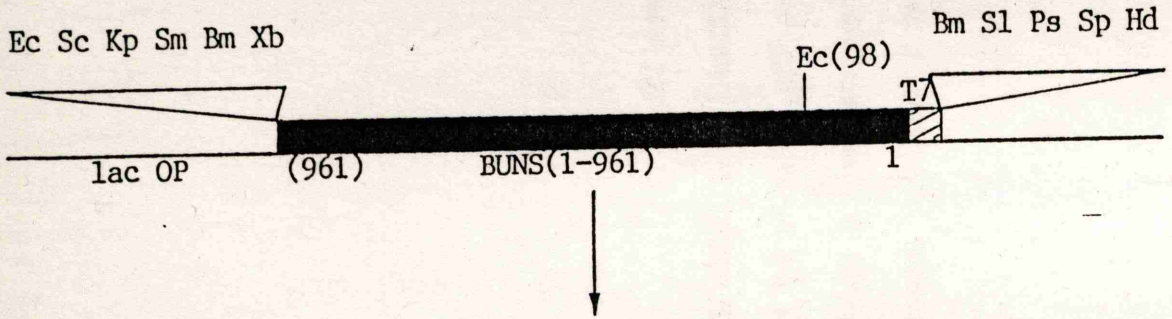


Figure 15.

Manipulations involved in the construction of BUNS 300 cDNA. An abbreviated BUNS cDNA essentially containing the 5' and 3'on coding regions of the BUNS segment was assembled under T7 promoter control. Two specific EcoRI sites, at positions 98 (A) and 768 (B), were introduced to the BUNS sequence by oligonucleotide site directed mutagenesis on the template M13mp18T7BUNS. (A) The first EcoRI site (position 98) was introduced by mutating position 103 from T to C.(B) The second EcoRI site (position 768) was introduced by mutating position 768 from A to G. Digestion with EcoRI allowed removal of the middle coding region of the BUN S cDNA (bases 99-768), and after religation of the 5' and 3'ends via the unique EcoRI site created M13mp18T7BUNS300 (C).

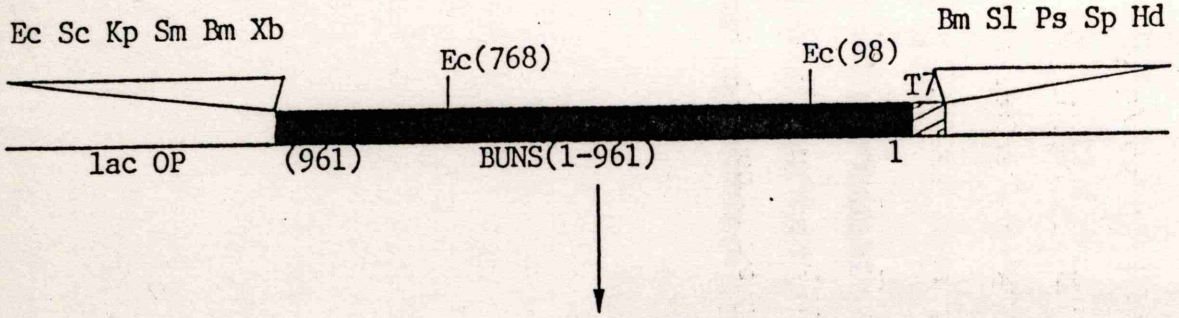


A

TGAGTTGGAATTTCATGATG
(91) (110)

TGAGTTGGAATTCCATGATG

EcoRI

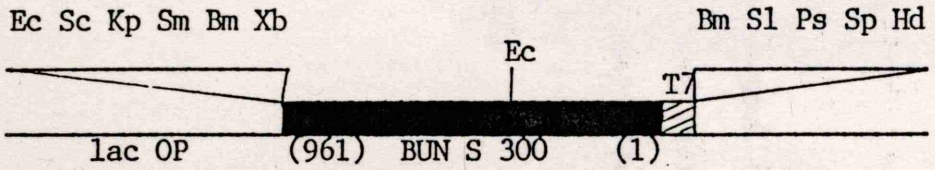


B

GGGACTTCCTTGCTAAATTCGG
(753) (775)

GGGACTTCCTTGCTGAATTCGG

EcoRI



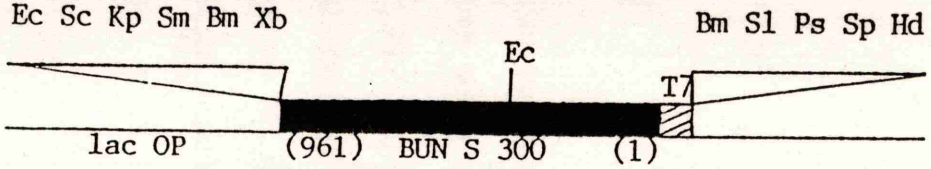
C

TGAGTTGGAATTCGGAATCAAC
(91) (782)

EcoRI
(98/768)

Figure 16.

Verification of BUNS 300 cDNA by sequencing across the internal EcoRI site. The junction of the ligated BUNS cDNA bases 769-961 with bases 1-98 was checked on the template M13mp18T7BUNS300 by dideoxy sequencing using a universal sequencing primer.



M13mp18T7BUNS300

TGAGTTGGAATTCGGAATCAAC
(91) (782)
EcoRI
(98/768)

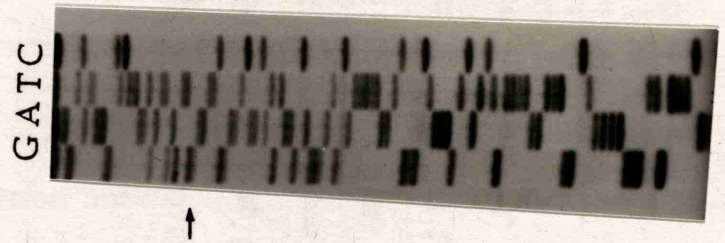
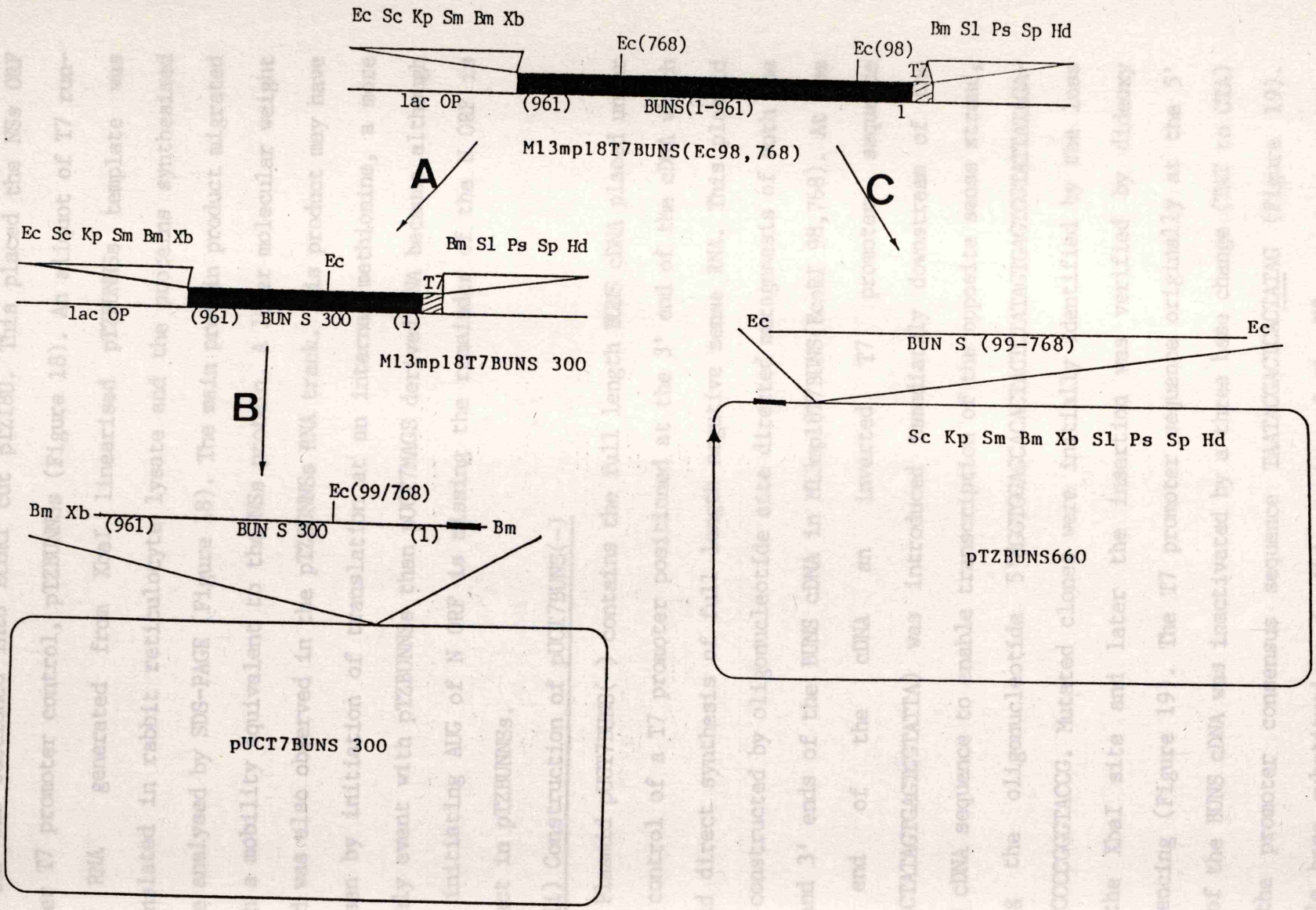


Figure 17.

Strategy to construct pUCT7BUNS 300 and pTZBUNS660. (A) EcoRI digestion and ligation of BUNS bases 961-769 and 1-98 to give mp18BUNS 300. (B) The BUNS 300 cDNA was subcloned as a BamHI fragment into pUC18 to give pUCBUNS 300. (C) EcoRI digestion and isolation of BUNS cDNA fragment containing bases 99-768 which was subcloned into pTZ18U linearised with EcoRI. The recombinant plasmid was designated pTZBUNS660.


Ec Sc Kp Sm Bm Xb
Ec(768)
Ec(98)
Bm Sl Ps Sp Hd
T7
lac OP
(961)
BUNS(1-961)
1
M13mp18T7BUNS(Ec98,768)
A
C
Ec Sc Kp Sm Bm Xb
Ec
Bm Sl Ps Sp Hd
T7
lac OP
(961)
BUN S 300
(1)
M13mp18T7BUNS 300
B
Ec
Ec
BUN S (99-768)
Sc Kp Sm Bm Xb Sl Ps Sp Hd
pTZBUNS660
Ec(99/768)
Bm Xb
(961)
BUN S 300
(1)
Bm
pUCT7BUNS 300

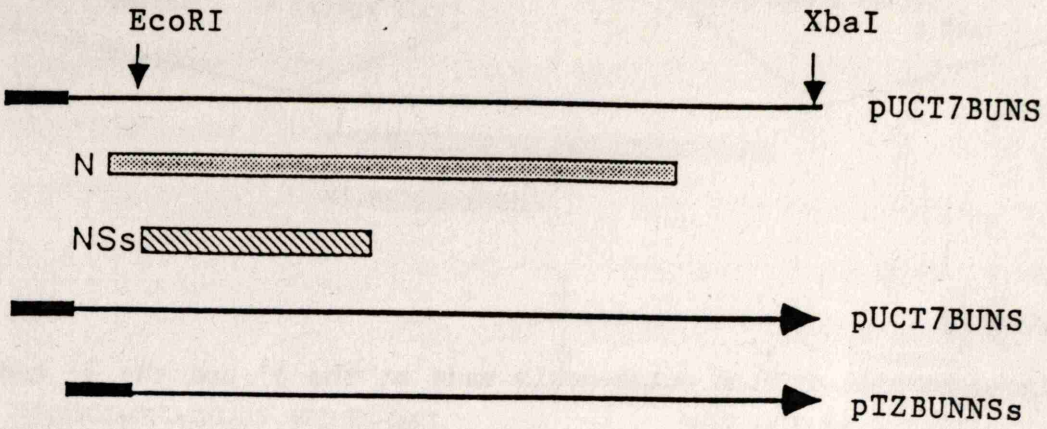
NSs ORF was subcloned into EcoRI cut pTZ18U. This placed the NSs ORF under T7 promoter control, pTZBUNNSs (Figure 18). An aliquot of T7 run-off RNA generated from XbaI linearised pTZBUNNSs template was translated in rabbit reticulocyte lysate and the proteins synthesised were analysed by SDS-PAGE (Figure 18). The main protein product migrated with a mobility equivalent to the NSs protein. A lower molecular weight band was also observed in the pTZBUNNSs RNA track. This product may have arisen by initiation of translation at an internal methionine, a more likely event with pTZBUNNSs than pUCT7MAGS derived RNA because although the initiating AUG of N ORF is missing the remainder of the N ORF is intact in pTZBUNNSs.

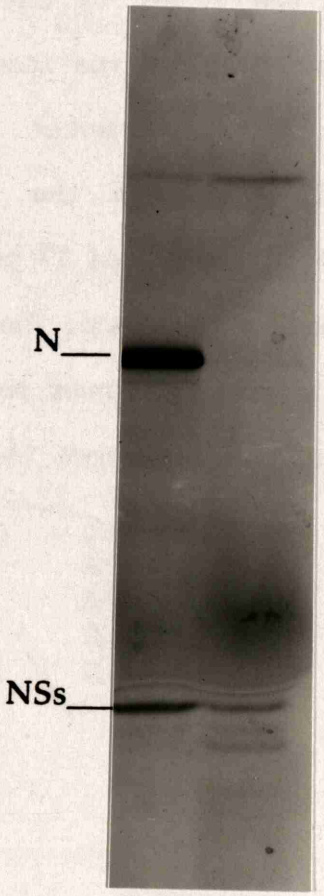
4F(ii) Construction of pUCT7BUNS(-)

Plasmid pUCT7BUNS(-) contains the full length BUNS cDNA placed under the control of a T7 promoter positioned at the 3' end of the cDNA which would direct synthesis of full length negative sense RNA. This plasmid was constructed by oligonucleotide site directed mutagenesis of both the 5' and 3' ends of the BUNS cDNA in M13mp18T7BUNS(EcoRI 98,768). At the 3' end of the cDNA an inverted T7 promoter sequence (5'dCTATAGTGAGTCGTATTA) was introduced immediately downstream of the BUNS cDNA sequence to enable transcription of the opposite sense strand, using the oligonucleotide 5'dGGTGGAGCACACTACTCTATAGTGAGTCGTATTATAGA-GGATCCCCGGGTACCG. Mutated clones were initially identified by the loss of the XbaI site and later the insertion was verified by dideoxy sequencing (Figure 19). The T7 promoter sequence originally at the 5' end of the BUNS cDNA was inactivated by a three base change (TAT to CTA) in the promoter consensus sequence TAATACGACTCACTATAG (Figure 19). Single base substitutions in this region of the T7 promoter sequence

Figure 18.

Cell free translation of pTZBUNNSs RNA. The upper part shows the encoded BUN S ORFs in pUCT7BUNS and pTZBUNNSs. The positions of the EcoRI site, which enabled the subcloning of the NSs ORF without the upstream N initiation codon, and the XbaI site, used to linearise the plasmid, are also indicated. Aliquots of RNA transcript derived from these templates were translated in rabbit reticulocyte lysate. Lower part: Labelled proteins were analysed by SDS-PAGE 20% gel. Lane: (1) pUCT7BUNS RNA programmed lysate; (2) pTZBUNNSs programmed lysate.

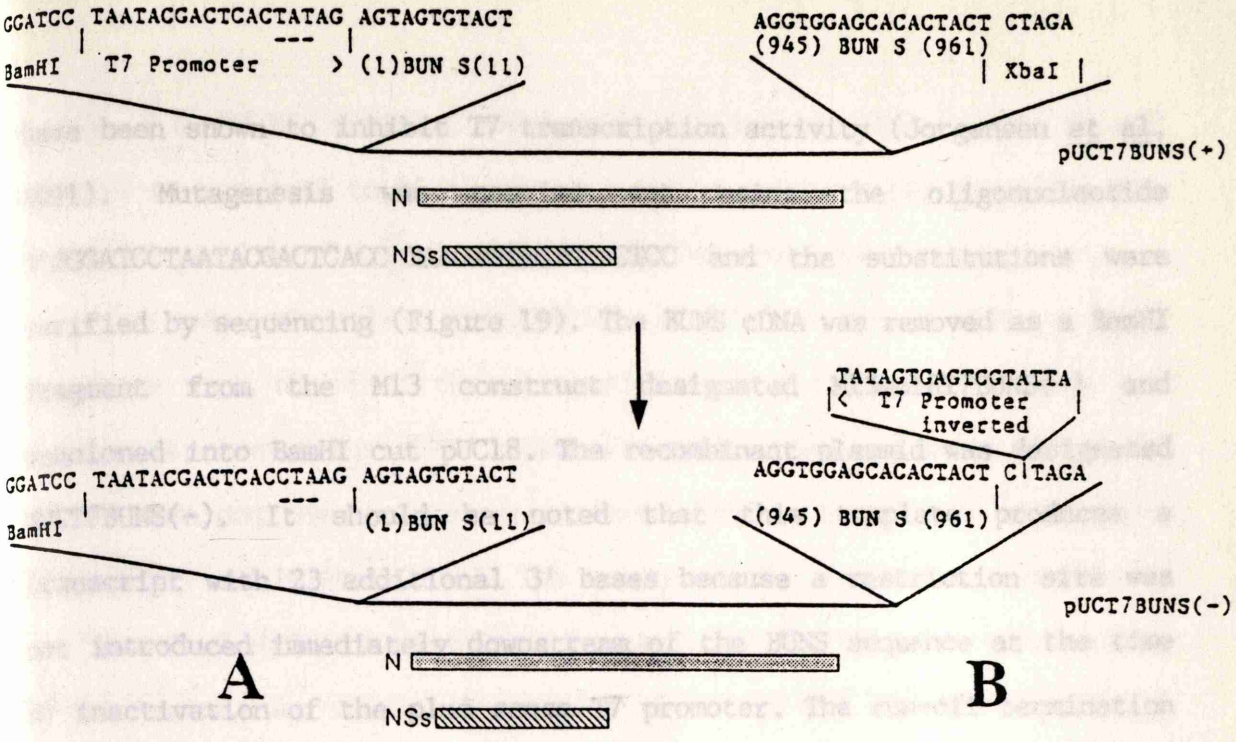


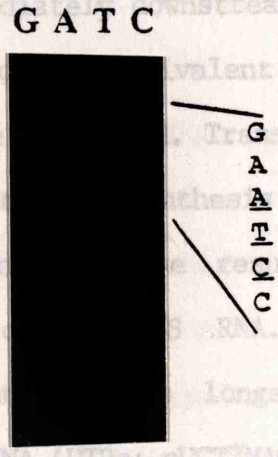


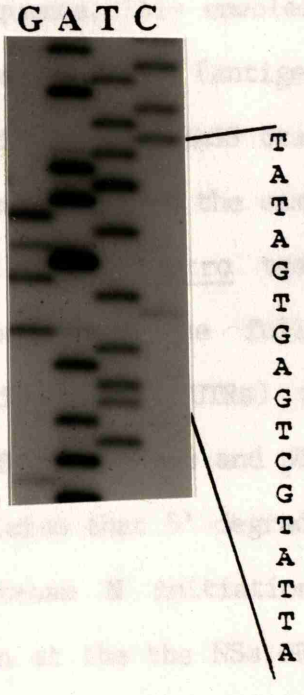
1 2

Figure 19.

Oligonucleotide directed mutagenesis made at the 5' and the 3' ends of BUNS cDNA during the construction of pUCT7BUNS(-). (Upper part) Summary of the alterations made at the 5' and 3' ends of the BUN S cDNA during the conversion from pUCT7BUNS(+) to pUCT7BUNS(-). (A) The core T7 promoter sequence positioned at the 5' end of the BUNS cDNA sequence (bases 1-961) cloned in vector pUCT7BUNS(+) was inactivated by a 3 base change of TAT to CTA in the T7 promoter consensus sequence (TAATACGACTCACTATAG). (B) At the 3'end of the BUNS cDNA sequence insertional mutagenesis introduced an inverted T7 promoter sequence to enable transcription of the opposite DNA strand. The insertion of the T7 promoter sequence disrupted the XbaI site. (Lower part) Dideoxy sequencing was carried out to verify sequence changes (A) 5' end and (B) 3' end.


