Replication of Bunyamwera virus in *Aedes albopictus* C6/36 cells: establishment and maintenance of a persistent infection

by

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Summary

Bunyamwera virus is a member of the Bunyaviridae, a large family of mainly arthropod-borne viruses which possess a tri-segmented RNA genome comprising three segments of single-stranded RNA, designated L (large), M (medium) and S (small). There are five recognised genera within the Bunyaviridae-*Bunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus*. Bunyamwera virus is the prototype virus of the *Bunyavirus* genus and each of its genome segments are of negative polarity.

In common with other arboviruses Bunyamwera virus replicates in its vector species (the mosquito) without causing overt detrimental effects and establishes a persistent infection which lasts for the life time of the mosquito. Upon transmission to a susceptible vertebrate host, Bunyamwera virus causes an acute infection. These disparate host-dependent outcomes of infection can be reproduced in tissue culture. Bunyamwera virus undergoes a lytic replication cycle in mammalian cells which results in cell death, but establishes a long-term, non-cytocidal, persistent infection in cultures of mosquito cells. In this thesis, the molecular mechanisms leading to the establishment of persistent Bunyamwera virus infection of a cloned mosquito cell line, *Aedes albopictus* C6/36 (C6/36) cells, and the factors responsible for the maintenance of the persistent infection, were investigated.

Viral protein and RNA synthesis were compared in BHK and C6/36 cells infected with Bunyamwera virus. In BHK cells, host protein synthesis was inhibited and viral protein synthesis was detected until the cells died. In C6/36 cells, host protein synthesis continued throughout the infection, but viral protein synthesis declined from maximum levels at 24 h pi to barely detectable levels at 36 h pi, despite the presence of translatable S mRNA. The levels of encapsidated L and M RNAs in the C6/36 cells declined after 24 h pi, but encapsidated S RNA species continued to accumulate. In the BHK cell infection, encapsidated L, M and S RNA species continued to accumulate up to 48 h pi. Overall, relatively more S segment RNA than L or M segment RNA accumulated in infected C6/36 cells compared to BHK cells. The decline in viral protein synthesis and genome replication observed in the C6/36 cells after 24 h pi, was paralleled by an accumulation of encapsidated S mRNA. Encapsidation of S mRNA may have a role to play in preventing the cytopathic effects of Bunyamwera virus infection in C6/36 cells by limiting translation of this viral mRNA.

A persistent infection of C6/36 cells was established with a biologically cloned stock of Bunyamwera virus (wtL$_9$BUN virus) and the persistently infected cell line (C6/36/BUN) was passaged at weekly intervals for over a year without cytopathic effects. The titre of virus released from the cells, the susceptibility of the cells to superinfection with homologous virus, and the level of viral RNA in the cells at different passages fluctuated markedly, but there was no simple relationship between virus titre, superinfectibility and

levels of viral RNA. There was an alteration in the plaque-phenotype of virus released from the persistently infected culture with increasing passage level. By passage 15 the virus released from the C6/36/BUN cell line produced small, cloudy plaques on BHK cell monolayers, in contrast to the clear lytic plaques produced by the wtL$_9$BUN virus.

Direct RNA sequence analysis was carried out on the S RNA segments of viruses plaque-purified from the supernatant culture fluid of the C6/36-PI LO cell line established by Elliott and Wilkie (Virology 150, p21-32, 1986). Only one nucleotide substitution, in a non-conserved region of the viral genome, was detected in viral isolate BUN18.10 (which was isolated from the persistently infected culture after it had been passaged for four months) suggesting that genetic drift during persistent Bunyamwera virus infection of C6/36 cells was minimal. However, Northern blot analysis of viral RNA extracted from different passage levels of the C6/36/BUN cell line revealed that polymerase errors which generated subgenomic L RNAs, and S RNA species larger than the standard S RNA segment, did occur in the persistently infected cells. The defective viral RNA species were not efficiently encapsidated by N protein. There was a strong selection for encapsidation of full-length S RNAs in the persistently infected cells and the majority of intracellular nucleocapsids contained standard-sized S RNA species.

Stable cell lines were established with cells cloned from the persistently infected C6/36/BUN cell line. The cloned cells were heterogeneous in their ability to produce virus, and their susceptibility to superinfection with wtL$_9$BUN virus, and in the levels of intracellular viral RNA they contained. Individual clones contained a single prominent defective L RNA species and resistance of the cells to superinfection appeared to correlate more with the levels of full-length viral genomic RNA resident in the cells than with the amount or occurence of defective viral RNAs.

Abbreviations

Viruses

ALMV	alfalfa mosaic virus
BUN	Bunyamwera virus
FMDV	foot and mouth disease virus
HTLV-1	human T-cell lymphotrophic virus
INSV	impatiens necrotic spot virus
LAC	La Crosse virus
MVM	minute virus of mice
NDV	Newcastle disease virus
NEV	Nephropathia Epidemica virus
PIV-1	parainfluenza virus-1
PIV-2	parainfluenza virus-2
PIV-3	parainfluenza virus-3
RSV	respiratory syncytial virus
RVFV	Rift Valley fever virus
SFV	Semliki Forest virus
SSH	snowshoe hare virus
SV-5	Simian virus-5
TMV	tobacco mosaic virus
TSWV	tomato spotted wilt virus
VSV	vesicular stomatitis virus
wtL$_9$BUN	triply plaque-purified stock of Bunyamwera virus

Cell lines

BHK	baby hamster kidney
C6/36	*Aedes albopictus* C6/36
C6/36/BUN	*Aedes albopictus* C6/36 cell line persistently infected with Bunyamwera virus (established during the course of this work)

C6/36-PI HI	*Aedes albopictus* C6/36 cell line persistently infected with Bunyamwera virus (established by Elliott & Wilkie, 1986)
C6/36-PI LO	*Aedes albopictus* C6/36 cell line persistently infected with Bunyamwera virus (established by Elliott & Wilkie, 1986)
C7/10	*Aedes albopictus* C7/10
U.4.4	*Aedes albopictus* U.4.4

Bunyaviridae genome segments

L	large (RNA segment)
M	medium (RNA segment)
S	small (RNA segment)

Bunyaviridae proteins

G1	the larger of the envelope glycoproteins
G2	the smaller of the envelope glycoproteins
L	the viral L protein (viral polymerase)
N	nucleocapsid protein
NSm	non-structural protein (encoded in the M RNA)
NSs	non-structural protein (encoded in the S RNA)

General

APS	antiviral polyclonal sera
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ci	Curie(s)
CIP	calf intestinal phosphatase
CNS	central nervous system
cpe	cytopathic effect
CSF	cerebrospinal fluid
ddNTP	2' 3' -dideoxyribonucleoside 5'-triphosphate

DI	defective interfering
DNase	deoxyribonuclease
DTT	dithiothreitol
dNTP	2' -deoxyribonucleoside 5' -triphosphate
E. coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
endo-H	endoglycosidase-H
GMEM	Glasgow modification of Eagle's medium
h	hour(s)
kb	kilobase(s)
kDa	kiloDalton(s)
L-15	Leibovitz' L-15 medium
min	minute(s)
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
NTP	ribonucleoside 5'-triphosphate
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RF	replicative form
pfu	plaque forming unit(s)
pi	post infection
RNase	ribonuclease
RNasin	ribonuclease inhibitor
RNP	ribonucleoprotein
rpm	revolutions per minute
S	Svedberg unit (sedimentation coefficient)
SDS	sodium dodecyl sulphate

SSPE	subacute sclerosing panencephalitis
TEMED	*N, N, N′, N′* -tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
ts	temperature-sensitive
UV	ultraviolet
v	volume
VRCs	vanadylribonucleoside complexes
vRNA	virion RNA
w	weight
W	Watts
wt	wild-type

Amino acids

Symbol	3-letter code	Amino acid	Codons
A	Ala	Alanine	GCA, GCC, GCG, GCU
C	Cys	Cysteine	UGC, UGU
D	Asp	Aspartate	GAC, GAU
E	Glu	Glutamate	GAA, GAG
F	Phe	Phenylalanine	UUC, UUU
G	Gly	Glycine	GGA, GGC, GGG, GGU
H	His	Histidine	CAC, CAU
I	Ile	Isoleucine	AUA, AUC, AUA
K	Lys	Lysine	AAA, AAG
L	Leu	Leucine	UUG, UUA, CUA, CUC, CUG, CUU
M	Met	Methionine	AUG
N	Asn	Asparagine	AAC, AAU
P	Pro	Proline	CCA, CCC, CCG, CCU
Q	Gln	Glutamine	CAA, CAG
R	Arg	Arginine	CGA, CGC, CGG, CGU, AGA, AGG
S	Ser	Serine	UCA, UCC, UCG, UCU, AGC, AGU
T	Thr	Threonine	ACA, ACC, ACG, ACU
V	Val	Valine	GUA, GUC, GUG, GUU
W	Trp	Tryptophan	UGG
Y	Tyr	Tyrosine	UAC, UAU

INTRODUCTION

The work presented in this thesis was aimed at elucidating the molecular mechanisms which enable Bunyamwera virus, the prototype virus of the *Bunyavirus* genus of the family Bunyaviridae, to persist in *Aedes albopictus* C6/36 cells. The literature review is divided into two sections: 'The Bunyaviridae' and 'Persistent viral infections'.

1 The Bunyaviridae

1.1 General biology

1.1.1 Taxonomy and classification of the Bunyaviridae

The Bunyaviridae comprises more than 300 viruses (Karabatsos, 1985). Members are assigned to the family by serological methods such as neutralization, haemagglutination inhibition, complement fixation and enzyme-linked immunosorbent assays (ELISA). In general, viruses within a genus share complement fixation antibodies and are segregated into serogroups on the basis of cross-neutralization and haemagglutination inhibition studies. The neutralization and haemagglutination inhibition tests are thought to be specific for antigenic determinants on the viral glycoproteins, while the complement fixation test is mainly directed against determinants on the nucleocapsid protein. For most member viruses the nucleocapsid protein is more conserved than the glycoproteins and the complementation fixation test will identify antigenic determinants which are shared by different viruses (Shope & Casey, 1962), while the neutralization and haemagglutination inhibition tests will distinguish between closely related viruses, particularly if monoclonal antibodies are employed (Gonzalez-Scarano *et al.*, 1982). Phleboviruses are exceptions in that the complement fixation test is group specific while the haemagglutination inhibition test is cross-reactive (Calisher *et al.*, 1981; Shope, 1985). Recent serological and sequencing data on the S segment of a second tospovirus isolate Impatiens necrotic spot virus (INSV) suggest that the tospoviruses may also be exceptions (Law *et al.*, 1991).

The classification of the Bunyaviridae has recently been reorganised with the merger of the former *Phlebovirus* and *Uukuvirus* genera and the inclusion of a new genus, *Tospovirus*. Hence the Bunyaviridae now consists of five genera: *Bunyavirus; Hantavirus; Phlebovirus; Nairovirus* and *Tospovirus* (Calisher, 1991). The fifth genus was created to include tomato spotted wilt virus (TSWV), an arthropod transmitted virus of plants, as the first plant virus in the family (Milne & Francki, 1984; de Haan *et al.*, 1989a, 1989b, 1990); a second isolate, INSV, has now been identified (Law & Moyer, 1989, 1990). Serological studies, and sequence analysis of the S RNAs of these viruses, support the separation of the two known tospoviruses into distinct serogroups (Law *et al.*, 1991). In addition at least 7 serogroups containing 19 viruses, as well as 22 ungrouped viruses, are considered to be

members of the family, but have yet to be assigned to a particular genus (Calisher, 1991). The recent reclassification of the *Phlebovirus* and *Uukuvirus* genera into a single genus arose in the light of sequencing studies, which showed that phleboviruses and uukuviruses express their S RNA via an ambisense coding strategy, distinct from the negative-sense coding strategy employed by the majority of the family. They have identical 5' and 3' terminal nucleotide sequences on their RNA segments, show a high degree of homology in their N proteins and low but significant homology between their glycoproteins. In addition, certain members of each group have been shown to be antigenically related to certain members of the other group (Simons *et al.*, 1990). A brief summary of the taxonomic groups in the family Bunyaviridae is given in Table 1.1. As more data become available and as new viruses are discovered the classification may change.

1.1.2 Characteristics of the Bunyaviridae

Some distinguishing features of the Bunyaviridae are listed below.

(i) The virions appear spherical or pleomorphic under the electron microscope, according to which fixation conditions are used, and are 80 -120 nm in diameter. They are enveloped by a 4-5 nm thick lipid bilayer which is cell-derived and accounts for 20-30% of the weight of the virus particle (Gonzalez-Scarano & Nathanson, 1990; Calisher, 1991).

(ii) Each virion contains four structural proteins, two glycoproteins (G1 and G2), a nucleocapsid protein (N) and the largest of the viral proteins, L, which is believed to be the viral transcriptase (Calisher,1991). The glycoproteins are embedded in the lipid envelope and project 5-10nm from the virion surface; they possess type-specific antigenic determinants and variations in these glycoproteins contribute unique external features to representatives of different genera (Martin *et al.*, 1985; Schmaljohn & Patterson, 1990).

(iii) Each virus particle contains three molecules (large [L], medium [M] and small [S]) of single-stranded RNA. Each RNA species has conserved complementary 3' and 5' ends which are genus specific (see Table 1.2). The ends of the RNAs are hydrogen-bonded to form circular RNA molecules (Calisher,1991).

(iv) The viral RNAs are present in the virion as nucleocapsids, each consisting of viral nucleoprotein, a unique, single-stranded, non-covalently closed circular genomic RNA segment and a transcriptase enzyme activity. The nucleocapsids are arranged as closed helical circles of ribonucleoprotein and have been designated small, medium and large (S, M and L) according to which of the three viral genome segments is enclosed. The circular nucleocapsids are 2.0-2.5nm in diameter, are sometimes supercoiled and can range in size from 0.2 to 3.0μm in length (Calisher, 1991; Bishop, 1990).

(v) Generally, viral morphogenesis occurs by budding into smooth-surfaced vesicles in or near the Golgi region (Calisher, 1991).

(vi) The viruses can interact genetically with serologically closely related viruses and recombinant viruses may arise by RNA segment reassortment (Bishop, 1990).

BUNYAVIRUS (162)	*PHLEBOVIRUS* (35)	*NAIROVIRUS* (33)	*HANTAVIRUS* (>6)	*TOSPOVIRUS* (2)
Antigenic groups. Anopheles A(12) Anopheles B (2) Bakau (5) Bunyamwera (32) Bwamba (2) C (14) California (13) Capim (10) Gamboa (8) Guama (12) Koongol (2) Minatitlan (2) Nyando (2) Olifantsvlei (5) Patois (7) Simbu (24) Tete (6) Turlock (4)	**Antigenic groups.** **Sandfly fever group (23)** Sandfly fever Naples (4) Bujaru (2) Candiru (6) Chilibre (2) Frijoles (2) Punta Toro (2) Rift Valley fever (3) Salehabad (2) **Uukuniemi group.** (Single serogroup containing 12 viruses)	**Antigenic groups.** Crimean-Congo hemorrhagic fever (3) Dera Ghazi Khan (6) Hughes (10) Qalyub (3) Sakhalin (7) Thiafora (2) Nairobi sheep disease (2)	**Antigenic group.** Hantaan (6)	**Antigenic Groups.** TSWV INSV

Table 1.1. Summary of the present classification of the Bunyaviridae. Numbers in brackets indicate the number of viruses in a given genus/antigenic complex (data from Calisher, 1991).

Genus	Sizes of RNA segments (nucleotides)			3' Terminal sequence	5' Terminal sequence
	L	M	S		
Bunyavirus	6,875 (1)	4,458 - 4,534 (4)	850 - 984 (6)	UCAUCACAUGA	UCGUGUGAUGA
Phlebovirus	6,423 (1)	3,231 - 4,330 (4)	1,746 - 1,904 (2)	UGUGUUUC	GAAACACA
Nairovirus	*11,400 - 14,400*	4,888 (1)	1,712 (1)	AGAGUUUCU	AGAAACUCU
Hantavirus	6,530 - 6,550 (2)	3,616 -3,682 (4)	1,675 - 1,785 (4)	AUCAUCAUCUG	AUGAUGAU
Tospovirus	8,897 (1)	4,972 -4,821 (2)	2,916 (1)	UCUCGUUAG	CUAACGAGA

Table 1.2. Sizes and consensus genus specific terminal sequences of the genomic RNAs of the Bunyaviridae. RNA segment sizes are as determined by sequencing of cloned cDNAs; numbers in brackets indicate the number of known sequences. Numbers in italics are size estimates from gel electrophoresis of the RNA where sufficent sequencing data are not available. For references see text.

1.1.3 Host range and transmission

The Bunyaviridae have a wide variety of animal hosts (see Table 1.3). Human to human transmission does not generally occur, and humans are dead end hosts, not producing sufficient viremia to transmit virus to a feeding mosquito (Gonzalez-Scarano & Nathanson, 1990). Crimean-Congo haemorrhagic fever nairovirus is exceptional amongst the Bunyaviridae in that person to person spread can occur and has lead to a number of hospital outbreaks (Gonzalez-Scarano & Nathanson, 1990). TSWV and INSV cause great yield losses worldwide in a large number of economically important crops; for example considerable losses in floral crops throughout North America due to INSV have been reported (Law & Moyer, 1989). The host range of TSWV includes at least 370 plant species in 50 botanical families, including *Nicotiania rustica, N. tabacum* and *N. benthamiana* as well as tomato plants (de Haan *et al.*, 1989b; Calisher, 1991; de Oliveira Resende *et al.*, 1991).

Excluding hantaviruses, all members of the family are transmitted by invertebrate vectors, including mosquitoes, ticks, biting midges (*Culicoides* species) and sandflies (*Phlebotomus* species). The Bunyaviridae are maintained and amplified in nature by biological transmission. This involves ingestion of the virus by the vector when feeding upon a viremic vertebrate host; after an extrinsic incubation period (see Section 1.1.5.1) the virus can be transmitted to another vertebrate from the salivary gland secretions of the vector as it seeks another blood meal (Turell, 1988). Another potential means of transmission is mechanical transmission, where the vector mouthparts become contaminated while feeding on a viremic host; if the vector feeds upon another host promptly, before the virus becomes inactivated, transmission may occur without a requirement for virus replication in the vector (Beaty & Calisher, 1991). The Bunyaviridae cause a persistent, lifelong infection in their invertebrate vectors.

During major epizootics of Rift Valley fever, the virus is likely to be amplified by mechanical transmission because of the high level of viremia prevalent in the infected vertebrate hosts (Meegan & Bailey, 1988). Members of the Bunyaviridae can also be maintained within the vector species by transovarial, transtadial and venereal transmission (see Section 1.1.5). TSWV is the only plant virus to date known to be transmitted by thrips (Sakimura, 1962). It has also been reported that TSWV can be vertically transmitted through the seeds of certain plant hosts (Mandahar, 1981). Hantaviruses are primarily rodent-borne and establish persistent infections in these animals. Transmission of hantaviruses is via aerosolized rodent excreta (Lee *et al.*, 1981a, 1981b). In nature individual members of the Bunyaviridae infect a limited number of arthropod and vertebrate hosts (Mclintock, 1978; Murphy *et al.*, 1975). Studies on La Crosse variant and revertant viruses suggest that the specificity is related to the larger of the two viral glycoproteins, G1, probably at the level of viral attachment to susceptible cells (Sundin *et al.*, 1987). The

Genus	Vector species	Natural vertebrate (or plant) host(s)	Mechanism of transmission to humans
Bunyavirus	Mosquitoes *(Aedes* species), midges *(Culicoides)* and ticks (tete group only).	Rodents, lagomorphs and ruminants (acute infection).	Insect bite.
Phlebovirus	Many genera of mosquitoes and *Phlebotomus* species.	Sheep and other domestic animals; Uukuniemi group viruses infect birds (acute infections).	Insect bite and also through contact with infected tissues. No human infections with Uukuniemi group viruses have been reported.
Nairovirus	Ixodid ticks, *Hyalomma* species.	Herbivores and lagomorphs (acute infection).	Insect bite and contact with infected tissues. Human to human transmission is believed to occur.
Hantavirus	None.	Rodents (persistent infection).	Aerosolized rodent excreta.
Tospovirus	Thrips.	Over 360 plant species belonging to 50 families (eg.tomatoes).	

Table 1.3. Host range and transmission of the Bunyaviridae (adapted from Gonzalez-Scarano & Nathanson, 1990 and Calisher, 1991).

molecular basis of the persistent, non-cytopathic infection of the vector species remains to be elucidated.

1.1.4 Disease and geographical distribution

Members of the Bunyaviridae have been isolated from every continent except Antartica, however serotypes tend to have a localised distribution (Bishop & Shope, 1979). A number of viruses belonging to the Bunyaviridae are serious human and veterinary pathogens, for example Crimean-Congo haemorrhagic fever virus (*Nairovirus*), Rift Valley fever virus (*Phlebovirus*), the California encephalitis viruses (*Bunyavirus*) and a number of hantaviruses. Hantaan and related viruses cause a severe haemorrhagic disease (haemorrhagic fever with renal syndrome) with significant mortality throughout Asia, and especially China (Elliott, 1990). Crimean-Congo haemorrhagic fever virus is the etiologic agent of another haemorrhagic fever which results in 10% to 20% mortality over a wide geographic area including Sub-Saharan Africa, eastern Europe, the Middle East and areas in central Asia (Gonzalez-Scarano *et al.*, 1991). An average of 75 cases of California encephalitis are reported annually in the United States; over 80% of patients recover fully after the acute disease, but evidence of residual central nervous system (CNS) infection is apparent in some patients. 10% of affected children develop epilepsy, but fatality is rare (only 0.3%; Kappus *et al.*, 1983). Rift Valley fever virus (RVFV) causes an acute disease in humans with a convalescense period which may be prolonged (Eddy & Peters, 1980). Serious complications do occur but are rare (Laughlin *et al.*, 1979). RVFV causes a more serious disease in domestic animals, sheep are more susceptible than cattle; pregnant ewes normally abort and over 90% mortality amongst infected lambs has been reported (Daubrey & Hudson, 1931; Meegan & Shope, 1981). Outbreaks of fetal abnormalities in Texan sheep have been associated with Cache Valley bunyavirus (Edwards *et al.*, 1989; Chung *et al.*, 1991). Other members of the Bunyaviridae, including Bunyamwera virus itself, cause transient fevers which although self-limiting in nature are important in terms of man-hours lost from work.

1.1.5 Invertebrate infection

Infection of invertebrates can take place via horizontal (oral and venereal transmission) or vertical (transovarial and transtadial transmission) mechanisms.

1.1.5.1 Oral infection

Electron microscopic studies have been used to trace the spread of California serogroup viruses through *Aedes* mosquitoes following a blood meal (Murphy *et al.*,1975). Ingestion of virus is followed by infection of the epithelial cells which line the mosquito mid-gut. An eclipse phase follows during which no infectivity can be detected. Virus first appears in the haemocoel just outside the basal lamina of the mid-gut. From here the virus has access to all body tissues, many of which are permissive. Fluorescent antibody studies

of *Aedes triseriatus* mosquitoes orally infected with La Crosse virus have demonstrated large amounts of viral antigen in the heart, the neural ganglia, fat body, ovaries and salivary glands (Beaty & Thompson, 1978). The salivary glands are one of the last tissues to become infected, and only a limited number of cells are antigen positive. The infected cells discharge large numbers of virions into the lumen of the gland, from where they can be injected into the vertebrate host during feeding (Murphy *et al.*, 1975). The interval from feeding until the mosquito is infectious, termed the extrinsic incubation period, is 7 to 14 days for La Crosse virus (Tesh & Beaty, 1983). The shorter the extrinsic incubation period, the greater is the likelihood of viral transmission. Typically, vectors with shorter extrinsic incubation periods are considered to be vector competent (Beaty & Calisher, 1991). With most arthropod-borne viruses, the duration of the extrinsic incubation period is a function of temperature; increased temperature reduces the time required (Turell, 1989).

Failure of arboviruses to disseminate from the mid-gut of non-permissive vector species has lead to the concept of a mid-gut barrier. Accumulation of viral antigen in the mid-gut epithelium has been observed in instances when ingestion of virus has not lead to completion of the extrinsic incubation period, and there has been no dissemination of the virus throughout the mosquito (Kramer *et al.*, 1981; Hardy *et al.*, 1983). Intrathoracic injection of the same virus can result in a disseminated infection (Miller, 1983; Hardy *et al.*, 1983). In support of the idea of a midgut barrier, Turell *et al.* (1984) demonstrated enhanced dissemination and transmission of arboviruses in mosquitoes when an infectious blood meal was contaminated with microfilarae. Microfilarae produce holes in the insect mid-gut through which virus may enter the haemocoel directly. The midgut barrier may also be breached by mechanical means; piercing the midgut with a needle has been shown to permit efficient infection of previously refractory mosquitoes (Hardy *et al.*, 1983). Individual species of invertebrate vectors display differential susceptibility to different viruses, ranging from being totally permissive for a particular virus to completely refractory for that virus (Mitchell, 1983; Hardy, 1988). Typically, there is a threshold level of infectious virus which must be present in an ingested blood meal for midgut infection to occur and with high virus titers most arthropods will become infected. The more susceptible the vector, the greater its competence to transmit virus (Mitchell, 1983). The factors involved in the midgut barrier are not known but a lack of midgut receptors, improper proteolytic processing of viral proteins, proteolytic inactivation of the virus in the non-preferred arthropod host, or an inability of the virus to fuse with the midgut cells are potential mechanisms (Hardy *et al.*, 1983; Turell, 1988; Beaty & Bishop, 1988).

1.1.5.2 Transovarial transmission

Infection of ovaries, including oocytes, occurs as part of the systemic infection and results in transovarial transmission to both male and female offspring (Tesh & Beaty, 1983; Watts *et al.*, 1973). Transovarial transmission has been demonstrated for most

members of the California serogroup and mosquitoes infected in this way can transmit virus by bite (Watts *et al.*, 1973; Miller *et al.*, 1977; Tesh, 1980; Tesh & Shroyer, 1980; Le Duc *et al.*, 1975; Le Duc, 1979; Turell *et al.*, 1982b; Patrician *et al.*, 1985). California serogroup viruses have been recovered from naturally infected mosquito larvae suggesting that transovarial transmission occurs in nature (Le Duc, 1979). Transovarial transmission is believed to serve as an overwintering mechanism by which virus is maintained during long periods of vector inactivity and as such is a major survival mechanism for the virus. San Angelo bunyavirus was shown to survive 3 months in dried *Aedes albopictus* eggs maintained at 28°C without any decrease in the infection rate of F1 offspring (Tesh, 1980), while California encephalitis bunyavirus survived storage in mosquito eggs for up to 19 months and withstood repeated freeze-thawing (Turell *et al.*, 1982c). Stabilized infections with highly efficient transovarial transmission from generation to generation have been reported for laboratory populations of mosquitoes infected with California serogroup viruses (Tesh & Shroyer, 1980; Turell *et al.*, 1982b). This mechanism, combined with venereal transmission of the virus, relieves much of the requirement for amplification through horizontal transmission (Turell & Le Duc, 1983). It has been proposed that virus survival rests mainly on vertical transmission (DeFoliart, 1983).

Transovarially infected mosquitoes have disseminated infections (Beaty & Thompson, 1976). Fluorescent antibody studies on infected larvae detected the highest levels of viral antigen in the alimentary tract, then the ganglia, malpighian tubules, muscles and salivary glands. Antigen was detected in identifiable organs immediately upon emergence from the egg. The same tissues remained infected through each developmental stage, including after metamorphosis. In pupae and adults, antigen was detected at high levels in the foregut, gonadal and associated tissues and in the salivary glands. Infection of gonadal tissue of both sexes provides mechanisms for further vertical transmission of the virus and for venereal spread within the mosquito population (Thompson & Beaty, 1978).

1.1.5.3 Venereal transmission

Venereal transmission of La Crosse virus has been demonstrated in *Aedes triseriatus* mosquitoes (Thompson & Beaty, 1978). Investigations with monoclonal antibodies revealed virus antigen in accessary sex gland fluid, but not within sperm (Thompson & Beaty, 1977). Direct paternal transfer of virus to progeny is therefore possible, since virus present in the spermathecal fluid could attach to and accompany sperm into the egg. Venereally infected females harbour viral antigen in ovaries, heart, gut and salivary glands amongst other organs, and were shown to be capable of transmission of La Crosse virus transovarially and by bite (Thompson & Beaty, 1978).

1.1.5.4 Transtadial transmission

A further mechanism of virus transmission within the vector population involves vertical transmission of the virus through the different stages of arthropod development following an initial horizontal infection of larvae. The initial infection might occur by ingestion of portions of infected dead larvae, ecdysed exoskeletons, or simply by contact with the virus shed from infected larvae. This mode of transmission has been demonstrated with California encephalitis bunyavirus in *Aedes dorsalis* and *Aedes melanimon* mosquitoes as well as with the flavivirus, St. Louis encephalitis virus (Collins, 1962; Turell *et al.*, 1982c).

Transtadially infected mosquitoes can transmit virus to vertebrates by bite. Recently Booth *et al.* (1991) examined tissue tropisms of Dugbe nairovirus in the tick vector *Amblyomma variegatum* by performing immunohistochemical analysis, *in situ* hybridization and infectivity assays on dissected tissue. They determined that the primary site of transtadial persistence of Dugbe virus was the hemocytes and proposed that these motile cells were important in the transmission of persistent virus infection from one cell or organ to another.

1.1.5.5 Effect of infection on the invertebrate host

Arbovirus replication in arthropods is characteristically apathogenic and no major untoward effects have been documented. However, studies carried out on experimentally infected mosquitoes have brought to light subtle effects of bunyavirus infection on the behaviour and development of mosquitoes. Grimstad *et al.* (1980) found that La Crosse virus infected *Aedes triseriatus* mosquitoes were less successful at taking a blood meal than were uninfected siblings. *Aedes albopictus* females infected transovarially with San Angelo virus produced 12% fewer viable eggs than did uninfected controls (Tesh, 1980) and transovarially infected larvae have been shown to take considerably longer to develop than their uninfected counterparts (Tesh, 1980; Turell *et al.*, 1982b). In one study mosquitoes infected with California serogroup viruses, either orally or by intrathoracic injection, were shown to have enhanced sensitivity to carbon dioxide; this sensitivity was inhibited by antiserum to California encephalitis virus (Turell & Hardy, 1980). Mosquitoes infected transovarially with California serogroup viruses do not develop carbon dioxide sensitivity (Turell & Hardy, 1980; Turell *et al.*, 1982a).

1.1.6 Evolution of the Bunyaviridae

Evolution of members of the Bunyaviridae is believed to occur by two major mechanisms, genetic drift and RNA segment reassortment. Until recently, evidence was weighted towards the view that the Bunyaviridae exhibit the genomic plasticity characteristic of RNA genomes (Domingo & Holland, 1988). Bishop and Shope (1979) calculated the spontaneous mutation rate of snowshoe hare virus to be 1 to 2% and, more recently,

Hewlett *et al.* (1992) have reported major sequence differences between two snowshoe hare virus isolates collected from mosquitoes in different areas of Canada in 1975 and 1976. Oligonucleotide fingerprint analysis of 30 field isolates of La Crosse virus yielded 30 non-identical fingerprints (El Said *et al.*, 1979; Klimas *et al.*, 1981), and Tesh & Gubler (1975) demonstrated the presence of small-plaque variants in populations of transovarially passaged La Crosse virus, indicative of a heterogeneous population. In addition, variation in the La Crosse virus genome in response to selective pressure has been demonstrated by the revertion of a monoclonal antibody selected variant, V22, which exhibited decreased oral infectivity for *Aedes triseriatus* mosquitoes amongst other altered biological properties, upon three oral passages in mosquitoes (Gonzalez-Scarano *et al.*,1983; Sundin *et al.*,1987).

Genetic drift occurs via the accumulation of point mutations and, less frequently, deletions, duplications and inversions in the genome. Oligonucleotide fingerprinting studies carried out by Bilsel *et al.* (1988) and Baldridge *et al.* (1989), suggested that the frequency of such events in persistently infected arthropods may be low. Bilsel *et al.* (1988) reported that Toscana phlebovirus underwent no detectable genomic change upon serial transovarial transmission through 12 generations of an experimentally infected laboratory colony of its natural sandfly host, *Phlebotomus pernicious*. Later, Baldridge *et al.* (1989) demonstrated that the La Crosse virus genome underwent no detectable change during transovarial transmission through two generations of a laboratory colony of *Aedes triseriatus* mosquitoes, nor during transfer between the insect and vertebrate host. Furthermore, the rate of evolution of the alphavirus Ross River virus during a 10-month outbreak of epidemic polyarthritis in a non-immune island population of humans was determined to be low by direct genomic sequencing of 1.6kb of the virus genome; only one nucleotide substitution was found in the virus isolates examined during the course of the epidemic (Burness *et al.*,1988). Taken together these observations suggest that arboviruses may exhibit a slower rate of evolution relative to non-arthropod-borne viruses. The molecular constraints imposed on arboviruses by their disparate hosts may act as a strong selective force for genomic stability by limiting the spectrum of viable and competitive variants in a cycling arbovirus population. If the laboratory observations of genomic stability of La Crosse and Toscana viruses during transovarial transmission reflect the situation in nature; and if it is true that certain arboviruses, including La Crosse virus, are principally maintained in nature within the invertebrate host (by transovarial and venereal transmission; Tesh & Shroyer 1980; Turell 1988), then genomic stability during transovarial transmission would confer a low rate of evolution upon these viruses. The genetic variability seen amongst field isolates of La Crosse virus (El Said *et al.*, 1979; Klimas *et al.*, 1981) and snowshoe hare virus (Hewlett *et al.*, 1992) might arise partly as a result of reproductively isolated mosquito populations, each maintaining a population of virus which may differ slightly from that maintained in the other mosquito populations.

Conservation of dominant variants in a given arbovirus population is reputed to be aided by physical and biological limitations imposed by small blood meal volumes, small injected saliva volumes and low virus titres during transmission between vertebrate and invertebrate hosts (Burness *et al.*, 1988; Yuill, 1983).

The evolutionary potential of the Bunyaviridae is enhanced by RNA segment reassortment, which has been documented experimentally both *in vitro* and *in vivo*, and in nature (Bishop & Beaty, 1986; Bishop *et al.*, 1987). Since only viruses from a single serogroup will reassort (Bishop, 1985; Pringle *et al.*, 1984) individual serogroups may potentially constitute divergently evolving virus pools. High frequency reassortment between mutant La Crosse viruses or mutant La Crosse and snowshoe hare viruses has been demonstrated in dually infected *Aedes triseriatus* mosquitoes following ingestion of mixtures of these viruses (Beaty *et al.*, 1985). The second infection must occur within one or two days of the initial infected blood meal (Beaty *et al.*, 1985). Interference to superinfection is apparent by three days post infection, once replication of the virus in the midgut has produced sufficient quantities of virus and virus antigen (Sundin & Beaty, 1988). Many mosquitoes exhibit interrupted feeding; if a mosquito is disturbed during engorgement it may finish feeding on an alternate host. Thus superinfection within a time frame which would preclude interference is possible and in the instance of dual infection with viruses from the same serogroup, reassortment could occur.

Transmission of reassortant viruses from mosquitoes to vertebrates was demonstrated by Beaty *et al.* (1985). They isolated reassortant viruses from mice fed upon by dually infected mosquitoes. More recently, Chandler *et al.* (1990) reported high frequency reassortment in *Aedes triseriatus* mosquitoes dually infected with temperature-sensitive variants of La Crosse and snowshoe hare bunyaviruses. Viral replication and genome segment reassortment in the ovaries of the infected mosquitoes were enhanced following ingestion of a blood meal; coinciding with high ovarian metabolical activity. Transovarial transmission of both parental and reassortant viruses was demonstrated; ten percent of infected progeny mosquitoes were infected with reassortant viruses and of these 50% were demonstrated to be able to transmit reassortant viruses to mice.

1.2 Molecular biology of the Bunyaviridae

1.2.1 The virion

Bunyaviridae particles appear spherical or pleomorphic when viewed by electron microscopy. They range in size from 80 to 120 nm in diameter and are usually surrounded by a fringe of glycoprotein spikes (Pettersson *et al.*, 1971; Murphy *et al.*, 1973; Smith & Pifat, 1982; Hung *et al.*, 1983; Pettersson & von Bonsdorff, 1987). Subtle differences have been observed in the surface morphology of different viruses which correlate with their genus (Martin *et al.*, 1985). Cryoelectron microscopy of vitrified-hydrated La Crosse virions has shown these virions to be uniformly spherical, varying in diameter between 75

to 115nm. They are surrounded by a lipid bilayer 4nm thick, in which are embedded 10nm long spike proteins (Talmon *et al.*, 1987). Other enveloped viruses, for example Sendai virus, have been found to be spherical under the neutral pH and 4°C conditions prevailing during cryoelectron microscopy. It is possible therefore, that Bunyaviridae virions are not pleomorphic as suggested by images of negative stained particles, but that the observed pleomorphism is a function of the desiccation which occurs during the specimen preparation required for this technique (Hewlett & Chiu, 1991).

The virus particles consist of four structural proteins: two internal proteins, the L (transcriptase component) and the N (nucleocapsid) proteins and two external glycoproteins, termed G1 and G2, which are inserted into the viral membrane. By convention, the larger molecular weight glycoprotein species is designated G1. Foulke *et al.* (1981) detected three glycoproteins in Hazara nairovirus virions suggesting that nairoviruses may be exceptions to this pattern. The Bunyaviridae do not encode an internal matrix protein. Direct interaction of the internal nucleocapsids with the membrane, or with the cytoplasmic domain of the inserted glycoproteins, may stabilize the virion structure (Pettersson & von Bonsdorff, 1987; Talmon *et al.*, 1987).

The three genome segments of members of the Bunyaviridae, designated small (S), medium (M) and large (L) are present in virions as circular, helical nucleocapsids (Calisher, 1991). In the virion these structures consist of a single segment of negative or ambisense viral RNA which is intimately associated with numerous molecules of the viral N protein and minor amounts of the viral L protein (Obijeski *et al.*, 1976a). Initial studies based on the chemical composition of La Crosse virions and the relative proportions of viral proteins present in La Crosse virions as judged by SDS-PAGE, estimated that each nucleocapsid contained one N protein for every 6 nucleotides of viral RNA (Obijeski *et al.*, 1976a). The RNA within nucleocapsids of members of the Bunyaviridae is inaccessible to reasonable concentrations of nucleases. Pettersson *et al.* (1971) reported that 1μg/ml of RNase at 37°C did not solubilize the RNA in Uukuniemi virus nucleocapsids and Hacker *et al.* (1989) demonstrated that La Crosse virus nucleocapsids were resistant to concentrations of RNase A or micrococcal nuclease far in excess of the concentration required to completely digest viral mRNA. The RNA within Bunyavirus nucleocapsids does not however appear to be as tightly complexed as the RNA within, for example, paramyxovirus nucleocapsids. The addition of oligonucleotides complementary to genomic or antigenomic La Crosse virus RNA to infected cell extracts, rendered up to 50% of the nucleocapsid RNA sensitive to RNase H (Bellocq & Kolakofsky, 1987; Kolakofsky *et al.*, 1987); paramyxovirus nucleocapsids were insensitive to this treatment (Kolakofsky & Hacker, 1991).

The precise structural arrangement of bunyavirus nucleocapsids is unknown. In order to explain the ability of the RNA within nucleocapsids to base pair with oligonucleotides it is necessary to assume that the N protein in the vicinity of the base

pairing is displaced from the RNA. The accessibility of the RNA to nucleases after the addition of oligonucleotides suggests that there are exposed regions of RNA. A nucleosome-like structure with the RNA arranged on the outside of the protein, or tobacco mosaic virus-like nucleocapsids with the RNA lying within a groove created by the assembled N have been proposed as possibilities (Kolakofsky & Hacker, 1991).

Each genome segment possesses complementary 3' and 5' termini with free 5' (pppA) (Gentsch *et al.*, 1977a; Obijeski *et al.*, 1976b; Pettersson *et al.*, 1977) and 3' ends (hydroxyl) ends (Obijeski *et al.*, 1976b). The terminal sequences are highly conserved (Clerx-van Haaster & Bishop, 1980; Obijeski *et al.*, 1980; Clerx-van Haaster *et al.*, 1982a, 1982b; Schmaljohn & Dalrymple, 1983; de Haan *et al.*, 1989b) and are genus specific (see Table 1.2). Circular and panhandle forms of naked viral RNAs have been observed by electron microscopy (Bouloy, 1973/1974; Samso *et al.*, 1976; Hewlett *et al.*, 1977; Pardigon *et al.*, 1982) and are thought to be a consequence of base pairing of the complementary ends of the RNAs. It is postulated that the complementary sequences may provide signals for recognition by the virus encoded polymerase or are involved in packaging of the viral genome. The terminal 11 bases of Bunyamwera virus L, M and S RNAs are conserved and complementary except for positions 9 and -9, with the following 20 or so bases showing segment specific complementarity (Elliott, 1990). The mismatch at positions 9 and -9 has been found in all bunyavirus RNAs sequenced so far, with the exception of the terminal sequences of the M and S segments of sandfly fever group phleboviruses, the M segments of uukuniemi group phleboviruses and in the hantavirus M and S segments. However, sequencing of the Hantaan virus M segment by Yoo & Kang (1987) revealed a mismatch between positions 12 and -12 in the terminal 18 complementary nucleotides of this RNA (Elliott, 1990). Unmatched and mismatched bases within double stranded regions of RNA are important for recognition by proteins (Wickens & Dahlberg, 1987; Raju & Kolakofsky, 1989). RNAs of positive- and negative-polarity would have significantly different structures due to G-U in genomic RNA being an "allowed" basepair, while C-A in the complementary RNA is not. If a protein (e.g. the N protein) could distinguish these structures this may be important in transcription and the selective packaging of genomic RNA into virions. A similar situation exists with influenza virus where Hsu *et al.* (1987) have shown that the negative-sense genomic RNA segments of influenza virus are circular. They suggested that the terminal basepaired structures were cis-acting regulatory elements for transcription and possibly the packaging of the influenza virus genome.

Nucleocapsids of members of the *Bunyavirus* genus and the uukuniemi serogroup of the *Phlebovirus* genus have been observed under the electron microscope as circular structures (Pettersson & von Bonsdorff, 1975; Samso *et al.*, 1975; Obijeski *et al.*, 1976b). Psoralen cross-linking studies carried out on La Crosse virus nucleocapsids derived from virions and from infected cells confirmed that base pair interactions occur between the

complementary ends of the viral RNA within nucleocapsids (Raju & Kolakofsky, 1989). The observation that viral genomic (-) and full-length complementary (+) viral RNAs are encapsidated, whereas viral mRNAs, which have 5' extensions and are shorter at their 3' ends, are usually not found in nucleocapsids, led to speculation that the conserved terminal sequences of the genome-length viral RNAs might harbour the encapsidation signal for the N protein (Raju & Kolakofsky, 1986). Subsequently, Raju and Kolakofsky (1987b) discovered minor amounts of encapsidated mRNA-like transcripts at late times post infection in La Crosse virus-infected cells along with some full-length (+) molecules with 5' extensions which were not encapsidated. The authors concluded that the nucleocapsid recognition sequence was at the 5' end of the RNA and had to be in the correct context for initiation of encapsidation.

1.2.2 Coding assignments

The trisegmented single-stranded RNA genome of the Bunyaviridae makes up 1% to 2% of the mass of the virion (Obijeski *et al.*, 1976b). The sizes of the RNA segments characteristic of each genus, as determined by sequencing of cloned cDNAs, or, where sequencing data are not available, as estimated by their electrophoretic migration in polyacrylamide or agarose gels, are shown in Table 1.2. The pattern of RNA segment size is distinctive for bunyaviruses, nairoviruses and TSWV, while the RNA profiles of the hantaviruses and phleboviruses are more similar.

For some viruses the mapping of viral proteins to genome segments has been achieved by genetic or biochemical methods (Bishop, 1985). Nucleotide sequence studies, along with N-terminal protein sequence data, have confirmed these results and revealed the coding strategies employed by individual genome segments (see Section 1.3.3.1). The L RNA segment encodes the L protein, the putative virion transcriptase, as verified for Bunyamwera virus by Jin and Elliott (1991). The M RNA segment encodes the two virion glycoproteins, G1 and G2, in the form of a putative precursor polypeptide which is thought to be processed by co-translational cleavage. Bunyavirus and phlebovirus M RNA segments also encode a non-structural protein (NSm) as part of the putative precursor polyprotein. The S RNA segment encodes the nucleocapsid protein (N), and in the case of bunyaviruses, phleboviruses and TSWV a non-structural protein (NSs). For bunyaviruses the N and NSs proteins are translated from overlapping reading frames (ORFs) in a single subgenomic mRNA species. All Bunyaviridae L and M segment RNAs with the exception of the M RNA segment of INS tospovirus, which has an ambisense M RNA segment (Law *et al.*, 1992), are of negative-polarity, as are the S RNA segments of bunyaviruses, hantaviruses and nairoviruses. The infecting genome segments must be transcribed into positive-sense mRNA prior to protein production. The S segments of phleboviruses and TSWV exhibit an ambisense coding strategy. For these viruses the N and NSs protein ORFs are in opposite orientations in the genomic S RNA segment. The N protein is

translated from a viral complementary-sense subgenomic mRNA whereas the NSs protein is translated from a viral-sense subgenomic mRNA. This expression strategy has an intrinsic requirement for a round of genome replication to provide the full-length viral-complementary template from which the NSs mRNA is transcribed; this may provide a degree of temporal control in the expression of the ambisense S segment specified proteins during infection.

1.2.3 Genetics

RNA segment reassortment occurs between closely related members of the Bunyaviridae. However, cross-genus and cross-serotype reassortment has not been reported and some viruses, even from within the same serogroup, appear to be incompatible with each other (Gentsch & Bishop, 1976; Gentsch *et al.*, 1977b, 1979; Ozden & Hannoun, 1978,1980; Iroegbu & Pringle, 1981; Rozhon *et al.*, 1981; Pringle & Iroegbu, 1982; Elliott *et al.*, 1984; Pringle *et al.*, 1984; Janssen *et al.*, 1986). Further restrictions on reassortment are evident in the co-segregation of the L and S RNA segments of Batai, Bunyamwera and Maguari bunyaviruses in heterologous crosses. This linkage was broken when heterologous reassortants were used as parental viruses (Elliott *et al.*, 1984; Pringle *et al.*, 1984). The alleviation of co-segregation in heterologous crosses suggests that there is no inherent incompatibility between the L, M and S RNAs of these viruses. Restriction may therefore be mediated at the gene product level, possibly involving interaction of the core proteins (N and L) with the RNA template, or protein-protein interactions between heterologous core proteins. This phenomenon has not been observed in heterologous crosses between California serogroup viruses (Bishop *et al.*, 1981; Gentsch *et al.*, 1977b), possibly due to the closer serological relationship which exists between La Crosse virus and snowshoe hare virus compared to between the Bunyamwera group viruses studied.

Genome segment reassortment has been demonstrated experimentally in dually infected mosquitoes (Beaty *et al.*, 1981, 1982, 1985; Shope *et al.*, 1981) and analysis of field isolates suggests that reassortment can also occur in nature (Klimas *et al.*, 1981; Ushijima *et al.*, 1981). The construction of reassortant viruses provides a powerful strategy for delineating the role of the gene products of individual viral RNA segments in determining the biological character of members of the Bunyaviridae. Laboratory studies with La Crosse, snowshoe hare and La Crosse-snowshoe hare reassortant viruses identified the middle RNA segment of La Crosse virus as the major determinant of infectivity for *Aedes triseriatus* mosquitoes, dissemination within the mosquito and transmission to mice (Beaty *et al.*, 1982; Beaty & Bishop, 1988). Recent studies with reassortant La Crosse-snowshoe hare and La Crosse-Tahyna viruses demonstrated the existence of distinct sites within the bunyavirus M RNA segment carrying genetic determinants for four different biological markers: subcutaneous and intracerebral mouse

virulence and oral and intrathoracic infection of mosquitoes (Gonzalez-Scarano *et al.*, 1988).

1.3 Replication of the Bunyaviridae

1.3.1 Attachment and entry

In common with other enveloped viruses, it is the glycoproteins which mediate the attachment of Bunyaviridae virions to host cells. Obijeski *et al.* (1976a) demonstrated a 10^5-fold reduction in infectivity when La Crosse virus particles were rendered "spikeless" by proteolytic enzyme treatment. Experiments using monoclonal and polyclonal antibodies to block infection or haemagglutinating activity have suggested that G1 is more actively involved in binding to vertebrate host cells than is G2 (Kingsford & Hill, 1981; Gonzalez-Scarano *et al.*, 1982; Grady *et al.*, 1983; Kingsford *et al.*, 1983). In addition, treatment of La Crosse virions with bromelain or pronase, which degrade portions of G1 but leave G2 unchanged, rendered the virus completely non-infectious (Kingsford & Hill, 1981). In contrast, neutralizing and haemagglutination inhibition sites have been found on both the G1 and G2 proteins of Hantaan (Arikawa *et al.*, 1989; Dantas *et al.*, 1986) and Punta Toro viruses (Pifat *et al.*, 1988) suggesting that both proteins may be required for attachment.

Efficient transmission, amplification and dissemination of arboviruses requires replication in both vertebrate and invertebrate hosts. As a result virions are exposed to two significantly different environments. Treatment of La Crosse virus with proteolytic enzymes such as those found in the mosquito midgut, eg. trypsin and pronase, increased virus affinity for mosquito cells (Ludwig *et al.*, 1989). These enzymes cleave the major envelope glycoprotein (G1) exposing the second glycoprotein (G2) which remains intact. Processing of La Crosse virus glycoproteins in the mosquito midgut therefore may be necessary to expose attachment proteins on the virion surface before attachment to and infection of midgut cells can occur. In line with the earlier work by Kingsford & Hill (1981), such enzyme treatment was shown to concurrently cause a significant decrease in viral attachment to Vero cells (Ludwig *et al.*, 1989). More recently, binding studies have been carried out using affinity purified La Crosse virus glycoproteins, labelled with [^{35}S]-methionine, to determine which glycoprotein is responsible for attachment to vertebrate and mosquito cell lines, as well as to freshly harvested mosquito midguts. Competition binding studies with radiolabelled glycoproteins suggested that La Crosse virus utilises a receptor mediated attachment mechanism for both vertebrate and invertebrate cells, G1 being important for vertebrate cell attachment and G2 for attachment to mosquito midgut cells (Ludwig *et al.*, 1991b).

Fusion of infected cells at acidic pH values has been reported for viruses in the Bunyaviridae (Gonzalez-Scarano *et al.*, 1984, 1985; Gonzalez-Scarano, 1985; Arikawa *et al.*, 1985). pH-dependent fusion is generally believed to relate to early events in the infection process, particularly the translocation of RNA and proteins into the cell

cytoplasm. Whether one or both of the glycoproteins are required for the fusion process has yet to be elucidated for most members of the family. Work carried out with the V22 mutant of La Crosse virus which exhibits a defective fusion function suggested that G1 mediated fusion (Gonzalez-Scarano *et al.*, 1985). This was supported by results which demonstrated that G1 of La Crosse virus underwent a conformational change at the pH of activation of the fusion function, resulting in both an alteration in the cleavage pattern produced by amino acid specific proteases and in a change in its antigenicity as defined by altered binding of monoclonal antibodies (Gonzalez-Scarano, 1985). In two well studied systems, the Orthomyxoviridae and the Paramyxoviridae, activation of the fusion function is accompanied by a conformational change in the fusion protein (Scheid & Choppin, 1977; Hsu *et al.*, 1981; Skehel *et al.*, 1982). In addition, anti-G1 monoclonal antibodies have been shown to inhibit fusion (Gonzalez-Scarano *et al.*, 1987). However, Pobjecky *et al.* (1989) reported that liposomes containing G1 were unable to induce fusion, whereas virus particles which had been protease treated so that only intact G2 was present at the virion surface were able to fuse liposomes. The authors suggested that a large hydrophobic domain in G2 is responsible for fusion and that G1 is concerned with interactions with the cell surface receptor rather than with the lipid bilayer directly.

Recently, Ludwig *et al.* (1991a) produced and characterised the first neutralizing monoclonal antibodies specific for the G2 glycoprotein of La Crosse virus. They also described three neutralizing monoclonal antibodies which bound both to G1 and G2. Amongst these was monoclonal antibody 807-22 which had been used previously by Gonzalez-Scarano *et al.* (1985) to select the fusion-defective La Crosse variant V22. In the course of their work, Ludwig *et al.* (1991a) found that monoclonal antibody 807-22 recognised both G1 and G2 of La Crosse virus, raising the possibility that the epitope recognised by this monoclonal antibody on G2 was at least part of the fusion domain previously assigned to G1 (Gonzalez-Scarano *et al.*, 1985). Additionally, Ludwig *et al.* (1991a) demonstrated that the G2 specific monoclonal antibodies had high neutralizing titres when assayed in mosquito cells but limited ability to neutralize virus in mammalian cells. The G1/G2 directed monoclonal antibodies neutralized virus infectivity both in vertebrate and invertebrate cells. These results support the hypothesis that G2 is involved in the interaction of virus with mosquito cells and suggest that G1 and G2 may share a common structural epitope relevant to their role as attachment proteins in vertebrate and mosquito cells.

Using electroncryomicroscopy, Wang *et al.* (1991) demonstrated that low pH conditions were sufficient to induce membrane fusion events between La Crosse virions. The ability of La Crosse virions to form aggregates at low pH lead these authors to propose that La Crosse virus may be capable of fusing to cell membranes without a requirement for a specific receptor.

1.3.2 RNA synthesis

1.3.2.1 Transcription

Transcriptase activity has been detected in detergent disrupted preparations of Lumbo (Bouloy & Hannoun, 1976), La Crosse (Patterson *et al.*, 1984), Germiston (Gerbaud *et al.*, 1987), Uukuniemi (Ranki & Pettersson, 1975) and Hantaan (Schmaljohn & Dalrymple, 1983) viruses.

(i) Initiation

The mRNAs of both bunyaviruses and phleboviruses are known to possess 5' terminal extensions, 12 to 18 nucleotides long, that are heterogeneous in sequence and are not templated from virion RNA (Bishop *et al.*, 1983; Patterson & Kolakofsky, 1984; Eshita *et al.*, 1985; Collett, 1986; Bouloy *et al.*, 1990; Simons & Pettersson, 1991). Patterson *et al.* (1984) demonstrated that oligonucleotides such as $(A)_nG$, cap analogues such as $m^7GpppAm$ and alfalfa mosaic virus (ALMV) RNA 4, stimulated the transcriptase activity associated with La Crosse virions. Primer extension studies of the polymerase products confirmed that these oligonucleotides were acting as primers (Patterson *et al.*, 1984). This was taken as indirect evidence that the non-templated nucleotides observed at the 5' ends of Bunyaviridae mRNAs were methylated, capped fragments of RNA parasitised from host cell mRNAs, analogous to those used to prime influenza virus mRNA synthesis (Krug *et al.*, 1987). More recently, Hacker *et al.*(1990) confirmed that La Crosse virus mRNAs are capped by selecting these mRNAs with anti-cap antibodies. Patterson *et al.* (1984) also detected a methylated cap-dependent endonuclease activity in La Crosse virions which specifically cleaved ALMV RNA 4, approximately 14 nucleotides from its 5' end.

Influenza virus primary transcription takes place in the host cell nucleus due to a requirement for host cell mRNAs synthesised *de novo* as a source of primers for mRNA initiation (Ishihama & Nagatak, 1988). The available evidence suggests that La Crosse virus transcription is quite different in this respect. Rossier *et al.* (1986) pulse-labelled La Crosse virus-infected cells at various times post infection and examined both cytoplasmic and nuclear fractions for labelled La Crosse virus mRNAs. The viral mRNAs were found exclusively in the cell cytoplasm. In addition, unlike influenza virus, La Crosse virus transcription is resistant to drugs such as actinomycin D, which inhibit DNA dependent RNA synthesis by inhibiting host RNA polmerases such as polymerase II (Obijeski & Murphy, 1977). It appears therefore that La Crosse virus acquires its primers from a stable pool of host mRNAs in the cytoplasm rather than from newly synthesised nuclear transcripts, and consequently La Crosse virus mRNA synthesis has no requirement for ongoing host RNA synthesis (Rossier *et al.*, 1986).

