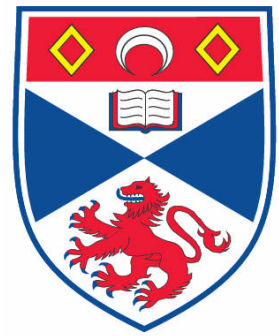
FUNCTIONAL ANALYSIS OF THE ORTHOBUNYAVIRUS NUCLEOCAPSID (N) PROTEIN

Saleh A. Eifan

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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Functional analysis of the orthobunyavirus nucleocapsid (N) protein

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A thesis submitted for the degree of Doctor of Philosophy in Molecular Virology
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Summary

Bunyamwera virus (BUNV) is the prototype of the family *Bunyaviridae*. It has a tripartite genome consisting of negative sense RNA segments called large (L), medium (M) and small (S). The S segment encodes the nucleocapsid protein (N) of 233 amino acids. The N protein encapsidates all three segments to form transcriptionally active ribonucleoproteins (RNPs). The aim of this project was to determine the domain map of BUNV N protein.

To investigate residues in BUNV N crucial for its functionality, random and site-specific mutagenesis were performed on a cDNA clone encoding the BUNV N protein. In total, 102 single amino acid substitutions were generated in the BUNV N protein sequence.

All mutant N proteins were used in a BUNV minigenome system to compare their activity to wt BUNV N. The mutant proteins displayed a wide-range of activity, from parental-like to essentially inactive. The most disruptive mutations were R94A, I118N, W134A, Y141C, L177A, K179I and W193A.

Sixty-four clones carrying single substitutions in the BUNV N protein were used in the BUNV rescue system in an attempt to recover viable mutant viruses. Fifty recombinant mutant viruses were rescued and 14 N genes were nonrescuable.

The 50 mutant viruses were characterized by*:* titration, protein labelling, western blotting, temperature sensitivity and host-restriction. Mutant viruses displayed a wide-range of titers between 10^3-10^8 pfu/ml, and three different plaque sizes large, medium and small. Protein labelling and western blotting showed that mutations in the N gene did not affect expression of the other viral genes as much as affecting N protein expression. It was demonstrated that single amino acid substitutions could alter N protein electrophoretic mobility in SDS- PAGE (*e.g.* P19Q and L53F).

Temperature sensitivity tests showed that recombinant viruses N74S, S96S, K228T and G230R were ts, growing at 33˚C but not at 37˚C or 38˚C, while the parental virus

grew at all temperatures. Using the northern blotting technique, mutant viruses N74S and S96G were shown to have a ts defect in genome-synthesis (late replication step), while mutant viruses K228T and G230R had a ts defect in antigenome-synthesis (early replication step).

Host-restriction experiments were performed using 5 different cell lines (Vero-E6, BHK-21, 2FTGH-V, A549-V and 293-V). Overall, the parental virus grew similarly in all cell lines. Likewise, the majority of mutant viruses follow this pattern except mutant virus Y23A. It showed a 100-fold reduction in titer in 2FTGH-V cells. Comparing the ratios of intracellular and extracellular particles revealed that only 15% of the total virus particles of mutant Y23A was released as extracellular particles compared to 30% of the parental virus.

Fourteen N genes were nonrescuable. They were characterized by (i) their activity in the BUNV minigenome system, (ii) their activity in BUNV packaging assay, (iii) their ability to form multimers, (iv) their ability to interact with L protein, and (v) their impact on RNA synthesis.

In summary, BUNV N protein was shown to be multifunctional and involved in the regulation of virus transcription and replication, RNA synthesis and assembly, via interactions with the viral L polymerase, RNA backbone, itself or the viral glycoproteins.

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Unless otherwise stated, all results were obtained by the authors own efforts.

This thesis is dedicated to the memory of my father

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Abbreviations

aa ..... amino acid
AMV ..... avian myeloblastosis virus
APS ..... ammonium persulfate
β-Me ..... β-mercaptoethanol
bp ..... base pair
BSA ..... bovine serum albumin
ºC ..... degree Celsius
cDNA ..... complementary DNA
Ci ..... Curie
CLB ..... Cell lysis buffer
Cm ..... centimetre
dATP ..... deoxyadenosine triphosphate
dCTP ..... deoxycytosine triphosphate
dGTP ..... deoxyguanosine triphosphate
dH_2O ..... distilled water
DMEM ..... Dulbecco's modified Eagle medium
DMSO ..... dimethylsulphoxide
DNA ..... deoxyribonucleic acid
dNTP ..... deoxynucleotide triphosphate
DSP ..... dithiobis [succinimidylpropionate]
E.coli ..... *Escherichia coli*
EDTA ..... ethylenediaminetetra-acetic acid
ER ..... endoplasmic reticulum
FCS ..... foetal calf serum
g ..... gram or gravity
HFRS ..... haemorrhagic fever with renal syndrome
HRP ..... horseradish peroxidase
IFN ..... interferon
IRES ..... internal ribosomal entry site
IRF-3 ..... interferon responsive factor 3
Kb ..... kilobase
kDa ..... kilodalton
mA ..... milli Ampere
mg ..... milligram
Mg ..... magnesium
ml ..... millilitre
µl ..... microlitre
mM ..... millimolar
MMLV ..... Moloney Murine Leukemia Virus
MOI ..... multiplicity of infection
mRNA ..... messenger RNA
NSV ..... negative strand RNA virus
ORF ..... open reading frame
PAGE ..... polyacrylamide gel electrophoresis
PBS ..... phosphate buffer saline

PCR.....polymerase chain reaction
PEG.....polyethylene glycol
Pmol .....picomol
pol II.....RNA polymerase II
RNA .....ribonucleic acid
RNase.....ribonuclease
RNP.....ribonucleoprotein
Rpm .....revolution per minute
RT.....reverse transcription
SDS.....sodium dodecyl sulphate
TAE .....Tris-acetate EDTA
TBE .....Tris-borate EDTA
U .....unit or uracil
UTR.....untranslated region
V .....volt
vRNA .....viral RNA
wt.....wild type
w/v.....weight per volume

Abbrevations of amino acids

A.....Alanine (Ala)
C.....Cysteine (Cys)
D.....Aspartic acid (Asp)
E.....Glutamic acid (Glu)
F .....Phenylalanine (Phe)
G .....Glycine (Gly)
H.....Histidine (His)
I .....Isoleucine (Ile)
K.....Lysine (Lys)
L .....Leucine (Leu)
M .....Methionine (Met)
N.....Asparagines (Asn)
P.....Proline (Pro)
Q .....Glutamine (Gln)
R.....Arginine (Arg)
S.....Serine (Ser)
T .....Threonine (Thr)
V.....Valine (Val)
W.....Tryptophan (Trp)
Y.....Tyrosine (Tyr)

Abbreviations of virus names

AINV.....Aino virus
AKAV.....Akabane virus
APEV.....Apeu virus
BATV.....Batai virus
BRUV .....Bruconha virus
BUNV .....Bunyamwera virus
BUTV.....Buttonwillow virus
CARV .....Caraparu virus
CCHFV.....Crimean-Congo haemorrhagic fever virus
CEV.....California encephalitis virus
CVV.....Cache valley virus
DOUV.....Douglas virus
DUGV.....Dugbe virus
EMCV.....encephalomyocarditis virus
FPV .....Facey's Paddock virus
GERV.....Germiston virus
GLV.....Gumbo Limbo virus
GROV.....Guaroa virus
GUAV.....Guaroa virus
HTNV .....Hantaa virus
INGV .....Ingwavuma virus
INKV.....Inkoo virus
ITAV .....Itaqui virus
JCV .....Jamestown Canyon virus
JSV.....Jerry slough virus
KAIV.....Kairi virus
KAKV.....Kaikalur virus
KEYV.....Keyston virus
LACV.....La Crosse virus
MADV.....Madrid virus
MAGV.....Maguari virus
MARV.....Maritube virus
MELV .....Melao virus
MERV.....Mermet virus
MDV .....Main Drain virus
MURV.....Murutucu virus
NEPV .....Nepuyo virus
NORV.....Northway virus
NSDV .....Nairobi sheep disease virus
ORIV .....Oriboca virus
OROV.....Oropouche virus
OSSV .....Ossa virus
PEA.....Peaton virus

PTV .....Punta Toro virus
PUUV .....Puumala virus
RESV .....Restan virus
RVFV.....Rift Valley fever virus
SABV.....Sabo virus
SAV .....San Angelo virus
SATV.....Sathuperi virus
SEOV .....Seoul virus
SDNV .....Serra do Navio virus
SHAV .....Shamonda virus
SHUV .....Shuni virus
SHV.....Snowshoe hare
SIMV .....Simbu virus
SNV.....Sin Nomber virus
SRV.....South River virus
SSHV .....Snowshoe here virus
TAHV.....Tahyna virus
TRIV .....Trivittatus virus
TSWV.....tomato spotted wilt virus
UUKV .....Uukuniemi virus
VINV.....Vinces virus
VSV.....vesicular stomatitis virus
YABV……..Yaba 7 virus

Chapter 1. Introduction

1.1. General introduction to bunyaviruses

The *Bunyaviridae* is one of the largest families of RNA viruses, containing more than 300 serotypes (Elliott *et al.,* 2000). The genome consists of three segments of negative sense RNA: the L (large), encodes L protein, the M (medium) encodes two glycoproteins (Gn and Gc) and the S (small) expresses nucleocapsid (N). Viruses of some genera also express non-structural proteins either on the M segment (called NSm) or the S segment (called NSs). On the basis of serological and morphological characteristics, bunyaviruses are classified into five genera, *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (plant-infecting viruses) (Table1.1).

The majority of these viruses are arthropod-borne with the exception of the hantaviruses which are transmitted by rodents. Virions have a spherical or pleomorphic shape, of 80-120 nm in diameter. On the outer surface are the two glycoproteins, Gn and Gc, which are embedded in the lipid bilayer envelope. The envelope, about 5nm thick, is usually derived from Golgi membranes of the host cell. The L, M and S segments are found within this envelope. Each segment is encapsidated by N protein and is associated with L polymerase to form ribonucleocapsids (RNP). Hence, three RNPs are found inside the virion (Figure1.1). Under the electron microscope, the RNPs are visualized as circular forms due to the complementarity of the 3' and 5' ends of the genome RNA (Figure1.2) to form a pan-handle structure (Hewlett *et al*., 1977). Typically, a bunyavirus virion (based on Uukuniemi virus (UUKV) estimates) consists of 2% RNA, 7% carbohydrates, 58% protein and 33% lipid. The virion density in sucrose is 1.16 - 1.18 g/cc, and in CsCl is 1.20 - 1.21 g/cc. Virions have sedimentation coefficients of 400 to 500 S (Obijeski and Murphy, 1977).

The orthobunyavirus Bunyamwera virus (BUNV) is the prototype of its genus and the entire *Bunyaviridae* family. BUNV is significantly an important tool for the study of

Genus	Serogroup	Representative Virus
Orthobunya-virus	19 groups containing 177 viruses. Example groups :	
	Bunyamwera group (32)	Germiston and Bunyamwera viruses (human pathogens) , and Cache Valley virus (pathogen of cattle and sheep)
	California group (14)	California encephalitis, La Crosse and snowshoe hare viruses (human pathogens)
	Simbu group (25)	Aino, Simbu and Oropouche Viruses (humans and animals pathogens)
Hantavirus	One group containing 10 viruses.	
	Hantaan group	Hantaan, Puumala, Seoul and Sin Nombre viruses (human pathogens)
Nairovirus	7 groups containing 34 viruses Example groups :	
	CCHF group (3)	Crimean-Congo hemorrhagic fever virus (human pathogenic)
	Nairobi Sheep disease group (2)	Dugbe and Nairobi sheep disease viruses (humans and animals pathogenic)
Phlebovirus	3 groups containing 51 viruses. Example groups :	
	Sandfly fever group (23)	Sandfly fever Naples , Punta Toro and Rift Valley fever viruses (human pathogenic)
	Uukuniemi (12)	Section 1.01 Uukuniemi virus
Tospovirus	2 groups containing 4 viruses	
	Tomato Spotted Wilt (3)	Tomato spotted wilt virus (plant pathogen)
	Impatiens necrotic spot (1)	Impatiens necrotic spot virus (plant pathogen)

Table 1.1. Classification of *Bunyaviridae* (Elliott *et al*., 2000).

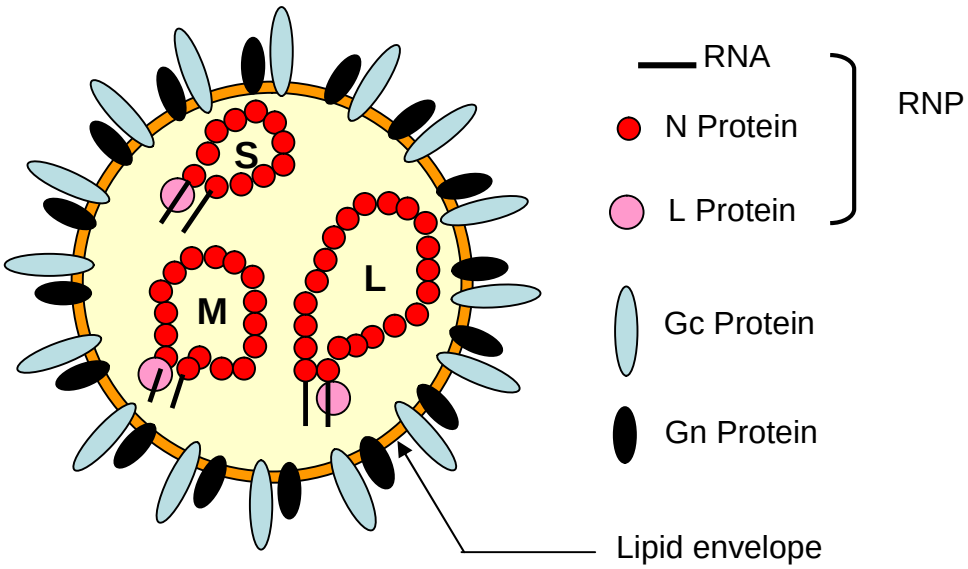


Figure 1.1. Schematic of bunyavirus particle.

The bunyavirus virion is 80-120nm in diameter. The three segments (L, M and S) within the viral envelope are encapsidated by the N protein and are associated with L polymerase to form RNPs. The two glycoproteins (Gn and Gc) are inserted into the viral envelope. Under the electron microscope, the RNPs are visualized as circular forms due to the complementarity of the 3' and 5' ends of the genome RNAs to form a pan-handle structure (Figure was provided by R.M.Elliott).

Orthobunyavirus	3'	UCAUCACAU---
	5'	AGUAGUGUG---
Hantavirus	3'	AUCAUCAUCUG---
	5'	UAGUAGUAUGC---
Nairovirus	3'	AGAGUUUCU---
	5'	UCUCAAAGA---
Phlebovirus	3'	UGUGUUUC---
	5'	ACACAAAG---
Tospovirus	3'	UCUCGUUA---
	5'	AGAGCAAU---

Figure 1.2. Consensus terminal sequences of the S, M, and L segments of bunyaviruses (taken from Elliott, 1996).

bunyaviruses for several reasons: it is relatively non-pathogenic, grows in many different cell lines, and produces high titres. The emergence of bunyaviruses has increased recently due to higher levels of travelling, animal transportation and globalisation. Rift Valley fever virus (RVFV) was previously restricted to the African continent, however, the recent outbreak of RVFV in Yemen and Saudi Arabia, is strong evidence of the ability of bunyaviruses to spread into new geographical areas (Balkhy and Memish, 2003).

1.2. Bunyaviruses and diseases

One of the most fascinating aspects of bunyaviruses is their ability to replicate in both vertebrate and invertebrate cells, with different outcomes of infection. In mammalian cells the infection is lytic and leads to cell death, whereas in invertebrate cells there is no cytopathology, and while high titres of virus are released early in infection, later the cells become persistently infected (Scallan and Elliott, 1992).

Members of the *Bunyaviridae* family cause several syndromes including: hemorrhagic fever, encephalitis and fatal respiratory syndrome (Table1.2). Hantaviruses are considered to be one of the most important among bunyaviruses due to their ability to cause two types of human illnesses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Among adults and children around 100 cases per year have been recorded for La Crosse virus (LACV) since it was discovered in 1960. It causes encephalitis in children (Gonzalez-Scarano *et al*., 1984). Another important disease is Crimean-Congo hemorrhagic fever (CCHF) which is caused by a nairovirus *via* a tick vector (Whitehouse, 2004). Bunyamwera virus causes a febrile illness with rash in children (Elliott, 1997). Moreover, RVFV causes different diseases in human such as febrile, retinal and fatal hemorrhagic diseases. It also causes abortion in domestic ruminants. RVFV is transmitted to humans and animals by sandflies or mosquitoes.

Globally, several bunyavirus epidemics have been recorded. In Egypt, 200,000 patients suffered from RVFV infection in 1977 and more than 200 human deaths were reported in Yemen and Saudi Arabia during a RVFV outbreak (Balkhy & Memish, 2003). In Moravia more than 20% of febrile diseases diagnosed were due to

GENUS/VIRUS	HOST:DISEASE	VECTOR	DISTRIBUTION
Orthobunyavirus			
Oropouche	Human: fever	Midge	SA
La Crosse	Human: encephalitis	Mosquito	NA
Tahyna	Human: fever	Mosquito	Europe
Akabane	Cattle: abortion	Midge	Africa, Asia
Cache Valley	Sheep: congenital defects	Mosquito	NA
Hantavirus			
Hantaan-like viruses	Human: severe HFRS	Rodent	Eastern Europe Asia
Seoul-like viruses	Human: moderate HFRS	Rodent	Worldwide
Puumala-like viruses	Human: mild HFRS	Rodent	Western Europe
Sin Nombre-like virus	Human: HPS	Rodent	NA & SA
Nairovirus			
Crimean-Congo hemorrhagic fever	Human: hemorrhagic fever	Tick	Eastern Europe, Africa, Asia
Nairobi sheep disease	Sheep, goats fever, hemorrhagic, gastroenteritis	Tick	E. Africa
Phlebovirus			
Rift Valley fever	Human: encephalitis, hemorrhagic fever, retinitis. Domestic ruminants: necrotic hepatitis, abortion	Mosquito	Africa
Sandfly fever Naples	Human: fever	Phlebotomine fly	Europe, Africa,
Tospovirus			
Tomato spotted wilt virus	Over 650 plant species	Thrips	Worldwide

Table1.2. The most important diseases due to members of the *Bunyaviridae* (taken from Elliott, 1997). NA, North America; SA, South America. HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome.

Tahyna bunyavirus (TAHV) infection (Gonzalez-Scarano *et al*., 1996). In Brazil, Oropouche virus (OROV) infects thousands of people (Pinheiro *et al*., 1981). In China about 100,000 patients are infected by hantaviruses annually (Elliott, 1997). In plants, tomato spotted wilt virus (TSWV), a member of *Tospovirus* genus, infects more than 650 plant species with different symptoms (Elliott *et al*., 2000).

1.3. Coding Strategies

1.3.1. The S segment

The S segment of viruses of the *Bunyaviridae* encodes the nucleocapsid N protein (Figure1.3a). In addition to the N protein, the S segment of orthobunyaviruses, phleboviruses and tospoviruses encodes a non-structural protein called NSs. In orthobunyaviruses, NSs is expressed in an overlapping reading frame using an alternative start codon to N from the same mRNA (Elliott, 1989b, Fuller and Bishop, 1982). In phleboviruses and tospoviruses, NSs is encoded by an ambisense strategy (de Haan *et al*., 1990) (Figure1.4). The N protein is encoded within the negative-sense half of the ambisense S RNA segment. The ambisense genomic segment serves as a template for synthesis of antigenomic RNA that acts as a template for the transcription of a second mRNA encoding non-structural protein NSs.

1.3.1.1. Nucleocapsid protein

The nucleocapsid protein of viruses of *Bunyaviridae* (size range 26-50 kDa) is the most abundant protein in virions and infected cells. The primary function of N protein is to encapsidate and protect the viral genome. The RNP of bunyaviruses is relatively resistance to digestion by RNase (Hacker *et al*., 1989; Kolakofsky and Hacker, 1991; Osborne and Elliott, 2000). The N protein is the major antigen, targeted by the humoral immune response during infection of Dugbe virus (El-ghorr *et al*., 1990), PUUV (Vapalahti *et al*., 1995) and TSWV (de Avila *et al*., 1990). Neither hantaviruses nor nairoviruses encode NSs, but, they encode the largest N protein in *Bunyaviridae* viruses 48 and 50kDa respectively.

High homology of the N protein within viruses from the same genus was reported whereas low homology was observed between the N proteins of viruses from different genera. For example, Bunyamwera serogroup viruses showed approximately 62% identity between N proteins and approximately 40% identity was observed with viruses in the California serogroup (Dunn *et al*., 1994; Elliott, 1995). Alignment of the N protein of 19 orthobunyaviruses from 3 serogroups (Bunyamwera, California and Simbu) demonstrates highly conserved regions in the middle and in the C-termini of the N proteins (Elliott, 1996) (Figure 1.5). Three phleboviruses display 39.5% identity in the N protein (Marriott and Nuttall, 1992) and the N proteins of five hantaviruses display 50% homology (Schmaljohn, 1996).The C-termini of hantavirus N proteins showed identities of 85% (Schmaljohn, 1996b). The N protein of some nairoviruses reveals 80% identity in the C-termini (Marriott and Nuttall, 1992).

1.3.1.2. NSs protein

Orthobunyaviruses, phleboviruses and tospoviruses are the only viruses of the family which encode NSs protein. There is no homology between NSs proteins from different genera and within viruses from the same genus, the similarity is lower than N protein. The size of NSs protein ranges from 11 to 52 kDa. Using a bunyavirus rescue system, a BUNV lacking NSs (BUNdelNSs) virus was generated (Bridgen *et al*., 2001). BUNdelNSs virus showed reduced shut-off in mammalian cells compared to wt BUNV. Furthermore, BUNdelNSs virus displayed a high induction of interferon (IFN) whereas wt BUNV does not. Upon infection with BUNdelNSs, cellular mRNA which encodes IFNβ was detected but not with wt BUNV. The NSs of BUNV was found to postpone cell death by inhibiting interferon responsive factor-3 (IRF-3) mediated apoptosis (Kohl *et al*., 2003). Thomas *et al*. (2004) found that BUNV NSs protein inhibits phosphorylation of cellular RNA polymerase II. A nonfatal human isolate of RVFV, designated clone 13, that expresses only 31% of the NSs ORF, was characterized (Muller *et al*., 1995). In interferon-deficient Vero cells clone 13 grows well, but grows poorly in interferon–competent cell lines. In mice lacking IFN receptors clone 13 was highly virulent and caused death within 2 days. In common with BUNdelNSs virus, clone 13 was a good inducer of INF, compared to wt virus. RVFV NSs protein caused host transcription inhibition (Billecocq *et al*., 2004).

Recently, the NSs of RVFV was shown to be disruptive to cellular RNA synthesis through interaction with cellular transcription factor TFIIH (Le May *et al*., 2004). TFIIH, one of the important transcription factors, consists of p44 and XPB proteins. The NSs of RVFV interacts with both p44 and XPB, preventing the assembly of TFIIH subunits which in return reduce concentration of TFIIH available for transcription. Leonard *et al*. (2006) found that inhibition of both host transcription and interferon response was linked to interaction of BUNV NSs protein with the MED8 protein (part of Mediator, a multiprotein complex necessary for mRNA synthesis). The interaction domain of MED8 mapped to the C-terminal aa 88-93 of the NSs protein. The NSs protein of UUKV interacts with 40S ribosomal subunit but the mechanism of this interaction has not yet been determined (Simons *et al*., 1992).

The inhibition of RNA silencing by NSs of LACV (Soldan *et al*., 2005) and TSWV (Takeda *et al*., 2002) was reported. The cellular machinery of several eukaryotic organisms such as mammals, plants and insects produces 21-25 nucleotides long RNAs called small interfering RNAs (siRNA) which target degradation of viral RNAs (Denli and Hannon, 2003). In the BUNV minigenome system, the NSs of BUNV, Guaroa and Lumbo orthobunyaviruses inhibits the viral polymerase activity (Weber *et al*., 2001). Similar BUNV minigenome utilizing mosquito cells was developed (Kohl *et al*., 2004b). In the latter system NSs does not inhibit the viral polymerase. Conversely, the NSs of RVFV was found to increase reporter gene activity (Ikegami *et al*., 2005).

1.3.2. The M segment

The M segment encodes two glycoproteins Gn and Gc, based on their position in the polyprotein precursor. The M segment of viruses from the *Orthobunyavirus*, *Phlebovirus* (except UUKV (Calisher, 1996)) and *Tospovirus* genera encodes a non-structural protein called NSm (Figure1.3b).

1.3.2.1. Glycoproteins

Bunyaviridae glycoproteins are type I membrane-spanning proteins, with the C-terminus of each facing towards the cytoplasm and the N-terminus oriented toward the ER (reviewed by Pettersson & Melin, 1996). The size of Gn ranges from 62-

110kD while the the Gc ranges from 32 to 67kD. Both glycoproteins are preceded by a signal peptide for translocation through the endoplasmic reticulum (ER) membrane (Eshita and Bishop, 1984; Lees *et al*., 1986; Schmaljohn *et al*., 1987; Arikawa *et al*., 1990; Pettersson & Melin, 1996). The presence of highly conserved cysteine residues in both Gn and Gc implies that disulfide bridge formation is a secondary structure determinant in these glycoproteins. Both glycoproteins are glycosylated at asparagine residues (at N60 in Gn and N624 and N1169 in Gc for BUNV) (Shi *et al*., 2005). The Gn of most bunyaviruses contains a Golgi retention signal and co-expression of both glycoprotiens is required for their transportation to the Golgi (Pettersson and Melin, 1996; Shi *et al*., 2004). In other words, Gn can localize at the Golgi apparatus by itself while Gc on its own is unable to, unless it is co-expressed with Gn (Lappin *et al*., 1994). Interactions of both glycoproteins in some hantaviruses are required for Golgi localisation. When both glycoproteins were expressed individually, they were retained in the ER, but coexpression of both Gn and Gc from separate plasmids resulted in localisation of both to the Golgi (Ruusala *et al*., 1992; Shi and Elliott, 2002). The Gn retention sequences of different bunyaviruses have been mapped. For example, the transmembrane domain of BUNV Gn protein (Shi *et al*., 2004), the transmembrane domain and cytoplasmic tail of RVFV Gn protein (Gerrard and Nichol, 2002), and the cytoplasmic domain of UUKV Gn protein (Andersson *et al.,* 1997). Heterodimerization of Gn and Gc of Hantaan, Uukuniemi and Punta Toro viruses in the ER was observed (Chen and Compans, 1991; Persson and Pettersson, 1991).

Although the polyprotein precursor has not been isolated from infected cells, the full length precursor has been detected by *in vitro* translation systems (Suzich and Collett, 1988). This observation can be explained as rapid co-translational cleavage of the polyprotein precursor (Nakitare and Elliott, 1993). To date, only one enzyme has been reported to be involved in processing CCHFV Gn protein, the cellular SK-1 protease (Vincent *et al*., 2003). Amino acids 860 -1442 of LACV Gc are crucial in fusion and entry (Plassmeyer *et al*., 2006) and similar regions were found in the E1 fusion protein of two alphaviruses, Sindbis and Semliki Forrest viruses.

1.3.2.2. NSm

The NSm protein of orthobunyaviruses is encoded from the middle of the genome, while in phleboviruses is encoded from the 3` end (Figure 1.3b). The NSm of tospoviruses is translated from mRNA transcribed from the antigenome RNA while the glycoproteins are transcribed from mRNA encoded on the genome RNA as they utilize an ambisense coding strategy. BUNV NSm is encoded as part of a precursor polyprotein. Its localisation in the Golgi complex has been reported (Nakitare and Elliott, 1993). The function of the NSm of orthobunyaviruses and phleboviruses is unclear. Although the NSm protein of BUNV is targeted to the Golgi, BUNV and LACV NSm proteins have no role in Gn and Gc localization in the Golgi (Bupp *et al*., 1996, Lappin *et al*., 1994). The NSm protein of TSWV was shown to be involved in the transport of RNPs *via* plant plasmodesmata (Storms *et al*., 1995). Significant reduction in Gc protein glycosylation occurred when NSm protein was deleted from the polyprotein (Shi and Elliott, 2004). The N-terminus (aa 300-350) of BUNV NSm is required for virus assembly while the C-terminal region (aa 460-480) is an integral signal for the Gc glycoprotein (Shi *et al*., 2006).

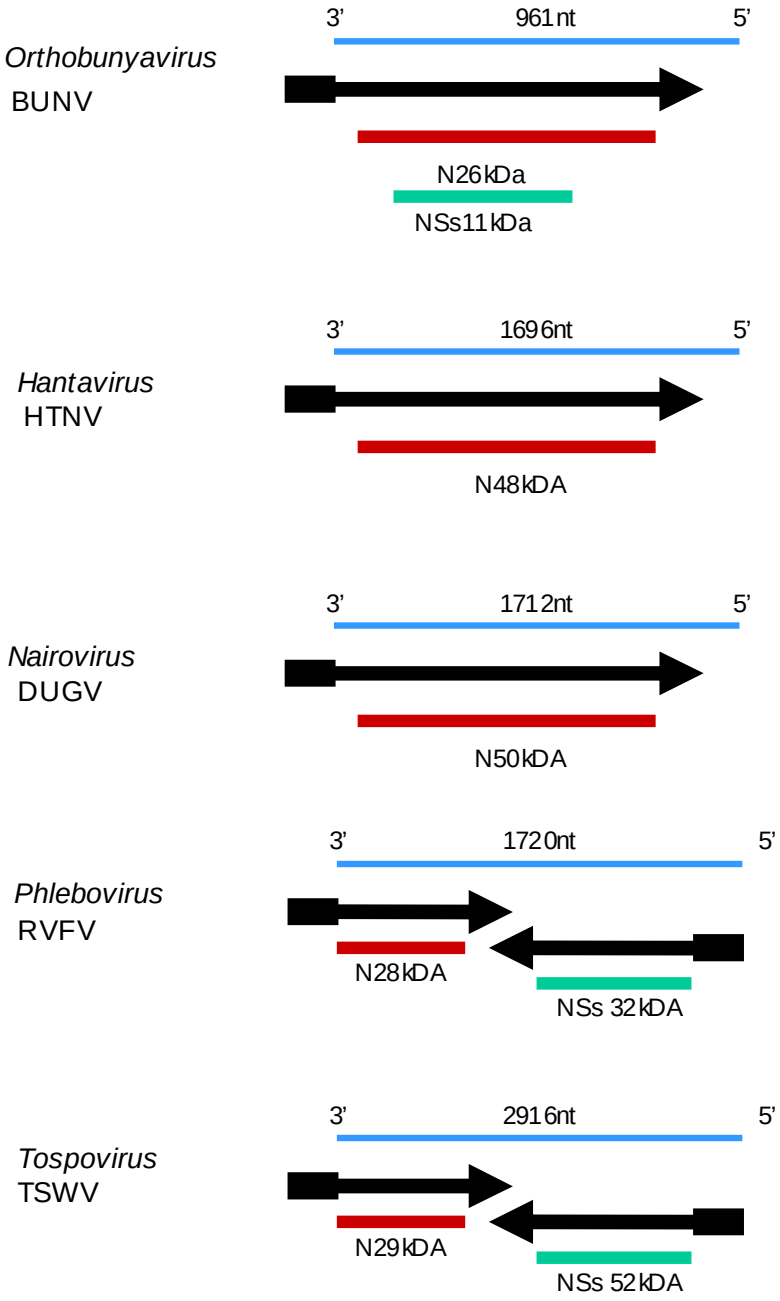
1.3.3. The L segment

The L segment of bunyaviruses encodes the RNA-dependent RNA polymerase or L protein (Figure1.3c). The L segment of orthobunyaviruses, hantaviruses and phleboviruses is about 6500 nucleotides (Elliott, 1989a, Elliott *et al*., 1992; Muller *et al*., 1994, Schmaljohn, 1990). Tospoviruses L segment is 8897 nucleotides in length and the longest L segment is 12225 nucleotides in Dugbe virus (DUGV) a nairovirus. Although the L segment of bunyaviruses contains one open reading frame (ORF), other smaller ORFs are predicted in the genome of orthobunyaviruses and hantaviruses; however, no additional product has been reported (Elliott, 1989a, Schmaljohn, 1990).

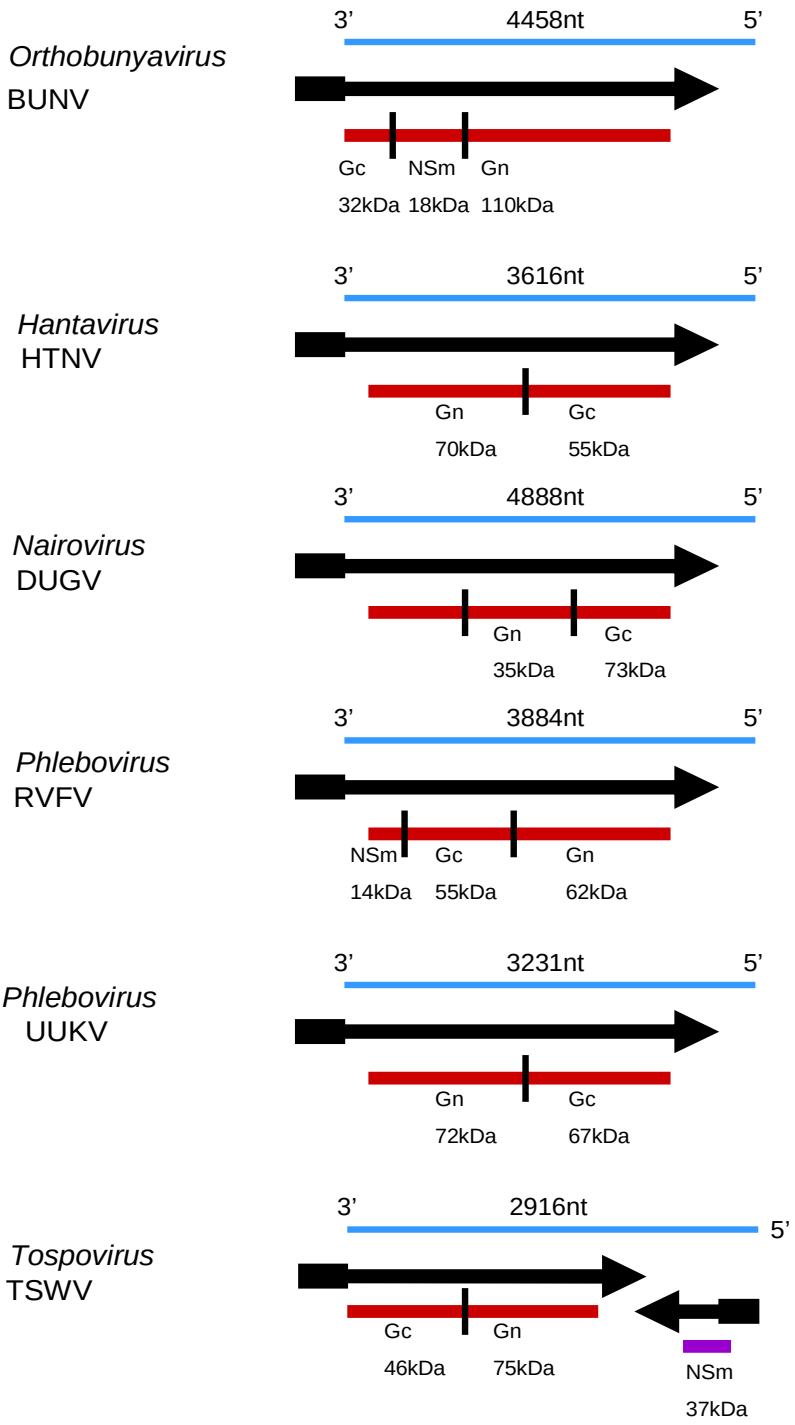
1.3.3.1. L protein

The RNA-dependent RNA polymerase is a structural protein involved in virus transcription and replication in concert with nucleocapsid N protein (Dunn *et al*.,

(a) S segment


3’
961nt
5’
Orthobunyavirus
BUNV
N26kDa
NSs11kDa
3’
1696nt
5’
Hantavirus
HTNV
N48kDA
3’
1712nt
5’
Nairovirus
DUGV
N50kDA
3’
1720nt
5’
Phlebovirus
RVFV
N28kDA
NSs 32kDA
3’
2916nt
5’
Tospovirus
TSWV
N29kDA
NSs 52kDA

(b) M segment


3’
4458nt
5’
Orthobunyavirus
BUNV
Gc
NSm
Gn
32kDa
18kDa
110kDa
3’
3616nt
5’
Hantavirus
HTNV
Gn
Gc
70kDa
55kDa
3’
4888nt
5’
Nairovirus
DUGV
Gn
Gc
35kDa
73kDa
3’
3884nt
5’
Phlebovirus
RVFV
NSm
Gc
Gn
14kDa
55kDa
62kDa
3’
3231nt
5’
Phlebovirus
UUKV
Gn
Gc
72kDa
67kDa
3’
2916nt
5’
Tospovirus
TSWV
Gc
Gn
46kDa
75kDa
NSm
37kDa

(c) L segment

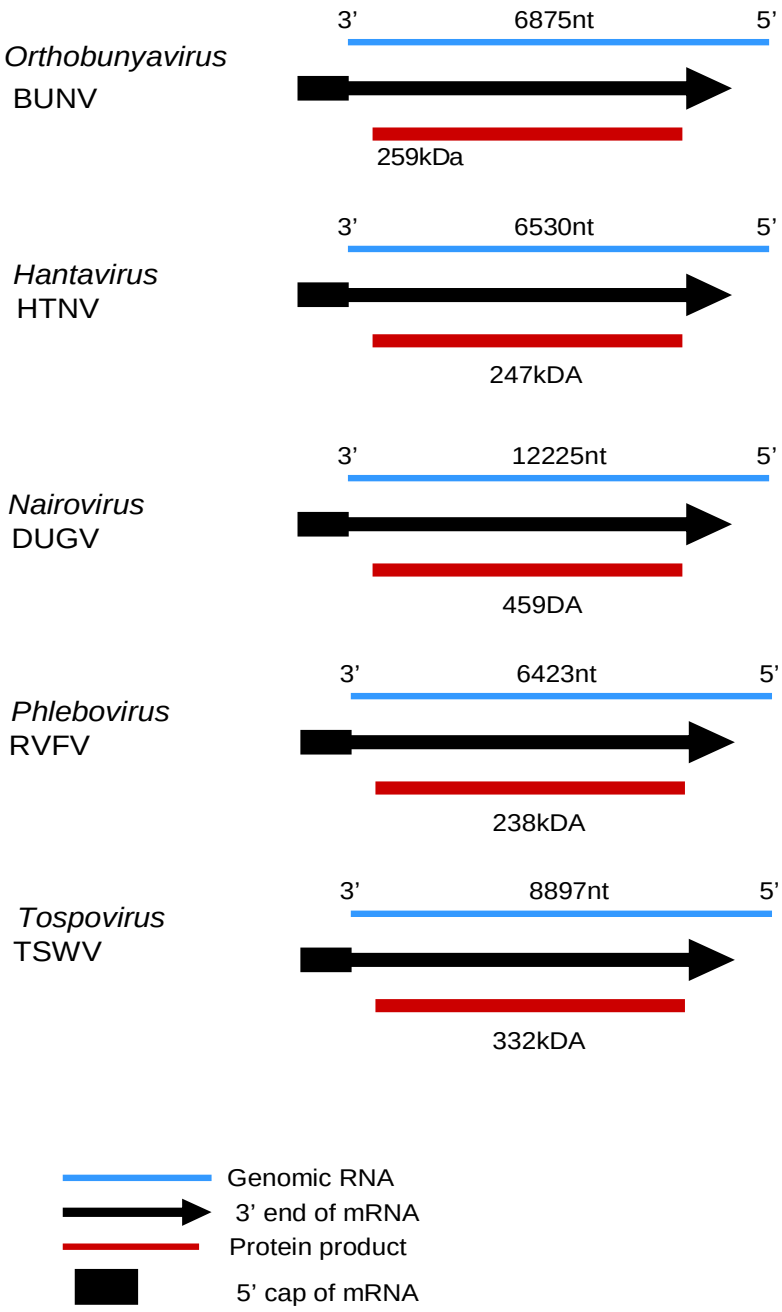


Figure 1.3. The coding strategies of bunyavirus genome segments. The S segment (a), M segment (b), and L segment (c) (adapted from Elliott, 1996).

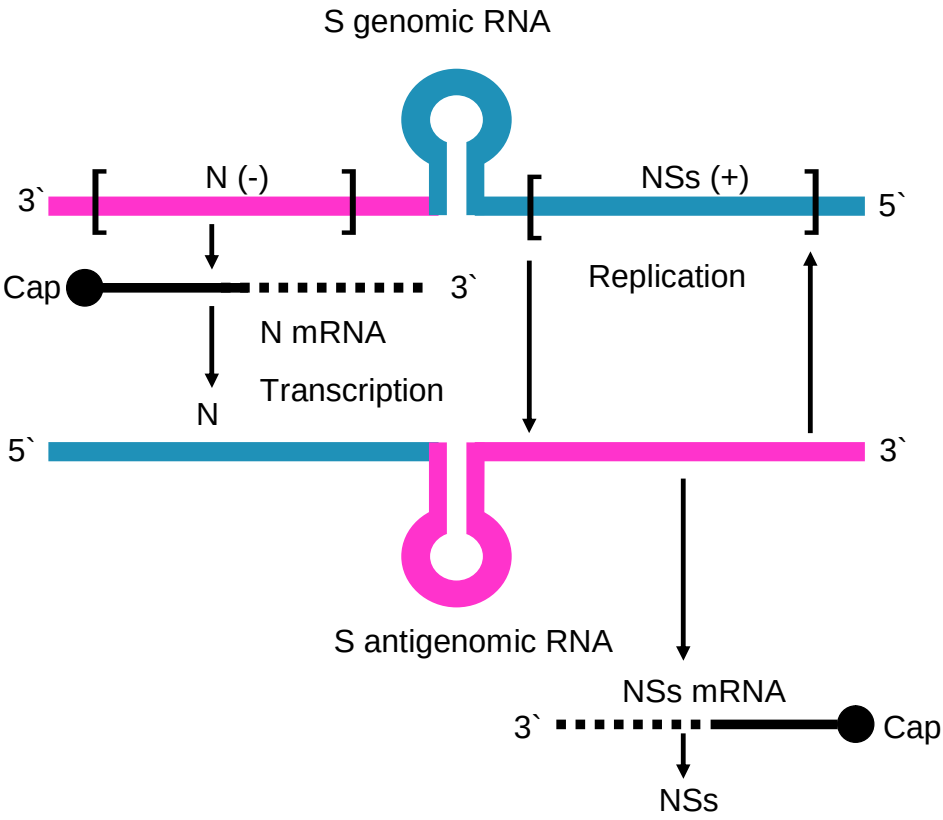


Figure 1.4. Ambisense strategy of phieboviruses and tospoviruses S segment. Viruses utilizing an ambi-sense codon strategy produce two mRNAs, one is transcribed from the viral genomic RNA and a second is produced from the viral antigenomic RNA (adapted from Wagner and Hewlett, 2004).

```
   1                                                                 100
LAC  .MsDLvFyDVastgangFDPDagYmdFcvknaeslnLaaVRIFFlnaakaKaaLsrkpErkanpkFGeWqVeViNnhFPGNRNnPIgnndLTiHRlSGYL
SSH  .MsDLvFyDVastgangFDPDagYmaFcvkyaesvnLaaVRIFFlnaakaKaaLsrkpErkanpkFGeWqVeVvNnhFPGNRNnPInsddLTiHRlSGYL
 CE  .MsDLvFyDVastgangFDPDagYvdFcakhgesinLaaVRIFFlnaakaKaaLsrkpErkanpkFGeWqVeVvNnhFPaNRNnPIgnndLTiHRiSGYL
LUM  .MsDLvFyDVastgangFDPDagYvdFcvkhgesinLhsVRIFFlnaakaKaaLarkpErkaspkFGeWqVeIvNnhFPGNRNnPIdnndLTiHRlSGYL
 JC  .MgDLvFyDVastgangFDPDagFvaFmadhgesinLsaVRIFFlnaakaKaaLarkpErkatpkFGeWqVeIvNnhFsGNRNnPIgnndLTiHRlSGYL
 JS  .MgDLvFyDVastgangFDPDagFvaFmadhgesinLsaVRIFFlnaakaKaaLarkpErkatpkFGeWqVeIiNnhFPGNRNnPIgnndLTiHRlSGYL
KEY  .MgDLvFyDVastgangFDPDagYvaFmanhgesisLstVRIFFlnaakaKaaLtrkpErkatpkFGeWqVeIvNnhFPGNRNnPIgnndLTlHRiSGYL
MEL  .MgDLiFyDVastgangFDPDagYlaFtiahgeainLsaVRIFFlnaakaKaaLsrkpErkatpkFGdWqVeIvNnhFPGNRNnPIgnndLTiHRlSGYL
TVT  .MsELvFyDapstgangFDPDagYvaFiaahagsydLsaVRIFFlnaakaKnaLsrkpEgkvsikFGeWsVeVvNnhFPGNRNnPIgnndLTiHRiSGYL
 CV  .MiELeFnDVaantsstFDPEiaYvnFkrihttglsYdhIRIFYikgreiKtsLtkrsEwevtlnLGgWkVtVfNtnFPGNRNsPVpddgLTlHRlSGYL
NOR  .MiELeFnDVaantsstFDPEvaYinFkrvyttglsYdhIRIFYikgreiKtsLtkrsEwevtlnLGgWkVtVfNtnFPGNRNsPVpddgLTlHRlSGFL
MAG  .MiELeFnDVaantsstFDPEiaYvnFkrihttglsYdhIRVLYikgreiKtsLtkrsEwevtlnLGgWkVaVfNtnFPGNRNsPVpddgLTlHRlSGFL
BAT  .MiELeFnDVaantsstFDPEvaYinFkriyttglsYdnIRIFYikgreiKtsLskrsEwevtlnLGgWkVtVfNtnFPGNRNsPVpddgLTlHRlSGFL
BUN  .MiELeFhDVaantsstFDPEvaYanFkrvhttglsYdhIRIFYikgreiKtsLakrsEwevtlnLGgWkItVyNtnFPGNRNnPVpddgLTlHRlSGFL
 MD  .MiELeFhDVaansstFDPEvaYasFkrvhttglsYdhIRIFYikgreiKtsLskrsEwevtlnLGgWkVaVfNtnFPGNRNsPVpddgLTlHRlSGFL
GER  .MlELeFeDVpnnigstFDPEsgYtnFqrnylpgvtLdqIRIFYikgreiKnsLskrsEwevtlnLGgWkVpVlNtnFPGNRNnaVpdygLTfHRiSGYL
GRO  .MaEieFfDVaqnatstFnPElqYatFkrtntglnYdnIRIFYlngkrsKdtLskrsEqsvvlnFGgWrIpVvNthFPGNRNsPVlddsFTlHRvSGYL
KRI  .MsEieFhDVtantsstFDPEagYaaFkrrhttglnYdhIRIFFlngkkaKdtLskrsEttitlnFGgWkIpVvNthFleNRNmsVpddgLTlHRvSGYL
AINO manqFiFqDVpqrnlatFnPEvgYvaFiakhgaqlnFdtVRfFFlnqkkaKmvLsktaqpsvdltFGgikftflvNnhFPqytanPVpdtaLTlHRlSGYL
 Con  -M--L-F-DV-------FDP---Y--F-----------------K--L----E------G-W-V---N--FPGNRN-P-----LT-HR-SG-L
```

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     101                                                                200
LAC  ARWvLDqynenddEsqhelIrttIINPiAEsNGVgWdsGpEIYLSFFPGtEMFLetFkFYPLtIGIhrVkqgmMDpqYLkKaLRQRYgtLtAdkWmsqKv
SSH  ARWvLEqykenedEsrrelIkttIINPiAEsNGVrWdsGaEIYLSFFPGtEMFLetFkFYPLtIGIYrVkqgmMDpqYLkKaLRQRYgsLtAdkWmsqKv
 CE  ARWvLEqykenedEsqrelVkttVINPiAEsNGIrWenGaEIYLaFFPGtEMFLetFkFYPLtIGIYrVkngmMDsqYLkKaLRQRYgsLtAekWmsqKt
LUM  ARWvLEqfkenedaaqrelIkttVINPiAEsNGIrWdnGaEIYLaFFPGtEMFLetFnFYPLtIGIYrVkhgmMDpqYLkKaLRrRYgsLtAdkWmsqKt
 JC  ARWvLEhfnsdddEsqrelIrstIINPiAEsNGIhWnnGpEIYLSFFPGtEMFLeiFkFYPLtIGIYrVkhgmMDpqYLkKaLRQRYgtLtAekWmaqKt
 JS  ARWvLEhftadddEsqrelIrstIINPiAEsNGIhWnnGpEIYLSFFPGtEMFLeiFkFYPLtIGIYrVkhgmMDpqYLkKaLRQRYgtLtAekWmaqKt
KEY  ARWvLEhfgegedEsqkelIkstVINPiAEsNGIrWgnGVEIYLSFFPGtEMFLelFkFYPLtIGIYrVkhgmMDaqYLkKaLRQRYgtLtAdkWmaqKt
MEL  ARWvLDlfkenedEsqkelIqstIINPiAEsNGIhWanGVEIYLSFFPGtEMFLeaFrFYPLtIGIYrVkhglMDpqYLkKaLRQRYgtLtAdkWmaqKt
TVT  ARWvLEefkgqddEaqkdiIrstIVNPiAEsNGIhWdsGaDaYLSFFPGtEMFLesFdFlPLaIGIYrVkngmMDvqYLkKaLRQRYgtMtAdkWmstKt
 CV  ARYlLEki.lkvsDpekviIkskIINPlAEkNGItwsdGeEVYLSFFPGsEMFLgtFkFYPLaIGIYkVqrkeMEpkYLeKtMRQRYmgLeAstWtisKv
NOR  ARYlLEki.lkvsEpekliIkskIINPlAEkNGItwtdGeEVYLSFFPGsEMFLgtFkFYPLaIGIYkVqrkeMEpkYLeKtMRQRYmgLeAstWtisKv
MAG  ARYlLEki.lkvsDpekliIkskIINPlAEkNGItwadGeEVYLSFFPGsEMFLgtFkFYPLaIGIYkVqkkeMEpkYLeKtMRQRYmgLeAatWtvsKv
BAT  ARYlLEki.lkvsDpekliIkskIVNPlAEkNGItwadGeEVYLSFFPGsEMFLgtFrFYPLaIGIYkVqrkeMEpkYLeKtMRQRYmgLeAstWtvsKl
BUN  ARYlLEkm.lkvsEpekliIkskIINPlAEkNGItwndGeEVYLSFFPGsEMFLgtFrFYPLaIGIYkVqrkeMEpkYLeKtMRQRYmgLeAatWtvsKl
 MD  ARYlLEki.lkvsEpekllIkskIINPlAEkNGItwadGeEVYLSFFPGsEMFLgiFkFYPLaIGIYkVqrkeMEpkYLeKtMRQkYmnMdAatWtvtqv
GER  ARYlLgky.laetEpeklimrtkIVNPlAEkNGItwesGpEVYLSFFPGaEMFLgtFrFYPLaIGIYkVqrkeMDpkFLeKtMRQRYlgidAqtWtttKl
GRO  ARYlLEry.ltvsapeqaiIrskIINPiAasNGItwedGpEVYLSFFPGtEMFLetFkFYPLaIGIYkVqkkmMEakYLeKtMRQkYagLdAsqWtqqKy
KRI  ARYlLDrv.ysagEpeklkIkttIINPiAashGItwddGeEVYLSFFPGsEMYLttFkFYPLaIGIYkVqrklMDpkYLeKtMRQRYmnLdAsqWtqkhf
AINO  AkWvaDqcktnqiklaeamek..IVmPlAEvkGctWteGltmYLgFaPGaEMFLetFeFYPLvIdmhrVlkdgMDvnFMrKvLRQRYgtLtAeqWmtqKi
 Con  AR-L-----------------------I-NP-AE-NGI-W--G-E-YLSFFPG-EMFL--F-FYPL-IGIY-V----MD--YL-K--RQRY--L-A--W---K-
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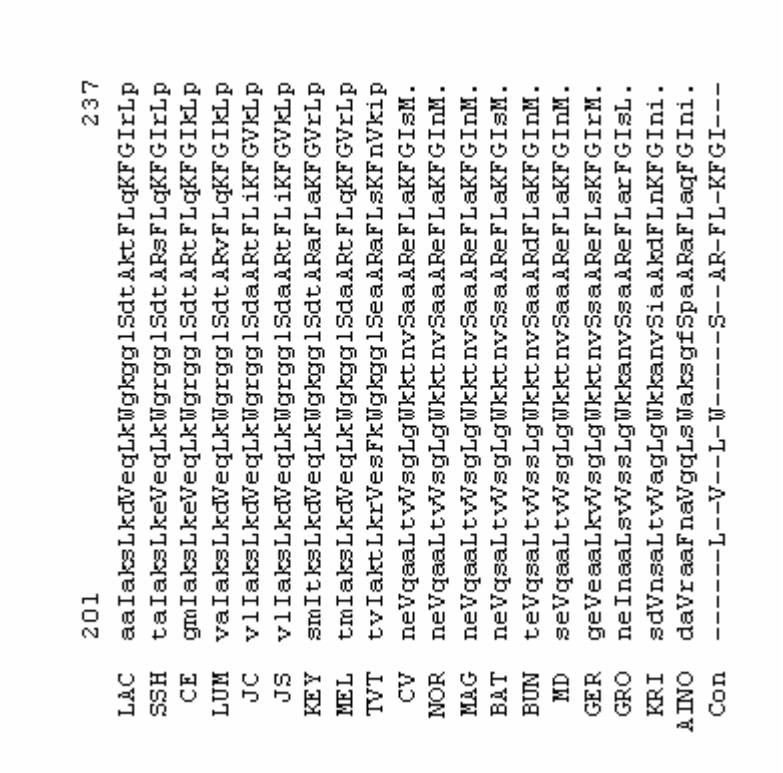

Figure 1.15. Alignment of 19 orthobunyavirus N protein sequences (taken from Elliott, 1996). “Con” indicates conserved amino acids.