GGATCC TAATACGACTCACTATAG AGTAGTGTACT
BamHI T7 Promoter > (1)BUN S(11)
AGGTGGAGCACACTACT CTAGA
(945) BUN S (961)
XbaI
pUCT7BUNS(+)
N
NSs
TATAGTGAGTCGTATTA
< T7 Promoter inverted
GGATCC TAATACGACTCACCTAAG AGTAGTGTACT
BamHI
(1)BUN S(11)
AGGTGGAGCACACTACT C TAGA
(945) BUN S (961)
pUCT7BUNS(-)
N
NSs
A
B


A
G A T C
G
A
T
C
C


B
G A T C
T
A
T
A
G
T
G
A
G
T
C
G
T
A
T
T
A

have been shown to inhibit T7 transcription activity (Jorgensen et al, 1991). Mutagenesis was carried out using the oligonucleotide 5'dGGATCCTAATACGACTCACC TAAGAGTAGTGTACTCC and the substitutions were verified by sequencing (Figure 19). The BUNS cDNA was removed as a BamHI fragment from the M13 construct designated M13mp18T7BUNS(-) and subcloned into BamHI cut pUC18. The recombinant plasmid was designated pUCT7BUNS(-). It should be noted that this template produces a transcript with 23 additional 3' bases because a restriction site was not introduced immediately downstream of the BUNS sequence at the time of inactivation of the plus sense T7 promoter. The run-off termination site for transcription was created by linearising the plasmid at a restriction site in the plasmid polylinker.

5 Discussion

Full length cDNAs of BUN and MAG virus S segments were assembled. In addition a T7 promoter was introduced immediately upstream and an XbaI site immediately downstream of each cDNA sequence. This enabled T7 run off transcripts equivalent to full length positive sense (antigenomic) S RNA to be generated. Translation in vitro of BUNS and MAGS transcripts demonstrated the synthesis of the N and NSs protein from the same S RNA. This reinforces the result obtained from the in vitro translation studies of pTZMAGS RNA. In addition, because these full length transcripts possess longer 5' untranslated regions (UTRs) than the pTZMAGS RNA (UTRs; pUCT7MAGS 73 bases, pUCT7BUNS 84 bases and pTZMAGS 52 bases) the results obtained negate the criticism that 5' degradation of the S RNA, and hence loss of the upstream N initiation codon, contributes to the initiation of translation at the the NSs ORF. Since translation of both capped and non-capped S RNA yielded equivalent

levels of N and NSs protein, and because the cap structure at the 5' end of mRNA is reported to block 5' exonuclease activity (Kozak, 1991) the perceived problem of 5' end mRNA degradation would seem unimportant.

The cap-independent translation of BUN and MAG S RNA *in vitro* contrasts with the fact that bunyavirus mRNAs in infected cells are capped i.e. bunyavirus mRNAs possess heterogeneous sequences at the 5' end of mRNA and these sequences snatched from host cell mRNA are capped (Kolakofsky and Hacker, 1991). The cap structure, as well as stabilising the 5' end of mRNA, is thought to be essential for the efficient binding of the translation initiation complex to the 5' end of cellular mRNA *in vivo* (Sonenberg,1991). Indeed among animal cells and viruses picornavirus mRNAs are some of the few mRNAs known to translate efficiently without a cap structure (Jackson, 1988; Kozak, 1991). It should be noted that picornavirus mRNAs do not rely on the scanning model for the initiation of translation and instead possess a sequence described as the "ribosomal landing pad" within the mRNA which enables internal initiation of translation (Sonenberg, 1990).

Bunyavirus mRNAs *in vivo* also possess additional heterogenous sequences of approximately 10-15 bases at the 5' end of the mRNA. These sequences are snatched from the 5' end of existing cellular mRNAs probably by the virus encoded endonuclease. The relative effect of the 5' heterogenous sequence in conjunction with the cap structure on the translation of bunyavirus S mRNA is unknown. Thus since heterogenous sequences at the 5' end of some clones of Germiston virus S mRNA have been obtained (Bouloy *et al*, 1990) it would be interesting to place these known sequences immediately upstream of either BUN S or MAG S cDNA under T7 promoter control. The translational efficiency of run-off

transcripts from these templates could then be compared with that of transcripts derived from either pUCT7MAGS or pUCT7BUNSS under cap plus or minus conditions.

A series of additional BUNS cDNA templates was created under T7 promoter control; pUCT7BUNS300, pTZBUNS660 and pUCT7BUNS(-). The most interesting of these templates is pUCT7BUNS300. This cDNA encodes the 5' and the 3' non-coding regions of the BUNS segment linked by a unique EcoRI site. The presence of this unique EcoRI would allow run-off transcripts to be made eqivalent in length to the 5' UTR. Such a transcript could be used in defining essential 5' UTR nucleocapsid-RNA binding sites. In addition the EcoRI site could be used to introduce a reporter gene such as chloramphenicol acetyl transferase (CAT) which would be flanked by BUN S 5' and 3' UTRs. Transcripts derived from this template could be used in nucleocapsid protein interaction studies in vitro and possibly in amplification, expression and packaging studies of viral RNA in bunyavirus infected cells, provided that the essential sequences are present in the 5' and 3' UTRs. This latter type of experiment has been successfully carried out with influenza virus (Luytjes et al, 1989).

All the S cDNA T7 transcription templates described in this chapter initiate transcription from a G residue, considered to be important for efficient transcription by T7 polymerase. However as this introduces an unwanted G residue at the 5' end of all transcripts it may be prudent in future experiments to remove this residue from the 3' end of the T7 promoter sequence to determine if loss of this residue significantly affects the yield of transcript. The removal of the 5' G residue would provide a further level of refinement to the S RNA transcripts in that they would be the authentic 5' terminus.

Chapter 5

Purification of Maguari virus nucleocapsid protein expressed by a recombinant baculovirus and analysis of RNA binding activity

1 Summary

This chapter describes: (a) characterisation of a recombinant baculovirus able to express Maguari virus nucleocapsid protein in infected insect cells; (b) purification of the recombinant nucleocapsid protein to homogeneity; and (c) analysis of the interaction of the purified nucleocapsid protein with *in vitro* transcribed bunyavirus S segment RNAs.

2 Introduction

In order to study the interaction of the bunyavirus nucleocapsid protein (N) with viral RNA a source from which the N protein could be obtained free of other viral components had to be developed. The baculovirus expression system was chosen for the production of recombinant protein as this system has been shown to produce high levels of recombinant proteins (Matsuura *et al*, 1987) and usually a large proportion of the expressed protein is soluble unlike recombinant proteins expressed in bacterial systems which are usually insoluble. Secondly, this system has been used to express successfully the nucleocapsid proteins of snowshoe hare (SSH), Punta Toro (PT) and Hantaan (HTN) viruses (Overton *et al*, 1987; Schmaljohn *et al*, 1988; Urakawa *et al*, 1988).

The baculovirus expression system involves a lytic, double-stranded DNA virus *Autographa california* nuclear polyhedrosis virus (AcNPV). Two types of virus progeny are produced during the life cycle of the virus: extracellular particles (non occluded viruses), and intracellular virus particles (occluded viruses). The non-occluded virus particles produced early in infection invade other cells either in culture or within an

insect larva, disseminating the infection. In the very late phase of the viral lytic cycle (approximately 18 hours post infection) the virus particles are occluded into proteinaceous structures called polyhedra, located within the nucleus of infected cells. These occlusion bodies are highly refractile and easily observed under the microscope. The polyhedrin protein (M_r 29K) is the major protein component of the occlusion bodies. Although the polyhedrin protein is essential for survival of the virus in nature it is not necessary for virus propagation in tissue culture. This feature has been exploited for the insertion of foreign genes into a recombinant baculovirus genome through genetic recombination at the polyhedrin gene. To produce a recombinant virus that expresses a protein of interest its gene is firsly cloned into a baculovirus shuttle vector such that the gene is flanked by polyhedrin specific sequences and is positioned downstream of the polyhedrin promoter. The recombinant vector is transfected along with purified wild type virus DNA into insect cells, Spodoptera frugiperda (Sf9). In a homologous recombination event the foreign gene is inserted into the virus genome at the polyhedrin gene locus generating a recombinant virus that expresses the foreign gene under polyhedrin promoter control. Cells infected with recombinant baculoviruses do not express polyhedrin protein and therefore lack occlusion bodies. This feature is used to distinguish wild type from recombinant baculovirus plaques after the transfection supernatant is used to infect monolayers of Sf9 cells.

3 Characterisation of a recombinant baculovirus

3(A) Construction of the AcNPV transfer vector

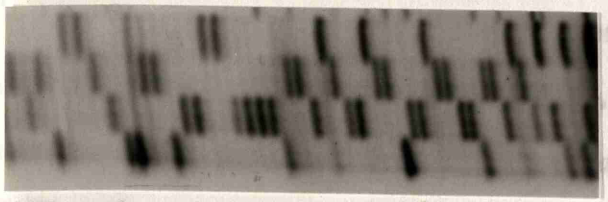
The shuttle vector used to transfer the entire MAGS cDNA coding region into a recombinant baculovirus genome was the plasmid pAcYM1 (Matsuura et al, 1987). This vector has a unique BamHI cloning site downstream of the polyhedrin promoter, but differs from other baculovirus shuttle vectors in that in addition to possessing the polyhedrin promoter sequence it also has the complete 5' untranslated leader sequence of the polyhedrin gene up to and including the initiating A of the ATG codon. The presence of this complete untranslated region is thought to enable a recombinant virus to express a foreign gene at or about the level obtained for wild type polyhedrin protein (Matsuura et al, 1987). In an earlier manipulation a MAG S cDNA (bases 61-937) had a BamHI linker introduced to its 3' end which enabled the MAGS cDNA to be subcloned into the BamHI site of various plasmids (Chapter 3, Section 4(i)). The MAGS cDNA was isolated from the plasmid pTZMAGS(BB) as a BamHI fragment and subcloned into the unique BamHI site of pAcYM1 just downstream of the polyhedrin promoter to generate pYM1MAGS. The recombinant plasmid was identified by BamHI digestion and the orientation of the cDNA insert verified by EcoRI and EcoRV double digests. The integrity of the reading frame of the MAGS cDNA BamHI insert was later checked by dideoxy sequencing of the recombinant plasmid using an oligonucleotide that primed on the baculovirus promoter approximately 40 bases upstream of the MAGS cDNA insert (Figure 1)

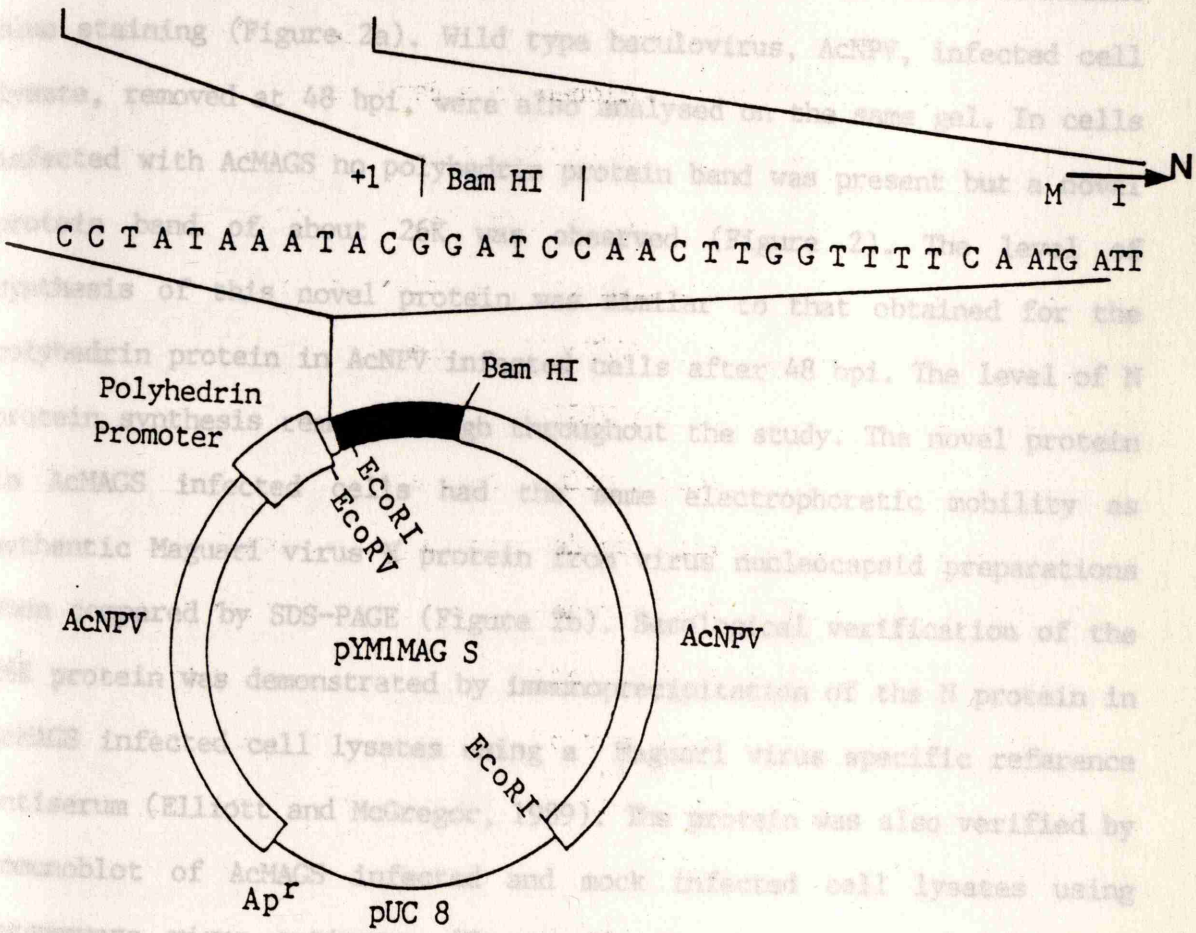
3(B) Generation of a recombinant Baculovirus (AcMAGS)

Generation and isolation of recombinant baculovirus AcMAGS was carried out by Dr. R.M. Elliott using established procedures (Overton et

Figure 1.

Construction of the AcNPV transfer vector. The shuttle vector used to transfer the entire MAGS cDNA coding region into a recombinant baculovirus genome was pAcYM1 (Matsuura et al, 1987). The MAGS cDNA (bases 61-937) was subcloned as a BamHI fragment into the unique BamHI cloning site on pAcYM1, immediately downstream of the polyhedrin promoter. The recombinant plasmid pYM1MAGS was identified by BamHI digestion and the orientation of the MAGS cDNA insert was verified by EcoRV/EcoRI double digests. The integrity of the reading frame of the MAGS cDNA insert was checked by dideoxy sequencing, using a primer that annealed to the polyhedrin promoter region. The sequence downstream of the BamHI site represents the MAGS cDNA sequence from the BamHI site to the start of the N ORF (indicated by an arrow). The sequence downstream of the BamHI site represents part of the polyhedrin gene untranslated region, +1 represents the A of the start codon of the polyhedrin ORF (Matsuura et al, 1987)


G A T C


+1
Bam HI
N
M I
C C T A T A A A T A C G G A T C C A A C T T G G T T T T C A ATG ATT
Bam HI
Polyhedrin
Promoter
EcoRI
EcoRV
AcNPV
pYM1MAG S
AcNPV
EcoRI
Ap r
pUC 8

al, 1987). Four plaques were isolated which had a polyhedrin negative phenotype. A recombinant virus stock was prepared from one of the plaque isolates and designated AcMAGS.

3(C) Expression of N protein in AcMAGS infected cells

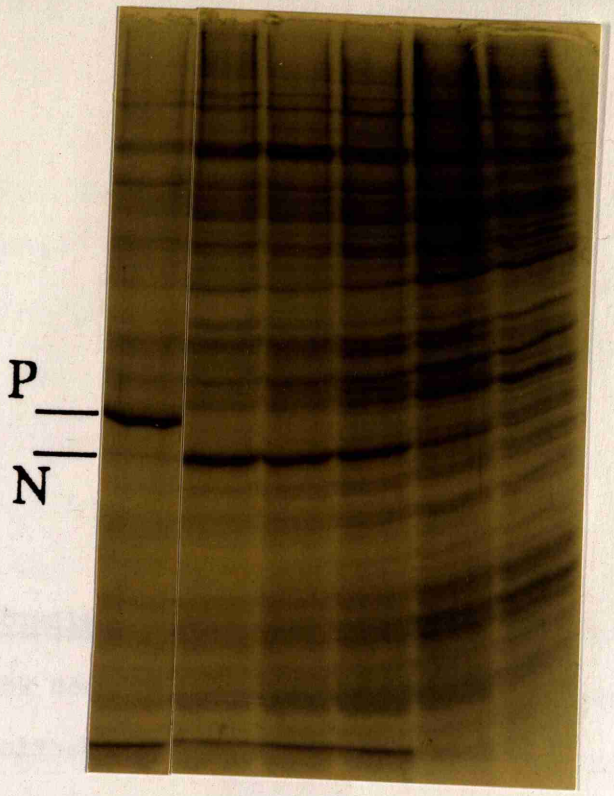
The expression of recombinant N protein in baculovirus infected cells was investigated by time course assay. Samples were taken at 24, 48, 72 and 96 hours post infection (hpi). Cell lysates were analysed by SDS-PAGE and protein bands visualised by staining with Coomassie brilliant blue staining (Figure 2a). Wild type baculovirus, AcNPV, infected cell lysate, removed at 48 hpi, were also analysed on the same gel. In cells infected with AcMAGS no polyhedrin protein band was present but a novel protein band of about 26K was observed (Figure 2). The level of synthesis of this novel protein was similar to that obtained for the polyhedrin protein in AcNPV infected cells after 48 hpi. The level of N protein synthesis remained high throughout the study. The novel protein in AcMAGS infected cells had the same electrophoretic mobility as authentic Maguari virus N protein from virus nucleocapsid preparations when compared by SDS-PAGE (Figure 2b). Serological verification of the 26K protein was demonstrated by immunoprecipitation of the N protein in AcMAGS infected cell lysates using a Maguari virus specific reference antiserum (Elliott and McGregor, 1989). The protein was also verified by immunoblot of AcMAGS infected and mock infected cell lysates using Bunyamwera virus antiserum (Figure 3). In the AcMAGS infected cell lysate tracks a single band was obtained corresponding to the recombinant N protein whereas the mock infected cell lysate tracks remained clear (Figure 3).

No novel bands corresponding to Maguari virus S segment encoded gene

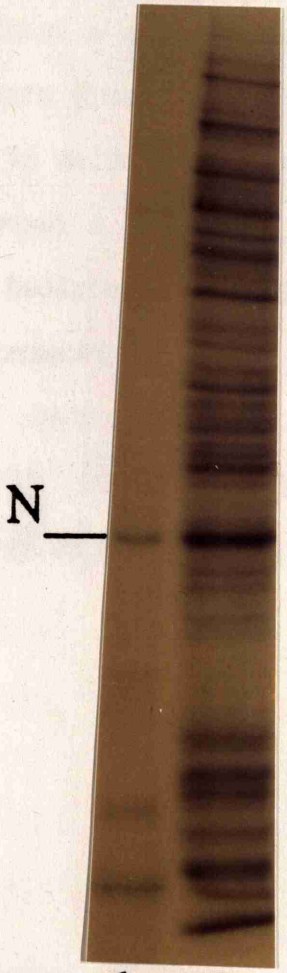
Figure 2.

(a) Time course analysis of the expression of N protein in AcMAGS infected cells. Spodoptera frugiperda (Sf9) cells in 30mm dishes were infected with recombinant baculovirus, AcMAGS, at a moi of 5 pfu. At 24, 48, 72 and 96 hours post infection (hpi) an infected cell monolayer was harvested by lysis in 500ul of dissociation mix. Equivalent volumes from the different time points were analysed by 15% SDS-PAGE and protein bands visualised by Coomassie brilliant blue staining. Equivalent volumes of additional samples of mock infected and AcNPV infected Sf9 cells were also analysed on the same gel. Lane (1) AcNPV infected cells at 48 hpi and lane (6) mock infected cells. Lanes (2) to (5) AcMAGS infected cells at 96, 72, 48 and 24 hpi respectively. The location of the nucleocapsid protein (N) and the polyhedrin protein (P) are indicated.