Bouloy *et al.* (1990) characterised the 5' extensions of a number of Germiston bunyavirus mRNAs. cDNA copies of single mRNA molecules were cloned into pBR327 and sequenced. Sequencing revealed that the primers have a high G and C content and invariably possess a C or a U residue adjacent to the viral sequence with U preferred. No

two primers were of identical sequence. In several cases the adenosine present at position +1 of the viral sequence was missing. This may be an artifact due to the cloning step and therefore it is difficult to ascertain whether mRNA molecules lacking the ultimate adenosine residue of the viral sequence exist in infected cells. However, similar observations have been reported for influenza virus (Beaton & Krug, 1981), snowshoe hare virus (Bishop *et al.*, 1983) and more recently Uukuniemi virus mRNAs (Simons & Pettersson, 1991).

The initiation of viral mRNA synthesis in cells infected by members of the Bunyaviridae may therefore proceed as follows. The viral polymerase, possibly attached to the 3' end of the genomic RNA, or alternatively free in the cell cytoplasm, binds to the cap group of a host mRNA. The viral exonuclease activity may then cleave the bound host mRNA 10 to 18 nucleotides downstream from the cap structure, exposing a 3' hydroxyl group on the capped fragment. The 3' end of the parasitised RNA may then be aligned at the 3' end of the template RNA and used as a primer by the virion transcriptase to proceed with mRNA synthesis. The first base incorporated by the polymerase would be expected to correspond to the penultimate nucleotide of the genomic template (Kolakofsky & Hacker, 1991).

Hacker *et al.* (1990) reported the existence of a heterogeneous population of 'anti-mRNAs' in La Crosse virus-infected cells. These subgenomic RNAs were transcribed from antigenomic RNA and possessed non-templated sequences at their 5' end. The ratio of anti-mRNAs to mRNAs in infected cells appeared to be similar to that observed with ambisense mRNAs.

(ii) Elongation

There are conflicting data on the requirement for ongoing host protein synthesis during primary transcription of bunyavirus genomes. A translational requirement has been reported for the primary transcription of a number of bunyaviruses in BHK cells including Bunyamwera (Abraham & Pattnaik, 1983); Akabane (Pattnaik & Abraham, 1983); La Crosse (Raju & Kolakofsky, 1986;1987a) and Germiston (Gerbaud *et al.*, 1987) viruses. In contrast, snowshoe hare bunyavirus has been reported to have no translational requirement for primary transcription in BHK cells (Vezza *et al.*, 1979; Eshita *et al.*, 1985). However, Kolakofsky and Hacker (1991) claimed that in the BHK cell line routinely used in their laboratory, snowshoe hare virus exhibited the same translational requirements as La Crosse virus. Another report from that laboratory claimed that there was only a very slight translational requirement for La Crosse virus mRNA synthesis in *Aedes albopictus* C6/36 cells (Raju *et al.*, 1989). It appears therefore that the translational requirement may be cell-type dependent and may even vary between different lines of BHK cells (Raju *et al.*, 1989; Kolakofsky & Hacker, 1991).

Further information has been obtained from *in vitro* studies. La Crosse virions were found to produce only incomplete transcripts in an *in vitro* transcription assay unless rabbit reticulocyte lysate was added to the system. In this coupled transcription-translation

system, drugs which inhibited protein synthesis also inhibited full-length mRNA synthesis and resulted in the reappearance of incomplete transcripts (Bellocq *et al.*, 1987). These results suggested that the translational requirement observed *in vitro* and in most cells *in vivo* was not for mRNA initiation, but to prevent premature termination of transcription. The replacement of guanosine with inosine in the nascent mRNA, which weakens RNA-RNA interactions, substituted for the translational requirement (Bellocq & Kolakofsky, 1987). This supported the idea that concurrent translation of the nascent mRNA was required to prevent interactions between the nascent chain and its RNA template which would otherwise lead to premature termination of transcription. Conversely, replacement of uridines in the nascent RNA with bromouridine was found to enhance premature termination of mRNA synthesis (Kolakofsky & Hacker, 1991). Raju *et al.* (1989) proposed that the translational requirement observed in BHK cells was due to a host factor present in BHK cells but absent from C6/36 cells. The addition of uninfected BHK cell extracts to an *in vitro* transcription system containing La Crosse virions and La Crosse virus nucleocapsids derived from infected mosquito cells resulted in an increased efficiency of initiation of mRNA synthesis and created a translational requirement for full-length mRNA synthesis. *In vitro* transcription of mosquito cell-derived La Crosse virus RNA in the absence of concurrent translation, or any additional factors, yielded full-length mRNAs (Raju *et al.*, 1989). Also, the addition of uninfected mosquito cell extracts to an *in vitro* transcription reaction containing BHK cell-derived La Crosse nucleocapsid RNA failed to relieve the requirement for concurrent translation. Pelleting BHK cell-derived La Crosse virus nucleocapsids through a sucrose cushion reduced the requirement for concurrent translation *in vitro*. It may be that this procedure removes the putative host component.

(iii) Termination

The 3' ends of mRNAs of several members of the Bunyaviridae have been determined. The S segment mRNAs of La Crosse, snowshoe hare, Akabane and Germiston bunyaviruses and Uukuniemi virus have been shown to be truncated at their 3' terminus by approximately 100 nucleotides as compared to virion RNA (vRNA; Cash *et al.*, 1979; Pattnaik & Abraham, 1983; Bouloy *et al.*, 1984; Patterson & Kolakofsky, 1984; Pettersson *et al.*, 1985). Similarly, the M segment mRNAs of snowshoe hare and Germiston bunyaviruses and the phlebovirus Rift Valley fever virus terminate 60, 80 and 112 nucleotides prior to the 3' end of the genomic RNA template, respectively (Eshita *et al.*, 1985; Collett, 1986; Bouloy *et al.*, 1990). Bunyavirus, phlebovirus and uukuvirus mRNAs are probably not polyadenylated, as judged by their inability to bind to oligo(dT)-cellulose (Ulmanen *et al.*, 1981; Abraham & Pattnaik, 1983; Pattnaik & Abraham, 1983; Bouloy *et al.*, 1984; Elliott, 1985; Emery & Bishop, 1987), but Cash *et al.* (1979) did claim to select a proportion of snowshoe hare bunyavirus mRNA molecules by this method.

The template sequences corresponding to the termination sites of six bunyavirus mRNAs, Rift Valley fever phlebovirus M mRNAs and Uukuniemi virus S mRNAs have been mapped by nuclease protection assays. The La Crosse virus S (Patterson & Kolakofsky, 1984) and L (Hacker *et al.*, 1990), snowshoe hare S (Eshita *et al.*, 1985) and Germiston M mRNAs (Bouloy *et al.*, 1990) all terminate near the sequence 3' GUUUUU 5', while Germiston S mRNA (Bouloy *et al.*, 1990) ends at a similar template sequence 3' GUUUGU 5'. The snowshoe hare (Eshita *et al.*, 1985) and Rift Valley fever virus (Collett, 1986) M mRNAs, however, end at the sequence 3' ACCCC 5'. While these sequences may signal transcription termination, this has not been proven. Indeed, the U-rich template sequences corresponding to the 3' ends of most of the above mentioned mRNAs seem unlikely to be the only termination signal recognised by the transcriptase, as similar U-rich sequences are present upstream of the termination site.

Stable secondary structures, such as hairpins, may be candidates for an additional signal, as has been proposed for Punta Toro phlebovirus ambisense S mRNAs (Emery & Bishop, 1987). These authors mapped the 3' termini of Punta Toro N and NSs mRNAs to a region of the viral S RNA between residues 977 and 1017, by Northern analyses using a series of [^{32}P]-labelled synthetic oligonucleotide probes. Computer analysis revealed a potential hairpin structure between nucleotides 866-1092 of the S RNA, and transcription of the N and NSs mRNAs appeared to terminate at either side of the top of this hairpin. The intergenic region of the S RNA of Uukuniemi virus has also been shown to contain a short palindromic sequence with the potential to form an A/U rich hairpin structure (Simons *et al.*, 1990), although the existence of secondary structure in this region and any role it might play in transcription termination remain to be determined. Simons and Pettersson (1991) reported that the 3' ends of the Uukuniemi virus S segment mRNAs overlap each other by approximately 100 nucleotides across this palindromic sequence and no other transciption termination signal was identified. Computer analysis of the 3' noncoding region of Germiston and La Crosse virus mRNAs failed to predict stable hairpin structures (Bouloy *et al.*, 1990), and no evidence has been found for potential secondary structure in the intergenic regions of the ambisense S segments of Rift Valley fever, Toscana, and sandfly fever Sicilian phleboviruses (Giorgi *et al.*, 1991). Thus, the precise mechanisms involved in mRNA transcription termination amongst the Bunyaviridae remain to be elucidated.

Little is known about the primary transcriptional properties of viruses in the *Hantavirus*, and *Nairovirus* genera or in the Uukuniemi serogroup of the recently expanded *Phlebovirus* genus. A manganese-dependent, virion-associated polymerase was found to produce Uukuniemi virus transcripts capable of hybridizing to viral RNA templates, although the transcripts appeared to be small (Ranki & Pettersson, 1975). A polymerase with similar divalent cation requirements was found in association with Hantaan virions (Schmaljohn & Dalrymple, 1983).

1.3.2.2 Genome replication

For members of the Bunyaviridae the initiation of genome replication requires a switch from the synthesis of subgenomic mRNA to the synthesis of full-length, complementary template RNA, and subsequently full-length virion RNA. The processes involved in making that switch have not been defined for any member of the Bunyaviridae. Genome replication and secondary transcription are prevented by translational inhibitors such as cycloheximide, indicating that continuous protein synthesis is required for replication of the genome. It is not known which protein(s) are required, but they are likely to be of viral origin. The virus non-structural proteins (NSs for example) may be potential candidates. The onset of genome replication requires that the mRNA transcription termination signals are suppressed and that the addition of host derived primers to newly synthesised viral-complementary RNAs is prevented. With vesicular stomatitis virus (VSV) the encapsidation of nascent viral RNAs by the viral nucleocapsid protein (N) seems to serve as an antitermination signal, thus allowing full-length genome synthesis (Arnheiter *et al.*, 1985). A role for VSV NS in controlling the availability of the N protein has also been suggested (Howard *et al.*, 1987).

1.3.3 Protein expression strategies and protein processing

Most of the current knowledge regarding the expression of Bunyaviridae proteins has been deduced from nucleotide sequence data. The L, M and S RNA segments of individual viruses employ distinct strategies for protein expression. In addition the gene organisation and expression strategies of each RNA segment, particularly the M and S RNA segments, varies between genera.

1.3.3.1 Expression strategies

(i) L segment proteins

To date, five L gene sequences are available, two from the *Hantavirus* genus and one from each of the *Bunyavirus*, *Phlebovirus* and *Tospovirus* genera (Elliott, 1989a; Schmaljohn, 1990; de Haan *et al.*, 1991; Elliott *et al.*,1992). Each sequence contains a large open reading frame (ORF) in the viral complementary-sense RNA which codes for a primary translation product (L protein) of approximately 2150 amino acids in length in the case of the hantaviruses, and 2238, 2104 and 2875 amino acids in length for Bunyamwera, Uukuniemi and tomato spotted wilt virus, respectively. Additional small ORFs have been identified in the viral-sense L RNAs of both Bunyamwera virus and Hantaan virus which potentially encode proteins of 129 and 221 amino acids in length. The significance of these small open reading frames during a natural infection is questionable since there is no homology between the two putative small polypeptides. In addition, neither the proteins themselves nor their mRNAs have been detected in infected cells and no corresponding ORF has been identified in the viral-sense L RNA of TSWV (de Haan *et al.*, 1991) or

Seoul 80-39 hantavirus (Antic *et al.*, 1991b). The viral L proteins are thought to be translated from a mRNA species just shorter than the full-length genomic RNA segment (de Haan *et al.*, 1991), but no information is available regarding processing of the L protein.

(ii) M segment proteins

With the exception of the *Nairovirus* genus, at least one complete M segment sequence is available for each genus of the Bunyaviridae. In general, a single large ORF encoding a polyprotein precursor to the two envelope glycoproteins (and in the case of bunyaviruses and phleboviruses the NSm protein) is contained within the viral complementary-sense M RNA. However, the M segment RNA of INSV has an ambisense gene arrangement (Law *et al.*, 1992).

The M RNA segments of La Crosse (Grady *et al.*, 1987), snowshoe hare (Eshita & Bishop, 1984), Bunyamwera (Lees *et al.*, 1986) and Germiston (Pardigon *et al.*, 1988) bunyaviruses have been sequenced, revealing ORFs which code for polypeptides ranging from 1433 to 1441 amino acids in length. By sequencing the amino and carboxy termini of the G1 and G2 glycoproteins of snowshoe hare virus, and analysing the reactivity of these glycoproteins with antibodies raised against specific peptides, Fazakerley *et al.* (1988) concluded that the order of the snowshoe hare virus M segment encoded proteins was NH_2-G2-NSm-G1-COOH.

Sequencing studies on a number of hantavirus M segments (Schmaljohn *et al.*, 1987; Yoo & Kang, 1987; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Antic *et al.*, 1991a) revealed that the hantavirus M segment polyproteins are approximately 1135 to 1148 amino acids in length. By direct sequencing of the amino termini of the individual glycoproteins the order of proteins within the precursor polyprotein encoded by Hantaan virus was determined to be NH_2-G1-G2-COOH (Schmaljohn *et al.*, 1987). The G2 protein of Hantaan virus was expressed efficiently by baculovirus and vaccinia virus recombinants which contained cDNA representing the Hantaan virus G2 sequence plus 32 upstream amino acids. The only possible initiation codon was a natural, inframe ATG located 17 amino acids upstream from the amino terminus of G2 (Schmaljohn *et al.*, 1987). Whether this independent initiation of translation of the G2 glycoprotein occurs in authentic viral infections is not known.

M segment sequences have been published for Punta Toro phlebovirus (Ihara *et al.*, 1985b), for three isolates of Rift Valley fever virus (Collett *et al.*, 1985; Takehara *et al.*, 1989) and for Uukuniemi virus (Ronnholm & Pettersson, 1987). The M segments of Punta Toro and Rift Valley fever viruses encode a nonstructural protein in their precursor polyprotein but Uukuniemi virus does not. The order of proteins in the precursor polypeptides has been determined to be NH_2-NSm-G1-G2-COOH for Punta Toro virus, NH_2-NSm-G2-G1-COOH for Rift Valley fever virus and NH_2-G1-G2-COOH for Uukuniemi virus. The apparent difference in the gene order of Punta Toro and Rift Valley fever virus

M segments is misleading and arises due to the convention of defining G1 as the larger of the two glycoproteins and the similarity in the sizes of the two phlebovirus glycoproteins.

Suzich *et al.* (1990) investigated the expression strategy of the Rift Valley fever virus (RVFV) M segment by performing site-directed mutagenesis on potential translation start sites in RVFV M segment-vaccinia virus recombinants and then expressing these recombinants. The RVFV M segment ORF contains four AUG codons between the AUG initiating the ORF and the amino terminus of mature G2 (Collett *et al.*, 1985). Suzich *et al.* (1990) revealed that RVFV M segment employs a relatively complex expression strategy which involves the use of at least two AUG codons in the same ORF to produce its full complement of gene products. In addition, the biosynthetic pathways for the two major viral glycoproteins, G2 and G1, appeared to be distinct from each other with a significant proportion of G1 synthesised independently of the other M gene products. Since Northern analysis of RNA isolated from cells infected with the recombinant viruses revealed only a single species of M segment sized mRNA which was found to possess preglycoprotein G1 and G2 coding sequences, the mechanism for independent production of G1 must occur at the post-transcriptional level. Conceivably, a proportion of RVFV G1 may be produced by internal initiation of translation downstream of the preglycoprotein region. Initiation of translation from the first ATG of the ORF appeared to predetermine the utilization of an N-linked glycosylation site within the preglycoprotein region and also precluded cleavage at the preglycoprotein-G2 junction (Kakach *et al.*, 1989). Conversely, initiation from the second ATG prevented glycosylation at this site but allowed proteolytic cleavage at the amino terminus of G2. Proteolytic cleavage at this junction is known to be independent of glycosylation (Kakach *et al.*, 1989) which suggests that the 37 amino acids between ATG codons 1 and 2 can influence proteolytic processing and glycosylation further along the polyprotein. Thus, a multisite translation initiation strategy may serve as a mechanism for controlling post-translational protein modifications.

In the absence of a complete nairovirus M segment sequence, information regarding the synthesis and processing of nairovirus glycoproteins is limited. The M RNA of nairoviruses generally encodes two glycoproteins, although Hazara nairovirus encodes three glycoproteins (Foulke *et al.*, 1981). Recently the first tospovirus M RNA sequence became available; revealing that the M RNA segment of INSV encodes a polyprotein precursor to glycoproteins G1 and G2 and a non-structural protein (NSm) in an ambisense arrangement (Law *et al.*, 1992). By *in vitro* transcription and translation the order of G1 and G2 in the precursor polyprotein was determined to be NH_2-G2-G1-COOH.

(iii) S segment proteins

The S RNA segments of hantaviruses and nairoviruses code only for the nucleocapsid protein (N), while those of the bunyaviruses, phleboviruses and tospoviruses code for two proteins, the N protein and a non-structural protein (NSs). Bunyavirus S segment proteins are translated from a single bicistronic mRNA species, while viruses of

the *Phlebovirus* and *Tospovirus* genera translate their S segment proteins from two monocistronic mRNAs of opposite polarity, which are transcribed from an ambisense S RNA (see Section 1.2.2). An additional ORF has been identified in the S RNA segments of Germiston and Maguari bunyaviruses. This is situated downstream of and in the same reading frame as the NSs protein (Gerbaud *et al.*, 1987; Elliott & McGregor, 1989). The product of this third ORF has been translated *in vitro* from RNA transcribed from cloned Maguari virus cDNA (Elliott & McGregor, 1989), but neither of these putative proteins have been detected in infected cells (Elliott & McGregor, 1989; Bouloy *et al.*, 1990).

Initiation of translation at the second AUG in the bicistronic bunyavirus S mRNA is thought to occur via "leaky scanning" of ribosomes along the mRNA (Kozak 1986a, 1986b). It has been proposed that a proportion of ribosomes scanning the 5' noncoding region can bypass the first AUG initiation codon and continue scanning until they find the AUG which initiates the second ORF. Kozak (1986b) defined the optimum consensus sequence for the initiation of translation as 5' (A/G)CCAUGG 3'; with a purine (preferentially an A residue) at position -3 and a G residue at position +4 (where the A of the AUG is designated +1) being the most important determinants. The leaky scanning model proposes that when the 5' proximal AUG in a bicistronic mRNA is in a suboptimal sequence context, ribosomal preinitiation complexes can bypass the first AUG and initiate at a downstream AUG which is in a more favourable context.

The NSs ORFs in Maguari and La Crosse bunyaviruses commence with tandem AUG codons (Cabradilla *et al.*, 1983; Elliott and McGregor, 1989). Which of these AUG codons is used for translation initiation remains to be determined.

	-3	+1	+4
Optimal	ACC	AUG	G
N	UCA	AUG	A
NSs (First)	UCA	AUG	A
NSs (Second)	AUG	AUG	U

Figure 1.1 Sequence contexts of the AUG codons initiating the N and NSs ORFs in the Maguari virus S RNA; including the optimum sequence context for initiation of translation as defined by Kozak (1986b). Adapted from Elliott and McGregor (1989).

Elliott and McGregor (1989) compared the sequence contexts of the AUG codons initiating the N and NSs ORFs in Maguari virus (see Figure 1.1) and found that the sequence context of the second AUG at the initiation site of NSs was the most favorable. The first AUG at the start of the NSs protein and the AUG initiating the N protein ORF were in identical sequence contexts.

The S RNA segment of Hantaan virus possesses a single ORF in the viral complementary-sense RNA which encodes an N protein 429 amino acids in length (twice as large as the N proteins of the other genera; Schmaljohn *et al.*, 1986b). The other known Hantaan S segment sequences: Sapporo rat virus (Arikawa *et al.*, 1990); Nephropathia Epidemica virus (NEV) strain Hallnas B1 (Stohwasser *et al.*, 1990) and Prospect Hill virus (Parrington & Kang, 1990) display a similar gene organisation. Additional small ORFs have been detected in the viral complementary-sense RNA of Hantaan virus, NEV Hallnas strain B1 and Prospect Hill virus and also in the virion-sense RNA of Sapporo rat virus. However, the products of these ORFs have never been detected in infected cells.

The only sequence information available for the *Nairovirus* genus is the complete sequence of the S RNA of Dugbe virus (Ward *et al.*, 1990). Dugbe virus S segment RNA contains one ORF in the viral complementary-sense RNA which encodes the N protein, and another small ORF in the virion RNA which may not be expressed. Five phlebovirus S segment sequences have been determined; Punta Toro (Ihara *et al.*, 1984), Sandfly fever Sicilian (Marriott *et al.*, 1989); Uukuniemi virus (Simons *et al.*, 1990) and Toscana and Rift Valley fever virus (Giorgi *et al.*, 1991). The N proteins of these viruses exhibit low sequence homology and the NSs proteins are even less well conserved.

De Haan *et al.* (1990) sequenced the S RNA segment of TSWV. They found two ORFs, one in the genomic-sense RNA, which encoded a 465 amino acid protein and another in the virus complementary-sense RNA, which coded for a smaller protein of 259 amino acids in length. The smaller of these two proteins was identified as the virus nucleocapsid protein by immunoprecipitation with antisera raised against purified TSWV nucleocapsids. Kormelink *et al.* (1991) made recombinant baculoviruses that expressed high levels of the larger of these two proteins and demonstrated by western blot analysis that this protein was absent from purified TSWV virions. This is the largest non-structural protein so far identified for any member of the Bunyaviridae. Conceivably, the TSWV S segment encoded non-structural protein (NSs) may contain an extra domain which is important for infection processes specific to plants. These authors observed a correlation between the levels of TSWV NSs protein in infected plants and the severity of disease symptoms. The nucleocapsid protein ORF of a second tospovirus isolate, INSV has now been sequenced (Law *et al.*, 1991). This ORF encodes a protein of 262 amino acids in length which is 67% homologous to TSWV at the amino acid level. (N protein)

1.3.3.2 The kinetics of viral protein synthesis in infected cells

Viral protein synthesis in cell cultures infected with members of the Bunyaviridae has been followed most commonly by pulse-labelling infected cells with [^{35}S]-methionine and fractionating radiolabelled cell extracts on SDS-PAGE gels. The viral N protein, being the most abundant viral protein in infected cells, is reliably detected by this procedure. The other viral proteins can be obscured if host cell protein synthesis is not inhibited by the

infection, but this problem can be overcome by immunoprecipitation of the viral proteins prior to electrophoresis (Watret & Elliott, 1985a; Ulmanen *et al.*, 1981). The G2 glycoprotein is not reliably detected on SDS-PAGE gels and this has recently been shown to be due to the heat-labile nature of this protein (R.M. Elliott, personal communication).

The viral glycoproteins are not detected in infected cells until after the onset of synthesis of the other viral proteins. This has been demonstrated with, for example, Bunyamwera virus (Pennington *et al.*, 1977), St. Abb's Head virus (Uukuniemi serogroup of the phleboviruses; Watret & Elliott, 1985a) and Uukuniemi virus (Ulmanen *et al.*, 1981). The rate of viral protein synthesis in cells infected with members of the Bunyaviridae depends to a degree on the moi used, but in general viral messages are transcribed and translated rapidly. High multiplicity infections usually result in maximum levels of viral protein synthesis being observed 3 to 5 h pi, as was observed in BSC-1 and BHK cell cultures infected with 40 pfu/cell of Bunyamwera and 50 pfu/cell La Crosse bunyaviruses respectively (Pennington *et al.*, 1977; Madoff & Lenard, 1982). Parker *et al.* (1984) detected RVFV N protein as early as 2 h pi in Vero cells which had been infected at a high moi.

Watret *et al.* (1985) determined the protein synthesis profiles for members of the *Bunyavirus, Phlebovirus* and *Nairovirus* genera in XTC-2 cells infected at a moi of 1 to 5 pfu/cell. They determined that Bunyamwera virus N protein was synthesised at high levels in XTC-2 cells by 4 h pi and continued to be translated at high levels up to the last time point tested (24h pi); host protein synthesis had been completely abolished by this time. Rates of viral protein synthesis were slower for nairoviruses and phleboviruses; neither Clo Mor nor Dugbe nairoviruses significantly inhibited host cell protein synthesis in XTC-2 cells, and the N protein of Clo Mor virus was not detected in infected XTC-2 cells until 8h pi. Low multiplicity studies (0.1 to 2 pfu/cell) with Dugbe nairovirus in BSC-1 cells also revealed slow kinetics of viral protein synthesis, with maximum levels of viral protein synthesis occurring between 24 and 31 h pi, and no significant inhibition of host protein synthesis (Cash, 1985). Sicilian sandfly fever phlebovirus failed to shut off host cell protein synthesis in XTC-2 cells, and viral protein synthesis was relatively slow (Watret *et al.*, 1985). The exact onset of N protein synthesis was not determined but significant levels of N protein were still being synthesised at 48 h pi. In a parallel infection of BSC-1 cells by Sicilian sandfly fever virus, significant levels of N protein were detected at 24 and 48 h pi and host protein synthesis was shut off. St. Abb's Head virus (Uukuvirus serogroup of the *phlebovirus* genus) did not shut off host cell protein synthesis in XTC-2 cells and also displayed slower kinetics of protein synthesis than bunyaviruses; only low levels of N protein were detected at 4 h pi but levels increased gradually throughout the period studied (up to 16 h pi). Similar slow rates of viral protein synthesis, without host cell protein synthesis shut off, have been observed during Uukuniemi virus infection of chick embryo

cells (Ulmanen *et al.*, 1981) and during hantavirus infection of Vero cells (Schmaljohn & Dalrymple, 1984).

1.3.3.3 Protein processing

The L and S segment encoded proteins of the Bunyaviridae do not require post-translational processing, as they are translated directly from individual mRNA species. Post-translational modification has only been reported for the NSs protein of Rift Valley fever virus which appears to be phosphorylated (Struthers *et al.*, 1984). In contrast, the M segment proteins are both processed and modified. With the exception of INSV, which has an ambisense M RNA segment (Law *et al.*, 1992), each Bunyaviridae M segment RNA encodes a single large ORF in the viral complementary sense from which a mRNA coding for a polyprotein consisting of the viral glycoproteins (and in the case of the bunyaviruses and phleboviruses the NSm protein) is transcribed.

Little is known regarding the processing of this polyprotein to yield the individual viral proteins. However, alanine specific proteases have been implicated in the cleavage events generating the amino termini of the G1 and G2 proteins of Rift Valley fever and Punta Toro phleboviruses (Ihara *et al.*, 1985b), and also in the cleavage events generating the carboxy terminus of G2 and possibly the amino terminus of G1 from bunyavirus precursor polypeptides (Elliott *et al.*, 1991). Precursor polyproteins have only been detected in a few instances, and only in nairovirus infected cells (Clerx & Bishop, 1981; Cash, 1985; Watret & Elliott, 1985b). The difficulty encountered in detecting the precursor polyproteins in infected cells suggests that processing occurs cotranslationally (Pennington *et al.*, 1977; Lees *et al.*, 1986; Schmaljohn *et al.*, 1986a; 1990; Bouloy, 1991). Attempts to translate bunyavirus M segment specific mRNA *in vitro* have been unsuccessful (Abraham & Pattnaik, 1983; Elliott, 1985). However Ulmanen *et al.* (1981) produced a 110kDa protein by *in vitro* translation of Uukuniemi virus M segment specific mRNA, which was cleaved in the presence of microsomal membranes to generate G1 and G2. Independent initiation of translation of the downstream glycoprotein has been observed *in vitro* with recombinant baculovirus and vaccinia virus constructs expressing M gene sequences from Hantaan and Rift Valley fever viruses (Schmaljohn *et al.*,1989; Suzich *et al.*, 1990). However, it is not known if independent initiation of translation of viral glycoproteins occurs *in vivo*.

All of the Bunyaviridae envelope proteins examined to date possess N-linked oligosaccharides. Bunyavirus glycoproteins contain relatively few potential glycosylation sites, with only four potential glycosylation sites evident in the M segment polyprotein encoded by Bunyamwera virus (Vorndam & Trent, 1979; Cash *et al.*, 1980; Lees *et al.*, 1986; Pardigon *et al.*, 1988). Studies with glycosylation inhibitors have confirmed that at least some of these sites are utilised in the mature glycoproteins (Cash *et al.*, 1980). The oligosaccharide side chains on bunyavirus glycoproteins consist mainly of endoglycosidase

H (endo-H) resistant complex glycans (Madoff & Lenard, 1982; Pesonen *et al.*, 1982), although some endo-H sensitive high mannose and some small endo-H-resistant intermediate-type glycans are found. The bunyavirus glycoproteins also contain covalently attached fatty acids (Madoff & Lenard, 1982).

Five N-linked glycosylation sites were identified in the glycoprotein coding sequences of Rift Valley fever and Punta Toro phleboviruses, however, the number of sites utilized in the mature glycoproteins was not defined (Collett *et al.*, 1985; Ihara *et al.*, 1985b). Uukuniemi virus glycoprotein coding sequences contain eight potential N-linked glycosylation sites (Ronnholm & Pettersson, 1987). Pesonen *et al.* (1982) determined that the G2 protein of Uukuniemi virus possesses mainly high mannose glycans, whereas G1 contains both complex and novel intermediate-type oligosaccharides. Hantaan virus glycoproteins possess a total of seven potential glycosylation sites, five were identified in G1 and two in G2. In this case the glycans were found to be mainly of the high mannose type (Elliot *et al.*, 1984; Schmaljohn *et al.*, 1986a; Schmaljohn *et al.*, 1987). Giebel *et al.* (1989) sequenced the M segment of Nephropathia Epidemica virus, strain Hallnas B1 (NEV) and found that all but one of the potential glycosylation sites observed in Hantaan virus were found in identical positions in NEV and two potential tyrosine sulphation sites (which are physiologically relevant only to proteins passing through, or located in the Golgi complex) were conserved between the two viruses. Recently, Law *et al.* (1992) identified nine putative N-linked glycosylation sites in the glycoprotein coding sequences of INSV.

All M segment gene products appear from their cDNA sequences to have a high cysteine content (5 - 7%). In related viruses the positions of these cysteine residues are conserved (Fazakerley *et al.*, 1988; Ihara *et al.*, 1985b; Ronnholm & Pettersson, 1987; Schmaljohn *et al.*, 1988). These findings suggest that there may be extensive disulphide bridge formation within the glycoproteins and the positions of the cysteines may be crucial for the correct folding of the protein. The molecular structure of the glycoprotein complexes at the surface of virions has not been determined. However, despite the abundance of cysteine residues in both G1 and G2, it appears from work with La Crosse virus that these proteins are not linked to each other by disulphide bonds (Gonzalez-Scarano & Nathanson, 1990).

1.3.3.4 Intracellular transport and targetting of the viral glycoproteins

The hydropathy profiles of the polyproteins of La Crosse, snowshoe hare, Bunyamwera and Germiston bunyaviruses are similar. Each begins with a hydrophobic region which is believed to act as a signal peptide for translocation of the polypeptide. Another hydrophobic domain towards the centre of the G2 molecule may serve as a transmembrane domain, while another signal-like sequence lies between NSm and G1. A hydrophobic domain at the carboxy terminus of G1 may function as a membrane anchor sequence.

The hydropathy profile of the Hantaan virus M segment polyprotein predicts a short hydrophobic leader sequence prior to the amino terminus of mature G1 and another short hydrophobic region preceding the amino terminus of G2 (Schmaljohn *et al.*, 1987). Giebel *et al.* (1989) sequenced the M segment of Nephropathia Epidemica nairovirus, strain Hallnas B1 (NEV). Comparison of the polyprotein coding sequences of Hantaan and NEV revealed a number of conserved features. The positions of the hydrophobic leader sequences preceeding Hantaan virus G1 and G2 glycoproteins were conserved in NEV, as was the amino acid sequence of a short hydrophilic domain within which cleavage of the hydrophobic leader is thought to occur to yield mature G2.

Chen *et al.* (1991) found that the G2 glycoprotein of Punta Toro phlebovirus did not contain an integral signal for Golgi retention but depended upon an association with G1. Subsequently, Chen and Compans (1991) reported that G1/G2 heterodimers were formed in Punta Toro virus infected cells within three minutes of the onset of viral protein synthesis.

Persson and Pettersson (1991) analysed the heterodimerization and intracellular transport of Uukuniemi virus G1 and G2 glycoproteins. They determined that G1 acquired its mature form within 10 minutes, having completed its disulphide bond formation, while completion of disulphide bond formation in G2 required up to 60 minutes. Pulse-chase experiments in the presence of cycloheximide indicated that the efficient transport of G2 to the Golgi required continuous synthesis of G1. It appeared that G1 and G2 were dependent upon each other for transport to the Golgi from the ER.

Ruusala *et al.* (1992) investigated the intracellular transport and targeting of the Hantaan virus glycoproteins by expressing the glycoproteins either individually or together from recombinant vaccinia viruses in Hela cells. Using immunofluorescence and subcellular fractionation these authors determined that G2 and G1 of Hantaan virus had to interact with each other in the endoplasmic reticulum (ER) in order to be transported to the Golgi complex. When either of the glycoproteins were expressed in the absence of the other they were retained in the ER. Studies with monoclonal antibodies suggested that G1 expressed alone did not acquire its correct conformation.

1.3.4 Assembly and virus release

The Bunyaviridae characteristically mature at Golgi membranes (Lyons & Heyduk, 1973; Murphy *et al.*, 1973; Kuismanen *et al.*, 1982, 1984; Smith & Pifat, 1982), although, Rift Valley fever phlebovirus has been observed to mature at the cell surface of infected rat hepatocytes (Anderson & Smith, 1987). Virus assembly appears to occur via an interaction between viral ribonucleoprotein (RNP) structures, which accumulate on the cytoplasmic side of Golgi membranes, and viral envelope proteins, which are displayed on the luminal side. Such interactions have only been observed across regions of Golgi membrane directly involved in the budding process. RNPs have not been found associated

with Golgi membranes devoid of viral glycoproteins, suggesting that transmembranal recognition between the viral glycoproteins and the N protein is a prerequisite for budding (Smith & Pifat, 1982). Accumulation of the viral glycoproteins in the Golgi complex causes progressive vacuolization (Kuismanen *et al.*, 1984), and this effect has been shown to be mediated by the glycoproteins alone (Gahmberg *et al.*, 1986a). The morphologically altered Golgi complex remains functionally active (Gahmberg *et al.*, 1986b). Expression of hantavirus and phlebovirus glycoproteins in recombinant vaccinia vectors has shown that Golgi targeting is an intrinsic property of the glycoproteins (Matsuoka *et al.*, 1988; Pensiero *et al.*, 1988). However, the signal responsible for Golgi localization has yet to be determined. After the virus particles bud into the Golgi cisternae, it is thought that they are released into the cell cytoplasm in small vesicles, in a manner analagous to secretory granules (Broadwell & Oliver, 1981; Rothman, 1981; Smith & Pifat, 1982). The virus particles are then believed to be released from the cell by exocytosis.

1.3.5 Effects of viral replication on host cells

The cytopathic effects observed in cell cultures infected with members of the Bunyaviridae vary widely, depending both on the virus and the type of host cell studied. With the exception of the *Hantavirus* genus all members of the Bunyaviridae are capable of alternate replication in vertebrate and invertebrate hosts. Generally the Bunyaviridae are cytolytic for their vertebrate hosts but cause little or no pathogenicity in their invertebrate hosts (James & Millican, 1986; Lyons & Heyduk, 1973).

Variation has been observed in the ability of members of the Bunyaviridae to induce host cell protein synthesis shut off in infected vertebrate cells. Bunyaviruses and phleboviruses have been shown to cause a progressive decrease in host protein synthesis during the course of infection in vertebrate cells. In Bunyamwera virus infected BSC-1 cells host cell protein synthesis declined rapidly between 5 and 7 h pi. In the absense of detectable host protein synthesis these cells continued to support virus replication. The synthesis of virus structural proteins was detected up to 22 h pi and infectious virus production continued up to 30 h pi (Pennington *et al.*, 1977). Similar results were obtained with La Crosse virus infected BHK-21 cells (Madoff & Lenard, 1982). Reduced host cell protein synthesis was observed in Rift Valley fever virus infected Vero cells and became gradually more pronounced between 4 to 20 h pi (Parker *et al.*, 1984). However, Sicilian sandfly fever phlebovirus did not significantly inhibit host cell protein synthesis in XTC-2 cells, although this virus efficiently shut off BSC-1 cell protein synthesis (Watret *et al.*, 1985). No reduction in host protein synthesis has been observed in mammalian cells infected with Uukuniemi virus or Clo Mor and Dugbe nairoviruses (Pettersson, 1974; Ulmanen, 1981; Watret *et al.*, 1985; Cash, 1985).

Hantaviruses cause no detectable reduction in host macromolecular synthesis (Elliot *et al.*, 1984; Schmaljohn & Dalrymple, 1984) and routinely establish persistent,

non-cytolytic infections in susceptible mammalian host cells, reflecting their non-pathogenic persistence in their natural rodent hosts (Lee *et al.*, 1981a). Interestingly, Dugbe and Hughes serogroup nairoviruses have been shown to readily establish persistent infections in mammalian cells (BSC-1 and Vero cells respectively; Gould *et al.*, 1983; Cash, 1985). In both virus-cell systems an initial acute phase of infection was superceded by a persistent infection. In the case of Dugbe virus, recovery of the cell monolayer was accompanied by a decline in virus induced cytopathic effect (cpe) and virus protein synthesis (Cash, 1985). Immunofluorescence studies on Vero cells persistently infected with Hughes serogroup nairoviruses demonstrated that 20 to 40% of these cells contained cytoplasmic viral antigen (Gould *et al.*, 1983). The arthropod-borne members of the Bunyaviridae cause no significant cytopathology in mosquito cell cultures and persistent infections are readily established (Newton *et al.*, 1981; Carvalho *et al.*, 1986; Elliott & Wilkie, 1986; Rossier *et al.*, 1988; de Haan *et al.*, 1990).

2 Persistent viral infections

Persistent viral infections can be categorized as slow, latent or chronic. Persistent infections described as slow are associated with the so-called "unconventional viruses" of which scrapie is the prototype. Following incubation periods of months to years these agents give rise to a unique set of degenerative brain diseases, the spongiform encephalopathies. These fatal diseases include scrapie in ruminants and rodents, bovine spongiform encephalopathy in cattle, and Creutzfeld-Jakob disease, Kuru and Gerstmann-Straussler syndrome in humans (Gajdusek, 1990). During a latent infection no infectious virus is present, for example herpes viruses and retroviruses persist latently by integration into the host genome (Weinberg, 1980; Roizman & Sears, 1987; Stevens, 1989). Chronic virus infections are productive persistent infections which can arise after an acute episode or an inapparent sub-clinical infection. Two well studied examples are hepatitis B virus infection of humans (Marion & Robinson, 1983) and lymphocytic choriomeningitis virus infection of mice (Lehmann-Grube *et al.*, 1983). It is with this latter category of persistent infection that the work in this thesis is concerned and any reference to persistent infection in the text refers specifically to chronic infections.

For a virus to persist in the course of a natural infection it must accomplish two things: first it must not be overtly cytolytic (for example most arenaviruses can replicate in host cells without adverse effects; Buchmeier *et al.*, 1980), or it must have available a means of attenuating its lytic potential (for example via restricted gene expression or by the generation of viral variants which are less cytolytic or interfere with the growth of wild-type virus; Chambers & Webster, 1987; Cattaneo *et al.*, 1988a, 1988b); second it has to avoid detection and elimination by the host's immune system. Despite the obvious importance of the immune system in modulating persistent infections *in vivo* it is likely that mechanisms fundamental to the establishment of persistence are to be found in the molecular interactions between the virus and its host cell. It is reasonable therefore to expect the mechanisms involved in the establishment and maintenance of persistent infections in tissue culture to resemble those underlying persistent infections *in vivo*. The readiness with which Bunyamwera virus establishes a persistent infection in *Aedes albopictus* C6/36 cells and the ease with which the persistent state can be maintained (Elliott & Wilkie, 1986) are two factors which make this virus-cell combination a useful system with which to study the molecular mechanisms leading to persistence, in the absence of the complexities of the host's immune response and clearance mechanisms.

As the work presented here was performed solely in tissue culture this review focuses mainly on *in vitro* studies but does include relevant *in vivo* investigations. While I have not attempted to cover the vast literature on the immunology of persistent infections (reviewed by Oldstone, 1989b; 1991), I occasionally refer to immunological aspects, for instance with regard to the mechanisms by which persistent infections might cause disease,

or with regard to the evolution of virus variants capable of escaping immune surveillance during the course of a persistent infection.

2.1 Involvement of persistent viral infections in human disease

There has been increasing interest in the possible involvement of persistent viral infections in a variety of human diseases. Chronic infections of the central nervous system (CNS) of man have been reported for measles virus, papovaviruses (JC and SV-40), rubella virus, cytomegalovirus, herpes simplex virus, adenovirus types 7 and 32, Russian spring-summer encephalitis (RSSE) virus, and the human retroviruses human T-cell lymphotrophic virus 1 and human immunodeficiency virus (Gajdusek, 1990).

Subacute sclerosing panencephalitis (SSPE) is a fatal, slowly progressing, inflammatory disease of the CNS. In 1967 Connolly *et al.* demonstrated that SSPE patients had extremely high measles antibody titres in their serum and cerebrospinal fluid (CSF). Subsequent immunofluorescence studies demonstrated that measles virus antigens were present in neurons and glial cells (Freeman *et al.*, 1967; Connolly, 1968). Infectious virus was obtained from these cells by co-cultivation or fusion with continuous cell lines susceptible to measles virus. In this way SSPE virus (a measles virus variant) was isolated (Horta-Barbosa *et al.*, 1969; Barbanti-Brodano *et al.*, 1970). This agent has been repeatedly obtained from both biopsy and autopsy material and from the lymph nodes of SSPE patients (Agnarsdottir, 1977).

Paget's disease is a slowly progressive, painful, deformity-inducing disease of bone. Electron microscopic studies revealed the presence of paramyxovirus nucleocapsids in abnormal osteoclasts taken from patients with Paget's disease (Mills & Singer, 1976; Rebel *et al.*, 1977; Harvey *et al.*, 1982). Both respiratory syncitial virus (RSV) and measles virus have been implicated in the etiology of this disease. Measles virus antigens were detected in bone sections, using immunofluorescence and peroxidase techniques (Rebel *et al.*, 1980; Basle *et al.*, 1985). In later experiments, Basle *et al.* (1986) demonstrated measles virus nucleic acid in both osteoclasts and osteoblasts (bone forming cells) of Pagetic bone, but not in control sections. RSV antigens were detected using fluorescent antibodies in abnormal bone osteoclasts in each of 12 patients with Paget's disease but not in osteoclasts from controls with other bone diseases (Mills *et al.*, 1981). Later, Mills *et al.* (1984) reported the simultaneous detection of both RSV and measles virus antigens in osteoclasts from the same patients. Other paramyxoviruses, simian virus 5 (SV-5) and parainfluenza virus 3 (PIV-3), have also been detected by immuno-fluorescence in Pagetic bone osteoclasts (Basle *et al.*, 1985).

Many viruses, including paramyxoviruses, have been proposed as etiological agents in multiple sclerosis. Measles virus, parainfluenza virus type-1 (PIV-1) and (SV-5), have all been isolated from tissues taken from multiple sclerosis patients (Russell, 1983).

Antibodies to measles virus, parainfluenza viruses 1, 2 and 3, and SV-5 have been detected in the cerebrospinal fluid of multiple sclerosis patients (Goswami *et al.*, 1987; Russell *et al.*, 1989; Vandvik & Norrby 1989), and levels of humoral antibodies against measles virus were much higher in multiple sclerosis patients than in controls (Adams & Imagawa, 1962; Albrect *et al.*, 1983). More recently, Reddy *et al.* (1989) demonstrated an association between the occurence of HTLV-1 related sequences in peripheral blood monocytes and macrophages from multiple sclerosis patients and the development of disease.

Progressive encephalitides develop in some individuals due to the persistence of certain echoviruses and polioviruses in the CNS (Wyatt, 1973; Davis *et al.*, 1977; Wilfert *et al.*,1977). Dalakas (1986) proposed that a post-poliomyelitis syndrome observed in some individuals was due to the persistence of poliovirus in these individuals. The case for the involvement of a persistent infection has been strengthened by the detection of an intrathecal immune response against poliovirus in patients with post-poliomyelitis syndrome (Sharief *et al.*, 1991). Currently, there is interest in the possible association between persistent enterovirus infection of muscle and post-viral fatigue syndrome (Archard *et al.*, 1988; Cunningham *et al.*, 1990).

2.1.1 Mechanisms by which persistent viral infections could cause disease

There are many possible mechanisms by which persistent viral infections could cause disease. Long term, low level genome replication within certain differentiated cell types or tissues may cause direct cell damage. Evidence is available which suggests that viruses can cause disease by altering the differentiated functions of a cell without affecting its vital functions (Holtzer, *et al* 1975; Oldstone, 1984; 1989a). Viral antigens exposed at the surface of cells may trigger immune system damage to the cells, while the interaction of viral proteins with "self" antigens may lead to the generation of "anti-self" responses; for example paramyxoviruses are known to break tolerance to cellular actin (Tyrrell *et al.*, 1987). The interaction of the M protein of Newcastle disease virus (NDV) with actin has been demonstrated to induce a conformational change in these proteins and to enhance the antigenicity of actin (Giuffre *et al.*, 1982; Tyrrell *et al.*, 1987). It is believed that the conformational change induced in actin by interaction with the M protein is sufficient to expose a new antigenic site at the carboxyl end of the actin molecule. It is possible that antigenic mimicry, where an antigenic epitope on a viral protein induces an antibody or T-cell clone which cross reacts with an epitope on a normal host cell protein, may have a role to play. Cross-reacting monoclonal antibodies for measles virus phosphoprotein and the intermediate filaments of neuronal cells (Fujinami *et al.*, 1983), and measles virus fusion protein and cellular stress protein (Sheshberadoran & Norrby, 1984), have been studied. In addition, persistent shedding of viral antigens could lead to immune complex disease and

infection of immunocytes could result directly in depression or hypereactivity of immune responses.

2.2 Characteristics of persistently infected cell cultures

Persistently infected cell cultures continue to shed virus after prolonged passage, although the levels of released virus may be very low. Sometimes infectious virus is not produced because mutations, host cell factors and defective interfering (DI) particles prevent virus maturation. Alternatively low levels of slowly replicating virus variants may be produced. Characteristically, persistently infected cultures harbour intracellular viral genomes, express viral antigens and are resistant to superinfection with homologous virus. The mechanisms involved in viral persistence are poorly understood. In the light of a multitude of studies carried out on a variety of virus-cell systems, persistence has been ascribed to:

(i) The evolution of viral mutants with a range of phenotypes. Temperature-sensitive and small-plaque mutants are commonly observed in association with persistent infections. Mutants defective in RNA synthesis and viruses which have lost their ability to switch off host protein synthesis are also believed to play a role in some persistent infections.

(ii) The inherent resistance of cells to virus.

(iii) The co-evolution of cells and virus.

(iv) The production of interferon or antiviral activity.

(v) The presence of DI particles.

While each of these factors is known to play a role in the establishment and maintenance of persistent viral infections, it is far from clear how these mechanisms operate, either alone or in combination with each other. The relative importance of each of the factors listed above appears to vary widely in different virus-cell systems and each will be discussed in the following sections.

2.2.1 Evolution of viral variants during persistent infections

Oligonucleotide fingerprinting and sequencing studies have revealed extreme variability amongst RNA genomes. Most natural isolates and clonal populations of RNA viruses consist of mixtures of variant genomes. Upon replication new variants constantly arise and the proportion of each of them increases or decreases in competition with other variants in the population. Thus a given RNA virus population is in a dynamic equilibrium defined by a "consensus" sequence. The composition of this consensus sequence is dependent on prevailing selective pressures (Domingo & Holland, 1988). Persistent infections might be expected to provide an excellent opportunity for the inherent high mutation rate of RNA genomes (due to the lack of proof reading activity associated with their encoded RNA polymerases) to be reflected in high rates of genome change. Genomic

*Also (Burrows, 1966).

sequence alterations during RNA virus persistence have been well documented. For instance, virus variants exhibiting an increased resistance to a particular species of DI RNA, relative to the original virus used to establish the persistent infection, have been observed in persistent infections established with rabies virus (Kawai & Matsumoto, 1977), VSV (Horodyski & Holland, 1980), and lymphocytic choriomeningitis virus (Jacobson & Pfau, 1980). Depolo *et al.* (1987) reported the continual, out of phase, co-evolution of DI RNAs and DI resistant variants of VSV, upon undiluted passage of this virus in BHK cells.

The introduction of a number of different variants into a cell which will support long-term replication provides an environment where intergenomic complementation could result in the propagation of even lethal mutations until further mutations suppress their lethality. Rapid changes in phenotypic properties are possible through the selection of variants present or arising in the heterogeneous population. Foot-and-mouth disease virus (FMDV), a picornavirus, shows remarkable antigenic variability. This virus causes an acute disease of domestic and wild cloven-hooved animals, and inapparent persistent infections are common in areas where the disease is enzootic (Burrows, 1966; Auge de Mello *et al.*, 1970). The virus may persist in the oesophageal pharyngeal region of cattle and other ruminants for up to several years without causing signs of disease (Condy *et al.*, 1985). Early studies showed that FMDV isolates from carrier animals differed from FMDV associated with acute disease in being temperature-sensitive and producing small plaques on cell monolayers (Straver & van Bekkum, 1972). More recent studies have indicated that the emergence of FMDV variants stems from the extreme genetic heterogeneity of the virus (Domingo *et al.*, 1980; Domingo *et al.*, 1985; Sobrino *et al.*, 1983), and the selection or random drift of variants continuously arising during viral replication (Rowlands *et al.*, 1983).

Gebauer and coworkers (1988) demonstrated the rapid selection of genetic and antigenic variants of FMDV during persistence in cattle. They established a carrier state experimentally and recovered virus from the oesophageal pharyngeal area of the animals up to 539 days postinfection. Their data, based on the analysis of viral capsid proteins by electrofocusing, and the electrophoretic mobility of the genomic poly(C)-rich tract of FMDV isolates, showed heterogeneity in several isolates and sequential dominance of viral subpopulations. Sequence analysis of the VP1-coding region (which codes for one of the virus structural proteins) of the parental FMDV RNA and of seven isolates from carrier cattle, revealed rates of fixation of mutations of 0.9×10^{-2} to 7.4×10^{-2} substitutions per nucleotide per year; 59% of the base changes lead to amino acid substitutions, some of which were located in a region involved in neutralization of FMDV. FMDV-neutralizing activity was detected in the oesophageal pharyngeal fluid samples, and antigenic variation amongst viral isolates from carrier cattle was demonstrated with monoclonal antibodies raised against the parental virus. Two isolates from the persistent infection displayed a 10^2 and 10^3 fold decreased neutralization with these monoclonal antibodies. Thus persistent

infection of ruminants with FMDV appears to promote the rapid selection of antigenically variant foot and mouth disease viruses.

These results were supported by a subsequent study performed in cell culture in the presence of antiviral polyclonal sera (Carrillo *et al.*, 1989). Cloned FMDV was serially passaged on secondary monolayers of bovine foetal kidney cells in the presence of subneutralizing antiviral polyclonal sera (APS). After a limited number of passages the virus population displayed increased resistance to neutralization by APS, altered electrophoretic mobility of VP1, a pronounced reduction in plaque size, temperature sensitivity and decreased pathogenicity for mice. Analysis of cloned virus harvested after 23 passages indicated that the temperature-sensitive character was displayed by the small-plaque variants. The evolution towards attenuated viruses with decreased pathogenicity demonstrated that under the appropriate conditions, less virulent virus variants can prevail over those which destroy the host (Steinhauer & Holland, 1987). Persistent infections were readily established with virus variants isolated from passage 23.

In contrast, isolates from clinical cases of SSPE and acute measles virus infections appeared to be virtually identical by classical serology (Stephenson & ter Meulen, 1982). However, sequencing studies revealed a 1% difference in two overlapping cDNA clones of viral RNA extracted from the brain of an SSPE patient, while the sequenced regions of either clone differed from the corresponding region of the reference Edmonston measles virus RNA in 2.8 and 3.7% of nucleotides. These results suggested that a rapid evolution of measles virus RNA occurs during persistence in the brain and, in conjunction with earlier results from oligonucleotide fingerprint analysis, strongly suggested that the viral RNAs in SSPE consist of related, non-identical nucleotide sequences (Stephenson & ter Meulen, 1982; Cattaneo *et al.*, 1986). Variant genomes with different tropisms and altered replication cycles may arise during the persistent infection and in conjunction with the host's immune response may induce SSPE in rare individuals.

Studies on persistent virus infections of tissue culture cells have provided considerable evidence that viral mutants can modulate the lytic potential of normally cytocidal viruses (Friedman & Ramseur, 1979; Younger & Preble, 1980; Wechsler & Meissner, 1982; Mahy, 1985). The mutants most commonly observed in association with persistent infections have temperature-sensitive and/or small-plaque phenotypes. Temperature-sensitive mutants appear to compete sucessfully with, and displace, the wild-type viruses in cultures maintained at relatively non-permissive conditions for these mutants (Preble & Youngner, 1975). In most of the studies with arboviruses in invertebrate cell culture, temperature-sensitive and small-plaque mutants accumulated after the onset of the persistent infection, suggesting they were not involved in the establishment of the persistent state (Newton *et al.*, 1981; Verani *et al.*, 1984). Cloned small-plaque variants of Bunyamwera virus released from persistently infected *Aedes albopictus* cells induce cytopathic effects in BHK cells or persistence in mosquito cells identically to wild-type

virus (Newton *et al.*, 1981). However, studies on reovirus persistence have demonstrated the importance of temperature-sensitive variants, and it has been shown that mutations in specific viral genes are crucial for the establishment and maintenance of persistent infection in mouse L-929 cells (Ahmed & Graham, 1977; Ahmed *et al.*, 1980; Ahmed & Fields, 1981, 1982). A genetic approach was used to determine the mutations which had a crucial role in persistence and it became clear that although attenuated viruses could contain multiple defects, the viral components and genetic changes responsible for initiating or maintaining persistence were highly specific (Ahmed & Fields, 1982; Kauffman *et al.*, 1983).

2.2.2 The role of the host cell in the establishment and maintenance of persistent viral infections

Persistent infections in tissue culture may be maintained either at the level of the individual cell or at the population level. The survival of cells following infection is the key to the former mode of persistence. In such "steady-state" infections, 100% of cells express viral antigen and the infected cultures cannot be cured easily with antiviral serum. For persistent infections maintained at the population level a minority of cells are susceptible to infection at any one time, either because a large proportion of cells in the culture is inherently resistant to the virus or because of the action of soluble inhibitors such as interferon. The spread of virus is limited so that cell death is counter-balanced by the production of new cells by cell division. A low and variable percentage of the cells are antigen-positive and such "carrier cultures" can be cured with antiviral serum (Walker, 1964; 1968; Mahy, 1985).