1995; Blakqori *et al*., 2003; Flick *et al*., 2003a; Flick *et al*., 2003b; Ikegami *et al*., 2005). The L protein of orthobunyaviruses, hantaviruses and phleboviruses is about 250kD. The L proteins of tospoviruses and nairoviruses are 332 and 459 kD respectively. The L polymerase cleaves the cap of cellular mRNAs (cap snatching) in order to prime the synthesis of S, M and L mRNAs. The process is similar to that which occurs in influenza virus (Krug *et al*., 1979, Plotch *et al*., 1981). Subsequently, extensions of 8 to 15 nucleotides are found at the 5` ends of the viral mRNAs that are not templated from the genomic RNA segments by the virus (Collett, 1986, Duijsings *et al*., 2001, Garcin *et al*., 1995, Gro *et al.*, 1992, Jin and Elliott,1993a, Patterson and Kolakofsky, 1984). Alignment of bunyavirus L proteins showed the presence of four consensus motifs, A, B, C and D (Poch *et al*., 1989). Site directed mutagensis of BUNV L protein revealed consensus amino acids in motifs A and C are crucial for the functionality of L protein. Mutation at residue N1119G which is conserved among orthobuyaviruses, hantaviruses and tospoviruses generated impaired L protein (Jin and Elliott, 1992). Two motifs conserved between bunyavirus and arenavirus L proteins were observed (Muller et al., 1994). Honig *et al*., (2004) predicted protease and homology sequence to helicase motifs in CCHFV L protein. Phylogenetic analysis of the L protein sequences of bunyaviruses revealed that the orthobunyaviruses and tospoviruses are more closely related (Roberts *et al*., 1995).

1.4. The role of N proteins in the virus replication cycle

The primary function of the N protein of many viruses is to protect the viral genome and antigenome from physical and chemical injuries, to prevent the formation of secondary structure, and to form ribonucleocapsids (RNPs) in negative-sense viruses that are transcriptionaly active. Furthermore, the N protein is suggested to regulate the activity of the viral polymerase, switching from mRNA synthesis to antigenome RNA synthesis. Moreover, lack of a matrix protein in bunyaviruses suggests that the RNPs interact with the C-tails of Gn or Gc to promote virus assembly underneath the Golgi membrane.

1.4.1. Positive-sense RNA viruses

The encapsidation process has been described best in tobacco mosaic virus (TMV) assembly (Figure 1.6). The genome RNA contains a stem-loop called the origin of assembly sequence (OAS) ≈ 1kb from the 3' end. Encapsidation is initiated when a two layered disc aggregate of 34 coat protein subunits binds to this site on the RNA. The OAS is inserted into the middle of the disc and melts to promote the rest of genome interacting with coat protein. As a result, a conformational change occurs in the discs converting them to a locked washer. The RNA genome is buried in the centre of the disc as more proteins are added to the helix. Encapsidation of the genome at the 3' end is slower as the travelling loop needs to be threaded through the central hole of the disc (Cann, 2005).

The N protein NCp7 of HIV-1 is involved in the annealing of the primer (tRNA) to the virus genome to initiate synthesis of (-) strand DNA, viral genome encapsidation and viral RNA dimerzation (Darlix *et al*., 1995). Furthermore, Berat *et al*., (1993) demonstrated that the reverse transcriptase of HIV was regulated by N protein. NCp7 interacts with encapsidation signal, a stem-loop designated SL3, triggering packaging of heterogenous sequences into virus-like particles (Hayashi *et al*., 1992). The core proteins of several positive-strand plant viruses have been reported to bind specifically or non-specifically to the RNA. Specific-binding to a secondary structure in the 3' UTR was observed in brome mosaic virus (Duggal and Hall, 1993), turnip crinkle virus (Wei & Morris, 1991) and alfafa mosaic virus (Reusken *et al*., 1994). The RNA-binding activity of N and the infectivity of infectious bronchitis virus (IBV) were dramatically reduced when either of R76 or Y94 in the N-terminus of N protein was substituted to Alanine (Tan *et al*., 2006).

1.4.2. Nonsegmented negative-strand RNA viruses

Vesicular stomatitis virus (VSV) and rabies virus (RV) (*Rhabdoviridae*) are nonsegmented NSVs. Their genomes contain leader RNA sequences at the 3' end thought to be an encapsidation signal. In both viruses, the N protein shows affinity for binding leader RNA 10-fold higher than that for nonviral RNA (Blumberg *et al*., 1983; Yang *et al*., 1998). The N protein of VSV (49kD) was thought to bind to a region of

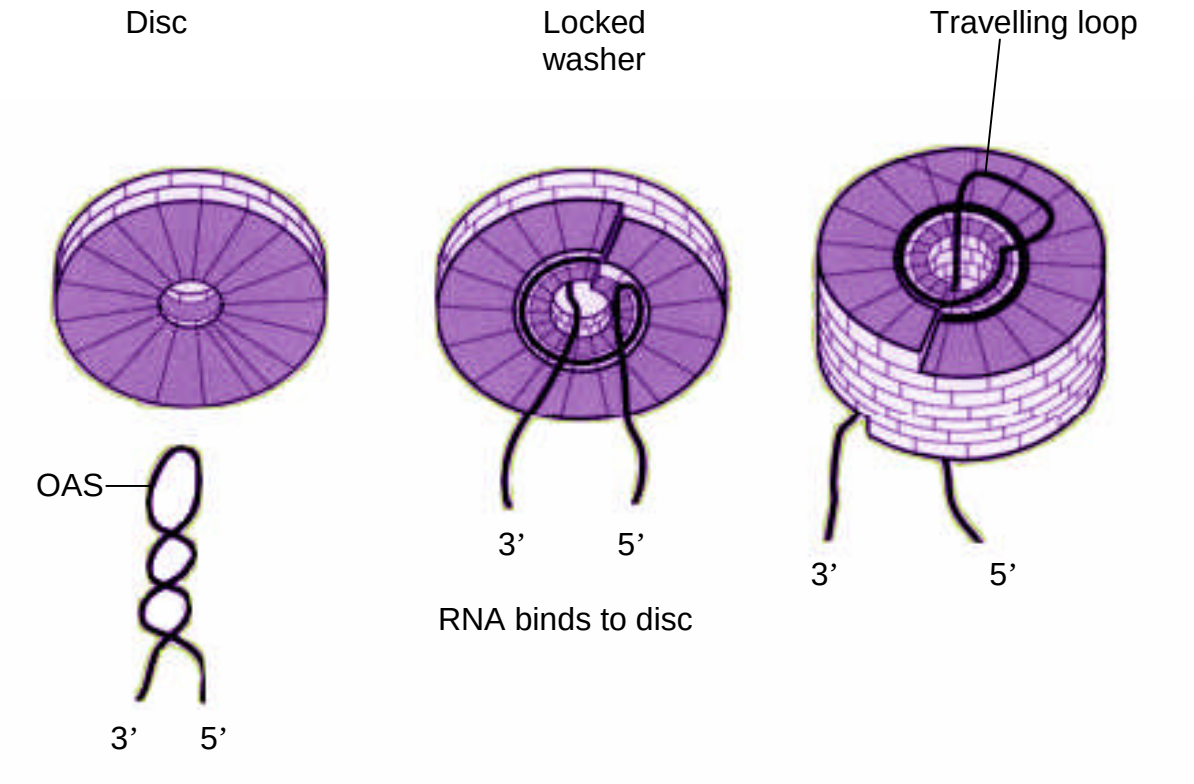

Figure 1.6. Assembly of TMV.

Assembly commences when the orgin of assembly sequence (OAS) interacts with a disc causing the disc to convert to a locked washer structure. Further discs add to this structure so RNA is buried in the centre of the discs (Taken from Cann, 2005).

the positive-sense leader RNA (14 -17nt) possessing an adenine at every third position. This region was implied to be the encapsidation initiation signal (Blumberg *et al*., 1983; Moyer *et al*., 1991; Smallwood and Moyer, 1993). RV N protein was suggested to bind adenine-rich sequences (20-30nt) on the leader RNA (Yang *et al*., 1998). Sendai virus (SeV) (*Paramixoviridae*) nucleoprotein NP protein (58kD) showed a similar pattern of encapsidation to that of VSV N protein. SeV NP binds to the 54nt leader RNA sequence specifically, commencing the encapsidation of virus RNA (Lamb and Kolakofsky, 1996). The RV, VSV and SeV RNPs are resistant to digestion by ribonuclease.

1.4.3. Segmented negative-strand RNA viruses

Influenza virus nucleoprotein (NP) (56kD) binds non-specifically to the RNA with high affinity to the 5' end (Bauding *et al*., 1994). It has been reported that NP protein binds RNA with a dissociation constant 2×10^{-8} M (Yamanaka *et al*., 1990; Bauding *et al*., 1994; Digard *et al*., 1999). *In vitro* translated NP displayed self-association in the absence of RNA with a dissociation constant of approximately 2×10^{-7}M (Elton *et al*., 1999a).This suggests that the NP binds RNA, triggering multimer formation. However, the replication efficiency reached the highest levels when even numbers of NP formed, suggesting that NP forms dimers. In infected cells dimeric and trimeric forms of NP were observed (Prokudina-kantorovich and Semenova, 1996). *In vitro*, multimerisation of NP was observed (Ruigrok and Bauding, 1995). Possessing a large number of positively charged residues of the NP implies that electrostatic interactions between the NP and RNA-back bone occur (Baudin *et al*., 1994). In contrast to RV, VSV and SeV, influenza virus RNPs are susceptible to digestion by ribonuclease, implying that the RNA is located on the outside of the NP (Duesberg, 1969; Pons *et al*., 1969; Baudin *et al*., 1994).

1.5. Encapsidation of bunyaviruses

In infected cells both the viral genome and antigenome but not the mRNA were found to be encapsidated by nucleocapsid N protein. Bunyavirus mRNAs are truncated by approximately 100nt at the 3' end compared to the antigenome RNA (Bishop *et al*., 1983; Jin and Elliott, 1993a; Patterson and Kolakofsky). The expression of BUNV N

protein as a 6-histidine-tagged fusion protein in *Echerichia coli* was reported by Osborne and Elliott (2000). Using metal chelate chromatography the 6-histidine-tagged-N protein was purified. To investigate binding of N protein *in vitro* an RNA probe containing the 5' terminal 32 and 3' terminal 33 nt of the S sgment was used. Gel electrophoretic mobility shift assay (GEMSA) showed multiple bands (ladder-like profile) on the protein gel when high concentration of N was added to the riboprobe. On the other hand, addition of low amount of N caused the formation of a single band. This suggests that the formation of multiple higher RNA-N complexes is due to binding of discrete N molecules to the riboprobe. RNA-N complexes were resistant to degradation with up to 1µg of RNase A per ml. Moreover, the N protein binds selectively to a predicted stem-loop at the 5' end of the S RNA, suggesting that the encapsidation initiation signal is located within 3-18 nt of the the 5' end. Likewise, the N protein of PUUV was expressed as a His-tagged forn in bacteria and the formation of complexes with ssRNA was confirmed. When the concentration of tagged-N increased, the complexes shifted further up the gel, indicating an increase in the size of the complexes. PUUV N protein binds preferentially to double-stranded RNA (Gott *et al*., 1993), suggesting a sequence-specific (panhandle or internal stem-loop) is involved in the encapsidation. The central conserved region (aa 175 -217) of HTNV N protein was found to mediate the interaction with genomic RNA (Xu *et al*., 2002). Trimeric forms of SNV N protein bound specifically with high affinity the 3' and 5' ends of the S genomic RNA.

In contrast, the monomeric and dimeric forms of SNV N protein bound semispecifically and were sensitive to high salt concentrations (Mir and Panganiban, 2004). Moreover, the trimeric form of N protein from Andes, PUU, Prospect Hill, SEO and SN viruses recognised both individual homologous and other hantavirus panhandles with high affinity. Dramatic affinity reduction was observed when panhandles from the *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* or *Tospovirus* genera were used (Mir *et al*., 2006). This is an indication of genus-specific recognition of the viral RNA panhandle by HTNV N protein.

1.6. Interaction of bunyavirus N protein molecules

Bunyavirus N protein shows the ability to multimerise in the presence or absent of viral RNA. Using a bacculovirus system, recombinant hantavirus N protein expressed in insect cells, showed a nucleocapsid-like structure with sedimentation and morphological characteristics similar to the viral N protein (Betenbaugh *et al*., 1995). The N proteins of three hantaviruses (Tula, PUU and Prospect Hill) were cross-linked and showed trimer formation (Alfadhli *et al*., 2001; Kaukinen *et al*., 2001). Kaukinen *et al*. (2004) suggested that the hantavirus N protein forms multimers by association of preformed trimers.

The first 10 and the last 17 residues of the BUNV N protein were found to be involved in N multimerisation and protein functionalty in the BUNV minigenome system (Leonard *et al*., 2005). Two deletion mutants in the N-terminal region of BUNV N protein were generated by PCR, BUNNdel1-10 and BUNNdel11-20. The latter mutant N displayed no activity in the minigenome system while mutant BUNdel1-10 showed very low activity and did not efficiently multimerize into oligomers beyond dimers compared to the native N protein. Furthermore, BUNNdel217-233 mutant and a double mutant lacking the first and the last 17 aa (called BUNdel1-10 and 217-233) were generated. Both mutant N proteins were inactive in the minigenome system. A cross-linking experiment showed that mutant BUNNdel217-233 was able to form dimers and weakly trimers but not higher multimers. In contrast, the double mutant (BUNNdel1-10 and 217-233) failed to form any multimers. This study suggested two possible interaction sites at the N-terminus and C-terminus of BUNV N protein, and also that BUNV N protein forms multimers from monomers built molecule by molecule. Likewise, TSWV N protein formed multimers by addition of one N molecule at a time (Uhrig *et al*., 1999). Several bunyavirus N protein domain maps have been determined (Figure1.7). Both Tula and SNV N proteins interact through the N-terminus and the C-terminus (Alfadhli *et al*., 2001; Kaukinen *et al*., 2003a; Uhrig *et al*., 1999). The central region and the C-terminal region of Seoul virus (SEOV) and Hantaan virus (HTNV) N proteins are involved in N molecule interactions (Yoshimatsu *et al*., 2003). Amino acids 100 -120 and 330 - 405 play a crucial role in PUUV N-N protein interactions (Lindgren *et al*., 2006).

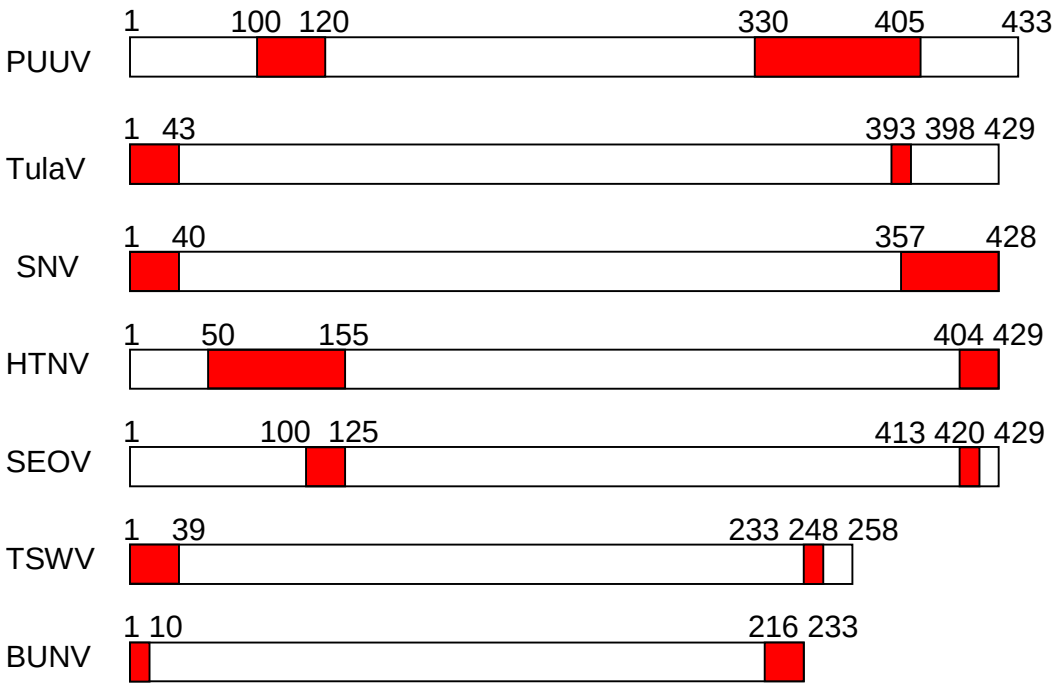

Figure 1.7. Linear domain maps of bunyavirus N proteins.The interaction domains are indicated by red boxes (adapted from Kaukinen *et a*l., 2003a).

1.7. Interactions of N protein with L polymerase

It has been suggested that the N protein of many negative-strand RNA viruse (*e.g.* SeV, measles) regulates viral transcription and replication through interactions with L polymerase. Bankamp *et al*. (1996) demonstrated that the N protein of measles virus binds the viral polymerase, regulating N protein assembly and replication. Influenza virus replication requires soluble NP, implicating possible interactions between the NP and the polymerase complex (Beaton and Krug, 1986; Shapiro and Krug, 1988). In contrast, virus transcription requires the binding of NP to the RNA (Medcalf *et al*., 1999). The 3D structure of recombinant influenza RNP showed that the viral polymerase binds directly to two monomers of the N protein (Martin-Benito *et al*., 2001).

Qanungo *et al*. (2004) reported that the L polymerase of VSV exists in two distinct complexes. The first consists of viral L and P proteins, and cellular translation elongation factor-1α, heat-shock protein 60 and guanylyltransferase, and is involved in the early synthesis of mRNA. The second complex is composed of L, P and N proteins, and is involved in the production of full-length antigenome and genome RNA.

The N protein of Kairi bunyavirus (KRI) does not function in the BUNV minigenome assay in concert with BUNV L protein and BUNV minigenome, whereas the N proteins of other bunyaviruses function almost as well as that of BUNV N protein (Dunn *et al*., 1995). Furthermore chimeras were made between these N proteins, fused at residues 96/97 and showed chimeras with the N-terminal half derived from KRI were non-functional, whereas chimeras with the KRI C-terminal half of N worked (RM.Elliott, personal communication).

1.8. Interactions of N protein with the glycoproteins

It is believed that interactions of the N proteins with the glycoproteins of several viruses promote virus budding and assembly. A Recombinant Semliki Forest virus (SFV), an alphavirus genome which lacks the N protein or the glycoproteins genes failed to be released from cells (Suomalainan *et al*., 1992). Using the mammalian

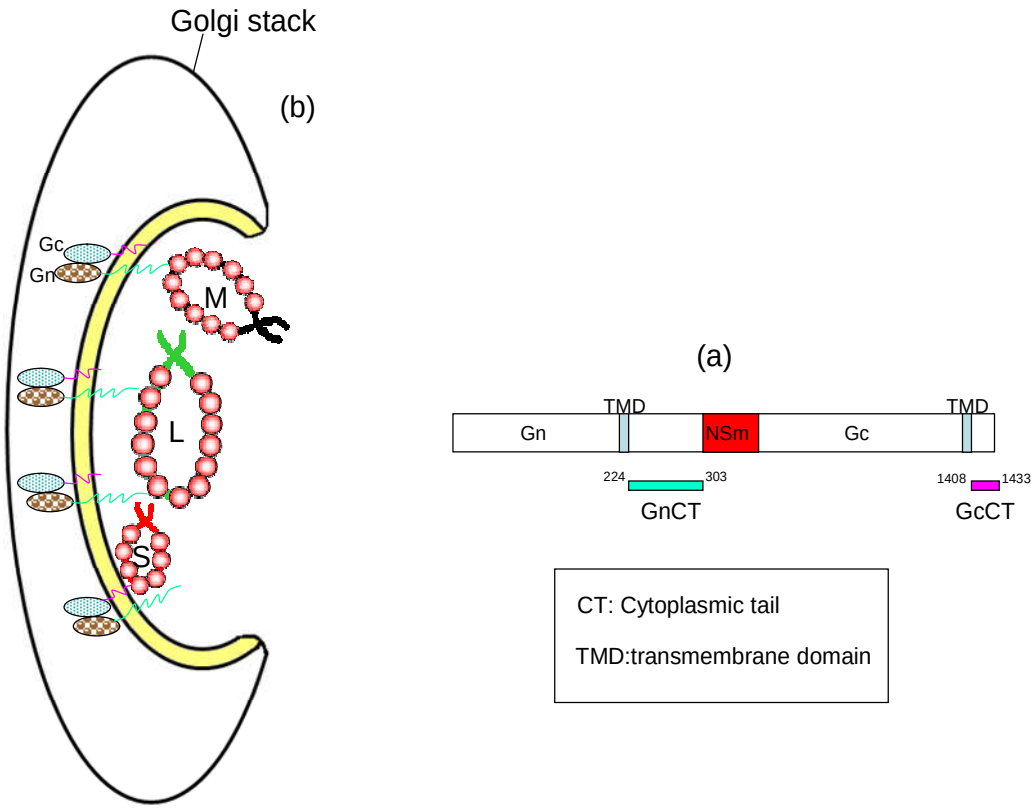

Figure 1.8. The CT domain map of Gn and Gc glycoprotein of BUNV (a). Possible interactions of the RNPs with the cytoplasmic tail of Gn or Gc underneath the Golgi membrane enhances virus assembly (b).

two-hybrid system, the interaction of severe acute respiratory syndrome, coronavirus (SARS-CoV) N protein with the membrane (M) protein was found to be an essential event for coronavirus assembly. Amino acids 168 -208 of SARS-CoV N protein were predicted to be the most important in this interaction (He *et al*., 2004). Moreover, the latter region is also involved in N multimerization.

Influenza virus matrix protein M1 interacts with the RNP structure and the viral glycoproteins to serve as a bridge between the different components (Nayak *et al*., 2004). Using the UUKV minigenome rescue system, which enables recovery of virus-like particles, mutational analysis showed that four amino acids in the Gn cytoplasmic tail, M76, L79, T80 and R81 are crucial for RNP packaging. These residues are thought to be involved in interactions with the nucleoprotein (Overby *et a*l., 2007).

Recently, the cytoplasmic tails (CT) of BUNV glycoproteins were identified (Gn is 78 residues and 25 residues for Gc) (Figure 1.8a). Deletion of the entire CT of either Gn or Gc disrupted the formation of virus-like particles (VLPs) in the BUNV packaging system. Alanine-scanning mutants were generated in both Gn and Gc CTs and were also tested in the BUNV packaging assay. It seems that the Gn CT domain has relatively more impact on virus assembly (Shi *et al*., 2007). Lack of a matrix protein in the bunyavirus virion suggests that possible interactions between the RNPs and the CT of the glycoproteins underneath the Golgi membrane promotes virus assembly and budding at the Golgi (Figure1.8b). The yeast two-hybrid system was used to test this hypothesis, however no obvious interactions between the N protein and Gn or Gc were detected (Leonard, 2005).

1.9. Replication cycle

The bunyavirus replication cycle can be divided into 4 stages: (i) adsorption and entry, (ii) transcription and translation, (iii) replication of viral genome, and (iv) budding, assembly, maturation and release (Figure1.9).

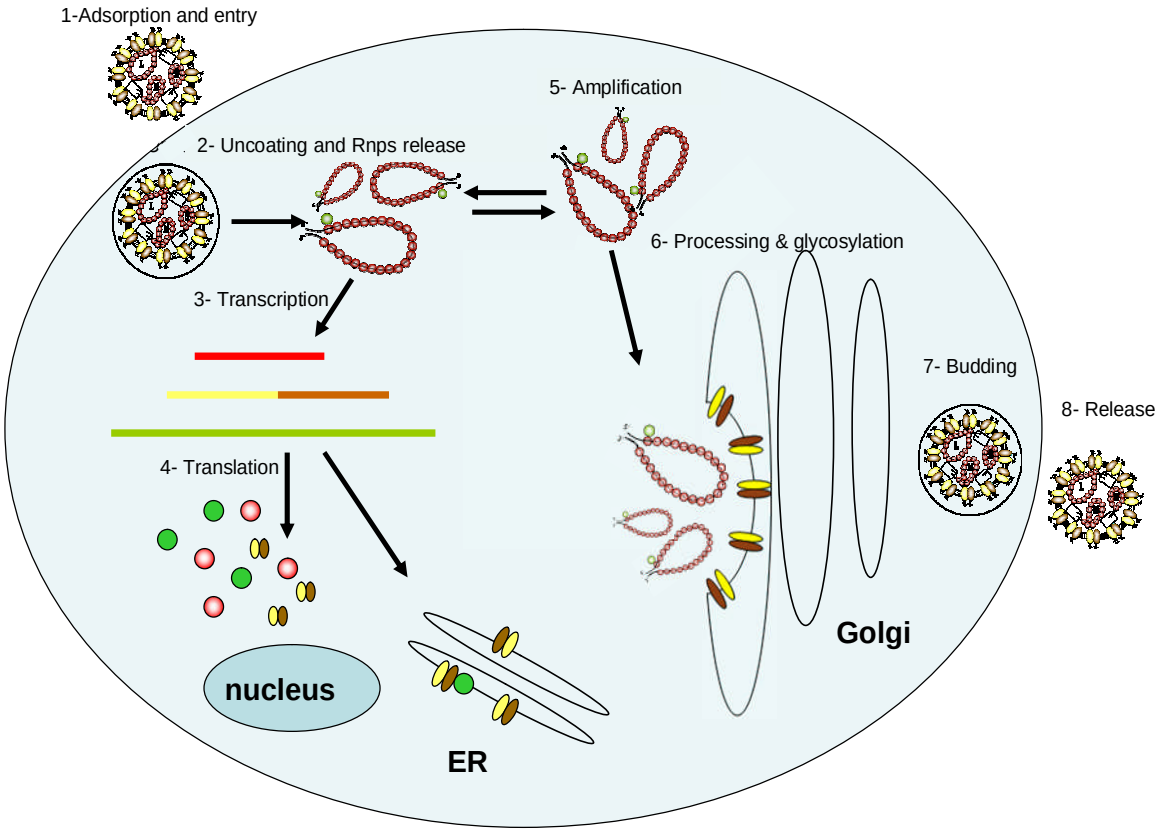

Figure 1.9. General schematic of a bunyavirus replication cycle.

1.9.1. Adsorption and entry

Bunyaviruses attach to cellular receptors *via* the viral glycoproteins. The Gc protein of LACV is thought to be involved in recognition of mammalian cells (Grady *et al*., 1983, Kingsford and Hill, 1983, Najjar *et al*., 1985), while the Gn protein is involved in recognition of mosquito cells (Ludwig *et al*., 1991). Protease-treated LACV displayed a high reduction in virus infectivity in mammalian cells due to the loss of the glycoprotein spikes, when compared to nontreated virus (Obijeski *et al*., 1976a). The infectivity of LACV for mammalian cells was also impaired when reacted with monoclonal antibodies raised against Gc glycoprotein indicating that Gc is crucial in virus attachment (Hacker *et al*., 1995; Kingsford and Hill, 1983). Kingsford and Hill (1983) confirmed the importance of Gc in virus attachment when LACV Gc was digested proteolytically while Gn was still intact. The virus again was unable to infect mammalian cells. On the other hand, the infectivity of LACV increased in insect cells when the Gc was removed, implying the involvement of Gn in invertebrate cell entry (Ludwing *et al*., 1989). So far, three cellular receptors, the β3 intgrins protein utilized by SNV and New York hantaviruses (Gavrilovskaya *et al*., 1998), and the g_1 and g_3 integrins utilised by non-pathogenic and pathogenic hantaviruses respectively (Gavrilovskaya *et al*., 2002), have been identified. After attachment, the virus particle is endocytosed leading to fusion of the viral envelope with the cellular endosomal membrane. Bunyavirus Gc proteins have been classified as a class II fusion protein (Gary and Gary, 2004). Fusion of the viral envelope with the endosomal membrane of the endosome relies on acidic pH 6.3 (Gonzalez-Scarano *et al*., 1984; Hacker and Hardy, 1997).

1.9.2. Transcription and translation

After release of the virus genome into the cytoplasm, the viral RNA-dependent-RNA polymerase starts transcription using the RNA genome as a template to produce mRNAs. The synthezised mRNAs which are not encapsidated (Bouloy *et al*., 1984), contain a methylated cap at the 5`end derived from cellular mRNA by the viral polymerase and are shorter than viral antigenome by 50-100 nucleotides at the 3`end (Bouloy *et al*., 1984, Cash *et al*., 1979, Eshita *et al*., 1985, Pattnaik and Abraham, 1983). The mechanism of bunyavirus mRNA synthesis is akin to that of influenza

viruses but with several exceptions. While bunyavirus mRNAs synthesis occurs in the cytoplasm, production of influenza virus mRNAs takes place in the nucleus. Bunyaviruses utilize only one enzyme (L polymerase), whereas, influenza virus uses three enzymes (PB1, PB2 and PA). Viral mRNAs contain non-templated RNA of 8-17 nt at the 5`end (Bishop *et al*., 1983; Eshita *et al*., 1985; Kormelink *et al*., 1992; Patterson and Kolakofsky, 1984).

All bunyavirus mRNA transcripts, from all five genera, contain preferential specific nucleotides at the 3` terminus of the non-viral sequence (Simons and Pettersson, 1991; Jin and Elliott, 1993a; Jin and Elliott, 1993b; Garcin *et al*., 1995a; Duijsings *et al*., 2001). Predominantly, BUNV transcript primers end with the sequence AGU3' or GGU3' that match the 5' end of viral RNA 5'…**G/AGU** AGU….3' where the primer sequence is highlighted in bold (Jin and Elliott, 1993a). It was proposed that the L polymerase slips backward after initiation of mRNA synthesis leading to a repeat of the first two or three bases on the template (Jin and Elliott, 1993b).The same mechanism (prime and realign model) was suggested for the initiation of HTNV genomic and mRNA synthesis (Garcin *et al*., 1995a) (Figure1.10). In the latter model, the 3' end of hantavirus genome is AUC AUC AUC, but primers were observed with a strong preference only for G at the 3' end. As a result, transcription commences by annealing the priming G to the C at +3 on the genome template, elongating a few bases and then realigning back 4 bases, relocating the G to position -1. Both *in vitro* and *in vivo* experiments showed that LACV transcripts terminated at nucleotide 175 (Raju and Kolakofosky, 1986 ; 1987a). When this termination site was disrupted read-through was observed. Termination sequences of bunyavirus S segments were also predicted. For instance, LACV and SSHV contain U-rich termination sequences whereas BUNV and GERV have GU-rich termination sequences (Patterson and Kolakofosky, 1984; Eshita *et al*., 1985; Bouloy *et al*., 1990; Jin and Elliott, 1992). The transciption termination signal of BUNV S segment mRNA was identified (Barr *et al*., 2007). A six nucleotide sequence (3'-GUCGAC-5') within a 33 nt region upstream of the 5' NTR of the S segment was found to play a crucial role in transcription termination. When the latter region was disrupted, a second region of five nucleotides downstream, which overlaps with the 6 nt major termination signal, was identified to be a minor functional termination signal in the absence of the major termination signal. The pentanucleotide sequence was also identified in the 5' NTR of the BUNV

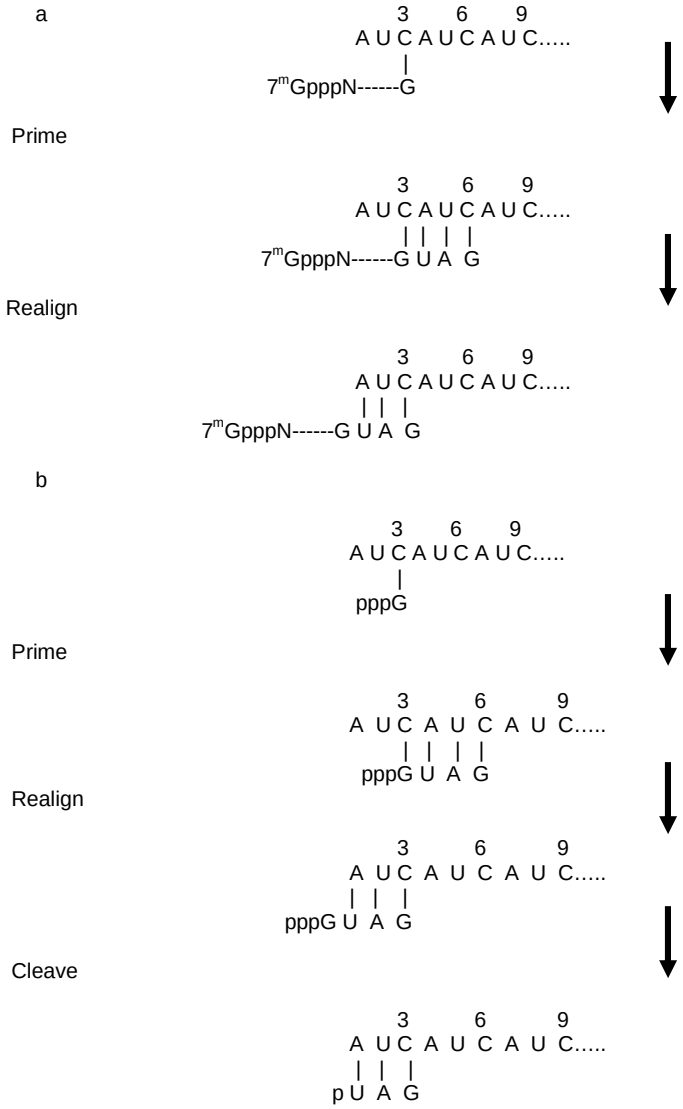

Figure 1.10. Prime and realign model for initiation of HTNV transcription and replication.

Initiation of mRNA synthesis (a). The 3' terminal G of a capped, host-derived primer anneals to the C at the +3 position and the primer is extended by a few nucleotides. The nascent mRNA then realigns on the template, skipping the priming G to the -1 position. Initiation of genome RNA synthesis (b). RNA synthesis begins with a GTP bound to the C at the +3 position. After a short elongation the nascent transcript is realigned to generate a 5' overhang that is to be cleaved later, leaving a monophosphorylated U at the 5' terminus (adapted from Garcin *et al*., 1995).

L RNA and other orthobunyaviruses. The coupling of BUNV transcription and translation to prevent truncated mRNA synthesis was demonstrated (Barr, 2007). After viral mRNA production, mRNAs derived from the L and S segments are translated by free ribosomes to generate the L and N proteins. The mRNAs synthesized from the M segment are translated by ER-bound cellular ribosomes to produce the glycoproteins (and for some bunyaviruses NSm protein).

1.9.3. Replication of the viral genome

Replication of bunyaviruses involves synthesis of full-length antigenome and genome RNAs. At some point, the L polymerase switches from mRNA transcription to antigenome synthesis using the genome as template (Figure1.11). The mechanism of switching from mRNA to antigenome RNA synthesis is not known, however, the N protein might play a role in this respect. Studies on VSV suggested that there is a biochemical difference between the polymerase (transcriptase) that drives mRNA synthesis from that responsible for antigenome and genome production (the replicase) (Qanungo *et al*., 2004). The latter hypothesis was supported by the finding that *in vitro* TSWV replication was enhanced by the addition of purified transcription factor (FoTF) but not viral transcription (de Medeiros *et al*., 2005). Subsequently, the antigenome is used as a template for production of genome RNA that can be utilized for a second round of transcription and replication. Priming of hantaviruses replication is similar to that described for transcription initiation (prime-and-realign) of HTNV. To date, no viral RNA polymerase starts RNA synthesis with a pyrimidine residue (C or U) but the 5’ base of HTNV is U, hence, prime-and-realign is a possible mechanism (Garcin *et al*., 1995).

1.9.4. Budding assembly,maturation and release

The bunyavirus packaging mechanism is not clear. In general, bunyaviruses extrude through the Golgi membranes. However, RVFV and some hantaviruses have been observed to bud through the plasma membrane (Anderson and Smith, 1987; Goldsmith *et al*., 1995; Ravkov *et al*., 1997). Furthermore, tospovirus assembly occurs in the ER (Goldbach and Peters, 1996). Both glycoproteins Gn and Gc together with N protein of UUKV accumulated within the Golgi, with the RNPs

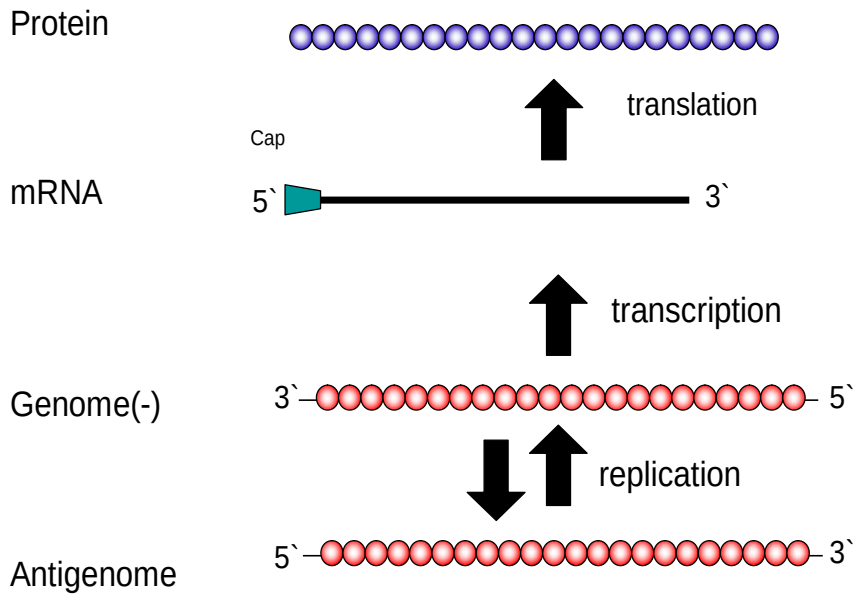

Figure 1.11. Transcription and replication of bunyavirus.

localized underneath the Golgi membrane (Kuismanen *et al*., 1984). Possible interaction between the cytoplasmic domain of Gc and or Gn and RNPs was suggested due to a lack of matrix protein in the bunyavirus particle. Hetero-dimerization of the glycoproteins is thought to take place in the ER, and they are then transferred to the Golgi (Pettersson and Melin, 1996). Studying the influenza virus packaging process revealed that the ratio of infectious to non-infectious particles produced during the infection support random packaging hypothesis (Lamb and Choppin, 1983). The ability of influenza virus to package more than 8 segments was reported (Enami *et al*., 1991). Likewise, packing of bunyavirus genome might occur randomly since the three segments are rarely purified from virions in equimolar ratios. The most predominant segment in bunyavirus virions is the S segment, usually with two copies, which explains the abundance of S RNA in the infected cell (Bouloy *et al*., 1973-4; Obijeski *et al*., 1976b; Gentsch *et al*., 1977).

It is not clear by which mechanism the genome RNPs can be distinguished from the antigenome RNPs. However, TSW, LAC, UUK virions contained small amounts of antigenome RNPs (Kormelink *et al*., 1992b; Raju and Kolakofosky, 1989; Simons *et al.,* 1990). Interestingly, the antigenome RNPs that were found to be incorporated in UUKV and TSWV were from the ambisense segments. This suggested that the packaging signal is linked to the promoter sequence which is present in both genome and antigenome segments as they utilize ambisense strategy. Budding in the Golgi apparatus results in virus particles being contained within a small vesicle; the vesicle migrates to the plasma membrane and the two membranes fuse, releasing the virus to the exterior.

1.10. Effect of bunyavirus infection on host cells

Members of *Bunyaviridae* generally cause cytopathic infection in vertebrates and plant hosts. However, hantavirus does not cause cytopathic effects in infected mammalian cells (Kraus *et al*., 2004; Meyer and Schmaljohn, 2000). In invertebrates, bunyaviruses cause persistent infection (Beaty and Calisher, 1991; Carvalho *et al*., 1986; Wijkamp et al., 1993).

1.11. Protein shut-off

Orthobunyaviruses and phleboviruses encode NSs protein that inhibits cellular protein expression. Recombinant BUNV lacking the NSs showed reduced shut-off in infected cells while wt BUNV shut-off host cells protein synthesis efficiently (Bridgen *et al*., 2001). In wt BUNV infected cells, phosphorylation of the C-terminal of cellular RNA polymerase II was impaired. When BUNdelNSs was used the previous observation did not occur (Thomas *et al*., 2004). Likewise, the NSs of RVFV inhibits cellular protein expression through interaction with the P44 subunit of transcription factor TFII H (Le May *et al*., 2004) resulting in inhibition of host mRNA synthesis. The C-terminal region of BUNV NSs protein was found to be involved in interaction with cellular MED8 protein causing the inhibition of host transcription and the interferon response (Leonard *et al*., 2005).

1.12. Bunyavirus and its arthropod vector

The life cycle of a mosquito-transmitted bunyavirus begins when the female mosquito bites infected or viraemic vertebrate host. The virus then establishes infection and replication in eipithelial midgut cells (Schmaljohn, 1996a). Release of virus from the midgut cells results in infection of the salivary glands and the ovaries. Virus can then be transmitted horizontally or vertically to another host. Bunyaviruses establish persistent infection in mosquitoes and some theories to account for this have been suggested. One is that high level of N protein leads to encapsidation of mRNA (Hacker *et al*., 1989). Another suggestion is that defective-interfering (DI) RNA drives the maintenance of persistent infection (Schmaljohn and Hooper, 2001). So far, it is not clear how persistent infection occurs but the obvious observations are reduction in viral replication and transcription in parallel with the presence of DI RNAs in infected mosquito cells (Newton *et al.*, 1981).

1.13. Defective-interfering (DI) RNAs

DI RNAs are internally-deleted RNA molecules having intact 5' and 3' non-coding regions of the parental virus. They are encapsidated, replicated and packaged by themselves. They take advantage by being shorter than viral genome in respect of replication and as a result interfere with viral genome replication. Bunyavirus DI RNAs have been identified as originality from the L segment (Inoue-Nagata *et al*., 1998; Patel and Elliott, 1992; Scallan and Elliott. 1992). It has been suggested that DI RNAs are produced when the RNA-dependent-RNA polymerase pauses during replication of the L segment causing a switch from the transcription to replication step. Secondary structure or short sequence repeats observed in deleted region of the L segment were though to be involved in the L polymerase pausing (Resende Rde *et al*., 1992). Meyer and Schmaljohn (2000) proposed a model for the establishment of SEOV persistent infection. They observed the accumulation of replication-incompetent, truncated genomes and antigenomes but not DI RNAs in Vero-E6 cells. They suggested that the deletions were due to the endonuclease activity of the viral L protein and the presence of these terminally deleted genomes and antigenomes interfered with virus replication.

1.14. BUNV minigenome system

BUNV minigenome system is an artificial system which mimics BUNV transcription and replication (Figure 1.12). It is a powerful tool for studying BUNV RNA synthesis. Previously, Dunn *et al*. (1995) developed a BUNV minigenome. A negative-sense chloramphenicol acetyltransferase (CAT) reporter gene was flanked by the authentic viral 5' and 3' noncoding regions (NCRs) of the BUNV S segment. When cells which express BUNV L and N proteins (either by recombinant vaccinia viruses or by the vaccinia virus-T7 system) were transfected with BUNV minigenome (BUNVSCAT) RNA, the activity of CAT could be measured, indicating that the negative-sense reporter RNA is transcribed and mRNA is produced and translated. Similar systems have been described for a number of negative-strand RNA viruses such as rhabdoviruses, paramyxoviruses and influenza viruses (Garcia-Sastre and Palese, 1993). The similarity among all these systems is the template RNA is driven from a

cDNA clone possessing authentic viral 5' and 3' NCRs sequences. However, several differences have been reported. In some systems, the viral proteins are provided by helper virus infection while some others *via* recombinant viruses. In influenza virus system, the RNA template is delivered in RNP complex form while RV minigenome transfected naked RNA. Different sequences are inserted between the authentic viral 5' and 3' ends sequences of the minigenome (reporter genes, authentic viral transcripts, defective-interfering RNA or mutated RNAs) (Neumann *et al*., 2002).

A BUNV minigenome system was modified to utilize the *Renilla* lucifrase a reporter gene instead of CAT (Kohl *et al*., 2004a).The *Renilla* gene was cloned in an antisense orientation between the 3' and 5' NCRs sequences of the BUNV L, M, or S segments under the control of T7 promoter. The minigenome cassette is inserted between a T7 promoter and hepatitis δ ribozyme, facilitating the expression of a virus-like genomic RNA by T7 RNA polymerase. Transfection of BSR-T7/5 cells, which stably express T7 RNA polymerase (Buhholz *et al*., 1999), with pTM1-based plasmids (encoding viral L and N proteins under the control of T7 promoters and an internal ribosome entry site sequence (IRES) of the encephalomyocarditis virus (EMCV) and BUNV minigenome expressing plasmid (pT7riboBUNM*Ren*(-)). Transfection efficiencies were normalized by cotransfection of the luciferase expressing plasmid pTM1-FF-luc. If the minigenome is encapsidated and mRNA is transcribed, *Renilla* luciferase activity can be measured. Analogous minigenome systems have been reported for other bunyaviruses, such as LACV, UUKV, HTNV, RVFV and CCHFV (Blakoqri *et al*., 2003; Flick & Pettersson, 2001; Flick *et al*., 2003a, b; Lopez *et al*., 1995).

Further modifications to BUNV minigenome using pT7ribo-based plasmds expressing viral L and N proteins instead of pTM1-based plasmids and using pT7-FF-luc rather than pTM1-FF-luc as an internal control have been reported (Lowen *et al*., 2004).

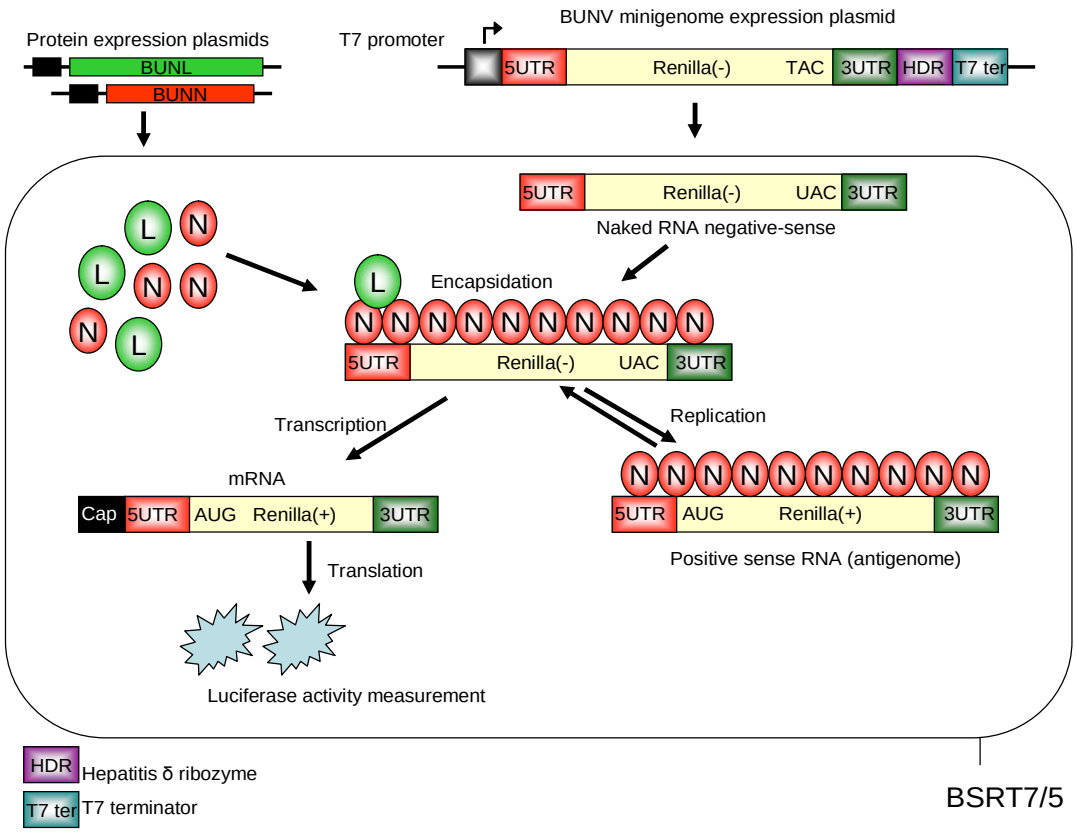

Figure 1.12. BUNV minigenome system.

BSR-T7/5 cells are transfected with a BUNV minigenome expression plasmid and two viral L and N proteins expression plsmids. The minigenome RNA is then encapsidated and the L protein starts transcription and replication. If mRNA is produced and translated, the luciferase activity can be measured.

1.15. BUNV minigenome packaging assay (virus-like particles,VLPs)

Little is known about the assembly of BUNV genome segments into virions. A BUNV minigenome packaging assay was developed to mimic BUNV assembly events (Figure 1.13). BSR-T7/5 cells are cotransfected with pTM1BUNL and pTM1BUNN, encoding viral L and N proteins, and a BUNV minigenome expressing plasmid (either pT7riboBUNLREN(-), pT7riboBUNMREN(-) or pT7riboBUNSREN(-)). The cells are then incubated for 24h to allow the formation of RNP structure. The cells are then infected with wt BUNV as a helper virus to provide the remaining viral proteins (Gn, NSm and Gc) required for VLP formation. 24h later, the growth medium is collected and used to infect BHK-21 cells. At 24h postinfection cells are lysed and luciferase assay carried out. If BUNV minigenome RNPs are packaged, released and the BHK-21 cells become infected with VLPs, reporter gene activity will be detected (Kohl *et al*., 2006).

Recently, BUNV minigenome packaging assay has been modified (Shi *et al*., 2006). BSR-T7/5 cells are cotransfected with three pTM1-based plasmids encoding the viral L, Gn, Gc and N proteins, BUN minigenome plasmid as well as pTM1-FF-Luc as internal control. At 24h post-transfection cells are lysed and assayed for luciferase activity as described by Kohl *et al*. (2004). The culture medium is cleared from cell debris and 1.5ml is transferred onto new BSRT7/5 cells transfected with pTM1BUNL and pTM1BUNN 3 - 5h earlier. Cells are incubated overnight and assayed for *Renilla* luciferase activity.

Further modifications of BUNV minigenome packaging assay have been introduced. First, rather than using pTM1-based clones to provide viral proteins, pT7-based plasmids encoding viral proteins are used. Second, instead of transferring the growth medium onto BSR-T7/5 pretransfected with pTM1BUNL and pTM1BUNN, the growth medium is transferred onto untransfected BSR-T7/5 cells (Shi *et al*., 2007).

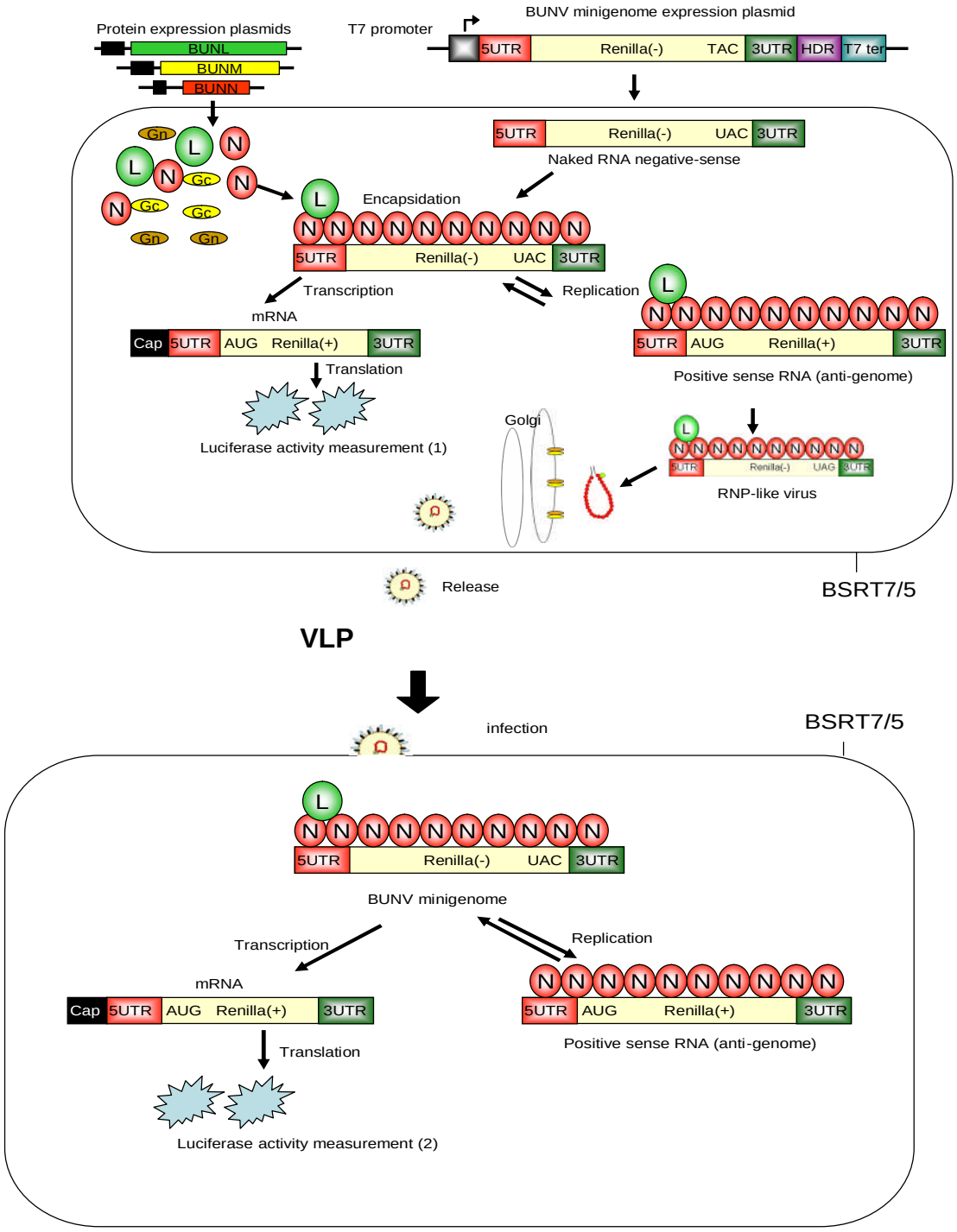

Figure 1.13. BUNV minigenome packaging assay. BSR-T7/5 cells are transfected with BUNV minigenome expression plasmid and three viral L, M and N protein expression plasmids lead to encapsidation, transcription, replication and packaging of the minigenome RNA into VLPs which be able to infect a new cells and the luciferase activity is measured.

