Developing a system for the application of reverse genetics to bunyaviruses

By

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Contents.

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Abbreviations

Nucleic acids:

DNA	2' deoxyribonucleic acid
RNA	ribonucleic acid
A	adenine (base in DNA/RNA)
G	guanine (base in DNA/RNA)
C	cytosine (base in DNA/RNA)
T	thymine (base in DNA)
U	uracil (base in RNA)
NTP(s)	ribonucleoside triphosphate(s)
ATP	adenosine 5' triphosphate
GTP	guanosine 5' triphosphate
CTP	cytidine 5' triphosphate
UTP	uridine 5' triphosphate
dNTP(s)	2' deoxyribonucleoside triphosphate(s)
dATP	2' deoxy adenosine 5' triphosphate
dGTP	2' deoxy guanosine 5' triphosphate
dCTP	2' deoxy cytidine 5' triphosphate
TTP	thymidine 5' triphosphate
ddNTP(s)	2',3' dideoxyribonucleoside triphosphate(s)
ddATP	2',3' dideoxy adenosine 5' triphosphate
ddGTP	2',3' dideoxy guanosine 5' triphosphate
ddCTP	2', 3' dideoxy cytidine 5' triphosphate
ddTTP	3' deoxy thymidine 5' triphosphate
cDNA	complementary DNA
mRNA	messenger RNA
vRNA	viral genomic RNA
cRNA	complementary RNA
RNP	ribonucleoprotein
ApG	Adenylyl 3',5' Guanosine (di nucleotide)
ss	single stranded nucleic acid
ds	double stranded nucleic acid

Chemicals and buffers:

^{32}P	radioisotope phosphorous-32
^{35}S	radioisotope sulphur-35
CsCl	caesium chloride
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetra acetic acid
NP-40	poly glycol ether (non ionic) surfactant type NP-40
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TBS	tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine

Physical units

°C	temperature in degrees Celcius
Ci	Curie (measure of radioactivity=3.7×10^{10} disintegrations/sec)
µCi	micro Curie
cpm	counts per minute (radioactivity)
cm^2	square centimetres area
g	gram mass or centrifugal force
mg	milligram (10^{-3}g)
µg	microgram (10^{-6}g)
ng	nanogram (10^{-9}g)
Hr(s)	hour(s)
min	minutes
kD	kilodalton(s)
kb	kilobase (pairs)
nt	nucleotide(s)
M	molar concentration
mM	milli molar concentration
p	pico (10^{-12})
pmole	pico mole quantity
pH	$-\log_{10}[H^+]$
rpm	revolutions per minute (centrifugation speed)
RT	room temperature (20–25°C)

Miscellaneous:

>	greater than
<	less than
~	approximately
λ	bacteriophage lambda
λ BstEII	DNA size ladder (λ digested with BstEII)
%	percent
%w/w	% weight of total weight
%w/v	% weight of total volume
A_{260}	Absorbance measured at 260nm
BHK	baby hamster kidney (cells)
CAT	chloramphenicol acetyl transferase (enzyme or gene)
MCN	micrococcal nuclease
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RT/PCR	coupled reverse transcription/PCR
RNAsin	placental ribonuclease inhibitor
RNAse A	ribonuclease A
T1	ribonuclease T1
Tn9	transposon 9 of *E. coli*
ts	temperature sensitive
wt	wild-type
T7 Pol.	T7 RNA Polymerase

Amino acids:

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartate	P	Pro	proline
E	Glu	glutamate	Q	gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

Viruses:

VTF7-3	vaccinia virus recombinant expressing T7 RNA polymerase
WR	non recombinant vaccinia virus strain WR
VSV	vesicular stomatitis virus
RSV	respiratory syncytial virus

Bunyaviruses:

AIO	Aino virus
BAT	Batai
BUN	Bunyamwera
Bun/Bun/Mag	reassortant Bunyamwera virus with Maguari virus S segment
CV	Cache valley
GER	Germiston
GRO	Guaroa
KRI	Kairi
LAC	La Crosse
LUM	Lumbo
MAG	Maguari
MD	Main drain
NOR	Northway
SSH	Snowshoe hare

Summary

For many positive strand RNA viruses, genomic RNA can initiate productive infection in the absence of preformed viral proteins when introduced into susceptible cells. RNA transcribed *in vitro* from cDNA copies of positive strand virus genomes is also infectious. This property has enabled the powerful techniques of recombinant DNA technology to be applied to produce viruses carrying defined mutations by manipulation of cDNA clones. In contrast, deproteinised genomes of negative strand RNA viruses are non infectious. The study of negative strand viruses has therefore been hampered by the absence of methodology enabling the manipulation of their genomes. The non infectious nature of negative strand genomes is due to the requirement of viral proteins to transcribe translation competent positive strand mRNA. The template for transcription by the RNA dependent RNA polymerases of negative strand viruses is a ribonucleoprotein complex (RNP). Production of infectious RNA from cDNA of negative strand RNA virus genomes is therefore thought to require correct assembly of the synthetic RNA into RNP which can then be expressed and replicated by viral RNA polymerase.

This thesis describes work carried out with the overall aim of establishing a system to produce Bunyamwera virus, a segmented negative strand RNA virus, carrying defined nucleotide alterations to its genome. Towards this goal, two broad approaches were investigated to attempt to present replicating virus with synthetic RNA in a form in which it could be recognised as a transcription template by the viral polymerase. The first approach involved transcribing viral-like RNA *in vivo* from transfected cDNA constructs in the presence of replicating Bunyamwera virus in the hope that the intracellular pool of viral proteins would enable encapsidation and replication of the synthetic RNA. The second approach involved attempts to reconstitute RNP *in vitro* by incubating synthetic RNA with nucleocapsid protein derived from Bunyamwera virus.

For *in vivo* transcription of bunyavirus-like RNA from transfected plasmids, a recombinant vaccinia virus (vTF7-3) which expresses T7 RNA polymerase was investigated to assess its suitability for producing a Bunyamwera virus small (S)

segment RNA from transfected cDNA in the presence of a replicating helper bunyavirus. The helper virus used was an existing reassortant virus (Bun/Bun/Mag) containing Bunyamwera virus large (L) and middle (M) genome segments and a Maguari virus S segment. Successful replication of the plasmid derived Bunyamwera S segment by the Bun/Bun/Mag helper virus would result in a proportion of progeny virus having all three gene segments of Bunyamwera virus.

Dual infection experiments were performed to investigate the compatibility of the component viruses of the rescue strategy. Metabolic labelling and Northern analysis suggested that the general strategy of rescuing a Bunyamwera virus S segment RNA, transcribed *in vivo* by vTF7-3, using the Bun/Bun/Mag reassortant virus, was feasible with regard to interactions of the component viruses.

Transcription of Bun S segment RNA *in vivo* from transfected cDNA by T7 RNA polymerase supplied by vTF7-3 was investigated by Northern blot analysis. It was not possible to detect RNA of the expected size. Transcription did occur since N protein could be detected in vTF7-3 infected cells transfected with a cDNA construct designed to produce positive strand S segment RNA from a T7 promoter.

In an attempt to rescue plasmid derived Bunyamwera virus S segment RNA into a Bun/Bun/Mag reassortant virus, supernatants were retained from cells which had been infected with vTF7-3 and the Bun/Bun/Mag reassortant virus and transfected with Bunyamwera virus S segment cDNA. 200 individual plaques of progeny virus were screened by metabolic labelling to determine the origin of their N protein. No Bun/Bun/Bun virus was recovered indicating that if rescue occured, it did so at a frequency of <1/200.

As an alternative to screening larger numbers of progeny virus, a counter-selectable rescue virus was sought to enable selection of any progeny virus containing plasmid derived (wild type) S segment RNA. Temperature sensitive (*ts*) mutants of Maguari virus were further characterised in attempt to identify a virus with a S segment lesion.

To enable the S segments of mutants from each of the three reassortment groups to be sequenced, a rapid PCR-based procedure was developed which allowed full-length S segment cDNA to be amplified in a single step from RNA isolated from crude preparations of virus. Using this method, full-length S

segment cDNAs were amplified and cloned from a representative of each of the three reassortment groups(*ts*6, *ts*17 and *ts*23) and from wild type Maguari virus. Three cDNA clones of each segment were sequenced by the Sanger di-deoxy chain termination method. A single point mutation was found in the S segment cDNA of Maguari virus*ts*23. This virus was used as a helper virus in attempt to replicate RNA transcribed *in vivo* from a Bunyamwera virus S segment cDNA by the vTF7-3 expression system. Progeny virus were amplified and grown at 38.5°C to suppress the *ts* helper virus. Any plaques forming were expected to contain a reassortant virus containing a Bunyamwera virus S segment (derived from cDNA) and Maguari virus L and M segments. No plaques formed even at the ( )permissive temperature indicating that the poorly growing *ts*23 mutant may not be suitable for this type of experiment.

The PCR based method developed for amplification of S segment cDNAs from the Maguari virus *ts* mutants was further exploited to clone several previously uncloned S segments from other bunyaviruses.

To increase the sensitivity of detection of replication of plasmid derived RNA, (if this was occuring), two reporter constructs were assembled containing Bunyamwera virus S segment non-coding sequences flanking the gene for chloramphenicol acetyl transferase (CAT). These were under control of T7 promoters and so could be transcribed *in vivo* using the vTF7-3 expression system.

The first of these constructs, pBunS(+)CAT, produces a Bunyamwera virus S segment cRNA-like transcript containing a message sense copy of the CAT gene. This construct was used in transfection experiments to determine suitable expression conditions. Packaging of the synthetic reporter RNA into Bunyamwera virus particles could not be demonstrated.

The second construct, pBunS(-)CAT, was designed to produce a vRNA like transcript containing an anti sense CAT gene and so was only expected to result in CAT activity if the T7 transcript was first copied by Bunyamwera virus proteins. However, transfection of this construct into vTF7-3 infected cells produced CAT activity even in the absence of Bunyamwera virus. This unpredicted activity could also be induced by infection with non recombinant (WR strain) vaccinia virus. Comparison of the construct sequence with the well characterised promoter sequences of vaccinia virus indicated a potential vaccinia virus late promoter-like sequence in the 5' non-coding region of the Bunyamwera virus S segment cDNA, just upstream of the protein coding

sequences. This sequence is thought to act as a cryptic promoter producing positive stranded (mRNA like) transcripts from pBunS(-)CAT in the presence of vTF7-3 and so account for the unexpected CAT activity. The potential cryptic promoter sequence was also present in the Bunyamwera virus S segment constructs discussed earlier and so would be expected to have given rise to incorrect RNA transcripts in these earlier studies and may therefore have been partly responsible for the failure of this approach.

Possible ways of overcoming the problems identified with the vTF7-3 expression system are discussed in context of a recent published precedent for the use of the system for *in vivo* replication of plasmid derived RNA.

Various approaches were investigated in attempts to reconstitute RNP *in vitro* using plasmid derived RNA. Successful *in vitro* RNP reconstitution might allow rescue of plasmid derived RNA by transfection of RNP into virus infected cells. Preformed continuous CsCl density gradients were used to assess formation of RNP which have a characteristic density of 1.31g/ml and so can be banded in 20–40% CsCl gradients while naked RNA forms a pellet.

Transcription of Bunyamwera virus S segment-like RNA in a rabbit reticulocyte lysate in which N protein was being translated failed to produce a complex of the density expected for RNP. Similarly, efforts to renature N protein from RNP denatured with guanidinium salt onto synthetic RNA failed. More promising results were obtained using nuclease treated RNP as a source of N protein. When analysed on CsCl gradients, reactions containing radiolabelled synthetic RNA and MCN treated RNP (with the nuclease inactivated by addition of EGTA) were found to contain a RNA/protein complex which banded at a density similar to that expected for native Bunyamwera virus RNP.

Using RNA transcribed *in vitro* from pBunS(-)CAT, which contains antisense CAT sequences flanked by S segment vRNA termini, several attempts were made to demonstrate Bunyamwera virus dependent CAT activity in cells transfected with reconstituted RNP. For all conditions tested no CAT activity could be detected. Comparison of the mobility on agarose gels of the RNA transcribed from linearised pBunS(-)CAT with RNA transcribed from theoretically identical template generated by PCR suggested that the linearised plasmid DNA may not be producing RNA of the correct length. This anomaly may be due to aberrant cleavage of the plasmid during transcription template preparation. Due to lack of time, this problem remains to be resolved but if an incorrect transcript was being produced this would account for the inability to

detect bunyavirus dependent CAT activity in cells transfected with the reconstituted RNP.

Preliminary evidence suggests that MCN treated RNP can be prepared which retains L protein activity. A combined CsCl/glycerol step gradient was designed to allow isolation of RNP in reduced concentrations of CsCl. RNP purified in this manner was digested with MCN and a ^{32}P CTP transcription mixture added to see if transcription, templated by the endogenous RNA fragments remaining after nuclease treatment, could be detected. When analysed on 20% acrylamide denaturing gels, a predominant signal could be detected by autoradiography. The strength of the signal could be increased by including dinucleotide ApG in the transcription reaction which is known to moderately stimulate bunyavirus transcription *in vitro*. The signal was stronger when the reaction was performed at 25°C than at 30°C and was weakest at 37°C. While formal proof that the signal represents an RNA transcript remains to be conducted, the observed stimulation by ApG and suppression by increasing temperature is consistent with the known properties of the L protein.

MCN digested RNP may therefore prove useful for *in vitro* transcription assays using short synthetic RNA transcription templates. Such technology should allow the promoter requirements for the L protein to be defined and would demonstrate that functional RNP can be assembled *in vitro* and therefore that rescue of synthetic RNA by transfection of *in vitro* reconstituted RNP into helper virus infected cells is feasible.

Chapter 1: RNA Viruses and their Genetic Manipulation.

1.1 Introduction.

In 1981 production of infectious poliovirus following transfection of a plasmid containing a DNA copy of the virus genome was reported (Racaniello & Baltimore 1981) and poliovirus became the first animal RNA virus to be recovered from cloned cDNA. It was immediately apparent that it would now be possible to produce virus which contained alterations deliberately introduced into a corresponding cDNA clone. This methodology provided a powerful tool for dissecting the functions of various components of the viral genome. It was speculated that such technology might also be developed for other RNA viruses. The success of rescuing poliovirus, and subsequently many other positive strand RNA viruses, from plasmid-derived RNA, was possible because a single RNA molecule could serve both as mRNA to produce viral proteins and as template for replication. As discussed in section 1.2, the RNA used as a replicative intermediate by negative strand RNA viruses is always different to mRNA. Until the recent advances made for influenza virus (Luytjes *et al* 1989), no DNA-derived RNA had been introduced into the genome of any negative strand RNA virus. Lack of such a system has prevented application of the powerful methods of recombinant DNA technology to produce defined mutants for functional analysis. The study of negative strand viruses has been greatly hindered by the absence of this methodology.

This thesis describes work carried out with the aim of establishing a system to produce a negative strand RNA virus (Bunyamwera virus) carrying defined nucleotide alterations to its genome. This ambitious goal remains to be achieved for Bunyamwera virus. However, it is hoped that the progress made and lessons learnt from this work, together with work on other negative strand RNA viruses from other groups working towards similar goals, will help towards achieving this goal for bunyaviruses in the near future.

1.1.1 Scope of this chapter

This chapter surveys selected animal viruses, briefly discussing some of the detailed knowledge known about the molecular mechanisms involved in expression and replication of their genomes. For the positive strand viruses much of this knowledge is due to the ability to produce defined mutants by manipulating cloned cDNA. For reasons of space and time this chapter does not set out to be comprehensive and covers only a few animal viruses. The choice of those covered reflects either direct relevance to the project (negative strand viruses) or personal choices which are felt to be interesting examples of engineering strategy (positive strand viruses).

No discussion of picornaviruses, retroviruses, bacteriophage or plant viruses is included even though a vast volume of molecular studies have been conducted on them. The interested reader is directed to any well stocked life science library for a life time of reading on these topics.

The positive strand alphaviruses have been discussed in preference to poliovirus since, in addition to having a genome which serves as mRNA, alphaviruses also produce subgenomic mRNA and therefore have additional control features making them perhaps more representative of positive strand RNA viruses. Also, alphaviruses have been engineered to produce efficient vectors for the expression of heterologous proteins and serve as a model for the type of RNA expression systems which may be developed for other RNA viruses.

Coronaviruses are included as an interesting plus stranded RNA virus for which genome manipulation has been achieved without it so far being possible to synthesise infectious RNA *in vitro*.

Reovirus, a double stranded segmented RNA virus, is included since, like the negative strand viruses, the genome RNA alone cannot establish infection without the presence of preformed viral proteins. Recent developments have enabled production of virus starting with naked RNA and similar methodology may prove applicable to some negative strand RNA viruses.

Moving to the negative strand RNA viruses, the rhabdovirus vesicular stomatitis virus and the paramyxoviruses are included as model non-segmented negative strand RNA viruses. For both these virus groups recent progress has established systems with the potential to investigate the *cis* acting signals involved in genome expression and replication. Although no report of production of rationally mutated virus has yet been forthcoming for either of these two virus groups, at least for VSV, major advances towards the goal of producing engineered virus have been made.

The remaining sections discuss influenza and Bunyamwera viruses. Both are negative strand segmented viruses and both share many distinctive features which set them apart from the other virus groups. For influenza virus it has recently been possible to produce viable virus containing genetic information derived from cDNA. This methodology has already been used to address interesting points concerning the replication of influenza virus which could not previously be directly tested. Establishment of an analagous system for members of the *Bunyaviridae*, the largest group of animal viruses, will undoubtedly enable similar studies to be conducted and should greatly increase our knowledge of the molecular characteristics of this group of viruses.

1.2 Key differences between positive and negative strand RNA viruses.

The genome of positive strand RNA viruses is also a functional mRNA. This property allows the input genome to be translated directly by cellular ribosomes to produce the viral proteins required for replication and production of progeny virus. These proteins use the input genome as a template to synthesise more genomes via a complementary negative strand replicative intermediate RNA, and package new genomes into virions (see figure 1.2.1). For this reason, naked viral genome RNA, if introduced into the suitable host cell, can establish replication in the absence of preformed viral proteins.

Conversely, negative strand RNA virus genomes are the complement of mRNA and therefore cannot be directly translated to give viral proteins without first being copied into complementary mRNA. RNA dependent RNA polymerase activity is required for this primary transcription and, since animal cells do not possess such an enzyme, this function must be supplied preformed in the input virion (Figure 1.2.2). For this reason the deproteinised genomic RNA of negative stranded viruses cannot initiate infection.

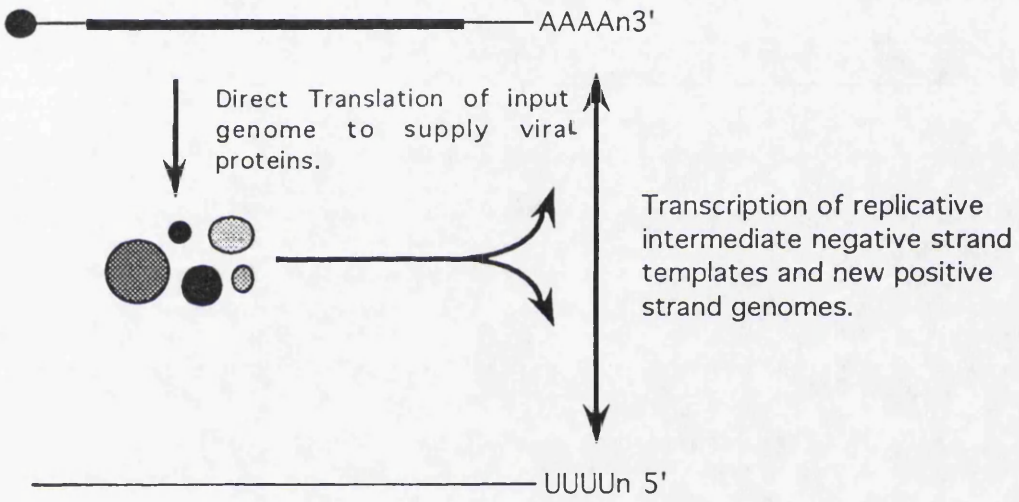


Figure 1.2.1 Replication of positive strand RNA virus genome. Upon infection, viral RNA is released from its nucleocapsid (an icosahedral structure for all positive strand viruses except the coronaviruses which have a helical nucleocapsid) and is free to serve as a messenger RNA. The input genome is usually modified at its 5' end either by the presence of a cap (as in the case of the alpha-, flavi- and corona-viruses) or by a viral peptide VPg (picorna and calici viruses). 3' ends of the genome are usually polyadenylated (exceptions being the nodaviruses—the only + stranded animal virus group with a segmented genome, and the flaviviruses). Primary translation of the input genome may produce all viral proteins, catalytic and structural, (e.g. poliovirus where a single polyprotein is encoded), or only replication proteins with structural genes being translated from subgenomic mRNA transcribed off negative strand replicative intermediate RNA (as occurs for the alphaviruses— see figure 1.3.1).

The picture is complicated further by the fact that mRNA transcribed from negative strand genomes is always different to the plus strand replicative intermediate used as a template for new genome production. These replicative intermediates resemble genomes in being always associated with viral proteins to form a ribonucleoprotein (RNP) complex which is the functional template of transcription. RNP cannot be translated by ribosomes. mRNA is not encapsidated to form RNP and can be translated but is never an exact complement of the genome. For the non-segmented negative strand viruses (the *Filoviridae, Paramyxoviridae* and *Rhabdoviridae*), several subgenomic mRNAs are produced while for the segmented negative strand viruses (*Arenaviridae, Bunyaviridae* and *Orthomyxoviridae*), mRNAs are 3' truncated relative to the positive strand replicative intermediate and also contain additional heterologous sequences at their 5' ends. Therefore, even if mRNA could serve as template for transcription, it would not contain all the viral specific information required for production of new genomes.

The infectivity of the positive strand virus genomes has made it possible, in many instances, to produce RNA molecules from cloned cDNA corresponding to viral sequences which can give rise to infectious virus when transfected into cells. Introducing the DNA step into virus production enables the use of recombinant DNA technology to alter the genome of the virus and so produce mutants carrying defined and rationally designed nucleotide alterations. Such technology has allowed the full force of reverse genetics to be applied to many positive strand viruses and has led to a rapid increase in our detailed understanding of the molecular biology of these viruses. For many years the absence of similar systems for manipulation of the negative strand viruses has hampered progress in defining their life cycles in molecular terms. Such systems are finally becoming available for negative strand viruses.

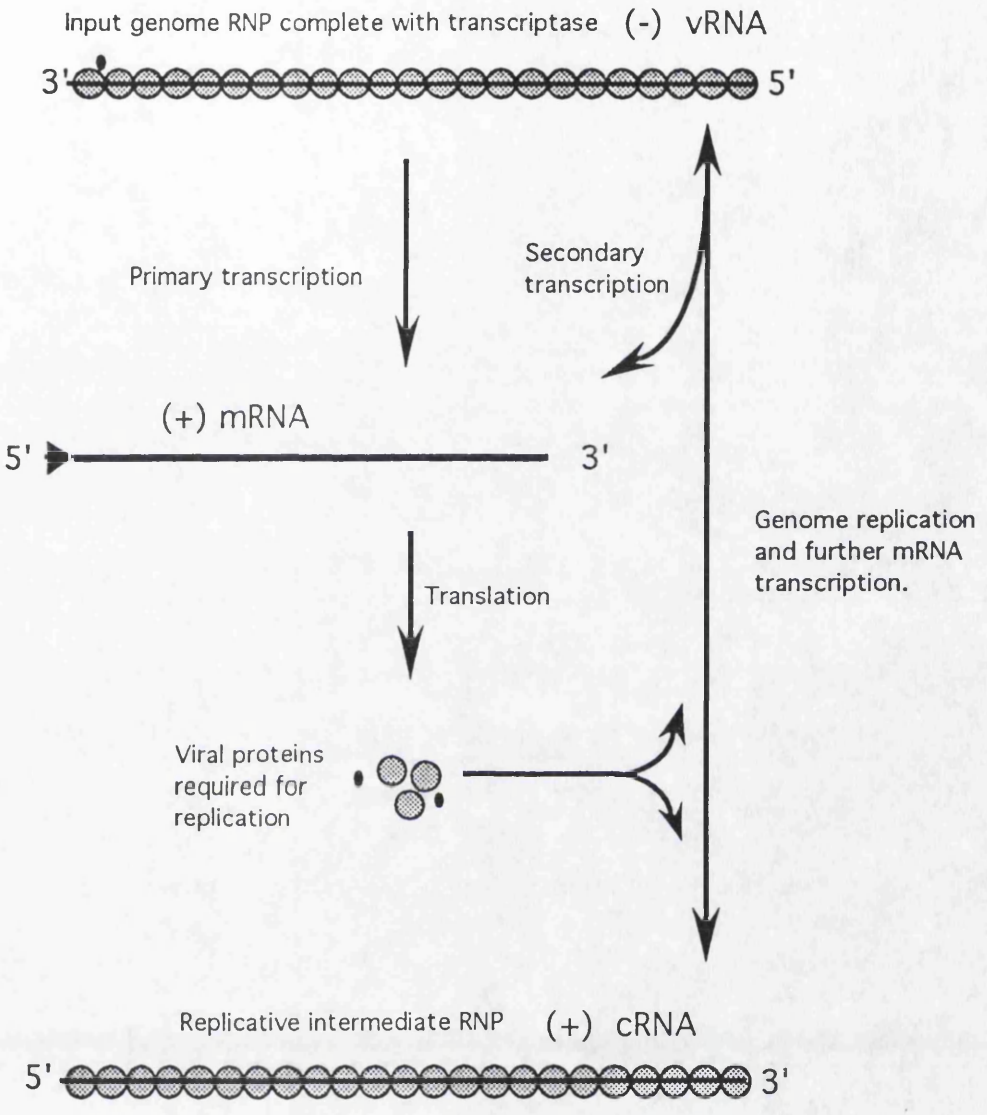


Figure 1.2.2 Replication of negative strand RNA virus genome. The input genome is a ribonucleoprotein (RNP) complex which cannot be translated. RNA dependent RNA polymerase is associated with the input genome and transcribes mRNA molecules as the first step to replication. Translation of primary transcripts produces proteins which can assemble full length + strand complementary RNA into RNP replicative intermediates. These can serve as templates for new genome synthesis. The mRNAs are truncated compared to the cRNA and are not encapsidated into RNP. Their 5' ends are capped whilst the 3' ends may or may not be polyadenylated depending on the virus.

1.3 Alphaviruses

The *Togaviridae* family is subdivided into the *Alphavirus* and *Rubivirus* genera (Schlesinger & Schlesinger 1990). Two alphaviruses, Sindbis virus and Semliki Forest virus, have been studied in great depth and both have been manipulated by reverse genetics. The *Alphavirus* genus also includes the equine encephalitis virus and Ross River virus.

1.3.1 Biology of alphaviruses

The alphaviruses are group A arboviruses, animal viruses having an arthropod host but also infecting mammals and avian species. In tissue culture, the alphaviruses grow lytically in vertebrate cells but readily establish persistent infections in mosquito cells.

The alphavirus genome is contained in an icosahedral core composed of a single type of capsid protein. The core is enclosed in a lipid bilayer containing two viral glycoproteins E1 and E2. Alphaviruses are taken into cells by endocytosis via clathrin coated pits (Marsh & Helenius 1990) followed by acid fusion (Edwards *et al* 1983), releasing the nucleocapsid into the cytoplasm where the RNA is uncoated and initiates replication. After genome replication and accumulation of structural proteins, virus is assembled by capsid protein subunits interacting with the genome RNA to form the icosahedral core. The core then associates with the viral glycoproteins inserted in the infected cell membrane (Fuller 1987) resulting in release, by budding, of the mature enveloped virion.

1.3.2 Molecular biology of alphaviruses

The alphaviruses have a non segmented single standed RNA genome of positive polarity. Genome RNA, free from all viral proteins, is infectious as is RNA transcribed *in vitro* from precise full length cDNA clones. The 12kb genome has a 7-methylguanosine 5' cap and a polyadenylated 3' tail (Strauss *et al* 1984). The input genome serves as a messenger RNA being directly translated to produce a polyprotein which is cleaved to give the nonstructural proteins required for expression of the structural proteins and genome replication (Figure 1.3.1). The AUG codon at nucleotide 59 is the only translation initiation site recognised on intact virion RNA (Cancedda *et al* 1975). The polyprotein is processed to give four nonstructural proteins, NSP1-4 (Strauss & Strauss 1990). These function in

transcription initiation (for full length negative strand RNA, full length positive strand RNA and subgenomic mRNA), elongation, 5' capping and methylation, 3' polyadenylation of positive strand RNA and self cleavage of the polyprotein as it is translated. The precise role of each nonstructural protein and any interactions required of host functions are not clearly understood (Schlesinger & Schlesinger 1990).

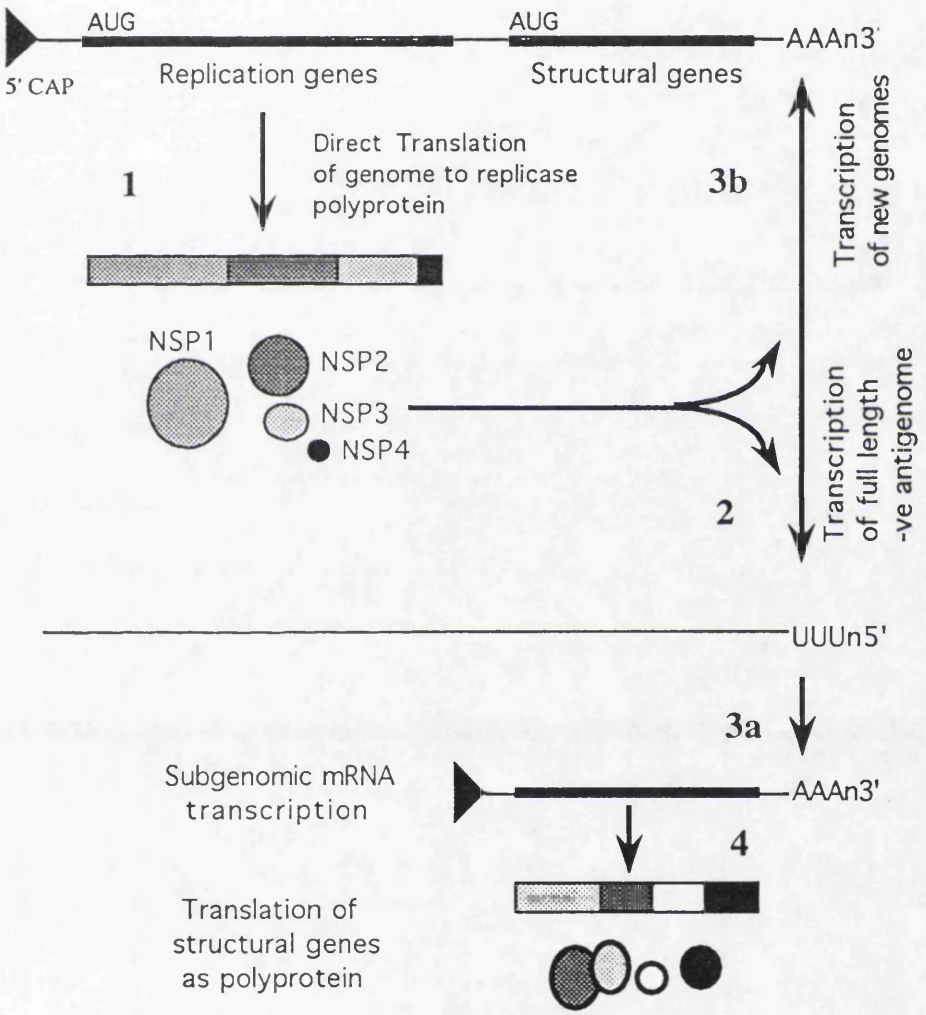


Figure 1.3.1 Expression and replication of the alphavirus genome. Input genome serves as a messenger RNA for translation of a self-cleaving polyprotein to provide replicase functions (1). The newly synthesised replicase complex (NSP 1-4) transcribes full length negative strand antigenome using the input genome as template (2). This replicative intermediate serves as a template for transcription of capped and polyadenylated subgenomic mRNA (3a) and full length positive strands to serve as new genomes (3b). The subgenomic mRNA is translated to a polyprotein (4) which is cleaved to give virion structural proteins.

The structural proteins are translated, as a polyprotein, from a subgenomic mRNA corresponding to the 3' most 4kb of the genome. This subgenomic mRNA is transcribed from full length negative strand RNA, initiation occuring at an internal promoter situated in the junction region between the nonstructural and structural genes (Bredenbeek & Rice 1992). The use of a subgenomic mRNA allows temporal control with the structural genes being expressed after translation of replicative genes and replication. The intergenic promoter also appears to be more active than the promoter involved in replication, resulting in a ten-fold higher abundance of subgenomic mRNA molecules compared to full length positive strand genome RNA (DL Sawicki *et al* 1981; SG Sawicki *et al* 1981). This observation has been exploited in designing recombinant alphaviruses to express foreign genes to high levels (Bredenbeek & Rice 1992)

Infectious cDNA clones have been constructed for several alphaviruses including Ross River virus (Kuhn *et al* 1991), Semliki Forest virus (Liljestrom *et al* 1991), Sindbis virus (Rice *et al* 1987) and Venezuelan equine encephalitis virus (Davis *et al* 1991). Reverse genetics has been used to investigate the *cis*-acting regulatory elements involved in genome replication, transcription and encapsidation. Comparison of several alphavirus nucleotide sequences (Strauss & Strauss 1986) identified conserved regions as potential *cis*-acting control elements (Figure 1.3.2). Site directed mutagenesis of infectious cDNA clones has been used to define these regions.

For genome and antigenome replication three conserved regions have been shown to be important. Two regions, one in the 5' nontranslated region of genomic RNA (Niesters & Strauss 1990a) and a 51 nucleotide stretch in the coding region of NSP1 (Niesters & Strauss 1990b) appear to control full-length positive strand transcription from negative strand template RNA while a single element, 19 nucleotides in length and situated adjacent to the 3' poly A tail, is the promoter for negatve strand synthesis (Kuhn *et al* 1990; Levis *et al* 1986).

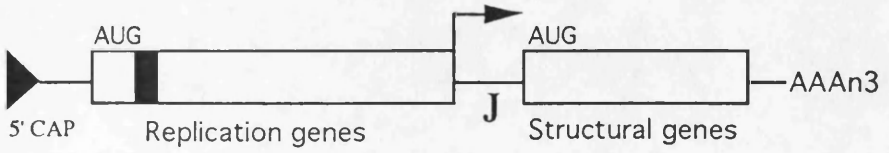


Figure 1.3.2 Map of the alphavirus genome showing the arrangement of protein coding regions and cis-acting regulatory elements. Genome and antigenome promoters reside in the termini. The internal promoter for expression of structural genes is situated in the junction region (J). The shaded box, inside the replication genes, represents sequences required for packaging RNA into virions.

Encapsidation signals are located in the NSP1 coding region between nucleotides 746 and 1226 of Sindbis virus genomic RNA (Weiss *et al* 1989). This region alone appears to provide specificity for encapsidation of positive strand full-length RNA molecules and excluding subgenomic mRNA and full-length negative strand molecules.

The internal promoter responsible for transcription of subgenomic mRNA from full-length negative strands is located in the junction region. A 24 nucleotide core promoter (Grakoui *et al* 1989; Levis *et al* 1990) is sufficient for subgenomic mRNA production. Flanking regions can be mutated resulting in enhanced (Raju & Huang 1991) or depressed (Rice *et al* 1987) transcription levels.

The availability of infectious cDNA clones has therefore contributed firm evidence relating to the control of transcription and replication of alphaviruses for which RNA sequence data alone could only allow speculation of function.

1.3.3 Heterologous gene expression in alphaviruses

The first expression of heterologous genes in alphaviruses used cDNA constructs based on defective interfering (DI) particles of Sindbis virus. DI particles can be replicated and packaged in the presence of wild-type helper virus and therefore must contain the necessary *cis*-signals for these functions. Levis *et al* (1987) constructed chimeric DNA templates containing the bacterial gene for chloramphenicol acetyl transferase (CAT) flanked by cDNA termini derived from a DI particle RNA of Sindbis virus. RNA transcribed *in vitro* from this construct, when transfected into avian cells, could be replicated by a wild-type helper Sindbis virus which provided replication enzymes and virion structural proteins in *trans*. Progeny virus stocks were shown to contain both helper virus genomes and the CAT/DI RNA. After several passages, the CAT RNA was the predominant species in infected cells although expression as measured by CAT activity was low. The poor level of translation was probably due to the reporter gene being expressed in an analagous manner to the replication genes of wild type genomes which provide catalytic functions and are therefore only required at low levels compared to structural proteins and maybe only early in infection (DL Sawicki *et al* 1981; SG Sawicki *et al* 1981).

To improve the low expression level, the reporter gene under control of the subgenomic mRNA promoter, was placed downstream of a truncated genome containing the 5' cap and untranslated region and a portion of the replicase gene containing the internal packaging signals (Levis *et al* 1990; Figure 1.3.3). Cotransfection of this recombinant RNA with wild-type helper RNA resulted in high level expression of the reporter gene. The chimeric RNA alone could also be

replicated and packaged by proteins supplied in *trans* from either recombinant vaccinia virus (Li *et al* 1991) or baculovirus (Buzan & Schlesinger 1992).

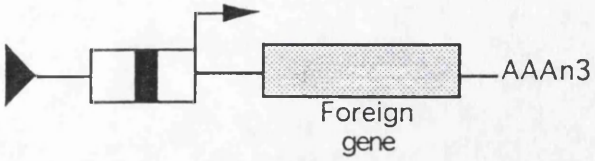


Figure 1.3.3 Expression of heterologous gene from alphavirus internal promoter. This construct has cis-replication/packaging signals and can be complemented by wild-type helper virus or recombinant virus to supply structural and replication proteins in trans. The internal promoter drives high level expression of the foreign gene. (See Fig 1.3.2 for alphavirus features).

Another approach was to build replication competent but packaging deficient chimeras by directly replacing the structural genes with the foreign gene (Figure 1.3.4 A). This was done for Semliki Forest virus (Liljestrom & Garoff 1991) and Sindbis virus (Xiong *et al* 1989), the strategy being originally developed for tobacco mozaic and bromegrass mozaic viruses (Takamatsu *et al* 1987; French *et al* 1986). After transfection into cells the 5' portion of the RNA is translated to give proteins required for replication. These transcribe negative strands which in turn are templates for amplification of positive strand genomes and efficient transcription of mRNA from the internal promoter. After 16 hours, high levels of foreign proteins could be detected, in the range of 10^8 protein molecules per transfected cell for Sindbis constructs (Xiong *et al* 1989). Having no structural genes, no RNA is packaged and so careful optimisation of transfection to establish replication in as many cells as possible was required.

