DEVELOPMENT OF A REVERSE GENETICS SYSTEM TO INVESTIGATE BUNYAMWERA VIRUS RNA SYNTHESIS

by

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January, 2000

Summary.

Bunyamwera (BUN) virus is the prototype type virus of the family *Bunyaviridae*. The virus has a genome composed of three single stranded RNA segments of negative polarity termed large (L), medium (M), and small (S). Described in this thesis, is the development of a reverse genetics reporter system to study the synthesis of BUN viral RNA and the proteins involved. The system utilises a recombinant RNA template, BUNSCAT, which comprises an antisense CAT gene flanked by the authentic BUN S 5' and 3' untranslated regions (UTRs). Transfection of *in vitro* transcribed BUNSCAT RNA into cells transiently expressing recombinant BUN viral proteins resulted in CAT activity only when both the L segment and the S segment proteins were expressed, indicating that the negative sense template had been transcribed into a positive sense, translatable RNA. Further analysis revealed that just BUN N and L proteins were sufficient for the transcription of BUNSCAT RNA. It was also demonstrated that the BUN L protein was able to transcribe BUNSCAT RNA in concert with the N proteins of certain heterologous bunyavirus S segments. Modifications to the BUNSCAT UTRs indicated that these terminal regions play an important role in transcription of BUNSCAT RNA. The addition of nucleotides to both the 3' and 5' termini were tolerated to an extent. While deletion of one nucleotide at the 3' terminus gave near wildtype CAT activity, any further deletions resulted in a significant drop in CAT activity, as did any deletion of the 5' terminus. Analysis of a series of single point and complimentary base-pair mutations to the 5' and 3' termini determined that sequence specificity for nucleotides 2-12 of the termini was essential while maintenance of base-pairing between nucleotides 13-15 of the termini was also important. Construction and comparison of BUNLCAT and BUNMCAT RNAs with the BUNSCAT reporter determined that the L UTRs gave a higher CAT activity than M UTRs which in turn were higher than S UTRs. Construction and analysis of a series of chimeric BUN segment UTR reporters (*e.g.* BUNL 3'-CAT-BUNM 5') showed that none of the chimeric RNAs were transcribed. A significant level of CAT activity was restored, however, to the BUNL/M-CAT reporter when the BUN L 5' UTR terminus was mutated to recreate partly M 5' UTR sequence, thereby restoring complementarity between the 18 terminal nucleotides.

Collectively, these results demonstrate that the reporter assay is a useful, reliable and convenient tool to study the molecular processes of Bunyamwera virus. Using this system, it is expected that further mutational analysis of the BUN RNA segments and replication proteins will provide insight into transcription and replication processes, and the *cis*-acting signals which control and orchestrate these important events.

Acknowledgements.

Firstly I would like to thank Professor Richard M Elliott for providing me with the opportunity to work in and make use of the facilities of the institute and his laboratory. I am also greatly indebted for his excellent supervision, critical reading, and patience, patience, patience during the preparation of this thesis.

I am very grateful for the friends I have been fortunate to make during my short spell (nine years) in Virology, which are now far to numerous to mention. Also I would like to thank the staff of the front office, washroom, media and store for their cheerfulness and support.

I want to thank my mother for her love and endless support over the years, even when it meant going back to school and college herself to make sure I got the grades, and to the rest of my family, Thomas, Graeme, Fiona and Morag, who never tired of asking "have you not finished that essay yet".

Most of all I want to thank Dr Susan Kane, for all her love and affection, without which nothing much would ever get done. Also for proof-reading, I know that some day the therapy will be over and the debt paid off.

These studies were carried out on a part-time basis while employed as an RA to Prof. RME. Unless otherwise stated, all results were obtained by the authors own efforts.

This thesis is dedicated to my mother Marion and to the memory of my father Thomas Dunn (15/3/99), I know they are both very proud of me.

CHAPTER 1.

INTRODUCTION.

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Abbreviations.

A_{260}	absorbance at 260 nm
acetyl CoA	acetyl coenzyme A
AP	alkaline phosphatase
ATP	adenosine 5'-triphosphate
bisacrylamide	N N'-methylene-bisacrylamide
bp	base-pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CF	cystic fibrosis
Ci	Curie
CIP	calf intestine phosphatase
cRNA	complementary RNA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythmidine triphosphate
dGTP	deoxyguanosine triphosphate
DI	defective interfering
DMEM	Dulbeccos modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dw	distiled water
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
ER	endoplasmic reticulum
Eth Br	ethidium bromide
FCS	fetal calf serum

g	gravity
GFP	green fluorescent protein
HPS	Hanatvirus Pulmonary Syndrome
IRES	internal ribosomal entry site
kb	kilobase
kDa	kilo Dalton
M	molar
mRNA	messenger ribonucleic acid
NBC	new born calf serum
NNS	nonsegmented negative strand
NSV	negative strand virus
nt	nucleotide
oligo	oligonucleotide
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque-forming units
Pol I	RNA polymerase I
Pol II	RNA polymerase II
poly(A)	polyadenylated
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein (nucleocapsid/ribonucleocapsid)
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate (buffer)
ssDNA	single stranded DNA
ssRNA	single stranded RNA
TAE	Tris/acetate (buffer)
Taq	*Thermus aquaticus* DNA (polymerase)
TBE	Tris/borate (buffer)
TCA	trichloroacetic acid
TE	Tris/EDTA (buffer)
TEMED	*N N N'N'*-tetramethyl-ethylenediamine
TEN	NaCl in TE buffer
TLC	thin-layer chromatography
tRNA	transfer RNA

UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
VLP	virus like particle
vRNA	viral genome RNA
wt	wild type
Xgal	5-bromo-4-chloro-3-indolyl-β-d-galactoside

Abbreviated virus names.

A/HK/8/86	Influenza A virus strain
A/WSN-HK	Influenza A virus strain
A/WSN/33	Influenza A virus strain
A/WSN/NA	Influenza A virus strain
AINO	Aino virus (bunyavirus)
BAT	Batai virus (bunyavirus)
BUN	Bunyamwera virus (bunyavirus)
CCMV	Cucumber Mosaic virus
CV	Cache Valley (bunyavirus)
DOB	Dobrava virus (bunyavirus)
DUG	Dugbe virus (nairovirus)
GER	Germiston virus (bunyavirus)
GRO	Guaroa virus (bunyavirus)
GRSV	Groundnut ringspot virus (tospovirus)
HDV	Hepatitis delta virus
HIV-1	Human immunodeficiency virus
hPIV-3	Human parainfluenza virus 3
HTN	Hantaan virus (hantavirus)
INK	Inkoo virus (bunyavirus)
KRI	Kairi virus (bunyavirus)
LAC	La Crosse virus (bunyavirus)
LUM	Lumbo virus (bunyavirus)
MAG	Maguari virus (bunyavirus)
MD	Main Drain virus (bunyavirus)
MeV	Measles virus
MVA-T7	host range restricted T7 pol expressing vaccinia virus
NOR	Northway virus (bunyavirus)
NY-1	New York 1 virus (hantavirus)

PT	Punto Toro virus (phlebovirus)
PUU	Puumala virus (hantavirus)
RSV	Respiratory Syncytial virus
RVF	Rift Valley fever virus (phlebovirus)
SEO	Seoul virus (hantavirus)
SeV	Sendia virus
SN	Sin Nombre virus (bunyavirus)
SSH	Snow Shoehare virus (bunyavirus)
TIN	Tinaroo virus (bunyavirus)
TRT	Trivattatus virus (bunyavirus)
TSWV	Tomato spotted wilt virus (tospovirus)
UUK	Uukuniemi virus (uukuvirus)
VSV	Vesicular Stomatitis virus
vTF7-3	recombinant T7 pol expressing vaccinia virus

Amino acids.

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

Chapter 1.

Introduction.

1.0.0 The *Bunyaviridae.*

1.0.1 Characteristics and taxonomy of the *Bunyaviridae.*

Members of the *Bunyaviridae* share characteristics such as molecular composition, particle morphology, mode of transmission, genome structure, and coding strategies (negative-sense and ambisense).

All viruses in the family are enveloped, spherical particles of approximately 100 nm in diameter (in the range of 80-120 nm). The envelope is composed of host derived lipid, and is approximately 4 to 5 nm thick (Bishop, 1996). The virus particles are composed of four viral encoded structural proteins, two glycoproteins termed G1 and G2 and two nucleocapsid associated proteins, termed the L protein (RNA-dependent RNA polymerase) and the N protein (nucleocapsid protein). The viral glycoproteins are embedded in the lipid envelope and project 5 to 10 nm from the virion surface. The glycoproteins possess type-specific antigenic determinants and these variations contribute unique external features to representatives of different genera (Martin *et al.*, 1985; Schmaljohn and Patterson, 1990: Schmaljohn, 1996b). Based on the structure and its components, the overall chemical composition is calculated to be 1-2% RNA, 58% protein, 33% lipid, and 7% carbohydrate (Objeski and Murphy, 1977). The envelope surrounds the virus genome which consists of three single stranded RNAs termed the small (S), medium (M), and large (L) segments, respectively. The three viral RNAs are present in the virions as nucleocapsids (RNPs). Each RNP consists of a single genomic RNA encapsidated by the viral N and L proteins. RNPs have a buoyant density of 1.31 g/ml (Kolakofsky *et al.*, 1991). The length of the genomic segments vary among the genera, ranging from 6.3 to 13 kb for the L segment, 3.5 to 5 kb for the M segment, and 0.8 to 2.9 kb for the S segment (Figures 1.0). The pattern of genome segment sizes is conserved within a genera. Each RNA species has conserved complementary 3' and 5' ends which are genus specific (see Figure 1.1). The 3' and 5' ends of the RNAs are thought to be hydrogen bonded to form closed circular structures as seen by electron microscopy (EM) (Hewlett *et al.*, 1977).

At present, the International Catalogue of Arboviruses lists more than 525 viruses, (Karabatsos, 1985). Of these, 313 have been placed in the family *Bunyaviridae*. These again have been subdivided into five genera on the basis of serological, biochemical and genotypic characteristics: 172 viruses in the *Bunyavirus* genus, 10 in the *Hantavirus* genus, 34 in the *Nairovirus* genus, 51 in the *Phlebovirus* genus (Table 1.1). In addition the *Tospovirus* genus consists of 4 viruses (Table 1.1) (Schmajohn *et al.*, 1985), another 19 viruses have characteristics of viruses of the family *Bunyaviridae* and have been placed in serogroups but unassigned to a genus, and another 23 are both ungrouped and unassigned within the family. Members of the same genera are also referred to by their vernacular term; bunyaviruses, hantaviruses, nairoviruses, phleboviruses and tospoviruses.

1.0.2 Host range, transmission and importance of *Bunyaviridae* diseases.

Four of the genera of the *Bunyaviridae, Bunyavirus*, *Hantavirus*, *Nairovirus* and *Phlebovirus*, contain vertebrate-infecting viruses while members of the *Tospovirus* genus infect plants. Most of the viruses are transmitted by arthropod vectors (and hence are known as arboviruses). In general, bunyaviruses are transmitted by mosquitoes or midges; nairoviruses by ticks, and phleboviruses by sandflys or ticks. Tospoviruses are transmitted (spread) by thrips. Hantaviruses do not have an arthropod vector but are maintained in nature as a persistent infection of rodents and are transmitted to humans and other rodents via aerosolised infectious rodent secretions.

A number of viruses belonging to the *Bunyaviridae* are serious human and veterinary pathogens (Elliott, 1997), for example Crimean-Congo haemorrhagic fever virus (nairovirus), Rift Valley fever virus (phlebovirus), La Crosse virus (bunyavirus) and a number of hantaviruses. Several hantaviruses including Hantaan (HTN), Seoul (SEO), Dobrava (DOB, and Puumala (PUU) viruses, are associated with haemorrhagic fever with renal syndrome of varying severity, ranging from HTN infection with 5 to 15% mortality to PUU virus infections with less than 1% mortality. These forms of the disease are currently recognised throughout Asia, the former Soviet Union, and northern Europe (McKee *et al.*, 1991). Recently, however, another hantavirus, Sin Nombre (SN) virus was recognised as a major etiological agent associated with the severest form of hantavirus pulmonary syndrome (HPS) occurring in the United States, with a mortality rate of approximately 60% (Nichol *et al.*, 1996). Crimean-Congo haemorrhagic fever virus is the etiologic agent of another haemorrhagic fever which results in 10 to 20% mortality over a wide geographical area including Africa, Eastern Europe, the Middle East, and Asia through the western provinces

Genus	Serogroups	Representative Viruses
Bunyavirus	19 groups containing 172 viruses. Example groups:	
	Bunyamwera group (32)	Germiston and Bunyamwera viruses (human pathogens), and Cache valley virus (pathogen of cattle and sheep).
	California group (14)	California encephalitis, La Crosse and Snowshoe hare viruses (human pathogens).
	Simbu group (25)	Aino and Simbu viruses and Oropouche virus (human pathogen)
Hantavirus	One group containing 10 viruses.	
	Hantaan group	Hantaan, Puumala, Seoul and Sin Nombre viruses (human pathogens).
Nairovirus	7 groups containing 34 viruses. example groups:	
	CCHF group (3)	Crimean-Congo haemorrhage fever virus (human pathogen).
	Nairobi sheep disease group (2)	Dugbi and Nairobi sheep disease viruses (pathogens of humans and sheep).
Phlebovirus	3 groups containing 51 viruses example groups:	
	Sandfly fever group (23)	Sandfly fever Naples, Punta Toro and Rift valley fever viruses (human pathogens).
	Uukuniemi (12)	Uukuniemi virus
Tospovirus	2 groups containing 4 viruses.	
	Tomato spotted wilt (3)	Tomato spotted wilt virus (plant pathogen).
	Impatiens necrotic spot (1)	Impatiens necrotic spot virus (plant pathogen).

Table 1.1 Classification of the *Bunyaviridae*

Table adapted from Calisher, 1996.

of China (Gonzalez-Scarano *et al.*, 1991, Gonzalez-Scarano and Nathanson, 1996). Rift Valley fever virus (RFV) causes an acute disease in humans with a convalescence period which may be prolonged (Eddy and Peters, 1980). RVF virus causes a more serious disease in domestic animals, and sheep are more susceptible than cattle; pregnant ewes normally abort and over 90% mortality amongst infected lambs has been reported (Meegan and Shope, 1981). La Crosse virus is responsible for the majority of paediatric viral encephalitis cases in the US. Infections are generally located around the Midwestern United states and are responsible for around 100 cases annually (Kappus *et al.*, 1983; Gonzalez-Scarano *et al.*, 1996).

Tospoviruses infect more than 800 species of plants from about 82 botanical families and result in great economic losses in many agricultural, horticultural, and ornamental crops (Prins and Goldbach, 1998).

1.0.3 Stages of virus replication.

The principle stages of the replication process for viruses in the family *Bunyaviridae* can be summarised simplisticaly as follows (Bishop, 1996; Shmaljohn, 1996a):

- Attachment of virus: this is mediated by an interaction of viral proteins and target host cell receptors.
- Entry and uncoating virions: this process is thought to occur via the endocytosis of virions and the subsequent fusion of the viral membrane with the endosomal membrane.
- Primary transcription: this step involves the synthesis of viral mRNA species from the genome templates by the virion associated polymerase using host cell derived primers.
- Primary translation: the L and S segment mRNAs are translated in the cytosol by free ribosomes, and the M segment mRNAs by membrane bound ribosomes on the rough endoplasmic reticulum. Processing, folding and primary glycosylation of the viral glycoproteins (G1 and G2) also occurs at the ER.
- Synthesis and encapsidation of antigenomes, also known as cRNA, to act as template for genomic RNA synthesis or, in some cases, as a template for subgenomic mRNA synthesis (*i.e.* a virus with an ambisense coding strategy).
- Genome replication and subsequent amplification.
- Secondary transcription: the amplified synthesis of the mRNA species and ambisense transcription.
- Continued translation and RNA replication.

- Morphogenesis: newly formed nucleocapsids accumulate under Golgi membranes into which glycosylated G1 and G2 have been inserted. By an undefined process, virus particles are formed by budding into the Golgi cisternae.
- Virus release: newly assembled virions are transported out of the Golgi lumen in exocytic transport vesicles. Fusion of these vesicles with the plasma membrane releases mature virions.

A number of the principle stages will be discussed further within this section.

1.0.4 Attachment and entry.

In common with other enveloped viruses, it is the viral glycoproteins which mediate the attachment of *Bunyaviridae* virions to host cell receptors. As yet the cellular receptor involved in attachment has not been identified for any member of the *Bunyaviridae* but β3 integrin is suspected to be involved with the attachment of several hantavirus.

Obijeski *et al.* (1976) demonstrated a five log reduction in infectivity of mammalian cells by La Crosse virus (LAC), when viral particles were rendered "spikeless" by proteolytic enzyme treatment. The viral proteins involved in attachment to vertebrate cells have been examined indirectly in experiments using monoclonal and polyclonal antibodies to block infection or haemagglutination activity. They have suggested that G1 is more actively involved in binding to vertebrate host cells than G2 (Kingsford and Hill, 1983; Grady *et al.*, 1983a and b; Kingsford *et al.*, 1983). In addition, treatment of La Crosse virions (LAC) with proteases, such as bromelain or pronase, which degrade portions of G1 but leave G2 unchanged, rendered the virus completely non-infectious to vertebrate cells (Kingsford and Hill, 1983).

There is some evidence which indicates that viruses within the California serogroup share a common receptor on vertebrate cells, and that this receptor may differ from the receptor used by other members of the *Bunyaviridae*. Pekosz *et al.* (1995), have shown that a truncated soluble form of La Crosse (LAC) virus G1 expressed from baculovirus was able to bind to cells permissive for LAC virus infection but not to nonpermissive cells. The truncated G1 was also able to block the entry and infection of LAC virus and other members of the California serogroup but not the infection of two other bunyaviruses. From this study the authors suggest that G1 is the viral attachment protein and that tropism for cultured cells occurs at the level of cell entry, as determined by RNA dot blots and virus binding assays (Pekosz *et al.*, 1995).

Efficient transmission and amplification of arboviruses requires replication in both vertebrate and invertebrate hosts. As a result virions are exposed to significantly different environments. Treatment of LAC virus with proteolytic enzymes such as those found in the mosquito midgut, *e.g.* trypsin and pronase, in line with earlier work by Kingsford and Hill (1983), cleave the G1 glycoprotein leaving the G2 glycoprotein intact; the treated virus was found to have an increased affinity for mosquito cells (Ludwig *et al.*, 1989). The same enzyme treatment was shown to concurrently cause a significant decrease in viral attachment to Vero cells (Ludwig *et al.*, 1989). Therefore the glycoprotein requirement for attachment to invertebrate and vertebrate cells maybe different.

With regard to the hantaviruses, which do not infect invertebrate hosts, it has recently been demonstrated by Gavrilovskaya *et al.* that β3-integrins facilitate the cellular entry of pathogenic HPS-associated hantaviruses New York-1(NY-1) and Sin Nombre (SN) viruses (Gavrilovskaya *et al.*, 1998). This study has shown that the treatment of permissive cells with a β3-integrin ligand, vitronectin, or with antibodies to β3 integrins, inhibited virus infection by 63-74%. Although virus infection was not completely inhibited by treatment with antibodies, the percentage of inhibition was within the range described for icosahedral viruses, which have previously been shown to enter cells via integrins. To further demonstrate that β3 integrins were indeed responsible for aiding cellular entry, the authors transfected a cell line (CHO), nonpermissive to SN and NY-1 infection with recombinant β3 integrins, and found that infectivity was increased by 19 to 39 fold. The authors also noted that since β3-integrins regulate vascular permeability and platelet function these findings may also correlate the use of β3-integrin with common elements of hantavirus pathogenesis (Gavrilovskaya *et al.*, 1998).

1.2.0 Genomes and coding strategies of the *Bunyaviridae.*

The genome of Bunyamwera virus was the first of the *Bunyaviridae* to be completely sequenced (Lees *et al.*, 1986, Elliott, 1989a, Elliott, 1989b). The total size of the Bunyamwera virus genome is 12.33 kb, of which 95.33% encodes polypeptides. Similar efficient utilisation of coding potential is observed for all other characterised members of the *Bunyaviridae* and for other negative strand RNA viruses.

The complete genomic RNA sequence for at least one representative of each genus has since been determined, allowing the coding strategies of each segment to be deduced (Figure 1.0).

1.2.1 L segment.

Bunyaviridae L segments range in size from 6.8 kb for Bunyamwera (BUN) virus, to 12.2 kb for Dugbe (DUG) virus; the coding capacity of this one genome segment is approximately equivalent to the entire BUN virus genome (12.3 kb). All the L segments encode the L protein (RNA dependent RNA polymerase) in a negative-sense manner (Figure 1.0(A)), and there is no evidence that other minor predicted ORFs within the segment encode additional polypeptides.

1.2.2 M segment.

All viruses of the *Bunyaviridae* possess two glycoproteins denoted G1 and G2, and these are always expressed by the M segment which ranges in size from 3.2 to 4.9 kb (Figure 1.0(B)). The M segment of all *Bunyaviridae* members encode a polyprotein precursor of G1 and G2 in a negative-sense manner. Bunyaviruses, tospoviruses and some members of the *Phlebovirus* genus encode a non structural protein, NSm. No function has been attributed to this protein, with the exception of tospovirus NSm. Tospoviruses are also the only members which have been shown to utilise an ambisense coding strategy for their M segment. Figure 1.0(B) shows the various M segment coding strategies of the *Bunyaviridae*.

1.2.3 S segment.

The length of virus genomic S segments range in size from 0.9 to 2.9 kb (Figure 1.0(C)), however a pattern of size is conserved within a genera. S segments for bunyavirus, nairovirus and hantaviruses code for proteins in a negative-sense manner, but unlike nairovirus and hantaviruses which encode only one protein (N), bunyaviruses encode two, N and NSs. The S segment of phleboviruses and tospoviruses, like bunyaviruses code for two proteins, one in the viral RNA complementary sense (N) and the other in the genomic RNA sense (NSs), a coding strategy referred to as ambisense. The two coding regions do not overlap, but are separated by an intergenic region (Figure 1.2(B)) extending between the two stop codons of the ORFs which varies in length (60 to 360 nucleotides) between different viruses.

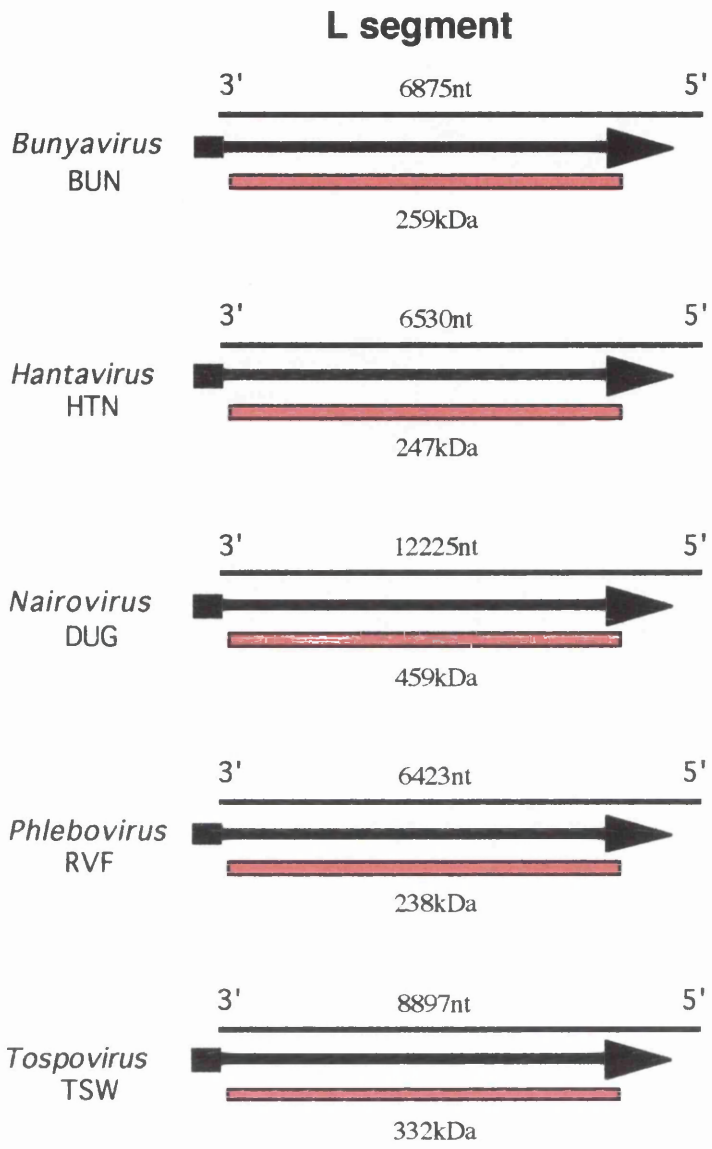


Figure 1.0 (A) The coding strategies for *Bunyaviridae* genome segments

Genomic RNAs are represented by thin lines (length in nucleotides is shown above) and mRNA are shown as arrows (■ indicates host derived primer sequences at 5' end, arrow head indicates 3' end). Gene products with their size in kilodaltons, are represented by coloured rectangles. Two examples of phelebovirus M segments are given which differ with respect to the presence of NSm. Virus abbreviations: BUN, Bunyamwera; HTN, Hantaan; DUG, Dugbe; RVF, Rift Valley fever; UUK, Uukuniemi; TSW, tomato spotted wilt. Figures adapted from Elliott, (1996b). Figure (A), L segments; (B), M segments; (C), S segments.

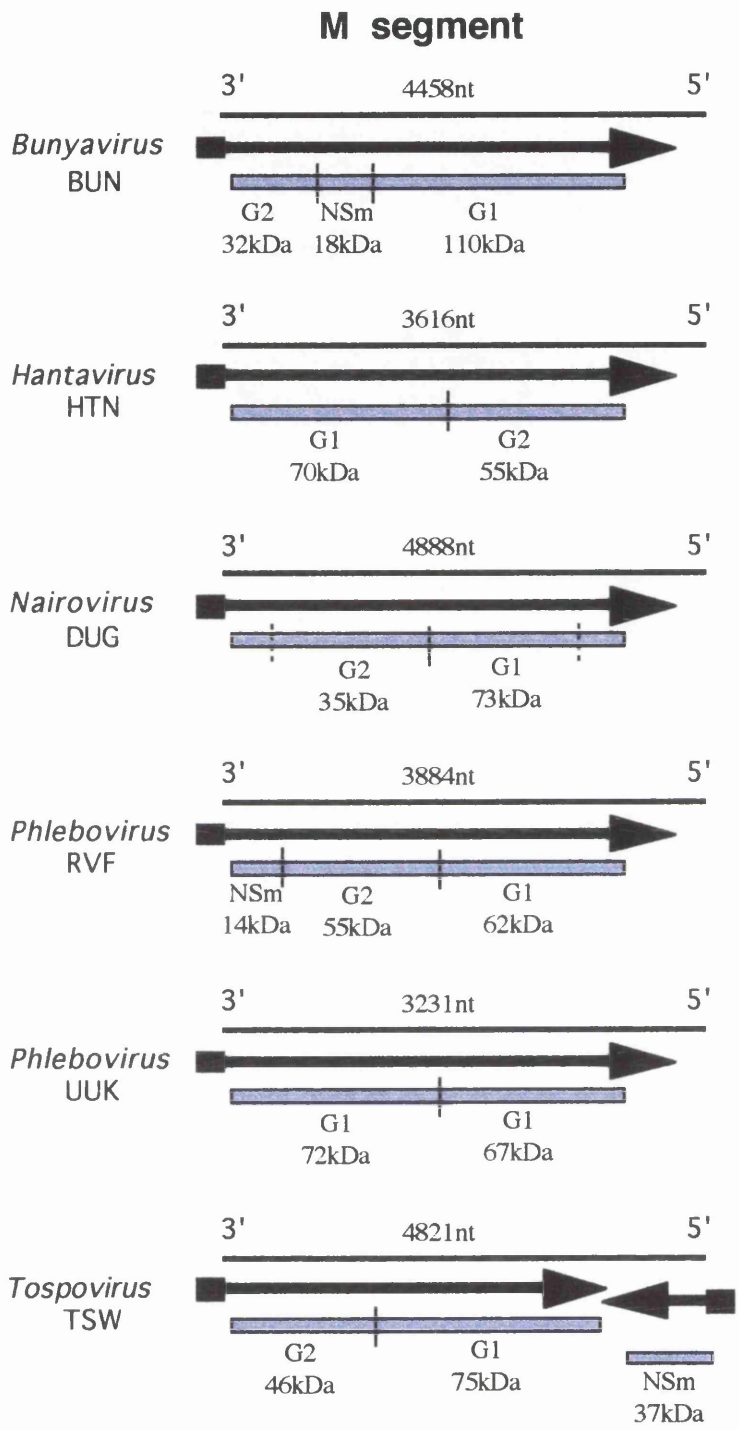

M segment
3'
4458nt
5'
Bunyavirus
BUN
G2
32kDa
NSm
18kDa
G1
110kDa
3'
3616nt
5'
Hantavirus
HTN
G1
70kDa
G2
55kDa
3'
4888nt
5'
Nairovirus
DUG
G2
35kDa
G1
73kDa
3'
3884nt
5'
Phlebovirus
RVF
NSm
14kDa
G2
55kDa
G1
62kDa
3'
3231nt
5'
Phlebovirus
UUK
G1
72kDa
G1
67kDa
3'
4821nt
5'
Tospovirus
TSW
G2
46kDa
G1
75kDa
NSm
37kDa

Figure 1.0 (B)

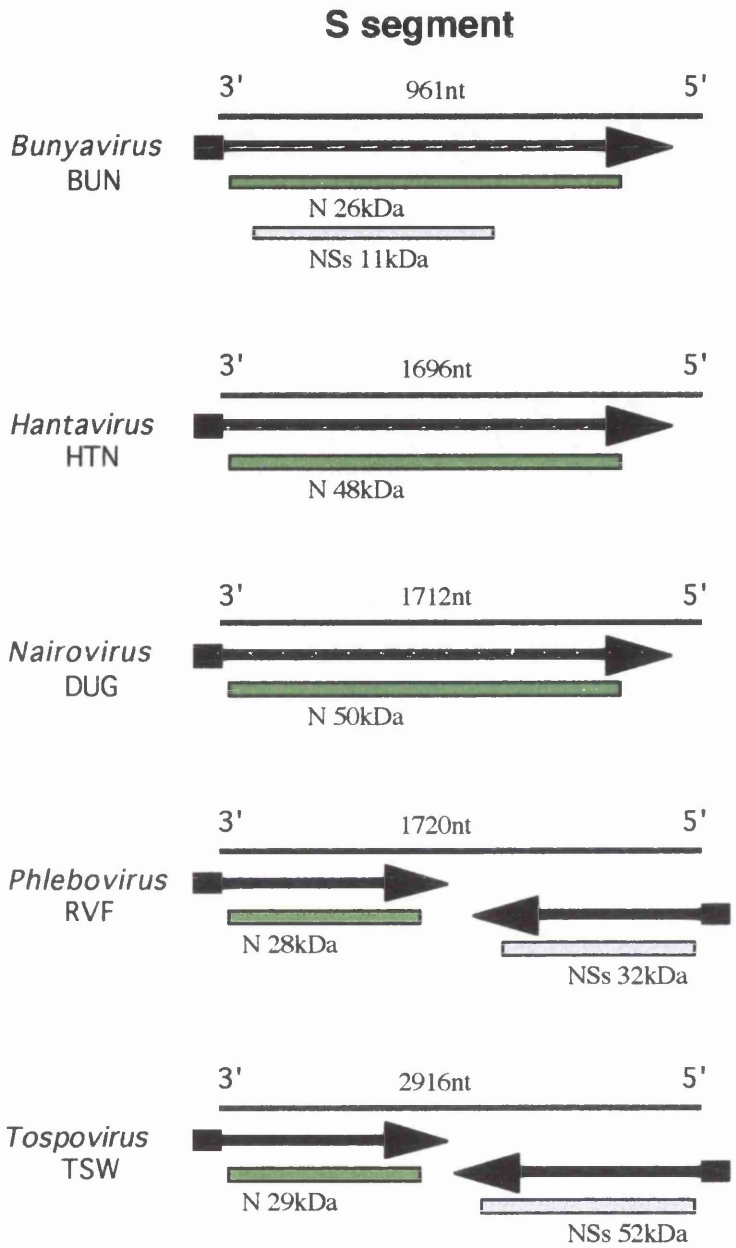

S segment
3'
961nt
5'
Bunyavirus
BUN
N 26kDa
NSs 11kDa
3'
1696nt
5'
Hantavirus
HTN
N 48kDa
3'
1712nt
5'
Nairovirus
DUG
N 50kDa
3'
1720nt
5'
Phlebovirus
RVF
N 28kDa
NSs 32kDa
3'
2916nt
5'
Tospovirus
TSW
N 29kDa
NSs 52kDa

Figure 1.0 (C)

Bunyavirus	3' **UCA UCA** CAU-----
Hantavirus	3' **AUC AUC AUC** UG-
Nairovirus	3' **AG AG** UUUCU-----
Phlebovirus	3' **UG UG** UUUCUG---
Tospovirus	3' **UC UC** GUUAG-----

Figure 1.1 3' Terminal consensus sequences of the five genera within the *Bunyaviridae*

The 3' terminal sequence conserved within each of the five genera is shown above. The di- or tri-nucleotide repeats within each terminus are highlighted (bold, underlined and spaced apart). The same complimentary terminal sequences are found at the 5' terminus of each genomic segment. Figure adapted from Elliott, (1996b).

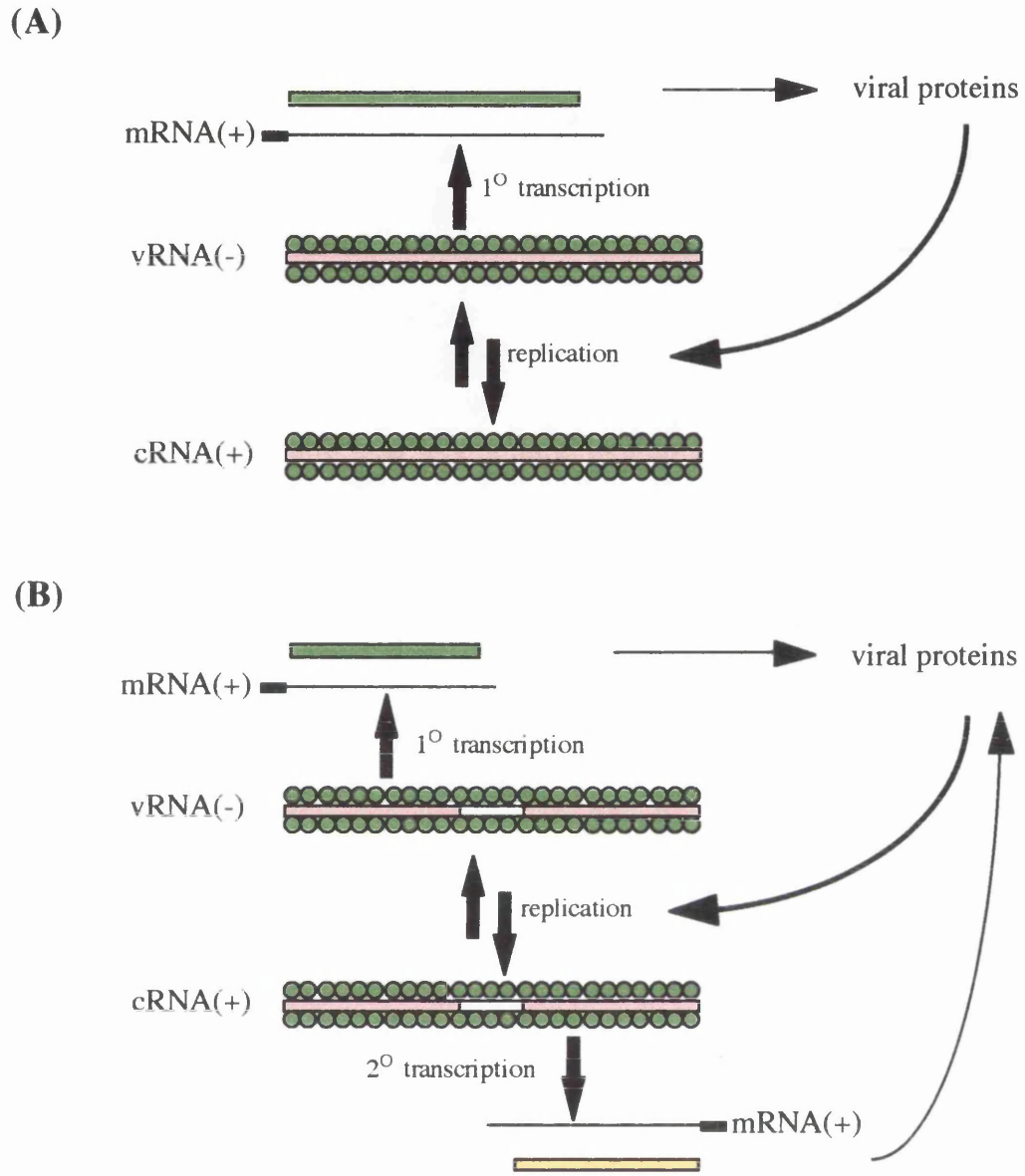


Figure 1.2 Transcription and replication strategies of the *Bunyaviridae* genome segments

Figure (A) describes a scheme for negative strand expression and (B) describes an ambisense strategy. (A) In the negative strand scheme the viral RNA (vRNA) is transcribed (1° transcription) to give a complimentary sense mRNA which expresses the viral protein. The vRNA is then replicated to give a full length complimentary RNA (cRNA) or antigenome, which in turn acts as a template for further replication. (B) For the ambisense coding strategy the vRNA is transcribed (1° transcription) to give a subgenomic viral complimentary mRNA which expresses the first protein. The vRNA is then replicated to give a full length cRNA. The cRNA is then transcribed (2° transcription) to give a viral sense subgenomic mRNA which expresses the second protein or replicated to give a vRNA. The mRNA in (A) and the two mRNAs in (B) each possess nontemplated sequences at their 5' end (black boxes; ■). The white boxes (B) in the vRNA and cRNA represents the intergenic region (IGR) between the two ORFs.

1.2.4 L segment protein.

The L proteins described for bunyaviruses, phleboviruses, and hantaviruses are predicted to be similar in length, approximately 240 to 260 kDa in size. In contrast, the tospovirus TSW L protein is somewhat larger at approximately 331 kDa. Studies utilising antibodies raised to the N- and C-terminal portions of the TSW L protein from cDNA, have established that the protein does not undergo any proteolytic cleavages, is found at the predicted size, and is present at 10 to 20 copies per mature virion (van Poelwijk *et al.*, 1993). Additionally, the L segment of the nairovirus, DUG, is predicted to encode a polypeptide of 460 kDa (Marriott and Nuttall, 1996a). The reason for the dramatic size differences in L protein size between the different genera is unclear, but may represent additional properties related to the different hosts and vectors utilised by the virus.

The function of the L protein as the viral polymerase was confirmed by using L protein expressed from vaccinia virus recombinants to transcribe BUN virus nucleocapsids (Jin and Elliott, 1991). Endonuclease activity was also demonstrated with the BUN L expressed protein, thus providing evidence that the L protein was also responsible for generating the capped primers needed for viral mRNA transcription (Jin and Elliott, 1993a).

Previous analysis of all available RNA polymerase sequences has identified four conserved amino acid motifs (termed A, B, C, and D) which lie in close proximity to each other, and have been coined the polymerase module. The conservation of this module throughout RNA polymerases from disparate sources provides evidence for a common ancestral protein (Poch *et al.*, 1989). Motif A is an acidic motif; motif B is the core motif for nucleotide binding; motif C is the core motif for catalytic function; and motif D is a basic motif (Poch et al., 1990; Ishihama and Barbier, 1994). When these motifs are aligned for *Bunyaviridae* L proteins, in motif C an SDD triplet is strictly conserved, this motif is also strictly conserved for all segmented RNA virus genomes (Elliott, 1996b), as opposed to the GDD or GDN triplet found in motif C for other viral RNA polymerases (Poch *et al.*, 1989).

With the availability of more L segments sequences for the family *Bunyaviridae*, further comparisons were achievable. Muller *et al.* (1994), described a further two conserved motifs, premotif A, found just upstream of motif A, and motif E found just down stream of D, which is strongly conserved only in segmented negative stranded virus polymerases.

The critical role of some of these residues conserved in the motifs initially described by Poch *et al.* (1989), Motifs A to D, has been tested by site directed mutagenesis of the Bunyamwera virus L protein (Jin and Elliott, 1992). It was shown that substitution of amino acids strictly

conserved among the L proteins of the *Bunyaviridae* family abolished the RNA synthesis activity of the L protein (Jin and Elliott, 1992), thus suggesting that the conserved regions may indeed represent the catalytic sites of the L protein. The results of this work will be discussed in further detail along with a diagram demonstrating the sites of mutagenesis in the four conserved polymerase motifs, results section Chapter 3.

1.2.5 M segment proteins.

The gene order of the BUN M segment is NH_2-G2-NSm-G1-COOH as shown in Figure 1.0(B). The first encoded glycoprotein, G2, is predicted to be 41 kDa in size, and the second encoded glycoprotein, G1, between 108 and 125 kDa. The short sequence between these two ORFs codes for, NSm, a short 174 amino acid peptide of unknown function. The exact cleavage sites between G2 and NSm (position 299) NSm and G1 (position 473) have only been determined for Snowshoe hare bunyavirus (SSH) (Fazakereley *et al.*, 1998).

1.2.6 Glycoprotein biogenesis.

Cotranslational cleavage of the M segment polyprotein is thought to be the general mechanism of glycoprotein biogenesis and that processing of bunyavirus glycoproteins occurs cotranslationally is inferred from the fact that no precursor has been found in pulse-chase experiments (Fazakerley *et al.*, 1988). Which trypsin-like proteases cleave the polyprotein precursor are unknown, but it is most likely that all cleavages are carried out by the ER luminal signal peptidases. None of the processing events involved in BUN glycoprotein biogenesis require the concomitant expression of any other viral proteins (Nakitare and Elliott, 1993).

Following synthesis, primary glycosylation and folding in the ER, it is presumed that G1 and G2 dimerise. This dimerisation allows transport to the Golgi, where the proteins are further processed. Transport is arrested at the Golgi, due to a retention signal in the G2 glycoprotein (Lapin *et al.*, 1994). All bunyavirus envelope proteins examined possess N-linked oligosaccharides. Two such potential sites are conserved in the G2 sequences of SSH, LAC, GER and BUN viruses, and either one or two in G1, depending on the virus, and none in NSm (Eshita and Bishop, 1984; Lees *et al.*, 1986; Grady *et al.*, 1987; Pardigon *et al.*, 1988). It has not been determined for all of the viruses which sites are actually utilised, but one study of SSH virus suggests that all of the sites on G2 are utilised, and at least one of the potential sites on G1 (Cash *et al.*, 1980; Eshita and Bishop, 1984; Fazakerley *et al.*, 1988).

All M segment gene products are predicted by nucleotide sequence analysis to have a high cysteine content, and the positioning of cysteine residues is highly maintained within individual genera (Eshita and Bishop, 1984; Grady *et al.*, 1987; Elliott, 1990). It has been suggested that these cysteine residues may give rise to extensive disulphide bridging between glycoproteins, which might be necessary for folding and adoption of correct structural conformation.

1.2.7 The NSm protein of tospoviruses.

The NSm protein of tospovirus TSWV is the only nonstructural protein for which there is convincing experimental evidence to attribute a function, that of a "movement" protein.

Immunogold EM analysis of TSWV infected plant tissues has demonstrated that the NSm protein associates with nonenveloped nucleocapsids, and the plasmodesmata (channel link between cells), where the protein assembles into tubular structures, estimated to be 40 to 50 nm in width (Storms *et al.*, 1995). These tubular structures extend from the cell wall into the cytoplasm at one side, providing firm evidence that they are involved in cell to cell translocation. Since intact viral particles have a diameter of between 90 and 110 nm, and would be therefore too large to cross the plasmodesmata normally, the intriguing possibility has been raised that TSWV is able to move from cell to cell along the tubule as free, nonenveloped nucleocapsids (Storms *et al.*, 1995).

1.2.8 S segment proteins.

The S RNA segment of bunyaviruses, phleboviruses and tospoviruses encode two proteins, the nucleocapsid protein (N) and a non-structural protein (NSs), while those of hantaviruses and nairoviruses encode only an N protein. Bunyavirus S segment proteins are translated from a single bicistronic mRNA species (Elliott *et al.*, 1989), while phleboviruses and nairoviruses translate their S segment proteins from two monocistronic mRNAs as a result of an ambisense coding strategy (Figure 1.0(C) and Figure 1.2). The S RNA segment of hantaviruses and nairoviruses possesses a single ORF in the viral complementary sense which encodes an N protein approximately twice the size of the other genera N proteins (Figure 1.0(C)). It is tempting to speculate that these larger N proteins may contain extra domains which would negate the need for a NSs protein. Additional small ORFs have been detected in the cDNA of hantaviruses, however, protein products from these ORFs have never been detected in infected cells (Parrington and Kang, 1990).

The N protein is the most abundant viral protein found both in the virion, and in infected cells. However, although the antigenic characteristics of N proteins of various members of the *Bunyaviridae* have been studied extensively (discussed in Elliott, 1996a), little is known of their basic functional properties.

Comparison of amino acid sequences of N proteins from viruses in different genera reveals a lack of homology between N proteins, suggesting that these proteins are highly divergent and may not share conserved functional motifs, such as RNA or polymerase binding motifs. Such amino acid homology, however, is evident in N proteins of viruses from within a specific genera. For bunyaviruses, there is 62% identity within the Bunyamwera serogroup, and >40% between the Bunyamwera and California serogroups (Dunn *et al.*, 1994; Elliott, 1995). Identical residues between amino acid sequences are not distributed randomly throughout the proteins, but are clustered, which may indicate maintenance of functional domains specific to viruses in a genus.

Gott *et al.* (1993) published a study on the RNA binding properties of the Puumala hantavirus N protein, in which it was shown that the N protein could bind co-operatively to a partially double-stranded substrate consisting of the exact 5' and 3' HTN S RNA complementary termini. The N protein bound noncooperatively to single-stranded (ss) RNA, and this noncooperative binding was shown to be inhibited by double-stranded competitor. From other experiments published in this study, it was also suggested that the carboxy-terminal portion of the N protein is involved in RNA binding (Gott *et al.*, 1993). However, in this study a large amount of non-specific binding was also observed which may compromise the interpretation of results from the binding experiments. Similarly, it was found that when HTN N protein was expressed by recombinant baculovirus in the absence of the correct RNA substrate, the HTN N protein was found to form nucleocapsid-like structures, possibly by binding with cellular or baculovirus nucleic acids, suggesting that the protein will bind non-specifically to available RNA (Betenbaugh *et al.*, 1995).

Recently, Richmond *et al.* (1998), have reported results which conflict with those found for HTN N protein. In this study it was shown that TSWV N protein did not bind to double stranded RNA but did bind to TSWV like-RNA and did so in a co-operative manner, and that this binding was stable at high ionic concentrations (≤ 0.3 M NaCl), suggesting that binding is not solely due to electrostatic interactions. The binding to TSWV ssRNA was not sequence specific since complex formation was seen with non-specific ssRNAs, yeast tRNA and CCMV RNA3, and even M13 ssDNA. However, if a nucleic acid competitor was added after complexes were allowed to form with TSWV ssRNA, then no competition was seen. In this study it was also demonstrated that both amino- and carboxy- regions of the N protein were capable of binding RNA, and they both did so in a co-operative manner. This was the

first demonstration of multiple RNA binding domains in a segmented negative strand virus nucleocapsid protein.

However, one important caveat that the authors of both these studies point out is that there may be a difference between the binding of N protein to its target RNA *in vitro* as opposed to *in vivo*, where other viral or host proteins may influence the process.

As both the HTN N and the TSWV N proteins have been shown experimentally to bind non-specifically to RNAs, it is possible that the N proteins of the *Bunyaviridae* may not be sequence specific in their binding to RNA *in vitro*, a feature which is common to other negative strand RNA virus nucleocapsid proteins (Richmond *et al.*, 1998) *e.g.* Sendai virus (Buchholz *et al.*, 1993); influenza virus (Kingsbury *et al.*, 1987); and VSV (Masters and Banerjee, 1988). The N protein of TSWV is predicted to have a charge of between +8 and +10 (Richmond *et al.*, 1998). Therefore, the interaction between the N protein and the RNA might simply be the result of a positively charged N protein binding to negatively charged RNA, and co-operative binding may be a function of the N protein itself. It might be speculated that the specificity with which the N protein recognises and binds with viral RNA in the cell, may be attributed to the presence or orchestration of another viral protein, specifically that of the L protein.

1.3.0 The nucleocapsid assembly process.

The processes involved in bunyavirus nucleocapsid assembly have not yet been fully elucidated. However, it is thought that control is exerted at the level of initiation of N protein interaction either with a specific sequence on the target RNA molecule, or with the polymerase/RNA complex. It is proposed that following such an initiation event, the encapsidation process would proceed by addition of further N protein molecules to newly formed RNA-N complex by (N-N) protein interactions. As the genome and antigenome are always found encapsidated (in contrast to the mRNAs which are not encapsidated) the sequence required for nucleoprotein assembly must be present at the 3' end of both the genome and antigenome. Therefore, it is likely that these sequences are contained in the conserved regions found at termini of each viral genomic segment (Kolakofsky and Hacker, 1991). It is conceivable that the polymerase binds to both termini to allow N protein binding, and its this binding of the L protein that orchestrates the addition of N proteins, possibly initially as a L-N binding, and continuing in a co-operative manner with further N-N bindings. If the polymerase does in fact need the presence of both termini to bind and subsequently initiate N protein binding, then this same hypothesis might also explain why viral mRNA is unencapsidated.

1.3.1 Packaging.

Equal numbers of genome segments (nucleocapsids) may not always be packaged into mature virions, as evidenced by varying, nonequimolar ratios of L, M, and S molecules extracted from purified virions (Bishop and Shope, 1979). Talmon *et al.* (1987) have speculated that the size differences of virion particles observed by electron microscopy may be directly related to the number of nucleocapsids incorporated into individual virions.

Interestingly, the phlebovirus, UUK, was found to package virus genome complementary (cRNA) as well as vRNA of the S segment (but not of the M segment) in a ratio of approximately 1:10 (Simons *et al.*, 1990). Similarly, both the S and M segment cRNA of TSW virus, a tospovirus, were found to be packaged (Kormelink *et al.*, 1992a), suggesting that encapsidation of cRNAs might relate to the ambisense coding strategies of these RNAs (*i.e.* that the genome packaging signal may be linked to a 3' UTR capable of promoting both transcription and replication, which for an ambisense coding strategy is both genome segments, vRNA and cRNA).

For LAC bunyavirus, S segment cRNA was detected in virions synthesised in insect cells, but not in mammalian cells (Raju and Kolakofsky, 1989). The significance of this finding is not clear.

1.3.2 Assembly, budding and release.

All members of the *Bunyaviridae* genera, with the possible exception of the tospoviruses, mature by budding through membranes of the Golgi complex (Pettersson, 1991; Hobman, 1993). The precise reason why this membrane is chosen over the more commonly used plasma membrane as the site for exit is not understood. However, the observation that the ionophore monensin inhibits the release of LAC virus has led to the idea that the lower pH of the Golgi compartment is necessary for at least one of the processes of assembly (Cash, 1982).

Bunyamwera virus, like other members of the *Bunyaviridae*, buds into Golgi membranes. BUN glycoproteins localise to Golgi membranes in infected cells. Additionally, accumulation of BUN glycoproteins at Golgi membranes has been shown to occur in HeLa cells expressing G1 and G2 from a full length BUN M cDNA (Nakitare and Elliott, 1993; Lappin *et al.*, 1994). In the presence of brefeldin A, the glycoproteins were redistributed to the ER. Results by Lappin *et al.* (1994) indicate that all three products of the M segment,

G1, G2 and NSm, are localised to the Golgi complex when expressed from full length cDNA. G2 and NSm when expressed independently also localised to the Golgi, while G1 expressed alone remained in the ER. It was shown that G1 localised to the Golgi if coexpressed with G2 but not with NSm, indicating that G2 facilitates the cotransport of G1 from the ER to the Golgi. Similar results have been reported for La Crosse bunyavirus glycoproteins (Bupp *et al.*, 1996). These results show that G1 requires coexpression of G2 to be transport competent, suggesting heterodimeric interactions between the two proteins. This study also indicates that at least G2 contains a Golgi retention signal (Pettersson and Melin, 1996). The function of the BUN NSm protein is unknown, but its Golgi localisation in BUN infected cells suggests a role in virus maturation (Lappin *et al.*, 1994).

The processes involved in assembly and release of bunyavirus particles are very poorly studied. Electron microscopic studies have visualised the maturation of the phleboviruses UUK (Kuismanen *et al.*, 1982), PT and Karimabad (Smith and Pifat, 1982), RVF (Anderson and Smith, 1987) and the nairovirus DUG (Booth *et al.*, 1991) at perinuclear membranes presumed to be Golgi cisternae. In other negative strand viruses such as members of the *Rhabdoviridae*, *Orthmyxoviridae*, and *Paramyxoviridae*, a viral matrix (M) protein bridges the gap between envelope viral glycoproteins and the nucleocapsids during virus assembly. Members of the *Bunyaviridae* lack a functional equivalent of the M protein. It has been shown that nucleocapsids accumulate under Golgi membranes in which glycoprotein projections are evident (Smith and Pifat, 1982; Parker *et al.*, 1984). Furthermore no nucleocapsid accumulation was observed to occur under membranes bearing no glycoprotein spikes. These observations may indicate a direct interaction of the nucleocapsids with the carboxy- tails of the glycoproteins, but there is no experimental evidence as yet to support this idea. However, if such a direct interaction does occur, then in the case of BUN virus, it is possible that the carboxy tail of G2, being longer than that of G1, would participate in the interaction.

It is thought that following viral budding into the Golgi, the particles are transported out of the cell in Golgi derived transport vesicles in a manner analogous to normal cellular exocytosis (Smith and Pifat, 1982).

1.4.0 Untranslated regions.

Each viral genomic segment can be described simply as consisting of two regions, a coding region (each of which have been discussed previously) and an untranslated region (UTR) which again can be subdivided into two regions, the conserved complementary termini and

the rest of the 3' and 5' UTRs flanking the ORFs which share no obvious complementarity between segments.

1.4.1 Bunyamwera virus genome termini.

Figure 1.3 shows the complementary sequences between the termini of Bunyamwera virus. This diagram also illustrates how the terminal eleven nucleotides at 5' and 3' termini of the three genomic RNAs are conserved both between all three segments and between all members of the *Bunyavirus* genus (Figure 1.1). A further three or four adjacent nucleotides are conserved for each viral segment within a BUN serogroup, but not between segment types (Elliott, 1990; Elliott *et al.*, 1991). These conserved regions between the 5' and 3' termini of the RNA molecule are also complementary. Further complementarity exists between 5' and 3' sequence adjacent to these 11 terminal nucleotides, although this complementarity occurs through sequence which is not conserved either between different viruses or between gene segments of the same virus. This additional complementary sequence results in a fairly long region at both ends of each gene segment with potential to form a double-stranded panhandle structure (see Figure 1.3), although whether this proposed panhandle structure is of significance to the structure and replication of the virus genome is unknown.

1.4.2 Possible function of genome segment UTRs.

It is believed that the 3' and 5' untranslated regions (UTRs) of the *Bunyaviridae* viral genome segments, also referred to as noncoding regions, contain signals which control the replication processes of the virus. These signals may include the viral recognition and promoter sequence, as well as packaging and cis-acting signals that may influence the level of transcription and replication of individual RNA segments. However, as discussed above, comparison of UTRs of bunyavirus genomes reveals very little conservation of sequence besides the terminal sequences. It is possible that this lack of conservation outwith the 11 terminal nucleotides might indicate evolutionary divergence in the controlling mechanisms of transcription and replication between the segments.

In Table 1.2, the length of the UTRs for a representative virus from each genus are shown. When the lengths of the different UTRs are compared two general striking features are apparent: the vRNA 5' UTR is at least twice the size of the 3' UTR, and the L segment UTRs are generally shorter than the M UTRs which in turn are shorter than the S UTRs.

		cRNA 5' UTR	cRNA 3' UTR
BUN	L	50	108
	M	56	103
	S	85	174
DUG	L	40	104
	M	47	185
	S	49	337
HTN	L	37	40
	M	40	168
	S	36	337
RVF	L	18	138
	M	20	271
	*S	38	34
TSWV	L	33	36
	*M	84	100
	*S	153	88

Table 1.2 The untranslated regions of the *Bunyaviridae*

A representative member from each genus, for which the complete genome sequence is known, was analysed to calculate the lengths of the untranslated regions (UTRs). The genome sequence accession numbers for each virus genome shown are referenced in Elliott (1996a). Those segments which have an ambisense coding strategy are indicated (*). The genera and example viruses were, *Bunyavirus*; Bunyamwera (BUN); *Nairovirus*, Dugbe (DUG); *Hantavirus*; Hantaan (HTN); *Phlebovirus*; Rift Valley fever (RVF); *Tospovirus*; Tomato spotted wilt (TSWV).

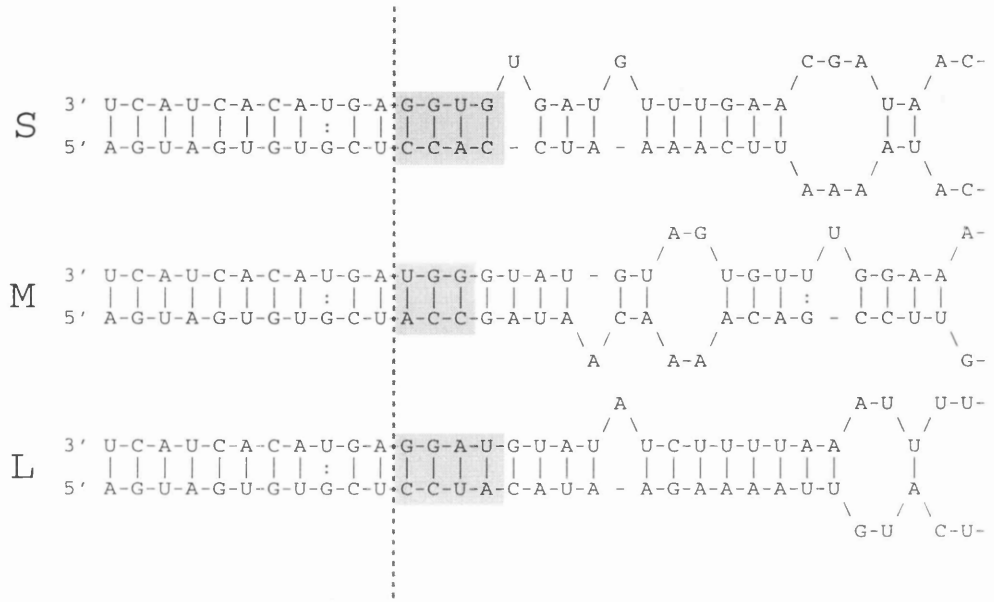


Figure 1.3 Complimentary sequences and possible base-pairing between the 3' and 5' termini of the Bunyamwera virus genome segments

The first eleven bases (left of dotted line) are conserved for all segments of all members of the *Bunyavirus* genus. The next three or four bases (shaded box) are conserved for each viral segment within the BUN serogroup. The remainder of the sequence is BUN segment specific but such regions of segment complementarity exist for all bunyaviruses. The G/U base pair (double dots, position 9) becomes a mis-matched A/C pair in cRNA which could result in disruption of the helical duplex possibly enabling discrimination of vRNA from cRNA for packaging purposes. Figure adapted from Elliott, (1990).

Those segments that do not hold true to these generalisations are those segments with an ambisense coding strategy (marked with an asterisk (*), Table 1.2).

The longer length of the 5' vRNA UTR may indicate the presence of a mRNA termination signal. Although there are no obvious conserved mRNA termination sequences maintained between the three segments, clearly mRNA termination does occur within this region and must be controlled by some un-apparent mechanism. The difference in length of the 5' vRNA UTR between the segments may also suggest the presence of further *cis*-acting signals which could regulate other aspects of segment expression.

1.4.3 Bunyavirus mRNA.

The mRNA transcripts of bunyaviruses contain non-templated heterologous capped leader sequences at their 5' ends (Figure 1.4) which have been obtained from the cytoplasmic pool of host cell mRNA by the viral polymerase (Bishop *et al.*, 1983; Patterson and Kolakofsky, 1984). Another defining feature of these viral mRNAs is that they are truncated at the 3' end by approximately 50-110 nucleotides compared to the full length cRNA (Patterson and Kolakofsky, 1984; Bouloy *et al.*, 1990; Jin and Elliott, 1993a). These features are shared with influenza virus mRNAs, however, unlike influenza virus, bunyavirus mRNA does not appear to be polyadenylated.

In vitro transcription studies using RNPs purified from La Crosse virus (Patterson *et al.*, 1984), demonstrated that transcription could be stimulated by dinucleotide ApG, A(n)G oligonucleotides and Alfalfa mosaic virus mRNA. This study also showed a methylated cap-dependent endonuclease activity which cleaved the plant virus mRNA 13 or 14 nucleotides from its 5' end. Direct evidence for the presence of capped structures at the terminus of the extensions in BUN viral mRNA was obtained by using anti-cap antibodies to immuno-select mRNAs (Hacker *et al.*, 1990; Vialat and Bouloy, 1992).

1.4.4 Leader primed bunyavirus mRNA synthesis.

The mechanism by which bunyaviruses initiate transcription of their mRNA is not well defined, but similarities do exist with the mRNA transcription process of the influenza virus, where capped and methylated oligonucleotides are cleaved from host cell mRNA by influenza virus proteins and are used to prime transcription from the viral genome. However, unlike influenza virus, the transcription of bunyavirus mRNA occurs in the cytoplasm (Rossier *et al.*, 1986).

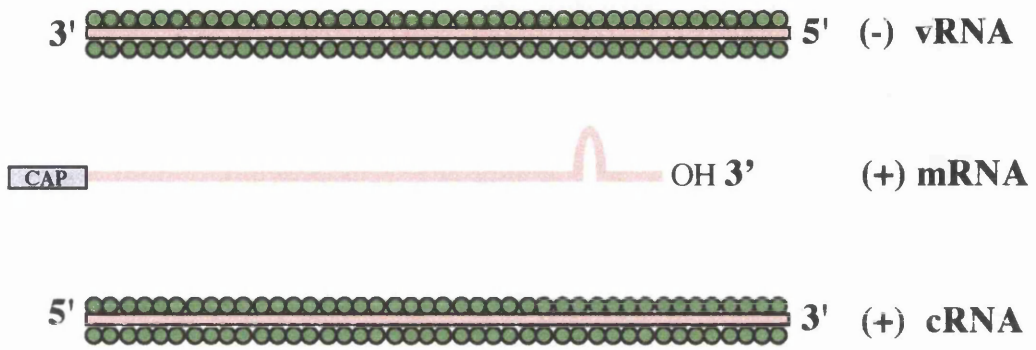


Figure 1.4 Infected cell viral RNA

The viral genomic (vRNA) and antigenomic (cRNA) viral RNAs are not found naked within the cell but encapsidated in N and L proteins. These RNAs are exactly complementary. The viral mRNA contains additional 5' sequences derived from host cell capped mRNA and is truncated at the 3' end compared to the template. Sequences just upstream of the 3' terminus of the mRNA (within the untranslated region) have the potential to form a stem-loop structure which may serve to stabilise the message (Kolakofsky and Hacker, 1991). The blue box at 5' of mRNA represents host derived leader sequence.

Although heterogeneous in sequence, the 5' terminal extensions described for various viruses in the family display preferences for specific mono, di, or tri-nucleotides at the -1 to -3 positions with respect to the 5' terminus of the mRNA (Figure 1.5).

The leader sequences of Bunyamwera virus S mRNA have been cloned and several independent 5' sequences determined (Jin and Elliott, 1993a). Of twenty one clones sequenced, all were found to have heterologous 5' leader sequences. The length ranged from 12 to 17 nucleotides (average 14 nucleotides) and they had a combined base composition of 62% G/C. The striking feature of the non viral sequences was the occurrence, in all but one of the clones, of a U residue at position -1 relative to the viral terminus. Ninety percent of the clones had G at position -2 and half had an A residue at -3. There appeared to be no marked base preference for sequences further upstream (Figure 1.5). These findings were similar to those reported for snow shoehare (SSH) bunyavirus (Eshita *et al.*, 1985) and Germiston bunyavirus (GER) (Bouloy *et al.*, 1990). Some evidence exists that these conserved nucleotides (A<G<U) are not present in the messages from which the leaders were cleaved (Jin and Elliott, 1993a). It seems an unlikely coincidence that the three conserved nucleotides are the same as the first three nucleotides of viral RNA.

A similar arrangement exists for the phlebovirus Uukuniemi (UUK). Instead of the double triplet 5'-AGU AGU-- found at the termini of bunyavirus segments, UUK termini have repeated 5'-AC AC--. For eight out of eleven NSs mRNAs which had all the viral terminal sequence intact, it was shown that all had C at -1 and six of these also had A at -2, and five had C again at -3 (Simons and Pettersson, 1991). No information is available as to whether these repeated bases were present in the cellular mRNA (and were somehow specifically selected for priming), or whether the bases were added by the viral polymerase, perhaps by a slippage mechanism using the 3' end of the vRNA as template. It is interesting to note that all sequenced UUK mRNAs which had the C as the last leader base, had intact 5'-AC AC-- starts to the viral sequence, whereas 9 out of 10 clones with the viral A (at nucleotide +1) missing had a G residue, with potential to hydrogen bond to the template 3' terminal U residue, as the last base of the leader.

Alternatively, similarities between the 3'-most nucleotides of the stolen host sequences and the 5' terminal viral sequences might result from a backward slippage of the viral polymerase on the template after the first two or three nucleotides are transcribed, resulting in a partial reiteration of the 5' terminal sequence (Jin and Elliott, 1993a & b) or as the result of a "prime-and-realign" mechanism (Garcin *et al.*, 1995a. This proposal will be discussed further in Chapter 7).

***Bunyavirus* (BUN)**

(mRNA capped leader)

-1 +1

^{M7}GpppXXXXXXXXAGU **AGU AGUGUA----- --3'**

/ | \

50% 90% 95%

***Nairovirus* (DUG)**

-1 +1

^{M7}GpppXXXXXXXXCUC **UC UCAAAGA------ --3'**

/ | \

46% 58% 96%

***Hantavirus* (HTN)**

-1 +1

^{M7}GpppXXXXXXXXUAG **UAG UAGUAGUG----3'**

/ | \

50% 90% 95%

***Phlebovirus* (UUK)**

-1 +1

^{M7}GpppXXXXXXXXAC **AC ACAAACUG-------- 3'**

/ |

55% 95%

Figure 1.5 Host derived sequences on viral mRNA

Shown is a schematic summary of four studies characterising the nontemplated sequences found upstream of the templated sequences of viral mRNA (i.e. host derived cap-snatched primer sequence). The genomic templated sequence is shown in bold and the terminal nucleotide is marked as +1. The nucleotide repeats within the termini are underlined. Those nucleotides found commonly at positions -1,-2 and -3 are shown (blue) along with the percentage of occasion, X means any nucleotide. The nucleotides most commonly found at positions -1 to -3 are identical to the genomic template terminal repeats. Results summarised from: (BUN) Jin and Elliott, 1993a; (DUG) Jin and Elliott, 1993b; (UUK) Simons and Pettersson, 1991; (HTN) Garcin *et al.*, 1995.

In Figure 1.5, the results of four individual studies are summarised, each of which examines one virus representative from four genera of the *Bunyaviridae*. No base preference at positions -1 to -3 were found in the nontemplated leader sequences of TSWV (tospovirus genus) N protein mRNA (van Poelwijk *et al.*, 1996).

Clearly more data will be needed, particularly concerning the downstream sequences of the cleaved cellular mRNA, before meaningful hypotheses concerning the mechanism of leader selection and priming can be made.

1.4.5 Translational requirement for mRNA transcription.

An unusual aspect of bunyavirus mRNA transcription is the requirement for ongoing translation (Abraham and Pattnaik, 1983; Pattnaik and Abraham, 1983; Raju and Kolakofsky, 1986; Gerbaud *et al.*, 1987). Studies of La Crosse virus have shown that the incorporation of radiolabelled NTP in *in vitro* transcription reactions was unaffected by cyclohexamide treatment, but that the *in vitro* transcripts formed terminated after only extending 175 nucleotides. If a reticulocyte lysate translation mix was added to the *in vitro* transcription reaction, normal length mRNA transcripts could be made (Bellocq *et al.*, 1987). Addition of cycloheximide to the *in vitro* transcription/translation mix resulted in the reappearance of the prematurely terminated transcripts. Messenger RNA transcripts which had terminated at nucleotide 175 could also be detected *in vivo* if cycloheximide was present (Raju and Kolakofsky, 1987).

The sequence around nucleotide 175, where the truncated transcripts end, was shown to have homology to the region near the normal termination site for mRNA transcription at nucleotide 886 (Bellocq *et al.*, 1987). Incorporation of inosine in place of guanosine in *in-vitro* transcription reactions, which would weaken any secondary structure involving G-C hydrogen bonds, allowed read through of the nucleotides 175 termination site and revealed other termination sites before the genuine one at nucleotide 886 (Bellocq and Kolakofsky, 1987). Conversely replacement of uridine by bromo-uridine, to create stronger hydrogen bonds, resulted in more pronounced termination. These results together suggest that in the absence of translation the nascent RNA forms secondary structure, probably with the template RNA, which causes premature termination of mRNA synthesis. For cRNA synthesis, co-transcriptional encapsidation with N protein would attenuate all premature termination sites and the genuine site at nucleotide 886, allowing transcription of full length replicative intermediates to nucleotide 983 (Kolakofsky *et al.*, 1987).

1.4.6 mRNA termination.

The termination sites for several bunyavirus segments have been mapped by nuclease protection assays. Bunyamwera virus S mRNA is predicted to terminate in or near a site 100 nucleotides in from the 5' end of template at 3'-UUGGGUGUUUU-5' (Jin and Elliott, 1992). For Germiston (GER) virus S mRNA (Bouloy *et al.*, 1990), 3'-GUUUGU-5' is present whereas for the S and L segments of La Crosse (LAC) virus (Patterson and Kolakofsky, 1984; Hacker *et al.*, 1990), and Germiston (GER) M (Bouloy *et al.*, 1990) mRNAs, 3'-GUUUUU-5' is present (reviewed in Kolakofsky and Hacker, 1991). This last sequence is similar to viral transcription termination signal employed by influenza virus. For influenza virus however, this site is also a polyadenylation signal. Polyadenylation is a result of polymerase slippage which is thought to result from steric hindrance to the polymerase by the double stranded pan handle which is immediately adjacent to the U-rich termination site (Luo *et al.*, 1991).

For bunyaviruses a possible pan handle structure lies much further downstream of the termination signal than for influenza virus so polyadenylation would not be expected to occur via a slippage mechanism. However the mRNAs of bunyaviruses are probably not polyadenylated, as evidenced by their low affinity for oligo (dT) columns (Cash *et al.*, 1979; Pattnaik and Abraham, 1983).

Located immediately after the predicted G-U rich termination site on the BUN genomic template lies a CA-rich sequence (3'-CCCACCCACCA/UA/UCCC-5'). This CA-rich sequence can be aligned and is conserved in all sequenced Bunyamwera (8) and California (10) serogroup S segments. It is conceivable that this purine rich sequence may play a role in transcription termination of bunyavirus S segment mRNA due to its proximity to the characterised termini, possibly by weakening the interaction of the polymerase with its template (Bishop *et al.*, 1982; Dunn *et al.*, 1994; Bowen *et al.*, 1995).

Another U rich stretch in the influenza virus vRNA promoter has been suggested to result in weak interaction of the polymerase to its template and can be mutated to result in premature termination (Seong and Brownlee, 1992b). In bunyaviruses the occurrence of U residues may therefore facilitate termination by weakening the contacts of the polymerase to its template which may be supplemented by the formation of secondary structures either with the nascent chain interacting with the template (Kolakofsky *et al.*, 1987) or within the new chain at sequences with potential to form a stem-loop structure just upstream of the 3' ends of mRNA (Kolakofsky and Hacker 1991). This picture is somewhat complicated by the occurrence in SSH and RVF phlebovirus M mRNAs of a C rich termination signal (3'-

ACCCC 5'), (Ihara *et al.*, 1985; Collett, 1986). The termination consensus may thus depend on a short homopolymeric region, not necessarily on a run of U residues to signal mRNA termination.

To complicate matters further a recent paper by Hutchinson *et al.* (1996), characterising mRNA transcripts of the Sin Nombre hantavirus (SN), suggests a different mechanism for the termination of each of the three viral mRNAs. In this study it was shown that the S and M segment mRNAs 3' termini were both truncated as described previously for mRNA termini, but that the M mRNA was found to be polyadenylated, and the L mRNA was untruncated. The truncated 3' end of the SN S mRNA mapped to a C rich region (ACCC) on the template RNA sequence, which is also situated just down stream of a CCCACCC motif. The authors were able to map this C-rich motif to 21 other hantavirus S segment sequences (Hutchinson *et al.*, 1996).

This motif is identical to a portion of the purine rich sequence down stream of the mRNA termination site in Bunyamwera and California serogroup viruses described above (shown underlined, 3'-CCCACCCACCA/UA/UCCC-5'). It is possible therefore, that these motifs are similar and function as a signal for mRNA termination, the purine rich sequence could cause the polymerase to pause which signals the polymerase to cleave the mRNA transcript via its endonuclease activity within this region (Kolakofsky and Hacker, 1991).

The finding that the L mRNA 3' end was a full length complement of the 5' end of the genomic RNA template was very surprising, however, the authors were confident that there was no contamination of L antigenome segments as no such contamination was found when studying the other two mRNAs. The other unexpected finding was that the 3' truncated end of the M mRNA was polyadenylated. The viral sequence at the 3' end mapped to a U8 sequence on the template, and this sequence was highly conserved among the other hantavirus M segment sequences (25 in all). To characterise the M mRNA 3' end, the authors used a reverse transcription/PCR approach with an oligonucleotide d(T) primer and a internal M segment specific primer. It is possible, though the authors rule out this reasoning, that during the RT reaction in the absence of polyadenylated mRNA, the oligonucleotide d(T) could in theory prime the stretch of 8A residues as opposed to a poly A tail which is then amplified during PCR.

Interestingly, when the polymerase protein of BUN virus, expressed with a recombinant vaccinia virus, was able to transcribe virion-derived nucleocapsids, it was found that some of the S mRNA transcripts terminated at the authentic transcription termination site, but a significant proportion did not and were presumably untruncated (Jin and Elliott, 1993a).

This however, may have been a side-affect of the expression system used, rather than an inherent property of the BUN mRNA termination mechanism.

1.4.7 Ambisense transcription.

For the S segment of phleboviruses and S and M segments of tospoviruses, transcription is complicated by their ambisense coding strategy (Figures 1.0(B and C) and 1.3(B)). As described above, both the N and NSs subgenomic mRNAs have the same sort of heterogeneous, nonviral, 5' terminal extensions like other *Bunyaviridae* mRNAs (Ihara *et al.*, 1985; Kromelink *et al.*, 1992b). Hybridisation studies on total cellular RNA from phleboviruses Punta Toro (PT) and UUK infected cells, confirmed earlier predictions based on sequence analysis that the NSs message was the same polarity as vRNA and the N message was complementary to vRNA (Ihara *et al.*, 1984; Simons *et al.*, 1990). Similarly for tospoviruses, both subgenomic NSs and NSm messages are the same polarity as vRNA (de Haan *et al.*, 1990; Law *et al.*, 1992). In the presence of cycloheximide, PT virus N mRNA was still detected, but NSs mRNA was not (Ihara *et al.*, 1985). These results are consistent with a model in which replication of full length, encapsidated cRNA must occur before synthesis of the NSs mRNA. This suggests that the phlebovirus or tospovirus NSs protein is not involved in the early stages of replication.

Transcription termination for the ambisense genomes may involve RNA secondary structure. The transcription termination sites for both N and NSs mRNAs of PT virus were mapped by hybridisation with a series of oligonucleotides corresponding to vRNA or cRNA messages. The results indicated that the 3' termini of both mRNAs were within 40 nucleotides of one another (Emery, 1987). Computer analysis of the intergenic region and the sequences encoding the 3' termini of the messages showed a long inverted complementary sequence that could potentially form a hairpin structure. Similar stable hairpin structures are predicted to occur in the intergenic regions of the S and M segments of tospoviruses (de Haan *et al.*, 1990; Law *et al.*, 1992).

1.5.0 Viral genome replication.

As the termini of all *Bunyaviridae* genomes described to date are exactly complementary for 8 or 9 nucleotides (Figure 1.1) (and mostly complementary for about 20 nucleotides (Figure 1.3), the 3' ends of genome and antigenome RNAs (the latter being the presumptive promoters for genome replication), are very similar. Genome and antigenome synthesis is

thus believed to initiate in the same manner on essentially the same sequence (Kolakofsky and Hacker, 1991).

In negative-strand viruses the change from primary transcription to replication requires a switch from mRNA synthesis to the synthesis of full length viral-complementary RNA templates and then viral RNA synthesis (Figure 1.4). The processes involved in these switches have not been defined for any member of the *Bunyaviridae* family. Genome RNA replication and subsequent secondary transcription can be prevented by translational inhibitors such as cycloheximide (Vezza *et al.*, 1979; Eshita *et al.*, I985), indicating that continuous viral protein synthesis is required for replication of the genome. While it is not known which protein(s) are responsible or required for the switch, they are likely to of viral origin, including N and L, and possibly the NSs protein.

The onset of genome replication requires that the viral polymerase does not attempt to cleave host derived capped primers to initiate mRNA transcription, but initiates RNA replication at the extreme 3' termini and that the recognition or action of mRNA transcription termination signals are suppressed, which may indeed be a result of not initiating transcription with a capped primer (Figure 1.4).

For the rhabdovirus vesicular stomatitis virus (VSV), the switch from RNA transcription to antigenome synthesis appears to be controlled by the availability of VSV N protein. Encapsidation by the VSV N also seems to serve as an antitermination signal, thus allowing full-length genome synthesis. It has been suggested that the VSV NS (or P protein) proteins are also involved and act to control the availability and delivery of N to the RNA species (Conzelmann, 1998; Wagner and Rose, 1996). A similar mechanism is plausible for the *Bunyaviridae*, whereby the bunyavirus N protein functions to regulate transcription and replication, possibly involving the NSs protein. The factors dictating how replicative RNAs react with N to form nucleocapsid structures, while mRNAs do not, are unknown. It has been suggested that the added (presumably capped) host cell sequences on the 5 ends of viral messages may somehow prevent encapsidation (Raju and Kolakofsky, 1986), or it could be the 3' truncation that inhibits active nucleocapsid formation.

Recently a BUN-like virus defective in the expression of NSs protein, BUN(-NSs), was rescued from the BUN virus *in vivo* rescue system (Bridgen and Elliott, 1996; Bridgen and Elliott, 1997; 1.8.5). This was achieved by mutating the NSs initiation codons to stop codons and number of amino acids within the NSs reading frame without affecting the N ORF of the S segment genome rescue plasmid cDNA. The resulting BUN(-NSs) virus was capable of infecting cells and producing infectious viral progeny, indicating that the BUN NSs protein is not essential for replication in tissue culture.

1.5.1 Persistent infections and defective RNAs.

Like all arboviruses, bunyaviruses and phleboviruses can replicate both in permissive vertebrate cells and in insect cells. The infection of cultured vertebrate cells is highly cytopathic and leads to cell death, whereas in cultured mosquito cells the infection is non-cytopathic and becomes persistent. This situation may be related to the inapparent infection of mosquitoes *in vivo* (Giorgi, 1996).
Mosquito cells (C6/36) infected with BUN virus have been shown to develop a persistent infection (Elliott and Wilkie, 1986). Upon analysis of total cell RNA from such cells, it was found that there was an over expression of S mRNA compared to L and M mRNAs. Additionally, the presence of subgenomic transcripts for the L segment but not for M and S was reported. Further investigation of the course of persistent infection of C6/36 cells by BUN virus by Scallan and Elliott (1992), confirmed the over-expression of S RNA. Initially in this study, no evidence of defective RNAs in the early phase of the infection was found, however, after prolonged propagation of the infected cultured cells several subgenomic L RNAs were identified. These subgenomic L RNAs were shown not to be defective interfering (DI) RNAs as they were not packaged into virions and their appearance did not correlate with infectious virus release titres (Scallan and Elliott, 1992).