1.16. Rescue of segmented negative-strand viruses

Recovery of several NSVs from cDNA clones has been reported (Table1.3). The first attempt towards rescue of NSVs was achieved by Luytjes *et al*. (1989), using a plasmid encoding influenza A minigenome (CAT gene negative-sense, flanked by influenza A UTRs) which can be linearized and transcribed in *vitro*. Combination of purified influenza A virus NP, PB1, PB2 and PA proteins with minigenome transcripts enables the formation of RNPs in *vitro*. This complex was then transfected into cells pre-infected with helper virus. Minigenome RNA was thus replicated and incorporated into some of the new progeny virions (Figure 1.14). However, it was not sustained in the genome beyond three passages. This obstacle was overcome using selective pressures, which enabled the isolation of recombinant viruses from helper viruses. Selection methods used include: neutralizing antibody selection (Enami and Palese, 1991; Horimoto and Kawaoka, 1994), host-range restriction (Enami *et al*., 1990; Subbarao *et al*., 1993), drug resistance (Castrucci and Kawaoko, 1995) and temperature sensitivity (Enami *et al*., 1991; Li *et al*., 1995; Yasuda *et al*., 1994).These methods allowed the generation of influenza viruses carrying a single cDNA-derived genome segment. Neumann *et al*. (1994) recovered influenza A viral RNPs using an RNA polymerase I expression system. Virus-like RNA (CAT gene negative sense flanked by viral untranslated region) was cloned into a plasmid contains RNA pol I promoter and terminator. Transfection of the latter plasmid into mammalian cells lead to generation of minigenome in the nucleus. Cells were then infected with helper virus to allow the encapsidation, transcription and packaging of the minigenome.

Further improvement was reported when the minigenome RNA was produced intracelluarly by using a T7 RNA polymerase–driven expression system. Importantly, the recombinant RNA must contain the authenic viral 3’ end. This was achieved by using hepatitis δ ribozyme which allows self-cleavage at the ribozyme sequence, producing authentic viral 3’ ends (Pattnaik *et al*., 1992).

Bridgen and Elliott (1996) reported the first segmented negative sense RNA virus to be rescued entirely from cDNA. In this system, HeLa cells were first infected with recombinant vaccinia virus vTF7-3 to express T7 RNA polymerase. Three viral

protein expression plasmids under control of T7 promoter were then transfected, followed by a second transfection of three ribozyme-based plasmids encoding the antigenome RNAs. In the latter constructs, BUNV sequences were flanked by the T7 promoter at the 5' end and a hepatitis δ ribozyme followed by the T7 terminator at the 3' end. Recoverd BUNV was purified by passaging the supernatant on C6/36 *Aedes albopictus* cells allowing BUNV growth but not that of vaccinia virus (Figure1.15). However, the yield was very poor with transfection of 10^7 cells only producing 10-100 infectious virus particles.

Recently, an improved and highly efficient BUNV rescue system has been developed (Lowen *et al*., 2004). BSR-T7/5 cells constitutively expressing T7 polymerase were transfected with three ribozyme-based plasmids expressing antigenome L, M and S RNAs using DAC-30 (transfection reagent) for 5 h at 37˚C. Fresh medium (4ml) was added and cells were incubated 4-5 days at 37˚C and supernatant was then used in plaque assay (Figure1.16).

Using a different protocol from that of BUNV rescue system, the recovery of influenza virus from cDNA has been reported (Fodor *et al*., 1999; Neumann *et al*., 1999). In this system, RNA Pol I promoter and terminator sequences were used for synthesis of viral RNAs while RNA Pol II promoters were also used for producing viral mRNA. Moreover, genome expression plasmids were used instead of using antigenome expression plasmids (Figure1.17). There are two reasons to explain the success of this system. First, lack of processing of Pol I transcripts and secondly, influenza virus replication takes place in the nucleus so no hybridization between the mRNAs and the viral genome would occur. In the latter system, influenza virus was recovered from transfection of either 12 or 17 plasmids. Cells were cotransfected with eight Pol I based plasmids encoding the genome segments and either four or nine protein expression plasmids. The yields was significantly higher (10^5 pfu/ml) when 17 plasmids were used compared to 10^3 pfu/ml obtained with 12 plasmids. Moreover, Hoffmann *et al*. (2000a) simplified the system by using Pol I/Pol II expression system which enables recovery of influenza virus from only eight plasmids. To produce a cDNA which can be used for transcription of both negative sense genomic RNA and mRNA, viral coding sequence was cloned in the positive sense between a pol II

Family	Genus	Species	Abbreviation	Reference
Rhabdoviridae	*Vesiculovirus*	Vesicular stomatitis virus	VSV	Lawson *et al*. (1995) Whelan *et a*l. (1995)
	Lyssavirus	Rabies virus	RV	Schnell *et al*. (1994)
Paramyxoviridae	*Henipavirus*	Nipah virus	NiV	Yoneda *et al*., 2006
	Morbillivirus	Measles virus	MV	Radecke *et al*. (1995)
		Rinderpest virus	RPV	Baron & Barrett (1997)
		Canine distemper virus	CDV	Gassen *eta*l.(2000)
	Respirovirus	Sendai virus	SeV	Gassen *et al*. (1995) Kato *et al*.(1996)
		Human parainfluenza virus type 3	hPIV3	Durbin *et al*. (1997a) Hoffman & Baneriee (1997)
		Bovine parainfluenza virus type 3	bPIV3	Haller *et a*l. (2000)
	Rubulavirus	Simian virus type 5	SV5	He *et al* (1997)
		Mumps virus		Clarke *et al*. (2000)
		Human parainfluenza virus type 2	hPIV2	Kawano *et al*. (2001)
		Newcastle disease virus	NDV	Peeters *et al*.(1999) Romer-Oberdorfer *et al*. (1999) Krishnamurthy *et al*. (2000)
	Peneumovirus	Human respiratory syncytial virus	hRSV	Collins *et al*. (1999)
		Bovine respiratory syncytial virus	bRSV	Buchholz *et al*. (1999)
Filoviridae	Ebola-like viruses	Ebola virus	EBoV	Volchkov *et a*l. (2000) Neumann *et al*. (2002)
Bunyaviridae	*Orthobunyavirus*	Bunyamwera, LaCrosse	BUNV, LACV	Bridgen & Elliott (1996) Blakqori & Weber (2005)
	Phlebovirus	Rift valley fever	RVFV	Ikegami *et al* (2006)
Orthomyxoviridae	Influenzavirus A	Influenza A virus		Neumann *et al*. (1999) Fodor *et al*.(1999)
	Thogotovirus	Thogoto virus	THoV	Wagner *et al*. (2001)

Table 1.3. Negative-strand RNA viruses recovered from cDNA clones (adapted from Neumann *et al*., 2002).

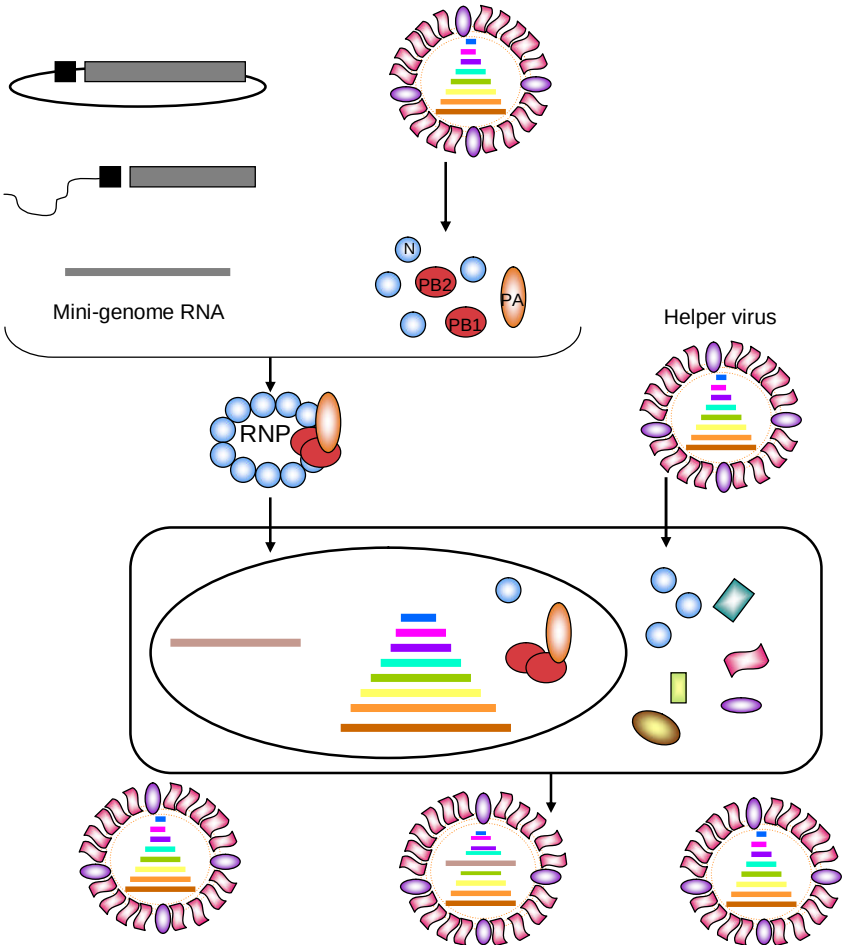

Figure 1.14. Influenza A virus RNP *in vitro* transfection method for recovery of viable virus. Influenza mini-genome RNA is transcribed *in vitro*. The influenza-like RNA is then combined with purified, virion-derived, NP, PB1, PB2 and PA proteins to allow RNP formation. The latter is transfected into cells pre-infected with helper virus. Some of the off-spring contain the heterologous segment (adapted from Luytjes *et al*., 1989).

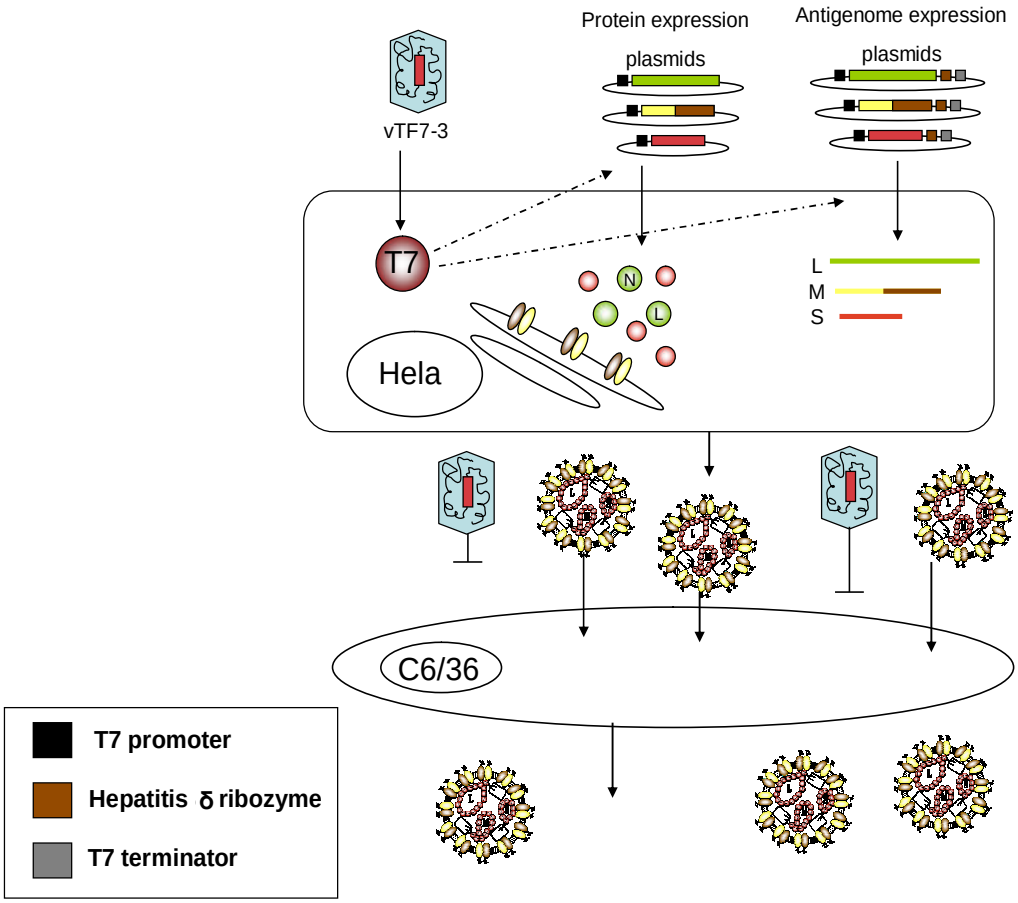

Figure 1.15. Bunyamwera virus rescue system.

HeLa cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase followed by transfection of three supported plasmids encoding the viral L, Gn, Gc and N proteins. Second transfection includes three ribozyme-based plasmids encoding the antigenome RNAs supernatant from transfected cells. This was passaged onto C6/36 mosquito cells to isolate BUNV (adapted from Bridgen and Elliott, 1996).

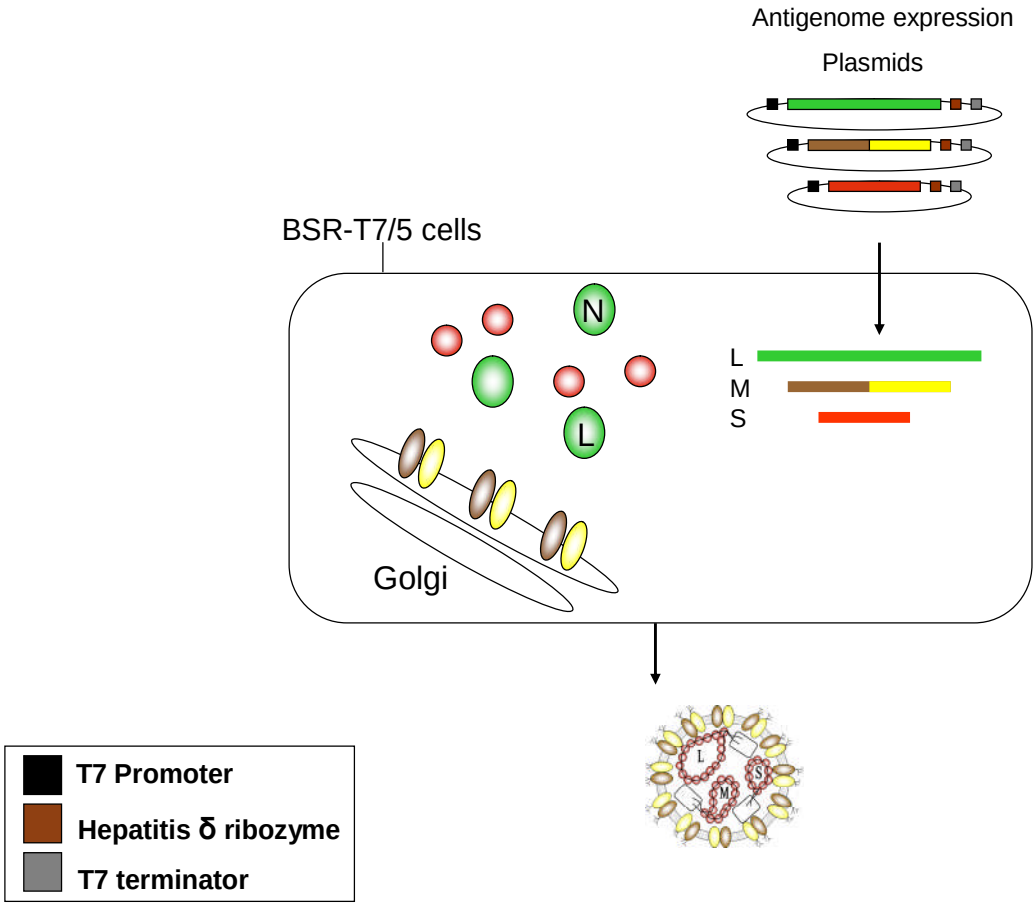

Figure 1.16. BUNV rescue system.

BSR-T7/5 cells which stably express T7 polymerase, are cotransfected with antigenomic expressing plasmids result in recovery of a viable virus.

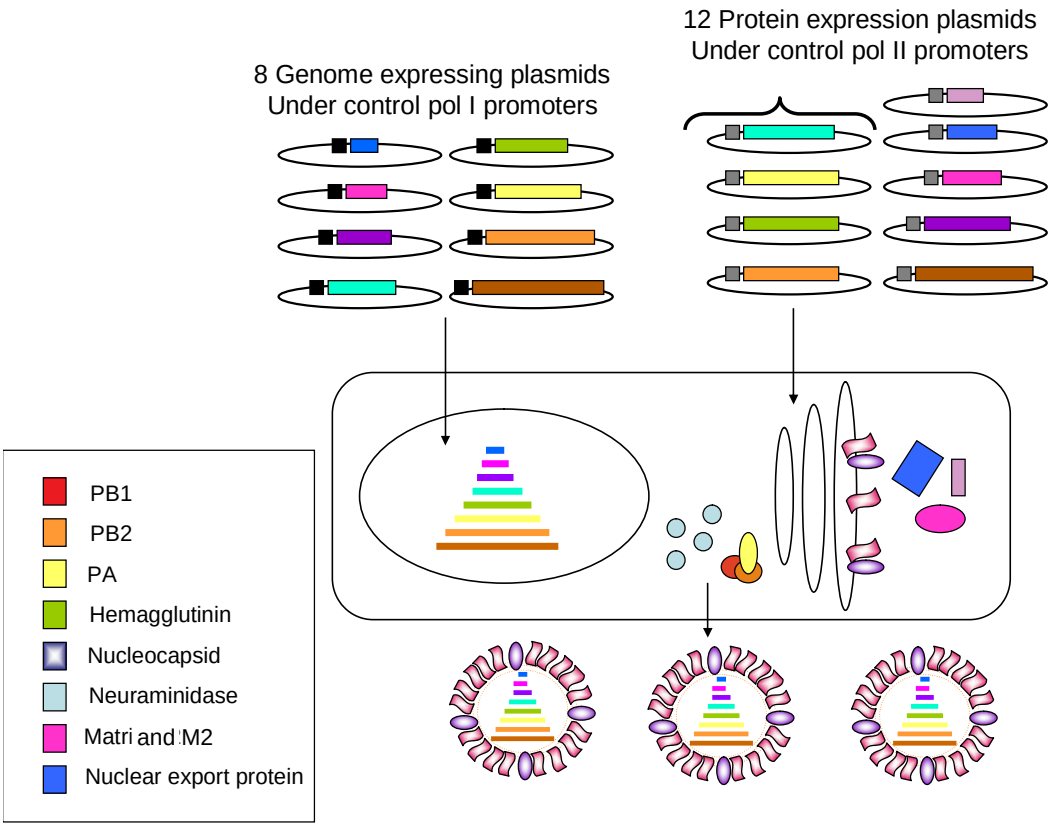

Figure 1.17. Influenza A virus rescue system utilizing 12 or 17 plasmids.

Cells are transfected with eight genome expressing plasmids followed by transfection of cells with either four (RNP) proteins or all nine structural proteins (adapted from Neumann *et al*., 1999).

promoter and polyadenylation signal and the latter cassette was then inserted in a negative sense between Pol I promoter and terminator sequences.

1.17. Rescue of non-segmented negative strand RNA viruses

Sendai virus (paramyxovirus) was rescued from synthetic naked viral-like RNA when transfected into cells pre-infected with helper virus (Park *et al*., 1991). Instead of using helper virus during recovery of recombinant viruses, viral protein expression plasmids have also been used. Pattnaik and Wertz (1990) reported using vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, to produce VSV proteins from cDNA clones under control of the bacteriophage T7 RNA polymerase promoter.

The first negative strand virus rescued was rabies virus (RV) from antigenome expression plasmids (Schnell *et al*., 1994). The RV rescue system consists of cells infected with vTF7-3. Transfecting these cells with a full length antigenome-expression plasmid and three viral protein expression plasmids (L, P and N) under the control of T7 promoter drives formation of active RNPs and produces viable virus (Figure1.18). Using a similar approach, several non-segmented negative viruses such as measles, SeV, respiratory syncytial virus (RSV) and VSV were recovered.

Several attempts have been made to recover negative strand viruses from genome expression plasmids but not all were successful. Sendai virus was rescued from genomic expression plasmids but with 100-fold less efficiency compared to using antigenomic expression plasmids (Kato *et al*., 1996). This might be attributed to hybridization of the genome and mRNA which could explain the deficiency of using genome expression plasmids in recovery of negative strand viruses. However, rescue of negative sense viruses from genome encoding plasmid was reported for some viruses such as Ebola virus and human parainfluenza virus-3 with equivalent virus yields from antigenome expression plasmids (Neumann *et al*., 2002).

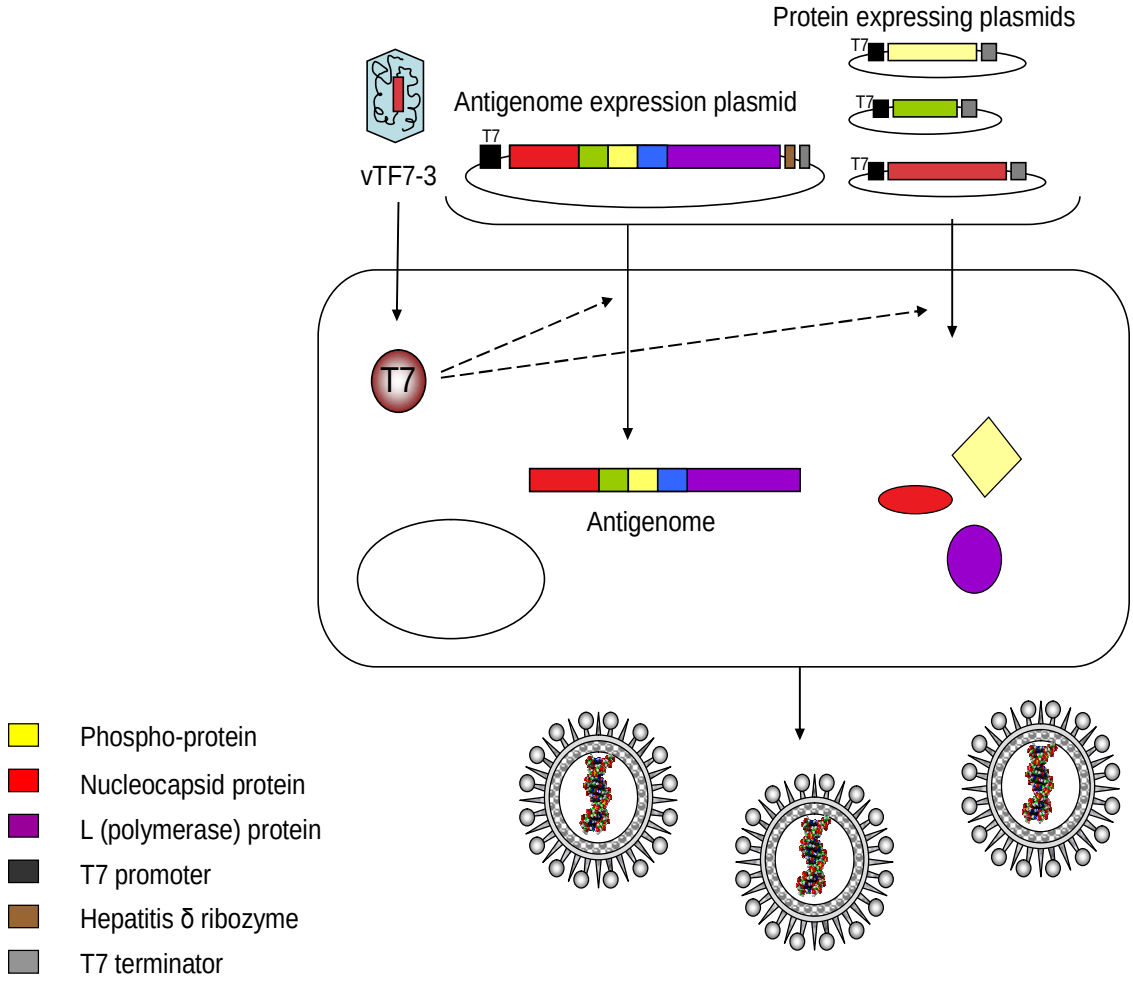

Figure 1.18. Rabies virus rescue system.

Transfection of four plasmids, three protein expression plasmids (encoding the viral L, N and P proteins) and ribozyme-based plasmid encodes antigenome onto cells preinfected with vTF7-3 (adapted from Schnell *et al*., 1994).

1.18. Bunyavirus mutants

Three snowshoe hare virus temperature-sensitive (ts) mutants were generated using 5-fluorouracil. All mutant viruses grew well at 33˚C but not at 39.5˚C, showing a greater than 1000-fold reduction in the yield (Gentsch and Bishop, 1976). Using the same mutagen, a total of 77 ts mutant bunyaviruses belonging to Bunyamwera serogroup (Batai, Bunyamwera and Maguari) viruses were generated (Iroegbu and pringle, 1981). Group I mutant viruses were thought to be due to mutations in the S segment while group II mutant viruses carried mutations in the M segment. A ts mutant of Maguari virus designated MAG*ts*23 (III), generated as above, was restricted on BS-C1 cells and displayed reduction at least 100-fold in the yield compared to wt virus (Pringle and Iroegbu, 1982). Nucleotide sequence analysis of the S segment of MAGts23 (III) showed that the mutation occurred at residue V85A in the N protein and F71L in the NSs protein (D.C.Pritlove and R.M.Elliott, unpublished data). Using BUNV reverse genetics, a mutant BUNV lacking NSs gene, called BUNdelNSs virus was generated (Bridgen *et al*., 2000). BUNdelNSs virus displays small plaque size, reduction of 10-fold in titer, and high induction of IFN compared to the wt BUNV. Recombinant mutant BUNV called BUN MLM, containing the L segment coding sequence flanked by the M segment UTRs, was generated using BUNV rescue system (Lowen *et al*., 2005). Characterization of BUN MLM virus revealed that, in mammalian cells the virus was attenuated displaying reduction in the L protein, L mRNA, L genomic and antigenomic RNA synthesis and produces small plaques. Mutants BUNV carrying mutations at residue N624 or N1168 or at both (sites for the attachment of N-linked glycans) on Gc were generated using BUNV reverse genetics (Shi *et al*., 2005). The glycans on Gc play a role in infection efficiency but are not important in virus replication. Two recombinant mutant viruses contain Alanine substitutions in the cytoplasmic tail of BUNV Gc were generated (Shi *et al*., 2007). They produce small plaque size and display more than 100-fold decrease in their titers compared to the wt virus.

1.19. Project Aim

The main objective of this project was to determine the linear domain map of BUNV N protein (233 amino acids). This was to be achieved through the introduction of single amino acid substitutions into BUNV S segment cDNA clone. The impact of the mutations in N protein functionality was to be analyzed by introducing mutant N genes into BUNV minigenome system to measure their activity compared to the wt BUN N protein. A number of mutant N genes were then to be introduced to BUNV rescue system in an attempt to recover viable viruses carrying mutations in the N gene. To understand the impact of mutations in the N protein in viral growth properties and determine any phenotypic differences, mutant viruses were to be characterized *via* (i) titration, (ii) plaque phenotype, (iii) protein labeling and Western blot, (iv) temperature sensitivity and (v) host-range. (vi) RNA synthesis by northern blotting.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Cell culture

Six different mammalian cell lines were used, BHK-21, BSR-T7/5, Vero-E6, 293-V, A549-V and 2FTGH-V. In general, BHK-21 cells were used for virus propagation, Vero-E6 cells were used for virus purification, titration, protein labelling and western blot. BSR-T7/5 cells were used for transfection purposes and virus rescue. BHK-21, Vero-E6, 293-V, A549-V and 2FTGH-V cells were used in host-range experiment. A full description of all cell lines and their maintenance shown in Table 2.1.

2.1.2. Plasmids

All plasmids are listed in Table 2.2.

2.1.3. Bacterial cultures

The bacterial strains used for plasmid propagation were:

(i) *E.coli* DH5α:Ø80d lacZΔM15, recA1, endA1, gyrA96,thi-1,hsdR17 (rk^-,mk^+), supE44,re1A1,deor, Δ (lacZYA-argF)U169,phoA.

(ii) *E.coli* JM109: endA1,recA1,gyrA96,thi-1.hsdr17(rk^-,mk^+), re1A1,supE44, Δ (lac-proAB),[F`,traD36,ProAB,$lacI^9$ZΔM15].

Cell line	Description	Reference
BHK-21	Baby hamster kidney cells were grown in GMEM supplemented with 10% (v/v) NCS and 20% (v/v) TPB.	Stoker and Macpherson, (1962)
BSR-T7/5	A modified BHK-21 cell line expressing T7 RNA polymerase constitutively. Cells were maintained in GMEM supplemented with 10 % (v/v) FCS and 10% (v/v) TPB and 1mg/ml G418-$So_{4.}$	Buchholz *et al*., (1999)
Vero-E6	An African green monkey cell line maintained in DMEM supplemented with 10% (v/v) FCS.	ATCC
293-V	Human embryo kidney cell line interferon-deficient maintained in DMEM containing 10% (v/v) FCS.	R.E Randall unpublished
A549-V	Human lung cell line interferon-deficient maintained in DMEM containing 10% (v/v) FCS.	R.E Randall unpublished
2FTGH-V	Human fibroblast diploid cell line interferon-deficient were grown in DMEM containing 10% (v/v) FCS.	Andrejeva *et al*. (2002)

Table 2.1. Mammalian cell lines.

Five different cell lines (BHK-21, Vero-E6, 293-V, A549-V and 2FTGH-V) were used in host-restriction examination. The cell lines 293-V, A549-V and 2FTGH-V were modified to express Simian Virus 5 V protein which blocks IFN signalling by targeting STAT1 for proteasome-mediated degradation (Andrejeva *et al*. (2002). Vero-E6 cells do not produce IFN due to loss of the IFN genes.

Plasmid	Description	Reference
pTM1	Vector contains a bacteriophage T7 promoter followed by IRES :used for protein expression	Moss *et a*l., (1990)
pTM1-BUNN	pTM1 plasmid contains the BUNN ORF. Point mutations were introduced to prevent expression of NSs protein	Weber *et al*., (2001)
pTM1-BUNS	pTM1 plasmid contains the coding sequence of BUNV S segment; expresses both N and NSs proteins.	Weber *et al*., (2001)
pTM1-BUNM	pTM1 construct contains the ORF of BUNV M segment	Weber *et al*., (2001)
pTM1-FF-luciferase	plasmid contains the firefly lucifrase ORF, was used as internal control of cells transfection	Weber *et al*., (2001)
pTM1-BUNL	pTM1 plasmid contains the coding sequence of BUNV L segment	Weber *et al*., (2001)
pT7ribo	A vector containing bacteriophage T7 promoter, followed by *StuI* and *SmaI* restriction enzyme sites and the hepatitis δ ribozyme sequence, is used for protein and antigenomic RNA expression	Dunn *et al*., (1995)
pT7riboBUNL (+)	pT7ribo encoding antigenomic BUNV L RNA	Bridgen and Elliott, (1996)
pT7riboBUNS (+)	pT7ribo containing the BUNV S segment ORF in positive sense allowing the expression of antigenome RNA segment	Bridgen and Elliott, (1996)

Plasmid	Description	Reference
pT7riboBUNN (+)	pT7ribo vector contains the full sequence of the BUNV S segment encodes only BUN N ORF, is cloned in the positive orientation driving the expression of antigenomic S RNA	Bridgen and Elliott, (1996)
pT7riboBUNM (+)	pT7ribo vector contains the full sequence of the BUNV M segment, cloned in the positive orientation driving the expression of antigenomic M RNA	Bridgen and Elliott, (1996)
pT7riboBUNS REN(-)	pT7ribo contains BUNV NCRs of the S segment and *Renilla* luciferase gene in the negative sense in place of the S segment ORF	Weber *et al*., (2001)
pT7riboBUNM REN(-)	pT7ribo contains BUNV NCRs of the M segment and *Renilla* luciferase gene in the negative sense in place of the M segment ORF	Weber *et al*., (2001)
pT7riboBUNL REN(-)	pT7ribo contains BUNV NCRs of the L segment and *Renilla* luciferase gene in the negative sense in place of the L segment ORF	Weber *et al*., (2001)
pT7-FF-luciferase	plasmid contains the firefly luciferase ORF, was used as internal control of cells transfection	R.M Elliott
pGEM-T Easy vector	A linear vector containing a 3` terminal thymidine at both ends. It was used for ligation PCR product for sequencing	Promega

Table 2.2. Plasmids used in this project.

2.1.4. Viruses

The wt BUNV supplied by RME and recombinant BUNdelNSs virus was rescued using same plasmid (pT7riboBUNN) described by (Bridgen *et al*., 2001) and recombinant vaccinia virus vTF7-3 that expresses the bacteriophage T7 RNA polymerase (Fuerst *et al*., 1986) were used in this project.

2.1.5. Antibodies

(i) Anti-BUNV N poly-clonal antibody raised in rabbit against N protein expressed in bacteria was provided by R.M.Elliott.

(ii) Anti-rabbit IgG horseradish peroxidase-linked antibodies were supplied by Cell Signalling Technology.

(iii) Anti-Digoxigenin-AP,Fab fragment was purchased from Roche.

2.1.6. Synthetic oligonucleotides

All oligonucleotides were supplied by Sigma-Genosys and are listed in Table 2.3.

2.1.7. Reagents, chemicals and solutions

All chemicals and reagents were supplied by Sigma chemicals Co or BDH chemicals Ltd unless otherwise stated.

2.1.7.1. Bacterial culture media

L-broth: 10g NaCl, 10g bactopeptone and 5g yeast extract/litre

LB agar: L-broth + 1.5% (w/v) agar.

TSB: L-broth + 10% (w/v) PEG (MW3350), 5% (w/v) DMSO, 10mM $MgCl_2$ and 10 mM $MgSO_4$.

TSB+glucose: 380mg glucose/ 100 ml TSB.

YTB: 16g tryptone, 10g yeast extract, and 5g NaCl / litre.

Ampicillin 0.1% (w/v) (Promega).

2.1.7.2. Virus manipulation

Agarose type HSA 1.2% (w/v) (park scientific limited).

Neutral red stain 0.1% (w/v) (BDH).

PBS: 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 , 0.68mM $CaCl_2$, 49mM $MgCl_2$.

Geimsa stain solution (BDH).

5% (v/v) formaldehyde PBS.

2.1.7.3. Enzymes

All enzymes are listed in Table 2.4.

2.1.7.4. Radiochemical

^{35}S L- methionine (40µCi/µl) was purchased from Amersham.

2.1.7.5. Transfection reagent

Lipofectamine-2000 (Invitrogen).

2.1.7.6. DNA gel electrophoresis

Agarose 1% (w/v) MP (multi-purpose) (Roche).

Ethidium bromide (0.04mg/100ml) (Promega).

DNA loading buffer: 2.5% (w/v) Ficoll 400; 11 mM EDTA; 0.017% (w/v) SDS; 0.015% (w/v) bromophenol blue; 3.3 mM tris-Hcl.

10× Tris-borate EDTA buffer (TBE): 1.0M Tris, 0.9M boric acid; 0.01M EDTA (Invitrogen).

2.1.7.7. RNA gel electrophoresis

Agarose 1.2% MP from (Roche).

Ethidium bromide (Promega).

RNA loading buffer: 25% (w/v) Ficoll 400; 0.4% (w/v) bromophenol blue; 0.4 % (w/v) xylene cyanol.

10×TAE buffer: 400mM Tris-acetate; 10mM EDTA (Invitrogen).

2.1.7.8. Northern blotting

RNA loading buffer (as above).

Maleic acid buffer: 0.1M maleic acid, 0.15M NaCl, pH7.5.

50% formamide hybridization buffer: 50% (v/v) deionised formamide (Sigma); 25% 5×SSC; 1% (w/v) N-lauroyl-sarcosine (Sigma); 0.02% (w/v) SDS; 1% (v/v) blocking solution.

20× Sodium chloride-sodium citrate (SSC): 3M NaCl, 0.3M sodium citrate, pH 7.0.

10× blocking buffer: 10g blocking reagent purchased from Roche were dissolved in 100ml maleic acid buffer.

Detection buffer: 0.1M Tris, 0.1M NaCl,pH 9.5.
Sodium dodecyl sulphate (SDS): 0.5% (w/v) in dH_20.

Washing buffer: 0.1M maleic acid, 0.15M NaCl, pH7.5; 0.3 % (v/v) Tween-20.

TRIzol reagent: a mono-phasic solution of phenol and guanidine isothiocyanate (Invitrogen).

2.1.7.9. Polyacrylamide gel electrophoresis (PAGE)

Western blotting and co-immunoprecipitation reagents:

Acrylamide/bis-acrylamide stock solution: 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide (Biorad).

Ammonium persulfate 10% (w/v) (APS) (Biorad).

Blocking buffer: 10% (w/v) skimmed milk powder, 0.1% (v/v) Tween-20 in PBS.

HRP substrate supersignal west pico chemiluminescent substrate system (Pierce).

DMEM without methionine solution (Sigma).

Gel fixation buffer: 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) dH_20.

Phosphate buffered saline (PBS) : 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.12-7.3,0.68mM $CaCl_2$,0.49mM $MgCl_2$ EnHance (Dupont).

Nitrocellulose membrane: Hybond-C extra (Amersham).

Coomassie blue stain: 10% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) coomassie blue.

Transfer buffer: 1×Tris-glycine, 20% methanol.

10×Tris-glycine running buffer: 2.4 M Tris, 1.9M glycine.
TEMED (N, N, N`, N` tetramethylethylenediamine) (Biorad).

1.5M Tris-HCl pH8.8: 90.9g Tris, 400ml dH_20, 7.3ml concentrated HCl, pH adjusted to 8.8 with HCl and volume made up to 500 ml with dH_20.

1M Tris-HCl pH6.8: 60.6g Tris, 400ml concentrated HCl, pH adjusted to 6.8 with HCl and volume made up to 500ml with dH_20.

Protein A sepharose (Sigma).

Protein loading buffer (2x) : 100mM Tris-HCl pH 6.8, 4% (w/v) SDS, 200mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue.

^{35}S methionine (Amersham).

Non-denaturing RIP buffer: 50mM Tris-HCl pH 7.4, 1% (v/v) Triton X-100,300mM NaCl, 5mM EDTA.

Complete protease inhibitor cocktail (Roche).

RIP wash buffer: 50mM Tris-HCl pH 7.4, 0.1% (v/v) Triton X-100, 300mM NaCl, 5mM EDTA.

Oligo	Sequence (5' → 3')	Coordinate Nucleotides of BUNV S
L4A	1) Fw GCGGAATTTCATGATGTCGC	95 → 113
	Rv TCAATCATTAAAGAGCCTT	94 → 76
F6A	Fw GCTCATGATGTCGCTGCTAACACC	102 → 125
	Rv TTCCAACTCAATCATTAAAGAGCC	101 → 78
D8A	2) Fw GACGTCGCTGCTAACACC	1) 108 → 126
Article II.	Rv ATGAAATTCCAACTCAATC	107 → 89
Y23A	Fw GCCGCTAACTTTAAGC	152 → 168
	Rv TGCGACCTCTGGGTCA	151 → 136
R40A	Fw GCAATCTTCTACATTAAAGGACGC	203 → 227
	Rv TATGTGGTCATAACTAAGCCCAGT	202 → 179
K50A	Fw GCAACTAGTCTCGCAAAAAGAAGTGAATGG	233 → 263
	Rv AATCTCGCGTCCTTTAATGTAGAAGATTCG	232 → 203
T91A	Fw GCCCTCCACCGCCTCAGTGG	356 → 376
	(b) Rv AAGACCATCGTCAGGAACTG	355 → 336
R94A	Fw GCCCTCAGTGGATTCCTTGCC	365 → 386
	Rv GTGGAGGGTAAGACCATCGTC	364 → 344
L104A	Fw GCGGAGAAGATGCTGAAAGTC	395 → 416
	Rv TAGGTACCTGGCAAGGAATCC	394 → 374
E105A	1) Fw GCGAAGATGCTGAAAGTC	398 → 416
	2) Rv AAGTAGGTACCTGGCAAG	397 → 380

Oligo	Sequence (5' → 3')	Coordinate Nucleotides of BUNV S
E112A	Fw GCACCAGAGAAATTGATTA Rv ACTGACTTTCAGCATCTTC	419 → 438 418 → 400
I118N	Fw AATAAATCAAAAATAATCAAC Rv AATCAATTTCTCTGGTTCAC	437 → 458 436 → 417
A127G	Fw GGAGAAAAGAATGGGATCAC Rv CAAAGGGTTGATTATTTTTG	464 → 484 463 → 444
E128A	Fw GCAAAGAATGGGATCACTTGGAA Rv AGCCAAAGGGTTGATTATTTTTG	467→ 490 466 → 444
P125H	Fw CATTTGGCTGAAAAGAATG Rv GTTGATTATTTTTGATTTAA	458 → 477 457 → 438
G131A	Fw TGGATCACTTGGAATGATG Rv ATTCTTTTCAGCCAAAGGG	476 → 495 475 → 457
W134A	Fw GCGAATGATGGAGAGGAAG Rv AGTGATCCCATTCTTTTCAG	485 → 504 484 → 465
G137A	3) Fw GCAGAGGAAGTTTATCTCTC 4) Rv ATCATTCCAAGTGATCCC	5) 494 → 514 493 → 476
Y141C	Fw TGTCTCTCTTTCTTCCCAG Rv AACTTCCTCTCCATCATTCC	506 → 525 505 → 486

Oligo	Sequence (5' → 3')	Coordinate Nucleotides of BUNV S
G147A	Fw GCATCAGAGATGTTCTTAGG	524 → 544
	Rv TGGGAAGAAAGAGAGATAAAC	523 → 503
M150A	Fw GCGTTCTTAGGAACTTTCAGA	534 → 555
	Rv CTCTGATCCTGGGAAGAAAGA	533 → 513
Y158N	Fw AACCCCTTAGCAATCGGG	557→ 575
	Rv GAATCTGAAAGTTCCTAAG	556 → 538
I162A	Fw GCCGGGATCTACAAAGTTCAGCGCAAG	569 → 596
	Rv TGCTAAGGGGTAGAATCTGAAAGTTCC	568 → 542
V167A	6) Fw GCGCAGCGCAAGGAAATGGAAC	7) 584 → 606
	Rv TTTGTAGATCCCGATTGCTAAG	583 → 562
M172A	Fw GCGGAACCAAAATACCTTGAG	599 → 621
	Rv TTCCTTGCGCTGAACTTTGTAG	598→ 577
Y176A	Fw GCCCTTGAGAAAACAATGC	611 → 630
	Rv TTTTGGTTCCATTTCCTTG	610 → 592
L177A	Fw GCGGAGAAAACAATGCGGC	614 → 633
	Rv GTATTTTGGTTCCATTTCC	613 → 595
K179I	Fw ATAACAATGCGGCAGAGGTAC	620 → 641
	Rv CTCAAGGTATTTTGGTTCC	619 → 639

Oligo	Sequence (5' → 3')	Coordinate Nucleotides of BUNV S
A190G	8) Fw GAAGCAACTTGGACTGTTAG 9) Rv TTCTAGTCCCATGTACCTC	10) 653 → 673 652 → 634
W193A	Fw GCGACTGTTAGTAAATTGACAG Rv AGTTGCTGCTTCTAGTCCCATG	662 → 684 661 → 640
L205A	Fw GCGACAGTTGTCTCTAGC Rv TGCAGACTGAACTTCTGTC	697 → 715 696 → 678
V 208A	Fw GCCTCTAGCTTAGGTTGGAAG Rv AACTGTCAGTGCAGACTGAAC	706 → 727 705 → 685
W213R	Fw CGGAAGAAAACCAATGTTAG Rv ACCTAAGCTAGAGACAACTG	722 → 742 721 → 702
S219A	11) Fw GCGGCAGCTGCCAGGGACTTC 12) Rv AACATTGGTTTTCTTCCAACC	13) 740 → 761 739 → 719
A222G	14) Fw GGCAGGGACTTCCTTGCTAAATTCG Rv AGCTGCACTAACATTGGTTTTCTTC	15) 749 → 774 748 → 724
L226A	Fw GCCGCTAAATTCGGAATCAAC Rv GAAGTCCCTGGCAGCTGCAC	761 → 782 760 → 741
I 231A	Fw GCCAACATGTAAGCAGGGATG Rv TCCGAATTTAGCAAGGAAGTC	776 → 797 775 → 755
Random* primers	Fw GACCATGATTACGAATTC Rv CGATTAAAAATGCATCCC	219 → 236 1063 → 1045

Table 2.3. The oligonuleotides were used in the mutagenesis of BUNV N cDNA clone. Example: oligo L4A was used to substituted aa L4 to Alanine. Fw: forward primer. Rv: reverse primer.

* **Random primers** were used to generate mutations in the BUNV N protein randomly.

Enzyme	Usage	Supplier
M-MLV reverse transcriptase	Two-step RT-PCR	Promega
AMV reverse transcriptase	Reverse transcription and amplification in one step	Promega
T4 DNA Ligase	Ligation	Invitrogen or Roche
KOD Hot Start polymerase	DNA amplification	Invitrogen
EcoR 1	Restriction enzyme	NEB
Nsi I	Restriction enzyme	NEB
Dpn I	Restriction enzyme	NEB
***Taq* DNA polymerase**	DNA amplification	Promega
Calf intestinal phosphatase (CIP)	Dephosphorylation	NEB
Hind III	Restriction enzyme	NEB
***PFU* DNA polymerase**	DNA amplification	Stratagene
Recombinant RNasin ribonuclease inhibitor	Cellular RNA inhibitor	Promega

Table 2.4. The enzymes were used in this project

NEB: New England Biolab

2.2. Methods

2.2.1. DNA manipulation and cloning procedures

2.2.1.1. Mini preparation of plasmid DNA

For small-scale plasmid preparation, miniprep Qiagen kit was used. Ten millilitres of LB broth containing 0.1mg/ml ampicillin was inoculated with a single colony and incubated in an orbital incubator overnight at 37ºC. The cells were pelleted and resuspended in 250 µl of buffer P1 containing RNase. Then, 250 µl of P2 (lysis buffer) containing SDS was added and mixed by inverting several times. A volume of 350 µl of N3 buffer was added and mixed gently. The sample was centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred to a miniprep column and centrifuged at 13,000 rpm for 1 minute. The flow through was discarded and DNA was washed and eluted with PBE buffer.

2.2.1.2. Maxi preparation of plasmid DNA

A 100 µl volume of overnight bacterial culture (above) was inoculated into 100 ml LB medium containing 0.1mg/ml ampicillin and the culture incubated overnight with shaking at 37ºC. The culture split into 50ml falcon tubes, centrifuged at 4000 rpm for 30 minutes and the supernatant discarded. The cells were resuspend in 10 ml buffer P1. Ten ml of buffer P2 was added, the solution mixed gently and incubated for 5 minutes at room temperature. Ten ml of chilled P3 buffer was added, the mixture transferred to a cartridge barrel and incubated for 10 minutes at room temperature. At the same time QIAGEN-tip 500 tubes were equilibrated with 10 ml buffer QBT and allowed tubes to empty by gravity flow. The supernatant was passed through the filter of the syringe onto the QIAGEN-tip which was washed twice with 30ml QC buffer. The DNA was eluted with 15ml buffer QF and precipitated by adding 10.5 ml isopropanol followed by centrifugation at 4000 rpm for 1.5 hour. The pellet was washed with 5 ml 70% ethanol, air-dried for 10 minute at room temperature and resuspended in 1ml dH_2O.

2.2.1.3. Restriction endonuclease digestion of DNA

For the analysis of mini-prep plasmid DNA, restriction digests were performed in a 20 µl reaction volume containing 2µg DNA, 2 µl 10x reaction buffer, 1 µl 100 x BSA, 1U of restriction enzyme and the volume was made up to 20 µl with dH_2O.The reaction was incubated as per manufacturers instructions.

2.2.1.4. Isolation of restriction fragments

Five µg DNA were digested with appropriate units of restriction enzyme in a 50 µl reaction volume and incubated overnight at optimal temperature. The total reaction volume was run out on an agarose gel and the desired fragment purified using a gel extraction kit according to manufacturer's instructions (Quigen).

2.2.1.5. Amplification of DNA by polymerase chain reaction (PCR)

Three DNA polymerases were used, KOD Hot start, *Pfu* turbo and *Taq*. Reactions were performed in thin-walled 0.5 ml tubes using a thermal cycler with heated lid.

KOD Hot Start DNA polymerase reaction:

10× KOD reaction buffer……..5µl
dNTPs (2 mM each)……..5µl
$MgSO_4$ (25mM).....2µl
Template DNA (25ng).....1µl
Primer 1 (10µM).....1µl
Primer 2 (10µM).....1µl
DMSO.....2µl
KOD polymerase (1U/ µl).....1µl
dH_2O……to 50µl

PCR programme:

Initial denaturing……..94°C……2mins

Strand separation…….94°C…….30 s
Annealing……variable…..30 s
Elongation…… 68°C…….variable
} 18-35 cycles

Final extension……..68°C…….10 mins

***Pfu* Turbo DNA polymerase reaction:**

10× *Pfu* reaction buffer……..5µl
dNTPs (10 mM)……...1µl
Primer 1 (10µM)..... 1µl
Primer 2 (10µM).....1µl
Template DNA (50ng).....1µl
Puf Turbo (2.5U/ µl)……1µl
dH_2O……. to 50µl

PCR programme:

Initial denaturing…….95°C…….3mins

Strand separation……..95°C…….30s
Annealing……variable…….30s
Elongation……...72°C……..3mins
} 30 cycles

Final extension…….. 72°C……..10mins

***Taq* DNA polymerase reaction:**

10× *Taq* reaction buffer with $MgCl_2$.....5µl
dNTPs (25 mM).....1µl
Primer 1(10µM).....1µl
Primer 2(10µM).....1µl
Template DNA (50ng).....1µl
Taq DNA polymerase (2U/ µl).....1µl
dH_2O.....to 50µl

PCR programme is similar to *Puf* Turbo programme.

2.2.1.6. PCR random mutagenesis

PCR random mutagenesis was performed using Stratagene's GeneMorph II random mutagenesis kit. Low (0-4) mutations/1kb frequency was used to introduce point mutations (substitution) in BUNV N cDNA clone. Briefly, pT7riboBUN N (template) was linearized with *HindIII* and purified using PCR purification kit (Qiagen) and 100 ng target DNA was then used for amplification.

PCR reaction:

10× Mutazyme II reaction buffer……5µl
DNA template (100ng)……1µl
dNTPs mix (40mM)…….....1µl
Primer mix (250 ng).....1µl
Mutazyme II DNA polymerase (2.5U/µl).....1µl
dH_2O..... to 50 µl

PCR programme:

Initial denaturing……95°C……2mins

Strand separation……..95°C……1min
Annealing……48°C……1min } 30 cycles
Extension……72°C……1min

Final extension……..72°C……10mins

The PCR product was gel extracted and double digested with *EcoR* I and *Nsi* I restriction enzymes. In parallel, pT7riboBUN N (vector) was digested in the same way and dephosphorylated using calf intestinal phosphatase enzyme (CIP).Vector segment then was gel purified and ligated with desired PCR product and transformed into competent *E.coli*.

2.2.1.7. Dephosphorylation of 5' end

To prevent self-ligation of a linearized vector during cloning, CIP enzyme was used. Addition of 20 U CIP directly to the digestion reaction and incubation for 2 hours at 37°C prevented self-ligation of the digested vector.

2.2.1.8. Quik-change PCR mutagenesis

The Quik-change protocol (Stratagene) was used to substitute single amino acids to Alanine or Glycine in BUNV cDNA clone. Two primers (Sigma-Genosys), forward and reverse were designed to anneal in both directions of the target site to generate desired mutation.

Quik-change reaction:

10× KOD Hot Start buffer……..5µl
dNTPs (2 mM)……..5µl
$MgSO_4$ (25mM)…….2µl
DMSO…….2µl
Template DNA (25 ng).....1µl
Primer 1(10µM).....1µl
Primer 2(10µM).....1µl
KOD polymerase (1U/ µl).....1µl
dH_2O…….. to 50µl

Quik-change PCR programme:

Initial denaturing…….94°C……2mins

Strand separation……...94°C…….35s
Annealing……..variable……...35s } 18 cycles
Elongation……68°C…….6 mins

Final extension……68°C…….10mins

The PCR reaction was digested with 5U of the restriction endonuclease *Dpn*1 for 1 hour at 37°C. This enzyme cleaves methylated parental DNA only. The PCR reaction was purified using PCR purification kit and transformed into competent *E.coli*.

2.2.1.9. Agarose gel electrophoresis

Digested DNA fragments or PCR products were resolved by agarose gel electrophoresis. One gram of agarose was melted in 100 ml 1x TBE buffer containing 0.04 µg/ml ethidium bromide. The mixture was poured into a horizontal gel tank

(5.7×8.3×0.4 cm) and the gel submerged in 1xTBE buffer. Samples containing 1x loading buffer were loaded into the wells and run at 100V for 30 minutes.

2.2.1.10. DNA ligation

2.2.1.10.1. Overnight ligation

Ligation reactions contained 100ng vector, 100ng insert DNA, 4µl 5x ligation buffer, 2U T4 DNA ligase and dH_2O to final volume of 20µl. Ligation reactions were incubated at room temperature overnight.

2.2.1.10.2. Rapid ligation

Roche's rapid ligation kit was used. Ligation reactions contained 100ng of insert DNA, 50ng of vector diluted in 1x DNA dilution buffer to a final volume of 10 µl. Ten microlitres of ligation buffer was then added and mixed thoroughly. Three units of T4 DNA ligase were added and the reaction incubated for 5 minutes before transformation into competent *E.coli.*

2.2.1.11. Competent *E.coli* preparation and Transformation

A single colony of *E.coli* strain DH5α or JM109 was used to inoculate 10ml LB and incubated with shaking at 37ºC overnight. One millilitre of the overnight culture was added to 100ml LB and incubated with shaking at 37ºC for 2 hours. The bacterial culture was centrifuged at 3500 rpm for 20 min then resuspended in 9.5 ml TSB+0.5 ml DMSO and incubated on ice for 10 min. Approximately 1µg of DNA was added to 200µl competent cells and mixed by flicking. After incubation on ice for 30 minutes, 800 µl TSB + glucose were added and mixed by flicking. Cells were incubated at 37ºC for 1 hour with shaking. For intact plasmid transformation, 200µl cells were plated onto LB agar + ampicillin.