Similar constructs were then complemented with replication gene-deficient recombinant RNA coding for structural proteins (Figure 1.3.4B). Following cotransfection the replicative functions from the chimeric RNA could replicate itself and the helper RNA containing the structural genes. The gene products of the helper RNA could package both the helper and chimeric RNA giving rise to virus containing two genome segments. For Sindbis virus, infectious particles with bipartite genomes could be purified and stably passaged (Geigenmuller-Gnirke *et al* 1991).

The logical progression from this system was to create helper RNA molecules which lacked the *cis*-signals required for packaging (Figure 1.3.4 C). This would prevent the helper molecule from being packaged while producing high titre stocks of packaged chimeric RNA capable of high levels of expression when used

to infect fresh cells. For Semliki Forest virus >10^8 infectious particles per ml, free of helper RNA, could be generated (Liljestrom & Garoff 1991).

Such an approach might one day be applied to deliver genes to humans for therapeutic purposes. Early attempts to establish the system using replicons based on Sindbis virus have resulted in stock titres up to 10^4 fold less than those obtained with Semliki Forest virus. This is because deletion of the packaging signals in Sindbis virus appears to interfere with subgenomic mRNA production (Weiss *et al* 1989) and therefore produces too little structural proteins to efficiently package progeny genomes.

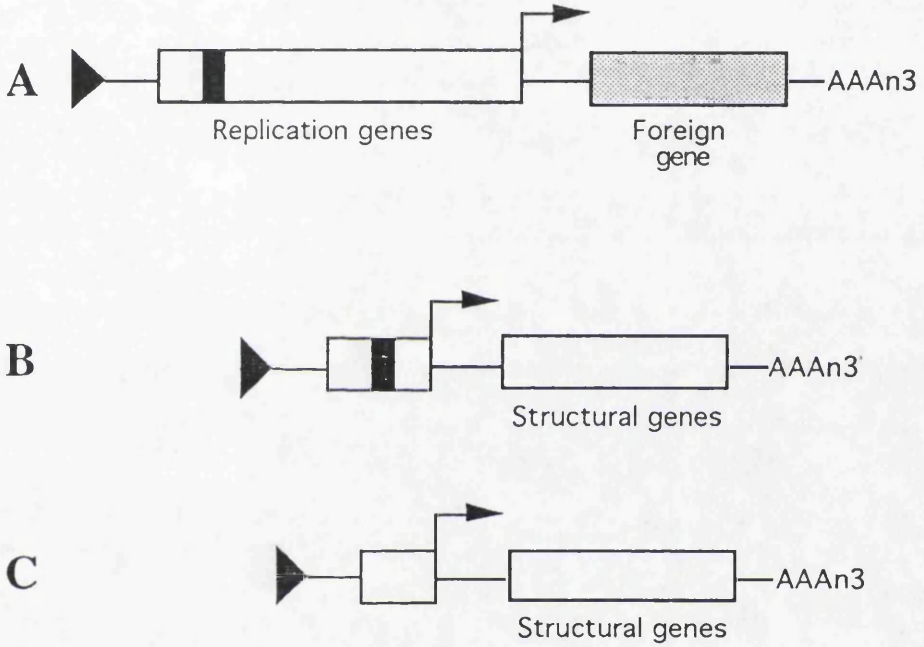


Figure 1.3.4 Packaging-deficient alphavirus vector (**A**) lacks internal structural genes but contains all *cis*-sequences required for replication and packaging. Alone, this RNA can replicate and express to high levels in transfected cells. The use of a helper construct carrying structural genes (**B**) enables packaging of both chimeric and packager RNA into viruses with bipartite genomes. An alternative helper RNA (**C**) has deleted packaging signals and so provides structural proteins which package the chimeric RNA alone. (Figure redrawn from Bredenbeek & Rice (1992), see text for original references).

Double subgenomic constructs have been produced which are complete alphavirus genomes with an extra, heterologous, gene inserted with a duplicated internal promoter either upstream or downstream of the structural genes (Figure 1.3.5). These constructs express well and give progeny titres of 10^8–10^9 pfu/ml

(Raju & Huang 1991; Hahn *et al* 1992). Inserted sequences of >3kb suffer deletions, possibly caused by packaging restrictions imposed by the icosahedral core although the 5' constructs appear to be more stable than those where the heterologous gene is downstream of the structural genes. Double subgenomic constructs have been used in a number of expression studies for human, *Drosophila* and viral genes (Bredenbeek & Rice 1992).

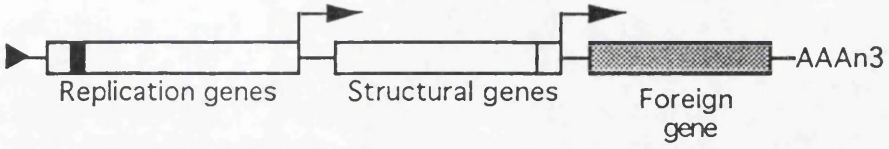


Figure 1.3.5 Double subgenomic alphavirus expression vector. The foreign gene is inserted into an intact alphavirus genome along with an additional junction region containing a second promoter for subgenomic mRNA production. In this example the foreign gene is downstream (3') of the structural genes but can also be inserted between the structural and replication genes.

Another strategy attempted was to insert the heterologous gene downstream of the structural genes and include a site for internal ribosome initiation derived from a picornavirus. This results in production of a di-cistronic subgenomic mRNA with the structural genes being translated as normal and the heterologous gene being translated by internal initiation. The system was found to produce heterologous protein to around one-fifth the level observed for double subgenomic constructs (Bredenbeek & Rice 1992).

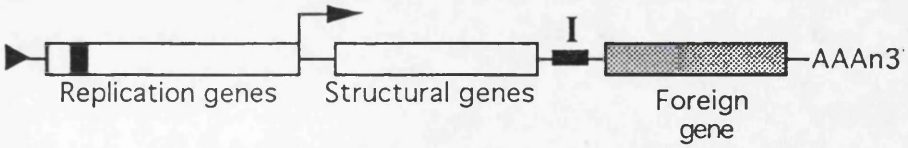


Figure 1.3.6 Alphavirus expression vector employing internal translation initiation signal. Viral transcription produces one subgenomic mRNA from the internal promoter (arrow). The mRNA is di-cistronic with the structural polyprotein being translated in a cap-dependent fashion and the foreign gene being translated by internal initiation directed by picornavirus ribosome-binding sequences (**I**) .

Because of the ease of manipulating alphavirus genomes at the cDNA level, their replication is well understood and fairly advanced expression systems for heterologous proteins have been developed. Establishment of such versatile methodology for other RNA viruses, particularly the negative strand viruses, will undoubtedly lead to a rapid growth in our understanding of their replication cycles and may ultimately play an important role in the development of new improved therapeutic agents.

1.4 Coronaviruses.

Although naked coronavirus RNA is infectious when transfected into cells (Schochetman *et al* 1977), infectious cDNA clones are not yet available. Useful progress enabling their genetic manipulation has however been achieved by exploiting the high recombination frequency of genomes during infection (section 1.4.3).

1.4.1 Biology of coronaviruses.

The coronaviruses are large enveloped viruses causing highly prevalent disease in humans and domestic animals. Each virus has a specific host range usually only infecting one or a few, closely related species or tissue types. Isolation of virus from an infected host can be difficult and may require differentiated cell lines from the same species (Larson *et al* 1980).

Coronavirus virions are enveloped. The lipid bilayer, derived from intracellular membranes, contains at least two viral glycoproteins. Glycoprotein E2 has a large external domain visible as spikes by electron-microscopy. E1, or matrix protein, extends through the membrane and has a significant domain inside the virion. This domain interacts with the large helical nucleocapsid formed from the genome RNA and a phosphorylated nucleocapsid protein (N). A third glycoprotein, E3, may be present, depending on the virus, and confers haemagglutination properties (Holmes 1990).

In tissue culture, coronaviruses grow either lytically or establish persistent infection depending on virus-host combination (Wege *et al* 1982). For some viruses cells may fuse to form syncytia.

After replication, newly transcribed genome RNA associates with N protein to form helical nucleocapsids (Stohlman *et al* 1988). These align and bud through membranes between the rough endoplasmic reticulum and the Golgi apparatus where viral glycoproteins have localised (Tooze *et al* 1984). Mature virions are

released by cell lysis or by transportation in secretory vesicles (Holmes *et al* 1984).

1.4.2 Molecular biology of coronaviruses.

The genome of coronaviruses is single stranded RNA of unusually large size which can exceed 30kb (Spaan *et al* 1988). The genome is positive stranded, 5' capped and 3' polyadenylated. Input genome is directly translated to give the non-structural proteins involved in replication. ~20kb at the 5' end of the genome is thought to be translated, by a ribosome frame-shifting mechanism, to yield a very large self-processing polyprotein (Brierley *et al* 1987).

The replication enzymes use input genomes as transcription templates to make full length negative strands which form a double stranded replicative intermediate with the positive strand template (Sawicki & Sawicki 1986).

The structural genes are expressed from 5-8 subgenomic mRNAs which form a 3' nested set (Stern & Kennedy 1980). Usually only the 5' most open reading frame is translated (Siddell 1983) although exceptions have been found (Holmes 1990).

The subgenomic mRNAs all share a common leader sequence of 60-70 bases which appears to be coded for at the 5' end of the genome (Lai *et al* 1982). This observation led to the proposal for discontinous, leader-primed transcription of mRNA. Leaders transcribed from the 3' end of the negative stand template would dissociate and anneal at intergenic regions, where short leader-complementary sequences are found, and prime mRNA synthesis (Baric *et al* 1985). The ability of the RNA dependent RNA polymerase to elongate annealed primers may account for the high recombination rate observed for coronaviruses which may be as high as 25% for the whole genome (Lai 1990).

1.4.3 Reverse genetics of coronaviruses

Two complete coronavirus sequences have been published (Boursnell *et al* 1987; Lee *et al* 1991). Naked RNA purified from virions is infectious when transfected into cells (Schochetman *et al* 1977) although recovery of complete virus from cDNA has not been achieved (Bredenbeek & Rice 1992). Two approaches have however been used to apply reverse genetics to coronaviruses.

Naturally occuring defective interfering (DI) particles, containing the *cis* elements required for replication and packaging, have been used to construct cDNA clones from which *in vitro* transcribed RNA can be replicated in the presence of wild-type helper virus. Such clones have been used to map *cis* acting signals (van der

Most *et al* 1991). In a recent study a DI cDNA construct was modified by insertion of coronavirus intergenic sequences (Makino *et al* 1991). In the presence of helper virus, transfected RNA transcribed from this construct could be replicated and used as a template for transcription of subgenomic mRNA, demonstrating the crucial role of the intergenic sequences in the transcription of mRNA from coronaviruses.

Reverse genetics has also been used to rescue a temperature sensitive (*ts*) coronavirus mutant containing a deletion in the nucleocapsid gene. Exploiting the high recombination frequency observed for coronavirus RNA (Lai 1990), an *in vitro* transcribed RNA containing wild-type nucleocapsid gene sequence was transfected into cells infected with the *ts* mutant. Progeny virus contained wild-type virus produced by replacement of the *ts* lesion by recombination of the transfected RNA sequences into the mutant genome (Koetzner *et al* 1992).

Since coronaviruses contain several genes which appear to be dispensable for growth in tissue culture (Yokomori & Lai 1991) it may prove possible to introduce foreign genes into coronavirus genomes by recombination of a plasmid derived RNA containing the foreign gene flanked by viral sequences directed against the non essential genes.

1.5 Reovirus

Reovirus has a segmented double stranded RNA genome (Wiener *at al* 1989). Purified reovirus RNA is non-infectious since double stranded RNA cannot be translated and eukaryotic cells do not possess RNA polymerases which can use RNA as a template for transcription. For these reasons, like negative strand viruses, reovirus has a virion associated RNA dependent RNA polymerase activity which is essential for viral replication.

Primary transcription of reovirus RNA occurs without release of the genome segments into the cytoplasm. The polymerase complex forms part of the virion and newly transcribed mRNA is passed out of the virion into the cytoplasm where it is translated and initiates replication (Shatin & Kozak 1983). The mRNA molecules are 5' capped but are otherwise identical to the + strands of the double stranded genome segments (Hay & Joklik 1971), and also serve as replicative intermediates for new genome synthesis. As such, mRNA representing all ten segments might be expected to be infectious if simultaneously introduced into cells. However, so far this approach to virus recovery has proved unsuccessful.

The use of a counter-selectable helper virus enabled rescue of naked RNA corresponding to the ten reovirus segments (Roner *et al* 1990). The ST2 strain of reovirus was used to infect cells after transfection of viral RNA. ST2 is a slow growing strain, not forming plaques until 10 to 12 days compared to five days for another reovirus, ST3. If both single stranded (ss) and double stranded (ds) RNA corresponding to the ten segments of reovirus ST3 (the faster growing variant), were transfected into cells which were then infected with ST2 virus, viable ST3 virus appeared in the supernatant at 48 hours post transfection. Both ss and ds RNA were required and either alone failed to produce virus.

If ss RNA was added to a rabbit reticulocyte lysate and the whole mix transfected into cells, virus could be recovered at the same efficiency as cotransfected ss and ds RNA which had not been translated. If melted ds RNA replaced ss RNA in the reticulocyte lysate, a twenty-fold increase in virus recovery was observed and where both ss and ds RNA were transfected in a primed reticulocyte lysate, the yield increased a further ten fold. The reason for the enhanced infectivity when both ds and ss RNA was present is not clear although it was demonstrated that the ds RNA increases infectivity of ssRNA and not the otherway round.

1.6 Paramyxoviruses

The *Paramyxoviridae* is subdivided into three genera: *Paramyxovirus*, which includes Sendai and mumps viruses; *Morbillivirus* including measles virus; and *Pneumovirus* which includes respiratory syncytial virus (Kingsbury 1990). The *Paramyxoviridae* share many features with another family of non-segmented negative strand RNA viruses the *Rhabdoviridae*. The *Paramyxo-* and *Morbillivirus* genera are remarkably similar in their structure and genome arrangement and differ in many respects to the *Pneumovirus* genus. This section will discuss briefly only Sendai and respiratory syncytial viruses. These viruses are of interest because systems with the potential of investigating *cis*-acting regulatory elements have been described for both.

1.6.1 Sendai virus

Sendai virus is enveloped and has three membrane proteins (HN, F_0 and M). The virus is thought to adsorb to cells via interactions of the haemagglutinin/ neuraminidase (HN) protein followed by fusion mediated by F_1/F_2 (cleavage products of F_0) resulting in delivery of the nucleocapsid into the cytoplasm. Replication occurs entirely in the cytoplasm (Kingsbury 1977).

The Sendai virus genome is non-segmented, single stranded RNA of negative polarity 15kb long. The genomic RNA is encapsidated in nucleocapsid protein (NP) to form a nuclease resistant ribonucleoprotein (RNP) complex. Two other proteins (termed P and L) are associated with the nucleocapsid and are presumed to form the RNA dependent RNA polymerase. During infection the virion RNP serves as template for transcription of a short leader RNA complementary to the 3' end of the genome (Leppert *et al* 1979), a feature shared with rhabdoviruses. Several mRNA are transcribed in the order NP, P, M, F_0, HN, L (Glazier *et al* 1977; Shioda *et al* 1983; Shioda *et al* 1986). The P mRNA also encodes two small non-structural proteins (C and C') of unknown function (Giorgi *et al* 1983). After translation has occured, full-length positive strand cRNA is produced which serves as a replicative intermediate for production of new genomes. This cRNA is always encapsidated as an RNP with encapsidation occuring co-transcriptionally—'Y-form' structures can visualised by electron microscopy which represent RNP templates with partially transcribed encapsidated cRNA still attached (Kingsbury 1977).

1.6.2 Rescue of synthetic RNA by Sendai virus

In vitro systems for studying transcription of Sendai virus had relied on using native RNP purified from infected cells or virus particles. These could be replicated and the newly transcribed RNA co-transcriptionally encapsidated in the presence of extracts made from infected cells (Carlsen *et al* 1985). NP alone could encapsidate preinitiated transcription products but was unable to initiate Sendai virus transcription for which the L and P proteins are required (Baker & Moyer 1988).

To enable investigation of the *cis* signals involved in transcription and replication, it was necessary to define conditions which would allow replication of plasmid transcribed RNA. This was done using a similar reporter system that had previously been applied to influenza virus (Luytjes *et al* 1989; see Section 1.8.5). A plasmid construct containing the bacterial gene for chloramphenicol acetyl transferase (CAT) flanked by cDNA corresponding to the Sendai 5' and 3' non-coding termini was built. This was used to produce transcripts *in vitro* which were Sendai virus genome-like RNAs with the whole of the protein coding regions replaced by an antisense copy of the CAT reporter gene (Park *et al* 1991). Transfection of this chimeric RNA into cells failed to produce CAT activity since the RNA is antisense to mRNA. Transfection of the same RNA into cells which were infected with Sendai virus resulted in CAT activity: the viral proteins acted in *trans* to encapsidate and express the transfected RNA. This was in marked contrast to studies with influenza virus where the RNA had to be assembled into a nucleocapsid structure prior to transfection for recognition and replication to occur (Section 1.8.5). For Sendai virus the level of expression of the synthetic RNA could be substantially enhanced by incubating the RNA with extracts of infected cells prior to transfection (Park *et al* 1991). Nuclease sensitivity data suggested that this enhancement was not due to the formation of synthetic Sendai virus RNP since these are highly resistant to nucleases in their native form. Further investigation demonstrated similar levels of enhancement if the RNA was incubated with extracts from uninfected cells, suggesting a role for cellular factors. Whether the cell extracts specifically aided recognition of the RNA by the helper virus or whether the enhancement was due to a non-specific effect or facilitated uptake by the cells is not clear. The CAT activity observed declined when high levels of extract were used, with the optimum conditions depending on whether infected or uninfected cell extract was employed.

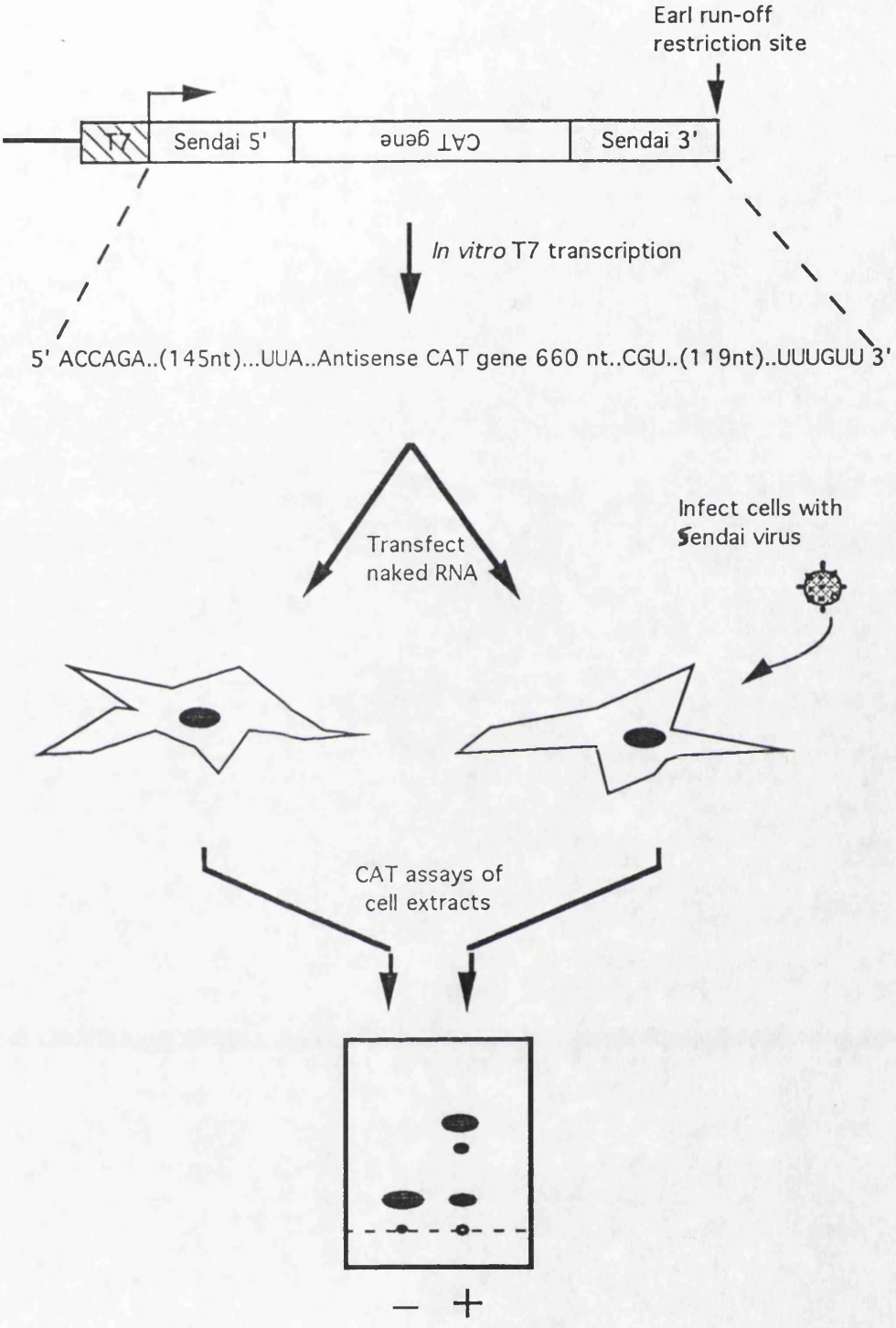


Figure 1.6.1 Rescue of *in vitro* transcribed RNA by Sendai virus.Sendai virus-like RNA containing an antisense copy of CAT was transcribed *in vitro* and transfected into cells. No CAT was produced in these cells unless they were also infected with Sendai virus to replicate and express the synthetic RNA. See text for details. (Figure based on Park *et al* 1991).

An RNAse protection assay, using a probe directed against negative strand Sendai virus RNA, was used to demonstrate that the input RNA was not only expressed to give CAT mRNA but also served as a template for replication and was thus amplified by the helper virus. Supernatants from the transfected cells contained virus particles which had packaged the synthetic CAT RNA. This was demonstrated by observing CAT activity after serial passage of the supernatant and by the ability to specifically abolish CAT activity by pretreating supernatants with anti Sendai virus sera but not with heterologous anti sera.

1.6.3 Respiratory syncytial virus

The *Pneumovirus* genus is relatively distinct from other genera of the *Paramyxoviridae* having extra mRNA and proteins, and differences in gene order and sequence (Collins 1991). However, using analagous constructs and procedures as those used for replication of synthetic Sendai virus RNA (section 1.6.2), rescue of synthetic respiratory syncytial virus-like RNA was achieved (Collins *et al* 1991) . *Cis*-acting replication and packaging signals were investigated by attempting to rescue RNAs having truncations or mutations of the viral sequences at their termini. Replacement of the 3' terminal 44 nucleotide vRNA leader region by the 50nt trailer complement, representing the 3' terminus of positive stranded replicative intermediate RNA, demonstrated interchangeability of the two 3' ends since rescue occured to a similar efficiency as for wild-type ends. This is in contrast to influenza virus where the vRNA and cRNA promoters have markedly different expression rates (see Section 1.8.4). Addition of 11 nucleotides of heterologous RNA to the 3' end prevented rescue, demonstrating the importance of the positioning of the *cis*-acting signals relative to the viral genome termini. This finding is in contrast to one influenza virus study (Yamanaka *et al* 1991) where efficient rescue of an RNA containing an additional stretch of 20-30 heterologous 3' nucleotides was reported. Despite high conservation between the positive and negative strands, the first five nucleotides of the RSV leader region did not appear to be critical since deletion of the first three nucleotides or mutation of the 4th and 5th nucleotides still resulted in efficient rescue.

The systems developed for Sendai virus and RSV provide a useful means to investigate the *cis*-acting signals of the genome termini which should yield

important information concerning promoter and packaging signals. Further progress has yet to be reported although preliminary data suggests that the system can be modified to study events occuring at the junctions between genes (Kolakofsky, unpublished data) which includes RNA editing as well as reinitiation of transcription required for expression of the various mRNAs. The stage is set for definition in molecular terms for the varied and interesting properties of this important group of viruses.

1.7 Vesicular stomatitis virus

The *Rhabdoviridae* contains over 100 viruses infecting a wide range of organisms including plants, fish, reptiles, crustaceans and mammals. The rhabdoviruses which infect mammals are classified, according to the type of disease they produce, into two genera: *Lyssavirus* which infect the central nervous system, the best known and most feared lyssavirus being rabies virus; and *Vesiculovirus* which infect epithelial cells, especially of the tongue, to cause vesicles. Vesicular stomatitis virus (VSV) is the prototype member of this genus and infects cattle resulting in epidemic disease. VSV is one of the most studied negative strand RNA viruses and has contributed to our knowlege of cellular processes such as protein processing and transport. The *Rhabdoviridae* have been recently reviewed (Wagner *et al* 1990) and will only be discussed briefly here with emphasis on recent progress in establishing a reverse genetics system for the study of this important group of viruses.

1.7.1 Molecular biology of VSV.

VSV has a non-segmented, negative stranded RNA genome 11kb long. Five viral proteins are encoded: the nucleocapsid protein (N); a phosphoprotein (P or NS); a matrix protein (M); a single glycoprotein (G) and the RNA dependent RNA polymerase (L). The genome of VSV is encapsidated in nucleocapsid protein as a ribonucleoprotein (RNP) complex. As with all negative strand RNA viruses studied, the RNP is the functional template for transcription, whereas naked RNA is not recognised by the viral polymerase. The VSV nucleocapsid has two minor proteins associated with it, the L and P proteins. This complex alone can initiate RNA synthesis *in vitro* and is infectious when introduced into cells (Szilagyi & Uryvaev 1973). Activity is lost if the L and P proteins are dissociated (Emerson & Yu 1975) but can be regained by addition of purified L and P proteins. For

processive transcription to occur excess N protein is required to facilitate read-through of termination signals (Patton *et al* 1984).

1.7.2 Replication of VSV DI particles by recombinant proteins.

RNP purified from DI particles of VSV could be replicated in cells transiently expressing the N, P and L proteins from recombinant DNA molecules (Pattnaik & Wertz 1990). This established an *in vivo* system capable of dissecting the functions of proteins required in replication. The system was developed further, still using purified DI RNP as template, but co-expressing all five VSV genes instead of just those required for replication. Where all five genes were expressed, the DI template could not only be replicated but also packaged into DI particles which were released from cells by budding and could infect other cells (Pattnaik & Wertz 1991). Using this system, reverse genetics can be applied to investigate the functions of all five VSV proteins.

The success of these types of experiments, where recombinant expressed proteins can mimick all the functions of a virus, gives an interesting insight into the evolution of VSV. The recombinant proteins expressed from DNA genes are produced with little, if any, quantitative or temporal control but can still function to perform all the essential replication and packaging functions required of a virus. This is in contrast to the VSV genome where the arrangement of genes and presumably *cis* signals, give controlled expression with proteins required in large amounts, e.g. the N protein, being expressed to much higher levels than those required at lower levels for catalytic roles, such as the L protein. While the recombinant proteins may not produce DI particles as efficiently as authentic virus infection would, they do function— demonstrating that a less highly controlled system could have existed as fodder for natural selection to improve to the organised arrangement found in VSV today.

1.7.3 Assembly of synthetic VSV RNPs

To enable dissection of *cis*-regulatory elements by introduction of nucleotide alterations, the RNP template would have to contain RNA derived from cDNA which requires the ability to reconstitute a functional RNP from naked RNA and proteins. For influenza virus, RNP could be reconstituted by mixing purified proteins with naked RNA while for Sendai virus, naked RNA transfected into infected cells could be encapsidated *in vivo*. Although functional RNP for VSV had been reconstituted *in vitro*, using extracts of infected cells to encapsidate naked RNA purified from DI particles (Mirakhur & Peluso 1988), no report of

replication of a plasmid derived RNA using this system was forthcoming. This is surprising since the study demonstrated *in vivo* replication of the reconstituted RNP in the presence of helper virus. Why studies involving plasmid derived RNA were not reported is unclear, especially since the authors commented on the importance of the work in the context of applying reverse genetics to study VSV (Mirakhur & Peluso 1988). More recently purified N protein has been used to encapsidate small synthetic RNAs *in vitro* which are competent transcription templates (Moyer *et al* 1991). The usefulness of this system is somewhat limited by the size of the RNA which can be encapsidated which is small even compared to DI genomes.

1.7.4 VSV DI genomes from cDNA

Plasmid derived RNA was successfully replicated by transcribing it *in vivo*, in the presence of VSV proteins (Pattnaik *et al* 1992). The cDNA corresponding to a DI genome was placed under control of bacteriophage T7 promoter such that transcription would initiate on the first DI-specific nucleotide. The 3' end of the T7 transcript was cleaved precisely at the last DI nucleotide by inclusion of ribozyme sequences adjacent to the DI RNA. Sequence-authentic DI RNA could be produced *in vivo* by transfecting this construct into cells previously infected with a recombinant vaccinia virus, vTF7-3 (Fuerst *et al* 1986), which produces T7 RNA polymerase *in vivo*. Co-transfection of further T7 constructs containing VSV genes enabled efficient encapsidation and replication of the DI RNA. When all VSV genes were present, the DI RNA could be packaged into VSV DI particles which budded from the cells (Figure 1.7.1). This work is a major achievement considering that the particle formed originates entirely from cDNA. This enables rescue, without VSV helper virus, of virus like particles and so opens the way for detailed mutagenic analysis of all *cis* and *trans* acting functions of VSV.

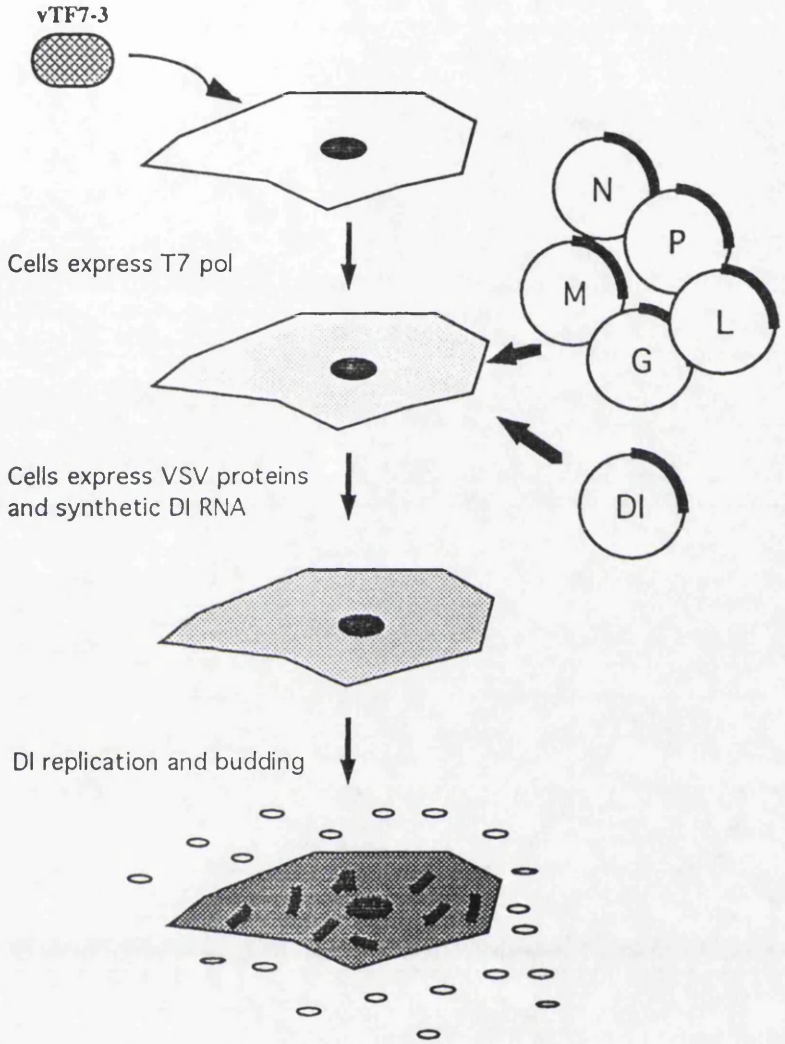


Figure 1.7.1 Production of VSV DI particles from cloned cDNA. BHK cells were infected with vTF7-3, a recombinant vaccinia virus which expresses bacteriophage T7 RNA polymerase. After accumulation of T7 polymerase, T7-expression constructs coding for the five VSV genes (N, M, L, P & G) were transfected along with a plasmid containing a cDNA corresponding to a VSV DI genome. The DI RNA is transcribed by T7 polymerase, encapsidated, replicated and packaged into budding virions by the co-expressed VSV proteins. (Figure redrawn from Pattnaik *et al* 1992)

1.8 Influenza A virus

1.8.1 Influenza virus

Influenza A virus is a member of the *Orthomyxoviridae* along with influenza B and C viruses. All three have segmented RNA genomes which are negative stranded and non infectious. Virus particles are enveloped and contain two types of membrane proteins (one for influenza C virus), the haemagglutinin (HA) and neuraminidase (NA) proteins. Internally, the eight genome segments (seven for influenza C virus) occur as helical nucleocapsids formed by association of the RNA with many copies of the nucleocapsid protein (NP) and a few copies of the polymerase complex (PB1, PB2 and PA for influenza A virus). Matrix protein occurs between the helical nucleocapsids and the viral envelope. (For review see Murphy & Webster 1990; Krug *et al* 1989; Kingsbury 1990).

Influenza A virus is an extremely important human pathogen causing pandemics which can lead to substantial loss of life. Influenza virus has several animal reservoirs. The inherent high mutation rate associated with RNA genomes results in antigenic drift and helps the virus evade the immune system. Having a segmented genome, reassortment of gene segments results in viruses with new genome combinations (antigenic shift) which emerge and cause world wide outbreaks of disease at strikingly regular intervals (Murphy & Webster 1990). Recently it has become possible to apply recombinant DNA technology to produce genetically altered influenza virus. This technology holds much promise for further investigating the molecular biology of this virus and may prove useful in producing improved vaccines.

1.8.2 RNA species of influenza virus infected cells

For each genome segment of influenza virus, three types of RNA are found in infected cells (see Figure 1.8.1): i) Viral genomic RNA (vRNA) is of negative polarity and is always associated with viral proteins as a ribonucleoprotein (RNP)

nucleocapsid; ii) mRNA is complementary to genomic RNA, has additional 5' heterogeneous sequences which are capped, is missing 17-20 3'-terminal nucleotides complementary to the genome RNA 5' end and is 3' polyadenylated. mRNA is not found in nucleocapsids or in virions; iii) genomic-complementary RNA (cRNA) is the same polarity as mRNA but is a faithful complement of genomic RNA lacking the capped leader and poly-A tail found in mRNA. cRNA, like vRNA, is only found in RNP form. Unlike vRNA, cRNA is found only in infected cells and not in the virus particle.

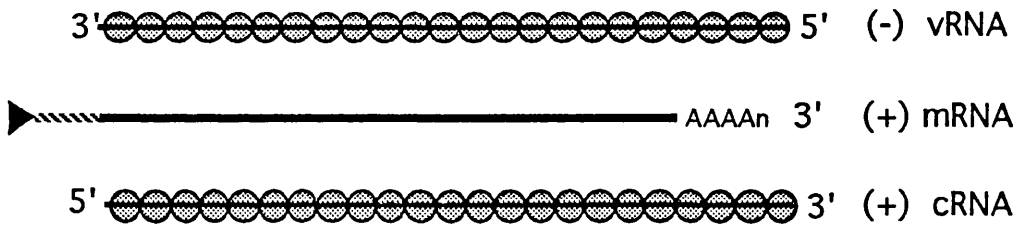


Figure 1.8.1 Infected cell RNAs of influenza virus. Both vRNA and cRNA are encapsidated in NP protein ( ) and are exactly complementary. mRNA contains additional 5' sequences derived from host cell capped mRNA ( ), are 3' truncated and polyadenylated.

The functional template for transcription in influenza virus is the RNP. Naked RNA is not recognised by the polymerase complex. Early studies of influenza transcription used native RNP from disrupted cells or virions (Beaton & Krug 1986; Takeuchi *et al* 1987; Shapiro & Krug 1988). Such studies were able to determine conditions under which synthesis of the different forms of RNA could occur *in vitro*. For example, mRNA production requires added capped heterologous mRNA from which short leaders are cleaved to prime transcription (Shapiro & Krug 1988). Addition of dinucleotide ApG (McGeoch & Kitron 1974), or the absence of added primer resulted in the synthesis of cRNA (Hay *et al* 1982). Full length cRNA synthesis is also dependent on the presence of an excess of NP protein (Beaton & Krug 1986; Honda *et al* 1988).

1.8.3.Transcription Initiation

The additional heterologous sequences found at the 5' end of influenza virus mRNA are cleaved from host cell mRNA in the nucleus (Krug *et al* 1989). The sensitivity of influenza virus transcription to α-amanitin, an inhibitor of cellular RNA-polymerase II transcription, demonstrates that newly synthesised transcripts are required (Mark *et al* 1979). PB2 is involved in binding host cell mRNA by cap recognition. A cap 1 structure (m7GpppNm) is prefered (Bouloy *et al* 1980). The cellular message is cleaved 10–15 nucleotides downstream of the cap at a purine residue. While priming does not require base pairing of the host derived leader to the genomic vRNA (Krug *et al* 1980), the first base to be added to the cleaved primer by the influenza PB1 protein appears to be G templated by a C next to the 3' teminal U residue of the vRNA (Braam *et al* 1983; see figure 1.8.4 for vRNA 3' terminal sequence). This suggests some interaction of the template terminal U with the 3' terminal nucleotide of the primer which was preferentially cleaved at a purine, A or G—both of which can form hydrogen bonds with U residues in RNA. Evidence of a role for hydrogen bonding of complementary nucleotides to initiate transcription, at least for dinucleotide-primed *in vitro* transcription, comes from the finding that RNP containing mutated termini can be primed for RNA synthesis if a complementary dinucleotide is included in the reaction but not if it is absent or replaced by a non-complementary dinucleotide (Seong & Brownlee 1992b).