Defective interfering particles, which are associated with autointerference in other negative-strand viruses, have been characterised for BUN virus (Patel and Elliott, 1992). Novel peptides and subgenomic RNAs were detected during the passaging of undiluted virus in mouse cells, but not in BHK cells. PCR cloning and sequencing of these RNAs determined that they comprised of only L segment sequences, and not M or S segment sequences. These L segment RNAs had intact 3' and 5' untranslated regions with a single internal deletion in the ORF, which accounted for 72% to 77% of the L RNA (Patel and Elliott, 1992).

DI RNAs have also been characterised for tospoviruses, TSW and groundnut ringspot virus (GRSV) (Resende *et al.*, 1991), and in this study, again, only L segment-like transcripts were found. Characterisation of the DI RNAs determined that all possessed the original 3' and 5' termini and a single internal deletion in the ORF which corresponded to 60% to 80% of the L RNA. Maintenance of an ORF suggests that these defective RNAs might encode protein. The comparison of DI sequences to published viral L segment sequence determined that the deletions in the ORF occurred between short sequence repeats (of up to five nucleotides), one of which is lost during the generation of the DI RNA (Resende *et al.*, 1992). On the basis of this, the authors of this study suggest that DI genomes may arise via a

"jumping" of the polymerase either internally on the template or even from one template to another.

1.5.2 Effects on host cell metabolism.

With the exception of viruses in 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 host but cause little or no pathological effect in their invertebrate host (James and Millican, 1986).

In vertebrate cells, bunyaviruses and phleboviruses have been shown to cause a progressive decrease in host cell protein synthesis. In Bunyamwera (BUN) infected BSC-1 cells, a decline in host protein synthesis was seen at 5 hours postinfection, by 7 hours, host protein synthesis was almost completely abolished (shutoff) (Pennington *et al.*, 1977). In the absence of host protein synthesis these cells continued to support virus replication. The synthesis of viral proteins was detected up to 22 hour postinfection and infectious virus production continued up to 30 hours postinfection (Pennington *et al.*, 1977). Similar results were obtained in La Crosse (LAC) virus infected BHK cells (Madoff and Lenard, 1982).

Hantavirus infections do not cause a detectable reduction in host cell macromolecular protein synthesis (Elliott *et al.*, 1984; Schmaljohn and Dalrymple, 1984), but routinely establish persistent, noncytolytic infections in susceptible mammalian host cells, a finding consistent with their nonpathogenic persistence in their natural rodent host (Lee *et al.*, 1981a).

1.5.3 Replication in vectors.

For several of the animal infecting members of the *Bunyaviridae* replication of the virus within the vector has been demonstrated (Calisher, 1991) but with no associated cytopathic effects (Beaty and Calisher, 1991), although evidence now exists that tospoviruses also replicate within their vectors and do cause some cytopathological effects and even mortality (Wijkamp *et al.*, 1993).

Hantaviruses, unlike the other members of the *Bunyaviridae*, are not transmitted by arthropod vectors as mentioned earlier. Instead, they are maintained through the persistent infection of rodents and small mammals: these are termed reservoirs. The reservoirs of hantaviruses do not show clinical signs of illness but excrete the virus in the urine and saliva for a long period of time (Lee, 1996; and references there in).

1.5.4 Evolution of the *Bunyaviridae.*

Evolution of members of the *Bunyaviridae* is believed to occur by two major mechanisms: by genetic drift which occurs via the acquisition of genomic changes through point mutations, deletions, duplications, inversions etc., and by genetic shift, which occurs via the reassortment of viral RNA segments as an outcome of a mixed infection.

Until recently, evidence was weighted towards the view that the *Bunyaviridae* exhibited the genomic plasticity characteristic of other RNA genomes (Domingo and Holland, 1988). Viral RNA-dependent RNA polymerases lack a proof-reading ability and thus have a high error rate. The result of a virus polymerase replicating its genome without an editing ability is a high inherent error rate and an accumulation of mutations. On the basis of this, Bishop and Shope (1979) calculated the spontaneous mutation rate of snowshoe hare bunyavirus to be 1 to 2 %. However, oligonucleotide fingerprinting studies carried out by Bilsel *et al.* (1988) and Baldridge *et al.* (1989), suggest that the frequency of such events in persistently infected arthropods may be very low. In support of this, Bilsel *et al.* have reported that Toscana phlebovirus underwent no detectable genomic change upon serial transovarial transmissions through 12 generations of experimentally infected laboratory colony of its natural sandfly host, *Phlebotomus perniciou* (Bilsel *et al.*, 1988).

It might be considered, however, that if intra cellular viral RNA had been examined in the above studies, as opposed to the RNA from infectious viral particles which have been subjected to the selective pressures exerted by the host (replication and infection within the host), then the level of errors found may have been closer to that predicted by Bishop and Shope (1979). Perhaps genomic plasticity is only evident in the genomic sequence of infectious viral particles when the virus adapts to changes in host.

The role of other genetic drift mechanisms such as deletions, duplications and inversions, is less well documented. One possible duplication (approximately 28 nucleotides) of sequence, reported by Dunn *et al.* (1994) and confirmed by Bowen *et al.* (1995), exists within the 3' untranslated region of the Lumbo bunyavirus S segment. The possible benefit or significance of this duplication is not known.

It is becoming clearer that the evolutionary potential of the *Bunyaviridae* is enhanced by genetic shift. The RNA segment reassortment possibilities for a virus with three genomic segments is equal to $2^3 - 2 = 6$ (*i.e.* the number of reassortant genotypes in progeny, less parental types). This has been documented experimentally both in cell culture and in mosquitoes (Bishop and Beaty, 1988; Beaty *et al.*, 1985). These genetic studies also

provided the first data on the coding assignments of the bunyavirus RNAs and proteins, and their biological role. It was determined that the M RNA segment is a major determinant of virulence and infectivity. Distinct sites within the M RNA segment code for different genetic determinants of biological markers, including subcutaneous and intercranial mouse virulence and oral and interthoratic infection of mosquitoes (Beaty *et al.*, 1982; Gonzalez-Scarano *et al.*, 1988). For the L segment, evidence was obtained which showed an association of the polymerase with mouse neurovirulence and neurovasiveness (Endres *et al.*, 1991).

Interestingly, it has been shown through reassortment experiments that only bunyaviruses from a single serogroup are able to reassort with each other (Pringle *et al.*, 1984). It is thought therefore, that individual serogroups may potentially constitute divergent evolving virus pools.

1.6.0 Reverse genetics of negative strand RNA viruses.

Negative single strand RNA viruses can be divided into two groups, those with segmented genomes, the family *Orthomyxoviridae* consisting of influenza A and B viruses (eight segments) and influenza C viruses (seven segments), the genus Thogoto-like viruses (six segments), the *Bunyaviridae* (three segments), and the *Arenaviridae* (two segments; *e.g.* Lassa fever virus) and those with non segmented genomes, the order *Mononegavirales.* The order *Mononegavirales* is subdivided into four families; *Bornaviridae* (Borna disease virus); *Rhabdoviridae* (*e.g.* vesicular stomatitis virus (VSV) and rabies); *Filoviridae* (*e.g.* Marburg and Ebola viruses); and the *Paramyxoviridae* which is again split into two subfamilies: *Pneumovirinae* (*e.g.* respiratory syncytial virus (RSV); and *Paramxyxovirinae* (*e.g.* Sendai virus (SeV) and measles virus (MeV)), (Francki *et al.*, 1991).

In the case of positive-strand RNA viruses, the naked RNA genome functions as a template to initiate translation and the infectious cycle. Therefore, either the introduction into cells of *in vitro* transcribed RNA from a cDNA clone or the intracellular expression of RNA following cDNA transfection, results in the production of infectious progeny. These approaches have established reverse technology for positive-sense viruses with relative ease (reviewed in Boyer *et al.*, 1994).

In contrast, the naked RNA genomes of negative strand viruses are non infectious, and they cannot initiate the production of infectious viruses. Neither the genomic (vRNA) or the antigenomic complementary RNA (cRNA) can serve as a mRNA template for the translation

of viral proteins. To direct viral protein synthesis, the positive sense genome must be transcribed into a positive-sense mRNA, a process which can only be achieved by the specific viral RNA polymerase (RNA dependent RNA polymerase, RdRp), since animal cells do not possess such an enzyme. Furthermore, to function as a template for transcription and replication, the viral genome must also be encapsidated with the viral nucleocapsid protein. Therefore the minimum replication unit of a negative strand virus is the genome complexed with nucleocapsid protein and the viral polymerase (complex) to form a ribonucleocapsid (RNP) (Szewczyk *et al.*, 1988; reviewed in Lamb and Krug, 1996).

Reverse genetic systems have now been successfully applied to all but two of the above mentioned families (*Arenaviridae* and *Bornaviridae*), and within the scope of this section I will not attempt to review all the current literature on the subject but highlight those reverse genetic systems and research that have allowed for the successful manipulation and rescue of negative strand genomes. Specifically the focus will be on that of influenza A virus research, as reverse genetic systems were established for this virus before other negative strand RNA viruses and have since helped propel their research. For futher information on the application and use of reverse genetic systems to study negative strand viruses the reader is directed to reviews by Conzelmann (1996), Palese *et al.* (1996), Garcia-Sastre (1998), and Conzelmann (1998).

1.6.1 Influenza A virus.

Influenza A virus is a negative strand RNA virus belonging to the *Orthomyxoviridae*. It has a segmented genome consisting of eight single stranded RNA molecules which collectively encode a total of ten genes (Krug *et al.*, 1989).

Following fusion of the viral and endosome membranes, the RNP complexes are released into the cytoplasm and migrate to the cell nucleus where transcription and replication of the viral genomes occur. The incoming RNPs are first transcribed into monocistronic mRNAs that are translated to yield viral proteins that are required for replication of the viral genome. Replication of the viral genome involves copying of the vRNA molecules into complementary RNA templates (cRNA) which in turn are used for synthesis of new vRNAs. Newly synthesised vRNAs are finally exported from the cell nucleus to the cytoplasm, and associate with other structural viral proteins to form virus particles that are budded at the plasma membrane and released into the cell medium (reviewed in Lamb and Krug, 1996).

1.6.2 Influenza A virus reverse genetic systems.

1.6.3 RNP reconstitution system.

The first successful attempt to reconstitute transcriptionally active influenza virus RNPs *in vitro*, used purified virus RNA mixed with purified viral proteins (Szewczyk *et al.*, 1988). This study utilised a method developed to allow renaturing of the NP and polymerase proteins (PB1, PB2 and PA) after isolation from intact virus particles by denaturing gel electrophoresis. After blotting onto membranes, the proteins were refolded by the bacterial protein thioredoxin. When genomic RNA purified from virions was mixed with the purified renatured proteins to reconstitute RNPs, transcription was detected. This study demonstrated for the first time that for transcription to occur all three proteins of the polymerase complex and NP were necessary. However this system was not developed further for the reconstitution of RNPs using synthetic RNAs derived from plasmid cDNA.

1.6.4 Reverse genetics of influenza viruses.

Influenza virus became amenable to the expression of foreign proteins when Luytjes *et al.* (1989) established the first reverse genetics system for negative strand RNA viruses (Figure 1.6(A)). A plasmid was engineered containing the chloramphenicol acetyltransferase (CAT) gene in negative polarity. The reporter gene was flanked by the 3' (26 nucleotides) and 5' (22 nucleotides) untranslated regions (UTRs) of the NS gene of influenza A/WSN/33 virus, and was under the transcriptional control of a T7 promoter. From this plasmid it was possible to generate *in vitro* an influenza virus-like RNA molecule with precise viral 3' and 5' termini, due to the presence of an appropriate restriction site at the 3' terminus and a truncated T7 promoter that starts transcription at the 5' terminus. The CAT-RNA molecules were then mixed with purified NP protein and viral polymerases, depleted of endogenous RNA by CsCl centrifugation. This would reconstitute active RNPs, which were then transfected into cells that were previously infected with an influenza helper virus. The helper virus was able to replicate, transcribe, and package the foreign CAT gene, and CAT expression was detected in infected cells. When either naked recombinant RNA alone in the presence of infecting helper virus or recombinant RNP complex in the absence of infecting helper virus were transfected into cells, no CAT activity was detected. Thus the helper viral proteins in this system were not able to elicit reporter activity from a naked RNA, nor were reconstituted RNPs alone able to initiate transcription (Luytjes *et al.*, 1989).

The role of the helper virus in this system (and in other systems that will be described later) is to provide *in trans* the viral proteins required for expression and amplification of the

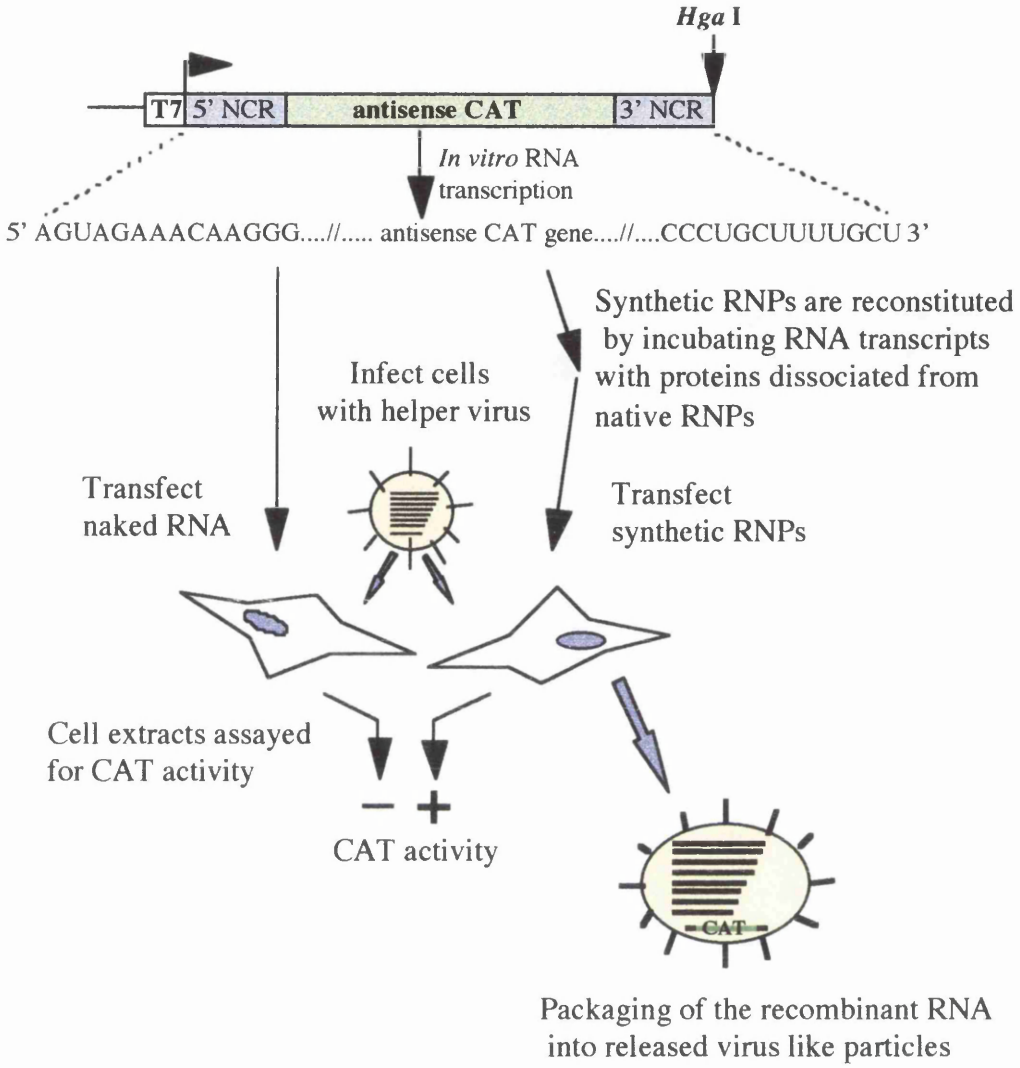


Figure 1.6(A) Synthetic RNP transfection, expression and packaging into virion like particles

Shown above is a diagram of the reporter construct pIVACAT1 (Luytjes *et al.*, 1989), and a schematic for RNP transfection. The *in vitro* transcribed RNA transcript from pIVACAT1 is essentially an antisense CAT ORF flanked by the authentic full length 5' and 3' UTRs of influenza virus segment 8 (see text for further details 1.6.4). The synthetic RNA is complexed with purified viral nucleocapside proteins to form RNPs before being transfected into influenza A virus infected cells. The helper viral proteins act in-*trans* to allow expression of the CAT gene, amplification, and packaging of the synthetic RNA into a viral-like particle.

synthetic RNP complex. An alternative procedure for RNP reconstitution was reported by Seong and Brownlee (1992a). In this procedure, the isolated viral RNPs of disrupted virion cores were freed from endogenous RNA molecules by digestion with micrococcal nuclease. The subsequent addition of EGTA to the preparation inactivated the calcium-dependent micrococcal nuclease enzyme. Reconstituted RNPs obtained after incubation of the nuclease treated cores with a synthetic viral RNA (Luytjes *et al.*, 1989) was shown to support transcription *in vitro* as well as *in vivo* and the subsequent rescue by a helper virus. CAT expression was also detected, although at low levels, when the RNP transfection was done in the absence of helper virus, a result differing from Luytjes *et al.*, suggesting that the reconstituted RNPs alone could initiate transcription of the synthetic RNA *in vivo*. This result may simply reflect the different methods used for preparing the viral cores for RNP reconstitution (Seong and Brownlee, 1992a). This system also allowed for the first time the use of short model templates. These short templates consist only of the 3' and 5' untranslated regions consensus sequences. When these short templates were reconstituted with the nuclease treated cores, all three influenza virus transcription activities were detected *in vitro*, *i.e.* capped primer dependent mRNA synthesis, primer-independent cRNA synthesis, and ApG-primed vRNA synthesis (Seong *et al.*, 1992). The use of the short template *in vitro* system has provided a wealth information on the functions and structure of the viral UTRs, some of which will be discussed in the context of other studies in future sections.

The systems described have been successfully used for the characterisation of *cis*-acting elements involved in replication and transcription of the influenza virus RNAs (reviewed in Garcia-Sastre and Palese, 1993). They all share one common problem, however, and that is that the foreign gene (segment) behaves like a defective RNA segment, and it is rapidly lost after a few passages of the virus. The generation of stable influenza virus vectors expressing foreign sequences was made possible with the establishment of methods to replace an influenza virus RNA segment with a synthetic RNA. In order to replace an influenza virus gene segment with a synthetic RNA, a negative selection method against the helper virus gene or a positive selection method for the transfected gene must be available.

1.6.5 Genetic manipulation of influenza virus genomes.

Enami *et al.* (1990) first described the rescue of a synthetic neuraminidase (NA) gene in which specific mutations had been introduced into infectious influenza viruses (Figure 1.6(B)). This was achieved by using a helper virus with a strong counter-selectable phenotype which was well characterised. Influenza virus A/WSN-HK is a reassortant containing seven segments from influenza A/WSN/33 and the neuraminidase gene segment

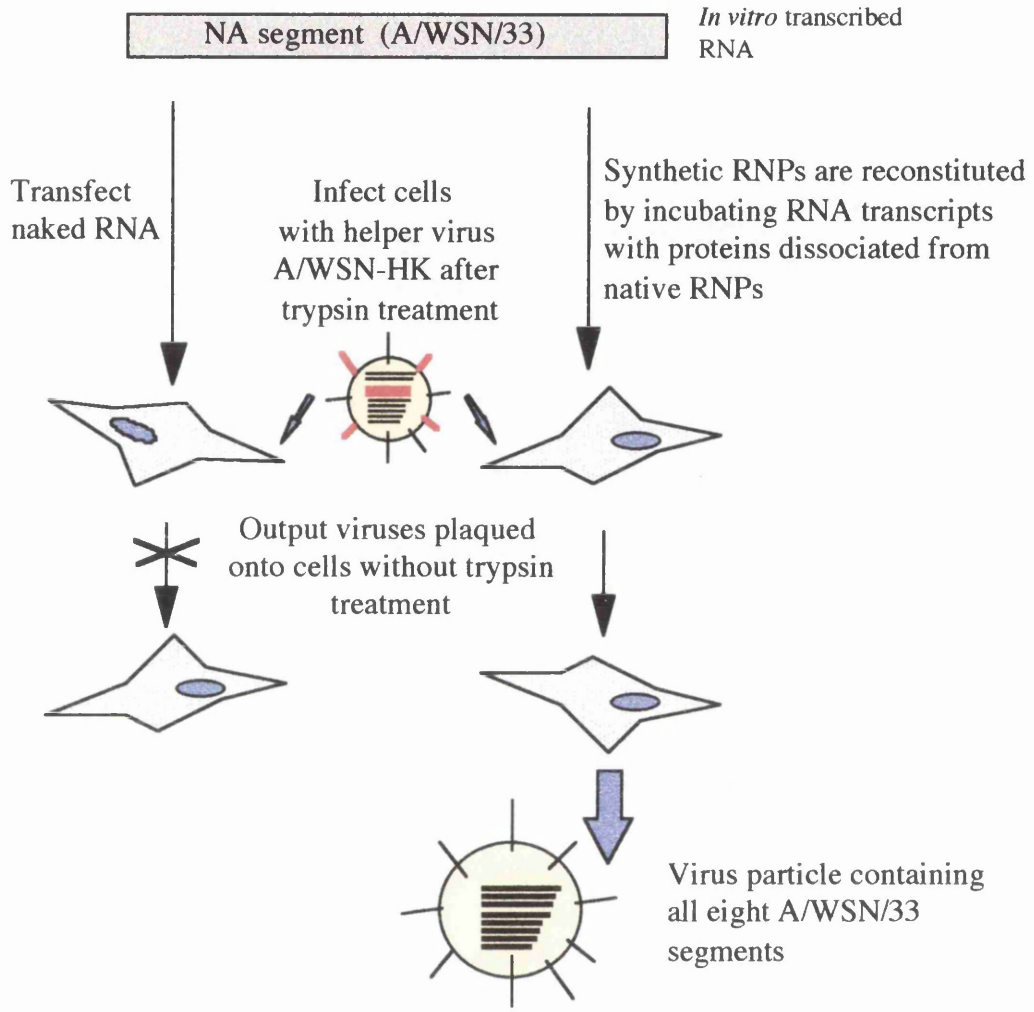


Figure 1.6(B) Rescue of a synthetic NA RNA segment by influenza virus

The schematic describes a method to allow substitution of one of the eight genomic RNA segments of the helper virus by a synthetic RNA. The synthetic NA RNA segment (A/WSN/33) transcribed *in vitro* from a cDNA is complexed with viral proteins to form RNPs. The RNPs are then transfected into cells infected with an influenza helper virus (trypsin treated). The RNP is amplified by the helper virus proteins and packaged into infectious progeny viruses. Appropriate selection conditions, in this case a lack of trypsin treatment, allows the rescue of an transfectant influenza virus containing an RNA derived from cDNA (Enami *et al.*, 1990).

from influenza virus A/HK/8/86. This reassortant virus can only form plaques in MDBK cells when the cell culture media is supplemented with a protease (Schulman and Palese, 1977), whereas the parent WSN/33 strain replicates and forms large plaques without the requirement for exogenous protease. The strategy was to provide WSN NA segment (and therefore protease-independence) in the form of a synthetic RNP. Any virus replicating and packaging the synthetic WSN/NA segment could easily be selected from the helper virus by omitting protease from the cell culture media. Using this procedure it was possible to obtain 2.5 x 10^2 pfu/ 35mm transfection dish formed by virus in which the neuraminidase segment was derived from the transfected synthetic RNP. To verify the plasmid origin of the WSN segment, further constructs were made into which five silent point mutations were introduced in the NA coding sequence. All the viruses recovered in the absence of protease contained the mutations, demonstrating that synthetic RNP had indeed been stably incorporated into an influenza virus (Figure 1.6(B)). Alternatively, a helper virus which only forms plaques in MDBK cells due to a deletion in the stalk region of its NA protein can be used to rescue synthetic NA genes after RNP transfection and passage in MDCK cells (again nonpermissive for the helper virus) (Li *et al.*, 1993b).

The efficiency of the system was considerably improved by performing the plasmid transcription reaction in the presence of purified influenza virus proteins (Enami and Palese, 1991) rather than mixing pre-transcribed RNA with the protein as had originally been done to effect reconstitution (Luytjes *et al.*, 1989). Using the optimised protocol, the rescue of synthetic WSN/NA segment into WSN/HK reassortant virus could be achieved at a much greater efficiency with at least 100-fold more virus being isolated (0.5-1 x 10^5 pfu/transfection compared with 2.5 x 10^2 observed earlier).

The improved efficiency enabled the exchange of the haemagglutinin (HA) and NS genes of influenza virus by cDNA derived RNAs. Selection of viruses containing the transfected HA gene was achieved by using neutralising antibodies that were directed against the HA protein of the helper virus and therefore suppress its infection and further replication. NS gene transfections have been performed using a temperature sensitive influenza virus strain with a defect in the NS gene, which meant transfections were performed at the permissive temperature (33°C), and selection of recombinant viruses was achieved by plaquing at the non-permissive temperature (38°C) (Enami and Palese, 1991).

The combination of improved RNP transfection technology and the use of appropriate selection methods has also allowed the introduction of specific mutations to the PB2 (Subbarao *et al.*, 1993), M (Castrucci and Kawaoka, 1995), and NP genes (Li *et al.*, 1995).

1.6.7 Plasmid based reverse genetics system: RNA polymerase I.

A method was recently developed to reconstitute a biologically active influenza virus RNP complex within the cell rather than *in vitro* (Neumann *et al.*, 1994). This approach circumvents the need for viral protein purification, *in vitro* transcription, and *in vitro* RNP reconstitution by taking advantage of the characteristics of RNA polymerase I (Pol-I) transcription. RNA Pol-I catalyses the synthesis of ribosomal RNA (rRNA) within the nucleus which lack a 5' cap and a 3' polyA tail, thereby resembling influenza virus RNA. An RNA Pol-I based reverse genetics system for influenza virus was therefore established by cloning a reporter cassette (Luytjes *et al.*, 1989) containing an antisense CAT gene flanked by the heamagglutinin (HA) UTRs between a mouse RNA Pol-I promoter and terminator sequences. Transcription by RNA Pol-I transcribes an RNA transcript that initiates and terminates with the authentic 5' and 3' termini, and this occurs within the nucleus, the site of influenza replication. Detectable CAT activity was found in mouse cells (NIH3T3) initially infected with a helper virus then transfected with the Pol-I construct (I-HA-CAT). Moreover, the HA-CAT vRNA was also shown to be packaged into progeny virions (Neumann *et al.*, 1994).

A similar system was also developed by Pleschka *et al.* (1996), but differed in using the human RNA Pol-I promoter to initiate transcription and the hepatitis delta ribozyme to generate the correct 3' end of the viral like RNA transcript. When the Pol-I driven synthetic NA cDNA plasmid is transfected into human cells infected with helper virus, transcription of reporter activity was detected, and packaging of the viral like transcript into virions was also reported. Pleschka *et al.* found that the efficiency of their system was enhanced by the cotransfection of plasmids that express the three polymerase proteins and the NP. These expression constructs were also specialised in their design, in that RNA transcription of each was driven by the RNA Pol-II promoter, thus producing mRNA like transcripts that are 5' capped and 3' polyA tailed to allow export from the nucleus and subsequent translation. These two systems provided the basis for a helper virus free system for the rescue of recombinant viral particles with segmented genomes (1.8.6).

1.6.8 Helper virus-free systems.

One consequence of using reverse genetic systems that rely on the use of helper virus to supply the viral proteins necessary for replication is that functional analyses of these replicative proteins is clearly precluded. Therefore, attempts have been made to develop a helper virus-free system. In one system the influenza virus proteins were expressed *in vivo* from recombinant vaccinia virus constructs (Huang *et al.*, 1990). When cells infected with

four recombinant vaccinia viruses expressing the polymerase subunits, (PB1, PB2 and PA) and the NP protein, were transfected with a synthetic RNP, CAT expression was detected when all four viral components were present. In a similar approach the polymerase subunits (PB1, PB2 and PA) and NP proteins were expressed from recombinant simian virus 40 (SV40) constructs (de la Luna *et al.*, 1993). As in the previous study it was demonstrated that only when COS-1 cells were infected with all four SV40 expression viruses and then transfected with PB2-CAT RNA transcripts reconstituted into RNPs, that CAT activity could be detected. However, unlike in the system described by Huang *et al.* (1990), naked RNA templates were expressed efficiently upon transfection into cells, and to a greater extent compared to when the RNA template was reconstituted into RNPs (de la Luna *et al.*, 1993).

Mena *et al.*, (1994), have also described a reverse genetics system based on the recombinant vaccinia virus (vTF7-3) T7-expression system (Fuerst *et al.*, 1986). In this system, four pGEM recombinant plasmids expressing the four influenza core proteins, allowed the expression of a synthetic influenza like CAT RNA (PB2CAT9). This was the first system to transiently express the viral proteins necessary to transcribe a synthetic RNA entirely from plasmids. Moreover, as in the SV40 system (de la Luna *et al.*, 1993), naked RNA could be transcribed and consistently yielded higher CAT activity levels compared to encapsidated RNA.

Both the SV40 and the vTF7-3 driven systems obviate the need to encapsidate template RNA, and the added advantage of the vTF7-3 system is the ease in which mutagenesis of the genes encoded by the expression plasmid cDNAs can be performed to allow functional studies of the individual viral proteins involved in transcription. The vTF7-3 system has since been further developed (Mena *et al.*, 1996), and it has been demonstrated that by supplying the vTF7-3 infected cell with plasmids containing cDNAs of all ten influenza encoded viral proteins, not only could the CAT RNA template be expressed but rescued into virus-like particles (VLPs). These VLPs budded into the supernate and resembled authentic influenza virions in EM studies. It was demonstrated that NS1 protein was dispensable for the detection of VLPs, which agrees with the fact that NS1 has never been detected in virus particles (Mena *et al.*, 1996). This system can be used to analyse the viral proteins involved in replication and virion particle formation.

1.6.9 Influenza virus as a vector for foreign epitopes and protein expression.

From the development of the systems described above to rescue synthetic viral genes into influenza A viruses, it has since become possible to generate chimeric viruses either containing foreign epitopes within a viral protein, or expressing a foreign protein.

Most of the antibodies generated during a influenza virus infection have been shown to be directed mainly against specific domains of the HA protein. These domains are hypervariable regions within the HA protein termed A, B, C, D and E (reviewed in Garcia-Sastre and Palese, 1995). The hypervariability of these regions allows them to be amenable as an insertion site for short protein sequences representing foreign B-cell epitopes. The B domain has been most successfully utilised.

Li *et al.* (1993a), constructed a chimeric influenza A virus by inserting a 12 amino acid sequence (IHIGPGRAFYTT) which was derived from the V3 loop of gp120 of HIV-1/MN into the antigenic site B of the HA protein of influenza virus A/WSN/33. They demonstrated that BALB/c mice immunised with the chimeric virus were able to elicit antibodies against the HIV peptide. The induced antibodies were able to neutralise the corresponding HIV-1 strain virus in tissue culture. Furthermore, the chimeric virus was able to induce production of HIV specific cytotoxic T cells. These results were the first to show that a influenza A virus can be used as a viral vector to induce B and T cell responses against other infectious agents. The highly conserved epitope (ELDKWAS) from the ectodomain of HIV-1 gp41 has been introduced into the antigenic B site of HA protein. The chimeric virus was also shown to induce neutralising antibodies which were also effective against different HIV-1 strains as well as inducing a mucosal immune response: high levels of HIV specific IgAs could be detected in mucosal excretions of immunised mice (Muster *et al.*, 1994).

Various other groups have successfully grafted foreign epitopes not only into other antigenic domains of the HA protein (site E; Castrucci *et al.*, 1992), but into the stalk region of the NA protein (Castrucci *et al.*, 1994; reviewed in Garcia-Sastre and Palese, 1995).

Transfectant influenza viruses can also protect animals from diseases caused by protozoa and bacteria. It has been demonstrated that a recombinant virus containing the B cell epitope of a human malaria (*Plasmodium falciparum*) antigen in the HA protein elicited a protective immunity in mice (Miyahira *et al.*, 1998). Also, Gilleland *et al.*, (1997) and Staczec *et al.*, (1998), have shown that immunisation of mice with recombinant influenza viruses expressing epitopes of the *Pseudomonus aeruginosa* protein F in the antigenic B site of HA, afforded protection against challenge. This work is potentially important for cystic fibrosis

(CF) sufferers as *P. aeruginosa,* an opportunistic pathogen, is a major cause of morbidity for CF sufferers (Gilleland *et al.*, 1997).

The success of foreign epitope grafting into a viral protein depends on the ability of the viral protein to accommodate the foreign sequence without compromising the viability of the chimeric virus. One approach to avoid this problem has been to engineer a virus segment to encode two proteins, an essential viral protein and a foreign protein, thus allowing the expression of long protein sequences without compromising the viability of the vector. Two such strategies are depicted in figure (1.7 A and B).

Percy *et al.* (1994) (Figure 1.7 A) engineered an NA segment encoding a polyprotein comprising the 2A self cleaving protease (17 amino acids) inserted in-frame between the coding regions of a CAT gene and the NA gene. Upon transcription and translation, this construct results in a CAT/2A/NA polyprotein which self-cleaves to release both the CAT-2A and NA proteins. It was shown that this recombinant NA segment could be rescued and CAT activity detected; notably the CAT activity was not lost after several passages of the virus.

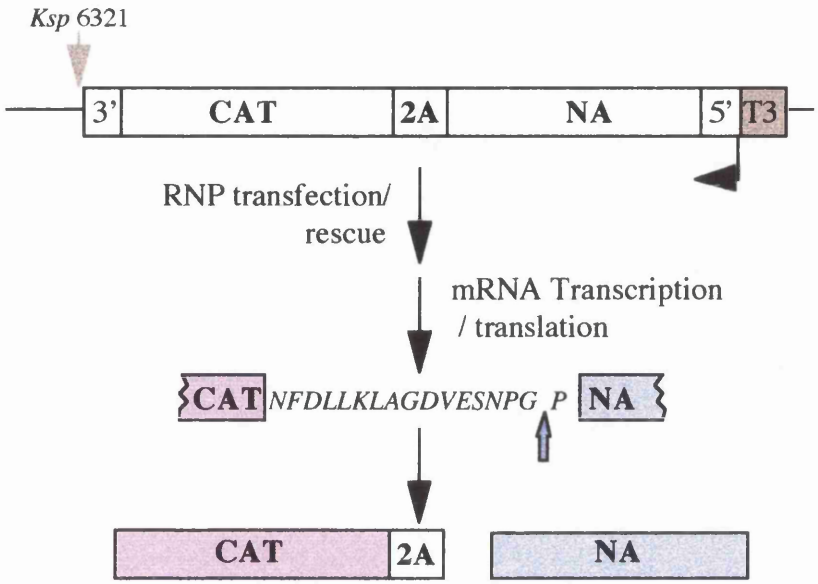
Using a different approach (Figure 1.7.B), Garcia-Sastre *et al.* (1994a) constructed and rescued a recombinant NA segment which transcribed a bicistronic mRNA. This was achieved by positioning an internal ribosomal entry site (IRES) between the foreign ORF, in this case the gp41 gene of HIV, and the NA gene. After transcription of the bicistronic mRNA, expression of the gp41 ORF was effected by cap-dependent translation initiation while the NA gene was expressed by cap-independent translation initiation via the IRES element.

1.7.0 Transcription and replication of influenza virus.

During the replication cycle of influenza virus in infected cells, the genomic viral RNA (vRNA) segments serve as templates for the synthesis of subgenomic mRNAs as well as for full length complement RNA (cRNA), which in turn acts as a template for vRNA synthesis.

The initial *in vivo* study by Luytjes *et al.* (1989) showed that the conserved 5' and 3' untranslated regions sequences were sufficient for the expression, replication, and packaging of genome like segments into influenza virus particles (Luytjes *et al.*, 1989). The development of RNP reconstitution systems and transfection systems has allowed detailed characterisation of the RNA signals involved in the regulation of transcription, initiation, termination, polyadenylation, and replication. All of these signals are known to reside in the

(A)



(B)

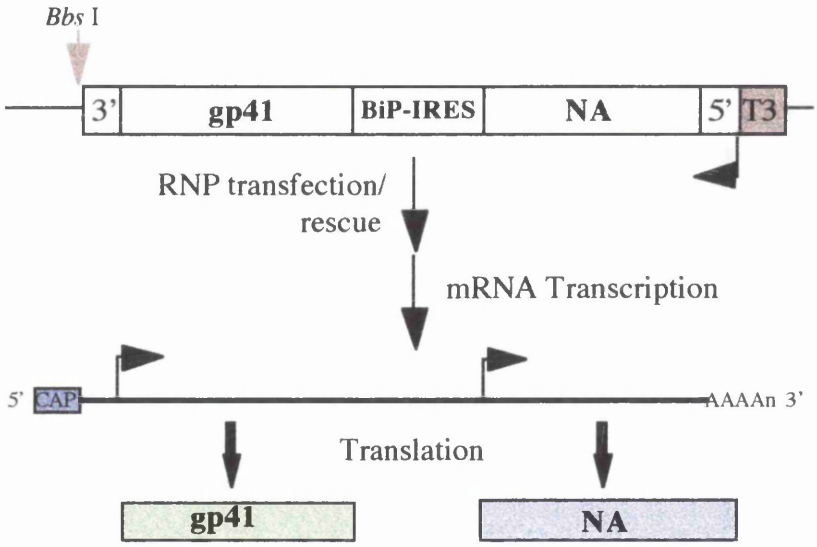


Figure 1.7 Strategies for the expression of full length foreign proteins from influenza virus vectors

(**A**) The foreign protein is expressed as part of a self cleaving polyprotein. The coding sequence for a self cleaving protease (shown in italics), 2A, of foot-and-mouth disease virus, is positioned in frame between the coding regions of CAT and NA. The resulting polyprotein CAT/ 2A / NA is autocatalyticaly cleaved by the protease *in trans* to release a CAT/2A fusion and the NA protein with an extra amino-terminal proline. The site of proteolytic cleavage is indicated by the arrow. Figure adapted from Percy *et al.* (1994). (**B**) The foreign protein is expressed from a bicistronic mRNA. The IRES element of the human immunoglobulin heavy chain binding protein (BiP) was inserted between the foreign protein gp41 and the NA gene, allowing gp41 to be expressed via cap-dependent translation while the NA is expressed via internal ribosomal initiation of translation. Figure adapted from Garcia-Sastre *et al.* (1994b).

terminal sequences of the vRNA segments (Luytjes *et al.*, 1989; Enami *et al.*, 1990). Specifically the vRNA 3' end is believed to be responsible for mRNA transcription and cRNA replication, while the signals for vRNA replication and mRNA termination/poly adenylation are thought to reside in the 5' end. Thus these regions have been major targets for mutational analysis using the reverse genetics systems.

1.7.1 Poly A signal.

Viral mRNAs are incomplete copies of the vRNA, whereas cRNAs are complete copies. Viral mRNA synthesis initiates at the 3' end on the vRNA template with a capped primer (derived from host mRNA) and terminates at a stretch of U residues 16 nucleotides (in the case of the NS gene) from the 5' end of the vRNA. Since the stretch of U residues is adjacent to the panhandle structure made by the termini it has been postulated that this sequence is responsible for polyadenylation, and that this process occurs by stuttering of the viral polymerase at the RNA duplex with repeated copying of the U sequence.

Both Luo *et al.* (1991) and Li and Palese (1994), have shown that for polyadenylation to occur an uninterrupted stretch of five to seven uridines must be located near the 5' end of the vRNA: a deletion of two uridines (from 6 to 4) decreased CAT expression to 4% of wild-type RNA. It has also been shown that the distance between the U stretch and the 5' end of the vRNA is crucial: 16 nucleotides optimal, and an increase of just one nucleotide reduced CAT expression to less than 1%. In addition, the base-paired region adjacent to the polyA signal (region II) was found to be crucial for its function, although a specific sequence in this region was not (Li and Palese, 1994).

Recent findings by Pritlove *et al.* (1998) and Poon *et al.* (1998) also indicate that a functional polymerase binding site at the 5' of the vRNA is essential for polyadenylation. Thus, for polyadenylation to occur, three elements are required: a uridine stretch near the 5' end of the vRNA, a base paired region at region II, and specific nucleotides in region I 5' end (Figure 1.8).

1.7.2 Noncoding regions.

The noncoding regions lie between the terminal partly double stranded promoter (conserved between genome segments) and the start and stop codon, respectively. The lengths and nucleotide composition of these regions vary among gene segments. Whereas the basic information for transcription and replication is located in the terminal promoter element, the noncoding regions are thought to contain *cis*-acting signals involved in the regulation of

these processes. Deletion of either, but not both, the 3' and 5' noncoding regions of the influenza NA gene did not effect the amount of NA RNAs (Zheng *et al.*, 1996). Also, transfectant viruses containing mutations in the noncoding regions showed a reduction in the corresponding vRNA (Bergmann and Muster, 1996). Together these data may indicate that the noncoding regions are not crucial for replication and transcription, but that they contain signals that regulate these processes.

1.7.3 Influenza virus promoter regions.

Whether it is essential that the viral promoter is situated at the extreme 3' end of the RNA template is unclear and seems dependent upon the assay system used. Parvin *et al.* (1989) reported that an RNA template containing 5 extra nucleotides at the 3' end was only transcribed *in vitro* at 33% the efficiency of authentic sequence. Similarly, templates with 1, 5, and 13 extra nucleotides at the 3' end of a cRNA CAT reporter retained only 55, 26 and 6% respectively of the promoter activity *in vitro* (Li and Palese, 1992). However, when these CAT cRNAs were tested in an *in vivo* system, only the template with one additional nucleotide was functional showing 20% wild type activity (Li and Palese, 1992). Piccone *et al.* (1993) reported that RNA templates containing 13 or 30 extra nucleotides or a deletion of one nucleotide at the 3' vRNA terminus showed a drastic reduction in either *in vitro* or *in vivo* systems. Taken together these results strongly suggest that optimal promoter activity *in vivo* and *in vitro* requires the precise 3' terminal sequence, without additional or deletion of terminal nucleotides. These results also suggest that *in vivo* assays are more sensitive to modifications at the 3' end.

The two main research groups who have undertaken extensive mutagenesis of the influenza promoters have used different systems. Brownlee's group have utilised an *in vitro* transcription system (Seong and Brownlee, 1992a, b; Fodor *et al.*, 1994, 1995, 1998; Pritlove *et al.*, 1995), while Hobom's group have used the Pol-I *in vivo* system (Neumann and Hobom, 1995; Flick *et al.*, 1996).

These extensive mutational studies have determined nucleotides crucial for promoter activity, however, which particular nucleotides were found to be critical depended on the assay system used in the investigation. In general, mutations in the vRNA or cRNA promoter affect *in vivo* expression of a CAT reporter gene more extensively than *in vitro* transcription. This maybe because *in vitro* systems measure a single step (*e.g.* the synthesis of cRNA from vRNA), whereas *in vivo* systems could involve multiple steps (*i.e.* transcription and replication of the vRNA template, as well as translation of the CAT mRNA). In fact, the discrepancies between results from the two systems suggest that nucleotides within the

promoter region are involved in additional functions required for CAT expression (*e.g.* stability, polyadenylation, transport, and or translation of the mRNA). Results of promoter studies obtained using various systems are therefore not strictly comparable, however, they do share general features.

The promoter sequence has been mapped to the first 12-14 nucleotides at the 3' end of the vRNAs, and to the first 11-13 nucleotides at the 3' end of the cRNAs, which correspond approximately to the conserved terminal nucleotides, although initially results from *in vitro* systems suggested that the promoter for polymerase binding and transcription initiation resided entirely in the 3' conserved sequence (Parvin *et al.*, 1989; Seong and Brownlee, 1992a). More recent studies using the vaccinia virus-T7 system suggest that the binding of the 5' ends of the vRNAs are a prerequisite for both endonuclease activity and transcription initiation of influenza virus polymerase (Hagen *et al.*, 1994; Tiley *et al.*, 1994; Cianci *et al.*, 1995). In fact the promoter activity of a construct containing a mutation within the 3' end of the vRNA could be restored by introducing a complementary mutation at the 5' end of the vRNA, or vice versa. Studies of the cRNA promoter produced similar results. Therefore, the vRNA and the cRNA promoters consist of both the 3' and 5' ends of the vRNA and the cRNA, respectively. Mutational analysis revealed that the vRNA and cRNA promoter can be divided into two elements: region I (nucleotides 1-9 at the 3' end and nucleotides 1-9 at the 5' end) and region II (nucleotides 10-15 and 11-16 at the 3' and 5' ends respectively; Fodor *et al.*, 1994; Fodor *et al.*, 1995; Flick *et al.*, 1996. These two regions may be connected by a flexible joint, formed by the unpaired A at position 10 at the 5' end of the vRNA (Flick *et al.*, 1996). Within region II base-pairing is crucial: mutations that result in the loss of base-pairing abolish promoter activity completely (Fodor *et al.*, 1994; Flick *et al.*, 1996; Kim *et al.*, 1997). Promoter activity can, however, be restored by introducing complementary mutations that restore base-pairing (Fodor *et al.*, 1994; Kim *et al.*, 1997; Flick *et al.*, 1996). By contrast, specific nucleotides are crucial for promoter activity in region I.

1.7.4 Structural models for the influenza promoter.

The panhandle was the first structural model to be proposed for influenza vRNA (Desselberger *et al.*, 1980; Hsu *et al.*, 1987) (Figure 1.8(A)). It is based on nuclease S1 protection assay and electron microscopy studies. The sequence shown in figure 1.8(A), is that of the influenza virus terminal consensus sequence. Initial attempts to characterise the interaction of the 3' and 5' ends by mutational analysis (Li and Palese, 1992; Piccone *et al.*, 1993) and by biochemical analysis (Baudin *et al.*, 1994) supported the notion of a panhandle structure. It was Fodor *et al.* (1994) who first proposed a new terminal structure, the fork model (Figure 1.8(B)), which predicts a single stranded conformation for region I and a

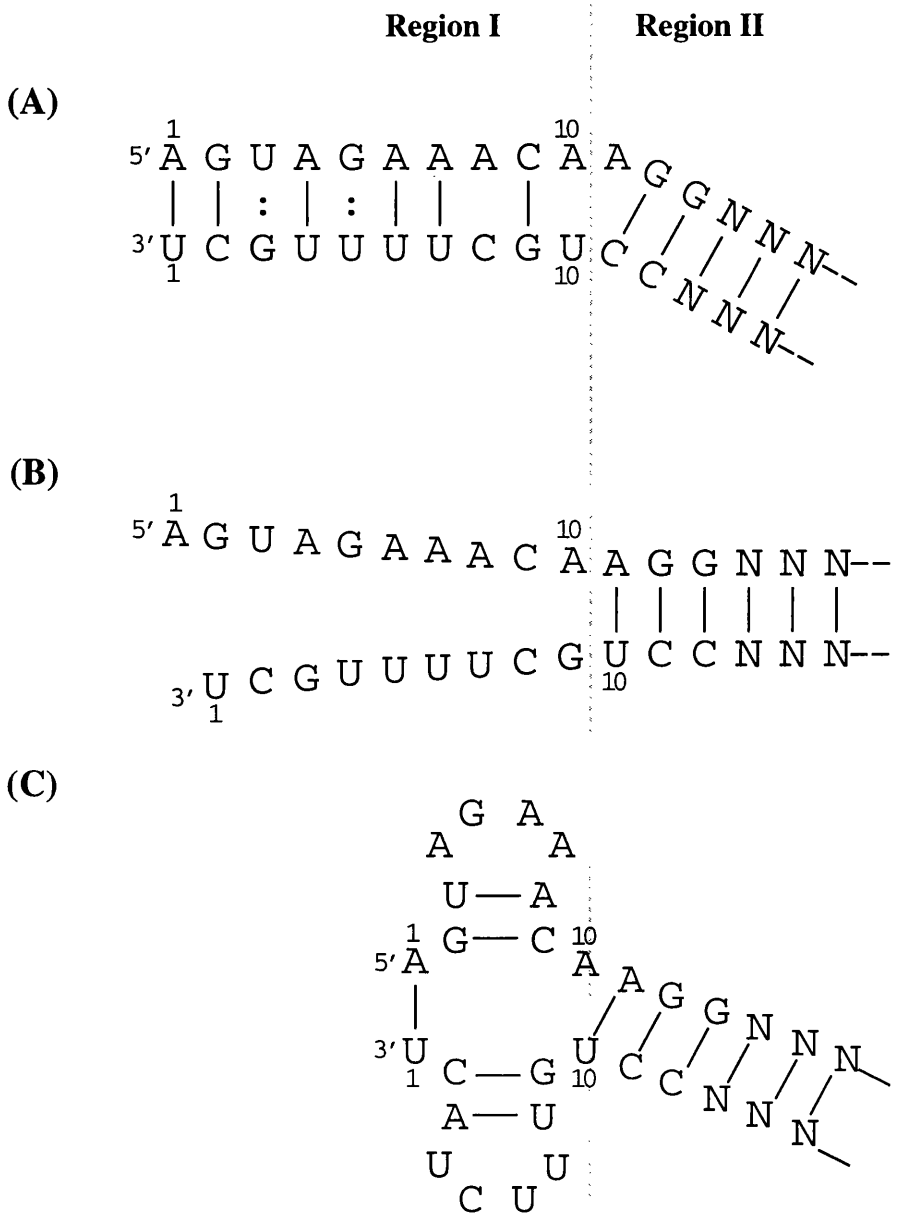


Figure 1.8 Proposed secondary structures for influenza A virus 3' and 5' ends

(A) The influenza A virus proposed panhandle secondary structure based on the consensus sequence. (B) and (C), secondary structures as proposed by patterns of single substitutions, and supported by complimentary double substitutions, (B) the fork model (Fodor *et al.*, 1994), and (C) the corkscrew model (Flick *et al.*, 1996). Watson and Crick base pairs are indicated by vertical lines. Figures (A) and (C) adapted from Flick *et al.* (1996), and Figure (B) is adapted from Fodor *et al.* (1994).

double stranded element in promoter region II. This fork model is based on findings that base pairing is crucial within region II (nucleotides 12-14, 3' and 11-13, 5'), but not at positions 1-8 (Fodor *et al.*, 1994; Fodor *et al.*, 1995; Kim *et al.*, 1997). Flick *et al.* (1996) proposed the corkscrew model (Figure 1.8(C)), which agrees that region II is base-paired, but differs from the other models in that it also predicts intra-strand base pairing within the 3' and 5' ends, rather than between them. The intra-strand base pairs are formed between the nucleotides at positions 2 and 9, and 3 and 8 at both ends of the viral RNA. These short base paired regions stabilise exposed single stranded RNA tetra-loop structures (positions 4 to 7). The strength of the cork screw model is supported by the finding that mutations that destroy base pairing within the short stem regions (positions 2:9, 3:8) reduce CAT activity, while if intra-strand base-pairing is maintained with a complementary mutation, reporter activity is restored (Flick *et al.*, 1996). The authors also suggest a two step model for the interaction of the polymerase complex with the vRNA terminal promoter sequence, where, initially the polymerase interacts with closed structure, as shown in figure 1.8(A), with the termini based paired together. Once protein has bound, it is proposed that the terminal structure then converts to an open complex interaction (corkscrew structure, Figure 1.8(C)) with the viral polymerase (Flick *et al.*, 1996).

1.8.0 Negative strand virus rescue systems.

1.8.1 Reverse genetics of nonsegmented viruses.

Following on from the successful rescue of influenza A virus genome segments by reconstituting synthetic RNPs *in vitro*, the same approach was tried in an attempt to rescue nonsegmented viruses. However, as yet, all attempts to encapsidate synthetic RNAs of nonsegmented viruses *in vitro* have failed (Garcin *et al.*, 1995(b); reviewed in Conzelmann, 1996). This maybe because these viruses have a tighter RNP structure or due to the length of RNAs involved (11-15 kb).

In previous attempts to rescue influenza A virus by transfecting naked influenza virus RNAs into virus infected cells, it was found that these RNAs were not recognised by the viral proteins (Luytjes *et al.*, 1989). In contrast, for some nonsegmented viruses it has been shown that *in vitro* transcribed synthetic RNAs when transfected into cells previously infected with the homologous helper virus were recognised by the viral nucleocapsid proteins, and were subsequently replicated, transcribed and rescued. However, the success of this method has been limited, achieving the rescue only of subgenomic RNAs for a small

number of nonsegmented viruses, *e.g.*, Sendai virus (SeV) (Park *et al.*, 1991), RSV (Collins *et al.*, 1991), and MeV (Sidhu *et al.*, 1995).

1.8.2 Ribozymes allow *in vivo* transcription of template RNAs.

Another major breakthrough in optimising reverse genetic systems exploited the use of the hepatitis delta virus (HDV) ribozyme. This ribozyme has autocatalytic activity which is indiscriminate with regard to sequences 5' of its cleavage site, and was therefore useful in developing plasmid vectors designed to allow intracellular generation of RNAs with defined 3' termini (Ball, 1992).

Initially, this system was applied to produce VSV interfering particles which originated entirely from cDNA clones (Pattnaik *et al.*, 1992). It had previously been shown that VSV DI particles could be replicated when infected into cells expressing the L, N, and NS proteins from plasmids via the vTF7-3 T7-system. If the M and G proteins were also expressed within the cell, the replicated DI genomes could be packaged into DI particles which budded from the cells (Pattnaik and Wertz, 1991). This system, as it stood, allowed structural and functional analysis of the interaction between the VSV proteins and DI template to be undertaken.

Pattnaik *et al.* (1992) placed a HDV ribozyme downstream of a cDNA corresponding to a DI genome under control of a T7 promoter, so that the resulting transcript was initiated at the first DI specific nucleotide, and when the ribozyme cleaved itself from the T7 transcript, it generated the authentic DI 3' nucleotide. As described previously, when all the VSV gene products were present in the cell, the *in vivo* transcribed DI RNA could be packaged into VSV DI particles (Pattnaik *et al.*, 1992). This work was a major achievement, and was the first report of a DI particle formed entirely from cDNA *in vivo*. The system also allowed for the mutational analysis of the VSV DI promoters and *cis*-acting sequences and formed the basis for the rescue of a full VSV genome (Pattnaik *et al.*, 1995).

The basis of this helper free system has been successfully utilised by nearly all other nonsegmented negative strand (NNS) virus reverse genetic systems (*e.g.*, human parainfluenza virus 3 (hPIV-3), De and Banerjee, 1993, Dimock and Collins, 1993; Rabies virus (RV), Conzelmann and Schnell, 1994; Sendai virus (SeV), Harty and Palese, 1995). However, at this point, all attempts to rescue a full length genomic RNA transcript were unsuccessful, and in fact, Conzelmann and Schnell (1994), had found that DI rescue efficiency decreased as the length of the transcripts increased, by approximately 10 fold with each additional kb.

1.8.3 Rescue of a negative strand virus from cDNA.

In 1994, Schnell *et al.*, reported the first successful recovery of a recombinant infectious rabies virus (RV), produced entirely from cDNAs. The authors used a similar system to that described by Pattnaik *et al.*, (1992), whereby cells infected with vaccinia virus vTF7-3, were transfected with T7-plasmids encoding the viral replication proteins N, P and L along with a T7-plasmid encoding the full length RV antigenomic viral RNA under the control of a T7 promoter placed at the 5' end, and with the HDV ribozyme placed at the 3' end. In this initial study only 1 to 3 plaque forming units were rescued from ~2 x 10^7 cells. The rescued viruses were separated from the high number of vTF7-3 particles by a filtration step after freezing and thawing. The key to the recovery of infectious RV was to express a full length antigenome transcript rather than previous attempts with genome templates.

Using the same experimental approach, the successful rescue of VSV was subsequently reported (Lawson *et al.*, 1995; Whelan *et al.*, 1995), and also in the same year, MeV (Radecke *et al.*, 1995), SeV (Garcin *et al.*, 1995b), and RSV (Collins *et al.*, 1995) (See Table 1.3). Although variations within the recovery systems were employed, each group found that successful recovery of recombinant viruses depended upon expression of the viral antigenomic RNAs.

The advantage of using antigenome transcripts to initiate encapsidation may be obvious. If naked genome sense transcripts were produced in the cytoplasm of cells that were also expressing mRNAs encoding viral proteins, the two could hybridise and prevent the critical assembly of the genome into an RNP structure. While starting with antigenomes templates, the mRNAs will not interfere in the encapsidation process. However, this has since been demonstrated not to be an absolute requirement for virus rescue (Table 1.3), as SeV and hPIV-3 viruses have both been rescued from genomic as well as antigenomic cDNA clones, albeit at a 10-100 fold lower level (SeV, Kato *et al.*, 1996; hPIV-3, Durbin *et al.*, 1997).

As can be seen from Table 1.3, vaccinia virus vTF7-3 was generally used in the earlier studies to drive the transient expression of viral proteins. One obstacle that was encountered in each of these studies was the requirement to separate the rescued virus from the vaccinia virus. This separation was accomplished either by a filtration step (Schnell *et al.*, 1994; Lawson *et al.*, 1995), by inhibiting vaccinia DNA replication with cytosine arabinoside (AraC) and rifampicin (Whelan *et al.*, 1995; Kato *et al.*, 1996; He *et al.*, 1997), or by passage in cells that were nonpermissive for vaccinia virus *e.g.* hens eggs (Garcin *et al.*, 1995(b) and Kato *et al.*, 1996) or mosquito cells (Bridgen and Elliott, 1996). An alternative

Nonsegmented Viruses	Rescue genome	Expression system	Reference
Rabies virus	(+)	vTF7-3	Schnell *et al.*, 1994
VSV	(+)	vTF7-3	Lawson *et al.*, 1995
VSV	(+)	vTF7-3	Whelan *et al.*, 1995
Measles virus	(+)	Cell line expressing T7 RNA pol, viral NP and P proteins	Radecke *et al.*, 1995
Sendai virus	(+)	vTF7-3	Garcin *et al.*, 1995
RSV	(+)	MVA-T7	Collins *et al.*, 1995
Sendai virus	(+) + (-)	vTF7-3	Kato *et al.*, 1996
RPV	(+)	MVA-T7	Baron and Barrett, 1997
hPIV-3	(+)	vTF7-3	Hoffman and Banerjee, 1997
hPIV-3	(+) + (-)	MVA-T7	Durbin *et al.*, 1997
SV5	(+)	MVA-T7	He *et al.*, 1997
RSV	(+)	MVA-T7	Jin *et al.*, 1998
Segmented Virus			
Bunyamwera virus	(+)	vTF7-3	Bridgen and Elliott, 1996
influenza A virus	(-)	Pol I and Pol II	Neumann *et al.*, 1999
influenza A virus	(-)	Pol I and Pol II	Fodor *et al.*, 1999

Table 1.3 The recovery of negative strand viruses from cDNA

The table lists in order the successful rescue of nonsegmented and segmented negative stranded RNA viruses entirely from cDNA. Polarity of the rescued RNA genome templates are indicated by (+) and (-) symbols. Recombinant vaccinia viruses expressing bacteriophage T7 RNA polymerase were, vTF7-3 (Fuerst *et al.*, 1986) and MVA-T7 (Wyatt *et al.*, 1995).

approach was to use a host-range restricted vaccinia virus (MVA-T7) that expresses T7 polymerase by early gene transcription (Wyatt *et al.*, 1995), and being deficient in late gene expression did not replicate in many mammalian cells (Collins *et al.*, 1995; Baron and Barrett, 1997).

Finally, a vaccinia virus-independent system has been developed by generating a 293 cell line constiitutively expressing T7 RNA polymerase along with viral helper proteins (Radecke *et al.*, 1995).

1.8.4 The rescue of segmented virus genomes.

1.8.5 Bunyamwera virus, the first segmented negative strand virus to be rescued.

The first segmented negative strand virus to be rescued from cDNA was Bunyamwera virus and was reported by Bridgen and Elliott (1996). This result marked a considerable achievement as each of three individual genomic segments had to be rescued. In this study, a similar approach was taken to that used by Schnell *et al.* (1994), but in this case six different plasmids had to be delivered to a single cell: three of these plasmids expressed the virus genomic segments and the other three included for the transient expression all of the viral proteins. The rescued BUN viruses were separated from the high number of vaccinia virus particles by infecting mosquito cells (C6/36) with transfectant supernates. These cells are permissive for BUN replication but nonpermissive to vaccinia virus replication, and so this step served to enrich for rescued BUN virus. As might be predicted from such a complex system, rescue levels were low at 10 pfu from 10^7 cells.

1.8.6 Influenza A virus rescued entirely from cDNAs.

Neumann *et al.* (1999) recently described the efficient rescue of influenza A virus entirely from cDNA clones. This was soon followed, independently, by another group describing a similar plasmid based rescue system for influenza A virus (Fodor *et al.*, 1999). Both groups describe a technique similar to that of Pleschka *et al.* (1996) who demonstrated the rescue of an artificial NA Pol-I transcript. In the Influenza virus A studies, both systems involved transfection of 293 cells with eight plasmids encoding the individual vRNA segments of influenza virus under the control of a truncated human RNA Pol-I promoter and mouse Pol-I transcription terminator. Cotransfection of four plasmids encoding the viral replication proteins under the control of an RNA Pol-II promoter (Figure 1.9) allowed vRNA

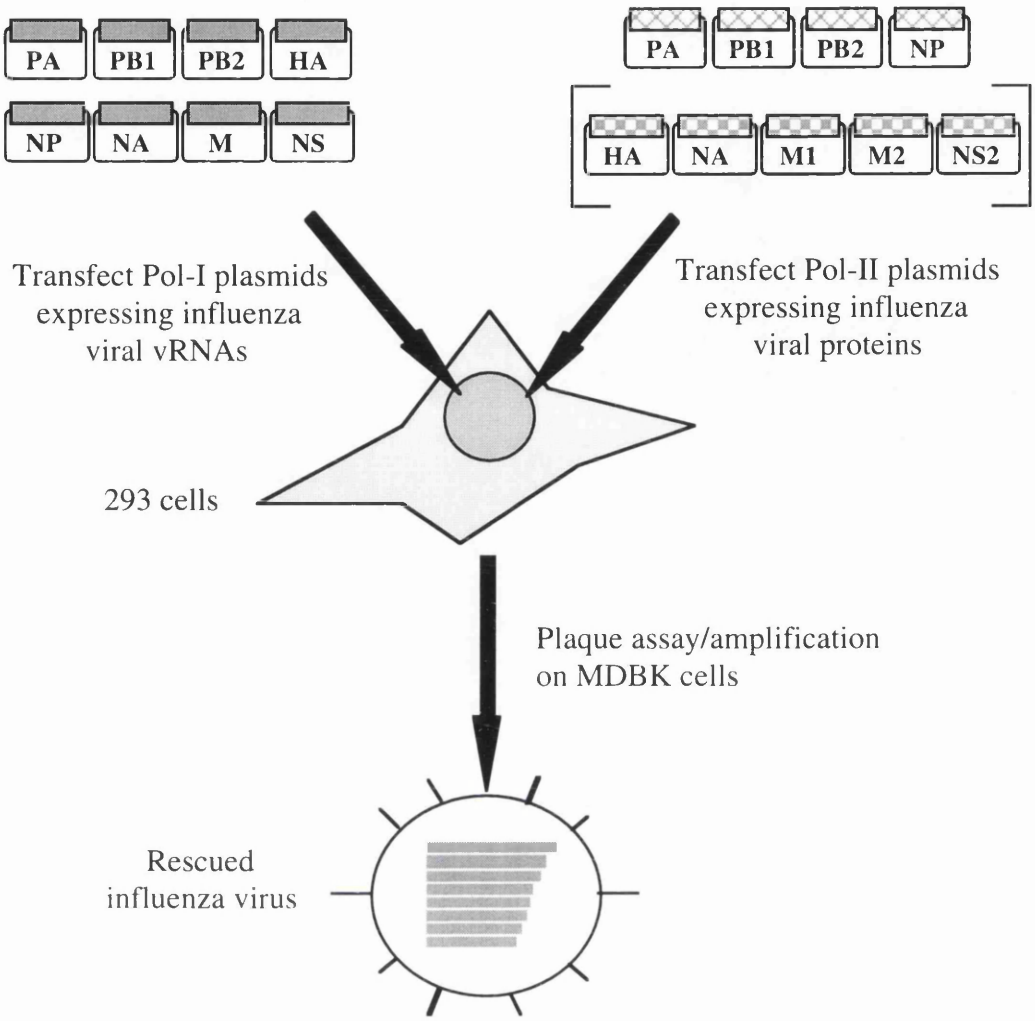


Figure 1.9 The rescue of influenza A virus entirely from cDNAs

This schematic describes a helper virus free plasmid based reverse genetics rescue system for influenza A virus. All eight viral genome segments (vRNAs) are expressed within the cell nucleus from Pol-I expression plasmids. The replication, amplification and expression of recombinant vRNAs is achieved by cotransfection of four Pol-II plasmids expressing the viral replication proteins (PA, PB1, PB2, and NP). Although recombinant influenza virus particles can be detected when only transfecting the Pol-II PA, PB1, PB2, and NP expression plasmids (Neumann *et al.* 1999; Fodor *et al.* 1999), expression of all remaining viral proteins (shown in brackets) increases the efficiency of virus rescue 100-fold (Neumann *et al.* 1999). Figure adapted from Neumann *et al.* (1999) and Fodor *et al.* (1999).

replication, amplification and subsequent RNP packaging and virion particle release. Neumann *et al.* (1999) described the efficient rescue of 10^3-10^7 pfu/ 10^6 cells (the highest reported level of NSV rescue) while Fodor *et al.* (1999) repeatedly obtained 10-20 pfu/ 10^7 cells. The marked differences in the rescue levels maybe due the protocol used by Neumann *et al.*, which utilised the mouse Pol-I terminator to terminate vRNA transcription with the correct 3' termini. Remarkably Neumann *et al.*, found that cotransfection of plasmids expressing all nine viraly encoded proteins (*i.e.* a 17 plasmid transfection) resulted in improved rescue levels of 100-fold. In contrast, Fodor *et al.*, used the HDV ribozyme to achieve the correct 3' termini and expressed only four of the viral proteins essential for replication.

In both influenza rescue systems vRNA templates were successful as opposed to the initial rescue attempts of NNS viruses and the rescue of BUN virus. It maybe that in the NNS and BUN virus rescue systems the (-) vRNA anneals to the vast amounts of (+) mRNA in the cytoplasm, and that this problem is overcome in the influenza system because it is host cell factors (*i.e.* RNA Pol-I and RNA Pol-II) that are responsible for the transcription of are vRNAs and recombinant mRNAs.

This plasmid based rescue system of influenza virus marks an important step which now allows investigation of all aspects of the influenza virus life cycle. It is expected that the system will permit the generation of transfectants with mutations in multiple gene segments or with severe growth defects (Neumann *et al.*, 1999).

Aims of the project.

The general aim of this project was to develop a reverse genetics system to investigate the molecular biology of the Bunyamwera virus. At the onset of this study no other reverse genetic systems had been developed for any member of the *Bunyaviridae,* nor had a plasmid based reverse genetics system been developed for the influenza A virus. For this reason some aspects of the work presented here should be regarded within the context of both scientific and technical advances that had been achieved in the field at the time.

To study the molecular processes of Bunyamwera virus it was decided to develop a reporter system that would be characterised by, and would benefit from, several key features. The reporter assay would be plasmid based, with the virus-like reporter RNA originating from an *in vitro* transcription reaction and the virus replication proteins being supplied by transfected cDNA containing plasmids. Such a system would allow all components of the assay to be manipulated with ease, allowing the study of signals within the viral RNA as well as the proteins involved in the transcription and replication processes.

Chapter 2.

2.0.0 Materials and Methods.

2.1.0 Materials.

2.1.1 Enzymes.

Most restriction enzymes, calf intestinal alkaline phosphatase (CIP) and *Taq* DNA polymerase were purchased from Boehringer Mannheim. The restriction enzymes *Bbs*I and *Hga*I, T4 DNA polymerase, T4 polynucleotide kinase and T4 RNA ligase were purchased from New England Biolabs. T7 RNA polymerase, SP6 RNA polymerase, RQ1 DNase, recombinant ribonuclease inhibitor RNasin®, M-MuLV and AMV reverse transcriptases were purchased from Promega. T7 DNA polymerase was purchased from Pharmacia. RNaseA and lysozyme were purchased from Sigma.

All enzymes reactions were performed in their appropriate reaction buffer, under the reaction conditions specified by the manufacture unless otherwise stated.

2.1.2 Radiochemicals.

With the exception of [^{14}C] chloramphenicol, which was supplied by DuPont NEN®, all other radiochemicals (listed below), were supplied by Amersham.

[^{14}C] chloramphenicol	(58.5 mCi/mmol, 0.1 μCi/μl)
[^{35}S] L-methionine	(≥800 Ci/mmol)
[α-^{35}S] dATP	(>1000 Ci/mmol)
[α-^{32}P] UTP	(~3000 Ci/mmol)

2.1.3 Synthetic oligonucleotides.

The M13/pUC sequencing primer (-40) 17-mer and the M13/pUC reverse sequencing primer (-48) 24-mer were obtained from New England Biolabs. Other oligonucleotides used as primers in this study were synthesized on a Cruachem PS250 automated synthesizer.

2.1.4 Expression vectors and plasmids.

The construction of T7 promoter-containing plasmids which express Bunyamwera virus proteins used in this study were described previously: pTF7-5BUNS, expressing N and NSs proteins (Jin and Elliott, 1991) and pTF7-5BUNL, expressing the L protein (Jin and Elliott, 1991).

Cloning vectors pUC118/119 and expression vectors pTZ18/19 were supplied by Prof. R. M. Elliott.

2.1.5 Bacterial strains.

The *E. coli* strain DH5α™ (f80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169) was the main host for maintenance and propagation of recombinant plasmids.

For plasmids carrying the *bla* resistance gene, maintenance and growth media were supplemented with 100 μg/ml ampicillin.

2.1.6 Viruses.

Bunyamwera and Maguari bunyaviruses were obtained from Prof. R M. Elliott, and vTF7-3 (Fuerst *et al.*, 1986), a recombinant vaccinia virus that expresses T7 RNA polymerase, was originally supplied by Dr. B. Moss.

2.1.7 Eucaryotic cells and tissue culture growth media.

Generally, unless otherwise stated, all reagents used in tissue culture were supplied by Gibco BRL.

BHK-21 Clone 13, a baby hamster kidney cell line, was used for growing stocks of Bunyamwera and Maguari viruses. The cells were maintained in Glasgow modified Eagle's medium supplemented with 10% new born calf serum and 5 mM L-glutamine, 10% tryptose phosphate broth and 14 ml /400 ml of 7.5% sodium bicarbonate.

CV-1, an African Green monkey kidney cell line, was used to grow vTF7-3. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal

calf serum. The two human cell lines, 293, and HeLa(T4) were grown in DMEM supplemented 10% foetal calf serum and 5 mM L-glutamine.

All cell media were supplemented with 100 units/ml penicillin and 100 units/ml streptomycin. Cells were grown at 37°C under 5% CO_2 in a humidified incubator.

2.1.8 Reagents.

Acrylamide/bis-acrylamide 30% stock solution: 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, final ratio 37.5: 1.

Agarose gel loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol.

AMV Reverse Transcriptase buffer (5x): 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine, (Promega).

***Bal* 31 reaction buffer (10x):** 200 mM Tris-HCl (pH 8.1), 125 mM $MgSO_4$, 125 mM $CaCl_2$, 6 M NaCl, 10 mM EDTA.

Carrier DNA: 10 mg/ml salmon sperm DNA in water, autoclaved, and stored at -20°C.

Denhart's solution 50x: 1% polyvinylpyrrolidone, 1% (w/v) BSA (Fraction V), 1% (w/v) Ficoll® (type 400).

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

Giemsa stain: 1.5% (w/v) Giemsa in glycerol, heated to 50°C for 2 hours and diluted with an equal volume of methanol.

Glyoxal gel loading buffer: 50% (v/v) glycerol, 10 mM Na-phosphate (pH 7.0), 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol.

GSCN solution: 4.2M guanidinium thiocyanate, 0.5% (w/v) N-lauroyl sarcosine, 100 mM, β-mercaptoethanol, 0.33% (v/v) antifoam-A emulsion, 50 mM Tris-HCl (pH 7.5) and 2 mM EDTA.

L-broth: 10g NaCl, 10g Bactopeptone and 5g yeast extract per litre.

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

***Pfu* PCR buffer (10x):** 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA.

Phenol: chloroform: isoamyl alcohol (25:24:1): 1 part TE-buffer saturated phenol mixed with 1 part chloroform: isoamyl alcohol (24:1).

Prehydridization/ Hybridization solution: 0.1% SDS, 50% Formamide, 5x SSC, 5x Denharts solution, 0.1% Sodium Pyrophosphate, 50 μg/ml carrier DNA.

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

RNaseA solution: 10 mg/ml pancreatic RNaseA in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, heated at 100°C for 15 minutes to inactivate DNases, cooled and stored at -20°C.

SSC 20x: 3M NaCl, 3M trisodium citrate, adjust to pH 7.0 with 1M HCl.

SSPE 20x: 3M NaCl, 0.18M NaH_2PO_4, 20 mM EDTA, adjust to pH 7.4 with 1M HCl.

Sequencing gel mix: 6-12% (w/v) acrylamide / bisacrylamide (57:3), 8M urea, 1 x TBE.

Solution 1 (Sol1): 50 mM glucose, 250 mM Tris-HCl (pH 8.0), 12 mM EDTA, 5 μg/ml Lysozyme.

Solution 2 (Sol2): 0.2N NaOH, 1% (w/v) SDS, freshly made.

Solution 3 (Sol3): 5M potassium acetate, adjust to pH 4.8 with 1M NaOH.

STET solution: 8% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 5% (v/v) Triton® X-100, filter sterilized and stored at 4°C.

T4 DNA polymerase buffer (10x): 500 mM NaCl, 100 mM Tris-HCl (pH 7.9), 100 mM $MgCl_2$, 10 mM DTT, (NEB).

T4 polynucleotide kinase buffer (10x): 700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT, (NEB).

T4 RNA Ligase NEBuffer (10x): 500 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP, (NEB).

T7 DNA polymerase enzyme dilution buffer: 20 mM Tris-HCl (pH 7.5), 5 mM DTT, 100 μg/ml BSA, 5% (v/v) glycerol, (Pharmacia).

T7 RNA polymerase optimised transcription buffer (5x): 200 mM Tris-HCl (pH7.5), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, (Promega).

TAQ DNA polymerase buffer (10x): 300 mM Tris-HCl (pH 9.0), 50 mM $MgCl_2$, 300 mM KCl, 0.5% (w/v) W-1® buffer.

TAE: 40 mM Tris-acetate (pH 8.0), 1 mM EDTA.

TBE: 90 mM Tris-HCl (pH 8.0), 90 mM boric acid, 1 mM EDTA (pH 8.0).

TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

TEN: 150 mM NaCl, 40 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

Trypsin solution: 0.25% (w/v) Difco trypsin dissolved in Tris-saline solution plus 0.005% (w/v) phenol red.

Versene solution: PBS supplemented with 0.6 mM EDTA and 0.0015% (w/v) phenol red.

2YT broth: 5g NaCl, 16g Bactopeptone and 10g yeast extract per litre.

2.1.10 Chemicals.

Unless specifically stated, all chemicals and reagents were obtained from BDH Chemicals Ltd or Sigma Chemicals Co. Ammonium persulfate was obtained from Bio-Rad. En^{3}Hance was obtained from DuPont. Temed was obtained from Bio-Rad.

2.1.11 Equipment and miscellaneous materials.

PerkinElmer Cetus DNA Thermal cycler 480 (Perkin-Elmer Corporation, Foster City, CA, USA), Blotting pads (11-14 cm) (BRL Life Technologies), GeneClean® II purification kit (Bio 101 Inc., La Jolla, CA, USA), X-Omat S film (Kodak Ltd), Tissue culture materials (Nunc), Hybond N+ nylon blotting membrane (Amersham), Light Mineral oil (Sigma), OptiMEM media (Gibco BRL), Nescofilm (Nipon, Bando Chemical Ind, Ltd), SDS-PAGE standards, low range (14.5-97.4 Kda) (BIO-RAD Laboratories Ltd), Spin-X® centifuge tube filter (Costar®), Polygram® SIL G (0.25 mm) TLC plates (Camlab), Sterile Acrodisc®, 0.2μm (Gelman Sciences).

2.2.0 Methods.

2.2.1 Virus growth and propigation.

2.2.2 Harvesting cell lines.

Cell monolayers were harvested by first washing the cell monolayer once with versene solution, then with a 5:1 mix of versene and trypsin solutions. Cells were then incubated at 37°C for 5 minutes before resuspension in 5 ml of media. Cells were routinely split 1:6 into flasks (Nunc, 80cm^2 or 175cm^2) or seeded at 4 x10^5 cell/ml of media in 35mm dishes and incubated at 37°C.

2.2.3 Growth and purification of recombinant vaccinia virus vTF7-3.

A confluent 175cm^2 flask of CV-1 cells was inoculated with vTF7-3 stock virus at 0.05 pfu/ cell, in 10 ml DMEM-5% FCS and incubated at 37°C for 1 hour with gentle agitation every 15 minutes. The medium was replaced with 30 ml DMEM-5% FCS and returned to 37°C for approximately 2 days or until cytopathic effects were visible. The infected cells were resuspended in their culture medium and centrifuged at 3,000 rpm before resuspension in 6 ml 10 mM Tris-HCl pH 9.0 at 4°C. The cell associated virus was released by 3 cycles of freeze-thawing (5 minutes in dry ice, 5 minutes at 37°C) before the nuclei and cellular debris were pelleted by centrifuging at 1,000 rpm for 5 minutes at 4°C, and then the supernatant was removed to a sterile tube. The cellular debris pellet was resuspended in 6 ml 10 mM Tris-HCl pH 9.0, recentrifuged and the two supernatants combined. Six milliliters aliquots were then layered onto 6 ml 36% (w/v) sucrose in 10 mM Tris-HCl pH 9.0 on ice and

centrifuged at 13,000 rpm in an TST41 rotor for 80 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 1 ml 10 mM Tris-HCl pH 9.0, aliquotted and stored at -70°C.

2.2.4 Titration of vTF7-3 stock virus.

Thirty-five millimeter petri dishes containing 5 x 10^5 CV-1 cells were infected with ten-fold serial dilutions of vTF7-3 stock virus. Dilutions were carried out in a total volume of 1 ml PBS and duplicate 0.1 ml aliquots (for each dilution from 10^{-5} to 10^{-9}) were used for titration. The cells were incubated at 37°C for 60 minutes with gentle agitation every 15 minutes to allow the virus to adsorb. The inoculum was removed, 2 ml DMEM-5% FCS added and the cells incubated at 37°C for 2 days. Then the medium was removed and the cells gently covered with CIDEX (a commercial glutaraldehyde preparation, diluted 1:1 with PBS) for 3-4 hours to fix the cells. The CIDEX was removed and the cells stained with Giemsa for 10 minutes before washing with tap water. Virus plaques were clearly visible as clear regions within purple-stained cells.

2.2.5 Growth and purification of Bunyamwera virus.

Confluent 175cm^2 flasks of BHK-21 cells were infected with Bunyamwera virus at a multiplicity of 0.01 pfu/ cell, in 10 ml GMEM-5% FCS and incubated at 37°C for 1 hour with agitation every 15 minutes. The medium was replaced with 30 ml GMEM-5% FCS and returned to 37°C for approximately 3 days until cytopathic effects were visible. The culture medium was harvested and clarified by centrifugation at 3,000 g for 15 minutes (Sorval GS3 rotor). The clarified fluid was aliquotted and stored at -70°C, and used as virus stock without further purification.

2.2.6 Titration of Bunyamwera stock virus.

Virus was titrated by plaque assay (Iroegbu and Pringle, 1981). Ten-fold serial dilutions of virus stocks were made in a final volume of 1 ml PBS/2% NBC serum. The growth medium was removed from confluent BHK cell monolayers in 35mm dishes and 0.1 ml of diluted virus (for each dilution from 10^{-4} to 10^{-7} in duplicate) was added to each dish. The dishes were incubated at 31°C or 37°C for 1 hour to allow the virus to adsorb to the cells. The inoculum was removed and the cells overlaid with Eagle's medium supplemented with 2% NBC serum containing 0.6% agarose. The dishes were incubated at 31°C for 3–5 days. Then to each dish, 2 ml of CIDEX was added (see 2.2.3) for 3-4 hours to fix the cells. The

CIDEX and agarose overlay was removed and the cells stained with Giemsa for 10 minutes before washing with tap water. Virus plaques were clearly visible as clear regions within purple-stained cells.

2.2.7 Bacterial glycerol stocks.

Duplicate glycerol stocks were made by mixing 800 µl of a fresh overnight culture with 200 µl 80% (w/v) glycerol in two sterile vials, which were then stored at -20°C and -70°C.