(b) Comparison of nucleocapsid protein mobility. The mobilities of nucleocapsid protein from Maguari virus nucleocapsid preparations and AcMAGS infected Sf9 cell lysate were compared by 15% SDS-PAGE. Protein bands were visualised by Coomassie brilliant blue staining. Lane (1) Maguari virus particles and lane (2) AcMAGS infected Sf9 cell lysate. The location of the nucleocapsid protein (N) is indicated.



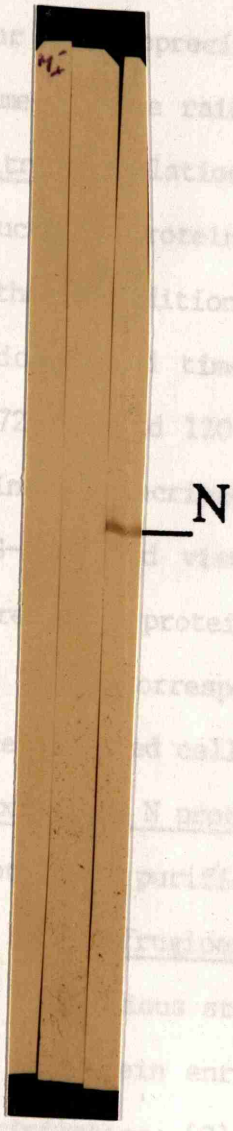
(a) Lane 1 2 3 4 5 6



(b) Lane 1 2

Figure 3.

Immunoblot of AcMAGS infected Spodoptera frugiperda (Sf9) cells. A confluent monolayer of Sf cells in 35mm dishes were infected with AcMAGS at moi of 5 pfu. At 48 hours post infection the cells were lysed with dissociation mix. A confluent monolayer of mock infected cells were similarly treated. Proteins from infected and mock infected cell lysates were resolved by electrophoresis (15% SDS-PAGE) and electroblotted to a nitrocellulose membrane. The blot was cut into strips and each strip incubated with rabbit anti-Bunyamwera serum (at a dilution of 1/100). Antibody protein reaction was detected by use of a secondary antibody conjugated with horseradish peroxidase as described in Materials and Methods. The location of the band corresponding to the recombinant nucleocapsid N protein is indicated. Strips (1) and (2) are equivalent tracks of uninfected Sf9 cells and strip (3) corresponds to AcMAGS infected Sf9 cells.


N
Strip 1 2 3

products NSs and 9.3K proteins were detected in the AcMAGS infected tracks by Coomassie brilliant blue staining because of the high background of other proteins. These proteins were not expected to be identified via immunoblot or immunoprecipitation assay since the antisera used in these experiments were raised against viral particles and as shown in earlier in vitro translation studies did not recognise the S segment encoded non-structural proteins (Chapter 3). Recombinant baculovirus expression of these additional proteins was further investigated by use of a radiolabelled time course assay of AcMAGS infected cells, where at 48, 72, 96 and 120 hpi AcMAGS infected cells were labelled with ^{35}S methionine as described in Materials and Methods. Proteins were analysed by SDS-PAGE and visualised by autoradiography (Figure 4). High levels of expressed N protein were detected throughout the time course study, but no bands corresponding to the NSs or 9.3K proteins could be seen above the infected cell protein profile.

4 Purification of baculovirus expressed N protein

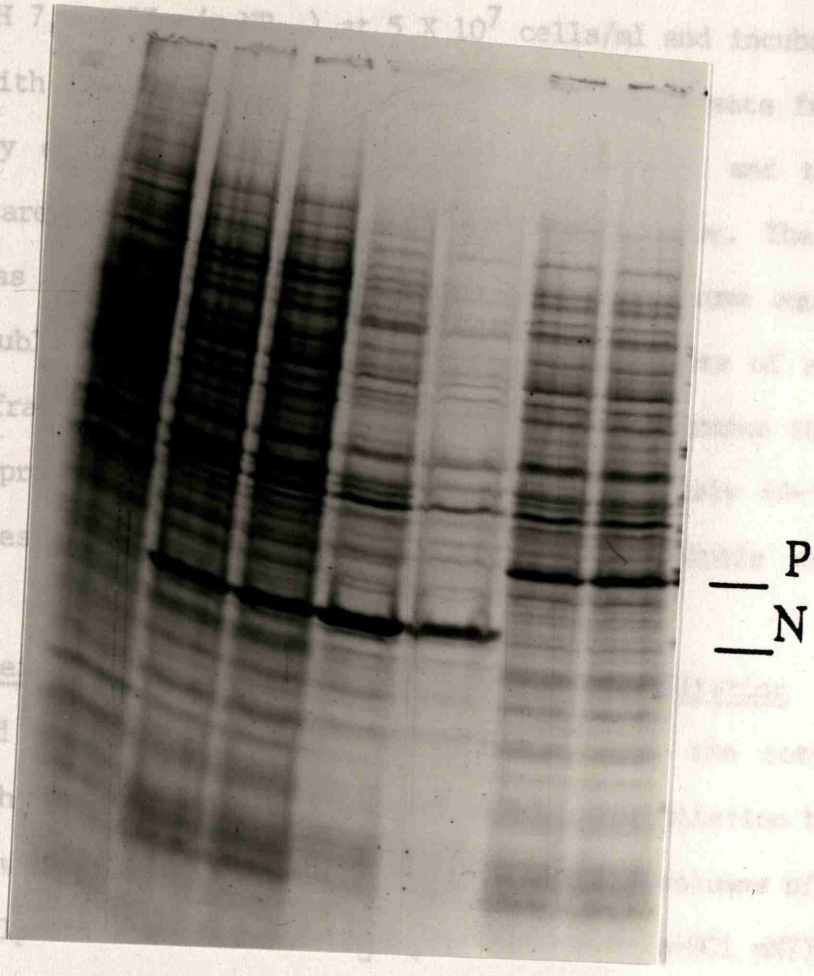
The following protocol was adopted for purification of N protein from a 500 ml spinner culture of 10^8 S.frugiperda cells infected with recombinant baculovirus AcMAGS. The various steps employed included: (A) cell harvesting and lysis; (B) N protein enrichment from soluble cell lysate by ammonium sulphate precipitation; (C) purification of N protein to homogeneity by ion exchange chromatography.

4(A) Cell harvesting and lysis

AcMAGS infected S.frugiperda cells were harvested 4-7 days post infection by centrifugation. Aliquots in 50 ml Falcon tubes were centrifuged at 3K for 15 min at 4 C. The culture supernatant was aseptically removed and stored at 4°C to be used as virus stock. The

Figure 4.

Time course of protein synthesis in AcMAGS infected Spodoptera frugiperda.(a) Sf9 cells in 35 mm dishes infected with AcMAGS or AcNPV at a moi of 5pfu per cell. Cells were labelled with ^{35}S methionine for 1 hour at 48, 72, 96 and 120 hours after infection for AcMAGS infected cells and at 48 and 72 hours for AcNPV infected cells. Labelled proteins were analysed by 15% SDS-PAGE and visualised by autoradiography. Lane (1) mock infected cells; lanes (2) to (5) AcMAGS infected cells at 48, 72, 96 and 120 hours post infection respectively. Lanes (6) and (7) AcNPV infected cells at 48 and 72 hours. The locations of recombinant nucleocapsid (N) protein and baculovirus polyhedrin protein (P) are indicated.


P
N
Lane 1 2 3 4 5 6 7

cell pellets were washed twice in 40 ml of PBS at 4°C. The cells were then subjected to hypotonic shock by resuspension in lysis buffer (10mM Tris-HCl pH 7, 0.05% v/v NP_{40}) at 5 X 10^7 cells/ml and incubated on ice for 1 hr with intermittent vortexing. The insoluble lysate fraction was pelleted by centrifugation at 3K for 5 min at 4 C and the soluble fraction carefully poured into another Falcon tube. The insoluble fraction was resuspended in dissociation mix in a volume equal to that of the soluble lysate fraction and equivalent aliquots of soluble and insoluble fractions were analysed by SDS-PAGE to determine the level of soluble N protein (Figure 5). On average approximately 40-50% of the total expressed N protein was present in the soluble cell lysate fraction.

4(B) N protein enrichment by ammonium sulphate precipitation

An enriched N protein fraction was obtained from the total soluble lysate by the established technique of protein precipitation by 'salting out'. This was achieved by the addition of precise volumes of saturated ammonium sulphate solution (4M NH_3SO_4 in 100 mM Tris-HCl pH7). In order to establish the conditions necessary to obtain a sufficiently enriched N protein fraction with low background protein contamination a series of precipitations was carried out on 1 ml of total lysate. This was accomplished by successively diluting the starting material with precise increasing volumes of saturated ammonium sulphate to obtain specific levels of saturation. After each dilution or cut any precipitate that formed was sedimented by microcentrifugation for 5 min. The supernatant was then poured off and to it was added more ammonium sulphate solution in order to obtain the next level of saturation. Ammonium sulphate cuts were carried out at 15%, 25%, 30%, 35%, 40%, 45% and 60% by the

Figure 5.

Analysis of insoluble and soluble fractions from lysed AcMAGS infected Sf9 cells. Cell pellets were lysed by hypotonic shock by suspension in lysis buffer (10 mM Tris-HCl, pH7, 0.05% v/v NP40) at a density of 5 X 10^7 cells/ml. After 1 hr incubation on ice the insoluble lysate fraction was pelleted by centrifugation and the soluble lysate carefully poured off. The insoluble fraction was resuspended in dissociation mix in a volume equal to that of the soluble lysate fraction and equivalent aliquots of soluble and insoluble fractions were analysed by 15% SDS-PAGE. Protein bands were visualised by Coomassie brilliant blue stain. The location of the N protein is indicated. Lane (1) total cell lysate, (2) cell lysate, insoluble fraction, (3) cell lysate, soluble fraction.


N
Lane 1 2 3

successive addition of predetermined volumes of ammonium sulphate as described in table 1. Precipitation was carried out for an hour on ice. The precipitated protein fractions were resuspended in equivalent volumes of 10 mM Tris-HCl, pH 7 and dialysed to remove the ammonium sulphate. Equivalent volumes from each sample were analysed by SDS-PAGE and protein bands visualised by Coomassie brilliant blue staining (Figure 6). This analysis revealed that the 15% to 25% cut gave the most favourably enriched N fraction with relatively low level of background proteins in comparison with the other fractions. However, the initial 0-15% cut did not precipitate a significant amount of protein and therefore it was decide that bulk N protein enrichment should be carried out by a direct one step 0-25% ammonium sulphate cut. This was carried out by the addition of 333 ul of 4M ammonium sulphate per ml of total soluble lysate in accordance with Table 1. After the addition of the ammonium sulphate the solution was incubated on ice for at least 1 hr with intermittent vortexing. The precipitated protein fraction was pelleted by centrifugation in a SS34 rotor at 10K for 20 min at 4°C and the pellet resuspended in a minimum volume of 10 mM Tris-HCl pH7 and dialysed o/n at 4°C against several changes of 10 mM Tris-HCl pH7 to remove any remaining ammonium sulphate. Samples taken from this fraction were analysed by 15% SDS-PAGE and by the Pierce Coomassie brilliant blue assay to determine the total protein present in the enriched fraction. On average the total protein concentration was approximately 1mg/ml. The relative proportion of N protein present was roughly determined by visual inspection of Coomassie brilliant blue stained acrylamide gels of protein samples. The N protein routinely was found to make up between 30-40% of the total protein present. Ammonium sulphate fraction

Figure 6.

Ammonium sulphate fractionation of total soluble lysate from AcMAGS infected Sf9 cells. In order to establish the optimum concentration of ammonium sulphate to be used to generate an enriched nucleocapsid protein fraction from total soluble cell lysate a series of sequential ammonium sulphate precipitations were carried out: a fraction of total soluble cellular lysate was mixed with a specific volume of 4 M ammonium sulphate; incubated on ice for 1 hr after which time precipitated protein was pelleted by centrifugation; the remaining soluble lysate transferred to a clean tube; more ammonium sulphate added and the process repeated. The protein precipitate obtained in the presence of a given percentage concentration of ammonium sulphate was referred to as a protein cut. Protein cuts of total soluble lysate were taken at 15, 25, 30, 40, 45 and 60% ammonium sulphate. Protein precipitates were re-dissolved in equal volumes, dialysed and equivalent fractions analysed by 15% PAGE in the presence of SDS and proteins visualised by Coomassie brilliant blue staining. The location of the N protein is indicated. Lane (1) total soluble lysate, (2) 0 to 15% cut, (3) 15 to 25% cut, (4) 25 to 30% cut, (5) 30 to 35% cut, (6) 35 to 40% cut, (7) 40 to 45% cut, (8) 45 to 60% cut.

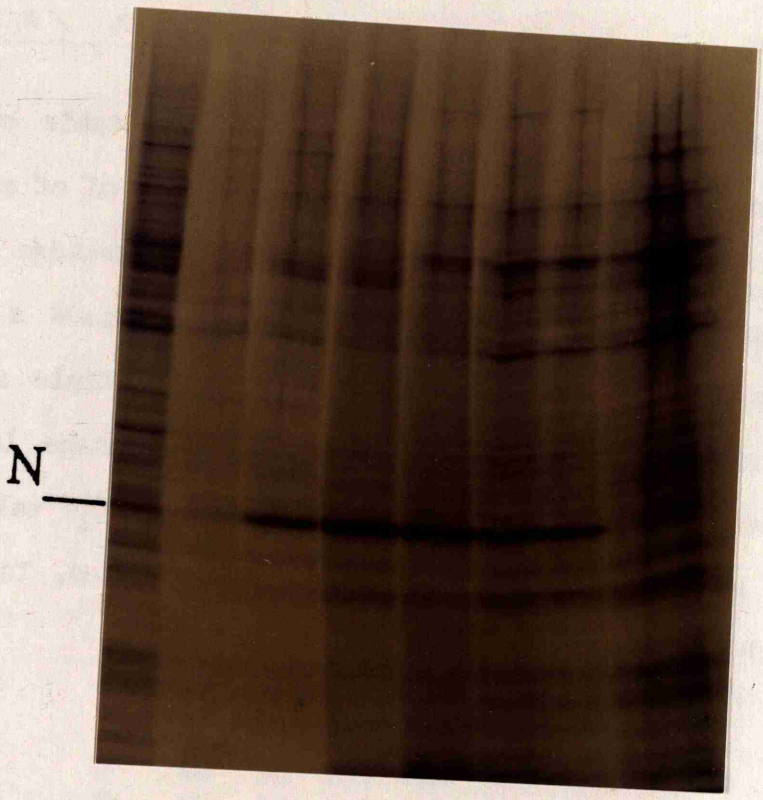

N
Lane 1 2 3 4 5 6 7 8

Table 1.

Ammonium sulphate percentage saturation table. The table shows the amount of saturated ammonium sulphate solution (µl per ml of sample) to be added to a sample, with a specific starting percentage level of ammonium sulphate saturation (C_1%), in order to achieve a specific higher percentage level of saturation (C_2%). In multiple rounds of stepped saturation to obtain subsequent higher percentage levels the obtained C_2% value is taken as the starting percentage C_1% value in the next round of saturation and the new C_2% value selected. Table taken from Scopes (1987).

Table I

From C_1% ↘ To C_2% →	5	10	15	20	25	30	35	40	45
0	52	111	176	250	333	428	538	666	818
5	55	117	187	266	357	461	583	727
10	58	125	200	285	384	500	636
15	62	133	214	307	416	545
20	66	142	230	333	454
25	71	153	250	363
30	76	166	272
35	83	181
40	90
45	50

From C_1%	50	55	60	65	70	75	80	85
0	1000	1222	1500	1857	2333	3000	4000	5666
5	900	1111	1375	1714	2166	2800	3750	5333
10	800	1000	1250	1571	2000	2600	3500	5000
15	700	888	1125	1428	1833	2400	3250	4666
20	600	777	1000	1285	1666	2200	3000	4333
25	500	666	875	1142	1500	2000	2750	4000
30	400	555	750	1000	1333	1800	2500	3666
35	300	444	625	857	1166	1600	2250	3333
40	200	333	500	714	1000	1400	2000	3000
45	100	222	375	571	833	1200	1750	2666
50	111	250	428	666	1000	1500	2333
55	125	285	500	800	1250	2000
60	142	333	600	1000	1666
65	166	400	750	1333
70	200	500	1000
75	250	666
80	333

saturation at other pH values (7.5 and 8.0) did not produce better levels of N enrichment than that obtained at pH 7.0 because of insolubility of other proteins in the presence of ammonium sulphate at these pH values.

4(C) Ion exchange chromatography

Purification of the N protein to homogeneity was achieved by application of the enriched N protein fraction to a 8 ml Mono Q HR5/5 Pharmacia FPLC anion exchange column. Immediately prior to application of the sample to the column, the sample was passed through a 0.45 μm and then a 0.2 μm filters to remove any remaining insoluble particles. This process did not affect levels of N protein in the sample fractions. The protein sample in a volume of 6 ml was applied to the Mono Q column under zero salt conditions in 10 mM Tris-HCl pH 7. The column was washed with 1 volume of 10mM Tris-HCl pH 7 and proteins eluted from the column over a 0-1 M NaCl step gradient in 10 mM Tris-HCl pH7 over 30 ml volume at a flow rate of 1 ml/min. Eluted fractions were collected in 1 ml aliquots and equal volumes analysed by SDS-PAGE and protein bands resolved by Coomassie brilliant blue stain. The N protein was eluted from the column above 0.55 M NaCl (Figure 7), but the first few N protein containing fractions were contaminated with other proteins. Holding the NaCl gradient at 0.45 M and again at 0.5 M NaCl for 5 ml at each NaCl concentration, prior to continuation of the gradient, resulted in the elution of N protein free from contaminating background proteins as determined by SDS-PAGE and visualisation of protein bands by the sensitive detection technique of silver staining (Figure 8).

A fraction of purified N protein was analysed by gel filtration chromatography to determine the overall structure of the protein in

Figure 7.

Purification of recombinant N protein by ion exchange chromatography. A 6 ml nucleocapsid protein enriched fraction from crude soluble lysate of AcMAGS infected cells, obtained by a 0-25% ammonium sulphate cut, was filtered through 0.45 and 0.2 um filters and chilled on ice before application to a FPLC Mono Q column HR5/5 (Pharmacia) at a flow rate of 1 ml per minute. The column was washed with one volume of application buffer (10 mM Tris-HCl, pH7.0). Proteins were eluted from the Mono-Q column in 10 mM Tris-HCl, pH 7.0 over a two part stepped NaCl gradient of 0 to 0.5 M NaCl over a 20 ml volume, held at 0.5 M NaCl for 3ml then continuing from 0.5 to 1.0 M NaCl over a further 10 ml. Samples were collected in 1 ml fractions and 50 ul aliquots analysed by 12% PAGE in the presence of SDS. Protein bands were resolved by Coomassie brilliant blue stain. The N protein was eluted from the column between 0.5 and 1.0 M NaCl. Samples from various fractions eluted between 0.45 and 1.0 M NaCl are shown, lanes (9) to (1). Lane (1) 0.9 M NaCl; (2) 0.8 M NaCl; (3) 0.7 M NaCl; (4) 0.6 M NaCl; (5) 0.5 M NaCl; (6) 0.5 M NaCl; (7) 0.5 M NaCl; (8) 0.475 M NaCl; (9) 0.45 M NaCl. Above 0.6M NaCl, lanes (4) to (1), N protein was eluted in the absence of other contaminating proteins. A sample of the starting material that was applied to the column is also shown, lane (10).