1.8.4 Synthetic influenza virus RNPs and their use in promoter analysis

To enable study of the *cis* sequences required for transcription and replication, it is necessary to use mutated RNAs as promoter elements. Since the template for transcription is not naked RNA but the RNP complex, mutational analysis can only occur if synthetic RNA can be reconstituted into transcription competent RNP. In recent years, various methods have been developed for *in vitro* reconstitution of active RNP. All these methods use proteins from native RNP, which have been separated from the influenza virus genome RNA, to complex with *in vitro* synthesised RNA of defined sequence.

1.8.4.1 Influenza virus RNP reconstitution using gel purified proteins.

The first successful attempt at reconstituting transcriptionally active influenza virus RNP used virus RNA mixed with proteins purified by denaturing gel electrophoresis (Szewczyk *et al* 1988). Virion RNP complexes were boiled in SDS under reducing conditions and the components separated by SDS PAGE. The gels were blotted to membranes from which bands containing each protein (PB1, PB2, PA and NP) were cut and the proteins eluted (see Figure 1.8.2). When genomic RNA, purified from virus by phenol extraction, was combined with the purified proteins in the presence of the bacterial enzyme thioredoxin to refold the proteins, transcriptionally active complexes formed. For transcription to occur the three polymerase proteins and NP were required suggesting that the polymerase complex could not recognise template RNA unless it was associated with NP. This system might have allowed detailed examination of *cis* signals involved in transcription although no further reports of its use have been forthcoming. Although the authors claimed restoration of 10–30% of the starting activity, the original activity of their RNP was much lower than that obtained by other workers (Krug *et al* 1989) and therefore the overall efficiency of the system may be questionable.

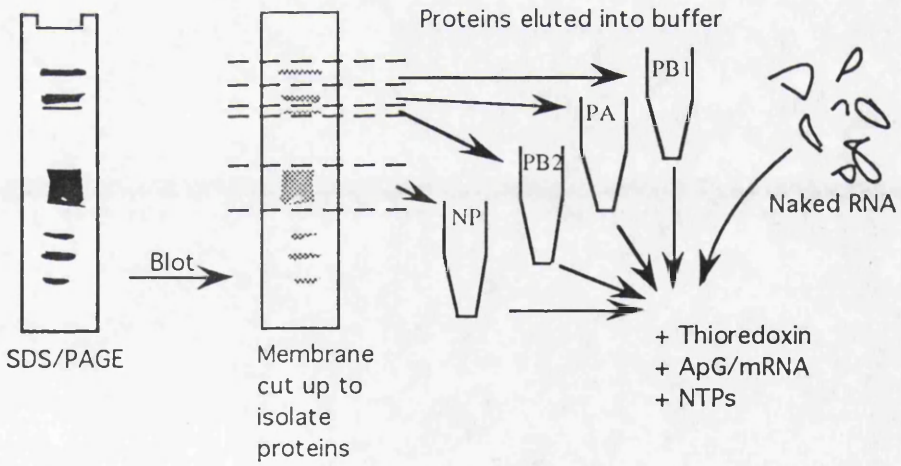


Figure 1.8.2 Thioredoxin renaturation of influenza virus RNP.

1.8.4.2 Reconstitution using gradient purified proteins

A more efficient procedure for reconstituting synthetic influenza virus RNP for *in vitro* transcription studies used NP and polymerase proteins depleted of viral RNA by CsCl/glycerol gradient centrifugation (Parvin *et al* 1989). Influenza virus RNP complexes centrifuged through CsCl/glycerol gradients dissociate into dense polymerase/RNA complexes and less dense free NP (Kato *et al* 1985). The polymerase/RNA complex at the bottom of the gradient was found to be active for transcription initiation when primed with ApG but produced only short transcripts of 12-19nt long unless free NP, purified from higher up the gradient, was added (Honda *et al* 1987; Honda *et al* 1988). The upper NP fractions (see Figure 1.8.3) contain no RNA but were found to contain small amounts of polymerase proteins (Parvin *et al* 1989).

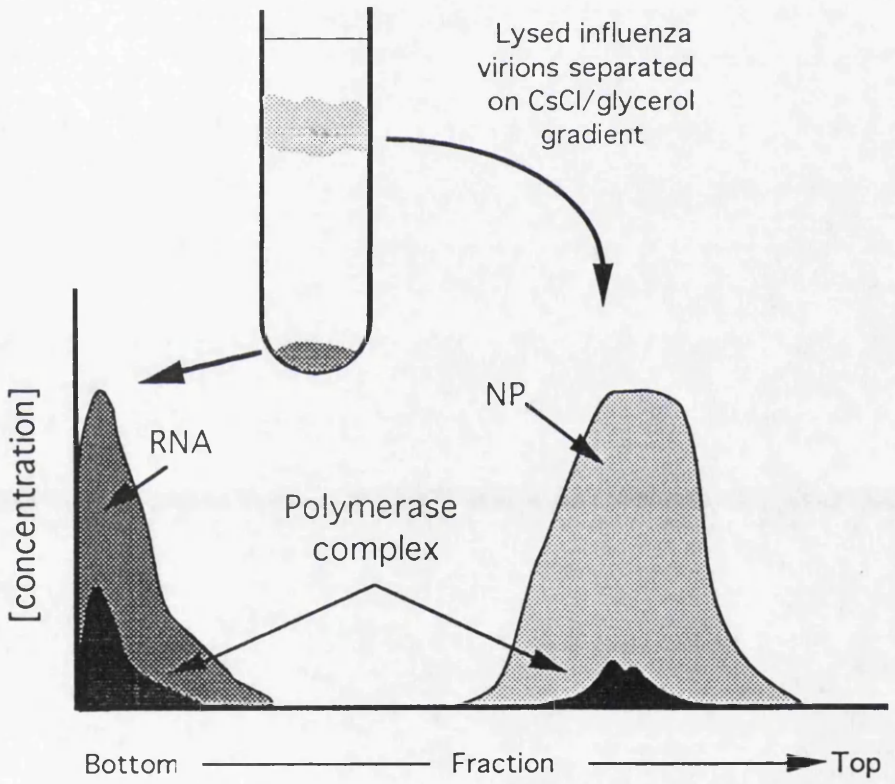


Figure 1.8.3 Gradient profile of disrupted influenza virions. NP dissocates from the RNP in high salt and bands free of RNA near the top of CsCl/glycerol gradients . The RNA, with polymerase proteins (PB1, PB2 and PA) is found at the bottom of the tube. Small amounts of polymerase complex is also found free of RNA in the NP band. See text for details. (Figure based on the findings of Parvin *et al* 1989).

Incubation of influenza virus naked RNA, either purified from virions by phenol extraction or transcribed from plasmids *in vitro*, with free NP/polymerase fractions, resulted in the formation of transcriptionally active complexes. The reconstituted RNP resembled native RNP in *in vitro* transcription reactions with regard to temperature and salt optima and kinetics of label incorporation.

Using short model RNA templates transcribed *in vitro* from plasmids containing mutated influenza virus sequences, a number of deductions could be made: Firstly, although it had long been speculated that the double-stranded panhandle formed by the termini of all influenza virus gene segments (Desselberger *et al* 1980; Hsu *et al* 1987) might be involved in polymerase recognition, it was found that 3' terminal sequences alone could optimally function as a promoter (Parvin *et al* 1989). The terminal 15nt were sufficient. Surprisingly, sequences corresponding to the 3' terminus of cRNA (i.e. the expected promoter for vRNA synthesis), which differs from the vRNA 3' terminus by three point differences and a single insertion (see Figure 1.8.4), was not an efficient template in this system—the activity was reduced to 20% of that observed for vRNA. Mutational analysis was used to define which differences resulted in the lower level observed for the vRNA promoter. Single changes at positions 5 or 8 of vRNA to their cRNA counterparts, had no effect on promoter activity although the double mutation resulted in only 30% activity. Position 3 seemed more critical with single mutation reducing activity to 40%. This activity was further greatly reduced by insertion of the additional U residue at position 10 (only 0.06% activity) although activity returned to 20% by the additional change C–U at position 8.

In the absence of *in vivo* data it is difficult to comment on the lower activity of cRNA compared to vRNA sequences in this assay. The observation suggests that vRNA sequences, which have to promote cRNA and high level mRNA synthesis, are inherently stronger than the cRNA sequences which only have to promote vRNA synthesis. An alternative possibility would be that transcription to produce genomic vRNA is temporally controlled to occur late in infection and efficient transcription requires accumulation or modification of viral proteins or the presence of a cellular factor.

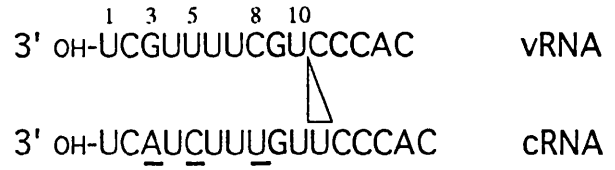


Figure 1.8.4 Influenza virus vRNA and cRNA 3' termini. The nucleotides are numbered from the 3' terminus for discussion in the text. The cRNA terminus differs from vRNA by three point differences (underlined) and by insertion of an extra U residue at position 11.

1.8.4.3 Reconstitution using nuclease treated RNP as protein source.

More recent *in vitro* studies mutated each of the twelve 3' terminal bases of influenza virus vRNA (Seong & Brownlee 1992b). Short model templates were reconstituted into transcriptionally active complexes using protein derived from disrupted virions which had been depleted of viral RNA by micrococcal nuclease digestion followed by inactivation of the calcium-dependent nuclease by chelation using EGTA (Seong & Brownlee 1992a). This system could mimick all three influenza virus transcription activities: capped primer dependent mRNA synthesis, primer-independent cRNA synthesis, and ApG-primed vRNA synthesis (Seong & Brownlee 1992a; Seong *et al* 1992). Surprisingly it was found that only three of the conserved twelve 3' terminal vRNA nucleotides were essential for all three activities. These formed a triplet (3' GUC 5') at position 9-11 bases in from the teminus. Mutation of any of these three nucleotides reduced activity to <10% of that observed for the wild-type sequence. At other positions mutations were found which led to premature termination of the transcript and some mutations had a stimulatory effect. For ApG-primed synthesis, mutations at positions 1 and 2 moderately reduced activity with position 1 being more important than for position 2—mutation of which could still give high level of activity if the corresponding complementary dinucleotide was used to prime instead of ApG. Mutation at positions 3 or 4 resulted in strong stimulation of unprimed RNA synthesis. Interestingly, position 4 is the only site where an alternative base is present in some segments of influenza virus (Robertson 1979; Winter & Fields 1980; Desselberger *et al* 1980; Allen *et al* 1980). Position 4 may therefore play a role in segment-specific regulation of expression. Based on the detailed *in vitro* analysis of the vRNA promoter, it was suggested that the polymerase complex specifically recognises two sites of the 12 nucleotide promoter—the 3' terminal three nucleotides and three or four nucleotides, adjacent to each other, starting at position 9. These two contact points are separated by a short stretch of U residues, mutation of which leads to premature termination of transcription (Seong & Brownlee 1992b).

1.8.5 *In vivo* expression, replication and packaging of synthetic influenza virus RNPs.

The successful *in vitro* reconstitution of transcriptionally active influenza virus RNPs (Parvin *et al* 1989) led to the system being applied to the rescue, into infectious virions, of RNA transcribed *in vitro* from cDNA (Luytjes *et al* 1989). A chimeric DNA version of influenza virus segment eight was constructed, in which the coding region for the NS gene was replaced by the CAT gene. *In vitro* transcription from an appropriately positioned T7 promoter to a run-off site formed by restriction enzyme cleavage of the plasmid resulted in a RNA molecule containing the terminal non-coding sequences, exactly matching those found in influenza virus segment eight, flanking an antisense copy of the CAT gene. Being of negative polarity, this RNA could not be translated to give active CAT protein unless it was first itself used as a template to make positive strand mRNA. This design gave a sensitive and absolute reporter system for influenza virus dependent transcription since eukaryotic cells contain neither CAT nor RNA dependent RNA polymerase activity. Using the same procedure that had been applied successfully to synthesise influenza virus RNPs active for *in vitro* transcription (Parvin *et al* 1989), RNP containing the chimeric CAT/segment eight RNA was synthesised and transfected into cells (Luytjes *et al* 1989). In the presence of replicating helper influenza virus, the synthetic RNP was expressed, replicated and packaged into infectious virions which could be serially passaged several times (see figure 1.8.5).

Amplification was necessary for expression to occur to detectable levels. This was demonstrated by including a similar chimeric CAT/segment eight RNA which differed by three point mutations at the 5' end which were known to interfere with synthesis of vRNA but not of cRNA or mRNA—the promoters for which lie entirely at the 3' terminus of vRNA. This mutant, although capable of primary transcription of the input template, did not allow measurable levels of CAT to accumulate suggesting that the chimera with wild type termini are replicated as well as expressed.

RNA was packaged into virions, as demonstrated by the ability of the media from transfection experiments to induce CAT activity in cells after serial passage even after RNase A treatment. CAT induction was however abolished by treating the medium with anti-influenza virus serum to aggregate virus particles. This report was the first to achieve packaging of genetically manipulated RNA of any negative strand RNA virus.

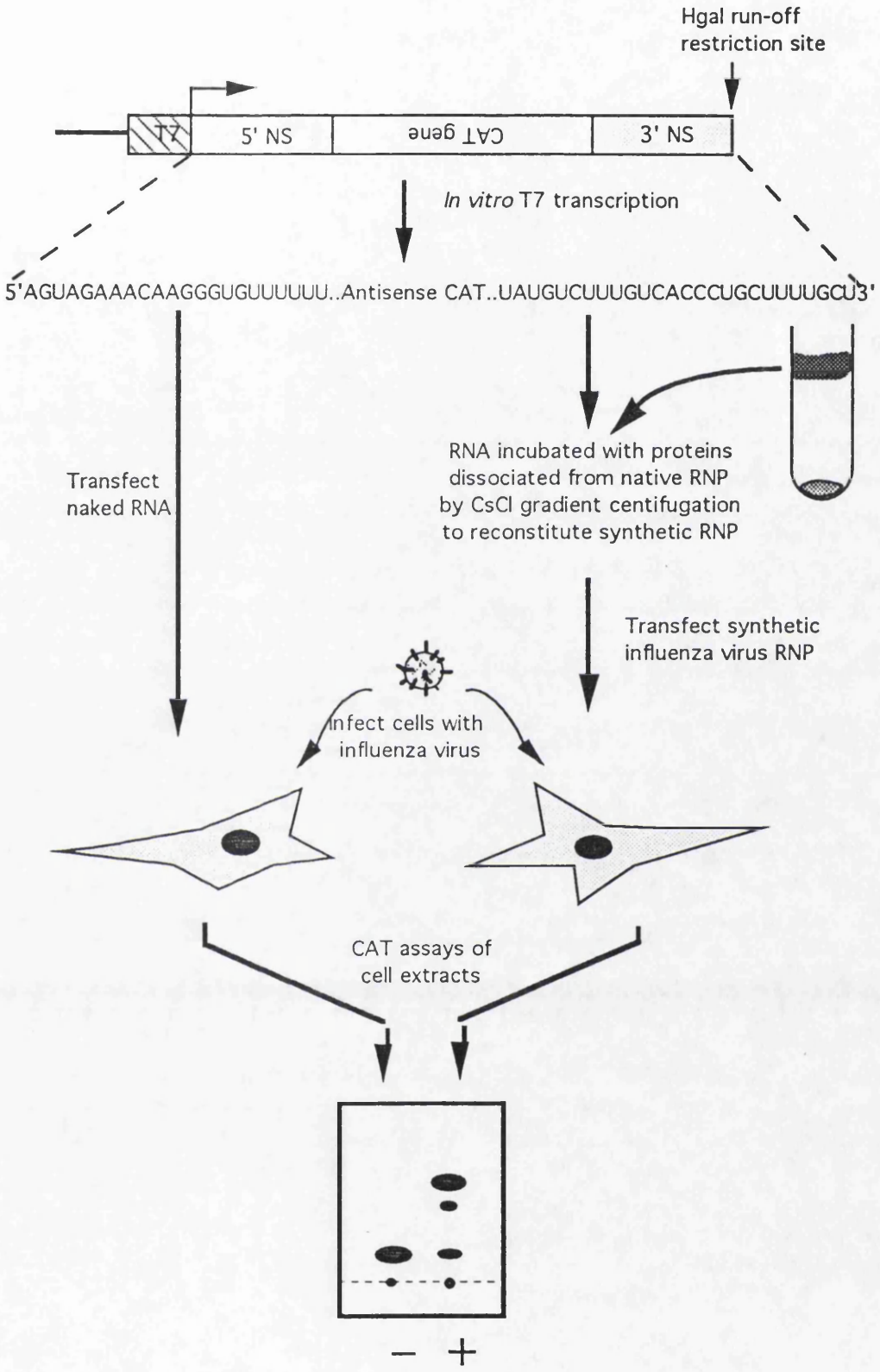


Figure 1.8.5 Rescue of *in vitro* transcribed RNA by influenza virus. See text for details. (Figure based on Luytjes *et al* 1989)

1.8.6 Introduction of mutations into the influenza virus genome.

The system for packaging plasmid transcribed RNA into influenza virus (Luytjes *et al* 1989) was applied to introduce site-specific mutations into the influenza virus genome (Enami *et al* 1990). This was achieved by using a helper virus with a strong counter-selectable phenotype which was well charaterised. Influenza virus A/WSN-HK is a reassortant containing seven segments from influenza A/WSN/33 and the neuraminidase gene segment from influenza virus A/HK/8/86. This reassortant can only form plaques in MDBK cells when the cell culture media is supplemented with a protease (Schulman & Palese 1977), whereas the parent WSN 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 for by omitting protease from the cell culture media. Using this procedure it was possible to obtain $2{\cdot}5 \times 10^2$ plaques/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 virus recovered in the absence of protease contained the mutations demonstrating that synthetic RNP had indeed been stably incorporated into an influenza virus.

1.8.6.1 Increased efficiency of synthetic RNP formation

The efficiency of the system was considerably improved by performing the plasmid transcription reaction in the presence of purified influenza virus proteins (Enami & Palese 1991) rather than mixing preformed 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 much greater efficiency with at least 100-fold more virus being isolated ($0{\cdot}5$–1×10^5 pfu/transfection compared with $2{\cdot}5 \times 10^2$ observed earlier).

1.8.7 Termination and polyadenylation of mRNA transcripts

Once capped-primer influenza virus mRNA synthesis has started, transcription continues until a stretch of 4–7 U residues close to the 5' end of the template vRNA is reached, where termination and polyadenylation occurs (Hay *et al* 1977; Robertson *et al* 1981). The termini of influenza virus gene segments all have conserved nucleotides which show extensive complementarity (Desselberger *et al* 1980). Cross-linking experiments demonstrated that the termini of genome RNAs can exist base paired as a pan handle structure in infected cells and virions (Hsu *et al* 1987). The proximity of the U stretch at which mRNA terminates to the predicted double stranded region of the pan handle suggested a polymerase slippage mechanism to account for polyadenylation. In this model the polymerase complex would be unable to melt the duplex region thus preventing it from transcribing sequences forming the pan handle, and would 'stutter' on the adjacent U tract resulting in poly A addition by repeatedly reading the U stretch.

The recent development of methodology enabling the application of reverse genetics to influenza virus (Luytjes *et al* 1989) has allowed models such as this to be tested (Luo *et al* 1991). In an *in vivo* transcription assay using synthetic RNP containing a CAT reporter gene flanked by influenza virus segment termini, the level of CAT activity was correlated with the level of polyadenylated mRNA. Thus for this system the level of CAT expression could be used as a measure of the abundance of mRNA (Luo *et al* 1991). Engineered RNA in which the uridine tract had been displaced away from the pan handle diminished CAT activity as did mutations introduced into the U tract. Mutations designed to prevent pan handle formation reduced activity, and this could be restored if compensatory changes were introduced to reform a pan handle of incorrect sequence. Finally, the activity of the RNA with the displaced U tract could be restored if an additional double stranded region was introduced immediately downstream of the U tract (see Figure 1.8.6).

These experiments demonstrate that both the sequence and its context relevant to the double stranded region are essential for expression of influenza virus mRNA. The observations are entirely consistent with a polymerase slippage mechanism of poly-A addition although they do not exclude other possibilities (formal proof that the differences of CAT expression were due to differences in polyadenylation of mRNA was not demonstrated in this work).

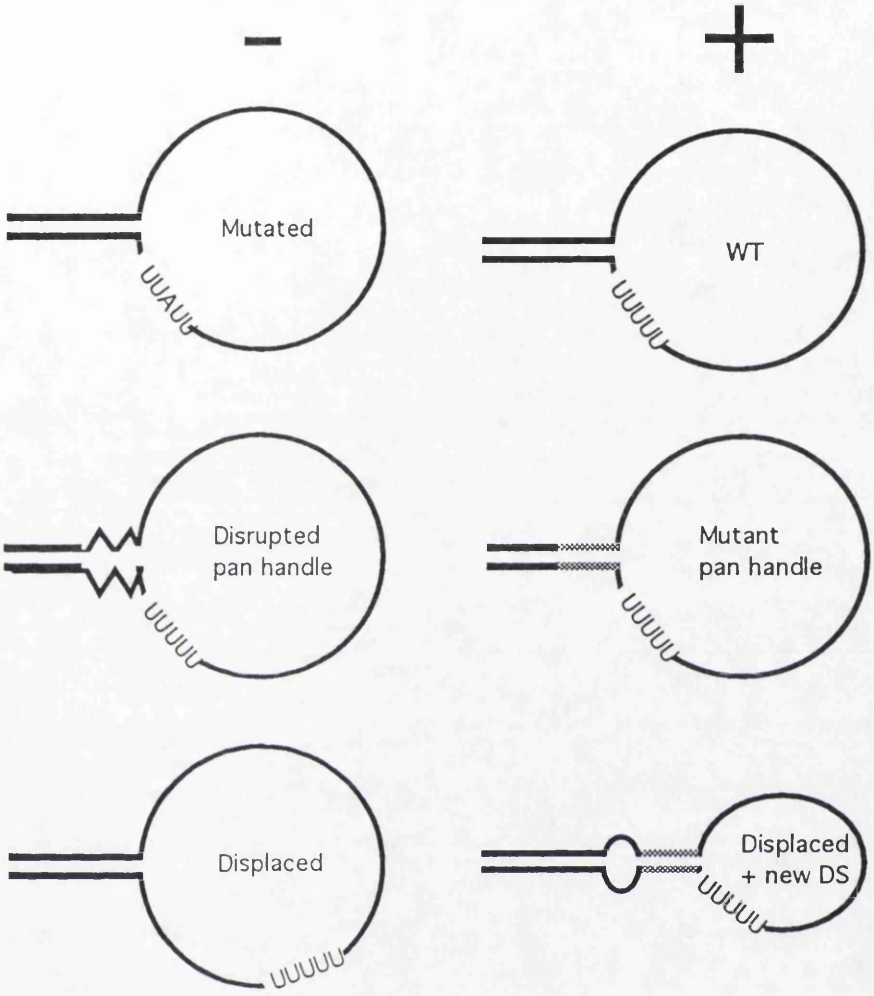


Figure 1.8.6 Influenza virus polyadenylation mutants. Diagramatic representation of RNAs containing alterations to the pan handle and/or U stretch and the effect on CAT expression. RNAs on the left (-) produced dramatically reduced levels of CAT while those on the right (+) gave high level expression. See text for details. (Representation of the results of Luo *et al* 1991).

1.8.8 Packaging of influnza virus genome segments.

Some aspects of packaging of gene segments into influenza virus can be deduced from observation. For example, RNP but not naked RNA is packaged into virus and so the ability of influenza virus to selectively package relevant RNA species can be controlled at one level by control of encapsidation by NP protein. Clearly more control is needed since influenza virus packages only RNP containing negative strand vRNA and not the replicative intermediate cRNA-containing RNP. The favoured hypothesis, for which there is no experimental evidence, is that diffences in the shape of the panhandle structure could account for selection

of only negative strand RNA. The ability of guanosine to base pair with uracil in RNA (in addition to cytosine) results in 'allowed' mismatches which can fit into a helix formed in double stranded regions of RNA such as is found in the panhandle of vRNA and cRNA. Conversely complementary pairing of cytosine with adenine cannot occur and results in disruption of the helix in a region of RNA duplex. Because the 3' ends of vRNA and cRNA are the complements of the 5' ends of cRNA and vRNA respectively, the presence of the point differences in these two regions (see Figure 1.8.4) result in the occurence of non-complementary bases appearing opposite each other in the panhandles formed between the termini of an RNP. Because of the template used for transcription of each strand (i.e. the complementary strand), where a G/U allowed mismatch occurs in one panhandle, a dis allowed C/A pair would be present in the panhandle of the opposite strand (see Figure 1.8.7). These differences could result in significant differences in the conformation of the panhandles for the two polarities of RNA which might allow recognition of only RNP containing negative strand RNA for packaging into virions.

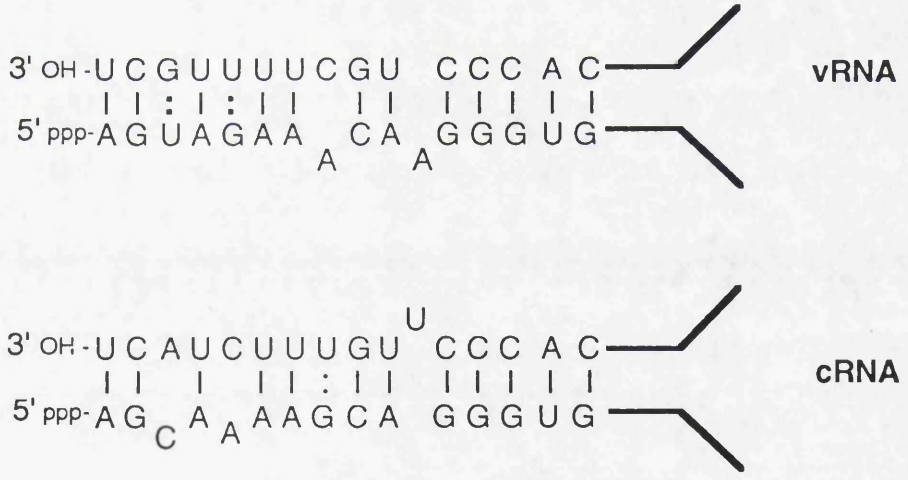


Figure 1.8.7 Differences in pan handles of vRNA and cRNA of influenza virus. Standard Watson-Crick base pairs are shown as vertical dashes, allowed G/U RNA base pairs are shown as double dots. Unpaired and misspaired bases are shown displaced from the duplex. These displacements occur at different positions for vRNA and cRNA and may result in a difference in conformation of the RNP for each panhandle.

1.8.8.1 Random vs selective packaging of influenza virus RNPs

Presuming the packaging mechanism provides a means to discriminate between vRNA and cRNA, influenza virus also has to be able to package the correct complement of vRNA gene segments. This aspect of packaging has been the focus of theoretical discussion and with the advent of systems enabling the rescue of artificial gene segments into influenza virus, has recently been addressed experimentally.

In order to establish infection, influenza virus particles must contain at least one of each of the eight types of gene segment. At the simplest level, either some means of selectively packaging the correct eight segments occurs or packaging is a stochastic event resulting in a proportion of gene-deficient viruses incapable of establishing infection. The observation that the ratio of different gene segments in infected cells differs from the ratio found in viruses (Smith & Hay 1982) supports a selective mechanism, since random choice should reflect the relative proportions present in the choice pool.

For random packaging, a high particle/pfu ratio would be expected if only eight RNP were packaged since most particles would contain the wrong combination. Random packaging could however be feasible if more than eight segments were packaged (Hirst 1973). Probability calculations predict that randomly selecting eight items from a large pool containing eight different types, would result in the occurence of eight different items being co-selected once in around 400 trials. For a stock of influenza virus where each particle contained only eight RNPs, this would result in 0.24% of particles having the genome complement required for infectivity (Enami *et al* 1991). This theoretical percentage rises to nearly 20% if fourteen segments were packaged. Comparison of the theoretical values for infectious particles obtained by stochastic packaging with observed particle/pfu ratios suggest random choice is a feasible mechanism for packaging of influenza gene segments into virions (Compans & Choppin 1975; Lamb & Choppin 1983). One piece of evidence that influenza virus could package more than eight segments came from the isolation of a reassortant virus which was heterozygous for two genome segments (Scholtissek *et al* 1978).

1.8.8.2 The application of reverse genetics to investigate packaging

The ability to assemble synthetic RNPs which could be rescued into infectious influenza virus was used to investigate the means by which influenza virus packages an infectious complement of genome segments (Enami *et al* 1991). The experiment involved the rescue of a *ts* mutant with a deletion in the NS1 gene. The *ts* mutant grows to $>10^7$ pfu/ml at the permissive temperature (33°C) but at the non-permissive temperature (38°C) no progeny virus can be detected. The NS segment encodes two proteins, NS1 and NS2, both of which are essential for replication. By supplying a synthetic RNP encoding NS1 alone it would be possible to rescue a virus with non *ts* phenotype only if nine segments were packaged since the original segment containing the NS1 deletion would still be required as the only source of NS2 protein.

Transfection of a synthetic RNP coding for only the NS1 protein did indeed result in apperance of non *ts* virus. Evidence from plaque assays of serially diluted virus indicated that complementation by coinfection of two or more virus particles, each with less than the required gene complement, was not responsible for the non *ts* phenotype nor was reversion of the original lesion. The ratio of the titre of the transfectant stock at 38°C (where nine segments are obligatory for productive infection) to the titre observed at 33°C (where the parental mutant can grow and so only eight segments are required) was consistent with that expected by calculations based on stochastic packaging of nine or eight segments respectively. While the number of segments normally packaged by influenza virus could not be definitively calculated, this study provided evidence that the correct complement of genome segments can be obtained by randomly choosing a numerical excess of RNPs.

1.8.9 Production of influenza A/B virus chimeras.

Using the same counterselectable WSN-HK helper reassortant described in section 1.8.6 (Enami *et al* 1990) it was possible to introduce a NA segment containing non coding regions from an influenza B virus gene segment into the influenza A virus genome (Muster *et al* 1991). This produced an entirely novel influenza virus since natural reassortment between influenza A and B viruses does not normally occur (Murphy & Webster 1990). The resulting virus has normal coding regions from influenza A virus in all segments but the non coding region of the neuraminidase gene segment is derived from the inflenza B virus NS gene segment. Interestingly synthetic RNP containing the non coding region of the NA gene of influenza B virus could not be rescued whereas if the non coding region of the influenza B virus NS segment was used to flank the wild type influenza A virus NA gene, the synthetic RNP could be rescued. The virus was found to be attenuated in mice and replication was restricted in the respiratory tract and partially so in the lungs (Muster *et al* 1990). Infection induced immunity to subsequent challenge with influenza virus. This work demonstrated the potential to produce new variants which are attenuated and show potential as live vaccines to protect against influenza virus infection.

1.8.10 Influenza virus engineering—concluding remarks.

Three years since the first report of *in vivo* replication, expression and packaging of synthetic influenza virus RNPs, influenza virus remains the only negative strand RNA virus for which stable genome alterations have been introduced by manipulating cDNA clones. Exploitation of this methodology has already furnished information about the replication cycle of influenza virus, such as those discussed here concerning the criteria for polyadenylation of mRNA and the mechanism of packaging RNPs into virus particles, which were not experimentally approachable prior to development of methods enabling the application of reverse genetics to influenza virus. The potential has also been demonstrated for producing novel strains of influenza virus which may prove useful as improved vaccines. This new technology has therefore paved the way for dissecting all aspects of the molecular biology of influenza virus and may find immense practical application to the development of new improved therapeutic agents.

1.9 Bunyamwera virus.

1.9.1 Taxonomy

Bunyamwera virus is the prototype of both the *Bunyavirus* genus and the *Bunyaviridae* family (Table 1.9.1). The *Bunyaviridae* is subdivided into five genera which together contain more than 300 viruses (Karabatsos 1985). Four of the five genera, *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* (Calisher *et al* 1990), contain many viruses with the fifth, *Tospovirus*, containing a single member. The *Bunyavirus* genus contains at least 18 serogroups (Beaty & Calisher 1991) which together include over 160 viruses (Bishop 1990). The Bunyamwera serogroup, of the *Bunyavirus* genus, contains 25 viruses including Bunyamwera virus, Maguari virus and Germiston virus.

Genus	Serogoups	Viruses
Bunyavirus	18 groups ~160 viruses Example groups:	
	Bunyamwera group	25 viruses including Maguari, Germiston and Bunyamwera viruses.
	California group	13 viruses including La Crosse virus
	Simbu group	21 viruses including Akabane virus
Nairovirus	6 groups ~20 viruses Example groups:	
	CCHF	4 viruses including Crimean-Congo Haemorrhagic fever virus.
	Dera Ghazi Khan	5 viruses
Phlebovirus	2 groups / 43 viruses	
	Phlebotomus fever group	37 viruses including Naples & Sicilian sand fly fever and Rift Valley fever viruses
	Uukuniemi group	6 viruses
Hantavirus	One group—Hantaan	5 viruses including Hantaan, Puumala and Seoul viruses
Tospovirus	One group one virus	Tomato spotted wilt virus

Table 1.9.1 Classification of the *Bunyaviridae*. (Table simplified from Beaty & Calisher 1991)

1.9.2 Biology of the *Bunyaviridae*

The natural history of the *Bunyaviridae* has been well reviewed elsewhere (Beaty & Calisher 1991 and references there in) and will only be covered briefly here. With very few exceptions, members of the *Bunyaviridae* are arthropod-borne viruses of vertebrates (arboviruses). The exceptions are the five hantaviruses which are rodent-borne with no known arthropod host (Schmaljohn & Dalrymple 1983) and tomato spotted wilt virus which is transmitted between plants by plant feeding arthropods called thrips (Milne & Francki 1984; de Haan *et al* 1989).

Susceptible vertebrate hosts are infected by blood-feeding arthropod vectors which appear to suffer no ill effects from the infection which is persistent and can be passed from insect to insect by horizontal (venereal) and vertical (transovarial) transmission. The bunyaviruses are transmitted principally by mosquitoes, nairoviruses by ticks and phleboviruses by sand flies (Phlebotomus group) or ticks (Uukuniemi group). Mammals and birds serve as the main animal reservoir hosts with different viruses principally infecting different species or groups of closely related species. Infected vertebrate hosts develop viraemia and serve to infect subsequent feeding arthropods and thus amplify the virus. Only rarely are humans or domestic animals amplifying hosts although the *Bunyaviridae* does contain a few members of human and veterinary importance such as Crimean-Congo haemorrhagic fever, Rift Valley fever, La Crosse and Hantaan viruses (Karabatsos 1985).

The human diseases include: several types of encephalitis (California group of bunyaviruses) which may be rare or endemic depending on the virus; epidemic fevers (Simbu bunyavirus, Rift Valley fever phlebovirus); endemic fevers (sand fly fever group of phlebovirus) or haemorrhagic fevers (hantaviruses and nairoviruses). These and related animal diseases have been recently reviewed (Gonzalez-Scarano *et al* 1991).

1.9.3 Bunyamwera virus genome structure.

Bunyamwera virus has a segmented genome consisting of three single stranded RNA molecules of negative polarity. Bunyamwera was the first member of the *Bunyaviridae* to be completely sequenced (Lees *et al* 1986; Elliott 1989a; Elliott 1989b). The total genome size is 12.3kb, 95% of which encodes polypeptides. Similar efficient utilization of coding potential is observed for other negative stranded RNA viruses.

The three genome segments, L, M and S, are 6875, 4458 and 961 nucleotides in length respectively. The L segment has a single open reading frame coding the 2238 amino acid L protein (Mr 259K) which is the virion associated transcriptase (Jin & Elliott 1991). The M segment encodes the two virion glycoproteins, G1 and G2, and a small non-structural protein (NSm) of unknown function. The protein products of the M segment are co-translationally cleaved from a larger polyprotein. The S segment encodes the nucleocapsid (N) protein and a non structural protein (NSs) of unknown function. These two proteins are coded for in overlapping reading frames (Fuller *et al* 1983) and are translated from a single type of mRNA (Bishop *et al* 1983; Elliott & McGregor 1989) by alternative translation initiation and not from separate RNAs as originally thought (Patterson *et al* 1983; Raju & Kolakofsky 1987b).

The terminal eleven nucleotides at each end of all three genomic RNAs are conserved for all members of the *Bunyavirus* genus. A further three or four adjacent nucleotides are also conserved for a given segment but with differences between segment types (Elliott 1990). These conserved regions are complementary between either end of the RNA molecule. Further complementary sequence, of non conserved sequence between different viruses and gene segments, is adjacent resulting in a fairly long region at both ends of each gene segment with potential to form a double stranded panhandle structure (see Figure 1.9.1).

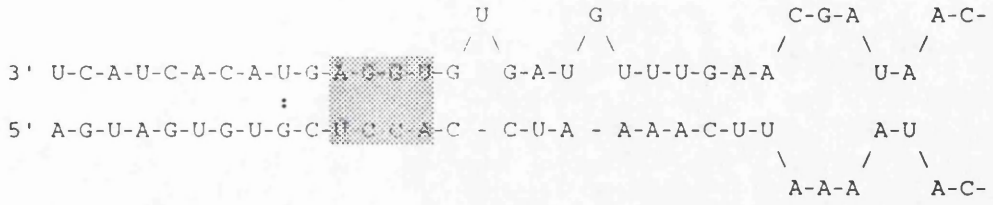


Figure 1.9.1 Complementary sequences at the termini of Bunyamwera virus S segment. The first eleven bases are conserved for all segments of all members of the bunyavirus genus. The next four bases (shaded box) are conserved for the S segment— different short conserved regions exist for other segments. The remainder of the sequence is Bunyamwera virus S segment specific but such regions of complementarity exist for all bunyaviruses. The G/U base pair (double dots) becomes a miss-matched A/C pair in cRNA which would result in disruption of the helical duplex possibly enabling discrimination of vRNA from cRNA for packaging purposes. (Figure redrawn from Elliott 1990).