2.2.8 Preparation of competent bacterial cells.

Ten milliliters of 2YT broth were inoculated with a single bacterial colony and incubated overnight at 37°C with shaking. One milliliter of the overnight culture was inoculated into 100 ml of 2YT broth in a 250 ml flask and shaken at 37°C until the cells reached an A_{550} of 0.4 (typically 2 hours). The cells were pelleted at 3,300 rpm for 5 minutes and then gently resuspended in 20 ml ice-cold 0.1M $CaCl_2$, and placed on ice for 30 minutes. Following this the bacteria were repelleted and resuspended in 5 ml of ice cold 0.1M $CaCl_2$ and stored at 4°C.

2.3.0 DNA manipulation and cloning procedures.

2.3.1 Plasmid DNA minipreparation via the boil lysis method.

A 20 ml glass universal containing 3 ml of YT broth (supplemented with 100 µg/ml ampicillin) was inoculated with a single bacterial colony, and incubated at 37°C with vigorous shaking for 12-16 hours. A 1.5 ml volume of the overnight culture was transferred to an 1.5 ml eppendorf tube and centrifuged at 13,000 rpm for 20 seconds. The medium was removed by aspiration leaving a bacterial pellet which was then vortexed for 5 seconds to break up the pellet before the bacteria were resuspended in 450 µl of STET lysis solution containing 200 µg lysozyme. The resuspended pellet was then placed on ice for 10 minutes before transferring to a boiling waterbath for 40 seconds and then centrifuged at 13,000 rpm for 20 minutes. The pelleted lysed bacterial debris were then picked out from the tube with a sterile toothpick before mixing the supernatant with 400 µl of isopropanol and freezing at -20°C for 30 minutes. The tube was then centrifuged at 13,000 rpm for 10 minutes, the supernatant removed by aspiration and the DNA/RNA pellet dried under vacuum for 10 minutes before the pellet was resuspended in 80 µl of dw and stored at -20°C.

2.3.2 Large scale plasmid DNA preparation.

A single bacterial colony picked from an agar plate was inoculated into 5 ml of LB broth, containing 100 μg/ml ampicillin, and incubated overnight at 37°C in a shaking incubator. The 5 ml overnight culture was used to inoculate 300 ml of LB-broth (containing 100 μg/ml ampicillin) in a 2l flask and shaken overnight at 37°C. Following centrifugation of the culture at 3,000 rpm (Sorval GS3 rotor) for 10 minutes at 4°C, the bacterial pellet was resuspended in 20 ml TE buffer and then repelleted again in the SS34 rotor at 3,000 rpm for 10 minutes at 4°C. This pellet was resuspended in 8 ml of Sol1 and incubated for 5 minutes at room temperature before the cells were lysed by the addition of 16 ml Sol2 and mixed by inverting the tube 10 times. The mixture was incubated for 10 minutes at room temperature followed by the addition of 12 ml of ice-cold Sol3 and a further 10 minute incubation on ice. Following centrifugation at 6,000 rpm (Sorval SS34 rotor) for 15 minutes at 4°C, the supernatant was transferred to Corex tubes and the plasmid DNA precipitated by the addition of 0.6 volumes of isopropanol for 15 minutes at room temperature followed by centrifugation at 10,000 rpm for 15 minutes at 15°C. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in 10 ml TE buffer. Ten grams of cesium chloride (CsCl) and 1 ml of a 10 mg/ml ethidium bromide (EtBr) solution were added to the DNA solution giving a final density of 1.55 to 1.60 g/ml. The solution was then transferred to a heat-sealable centrifuge tube and centrifuged at 40,000 rpm (Beckman T1270 or Ti-50 rotor) for 48 hours at 19°C. Supercoiled plasmid DNA appeared as a red band (due to the intercalated ethidium bromide) and was removed from the gradient using a syringe fitted with a wide-bore needle (18-G) through the side of the tube. EtBr was removed from the plasmid DNA solution by at least five extractions with an equal volume of isopropanol (equilibrated with 5M NaCl). The CsCl was then removed by dialyzing the solution twice against 4l of 0.5% TBE buffer at 4°C after which the DNA was precipitated by the addition of one tenth of a volume 3M NaOAc and 3 volumes EtOH and stored at -20°C for 2-24 hours. The DNA was then pelleted, dried under vacuum, resuspended in a suitable volume of dw and stored at -20°C. The concentration of plasmid DNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer, assuming that $1A_{260}$= 50 μg/ml in water for dsDNA.

2.3.3 Restriction enzyme digestion of DNA.

For the analysis of plasmid DNA from minipreparations, restriction enzyme digests were set up in 10 μl volumes containing 2 μl miniprep DNA, 1 μl specific reaction buffer, 2 units restriction enzyme, 0.2 μl RNaseA (10 μg/ml) and made up to volume with dw and incubated at the appropriate temperature, depending on the enzyme for 2 hours. After

incubation, 0.1 volume of loading dye was added and the DNA fragments were separated by electrophoresis on a non-denaturing 1% agarose TBE gel.

For the preparation of a vector backbone or DNA fragments for cloning and subcloning, 5-20 μg DNA were digested in 50-100 μl reaction volume in the presence of a specific restriction enzyme buffer, 2 units restriction enzyme/μg DNA and incubated for 2-4 hours before electrophoresis in a 1-1.2% agarose TAE gel.

2.3.4 Dephosphorylation of linearised plasmid DNA.

The terminal 5' phosphates of digested vector were removed by treatment with calf intestinal alkaline phosphatase (CIP). The reaction was as follows, DNA (typically 3-5 μg) was diluted to 43 μl with dw and mixed with 5 μl 10x CIP buffer, 2 μl CIP (1 U/μl) and then incubated at 37°C for 30-60 minutes. The dephosphorylated vector was then purified by the GeneClean® protocol (2.3.7), and recovered in a final volume of 30 μl.

2.3.5 Agarose gel electrophoresis of DNA.

Electrophoresis of DNA was performed in horizontal slab gels, either 14 x 11 x 0.5 cm (BRL gel electrophoresis apparatus Model H5), or 5.7 x 8.3 x 0.3 cm (BRL gel electrophoresis Horizon 58), containing 1-1.2% (w/v) agarose in 1x TBE or 1x TAE containing 0.5 μg/ml ethidium bromide. DNA samples were mixed with a tenth volume loading dye before loading an appropriate volume into the gel. Electrophoresis was carried out at 50-100 V in 1x TBE or 1x TAE containing 0.5 μg/ml ethidium bromide.

2.3.6 Transformation of competent bacterial cells.

Two hundred microlitres of competent bacterial cells were incubated with half of a ligation reaction (10 μl) for 60 minutes on ice, followed by a heat shock at 42°C for 2 minutes to allow uptake of the ligated DNA by the *E. coli* cells. The reaction tube was returned to the ice bath to cool before 0.5 ml of 2YT broth was added and the cells incubated for a further 60 minutes at 37°C. The cells were then plated onto LB agar plates, containing 100 μg/ml ampicillin, and incubated for 16 hours at 37°C to allow single colonies to form. For vectors containing the *lacZ* gene, 45 μl of X-Gal (40 μg/ml) was added to the cells before plating out to allow blue/white colour selection of recombinants.

2.3.7 DNA purification from agarose gels by silica matrix adsorption.

DNA fragments produced by restriction enzyme digestion or by PCR were first resolved by electrophoresis through a 1-1.2% agarose TAE gel containing 0.5 μg/ml ethidium bromide. DNA fragments were visualised by long-wave UV illumination, and a gel slice was removed and mixed with 3 volumes of NaI solution (GeneClean® kit) and incubated at 55°C until the agarose slice has completely dissolved (10-15 minutes). Glassmilk® (GeneClean® kit), a silica matrix solution, was added (5 μl for up to 5 μg of DNA) and the mixture incubated for 10-20 minutes at room temperature and vortexed intermittently to allow the DNA to bind to the matrix. Following a 5 seconds spin at 13,000 rpm, the supernatant was removed and the pellet (containing DNA bound to silica particles) washed 3 times by repeated resuspension/centrifugation cycles using NEW Wash™ solution (a Tris-buffered mixture of NaCl, ethanol and water). The DNA was eluted from the silica matrix by resuspending the pellet in 20-80 μl water, incubating for 3 minutes at 55°C and pelleting the glass milk at 13,000 rpm for 2 minutes before removing the DNA solution and storing at -20°C.
The GeneClean® protocol was also used to purify DNA from enzyme reactions and oligonucleotides when electrophoresis was not required.

2.3.8 End repair of DNA fragments.

Linearised vectors and DNA fragments were blunt-ended by resuspending the DNA in T4 DNA polymerase 1x buffer (NEB) containing 150 μM of each dNTP, 0.1 mg/ml BSA and 5 units T4 DNA polymerase (NEB)/μg of DNA in a final volume of 50 μl. The reaction was incubated at 37°C for 20 minutes before purifying the repaired DNA by the GeneClean® protocol (2.3.7).

For the end repair of PCR fragments, the DNA was resuspended in 100 μl of 1x T4 polynucleotide kinase buffer (NEB) which contained 150 μM of each dNTP, 0.1 mg/ml BSA, 2 mM ATP, 5 units T4 DNA polymerase (NEB)/μg DNA and 10 units T4 polynucleotide kinase (NEB). The reaction was incubated at 37°C for 30 minutes then the repaired DNA was purified by the GeneClean® protocol (2.3.7).

2.3.9 DNA ligation.

Ligation reactions were set up at two ratios (1:1 and 1:5) of linerised vector DNA (100 ng/μl) to insert DNA (50 ng/μl) in a 20 μl reaction volume (DNA, 5 μl 5x Ligation buffer (Gibco-BRL), 1 unit T4 DNA Ligase (Gibco-BRL) made up to volume with dw) and incubated at 16°C overnight, then stored at -20°C.

2.3.10 Purification of synthetic oligonucleotides.

Synthetic oligonucleotides were removed from the column by pushing 1.5 ml ammonia through the column between two 2 ml syringes slowly over 1 hour. The solution was heat treated for 5-16 hours at 55°C to remove the base protecting groups and then the ammonia was removed by lyophylisation. The dried oligonucleotide pellet was dissolved in 200 µl of dw, mixed with 100 µl 95% formamide and electrophoresed on an 12% polyacrylamide gel containing 8M urea. The gel was then viewed under short wave UV light and oligonucleotide bands were visualized as dark shadows against an intensifying screen. Gel slices containing each full length oligonucleotide species were excised with a scalpel blade and the oligonucleotide was eluted in 1.5 ml of water at 42°C overnight with shaking. Supernatants were transferred to a Spin®-X centrifuge tube (Costar®) containing a 0.45 µm cellulose acetate filter and centrifuged for 2 minutes to remove the acrylamide gel pieces. The oligonucleotide in the filtrate was precipitated with 3 volumes of ethanol in the presence of 0.2M NaCl overnight at -20°C. The oligonucleotide precipitate was pelleted, washed with 70% ethanol, dried under vacuum and dissolved in 200 µl dw. Spectrophotometry was used to determine the concentration of the oligonucleotide in solution, assuming that 1 A_{260} = 20 µg/ml for ssDNA.

2.3.11 Polymerase chain reaction (PCR) amplification of DNA.

All PCR reactions were set up using the 10x PCR buffer and 50 mM $MgCl_2$ stock solution supplied by the manufacturer of the *Taq* DNA polymerase used in the reactions.

Each reaction consisted of the following components which were added and mixed in the following order, on ice, in a thin-walled 0.5 ml reaction tube. (When working with multiple samples, a master mix containing all but the DNA template and primers was assembled.)

Nuclease free dH_2O	
(to a final volume of 50 µl)	xµl
10x PCR buffer (Gibco-BRL)	5 µl
4dNTPs (10 mM)	1 µl
50 mM $MgCl_2$ (Gibco-BRL)	2 µl
Taq DNA polymerase (5 U/µl) (Gibco-BRL)	1U
downstream primer	50 ng
upstream primer	50 ng
template DNA (10-50 ng/µl)	1 µl

The reaction was overlaid with 1-2 drops nuclease-free light mineral oil (Sigma) (to overlay the top of the reaction mixture), centrifuged briefly, then placed in the heating block of the Perkin Elmer Cetus DNA Thermal cycler 480. The thermal cycling profile was 93°C for 30 sec, 56°C for 20 sec, 72°C for 80 sec (80 sec/kb) and cycled 25-30 times unless otherwise stated. PCR reactions were run on a 1% TAE agarose gel and fragments of interest were purified as in protocol (2.3.7).

PCR was also carried out using the proofreading DNA polymerase *Pfu* . Reactions were set up using the 10x *Pfu* PCR buffer supplied with the enzyme (Stratagene) as illustrated below.

Nuclease free dH_2O	
(to a final volume of 50 µl)	xµ
10x *Pfu* PCR buffer	5 µl
4 dNTPs (10 mM)	1 µl
upstream primer	100-250 ng
downstream primer	100-250 ng
Pfu DNA polymerase (5 U/µl)	1 µl
Template DNA (~ 100 ng/µl)	1 µl

The actual reaction was carried out in the same manner as for *Taq* PCR reactions but with a strand separation temperature of 94°C and a strand elongation temperature of 73°C (4 minutes/Kb).
PCR reactions were electrophoresed through a 1% TAE gel (2.3.5) and purified using a silica matrix as in protocol (2.3.7).

2.3.12 PCR driven site-directed mutagenesis.

The QuikChange™ site-directed mutagenesis kit from Stratagene was utilised for PCR driven site-directed mutagenesis. All reactions were set up following the manufacturers protocol and with the supplied reagents (10x *Pfu* reaction buffer, dNTP mix and *Pfu* DNA polymerase) as indicated below.

10x Pfu reaction buffer	5 µl
dNTP mix	1 µl
dsDNA template (50 ng/µl)	1 µl
primer 1 (125 ng)	1 µl
primer 2 (125 ng)	1 µl
ddH_2O	41 µl

Then add

Pfu DNA polymerase (2.5 U/µl)	1 µl

After the addition *Pfu* DNA polymerase, the reaction was overlaid with 30 µl nuclease-free light mineral oil (Sigma) centrifuged briefly, then placed in the heating block of the Perkin Elmer Cetus DNA Thermal cycler 480. The thermal cycling profile was 95°C 30 sec for one cycle followed by 12 cycles of 95°C 30 sec, 55°C 30 sec, and 68°C 16 minutes. After temperature cycling, the reaction was cooled on ice for 2 minutes before 1 µl of restriction enzyme *Dpn* I (10 U/µl) was added below the mineral oil layer with a fine pipette tip. The reaction was then incubated at 37°C for 1 hour to digest the parental DNA. After which, 1 µl of the *Dpn* I-treated DNA was mixed with 50 µl Epicurian Coli XL1-Blue supercompetent cells and the transformation reaction incubated on ice for 30 minutes and then heat-shocked at 42°C for 45 seconds before cooling on ice for a further 2 minutes. 0.5 ml of NZY+ broth (see materials) preheated to 42°C was added to the transformation reaction and incubated at 37°C for 1 hour before plating the entire transformation reaction onto an agar (*amp*) plate. The agar plate was then incubated at 37oC for approximately 16 hours.

2.3.13 Double stranded DNA Sequencing.

Sequencing of dsDNA by dideoxynucleotide chain termination (Sanger *et al.*, 1977) was performed in accordance with the methods used by Tabor and Richardson (1987) utilising a modified T7 DNA polymerase (Pharmacia LKB) which lacks a 3' to 5' exonuclease. The method was as follows:-

Preparation of single strand template- In an 1.5 ml eppendorf tube, 10 µl of plasmid miniprep DNA (approx. 2-5 µg) were denatured by the addition of 2.5 µl 2M NaOH and incubated at 42°C for 10 minutes. The reaction was then neutralized and the ssDNA precipitated by adding 3.75 µl of 3M NaOAc (pH 4.5), 9 µl H_2O and 65 µl absolute ethanol. The tube was then placed in dry ice for 15 minutes or at -20°C for 1 hour, centrifuged at 13,000 rpm for 10 minutes, the supernatant removed and the DNA pellet dried under vacuum for 15 minutes. The pellet was then resuspended in 10 µl water.

Annealing Reaction- To 10 µl denatured template were added 2 µl primer (5 ng/µl, ~0.8 µM), 2 µl of annealing buffer (280 mM Tris-HCl (pH 7.5) and 100 mM $MgCl_2$, 350 mM $NaCl_2$), and this was incubated at 42°C for 10 minutes, then at 4°C for a further 10 minutes, or frozen at -20°C.

Labeling Reaction- To the annealing reaction (14 µl) 6 µl of labeling mixture were added (2 µl of 3dNTPs (2 µM each dGTP, dTTP and dCTP), followed by 1 µl of 0.3M dithiothreitol (DTT), 0.5 µl a-^{35}S-dATP, 2 units T7 DNA polymerase (Pharmacia) diluted in

enzyme dilution buffer (Pharmacia) to give a final volume of 6 µl) mixed and incubated at room temperature for 5 minutes.

Extension-Termination Reaction- Four separate dideoxy "termination" mixtures were prepared, each containing 150 µM 4dNTPs and 15 µM ddNTP where the ddNTP is ddGTP (G), ddATP (A), ddTTP (T) or ddCTP (C) in 40 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl. While the labeling reaction was incubating at room temperature the termination reactions were set up in a 96 well roundbottom micro-well plate by pipetting 2.5 µl of each termination mixture into individual wells, in the order G, A, T, C to which 4.5 µl of the incubated labeling reaction was aliquoted into each. The reactions were mixed by centrifuging the plate at 1000 rpm for 10 seconds, incubated at 37°C for 5 minutes, after which the reactions were stopped by the addition of 5 µl of stop mix (95% formamide, 20 mM EDTA (pH7.5), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol). The reactions were incubated at 85°C for 2 minutes prior to loading (3 µl) onto a 6% sequencing gel (assembled and poured as described in the manufacturer's manual) in the order G, A, T, C. The samples were run at a constant power of 60 watts for 2-4 hours, the gel was transferred from the glass plates, onto 3MM paper and dried under vacuum for 2 hours. The dried sequencing gel was then exposed directly against a sheet of X-Omat S film for 16-24 hours at room temperature.

2.3.14 Nuclease *Bal* 31 digestion of linearised DNA.

Approximately 10 µg of linearised DNA was digested with *Bal* 31 nuclease (0.1 units/µg) in 1x *Bal* 31 buffer in a final volume of 100 µl, at 30°C. At various time points 20 µl aliquots were removed and the nuclease reaction stopped by the addition of EGTA to give a final concentration of 20 mM. Reactions were then run on a 1% TAE gel, and the DNA bands excised and purified by GeneClean (2.3.4), prior to DNA end repair with T4 DNA polymerase (2.3.8).

2.4.0 RNA manipulations.

2.4.1 T7 RNA polymerase *in vitro* transcription reaction.

Transcription reactions were set up in the following in order at room temperature.

Nuclease free water	
(to a final volume of 100 µl)	47.5 µl
5x Transcription buffer	20.0 µl
DTT, 100 mM	10.0 µl
10 mM rNTP mix	5.0 µl
RNasin®, 40 U/µl	2.5 µl
T7 RNA Polymerase (20 U/µl)	5.0 µl
linearised DNA template, 100 ng/µl	10.0 µl

The reaction mixture was incubated at 37°C for 2 hours after which 4 µl were removed and run on a 1% agarose TBE gel to check RNA quality. To the remainder of the reaction 4 µl of RQ1 DNase 1U/µl (Promega) were added and this was then incubated at 37°C for 15 minutes. The transcribed RNA was either utilised immediately or stored in liquid nitrogen.

2.4.2 Measuring radioactivity in RNA transcripts by acid precipitation.

When studying the pBUNSCAT single point mutants it was essential that equivalent amounts of RNA transcripts were transfected. To achieve this a trace amount of ^{32}P-UTP (0.2 µl) was added to the transcription mix (2.4.1). Each template has approximately the same efficiency incorporating ^{32}P-UTP and allowed comparable quantification of each RNA transcript by acid precipitation.

An aliquot (typically 4 µl) of the reaction mixture was mixed with 50 µl salmon sperm DNA (10 mg/ml) in a 1.5 ml eppendorf tube, 500 µl ice-cold 10% TCA solution were added, and the tube placed on ice for 10 minutes. The contents were mixed and the precipitate collected by filtering through a Whatman GF/C glass-fiber disc (2.4 mm diameter). The filter was washed 5 times with 4 ml ice-cold 10% TCA solution then once with 5 ml ethanol. It was then allowed to dry briefly before being transferred to a scintillation vial containing 5 ml of a toluene-based scintillation fluid. Radioactivity was measured in a scintillation counter set to count ^{32}P for 30 seconds.

2.4.3 Extraction of total cellular RNA.

RNA was extracted from cell monolayers by the method described by Chomczynski and Sacchi (1987). Cell monolayers in 35mm dishes were washed with PBS and lysed in 500 µl GSCN solution. The lysate was transferred to a 1.5 ml microfuge tube and 50 µl of 2M NaOAc pH 4.0, 500 µl of water-saturated phenol, and 100 µl chloroform/isoamylalcohol (49:1) were added. After vortexing for 10 seconds the tubes were incubated on ice for 15 minutes before centrifuging for 10 minutes at 13,000 rpm in a microcentrifuge. The aqueous phase was mixed with an equal volume of isopropanol and the RNA precipitated at -20°C for 1 hour followed by centrifugation for 10 minutes at room temperature. The RNA pellet was resuspended in 300 µl of GSCN solution, mixed with 300 µl isopropanol and reprecipitated. After centrifugation for 10 minutes, the pellet was dissolved in 200 µl of water by heating for 5 minutes at 60°C and extracted with an equal volume of a 4:1 mixture of chloroform: butanol. The RNA was precipitated with 3 volumes of ethanol in the presence of 0.3M NaOAc and pelleted by centrifugation for 10 minutes at 13,000 rpm in a microcentrifuge. The RNA pellet was washed once with 70% ethanol, partly vacuum dried and dissolved in water before use or storage at -70°C.

2.4.4 Purification of RNA transcripts by spin columns.

The RNeasy Mini spin column kit protocol for RNA clean up was essentially as described in the RNeasy Mini hand book (QIAGEN). Three hundred and fifty microlitres of RLT™ buffer (supplied in the kit) were added to the transcription reaction after DNase 1 treatment (methods 2.4.1) and mixed thoroughly. A further 250 µl of ethanol (100%) were added to the reaction mixture and vortexed before applying the sample, now 700 µl, to an RNeasy mini spin column and centrifuging at 10,000 rpm for 15 seconds. The RNeasy column was then transferred to a new 2 ml collection tube (supplied in the kit) and then washed three times by the addition of 500 µl RPE buffer (supplied in kit) to the column and centrifuged for 15 seconds at 10,000 rpm. After each wash, the flow-through was discarded before the next wash. After the final wash the RNeasy column was transferred to a new 1.5 ml collection tube and centrifuged at 13,000 rpm to remove any residual ethanol from the wash. The RNeasy column was then transferred to a new 1.5 ml collection tube (supplied by kit) and 100 µl of RNase free-water (supplied by kit) pipetted directly onto the RNeasy membrane and centrifuged at 10,000 rpm to elute the RNA transcripts. The eluted RNA transcripts were either utilised immediately or aliquoted and stored at -70°C.
The concentration of the RNA transcripts were determined by measuring the absorbance at 260nm (A_{260}) in a spectrophotometer, given that an absorbance of 1 unit at A_{260} corresponds to 40 µg of RNA/ ml in water (A_{260} = 1 = 40 µg/ml).

2.4.5 Northern blot hybridization of RNA denatured by glyoxal/DMSO treatment.

RNA was denatured by treating the RNA samples with glyoxal and DMSO prior to running in a phosphate buffer/ agarose gel. The RNA was denatured by mixing 11 µl of RNA solution with 4.5 µl 100 mM sodium phosphate (pH 7.0), 22.5 µl DMSO and 6.6 µl 6M de-ionized glyoxal and incubating for 1 hour at 50°C. The sample was then cooled on ice and 12 µl glyoxal loading buffer were added. Electrophoresis of the denatured RNA samples was carried out on a 1% agarose gel buffered with 10 mM sodium phosphate (pH 7.0) and run at 4 V/cm with constant recirculation of buffer so to maintain a pH below pH8.0. Once the bromophenol blue dye had migrated two thirds of the length of the gel electrophoresis was halted and the gel blotted (Northern transfer) onto a nylon membrane (Hybond-N+, Amersham). The gel immersed for 30 minutes in a tray containing 20x SSC. Meanwhile all components of the blotting apparatus were also presoaked in 20x SSC. Two pieces of Whatman® 3MM paper were layered over a platform in a tray containing a reservoir of 20x SSC so that the paper was over the edge of the platform into the 20x SSC buffer to act as a wick. A presoaked blotting pad (the same size as the gel, BRL) was placed on top of the 3MM paper followed by the gel which was placed face down on top of the pad. A sheet of presoaked nylon membrane cut to the same size as the gel was then placed on top of the gel followed by another presoaked blotting pad. At all stages any air bubbles were removed by gently rolling a 10 ml pipette over the surface. A further ten blotting pads (dry) were placed on top followed by a glass plate and a weight (0.5kg). The setup is left overnight at room temperature to allow transfer to proceed.

Once transfer was complete the apparatus was dismantled carefully and the nylon membrane rinsed gently in 5x SSC for 5 minutes and allowed to air dry RNA side up. The RNA was immobilised to the nylon membrane (RNA side up) by UV crosslinking in a Stratalinker® UV Crosslinker set to irradiate at 120mJoules. The membrane was stored between two sheets of 3MM Whatman® paper prior to hybridization.

The components of the riboprode reaction mix were set up at room temperature as follows:-

Transcription 5x buffer	4.0 µl
DTT 100 mM	2.0 µl
Rnasin®(40 U/µl)	0.5 µl
ATP, CTP and GTP (2.5 mM each)	4.0 µl
100 µM UTP	2.5 µl
linearised DNA template (200 ng/µl)	1.0 µl
T7 RNA Polymerase (20 U/µl)	1.0 µl
[α-^{32}P] UTP (~1000 Ci/mmol)	5.0 µl

The reaction mixture was incubated at 37°C for 60 minutes then 80 µl dw, 50 µl 7.5M ammonium acetate and 250 µl of 100% ethanol were added and the mixture placed in dry-ice for 30 minutes. Centrifugation was carried out at 13,000 rpm for 5 minutes, the supernate was removed and RNA pellet dried under vacuum. The pellet was resuspended in 100 µl nuclease free dw and frozen at -70°C until required.

To allow hybridisation of the probe to the membrane, the membrane was first immersed briefly in 6xSSC, then placed (RNA side in) into a hybridization tube containing 15 ml prehybridisation solution and incubated for 3 hours at 42°C. The prehybridisation solution was removed, and 15 ml hybridisation solution (preheated to 60°C) were added, followed by the addition of 50 µl riboprobe. The incubation was continued at 60°C overnight.

The following day, hybridization solution was removed and the membrane washed with 30 ml 2xSSC/0.1% SDS for 2x 10 minutes. A low-stringency (0.2xSSC, 0.1%SDS) wash was then carried out, and the incubation continued for 15 minutes at room temperature, the wash then changed and repeated. The blot was removed from the tube, rinsed once in 2xSSC and allowed to air dry for 5 minutes before being wrapped in cling film and exposed against X-Omat S film at -70°C for 2-24 hours. If there was a high background the blot was washed two more times with high-stringency wash solution at 68°C for 15 minutes.

2.4.6 T4 RNA Ligase reactions.

The ligation of single-stranded RNA transcripts was achieved by treatment with T4 RNA ligase (NEB). The reaction conditions were as follows.

ssRNA transcript	1.0 µg
10x RNA Ligase NEBuffer	2.0 µl
RNasin (40 U/µl)	0.5 µl
T4 RNA Ligase (2,000 U/µl)	4.0 µl
Nuclease free water	
to a final volume of	20.0 µl

Reactions were incubated at 17°C for 6 hours, and subsequently stored frozen at -70°C, or utilised immediately as a template for first strand cDNA synthesis (Methods 2.4.7).

2.4.7 First strand cDNA synthesis.

First strand cDNA synthesis was achieved using AMV Reverse transcriptase under the reaction conditions described below. Initially 14 µl of a RNA Ligation reaction (Methods 2.4.6) was mixed with 1 µl of first strand primer (0.5 µg/µl), and incubated at 70°C for 10 minutes, then chilled on ice before the addition of further reaction components in the order shown.

(RNA template/ first strand primer	15.0 µl)
5x AMV buffer	5.0 µl
RNasin® (40 U/µl)	1.0 µl
4 dNTPs, 10 mM	0.5 µl
AMV-RT (20 U/µl)	1.0 µl
Nuclease free water	2.5 µl

The reaction was incubated at 42°C for 1 hour then chilled on ice before using 5 µl of the first strand synthesis reaction as a template for second strand synthesis with the appropriate primer and PCR amplification by TAQ DNA polymerase (methods 2.3.11).

2.5.0 Introduction of nucleic acids into eucaryotic cells.

2.5.1 Preparation of cationic liposomes.

This method is based on that described by Rose *et al* . (1991).
Dimethyldioctadecyl ammonium bromide (DDAB) was diluted to 4 mg/ml in chloroform (stored at -20°C) and 1 ml was mixed with 1 ml of dioleoyl L-α-phosphatidyl ethanolamine (DOPE: 10 mg/ml in chloroform) in a glass universal. The chloroform was removed by evaporation with a gentle stream of nitrogen gas. Lyophilization was carried out for 4 hours to remove any further traces of chloroform and the dried lipids were resuspended in 10 ml sterile dw by sonication in a sonicating water bath. The lipids were further sonicated with a soniprobe (on ice) set at maximum power until the suspension cleared (~4 minutes.). The final suspension had a 1:2.5 ratio by weight of DDAB to DOPE and could be stored at 4°C for up to one month.

2.5.2 Liposome mediated transfection of nucleic acids into mammalian cells.

Cationic liposomes were used for the transfection of plasmid DNA and *in vitro*-transcribed RNA transcripts into mammalian cells.

Thirty five millimetre dishes containing ~80% confluent cell monolayers were infected with the recombinant vaccinia virus, vTF7-3, at 5-10 pfu/cell in 400 µl OptiMEM®(Gibco-BRL) for 1 hour at 37°C. The inoculum was removed and the monolayer washed once in OptiMEM® before the addition of 500 µl DNA-transfection mix. Transfection mixes were set up in 15 ml Falcon tubes and contained plasmid DNA (1-10 µg) which was diluted in 250 µl OptiMEM® before mixing with 250 µl diluted liposomes (15 µl cationic liposomes mixed with 235 µl OptiMEM®). The transfection mixture was allowed to stand for 10-15 minutes at room temperature before addition to cell monolayer. The monolayers were then incubated at 37°C for 2.5 hours. Following this, RNA transfection mix (10 µl in vitro transcribed RNA and 15 µl liposomes were diluted to 500 µl OptiMEM®, vortexed and added immediately) was added, and incubation was continued for a further 3 hours at 37°C with periodic agitation. Five hundred microlitres of DMEM-10% FCS were subsequently added and incubation continued for a further 16-40 hours, after which cell lysates were then assayed for CAT activity (2.5.3).

2.5.3 Chloramphenicol acetyltransferase (CAT) assay.

The CAT enzyme assay system is an adaptation of that described by Gorman (1982) and Cullen (1987).

Cell extracts were prepared by first scraping cells into their medium before transferring to an 1.5 ml eppendorf and centrifuging at 6,500 rpm for 30 seconds. The supernatant was removed and cells washed once in 500 µl TEN buffer, repelleted at 13,000 rpm for 2 minutes and the supernatant removed. The cell pellets were then resuspended in 75 µl of 250 mM Tris-HCl (pH7.5) and subjected to 3 freeze/thaw cycles (dry ice 5 minutes / 37°C 5 minutes). Nuclei and cell debris were removed by centrifuging samples at 13,000 rpm for 2 minutes. Extracts were removed to another tube and assayed immediately or stored at -20°C. CAT assay reactions were set up by mixing 2-25 µl cell extract with 1 µl [^{14}C] chloramphenicol (0.1 µCi/µl), 1µl 50 mM acetyl CoA and 0.25M Tris-HCl (pH7.5) to a final volume of 50 µl. Incubations were carried out at 37°C for 2-16 hours (if incubating for 16 hours, the cell extract was first heated to 60°C for 10 minutes to inactivate any endogenous deacetylase activity). After incubation chloramphenicol was extracted by first

vortexing the reaction mixture for 20 seconds with 250 µl ethyl acetate, and centrifuging at 13,000 rpm for 5 minutes to achieve phase separation. The upper organic phase was removed to a new tube and dried under a vacuum. The dried residue of chloramphenicol was resuspended in 25 µl ethyl acetate and spotted 2cm from the base of a silica gel TLC plate and allowed to air dry. The TLC plate was then placed in a chromatography tank which had been pre-equilibrated for 30 minutes with chloroform: methanol (95:5), and the chromatography allowed to proceed until the solvent was 0.5cm from the top of the plate before removing the plate to air dry. Once dry, the TLC plate was then exposed directly against a sheet of Kodak X-omat S film for 16-24 hours, or a phosphor-imager plate (3-16 hours) for phosphor-imager scanning.

2.6.0 Protein analysis.

2.6.1 Coupled *in vitro* transcription / translation reactions.

Cell-free protein synthesis was carried out using the TnT® Coupled Transcription/ Translation system (Promega). The TnT® reactions were set up on ice in accordance with the protocol supplied.

Nuclease free dH_2O	
(to a final volume of 25 µl)	5.9 µl
TnT® Rabbit Reticulocyte Lysate	12.5 µl
TnT® Reaction buffer	2.0 µl
TnT® T7 RNA polymerase	0.5 µl
Amino acid mixture Minus methionine	0.5 µl
[^{35}S] methionine (15µCi/µl)	2.0 µl
RNasin® (40 U/µl)	0.6 µl
DNA template (0.5 µg/µl)	1.0 µl

The reaction was incubated at 30°C for 60 minutes after which 50 µl protein dissociation mix was added. The reactions were stored at -20°C or immediately analysed by SDS-PAGE (Methods 2.6.4).

2.6.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins.

Protein samples were fractionated by electrophoresis through a polyacrylamide gel containing SDS using a discontinuous buffer system (Laemmli, 1970). The gel apparatus

and glass plates were assembled following the manufacturer's instructions. The resolving gel mix 12% or 15% acrylamide solution (recipes as below, 10% APS and TEMED added prior to pouring gel) was poured between the assembled glass plates, with sufficient space left at the top for the stacking gel (1 cm of stacking gel is below the loading well) to be added later. The resolving gel was immediately overlaid with 2 ml isopropanol to leave a smooth interface after polymerisation.

Resolving gel mixes:

	12%	15%	18%
acrylamide/bis stock solution (37.5:1)	14.4 ml	18.0 ml	21.6 ml
1M Tris-HCl (pH8.8)	16.8 ml	16.8 ml	16.8 ml
dH_2O	12.9 ml	9.3 ml	5.7 ml
10% SDS	0.45 ml	0.45 ml	0.45 ml
10% APS	0.45 ml	0.45 ml	0.45 ml
TEMED	50.0 µl	50.0 µl	50.0 µl

Once the gel had polymerised, the isopropanol was poured off and the surface was rinsed once with water, and once with unpolymerised stacking gel solution. The stacking gel (1.0 ml acrylamide stock solution, 0.63 ml 1MTris-HCl (pH6.8), 8.0 ml dH_2O, 100 µl 10% SDS, 100 µl APS, 5.0 µl TEMED) was poured onto the resolving gel surface and a comb inserted to form the loading wells. Once the stacking gel had polymerised the comb was removed and the wells rinsed once with running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). The gel was placed in the electrophoresis apparatus and the reservoirs filled with running buffer. Samples in protein dissociation mix were boiled for 10 minutes before being loaded into the loading wells along with mid-range protein molecular markers (14.5-97.4 KDa, BIO-RAD) and the gel run at 45mA until the bromophenol blue reached the bottom of the resolving gel. The gel was removed from the glass plates after electrophoresis and immersed in gel fix solution for 30 minutes and then treated with gel En^{3}Hance solution for 30 minutes. After treatment with En^{3}Hance, the gel was washed in water with several changes for a further 30 minutes before being dried under vacuum for 2 hours and placed in contact with X-Omat S film over night at -70°C.

2.6.5 Protein concentration determination.

The protein concentration of cell lysates to be used in CAT assays were determined using the method described by Bradford (1976).

The protocol for determining protein concentration was essentially as described in the manual supplied with the Bradford reagent (Bio-Rad). Four microlitres of cell extract and 4 µl 10 mM Tris pH 7.5 were pipetted directly onto the bottom of a 96 flat bottom microtiter plate (Nunc), to which 192 µl of 1x Bradford reagent (5x Bradford reagent (BioRad) diluted in 10 mM Tris pH 7.5 to give a 1x working solution, stored at 4°C) was added. The wells of the plate were sealed with a plate sealer and, to allow efficient mixing of the reaction, the plates were inverted several times before centrifuging at 300 rpm for 20 seconds.

Protein concentration standards were made by the dilution of BSA 100 mg/ml (NEB) between the range 0.1 to 1.5 mg/ml in 10 mM Tris pH 7.5. Eight microlitres of diluted protein standards were pipetted onto the bottom of a flat bottom well microtiter plate to which were then added, 192 µl of 1x Bradford reagent. The Bradford reactions were incubated at room temperature for 10 minutes before the absorbance at A_{595} was read in a automated plate reader. The A_{595} values for the BSA standards were plotted against protein concentration to give a standard curve from which the absorbances of the test samples could be applied to determine their protein concentrations.

2.7 Quantification of CAT assays.

The calculation of CAT activity from cell extracts was done exactly as described in Current Protocols in Molecular Biology (9.7.4 supplement 29). The air dried TLC plate (methods 2.5.3) was placed in direct contact with a Molecular Dynamics PhosphorImager screen and placed at room temperature overnight. The screen was then loaded into a Molecular Dynamics PhosphorImager and the image from the screen scanned. The ImageQuant software (version 3.2) was used to analyse the scanned image. Background volume values were taken before determining the volumes of the acetylated and unacetylated chloramphenicol forms. The background value boxed areas were equal to the boxes used to calculate the volume of the chloramphenicol forms. The background values were subtracted from the sample values before any calculations were carried out.

$$\% \text{ acetylated} = \frac{\text{acetylated volume}}{\text{acetylated volume} + \text{non-acetylated volume}}$$

The acetylated forms were only read when the reaction was in the linear range.

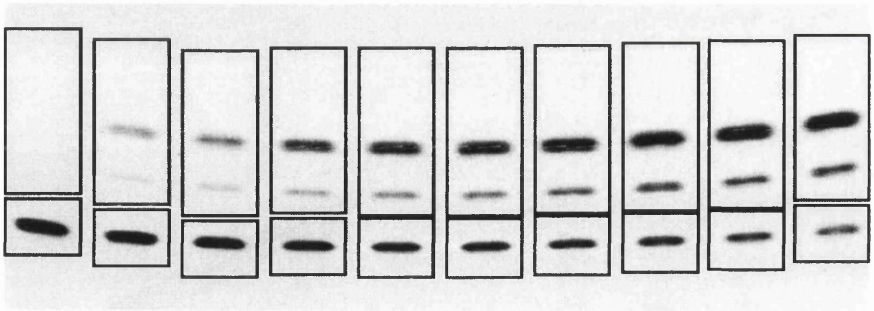


Figure 2.1 Calculation of CAT activity from cell extracts.

Increasing amounts of cell lysate were tested for CAT activity. Using ImageQuant software, the nonacetylated CAT signal (lower small box) and the monoacetylated CAT signal were measured. For subsequent experiments which required quantification, CAT activity was only measured in the linear range, where the monoacetylated species is between 20 to 40% of total counts.

Chapter 3.

3.0 Establishment of a reporter assay to study transcription and replication of Bunyamwera virus.

3.0 Introduction.

This chapter describes the establishment and optimisation of a CAT reporter assay designed to investigate the transcription and replication processes of Bunyamwera virus. The intention was to develop a reporter assay which would allow the characterisation of the transcription/replication signals encoded within the S segment of the virus genome, and which would also facilitate investigation of the viral proteins involved in these processes.

In this system, it was planned that a recombinant bunyavirus-like RNA transcript carrying a reporter gene would be transfected into cells, and be recognised and transcribed by transiently expressed recombinant bunyavirus proteins. To provide an RNA template for this assay, a reporter construct based on the Bunyamwera virus (BUN) S segment was designed (Pritlove, 1993). The design of this construct was based on a reporter construct (pIVACAT), originally described by Luytjes *et al.* (1989) which was used in the investigation of influenza virus transcription. Luytjes *et al.* constructed a recombinant RNA molecule which consisted of an antisense CAT gene flanked by the authentic 3' and 5' noncoding regions of the NS gene RNA of influenza virus A/AR18/34. This RNA transcript was mixed with purified influenza virus nucleocapsid proteins and transfected into cells. When these cells were subsequently infected with influenza A/WSN/31 virus, CAT activity was detected. This indicated that the recombinant RNA transcript was recognised by influenza viral proteins to and transcribed at least, to give a functional CAT mRNA. These workers also demonstrated that the recombinant RNA was not only transcribed, but was replicated and packaged correctly.

Given the success of this reporter construct, it was decided to utilise a similar design to build the Bunyamwera virus (BUN) S segment reporter construct.

3.1 Design of the reporter construct.

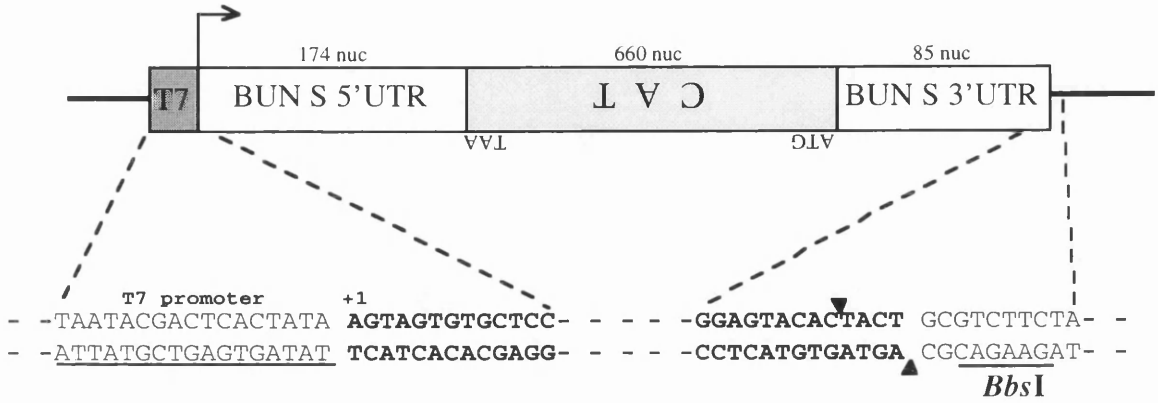
The reporter construct designed for this assay was named pBUNSCAT, and is shown diagramatically in Figure 3.1. The reporter plasmid contained an antisense sequence encoding the open reading frame (ORF) of the chloramphenicol acetyl transferase (CAT) gene (the reporter gene). The antisense CAT gene replaced the coding region in a negative sense BUN S segment cDNA. Essentially, therefore, the antisense CAT gene was flanked by the complete 3' and 5' BUN S segment untranslated regions (UTRs). Since the complete 3' and 5' UTRs of the BUN S segment are thought to contain the signals for encapsidation, transcription and replication of the RNA transcript (Chapter 1), it was expected that transcripts from the reporter construct would behave as authentic viral RNAs.

It was thought to be important that the reporter RNA transcripts should have the exact 3' and 5' termini of the BUN S segment since these sequences are strictly conserved among different bunyaviruses. To achieve the exact 5' terminus, the reporter construct was cloned immediately downstream of a truncated T7 RNA promoter (Parvin *et al.*, 1989) so that transcription would initiate at the 5' A residue of the BUN S 5' terminus. The rationale behind using the truncated T7 promoter to initiate transcription of the reporter construct was based on previous work by others (Luytjes *et al.*, 1989; Seong and Brownlee, 1992a), in which it was demonstrated that the truncated T7 promoter initiates transcription on an A residue at nucleotide position +1. These studies also demonstrated that an influenza-like RNA transcript initiating on an A residue (+1 AGUAG---3') was replicated by viral proteins, which suggested that this arrangement should also function to produce bunyavirus-like transcripts which initiate on the correct nucleotide.

To achieve the exact 3' end in the RNA transcript, a *Bbs*I restriction enzyme site was engineered downstream of the 3' terminus. This enzyme was chosen as there are no *Bbs*I sites either in the CAT gene, or in the BUN UTRs. Additionally, *Bbs*I cuts away, *i.e.* upstream of its recognition site, and therefore, it was possible to position the recognition sequence of this enzyme, so that linearisation of the pBUNSCAT plasmid with this enzyme allowed run off transcripts to terminate exactly at the 3' nucleotide of the BUN S 3' terminus.

RNA transcripts derived from the pBUNSCAT plasmid, therefore, would have the authentic ends of the BUN S segment RNA, and would contain, in the 5' to 3' order, the 174 nucleotides of the 5' untranslated region of the BUN S segment, the entire CAT gene in a negative-sense polarity, and the 85 nucleotides BUN S segment 3' untranslated region.

(A)



(B) 5' AGUAGUGUGCUCC- - /-/ - -GGAGUACACUACU 3'

Figure 3.1 Diagram of the reporter construct pBUNSCAT.

(A) The plasmid pBUNSCAT, contains a CAT gene in the antisense orientation (indicated on the diagram by "CAT" -written upside down and backwards), flanked by the complete 5' (174 nucleotides) and 3' (85 nucleotides) non-coding regions (UTRs) of the Bunyamwera genomic S segment in a pUC118 backbone. The truncated T7 promoter initiates transcription on the first base (+1 A) of the BUN S 5' UTR. Linearisation of the template prior to transcription with the restriction endonuclease *Bbs*I (recognition sequence underlined, and restriction sites marked by black triangles), allows run-off transcripts to terminate at the precise 3' terminal nucleotide (nucleotide U) of the BUN S 3' UTR.
(B) Run-off transcripts from pBUNSCAT will encode an antisense CAT gene flanked by the complete BUN S 5' and 3' UTRs with the authentic 5' and 3' termini.

3.2 *In vitro* transcription of run-off BUNSCAT RNA transcripts.

To allow the production of RNA transcripts from pBUNSCAT, the plasmid DNA was initially linearised by digestion with the restriction enzyme *Bbs*I (Methods 2.3.3). The linearised DNA was isolated by agarose gel electrophoresis, and purified by silica matrix absorption (Methods 2.3.7). The purified linear DNA was used as a template in an *in vitro* transcription reaction, performed exactly as described in Methods 2.4.1. The quality of the RNA was assessed by agarose gel electrophoresis. The RNA was treated with DNase I, assessed again for integrity by agarose gel electrophoresis, and if suitable, was used for transfection. Quantitation of the RNA was performed according to Methods 2.4.2. Figure 3.2 shows aliquots of purified *Bbs*I-digested template DNA, run off RNA transcripts prior to DNase I treatment, and the RNA transcripts after DNase I treatment (Lanes 2, 3, and 4 respectively).

3.3 Expression of recombinant BUN virus proteins.

To allow *in vivo* transcription/translation of the reporter RNA, it was necessary to supply the cells with the BUN virus proteins which were presumed to be involved in virus replication, *i.e.* the proteins encoded by the L and S segments. In setting up this reporter assay, two approaches were initially utilised to transiently express recombinant BUN virus proteins in cells: either by recombinant vaccinia virus, or by transfected plasmids which encoded BUN virus proteins, the expression of which was driven by the vaccinia virus-T7 system (vTF7-3) (Fuerst *et al.*, 1986).

3.4 Expression of BUN virus proteins by recombinant vaccinia viruses.

The recombinant vaccinia viruses vSC11BUNL and vSC11BUNS were used to supply BUN virus replication proteins to the assay. Recombinant vaccinia virus vSC11BUNL has been described previously (Jin and Elliott, 1992), and contains the entire open reading frame of the BUN L segment therefore expressing the BUN L protein, the RNA-dependent RNA polymerase (RdRp). vSC11BUNS was also constructed previously (Elliott and Page, unpublished work), and contains the entire open reading frame of the BUN S segment, therefore expressing the BUN nucleocapsid (N) and non-structural (NSs) proteins. Both the L and the S genes were cloned, using the transfer vector pSC11 (Chakrabarti *et al.*, 1985), downstream of the vaccinia virus *P*7.5 promoter.

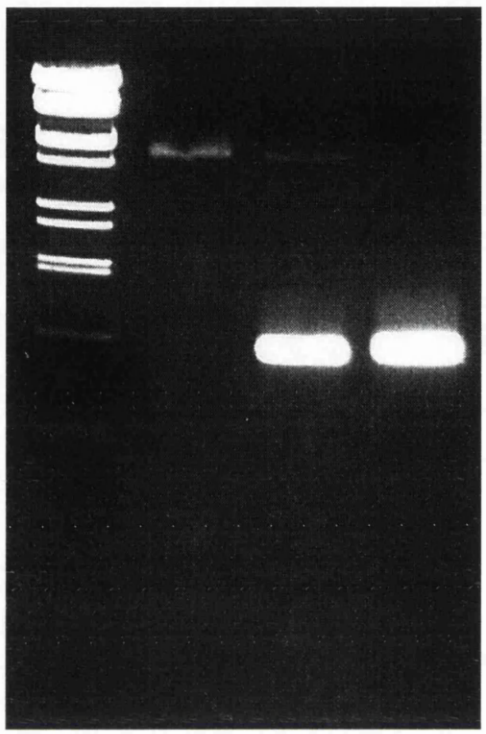


Figure 3.2 *In vitro* transcription of *Bbs*I linearised pBUNSCAT.

A 1% agarose TBE gel was loaded with the following samples: 100ng of *Bbs*I linearised pBUNSCAT DNA (Lane 2), 4µl of *Bbs*I linearised pBUNSCAT transcription reaction (methods 2.4.1) after 2 hours incubation at 37°C (Lane 3), 4µl of the same pBUNSCAT transcription reaction after a further incubation with 5 units DNaseI for 20 minutes at 37°C (Lane 4). λ *Bst*EII markers (0.5µg) are shown in lane 1.

3.5 Expression of BUN virus proteins by plasmids.

The plasmids used to allow transient expression of BUN virus proteins were pTFBUNL and pTFBUNS. The construction of pTFBUNL has been described previously (Jin and Elliott, 1992). This plasmid contains the entire open reading frame of the BUN L segment under the control of the T7 promoter in pTF7-5 (Fuerst *et al.*, 1986). pTFBUNS was also constructed previously (Elliott and McGregor, unpublished work), and contains nucleotides 86 to 961 of the BUN S segment cDNA, cloned downstream of a T7 promoter in pTF7-5, and therefore expresses both the BUN N and NSs proteins.

3.6 Introduction of plasmid DNA into cell cytoplasm by cationic-lipid transfection.

The chosen method of delivery of plasmid DNA into the cell cytoplasm was by a cationic liposome mediated DNA transfection procedure. This method was originally described by Felgner *et al.* (1987), and then later adapted by Rose *et al.* (1991). Both groups were able to demonstrate efficient transfection of plasmid DNA into a variety of eucaryotic cell types. Furthermore, Rose *et al.* also demonstrated the efficient cytoplasmic expression of a reporter construct, delivered by this transfection method, using the vaccinia virus-T7 expression system.

The method for the preparation of the cationic liposomes used in these experiments is described in Methods 2.5.1, and is adapted from Rose *et al.* (1991). The conditions of the transfection protocol used in these experiments are described below (see also methods 2.5.2). These conditions were adapted from those originally described by Felgner *et al.* (1987), and Rose *et al.* (1991).

3.7 Transfection of BUNSCAT RNA into 293 cells transiently expressing recombinant BUN virus proteins.

The following describes the protocol used for the transfection of cells used in the CAT reporter assay, as outlined in Figure 3.3. Subconfluent monolayers of 293 cells were infected with the recombinant vaccinia virus vTF7-3 in serum-free medium (OptiMEM), to allow expression of T7 RNA polymerase in the cytoplasm of infected cells. The cells were then transfected with the plasmids pTFBUNS and pTFBUNL, via liposome mediated transfection (methods 2.5.1). Incubation at 37°C for 3 hours allowed for expression and accumulation of recombinant BUN proteins. Following this, the cells, now transiently expressing recombinant Bunyamwera virus proteins, were transfected with *in-vitro*

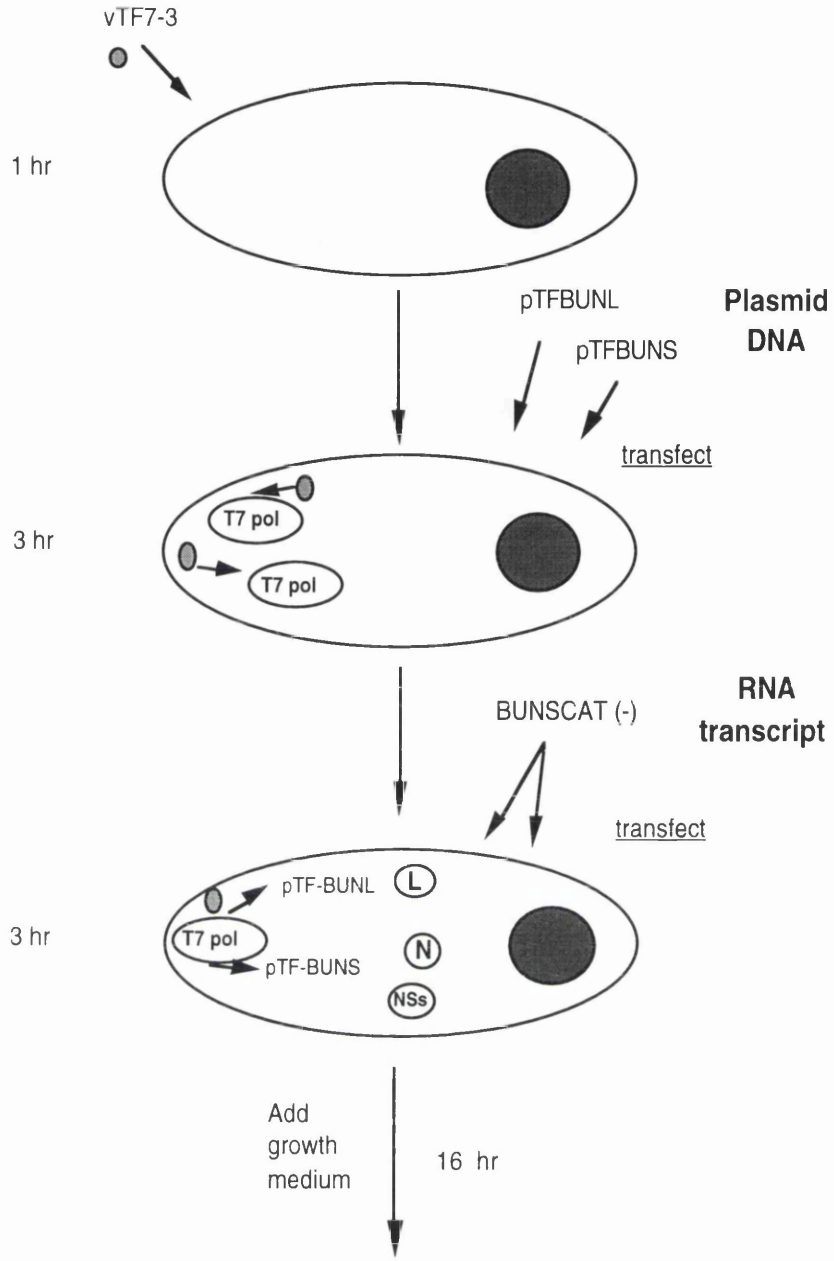


Figure 3.3 Schematic describing the transfection and detection of CAT activity from BUNSCAT RNA.

Subconfluent monolayers of 293 cells were infected with vTF7-3 at 10 pfu/cell, and incubated at 37°C for 1 hour. The monolayers were then washed once with 500µl of OptiMEM prior to the transfection of plasmid DNA in 500µl OptiMEM with 15µl liposomes. After a 3 hour incubation to allow expression and accumulation of recombinant bunyavirus proteins, 10µl of *in-vitro* transcribed BUNSCAT RNA was transfected into the cells in 500µl OptiMEM with 15µl liposomes. The incubation was continued for a further 3.5 hours before the addition of 500µl of growth medium. The cells were incubated at 37°C for a further 16 hours before harvesting, and cell extracts assayed for CAT activity (Methods 2.5.3).

transcribed BUNSCAT RNA (Figure 3.2), and the incubation continued for a further 3.5 hours before the addition of growth medium. The experiment was incubated for a further 16 hours, after which the cells were harvested, and cell extracts assayed for CAT activity by thin layer chromatography (Methods 2.5.3).

When Bunyamwera virus proteins were supplied by recombinant vaccinia viruses, the assay was performed as follows: subconfluent monolayers of 293 cells were infected with the recombinant vaccinia viruses vSC11BUNS and vSC11BUNL for 1 hour, prior to liposome mediated transfection of the cells with *in-vitro* transcribed BUNSCAT RNA. Following a 3.5 hour incubation, growth medium was added, and the incubation continued for a further 20 hours. The cells were harvested and cell extracts assayed for CAT activity as described in Methods 2.5.3.

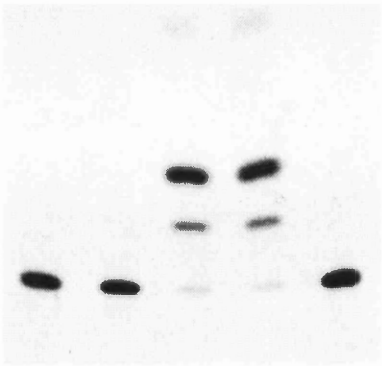
3.8 Demonstration that BUNSCAT RNA transcripts could act as a template for transcription by recombinant BUN virus proteins.

It was first necessary to determine whether the BUNSCAT RNA transcripts derived from the *Bbs*I linearised pBUNSCAT could act as a template for transcription by recombinant BUN virus proteins.

Figure 3.4 (A) shows the results of the reporter assay when the BUN virus proteins were supplied by recombinant vaccinia viruses. CAT activity could only be detected in cells expressing both the L and S segment gene products (Lanes 3 and 4). No CAT activity was detected in cells infected with only one of the recombinant viruses (Lanes 2 and 5).

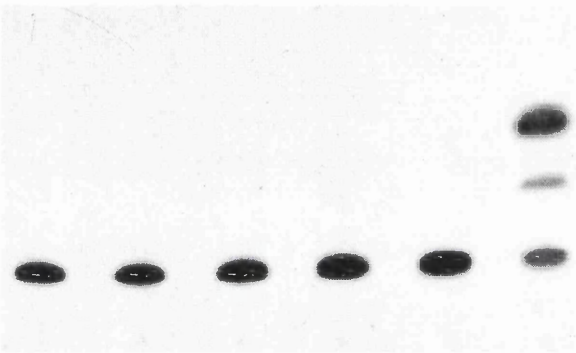
To determine whether BUN virus proteins could be supplied by transfected plasmids, the vTF7-3 T7 expression system was employed. In this system, the recombinant vaccinia virus vTF7-3 expresses T7 RNA polymerase (Figure 3.3). Thus infection with this virus prior to plasmid transfection results in expression of T7 RNA polymerase in the cytoplasm of infected cells. The T7 RNA polymerase drives the transcription of mRNAs from plasmids encoding the BUN S and L segment ORFs, under the control of the T7 promoter (Figure 3.3). The results of the experiment shown in Figure 3.4 (B) show that the transcripts derived from BUNSCAT could act as templates for transcription by transiently expressed BUN virus proteins, when the proteins were supplied by transiently transfected plasmids. Measurable CAT activity could only be detected in cells which had been transfected with both plasmids, and were therefore expressing both the L and S segment gene products (Figure 3.4 B, Lane 11). No CAT activity was detected in cells which were transfected with only one of the plasmids (Lanes 9 and 10), or in control cells which had been infected with vTF7-3, and

(A)



BUNSCAT RNA	+	+	+	+	+
vSC11BUNS	-	+	+	+	-
vSC11BUNL	-	-	+	+	+
Lane	1	2	3	4	5

(B)



BUNSCAT RNA	-	+	+	+	+	+
vTF7-3	-	-	+	+	+	+
pTFBUNS	-	-	-	+	-	+
pTFBUNL	-	-	-	-	+	+
Lane	6	7	8	9	10	11

Figure 3.4 Transcription of BUNSCAT RNA transcripts by transiently expressed bunyavirus proteins.

(A) Cells were infected with 10 pfu/cell of vSC11BUNL, vSC11BUNS, or both viruses, as indicated, and subsequently transfected with 10µl BUNSCAT RNA. Cell extracts were harvested 20 hours post infection, and assayed for CAT activity by thin layer chromatography (TLC) (methods 2.5.3). (B) Cells were infected with vTF7-3 at 10 pfu/cell, and then transfected with 5µg pTFBUNL, 5µg pTFBUNS, or 5µg of both plasmids, as indicated, then subsequently transfected with 10µl BUNSCAT RNA. Cell extracts were harvested 24 hours post infection and assayed for CAT activity by thin layer chromatography (TLC). As can be seen from lanes 3, 4 and 11, CAT activity was dependent on expression of both L and S segment gene products, regardless of whether these proteins were supplied by recombinant vaccinia viruses or by transfected plasmids using the vaccinia virus-T7 expression system.

mock transfected (Lanes 7 and 8). Comparison of figure 3.4 A and B, which represent similar cell equivalents suggest that CAT expression was greater when BUN proteins were supplied by recombinant vaccinia viruses compared to recombinant plasmids.

The results of these experiments indicated that proteins from both the BUN L and the S segments were required for successful transcription of the reporter BUNSCAT RNA. Furthermore, these proteins could be supplied either via recombinant vaccinia viruses, or via the transfection of plasmids using the vaccinia T7 expression system.

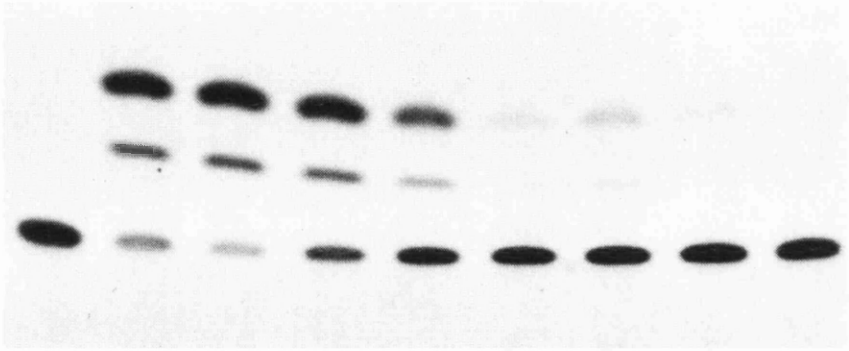
3.9 Optimisation of the expression of BUN virus proteins by plasmids.

The ultimate aim of the experiments in this section was to develop a reporter assay system which would not only allow us to study bunyavirus transcription, but also enable the analysis of the effects of specific mutations in the BUN viral proteins. Therefore, it was decided that, despite higher CAT levels obtained with the recombinant vaccinia virus system, the plasmid expression system would be more convenient. The main advantage this system has over recombinant vaccinia viruses is the relative ease with which mutated BUN proteins could be produced via specific mutagenesis of plasmid encoded cDNA.

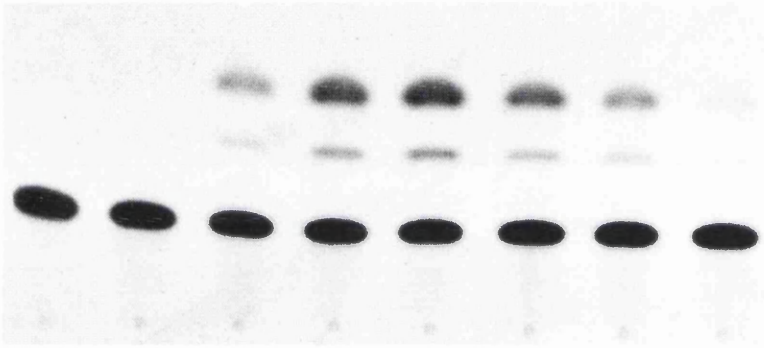
Experiments were performed to optimise the expression of BUN viral proteins from plasmids. As shown in Figure 3.5, pTFBUNL and pTFBUNS were titrated against each other in order to optimise the ratio of each plasmid in the reporter assay. Different amounts of pTFBUNS DNA (0.5 to 20μg) and 5μg transfected pTFBUNL DNA were used, thereby increasing the amount of S segment proteins expressed against a constant level of BUN L protein. By simple visual assessment of the CAT signal, it was judged that the optimal amount of pTFBUNS transfected was 1μg (Figure 3.5 A). By varying the amounts of pTFBUNL transfected (0.5 to 15μg) with 1μg pTFBUNS, thereby increasing the amount of BUN L protein expressed against a constant level of S segment proteins. , it was found that the optimal amount of this plasmid for transfection was 5μg (Figure 3.5 B). Therefore, the highest measurable CAT activity obtained (as judged by visual assessment) from the reporter RNA was achieved when 5μg pTFBUNL were transfected with 1μg pTFBUNS. Hence, in all subsequent experiments these amounts of plasmid DNA were used (unless otherwise stated).

3.10 Optimisation of the incubation time before cell extract harvesting.

In an effort to determine the optimal time point at which harvesting the cells from the reporter assay would result in the highest and most consistent level of CAT activity, a time course



pTF-BUNS	0	0.5	1.0	2.5	5.0	7.5	10.0	15.0	20.0
pTF-BUNL	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0



pTF-BUNS	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pTF-BUNL	0	0.5	1.0	2.5	5.0	7.5	10.0	15.0

Figure 3.5 Titration of the pTFBUNL and pTFBUNS plasmids in the CAT reporter system.

(A) Cells infected with vTF7-3 were transfected with 5μg pTFBUNS and varying amounts of pTFBUNL as indicated (in μg). (B) Cells were transfected with 5μg pTFBUNL, and varying amounts of pTFBUNS, as indicated (in μg). After transfection of plasmid DNA, cells were then transfected with an equal quantity of BUNSCAT RNA from the same transcription reaction. Cell extracts were harvested 24 hours post infection and assayed for CAT activity by thin layer chromatography.

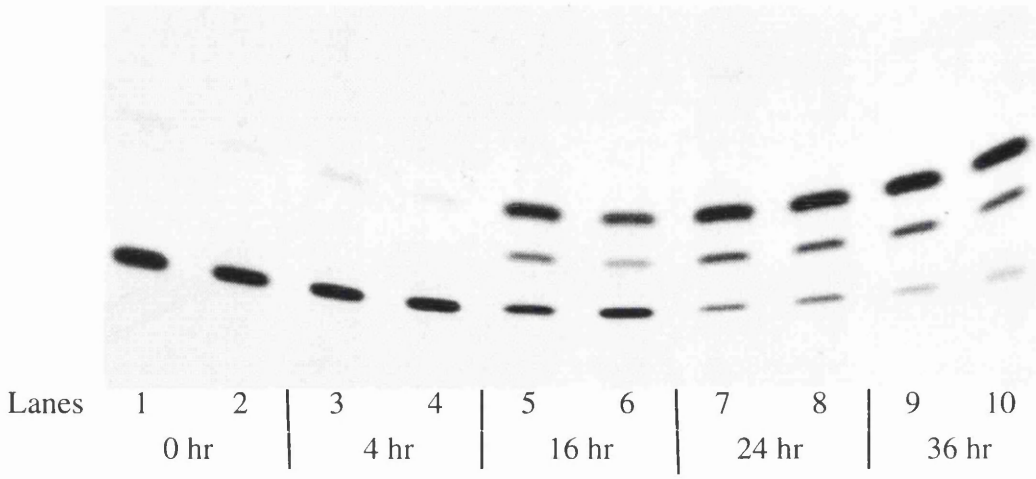


Figure 3.6 The effect of incubation time on CAT expression.

Duplicate dishes of cells transiently expressing the BUN L and S segment gene products were transfected with BUNSCAT RNA for 3.5 hours at 37°C, prior to the addition of 0.5 ml growth medium (DMEM/10% FCS). The cells were subsequently incubated at 37°C for varying times (as indicated) before extracts were harvested and assayed for CAT activity. The addition of growth medium was taken as time zero (0 hours). Low levels of CAT activity were detected at 0 hours and 4 hours incubation. A strong CAT signal was detected at 16 hours, and this increased to 36 hours.

experiment was performed, the results of which are shown in Figure 3.6. A series of replicate BUNSCAT RNA transfections were set up as described previously, where cells transiently expressing recombinant BUN proteins were transfected with identical amounts of BUNSCAT RNA. The transfected cells were then incubated at 37°C after the addition of growth media (time 0 hours) for various time points (0, 4, 16, 24 and 36 hours post RNA transfection) when cell extracts were assayed for CAT activity.

As shown in figure 3.6, low levels of CAT activity could be detected at 0 and 4 hours incubation. These levels increased markedly to give a strong CAT signal at 16 hours, which was increased slightly at 36 hours incubation. The half life of CAT is relatively long (approximately 50 hours). Therefore, it was thought that the increase observed between 24 hours and 36 hours post RNA transfection was due not to a continued linear increase in translation of the reporter RNA, but to a small amount of continued translation and a slow accumulation of CAT enzyme. On this basis, therefore, it was decided that the level of CAT activity obtained between 16 and 24 hours post RNA transfection, was both consistent and sufficiently high for accurate and meaningful quantitation. Hence, in all subsequent experiments the 20 hour time point was used (unless otherwise stated).

3.11 Optimisation of the amount of BUNSCAT RNA used in transfections.

To determine the optimal quantity of transfected BUNSCAT RNA to produce a consistent and quantifiable CAT signal, the following experiment was performed. Cells transiently expressing recombinant BUN proteins were transfected with various amounts of BUNSCAT RNA ranging between 0.5μg and 5μg. It can be seen in Figure 3.7, CAT activity increased with increasing amounts of BUNSCAT RNA transfected.

It was decided, that transfection of 3μg of BUNSCAT RNA gave a CAT signal that was both consistent and sufficiently high for the purposes both of detection and for accurate and meaningful quantitation, this amount was used in all subsequent experiments (unless otherwise stated).

3.12 The effect of using different cell lines in the reporter assay.

To determine if different levels of CAT activity would be produced in the reporter assay by using different cell lines, three other cell lines HeLa(T4), CV-1 and BHK-21, cells were tested and compared to 293 cells. Dishes of each of the four cell lines, transiently expressing the BUN viral proteins of the L and S segments, were transfected with identical amounts of BUNSCAT RNA and assayed for CAT activity as described previously. From the CAT

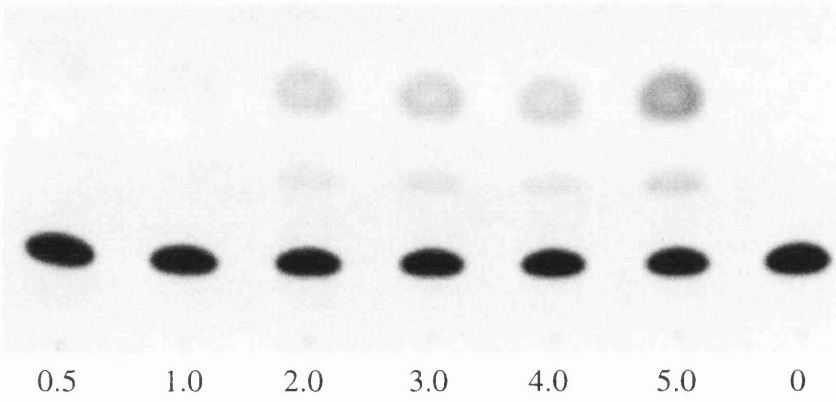


Figure 3.7 CAT activity in cells transfected with varying amounts of BUNSCAT RNA.

Cells transiently expressing the gene products of the BUN L and S segments were transfected with varying amounts of BUNSCAT RNA as indicated, (in µg), in 0.5 ml OptiMEM containing 15µl liposomes. Cells were harvested 24 hours post infection, and cell extracts were assayed for CAT activity. CAT activity was detected in cells transfected with 0.5 to 5.0 µg BUNSCAT RNA. CAT activity increased in accordance with amount of RNA transfected.

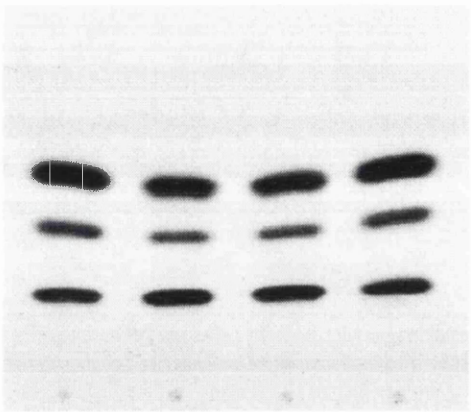


Figure 3.8 The effect of using different cell lines on CAT expression.

Four different cell lines (as indicated above) transiently expressing the gene products of the BUN L and S segments were transfected with identical amounts of BUNSCAT RNA. Cell extracts were harvested 16 hours later and assayed for CAT activity. As judged by visual assessment all four cell lines gave similar CAT activities.

results in figure 3.8, it can be seen that all four cell lines gave similar CAT activities. It was decided that because they were easier to maintain and grow, CV-1 cells would be used for all future reporter assays.

3.13 The effects of a specific point mutation in the 5' UTR terminal sequence in pBUNSCAT.

During the course of constructing pBUNSCAT, a clone was isolated which had a single C to G mutation at position 12 in the BUN S 5' UTR terminal sequence. This was named pBUNSCATmut-12 (Figure 3.9). This mutation lies within the terminal 15 nucleotides which have been found to be conserved in all the available sequenced bunyavirus S segments (Dunn *et al.*, 1994). The effect of the mutation was predicted to result in a slight disruption in the potential base-paired structure between the 5' and 3' sequences of the BUN S segment termini (Elliott, 1990), as indicated in Figure 3.9.

Transcripts derived from this mutant clone were tested in the reporter assay, and the results shown in Figure 3.10. It was demonstrated that transfection of the mutant RNA transcripts resulted in barely detectable CAT activity, when BUN virus proteins were supplied by either transfected plasmids (Lane 6) or recombinant vaccinia viruses (Lane 8). Equivalent amounts of wildtype (wt) BUNSCAT RNA (Lanes 7 and 9) gave a high CAT signal. No CAT activity was seen for either RNA when transfected into vTF7-3 infected cells (Lanes 4 and 5). However, CAT activity could be measured following transfection of either plasmid DNA into vTF7-3 infected cells (Lane 4 and 5). This result illustrates the promiscuity of vaccinia virus RNA polymerase in utilizing the plasmid DNA as a template, and also demonstrates that the CAT gene in pBUNSCATmut-12 was functional. More importantly, the experiment shows that a mutation in the conserved terminal sequence drastically affects recognition of the template by the BUN virus proteins.

3.14 The effects of specific mutations in the BUN L protein using the BUNSCAT reporter assay.

In a previous paper from this laboratory, the construction of a series of specifically mutated BUN L proteins expressed from the plasmid pTF7.5 was described (Jin and Elliott, 1992). Using a nucleocapsid transfection assay it was demonstrated that certain mutations in the putative polymerase motifs, which are conserved among all RNA-dependent RNA polymerases (Chapter 1.2.4 and Figure 3.12), abolished the RNA synthesis capability of L protein, whereas mutations at unconserved sites still gave rise to functional L protein (Jin and Elliott, 1992). It was decided to investigate the effects of these mutated L proteins in the

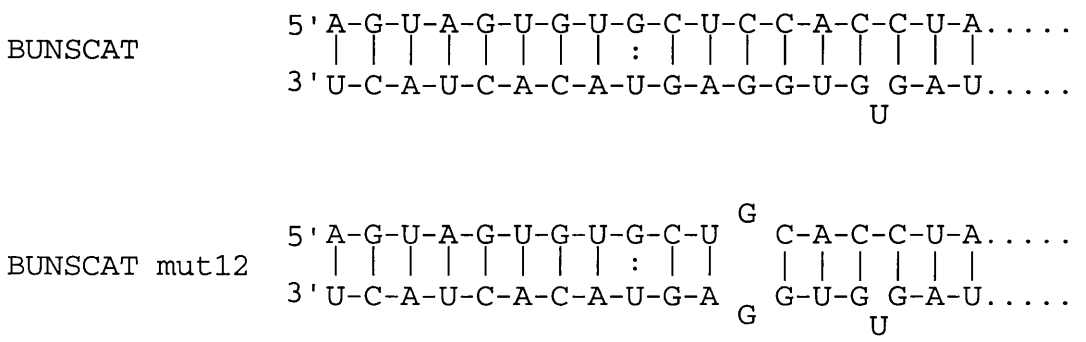
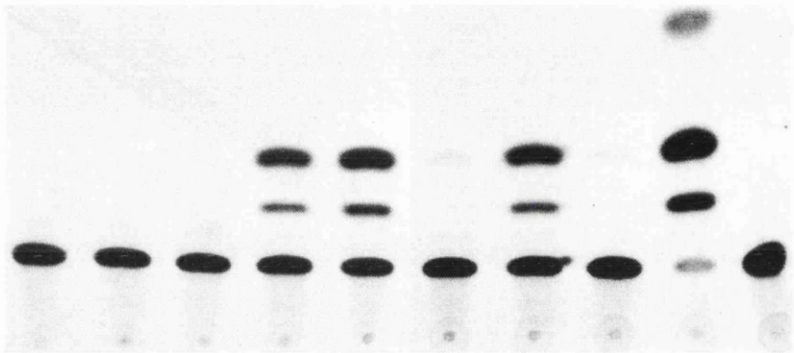


Figure 3.9 Comparison of the predicted base pairing between the 3' and 5' termini of RNAs transcribed from pBUNSCAT and pBUNSCATmut-12.



vTF7-3	+	+	+	+	+	+	+	-	-	-
BUNSCATmut12 RNA	-	+	-	-	-	+	-	+	-	-
BUNSCAT RNA	-	-	+	-	-	-	+	-	+	-
pBUNSCATmut12 DNA	-	-	-	+	-	-	-	-	-	-
pBUNSCAT DNA	-	-	-	-	+	-	-	-	-	-
pTFBUNS	-	-	-	-	-	+	+	-	-	-
pTFBUNL	-	-	-	-	-	+	+	-	-	-
vSC11BUNS	-	-	-	-	-	-	-	+	+	-
vSC11BUNL	-	-	-	-	-	-	-	+	+	-
Lane	1	2	3	4	5	6	7	8	9	10

Figure 3.10 The effects of a specific mutation in the BUNSCAT 5' UTR terminal RNA sequence.

BUN L and S segment proteins were expressed either by the vaccinia virus-T7 transient system (Lanes 6 & 7), or by infection with recombinant vaccinia viruses (Lanes 8 & 9) as indicated. Control cells were infected with vTF7-3 alone (Lanes 1-5) or were mock infected (Lane 10). The cells were then transfected with BUNSCAT RNA (Lanes 3, 7 & 9), BUNSCATmut-12 RNA (Lanes 2, 6 & 8), or the respective plasmid DNAs (Lanes 4 & 5). Cell extracts were assayed for CAT activity as described. CAT activity in cells transfected with BUNSCATmut-12 RNA was approximately 0.5% of that in cells transfected with BUNSCAT RNA (Lanes 6 vs. 7, 8 vs. 9), whereas similar levels of CAT activity were observed in vTF7-3 cells transfected with the respective plasmid DNAs (Lanes 4 vs. 5).

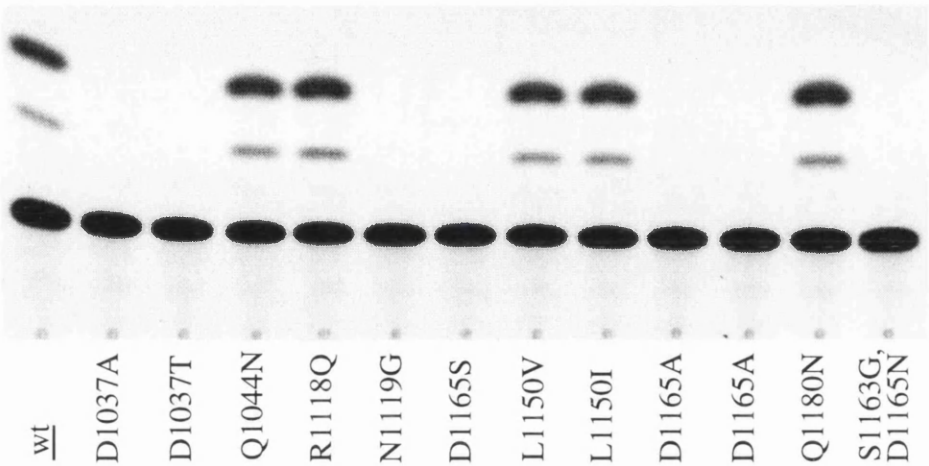


Figure 3.11 Effects of specific amino acid substitutions in the Bunyamwera virus L protein.