Igarashi *et al.* (1977) studied an *Aedes albopictus* cell line persistently infected with Sindbis alphavirus. They cloned individual cells from the persistently infected cell culture and found that about 50% of the cells released infectious virus. Recloning of the virus-positive clones yielded both infected and uninfected cultures (62% of the recloned cells released infectious virus). Immunofluorescence studies indicated that 40 to 80% of cells in the persistently infected cultures contained viral antigen, a result largely consistent with the cell cloning experiments. The existence of virus negative clones suggested that these cells had a self-curing mechanism. The argument for a self-curing mechanism was strengthened by the observation that growth of the persistently infected cultures for 24 weeks in the presence of antiviral serum (10% v/v) resulted in cultures remaining consistently virus negative through 16 subsequent serial transfers without antisera. Curing in the presence of antiviral serum suggested that extracellular virus was required to maintain the long-term infection and that the infection could not be maintained indefinitely in the culture by vertical transmission from parent cell to daughter cell.

Riedel and Brown (1977) proposed a hypothetical scheme for the maintenance of the persistent state established by Sindbis virus in *Aedes albopictus* cells, based on a

number of observations. Differences in the process of virus morphogenesis in vertebrate and invertebrate cultures had previously been demonstrated by Gliedman *et al.* (1975). Using electron microscopy these authors observed membranous cytoplasmic vacuoles in Sindbis virus infected *Aedes albopictus* cells, which were not present in uninfected cells, and which contained Sindbis virions. Viral nucleocapsids at various stages of envelopment were observed in the vesicles, suggesting that envelopment takes place in these internal structures. In addition, virus release appeared to take place via fusion of these vesicles with the plasma membrane. Few free nucleocapsids were seen in the cytoplasm of infected mosquito cells and virions were rarely observed budding from the surface of these cells. The confinement of stages of viral replication to discrete areas of invertebrate cells may afford the cell protection from the toxic effects of virus infection, whereas these effects cause death in infected vertebrate cells.

In their work Riedel and Brown (1977) demonstrated that during the initial acute phase of Sindbis virus infection in *Aedes albopictus* cells, 100% of the cells released infectious virus (resulting in relatively high yields of virus) and expressed viral antigens. No cytopathic effects were observed during the acute phase of infection, yet once persistence was established only a small percentage of the cultured cells contained detectable viral antigen as monitored by immunofluorescence, and less than 1% of the cells released infectious virus as measured by an infectious centres assay. Relatively low levels of virus were released from the persistently infected cells. The culture as a whole however, was as resistant to homologous superinfecting virus as cells in the acute phase of infection. Elimination of the extracellular virus, by growing the cells in the presence of Sindbis virus antiserum, resulted in curing of the culture, such that the culture responded to infection as would an uninfected culture. It was clear therefore that the presence of extracellular virus was important to the maintenance of the persistent state.

Combining these observations it appeared that when mosquito cells were infected with Sindbis virus the cells entered a phase of maximum virus synthesis (acute infection). A large number of virus-producing vesicles were generated in the cell cytoplasm, each being, to an extent, isolated biochemically and genetically from other vesicles and from the host cell. This proposed compartmentalisation of infection was supported by observations made during two earlier studies. Renz and Brown (1976) had observed that many of the temperature-sensitive mutants of Sindbis virus which complement each other readily in vertebrate cells failed to do so in cultured mosquito cells, and Igarashi and Stollar (1976) had demonstrated that DI particles of Sindbis virus could neither be propagated, nor exert their inhibitory action, in mosquito cells. These findings were interpreted as reflecting the isolation of the replication of individual infecting virions in the cytoplasm of infected cells, resulting in an inability of normal particles to provide the necessary helper functions for defective virion synthesis in the mosquito cells. The large amounts of virus released during the first 48 hours post infection from Sindbis virus infected mosquito cells suggested that

many of the internal vesicle structures were lost by this time. If the virus induced vesicles were released into the surrounding medium the cells would have had less virus synthesizing capability and contained less viral antigen; the presence of a single vesicle might not have been detectable by immunofluorescence or infectious centres assay but might have been adequate to maintain resistance to superinfection.

The progressive loss of virus-induced vesicles from the infected cells coupled with normal cell division would result in the appearance of uninfected cells in the culture; these cells would be susceptible to reinfection by virions in the medium and the cycle from the acute to the persistent phase of infection would be repeated for these particular cells. From the observations of Riedel & Brown (1977) it appeared that at any given time a very small percentage of the cells in the persistently infected Sindbis virus/*Aedes albopictus* culture were in the acute phase of infection. Using immunofluorescence these authors detected a decrease in the rate of synthesis of virus components in the persistently infected cells, which suggested that the persistently infected cells might possess some mechanism for specifically retarding virus production without interfering with normal cell function. The persistently infected cells did however support the replication of West Nile virus, a non-homologous superinfecting virus, indicating that the observed suppression of virus synthesis was not the result of a classical interferon-like agent (see Section 2.3.2).

Other morphological profiles of alphavirus growth in *Aedes albopictus* cells have been presented. Stollar *et al.* (1979) observed Sindbis virus nucleocapsids in the cytoplasm of infected *Aedes albopictus* cells and saw virions budding at the surface of these cells; suggestive of a mode of replication more akin to that observed in Sindbis virus infected vertebrate cells. Simizu and Maeda (1981) examined the morphogenesis of western equine encephalitis alphavirus in the C6/36 clone of *Aedes albopictus* cells. These authors found cells containing virus inclusions similar to those described by Gliedman *et al.* (1975) in addition to virus budding from the cell surface. The different profiles of virus development observed for alphaviruses in *Aedes albopictus* cells may be due to differences between the subclones of *Aedes albopictus* cells used (see Section 2.3). It is possible that both modes of virus development co-exist in the mosquito. The maturation pathway described by Gliedman *et al.* (1975) may be important in the establishment of inapparent persistent infections, essential for maintenance of the life-long infection of the mosquito, whereas the pathway described by Stollar *et al.* (1979) may be important for the production of large amounts of extracellular virus in tissues such as the salivary gland of the mosquito.

2.2.3 Inherent resistance of cells to virus

Vogt & Dulbecco (1958) studied the properties of a cloned HeLa cell line (S3) which exhibited an increased resistance to poliovirus type 3. Although no evidence was obtained which suggested that this culture could become persistently infected, this study revealed the existence of individual cells within the population which displayed different

degrees of resistance to the infecting virus. Repeated exposure of the standard culture to virus resulted in a cell line with increased resistance to poliovirus infection. The "resistant" culture contained a higher proportion of cells displaying resistance to poliovirus than did the original culture and was proposed to arise by the selection of pre-existing "resistant" variants in the S3 population. More recently, Kaplan *et al.* (1989) isolated and characterised HeLa cell lines blocked at different stages in the poliovirus life-cycle. They detected lower levels of expression of the poliovirus receptor on HeLa cell clones resistant to poliovirus infection.

Takemoto & Habel (1959) established a carrier-culture of HeLa cells infected with Coxsackie A9 virus which was passaged for many cell generations with consistent production of virus. Clonal analysis of the persistently infected culture revealed that only a small proportion of the cells were infected. Prolonged cultivation of the carrier-culture selected for cells with increased resistance to Coxsackie A9 virus and also generated virus with altered antigenic composition.

Mizzen *et al.* (1983) proposed that persistence of mouse hepatitis virus (coronavirus) in mouse LM cells was maintained by subsets of host cells which could resist infection and withstand cytopathic effects, i.e. cell fusion. Persistence was proposed to be a feature of the entire culture, not of individual cells, dependent upon a dynamic balance between infected and uninfected cells.

Lymphotrophic minute virus of mice (a parvovirus) establishes a persistent infection in mouse L cells which is believed to be maintained by co-evolution of cells and virus in the carrier culture (see Section 2.2.4). Spontaneous curing of the culture occurs after 20 to 30 passages (Ron *et al.*, 1984; Ron & Tal, 1985). Ron and Tal (1986) demonstrated that curing occurred via the selection of virus-resistant cells in the culture. Resistance to virus replication was shown to be due to an intracellular block, and the data presented suggested that the replication of minute virus of mice required host-coded helper functions for at least two stages of its replication cycle. These authors hypothesized that during the persistent infection, either the cell-coded helper functions were modified or their expression was reduced.

2.2.4 Co-evolution of cells and virus

Ahmed and coworkers (1981) performed a detailed study of reovirus persistence in mouse L cells. Their persistently infected culture was established by infecting a monolayer of mouse L cells with a serially passaged stock of reovirus tsC(447) (Ahmed & Graham, 1977). Cell clones free of virus (LR cells) were obtained by growing the persistently infected L cells in the presence of anti-reovirus serum for 30 days and isolating cell clones from the cured culture. Infection of the LR cells with reovirus type-3, or with reovirus isolated from the carrier culture, readily gave rise to a persistent infection. In contrast, infection of L cells with reovirus type-3 is known to result in inhibition of host

cell macromolecular synthesis and complete cell destruction (Zweerink & Joklik, 1970). Infection of L cells with reovirus isolated from persistently infected cultures also resulted in a lytic infection with no surviving cells. Ahmed *et al.* (1981) argued that the mutant cells evolved during the course of persistence, rather than having been selected from a sub-population of cells, on the basis that such a sub-population of cells should always survive and become persistently infected.

The LR cells were partially resistant to infection with reovirus type-3 resulting in a low level persistent infection with inefficient virus replication. The replication of reovirus type-3 in the LR cells appeared to be restricted at an early step such as absorption, penetration or uncoating. Most of the LR cells were morphologically distinct from the parental L cells, generally appearing larger and less fibroblast-like. Preliminary observations suggested that LR cells had an altered cytoskeleton and contained a large number of lysosome-like structures in the cytoplasm. The LR cells were also resistant to vesicular stomatitis virus which is extremely cytopathic for L cells. The evolution of virus was evident in so far as virus released from later passages of the persistently infected cells grew better in LR cells than reovirus type-1 and type-2 and displayed complete dominance in co-infection experiments. By infecting mutant cells with mutant virus it was possible to construct the original carrier culture. Carrier cultures established with LR cells and wild-type reovirus produced less virus and contained fewer infected cells than the cultures created with mutant cells and mutant virus.

Co-evolution of cells and virus has also been proposed as the mechanism whereby lymphotrophic minute virus of mice (MVM(i); parvovirus) persists in L cells (Ron & Tal, 1985) and FMDV persists in BHK-21 cells (de la Torre *et al.*, 1988). In the former study, infection of L cells with MVM(i) resulted in the emergence of host-range mutant viruses. Parental MVM(i) grew well in T-lymphocyte derived cell lines but its replication in L cells was restricted. Mutant viruses which arose after infection of L cells were capable of a lytic infection which destroyed most of the initially restrictive parental L cells. However the culture was not completely lysed and a persistent infection was established. Throughout the persistent infection extensive changes occurred in both the tissue tropism of the progeny virus and in the phenotypic properties of the cells. Mutant cells were selected which were increasingly restrictive to the replication of the resident virus, but concomitant changes in the virus enabled it to replicate in a subpopulation of these cells. Infection of mutant cells with mutant virus resulted in a persistent infection, whereas neither infection of parental cells with mutant virus nor infection of mutant cells with parental virus led to persistence.

De la Torre *et al.* (1988) studied the evolution of cells and virus during serial passage of a cloned BHK-21 cell line persistently infected with FMDV C-S8c1. The persistently infected cells termed C1-BHK-Rc1 (de la Torre *et al.*, 1985) became constitutively resistant to the parental FMDV C-S8c1. Treatment of persistently infected

cells from late passages of the carrier culture with ribavirin cured the cells of virus (de la Torre *et al.*, 1987) but did not restore sensitivity to FMDV C-S8c1. The resistance of C1 BHK-Rc1 cells to FMDV C-S8c1 was due to an intracellular block which resulted in a 100-fold decrease in the amount of FMDV RNA in the infected cells. Virus isolated from late-passage carrier cells (FMDV R59) partly overcame this cellular block and was also more cytolytic than the parental FMDV for BHK-21 cells. Sequencing of the VP1 gene from nine viral clones arising from C1-BHK-21 cells revealed genetic heterogeneity between these isolates.

C1-BHK-Rc1 cells had a characteristic round cell morphology, and grew faster than BHK-21 cells in liquid culture. They were less subject to contact inhibition of growth, and had an increased ability to form colonies in semi-solid agar. The persistent state was readily reconstituted with late-passage C1-BHK-Rc1 cells and FMDV C-S8c1 or FMDV R59. Clonal analysis of the cells in the C1-BHK-Rc1 carrier culture revealed a range of cell phenotypes coexisting in the cell population (de la Torre *et al.*, 1989b). A total of 248 stable cloned cell lines were derived from 294 individual cells isolated from the original carrier culture. None of the stable cloned cell lines tested released infectious FMDV, or contained levels of viral RNA detectable by dot blot hybridization using cDNA probes. FMDV was present in the culture medium of 8 of 177 single cells isolated from pass 19 of the carrier culture, none of which gave rise to stable cell clones, suggesting that a small proportion of cells in the carrier culture (approximately 4%) were lytically infected. In fact the proportion of lytically infected cells in the persistently infected culture probably fluctuated between 4%-20% based on the above observation and the fact that 80% of cells isolated from the carrier culture gave rise to stable cell clones. At least six distinct cell phenotypes with regard to cell morphology, resistance to FMDV strain C-S8c1 and cell growth characteristics, were present in the cloned cell cultures. The increased resistance to FMDV C-S8c1 and enhanced transformation phenotype exhibited by late passages of C1-BHK-Rc1 cells (de la Torre *et al.*, 1988) would appear therefore to be the additive effect of a range of cell responses within the population. A number of cell clones were demonstrated to retain their distinct phenotype after 20 serial passages, subcloning and further propagation. Overall the results suggested that the altered phenotypes of individual cell clones were the result of inheritable cell modifications. De la Torre *et al.* (1989b) suggested that the origin of this cell diversity may lie in the selection of increasingly transformed cells in the culture or may be due to DNA hypermutability which has been described in association with other systems: for example shuttle plasmid vectors during their replication in mammalian cells (Razzaque *et al.*, 1983) and immunoglobulin gene segments (O'Brien *et al.*, 1987). The FMDV carrier culture (C1-BHK-Rc1) appears to exist as a dynamic interaction between an evolving heterogeneous population of FMDV (Steinhauer & Holland, 1987; de la Torre *et al.*,1988; Domingo & Holland,1988) and multiple cell variants. It is likely that cell heterogeneity confers a selective advantage for long-term virus

and cell survival by providing the cell population with a range of responses towards FMDV.

In later experiments de la Torre and co-workers (1989a) transformed two cell clones from pass 74 of the C1-BHK-Rc1 culture, which were highly resistant to FMDV C-S8c1, to puromycin resistance (Pur^r) and fused these cells to BHK-21 cells transformed to neomycin resistance (Neo^r). The hybrid Neo^r Pur^r cells displayed the specific resistance to FMDV C-S8c1 characteristic of the original clones. The results therefore suggested that resistance to FMDV C-S8c1 was mediated by dominant *trans*-acting cellular products.

2.2.5 Interferon

Interferon may be involved in the maintenance of a number of persistent infections of vertebrate cell lines. For example, interferon has been detected in cultures of mouse L cells persistently infected with Semliki Forest virus (Meinkoth & Kennedy, 1980); VSV (Ramseur & Friedman, 1977; Nishiyama *et al.*, 1978) and Sindbis virus (Inglot *et al.*, 1973). However, it is likely that interferon plays a subsiduary rather than a principal role in these persistent infections (Friedman & Ramseur, 1979). Interferon has never been detected in persistently infected invertebrate cell cultures, although antiviral activity, distinct from interferon in the specificity of its action, has been detected in mosquito cell cultures persistently infected with Sindbis and Semliki Forest alphaviruses (see Section 2.3.2).

2.2.6 Defective interfering particles

Defective interfering (DI) particles were first observed by von Magnus in 1947 as "incomplete" virus particles generated on passaging influenza virus serially undiluted in embryonated chicken eggs. They have subsequently been observed in association with most well studied viruses. The best characterised DI particles are those of the negative-strand RNA viruses vesicular stomatitis virus (VSV; rhabdovirus), influenza virus (orthomyxovirus) and Sendai virus (paramyxovirus). DI particles of positive-strand RNA viruses such as poliovirus and FMDV (Picornaviridae) and of reoviruses, with their double-stranded RNA genome, have also been well studied. In the case of icosahedral viruses such as the Picornaviridae, the structural similarity between standard virus and DI particles extends to overall shape and size; in most cases the defective viruses contain less total RNA than the parent virus and can be separated by density gradient centrifugation. Defective forms of rhabdoviruses such as VSV can be separated from standard virus relatively easily on the basis of size difference since the length of the viral RNA governs the overall size of these bullet-shaped viruses and defective viruses contain shorter RNAs. In more pleomorphic viruses, such as influenza and measles viruses, DI virus particles tend to be smaller than the standard particle, but the variability in size of the virion population as a whole makes it difficult to achieve clean separation (Huang & Baltimore, 1977). Because

of the relative ease of isolation of VSV DI particles most of the early work on the general properties of DI particles was carried out with this virus.

Huang and Baltimore (1970) coined the term "DI particle" having recognised the ubiquitous nature of DI viruses. They defined these entities as defective viruses which lacked an essential portion of the viral genome, required homologous parental virus as helper for replication, contained virus structural proteins and antigens; and exhibited the capacity to interfere specifically with homologous helper virus, by replicating at its expense in doubly infected cells. In the case of non-segmented viruses such as VSV defective RNAs are the basis of defective interfering virus particles. The DI particles of segmented RNA viruses such as influenza virus generally contain an increased proportion of lower molecular weight RNAs and fewer high molecular weight RNA species. In addition to varying amounts of the 8 standard RNA segments, novel RNAs (DI RNAs) have been observed in influenza virus DI preparations (Nayak *et al.*, 1989).

The majority of DI RNAs originate from the polymerase genes of the parental virus. For instance, 88% of influenza virus DI RNAs originate from the three polmerase genes (Nayak *et al.*, 1989). The same progenitor gene can give rise to multiple DI RNAs of varying lengths, and the smaller DI RNAs are not always a subset of the larger DI RNAs. It is worth noting that defective RNAs which are not interfering, but which are subgenomic RNAs arising from polymerase errors, have also been recognised. Comparative sequence analysis of DI RNAs and subgenomic RNAs has not revealed any distinguishing features between the two species (Nayak *et al.*, 1989).

2.2.6.1 Generation of DI RNAs

Sequencing studies have revealed four major types of DI RNAs of negative-strand RNA viruses: internally deleted RNAs, which retain their parental 3'and 5' ends but display one or more internal deletions; stem or panhandle RNAs, where the parental 5' terminus is retained but the 3' terminus is complementary to the 5' terminus; snapback or hairpin RNAs, where the 3' half of the RNA is complementary to the 5' half; and mosaic RNAs, where the defective RNAs can possess either of the terminal arrangements detailed above, but due to numerous intermolecular or intramolecular recombinations have relatively complex sequence alterations. DI RNAs are believed to arise as a consequence of polymerase errors, as originally suggested by Huang (1977) and Leppert *et al.* (1977). These authors suggested that DI genomes are created by genome rearrangements or recombinations resulting from "jumping" of the viral replicase from one viral RNA template to another, or from one segment of a template to another. The RNA replicase would carry the incomplete nascent strand to the new template and then use this RNA as a primer for resumption of chain elongation. This "polymerase copy-choice" mechanism for DI RNA generation was generalised by Lazzarini *et al.* (1981) and Perrault (1981) to explain the generation of all known forms of DI particles, and was subsequently verified

experimentally for polioviruses by Kirkegaard and Baltimore (1986) and for coronaviruses by Lai (1990). The incorporation of cellular transfer RNA into the 5' termini of a class of Sindbis virus DI particles has been observed (Schlesinger, 1988). The ease with which DI RNAs are generated via the polymerase copy-choice mechanism is dependent upon the ability of the replicase, carrying the nascent RNA strand, to jump between templates or between different sites on the same template. The efficiency of DI generation must therefore be inversely proportional to the processivity of the polymerase, that is its tendency to continue transciption on its original template (Roux *et al.*, 1991). Variant viruses which generate VSV DI RNAs with increased efficiency have been reported (Depolo & Holland, 1986). These variants might be expected to possess a decreased processivity and/or an increased ability to bind to a new template.

The Wa-182 strain of influenza virus, a single-gene reassortant containing the NS gene from the A/Aichi/2/68 (H3N2) wild type (wt) strain of influenza virus against an A/WSN/33 (H1N1) wt background, and which harbours three point mutations in its NS2 gene compared to the wild-type A/WSN/33 (H1N1), is readily converted to a DI form by a single high-multiplicity infection (Odagiri & Tobita, 1990a). In contrast, wild-type A/WSN/33 (H1N1) virus requires at least three consecutive high-multiplicity passages to generate a comparable amount of DI particles (Janda *et al.*, 1979). This reassortant was generated by Odagiri and Tobita (1990a) who demonstrated that the three point mutations in the NS2 gene of Wa-182 were responsible for it's enhanced efficiency of DI generation. The DI RNAs of Wa-182 were formed by deletion of the PA gene of the virus. Sequence analysis revealed no mutations in the PA gene of Wa-182 when compared to the progenitor gene of A/WSN/33 (H1N1) wt (Odagiri & Tobita, 1990b), suggesting that a mutation or unique region of the nucleotide sequence, such as a uracil-rich region on the polmerase genes (Fields & Winter, 1982), is not a prerequisite for DI RNA generation. This is consistent with the study carried out by Jennings *et al.* (1983) in which no consensus sequences were found which might favor the generation of DI RNAs. In addition, determination of the sequences flanking the deleted regions of the progenitor genes failed to reveal significant homology with consensus cellular RNA splicing sequences (Fields & Winter, 1982; Nayak *et al.*, 1982; Sivasubramanian & Nayak, 1983). Odagiri and Tobita (1990a) postulated that the non-structural influenza virus protein NS2 plays an important role in the synthesis of intact viral genomic RNAs. It may be a component of the replicase, determining the balance between the processivity of the polymerase and its ability to continue chain elongation on a new template.

2.2.6.2 Interference

DI particles amplify their genomes at the expense of homologous helper virus, which encodes the replication and encapsidation proteins essential for the replication of both. The interfering effects of DI particles can reduce the yield of virus to zero in doubly

infected cells (Sekellick & Marcus, 1980). The helper virus must compete against the DI particles for its own gene products and consequently is hampered in its replication. Often the genomes of DI particles are rearranged in a manner which enhances their ability to replicate and to compete for replication and encapsidation proteins. This results in dominant replication of the DI particle and concomitant interference with the replication of the infectious helper virus. For example, VSV DI particles usually contain rearranged termini which favour replication and preclude transcription. Therefore, while helper virus genomes are engaged mainly in transcription DI genomes, which are devoted solely to replication, become dominant in the viral RNA population. Re and Kingsbury (1986, 1988) investigated the factors influencing the replication "fitness" of Sendai virus DI RNA species. They determined that there was a minimum size of about 450 bases for a defective RNA to be viable. However, any DI RNAs smaller than 1600 nucleotides (10% of the genome size) were enveloped with decreasing efficiency as their size decreased. This was thought to be due to limited membrane flexibility: as the limit of curvature is approached the efficiency of particle budding would be expected to decrease and smaller nucleocapsids would be discriminated against. For RNAs larger than 1600 nucleotides, the data were consistent with smaller RNAs being replicated more quickly. Additionally, superimposed on the size constraints, were the findings that copyback 3' termini were more competitive for replication than genomic 3' termini and non-transcribing RNAs were replicated more efficiently than transcribing RNAs.

DI genomes which are transcriptionally and/or translationally active can interfere at a level other than replication. For instance, relatively stable defective polypeptides of PB2 gene origin have been detected in influenza virus infected cells (Akkina *et al.*, 1984a, 1984b; Chambers *et al.*, 1984; Penn & Mahy, 1985; Akkina & Nayak, 1987). These polypeptides can bind to RNP complexes and by competing with functionally active PB2 render the complexes transcriptionally or replicationally defective. Despite being made in large amounts these polypeptides are not assembled into mature virions, and neither are the RNP complexes bound to defective PB2. Interference may therefore occur at the level of assembly leading to decreased virus production (Akkina & Nayak, 1987). DI particles are also capable of interfering indirectly with virus, for example by interferon induction (Johnson, 1981), modulation of immune responses (Dimmock *et al.*, 1986), or restriction of cell surface expression of virus proteins. In addition, the presence or absence of encapsidation or packaging signals in the DI RNAs determines their relative efficiency of maturation.

2.2.6.3 Influence of host cell type

The variability of DI particle effects within different cell types has been reported. DI particles which interfere strongly in one cell type may interfere only weakly, or not at all, in another. In some cases this is due to poor replication and amplification of the DI

particles in particular cell types, but in some cell types the DI particles may replicate efficiently, but exert weak interference with helper virus replication (Choppin, 1969; Huang, 1988; Brinton *et al.*, 1984; Cave *et al.*, 1985; Kang *et al.*, 1981; Holland, 1987; Gillies & Stollar, 1980; Barrett & Dimmock, 1986).

2.2.6.4 Cell protection, disease attenuation and persistence

In addition to indirect cell-sparing effects such as interferon induction, alteration of immune responses and reduction of early virus yields, it has been proposed that DI particles exhert a direct cell-sparing effect (Huang & Baltimore, 1970). To date, numerous DI particles have been demonstrated to facilitate the establishment and maintenance of persistent infections by a wide variety of animal viruses in tissue culture (Huang & Baltimore, 1977; Holland *et al.*, 1980; Barrett & Dimmock, 1986; Holland, 1987; Schlessinger, 1988). In a number of systems an initial high multiplicity infection or undiluted passage of a normally cytolytic virus will result in a persistent infection. High multiplicity infection or undiluted passage of virus increases the probability of introducing DI viruses into the host cell or animal, as it is very difficult to prepare virus totally free of DI particles (Holland, 1987). Also, co-infection of DIs and a homologous cytolytic virus can convert a normally lytic infection to a persistent one, as reported for Sendai virus in BHK cells (Roux & Holland, 1979).

In vivo studies with influenza virus have demonstrated that influenza A/chicken/Pennsylvania/1/83 (H5N2), a natural isolate consisting primarily of DI particles (Chambers & Webster, 1987), protects chickens from the virulent strain of the virus (influenza A/chicken/Pennsylvania/1370/83) upon co-inoculation. Chambers and Webster (1991) investigated the biological basis of this effect and found that protection correlated with a complete inhibition of virulent virus spread to the brains of infected chickens. It appeared that the protective effect was afforded by DI particle-mediated interference with virulent virus multiplication within the respiratory tract of infected chickens. The defective viral isolate had to be administered within 20 hours of the virulent inoculum for protection to be achieved.

2.3 Persistent infections established by arboviruses

Arboviruses characteristically replicate in both vertebrate and invertebrate hosts, displaying very different outcomes of infection. Usually an inapparent persistent infection is established upon infection of an invertebrate vector, but on transmission to the vertebrate host an acute cytocidal infection ensues. These disparate outcomes of infection can be reproduced in tissue culture. In particular the replication of members of the Bunyaviridae and Alphaviridae in mosquito cell cultures has been extensively studied.

Persistent infections have been established in mosquito cell cultures with Marituba, Germiston, La Crosse and Bunyamwera bunyaviruses (Carvalho *et al.*, 1986;

Delord *et al.*, 1989; Rossier *et al.*, 1988; Newton *et al.*, 1981; Elliott & Wilkie, 1986) and with Toscana phlebovirus (Nicoletti & Verani, 1985). In addition, mosquito cell cultures persistently infected with Sindbis and Semliki forest alphaviruses have been well studied (Davey & Dalgarno, 1974; Shenk *et al.*, 1974; Gliedman *et al.*, 1975; Igarashi *et al.*, 1977; Riedel & Brown, 1977; Eaton, 1981; Stalder *et al.*, 1983; Brown & Condreay, 1986). In each case, an initial acute phase of infection, on average of about 3-4 days duration, was followed by a long-term persistent infection.

Invertebrate cell cultures first became available in 1967 when Singh derived *Aedes albopictus* cells from the macerated tissues of whole mosquito larvae (Singh, 1967). While infection of cultured *Aedes albopictus* cells with arboviruses has consistently been reported to lead to the establishment of persistent infections, different laboratories have reported varying degrees of cytopathic effect during the acute phase of infection. Tooker and Kennedy (1981) cloned subpopulations of cells from an *Aedes albopictus* cell line. Of 115 clones, they found that 70% produced poor yields of Semliki Forest virus and exhibited no cpe while 30% produced high virus yields and exhibited extensive cpe. In contrast, Brown and coworkers (Gliedman *et al.*, 1975; Riedel & Brown, 1977,1979) did not observe cpe at any time after infection of their *Aedes albopictus* cells with Sindbis virus; repeated attempts to subclone cells which demonstrated cpe, from this particular cell line, were unsuccessful (Brown & Condreay, 1986).

All *Aedes albopictus* cell lines currently in use originated from the parental Singh line (Singh, 1967). This original culture probably contained a diverse population of partially differentiated cells destined for development into particular tissues in the mature mosquito. Variation between *Aedes albopictus* cell lines in different laboratories may have arisen as a consequence of subtle differences in tissue culture materials and cell-handling techniques, imposing slightly different selection pressures on the cell lines. The individual tissues of the mature mosquito are known to be differentially susceptible to arbovirus infection. While the mosquito as a whole shows no deleterious effects of virus infection and transovarial transmission to offspring has been reported (for both bunyaviruses and alphaviruses) without any indication of virus in the abdomen or thorax (Leake, 1984), the salivary glands produce large amounts of mature virus throughout the life time of the mosquito.

Studies with cell lines subcloned from Singh's original *Aedes albopictus* cultures (u4.4 cells, C6/36 cells and C7/10 cells) have demonstrated heterogeneity in the response of individual cell clones to arbovirus infection. Each of these cell lines produce different amounts of virus and display correspondingly different degrees of cytopathology following infection with Sindbis virus (Miller & Brown, 1992). In addition, these cell lines respond differently to homologous interference (Condreay & Brown, 1986) and actinomycin D treatment (Condreay *et al.*, 1988; see Section 2.3.2), and vary in their ability to produce and respond to an antiviral factor (Condreay & Brown, 1988; see Section 2.3.2).

There is evidence that host cell functions participate in the replication of Sindbis virus in cultured invertebrate cells. Studies with cloned *Aedes albopictus* cell lines, including treatment with actinomycin D (u.4.4 and C6/36 cells), and enucleation (u.4.4 cells) have been shown to interfere with Sindbis virus production (Scheefers-Borchel *et al.*, 1981; Erwin & Brown, 1983). Differences in the participation of the host cell in virus replication in each of these cell types could be responsible for the variability in virus production exhibited by these cells as well as the differences seen in the establishment of homologous interference and superinfection exclusion. By studying the effects of actinomycin D and cycloheximide on the synthesis of viral RNA and the production of mature virus in u.4.4, C6/36 and C7/10 cells, Condreay *et al.* (1988) demonstrated that host components were involved in at least two distinct steps in the production of Sindbis virus by mosquito cells: in the production of viral RNA and in the synthesis of viral structural polypeptides.

Vertebrate cell cultures which are permissive for bunyavirus replication usually perish within 72 hours of infection (Bishop & Shope, 1979). Uukuniemi virus (Pettersson, 1974) and Dugbe virus (David-West & Porterfield, 1974) have been shown to be exceptions. Uukuniemi virus did not affect host macromolecular synthesis in either resting BHK-21 or chick embryo cells, while Dugbe virus produced only very slight cpe in three continuous vertebrate cell lines (a pig kidney cell line, African green monkey cells and Rhesus monkey kidney cells). Viruses which are normally cytocidal for a particular cell type can be made to establish persistent infections under certain conditions. An example from the Bunyaviridae is the persistent infection established by Toscana phlebovirus upon undiluted passage in Vero cells (Verani *et al.*, 1984).

Studies on arbovirus persistence in invertebrate cell cultures have suggested that DI particles and antiviral proteins produced by the host cell may promote the persistent state. Evidence for the involvement of these factors in arbovirus persistence will be discussed in the following sections.

2.3.1 Defective interfering particles associated with arboviruses

2.3.1.1 The Bunyaviridae

Kascsak and Lyons (1978) demonstrated the generation of DI virus particles upon high multiplicity passage of Bunyamwera virus in BHK cells. Two major classes of virus particle were detected: standard virus which banded during isopycnic centrifugation at a buoyant density of 1.19 g/cm^3 and non infectious particles with a buoyant density of 1.17 g/cm^3. The latter particles contained only S segment sized RNA and interfered with the replication of standard Bunyamwera virus. Infection of BHK cells with a mixture of standard and defective Bunyamwera virus resulted in an alteration in the intracellular profile of viral RNA. Incorporation of radiolabelled precursors into the L and M RNAs was greatly reduced whereas labelling of the S RNA species was unaffected. The interference

observed in this virus-cell system appeared therefore to stem from an inhibitory effect exerted at the level of viral genome replication. Defective viral stocks only inhibited the replication of homologous standard virus and this interference was host cell dependent. Studies on autointerference (defined as the inhibition of infectious virus yield at a high moi) in MDBK, *Aedes albopictus,* BHK and Vero cells, identified BHK and Vero cells as "high interference" cell lines, while MDBK and *Aedes albopictus* cells were designated "low interference" cell lines. The defective virus used in these studies was grown in BHK cells from virus which had been plaque-purified on Vero cell monolayers.

There are two reports to date of studies on the establishment and maintenance of persistent infection by Bunyamwera virus in *Aedes albopictus* mosquito cell lines. In the earlier paper Newton *et al.* (1981) used an uncloned *Aedes albopictus* cell line and no evidence was presented for the presence of defective virus. However, in a more recent study using the cloned mosquito cell line *Aedes albopictus* C6/36, Elliott and Wilkie (1986) described interfering particles which were released from the persistently infected cells and which contained full-length S RNA segments but no L or M segment RNA. In addition, viral S RNA was the major viral RNA species detected in the persistently infected cells. Some subgenomic RNAs were observed which originated from the L RNA segment.

More recently in a study carried out by Cunningham and Szilagyi (1987) on the replication of Germiston bunyavirus in BHK cells, discrete truncated RNAs (both genomic and antigenomic and of the same mobility on agarose-urea gels) were detected and shown to be derived from the L RNA. The genomic form was packaged into virions and released from infected cells. Defective virus particles were therefore expected to be present but interfering activity was not tested for. These RNAs represented 20% of the large genomic RNA and first became detectable in infected cells four hours after infection with 50 pfu/cell of Germiston virus. When two strains of Germiston virus were compared each gave rise to a unique size of subgenomic L RNA, suggesting that the defective replication event giving rise to these RNAs was a non-specific event. The positive-sense subgenomic L RNAs (which were not packaged into virions) were most likely to be replicative intermediates for replication of the defective L RNAs.

Recently, de Oliveira Resende *et al.* (1991) investigated the effect of repeated mechanical transfer of TSWV between plants on the genome constitution of the virus. Hybridization with an L RNA specific probe revealed subgenomic L RNA species after 20 or so mechanical passages. By 6 days post infection with the defective isolates, more defective L RNAs than full-length L RNAs were detected in the plants, suggesting that replication of the defective forms interfered with standard genome replication. While both negative- and positive-sense full-length and truncated L RNAs were detected in intracellular nucleocapsids, only negative-sense RNA species, both full-length and truncated, were detected in virus particles. It appeared therefore that negative-sense full-length and truncated L RNAs were exclusively packaged into envelopes. Interestingly, the generation

of defective L RNAs was associated consistently with symptom attenuation. Differential hybridization suggested that deletions may have occured in different regions of the L RNA as has already been described for other viruses (Holland, 1986).

In addition to the defective L RNA containing isolates, another distinct class of mutants was generated which were morphologically deficient in that they were incapable of producing enveloped particles and lacked the ability to synthesise the membrane glycoproteins. RNA analysis failed to reveal any large deletions in the three genomic RNA species of this isolate. Indeed the M RNA segment appeared to be full-length. The morphological defect may therefore be due to point mutations or very small deletions in the M RNA which do not affect the size of this molecule. Truncated L RNAs were not detected in the morphologically defective isolate.

Biological evidence has been presented for the involvement of DI particles in persistent infections established by Dugbe nairovirus in pig kidney cells (David-West & Porterfield, 1974) and Toscana phlebovirus in Vero cells (Verani *et al.*,1984), but no direct biochemical proof was obtained. Carvalho *et al.* (1986) examined Marituba bunyavirus replication in cultured *Aedes albopictus* cells and in a mouse fibroblast cell line (L-A9) but found no evidence for autointerference during primary infection of the invertebrate cells. However, studies carried out by Volkmer *et al.* (1983) revealed that Marituba virus replication in L-A9 cells generated DI particles. Marituba virus DI particles interfered with the replication of homologous but not heterologous virus.

2.3.1.2 The Alphaviridae

DI particles of alphaviruses have been generated in both vertebrate (Johnson *et al.*, 1975; Guild & Stollar, 1977; Stark & Kennedy, 1978; Barrett *et al.*,1984) and invertebrate cell lines (Brown & Gliedman, 1973; Logan, 1979; King *et al.*, 1979; Tooker & Kennedy, 1981; Stalder *et al.*, 1983). Serial undiluted passage of alphaviruses in vertebrate cells has been demonstrated to generate DI particles with a host cell-restricted ability to interfere with standard virus. While these DI particles interfere with standard virus replication in vertebrate cells, they do not interfere with the replication of standard virus in invertebrate cell lines (Eaton, 1975; Igarashi & Stollar, 1976; King *et al.*, 1979). Co-inoculation of standard and defective alphaviruses into vertebrate cells has been demonstrated to result in a persistent infection (Stollar, 1980).

Igarashi *et al.* (1977) and Eaton (1981) studied *Aedes albopictus* cell lines persistently infected with Sindbis virus. These authors reported the generation of Sindbis DI particles 7 to 10 weeks after the initiation of the persistent infection. The relatively late appearance of these DI particles suggested that they did not have an essential role to play in the establishment of the persistent infection. Subsequently, Tooker and Kennedy (1981) and Stalder *et al.* (1983) detected DI RNAs in cloned *Aedes albopictus* cell lines persistently infected with Semliki Forest virus. In both studies DI RNAs were observed within 24 h of

the initial infection. The cell lines underwent cytopathic crises during the acute phase of infection and 10% of the cells survived to establish the persistently infected cultures. It is possible that the rapid generation of DI RNAs prevented cell death in a subpopulation of the cells and so enabled transition to the persistent infection. Stalder *et al.* (1983) reported that the DI RNAs generated in their *Aedes albopictus* C6/36 cell line were not efficiently packaged into virions.

2.3.2 Antiviral activity associated with persistent arbovirus infections of invertebrate cell lines

Newton *et al.* (1981) could not detect any interferon-like activity in their persistently infected *Aedes albopictus* cell line. However an antiviral activity, distinct from interferon in the specificity of its action, has been shown to be produced by *Aedes albopictus* mosquito cells infected with Sindbis and Semliki Forest alphaviruses and also Banzi flavivirus (Riedel & Brown, 1979; Newton & Dalgarno, 1983; Condreay & Brown, 1986; 1988; Lee & Schloemer 1981a,b). Riedel and Brown (1979) described a low molecular weight antiviral protein in the culture medium of *Aedes albopictus* cells infected with Sindbis virus. Treatment of mosquito cell cultures with culture fluid harvested from Sindbis virus infected *Aedes albopictus* cells, prior to infection with Sindbis virus, reduced virus yields and gave rise to levels of virus production and percentages of infectious centres characteristic of persistently infected cell populations. The maximal effect was achieved by incubating the cells in medium containing the antiviral activity 48 hours prior to infection. The antiviral activity was insensitive to antisera against BHK cell derived Sindbis virus and was rapidly inactivated at 56°C. The factor was sensitive to proteinase K but insensitive to α- and β-galactosidases, phospholipase C and a range of DNases and RNases.

The most striking observation was the specificity of action of this antiviral protein. The antiviral activity produced by Sindbis virus-infected mosquito cells did not interfere with Sindbis virus production in BHK-21 cells, nor did it interfere with the replication of Semliki Forest virus or West Nile virus in mosquito cells. Newton and Dalgarno (1983) identified an antiviral activity produced by Semliki Forest virus infected mosquito cells. This antiviral activity was also cell and virus specific, sensitive to proteinase K and heat sensitive. They demonstrated that no analogous antiviral activity was produced by *Aedes albopictus* cells persistently infected with Kunjin flavivirus or Bunyamwera bunyavirus, which suggested that the activity might be specific to alphaviruses with little significance in relation to infections of arthropod cells by other arboviruses. However, another group described an antiviral activity specific for Banzi flavivirus produced by infected mosquito cells (Lee & Schloemer 1981 a,b).

Based on experiments where RNA synthesis was measured as incorporation of [^{3}H]-uridine into cells treated with actinomycin D, Newton and Dalgarno (1983) concluded that the antiviral activity released from Semliki Forest virus infected *Aedes albopictus* cells

blocked viral RNA synthesis, and that host transcription was required to establish the antiviral state. The validity of these results was brought into question when it was realised that actinomycin D itself efficiently arrests virus protein and RNA synthesis in cultured insect cells (Scheefers-Borchel, 1981; Condreay & Brown, 1988).

Since the observed repression of [^{3}H]-uridine incorporation into RNA could have resulted from the presence of actinomycin D, Condreay and Brown (1988) studied the effect of the antiviral protein produced in Sindbis virus infected mosquito cells on virus specific RNA synthesis using a procedure not requiring actinomycin D. They studied the sensitivity to and the production of the antiviral protein in three independently subcloned *Aedes albopictus* cell lines, u.4.4, C6/36 and C7/10. The activity produced by infected u.4.4 cells was demonstrated to inhibit total viral RNA synthesis in these cells and in C6/36 cells, in a time dependent manner. Its effect was maximally realised when the cells were treated with the activity 48 hours before infection. Interestingly, Sindbis virus-infected C7/10 cells did not produce an antiviral protein and were not sensitive to the antiviral factor produced by Sindbis virus-infected u.4.4 cells. The data were consistent with an antiviral state induced by the antiviral protein which prevented the formation or efficient functioning of viral RNA-synthesising complexes in treated cells. Northern blot analysis showed that both 49S and 26S RNA species were reduced in Sindbis virus infected u.4.4 cells treated with the antiviral activity. It was not determined whether the activity affected the stability or the synthesis of the viral RNA. Experiments with a mutant of Sindbis virus which was known to be able to overcome an actinomycin D induced block in RNA synthesis in vertebrate cells (Baric *et al.*, 1983), demonstrated that the block to viral replication induced by the antiviral factor was distinct from that induced by actinomycin D. This mutant was shown to bypass the actinomycin D induced block of viral RNA synthesis in invertebrate cells (Condreay *et al.*, 1988) but did not overcome the block induced by the antiviral agent (Condreay & Brown, 1988).

2.3.3 The value of studying arbovirus persistence

A multitute of studies on a variety of virus-cell (or indeed virus-host) systems have created a wealth of published observations on persistent viral infections, but no real understanding of the mechanisms underlying the establishment and maintenance of the persistent state has been achieved. Persistent viral infections are difficult to study particularly *in vivo*, where the role of the virus, the infected tissue/cell and the host's immune response must be evaluated. *In vitro* models are therefore important tools with which to unravel the complexities of viral persistence. A number of tissue culture systems have been developed in which persistence is established by introducing interference at an early stage in infection. This can be achieved by using a high moi or serially passaged virus preparations (both of which contain a mixture of standard and defective virus), or by co-inoculation of a standard virus preparation and homologous defective interfering

particles. Viral variants generated during persistent infections established by such manipulations are then often capable of establishing persistence by themselves. In arbovirus-invertebrate cell systems persistence is the natural course of infection. Since no manipulation of the system is required to establish persistence this is an ideal system with which to investigate the molecular mechanisms fundamental to the establishment of persistence.

2.4 Aims of this study

A detailed study of Bunyamwera virus persistence in C6/36 cells was carried out to gain some insight into the mechanisms underlying the establishment and maintenance of the persistent infection. The major questions addressed were as follows:

- What early molecular events limit the acute phase of Bunyamwera virus infection in C6/36 cells?
- How do levels of extracellular virus and intracellular viral RNAs vary during the course of persistence?
- Are defective viral RNAs generated, and if so are they fundamental to the maintenance of the persistent state?
- Is there heterogeneity in the response of individual cells within the culture to the persistent infection?

* Then subsequently from Dr Paul Young (London School of Hygiene and Tropical Medicine, London, U.K.).

3 Materials and Methods

3.1 Cells and virus

3.1.1 Cells

***Aedes albopictus* C6/36** cells (Igarashi, 1978) were obtained originally by Dr. R. M. Elliott from Dr. D. L. Knudson (Yale Arbovirus Research Unit, New Haven, Connecticut, USA). *

BHK-21 cells (MacPherson & Stoker, 1962) were obtained from the Cytology department of this Institute.

3.1.2 Virus

Bunyamwera virus (Watret et al., 1985) was three times plaque-purified in BHK cells from a laboratory stock originally obtained from Dr. N. Karabatsos, Vector-borne Diseases Laboratory, Center for Disease Control, Fort Collins, Co., USA. Working stocks of virus were only two passages in BHK cells away from the triple plaque-purified stock.

3.2 Tissue culture materials

Materials marked with an asterisk were obtained from the media department of this Institute (details are given in Section 3.2.2). "HSA" grade agarose, which was used for the plaque-assay overlays, was obtained from Park Scientific Ltd., Northampton, U.K., and Cidex fixative (activated glutaraldehyde solution) from Johnson & Johnson Co. The remaining tissue culture materials were obtained from Life Technologies, Paisley.

3.2.1 Tissue culture media

Glasgow Minimal Essential medium (GMEM) containing 10% newborn calf serum and 10% tryptose phosphate broth.

Leibovitz L-15 medium (Leibovitz, 1963) supplemented with 5% heat-inactivated foetal calf serum and 10% tryptose phosphate broth.

3.2.2 Tissue culture solutions

All solutions were obtained from the Institute's in-house media department.

Amphotericin B: 0.025% (w/v) amphotericin B in distilled water.

Eagles A: 1.6mM $CaCl_2.2H_2O$; 1mM $MgSO_4.7H_2O$; 0.001% (v/v) HCl.

Eagles B (without phenol red): Solution of salts, amino acids and vitamins in distilled water, with final concentrations as follows: 0.88M NaCl; 43mM KCl; 6mM $NaH_2PO_4.2H_2O$; 0.2M glucose; 0.0001% ferric nitrate; 0.26M $NaHCO_3$; 0.02% (w/v) L-glutamine; $8x10^5$ Units of penicillin; 0.08% (w/v) streptomycin; 0.0001% (w/v) antimycotic; 0.033% (w/v) L-arginine mono HCl; 0.019% (w/v) L-cysteine; 0.015% (w/v) L-histidine mono HCl; 0.041% (w/v) Isoleucine; 0.041 (w/v) L-leucine; 0.056% (w/v) L-

lysine mono HCl; 0.026% (w/v) L-phenylalanine; 0.038% (w/v) L-threonine; 0.006% (w/v) L-tryptophan; 0.028% (w/v) L-tyrosine; 0.037% (w/v) L-valine; 0.012% (w/v) L-methionine; 0.003% (w/v) inositol; 0.002% (w/v) each choline chloride, folic acid, nicotinamide; DL pantothenic acid (calcium salt); pyridoxal HCl; thiamine HCl and 0.0002% riboflavin. Carbon dioxide was bubbled through the solution until the pH reached 6.5 and the solution was filter sterilised.

Giemsa stain: 1.5% suspension of Giemsa stain (BDH) in glycerol, heated at 56°C for 2 hours, diluted with an equal volume of methanol, and diluted 1:10 in buffered distilled water before use.

Neutral red: 0.4% (w/v) solution of neutral red chlorine in distilled water.

PBS working solution: 137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.7H_2O$; 1.4mM KH_2PO_4, pH 7.3.

Trypsin: 0.25% (w/v) Difco 1: 250 trypsin in Tris-saline (136mM NaCl; 5.4mM KCl; 0.4mM Na_2HPO_4; 5.6mM dextrose [D-glucose]; 24.8mM Tris (hydroxymethyl) aminomethane). HCl was added to bring the pH to 7.7 and 0.002% (w/v) phenol red was added along with 100,000 Units of penicillin and 0.01% streptomycin. The trypsin was added last, prior to filter sterilization.

Tryptose phosphate broth: 3% (w/v) Difco Bacto tryptose phosphate in distilled water.

Versene: 0.5mM EDTA; 137mM NaCl; 2.6mM KCl; 4.3mM $Na_2HPO_4.7H_20$; 2.4mM KH_2PO_4 (pH 7.12 to 7.3) plus 0.002% (w/v) phenol red.

3.2.3 Overlay for plaque titrations

310ml	Eagles A*
65ml	Eagles B (without phenol red)*
20ml	Newborn calf serum
125ml	Agarose (2.4% in Eagles A w/o phenol red)*

3.2.4 Neutral red overlay

310ml	Eagles A*
65ml	Eagles B (without phenol red)*
7ml	Neutral red (0.4%)*
125ml	Agarose (2.4% in Eagles A w/o phenol red)*

3.2.5 Tissue culture plasticware

Tissue culture plasticware was obtained either from Life Technologies Ltd. (flasks, Limbro well plates and 35mm diameter Petri dishes) or Sterilin Ltd. (50mm and 100mm diameter Petri dishes).

3.3 Bacteria

Escherichia coli strain JM101 (*supE44*, *thi*, Δ (*lac-proAB*) / *F'*, [*traΔ36*, *proAB*$^+$, *lac I*^{q} *lacZ ΔM15*]; Messing, 1979) was used throughout the course of this work.

3.4 Bacterial culture materials

Escherichia coli strain JM101 was grown in 2YT broth.

2YT broth: 85mM NaCl, 10g/l Difco Bactotryptone, 10g/l yeast extract.

L-broth: 117mM NaCl, 10g/l Difco Bactopeptone, 5g/l yeast extract (pH 7.5 prior to sterilization).

Top agar: 1% (w/v) agar in water.

Agar plates contained 1.5% w/v agar in L-broth.

Petri dishes were obtained from Bibby Sterilin Ltd., U.K.

3.5 Plasmids and vectors

3.5.1 Plasmids

pMG1: Mouse cytoskeletal γ-actin cDNA cloned in pBR322 (Peter et al., 1988); kindly provided by Jasmine Daksis. The [^{32}P]-labelled actin probe was prepared from *Pst* I-digested pMG1 DNA.

pBUN127: The Bunyamwera virus M gene sequences inserted into M13mp18 bacteriophage during the construction of templates for the synthesis of labelled cDNA probes were obtained from pBUN127 (Lees et al., 1986). This recombinant plasmid contained a G/C tailed cDNA of approximately 3600 nucleotides derived from the Bunyamwera virus M segment cloned into the *Pst* I site in the ampicillin resistance gene of pBR322.

pTZ18R-BUN L: positive-sense (L RNA specific) riboprobes were synthesised from a full-length L segment cDNA cloned into the *Sma* I site of pTZ18R, under the control of the bacteriophage T7 promoter (Jin & Elliott, 1991; unpublished data).

pT7T3 M: positive-sense (M RNA specific) riboprobes were transcribed from a full-length M segment cDNA cloned into the *Pst* I site of pT7T319U, under the control of the T3 promoter, obtained from George Nakitare.

pUC9 BUNS 378/79: positive-sense (S RNA specific) riboprobes were transcribed from a full-length S segment cDNA inserted into pUC9, under the control of the T7 promoter, obtained from Alastair McGregor.

3.5.2 Cloning Vectors

M13mp18 bacteriophage vectors (Messing & Vieira, 1982) were used for the production of single-stranded DNA. *Hinc* II fragments from pBUN127 (see later) were cloned into the double-stranded replicative form of M13mp18.

3.5.3 Recombinant M13mp18 bacteriophage

Recombinant M13mp18 bacteriophage, containing fragments of cDNA to Bunyamwera virus S (Elliott, 1989b), M (Lees et al., 1986) or L (Elliott, 1989a) RNA inserted into the lacZ coding sequence of the vector, were used as templates from which [^{32}P]-labelled strand specific cDNAs were synthesised for use as hybridization probes. For the L and S segment specific probes, single-stranded template DNA was prepared from selected laboratory stocks of recombinant bacteriophage which had been established during the sequencing of the Bunyamwera virus L and S segments (Elliott, 1989a, 1989b). M13mp18 recombinants containing fragments of the Bunyamwera virus M RNA were constructed from the cDNA insert from pBUN127 (Lees et al., 1986; see Section 3.12.1). Details of the recombinant bacteriophage DNAs used as templates and the probes synthesised from them are given in Table 3.1.

Template DNA	Strand detected	Bases covered	Size (nuc)	Probe name
pBUN357.A8	Genomic L	997-1436	439	L5
pBUN363.86	Antigenomic L	5664-5864	200	L7
pBUN308.6	Genomic S	10-400	390	S8
pBUN308.15	Antigenomic S	550-944	394	S9
pBUN.M1	Genomic M	2233-2517	285	M1
pBUN.M6	Antigenomic M	2517-2233	285	M6

Table 3.1 Details of the recombinant bacteriophage DNAs used as templates to synthesise radiolabelled cDNA probes

3.6 Chemicals, radiochemicals, miscellaneous reagents and enzymes

3.6.1 Chemicals

Chemicals were obtained from either BDH Ltd. or Sigma Chemical Co. Ltd., with the following exceptions: Seakem GTG Agarose (FMC Bioproducts); ammonium persulphate and TEMED [N,N,N'N' tetramethylethylenediamine] (Bio-Rad Laboratories Ltd.); caesium chloride [99.9%] (Melford Laboratories Ltd.); methylmercury (II) hydroxide [1M in water] (ALFA Products); En^{3}hance (NEN Research Products); formamide (Fluka Chemicals Ltd.); Sephadex G-50, Fine [20-80μm] (Pharmacia Fine Chemicals); IPTG and X-gal (BRL, Life Technologies Inc.).

3.6.2 Radiochemicals

Radioisotopes were supplied by Amersham International plc. at the following specific activities:

[^{35}S]-L-methionine	800mCi/mmol	(approx.)
5' α-[^{32}P] dNTPs	3000 Ci/mmol	(10μCi/μl)
5' α-[^{32}P] CTP	400 Ci/mmol	(10μCi/μl)
γ-[^{32}P] dATP	5000 Ci/mmol	(10μCi/μl)

3.6.3 Miscellaneous materials

Dialysis membrane (A & J Beverage Ltd); nitrocellulose membranes [0.2 or 0.45 μm pore size] (Schleicher & Schuell, Inc.); RNasin (Promega); Millipore disposable filters (Nalge Company); Spin-X centrifuge filter units (Costar); Falcon tubes (15 and 50 ml) (Becton Dickinson Labware); rabbit reticulocyte lysate (New England Nuclear); Multiprime DNA labelling system (Amersham International plc.); and Sequenase DNA sequencing kit (United States Biochemicals).

3.6.4 Enzymes

Restriction enzymes and buffers and calf intestinal phosphatase (CIP) were supplied by Boehringer-Mannheim. Lysozyme was obtained from Sigma; AMV reverse transcriptase from Life Sciences; T7 RNA polymerase and RQ1 RNase-free DNase from Promega; T4 polynucleotide kinase from Northumbrian Biologicals Ltd. (NBL); T7 DNA polymerase from Pharmacia LKB Ltd., while large fragment DNA polymerase I (Klenow enzyme), DNA polymerase I, and T4 DNA ligase were supplied by Bethesda Research Laboratories (BRL).

3.7 Commonly used solutions

3.7.1 Reaction buffers

***In vitro* transcription buffer (5x):** 200mM Tris.HCl (pH 7.5), 30mM $MgCl_2$, 10mM spermidine, 50mM NaCl.

Kinase buffer (5x): 250mM Tris.HCl (pH 7.6), 50mM $MgCl_2$, 25mM DTT, 0.5mM spermidine.

Ligase buffer (BRL; 5x): 0.25M Tris.HCl (pH 7.6); 50mM $MgCl_2$; 5mM ATP; 5mM DTT; 25% (w/v) polyethylene glycol-8,000.

NT buffer (10x): 0.5M Tris.HCl (pH 7.5), 0.1M $MgSO_4$, 10mM DTT, 0.5mg/ml BSA.