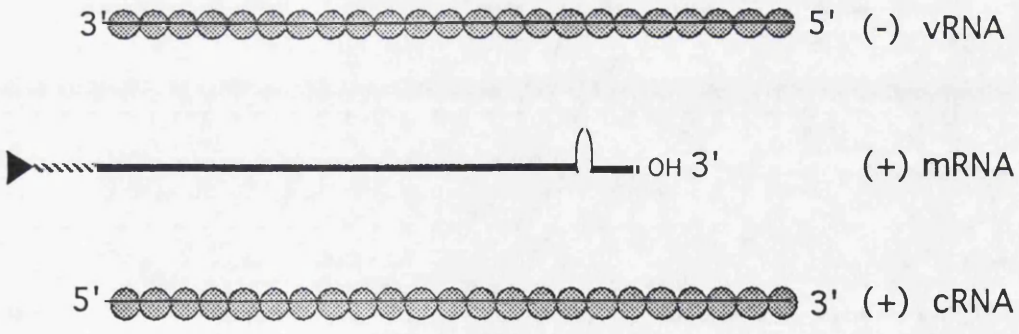


Figure 1.9.2 Infected cell RNAs of bunyaviruses. Both vRNA and cRNA are encapsidated in N protein and are exactly complementary. mRNA contains additional 5' sequences derived from host cell capped mRNA. Bunyavirus mRNA differs from influenza virus mRNA in being shorter compared to the template and lacking a poly A tail. (See Figure 1.8.1 for influenza virus RNA). Bunyavirus mRNA also has sequences just upstream of its 3' terminus with potential to form a stem-loop structure which may serve to stabilise the message (Kolakofsky & Hacker 1991).

1.9.4 Bunyavirus mRNA.

The mRNA transcripts of bunyaviruses contain non templated heterologous sequences at their 5' ends which are capped (Bishop *et al* 1983; Patterson & Kolakofsky 1984). These are also truncated 50-110 nucleotides compared to the full length cRNA (Patterson & Kolakofsky 1984; Bouloy *et al* 1990; Jin & Elliott 1993). These features are shared with influenza virus mRNAs but unlike influenza virus, bunyavirus mRNA does not appear to be polyadenylated (see Figure 1.9.2). *In vitro* transcription studies using RNPs from purified 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 methylated cap-dependent endonuclease activity which cuts the plant virus mRNA 13 or 14 nucleotides from its 5' end. Studies using anti-cap antibodies demonstrated that bunyavirus mRNAs are also capped *in vivo* (Hacker *et al* 1990).

1.9.4.1 Leader primed bunyavirus mRNA synthesis.

Recently the leader sequences of Bunyamwera virus S mRNA were cloned and several independent 5' sequences determined (Jin & Elliott 1993). mRNA was purified from infected cells by pelleting through CsCl gradients to remove encapsidated genome and antigenome RNP. . The S mRNA 5' termini were then specifically amplified using a PCR-based procedure developed for cloning 5' ends of RNA (Dumas *et al* 1991). The strategy used is shown in Figure 1.9.3.

Of twenty one clones sequenced, all were found to have heterologous 5' leader sequence. The length ranged from 12 to 17 nucleotides (average 14nt) and they had a combined base composition of 62% G/C. The striking feature of the non viral sequences was the occurence in all but one of the clones of a U residue at position -1 relative to the viral terminus. 90% of the clones had G at position -2 and half had an A residue at -3. Sequences further upstream did not show any marked base preference (see Figure 1.9.4). These findings were similar to those reported for snowshoe hare bunyavirus (Eshita *et al* 1985) and Germiston bunyavirus (Bouloy

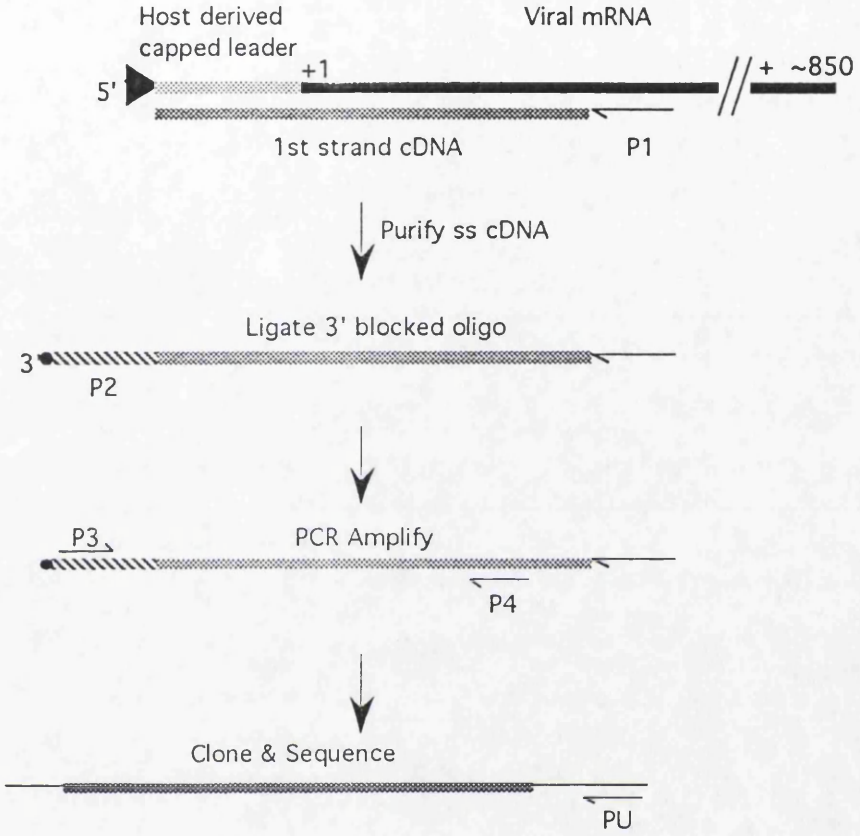


Figure 1.9.3 PCR cloning of Bunyamwera virus mRNA leader sequences. Reverse transcription was primed with oligo P1 to make ss cDNA across the host derived leader RNA. After purification, oligo P2 was ligated to the 3' terminus of the ss cDNA using T4 RNA ligase. P2 was 3' blocked to prevent multimeric ligation. The cDNA population was then amplified by PCR using oligonucleotides complementary to P2 and internal to Bunyamwera S segment sequence. After cloning individual RNA leaders could be sequenced off the Universal phagemid primer (PU). (Figure adapted from Jin & Elliott 1993; Method based on Dumas *et al* 1991)

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 & Elliott 1993). 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 Uukuniemi uukuvirus. Instead of the double triplet 5' AGUAGU-- found at the termini of bunyavirus segments, uukuviruses have a paired repeat 5' ACAC--. For eight out of eleven NSs mRNAs having all the viral terminal sequence intact, all had C at -1 and six of these also had A at -2, and five had C again at -3 (Simons & Petterson 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 uukuvirus mRNAs having the C as the last leader base, had intact 5' ACAC-- 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.

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.

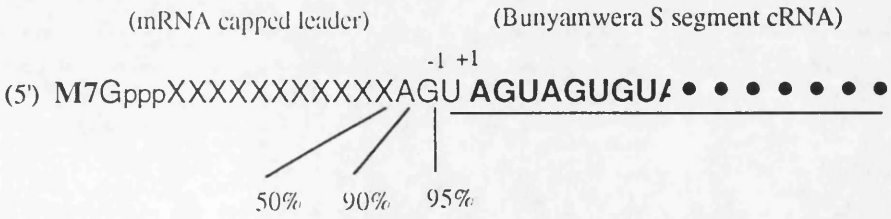


Figure 1.9.4 Host derived leaders of Bunyamwera virus S segment mRNA showing the commonly occuring bases at positions -1, -2 and -3 relative to the start of viral sequences. (Figure based on the results of Jin & Elliott 1993) see text for details.

1.9.5 Translational requirement for mRNA transcription

An unusual aspect of bunyavirus mRNA transcription is the requirement for ongoing translation (Abraham & Pattnaik 1983; Pattnaik & Abraham 1983; Raju & Kolakofsky 1986; Gerbaud *et al* 1987) for La Crosse virus. Although radiolabelled NTP incorporation in *in vitro* transcription reactions was unaffected by cycloheximide, it was found 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. mRNA transcripts which had terminated at nucleotide 175 could also be detected *in vivo* if cycloheximide was present (Raju & Kolakofsky 1987a).

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 nt 175 termination site and revealed other termination sites before the genuine one at nucleotide 886 (Bellocq & 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 (see Figure 1.9.5; Kolakofsky *et al* 1987).

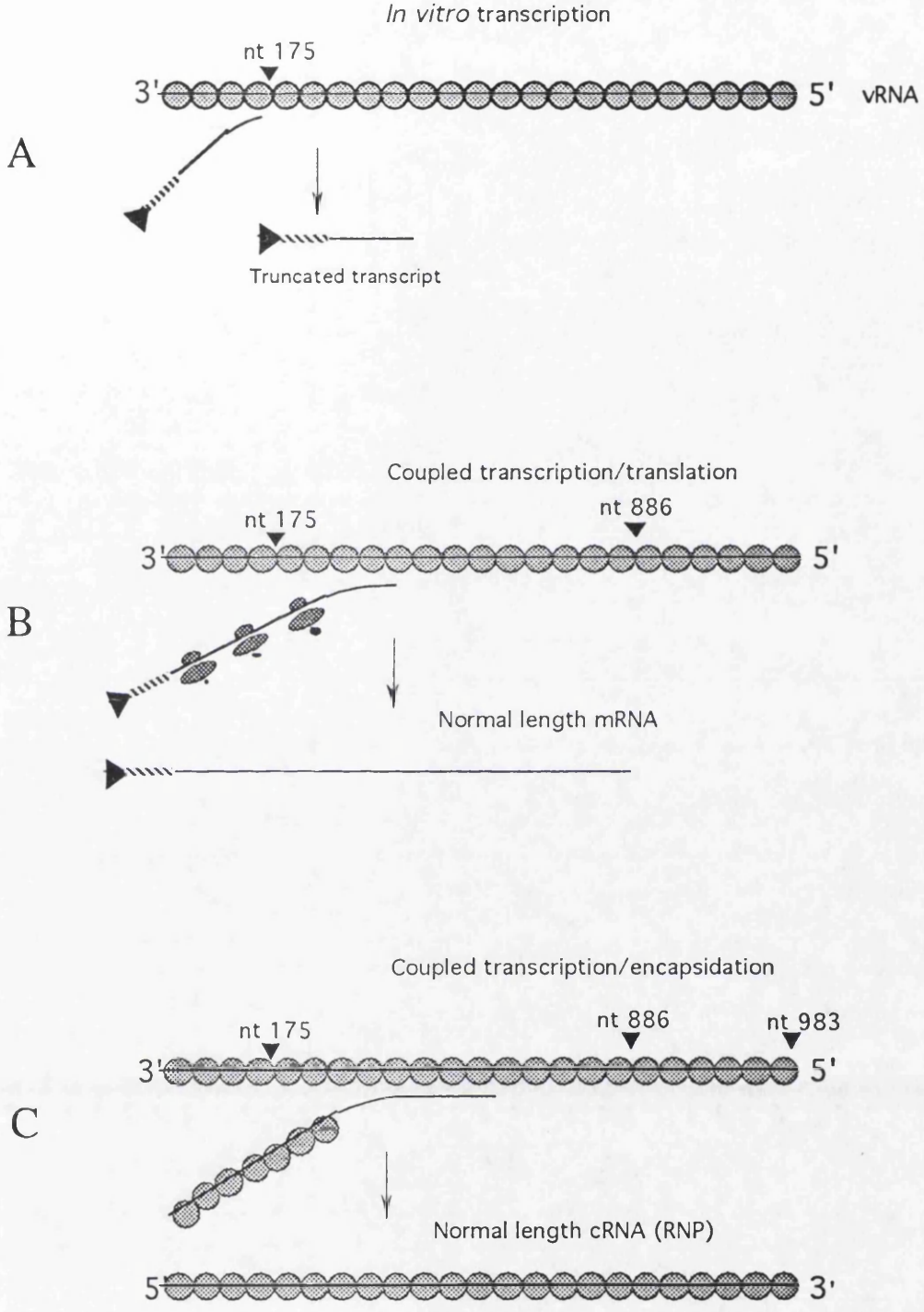


Figure 1.9.5 Translational requirement for La Crosse virus mRNA transcription. During *In vitro* transcription, transcripts terminate at nucleotide 175 (**A**) unless transcription is performed in the presence of reticulocyte lysate (**B**) which attenuates the 175 stop site. Coupled encapsidation is thought to attenuate premature stop sites and the usual mRNA terminator (**C**) to allow production of full length antigenomes. See text for details and references.

1.9.6 mRNA termination

The mRNA termination sites for several bunyavirus segments have been mapped. 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 & Elliott 1993). For Germiston virus S mRNA, 3'-GUUUGU-5' is present whereas for the M segment and the La Crosse virus S segment, 3'-GUUUUU-5' is present (reviewed in Kolakofsky & Hacker 1991). This last sequence is similar to that employed by influenza virus (see Section 1.8.7) although for influenza virus the site is also implicated in polyadenylation by slippage which is thought to result from 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 the pan handle lies much farther downstream of the termination signal than for influenza virus so polyadenylation would not be expected to occur.

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 & Brownlee 1992b). In bunyaviruses the occurence 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 & Hacker 1991). This picture is somewhat complicated by the occurence in snowshoe hare bunyavirus and Rift Valley fever phlebovirus of a C rich termination signal (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.

1.9.7 Bunyavirus nucleocapsids.

As for all negative strand RNA viruses, genomes and complementary replicative intermediates are associated with viral proteins as ribonucleoprotein (RNP) structures or nucleocapsids. The RNP is the functional template for transcription.

Bunyavirus RNPs are stable in high salt concentrations and can be separated away from non-encapsidated viral or cellular mRNAs by centrifugation through preformed CsCl density gradients (Obijeski *et al* 1976). High salt stability is a property shared with all other negative strand RNA viruses except influenza virus where high salt concentrations dissociate the nucleocapsid protein from the genome segments (see Section 1.8.4).

In CsCl density gradients, bunyavirus RNPs band at a density of 1.31g/ml whereas naked RNA is found in the pellet (>1.35g/ml). The RNP density is the same as that found for paramyxoviruses and rhabdoviruses and tobacco mosaic virus. Applying stoichiometries of protein:RNA found for these other nucleocapsids, and taking account of the density and M_r of the bunyavirus N protein, it has been estimated that one N protein monomer is present for each three nucleotides (Kolakofsky & Hacker 1991). However, estimation of protein ratios from SDS-PAGE compared to the chemical composition of purified virus, put this figure at one N protein molecule per six nucleotides (Obijeski *et al* 1976).

Nucleocapsids can be found as circular forms in cells and virus (Samso *et al* 1975) as can deproteinised genomic RNA *in vitro* (Hewlett *et al* 1977). The observation that genomic RNA had triphosphate at its 5' end (Gentsch *et al* 1977) and a free hydroxyl group at the 3' end (Obijeski *et al* 1976), suggested that the observed circular configuration of RNPs would not be due to covalent linkage and may therefore be due to hydrogen bonding. The conserved and complementary nucleotides found at the termini of bunyavirus gene segments (Elliott 1990), can indeed be found hydrogen bonded in nucleocapsides (Raju & Kolakofsky 1989).

N protein encapsidated bunyavirus RNA is moderately resistant to nuclease digestion (Pettersson *et al* 1971) but not as resistant as for rhabdovirus or paramyxovirus RNPs (Obijeski *et al* 1976). Another difference is the hydrogen bonding potential of the encapsidated RNA. For bunyaviruses, encapsidated sequences are sufficiently exposed to base pair with complementary sequences, as judged by their sensitivity to RNAse H in the presence of added complementary

oligonucleotide (Bellocq & Kolakofsky 1987). Similar studies using paramyxovirus RNPs suggested that these encapsidated RNAs had a greatly diminished base pairing potential (Kolakofsky & Hacker 1991). The ability of the bunyavirus RNPs to form pan handle structures (Samso *et al* 1975; Obijeski *et al* 1976) by complementary base pairing of segment termini (Obijeski *et al* 1980) is further evidence of the 'open' nature of the RNP. This latter property is shared with influenza virus RNPs as is relative nuclease sensitivity—both properties contrast to the non segmented negative strand RNA viruses.

1.9.7.1 Bunyavirus encapsidation signals.

Except in the case of persistent infection of mosquito cells (Hacker *et al* 1989) and for a small fraction in BHK cells (Raju & Kolakofsky 1987b), bunyavirus mRNA is not encapsidated nor are cellular mRNAs. Encapsidation must therefore specifically discriminate between mRNA and genome/antigenome RNA. mRNA is 3' truncated relative to full length complementary RNA and so would not be capable of pan handle formation (Figure 1.9.2). This difference however would not account for the differential encapsidation since abnormal antigenomes of La Crosse bunyavirus occur which are truncated to the same point as mRNA, have a complete 5' end but no host derived leader, but which are only found in RNP form (Raju & Kolakofsky 1987b). Another unusual transcript which has a capped leader but ends as a full length antigenome is mostly found not associated with N protein (see Figure 1.9.6).

These observations suggest that the 5' terminal nucleotides are the recognition signal for N protein binding and that to function correctly these nucleotides must be precisely located at the terminus and not preceeded by the capped leader as found in mRNA. As all segments and both polarities of full length RNA are found encapsidated, the encapsidation signal must be common to all 5' ends. This reasoning implicates at least some of the conserved 11 nucleotides as encapsidation signals (see Figure 1.9.1).

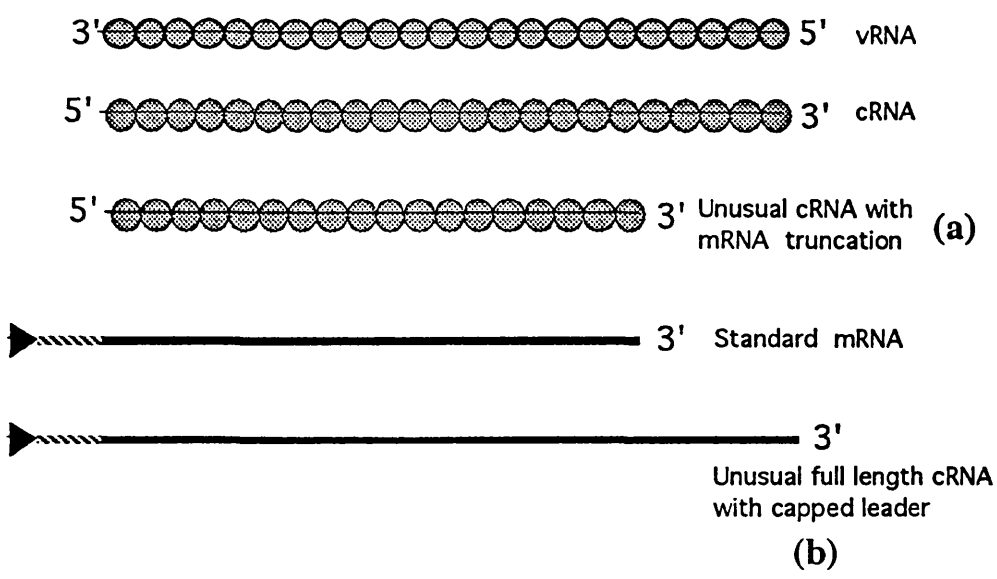


Figure 1.9.6 Clues to N protein encapsidation signals from La Crosse virus RNAs. In addition to the usual genome antigenome and mRNA, unusual transcripts can be found in La Crosse virus infected cells. Among others, two species give a clue to the encapsidation signals. One of these (a) is a truncated cRNA which exists in RNP form and the other (b) is a full length cRNA with additional 5' sequences, normally found only in truncated mRNA, which is mostly non encapsidated. See text for discussion and references.

1.9.8 Reassortment of bunyavirus gene segments.

Having a segmented genome, members of the *Bunyaviridae* would be expected to be able to reassort gene segments during dual infection. In the laboratory, coinfection of cells with closely related bunyaviruses can result in a high proportion of reassortants amoungst progeny virus (Bishop & Shope 1979; Iroegbu & Pringle 1981a). The property of segment reassortment has been exploited to assign gene products to their segments by comparing the protein profiles of reassortant viruses to their gene segment complements (Bishop & Shope 1979). Reassortant viruses have also been used to define determinants of *in vivo* virulence and tissue tropism (Beaty *et al* 1982).

1.9.8.1 Natural reassortment

For reassortment to occur in nature, dual infection of a single host would be necessary. Some examples of naturally occuring reassortants have been reported. An isolate of La Crosse virus from Minnesota was shown to have the expected RNase T1 fingerprints for the L and M segments but had a S segment of clearly different origin (Klimas *et al* 1981). Two distinct bunyaviruses, Shark river and Pahayokee viruses had similar L and S segments but had M segments with easily distiguishable RNA fingerprints suggesting a divergence by reassortment (Ushijima *et al* 1981). This ability to reassort gives the *Bunyaviridae* a great evolutionary potential as demonstrated by the large number of distinct viruses in the family (Karabatsos 1985).

1.9.8.2 Bunyavirus Temperature Sensitive mutants.

Over 200 *ts* mutants have been isolated, mostly from the Bunyamwera and California groups of the *Bunyavirus* genus (Pringle 1991). These have been useful tools to study reassortment and identify the limitations of genome segment exchange. Chemical mutagenesis using 5-flurouracil, 5-azacytidine or N-methyl-N'nitrosoguanidine has mostly been employed to generate *ts* mutants (Ozden & Hannoun 1978) although spontaneous mutants of La Crosse, snowshoe hare and Maguari bunyaviruses have been isolated at frequencies ranging from 1% to 2.7% (Gentsch *et al* 1977; Iroegbu 1981).

Reassortment of gene segments between different *ts* mutants can result in progeny virus with non *ts* phenotype during dual infection provided that the *ts* lesions are in different segments for the two viruses. For example coinfection by two M segment *ts* mutants could not result in a non *ts* virus unless molecular recombination occured, and no such recombination has been reported. This property has been exploited to assign *ts* mutants to reassortment groups with viruses capable of reassorting with each other to lose their *ts* phenotype being placed into separate groups.

Having three genome segments, bunyaviruses would be expected to form three reassortment groups representing lesions in each of the three segments. However, the vast majority of *ts* mutants fall into only two distinct reassortment groups (Pringle 1991). For the bunyavirus genus, of 197 *ts* mutants, all but ten belong to two reassortment groups. Eight of these are so far ungrouped. Of the remaining two, a Guaroa virus isolate has been tentatively assigned to a third group (Bishop 1979) and a Maguari virus, *ts*MAG23, is a genuine member of a third group based on its ability to reassort with all group I and II Maguari virus mutants and with two viruses thought to be double group I/II mutants (Pringle & Iroegbu 1982).

1.9.8.3 Heterologous reassortment in bunyaviruses

Reassortment only occurs between certain members of a serogroup, for example, Bunyamwera, Maguari and Germiston bunyaviruses can exchange gene segments with each other but not with Guaroa or Main Drain viruses which are also in the Bunyamwera serogroup (Pringle 1991). Reassortment does not occur between different serogroups such as between the California group of bunyaviruses (e.g. La Crosse and snowshoe hare viruses) and the Bunyamwera serogroup (e.g. Bunyamwera and Maguari viruses).

Because each virus has a distinguishable protein profile and RNA fingerprint, it has been possible to use heterologous reassortment to assign gene products to their segments. In general consistent assignments could be made. For some virus combinations, segment exchange was not reciprocal or was restricted. Co-segregation of the L and S segments was often observed. Heterologous reassortment has been recently reviewed elsewhere (Pringle 1991) and therefore the complex crosses and their sometimes confusing outcomes will not be discussed further here except for the case of *ts*MAG23 which will be discussed in Chapter 4.

	Virus	*ts* mutants	Reassortment groups I	II	III
Bunyamwera serogroup	Batai	5	1	4	0
	Bunyamwera	8	5	3	0
	Germiston	8	2	6	0
	Maguari	46	12	31	1
	Guaroa	13	5	7	1?
California serogroup	La Crosse	20	6	14	0
	Lumbo	17	3	14	0
	Snowshoe hare	48	26	20	0
	Tahyna	20	1	17	0
	Trivittatus	12	4	6	0

Table 1.9.2 Temperature-sensitive bunyavirus mutants.The Table lists the number of *ts* mutants isolated for each virus and their assignments to each of three possible reassortment groups. Heavily lined boxes group viruses capable of heterologous reassortment. Members of one box cannot reassort with those of another even within a serogroup (e.g. Guaroa virus cannot reassort with Bunyamwera virus). Temperature sensitive members of one reassortant group (I,II or III) can reassort with members of other reassortant groups to produce non-*ts* progeny by segment exchange. (Table modified from Pringle 1991).

1.9.9 Recombinant Bunyamera virus L protein

Functional Bunyamwera virus L protein has been expressed from cloned cDNA (Jin 1991; Jin & Elliott 1991; Jin & Elliott 1992). Two recombinant vaccinia virus expression systems were investigated (Jin 1991). The first of these expresses the foreign protein constitutively and to high levels from the vaccinia virus p7.5 promoter (Chakrabarti *et al* 1985). The second system involves placing the foreign gene under control of a bacteriophage T7 promoter in the TK locus of vaccinia virus. Expression of the foreign protein is dependent on, and can therefore be controlled by, coinfection with a second recombinant vaccinia virus, vTF7-3, which expresses T7 RNA polymerase (Fuerst *et al* 1986).

Both systems were successful at producing antigenically authentic L protein *in vivo* (Jin 1991; Jin & Elliott 1991). The functionality of the recombinant L protein was assessed in an *in vivo* transcription assay using transfected RNPs. Native Bunyamwera virus RNP was isolated from infected cells by CsCl gradient centrifugation and transfected into CV1 cells using calcium phosphate. Under these conditions, transfection of RNP alone resulted in only very weak signals by Northern blot analysis. In the presence of recombinant vaccinia virus expressing L protein, the RNPs served as templates for efficient amplification of S segment RNA. The use of strand specific probes revealed that both positive and negative strand RNA were produced from the RNP templates which were of mixed polarity. Metabolic labelling using ^{35}S methionine showed that the transcribed RNA could be translated to produce N protein. RNP purified from virus, and therefore containing essentially only negative polarity RNA, was also transfected and used as transcription template by the recombinant L protein. Both polarities of RNA were detected demonstrating replication and implying that newly transcribed RNA could be encapsidated in newly synthesised N protein to form template-competent RNPs.

Characterisation of the RNA transcribed and replicated by the recombinant L protein demonstrated that the unencapsidated mRNA was primed by capped leaders scavenged from the intracellular pool of mRNA as occurs during normal infection. The chief difference in the host leaders was that they were unusually AU rich compared to those found during Bunyamwera virus infection. This anomaly was thought to be due to the abundance of AU rich vaccinia virus mRNAs in the cytoplasm. Sequence comparison of some of the leader sequences to the vaccinia virus genome suggested that this indeed may be the case (Jin & Elliott 1993). An

unexplained difference was the heterogeneous termination site of the mRNA transcribed by the recombinant L protein.

Expression of functional recombinant L protein was the first direct evidence that the L protein of bunyaviruses was the RNA polymerase, before this could only be assumed by deduction based on functional assignments to other segments (Elliott 1990).

1.9.9.1 Mutational analysis of the L protein

The system for expressing functional L protein was modified to enable transient expression from plasmid constructs under control of the T7 promoter. The system involved transfecting template RNP and L gene-containing plasmids (under control of a T7 promoter) into cells which had been infected with vTF7-3 to supply T7 RNA polymerase (Jin & Elliott 1992). This system enabled mutations to be introduced into the L protein which could be tested for *in vivo* activity without the need to create recombinant vaccinia viruses for each mutant.

Four conserved motifs had been identified in RNA dependent RNA polymerases (Poch *et al* 1989) and were found to correspond to regions of similarity noted in the Bunyamwera virus L protein and the influenza virus PB1 protein (Elliott 1989b). The growing availability of more sequence data from L segments of other members of the *Bunyaviridae*, made sequence alignment of seven members possible and revealed highly conserved nucleotides in all four putative polymerase motifs (Jin & Elliott 1992). Some of these were mutated and tested for *in vivo* activity using the RNP transcription assay. In general highly conserved nucleotides were essential for L protein activity whereas poorly conserved ones were not. An amino acid triplet SDD, present in all available *Bunyaviridae* sequences was mutated to GDN, the invariant counterpart found in the RNA polymerases of non segmented negative strand RNA viruses. This change abolished activity of the L protein. No attempt to systematically mutate all bases of the conserved regions was undertaken but important deductions could be made. This report (Jin & Elliott 1992) was the first to investigate rationally introduced mutations in the RNA polymerase of any negative strand virus and establishes a system of great potential to investigate the transcription and replication of bunyaviruses.

1.10 Aims of project.

The overall goal of the project was to attempt to define conditions under which RNA transcribed from cDNA could be replicated by Bunyamwera virus. Two approaches were devised to attempt to present the virus with synthetic RNA in a form in which it could be recognised as a transcription template. The first of these involved transcribing Bunyamwera virus S segment RNA *in vivo* in the presence of a replicating helper virus. It was hoped that this approach might allow encapsidation of nascent RNA into the RNP form required for viral recognition. The second approach involved attempts to reconstitute RNP *in vitro* from synthetic RNA and N protein of viral origin. RNP reconstituted *in vitro* and transfected into Bunyamwera virus infected cells might be replicated by the virus.

For the *in vivo* transcription approach, the project set out to characterise suitable helper virus and *in vivo* transfection systems before attempting rescue of S segment RNA derived from cDNA. For the *in vitro* reconstitution, investigation was aimed at determining conditions under which synthetic RNA could associate with N protein to reconstitute RNP.

Chapter 2: Materials and Methods.

2.1 Materials.

2.1.1. Enzymes.

Restriction endonuclease enzymes, DNA polymerase I and T4 DNA ligase were purchased from Bethesda Research Labs (BRL) or Boehringer Mannheim; T7 DNA polymerase from Pharmacia LKB Ltd; T7 RNA polymerase, RQ1 DNaseI and RNasin ribonuclease inhibitor from Promega; Vent™ thermostable DNA polymerase, T4 polynucleotide kinase, MuLV reverse transcriptase and mung bean nuclease from New England Biolabs; calf intestinal phosphatase from Boehringer Mannheim; micrococcal nuclease and ribonucease A from Sigma and Taq DNA polymerase from Cetus.

2.1.2. Radiochemicals.

With the exception of ^{14}C chloramphenicol which was supplied by DuPont, radiochemicals were supplied by Amersham International plc. at the following specific activities:

^{35}S-L-methionine	approximately 800 Ci/mmol (15μCi/μl)
α-^{32}P-TTP	3,000 Ci/mmol (10μCi/μl)
α-^{32}P-dATP	3,000 Ci/mmol (10μCi/μl)

^{35}S (αS)-dATP	>1,000 Ci/mmol (10μCi/μl)
^{35}S (αS)-CTP	>800 Ci/mmol (40μCi/μl)
α-^{32}P-CTP	400 Ci/mmol (10μCi/μl)
α-^{32}P-UTP	400 Ci/mmol (10μCi/μl)
γ-^{32}P-ATP	>5,000 Ci/mmol (10μCi/μl)
^{14}C chloramphenicol	58.5 mCi/mmol (0.1μCi/μl)

2.1.3. Synthetic oligonucleotides.

Synthetic oligonucleotides were either produced in house using a Biosearch model 8600 DNA synthesiser or were gifts from C ruachem Ltd..

2.1.4. Plasmids.

Phagemid cloning vectors pUC118 / pUC119 (Vieira and Messing, 1987) were supplied by Dr. R.M. Elliott. Recombinant plasmids containing full length Bunyamwera virus S segment in pUC9 and pUC18 (pUC9BunS+ and pUC18BunS-) and a T7 expression construct for synthesis of Bunyamwera virus N protein (pTF/BunN) were obtained from A. McGregor of this Institute.

2.1.5. Bacterial and bacteriophage strains.

E. coli strain XL-1 (*supE44 hsdR17 recA1 endA1 gryA46 thirelAl lac- F-;* Bullock *et al.*, 1987) was the usual host for the propagation of recombinant plasmids and was also used for the growth of phagemids. Bacteriophage M13K07 supplied by Dr. R. M. Elliott is a derivative of bacteriophage M13 that carries a mutated version of gene II, a plasmid origin of replication, and the kanamycin resistance gene from Tn903 (Vieira and Messing, 1987). M13KO7 was used to rescue single-stranded phagemids from XL-1 host bacterium.

2.1.6. Bacterial culture media.

The following bacterial culture media were used:

LB-Broth (LB)	10g NaCl, 10g Bactopeptone, 15g yeast extract per litre.
LB-agar:	LB-Broth plus 1.5% (w/v) agar.
2 x YT broth:	5g NaCl, 16g Bactopeptone, 10g yeast extract per litre.

Ampicillin was added at 75µg/ml to the medium where appropriate.

2.1.7. Cells and tissue culture media.

BHK-21.C-13 cells, a continuous cell line derived from baby hamster kidneys (MacPherson and Stoker, 1962), were used for growing Bunyamwera virus in Glasgow modified Eagle's medium supplemented with 10% new born calf serum (10% GMEM, Stoker and MacPherson, 1961). GMEM was supplied as a 10 x liquid concentrate. This was diluted in distilled water and supplemented with 5mM L-glutamine, 2.75g/l $NaHCO_3$, 100 unit/ml penicillin, 100µg/ml streptomycin and 10% tryptose phosphate broth.
CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal calf serum.
293 cells, a human cell line transformed by DNA from human adenovirus type 5 (Graham *et al* 1977), were grown in DMEM containing 5% foetal calf serum.
Cells were grown at 37°C under 5% CO_2 in a humidified incubator. Routine maintenance and passaging of cells was done by Joyce Mitchell of the cytology department of this Institute.

2.1.8. Viruses.

Bunyamwera virus, originally isolated from a suckling mouse brain homogenate, was obtained from Dr. N. Karabasos (Vector-borne Diseases Laboratory, Centre for Disease Control, Fort Collins, Colorado, USA.) and subsequently triple-plaque purified in BSC-1 cells by Prof. C.R. Pringle in this Institute. The temperature sensitive mutants of Maguari virus (*ts*6, *ts*17 and *ts*23) were isolated by C.U. Iroegbu (Iroegbu and Pringle, 1981a).

vTF7-3, a recombinant vaccinia virus which expressed T7 RNA polymerase was obtained from Dr. B. Moss (Fuerst *et al* 1986). Vaccinia virus WR strain was obtained from Dr. A. Patel of this Institute.

2.1.9. Antiserum.

Anti-Bunyamwera virus antiserum (Watret, 1985) was obtained from Dr. R.M. Elliott.

2.1.10. Commonly used solutions.

Agarose gel loading buffer:	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol.
10 x EM buffer:	0.05M sodium borate, 0.1M sodium sulphate, 0.01M EDTA, 0.15M boric acid.
2 x RNA gel sample buffer:	20% glycerol, 0.05% bromophenol blue, 2 x EM buffer.
Carrier DNA:	10mg/ml salmon sperm DNA, autoclaved and stored at -20°C.
50 x Denhart's solution:	1% polyvinylpyrrolidone, 1% BSA (Pentex fraction V), 1% Ficoll.
Giemsa stain:	1.5% Giemsa in glycerol, heated to 50°C for 2 hr and diluted with an equal volume of methanol.
5% milk:	5% dried non-fat milk in water and stored at -20°C.
PBS:	170mM NaCl, 3.4mM KCl, 10mM HPO_4, 1.8mM KH_2PO_4, pH 7.2, 6.8 mM $CaCl_2$, 4.9mM $MgCl_2$

Protein dissociation mix:	100mM Tris-HCl pH 6.8, 4% SDS, 200mM β-mercaptoethanol, 20% glycerol. 0.2% bromophenol blue,
RNase A solution:	10mg/ml pancreatic RNase A in 10mM Tris-HCl, pH 7.5, 15mM NaCl, heated at 100°C for 15 min to inactivate DNases, cooled and stored at -20°C.
Sequencing gel mix:	6-12% acrylamide / bisacrylamide (57:3), 8M urea, 1 x TBE.
20 x SSC:	3M NaCl, 3M trisodium citrate.
20 x SSPE:	3M NaCl, 0.18M NaH_2PO_4, 20mM EDTA, pH 7.4.
TAE:	40mM Tris-acetate, pH 8.0, 1 mM EDTA.
TBS^+ buffer:	25mM Tris-HCl, pH 7.4, 135mM NaCl, 50mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$.
TBE:	90mM Tris-HCl, 90mM boric acid, 1mM EDTA, pH 8.
TE:	10mM Tris-HCl, pH 8, 1mM EDTA.

2.1.11 Miscellaneous materials.

Dialysis membrane (Medical International Ltd.).
Tissue culture disposable plasticware (Gibco Ltd.; Sterilin Ltd.).
Hybond N nylon blotting membrane (Amersham).
Gene Clean DNA purification kit (Bio 101).
Qiaex DNA purification kit (Qiagen).
RNaid RNA purification kit (Bio 101).
X-omat S film for autoradiography (Kodak Ltd.).
Disposable plastic reaction vials & pipette tips (Scotlab).

2.2 DNA cloning procedures.

2.2.1 Large scale preparation and purification of plasmid DNA.

Single colonies of the appropriate *E. coli* strain were picked from agar plates and inoculated into 10ml aliquots of 2 x YT broth, containing 75µg/ml ampicillin, and incubated overnight at 37°C in a shaking incubator.

Supercoiled plasmid DNA was purified on caesium chloride / ethidium bromide gradients as described by Maniatis *et al.* (1982). A 5ml overnight culture was inoculated into 300ml of LB-broth in a 2-litre flask (containing 75µg/ml of ampicillin where appropriate) and shaken for 20 hours at 37°C. Following centrifugation of the cultures at 3,000 rpm (Sorval GS3 rotor) for 10 minutes at 4°C, the bacterial pellet was by washed by resuspending it in 10ml of TE buffer and repelleting in the SS34 rotor at 3,000 rpm for 10 minutes at 4°C. The pellet was resuspended in 8ml of 50mM glucose, 250mM Tris-HCl pH 8 and 12mM EDTA containing 5µg/ml lysozyme, and was incubated for 5 minutes at room temperature before lysing the cells by the addition of 16ml of 0.2N NaOH/1% SDS (freshly made). The mixture was incubated for 10 minutes on ice followed by addition of 12ml of ice-cold 5M potassium acetate pH 4.8, and a futher 10 minute incubation on ice. Following centrifugation at 8,000 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 8,000 rpm for 15 minutes at 15°C. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in 10ml TE pH 8.0. 10g of caesium chloride and 1ml of a 10 mg/ml solution of ethidium bromide was added to the DNA solution giving a final density of 1.55 to 1.60 g/ml. The solution was then transferred to a Ti 50 heat-sealable centrifuge tube and centrifuged at 40,000 rpm (Beckman Ti 65 or 50 rotor) for 36 hours at 15°C. The plasmid DNA appeared as a red band (due to the intercalated ethidium bromide) and was removed from the gradient using a needle and syringe through the side of the tube.