Cells were infected with vTF7-3, then transfected with pTFBUNS and 5µg of either wild type (wt) pTFBUNL or derivative containing specific mutations as indicated. The mutations are designated by amino acid in wt L protein/ position/ amino acid in mutant. The cells were subsequently transfected with aliquots of the same preparation of BUNSCAT RNA, and cell extracts were prepared for CAT assay 16 hours later. Mutation of residues conserved between negative strand virus polymerases (Figure 3.12) resulted in nonfunctional L protein and hence no CAT detectable CAT activity, (Lanes 2, 3, 6, 7, 10, 11 and 13). Mutations to the nonconserved residues were found to still be positive for CAT activity, (Lanes 4, 5, 8, 9 and 12) and summerised in Figure 3.12.

Figure 3.12 Alignment of the putative polymerase domains of Bunyaviridae L proteins.

The above figure is an adapted from Jin and Elliott, (1992) and shows an alignment of the available Bunyaviridae L protein amino acid sequences using the programs PILEUP and PRETTY in the GCG Wisconsin package. Regions corresponding to the four motifs (A to D) identified by Poch *et al*, (1989) (1.2.4) are overlined. 'Con' shows the residues conserved in all sequenced L segments of the *Bunyaviridae* and the arrows indicate the position and the resulting change to the amino acid residues in the Bunyamwera virus L protein (BUN sequence, bold). The plus and minus signs indicate the ability of the mutant L proteins to function, both in the nucleocapsid assay (Jin and Elliott, 1992) and in the CAT reporter system discussed here (Figure 3.11).

CAT reporter system by substituting plasmids which encoded specifically mutated L proteins, and to compare the results from these experiments to the previous results from the nucleocapsid transfection assay.

The results of the CAT assays shown in Figure 3.11, are entirely in accord with those obtained previously with the nucleocapsid transfection assay (Figure 3.12). Thus, mutation of the conserved Asp at position 1037 in motif A, Asn at position 1119 in motif B, or Asp at position 1165 in motif C gave L proteins that were inactive in transcribing BUNSCAT RNA (Figure 3.11, Lanes 2, 3, 6, 7, 10, and 11 respectively). Similarly, changing the SDD triplet (motif C, Figure 3.12) which is characteristic of the polymerases of negative strand viral polymerases, also resulted in nonfunctional L protein (Figure 3.11, Lane 13). In contrast, mutation of nonconserved residues, such as Gln at position 1044, Leu at position 1150, and Gln at position 1180, yielded L proteins which retained polymerase activity (Figure 3.11, Lanes 4, 5, 8, 9 and 12 respectively, and Figure 3.12).

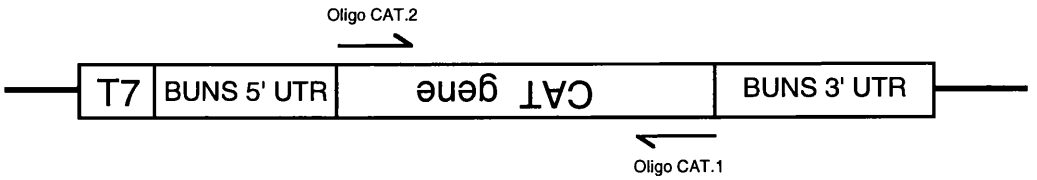
At the time of conducting this study, it was decided that characterisation of the signals encoded in the RNA transcript was of primary importance, and that study of the interaction of the RNA transcript with the replication proteins should be pursued at a later date. The BUN L protein mutants, therefore, were not investigated further using the reporter assay. However, the importance of motif C in RNA-dependent RNA polymerases has since been verified by several other groups working on other negative strand RNA viruses such as VSV (Canter *et al.*, 1993), and rabies virus (Schnell and Conzelmann, 1995).

In conclusion therefore, the observation that the results of testing the L protein mutants in the CAT reporter assay were entirely in accord with results from previous experiments using the nucleocapsid transfection assay substantiates the authenticity of this system.

3.15 The BUNSCAT transcript is transcribed, but is it also replicated?

The detection of CAT activity from cell extracts, shown in Figure 3.4, indicates that the BUNSCAT RNA transcript (which is of negative polarity) had been transcribed by the transiently expressed BUN virus proteins into a positive sense RNA transcript, and this positive sense transcript was subsequently translated to yield CAT protein. However, these experiments could not distinguish whether the positive-sense RNA was mRNA-like or antigenome-like, or whether BUNSCAT could act as a template for both authentic transcription (primer dependent mRNA production) and replication (primer independent production followed by further negative strand synthesis). To this end Northern blot analysis

(A)



(B)

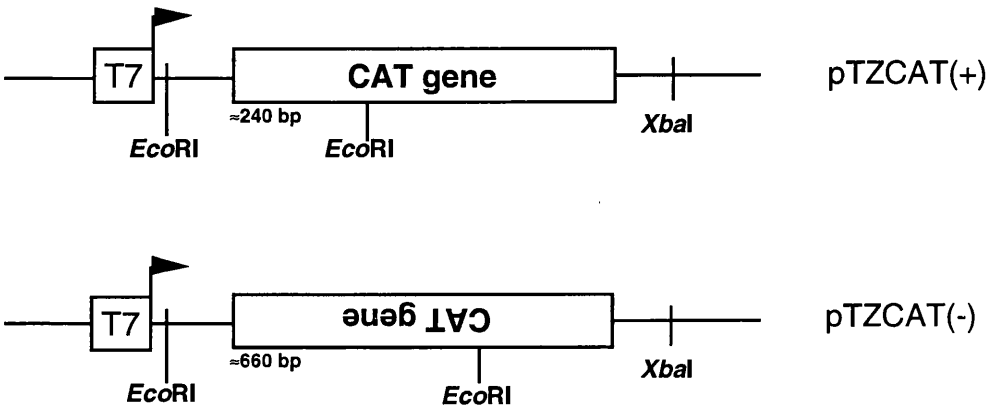


Figure 3.13 Construction of riboprobe constructs pTZCAT(-) and pTZCAT(+).

(A) Primers were designed to amplify the CAT ORF of pBUNSCAT by PCR (methods 2.3.11). The CAT.1 primer sequence was homologous to nucleotides 1 to 27 of the CAT ORF :5'-ATGGAGAAAAAAATCACTGGATATACC. The CAT.2 primer sequence was complementary to nucleotides 660 to 636 of the CAT ORF: 5'-TTACGCCCCGCCCTGCCACTCATCGCA. The resulting PCR fragment was end-repaired, phosphorylated (Methods 2.3.8) and cloned into *Sma*I cut pTZ18U to allow the cloning in both orientations. **(B)** The orientation of the CAT gene in the resulting constructs was characterised by restriction digestion with *EcoR*I. The CAT riboprobe constructs were called pTZCAT(+) and pTZCAT(-).

was performed on total cellular RNA extracts from transfected cells, since mRNA and antigenome RNAs would have different mobilities on agarose gels.

Duplicate transfection reactions were performed as described in Figure 3.4. One set of transfected dishes was analysed for CAT activity, while total cellular RNA was extracted from the other using the acid-guanidinium method as described in Methods 2.4.3. The total cellular RNA was subjected to Northern blot analysis according to the method described in Methods 2.4.5.

To detect BUNSCAT negative and positive sense transcripts, two CAT gene containing plasmids were constructed (pTZCAT(+) and pTZCAT(-)) for the purpose of generating riboprobes. These are shown in Figure 3.13. CAT probes were used instead of BUN S segment probes to remove the possibility that a background signal would be detected from transcripts transcribed from the pTFBUNS support plasmid.

A ^{32}P-UTP labeled riboprobe was transcribed from *Xba*I linearised pTZCAT(-) (Figure 3.13). This probe directs transcription of a minus sense CAT transcript, and was therefore used to probe for positive sense transcripts transcribed from BUNSCAT, *i.e.* antigenomic and messenger RNA. A ^{32}P-UTP labeled riboprobe was transcribed from *Xba*I linearised pTZCAT(+) (Figure 3.13). This probe transcribes a positive sense CAT transcript, and was therefore used to detect the negative sense, *i.e.* genomic sense, the BUNSCAT RNA transcript.

As is shown in Figure 3.14, when northern blots (A) and (B), were probed with the CAT(-) riboprobe, the positive sense CAT control marker CAT(+) RNA, could be detected in (A) lane 12 and (B) lane 10, however, no positive sense transcripts were detected (as hoped for) in (A) lanes 7 and 8 and in gel (B) lanes 5 and 6 which would have indicated transcription of a positive sense transcript. When the northern blots were probed with the CAT(+) riboprobe, BUNSCAT RNA could be detected in those dishes that had been transfected with RNA, (A) lanes 3 to 8 and (B) lanes 2 to 6, the BUNSCAT control marker (Lanes (A) 10 and (B) 8) and the CAT(-) control marker (Lanes (A) 11 and (B) 9). However, there was also no indication of an increase in the amount of BUNSCAT RNA detected. Had an increase in BUNSCAT RNA been detected in (A) lanes 7 and 8 and (B) lanes 5 and 6, this would have indicated that replication of the BUNSCAT template had occurred.

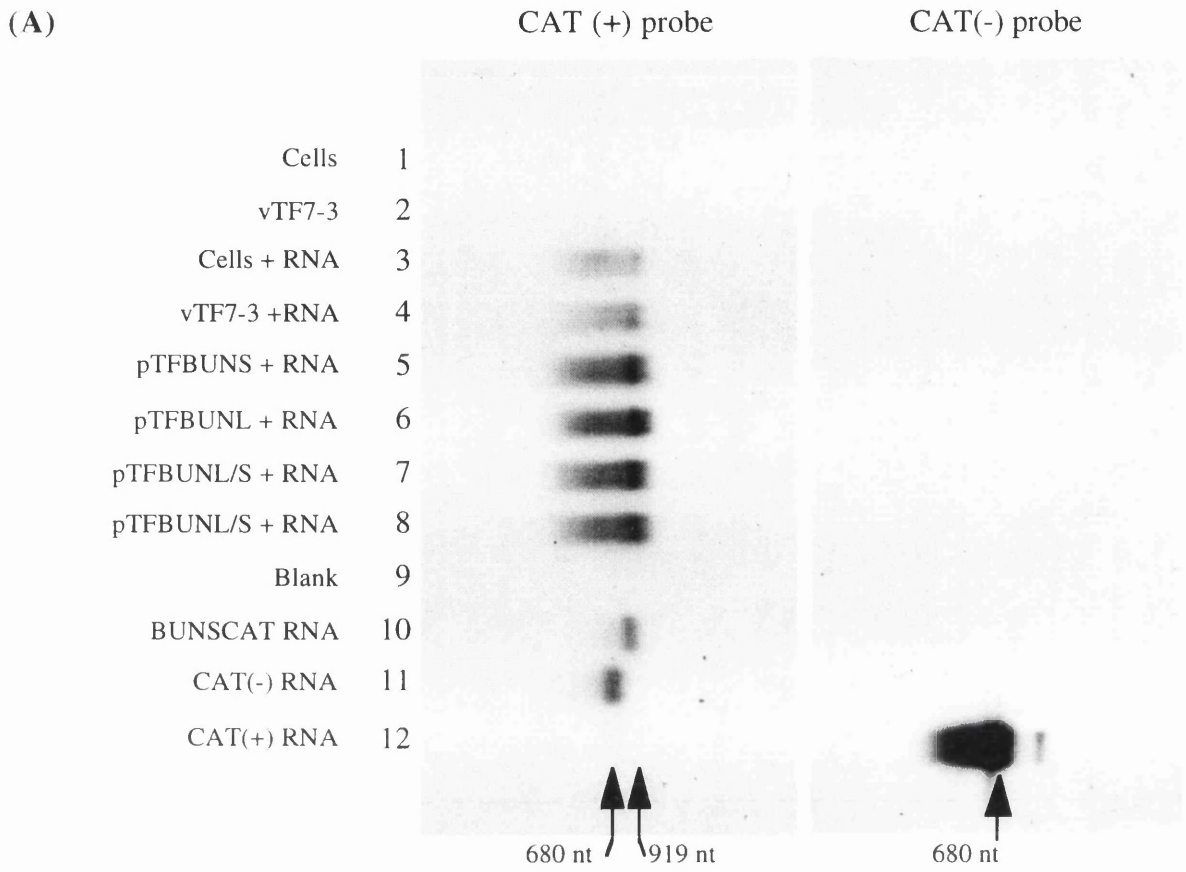


Figure 3.14 (A and B) Northern blot analysis of total cellular RNA extracts.

Total cellular RNA was harvested by the acid guanidinium method (methods 2.4.3) from a series of transfection experiments (shown above) performed as described previously (Figure 3.4). The purified total cellular RNA was denatured by glyoxal treatment, and then fractionated on duplicate 1.2% agarose gels and transferred to nylon membranes (methods 2.4.5). The membranes were either hybridised with a negative sense (-) CAT riboprobe, transcribed from *Xba*I linearised pTZCAT(-) to detect positive sense CAT transcripts, or with a positive sense (+) CAT riboprobe, transcribed from *Xba*I linearised pTZCAT(+) (Figure 3.13) to detect negative sense CAT transcripts.

(A) The CAT (+) riboprobe detected transcripts only from cells which had been transfected with BUNSCAT (RNA) (Lanes 3 to 8) as well as detecting the BUNSCAT RNA template control in lane 10 and cold CAT(-) RNA in lane 11. The CAT(-) riboprobe was only able to detect a positive sense CAT transcript in the control lane 12, containing cold CAT(+) RNA. No positive sense transcripts were detected in lanes 7 and 8.

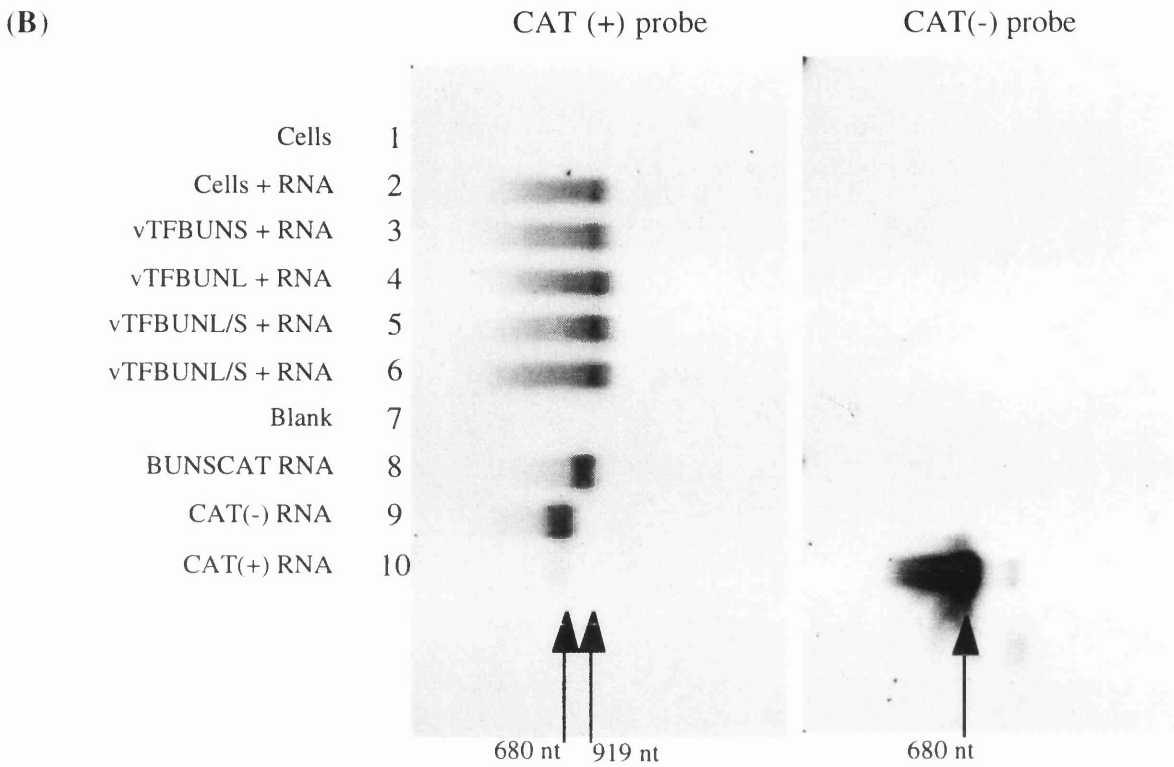


Figure 3.14 (B).

(B) Similarly, the CAT(+) riboprobe detected transcripts only from cells which had been transfected with BUNSCAT (RNA) (Lanes 2 to 6) as well as detecting the BUNSCAT RNA template control in lane 8 and cold CAT(-) RNA in lane 9. No positive sense CAT transcripts again were detected (Lanes 5 and 6) except for the cold CAT(+) probe control (Lane 10) when probed with the CAT(-) riboprobe.

3.16 Discussion.

In this chapter, I have described the establishment and characterisation of a convenient *in vivo* transient expression system which utilised a chimeric, Bunyamwera virus-like S segment RNA transcript carrying an antisense CAT reporter gene. Transfection of this reporter RNA into cells expressing recombinant BUN virus proteins resulted in the detection of CAT activity. This indicated that the negative sense BUNSCAT RNA transcript, which could not have acted as a template for translation, was recognised by, and had interacted with, the recombinant BUN virus proteins to form a functional bunyavirus ribonucleoprotein (RNP)-like complex. Formation of this complex allowed the subsequent transcription of a positive sense RNA transcript from the BUNSCAT reporter RNA transcript. This is shown schematically in (Figure 3.15).

It was also shown that detection of CAT activity from the reporter transcript was dependent upon expression of proteins from both the BUN L and S segments. These proteins could be supplied by transient expression, either from recombinant vaccinia viruses, or from transfected plasmids using the vaccinia virus-T7 system.

Titration of the amounts of pTFBUNL and pTFBUNS plasmids added to the system determined optimal conditions for the assay. This suggests CAT expression was dependent on the amounts and the ratio of the plasmids transfected into the cells. It could be argued that a "checkerboard" style titration may have offered a finer optimisation of the system. However, this was not considered necessary, in view of the sufficient level of CAT activity that was obtained.

A time course experiment was performed to determine the optimal time for cell extract harvesting. It was decided that collection of cell extracts between the time points of 16 and 24 hours post RNA transfection, gave a suitably strong CAT signal. Although the CAT signal increased slightly after 24 hours, until 36 hours post RNA transfection, it must also be considered that the CAT enzyme has a relatively long half life of 50 hours. Therefore, the increase noted at 36 hours time point may have been due to accumulation of the protein, rather than continued optimal expression. All further assays were thereby harvested at 24 hours post infection. It was also shown that transfection of 3μg of BUNSCAT RNA gave a CAT signal that was consistent and sufficient for efficient detection and quantitation.

In order to demonstrate directly that the BUNSCAT RNA was being replicated in the assay, detection of positive sense transcripts was attempted using Northern Blot analysis. This proved unsuccessful. The inability to detect BUNSCAT positive sense RNA transcripts (*i.e.* BUNSCAT mRNA and antigenome RNA) by Northern blotting, despite the fact that CAT

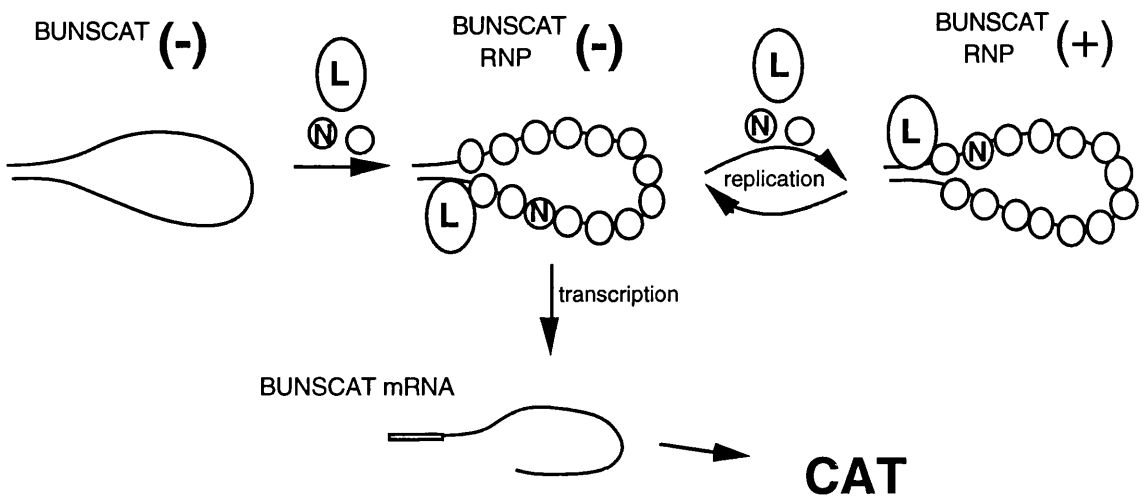


Figure 3.15 Proposed recognition and transcription of BUNSCAT RNA by recombinant BUN viral proteins.

The diagram above describes how naked RNA is encapsidated by N protein, with a possible association of L protein, to form the RNP complex. The RNP can act as the template either for subsequent transcription to produce mRNA, or it can act as a template for replication.

activity, indicative of the presence of positive sense transcripts, was obtained, may be explained by the fact that the CAT assay, because it is an enzymatic reaction, is considerably more sensitive than direct RNA detection. The level of replication in transfected cells may have been low, which would have resulted in a low number of genomic RNA transcripts, undetectable by RNA detection methods. Additionally, it is possible that only a small proportion of the cells from the given population (*i.e.* from any one dish) actually became successfully transfected with the BUNSCAT RNA transcript. This would have resulted in a low copy number of mRNA transcripts within the whole cell population, again possibly rendering positive sense transcripts undetectable by the RNA detection methods employed.

Finally, experiments with the BUNSCATmut-12 transcript, and the mutated L proteins, demonstrated the versatility of the CAT reporter system to study the effects of mutations in both the input reporter RNA, and the interacting recombinant BUN viral proteins.

Chapter 4.

4.0 Protein requirements for transcription of BUNSCAT RNA.

4.0 Introduction.

As described in Chapter 3, transcription of the reporter BUNSCAT RNA required the protein products of the Bunyamwera virus L and S segments. These proteins are presumed to be necessary for the formation of a replication competent RNP complex. The bunyavirus L segment codes for only one structural protein, the L protein, which has been demonstrated to be the viral RNA-dependent RNA polymerase (Jin and Elliott, 1992). The S segment, however, encodes two polypeptides, N and NSs, which are translated from overlapping reading frames in a single mRNA species (Bishop *et al.*, 1982). As discussed previously, the N protein is the nucleocapsid protein while the function of the non-structural NSs protein is unknown. Both of these proteins are synthesised in the reporter assay from expression of the S segment, and therefore it was not known if both proteins are required for transcription of the reporter RNA.

This chapter describes experiments to determine whether both BUN S segment gene products, N and NSs, are required for transcription of the reporter RNA.

4.1 Supplying the N and NSs proteins from individual plasmids.

In order to investigate whether both N and NSs proteins were required for transcription in the reporter assay, it was necessary to obtain plasmids which expressed these proteins individually.

Initially it was planned to use plasmids which were designed previously in our laboratory to produce only N protein or NSs protein from the S segments of Bunyamwera virus (BUN) and Maguari virus (MAG), another member of the Bunyamwera virus serogroup. The strategy used to produce these N-expressing clones was to mutate the tandem ATG initiation codon (ATGATG) of the NSs ORF to AAGACG (at nucleotides 105-110 of the BUN virus S segment cDNA, and at nucleotides 93-98 for the MAG S segment cDNA). This mutation however, also caused a change in the amino acid residue (His to Glu) at position 7 in the overlapping N ORF. The resulting cDNAs were cloned under the control of a T7 promoter

resulting in the plasmids pGEMBUNN (Elliott and Page, unpublished work), and pTZMAGN (Elliott and McGregor, 1989).

Plasmids which express only NSs were constructed by subcloning appropriate portions of the cDNAs, omitting the N protein initiation codon, downstream of a T7 promoter, to give plasmids designated pTZBUNNSs and pTZMAGNSs (Elliott and McGregor, 1989).

4.2 Verifying previously constructed plasmids, pGEMBUNN, pTZMAGN, pTZBUNNSs, pTZMAGNSs, by *in vitro* transcription /translation reactions.

The TNT coupled transcription/ translation system (Promega) was used to confirm that these constructs expressed the expected proteins. Reticulocyte lysates were programmed with pGEMBUNN, pTZMAGN, pTFBUNS pTZMAGS, pTZBUNNSs and pTZMAGNSs plasmid DNAs. The products of these reactions were analysed by SDS-PAGE (Figure 4.1(A) and (B)). It can be seen that N protein was translated in reactions programmed with pGEMBUNN (Figure 4.1(A), Lane 4), pTZMAGN (Figure 4.1(B), Lane 2), pTFBUNS (Figure 4.1(A), Lane 1), and pTZMAGS (Figure 4.1(B), Lane 4), as expected. The NSs expressing plasmids (pTZBUNNSs and pTZMAGNSs) also produced proteins which migrated at the same rate as the NSs proteins expressed from the intact S segment plasmids (pTFBUNS and pTZMAGS). However, in reactions programmed with both pGEMBUNN and pTZMAGN (Lanes 4 (Figure 4.1(A)) and 2 (Figure 4.1(B)) small amount of a protein corresponding in size to NSs was detected. It is possible that translation of NSs protein was initiated at a ACG codon, (the product of mutating the NSs ORF tandem ATG from ATGATG to AAGACG), this would explain the expression of the NSs-like protein. The use of ACG as a translation initiation codon is rare in eukaryotic systems, but has been reported for the initiation of a adeno-associated virus coat protein (Becerra *et al.*, 1985) and the Sendai virus C' protein (Curren and Kolakofsky, 1988; Gupta and Patwardhan, 1988). The possibility that even a small quantity of NSs protein could be translated from the mutated BUN and MAG virus N clones would have compromised the validity of the experiment. Similarly, the generation of the histidine to glutamine amino acid substitution at position 7 of the BUN N protein amino acid sequence was not ideal. Therefore, a different strategy for the production of N protein without NSs was adopted.

4.3 Construction of new BUN and MAG S segment plasmids expressing N but not NSs protein.

Instead of mutating the tandem initiation codon, the third codon of the NSs ORF was mutated to a stop codon (TAG, Amber), which results in the termination of NSs translation,

without mutation of the N protein overlapping reading frame (Figure 4.3). S segments of both BUN and MAG viruses, expressing N protein without NSs, were constructed using this strategy. These plasmids were named pBUNS(-NSs) and pMAGS(-NSs), respectively. The details of the PCR used to introduce these mutations, and the cloning of the constructs into the pTZ18U plasmid vector, are shown in Figure 4.3, and are described below.

The template for amplification of BUNS(-NSs) was pTFBUNS, and for MAG(-NSs) the template was pT7MAGS-R-Tϕ (Dunn and Elliott, unpublished work) which contains the complete MAG S segment cDNA under control of a T7 promoter. These cDNA were amplified with oligonocleotides designed to mutate the third codon of the NSs ORF to a stop codon (TAG, (amber)). For BUN the mutating oligonucleotide was ED.13 (5'CGCGGATCC**ACCATGATTGAGTTGGAATTTCATGATGTAGCTGCTAAC**), which is complementary to bases 86 to 121 (bold) of BUN S cDNA and introduces a stop codon in the NSs ORF by a C to A change (underlined) at position 112. The mutating oligonucleotide for MAG was ED.14 (5'GCGGGATCC**ACCATGATTGAACTTGAATTCAATGATGTAGCT GCTAAC**) which is complementary to bases 74 to 109 (bold) of MAG S cDNA and introduces a stop codon in the NSs ORF by a C to A change at position 100. Both mutating oligonucleotides also contain a *BamHI* restriction enzyme site upstream of the cDNA complementary sequence to facilitate subsequent cloning.

The cDNAs were amplified using appropriate 5' oligonucleotide and the same 3' oligonucleotide BUN-S4 (5' AGTAGTGTGCTCCACCT) which comprises the conserved 3' terminal 17 nucleotides of BUN and MAG S segments. PCR involved 25 cycles of heating at 93°C for 15 seconds, 40°C for 20 seconds, and 72°C for 70 seconds. The cDNA fragments were resolved by electrophoresis through a 1% agarose TAE gel and the correct sized fragment removed as a gel slice for further purification by GeneClean. The purified DNA fragments were end-repaired and phosphorylated, then digested with *BamHI* before being cloned by directional ligation into *Bam*HI/*Hinc*II digested pTZ18U. Clones were characterized by restriction enzyme digestion, and then sequenced to confirm their mutations. Suitable plasmid clones were selected and designated as plasmids pTZBUNS(-NSs) and pTZMAGS(-NSs).

4.4 Verifying proteins expressed by pTZBUNS(-NSs) and pTZMAGS(-NSs), by *in vitro* transcription/translation reactions.

The new Bunyamwera and Maguari S segment constructs, pTZBUNS(-NSs) and pTZMAGS(-NSs), were used to program TNT reticulocyte lysates, and the results shown in Figures 4.1 (Lane 2), and 4.2 (Lane 3). As can be seen from the lane 2 (Figure 4.1) and lane

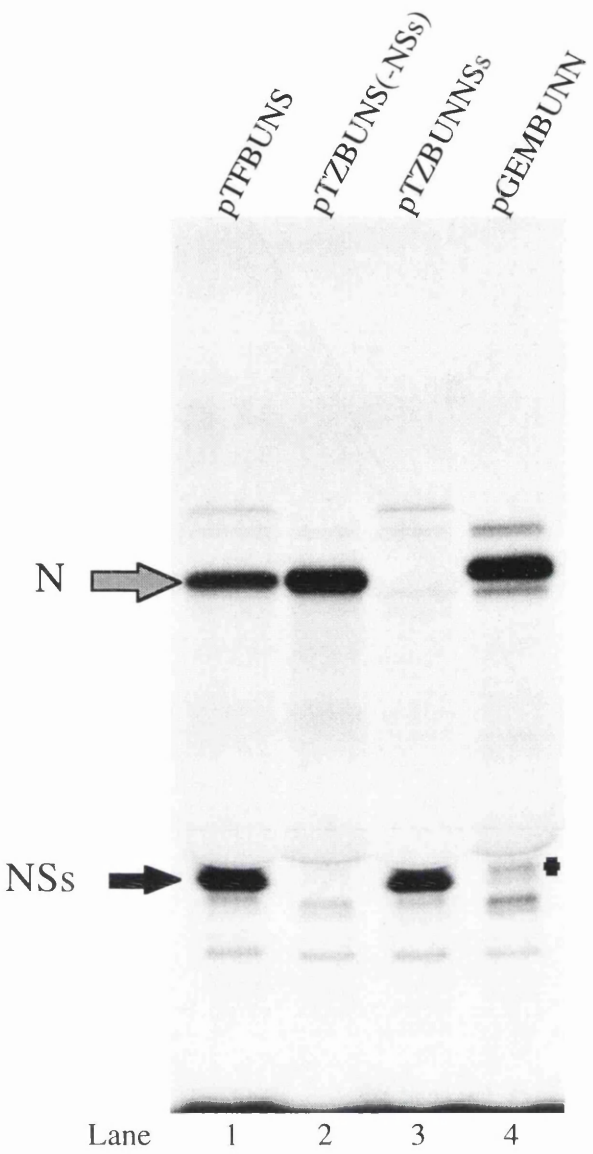


Figure 4.1 (A) Protein expression from BUN S cDNA expression clones in TNT (Promega) coupled *in vitro* transcription/translation reactions.

DNA from Bunyamwera virus S cDNA clones were used to program coupled *in vitro* transcription-translation reactions. The radiolabeled translation products were analysed on a 15% SDS-polyacrylamide gel. The positions of the N and NSs proteins are indicated. Reactions were programmed with pTFBUNS (Lane 1), pTZBUNS(-NSs) (Lane 2), pTZBUNNSs (Lane 3) and pGEMBUNN (Lane 4). A band produced from reaction lysates programmed with pGEMBUNN, migrating at the same rate as the BUN NSs, is indicated on the gel above with a cross. This possible BUN NSs-like protein may have been produced as a result of translation initiation on the mutated initiation site ACG. No such band is present in lysates from reactions programmed with pTZBUNS(-NSs) (Lane 2).

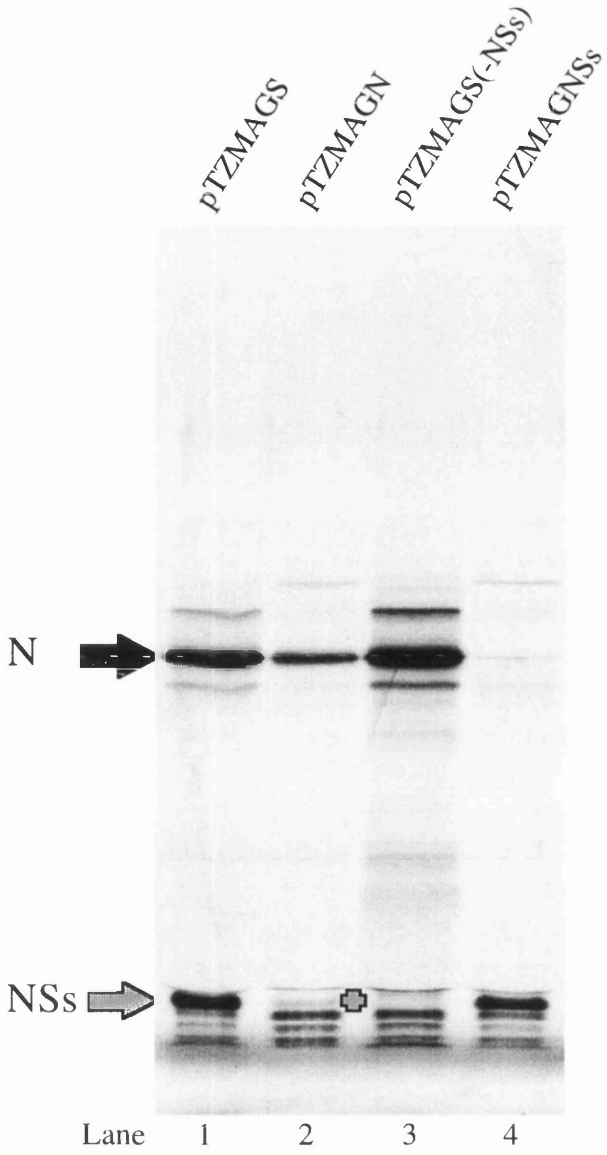


Figure 4.1 (B) Protein expression from Maguari virus S segment expression clones.

DNA from Maguari S segment cDNA clones were used to used program coupled *in-vitro* transcription-translation reactions. The radiolabeled translation products were analysed on an 18% SDS-polyacrylamide gel. The positions of the N and NSs proteins are indicated. Reactions were programmed with pTZMAGS (Lane 5), pTZMAGN (Lane 6), pTZMAGS(-NSs) (Lane 7), and pTZMAGNSs (Lane 8). A band produced from reaction lysates programmed pTZMAGN, migrating at the same rate as Maguari virus NSs, is indicated on the gel above with a cross. This possible Maguari NSs-like protein may have been produced as a result of translation initiation on the mutated initiation site ACG. No such band is present in lysates from reactions programmed with pTZMAGS(-NSs) (Lane 7).

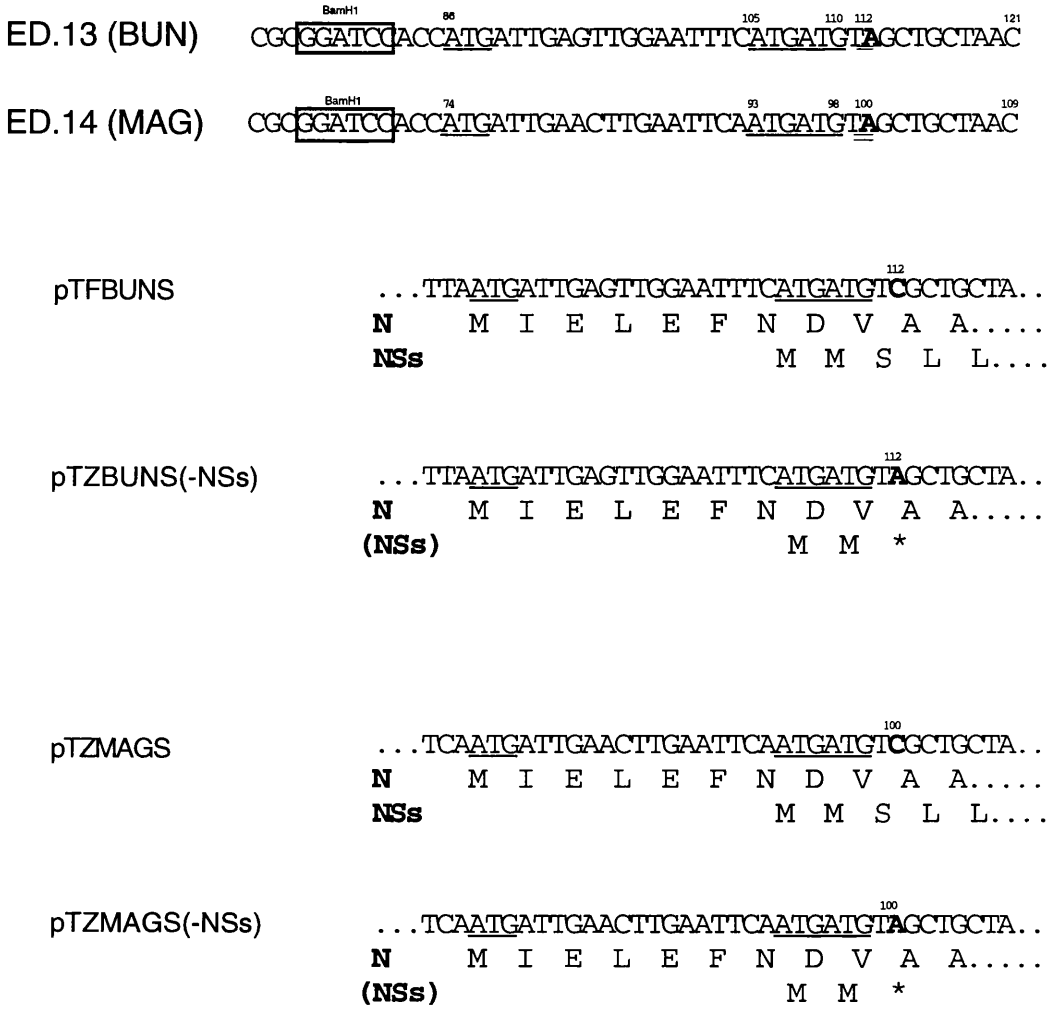


Figure 4.2 Strategy employed to disrupt the NSs coding sequence of Maguari and Bunyamwera virus S segments.

(A) Schematic of oligonucleotides ED.13(BUN) and ED.14(MAG). (B) Nucleotide and amino acid sequences of the wild type and NSs-minus mutants of Bunyamwera virus (nucleotides 93-119), and Maguari virus (nucleotides 91-107) S segments. The ATG initiation codons of N and NSs ORFs are underlined, and the C to A mutations, which results in the introduction of a stop codon in the NSs ORF of both proteins, are denoted in bold type and underlined twice. For the Bunyamwera virus S segment, the mutated nucleotide is at position 112 (A), and for the Maguari virus S segment, position 100 (A).

3 (Figure 4.2), no NSs protein was produced from either of the new constructs pTZBUNS(-NSs) and pTZMAGS(-NSs). These plasmids were then used in conjunction with pTFBUNL, to determine the S segment protein requirements for transcription of the BUNSCAT reporter RNA.

4.5 Determination of the minimum protein requirements for CAT activity in the BUNSCAT reporter assay.

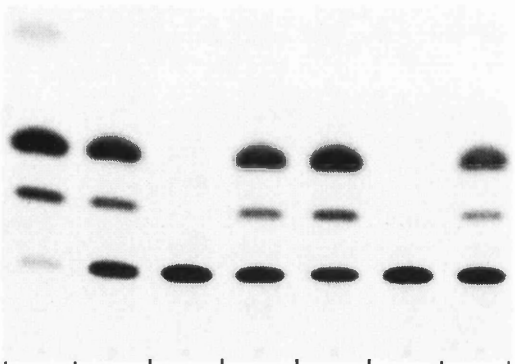
The N and NSs expressing plasmids were incorporated into the BUNSCAT reporter assay to determine which proteins were required for the production of CAT activity. The BUNSCAT reporter assay was performed as described previously (Chapter 3), with the exception that the S segment proteins were supplied by the new constructs. N and NSs expressing plasmids were transfected into vTF7-3 infected cells at two concentrations: 2.5μg and 5μg DNA. pGEM11 DNA was also added, as required, to maintain a consistent amount of total DNA transfected.

The results of this experiment are shown in Figure 4.4. CAT activity was detected when the BUN L protein was expressed in conjunction with just the BUN N protein *i.e.* in the absence of the BUN NSs (Lanes 2 and 5). No CAT activity was detectable when the BUN L protein was expressed with just the BUN NSs (Lanes 3 and 6). This result indicates that the L and the N proteins of Bunyamwera virus are both necessary and sufficient for the transcription of BUNSCAT RNA in the reporter assay. These results also indicate that there is no requirement, in the reporter assay, for the expression of NSs.

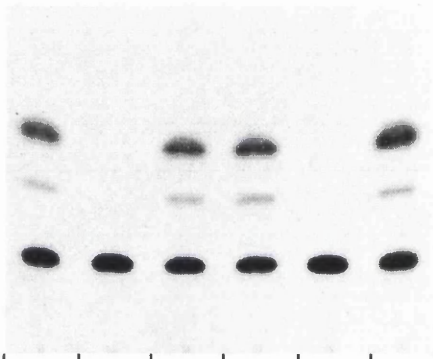
Similar results were obtained with Maguari virus S segment cDNAs : BUN L protein expression would only result in measurable CAT activity when co-expressed with MAG N protein (Lanes 8 and 11), and not with MAG NSs protein (Lanes 9 and 12). Interestingly, in addition to supporting the observation that NSs is not required for production of CAT activity from the reporter assay, this experiment also demonstrates that the BUNSCAT RNA could be transcribed by the BUN L protein in cooperation with a heterologous N protein, *i.e.* that of MAG virus S segment cDNA.

4.6 Discussion.

The findings from this study, that the L and the N proteins of bunyavirus are both necessary and sufficient for the transcription of BUNSCAT RNA in the reporter assay, can be extrapolated to suggest that the only L and N proteins are required, in conjunction with an



pTZBUNS(-NSs)	-	2.5	-	2.5	5.0	-	5.0
pTZBUNNSs	-	-	2.5	2.5	-	5.0	5.0
pGEM11	-	2.5	2.5	-	5.0	5.0	-
pTFBUNS	1.0	-	-	-	-	-	-
Lane	1	2	3	4	5	6	7



pTZMAGS(-NSs)	2.5	-	2.5	5.0	-	5.0
pTZMAGNSs	-	2.5	2.5	-	5.0	5.0
pGEM11	2.5	2.5	-	5.0	5.0	-
Lane	8	9	10	11	12	13

Figure 4.3 Protein requirements for transcription of BUNSCAT RNA.

CV-1 cells were infected with vTF7-3, then transfected with 5μg pTFBUNL and varying amounts (indicated above, in μg), of Bunyamwera or Maguari virus S segment cDNA expressing plasmids (Lanes 1 to 7, and Lanes 8 to 13, respectively). pGEM11 DNA was added to some samples, as required, to maintain the overall amount of transfected DNA. The cells were subsequently transfected with the same preparation of BUNSCAT RNA, and the cell extracts were assayed for CAT activity 24 hours post infection. It can be seen from Lanes 2 and 5, that CAT activity was detected in cells expressing only BUN L and N proteins. No significant increase or decrease is noted when cells are supplied with BUN NSs protein, in addition to L and N proteins (Lanes 4 and 7). No CAT activity was detected in cells expressing BUN L and NSs proteins (Lanes 3 and 6). In Lanes 8 and 11, it can be seen that Maguari virus N protein can co-operate with Buyamwera virus L protein to produce detectable CAT activity from BUNSCAT RNA.

appropriate RNA molecule, to reconstitute a transcriptase active RNP structure. This is in agreement with the observations of others. It has been shown previously that transcriptase activity can be detected in detergent disrupted bunyavirus preparations (BUN, Jin and Elliott, unpublished results; La Crosse virus (LAC), Patterson *et al.*, 1984; GER, Gerbaud *et al.*, 1987). Given that NSs is a putative non-structural protein, and detergent disrupted preparations made from intact virus particles would presumably contain only structural proteins, the suggestion is that there is no requirement, at least in the stages of primary transcription, for the presence of NSs.

Recently, Lopez *et al.* (1995) described a similar CAT reporter system to study Rift Valley fever virus (RVF: *Bunyaviridae*; *Phlebovirus* genus) RNA transcription. They used a reporter RNA construct, based on the RVF S segment designated CAT-RVF-Sg, which consisted of an antisense CAT gene, flanked by the 5' and 3' terminal untranslated regions of the RVF S segment. Normally, the S segment of RVF encodes a nucleocapsid, N protein and a non-structural protein, NSs, but the reading frames for these proteins are in an ambisense arrangement. The reporter template however, was designed so that it contained only a single ORF, *i.e.* that of the antisense CAT gene, rather than two ambisense ORFs. Using this reporter system, Lopez *et al.* demonstrated that RVF L and N proteins were necessary and sufficient for transcription of the RNA template, and that co-expression with RVF NSs protein had neither an additive, nor an inhibitory effect on the level CAT activity obtained. This may have been expected as the biology of RVF virus dictates that NSs expression in a virus-infected cell does not result from primary transcription, but from secondary transcription from the viral complementary RNA (cRNA), *i.e.* after replication (Giorgi, 1996).

However, it should be noted that in replacing the ambisense coding regions with the CAT open reading frame, the intergenic region between the N and NSs ORFs was omitted (Figure 1.2). Therefore, the RVF reporter transcript did not contain the authentic intergenic sequences of the RVF S segment. It has been suggested that both the sequence and structure of the intergenic region are thought to play some role in the transcription termination mechanism of mRNA in Punta Toro and Toscana Phleboviruses (Emery and Bishop, 1987; Gro *et al.*, 1992; Giorgi, 1996; Qin *et al.*, 1998), recently provided evidence that demonstrates the importance of this region. They showed that a mutant tomato spotted wilt virus (TSWV-10), with an S segment which contained a 33 nucleotide duplication in the intergenic region of this segment, suffered a selective disadvantage in forming genome reassortants *i.e.* in a mixed virus infection, this mutated S segment was not selected into reassortant viruses. Thus the authors concluded that the intergenic region correlated with the competitiveness of this genome segment in reassortment. If the intergenic region is important in this context, then the mRNA transcripts produced from the RVF reporter system would

not have the correct 3' terminus, and would possibly resemble a capped antigenome-like transcript.

Nevertheless, assuming NSs is truly a non-structural protein of RVF virus, the implication would be that it is not necessary for either primary transcription or replication events, although this does not rule out a regulatory role for the protein. It should be considered, however, there is no evidence as yet to suggest that NSs of RVF and NSs of BUN are functionally homologous.

The results presented in this chapter have also demonstrated that Bunyamwera virus L protein was able to transcribe BUNSCAT RNA in the presence of heterologous N protein, expressed from a Maguari virus S segment cDNA. This may not be altogether surprising in light of the observations made previously by Pringle *et al.* (1991) (Pringle, 1996), that Bunyamwera and Maguari viruses can form reassortments (for example BUN(L), BUN(M), MAG(S)), as a result of a mixed infection.

Chapter 5.

5.0 The interaction of various heterologous S segment encoded proteins with Bunyamwera virus L protein.

5.0 Introduction.

Previously, we reported the cloning and sequencing of S segments from a number of bunyaviruses from the Bunyamwera serogroup (Batai (BAT), Cache Valley (CV), Guaroa (GRO), Kairi (KRI), Main Drain (MD) and Northway virus (NOR)), and also one virus from the California serogroup (Lumbo virus (LUM)) (Dunn *et al.*, 1994). It has been demonstrated previously that a number of these viruses could form reassortments by the exchange of genome segments as a result of mixed infections (reviewed in Pringle, (1991) and Elliott, (1996)).

The purpose of the experiments presented in this chapter was to investigate whether the Bunyamwera virus L segment protein could act in concert with heterologous S segments proteins to transcribe the BUNSCAT reporter RNA.

5.1 Construction of heterologous S-segment expression plasmids.

In order to use the BUNSCAT reporter assay to investigate the interaction of different heterologous N and NSs proteins with the Bunyamwera L protein, it was necessary to subclone the heterologous S segments described in Dunn *et al.* (1994) into an expression plasmid under the control of a T7 promoter. Therefore, the full length cDNAs of various heterologous S segments were excised as *Xba*I/*Sac*I fragments from their original pUC118 plasmid, and subcloned into pTZ18U. The resulting clones were digested with appropriate restriction enzymes and sequenced to confirm the presence and orientation of the insert. These were denoted: pTZBATS (Batai); pTZCVS (Cache Valley); pTZGROS (Guaroa); pTZKRIS (Kairi); pTZLUMS (Lumbo); pTZMDS (Main Drain); and pTZNORS (Northway).

Full length Bunyamwera and Maguari S segment cDNAs were also cloned into pTZ18U. To achieve this, full length cDNA fragments were amplified from pT7-BUNS-R-Tϕ and pT7-MAGS-R-Tϕ (Dunn and Elliott, unpublished work) with the bunyavirus S segment specific

5' terminal primer BUN-S4 (5' AGTAGTGTACTCCACAC), and the 3' terminal primer BUN-S3 (5' AGTAGTGTGCTCCACCT) by PCR. PCR fragments were purified and end repaired/ kinased, and cloned into *Sma*I cut pTZ18U. Clones were digested with appropriate restriction enzymes and sequenced to confirm the presence and orientation of the insert. The resulting clones were named pTZBUNS and pTZMAGS.

5.2 *In vitro* expression of N and NSs proteins from various bunyavirus S segment clones.

The expression of N and NSs proteins from these plasmids was confirmed by *in vitro* transcription/translation reactions (Figure 5.1). Plasmid DNA from each clone was used to program a TNT coupled transcription/ translation reaction (Methods 2.6.1), the products of which were analysed by SDS-PAGE electrophoresis. As indicated in Figure 5.1, all constructs expressed both N and NSs proteins. The slight differences in electrophoretic mobility detected between the Bunyamwera serogroup virus N proteins, which all have 233 amino acids, has been noted previously for proteins detected in infected cell extracts and is thought to be due to differences in amino acid composition (Elliott, 1985). LUM virus (which is from the California serogroup) encodes an N protein of 235 amino acids in length, which is the case for all California serogroup viruses thus explaining its slightly slower migration.

All S segment clones gave detectable NSs proteins. The NSs protein of GRO virus (indicated by the arrow shown in Lane 7) has a notably faster migration than the NSs proteins produced by other clones, as described previously (Dunn *et al.*, 1994). The NSs protein of KRI virus is larger than the others (109 amino acids). This protein appears as a smear on the gel (as indicated by the arrow shown in Lane 8). This smearing is an artifact of the reticulocyte lysate reaction, and due to the protein co-migrating with globin, a constituent of reticulocyte lysate.

5.3 Transfection of heterologous S segment RNAs into the BUNSCAT reporter assay.

The protocol was essentially the same as that described in Figure 3.3. Following infection with vTF7-3, cells were transfected with pTFBUNL and varying amounts of the appropriate heterologous S segment plasmid. This was followed by transfection with equal amounts of BUNSCAT RNA, transcribed from the same transcription reaction.

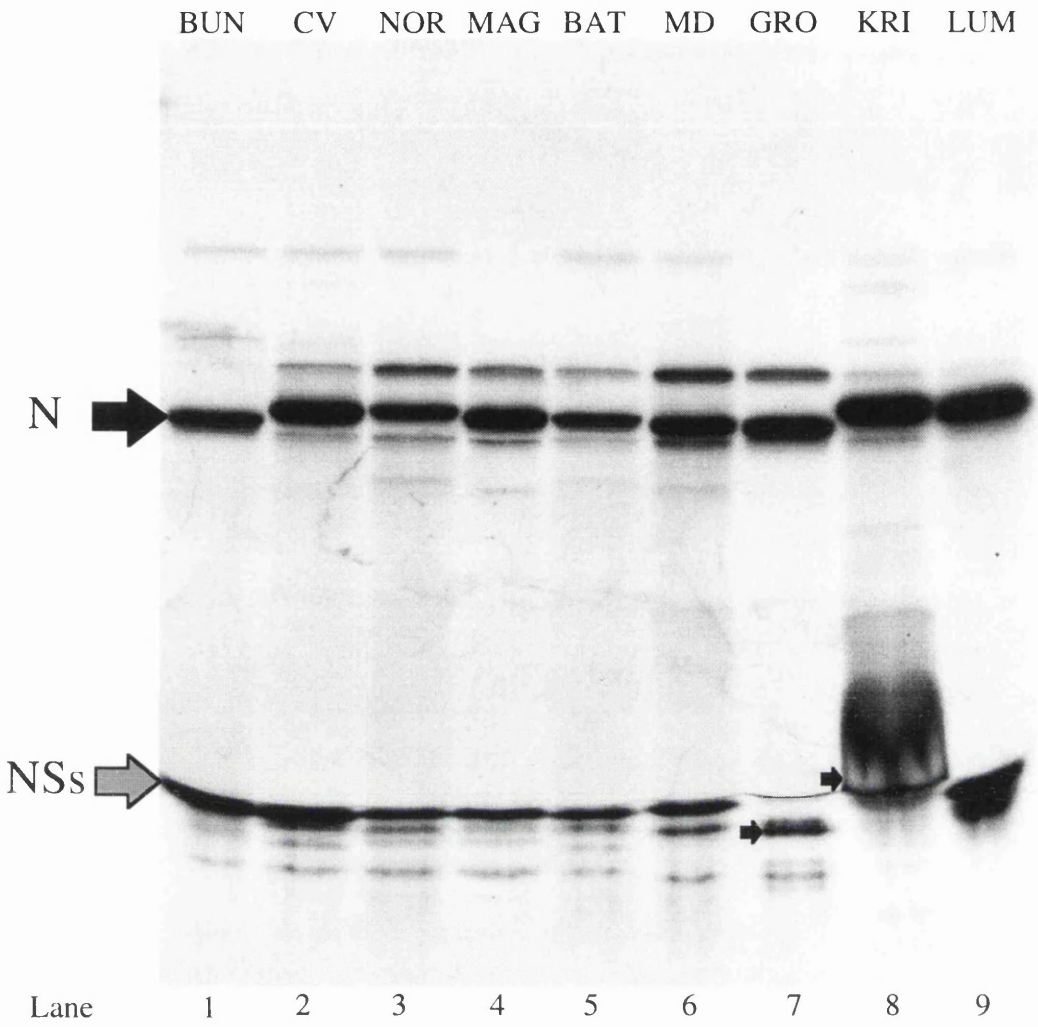
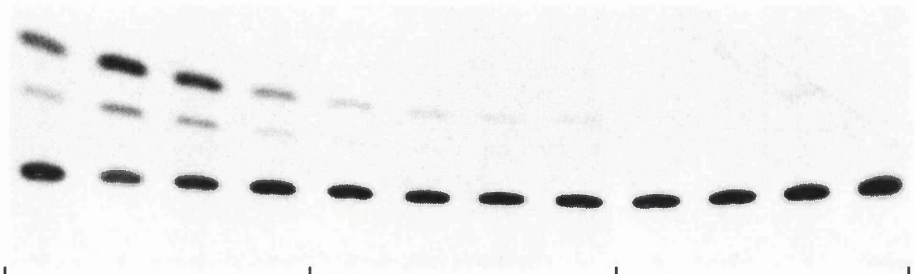
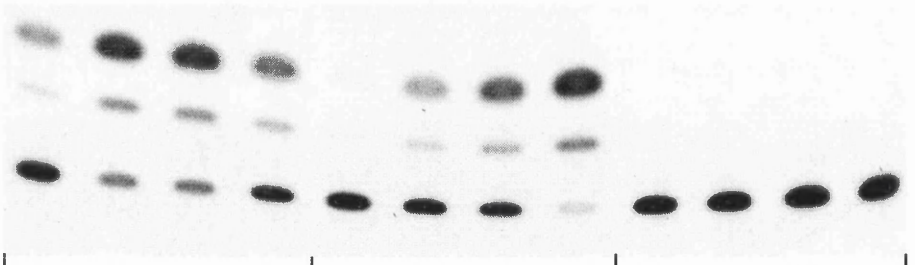


Figure 5.1 Expression of various bunyavirus S segment clones.

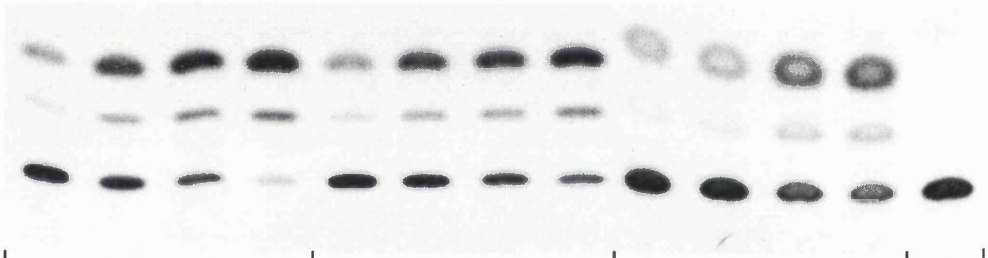
The DNA from the different S segment clones in T7-promoter plasmids, were used to program *in vitro* transcription/translation reactions. The radiolabeled translation products were analysed by electrophoresis on a 15% SDS-polyacrylamide gel. Samples were loaded on the gel as follows: pTFBUNS (Lane 1); pTZCVS (Lane 2); pTZNORS (Lane 3); pTZMAGS (Lane 4); pTZBATS (Lane 5); pTZMDS (Lane 6); pTZGROS (Lane 7); pTZKRIS (Lane 8) and pTZLUMS (Lane 9). The positions of the N and NSs proteins are indicated at the side of the gel (denoted by large arrows). The faster migrating NSs of GRO is indicated at the side of the gel (indicated by a small arrow, Lane 7). The NSs protein of the KRI S segment, migrates slower on the gel and appers as a smear (indicated by a small arrow, Lane 8). This is due to co-migration with the heavy globin protein band, a component of the *in vitro* transcription/translation reaction.



µg DNA	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0
S segment		MD				KRI				GRO		
% homology		90				71				69		
% CAT		45				2				1		



µg DNA	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0
S segment		CV				NOR				LUM		
% homology		91				93				43		
% CAT		50				53				<0.5		



µg DNA	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0	0
S segment		BUN				MAG				BAT			-
% homology		100				91				92			-
% CAT		100				42				50			0

Figure 5.2 CAT activity in cells expressing BUN L protein and heterologous S segment proteins.

Transfections were set up as described (Methods 2.5.2). vTF7-3 infected cells were transfected with 5 µg pTFBUNL and varying amounts, -either 1µg, 2.5µg, 5µg, or 10µg heterologous S segment plasmid DNA (as indicated above). Cell extracts were harvested and assayed for CAT activity 16 hours post transfection with BUNSCAT RNA, as described previously. It was found that the maximum CAT activity (over the range of transfected DNA), for each heterologous S segment tested, was directly related to the percentage homology of each particular S segment to the BUN S sequence. Also shown in the figure are the CAT activities measured for each heterologous S segment, expressed as a percentage of the CAT activity measured for the "wt" control, the homologous BUN S : BUN L combination.

Cells were transfected with varying amounts of DNA in order to optimise the ratio of heterologous S to BUN L segment gene products expressed. The maximum CAT activity obtained for each heterologous S : BUN L segment combination was compared to the activity gained from the BUN S : BUN L segment combination. The results of this are also shown in Figure 5.2. Only half of the amount of cell extract (protein), was assayed for the control samples (*i.e.* BUN S : BUN L assays) compaired to the herologous S segments. This was taken into consideration when the overall percentages of CAT activity were calculated for the heterologous S segment combinations.

It was found that the Bunyamwera virus L protein was able to transcribe the reporter BUNSCAT RNA, resulting in a significant level of CAT activity, when acting in concert with the S segment proteins from BAT, NOR, MAG and MD viruses, but not with the S segment proteins of KRI, GRO and LUM viruses.

With the exception of the MD virus, the S segment of which is able to interact in the reporter assay, but fails to produce reassortments, the observed compatibility of bunyavirus S segment proteins in the CAT reporter system, reflects the ability to form reassortments by genome segment exchange in the course of a mixed infection.

Figure 5.3 displays a dendrogram adapted from Dunn *et al.*(1994), showing the relationship between the N protein amino acid sequences of 15 bunyaviruses. The distance along the horizontal axis of the dendrogram is proportional to the similarity between sequences. The viruses whose S segments can successfully interact in the reporter assay, and can also form reassortments as a result of mixed infection, are also found closely grouped together on this diagram. Additionally, those unable to interact in the reporter assay are found outwith the close groupings. In other words, the degree of homology of the N proteins from these viruses correlate with their ability to interact with BUN polymerase in the reporter assay.

5.4 Discussion.

The results shown in this chapter demonstrate that the Bunyamwera virus L protein was able to transcribe the reporter BUNSCAT RNA, in concert with certain heterologous bunyavirus S segment proteins. Some of the heterologous S segments studied, were unable to mediate the transcription reaction. The heterologous S segments that produced significant levels of CAT activity, were those in which the N protein was most closely related to Bunyamwera virus N protein, at the amino acid level (Figure 5.3).

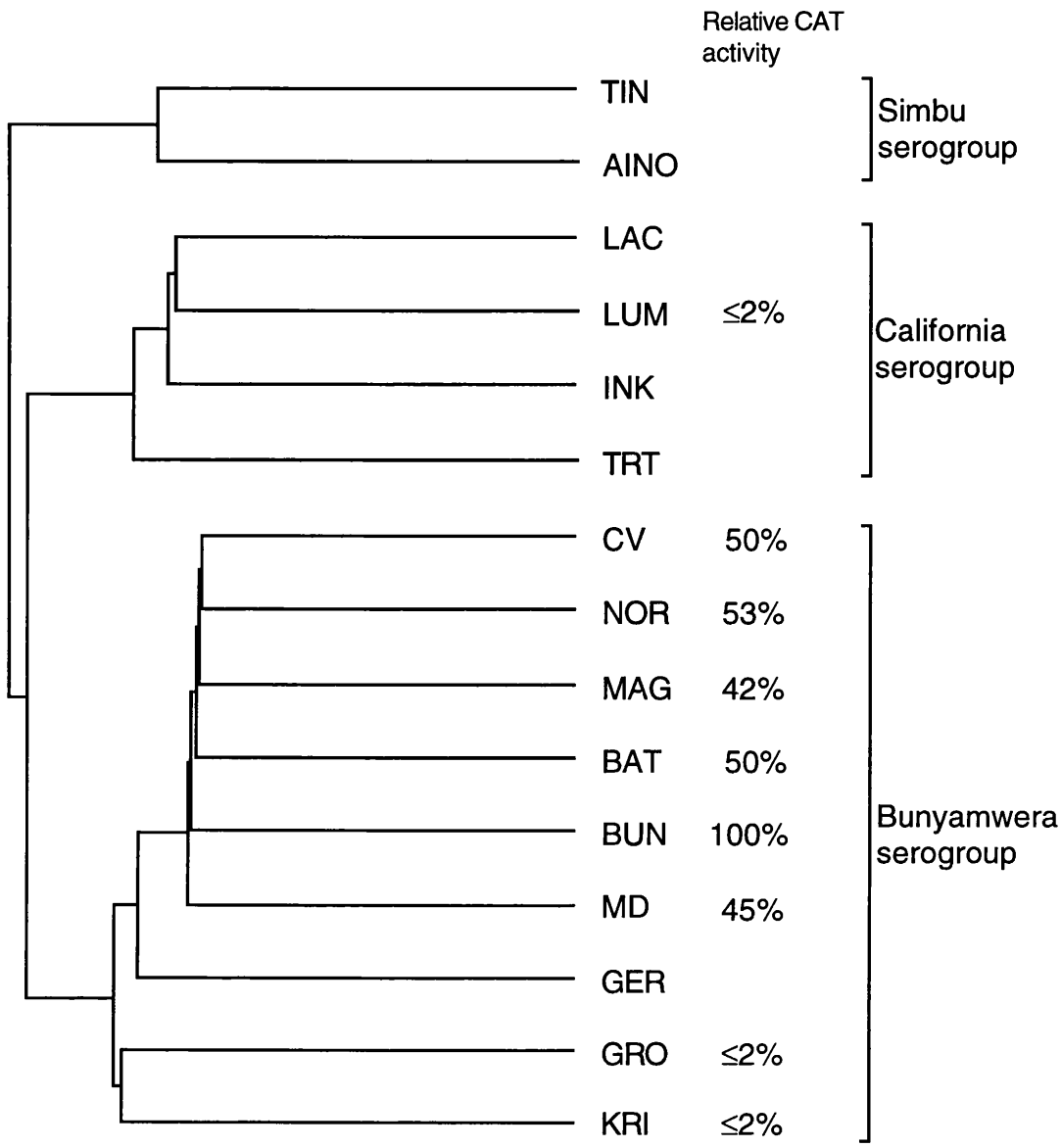


Figure 5.3 Dendrogram showing the relationship between the bunyavirus N protein amino acid sequences.

The diagram above displays a dendrogram (adapted from Dunn *et al.*, (1994)), showing the relationship between the N protein amino acid sequences of 15 bunyaviruses from three serogroups was generated by the PILEUP program in the GCG Wisconsin Package (Version 9)™. The dendrogram represents the clustering order of similarity between the N proteins after pairwise comparisons by the program to create the alignment figure shown above. The distance along the horizontal axis of the dendrogram is proportional to the similarity between sequences. The figure also displayes the relative CAT activity detected from those S-segments above that were applied to the reporter system. Bunyaviruses not previously described in this chapter were Aino virus (AINO) and Tinaroo virus (TIN) of the Simbu serogroup, La Crosse virus (LAC), Inkoo virus (INK) and Trivittatus virus (TVT) of the California serogroup and Germiston virus (GER) another member of the Bunyamwera serogroup.

With one exception, compatibility in the CAT reporter system, correlated with the ability of certain bunyaviruses to form reassortments by genome segment exchange in the course of a mixed infection (reviewed in Pringle, 1991). Therefore, as BUN has been shown to reassort with BAT, MAG, NOR, so have the BUN L segment gene products been shown here to cooperate with the S-segment gene products of these viruses to produce transcription of the BUNSCAT reporter RNA. Similarly, as BUN has been observed to be unable to produce reassortments with KRI, GRO, or LUM, so do the products of the BUN L segment fail to interact with the S-segment product of these viruses in the BUNSCAT reporter system. Furthermore, it was shown that this compatibility also correlated with a high percentage of sequence homology with the BUN N protein.

It is not clear at what level the KRI, GRO and LUM S segment proteins fail to interact with the BUN L protein to result in non-transcription of BUNSCAT RNA. The possibilities are as follows: either a failure of the heterologous N protein to encapsidate reporter RNA transcripts, or possibly a failure of the heterologous N protein to interact with the BUN L protein.

5.6 Future work.

To determine if the failure to form a functional nucleocapsid occurred at the level of RNA-N protein interaction, the following experiment could be performed. A radioactive BUNSCAT transcript could be transfected into cells expressing BUN L and one of the heterologous S segments. UV crosslinking, followed by SDS-PAGE analysis of cell lysates, could then be performed to determine if the N protein was associated with the BUNSCAT RNA. To determine if the failure occurred at the level of L protein-N protein interaction, the same transfection experiment could be performed, although with radiolabeled proteins, and the resulting lysates probed by chemical crosslinking.

In future work, the fact that some heterologous N proteins are not transcriptionally active in the reporter system (*i.e.* KRI, GRO and LUM), could be exploited to our advantage. It may be possible to map regions of importance in the BUN N protein, by creating chimeric N proteins between BUN N and the non-active heterologous N proteins. Figure 5.4 explains how one of these chimeras could be achieved. The S segments from both BUN and KRI share a unique restriction site within the N coding region, therefore BUN/KRI and KRI/BUN chimeras can be easily constructed. The use of the *Kpn*I site at nucleotides 387-392 in BUN S segment cDNA, and nucleotides 383-388 in KRI S segment cDNA, along with a site unique to the multiple cloning site will allow the construction of chimeras BUN/KRI N, and a KRI/BUN N protein, by the exchange of 102 amino acids between the

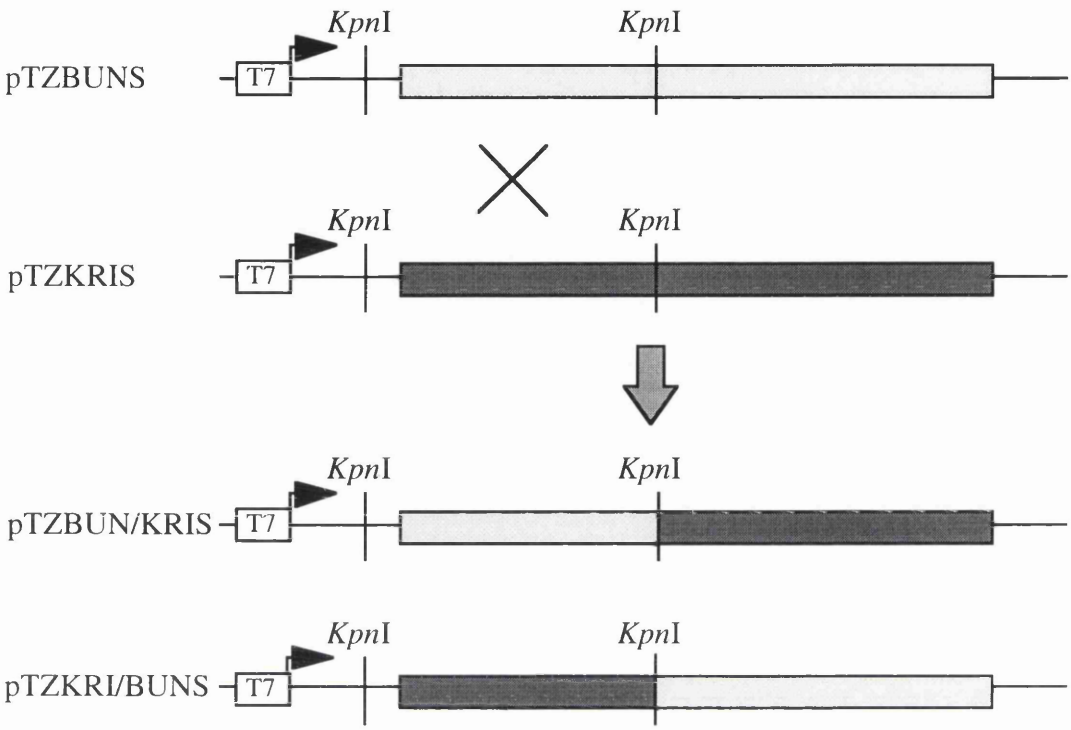


Figure 5.4 Construction of the proposed N protein chimeras of BUN and KRI N proteins.

The initial construction of a chimeric BUN/KRI N protein, and a KRI/BUN N protein could be easily achieved by the subcloning of a *Kpn*I fragments from pTZKRIS and pTZBUNS. The orientation of the insert could be checked by restriction digestion, and fidelity of sequence and reading frames, by sequencing.

cDNAs of each of these segments, without altering the reading frame of the N and NSs proteins (Figure 5.4).

Chapter 6.

6.0 Construction and expression of an antigenomic BUNSCAT construct, pBUNSCAT(+).

6.0 Introduction.

As has been discussed previously, all bunyavirus S segment sequences are conserved for the first 15 nucleotides at the 3' and 5' termini, and with the exception of one mismatch at position 9, the 3' and 5' sequences are complementary. It is presumed that the promoters and *cis*-acting signals required for replication and transcription reside in these regions. The genomic RNA promoter stimulates transcription with capped primers, whereas the antigenomic promoter does not (reviewed in Elliott, 1996), pointing to a mechanism whereby the BUN polymerase can distinguish between them.

In this chapter, an antigenome CAT reporter construct is described, which was designed to investigate the mechanism of differentiation between the two promoters.

6.1 Construction of pBUNSCAT(+).

Two oligonucleotides (ED.17 and ED.18), complementary to the 3' and 5' BUN S termini respectively, were designed to amplify the BUNSCAT reporter construct from pBUNSCAT (Figure 6.1). The resulting PCR fragment was digested with *Xba*I and *Sac*I, and directionally cloned into appropriately cut pUC118. The resulting clones were sequenced into the CAT open reading frame to confirm the orientation and fidelity of the insert sequence. After sequencing, three suitable clones were selected which had the insert in the correct orientation, with the T7 promoter upstream of the coding sequence, and which had the *Bbs*I site intact, and had no spurious misincorporations. These constructs were denoted pBUNSCAT(+), and the clones numbered 11, 12 and 13.

pBUNSCAT(+), therefore, contained the complementary sequence of pBUNSCAT under the control of the T7 promoter, such that transcripts from this construct would be of message sense (*i.e.* positive polarity). These message sense transcripts would essentially be the equivalent of BUN S antigenomic transcripts. Again, as in the case of pBUNSCAT, transcription of pBUNSCAT(+) would initiate from a truncated T7 promoter at the 5' A

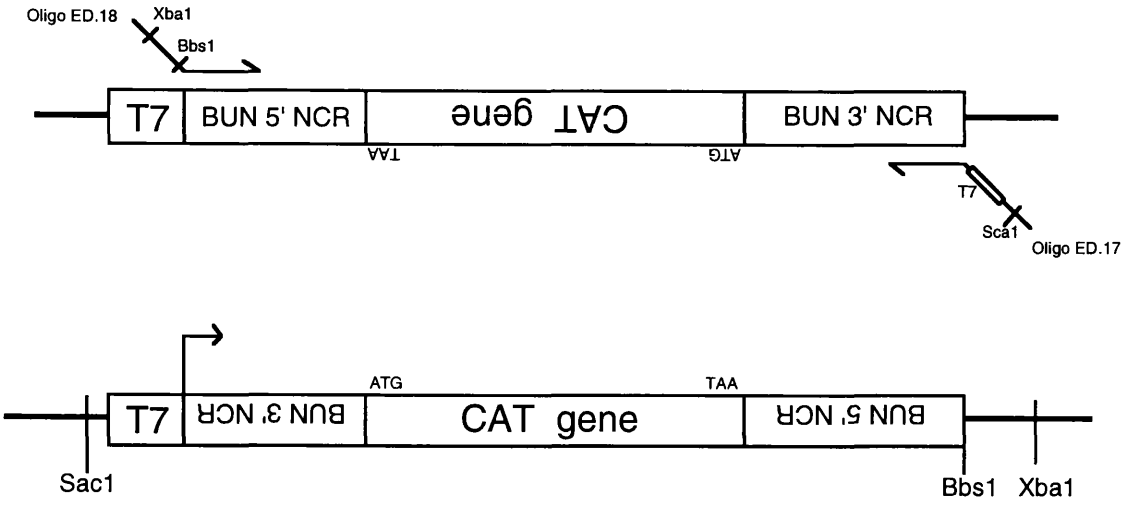


Figure 6.1 Templates and primers used in the construction of pBUNSCAT(+).

pBUNSCAT(+) was constructed by PCR using pBUNSCAT as the template, and the primers ED.17 (5' ATTCGAGCTCTAATACGACTCACTATAAGTAGTGTACTCCACA CTACAAAC) and ED.18 (5' AGACTCTAGAAGACGCAGTAGTGTGCTCCACCTAA). The region of sequence of primer ED.17 which is underlined, corresponds to the complement of the last 24 nucleotides of the BUN S segment 3' UTR. This sequence is immediately preceded by a T7 promoter, and a *Sac*I restriction site, included for cloning purposes. The region of sequence of primer ED.18 which is underlined, corresponds to the first 26 nucleotides of the BUN S segment 5' UTR. The remainder of the sequence an *Xba*I site, included for cloning purposes, and an inverted *Bbs*I site. This site allows run-off transcripts to terminate at the correct 3' nucleotides. The PCR conditions were 93°C for 30 seconds, 62°C for 30 seconds, and 72°C for 80 seconds, for 30 cycles. The resulting PCR fragment was purified from a 1% TAE gel by silica matrix absorption, digested with *Xba*I and *Sac*I, and ligated into *Xba*I/*Sac*I digested pUC118. Three clones were selected for screening. These were denoted pBUNSCAT(+) -11, -12, and -13.

residue of the BUN S antigenome, and would terminate at the exact 3' residue of the BUN S antigenome, due to linearisation of the plasmid by *Bbs*I. Thus T7 transcripts derived from pBUNSCAT(+) would have the authentic termini of the BUN S antigenome, and would contain a CAT ORF, flanked by the 5' UTR (85 nucleotides) and 3' UTR (174 nucleotides) of a BUN S antigenome.

6.2 Confirmation that the CAT gene in the pBUNSCAT(+) reporter construct was active.

To confirm that the CAT gene in the pBUNSCAT(+) gene was active, 1μg of plasmid DNA was transfected into CV-1 cells which had been pre-infected with vTF7-3. At 20 hours post infection, cells were harvested and cell lysates assayed for CAT activity. The results are shown in Figure 6.2, and it can be seen from Lanes 2, 3 and 4, that all three pBUNSCAT(+) clones contained an active CAT gene.

6.3 Transfection of BUNSCAT(+) RNA transcripts.

Run-off RNA transcripts were transcribed from *Bbs*I linearised pBUNSCAT(+) constructs (Figure 6.3). The RNA transcripts from each reaction were quantified by TCA precipitation, and ^{32}P counting (Methods 2.4.2).

The RNA transcripts from three independent clones of pBUNSCAT(+), along with RNA transcripts from pBUNSCAT included as a control, were used in the reporter assay. Equivalent amounts of each transcript were transfected into either mock infected cells, cells infected with vTF7-3, or cells transiently expressing the viral proteins of the BUN L and S segments. Cells were harvested, and extracts assayed for protein concentration as described previously, before equivalent amounts were assayed for CAT activity. Thus CAT activity per μg of protein was calculated for each transfection.

It was thought that the BUNSCAT(+) RNA transcripts, when transfected into cells, could in theory, act as a template for translation. However, as can be seen from Figure 6.4 (A) (Lanes 2, 3 and 4), no CAT activity was detected when BUNSCAT(+) RNA was transfected into mock infected cells, suggesting therefore, that translation did not occur. CAT activity was detected in cells which had been infected with vTF7-3, in the absence of BUN L and S segment proteins (Figure 6.4 (A), Lanes 10, 11 and 12). This observation suggests that a vaccinia virus encoded activity, such as mRNA capping by guanylyltransferase, enables translation of the BUNSCAT(+) RNA by host cell proteins. However, in cells which had been supplied with BUN L and S segment proteins, CAT activity was markedly increased,

Figure 6.2 Confirmation that the CAT gene in the pBUNSCAT(+) reporter construct is active.

CV-1 cells were infected with vTF7-3, and then transfected with 1μg of plasmid DNA. Following a 3 hour incubation at 37°C, 1ml of growth medium was added, and the incubation continued overnight. At 20 hours post infection, cells were harvested and cell lysates assayed for CAT activity. In lanes 2, 3 and 4, cells have been transfected with DNA from pBUNSCAT(+) reporter construct, clones 11, 12 and 13 respectively. In lane 1, to provide a positive control, cells have been transfected with pBUNSCAT DNA. As can be seen in lanes 2, 3 and 4, all three pBUNSCAT(+) clones contained an active CAT gene.

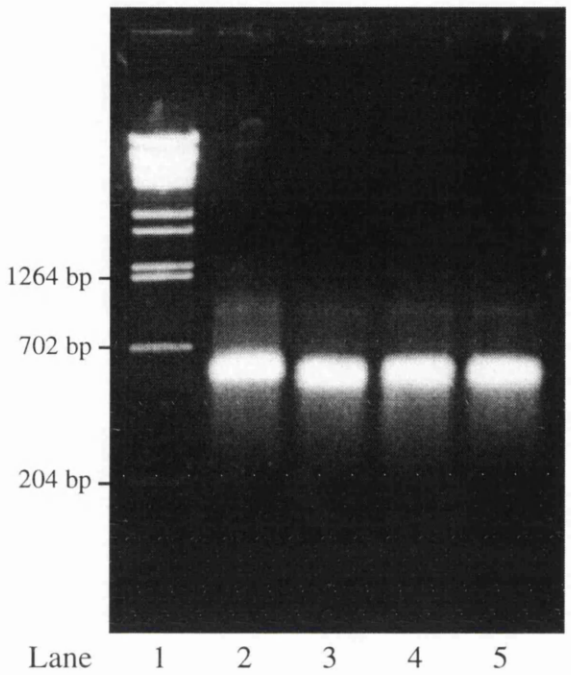


Figure 6.3 *In vitro* transcription of reporter RNA from *Bbs*I linearised pBUNSCAT(+) templates.