For ligation, cells were pelleted by centrifugation at 13000 rpm for 3 minutes and 800 µl from the supernatant was removed and the cells were resuspended in the remaining 200 µl LB and plated on LB agar +ampicillin. Plates were incubated at 37 ºC overnight.

2.2.1.12. DNA gel extraction

Digested DNA was subjected to gel electrophoresis. The desired band was cut from the gel and extracted using the Qiaquick gel extraction kit (Qiagen).
The digestion was performed according to the manufacturer's instructions followed by agarose gel electrophoresis.

2.2.1.13. T/A cloning

For sequencing purpose, 50ng cDNA obtained with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase was amplified using *Taq* DNA polymerase. The PCR product was gel purified and cloned into pGEM-T Easy vector according to the manufacturer's instructions. The ligation reaction was set up as following:

pGEM-T Easy vector (50ng)……1µl
PCR product (100ng)……1µl
2× ligation buffer……..10µl
dH_2O……7µl
T4 DNA ligase (3U/ µl).....1µl

The reaction was incubated overnight at room temperature and 1-5 µl transformed into competent *E.coli.* If the PCR product generated with blunt ends, an A-tailing procedure was carried out as following:

PCR product…….. .1-7µl
Taq DNA polymerase 10× reaction buffer with $MgCl_2$…… 1µl
dATP (2mM)……1µl
Taq DNA polymerase (5U/ µl).....1µl
dH_2O……...to 10µl

The reaction was incubated for 30 minutes at 70 ºC and 1-2 µl used in a ligation reaction with pGEM-T Easy vector.

2.2.1.14. Digestion of DNA

To confirm the insert, DNA was digested using *EcoR*1 restriction enzyme:

10× NEBuffer……2µl
DNA (300ng)……..1µl
10× bovine serum albumin (BSA)……...2µl
*EcoR*1 restriction enzyme (3U/ µl)……..1µl
dH_2O……..to 20µl

The reaction was incubated at 37˚C for 1 h and 5µl was run in gel.

2.2.2. RNA manipulation

2.2.2.1. Total cellular RNA isolation using TrIzol

Total RNA was isolated from cell monolayers in 35mm dishes. First, the culture medium was removed and 1ml TrIzol reagent (Invitrogen) was added to the monolyers and incubated for 5 min before resuspension by pipetting several times. The cell lysate was then transferred to a 1.5ml tube and left at room temperature for 5 min. 200µl chloroform was then added and mixed vigorously for 15s. The sample was incubated for 3 min at room temperature and then centrifuged at 13000rpm for 15 minutes at 4ºC. The upper colourless aqueous layer was transferred to a new 1.5ml tube, 0.5ml Isopropanol added and mixed by gently inverting. The sample was incubated at room temperature for 15 min and the RNA pelleted by centrifugation at 13000rpm for 15 min at 4ºC. The supernatant was removed and replaced with 1ml 75% (v/v) ethanol. RNA samples were stored at -70ºC, or pelleted by centrifugation at 11000 rpm for 7 min at 4ºC. The ethanol was aspirated and the pellet allowed to air dry for ½ hour. The pellet was resuspended in 30µl nuclease-free water.

2.2.2.2. Viral RNA extraction from virions

At 20 h post-infection, growth medium was collected and centrifuged at 26,000 rpm for 1.5 h in SW40 rotor at 4ºC. The supernatant was removed and virus pellet were resuspended in 1ml Trizol reagent. The samples were treated as described above for total RNA extraction.

2.2.2.3. RNA reverse transcription

For analysis purposes, viral RNA was converted to cDNA by using either the Avian Myeloblastosis Virus (AMV) reverse transcriptase or Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega).

2.2.2.3.1. One-step RT-PCR (AMV)

After RNA extraction, RT-PCR reaction was prepared as follows:

AccessQuick Master Mix, 2x……25µl
Upstream primer (10µM)……1µl
Downstream primer (10µM)……1µl
RNA template (1µg).....1µl
rRNasin RNase inhibitor (25U).....0.5µl
AMV reverse transcriptase (5U)……1µl
Nuclease-free water……..to 50µl

PCR programme:

Reverse transcription step……..42ºC……1h

Denaturation……..94ºC……5mins

Strand separation……94ºC……...30s
Annealing……..variable……30s } 35 cycles
Elongation……..72ºC……1min

Final extension……..72ºC……10mins

2.2.2.3.2. Two-step Reverse transcription (M-MLV)

After RNA extraction RT-PCR reaction was prepared as follows:

RNA sample (2µg)…….2µl
Forward primer (10µM)…….1µl
Reverse primer (10µM)…….1µl
Nuclease-free water…….to 15µl

The reaction was mixed and heated at 70ºC for 5 min and cooled immediately on ice. The following components were added to the annealed primer/template reaction:

M-MLV 5x reaction buffer……5µl
dNTPs mix (10mM each)……...1.25µl
Recombinant rRNasin inhibitor (25U)……0.5µl
M-MLV RT enzyme (200U).....1µl
Nuclease-free water…….to 50µl

The reaction was incubated at 42ºC for 1 hour and 50-100ng cDNA used for amplification.

2.2.2.4. Northern blotting

2.2.2.4.1. Preparation of an agrose gel

Agarose gel 1.5% (w/v) was prepared by melting 1.5 g agarose in 85ml 1xTAE buffer and cooled to 55ºC in a water bath. Agarose gel was then poured into a gel casting tray with comb to produce an 11×15×0.6 cm slab gel with ×14 of 31 µl wells. Once the gel had solidified, it was submerged in 750ml 1×TAE buffer (Masek *et al*., 2005).

2.2.2.4.2 Preparation of RNA samples

3 to 5 µg total cellular RNA was diluted to 10 µl dH_2O. Seventeen microlitres of deionised formamide, was added and mixed. The samples were heated at 65ºC for 15 min, rapidly cooled on ice and then centrifuged briefly to collect the solution at the bottom of the tube. Three microlitre RNA loading buffer was added and 30µl loaded per well. The gel was run at 100V for 1½ hours for RNA genome detection or 2½ hours for antigenome/mRNA detection.

2.2.2.4.3 Capillary blotting

The gel was washed twice for 25 min with 10×SSC. A gel casting tray was placed upside-down in a shallow tray. Two strips of 3MM (Whitman) paper (28 cm ×14 cm) were placed on the top of the tray and were soaked with 20 ×SSC until the shallow tray was full. The gel was placed on top of the wick on the upside-down gel casting tray. The whole tray was then covered with cling film. A scalpel was used to cut a window in the cling film around the gel. A positive charged nylon membrane (Roche) was cut to the size of the gel, soaked in dH_2O and then placed on top of the gel. Three pieces of 3MM paper were cut to size of 14cm ×11.5cm, pre-soaked in 20×SSC and then placed on top of the nylon membrane. Bubbles were removed by rolling with a plastic pipette. A Pad papers were placed on the top of the chromatography paper. Finally, a 1kg weight was placed on the top stack overnight to allow the capillary-driven transfer of RNA from the gel to membrane.

2.2.2.4.4. UV cross-linking of RNA to membrane

After blotting overnight, the orientation of the membrane was marked by cutting the top, right corner. The membrane was removed, incubated for 10 min in 2×SSC, and then hung to dry completely. The RNA was then cross-linked to the membrane using 2UV transilluminator (302 nm for 3 mins).The membrane was stored at 4ºC until used for hybridization.

2.2.2.4.5. Hybridization of DIG-labelled RNA probes

The membrane was placed in a cylindrical glass bottle (Techne FHB11) and 10ml pre-warmed 50% formamide hybridization buffer at 68ºC added. The membrane was incubated for 2 hours. Digoxigenin (DIG)-labelled RNA probe was denatured by boiling for 5 min then cooling rapidly on ice. Approximately 150ng of each probe was added to 10ml 50% formamide hybridization buffer at 68 ºC. The pre-hybridization buffer was removed and replaced with the probe-containing hybrization buffer and left at 68 ºC overnight, with rotation.

2.2.2.4.6 Washing

To remove excessive nonbinding probes, membrane was washed as following:

2×10min in 100ml 2×SSC, 0.1% (v/v) SDS at room temperature.
2×25 min in 100ml 0.1× SSC, 0.1% (v/v) SDS at 68 ºC.

2.2.2.4.7 Detection

As described by DIG Northern Starter kit (Roche) DIG-labelled probes were detected with slight modifications in incubation times to reduce signals background:

10 min in 50ml washing buffer
45 min in 50ml 1×blocking buffer
45 min in 50ml 1×antibody solution
2×45 min in 60ml washing buffer
5min in 50ml detection buffer

The membrane was then transferred to translucent plastic sheet and 1ml CDP star solution (Roche) was added drop-wise and incubated for 5 min at room temperature before excessive liquid was removed. The plastic sheet was sealed and the membrane was then exposed to x-ray film (18×24 MOL7016) for 10s-5min.

2.2.2.5. Preparation of SDS-polyacrylamide gel

The resolving gel solution was prepared (below) then, poured into clean glass cassette plates. A space equivalent to 2 comb-depths was left for the stacking gel. To create a flat surface, drops of isopropanol were added. Once the resolving gel had polymerized, the isopropanol was removed and washed with dH_2O to remove traces of isopropanol. The stacking gel was prepared (below) and poured on top of resolving gel. Immediately, a comb was inserted and the gel was left to polymerize.

Resolving 12%

H_2O……5.0ml
30% Acrylamide mix……6.0ml
1.5M Tris-HCl (pH8.8)……3.8ml
10% SDS……150µl
10% APS……150µl
TEMED……6 µl

Stacking

H_2O……2.7ml
30% Acrylamide mix……...670µl
1M Tris-HCl (6.8pH)……0.5ml
10% SDS……40µl
10% APS……40µl
TEMED……..4µl

The glass plates were then placed into a mini-gel tank (8×7.3cm) (Biorad) filled with 1×Tris-Glycine running buffer. The comb was removed gently and wells were washed by syringe to be ready for use.

2.2.2.6. *In vivo* protein labelling

Using 35mm-diameter dishes, Vero-E6 cells were infected at 0.01-1.0 MOI and incubated at 33ºC overnight. Next day, the cells were starved of methionine by replacing the culture medium with 1ml DMEM minus methionine and incubated for 1.5 hours at 37 ºC. Medium was then removed and replaced with 1ml fresh DMEM containing 40µCi ^{35}S-methionine/ml and cells were incubated at 37ºC for 2 hours. The labelling medium was then removed and 200µl 1×protein dissociation buffer added to each dish and cells were incubated 10 minutes at room temperature. Cells were then harvested using a rubber policeman and transferred to 1.5ml tubes. The samples were boiled for 5 mins and cooled on ice. Ten to 15 microlitres of samples were loaded into 12% gel wells in parallel with protein marker (PageRuler prestained protein ladder, Fermentase) .The samples were electrophoresed at 100V in 1×Tris-Glycine running buffer until the bromophenol blue band reached the bottom of the gel. The gel was incubated in fixation buffer for 1 hour at room temperature. The gel was transferred to chromatography paper, covered with cling-film, dried and exposed to x-ray film overnight.

2.2.2.7. Western blotting

Vero-E6 or BHK cells in 35mm-diameter dishes were infected at an appropriate MOI for 1h at 37ºC and incubated at 33ºC overnight. Cells were lysed by adding 200µl 1×protein dissociation buffer/dish and incubated for 20 min at room temperature. Cells were detached using cell scraper and the lysates collected into 1.5ml tubes. Samples were boiled for 5min. Forty microlitres of the lysate was loaded onto a 14-well, 12% acrylamide gel. The samples were electrophoresed at 100V in 1×Tris-Glycine running buffer for 1 hour. The gel was incubated in transfer buffer for 10min, as well as the nitrocellulose membrane and two blotting pads. First, a piece of paper pad was placed on a semi-dry transfer cell (Bio-Rad Trans-Blot SD), the membrane then replaced on top of first paper pad, and the gel on top of the membrane and finally the second blotting pad on top. Bubbles were removed by rolling a pipette over the stack. Transfer was carried out at 0.8mA/cm^2 for 1 hour. The membrane was then incubated 1 hour at room temperature in 100ml blocking buffer.

The primary antibodies (anti-N) were added at a 1/1000 dilution and the membrane incubated for 2 hours. The membrane was washed three times for 5 minutes with blocking buffer. Secondary antibody HRP-linked anti-rabbit IgG (Cell Signalling Technology) at a 1/1000 dilution in blocking buffer was added and membrane was incubated for 1 h. The membrane was then washed 3 times for 20 minutes with blocking buffer and once with PBS. The PBS was poured off and enhanced chemiluminescent substrate were added to the membrane and incubated for 3 minutes at room temperature. Any excessive substrate was removed and the membrane covered by a sheet of plastic and exposed to X-ray film.

2.2.2.8. Co-immunoprecipitation

2.2.2.8.1. Metabolic [^{35}S] methionine radiolabeling of BUNV proteins

Sub-confluent BSR-T7/5 cells grown in a 35mm dish were transfected with 0.1µg of pTM1BUNN or mutant, pTM1BUNL and 0.2µg of pT7riboBUNM*Renilla* (-) using Lipofectamine-2000 (Invitrogene) for 4 - 5 hours at 37˚C with shaking every 15 mins. Transfection mixture was removed and replaced with fresh medium. Cells were incubated at 33˚C overnight. The culture medium was removed and replaced with 1ml methionine-deficient medium and cells incubated for 1½ hours at 37ºC. The medium was removed and replaced with protein labelling medium containing 40µCi/ml [^{35}S] methionine/ml and cells were incubated overnight at 33ºC.

2.2.2.8.2. Preparation of cell lysate for immunoprecipitation

The medium was removed from the previous dishes and cells rinsed once with 1ml ice-cold PBS and 300µl RIP buffer containing protease inhibitor cocktail was added and then cells incubated on ice for 10 min. The cell lysate was harvested with a cell scraper and was collected into 1.5ml tube. The cell lysate was vortexed for 5 seconds and incubated on ice for 10 min. The samples were centrifuged at 16,000g for 10 min at 4ºC to remove the cell debris and nuclei. The supernatant was transferred to a new tube and placed on ice or stored at -20ºC.

2.2.2.8.3. Preparation of antibody conjugated protein A-agarose beads

To prepare 50% (w/v) protein A-agrose beads, 100mg beads were mixed with 800µl RIP buffer and incubated for at least 30 minutes at room temperature before use. Thirty microlitres of 50% (v/v) protein A-agrose beads were combined with 1µl anti-BUNV N poly-clonal antibody and 0.5ml ice cold PBS. The mixture was incubated overnight at 4ºC with rotating. The beads were spun down for 5 sec at 16,000g, 4ºC and washed 3 times with 1ml ice-cold RIP wash buffer and once with RIP buffer.

2.2.2.8.4. Immunoprecipitation

Ten microliters of 10% (w/v) BSA and 300µl of cell lysate were added to antibody conjugated beads and incubated overnight at 4ºC on a rotating wheel. The samples were centrifuged for 5 seconds at 16,000g and the supernatant was removed carefully. The samples were washed 4 times with 1ml of ice-cold RIP wash buffer and once with 1ml of cold PBS. Supernatant was removed and 30µl of 2x protein loading buffer was added and samples were boiled for 3 min. The samples was electrophresed in SDS-PAGE and the gel was subjected to same procedure mentioned in 2.2.2.6.

2.2.3. Manipulation of higher eukaryotic cells

2.2.3.1. Maintenance of mammalian cell lines

Cells were grown in large (175cm^2) or medium (80cm^2) tissue flasks with regular passaging: BHK-21, Vero-E6, 293T-V, A549-V and 2FTGH-V cells were split every 3 or 4 days. BSR-T7/5 cells were split every 2 or 3 days. The monolayer was first washed with 10ml trypsin-EDTA solution (large flask) or 5ml (medium flask). Next, 2ml (large flask) or 1ml (medium flask) trypcine-EDTA solution was added and the flask was incubated at 37ºC for 5min. Cells were resuspended by rigorously pipetting in 10ml growth medium. Cells were spun down at 1700rpm for 3 minutes, the supernatant discarded, and cells resuspended in 10ml fresh medium. Flasks were seeded using 1ml of cell stock in 30ml medium for large flask or 0.5ml in 20ml medium for a medium flask. Antibiotic G-418 So_4 1mg/ml was added to BSR-T7/5 cells.

2.2.3.2. Transfection of mammalian cells

Sub-confluent BSR-T7/5 cells were used for transfection, using Lipofectamine-2000 (Invitrogene) as transfection reagent. Thirty-five mm-diameter dishes were seeded with 3.5×10^5 cells, 24 hours prior to transfection. All plasmids to be transfected were diluted in 100µl Opti-MEM. In a separate tube, 2.5µl lipofectamine-2000 for each 1 µg of DNA was diluted in 100 µl Opti-MEM, mixed and incubated for 5 min at room temperature. The diluted Lipofectamine-2000 was added to the DNA solution mixed, and incubated for 20 minutes at room temperature. The medium were removed and 200 µl of DNA/lipofectamine mixure added to a 35mm dish. Dishes were incubated at 37ºC for 4 hours with rocking every 15 min. The DNA/lipofectamine mixture was removed, 2ml fresh growth medium added and cells were incubated at 33ºC overnight.

2.2.3.4. BUNV minigenome assay

The BUNV minigenome system mimics BUNV transcription and replication. BSR-T7/5 cells grown in 35mm-diameter dishes were co-transfected with a plasmid expressing a luciferase based mini-genome pT7riboBUNS, M or L*Renilla* (-), two pT7ribo-based plasmids encoding BUNV N and L proteins and pT7-FF-luciferase as internal control of transfection efficiency. Transfection was performed as described in section 2.2.2.8.2. Eighteen hours post-transfection cells were lysed and luciferase assay performed (Weber *et al*., 2001).

2.2.3.5. Luciferase assay

Using the *Renilla* luciferase Assay Kit (Promega), the activity of BUNV minigenome was measured. At 18 hrs post-transfection, BSR-T7/5 cells in 35mm-diameter dishes were lysed by addition 200µl 1× passive lysis buffer and incubating for 20 minutes at room temperature with rocking. The lysate was transferred to 1.5ml tube and centrifuged for 1 min at 1700xg. Five microlitres lysate was diluted 1:20 in water. Stop & Glo substrate (50x) was diluted to 1× in Stop & Glo buffer. Seventy microlitres of luciferase assay buffer II (LARII) was added to a luminometer cuvette and

combined with 5µl of diluted lysate and the first luminometer reading taken (the firefly). Seventeen microlitres of Stop & Glo solution was added to the previous combination, mixed and *Renilla* activity taken.

2.2.3.6. BUNV minigenome packaging assay

BSR-T7/5 cells were transfected with five plasmids: pTM1BUNL (0.1µg), pTM1BUNM (0.1µg), pTM1BUNS (0.05µg), pT7riboBUNM*Renilla* (-) (0.1µg) and pTM1-FF-luciferase (0.05µg) as internal control. At 24 h post-transfection, the supernatant was used to infect new monolayers of BSR-T7/5 cells. *Renilla* luciferase activity was measured after 24 h of incubation (Shi *et al*., 2007).

2.2.4. Bunyavirus manipulation

2.2.4.1. Rescue of BUNdelNSs

BUNV lacking NSs protein was rescued from cloned DNA by transfecting approximately 10^7 BSR-T7/5 cells grown in 60 mm-diameter dishes with pT7riboBUNL (1.0µg), pT7riboBUNM (1.0µg) and pT7riboBUNN (1.0 µg). Five days post-transfection, culture medium was collected into 15 ml falcon tubes and centrifuged at 1700 rpm for 3 minutes to remove cell debris. The supernatant was aliquoted into 1ml virus storage tubes at -20ºC. One hundred microlitres were used in a plaque assay on Vero–E6 cell line for virus isolation purpose. Vero-E6 cells were infected using serial dilutions for 1 hour at 37ºC with rocking every 15 min. The virus was removed and replaced with fresh medium and cells incubated at 33ºC for 6 days.

2.2.4.2. Plaque purification

Cells were stained with 1ml of 0.1% (w/v) neutral red/PBS and incubated for 1 hour at 37ºC. Excessive stain was removed and cells incubated for 3-4 hours at 37ºC. A single plaque was then picked by 1000 ml pipette tip and resuspended into 0.5ml medium and used to inoculate BHK-21 cells grown in a medium flask for propagation purpose.

2.2.4.3. Plaque assay

Recombinant virus titres were determined using 100µl from the original stock which was diluted 10^{-1} to 10^{-6} in 900µl PBS with 2% (v/v) FCS. A 200µl aliquot of each dilution was used to infect 90% confluent Vero-E6 cells. Cells were then incubated at 37ºC for 1 h with rocking every 15 min. The virus aliquot was then removed and 2ml agarose overlay was added, and left 10 min to solidify. The dishes were incubated at 33ºC for 6 days. To prepare agarose overlay, 1.2g of agarose type HSA was melted in 100ml dH_2O and autoclaved. Ten mililitres of FCS was added to 250 ml 2x MEM to give 2% FCS. Before performing a plaque assay 1.2% agarose was melted in a microwave oven and cooled to 55ºC in a water bath. Twenty five ml agarose was added to 25ml 2×MEM containing 2% FCS, mixed and 2ml added to each dish. Dishes were left 10 min at room temperature to solidify the agarose overlay. After 6 days of incubation at 33ºC, cells were fixed by adding 2ml 5% (w/v) formaldehyde in PBS to each dish and incubated overnight at room temperature. The overlay was then detached with a scalpel and cells were stained by adding 1ml Giemsa stain solution per dish. The stain was incubated 20 min and washed with water. Plaques were visualized as a clear circle area within blue monolayer surface.

2.2.4.4. Freeze-thawing

Freeze-thawing experiment was used for two purposes. First, to explore possibility of some recombinant viruses were rescued but they were unable to be released from cells. BSR-T7/5 cells were transfected with pT7riboBUNL (1.0µg), pT7riboBUNM (1.0µg) and pT7riboBUNN or mut (1.0 µg). Five days post-transfection the supernatant was used in plaque assay and cells detached in 1ml PBS using a cell scraper. Cells were then subjected to 3 cycles of freeze-thaw (dry ice/waterbath 37ºC). The supernatant was cleared from cell debris and used in plaque assay. Second, to compare the ratio between the intracellular and extracelluar virus particles. 2FTGH-V cells were infected at MOI of 1 with the parental virus and mutant virus. At 20h post-infection, supernatant was collected, cleared from cells debris and used in plaque assay (extracellular particles).Cells were detached in 1ml PBS, subjected to 3 cycles of freeze-thaw. The supernatant was used in plaque assay (intracellular particles).

2.2.4.5. Immunofluorescence

Approximately 8 $\times 10^4$ BHK-21 cells were grown on coverslips placed in 24-well dishes. After infection with 200µl rescue supernatant, the cells were incubated overnight at 37ºC. Cells were washed twice with cold PBS and fixed with 100% methanol for 5 minutes at room temperature. Cells were washed twice with PBS. The coverslips were removed from dishes and incubated for 30 minutes at room temperature in PBS containing the appropriate dilution of the primary antibody (Anti-N antibody). The cover slips were then washed 5 times with PBS and incubated with secondary antibodies (Anti-sheep IgG FITC) for 30 minutes at room temperature. Cells were washed 5 times with PBS. The coverslips were then mounted face down on a drop of moviol and tested using a confocal microscope.

2.2.4.6. Cross-linking of BUNV N protein

Subconfluent Vero-E6 cells grown in 35mm dishes were infected with vaccinia virus vTF7-3 at MOI of 1 for 1 hour at 37ºC. The virus mixture was then removed and cells were transfected with 2 µg of pT7riboBUNN or pTM_1BUNN. Cells were incubated 3-5 hours at 37ºC. The transfection mixture was removed and replaced with fresh medium. Cells were incubated over night at 37ºC. The cells were washed once with 0.5 ml PBS and treated with 200 µl of PBS containing 1mM Dithiobis [succinimidylpropionate] (DSP) cross-linking agent (Figure 2.1) for 20 minutes. DSP is water insoluble, creates a disulfide bridge between two reactive primary amines. This bridge can be broken by β-mercaptoethanol. It is membrane permeable can be used for intracellular and intramembrane cross-linking. Cells were detached with a cell scraper and transferred to an eppendorf tube and incubated 20 minutes at room temperature. The samples were then subjected to freeze-thaw 3 times. The samples were then incubated 5 minutes at room temperature before adding 5µl of 1M Tris-HC1 pH 7.5 to stop the cross-linking reaction. The samples were then incubated 15 minutes at room temperature. The samples were diluted in 2× protein loading buffer without β-mercaptoenhanol (negative control). The samples were boiled and electrophoresed by SDS-PAGE. Western blot was then carried out.

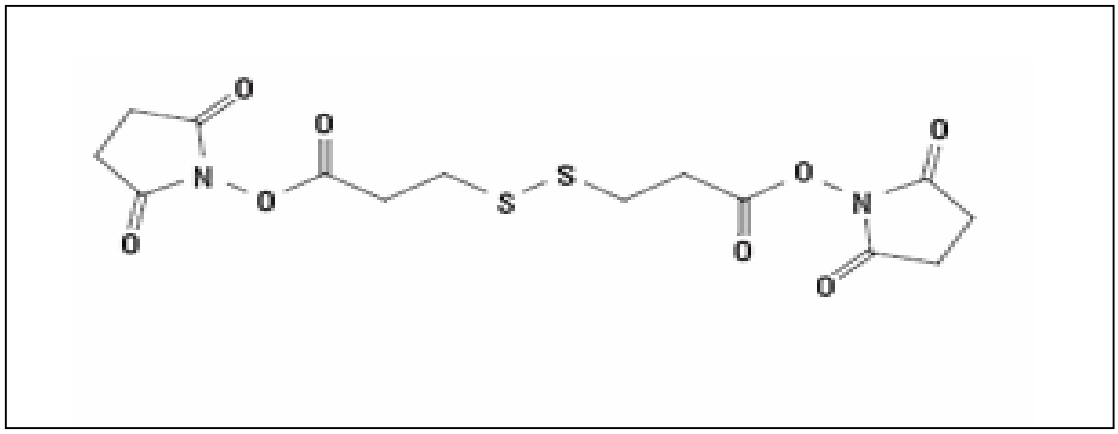

Figure 2.1. Dithiobis[succinimidylpropionate] (DSP) cross-linking agent with molecular weight of 404.42g/mol (Taken from Pierce instructions).

DSP is a water insoluble molecule that creates a disulfide bound between two reactive primary amines. The x-links can be broken by β-mercaptoethanol since the bridge contains thiol-thiol interaction.

Results and Discussion

Chapter 3. Mutagenesis of Bunyamwera virus (BUNV) N gene

3.1. Introduction

The orthobunyavirus N proteins sequenced to date range from 233-235 aa in length. Alignment of 8 orthobunyavirus N proteins from the Bunyamwera serogroup shows that at least 159 residues are conserved, too many to help in identifying the domain map of N protein. The number of conserved amino acids decreases when 20 closely related orthobunyavirus N proteins from the Bunyamwera and California serogroups were aligned, showing 92 residues are well conserved, again still too many to be mutated. A global alignment of 51 orthobunyavirus N proteins from four serogroups (Bunyamwera, California, Group C and Simbu) showed that 58 residues are highly conserved, and hence are more likely to be of functional significance (Figure 3.1).

To investigate the residues crucial for N protein functionality, several PCR-based approaches were available to mutate the BUNV N gene including random or specific site-directed mutagenesis. To minimize the scale of disruption of N protein function, it was decided to produce mutants where single amino acids were substituted in the BUNV N protein sequence. The two methods of substitution above were used. Lack of sufficient data about the domain map of the orthobunyavirus N protein suggested that the random approach should to be used first to make as many mutations across the N ORF as quickly and cheaply as possible, then site-directed mutagenesis to cover the whole region of the N protein and to fill in gaps.

Several differences between both approaches can be highlighted. Random mutagenesis was performed using the GeneMorph II random mutagenesis kit (Stratagene). It utilizes two error-prone DNA polymerases (Mutazyme I and *Taq* mutant) that display increased misinsertion and misextension frequencies compared to the wt *Taq*. They have been combined to minimise mutational bias and produce equivalent mutation rates at A's and T's vs. G's and C's. The protocol can be adjusted to give 3 levels of mutation frequency, low, medium and high, based on the amount of the initial target DNA to be amplified and the number of cycles (Table 3.1). Unique primers (forward and reverse) binding immediately upstream of the T7 promoter sequence and downstream of the N ORF sequence and containing two

restriction sites, *EcoRI* and *NsiI* respectively (Figure 3.2), were used to ease subcloning the insert (N gene) into the vector (pT7ribo) after mutagenesis. Site-directed mutagenesis was performed using the Quik-change site-directed mutagenesis kit (Stratagene). It was performed using KOD Hot Start DNA polymerase which replicates both plasmid strands with high fidelity (Figure 3.3). Two synthetic primers containing the desired mutation were designed, each complementary to the opposite strand. Only 25ng of the template plasmid (pT7riboBUNN) was amplified (18 cycles) and the product was treated with *DpnI* to digest parental plasmid, the DNA self-ligated and transformed into competent *E.coli* cells.

In both approaches, mutagenesis was performed using the pT7riboBUNN clone rather than pT7riboBUNS. The reasoning behind this was that as BUNV S segment encodes both N and NSs proteins in overlapping reading frames, a mutation in the N ORF would likely also affect the NSs ORF in the overlap region, making it difficult to determine whether any phenotype was due to an affect on the N or NSs protein. Hence, a pT7riboBUNN clone expressing only the N protein gene was produced by introducing a stop codon immediately after the first start codon of NSs protein gene (Bridgen *et al,* 2001). Another vector expressing only the N protein, designated pTM1BUNN, was also available. In pT7riboBUNN, the full length viral cDNA sequence is cloned in the positive sense between T7 promoter and hepatitis δ ribozyme sequences, followed by the T7 terminator immediately downstream, allowing transcription of antigenome S by T7 RNA polymerase.
On the other hand, pTM1BUNN contains the N ORF cloned between the IRES sequence and T7 terminator, and hence, it is a protein expressing plasmid only. pTM1BUNN expresses about 10-fold more N protein than pT7riboBUNN due to the IRES sequence. However, pT7riboBUNN supports BUNV minigenome activity (Lowen *et al*., 2004).
It would be more appropriate to use pT7riboBUNN clone as a template for mutagenesis of BUNV N gene as it could be used in the BUNV rescue system without further modification.

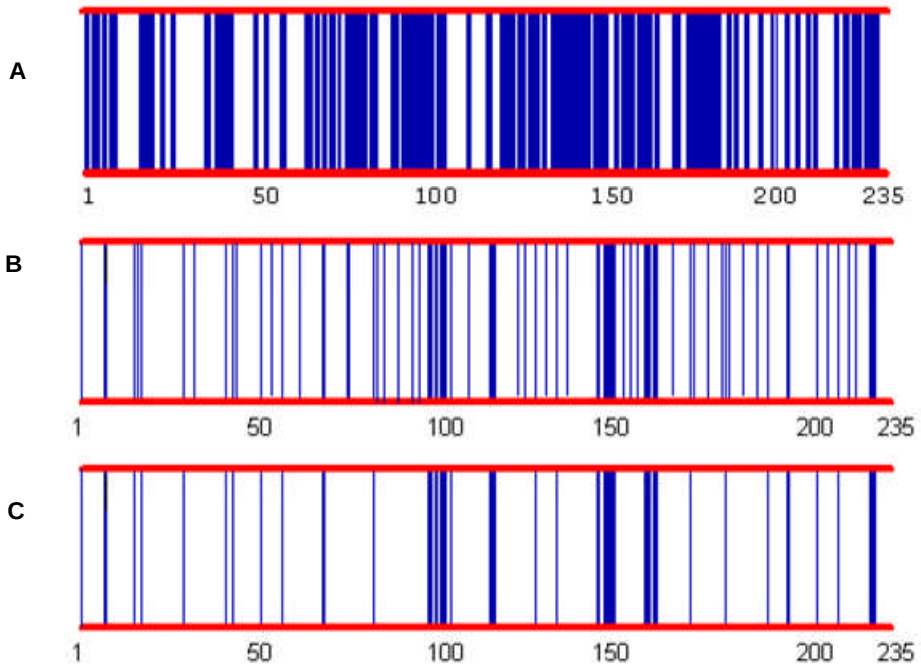

Figure 3.1. Alignment of orthobunyavirus N proteins.

(A) Alignment of 8 orthobunyavirus N proteins belong to Bunyamwera serogroup shows at least 159 residues are conserved. (B) Alignnment of 20 orthobunyavirus N proteins from two closely related serogroups (Bunyamwera and California) displays 92 conserved amino acids. (C) A global alignment of 51 orthobunyavirus N protens from (Bunyamwera, California, Group C and Simbu serogroups) shows 58 residues are highly conserved. Each blue line indicates a conserved amino acid.

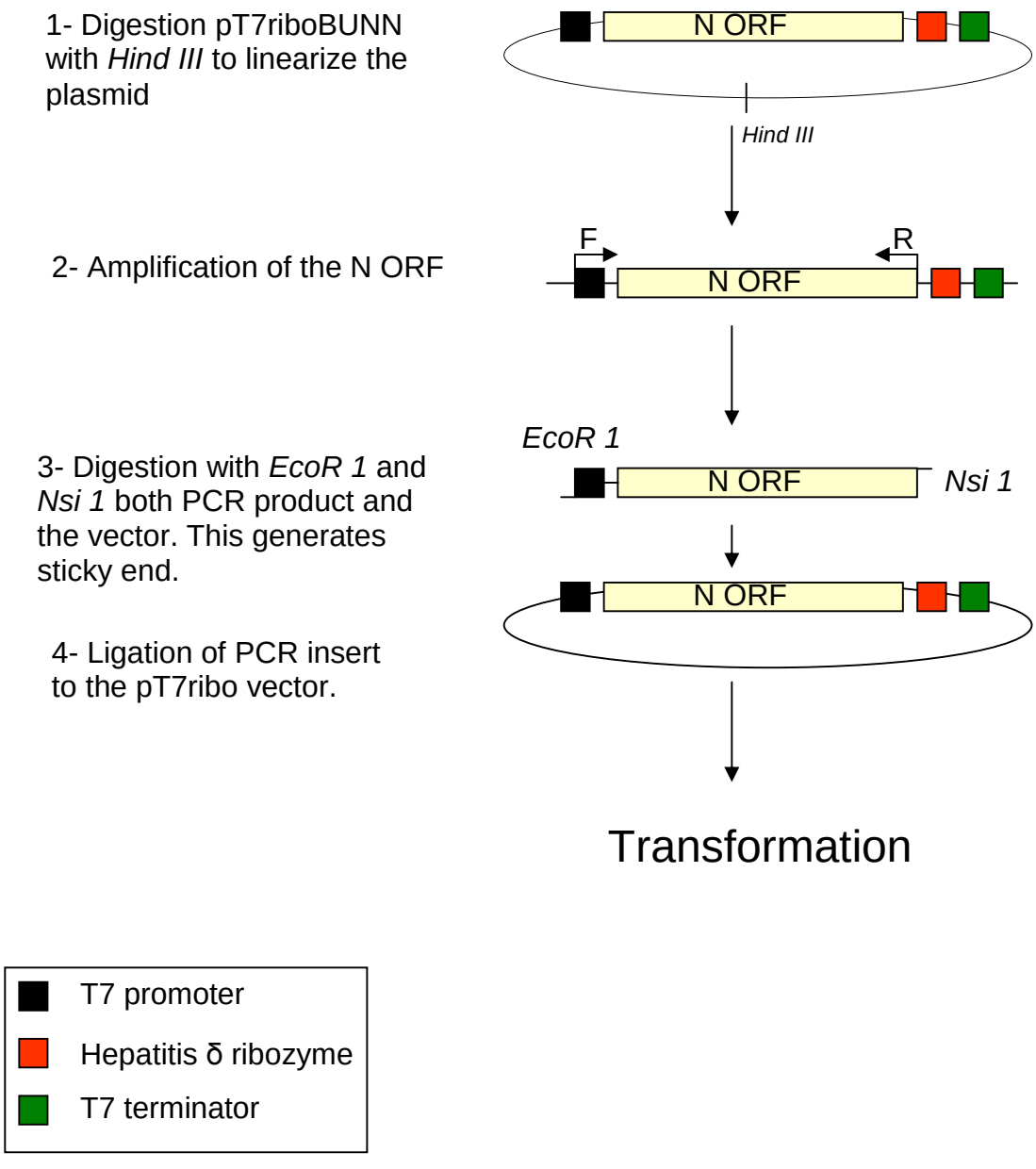

Figure 3.2. PCR random mutagenesis was performed using GeneMorph II kit. To ease the primers binding pT7riboBUNN was linearized using *Hind III* restriction enzyme. The forward primer binds immediately upstream of T7 promoter (nucleotides 219 - 236 of the vector sequence), contains *EcoR 1* restriction enzyme site and reverse primer binds immediately downstream of N ORF (nucleotides 1063–1046 of the NCR), possesses *Nsi 1* restriction enzyme site.

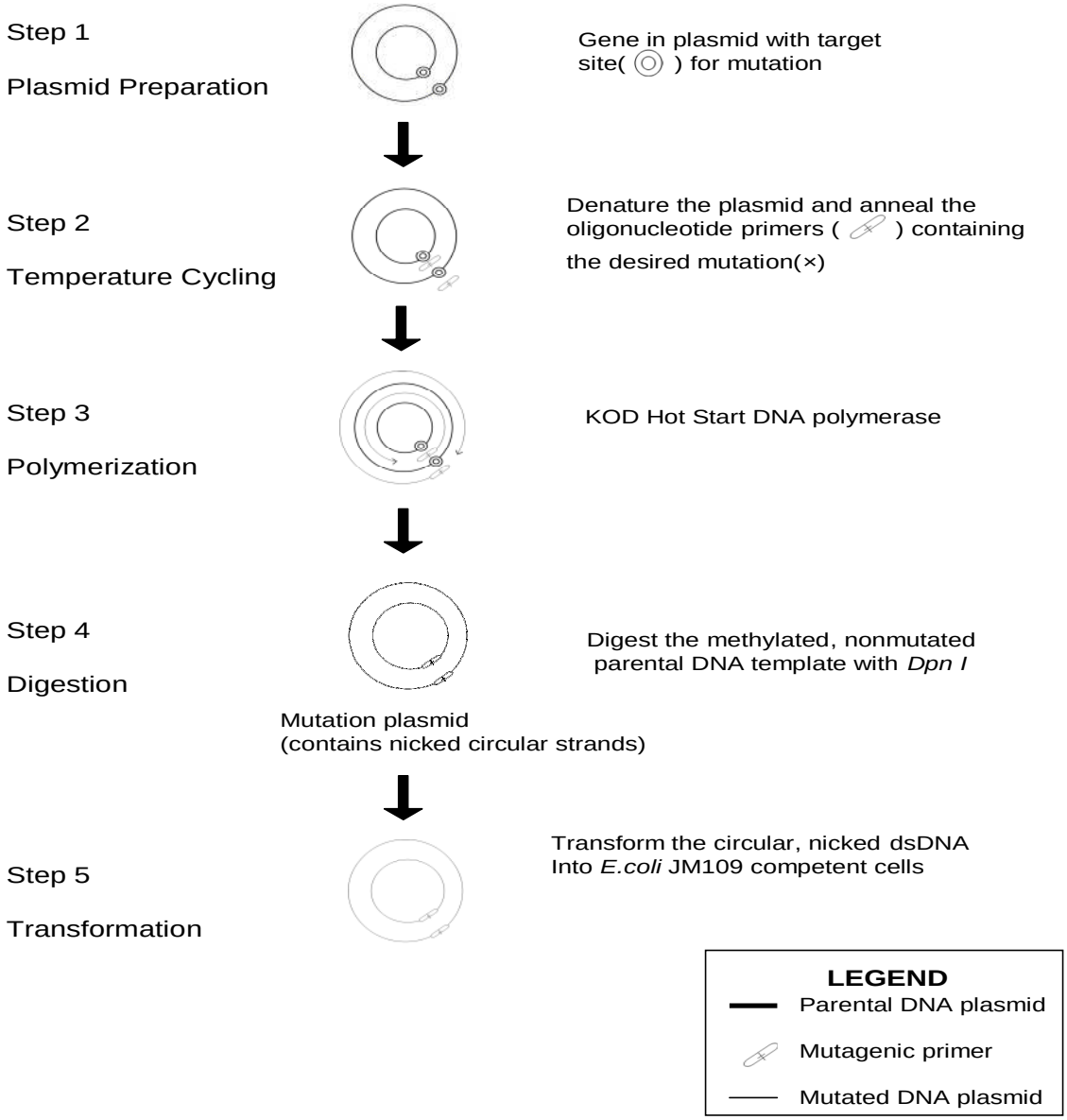

Figure 3.3. Quik-change PCR site-directed mutagenesis. Two designed primers bind at the target site. Amplification of the new plasmid was performed using KOD Hot Start polymerase. After the production of the nicked plasmid, PCR reaction was treated with *Dpn 1* to digest the methylated parental plasmid. The mutant plasmid was then transformed into competent cells (taken from Promega instruction manual).

3.2. PCR random mutagenesis of BUN N gene

PCR random mutagenesis was performed using the GeneMorph II kit. According to the manufacturer's instructions, low mutation frequency (0 - 4.5 mutation/ kb) could be achieved either by using high concentrations of the template (Table 3.1) or by reducing the number of cycles (Table 3.2). The initial amount of target DNA required to achieve a low mutation frequency refers to the amount of target DNA (N ORF), not the total amount of plasmid DNA template (pT7riboBUNN) added to the reaction. As the N gene is about 700 bases and the total construct is about 4300 bases, the target DNA (N ORF) occupies ≈ 16% of the pT7riboBUNN. Accordingly, the amount of target DNA was calculated from this perspective when PCR reaction was prepared.

When the manufacturer's protocol was followed using 100ng target DNA and 20-25 cycles or 500ng target DNA and 30 cycles almost all the clones obtained were the same as the input DNA and did not contain mutations. Therefore, slight modifications were introduced to produce as many clones as possible carrying single mutations in the N gene. When 100ng of target DNA was amplified for 30-35 cycles the result was much better, and 2 in 10 clones carried single mutations.

To perform PCR random mutagenesis on the pT7riboBUNN clone, the plasmid was first digested using *HindIII* restriction enzyme to linearize the template to ease primer binding and prevent primer rotation. Then, 100ng of target DNA (N ORF) was amplified for 30 – 35 cycles. The PCR product was then digested with *EcoR1* and *Nsi1* restriction enzymes to facilitate insertion into the pT7ribo vector. pT7riboBUNN plasmid was digested with *EcoR1* and *Nsi1* restriction enzymes, the pT7ribo fraction gel purified and the insert ligated similarly into digested vector backbone of pT7riboBUNN to generate mutant clones carry single mutations ready to be used in BUNV minigenome and rescue systems for further investigation. This approach overcomes an effects of mutations in non-coding sequences of the N clone as might occur if the whole plasmid was used.To confirm the size of the insert (≈ 700 bases), the clone was digested with *EcoR 1* and *Nsi1* restriction enzymes and electrophoresed on an agarose gel. From 10 PCR reactions, three hundred and twenty eight colonies were picked at random to generate as many single mutations in the BUNV N gene as possible. Of the 328 miniprep clones sequenced, 72 were

Mutation rate	Mutation frequency (mutations/kb)	Initial target Amount (ng)	Recommended fold Amplification
Low	0 - 4.5	500 -1000	1.5 -10
Medium	4.5 - 9	100 - 500	10 -100
High	9 -16	0.1 -100	100 -10,000

Table 3.1. Mutation frequencies vs. initial target DNA quantity using the GeneMorph II kit.

The amount of template indicated is the amount of target DNA to be amplified not the total amount of DNA (vector). Low mutation frequency can be achieved by using higher amounts of target DNA (taken from Stratagene's instruction manual).

Mutation frequency (mutation/kb)	Cycle number	Initial target amount
0-4.5	20-25	100ng
	30	500ng-1000ng

Table 3.2. Alternative way of achieving low mutation frequency. Using fewer cycle numbers (taken from manufacturer's instruction manual).

found to carry single mutations (20 in the N-terminal region, 22 in the middle and 30 in the C-terminal region) of the BUNV N protein (Figure 3.4), 17 clones contained double mutations and 2 clones carried triple mutations. Twenty-two single substitutions occurred at globally conserved amino acids, 14 mutations occurred at conserved residues between at least 20 orthobunyavirus N proteins, 10 single mutations were at BUNV-specific and 26 mutations were Bunyamwera serogroup-specific residues. The majority of clones carrying double mutations showed the same pattern, carrying one mutation at a nonconserved residue and a second at a conserved amino acid. Three clones contained mutations at two conserved residues. Another 3 clones carried double mutations at nonconserved positions. Triple mutations occurred at two nonconserved residues and the third mutation was at conserved residue.

3.3. PCR specific site-directed mutagenesis of BUNV N gene

Not all regions of BUNV N protein sequence were mutated by the random approach. To produce further mutations in the N gene, targeted mutagenesis was performed. Thirty globally conserved amino acids (6 residues in the N-terminal region, 11 amino acids in the middle and 13 positions in the C-terminal region) were mutated to Alanine or Glycine to minimize any disruption that could occur (Figure 3.5). Two oligonucleotides were designed to each targeted site and the whole template (pT7riboBUNN) was then amplified. The PCR product was then digested with restriction enzyme *Dpn1* to remove methylated template DNA (input plasmid), self-ligated and used to transform competent *E.coli*. The desired mutation was confirmed by sequencing.

3.4. PCR random and specific mutagenesis of BUNV N gene

In total, 102 single mutations were generated randomly or deliberately covering the whole region of BUN N gene sequence (Figure 3.6). Forty single mutations occurred at globally conserved amino acids of orthobunyavirus N proteins, 26 single mutations occurred at conserved residues among at least 20 orthobunyavirus N proteins, 26 single mutations were at Bunyamwera serogroup-specific residues and 10 single mutations were at BUNV-specific residues.

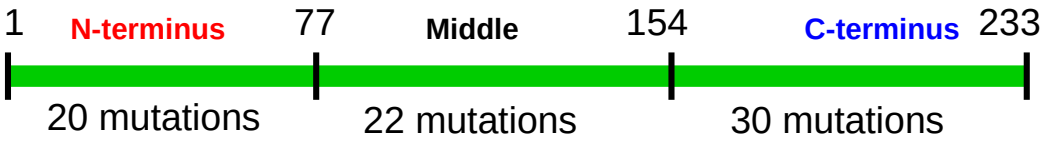

Figure 3.4. The BUNV N protein has been divided into three regions, the N-terminus (aa 1-77), the middle (aa 78-154) and the C-terminus (aa 155-233).

Using random mutagenesis approach, 72 single mutations were generated in the BUNV N protein, 20 mutations occurred in the N-terminus, 22 mutations were generated in the middle and 30 mutations in the C-terminus.

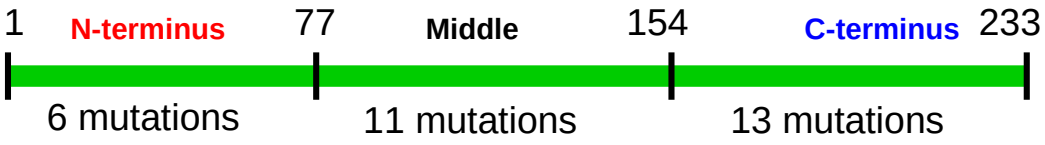

Figure 3.5. Thirty globally conserved amino acids among orthobunyavirus N proteins were substituted to Alanine or Glycine using PCR site-directed mutagenesis approach. Six mutations occurred in the N-treminal region, 11 mutations in the middle and 13 mutations in the C-terminal region.