NT buffer (10x) & dNTP working solution: 500μl of 10x NT buffer; 5μl of 4x 5mM dNTPs.

NTE buffer: 100mM NaCl, 10mM Tris, 1mM EDTA.

TA buffer (10x): 330mM tris acetate pH 7.9, 660mM potassium acetate, 100mM magnesium acetate, 1mg/ml nuclease free bovine serum albumin, 5mM DTT.

TE buffer: 10mM Tris.HCl pH 8.0, 1mM EDTA pH 8.0.

TM buffer (10x): 100mM Tris.HCl pH 8.0, 100mM $MgCl_2$.

3.7.2 Buffers for RNA extractions

Guanidinium thiocyanate (GSCN) buffer: 4M guanidinium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% (w/v) sarcosyl; 0.1M β-mercaptoethanol; 0.33% (v/v) Antifoam A. The guanidinium thiocyanate, sodium citrate, sarcosyl and Antifoam A were dissolved in 40ml of sterile distilled water, the volume was adjusted to 100ml and the solution was filtered through a disposable millipore filter. Finally the β-mercaptoethanol was added.

Lysis buffer (to disrupt virions): 50mM Tris.HCl pH 8.0; 10mM NaCl; 0.6% NP40.

Lysis buffer (to disrupt infected cells): 50mM Tris.HCl pH 7.5; 150mM NaCl; 0.6% NP40.

3.7.3 Buffers for direct RNA sequencing

5x Transcription buffer: 120mM Tris.HCl pH 8.3; 80mM $MgCl_2$; 40mM DTT; 2mM dATP, dCTP and dTTP; 4mM dGTP; 500μg/ml actinomycin D.

5x Annealing buffer: 50mM Tris.HCl pH 8.3; 5mM EDTA; 1.25M KCl. (Both were stored frozen at -20°C).

3.7.4 Sample buffers (containing dyes to mark progress of electrophoresis)

Formamide dyes: 100% formamide; 0.3% bromophenol blue; 0.3% xylene cyanol F.F.

Protein Dissociation buffer: 4% (w/v) SDS; 1.15M β-mercaptoethanol; 0.16M Tris.HCl pH 6.8; 25% glycerol and 0.05% bromophenol blue.

Sample Buffer (2x) for methylmercury gels: 20% glycerol; 0.05% bromophenol blue; 2x EM buffer.

3.7.5 Electrophoresis buffers

EM buffer (10x): 0.05M sodium borate; 0.1M sodium sulphate; 0.01M EDTA; 0.15M boric acid.

TBE buffer (10x): 0.89M Tris; 0.89M boric acid; 0.002M EDTA.

Tris-glycine (10x): 1.9M Glycine; 0.25M Tris.

(SDS-PAGE running buffer consisted of 1x Tris-glycine made 0.1% with respect to SDS)

3.7.6 Solutions for hybridization/Northern blotting

Denhardt's (100x): 2% (w/v) Ficoll 400; 2% (w/v) polyvinylpyrrolidone; 20mg/ml bovine serum albumin (Pentex fraction V, Miles Laboratories). Dissolved in sterile distilled water, filtered and stored at -20°C (Adams et al., 1990).

SSC (20x): 3M NaCl; 0.3M sodium citrate, pH adjusted to 7.0 with 1M HCl.

SSPE (20x): 3.6M NaCl; 200mM NaH_2PO_4 (pH 7.4); 20mM EDTA (pH 7.4).

3.7.7 Other solutions

Gel fix: 50% (v/v) methanol; 10% (v/v) acetic acid; 40% water.

3.8 Synthetic oligonucleotides

Oligonucleotides were synthesised by Dr. J. McLauchlan using a Biosearch DNA synthesiser, model 8600. Six 20mer oligonucleotides were used during the course of this work, details of which are given in Table 3.2. Tubes containing freshly synthesised oligonucleotides were heated at 55°C for 5 hours and opened on ice in a fume hood to release ammonia vapours. The oligonucleotides were transferred to labelled 1.5ml reaction vials and lyophilised for a minimum of 5 hours in a vacuum concentrator.

Oligo No.	Annealing temp. (T°C)	Sequence
1	51	5'-CAACAGAAGGTCATTAAAGG-3'
2	51	5'-TTCTACATTAAAGGACGCGA-3'
3	61	5'-CTCCACCGCCTCAGTGGATG-3'
4	55	5'-CTCTCTTTCTTCCCAGGATC-3'
5	55	5'-GACAGAAGTTCAGTCTGCAC-3'
6	53	5'-AGGGTTGTTTCAACCCACAA-3'

Table 3.2 Sequences and annealing temperatures [T (°C) = 4 (G+C) + 2 (A+T) - 5°C] of the oligonucleotides used for direct RNA sequencing.

3.9 Cell manipulations

3.9.1 Maintenance of cells

***Aedes albopictus* C6/36 cells** were maintained at 28°C in L-15 medium (Leibovitz, 1963) supplemented with 5% heat inactivated foetal calf serum and 10% tryptose phosphate broth. The cells were subcultured at a 1/20 split at approximately 6 day intervals and were regularly tested for mycoplasma contamination.

BHK-21 cells were maintained at 37°C in Glasgow minimal essential medium (GMEM) supplemented with 10% newborn calf serum and 10% tryptose phosphate broth

(Stoker & MacPherson, 1961). The BHK cells were not routinely subcultured in our laboratory but were obtained regularly from the cytology department of this Institute.

3.9.2 Establishment of an *Aedes albopictus* C6/36 cell line (C6/36/BUN) persistently infected with Bunyamwera virus

Aedes albopictus C6/36 cells were grown to 90% confluency and infected with 5 pfu/cell of Bunyamwera virus. After four days the cells were subcultured at a 1/10 split and thereafter at a 1/20 split at approximately 6 day intervals. The cells were maintained at 28°C in supplemented L-15 medium.

3.9.3 Cloning of cells from the C6/36/BUN cell line

Persistently infected *Aedes albopictus* C6/36 cells were cloned from a given pass of the C6/36/BUN cell line, essentially as described by Igarashi et al. (1977). The cells were dispersed into a single-cell suspension in L-15 medium and aliquots containing approximately 20 single cells were seeded onto 100mm diameter tissue culture plates. The absence of aggregates of cells was confirmed by examination of an aliquot of the suspension under the light microscope. The cells were maintained at 28°C in L-15 medium and within two weeks colonies of cells were visible to the eye. The medium was carefully removed from the plates and isolated colonies were circled on the underside of the plates using a marker pen. Small ($2mm^2$) pieces of sterile filter paper were immersed in a 1:4 mixture of trypsin:versene. Circled colonies were overlaid with a piece of saturated filter paper for a few minutes while the trypsin dislodged the cells from the plate. The cells were transferred attached to the filter paper, using a sterile syringe needle, into 1ml of L-15 medium contained in individual wells (15mm diameter) of a 24 well plate. The 24 well plate was then incubated at 31°C until confluent monolayers were observed for each clone. Transfer of confluent monolayers from multiwell plates to small ($25cm^3$) tissue culture flasks was carried out by washing the cells off the well surface using a sterile Pasteur pipette and L-15 medium. Transfer of cells to medium ($80cm^3$) and large ($175cm^3$) tissue culture flasks was achieved by shaking the cells into fresh medium. These cloned cell lines were maintained in an identical manner to the uninfected *Aedes albopictus* C6/36 cell line.

3.9.4 Cryopreservation of cell cultures

Liquid-nitrogen stocks of cells from each pass of the C6/36/BUN cell line and of cloned cell lines derived from this cell line were established. The cells were frozen at a concentration of approximately $1x10^7$ cells/ml in Leibovitz's L-15 medium, containing 10% dimethyl sulphoxide (DMSO) which had been filter sterilized. The cell suspension was transferred to 2ml plastic cryotubes and stored at -70°C overnight before being immersed in liquid-nitrogen for long-term storage.

3.9.5 Revival of liquid-nitrogen cell stocks

Ampules of cells were thawed at 37°C and decanted into small ($25cm^3$) tissue culture flasks along with 5ml of Leibovitz's L-15 medium. The cells were left to settle for 4 h at 28°C after which time the culture fluid was replaced with fresh Leibovitz's L-15 medium.

3.10 Virus manipulations

3.10.1 Plaque-purification of virus

Fifty millimetre diameter tissue culture dishes were seeded with $2x10^6$ BHK cells/dish in 4ml of GMEM and incubated overnight at 37°C. Serial ten fold dilutions of virus, in GMEM, were prepared to 10^{-6} dilution and 100µl of each dilution was used to infect individual monolayers. Each dilution was plated out in duplicate. The virus was left to adsorb for one hour at 31°C. The inoculum was then removed and replaced with 4ml of agarose overlay (see Section 3.2.3). After four days at 31°C, a second agarose overlay containing 0.01% neutral red (see Section 3.2.4) was applied to the plates and the plates were returned to the 31°C incubator for a further 24 hours. At the end of the incubation period plaques were visible as clear round areas against a red background. Well separated plaques were picked into 0.5ml of medium using a sterile Pasteur pipette and stored frozen at -20°C. The second and third successive plaque picks were carried out as above, with the inoculum comprising ten-fold dilutions (to 10^{-3}) of 30µl of the preceeding plaque isolate.

3.10.2 Assay of viral infectivity by plaque-titration

Virus titres were determined by plaque-titration on BHK cell monolayers of 90% confluency. Fifty millimetre diameter tissue culture dishes were seeded with $2x10^6$ BHK cells/dish in 4ml of GMEM and incubated overnight at 37°C. Serial ten fold dilutions of virus in GMEM were prepared to 10^{-8} dilution. 100µl of each dilution was used to infect a single monolayer taking care to distribute the inoculum over the entire monolayer. Each dilution was assayed in duplicate. The virus was left to adsorb for 1 hour at 31°C after which time the inoculum was removed and replaced with 4ml of agarose overlay (see Section 3.2.3). The overlaid, infected cell monolayers were incubated at 31°C for 4 to 5 days and then fixed with activated Cidex (1% glutaraldehyde) for 4 hours. The monolayers were stained with Giemsa stain for 10 minutes, excess stain was washed off and the plates were left to air dry prior to counting the plaques.

3.10.3 Preparation of elite stocks of virus

Small ($25cm^2$) tissue culture flasks were seeded with $2x10^6$ BHK cells in 15 ml of GMEM and incubated overnight at 37°C. The next day the medium was removed from the flasks and the cells were infected at a multiplicity of 0.001 to 0.01 pfu/cell with triple plaque-purified virus (200µl out of the 500µl total plaque isolate). The flasks were shaken gently to disperse the virus over the cells and incubated at 31°C for one hour while virus

adsorbed to the cells. The inoculum was then removed and 5ml of GMEM was added to each flask. The infection was allowed to proceed at 31°C until cpe was evident (usually 48 to 72 hours). The medium was then decanted from each flask into a 15 ml Falcon tube and clarified by centrifugation in an MSE Coolspin centrifuge at 3,000 rpm for 8 minutes at 4°C. The clarified virus suspension was divided into 2.5ml aliquots and stored frozen at -70°C. Each elite stock was titrated in BHK cells.

3.10.4 Preparation of virus working stocks

Large (175cm^2) tissue culture flasks were seeded with 1x10^7 BHK cells/flask and incubated overnight at 37°C. The resulting 90% confluent cell monolayers were infected with 0.001 to 0.01 pfu/cell of Bunyamwera virus elite stock and the virus was adsorbed for one hour at 31°C. Fifteen millilitres of GMEM was added to each flask and the infection was allowed to proceed at 31°C for 4 to 5 days, by which time extensive cpe was evident. The culture fluid was harvested and clarified by centrifugation in an MSE Coolspin centrifuge at 3,000 rpm for 8 minutes at 4°C. The virus suspension was divided into 5ml aliquots and stored frozen at -70°C. Each working stock was titrated in BHK cells.

3.10.5 Assay for resistance to superinfection

Cells from a chosen pass of the persistently infected C6/36/BUN cell line, or from cloned cell lines derived from this culture, were harvested into L-15 medium and dispersed into a single-cell suspension. Duplicate 35mm diameter tissue culture plates were seeded with 1.5x10^6 cells/plate and the cells were left at 28°C overnight to grow to 90% confluency. The cell monolayers were either infected with 5 pfu/cell of wt Bunyamwera virus or mock infected with 250µl of L-15 medium, and incubated for 1 hour at 28°C. After 1 hour the inoculum was removed, the monolayers were washed 2x with PBS and 2.5ml of fresh L-15 medium was added to each plate. The plates were maintained at 28°C for 48 hours. The supernatant was then removed for plaque-titration in BHK cells, as described previously.

3.10.6 Infectious centres assay

Cells from passage 6 of the persistently infected C6/36/BUN cell line were washed twice in PBS, and harvested as a single-cell suspension in 10ml of Leibovitz L-15 medium. The concentration of the cell suspension was calculated by counting the number of cells in an aliquot, using a haemocytometer. Ten-fold dilutions of the cell suspension (down to 10^{-6}) were prepared in supplemented GMEM medium and 200µl aliquots of each dilution were plated onto 90% confluent BHK cell monolayers in 50mm diameter tissue culture dishes. The C6/36 cells were left to adsorb to the BHK cell monolayers at 31°C for one hour. The plates were then overlaid with 4ml of agarose overlay (see Section 3.2.3) and incubated at 31°C for 4 to 5 days. The cell monolayers were subsequently fixed and

stained as detailed in Section 3.10.2. Plaques were counted and the proportion of C6/36 cells releasing infectious virus was calculated.

3.11 Bacterial manipulations

3.11.1 Preparation of competent bacteria

A single colony of *E. coli* JM101 was picked off an agar plate (which had been stored sealed at 4°C) and was inoculated into 10ml of 2YT broth. The culture was shaken overnight at 37°C. 0.5ml of the overnight culture was inoculated into a conical flask containing 50ml of 2YT broth and incubated at 37°C with shaking for 2.5 to 3 hours, until the optical density reading at 590nm was 0.3. The flask was then placed on ice for a few minutes. The bacterial culture was transferred to a 50ml Falcon tube and the bacteria were pelleted by centrifugation at 3,000 rpm for 8 minutes in a Wifug 500E bench centrifuge. The supernatant was discarded and the bacterial pellet was resuspended in 20ml of cold 0.1M $CaCl_2$. The bacterial suspension was left on ice for 20 minutes to 2 hours before being pelleted as before. The bacterial pellet was resuspended in 3ml of cold 0.1M $CaCl_2$ and stored on ice until required.

3.11.2 Transformation of *E. coli* strain JM101

Selected stocks of M13mp18 replicative form (RF) DNA were diluted in 2YT medium and 50 to 500 ng of DNA was used to transform *E. coli* JM101 bacteria. Competent cells and DNA were mixed as follows:

300µl Competent cells

1.0µl DNA (0.05 to 0.5 mg/ml)

The mixture was incubated on ice for one hour, and then heat-shocked at 42°C for 40 seconds. Meanwhile 1ml of an *E. coli* JM101 overnight culture was added to 10ml of 2YT broth and shaken at 37°C for one hour. A bottle of top agar was melted and equilibrated at 42°C and the required number of LB agar plates were dried at 37°C. Each transformation mix was added to a prewarmed tube (42°C) containing the following:

250µl Freshly grown JM101

25µl IPTG (100mM)

25µl X-gal (20mg/ml in dimethylformamide)

3ml Top Agar

The contents of the glass tubes were mixed and poured immediately onto dry, prewarmed LB agar plates. The plates were tipped gently from side to side to ensure an even distribution of bacteria and top agar and left at room temperature for 5 minutes to set. The plates were incubated inverted at 37°C overnight. Clear plaques, indicative of bacteriophage containing insertions into the vector cloning sites within lacZ coding sequences, were visible the next day and isolated plaques were picked.

3.12 DNA manipulations

3.12.1 Synthesis of recombinant M13mp18 containing cDNAs to the Bunyamwera virus M segment

The 3.6 kb Bunyamwera virus M segment cDNA from pBUN127 was excised with *Pst* I and digested for two hours at 37°C with 20 Units of *Hinc* II (see Section 3.12.6). After phenol/chloroform extraction, the DNA fragments were ethanol precipitated and resuspended in 20µl of water. The fragments were then ligated into M13mp18 (previously linearised with *Sma* I and dephoshorylated using calf intestinal phosphatase) by incubation with T4 DNA ligase at 15°C overnight. Three reactions were set up as shown in Table 3.3. After the overnight incubation, the recombinant phage were extracted once with phenol and once with chloroform, ethanol precipitated (see Section 3.12.5) and dissolved in 20µl of water.

	1	2	3
Hinc II digest.	1µl	5µl	10µl
5x Ligase buffer	4µl	4µl	4µl
T4 DNA ligase	0.5U	0.5U	0.5U
Sma/cap M13mp18	1µl	1µl	1µl
Water	To make total volume to 20µl		

Table 3.3 Ligation reactions set up to generate Bunyamwera virus M segment cDNA/M13mp18 recombinants.

3.12.2 Preparation of single-stranded template DNA

A 100ml bottle of 2YT broth was prewarmed to 37°C and 1ml of a fresh overnight culture of *E. coli* JM101 bacteria was added. Single, isolated plaques, freshly picked with a sterile cocktail stick, were added to 1.5ml aliquots of this suspension in sterile universals (the cocktail stick was left in the universal) and the cultures were incubated in an orbital shaker, set at 100-200 rpm and 37°C, for 5-6 hours. One ml of the final infected culture was transferred to a 1.5ml Eppendorf tube and the bacteria were pelleted by centrifugation at 13,000 rpm for 5 minutes in a microcentrifuge. The supernatants were decanted into a fresh Eppendorf tube (taking care not to transfer any of the pelleted bacteria) and 150µl of 20% polyethylene glycol in 3.5M potassium acetate (or 2.5 M NaCl) was added. The samples were mixed well by several vigorous inversions and left at room temperature for at least 30 minutes, or at 4°C overnight. The bacteriophage were pelleted at 13,000 rpm for 5 minutes in a microcentrifuge and the supernatant fluid was removed using a sterile Pasteur pipette attached to a suction pump. The bacteriophage pellets were forced to the other side of the Eppendorf tubes by centrifugation at 13,000 rpm for 30 seconds, and the last traces of supernatant were removed as before. The

bacteriophage were resuspended in 100µl of TE and 50µl of TE-equilibrated phenol was added. The samples were vortexed for 10 seconds, left on the bench for 5 minutes and vortexed again briefly before centrifugation at 13,000 rpm for 10 minutes. The upper aqueous phase containing the phage DNA was transferred into a fresh tube and the DNA was precipitated by the addition of 10µl 3M sodium acetate pH 5.5 and 250µl of ethanol. The samples were vortexed briefly and left overnight at -20°C or on dry ice for 30 minutes. The DNA was pelleted by centrifugation at 13,000 rpm for 5 minutes. The supernatant was removed using a Pasteur pipette and the pellet of single-stranded bacteriophage DNA was washed with 70% ethanol. The DNA was lyophilised and dissolved in 30µl of sterile distilled water. The templates were stored at -20°C.

3.12.3 Quantitation of nucleic acid

The concentration of RNA and DNA preparations was determined from optical density measurements at 260nm (OD_{260}), measured using Beckman microcell 8mm high quartz cuvettes in a Beckman DU-62 spectrophotometer. It was assumed that an OD_{260} of one was equivalent to a concentration of 50µg/ml for double-stranded DNA, 40µg/ml for single-stranded DNA and RNA, and 20µg/ml for single-stranded oligonucleotides.

3.12.4 Assessment of the purity of nucleic acid preparations

Two optical density measurements were taken for each preparation, one at 260nm and another at 280nm. Calculation of the OD_{260}/OD_{280} ratio provided an assessment of the purity of a given preparation. Ratios of 1.8 and 2.0 are indicative of relatively pure DNA and RNA preparations respectively. Contamination with phenol or protein lowers the ratio.

3.12.5 Ethanol precipitation

Nucleic acids were precipitated with one tenth volume of 3M NaOAc pH 5.5 and 2.5 volumes of ice-cold ethanol. The samples were mixed well and frozen at -20 or -70°C for at least one hour. The precipitate was pelleted by centrifugation at 12,000 x g for 20 minutes and the supernatant was removed. The pellet was washed with 70% ethanol and recentrifuged briefly. Finally the supernatant was removed, the pellet was dried in a vacuum concentrator and resuspended in the appropriate buffer.

3.12.6 Restriction enzyme digestion

Restriction digests were set up using 2 units of enzyme per µg of double-stranded DNA (BRL enzymes and buffers were used). A typical reaction would be:

10µg	double-stranded DNA
6µl	10x restriction enzyme buffer
2µl	(20 U) restriction enzyme
	H_2O to 60µl

Digestion usually required a 2-5 hour incubation at the optimum temperature for the restriction enzyme. The extent of digestion was assessed by running 1/10th of the sample on an agarose minigel with ethidium bromide in the running buffer, and visualizing the digestion pattern with a long wave UV source. The remainder of the sample was phenol extracted, ethanol precipitated and taken up in 10µl of H_2O.

3.12.7 Gel purification of oligonucleotides

A 16% denaturing polyacrylamide/urea gel was prepared as follows:

25ml	16% sequencing gel mix (see Section 3.18.4)
0.25ml	10% ammonium persulphate
25µl	TEMED

The gel was poured to 0.3mm thickness. Formamide dyes were added to each sample (1/10th of the sample volume) and the gel was loaded. Electrophoresis was carried out at 37W for approximately 3 hours or until the bromophenol blue had migrated 3/4 of the way down the gel. The glass plates were removed from the gel which was then covered with "Clingfilm". The gel was placed face upward on a fluorescent thin-layer chromatography (TLC) plate and the oligonucleotides were visualized by UV shadowing. Oligonucleotides have a UV absorption peak at 260nm; when the electrophoresed gel was placed on the fluorescent TLC plate and illuminated from above by short wave UV light the oligonucleotides were visible as dark bands against the light surface. The oligonucleotides were excised from the gel using a clean scalpel and transferred to a 15ml Falcon tube. The gel slices were covered with water and the oligonucleotides were eluted into the water by shaking at 37°C overnight. The water containing the oligonucleotides was then spun through a Spin-X centrifuge filter unit to remove gel pieces and the oligonucleotide solutions were lyophilised. Finally each oligonucleotide was dissolved in 50µl of water and a 1µl aliquot was removed to determine the concentration and purity of the preparation by spectroscopy.

3.13 DNA sequencing

3.13.1 Sequencing and size determination of recombinant M13mp18 cDNAs

All single-stranded templates were sequenced using Sequenase. The relative sizes of the cDNA inserts were checked by generating prime-cut probes and running them side by side on a 6% denaturing polyacrylamide/urea gel (see Section 3.18.4). Details of the recombinant bacteriophage selected from laboratory stocks, or constructed from pBUN.127, and the probes synthesised from them are given in Table 3.1.

3.13.2 Single-stranded DNA sequencing (using Sequenase)

(i) Annealing reaction

For each set of four sequencing lanes one annealing reaction was set up as follows:

1µl Primer (provided in kit)

2µl Sequencing buffer (provided in kit)

2µg DNA (reaction volume made up to 10µl with H_2O)

Samples were heated to 65°C for two minutes and allowed to cool slowly to room temperature over a period of 30 minutes (using a beaker of water heated initially to 65°C).

(ii) Labelling reaction

Each labelling reaction was set up as follows:

10µl Template-primer

1µl 0.1M DTT

2µl Labelling mix (diluted five-fold with distilled H_2O)

0.5µl α-[^{32}P] dATP

2µl Sequenase enzyme (diluted 1:8 with ice cold TE)

The samples were mixed thoroughly and incubated at room temperature for 5 to 10 minutes.

(iii) Termination reaction

For each sequence determination four Eppendorf tubes were labelled G, A, T or C and 2.5µl of ddGTP, ddATP, ddTTP or ddCTP was added to the appropriately labelled tube. The chain terminating nucleotides were prewarmed at 37°C for at least 1 minute and 3.5µl of the above labelling incubation was transferred to each tube. The samples were mixed, spun down briefly in a microcentrifuge and incubated for a further 5 minutes at 37°C. Finally, 4µl of stop solution was added to each tube, the samples were mixed thoroughly and stored on ice until they were loaded onto the gel. Just prior to loading the samples were heated to 80°C for 2 minutes and 2 to 3µl of each sample was loaded onto an 8% sequencing gel (see Section 3.18.4).

3.14 Radiolabelling procedures

3.14.1 Prime cut probes

M13 Universal primer was annealed to recombinant M13 DNA in TM buffer for one hour at 60°C. One microlitre of a 0.5mM solution of dCTP, dTTP and dGTP was added along with 10µCi of α-[^{32}P]-dATP (10mCi/ml) and 1 unit of large fragment DNA polymerase (Klenow); the sample was incubated at room temperature for 10 minutes. A chase mix consisting of 1µl of 4 x 0.5mM dNTP was added to the reaction and the sample was incubated at room temperature for 5 minutes. The large fragment DNA polymerase was then inactivated by heating at 70°C for 10 minutes. The appropriate restriction enzyme

was added to the reaction mixture and the newly generated double-stranded DNA was digested with 10U of restriction enzyme for 90 minutes at the appropriate temperature. Labelled DNA fragments were purified by electrophoresis through a thin (0.3mm) denaturing 6% polyacrylamide/urea gel (see Section 3.18.4). Prior to loading the gel, 0.75µl of 0.5M EDTA and 25µl of formamide dyes were added to the DNA and each sample was boiled for 2 minutes. Each sample was loaded onto the gel and electrophoresed at 37 W for 4 hours or until the bromophenol blue band was 3/4 of the way down the gel. The probe was located by autoradiography, excised from the gel and the gel slice was broken into small pieces by centrifugation into a 1.5ml Eppendorf tube through a hole in the bottom of a 0.5ml Eppendorf tube. The gel pieces were transferred to a 15ml Falcon tube in 1ml of water and the probe was eluted into the water by shaking overnight at 37°C. The gel pieces were removed by centrifugation through a Spin-X centrifuge filter unit.

3.14.2 Nick translation

The labelling reaction, in a total volume of 50µl, was set up using 100-200ng of template DNA to which the following were added:

5µl 10x NT buffer & dNTP

4µl 4 x [^{32}P]-dNTPs.

2µl 10^{-4} dilution of DNase 1 (stock solution concentration = 1,000 U/ml).

1µl DNA polymerase 1 (10 U).

The reaction was incubated at room temperature for 30 minutes. Labelled DNA was separated from unincorporated nucleotides by chromatography on a 1ml column of Sephadex G-50. Ten microlitres of Orange-G was added to the sample prior to loading onto the column; this dye chromatographed with the unincorporated dNTPs. The sample was applied to the top of the column and the labelled DNA was eluted with water until the orange-G had almost reached the bottom of the column. The probe was denatured prior to use by boiling for 5 minutes.

3.14.3 Generation of single-stranded α-[^{32}P]-labelled cDNAs (using the Amersham 'rapid multiprime DNA labelling system')

Recombinant M13mp18 bacteriophage containing fragments of cDNA to the Bunyamwera virus S, M or L segment RNAs were used as templates to make strand specific probes. Single-stranded template DNA was stored at -20°C at a concentration of 25 µg/ml in sterile distilled water. For each probe, 25ng of template DNA was denatured by heating to 95 to 100°C for two minutes followed by incubation on ice. The labelling reaction was set up on ice as follows:

4µl Each unlabelled dNTP (omitting ones to be used as label)
5µl Buffer provided in kit
5µl Primer provided in kit
5µl Combined volume of α-[^{32}P]-dNTPs (3000 Ci/mmol; 10mCi/mmol)
2µl Enzyme
15µl H_2O

The reaction mix was vortexed briefly and spun down in a microfuge for a few seconds before incubation at 37°C for 30 minutes. Unincorporated nucleotides were removed by gel filtration through a Sephadex G-50 mini column as described in the preceeding section on nick translation. Before use, the labelled DNA was denatured at 95 to 100°C for two minutes and chilled on ice. The probe can be stored at -20°C after the addition of EDTA to 20mM.

3.14.4 End-labelling of oligonucleotides with γ-[^{32}P]-dATP

The reaction was set up as follows:

1µl 0.1 µg/µl oligonucleotide
4µl 5x kinase buffer
10µl γ-[^{32}P]-dATP (100µCi)
1.4µl T4 polynucleotide kinase (7U)
3.6µl H_2O

Each sample was incubated at 37°C for 30 minutes, followed by a 5 minute incubation at 65°C to inactivate the T4 polynucleotide kinase. The radiolabelled oligonucleotides were stored frozen at -20°C; 1µl (5ng of labelled oligonucleotide) was sufficient for one primer extension reaction.

3.14.5 Synthesis of Riboprobes

Riboprobes were synthesised from full-length L, M and S segment cDNAs cloned under the control of the bacteriophage T7 promoter (Jin and Elliott, 1991 and unpublished data), using T7 RNA polymerase (Promega) and 50µCi α-[^{32}P]-CTP in an *in vitro* transcription reaction as detailed below.

(i) Standard transcription protocol

The template DNA was linearised and the reaction was set up as follows:

1.0µg Linearised template DNA
4.0µl 5x transcription buffer
2.0µl 100mM DTT
20 U RNasin
1.0µl 10mM ATP
1.0µl 10mM GTP
1.0µl 10mM UTP

2.4µl 100µM CTP

5.0µl α-[^{32}P]-CTP (50µCi at 10mCi/ml)

1.0µl T7 RNA polymerase (at 15-20 Units/µl)

The reaction volume was adjusted to 20µl with water and the samples were incubated at 37 to 40°C for 60 to 120 minutes.

(ii) Digestion of the DNA template and removal of unincorporated nucleotides following transcription.

After the transcription reaction, RQ1 RNase-free DNAse was added to a concentration of 1 unit/µg of template DNA and the sample was incubated for 15 minutes at 37°C. The radiolabelled RNA was phenol/chloroform extracted and recovered by ethanol precipitation in the presence of 1M NH_4OAc. For the precipitation step each sample was mixed thoroughly with 1/4 volume of 5M NH_4OAc and 3 volumes of ethanol and placed at -20°C for 30 minutes. The RNA was pelleted by centrifugation at 12,000 x g for 30 minutes and the supernatant was decanted. The RNA was resuspended in 50µl of water which was then made 1M with respect to NH_4OAc and a second precipitation step was carried out as described above. The pellet was dried under vacuum and resuspended in TE buffer for storage at -70°C.

3.14.6 Pulse-labelling with [^{35}S]-methionine

Protein synthesis in infected cells was monitored by labelling the cells with [^{35}S]-methionine during a one hour pulse. Confluent monolayers of *Aedes albopictus* C6/36 or BHK cells, in 35mm diameter tissue culture plates, were infected at a moi of 5 pfu/cell with Bunyamwera virus and incubated at 28°C and 31°C respectively. Uninfected controls were included for both cell types. At designated time points post infection the culture medium from infected cell monolayers was replaced with 500µl of PBS containing 50µCi of [^{35}S]-methionine and incubation was continued for one hour. The labelling solution was removed and the monolayers were washed twice with PBS. The cells were scraped into 80µl of protein dissociation buffer and stored at -20°C prior to SDS-PAGE. The samples were boiled for 2 minutes and spun briefly in a microcentrifuge before loading 1/3rd of each sample onto a polyacrylamide gel.

3.15 RNA isolation

3.15.1 Preparation of virion RNA

Culture fluid from Bunyamwera virus infected BHK cells was harvested and clarified by centrifugation at 3,000 rpm, for 10 minutes, in an MSE Coolspin centrifuge, maintained at 4°C. The clarified supernatant was centrifuged at 35,000 rpm for 3 hours in a Sorvall TST41 rotor, again at 4°C. Pelleted virus was resuspended in lysis buffer comprising 50mM Tris HCl, pH8, 10mM NaCl and 0.6% NP40, and left for one hour at 4°C. The lysed virus suspension was centrifuged at 6,500 rpm in a Sorvall SS34 rotor, the

supernatant was harvested and EDTA was added to a concentration of 6mM. This suspension was layered onto a preformed CsCl gradient overlaid with 2.5ml of 5% sucrose in 50mM NaCl, 25mM Tris and 2mM EDTA. The CsCl gradient consisted of 6ml of 20-40% (w/w) CsCl in 50mM NaCl, 25mM Tris and 2mm EDTA. The loaded gradients were centrifuged at 35,000 rpm at 10°C in a Sorvall TST-41 rotor for 15 hours . The viral nucleocapsids formed a sharp band in the middle of the CsCl gradient and were harvested through the side of the centrifuge tube using a sterile syringe and needle. The nucleocapsid material was diluted in 1x NTE and concentrated by centrifugation at 39,000 rpm for 2 hours at 4°C in a Sorvall TST-41 rotor. RNA was extracted from the pelleted nucleocapsids using the acid-phenol-guanidinium thiocyanate protocol of Chomczynski & Sacchi (1987), as outlined below.

3.15.2 Preparation of fractionated infected cell RNA

Viral nucleocapsids were separated from the remainder of the infected cell RNA as described by Leppert et al., (1979) and Rossier et al., (1988). Infected cell monolayers were washed twice with PBS and scraped into PBS using a rubber policeman. The cells were pelleted by centrifugation at 3,000 rpm in an MSE Coolspin centrifuge, resuspended in PBS and repelleted. The cells were lysed on ice for 15 minutes in buffer containing 150mM NaCl, 50mM Tris.HCl pH 7.5 and 0.6% NP40. The cells were further disrupted by vortexing for 2 minutes. Nuclei and cell debris (membranes) were removed from the suspension by centrifugation at 8,000 rpm for 5 minutes at 4°C in a Sorvall SS34 rotor. The cytoplasmic extract was made 6 mM with respect to EDTA and centrifuged through a preformed 20-40% (w/w) CsCl gradient overlaid with 5% sucrose, as described in section 3.15.1. Bunyamwera virus nucleocapsids appeared as a sharp band in the centre of the gradient and the remainder of the infected cell RNA pelleted. The pelleted RNA was used as the source of viral mRNA after a further extraction was performed using the acid-phenol-guanidinium thiocyanate protocol of Chomczynski and Sacchi (1987). The nucleocapsids were harvested using a syringe, diluted with 1x NTE buffer (100mM NaCl, 10mM Tris.HCl (pH 7.5), 1mM EDTA) and concentrated by centrifugation at 39,000 rpm for 2 hours at 4°C in a Sorvall TST-41 rotor. RNA was extracted from the purified nucleocapsids using the acid-phenol-guanidinium thiocyanate protocol of Chomczynski and Sacchi (1987).

3.15.3 Preparation of total cell RNA

Total cellular RNA was extracted by the acid-phenol-guanidinium thiocyanate protocol of Chomczynski and Sacchi (1987). The reagent volumes specified below were sufficient to harvest RNA from 1 x 10^6 cells and were scaled up as appropriate. Infected cell monolayers were washed twice with PBS before lysis with 100µl of GSCN buffer (see Section 3.7.2). The lysed cells were transferred to an Eppendorf tube to which 10µl of 2M

NaOAc pH 4.0, 100µl of water-equilibrated phenol, and 20µl of a 49:1 mixture of chloroform/amyl alcohol were added with thorough mixing after the addition of each reagent. Each sample was then vortexed for 10 seconds and left on ice for 15 minutes. The aqueous and organic phases were separated by centrifugation at 10,000 x g for for 15 minutes at 4°C. The upper aqueous phase was harvested and mixed with 100µl of isopropanol and the samples were incubated at -20°C for one hour. The RNA was pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C. The pellet was dissolved in 300µl of GSCN buffer and mixed with 300µl of isopropanol. The samples were incubated at -20°C for one hour and centrifuged at 10,000 x g for 15 minutes. The pelleted RNA was dissolved in water (brief heating to 60°C was sometimes necessary to ensure that the RNA had dissolved completely). An equal volume of a 4:1 mixture of chloroform:butan-1-ol was added and the sample was vortexed to an emulsion and then centrifuged briefly at 10,000 x g to separate the phases. The upper aqueous phase was harvested and the organic phase was back extracted with an equal volume of water. Finally the RNA was recovered from the pooled aqueous phases by precipitation with 1/10 volume of 3M NaOAc pH 5.5 and 2.5 volumes of ice-cold ethanol (see Section 3.12.5).

3.16 RNA manipulations

3.16.1 Slot blot analysis

Each RNA sample was mixed with 100µl of 20x SSC and applied under vacuum to nitrocellulose using a Schleicher and Schuell Minifold II. The nitrocellulose (Schleicher and Schuell) and two pieces of filter paper were pretreated by emersion first in sterile distilled water for 5 minutes and then in 10x SSC for 5 minutes. The manifold was assembled as per the manufacturer's instructions. After application of the RNA, the nitrocellulose was baked at 80°C under vacuum for two hours.

3.16.2 Northern blot analysis

Electrophoresis was performed in 1.0, 1.5, or 1.8% agarose gels containing 5mM methylmercury hydroxide (see Section 3.18.3). Non-submarine gels were electrophoresed in 1x EM buffer, for five hours at 75V, with recirculation of the tank buffer (Perbal, 1988). Transfer of RNA to nitrocellulose was by capillary transfer in 20x SSC, without pretreatment of the gel. Prior to transfer, the nitrocellulose (which had been cut so that it was 1mm longer and wider than the gel) was soaked in sterile distilled water for 5 minutes and then in 10x SSC for 5 minutes. The gel was placed on a sheet of 3MM paper which acted as a buffer bridge (having been wet through with 20x SSC and arranged on a glass plate support spanning a tray containing 20x SSC). The ends of the 3MM paper were immersed in the 20x SSC. The nitrocellulose was placed on top of the gel so that it just overlapped the sides of the gel but was not in danger of contacting the filter paper underneath (which would have short circuited the flow of buffer through the gel). Care

was taken to avoid air bubbles either underneath the 3MM paper or between the paper and the gel, or the gel and the nitrocellulose; any air bubbles were removed by rolling a 10ml pipette over the surface, applying gentle pressure. Two pieces of filter paper which had been cut just smaller than the nitrocellulose and had been presoaked as per the nitrocellulose were placed on top, followed by an 8cm stack of absorbant paper towels. A 500g weight was placed on top of the blot to aid capillary transfer and the blot was left overnight. Finally the nitrocellulose was baked at 80°C in a vacuum oven for two hours.

3.16.3 Hybridization conditions

Prehybridization and hybridization were carried out at 42°C for 4 and 16 hours respectively. The buffer conditions used with particular probes are set out in Table 3.4.

Buffer constituents	Riboprobes		Prime-cut or rapid multiprime generated DNA probes	
	Prehybridization	Hybridization	Prehybridization	Hybridization
SSPE	6x	6x	5x	5x
Formamide	50%	50%	50%	50%
Low fat milk	----------	----------	0.5%	0.1%
Carrier DNA	0.05 mg/ml	0.05mg/ml	0.1 mg/ml	0.1 mg/ml
Yeast tRNA	0.05 mg/ml	0.05 mg/ml	----------	----------
SDS	0.1%	0.1%	----------	----------
Denhardt's	5x	1x	----------	----------

Table 3.4 Recipes for prehybridization and hybridization buffers.

3.16.4 Post hybridization washes

After hybridization the filters were washed several times at room temperature. Filters which had been probed for viral RNA were washed twice in 2x SSC, 0.5% SDS and twice at higher stringency (0.1x SSC, 0.1% SDS). Filters probed with the actin specific probe were washed twice at the lower stringency only.

3.16.5 *In vitro* translation

In vitro translation reactions were carried out with New England Nuclear (NEN) rabbit reticulocyte lysate. Each reaction contained 2µg of total infected cell RNA or the cell equivalent of nucleocapsid derived RNA, adjusted to 2µl total volume. For each set of reactions a premix was prepared:-

Per reaction:

5µl [^{35}S]-methionine.
5.5µl translation cocktail
2.0µl 1M potassium acetate
0.5µl 32.5mM magnesium acetate

Separate reactions were then set up as follows:

10µl lysate
13µl premix
2µl RNA/H_2O

The samples were incubated at 37°C for 90 minutes and then 25µl of protein dissociation buffer was added to each. The *in vitro* translation products were analysed by SDS-PAGE.

3.17 Direct RNA sequencing

Direct RNA sequencing was performed as described by Geliebter et al. (1986). This method is based on enzymatic extension of [^{32}P]- end-labelled oligonucleotide primers by reverse transcriptase, in the presence of dideoxynucleoside triphosphates, which function as chain terminators during the reverse transcription step. Direct RNA sequencing yields the consensus sequence of the RNA population under study. Both virion RNA and total infected cell RNA were used as templates for sequencing.

(i) Annealing reaction:

Each annealing reaction was set up:-

1µl (5ng) [^{32}P]-labelled oligonucleotide primer
10µg total infected cell RNA or 1µg of virion RNA
2µl 5x annealing buffer

The reaction volume was adjusted to 10µl with water and the nucleic acid was denatured by heating to 80°C for 3 minutes. Radiolabelled oligonucleotides were annealed to the template RNA by incubation for one hour at the annealing temperature (T°C), defined as five degrees below the denaturation temperature for the oligonucleotide. Denaturation temperature is determined by nucleotide composition and T°C was calculated as shown below:

$$T\,(^{o}C) = 4\,(G + C) + 2\,(A + T) - 5^{o}C$$

(ii) Primer extension reaction

Primer extension reactions were set up in quadruplicate (one reaction for each chain terminator) as follows:-

2.0µl annealed RNA-Primer
1.0µl 5x transcription buffer
3 U reverse transcriptase
0.5µl ddNTP (either 1.5mM ddATP, ddCTP or ddGTP or 3.0mM ddTTP)

The samples were incubated at 50°C for one hour, then 2µl of formamide dyes were added to each. The samples were boiled for 3 minutes and half of each sample was loaded onto an 8% sequencing gel (see Section 3.18.4).

3.18 Electrophoresis

3.18.1 SDS-PAGE

	15% Resolving Gel	3% Stacking Gel
Acrylamide: Bis (75:1) [37.5 and 0.5% (w/v) stock]	24.9ml	1.0ml
1M Tris HCl pH 8.8	24.0ml	---------
1M Tris HCl pH 6.8	---------	625µl
H_2O	14.7ml	8.0ml
10% SDS	650µl	100µl
10% Ammonium persulphate	650µl	100µl
TEMED	60µl	5µl

Table 3.5 Recipe for 15% resolving and 3% stacking portions of SDS-PAGE gels

SDS-PAGE gels were poured between two clean 20 x 22cm glass plates separated by 1mm thick spacers on either side. The gel plates were sealed with Kapton tape. The 15% resolving gel mix was prepared as shown in Table 3.5, adding the ammonium persulphate and TEMED last. Using a 10ml glass pipette the resolving gel was poured to a height approximately two sample depths below the bottom of the sample wells, taking care to avoid air bubbles. A small volume of butan-1-ol was applied to the top of the gel to ensure a level interface with the stacking gel and to aid polymerization by excluding air. The gel was left to set for 15 minutes. Meanwhile the 3% stacking gel mix was prepared as indicated in Table 3.5, the butan-1-ol was poured off the set stacking gel and the top of the gel was rinsed with water. The stacking gel was poured to the top of the gel plates and the gel comb was inserted to form the sample wells. The gel was wrapped in damp tissues, covered in cling film and left to set a few degrees from the horizontal. Best results were obtained if the gels were left at 4°C overnight prior to electrophoresis. Electrophoresis was performed in 1x Tris/glycine buffer containing 0.1% SDS. The gel comb was removed, the sample wells were rinsed with electrophoresis buffer and the gel was pre-electrophoresed for 30 minutes at a constant current of 60mA. Samples were boiled for two minutes and centrifuged briefly in a microcentrifuge prior to loading onto the gel.

Electrophoresis was performed at 4°C at a constant current of 60mA for approximately 3.5 hours (until the bromophenol blue in the sample buffer had migrated to between 2 and 3cm from the bottom of the gel).

3.18.2 Agarose gels for electrophoresis of DNA

A molten solution of 1% agarose in 1x TBE (25ml/gel) was left to cool slightly and was poured to form a horizontal slab gel in a BRL Horizon-58 electrophoresis tank, the gel comb was put in place and the gel was left to set for 5 minutes. The gel was emersed in 1x TBE running buffer containing 0.5µg/ml ethidium bromide to enable visualisation of the DNA under UV illumination after electrophoresis. Samples containing 1/10th volume of glycerol dyes were loaded onto the gel and electrophoresis was carried out at 80 to 100V for 30 minutes, or until the bromophenol blue was three quaters of the way down the gel.

3.18.3 Agarose gels containing 5mM methylmercury hydroxide

	Gel Concentration		
	1%	1.5%	1.8%
Agarose	0.8g	1.2g	1.44g
1x EM buffer	80ml	80ml	80ml
1M methylmercury hydroxide	400µl	400µl	400µl

Table 3.6 Composition of the agarose gels containing methylmercury hydroxide, used for electrophoresis of viral RNAs.

The appropriate quantity of agarose was melted in 1x EM buffer as indicated in Table 3.6. The molten agarose was cooled to 60-62°C before the addition of 400µl of 1M methylmercury hydroxide; every step after and including the addition of methylmercury hydroxide was performed in a fumehood. Horizontal gels were poured in a BRL Horizon 11.14 electrophoresis tank. Each sample was mixed with an equal volume of 2x sample buffer and 1µl of 0.1M methylmercury hydroxide, vortexed briefly and centrifuged for 1 second at 12,000 rpm in a microfuge. The samples were loaded quickly onto the gel to avoid diffusion and nonsubmarine gels were run in 1x EM buffer at 75 V. A peristaltic pump was used to recirculate the buffer between the two reservoirs of the electrophoresis tank.

3.18.4 Polyacrylamide/urea denaturing gels

	Gel Concentration		
	6%	8%	16%
Urea	288g	288g	288g
Acrylamide	34.2g	45.6g	91.2g
Bisacrylamide	1.8g	2.4g	4.8g

Table 3.7 Sequencing gel mixes

Each of the above was dissolved in sterile distilled water to a total volume of 500ml. 20g of Amberlite was added and the solution was stirred for 30 minutes. The gel solution was filtered, 60ml of 10x TBE was added and the volume was adjusted to 600ml. The sequencing gel mixes were stored at 4°C.

These gel mixes were used in the preparation of 0.3mm thick denaturing polyacrylamide/urea gels required for sequence determinations (8% mix); gel purification of 'prime-cut' probes (6% mix) and gel purification of oligonucleotides (16% mix). In each case the gels were prepared with the same relative proportions of sequencing mix, 10% ammonium persulphate and TEMED (100:1:0.1). The total volume of gel required was generally 60ml for full-size sequencing gels while 25ml was sufficent to prepare gels for the purification of oligonucleotides and probes. Pre-electrophoresis of the gels was carried out at 37W for 30 minutes. The sample wells were washed out with electrophoresis buffer (1x TBE) to remove any local concentration of urea which would prevent the samples from settling in the wells. Formamide dyes were added to each sample (1/10th of the sample volume) and the sequencing and 'prime-cut' probe samples were boiled for 2 minutes prior to loading onto the gel. Electrophoresis was carried out at 60W for sequencing gels and at 37W for the smaller denaturing gels for 2-3 hours, or until the bromophenol blue had travelled most of the way down the gel. For direct RNA sequence analysis longer electrophoretic runs were regularly performed inorder to obtain the maximum amount of sequence information from a given primer.

3.18.5 Treatment of gels after electrophoresis

SDS-PAGE gels of [^{35}S]-methionine labelled proteins were fixed overnight. For gels of proteins translated *in vitro* the gel fix solution was changed several times during the first hour to reduce background due to residual free [^{35}S]-methionine in the gel. After fixation, all [^{35}S]-methionine labelled gels were soaked in En^{3}Hance at room temperature with shaking for 50 minutes, and then washed in water for a further 30 minutes. SDS-PAGE gels were dried under vacuum in a Bio-Rad gel drier set at 80°C for 2.5 hours.

Polyacrylamide/urea gels were transferred directly to 3MM paper. Immediately after electrophoresis one of the glass plates was removed and a single sheet of 3MM paper was pressed firmly onto the exposed gel. The paper was slowly peeled away from the plate with the gel attached. The gel was dried onto the 3MM paper under vacuum in a Bio-Rad gel drier set at 80°C for 1 hour.

3.19 Autoradiography and fluorography

Gels of [^{32}P]-labelled material were exposed against Kodak X-Omat film, at room temperature or at -70°C if an intensifying screen was used to enhance the signal. [^{35}S]-methionine labelled gels which had been treated with En^{3}Hance (fluorography) were exposed against Kodak X-Omat film at -70°C.

3.20 Densitometric analysis

Densitometric analysis of autoradiographic images was carried out using a GS 300 Transmittance/Reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco). The relative intensities of individual autoradiographic signals were calculated using the GS 360 data system (IBM).

4 Bunyamwera virus (wtL9BUN): plaque-purification and determination of the consensus population sequence of the genomic S RNA segment

4.1 Production of a triply plaque-purified elite stock of Bunyamwera virus

A triply plaque-purified stock of Bunyamwera virus was prepared from a laboratory stock of virus to provide a pedigreed stock for all future experiments. The laboratory stock was originally plaque-purified in BSC-1 cells from a suckling mouse brain homogenate, and subsequently passaged a number of times in BHK cells (Watret *et al.*, 1985; see Section 3.1.2). Some heterogeneity in plaque size was evident within the laboratory stock of Bunyamwera virus prior to plaque-purification and one of the larger plaques (designated wtL9BUN) was selected. After three successive rounds of plaque-purification, an elite stock of wtL9BUN virus was prepared and titrated. BHK cell monolayers were used for the plaque-purification steps, for growing the elite stock of virus and for plaque-titration of the elite stock. The titre of the elite stock was 2 x 10^7 pfu/ml and working stocks of wtL9BUN virus were prepared from the elite stock by a single low multiplicity passage in BHK cells (see Section 3.10.4).

4.2 Direct RNA sequence determination of the small (S) genomic RNA segment of wtL9BUN virus

To determine how representative the cDNA derived sequence for the small (S) genomic RNA segment of Bunyamwera virus (Elliott, 1989b) was of the consensus population sequence, the genomic S RNA segment of wtL9BUN virus was sequenced directly, by primer extension of γ-[^{32}P]-labelled oligonucleotides in the presence of dideoxynucleoside triphosphates (see Section 3.17). On the basis of the cDNA derived sequence, six 20mer oligonucleotides complementary to regions of Bunyamwera virus genomic S RNA were synthesised for use as primers (see Figure 4.1). The priming sites were spaced at intervals of between 130 and 150 nucleotides along the S RNA, and up to 200 bases of sequence could be read from each primer. Nucleotide positions are numbered with respect to the positive-sense RNA, thus corresponding directly to the numbering of the cDNA (Elliott, 1989b). The consensus population sequence agreed with the cDNA derived sequence although the detection of adenosine alongside uridine at position 923 within the 3' non-coding region of the positive-sense S RNA suggested that there might be some heterogeneity within the virus preparation (see Figure 4.2). Since this nucleotide substitution was within the 3' non-coding region and outside the conserved complementary terminal sequences of Bunyamwera virus S RNA (Elliott, 1990) it was unlikely to be of consequence. Its presence does however illustrate the difficulty in obtaining completely

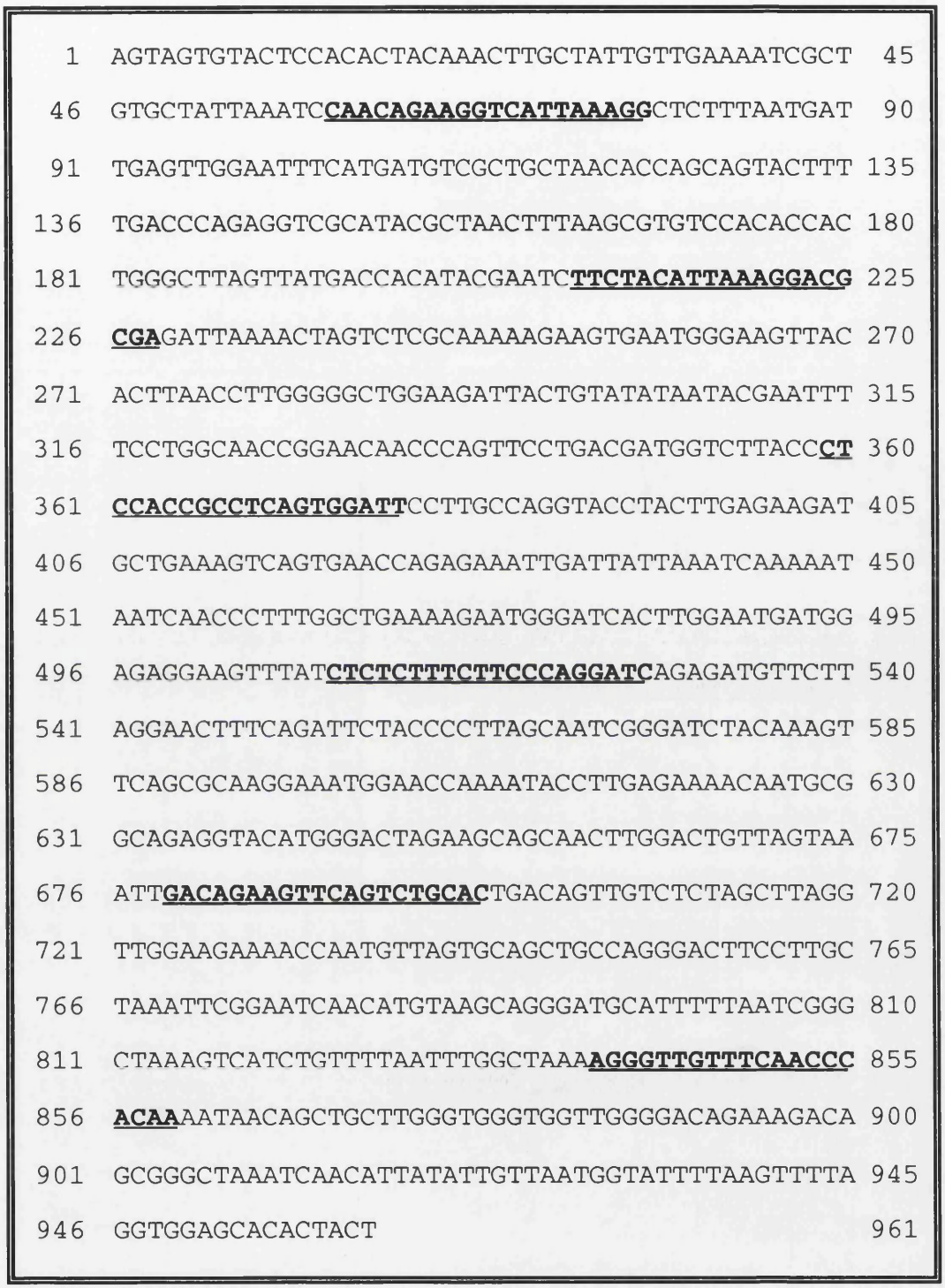

Figure 4.1. The cDNA sequence of the S RNA segment of Bunyamwera virus presented as the complementary positive RNA strand, written as DNA (from Elliott, 1989b). The positions of the oligonucleotides used for direct RNA sequencing are shown in **bold** and underlined.