A 1% TBE agarose gel was loaded with 4µl of a transcription reaction which had been incubated for 20 minutes at 37°C with 5 units of RQ1DNase. Lanes 3, 4 and 5 contain the RNA transcribed from *Bbs*I linearised pBUNSCAT(+) constructs, clones 11, 12 and 13, respectively. Lane 2 contains RNA transcribed from pBUNSCAT (also DNaseI treated). Lane 1 contains λ*BstE*II markers (0.5 µg). The RNA transcribed in each reaction was quantified by TCA precipitation, and ^{32}P counting (Methods 2.4.2).

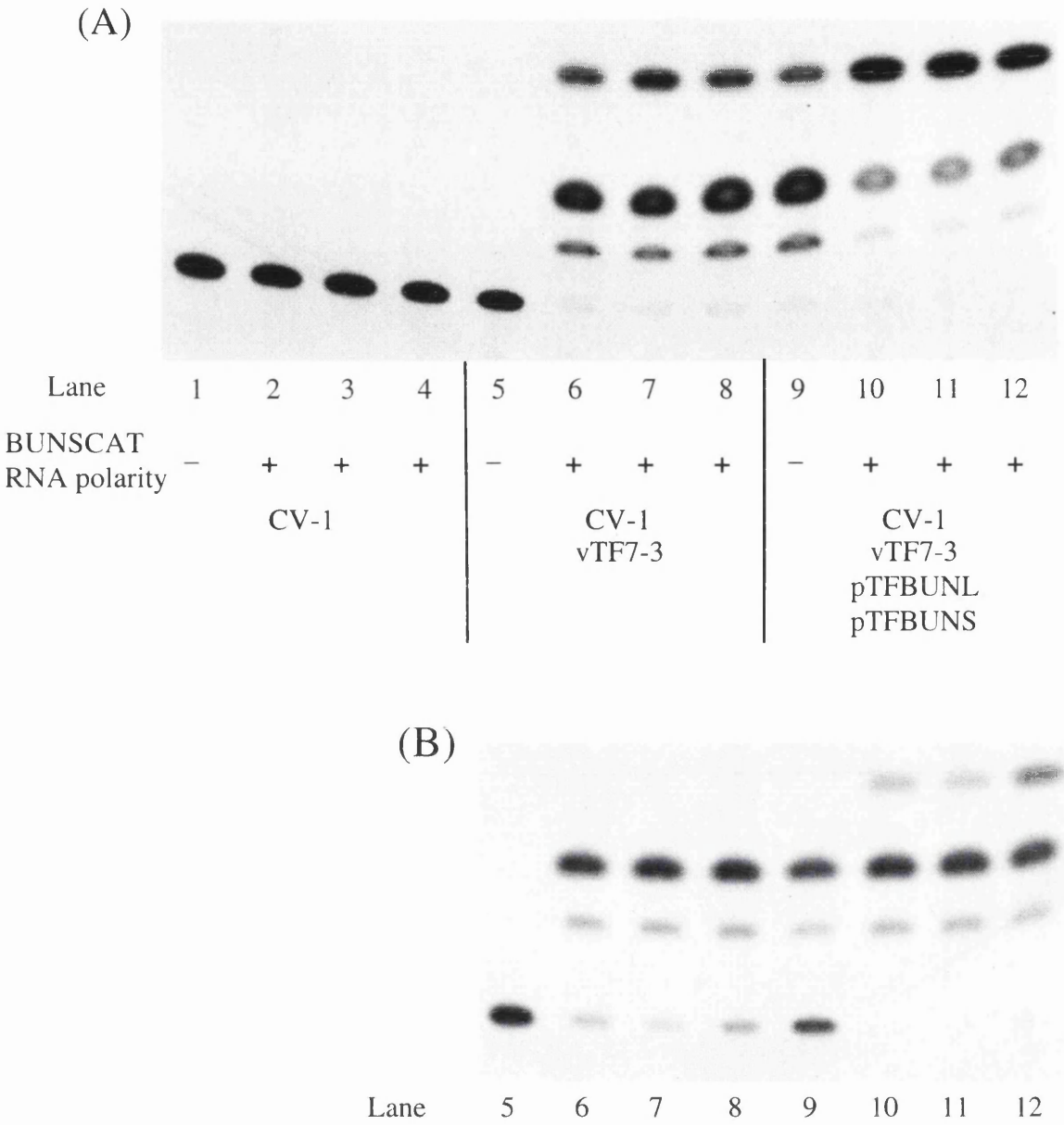


Figure 6.4 Replication/Transcription of BUNSCAT(+) RNA by recombinant BUN viral proteins.

Equivalent amounts of three independent BUNSCAT(+) RNA transcripts, and a control BUNSCAT RNA transcript, were transfected into **(A)** mock infected cells (Lanes 1-4), vTF7-3 infected cells (Lanes 5-8), and vTF7-3 infected cells transiently expressing recombinant BUN L and S segment proteins (Lanes 9-12). Cell extracts were harvested 20 hours post infection, and CAT protein concentration determined prior to assaying for CAT activity. No CAT activity was detected when the positive sense BUNSCAT(+) transcripts were transfected into mock infected CV-1 cells. In vTF7-3 infected cells, CAT activity was detected following transfection of BUNSCAT(+) RNA (Lanes 6, 7 and 8). Levels of CAT activity were markedly increased when BUN proteins were transiently expressed in vTF7-3 infected cells (Lanes 10, 11 and 12). **(B)** To confirm that BUNSCAT(+) transfected cell extracts truly exhibited a higher CAT activity than vTF7-3 infected samples, cell extracts from lanes 5-12 shown above, were diluted 1 in 10, and re-assayed. Lanes 5-12 on this TLC plate highlight the increase in CAT activity in cells expressing BUN proteins.

such that almost all the chloramphenicol was completely acetylated. The cell lysates from Lanes 5 to 12, were re-assayed, with just one tenth of the quantity of extract tested previously, to highlight the increase in CAT activity (Figure 6.4 (B)).

6.4 Attempted BUN virus rescue of BUNSCAT(+) (anti genome) and BUNSCAT (genome) transcripts.

It had been determined from previous experiments that both the BUNSCAT and the BUNSCAT(+) RNA transcripts can be recognised, encapsidated and expressed by recombinant BUN viral proteins (Chapter 3 and this chapter). Therefore, it might also be possible, during an ongoing infection of a cell by Bunyamwera virus, to introduce the BUN-like RNA transcripts into the cytoplasm of cells, so that these RNAs might be recognised by viral proteins, encapsidated, transcribed and possibly packaged into virus like particles. If so this would provide a means to study signals encoded by the reporter transcript for these events.

To investigate whether Bunyamwera virus dependent transcription of BUNSCAT and BUNSCAT(+) RNA was possible, CV-1 and C6/36 cells were either mock infected or infected with BUN virus at 10 pfu/cell for 3 hours before the inoculum was removed. Monolayers were washed once with OptiMEM before 3 μg of BUNSCAT or BUNSCAT(+) were transfected into the cell monolayers as described previously. Cells and supernates were harvested 24 hours later, and cell extracts assayed for CAT activity (Figure 6.5).

As shown in figure 6.5, no CAT activity was detected from mock infected or BUN infected CV-1 cell lysates when transfected with either transcript.

When C6/36 cells were either mock transfected or transfected with BUNSCAT, similarly, no CAT activity was detected. Transfection of BUN infected C6/36 cells with BUNSCAT(+) resulted in a small but detectable amount of CAT activity. This signal, however, was also present in C6/36 cells which had not been infected with helper virus although at a lower level (Figure 6.6A). Since the CAT signal from cells which had been infected with helper virus was slightly higher than cells which had no helper virus, it was decided to investigate whether the viral-like BUNSCAT(+) transcript might have been replicated and packaged into virus-like particles in the C6/36 cells. Therefore, the supernatant from the transfected cells the which had also been infected with helper virus, was used to infect fresh plates of C6/36 cells. The lysates from these cells were subsequently assayed for CAT activity, but no signal was detected, indicating that replication and packaging was unlikely to have occurred (Figure 6.6B).

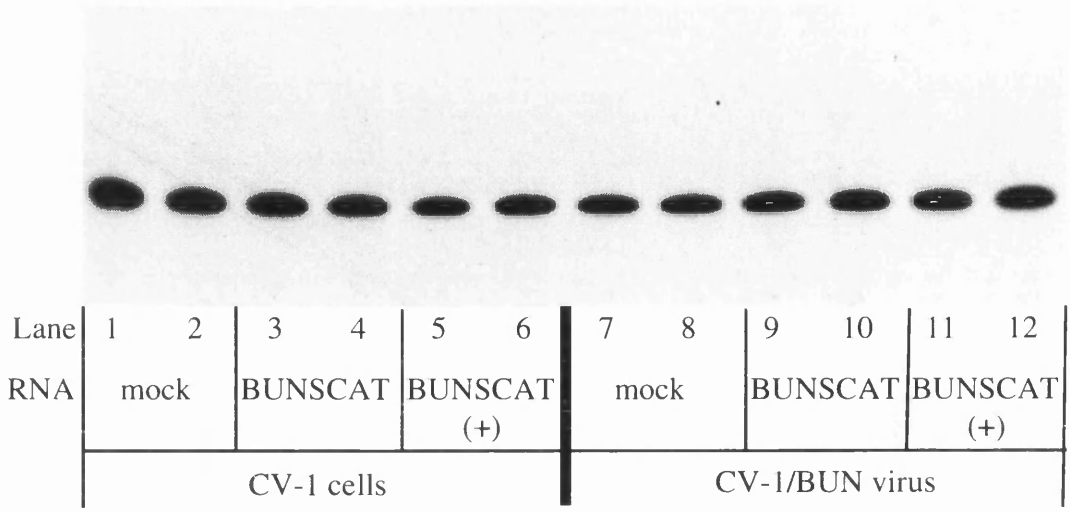
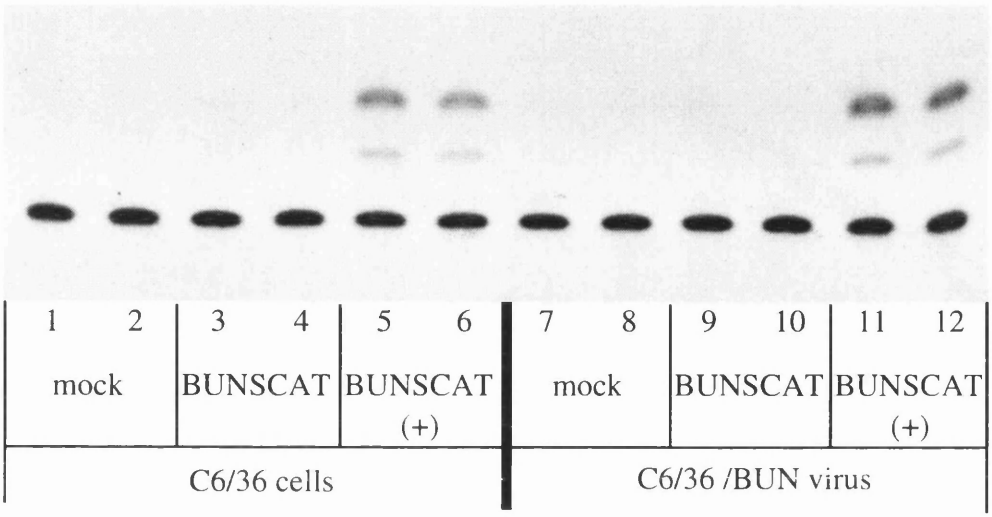
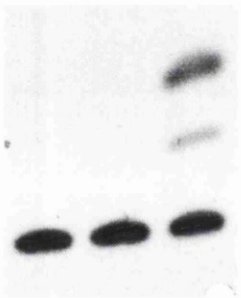


Figure 6.5 Expression of BUNSCAT and BUNSCAT(+) RNA during a BUN virus infection of CV-1 cells.

CV-1 cells were either mock infected or infected with BUN virus at 10 pfu/cell for 3 hours before the inoculum was removed. Monolayers were washed once with OptiMEM before 3 µg of BUNSCAT (Lanes 3, 4, 9 and 10) or BUNSCAT(+) (Lanes 5, 6, 11 and 12) were transfected into the cell monolayers as described previously. Cells and supernates were harvested after 24 hours, and cell extracts assayed for CAT activity.

(A)





(B) Lane 13 14 15

Figure 6.6 Expression of BUNSCAT and BUNSCAT(+) RNA during a BUN virus infection of C6/36 cells.

(A) C6/36 cells were either mock infected or infected with BUN virus at 10 pfu/cell for 3 hours before the inoculum was removed. Monolayers were washed once with OptiMEM before 3 μg of BUNSCAT (Lanes 3, 4, 9 and 10) or BUNSCAT(+) (Lanes 5, 6, 11 and 12) were transfected into the cell monolayers as described previously. Cells and supernates were harvested after 24 hours, and cell extracts assayed for CAT activity. (B) To determine if BUNSCAT(+) had been packaged into virion like particles, the media from dishes 5+6 as a control and the media from dishes 11+12 were used to infect fresh C6/36 cell, lanes 13 and 14 respectively. Cell lysates were harvested 24 hours postinfection and assayed for CAT activity (Lanes 13 and 14). Lane 15 is a repeat of lane 12.

That the BUNSCAT and BUNSCAT(+) transcripts were not recognised by the BUN helper virus proteins is perhaps not surprising, as similar results were found for other *Bunyaviridae* genus reporter systems (HTN virus, Schmaljohn (personal communication); RVF virus, Lopez and Bouloy, (personal communication); and PUU virus, (Shi and Elliott, personal communication). Luytjes *et al.* (1989), investigating influenza A virus, also reported that naked RNA transfected into cells which were subsequently infected with helper virus, failed to give rise to reporter activity.

However, the observation that transfection of the BUNSCAT(+) transcripts into C6/36 cells resulted in a small but detectable CAT signal, both in the presence and the absence of helper virus, is not easily explained. In theory, eukaryotic cells require a cap structure to be present at the 5' end of the mRNA transcript to achieve efficient translation. One hypothetical explanation might be that C6/36 cells may not have such a strict requirement for a capped structure, and that cap-independent translation may be possible from the BUNSCAT(+) RNA transcript.

In conclusion therefore, it was found that the BUNSCAT and BUNSCAT(+) constructs proved were not useful as reporter constructs for helper virus rescue studies.

6.5 Discussion.

In this chapter, a pBUNSCAT(+) reporter construct was described which was designed to allow the generation of positive sense RNA transcripts. These transcripts would represent the antigenomic version of BUNSCAT RNA (Chapter 3).

It was found that when BUNSCAT(+) transcripts were transfected into cells expressing the gene products of the BUN L and S segments, a high level of CAT activity was obtained. This CAT activity was elevated above the background CAT activity obtained from transfection of BUNSCAT(+) RNA into vTF7-3 infected cells.

In an authentic bunyavirus infection, the antigenomic (cRNA) transcripts are not naked, but are encapsidated in viral proteins to form nucleocapsid structures within the cell. Antigenomic transcripts, incorporated within nucleocapsids, represent an intermediate step in the replication process of the virus genome. Therefore, the elevated CAT activity detected in cells transiently expressing BUN L and S segment proteins suggests that the BUNSCAT(+) transcripts were recognised by these proteins, and interacted with them to form functional nucleocapsids: these nucleocapsids then served as templates for the transcription of a

negative sense BUNSCAT RNA transcript. The negative sense BUNSCAT RNA then acted as a template for transcription of BUNSCAT(+) mRNA, which was subsequently translated, giving rise to the observed elevated CAT activity. This series of events is described schematically in Figure 6.7.

A reporter system to study the replication events of Rift Valley fever (RVF) virus, first described by Lopez *et al.* (1995), was utilised by Prehaud *et al.* to try to demonstrate replication and expression of CAT activity from the reporter construct CAT-RVF-Sag(+) (Prehaud *et al.*, 1997). CAT-RVF-Sag(+) contained the antigenome of the RVF S segment, with a CAT gene in a positive sense orientation. This group were unable to demonstrate replication of the CAT-RVF-Sag(+) reporter construct (*i.e.* the positive sense reporter RNA), as CAT activity was not raised above background level when transfected into cells expressing RVF L and N proteins. They also noted that transfection of CAT-RVF-Sag(+) gave rise to detectable CAT activity in cells which had been previously infected with wild type vaccinia virus, but were not expressing the RVF proteins. This was interpreted to be a consequence of expression of vaccinia virus encoded proteins, which allowed translation of the transfected CAT-RVF-Sag(+) RNA transcripts.

The effect of vaccinia virus encoded proteins on positive sense reporter transcripts has also been noted previously by other groups, who have used recombinant vaccinia viruses both to drive expression of their reporter transcript (Stillman *et al.*, 1995), and to express recombinant proteins (Prehaud *et al.*, 1997). Stillman *et al.* have used the recombinant vaccinia virus-T7 (vTF7-3) in a reporter system to study synthetic VSV minigenomes. In this study, it was suggested that expression from antigenomic replicative intermediates (RI) may have occurred inadvertently as a consequence of vaccinia virus encoded proteins. An alternative explanation was also given for this expression: there may have been insufficient N protein in some cells to allow efficient encapsidation of the RI transcripts, thus enabling these transcripts to be translated.

Further experiments to study the antigenomic promoter of the BUN S segment were not pursued. This was largely due to the high level of background CAT activity detected in vTF7-3 infected cells. Thus, no attempt was made either to investigate the antigenomic promoter by mutational analysis, or to determine whether the bunyavirus NSs protein was necessary for the elevated CAT expression observed when transfected cells express L and S segment proteins.

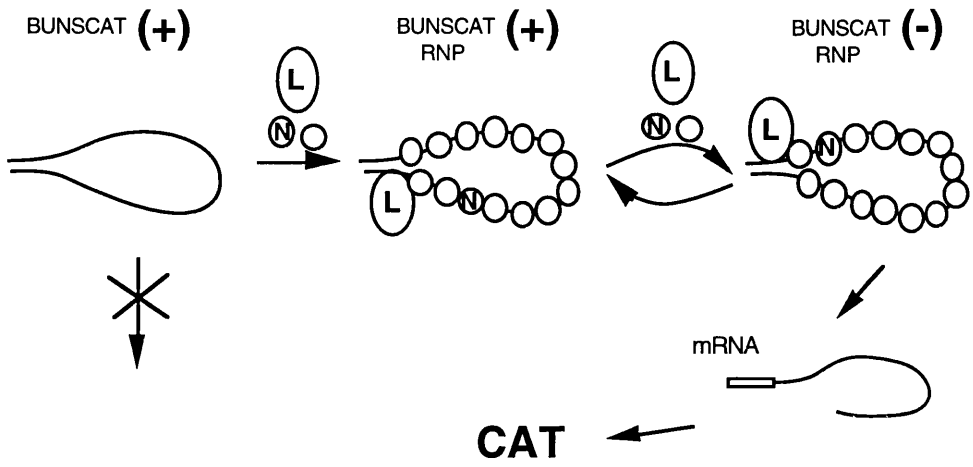


Figure 6.7 Proposed recognition and transcription of BUNSCAT(+) RNA.

The schematic describes the mechanism by which BUNSCAT(+) RNA may be recognised by recombinant BUN viral proteins, to give rise to a nucleocapsid structure, from which BUNSCAT (negative polarity) RNA can be transcribed. The resulting encapsidated negative sense RNA transcript then can act as a template for the transcription of a message sense transcript, to result in translation of the CAT gene.

6.5 Future Work.

Due to the background CAT activity discussed previously, it would be difficult to use the reporter system as it stands to study the promoter and the viral proteins involved in the replication of the antigenomic BUNSCAT(+) transcript to genomic sense (BUNSCAT) transcripts. To circumvent this problem, expression of BUN viral proteins could be achieved via a vaccinia-independent system. It may be possible, for example, to establish a stably transformed cell line which constitutively expresses T7 RNA polymerase, or alternatively, a stably transformed cell line expressing the BUN L and S segment gene products would also remove the requirement for vTF7-3. Semliki Forest Virus vectors (Gibco), constructed to allow cytoplasmic expression, have also been successfully utilised by others to express recombinant viral proteins *in trans* (Rolls *et al.*, 1994).

Chapter 7.

7.0 Effect of modifications to the termini of BUNSCAT RNA.

7.0 Introduction.

It has been reviewed previously in this thesis that the terminal sequences of bunyavirus RNA segments are conserved and complementary, and that these sequences are important for recognition by the viral polymerase and encapsidation by N protein (Kolakofsky and Hacker, 1991). Presented in this chapter is a series of experiments designed to investigate the effect of deletion or addition of nucleotides at the extreme 3' and 5' termini of the BUNSCAT reporter RNA.

7.1 Production of BUNSCAT RNA transcripts with modified termini.

To produce BUNSCAT reporter RNA containing the desired deletions or extensions to the 3' terminus, pBUNSCAT plasmid DNA was digested with suitable restriction enzymes as described in Figure 7.1, and subsequently used in *in-vitro* transcription reactions. Linearisation of the pBUNSCAT template with *Bbs*I produced a transcript which had wild type 5' and 3' termini, *i.e.* termini in which the full complement of the eleven conserved nucleotides were present. Linearisation of the pBUNSCAT template with *Xba*I produced a BUNSCAT RNA in which the 3' terminus had an additional 11 nucleotides at the extreme end. Similarly, digestion with *Hind*III produced an RNA with an additional 35 nucleotides whereas digestion with *Hga*I produced an RNA with 5 nucleotides deleted from the 3' terminus.

The addition of nucleotides at the 5' end was achieved by transcribing the BUNSCAT RNA from the transcription template, pT7BUNSCAT-Ribo, instead of pBUNSCAT. RNA transcribed from this construct is identical to that from pBUNSCAT except that it contains 2 additional G residues at the 5' end before the conserved terminal BUN sequence, and has a 2',3' cyclic phosphate at the 3' terminus generated by ribozyme (Ribo) cleavage (Kuo *et al.*, 1988). Transcripts from this template were synthesised *in-vitro* under standard conditions, and the DNA template was removed by DNaseI digestion. Agarose gel electrophoretic analysis showed that about 50% of the primary transcripts were processed by self cleavage by the ribozyme (Figure 7.2), however, it was not possible to purify, and thereby quantify,

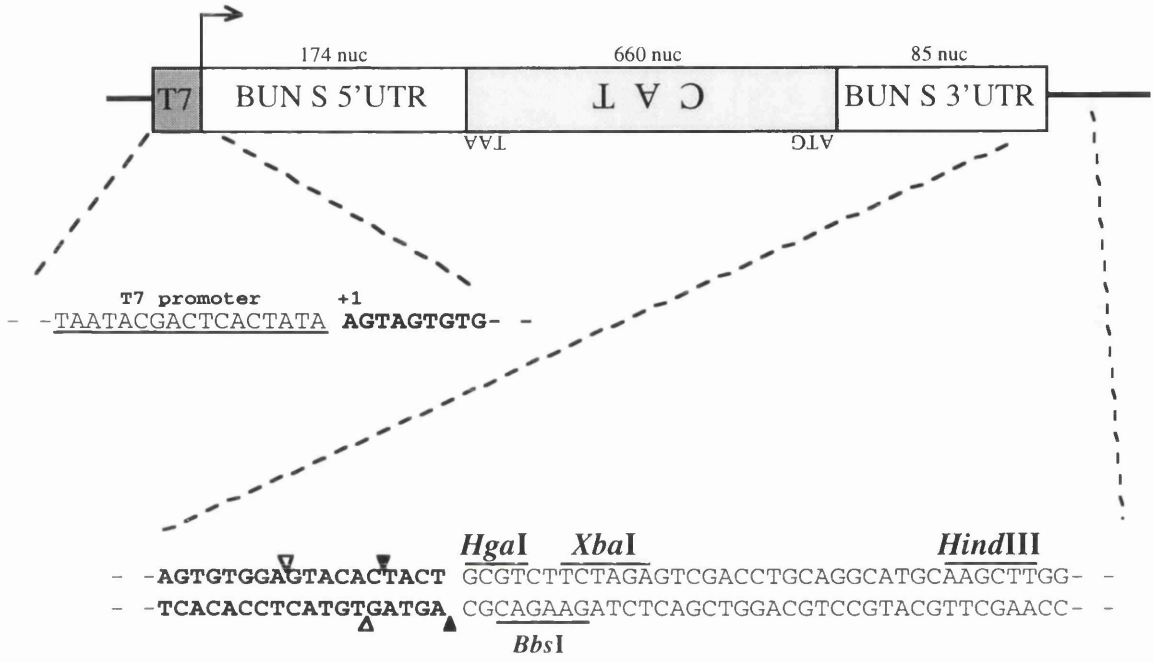


Figure 7.1 Restriction digestion of pBUNSCAT to result in transcripts with modified 3' termini.

pBUNSCAT plasmid DNA was digested with suitable restriction enzymes (these enzyme sites are indicate in the diagram above), and subsequently used in *in-vitro* transcription reactions to produce run-off BUNSCAT RNA transcripts with modified termini. Linearisation of the pBUNSCAT DNA template with *Bbs*I (shown as black triangles) produced a transcript which had the exact wild type 5' and 3' termini. Linearisation of the pBUNSCAT template with *Hga*I (shown as open triangles), *Xba*I or *Hind*III, produced run-off transcripts with deletions or additions at the 3' termini of -5, +11 and +35 bases respectively.

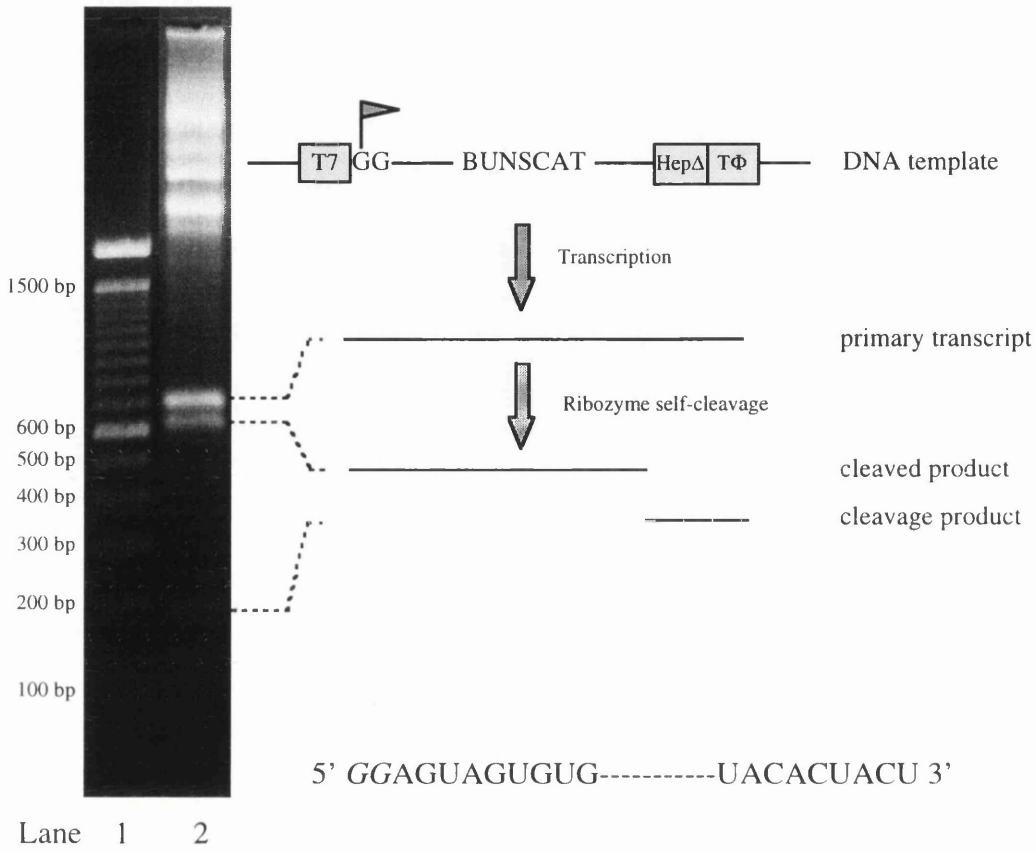


Figure 7.2 Mechanism of ribozyme cleavage to release BUNSCAT plus two extra 5' G residues.

The above diagram describes the self cleavage event, mediated by the Hepatitis delta ribozyme (HepΔ) sequence present at the 3' terminus of the primary transcript, which results in the release of a BUNSCAT RNA with two additional G residues at the 5' terminus. One hundred basepair (bp) ladder markers are shown in lane 1. In lane 2 the primary transcript is evident, running between the 700 and the 800 bp markers, and the self cleaved BUNSCAT RNA is present as the lower band, which runs between the 600 and the 700 bp markers. The ribozyme fragment is also visible on the gel, running around the 200 bp marker.

the fraction of correctly processed RNA. Therefore, the RNA transfected into cells contained a mixture of processed and unprocessed transcripts.

7.2 Effect of modified template RNAs in the reporter assay.

Run off BUNSCAT RNA transcripts with modified ends, generated as described above, were transfected into the reporter system using the same protocol as described earlier (Section 3.1). CV-1 cells, transiently expressing the viral proteins of the BUN S and L segments, were transfected with equal amounts of each transcript (as determined by incorporation, Methods 2.4.2). The cells were harvested 20 hours post transfection and the extracts collected as described previously (Section 3.1). The protein concentration in each cell extract was determined and equal amounts were used for determination of CAT activity. The results of which are shown in Figure 7.3.

It can be seen from Figure 7.3 that CAT activity was obtained from BUNSCAT RNAs which had additional nucleotides at either the 5' terminus or the 3' terminus. Lanes 1 and 2 show the CAT signal obtained from BUNSCAT RNA with 2 additional nucleotides at the 5' terminus, which was comparable to wild type BUNSCAT. It should be noted that since it was not possible to quantify the amount of processed RNA produced by the self cleavage reaction, it was also not possible to estimate the efficiency of CAT production from pT7riboBUNSCAT(-) RNA compared to BUNSCAT RNA. Essentially, however, this is irrelevant, and the fact that a high CAT signal was obtained from this construct, regardless of a possible difference in CAT production between this and the wild type BUNSCAT transcript, indicates that the addition of 2 G residues to the 5' UTR produced no gross change in the ability of the polymerase to recognise and interact with this modified terminus.

Lanes 7 and 8 show the CAT signal obtained from BUNSCAT RNA with 11 additional nucleotides at the 3' terminus, and Lanes 9 and 10 show the signal obtained from BUNSCAT RNA with 35 additional nucleotides at the 3' terminus. The CAT signal obtained from RNAs with 11 and 35 additional nucleotides at the 3' terminus, although lower than wild type BUNSCAT RNA was still detectable. However, deletion of 5 nucleotides from the 3' terminus effectively abolished CAT activity (Lanes 5 and 6).

That CAT activity was obtained from BUNSCAT RNA with 2 additional nucleotides at the 5' terminus indicates that the presence of 2 G residues 5' to the conserved terminal BUN sequence still allowed recognition by the transcriptase complex. Similarly, the results obtained from BUNSCAT RNAs with additional nucleotides at the 3' terminus suggest that the RNA can still be recognised by the transcriptase complex and initiate transcription,

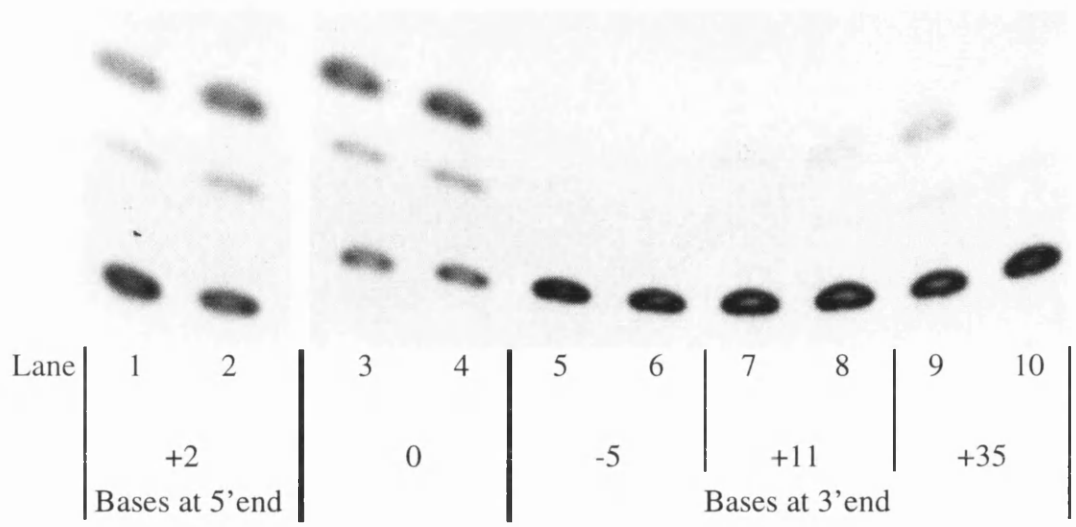


Figure 7.3 The effect of a deletion and extensions to the BUNSCAT termini on CAT activity.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of the BUNSCAT RNAs with modified termini and BUNSCAT RNA with authentic (i.e. wild type) termini, included as a control (lanes 3 and 4). Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration. Equivalent amounts of protein from each cell extract were assayed for CAT activity. Lanes 1 and 2 show the CAT signal obtained from transfection of BUNSCAT RNA which had an additional 2 nucleotides at the 5' terminus. Lanes 5 and 6 show the CAT signal obtained from transfection of BUNSCAT RNA with 5 nucleotides deleted from the 3' terminus, Lanes 7 and 8, BUNSCAT RNA with 11 nucleotides added at the 3' terminus, and Lanes 9 and 10, BUNSCAT RNA with 35 nucleotides added at the 3' terminus. Lanes 3 and 4 represent the CAT signal from BUNSCAT with authentic termini as a control.

although the efficiency of the interaction may be reduced by the presence of the extra sequence. Therefore, it appears that the viral polymerase and the N protein can recognise and can interact, at least to some extent, with specific sequences present in the 5' and 3' termini, whether these signals are presented at the extreme termini, or whether they are internal to other sequence.

The deletion of 5 nucleotides at the 3' terminus abolished CAT activity, suggesting that although it is possible to add extra nucleotides on to the 5' and 3' termini, it may not be possible to delete nucleotides from these regions, presumably as this would remove part of the sequences recognised by the viral proteins.

Further experiments, described below, were performed to determine if deletions of less than 5 nucleotides from the 3' terminus would result in a functional RNA template, and to investigate the effect of deletion of nucleotides from the 5' terminus.

7.3 Construction of terminal deletion mutants.

The introduction of terminal deletions to the 3' and 5' termini of BUNSCAT transcripts was performed in a stepwise fashion by oligonucleotide directed PCR mutagenesis. A series of oligonucleotides were synthesised as primers to introduce deletions to the BUNSCAT 3' and 5' termini by PCR mutagenesis (Figure 7.4).

The pBUNSCAT 3' terminal deletions were constructed by PCR, using the 3' deletion primers ED.37, 38, 39 (Figure 7.4 A) and an unmutated 5' terminal primer (shown as wt sequence, Figure 7.4 B) as the distal primer. The pBUNSCAT 5' terminal deletion mutants were constructed using the 5' terminal deletion primers ED.41, 42, 43 (Figure 7.4 B) and an unmutated 3' terminal primer (shown as wt sequence, Figure 7.3 A) as the distal primer. A similar PCR reaction was performed, using primers ED.37 and ED.41, to construct a pBUNSCAT clone with a deletion of 1 nucleotide at both the 3' and 5' termini.

The resulting PCR fragments were digested with restriction enzymes *Sac*I and *Xba*I and then ligated into *Sac*I and *Xba*I digested pUC118. Clones positive for a insert were sequenced into the CAT open reading frame to confirm the sequences of the T7 promoter, *Bbs*I site, to check that no spurious mutations had occurred during PCR, and to confirm that the desired deletion to the 3' or 5' BUNSCAT termini was present.

The terminal deletion clones were denoted pBUNSCAT(37)(3'-1), pBUNSCAT(38)(3'-2), pBUNSCAT(39)(3'-3), pBUNSCAT(41)(5'-1), pBUNSCAT(42)(5'-2), pBUNSCAT(43)-(5'-3') and pBUNSCAT (3'-1,5'-1)

7.4 Use of BUNSCAT terminal deletion RNA transcripts in the reporter assay.

The RNAs transcribed from the pBUNSCAT terminal deletion clones were applied to the reporter system using the same protocol as described earlier (Section 3.1). CV-1 cells, transiently expressing the BUN viral proteins of the BUN S and L segments, were transfected with equal amounts of each transcript. The cells were harvested 20 hours post transfection and the extracts collected as described previously (Section 3.1). The amount of cell extract assayed was adjusted for protein concentration before assaying for CAT activity and quantification (Figure 7.5).

From the results shown in Figure 7.5 (Lanes 13 and 14), it can be seen that transfection of BUNSCAT RNA with 1 nucleotide deleted from the 3' terminus, as in construct 3(-1), resulted in a level of CAT activity which was comparable to wild type BUNSCAT RNA. Quantification of this CAT signal (Figure 7.9) by phosphorimager analysis showed that it represented 98% of the activity obtained from transfection of BUNSCAT RNA. However, the CAT signal obtained from BUNSCAT RNA with 2 nucleotides deleted from the 3' terminus, as in construct 3'(-2), was drastically reduced (Figure 7.5, Lanes 11 and 12), and when quantified (Figure 7.9), it was found to represent only 6% of the signal obtained from wild type BUNSCAT RNA. Similarly, deletion of 3 nucleotides from the 3' terminus, as in construct 3'(-3), also drastically reduced the CAT signal (Figure 7.5, Lanes 9 and 10). This signal was found to represent less than 3% of that obtained from the wild type BUNSCAT RNA (Figure 7.9).

Deletion of nucleotides from the 5' terminus was found to be less well tolerated than the 3' deletions. Removal of 1 nucleotide, as in construct 5'(-1), resulted in a CAT signal which was only 8% of the wild type BUNSCAT signal (Figure 7.5, Lanes 3 and 4, and Figure 7.5). Similarly, removal of 2 or 3 nucleotides (Figure 7.5, Lanes 5 and 6, and Lanes 7 and 8, respectively), as in constructs 5'(-2) and 5'(-3), resulted in CAT activity which was less than 3% of wild type BUNSCAT RNA CAT activity.

The removal of 1 nucleotide from both the 3' and the 5' termini also resulted in a drastic loss of CAT activity (Figure 7.6), and the signal from this construct, construct 3'(-1)5'(-1), was quantified to represent 8% of the wild type BUNSCAT RNA signal (Figure 7.9). This is

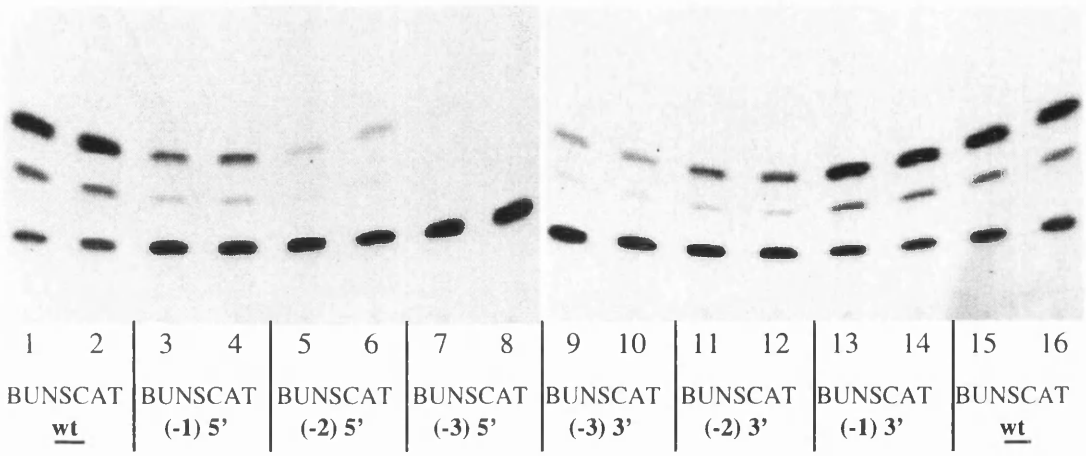


Figure 7.5 The effect of terminal deletions to the BUNSCAT termini on CAT activity.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of the BUNSCAT RNAs with modified termini and BUNSCAT RNA with authentic (*i.e.* wild type) termini included as a control (lanes 1 and 2 and 15 and 16). Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration. Equivalent amounts of protein from each cell extract were assayed for CAT activity.

Lanes (1 and 2) and (15 and 16) show the CAT signal obtained from transfection of wild type BUNSCAT RNA, and Lanes 3 and 4 show the CAT signal obtained from BUNSCAT RNA with 1 nucleotide deleted from the 5' terminus. Lanes 5 and 6 show the CAT signal from BUNSCAT RNA with 2 nucleotides deleted, and Lanes 7 and 8, BUNSCAT RNA with 3 nucleotides deleted at the 5' terminus. Lanes 9 and 10, Lanes 11 and 12, and Lanes 13 and 14 show the CAT signal from BUNSCAT RNA with deletions at the 3' terminus of -3, -2 and -1, respectively. The resulting levels of CAT activity are summerised in Figure 7.9

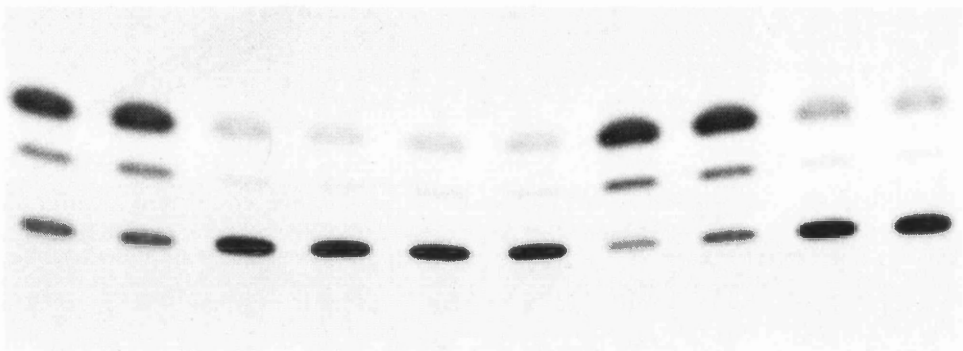


Figure 7.6 Reporter gene expression from BUNSCAT with terminal deletions.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of the BUNSCAT RNAs with modified termini and BUNSCAT RNA with authentic (*i.e.* wild type) termini, included as a control (lanes 1 and 2). Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration. Equivalent amounts of protein from each cell extract were assayed for CAT activity. Lanes 3 and 4 show the CAT signal obtained from transfection of BUNSCAT RNA which possessed a deletion of 1 nucleotide at the 3' terminus and also 1 nucleotide at the 5' terminus. Lanes 5 and 6 contain extracts from cells transfected with a different clone of this same construct. The CAT signals obtained from transfection of BUNSCAT RNAs which possessed wild type 3' termini, and which had a deletion of 1 nucleotide at the 3' terminus or the 5' terminus, are shown in Lanes 1 and 2, Lanes 7 and 8, and Lanes 9 and 10, respectively.

comparable to the removal of 1 nucleotide from only the 5' terminus. Furthermore, as the complementarity of sequence, and flushness of the termini was restored in this construct, this result also demonstrates that the reduced CAT activity obtained from the BUNSCAT RNA with a deletion of 1 nucleotide from the 5' terminus was not due to the inhibitory presence of a 3' overhang, but was truly the effect of deletion of the 5' nucleotide.

7.5 Can T7 RNA polymerase initiation transcription with a uridine?

In a previous study which characterised mRNA transcripts from a large number of viral RNA polymerases by direct mRNA sequencing, it was found that mRNA transcription initiation always occurred with a purine (G or A) and not with a pyrimidine (U or C), (Banerjee, 1980). This study suggests the possibility that all viral RNA polymerases initiate mRNA transcription with a purine.

After analysis of the pBUNSCAT 5' terminal deletion clones CAT results (Figure 7.5), the question arose as to whether T7 RNA polymerase (a DNA dependent RNA polymerase) can initiate transcription at the +1 residue of BUNSCAT 5'(-2) transcript which initiates with a uridine (U) (Figure 7.7B). It has been shown previously that T7 RNA polymerase initiates transcription of its own viral genes with a purine, either a G or a A, as the first encoded nucleotide (Dunn and Studier, 1983). However, evidence that T7 RNA polymerase may be able initiate transcription with a pyrimidine was reported in a paper by Chapman and Burgess, (1987). In this study a series of constructs was built which contained single point mutations within the a full length T7 promoter. Of the mutants characterised, one contained a G to T substitution at nucleotide position +1, therefore transcription from this construct would theoretically initiate with a U +1. The authors found that this mutant gave approximately 80% transcription activity compared to the control sequence. However, this study examined only the quantity of transcripts produced, and no attempt was made to determine the quality or the terminal 5' nucleotides of the mutant transcripts. It has previously been reported that T7 RNA polymerase run-off transcripts can demonstrate a degree of heterogeneity of the 5' termini, depending on the sequence downstream of the T7 promoter (Pleiss *et al.*, 1998), suggesting that T7 RNA polymerase can initiate transcription at various nucleotides downstream of its promoter. Therefore, it is possible that transcription from the position +1 mutant in Chapman and Burgess's study was actually initiated from the purine at position +2 (*i.e.* G).

Clearly from the literature, the question of whether T7 RNA polymerase can initiate transcription at position +1 with a pyrimidine has not yet fully been resolved, however, if the requirement is in fact for a purine, then this has implications for the pBUNSCAT 5'(-2)

(A) BUNSCAT

```
                        +1 ⇒
                        AGU AGU GUG---3'   RNA
ATTATGCTGAGTGATAT TCA TCA CAC---5'   DNA
                 -1
```

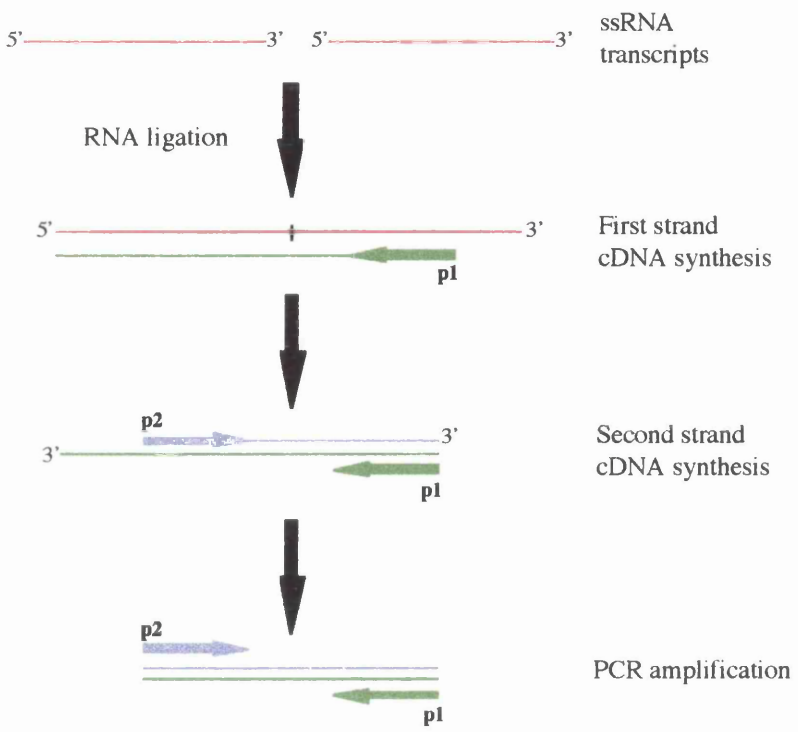
(B) BUNSCAT 5'(-2)

```
                        +1  +2 ⇒
                        U AGU GUG---3'   RNA
ATTATGCTGAGTGATAT A TCA CAC---5'   DNA
                 -1
```

Figure 7.7 T7 RNA polymerase transcription initiation.

The above figure demonstrates how T7 RNA polymerase may initiate transcription down stream of the T7 promoter sequence (underlined) at position +1 with respect to the promoter. In (A) BUNSCAT transcription initiates with a A residue at position +1, and in (B) deletion mutant BUNSCAT 5'(-2) transcripts may initiate with an A at position +2 rather than with a U residue at position +1.

(A)



(B)

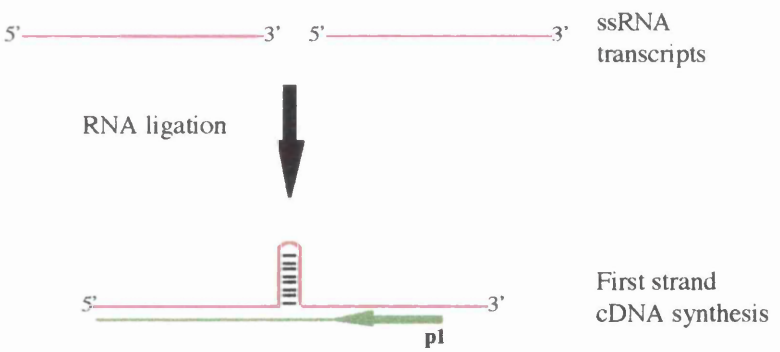


Figure 7.8 Characterisation of 5' terminal nucleotides.

Figure (A) describes schematically the methods utilised to characterise the terminal 5' nucleotides of BUNSCAT transcripts, where primer p1 is nucleotides 646 to 660 of the CAT gene, and p2 is the compliment of nucleotides 15 to 29 of the CAT gene. (B) The ligation of the template RNA 3' and 5' termini may allow formation of a secondary structure due to the high degree of complimentarity of the sequence, which is deleted during first strand synthesis.

Clone		BUNSCAT Terminal sequences		relative CAT activity
wt	5'	AGUAGUGUGCUCC------//-------GGUGAUCACUACU	3'	100%
3'(-1)	5'	AGUAGUGUGCUCC------//-------GGUGAUCACUAC	3'	98%
3'(-2)	5'	AGUAGUGUGCUCC------//-------GGUGAUCACUA	3'	6%
3'(-3)	5'	AGUAGUGUGCUCC------//-------GGUGAUCACU	3'	<3%
wt	5'	AGUAGUGUGCUCC------//-------GGUGAUCACUACU	3'	100%
5'(-1)	5'	GUAGUGUGCUCC------//-------GGUGAUCACUACU	3'	8%
5'(-2)	5'	UAGUGUGCUCC------//-------GGUGAUCACUACU	3'	**<3%**
5'(-3)	5'	AGUGUGCUCC------//-------GGUGAUCACUACU	3'	<3%
3'(-1), 5'(-1)	5'	GUAGUGUGCUCC------//-------GGUGAUCACUAC	3'	8%

Figure 7.9 Summary of the CAT activity results obtained from the BUNSCAT terminal deletion mutants.

The above diagram summarises the results obtained from the BUNSCAT terminal deletion mutants. The mutants are denoted as follows: clone 3'(-1), for example, possesses a 3' terminus which has a deletion of 1 nucleotide, and has a wild type 5' terminus, and clone 5'(-1) possesses a 5' terminus which has a deletion of 1 nucleotide, and has a wild type 3' terminus. Clone 3'(-1) 5'(-1) has 1 nucleotide deleted at both the 5' and the 3' termini. The sequence of the termini of each mutant is also shown in the diagram. The % CAT activity from each of the modified BUNSCAT RNAs compared to wild type BUNSCAT RNA is listed to the right of the figure. The CAT activity from transcript BUNSCAT 5'(-2) is shown in bold and underlined as the validity of this result is discussed in section 7.5.

clone used in the study presented here. It is possible that transcription from this clone may not initiate with a pyrimidine (U) at position +1 but with a purine (A) at positions +2 with respect to the T7 RNA promoter (Figure 7.7B), rendering it impossible under the present system to characterise the effect of a two nucleotide deletion at the 5' terminus of BUNSCAT RNA.

In an attempt to determine the nature of the nucleotides at the 5' terminus of transcripts from the pBUNSCAT 5'(-2) clone, RNA ligation of the resulting run-off transcripts was performed (methods 2.4.6), followed by reverse transcription (methods 2.4.7) and PCR with the appropriate primers. Figure 7.8A describes schematically the methods used in this protocol. As a control, BUNSCAT transcripts was also treated in this manner.

Various attempts were made to analyse these transcripts in this way, but without success, since sequencing of the resulting cDNAs showed that all contained deletions spanning the predicted region of ligation between the 3' and 5' termini of RNA transcripts of approximately 30 to 50 nucleotides. It is possible that deletion of these sequences was due to the reverse transcriptase transcribing over this region due to secondary structures, formed between the complementary ligated 3' and 5' termini (Figure 7.8 (B)). In an attempt to inhibit the formation of secondary structures, DMSO was included in the ligation reaction and subsequent reverse transcription reaction up to a final concentration of 20% v/v (Romaniuk and Uhlenbeck, 1983), but without improvement. No further attempts were performed to determine the 5' nucleotide of BUNSCAT 5'(-2) RNA transcripts.

In other studies characterising the promoter regions of influenza virus segments, the problem of RNA transcription initiation was either not a consideration, as in the *in-vivo* RNA pol-I catalysed transcription system used by Flick *et al.* (1996), or circumvented by the chemical synthesis of short RNA oligonucleotides as templates as used in the study by Fador *et al.* (1994).

As it was not possible to determine if the BUNSCAT 5' (-2) transcript contained a U residue at its 5' terminus, the results from the pBUNSCAT 5'(-2) clone are not valid at present until further characterisation of the 5' terminal can be achieved. In future work, this could possibly be done using a synthesised short ^{32}P-UTP labeled transcript containing only one uridine at position +1.

7.6 Discussion.

The first 11 nucleotides of the 5' and 3' terminal sequences of bunyavirus RNA segments are conserved and complementary, both between genomic segments and between different bunyaviruses. The aim of the experiments presented in this chapter was to investigate the effect of adding or removing nucleotides from the extreme ends of these termini.

It was found that the addition of nucleotides at the end of either the 5' or the 3' termini of BUNSCAT reporter RNA resulted in a transcript which gave detectable CAT activity in the reporter assay. This result suggests that the signals encoded within the conserved terminal BUN sequence were recognised by the transcriptase complex, and that these additional nucleotides were tolerated because they did not completely abolish viral polymerase recognition and interaction. The viral polymerase, therefore, can probably recognise and interact, at least to some extent, with specific sequences which are present in the 5' and 3' termini, whether these signals are presented at the extreme termini, or whether they are internal to other sequence. This result is in accordance with evidence offered by Prehaud *et al* (1997), that addition of 4 nucleotides to the 3' terminus of a reporter RNA based on the Rift Valley fever virus S segment, exhibited measurable CAT activity. This suggested that the polymerase could still recognise and interact with the promoter at the 3' UTR, without it being presented at the extreme end of the terminus. However, this study also reported that an addition of 16 nucleotides to the 3' terminus abolished CAT activity from the reporter RNA, a result which differs slightly from the finding here, that an additional 35 nucleotides could be added to the 3' terminus of the BUNSCAT RNA without complete disruption of function.

The specific mechanism of how the polymerase recognises and interacts with the termini, and initiates transcription of the bunyavirus segments, is not understood. However, on the basis of recent work on influenza virus it has been proposed that the it is the 5' terminus that is initially recognised by the polymerase complex, and then the 3' terminus binds to initiate transcription. Li *et al.* (1998) have offered evidence in support of this model, the mechanics of which are described in Figure 7.10 (A). Binding of the 5' vRNA terminus to the PB1 component of the influenza virus transcriptase complex induces a conformational change in this protein which then allows the PB2 component to bind the capped 5' end of host mRNA. Once the capped mRNA is bound, PB1 can then bind the 3' terminus of the vRNA, and it is the binding of the 3' terminus which activates enzymic activity of the complex to result in cleavage of the cap-group and 10-13 nucleotides from the host mRNA. This capped RNA fragment then serves as a primer for mRNA synthesis by the PB1 protein. Throughout chain elongation, the polymerase complex tethers the 5'-end vRNA sequence, and as a result it is unable to transcribe through the bound region at the extreme 5' vRNA terminus because of

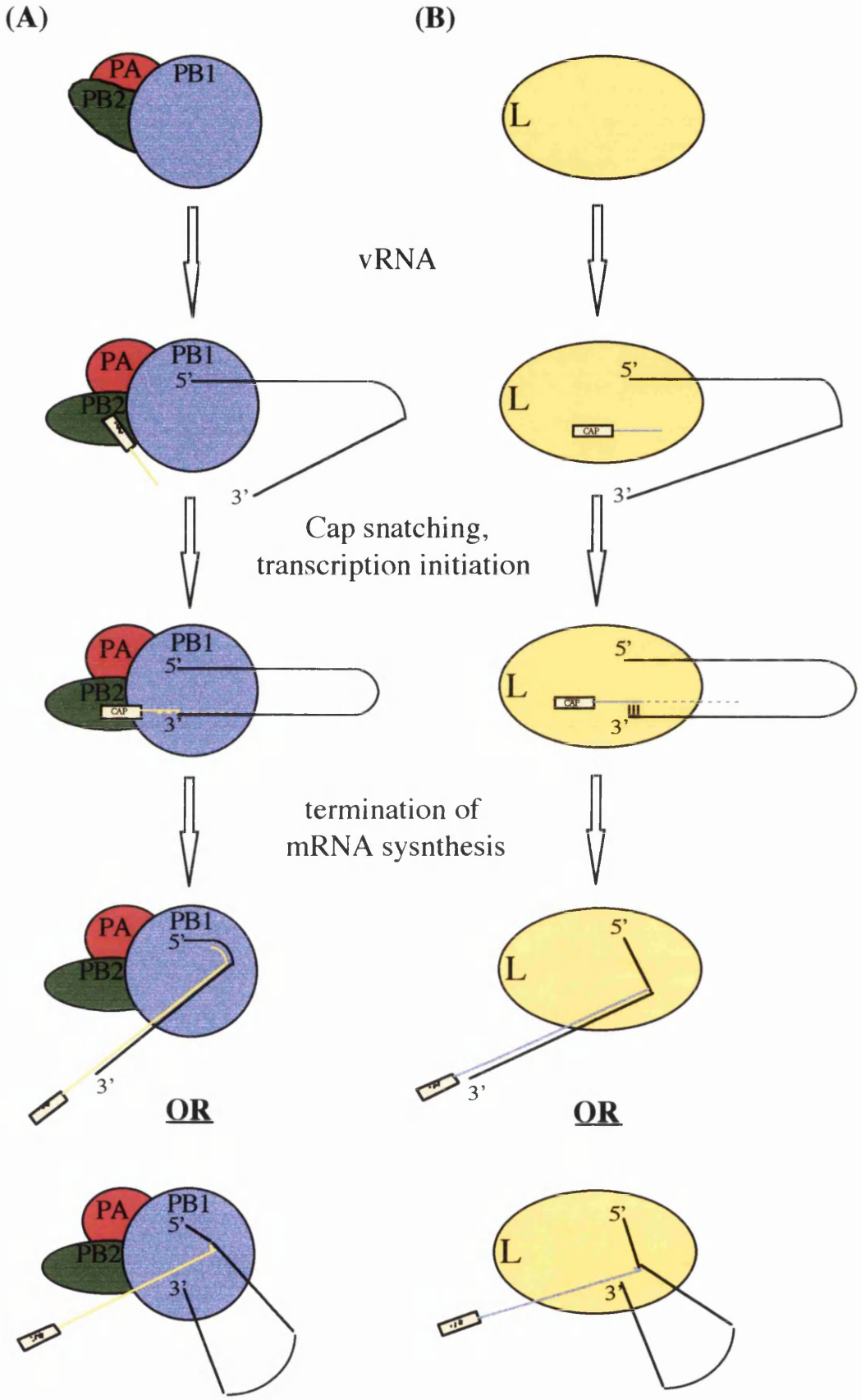


Figure 7.10 Proposed models for the initiation of influenza virus and bunyavirus mRNA transcription.

Figure 7.10 Proposed models for the initiation of influenza virus and bunyavirus mRNA transcription.

(A) Model for the proposed mechanism for RNA transcription initiation in influenza virus. The 5' terminus is initially recognised by the polymerase complex, and the 3' terminus consequently binds to initiate transcription. Binding of the 5' vRNA terminus to PB1 induces a conformational change in this protein which allows PB2 to bind 5'-capped host mRNA. PB1 can then bind the 3' terminus of the vRNA, which activates enzymic activity of the complex to result in cleavage of the cap-group and 10-13 nucleotides from the host derived mRNA. The capped RNA fragment serves as a primer for mRNA synthesis by the PB1 protein. Throughout chain elongation, the polymerase complex tethers the 5'-end vRNA sequence, and is unable to transcribe through this bound sequence region at the extreme 5' terminus due to steric hindrance. This leads to reiterative copying of the poly-U tract, resulting in the addition of a poly(A) tail to the mRNA transcript.

(B) This describes a similar model for mRNA initiation by the BUN L protein where the 5' terminus of the BUN genomic segment is tethered by the BUN L protein. This induces a conformational change in the polymerase which allows it to bind host derived capped mRNA. This facilitates an interaction of the polymerase with the 3' terminus of the segment, thereby allowing the polymerase to cleave off a capped leader sequence from the host mRNA (via endonuclease activity), to prime transcription initiation via the prime-and-realign mechanism. As with the influenza virus model, the L protein transcribes along the template, whilst constantly tethering the 5' terminus and is consequently unable to transcribe through the bound 5' terminal sequence region due to steric hindrance. However, unlike the influenza virus polymerase, which stutters at the poly-U tract and results in the addition of a poly(A) tail, the bunyavirus L protein cleaves the nascent transcript, possibly via the same endonuclease activity used for cap-snatching, to result in termination of transcription.

steric hindrance. This leads to reiterative copying of the poly-U tract, resulting in the addition of a poly(A) tail to the mRNA transcript.
A similar model can be imagined for mRNA initiation by the BUN L protein to which all viral encoded enzymic functions have been attributed (Figure 7.10 B). It is possible that the 5' terminus of the BUN genomic segment interacts with the BUN L protein, and in doing so induces a conformational change in the polymerase which allows it to bind host capped mRNA. This may facilitate an interaction of the polymerase with the 3' terminus of the segment, thereby allowing the polymerase to cleave off a capped leader sequence from the host mRNA (via endonuclease activity), and use it to prime transcription initiation via the prime-and-realign mechanism proposed by Garcin *et al.* (1995a). In accordance with the influenza virus model, the L protein would transcribe the template, whilst constantly tethering the 5' terminus. As a result, the L protein would be unable to transcribe through the 5' vRNA terminal sequence region to which it is bound because of steric hindrance. However, unlike the influenza virus polymerase, which stutters at the poly-U tract and results in the addition of a poly(A) tail, the bunyavirus L protein might cleave the nascent transcript, possibly via the same endonuclease activity used for cap-snatching at a transcription termination signal which is upstream of the 5' end of the template, to result in termination of transcription .

The results of the experiments presented in this chapter would lend support to such a model for bunyavirus transcription, where the 5' terminus is involved in recognition by the L protein, and the 3' is involved in transcription initiation.

It was found that deletion of any number of nucleotides from the 5' UTR was not tolerated, indicating that the terminal conserved nucleotides of the 5' UTR are absolutely essential for interaction of some description with the polymerase. Deletion of one nucleotide from the 3' terminus was better tolerated, resulting in CAT activity that was equivalent to "wild type" BUNSCAT RNA. However, a deletion of 2 nucleotides drastically reduced CAT activity. This is interesting, and can be explained if we consider that the 3' UTR is involved in transcription initiation.

Analysis of *Bunyaviridae* mRNA 5' leader sequences has indicated a preference for certain bases at positions -1 to -3. These nucleotides have been found to be a reiteration of the terminal nucleotides of the genomic template (Chapter 1.4.4). On the basis of this observation, a model has been proposed which describes a mechanism for *Bunyaviridae* transcription initiation. The mechanism, known as "prime-and-realign", is shown in Figure 7.11, and is based on the evidence offered by Garcin *et al* .(1995a). According to this model, the viral polymerase primes transcription with a cap-snatched primer ending in a G residue (the endonuclease activity has a bias for cleavage after a G), and initiates internally

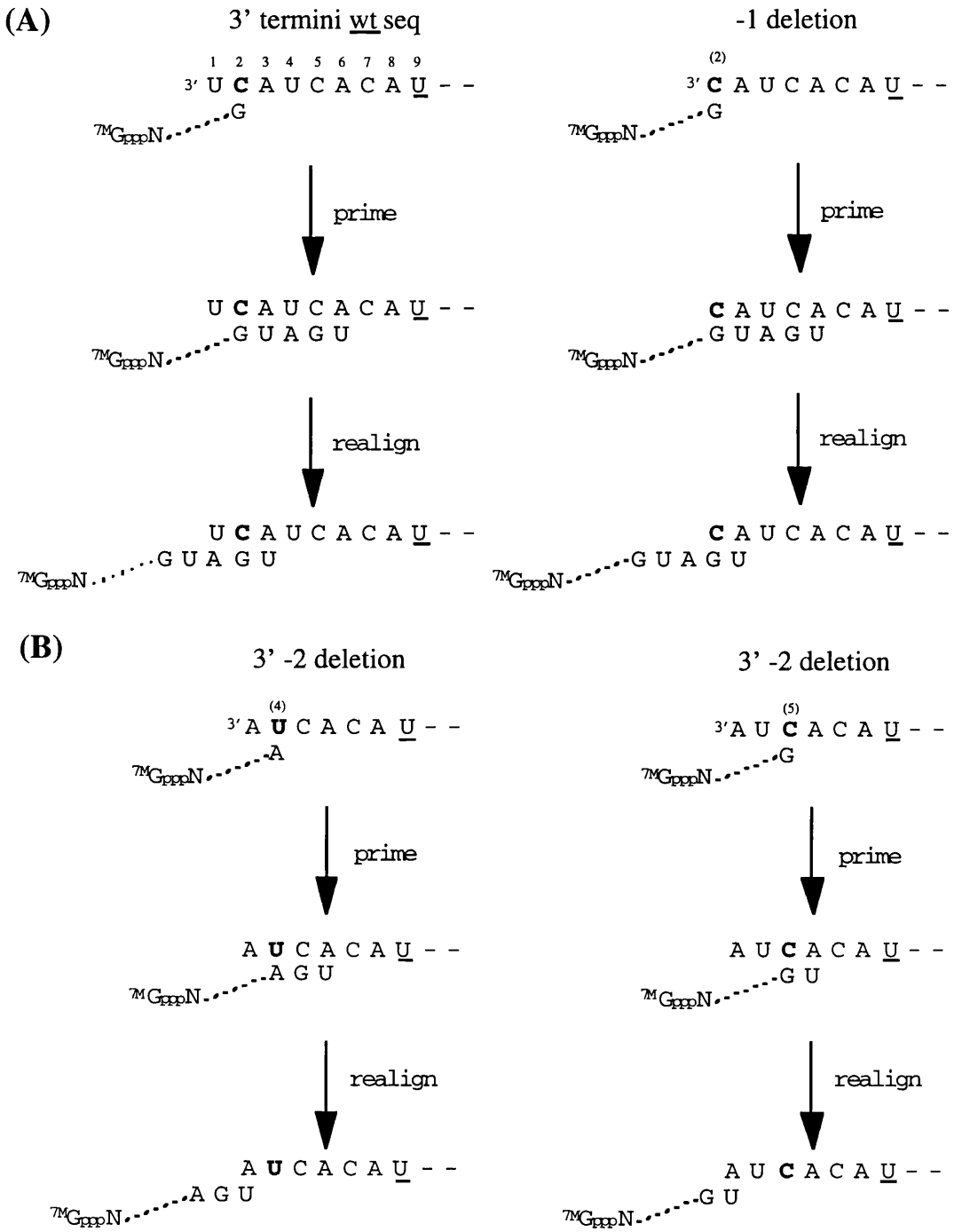


Figure 7.11 Initiation of mRNA transcription by the "prime-and-realign " mechanism.

The above figures describe the possible mechanism for bunyavirus mRNA initiation as described by Garcin *et al.*(1995). **(A)** Predicted mRNA initiation and synthesis mechanism for authentic BUN termini and for a deletion of -1 nucleotide. **(B)** Possible initiation of mRNA synthesis with a -2 deletion.

on the vRNA template at a C residue, possibly at position +2 (3' U**C**A UCA--5'). After incorporation of a few nucleotides, the newly transcribed strand slips back and realigns on the template, as shown, before further elongation of the mRNA transcript (Figure 7.11 A).

This model would account for the observation that a deletion of 1 nucleotide from the 3' UTR was well tolerated. Deletion of 1 nucleotide would still allow the primer to anneal at the C at position 2, and to initiate with a G in the usual manner, and that the prime-and-realign mechanism could proceed without disruption. A deletion of 2 and 3 nucleotides was found to drastically reduce transcription from the BUNSCAT reporter RNA, but the low level of activity that was detected may have risen from transcription initiation further upstream of the deleted 3' UTR, either on the U at position +4, or the C at position +5, as shown in Figure 7.11 B.

Results from studies conducted by Prehaud *et al.* (1997) lend support to this explanation of transcription from the bunyavirus 3' deletion constructs. In their study, it was found that a deletion of 1 or 2 nucleotides from the RVF S segment reporter RNA was tolerated, whereas deletion of more than 2 nucleotides was not. Again, the prime-and-realign mechanism is thought to be used for transcription initiation in RVF, and the snatched CAP-primer which the virus uses to initiate transcription from the 3' UTR initiates with an A at position 3 (3' U^1 G^2 **U^3** G^4 U^5 U^6 U^7- - 5') of the vRNA template. This would explain how a deletion of 1 and 2 nucleotides can be tolerated by the viral polymerase, which is still able to initiate transcription on the preferred nucleotide at position 3.

The models described above have largely discussed the transcription of mRNA. However, it is thought that the same mechanisms also operate for viral genomic replication of hantaviruses (Garcin *et al.*, 1995a), although this process is thought not to employ the use of a host derived capped primer RNA. Instead, the polymerase initiates transcription internally without the use of a primer sequence, but primes with a single purine (GTP or ATP) and the prime-and-realign model continues as usual. To date, all RNA polymerases that initiate chain transcription with triphosphates, have a preference for purines (Banerjee, 1980; Garcin *et al.*, 1995a).

Therefore, transcription of bunyavirus genomic RNA could initiate internally on the C at position +2, as shown in Figure 7.11. Initial chain elongation, slippage and realignment, would then produce a 2 nucleotide overhang in the newly transcribed RNA. This is thought to be excised by the same endonuclease activity responsible for cap-snatching, before the polymerase continues elongation.

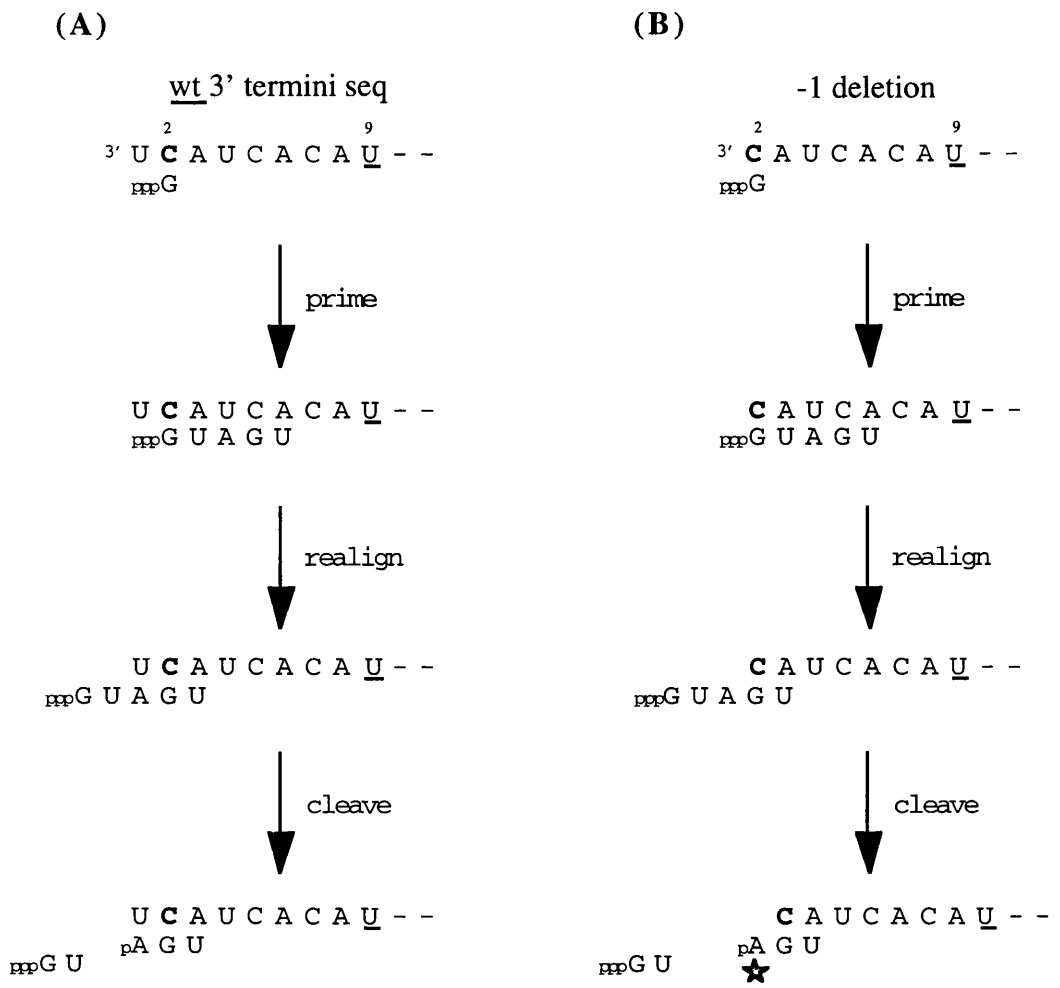


Figure 7.12 Initiation of genome replication by the "prime-and-realign" mechanism.

The model shown in (A) describes the proposed mechanism for initiation of bunyavirus replication. This model is based on studies of hantavirus replication (Garcin *et al.*, 1995). It is thought that the polymerase initiates transcription internally by priming the template with a single purine, in this case a GTP, and the "prime-and-realign" mechanism continues, as described previously. The L protein initiates transcription internally on the C at position +2, as shown. Initial chain elongation, slippage and realignment, would produce a 2 nucleotide overhang in the newly transcribed RNA. This could possibly be excised by the same endonuclease activity responsible for cap-snatching, thereby leaving a pA at the 5' terminus, before the polymerase proceeds with elongation. This mechanism also has a potential ability to repair damaged genome ends by restoring small terminal deletions or mutations in the resulting complementary segment, as shown in (B). The figure demonstrates a possible mechanism by which a single 3' deletion could be repaired in the resulting complementary strand by the "prime-and-realign" mechanism. The polymerase would again prime at the C residue (now position +1) with GTP, by "prime-and-realign" as described previously, then cleave off the non-viral terminal sequence before continuing elongation, thus restoring a 5' A residue (highlighted with star).

Another feature of this mechanism is that it has a potential ability to repair damaged genome ends by restoring small terminal deletions or mutations. The reiteration of sequence at the 3' terminus, in conjunction with the prime and realign mechanism, would mean that segments which lack a few nucleotides at the 3' end could feasibly be repaired, as shown in Figure 7.11 B. This is achieved by a slipping back of the new transcript, primed from reiterated sequence found at the 3' terminus, followed by further transcription through the template, which thereby restores the correct nucleotide sequence at the terminus.

This is also shown in Figure 7.12, and interestingly, this mechanism could possibly account for the results of the 3' terminal deletion studies presented in this chapter, where it was shown that a deletion of 1 nucleotide from the 3' terminus of the reporter RNA produced no significant reduction in function of this RNA. This being so, the polymerase would have to initiate transcription on the first nucleotide of the 3' terminus, rather than initiating internally as in the model.

7.7 Future work.

It would be interesting to try and determine if transcripts made off deleted templates have had their ends repaired. This would support the idea of the "prime-and -realign" mechanism and help determine the function of the conserved reiterated terminal sequences.

Chapter 8.

8.0 Internal deletions within the BUNSCAT RNA 5' UTR sequence.

8.0 Introduction.

The 5' UTR of the Bunyamwera S segment vRNA is 174 nucleotides in length: this is about twice as long as the 3' UTR, which is 85 nucleotides. It is proposed that this difference in length is relevant in terms of encoded signals and *cis* acting sequences involved in transcription, specifically, signals involved in the termination of mRNA transcription (Kolakofsky and Hacker, 1991). A comparison of the S vRNA 5' UTRs of nine different BUN serogroups and three different CAL serogroup viruses (Figure 8.1, Dunn *et al.*, 1994), and twelve different CAL serogroups viruses (Bowen *et al.*, 1995), revealed a highly conserved purine (A-C) rich region stretching between nucleotide positions 74 and 89, with respect to the BUN S 5' UTR. This motif is found in close proximity to the region of the 3' termini of viral mRNA, which has been characterised for BUN, GER, LAC and SSH viruses (Jin and Elliott, 1993; Bouloy *et al.*, 1990; Patterson and Kolakofsky, 1984; Eshita *et al.*, 1985, respectively). This region, which stretches between nucleotides 100 to 112 with respect to the BUN S 5' UTR, is a U-G rich region which can be aligned for all the viruses mentioned above (Dunn *et al.*, 1994)

To analyse the possible significance of these motifs, it was decided to produce a series of deletion mutants within the BUN S vRNA 5' UTR. These deletions would be within the 5' UTR, between the stop codon of the CAT ORF and the 5' terminus. To achieve this, the exonuclease *Bal* 31 was used to degrade pBUNSCAT DNA, linearised previously with *Nsi*I. The unique *Nsi*I site is downstream of the CAT stop codon, and within the 5' UTR (Figure 8.2).

8.1 Introduction of deletions within the BUN S 5' UTR.

Construction of the deletions within the BUN S 5' UTR is described below, and is also outlined schematically in Figure 8.2. *Nsi*I linearised pBUNSCAT DNA was digested with *Bal* 31 endonuclease for varying time after which the DNA was separated on a 1% TAE

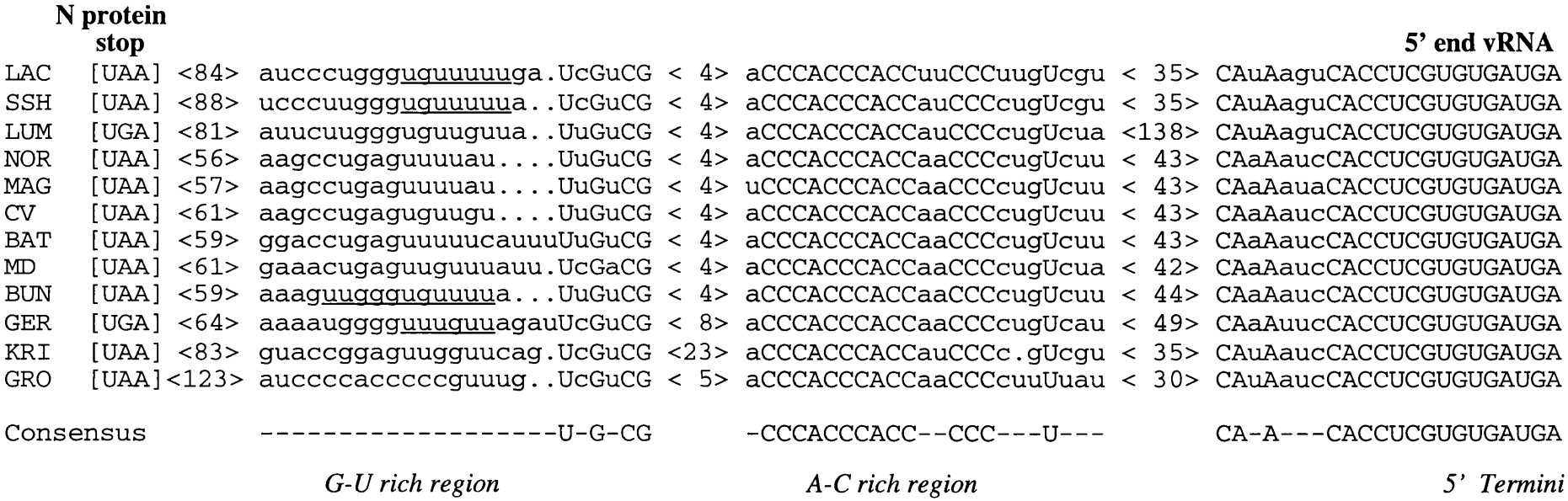


Figure 8.1 Nucleotide sequence comparison of the 5' end of viral RNA.