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     1                                                          59
CAR -MIELIFKDVASFTDNTFDPEAAYLRFESDYGSQLESAIVRVFYINAAKVKAYLRRSSEL
ORI -MIELIFNDVASFTDNTFDPEAAYLRFESDYGSQLESAIVRVFYINAAKVKAYLRRSSEL
MUR -MIELIFKDVASFTDNTFDPEAAYLRFESDYGSQLESAIVRVFYINAAKVKAYLRRSSEL
RES -MIELIFKDVASFTDNTFDPEAAYLRFESAYGSQLESAIVRVFYINAAKVKAYLRRSSEL
BRU -MIELIFKDVASFTDNTFDPEAAFLRFESAYGSQLVSAVVRVFYINAAKVKAYLSRSSEL
OSS -MIELIFKDVASFTDNTFDPEAAFLRFESAYGSQLVSAVVRVFYINAAKVKAYLSRSREL
ITA -MIELIFKDVPSFTDNTFDPEAAFLRFESAYGRQLVSAVVRVFYINAAKVKAYLSRSSEL
VIN -MIELIFKDEPSFTDNTFDPEAAFLRFESVYGQQLVAAVVRVFYINAAKVKAYLRRSREL
APE -MIELVFKDVASFTDNTFDPEAAYVRFKPAYGSELESSIVRVFYINAAKVKAYLRRSSEL
MAR -MIELVFKDVASFTDNTFDPEAAYVRFKPAYGSELESSIVRVFYINAAKVKAYLRRSSEL
NEP -MIELVFKDEASFTDNTFDPEAAYVRFKPAYGSELESAIVRVFYINAAKVKAYLRRSSEL
Gum -MIELVFKDETSFADNTFNPEAAFVAFKPTHGSELESSTVRIFYINAAKVKAYLRRSAEQ
MAD -MSELEFKDVPSFADNTFNPEAQYVTFIPRNGRVLEATIVRIFYINAAKVKAYLHRSSEQ
AIN MANQFIFQDVPQRNLATFNPEVGYVAFIAKHGAQLNFDTVRFFFLNQKKAKMVLSKTAQP
PEA MANQFIFQDVPQRNLATFNPEVGYVAFIAKYGAQLNFDTVRVFFLNQKKAKMVLSKTAQP
SHU MANQFIFQNVPQRNLATFNPEVGYVAFIAKHGAQLNFDTVRVFFLNQKKAKMVLSKTAQP
KAI MATHFIFQDVPQRNLATFNPEVGYVAFIAKHGAQLNFDTVRVFFLNQKKAKMVLSKTAQP
YAB MANQFVFNDVPQRNAATFNPDTGYVAFISKYGQHLNFSVARVFFLNQKKAKMVLHKTPQP
AKA MANQFIFNDVPQRNAATFNPDAGYVAFISKYGQQLNFTVARVFFLNQKKAKMVLHKTPQP
SAB MASQFIFDDVPQRNAATFNPDTGYVAFISKYGQHLNFTVARVFFLNQKKAKMVLHKTAQP
SIM MANQFIFEDVPQRNLSTFSPEAGYVAFIGRYGQQLNFSVVRVFFLNQKKAKMVLHKTAQP
SAT MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHKTAQP
SHA MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHKTAQP
DOU MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFSVARVFFLNQKKAKMVLHKTAQP
BUT -MSEFIFNDVPQRSTSTFDPEAAYVAFEAQYRAXLSVSVARIFFLNQKKAKDRLRQTSQP
Fac -MTDFVFNDVPQRATSTFDPEAAYVAFENRFRANLTVDVARIFFLNQKKAKDRLAKTARA
ING MAEAIVFNDVPQRTQSTFDPEAQYVVFENTYRANLTVNTARIFFLNQKRAKDTLRQTPRP
MER MADAIVFNDVPQRAQSTFDPEAQYVVFENTYRANLTINTARIFFLNQKRAKDTLRQTPRP
ORO -MSEFLFNDVPQRTTSTFDPEAAYVAFEARYGQVLNAGVVRVFFLNQKKAKDVLRKTSRP
CV  -MIELEFNDVAANTSSTFDPEIAYVNFKRIHTTGLSYDHIRIFYIKGREIKTSLTKRSEW
MAG -MIELEFNDVAANTSSTFDPEIAYVNFKRIHTTGLSYDHIRVLYIKGREIKTSLTKRSEW
NOR -MIELEFNDVAANTSSTFDPEVAYINFKRVYTTGLSYDHIRIFYIKGREIKTSLTKRSEW
BAT -MIELEFNDVAANTSSTFDPEVAYINFKRIYTTGLSYDNIRIFYIKGREIKTSLSKRSEW
BUN -MIELEFHDVAANTSSTFDPEVAYANFKRVHTTGLSYDHIRIFYIKGREIKTSLAKRSEW
MD  -MIELEFHDVAANSSSTFDPEVAYASFKRVHTTGLSYDHIRIFYIKGREIKTSLSKRSEW
GER -MLELEFEDVPNNIGSTFDPESGYTNFQRNYLPGVTLDQIRIFYIKGREIKNSLSKRSEW
KAI -MSEIEFHDVTANTSSTFDPEAGYAAFKRRHTTGLNYDHIRIFFLNGKKAKDTLSKRSET
GUA -MAEIEFFDVAQNATSTFNPELQYATFKRTNTTGLNYDNIRIFYLNGKRSKDTLSKRSEQ
SSH -MSDLVFYDVASTGANGFDPDAGYMAFCVKYAESVNLAAVRIFFLNAAKAKAALSRKPER
LAC -MSDLVFYDVASTGANGFDPDAGYMDFCVKNAESLNLAAVRIFFLNAAKAKAALSRKPER
Cal -MSDLVFYDVASTGANGFDPDAGYVDFCAKHGESINLAAVRIFFLNAAKAKAALSRKPER
TAH -MSDLVFYDVASTGANGFDPDAGYVDFCIKHGEAINLHSVRIFFLNAAKAKAALARKPER
SA  -MSELVFYDVASTGANGFDPDAGYLAFCVKHGESISLSSVRIFFLNAAKAKAALSRKPER
JS  -MGDLVFYDVASTGANGFDPDAGFVAFMADHGESINLSAVRIFFLNAAKAKAALARKPER
Jam -MGDLVFYDVASTGANGFDPDAGFVAFMADHGESINLSAVRIFFLNAAKAKAALARKPER
INK -MGDLVFYDEASTGANGFDPDAGFVAFMADHGESINLAAVRIFFLNAAKAKAALARKPER
SR  -MGDLVFYDVASTGANGFDPDAGFVAFMADHGESINLSAVRIFFLNAAKAKAALARKPER
SDN -MGDLVFYDVASTGANGFDPDAGFVAFMAGHGESINLTAVRIFFLNAAKAKAALSRKPER
KEY -MGDLVFYDVASTGANGFDPDAGYVAFMANHGESISLSTVRIFFLNAAKAKAALTRKPER
MEL -MGDLIFYDVASTGANGFDPDAGYLAFTIAHGEAINLSAVRIFFLNAAKAKAALSRKPER
TRI -MSELVFYDAPSTGANGFDPDAGYVAFIAAHAGSYDLSAVRIFFLNAAKAKNALSRKPEG

Con  ------F-DV-------F-P------F-------------R-F-------K--L------
```

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     60                                                                  118

CAR LVLLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLARWALYLIRPSP-ESDIGLV
ORI LVLLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLARWALYLIRPSP-ESDIGLV
MUR LVSLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLSRWALYLIRPSP-ESEIGLV
RES LVSLTLGGVNLAVLNTHFPGTQSIPLPDYGLTLHRLSGYLARWALYLIRPSP-ESDIGLV
BRU LVFLTLGGVNLAVLNTHFPGTQSIPLPDYGLTLHRISGYLARWALYLIRTNP-ESDIALV
OS  LVFLTLGGVNLAVLNTHFPGTQSIPLPDYGLTLHRISGYLARWALYLIRTNP-ESDIALV
ITA LVFLTLGGVNLAVLNTHFPGTQSIPLPDYGLTLHRISGYLTRWALYLIKTNP-ESDIALV
VIN LVFLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRISGYFSRWALYLIATNP-ESDIALV
APE LVLLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLARWALYLIYIIP-ESDIDLV
MAR LVLLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLSRWALYLIYIIP-ESEIDLV
NEP LVSLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLARWALYLICPIP-DSDIDLV
Gum LVSLTLGGVNLAVLNTHFPGTQSIPLPDYGLTIHRLSGYLARWALYLICPSP-DSSIGLV
MAD LVLLTLGGVNLAVLNTHFPGNQSIPLPDYGLTIHRVSGYLARWALYLIAPSP-YSDRDIV
AIN SVDLTFGGIKFTLVNNHFPQYTANPVPDTALTLHRLSGYLAKWVADQCKTN--QIKLAEA
PEA SVDLTFGGIKFTLVNNHFPQYTANPVPDTALTLHRLSGYLAKWVADQCKTN--QIKLAEA
SHU SVDLTFGGIKFTLVNNHFPQYTANPVPDTALTLHRLSGYLAKWVADQCKTN--QIKLAEA
KAI SVDLTFGGIKFTLVNNHFPQYTANPVPDTALTLHRLSGYLARWVADQCKTN--QIKLAEA
YAB SVDLTFAGVKFTVVNNHFPQYTANTVSDTAFTLHRISGYLARWVAEQCKGN--QIKLAEA
AKA SVDLTFAGVKFTVVNNHFPQYTANPVSDTAFTLHRISGYLARWIAEQCKAN--QIKFAEA
SAB SVDLTFGGVKFTVVNNHFPQYIANPVPDTALTLHRMSGYLARWVAELCRGN--QIKLAEA
SIM NVDLTFGGVKFTLVNNNFPQYTANPVPDNALTLHRLSGYLARWTAEQVKNN--QVKLAEA
SAT SVDLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWVADTCKAS--VLKLAEA
SHA SVDLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWIADTCKAS--VLKLAEA
DOU SVDLTFGGVKFTVVNNHFPQYVSNPVPDNAITLHRMSGYLARWVADTCKAS--VLKLAEA
BUT NVNLTFGQSVFPVVNNHFPQFQSNPVPDNGLTLHRLSGYLARWLMDQIXVGG-SVREAEI
Fac TVDITFGGVVFPVVNNHYPEYQRNPVPDDGLTLHRLSGYLARWLIDQCNAS--PVRMTEI
ING TVNLTFGQCTFPVVNNHYPQFQSNPVDDSALTLHRLSGYLARWVMTFYAQS--PVNQAAV
MER TVNLTFGQCTFPVVNNHFPQFQSNPVADTALTLHRLSGYLARWLMTFYSQS--PVNQAAV
ORO MVDLTFGGVQFAMVNNHFPQFQSNPVPDNGLTLHRLSGYLARWAFTQMRSP---IKQAEF
CV  EVTLNLGGWKVTVFNTNFPGNRNSPVPDDGLTLHRLSGYLARYLLEKILKVS-DPEKVII
MAG EVTLNLGGWKVAVFNTNFPGNRNSPVPDDGLTLHRLSGFLARYLLEKILKVS-DPEKLII
NOR EVTLNLGGWKVTVFNTNFPGNRNSPVPDDGLTLHRLSGFLARYLLEKILKVS-EPEKLII
BAT EVTLNLGGWKVTVFNTNFPGNRNSPVPDDGLTLHRLSGFLARYLLEKILKVS-DPEKLII
BUN EVTLNLGGWKITVYNTNFPGNRNNPVPDDGLTLHRLSGFLARYLLEKMLKVS-EPEKLII
MD  EVTLNLGGWKVAVFNTNFPGNRNSPVPDDGLTLHRLSGFLARYLLEKILKVS-EPEKLLI
GER EVTLNLGGWKVPVLNTNFPGNRNNAVPDYGLTFHRISGYLARYLLGKYLAET-EPEKLIM
KAI TITLNFGGWKIPVVNTHFLENRNMSVPDDGLTLHRVSGYLARYLLDRVYSAG-EPEKLKI
GUA SVVLNFGGWRIPVVNTHFPGNRNSPVLDDSFTLHRVSGYLARYLLERYLTVS-APEQAII
SSH KANPKFGEWQVEVVNNHFPGNRNNPINSDDLTIHRLSGYLARWVLEQYKENEDESRRELI
LAC KANPKFGEWQVEVINNHFPGNRNNPIGNNDLTIHRLSGYLARWVLDQYNENDDESQHELI
CAL KANPKFGEWQVEVVNNHFPANRNNPIGNNDLTIHRISGYLARWVLEQYKENEDESQRELV
TAH KASPKFGEWQVEVVNYHFPGNRNNPIDNNDLTIHRLYGYLARWVLEQFKENEDAAQRELI
SA  KANPKFGEWQVEIVNNHFPGNRNNPIDNLDLTIHRLSGYLARWVLEQFIENEDESQRELI
JS  KATPKFGEWQVEIINNHFPGNRNNPIGNNDLTIHRLSGYLARWVLEHFTADDDESQRELI
Jam KATPKFGEWQVEIVNNHFSGNRNNPIGNNDLTIHRLSGYLARWVLEHFNSDDDESQRELI
INK KATPKFGEWQVEIVNNHFPGNRNNPIGNNDLTIHRLSGYLARWVLEHFTEDDDESQRELI
SR  KANPKFGEWQVEIVNNHFPGNRNNPIGNNDLTIHRLSGYLARWVLEHFTEDEDESQRELI
SDN KATPKFGDWQVEIVNNHFPGNRNNPIGNNDLTIHRISGYLARWTLEHFREGGDEAQKELI
KEY KATPKFGEWQVEIVNNHFPGNRNNPIGNNDLTLHRISGYLARWVLEHFGEGEDESQKELI
MEL KATPKFGDWQVEIVNNHFPGNRNNPIGNNDLTIHRLSGYLARWVLDLFKENEDESQKELI
TRI KVSIKFGEWSVEVVNNHFPGNRNNPIGNNDLTIHRISGYLARWVLEEFKGQDDEAQKDII

Con ------G-------N--FP-----P-----LT-HR-SGYLAR-----------------
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     119                                                                 178
CAR RSTFIVPLAEENGITWSDGVGMYLAFLPGAEMFMSTFTFYPLTIGMYRVRRWSMDPNCLE
ORI RSTFIVPLAEENGITWSDGVEMYLAFLPGAEMFMSTFTFYPLTIGMYRVRRWSMDPNCLE
MUR RSTFIVPLAEENGITWSDGVGMYLAFLPGAEMFMSTFTFYPLTIGMYRVRRWSMDPNCLE
RES RSTFIVPLAEENGITWSDGVGMYLAFLPGAEMFMSTFAFYPLTIGMYRVRRWSMDPNCLE
BRU RSTFVVPLAEENGITWRDGVEMYLAFLPGAEMFMSTFEFYPLTIGMYRVRRWSMDPNCLE
OSS RSTFVVPLAEENGITWRDGVEMYLAFLPGAEMFMSTFEFYPLTIGMYRVRRWSMDPNCLE
ITA RSTFVVPLAEENGITWRDGVEMYLAFLPGAEMFMSTFEFYPLTIGMYRVRRWSMDPNCLE
VIN RSTFVVPLAEENGITWRDGVEMYLAFLPGAEMFMSTFEFYPLTIGMYRVRRWSMDPNCLE
APE RTTFIVPLAEENGITWTDGVAMYLAFLPGAEMFMSTFKFFPLTIGMYRVRRWSMDPNCLE
MAR RTTFIVPLAEENGITWTDGVAMYLAFLPGAEMFMSTFKFFPLTIGMYRVRRWSMDPNCLE
NEP RTTFIVPLAEENGITWTDGVAMYLAFLPGAEMFMSTFTFFPLTIGMYRVRRWSMDPNCLE
Gum RATFVVPLAEENGITWCDGVAMYLAFLPGAEMFMSTFAFFPLTIGMYRVRRWTMDPQCLE
MAD RTTFIVPIAEKNGITWCDGVTMYLAFLPGSEMFMSTFRFFPLTIGMYKVQRWGMDPKCLE
AIN MEKIVMPLAEVKGCTWTEGLTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
PEA MEKIVMPLAEVKGCTWTEGLTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
SHU MEKIVMPLAEVKGCTWTEGLTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
KAI MEKIVMPLAEVKGCTWTEGLTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
YAB AATIVMPLAEIKGCTWNDGYTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
AKA AATVVMPLAEVKGCTWSDGYAMYLGFAPGAEMFLETFEFYPLVIDMHRVIKDGMDVNFMR
SAB ATLIVMPLAEVKGCTWNDGYTMYLGFAPGAEMFLETFEFYPLVIDMHRVLKDGMDVNFMR
SIM TAAIVMPLAEVKGCTWNDGYTMYLGFAPGAEMFLETFEFFPLVIDMHRVLKDGMDVNFMR
SAT SAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNFMK
SHA SAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNFMK
DOU SAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDFYPLVIEMHRVLKDNMDVNFMK
BUT RGAIVIPLAEIKGCTWNDGNAMYLAFAAGTEMFLQTFTFFPLAIEMQRVLKDGMDVNFMK
Fac RTKVIIPLAEVKGCTWNDGASMYLGFAAGAEMFLQSFTFYPLVIEMQRVLKDGMDVNFMR
ING REAVVIPLAEVKGCSWNDGPALYLGFAAGAEMFLQTFTFFPLVIEMHRVLKDGMEVNFMR
MER REAVVIPLAEVKGCSWDDGPPFYLGFASGAEMFLQTFTFFPLVIEMHRVLKDGMDVNFMR
ORO RATVVVPLAEVKGCTWNDGDAMYLGFAAGAEMFLQTFTFFPLVIEMHRVLKDGMDVNFMK
CV  KSKIINPLAEKNGITWSDGEEVYLSFFPGSEMFLGTFKFYPLAIGIYKVQRKEMEPKYLE
MAG KSKIINPLAEKNGITWADGEEVYLSFFPGSEMFLGTFKFYPLAIGIYKVQKKEMEPKYLE
NOR KSKIINPLAEKNGITWTDGEEVYLSFFPGSEMFLGTFKFYPLAIGIYKVQRKEMEPKYLE
BAT KSKIVNPLAEKNGITWADGEEVYLSFFPGSEMFLGTFRFYPLAIGIYKVQRKEMEPKYLE
BUN KSKIINPLAEKNGITWNDGEEVYLSFFPGSEMFLGTFRFYPLAIGIYKVQRKEMEPKYLE
MD  KSKIINPLAEKNGITWADGEEVYLSFFPGSEMFLGIFKFYPLAIGIYKVQRKEMEPKYLE
GER RTKIVNPLAEKNGITWESGPEVYLSFFPGAEMFLGTFRFYPLAIGIYKVQRKEMDPKFLE
KAI KTTIINPIAASHGITWDDGEEVYLSFFPGSEMYLTTFKFYPLAIGIYKVQRKLMDPKYLE
GUA RSKIINPIAASNGITWEDGPEVYLSFFPGTEMFLETFKFYPLAIGIYKVQKKMMEAKYLE
SSH KTTIINPIAESNGVRWDSGAEIYLSFFPGTEMFLETFKFYPLTIGIYRVKQGMMDPQYLK
La  RTTIINPIAESNGVGWDSGPEIYLSFFPGTEMFLETFKFYPLTIGIHRVKQGMMDPQYLK
CAL KTTVINPIAESNGIRWENGAEIYLAFFPGTEMFLETFKFYPLTIGIYRVKNGMMDSQYLK
TAH KTTVINPIAESNGIRWDNGAEIYLAFFPGTEMFLETFNFYPLTIGIYRVKQGMMDPQYLK
SA  KTTVINPIAESNGIKWENGAEIYLSFFPGTEMFLEIFKFYPLTIGIYRVKQGMMDPQYLK
JS  RSTIINPIAESNGIHWNNGPEIYLSFFPGTEMFLEIFKFYPLTIGIYRVKHGMMDPQYLK
Jam RSTIINPIAESNGIHWNNGPEIYLSFFPGTEMFLEIFKFYPLTIGIYRVKHGMMDPQYLK
INK RSTIINPIAESNGIHWNNGPEIYLSFFPGTEMFLEVFKFYPLTIGIYRVKHGMMDPQYLK
SR  RSTIINPIAESNGIHWNNGPEIYLSFFPGTEMFLEAFKFYPLTIGIYRVKHGMMDPQYLK
SDN RTTIINPIAESNGIHWANGAEIYLSFFPGTEMFLEAFKFYPLTIGIYRVKHGMMDAQYLK
KEY KSTVINPIAESNGIRWGNGVEIYLSFFPGTEMFLELFKFYPLTIGIYRVKHGMMDAQYLK
MEL QSTIINPIAESNGIHWANGVEIYLSFFPGTEMFLEAFRFYPLTIGIYRVKHGLMDPQYLK
TRI RSTIVNPIAESNGIHWDSGADAYLSFFPGTEMFLESFDFLPLAIGIYRVKNGMMDVQYLK

Con ------P-AE—G---W--G---YL-FFPG-EMF---F-F-PL-I----V----M------
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      179                                                                233
CAR   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWARTAFTSSAKEFVSRFGVRLA
ORI   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWAKTAFTSSAREFVSRFGIRLA
MUR   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWAKTAFTSSAREFVSRFGIRLA
RES   KAIWQRYMGIGADQWITKETLAILTRFEDVEKLDWAKTAFTSAAREFVSSFGIRLA
BRU   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWARTAFTSAAREFVSRFGIRLA
OSS   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWARTAFTSAAREFVSRFGIRLA
ITA   KAIWQRYMGIGADQWITKETVAILTRFEDVEKLDWARTAFTSAAREFVSRFGIRLA
VIN   KAIWQRYMGIGADQWITKETVAILTRFDAVEKLDWARTAFTSAAREFVIQFGIRLA
APE   KAIWQRYMGIGADDWITKETVTILARFEDVEKLDWARTAFTSSAREFVSQFGIKLA
MAR   KAIWQRYMGIGADDWITKETVTILARFEDVEKLDWARTAFTSSAREFVSQFGIKLA
NEP   KAIWQRYMGIGADDWITKETVTILARFEDVEKLDWARTAFTSSAREFVSQFGIKLA
Gum   KAIWQRYMGIGADEWITKETVSILARFEDVEKLDWAKTAFTSAAREFVSKFGIRLA
MAD   KAIWQRYAGIGADTWIQKEYADINARFSVVESLDWARTAFTSTAREFVAQFGIKLA
AIN   KVLRQRYGTLTAEQWMTQKIDAVRAAFNAVGQLSWAKSGFSPAARAFLAQFGINI-
PEA   KVLRQRYGTLTAEQWMTQKIDAVRAAFNAVGQLSWAKSGFSPAARTFLAQFGINI-
SHU   KVLRQRYGTLTAEQWMTQKIDAVRAAFNAVGQVNWAKIRILPAARTFLAQFGINI-
KAI   KVLRQRYGTLTAEQWMTQKIDAVRAAFNAVGQLSWAKSGFSPAARAFLAQFGINI-
YAB   KVLRQRYGQLTAEEWMTTKVDAVKAAFGSVAQISWAKSGFSPAARAFLTQFGIQI-
AKA   KVLRQRYGQLTAEEWMTSKLDAVKAAFGSVAQISWAKSGFSPAARAFLAQFGIQI-
SAB   KVLRQRYGTLTAEEWMTTKIEAVRAAFNSVAQISWAKTGFSPAARAFFANFGIQI-
SIM   KALRQRYGLLTAEQWMTQKIVEVKAAFDAVGQIAWAKSGFSPAARAFLQQFGFTG-
SAT   KVLRQRYGTMTAEEWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI-
SHA   KVLRQRYGTMTAEEWMTQKITEIKAAFNSVGQLAWAKSGFSPAARTFLQQFGINI-
DOU   KVLRQRYGTMTAEEWMTQKITEIKAAFKFVGQLAWAKSGFSPAVRTFLQEFGINI-
BUT   KVIRQRYGTLTAEQWMRHEVTAVTAAFNAGGLLPWARSGFSSMARKFLRNFGINI-
Fac   KVLRQRYGTKTAEQWMRDDIVAVKTAFEAINTISWARSGFSPAAREFLRQFGINI-
ING   KVLRQRYGALTAEQWMRQEIVAIRAAFDAVGALPWAKSGFFPFAREFLAQFGIKI-
MER   KVLRQRYGALTAEQWMRQELVAIRAAFEAVGALPWAKTGFFSAAREFLAQFGIRI-
ORO   KVLRQRYGQKTAEQWMREEIVAVRAAFEAVGTLAWARTGFSPAGRDFLRQFGIGI-
CV    KTMRQRYMGLEASTWTISKVNEVQAALTVVSGLGWKKTNVSAAAREFLAKFGISM-
MAG   KTMRQRYMGLEAATWTVSKVNEVQAALTVVSGLGWKKTNVSAAAREFLAKFGINM-
NOR   KTMRQRYMGLEASTWTISKVNEVQAALTVVSGLGWKKTNVSAAAREFLAKFGINM-
BAT   KTMRQRYMGLEASTWTVSKLNEVQSALTVVSGLGWKKTNVSSAAREFLAKFGISM-
BUN   KTMRQRYMGLEAATWTVSKLTEVQSALTVVSSLGWKKTNVSAAARDFLAKFGINM-
MD    KTMRQKYMNMDAATWTVTQVSEVQAALTVVSGLGWKKTNVSAAAREFLAKFGINM-
GER   KTMRQRYLGIDAQTWTTTKLGEVEAALKVVSGLGWKKTNVSSAAREFLSKFGIRM-
KAI   KTMRQRYMNLDASQWTQKHFSDVNSALTVVAGLGWKKANVSIAAKDFLNKFGINI-
GUA   KTMRQKYAGLDASQWTQQKYNEINAALSVVSSLGWKKANVSSAAREFLARFGISL-
SSH   KALRQRYGSLTADKWMSQKVTAIAKSLKEVEQLKWGRGGLSDTARSFLQKFGIRLP
LAC   KALRQRYGTLTADKWMSQKVAAIAKSLKDVEQLKWGKGGLSDTAKTFLQKFGIRLP
CAL   KALRQRYGSLTAEKWMSQKTGMIAKSLKEVEQLKWGRGGLSDTARTFLQKFGIKLP
TAH   KALRQRYGSLTADKWMSQKTTAIAKSLKDVEQLKWGRGGLSDTARTFLQKFGIRLP
SA    KALRQRYGSLTADKWMSQKVGAIAKSLKDIEQLKWGKGGLSDTARTFLQKFGIKLP
JS    KALRQRYGTLTAEKWMAQKTVLIAKSLKDVEQLKWGRGGLSDAARTFLIKFGVKLP
Jam   KALRQRYGTLTAEKWMAQKTVLIAKSLKDVEQLKWGRGGLSDAARTFLIKFGVKLP
INK   KALRQRYGTLTAEKWMAQKTVLIAKSLKDVEQLKWGRGGLSDAARTFLIKFGVKLP
SR    KALRQRYGTLTAEKWMAQKTVLIAKSLKDVEQLKWGRGGLSDAARTFLIKFGVKLP
SDN   KALRQRYGTLTADEWMAQKTVLITKSLKDVEQLKWGRGGLSDAARTFLSKFGVKLP
KEY   KALRQRYGTLTADKWMAQKTSMITKSLKDVEQLKWGKGGLSDTARAFLAKFGVRLP
MEL   KALRQRYGTLTADKWMAQKTTMIAKSLKDVEQLKWGKGGLSDAARTFLQKFGVRLP
TRI   KALRQRYGTMTADKWMSTKTTVIAKTLKRVESFKWGKGGLSEAARAFLSKFNVKIP

Con   K---QRY----A-W---------------V----W--------AR-F---FG----
```

Figure 3.6. Alignment of 51 orthobunyavirus N protein sequences. 102 residues were substituted randomly or deliberately in the BUNV N protein and are highlighted in red. “Con” indicates conserved amino acids.

3.5. Activity of mutant N proteins in a BUNV minigenome system

The BUNV minigenome replication system was described previously (see section 1.14). Briefly, a virus-like RNA (minigenome) containing the sequence of the reporter gene (*Renilla* lucifrase) flanked by authentic viral 5` and 3` ends, is transcribed in BSR-T7/5 cells expressing T7 polymerase. Provision of both BUNV N and L protein leads to encapsidation of the minigenome and formation of virus-like RNP. The L polymerase binds the RNP commencing transcription of the RNP and producing mRNA. If the latter is produced and translated, the reporter gene is expressed and luciferase activity can be measured (Kohl *et al*., 2004a).

3.5.1. Single amino acid substitutions

The mutant N genes were introduced to the BUNV minigenome system to measure the activity of mutant N proteins compared to wt N protein. An example of the activity of mutant N proteins is given in Figure 3.7. It seems that mutations at serogroup or globally conserved residues are more disruptive than non-conserved residues. Seventy-two mutant N proteins (Table 3.3) displayed 70 -100% of the parental N activity, although 36 of these mutations occurred at conserved amino acids. Mutant N proteins that showed 50% or less of parental N protein activity carry mutations in the N-terminal region (E20G, G66R and W68R), in the middle of N protein sequence (V85I, R94A, L104A, I118N, G131W, W134A, G137A, Y141C and M150A) and finally in the C-terminal region (F157I, Y158N, I162A, L177A, K179I, W193A, W213R and L226A), all of which are highly conserved residues in orthobunyavirus N proteins. The most disruptive mutations occurred in the middle of N protein sequence at globally conserved residues among all orthobunyavirus N proteins, R94A, I118N, W134A, Y141C, L177A, K179I and W193A. Their activity in the BUNV minigenome system was less than 15% of the wt BUNV N protein activity. In particular, mutations R94A, W134A and Y141C were almost inactive in the BUNV minigenome system, displaying 0.17, 1.9 and 1.8 % of the activity of the wt BUNV N protein respectively. These residues are totally conserved among all orthobunyavirus N proteins. Overall, mutant N proteins displayed a wide-range of activity, parental-like, moderate, or weak or very low (Table 3.4).

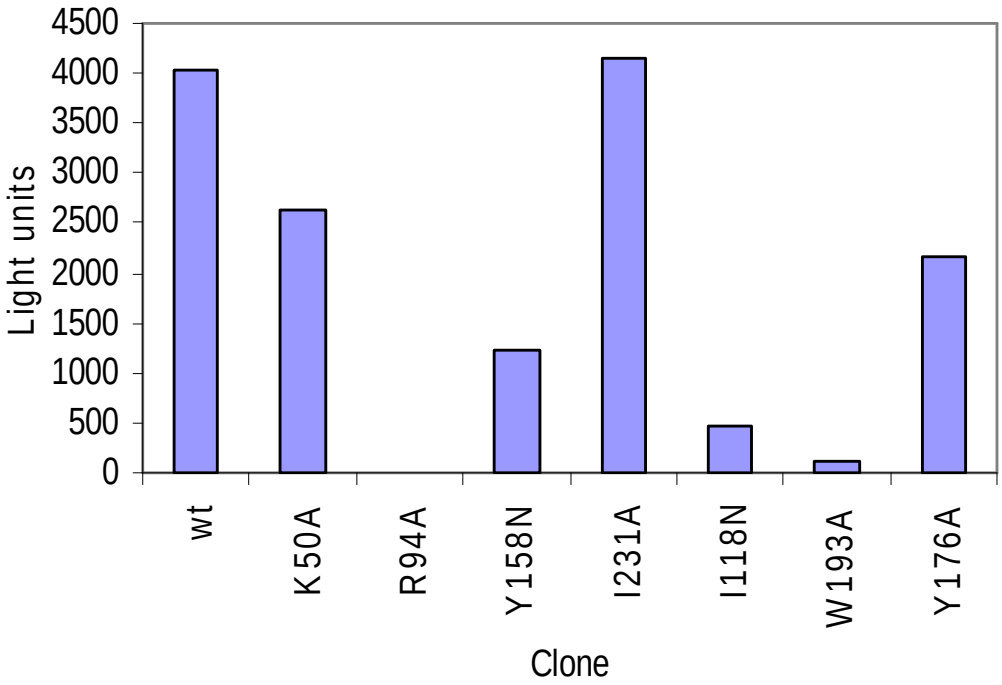


Figure 3.7. Examples of activity of mutant N proteins in the BUNV minigenome system .

BSR-T7/5 cells were transfected with 0.2 µg pT7riboBUNN or mutant clone, 0.2 µg pT7riboBUNL, 0.2 µg pT7riboBUNM*Renilla* (-) and 0.1 µg pT7-FF-luc as internal control of the trasfection efficiency. Cells were incubated overnight at 33˚C and luciferase activities were measured. The transfection was performed in duplicate and luciferase activity was normalised. For example, wt N protein produces 4042 light/units which is considered as 100% and mutant N K50A produces 2643 light/units so mutant K50A produces 2643/4043 × 100 = 65% of the wt N activity in BUNV minigenome system.

Clone	Mutation position	Nature of change	Con*	Activity (%)
L4A	95,T → C 96,T → G	Nonpolar (hydrophobic) →nonpolar (small)	++	85
E5D	100 A → T	Polar - → Polar –	-	97
F6A	101,T → G 102, T → C	Aromatic → nonpolar (small)	++++	92
D8A	108 A → C	Polar - → nonpolar (small)	++++	65
V9I	110, G → A 112, C → A	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	++++	94
A10V	114,C → T 115,T → C	Nonpolar (small) → nonpolar (hydrophobic)	-	100
T13A	122 A → G	Polar = (nucleophilic) → nonpolar (small)	-	85
P19Q	141 C → A	Polar = (hydrophobic) → polar = (amide)	++++	76
E20G	144 A → G	Polar - → nonpolar (small)	++	55
V21I	146 G→A	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	-	95
Y23A	152, T →G 153, A → C	Aromatic → nonpolar (small)	+++	88
A24V	156 C → T	Nonpolar (small) → nonpolar (hydrophobic)	-	92
K27E	164 A → G	Polar+ → polar –	-	95
R28G	167 C →G	Polar+ → nonpolar (small)	-	73
R40A	203, C →G 204, G → C	Polar+ → nonpolar (small)	++++	85
K45N	220 A →C	polar+ → polar = (amide)	-	93
K50A	233, A → G 234, A → C	polar+ → nonpolar (small)	++++	65
L53F	242 C → T	Nonpolar (hydrophobic) →aromatic	++++	90

Clone	Mutation position	Nature of change	Con*	Activity (%)
E58D	259 A → T	Polar - → polar –	++	100
W59C	262 G → T	Aromatic → polar = (nucleophilic)	-	82
E60V	263 A → T	Polar - →nonpolar (hydrophobic)	-	68
V61A	267 T → C	Nonpolar (hydrophobic) →nonpolar (small)	-	93
G66R	281 G → A	Nonpolar (small) → polar +	++++	54
G67D	285 G → A	Nonpolar (small) → polar –	-	90
W68R	287 T → C	Aromatic → polar +	+	50
K69E	290 A → G	Polar + → Polar –	-	80
I70V	293 A → G	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	-	68
I70T	293 T → C	Nonpolar (hydrophobic) → Polar = (nucleophilic)	-	100
N74S	306 A → G	Polar = (amide) → polar = (nucleophilic)	++++	99
P78H	318 C → A	Polar = (hydrophobic) → polar +	++++	81
N80S	324 A → G	Polar = (amide) → polar = (nucleophilic)	+	89
N82D	329 A → G	Polar = (amide) → Polar –	+	71
V85A	339 T → C	Nonpolar (hydrophobic) → nonpolar (small)	+	95
V85I	338 G → A	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	+	50
T91A	356 A → G	Polar = (nucleophilic) → nonpolar (small)	++++	62
H93Q	364 C → G	Polar + → polar = (amide)	++++	86

Clone	Mutation position	Nature of change	Con*	Activity (%)
R94A	365, C → G 366, G → C	Polar+ → nonpolar (small)	++++	0.17
S96G	371 A → G	Polar = (nucleophilic) → nonpolar (small)	++++	100
A100V	384 C →T	Nonpolar (small) → nonpolar (hydrophobic)	++++	100
L104A	395, C → G 396, T → C 397, T → G	Nonpolar (hydrophobic) → nonpolar (small)	++	49
E105A	399 A → C	Polar - → nonpolar (small)	+	79
M107I	406 G → A	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	-	100
L108M	407 C → A	Nonpolar (hydrophobic) → Nonpolar (hydrophobic)	-	96
K109E	410 A → G	Polar + → Polar –	-	100
V110A	414 T → C	Nonpolar (hydrophobic) → nonpolar (small)	-	97
S111N	417 G → A	Polar = (nucleophilic) → Polar = (amide)	-	92
E112A	420 A → C	Polar - → nonpolar (small)	+	58
I118N	438 T → A	Nonpolar (hydrophobic) → polar = (amide)	+	11
K121N	448 A → C	Polar + → polar = (amide)	-	98
P125H	459 C → A	Polar = (hydrophobic) → polar +	++++	66
A127G	465, C → T 466, T → A	Nonpolar (small) → Nonpolar (small)	++++	100
E128A	468 A → C	Polar - → Nonpolar (small)	++++	105
G131W	476 G → T	Nonpolar (small) → aromatic	++++	40

Clone	Mutation position	Nature of change	Con*	Activity
W134A	485, T → G 486, G → C	Aromatic → nonpolar (small)	++++	1.9
N135Y	488 A → T	polar = (amide) → aromatic	-	90
G137A	495 G → C	Nonpolar (small) → nonpolar (small)	++++	36
Y141C	507 A → G	Aromatic → polar = (nucleophilic)	++++	1.8
F145I	518 T → C	Aromatic → nonpolar (hydrophobic)	+	93
G147A	525 G → C	Nonpolar (small) → nonpolar (small)	++++	79
M150A	533, A → G 534, T → C	Nonpolar (hydrophobic) → nonpolar (small)	++++	48
F155L	550 C → A	Aromatic → Nonpolar (hydrophobic)	++++	75
F157I	554 T → A	Aromatic → nonpolar (hydrophobic)	++++	33
Y158N	557 T → A	Aromatic → polar = (amide)	++++	30
I162A	569, T → C 570, T → G	Nonpolar (hydrophobic) → nonpolar (small)	++++	60
K166R	582 A → G	Polar + → polar +	-	100
V167A	585, T → C 586, T → G	Nonpolar (hydrophobic) → nonpolar (small)	++++	79
R169G	590 C → G	polar + → Nonpolar (small)	-	78
R169H	591 G → A	polar + → polar +	-	73
M172A	599, A → G 600, T → C	Nonpolar (hydrophobic) → nonpolar (small)	++++	90
K175E	608 A → G	Polar + → Polar –	-	100
Y176A	611, T → G 612, A → C	Aromatic → nonpolar (small)	+	53

Clone	Mutation position	Nature of change	Con*	Activity (%)
L177A	613, C → G 614, T → C 615, T → G	Nonpolar (hydrophobic) → nonpolar (small)	++	10
K179I	621 A → T	Polar + → nonpolar (hydrophobic)	++++	4
T180S	623 A → T	Polar = (nucleophilic) → Polar = (nucleophilic)	-	91
R184M	636 G → T	polar + → Nonpolar (hydrophobic)	++++	100
M186L	642 A → T	Nonpolar (hydrophobic) → nonpolar (hydrophobic)	-	100
G187E	644 G → A	Nonpolar (small) → Polar –	-	105
A190G	654 C → G	Nonpolar (small) → nonpolar (small)	++++	98
A191E	657 C → A	Nonpolar (small) → Polar –	-	100
W193A	662,T → G 663, G → C	Aromatic → nonpolar (small)	++++	3
T194I	666 C → T	Polar = (nucleophilic) → nonpolar (hydrophobic)	-	61
T194A	665 A → G	Polar = (nucleophilic) → Nonpolar (small)	-	74
K197T	675 A → C	Polar + → Polar = (nucleophilic)	++	89
K197E	674 A → G	Polar + → Polar –	++	91
L198M	677 T → A	Nonpolar (hydrophobic) → Nonpolar (hydrophobic)	-	100
S203F	693 C → T	Polar = (nucleophilic) → Aromatic	-	82
A204T	695 G → A	Nonpolar (small) → Polar = (nucleophilic)	-	72
L205A	698, G → G 699, C → T	Nonpolar (hydrophobic) → nonpolar (small)	-	75

Clone	Mutation position	Nature of change	Con*	Activity (%)
T206I	701 C → T	Polar = (nucleophilic) → nonpolar (hydrophobic)	-	84
V208A	708 T → C	Nonpolar (hydrophobic) → nonpolar (small)	++++	100
S210N	714 G → A	Polar = (nucleophilic) → polar = (amide)	-	87
W213R	722 T → C	Aromatic → Polar +	++++	28
K215R	729 A → G	Polar + → polar +	-	71
T216I	732 C → T	Polar = (nucleophilic) → nonpolar (hydrophobic)	-	100
S219A	740, A → G 741, G → C 742, T →G	Polar = (nucleophilic) → nonpolar (small)	++	61
A222G	750 C → G	Nonpolar (small) → nonpolar (small)	++++	98
L226A	761, C → G 762, T → C 763, T → C	Nonpolar (hydrophobic) → nonpolar (small)	++++	26
A227V	765 C → T	polar + → Nonpolar (hydrophobic)	-	78
K228T	768 A → C	Polar + → polar = (nucleophilic)	+	80
F229L	772 C → A	**(ii) Aromatic → nonpolar (hydrophobic)**	++++	100
G230R	773 G → A	Nonpolar (small) → polar +	++++	66
I231A	775, A →G 776,T → C	Nonpolar (hydrophobic) → nonpolar (small)	+++	95

Table 3.3. Activity of mutant N proteins (carry single mutations) in a BUNV minigenome system.

Clones highlighted **in bold** were generated using Quik-change site-directed mutagenesis.
*****Con: conserved aa, (-) non-conserved aa, (+) conserved aa among at least 20 orthobunyavirus N proteins, (++) conserved aa among at least 30 orthobunyavirus N proteins, (+++) conserved aa among at least 40 orthobunyavirus N proteins, (++++) highly conserved aa among all orthobunyavirus N proteins.
%: Activity of mutant N proteins compared to 100% activity of wt BUNV N protein.

Activity (%)	**70-100 Parental-like**	**50-69 Moderate**	**15-49 Weak**	**<15 Very low**
	72 clones	13 clones	10 clones	7 clones

Table 3.4. Summary of the activity of mutant N proteins in the BUNV minigenome system.

Four levels of activity were observed (parental-like, moderate, weak and very low). The majority of mutant N proteins display parental-like activity even though some mutations occurred at highly conserved residues.

3.5.2. Double mutations

As low mutation frequency could generate 0-4.5 mutations/kb when random mutagenesis was performed, double and triple mutations were generated in the BUNV N gene. Seventeen N genes carried double mutations at different positions. Mutant N proteins with double mutations showed a wide-range of activity in the BUNV minigenome system. Although it was speculated that they might be dysfunctional in the minigenome system, as several single mutations were shown to disrupt the N protein function in the BUNV minigenome system previously, 6 double mutant N proteins were functional even though some of the mutations were at conserved residues. Four double mutant N proteins were weak, and 5 double mutant N proteins were almost inactive (Table 3.5).

By targeting the conserved residue of these inactive double mutant N proteins, I was able to explore whether the disruption was due to mutation at this residue or not. For example, mutant N protein R94S/K109N, carries mutations in the middle of N protein sequence. Mutant N protein K109E was generated previously and its activity in the minigenome system was similar to parental N protein (Table 3.3). Therefore, Arginine 94 was targeted and substituted to Alanine. It was dysfunctional in the BUNV minigenome which reflects the importance of the R94 residue. Similarly, mutant N protein E105G/A204E was also inactive in the BUNV minigenome system. As mutant N protein A204T was functional in the BUNV minigenome system, residue E105 was mutated to Alanine to generate mutant N protein E105A. Its activity in the BUNV minigenome system was almost like the parental, a result that was totally unexpected. Not all N genes carrying double mutations and inactive in BUNV minigenome were investigated in the same way since some genes were already generated carrying single mutations and the activity of mutant N proteins was weak or inactive.

3.5.3. Triple mutations

For the same reason mentioned above, the generation of mutant N protein with triple mutations was expected. Two mutant N proteins carrying triple mutations were produced and they were inactive in BUNV minigenome (Table 3.6). Mutant N protein

Clone	Mutation position	Section 2.02 Nature of change	Con	Activity (%)
I2N/H93R	89, T → A	Nonpolar (hydrophobic)→ polar = (amide)	-	15
	363, A → G	Polar + → Polar +	++++	
A11P/S15N	116, G → C	Nonpolar (small) → Polar = (hydrophobic)	-	60
	118, T → C		-	
	130, G → A	Polar = (nucleophilic) → polar = (amide)		
A22T/K197R	149, G → A	Nonpolar (small) → polar = (nucleophilic)	-	35
	275, A → G	Polar + → Polar +	++	
E58D/N82S	259, A → T	Polar - → Polar -	++	91
	330, A → G	polar (amide) → Polar = (nucleophilic)	+	
T62A/P125L	269, A → G	polar = (nucleophilic) → Nonpolar (small)	-	98
	459, C → T	Polar = (hydrophobic) → Nonpolar (hydrophobic)	++++	
N74I/P113S	306, A → T	Polar = (amide)→ Nonpolar (hydrophobic)	++++	25
	422, C → T	Polar = (hydrophobic) →Polar = (nucleophilic)	-	
V85A/L177I	339, T → C	Nonpolar (hydrophobic) → Nonpolar (small)	+	50
	614, C → A	Nonpolar (hydrophobic)→Nonpolar (hydrophobic)	++	
T206S/G212V	701, A → T	polar = (nucleophilic) → Polar = (nucleophilic)	-	0.11
	720, G → T	Nonpolar (small)→ Nonpolar (hydrophobic)	-	
E128K/L226R	467, G → A	Polar -→ Polar +	++++	0.05
	762, T → G	Nonpolar (hydrophobic) → Polar +	++++	

Clone	Mutation position	Section 2.03 Nature of change	Con	Activity (%)
D87E/K121N	346, C → A	Polar - → Polar -	-	39
	448, A → T	Polar + → polar = (amide)	-	
L92I/V208A	359, C → A	Nonpolar (hydrophobic) →Nonpolar (hydrophobic)	-	95
	708, T → C	Nonpolar (hydrophobic) → Nonpolar (small)	++++	
R94S/K109N	365, C → A	Polar + → Polar = (nucleophilic)	++++	0.09
	413, A → T	Polar + → polar = (amide)	-	
E105G/A204E	398, A → G	Polar - → Nonpolar (small)	+	0.35
	695, C → A	Nonpolar (small) → Polar -	-	
N67I/L99H	312, A → T	Polar = (amide) → Nonpolar (hydrophobic)	-	100
	381, T → A	Nonpolar (hydrophobic) → Polar +	++++	
G79S/L188V	320, G → A	Nonpolar (small) → Polar = (nucleophilic)	++	97
	647, C → G	Nonpolar (hydrophobic)→Nonpolar (hydrophobic)	+	
V21D/K166E	147, T → A	Nonpolar (hydrophobic) → Polar -	-	90
	582, A → G	Polar + → Polar -	-	
E178G/A222T	618, A → G	Polar - → Nonpolar (small)	-	0.05
	749, G → A	Nonpolar (small) → polar = (nucleophilic)	++++	

Table 3.5. Activity of mutant N proteins (carry double mutations) in the BUNV minigenome system.

Clone	Mutation position	Nature of change	Con	Activity (%)
A10T/K27M/ L226P	113 , G → A	Nonpolar (small) → polar = (nucleophilic)	-	0.07
	115, T → C	Polar + → Nonpolar (hydrophobic)	-	
	164, A →T		++++	
	762, T → C	Nonpolar (hydrophobic) → Polar = (hydrophobic)		
A24T/E105V /P113S	154, G → A	Nonpolar (small) → polar = (nucleophilic)	-	0.05
	398, A → T	Polar - → Nonpolar (hydrophobic)	+	
	422, C → T	Polar = (hydrophobic) → Polar = (nucleophilic)	-	

Table 3.6. Activity of mutant N proteins (carry triple mutations) in the BUNV minigenome system.

A10T/K27M/L226P carries two mutations at non-conserved residues in the N-terminal region and a third mutation at a globally conserved residue in the C-terminal region of BUNV N protein. All the three mutations above were already generated as single substitutions in BUNV N protein. Mutant N proteins A10V and K27E were functional in BUNV minigenome system, displaying 100% and 95% activity respectively while mutant N protein L226A shows 26% of the wt BUNV N protein activity (Table 3.3). Mutant N protein A24T/E105V/P113S contains a mutation in the N-terminal region at a non-conserved residue and two mutations at conserved and non-conserved residues in the middle of BUNV N protein. Both residues A24 and E105 were obtained as single mutations and mutant N proteins carrying these mutations were active in BUNV minigenome displaying 92% and 79% of the wt BUNV N protein activity respectively (Table 3.3).

3.6. Effect of changing the amount of transfected DNA in the activity and expression of N protein in BUNV minigenome system

Mutant N protein Y141C was selected as an example of an inactive mutant N protein in BUNV minigenome assay, displaying only 1.8% of the activity of wt N protein. To investigate whether increasing the amount of transfected DNA would increase its activity, BSR-T7/5 cells were transfected with different amounts (0.2, 0.4, 0.6, 0.8 and 1.0µg) of pT7riboBUNN or mutant pT7riboBUNN (Y141C). The amount of pT7riboBUNL (0.2µg), pT7riboBUNM*Renilla* (-) (0.2µg) and pT7-FF-lucifrase (internal control, 0.1µg) were fixed in the experiment. Cell lysates were used in both the luciferase assay and western blot. The activity of wt N protein was not affected by altering the amount of DNA (Figure 3.8). Moreover, the levels of BUNV N protein expressed in the BUNV minigenome system seemed roughly equivalent despite the use of different amounts of pT7riboBUNN (Figure 3.10A). It seems that using high amounts of pT7riboBUNN does not increase the activity or expression of wt BUNV N protein in the BUNV minigenome system. On the other hand, the activity of mutant N protein Y141C was significantly increased by increasing the amount of transfected DNA. The highest activity was obtained when 1.0 µg of the DNA was used, displaying a 5-fold increase in activity compared to 0.2 µg DNA (Figure 3.9).

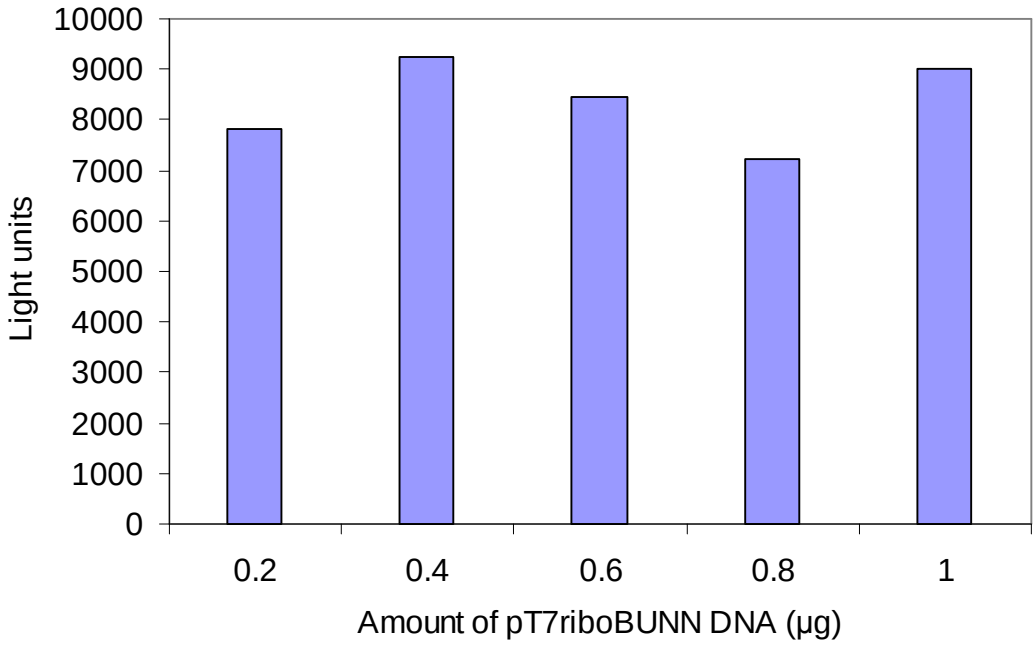


Figure 3.8. Affect of changing the amount of pT7riboBUNN plasmid in the activity of wt BUNV N protein in the BUNV minigenome system.

BSRT7/5 cells were transfected with 0.2 µg pT7riboBUNL, 0.2 µg pT7riboBUNM*Renilla* (-) and various amounts of pT7riboBUNN (0.2, 0.4, 0.6, 0.8 and 1.0 µg). The highest activity was obtained when 0.4 µg of pT7riboBUNN was used. In general, there were not considerable differences in the activity of N protein.

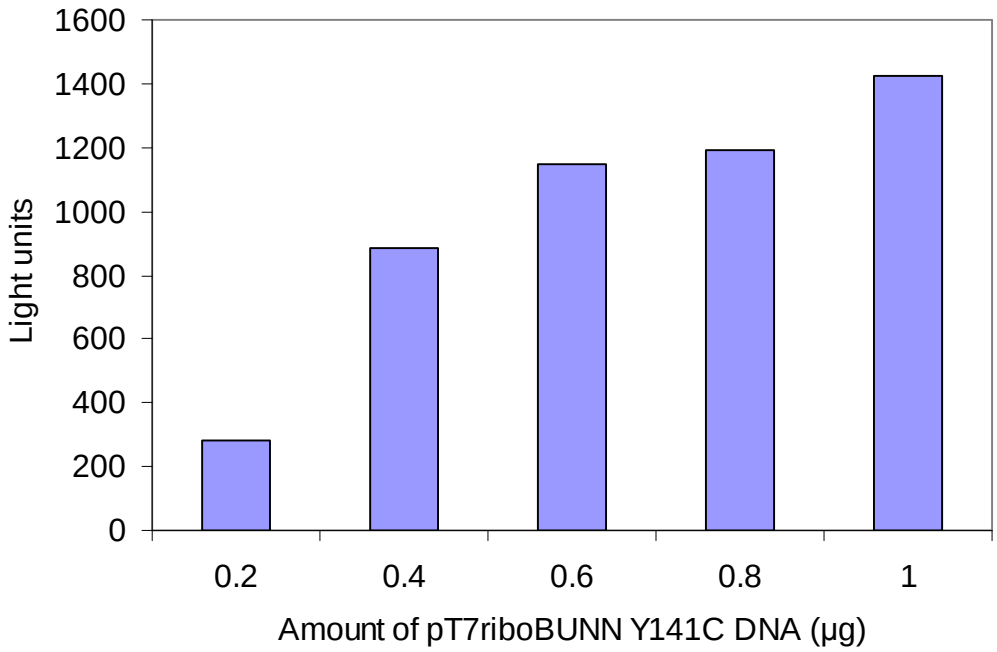


Figure 3.9. Affect of changing the amount of mutant pT7riboBUNN Y141C plasmid in the activity of mutant Y141C N protein in the BUNV minigenome system.

BSR-T7/5 cells were transfected with 0.2 µg pT7riboBUNL, 0.2 µg pT7riboBUNM*Renilla* (-) and various amounts of mutant pT7riboBUNN Y141C (0.2, 0.4, 0.6, 0.8 and 1.0 µg). The activity of mutant Y141C N protein increased dramatically as the amount of DNA increased. The highest activity was observed when 1.0 µg of DNA was used, displaying greater than 3-fold increase compared to 0.2 µg.

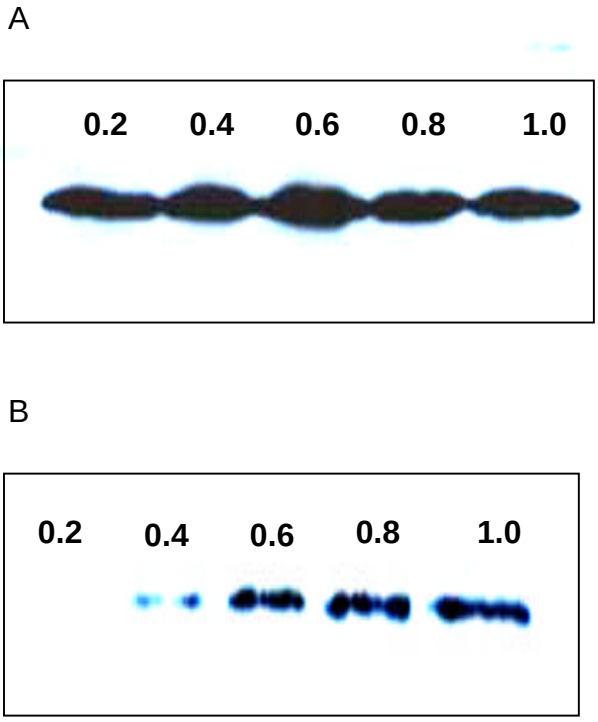


Figure 3.10. Western blot of wt BUN N protein and mutant Y141C N protein using the minigenome cell lysate.

BSRT7/5 cells were transfected with 0.2 µg of pT7riboBUNL, 0.2 µg of pT7riboBUNMRenilla (-) and various amounts (0.2, 0.4, 0.6, 0.8 and 1.0 µg) of pT7riboBUNN or pT7riboBUNN Y141C. It seems that using high amount of pT7riboBUNN did not increase levels of expression (A).The mutant Y141C N protein was not detected when 0.2 µg of DNA was used. However, the levels of expression increased gradually as the amount of DNA increased (B).

No Y141C N protein was detected when 0.2 µg of mutant plasmid was used (Figure 3.10B). However, the total amount of mutant Y141C N protein was increased when the amount of transfected DNA increased.

3.7. Discussion

Two PCR mutagenesis approaches, random and targeted, were used to mutate BUNV N gene. First, PCR random mutagenesis was performed using low mutation frequency (0 - 4.5 mutations/kb). To generate as many clones as possible carrying single mutations, a slight modification of the manufacturer's instructions was introduced, using 100ng rather 500ng of the initial target DNA, and 30 - 35 cycles rather than 20 - 25 cycles. The only explanation why the low mutation frequency did not work efficiently when the manufacturer's protocol was used, is the GenMorph kit was designed to mutate fragments bigger than 1kb while the N target is about 700 bases.

In total, 328 clones were sent for sequencing. Of these, 72 clones carried single mutations, 17 clones contained double mutations and 2 clones carried triple mutations in BUNV N protein. The efficiency of this method to generate single mutations is 79% when the number of double and triple mutations are taken into account. However, if the total number of clones (328) sent for sequencing is considered, the efficiency is 22% for clones carrying single mutations, 5% for clones carrying double mutations, and 0.6% for clones containing triple mutations. Two-hundred and thirty-seven clones (74%) had the same sequence as input parental clone. Eighteen globally conserved residues between 51 orthobunyavirus N proteins were substituted randomly. The remaining single mutations (10) occurred at BUNV-specific, 26 mutations at Bunyamwera serogroup-specific residues, 18 mutations occurred at closely related orthobunyaviruses N protein residues from two serogroups (Bunyamwera and California).

To produce adequate mutational coverage along the BUNV N protein, 30 further residues were targeted using PCR site-directed mutagenesis. Twenty-two residues are conserved globally among 51 orthobunyavirus N proteins and 8 residues are conserved between at least 20 orthobunyavirus N proteins. During both types of

mutagenesis, 102 single mutations were generated in BUNV N protein covering the whole region. Of these, 40 globally conserved amino acids were substituted and this gives a percentage of 68% of global conserved amino acids of orthobunyavirus N proteins.

A BUNV minigenome system was exploited by introducing all mutant N genes to test the activity of mutant N proteins compared to the wt BUNV N protein. Four levels of activity (parental-like, moderate, weak and very low) of mutant N proteins in BUNV minigenome system were observed. Approximately 70% of mutant N protein behaved like the wt BUNV N protein even though some mutations occurred at globally conserved amino acids. This implies that such residues are not involved in the functionality of N protein in this assay. In other words, the mutant N proteins are able to encapsidate the BUNV minigenome, interact with the BUNV L polymerase and produce mRNAs, indicating that transcription and replication of the BUNV minigenome are competent. These data suggest that interactions of N-L, N-N and N-RNA are intact. However, it is still possible that they may play a crucial role in other steps in the virus life cycle *e.g.* entry, assembly, maturation or release.

Thirteen mutations caused moderate disruption (50-69%) of the N protein activity in the minigenome system. It is possible that they could affect primary structure (linear peptide) of the N protein. However, their activity can still be measured in the BUNV minigenome system. The possibility that such residues affect other steps of the virus cycle is higher than those functions like the parental N in the BUNV minigenome system. All mutations resulting in high disruption (greater than 50%) occurred in the middle (4 mutations) and in the C-terminus (7 mutations) of the N protein. Mutations in the middle of N protein which caused high disruption might be involved in protein folding while those in the C-terminal could be involved in multimerization of N protein (Leonard *et al*., 2006).