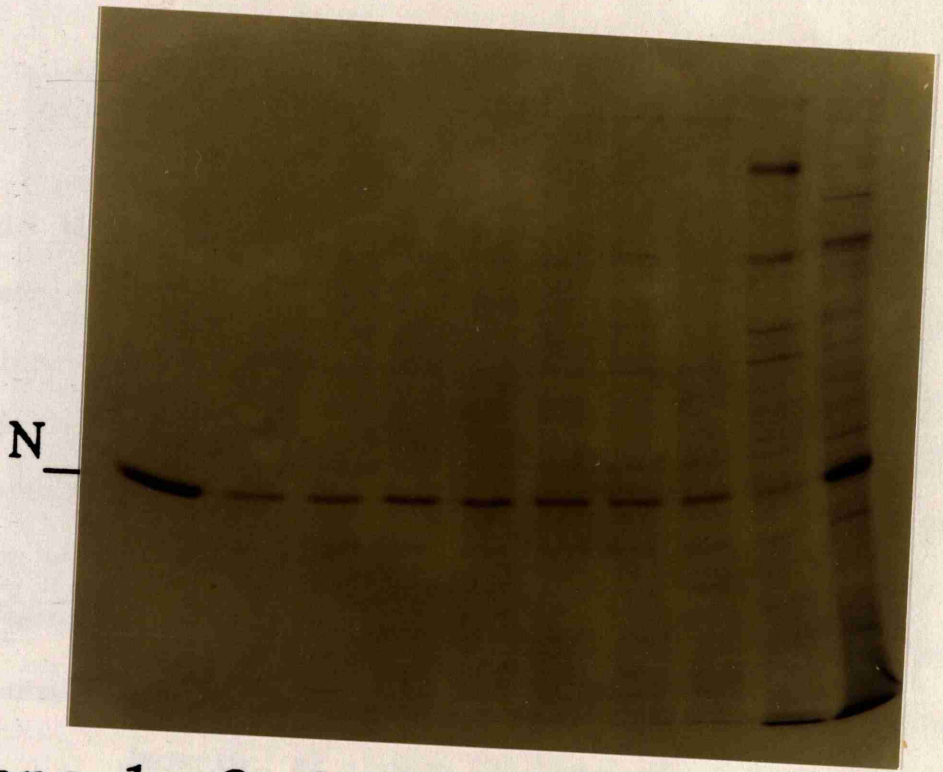

N
Lane 1 2 3 4 5 6 7 8 9 10

Figure 8.

Analysis of purified nucleocapsid protein fractions by silver staining. Ten microliter samples of 1 ml nucleocapsid protein purified fractions eluted from a Mono Q FPLC column were analysed by 12% PAGE in the presence of SDS and the proteins visualised by silver staining as described in Materials and Methods. Lanes (1) to (7) are purified N protein fractions eluted from the column above 0.6 M NaCl and lane (8) is a typical fraction of the starting material before application to the Mono Q column. The location of the nucleocapsid (N) protein is indicated.


N
Lane 1 2 3 4 5 6 7 8

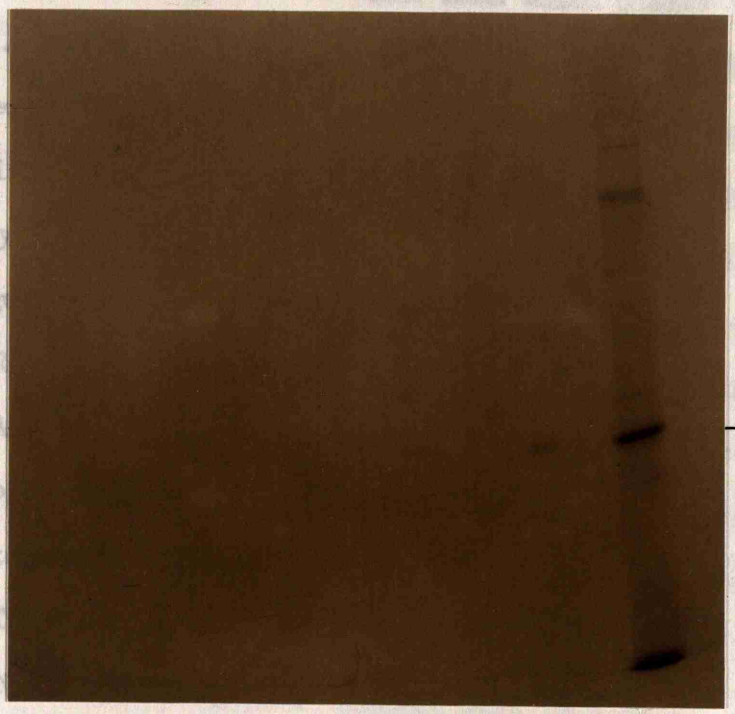
solution. Gel filtration was carried out on a calibrated Superose 12 FPLC column. The N protein fraction was desalted by dialysis prior to application to the column. The filtration was carried out in 10mM Tris-HCl pH7 at a flow rate of 25ml/hr. Eluted fractions were collected in 1ml aliquots and equivalent fractions were analysed by 12% SDS-PAGE and protein bands visualised by Coomassie brilliant blue stain (Figure 9). A faint band corresponding to the N protein was observed in a single fraction corresponding to a high M_r value. Further analysis of the eluted fractions on a silver stained gel revealed that the N protein was present in all fractions indicating that the N protein in solution was forming oligmers of various sizes.

The purified N protein fractions were analysed by spectroscopy to determine the level of nucleic acid contamination (Layne, 1957). Analysis of the $A_{260/280nm}$ ratios of the purified N protein fractions revealed that there was no significant contamination of the purified N protein with nucleic acid. It is possible that N protein heavily contaminated with nucleic acid was retained in the insoluble cell lysate fraction as this contained nuclear material.

Overall, the yield of purified N protein was low in comparison to the amount of N protein present in the start material applied to the column ie approximately 100 ug of purified N protein from 2 mg of crude N protein fraction. A variety of changes were made to the basic protocol in an attempt to increase the yield of N protein. Increasing the ion exchange NaCl gradient from 1 M to 2 M NaCl failed to yield any additional N protein. An attempt at reducing protein aggregation by the inclusion of a zwiterionic detergent, CHAPS, at 8 mM in all solutions used in the cell lysis and N protein purification stages also failed to

Figure 9.

Gel filtration chromatography of purified N protein. A pre-calibrated Superose 12 FPLC column was used for gel filtration analysis of N protein in 10 mM Tris HCl pH 7. Approximately 2 µg of purified N protein was desalted in 10mM Tris HCl pH 7 and then applied to the column in a 100 ul volume. The column was run at a flow rate of 25 ml/hr and and the eluted material collected in 1ml fractions. Equivalent fractions were anlaysed by 12% PAGE in the presence of SDS and protein bands visualised by Coomassie brilliant blue staining. The N protein was detected in a single fraction (lane 12) eluted from the gel at around the same point as BSA protein (69 kDa). However, silver staining of a duplicate gel revealed that N protein was present in all eluted fractions (25 - >200 kDa), which implied that N protein was forming multimers of varying sizes. Lanes 1-13 eluted column fractions. Lane 14 is a control track of partially purified N protein.



Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

affect the yield of purified N protein.

Analysis of nucleocapsid protein RNA binding activity

It is known that N protein exclusively encapsidates viral RNA in infected cells and that this selectivity is for positive or negative sense full length RNA as opposed to viral mRNA which posses different 5' and 3' termini. By studying in vitro the interaction of N protein with S segment derived RNA it should be possible to determine the nature of this selectivity. Recombinant nucleocapsid RNA binding activity was investigated by three different assay systems: North-west blot; filter binding; and gel retardation assay. The RNA used in these assays were derived by T7 RNA polymerase directed run-off transcription reactions of BUN S cDNAs. As previously stated BUN and MAG S segments share a high sequence homology and transcripts derived from BUN S templates were used in preference to MAG S templates because both positive and negative full length BUN S T7 transcription templates were available, as well as an abbreviated BUN S cDNA template, whereas only a positive sense full length transcription template was available for MAG S.

5. Filter binding assay

Filter binding assays were carried out to initially establish optimum conditions for purified nucleocapsid protein RNA binding. This assay system was developed originally to study the interaction of bacteriophage coat protein with RNA (Carey et al, 1983) and has subsequently been successfully employed in defining a number of RNA protein interactions. The assay is based on the principle that RNA/protein complexes are retained on a nitrocellulose filter whereas free RNA passes through the filter. The conditions employed were based on those used by Yamanaka et al (1990) to investigate influenza virus

nucleoprotein (NP) RNA binding activity and are described in Materials and Methods. The RNA binding activity of N protein was examined using ^{32}P labelled full length (+) and (-) sense S RNA derived by *in vitro* transcription of plasmid templates pUCT7BUNS(+) and pUCT7BUNS(-). The RNA binding activity of recombinant N protein was investigated under different temperatures, sodium chloride concentrations and pH values. Specificity of interaction was also investigated by comparing the interaction of N protein with bunyavirus specific S RNA and non-specific RNA.

5(A) pH

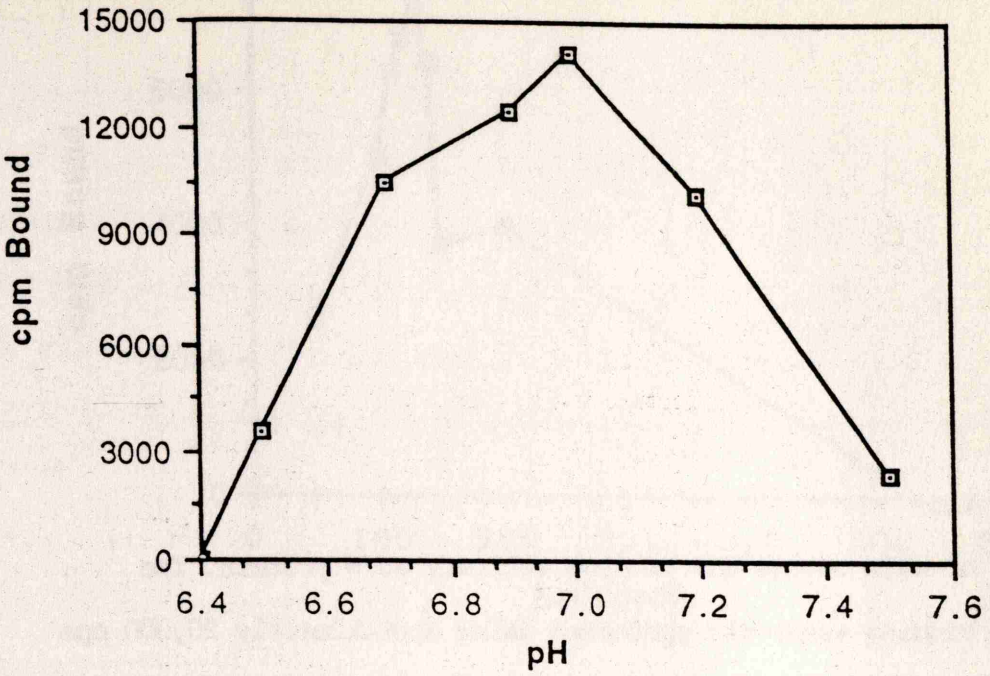
The effect of pH on N protein RNA binding was investigated over a pH range of 6.4 to 7.5 (Figure 10). Maximal levels of binding was observed at pH 7 with a relatively sharp decline in binding levels above or below this value. These results indicate that *in vitro* optimum N protein binding is restricted to around pH 7.0. This contrasts with bunyavirus nucleocapsids structures which are stable over a wider pH range. *In vitro* influenza virus nucleoprotein is able to bind RNA over a broader pH range (Yamanka *et al*, 1990).

5(B) Salt

Nucleocapsid protein-RNA binding was investigated over salt concentrations from 50 to 500 mM sodium chloride (Figure 11). The highest level of bound RNA was observed within a narrow range of 120 to 140 mM NaCl and was at a maximal value at 130 mM. High concentrations of salt (500mM) significantly depressed N protein binding, possibly by impeding N-N interaction that may be essential for maximal binding. The peak level of N-RNA binding occurs at concentrations similar to endogenous cellular levels of salt and are similar to the optimum value

Figure 10.

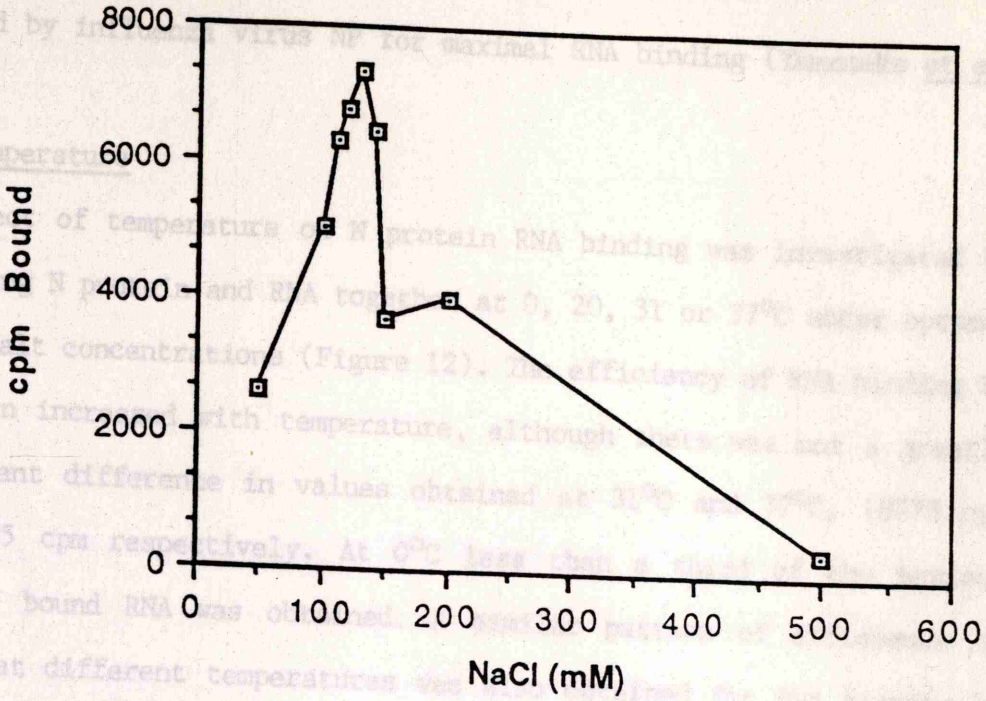
Effect of pH on the RNA binding activity of N protein. The filter binding assay was performed using approximately 20,000 cpm of ^{32}P labelled full length BUN S (+) RNA incubated in the presence of 120 ng of N protein for 20 minutes at 31°C in 130mM NaCl in a 50µl reaction under conditions described in Materials and Methods over a pH range from 6.4 to 7.5. The number of counts retained on the filter are shown and plotted against pH. The cpm of RNA bound were obtained after the subtraction of background counts for filtered RNA samples incubated in the absence of N protein.



pH	cpm Bound
6.4	0.0
6.5	3586.7
6.7	10458.7
6.9	12468.2
7.0	14109.2
7.2	10155.7
7.5	2437.2

Figure 11.

Effect of salt on the RNA binding activity of N protein. The filter binding assay was performed using approximately 20,000 cpm of ^{32}P labelled full length BUNS (+) RNA in the presence of 120ng of N protein under optimum binding conditions as described in Materials and Methods except that samples were incubated at 31°C in different concentrations of sodium chloride for 20 minutes prior to filtration. The number of counts retained on the filter at the different concentrations of sodium chloride are shown. The values shown were obtained after the subtraction of background counts for filtered RNA samples incubated in the absence of N protein at the different NaCl values.



[NaCl] mM	cpm Bound
50.0	2601.2
100.0	5026.6
110.0	6296.1
120.0	6781.5
130.0	7331.9
140.0	6456.1
150.0	3700.1
200.0	3979.7
500.0	348.2

required by influenza virus NP for maximal RNA binding (Yamanaka et al, 1990).

5(C) Temperature

The effect of temperature on N protein RNA binding was investigated by incubating N protein and RNA together at 0, 20, 31 or 37°C under optimum pH and salt concentrations (Figure 12). The efficiency of RNA binding by N protein increased with temperature, although there was not a greatly significant difference in values obtained at 31°C and 37°C, 18775 cpm and 19885 cpm respectively. At 0°C less than a third of the maximum level of bound RNA was obtained. A similar pattern of efficiency of binding at different temperatures was also obtained for RNA binding by influenza virus NP (Yamanaka et al (1990).

5(D) N protein concentration

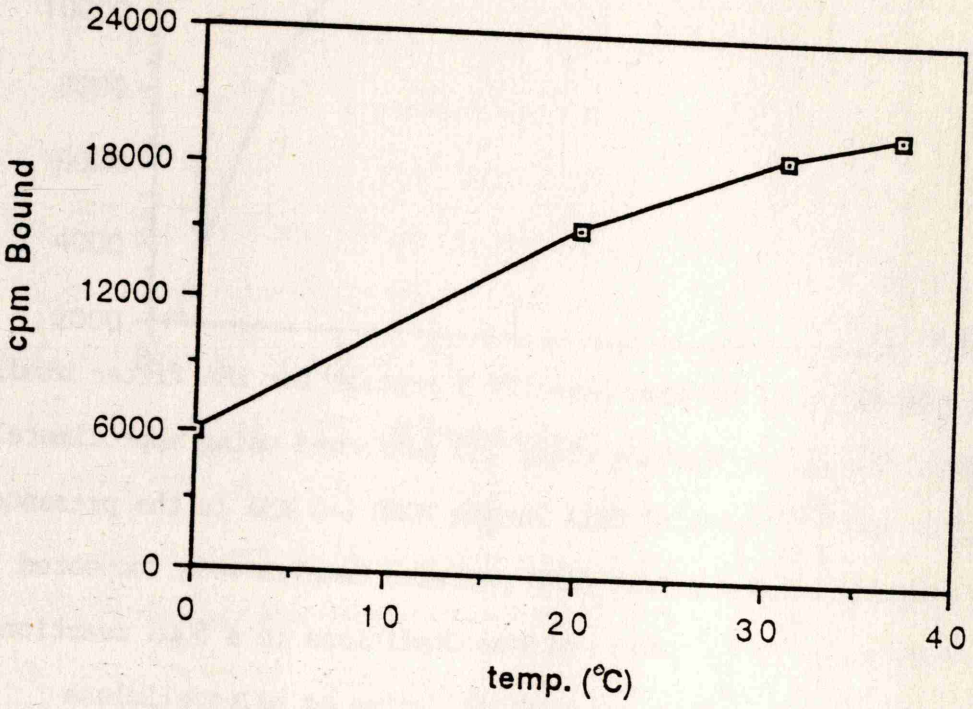
The effect of different concentrations of N protein on the RNA binding efficiency of a fixed amount of RNA was investigated at 31°C under optimum conditions (Figure 13). RNA binding efficiency proportionally increased with increasing amounts on N protein. In the presence 52 ng N protein approximately 67% (12404 cpm) of total counts were bound. The total percentage of counts bound did not significantly rise after that point with only a 2.5% increase in bound RNA (13902 cpm) when the concentration of N was doubled to 102 ng or tripled to 153 ng (13909 cpm). These results indicate that free RNA is rapidly bound by N protein but requires a minimum concentration of N protein before maximal levels of bound RNA are obtained. This may be due to a co-operative N-N interaction upon initial RNA-N interactions.

5(E) RNA Template specificity

The specificity of interaction of N protein with RNA was investigated by

Figure 12.

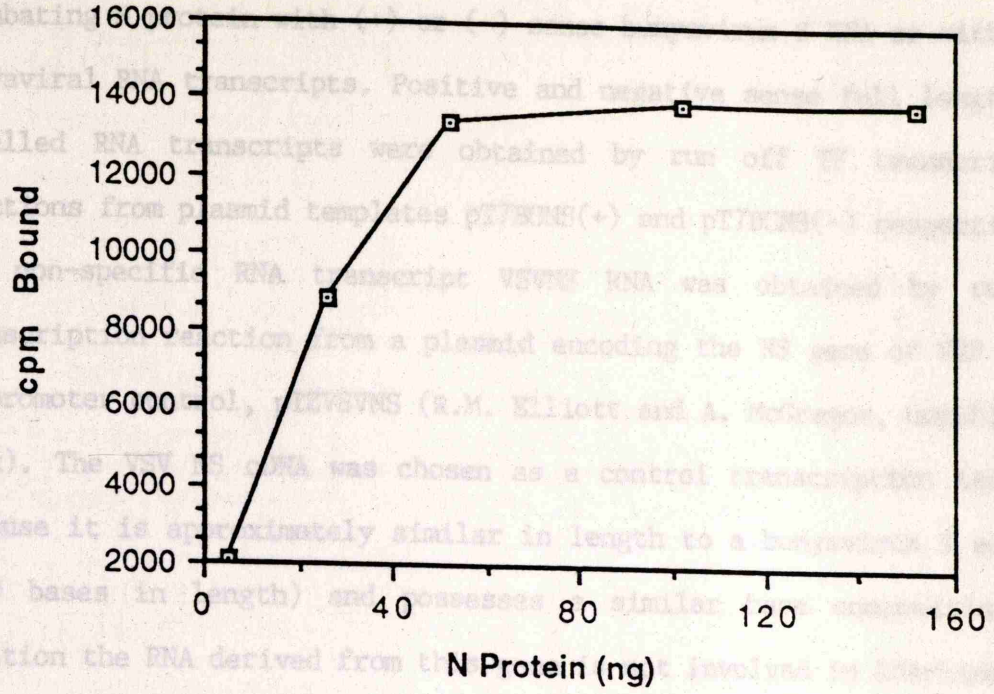
Effect of temperature on the RNA binding activity of N protein. The filter binding assay was performed using approximately 20,000 cpm of ^{32}P labelled full length BUNS (+) RNA in the presence of 120ng of N protein under optimum conditions in a 50µl reaction as described in Materials and Methods except that RNA complexes were allowed to occur at 0, 20, 31 or 37°C for 20 minutes prior to filtration. The values shown were obtained after subtraction of background counts for filtered RNA samples incubated in the absence of N protein at these various temperatures.