Up to seven extractions with an equal volume of isoamyl alcohol (equilibrated with 5M NaCl) were carried out to remove the ethidium bromide. Following dilution of the solution with 3 volumes of water to prevent precipitation of CsCl, the DNA was precipitated by adding 2 volumes of ethanol. Following centrifugation at 6,000 rpm (SS34 rotor) for 10 minutes, the DNA pellet was air-dried, dissolved in water and stored at -20°C. The concentration of the plasmid DNA preparation was determined by measuring the absorbance of the solution at 260nm and assuming $1A_{260}$= 50µg/ml for a 1cm light pathlength cuvette.

2.2.2. Small scale preparation of plasmid DNA.

Single colonies of transformed *E. coli* strain XL1 were picked from transformation plates using a cocktail stick and inoculated into 2ml aliquots of 2 x YT broth containing 75µg/ml of ampicillin and cultures were grown overnight at 37°C with shaking. The bacterial cells from ~1.4ml of culture were pelleted (in 1.5 ml Eppendorf tubes) for 1 minute and the supernatant removed by aspiration. The pellet was resuspended in 100µl of an ice-cold solution containing 50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA and incubated on ice for five minutes. The bacterial cells were then lysed by the addition of 200µl of 0.2N NaOH/1% SDS. After 5 minutes at room temperature, 150µl of 5M potassium acetate (pH 4.8) was added to the tubes and incubation continued for a further 5 minutes. The supernatant produced following centrifugation for 5 minutes at 13,000 rpm in a microfuge, was extracted with phenol / chloroform and ethanol precipitated to yield a pellet containing plasmid DNA. This was washed with 70% ethanol, dried briefly under vacuum and dissolved in 40µl of water.

2.2.3 Restriction enzyme digestion of DNA.

Appropriate restriction enzymes were used at 1 unit per µg DNA in buffers supplied by the manufacturers (BRL or Boehringer Mannheim). Restriction digest analysis of small scale DNA preparations used 5µl of DNA, digested in a 10µl reaction volume containing 20µg/ml RNase A. Digestions were for 1–4 hours at 37°C after which DNA fragments were separared by electrophoresis on agarose gels.

2.2.4. Agarose gel electrophoresis of DNA.

Electrophoresis of DNA was performed on horizontal slab gels (14 x 11 x 0.5 cm, BRL gel electrophoresis apparatus Model H5, of 5.7 x 8.3 x 0.3 cm, BRL gel electrophoresis apparatus Horizon 58). 1% (w/v) agarose in 1 x TBE or 1 x TAE containing 0.5 μg/ml ethidium bromide was used. DNA samples were loaded to the gel in 5% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol. Electrophoresis was carried out at 50-100 V in 1 x TBE or 1 x TAE containing 0.5 μg/ml ethidium bromide.

2.2.5. DNA purification from agarose gels by silica matrix adsorption.

DNA fragments produced by restriction enzyme digests and PCR reactions were separated by electrophoresis through 1% agarose TAE gels containing 0.5μg/ml ethidium bromide. Long wave UV illumination allowed visualization of the DNA bands. Two commercial kits, Geneclean (BIO 101 Inc, La Jolla, CA) and Qiaex (Qiagen Inc, Chatsworth, CA), were used for retrieval of DNA from agarose blocks excised from the gels. Both kits contain a silica matrix that binds DNA in the presence of high concentrations of sodium iodide (Vogelstein & Gillespie, 1979). Blocks containing the required DNA fragments were excised from 1 x TAE agarose gels and mixed with 3 volumes of saturated NaI solution and incubated for 5 minutes at 55°C until the agarose had completely dissolved. Silica matrix was added (5μl for up to 5μg of DNA) and the mixture incubated for 5 minutes on ice to allow the DNA to bind to the matrix. Following a 5 second microcentrifuge spin, the pellet (containing DNA bound to silica particles) was washed with buffers supplied with the kits. For Geneclean three washes were performed by repeated resuspension / centrifugation cycles using 'NEW' wash (a Tris-buffered mixture of NaCl, ethanol and water). For Qiaex, two washes in 8M $NaClO_4$ followed by two washes in buffer QX3 (70% ethanol, 100mM NaCl, 10mM Tris/HCl, 1mM EDTA, pH7.5) were used. For both kits, the DNA was eluted from the silica matrix by resuspending the pellet in 20μl water and incubating for 2 minutes at 55°C. After a short centrifugation, the DNA solution was removed and stored at -20°C.

In general the Qiaex kit was used for fragments <500bp which were not efficiently recovered using the Geneclean kit. For larger fragments Geneclean was used as it involved slightly simpler wash steps.
Both kits were also used to purify DNA away from enzymes, oligonucleotides and other unwanted buffer components. For this application electrophoresis was not required.

2.2.6. End repair and DNA ligation.

DNA fragments (0.1 µg) and vector DNA (0.01-0.05 µg), which had been digested previously with the appropriate restriction enzyme(s), were ligated together in a 20µl mixture containing 0.1M Tris-HCl pH 7.4, 50mM $MgCl_2$, 1mM spermidine, 1mM ATP, 100µg/ml BSA and 1 unit of T4 DNA ligase for 16 to 24 hours at room temperature.

For blunt end DNA ligations, DNA fragments with sticky ends were converted to blunt ends by incubating the DNA fragment (up to 1µg) in a reaction buffer containing 33mM Tris. OAc pH 7.9, 66mM KOAc, 10mM $MgCl_2$, 0.5mM DTT, 0.1mg/ml BSA, 0.2mM of each dNTP, 0.1mM ATP and 3 units of T4 DNA polymerase for 30 minutes at 37°C. Following phenol / chloroform extraction, the DNA was resuspended in 20µl of NT buffer with 0.2mM dNTPs, and incubated with 1 unit of Klenow fragment of DNA polymerase I for 30 minutes at 20°C. The DNA was again phenol / chloroform extracted before ethanol precipitation and resuspension in 20µl of water. Ligation was carried out as above except that the ATP concentration was reduced to 0.05mM.

2.2.7. Preparation of competent bacterial cells.

0.5ml of an overnight *E. coli* culture was diluted into 50ml of 2 x YT broth in a 250ml flask and shaken for approximately 2 hours at 37°C until the culture OD_{550} reached 0.2. The bacterial cells were harvested by centrifugation at 3,000 rpm for 10 minutes, the pellet was gently resuspended in 10ml of 0.1M $CaCl_2$ and placed on ice for at least 20 minutes. The suspension was centrifuged at 3,000 rpm for 5 minutes and the pellet resuspended in 3ml of 0.1M $CaCl_2$. The resulting competent bacterial cells were either used directly for transformation or were stored for up to 48 hours at 4°C.

2.2.8. Transformation.

200µl of competent cells were incubated with half of a ligation reaction (10µl) for 60 minutes on ice, followed by a 1 minute 42°C heat shock to allow uptake of the ligated DNA by the *E. coli* cells. The reaction tube was returned to the ice bath, 0.5ml of 2 x YT broth added and the cells incubated for a further 60 minutes at 37°C. The cells were then plated onto LB agar, containing 75µg/ml ampicillin, and incubated for 12 to 20 hours at 37°C to allow single colonies to form. For vectors containing the LacZ gene, 20µl of 100mM IPTG and 20µl of 2mg/ml X-Gal was added to the cells before plating out to allow blue/white colour selection of recombinants.

2.2.9 Purification of synthetic oligonucleotides.

Synthetic oligonucleotides were produced by the phosphoramidite method and supplied in ammonium hydroxide; heat treatment for 5 hours at 55°C removed the base protecting groups and the ammonia was removed by lyophylisation. The oligonucleotide pellet was dissolved in 0.2ml of water and loaded in 95% formamide onto an 8% or 12% polyacrylamide gel containing 8M urea. The gels were viewed under UV light following electrophoresis and oligonucleotide bands were visualized as dark shadows against a fluorescent thin-layer chromatography plate. Gel slices containing the full length oligonucleotide species were excised with a scalpel blade and eluted in 1–2 ml of water at 42°C overnight with shaking. Supernatants were transferred to Spin-X centrifuge units (COSTAR) which contain a 0.45µm cellulose acetate filter in a microfuge tube. Centrifugation for 2 minutes in a microcentrifuge removed the acrylamide gel pieces, the oligonucleotides in the filtrate were then precipitated with 3 volumes of ethanol in the presence of 0.2M NaCl overnight at -20°C. The oligonucleotide pellets were washed with 70% ethanol, dried under vacuum and dissolved in water. Spectrophotometry was used to quantify the concentration of the oligonucleotide solutions, assuming that an $1A_{260}$= 20µg/ml for a 1cm pathlength cuvette.

An alternative rapid deprotection/purification method (Cruachem technical bulletin no. 41; Sawadago & Van Dyke 1991) was employed for oligonucleotides to be used for PCR primers. 50µl of triethylamine was added to 1ml oligonucleotide in

ammonia (as supplied). Deprotection could then be achieved by heating to 80°C for 30 minutes instead of 5 hours at 55°C. Instead of lyophilisation, ammonia was removed by precipitating the oligonucleotide by the addition of 9 volumes of dry butan-2-ol followed by centrifugation at 13,000 rpm for 1 minute in a microfuge. The oligonucleotide pellet was resuspended in 100µl of water and butanol-precipitated a further three times to remove the last traces of ammonia. The final oligonucleotide pellet was resuspended in water and diluted appropriately for use in PCR reactions without further treatment. In general ~1/5,000th of a complete 0.2µmole column synthesis was used per PCR reaction giving between 40 picomoles (theoretical 100% recovery) and 10 picomoles (25% recovery). If problems were encountered, concentrations were re-determined spectrophotometrically.

2.2.10 Polymerase chain reaction (PCR) amplification of DNA.

PCR primers were typically 15–30 nucleotides long. 10–50 picomoles of each primer were used in reaction volumes ranging from 10µl to 50µl. When plasmid DNA was used as template, triplicate reactions were set up containing ~10, 1 and 0.1ng of template. Further replicates allowed reactions to be halted after different numbers of cycles to minimise the number of copying steps and so reduce the possibility of accumulating point mutations. Standard reactions (Innis *et al* 1990) contained, in addition to primers and templates (above): 50mM KCl, 20mM Tris-HCl pH 8.4 at 25°C, 2.5mM $MgCl_2$, 1mg/ml BSA, 200µM dNTPs and 1 unit of Taq thermostable DNA polymerase. Reactions were overlaid with 1 drop of mineral oil and cycled for between 10 and 30 PCR rounds. Typical cycling parameters were duplex melting at 94°C for 30 seconds, primer annealing at 54°C for 30 seconds and primer elongation by Taq polymerase at 72°C for 1 minute. These conditions were altered empirically where improvement was required. Reactions were performed either in the Cambio Intelligent Heating Block or the Perkin-Elmer DNA Thermal cycler 460.

2.2.11 Coupled reverse transcription/PCR for cDNA amplification starting from RNA.

The following protocol for coupled reverse transcription/PCR was devised by combining methods based on Kawasaki and Wang (1989). Template RNA was purified from pelleted virus by two phenol extractions. Following ethanol

precipitation and resuspension in water, the RNA was added to the standard PCR mix (above) along with 20 units of placental ribonuclease inhibitor ('RNasin'—Promega), and 100 units MuLV reverse transcriptase (NEB). For bunyavirus S segment amplification two 15mer primers were used which anneal to the 3' ends of positive and negative strand S segment RNA (5' AGTAGTGTACTCCAC and 5' AGTAGTGTGCTCCAC) The reaction was overlaid with mineral oil and incubated at 42°C for 45 minutes before being cycled (30 rounds 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 1 minute). The products of the amplification were separated by 1% TAE agarose gel electrophoresis.

2.2.12 Cloning of PCR-generated DNA fragments.

DNA fragments were retrieved from agarose gels as described in Section 2.2.5. Prior to ligation into suitably cleaved vector DNA, the fragments were treated to ensure the ends were flush and to add a phosphate group to the 5' ends in order to allow ligation. The end-repair/kinasing steps were combined into a single reaction. The DNA fragment was added to a reaction mix containing 50mM Tris-acetate pH7.8, 50mM KOAc, 10mM $MgCl_2$, 1mM DTT, 200mM each dNTP, 1mM ATP, 1 unit of T4 DNA polymerase and 5 units of polynucleotide kinase. After incubation at 37°C for 30 minutes the DNA was purified by binding to silica matrix and ligated to SmaI cut vector, transformed to XL1 *E. coli* cells and plated onto LB-agar plates containing 75μg/ml ampicillin.

2.3 DNA sequence determination.

2.3.1 Preparation of ssDNA templates from phagemid vectors

10ml cultures of *E. coli* strain XL1 harbouring phagemid vectors (pUC118 or pUC119) containing cloned inserts were grown in 2 x YT broth for two hours at 37°C in the presence of 75μg/ml ampicillin to maintain the vector. Helper phage M13KO7 was then inoculated into each universal and shaken for 1hr at 37°C before addition of Kanamycin to select cells infected with M13KO7. Incubation was continued at 37°C with shaking, overnight. After growth, the cultures were transferred to 12ml reaction tubes and centrifuged for 5 minutes at 3,000 rpm (Sorval SM24 rotor) at 4°C. The supernatant was removed to a fresh tube and

1.5ml of 20% PEG / 2.5 M NaCl solution added, mixed and incubated for 15 minutes on ice. After centrifugation for 10 minutes at 8,000 rpm, the supernatant was removed by aspiration using a drawn-out Pasteur pipette; followed by removal of any remaining traces of PEG by centrifugation and aspiration. The visible pellet, containing single stranded phagemid vector packaged into M13-like particles by M13KO7, was resuspended in 500µl of water and twice extracted with 500µl of TE-saturated phenol followed by a single chloroform extraction. The upper phase was transferred to a fresh tube and the DNA was ethanol precipitated and dissolved in 100 µl of water.

The procedure could be scaled down to use microcentifuge tubes. This produced sufficient template for 1 or 2 sequencing reactions. The use of the SM24 rotor method provided sufficient template for at least 10 sequencing reactions and allowed processing of 24 tubes per spin instead of 12 for the microcentrifuge based method.

2.3.2. Dideoxynucleotide sequencing reactions.

The dideoxynucleotide chain termination sequencing method (Sanger *et al.*, 1977), according to the protocol of Pharmacia LKB, was used to determine DNA sequence from single stranded phagemid vector DNA templates.

10µl of single stranded DNA (approx 2-10µg), was mixed with 2µl of sequencing oligonucleotide primer (0.8µM) and 2µl of annealing buffer (200mM Tris-HCl, 100mM $MgCl_2$, 250mM NaCl, pH 7.5). Annealing reactions were carried out for 10 minutes at 60°C followed by incubation for 10 minutes at room temperature. For labelling, 6µl of enzyme premix (5 µCi of α-^{32}P-dATP at 3,000 Ci/mmol, or α-^{35}S-dATP at >1,000 Ci/mmol, 0.57µM each dGTP, dCTP and TTP, and 3 units of T7 DNA polymerase) was added to the annealed DNA/primer and incubated for 5 minutes at room temperature. Each reaction was divided into four wells of a prewarmed (37°C) 96 well reaction plate (Nunc) for the four chain termination reactions. For each 1/4 reaction, 2.5µl of termination mix (150µM each of the 4 dNTPs, 15µM of the required ddNTP chain terminator) was pipetted into the relevant well. mixing was achieved by centrifugation at 1,000 rpm for 10 seconds. The plate was incubated for 5 minutes at 37°C, 5µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% Xylene cyanol) was added to each well, and the plate was heated for 2 minutes at 80°C immediately before loading onto a 6% sequencing gel.

2.3.3 End-labelling of sequencing primers

When sequence close to the primer binding site was to be determined, the sequencing primer was labelled at the 5' end to allow terminations immediately following the primer to be detected. The labelling reaction was omitted if end labelled sequencing primer was used. End labelling was achieved by incubating 10pmole of primer in a 20µl reaction containing 10pmole γ-^{32}P ATP (5µl at 10mCi/ml, 5,000Ci/mmole) and 5 units of polynucleotide kinase in 1x kinase buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 5mM DTT and 1mM spermidine) The reaction was incubated at 37°C for 10 minutes before being heated to 90°C for 2 minutes to inactivate the enzyme. The reaction was used without further treatment (2µl containing 1 picomole of end labelled primer, per template). Excess labelled primer was stored for up to 2 weeks at -20°C. Annealing was performed as for the standard sequencing reaction but the labelling step was omitted. Annealed template/end labelled primer was then used directly in the termination reactions.

2.3.4 Denaturing electrophoresis of the products of DNA sequencing reactions.

6% acrylamide sequencing gel mix was made by dissolving 480g urea, 57g acrylamide and 3g bisacrylamide in 800ml of distilled water. The solution was deionised by stirring with Amberlite ion exchange resin for 30 minutes and then filtering through Whatman 3MM filter paper. 100ml of 10 x TBE was then added and the volume adjusted to 1 litre with water.

The BRL model S2 electrophoresis apparatus (44.5 x 42.2 x 21.6 cm) was used to run sequencing gels. One pair of glass plates (one is 2 cm shorter) were siliconized and assembled with one spacer (0.4 mm thick) on each side. 70ml of 6% sequencing gel mix was used for one gel and poured between the sealed plates immediately after having added the ammonium persulfate and TEMED to 0.1%. The smooth interface was formed by inserting the flat edge of the sharkstooth comb between the plates to a depth of 2-3 mm below the short plate. The gel sandwich was assembled in the gel apparatus and the comb inserted between the plates until it just made contact with the surface of the gel. Sequencing reactions (2–2.5µl) were loaded using a 'crystal tip' fitted to a 10µl Eppendorf pipette.

Electrophoresis was performed at 60–80 watts for 2–4 hours and the smaller plate was carefully removed from the gel. The gel was transferred to a sheet of Whatman 3MM paper, covered with 'cling film' and dried on a vacuum gel drier. The cling film was removed and the gel exposed to X-ray film for several hours for ^{32}P labelled DNA, and 1–3 days for ^{35}S labelled DNA.

2.4 Virus Culture.

2.4.1. Bunyamwera virus growth and titration.

BHK-21, clone 13, cells (MacPherson and Stoker, 1962) were used for growing Bunyamwera virus stocks. Confluent BHK-21 cells grown in 10% GMEM in 80oz roller bottles were infected with Bunyamwera virus at multiplicity of 0.01 pfu/cell and incubated at 31°C for 2–3 days. The culture fluids were harvested and clarified by centrifugation at 3,000 rpm for 15 minutes (Sorval GS3 rotor). The clarified fluids were aliquoted and stored at -70°C and used virus stocks without further purification.

Virus was titrated by plaque assay (Iroegbu and Pringle, 1981a). Ten-fold serial dilutions of virus stocks were made in PBS/2% serum. The growth medium was removed from confluent BHK monolayers in 50 mm dishes and 0.1ml of diluted virus 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% calf serum containing 0.6% agarose. The dishes were incubated at 31°C for 3–5 days. The cell monolayers were fixed with 1% glutaraldehyde in PBS for 2 hours and plaques were counted after Giemsa staining.

2.4.2 Growth and titration of vaccinia viruses

Crude stocks of vaccinia viruses were grown as described by Macket *et al* (1985). A single plaque was picked and used to inoculate an almost confluent monolayer of CV-1 cells grown in a small (25cm^2) flask. After growth for 48 hours at 37°C the supernatant was harvested and used as seed stock to grow working stocks.

Large flasks (150cm^2) containing almost confluent monolayers were infected with seed stock using 1/16 of the supernatant from a 25cm^2 infected monolayer per large flask. After virus adsorption for 1 hour, DMEM containing 5% foetal calf serum was added and the flasks incubated at 37°C for 48 hours. The infected cells were then scraped into the tissue culture medium and pelleted by centrifugation 3,000 rpm for 5 minutes (Sorval SS34 rotor). The pellet was resuspended in 10mM Tris-HCl pH9 at a rate of 2ml per large flask. The cell associated virus was released by three cycles of freeze/thawing and the nuclei removed by centrifuging at 1,000 rpm for 5 minutes. The supernatants were titrated by plaque assay on CV-1 cell monolayers and stored at -20°C for use as working stocks of vaccinia virus.

2.5 Bunyamwera virus RNP purification and analysis.

2.5.1. Preparation of Bunyamwera virus nucleocapsids (RNP).

The method was based on Leppert *et al.* (1979). Bunyamwera virus infected cells were scraped into the medium and pelleted by centrifugation at 3,000 rpm for 30 minutes in a 50ml Falcon tube. The cell pellet was suspended in cold lysis buffer (0.15M NaCl, 0.05M Tris-HCl pH 7.5, 0.6% NP-40) using 2ml per roller bottle of infected cells. The suspension was incubated on ice for 5 minutes before being vortexed for 2 minutes to break open the cells. Nuclei and cell debris were pelleted by centrifugation at 4,000 rpm (Sorval SS34 rotor) for 10 minutes at 4°C. EDTA was added to the supernatant to give a final concentration of 6mM. Up to 5ml of extract was loaded onto each CsCl gradient.

The preformed continuous CsCl gradient contained 6 ml of 20–40% (w/w) CsCl overlaid with 2ml of 5% (w/v) sucrose in 50mM NaCl, 25mM Tris-HCl pH 7.5, in a TST41 centrifuge tube. Nucleocapsids were separated by centrifugation at 32,000 rpm (TST41 rotor) overnight at 12°. After centrifugation, the nucleocapsid band was visible in the middle of the gradient and was harvested with a needle and syringe through the side of the tube. CsCl was removed by dialysis against three 1litre volumes of 50mM NaCl, 25mM Tris pH7.5 and 1mM EDTA allowing 1 hour between changes. RNP preparations were stored at -20°C.

2.5.2 Micrococcal nuclease treatment of Bunyamwera virus RNP.

This method was used by Seong and Brownlee (1992a) for production of influenza virus proteins capable of transcribing synthetic influenza virus-like RNA *in vitro*. Bunyamwera virus RNP was dialysed against 50mM Tris-HCl pH8, 50mM NaCl and 1mM $CaCl_2$. RNP (50µg protein content) was added to a reaction containing 25mM Tris HCl pH8, 25mM NaCl, 1mM $CaCl_2$ and 5 units of micrococcal nuclease (Sigma). Digestion was for 5 hours at room temperature after which time the nuclease was inhibited by addition of EGTA to 5mM (Cautrecasas *et al* 1967; Pelham & Jackson 1976). For each batch, degradation of the RNA to smaller fragments was confirmed by Northern analysis using S segment specific probes. The nuclease treated RNP preparations were supplemented with 20% glycerol and stored at -20°C prior to use in *in vitro* reconstitution studies.

2.5.3 *In vitro* Bunyamwera virus RNP reconstitution.

Micrococcal nuclease treated Bunyamwera virus RNP was mixed with RNA transcribed *in vitro* from the relevant plasmid constructs using T7 RNA polymerase. 1µg (protein content) digested RNP was incubated with 50–100ng of RNA at 30°C for 1 hour followed by incubation on ice for a further 1 hour (method based on Seong & Brownlee (1992a)). Reconstituted RNP was then either transfected into cells for expression studies or analysed by CsCl gradient centrifugation.

2.5.4 Fractionation of CsCl gradients.

To monitor reconstitution of synthetic RNP containing radiolabelled RNA, reactions were centrifuged through preformed 20–40% CsCl gradients as described in Section 2.5.1, fractionated and the radioactivity of each fraction determined by liquid scintillation counting. After centrifugation, a capillary tube with flexible tubing attached, was inserted through the gradient from the top of the tube until its open end was just above the base of the tube. The capillary was held firmly in this position using modelling clay. The tubing attached to the glass capillary was fed through a peristaltic pump which was then used to remove the

gradient from the bottom upwards. Fractions were collected into Eppendorf tubes. Typically each fraction contained ~150µl (6-10 drops). A portion of each fraction (20–50 µl) was removed to scintillation vials containing 5ml Ecoscint water-compatible scintillation fluid and the radioactivity of the sample determined using the appropriate channel of the scintillation counter.

The density of each fraction was determined by refractometry. 20µl samples were pipetted onto the glass stage of a refractometer and the edge of the refraction shadow aligned with the graticule before reading the refractive index from the scale. This figure was converted to density for CsCl solutions by consulting the appropriate data tables (Dawson *et al* 1986).

2.6 Introduction of nucleic acid into mammalian cells.

2.6.1 Liposome mediated transfection

A commercially available liposome preparation, Transfectace™ (BRL), was used for transfection of DNA, RNA and the products of *in vitro* reconstitution reactions, into cells. Confluent monolayers of cells were washed once with Opti-MEM™ (Gibco) low serum medium prior to application of the transfection mix. Nucleic acid (0.5–5µg) was diluted to 250µl in Opti-MEM and combined with separately diluted Transfectace (10–50µg diluted to 250µl opti-MEM). The mixture was incubated at room temperature for 15 minutes before addition to the cell monolayers (0.5ml per 35mm dish). After 4 hours, 2ml DMEM containing 5% serum was added and incubation continued for a further 20–48 hours before harvesting the cells for analysis.

2.6.2 DEAE dextran mediated transfection.

For transfection of the products of *in vitro* RNP-reconstitution reactions the DEAE dextran transfection method, as used for transfection of influenza virus synthetic RNP (Luytjes *et al* 1989), was used. 35mm dishes containing semi-confluent cell monolayers (~$5x10^5$ cells) were washed with PBS and treated for 30 minutes at room temperature with 1ml of a solution containing 300µg/ml DEAE dextran, 0.5% DMSO and 0.1mg/ml gelatin in PBS. Reconstituted RNP

was diluted to 200µl in PBS containing 0.1mg/ml gelatin and 80 units RNasin (promega). This mixture was added to the cell monolayer after removal of the dextran/DMSO solution. Following incubation at 37°C for 1 hour, the solution was removed and DMEM containing 5% serum added. Incubation was continued at 37°C for 20–48 hours until the cells were harvested.

2.6.3 Calcium phosphate mediated transfection.

Transfection of *in vitro* reconstituted RNP was also attempted by the calcium phosphate method used by Jin and Elliott (1991) for transfection of Bunyamwera virus nucleocapsids. Almost confluent cell monolayers (~10^6 cells per 35mm dish) were washed with TBS^+ (25mM Tris-HCl pH 7.4, 135mM NaCl, 50mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$). *In vitro* RNP reconstitution reactions (up to 50µl) were diluted to 250µl in transfection solution containing 140mM NaCl, 5mM KCl, 0.75mM $Na_2HPO_4 \cdot 2H_20$, 6mM dextrose, 25mM HEPES buffer pH 7.05, and 125mM $CaCl_2$. This mixture was applied to the cell monolayers and incubated at room temperature for 30 minutes. 2ml of DMEM containing 5% foetal calf serum was then added and incubation continued a further 4 hours at 37°C. The supernatant was then removed and the cells were shocked with 10% glycerol (in TBS^+) for 1 minute. After two washes with TBS^+, 2ml of DMEM containing 5% serum was added and the cells incubated at 37°C until they were harvested.

2.7 RNA manipulations.

2.7.1 T7 RNA polymerase *in vitro* transcription.

For synthesis of µg quantities of RNA for RNP reconstitution experiments ~1µg of linearised plasmid DNA was mixed with the following components in a final volume of 50µl: 1mM each ATP, GTP, CTP and UTP, 20 units placental ribonuclease inhibitor (RNasin), 50 units of T7 RNA polymerase (Promega) and a buffer containing 10mM DTT, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl and 40mM Tris-HCl pH 7.5. Trace quantities of $\alpha^{32}P$ CTP, $\alpha^{32}P$ UTP or ^{35}S (αS) CTP (~0.5µCi) were included to enable quantification of RNA (by % NTP

incorporation calculated according to the ratio of TCA precipitatable/total counts) and for analysis of reconstituted RNP by CsCl gradient fractionation (see section 2.5.4). The transcription reaction was incubated for 1–2 hours at 37°C before 1 unit of DNaseI (Promega) was added and incubation continued for a further 15 minutes to degrade the DNA template. The reaction was phenol extracted and chloroform extracted once to remove enzymes. 0.5 volumes of 7.5M ammonium acetate was added to the supernatant followed by 2.5 volumes of ethanol. The RNA was precipitated on dry ice for 15 minutes and pelleted by centrifuging at 13,000 rpm in a microfuge for 10 minutes. The RNA pellet was rinsed with 70% ethanol, air dried and resuspended in water. *In vitro* transcribed RNA was used immediately or stored at -70°C.

2.7.2. Extraction of total cellular RNA.

RNA was extracted from virus infected or uninfected cell monolayers by the method described by Chomczynski and Sacchi (1987). Cell monolayers in 35mm dishes were washed with PBS and lysed in 0.5ml GSCN solution (4.2M guanidinium thiocyanate, 0.5% sarcosine, 100mM β-mercaptoethanol, 0.33% Antifoam A emulsion, 50mM Tris-HCl pH 7.5 and 2mM EDTA). The lysate was transferred to a microfuge tube and 50µl of 2M NaOAc pH 4.0, 500µl of water-saturated phenol, and 100µl 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 1hour followed by centrifugation for 10 minutes at room temperature. The RNA pellet was resuspended in 300µl of GSCN solution, mixed with 300µl isopropanol and reprecipitated. After centrifugation for 10 minutes, the pellet was dissolved in 200µl of water by heating for 5 minutes at 60°C and extracted with an equal volume of a 4:1 mixture of chloroform:butan-2-ol. 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 with 70% ethanol, briefly vacuum dried and dissolved in water before use or storage at -70°C.

2.7.3. Electrophoresis of RNA

Two methods were used for denaturing electrophoresis of RNA through agarose. The use of methylmercury hydroxide as a denaturant was initially used but was later superseded by the glyoxal/DMSO method principally to avoid the handling of the volatile and highly toxic mercury compound.

2.7.3.1 Methylmercury hydroxide method.

RNA samples were separated on an agarose gel containing 5mM methylmercuric hydroxide, as described by Perbal (1988). Electrophoresis was performed on a 1% or 1.2% agarose horizontal slab gel (14 x 11 x 0.5 cm) containing 1 x EM buffer (5mM sodium borate, 10mM sodium sulphate, 1mM EDTA, 15mM boric acid) and 5mM methylmercuric hydroxide as denaturant. Agarose was melted in 1 x EM buffer and cooled to 60°C prior to the addition of methylmercuric hydroxide. RNA samples were mixed with an equal volume of 2x sample buffer (20% glycerol, 0.05% bromophenol blue, 2 x EM buffer) and loaded onto the gel. Electrophoresis was carried out in 1 x EM buffer at 75 volts for 4 hours until the bromophenol blue had migrated to the bottom of the gel. All manipulations involving methylmercuric hydroxide were performed under a fume hood.

2.7.3.2 Glyoxal method.

RNA was denatured in glyoxal/DMSO according to Sambrook *et al* (1989). RNA was added to a mixture containing 5.4µl deionised glyoxal, 16µl DMSO and 0.1M sodium phosphate pH7 in a total volume of 30µl and heated at 50°C for 1 hour. Agarose gels (1–1.4%) were prepared in 10mM sodium phosphate pH7 which was also used as running buffer. 0.1 volumes of loading buffer (50% glycerol, 10mM sodium phosphate pH7, 0.25% bromophenol blue and 0.25% xylene cyanol) was added to the heated RNA/glyoxal/DMSO mixture before loading the samples to the gel. Electrophoresis was at 3–4 volts/cm with the buffer being circulated between the two buffer tanks to maintain the pH within acceptable limits and so prevent the glyoxal dissociating from the RNA. When the bromophenol blue dye front had migrated ~4/5 the length of the gel, electrophoresis was halted and the gel removed for Northern transfer.

2.7.4 RNA purification by silica matrix adsorption.

RNA fragments were located in agarose gels by ethidium bromide staining and retrieved from excised gel blocks using a commercial Kit RNaid™ (BIO 101 Inc, La Jolla, CA). RNaid™ works on the same principle, binding nucleic acid to silica particles in the presence of high salt concentrations, as the Geneclean and Qiaex kits used for DNA purification (section 2.2.5). Agarose blocks containing the required RNA fragments were solubilised in concentrated RNase-free $NaClO_4$ solution ('RNA binding salt') before adding 5µl of the binding matrix and allowing adsorption for 5 minutes on ice. Following three washes in RNase-free ethanolic RNA wash (resuspension/centrifugation cycles), RNA was eluted into 30µl distilled water.
RNaid™ was also used to purify RNA from solutions containing enzymes or radiolabel.

2.7.5. Northern transfer of RNA.

RNA samples separared by denaturing agarose gel electrophoresis were transferred to a charged nylon membrane (Hybond N, Amersham Plc.) by a standard method (Sambrook *et al.* 1989). Two strips of Whatman 3MM paper were wrapped around a glass plate which was put on a rack in a tray containing 20 x SSC. The gel was placed upside down on the Whatman 3MM paper and covered with a sheet of nylon membrane which had been prewetted with 20 x SSC. The nylon membrane was then covered by a stack of blotting pads (BRL) about 10 cm high and a weight (~0.5Kg). Transfer was performed overnight. The nylon membrane was then air-dried and the transferred RNA cross-linked to it by ultraviolet radiation (150 mJ/cm^2) in a Stratalinker™ uv cross-linking apparatus, prior to hybridisation.

2.7.6 Radiolabelling of nucleic acid for use as hybridisation probes

2.7.6.1. Nick translation labelling of DNA

Plasmid DNA was labelled *in vitro* with ^{32}P by nick translation as described by Maniatis *et al.* (1982). 0.2–0.5µg of plasmid DNA was added to a 50µl reaction,

containing NT buffer (100mM Tris-HCl pH 7.5, 10mM $MgSO_4$, 1mM DTT, 0.05mg/ml BSA), 10μCi of α-^{32}P-dATP or 10μCi of α-^{32}P-TTP, 20μM each of the four unlabelled dNTPs, 1 unit of *E. coli* DNA polymerase I and 10^{-3}μg/ml DNase I. This reaction was incubated for 1hour at room temperature. The radiolabelled plasmid DNA was separated from unincorporated label by gel filtration chromatography through a Sephadex G-50 column. The Sephadex G-50 beads were equilibrated in TE buffer and the column prepared in a 1ml plastic syringe with a siliconised glass wool plug. Orange G dye was added to the reaction as a small-solute mobility marker. The DNA was applied to the column and fed through with water. The eluted DNA was collected before the orange G, and therefore unincorporated radiolabel, had reached the bottom of the column. The probes were heated at 90-100°C for 2 minutes before hybridisation.

2.7.6.2 Riboprobes from *in vitro* transcription.

Recombinant plasmid DNA with cDNA insert under the control of a T7 promoter was linearised with an appropriate restriction enzyme and the DNA used as template for *in vitro* transcription by T7 RNA polymerase (Promega) as described by Nielsen and Shapiro (1986). 0.5–1μg of linearized DNA template was added to an *in vitro* transcription reaction containing 40mM Tris-HCl pH 8.0, 25mM NaCl, 8mM $MgCl_2$, 2mM spermidine.HCl, 5mM DTT, 0.5mM ATP, GTP, UTP, 1.2μM CTP, 50 μCi/ml α-^{32}P-CTP (400 Ci/mmol), 40 units of RNasin and 25 units of T7 RNA polymerase in a total volume of 20μl. The reaction was incubated for 1 hour at 37°C followed by the addition of 1 unit of RQ1 DNase I (Promega) to degrade the DNA template. After 15 minutes at 37°C the reaction mix was extracted with phenol / chloroform. The RNA was precipitated from the aqueous phase by adding 3 volumes of ethanol in the presence of 1M ammonium acetate and pelleted in a microcentrifuge.The labelled RNA pellet was dried briefly under vacuum before being dissolved in 50μl of water and either used immediately or stored at -70°C.

2.7.7. Hybridisation of radiolabelled probes to nucleic acids immobilized on nylon membranes.

When ^{32}P-labelled cDNA was used as a probe, the nylon membrane with transferred nucleic acid was prehybridised in a heat-sealed polythene bag containing 5 x SSPE, 0.5% non-fat milk, 0.2μg/ml salmon sperm DNA, and 50% formamide. Prehybridisation was continued for 2–4 hours at 42°C. The

prehybridisation solution was then replaced by hybridisation solution containing 5 x SSPE, 0.1% milk. 0.2% carrier DNA, and 50% formamide. ^{32}P labelled cDNA probes were denatured by heating for 5 minutes at 100°C, quick-chilled on ice and added to the hybridisation bag which was then sealed and submerged in a water bath at 42°C and incubated with agitation for 12–18 hours.

When ^{32}P labelled riboprobes were used, the prehybridisation solution was 6 x SSPE, 5 x Denhardt's reagent (0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin) (Maniatis *et al.* 1982), 0.2mg/ml carrier DNA, 0.1% SDS, 50% formamide. The hybridisation solution contained 6 x SSPE, 1 x Denhardt's solution, 0.1mg/ml carrier DNA, 0.1mg/ml yeast tRNA and 50% formamide. Hybridisation was performed at 42°C as described above.

After hybridisation, the membrane was removed from the bag and immediately submerged in a tray containing 200ml of 2 x SSC and 1% SDS. The filter was washed for 30 minutes at room temperature with constant agitation with one change of the wash solution. The filter was then further washed with 0.1% SSC and 1% SDS for 30 minutes at 42°C. The filter was then placed on a pad of paper towels to remove most of the liquid, wrapped with 'cling film' and exposed to X-ray film at -70°C with an intensifying screen.

2.8 Protein analysis.

2.8.1. Protein concentration determination.

The protein concentrations of RNP preparations and cell extracts to be used in CAT assays were determined using the method of Bradford (1976). Protein samples and solutions of BSA for use as standards were adjusted to 100µl volumes using distilled water (BSA standards contained between 0.5 and 15µg of protein). 900µl of Bradford reagent (100µg/ml Coomassie brilliant blue, 8.5% phosphoric acid, 50% ethanol, filtered and stored at room temperature) was added and mixed. After 5 minutes the absorbances at 595nm were determined in a spectrophotometer. 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 the unknown protein concentrations.