The most important residues were R94A, I118N, W134A, Y141C, L177A, K179I and W193A. All of these are globally conserved residues among 51 orthobunyavirus N proteins, except I118N which is conserved between 22 orthobunyavirus N proteins (Figure 3.4). Mutations I118N and L177A converted hydrophobic residues to a hydrophilic and small nonpolar amino acids respectively. This would suggest strong

disruption of its function. Mutations R94A and K179I occurred at positively charged basic amino acids, and their function could be to interact with the RNA backbone. The remaining disruptive mutations occurred at aromatic amino acids. Two residues (W134A and Y141C) are in the middle of the N protein and W193A in the C-terminal region. Alteration of the latter residues would definitely disturb protein structure since they have a benzene-like ring. Coimmunopreciptation could reveal the intactness of interaction between N and L proteins. On the other hand, cross-linking would show failure in interaction of N protein molecules themselves. If there is no evidence of a protein interaction failure, protein-RNA interaction might be examined.

Some clones carrying double mutations at conserved residues of the BUNV N protein were still functional in the BUNV minigenome system. This could be explained *via* either one mutation counteracts the impact of the other or both residues are not crucial in the functionality of N protein in this assay. It seems that BUNV N protein is very sensitive to triple mutations. Clones carrying double or triple mutations were excluded from any further investigation as this project is concerned with the function of individual residues and also more difficult to interpret.

The affect of changing the amount of DNA in the activity and expression of wt BUNV N protein and inactive mutant Y141C as an example were studied. The wt BUNV N protein displays no considerable differences in its activity and levels of expression in the BUNV minigenome system when various amount of DNA were used. On the other hand, the activity and expression of mutant N protein Y141C significantly increased when high amount of DNA were used. No mutant N Y141C protein was detected when 0.2 µg of mutant DNA was used. Its activity was 5-fold greater when 1.0 µg of the DNA was used. This is an indication of the impact of mutation at residue Y141 on the activity and level of protein expression. Also mutagenesis strategy reduced the possibility of introduction of mutations in the plasmid backbone or control sequences, so that effects on N protein stability would be due to the mutation alone.

Residue Y141 might be involved in interactions with L polymerase during transcription process. This would be investigated using coimmunopreceptation. Otherwise, mutation Y141C might affects N protein stability.

3.8. Summary

1- Using PCR random and targeted mutagenesis, 102 single substitutions were generated in the BUNV N gene.
2- Mutant N genes were introduced to the BUNV minigenome to measure the activity of mutant N proteins compared to the wt BUN N protein. They displayed a wide-range of activity, parental-like, moderate, weak or inactive.
3- Mutations R94A, W134A and Y141C caused severe disruption in the activity of N protein in the BUNV minigenome system.
4- In the BUNV minigenome system, the levels of activity and expression of BUNV N protein was not significantly affected by changing the amount of DNA. However, mutant Y141C, an example of an essentially inactive mutant N protein showed dramatic increase in the activity and level of expression when the amount of DNA increased which, reflects the impact of single mutation in BUNV N protein functionality in this assay.

Chapter 4. Rescue of recombinant viruses carrying mutations in the N gene

4.1. Introduction

As mentioned previously, the BUNV S segment encodes the nucleocapsid and NSs proteins in overlapping reading frames. A mutation on the BUNV S RNA could affect the either N, NSs or both genes which would make it hard to distinguish whether the phenotype of the resulting virus was due to mutation of the N or NSs ORF.

An attempt to recover a BUNV with 4 segments was carried out previously (Bridgen *et al.*, 2001). The purpose of that study was to rescue a recombinant BUNV which encodes the N and NSs proteins from separate segments which offers the opportunity to manipulate each gene individually. Two S-like segments were constructed from pT7riboBUNS plasmid. To produce a plasmid which encodes only the N protein (pT7riboBUNN), five silent point mutations were generated in pT7riboBUNS to abrogate NSs protein expression. The first two start codons in NSs ORF were both mutated to ACG, codon 3 was substituted from TCG (Ser) to TAG (stop codon), and codons 4 and 5 were mutated from Leucine to Proline codons. The second plasmid (pT7riboBUNNss) was constructed to encode only the NSs protein ORF by introducing a single point mutation in the N protein initiation codon to be converted to stop codon and 377 nucleotides were deleted at the 3' end of the N ORF. Hence, pT7riboBUNN expresses full length BUNV S segment (961bases) while pT7riboBUNNSs encodes a smaller S-like RNA of 584 bases. However, only a 3 segment virus was recovered designated BUNdelNSs, indicating that NSs was not essential for growth in tissue culture.

As it is possible to use only pT7ribo constructs in BUNV rescue system without any support plasmids (Lowen *et al*., 2005), BSR-T7/5 cells grown in 60 mm-diameter dishes were transfected with 1µg pT7riboBUNL, 1µg pT7riboBUNM and 1µg pT7riboBUNN using lipofectamine-2000 transfection reagent. Cells were incubated for 4 - 5 hours at 37ºC and the transfection mixture removed and replaced with 4 ml fresh medium. Cells were incubated for 5 days at 33ºC. Cells were harvested and the

supernatant was used in a plaque assay on Vero-E6 for isolation of recombinant virus. The BUNdelNSs virus was then propagated on BHK-21 cells and titered.

4.2. Affect of mutations at conserved or non-conserved residues of BUNV N protein in rescue of recombinant mutant viruses

In the previous chapter I described the generation of 102 single mutations in BUNV N gene. The first step was to study the impact of such mutations in the N protein by measuring their activity in the BUNV minigenome system. However, limited information can be obtained from this assay as it can only mimic virus transcription and replication. To produce a better understanding of the impact of mutations in the N protein functionality, it would be more instructive to attempt to rescue viable viruses carrying mutations in the N gene. The activity of N protein in the BUNV minigenome system was shown to be severely affected by mutations at highly conserved residues while mutations at nonconserved residues had no affect in the functionality of N protein in this assay.

To investigate this observation in the BUNV rescue system, five mutant N genes (E5D, AI0V, G67D, N135Y and A227V) carrying single mutations at nonconserved residues in parallel with 5 mutant N genes (F6, E20G, G66R, W134A and K228T) carrying single mutations at well-conserved amino acids in orthobunyavirus N proteins were first introduced to BUNV rescue system to explore whether mutations at conserved or non conserved residues could affect virus recovery. Several preliminary data supported focusing on mutations at conserved residues (Table 4.1). Mutant viruses carrying mutations at nonconserved residues were recovered from the first attempt like the parental virus while mutant viruses that contain mutations at conserved residues were rescued after at least two attempts. Parental plaque size (large) was produced by mutant viruses carrying mutations at non-conserved residues and small plaque size was shown by mutant viruses carrying mutations at conserved residues. Recovery of mutant viruses with mutations at nonconserved residues took 4-5 days, similar to the parental virus, while mutant viruses containing mutations at conserved residues took more than 5 days. The recovery of viruses carrying mutations at nonconserved residues was between 10^5-10^6 pfu/ml, while

Clone	Attempt	Rescuable	Incubation period (days)	Plaque size	Recovery pfu/ml
Parental	1	+	5	large	10^7
E5D	1	+	5	large	10^5
F6A	2	+	8	small	10^2
A10V	1	+	5	medium	10^6
E20G	3	+	7	medium	10^2
G66R	3	+	8	small	10^0
G67D	1	+	5	large	10^5
W134A	3	-	13	-	-
A227V	1	+	5	large	10^5
K228T	2	+	5	large	10^2

Table 4.1. Preliminary data of the affect mutations at conserved or non-conserved residues of BUNV N protein in recovery of viable virus.

BSR-T7/5 cells were transfected with 1.0 µg each of pT7riboBUNN or mutant, pT7riboBUNL and pT7riboBUNM using lipofectamine-2000 for 4 – 5 h at 37˚C. Transfection mixture was then removed and replaced with fresh medium. Cells were incubated at 33˚C for 4-5 days before plaque assay was performed in Vero-E6 cells.

Clones highlighted **in bold** carry mutations at conserved residues

mutant viruses with mutations at conserved residues yielded only 10^2 pfu/ml. Moreover, mutant N gene W134A, a globally conserved residue, was nonrescuable (Table 4.1). This suggests that it would be more appropriate to focus only on mutations at conserved residues in BUNV N protein. A further 59 mutant N genes carrying single mutations at conserved residues between 20 closely related viruses (Bunyamwera and California) serogroups or between 51 orthobunyavirus N proteins from 4 serogroups (Bunyamwera, California, Group C and Simbu) were introduced to BUNV rescue system.

4.3. Attempt to rescue recombinant viruses carrying single mutations in the N protein covering a wide-range of minigenome activities

Of 64 mutant N genes spanning a range of minigenome activities introduced in parallel with parental N genes as a (+ve) control into BUNV rescue system, 50 recombinant viruses were recovered and 14 mutant N genes were nonrescuable. The efficiency of BUNV rescue system to recover recombinant viruses carrying mutations in N gene is 78% if the total number of selected genes is to be considered. The parental virus and mutant V85A plaques were picked up from dilution 10^{-7}, 20 mutant viruses were purified by picking single plaques from dilution10^{-6}, 6 recombinant viruses were purified from dilution 10^{-5}, 5 mutant viruses were purified from dilution 10^{-4}, 7 mutant viruses plaques were picked from dilution 10^{-3}, single plaques of 7 recombinant viruses were picked from dilution10^{-2} and 3 mutant viruses carrying mutations at conserved residues in N-terminal region of N protein (K50A, G66R and W68R) were purified from undiluted supernatant (Table 4.2 and figure 4.1). Three individual plaques of each mutant virus were picked.

4.4. Recombinant viruses with single, double or triple mutations in N gene

To confirm the input mutations in the BUNV N gene, a single plaque of each mutant virus was grown up, RNA extracted and used in RT-PCR in order to determine the sequence of the S segment. Forty mutant viruses were found to carry the input mutations, 5 recombinant viruses (D8A, E20G, R40A, G66A and W68R) contained the input mutations and as well as second mutations (Table 4.3). Two recombinant

Virus	pfu/ml	Virus	pfu/ml	Virus	pfu/ml	Virus	pfu/ml	Virus	pfu/ml
Parental	10^7	K50A	10^0	V85I	10^6	F145I	10^6	V208A	10^3
L4A	10^2	L53 F	10^6	T91A	10^3	G147A	10^3	S219A	10^6
E5D	10^5	E58D	10^6	H93N	10^2	M150A	10^3	A222G	10^6
F6A	10^2	G66R	10^0	S96G	10^6	F155L	10^5	**A227V**	10^5
D8A	10^6	**G67D**	10^5	A100V	10^4	I162A	10^2	K228T	10^2
V9I	10^6	W68R	10^0	L104A	10^2	V167A	10^4	F229L	10^3
A10V	10^6	N74S	10^6	E105A	10^6	M172A	10^4	G230R	10^5
P19 Q	10^6	P78H	10^6	E112A	10^3	R184M	10^3		
E20G	10^2	N80S	10^6	A127G	10^5	A190G	10^4		
Y23A	10^6	N82D	10^6	E128A	10^6	K197T	10^4		
R40A	10^6	V85A	10^7	**N135Y**	10^6	L205A	10^3		

Table 4.2. Recovery of mutant bunyaviruses.

The titer of each virus presents the dilution from which single plaques were picked. (10^0) indicates single plaque was picked from nondiluted supernatant. Mutant viruses highlighted **in bold** carry mutations at nonconserved residues.

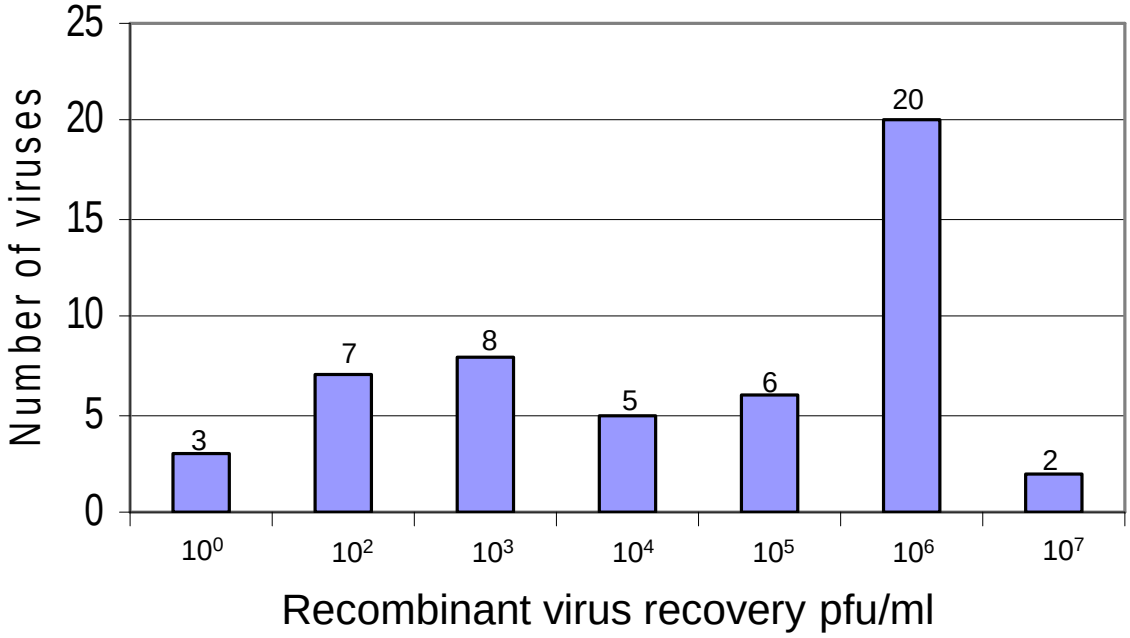


Figure 4.1. Recovery of fifty recombinant viruses.

Different serial dilutions of rescue supernatants were used in plaque assay to pick single plaques for each mutant virus. Three mutant viruses (K50A, G66R and W68R) were purified from nondiluted supernatants (10^0).

viruses (E112 and R184M) contained 2 mutations in addition to the input changes. (Table 4.4). The second mutations in the N genes of recombinant viruses D8A, E20G and W68R were at the same nonconserved residue with same substitution K109E. In the N gene of mutant E112A, two additional mutations occured in the N-terminal region at non-conserved residues K27E and T62A. Mutation R184M occurred in the C-terminus and an extra two mutations were introduced, one in the N-terminus at conserved amino acid D8G and the other in the middle at a non-conserved residue of N protein.

4.5. Revertant viruses

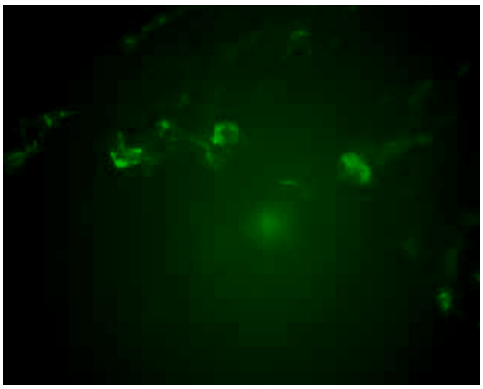
Mutant genes R94A, I118N, P125H, G131W, W134A, G137A, Y141C, F157I Y158N, Y176A, L177A, K179I, W193A, W213R, L226A and I231A contain changes at globally conserved residues in the middle and the C-terminus of the N protein. No viruses were recovered when plasmids containing these mutations were used in the rescue system. Mutant F157I is an example of a nonrescuable gene that was used in an immunofluorecent experiment to explore whether the recombinant virus had been rescued but was unable to grow. The rescue supernatants of F157I and the parental virus were used to infect BHK-21 cells. The mutant F157I N protein was detected by immunofluorecent staining, however, the signal was very weak compared to wt N protein (Figure 4.2). This result confirms the recovery of mutant F157I, however, the recombinant virus seems to be very debilitated. The rescue supernatant was used to infect BHK-21 cells in order to propagate the virus but without success. Such observations suggest that extending the incubation period would give the virus a better chance to grow. When the rescue experiment was performed again and the cells incubated for 12 days, a virus was eventually rescued. However, nucleotide sequencing showed that the recovered virus was the parental virus not F157I. All nonrescuable mutations were subjected to the same incubation period. G137A was rescued but nucleotide sequence analysis confirmed it had reverted to the parental virus sequence.

Recombinant virus	Input mutation	Second mutation
D8A	D8A	K109E
E20G	E20G	K109E
R40A	R40A	T71S
G66A	G66A	R28H
W68R	W68R	K109E

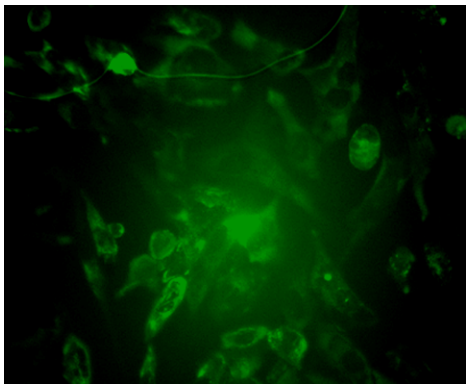
Table 4.3. Five recombinant viruses shown to carry double mutations although the input mutation was single.

Recombinant virus	Input mutation	Second mutation	Third mutation
E112A	E112A	K27E	T62A
R184M	R184M	D8G	F97S

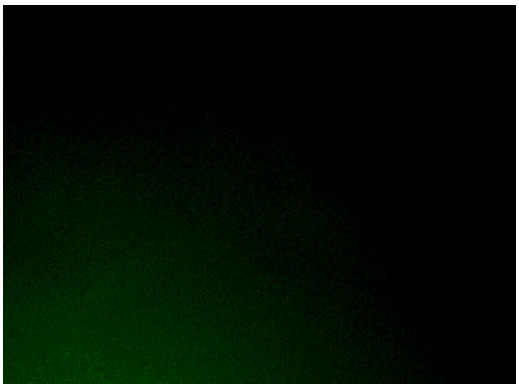
Table 4.4. Two mutant viruses carry triple mutations .



F157I



Parental virus



Mock

Figure 4.2. Immunofluorescent experiment .

BSR-T7/5 cells were transfected with 1.0 µg each of pT7riboBUNN or mutant F157I N clone, pT7riboBUNL and pT7riboBUNM using lipofectamine-2000 for 4-5 h at 37˚C. Transfection mixture was then removed and replaced with fresh medium. Cells were incubated at 33˚C for 4-5 days. Cells were harvested and the supernatants were used to infect 8×10^4 BHK-21 cells. After overnight of incubation at 33˚C immunofluorescent experiment was performed in the samples using Anti-BUNV N antibody.

4.6. New strategies to attempt to recover the nonrescuable N genes

In addition to extending the rescue incubation period, several attempts to recover the remaining nonrescuable genes were carried out. Previously, the activity and expression of mutant Y141C (an example of an inactive mutant N protein) increased dramatically in the BUNV minigenome system when higher amounts of DNA (1.0 µg) was used. This suggests that if the amount of DNA of nonrescuable genes were increased, it might help to recover the viruses. To explore these possibilities, BSR-T7/5 cells were transfected with various amounts of pT7riboBUNN or mutant clone (pT7riboBUNN Y141C) (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 µg) and fixed amount (1.0 µg) each of pT7riboBUNL and pT7riboBUNM. The recovery of the parental virus was 10^8 pfu/ml for all combinations (Table 4.5). However, mutant virus Y141C was still not recovered. The support plasmid (pTM1BUNN) which expresses wt N protein was then included in the rescue mixture to overcome the possibility of low level of expression of mutant N protein. In this experiment, BSR-T7/5 cells were transfected with 1.0µg each of pT7riboBUNN, pT7riboBUNM and pT7riboBUNL and different amounts of pTM1BUNN (0.01, 0.05, 0.1 and 0.5 µg). Massive CPE was observed but no virus was recovered.

4.7. Discussion

The BUNV rescue system provides the opportunity to generate viable viruses carrying desired mutations from cDNAs. 102 mutations were generated in BUNV N protein and their activity measured in the BUNV minigenome system. The second step was to introduce mutant N genes to the BUNV rescue system in attempt to recover viable viruses carrying mutations in BUNV N protein. It was not feasible to attempt to rescue all these mutations, so, 64 mutant N genes carrying single mutations covering the whole region of BUNV N ORF and display a wide-range of activity in BUNV minigenome were selected for further investigation. Preliminary data from the activity of mutant N proteins in minigenome system suggested high and severe disruption was caused by mutations at conserved residues in BUNV N protein while mutations at nonconserved amino acids cause less or no disruption.

pT7riboBUNN T (µg)	Yield (pfu/ml)	pT7riboBUNNY141C (µg)	Yield (pfu/ml)
0.2	3.6 ×10^8	0.2	-
0.4	1.7 ×10^8	0.4	-
0.6	3.7 ×10^8	0.6	-
0.8	2.2 ×10^8	0.8	-
1.0	1.5 ×10^8	1.0	-
1.5	4.7 ×10^8	1.5	-
2.0	4.5 ×10^8	2.0	-

Table 4.5. Affect of changing amount of pT7riboBUNN and mutant pT7riboBUNNY141C DNA in the recovery of the parental virus and mutant Y141C virus.

BSR-T7/5 cells were transfected with various amount of pT7riboBUNN DNA (0.2, 0.4, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 µg) and 1.0 µg each of pT7riboBUNL and pT7riboBUNM. From all combinations virus yields are within the same log

To explore this observation in terms of the ability to rescue mutant viruses, 5 mutant N genes carrying single mutations at nonconserved amino in parallel with 5 other mutant N genes carrying single mutations at conserved residues were introduced to BUNV rescue system (Table 4.1). Recombinant viruses carrying single mutations at nonconserved residues of N protein displayed no phenotypic differences from the parental virus while recombinant viruses with single mutations at conserved residues in the N protein displayed phenotypic differences (small plaque, low titer, nonrescuable).

It was clear that mutations at conserved amino acids of BUNV N protein affected virus recovery. Hence, all mutant N genes (59) carrying single mutations at conserved amino acid were introduced to BUNV rescue system.
Recombinant viruses were recovered at a wide-range of efficiecies (Table 4.2). This could be used as evidence of the impact of mutations in BUNV N functionality. Some viruses showed low or very poor recovery while others displayed high recovery. This variation could be explained in a number of ways, some residues are not involved in BUNV N functionality while other residues may affect some aspect of virus replication and growth. In some cases the poor recovery (rescuable N gene cDNAs) could be due to experimental conditions (e.g. DNA quality, cell viability, transfection reagent etc). For example, mutant K228T showed recovery of 10^2 pfu/ml. When mutant K228T was purified, the mutant virus grew to 10^8 pfu/ml. The S gene nucleotide sequence of virus K228T was confirmed as only carrying the desired mutation.

The majority of recovered viruses contained the input mutations and hence such mutations could be classified as tolerant mutations. However, they might be conditional lethal when growth conditions *e.g.* temperature, cell line etc are changed.
Second and third site mutations were introduced into BUNV N gene of some mutant viruses. RNA viruses have high rates of mutations due to lack of proof-reading activity of their RNA polymerases. The manipulation of the BUNV N protein might enhance the viruses to mutate further. A common feature was observed during rescue of these mutant viruses. They were rescued after 7-9 days of incubation while the parental requires a period of 5 days. The longer incubation period might give the virus a chance, first to recover, and then to overcome the debilitating mutation by introducing a second or third mutation to counteract the first mutation.

Two mutant viruses, G137A and F157I, reverted back to the parental virus S RNA sequence. Both residues are globally conserved (Figure 3.4). They are in the middle of N protein within a cluster of conserved residues. The data obtained from the immunofluorescence experiment suggests that the viruses had recovered but grew poorly. When the incubation period was extended this might have given the virus more chance to correct the input mutation culminating in the recovery of the parental virus. Six mutant genes carrying mutations in the same region of N were nonrescuable. It seems that this region therefore plays a crucial role in BUNV N protein functionality.

4.8. Summary

1- 64 mutant N genes carrying single mutations were introduced into BUNV rescue system in an attempt to recover viable viruses.
2- 50 recombinant viruses were rescued; 14 mutant N genes were nonrescuable.
3- Mutant viruses were recovered over a wide-range of efficiency.
4- In general, mutations at conserved residues severely affect virus recovery compared to mutations at nonconserved residues of N gene.
5- Revertant viruses or viruses with additional mutations were also recovered for some mutant N clones.

Chapter 5. Characterization of mutant viruses

5.1. Introduction

102 mutations were generated in the BUNV N protein randomly or specifically. The activity of mutant N proteins was measured in the BUNV minigenome system. They displayed a wide-range of activity. Sixty-four plasmids carrying single mutations (59 mutations at conserved residues and 5 at nonconserved residues) in the BUNV N protein were used in the BUNV rescue system in an attempt to recover viable viruses carrying mutations in the N protein gene. Fifty recombinant viruses were rescued; no virus was recovered from the other 14 clones. These preliminary data indicate that the recovery of recombinant mutant viruses from clones that carried mutations at conserved residues was poorest. Mutations in the N protein gene could affect the virus life cycle at any step (entry, uncoating, transcription, replication, assembly, maturation or release).

The impact of such mutations in the N protein could be observed *via* virus growth properties. It is possible that mutations in the N protein could produce an attenuated virus with very low titre. Viruses carrying mutations in the N protein might show different plaque phenotypes (small or large). Mutations in the BUNV N protein gene could also affect N protein expression, however, it is also possible that mutations in the N protein gene could affect expression of other viral genes as the N protein encapsidates all the three segments. The generation of temperature-sensitive virus, is another possibility from viruses carrying mutations in the N protein gene since the N protein is involved in virus transcription and replication. Moreover, if the N protein interacts with cellular proteins, mutations in the N protein might affect these interactions and a new phenotype could be observed. All the above possibilities were investigated through the characterisation of the mutant viruses by: virus titration and plating efficiency, plaque phenotypes, protein labelling, western blotting, temperature sensitivity and host restriction.

5.2. Titration of mutant viruses

All mutant viruses were grown under the same conditions and stock viruses were used in plaque assay on Vero-E6 cells. The mutant viruses grew to different titres (Table 5.1). The majority of recombinant viruses (16) produced about 10^7 pfu/ml, 13 mutant viruses behaved like the parental virus yielding about 10^8 pfu/ml, 7 mutant viruses showed about 10^6 pfu/ml, 8 mutant viruses displayed about 10^5 pfu/ml, 3 mutant bunyaviruses (R40A, A100V and V208A) released about 10^4 pfu/ml and 4 recombinant viruses (F6A, G147A, M150A and I162A) were debilitated and showed titres of about 10^3 pfu/ml. The latter viruses carry mutations at universally conserved amino acids, one in the N-terminus and the rest in the middle of N protein. Not all conserved residues affected virus titration since 12 mutant viruses (L53F, N74S, P78H, S96G, E105A, E112A, E128A, F145I, S219A, A222G, K228T and G230R) carrying mutations at well conserved residues grew to titres similar to that of the parental virus, about 10^7-10^8 pfu/ml. A summary of mutant virus titres is given in Figure 5.1.

5.3. Plaque phenotypes of mutant viruses

In Vero-E6 cells mutant viruses showed 3 types of plaque size: large, medium or small after an incubation of 6 days at 33˚C (Figure 5.2). The majority of recombinant viruses (22) produce parental plaque sizes (large, 4-5mm) even when mutations occurred at conserved residues. Medium size plaques (2mm) were shown by 9 mutant viruses, and 11 recombinant viruses displayed pinpoint plaque size (<1mm). Mixed plaque sizes were observed in 8 recombinant viruses. Nineteen mutant viruses showed plaque size and titers similar to the parental virus. Mutant viruses A100V and L205A produced large plaque sizes, however, their titres were 2.8×10^4 and 5.5×10^5 pfu/ml respectively. This means that they displayed a 10000 and 1000-fold reduction, respectively, in virus yield compared to the parental virus. Recombinant viruses Y23A and E128A showed pin point plaque sizes although they grew to 10^7- 10^8 pfu/ml. Mutant viruses F6A, R40A, G147A, M150A, I162A and V208A displayed pin point plaque sizes and poor titrations with reductions of 10000 or 100000-fold below the parental virus titre. Mutant viruses which displayed medium

Virus	Titre pfu/ml	Virus	Titre pfu/ml	Virus	Titre pfu/ml	Virus	Titre pfu/ml
Parental	7.4×10^8	E58D	3.5×10^8	A100V	2.8×10^4	M172A	2.0×10^6
L4A	1.0×10^7	G66R	2.0×10^7	L104A	6.0×10^5	R184M	4.5×10^5
E5D	2.5×10^7	**G67D**	9.0×10^7	E105A	6.5×10^7	A190G	2.5×10^6
F6A	4.0×10^4	W68R	3.5×10^7	E112A	1.5×10^8	K197T	1.0×10^5
D8A	8.0×10^7	N74S	4.0×10^8	A127G	2.0×10^6	L205A	5.5×10^5
V9I	1.0×10^7	P78H	3.3×10^8	E128A	1.1×10^8	V208A	1.5×10^4
A10V	6.7×10^8	N80S	2.8×10^8	**N135Y**	3.0×10^7	S219A	7.5×10^7
P19Q	1.4×10^6	N82D	1.0×10^8	F145I	2.5×10^8	A222G	4.0×10^8
E20G	5.0×10^7	V85A	1.5×10^8	G147A	2.0×10^3	**A227V**	1.5×10^7
Y23A	7.0×10^6	V85I	4.0×10^7	M150A	5.0×10^3	K228T	3.0×10^7
R40A	5.0×10^4	T91A	1.0×10^5	F155L	$2 .0 \times 10^5$	F229L	9.5×10^5
K50A	6.0×10^5	H93Q	1.5×10^6	I162A	5.0×10^3	G230R	1.8×10^7
L53 F	4.8×10^8	S96G	5.5×10^7	V167A	2.0×10^6		

Table 5.1. Titres of recombinant viruses in Vero-E6 cells.

Recovery supernatants of mutant viruses were used in plaque assay in Vero-E6 cells. After 6 days of incubation at 33˚C, cells were stained with neutral red and three single plaques for each mutant virus were picked and one single plaque was inoculated into BHK-21 cells to grow a stock. After 6 days of incubation at 33˚C, the supernatants were used in plaque assay in Vero-E6 cells to determine their titre.
Mutant viruses carry mutations at nonconserved residues are highlighted **in bold**.

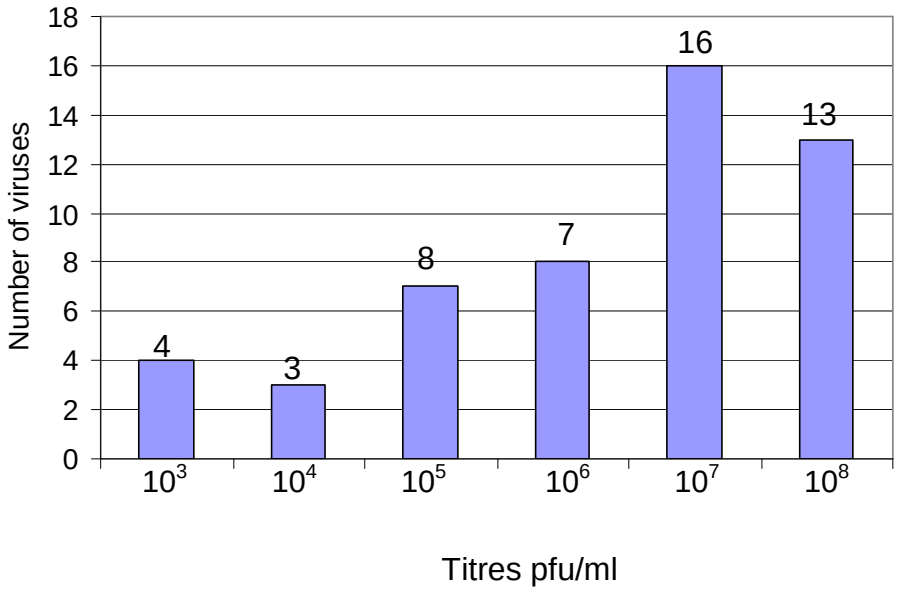
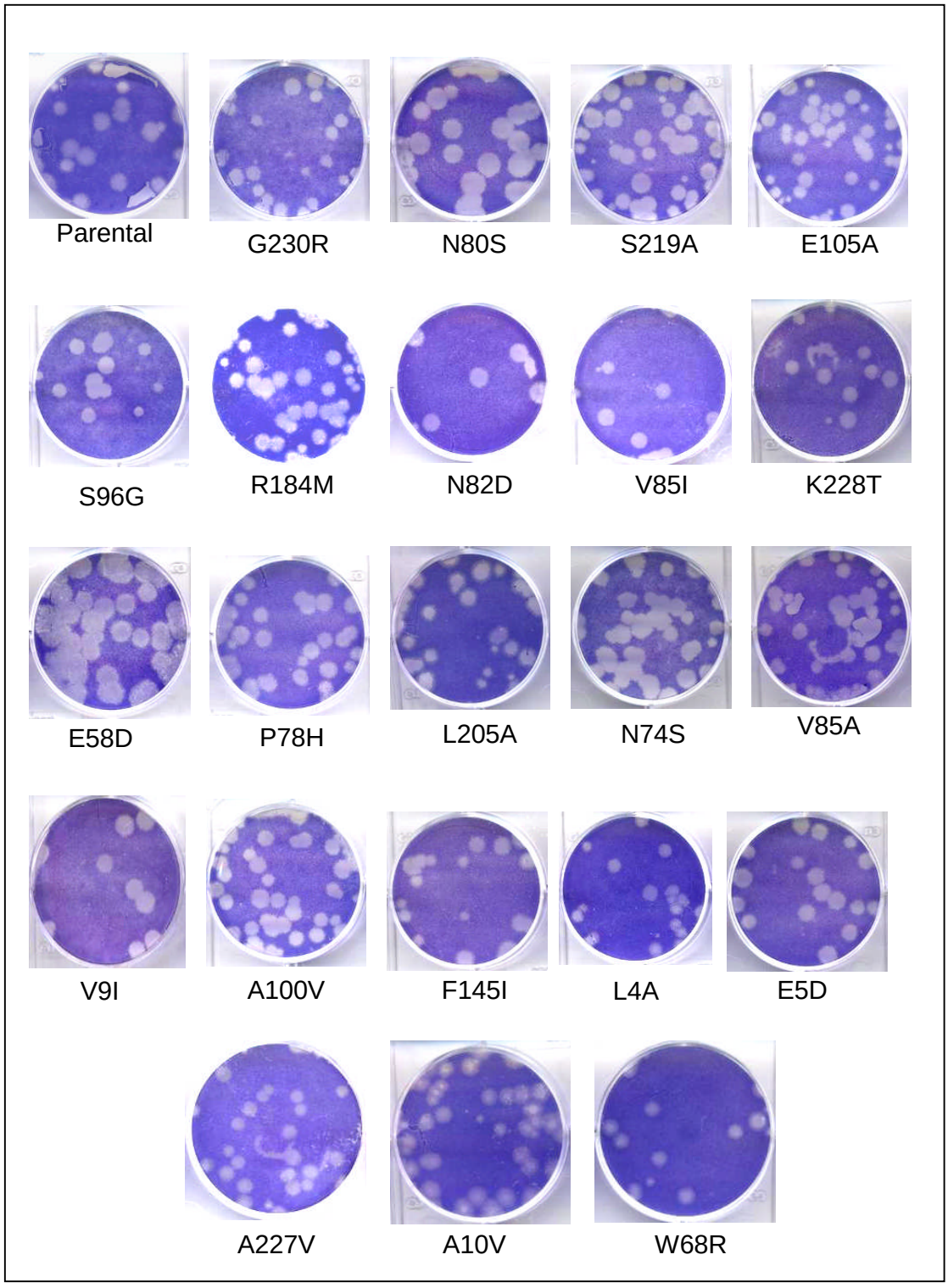
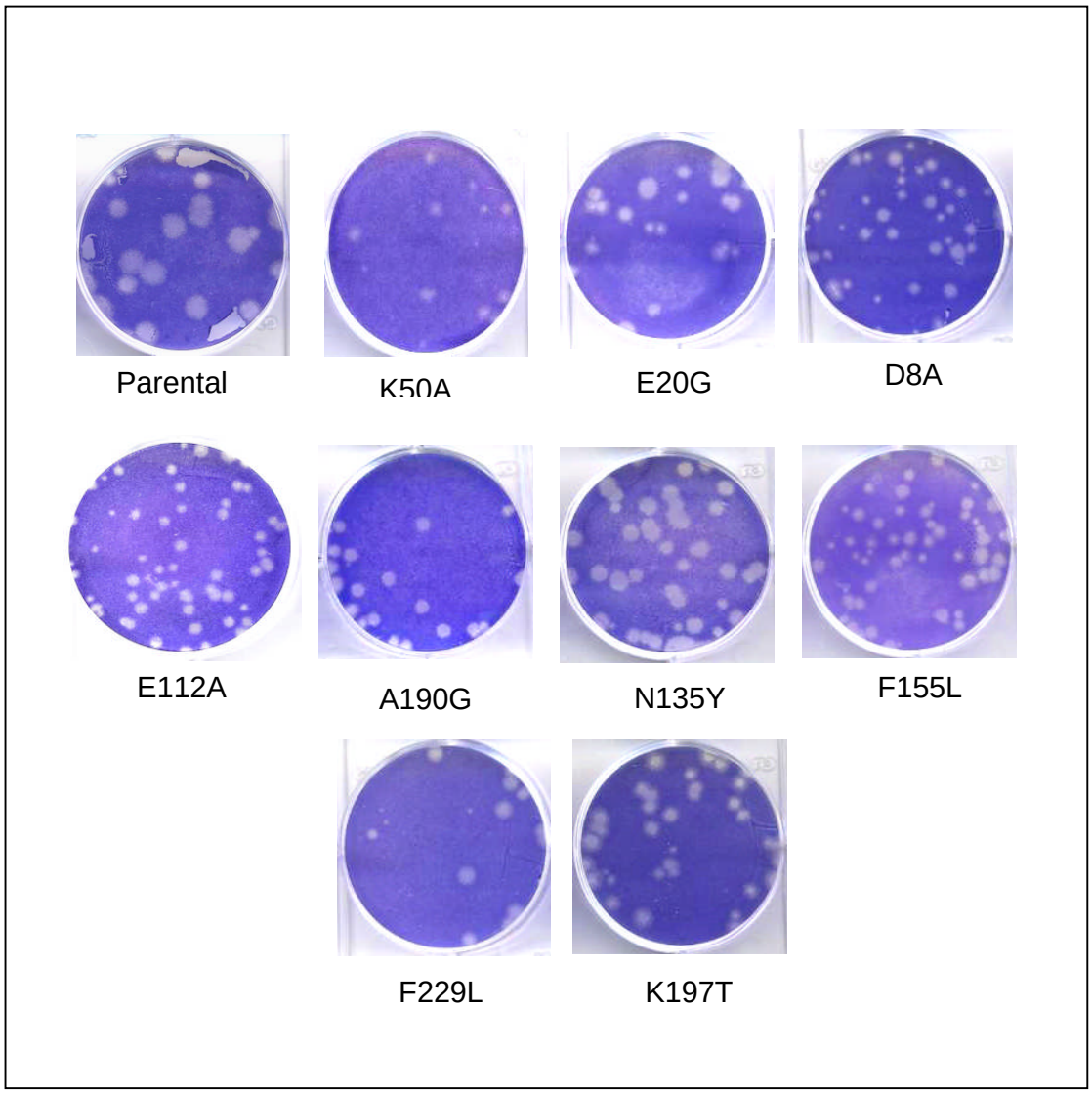


Figure 5.1. Summary of recombinant mutant virus titres.

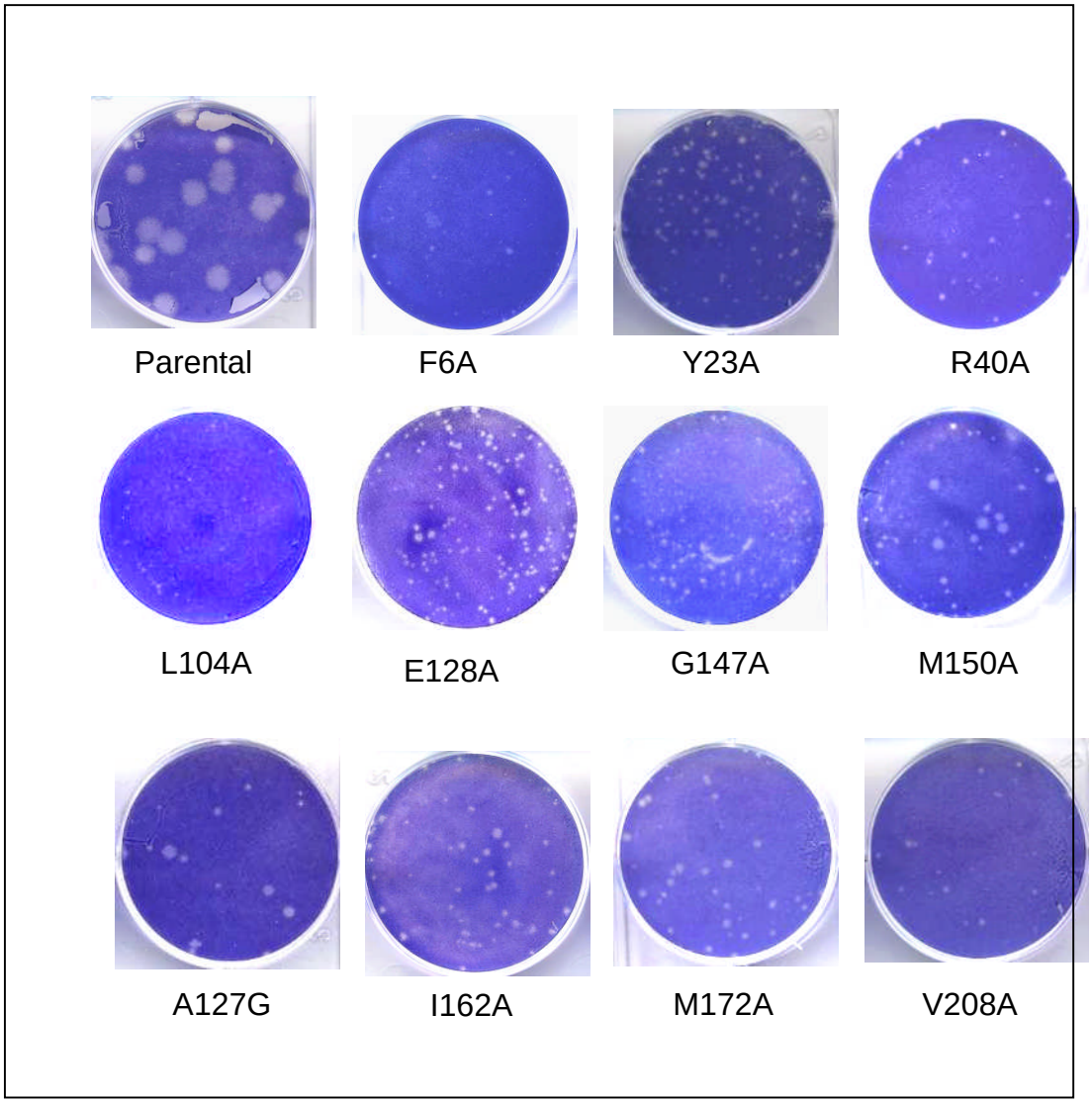
(A)


Parental
G230R
N80S
S219A
E105A
S96G
R184M
N82D
V85I
K228T
E58D
P78H
L205A
N74S
V85A
V9I
A100V
F145I
L4A
E5D
A227V
A10V
W68R

(B)



(C)


Parental
F6A
Y23A
R40A
L104A
E128A
G147A
M150A
A127G
I162A
M172A
V208A

(D)

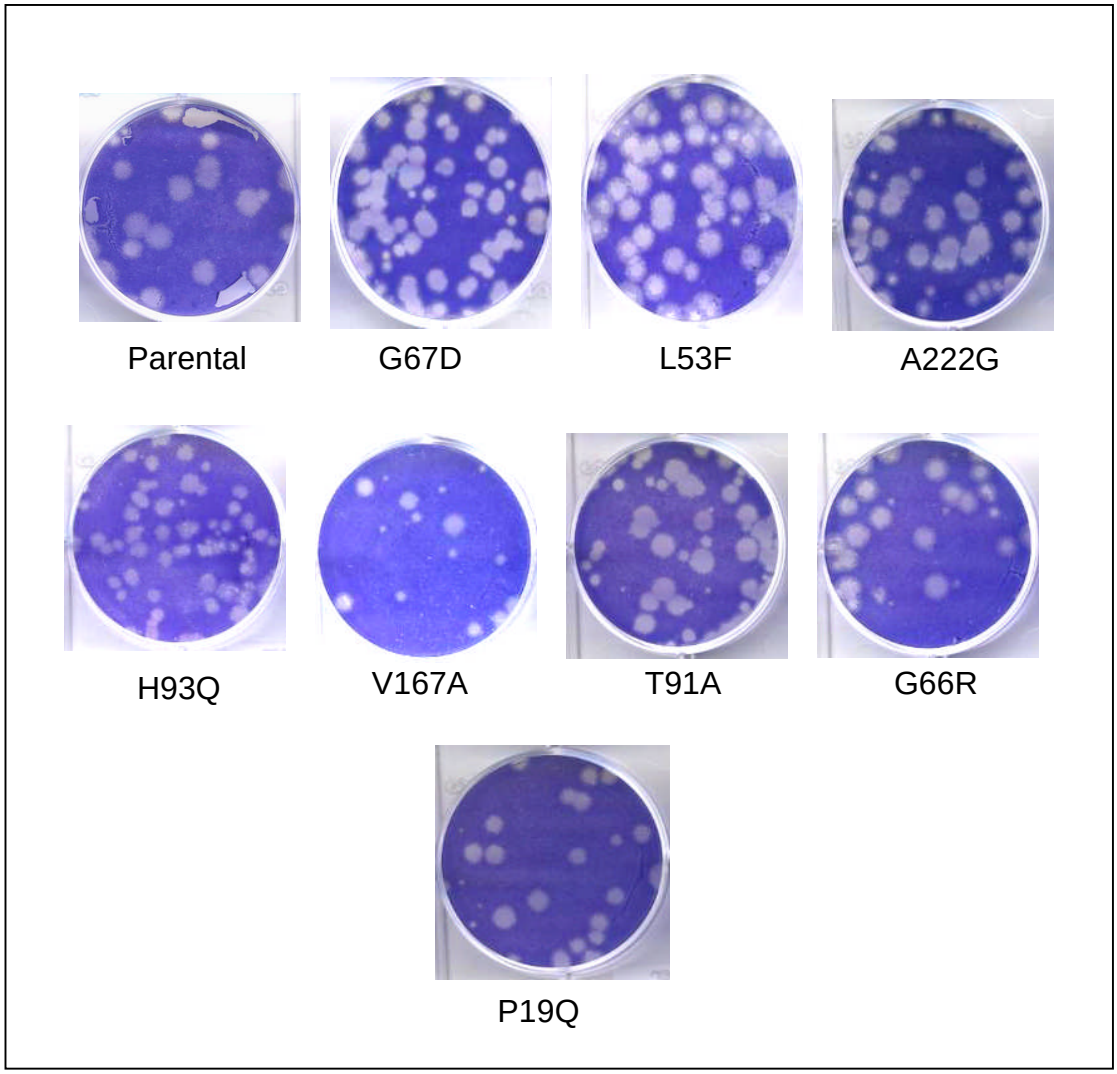


Figure 5.2. Plaque phenotypes of mutant viruses.

After mutant viruses were purified, stock viruses were used in plaque assay. Vero-E6 cells were infected with serial dilution (10^{-1} - 10^{-7}) of stock virus for 1 h at 37°C. The virus was removed and replaced with 2ml agarose overlay and incubated at 33°C for 6 days. Cells were then fixed with 5% formaldehyde for at least 2 hrs, stained with Geimsa and plaques were examined. The viruses were classified into 3 size classes, large (A), medium (B) and small (C), while some mutants produced mixed sizes of plaque (D).

plaque sizes yielded titres of 10^6 -10^8 pfu/ml except recombinant viruses K50A, F155L and F229L which produced titres of 10^5 pfu/ml, 1000-fold less than the parental virus titre. Those recombinant viruses which displayed mixed plaques produced 10^6 -10^8 pfu/ml, except mutant T91A which yielded 10^5 pfu/ml, 1000-fold reduction compared to the parental virus.

5.4. Protein labelling and western blotting

Mutations in the N gene could affect not only N protein expression, they might affect expression of other viral genes. To investigate the impact of mutations on N protein expression as well as expression of the other viral proteins, protein labeling and western blotting were performed (an example of a whole western blot is given in Figure 5.3). Different MOI (0.01, 0.1 or 1.0) were used in the latter experiments due to the poor titers shown by some mutant viruses which made it difficult to use a high MOI. However, similar MOI of the parental virus was used with each group of viruses (Figure 5.4). Several mutant viruses (F6A, R40, T91A, A100V, F155L, E112A, K197T, V167A, M172A, and V208A) showed a dramatic decline in the level of N protein expression. These results can be correlated to the titre of mutant viruses since they displayed 1000 to 10000-fold reduction compared to the parental virus (Table 3.10). In other cases, mutant viruses K50A, L205A, V208A and F229L showed no deficiency in N protein expression which is consistent with results obtained from the BUNV minigenome system; however, their titres showed significant reduction 3 to 4 logs below the parental virus titre.

No significant differences in viral gene expression were observed. In general, it seems that mutations in the N protein only affected the level of N protein expression while the levels of other viral proteins were not affected. Some mutations in the N protein affect protein mobility. This was observed in mutant viruses P19Q and L53F (Figure 5.4A and B).

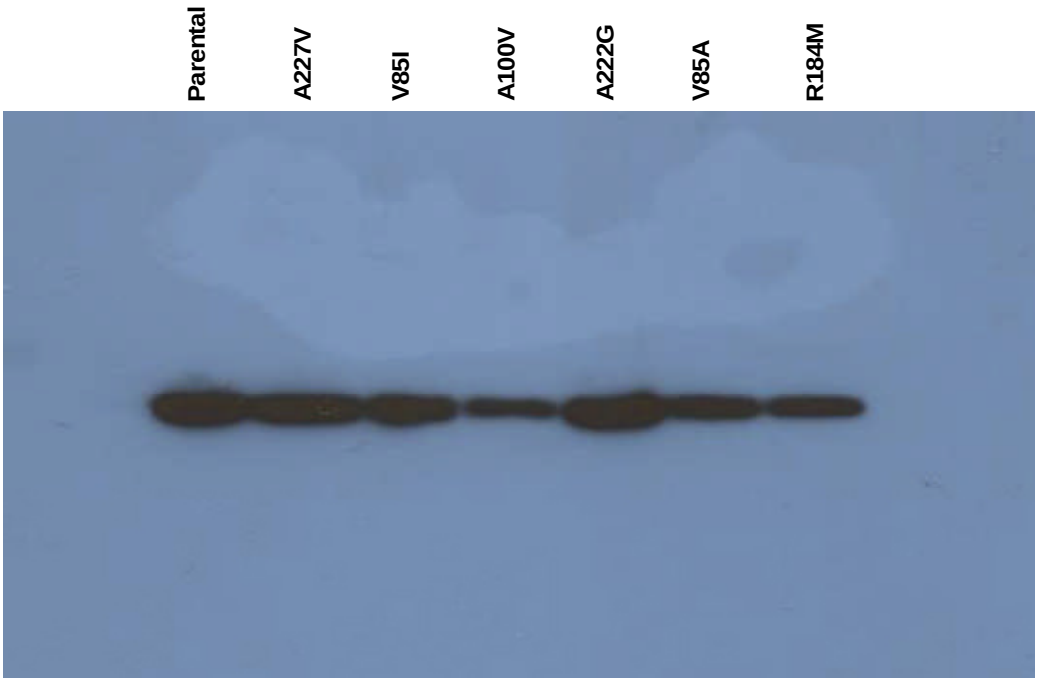
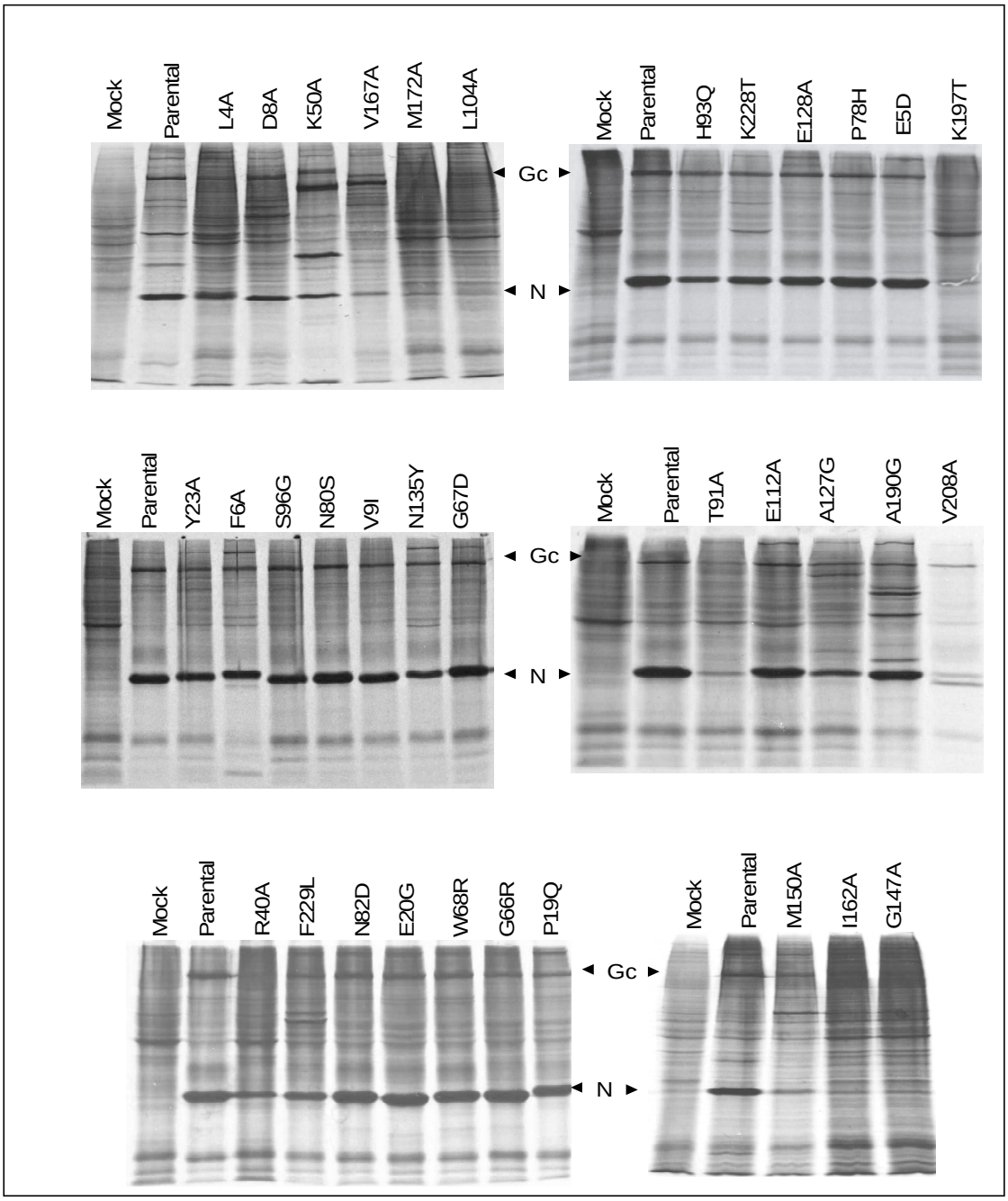
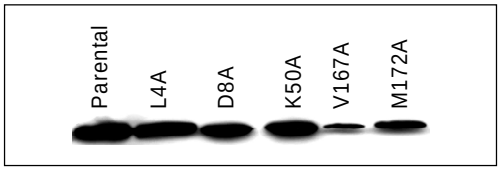
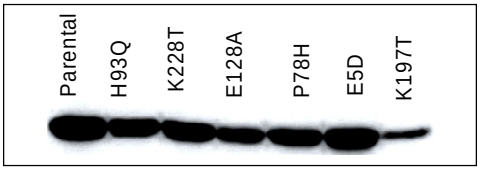


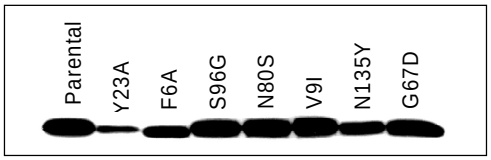
Figure 5.3. An example of the whole blot (membrane size 11×7cm) Using anti-N polyclonal rabbit antibody.