Temp (°C)	cpm Bound
0.0	6000.0
20.0	15450.3
31.0	18775.1
37.0	19885.2

Figure 13.

Determination of optimum level of N protein for RNA filter binding assay. The filter binding assay was performed using approximately 20,000 cpm ^{32}P labelled full length BUNS (+) RNA in the presence of varying concentrations of N protein. Samples were incubated for 20 minutes at 31°C, under optimum conditions in a 50µl reaction as described in Materials and Methods, prior to nitrocellulose filtration. The number of counts retained on the filter are shown and plotted against amount of N protein (ng) used. The cpm of RNA bound were obtained after the subtraction of background counts for filtered RNA samples incubated in the absence of N protein.



N (ng)	cpm Bound
5.2	2045.6
26.0	8837.7
52.0	13404.2
102.0	13902.2
152.0	13909.5

incubating N protein with (+) or (-) sense bunyavirus S RNA or with non-bunyaviral RNA transcripts. Positive and negative sense full length ^{32}P labelled RNA transcripts were obtained by run off T7 transcription reactions from plasmid templates pT7BUNS(+) and pT7BUNS(-) respectively. The non-specific RNA transcript VSVNS RNA was obtained by run-off transcription reaction from a plasmid encoding the NS gene of VSV under T7 promoter control, pTZVSVNS (R.M. Elliott and A. McGregor, unpublished work). The VSV NS cDNA was chosen as a control transcription template because it is approximately similar in length to a bunyavirus S segment (860 bases in length) and possesses a similar base composition. In addition the RNA derived from this gene is not involved in RNA-binding.

In all cases the assay was carried out with a fixed amount of ^{32}P labelled RNA and a range of N protein concentrations under optimal pH and salt conditions at 31°C. The results are shown in Figure 14. Both (+) and (-) sense BUNS RNA were bound by N protein in a similar manner in separate assays. The non-specific VSVNS RNA was also bound by N protein with similar affinity. This would imply that RNA binding by N protein as monitored by filter binding assay is non-specific. Subsequent filter binding competition experiments were therefore not pursued.

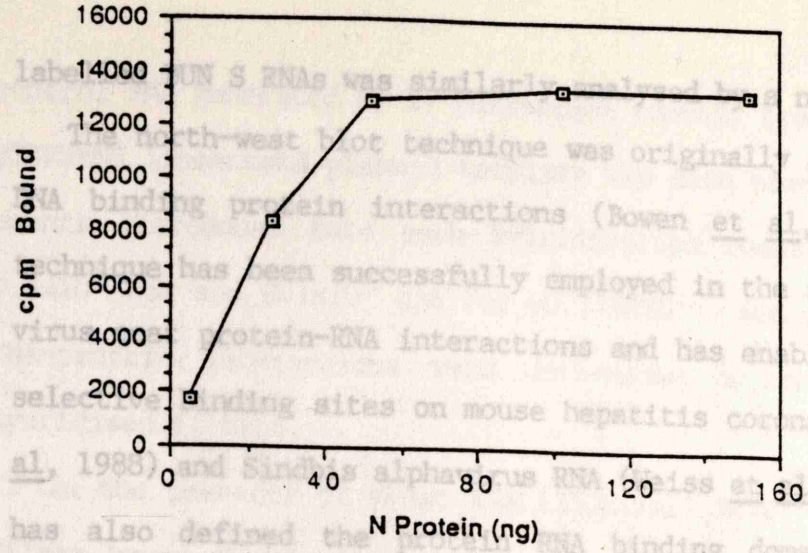
6. North-west blot assay

The interaction between recombinant baculovirus expressed Maguari virus N protein and Bunyamwera virus S RNA was investigated by north-west blot assay. In this technique proteins from recombinant baculovirus (AcMAGS) infected Sf9 cell lysates were separated by SDS-PAGE, trans-ferred onto nitrocellulose filters and hybridised with ^{32}P labelled RNAs generated *in vitro*. The Maguari virus N protein was also expressed in bacterial cells as a β-gal fusion protein and the interaction of this protein with

Figure 14.

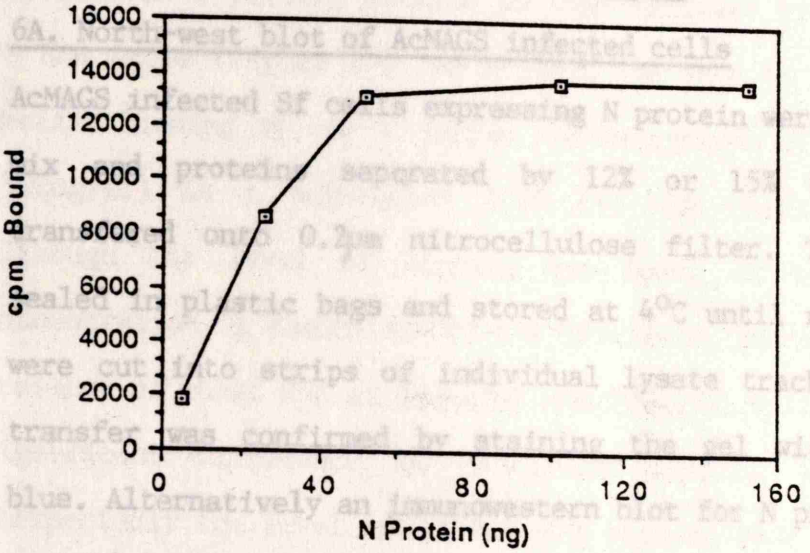
RNA binding activity of N protein for specific and non-specific RNA templates. RNA binding assays were performed using either ^{32}P labelled full length BUNS (+) or BUNS (-) RNA transcripts derived by run-off *in vitro* transcription reaction from 3' linearised plasmid templates pT7BUNS (+) and pT7BUNS (-) respectively. A non-specific ^{32}P labelled RNA template VSV NS RNA was derived by run-off transcription reaction from 3' linearised plasmid template pTZVSVNS as described in Materials and Methods. RNA binding was performed with approximately 20,000 cpm of ^{32}P labelled RNA (BUNS (+), BUNS(-) or VSVNS RNA) incubated with 5.2, 26, 52, 102 and 152 ng of N protein at 31°C for 20 minutes in a 50µl reaction under optimum salt and pH conditions prior to filtration as described in Materials and Methods. The number of counts retained on the filter are shown and plotted against concentration of N protein (ng). The cpm shown were obtained after the subtraction of background counts for filtered RNA samples incubated in the absence on N protein. Graph: (a) cpm BUNS (+) RNA bound vs N protein concentration; (b) cpm BUNS (-) RNA bound vs N protein concentration; (c) cpm VSVNS RNA bound vs N protein concentration.

(a)



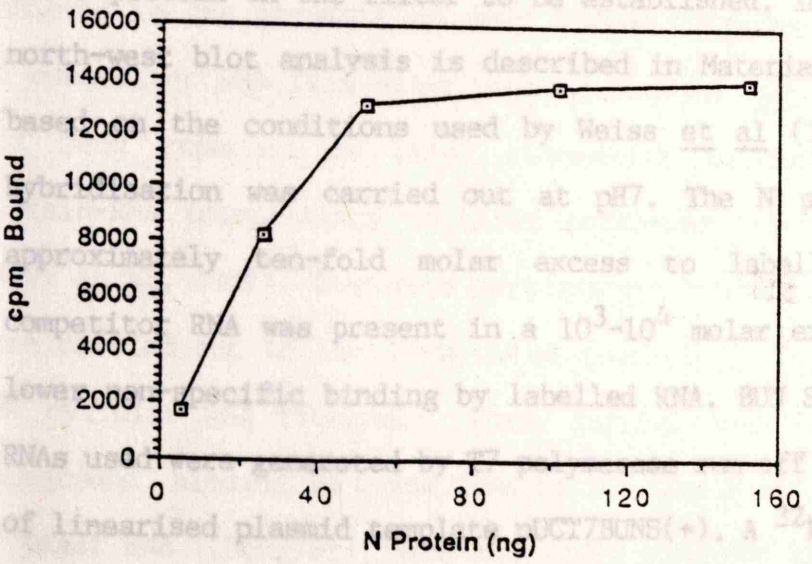
N (ng)	cpm Bound
5.2	1731.1
26.0	8231.3
52.0	12981.1
102.0	13731.4
152.0	13901.2

(b)



N (ng)	cpm Bound
5.2	1809.7
26.0	8579.4
52.0	13079.9
102.0	13757.2
152.0	13774.2

(c)



N (ng)	cpm Bound
5.2	1757.4
26.0	8407.6
52.0	12907.3
102.0	13464.1
152.0	13481.8

labelled BUN S RNAs was similarly analysed by a north-west blot.

The north-west blot technique was originally applied to the study of DNA binding protein interactions (Bowen et al, 1980). However, this technique has been successfully employed in the study of eukaryotic RNA virus coat protein-RNA interactions and has enabled the localisation of selective binding sites on mouse hepatitis coronavirus RNA (Stohlman et al, 1988) and Sindbis alphavirus RNA (Weiss et al, 1989). This technique has also defined the protein RNA binding domain of hepatitis delta antigen to hepatitis delta RNA (Lin et al, 1990).

6A. North-west blot of AcMAGS infected cells

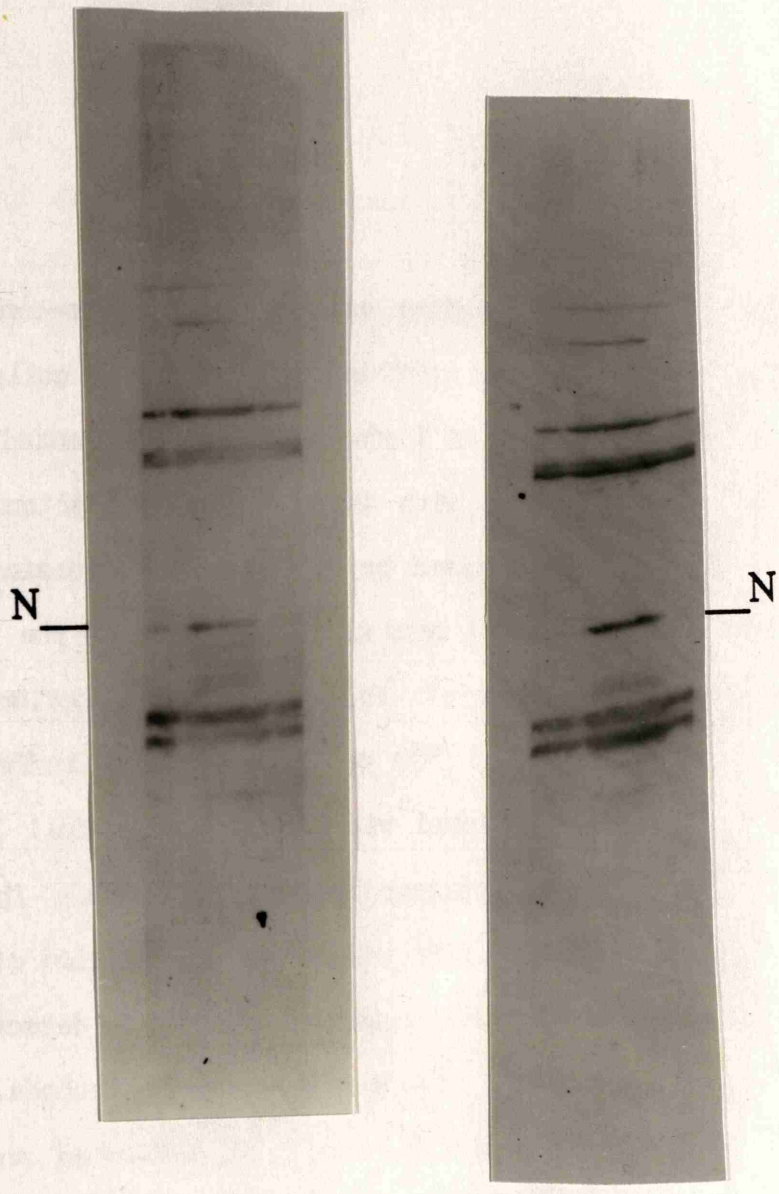
AcMAGS infected Sf cells expressing N protein were lysed in dissociation mix and proteins separated by 12% or 15% SDS-PAGE before being transfered onto 0.2µm nitrocellulose filter. The filters were then sealed in plastic bags and stored at 4°C until required whereupon they were cut into strips of individual lysate tracks before use. Protein transfer was confirmed by staining the gel with Coomassie brilliant blue. Alternatively an immunowestern blot for N protein was performed on a sample strip of the filter. This additionally enabled the location of the N protein on the filter to be established. The protocol adopted for north-west blot analysis is described in Materials and Methods and was based on the conditions used by Weiss et al (1989), except that RNA hybridisation was carried out at pH7. The N protein was present at approximately ten-fold molar excess to labelled RNA. Non-specific competitor RNA was present in a 10^3-10^4 molar excess in an attempt to lower non-specific binding by labelled RNA. BUN S specific ^{32}P labelled RNAs used were generated by T7 polymerase run-off transcription reaction of linearised plasmid template pUCT7BUNS(+). A ^{32}P-labelled non-specific

control RNA generated by T7 polymerase run-off transcription reaction of pTZVSVNS linearised plasmid template was also used. The total number of counts introduced into each hybridisation reaction was approximately $0.5x10^6$ cpm and $0.1x10^6$ cpm for pUCT7BUNS(+) and pTZVSVNS respectively. RNA-protein interactions were determined by autoradiography of the hybridised filters.

In the presence of yeast RNA (250µg/ml) BUNS(+) RNA interacted with filter bound N protein and other unidentified cellular or baculovirus proteins in a pattern similar to that achieved by the non specific VSV NS RNA probe (Figure 15). An attempt was made to lower background non-specific binding by use of higher concentrations of competitor yeast RNA (500µg/ml) in a subsequent BUNS (+) RNA north-west blot. However, although the level of binding was lower at the higher yeast RNA concentration, a comparison of the hybridisation results obtained in the presence of yeast RNA at 250µg/ml and 500µg/ml failed to demonstrate any significant difference in background binding pattern (Figure 16). Unexpectedly the level of BUNS(+) RNA binding to N protein was halved in the presence of yeast RNA at 500µg/ml in comparison to the level achieved in the presence of yeast RNA at 250µg/ml (Figure 16). The use of competitor RNA from other sources such as E.coli or mammalian cells had been reported as being successful in depressing non-specific protein-RNA interactions in other north-west blots (Lin et al, 1990; Weiss et al, 1989). Therefore northwestern hybridisations of BUNS(+) RNA were carried out in the presence of competitor E.coli RNA (100µg/ml) or BHK cellular RNA (50µg/ml). These carrier RNAs succeeded in reducing background binding in comparison to the levels obtained in the presence of yeast RNA (250µg/ml). However, their presence also reduced N protein

Figure 15.

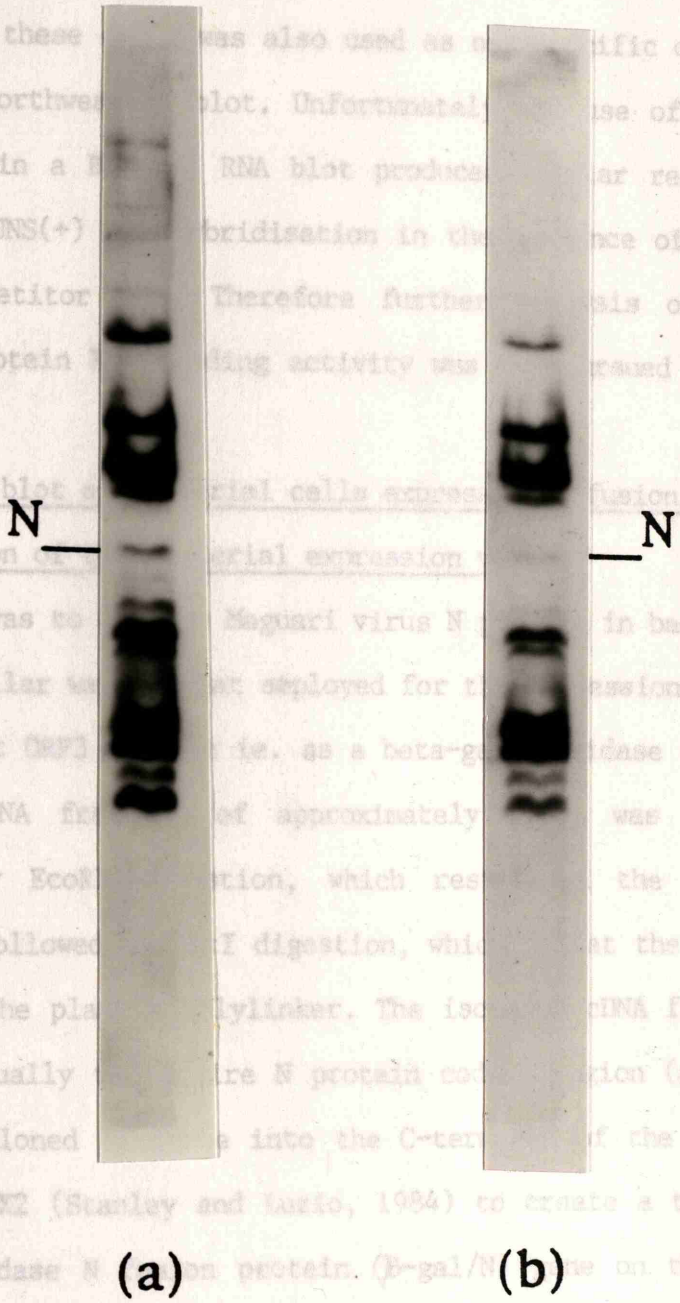
North-western blot showing binding of specific and non-specific RNA transcripts to recombinant nucleocapsid protein. Aliquots of Sf9 cell lysate infected with AcMAGS were electrophoresised by 12% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose and the filter cut up into single track strips. Following pre-incubation with E.coli RNA at 250 µg/ml in binding buffer the nitrocellulose strips were probed with ^{32}P-labelled RNA transcripts: BUNS(+) RNA (approximately 0.5 x 10^6 cpm) and VSVNS RNA (approximately 0.1 x 10^6 cpm). The virus specific probe (BUNS(+) RNA) and non-specific probe (VSVNS RNA) were generated by T7 run-off transcription reactions of linearised plasmid templates pT7BUNS(+) and pTZVSVNS respectively. RNA-protein bands were detected by autoradiography as described in Materals and Methods. The location of the N protein on the filter is indicated and was determined by immunowestern blot of an equivalent strip using Bunyamwera virus specific antiserum. Exposure times at -70°C for blots are o/n for VSVNS probed blot and 1hr for BUNS (+) probed blot.



Probe VSV NS RNA BUN S (+) RNA

Figure 16.

North-western blot showing binding of BUNS RNA with recombinant nucleocapsid protein. A 30 mm confluent plate of Sf9 cells were infected with AcMAGS at a moi of 5 pfu. Cells were harvested at 48 hours post infection by lysis with dissociation mix. Aliquots of cell lysate were electrophoresised by 12% SDS-PAGE. Proteins were electrotransferred from the gel onto nitrocellulose and the filter cut up into single track strips. Following pre-incubation with E.coli RNA at (a) 250 or (b) 500 µg/ml in binding buffer the nitrocellulose strips were probed with ^{32}P labelled full length BUNS (+) RNA transcript (approximately 0.5 X 10^6 cpm). The RNA transcript was obtained by run-off transcription reaction of XbaI linearised template pT7BUNS(+). RNA-protein bands were detected by autoradiography as described in Materials and Methods. The location of the N protein on the filter is indicated and was determined by immunowestern blot of an equivalent strip using Bunyamwera virus specific antiserum.


N
N
(a)
(b)

binding by the same margin giving an approximate overall 20-fold reduction in binding. Since MAG N protein was expressed in Sf9 cells, total RNA from these cells was also used as non-specific competitor RNA in a BUNS(+) northwestern blot. Unfortunately the use of Sf9 cellular RNA (50µg/ml) in a BUNS(+) RNA blot produced similar results to that obtained for BUNS(+) RNA hybridisation in the presence of mammalian or bacterial competitor RNA. Therefore further analysis of baculovirus expressed N protein RNA binding activity was not pursued by north-west blot assay.