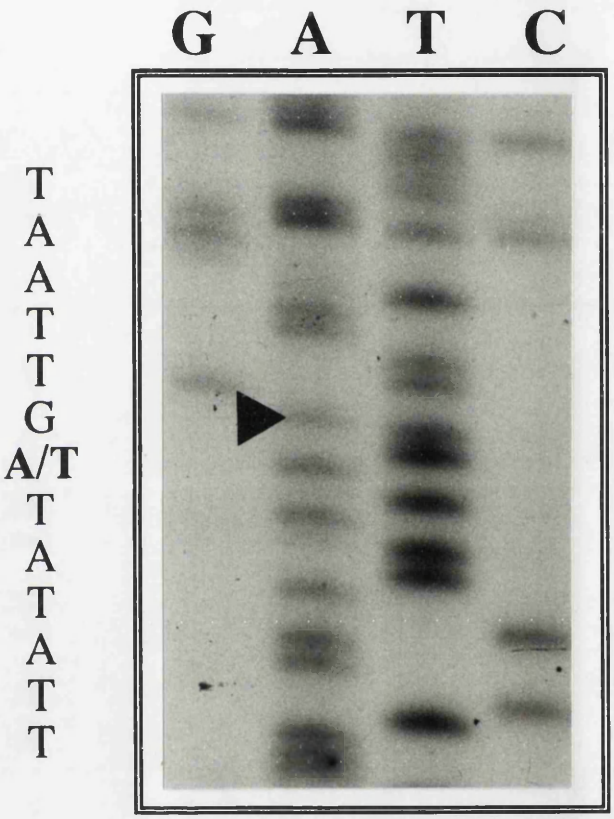

Figure 4.2. Direct RNA sequencing of the genomic S RNA segment of wtL9BUN virus by primer extension from oligonucleotide number 6. The sequence shown is from nucleotide 908 to 939. The sequence from nucleotide 917 to 929 is highlighed, with the nucleotide heterogeneity at position 923 shown in **bold**.

homogeneous preparations of RNA viruses due to the inherent high mutation rate associated with their encoded RNA polymerases (see Section 2.2.1).

In the course of determining the cDNA sequence of Bunyamwera virus genomic S RNA, discrepancies were encountered between the sequence of the largest cDNA clone (pBUN S14) and three other cDNA clones (pBUN308, pBUN309 and pBUN3/59) at nucleotide positions 378 and 379 (Elliott, 1989b; see Figure 4.3). The pBUNS14 cDNA contained guanosine and thymidine at nucleotide positions 378 and 379, which created codons for cysteine at amino acid position 98 of the N protein and valine at amino acid position 92 of the NSs protein respectively. Earlier radiolabelling studies on Bunyamwera virus-infected cells had indicated that the N protein was deficient in cysteine residues (Elliott, 1985), and four overlapping cDNAs (pBUN308, pBUN309 and pBUN3/59) contained thymidine at nucleotide position 378 and cytidine at nucleotide position 379, coding for phenylalanine and serine at amino acid positions 98 and 92 in Bunyamwera virus N and NSs proteins respectively. Derivation of the consensus population sequence of wtL9BUN virus S RNA, by primer extension sequencing on virion RNA, confirmed the presence of uridine (thymidine in the cDNA) and cytidine at nucleotide positions 378 and 379, respectively (see Figure 4.4).

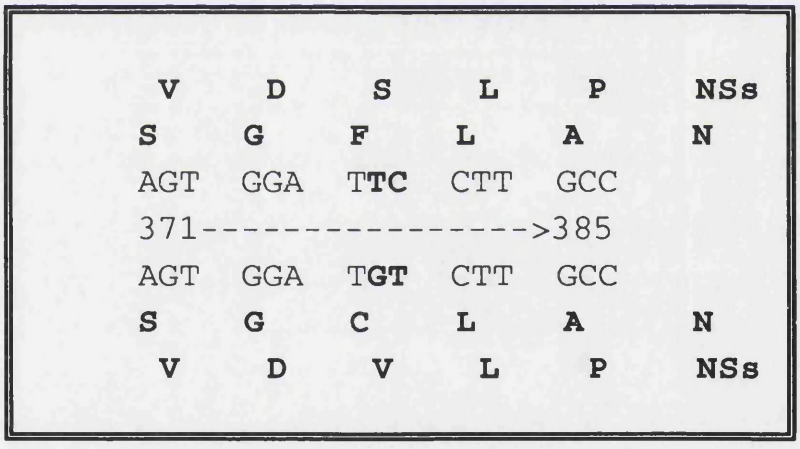

Figure 4.3 Differences between the sequences of the cDNA clone pBUN S14 and the cDNA clones pBUN308, pBUN309 and pBUN3/59 used to sequence Bunyamwera virus S segment RNA (Elliott, 1989b). ------> represents positive-sense Bunyamwera virus S RNA, nucleotides 371-385; the upper half of the figure shows the nucleotide sequence and the deduced amino acid sequences obtained from clones pBUN3/59, pBUN308 and pBUN309; the lower half of the figure shows the corresponding sequences obtained from the cDNA clone pBUN S14 (figure adapted from Elliott, 1989).

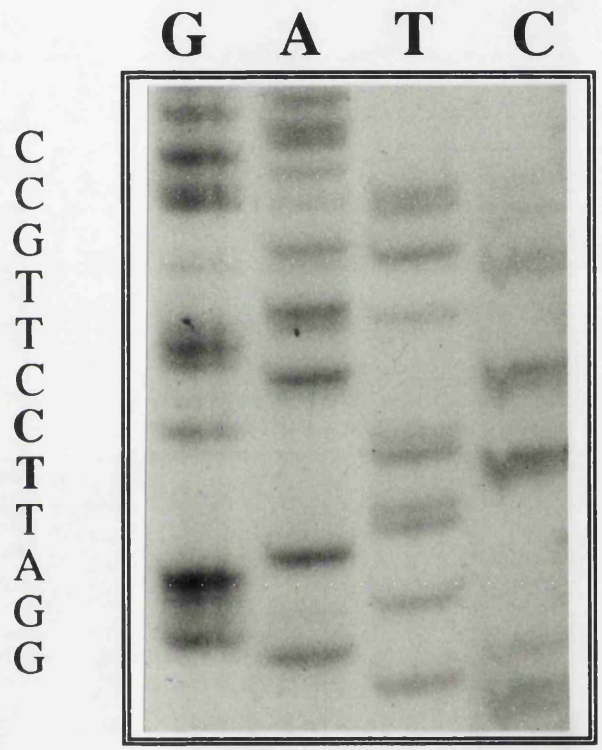

Figure 4.4. The consensus population sequence of the genomic S RNA segment of wtL9BUN virus, from nucleotide 367 to 393. The sequence between nucleotide 374 and 385 is highlighted with the **T** at position 378 and **C** at position 379 shown in **bold**.

4.3 Summary

Determination of the consensus sequence of the genomic S RNA segment of the wtL$_9$BUN virus preparation confirmed that this triply plaque-purified stock of Bunyamwera virus was homogeneous. The only sequence heterogeneity detected was at nucleotide position 923 within the 3' non-coding region of the viral complementary-sense S RNA. The consensus sequence of the genomic S RNA segment of the wtL$_9$BUN virus preparation was otherwise identical to the published cDNA derived sequence (Elliott, 1989b). A discrepancy between the sequence of the cDNA clone pBUNS14 and three other cDNA clones from which the Bunyamwera virus S sequence was derived, was resolved by primer extension sequencing on virion RNA. It appeared that pBUNS14 may have represented a minor variant in the RNA population, or may have arisen due to a reverse transcriptase error during cDNA synthesis.

5 Comparative study of macromolecular syntheses in Bunyamwera virus infected *Aedes albopictus* C6/36 and BHK cells

The patterns of viral protein and RNA syntheses leading to cell death in Bunyamwera virus-infected BHK cells, and to inapparent, persistent infection in *Aedes albopictus* C6/36 (C6/36) cells were compared by SDS-PAGE analysis of [^{35}S]-methionine pulse-labelled infected cell extracts and Northern analysis of infected cell RNA respectively. Infections were initiated with 5 pfu/cell, using the same stock of virus (wtL$_9$BUN virus), and viral protein synthesis and RNA accumulation were followed for 72 hours post infection (h pi).

5.1 Protein synthesis in Bunyamwera virus-infected BHK and C6/36 cells

Infected BHK and C6/36 cells were pulse-labelled for one hour with [^{35}S]-methionine at intervals over 72 h post infection. The pattern of protein synthesis observed in each infected cell type was strikingly different (Figure 5.1). In BHK cells host protein synthesis diminished drastically between 12 and 16 h pi such that, by 20 h pi the viral L, G1, N and NSs proteins (first detected 6 to 8 h pi) were virtually the only proteins detected. Between 16 and 24 h pi viral protein synthesis continued at high levels in the absence of significant host protein synthesis. By 36 h pi only very low levels of viral protein synthesis were detectable during a one hour pulse with [^{35}S]-methionine. The G2 and NSm proteins, which are not reproducibly detected by SDS-PAGE (Elliott, 1985), were not identified in this experiment. [It has subsequently been discovered that G2 is heat-labile and can be detected by not boiling the Bunyamwera virus-infected cell extracts prior to loading SDS-PAGE gels (R.M. Elliott, personal communication)].

In C6/36 cells there was little decrease in host protein synthesis, and only G1, N and NSs could be identified above host background. Overall, Bunyamwera virus infection proceeded more slowly in C6/36 cells. G1 was detected between 16 and 36 h pi, N between 8 and 24 h pi (weakly at 36 h pi) and NSs between 12 and 24 h pi. While G1 and N were detectable at comparable levels in both cell types, the level of NSs was much lower in C6/36 cells relative to BHK cells. No viral proteins were detected after 36 h pi in the C6/36 cells and host protein synthesis continued at a slightly reduced rate. Thus, against a background of continuing host protein synthesis, Bunyamwera virus protein synthesis was down-regulated. Although comigrating host bands made it difficult to determine the precise onset of synthesis of L and G1, the synthesis of N and NSs did appear to preceed that of G1, particularly in C6/36 cells. This illustrates a degree of temporal control of bunyavirus induced polypeptide synthesis as first reported by Pennington *et al.* (1977).

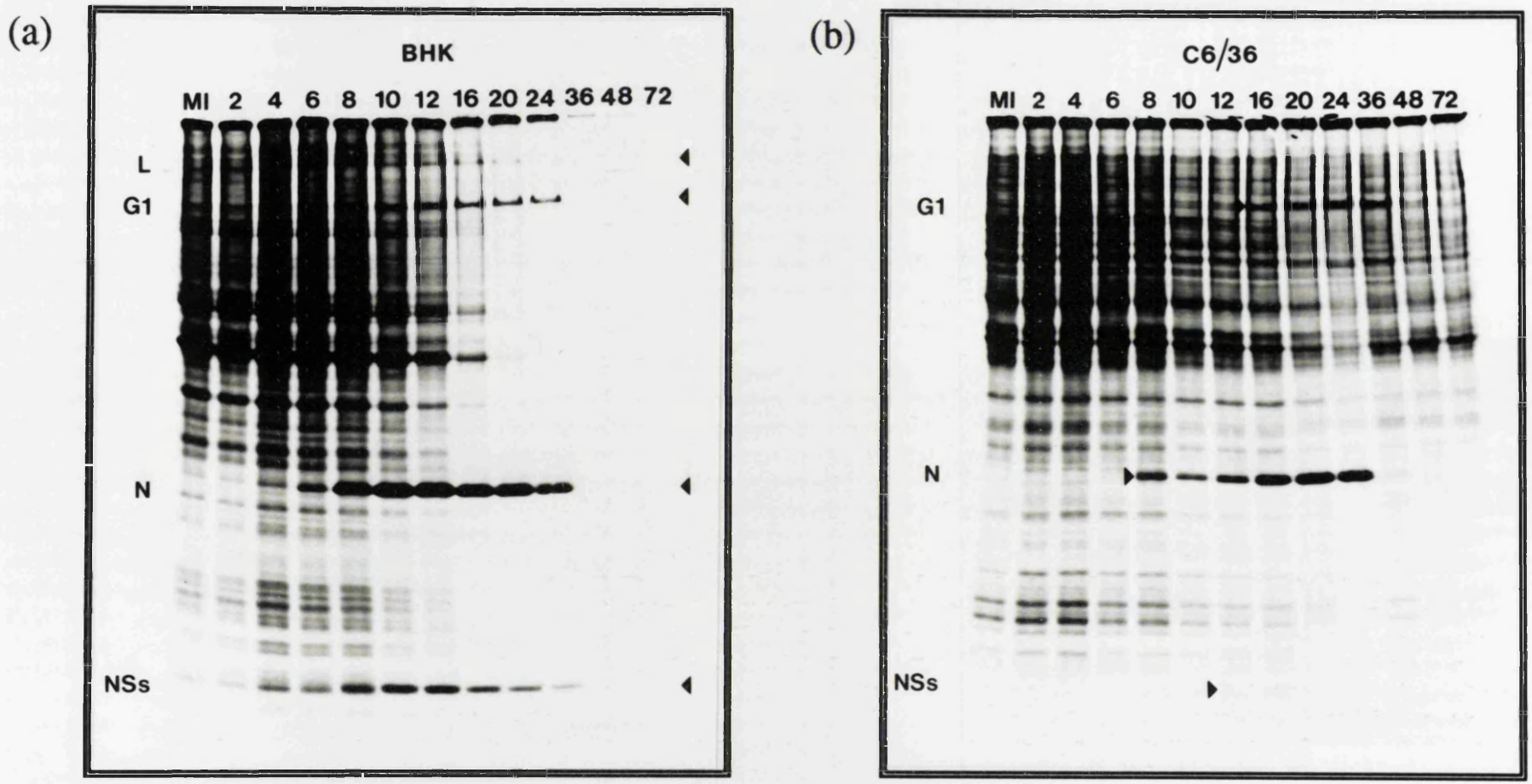

Figure 5.1. Protein synthesis in Bunyamwera virus-infected (a) BHK and (b) C6/36 cells. Cells were infected with 5 pfu/cell of Bunyamwera virus, and the infected cultures were pulse-labelled for 1 h with [^{35}S]-methionine at the times indicated (h pi). MI indicates mock infected cell lysate. Cell lysates were fractionated on 15% SDS-PAGE gels. The positions of the viral proteins L, G1, N and NSs are indicated.

5.2 S segment mRNA levels in infected C6/36 cells

Slot-blot hybridization was employed to measure the steady-state levels of Bunyamwera virus S mRNA (BUN S mRNA) in infected C6/36 cells, over 72 h pi, to determine if the decline in viral protein synthesis observed in these cells after 24 h pi was due to altered levels of viral mRNAs. Bunyavirus-infected cells contain both encapsidated and unencapsidated viral RNA species. The former are the viral nucleocapsids in which, usually, full-length genomic and antigenomic RNAs are complexed with the viral N protein, while the latter consist of the viral mRNAs. Because viral mRNA and antigenomic RNA are the same sense, they must be separated before mRNA levels can be determined. These populations are readily separated (fractionated) on the basis of their different buoyant densities in CsCl gradients (Leppert *et al.*, 1979; Rossier *et al.*, 1988; see Section 3.15.2). Encapsidated viral RNA species form a sharp band in the middle of the gradient while the remainder of the infected-cell RNA including the viral mRNAs pellet.

Slot blot analysis of the pellet RNA fraction from Bunyamwera virus-infected C6/36 cells (Figure 5.2) revealed that significant levels of Bunyamwera virus S mRNA were present in the infected C6/36 cells beyond 36 h pi, when N protein synthesis could no longer be detected by [^{35}S]-methionine pulse-labelling *in vivo* (Figure 5.1). Maximum levels of S mRNA were detected 24 to 48 h pi in the C6/36 cells by slot-blot analysis and significant levels were detected up to the last time point tested (72 h pi; Figure 5.2). The presence of apparently intact S mRNA, up to 72 h pi in infected C6/36 cells, was confirmed by Northern blot analysis of pellet RNA extracted during a separate experiment (Figure 5.3).

In order to preserve the nucleocapsid structures so that encapsidated and unencapsidated viral RNA species could be isolated separately, cytoplasmic extracts were prepared using a relatively mild procedure based on detergent disruption of infected cells. Examination of a number of Northern blots of pelleted RNAs from both infected cell types revealed that the viral mRNA species, particularly the infected C6/36 cell derived viral mRNAs were susceptible to nuclease degradation during this extraction procedure. The greater susceptibility of C6/36 cell derived viral mRNAs to degradation suggested that C6/36 cells may be richer in ribonucleases, or that the viral mRNAs synthesised in the two cell types differ in their susceptibility to nuclease degradation, possible due to the nature of their host derived primers. The relatively poor detection of C6/36 cell derived BUN S mRNA by Northern analysis, compared to the strong positive signals obtained by slot-blot hybridization (using the same probe; see Figures 5.2 and 5.3), may be explained, at least partly, by the fact that slot-blot hybridization does not rely on the complete integrity of the target RNA for its detection. This may also have contributed to the different patterns of RNA accumulation observed by the slot blot and Northern blot analyses detailed above (Figures 5.2 and 5.3 respectively); although some variation between two separate time-course RNA extractions might be expected.

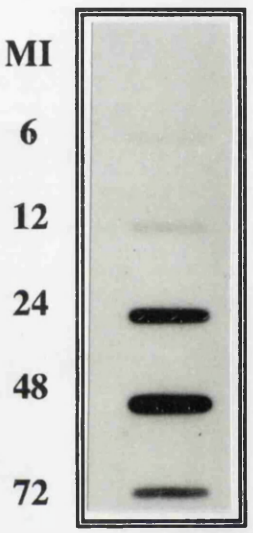

Figure 5.2. Slot-blot analysis of the pellet RNA fraction from Bunyamwera virus infected C6/36 cells. 12μg of CsCl pellet RNA, extracted at the indicated h pi, was immobilized onto nitrocellulose and hybridized with a [^{32}P]-labelled cDNA probe (S9) specific for positive-sense Bunyamwera virus S RNA.

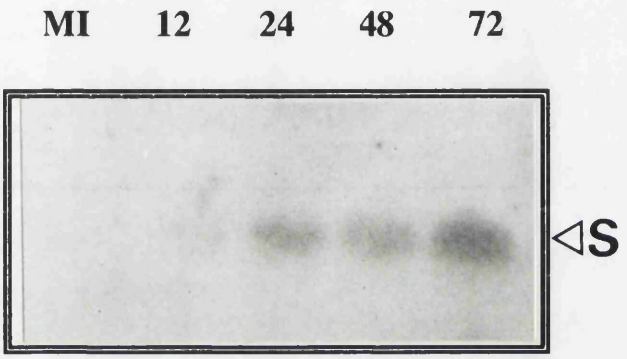

Figure 5.3. Northern blot analysis of the pellet RNA fraction from Bunyamwera virus-infected C6/36 cells. 20μg of CsCl pellet RNA, extracted at the indicated h pi, was fractionated in a 1% agarose gel containing 5mM methylmercury hydroxide and blotted to a nitrocellulose filter. The filter was hybridized with a [^{32}P]-labelled cDNA probe (S9) specific for positive-sense Bunyamwera virus S RNA.

* Assuming that a similar ratio of antigenomic S RNA to S mRNA exists in Bunyamwera virus infected BHK cells.

It was clear, from the slot blot and Northern blot analyses, that the decline in virus protein synthesis observed between 24 and 36 h pi in the C6/36 cells was not paralleled by a decline in S mRNA levels. Interestingly, these observations are similar to those reported by Rossier *et al.* (1988) who detected maximum levels of La Crosse bunyavirus S mRNA in infected C6/36 cells at 24 h pi; although levels of S mRNA remained relatively high up to 80 h pi, N protein synthesis could not be detected after 24 h pi by pulse-labelling of infected cells (Rossier *et al.*, 1988).

5.3 *In vitro* translation

Total cell RNA, prepared from Bunyamwera virus-infected BHK and C6/36 cultures at time points up to 72 h pi, was used to programme *in vitro* translation reactions (see Section 3.16.5 and Figure 5.4). Maximum levels of translation were achieved with RNA extracted from BHK cells at 12 h pi and from C6/36 cells at 24 h pi, reflecting the slower progress of Bunyamwera virus infection in C6/36 cells. Virtually no host cell protein translation was obtained with RNA samples prepared from infected BHK cells after 12 h pi. The decrease in host cell protein translation *in vitro* paralleled the decline in host cell protein synthesis observed *in vivo* (see Figure 5.1a) and can be attributed to virus-induced mRNA instability in these cells (Raju & Kolakofsky, 1988).

Fractionated RNA preparations from Bunyamwera virus-infected BHK cells were also used to programme *in vitro* translation reactions (Figure 5.5). High levels of translation were achieved *in vitro* with CsCl pellet RNA extracted from BHK-21 cells up to 24 h pi, but little or no translatable free S mRNA was evident in the 48 and 72 h pi samples (Figure 5.5a). At all time points tested low levels of translation were observed from nucleocapsid derived RNA from Bunyamwera virus-infected BHK cells (Figure 5.5b). Raju & Kolakofsky (1987b) reported that approximately 2% of La Crosse bunyavirus mRNAs were encapsidated in infected BHK cells, and Bunyamwera virus S mRNA could be detected as early as 12 h pi amongst the encapsidated RNA population in infected BHK cells by Northern blot analysis (Figure 5.6). The ratio of La Crosse virus S mRNA to antigenomic S RNA in infected BHK cells can be estimated from available data to be 35:1 (Rossier et al., 1986; 1988). Comparison of the levels of antigenomic S RNA and S mRNA in the banded fractions displayed in Figure 5.6 suggests that a similar proportion of Bunyamwera virus mRNAs was encapsidated during infection of BHK cells. The ratio of S mRNA to antigenomic S RNA in the encapsidated RNA fraction from Bunyamwera virus infected BHK cells remained constant up to 72 h pi. It is probable therefore that the low levels of translation observed *in vitro* with nucleocapsid-derived RNA extracted from Bunyamwera virus BHK cells (Figure 5.5b) resulted from translation of the small proportion of viral messages which were encapsidated in these cells.

A slot blot hybridization was performed to estimate the levels of positive-sense S RNA in each of the infected-BHK cell samples used in the *in vitro* translation reactions

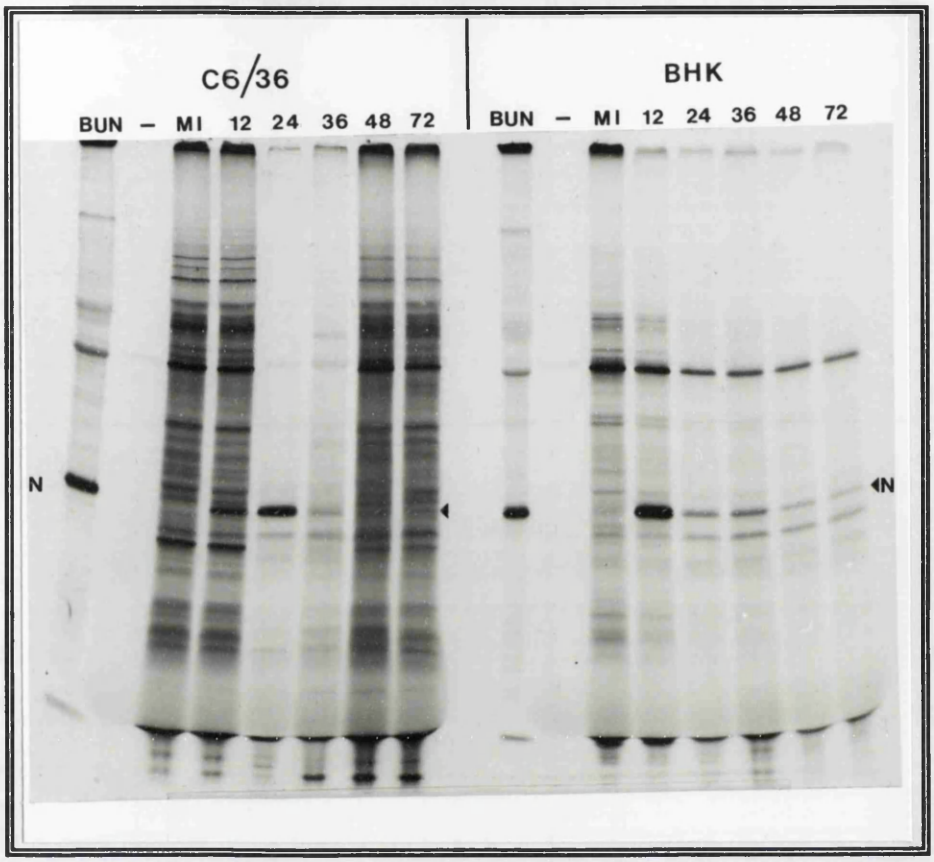

Figure 5.4. 2μg of total cell RNA, extracted from Bunyamwera virus-infected C6/36 and BHK cells at the indicated h pi, was translated *in vitro* using the NEN rabbit reticulocyte lysate. BUN, marker track of [^{35}S]-methionine-labelled, infected BHK cell extract, prepared at 18 h pi; -, control reaction without added RNA; MI, mock-infected cell RNA. The viral N protein is indicated; in this gel NSs was obscured by the globin band.

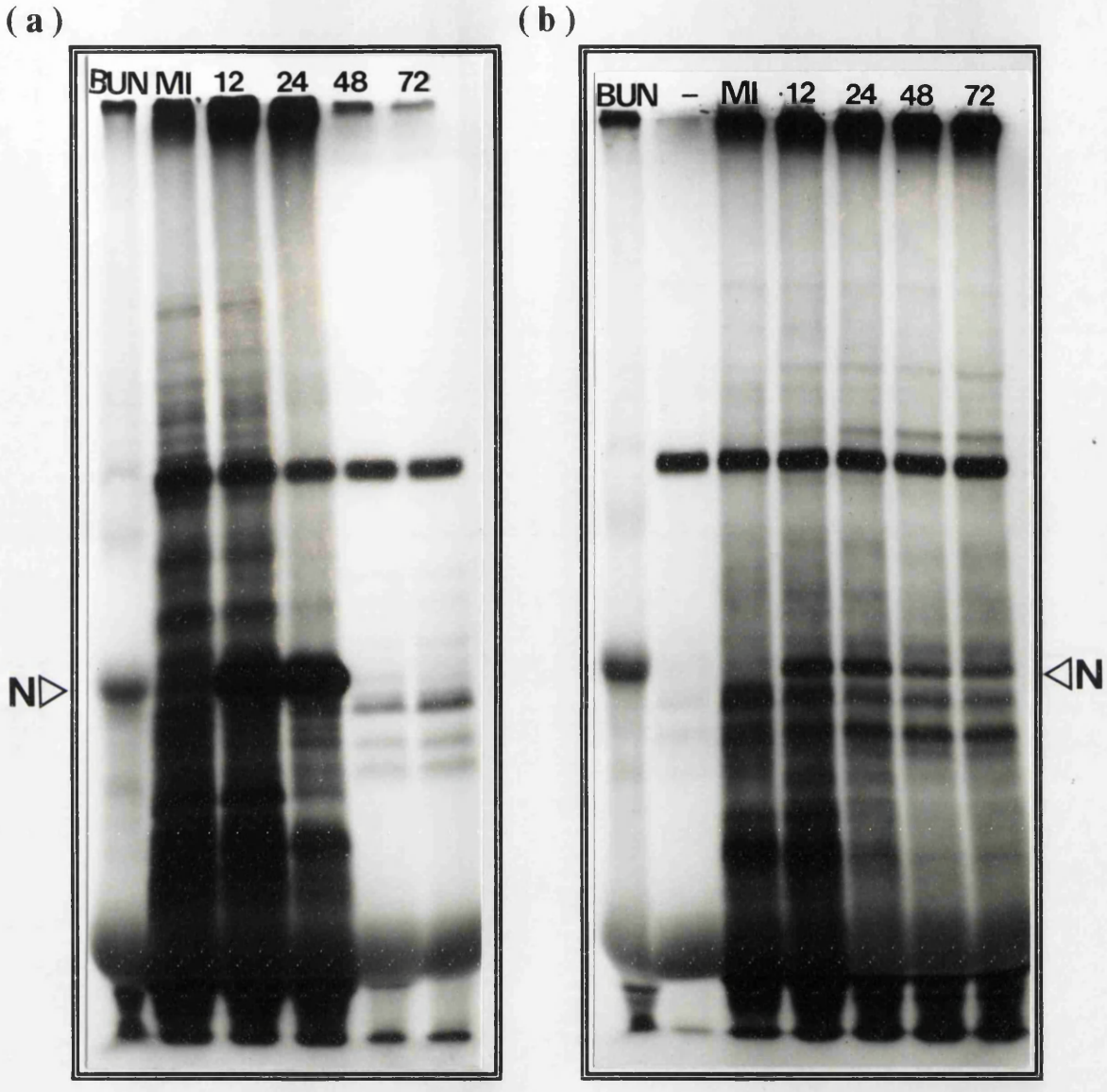

Figure 5.5. (a) 1.5µg of CsCl pellet RNA and (b) 1.5µg of nucleocapsid-derived RNA, extracted from Bunyamwera virus-infected BHK cells at the indicated h pi, were translated *in vitro* using the NEN rabbit reticulocyte lysate. BUN, marker track of [^{35}S]-methionine-labelled, infected BHK cell extract, prepared at 18 h pi; MI, mock-infected cell RNA. The viral N protein is indicated; in these gels NSs was obscured by the globin band.

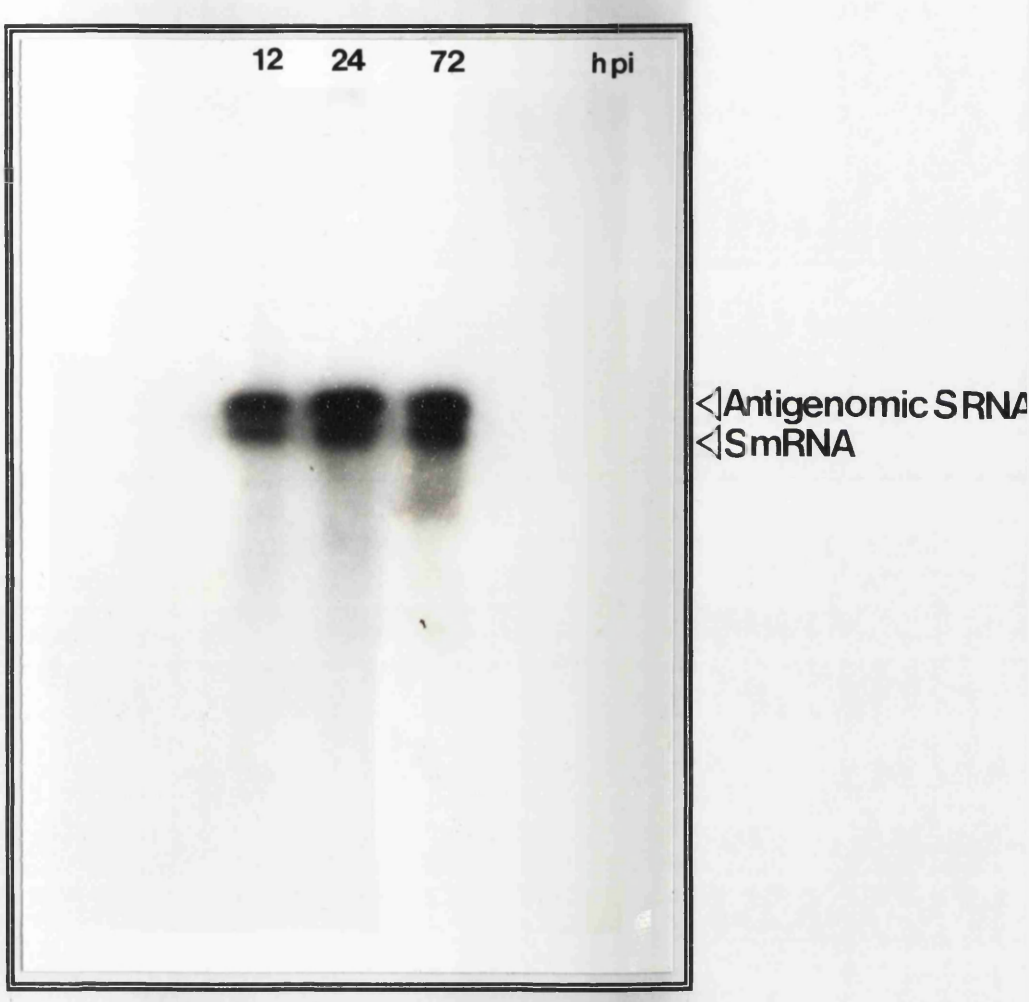

Figure 5.6. Northern blot of nucleocapsid-derived RNA extracted from Bunyamwera virus-infected BHK cells at the indicated h pi. 10μg of RNA was fractionated on a 1.8% agarose gel, containing 5mM methylmercury hydroxide, and transferred to a nitrocellulose filter. The filter was probed for positive-sense Bunyamwera virus S RNA using the [^{32}P]-labelled cDNA probe S9.

(data not shown)
described above. The 48 and 72 h pi banded RNA samples contained four-fold more positive-sense S RNA than the earlier time point samples yet, while N protein was translated from RNA samples extracted up to 72 h pi, the levels translated from RNA extracted from 48 h onwards were 50% lower than at the 12 and 24 h pi time points. It was also clear that the 12 h pi pelleted RNA sample from BHK cells and the 24 h pi banded RNA sample from the same cells contained the same amount of positive-sense S RNA, yet more N protein (greater than ten-fold more) was translated from the pelleted RNA than from the banded RNAs. It appeared therefore that infected-cell derived S antigenomic RNA was translated poorly (if at all) *in vitro* compared to S mRNA. In contrast, *in vitro* translation from the equivalent of full-length positive-sense Bunyamwera virus S RNA, transcribed from a full-length cDNA clone, has been achieved in our laboratory (R. M. Elliott, personal communication).

The levels of host protein translation observed *in vitro* with total cell RNA prepared at 12, 48 and 72 h pi from Bunyamwera virus-infected C6/36 cells were comparable to the levels seen with RNA extracted from mock infected C6/36 cells (Figure 5.4), and were similar to the levels of host protein synthesis observed by pulse-labelling *in vivo* at the same time points. However, host protein translation *in vitro* diminished drastically between 12 and 24 h pi and remained low at 36 h pi. This was in marked contrast to the *in vivo* situation, where no significant decrease in host cell protein synthesis was observed during the course of infection (see Figure 5.1a). It is conceivable that the relatively high ratio of viral to host protein translation observed *in vitro* with the 24 h pi RNA sample represents the true ratio of viral to host mRNAs in Bunyamwera virus infected C6/36 cells at this time. Competition between viral mRNAs (at their maximum level) and host mRNAs for the limited components of the *in vitro* translation reaction could result in the low levels of host protein translation observed. The lower ratio of viral to host proteins synthesised *in vivo* at 24 h pi in infected C6/36 cells may be a consequence of a translational block imposed selectively on viral mRNAs. The absence of this block in the *in vitro* translation reactions suggests that it is removed by the RNA extraction procedure and may be proteinaceous. The possibility that a transient mRNA instability was induced in these cells by the virus infection was not supported by the *in vivo* pulse-labelling data (see Figure 5.1).

Translatable S mRNA was present in infected C6/36 cells up to the last time point tested (72 h pi; see Figure 5.4) providing further evidence that the decline in viral N protein synthesis observed after 24 h pi *in vivo* (Figure 5.1) could not be explained solely by the absence of translatable S mRNA. However, at later time points (48 and 72 h pi), the levels of N protein synthesised *in vitro* were not representative of the levels of S mRNA estimated to be present by slot blot and Northern blot analyses; Figures 5.2 and 5.3). Judging from the ratio of viral to host proteins translated *in vitro*, the virus specific mRNAs did not appear to compete as successfully with the host cell mRNAs for translation as they had

earlier in infection. This suggested that there may also be some alteration in the viral mRNA molecules synthesised later in infection in Bunyamwera virus-infected C6/36 cells which affects their translatability.

Attempts were made to prepare intact fractionated RNA samples from Bunyamwera virus-infected C6/36 cells which could be used to programme *in vitro* translation reactions. Vanadylribonucleoside complexes (VRCs; transition state analogues that bind to and inhibit many RNases; Berger & Birkenmeier, 1979) were added to the detergent-based lysis buffer, and were maintained at a concentration of 10mM through all stages of extraction and purification of the RNAs. Five μg (12% of the total yield) of the pellet RNA fraction and an equal proportion of the total yield of nucleocapsid-derived RNA, extracted from Bunyamwera virus-infected C6/36 cells at 48 h pi, were translated *in vitro* using the NEN rabbit reticulocyte lysate. The results suggested that by 48 h pi a significant amount of translatable Bunyamwera virus S mRNA was encapsidated in Bunyamwera virus-infected C6/36 cells (Figure 5.7).

5.4 Accumulation of viral RNA species in Bunyamwera virus infected *Aedes albopictus* C6/36 and BHK cells

The accumulation of positive-sense viral RNA species in Bunyamwera virus-infected C6/36 and BHK cells was compared by Northern analysis of total cell RNA (see Section 3.16.2) and of fractionated RNA samples (see Section 3.15.2) prepared from infected cultures at intervals up to 72 h pi. Hybridization was carried out with [^{32}P]-labelled cDNA probes (L7, M6 and S9) specific for positive-sense Bunyamwera virus L, M and S RNA species, respectively. The size differences between full-length L and M RNA species and their respective subgenomic mRNAs were not sufficient to resolve these RNAs by electrophoresis, but Bunyamwera virus S mRNA could be separated from antigenomic S RNA by electrophoresis in 1.5% denaturing agarose gels (see later). Each set of filters containing C6/36 and BHK cell derived RNA samples from parallel time courses were hybridized at the same time, using the same probes so that direct comparisons of the relative levels of each viral RNA species at a given time point during the lytic and persistent infections could be made. However, the specific activities of the segment specific probes used were not determined and therefore interpretations regarding the absolute ratio of the individual RNA segments within any one sample could not be made.

Northern analysis of total cell RNA revealed different patterns of viral RNA accumulation in the two cell types (Figure 5.8). In infected BHK cells, positive-sense Bunyamwera virus L, M and S RNA species were detected at 12 h pi and accumulated, in parallel, up to 48 h pi. Between 48 and 72 h pi levels of each of the positive-sense RNA species declined slightly. In infected C6/36 cells, positive-sense viral RNA species were not detected until 24 h pi. The levels of each of the positive-sense viral RNA species detected at 24 h pi represented the maximum levels reached, since no further accumulation

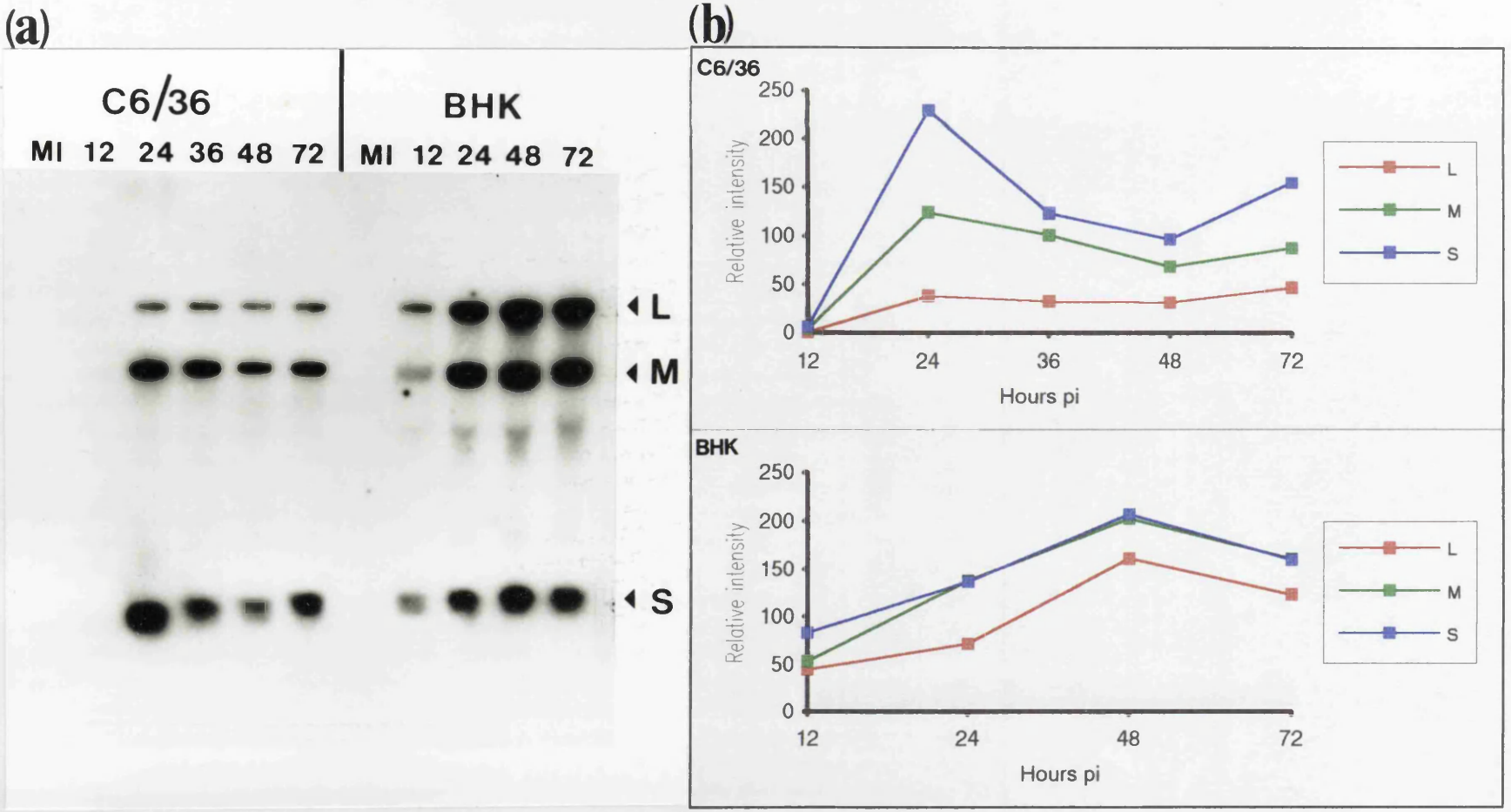

Figure 5.8. Positive-sense viral RNA synthesis in Bunyamwera virus-infected C6/36 and BHK cells: (a) 10μg of total cell RNA, extracted from Bunyamwera virus-infected C6/36 and BHK cells at the indicated h pi was fractionated on a 1.0% agarose gel containing 5mM methylmercury hydroxide and transferred to a nitrocellulose filter. The filter was hybridized with a mixture of [^{32}P]-labelled cDNA probes, L7, M6 and S9, specific for Bunyamwera virus positive-sense L, M and S RNA species, respectively; (b) Graphs showing the pattern of accumulation of each of the positive-sense viral RNA species in Bunyamwera virus infected C6/36 and BHK cells as determined by densitometric analysis of the Northern blot shown in (a).

of viral RNAs was observed in the C6/36 cells. The ratio of Bunyamwera virus L, M and S RNA segments synthesised in the two cell types differed; relatively more L and M RNA compared to S RNA was synthesised in BHK cells. Notably, the absence of viral RNA accumulation in the infected C6/36 cells, beyond 24 h pi, coincided with the downregulation of viral protein synthesis observed in these cells (see Section 5.1).

Northern analysis of fractionated RNA samples from both cell types facilitated a more detailed comparison of RNA accumulation in the two systems. Separate analysis of encapsidated and unencapsidated viral RNA species enabled viral RNA replication and mRNA transcription to be considered independently. Despite the problems encountered with ribonuclease digestion of the pellet RNA fraction from infected C6/36 cells (see Section 5.2), all three size classes of Bunyamwera virus mRNA were detected in the CsCl pellet RNA fraction from these cells up to late times post infection (albeit at low levels). Figure 5.3 illustrates that S mRNA was present in these cells up to 72 h pi, while Figure 5.9 shows the upper portion of another Northern blot demonstrating that L and M segment mRNAs were also present in these cells up to 72 h pi.

All three mRNA species were detected in the CsCl pellet RNA fraction from infected BHK cells (Figure 5.10). Maximum levels of S mRNA were present at 12 h pi and declined thereafter as would be expected as a result of the general mRNA instability induced in these cells after bunyavirus infection (Raju & Kolakofsky, 1988). Accumulation of the L and M mRNA species lagged behind accumulation of the S mRNA; L and M mRNAs were first detected at 12 h pi, whereas S mRNA first became detectable at 6 h pi. After maximum levels of each of the mRNA species had been reached (12 h pi for Bunyamwera virus S mRNA and 24 h pi for the L and M mRNAs, respectively), S mRNA levels declined rapidly, while levels of the L and M mRNAs decreased more slowly. These results are similar to those reported by Rossier *et al.* (1988) regarding La Crosse virus replication in BHK cells. Although, because La Crosse virus replicates more rapidly than Bunyamwera virus, viral mRNAs were detected earlier during the La Crosse virus infection, and a time interval between the attainment of maximum levels of S mRNA and L and M mRNAs was not observed. The higher multiplicity of infection used by Rossier and co-workers (20 to 50 pfu/cell compared to the 5 pfu/cell used in the work presented here) may have contributed to the differences observed. Figure 5.11 shows a Northern blot of the CsCl pellet RNA fraction from Bunyamwera virus-infected BHK cells, probed for positive-sense Bunyamwera virus S RNA species only. It is interesting to note that at 48 and 72 h pi, when S mRNA levels were low, the slower migrating, full-length antigenomic S RNA was detected in the pellet RNA fraction from infected BHK cells. There is some evidence to suggest that encapsidation of antigenomic RNA occurs concurrently with its synthesis (Raju & Kolakofsky, 1987a; Hacker *et al.*, 1989). If this is the case, the presence of antigenomic S RNA in the pellet RNA fraction suggests that insufficient N

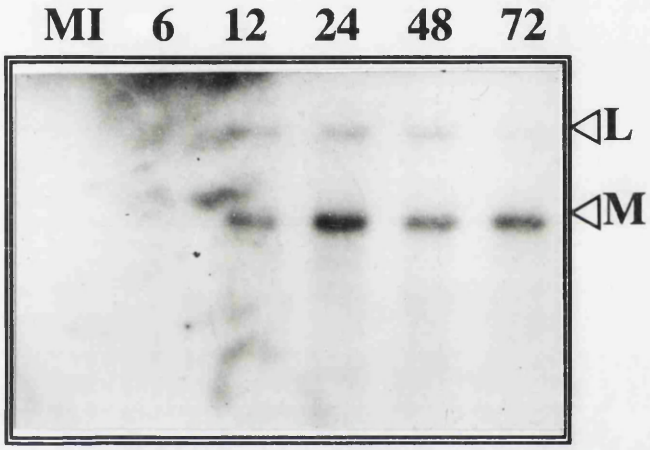

Figure 5.9. Unencapsidated positive-sense L and M RNA species in Bunyamwera virus-infected C6/36 cells up to 72 h pi. 40μg of CsCl pellet RNA, prepared from Bunyamwera virus-infected C6/36 cells at the indicated h pi, was fractionated on a 1.8% agarose gel containing 5mM methylmercury hydroxide and blotted to a nitrocellulose filter. Bunyamwera virus positive-sense L and M RNA species were detected using the [^{32}P]-labelled cDNA probes, L7 and M6 respectively.

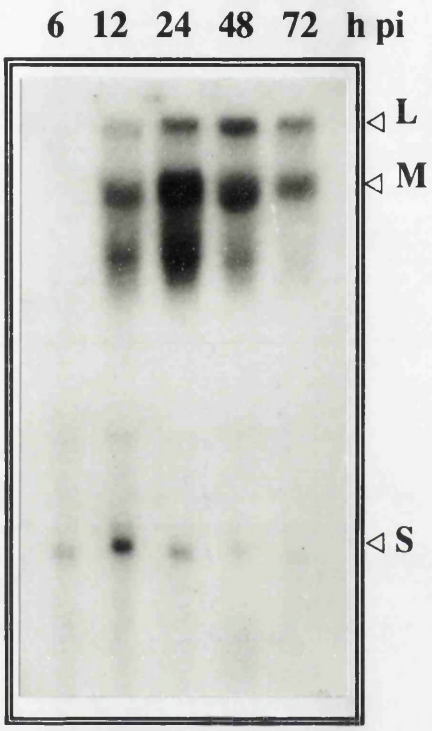

Figure 5.10. Unencapsidated positive-sense viral RNA species in Bunyamwera virus-infected BHK cells up to 72 h pi. 40μg of CsCl pellet RNA, prepared from Bunyamwera virus-infected BHK cells at the indicated h pi, was fractionated on a 1.8% agarose gel containing 5mM methylmercury hydroxide and blotted to a nitrocellulose filter. The filter was hybridized with a mixture of [^{32}P]-labelled cDNA probes, L7, M6 and S9, specific for Bunyamwera virus positive-sense L, M and S RNA species, respectively.

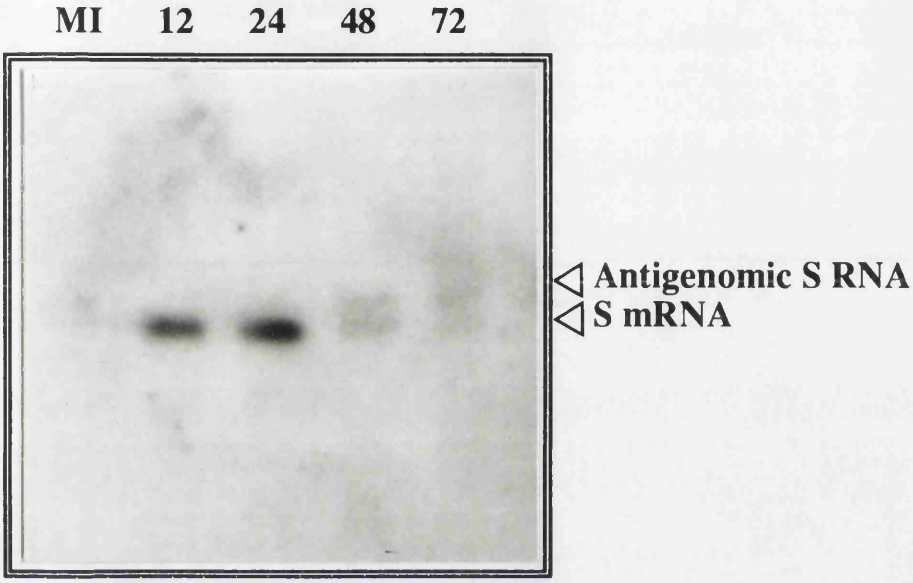

Figure 5.11. Northern blot of CsCl pellet RNA prepared from Bunyamwera virus-infected BHK cells at the indicated h pi. 10μg of RNA was fractionated on a 1.8% agarose gel, containing 5mM methylmercury hydroxide, and transferred to a nitrocellulose filter. The filter was probed for positive-sense Bunyamwera virus S RNA using the [^{32}P]-labelled cDNA probe S9.

protein is present in Bunyamwera virus-infected BHK cells at 48 h pi to fully encapsidate all of the antigenomic S RNA synthesised at that time.

Nucleocapsid-derived RNAs from both infected cell types remained intact throughout the isolation procedure (see Figure 5.12). The encapsidation of bunyavirus RNAs by N protein has been demonstrated to protect the RNA against ribonuclease digestion. Hacker *et al.* (1989) demonstrated that bunyavirus nucleocapsids are resistant to concentrations of RNase A far in excess of those required to completely digest viral mRNAs. During lytic infection in BHK cells, Bunyamwera virus RNA replication continued up to 48 h pi. After 48 h pi, levels of positive-sense L and M RNA declined slowly. However, positive-sense Bunyamwera virus S RNA continued to be encapsidated in the BHK cells between 48 and 72 h pi (see Figure 5.12). By 48 h pi, host protein synthesis had been completely abolished in these cells, viral protein synthesis was barely detectable (see Figure 5.1) and the cells had begun to display cpe. The appearance of antigenomic S RNA in the pellet RNA fraction from infected BHK cells at 48 h pi (see Figure 5.11) suggested that levels of unassembled N protein may be low in these cells by 48 h pi. This was to be expected given that levels of S mRNA were low in the BHK cells at this time. Under conditions where levels of N protein are limiting, the L, M and S segment RNAs would have to compete for the available unassembled N protein. Smaller RNAs would be encapsidated more rapidly, and hence the S segment RNA would be expected to be preferentially assembled into stable nucleocapsid structures later in infection in BHK cells. In contrast, viral RNA replication declined relatively early during Bunyamwera virus infection of C6/36 cells (Figure 5.12). After 24 h pi levels of encapsidated positive-sense L and M RNA species declined to low levels, but encapsidated positive-sense S RNA continued to accumulate up to 72 h pi. When the same infected C6/36 cell derived RNA samples were fractionated on a 1.5% denaturing agarose gel, prior to Northern analysis, the S RNA specific signal at 48 and 72 h pi was resolved into two bands corresponding to antigenomic S and S mRNA sized RNA species (Figure 5.13). It appeared that beyond 24 h pi, after genome replication had declined in Bunyamwera virus-infected C6/36 cells, S mRNA species were encapsidated by the viral N protein. The encapsidation of S mRNA may be responsible for the down-regulation of N protein synthesis observed in these cells beyond 24 h pi.

5.5 Summary

Bunyamwera virus infection induced shut off of host cell protein synthesis in BHK cells but not in C6/36 cells. The overall rate of viral protein synthesis appeared to be slower in C6/36 cells and NSs appeared to be synthesised at lower levels in these cells. Against a background of continuing host protein synthesis, levels of viral protein synthesis decreased after 24 h pi in the infected C6/36 cells; no viral protein synthesis was detected in these cells beyond 36 h pi. The decline in viral N protein synthesis was not paralleled by a

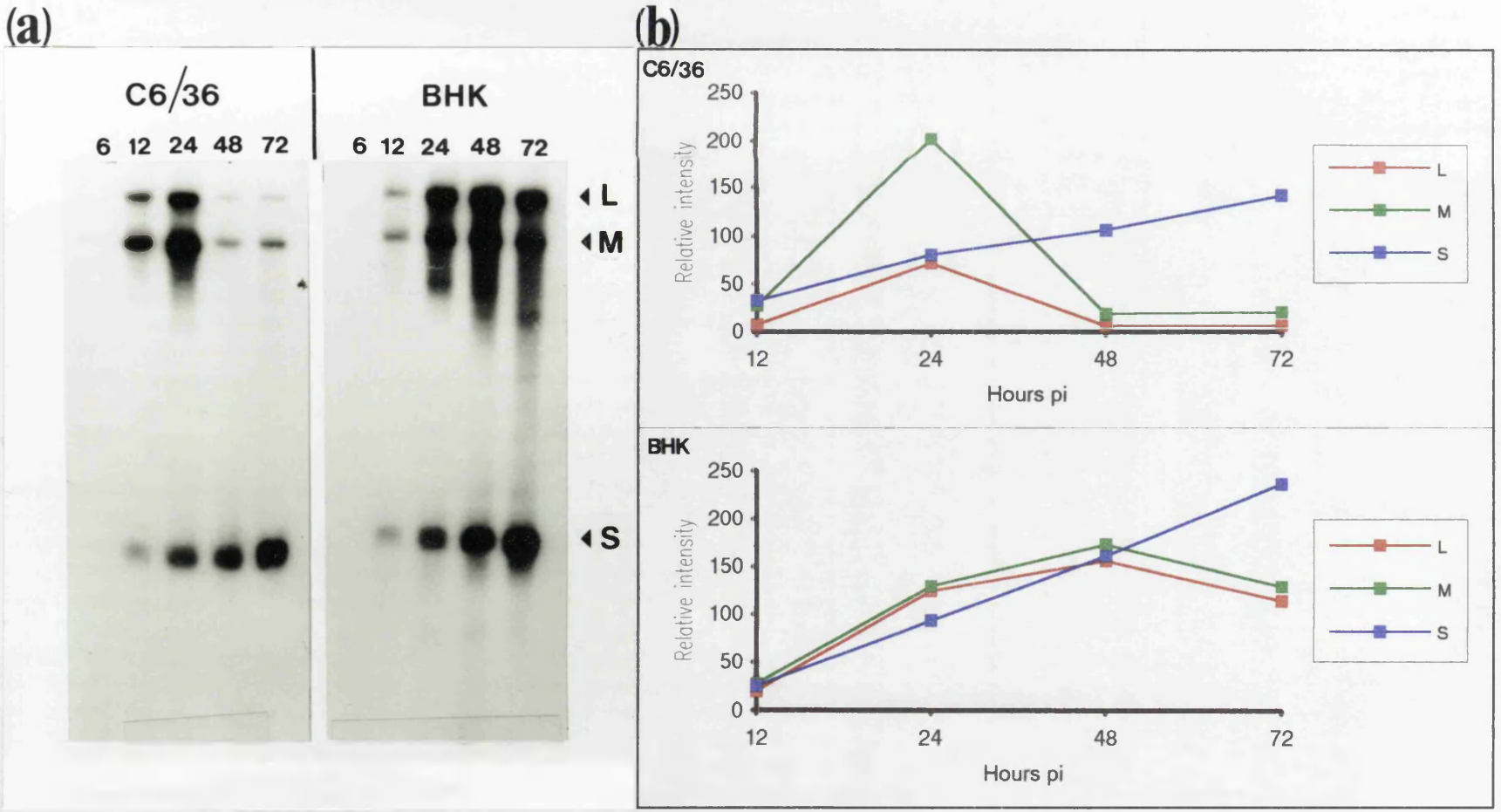

Figure 5.12. Patterns of accumulation of encapsidated positive-sense viral RNA species in Bunyamwera virus-infected C6/36 and BHK cells: (a) 0.3µg of RNA extracted from CsCl-banded nucleocapsids at the indicated h pi was fractionated on a 1.8% agarose gel containing 5mM methylmercury hydroxide and transferred to nitrocellulose filters. The filters were hybridized with a mixture of [^{32}P]-labelled cDNA probes, L9, M6 and S9, specific for Bunyamwera virus positive-sense L, M and S RNA species, respectively; (b) Graphs showing the pattern of accumulation of encapsidated positive-sense viral RNA species in Bunyamwera virus-infected C6/36 and BHK cells as determined by densitometric analysis of the Northern blot shown in (a).