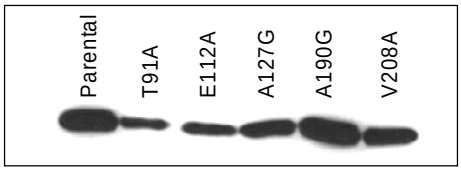
(a)

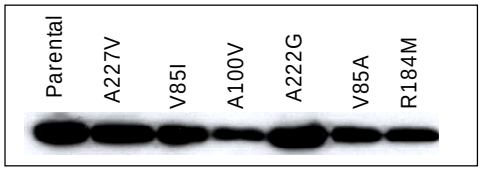

Mock
Parental
L4A
D8A
K50A
V167A
M172A
L104A
Gc
N
Mock
Parental
H93Q
K228T
E128A
P78H
E5D
K197T
Mock
Parental
Y23A
F6A
S96G
N80S
V9I
N135Y
G67D
Gc
N
Mock
Parental
T91A
E112A
A127G
A190G
V208A
Mock
Parental
R40A
F229L
N82D
E20G
W68R
G66R
P19Q
Gc
N
Mock
Parental
M150A
I162A
G147A


Parental
L4A
D8A
K50A
V167A
M172A

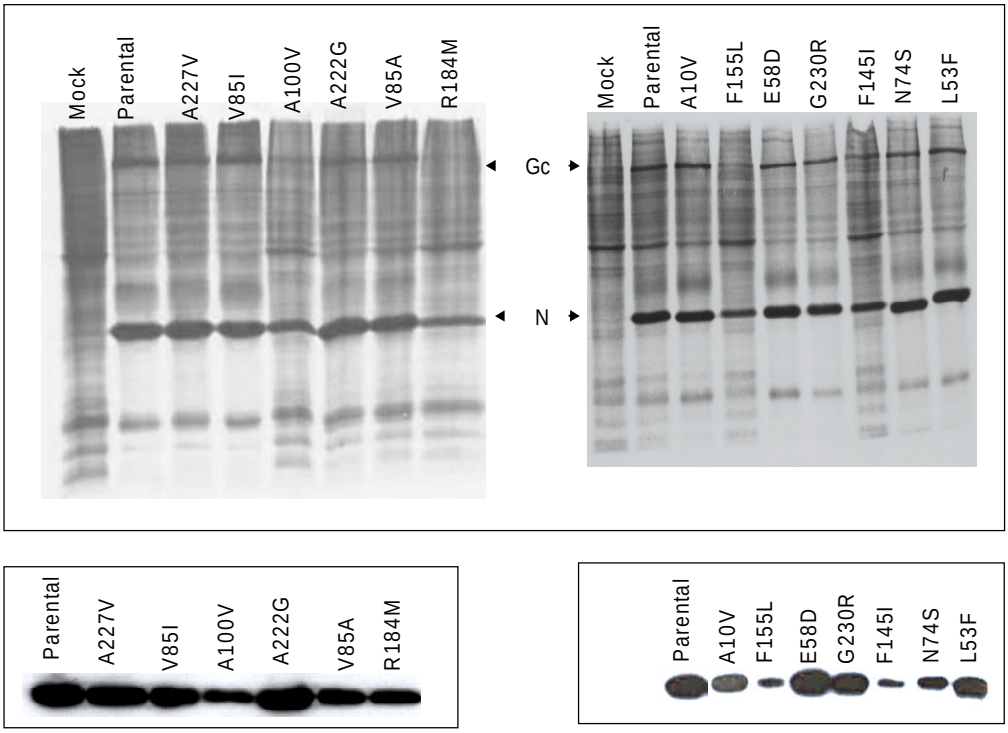

Parental
H93Q
K228T
E128A
P78H
E5D
K197T


Parental
Y23A
F6A
S96G
N80S
V9I
N135Y
G67D


Parental
T91A
E112A
A127G
A190G
V208A


Parental
A227V
V85I
A100V
A222G
V85A
R184M

(B)


Mock
Parental
A227V
V85I
A100V
A222G
V85A
R184M
Gc
N
Mock
Parental
A10V
F155L
E58D
G230R
F145I
N74S
L53F
Parental
A227V
V85I
A100V
A222G
V85A
R184M
Parental
A10V
F155L
E58D
G230R
F145I
N74S
L53F

(C)

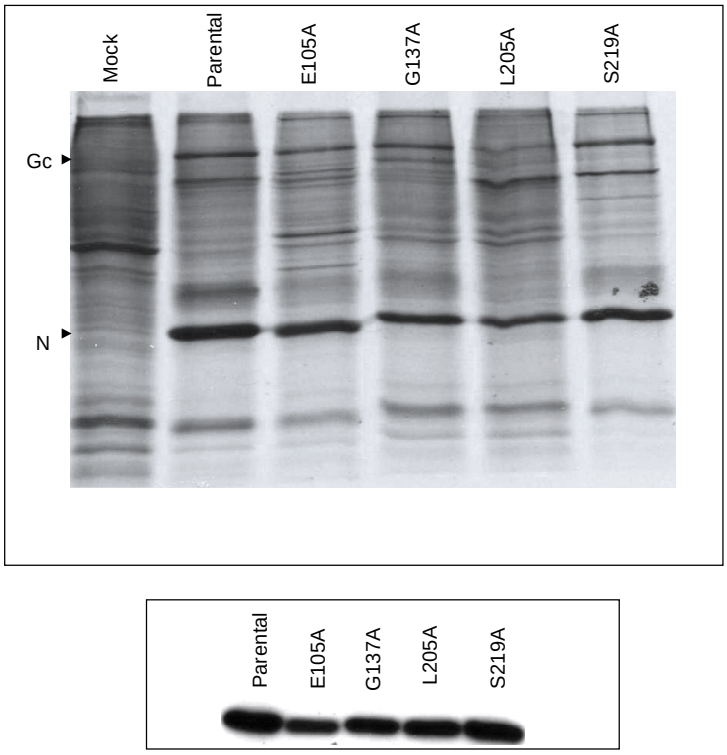


Figure 5.4. Protein labeling and western blot of mutant viruses.

Since mutant viruses have different titres and some mutant viruses were debilitated making it difficult to infect at high MOI, Vero-E6 cells were infected at MOI 0.01 (a), 0.1 (b) or 1 (c) in duplicate, and incubated overnight at 33ºC. Protein labeling and western blot were performed. Position of viral Gc and N proteins are indicated.

5.5. Temperature-sensitivity

As mentioned previously, all recombinant viruses were rescued at 33ºC. This would increase the probability of recovering ts mutants which can grow only at lower temperatures. The mutant viruses were tested for their ability to form plaques at 33ºC, 37ºC and 38ºC on Vero-E6 cells (Table 5.2). The parental virus behaved similarly at all temperatures, producing almost the same titre and the same plaque size (Table 5.2 and Figure 5.5). The majority of mutant viruses (23) displayed a reduction of 10-fold in their titers at 37ºC or 38ºC or at both. Three mutant viruses (F6A, G147A and V208A) functioned poorly at 33ºC, and displayed titers of 10^3-10^4 pfu/ml with pin point plaques size and did not grow at 37ºC or 38ºC. Another 4 mutant viruses (L104A, M150A, I162A and M172A) were debilitated, growing at 33ºC and 37ºC only.

Using the titration data the reduction in virus titer (log_{10} pfu/ml) and the efficiency of plating (EOP) of the mutant viruses were calculated (Table 5. 2). The parental virus displayed reduction of ≥ 0.3 log_{10} pfu/ml in virus titre and plating efficiency of 0.5. Twenty-nine mutant viruses showed reduction of ≤ 2.0 log_{10} pfu/ml and their plating efficiency was 0.01-0.3, 13 mutant viruses displayed reduction of 2.1-2.6 log_{10} pfu/ml and plating efficiencies between 0.003-0.009, 8 recombinant viruses showed greater than 3.0 log_{10} reduction in titer and plating efficiency between 0.0-0.0004. Based on the plaque phenotypes, titres and plating efficiency at various temperatures of mutant viruses, temperature sensitivity was defined as production of large plaque size and high titre (10^8 pfu/ml) at the permissive temperature (33°C) while at 38ºC (non-permissive temperature) ts mutant virus produce small plaque size, reduction of >2.0 log_{10} in virus titre and plating efficiency ≤0.009. Hence, mutants N74S, S96G, K228T and G230R are ts mutant viruses. Although mutant viruses F6A, K50A, L104A, G147A, M150A, I162A, M172A and V208A displayed reduction of >2.0 log_{10} in virus titer and plating efficiency <0.008, they were not classified as ts mutant either because they displayed small plaque size and grew poorly at 33°C.

virus	Titre (log_{10} pfu/ml)					virus	Titre (log_{10} pfu/ml)				
	33°C	**37°C**	**38°C**	Δ	**EOP**		**33°C**	**37°C**	**38°C**	Δ	**EOP**
Parental	8.5	8.3	8.2	0.3	0.5	G66R	7.2	6.2	6.0	1.2	0.06
L4A	6.8	6.5	5.5	1.3	0.05	G67D	7.1	6.7	6.6	0.5	0.3
E5D	7.4	7.2	6.7	0.7	0.2	W68R	7.8	6.4	6.0	1.8	0.02
F6A	4.5	0.0	0.0	4.5	0.0	**N74S**	8.5	7.9	6.3	2.2	0.007
D8A	7.8	7.2	6.2	1.6	0.02	P78H	8.0	7.9	6.7	1.3	0.06
V9I	6.5	5.5	5.5	1.0	0.09	N80S	8.1	7.5	7.4	0.7	0.2
A10V	8.4	7.8	7.8	0.6	0.2	N82D	7.7	7.1	6.5	1.2	0.05
P19Q	5.9	3.5	3.3	2.6	0.003	V85A	7.9	7.2	6.5	1.4	0.04
E20G	7.0	5.7	5	2.0	0.01	V85I	6.7	6.5	5.9	0.8	0.2
Y23A	7.4	5.5	5.2	2.2	0.006	T91A	4.3	3.5	3.3	1.0	0.1
R40A	4.5	2.2	2	2.5	0.003	H93Q	5.8	3.5	3.5	2.3	0.005
K50A	5.7	4.4	2.3	3.4	0.0004	**S96G**	8.1	6.7	6.0	2.1	0.008
L53 F	8.4	8.1	7.2	1.2	0.06	A100V	3.9	2.6	2.5	1.4	0.04
E58D	8.5	7.2	7.2	1.3	0.06	L104A	5.5	5.1	0.0	5.5	0.0

virus	Titre (log_{10} pfu/ml)					virus	Titre (log_{10} pfu/ml)				
	33°C	**37°C**	**38°C**	**Δ**	**EOP**		**33°C**	**37°C**	**38°C**	**Δ**	**EOP**
E105A	8.0	7.0	6.4	1.6	0.03	R184M	5.7	3.6	3.5	2.2	0.008
E112A	7.5	6.8	6.3	1.2	0.06	A190G	5.4	4.8	4.5	0.9	0.1
A127G	6.0	4.3	3.8	2.2	0.006	K197T	7.4	7.0	6.7	0.7	0.2
E128A	7.7	6.8	5.3	2.4	0.004	L205A	5.3	4.4	3.2	2.1	0.008
N135Y	6.6	6.0	5.9	0.7	0.2	V208A	3.7	0.0	0.0	3.7	0.0
F145I	8.3	7.2	7.2	1.1	0.08	S219A	7.9	6.2	6.1	1.8	0.02
G147A	3.4	0.0	0.0	3.4	0.0	A222G	8.0	7.2	6.5	1.5	0.03
M150A	3.9	1.7	0.0	3.9	0.0	A227V	7.5	5.7	5.7	1.8	0.02
F155L	6.4	5.0	4.3	2.1	0.008	**K228T**	8.4	7.7	6.3	2.1	0.009
I162A	3.7	2.8	0.0	3.7	0.0	F229L	5.3	3.6	3.6	1.7	0.02
V167A	6.5	6.3	5.3	1.2	0.07	**G230R**	8.3	7.2	6.2	2.1	0.007
M172A	5.0	3.7	0.0	5.0	0.0						

Table 5. 2. Temperature sensitivity and plating efficiency of mutant viruses

The Δ indicates the reduction in virus titre (log_{10} pfu/ml) at 38°C compared to that for the permissive temperature (33°C).
EOP: efficiency of plating determined via: titer at 38°C/titer at 33°C
Underlined values indicate a reduction of ≥2.0 log_{10} pfu/ml in virus titre at 38°C compared to that of the titre at the permissive temperature (33°C).
Mutant viruses highlighted **in bold** display *ts* phenotype.

5.6. Characterization of ts mutant viruses

The four mutant viruses (N74S, G96S, K228T and G230R) designated as ts displayed different behaviours. They grew only at 33°C while the parental virus grew similarly at all temperatures, 33°C, 37°C and 38°C (Table 5.3 and Figure 5.5). They displayed greater than 100-fold reduction in their titers, produced pinpoint plaque sizes and showed plating efficiency ≤0.009 at 38°C compared to the parental virus. Three stages in viral RNA synthesis could be ts: it could be a transcription step (mRNA synthesis), early replication (antigenomic RNA synthesis) or late replication step (genomic RNA synthesis).

To characterize these mutant viruses, protein labelling, western blotting and northern blotting were performed at various temperatures, 33°C, 37°C and 38°C to test viral gene expression, total amount of N protein expressed and RNA synthesis. Vero-E6 cells were infected with mutant viruses or parental virus at MOI of 1 for 1 hour at 37°C in triplicate, and incubated at 33°C, 37°C or 38°C overnight. The results showed that none of the mutations (N74S, G96S, K228T and G230R) affected N protein expression significantly (Figure 5.6). In other words, mRNA synthesis was competent. This would suggest that these mutations could affect antigenomic or genomic RNA synthesis.

To explore this possibility northern blotting is the best available technique. Total RNA was extracted from infected cells using TrIzol reagent (Invitrogen). Between 3 - 5µg of total RNA were run on an agarose gel using 1× TAE buffer (Masek *et al*., 2005) for 2 hrs (genome detection) or 3 - 4 hrs (antigenomic and mRNA detection). By using a negative-sense DIG-labelled probe, mRNA and antigenome RNA can be detected while a positive-sense DIG-labelled probe enables detection of the RNA genome (Figure 5.7). The reasoning behind using probes for BUNV S segment rather for M or L sgments is that it is possible to separate the S mRNA from the S antigenomic RNA since the mRNA is 100 bases shorter than antigenomic RNA which represents 10% of the size of antigenomic RNA. Two mutant viruses (N74S and S96G) were shown to be deficient in genome-RNA synthesis (late replication step). Although both mutant viruses were genome-synthesis deficient at 37°C and 38°C, they behaved slightly differently. Mutant virus N74S showed decreased genomic RNA synthesis at 38°C

virus	Titre pfu/ml				
	33°C	**37°C**	**38°C**	Δ	**EOP**
Parental	6.0×10^8	3.5×10^8	3.0×10^8	0.3	0.5
N74S	3.0×10^8	7.5×10^7	2.0×10^6	2.0	0.007
S96G	1.2×10^8	5.0×10^6	1.0×10^6	2.1	0.008
K228T	2.5×10^8	4.5×10^7	2.4×10^6	2.0	0.009
G230R	2.2×10^8	1.5×10^7	1.2×10^6	2.2	0.006

Table 5. 3. Temperature sensitivity and platting efficiency of ts mutant viruses. The Δ indicates a reduction in virus titre (log_{10} pfu/ml) at 38°C compared to that for the permissive temperature (33°C). **EOP**: efficiency of platting.

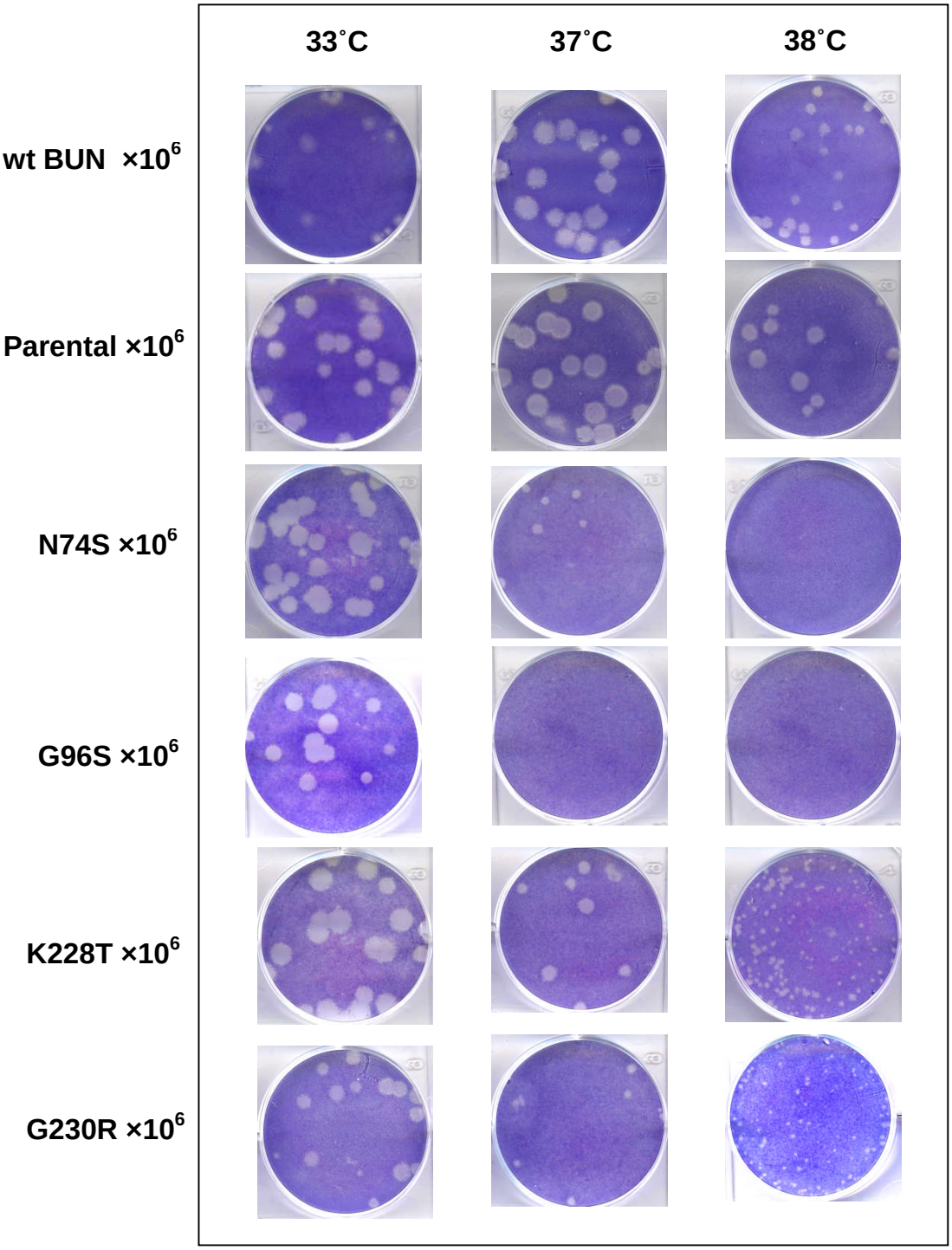
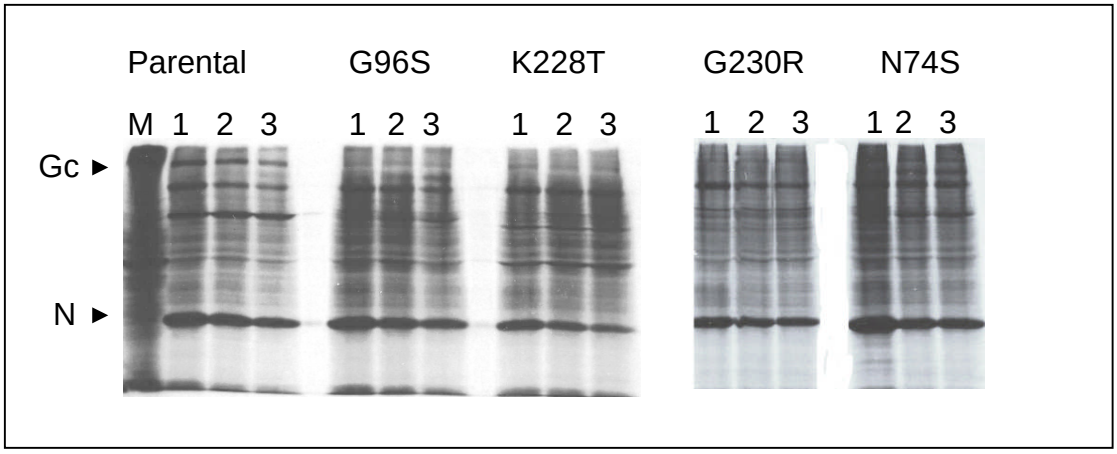


Figure 5.5. Plaque phenotypes of ts mutant viruses .

The same stock of virus was assayed at different temperatures. Vero-E6 cells were infected with serial dilution (10^{-1} - 10^{-7}) of stock virus for 1 h at 37°C. The virus was removed and replaced with 2ml agarose overlay and incubated at 33°C, 37°C or 38°C for 6 days. Cells were then fixed with 5% formaldehyde for at least 2 hrs, stained with Geimsa and plaques were examined.

(A)



(B)

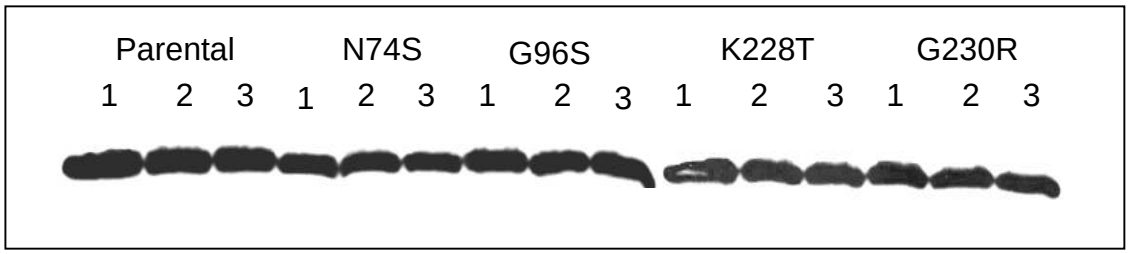


Figure 5.6. Protein labelling and western blots of ts mutant viruses at various temperatures.

Vero-E6 cells were infected in duplicate at MOI of 1 for 1 hour at 37°C and incubated overnight at 33°C (lane 1), 37°C (lane 2) or 38°C (lane 3). Protein labelling (A) and western blotting for N protein (B) were performed on the samples. Viral Gc and N proteins are indicated. Mock (M).

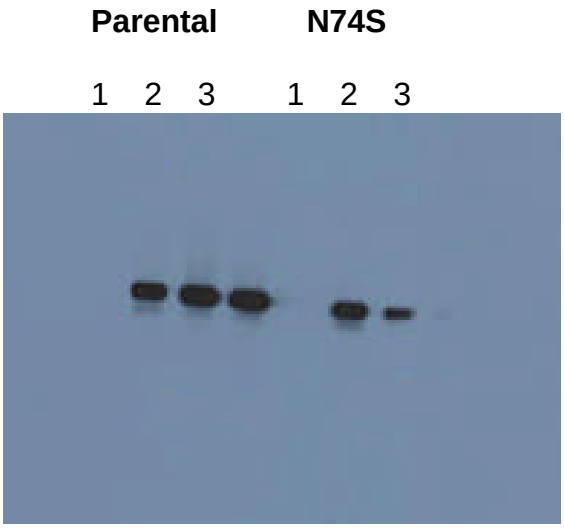


Figure 5. 7. An example of the whole blot of S segment genome RNA detection using northern blotting at 33°C (lane 1), 37°C (lane 2) or 38°C (lane 3).

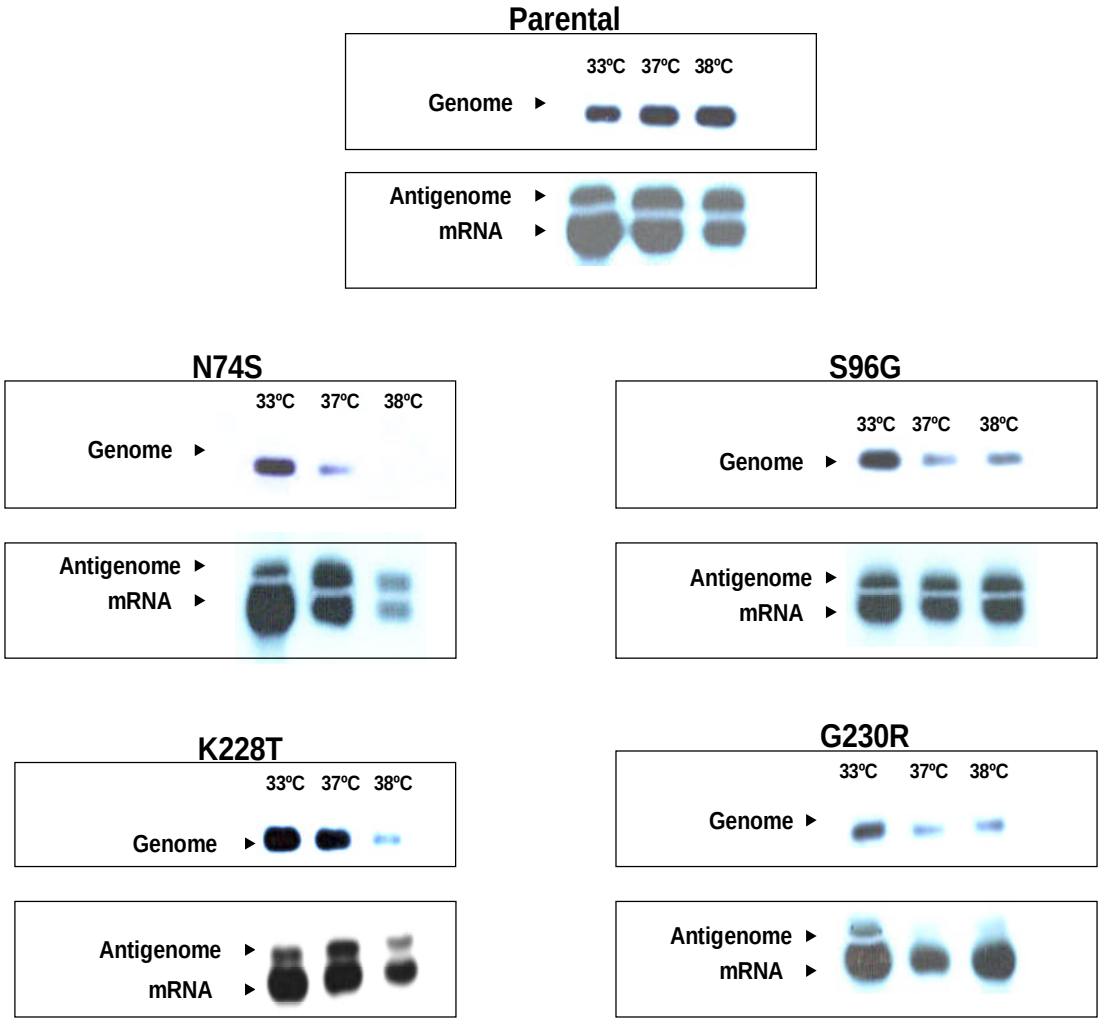


Figure 5.8. RNA synthesis of ts mutant viruses.

Vero-E6 cells were infected with mutant virus at MOI of 1 at 37°C in triplicate. Cells were then incubated overnight at 33°C, 37°C or 38°C. Total RNA was extracted fron cells using TrIzol reagent and northern blotting was carried out. To detect S genomic RNA positive DIG-labelled probe was used and negative DIG-labelled probe was used for S mRNA and antigenome detection.

but also mRNA and antigenome synthesis declined. Recombinant virus S96G shows no deficiency in mRNA and antigenome synthesis at all temperatures and only the genome synthesis was deficient at 37°C and 38°C. Mutant viruses K228T and G230R were ts at early replication step (antigenome-synthesis deficient). Mutant virus G228T was early replication step strictly at 38°C while mutant G230R displayed decreased antigenome-RNA synthesis at both 37°C and 38°C (Figure 5.8).

5.7. Host range restriction

In an attempt to detect host-restricted mutant viruses, plaque-forming ability of all mutant viruses was tested on 5 different cell lines, Vero-E6, BHK-21, 2FTGH-V, A549-V and 293T-V (Table 5.4). As BUNdelNSs virus was used in this project, it was necessary to use interferon-deficent cell lines (for full details see Table 2.1). Host-restriction could be defined as at least 100-fold reduction in titre on a specific cell line compared to other cell lines. The parental virus displayed no differences in all cell lines, showing titers of 8.5-8.8 $\log_{10}$ pfu/ml. Most mutant viruses followed this pattern. The most permissive cell line was A549-V, even for those mutant viruses that displayed poor titers in other cell lines. Mutant viruses F6A, M150A and G147A produced pinpoint plaques in Vero-E6 and A549-V cells but did not plaque in 2FTGH-V or 293T-V cells. In general, the parental virus produced smaller plaques in BHK-21, 2FTGH-V and 239-V than in Vero-E6 and A549-V cells (Figure 5.9) which could explain the inability of the latter mutant viruses to form plaques in 2FTGH-V or 293-V since they produced pinpoint plaque size in Vero-E-6 cells. Based on the definition of host restriction above, mutant virus Y23A is defined to be host-restricted in 2FTGH-V cells. It displayed greater than a 100-fold reduction in virus titre compared to other cell lines. In Vero-E6, BHK-21, A549-V and 293T-V cell lines, the mutant virus yielded 7.3-8.1 $\log_{10}$ pfu/ml. Further investigations were carried out on mutant virus Y23A to determine why it behaved differently only in 2FTGH-V cells.

Comparison of the ratio of intracellular and extracellular virus particles would reveal whether the mutant virus Y23A had a deficiency in release from cells. 2FTGH-V cells were infected with the parental or mutant virus at MOI of 1 overnight. The overnight supernatant was collected and cell debris cleared by centrifugation. Cells were harvested in PBS and subjected to 3 cycles of freeze-thaw. The lysate was then

virus	Titre (log_{10} pfu/ml)				
	Vero-E6	**BHK-21**	**2FTGH- V**	**A549-V**	**293-V**
Parental	8.5	8.7	8.5	8.8	8.5
L4A	7.0	6.8	5.8	7.3	6.9
E5D	7.3	7.9	6.7	8.0	7.5
F6A	3.7	4.5	-	5.0	-
D8A	8.2	7.7	7.6	8.6	8.0
V9I	7.9	7.7	7.3	8.3	7.7
A10V	8.3	7.7	7.0	8.3	8.2
P19Q	7.1	6.6	6.2	7.4	6.9
E20G	7.9	7.3	7.4	8.3	7.7
Y23A	7.7	7.0	6.0	8.2	7.3
R40A	7.2	5.3	3.2	5.0	5.5
K50A	5.8	5.3	5.5	6.7	6.3
L53 F	8.5	7.9	7.3	8.4	8.4
E58D	8.0	8.2	7.5	8.7	7.9
G66R	7.0	6.6	6.3	7.4	7.3
G67D	7.7	6.5	6.5	7.4	7.0
W68R	7.8	7.6	7.0	8.7	7.9

virus	Titre (log_{10} pfu/ml)				
	Vero-E6	**BHK-21**	**2FTGH- V**	**A549-V**	**293-V**
N74S	8.7	8.7	8.0	9.0	8.3
P78H	7.6	7.0	5.9	7.5	6.8
N80S	8.5	8.3	8.1	8.6	8.3
N82D	8.2	8.3	7.2	8.7	7.6
V85A	8.3	8.2	8.0	8.2	8.4
V85I	7.2	7.4	6.0	7.5	7.2
T91A	4.3	3.7	3.9	4.4	4.5
H93Q	7.4	7.2	6.6	7.5	7.7
S96G	8.0	8.3	7.5	8.5	7.6
A100V	4.5	4.0	4.0	4.7	4.7
L104A	5.9	6.5	3.3	6.4	5.9
E105A	7.6	7.7	6.4	7.5	7.0
E112A	7.8	7.5	6.7	7.8	7.7
A127G	5.9	6.0	4.7	6.7	5.0
E128A	7.7	8.1	6.7	8.2	7.8
N135Y	7.7	7.4	7.3	7.6	7.4
F145I	8.1	8.7	7.6	8.8	8.5

virus	Titre (log_{10} pfu/ml)				
	Vero-E6	**BHK-21**	**2FTGH- V**	**A549-V**	**293-V**
G147A	3.0	-	-	3.4	-
M150A	2.5	2.4	1.7	4.2	3.0
F155L	7.5	7.4	6.5	7.4	7.6
I162A	4.4	4.4	2.9	5.0	4.3
V167A	6.9	6.9	5.2	7.3	6.5
M172A	5.2	6.0	4.5	6.5	5.7
R184M	5.5	5.6	3.6	6.2	6.2
A190G	5.9	5.4	5.0	5.5	6.8
K197T	8.1	6.5	6.7	8.2	8.0
L205A	7.5	6.9	7.4	7.8	7.4
V208A	4.0	5.0	4.3	5.9	4.4
S219A	7.5	7.7	7.0	7.8	7.7
A222G	7.9	7.8	6.6	8.2	7.4
A227V	7.5	8.0	8.2	8.3	8.0
K228T	8.3	8.2	7.7	8.4	8.0
F229L	5.8	5.4	4.1	6.0	5.5
G230R	8.2	8.0	7.7	8.4	7.8

Table 5.4. Host-restriction experiment

Stock of each mutant virus was assayed on five different cell lines (above) using plaque assay technique. After an incubation of 6 days at 33°C, mutant virus titre was counted. (-) indicates that mutant virus can not plaque even when undiluted stock virus was used.

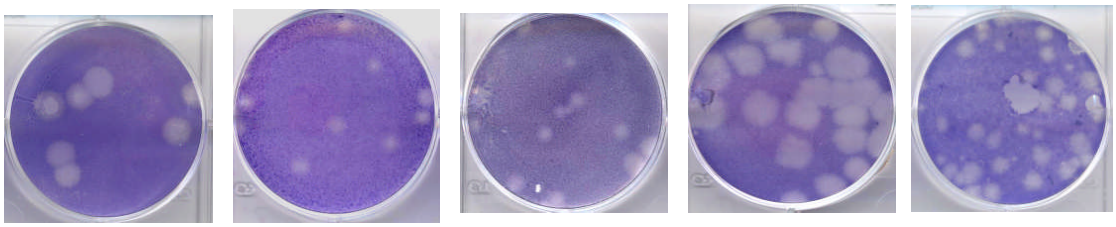


Figure 5. 9. Parental virus plaque phenotypes in 5 different mammalian cell lines.

Virus	Intracellular particles pfu/ml	Extracellular particles pfu/ml	Release efficiency %
Parental	3.5×10^7	1.5×10^7	30
Y23A	8.5×10^5	1.5×10^5	15

Table 5. 5. Comparison the ratio of the intracellular and extracellular virus particles of mutant Y23A.

Vero-E6 cells were infected with parental virus or mutant virus Y23A at MOI of 1 and were incubated for 16 hrs at 33°C. The supernatants were collected and cells were subjected to 3 cycles of freeze-thaw. The overnight supernatant medium and freeze-thaw supernatant were used in a plaque assay.
The release efficiency was calculated *via* formula =

$$\frac{\text{Extracellular titer}}{\text{Total amount of virus}} \times 100$$

centrifuged to remove cell debris and both supernatants used in a plaque assay on Vero-E6 cells. Mutant Y23A showed a 100-fold reduction in both intracellular and extracellular virus particles compared to the parental virus (Table 5.5). These results are consistent with the previous results obtained from host-range experiment (Table 3.14). Moreover, mutant virus Y23A not only had replication deficits but also showed reduced release from cells. Comparison of the ratio of intracellular to extracellular particles showed that only 15% of the total amount of mutant virus Y23A particles were released compared to 30% of the parental virus particles.

5.8. Discussion

The growth properties of 50 recombinant viruses carrying mutations in the BUNV N protein were characterized *via* titration, protein labelling, western blotting, temperature sensitivity and host-restriction experiments. The majority of mutations had little effect on virus growth properties. However, it was shown that single substitutions in the BUNV N protein (*e.g.* G147A, M150A and I162A) reduced the virus titer 100000-fold compared to the parental virus. These residues are highly conserved in the middle of orthobunyavirus N proteins. Although mutant viruses G147A, M150A and I162A were repropagated several times, they did not yield titres more than 10^3 pfu/ml. The latter mutation might affect virus spread from cell to cell since they produced pinpoint plaques. Overall, the titration of mutant viruses was a good indication of the impact of mutations in the BUNV N protein which could affect the virus life cycle at any step. Several mutant viruses (*e.g.* L4A, E20G and K228T) showed poor recovery, about 10^2 pfu/ml (Table 4.2), however, when they were purified they produced 10^5-10^7 pfu/ml (Table 5.1). The poor recovery could be attributed to poor rescue conditions, DNA quality (*e.g.* purity, age, freez-thaw), cell viability (*e.g.* passages) or high concentration of DI particles. Mutant viruses K50, G66R and W68R displayed the poorest recovery and could only be isolated from nondiluted rescue supernatant, but after they were purified and propagated, they grew to titers 10^5-10^7 pfu/ml. Sequence analyses of the S segment of mutant viruses G66R and W68R revealed second site mutations had been introduced at nonconserved residue R28H and K109E respectively. In the latter case the poor recovery might have been due to effects of the actual mutation rather than the rescue conditions mentioned above.

The recombinant viruses showed 3 types of plaque size, large, medium, and small (Figure 5.2). Twenty-two mutant viruses produced parental-like plaque sizes (large) (Figure 2.15A). These mutant viruses had no difficulty in spreading from cell to cell, however, not all these mutant viruses produced high titers. Mutant viruses A100V and L205A produced large plaques but their titers were 10^4 -10^5 pfu/ml more than a 1000-fold less than the parental virus. The latter residues affected virus replication but not the spread from cell to cell. In contrast, mutant viruses Y23A and E128A produced high titers of 10^7-10^8 pfu/ml but displayed pinpoint plaques (Figure 5.2C). It seems that these mutations affected virus spread from cell to cell, but replication was not affected. Mutant viruses F6A, R40A, G147A, M150A, I162A and V208A gave pinpoint plaques and low titers (10^3-10^4 pfu/ml). Such mutations might affect both virus replication and spread. Those mutant viruses which displayed mixed plaque sizes (Figure 5.2D) might be due to the presence of DI particles or revertant viruses.

Protein labelling and western blotting results showed that mutations in the BUNV N protein did not affect the expression of other genes specifically, but could affect N gene expression in particular or all gene expression, as the N protein encapsidates all three segments. N protein mobility in SDS-PAGE was affected by mutations P19Q, L53F, G67D and N135Y. The latter substitutions increased the MW of BUNV N by 31, 34, 58 and 45. As a result of the increase of the MW of mutant N proteins, their migration in the polyacrylamyde gel was slower than the wt BUNV N protein (Figure 5.4a, b).

Temperature-sensitivity experiments showed that 4 recombinant mutant viruses (N74S, S96G, K228T and G230R) were ts, growing at 33°C, but not at 37°C or 38°C while the parental virus grows at all temperatures. At permissive temperature (33°C) they displayed large plaques and grew to titers of 10^8 pfu/ml. At nonpermissive temperatures (37°C or 38°C) they showed pinpoint plaques, displayed a reduction of >2.0 $\log_{10}$ pfu/ml in virus titer and plating efficiency ≤0.009 at 38°C (Table 5.3). Three possible steps in RNA synthesis could be affected by these mutations at nonpermissive temperature: mRNA, antigenome or genome synthesis. Protein labelling and western blot analysis (Figure 5.6) shows no significant deficiency in N protein expression in all ts mutant which is a preliminary indication of competent mRNA synthesis.

Using the northern blotting technique, the ts step of each mutant N protein was determined. Two mutant viruses N74S and S96G were affected at a late replication step (genome-deficient synthesis) with slight differences. Mutant N74S showed genome-deficient synthesis strictly at 38°C but also mRNA and antigenome synthesis declined compared to mutant virus S96G which displayed consistent production of mRNA and antigenome (Figure 5.8). This could be explained by the fact N74S is more sensitive at 38°C. Mutant viruses K228T and G230R are early replication step (antigenome-deficient synthesis). While mutant G230R is early replication step at both 37°C and 38°C, mutant K228T only at 38°C. These results demonstrate that BUNV N protein plays a role in regulation of virus transcription and replication.

It is worth noting that only one ts mutant previously had been mapped to the S segment of bunyaviruses designated MAG*ts*23(III) (Pringle and Iroegbu, 1982). It displayed plating efficiency of 0.001 (plaques at 38°C /plaque at 31°C). However, nucleotide sequence analysis of the S segment of MAG*ts*23 (III) showed that the mutation occurred at residue V85A in the N protein (conserved residue) and F71L in the NSs protein (conserved residue) (D.C.Pritlove and R.M.Elliott, unpublished data). At this stage, it is not clear whether the phenotype of MAG*ts*23 (III) was due to substitution in N ORF or NSs ORF or both. Mutation at residue V85A of BUNV N protein was generated randomly and viable virus carries this mutation was recovered. Temperature sensitivity results showed that mutant V85A showed reduction of 1.4 $\log_{10}$ pfu/ml in virus titer and plating efficiency of 0.04. These results suggest that MAG*ts*23(III) was not ts due to the mutation in the N ORF only but also due to the mutation in the NSs ORF.

Host-restriction tests showed that the parental virus grew similarly in all cell lines with titers of 8.5 - 8.8 $\log_{10}$ pfu/ml (Table 5.4). Mutant virus Y23A was host-restricted in 2FTGH-V cells. It displayed greater than 100-fold reduction in virus titer in 2FTGH-V cells compared to the titers in other cell lines. The ability of mutant virus Y23A to be released from 2FTGH-V cells was measured by comparing the ratio between the intracellular and the extracellular particles (Table 5. 5). Only 15% of the total amount of mutant virus Y23A particles were released as extracellular particles compared to 30% released by the parental virus. These results suggest that mutant Y23A grew poorly in 2FTGH-V cells since it showed greater than 2.0 $\log_{10}$ pfu/ml reduction in titer and also has particle release deficiency since 85% of the total amount of the virus

was still trapped inside the cells. MAG*ts*23(III) was host-restricted on BS-C-1 cells displaying at least 100-fold reduction in virus plaque-forming ability compared to on BHK-21 cells. Mutant V85A produced similar titers (8.0-8.4 $\log_{10}$ pfu/ml) in all cell lines used. It is possible that the host-restriction of MAG*ts*23(III) was due to mutation at residue F71L of NSs ORF, however, unless the two ORFs of the N and NSs proteins can be reconstructed separately it is hard to tell at this stage whether the phenotypic changes displayed by MAG*ts*23(III) was due to mutation in N or NSs ORF.

5.9. Summary

1- Single substitutions (*e.g.* G147A, M150A, I162A) in the BUNV N protein gene reduce virus titre 100000-fold below the parental virus titre.
2- Single substitutions (*e.g.* F6A, Y23A, and E128A) in the BUNV N protein gene generate mutant viruses that display 3 plaque phenotypes: large, medium and pinpoint.
3- Single substitutions (N74S, S96G, K228T and G230R) in the BUNV N protein gene produce ts mutant viruses with different phenotypes.
4- Single substitution Y23A in the BUNV N protein gene produce host-restricted mutant virus in 2FTGH-V displaying greater than 2.0 $\log_{10}$pfu/ml reduction in virus titre compared to other cell lines.

Chapter 6. Characterization of the nonrescuable N genes

6.1. Introduction

Fourteen mutant N genes carrying single mutations at highly conserved residues in the BUNV N protein were nonrescuable (Figure 6.1). Six of these involved residues that are conserved in the middle of N protein while eight amino acids are conserved in the C-terminal region of BUNV N protein. The mutant N proteins displayed a wide-range of activity in the BUNV minigenome system (Table 6.1 and Figure 6.2). Mutant N proteins P125H, G131W, Y158N, Y176A, W213R and L226A were functional, displaying 66%, 30%. 25%, 53%, 28% and 26% of the wt N protein activity respectively. Moreover, mutant I231A showed activity similar to wt N protein activity. The other mutant N proteins were weakly active or inactive in the BUNV minigenome system. The BUNV N protein is multifunctional and several interactions can be speculated: interaction between N molecules to form a chain of N proteins, interaction with L polymerase during virus transcription and replication, interaction with the genomic and antigenomic RNA backbone and interaction with the C-tails of the glycoproteins during virus assembly. Hence, it is possible that some of these mutations could affect one or more of the above interactions making the recovery of the virus unachievable.

Mutant N proteins which were weakly active (I118N and L177A) or inactive (R94A, W134A, Y141C, K179I and W193A) in the BUNV minigenome system might be affected in interaction with L polymerase, and hence coimmunoprecipitation could be used to test N-L interactions. Another possibility was an affect on N protein multimerization, that could be examined by cross-linking experiments. Mutant N proteins P125H, Y176A and I231A, which displayed high activity in the BUNV minigenome system, may have affected another step such as RNP packaging. Therefore these mutant N genes were introduced into the BUNV packaging assay to reveal whether the mutant N protein was able to package the BUNV minigenome into virus-like particles.
Finally, any effect on RNA synthesis could be assayed by northern blotting of RNA extracted from the cells transfected with plasmids for attempted rescue.

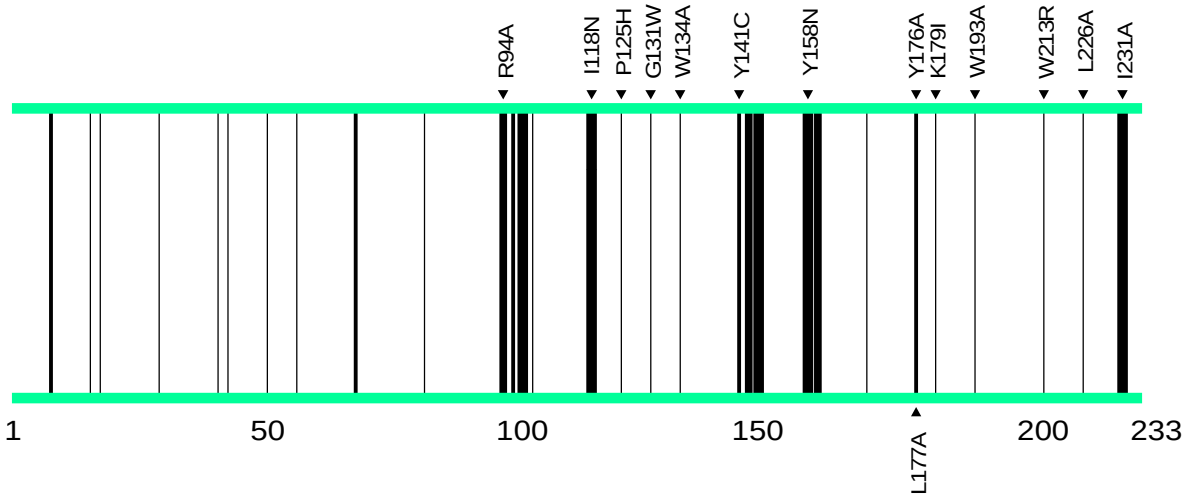


Figure 6.1. Positions of nonrescuable mutations in the BUNV N protein sequence. Six residues are conserved in the middle of N protein while eight are conserved in the C-terminal domain. Black lines indicate the conserved residues among 51 orthobunyavirus N proteins.

N gene	Mutation position	Nature of change	Con*	Activity (%)
R94A	365, C → G 366, G → C	Polar+ → nonpolar (small)	++++	0.17
I118N	438 T → A	Nonpolar (hydrophobic) → polar = (amide)	+	11
P125H	459 C → A	Polar = (hydrophobic) → polar +	++++	66
G131W	476 G → T	Nonpolar (small) → aromatic	++++	30
W134A	485, T → G 486, G → C	Aromatic → nonpolar (small)	++++	1.9
Y141C	507 A → G	Aromatic → polar = (nucleophilic)	++++	1.8
Y158N	557 T → A	Aromatic → polar = (amide)	++++	25
Y176A	611, T → G 612, A → C	Aromatic → nonpolar (small)	+	53
L177A	613, C → G 614, T → C 615, T → G	Nonpolar (hydrophobic) → nonpolar (small)	++	10
K179I	621 A → T	Polar + → nonpolar (hydrophobic)	++++	4
W193A	662,T → G 663, G → C	Aromatic → nonpolar (small)	++++	3
W213R	722 T → C	Aromatic → Polar +	++++	28
L226A	761, C → G 762, T → C 763, T → C	Nonpolar (hydrophobic) → nonpolar (small)	++++	26
I231A	775, A →G 776,T → C	Nonpolar (hydrophobic) → nonpolar (small)	+++	102

Table 6.1. Activity of nonrescuable N genes in the BUNV minigenome system.

N genes highlighted **in bold** were mutated specifically.
"Con" indicates conserved residue. (+) conserved residue among at least 20 orthobunyavirus N proteins, (++) conserved residue among at least 30 orthobunyavirus N proteins, (+++) conserved residue among at least 40 orthobunyavirus N proteins, (++++) conserved residue among all orthobunyavirus N proteins.
%: Activity of mutant N proteins compared to 100% activity of wt BUNV N protein.

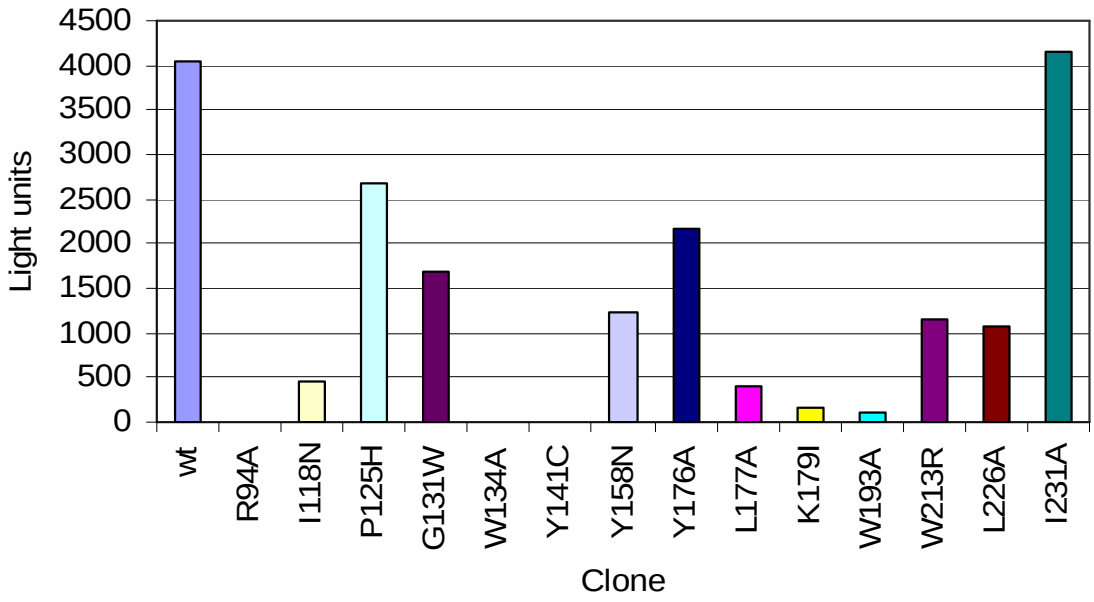


Figure 6.2. Activity of nonrescuable mutant N genes in the BUNV minigenome system.

BSR-T7/5 cells were transfected with 0.2µg of pT7riboBUNN or mutant clone, 0.2µg of pT7riboBUNL, 0.2µg of pT7riboBUNM*Renilla* (-) and 0.1µg pT7-FF-luciferase. Cells were incubated overnight at 33˚C and were lysed and luciferase activity was measured.

6.2. Quick-Change Directed mutagenesis on pTM1BUNN construct

As mentioned previously, both random and targeted mutagenesis were performed on plasmid pT7riboBUNN rather than plasmid pTM1BUNN. The pT7riboBUNN construct is an antigenome expression plasmid which contains the viral sequence cloned in the positive sense between the T7 promoter and hepatitis δ ribozyme, followed by the T7 terminator immediately downstream. It is designed for use in the BUNV rescue system to further investigate the functionality of the N protein. The pTM1BUNN plasmid contains the N ORF cloned between the EMCV IRES and T7 terminator. It is designed for protein expression and its expression is 10-fold higher than pT7riboBUNN due to the IRES sequence. For protein-protein interaction studies the pTM1BUNN plasmid was considered more appropriate, and hence a new series of mutagenesis reactions was performed on pTM1BUNN to generate the same nonrescuable N proteins.