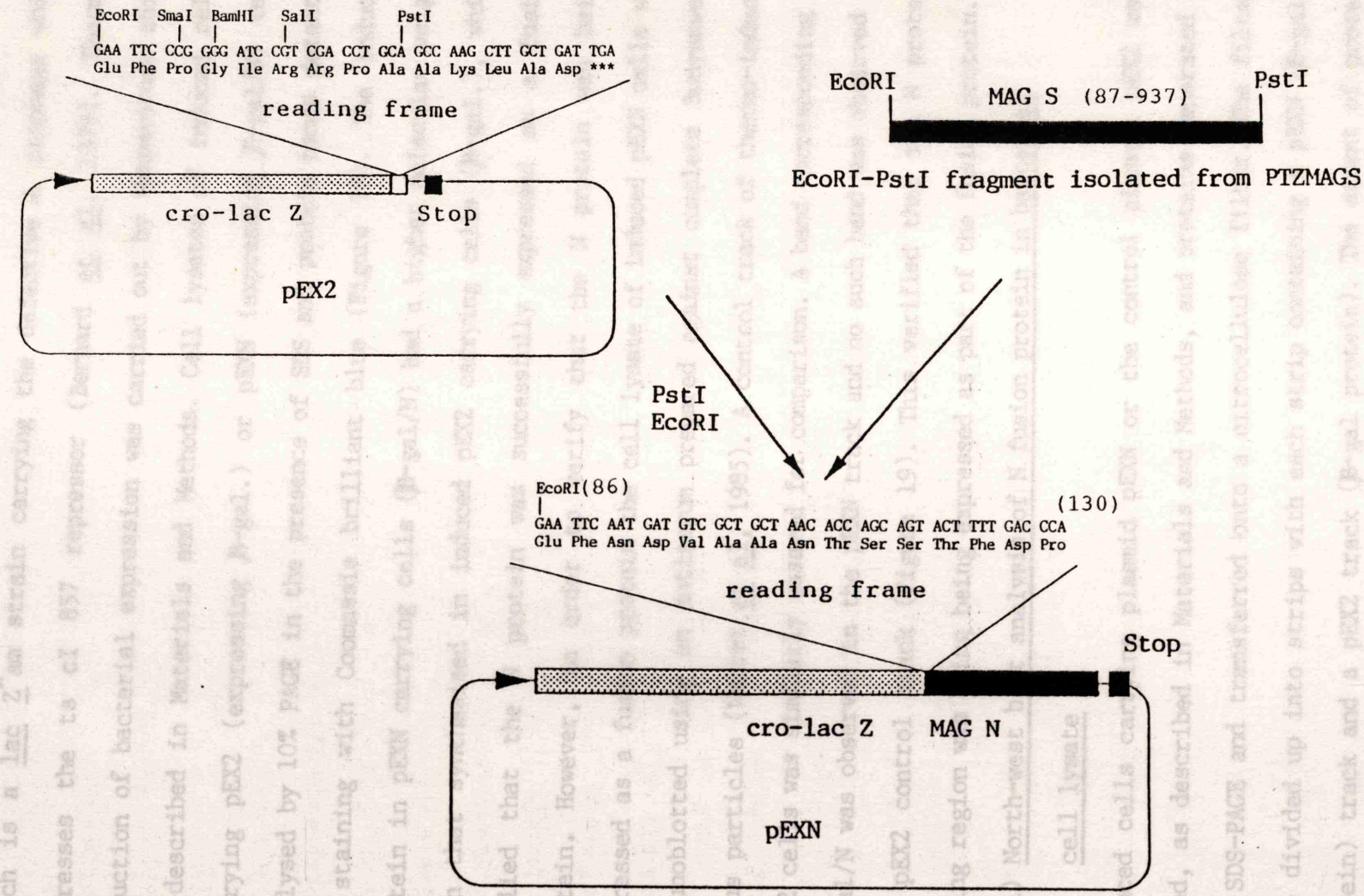
6B. North-west blot of bacterial cells expressing N fusion protein

(i) Construction of the bacterial expression vector

The intention was to express Maguari virus N protein in bacterial cells in a similar way to that employed for the expression of Maguari virus S segment ORF3 protein ie. as a beta-galactosidase (β-gal) fusion protein. A cDNA fragment of approximately 850bp was isolated from pTZMAGS(BP) by EcoRI digestion, which restricted the MAGS cDNA at position 86, followed by PstI digestion, which cut at the 3' end of the MAGS cDNA on the plasmid polylinker. The isolated cDNA fragment, which contained virtually the entire N protein coding region (amino acids 4-233), was subcloned in frame into the C-terminus of the lac Z gene on the plasmid pEX2 (Stanley and Luzio, 1984) to create a thermoinducible beta galactosidase N fusion protein (β-gal/N) gene on the recombinant plasmid pEXN. Recombinant plasmids were verified by EcoRI-PstI double digest which released the MAGS cDNA insert. A diagram summarising the construction of pEXN is shown in Figure 17.

Figure 17.

Construction of pEXN. A fragment of Maguari virus S segment cDNA bases (87-937) was isolated by EcoRI digestion which cuts at the internal EcoRI site and PstI digestion which cuts 3' of the cDNA in the polycloning site of PTZMAGS. This fragment encoded the nucleocapsid protein ORF except the N-terminal 4 amino acids. The MAGS cDNA was cloned in-frame into the multi-cloning site in the lac Z gene on pEX2 which was linearised by PstI and EcoRI digestion. Recombinant clones were verified by PstI and EcoRI double digestion which released the MAGS cDNA insert. The recombinant clone was designated pEXN. The reading frame across the EcoRI site, the junction between the lac Z reading frame and the start of the nucleocapsid protein reading frame is shown on pEXN.


EcoRI SmaI BamHI SalI PstI
GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC AAG CTT GCT GAT TGA
Glu Phe Pro Gly Ile Arg Arg Pro Ala Ala Lys Leu Ala Asp ***
reading frame
cro-lac Z
Stop
pEX2
EcoRI
MAG S (87-937)
PstI
EcoRI-PstI fragment isolated from PTZMAGS
PstI
EcoRI
EcoRI(86)
(130)
GAA TTC AAT GAT GTC GCT GCT AAC ACC AGC AGT ACT TTT GAC CCA
Glu Phe Asn Asp Val Ala Ala Asn Thr Ser Ser Thr Phe Asp Pro
reading frame
Stop
cro-lac Z
MAG N
pEXN

(ii) Expression of the N fusion protein in bacterial cells

The pEXN plasmid was maintained in the bacterial strain K-12ΔHIΔtrp which is a lac Z⁻am strain carrying the defective λ prophage which expresses the ts cI 857 repressor (Bernard et al, 1979). Thermal induction of bacterial expression was carried out by temperature shift as described in Materials and Methods. Cell lysates of induced cells carrying pEX2 (expressing β-gal.) or pEXN (expressing β-gal/N) were analysed by 10% PAGE in the presence of SDS and protein bands detected by staining with Coomassie brilliant blue (Figure 18). The induced protein in pEXN carrying cells (β-gal/N) had a higher molecular weight than that synthesised in induced pEX2 carrying cells (β-gal.), which implied that the N protein was successfully expressed as a fusion protein. However, in order to verify that the N protein was being expressed as a fusion protein the cell lysate of induced pEXN cells was immunoblotted using an antiserum prepared against complete Bunyamwera virus particles (Watret et al, 1985). A control track of thermo-induced pEX2 cells was similarly assayed for comparison. A band corresponding to β-gal/N was observed in the pEXN track and no such band was observed in the pEX2 control track (Figure 19). This verified that the N protein coding region was being being expressed as part of the fusion protein.

(iii) North-west blot analysis of N fusion protein in bacterial cell lysate

Induced cells carrying plasmid pEXN or the control plasmid pEX2 were lysed, as described in Materials and Methods, and proteins separated by 10% SDS-PAGE and transferred onto a nitrocellulose filter. The filters were divided up into strips with each strip containing a pEXN (β-gal/N protein) track and a pEX2 track (β-gal protein). The extent of protein

Figure 18.

Expression of beta-galactosidase nucleocapsid fusion protein in bacterial cells carrying pEXN. Beta-galactosidase nucleocapsid fusion protein (β-gal fusion) synthesis was induced in pEXN carrying cells by temperature shift as described in Materials and Methods. Beta-galactosidase protein synthesis was also induced in control pEX2 carrying cells. Bacterial cells were lysed and proteins separated by 12% PAGE in the persence of SDS. Proteins were visualised by Coomassie brilliant blue staining. Lane (1) induced pEX2 carrying cells. Lanes (2) and (3) induced pEXN carrying cells. β-gal and β-gal fusion proteins are indicated.

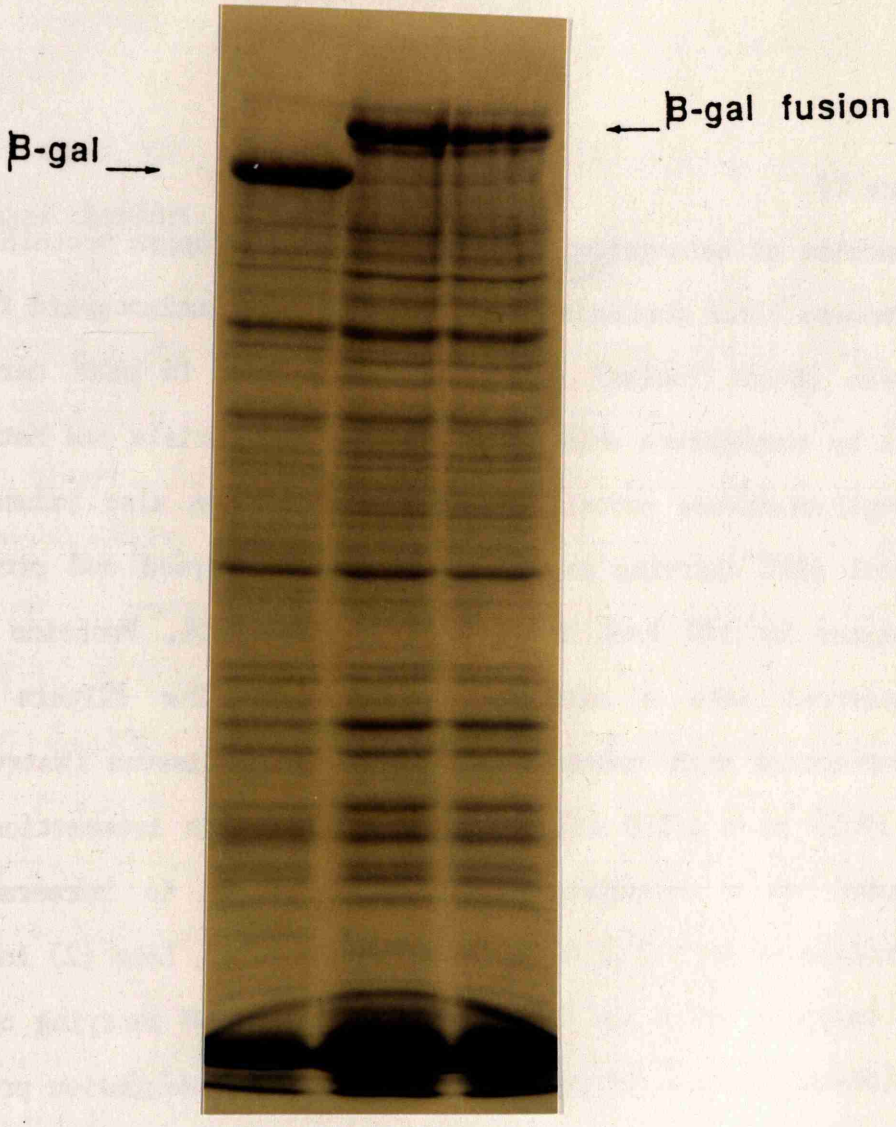
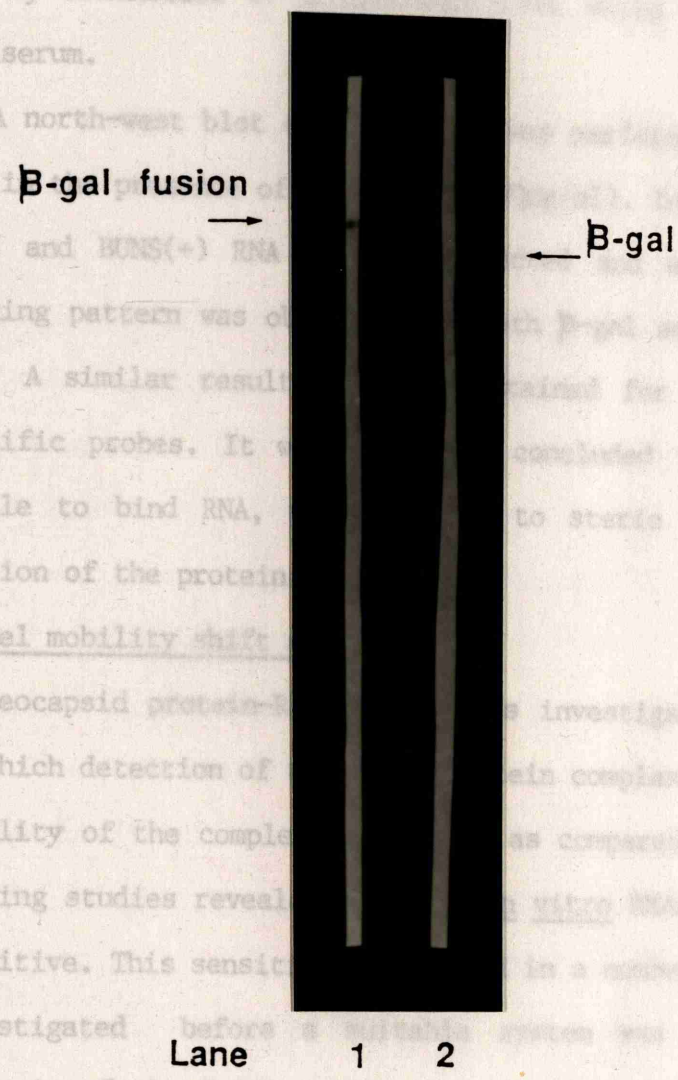

β-gal
β-gal fusion
Lane 1 2 3

Figure 19.

Immunoblot of beta-galactosidase nucleocapsid fusion protein with Bunyamwera virus antiserum. Beta-galactosidase nucleocapsid fusion protein (β-gal fusion) synthesis was induced in pEXN carrying cells by temperature shift as described in Materials and Methods. Beta-galactosidase protein (β-gal) synthesis was also induced in control pEX2 carrying cells. The cells were lysed and proteins separated by 10% PAGE in the presence of SDS. Proteins were transferred onto a nitrocellulose filter. The filters were immunoblotted with Bunyamwera virus rabbit antiserum (Watret et al, 1985) at a 1/100 dilution. Antibody protein interaction was detected by a secondary antibody conjugated to horseradish peroxidase as described in Materials and Methods. Lane (2) induced pEX2 carrying cells and lane (1) induced pEXMAGN carrying cells. The location of the beta-galactosidase nucleocapsid fusion protein (β-gal fusion) is indicated.


B-gal fusion
B-gal
Lane 1 2

transfer to the filter and localisation of the βgal/N protein on the filter were determined by staining the gel with Coomassie brilliant blue and by immunoblot of a nitrocellulose strip with Bunyamwera specific antiserum.

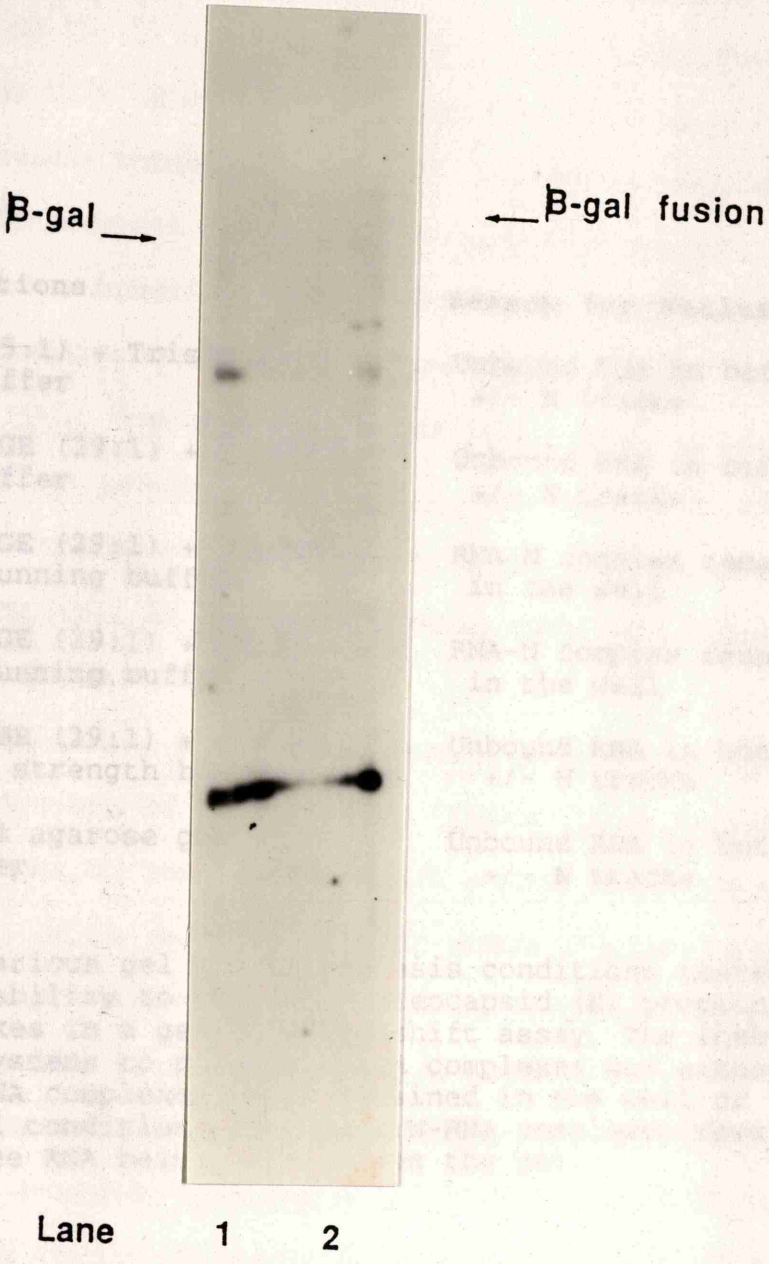
A north-west blot of the filter was performed using labelled BUNS(+) RNA in the presence of yeast RNA (250µg/ml). Interaction between βgal/N band and BUNS(+) RNA was not detected and an identical non-specific binding pattern was obtained for both βgal and βgal/N tracks (Figure 20). A similar result was also obtained for other specific and non-specific probes. It was therefore concluded that βgal/N protein was unable to bind RNA, possibly due to steric hinderance by the βgal portion of the protein.

7. Gel mobility shift assay

Nucleocapsid protein-RNA binding was investigated by "gel shift" assay in which detection of the RNA-N protein complex is based on the retarded mobility of the complex in the gel as compared to free RNA. The filter binding studies revealed that the *in vitro* RNA-N protein complex was pH sensitive. This sensitivity resulted in a number of gel conditions being investigated before a suitable system was discovered that enabled analysis of the N-RNA complex by gel electrophoresis. The unsuccessful gel electrophoresis conditions tested are listed in Table 2. The majority of the gel electrophoresis systems tested resulted in either the disruption of the complex with only unbound RNA being resolved on the gel or alternatively the complex being retained in the well of the gel. A phoshate buffered agarose gel system proved to be the most successful system with which to investigate *in vitro* formed bunyavirus N-RNA complexes and the protocol adopted is desribed more fully in

Figure 20.

North-west blot showing binding of BUNS(+) RNA with proteins from lysed bacterial cells carrying expression plasmids pEX2 or pEXN. Lysates of cells expressing beta-galactosidase (β-gal) or beta-galactosidase nucleocapsid fusion protein (β-gal fusion) were analysed by 10% SDS-PAGE and proteins transferred onto nitrocellulose. Following pre-incubation with E.coli RNA (250μg/ml) in binding buffer as described in Materials and Methods, the filter was probed with ^{32}P labelled full length BUNS(+) RNA (approximately 0.5 x 10^6 cpm). The RNA transcript was obtained by run-off transcription reaction of XbaI linearised template pT7BUNS(+). The approximate location of β-gal and β-gal fusion are indicated. Lane (1) pEXN bacterial cell lysate and lane (2) pEX 2 bacterial cell lysate.