Figure 5.13. Northern blot analysis of nucleocapsid-derived RNA from Bunyamwera virus-infected C6/36 cells. 1μg of RNA, extracted from CsCl-banded nucleocapsids at the indicated h pi, was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with a mixture of [^{32}P]-labelled cDNA probes, L9, M6 and S9, specific for Bunyamwera virus positive-sense L, M and S RNA species respectively.

decline in the levels of translatable SmRNA. Northern blot analysis of encapsidated viral RNA species extracted from infected C6/36 cells revealed the presence of S mRNA-sized RNA in viral nucleocapsid structures at 48 and 72 h pi whereas only full-length viral RNA species were encapsidated up to 24 h pi. Encapsidation of S mRNA in infected C6/36 cells may promote the establishment of persistent infection by limiting the translation of viral proteins.

Different patterns of viral RNA accumulation were observed in the two cell types following infection with Bunyamwera virus. Viral RNA synthesis proceeded more slowly in C6/36 cells, and L and M RNAs were less abundant relative to S RNA in these cells compared to BHK cells. Northern blot analysis of encapsidated viral RNA species isolated from infected C6/36 cells suggested that levels of viral genome replication were severely reduced after 24 h pi. Levels of encapsidated positive-sense L and M RNA declined to low levels in these cells between 24 and 48 h pi, however, positive-sense S RNA continued to accumulate in nucleocapsid structures up to the last time point tested (72 h pi). The encapsidation of S mRNA may account, at least partly, for the continuing accumulation of encapsidated positive-sense S RNA species in the C6/36 cells.

6 Studies on *Aedes albopictus* C6/36 cell lines persistently infected with Bunyamwera virus

6.1 Screening of viruses released from two persistently infected cell lines established by Elliott and Wilkie (1986)

Elliott and Wilkie (1986) established two lines of mosquito cells persistently infected with Bunyamwera virus by infecting cultures of *Aedes albopictus* C6/36 cells with 5 or 0.5 pfu/cell of Bunyamwera virus. These cell lines, termed C6/36-PI HI and C6/36-PI LO respectively, were passaged at approximately weekly intervals through more than 40 passages and at no time was cpe observed. Supernatant culture fluid was harvested from each pass immediately before subculture and stocks of virus released at different passage levels of both persistently infected cell lines were stored at -20°C. Initial studies on these cell lines had shown that S segment specific RNAs were dominant both in the persistently infected cells and in virus released from these cells, almost to the exclusion of the L and M segment specific RNAs. It was suggested that the persistent virus released from both cell lines consisted mainly of variants possessing only the genomic S segment RNA. These studies also revealed that the nucleocapsid protein (N) encoded by virus released from one of the later passages of the C6/36-PI LO cell line (only pass 17 was tested) had a slower electrophoretic mobility on SDS-PAGE gels than the N protein of wild-type Bunyamwera virus (Elliott & Wilkie, 1986).

To investigate further the role of the S segment RNA and the evolution of virus variants in the maintenance of the persistent state, all the laboratory stocks of supernatant tissue culture fluid from the C6/36-PI HI and PI LO cell lines were screened for viruses producing variant N proteins. Initially it was important to determine the passage level at which the variants first appeared and to assess the extent of their occurence. Culture fluid from each passage level of both PI cell lines was used to infect BHK cell monolayers, which were pulse-labelled with [^{35}S]-methionine for 1 h at 24 h pi. SDS-PAGE analysis of the radiolabelled cell extracts revealed the emergence of three distinct N protein phenotypes during the course of the persistent infection (Figures 6.1 and 6.2). Variation was apparent in the levels of viral protein synthesis and in the extent of host cell protein synthesis shut-off observed with individual samples. This variation paralleled the fluctuating titres of extracellular virus recorded for the different passage levels of the persistently infected cell lines by Elliott & Wilkie (1986) and therefore probably reflected the faster progress of infections initiated at higher multiplicities. In relatively advanced infections, where host protein synthesis had been severely reduced, the viral L, G1, N and NSs proteins were easily identified. In the instances where host protein synthesis was not yet shut off, only the viral N protein could be identified against a background of comigrating host proteins. Both cell lines yielded viruses encoding N proteins with wild-

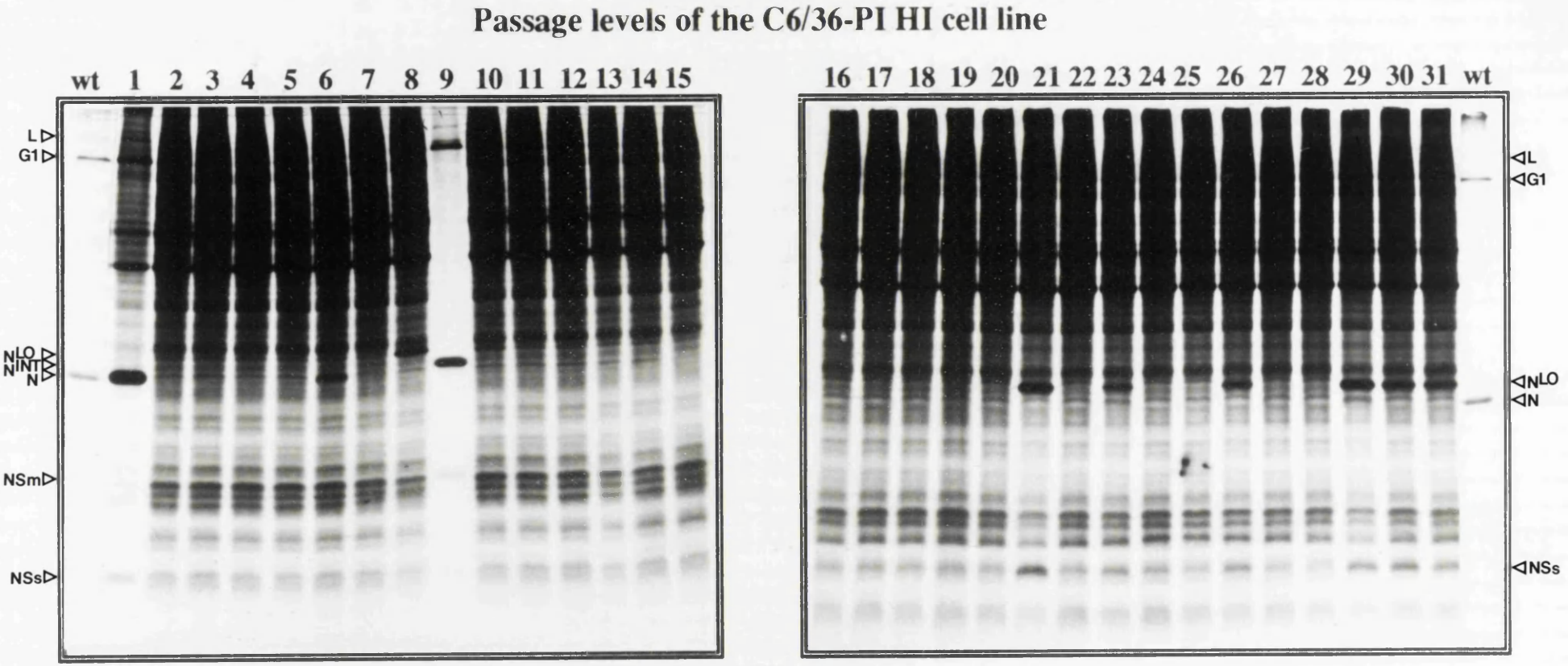

Figure 6.1 Screening of the virus released from the C6/36-PI HI cell line for viral variants encoding N proteins with altered electrophoretic mobility. BHK cell monolayers were infected with culture fluid from each passage level (1-31) of the C6/36-PI HI cell line. At 24 h pi the monolayers were pulse-labelled for 1 h with [^{35}S]-methionine. Labelled cell extracts were analysed by SDS-PAGE. **wt**: marker track of [^{35}S]-methionine labelled cell extract from wt Bunyamwera virus infected BHK cells.

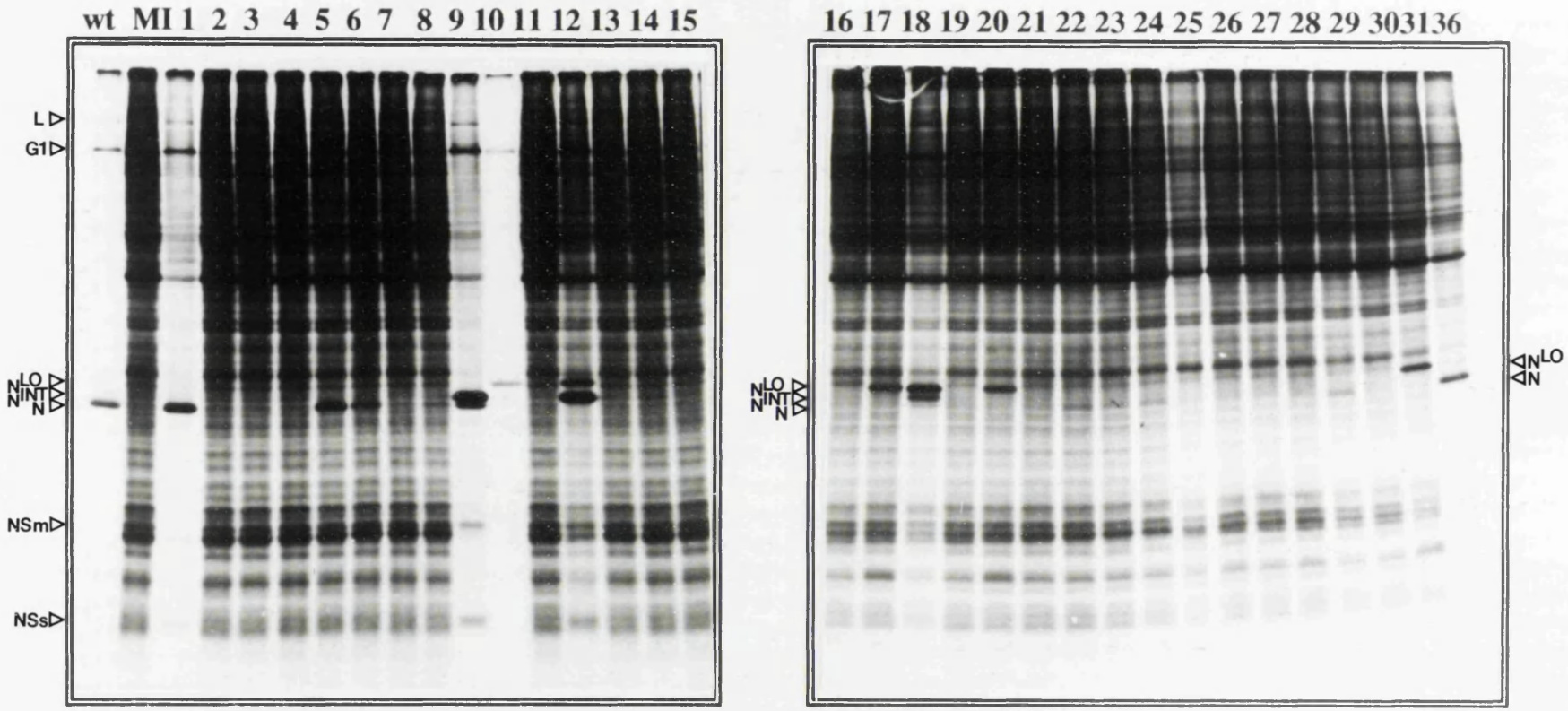

Figure 6.2 Screening of the virus released from the C6/36-PI LO cell line for viral variants encoding N proteins with altered electrophoretic mobility. BHK cell monolayers were infected with culture fluid from each passage level (1-31 and 36) of the C6/36-PI LO cell line. At 24 h pi the monolayers were pulse-labelled for 1 h with [^{35}S]-methionine. Labelled cell extracts were analysed by SDS-PAGE. **wt**: marker track of [^{35}S]-methionine labelled cell extract from wt Bunyamwera virus infected BHK cells. **MI**: labelled cell extracts from uninfected BHK cells.

type mobility (N), as well as variants encoding N proteins with intermediate mobility (N^{INT}), and slow mobility (N^{LO}), on SDS-PAGE gels (Figures 6.1 and 6.2). Variant N protein containing viruses first appeared at passages 8 and 9 of the C6/36-PI HI and C6/36-PI LO cell lines respectively. At passage 8 of the C6/36-PI HI cell line the viral N protein synthesised appeared to be of the same mobility as the N^{LO} species which had been detected at passage 17 of the C6/36-PI LO cell line by Elliott and Wilkie (1986). By passage 9 another N protein phenotype was evident which displayed a mobility on SDS-PAGE gels between that of wild-type Bunyamwera virus N protein and N^{LO} (N^{INT}). In addition, at passage 9 two G1 protein species were evident one with wild-type mobility and a slower migrating species. At later passages N^{LO} was most prevalent, appearing at significant levels at passages 10, 21, 23, 26 and 29 to 31 (Figure 6.1). In the C6/36-PI LO cell line, N proteins of all three phenotypes (N, N^{INT} and N^{LO}) were detected concurrently at passage 9; in addition, two G1 species were synthesised at passage 9, as observed for the same passage level of the C6/36-PI HI cell line. At passages 10 and 12 both N^{LO} and N^{INT} were evident. At later passages the dominant N protein phenotype varied: at passage 14 relatively low levels of N^{INT} were observed; at passage 17 only N^{LO} was observed, in agreement with previous observations (Elliott & Wilkie, 1986); at passage 18, N^{LO}, an N species of intermediate mobility (possibly distinct from the other N^{INT} species) and traces of wild-type N protein were synthesised. Then at passage 20 only N^{LO} was evident; through passages 22 to 24 and also at passage 29, N protein of wild-type mobility was synthesised; at passage 31, N^{LO} was the dominant species but at passage 36 wild-type N protein was once again the only species detected (Figure 6.2). At passages 9 and 10 of the C6/36-PI HI and C6/36-PI LO cell lines respectively, a protein corresponding in size to NSm was observed. Thus variant viruses were detected relatively early during the persistent infection and appeared to co-exist with wild-type virus in the persistently infected cells. The relative level of each viral species fluctuated during the course of persistence.

In order to further characterise the variants which emerged during the persistent infection, virus released at passages 8, 18, 20, 29 and 30 of the C6/36-PI LO cell line were selected for further investigation. Supernatant tissue culture fluid from each of these passage levels was titrated on BHK cell monolayers and a minimum of twelve isolated plaques were picked from each passage. The protein synthesis profile produced by each isolate upon infection of BHK cells was determined. All 12 plaque isolates from passage 8 encoded N proteins of wild-type mobility. Of 12 isolates plaque-purified from passage 18, 11 possessed wild-type N proteins, while 1 isolate encoded an N protein of N^{LO} phenotype. The ratio of the different virus phenotypes selected from this population of persistent viruses did not reflect the relative proportion of each N protein phenotype synthesised during the initial screen (Figure 6.2). No viruses encoding N^{INT} proteins were recovered from passage 18 despite their presence at levels significantly higher than the wild-type phenotype as determined by the initial screen. In addition the initial screen

suggested that N^{LO} encoding variants were more abundant relative to viruses encoding wild-type N proteins than was apparent upon screening the plaque isolates. Sixteen plaque isolates were picked from passage 20, and of these 11 displayed N^{LO} phenotype and 5 possessed wild-type mobility N proteins. Of 14 isolates plaque-purified from passages 29 and 30, 7 and 9 respectively exhibited N^{LO} phenotype. The phenotype(s) of the other isolates fom these passage levels could not be determined due to low levels of viral protein synthesis against a background of high levels of host protein synthesis at the time of pulse-labelling. However, once again, the abundance of N^{LO} encoding viruses recovered from passage 29 was not in line with predictions based upon the initial screen (Figure 6.2) during which only wild-type N protein encoding viruses were detected at this passage level.

On the basis of this second screen, two isolates from each passage level were selected for a further two rounds of plaque-purification. Each plaque isolate was identified by passage and plaque number. For instance the ninth plaque isolated from passage 18 was labelled BUN18.9. Elite stocks of triple-plaque purified viruses encompassing the N and N^{LO} phenotypes were then grown on BHK cell monolayers to provide a source of biologically cloned virus which could be characterised further, and from which virion RNA could be prepared for molecular analysis (see Section 6.3). Figure 6.3 shows the protein synthesis profiles of the elite stocks of virus established from the C6/36-PI LO cell line.

6.2 Determination of the temperature-sensitivity of variants arising in the C6/36-PI LO cell line

Elliott and Wilkie (1986) reported the appearance of temperature-sensitive (ts) virus during the establishment of persistence in the C6/36-PI HI and C6/36-PI LO cell lines. By passage 8 the virus released from both persistently infected cell lines displayed temperature-sensitivity. As detailed above, the viruses encoding variant N proteins first appeared in both persistently infected cell lines around this time (see Figures 6.1 and 6.2). To determine if the ts phenotype could be correlated with the altered N protein mobility on SDS-PAGE gels, the temperature-sensitivity of selected elite stocks of virus derived from the C6/36-PI LO cell line was assayed by plaque titration on BHK cell monolayers. Plaque assays were performed at 31, 37 and 38.5°C. Viruses with variant N proteins grew less well (BUN18.9) or not at all (BUN20.2, BUN20.4, BUN29.9 and BUN29.10) at 38.5°C compared to 31 and 37°C, while viruses with wild-type-like N proteins grew equally well at the three temperatures tested (Table 6.1). It appeared that the viruses with variant N proteins also exhibited a ts phenotype.

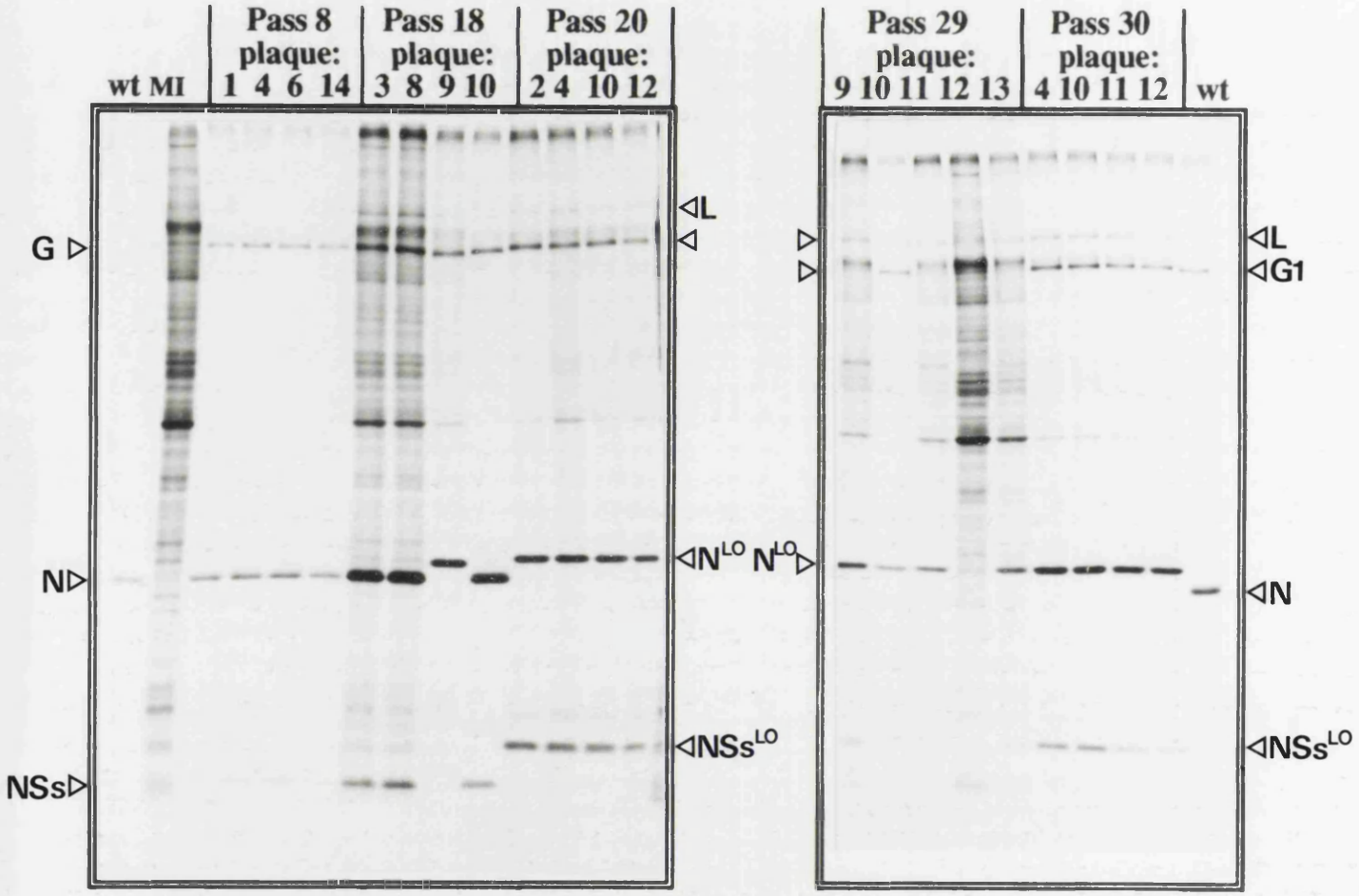

Figure 6.3 Protein profiles of BHK cells infected with each of the elite stocks of triple plaque-purified virus isolated from the C6/36-PI LO cell line. BHK cell monolayers were infected with the elite stocks of virus at a moi of 10 pfu/cell. At 48 h pi the infected monolayers were pulse-labelled for 1 h with [^{35}S]-methionine and the labelled cell extracts were analysed by SDS-PAGE. **wt**, [^{35}S]-methionine labelled cell extract from wild-type Bunyamwera virus infected cells; **MI**, [^{35}S]-methionine labelled cell extract from uninfected BHK cells. The elite stocks are identified by passage number and plaque number from the first round of plaque-purification.

Virus isolate		Virus titre (pfu/ml)			ts phenotype	N protein phenotype
Passage	Plaque	31°C	37°C	38.5°C		
8	4	1.2×10^7	1.1×10^7	4.5×10^7	no	wt
	6	2.1×10^7	2.8×10^7	2.9×10^7	no	wt
18	9	2.0×10^6	1.2×10^6	2.5×10^4	yes	N^{LO}
	10	3.2×10^7	3.9×10^7	3.8×10^7	no	wt
20	2	2.9×10^7	4.0×10^7	No plaques	yes	N^{LO}
	4	3.4×10^7	1.8×10^7	No plaques	yes	N^{LO}
29	9	1.1×10^6	1.1×10^6	No plaques	yes	N^{LO}
	10	2.2×10^7	2.2×10^7	No plaques	yes	N^{LO}
wt	L_9	4.1×10^7	6.0×10^7	1.9×10^7	no	wt

Table 6.1 Results of the temperature-sensitivity assay performed on BHK cell monolayers with viruses plaque-purified from the supernatant tissue culture fluid of *Aedes albopictus* C6/36 cells persistently infected with Bunyamwera virus.

6.3 Sequence analysis of the genomic S RNA segments of viruses released from the C6/36-PI LO cell line

To determine the molecular basis for the altered electrophoretic mobility of the N proteins encoded by the variant viruses arising during the persistent infection, and to investigate the overall rate of nucleotide change in the Bunyamwera virus S segment RNA during the persistent infection, attempts were made to sequence the S segments of isolates plaque-purified from passages 8, 18, 20 and 29 of the C6/36-PI LO cell line. Virion RNA was prepared (see Section 3.15.1) and sequenced by primer extension from γ-[^{32}P]-labelled oligonucleotides. The oligonucleotides used were those used to sequence the S RNA segment of wtL_9BUN virus (see Figure 4.1 and section 3.17).

Sequence analysis of the S segment RNA of viruses BUN8.6 and BUN18.10, which produced wild-type mobility N proteins, demonstrated that the S RNA of the BUN8.6 isolate was identical in sequence to the S RNA of wild-type Bunyamwera virus, while the S RNA segment of the BUN18.10 isolate had undergone a single nucleotide substitution (see Figure 6.4). The S RNA segment of BUN18.10 had a guanosine to adenosine transition at nucleotide position 255 in the positive-sense S RNA. This nucleotide change resulted in amino acid substitutions in the N and NSs proteins encoded by this virus. Asparagine replaced serine at amino acid position 57 of the N protein and

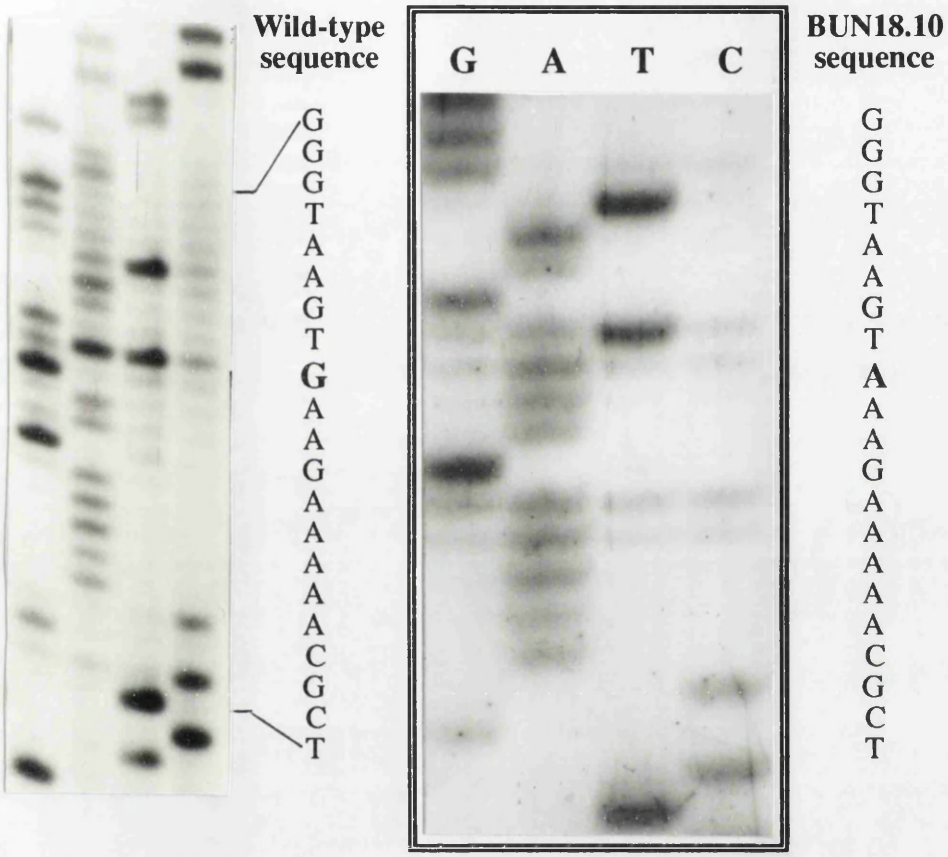

Figure 6.4. Direct RNA sequence determination of the genomic S RNA segment of the persistent viral isolate BUN 18.10, by primer extension sequencing from oligonucleotide number 2. The sequence shown covers nucleotides 243 to 263 inclusive, and is written (together with the corresponding Bunyamwera virus S segment sequence; Elliott, 1989b) as the cDNA, alongside. The guanosine to adenosine transition at nucleotide position 255 is indicated in **bold**.

On the left is shown the sequence of the corresponding region of the wtL$_9$BUN virus S RNA. Note the A/T ambiguity at position 256, which is a T residue in the cDNA derived sequence.

methionine replaced valine at amino acid position 51 of the NSs protein. Both were relatively conservative amino acid changes and neither of these changes occured in conserved regions of the N or NSs proteins as defined by a consensus amino acid sequence composed by alignment of the six known bunyavirus S gene sequences (Figure 6.5).

Problems were encountered during attempts to sequence the S RNA segment of each of the variant viruses, so a primer extension reaction in the absence of dideoxy nucleotides was performed. This too failed to work suggesting that the problem lay in the ability of the oligonucleotides to prime the extension reaction. Eventually, primer extension from oligonucleotide number 2, annealed to BUN18.9 RNA, yielded a stretch of sequence identified as Germiston virus S RNA bases 233 to 433 (of the positive-sense full-length S RNA). Comparison of the published sequences for the S RNAs of Germiston and Bunyamwera bunyaviruses (Gerbaud et al., 1987; Elliott, 1989) revealed a 20 nucleotide stretch of Germiston virus S RNA, between bases 211 and 230, which was identical to the stretch of sequence from bases 209 to 228 inclusive of Bunyamwera virus genomic S RNA to which oligonucleotide 2 was the exact complement. This was confirmed by using the FIND program (Devereux et al., 1984) to scan the Germiston virus S gene sequence for potential priming sites. The perfect match between oligonucleotide 2 and the afore mentioned sequence was detected while none of the remaining 5 oligonucleotides had complementary sites with fewer than 3 mismatched bases within the Germiston virus S gene sequence.

Figure 6.6 shows a portion of the sequencing data obtained by priming with oligonucleotide number 2 on BUN18.9 virion RNA and wtL$_9$BUN virion RNA. As priming was not achieved with oligonucleotides numbers 2, 3 or 4 on BUN20.2 or BUN29.10 virion RNA, whereas parallel reactions, under identical conditions, yielded sequencing data from BUN8.6 and BUN18.10, it appeared that the BUN20.2 and BUN29.10 isolates were also contaminating viruses in the persistently infected C6/36 cell line. It appeared therefore that the variant N protein producing viruses, observed in association with the persistently infected *Aedes albopictus* C6/36 cell line established and passaged by Elliott and Wilkie (1986), originated by contamination of the cell line with temperature-sensitive viruses which were in use in the laboratory at that time. It is interesting that Bunyamwera virus persistently infected C6/36 cells were susceptible to infection by other bunyaviruses. Verani et al. (1984) reported that Toscana virus persistently infected cells were resistant to infection with other phleboviruses but remained susceptible to viruses within other genera of the Bunyaviridae. However, Newton et al. (1981) reported that Barmah Forest bunyavirus did replicate in their Bunyamwera virus persistently infected uncloned *Aedes albopictus* cells.

From the sequencing data on the BUN8.6 and BUN18.10 virus isolates, both of which encoded wild-type mobility N proteins, the rate of nucleotide change in the viral genome during the course of the persistent infection appeared to be slow. No nucleotide

(a)

```
                1                                                        *60
Bunyamwera      .mieLeFhDVaantsstFdPEvaYanFkrvhttglsYdhIRiFYikgreiKtsLakrsew
Maguari         .mieLeFnDVaantsstFdPEiaYvnFkrihttglsYdhIRvLYikgreiKtsLtkrsew
Germiston       .mleLeFeDVpnnigstFdPEsgYtnFqrnylpgvtLdqIRiFYikgreiKnsLskrsew
La Crosse       .msdLvFyDVastgangFdPDagYmdFcvknaeslnLaaVRiFFlnaakaKaaLsrkper
snowshoe hare   .msdLvFyDVastgangFdPDagYmaFcvkyaesvnLaaVRiFFlnaakaKaaLsrkper
Aino            manqFiFqDVpqrnlatFnPEvgYvaFiakhgaqlnFdtVRfFFlnqkkaKmvLsktaqp
  Consensus     ----L-F-DV-------F-PE--Y--F---------L---R-F-------K--L------

                61                                                      120
Bunyamwera      evtlnLGgwkitvyNtnFPgnrnnpVpddgLTlHRlSGFLArYllekm.lkvsepeklii
Maguari         evtlnLGgwkvavfNtnFPgnrnspVpddgLTlHRlSGFLArYlleki.lkvsdpeklii
Germiston       evtlnLGgwkvpvlNtnFPgnrnnaVpdygLTfHRiSGYLArYllgky.laetepeklim
La Crosse       kanpkFGewqveviNnhFPgnrnnpIgnndLTiHRlSGYLArWvldqynenddesqheli
snowshoe hare   kanpkFGewqvevvNnhFPgnrnnpInsddLTiHRlSGYLArWvleqykenedesrreli
Aino            svdltFGgikftlvNnhFPqytanpVpdtaLTlHRlSGYLAkWvadqcktnqiklaeam.
  Consensus     ------G-------N--FP------V----LT-HR-SGYLA------------------

                121                                                     180
Bunyamwera      kskIInPlAEknGitWndGeevYLsFfPGsEMFLgTFrFYPLaIgiykVqrkeMEpkYLe
Maguari         kskIInPlAEknGitWadGeevYLsFfPGsEMFLgTFkFYPLaIgiykVqkkeMEpkYLe
Germiston       rtkIVnPlAEknGitWesGpevYLsFfPGaEMFLgTFrFYPLaIgiykVqrkeMDpkFLe
La Crosse       rttIInPiAEsnGvgWdsGpeiYLsFfPGtEMFLeTFkFYPLtIgihrVkqgmMDpqYLk
snowshoe hare   kttIInPiAEsnGvrWdsGaeiYLsFfPGtEMFLeTFkFYPLtIgiyrVkqgmMDpqYLk
Aino            .ekIVmPlAEvkGctWteGltmYLgFaPGaEMFLeTFeFYPLvIdmhrVlkdgMDvnFMr
  Consensus     ---II-P-AE--G--W--G---YL-F-PG-EMFL-TF-FYPL-I----V----MD--YL-

                181                                                   237
Bunyamwera      KtMRQRYmgleAatWtvsKlteVqsaLtvVssLgWkktnvSaaArdFLakFGInm
Maguari         KtMRQRYmgleAatWtvsKvneVqaaLtvVsgLgWkktnvSaaAreFLakFGInm
Germiston       KtMRQRYlgidAqtWtttKlgeVeaaLkvVsgLgWkktnvSsaAreFLskFGIrm
La Crosse       KaLRQRYgtltAdkWmsqKvaaIaksLkdVeqLkWgkgglSdtAktFLqkFGIrlp
snowshoe hare   KaLRQRYgsltAdkWmsqKvtaIaksLkeVeqLkWgrgglSdtArsFLqkFGIrlp
Aino            KvLRQRYgtltAeqWmtqKidaVraaFnaVgqLsWaksgfSpaAraFLaqFGIni
  Consensus     K--RQRY----A--W---K---V---L--V--L-W-----S--A--FL--FGI--
```

(b)

```
                1                                                  *    60
Bunyamwera      mMslltpavlLtqrshtltlsvstplglvmttYeSStlkdaRlklvSqkevngklhLtLg
Maguari         mMslltpavlLtqrlhtltlsvstplglvmttFeSStlkdaRlklvSqkevsgrlrLtLg
Germiston       .MslitsgvlLtqsqdtltfsvttcqglrltkFaSStlkdaRlkivSqkevngklrLtLg
La Crosse       mMshqqvqmdLilmqgiwtsvlkmqnhstllqLgSSssmlqRprllSrvsqrgrltLnLe
snowshoe hare   mMshqqvqmdLilmqgiwhsvlnmqnqsillqLgSSssmpqRprllSrvsqrgrqiLnLe
Aino            .MflngislrLtrrsgmwhlllnmgpnsisipLdSSssirrRprwySvrrhnqvliLhLv
  Consensus     -M--------L--------------------L-SS-----R----S---------L-L-

                61                                                  111
Bunyamwera      agrllyiIrIflaTgttqfltmvlpStasvdslpgtylrrc
Maguari         agrllylIqIflaTgtvqfqtmvlpStdsvdslpgtylrkf
Germiston       agrylysIrIsleTgtmqclttvlpStvsvdtlpgtylestlqrqnqkss
La Crosse       sgrwrlsIiIfleTgttqlvttilpStdylgi
snowshoe hare   sgrwrlsIiIfleTgtiqltatilpStdcqdi
Aino            asslhwlItIfpnTqqilcqtlpslSivsqdi
  Consensus     -------I-I---T-----------S------
```

Figure 6.5. Six-way alignment of (a) the nucleocapsid (N) proteins and (b) the non-structural (NSs) proteins of six different bunyaviruses. The alignments were generated with the GCG programs PILEUP and PRETTY. The consensus sequence shows residues completely conserved in all six sequences. * indicates the position of the amino acid substitutions found in the N and NSs proteins of the BUN 18.10 isolate.

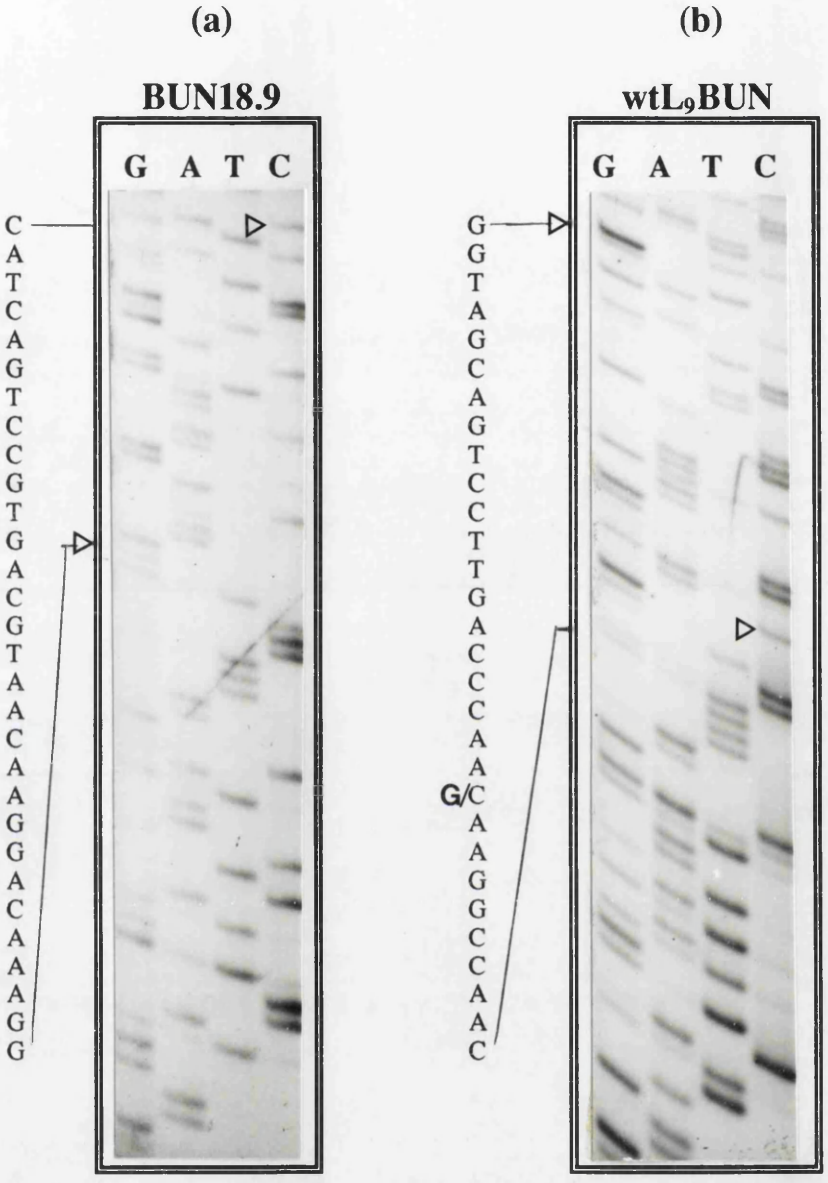

Figure 6.6. Direct RNA sequence determination of the genomic S RNA segments of (a) the persistent viral isolate BUN18.9 and (b) wtL$_9$BUN virus, by primer extension sequencing from oligonucleotide number 2. The sequences shown are from nucleotide 290 to 351 of BUN18.9 S RNA (identified as Germiston virus S RNA) and nucleotide 288 to 360 of wtL$_9$BUN virus S RNA. The sequence from nucleotide 322 to 351 of both RNAs is written as cDNA alongside.

changes were observed in the consensus S segment sequence for isolate BUN8.6 which had been plaque-purified from the persistently infected culture after it had been passaged for over two months. The BUN18.10 isolate was plaque-purified from the supernatant tissue culture fluid of the persistently infected culture after it had been passaged for over four months and only one nucleotide substitution, resulting in conservative amino acid substitutions in N and NSs, was observed. The constraints on the evolution of the Bunyavirus S segment RNA may be greater than for the other genome segments due to the overlapping reading frames encoded in the S RNA. Hence the rate of change of the Bunyamwera virus genome as a whole, during the persistent infection, may have been greater than is suggested by the relative stability of the S segment RNA.

6.4 Establishment of an *Aedes albopictus* C6/36 cell line persistently infected with wtL$_9$BUN virus

Since the available persistently infected cell lines were apparently contaminated with at least one other bunyavirus, further studies on the molecular basis of Bunyamwera virus persistence in C6/36 cells required that another persistently infected cell line be established. *Aedes albopictus* C6/36 cells were infected with wtL$_9$BUN virus at a moi of 5 pfu/cell. The cells were subcultured at a 1 in 10 split four days post infection and thereafter at a 1 in 20 split every 6 days. The resultant persistently infected culture (C6/36/BUN) grew at a similar rate to both uninfected C6/36 cells and to the C6/36-PI HI and C6/36-PI LO cell lines. Like the former persistently infected cell lines, the C6/36/BUN cell line was passaged for over a year without observable cytopathic effects. Extracellular virus titres were determined and the plaque-phenotypes and protein synthesis profiles, produced in BHK cells, by virus released at different passage levels of the C6/36/BUN cell line were monitored. In addition, total cell RNA and fractionated RNA samples (see Section 3.15) were prepared from the first 23 passages of the C6/36/BUN cell line. These samples were probed with Bunyamwera virus segment specific riboprobes to follow the synthesis and encapsidation of viral RNA species throughout the persistent infection.

6.5 Virus release from the C6/36/BUN cell line

Supernatant culture fluid from each of the first 30 passages of the C6/36/BUN culture was routinely sampled immediately before subculture and titrated on BHK cell monolayers. During the persistent infection the titre of released virus fluctuated between 10^2 and 10^7 pfu/ml but no regular periodicity was observed (see Figure 6.7). There was a trend towards lower levels of extracellular virus with increasing passage level of the culture and at passages 24, 25 and 28 levels of extracellular virus fell below the threshold of detection of the plaque assay (10 pfu/ml). Superimposed on this general trend towards decreased virus production, occasional increases in levels of released virus were observed;

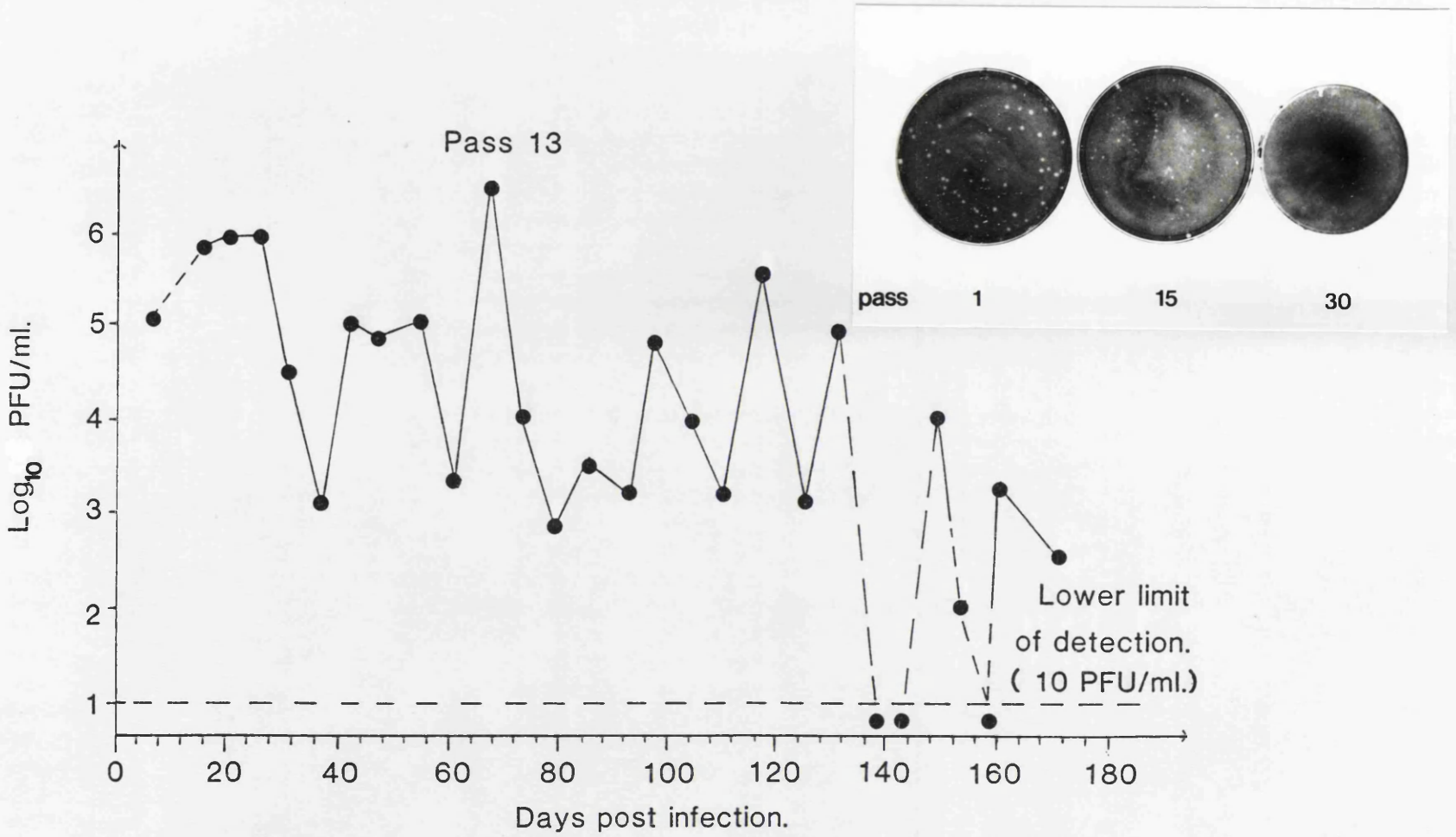

Figure 6.7. Fluctuations in extracellular virus titres during the first 30 passages of the C6/36/BUN cell line. Supernatant culture fluid was sampled immediately before subculture of each pass and titrated on BHK cell monolayers. The inset shows the change to small, cloudy plaque-phenotype virus observed with increasing passage level of the C6/36/BUN cell line.

for instance, marked increases in the levels of extracellular virus were detected at passages 13 and 21 (reaching titres of 3 x 10^6 and 4 x 10^5 pfu/ml respectively) and virus production returned to detectable levels at passages 26 and 29 after declining to levels too low to be detected by plaque assay on BHK cells. There was also a marked change in the plaque morphology (in BHK cells) of extracellular virus with increasing passage level; the original well defined, round, lytic-plaque morphology was gradually replaced by a small, cloudy-plaque phenotype. The cloudy-plaque variants arose before passage 5 and became dominant by passage 15 (see Figure 6.7). The decreasing plaque-size made counting of plaques difficult and may have contributed to the low titres recorded for later passages.

An infectious centres assay carried out on cells from passage 6 of the C6/36/BUN cell line revealed that 2% of the cells shed infectious virus. Infectious centres and immunofluorescence studies carried out on an *Aedes albopictus* C6/36 cell culture persistently infected with Semliki Forest virus (Davey and Dalgarno, 1974) also revealed that only 2% of the cells within the persistently infected culture released infectious virus, while 100% of the cells appeared to be productively infected during the acute phase of infection. Immunofluorescence studies carried out with the C6/36/BUN cell line yielded inconclusive results.

6.6 Protein profiles in BHK cells infected with culture fluid from the persistently infected C6/36/BUN cell line

Supernatant culture fluid from the first 30 passages of the C6/36/BUN cell line was used to infect BHK cell monolayers. Initially each monolayer was infected with 400µl of supernatant culture fluid, after 30 minutes at 31°C the culture fluid was replaced with another 400µl of culture fluid from the same passage of the persistently infected cell line. The inoculum was removed after a further 30 minute absorption period and the infected cells were maintained in supplemented GMEM (see Section 3.2.1). At 48 h pi the cells were pulse-labelled for 1 h with [^{35}S]-methionine and labelled cell extracts were analysed by SDS-PAGE (Figure 6.8). No variant N protein-containing viruses were detected, but novel polypeptides, potentially of viral L segment origin, were observed in infections initiated with virus released at passage levels 18, 21, 23, 26 and 29 of the C6/36/BUN culture (see Section 8.4). The levels of viral protein synthesis and host cell shut-off observed for each passage correlated with fluctuations in the levels of extracellular virus in the tissue culture supernatant of the C6/36/BUN culture (see Figure 6.7). It was interesting to note that while the infections initiated with culture fluid from passages 24, 25 and 28 of the C6/36/BUN cell line (which contained levels of virus below the threshold of detection by plaque assay on BHK cell monolayers) were slow, the synthesis of viral N protein was observed in the infected BHK cells, demonstrating that infectious virus was released at these passage levels.

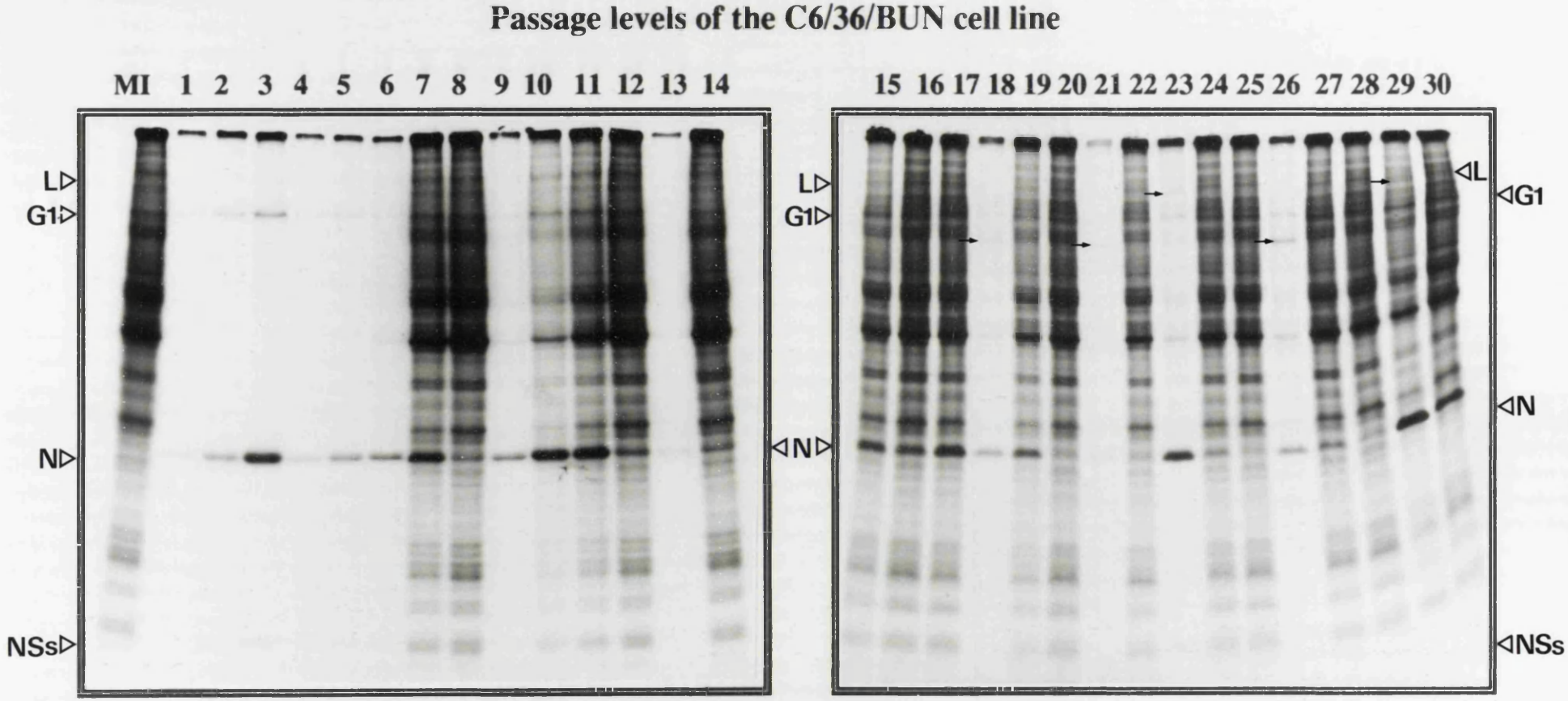

Figure 6.8 Protein profiles of BHK cells infected with supernatant tissue culture fluid from the first 30 passages of the C6/36/BUN cell line. BHK cell monolayers were infected with culture fluid from each passage level of the persistently infected cell line, and were maintained in supplemented GMEM at 31°C for 48 h. The cells were then pulse-labelled with [^{35}S]-methionine and the labelled cell extracts were analysed by SDS-PAGE. **MI**, mock infected BHK cell extract.

6.7 Levels of intracellular viral RNAs in the persistently infected cell line

Total cell RNA was extracted at every passage level (up to passage 23) of the C6/36/BUN cell line. Each sample was probed for Bunyamwera virus genomic L, M and S RNAs and for Bunyamwera virus complementary-sense S RNA by slot blot analysis using segment- and sense-specific riboprobes (as detailed in Section 3.16.1). An additional filter was hybridized with a [^{32}P]-labelled cDNA probe specific for mouse actin mRNA to monitor the amount of cellular RNA applied with each sample. The films were scanned by densitometry and each virus-specific signal was normalised against the corresponding actin signal. On the assumption that the actin mRNA levels remained relatively constant at each passage level, fluctuations in the levels of each of the viral RNA species relative to actin mRNA could be expressed as changes in the abundance of each of the viral RNA species per cell in the culture (see Figure 6.10). Levels of each of the viral RNA species varied between passage levels without regular periodicity and no simple correlation between the fluctuating levels of each of the viral RNA species and the titres of virus released from these cells was evident (Figure 6.9). Furthermore, the level of each viral RNA species appeared to vary independently (Figure 6.10). For example, at passage 2 the signal for genomic S RNA was stong relative to the signals for the genomic L and M RNAs; by passage 4 the level of M RNA had increased 3-fold, levels of L RNA had doubled and S RNA levels remained approximately the same. By passage 6, levels of genomic L RNA had increased considerably but levels of M and S genomic RNA remained relatively low. At passage 13, both the L and S genomic RNA segments gave considerably stronger signals than the M genomic RNA segment. The relatively poor signals obtained while probing for Bunyamwera virus genomic M RNA appeared to be due, at least in part, to problems with the probe as a control sample consisting of nucleocapsid derived RNA extracted from Bunyamwera virus-infected C6/36 cells at 24 h pi (which was known to contain significant quantities of M RNA) also gave a weak signal with this M segment specific riboprobe. Viral complementary-sense S RNA was detected at all passage levels (Figure 6.9). Levels fluctuated irregularly between passages and maximum levels were observed at passages 8, 9 and 18. Levels of viral complementary-sense S RNA appeared to vary independently of the levels of genomic S RNA (Figure 6.11).