6.3. Co-immunoprecipitation

Mutant N proteins R94A, W134A, Y141C, K179I and W193A were inactive in the BUNV minigenome system, displaying less than 5% of the wt BUNV N protein activity. One reason might be that these residues are involved in interaction with L polymerase. To test this hypothesis, BSR-T7/5 cells were transfected with 0.1µg each of pTM1BUNN or mutant clone, pTM1BUNL and 0.2 µg of pT7riboBUNM*Renilla* (-) using Lipofectamine-2000 for 5 hours at 37˚C. The transfection mixture was replaced with fresh medium, and the cells were incubated at 33˚C overnight. Coimmunoprecipitation was performed using antibodies against N protein as described in section 2.2.2.8. Two strong bands L and N were visualised in BUNV-infected cells or cells transfected with wt BUNV N and L clones, indicating that N protein interacts effectively with the L protein (Figure 6.3). The band above the L band was observed in all transfected cells including the mock-transfected cells which suggests it is a cellular band. In general, the mutant N proteins interacted less effectively with L protein compared to the wt N protein. However, the weakest interactions were observed when mutant N proteins I118N, W134A and Y141C were used.

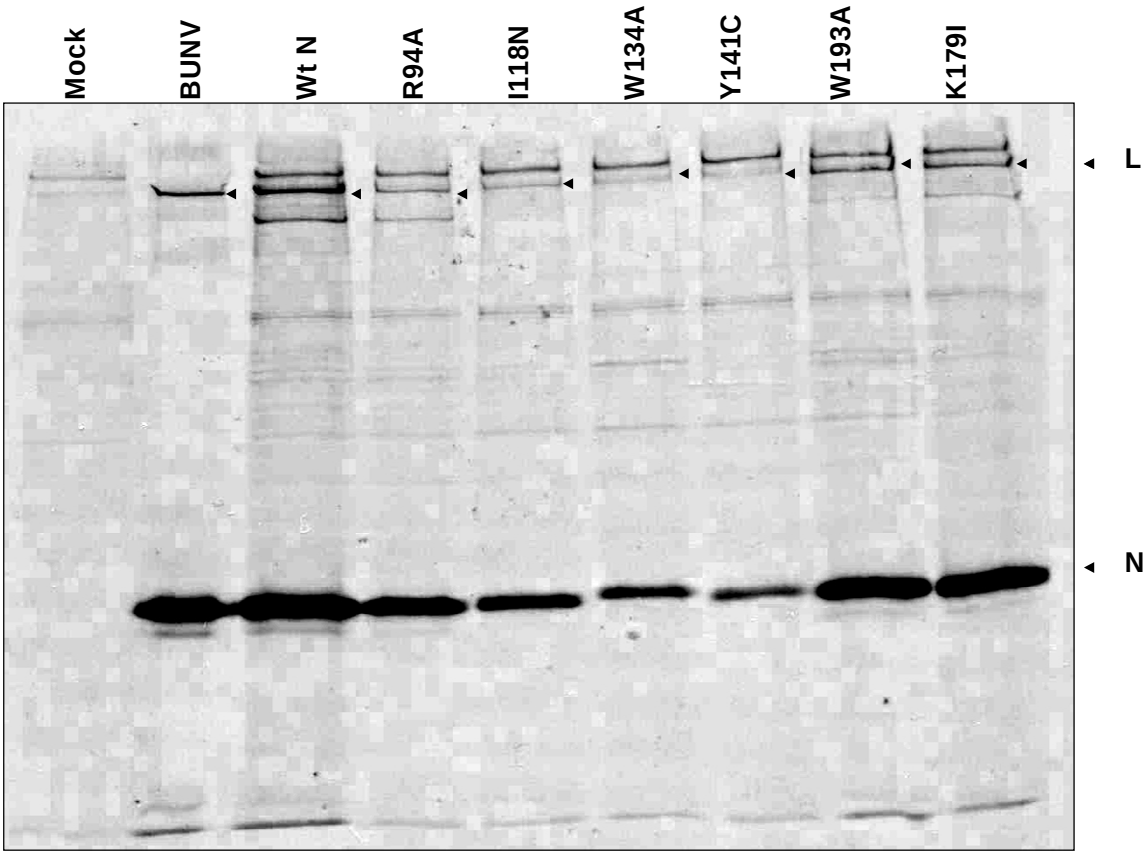


Figure 6.3. Co-immunoprecipitation of BUNV L and N proteins.

BSR-T7/5 cells were transfected with 0.1 µg of pTM1BUNN or mutant clone, 0.1 µg of pTM1BUNL and 0.2µg of pT7riboBUNM*Renilla* (-). Cells were incubated overnight at 33˚C and coimmunoprecipitation performed using anti BUNV N protein antibodies.
Mock: mock transfected BSR-T7/5 cells
BUNV: BUNV -infected Vero-E6 cells was used as marker
Band above L is cellular band

	L band density AU/mm^2	**N band density AU/mm^2**	**Ratio N/L**	**Amount of N** (%)
wt N	109461.6	335845.7	3.0:1.0	100
R94A	34957.5	303406.5	9.0:1.0	90
I118N	33960.4	210920.3	6.0:1.0	63
W134A	27863.6	162885	6.0:1.0	48
Y141C	24420.1	121062.6	5.0:1.0	36
W193A	67649.7	322747.5	5.0:1.0	96
K179I	69049.9	307746.6	5.0:1.0	91

Table 6.2. Ratio density of N to L bands and amount of mutant N proteins to wt N protein using densitometry.

AU: absorbent units

At this stage, it was not clear whether the poor interactions observed were due to low amounts of mutant N proteins expressed or to binding deficiency to L. To determine the latter hypothesis, the ratio of the density of N to L bands, and density of mutant N protein bands to wt N protein band were measured (Table 6.2). The ratio of wt N to wt L bands was 3:1. Overall, mutant N proteins displayed ratio ≥ 5:1. The highest ratio was found when mutant N protein R94A was used. It displayed ratio 9:1 although the amount of mutant N protein R94A expressed represented 90% of the amount of wt N protein. Mutant N proteins I118N, W134A and Y141C showed the lowest amount of N protein expressed 63%, 48% and 36% respectively of the amount of wt N protein. They showed almost similar ratios to L of 5-6:1. Conversely, mutant N proteins K179I and W193A showed 91% and 96% of the amount of wt N protein respectively but they displayed similar ratios to mutant N proteins I118N, W134A and Y141C.

6.4. Multimerization of nonrescuable mutant N proteins

The ability of individual N molecules to form multimers was tested using a cross-linking experiment (Leonard *et al*., 2005). Vero-E6 cells were infected with vaccinia virus vTF7-3 at MOI of 1 for 1 hour at 37˚C. The virus was removed and cells were transfected with 1 µg of pT7riboBUNN or mutant clone. The cells were treated with 1mM DSP, a cross-linking agent that creates a disulfide bridge between two reactive primary amines. As the bridge contains thiol-thiol interactions it can be broken by the addition of β-mercaptoethanol to the protein loading buffer.

Using the DSP cross-linking agent wt BUNV N protein shows a ladder of protein bands, monomers, dimers, trimers, tetramers and higher multimers towards the top of the gel, with molecular weights corresponding to multiples of 25kDa. Five mutant N proteins (R94A, I118N, W134A and Y141C) were shown to be unable to form multimers (Figure 6.4). In common, all of these are inactive in the BUNV minigenome system so the multimerization results were expected. Mutant N protein W193A was able to form multimers although it was inactive in the BUNV minigenome. Mutant N

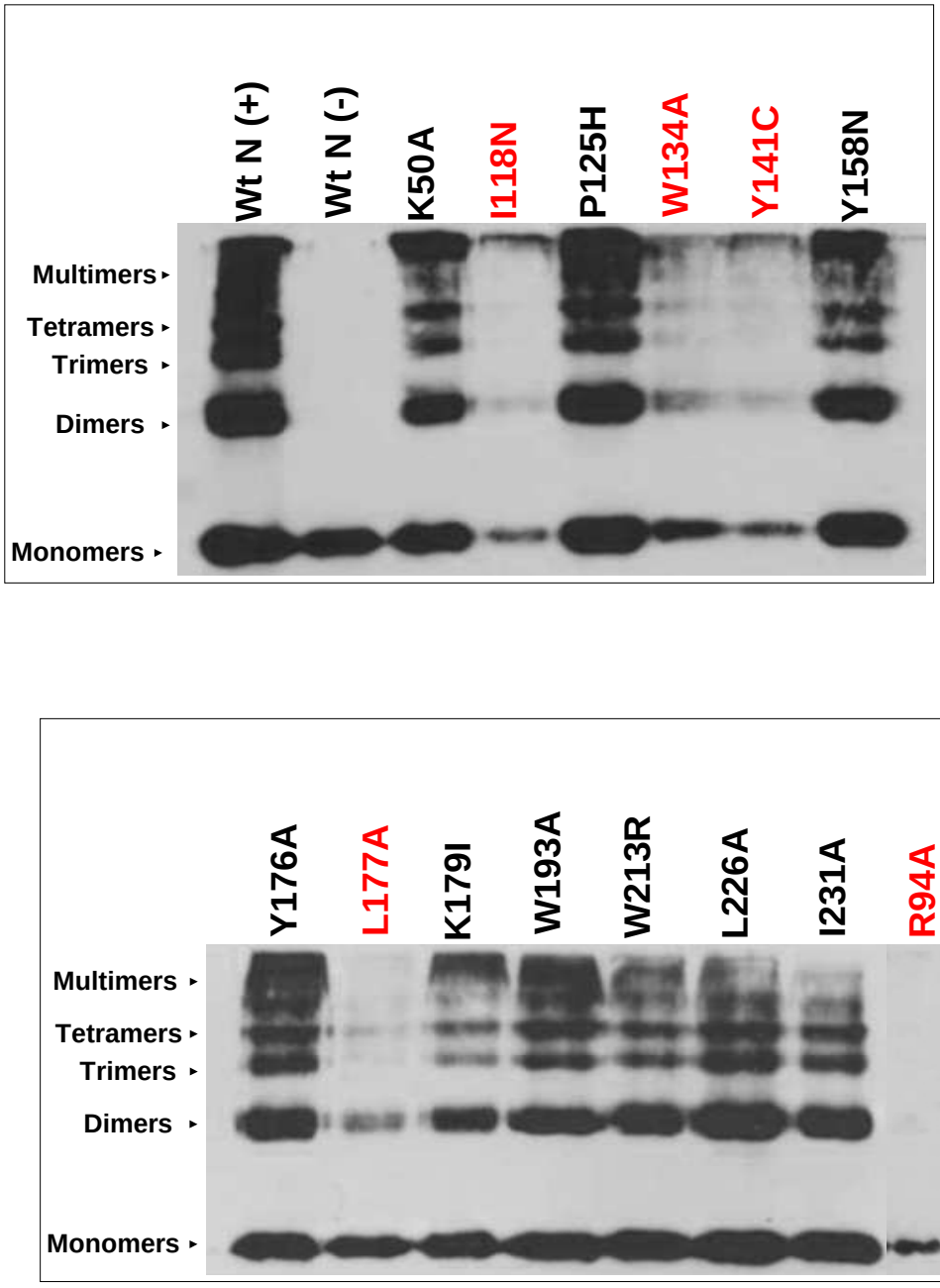


Figure 6.4. Cross-linking of nonrescuable mutant N proteins.

Vero-E6 cells were infected with vTF7-3 and transfected with 1 µg of pT7riboBUNN or mutant plasmid. N proteins were cross-linked using PBS containing 1mM DSP. The samples were then electrophoresed on a 12% protein gel. To break down x-links, β-mercaptoethanol was added to the protein loading buffer as negative control lane Wt N (-).

	N monomer band density AU/mm^2	**Mutant N / wt N (%)**
wt	435655	100
R94A	140246	32
I118N	73795	17
P125H	425701	98
W134A	197001	45
Y141C	102100	23
Y158N	400423	92
Y176A	296304	68
L177A	230255	53
K179I	370759	85
W193A	338177	77
W213R	35478	81
L226A	393326	90
I231A	356695	82

Table 6.3. Relative expression levels of mutant N protein monomers using densitometry.

proteins L177A, K179I and I231A were seen to be deficient in higher multimerization. Mutant N protein L177A formed a decent monomer signal and a very weak signal of dimmers, but no further multimers. Mutant N proteins K179I and I231A were not able to form oligomers. All functional mutant N proteins in the BUNV minigenome system were shown to be multimerization competent.

Measuring the density of the N monomer bands by densitometry as a measure of expression efficiency (Table 6.3) showed that mutant N proteins R94A, I118N, W134A and Y141C displayed 32%, 17%, 45% and 23% of the amount of the wt N protein respectively. These mutant N proteins that failed to form high multimers (L177A, K179I and I231A) showed no expression deficiency displaying 53%, 85% and 82% of the amount of the wt N protein respectively.

6.5. RNA synthesis by nonrescuable mutant N genes

It is possible that some mutant N genes were not recovered due to RNA synthesis deficiency during the rescue experiment. In the BUNV rescue system, the pT7riboBUN-N, -M and -L constructs are transcribed by the T7 polymerase to produce full length transcripts. The transcripts contain hepatitis δ ribozyme sequence which promotes a self-cleave to produce the full length antigenomic viral RNAs. The latter is encapsidated by the N protein and is used as a template to produce full length genomic RNAs.

To investigate whether there was a deficiency in RNA synthesis, BSR-T7/5 cells were transfected with 0.2 µg of each pT7riboBUNN or mutant clone, pT7riboBUNM and pT7riboBUNL using Lipofectamine-2000 (Invitrogene) for 5 hours at 37˚C. The transfection mixture was then removed and replaced with fresh medium. The cells were incubated overnight at 33˚C. RNA extraction was performed using TrIzol reagent and a northern blotting analysis was carried out. Using a positive-sense DIG-labelled probe the S genomic RNA can be detected. In a separate blot a negative-sense DIG-labelled probe was used to detect the S primary T7 transcript, and the antigenomic RNA produced after processing by the ribozyme.

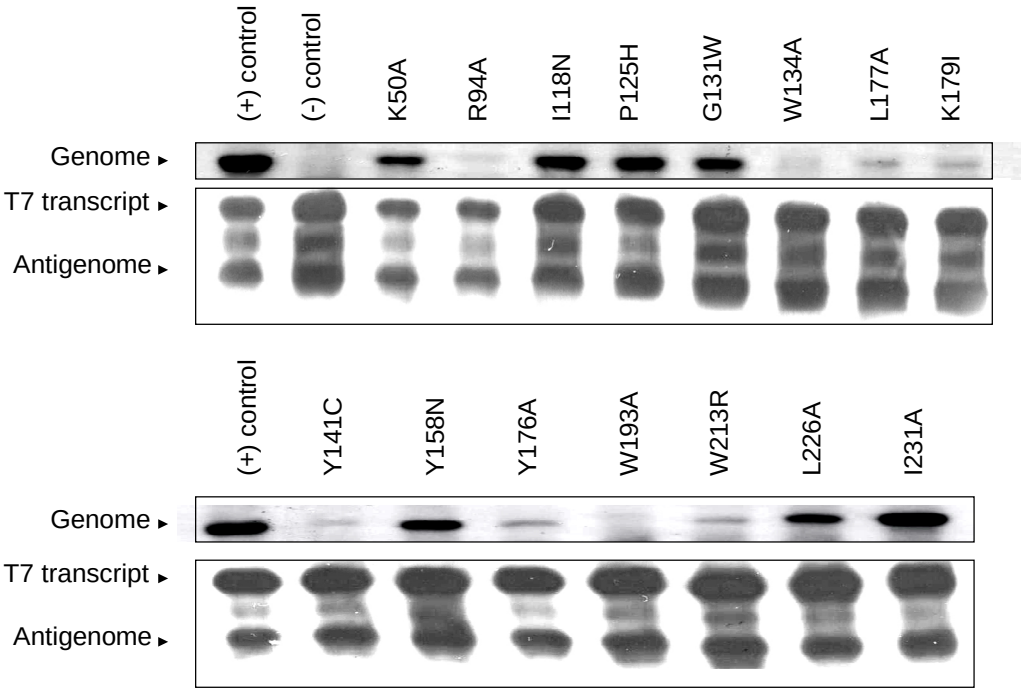


Figure 6.5. RNA synthesis by nonrescuable mutant N genes.

BSR-T7/5 cells were transfected with 0.2 µg of each pT7riboBUNN or mutant clone, pT7riboBUNM and pTriboBUNL. After an overnight incubation at 33˚C total RNA was extracted using TrIzol reagent and northern blotting analysis was performed. To detect the S segment primary transcript and antigenomic RNA, a negative DIG-labelled probe was used. Using a separate blot, a positive DIG-labelled probe was used to detect the S genomic RNA. As a negative control of the genomic RNA synthesis, BSR-T7/5 cells were transfected only with pT7riboBUNN. After an overnight incubation at 33˚C total RNA was extracted using TrIzol reagent and northern blotting analysis was performed. Using a negative DIG-labelled probe primary T7 transcript and antigenome can be detected. Genomic RNA can not be detected when a positive DIG-labelled probe was used since there was no replication took place.

All constructs produced both the unprocessed T7 transcript and the processed antigenome RNA An additional band was detected, when the negative-sense DIG-labelled probe was used, between the full length transcript unprocessed by ribozyme and the full length genomic RNA; the origin of this band is unknown (Figure 6.5).

Six mutant N genes (I118N, P125H, G131W, Y158N, L226A and I231A) showed no deficiency in genomic RNA synthesis. However, mutations at residues R94A, W134A, Y141C, Y176A, L177A, K179I, W193A and W213R affected genomic RNA synthesis in that the hybridization signal was weak or badly detectable.

6.6. Modification of a BUNV packaging assay

A BUNV packaging assay was described previously (Shi *et al*., 2007). Briefly, BSR-T7/5 cells are cotransfected with three pTM1-based plasmids encoding the viral structural proteins, pT7riboBUNM*REN*(-) (BUNV minigenome) as well as pTM1-FF-Luc as an internal control. At 24h post-transfection, cells are lysed and assayed for luciferase activity as described by Kohl *et al*. (2004). The culture medium is cleared of cellular debris and 1.5ml is transferred onto new BSR-T7/5 cells transfected 3 - 5 hrs earlier with pTM1BUNL and pTM1BUNN. Cells are incubated overnight and assayed for *Renilla* luciferase activity.

This assay was modified as follows; BSR-T7/5 cells were transfected with three pT7ribo-based plasmids encoding L, M and N viral proteins, the BUNV minigenome plasmid and pT7-FF-Luc as an internal transfection control. After 24h incubation at 33˚C, cells were lysed and assayed for luciferase activity. The culture medium was cleared of cellular debris and 1.5ml used to infect naive BSR-T7/5 or BHK-21 cells (*i.e*. cells that had not been pretransfected as in the original method). The cells were incubated overnight at 33˚C and assayed for *Renilla* luciferase activity. Firstly, the affect of using mock or pretransfected BSR-T7/5 cells on *Renilla* luciferase activity was compared (Figure 6.6). *Renilla* luciferase activity was easily measured when the infectious supernatant was transferred to mock BSR-T7/5 cells rather cells pretransfected with L and N plasmids, giving about 40% activity obtained when viral L and N proteins were previously expressed. The modified protocol was used as it reduces the amount of plasmids needed and saves time.

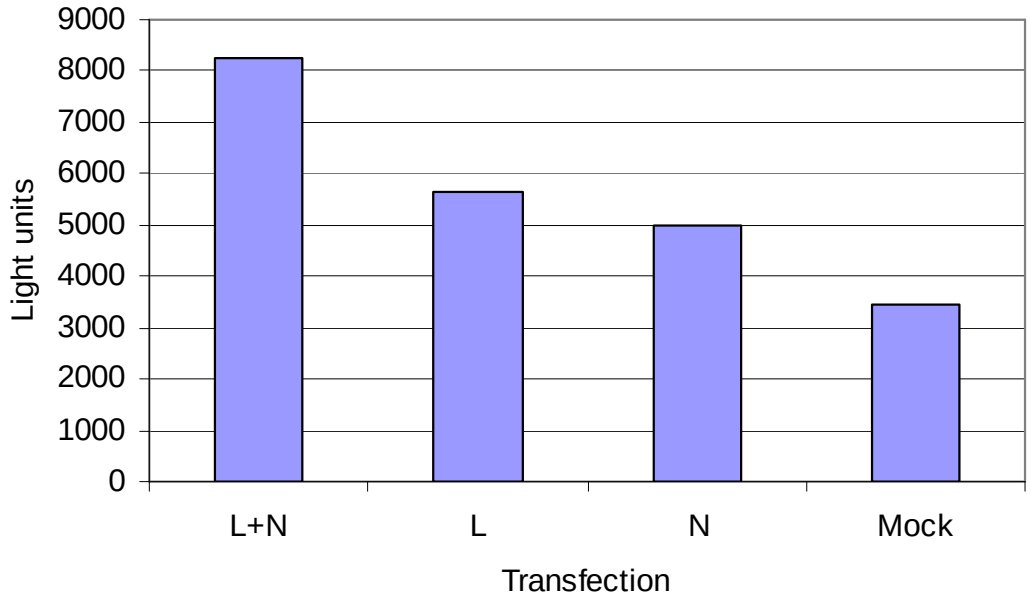


Figure 6.6. The affect of using mock or pretransfected BSR-T7/5 cells on the activity of BUNV minigenome.

BSR-T7/5 cells were transfected with 0.2µg of each pT7riboBUNN, pT7riboBUNL, pT7riboBUNMREN(-), 0.1µg pT7riboBUNM and 0.1µg pT7-FF-luc. At 24hr postinfection, the supernatant was clarified and 1.5ml was transferred onto BSR-T7/5 cells pretransfected with pT7riboBUNL and N, just pT7riboBUNL, just pT7riboBUNN or mock transfected. Luciferase activity was measured 20 hrs later.

6.7. Activity of nonrescuable mutant N proteins in BUNV packaging assay (virus-like particle assay)

Only the functional mutant N proteins (P125H, G131W, Y158N, Y176A, W213R, L226A and I231A) in the BUNV minigenome system were used in the BUNV packaging assay to test their ability to form infectious virus-like particles (VLPs) (Table 6.4 and Figure 6.7). Packaging activity was taken as 100% when the wt N protein was used. The weakest packaging activity was observed when mutant N proteins Y158N, W213R or I231A were used in this assay displaying only 12% of its transcription and replication activity. Mutant N proteins P125H, G131W, Y176A and L226A displayed packaging activity of 29%, 42%, 36% and 66% respectively.

6.8. Discussion

Fourteen mutant N genes were nonrescuable. Seven mutant N proteins (R94A, I118N, W134A, Y141C, L177A, K179I and W193A) were very weak or inactive in BUNV minigenome system, displaying between 0.17-11% of the wt N protein activity. The remaining 7 mutant N proteins (P125H, G131W, Y158N, Y176A, W231R, L226A and I231A) were functional in BUNV minigenome system, showing between 25-95% of the wt N protein activity (Table 3.16 and Figure 2.24). The failure to rescue viruses containing these mutations in the N protein could be due to deficiency in N-L interaction, N-N molecules interaction, N-RNA interaction or N-glycoproteins interaction.

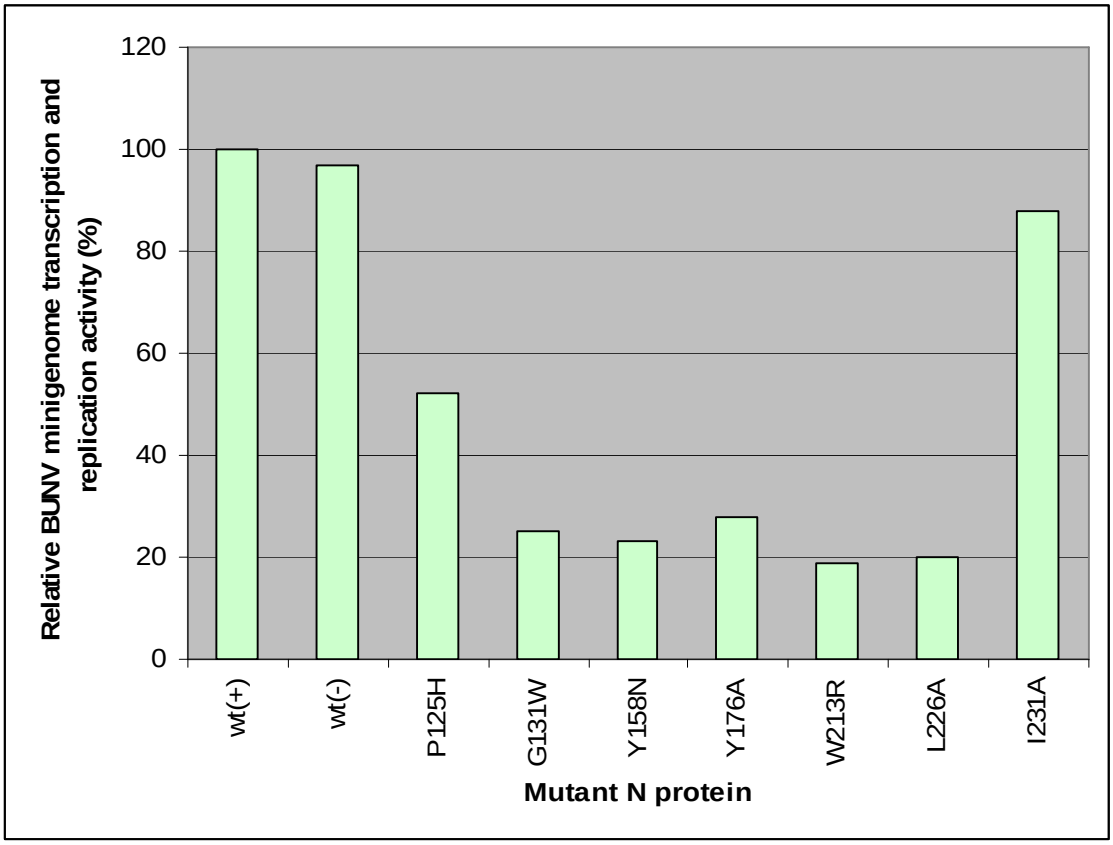
The residues thought to be involved in interaction with BUNV L polymerase were R94A, I118N, W134A, Y141C, K179I and W193A since they were almost inactive in the BUNV minigenome system. Coimmunoprecipitation results (Figure 2.25) showed the latter mutant N proteins have deficiency in interaction with L protein but to different degrees. Interaction of mutant N proteins I118N, W134A and Y141C with L protein was very weak. Two possibilities may explain this weakness, either these mutations affected N-L interaction directly or the mutant N proteins were being degraded which affected the amount of N protein which in turn affected N-L interaction. The residues most probably affecting interaction of N-L were R94A, K179I and W193A since the N bands of these mutant protein were almost equivalent

N gene	Minigenome transcription and replication activity Light units (a)		Minigenome packaging activity Light units (b)		
	Firefly	*Renilla*	Firefly	*Renilla*	(%)
wt (+)	2242	3258	2.0	2458	100
wt (-)	2239	3164	1.4	5.5	0.26
P125H	1723	1680	1.3	375	29
G131W	2290	810	1.7	258	42
Y158N	1860	736	1.1	69	12
Y176A	2313	915	1.4	256	36
W213R	1886	630	1.0	56	12
L226A	2303	639	1.2	321	66
I231A	2140	2390	1.1	213	12

Table 6.4. Normalized BUNV minigenome packaging activity

BSR-T7/5 cells were transfected with pT7riboBUNN or mutant clone, pT7riboBUNM and pT7riboBUNL plasmids encoding BNUV N, M and L viral proteins, pT7riboBUNMRenilla(-) (minigenome) and pT7-FF-luciferase as internal control of transfection efficiency using Lipofectamine-2000 reagent for 5 hrs at 37˚C. Transfection mixture was then removed and replaced with 2ml fresh medium and cells were incubated overnight at 33˚C. The supernatant was then collected and cleared from cell debris by centrifugation. Luciferase assay was performed on cells and the results represent BUNV minigenome transcription and replication activity (a). The previous supernatant was transferred onto mock BSR-T7/5 cells and incubated overnight at 33˚C. Cells were lysed and luciferase assay was performed. The obtained results represent BUNV minigenome packaging activity (b). The negative control of the packaging activity parental (-) was transfection of all the above plasmids except pT7riboBUNM which encodes BUNV glycoproteins so only BUNV minigenome transcription and replication can be measured and no VLPs are produced.

(a)


Relative BUNV minigenome transcription and replication activity (%)
120
100
80
60
40
20
0
wt(+)
wt(-)
P125H
G131W
Y158N
Y176A
W213R
L226A
I231A
Mutant N protein

(b)

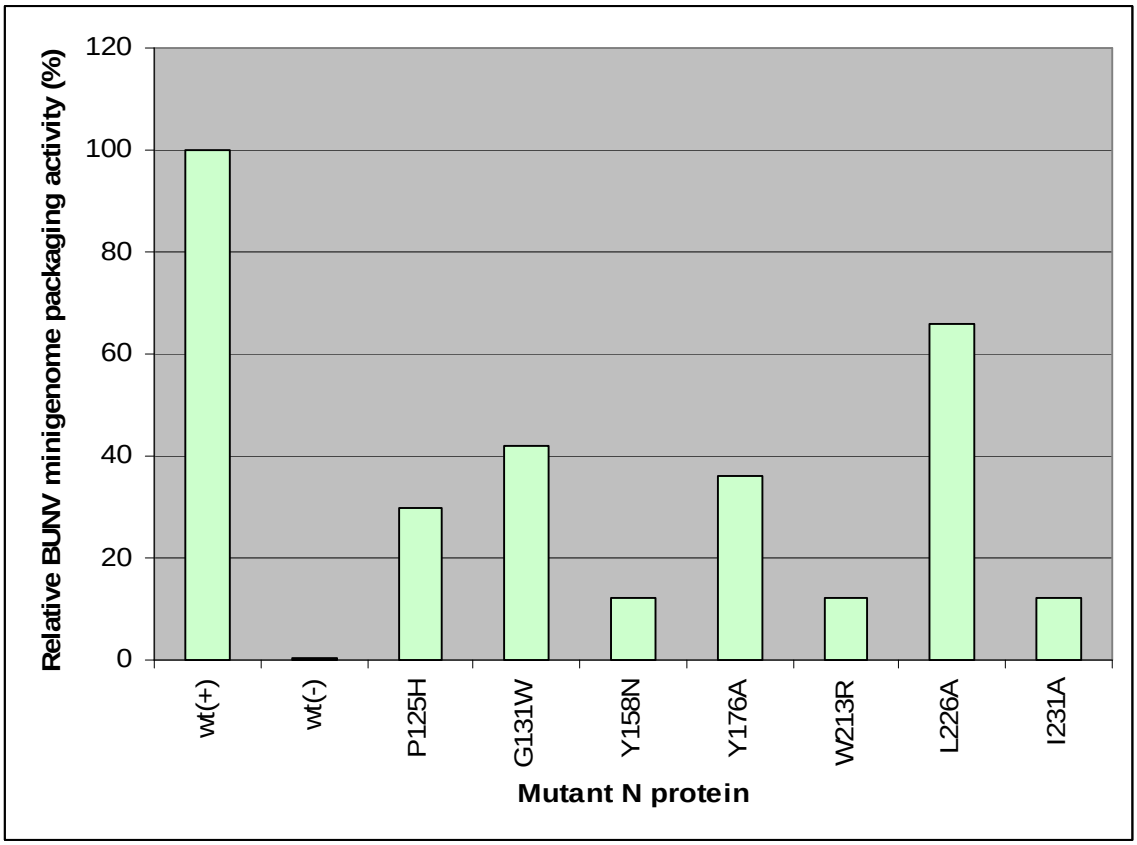


Figure 6.7. BUNV minigenome transcription and replication activity (a), BUNV minigenome packaging activity (b).

to the wt N band but the L protein bands were weaker than those produced when the wt N protein was used . To confirm the above hypothesis, the ratio of the density of the bands (N to L) calculated by densitometry and density of mutant N proteins bands compared to wt N protein band, were determined (Table 3.17). The weakest N-L interaction was observed when mutant N R94A was used, since the ratio of mutant N protein R94A to L protein was about 9:1, that is three fold higher than the ratio of wt N protein to L protein (3:1). The amount of mutant N protein R94A presented 90% of the amount of the wt N protein. This suggests that residue R94A affects interaction of N-L directly. Mutant N proteins I118N, W134A and Y141C showed similar ratios 5-6:1, which is twice that shown by wt N protein, but the amounts of mutant N protein were 63%, 48% and 36% respectively of the amount of wt N protein. This could explain why weak interaction was observed between the latter mutant N proteins and L protein. These mutant N proteins might be degraded and further investigation needs to be done, for example using the proteosome inhibitor MG-132 to find out whether the low amount of mutant N protein was due to cellular degradation or expression deficiency in the first place. Residues K179I and W193 apparently seem less involved in interaction with L protein than residue R94A, and displayed ratio of 5:1 although expression of the mutant N proteins was almost equivalent to wt N protein.

Using the cross-linking reagent DSP, Leonard *et al*. (2005) found that deletion of the first 10 aa of BUNV N protein affected N multimerization. BUNNdel1-10 was unable to form oligomers beyond dimers compared to the wt N protein. Furthermore, a mutant BUNV N protein lacking the last 17 aa was generated. BUNNdel217-233 showed the ability to form dimers and weak signals of trimers but not higher multimers. This study suggested that BUNV N protein molecules form head-head and tail-tail interactions. The ability of the nonrescuable mutant N proteins to form multimers was tested using cross-linking experiment (Figure 2.26). Mutant N proteins R94A, I118N, W134A and Y141C were weakly active or inactive in BUNV minigenome system and their ability to form oligomers was unlikely since they might affect protein expression or stability in the first place. This was confirmed by measuring the density of the N monomer bands of mutant N proteins as a measure of expression efficiency. They displayed 32%, 17%, 45% and 23% of the amount of the wt N protein respectively. Surprisingly, mutant N protein W193A was inactive in

BUNV minigenome but still able to multimerize. Previously, coimmunoprecipitation showed interaction of mutant N protein W193A with L was weak which could explane the disfunctionality of mutant N protein W193A in BUNV minigenome system. Clearly residue W193 is not involved in multimerization of BUNV N protein. Mutant N proteins L177A, K179I and I231A were unable to form high multimers. The amount of mutant N proteins expressed were 53%, 85% and 82% respectively. The failure of mutant N protein L177A in multimerization could be due to low level of expression but this is not the case for mutant N proteins K179I and I231A since they displayed more than 80% of the amount of the wt N protein.

RNA synthesis of the nonrescuable mutant N genes was carried out. It was though, those mutant N proteins that fail to form multimers (R94A, I118N, W134A, Y141 and L177A) were more likely to show deficiency in RNA synthesis since the antigenomic RNA must be encapsidated by N protein to be used as a template for genomic RNA synthesis, a finding that was subsequently confirmed with exception of mutant N protein I118N (Figure 2.27). Although mutant N protein I118N showed an inability to form multimers, it had no affect on genomic RNA synthesis. The latter residue might affect mRNA synthesis since it was very weak in BUNV minigenome system. Despite mutant N protein W193A being able to form multimers, mutant N gene carrying this mutation was incompetent for genomic RNA synthesis. Moreover, mutant N proteins Y176A and W213R were functional in BUNV minigenome, competent in multimerization but they affected genomic RNA synthesis. As mutant N proteins R94A, I118N, W134A, Y141C, L177A, K179I and W193A were inactive in the BUNV minigenome system and affected genomic RNA synthesis , there was no need to introduce them to the BUNV packaging assay since it was obvious that these mutations affect the N protein functionality prior to the packaging step (transcription or replication). On the other hand, the nonrescuable mutant N proteins (P125H, G131W, Y158N and I231A) were functional in the BUNV minigenome system, able to form multimers and had no impact on RNA synthesis. This suggested that these residues might affect N protein functionality beyond transcription and replication.

In other words, these mutant N proteins encapsidate the BUNV minigenome RNA and transcriptionally active RNP is produced. To test the ability of RNP to be packaged into VLPs, the above mutant N proteins were used in the BUNV packaging assay (Table 3.18 and Figure 2.29). BUNV minigenome showed a dramatic decline

in its packaging activity when mutant N proteins Y158N, W213R and I231A were used, displaying only 12% of the initial activity (transcription and replication activity). The latter residues might be involved in interactions with the C-tails of the glycoprotein. Residue L226 had no impact in BUNV minigenome packaging activity since its packaging activity 3-fold higher than transcription and replication activity. Likewise, residue G131W displayed no impact in BUNV minigenome packaging activity. When mutant N protein G131W was used in this assay BUNV minigenopme displayed packaging activity twice its transcription and replication activity. In general, mutant N proteins G131W, Y176A and L226A did not affect BUNV minigenome packaging activity since the minigenome displayed packaging activity higher than transcription and replication activity. In common, mutant N proteins G131W, Y176A and L226A behave almost similarly in BUNV minigenome system displaying 24%, 28% and 20% respectively of the wt N protein activity, were able to multimerize, did not affect genomic RNA synthesis (except Y176A) and did not affect packaging of the minigenome. Residues G131W and L226A might be involved in N-RNA interaction.

6.9. Summary

The nonrescuable mutant genes were characterized *via* their activity in the BUNV minigenome system, their ability to form multimers, their ability to interact with L protein, their impact in RNA synthesis and their impact in packaging of the minigenome (Table 6.5).

1- Residues W134A and Y141C might affect N protein stability.
2- Residue I118N might affect mRNA synthesis
3- Residue R94A could be involved in interaction with L protein.
4- Residues Y176A, L177A, K179I, W193A and W213R are involved in genomic RNA synthesis.
5- Residues G131W, Y176A and L226A might be involved in N-RNA interaction.
6- Residues Y158N, W213R and I231A might be involved in interactions with the C-tail of the glycoproteins.

N gene	Con (%)	Minigenome transcription & replication Activity (%)	Ability to form multimers	Genomic RNA synthesis	Mimigenome packaging activity (%)
R94A	100	0.17	-	-	-
W134A	100	1.9	-	-	-
Y141C	100	1.8	-	-	-
L177A	68	10	-	-	-
K179I	100	4.0	-	-	-
I118N	43	11	-	+	-
I231A	78	102	-	+	12
Y176A	41	53	+	-	36
W193A	100	3.0	+	-	-
W213	100	28	+	-	12
P125H	100	66	+	+	29
G131W	100	30	+	+	42
Y158N	78	25	+	+	12
L226A	72	26	+	+	66

Table 6.5. Summary of characterization of nonrescuable mutant N proteins.
Con: Conservation among 51 orthobunyavirus N proteins

Chapter 7. Conclusions

7.1. BUNV N protein functional domains in advance of its 3D structure

Previously, it has been demonstrated that the first 10 aa and the last 17 aa of BUNV N protein are important for N molecule multimerization (Leonard *et al*., 2006). The aim of this project was to determine the linear domain map of BUNV N protein in advance of its 3D structure determination. The introduction of 102 single amino acid substitutions in BUNV N protein sequence enabled me to determine some crucial residues in BUNV N protein functionality (Figure 7.1). Using several established techniques, this work demonstrates that single amino acid changes in the BUNV N protein can cause:

- Disruption of BUNV minigenome transcription and replication (e.g. W134A, Y141C)
- Disruption of BUNV-like particle assembly (e.g. I231A).
- Disruption of BUNV genomic RNA synthesis (e.g. W193A, K179I).
- Reduction of virus titer 100000-fold (e.g. F6A, M150A).
- Production of distinct plaque phenotypes (small, medium or large).
- Generation of ts mutant viruses (e.g. N74S, K22T).

It is very clear from the above observations that the BUNV N protein is multifunction, being involved in several interactions: N-N molecules to form chain of N molecules, N-L polymerase during virus transcription and replication, N- RNA backbone and N-C tail of the glycoproteins during virus assembly underneath the Golgi membrane.
It is possible that residues in the C-terminus of N protein come together with residues in the middle performing the same function when the N protein is folded. The 3D structure determination is only the way to demonstrate this hypothesis. So far, residues in the middle of BUNV N protein (aa 94 -158) and the C-terminus (aa176-231) appear crucial for N protein functionality with single mutations in these regions causing marked disruption to several specific functions of BUNV N protein.

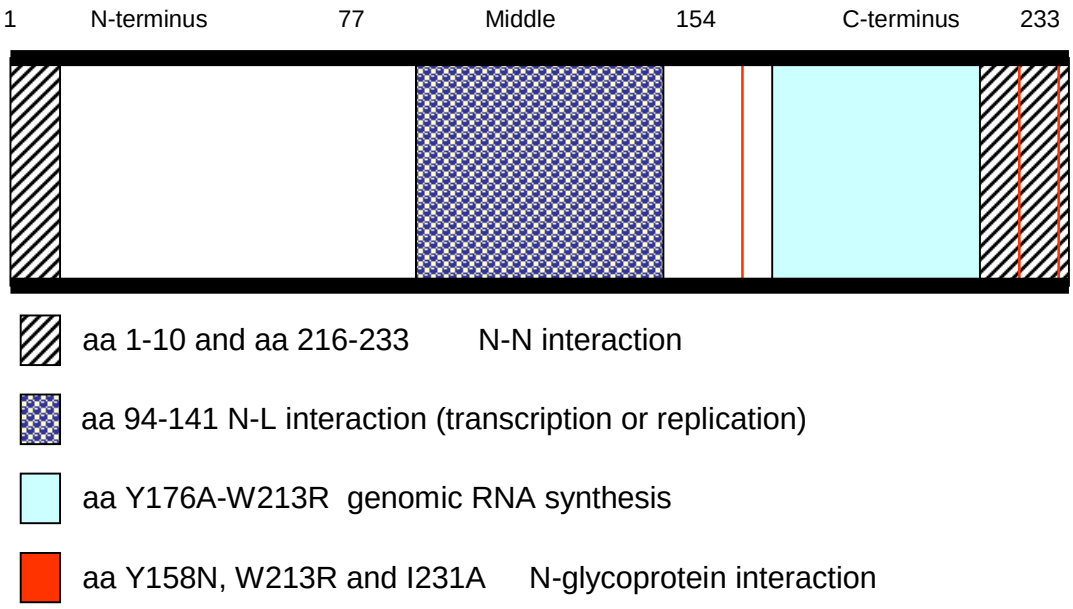


Figure 7.1. Preliminarily BUNV N protein domain map

7.2. 3D structures of NSVs

The molecular design of all single-stranded, negative-sense RNA viruses is essentially similar. Segmentation of the virus genome is a common, although not universal, feature of such viruses. The RNA genome and a basic nucleocapsid (N) protein condense together in the infected cell to form a helical structure. The individual N protein monomer covers about nine nucleotides of the RNA genome and protects it from chemical and physical injuries. An outer lipid envelope derived from the host cell membrane surrounds the nucleocapsid core.

This category includes many of the best known human pathogens such as influenza virus (*Orthomyxoviridae*), rabies virus (*Rhabdoviridae*), and measles virus (*Paramyxoviride*). By comparing the nucleocapsid proteins of these infectious agents we may finally speculate about the 3D structure of the BUNV N protein.

Influenza A virus is a segmented negative sense RNA virus. The N protein (55kDa) is transported from the cytoplasm of infected cells to the nucleus *via* two nuclear localization signals, residues 1-38 (Neumann *et al*., 1997) and residues 198-216 (Weber *et al*., 1998). Using electron microscopy, viral RNPs were observed as supercolied structures containing a terminal loop (Compans *et al*., 1972; Heggeness *et al*., 1982). The genome RNA is susceptible to RNase digestion (Baudin *et al*., 1994). The three-dimensional reconstitution reveals that each ring consists of 9 N protein molecules, and the viral polymerase was visualized as binding directly to two subunits of the ring (Martin-Benito *et al*., 2001) (Figure 7.2). The N molecules of influenza A virus interact through one point of contact (Figure 7.5a) which gives the RNPs more flexibility (Schoehn *et al*., 2004).

RV is an enveloped, non-segmented negative-sense RNA virus. The N-terminus of N is essential for N-N and N-RNA interactions while the C-terminus is involved in interactions with phosphoprotein (P). The 3D structure of RV RNP (Figure 7.3) shows that each ring consists of 10 N molecules (Schoehn *et al*., 2001). The N molecules are bi-lobed in shape with two binding sites (Figure 7.5b) at the top and bottom of each lobe while the middle of each monomer is not involved in N protein

molecule interactions. They assemble into ring structures, each ring consisting of 9 to 11 monomers. 3D reconstruction of N-RNA complexes show the RNA is located on the top of the rings, however, conformational change occurs in N which leads to relocation of the RNA inside N protein. Major differences between N-RNA complexes in infected cells and virions have been observed (Newcomb *et al.,* 1982; Schoehn *et al*., 2001). In the infected cells each ring consists of 15 monomers instead of 10, a result of the longer RNA converting the shape to a loose helical structure. In virions, condensation of the helix causes monomers to from tighter structures of 54 monomers per turn. The latter observation is though to be due to interaction of the matrix protein with the RNP (Newcomb and Brown, 1981, Newcomb *et al*., 1982).
The RNP is resistant to RNase treatment and each N molecule covers 9 nucleotides. Trypsin treatment of RNP caused removal of the C-terminal domain of N, but the RNP structure shows major changes (Kouznetzoff *et al*., 1998) and does not linearize (Iseni *et al*., 1998).

Measles virus (*Paramyxoviridae*) is an enveloped, nonsegmented negative-strand RNA virus. It has been found that the N-terminus of N protein is crucial in N molecule interactions and RNA binding (Buchholz *et al*., 1993). In measles virus N protein, deletion of aa 189-373 disrupted N self-association while aa 240-303 were essential for N protein stability (Bankamp *et al*., 1996). Furthermore, Liston *et al*. (1997) found that two regions of measles N protein (aa 267-367 and the C-terminal 122 amino acids) were involved in interactions with phosphoprotein (P). It has been speculated that the presence of the P protein reduces the possibility of encapsidation of non-viral RNA by N protein (Spehner *et al*., 1997). Study of measles virus N protein by electron microscopy showed that each helical turn consists of 13 N protein molecules (Figure 7.4). Two sites of interaction were detected between each two monomers of N protein (Figure 7.5c). The N-RNA stoichiometry is 6 bases per N molecule (Egelman *et al*., 1989). As for RV, the RNP of paramyxovirus is resistant to RNase degradation.

It is possible that the 3D structure of BUNV has elements of both influenza virus and rabies virus 3D structures since BUNV is segmented and the genome is wrapped by the N protein. BUNV N protein has been preliminarily crystallised by sitting-drop in

solution containing 0.1M acetate buffer pH 6.0, 20% PEG 4K and 0.2M ammonium sulphate (Figure 7.6). Further optimisation for better X-ray diffraction quality crystals is ongoing (P.Li and R.M.Elliott, personal communication). It will be more instructive to map all mutations with new phenotypes in the 3D structure of BUN N protein to produce a clear insight of the real position of such residues. Such data would confirm several hypotheses such as interaction of N molecule by head-to-head and tail-to-tail association (Leonard *et al*., 2005). Moreover, 3D structure determination of BUNV N protein would provide information on the location of the RNA as well as the mechanism of transcription and replication by viral L polymerase.

9-mers ring L polymerase

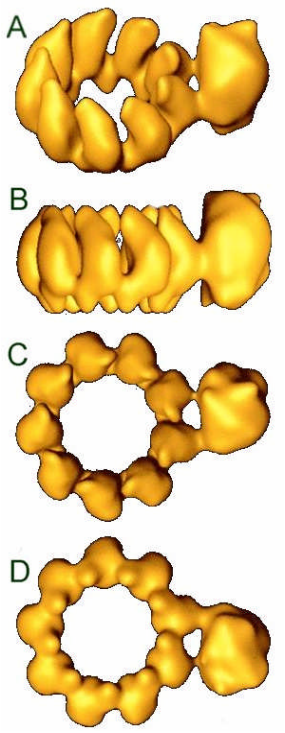
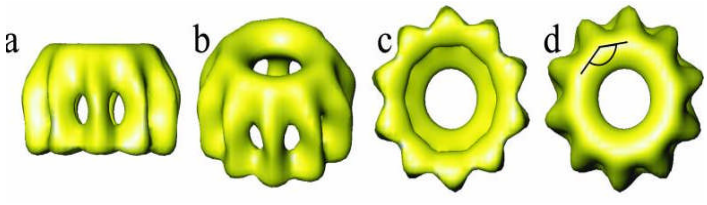


Figure 7.2. Three-dimensional model of a recombinant influenza virus mini-RNP. (A) Perspective view. (B) Side view. (C)Top view. (D) Bottom view (taken from Martin-Benito *et al*., 2001).



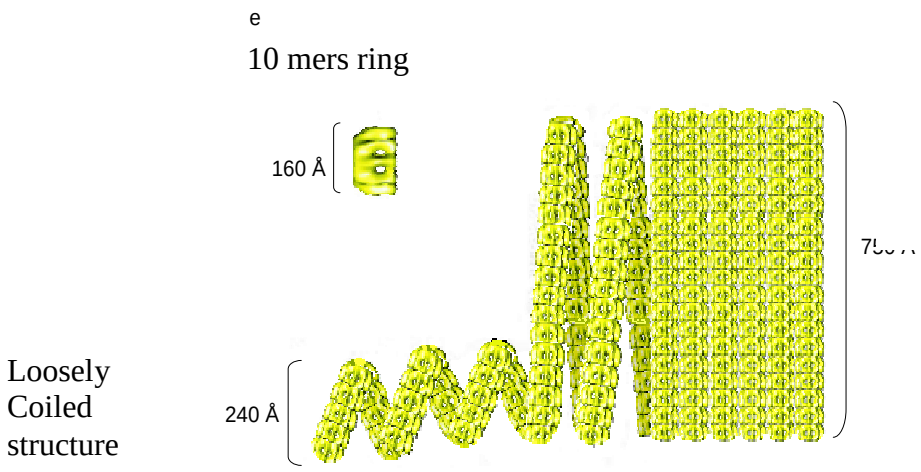
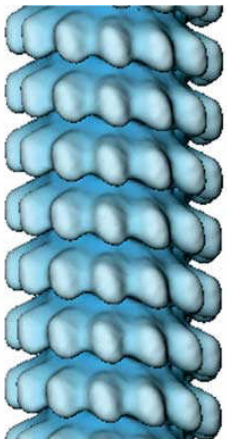


Figure 7.3. Rabies virus N-RNA complexes. 3D of N from the side (a), tilted (b), from the bottom(c), and from above (d), helical structure side view with an indication of the diameters (e) (taken from Schoehn *et al*., 2001).

a



b

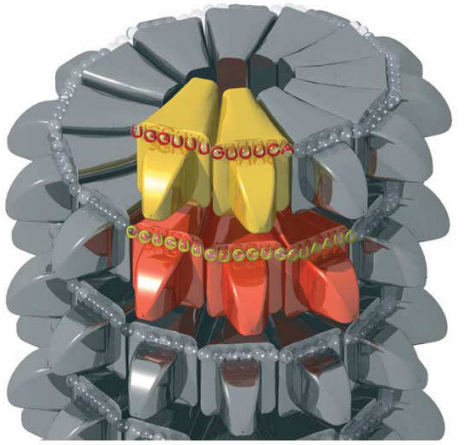


Figure 7.4. 3D structure of recombinant measles virus nucleocapsid protein. Helical structure from the surface with 54Å pitch nucleocapsid comprising 13.04 subunits per turn (a), schematic representation of measles RNP (b) (taken from Behlla *et al*., 2004).

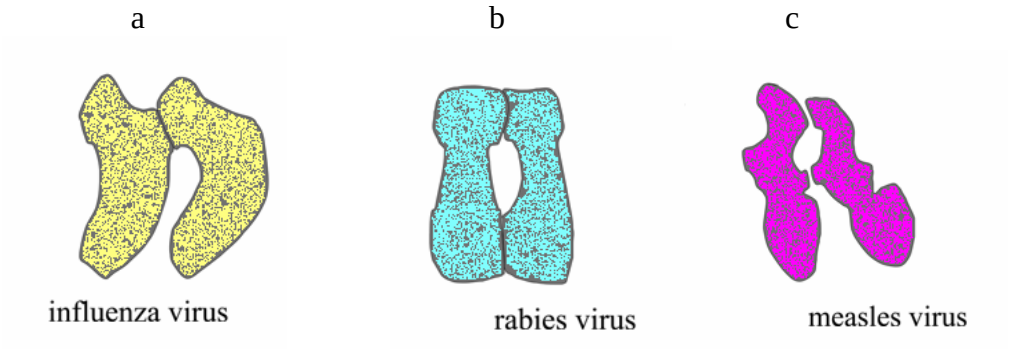


Figure 7.5 . Interaction domains of NSV N proteins. Influenza virus N molecules interact through a single point of contact (a), there are two points of contact between the N molecules of rabies and measles viruses (b and c) (taken from Schoehn *et al*., 2004).

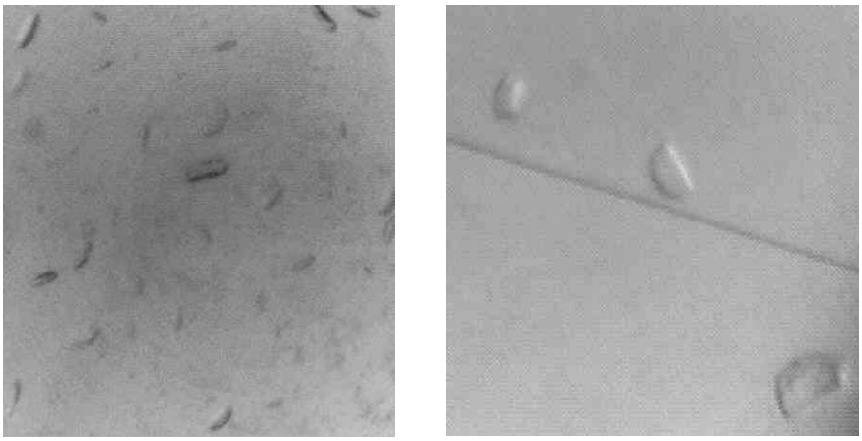


Figure 7.6. BUNV N protein crystals (Courtesy of Dr. Ping Li).

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