2.8.2. Metabolic labelling of intracellular proteins.

Monolayers of cells in 35 mm dishes were radiolabelled with ^{35}S-methionine in 1ml of PBS (30μCi/ml) for 2 hours at 37°C. The radioactive solution was then removed, the monolayers washed with PBS and the cells lysed in 200μl of protein dissociation mix (0.125M Tris-HCl pH 6.8, 4% SDS, 10% β–mercaptoethanol, 20% glycerol and 0.1% bromophenol blue). Cell lysates were heated at 100°C for 5 minutes before loading onto polyacrylamide gel containing SDS (see Section 2.8.4).

2.8.3. Immunoprecipitation of radiolabelled proteins.

Cell monolayers were radiolabelled with ^{35}S-methionine (30 μCi/ml) in 1 ml methionine free minimal essential medium for 2 to 16 hours. The cells were washed with PBS and 1ml of RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.02% SDS) was added to each dish to lyse the cells. The dishes were incubated on ice for 30 minutes and the solution transferred to microcentrifuge tubes. Nuclei and cellular debris were removed by centrifugation for 5 minutes at 13,000 rpm and the lysate precleared by incubation with 10μl of preimmune serum for 30 minutes at 4°C followed by a second 30 minute incubation at 4°C after adding 50μl of a 10% suspension of formalin fixed *Staphylococcus aureus cells*. The *S. aureus* cells and bound antibody from the preimmune serum, were removed by centrifugation. The cleared supernatant was then reacted with 5μl of antiserum for 4–16 hours at 4°C on a rotating mixer. Immune complexes were collected by incubation with 50μl of *S. aureus* cells for 30 minutes at 4°C and pelleted by centrifugation for 30 seconds. The pellet was washed three times with 0.5M LiCl / 0.1M Tris-HCl pH 8, resuspended in 50μl of protein dissociation mix and boiled for 5 minutes prior to electrophoresis on SDS-polyacrylamide gels.

2.8.4 SDS polyacrylamide gel electrophoresis (SDS PAGE) of proteins

Denatured proteins were fractionated by electrophoresis through polyacrylamide gels containing SDS (Laemmli 1970). The resolving gel contained 10% or 12% polyacrylamide in which the acrylamide was crosslinked with N, N'-methylene bisacrylamide in a ratio of 75:1 (w/w) in resolving gel buffer (0.375M Tris-HCl pH 8.8, 0.1% SDS). Ammonium persulphate and TEMED were added to a final concentration of 0.1% (v/v) immediately prior to pouring the gel. The gel was overlaid with butan-2-ol to leave a smooth interface after polymerisation. The butanol was removed when the gel had polymerised and the interface rinsed once with unpolymerised stacking gel solution. The stacking gel (3.8% polyacrylamide, 62.5mM Tris-HCl pH 6.8, 0.1% SDS 0.1% ammonium persulfate and TEMED) was layered on top of the resolving gel and the required comb inserted to form wells. The stacking gel was allowed to polymerise for 1 hour before being assembled in the electrophoresis tank which was then filled with running buffer (53mM Tris, 53mM glycine, 0.1% SDS). Samples in protein dissociation mix were heated at 100°C prior to loading to the gel. Electrophoresis was carried out at 40 mA until the bromophenol blue had reached the bottom of the resolving gel.

The gel was removed from the glass plates after electrophoresis and either stained with 0.2% Coomassie brilliant blue for 30 minutes followed by destaining with several changes of gel fix (7% acetic acid, 20% methanol) or dried under vacuum and placed in contact with Kodak X-Omat S film for autoradiography (flourography was not used).

2.8.5 Chloramphenicol acetyl transferase (CAT) assays.

CAT assays were adapted from Gorman *et al* (1982). Cell monolayers were washed once with, and scraped into, PBS. Cells were pelleted by low speed centrifugation (500 rpm for 5 minutes) and resuspended in 75µl of 250mM Tris-HCl pH 7.5. Three freeze–thaw cycles were performed (dry ice/ethanol bath – 37°C water bath) to disrupt the cells. Nuclei were removed by centrifugation at

13,000 rpm for 1 minute in a microcentrifuge. The supernatant was then heated to 60°C for 10 minutes to inactivate any deacetylase enzymes present. Extracts were then stored at -20°C or assayed immediately.

The CAT assays were performed for between 2 and 20 hours at 37°C in a total volume of 75µl. The reaction contained up to 50µl cell extract (equivalent to approximately half a cell monolayer from a 35mm dish), 2µl of 50mM acetyl coenzyme A and 1µl of 14C chloramphenicol (0.1 µCi/µl; 58.5 mCi/mmole) in 250mM Tris-HCl pH 7.5.

After incubation the reactions were extracted with 200µl of ethyl acetate. Following centrifugation at 13,000 rpm for 1 minute in a microfuge, the upper organic phase was removed and dried under vacuum in a 'speedi-vac' centrifuge. The residue of chloramphenicol was redissolved in 20µl ethyl acetate and applied as a spot near the base of a thin layer chromatography plate. Ascending chromatography was performed using 95% chloroform/ 5% methanol until the solvent front had reached the top of the plate. The chromatographs were then removed from the tank, dried in a fume hood for 5 minutes and placed directly against Kodak X-omat S film for exposure (12 hours to 5 days).

For quantitation, the regions of the TLC plate containing acetylated chloramphenicol were located by autoradiography, cut out and scintillation counted. Based on the specific activity of the ^{14}C chloramphenicol used and the amount of protein in each assay (judged by Bradford assay) CAT activity was expressed as pico moles chloramphenicol acetylated per mg protein per minute.

Chapter 3: Attempted Rescue, of RNA Transcribed *in vivo*, by a Reassortant Virus.

3.1 Strategy.

The original strategy for rescuing plasmid derived RNA into a bunyavirus was to exploit the property of genome segment reassortment to replace a segment from a natural virus with a distinguishable segment derived from cDNA. The S segment was chosen since full length cDNA clones were available for Bunyamwera virus S segment (see Figure 3.1) and a reassortant virus, with an easily distinguishable N protein, was available which would be suitable as a helper virus to replicate and package the synthetic RNA. The reassortant virus (Bun/Bun/Mag) contained the L and M segments of Bunyamwera virus and the S segment of Maguari virus. Although the N proteins of Bunyamwera virus and Maguari virus contain the same number of amino acids, they have different electrophoretic mobilities when analysed by SDS PAGE. This difference, and the fact that N protein is abundant in infected cells compared to other viral products, would allow screening of genotype and make the S segment the ideal target for segment replacement. Another possible advantage was that using the Bun/Bun/Mag reassortant as a helper virus to rescue a synthetic Bunyamwera virus S segment would result in wild type Bunyamwera virus, which would not be expected to suffer any growth disadvantage compared with the helper virus.The plan involved transcribing the synthetic Bunyamwera virus RNA *in vivo* from transfected cDNA in the presence of the replicating helper reassortant virus. This was felt to increase the chances of the plasmid derived RNA being encapsidated and replicated since encapsidation is thought to occur co-transcriptionally during the normal life cycle of bunyaviruses. *In vivo* DNA-templated transcription was to be achieved by co-infecting the cells with the recombinant vaccinia virus vTF7-3 (Fuerst *et al* 1986), which expresses T7 RNA polymerase, which could transcribe from an appropriately positioned T7 promoter in the plasmid. The overall strategy is depicted in Figure 3.2.

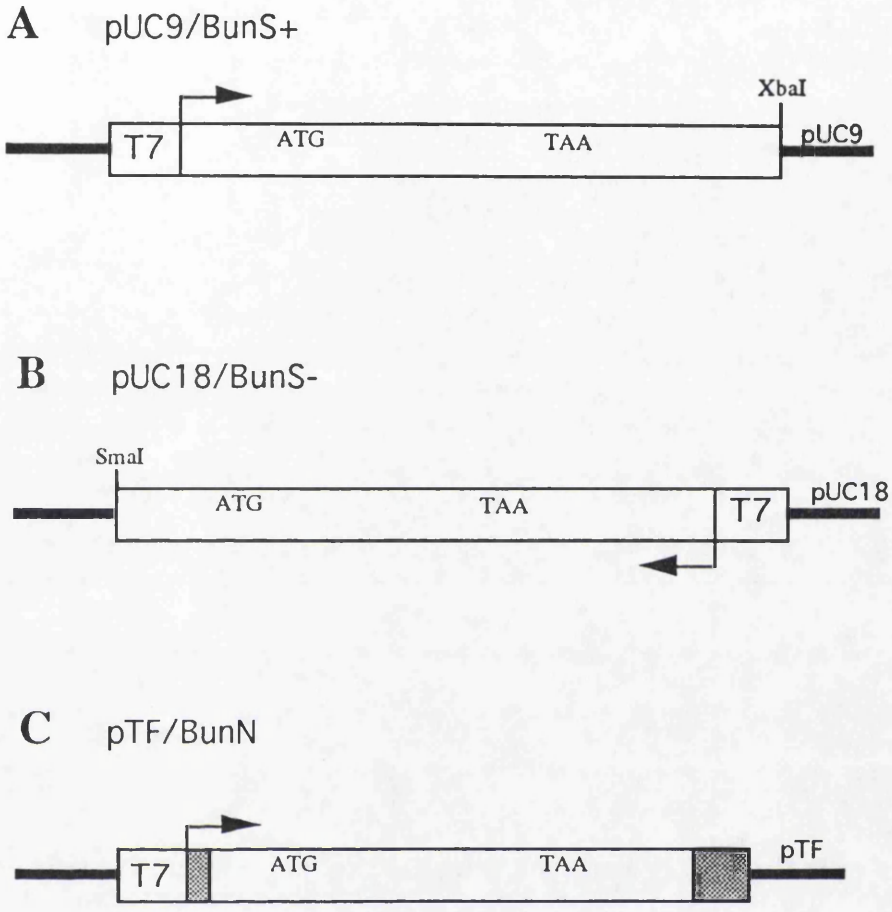


Figure 3.1 Bunyamwera virus S segment cDNA constructs. Vector DNA is shown as a thick black line, inserts as boxes. The start and stop codons of the N protein open reading frame are indicated. Transcription st art sites from the T7 promoters are indicated by arrows. pUC/BunS+ (A) contains the complete S segment cDNA. Xba I digestion followed by mungbean nuclease digestion of the resultant 5' overhang, results in a template for run-off transcription to produce a positive stranded RNA terminated at exactly the last viral base. The 5' end of the transcript is determined by T7 promoter position and for this plasmid adds one additional nucleotide (G) to the 5' end of the transcript. The inclusion of the extra G molecule was to improve transcription yield since T7 polymerase prefers to initiate on a G residue. pUC18/BunS- (B) contains the complete S segment cDNA. Transcription following SmaI digestion results in a negative stranded complete S RNA with Three additional C residues at its 3' end and the one additional G residue at the 5' end. pTF/BunN (C) is an expression construct containing the N protein open reading frame and a portion of 3' and 5' S segment untranslated regions flanked by bacteriophage T7 sequences (shaded boxes) including a transcription termination signal. All three constructs were designed and built and generously provided by A. McGregor of this Institute. The pTF/BunN plasmid is usually referred to as pTF/BunS, this term is avoided here to distinguish this construct from those that contain complete S segment sequences.

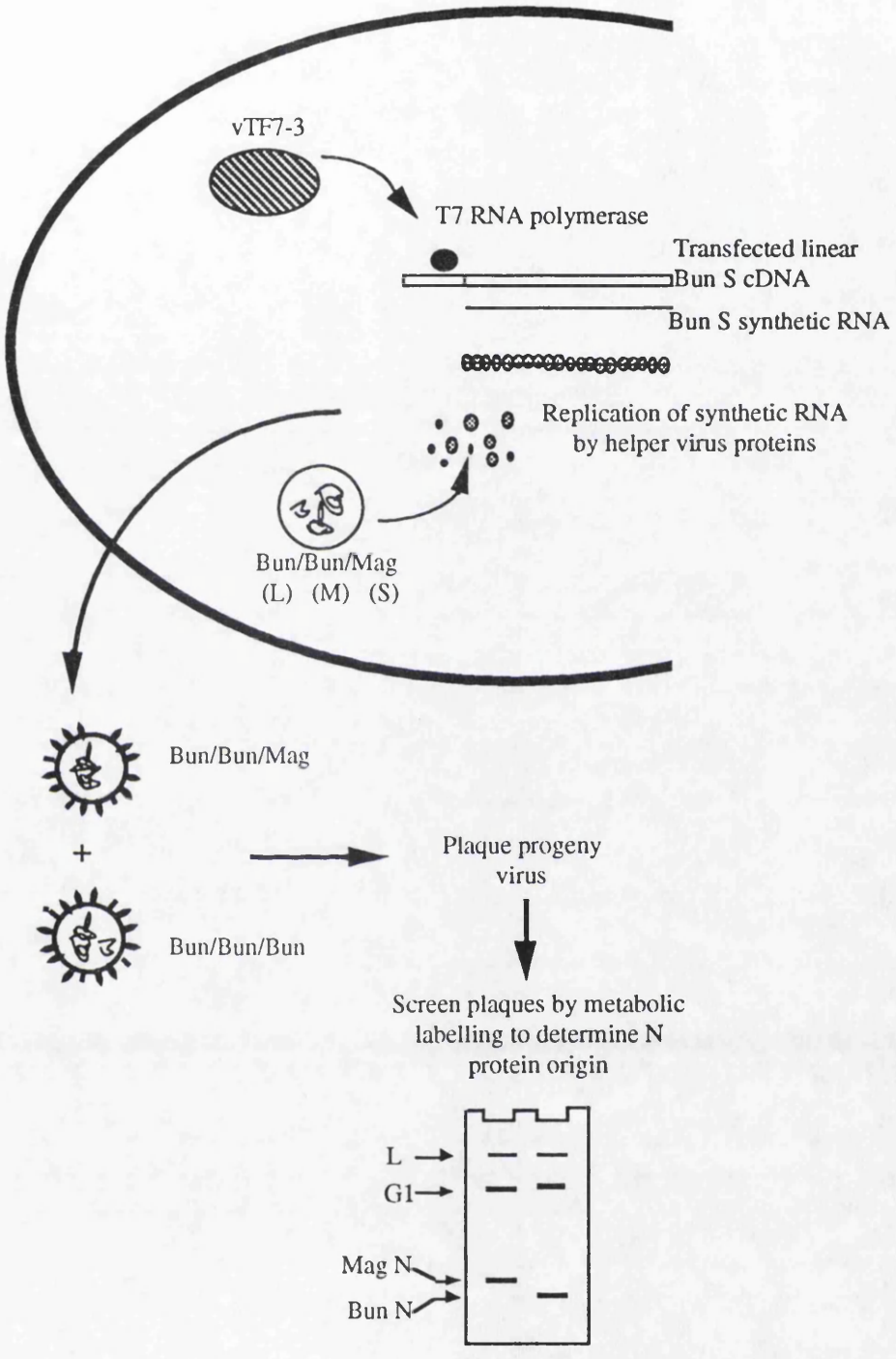


Figure 3.2 Strategy for attempted rescue of synthetic Bunyamwera virus S RNA transcribed *in vivo*. vTF7-3 is a recombinant vaccinia virus which expresses T7 RNA polymerase. The transfected linear cDNA produces Bun S RNA by T7 run-off transcription (see Figure 3.1). The replicating helper virus supplies proteins to encapsidate, replicate and package the synthetic RNA. Progeny viruses are screened to identify any carrying Bunyamwera virus N protein, which is distinguishable from the Maguari virus N protein encoded by the reassortant rescue virus.

3.2 Protein synthesis during dual infection.

Of key importance to the design of this rescue strategy is the ability of bunyaviruses to replicate in cells which are infected with vaccinia virus. This property was assessed by examining protein production in dually infected cells (see Figure 3.3). This method was also applicable to assessing whether any growth advantage was apparent between the parent or reassortant viruses.

Bunyamwera virus N and G1 proteins are clearly distinguished from their Maguari virus counterparts by their slightly faster electrophoretic mobility (Figure 3.3, lanes 11 and 10). Both were expressed to higher levels in the absence of co-infecting vaccinia virus vTF7-3 (compare lanes 9, 10, 11 with lanes 2, 3, 4 respectively). The difference seen for the the Bun/Bun/Mag reassortant is due to the slightly lower multiplicity (2–5 pfu/cell) used for infection compared with the parent viruses (10 pfu/cell).

Co-infection of cells with Bunyamwera and Maguari viruses resulted in the Maguari virus G1 and N proteins predominating over the Bunyamwera proteins (compare lane 8 to 10 and 11). Maguari virus G1 protein also predominated over Bunyamwera G1 protein coded for by the Bun/Bun/Mag reassortant virus (lane 6). The former result is unlikely to be due to errors in titration of the viruses since in single infections, using the same volume of stock viruses as used for the dual infections, Bunyamwera virus produces at least as strong a signal as Maguari virus (lanes 11 and 10). Advantage by Maguari virus over Bunyamwera virus during dual infection has been reported by Pringle (1991). Regardless of any growth advantage Maguari virus may have over Bunyamwera virus during dual infection, Bunyamwera virus N protein is efficiently produced when Bunyamwera virus is replicating in the presence of the Maguari virus S segment supplied by the Bun/Bun/Mag reassortant virus (lane 7). This observation is evidence that if encapsidated, the plasmid transcribed Bunyamwera virus S segment would not be dissadvantaged in the reassortant virus infected cell.

The co-infection experiments were carried out at a multiplicity of infection of 10 pfu/cell for each virus to increase the proportion of cells co-infected. Although it is formally possible that in individual cells one or other of the viruses predominates, the ability to isolate reassortant viruses following dual infection with Bunyamwera and Maguari viruses demonstrates that both viruses can multiply in the same cell. Therefore this experiment is felt to genuinely reflect the expression of co-replicating viruses.

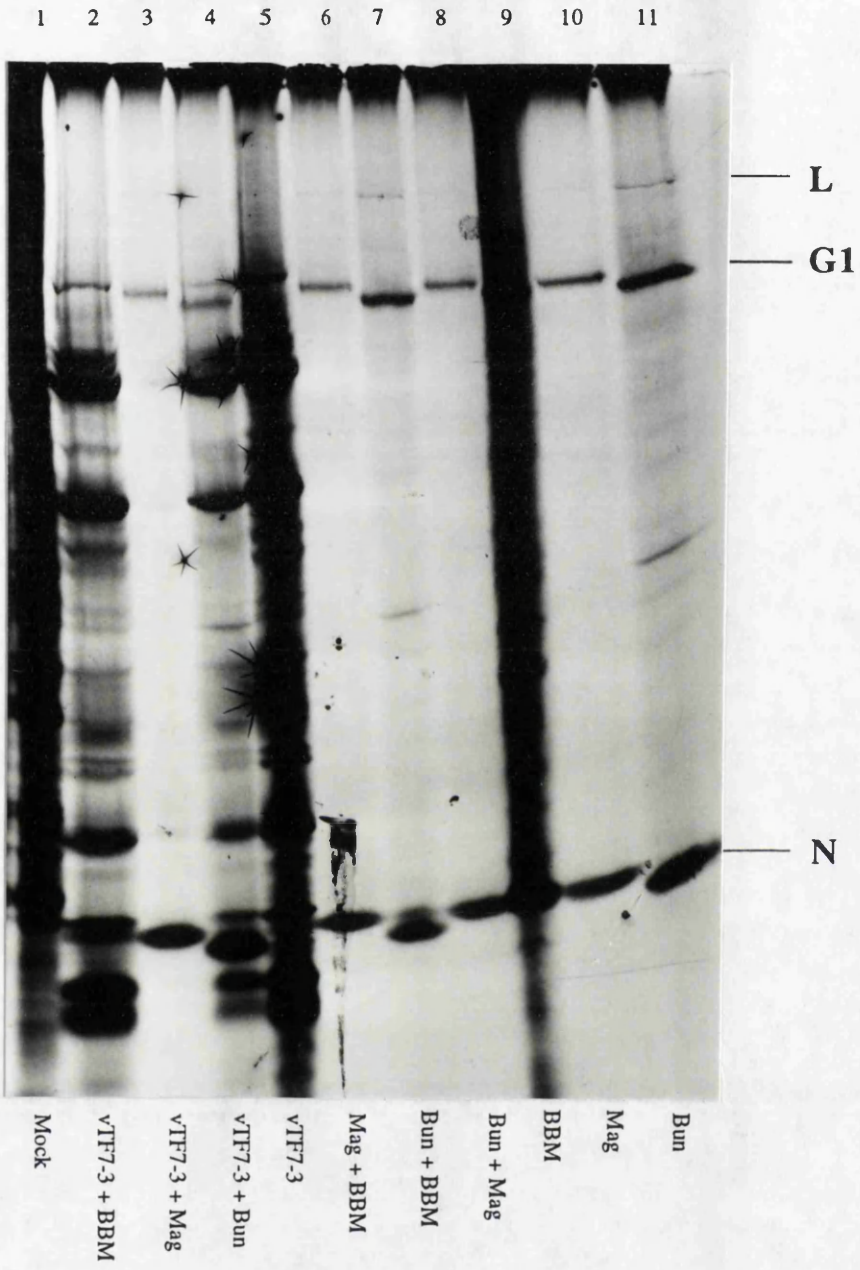


Figure 3.3 Protein profiles of bunyavirus and vaccinia virus infected cells. BHK cell monolayers were infected at 10pfu/cell for each virus, except for the Bun/Bun/Mag reassortant virus which was used at 2–5 pfu/cell. 24hrs post-infection cells were labelled with 15μCi ^{35}S methionine for 2hrs before being washed and harvested. Cell lysates were denatured by heating in SDS/β–mercaptoethanol before being analysed by SDS PAGE. Gels were dried and autoradiographed overnight. **Lane 1**: Mock infected. **Lane 2**:Vaccinia virus vTF7-3 coinfected with the Bun/Bun/Mag reassortant virus (BBM). **Lane 3**: vTF7-3 plus Maguari virus. **Lane 4**: vTF7-3 plus Bunyamwera virus. **Lane 5**: vTF7-3 alone. **Lane 6**: Maguari plus reassortant virus. **Lane 7**: Bunyamwera plus reassortant virus. **Lane 8**: Bunyamwera plus Maguari virus. **Lanes 9** Reassortant virus, **10** Maguari virus and **11** Bunyamwera virus alone. Bunyamwera virus N and G1 proteins migrate faster than the corresponding Maguari virus proteins.

Efficient shut-off of host cell protein synthesis by bunyavirus infection is observed (Figure 3.3 lanes 6–11 compared to lane 1)—the partial exception being for the Bun/Bun/Mag reassortant (lane 9) which is accountable by a lower multiplicity of infection being employed for this virus. Similar shut off is also observed for vaccinia virus proteins—several strong vaccinia virus specific proteins occur (compare lane 7 to lane 1) but signals for these are reduced to varying extents by co-infection with one or other of the bunyaviruses (lanes 2, 3, 4). T7 RNA polymerase production from the vaccinia virus to adequate levels in the presence of co-infecting bunyavirus is not addressed by this experiment.

This experiment appears to confirm that protein production by the bunyaviruses used is not significantly affected by co-infection with the recombinant vaccinia virus. Moreover, at least for the relevant Bun/Bun/Mag rescue virus, efficient expression of the N protein from Bunyamwera virus S segment, in the presence of the Maguari S segment, could be achieved. This experiment also demonstrates that the two N proteins can easily be distinguished and therefore screening by metabolic labelling would be able to discriminate between the rescue reassortant and any progeny virus carrying the plasmid derived Bunyamwera virus S segment.

3.3 Rescue attempt

BHK cell monolayers were infected with the Bun/Bun/Mag reassortant and the T7 polymerase expressing vTF7-3 recombinant vaccinia virus at 10 pfu/cell each and then transfected with linearised pUC9/BunS+ DNA. After 24 hours supernatant fluid was harvested and used to infect fresh monolayers. Plaques were allowed to develop for five days under overlays containing phosphonoacetic acid at 300 µg/ml. This reagent had been previously shown to inhibit plaque formation by vaccinia virus while allowing bunyaviruses to form plaques (Jin 1991). Plaques were visualised by staining with neutral red and were transferred individually to BHK monolayers in 24-well limbro plates. Conditions were determined under which N protein could be detected by metabolic labelling using ^{35}S methionine followed by SDS PAGE and autoradiography. Where host shut-off had not occurred sufficiently for identification of N protein, fresh infections were made using the supernatants from the first infection and rescreened. Gels included labelled extracts from cells infected with either Bunyamwera virus or Maguari virus to provide appropriate N protein markers. Of 200 plaques screened, all displayed N protein mobilities consistent with those expected for Maguari virus and were therefore judged to be formed by the amplified input Bun/Bun/Mag reassortant virus. It was decided to further characterise the expression system before continuing.

3.4 RNA analysis

RNA accumulation in infected and transfected cells was examined by Northern analysis, using nick-translated Bunyamwera S segment cDNA as a probe (Figure 3.4). The RNA extraction method employed did not exclude carry over of plasmid DNA. The probe detects S RNA of both Bunyamwera and Maguari virus and the mobilities of each are indistinguishable in this gel system (Lanes 6 & 4).

For both Bunyamwera and Maguari viruses, co-infection with the recombinant vaccinia virus appeared to slightly reduce accumulation of S segment RNA (compare lane 6 with 5 and lane 4 with 3 for Bunyamwera and Maguari viruses respectively). The reduction in signal is thought to be due to different harvesting efficiencies—the cell sheets infected with vaccinia virus detached more readily than those without and so more losses occured during washing.

Transfection of cells with linearised or circular plasmids containing the Bunyamwera virus S segment cDNA resulted in detection of a major, slow migrating band of similar mobility in both cases (lanes 8 &10). These signals most likely represent DNA not RNA since no vTF7-3, and therefore no T7 RNA-polymerase was present. The occurence of bands of similar mobility was not as expected since under the denaturing conditions employed, linear DNA would migrate as two (possibly co-migrating) single stranded molecules while circular DNA would be in the form of two covalently inter-linked single stranded circular molecules. Nicks in the circular plasmid DNA may account for the similar mobility. The marked difference in signal strength for linear versus circular DNA (lanes 8–10) could reflect differential transfection or harvesting efficiencies or differences in degradation rates for the two species.

When linearised DNA was transfected in the presence of recombinant vaccinia virus vTF7-3, no convincing S segment-sized RNA was detected (lane 7) (a slight shadow was present around the position for S segment sized RNA on the original autoradiograph but a stronger equivalent signal is also present in lane 9 where circular plasmid was transfected).

This experiment therfore raised concern about the transcription system employed but did confirm the expectation from the protein labelling study of co-infected cells that the vaccinia vTF7-3 recombinant does not significantly interfere with RNA production from at least the S segments of the bunyaviruses tested.

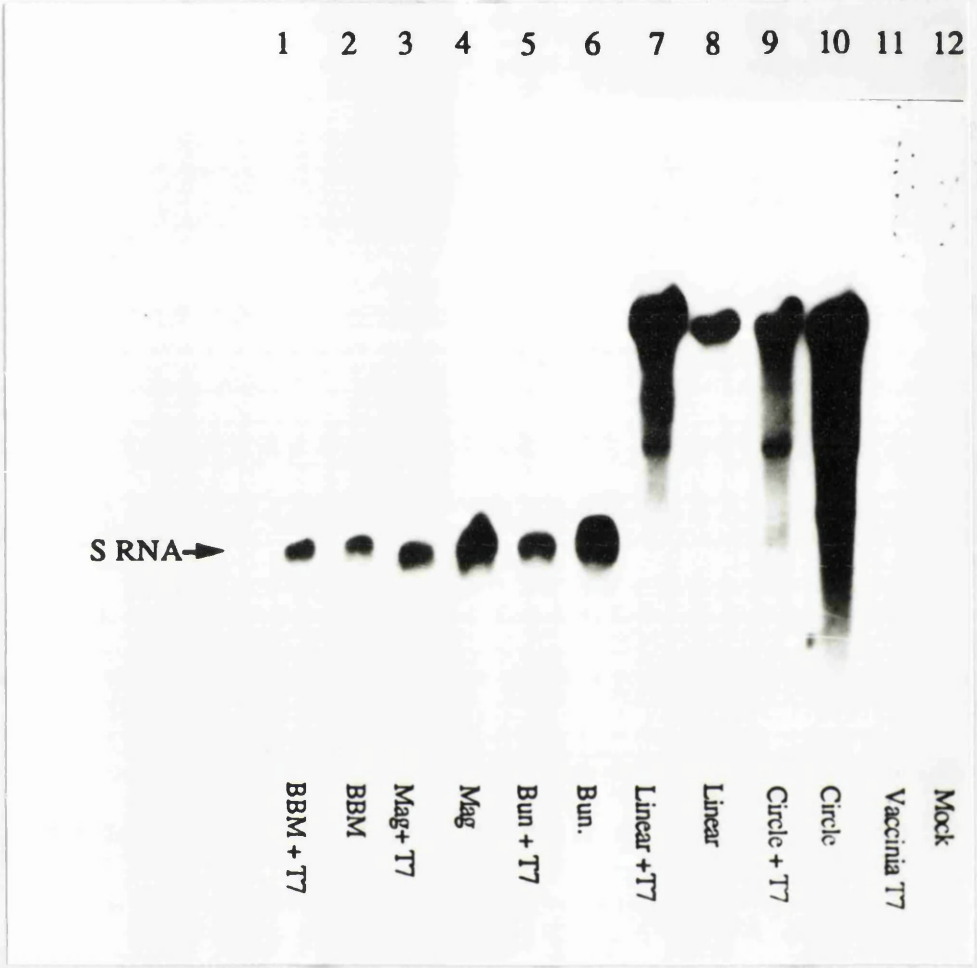


Figure 3.4 RNA profiles of infected and transfected cells. Infections were performed at 10pfu/cell. For transfections, 5µg of circular or XbaI/Mung bean nuclease digested pUC9/BunS+ DNA was lipofected into BHK monolayers 1hr after virus infection. RNA was extracted from washed monolayers 24 hours later by the acid-guanidinium method without DNAse treatment. RNA was electrophoresed through 1% agarose gels containing methylmercury hydroxide as denaturant, transfered to nylon membranes and probed using ^{32}P-dNTP labelled nick translated Bunyamwera virus S segment cDNA. Single infections with Bunyamwera, Maguari or the Bun/Bun/Mag reassortant—lanes 6, 4 and 2 respectively. The same bunyaviruses in dual infection with vaccinia virus vTF7-3 (T7)—lanes 5, 3 and 1 respectively. Note the reduction of S RNA signal when vTF7-3 is present, most noted for Bunyamwera and Maguari viruses (Lanes 3 through to 6). Transfection of linear (lanes 7 and 8) or circular (lanes 9 and 10) pUC9/BunS+ in the presence (7 and 9) or absence (8 and 10) vTF7-3. The low mobility band and smear for lanes 7 through to 10, is carry over DNA as it occurs independently of vTF7-3.

3.5 Protein production from transfected plasmids.

The inability to detect S segment RNA transcribed from transfected plasmids could have been due to a number of causes including correct transcription simply not occuring (e.g. promoter or run-off restriction site incorrectly placed, vaccinia virus vTF7-3 not functioning properly) or to degradation of the RNA. While most of the former possibilities were excluded by *in vitro* analysis of the construct, further investigation was performed to help clarify the situation. Metabolic labelling was performed to see if N protein could be detected from transfected plasmids. This would demonstrate that RNA was transcribed in the system and that some other property of the system was responsible for the inability to detect RNA.

As controls, lysates from mock, Bunyamwera virus, Maguari virus and vaccinia virus infected cells were immune-precipitated using anti-Bunyamwera virus antiserum (Figure 3.5, lanes 1 to 4). Bands of the expected different mobilities for Bunyamwera and Maguari viruses N proteins were detected. No equivalent signals were observed in the vaccinia virus- or mock-infected cell lysates.

In a separate experiment protein expression from transfected cDNA was assessed. Maguari virus infection was used as a control for N protein detection to avoid possible cross contamination to the transfected cells as Maguari virus N protein is of different mobility to Bunyamwera virus N protein. In addition to the pUC9/BunS+ construct which produces a full-length S segment of positive polarity, a second expression construct was included. pTF/BunN contains an incomplete S segment under T7 promoter control followed by a downstream T7 RNA polymerase terminator sequence (see Figure 3.1). This construct, when transfected into cells infected with vTF7-3 to supply T7 RNA polymerase, produces RNA easily detectable by Northern blot and Bunyamwera virus N protein (A. McGregor & R.M. Elliott, personal communication). The ability to produce N protein from these two plasmids was assessed (Figure 3.5 lanes 5 to 8).

Transfection of pUC9/BunS+ construct, which contains the complete Bun S segment, gave similar levels of N protein to those observed for pTF/BunN transfection and infection with the Bun/Bun/Mag reassortant virus. This experiment demonstrates that transcription from pUC9/BunS+ must occur even though no unique RNA species could be detected by Northern analysis.

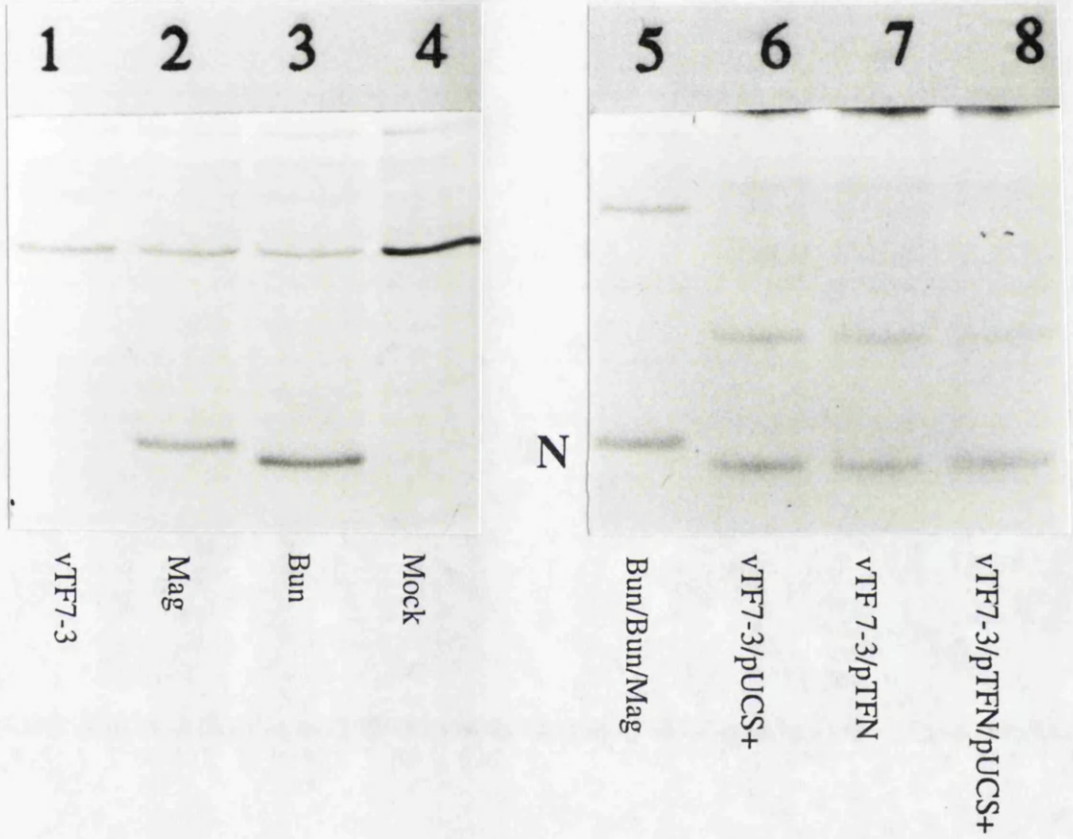


Figure 3.5 N protein production from bunyaviruses and transfected cDNAs. Infections, transfections and labelling were as described for Figure 3.4. Cell lysates were reacted with anti-Bunyamwera virus antiserum to immune-precipitate Bunyamwera and Maguari virus N proteins. SDS PAGE followed by autoradiography of dried gels revealed signals for N protein. Virus and mock infected cells demonstrate the specificity of the IP procedure (lanes 1 to 4). No N protein signal is seen in lysates from vTF7-3 infected or mock infected cells (lanes 1 and 4 respectively). Maguari and Bunyamwera viruses produce N protein of distinguishable mobility (lanes 2 and 3 respectively). Lane 5: marker for N protein (Maguari virus N). Lanes 6 to 8, transfections of cDNA into vTF7-3 infected cells: Lane 6— pUC9/BunS+; lane 7— pTF/BunN; lane 8— cotransfection of both plasmids. (See Figure 3.1 for plasmid maps).

3.6 Transcription of S RNA in the presence of N protein.

Because N protein could be detected in cells transfected with cDNA clones, degradation of the RNA immediately following translation may have been the cause of the inability to detect RNA. A further experiment attempted to protect newly transcribed RNA by transcribing it in the presence of an intracellular pool of N protein. pTF/BunN has already been shown to produce levels of N protein similar to those found in virus infected cells (Figure 3.5) and the mRNA produced would not be expected to bind N protein since it lacks S segment terminal sequences and is flanked by heterologous RNA. This construct was used to provide an intracellular pool of N protein in which a Bunyamwera virus S segment could be transcribed from cDNA and possibly encapsidated. To facilitate specific detection of the S segment RNA without interference from the N protein mRNA from pTF/BunN, a second S segment cDNA construct, pUC18/BunS- (see Figure 3.1), was used in which the T7 promoter is oriented such that negative stranded S segments would be produced. This would allow strand specific riboprobes to discriminate between the two species.

When probed to detect positive stranded S RNA (Figure 3.6, panel A) a strong signal was detected in Bun/Bun/Mag infected cell RNA (lane 2). For pTF/BunN and pUC18/BunS- dually transfected cells, a faint doublet band could be detected in the absence of vTF7-3 (lane 3). The origin of the polymerase responsible for transcription here is unknown but it may recognise promoter and termination signals at or very close to those for T7 RNA polymerase as judged by the similar mobilities to T7 transcripts from pTF/BunN (lane 4). This doublet was many times stronger when the transfected cells were also infected with vTF7-3 to supply T7 RNA polymerase. The occurence of a doublet signal from this construct is reproducible (A. MacGregor personal communication) although the origin of the two species is unknown. The strength of the signal diminished again when the full length cDNA in pUC18/BunS- was co-transfected (lane 5). Why co-transfection should diminish this signal is not clear—competition for T7 RNA polymerase would be expected to be greatest from the pTF construct which has more efficient transcription initiation nucleotides (5'-GGG...) for T7 transcription than the full length cDNA (5'-GAGU...). Any hybrids formed between the mRNA and full length genome RNA would not be expected to interfere with probe hybridisation on the blot since the gel was run under strong denaturing conditions.