The 5' UTR sequences are written 3' to 5' for genomic sense RNA, and residues conserved in all 12 sequences are given in capitals and in the "consensus" sequence. The mRNA transcription termination sites that have been experimentally mapped (Introduction 1.4.6) are underlined. LAC, SSH, and LUM are members of the California (CAL) serogroup, while NOR, MAG, CV, BAT, MD, BUN, GER, KRI, and GRO are members of the Bunyamwera (BUN) serogroup. Figure adapted from Dunn *et al.*, 1994.

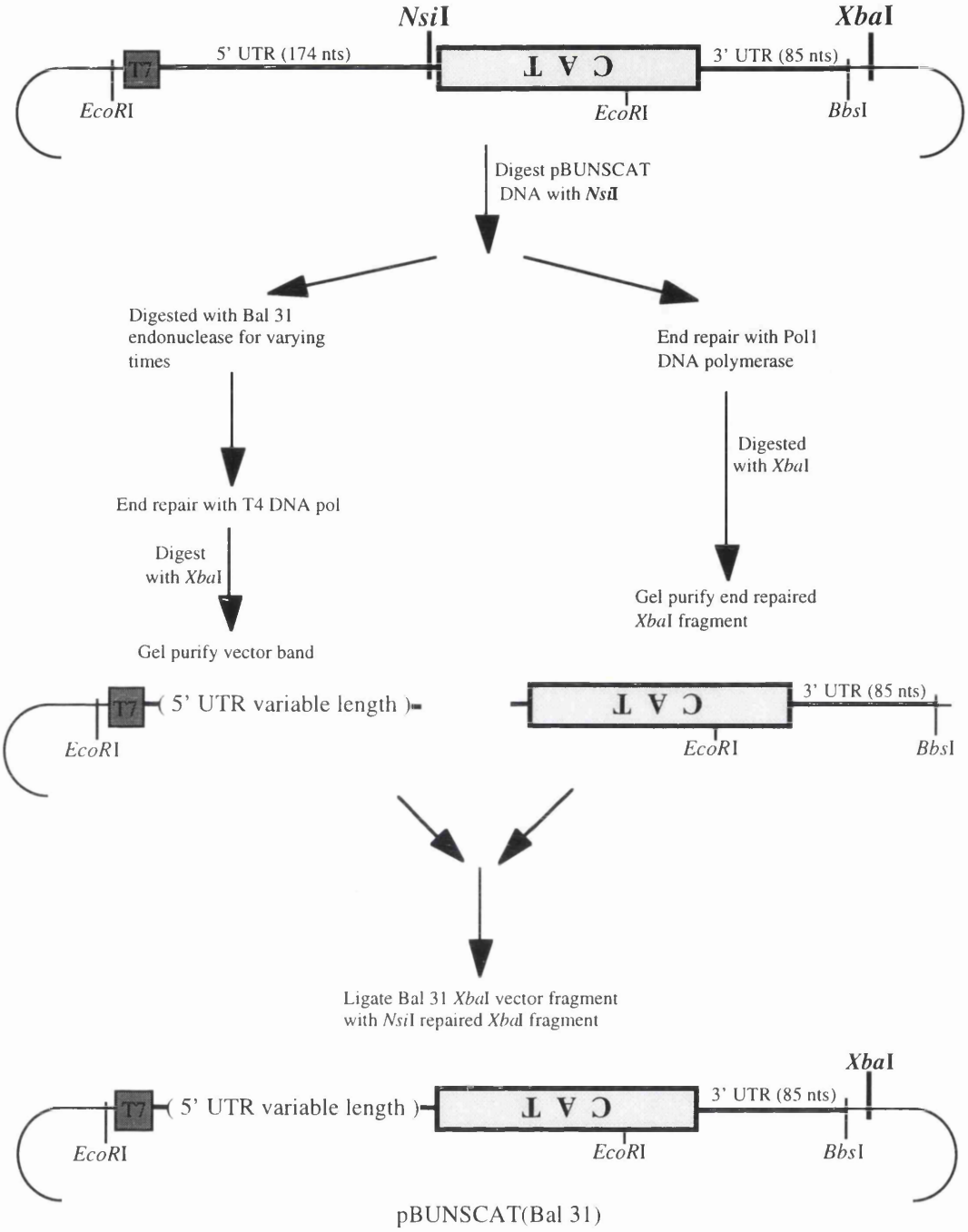


Figure 8.2 Schematic describing the protocol for the generation of pBUNSCAT(*Bal* 31) mutants.

agarose gel (Figure 8.3) and then purified by silica matrix absorption before the ends of the DNA were repaired with T4 DNA polymerase.
As *Bal* 31 nuclease would digest the DNA bidirectionaly at the *Nsi*I site, *i.e.* upstream, into the 5' UTR (as intended), and also downstream, into the CAT ORF. Therefore, to ensure a complete CAT ORF would be present in the resulting construct, the entire CAT ORF and the BUN S 3' UTR from the *Bal* 31 treated DNA was excised by digestion with *Xba*I, and replaced with an intact antisense CAT ORF and BUN S 3' UTR. To produce the intact CAT ORF and BUN S 3' UTR fragment, a portion of the *Nsi*I digested pBUNSCAT DNA was end repaired by treatment with DNA polymerase I prior to digestion with *Xba*I. The *Xba*I fragment, which contained the complete antisense CAT ORF and the 3' BUN S UTR, was then gel purified, prior to ligation into the *Bal* 31 digested/ *Xba*I vector fragment. The products of this ligation were used to transform competent DH5α cells, and small scale plasmid preparations were made from ampicillin resistant clones. Clones which were found to be positive by digestion with *Eco*RI were then sequenced through their T7 promoter region, and into the CAT ORF, to confirm the extent of the *Bal* 31 deletions within the BUNS 5' UTR.

Out of a range of positive clones generated in this way, five were selected for initial testing on the basis that the deleted sequences covered the regions of interest, namely, the A-C rich motif (nucleotides 73 to 88), and the site of the predicted mRNA terminus (nucleotides 100 to 112). Additionally, the selected clones contained sequential deletions, thus providing a range of mutants for this study. The clones that were chosen were denoted pBUNSCAT(*Bal* 31), clones -(75), -(2), -(7), -(15), and -(10).

pBUNSCAT(*BAL* 31) clone (75) has a deletion of 29 nucleotides upstream of the *Nsi*I site, clone (2), 57 nucleotides upstream, clone (7), 71 nucleotides upstream, clone (15), 93 nucleotides upstream, and clone (10), 156 nucleotides upstream. The deleted regions within these clones is highlighted in Figure 8.5.

A higher than expected number of the deletion clones (which were not selected for further study) were found to have deletions which mapped to the GU rich region. It is possible that the GU rich sequence may have caused the *Bal* 31 exonuclease to pause, resulting in a number of clones with the same deletion (Berger and Kimnel, 1987).

8.2 Testing the pBUNSCAT *Bal* 31 clones in the reporter system.

It was important that an equal number of transcripts from each clone were transfected into the reporter system. Therefore, the amount of transfected RNA was adjusted to take into account

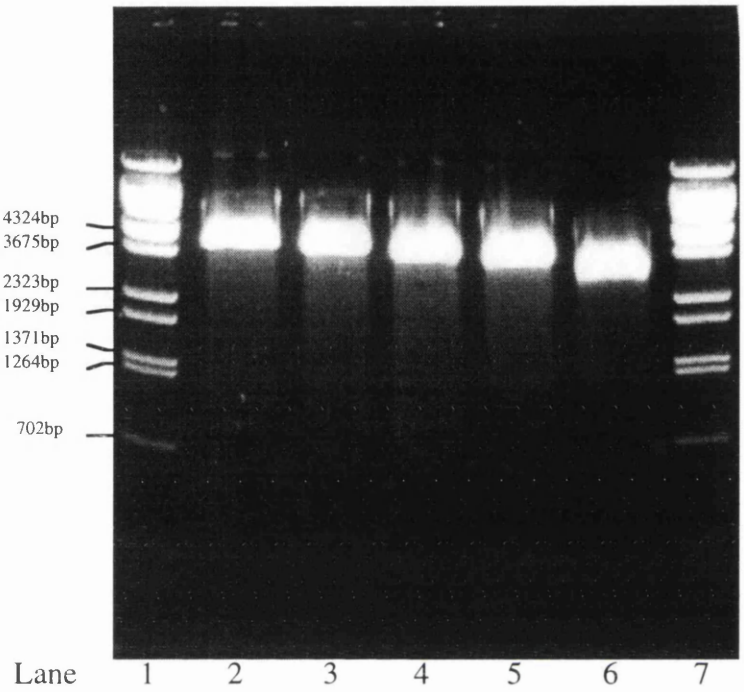


Figure 8.3 *Bal* 31 digestion of *Nsi*I linearised pBUNSCAT DNA.

Approximately 10 µg *Nsi*I linearised pBUNSCAT DNA was digested with *Bal* 31 at 0.1 units/µg, at 30°C, for varying times as follows: lane (2) 30 sec.; lane (3) 1 minutes 30 sec.; lane (4) 3 minutes; lane (5) 4 minutes 30 sec.; lane (6) 6 minutes. Reactions were stopped by the addition of EDTA and run on a 1% TAE gel. DNA bands were excised and purified by Geneclean, prior to incubation with T4 DNA polymerase at 11°C for 30 minutes, to repair the ends. Reactions were then digested with *Xba*I before purifying the vector band. Lanes 1 and 7 on the gel shown above contain 1µg of λ *Bst*EII markers. A reduction in the size of *Nsi*I linearised pBUNSCAT can be seen as the incubation time of *Bal* 31 digestion is increased.

the size difference in the deletion mutants. Thus, an equimolar amount of each transcript was transfected (Table 8.1).

Clone Number	transcript length nucleotides	% in length of BUNSCAT	amount of RNA transfected
pBUNSCAT wt	919	100%	2.50μg
pBUNSCAT(*Bal* 31)75	890	96.8%	2.44μg
pBUNSCAT(*Bal* 31)2	862	93.4%	2.34μg
pBUNSCAT(*Bal* 31)7	848	92.7%	2.32μg
pBUNSCAT(*Bal* 31)15	826	89.9%	2.25μg
pBUNSCAT(*Bal* 31)10	763	83.0%	2.08μg

Table 8.1 Transfection of BUNCAT *Bal* 31 clone transcripts.
The above table demonstrates the adjustments made to the amount of RNA transfected when taking the length of the transcript into consideration, ensuring an equimolar amount of each transcript was transfected.

The results of the CAT assay using the *Bal* 31 deletion mutants are shown in Figure 8.4, and summarised in Figure 8.5. These results show that a construct containing an internal deletion of 29 nucleotides in the 5' UTR (clone 75), resulted a level of CAT activity in the reporter assay that was comparable to that obtained with full length BUNSCAT. Similarly, in the constructs where the deleted sequence was extended to 57 nucleotides (clone 2), 71 nucleotides (clone 7), and 93 nucleotides (clone 15), all of which resulted in the gradual removal of the U-G rich and the A-C rich motifs, the level of CAT activity obtained was still comparable to full length BUNSCAT. In fact, the only deletion mutant which showed any deviation from wild type BUNSCAT behaviour was clone 10 with a CAT expression level <1% that of wild type; this construct had a deletion of 156 nucleotides, leaving only eleven nucleotides at its 5' terminus.

8.3 Discussion.

As demonstrated in Figure 8.5, reporter construct RNAs in which the A-C and G-U rich motifs were deleted gave rise to a level of CAT activity indistinguishable from wild type BUNSCAT RNA, when transfected into the reporter assay. It was expected that deletion of the motifs might have caused a reduction in the CAT activity, thereby explaining the sequence conservation in this region.

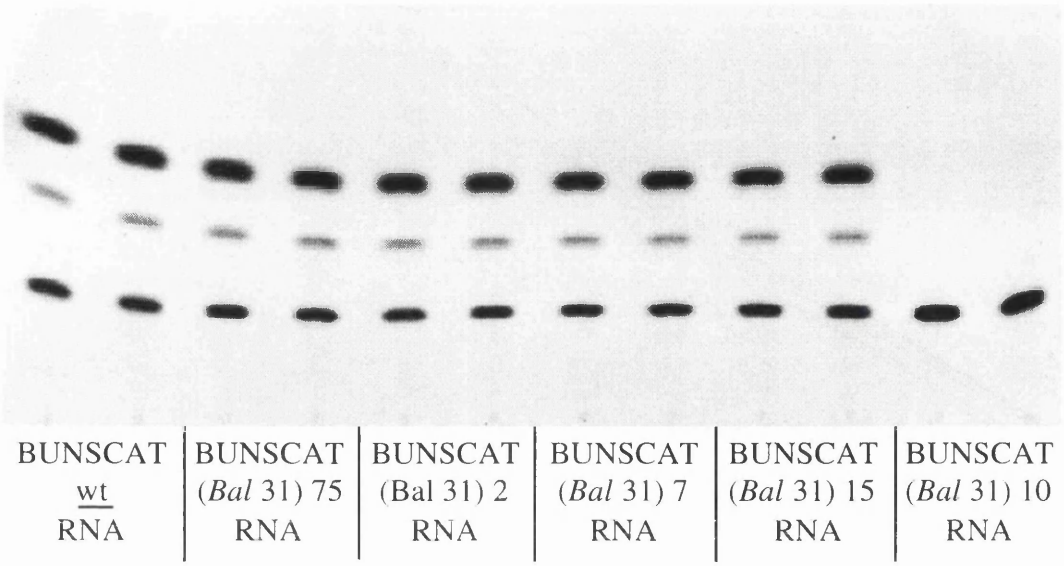


Figure 8.4 CAT expression from *Bal* 31 deletion pBUNSCAT clones.

CV-1 cells expressing BUN S and L segment proteins were transfected with equimolar amounts of each RNA transcript (Table 8.1). The cells were incubated at 37°C for 3 hours before the addition of growth medium, after which the incubation was continued overnight. At 24 hours post infection the cells were harvested and the cell extracts assayed for protein concentration. The volume of cell extracts which were assayed was adjusted, so that the amount of protein tested was equivalent to that in the BUNSCAT extract. CAT activity comparable to BUNSCAT wild type was detected in extracts from cells transfected with BUNSCAT (*Bal* 31)-clones 75, 2, 7 and 15. The deleted sequences in these clones ranged from 28 to 93 nucleotides, and encompassed the G-U rich motif. CAT activity less that 1% that of wild type BUNSCAT was detected for clone 10: this construct has a deletion of 156 nucleotides. Therefore, sequences important for CAT expression, within the reporter system, lie between nucleotides 11 and 74 in the BUN S 5' UTR.

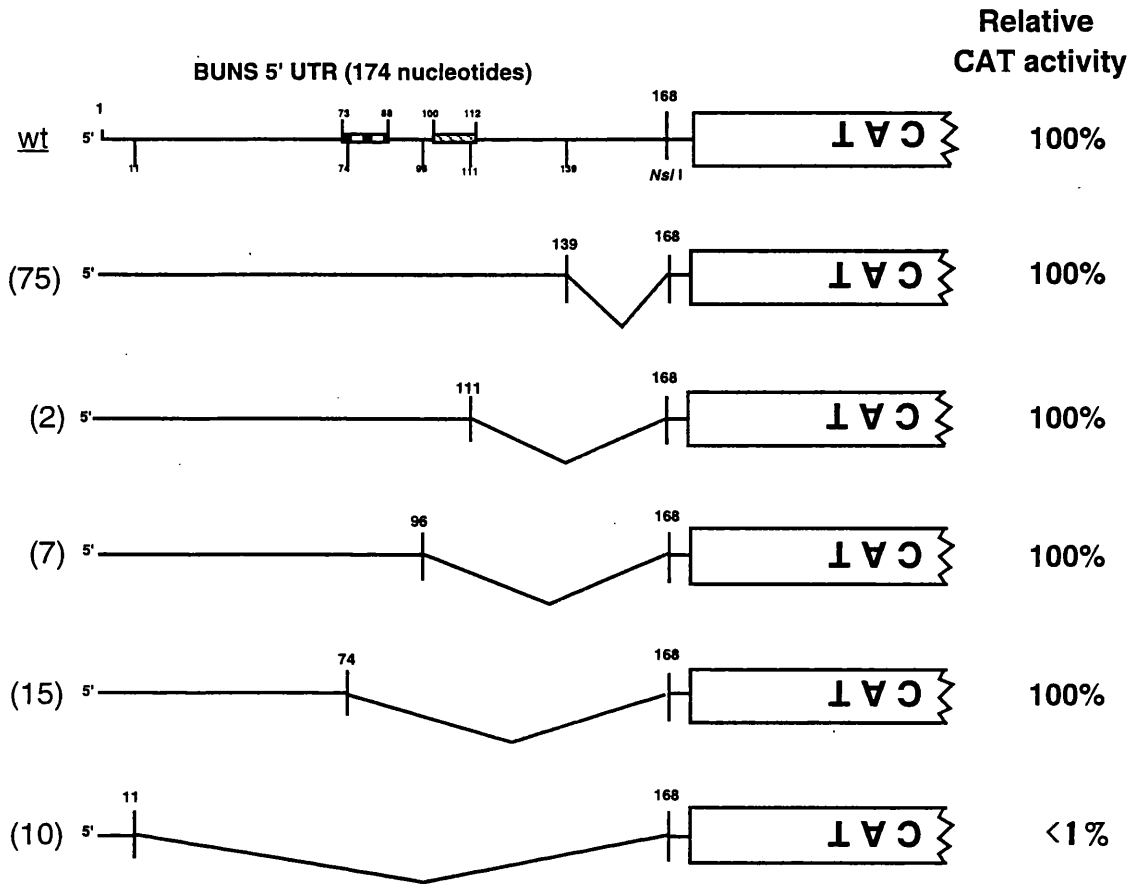


Figure 8.5 Schematic showing the extent of the 5' UTR internal *Bal* 31 deletions.

The above diagram shows a series of pBUNSCAT clones with deletions within the 5' UTR. The deleted regions range from 28 to 156 nucleotides. The purine rich motif within the 5' UTR at position 100-112 is marked by a hatched box, and the predicted mRNA termini is marked by a striped box at position 73-88. Also shown on the diagram is the CAT activity obtained from transfection of transcripts from each of the mutants into the reporter assay. The CAT activity is noted as a % of that obtained from transfection of "wild type" BUNSCAT transcripts. It can be seen that deletions of between 28 and 93 nucleotides result in no detectable drop in the CAT signal obtained compared to "wild type" BUNSCAT. Deletion of 156 nucleotides, however, results in a drastic reduction in CAT activity to less than 1% wild type.

It was observed from transfection of clone 10 that a deletion of 156 nucleotides completely abolished all detectable CAT activity, thus demonstrating that the sequence between nucleotides 11 and 74 is essential for transcription of BUNSCAT RNA. To determine the exact nucleotide at which CAT activity is lost, further deletion analysis of the region between nucleotides 11 and 74 of pBUNSCAT would be required.

The lack of detectable CAT activity in clone 10 may have been the result of removing sequence that is conserved between all the Bunyavirus S segments. Although clone 10 still retained eleven terminal nucleotides common to all three BUN segments, the four preceding nucleotides, CCAC (Figure 1.3), which are common to all S segments of the Bunyavirus genus, were not present in this clone. These four nucleotides may form part of a signal, or more likely, may be required to stabilise the formation of a panhandle structure in which the first eleven nucleotides of the BUN S segment are involved.

The purine rich region has previously been characterised as the 3' termini of mRNA. If the signal for termination of mRNA transcription was located in the G-U rich region, then when this region is removed, as it is in clones (7) and (15), the resulting CAT activity may be produced by translation of a pseudo-mRNA transcript. There are two possible scenarios by which this could occur: either the BUN L polymerase could terminate transcription at a pseudo-termination site or, more likely, the resulting transcript, which would consist of a 5' capped leader sequence and an intact antigenomic 3' BUN S termini (with the potential to form a panhandle), could act as a mRNA within this reporter system.

It is possible that the capped leader sequence stops or interferes with the encapsidation of the transcript by the nucleocapsid protein. Alternatively, as the polymerase would be primed to transcribe a mRNA transcript, it might not be able to orchestrate the encapsidation of the transcript, or to recognise the 5' termini due to the presence of the cap leader sequence. Analysis of the 3' ends of the message sense chimeric CAT RNA would help determine what effect the various *Bal* 31 deletions have on mRNA transcription.

Similar work has been performed previously by a group working on influenza B virus (Barclay and Palese, 1995). In these experiments, a full length cDNA of the influenza B virus HA (MdHAX) segment was engineered, carrying a silent mutation encoding an *Xba*I site to differentiate this segment from wild type RNA. A CAT reporter construct based on the same HA segment termed HABCAT was also engineered. A series of deletion mutants were then generated from both the MdHAX and the HABCAT constructs. The deleted regions were in the 5' UTR of both the MdHAX and the HABCAT constructs, and were of 10, 33

and 67 nucleotides in length; between the termination codon and the poly-U stretch which is thought to act as a polyadenylation signal.

It was found that transcripts from all the HABCAT mutants produced a comparable CAT signal to undeleted HABCAT transcripts in a reporter assay. They also found that these transcripts could be rescued for one passage. However, with the MdHAX segment, it was found that it was only possible to rescue viruses which had deletions of 10 and 33 nucleotides. No viruses could be rescued containing the MdHAX segment with 67 deleted nucleotides. This implies that a deletion of 67 nucleotides in the 5' UTR of the MdHAX segment is lethal to the virus, despite the lack of effect of this deletion on the similar reporter construct, HABCAT.

This result is important as it highlights the fact that although the CAT reporter assay can provide insight into many aspects of the replicative processes of bunyavirus, it may not be comprehensively informative, and will be unable to measure certain effects of mutations on the *in vivo* events occurring during virus infection.

8.4 Future Work.

The results from the BUNSCAT *Bal* 31 deletion mutants showed that mutation of the A-C and G-U rich motifs resulted in constructs which displayed a level of CAT activity in the reporter assay that was indistinguishable from wild type BUNSCAT RNA. It is possible that the inability to characterise a possible BUN S mRNA termination signal or site was made difficult due to vaccinia virus encoded proteins modifying the resulting transcripts, possibly by polyadenylation of the resulting transcripts, thus masking any detectable drop in CAT activity brought about by the BUN S 5' UTR deletion. An alternative explanation might be that mRNA was not transcribed in the reporter system, only antigenomic RNA, which may not have become encapsidated.

An alternative way to isolate a possible mRNA termination signal would be to insert regions of the 5' UTR after the CAT ORF, but before the 3' UTR (Figure 8.6). Thus if a specific inserted region caused a drop in CAT activity, this would signify that the insertion of the sequence had caused a premature termination of the transcript, resulting in a drop in CAT activity. Active CAT genes would be verified as before, by transfecting the construct DNA into vTF7-3 infected cells.

Chapter 9.

9.0 Mutagenesis of the terminal regions of the 3' and 5' BUNS UTRs.

9.0 Introduction.

As was discussed in Chapter 3, transcripts derived from a pBUNSCAT clone, pBUNSCATmut-12, which contained a single point mutation in the BUNS 5' terminus (C to G, at position 12), produced a level of CAT activity in the reporter assay that was significantly lower than that obtained from "wild type" BUNSCAT transcripts. That a single point mutation could exert such an effect on the functioning of the reporter RNA, suggested that conservation of the sequence and/or the structure of the BUN segment UTRs might be of great importance.

Electron microscopy studies performed on both influenza virus and bunyavirus RNPs have led to the suggestion that the 5' and 3' UTRs of a genome segment might interact, possibly through base-pairing of the complementary sequences in the termini, to form a "pan-handle" structure (Raju and Kolakofsky, 1989; Kolakofsky and Hacker, 1991). In the pan-handle model, the "handle" is formed by the interacting 5' and 3' termini, and the "pan" is formed by the coding sequence of the segment, tightly bound up with N protein. Although there is no direct evidence, it is thought that the polymerase is also associated with the RNP and may be bound between the termini of the UTRs: this suggestion is based purely on the premise that the polymerase would probably be excluded from binding to the coding sequence of the segments due to their tight association with N protein.

It is not known if the mutation in clone pBUNSCATmut-12 which disrupted the functioning of the RNA transcript did so because it interfered with a recognition or transcription signal present in the 5' UTR, or because it affected the structural stability of the putative panhandle.

Therefore, in an effort to address this, a series of mutations were made to the 5' and 3' UTRs of the BUNSCAT reporter RNA, to investigate both the effect of changing the sequence content of the termini, and also to question whether disruption of the hypothetical panhandle structure would result in loss of CAT activity from the reporter RNA. These experiments involved the construction of a large range of mutants which contained individual

nucleotide substitutions for each of the terminal 15 nucleotides of either the 5' or 3' UTR. This series of constructs was designed to investigate the effect of changing the sequence content of the termini. Further to this, a second series of mutants was constructed which contained individual substitutions to nucleotides of the 5' terminus with additional, corresponding mutations in the 3' terminus, so that the base pairing potential between the termini was maintained. It was hoped that analysis of these mutants using the reporter assay would resolve whether it was more important to maintain sequence specificity in the termini or whether base pairing potential was sufficient.

Whilst in the process of this work, a new model was proposed for influenza A virus genome segments. This model was based on secondary structure predictions from results of a mutagenesis study of the 3' and 5' termini, and described the possible interaction between the 3' and 5' ends (Flick *et al.*, 1996). The model, termed the "corkscrew" model (Chapter 1.7.4), differs from previous models as it predicts both intra-strand base-pairing within each of the 3' and 5' termini, resulting in a tetra stem-loop structure, as well as base-pairing between the termini.

From examination of the 3' and 5' termini of BUN S it was noticed that a similar hypothetical structure could be drawn, where base-pairing within the 3' and 5' termini formed stem-loops, as well as base pairing between the termini. Three possible tri-stem-loop structures, each with increasing "stem" length are shown in figure 9.6.

Therefore, to test these models a series of complementary intra-strand mutations were made at base-paired regions shown in each of the three proposed models for the BUN S 5' and 3' termini (Figure 9.6).

9.1 Construction of the single 5' and 3' terminal substitution mutants.

This first series of BUNSCAT mutants contained single, individual nucleotide substitutions in either the 5' or the 3' termini. Construction of these mutants was achieved by random nucleotide substitution mutagenesis PCR, using spiked oligonucleotides as primers with the pBUNSCAT reporter construct as a template

By synthesising each oligonucleotide in two stages, on two separate oligonucleotide synthesising machines, it was possible to produce a primer that contain a spiked region for the mutagenesis of the 3' and 5' termini, and an un-spiked region to encode the T7 promoter, *Bbs*I site and restriction sites for cloning (*Xba*I and *Sac*I) (Figure 9.1).

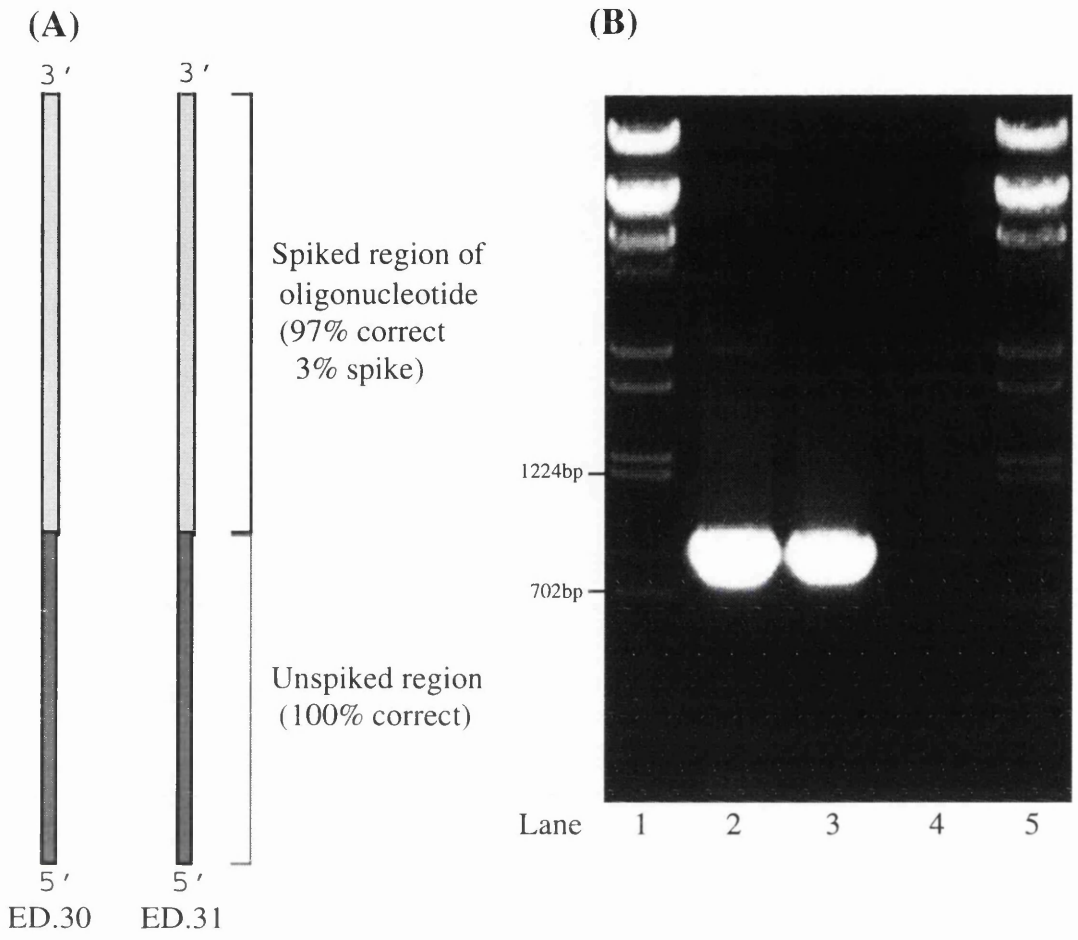


Figure 9.1 Construction of 5' and 3' terminal mutants.

(A) Spiked oligonucleotides ED.30 (5' ATTC*GAGCTC*TAATACGACTCACTATA**AGTAGTGTG CTCCACCTAAAACTTAAAA**) and ED.31 (5' AGAC*TCTAGA*AAGACGC**AGTAGTGTACTCC ACACTACAAACTTGC**) were synthesised as primers. The spiked region, *i.e.* the region which contained the BUNS terminal sequences (shown in bold), was synthesised using a nucleotide ratio of 97% specific and 3% others (*i.e.* 1% of each), while the un-spiked region, *i.e.* the region encoding the T7 promoter, *Bbs*I site (underlined) and the restriction sites for cloning, *Sac*I and *Xba*I (shown in italics) were synthesised using 100% specific nucleotides. (B) PCR reactions were set up with pBUNSCAT as the template, with either the spiked primer ED.30 (BUNS 5' UTR) and an un-spiked version of ED.31 as the distal primer (Lane 2) or the spiked primer ED.31 (BUNS 3' UTR) and an un-spiked version of ED.30 as the distal primer (Lane 3). Lane 4 represents a control PCR reaction with the two spiked oligonucleotides but with no template. The resulting PCR fragments were purified from a 1% TAE gel by silica matrix absorption, and subsequently digested with restriction enzymes *Xba*I and *Sac*I. The digested PCR fragments were then ligated into similarly digested pUC118. Clones which were positive for the BUNSCAT insert were then sequenced in both orientations to determine the possible mutations to the 3' and 5' UTRs (Figure 9.2).

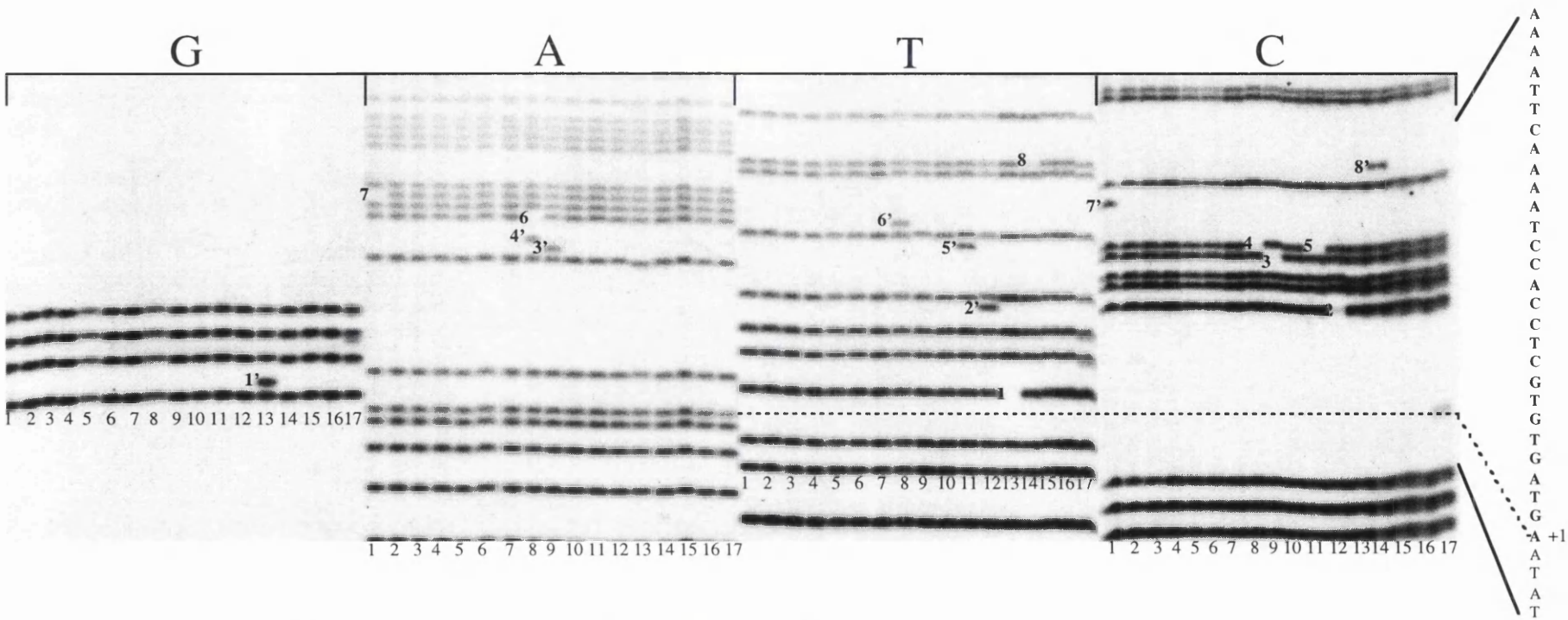


Figure 9.2 Screening of pBUNSCAT 5' UTR termini panhandle mutants.

Shown above is an example of 17 clones which were sequenced with the reverse primer to determine point mutations in the BUNSCAT 5' UTR. To facilitate identification of the point mutations in the termini, the G, A, T, and C termination reactions were run together in blocks. As can be seen from the image, seven of the 17 clones sequenced contain single point mutations to the BUNS 5' UTR. For example, clone 13 has a T(1) to G(1') change at position 3 of the 5' UTR, so that the sequence from this clone now reads 5' AG**G**AGTGTG-- as opposed to reading 5' AG**T**AGTGTG-- initially. Clone 1 has a A to C change at position 20, clone 9 has a C to A change at position 15, clone 11 has a C to T change at position 16, clone 12 has a C to T change at position 10, clone 14 has a T to C change at position 24, and clone 8 has two changes, a C to A at position 16 and a A to T at position 18. Clones 2, 3, 4, 5, 6, 7, 10, 15, 16, and 17 have wild type 5' terminal sequences.

The spiked region of the oligonucleotide was synthesised with a nucleotide ratio of 97% specific and 3% others, as described by Blacklow and Knowles (1991). This protocol results in the spiked region of the oligonucleotide having a 3% probability of an incorrect base at any particular position. After synthesis of the spiked region, the oligonucleotide columns were transferred to another machine to continue synthesis with un-spiked nucleotides (100% specific). Thus the resulting oligonucleotides ED.30 (to mutate the BUNSCAT 3' termini) and ED.31 (to mutate the BUNSCAT 5' termini), shown in Figure 9.1, were composed of a 3' spiked region and a 5' un-spiked region. The oligonucleotides were then used as primers in conjunction with an un-spiked version of the opposite primer (*i.e.* ED.30 was paired with an un-spiked version of ED.31), to amplify the BUNSCAT reporter from pBUNSCAT (Figure 9.1). The resulting PCR fragments were digested with *Xba*I/*Sac*I and ligated into similarly digested pUC118, those clones positive for insertion were sequenced to determine if they carried a mutation to the 3' or 5' termini (Figure 9.2).

Of 200 clones screened, only 20% encoded a single point mutation to the 3' or 5' termini of the BUNSCAT. This was lower than expected and may have been caused by inaccurate spiking of the nucleotides to synthesis the oligonucleotides, or it is possible that the annealing temperature during PCR affected the outcome. Due to the expense of synthesising spiked oligonucleotides of this nature, no attempts were made to increase the ratio of spike/ nucleotide during synthesis.

9.2 Construction of complementary base-paired mutations.

To determine if base pairing potential between the terminal sequences was important, a series of complementary double mutations were made within the panhandle region that maintained base-pairing. Initially, where possible, this was done by pairing specific 3' and 5' single point mutants. This was achieved by subcloning the 5' termini mutant (utilising the unique *Sac*I site upstream of the T7 promoter and the unique *Nsi*I site within the CAT gene) into the similarly digested 3' termini mutant. Where this was not possible, oligonucleotides based on ED.30 and ED.31 were synthesised carrying specific mutations for PCR (Figure 9.1).

9.3 Construction of complementary intra-strand double nucleotide substitution mutants.

Complementary double mutations to the possible stem-loop structures (Figure 9.6), were achieved by synthesising oligonucleotides as mutating primers similar to ED.30 and ED.31, but carrying specific nucleotide changes (the position of these nucleotide changes are shown in Figure 9.6). PCR mutagenesis was performed on the pBUNSCAT template with a

specific mutating primer and the distal wt sequence primer (*i.e.* ED.30 or ED.31, Figure 9.1).

In the 5' stem-loop, primers ED.5'db3 and ED.5'db4 mutated the nucleotides at positions 3 and 9 from U-G to C-A, and at positions 4 and 8 from a A-U to a G-C base-pair respectively. In the 3' stem-loop, primers ED.3'db3 and ED.3'db4 mutated the nucleotides at positions 3 and 9 from a A-U to a G-C base-pair, and at positions 4 and 8 from a U-A to a C-G base-pair respectively. The consequence of these changes are also shown in figure 9.6. The resulting mutants were named after the mutating oligonucleotide, *e.g.* pBUNSCAT (3'db3).

9.4 Analysis of the single 5' and 3' terminal substitution mutants and complementary double mutants using the reporter assay.

DNA from each of the single 5' and 3' terminal substitution mutants and complementary base-pair mutants were each linearised by digestion with *Bbs*I and used as a template for *in vitro* transcription. After DNaseI treatment, the resulting RNA transcripts were purified from the transcription reaction using the RNeasy Mini Spin column (QIAGEN) clean-up protocol (methods 2.4.4), and resuspended in RNase-free water. The transcribed mutant BUNSCAT RNAs were applied to the reporter system using the same protocol as described earlier (Section 3.1). CV-1 cells, transiently expressing the BUN viral proteins of the BUN S and L segments, were transfected with equimolar amounts of each transcript. The cells were harvested 20 hours post transfection and the extracts collected as described previously (Section 3.1). The quantity of cell extract assayed was adjusted for protein concentration before assaying for CAT activity and quantification. The results of these experiments are summarised in Figures 9.3 and 9.4.

9.5 Results.

The effect of single point mutations and complementary double mutations within the 5' and 3' termini of BUNSCAT RNA on the resulting CAT activity are summarised in Figures 9.3 and 9.4 respectively.

From Figure 9.3 it can be seen that a large number of the single point mutations to the 5' and 3' termini resulted in a marked reduction in CAT activity (<4%). This is in keeping with the observation discussed earlier, that a spurious mutation (C->G nucleotide 12) in the 5' termini of construct pBUNSCATmut-12 resulted in resulted in a drastic loss of CAT activity. In the terminal 14 nucleotides of the putative panhandle, only the single nucleotide changes at

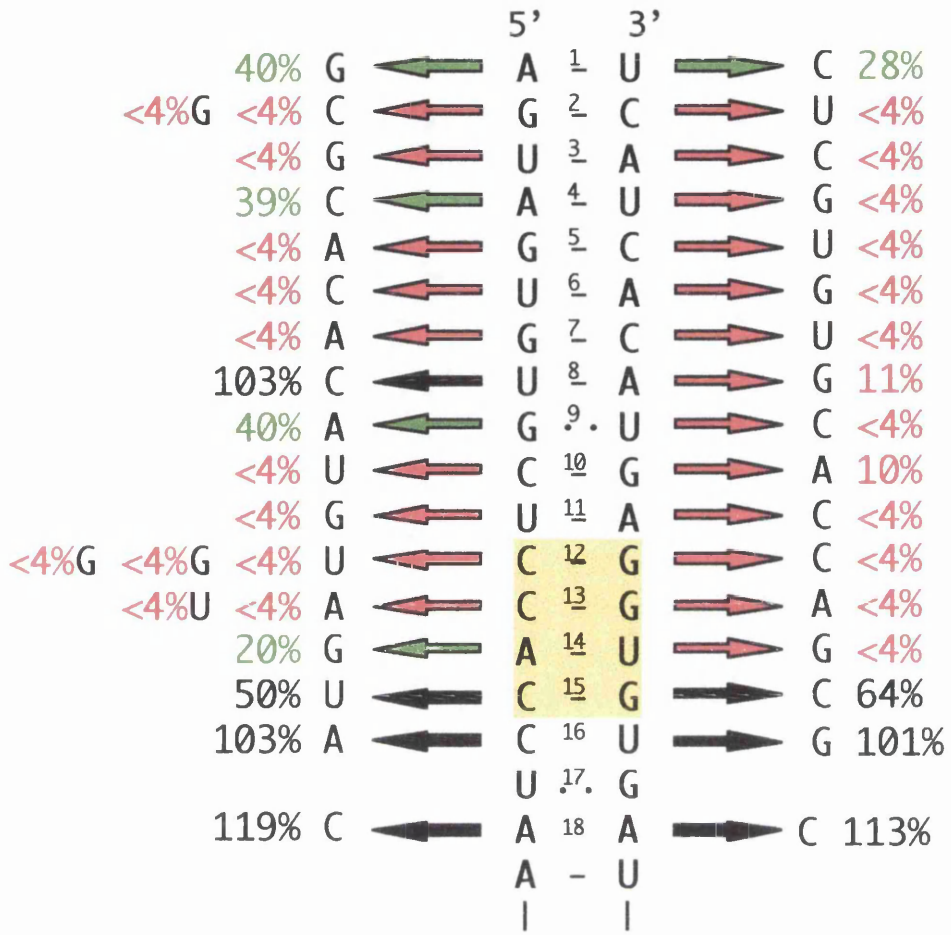


Figure 9.3 The effect of single point mutations in the BUN S 5' and 3' termini.

The above figure describes a series of single point mutations to the 5' and 3' termini of the BUNSCAT RNA. The effect of each mutation, as assayed in the reporter system, is expressed as a % activity of wild type BUNSCAT reporter RNA. Each point mutation is indicated by an arrow and the specific nucleotide change. Also shown beside each mutation is the resulting CAT activity, for example, the nucleotide at position 1 of the 5' UTR is changed from A to G, and the CAT activity from this mutant is 40% that of wt. The mutants which produced a level of CAT activity less than 20% are shown in red, those which gave a signal between 20% and 49% are shown in green, and those equal to or greater than 50% are shown in black. Nucleotides 12 to 15, shown as the boxed yellow region, represents those nucleotides that are conserved between bunyavirus S segments.

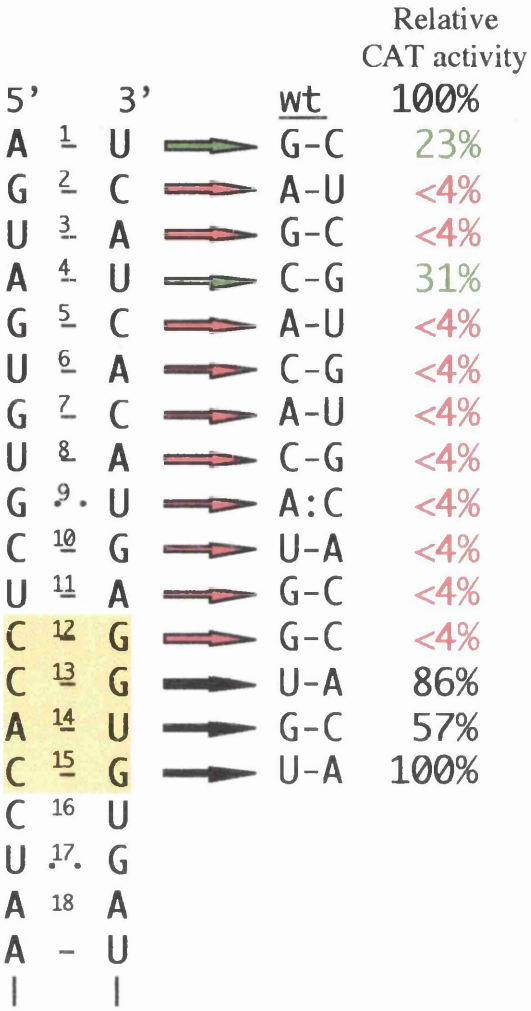
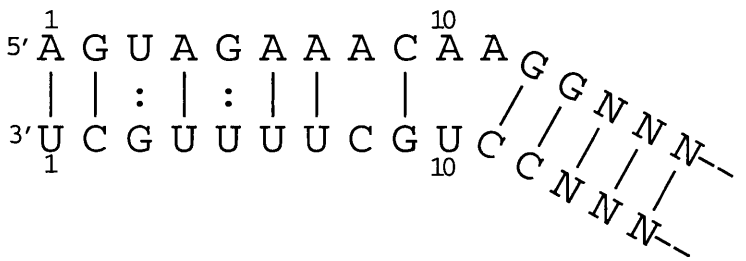


Figure 9.4 The effect of complementary base-paired mutations in the BUN S 5' and 3' termini.

The above figure describes a series of complementary base-paired mutations to the 5' and 3' termini of the BUNSCAT RNA. The effect of each mutation, as assayed in the reporter system, is expressed as a % activity of wild type BUNSCAT reporter RNA. Each mutation is indicated by an arrow and the specific base-pair changes. Also shown beside each mutation is the resulting CAT activity, for example, the base-pair at position 1 of paired termini is changed from A-U to G-C, and the CAT activity from this mutant is 23% that of wt. The mutants which produced a level of CAT activity less than 20% are shown in red, those which gave a signal between 20% and 49% are shown in green, and those equal to or greater than 50% are shown in black. Nucleotides 12 to 15, shown as the boxed yellow region, represents those nucleotides that are conserved between bunyavirus S segments.

(A)



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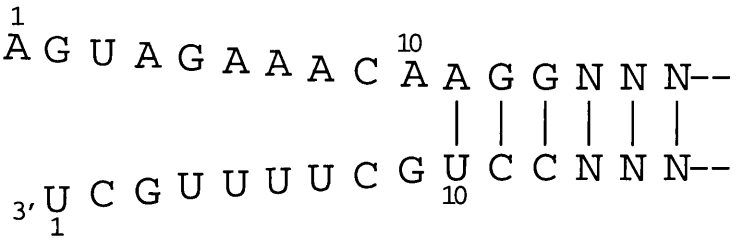

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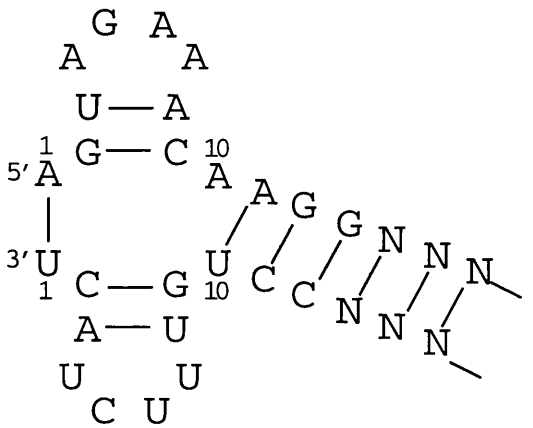


Figure 9.5 Proposed secondary structures for influenza A virus 3' and 5' ends.

(A) The influenza A virus proposed panhandle secondary structure based on the consensus sequence. (B) and (C), secondary structures as proposed by patterns of single substitutions, and supported by complementary double substitutions, (B) the fork model (Fodor *et al.*, 1994), and (C) the corkscrew model (Flick *et al.*, 1996). Watson and Crick base pairs are indicated by vertical lines. Figures (A) and (C) adapted from Flick *et al.* (1996), and Figure (B) is adapted from Fodor *et al.* (1994).

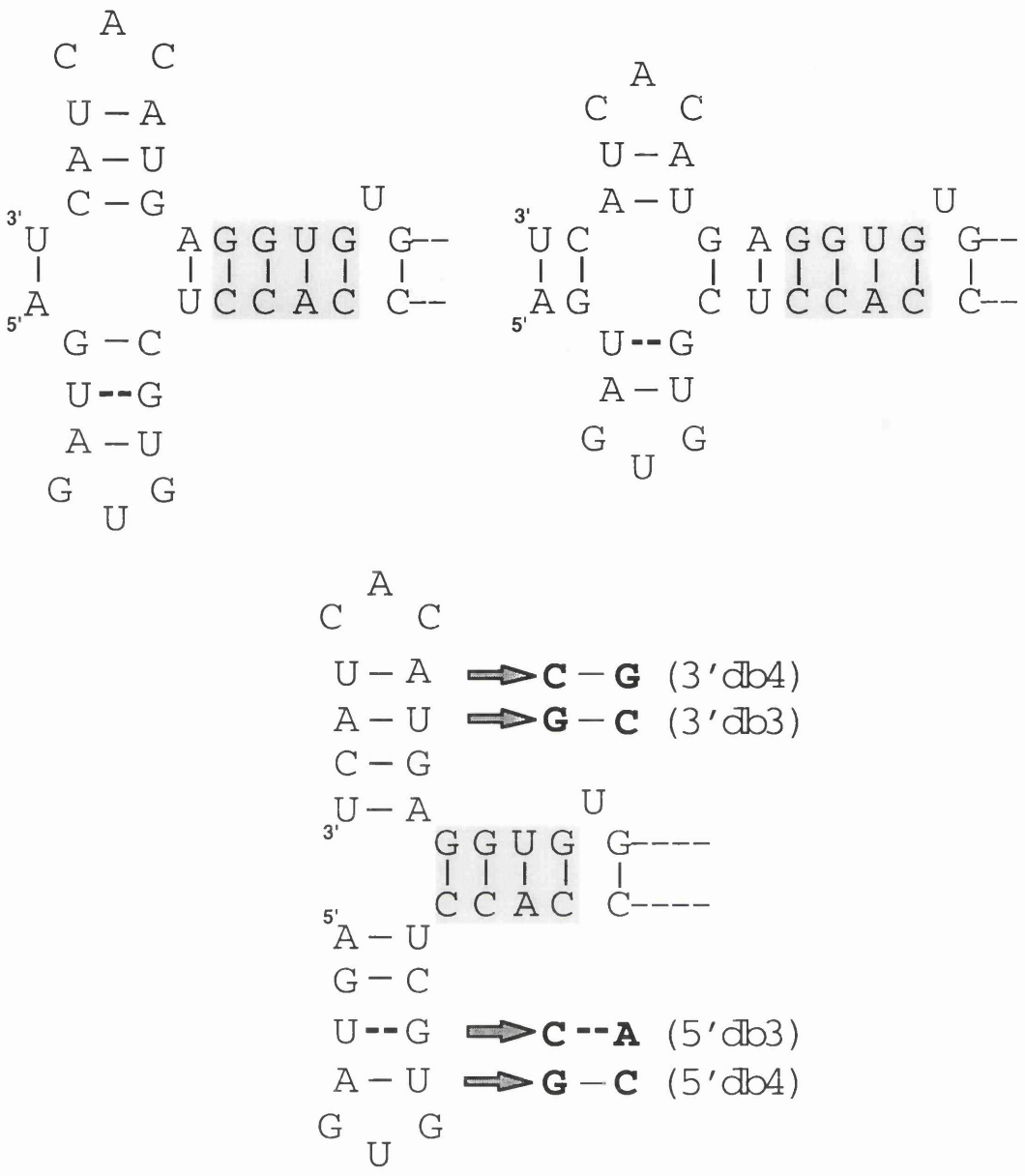
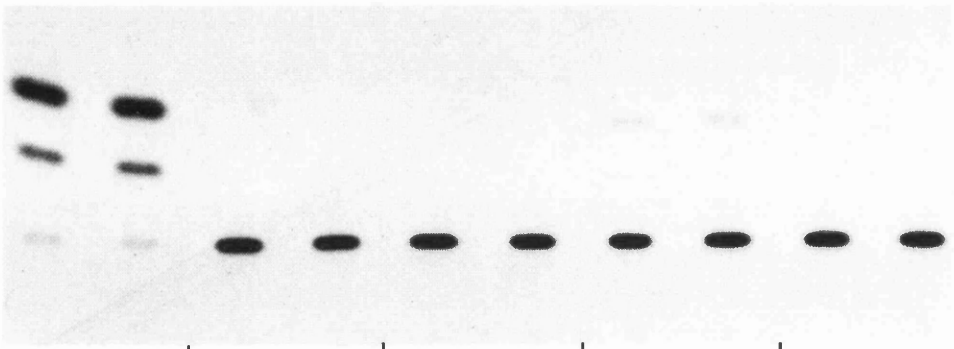


Figure 9.6 Hypothetical secondary structures formed between the 5' and 3' termini of the BUN S segment.

The above figure represents three hypothetical secondary structures formed between the 5' and 3' termini of the BUN S segment: these are based on the "corkscrew" model proposed for influenza A virus (Flick *et al.*, 1996), shown in Figure 9.5. Each model predicts a tri-stem loop structure formed by varying lengths of intra-strand base-pairing as well as base-pairing between the termini. Shown in the diagram are the positions of four complementary base-pair changes, each designed to maintain the possible intra-strand base-pairing in all three models. The notation is such that (3'db4) BUNSCAT contains the mutation U-A to C-G.



Lane	1	2	3	4	5	6	7	8	9	10
RNA template	BUNSCAT wt		BUNSCAT 5'db3		BUNSCAT 5'db4		BUNSCAT 3'db3		BUNSCAT 3'db4	

Figure 9.7 The effect of intra-strand complementary base-pair mutations within the BUN S 5' and 3' termini.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of the BUNSCAT RNA containing the intra-strand complementary base-pair mutations. Wild type BUNSCAT RNA was included as a control (Lanes 1 & 2). Lanes 3 & 4, 5 & 6, 7 & 8, and 9 & 10, show the CAT results obtained from the BUNSCAT mutants 5'db3, 5'db4, 3'db3, and 3'db4, respectively. All mutations resulted in CAT activities <2% wildtype level.

positions 1, 4 and 8 of the 5' termini and position 1 of the 3' termini did not result in a drastic reduction in CAT activity (40%, 39%, 103% and 28% respectively). Single nucleotide changes within positions 15-18 in the 5' termini and 3' termini did not markedly reduce the CAT signal. This may reflect a greater necessity for base pairing between the terminal 14 nucleotides of the UTRs or of nucleotide specificity.

Figure 9.4 summarises the CAT activities from reporter BUNSCAT RNAs containing the complementary double mutations to the terminal 15 nucleotides of the 5' and 3' termini. It can be seen that, as in the case of single point mutations within this region, the majority of changes resulted in a drastic loss in CAT activity (<4%). Only at positions 1 and 4 was a moderate level of CAT activity detected at 23% and 31% respectively, while complementary double mutations at positions 13 to 15 of the panhandle produced relatively high levels of CAT activity, at 86%, 57% and 100% respectively.

Figure 9.7 shows the resulting CAT activities from a series of complementary intra-strand double mutations based on the structure in Figure 9.6. These results show that all intra-strand complementary double nucleotide changes resulted in a CAT activity less than 4% of the BUNSCAT wt level.

9.6 Discussion.

Figure 9.3 shows the CAT activities from the series of mutant BUNSCAT reporter RNAs containing single point changes in the 5' and 3' termini. This series of mutants was constructed in an attempt to determine if base-pairing potential between the termini was important for function of the reporter RNA. Since most of the changes to the terminal 14 nucleotides of either the 5' or the 3' UTRs drastically reduced CAT activity, the initial conclusion from this experiment was that the base pairing potential between the termini might be important for activity. However, Figure 9.4 summarises the CAT results from a series of pBUNSCAT mutants containing complementary double exchanges in the 3' and 5' termini. These mutants were designed to address the requirement for sequence specificity while maintaining base-pairing within the proposed panhandle structure. From Figure 9.4 it can be seen that only nucleotide "pairs" at position 1 and 4 in the first 12 nucleotides can be varied without abolishing CAT activity, demonstrating the importance not only of base pairing potential but also of sequence specificity within the termini. Whether these features of the termini are necessary for the processes of transcription and/or encapsidation is not clear.

The complementary mutations at positions 13, 14 and 15 result in CAT activity between 57% and 100% that of wild type (Figure 9.4). However, a number of the point mutations which disrupt base-pairing in this region abolish CAT activity (Figure 9.3). Although a limited number of complementary mutations were made within nucleotides 12 to 15 of the 3' and 5' termini, the suggestion from the results so far is that the maintenance of base-pairing within this region might be more important than specificity of sequence. Interestingly, these nucleotides correspond to the region of sequence shared between bunyavirus S segments but not between the bunyavirus M and L genome segments (Figure 1.3), and so it might be predicted that base-pairing in this region will also be essential for transcription from the L and M BUN segments.

Mutational studies of the influenza A virus 3' and 5' termini has predicted two models, "the fork " and the "corkscrew" models. In both models a region of base-pairing between the termini was predicted (Fodor *et al.*, 1994, 1995: Flick *et al.*, 1996; Kim *et al.*, 1997). The first three base-pairs formed by nucleotides 11 to 13 at the 5' end and nucleotides 10 to 12 at the 3' end, are strictly conserved among different vRNA segments of all influenza A viruses. In addition, the panhandle base-pairing may be extended by nonconserved nucleotides at the 3' and 5' ends. Thus nucleotides 14 to 16 at the 5' end are generally complementary to nucleotides 13 to 15 at the 3' end. These nucleotide positions, although nonconserved among the different vRNA segments, are in most instances highly conserved for the same segment of different influenza virus strains (Desselberger *et al.*, 1980).

In the mutational studies that predict the two models, the authors suggest that within the base-paired region, secondary structure is more important than the actual sequence of the residues (Fodor *et al.*, 1994, 1995; Flick *et al.*, 1996)) Within the "corkscrew" model (Flick et al., 1996) intra-strand base-pairing was predicted within the 3' and 5' ends.

From examination of the BUN S termini sequence, it was thought that similar secondary structures might be formed, and therefore the effect of complementary intra-strand mutations was tested on these theoretical models. Unfortunately, each the four complementary intra-strand 3' and 5' mutations abolished CAT activity, a result which might indicate that the termini do not form such structures. However, it is still possible that intra-strand pairing does occur and that the lack of CAT activity from the mutants might simply be the result of changing the sequence specificity.

9.7 Future Work.

Future work would be to continue the single point mutagenesis of the 3' and 5' BUN S termini to predict those nucleotides essential for transcription and possibly predict a secondary structure complex formed between the 3' and 5' termini.

Chapter 10.

10.0 The effect of modifications to the untranslated regions of BUNSCAT RNA.

10.0 Introduction.

The S segments from viruses in the Bunyamwera serogroup whose genomes have been sequenced so far vary in length from 945 to 992 nucleotides. This variation is not attributed to a size difference in the open reading frames of these segments as they are identical, encoding a protein of 233 amino acids. Rather, the difference in length of the S segments is directly due to variations in the length of the untranslated regions (UTRs). Both the individual 5' and 3' UTRs of the S-segments vary in size: the 5' UTR ranges from 26 to 87 nucleotides and the 3' UTR from 169 to 226 nucleotides in length. It is the UTRs which are thought to contain the *cis*-acting signals for the initiation of transcription and replication, for nucleocapsid assembly, and for packaging into virions. As each of the S-segment UTRs is expected to encode the equivalent signals, the variation in length of the UTRs may represent a different spacing of these important signals.

To determine the minimum length of the 5' and 3' UTRs needed for the expression of CAT activity, a series of BUNSCAT mini-reporter constructs was engineered: these constructs featured an antisense CAT gene flanked by varying lengths of the BUN S termini.

10.1 Construction of pBUNS-13CAT, pBUNS-20CAT, and pBUNS-32CAT mini-reporter constructs.

The possible base-pairing between the 3' and 5' termini of the BUN S segment is shown in Figure 10.1. It was on the basis of these predicted base pairings that specific regions of the 5' and 3' UTRs were selected for investigation.

Importantly, the figure highlights the possibility of base pairing of the first 11 nucleotides of the 5' UTR, with the last 11 nucleotides of the 3' UTR. These are conserved between the S segment termini of all bunyavirus segments sequenced thus far, which suggests that this region must be highly important if not essential. Therefore, in designing the mini-reporter constructs, it was thought crucial that this conserved region be maintained in the flanking sequences.

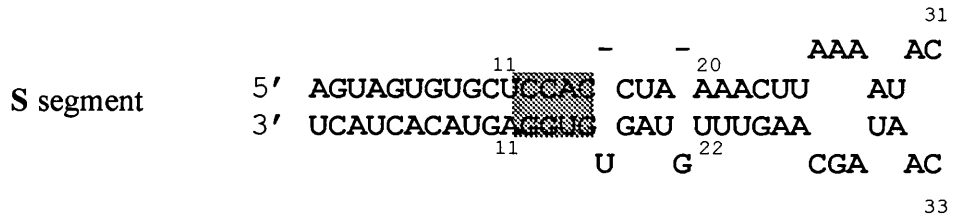
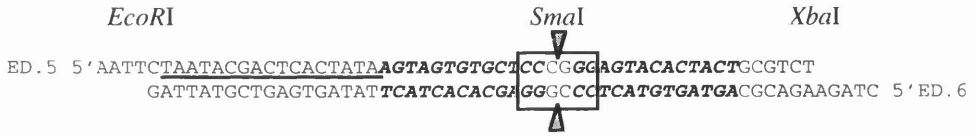


Figure 10.1 Possible complementary base-pairing between the 3' and 5' termini of the Bunyamwera S segment.

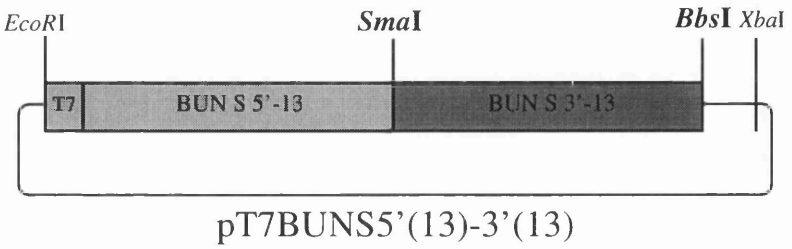
Figure 10.1 also depicts the observed high degree of complementary between termini, which is maintained for approximately the first 31 nucleotides of the 5' and 3' UTRs of the BUN S segment. Specifically, for the BUN S segment, there are 31 nucleotides at the 5' terminus which may possibly form a structure with a high degree of base-pairing with 33 nucleotides of the 3' terminus.

Therefore, two mini-reporter constructs were built initially, one contained a CAT ORF flanked by the terminal 11 nucleotides, and another contained a CAT ORF flanked by the first 31 nucleotides of the BUNS 5' UTR and the last 33 nucleotides of the 3' BUNS termini: these sequences encompassed the regions of high complementary between the termini. Details of the construction of the mini-reporters are shown in Figure 10.2 and 10.3; however, to achieve the final constructs it was necessary to use purpose-built cloning cassettes. These cassettes contained a restriction site (*Sma*I), placed to allow insertion of a reporter gene, so that the reporter gene would be flanked by varying lengths of the 5' and 3' BUNS termini (Figure 10.2 and 10.3). Consequently, due to the use of the *Sma*I restriction site to introduce the CAT ORF into the cloning cassettes, the number of flanking BUN S specific nucleotides in the first construct increased from 11 to 13 nucleotides (in both termini), and the number of flanking nucleotides in the second construct increased from 31 to 32 nucleotides (of the 5' termini). The constructs were denoted pBUNS-13CAT and pBUNS-32CAT (Figure 10.2 and 10.3).

Another mini-reporter construct was built which contained the antisense CAT gene flanked by 20 nucleotides of the 5' and 3' BUN S termini. Twenty nucleotides of the BUN S termini were chosen as it served as an intermediate between the pBUNS-13CAT and pBUNS-32CAT mini-reporters. This mini-reporter was denoted pBUNS-20CAT, and details of the construction are shown in Figure 10.4.



(B)



(C)

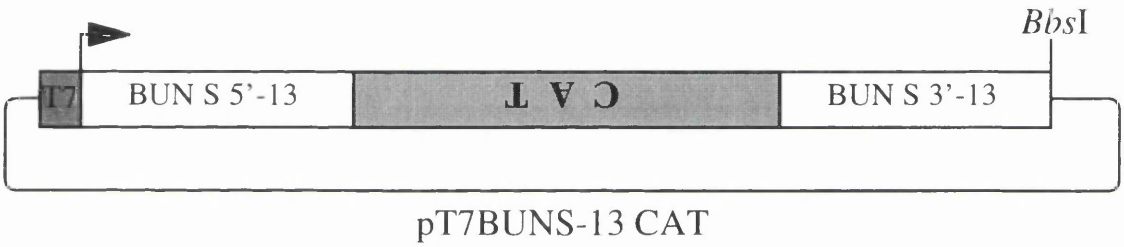


Figure 10.2 Primer design and construction of pBUNS-13CAT.

Complementary oligonucleotides ED.5 and ED.6 (A), were annealed by the following method: 0.5µg of each oligonucleotide were diluted to a final volume of 49µl in 1x T4 kinase buffer and heated for 5 minutes at 94°C, then slowly cooled to room temperature. The resulting annealed oligonucleotides were treated with T4 DNA kinase before being ligated into *Eco*RI/*Xba*I cut pUC118 (B), resulting in the construct pT7BUNS5'(13)-3'(13)CAT. A full length CAT ORF, as described in Figure 3.11, was ligated into *Sma*I digested pT7BUNS5'(13)-3'(13). Resulting clones were screened by restriction digestion to confirm the presence and orientation of the CAT gene, and then sequenced to check the fidelity of sequence of the flanking regions. The final construct, shown in (C), contained an antisense CAT gene flanked by 13 nucleotides of the BUN S 5' and 3' termini, and was denoted pT7BUNS-13CAT.

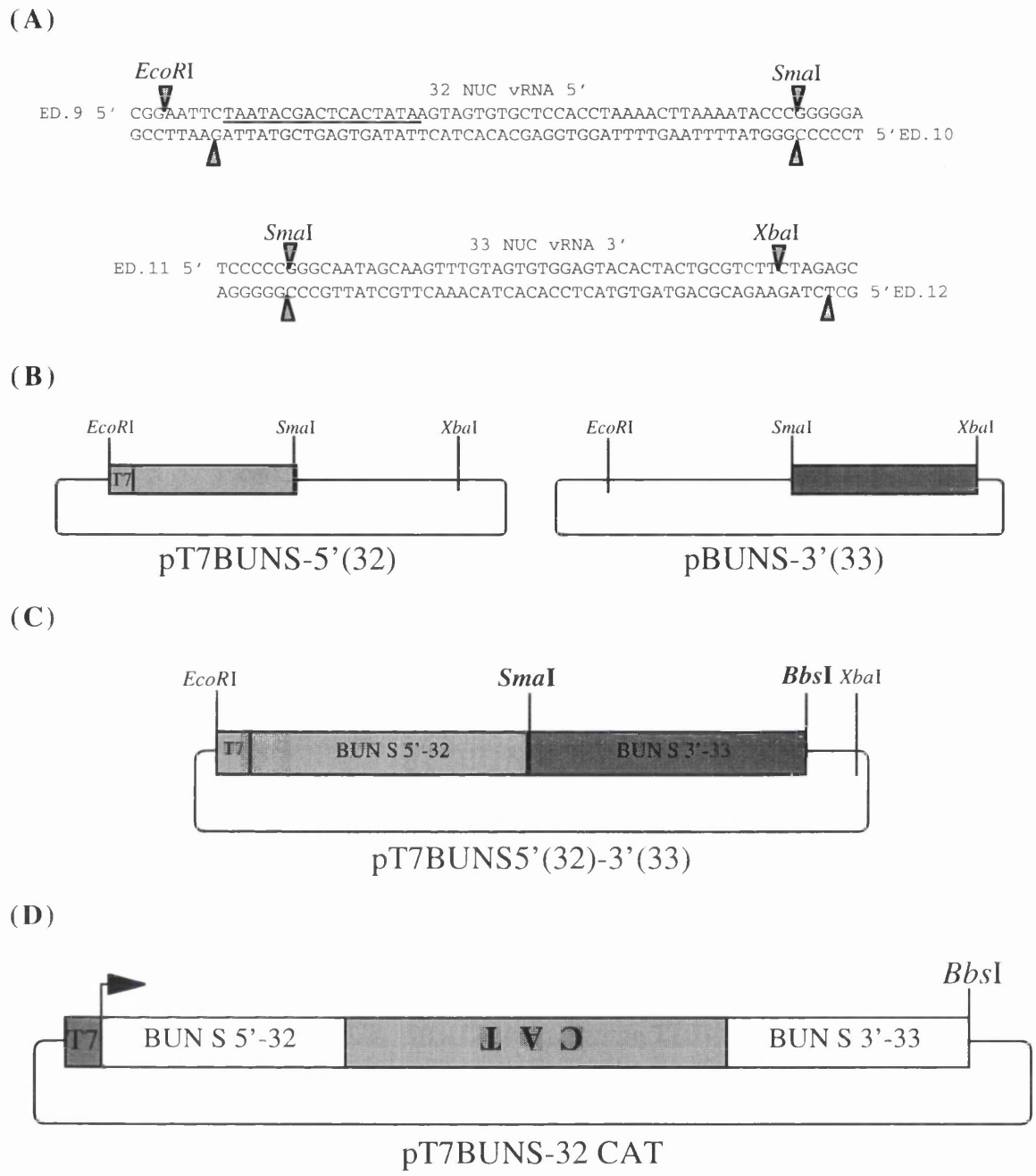


Figure 10.3 Construction of pT7BUNS-32CAT.

Generation of the cloning cassette shown in (C), was achieved by a two-step procedure. Two sets of complementary oligonucleotides, ED.9 & ED.10, and ED.11 & ED.12, were annealed as described earlier (Figure 10.1), and subsequently digested with *Eco*RI/*Sma*I, and *Sma*I/*Xba*I, respectively. The resulting fragments were then purified by silica matrix absorption, and ligated into similarly digested pUC118, to produce the constructs pT7BUNS-5'(32) and pBUNS-3'(33), as shown in (B). The *Sma*I/ *Xba*I fragment from pBUNS-3'(33) was removed and ligated into pT7BUNS-5'(32), to result in pT7BUNS5'(32)-3'(33), as shown in (C). The CAT ORF was then cloned into the *Sma*I site of pT7BUNS5'(32)-3'(33). Resulting clones were digested and sequenced to confirm the presence and orientation of the CAT gene, and the fidelity of sequence of the flanking regions. The resulting construct (D), which contained a full length CAT ORF, flanked by 32 nucleotides of the BUNS 5' UTR and 33 nucleotides of the BUNS 3' UTR, was denoted pT7BUNS-32CAT.

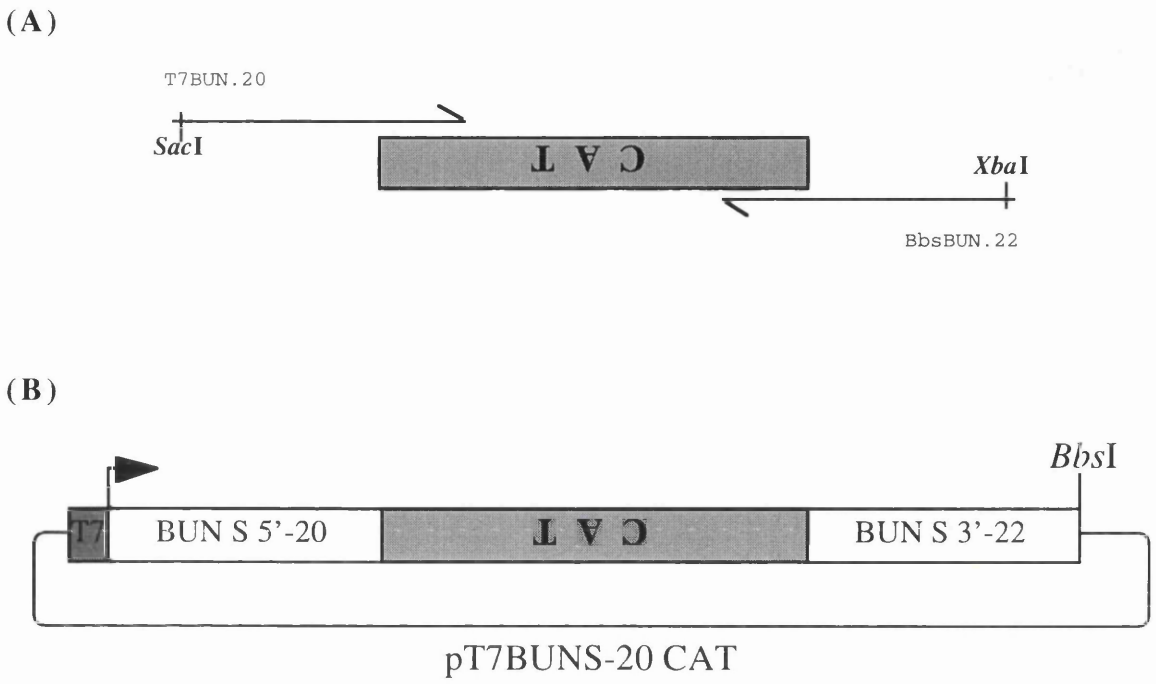


Figure 10.4 Construction of pT7BUNS-20CAT.

Two oligonucleotides were designed as primers to amplify and flank a CAT ORF with BUNS termini sequence by PCR. (A). Oligonucleotide T7BUN.20 (5' GGGGAGCTCTAATA CGACTCACTATAAGTAGTGTGCTCCACCTAAA**TTACGCCCCGCCCCTGCCA**) encoded, in the 5' to 3' direction, a *Sac*I site for cloning, a T7 promoter (underlined), the first 20 nucleotides of the BUNS 5' UTR and then the complement of the last 19 nucleotides of the CAT ORF (bold, stop codon underlined). Oligonucleotide BbsBUN.22 (GGGTCTAGAAGAC GCAGTAGTGTACTCCACACTACAA**ATGGAGAAAAAAATCACTGGA**) encoded, in the 5' to 3' direction, a *Xba*I site for cloning, *Bbs*I site, the terminal 22 nucleotides of the BUNS 3' UTR followed immediately by the first 21 nucleotides of the CAT ORF (bold, start codon underlined). The resulting PCR fragment was digested with *Sac*I/ *Xba*I, and cloned into similarly digested pUC118 (B). Positive clones were sequenced to confirm fidelity of sequence of the CAT gene and the flanking regions. The resulting construct contained a full length antisense CAT ORF flanked by 20 and 22 nucleotides of the BUNS 5' and 3' termini, and was denoted pBUNS-20CAT.

10.2 Production of run-off transcripts from the mini-reporter constructs.

As in the case of pBUNSCAT, transcription of RNA from the mini-reporter constructs initiated from a truncated T7 promoter at the 5' A residue of the BUN S 5' terminus and terminated at the authentic 3' U residue due to the engineered *Bbs*I downstream of the 3' terminus.

RNA transcripts derived from the pBUNS-13CAT plasmid, therefore, possessed the authentic ends of the BUN S segment RNA, and contained, in the 5' to 3' order, the 13 nucleotides of the 5' UTR of the BUN S segment, the entire CAT gene in a negative-sense polarity, and 13 nucleotides of the BUN S segment 3' UTR. Similarly transcripts derived from the BUNS-20CAT plasmid contained, in the 5' to 3' order, 20 nucleotides of the 5' UTR of the BUN S segment, the entire CAT gene in a negative-sense polarity, and 20 nucleotides of the BUN S segment 3' UTR, and transcripts from the pBUNS-32CAT plasmid contained, in the 5' to 3' order, the 32 nucleotides of the 5' UTR of the BUN S segment, the entire CAT gene in a negative-sense polarity, and 33 nucleotides of the BUN S segment 3' UTR.

In-vitro synthesis of run-off RNA transcripts from each the mini-reporters was achieved as described previously. After DNaseI treatment, the resulting RNA transcripts were purified from the transcription reaction using the RNeasy Mini Spin column (QIAGEN) clean-up protocol (Methods 2.4.4), and resuspended in RNase-free water. The RNA concentration for each of the transcripts was determined by spectrophotometery as described previously. Figure 10.5 shows RNA transcripts derived from pBUNS-13CAT, pBUNS-32CAT and pBUNS-20CAT, shown alongside RNA derived from pBUNSCAT for comparison.

10.3 CAT activity detected from BUNS-13CAT, BUNS-20CAT and BUNS-32CAT mini-reporter RNA transcripts in the reporter assay.

It was important that an equal number of transcripts from each clone were transfected into the reporter system, and therefore the amount of transfected RNA was adjusted to take into account the size difference of the transcripts. Thus, an equimolar amount of each transcript was transfected (Table 10.1).

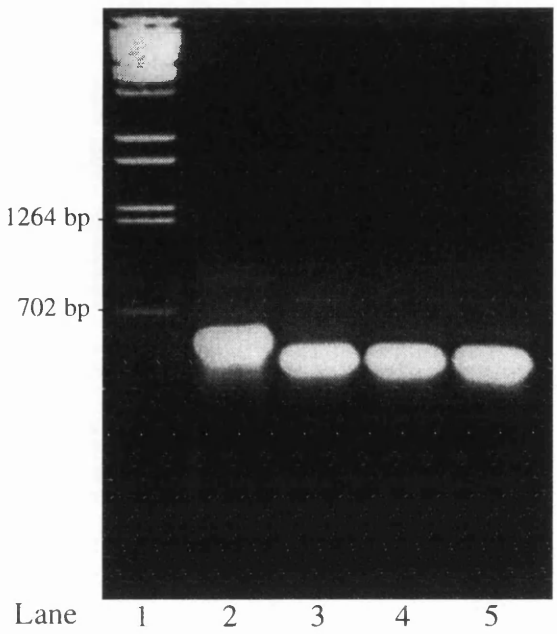


Figure 10.5 Minireporter run-off transcripts.

The run-off transcripts from *Bbs*I linearised pBUNS-13CAT (Lane 5), pBUNS-20CAT (Lane 4) and pBUNS-32CAT (Lane 3) were compared with pBUNSCAT RNA transcripts (Lane 2). λ *Bst*EII markers (0.5 μg) are shown in lane 1.

BUNS-CAT clone	transcript length in nucleotides	% in length of BUNSCAT	amount of RNA transfected
pBUNSCAT	919	100%	2.50μg
pBUNS-32CAT	728	79.2%	1.98μg
pBUNS-20CAT	702	76.4%	1.91μg
pBUNS-13CAT	688	74.9%	1.87μg

Table 10.1 Transfection of BUNSCAT mini-reporter RNAs.
This table describes the adjustments made to the amount of RNA transfected when the length of the transcript is taken into consideration. This ensured that equimolar amounts of each transcript were transfected onto cell expressing the BUN viral proteins.

The RNAs transcribed from the pBUNS-13CAT and pBUNS-32CAT, and pBUNS-20CAT mini-reporter clones were used in the reporter system along with BUNSCAT RNA as a control.

A comparison of the level of reporter gene expression obtained from BUNS-13CAT, BUNS-32CAT, and BUNS-20CAT mini-reporter RNA transcripts in the reporter assay is shown in Figure 10.6. Extracts from cells transfected with BUNSCAT RNA are shown in lanes 1, 2, and 3, extracts from cells transfected with BUNS-32CAT are shown in lanes 4, 5, and 6, extracts from cells transfected with BUNS-20CAT are shown in lanes 7, 8, and 9, and extracts from cells transfected with BUNS-13CAT are shown in lanes 10, 11, and 12,

From this experiment it was apparent after quantification, that the highest level of CAT activity was obtained from BUNSCAT RNA; this was taken as 100%. Transfection of BUNS-32CAT RNA by comparison, was calculated to be between 63% and 70% of "wild type" *i.e.* BUNSCAT CAT activity. Both BUNS-20CAT and BUNS-13CAT produced levels of CAT activity that were extremely low (< 5%), and since sequencing confirmed that the CAT gene of these constructs was intact and without errors, it was concluded that this result indicated a drastic loss of function in these RNA transcripts.