A more detailed study of intracellular viral RNAs in the C6/36/BUN cell line was carried out by Northern analysis. Initially, total cell RNA from passages 4 and 13 of the persistently infected cell line (chosen because at these passage levels titres of released virus and levels of intracellular viral RNA were relatively high) was analysed using partial cDNA probes (L5, L7, S8, S9, M1 and M6) synthesised from the template cDNAs detailed in Section 3.5.3. In agreement with the slot blot data, genomic M RNA was more abundant relative to genomic L and S segment RNAs at passage 4 than at passage 13 (Figure 6.12a).

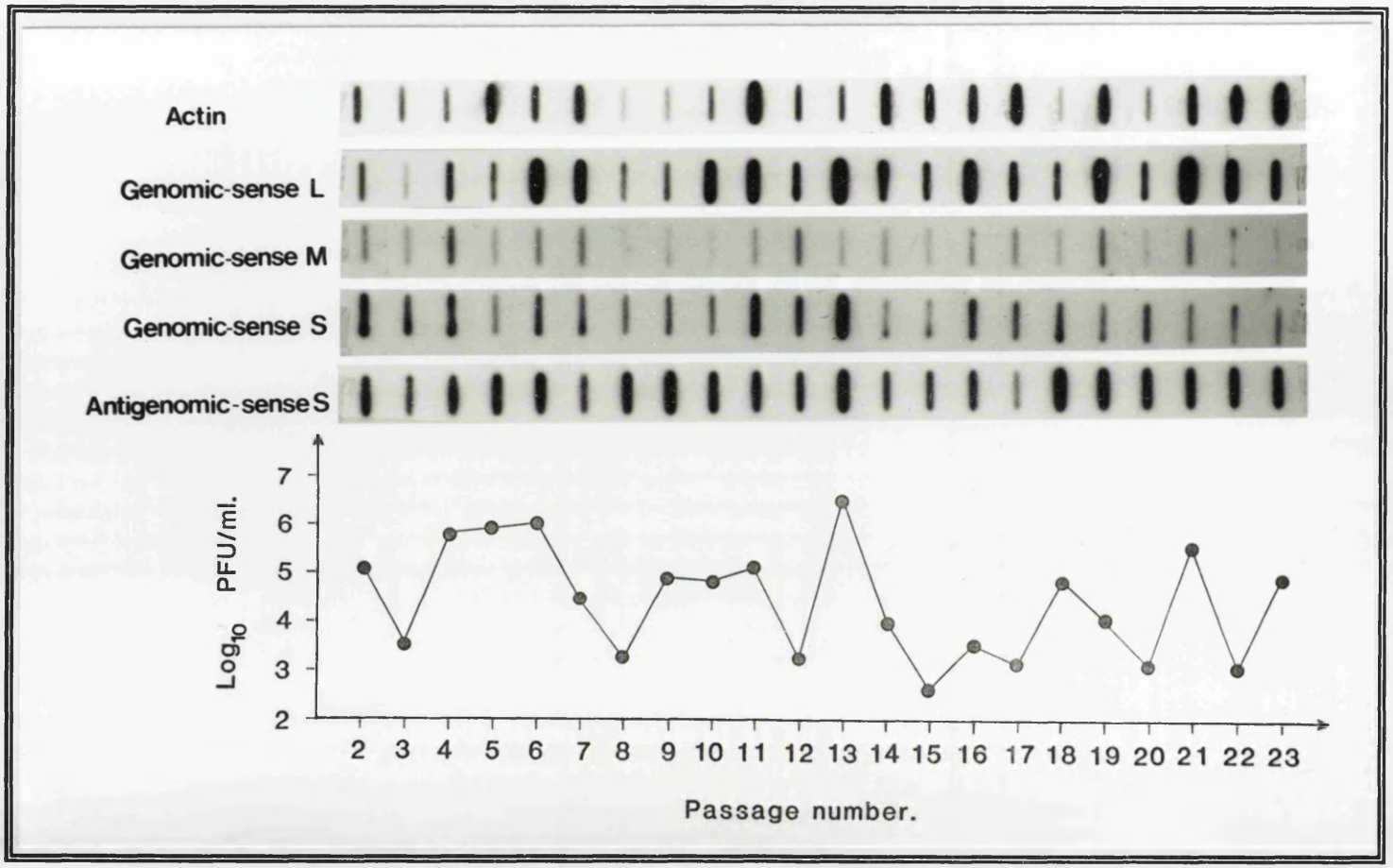

Figure 6.9. Analysis of viral RNA levels in *Aedes albopictus* C6/36 cells persistently infected with Bunyamwera virus. Total cellular RNA was extracted from the first 23 passages of the C6/36/BUN cell line and aliquots were applied to nitrocellulose using a vacuum manifold. Five replicate nitrocellulose filters were prepared, four were hybridized individually with [^{32}P]-labelled riboprobes specific for Bunyamwera virus genomic L, M and S RNA or positive-sense S RNA species, and the fifth filter was hybridized with a [^{32}P]-labelled actin gene cDNA probe to estimate the relative amount of RNA applied in each sample. The titre of Bunyamwera virus released at each passage level is shown below the slot blot filters.

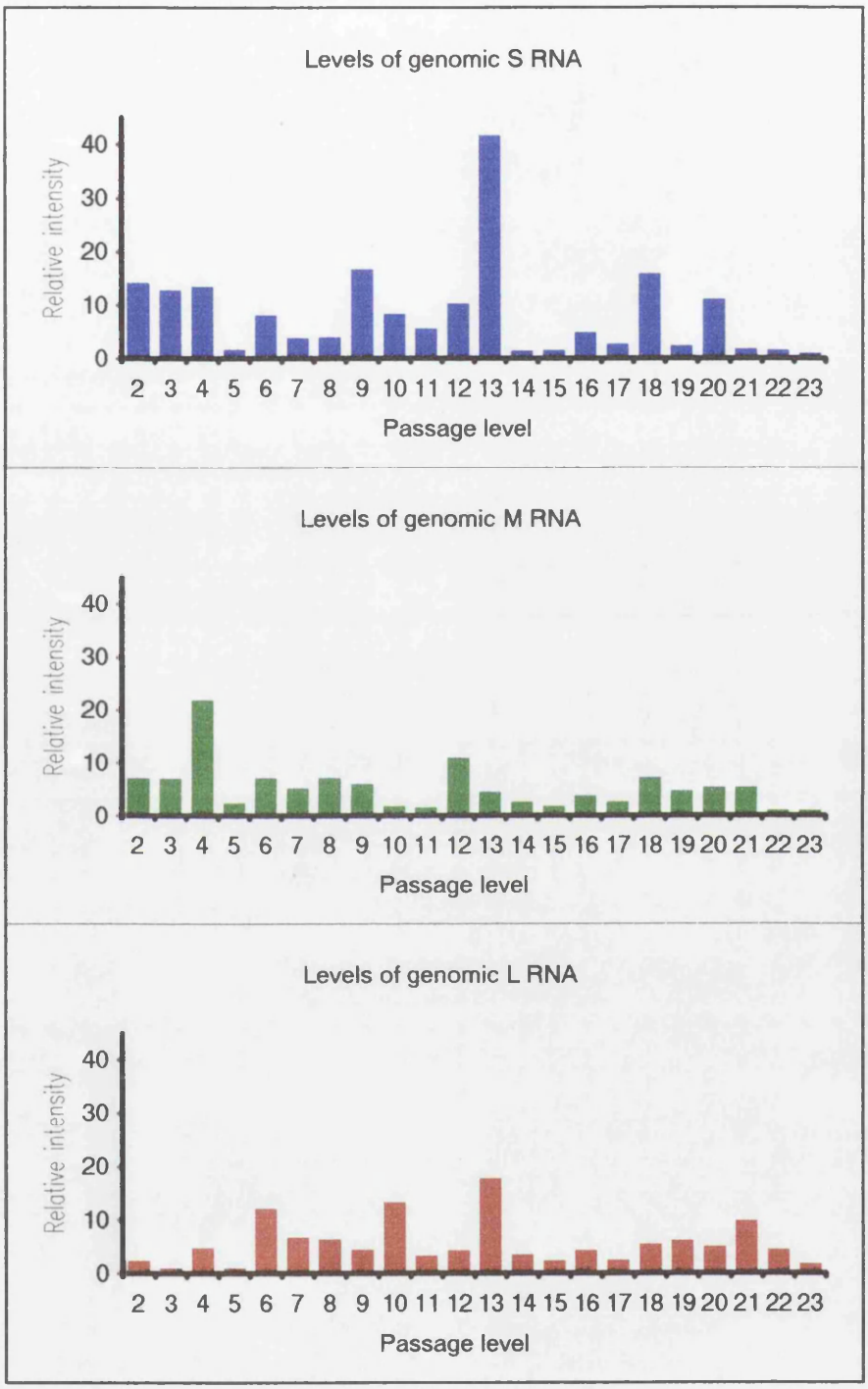

Figure 6.10. Levels of Bunyamwera virus genomic L, M and S RNA, relative to actin mRNA levels, resident in the C6/36/BUN cell line during the first 23 passages as determined by densitometric analysis of the slot blot data presented in Figure 6.9.

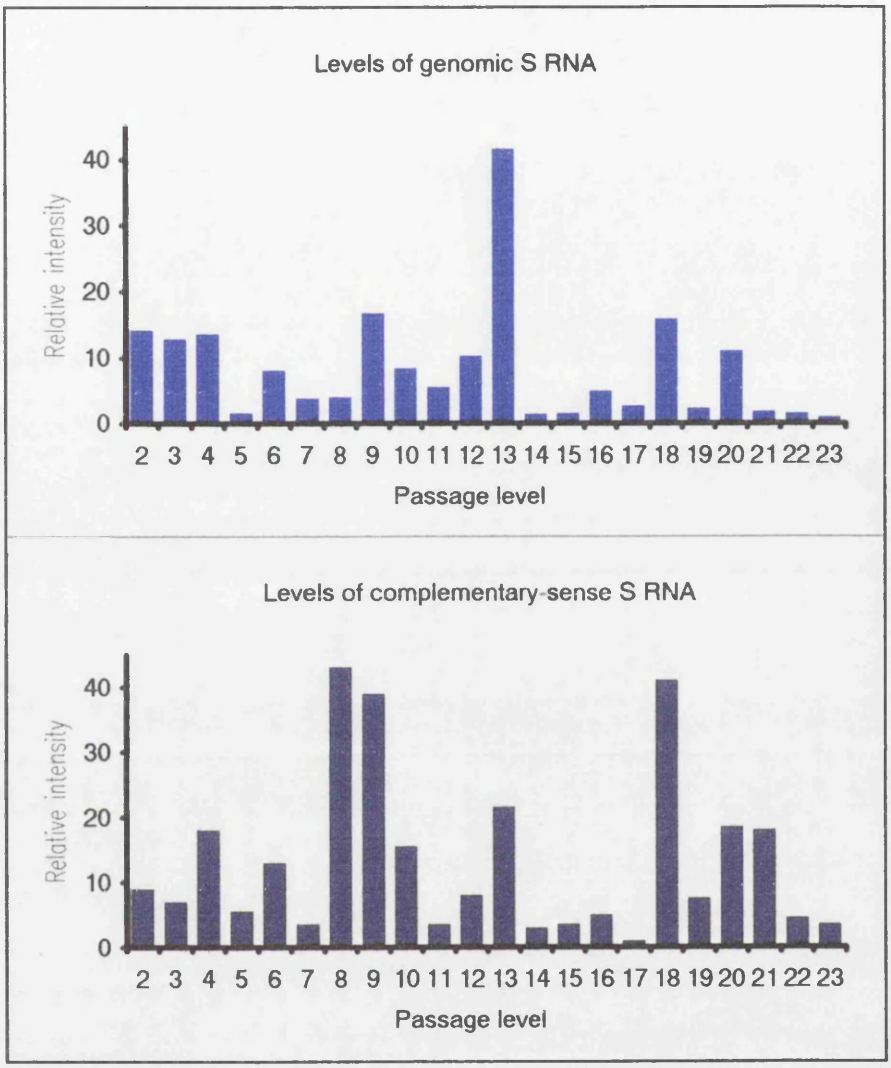

Figure 6.11. Levels of Bunyamwera virus genomic and complementary-sense S RNA, relative to actin mRNA levels, resident in the C6/36/BUN cell line over the first 23 passages as determined by densitometric analysis of the slot blot data presented in Figure 6.9.

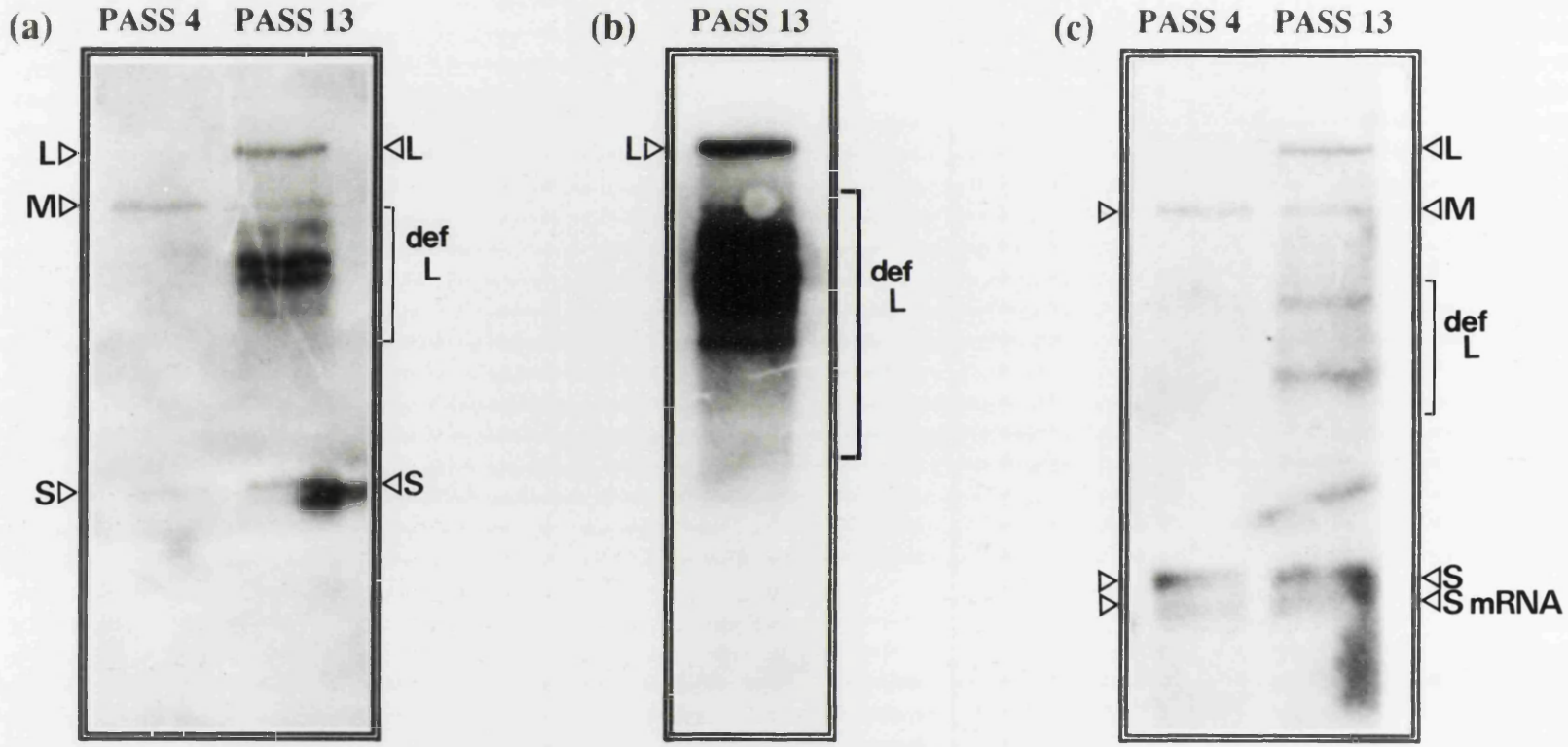

Figure 6.12 Northern blot analysis of total infected cell RNA extracted from passage levels 4 and 13 of the C6/36/BUN cell line. Each RNA sample was fractionated by electrophoresis through a 1.5% agarose gel containing 5mM methylmercury hydroxide. Following capillary transfer to nitrocellulose, the RNA was hybridized with Bunyamwera virus specific [^{32}P]-labelled cDNA probes. (a) 20μg of RNA from both passage levels was probed with [^{32}P]-labelled cDNA probes (L5, M1 and S8) specific for Bunyamwera virus genomic L, M and S RNA species; (b) 20μg of RNA extracted from passage level 13 of the C6/36/BUN cell line was probed with a [^{32}P]-labelled cDNA probe (L5) specific for Bunyamwera virus genomic L RNA; (c) 20μg of RNA from passage levels 4 and 13 was probed with [^{32}P]-labelled cDNA probes (L7, M6 and S9) specific for positive-sense Bunyamwera virus L, M and S RNA species.

In addition to the full-length viral RNA species, subgenomic viral RNAs were observed at passage 13. These subgenomic (defective) RNAs were identified as L segment derived by hybridization with a [^{32}P]-labelled cDNA probe (L5) specific for Bunyamwera virus genomic L RNA (Figure 6.12b). A different spectrum of defective RNAs was observed when the filter was hybridized with a mixture of [^{32}P]-labelled cDNA probes (L7, M6 and S9) specific for positive-sense L, M and S RNAs (Figure 6.12c). Since the L segment specific partial cDNA probes (L5 and L7) were synthesised from non overlapping clones (see Section 3.5.3) they may have detected different subpopulations of defective RNAs. S mRNA sized RNA was detected at both passage levels by Northern analysis, coinciding with the detection of significant levels of viral positive-sense S RNA by slot blot hybridization (see Figure 6.11).

6.8 Defective viral RNAs in the persistently infected cells

The occurence of defective viral RNAs in the persistently infected cells was investigated further. Total cell RNA extracted from passages 2, 6, 18, 21 and 58 of the persistently infected culture was fractionated in a denaturing agarose gel and transferred to a nitrocellulose filter. The filter was hybridized sequentially with first an L segment specific riboprobe and then an S segment specific riboprobe, in both cases to detect genomic-sense RNA (Figure 6.13). Subgenomic L segment derived RNAs were just detectable at passages 2 and 6, and were abundant at passage 21 of the persistently infected cell line. The ratio of defective L segment RNA to full-length L RNA varied between different passage levels, and at passage 58 defective L RNAs were much more abundant than the full-length L RNA. The pattern of defective L RNAs changed with increasing passage level. Most, but not all, of the size classes of defective RNAs appeared to be stably maintained in the cell line and new species arose with increasing passage level. No subgenomic S segment RNAs were observed, but interestingly, at passage 21, a more slowly migrating S segment RNA was detected. Figure 6.14 compares the Northern profiles of total cell RNA from passages 21 and 58 of the persistently infected cell line, probed for genomic-sense S RNA only. At passage 21 the defective S RNA was far more abundant than the authenic genomic S RNA, but at passage 58 the normal sized genomic S RNA was the only negative-sense S RNA species detected. Slower migrating genomic-sense S RNA species were also detected at passages 19 and 22. The defective S RNA species present at passage 19 was intermediate in mobility between authentic sized genomic S RNA and the larger S RNA species observed at passages 21 and 22 (Figure 6.15). Clearly, the population of defective viral RNAs resident in the persistently infected C6/36/BUN cell line altered as the culture matured.

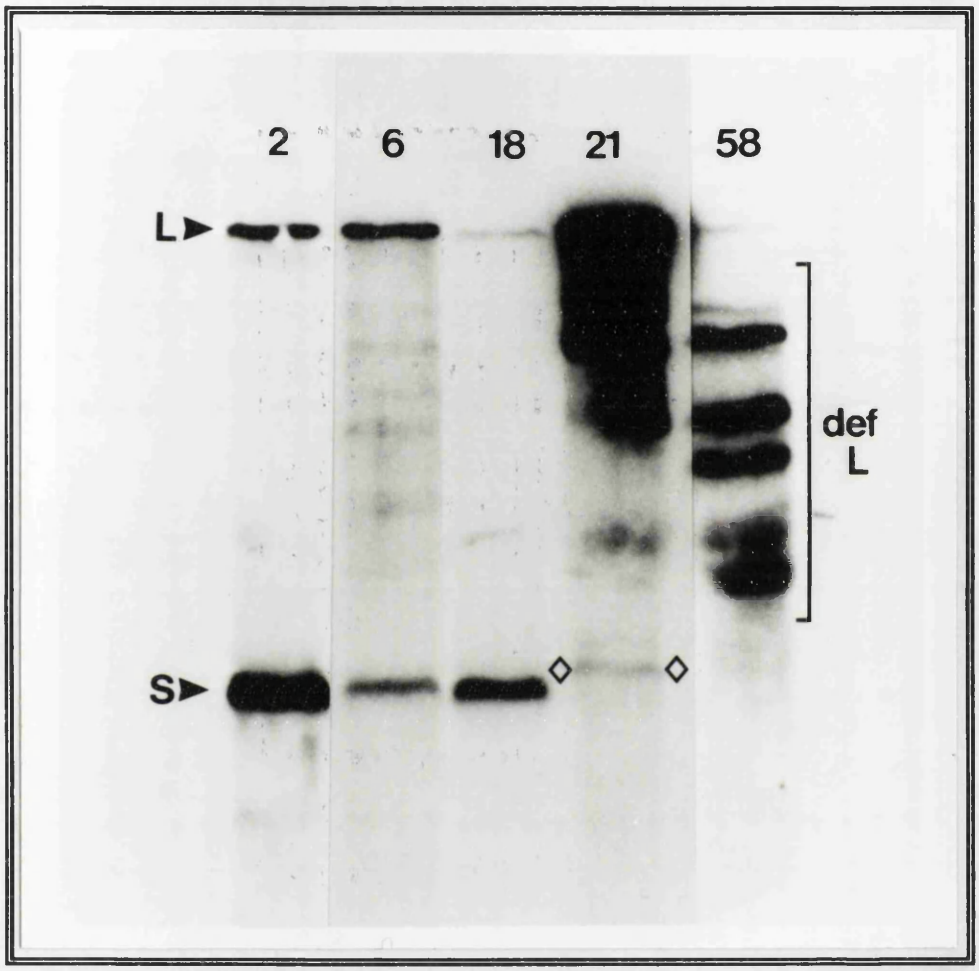

Figure 6.13. Northern blot analysis of total cell RNA extracted from passage levels 2, 6, 18, 21 and 58 of the C6/36/BUN cell line. 20μg of RNA from each passage level was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide and transferred to a nitrocellulose filter. The filter was hybridized sequentially, first with a genomic L segment specific [^{32}P]-labelled riboprobe, and then with a genomic S segment specific [^{32}P]-labelled riboprobe. ◇ indicates S RNA species migrating more slowly.

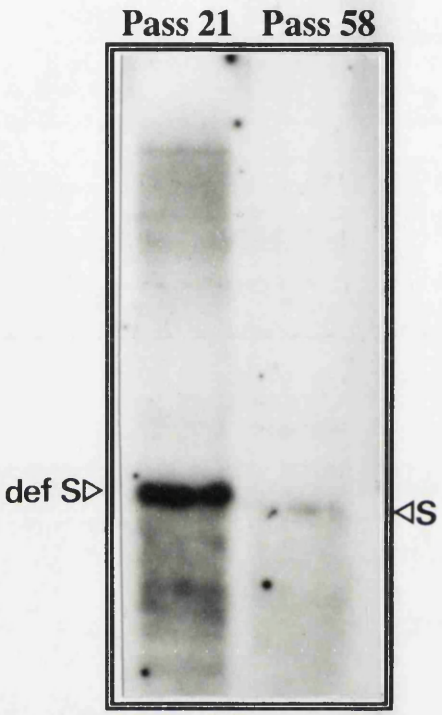

Figure 6.14. Northern blot analysis of total cell RNA from passage levels 21 and 58 of the C6/36/BUN cell line. 20μg of RNA from each of these passage levels was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with a [^{32}P]-labelled riboprobe specific for Bunyamwera virus genomic S RNA.

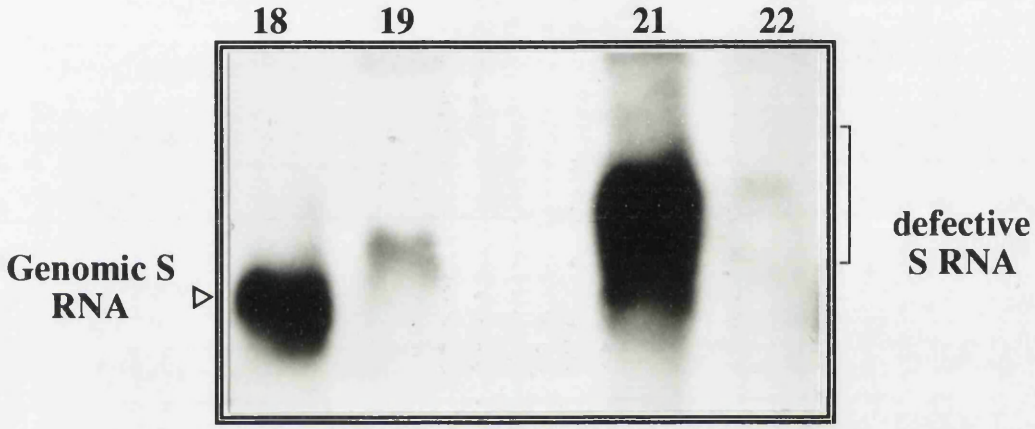

Figure 6.15. Northern blot analysis of total cell RNA extracted from passage levels 18, 19, 21 and 22 of the C6/36/BUN cell line. 15μg of RNA from each passage level was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with a [^{32}P]-labelled riboprobe specific for Bunyamwera virus genomic S RNA.

6.9 Encapsidation of viral RNA species in the C6/36/BUN cell line

In addition to preparing total cell RNA from each passage level of the C6/36/BUN cell line, encapsidated viral RNA was isolated by banding intracellular viral nucleocapsids on CsCl gradients and subsequent extraction using the acid-phenol-guanidinium-thiocyanate protocol of Chomczynski and Sacchi (1987). Encapsidation of viral RNAs in the persistently infected cells was investigated by comparing Northern blots of total cell RNA and nucleocapsid derived viral RNA from the same passage level of the C6/36/BUN cell line.

Analysis of nucleocapsid derived RNA from passage level 18 revealed that the only positive-sense viral RNA species detected in intracellular nucleocapsids was positive-sense S RNA (Figure 6.16a). Similarly analysis of nucleocapsid derived RNA from passage levels 18 and 21 revealed that genomic S RNA was preferentially encapsidated over the other negative-sense viral RNA segments (Figure 6.16b). Interestingly, there appeared to be a strong selection for packaging of authentic sized genomic S RNA in the persistently infected cells; at passage 21, when full-length and defective L RNA species and an aberrant S RNA species were abundant (see Figure 6.13), only authentic sized S RNA, which was far less abundant than either of the former RNA species, was encapsidated (Figure 6.17). It appeared therefore that the defective L RNAs derived from the L RNA segment were not efficiently assembled into nucleocapsids. To investigate this further, total cell RNA and nucleocapsid derived RNA samples from passages 18, 19, 21 and 22 of the C6/36/BUN cell line were fractionated on a denaturing agarose gel and transferred to a nitrocellulose filter which was probed sequentially, first with a genomic L segment specific riboprobe, and then a genomic S segment specific riboprobe (Figure 6.18). The result further emphasised the exclusive encapsidation of full-length genomic S RNA at passage level 21, despite its low abundance, while the defective viral RNAs prevalent at this passage level and full-length genomic L RNA remained uncomplexed with N protein.

In this analysis full-length genomic L RNA and some defective L segment derived RNAs were detected amongst the encapsidated RNA species extracted from passage level 18. Indeed when nucleocapsid derived RNA from passage 18 was probed solely for genomic L RNA sequences a range of size classes of defective L RNAs were detected, including a defective RNA not much larger than 1kb in length, and possibly an L RNA species larger than full-length Bunyamwera virus genomic L RNA (Figure 6.19). The encapsidated L segment specific RNAs observed at passage 18 were however present in low abundance relative to encapsidated genomic S RNA (Figure 6.18). Clearly there was a strong selection for encapsidation of standard viral RNA species in the persistently infected cells. In addition full-length S RNA species predominated over full-length L and M RNA species in intracellular nucleocapsids.

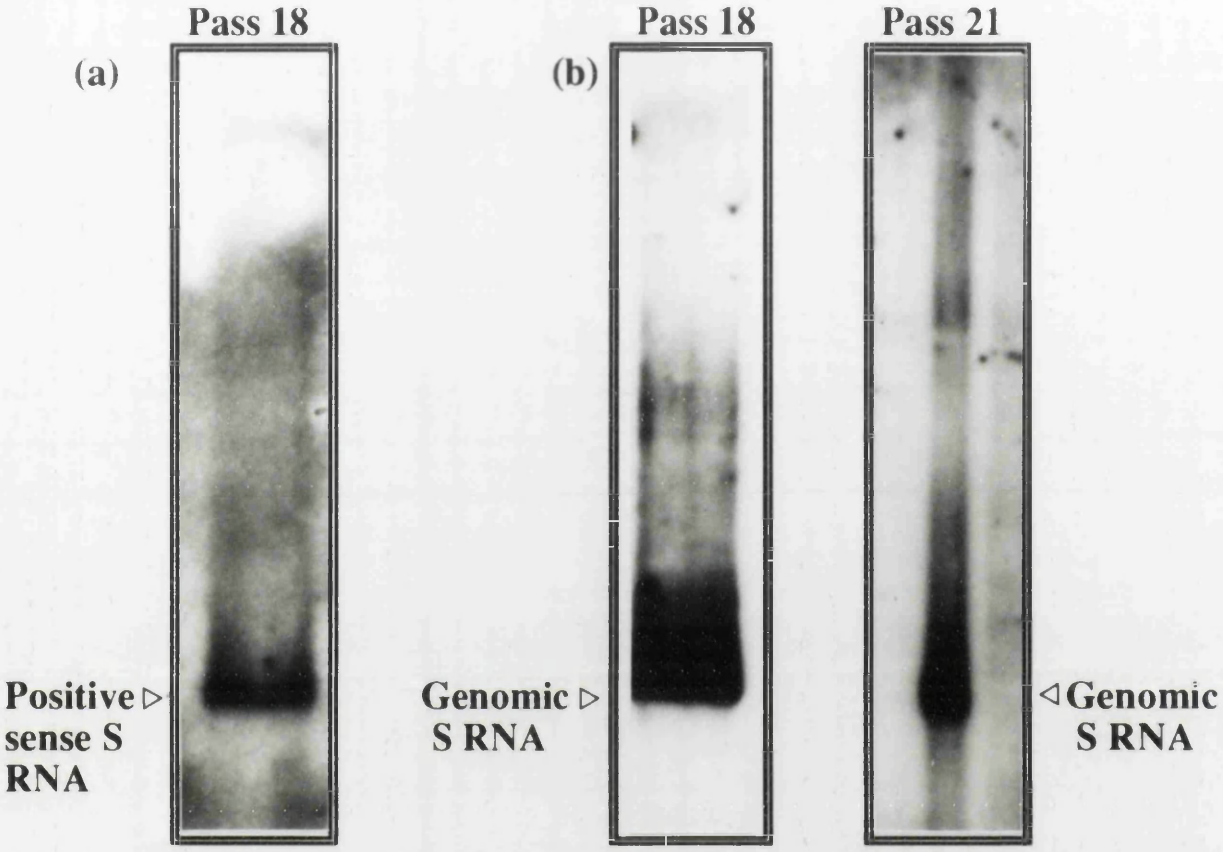

Figure 6.16 Northern blot analysis of encapsidated viral RNA species in the C6/36/BUN cell line. Samples of intracellular-nucleocapsid derived RNA were fractionated on a 1.5% agarose gel containing 5mM methyl mercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with segment and sense-specific [^{32}P]-labelled probes: (a) One tenth of the total yield of encapsidated RNA from passage level 18 was probed with [^{32}P]-labelled cDNA probes (L7, M6 and S9) specific for positive-sense Bunyamwera virus L, M and S RNA species; (b) One tenth of the total yield of encapsidated RNA from passage levels 18 and 21 was probed with [^{32}P]-labelled riboprobes specific for Bunyamwera virus genomic L, M and S RNA species.

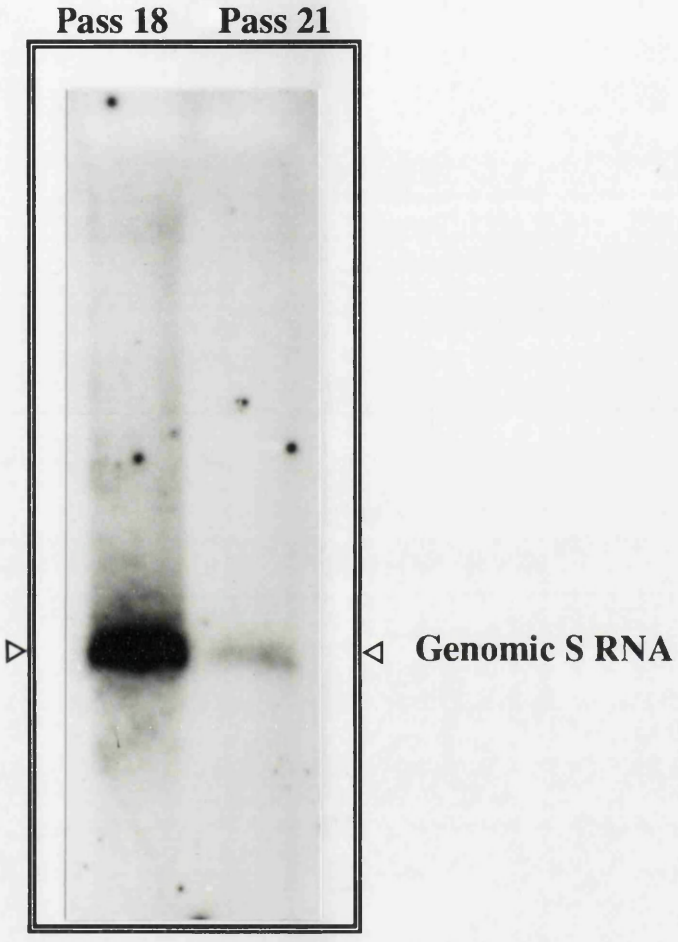

Figure 6.17. Northern blot analysis of nucleocapsid-derived RNA from passage levels 18 and 21 of the C6/36/BUN cell line. One tenth of the total yield of nucleocapsid-derived RNA from each passage level was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with a [^{32}P]-labelled riboprobe specific for Bunyamwera virus genomic S RNA.

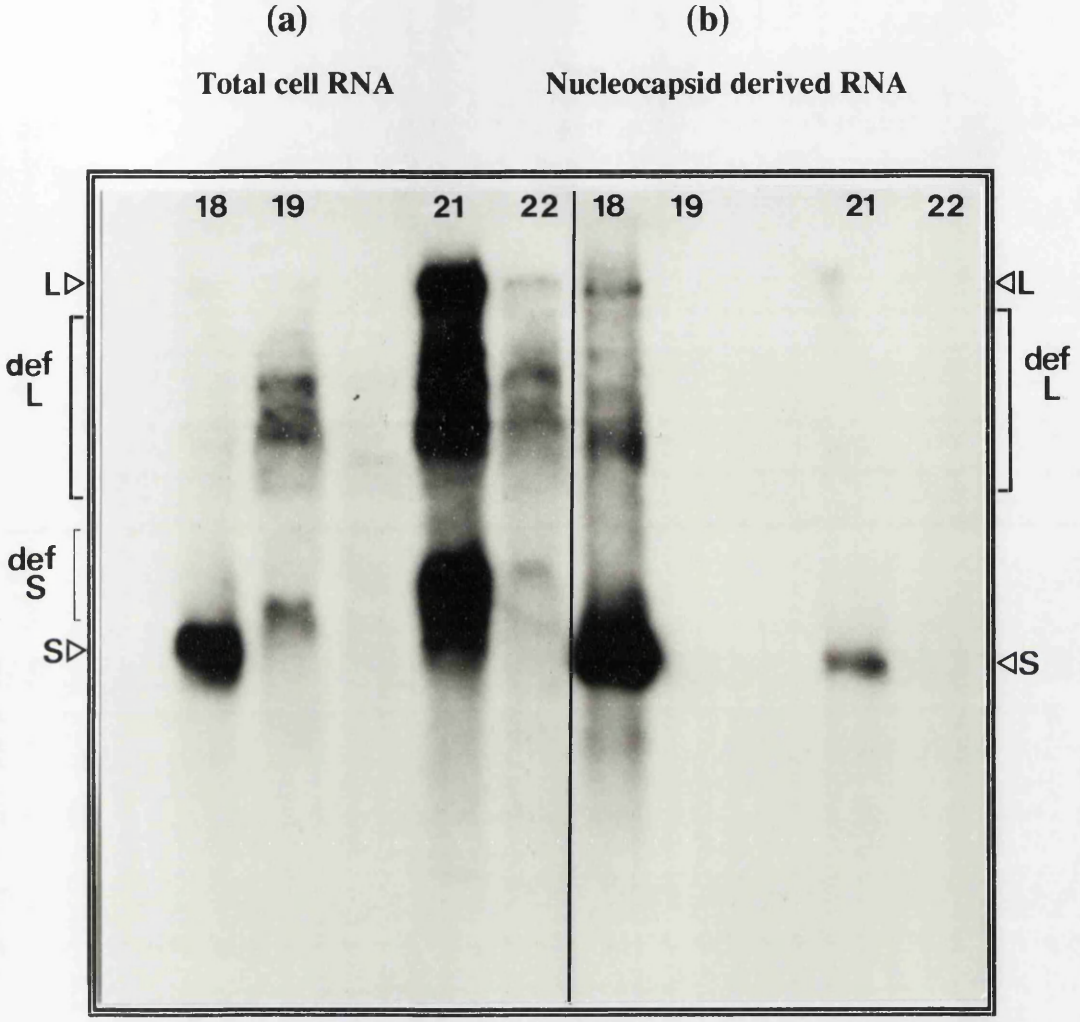

Figure 6.18. Northern blot analysis of (a) total cell RNA and (b) nucleocapsid-derived RNA from passage levels 18, 19, 21 and 22 of the C6/36/BUN cell line. 12μg of total cell RNA (one twenty-fifth of the total yield), and one tenth of the total yield of nucleocapsid-derived RNA, from each passage level were fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized sequentially first with a Bunyamwera virus genomic L segment specific riboprobe and then a genomic S segment specific riboprobe. Both riboprobes were labelled with [^{32}P].

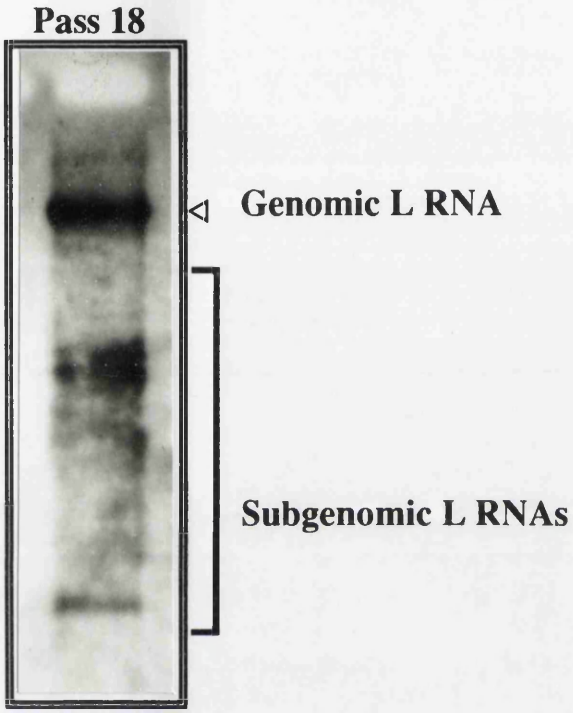

Figure 6.19. Northern blot analysis of nucleocapsid derived RNA from passage level 18 of the C6/36/BUN cell line. One tenth of the total yield of intracellular-nucleocapsid derived RNA from passage level 18 was fractionated on a 1.5% agarose gel containing 5mM methylmercury hydroxide. The RNA was transferred to a nitrocellulose filter which was hybridized with a [^{32}P]-labelled riboprobe specific for Bunyamwera virus genomic L RNA.

The detection of encapsidated full-length and defective L RNA species at passage level 18 (albeit at low levels relative to the levels of encapsidated genomic S RNA), coincided with relatively high levels of positive-sense S RNA in the C6/36/BUN cell line (see Figure 6.11). High levels of positive-sense S RNA probably represent high levels of S mRNA, and if viral mRNAs were efficiently translated in the persistently infected cells at this time, relatively high levels of N protein would be present in the cells at passage level 18. This would relieve any competition for encapsidation which might exist in the persistently infected cells when N protein availability is limited. Elliott and Wilkie (1986) reported that levels of Bunyamwera virus N protein did fluctuate between passage levels of their C6/36-PI HI and C6/36-PI LO cell lines. Thus the changing profiles of encapsidated RNA species in the persistently infected cells may to some extent reflect the relative abundance of the viral N protein at each passage level.

6.10 Summary

Direct RNA sequence analysis of the S RNA segment of viruses plaque-purified from culture fluid of the C6/36-PI LO cell line (Elliott & Wilkie, 1986) revealed a low level of genetic drift in the S RNA of Bunyamwera virus during persistent infection of C6/36 cells. The variant N protein-containing viruses reported to arise during propagation of the persistently infected C6/36-PI LO cell line (Elliott & Wilkie, 1986) were determined to be contaminating temperature-sensitive bunyaviruses, not endogenous to the persistently infected cell line.

A new C6/36 cell line persistently infected with Bunyamwera virus (C6/36/BUN) was established, and passaged for over a year without observable cpe. Once the persistent infection was established, levels of released virus fluctuated between 10^2 and 10^7 pfu/ml. Small, cloudy plaque-phenotype variants were detected in the culture fluid of the C6/36/BUN cell line before passage 5 and predominated by passage 15. An infectious centres assay carried out at passage 6 indicated that 2% of the cells in the persistently infected culture shed infectious virus.

Levels of intracellular viral genomic RNA varied between passage levels. Each species of viral RNA varied independently and no simple correlation between the fluctuating levels of viral RNA and titres of released virus was evident. Levels of positive-sense S RNA varied independently of levels of genomic S RNA. Positive-sense S RNA was detected at all passage levels with maximum levels at passages 8, 9 and 18.

Subgenomic L segment derived RNAs were detected in the C6/36/BUN cell line. These defective RNAs were present at low levels at passage 2 and were dominant over full-length viral RNA species by passage 58. Aberrant S RNA species which were larger than the standard genomic RNAs were detected at passages 19, 21 and 22.

Full-length S RNA species (both positive- and negative-sense) were preferentially encapsidated over full-length L and M segment RNAs. However the aberrant S RNAs detected at passage levels 19, 21 and 22 were never observed in nucleocapsid structures.

The defective L RNAs were inefficiently encapsidated by the viral N protein, and were observed in nucleocapsid structures only at passage level 18, coinciding with high levels of positive-sense S RNA. It is possible that high levels of positive-sense S RNA represent elevated levels of S mRNA and thus increased levels of N protein, which may allow encapsidation of the defective L RNAs in addition to the more efficiently encapsidated viral RNA species (in particular full-length S RNA). Therefore the availability of N protein, rather than the absence of a specific encapsidation signal, may determine whether or not defective viral RNAs are encapsidated and possibly packaged into defective virions. The observation that full-length Bunyamwera virus S RNA is the most efficiently encapsidated viral RNA species in persistently infected C6/36 cells agrees with the observation that virions, containing predominantly S RNA, are released from persistently infected C6/36 cells (Elliott & Wilkie, 1986).

7 Analysis of cell lines cloned from the C6/36/BUN culture

The C6/36 cell line used in the course of this work was cloned by Igarashi (1978) from an *Aedes albopictus* cell line derived from the macerated tissues of mosquito larvae (Singh, 1967). While the original uncloned Singh cell line was likely to have consisted of a mixed cell population containing partially differentiated cells which were destined for particular tissues of the mature mosquito, the cloned C6/36 cell line was expected to consist of a homogeneous cell population. However, microscopic examination of our uninfected (C6/36) and persistently infected (C6/36/BUN) mosquito cell cultures revealed variation in cell morphology within both cultures. This variation may have been due to individual cells being at different stages in the cell cycle, but may also have reflected cell heterogeneity within the cultures. Variations within other clonal cell lines have been reported (Varma et al., 1979).

Cell heterogeneity can arise during the course of a persistent infection, as exemplified by the rapid generation of cell heterogeneity in cultures of cloned BHK-21c1 cells persistently infected with FMDV (de la Torre et al., 1985). To determine if there was heterogeneity in the response of individual cells within the C6/36/BUN culture to persistent Bunyamwera virus infection, cells were cloned from four different passages of the C6/36/BUN cell line: pass 12 (p12), pass 20 (p20), pass 30 (p30) and pass 58 (p58). Those cells which gave rise to stable cloned cell lines were tested for their ability to produce infectious virus and to resist superinfection by wtL_9BUN virus. In addition the levels of viral RNA resident in each of the cloned cell lines derived from p20 and p58 of the C6/36/BUN culture were determined.

7.1 The ability of cells cloned from p12, p20, p30 and p58 of the C6/36/BUN culture to give rise to stable cell lines

Cells were cloned directly from the ongoing pass 58 of the C6/36/BUN cell line, while cells from the earlier passes (p12, p20 and p30) had to be revived from liquid nitrogen stocks. The cells recovered well from storage and confluent (80cm^2) monolayers were grown within one week. The resultant cell lines, p12', p20' and p30' continued to grow well and were subcultured at approximately weekly intervals; at no time was generalized cpe observed. The release of small, cloudy plaque-phenotype virus from the cells and the detection of Bunyamwera virus genomic RNA in these cells indicated that the cultures remained persistently infected (Figure 7.1).

Initial attempts to clone individual cells from p58 by seeding single cells into individual wells of a 96 well plate were unsuccessful. Numerous wells were successfully seeded with single cells, but only wells seeded with two or more cells gave rise to monolayers. Isolated single cells were prone to cytolysis. It would be interesting to

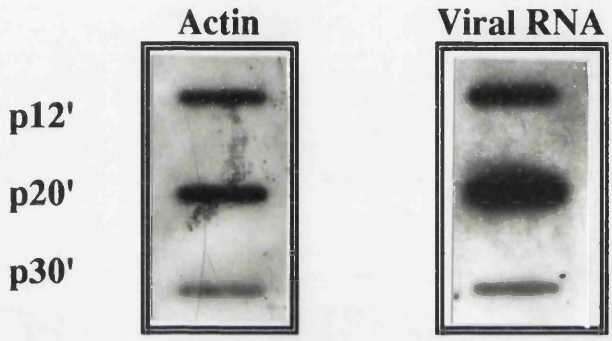

Figure 7.1. Levels of Bunyamwera virus genomic RNA resident in cells revived from frozen stocks (originating from passes 12, 20 and 30 of the C6/36/BUN cell line). Total cell RNA was prepared from each of the revived cell lines and aliquots of RNA were applied in duplicate to nitrocellulose using a vacuum manifold. Replicate strips of nitrocellulose were then hybridized either with a mixture of [^{32}P]-labelled cDNA probes specific for each Bunyamwera virus genomic RNA segment (i.e. to detect negative-sense L, M and S RNA) or with a [^{32}P]-labelled actin gene cDNA probe to estimate the relative amount of RNA applied with each sample.

determine if cell death in these instances was due to virus induced lysis of isolated cells, but this question was not persued during the course of this work.

Clonal cultures were obtained by seeding 100mm diameter tissue culture plates with at least 20 cells from a single-cell suspension (see section 3.9.3). The cells were left at 28°C for two weeks after which time colonies of cells were visible to the eye. The colonies were then transferred to individual wells of a 24 well plate. After transfer most of the clones, but not all, grew well giving rise to stable cloned cell lines. Some variation was observed in the rate of growth of individual clonal cell lines, for instance the clones derived from p20' grew more slowly (at least initially) than clones from p12', p30' and p58. A small minority of the cloned cell lines exhibited cpe. One of the p58 clones (p58c9) displayed extensive cpe after transfer to the multiwell plate, but recovered over the course of 10 days and gave rise to a stable cell line. Two out of 18 clones derived from p20 failed to establish stable cell lines but exhibited extensive cpe leading to total cell destruction after transfer to the multiwell plate. 100% of the cloned cells from p12' and p30' gave rise to stable cell lines without any signs of generalised cpe.

7.2 Virus release from the cloned cell lines

Samples of supernatant culture fluid from the revived parental cell lines p12', p20'and p30', from pass 66 of the C6/36/BUN culture and from each of the cloned cell lines were assayed for infectious virus. The culture fluid was sampled 48 h after subculture of the cell lines and was titrated on BHK cell monolayers (see Table 7.1). Only small, cloudy plaque-phenotype virus was detected. Overall, the proportion of the cloned cell lines which released detectable levels of virus decreased with increasing passage level of the parental cell lines. p12' and p30' released similar levels of virus ($<1 \times 10^2$ pfu/ml) however, 57% of p12' derived clones released detectable levels of virus compared to only 6% of p30' derived clones. At p66 no virus was detected in the culture fluid of the C6/36/BUN culture by plaque assay on BHK cell monolayers. However, supernatant culture fluid from p55 of C6/36/BUN cell line did produce cpe in BHK cell monolayers. All of the cloned cell lines derived from p20' released relatively high levels of virus (on average 2×10^5 pfu/ml). Given the irregular fluctuations in the titre of virus released over the first 30 passages of the C6/36/BUN culture (see Figure 6.7) it is not suprising that variation was also observed in the levels of virus released from each set of cloned lines.

7.3 Determination of the susceptibility of the cloned cell lines to superinfection with wtL$_9$BUN virus

Each of the cloned cell lines as well as the parental cell lines p12', p20', p30' and the ongoing pass of the C6/36/BUN cell line (p66) were tested for their ability to support the replication of wtL$_9$BUN virus. 48 h after superinfection, the supernatant culture fluid was harvested and titrated on BHK cell monolayers, along with control culture fluid from

	Virus release from the cloned cell lines (pfu/ml)			
	p12'	**p20'**	**p30'**	**p58**
Clone 1	1×10^2	2×10^3	——	——
Clone 2	——	3×10^5	——	——
Clone 3	1×10^2	1×10^5	——	——
Clone 4	——	3×10^5	——	——
Clone 5	——	6×10^5	——	——
Clone 6	——	1×10^5	——	——
Clone 7	$< 1 \times 10^2$	2×10^5	——	——
Clone 8	——	3×10^5	——	——
Clone 9	——	2×10^5	——	——
Clone 10	$< 1 \times 10^2$	2×10^4	——	——
Clone 11	$< 1 \times 10^2$	2×10^5	——	10
Clone 12	$< 1 \times 10^2$	1×10^5	——	——
Clone 13	$< 1 \times 10^2$	4×10^5	——	N/A
Clone 14	$< 1 \times 10^2$	10	10	N/A
Clone 15	N/A	8×10^5	——	N/A
Clone 16	N/A	1×10^5	——	N/A
Average titres of virus released from the clones	$< 1 \times 10^2$	2×10^5	——	——
% of cloned cell lines releasing virus	57%	100%	6%	8%
Virus release from parental cell lines	**(p12')** $< 1 \times 10^2$	**(p20')** 6×10^4	**(p30')** $< 1 \times 10^2$	**(p66)** ——

Table 7.1. Virus titres in the supernatant culture fluid of each of the cloned cell lines 48 h after subculture. The indistinct plaque-phenotype of the virus produced by the persistently infected cells made accurate counting difficult and therefore the titres given are approximate. The limit of detection of the plaque assay was 10 pfu/ml (—— denotes levels of virus below the limit of detection; N/A, not applicable).

non-superinfected cultures. Cell-released wtL$_9$BUN virus was easy to distinguish from background virus released from the persistently infected cultures due the marked contrast in plaque-phenotype. The results of the assay are displayed in Table 7.2.

The parental cell lines p12' and p30' displayed significant resistance to superinfection with wtL$_9$BUN virus and produced 9 x 10^2 and 8 x 10^2 pfu/ml of wtL$_9$BUN virus respectively, compared to 1.4 x 10^9 pfu/ml produced by uninfected C6/36 cells. However, the stable cloned cell lines derived from p12' and p30' did not display this degree of resistance; the most marked reduction in virus yield compared to uninfected cells was a 2 Log reduction exhibited by two p12' clones, p12'c11 and p12'c12. The parental cell line p20' also displayed resistance to superinfection although not to the same extent as p12' and p30'; superinfected p20' cells produced 1.1 x 10^5 pfu/ml of wtL$_9$BUN virus, 4 Logs less than uninfected C6/36 cells, but 3 Logs above the levels of virus produced by the p12' and p30'cell lines. However, all of the cloned cell lines derived from p20' were resistant to superinfection, although again to varying degrees. By pass 66 of the C6/36/BUN cell line the persistently infected culture no longer displayed resistance to superinfection with homologous virus but supported the replication of wtL$_9$BUN virus, producing relatively high yields (1.5 x 10^8 pfu/ml). The levels of the wild-type virus produced by p66 of the C6/36/BUN cell line were one Log lower than the yields from the cloned cell lines derived from p58.

7.4 Presence/absence of Bunyamwera virus RNA in the cloned cell lines

After 10 passages of the cloned cell lines, total cell RNA was extracted from each of the lines derived from p20' and p58 using the method of Chomczynski and Sacchi (1987). The levels of viral genomic RNA/cell in each cell line were compared by performing parallel hybridizations with virus and cell specific RNA probes. Duplicate samples of RNA (of approximately 8μg) were immobilised onto nitrocellulose using a Schleicher and Schuell Minifold II slot blot apparatus and were hybridized either with a mixture of [^{32}P]-labelled cDNA probes specific for Bunyamwera virus L, M and S genomic RNA or with a [^{32}P]-labelled probe specific for actin mRNA. Determination of the relative levels of actin mRNA in each sample (assuming that actin mRNA levels remain relatively constant) enabled variations in the quantity of total cell RNA applied to the nitrocellulose to be monitored and corrected for. After autoradiography the slot blots were scanned by densitometry and variations in the levels of viral genomic RNA were normalized against actin mRNA levels. The results are presented graphically in Figure 7.2.