When probed to detect negative strands (Figure 3.6, panel B), apart from a weak aberrant signal in the mock infected track (lane 1) and the strong signal expected from virus infected cells (lane 2), no negative stranded S segment RNA could be detected either in the presence or absence of co-transfected pTF/BunN plasmid as a source of N protein (Lanes 5 & 6).

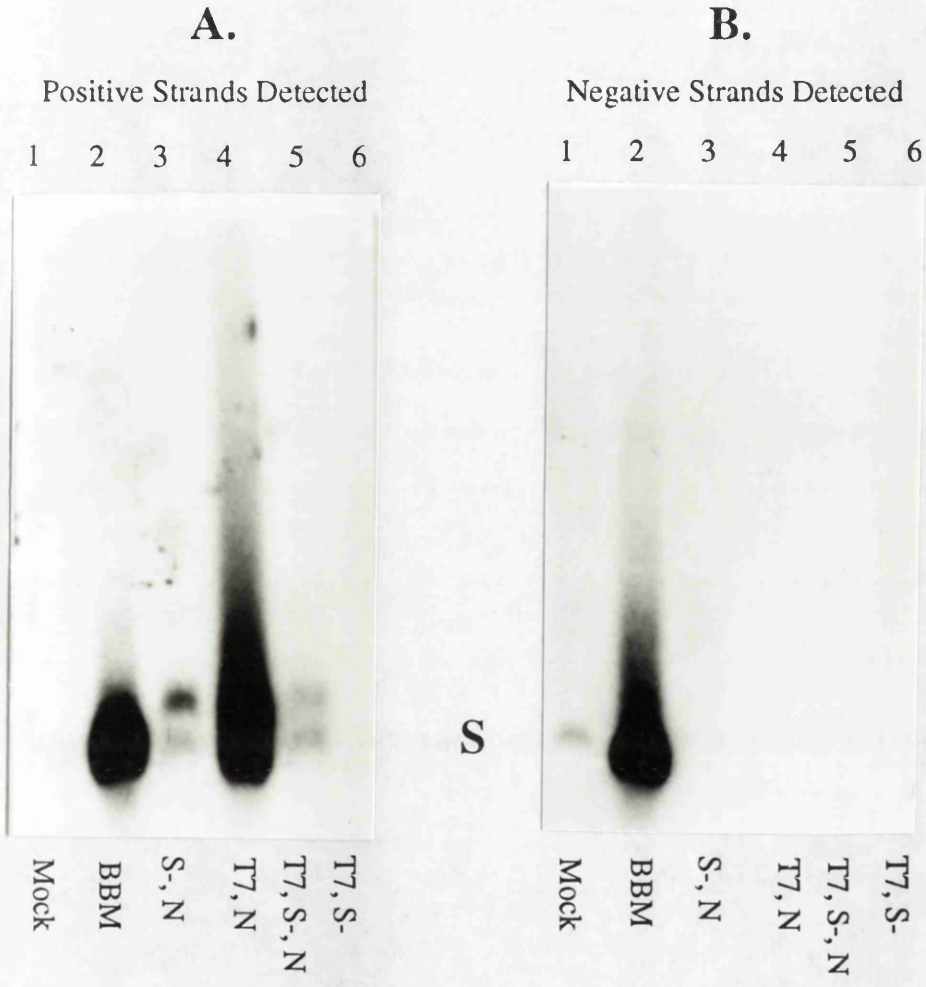


Figure 3.6 Attempted *in vivo* transcription in the presence of N protein. Transfections and infections were carried out at 5µg and 10pfu per cell respectively. Electrophoresis and blotting was as described for Figure 3.4 except filters were probed with strand specific ^{32}P-labelled riboprobes to specifically detect positive (panel A) or negative (panel B) S segment RNA. For both panels, lane assignment is as follows. **Lane 1**: Mock infected; **Lane 2**: Infected with Bun/Bun/Mag reassortant virus.(BBM) **Lane 3**: Transfected with pUC18/BunS- (S-) and pTF/BunN (N) without vTF7-3 infection. **Lane 4**: Transfected with pTF/BunN and infected with vTF7-3 (T7). **Lane 5**: Transfected with both pUC18/BunS- and pTF/BunN and infected with vTF7-3. **Lane 6**: Transfected with pUC18/BunS- and infected with vTF7-3.

3.7 Why no RNA?

The inability to detect S segment RNA even though its gene product N protein could be detected suggested either rapid degradation of the RNA or the existence of heterogeneous length transcripts which were sufficiently variable to be undetectable by Northern analysis. A possible explanation for the observation, which might indeed produce a wide range of transcript sizes, is that the potent ligase activity encoded by vaccinia virus could recircularise the transfected plasmids. In the absence of any T7 terminator sequence, the resultant loss of the run-off end of the transfected DNA would cause transcription to continue until the polymerase fell off the template. If termination was random, a whole range of RNAs of very variable length would be produced which would be too diffuse to detect on Northern blots but which could still be translated to give N protein. The observation of similar mobilities for plasmid DNA isolated from cells transfected with either linear or circular plasmids is consistent with this hypothesis. However, the vaccinia virus alone could not be implicated since the mobilities were still similar when the cells had not been infected with vaccinia virus. (lanes 7–10 Figure 3.4). In more recent work (Pattnaik *et al* 1992) the difficulties of making run-off transcripts *in vivo* using this sytem were commented on and overcome by incorporating ribozyme sequences to tailor a larger transcript to the required length.This approach may be applicable to the present problem and this is discussed further in Chapter 7.

To help discourage recircularisation of transfected linearised plasmids treatment with alkaline phosphatase could be performed prior to transfection. Even when this precaution was taken, no S segment sized transcript could be detected (data not shown). Other workers (D. Kolakofsky personal communication) have found recircularisation of linearised plasmids transfected into vaccinia virus infected cells, even when the ends of the linear molecule were cut with different restriction enzymes such that opposing single stranded extensions were generated (i.e. a 5' single strand extension at one end and a 3' single strand extension at the other). In principle such treatment should make it exceedingly difficult for the two ends to be arranged in a position suitable for ligation. Other effects may occur such as degradation of single strand extensions, which might allow ligation. For blunt-ended, dephosphorylated termini, phosphorylation would be required for ligation either as a direct enzyme activity or by cleavage of terminal bases to expose a phosphate.

Clearly production of RNA from templates which were circular would not produce transcripts which mimicked natural S segments and so the rescue of such transcripts into virus would be most unlikely.

With the system as it stands, if any plasmid derived RNA transcripts are packaged into virus, the frequency at which this occurs (<1 per 200 progeny virus) is sufficiently low to make screening of individual isolates by metabolic labelling impractical. Screening pooled samples by PCR was considered as was screening plaques by hybridisation to filter lifts. Preliminary studies indicated that both these approaches would prove difficult due to the extremely similar nucleotide sequences of Bunyamwera and Maguari viruses which made production of discriminatory oligonucleotides very difficult. Efforts were instead directed towards modifying the strategy to allow counter-selection of a rescue virus and so aid detection of any, rare, viruses carrying the S segment derived from cDNA. The most obvious counter-selectable helper virus would be one carrying a temperature sensitive lesion in its S segment. Since unambigous assignment of lesions to the S segment had not yet been made, this approach required some preliminary characterisation of available *ts* mutants. Such work and rescue attempts using a likely S segment *ts* mutant are discussed in the next chapter.

3.8 Conclusions.

Protein and RNA profiles of co-infected cells suggested that the vaccinia virus vTF7-3 slightly reduced bunyavirus expression levels although the reductions were not thought to be significant. Maguari virus appeared to have a slight growth advantage compared with Bunyamwera virus as judged by N protein synthesis in co-infected cells. However, similar experiments using Bunyamwera virus and the Bun/Bun/Mag reassortant virus suggested that the Bunyamwera virus S segment would not be dissadvantaged in the presence of co-expressed Maguari virus S segment supplied by the reassortant virus. These results suggested the general strategy of rescuing a Bunyamwera virus S segment RNA, transcribed *in vivo* by vTF7-3, using the Bun/Bun/Mag reassortant virus, was feasible with regard to interactions of the component viruses.

It was not possible to detect RNA transcribed *in vivo* from transfected linearised plasmids using recombinant vaccinia virus vTF7-3 as a source of T7 RNA polymerase. Transcription did occur since N protein could be detected in transfected cells. Attempts to stabilise transcripts by co-expressing N protein still did not allow detection of a unique RNA species. Signals present on Northern blots representing transfected DNA, suggested that the transfected linearised plasmids

were religated to form a circular template which would not be expected to yield a discrete transcript but which would be expected to be translatable.

In an attempt to rescue plasmid derived Bunyamwera virus S RNA into a Bun/Bun/Mag reassortant virus, 200 plaqued progeny virus were screened by metabolic labelling to determine the origin of their N protein. No Bun/Bun/Bun virus was recovered indicating that if rescue occured, it did so at a frequency of <1/200. This makes screening of individual plaques impractical and preliminary attempts to detect packaged S segment in pooled virus progeny, using oligonucleotides as PCR primers or probes, suggested the high degree of conservation between Bunyamwera and Maguari virus S segments would prevent such an approach. An alternative, selectable rescue virus was felt more likely to allow detection of rescued RNA if it occured using the present system. The use of a temperature sensitive rescue virus is discussed in Chapter 4.

Chapter 4: Temperature Sensitive Rescue Virus

As discussed in Section 1.9.8.2, bunyavirus *ts* mutants can be assigned to reassortment groups based on their ability to reassort only with members of a different group, producing viruses with a non-*ts* phenotype. Three such reassortment groups would be expected for bunyaviruses, representing lesions in each of the three gene segments. Contrary to this expectation, almost all mutants can be placed into two reassortment groups with only one isolate, Maguari virus *ts*23, definitely belonging to group III (see Table 1.9.2). Since *ts* mutants are available for closely related bunyaviruses, heterologous crosses could be used to assign each group to a segment in which the lesion is present for that group. This would involve selecting non-*ts* progeny from heterologous crosses between for example, a Bunyamwera virus *ts* mutant and a Maguari virus *ts* mutant and determining the parental origin of each of the non *ts* gene segments by RNA or protein profiles. Alternatively, the phenotype of non-selected reassortants from heterologous crosses between wild-type and *ts* viruses can be compared to their genotype and *ts* status. Such studies have clearly demonstrated that the Maguari virus group II *ts* mutants contain lesions in the M segment (Murphy & Pringle 1989). The segment assignment for group I Maguari virus mutants (the other well represented group) could not be unequivocally determined. Most available evidence pointed to the group I mutants having lesions in the S segment. For example when group I Maguari mutants were crossed with Bunyamwera virus, reassortants displaying a *ts* phenotype were found to include a Mag/Bun/Mag genotype while a

non-*ts* Mag/Bun/Bun virus was also isolated (Murphy and Pringle 1987). This finding is not as expected for the well represented group I since the relative proportion of mutants isolated for each of a given set of target sequences is expected to reflect the relative sizes of those target sequences. This expectation is largely fulfilled for other virus systems (Pringle 1987; Pringle 1990). More convincing contrary evidence comes from the extremely thorough crosses performed with *ts* mutants of California group bunyaviruses (Gentsch *et al* 1979). The observed progeny reassortants from these crosses unambiguously suggest that the two common reassortant groups of California group viruses represent lesions in the L and M segments.

Since a *ts* S segment mutant would be an ideal helper virus to rescue a cDNA-derived S RNA, further characterisation of the Maguari virus *ts* mutants was undertaken to try to resolve the actual assignment for the group I and group III Maguari virus mutants. Identification of a potential S segment mutant would enable it to be used as a rescue virus in the same strategy discussed in Chapter 3 but with an additional step involving temperature selection to supress growth of the helper virus and so increase the chance of identifying rare reassortants carrying the synthetic S segment.

4.1 Progeny virus titre and plaque formation

Plaque purified virus stock for each of the *ts* mutants was used to infect BHK cell monolayers in large flasks and grown at 31°C or 38·5°C for five days before harvesting the viral supernatants. Titrations were performed on the resultant stocks by plaquing onto BHK cells. Incubation was at 31°C for five days before staining and counting plaques. The three *ts* mutants were all found to be restricted at 38·5°C compared to 31°C and all grew to lower titres at 31°C than similarly inoculated wild type virus (see Table 4.1).

The group I and II viruses gave slightly smaller plaques than wild type virus (2–3 mm) while the plaques of the group III mutant were barely visible after five days incubation (<0·5mm). Subsequent attempts to improve yields or plaque size by longer incubations failed and the low titres available for the mutant made useful studies very difficult.

Virus	Reassortment group	pfu/ml	
		31°C	38·5°C
Maguari virus (wt)	-	$1{\cdot}2 \times 10^6$	$1{\cdot}15 \times 10^6$
Maguari virus *ts*6	I	$3{\cdot}5 \times 10^5$	$<1 \times 10^2$
Maguari virus *ts*17	II	$2{\cdot}8 \times 10^5$	$<1 \times 10^2$
Maguari virus *ts*23	III	$2{\cdot}5 \times 10^3$	$<1 \times 10^2$

Table 4.1 Wild type and *ts* Maguari virus titrations. Stocks were grown for five days at either 31°C or 38·5°C before being titrated on BHK monolayers at 31°C. Cell sheets were fixed in glutaraldehyde and stained with Giemsa stain to visualise the plaques.

4.2 Protein and RNA synthesis by Maguari virus *ts* mutants

Members of each of the three Maguari virus *ts* reassortant groups were investigated to see if any differences in protein or RNA synthesis were apparent which might provide evidence as to which segment was mutant for each. Profiles were compared for each virus following growth at 31°C or 38·5°C.

Lysates from cells radiolabelled with ^{35}S methionine were reacted with anti Bunyamwera virus serum and analysed by SDS PAGE. Wild type Maguari virus infection gave a clear strong band for N protein at both temperatures (Figure 4.1; lanes 4 & 9). Other proteins are cellular, remaining from the low-stringency IP washes, as shown by their presence in uninfected cells (lanes 5 & 10). Slightly less labelled N protein was precipitated from virus cultured at 38·5°C (lane 9) than at 31°C (lane 4) although the difference is minimal.

The apparent partial restriction of N protein synthesis seen for all three *ts* mutants at the permissive temperature (compare lanes 1, 2, 3 with lane 4), is partly artefactual due to different multiplicities of infection used for the viruses. This was caused by difficulties in growing high titre stocks of the *ts* mutants (see Section 4.1). The group II mutant (lane 2) is probably genuinely partially restricted at the permissive temperature since it was infected at the same multiplicity as the group I mutant (lane 1). The multiplicity used for the group III mutant *ts*23 was only ~0·01 pfu/cell due

to the extremely low titres obtained for stocks grown at 31°C. This resulted in the N protein for this mutant being undetectable even at the permissive temperature.

For the group I mutant, complete shut-off of N protein synthesis is observed when grown at 38.5°C (compare lane 1 to lane 6). Temperature dependent shut-off is consistent with the group I mutant being *ts* in either the L or S segments since the products of both segments are required for amplified gene expression and the low multiplicities used would not allow detection of primary transcripts.

For the group II mutant, a small amount of N protein is still detected at the raised temperature (lane 7). Group II mutations are known to map to the M segment which encodes the glycoproteins. A lesion in the glycoproteins would not be expected to interfere with gene expression. The apparent restriction in N protein synthesis is probably therefore due to the mutant being unable to establish secondary infections in neighbouring cells due to the glycoprotein defect. At low multiplicity infection, this effectively reduces the number of cells infected and would account for the observed lower level of N protein. If increasing multiplicities were used the effect would be predicted to be less marked.

RNA accumulation was assessed by Northern analysis using S segment ^{32}P labelled cDNA as a probe. The results obtained following growth of the mutants at permissive and non-permissive temperatures (Figure 4.2) largely mirror those found for protein synthesis. Maguari virus *ts*23 S segment RNA could not be detected at either temperature (lanes 3 & 8)—again presumably due to the low multiplicity used to infect. The group II mutant produced RNA at both temperatures (compare lane 2 to lane 7) while the group I mutant showed undetectable levels of RNA at 38·5°C (lane 1 compared to lane 6).

Since the L protein is the viral RNA polymerase (Jin & Elliott 1991), L segment mutants would be expected to display reduced RNA, and consequently reduced protein, levels when grown at the non-permissive temperature. N protein, encoded by the S segment would not be required for primary transcription but is required for amplification of transcription templates since it forms an essential component of the viral RNP. If the multiplicity of infection were high enough to allow detection, S segment mutants might allow production of mRNA by primary transcription of input genomes (providing that the preformed template did not alter conformation at the non permissive temperature) but would result in reduced genome replication and therefore little secondary mRNA production. This investigation was severly compromised by the inability to grow sufficient seed stocks of mutant for meaningful study—in the case of *ts*23, no RNA or protein could be detected even at

the permissive temperature. Depending on how leaky the phenotype of the mutants are, it might be possible to specifically detect primary transcrips in the S segment mutant by PCR using a reverse transcription primer directed against positive stranded RNA. For the L segment mutant this approach should result in the absence of a signal if primary transcription is completely abolished and if the inoculum does not contain detectable quantities of positive stranded RNA. This approach would only yield informative results if a) the L segment mutant was completely restricted for transcription at 38.5°C and b) if RNP of the S segment mutant formed at the permissive temperature was still a functional transcription template at 38.5°C.

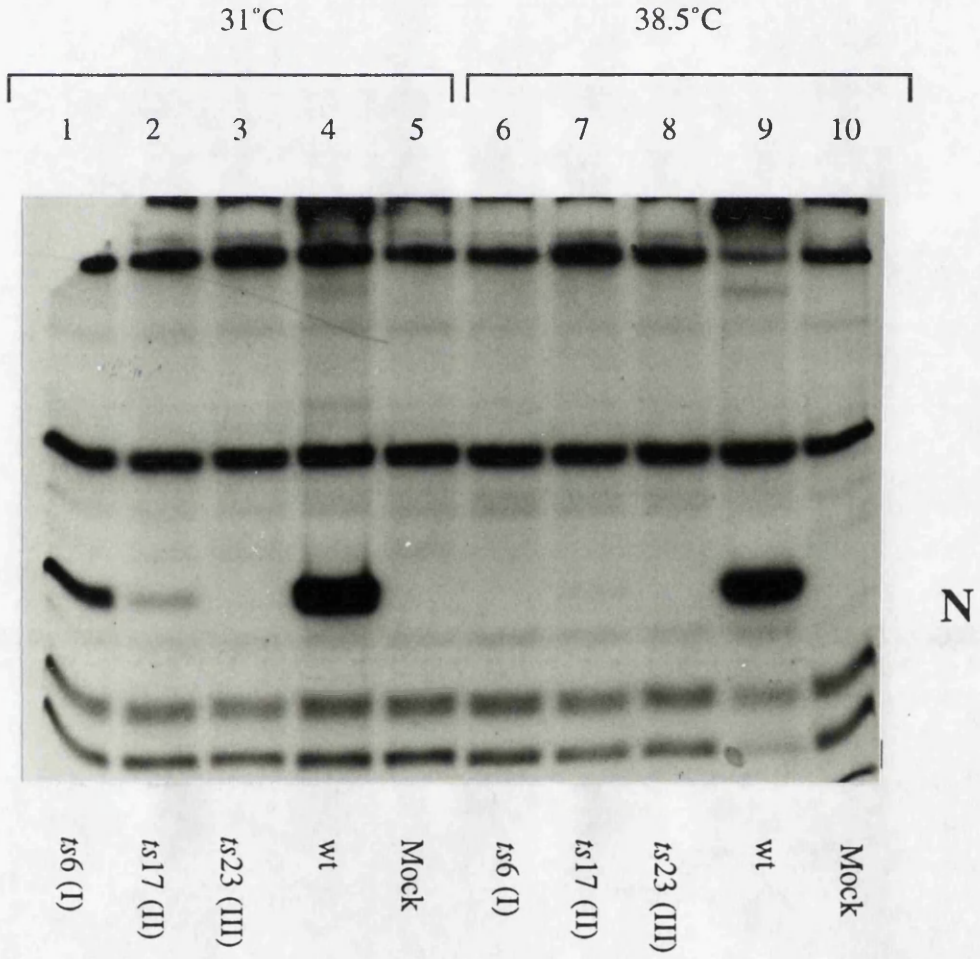


Figure 4.1 Protein synthesis by Maguari virus and *ts* mutants. BHK monolayers were infected at 10pfu/cell (wild type Maguari virus), 1pfu/cell (*ts*6 and *ts*17) and 0.01pfu/cell (*ts*23). Different multiplicities were used because of the difficulty of growing high titre stocks for the mutants. For *ts*23 (lanes 3 and 8) insufficient virus was present to detect N protein even at the permissive temperature. For wild type virus, a strong N protein band is present at both temperatures (lanes 4 and 9). A faint band is seen for the group II mutant even at 38·5°C (lane 7). A slightly stronger band is visible at the lower temperature (lane 2). The group I mutant (lanes 1 and 6) shows marked shut off at the higher temperature.

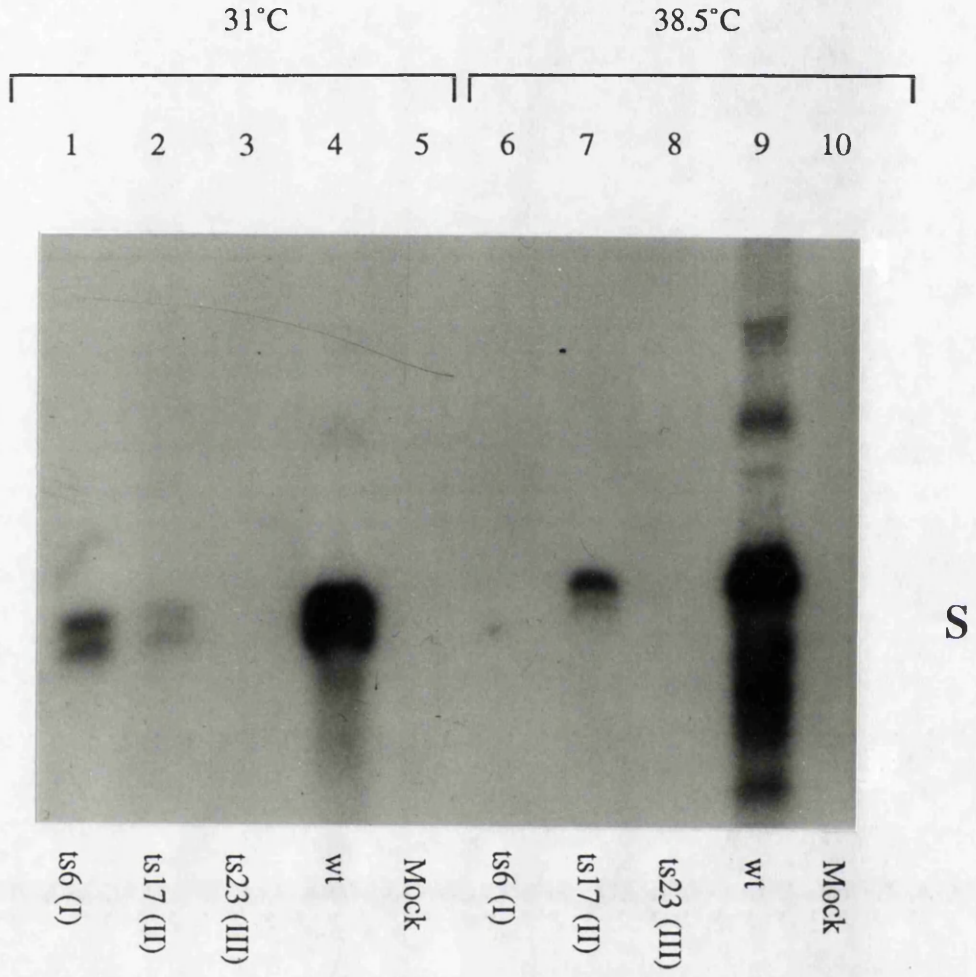


Figure 4.2 RNA profiles of wild type and *ts* mutant Maguari virus infected cells. BHK monolayers were infected at the same multiplicities described for Figure 4.1. At 24Hrs post infection, RNA was purified from cells and separated on 1% agarose gels under denaturing conditions. Northern blots were probed using negative strand riboprobes transcribed *in vitro* from S segment cDNA to detect positive sense RNA. S RNA could be separated into full length (antigenome) and shorter (mRNA) species resulting in a doublet band. For *ts*23, the low multiplicity prevented detection of RNA at both temperatures (lanes 3 and 8). For wild type virus similar levels of RNA accumulated at both temperatures (lanes 4 and 9). The group II mutant produced RNA at both temperatures (lanes 2 and 7) with a strong signal seen for full length anti genome RNA at 38.5°C (upper band lane 7). No RNA could be detected for the group I mutant grown at 38.5°C (lane 6 compared to lane 1).

4.3 S segment sequencing.

The RNA and protein analysis of cells infected with the Maguari virus *ts* mutants were not helpful in assigning the group I and group III mutants to their segments. Further characterisation was required and it was decided to attempt to identify sequence differences between the S segments in the hope that any lesion would be unique to one group and would therefore likely be the cause of the *ts* phenotype.

Various methods could have been employed to identify which S segments contained mutations. One potential approach would be to anneal S RNA from each of the mutants to wt cDNA and treat with RNase to nick at mismatched nucleotides. Such nicks could then be identified by electrophoresis to separate the resultant fragments (Sambrook *et al* 1989). Another approach, which has been successfully used to discriminate sequences containing single point differences, is denaturing gradient gel electrophoresis (Fischer & Lerman 1979) where hybrids of the two sequences being compared are electrophoresed through a two-dimensional system which employs a denaturing gradient which at some point (depending on the degree of difference between the two strands of the hybrid), will separate the duplex into two single strands. While both techniques would in principle have been applicable to the present problem, and might be extremely useful if many mutants were to be screened, a potential draw back would be that since the mutants had been generated by chemical mutagenesis, inconsequential mutations may occur in S segments which were not *ts*. It was therefore decided to clone and sequence the S segments from each of the three mutants and wild type virus. The ideal outcome would be to find no mutations in any but one segment—the genuine *ts* segment. Even if this ideal were not attained, the availability of sequence data might enable probable assignment based on the degree of conservation of the affected nucleotide with other bunyaviruses.

A rapid coupled reverse transcription/PCR procedure was developed which allowed production of full-length S segment cDNAs starting with crude virus preparations. Virus from infected cell supernatant fluid was pelleted by high speed centrifugation and RNA purified by double phenol extraction. Sufficient virus was present in the 5ml supernatant of an infected BHK cell monolayer grown in one 50mm petri dish to allow S segment cDNA amplification from even the poorly growing *ts*23 mutant. Following ethanol precipitation and resuspension in water, the viral RNA was added to a 50µl reaction mix (50mM KCl, 20mM Tris.HCl —pH 8.4 at room temperature, 2.5mM $MgCl_2$, 1mg/ml BSA and 10 units RNasin). 100 units of MuLV reverse transcriptase (BRL) and 1 unit of Taq thermostable DNA

polymerase (Cetus) was added along with 30 pmole of each of two synthetic oligonucleotides for cDNA and PCR priming. The oligonucleotides used were complementary to the 15 terminal nucleotides at the 3' ends of genome and anti genome Maguari virus S segment RNA (5' AGTAGTGTACTCCAC and 5' AGTAGTGTGCTCCAC respectively). These primers are specific for S segments of all members of the *Bunyavirus* genus (Elliott 1990). Although the oligonucleotides differ from each other by only one nucleotide (position 9) they correctly primed their specific ends when used in combination. If used alone, either primer was able to prime amplification by annealing to both ends of the segment and introducing a point mutation nine nucleotides in from one end. The assembled reaction mixture was incubated at 42°C for 45 minutes to allow first strand cDNA synthesis. This was followed by 30 rounds of temperature cycling (94°C for 30 seconds, 54°C for 30 seconds and 72°C for 1 minute) during which duplex melting, primer annealing and elongation could occur.

The reaction products were electrophoresed through 1% agarose and S segment cDNA isolated and cloned to pUC118 for sequencing. This procedure enabled even the low abundance *ts* 23 RNA to be cloned (see Figure 4.3). Several clones were isolated from three independent RT/PCR amplifications and were sequenced using primers spanning the S segment. For wild type and and *ts* mutants 6 and 17 (the group I and II mutants), no mutations were found. For *ts*23 (the group III mutant) a point mutation was found in each of three independent clones. The mutation was also found when uncloned PCR amplified DNA was used as sequencing template and no alternate base was present at that position as might be expected if the mutation had arisen during PCR amplification (see Figure 4.4).

The mutation, a U to C transition, found in Maguari virus *ts* 23 occurs at nucleotide 327 and therefore affects the amino acid sequence of both the N and NSs proteins resulting in a valine to alanine change in the former and a phenylalanine to leucine change in the latter. In thirteen available S segment cDNA sequences, nucleotide 327 is invariant with the *ts*23 mutant being the only exception. Table 4.2 shows nucleotide and amino acid sequence comparisons for different bunyaviruses at this region of the S segment. The alanine introduced into the N protein of *ts*23 is not present in any of the other viruses which have a conserved valine except for three California group viruses which have isoleucine—an amino acid of similar size and hydrophobicity as valine. For NSs the relevant amino acid is less conserved and the leucine present in *ts*23 is also present in the three California group viruses. Based on these comparisons it is possible that the alteration found in the N protein would be of more significance to phenotype than the NSs change.

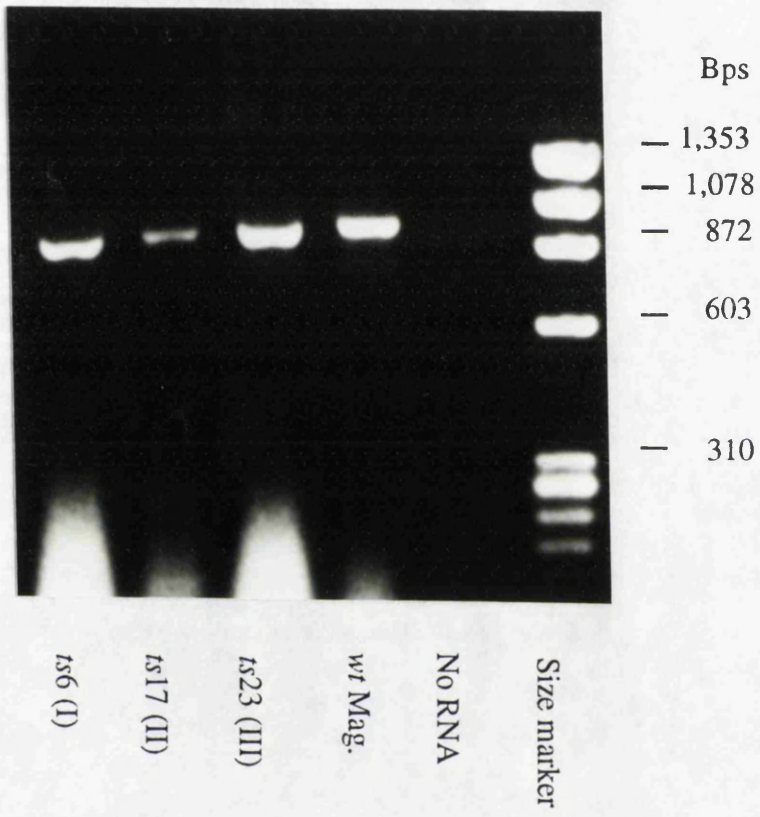


Figure 4.3 cDNA amplified from wild-type and temperature sensitive mutant Maguari viruses. RNA was purified from pelleted virus and cDNA amplified in a coupled reverse transcription / PCR reaction. 15mer primers, complementary to the 3' termini of genome and antigenome RNA, were annealed to RNA, mixed with reverse transcriptase, Taq DNA polymerase and dNTPs. The reaction was incubated at 42°C for 45 minutes to allow reverse transcription to occur. This was followed by 30 cycles of denaturing (94°C for 30 seconds), annealing (54°C for 30 seconds) and extension (72°C for 1 minute). The cDNA produced was separated on 1% agarose gels and visualised by staining with ethidium bromide. DNA was purified from gels by adsorption to silica, end-repaired, kinased and cloned to phagemid pUC118 for sequencing. PCR populations were also sequenced directly by boiling in the presence of excess sequencing primer and cooling to room temperature before standard di-deoxy termination reactions were performed. (DNA size marker is HaeIII digested ϕx174).

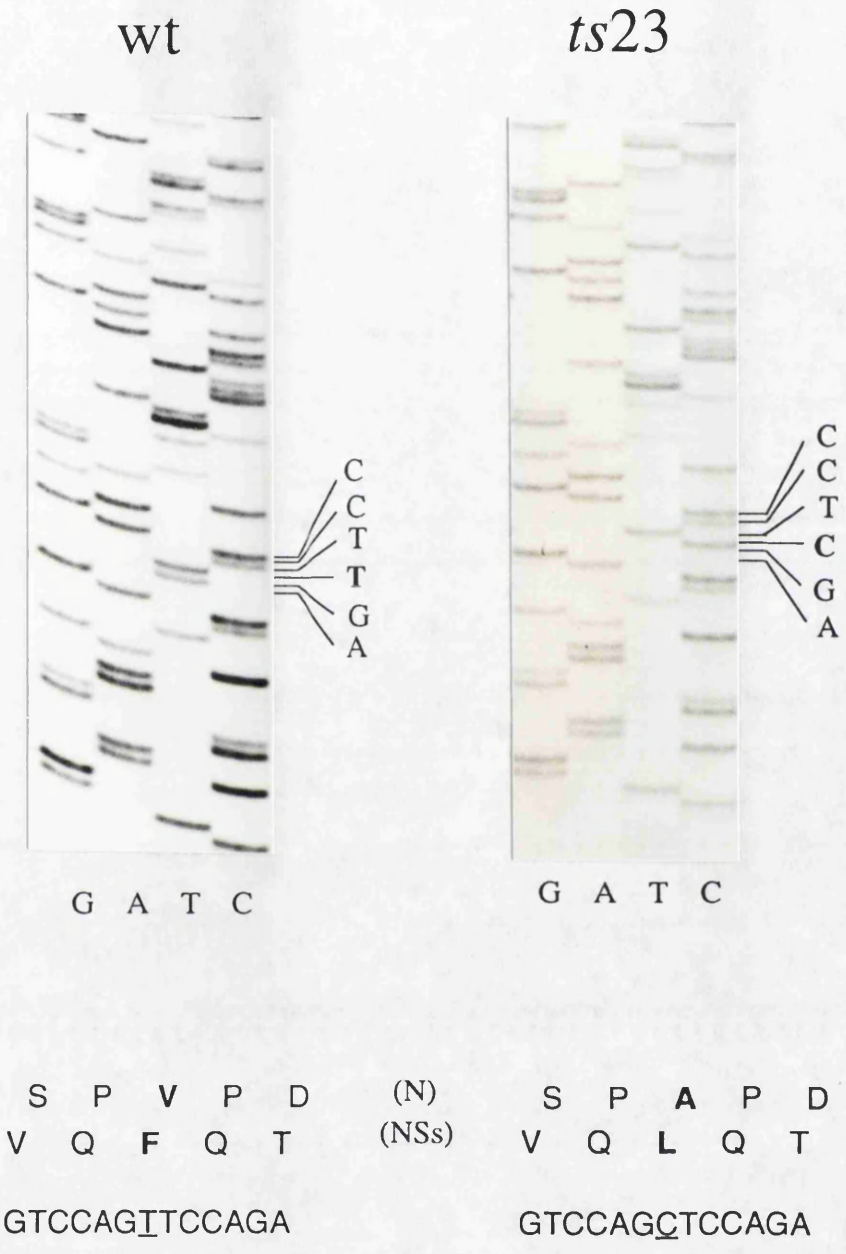


Figure 4.4. Sequence of Maguari virus *ts*23 S segment mutation. Left panel shows sequence of cloned wild type Maguari virus S segment cDNA determined by the Sanger di-deoxy chain termination method. Right panel shows *ts*23 sequenced using uncloned PCR product as template. The T to C transition at nucleotide 327 (cDNA sense strand) is shown in bold type. The deduced effect of the mutation on the primary sequence for N and NSs proteins is shown. See abbreviations section for amino acid symbols.

nucleotides	virus	N protein	NSs protein
GTCCACCTCCAGAC	*ts*23	NSPAPDD	VQLQT
GTCCACTTCCAGAC	MAG	NSPVPDD	VQFQT
GTCCACTTCCAGAC	NOR	NSPVPDD	VQFQT
GTCCACTTCCAGAC	CV	NSPVPDD	VQFQT
GTCCACTTCCAGAC	MD	NSPVPDD	VQFQT
ACCCACTTCCTGAC	BUN	NNPVPDD	TQFLT
GTCCACTTCCAGAC	BAT	NSPVPDD	VQFQT
ATGCACTGCCTGAC	GER	NNAVPDY	MQCLT
TGTCACTTCCAGAT	KRI	NMSVPDD	CQFQM
GTCCACTCCTCGAT	GRO	NSPVLDD	VQSSM
ACCCAATTGGTAAC	LAC	NNPIGNN	TQLVT
ATCCAATTAACAGC	SSH	NNPINSD	IQLTA
ACCCAATTGATAAC	LUM	NNPIDNN	TQLIT
ATCCTGTGCCAGAC	AIO	ANPVPDT	ILCQT

Table 4.2 Sequence alignment of *ts*23 mutation with other bunyavirus S segments. Numbered for Maguari virus S segment sense strand cDNA, nucleotide 327 (boxed) is invariant in all S segments of the bunyavirus genus so far sequenced except for the T to C transition found in the Mag *ts*23 S segment cDNA. The deduced effect on N and NSs amino acid sequences are shown and compared to the sequences present in other viruses. At the relevant position in N protein, valine occurs in all bunyaviruses except those of the California serogroup (La Crosse, snowshoe-hare and Lumbo viruses) which have isoleucine. The *ts*23 mutant has alanine at this residue. For NSs the amino acids at this position are more variable and the leucine found in *ts*23 is also present in the California group viruses although in a different context. See abbreviations section for amino acid and virus symbols. (Data compiled by, and includes the unpublished results of, E. Dunn and R.M. Elliott).