10.4 Discussion.

It was discussed earlier in this chapter that there may be possible base-pairing between the 3' and 5' termini of the BUN S, M and L-segments. Additionally, the first 11 nucleotides of the

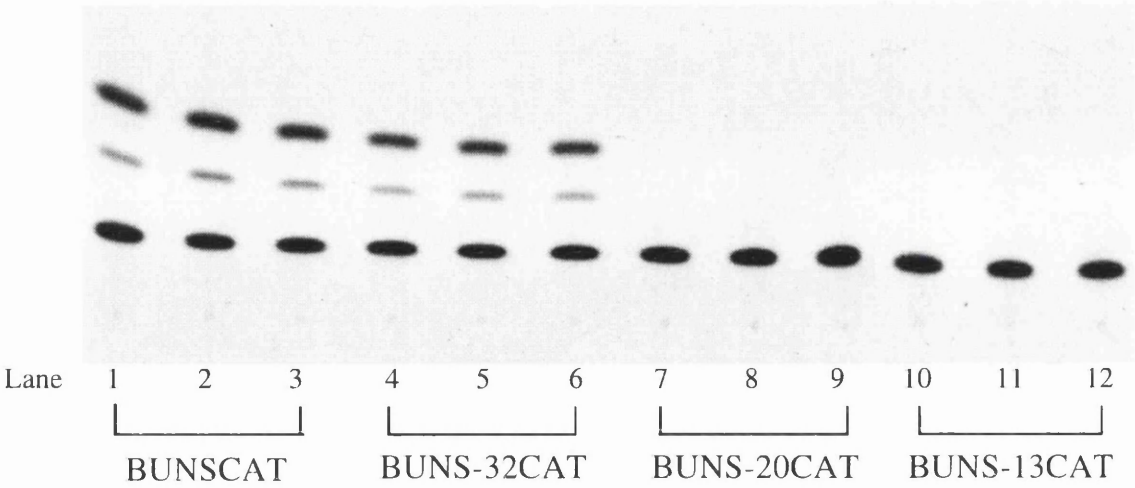


Figure 10.6 Comparison of reporter gene expression from BUNS-CAT, BUN-32CAT, BUN-20CAT and BUNS-13CAT RNA transfections.

CV-1 cells transiently expressing BUN viral proteins were transfected with equal quantities of BUNSCAT (Lanes 1, 2 and 3), BUNS-32CAT (Lanes 4, 5 and 6) BUNS-20CAT (Lanes 7, 8 and 9) and BUNS-13CAT (Lanes 10, 11 and 12) RNA transcripts. This was achieved by adjusting the amount of transfected RNA to account for the length of each transcript (BUNSCAT (919 nucleotides), BUNS-32CAT (728 nucleotides) BUNS-20CAT (702 nucleotides) and BUNS-13CAT (688 nucleotides)) so that a equivalent number of molecules for each transcript was transfected. The ratio of transfected BUNSCAT: BUNS-32CAT: BUNS-20CAT: BUNS-13CAT, in μg, was 1: 0.792: 0.764: 0.749 respectively. Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration before equivalent amounts of cell protein in each cell extract were assayed for CAT activity.

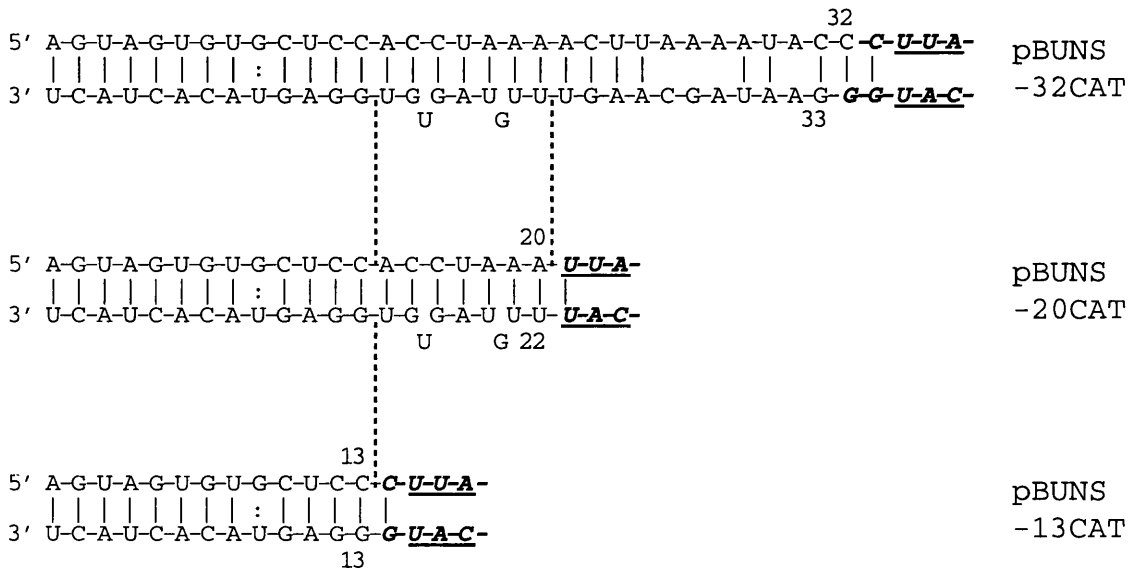


Figure 10.7 Comparison of the termini from the BUNSCAT mini reporter constructs.

The portions of the BUNS UTR flanking the CAT gene of the mini reporter constructs are shown schematically above. The diagram also depicts the possible complementary base pairing of the BUNS 5' and 3' termini flanking the CAT gene. Non-BUNS UTR sequences are shown in bold italics, and the CAT initiation stop codons are underlined.

segment 5' UTR, and the last 11 nucleotides of the 3' UTR are conserved between the termini of all bunyaviruses sequenced thus far, which suggests that this region must be highly important, if not essential.

On the basis of the predicted base pairings, and the observation that the terminal 11 nucleotides of the S segment are conserved, it was decided that these regions should be investigated. Therefore, three mini-reporter constructs were built, pBUNS-13CAT, pBUN-20CAT and pBUNS-32CAT, which contained the antisense CAT gene flanked by various lengths of the BUNS termini. Figure 10.7 depicts a diagram which highlights the differences in length, and the possible complementary base pairing of the termini of the minireporter RNAs.

When the RNA transcripts from the mini-reporter constructs were used in the reporter assay, it was found that transfection of RNA transcripts from pBUNS-13CAT and pBUNS-20CAT resulted in no significant CAT activity. This finding indicated that flanking sequences of either 13 or 20 nucleotides of the 5' and 3' termini, were insufficient to allow transcription of the reporter gene.

CAT activity which was between 63 and 71% of "wild type", *i.e.* the BUNSCAT signal, was detected from transfection of the BUNS-32CAT RNA. This suggested that 32 nucleotides of the 5' BUN S UTR and 33 nucleotides of the 3' BUN S UTR were sufficient for the recognition and subsequent transcription of the reporter RNA by BUN viral proteins. However, given that this reporter RNA gave a signal which was not wild type level, it is suggested that sequences outwith this region may also be important for full transcriptional activity from the reporter. It can also be concluded from this experiment that the minimum requirement of nucleotides of the 5' and 3' BUN S UTRs necessary for recognition and transcription, is more than 20, and may be less than 32.

A paper from this laboratory (Bridgen and Elliott, 1996), published subsequently to this study, demonstrated the rescue of a recombinant Bunyamwera virus from three full length cDNA clones. This important paper is discussed in more depth in Chapter 1 section 1.8.5. However, in this study, on the basis of the results obtained with the pBUNS-32CAT minireporter, this construct was utilised to build similar construct termed pT7riboBUNSGFP(-). This minireporter contained an antisense GFP (Green Fluorescent Protein) ORF in place on the CAT gene, and was used essentially to optimise the transfection protocols in the rescue procedures used in these experiments. RNA transcripts transcribed *in-vivo* from pT7riboBUNSGFP contained, in the 5' to 3' direction, two G residues derived from the cloning site, 32 nucleotides of the BUNS 5' UTR, the antisense GFP ORF, and the 33 nucleotides of the BUNS 3' UTR.

Transfection of pT7riboBUNSGFP(-) into cells expressing the proteins from the BUNL, M, and S-segments, resulted in the expression and subsequent detection of GFP. This was in agreement with the pBUNS-32 CAT results, and demonstrated that an antisense reporter ORF flanked by 32 nucleotides of the BUNS 5' and 33 nucleotides of the 3' UTRs, could be recognised and transcribed by the recombinant BUN viral proteins to result in expression of the reporter protein.

It was also demonstrated in this study that when extracts prepared from these cells were subsequently overlaid onto cells expressing BUN viral proteins (either by BUN virus infection or by transient expression), some of the new cells were observed to express GFP. No GFP was detected in cells which were not expressing BUN viral proteins. From this result, it was suggested that the BUNSGFP RNA transcripts had been packaged into BUN virus-like particles that were capable of infecting new cells.

This result would suggest therefore, that the terminal 32 nucleotides and 33 nucleotides of the BUNS 5' and 3' UTRs are sufficient, and contain the appropriate *cis* acting signals, not only for transcription and replication of the reporter RNA, but also for packaging of the transcript into virus-like particles.

Chapter 11.

11.0 Investigation of the untranslated regions in the BUN genomic segments.

11.0 Introduction.

Throughout the course of a bunyavirus infection, viral mRNA transcripts of the L, M and S segments can be detected at different levels in molar terms within the cell. The S mRNA species is more abundant than the M mRNA species, which in turn is more abundant than the L mRNA species; it has been estimated that the ratios are 40: 10: 1 respectively (Vezza *et al.*, 1979; Rossier *et al.*, 1988). The exact reasons for these differences are unknown, but it is thought that *cis*-acting signals encoded within the untranslated regions of each of the three genomic segments may control or influence transcription and replication events.

Alignment and comparison of the BUN genomic full length UTRs, shown in Figure 11.1, highlights that other than the terminal 11 conserved nucleotides, there are few residues in the remaining regions of the 5' and 3' UTRs that are conserved between the BUN segments. There are no other obvious sequence motifs common to all three segments to which *cis*-acting functions, related to transcription and replication, could be attributed.

To investigate the possibility that control of transcription and replication is mediated by signals in the untranslated regions CAT reporter constructs, containing the BUN L and M segment UTRs were constructed. Since measurement of CAT activity is essentially an indication of the amount of CAT protein translated from mRNA transcripts, it was hoped that use of these constructs in the reporter system would determine the influence of the BUN L and M UTRs on the level of CAT mRNA transcript .

11.1 Construction of pBUNLCAT and pBUNMCAT reporter constructs.

The pBUNLCAT and pBUNMCAT reporter constructs were built using a PCR approach similar to that used in the construction of pBUNSCAT , (Chapter 3.1). Again, as in the case of pBUNSCAT, transcription of RNA from pBUNLCAT and pBUNMCAT initiated from a truncated T7 promoter at the 5' A residue of the BUN L and M genome, and terminated at the exact 3' residue of the BUN genomes, due to linearisation of the plasmids by the

(A)

```
                1                                                    50
ncr5.msf{BunL}  AGTAGTGTGC TCCtaCatAA gaaaAttgTA CttTTttgAA TgCtGTtaTc
ncr5.msf{BunM}  AGTAGTGTGC T~~~~~~~~~ ~~~~~~~~~A CCgaTAACAA aACAGccTTG
ncr5.msf{BunS}  AGTAGTGTGC TCCacCtaAA acttAaaaTA CCaTTAACAA TAtAaTgTTG
     Consensus  AGTAGTGTGC TCC--C--AA -a-tAt--TA CC-TTAACAA TACAGT-TTG
                ********** *                   * *   *   **         *
                51                                                  100
ncr5.msf{BunL}  ATTTTTGCCA CAtaGTGctT tTggaAtgTC caAtTaaATA aAcCAATGAA
ncr5.msf{BunM}  tTTTTTGaCA CAaTGTGTCa aTatgAAtTt tgAaTgtATt tAACAATGAA
ncr5.msf{BunS}  ATTTagcCCg CtgTcTtTCT gTccccAacC acccacccaA gcAgctgttA
     Consensus  ATTTTTGCCA CA-TGTGTCT -T---AA-TC --A-T--ATA -AACAATGAA
                 ***    *  *    *      *                          *
                101                                                 150
ncr5.msf{BunL}  gaTATgtt~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
ncr5.msf{BunM}  TTaATaaccg TctAAttt~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
ncr5.msf{BunS}  TTTtgtgggt TgaAAcaacc cttttagcca aattaaaaca gatgacttta
     Consensus  TTTAT----- T--AA----- ---------- ---------- ----------

                151                  174
ncr5.msf{BunL}  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~
ncr5.msf{BunM}  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~
ncr5.msf{BunS}  gcccgattaa aaatgcatcc ctgc
     Consensus  ---------- ---------- ----
```

(B)

```
                1                                                    50
ncr3.msf{BunL}  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~AcTcC taCTGgTTaT
ncr3.msf{BunS}  taaagagcct ttaatgacct tctgttggat TTaatAgcAC AGCgaTTTtc
ncr3.msf{BunM}  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~c TTggaAaTAa AGaTGTgTcT
     Consensus  ---------- ---------- ---------- TT---A-TAC AGCTGTTT-T
                                                      *            *
                51                                    85
ncr3.msf{BunL}  ATtttTAaaT TtTcTaTAtg taGGAGTACA CTACT
ncr3.msf{BunS}  AacAATAGca aGTtTgTAgT gtGGAGTACA CTACT
ncr3.msf{BunM}  cTgAAagGtT TGTgatgtaT cgGtAGTACA CTACT
     Consensus  AT-AATAG-T TGT-T-TA-T --GGAGTACA CTACT
                           *          * ****** *****
```

Figure 11.1 Conserved nucleotides in the 3' and 5' UTRs of the BUN virus genomic segments.

Alignments of the BUN L, M and S segment UTRs were generated using the PILEUP program of the GCG Wisconsin Package™ (version 9). Sequences were aligned in the genomic RNA sense in a 5' to 3' manner. Capital letters highlight residues conserved in two or more segments and the consensus sequence, while those residues conserved in all three segments are marked with an asterisk. Figure (A) shows the alignment of the 5' UTRs, while (B) shows the alignment of the 3' UTRs. The figure demonstrates that besides the terminal eleven nucleotides (conserved in all bunyaviruses), few residues are conserved between all three BUN segments.

restriction enzyme *Bbs*I. Thus T7 transcripts derived from pBUNLCAT and pBUNMCAT contained the authentic termini of the genome segments, with a CAT ORF of negative polarity, flanked by the 5' UTR (108 nucleotides) and 3' UTR (50 nucleotides) of the BUN L genome or alternatively, flanked by the 5' UTR (103 nucleotides) and 3' UTR (56 nucleotides) of the BUN M genome segment.

11.2 The design of primers to construct pBUNLCAT and pBUNMCAT.

The oligonucleotides L-START and L-STOP were designed as primers to amplify the CAT open reading frame from pTZCAT(-) and tail it with short regions of the Bunyamwera virus L segment UTR which would normally flank the L protein coding region. The oligonucleotides T7-L and *Bbs*I-L were designed as primers to amplify the 5' and 3' UTRs respectively. T7-L placed a T7 promoter upstream of the 5' UTR such that transcription initiated at +1 of the BUN L 5' UTR. The primer BbsI-L positioned a *Bbs*I restriction site down stream of the 3' UTR so that T7-directed run-off transcription terminated at the exact nucleotide of the 3' UTR (Figure 11.2A).

Oligonucleotides were designed in a similar manner to act as primers in the construction of pBUNMCAT: these were denoted M-START, M-STOP, T7-M and BbsI-M (Figure 11.3).

11.3 Generation of BUN L and M 5' and 3' UTRs containing cDNA restriction fragments.

cDNA templates containing the complete untranslated regions of the BUN M and BUN L segments were obtained as follows: pT7-BUNM-Ribo(-) (Bridgen and Elliott, unpublished work) was digested with *Cla*I and *Xba*I to produce a fragment containing the complete BUN M 3' UTR and plasmid pT7-BUNM-Ribo(+) (Bridgen and Elliott, 1996) was digested with *BamH*I to obtain a fragment containing the complete BUN M 5' UTR. To obtain cDNA fragments containing the complete BUN L 3' and 5' UTRs, pT7-BUNL(M7)-Ribo(+) (Bridgen and Elliott, 1996) was either digested with *Eco*RI, to obtain the BUN L 3' UTR fragment, or *Xba*I to obtain the BUN L 5' UTR fragment. Digestion reactions were run on a 1% agarose TAE gel, the desired cDNA fragments were removed and purified by silica matrix absorption.

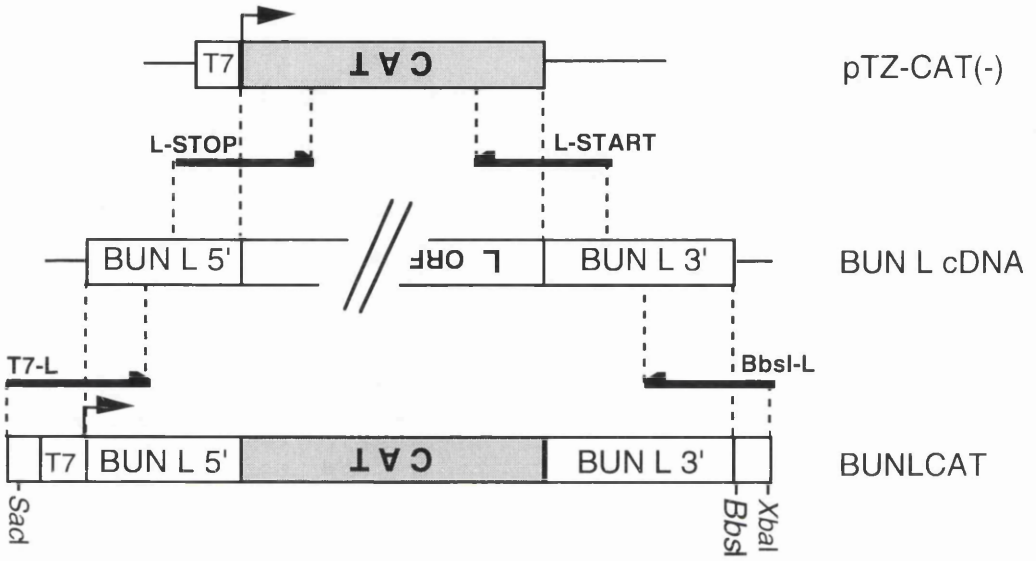


Figure 11.2 (A) Construction of pBUNLCAT.

The sequence of the primers were as follows:
L-STOP: 5'AACCAATGAAGATATGTT**TCA**CGCCCCGCCCTGCCACTC. The underlined sequence shown above, corresponds to BUN L segment cDNA nucleotides 6785-6768, which immediately follow the L protein reading frame. The remainder of the sequence is the complement of the last 21 nucleotides of the CAT reading frame starting with the complement of the stop codon (bold).
L-START: 5' AATATAACCAGTAGGAGT**ATG**GAGAAAAAAATCACTGGA.
The underlined sequence corresponds to BUN L segment cDNA nucleotides 33-50 which immediately precede the L protein reading frame. The remainder of the sequence is the first 21 nucleotides of the CAT reading frame starting with the initiation codon (bold).
T7L(-):5' ATTCGAGCTC**TAATACGACTCACTATA**AGTAGTGTGCTCCTACATA AGAAAATT. The underlined sequence corresponds to the complement of the last 27 nucleotides of the Bunyamwera virus L segment cDNA. This is immediately preceded by a truncated T7 promoter (17 nucleotides ending TATA (bold)) and an *Sac*I restriction site for cloning.
Bbs1-L(-): 5' AGACTCTAGAAGACGCAGTAGTGTACTCCTACATATAGAAAATT. The underlined sequence corresponds to the first 28 nucleotides of the Bunyamwera virus L segment cDNA. The remainder of the sequence codes for a *Xba*I restriction site for cloning and an inverted *Bbs*I site to allow cleavage at the viral cDNA terminus for run-off transcription.

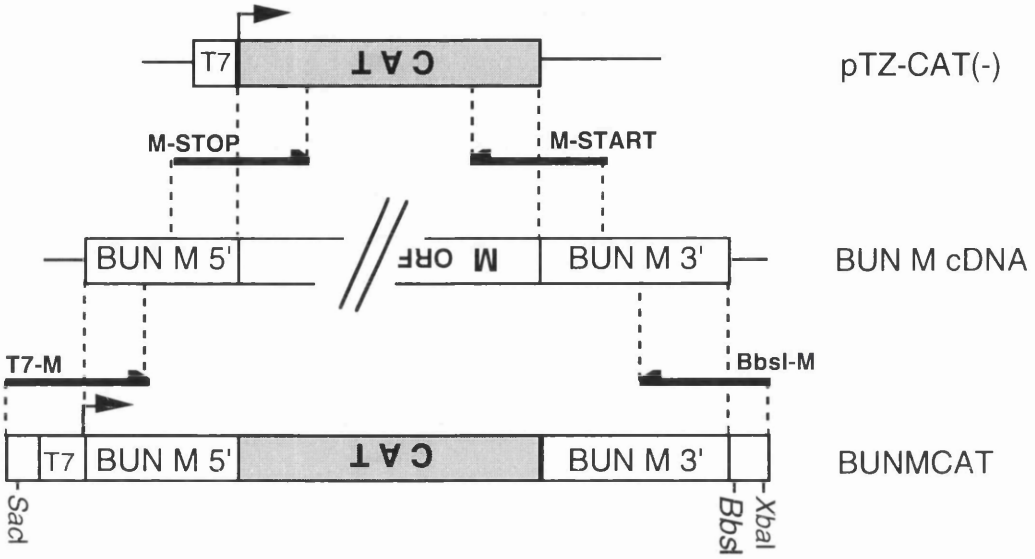


Figure 11.2 (B) Construction of pBUNMCAT.

The sequence of the primers are as follows:
M-STOP: 5' ATAACCGTCTAATTT**TCA**CGCCCCGCCCTGCCACTC. The underlined sequence shown above, corresponds to BUN M segment cDNA nucleotides 4359-4373 which immediately follow the M segment reading frame. The remainder of the sequence is the complement of the last 23 nucleotides of the CAT reading frame starting with the complement of the stop codon (shown in bold).
M-START: 5' CACATCTTTATTTCCAAG**ATG**GAGAAAAAAATCACTGGA. The underlined sequence corresponds to BUN M segment cDNA nucleotides 39-56, which immediately precede the M segment reading frame. The remainder of the sequence is the first 20 nucleotides of the CAT reading frame starting with the initiation codon (bold).
T7-M(-): 5' ATTCGAGCTC**TAATACGACTCACTATA**AGTAGTGTGCTACCGAT AACAAAACAG. The underlined sequence corresponds to the complement of the last 27 nucleotides of the Bunyamwera virus M segment cDNA. This is immediately preceded by a truncated T7 promoter (17 nucleotides ending TATA (bold)) and an *Sac*I restriction site for cloning.
Bbs1-M(-): 5' AGACTCTAGAAGACGCAGTAGTGTACTACCGATACATCACAAAC. The underlined sequence corresponds to the first 28 nucleotides of the Bunyamwera virus M segment cDNA. The remainder of the sequence codes for a *Xba*I restriction site for cloning and an inverted *Bbs*I site to allow cleavage at the viral cDNA terminus for run-off transcription.

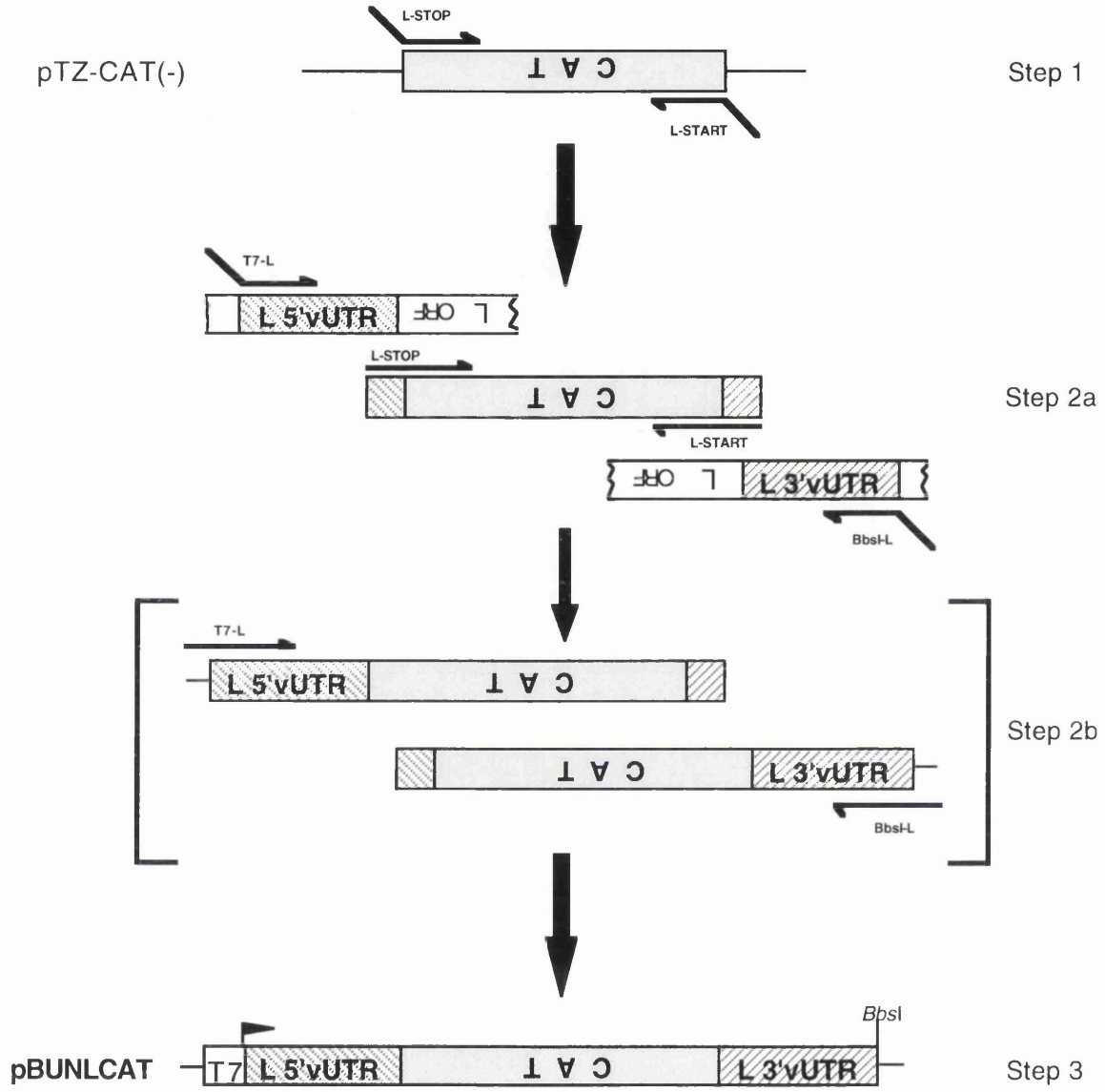


Figure 11.3 Schematic protocol for the construction of pBUNLCAT.

The same protocol was used to construct pBUNMCAT, but with M segment cDNAs and the oligonucleotides described in figure 11.2B.

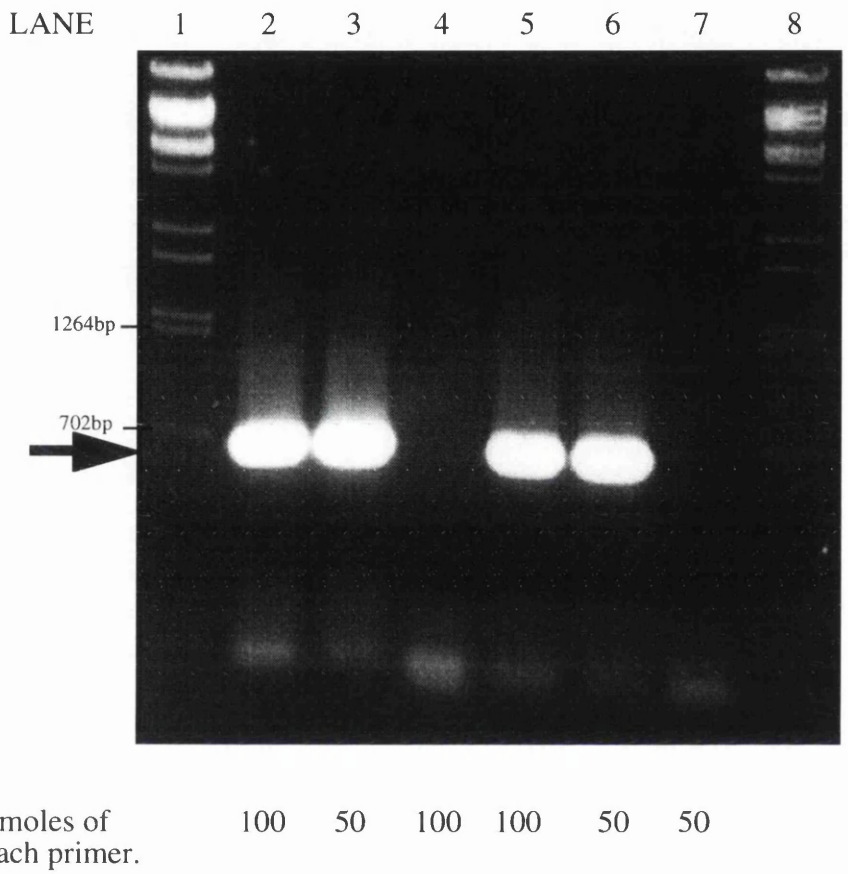


Figure 11.4 Amplification of the CAT open reading frame from pTZCAT(-).

A DNA fragment flanked by short sequences from the BUN M or the BUN L segments was generated by PCR from pTZCAT(-) (100ng), using the primer pairs M-START and M-STOP (Lanes 2 and 3), and L-START and L-STOP (Lanes 5 and 6). Lanes 4 and 7 shows a control PCR reaction in which all four primers were included, but no template. Lanes 1 and 8 contain λ *Bst*EII size markers (0.5 μg). The PCR generated cDNA fragments in lanes 3 and 6 (position marked by arrow) were cut from the gel and purified by silica matrix adsorption (methods 2.3.7), then used as templates in further PCR reactions (see figure 11.2 (B)).

11.4 Generation of pBUNLCAT and pBUNMCAT.

The strategy used in the construction of pBUNLCAT is shown schematically in Figure 11.3. Note that pBUNLCAT and pBUNMCAT were constructed in a similar manner. In the first step, a CAT open reading frame was amplified by PCR using oligonucleotides L-START and L-STOP, which were designed to tail the CAT sequence with short regions identical to those flanking the BUN L or BUN M open reading frames: The resulting PCR products of approximately 696 base-pairs are shown in Figure 11.4, Lanes 2, 3 and 5, 6 respectively.

For the next step (Figure 11.3, step 2a), in a "three way" PCR reaction, two BUN L segment termini cDNA fragments were recombined with the BUN-tailed CAT gene. Initially this reaction (Figure 11.3 step 2b) produced two overlapping fragments which both contained the CAT coding sequence recombined by PCR with the 3' or 5' untranslated L segment sequences, together with the relevant terminal modifications (Figure 11.3, step 2b). These fragments were then combined and amplified with the terminal primers to produce the final full length expression cassette, to result PCR products of approximately 825 base-pairs as shown in Figure 11.5 (Lanes 4 and 7), (Figure 11.3, step 3). This final fragment was digested with restriction enzymes *Sac*I and *Xba*I and then ligated into *Sac*I and *Xba*I digested pUC119. Sequencing of each clone into the CAT open reading frame confirmed the sequences of the T7 promoter, *Bbs*I site and the BUN termini and that no spurious mutations had occurred during PCR. Two clones for each construct were selected, and were denoted pBUNMCAT (2) and (3) and pBUNLCAT (22) and (23).

11.5 Production of run-off RNA transcripts from pBUNMCAT and pBUNLCAT clones.

DNA from each of the clones pBUNMCAT (2) and (3) and pBUNLCAT (22) and (23) was linearised by digestion with *Bbs*I to act as a template for *in vitro* transcription (Figure 11.6). After DNaseI treatment, the resulting RNA transcripts were purified from the transcription reaction using the RNeasy Mini Spin column (QIAGEN) clean-up protocol (Methods 2.4.4), and resuspended in RNase-free water.

The RNA concentration for each of the transcripts was determined by spectrophotometery as described previously (Methods 2.4.4). It was important that an equal number of transcripts from each clone were transfected into the reporter system, therefore, the amount of transfected RNA was adjusted to take into account the size difference of the transcripts. Thus, an equimolar amount of each transcript was transfected (Table 11.1).

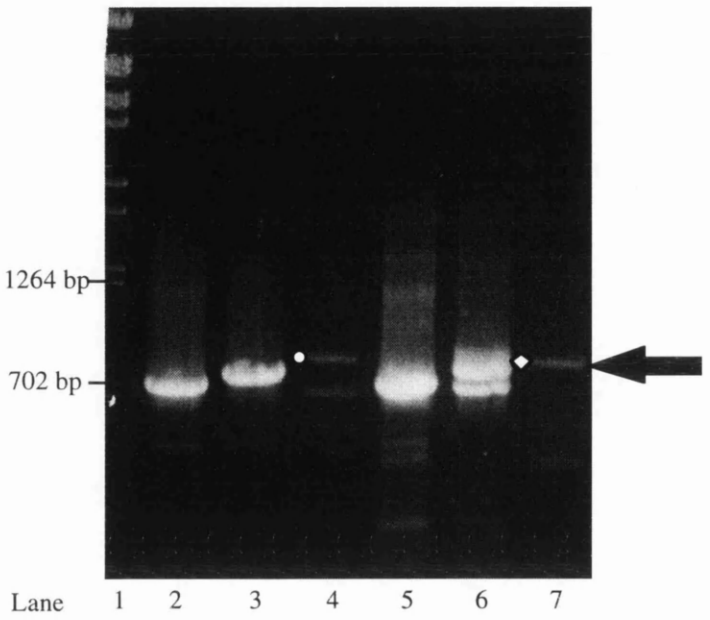


Figure 11.5 Recombinant PCR of the M and L tailed CAT gene with BUNM and BUNL segment termini.

A 1% TAE agarose gel was loaded with the products of PCR reactions set up with the following template and primer combinations:- Lane 2 contained two templates, the M-CAT tailed fragment and the M 3' UTR(*Cla*I, *Xba*I) fragment. Primers in this reaction were M-STOP and BbsI-M. Lane 3 contained two templates, the M-CAT tailed fragment and M 5' UTR(*Bam*HI) fragment. Primers in this reaction were M-START and T7-M. Lane 4 contained three templates, the M-CAT tailed fragment, M 3' UTR(*Cla*I, *Xba*I) fragment and the M 5' UTR(*Bam*HI) fragment. The primers in this reaction were M-STOP, M-START , BbsI-M and T7-M. Lane 5 contained two templates, the L-CAT tailed fragment and the L 3' UTR(*Eco*RI) fragment. Primers in this reaction were L-STOP and BbsI-L. Lane 6 contained two templates, the L-CAT tailed fragment and L 5' UTR(*Xba*I) fragment. Primers in this reaction were L-START and T7-L. Lane 7 contained three templates, the L-CAT tailed fragment, L 3' UTR(*Eco*RI) fragment and the L 5' UTR(*Xba*I) fragment. The primers in this reaction were L-STOP, L-START, BbsI-L and T7-L. Lanes 2 and 5, and lanes 3 and 6 represent the intermediate PCR fragments generated by the addition of the 3' and 5' UTRs onto the M and L tailed CAT genes. These PCR reactions served as controls to ensure intermediate fragments of the correct size would be generated in the three way PCR reactions (Lanes 4 and 7). Lanes 4 and 7 contain the final full length PCR products marked of approximately 850 basepairs, BUNMCAT(circle) and BUNLCAT(diamond) respectively. The cDNA fragments in lanes 4(circle) and 5(diamond) were removed from the gel, purified and digested with *Sac*I and *Xba*I before being ligated into *Sac*I, *Xba*I cut pUC119 vector.

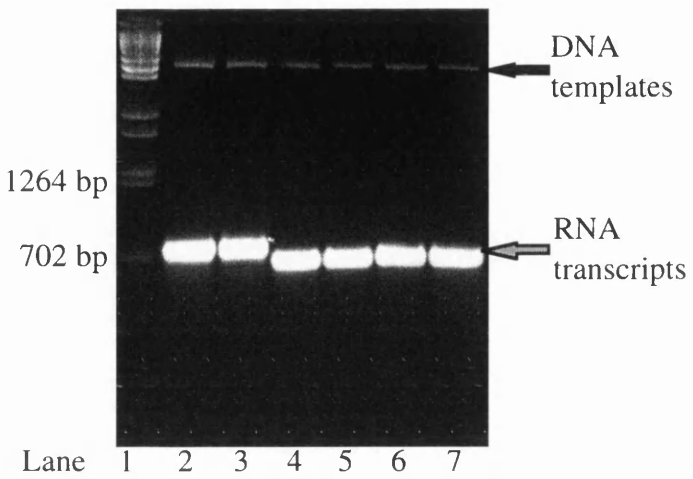


Figure 11.6 *In vitro* transcription from *Bbs*I linerised templates.

A 1% TBE agarose gel was loaded with a 4 µl aliquot from each 100 µl run-off transcription reaction, before the addition of RQ1 DNase. Lanes 2 and 3 contain RNA transcripts from *Bbs*I cut pBUNSCAT. Lanes 4 and 5 contain RNA transcribed from *Bbs*I cut pBUNMCAT. Lanes 6 and 7 contain RNA transcribed from *Bbs*I linearised pBUNLCAT. Lane 1 contains λ *Bst*EII size markers (0.5 µg).

BUN-CAT clone	transcript length nucleotides	% in length	amount of RNA transfected
pBUNSCAT	919	100%	2.50 μg
pBUNMCAT	819	89.12%	2.23 μg
pBUNLCAT	818	89.01%	2.23 μg

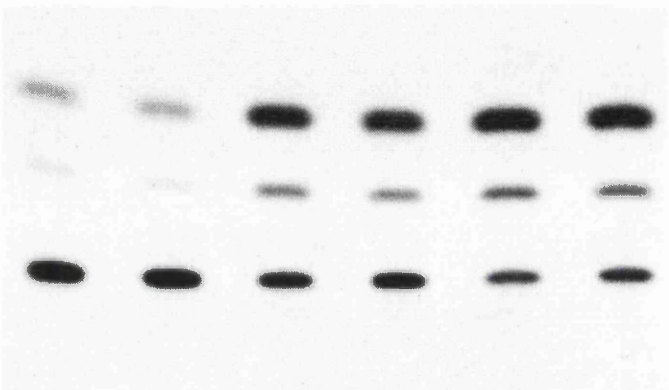
Table 11.1 Transfection of genomic BUNCAT transcripts.
The above table demonstrates the adjustments made to the amount of RNA transfected when taking the length of the transcript into consideration, ensuring an equimolar amount of each transcript was transfected.

11.6 Comparison of CAT activity from (genomic CAT constructs) BUNLCAT, BUNMCAT and BUNSCAT RNA in the reporter assay.

The RNAs transcribed from the pBUNLCAT, pBUNMCAT and pBUNSCAT clones (Figure 11.6) were applied to the reporter system using the same protocol as described earlier (Section 3.1). The quantity of cell extract assayed was adjusted for protein concentration before assaying for CAT activity and phosphorimager quantification.

A comparison of the level of reporter gene expression obtained from transfecting BUNSCAT, BUNMCAT and BUNLCAT RNA in the reporter assay is shown in Figure 11.7. From this experiment it was apparent that the highest level of CAT activity was obtained from transfection of BUNLCAT RNA, and the lowest level from transfection of BUNSCAT RNA. If the level of CAT expression from transfection of BUNLCAT was taken as 100%, then the CAT activity from BUNMCAT RNA was between 60 and 70%, and from BUNS-CAT, between 3 and 10%. Given that equimolar amounts of RNA of each construct were transfected into the reporter system, the efficiency of expression of the reporter gene in the context of each of the BUN segments is in the order of BUNLCAT>>BUNMCAT>>BUNSCAT.

It was not known whether the different levels of CAT expression from each of the three genomic CAT constructs was a result of differences in efficiency of transcription or replication by the BUN viral proteins, or even possibly the efficiency of translation of the mRNA transcripts by host cell proteins (although this last factor was not initially considered to be of issue here). Therefore, in an effort to establish if control of expression of the reporter occurred at the transcriptional level, Northern blot analysis was used to probe total RNA extracts from cells transfected with genomic BUN-CAT transcripts in the reporter assay. Probing was performed as described in Chapter 3.1. However, as was found



Lane	1	2	3	4	5	6
RNA template	BUNSCAT		BUNMCAT		BUNLCAT	

11.7. Comparison of reporter gene expression from BUNS-CAT, BUNM-CAT and BUNL-CAT RNA transfections.

CV-1 cells transiently expressing BUN viral proteins were transfected with equal quantities of BUNSCAT (Lanes 1 and 2), BUNMCAT (Lanes 3 and 4) and BUNLCAT (Lanes 5 and 6) RNA transcripts. This was achieved by adjusting the amount of transfected RNA to account for the length of each transcript i.e., (BUNSCAT (100%), BUNMCAT (89.1%) and BUNLCAT (89%)) so that an equivalent number of molecules for each transcript was transfected. The amount of transfected BUNSCAT, BUNMCAT, BUNLCAT RNA in μg, was 2.50, 2.23 and 2.23 μg respectively. Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration before equivalent amounts of cell protein in each cell extract were assayed for CAT activity.

previously, Northern Blot analysis was unsuccessful in detecting the presence of positive sense transcripts.

11.7 The effect on translational efficiency by changing the sequence context of the AUG initiation codon.

It has been shown previously, that the sequence context of the initiation codon is important for translational efficiency (Kozak, 1986; Kozak, 1996). Since Northern blot analysis was unsuccessful, it was not clear if the observed differences in expression of the CAT reporter gene from each of the genomic BUN-CAT constructs were due to transcriptional control, and thus the possibility arose that translational control might also be responsible for the differing levels of CAT activity obtained from the BUN-CAT constructs. Therefore, it was decided to examine the sequences around the AUG initiation codon in each of the three BUN antigenomic segments for Kozak consensus similarity.

Figure 11.8 (A) shows a comparison of the sequences surrounding the AUG of each of the antigenomic BUN segment RNAs. These are compared against the optimal translation initiation signal consensus, Kozak, (1986). Figure 11.8 (B) shows the same comparison of the antigenomic BUN-CAT RNAs, where the sequence context of the AUG is slightly different due to the presence of the reporter gene in place of the segment ORFs. Again, this is compared against the Kozak sequence. It has been shown previously that the strongest determinant for efficient translation initiation is the presence of a purine (**R**) at position -3, followed by a **G** at position +4 (*i.e.* **R**NNATG**G**) with respect to the initiation codon (Kozak, 1986). It can be seen in Figure 11.8 (A) that the context of the AUG in the BUN L segment antigenomic RNA has the highest Kozak similarity, with an A residue at position -3, and a G residue at position +4. By comparison, the BUN M segment antigenomic RNA has an A at position -3, but no G at position +4, and the BUN S segment antigenomic RNA has neither of these important residues.

However in Figure 11.8 (B), which shows the context of the AUG in the BUN-CAT constructs, it can be seen that each of the BUN-CAT sequences now have a G at position +4. This increases the Kozak similarity of the context of the BUNMCAT initiation codon, to that of the BUNLCAT AUG. Since the BUNMCAT antigenomic RNA also possesses minor Kozak determinants at positions -4 and -5, it might be expected that this transcript would be translated most efficiently to result in the highest CAT signal.

Since the BUNLCAT and BUNMCAT antigenomic RNAs exhibited more similarity to the Kozak consensus than the BUNSCAT antigenomic RNA, it was possible that these

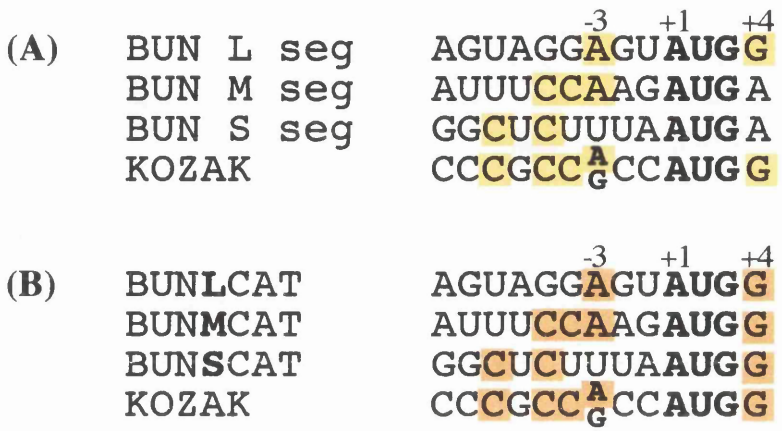


Figure 11.8 Comparison of BUN sequences upstream and downstream of the AUG initiation codon.

The above diagram shows a comparison of BUN sequences upstream and downstream of the initiation codon, specifically, from nucleotides at position -9 to +1, with respect to the AUG initiation codon. This is shown both for each BUN virus antigenome segment (A), and each BUN-CAT antigenome transcript (B). Both of these are shown compared with the optimum eukaryotic translation initiation signal 'KOZAK' (Kozak, 1996). Nucleotides which are conserved between the BUN segments and the Kozak sequence are highlighted.

sequences accounted for the observed differences in expression of the CAT gene from each of these constructs. This seemed possible considering that BUNLCAT and BUNMCAT gave much higher expression than BUNSCAT (10 and 7 fold, respectively). To investigate this, it was decided to mutate nucleotides at position -3, with respect to the initiation codon, in the BUNLCAT and BUNSCAT transcripts, to attempt to increase and decrease, respectively, their similarity to the Kozak sequence. Therefore the U residue at position -3 in the BUNSCAT transcript was mutated to an A, and the A at the same position in the BUNLCAT transcript was changed to a U.

11.8 Construction of BUN-CAT "Kozak" mutants.

Mutation of position -3 residues in the pBUNSCAT and pBUNLCAT reporter constructs was achieved using the Quik Change™ mutagenesis kit by Stratagene. This method was chosen as it offered a low spurious error rate. The protocol entailed designing two sets of complementary oligonucleotides (CAT.6 and CAT.7, CAT.8 and CAT.9) encoding the desired nucleotide change at position -3. These are shown in Figure 11.9. All other methodology was performed exactly according to instructions supplied by the manufacturers of this kit (Methods 2.3.12).

Positive colonies generated using this system were selected and sequenced to confirm the presence of the desired mutations. The resulting constructs were denoted pBUNSCATkozak, which contained a U-->A change at position -3, and pBUNLCATkozak, which encodes a A-->U change at position -3, with respect to their CAT ORF initiation codon. Figure 11.9 shows samples of sequence from clones positive for the desired mutants.

11.9 Transfection of BUNSCATkozak and BUNLCATkozak RNA in the reporter assay.

Run-off RNA transcripts were obtained from *Bbs*I linerised pBUNSCATkozak and pBUNLCATkozak by the same methods described previously.

As described previously, it was important that equivalent amounts of each transcript were transfected to allow comparison of the resulting CAT expressions from the transcripts. Therefore, adjustments were made to take transcript length into account. These adjustments were essentially the same as those made for transcripts from the parental constructs (Table 11.1).

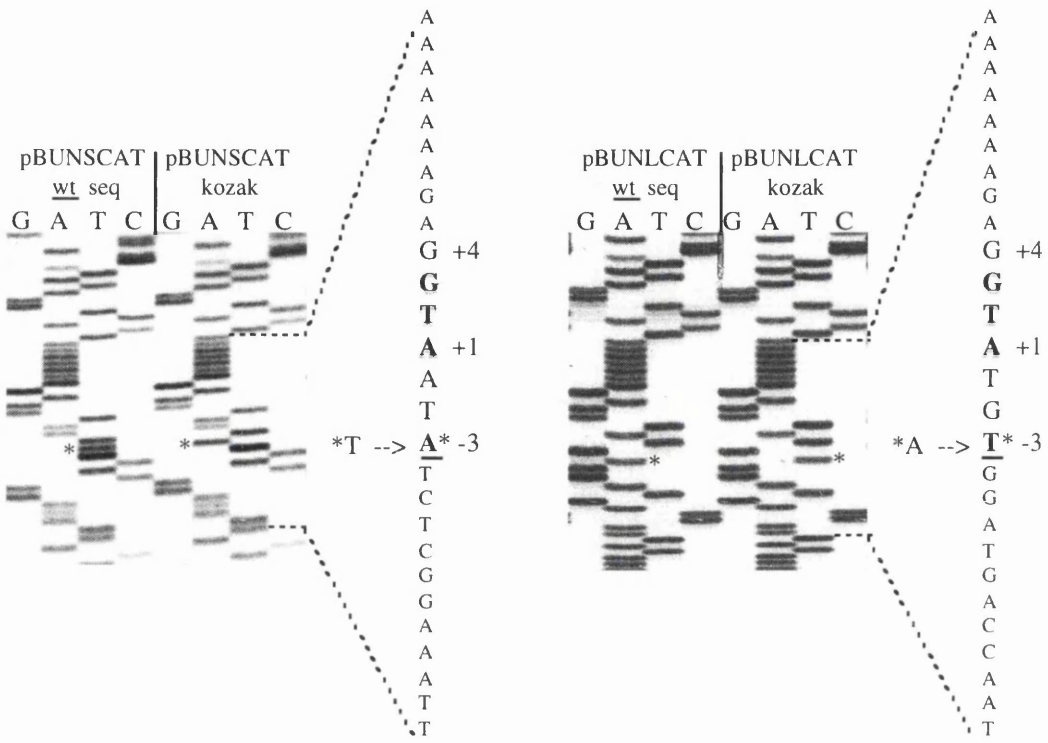


Figure 11.9 Construction of Kozak consensus mutants pBUNSCATkozak and pBUNLCATkozak.

To construct the BUNSCAT and BUNLCAT Kozak consensus mutants the Quik Change ™ site-directed mutagenesis kit (Stratagene) was used (Methods 2.3.12). The oligonucleotides used as primers to construct the BUNSCAT Kozak mutant, were CAT.6 (5'CTCCATTATA GAGCCTTTAATG) and CAT.7 (5'CATTAAAGGCTCTATAATGGAG), and oligonucleotides CAT.8 (5'CTCCATACACCTACTGGTTA) and CAT.9 (5'TAACCAGTA GGTGTATGGAG) were used to construct the BUNLCAT Kozak mutant. The resulting constructs were termed pBUNSCATkozak and pBUNLCATkozak.

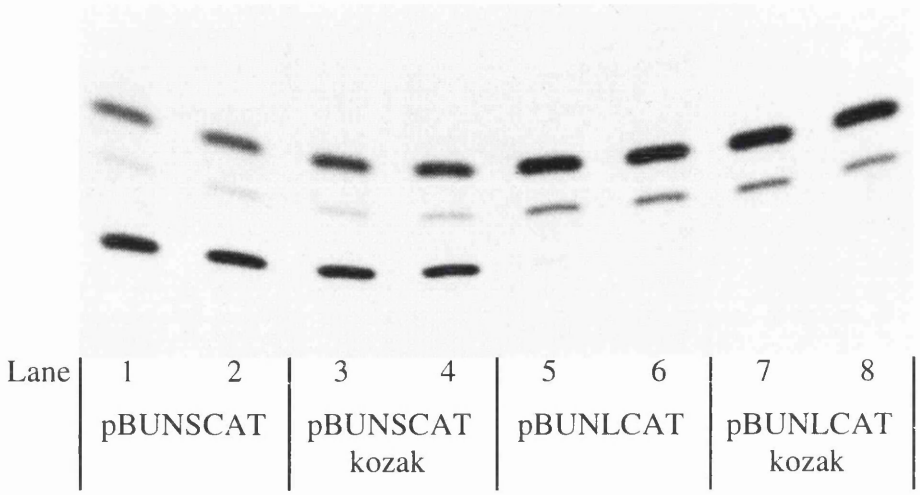


Figure 11.10 Reporter gene expression from mutants pBUNSCATkozak and pBUNLCATkozak.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of RNA transcripts from the mutants BUNSCATkozak and BUNLCATkozak as indicated above (Lanes 3 and 4, and 7 and 8), and the BUNSCAT and BUNLCAT transcripts as controls (Lanes 1 and 2, 5 and 6). Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration before equivalent amounts of cell protein in each cell extract were assayed for CAT activity.

Transcripts of the Kozak mutants pBUNSCATkozak and pBUNLCATkozak were transfected into the reporter system by methods described previously. Transcripts from the parental constructs, pBUNSCAT and pBUNLCAT, were also transfected into the reporter system as controls. Cell extracts were harvested and assayed for protein concentration, before CAT activity was measured and quantified by methods described previously.

The results of this experiment are shown in Figure 11.10, where it can be seen that mutation of the U residue at position -3 to an A residue in the BUNSCAT antigenomic RNA (*i.e.* as in the pBUNSCATkozak mutant) resulted in no significant change in the expression of the CAT gene when compared to the parental control (*i.e.* as in the pBUNSCAT). After quantitation, the CAT activity obtained from the BUNSCAT RNA was taken to be 100%, and by comparison, the activity from the BUNSCATkozak mutant was 104%.

Similarly, mutation of the A residue at position -3 to a U residue in the BUNLCAT antigenomic RNA (*i.e.* as in the pBUNLCATkozak mutant), also produced no significant change in the CAT activity detected, as compared to the parental control construct (*i.e.* as in pBUNLCAT "wild type"). If, after quantitation, the CAT activity from BUNLCAT was taken to be 100%, then the resulting CAT activity from BUNLCATkozak mutant was also found to be 100%.

Therefore, mutation of the nucleotide at position -3 in pBUNSCATkozak from an A to a U, a mutation which would have made the context of the initiation codon of the CAT gene more Kozak-like, did not result in a significant rise in CAT activity, as may have been expected. Furthermore, a mutation at the -3 position in pBUNLCATkozak from a U to an A, a mutation which would have made the context of the initiation codon of the CAT gene less Kozak-like, did not result in a significant reduction in CAT activity, as may have been expected.

Taken together, these results indicated that the sequence at the -3 position with respect to the initiation codon of the CAT ORF does not influence the level of CAT expression from BUN-CAT mRNA transcripts in this reporter system. This experiment does not rule out the possibility that translation efficiency may be affected by other sequences outwith nucleotides -9 to +1, in the context of the initiation codon. However, the data collected here lends support to the initial premise that CAT activity is an indication of CAT protein production, which is in turn an indication of CAT mRNA transcript levels, and furthermore, that the marked difference in the level of CAT activity detected between BUNLCAT and BUNSCAT is not due to differences in translational efficiency.

11.10 Construction of chimeric BUN-CAT reporter constructs.

Northern blot analysis was unsuccessful in determining whether the differences in CAT activity from each of the BUN-CAT reporter constructs was due to replicational or transcriptional control. Therefore, it was decided to make a series of chimeric BUN segment CAT reporter constructs, designed to determine the reason for the elevated CAT activity of BUNLCAT. The rationale behind this was that by separating the BUN L UTRs, *i.e.* by making chimeras with the CAT reporter gene flanked by either the 3' or the 5' UTR of the L segment (with the opposite termini from another segment), it would be possible to determine which of the BUN L UTRs was responsible for the elevated expression CAT from the BUNLCAT transcript. Essentially, it was hoped that this experiment would also allow the isolated study of the replication and the transcription promoters of the BUN L segment (*i.e.* 5' UTR for anti genome replication (anti-vRNA) and mRNA transcription, the 3' UTR for genome replication (vRNA)). In addition, the generation of chimera with termini from the other segments would also allow investigation into how pairing of heterologous termini affects CAT expression from these constructs. In pairing the different combinations of termini of the BUN L, M and S segments, it was expected that a pattern in the levels of CAT expression would suggest which of the termini, 3' or 5', and therefore which promoter, was responsible for the characteristic level of CAT activity observed for each chimeric construct. As each terminus must contain the proper signals *i.e.* the authentic viral signals, potentially required for recognition by the BUN viral proteins and expression during a viral infection, it was presumed that the termini from each segment would be able to co-operate, at least to some extent, with other heterologous termini.

11.11 Generation of chimeric BUN CAT constructs.

The chimeric UTR BUN-CAT constructs were engineered by taking advantage of the unique *Nco*I restriction sites within the CAT gene of the reporter constructs and the *Xba*I site downstream of the *Bbs*I site, downstream in the vector sequence (Figure 11.11 A).

Therefore, ligation of the different *Xba*I/*Nco*I fragments containing each of the BUN segment 3' UTRs into the different *Xba*I/*Nco*I BUN segment 5' UTR vector fragments resulted in a series of constructs covering all possible combinations of the three segments UTRs. All positive clones were sequenced to confirm that no mutations had been introduced during cloning.

The resulting chimeric BUN termini-CAT constructs were termed pBUNS/M-CAT, pBUNS/L-CAT, pBUNM/S-CAT, pBUNM/L-CAT, pBUNL/S-CAT and pBUNL/M-CAT.

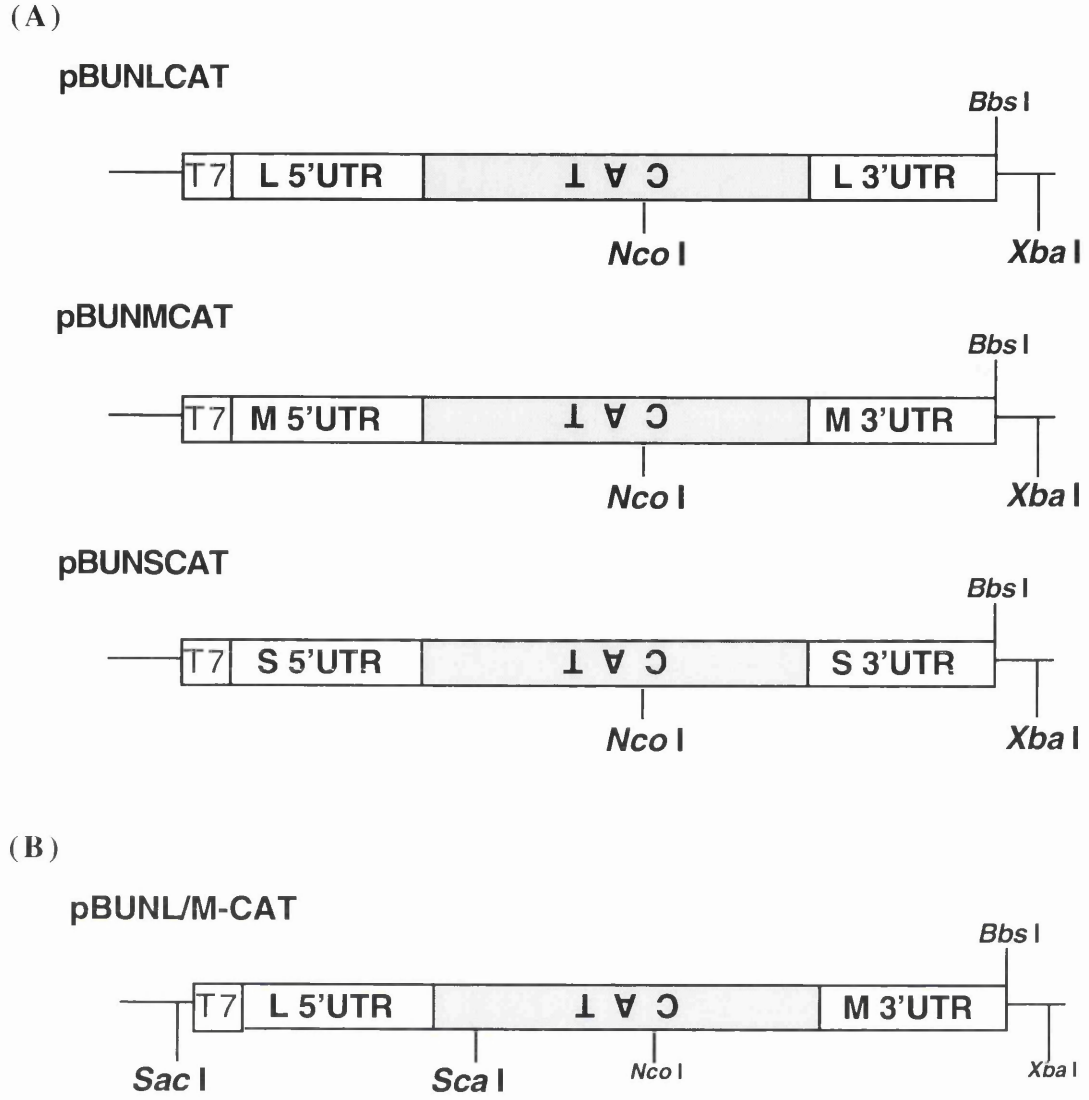


Figure 11.11 Construction of chimeric UTR BUN-CAT constructs.

(A) Each of the genomic CAT reporter plasmids were digested with *Xba*I and *Nco*I and the resulting fragments purified from a 1% TAE gel by silica matrix adsorption. The three different *Xba*I/*Nco*I fragments containing the 3' UTRs were ligated into the three different *Xba*I/*Nco*I 5' UTR vector fragments, resulting in a series of constructs covering all possible combinations of the three segments non-coding regions. The resulting chimeric BUN termini CAT constructs were termed pBUNS/M-CAT, pBUNS/L-CAT, pBUNM/S-CAT, pBUNM/L-CAT, pBUNL/S-CAT and pBUNL/M-CAT. **(B)** For example, pBUNL/M-CAT contains an antisense CAT gene flanked by the BUNL 5' UTR and the BUNM 3' UTR. All positive clones were sequenced to confirm the non-coding regions and to eliminate the possibility of any changes to sequence at the cloning sites.

The clones were given names according to a system which referred first to the origin of the 5' UTR, then the 3' UTR. For example, pBUNL/M-CAT contained an antisense CAT gene flanked by the BUNL 5' UTR and the BUNM 3' UTR. This chimeric BUN termini construct described diagrammatically in Figure 11.11 B.

11.12 CAT expression from chimeric BUN termini-CAT RNA transcripts.

The run-off transcripts from each of the six chimeric BUN termini-CAT constructs were transfected into cells transiently expressing the products of the S and L segments. The BUNL-, M- and S-CAT constructs were also transfected to serve as comparative controls. For clarity, the BUNL-, M- and S-CAT constructs will be referred to forthwith in this chapter as the genomic BUN-CAT constructs. The transfection protocol was as described previously.

As demonstrated in Figure 11.12, CAT activity was detected in the cell lysates from cells transfected individually with the genomic BUN-CAT transcripts as described previously, but surprisingly no CAT activity was detectable in the lysates from cells transfected with any of the six chimeric BUN-CAT transcripts. All chimeric constructs, pBUNS/M-CAT, pBUNS/L-CAT, pBUNM/S-CAT, pBUNM/L-CAT, pBUNL/S-CAT and pBUNL/M-CAT, were completely negative for CAT activity.

That every chimeric construct would be negative for CAT activity in the reporter assay was unexpected. Therefore, to check that the CAT ORF was active in each of the chimeric reporter constructs, the chimeric DNAs were transfected into vaccinia (vTF7-3)-infected cells (Figure 11.13). As described previously transfection of BUN-CAT DNA into cells infected with vTF7-3 results in expression of the CAT gene, mediated by vaccinia associated proteins. This observation inadvertently gave rise to a convenient means by which the CAT ORF could be checked (section 3.1).

Of the constructs tested, it was found that those which contained a BUNM 3' UTR were all found to be negative for CAT activity while all others were positive for CAT activity (Figure 11.13). However, the CAT gene of pBUNMCAT had previously been shown to be active, as demonstrated by the results shown in Figure 11.7, where the transfection of BUNMCAT RNA gave rise to CAT activity. This result was also surprising, and it was not clear why CAT activity was not generated by transfection of the chimeric DNA.

One explanation of this is that it is possible that a vaccinia virus pseudo transcription termination signal is encoded within the BUNM 3' UTR sequence. Only one such

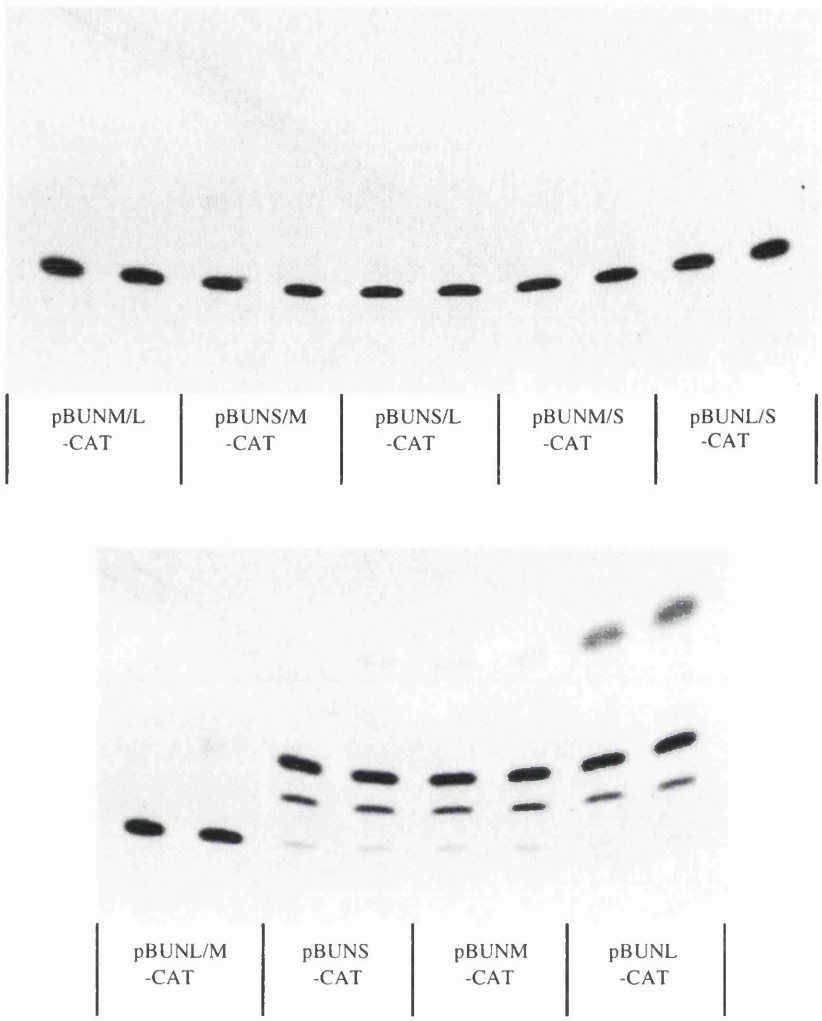


Figure 11.12 Transfection of chimeric BUN-CAT RNA transcripts.

Run-off RNA transcripts from *Bbs*I linearised templates from each of the chimeric CAT constructs and the three BUN segment CAT constructs were transfected into CV-1 cells transiently expressing BUN viral proteins. An equal quantity of each transcript was transfected in duplicate.

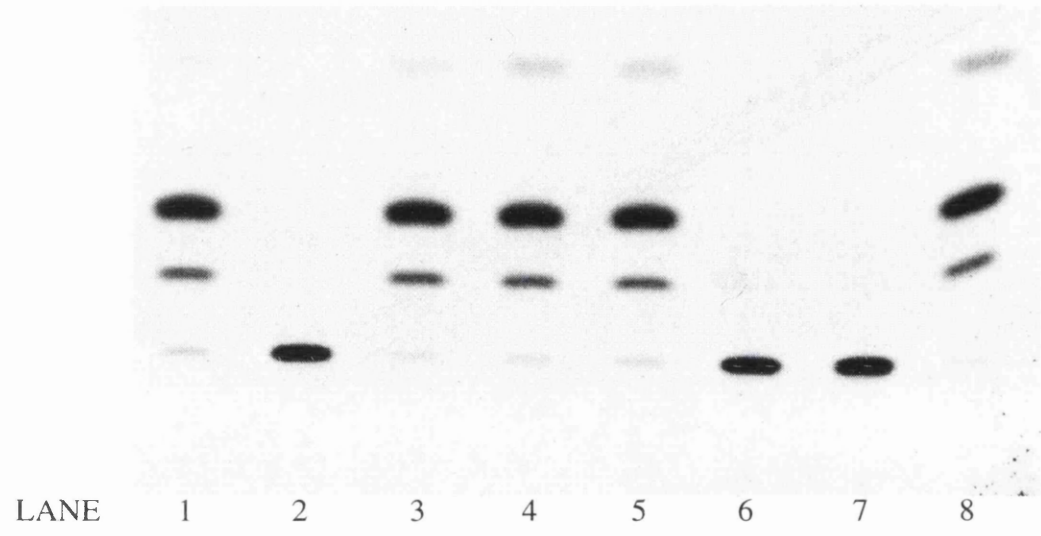


Figure 11.13 Expression of CAT from chimeric pBUN-CAT DNAs.

CV-1 cells infected with vTF7-3 at 5 pfu/cell were transfected with 2 μg of plasmid DNA to confirm that the CAT gene was active in each. Lanes 1 to 6 show extracts from cells which had been transfected with chimeric constructs pBUNM/L-CAT (Lane 1), pBUNS/M-CAT (Lane 2), pBUNS/L-CAT (Lane 3), pBUNM/S-CAT (Lane 4), pBUNL/S-CAT (Lane 5) and pBUNL/M-CAT (Lane 6). Lane 7 shows extract of cells transfected with pBUNMCAT and lane 8 with pBUNLCAT as control positives.

transcription termination mechanism has been described for vaccinia virus early genes (Rohrmann *et al.*, 1986), and in this case, the appearance of a **UUUUUNU** sequence terminated transcription of the nascent RNA transcript (Shuman *et al.*, 1988). No such signal exists within the BUNM 3' UTR, however, there is a pyrimidine-rich stretch, **UUUAUUU** (at nucleotides 4408 to 4414 of the BUN M segment 3' UTR). It is possible that this sequence has enough similarity to a vaccinia virus termination sequence so as to terminate transcription from the plasmid template, resulting in no mRNA and thus no background CAT activity.

11.13 Comparison of the predicted base-pairing of the termini.

The possible secondary structure of the termini of both the BUN genomic segments and the chimeric constructs were predicted by the FoldRNA (Zuker *et al.*, 1991) program of the GCG Wisconsin Package. The terminal 40 nucleotides of the 5' and 3' termini were paired together in a 5' to 3' manner, and their putative secondary structures predicted

The results of the predictions are shown in Figure 11.14 (A and B). In the diagram shown in this figure, the sequences to the left of the dotted line comprise the 11 conserved terminal nucleotides in the BUN genomic termini. It can be seen that the nucleotides to the right of the dotted line, *i.e.* to the right of the 11 conserved terminal nucleotides in the BUN genomic termini, base-pair up to nucleotide 15 (5' termini) in the S segment (S/S), nucleotide 18 (5' termini) in the M segment (M/M) and nucleotide 19 (5' termini) of the L segment (L/L).

However, it can also be seen from Figure 11.14 (A and B), that in the predicted folding for the termini of all of the chimeras, some mismatches occur in the paired regions. A consistent feature is disruption of base-pairing at the segment specific regions from nucleotides 12 to 14 or 15 which are conserved on a segment specific basis in all the available sequenced bunyavirus RNA segments. These are highlighted in Figures 11.14 (A) and (B).

11.14 Mutagenesis of the BUN L 5' UTR of the pBUNL/M-CAT construct.

From the results of transfecting the chimera into the reporter assay it was found that the termini of heterologous segments were unable to co-operate. It was possible that there was a requirement for base pairing of nucleotides proceeding the 11 terminal conserved nucleotides, specifically those nucleotides conserved within segments, between bunyaviruses, *i.e.*, the terminal nucleotides 12-14 of the M-segment 5' and 3' UTRs and the terminal nucleotides 12 to 15 of the S and L segment 5' and 3' UTRs.

(A)

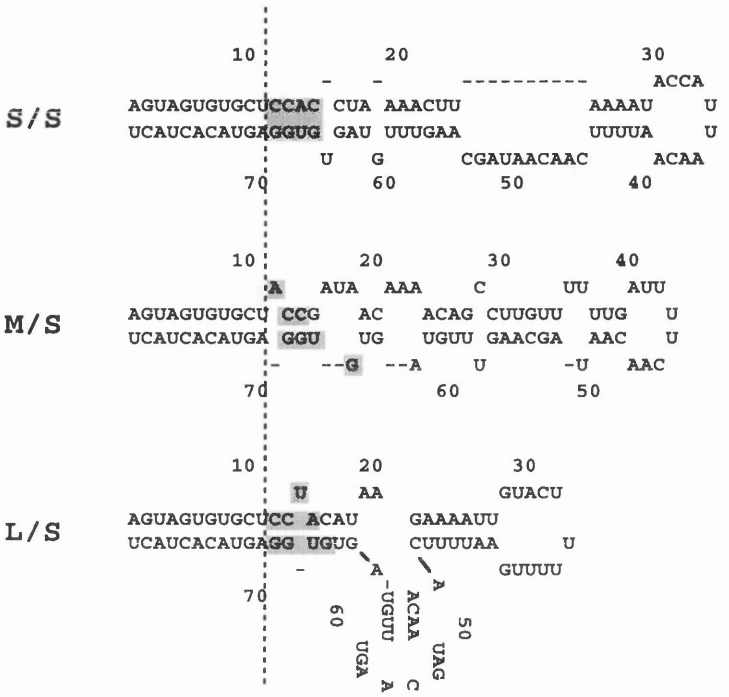


Figure 11.14 (A) and (B) Possible base-paired structures between the 5' and 3' termini of the BUN genomic segments and the chimera RNA transcripts.

The terminal 40 nucleotides of the 5' and 3' termini from each of the Bunyamwera virus genome segments and the chimera constructs were paired together in a 5' to 3' manner in the genomic sense. The putative base pairing and secondary structure of these sequences were predicted by the FoldRNA (Zuker *et al.*, 1991) program in the GCG Wisconsin Package (Version 9)™. The dotted line marks the boundary of the 11 terminal nucleotides which are conserved in all genomic segments. The shaded regions highlight the nucleotides which are conserved on a segment specific basis in all available sequenced bunyavirus RNA segments.

(B)

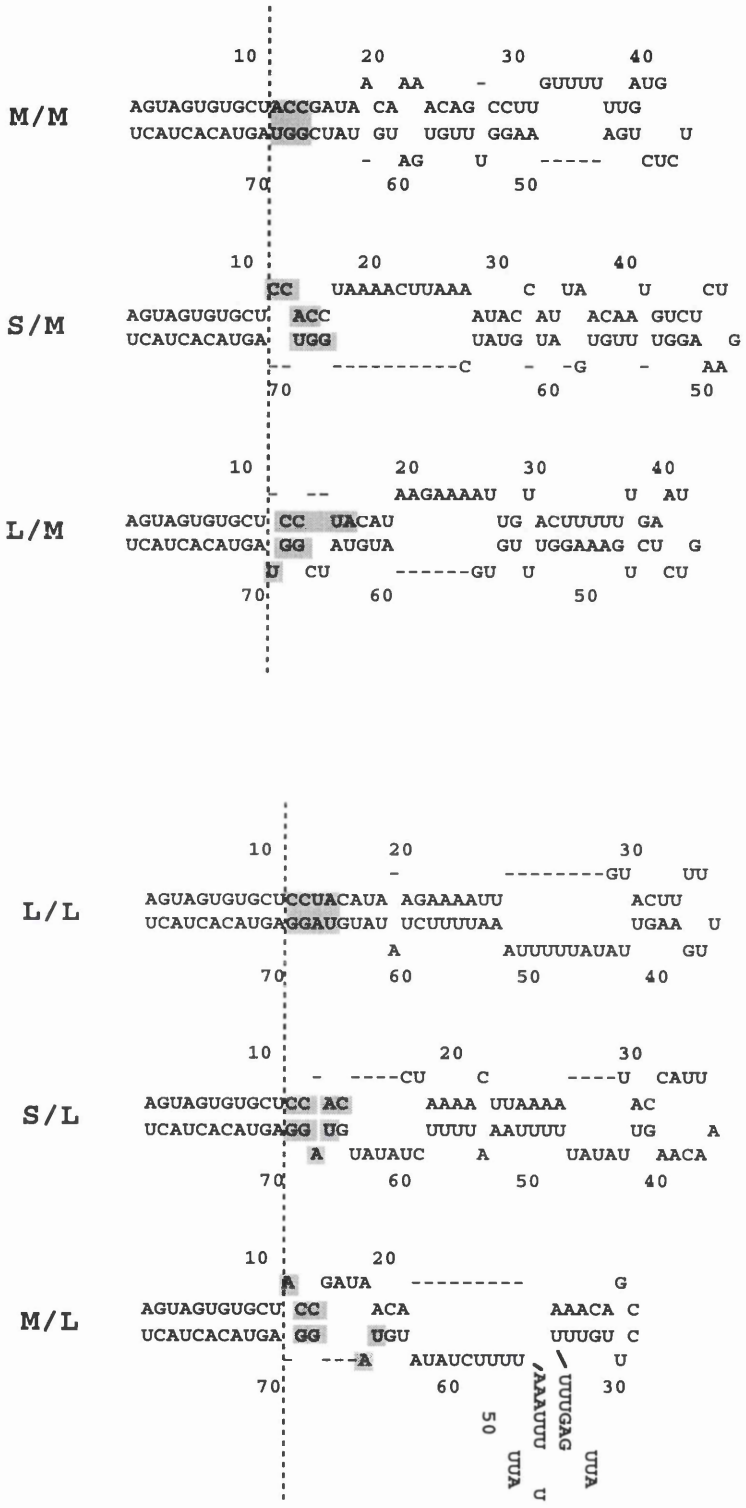


Figure 11.14 (B) continued.

To test this possibility, mutagenesis was performed on the BUNL/M-CAT sequence, so that the 5' UTR (L 5' UTR) was mutated to the sequence of the BUN M 5' terminal sequence. It was decided to initially perform mutagenesis on pBUNL/M-CAT construct as the BUN L and M 5' UTRs are of a similar length

A series of mutations were made to the BUNL 5' UTR of pBUNL/M-CAT, with the aim of increasing the amount of possible complementary base pairing between the 5' and 3' termini. This was achieved by mutating sequences within the L 5' UTR termini sequence to those of BUNM 5' UTR termini sequence, thereby increasing the complementarity between the 3' and 5' termini (Figure 11.15).

To mutate the BUN L 5' UTR of pBUNL/M-CAT, five oligonucleotides were designed and synthesised as primers (mutations shown in italics, bold, underlined), Primer L(IA-12M) inserted a ***A*** residue between nucleotides 11 and 12, Primer L(12-14M) mutated nucleotides 12-14 to the corresponding M UTR sequence (CTT to ***ACC***), Primer L(12-16M) mutated nucleotides 12-16 to the corresponding M UTR sequence (CTTAC to ***ACCGA***), Primer L(12-18M) mutated nucleotides 12-18 to the corresponding M UTR sequence (CTTACAT to ***ACCGAUA***), and Primer L(12-21M) mutated nucleotides 12-21 to the corresponding M UTR sequence (CTTACATAAG to ***ACCGAUAACA***).

The sequence of the primers were:

+1

L(I-12M): 5'GGGGAGCTCTAATACGACTCACTATAAGTAGTGTGCT***A***CCTACATAAGAAAATT

L(12-14M):5'GGGGAGCTCTAATACGACTCACTATAAGTAGTGTGCT***ACC***ACATAAGAAAATT

L(12-16M):5'GGGGAGCTCTAATACGACTCACTATAAGTAGTGTGCT***ACCGA***ATAAGAAAATT

L(12-18M):5'GGGGAGCTCTAATACGACTCACTATAAGTAGTGTGCT***ACCGAUA***AAGAAAATTG

L(12-21M):5'GGGGAGCTCTAATACGACTCACTATAAGTAGTGTGCT***ACCGAUAACA***AAAATTG

PCR reactions were set up with the mutating primers and the UNIversal primer (which anneals to the vector sequence) as the distal primer, and pBUNL/M-CAT was the DNA template. For the construction of L(12-18M) and L(12-21M) mutants, pBUNL/M-CAT (12-16M) was used as the template for PCR.