Variation was observed in the levels of viral RNA resident in individual clones derived from the same passage level of the C6/36/BUN cell line. Seven of the sixteen p20' clones hybridized strongly for viral genomic RNA, while only one of the fifteen p58 derived cloned cell lines appeared to contain relatively high levels of viral RNA. In

	Yields of wtL$_9$BUN virus (pfu/ml)			
	p12'	**p20'**	**p30'**	**p58**
Clone 1	3 x 10^8	**2 x 10^5**	1 x 10^9	4 x 10^9
Clone 2	2 x 10^8	**3 x 10^4** *	2 x 10^9	2 x 10^9
Clone 3	7 x 10^8	4 x 10^7 *	2 x 10^9	3 x 10^9
Clone 4	3 x 10^8	**6 x 10^4**	2 x 10^9	3 x 10^9
Clone 5	1 x 10^9	**3 x 10^4**	4 x 10^8	2 x 10^9
Clone 6	3 x 10^8	**3 x 10^4**	2 x 10^9	2 x 10^9
Clone 7	1 x 10^9	**6 x 10^4**	7 x 10^8	3 x 10^9
Clone 8	9 x 10^8	**1 x 10^4**	7 x 10^8	4 x 10^9
Clone 9	5 x 10^8 *	1 x 10^7 *	9 x 10^8	4 x 10^9
Clone 10	2 x 10^9 *	**2 x 10^4**	ND	2 x 10^9
Clone 11	3 x 10^7 *	**8 x 10^4**	3 x 10^9	5 x 10^9
Clone 12	7 x 10^7 *	**4 x 10^4**	5 x 10^8	1 x 10^9
Clone 13	8 x 10^8 *	**2 x 10^4**	7 x 10^8	N/A
Clone 14	1 x 10^9 *	**3 x 10^4**	3 x 10^9	N/A
Clone 15	N/A	**4 x 10^4**	1 x 10^8	N/A
Clone 16	N/A	**5 x 10^4**	1 x 10^9	N/A
Parental cell lines	**(p12')** **9 x 10^2**	**(p20')** **1 x 10^5**	**(p30')** **8 x 10^2**	**(p66)** 2 x 10^8
Yield from uninfected *Aedes albopictus* C6/36 cells = 1.4 x 10^9 pfu/ml				

Table 7.2. Susceptibility of the parental and cloned cell lines to superinfection with wtL$_9$BUN virus, expressed as extracellular levels of wild-type Bunyamwera virus in the supernatant culture fluid (pfu/ml). Bold type denotes a greater than 4 log reduction in virus yield relative to uninfected C6/36 cells; ND (no data); N/A (not applicable). The cloned and the parental cell lines were passaged a number of times prior to the superinfection assay: p12' derived clones c1 to c8 and all of the p20' derived clones apart from c2, c3 and c9 were passaged 3 times; p12' derived clones c9 to c14 and p20' c2, c3 and c9 were passaged 7 times (*), while p30' and p58 derived clones went through 9 and 12 passages respectively. The revived parental cell lines: p12', p20'and p30' were passaged 12 times prior to the superinfection assay. The ongoing pass of the C6/36/BUN cell line (p66) was also tested for resistance to superinfection.

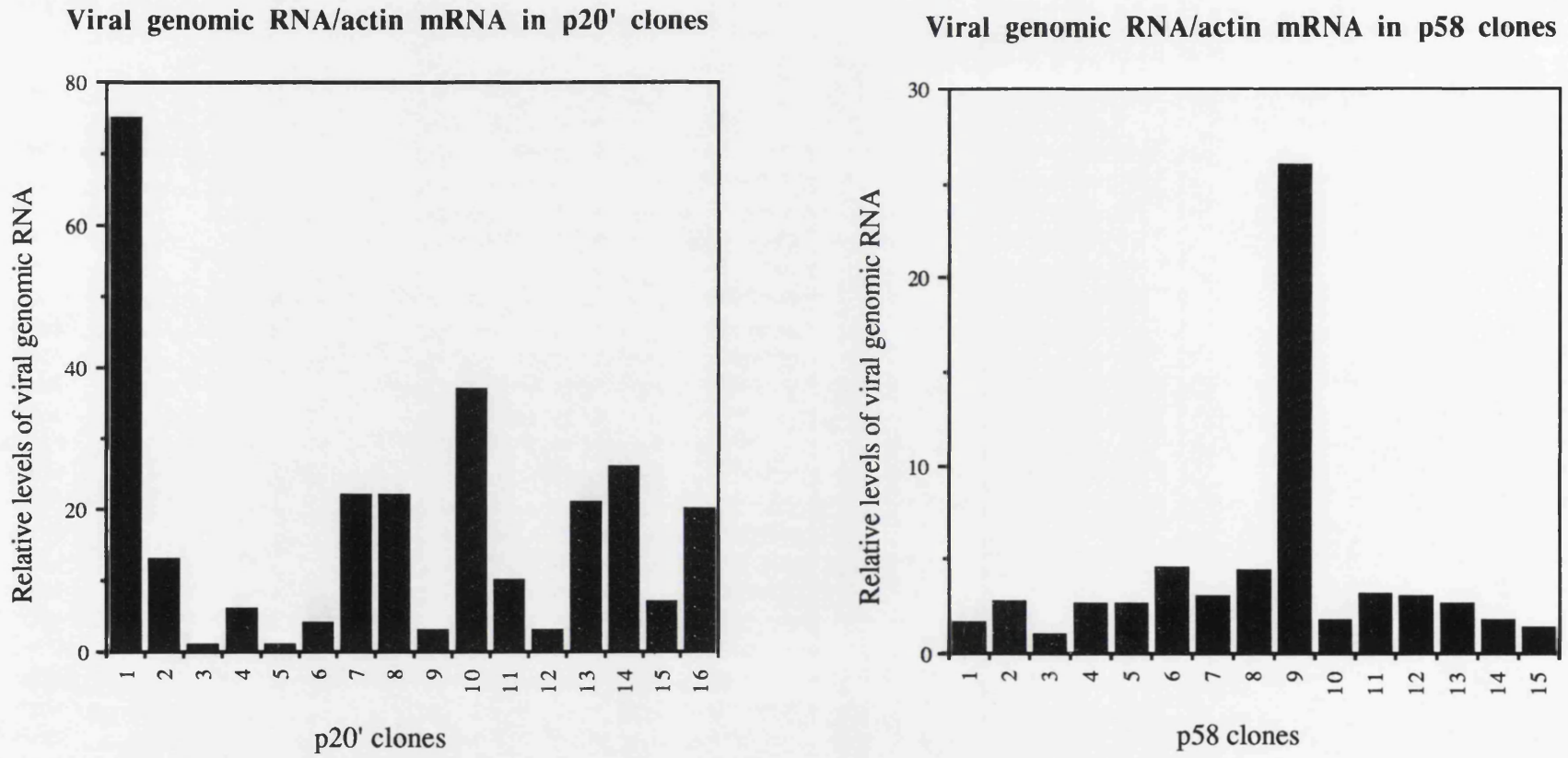

Figure 7.2. Relative levels of Bunyamwera virus genomic RNA, relative to actin mRNA, resident in each of the cloned cell lines derived from pass 20' and pass 58 of the C6/36/BUN culture. RNA levels were determined by densitometric analysis of slot blot data. The slot blot analysis was carried out on duplicate samples of total cell RNA (of approximately 8μg) which were immobilised onto nitrocellulose using a vacuum manifold. Replicate strips of nitrocellulose were then hybridized with a mixture of [^{32}P]-labelled cDNA probes specific for Bunyamwera virus genomic RNA, or with a [^{32}P]-labelled actin gene cDNA to estimate the relative amount of RNA applied in each sample.

general, the levels of viral RNA observed in the p20' clones were significantly higher than for the p58 clones and while most of the p20' derived cloned cell lines clearly harboured viral RNA sequences some of the cloned cell lines derived from passage 58 gave weak hybridization signals which were not significantly above background levels (see Figure 7.2).

Four of the p20' cloned cell lines (c1, c3, c5 and c8), which represented cultures containing high (c1 and c8) and low (c3 and c5) levels of Bunyamwera virus genomic RNA, were selected for Northern analysis to determine the relative levels and the nature of the genomic L and S RNA species resident in each of these cell lines (Figure 7.3). Full-length Bunyamwera virus genomic L RNA was present at significant levels in p20' c5 and p20' c8 and at very low levels relative to the defective L RNA in p20' c1 while the levels of full-length genomic L RNA in p20' c3 were too low to be detected by Northern analysis. However, each of the four cloned lines contained a single species of defective L RNA. For three out of the four clones the defective L RNA predominated over full-length genomic L RNA but in p20' c8 similar levels of both L RNA species were present. Three distinct size classes of subgenomic L RNA were observed amongst the four cloned cell lines.

The levels of actin mRNA contained in each of the RNA samples varied (Figure 7.3c), indicating that more total cell RNA was examined from p20' c3 and p20' c5 than from p20' c1 and p20' c8. Consequently the viral RNA species in p20' c3 and p20' c5 were over represented on the Northern blots relative to p20' c1 and p20' c8. Taking this into account the relative levels of Bunyavirus genomic L and S RNAs observed in the cloned cell lines by Northern analysis were in overall agreement with the data from the slot blot hybridizations carried out with partial [^{32}P]-labelled cDNA probes (Figure 7.2). There may be some discrepancy between the results for p20' c5 which could be a consequence of the different probes used, since it is possible that the small defective L RNA in p20'c5 might not have been detected by the [^{32}P]-labelled cDNA probe used for the initial slot blot analysis. The cDNA probe was specific for sequences between nucleotides 997 and 1436 of Bunyamwera virus genomic L RNA (see Section 3.5.3) which may be deleted from the defective L RNA in the p20' c5 cell line. It would be interesting to hybridize a Northern blot of p20' c5 RNA with the partial probe to determine if this region is deleted from the defective L RNA.

Full-length Bunyamwera virus genomic S RNA was detected in the p20' c5 and p20' c8 cell lines but not in the other two cloned cell lines (Figure 7.3b). The presence of detectable levels of full-length genomic S RNA coincided with the highest levels of full-length genomic L RNA, although the ratio of Bunyamwera virus negative-sense L RNA species to negative-sense S RNA species varied between the clones. In p20' c8, genomic S RNA was more abundant relative to both of the L RNA species compared to the RNA profile for p20' c5.

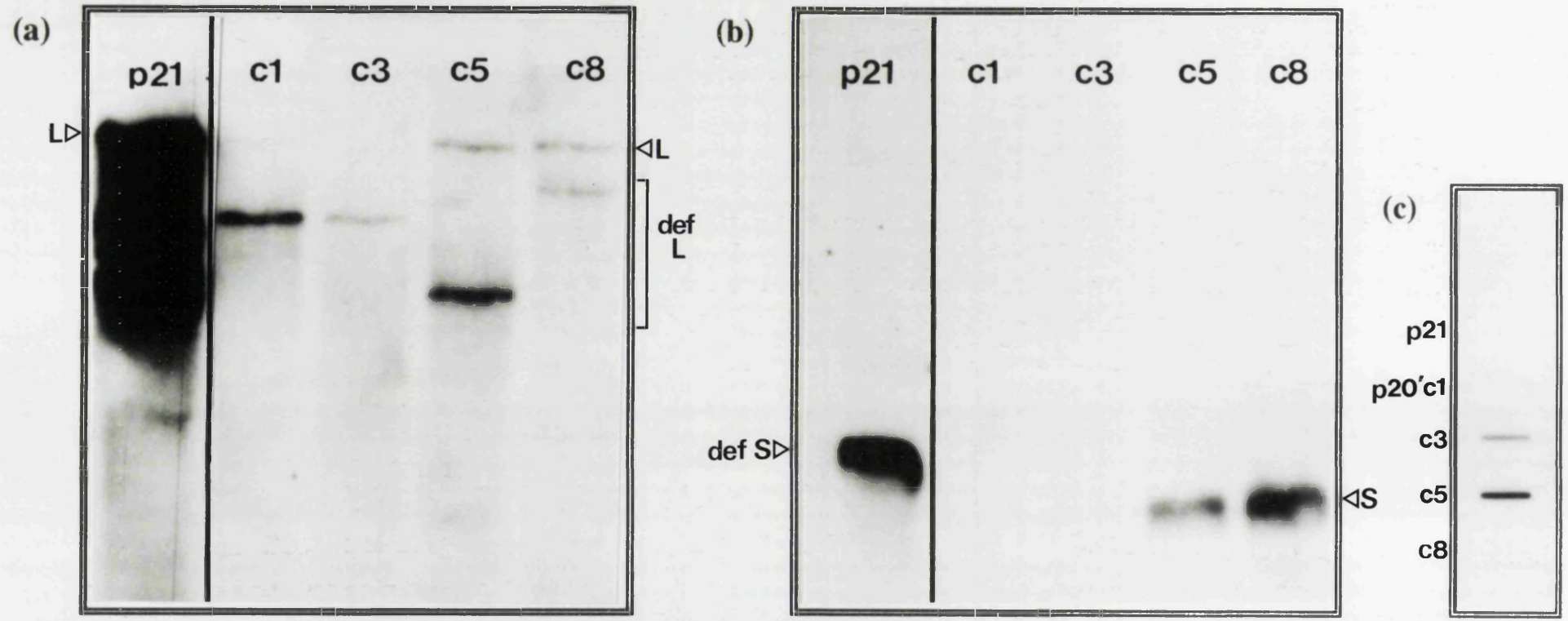

Figure 7.3 Northern analysis of Bunyamwera virus genomic L and S RNA species resident in the cloned cell lines: p20'c1, p20'c3, p20'c5 and p20'c8; derived originally from passage 20 of the C6/36/BUN cell line. Duplicate samples of total cell RNA from each of these cloned cell lines were fractionated on 1.5% agarose gels containing 5mM methylmercury hydroxide. The RNA was then transferred to nitrocellulose filters which were hybridized with [^{32}P]-labelled riboprobes specific for Bunyamwera virus genomic L RNA (a) or genomic S RNA (b). An equal volume of each sample was applied to a nitrocellulose filter using a vacuum manifold. This filter was hybridized with a [^{32}P]-labelled actin gene cDNA probe to estimate the total amount of RNA applied in each sample (c). Total cell RNA from passage 21 was included in each blot to function as a positive control and a marker track.

Superinfection experiments (see Section 7.3) showed that clones p20' c5 and p20' c8 yielded less virus ($3x10^4$ pfu/ml and $1x10^4$ pfu/ml respectively) than p20' c1 ($2x10^5$ pfu/ml) and p20' c3 ($4x10^7$ pfu/ml); the yield from normal C6/36 cells was $1x10^9$ pfu/ml. Hence resistance of the persistently infected cells to superinfection with Bunymawera virus appeared to correlate with levels of full-length viral RNA present in the cells and not with the amount or occurence of defective L RNAs.

7.5 Summary

Stable cell lines were established from cells cloned from passages 12, 20 and 30 of the persistently infected C6/36/BUN cell line, which had been stored frozen. The revived cell lines (p12', p20' and p30') remained persistently infected as judged by their resistance to superinfection with wtL_9BUN virus, the presence of intracellular viral RNA sequences and the release of small, cloudy plaque-phenotype virus. All of the cell lines were passaged a number of times before they were analysed and consequently no longer represented passages 12, 20 and 30 of the original C6/36/BUN cell line.

Levels of virus released from the p12', p20' and p30' parental cell lines varied from below $1x10^2$ pfu/ml for the p12' and p30' cell lines, to $6x10^4$ pfu/ml for the p20' cell line. The percentage of virus producing clones derived from each parental cell line also varied; p12' (57%), p20' (100%), p30' (6%) and p58 (8%).

The ability of the parental and cloned cell lines to support the replication of wtL_9BUN virus was tested in superinfection experiments. wtL_9BUN virus replicated to high titres in cells from passage 66 of the C6/36/BUN culture and in cloned cell lines derived from passage 58 of the C6/36/BUN cell line, suggesting that the persistently infected culture had lost its ability to exclude superinfecting homologous virus at these higher passage levels. However the p12', p20' and p30' cell lines, and the cloned cell lines derived from them, displayed a range of magnitude of resistance to superinfection. The results suggested that (at least at relatively early passage levels) resistance to superinfection varied just as levels of released virus and levels of intracellular viral RNA varied in the persistently infected culture (see Chapter 6). No simple correlation between levels of production of the persistent, cloudy plaque-phenotype virus and superinfectability of the cell cultures was evident.

The level of viral genomic RNA resident in each of the p20' and p58 cloned cell lines was measured by slot blot analysis. In general, higher levels of viral RNA were detected in the p20' clones. Seven out of sixteen p20' clones contained relatively high levels of viral genomic RNA compared to one out of fifteen of the p58 clones. Northern blot analysis of RNA extracted from four of the p20' clones (p20' c1, p20' c3, p20' c5 and p20' c8) revealed that each clone harboured a single prominent species of defective L RNA. However, resistance to superinfection appeared to correlate more with the level of full-

length viral RNAs resident in the cloned cell lines than with the amount or occurence of defective L RNAs.

8 Discussion

During the course of this work, the molecular mechanisms leading to the establishment of persistent Bunyamwera virus infection of *Aedes albopictus* C6/36 cells, and the factors responsible for the maintenance of the persistent infection, were investigated. In this section, the results presented in the preceeding chapters will be discussed in the light of the findings of other research groups, particularly during the period since the commencement of this work. Possible directions for future research will be outlined.

8.1 Establishment versus maintenance of persistent viral infections

For some time it has been recognised that the establishment and maintenance of persistent viral infections should be considered independently. Recently, Borzakian *et al.* (1992) demonstrated that the establishment and maintenance of poliovirus persistence in a non-neural human cell line (HEp-2c) involve distinct mechanisms. Wild-type as well as attenuated strains of poliovirus can establish persistent infection in human neuroblastoma cells, and poliovirus mutants with altered biological properties can be isolated from these cells (Colbere-Garapin *et al.*, 1989). These variants have a highly mutated genome and an altered cell specificity, and can induce a secondary persistent infection in cells of non-neural origin (Pelletier *et al.*, 1991). Borzakian *et al.* (1992) used poliovirus mutants, derived from wild-type and attenuated strains of poliovirus by selection in neuroblastoma cells, to establish persistent infections in HEp-2c cells. They determined that while the establishment of persistence was dependent upon the virus strain and moi used, the maintenance of the persistent infection was dependent upon the evolution of mutant host cells within the persistently infected culture in which poliovirus multiplication was restricted.

In the above study, viral variants were used to establish the persistent infection. Other popular means of establishing persistent viral infections with RNA viruses are to co-infect cells with defective interfering particles and homologous standard virus preparations, or to use serially passaged stocks of virus and/or a high moi to initiate the infection. Each of these procedures increases the probability of the occurence of interference between standard and defective virus in the infected cells, which could then lead to persistence (see Section 2.2.6.4 and references therein). The widely documented use of defective interfering virus particles to establish persistent viral infections (e.g.Roux & Holland, 1979; Meinkoth & Kennedy, 1980; Weiss *et al.*, 1980; Weiss *et al.*, 1983; Cave *et al.*, 1985; Atkinson *et al.*, 1986; Poidinger *et al.*, 1991) and reports on the generation of defective interfering (DI) particles during the course of persistence in a number of *in vivo* and *in vitro* systems (Nuesch *et al.*, 1988; Nuesch *et al.*, 1989; Moscana, 1991) suggest that DI particles may have a role to play in many persistent viral infections. However,

arboviruses establish persistent infections in invertebrate cell cultures without co-inoculation of standard and defective viruses. When defective viral RNAs or DI particles have been detected during these infections, the defective species were usually not evident until well after the establishment of the persistent infection (Igarashi *et al.*, 1977; Eaton, 1981; Kascsak & Lyons, 1978; Elliott & Wilkie, 1986). There have been reports of the detection of Semliki Forest virus (SFV) DI RNAs within 24 h of infection of *Aedes albopictus* C6/36 cells (Tooker & Kennedy, 1981; Stalder *et al.*, 1983). However, since cloned virus stocks were not used in these studies, the possibility exists that the defective SFV RNAs were introduced with the virus inoculum.

In the system described in this thesis, working stocks of biologically cloned Bunyamwera virus readily established persistent infection in *Aedes albopictus* C6/36 cells. The newly initiated persistent infection mimicked that reported previously (Elliott & Wilkie, 1986), demonstrating the reproducibility of the bunyavirus system. Working stocks of Bunyamwera virus were prepared by a total of two low multiplicity passages, in BHK cells, of a triply-plaque purified viral isolate (wtL$_9$BUN). The low moi used, and the limited passage history of the wtL$_9$BUN virus preparation, were designed to limit the possibility of introducing defective virus into the stocks. In addition, defective viral RNAs could not be detected in the initial virus stocks by Northern blot analysis of virion RNA. The consensus sequence of the genomic S segment RNA of this virus preparation (which was obtained by primer extension sequencing using virion RNA as template and consequently represented virus which had undergone a total of three low multiplicity passages in BHK cells) revealed that the virus preparation remained homogeneous after these low multiplicity passages. The readiness with which this biologically cloned Bunyamwera virus preparation established a persistent infection of *Aedes albopictus* C6/36 cells, at a relatively low moi (5 pfu/cell), suggests that neither defective interfering virus nor viral variants are essential to the initiation of this persistent infection. Similar conclusions have been reached for the establishment of persistent La Crosse virus infection of *Aedes albopictus* C6/36 cells (Rossier *et al.*, 1988; Hacker *et al.*, 1989). However, certain characteristics of the persistently infected C6/36/BUN cell culture described herein, and of mosquito cell cultures persistently infected with other bunyaviruses, such as fluctuating titres of released virus and resistance to superinfection with homologous virus, suggest that DI genomes may arise once the persistent infection has been established (Newton *et al.*, 1981; Elliott & Wilkie, 1986; Holland, 1990; see Section 8.4).

8.2 Molecular events underlying the establishment of persistent viral infections

One of the difficulties encountered in studying persistent viral infections is distinguishing mechanisms fundamental to the maintenance of the persistent state from other phenomena which arise due to the long-term residence of virus in the host cell, but

which are not essential to the maintenance of the persistent infection. It therefore seemed pertinent to consider the early molecular events underlying the establishment of Bunyamwera virus persistence in *Aedes albopictus* C6/36 cells. Arboviruses are particularly valuable in this respect, as comparative analyses of the replication of these viruses in vertebrate and invertebrate cell cultures facilitates a direct comparison of the molecular mechanisms leading to the expression of the lytic and persistent phenotypes (respectively) of the same virus. The Alphaviridae and Bunyaviridae together account for most of the described arboviruses. More extensive molecular studies have been carried out on alphavirus persistence in invertebrate cell culture than on bunyavirus persistence, but in the past five years the balance has been redressed to some extent by a number of publications on La Crosse bunyavirus persistent infection of *Aedes albopictus* C6/36 cells (Rossier *et al.*, 1988; Hacker *et al.*, 1989). Besides the work presented here, only one other study has been undertaken to directly compare Bunyamwera virus infection of vertebrate and invertebrate cell lines. Newton *et al.* (1981) investigated Bunyamwera virus infection of BHK cells and an uncloned mosquito cell line (*Aedes albopictus* cells) at 37°C and 26°C respectively and followed gene expression, in both infected cell types, by metabolic labelling.

As reported in Chapter 5, detailed examination of viral and host protein synthesis, and of viral RNA synthesis, in Bunyamwera virus-infected BHK and C6/36 cells over 72 h pi, revealed a number of differences underlying the divergent, host-dependent, outcomes of infection. Following infection with Bunyamwera virus, host protein synthesis was inhibited in BHK cells but not in C6/36 cells, in agreement with the observations of Newton *et al.* (1981). Between 48 and 72 h pi the BHK cell cultures exhibited extensive cytopathic effects. However, the infected mosquito cell cultures continued to grow without any obvious cytopathic effects. The protein synthesis profile obtained for Bunyamwera virus-infected BHK cells was similar to the pattern of protein synthesis observed in La Crosse virus infected BHK cells by Raju & Kolakofsky (1988). In agreement with this report, the onset of the decline in host protein synthesis in Bunyamwera virus-infected BHK cells coincided with maximum levels of viral gene expression. However, in the Bunyamwera virus system, the rapid decrease in host protein synthesis observed between 10 and 16 h pi was not paralleled by an equivalent decline in viral protein synthesis. Viral proteins continued to be synthesised at high levels for a further 12 h (up to 24 h pi). In contrast, Raju & Kolakofsky (1988) reported that viral and cellular protein synthesis declined in parallel following La Crosse virus infection of BHK cells. They determined the steady-state levels of viral and host mRNAs in La Crosse virus-infected BHK cells and concluded that the decline in viral and cellular protein synthesis was due to a general mRNA instability induced in these cells following infection with La Crosse virus.

La Crosse virus infection of BHK cells (Raju & Kolakofsky, 1988) proceeds more rapidly than Bunyamwera virus infection, and the higher multiplicity of infection used

by Raju & Kolakofsky (20 to 50 pfu/cell) may also have contributed to the different rates of viral replication observed in the two systems. The slower progress of the Bunyamwera virus infection (initiated at 5 pfu/cell) enabled the decay in viral and cellular protein synthesis to be examined in more detail. In our hands, viral protein synthesis did not decay with the same kinetics as cellular protein synthesis in Bunyamwera virus-infected BHK cells. Whether or not this reflected a greater stability of viral mRNAs in Bunyamwera virus-infected BHK cells relative to host cell mRNAs is open to question. Raju & Kolakofsky (1988) demonstrated that viral mRNA synthesis continued at significant levels in La Crosse virus-infected BHK cells beyond 12 h pi (well after the decline in viral protein synthesis was observed). These authors suggested that, in these cells, the decrease in viral protein synthesis arose due to a net decrease in the rate of viral mRNA synthesis relative to mRNA degradation with time.

Raju & Kolakofsky (1988) presented evidence that the mRNA instability induced in BHK cells following La Crosse virus infection was not mediated via an interferon-activated latent cellular RNase. They demonstrated that the activity reponsible for the degradation was operative only when viral replication was at near maximal levels (in this case at 6 h pi) and was specific for mRNAs. They proposed that the endonuclease activity detected in La Crosse virions (Patterson *et al.*, 1984) might be responsible. The viral endonuclease activity is presumed to be a function of the viral polymerase (L protein) and the fact that the induction of mRNA instability paralleled viral gene expression supports a role for this enzyme in the induction of mRNA instability.

It is interesting, therefore, to speculate on the apparent lack of mRNA instability induction following bunyavirus infection of C6/36 cells. These cells support high levels of viral gene expression during the acute phase of infection (up to 24 h after infection with 5 pfu/cell of wtL$_9$BUN virus) without any overt detrimental effects upon the host cell. C6/36 cell derived bunyavirus mRNAs have heterogeneous non-templated primers at their 5' end (Hacker *et al.*, 1989), which are presumably derived from host cell mRNAs, as in infected BHK cells. The virion associated endonuclease activity is believed to perform the 'cap-snatching' activity responsible for generating primers from host mRNAs (Patterson *et al.*, 1984; Patterson & Kolakofsky, 1984). Therefore the virion associated endonuclease must function in infected C6/36 cells. If this activity is responsible for inducing mRNA instability in bunyavirus-infected mammalian cells what modulates this activity in C6/36 cells ?

The analysis of positive-sense viral RNA species in Bunyamwera virus-infected C6/36 cells and BHK cells (presented in Chapter 5) revealed a relatively low abundance of positive-sense L RNA species in infected C6/36 cells compared to BHK cells. This probably reflects lower levels of the L mRNA in Bunyamwera virus-infected C6/36 cells relative to BHK cells. Lower levels of La Crosse virus L mRNA were also detected in infected C6/36 cells relative to BHK cells infected under the same conditions (Rossier *et*

al., 1988). Lower levels of expression of the viral L protein in C6/36 cells would be consistent with mRNA instability in bunyavirus-infected mammalian cells being induced by the viral endonuclease activity. The destruction of host mRNAs would be expected to result ultimately in cell death, as is observed following bunyavirus infection of BHK cells.

Viral RNA replication and gene expression in Bunyamwera virus-infected C6/36 cells declined after 24 h pi, while levels of host protein synthesis remained relatively constant. This observation agreed with that of Newton *et al.* (1981) regarding Bunyamwera virus infection of uncloned *Aedes albopictus* cells. Measurement of S mRNA levels, by slot blot and Northern blot analyses, suggested that the levels of viral transcription were not dramatically altered at this stage in the infection. Rossier *et al.* (1988) reported similar findings for La Crosse virus infection of C6/36 cells. These authors judged that the transition from the acute to the persistent phase of La Crosse virus infection of C6/36 cells took place at 24 h pi. At this time, the synthesis of La Crosse virus genomic RNA and N protein was severely reduced, although S mRNA levels remained high. By pulse-labelling infected cultures with [^{3}H]-uridine, Rossier *et al.* (1988) determined that S mRNA synthesis declined relatively gradually during the initial days of the persistent infection and was still at 50% of its maximum level at 5 days pi. In contrast, genome and antigenome synthesis declined rapidly and became undetectable by 2 days pi. Levels of La Crosse virus S mRNA were still approximately 65% of the maximum levels (observed on day 1) on day 3 pi. However, despite significant levels of S mRNA synthesis, S mRNA levels fell to low levels, between 3 and 5 days post infection, in the infected C6/36 cells. Presumably the fall in S mRNA levels represented an increased rate of turnover with time.

Northern blot analysis of encapsidated RNA species in Bunyamwera virus-infected C6/36 cells revealed a dramatic decrease in the levels of encapsidated, positive-sense L and M RNAs between 24 and 48 h pi. However, encapsidated positive-sense S RNA continued to accumulate throughout the three days monitored (Figure 5.12). Northern blot analysis and *in vitro* translation of CsCl fractionated RNA samples from Bunyamwera virus-infected C6/36 cells revealed that by 48 h pi significant levels of Bunyamwera virus S mRNA were encapsidated. This may account, at least partly, for the continuing accumulation of encapsidated positive-sense S RNA species in the infected C6/36 cells.

Hacker *et al.* (1989) similarly found that within 24 to 48 h of La Crosse virus infection of C6/36 cells, 75% of the S mRNA was encapsidated. The encapsidated S mRNA could not be translated *in vitro* unless the N protein was first dissociated from the RNA, but, once free of N protein, the S mRNA was translatable. These findings are in line with the *in vitro* translation data shown in Figure 5.4; total infected-cell RNA samples were used. The total cell RNA samples were prepared using the acid-phenol-guanidinium-thiocyanate protocol of Chomczynski and Sacchi (1987) which will dissociate RNA-protein

interactions. The high ratio of viral to host protein translation observed *in vitro* compared to *in vivo* using the 24 and 36 h pi RNA samples from Bunyamwera virus-infected C6/36 cells may reflect the release of translatable S mRNA from nucleocapsid structures.

Hacker *et al.* (1989) examined the S mRNA-N protein complexes in some detail. They determined that the binding of N protein to its own mRNA was highly specific (no host cell mRNAs appeared to be associated with the viral N protein). N protein assembly occurred on preformed mRNAs, rather than concurrently with their synthesis, and in some cases the primers on the mRNAs were also encapsidated. By 16 h pi in La Crosse virus-infected C6/36 cells, significant levels of viral genome replication continued in the absence of new protein synthesis, yet the genomes which were made were all encapsidated. The continued availability of N protein in the absence of detectable levels of N protein synthesis suggested that a pool of N protein had accumulated in La Crosse virus-infected C6/36 cells by 16 h pi. It may be the existence of this N protein pool which facilitates the encapsidation of S mRNA. With time, the S mRNA appeared to become the preferred target for encapsidation. Potentially, some alteration in the S mRNA molecules which are transcribed later in infection may allow them to compete efficiently with the full-length viral RNA species for encapsidation. Assuming that bunyavirus genome replication and encapsidation are coupled, as suggested by studies on La Crosse virus infection of BHK cells (Patterson & Kolakofsky, 1984; Raju and Kolakofsky, 1987a), depletion of the N protein pool might then result in a decline in genome replication. However, the affinity of the N protein for S mRNA does not appear to be particularly strong, since the mRNA remains unencapsidated for up to 4 h after its synthesis (Hacker *et al.*, 1989). It seems more likely that the decline in genome replication is the initial event. The lack of genome synthesis might then lead to accumulation of a pool of unassembled N protein which can interact with the S mRNA. An early event in the establishment of persistent infection of C6/36 cells would therefore appear to be the restriction of genome replication.

Hypothetically, before the viral polymerase (L protein) can synthesise full-length viral RNAs, some factor (viral or cellular) may be required to promote the association of the viral L and N proteins so that genome replication and encapsidation are coupled. Depletion or exhaustion of this putative factor in the infected C6/36 cells may release the L protein from its replicase activity so that it functions solely as a transcriptase. In this situation viral RNA species may still be encapsidated, but not concurrently with their synthesis. It is also possible that a factor involved in the initiation of genome replication (i.e. initiation with ATP rather than with a primer) which modifies the activity of the L protein from a transcriptase to a replicase may be present in limiting amounts in infected C6/36 cells. In this respect, it is interesting to note the relatively low levels of expression of the NSs protein in infected C6/36 cells, compared to infected BHK cells. The function of the NSs protein has not yet been elucidated, but a role in modulating the activity of the

viral polymerase is possible. Alternatively, a factor which inhibits genome replication may accumulate in bunyavirus-infected C6/36 cells.

Encapsidation of the S mRNA may act as a secondary level of control in the persistent infection. By blocking translation of the S mRNA, this association of N protein with S mRNA may prevent cytotoxic effects due to the accumulation of viral N protein, which might begin to interact non-specifically with host mRNAs or become insoluble.

Before the molecular events underlying bunyavirus persistence in C6/36 cells can be understood, we need to know more about the molecular biology of genome replication and transcription of viral mRNAs in this system. In particular, a detailed understanding of the function of the viral polymerase and the role of any accessory factors (viral or cellular) will pave the way towards elucidating the mechanisms fundamental to the establishment of persistence. The sequence of the Bunyamwera virus L segment is available (Elliott, 1989a), and functional Bunyamwera virus L protein has been expressed from recombinant vaccinia viruses (Jin & Elliott, 1991). More recently mutagenesis studies, using Bunyamwera virus L gene-vaccinia virus recombinants, have highlighted regions of the L protein which are functionally important (Jin & Elliott, 1992). The Bunyamwera virus M segment proteins (G1, G2 and NSm) and S segment proteins (N and NSs) have also been cloned and expressed (Nakitare, 1992 (PhD thesis); R. M. Elliott, personal communication). Thus, the individual constructs required to develop a system in which Bunyamwera virus RNA synthesis is driven by plasmid generated viral proteins are available and could facilitate the elucidation of the role of each of the Bunyamwera virus proteins in RNA synthesis. The development of an infectious Bunyamwera virus cDNA system is also underway.

Relatively recently, Curran *et al.* (1991) proposed a model for Sendai virus RNA replication, based on deletion studies using an *in vivo* transcription system. Within this model there is potential for negative-regulation of genome replication, which could lead to persistent infection. These authors determined that the Sendai virus P protein is a modular protein which is essential for genome replication. The N-terminal domain appears to bind the viral polymerase (L) protein, whereas the C-terminal domain binds the viral nucleoprotein (NP). Earlier studies revealed that the P gene of Sendai virus expresses up to eight proteins; two of these proteins (V and W) are translated from edited mRNAs but have N-terminal domains identical to the N-terminal domain of the P protein (Pelet *et al.*, 1991). The V protein had been suspected to play a role in RNA synthesis since it contains the N-terminal half of P and is strongly conserved across the majority of paramyxoviruses. Contrary to expectations that V might enhance RNA synthesis, genome replication was inhibited in a dose dependent manner when V was co-expressed with NP, P and L (the later three being essential for paramyxovirus genome replication). It appeared that both V and W could bind to nucleocapsids and inhibit the functional interaction between P protein and

nucleocapsids. It is possible that V and/or W are components of a negative-regulatory mechanism which restricts viral replication during the establishment of persistent infection.

Paramyxovirus genome replication is thought to differ from transcription in its requirement for unassembled NP protein. During genome replication, NP protein initiates nucleocapsid assembly on the nascent leader RNA. Concurrent encapsidation of the growing nascent RNA suppresses polymerase termination signals at the gene junctions, so that full-length viral RNAs are synthesised as opposed to mRNAs. From studies with vesicular stomatitis virus, N is thought to function in genome replication as a complex with P (Peluso & Moyer, 1988; Howard & Wertz, 1989).

Curran *et al.* (1991) proposed that for Sendai virus RNA synthesis, P must contact both the NP of the template and the L protein. Their model makes two assumptions: firstly one L protein molecule must interact with two molecules of P and, secondly, NP protein must be associated with the P protein before it will assemble nascent RNA. When the levels of unassembled NP are low, both P proteins (associated with the L protein) would contact the assembled NP of the genome template as RNA synthesis is initiated. In this situation the polymerase (L) would recognise the gene termination signals, on the nascent RNA, yielding leader and mRNAs. When the level of unassembled NP protein rises, P-NP complexes can form off the template. The availability of these 'free' P-NP complexes means that the nascent RNA can be encapsidated concurrently with its synthesis and consequently full-length viral RNA molecules will be synthesised. V and W could potentially interfere with the P-L complex by sequestering L in a form which is non-functional for genome replication. Thus the relative levels of the Sendai virus V and P proteins might control genome replication. Hypothetically, these levels may be controlled by the frequency of RNA editing.

The relative ratios of paramyxovirus mRNAs can also be influenced by the host cell. Paramyxoviruses display a gradient of decreasing mRNA abundance with increased distance of the gene from the 3' end of the genome template. The steepness of this gradient differs in different host backgrounds (Cattaneo *et al.*, 1987; Schneider-Schaulies *et al.*, 1989). The gradient is relatively shallow during acute infection of epithelial cells and fibroblasts but steep in cells such as neurons, in which infection is slow and persistent. Since the L and P genes are distantly spaced on the paramyxovirus transcriptional map, their ratios will be strongly affected by changes in this gradient. The ability of V to inhibit RNA synthesis is dependent upon limiting levels of L. It is possible, therefore, that V exerts a strong negative effect on RNA replication only when L levels are relatively low, such as in neurons. This fits with the pattern of persistence observed with paramyxoviruses: acute infection of epithelial cells and persistent infection of neurons.

8.3 Analysis of *Aedes albopictus* C6/36 cells persistently infected with Bunyamwera virus

Preliminary sequencing studies revealed that Bunyamwera virus undergoes minimal genetic drift during persistent infection of C6/36 cells. The S RNA segments of two viral isolates (8.6 and 18.10), which were plaque-purified from passage levels 8 and 18, respectively, of the C6/36-PI LO cell line, established by Elliott & Wilkie (1986), were sequenced by primer extension sequencing of virion RNA. No nucleotide changes were evident in the 8.6 isolate and a single nucleotide substitution was detected in the 18.10 isolate. The mutation in the 18.10 isolate resulted in conservative amino acid changes in both the N and NSs proteins of this virus. Neither of these amino acid substitutions occurred in conserved regions of the N or NSs proteins (see Section 6.3). It is possible that the constraints on the evolution of the S RNA segment are greater than on the other genome segments, due to the overlapping reading frames encoded in the S RNA. The rate of evolution of the Bunyamwera virus genome as a whole may therefore be greater than is suggested by the relative stability of the S RNA. It has, however, been suggested that arboviruses exhibit a slower rate of evolution relative to non-arthropod-borne RNA viruses (Bilsel *et al.*, 1988; Baldridge *et al.*, 1989), and Poidinger *et al.* (1991) reported that Murray Valley encephalitis flavivirus underwent few genomic changes during 300 days of persistent infection in Vero cells. These results are in contrast to the rapid rates of virus evolution observed in cell cultures persistently infected with FMDV, VSV and influenza virus (de la Torre *et al.*, 1988; Depolo *et al.*, 1987; Frielle *et al.*, 1984).

Sequencing studies also revealed that the variant-N protein containing viruses reported to be present in *Aedes albopictus* C6/36 cells persistently infected with Bunyamwera virus (Elliott & Wilkie, 1986) were not endogenous to the persistently infected culture, but represented contaminating temperature-sensitive bunyaviruses.

As mentioned above, cloned stocks of Bunyamwera virus (wtL$_9$BUN virus) readily established persistent infection in *Aedes albopictus* C6/36 cells. The persistently infected cells were indistinguishable from uninfected C6/36 cells in terms of cell morphology and growth rate, and were passaged for over a year without observable cytopathic effect. The newly established persistent infection mimicked that reported previously in C6/36 cells (Elliott & Wilkie, 1986) and also the persistent Bunyamwera virus infection established in an uncloned *Aedes albopictus* cell line by Newton *et al.* (1981).

Levels of virus released from the persistently infected culture fluctuated between 10^2 and 10^7 pfu/ml. The persistently infected culture also displayed resistance to superinfection with wtL$_9$BUN virus, which varied in magnitude at different passage levels. There was a marked change in the plaque-morphology of released virus with increasing passage level: well defined lytic plaques were gradually replaced by small, cloudy plaques.

Each of the above characteristics were also observed by Newton *et al.* (1981) and Elliott & Wilkie (1986), and are generally associated with the presence of defective interfering particles in the culture (Holland, 1990).

The relative levels of each of the viral genomic RNAs, and of positive-sense S RNA, at different passage levels of the C6/36/BUN cell line were determined. The levels of each of the viral RNA species varied between passage levels, and each of the viral RNAs appeared to vary independently. No simple correlation between the fluctuating levels of each of the viral RNAs and the titres of virus released from the C6/36 cells was evident. More detailed examination of the viral RNAs resident in the C6/36/BUN culture, by Northern blotting, revealed the presence of defective viral RNAs.

8.4 Bunyaviridae DI particles

Prior to the study presented in this thesis, there had only been two reports on Bunyaviridae DI particles. Kascsak & Lyons (1978) reported the generation of defective Bunyamwera virus particles upon high multiplicity passage of Bunyamwera virus in BHK cells. These particles contained predominantly S segment-sized RNA and interfered with the multiplication of homologous virus. Elliott & Wilkie (1986) observed the generation of defective Bunyamwera virus particles, which contained only S segment sequences, during persistent Bunyamwera virus infection of *Aedes albopictus* C6/36 cells. These particles also interfered with the replication of homologous virus. In neither case were novel viral RNA species (DI RNAs or defective RNAs without interfering activity) observed in the defective interfering virus preparations. More recently, subgenomic RNAs derived from the L RNA segment of Germiston bunyavirus and tomato spotted wilt tospovirus (TSWV) have been detected (Cunningham & Szilagyi, 1987; de Oliveira Resende *et al.*, 1991; see Section 2.3.1.1). Interfering activity was inferred, but not proven, in the former case. TSWV defective L RNAs out-compete full-length L RNAs in infected *Nicotiana rustica* plants and are associated with disease attenuation, suggesting that these DI RNAs possess interfering activity.

A range of size classes of subgenomic RNAs derived from the Bunyamwera virus L RNA segment were observed in the persistently infected C6/36/BUN cell line. Low levels of the defective RNAs were first detected (by Northern blot analysis) at passage level two of the persistently infected culture and they were clearly detectable by passage level 21. By passage level 58 a wider spectrum of defective L RNAs were seen and defective RNA was much more abundant than the full-length L RNA segment. No subgenomic M segment RNA or S segment RNA was observed, but interestingly at passages 19, 21 and 22 more slowly migrating S segment RNA species were detected.

When passage levels 18, 19, 21 and 22 of the C6/36/BUN cell line were probed for encapsidated Bunyamwera virus genomic RNAs, only passages 18 and 21 contained detectable levels of encapsidated viral RNAs. Defective L RNAs were observed in

nucleocapsid structures, at passage 18 only, while no encapsidated viral RNAs were detected at passages 19 and 22. It is interesting to compare the levels of positive-sense S RNA in the persistently infected C6/36/BUN cell culture at these passage levels. At passage 18, relatively high levels of intracellular positive-sense S RNA coincided with significant levels of encapsidated defective L RNAs. At passage 21, the levels of positive-sense S RNA were approximately 40% of the levels observed at passage 18, and only full-length genomic S RNA was observed in viral nucleocapsids, despite an abundance of defective L RNAs being present in these cells. At passages 19 and 22, the levels of positive-sense S RNA were low and no encapsidated viral RNAs were detected in these cells.

High levels of positive-sense S RNA most probably represent high levels of S mRNA and, in the absence of a translational block, relatively high levels of N protein. Encapsidation of the defective L RNAs, generated in the C6/36/BUN cell line, may therefore be dependent upon the levels of N protein in the persistently infected cells at a particular passage level, rather than the absence of encapsidation signals in the subgenomic RNAs. However, the larger S RNA species detected at passages 19, 21 and 22 may lack encapsidation signals, since these RNAs appeared not to be encapsidated. Stalder *et al.* (1983) reported the generation of SFV DI RNAs, which were inefficiently encapsidated, during persistent infection of C6/36 cells. Sequence comparisons between the defective S RNAs and standard Bunyamwera virus genomic S RNA may identify genomic signals required for efficient encapsidation and packaging of the viral genome. The defective S RNA at passage level 21 was particularly abundant relative to the standard genomic S RNA segment, but only the authentic S RNA was encapsidated (Figure 6.18). There does appear to be a strong selection for encapsidation of full-length S RNA species in the persistently infected cells, and full-length S RNA species predominate over full-length L and M RNA species in intracellular nucleocapsids. This may be due to the greater efficiency with which smaller RNA molecules can be encapsidated and is in line with early reports of Bunyamwera virus DI particles containing only S RNA (Kascsak & Lyons, 1978; Elliott & Wilkie, 1986). Rossier *et al.* (1988) failed to detect defective viral RNAs in their culture of *Aedes albopictus* C6/36 cells persistently infected with La Crosse bunyavirus; this may reflect the fact that they examined relatively early passage levels (4, 6 and 8), when the levels of defective viral RNAs are low.

Recently, there have been two reports of the characterisation of Bunyaviridae DI RNAs (Patel & Elliott, 1992; de Oliveira Resende *et al.*, 1992). In both instances the DI RNAs were subgenomic species, derived from the L RNA segment. Patel & Elliott (1992) characterised Bunyamwera virus DI RNAs generated in mouse L cells. These defective L RNAs were efficiently packaged into nucleocapsids. In addition, the DI particle preparations produced novel polypeptides of various sizes in infected BHK and mouse L cells. These novel polypeptides could be precipitated with antisera raised against either the

N or C terminus, or both, of the Bunyamwera virus L protein. Nucleotide sequence analysis of cloned cDNAs to the most abundant DI RNAs revealed that in each case a single internal deletion of the L segment had taken place, removing between 72% and 77% of the L RNA. The 5' and 3' terminal sequences were retained.

De Oliveira Resende *et al.* (1992) analysed four TSWV DI L RNAs by hybridization studies, PCR cloning and sequence determination. They determined that, like the Bunyamwera virus DI L RNAs characterised by Patel & Elliott (1992), these RNAs belonged to the 5'-3' class of DI RNAs (Lazzarini *et al.*, 1981). The TSWV DI L RNAs retained both genomic termini and displayed a single extensive internal deletion of 60% to 80% of the standard genomic L RNA. All four TSWV DI L RNAs examined contained ORFs. In each case the encoded protein retained the original C-terminal amino acid sequence of the viral polymerase (L). De Oliveira Resende *et al.* (1992) also determined the junction sites within each DI L RNA. Analysis of the nucleotide sequences surrounding the recombination sites of the TSWV DI L RNAs revealed the involvement of identical sequences (such as UA, UAG or CCACU) at the release and reinitiation sites of individual DI L RNAs. It is thought that the repeat sequences may enable the viral polymerase to jump from one site to another on the L RNA molecule during virus replication. This hypothesis fits with the copy-choice mechanism of DI RNA generation (Lazzarini *et al.*, 1981; Perrault, 1981; Re *et al.*, 1985; Cascone *et al.*, 1990). Meier *et al.* (1984) proposed that repeat sequences in the vesicular stomatitis virus genome were involved in defining junction sites during the generation of vesicular stomatitis virus DI RNAs. However, in this case, the repeated sequences are not immediately adjacent to the junction site, as in TSWV DI L RNAs.

Comparison of the nucleotide sequences immediately preceeding and at the 3' end of the deleted region of the Bunyamwera virus DI L RNAs characterised by Patel and Elliott (1992), revealed the occurence of similar repeat sequences. In two of the DI RNAs (DI-M5 and DI-M7) the sequences AUA and AA, respectively, were found immediately preceeding the polymerase dissociation and reattachment sites. In the third DI RNA (DI-M3) the sequence GGA was repeated prior to the termination and reinitiation sites, however, in this case the sequence was close to, but not immediately adjacent to the recombination site. Engelhorn *et al.* (1993) recently characterised a Sendai virus internal deletion defective RNA (E307). Examination of the sequences preceeding the deletion and recombination sites required to generate this defective RNA, revealed the sequence UAC close to the polymerase dissociation site and immediately preceeding the reattachment site.

These short sequences are likely to occur at other sites in the viral genome. This was found to be the case with the short sequences repeated at the junction sites of TSWV DI L RNAs. It is possible therefore that, in addition to the repeat sequences, secondary structure(s) in the L RNA, or higher order structures in the ribonucleoprotein, cause the viral polymerase to "jump" from one site to the other. Computer models predict hairpin

structures at the release and reinitiation sites on the TSWV genome. The first of the pair of repeat sequences is predicted to occur in the end loop of the first hairpin, while the repeat sequence at the release site is located in unpaired sequences in the stem of another hairpin structure. These conformations, within the higher order structure of the ribonucleoprotein, may expose the repeat sequences at the recombination sites, allowing the polmerase to jump from one to the other. In all TSWV DI L RNAs characterised so far, and in the Bunyamwera virus, VSV and Sendai virus examples cited above, one of the repeat sequences defining the release and reinitiation sites is lost during the formation of the DI RNA. This is consistent with a model in which sequences in the nascent RNA which are complementary to sequences at the release site in the L RNA template, anneal to the repeat sequence at the reinitiation site. Upon reinitiation of RNA synthesis, the second sequence will be lost. The existence of repeat sequences in the viral genome is likely to increase the probability of the viral polymerase jumping between between these sites and deleting the intervening sequences.

Analysis of the defective Bunyamwera virus L RNAs produced in the C6/36/BUN cell line suggested that these defective RNAs were inefficiently encapsidated. However, when BHK cells were infected with virus released from each of the first thirty passages of the C6/36/BUN cell line, novel polypeptides were observed in infections initiated with virus released at passage levels 18, 21, 23, 26 and 29 (Figure 6.8), when host protein synthesis was efficiently inhibited. Immunoprecipitation studies are required to determine if these polypeptides are virus-specified and akin to those observed by Patel & Elliott (1992). If these proteins are virus specific, a small proportion of the defective L RNAs in the C6/36/BUN cell line may be encapsidated and packaged into virions. As discussed earlier, the number of defective virions released from the C6/36/BUN culture might vary according to the level of unassembled N protein in the cells at a particular passage level.

8.5 Cell heterogeneity within persistently infected cell cultures

Cells were cloned from passages 12, 20, 30 and 58 of the persistently infected C6/36/BUN cell line. When the cell clones, which gave rise to stable cell lines, were examined for levels of intracellular viral RNA, levels of released virus, and their ability to resist superinfection with wild-type Bunyamwera virus, it became clear that there was heterogeneity within the persistently infected cell culture. Individual cells harboured different defective L RNAs and responded differently to superinfection with homologous virus. In addition, no Bunyamwera virus RNA was detected in some clones, and a minority of cells cloned from the C6/36/BUN cell line displayed cpe and failed to establish stable cell lines.

Similar observations have been made with other persistently infected cell cultures, for example BHK cells persistently infected with FMDV (de la Torre *et al.*, 1989b). Clonal analysis of this persistently infected cell line revealed that none of the stable cloned cell lines, derived from the original persistently infected culture, released infectious FMDV, or contained levels of viral RNA detectable by dot blot hybridization with cDNA probes. Eight out of a total of 177 single cells isolated from the original persistently infected culture (termed C1-BHK-Rc1), released FMDV, but none of these cells gave rise to stable cell clones. Presumably these cells were lytically infected. On the basis of their studies, de la Torre *et al.* (1989b) estimated that between 4 to 20% of the cells in the C1-BHK-Rc1 culture were lytically infected. Heterogeneity was evident in the morphology of individual cells within the culture, in the resistance of individual cells to superinfection with homologous virus, and in the growth characteristics of individual cells. Upon serial passage of the persistently infected culture, cells with increased resistance to FMDV were selected. When cloned cells derived from this culture were infected with the original FMDV, the virus yields were 10^3 to 10^5 fold lower than those obtained with BHK-21 cells (de la Torre *et al.*, 1989b). There was no evidence for DI involvement (de la Torre *et al.*, 1988; 1989b). The resistance was specific for FMDV and appeared to be due to an intracellular block which resulted in a 100-fold decrease in the levels of FMDV RNA relative to infected BHK-21 cells. As stated earlier, no viral RNA was detected in the resistant cells, and the resistance appeared to be mediated by trans-acting cellular factors (de la Torre *et al.*, 1989a).

Two, out of a total of 58, single cells isolated from the persistently infected C6/36/BUN cell line, exhibited cpe and failed to establish stable cell clones. Since it was not ascertained if infectious virus was released from these cells, we cannot be certain that cell death was due to virus-induced lysis of the cells. In contrast to the C1-BHK-Rc1 persistently infected culture, most of the single cells isolated from the C6/36/BUN cell line gave rise to stable cell clones which harboured intracellular viral RNA. The majority of the cloned cell lines derived from passages 12 and 20 released detectable levels of infectious virus, while 2 out of 28 cell clones derived from passages 30 and 58 of the C6/36/BUN culture, released low, but detectable, levels of infectious virus. Thus, by passage 12 of the persistently infected C6/36/BUN cell line, the majority of individual cells within the culture appeared to able to withstand the cytopathic effects of Bunyamwera virus infection. The virus released from each of the cloned cell lines, and from later passage levels of the parental C6/36/BUN cell line had a cloudy plaque morphology, suggestive of interfering activity (Welsh & Pfau, 1972).

In contrast to the C1-BHK-Rc1 culture, the C6/36/BUN cell line no longer exhibited resistance to infection with wild-type Bunyamwera virus by passage level 58. Northern blot analysis of total cellular RNA from passage level 58 of the C6/36/BUN cell line revealed mainly defective L RNAs in these cells. In addition, resistance to

superinfection appeared to vary at different passage levels of the persistently infected culture, and between individual cloned cell lines derived from a given passage level of the culture. Northern blot analysis of total cellular RNA from a selection of cloned cell lines derived from passage 20 of the C6/36/BUN culture, showed that individual cells harboured different defective L RNAs. All of the clones examined contained a single prominent defective L RNA species. The resistance of the cloned cells to superinfection with Bunyamwera virus appeared to correlate with the amount of full-length L and S RNA present, and not with the amount or occurence of defective L RNA.

8.6 Directions for future work

The mechanisms by which persistent viral infections are established and maintained are still far from clear. With the bunyavirus system a more detailed understanding of the molecular biology of infection, and in particular the functioning of the viral polymerase and the function/role of the viral non-structural proteins must precede advances in our understanding of the establishment of persistent bunyavirus infections.

The role of the host cell in persistent viral infections must be addressed. The expression of the lytic and persistent phenotypes of Bunyamwera virus, upon infection of BHK and C6/36, respectively, may well depend upon host cell factor(s) present in one cell type but not the other. Recently, Levine *et al.* (1993) demonstrated that Sindbis alphavirus infection of a number of vertebrate cell lines (BHK, mouse neuroblastoma (N18) and rat prostatic adenocarcinoma (AT-3) cells) induces an active cell suicide process (apoptosis). In contrast to the lytic replication cycle observed in these vertebrate cell lines, Sindbis virus establishes a persistent infection in differentiated primary cultured rat neurons (Levine *et al.*, 1991). However, Sindbis virus infection of undifferentiated primary cultures of embryonic rat neurons results in cell death. This differential susceptibility to virus induced cell death was associated with levels of expression of the proto-oncogene bcl-2 which is known to block apoptosis. Levine *et al* (1993) also generated stable transfectant AT-3 cell clones expressing the human bcl-2 protein (AT3Bcl2). They demonstrated that expression of the bcl-2 protein protected the AT-3Bcl2 cells from Sindbis virus-induced cell death and lead to the establishment of a persistent infection. It would be interesting to investigate whether or not Bunyamwera virus infection kills vertebrate cells via apoptosis. Since Sindbis and Bunyamwera virus readily establish persistent infections in mosquito cell cultures (Newton *et al.*, 1981; Stalder *et al.*, 1983; Elliott & Wilkie, 1986), it would be interesting to determine if mosquito cell cultures express bcl-2, or an equivalent protein which promotes cell survival.

A role for DI particles in maintaining bunyavirus persistent infections seems likely, but we are still some way from understanding how DI particles exert their effects. In the meantime DI genomes generated during persistent infections may serve as tools with which to identify functionally important regions of the virus genome. For instance,

characterisation of the larger S RNA species detected in the C6/36/BUN culture, which appeared not to possess the signals required for encapsidation, may help identify RNA encapsidation signals. In addition, sequence analysis of the defective L RNA species generated in the C6/36/BUN cell line may increase our understanding of the mechanisms of DI RNA generation.

An understanding of invertebrate cell tolerance to arbovirus infection may lead to the development of novel antiviral therapies. In particular, the purification and characterisation of the antiviral proteins produced by alphavirus-infected invertebrate cells (see Section 2.3.2) could lead to the development of therapeutic agents for the control of arbovirus-induced disease.

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