β-gal
β-gal fusion
Lane 1 2

Gel Conditions	Reason for Failure
5% PAGE (75:1) + Tris-Glycine running buffer	Unbound RNA in both +/- N tracks
4% & 6% PAGE (29:1) + 0.5xTBE running buffer	Unbound RNA in both +/- N tracks
4% & 6% PAGE (29:1) + 0.5xTBE (pH 7.5) running buffer	RNA-N complex remained in the well
4% & 6% PAGE (29:1) + HEPES (pH 7.5) running buffer	RNA-N complex remained in the well
4% & 6% PAGE (29:1) + low and high ionic strength buffers	Unbound RNA in both +/- N tracks
0.7% & 1.7% agarose gel + 1xTBE buffer	Unbound RNA in both +/- N tracks

Table 2
Table of various gel electrophoresis conditions tested for their ability to resolve nucleocapsid (N) protein RNA complexes in a gel mobility shift assay. The inability of these systems to resolve N-RNA complexes was either due to the N-RNA complexes being retained in the well or because gel conditions disrupted N-RNA complexes resulting in only free RNA being resolved on the gel.

Materials and Methods. Basically ^{32}P labelled RNA was incubated in the presence or absence of a fixed concentration of N protein under optimum binding conditions for 20 mins at 31°C. Samples were electrophoresed on either a 1.7% or 0.7% agarose gel (dependent on the size of the RNA molecule) in phospate buffer and bands were resolved by autoradiogaphy of dried gels. Nucleocapsid interaction with bunyavirus specific RNA and non-specific RNA was investigated. Bunyavirus specific RNA consisted of genomic length positive sense BUN S RNA and abbreviated BUN S 300 RNA transcribed *in vitro* from pUCT7BUNS(+) and pUCT7BUNS300 respectively. Non-specific RNA was generated by *in vitro* transcription of pTZVSVNS. Labelled S RNA incubated in the presence or absence of N protein under optimum conditions used in the filter binding assay were subsequently analysed by this system.

A band corresponding to the N-RNA complex could be observed when different concentrations of genomic length (Figure 21) or abbreviated S segment RNAs (Figure 22) were incubated with a fixed concentration of N protein. However, unlike gel mobility shift assays of other RNA-protein interactions, eg influenza virus genomic RNA and NP protein (Yamanaka *et al*, 1990), a specific band corresponding to unbound RNA could not be observed in tracks where RNA was incubated in the absence of N protein. Instead a smear extending from the well to above the RNA-N band was observed in these tracks. One possibility is that under the favourable pH 7 conditions the RNA molecules were able to form a variety of secondary structures, with base pairing occurring within RNA strands and between RNA strands, resulting in RNA molecules migrating under electrophoresis with a variety of mobilities. Analysis of N protein binding with non-specific VSV NS RNA by gel mobility shift assay (Figure 23)

produced similar results to those obtained using viral specific template. It was therefore decided that further investigations of N protein RNA binding activity was not worth pursuing by this gel assay system since no specificity of interaction could be demonstrated.

Figure 21.

Gel mobility shift assay of genomic length BUN S RNA. ^{32}P labelled RNA, derived by T7 transcription of Xba I linearised plasmid template pT7BUNS(+), was incubated at 31°C for 20 minutes in presence or absence of purified recombinant nucleocapsid (N) protein (80ng) at pH7 in 130mM NaCl in a 25µl reaction as described in Materials and Methods. Samples were then electrophoresised at room temperature in a 0.7% agarose gel containing 10mM sodium phosphate. The gel was run semi-submerged and the running buffer constantly recirculated as described in Materials and Methods. RNA bands were visualised on dried gels by autoradiography. Bands corresponding to N-RNA complexes are indicated (N-RNA). The wells of the gel are indicated by the origin. Lane (1) 5 pg BUNS(+) RNA + 80 ng N; (2) 10 pg BUNS(+) RNA + 80 ng N; (3) 5 pg BUNS(+) RNA; (4) 10 pg BUNS(+) RNA. Exposure time o/n at -70°C.

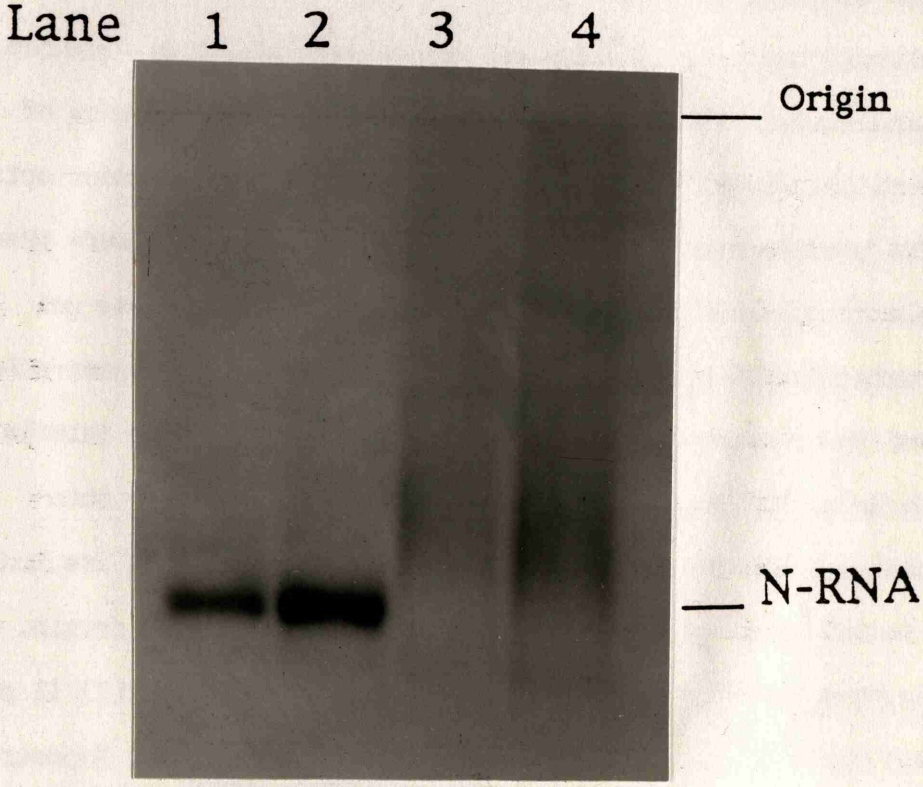

Lane 1 2 3 4
Origin
N-RNA

Figure 22.

Gel mobility shift assay of abreviated S RNA segment. ^{32}P labelled RNA was derived by T7 transcription reaction of XbaI linearised plasmid template pT7BUNS 300. RNA at various concentrations was incubated at 31°C for 20 minutes in presence or absence of purified recombinant nucleocapsid protein (80 ng) under optimum RNA binding conditions in a 25µl reaction. Samples were then electrophoresed at room temperature in a 1.7% agarose gel containing 10 mM sodium phosphate. The gel was run semi-submerged and the running buffer recirculated as described in Materials and Methods. RNA bands were visualised on dried gels by auto-radiography. Bands corresponding to N-RNA complexes are indicated (N-RNA). The wells of the gel are indicated by the origin. (1) 23 pg BUNS 300 RNA; (2) 23 pg BUNS 300 RNA + 80 ng N; (3) 11 pg BUNS 300 RNA + 80 ng N; (4) 2 pg BUNS 300 RNA + 80 ng N. Exposure time o/n at -70°C.

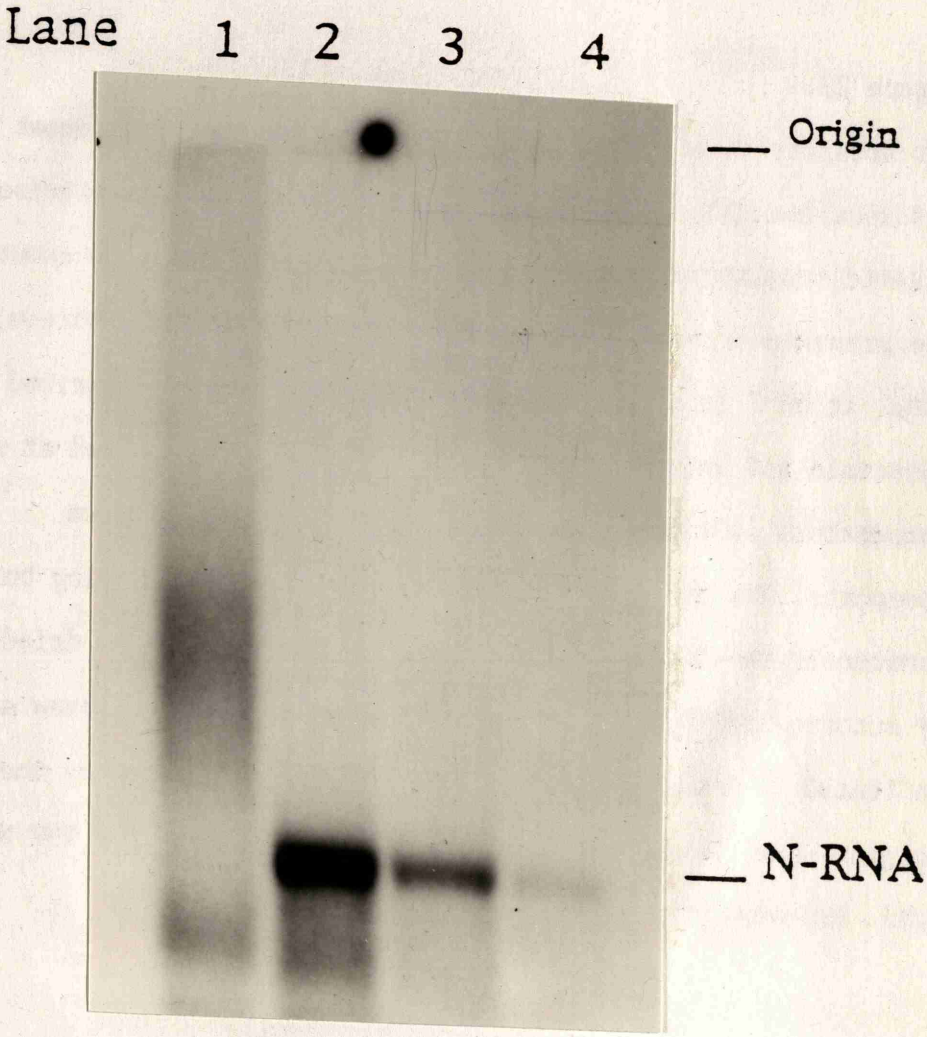
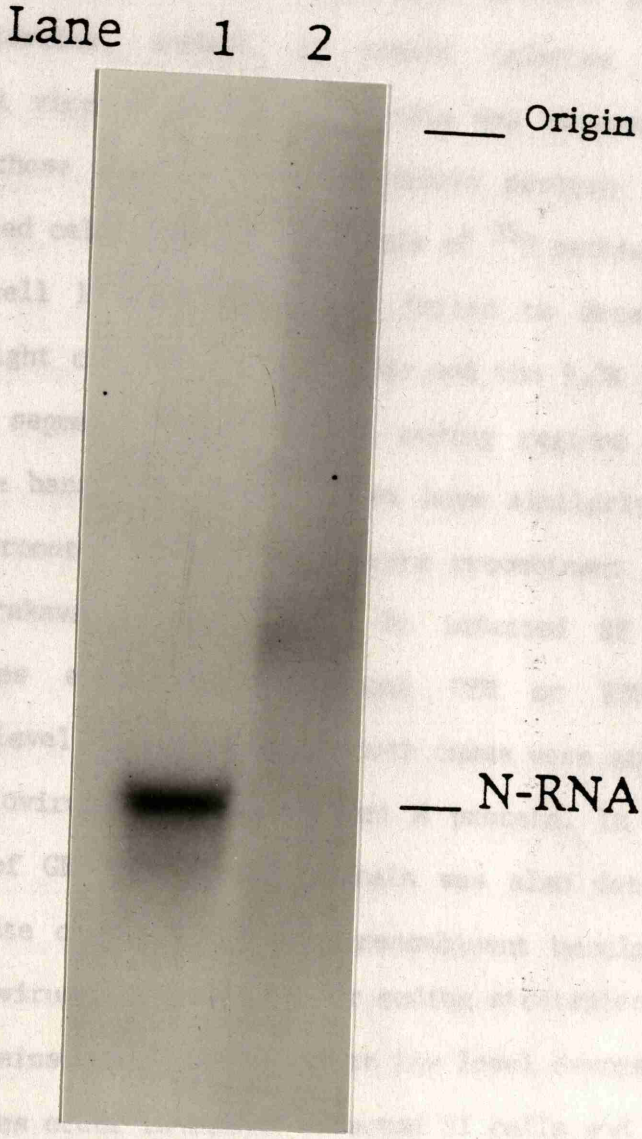

Lane 1 2 3 4
Origin
N-RNA

Figure 23.

Gel mobility shift assay of non-specific nucleocapsid target RNA. ^{32}P labelled RNA, derived by T7 transcription of 3' linearised plasmid template pTZVSVNS, was incubated at 31°C for 20 minutes in the presence or absence of purified nucleocapsid (N) protein, 80ng, at pH 7 in 130 mM NaCl in a 25µl reaction as described in Materials and Methods. Samples were then electrophoresed at room temperature in a 0.7% agarose gel containing 10mM sodium phosphate. The gel was run semi-submerged and the running buffer constantly re-circulated. RNA bands were visualised on dried gels by autoradiography. Bands corresponding to N-RNA complexes are indicated (N-RNA). The wells of the gel are indicated by the origin. Lane (1) 10pg VSV NS mRNA + 80 ng N; (2) 10 pg VSV NS mRNA. Exposure time o/n -70°C.


Lane 1 2
Origin
N-RNA

8 Discussion

Expression and purification of baculovirus expressed N protein

The entire coding region of the Maguari bunyavirus S segment was placed under the control of the polyhedrin protein promoter in a recombinant baculovirus, AcMAGS. In AcMAGS infected insect cells recombinant Maguari virus nucleocapsid protein was expressed at levels comparable with those obtained for polyhedrin protein in wild type baculovirus infected cells. However, analysis of ^{35}S methionine labelled AcMAGS infected cell lysate by SDS-PAGE failed to detect any novel proteins, which might correspond to the NSs and the 9.3K proteins also encoded on the S segment. The S segment coding regions of Germiston (GER) and snowshoe hare (SSH) bunyaviruses have similarly been placed under polyhedrin promoter control in separate recombinant baculoviruses (Bouloy, 1991; Urakawa *et al*, 1988). In infected Sf cells these recombinant viruses expressed recombinant GER or SSH N protein respectively. The level of expression in both cases were similar to that obtained for baculovirus expressed Maguari N protein. In addition low level expression of GER or SSH NSs protein was also detected in the infected cell lysate of the respective recombinant baculovirus. As all three bunyaviruses possess similar coding strategies for N and NSs proteins it is possible that low level expression of the NSs protein does occur in AcMAGS infected Sf cells and perhaps subtle differences in cell culture, conditions of labelling or PAGE may allow detection of NSs in AcMAGS infected Sf cells. A similar assumption can also be applied to the 9.3K protein since it is also able to be translated *in vitro* from mRNA encoding N and NSs proteins. The most desirable way to ascertain the expression status of the NSs and 9.3K

proteins in AcMAGS infected cells would be by immunodetection with monospecific antisera. Since such antisera are at present unavailable an alternative method of detecting low level expression may be accomplished by the reduction of background host cell mRNA translation by pre-treatment of AcMAGS infected cells with alpha-amanitin prior to pulse labelling the cells with ^{35}S methionine. Alpha-amanitin would inhibit host cell RNA synthesis and thus lower the level of host cell mRNA available for translation. As the baculovirus late genes, including the polyhedrin gene, are transcribed by a viral alpha-amanitin resistant RNA polymerase (Grula et al, 1981; Huh and Weaver, 1990) the S segment cDNA under polyhedrin promoter control would continue to be transcribed. The effectiveness of such a strategy would however be dependent upon the length of the half-life of pre-synthesised host cell mRNAs and the lack of baculovirus late gene encoded protein products with M_r values similar to NSs or the 9.3K protein.

Relatively high levels of expression have been reported for recombinant baculoviruses encoding the ambisense NSs proteins of PT and TSWV Bunyaviridae (Kormelink et al, 1991; Overton et al, 1987). Therefore, expression of the NSs protein could be further investigated by by the creation of a second recombinant baculovirus in which the upstream N initiation codon was removed by restriction digestion at the unique EcoRI site between the N initiation codon and the NSs tandem ATG on the MAGS cDNA placing the NSs initiation codon directly downstream of the polyhedrin promoter. In the case of SSH virus removal of the N initiation codon and a small region of the N coding sequence upstream of the NSs ORF on the S cDNA resulted in the generation of a baculovirus able to express NSs protein at increased levels in infected cells.

However the level of synthesis was only increased from 1% to 2% of total cell proteins in comparison to levels of 40% of total cell protein obtained for recombinant N protein in the original SSH S cDNA recombinant baculovirus (Urakawa et al, 1987).

The recombinant N protein was purified to homogeneity in a three stage process of hypotonic cell lysis N fraction enrichment of suitable cell lysate by ammonium sulphate precipitation and FPLC Mono Q exchange chromatography. Although the final protein was taken to be 100% pure, within the limits of detection, the yield of pure N protein of 100 ug per 6 X 10^7 cells was low in comparison to the level of expressed N protein. The most significant loss of N protein occurred at the stage of purification on the Mono Q column. Ultrafiltration of purified N protein revealed that it was forming oligomers of various sizes. Formation of large aggregates within the column may have been the reason for the retention of the majority of the protein within the column. Elution over higher salt gradients failed to counteract charge interaction between N protein molecules possibly due to protein precipitation within the column. Protein aggregation may have been reduced if the column purification was carried out at 4^oC; certainly N-RNA interaction at 4^oC is reduced at lower temperature. Therefore if N-N interaction were similarly affected by temperature then the extent of N-N aggregation would be reduced and hence the yield of N protein eluted from the column would be increased. However, facilities for low temperature FPLC-Mono Q runs were unavaliable to allow testing of this hypothesis. Alternatively N-N interaction could be minimised if crude N protein fractions were introduced to the Mono Q column under denaturing conditions of 6 M urea. The lack of S bonds and relatively small size of the protein should in

theory mean that the N protein would renature after dialysing out the urea from eluted N protein fractions. However this strategy may itself affect the point of elution on the salt gradient of the N protein and other proteins present in the crude extract, possibly resulting in the elution of N protein with background contaminating proteins.

An alternative means of improving the final yield of purified N protein would be to adopt the use of an expression system with a relatively simple standardised system for purification of recombinant expressed protein such as the pGEX(GST) recombinant protein expression system (Smith and Johson, 1988), and the polyhis fusion protein expression metal chelate purification system (Gentz et al, 1989). Although initially developed for bacterial expression of foreign proteins baculovirus vector counterparts for these systems are now available. In general these systems work on the principal of introducing a leader sequence upstream of the foreign gene to be expressed. This allows the expression of a fusion protein with high affinity for a specific resin substrate to allow a one step purification of the fusion protein by chromatography. The leader peptide is then usually removed by use of a protease which cuts the protein at specifically engineered site between the leader peptide and the start of the foreign protein amino acid sequence. The use of such systems might allow the purification of large amounts of N protein to homogeneity for studies such as X-ray crystallography. In addition such expression systems would allow the rapid purification of a diversity of N protein mutants by the same protocol under non-denaturing conditions and even the purification of separately expressed amino and carboxy terminal halves of the N protein.