Initially, *Pfu* polymerase was utilised in the PCR reactions, however, no visible products were generated with this enzyme. This problem was solved by generating the PCR products with *Taq* polymerase. However, to limit the risk of spurious mutations resulting from *Taq* errors, the PCR products were digested with *Sac*I and *Sca*I to release a small 200 base-pair fragment which was then subcloned into *Sac*I, *Sca*I digested pBUNL/M-CAT (The

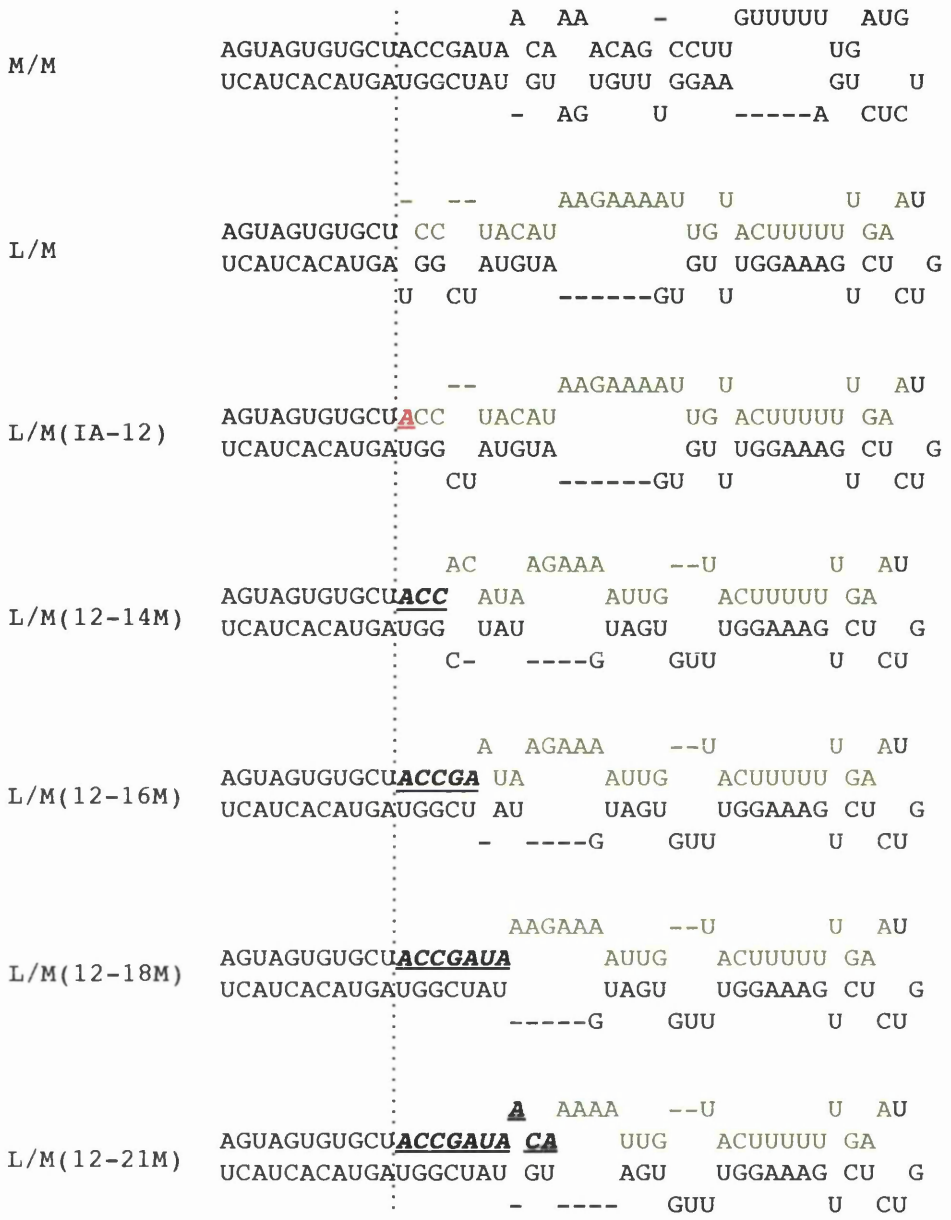


Figure 11.15 Mutation of the BUNL/M-CAT chimera L 5' UTR to increase the possible basepairing between the 3' and 5' termini.

The above figure demonstrates schematically the FoldRNA secondary structure predictions for a series of BUNL 5' UTR mutants in the pBUNL/M-CAT reporter construct. The aim of the mutations was to increase the complementarity between the 3' and 5' termini by mutating terminal regions of the L UTR sequence to that of M UTR sequence. The conserved terminal nucleotides to the left of the dotted line are shown in black, as is the M-segment sequences. The BUN L-segment sequences are shown in green. The insertion mutation of L/M(IA-12) is shown bold red (italic and underlined), while the mutations in L/M(12-14M), L/M(12-16M), L/M(12-18M) and L/M(12-21M) are shown in bold (italics and underlined).

positioning of these restriction sites in the chimeric BUN-CAT constructs can be seen in Figure 11.11 (B)). Clones positive for insertion were sequenced to confirm the desired mutations of the L 5' UTR and to check that no spurious mutations occurred during *Taq* driven PCR and subcloning. Clones representing each mutation were selected and denoted pBUNL/M-CAT (I-12), pBUNL/M-CAT (12-14M), pBUNL/M-CAT (12-16M), pBUNL/M-CAT (12-18M), pBUNL/M-CAT (12-21M).

11.15 Transfection of the pBUNL/M-CAT (BUNL 5' UTR) mutants into the reporter assay.

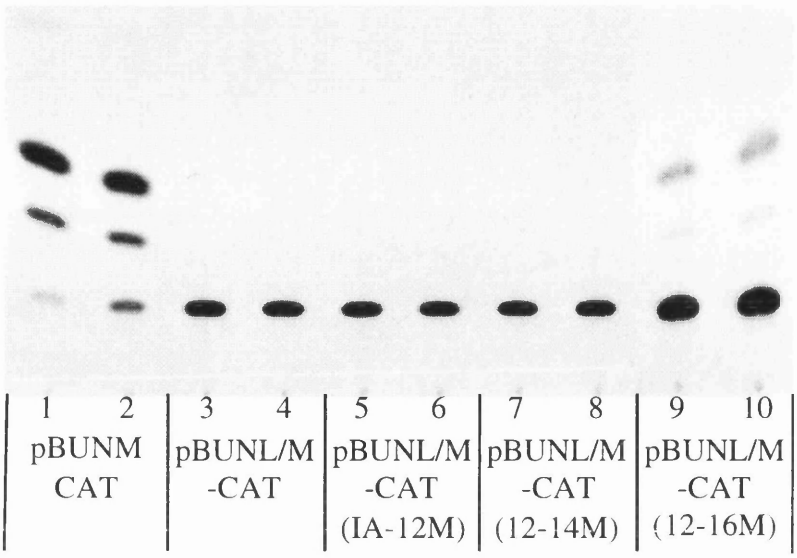
Run-off transcripts from each of the *Bbs*I linearised mutants were tested in the reporter system by methods described previously, and the results shown in Figure 11.16.

From quantitation of the CAT signals shown in Figure 11.16, it was found that the insertion of an A residue between positions 11 and 12 of the BUN L 5' UTR, as in mutant pBUNL/M-CAT (IA-12M), failed to restore CAT activity. Furthermore, when nucleotides 12-14 of the L 5' UTR were mutated to that of the M 5' UTR, as in mutant pBUNL/M-CAT (12-14M), still no CAT activity was detected. CAT activity was detected when nucleotides 12 -16 of the L 5' UTR were mutated to those of the M 5' UTR (as in mutant pBUNL/M-CAT (12-16M)), although this activity was calculated to be only 6% of the BUNMCAT "wildtype" level. However, when the mutated region was extended to 12-18 nucleotides of the M 5' UTR, as in mutant pBUNL/M-CAT (12-18M), or to 12-21 nucleotides of the M 5' UTR, as in mutant pBUNL/M-CAT (12-21), then CAT activity was observed to increase dramatically, to 71% and 65% respectively.

The results from this experiment indicated therefore that to achieve CAT activity from the reporter construct, base pairing was required, not only between the conserved 11 terminal nucleotides at the 5' and 3' termini, but between the terminal 18 nucleotides of the 5' and 3' termini, at least in the BUNM segment.

However, since the CAT activity detected by mutating nucleotides 12 -21 of the L 5' UTR in pBUNL/M-CAT was still approximately 30% lower than that detected from pBUNMCAT, this indicates that further mutagenesis may need to be performed to increase CAT activity to wild type (*i.e.* pBUNMCAT) levels.

(A)



(B)

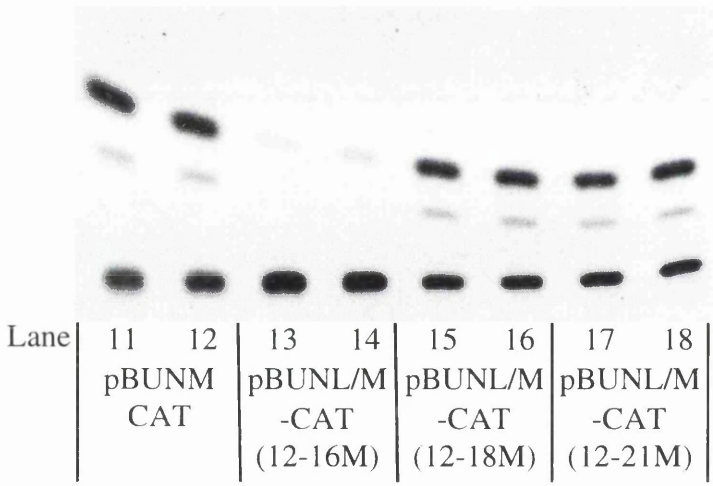


Figure 11.16 Reporter gene expression from the pBUNL/M-CAT BUNL 5' UTR mutants.

CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of RNA transcripts from the BUNL/M-CAT mutants as indicated above (Lanes 3 to 10, and 13 to 18), BUNMCAT and BUNL/M-CAT as controls (Lanes 1 to 4, 11 and 12). Cells were harvested 20 hours post transfection and the cell extracts assayed for protein concentration before equivalent amounts of cell protein in each cell extract were assayed for CAT activity.

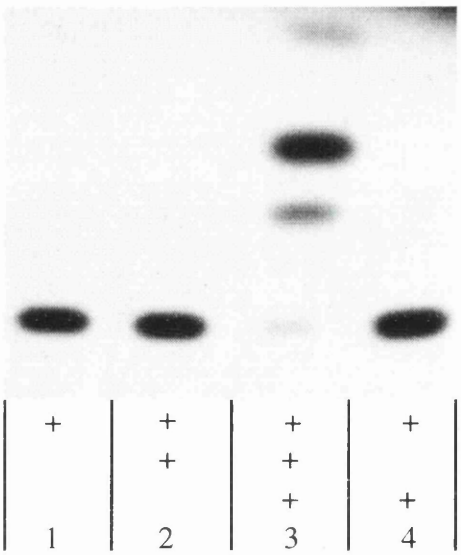


Figure 11.17 CAT activity obtained from transfection of cells expressing bunyavirus proteins with pBUNMCAT-R-Tϕ DNA.

Cells were infected with vTF7-3 at 5 pfu/cell, and then transfected with 1 μg pTFBUNS (Lane 2), 5 μg pTFBUNL (Lane 4), or both plasmids (Lane 3), as indicated, then subsequently transfected with 1 μg pBUNMCAT-R-Tϕ DNA. Cell extracts were harvested 24 hours post infection and assayed for CAT activity by thin layer chromatography (TLC). CAT activity was detected when the proteins from both recombinant plasmids were supplied (Lane 3), but not individually (Lanes 2 and 4). No CAT activity was detected from vaccinia infected cells when transfected with pBUNMCAT-R-TΦ DNA.

11.16 Construction of pBUNMCAT-Ribo-TΦ.

A fragment encoding the BUNMCAT reporter construct was amplified by PCR from the pBUNMCAT plasmid, using BUNM specific terminal primers, and ligated into *StuI*/*Sma*I sites of the ribozyme construct, pT7-Ribo-TΦ. The resulting construct, pBUNMCAT-Ribo-TΦ, was sequenced to determine the orientation of the insert and to check that no spurious mutations had occurred during PCR and cloning.

RNA transcripts derived from pT7-Ribo-TΦ have two extra nucleotides (GG) at the 5' end. Another feature of this vector is that it contains a hepatitis delta virus ribozyme sequence. Thus, RNA transcripts derived from pBUNMCAT-Ribo-TΦ contained, in the 5' to 3' order, two G residues followed by BUNMCAT, a hepatitis delta virus ribozyme sequence, then a portion of the T7 terminator sequence (TΦ). Autolytic cleavage by the ribozyme releases itself, to generate the authentic 3' BUNM termini.

When DNA from this construct was transfected into cells pre-infected with vTF7-3, no CAT activity could be detected, but when transfected into cells transiently expressing the L and S segment proteins, CAT activity was detected (Figure 11.17).

As there is no problem of background CAT activity from transfection of the DNA from this construct, and since transfection of DNA into eukaryotic cells is a much simpler procedure than transfection of *in vitro* transcribed RNA, pBUNMCAT-Ribo-TΦ could be used as a template for construction of mutants designed to investigate the effects of mutations to the 5' terminus of the M segment: the plasmid DNA of these constructs could be transfected directly into cells, obviating the need for transfection of *in vitro* transcribed RNA.

11.17 Discussion.

As was discussed earlier in this chapter, upon infection with bunyavirus, viral mRNA transcripts of the L, M and S segments, can be detected at different levels within the cell, with the S mRNA species, the M mRNA species and the L mRNA species in a molar ratio of 40: 10: 1. Reasons why this occurs are not clear, but it was suggested that sequences encoded within the UTRs of each segment may influence or control the transcription of that segment. To investigate this possibility, reporter constructs based on the BUN L and M segments which contained the CAT gene flanked by either the BUN L or M UTRs were constructed. It was intended that use of RNA transcripts derived from these plasmids in the

reporter system would determine the effect of the BUN L and M UTRs on the level of CAT expression.

The CAT signal obtained from the reporter assay is a direct measurement of the amount of CAT protein translated from CAT mRNA transcripts. Therefore, for the purpose of this experiment, CAT activity was initially presumed to be an indirect measurement of actual level of CAT mRNA transcripts. However, the results from this experiment showed that the highest level of CAT activity was obtained from transfection of BUNLCAT RNA, and the lowest level, from transfection of BUNSCAT RNA. The efficiency of expression of the reporter gene in the context of each of the BUN segments, was in the order of BUNLCAT (taken as 100%) > BUNMCAT (60-70%) >> BUNSCAT (3-10%), a complete inversion of the ratios of mRNA transcripts found in infected cells.

The obvious disparity between the observed ratio of L, M, and S segment transcripts found in the cell throughout infection with bunyavirus, and the ratio of expression from each of the BUN-CAT reporters was surprising, and suggested that other factors must influence the transcript levels in the context of the infected cell.

During an infection, there are a number of other factors which may influence the gene expression of viral proteins which cannot be addressed with the reporter system. It is possible that the length of each genomic segment may influence the resulting levels of transcription and gene expression from that segment. Since each of the BUN genomic segments differ in length, it might be predicted that the S segment (961 nucleotides) for example, which is approximately seven times shorter than the L segment (6875 nucleotides), would be replicated, transcribed and encapsidated by viral proteins at a more efficient rate.

Another factor which also must be considered in the context of the viral infection is the involvement of the coding sequences of the BUN segments in the control of expression of their gene products. In work that was published subsequently to this study, Zheng *et al.* (1996), engineered two influenza A (WSN) virus NA-RNA segments, in which the UTRs were substituted by the corresponding UTRs of the PB1- and NS-RNA segments. Using these constructs, it was demonstrated via RNP transfection of these RNAs, that transfectant viruses NA/PB1 and NA/NS could be rescued from the experiment. However, transfectant viruses containing an NA-RNA segment with PB1- or NS-specific UTRs showed a reduction in NA-specific vRNA levels of about 10 and 50 times, respectively, both in the released virus population and in infected cells while the levels of both the PB1 and NS segments were unaffected. This work suggests that RNA synthesis is influenced by both the coding region and noncoding regions of the viral RNA, and that the UTRs of influenza virus have evolved in such a way that each RNA segment contains a UTR which matches its

corresponding coding region in a manner that ensures optimal replication and transcription of that segments gene product(s). Furthermore, the discovery that sequences within a coding region can influence their own gene expression is not unique to influenza virus. The nucleotide sequences of the Hepatitis C virus capsid protein ORF, for example, has been shown to be involved and essential for IRES activity (translation initiation) (Honda *et al.*, 1996).

Therefore it is highly possible that the coding sequence of the BUN segments play a role in the control of replication and transcription of its specific segment (*i.e.* the N ORF of the S segment may increase expression of itself).

Another feature of replication and transcription of the BUN segments that cannot be measured by the CAT reporter system is the possibility that each segment exerts an effect on the others, by competing for interaction with the viral replication proteins during a viral infection. Analysis of transcription and replication signals in the UTRs of one segment in isolation may not illuminate the effects of competition from other segments, in what is essentially a series of enzymic reactions. It is possible, for example, that the S segment has a selective advantage over the L and M segments, in the context of a viral infection, that accounts for, or contributes to, the high levels of this transcript found in infected cells.

Therefore, it must be considered that various aspects of viral replication are not measurable in this system, and that possibly, one or more of these factors may influence or regulate the levels of segment replication, transcription and gene expression during bunyavirus infection, to result in the observed ratio of segment mRNA transcripts.

However, the fact that these other factors cannot be measured by the reporter assay, could be used to the advantage of this study, to allow the isolated investigation of the signals which reside in the BUN segment UTRs. Since each of the BUNCAT reporter constructs encoded the same reporter gene, the potential influence of length of the coding region, and potential involvement of the coding region in reporter gene expression, were eliminated from the assay.

The results obtained here indicated that when the involvement of other factors was removed, the ratio of gene expression from the BUN L, M and S segments in the reporter assay was completely inverted, with respect to mRNA transcript levels found in BUN infected cells. It was perhaps even more striking that the BUN segment UTRs possessed signals which strictly and specifically regulated reporter gene expression, albeit with a different pattern than might have been expected. At this point therefore, an exact explanation was not apparent of how the BUN segment UTRs mediated this regulation of CAT gene expression, whether via

replicational or transcriptional control, or if level of CAT protein expression could be influenced by translational efficiency.

In an attempt to determine if expression of the CAT gene from the BUN-CAT constructs was under transcriptional control, Northern blot analysis of the BUN-CAT RNA transcripts formed in the reporter assay was attempted. Again, as in the experiments presented in Chapter 3, I was unsuccessful in detecting the presence of positive sense transcripts. Possible reasons for the failure of this technique are discussed elsewhere in this thesis.

Since Northern blot analysis of the RNAs transcribed from each reporter construct within the reporter system was unsuccessful, alternative routes were chosen to determine the reason for the observed ratio of CAT activity from the BUNCAT constructs. To investigate the possibility that expression from the CAT gene in the BUN-CAT constructs was influenced by translational control, the sequences around the AUG initiation codon in each of the three BUN antigenomic segments were examined for Kozak-similarity. It was found that the sequence context of the initiation codon in the L, M, and S segment antigenomic RNAs showed some degree of Kozak similarity (Figure 11.8), the L segment exhibiting the highest, and the S segment showing the lowest degree of similarity. The sequence context of the initiation codon in the BUN-CAT constructs sequence was slightly different due to the replacement of the coding sequence with the CAT gene, and the Kozak similarity of the BUNMCAT and the BUNSCAT was increased slightly due to the insertion of one of the major Kozak determinants (*i.e.* a G residue at position -4). However, the BUNLCAT construct still exhibited a higher Kozak similarity compared to BUNSCAT.

Mutagenesis was performed on the BUNLCAT to produce a lower degree of Kozak similarity, resembling that found in the BUNSCAT construct. Similarly, the BUNSCAT construct was mutated to possess a higher degree of Kozak sequence similarity, thus resembling the BUNLCAT construct. Study of these mutants in the reporter assay however, revealed no major change in expression of the CAT gene as a result of the changes in Kozak similarity, and it was concluded that differences in the level of CAT activity between the BUNLCAT and BUNSCAT constructs was not due to the extent of Kozak consensus similarity within the sequences surrounding the initiation codon.

To determine if either the replication or transcription promoters of each of the BUN segments (*i.e.* 5' UTR for anti genome replication (anti-vRNA) and mRNA transcription, the 3' UTR for genome replication (vRNA)), influenced the level of CAT activity produced from each of the BUN-CAT constructs, a series of chimeric BUN segment CAT reporter constructs were engineered where the CAT reporter gene was flanked by either the 3' or the 5' UTR of one particular segment, with the opposite termini from another segment. It was presumed that the

termini from each segment would be able to co-operate, at least to some extent, with other heterologous termini. However, the results of the chimeric BUN UTR constructs demonstrate that the 3' and 5' termini of the three BUN segments cannot co-operate, possibly either in forming the correct secondary structure, or in supplying the proper recognition or transcription signals. This was surprising, as the termini from each of chimeric transcripts must contain the correct BUN virus promoter sequences for transcription. These results do suggest however, that the 3' and 5' termini of each BUN segment have co-evolved so that the correct secondary structure between the 3' and 5' termini is maintained to present the cis acting signals encoded in the correct conformation.

The finding that the termini of the three BUN segments were not able to co-operate, either to form the correct secondary structure, or to supply the proper recognition or transcription signals, is also surprising in light of recent work by Odagiri and Tashiro (1997) on influenza A virus, which provided evidence, not only for reporter gene expression, but for the efficient viral rescue of chimeric UTR CAT RNA transcripts. In this study the CAT gene was flanked either by the PA segment 3' UTR and the PB2 5' UTR (3' PA/ 5' PB2) or the PB2 3' UTR and the PA 5' UTR (3' PB2/ 5' PA). Therefore, this work demonstrated that, in the case of influenza A virus at least, chimeric RNA segments which possessed a 3' UTR and a 5' UTR each derived from different segments were capable of being expressed and packaged into viral particles.

Upon comparison of the possible secondary structures formed between each of the 3' and 5' termini of the genomic transcripts and the chimera transcripts, it was found, not unsurprisingly, that there was a greater degree of complementarity maintained between the termini of the genomic segments compared to the termini of the chimera segments (Figure 11.14).

To investigate whether the reduced complementarity between the termini of the chimera transcripts was responsible for the lack of CAT activity, a series of mutants was constructed, which increased gradually the degree of complementarity between the 3' and 5' termini. The L/M-CAT chimera was chosen for further investigation as the L and M segments have UTRs of a similar length. The complementarity between the termini was increased gradually by mutating the L 5' UTR terminal sequence to that of the M segment 5' UTR sequence. As was shown in Figure 11.16, CAT activity was not detected until the terminal 16 nucleotides were complementary. Furthermore, when the restored complementarity between the 5' and 3' termini was increased to 18 and 21 nucleotides, the CAT signal increased to approximately 70% the level of BUNMCAT CAT activity.

These results indicate that complementarity between the 5' and 3' termini, greater than the terminal 11 nucleotides, may be critical for creating the correct secondary structure at the termini to allow transcription and subsequent expression of the CAT reporter construct.

It has been proposed previously by several bunyavirus groups (Elliott, 1990; Kolakofsky and Hacker, 1991), that the sequences conserved between the termini of the 3 genomic segments (*i.e.* the first 11 nucleotides in the case of bunyaviruses), are important for viral protein recognition and encode the viral promoters necessary for transcription and replication (Chapter 1.4.1). Furthermore, it is thought that these conserved sequences may form a panhandle structure. However, it is possible that the nonconserved nucleotides flanking these regions (*i.e.* nucleotides 12-18 of the BUN M termini) may be involved in stabilising the panhandle structure, possibly by extending the complementarity and base-pairing between the termini (Figure 1.3).

These results also imply that further mutation of the L 5' UTR within pBUNL/M-CAT to that of M 5' UTR would presumably restore CAT activity equivalent to BUNMCAT levels.

It is also possible that the non-conserved nucleotides may be involved in the formation of more complex RNA structures, such as pseudoknots, which might be required for interaction with cellular factors during RNA replication. Alternatively, nucleotides close to this structure may enhance or inhibit the formation of the panhandle structure, either by directly interacting with the conserved nucleotides or by interacting with internal sequences.

Conclusions

I have described the establishment and characterisation of a convenient *in vivo* transient expression reporter system to investigate Bunyamwera viral RNA synthesis and the proteins involved by reverse genetics. The reporter system initially utilised a chimeric BUN virus-like S segment RNA transcript carrying an antisense CAT reporter gene. Transfection of this reporter RNA into cells expressing recombinant BUN virus proteins resulted in the detection of CAT activity. This indicated that the negative sense BUNSCAT RNA transcript, which could not have acted as a template for translation, was recognised by, and had interacted with the recombinant BUN virus proteins to form a functional bunyavirus ribonucleoprotein (RNP)-like complex. Formation of this complex allowed the subsequent transcription of a positive sense RNA from the transfected BUNSCAT reporter RNA transcript. It was also shown that detection of CAT activity from the reporter transcript was dependent upon expression of proteins from both the BUN L and S segments. These proteins could be supplied by transient expression, either from recombinant vaccinia viruses, or from transfected plasmids using the vaccinia virus-T7 RNA polymerase system.

Further analysis of the S segment proteins determined that the N protein and the BUN L protein are sufficient for the transcription of BUNSCAT RNA in the reporter assay. This suggests that only the L and N proteins are required, in conjunction with an appropriate RNA molecule, to reconstitute a transcriptionally active RNP structure. The function of the BUN S segment NSs protein still remains unclear.

It was also demonstrated that BUN L protein was able to transcribe BUNSCAT RNA in concert with certain heterologous bunyavirus S segment proteins. The heterologous S segments that produced significant levels of CAT activity were those in which the N protein was most closely related to BUN N protein at the amino acid level.

Analysis of BUN viral-like RNA transcripts produced within the reporter system by Northern blotting, was unsuccessful in determining whether the BUNSCAT transcript was being replicated. However, the use of a BUNSCAT(+) RNA in the reporter system which gave an elevated CAT level above background when the BUN L and S segment proteins were supplied, indicated that BUNSCAT(+) RNA was replicated to BUNSCAT (negative sense) which subsequently acted as a template for mRNA transcription. This suggests that the reporter system does indeed support replication of BUN S-like transcripts.

When the effect of adding or removing nucleotides from the extreme ends of the BUNSCAT RNA termini was tested, it was found that the addition of nucleotides at either the end of the 5' or the 3' termini of BUNSCAT reporter RNA resulted in a transcript which gave detectable CAT activity in the reporter assay. This suggests that the viral polymerase can probably recognise and interact, at least to some extent, with specific sequences which are present in the 5' and 3' termini, whether these signals are presented at the extreme termini, or whether they are internal to other sequence. Deletion of any number of nucleotides from the BUNSCAT 5' UTR was not tolerated, indicating that the terminal conserved nucleotides of the 5' UTR are absolutely essential for interaction with the polymerase. Deletion of one nucleotide from the 3' terminus were better tolerated, resulting in CAT activity that was equivalent to "wild type" BUNSCAT RNA. However, a deletion of 2 nucleotides drastically reduced CAT activity. This maybe explained if we consider that the 3' UTR would be acting as the template for transcription initiation while the 5' UTR maybe important for in polymerase recognition or positioning of the template RNA.

Garcin *et al.* (1995), proposed a mechanism for the synthesis of viral mRNA termed the "prime-and-realign" model. This model could account for the observation that a deletion of 1 nucleotide from the 3' UTR was tolerated, as a deletion of 1 nucleotide would still allow the capped-primer to anneal at the C at position 2, and to initiate with a G in the usual manner, and that the prime-and-realign mechanism could proceed without disruption. The reiteration of sequence at the 3' terminus, in conjunction with the "prime-and-realign" mechanism, would mean that genome segments which lack a nucleotide at the 3' end are still active for transcription and could feasibly be repaired during replication, as shown in Figure 7.11 B.

In an attempt to determine the function of two conserved sequence motifs which are present within the 5' UTR of all sequenced bunyavirus S segments, a series of deletions were made within the BUNSCAT 5' UTR. When the RNA transcripts from these mutants were tested in the reporter system only those with an internal deletion of 156 nucleotides, leaving only 11 terminal nucleotides were inactive in the assay. It was thought that the drop in CAT activity was not due to the removal of conserved sequenced motifs in the RNA, but rather of nucleotides 12-15 which are conserved between all bunyavirus S segments.

To determine the importance and function of the conserved sequences found at the BUN 3' and 5' termini a series of mutations was made. In the first series of BUNSCAT mutants, the results of single nucleotide substitutions in either the 5' or the 3' terminus suggested that sequence specificity and/or base-pairing between the termini was essential. To investigate the importance of base-pairing between the termini a series of complementary base-paired mutations were tested; these mutations also demonstrated that the majority of changes to the terminal 12 nucleotides were detrimental. Only changes to nucleotides 13-15 were tolerated

suggesting that the maintenance of base-pairing in this region is more important than specificity of sequence. Interestingly, mutations to the very terminal nucleotides were moderately tolerated which again suggests a role for the "prime-and-realign" model to overcome changes in a terminal nucleotide.

Mini-BUNSCAT reporters were constructed to determine the minimum lengths of the BUN S 3' and 5' UTRs required for the expression of CAT activity. It was shown that the termini had to be more than 20 terminal nucleotides in length for functional activity. Termini which were 13 nucleotides in length and therefore contained the conserved terminal 11 nucleotides, were insufficient in length to allow transcription of the reporter gene. Furthermore, termini which were 20 nucleotides in length and therefore contained both the conserved terminal sequence and those sequences conserved between bunyavirus S segments were similarly insufficient for functional activity. However, a mini reporter with 32 5' and 33 3' terminal nucleotides elicited CAT activity, which suggests that a length of between 20 and 33 terminal nucleotides is sufficient for the formation of an active RNP structure.

Comparison of the level of CAT activity within the reporter system from all three BUN genomic CAT reporters found that the BUNLCAT reporter was greater than the BUNMCAT which in turn was greater than BUNSCAT. To determine which UTR, 3' or 5', was responsible for controlling CAT levels a series of chimeric BUN UTR CAT reporter was constructed. The results from these chimeric RNAs demonstrated that the 3' and 5' termini of different BUN segments cannot co-operate, possibly either in forming the correct secondary structure, or in supplying the proper recognition or transcription signals. This was surprising, as the termini from each of the chimeric transcripts must each contain the correct BUN virus promoter sequences for transcription of its segment, and suggests that complementarity greater than the terminal 11 nucleotides may be critical for creating the correct secondary structure at the termini to allow transcription and subsequent expression of the CAT reporter construct.

Further to this, it was shown that when nucleotides 12-18 of the L 5' UTR in the chimeric reporter pBUNL/M-CAT were mutated to M 5' UTR sequence, thereby increasing complementarity between the termini of the chimeric RNA, a significant level of CAT activity was restored. This suggests that not only is complementarity required between the terminal 11 nucleotides of the termini, but also between the terminal 18 nucleotides of the 3' and 5' termini, at least in the case of the BUN M segment. These nonconserved nucleotides that flank the conserved terminal 11 nucleotides in genomic segments (*i.e.* nucleotides 12-18 of the BUN M termini) may be involved in stabilising the panhandle structure, possibly by extending the complementarity and base-pairing between the termini (Figure 1.3).

The BUN-CAT reporter system as it stands is a very useful reliable and convenient tool for investigating BUN RNA synthesis and specifically the proteins involved in the replication and transcription events. However, one potential problem with the reporter system which was not experimentally addressed in this study was the effect of vaccinia virus (vTF7-3) encoded proteins on the transfected RNAs and the resulting BUN viral-like transcripts produced within the system. It is possible that vaccinia virus encoded proteins might cap and polyadenylate BUN viral-like transcripts: if this were so then the degree of polyadenylation may have made it difficult to detect individual BUN transcripts as discrete bands by Northern blotting.

Recently Agapov *et al.* (1998) described a series of noncytopathic Sindbis virus vectors capable of generating cell lines for the long term expression of foreign genes. One such vector, SINrep19/T7pol which expresses the T7 RNA polymerase, could act as an alternative to vaccinia virus vTF7-3 in the BUN reporter system for *in vivo* T7-expression.

Employment of a different T7-expression system could greatly enhance the usefulness of the BUN reporter system, by allowing the detection and further characterisation of RNA transcripts produced within the assay. Such characterisation might provide further insight into specific aspects of BUN replication and transcription such as CAP snatching, transcription initiation and mRNA termination.

References.

Abraham, G. & Pattnaik, A. K. (1983). Early RNA synthesis in Bunyamwera virus-infected cells. *J Gen Virol* **64**, 1277-90.

Agapov, E. V., Frolov, I., Lindenbach, B. D., Pragai, B. M., Schlesinger, S. & Rice, C. M. (1998). Noncytopathic Sindbis virus RNA vectors for heterologous gene expression [see comments]. *Proc Natl Acad Sci U S A* **95**, 12989-94.

Anderson, G. W., Jr. & Smith, J. F. (1987). Immunoelectron microscopy of Rift Valley fever viral morphogenesis in primary rat hepatocytes. *Virology* **161**, 91-100.

Banerjee, A. K. (1980). 5'-terminal cap structure in eucaryotic messenger ribonucleic acids. *Microbiol Rev* **44**, 175-205.

Baldridge, G. D., Beaty, B. J. & Hewlett, M. J. (1989). Genomic stability of La Crosse virus during vertical and horizontal transmission. *Arch Virol* **108**, 89-99.

Ball, L. A. (1992). Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. *J Virol* **66**, 2335-45.

Barclay, W. S. & Palese, P. (1995). Influenza B viruses with site-specific mutations introduced into the HA gene. *J Virol* **69**, 1275-9.

Baron, M. D. & Barrett, T. (1997). Rescue of rinderpest virus from cloned cDNA. *J Virol* **71**, 1265-71.

Baudin, F., Bach, C., Cusack, S. & Ruigrok, R. W. (1994). Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. *EMBO J* **13**, 3158-65.

Beaty, B. J. & Calisher, C. H. (1991). Bunyaviridae--natural history. *Curr Top Microbiol Immunol* **169**, 27-78.

Beaty, B. J., Miller, B. R., Shope, R. E., Rozhon, E. J. & Bishop, D. H. (1982). Molecular basis of bunyavirus per os infection of mosquitoes: role of the middle-sized RNA segment. *Proc Natl Acad Sci U S A* **79**, 1295-7.

Beaty, B. J., Sundin, D. R., Chandler, L. J. & Bishop, D. H. (1985). Evolution of bunyaviruses by genome reassortment in dually infected mosquitoes (Aedes triseriatus). *Science* **230**, 548-50.

Becerra, S. P., Rose, J. A., Hardy, M., Baroudy, B. M. & Anderson, C. W. (1985). Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. *Proc Natl Acad Sci U S A* **82**, 7919-23.

Bellocq, C. & Kolakofsky, D. (1987). Translational requirement for La Crosse virus S-mRNA synthesis: a possible mechanism. *J Virol* **61**, 3960-7.

Bellocq, C., Raju, R., Patterson, J. & Kolakofsky, D. (1987). Translational requirement of La Crosse virus S-mRNA synthesis: in vitro studies. *J Virol* **61**, 87-95.

Berger, S. L. & Kimmel, A. R. (1987). Guide to molecular cloning techniques. In *Methods in Enzymology*: Academic Press.

Bergmann, M. & Muster, T. (1996). Mutations in the nonconserved noncoding sequences of the influenza A virus segments affect viral vRNA formation. *Virus Res* **44**, 23-31.

Betenbaugh, M., Yu, M., Kuehl, K., White, J., Pennock, D., Spik, K. & Schmaljohn, C. (1995). Nucleocapsid- and virus-like particles assemble in cells infected with recombinant baculoviruses or vaccinia viruses expressing the M and the S segments of Hantaan virus. *Virus Res* **38**, 111-24.

Bilsel, P. A., Tesh, R. B. & Nichol, S. T. (1988). RNA genome stability of Toscana virus during serial transovarial transmission in the sandfly Phlebotomus perniciosus. *Virus Res* **11**, 87-94.

Bishop, D. H. & Beaty, B. J. (1988). Molecular and biochemical studies of the evolution, infection and transmission of insect bunyaviruses. *Philos Trans R Soc Lond B Biol Sci* **321**, 463-83.

Bishop, D. H., Gay, M. E. & Matsuoko, Y. (1983). Nonviral heterogeneous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA. *Nucleic Acids Res* **11**, 6409-18.

Bishop, D. H., Gould, K. G., Akashi, H. & Clerx-van Haaster, C. M. (1982). The complete sequence and coding content of snowshoe hare bunyavirus small (S) viral RNA species. *Nucleic Acids Res* **10**, 3703-13.

Bishop, D. H. L. & Shope, R. E. (1979). Bunyaviridae. *Compr Virol* **14**, 1-156.

Bishop, D. H. L. (1996). Biology and molecular biology of bunyaviruses. In *The Bunyaviridae*, pp. 19-53. Edited by R. M. Elliott. New York: Plenum Press.

Blacklow, S. C. & Knowles, J. R. (1991). Spiked oligonucleotide mutagenesis. In *Directed Mutagenesis*, pp. 177-98. Edited by M. J. McPherson. Oxford: Oxford University Press.

Booth, T. F., Gould, E. A. & Nuttall, P. A. (1991). Structure and morphogenesis of Dugbe virus (Bunyaviridae, Nairovirus) studied by immunogold electron microscopy of ultrathin cryosections. *Virus Res* **21**, 199-212.

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. & Girard, M. (1990). Characterisation of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (Bunyavirus). *Virology* **75**, 50-58.

Bowen, M. D., Jackson, A. O., Bruns, T. D., Hacker, D. L. & Hardy, J. L. (1995). Determination and comparative analysis of the small RNA genomic sequences of California encephalitis, Jamestown Canyon, Jerry Slough, Melao, Keystone and Trivittatus viruses (Bunyaviridae, genus Bunyavirus, California serogroup). *J Gen Virol* **76**, 559-72.

Boyer, J. C. & Haenni, A. L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* **198**, 415-26.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.

Bridgen, A. & Elliott, R. M. (1996). Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proceedings of the National Academy of Sciences, USA* **93**, 15400-15404.

Bridgen, A. & Elliott, R. M. (1997). Unpublished.

Buchholz, C. J., Spehner, D., Drillien, R., Neubert, W. J. & Homann, H. E. (1993). The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. *J Virol* **67**, 5803-12.

Bupp, K., Stillmock, K. & Gonzalez-Scarano, F. (1996). Analysis of the intracellular transport properties of recombinant La Crosse virus glycoproteins. *Virology* **220**, 485-90.

Calisher, C. (1991). Classification and nomenclature of viruses. Fifth report of the international committee on taxonomy of viruses. *Arch Virol* **suppl 2**, 273-283.

Calisher, C. H. (1996). History, classification, and taxonomy of viruses in the family Bunyaviridae. In *The Bunyaviridae*, pp. 1-15. Edited by R. M. Elliott. New York: Plenum Press.

Canter, D. M., Jackson, R. L. & Perrault, J. (1993). Faithful and efficient in vitro reconstitution of vesicular stomatitis virus transcription using plasmid-encoded L and P proteins [published erratum appears in Virology 1993 Nov;197(1):506]. *Virology* **194**, 518-29.

Cash, P. (1982). Inhibition of La Crosse virus replication by monensin, monovalent ionophore. *J Gen Virol* **59**, 193-6.

Cash, P., Hendershot, L. & Bishop, D. H. (1980). The effect of glycosylation inhibitors on the maturation and intracellular polypeptide synthesis induced by snowshoe hare bunyavirus. *Virology* **103**, 235-40.

Cash, P., Vezza, A. C., Gentsch, J. R. & Bishop, D. H. (1979). Genome complexities of the three mRNA species of snowshoe hare bunyavirus and in vitro translation of S mRNA to viral N polypeptide. *J Virol* **31**, 685-94.

Castrucci, M. R., Bilsel, P. & Kawaoka, Y. (1992). Attenuation of influenza A virus by insertion of a foreign epitope into the neuraminidase. *J Virol* **66**, 4647-53.

Castrucci, M. R., Hou, S., Doherty, P. C. & Kawaoka, Y. (1994). Protection against lethal lymphocytic choriomeningitis virus (LCMV) infection by immunization of mice with an influenza virus containing an LCMV epitope recognized by cytotoxic T lymphocytes. *J Virol* **68**, 3486-90.

Castrucci, M. R. & Kawaoka, Y. (1995). Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. *J Virol* **69**, 2725-8.

Chakrabarti, S., Brechling, K. & Moss, B. (1985). Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol Cell Biol* **5**, 3403-9.

Chapman, K. A. & Burgess, R. R. (1987). Construction of bacteriophage T7 late promoters with point mutations and characterization by in vitro transcription properties. *Nucleic Acids Res* **15**, 5413-32.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* **162**, 156-9.

Cianci, C., Tiley, L. & Krystal, M. (1995). Differential activation of the influenza virus polymerase via template RNA binding. *J Virol* **69**, 3995-9.

Collett, M. S. (1986). Messenger RNA of the M segment RNA of Rift Valley fever virus. *Virology* **151**, 151-156.

Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M. & Murphy, B. R. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proc Natl Acad Sci U S A* **92**, 11563-7.

Collins, P. L., Mink, M. A. & Stec, D. S. (1991). Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc Natl Acad Sci U S A* **88**, 9663-7.

Conzelmann, K. K. (1996). Genetic manipulation of non-segmented negative-strand RNA viruses. *J Gen Virol* **77**, 381-9.

Conzelmann, K. K. (1998). Nonsegmented negative-strand RNA viruses: genetics and manipulation of viral genomes. *Annu Rev Genet* **32**, 123-62.

Conzelmann, K. K. & Schnell, M. (1994). Rescue of synthetic genomic RNA analogs of rabies virus by plasmid- encoded proteins. *J Virol* **68**, 713-9.

Cullen, B. R. (1987). Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol* **152**, 684-704.

Curran, J. & Kolakofsky, D. (1988). Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. *EMBO J* **7**, 245-51.

De, B. P. & Banerjee, A. K. (1993). Rescue of synthetic analogs of genome RNA of human parainfluenza virus type 3. *Virology* **196**, 344-8.

de Haan, P., Wagemakers, L., Peters, D. & Goldbach, R. (1990). The S RNA segment of tomato spotted wilt virus has an ambisense character. *J Gen Virol* **71**, 1001-7.

de la Luna, S., Martin, J., Portela, A. & Ortin, J. (1993). Influenza virus naked RNA can be expressed upon transfection into cells co-expressing the three subunits of the polymerase and the nucleoprotein from simian virus 40 recombinant viruses. *J Gen Virol* **74**, 535-9.

Desselberger, U., Racaniello, V. R., Zazra, J. J. & Palese, P. (1980). The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. *Gene* **8**, 315-28.

Dimock, K. & Collins, P. L. (1993). Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. *J Virol* **67**, 2772-8.

Domingo, E. & Holland, J. J. (1988). . In *RNA Genetics*, pp. 3-36. Edited by E. Domingo, J. J. Holland & P. Ahlquist. Boca Raton: CRC Press.

Dunn, E. F. & Elliott, R. M. E. Unpublished work; construction of pT7MAGS-R-T: Institute of Virology, University of Glasgow.

Dunn, E. F., Pritlove, D. C. & Elliott, R. M. (1994). The S RNA genome segments of Batai, Cache Valley, Guaroa, Kairi, Lumbo, Main Drain and Northway bunyaviruses: sequence determination and analysis. *J. Gen. Virol.* **75**, 597-608.

Dunn, J. J. & Studier, F. W. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J Mol Biol* **166**, 477-535.

Durbin, A. P., Hall, S. L., Siew, J. W., Whitehead, S. S., Collins, P. L. & Murphy, B. R. (1997). Recovery of infectious human parainfluenza virus type 3 from cDNA. *Virology* **235**, 323-32.

Eddy, G. A. & Peters, C. J. (1980). The extended horizons of Rift Valley fever: current and projected immunogens. *Prog Clin Biol Res* **47**, 179-91.

Elliott, L. H., Kiley, M. P. & McCormick, J. B. (1984). Hantaan virus: identification of virion proteins. *J Gen Virol* **65**, 1285-93.

Elliott, R. M. (1985). Identification of nonstructural proteins encoded by viruses of the Bunyamwera serogroup (family Bunyaviridae). *Virology* **143**, 119-26.

Elliott, R. M. (1989a). Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *Virology* **173**, 426-36.

Elliott, R. M. (1989b). Nucleotide sequence analysis of the small (S) RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *J Gen Virol* **70**, 1281-5.

Elliott, R. M. (1990). Molecular biology of the Bunyaviridae. *J. Gen. Virol.* **71**, 501-522.

Elliott, R. M. (1995). Evolution of bunyaviridae. In *Coevolution of viruses and their hosts*, pp. 321-337. Edited by A. J. Gibbs, C. H. Calisher & F. Garcia-Arenal. Cambridge: Cambridge University Press.

Elliott, R. M. (1996a). The Bunyaviridae. In *The Viruses*: Plenum Press.

Elliott, R. M. (1996b). The Bunyaviridae: concluding remarks and future prospects. In *The Bunyaviridae*, pp. 295-332. Edited by R. M. Elliott. New York: Plenum Press.

Elliott, R. M. (1997). Emerging viruses: the Bunyaviridae. *Molecular Medicine* **3**, 572-577.

Elliott, R. M. & McGregor, A. Unpublished work; construction of pTFBUNS: Institute of Virology. University of Glasgow.

Elliott, R. M. & McGregor, A. (1989). Nucleotide sequence and expression of the small (S) RNA segment of Maguari bunyavirus. *Virology* **171**, 516-24.

Elliott, R. M. & Page, K. Unpublished work; construction of pGEMBUNN: Institute of Virology. University of Glasgow.

Elliott, R. M. & Page, K. Unpublished work; construction of vSC11BUNS: Institute of Virology. University of Glasgow.

Elliott, R. M., Schmaljohn, C. S. & Collett, M. S. (1991). Bunyaviridae genome structure and gene expression. *Curr Top Microbiol Immunol* **169**, 91-141.

Elliott, R. M., Smith, M. & McGregor, A. (1989). Expression of the bunyavirus S RNA segment. In *Genetics and Pathogenicity of Negative Strand Viruses*, pp. 67-73. Edited by B. W. J. Mahy & D. Kolakofsky. Amsterdam: Elsevier Biomedical Press.

Elliott, R. M. & Wilkie, M. L. (1986). Persistent infection of Aedes albopictus C6/36 cells by Bunyamwera virus. *Virology* **150**, 21-32.

Emery, V. C. & Bishop, D. H. L. (1987). Characterisation of Punta Toro S mRNA species and identification of an inverted complementary sequence in the intergenic region of Punta Toro virus phlebovirus ambisense S RNA that is involved in mRNA transcription termination. *Virology* **156**, 1-11.

Enami, M., Luytjes, W., Krystal, M. & Palese, P. (1990). Introduction of site-specific mutations into the genome of influenza virus. *Proc Natl Acad Sci U S A* **87**, 3802-5.

Enami, M. & Palese, P. (1991). High-efficiency formation of influenza virus transfectants. *J Virol* **65**, 2711-3.

Endres, M. J., Griot, C., Gonzalez-Scarano, F. & Nathanson, N. (1991). Neuroattenuation of an avirulent bunyavirus variant maps to the L RNA segment. *J Virol* **65**, 5465-70.

Eshita, Y. & Bishop, D. H. (1984). The complete sequence of the M RNA of snowshoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoprotein. *Virology* **137**, 227-40.

Eshita, Y., Ericson, B., Romanowski, V. & Bishop, D. H. (1985). Analyses of the mRNA transcription processes of snowshoe hare bunyavirus S and M RNA species. *J Virol* **55**, 681-9.

Fazakerley, J. K., Gonzalez-Scarano, F., Strickler, J., Dietzschold, B., Karush, F. & Nathanson, N. (1988). Organization of the middle RNA segment of snowshoe hare Bunyavirus. *Virology* **167**, 422-32.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. & Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* **84**, 7413-7.

Flick, R., Neumann, G., Hoffmann, E., Neumeier, E. & Hobom, G. (1996). Promoter elements in the influenza vRNA terminal structure. *RNA* **2**, 1046-57.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J Virol* **73**, 9679-82.

Fodor, E., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. (1998). Attenuation of influenza A virus mRNA levels by promoter mutations. *J Virol* **72**, 6283-90.

Fodor, E., Pritlove, D. C. & Brownlee, G. G. (1994). The influenza virus panhandle is involved in the initiation of transcription. *J Virol* **68**, 4092-6.

Fodor, E., Pritlove, D. C. & Brownlee, G. G. (1995). Characterization of the RNA-fork model of virion RNA in the initiation of transcription in influenza A virus. *J Virol* **69**, 4012-9.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L. & Brown, F. (1991). Classification and nomenclature of viruses; Fifth report of the international committee on taxonomy of viruses. In *Arch. Virol. (Suppl. 2)*.

Fuerst, T. F., Earl, P. L. & Moss, B. (1987). Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Molecular and Cellular Biology* **7**, 2538-2544.

Fuerst, T. F., Niles, E. G., Studier, F. W. & Moss, B. (1986). Eukaryotic expression system based on recombinant vaccinia virus that synthesises bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences, USA* **83**, 8122-8126.

Garcia-Sastre, A. (1998). Negative-strand RNA viruses: applications to biotechnology. *Trends Biotechnol* **16**, 230-5.

Garcia-Sastre, A., Muster, T., Barclay, W. S., Percy, N. & Palese, P. (1994a). Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. *J Virol* **68**, 6254-61.

Garcia-Sastre, A. & Palese, P. (1993). Genetic manipulation of negative-strand RNA virus genomes. *Annu Rev Microbiol* **47**, 765-90.

Garcia-Sastre, A. & Palese, P. (1995). Influenza virus vectors. *Biologicals* **23**, 171-8.

Garcia-Sastre, A., Percy, N., Barclay, W. & Palese, P. (1994b). Introduction of foreign sequences into the genome of influenza A virus. *Dev Biol Stand* **82**, 237-46.

Garcin, D., Lezzi, M., Dobbs, M., Elliott, R. M., Schmaljohn, C., Kang, C. Y. & Kolakofsky, D. (1995a). The 5' ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. *J. Virol.* **69**, 5754-5762.

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J. & Kolakofsky, D. (1995b). A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. *EMBO J* **14**, 6087-94.

Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. & Mackow, E. R. (1998). beta3 Integrins mediate the cellular entry of hantaviruses that cause respiratory failure. *Proc Natl Acad Sci U S A* **95**, 7074-9.

Gerbaud, S., Vialat, P., Pardigon, N., Wychowski, C., Girard, M. & Bouloy, M. (1987). The S segment of the Germiston virus RNA genome can code for three proteins. *Virus Res* **8**, 1-13.

Gilleland, H. E., Jr., Gilleland, L. B., Staczek, J., Harty, R. N., Garcia-Sastre, A., Engelhardt, O. G. & Palese, P. (1997). Chimeric influenza viruses incorporating epitopes of outer membrane protein F as a vaccine against pulmonary infection with Pseudomonas aeruginosa. *Behring Inst Mitt*, 291-301.

Giorgi, C. (1996). Molecular biology of phleboviruses. In *The Bunyaviridae*, pp. 105-123. Edited by R. M. Elliott. New York: Plenum Press.

Goldbach, R. & Peters, D. (1996). Molecular and biological aspects of tospoviruses. In *The Bunyaviridae*, pp. 129-153. Edited by R. M. Elliott. New York: Plenum Press.

Gonzalez-Scarano, F., Beaty, B., Sundin, D., Janssen, R., Endres, M. J. & Nathanson, N. (1988). Genetic determinants of the virulence and infectivity of La Crosse virus. *Microb Pathog* **4**, 1-7.

Gonzalez-Scarano, F., Bupp, K. & Nathanson, N. (1996). Pathogenesis of diseases caused by viruses of the Bunyavirus genus. In *The Bunyaviridae*, pp. 227-244. Edited by R. M. Elliott. New York: Plenum Press.

Gonzalez-Scarano, F., Endres, M. J. & Nathanson, N. (1991). Bunyaviridae: Pathogenesis. *Curr Top Microbiol Immunol* **169**, 217-49.

Gonzalez-Scarano, F. & Nathanson, N. (1996). Bunyaviridae. In *Fields Virology*, pp. 1473-94. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2**, 1044-51.

Gott, P., Stohwasser, R., Schnitzler, P., Darai, G. & Bautz, E. K. (1993). RNA binding of recombinant nucleocapsid proteins of hantaviruses. *Virology* **194**, 332-7.

Grady, L. J., Sanders, M. L. & Campbell, W. P. (1983a). Evidence for three separate antigenic sites on the G1 protein of La Crosse Virus. *Virology* **126**, 395-7.

Grady, L. J., Sanders, M. L. & Campbell, W. P. (1987). The sequence of the M RNA of an isolate of La Crosse virus. *J Gen Virol* **68**, 3057-71.

Grady, L. J., Srihongse, S., Grayson, M. A. & Deibel, R. (1983b). Monoclonal antibodies against La Crosse virus. *J Gen Virol* **64**, 1699-704.

Gro, M. C., Di Bonito, P., Accardi, L. & Giorgi, C. (1992). Analysis of 3' and 5' ends of N and NSs messenger RNAs of Toscana Phlebovirus. *Virology* **191**, 435-8.

Gupta, K. C. & Patwardhan, S. (1988). ACG, the initiator codon for a Sendai virus protein. *J Biol Chem* **263**, 8553-6.

Hacker, D., Rochat, S. & Kolakofsky, D. (1990). Anti-mRNAs in La Crosse bunyavirus-infected cells. *J Virol* **64**, 5051-7.

Hagen, M., Chung, T. D., Butcher, J. A. & Krystal, M. (1994). Recombinant influenza virus polymerase: requirement of both 5' and 3' viral ends for endonuclease activity. *J Virol* **68**, 1509-15.

Harty, R. N. & Palese, P. (1995). Mutations within noncoding terminal sequences of model RNAs of Sendai virus: influence on reporter gene expression. *J Virol* **69**, 5128-31.

He, B., Paterson, R. G., Ward, C. D. & Lamb, R. A. (1997). Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237**, 249-60.

Hewlett, M. J., Pettersson, R. F. & Baltimore, D. (1977). Circular forms of Uukuniemi virion RNA: an electron microscopic study. *J Virol* **21**, 1085-93.

Hobman, T. C. (1993). Targeting of viral glycoproteins to the Golgi complex. *Trends Microbiol* **1**, 124-30.

Honda, M., Brown, E. A. & Lemon, S. M. (1996). Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* **2**, 955-68.

Hsu, M. T., Parvin, J. D., Gupta, S., Krystal, M. & Palese, P. (1987). Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proc Natl Acad Sci U S A* **84**, 8140-4.

Huang, T. S., Palese, P. & Krystal, M. (1990). Determination of influenza virus proteins required for genome replication. *J Virol* **64**, 5669-73.

Hutchinson, K. L., Peters, C. J. & Nichol, S. T. (1996). Sin Nombre virus mRNA synthesis. *Virology* **224**, 139-49.

Ihara, T., Akashi, H. & Bishop, D. H. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro Phlebovirus S RNA. *Virology* **136**, 293-306.

Ihara, T., Matsuura, Y. & Bishop, D. H. (1985). Analyses of the mRNA transcription processes of Punta Toro phlebovirus (Bunyaviridae). *Virology* **147**, 317-25.

Iroegbu, C. U. & Pringle, C. R. (1981). Genetic interactions among viruses of the Bunyamwera complex. *J Virol* **37**, 383-94.

Ishihama, A. & Barbier, P. (1994). Molecular anatomy of viral RNA-directed RNA polymerases. *Arch Virol* **134**, 235-58.

James, W. S. & Millican, D. (1986). Host-adaptive antigenic variation in Bunyaviruses. *J Gen Virol* **67**, 2803-6.

Jin, H. & Elliott, R. M. (1991). Expression of functional Bunyamwera virus L protein by recombinant vaccina viruses. *J. Gen. Virol.* **65**, 4182-4189.

Jin, H. & Elliott, R. M. (1992). Mutagenesis of the L protein encoded by Bunyamwera virus and production of monospecific antibodies. *J Gen Virol* **73**, 2235-44.

Jin, H. & Elliott, R. M. (1993a). Characterisation of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. *J. Virol.* **67**, 1396-1404.

Jin, H. & Elliott, R. M. (1993b). Non-viral sequences at the 5' ends of Dugbe nairovirus mRNAs. *J. Gen. Virol.* **74**, 2293-2297.

Kappus, K. D., Monath, T. P., Kaminski, R. M. & Calisher, C. H. (1983). Reported encephalitis associated with California serogroup virus infections in the United States, 1963-1981. *Prog Clin Biol Res* **123**, 31-41.

Karabatsos, N. (1985). International catalogue of Arboviruses including other viruses of vertebrates, 3rd edn. San Antonio: Am. Soc. Trop. Med. Hyg.

Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M. & Nagai, Y. (1996). Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* **1**, 569-79.

Kim, H. J., Fodor, E., Brownlee, G. G. & Seong, B. L. (1997). Mutational analysis of the RNA-fork model of the influenza A virus vRNA promoter in vivo. *J Gen Virol* **78**, 353-7.

Kingsbury, D. W., Jones, I. M. & Murti, K. G. (1987). Assembly of influenza ribonucleoprotein in vitro using recombinant nucleoprotein. *Virology* **156**, 396-403.

Kingsford, L. & Hill, D. W. (1983). The effect of proteolytic cleavage of La Crosse virus G1 glycoprotein on antibody neutralization. *J Gen Virol* **64**, 2147-56.

Kingsford, L., Ishizawa, L. D. & Hill, D. W. (1983). Biological activities of monoclonal antibodies reactive with antigenic sites mapped on the G1 glycoprotein of La Crosse virus. *Virology* **129**, 443-55.

Kolakofsky, D., Bellocq, C. & Raju, R. (1987). The translational requirement for La Crosse virus S-mRNA synthesis. *Cold Spring Harb Symp Quant Biol* **52**, 373-9.

Kolakofsky, D. & Hacker, D. (1991). Bunyavirus RNA synthesis: genome transcription and replication. *Curr Top Microbiol Immunol* **169**, 143-59.

Kormelink, R., de Haan, P., Peters, D. & Goldbach, R. (1992a). Viral RNA synthesis in tomato spotted wilt virus-infected Nicotiana rustica plants. *J Gen Virol* **73**, 687-93.

Kormelink, R., van Poelwijk, F., Peters, D. & Goldbach, R. (1992b). Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. *J Gen Virol* **73**, 2125-8.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283-92.

Kozak, M. (1996). Interpreting cDNA sequences: some insights from studies on translation. *Mamm Genome* **7**, 563-74.

Krug, R. M., Alonso-Caplen, F. V., Julkunen, I. & Katze, M. (1989). Expression and replication of the influenza virus genome. In *The Influenza Viruses*, pp. 89-152. Edited by R. M. Krug. New York: Plenum Press.

Kuismanen, E., Hedman, K., Saraste, J. & Pettersson, R. F. (1982). Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. *Mol Cell Biol* **2**, 1444-58.

Kuo, M. Y., Sharmeen, L., Dinter-Gottlieb, G. & Taylor, J. (1988). Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. *J Virol* **62**, 4439-44.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Lamb, R. A. & Krug, R. M. (1996). Orthomyxoviridae: the viruses and their replication. In *Fields Virology*, 3rd edn, pp. 1353-87. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Lappin, D. F., Nakitare, G. W., Palfreyman, J. W. & Elliott, R. M. (1994). Localisation of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not NSm. *J. Gen. Virol.* **75**, 3441-3451.

Law, M. D., Speck, J. & Moyer, J. W. (1992). The M RNA of impatiens necrotic spot Tospovirus (Bunyaviridae) has an ambisense genomic organization. *Virology* **188**, 732-41.

Lawson, N. D., Stillman, E. A., Whitt, M. A. & Rose, J. K. (1995). Recombinant vesicular stomatitis viruses from DNA [published erratum appears in Proc Natl Acad Sci U S A 1995 Sep 12;92(19):9009]. *Proc Natl Acad Sci U S A* **92**, 4477-81.

Lee, H. W. (1996). Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome. In *The Bunyaviridae*, pp. 253-267. Edited by R. M. Elliott. New York: Plenum Press.

Lee, H. W., French, G. R., Lee, P. W., Baek, L. J., Tsuchiya, K. & Foulke, R. S. (1981a). Observations on natural and laboratory infection of rodents with the etiologic agent of Korean hemorrhagic fever. *Am J Trop Med Hyg* **30**, 477-82.

Lees, J. F., Pringle, C. R. & Elliott, R. M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: conservation of structural features in the bunyavirus glycoprotein gene product. *Virology* **148**, 1-14.

Li, M. L., Ramirez, B. C. & Krug, R. M. (1998). RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. *EMBO J* **17**, 5844-52.

Li, S., Polonis, V., Isobe, H., Zaghouani, H., Guinea, R., Moran, T., Bona, C. & Palese, P. (1993a). Chimeric influenza virus induces neutralizing antibodies and cytotoxic T cells against human immunodeficiency virus type 1. *J Virol* **67**, 6659-66.

Li, S., Schulman, J., Itamura, S. & Palese, P. (1993b). Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J Virol* **67**, 6667-73.

Li, S., Xu, M. & Coelingh, K. (1995). Electroporation of influenza virus ribonucleoprotein complexes for rescue of the nucleoprotein and matrix genes. *Virus Res* **37**, 153-61.

Li, X. & Palese, P. (1992). Mutational analysis of the promoter required for influenza virus virion RNA synthesis. *J Virol* **66**, 4331-8.

Li, X. & Palese, P. (1994). Characterization of the polyadenylation signal of influenza virus RNA. *J Virol* **68**, 1245-9.

Lopez, N., Muller, R., Prehaud, C. & Bouloy, M. (1995). The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. *J Virol* **69**, 3972-9.

Ludwig, G. V., Christensen, B. M., Yuill, T. M. & Schultz, K. T. (1989). Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus- vector infection model. *Virology* **171**, 108-13.

Luo, G. X., Luytjes, W., Enami, M. & Palese, P. (1991). The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. *J Virol* **65**, 2861-7.

Luytjes, W., Krystal, M., Enami, M., Pavin, J. D. & Palese, P. (1989). Amplification, expression, and packaging of foreign gene by influenza virus. *Cell* **59**, 1107-13.

Madoff, D. H. & Lenard, J. (1982). A membrane glycoprotein that accumulates intracellularly: cellular processing of the large glycoprotein of LaCrosse virus. *Cell* **28**, 821-9.

Marriott, A. C. & Nuttall, P. A. (1996a). Large RNA segment of Dugbe nairovirus encodes the putative RNA polymerase. *J Gen Virol* **77**, 1775-80.

Marriott, A. C. & Nuttall, P. A. (1996b). Molecular biology of nairoviruses. In *The Bunyaviridae*, pp. 91-102. Edited by R. M. Elliott. New York: Plenum Press.

Martin, M. L., Lindsey-Regnery, H., Sasso, D. R., McCormick, J. B. & Palmer, E. (1985). Distinction between Bunyaviridae genera by surface structure and comparison with Hantaan virus using negative stain electron microscopy. *Arch Virol* **86**, 17-28.

Masters, P. S. & Banerjee, A. K. (1988). Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. *J Virol* **62**, 2658-64.

McKee, K. T., LeDuc, J. W. & Peters, C. J. (1991). Hantaviruses. In *Textbook of human virology*, pp. 615. Edited by R. B. Belshe. St. Louis: Mosby-Year Book.

Meegan, J. & Shope, R. E. (1981). emerging concepts on Rift Valley fever. In *Perspectives in Virology*.

Mena, I., de la Luna, S., Albo, C., Martin, J., Nieto, A., Ortin, J. & Portela, A. (1994). Synthesis of biologically active influenza virus core proteins using a vaccinia virus-T7 RNA polymerase expression system. *J Gen Virol* **75**, 2109-14.

Mena, I., Vivo, A., Perez, E. & Portela, A. (1996). Rescue of a synthetic chloramphenicol acetyltransferase RNA into influenza virus-like particles obtained from recombinant plasmids. *J Virol* **70**, 5016-24.

Miyahira, Y., Garcia-Sastre, A., Rodriguez, D., Rodriguez, J. R., Murata, K., Tsuji, M., Palese, P., Esteban, M., Zavala, F. & Nussenzweig, R. S. (1998). Recombinant viruses expressing a human malaria antigen can elicit potentially protective immune CD8+ responses in mice. *Proc Natl Acad Sci U S A* **95**, 3954-9.

Muller, R., Poch, O., Delarue, M., Bishop, D. H. & Bouloy, M. (1994). Rift Valley fever virus L segment: correction of the sequence and possible functional role of newly identified regions conserved in RNA- dependent polymerases. *J Gen Virol* **75**, 1345-52.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., Palese, P. & Katinger, H. (1994). Cross-neutralizing activity against divergent human immunodeficiency virus type 1 isolates induced by the gp41 sequence ELDKWAS. *J Virol* **68**, 4031-4.

Nakitare, G. W. & Elliott, R. M. (1993). Expression of the Bunyamwera virus M genome segment and intracellular localisation of NSm. *Virology* **195**, 511-520.

Neumann, G. & Hobom, G. (1995). Mutational analysis of influenza virus promoter elements in vivo. *J Gen Virol* **76**, 1709-17.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., Hobom, G. & Kawaoka, Y. (1999). Generation of influenza A viruses entirely from cloned cDNAs [see comments]. *Proc Natl Acad Sci U S A* **96**, 9345-50.

Neumann, G., Zobel, A. & Hobom, G. (1994). RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* **202**, 477-9.

Nichol, S. T., Ksiazek, T. G., Rollin, P. E. & Peters, C. J. (1996). Hantavirus pulmonary syndrome and newly described hantaviruses in the United States. In *The Bunyaviridae*, pp. 269-279. Edited by R. M. Elliott. New York: Plenum Press.

Obijeski, J. F., Bishop, D. H. L., Palmer, E. & Murphy, F. A. (1976). Segmented genome and nucleocapsid of La Crosse virus. *Journal of Virology* **20**, 664-675.

Obijeski, J. F. & Murphy, F. A. (1977). Bunyaviridae: recent biochemical developments. *J Gen Virol* **37**, 1-14.

Odagiri, T. & Tashiro, M. (1997). Segment-specific noncoding sequences of the influenza virus genome RNA are involved in the specific competition between defective interfering RNA and its progenitor RNA segment at the virion assembly step. *J Virol* **71**, 2138-45.

Palese, P., Zheng, H., Engelhardt, O. G., Pleschka, S. & Garcia-Sastre, A. (1996). Negative-strand RNA viruses: genetic engineering and applications. *Proc Natl Acad Sci U S A* **93**, 11354-8.

Pardigon, N., Vialat, P., Gerbaud, S., Girard, M. & Bouloy, M. (1988). Nucleotide sequence of the M segment of Germiston virus: comparison of the M gene product of several bunyaviruses. *Virus Res* **11**, 73-85.

Park, K. H., Huang, T., Correia, F. F. & Krystal, M. (1991). Rescue of a foreign gene by Sendai virus. *Proc Natl Acad Sci U S A* **88**, 5537-41.

Parker, M. D., Smith, J. F. & Dalrymple, J. M. (1984). Rift Valley fever virus intracellular RNA: a functional analysis. In *Segmented negative strand viruses*, pp. 21-28. Edited by R. Compans & D. Bishop. Orlando, FL: Academic.

Parrington, M. A. & Kang, C. Y. (1990). Nucleotide sequence analysis of the S genomic segment of Prospect Hill virus: comparison with the prototype Hantavirus. *Virology* **175**, 167-75.

Parvin, J. D., Palese, P., Honda, A., Ishihama, A. & Krystal, M. (1989). Promoter analysis of influenza virus RNA polymerase. *J Virol* **63**, 5142-52.

Patel, A. H. & Elliott, R. M. (1992). Characterisation of Bunyamwera virus defective interfering particles. *J. Gen. Virol.* **73**, 389-396.

Patterson, J. L. & Kolakofsky, D. (1984). Characterisation of La Crosse virus small-genome transcripts. *J Virol* **49**, 680-5.

Patterson, J. L., Holloway, B. & Kolakofsky, D. (1984). La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *J Virol* **52**, 215-22.

Pattnaik, A. K. & Abraham, G. (1983). Identification of four complementary RNA species in Akabane virus- infected cells. *J Virol* **47**, 452-62.

Pattnaik, A. K., Ball, L. A., LeGrone, A. & Wertz, G. W. (1995). The termini of VSV DI particle RNAs are sufficient to signal RNA encapsidation, replication, and budding to generate infectious particles. *Virology* **206**, 760-4.

Pattnaik, A. K., Ball, L. A., LeGrone, A. W. & Wertz, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**, 1011-20.

Pattnaik, A. K. & Wertz, G. W. (1991). Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles. *Proc Natl Acad Sci U S A* **88**, 1379-83.

Pekosz, A., Griot, C., Nathanson, N. & Gonzalez-Scarano, F. (1995). Tropism of bunyaviruses: evidence for a G1 glycoprotein-mediated entry pathway common to the California serogroup. *Virology* **214**, 339-48.

Pennington, T. H., Pringle, C. R. & McCrae, M. A. (1977). Bunyamwera virus-induced polypeptide synthesis. *J Virol* **24**, 397-400.

Percy, N., Barclay, W. S., Garcia-Sastre, A. & Palese, P. (1994). Expression of a foreign protein by influenza A virus. *J Virol* **68**, 4486-92.

Pettersson, R. & Melin, L. (1996). Synthesis, assembly, and intracellular transport of Bunyaviridae membrane proteins. In *The Bunyaviridae*, pp. 159-188. Edited by R. M. Elliott. New York: Plenum Press.

Pettersson, R. F. (1991). Protein localization and virus assembly at intracellular membranes. *Curr Top Microbiol Immunol* **170**, 67-106.

Piccone, M. E., Fernandez-Sesma, A. & Palese, P. (1993). Mutational analysis of the influenza virus vRNA promoter. *Virus Res* **28**, 99-112.

Pleiss, J. A., Derrick, M. L. & Uhlenbeck, O. C. (1998). T7 RNA polymerase produces 5' end heterogeneity during in vitro transcription from certain templates. *RNA* **4**, 1313-7.

Pleschka, S., Jaskunas, R., Engelhardt, O. G., Zurcher, T., Palese, P. & Garcia-Sastre, A. (1996). A plasmid-based reverse genetics system for influenza A virus. *J Virol* **70**, 4188-92.

Poch, O., Blumberg, B. M., Bougueleret, L. & Tordo, N. (1990). Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J Gen Virol* **71**, 1153-62.

Poch, O., Sauvaget, I., Delarue, M. & Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J* **8**, 3867-74.

Poon, L. L., Pritlove, D. C., Sharps, J. & Brownlee, G. G. (1998). The RNA polymerase of influenza virus, bound to the 5' end of virion RNA, acts in cis to polyadenylate mRNA. *J Virol* **72**, 8214-9.

Prehaud, C., Lopez, N., Blok, M. J., Obry, V. & Bouloy, M. (1997). Analysis of the 3' terminal sequence recognized by the Rift Valley fever virus transcription complex in its ambisense S segment. *Virology* **227**, 189-97.

Pringle, C. R. (1991). The Bunyaviridae and their genetics--an overview. *Curr Top Microbiol Immunol* **169**, 1-25.

Pringle, C. R. (1996). Genetics and genome segment reassortment. In *The Bunyaviridae*, pp. 189-220. Edited by R. M. Elliott. New York: Plenum Press.

Pringle, C. R., Clark, W., Lees, J. F. & Elliott, R. M. (1984). Restriction of sub-unit reassortment in the Bunyaviridae. In *Segmented Negative Strand Viruses*, pp. 45-50. Edited by R. W. Compans & D. H. L. Bishop. New York: Academic Press.

Prins, M. & Goldbach, R. (1998).The emerging problem of tospovirus infection and nonconventional methods of control. *Trends Microbiol* **6**, 31-4.

Pritlove, D. C. (1993). Developing a system for the application of reverse genetics to bunyaviruses. In *Institute of Virology*. Glasgow: University of Glasgow.

Pritlove, D. C., Fodor, E., Seong, B. L. & Brownlee, G. G. (1995). In vitro transcription and polymerase binding studies of the termini of influenza A virus cRNA: evidence for a cRNA panhandle. *J Gen Virol* **76**, 2205-13.

Pritlove, D. C., Poon, L. L., Fodor, E., Sharps, J. & Brownlee, G. G. (1998). Polyadenylation of influenza virus mRNA transcribed in vitro from model virion RNA templates: requirement for 5' conserved sequences. *J Virol* **72**, 1280-6.

Qiu, W. P., Geske, S. M., Hickey, C. M. & Moyer, J. W. (1998). Tomato spotted wilt Tospovirus genome reassortment and genome segment- specific adaptation. *Virology* **244**, 186-94.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G. & Billeter, M. A. (1995). Rescue of measles viruses from cloned DNA. *EMBO J* **14**, 5773-84.

Raju, R. & Kolakofsky, D. (1986). Inhibitors of protein synthesis inhibit both La Crosse virus S-mRNA and S genome syntheses in vivo. *Virus Res* **5**, 1-9.

Raju, R. & Kolakofsky, D. (1987). Translational requirement of La Crosse virus S-mRNA synthesis: in vivo studies. *J Virol* **61**, 96-103.

Raju, R. & Kolakofsky, D. (1989). The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired. *J Virol* **63**, 122-8.

Resende, R. d. O., de Haan, P., de Avila, A. C., Kitajima, E. W., Kormelink, R., Goldbach, R. & Peters, D. (1991). Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. *J Gen Virol* **72**, 2375-83.

Resende, R. d. O., de Haan, P., van de Vossen, E., de Avila, A. C., Goldbach, R. & Peters, D. (1992). Defective interfering L RNA segments of tomato spotted wilt virus retain both virus genome termini and have extensive internal deletions. *J Gen Virol* **73**, 2509-16.

Richmond, K. E., Chenault, K., Sherwood, J. L. & German, T. L. (1998). Characterization of the nucleic acid binding properties of tomato spotted wilt virus nucleocapsid protein. *Virology* **248**, 6-11.

Rohrmann, G., Yuen, L. & Moss, B. (1986). Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. *Cell* **46**, 1029-35.

Rolls, M. M., Webster, P., Balba, N. H. & Rose, J. K. (1994). Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. *Cell* **79**, 497-506.

Romaniuk, P. J. & Uhlenbeck, O. C. (1983). Joining of RNA molecules with RNA ligase. *Methods Enzymol* **100**, 52-9.

Rose, J. K., Buonocore, L. & Whitt, M. A. (1991). A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques* **10**, 520-5.

Rossier, C., Patterson, J. & Kolakofsky, D. (1986). La Crosse virus small genome mRNA is made in the cytoplasm. *J Virol* **58**, 647-50.

Rossier, C., Raju, R. & Kolakofsky, D. (1988). LaCrosse virus gene expression in mammalian and mosquito cells. *Virology* **165**, 539-48.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463-7.

Scallan, M. F. & Elliott, R. M. (1992). Defective RNAs in mosquito cells persistently infected with Bunyamwera virus. *J. Gen. Virol.* **73**, 53-60.

Schmaljohn, C. S. (1996a). Bunyaviridae: The virus and their replication. In *Fields Virology*, 3rd edn, pp. 1447-71. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Schmaljohn, C. S. (1996b). Molecular biology of hantaviruses. In *The Bunyaviridae*, pp. 63-86. Edited by R. M. Elliott. New York: Plenum Press.

Schmaljohn, C. S., Beaty, B. J., Calisher, C. H., Dalrymple, J. H., Elliott, R. M., Karabatsos, N., Kolakofsky, D., Lee, H. W., Lvov, D. K., Marriott, A. C., Nuttall, P. A., Peters, D., Pettersson, R. F. & Shope, R. E. (1985). Bunyaviridae. In *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*, pp. 300-315. Edited by F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo & M. D. Summers. Vienna: Springer-Verlag.

Schmaljohn, C. S., Chu, Y. K., Schmaljohn, A. L. & Dalrymple, J. M. (1990). Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J Virol* **64**, 3162-70.

Schmaljohn, C. S. & Dalrymple, J. M. (1984). Biochemical characterisation of Hantaan virus. In *Segmented negative strand viruses*, pp. 117-124. Edited by R. W. Compans & D. H. L. Bishop. New York: Elsevier.

Schnell, M. J. & Conzelmann, K. K. (1995). Polymerase activity of in vitro mutated rabies virus L protein. *Virology* **214**, 522-30.

Schnell, M. J., Mebatsion, T. & Conzelmann, K. K. (1994). Infectious rabies viruses from cloned cDNA. *EMBO J* **13**, 4195-203.

Schulman, J. L. & Palese, P. (1977). Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. *J Virol* **24**, 170-6.

Seong, B. L. & Brownlee, G. G. (1992a). A new method for reconstituting influenza polymerase and RNA in vitro: a study of the promoter elements for cRNA and vRNA synthesis in vitro and viral rescue in vivo. *Virology* **186**, 247-60.

Seong, B. L. & Brownlee, G. G. (1992b). Nucleotides 9 to 11 of the influenza A virion RNA promoter are crucial for activity in vitro. *J Gen Virol* **73**, 3115-24.

Seong, B. L., Kobayashi, M., Nagata, K., Brownlee, G. G. & Ishihama, A. (1992). Comparison of two reconstituted systems for in vitro transcription and replication of influenza virus. *J Biochem (Tokyo)* **111**, 496-9.

Shuman, S. & Moss, B. (1988). Factor-dependent transcription termination by vaccinia virus RNA polymerase. Evidence that the cis-acting termination signal is in nascent RNA. *J Biol Chem* **263**, 6220-5.

Sidhu, M. S., Chan, J., Kaelin, K., Spielhofer, P., Radecke, F., Schneider, H., Masurekar, M., Dowling, P. C., Billeter, M. A. & Udem, S. A. (1995). Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. *Virology* **208**, 800-7.

Simons, J. F., Hellman, U. & Pettersson, R. F. (1990). Uukuniemi virus S RNA segment: ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus Phlebovirus. *J Virol* **64**, 247-55.

Simons, J. F. & Pettersson, R. F. (1991). Host-derived 5' ends and overlapping complementary 3' ends of the two mRNAs transcribed from the ambisense S segment of Uukuniemi virus. *J Virol* **65**, 4741-8.

Smith, J. F. & Pifat, D. Y. (1982). Morphogenesis of sandfly viruses (Bunyaviridae family). *Virology* **121**, 61-81.

Staczek, J., Gilleland, H. E., Jr., Gilleland, L. B., Harty, R. N., Garcia-Sastre, A., Engelhardt, O. G. & Palese, P. (1998). A chimeric influenza virus expressing an epitope of outer membrane protein F of Pseudomonas aeruginosa affords protection against challenge with P. aeruginosa in a murine model of chronic pulmonary infection. *Infect Immun* **66**, 3990-4.

Stillman, E. A., Rose, J. K. & Whitt, M. A. (1995). Replication and amplification of novel vesicular stomatitis virus minigenomes encoding viral structural proteins. *J Virol* **69**, 2946-53.

Storms, M. M., Kormelink, R., Peters, D., Van Lent, J. W. & Goldbach, R. W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* **214**, 485-93.

Subbarao, E. K., Kawaoka, Y. & Murphy, B. R. (1993). Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation. *J Virol* **67**, 7223-8.

Szewczyk, B., Laver, W. G. & Summers, D. F. (1988). Purification, thioredoxin renaturation, and reconstituted activity of the three subunits of the influenza A virus RNA polymerase. *Proc Natl Acad Sci U S A* **85**, 7907-11.

Tabor, S. & Richardson, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc Natl Acad Sci U S A* **84**, 4767-71.

Talmon, Y., Prasad, B. V., Clerx, J. P., Wang, G. J., Chiu, W. & Hewlett, M. J. (1987). Electron microscopy of vitrified-hydrated La Crosse virus. *J Virol* **61**, 2319-21.

Tiley, L. S., Hagen, M., Matthews, J. T. & Krystal, M. (1994). Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5' ends of the viral RNAs. *J Virol* **68**, 5108-16.

van Poelwijk, F., Boye, K., Oosterling, R., Peters, D. & Goldbach, R. (1993). Detection of the L protein of tomato spotted wilt virus. *Virology* **197**, 468-70.

van Poelwijk, F., Kolkman, J. & Goldbach, R. (1996). Sequence analysis of the 5' ends of tomato spotted wilt virus N mRNAs. *Arch Virol* **141**, 177-84.

Vezza, A. C., Repik, P. M., Cash, P. & Bishop, D. H. (1979). In vivo transcription and protein synthesis capabilities of bunyaviruses: wild-type snowshoe hare virus and its temperature- sensitive group I, group II, and group I/II mutants. *J Virol* **31**, 426-36.

Vialat, P. & Bouloy, M. (1992). Germiston virus transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *J Virol* **66**, 685-93.

Wagner, R. R. & Rose, J. K. (1996). Rhabdoviridae: The viruses and their replication. In *Fields Virology*, 3rd edn, pp. 1121-35. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott-Raven.

Whelan, S. P., Ball, L. A., Barr, J. N. & Wertz, G. T. (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc Natl Acad Sci U S A* **92**, 8388-92.

Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R. & Peters, D. (1993). Multiplication of tomato spotted wilt virus in its insect vector, Frankliniella occidentalis. *J Gen Virol* **74**, 341-9.

Wyatt, L. S., Moss, B. & Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**, 202-5.

Zheng, H., Palese, P. & Garcia-Sastre, A. (1996). Nonconserved nucleotides at the 3' and 5' ends of an influenza A virus RNA play an important role in viral RNA replication. *Virology* **217**, 242-51.

Zuker, M., Jaeger, J. A. & Turner, D. H. (1991). A comparison of optimal and suboptimal RNA secondary structures predicted by free energy minimization with structures determined by phylogenetic comparison. *Nucleic Acids Res* **19**, 2707-14.