RNA binding activity of recombinant N protein

Investigations into the RNA binding activity of purified recombinant N protein were able to determine optimum conditions for *in vitro* RNA binding by filter binding assay and the visualisation of RNA-N interaction by gel mobility shift assay. However, these interactions were found to be non-specific. Failure to demonstrate specificity of N-RNA interaction contrasts sharply with the situation that exists in virus infected cells where genomic length positive and negative sense RNA is encapsidated in preference to viral mRNA and host cell mRNAs. Recently two hantavirus nucleocapsid proteins (Hantaan 76-118 and Puumala viruses) have been expressed in *E.coli*, purified and *in vitro* RNA binding studies with hantaviral S segment RNA carried out (Gott *et al*, 1993). Although hantaviral and bunyaviral nucleocapsid proteins differ in size (49K compared with 26K) and share no significant amino acid sequence homology it is thought that they bind their respective target genomic RNAs in a similar manner. Binding studies with hantaviral nucleocapsid protein demonstrated RNA binding activity and localised the binding domain to a conserved 95 amino acid region at the carboxy terminus. This region although strongly conserved among the hantavirus N proteins exhibited no known RNA binding consensus sequence nor obvious homology to other N proteins of the *Bunyaviridae* family. However bunyavirus nucleocapsid proteins do have a strongly conserved carboxy terminal region (Elliott, 1989a) which may be important for RNA binding activity. Although hantavirus capsid protein specificity of interaction with target RNA was not observed a preference of hantaviral nucleocapsid protein for double stranded RNA was successfully demonstrated, perhaps emphasising the importance of genomic RNA possesing complementary 5' and

3' termini. The presence of the terminal 23bp inverted repeats resulted in a two fold increase in the amount of RNA capsid protein complex made over complexes consisting of template S RNA lacking these complementary sequences. However, the investigators concluded that it was the presence of additional internal stem loop structures that allowed a significant proportion of RNA-N complex to be made without the presence of the terminal base pair repeats. The authors proposed that encapsidation of viral RNA by the N protein may initiate at the terminal panhandles or at the internal stem loop structures followed by a co-operative binding consisting of N-N and N-RNA interactions, resulting in the encapsidation of RNA segment. If Maguari virus nucleocapsid protein also exhibited a similar preference for double stranded RNA this would add weight to such a theory but would not explain the apparent disparity in specificity of RNA binding that is obtained in virus infected cells but not *in vitro*. This contradiction is not restricted to members of the *Bunyaviridae* family but is observed in many other negative sense RNA viruses.

The complementary non-coding 5' and 3' termini of influenza virus have been shown to be sufficient for the *in vivo* encapsidation and replication of heterogenous RNAs that posses these terminal complementary sequences flanking a reporter gene in virus infected cells (Luytjes *et al*, 1989). In contrast *in vitro* RNA binding studies of influenza virus nucleoprotein (NP) with genomic RNA, although able to demonstrate optimum conditions for RNA binding, failed to demonstrate NP protein specificity for genomic influenza RNA (Yamanaka *et al*, 1990). Specificity of binding of nucleocapsid proteins in negative sense RNA viruses has only been successfully demonstrated for the N protein of vesicular stomatitis virus (VSV), where specificity has been localised

to the leader sequence at the 5' end of both negative and positive sense genomic length RNA (Moyer et al, 1991). However this specificity of binding found in virus infected cells could only be duplicated in vitro in the presence of rabbit reticulocyte lysate or cytoplasmic extract. It is presumed that proteins present in these extracts enable the protein to specifically bind target RNA sequences. This may be achieved by reducing N-N aggregation to a level that permits specific N-RNA interaction. Alternatively, cellular factors may act as chaperone proteins that would enable the N protein to assume the correct folding that allows the the protein to encapsidate the RNA correctly. Chaperone proteins from rabbit reticulocyte have been reported as necessary for the correct folding of cytoplasmic actin-like proteins (Gao et al, 1992). The presence of such host cell factors may similarly be required by Magauri virus nucleocapsid protein to enable specificity of binding. Alternatively specificity of binding may be dependent upon the presence of other viral proteins with which N protein must interact with before the initial N-RNA specific interaction can occur. The NS1 protein of influenza virus has been reported to have specific affinity for the 5' and 3' terminal regions of minus sense RNA segments and may be required to bind viral RNA to enable specific RNA encapsidation by the NP protein (Hatada et al, 1992). Therefore specificity for RNA encapsidation by N protein may be mediated by association of N with L protein bound to the conserved termini of the RNA segment. An association of N and L proteins in Bunyamwera virus has already been demonstrated by the ability of monospecific antiserum to L protein to immunoprecipitate both L and N protein from viral particles (H. Jin and R.M. Elliott, unpublished results). Alternatively NSs protein may play a significant role in

allowing N protein to bind RNA in a specific manner. The requirement of additional cellular or viral proteins to enable specificity of encapsidation to occur remains to be investigated.

Chapter 6

Outlook

This project had two major aims: (1) To examine the expression strategy of the bunyavirus S segment encoded gene products; and (2) To study the interaction of bunyavirus nucleocapsid protein with its target viral RNA.

The first aim was achieved by examining the the expression strategy of the Maguari virus S segment (Chapter 3). Most bunyavirus S segments encode two proteins, N and NSs, in overlapping reading frames. Maguari virus in addition encodes a predicted third ORF (ORF 3) and its expression was also investigated. Cell free translation experiments revealed that all three encoded proteins were as efficiently expressed from polycistronic mRNAs as from monocistronic mRNA counterparts. A similar result was obtained for the cell free translation of N and NSs proteins encoded by the Bunyamwera virus S RNA segment (Chapter 4). These results imply that bunyavirus S segment encoded proteins are translated from one species of bicistronic mRNA in virus infected cells. This hypothesis is further substantiated by the fact that only bicistronic S mRNA have been isolated from bunyavirus infected cells (Bishop et al, 1983; Bouloy et al, 1984, 1990). It is presumed that initiation of translation of the bunyavirus S segment overlapping reading frames on these mRNAs is accomplished by the leaky scanning model (Kozak, 1986) where initiation of translation of the downstream initiation codon (NSs) is dependent upon the upstream initiation codon (N) lying in an inefficient sub-optimal sequence context to allow a proportion of the scanning ribosomes to by-pass the N initiation codon and initiate translation at the NSs initiation codon. The Maguari virus NSs reading frame has a tandem AUG initiation codon at the start of the

reading frame, a feature common to 11 out of 13 bunyavirus S segments so far sequenced (Dunn et al, 1994). Cell free translation experiments (Chapter 3) demonstrated that both AUGs were equally capable of initiating translation. The feature of tandem initiation codons may allow maximal levels of NSs protein to be translated in the presence of the upstream N initiation codon. However, the NSs expresion levels may be affected if the N initiation codon lay in a more optimal context. It may be prudent in future experiments to address this question especially since in Bunyaviridae that do not encode an NSs in overlapping reading frames, ie phlebo- and tospo- viruses, or do not encode an NSs protein, ie hantaviruses and nairoviruses, the N initiation codon is in near optimal context (Chapter 3, Figure 22). These studies could be accomplished by altering the context of the N initiation codon on different MAG S cDNAs to optimal or near optimal status and performing similar transcription/ translation experiments on these templates to those described in Chapter 3.

It should be noted that the S mRNA templates employed in the transcription/ translation experiments do not possess the 5' and 3' termini of S mRNA found in bunyavirus infected cells. These S mRNAs have additional non-viral sequences of 10-18 nucleotides in length preceding the complete S segment 5' non-coding region, and the 3' end of the mRNA pre-terminates approximately 60-110 nucleotides before the end of the genomic segment (Chapter 1). The S mRNA non-viral 5' nucleotide sequences and 3' transcription termination site were recently determined for Bunyamwera virus (Jin and Elliott, 1993) it may be of interest to create S mRNA templates that incorporate these 5' and 3' features, either individually or together, to determine if they affect either the

stability or translational efficiency of the S mRNA.

Although a significant amount of information has and could continue to be generated from cell free translation systems it would undoubtedly be desirable to perform similar experiments in vivo. Although transient expression constructs were made (Chapter 3) and preliminary cellular expression assays carried out (Elliott et al, 1989; R.M. Elliott and K.W. Page, unpublished work) further investigations were hampered by lack of antisera to NSs and ORF 3 proteins which would have allowed positive identification of these proteins in cell lysates. Antisera to NSs and ORF 3 proteins would enable the monitoring of expression levels in comparison with N protein in cellular transient expression assays and would additionally allow analysis of the cellular localisation, by immunofluorescence, of these proteins when expressed by themselves or in conjunction with N or other viral proteins. Such an approach might provide an insight into the function of either the NSs or ORF 3 proteins. The development of antisera to NSs and ORF 3 proteins is therefore a pre-requisite to any intended future investigations. An attempt was made to raise antiserum to a recombinant ORF 3 beta-galactosidase fusion protein in rabbits (Chapter 3). However, this antiserum did not possess activity to the ORF 3 part of the fusion protein. A similar approach was also unsuccessful in raising antiserum to NSs fusion protein (R.M. Elliott and K.W. Page, unpublished results). One possible future approach to raising antisera to these proteins would be to chemically synthesise oligopeptides corresponding to likely epitope sequences, determined through hydropathic profiles of the amino acid sequences of NSs and ORF 3 proteins. Alternatively polypeptides with the predicted amino acid sequence of the complete NSs or ORF 3

proteins could be synthesised. In either case these peptides could then be used to raise antisera in animals. The effectiveness of resulting antisera would however be dependent upon the immunogenicity of NSs and ORF 3 proteins. If these proteins are poorly immunogenic a second possible strategy of tagging the NSs or ORF 3 protein at the carboxy terminal, with an epitope sequence to which antibodies were available, could be employed. A similar approach was successfully employed in HSV-1 UL 26 ORF expression studies (Liu and Roizman, 1991). The epitope employed in these studies corresponded to a 20 amino acid sequence on human CMV glycoprotein A. An oligonucleotide encoding this epitope sequence could be used to introduce, in frame, this epitope into the reading frame of either the NSs or ORF 3 protein by site directed insertional mutagenesis. The use of expression vectors encoding these tagged proteins would allow the subsequent analysis of the transient expression of these proteins *in vivo* by using monoclonal antibody directed against the CMV glycoprotein epitope.

The question of the functional significance of the protein encoded in ORF 3 has recently been heightened by the report of similar third overlapping reading frames encoded in two more bunyavirus S segments (Dunn *et al*, 1994). One classical way in which to determine whether a reading frame encodes a functional protein would be to create a mutant virus lacking this protein. Until recently it has not been possible to engineer genetic changes into negative stranded RNA genomes because unlike positive stranded RNA viruses their genomic RNA is not infectious and thus RNA transcribed from full-length cDNA clones of negative stranded RNA genomes is not by itself capable of initiating infection in transfected cells. In order to be infectious the RNA must be

encapsidated with nucleocapsid protein and the viral RNA polymerase supplied to enable mRNA sythesis to occur. Influenza virus was the first negative strand RNA virus for which a system to successfully introduce RNA derived from transcription of a cDNA clone has been described. The RNA contained the regulatory 5' and 3' terminal sequences of an influenza virus genome segment flanking a reporter gene and was transfected into cells in the presence of helper virus to amplify and express the recombinant segment (Luytjes et al, 1989). This was followed by a report of the generation of reassortant influenza viruses in which 5 mutations introduced into to an individual segment at the cDNA level were engineered back into viruses by transfection into cells of RNA transcripts, derived from the mutated cDNA and assembled into RNPs containing RNA polymerase. Mutant virus was released when the cells were superinfected with helper influenza virus (Enami et al, 1990). A similar approach could be applied in the creation of a mutant Maguari virus, where the ORF 3 initiation codon on the S segment was altered to abolish protein translation. A mutated genomic S segment RNA could be transcribed from a cDNA template using T7 RNA polymerase by introducing an inverted T7 promoter at the 3' end of the MAG S cDNA, as in pUCT7BUNS(-) (Chapter 4), and a unique restriction site at the 5' end of the cDNA template, to define the end of the S segment. The genomic S RNA would then be mixed with recombinant nucleocapsid protein to generate RNPs, transfected into cells and the cells superinfected with a helper virus in the form of a reassortant virus, consisting of Maguari L and M segments and a Bunyamwera S segment. Progeny mutant viruses would be identified by the presence of the Maguari S segment. The isolated mutant could be used in comparative growth experiments with wild type virus to

determine a possible functional feature that could be assigned to the ORF 3 protein.

In bunyavirus infected cells N protein specifically binds to viral RNA. This interaction was studied *in vitro* using purified recombinant Magurai virus N protein, expressed by a recombinant baculovirus in insect cells (Chapter 5), and target RNA transcripts derived from various Bunyamwera virus S segment cDNA templates by T7 polymerase directed run-off transcription reactions (Chapters 4 and 5). Although optimal conditions for RNA binding were established, specificity of the interaction could not be demonstrated in various assay systems (Chapter 5). Non-specific RNA binding has also been reported recently for bacterially expressed nucleocapsid proteins of other Bunyaviridae, Hantaan (HTN) and Puumala (PUU) hantaviruses (Gott *et al*, 1993). The recombinant expressed HTN and PUU N proteins exhibited lack of *in vitro* RNA binding specificity for their target viral RNAs and this phenomenon has been reported for other negative sense RNA viruses eg NP protein of influenza and N protein of VSV (Das and Banerjee, 1993; Moyer *et al*, 1991; Yamanaka *et al*, 1990) despite these proteins demonstrating specificity of interaction in virus-infected cells.

In the case of the VSV N protein specificity of interaction was obtained *in vitro* in the presence of a cellular cytoplasmic extract which presumably supplied necessary host factor(s) (Das and Banerjee, 1993; Moyer *et al*, 1991). A similar approach could be adopted to future studies on bunyavirus N protein RNA binding by the inclusion of cytoplasmic extract. The source of cytoplasmic extract could be from non-infected, virus infected cells, or cells transiently expressing virus proteins via recombinant expression vectors in an attempt to

identify whether additional factors, host or virus derived, are required.

It is not known if the complete bunyavirus N protein sequence is required for RNA binding activity, or if the RNA binding domain is restricted to one particular region of the protein. Recent sequence information of nairovirus S segments indicate that the carboxy region of the encoded N protein is not necessary for full N protein activity since one of the nairovirus S segments (DUG virus) has a 40 amino acid deletion at the carboxy terminal of the N protein sequence in comparison with the N protein sequences of CCHF and HAZ nairovirus N proteins, whereas the remainder of the N protein sequences exhibit high homology (Marriott and Nutall, 1992). In contrast although the complete N protein sequence is strongly conserved between hantaviruses (Figure 1) it has been demonstrated that only the carboxy terminal 100 amino acids, the most conserved region, is necessary for RNA binding activity (Gott et al, 1993). This may indicate that although the RNA binding domain can be located to a specific region on the protein sequence that location may vary between genera.

The hantavirus RNA binding domain does not exhibit any recognised RNA binding motif and does not correspond with the distribution of basic residues, a possible indicator of the location of an RNA binding domain, which are present in greater numbers at the amino terminal of the protein (Chapter 1, Figure 8). In addition the RNA binding domain does not exhibit significant homology to other N proteins of the Bunyaviridae family (Gott et al, 1993) indicating that although the RNA binding motif may be conserved within a genus it is not conserved between genera. The Maguari virus N protein possesses sequences that are

Figure 1.

Comparison of the amino acid sequences on hantavirus N proteins.

The N protein amino acid sequences of Hantaan, SR-11 and the Hallnas strain of Puumala virus were compared. The first line of symbols under the amino acids designate identical (*) or conservative (:) amino acid differences detected between Hantaan and SR-11 viruses. The second line of symbols designate identical (*) or conservative (:) amino acid differences detected among all three viruses. The numbers correspond to Hantaan amino acids, starting from the first initiation codon of the S segment ORF. Taken from Arikawa et al (1990).

```
                1
Hantaan:        MATMEELQREINAHEGQLVIARQKVRDAEKQYEKDPDELNKRTLTDREGVAVSIQAKIDE
SR-11   :       MATMEEIQREISAHEGQLVIARQKVKDAEKQYEKDPDDLNKRALHDRESVAASIQSKIDE
Hallnäs:        MSDLTDIQEEITRHEQQLVVARQKLKDAERAVEVDPDDVNKSTLQARQQTVSALEDKLAD
                ******:**** ************:**********:**** * *** ** *** ****
                *  : :::* **  ** ***:****::***:  * ***::**  *  *:     :: *:: :
                61                                                          120
Hantaan:        LKRQLADRIATGKNLGKEQDPTGVEPGDHLKERSMLSYGNVLDLNHLDIDEPTGQTADWL
SR-11   :       LKRQLADRIAAGKNIGQDRDPTGVEPGDHLKERSALSYGNTLDLNSLDIDEPTGQTADWL
Hallnäs:        YKRRMADAVSRKKMDTKPTDPTGIEPDDHLKERSSLRYGNVLDVNAIDIEEPSGQTADWY
                ********** ***:* : ***************** ***** **** ***************
                 ** :** :   *      ****:** ******* * *** **:* :**:** ******
                121                                                         180
Hantaan:        SIIVYLTSFVVPILLKALYMLTTRGRQTTKDNKGTRIRFKDDSSFEDVNGIRKPKHLYVS
SR-11   :       TIIVYLTSFVVPIILKALYMLTTRGRQTSKDNKGMRIRFKDDSSYEDVNGIRKPKHLYVS
Hallnäs:        TIGVYVIGFTIPIILKALYMLSTRGRQTVKENKGTRIRFKDDTSFEDINGIRRPKHLYVS
                ************:*************** ***** **********:***************
                * **:  * :**:******* ****** *:*** ******* *:**:*****:*******
                181                                                         240
Hantaan:        LPNAQSSMKAEEITPGRYRTAVCGLYPAQIKARQMISPVMSVIGFLALAKDWSDRIEQWL
SR-11   :       MPNAQSSMKAEEITPGRFRTAVCGLYPAQIKARNMVSPVMSVVGFLALAKDWTSRIEEWL
Hallnäs:        MPTAQSTMKAEELTPGRFRTIVCGLFPTQIQVRNIMSPVMGVIGFSFFVKDWPEKIREFM
                :**************:*************** *:******:********* ***:**
                :* *** *****:****:** ****:* **  * ::**** *:**  : ***  :* : :
                241                                                         300
Hantaan:        IEPCKLLPDTAAVSL----LGGPATNRDYLRQRQVALGNMETKESKAIRQHAEAAGCSMI
SR-11   :       GAPCKFMAESLIAGS----LSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTLV
Hallnäs:        EKECPFIKPEVKPGTPAQEVEFLKRNRVYFMTRQDVLDKNHVADIDKLIDYAASGDPTSP
                 ***:: :          * *   ****:**** **  ** **  *:***   *** ::
                  * ::            :    ** *  **  *       :  : :
                301                                                         360
Hantaan:        EDIESPSSIWVFAGAPDRCPPTCLFIAGIAELGAFFSILQDMRNTIMASKTVGTSEEKLR
SR-11   :       EHIESPSSIWVFAGAPDRCPPTCLFVGGMAELGAFFSILQDMRNTIMASKTVGTADEKLR
Hallnäs:        DDIESPNAPWVFACAPDRCPPTCIYVAGMAELGAFFSILQDMRNTIMASKTVGTAEEKLK
                * ************************: *:****************************
                : ****   **** *********::: *:****************************** :****
                361                                                         420
Hantaan:        KKSSFYQSYLRRTQSMGIQLGQRIIVLFMVAWGKEAVDNFHLGDDMDPELRTLAQSLIDV
SR-11   :       KKSSFYQSYLRRTQSMGIQLDQRIIVMFMVAWGKEAVDNFHLGDDMDPELRSLAQILIDQ
Hallnäs:        KKSSFYQSYLRRTQSMGIQLDQRIILLYMLEWGREMVDHFHLGDDMDPELRGLAQSLIDQ
                ********************** *****:************************ *** ***
                ********************** ****:::*: **:* **:************ *** ***
                421       429
Hantaan:        KVKEISNQEPLKL
SR-11   :       KVKEISNQEPMKL
Hallnäs:        KVKEISNQEPLKI
                ***********:**
                ***********:*:
```

strongly conserved with other bunyavirus N proteins, but these regions of conservation are spread over the length of the protein (Chapter 1, Figure 6; Chapter 4, Figure 2). Therefore in order to investigate if the complete Maguari N protein or specific regions are necessary to maintain RNA binding activity it would be necessary to alter the amino acid sequence within the conserved regions by oligonucleotide site directed mutagenesis and to create a series of amino and/or carboxy terminally deleted N proteins for use in existing RNA binding assays. In order to successfully purify these mutant N proteins a rapid standardised method of purification of these recombinant mutant proteins will have to be adopted. As discussed in Chapter 5 recent developments in recombinant protein expression and purification such as histidine tagging and metal chelate affinity chromatography make this strategy a possibility, especially since these protocols have the added advatage of being able to purify proteins under both denaturing and non-denaturing conditions, which would overcome any possible insolubility problems associated with any of the prospective mutant N proteins. The production of a range of altered or truncated N proteins will hopefully aid in the definition of the bunyavirus N protein RNA binding domain and perhaps an insight into the RNA-protein binding site.

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