Since the terminal 15nt at both ends of the clones are formed by the primers used for PCR, it is formally possible that a mutation could exist in this region which might affect promoter or packaging functions of the segment. Direct RNA sequencing to confirm the absence of such mutations in this region was not undertaken due to the small amounts of RNA obtainable from the poorly growing mutants. Using the PCR based method recently applied to sequence the 5' terminal sequences of Bunyamwera virus mRNA (Jin & Elliott 1993; see Section 1.9.4.1), it should now prove possible to check the terminal sequences exactly as depicted in Figure 1.9.3 using CsCl banded RNP as a source of RNA. However, although a *ts* mutant carrying a lesion in a non-coding region has been reported for poliovirus (Racaniello & Meriam 1986), mutations resulting in a *ts* phenotype almost always occur in protein coding regions of a genome.

The finding here of a point mutation in the S segment of *ts*23, with no mutations being found in the S segment of other mutant virus groups compared to wild type, is evidence that the group III Maguari virus mutants, for which *ts*23 is the only available member, do indeed represent lesions in the S segment. As discussed earlier this suggestion contradicts the results of reassortment studies (Murphy & Pringle 1986) but is in agreement with the assignment expected based on target sequence size and the unambiguous findings for the California group bunyaviruses (Gentsch *et al* 1979). Further sequencing studies to identify lesions in the L segments of group I mutants would be useful additional evidence for group assignment as would complete sequence determination of the L and M segments of *ts*23. Complementation with a wild type segment would provide the strongest evidence of which segment contains the *ts* lesion.

During growth of *ts*23 no revertants were observed. This prevented investigation of nucleotide 327 in a revertant which should have demonstrated that the mutation identified was no longer present. *Ts*23 produces only very small plaques at the permissive temperature. It is not known whether the small plaque phenotype is a result of the same mutation as the *ts* phenotype or whether another mutation is responsible. Revertants of a small plaque mutant would have been easy to spot had they occured and their absence is puzzling considering the growth advantage they would be expected to have especially as a single point mutation is all that would be required for reversion if the identified lesion was the cause of the small plaque phenotype.

4.4 Rescue experiments

Several attempts were made to rescue the Maguari virus *ts*23 mutant using plasmid derived RNA. As described in Chapter 3, linearised cDNA designed to produce T7 transcripts corresponding to Bunyamwera virus S RNA, was transfected into cells infected with vTF7-3, to supply T7 RNA polymerase, and the *ts* mutant. Cells were then incubated at 31°C or 38·5°C for 24 or 48 hours before supernatants were harvested and inoculated onto BHK monolayers and then grown at either temperature for plaques to form. Phosphonoacetic acid was included in overlays to selectively inhibit vaccinia virus replication (Jin 1991). Any plaques forming at 38·5°C would be expected to be formed by a reassortant virus, Mag/Mag/Bun, with the S segment being derived from the plasmid transcribed RNA (see Figure 4.5).

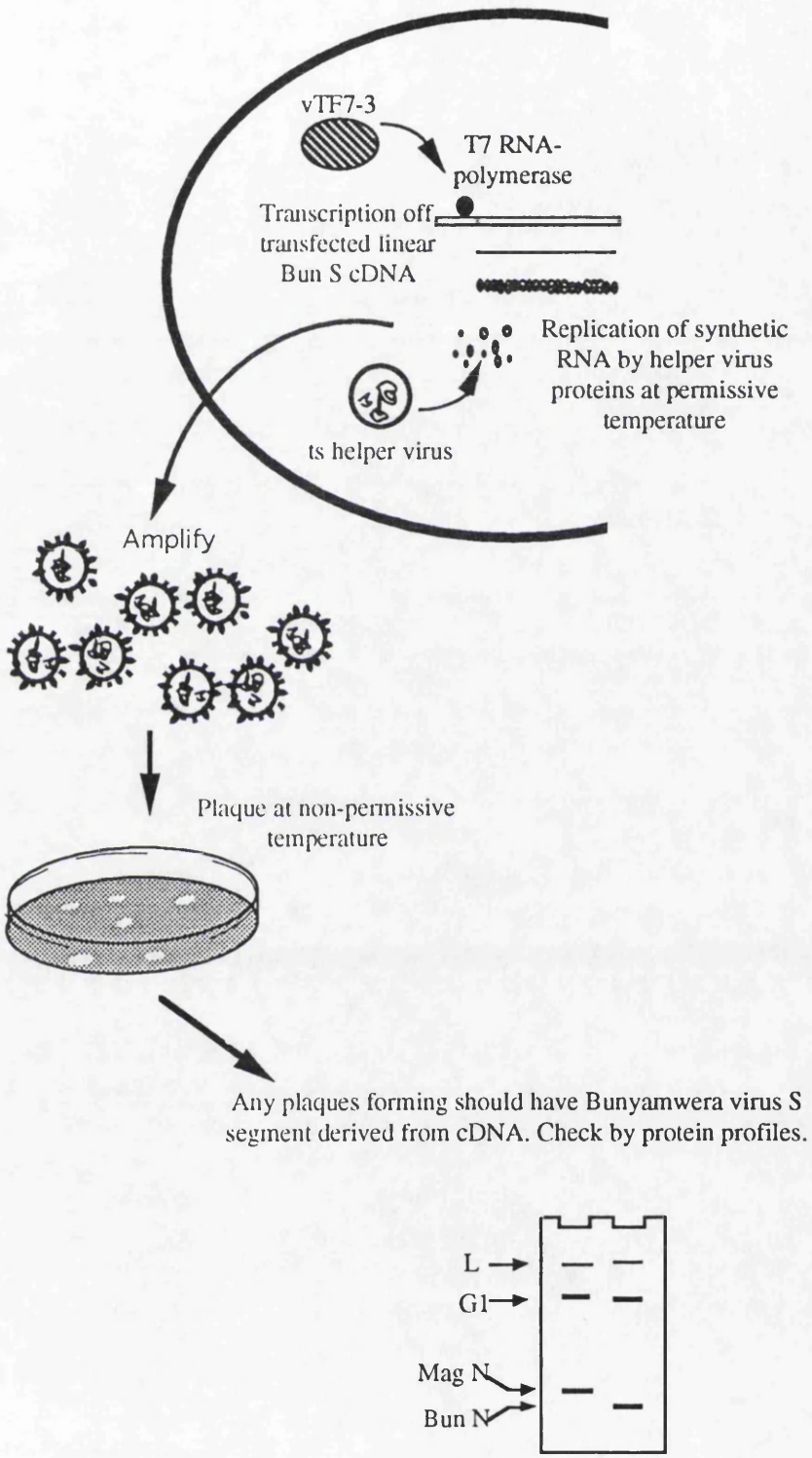


Figure 4.5 Rescue strategy using Maguari virus *ts*23 as a counter-selectable helper.

No plaques were obtained at either temperature. The *ts*23 mutant is severely restricted even at 31°C and in coinfection with vaccinia virus was not able to form plaques in this study. Plaquing of non *ts* control virus was achieved in the presence of vTF7-3 and so if rescue of *ts*23 had occured, the resultant virus should have been able to form plaques. Although the Maguari virus *ts*23 appears not to be suitable for a rescue strategy involving vaccinia virus it may still prove useful for rescue of S segment RNP assembled *in vitro* since the only competing virus present would be the non-*ts* virus which replication of the synthetic S segment would produce.

Establishment of a rescue system involving Maguari virus *ts*23 would enable some interesting studies to be conducted. With the identified lesion causing alteration of two proteins, it would be expected that one lesion may be more important than the other. For example if, the Val to Ala mutation in the N protein were more damaging than the Phe to Leu change in the NSs protein, it might be possible to rescue a less restricted virus by supplying just the N protein in a synthetic S segment. Such separate expression of proteins usually encoded by overlapping reading frames was successfully achieved for influenza virus and resulted in a virus which packaged an additional segment (Luo *et al* 1991; see Section 1.8.8.2). The success of such an experiment would not only demonstrate that bunyaviruses can package more than three segments but might provide useful information concerning the function of N or NSs by comparing phenotypes of viruses known to be mutant only in one or other of the proteins, with that of wild type virus.

4.5 Why so few S segment mutants?

In addition to the smaller target size for mutagenesis already discussed, the overlapping coding strategy employed by the S segment might place tight constraints on sequence variation. This is because a significant proportion of S segment mutations would be expected to affect two genes, those coding for the N and NSs proteins, and this might result in a higher proportion of mutations being lethal. The validity of this argument could be assessed by isolating a reasonable number of *ts* mutants for a representative of another genus other than the *Bunyavirus* genus which is the only group to employ overlapping reading frames for N and NSs. If the presence of overlapping genes did limit the proportion of S segment bunyavirus mutants, analagous limitation would not be expected for these other groups of viruses since their S segment coding sequences do not overlap. Five *ts* mutants of Uukiniemi virus have been assigned to one of two reassortant

groups . Such a small number might not be expected to contain any S segment mutants due to the smaller target size and therefore more mutants would need to be characterised for this suggestion to be investigated.

For influenza virus, direct evidence exists that more than the usual eight segments can be packaged (see Section 1.8). Some evidence exists that this may also be the case for bunyaviruses. In preparations of purified bunyaviruses it is rarely possible to achieve recovery of each of the three gene segments in equimolar amounts (Elliott 1990). For Germiston bunyavirus, a ratio of 1:1:2·5 for the L, M and S segments has been measured from labelled RNP separated by sucrose gradient centrifugation (J. Szilagyi personal communication). Also electron microscopy of vitrified virus has demonstrated variation in virion size which might reflect variable packaging (Talmon *et al* 1987). While these observations are consistent with an 'overpacking' hypothesis, they are also the results expected if a proportion of virions contained less than the complete genetic complement of a viable virus .

More compelling evidence comes from the isolation of several reassortant viruses which appear to be heterozygous for the S segment (Iroegbu & Pringle 1981a and 1981b). Protein analysis of end point dilution infections provides evidence that such viruses are genuine heterozygotes and not merely the result of contamination with a reassortant virus (C.R. Pringle personal communication). Heterozygosity requires packaging of at least two segments of the same type. Assuming random segregation of two distinguishable S segments in a heterozygote, estimation of S segment copy number might be possible by analysing plaqued progeny and

determining the proportion of each genotype present. For example, if two S segments are packaged and can freely segregate to progeny from a heterozygote, 50% of plaques would be expected to remain heterozygous (S1 *and* S2 *or* S2 *and* S1 being the possible choices leading to heterozygosity) with 25% of each homozygote also occuring (S1 *and* S1 and S2 *and* S2 being pakaged for each). The more segments packaged the lower the expected frequency of homozygous progeny. Also for multi segment packaging, any bias of segment frequency in the parent would be reflected by the proportion of each homozygote found in progeny virus.

The absence of revertants of *ts*23 may be explicable if the mutant protein had a dominant interfering effect. This is feasible for a protein such as the N protein which has to polymerise onto viral RNA to form a functional transcription template. A defect might enable N protein to bind RNA or an existing N protein nucleation centre already formed on the RNA, but prevent further binding of N protein even of the correct conformation. By acting as a terminator of coordinate binding, a very small dose of mutant to wildtype protein could effectively block replication at the stage of transcription template assembly and so when revertant genomes occured they would be unable to complement the defect. If the small plaque and *ts* phenotypes are caused by the same lesion, any dominant-interfering affect of the *ts*23 gene products could be investigated by assessing the plaque forming ability of wild type virus in a cell sheet which had previoulsly been infected with *ts*23. A dominant interfering effect would restrict formation of plaques by wild type virus.

4.6 Exploitation of S segment amplification for other bunyaviruses.

The ease and apparent lack of error with which the S segments of the Maguari virus *ts* mutants were cloned led to the technique being applied to isolate full length cDNA clones for the S segments of other viruses. Since for all available sequences, the bunyaviruses share common 5' and 3' nucleotides at the termini of the S segments for fifteen nucleotides, it was possible to use the technique without modification. RNA was purified from crude virus preparations of Northway, Cache Valley, Main Drain, Batai, Kairi, Guaroa and Lumbo viruses and cDNA obtained by the coupled RT/PCR used for the Maguari virus mutants. For all but Guaroa virus, single DNA bands of the expected size for the S segment were obtained (see Figure 4.6) and cloned in phagemid vectors for sequencing. Guaroa virus S segment cDNA was later amplified and cloned using fresh RNA and slightly shorter primers (R.M. Elliott personal communication). Sequencing (performed by E. Dunn) confirmed the clones for different viruses to be different

from each other and demonstrated the usefulness of the technique which doubled the number of complete S segment cDNAs available. The sequence data and comparisons with existing data will be discussed elsewhere (E. Dunn, D. Pritlove and R.M. Elliott.; Manuscript in preparation).

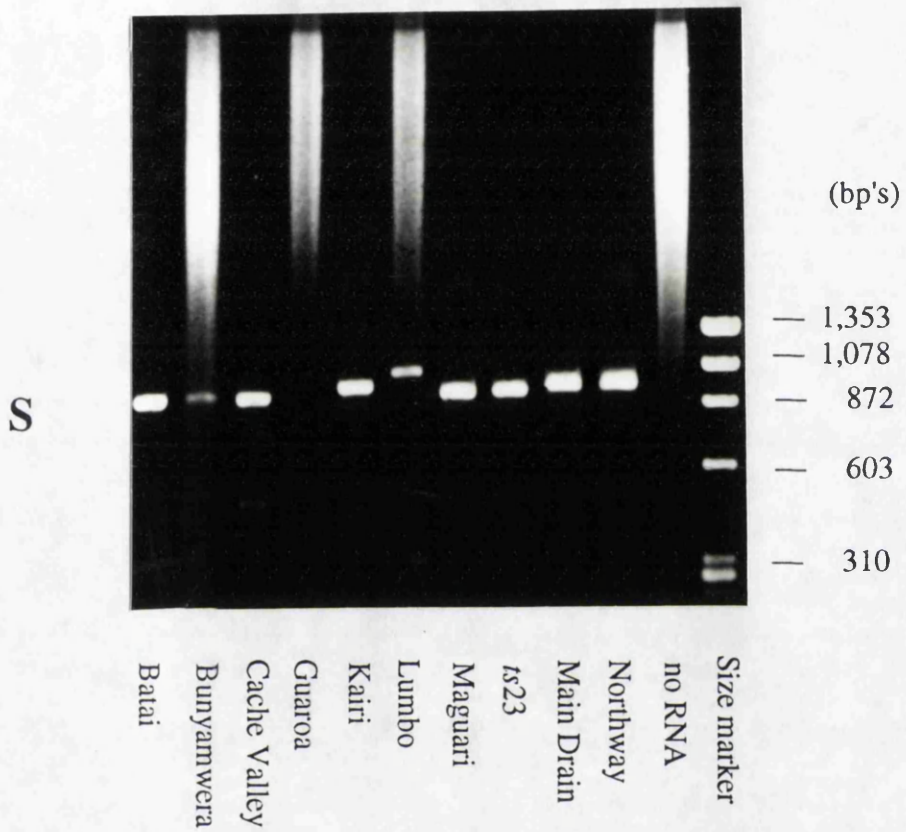


Figure 4.6 Bunyavirus S segment cDNAs. A 1% agarose gel stained with ethidium bromide was used to visualise S segment cDNAs amplified by coupled-reverse transcription /PCR (DNA size marker is HaeIII digested ϕX174). See text for details.

4.7 Chapter 4 summary.

In an attempt to identify a counter-selectable virus for rescue of plasmid derived S segment RNA, representatives of each of the three *ts* reassortment groups of Maguari virus (Pringle 1990) were examined to identify a *ts* virus with an S segment lesion. Previous characterisation had assigned group II mutations to the M segment, suggested that the group I mutants carried S segment mutations and that the single group III mutant, *ts*23, resulted from a lesion in the L segment (Murphy and Pringle 1987). Because of the relative frequencies with which mutants belonging to each of the three groups could be generated in random mutagenesis studies, this assignment was not as expected based on the assumption that genes of larger target size would produce more mutants in such experiments than those of smaller size (Pringle 1990). Conversely, thorough studies of *ts* mutants of California group bunyaviruses (Gentsch *et al* 1979) demonstrated that the two well represented reassortment groups carried lesions in the L and M segments not the M and S segments as suggested for Maguari virus mutants.

Attempts to compare the levels of RNA and protein synthesised in cells infected with mutants at the permissive (31°C) or non-permissive (38.5°C) temperatures were hampered by difficulties in growing stocks of sufficiently high titre to enable infections to be established at high multiplicity.

To enable the S segments of mutants from each of the three reassortment groups to be sequenced, a rapid PCR-based procedure was developed which allowed full-length S segment cDNA to be amplified in a single step from RNA isolated from crude preparations of virus. Using this method, full-length S segment cDNAs were amplified and cloned from three ts mutants (*ts*6, *ts*17 and *ts*23; representing reassortment groups I, II and III respectively) and from wild type Maguari virus. Three cDNA clones of each segment were sequenced by the Sanger di-deoxy chain termination method. No mutations were found in the *ts*6 or *ts*17 S segment cDNAs compared to wild type. A single point mutation was found in the S segment cDNA of *ts*23 which was also apparent when the non-cloned PCR population was sequenced directly. The mutation was at nucleotide 327 (sense strand cDNA) and altered a thymidine (invariant in all bunyavirus S segment cDNAs so far sequenced) to a cytosine. This mutation is expected to alter the primary amino acid sequences of both the N and NSs proteins which overlap this region of the S segment. The

predicted change in the N protein alters a valine residue (invariant in all sequenced members of the Bunyamwera group viruses) to an alanine residue. For NSs this region is slightly less conserved for different bunyaviruses. Phenylalanine is replaced by leucine which is also found in the equivalent position in the NSs proteins of three California group bunyaviruses.

Maguari virus *ts*23 was used as a helper virus in attempt to replicate RNA transcribed *in vivo* from a Bunyamwera virus cDNA by the vTF7-3 T7 polymerase expression system. Progeny virus were amplified and grown at 38.5°C to suppress the *ts* helper virus. Any plaques forming were expected to contain a reassortant virus containing a Bunyamwera virus S segment (derived from cDNA) and Maguari virus L and M segments (derived from *ts*23). No plaques formed even at the non permissive temperature indicating that the poorly growing *ts*23 mutant may not be suitable for this type of experiment.

The PCR based method developed for amplification of S segment cDNAs from the Maguari virus *ts* mutants was exploited to clone several previously uncloned S segments from other bunyaviruses.

Chapter 5: Reporter constructs and *in vivo* expression

The inability to detect rescue of synthetic RNA corresponding to a distinguishable but *bone-fida* bunyavirus S segment could have been due to the low efficiency of the system. Bunyavirus-like RNA containing a reporter gene might increase the level of detection of rescued RNA and so enable conditions to be optimised to an efficiency which would allow rescue and detection of virus-like RNA.

Two reporter gene containing constructs were made, both employing T7 RNA-polymerase promoter and a run-off transcription site to produce RNA with authentic bunyavirus termini flanking the reporter gene. The first construct makes positive stranded RNA containing message-sense reporter gene RNA flanked by Bunyamwera virus S segment cRNA termini, while the second makes a vRNA-like transcript containing antisense reporter gene RNA.

No measure of the proportion of cells in a monolayer which were transfected or infected was made in the studies reported in this thesis. Immunofluorescence could be employed to help optimise infection/transfection conditions to maximise the number of cells receiving both virus and nucleic acid.

5.1 Construction of pUC118/T7BunS(+)30/29CAT.

Two synthetic oligonucleotides were used to create a 102bp fragment containing the terminal 30 (5') and 29 (3') nucleotides of the Bunyamwera virus S segment cRNA, separated by a restriction site into which a reporter gene could be cloned. The overall cloning strategy is shown in Figure 5.1. The cDNA was flanked by a T7 promoter at one end and a XbaI restriction site at the other to allow run-off trascripts to be made (Figure 5.2). A 15mer (5' CCTGCAGCCT CTAGA) was annealed to the 3' end of a 102mer (5' CGAATTCTAATACGA CTCACTATAAGTAGTGTACTCCACACTACAAACTTGCTAAGCTTATTTTA AGTTTTAGGTGGAGCACACTACTCTAGAGGCTGCAGG) and extended using T7 DNA polymerase to make a double stranded fragment 102 base pairs long. This fragment was digested with EcoRI and 5' phosphorylated resulting in a blunt/EcoRI phosphorylated fragment. This was ligated to pUC118 which had been cut with HindIII and end-filled (destroying the HindIII site and generating a blunt end) followed by EcoRI digestion and dephosphorylation. The ligation reaction was transformed into *E.coli* strain XL1 and recombinants selected by ampicillin resistance and white colony phenotype in the presence of X-gal. Single-stranded DNA generated from XL1 cells harbouring the plasmid, by infection with helper phage M13K07, was sequenced to confirm the presence of correct insert (Figure 5.3).

A plasmid containing the correct insert was cleaved with HindIII at the unique site between the Bun S terminal sequences and ligated to a HindIII fragment containing the CAT coding sequence and some Tn9 flanking sequence. Sequencing was used to identify clones containing the CAT gene in the correct orientation. The final construct map is shown in Figure 5.4. For brevity, pUC118/T7BunS(+)30/29CAT is abbreviated to pBunS(+)CAT.

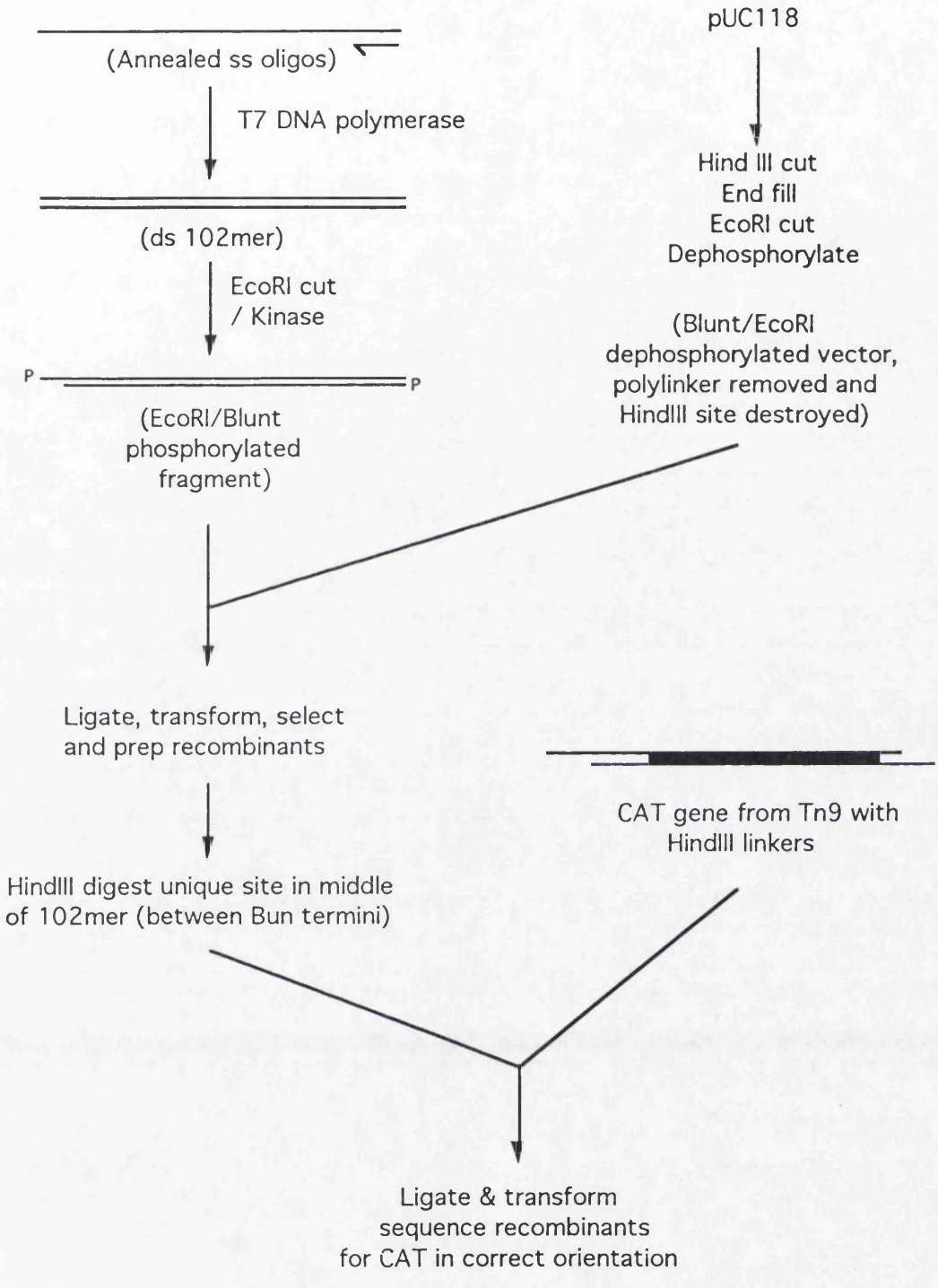


Figure 5.1. Construction of pUC118/T7BunS(+)30/29 CAT. A 102mer DNA fragment was assembled from two overlapping oligonucleotides (a 102mer and a complementary 15mer). This fragment contains (5' to 3' for the ss 102mer): an EcoRI restriction site followed by T7 promoter followed by 30nt Bunyamwera virus S segment sequence (cRNA 5' terminus) followed by a HindIII cloning site followed by 29nt Bun S sequence (cRNA 3' terminus) followed by XbaI and PstI restriction sites. When cloned into pUC118 (in which the HindIII site had been destroyed) the insert was cleaved at the HindIII site between the Bun termini and a fragment of Tn9 containing the CAT coding sequence was ligated. Clones were sequenced to identify those containing the CAT open reading frame in the correct orientation. The Map and partial sequence of the construct is shown in Figure 5.4.

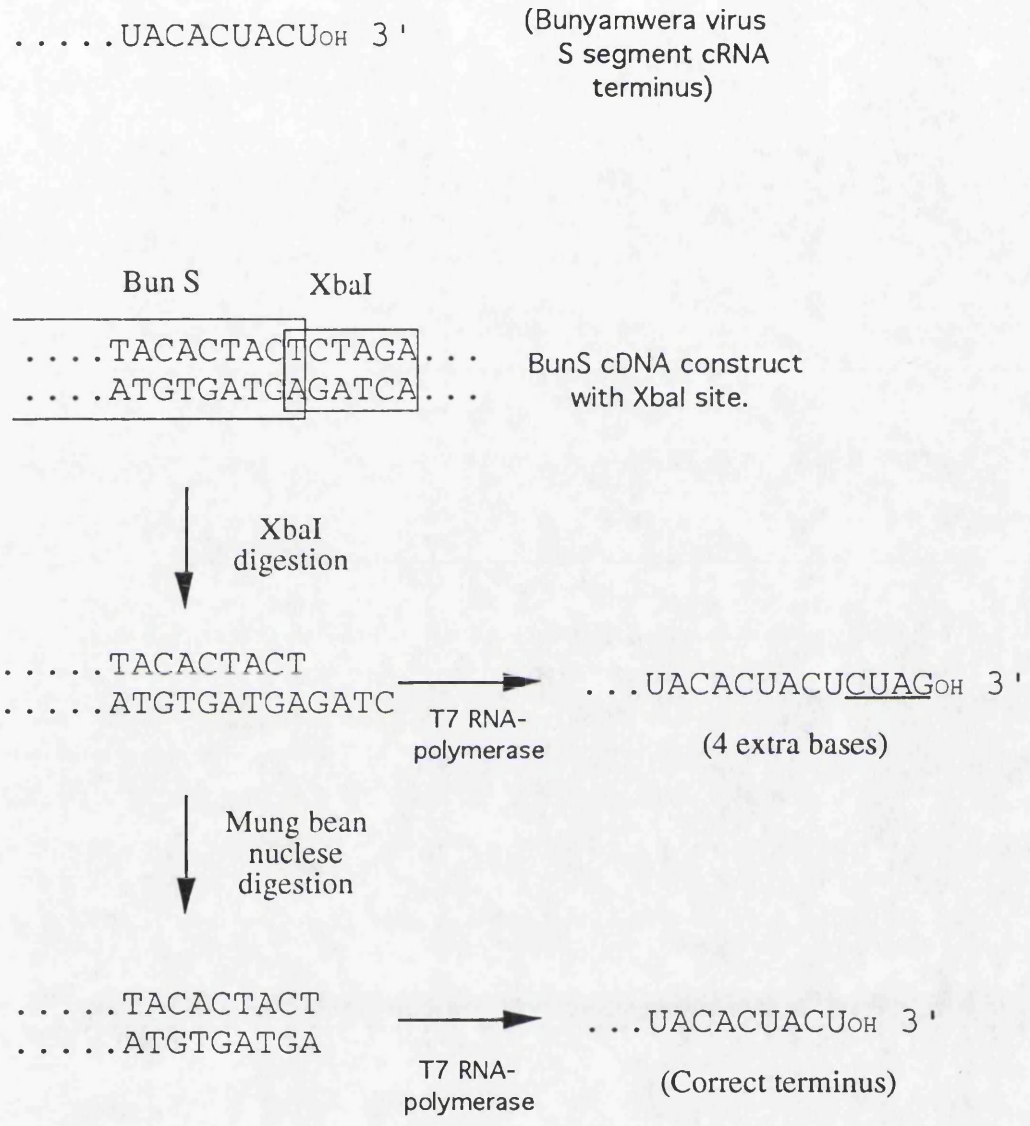


Figure 5.2 XbaI run-off transcription end for Bunyamwera virus cRNA 3' terminus. DNA construct sequences are shown on the left as double strands with the top strand written 5'–3'. Single stranded RNA 3' run off transcription termini are shown to the right of their relevant DNA templates. This XbaI run off site is used for pUC9/BunS+ (Chapter 3) and the pBunS(+)CAT construct of this chapter.

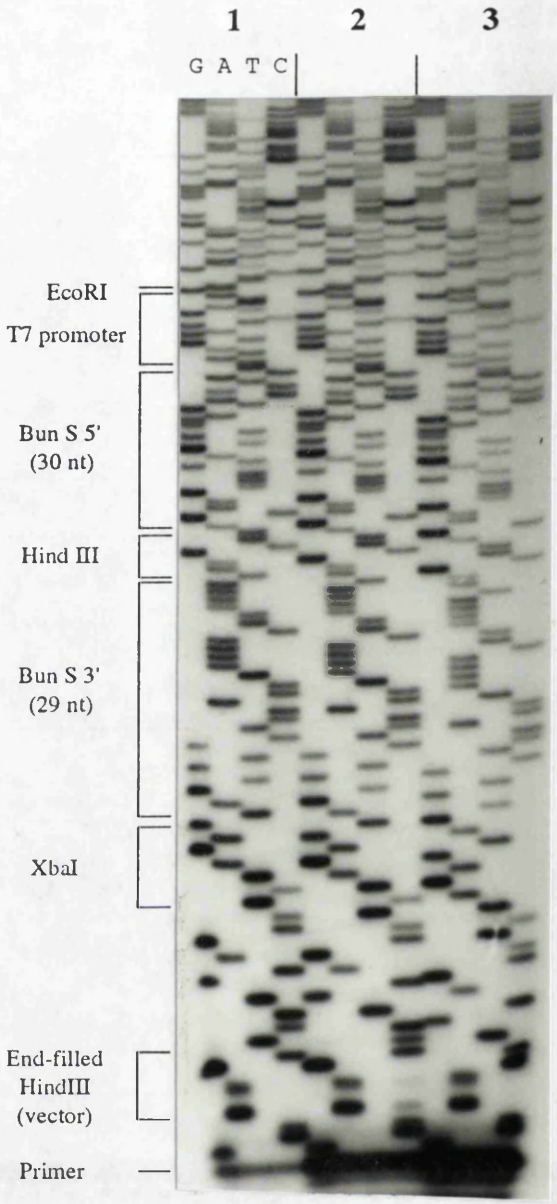


Figure 5.3. DNA sequence of cloned 102mer oligonucleotide. Three clones are shown each with the HindIII site of the vector end-filled to a different base. ^{32}P end-labelled universal primer and a doubled ratio of dideoxy:deoxy NTP were used to allow sequence close to the primer to be read. The first base after the primer is easily read here (see Figure 5.4 for the relationship of vector to insert and position of universal primer binding). Clone 1 above was digested with HindIII (between the Bun S termini) to allow ligation of a HindIII fragment containing the CAT open reading frame resulting in pUC118/T7BunS(+)30/29CAT (see Figures 5.1 and 5.4).

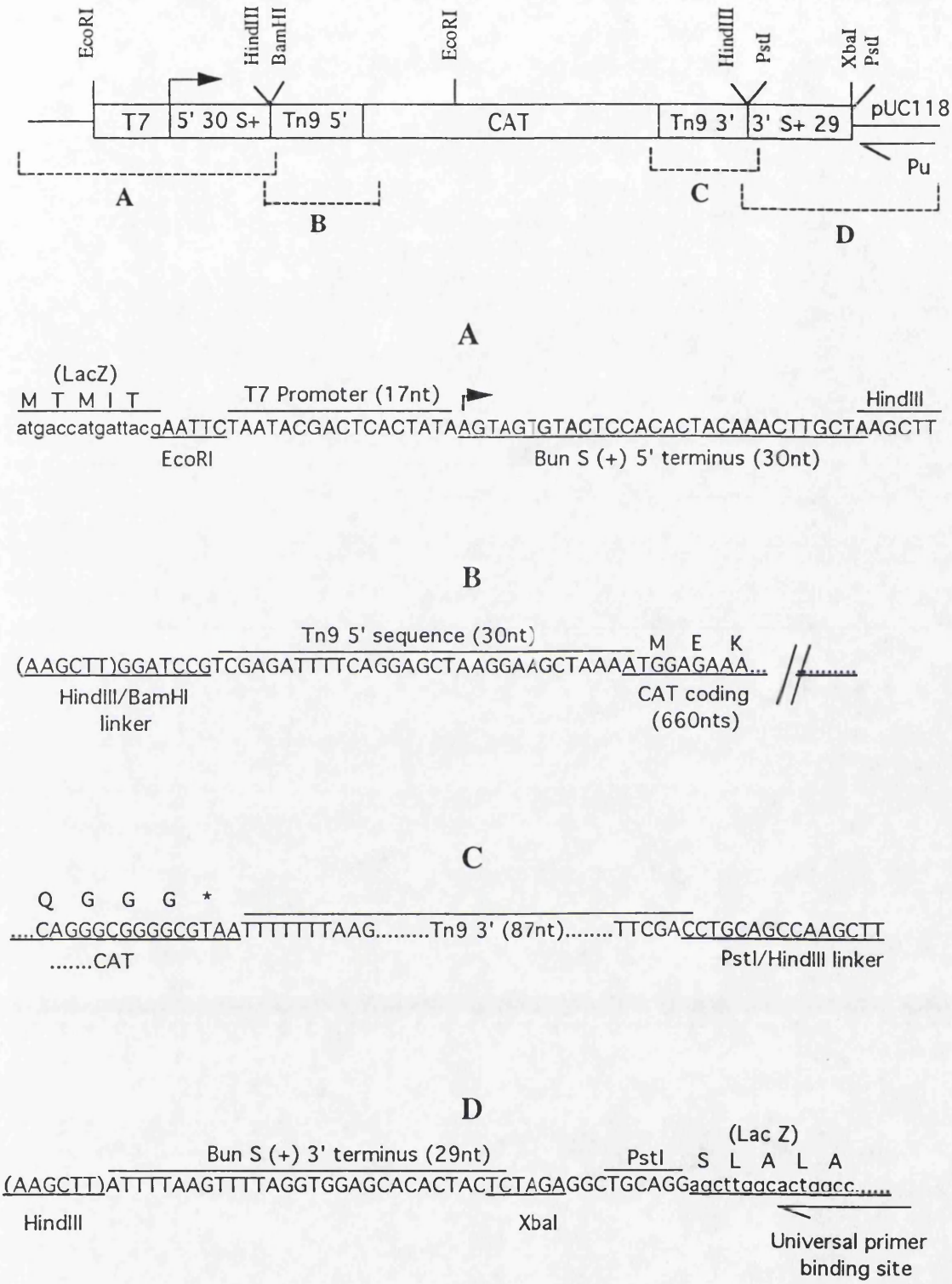


Figure 5.4 Map of pUC118/T7BunS(+)30/29 CAT. Construct map (top—not to scale) shows the components of pBunS(+)CAT including sites for common restriction enzymes and transcription start site from the T7 promoter (arrow). Under the map is sequence detail of various junctions including flanking vector sequence (lower case letters). Bracketed sequence at the start of B and D are repeated from A and C respectively. T7 run off transcription would produce a transcript 863 nt long with Bunyamwera virus S segment cRNA termini.

5.2 Transfection and infection conditions.

Optimisation of transfection and vaccinia virus infection conditions was performed using pBunS(+)CAT and assaying cell lysates for CAT activity. Two transfection methods were investigated: Calcium phosphate mediated transfection (using two batches of calcium chloride) and lipofection using commercially available Transfectace™. Initially, cells were infected with recombinant vaccinia virus vTF7-3, at 2 or 20 pfu/cell and transfected with 5µg uncut pBunS(+)CAT. As a control for DNA transfection and CAT detection, plasmid pRR55, which constitutively expresses CAT under control of a cytomegalovirus promoter and enhancer (M. McFarlane personal communication), was included. 24 hours post transfection, cell sheets were harvested and used to make extracts which were then assayed for CAT activity as described in Chapter 2. Because of the varying harvesting efficiencies, caused by different degrees of cell losses for the different infection and transfection conditions, CAT activity was standardised by correcting for protein content of the samples (determined by Bradford assay) and expressing CAT activity as picomoles chloramphenicol acetylated/ min/ milligram protein.

Figure 5.5 shows the variable activities obtained for the two batches of calcium chloride used. In both cases infection with vTF7-3 at 20pfu/cell resulted in greater CAT activity than infection at 2 pfu/cell. Expression from pBunS(+)CAT by vTF7-3 at either multiplicity resulted in greater CAT activity than that obtained from the constitutively expressing plasmid pRR55 when calcium phosphate was used as transfection facilitator. Lipofection greatly increased CAT levels from pRR55 and pBunS+CAT at the higher vTF7-3 multiplicity while with vTF7-3 at 2 pfu/cell one of the batches of calcium chloride produced a higher level than obtained with Transfectace™. Tranfection of pBunS(+)CAT in the absence of vTF7-3 gave no detectable CAT activity as expected. Similarly, vTF7-3 infection alone failed to produce any CAT activity. Based on these results lipofection was adopted to investigate optimum vTF7-3 infection conditions since a difference in levels of CAT activity was observable for the two multiplicities used.

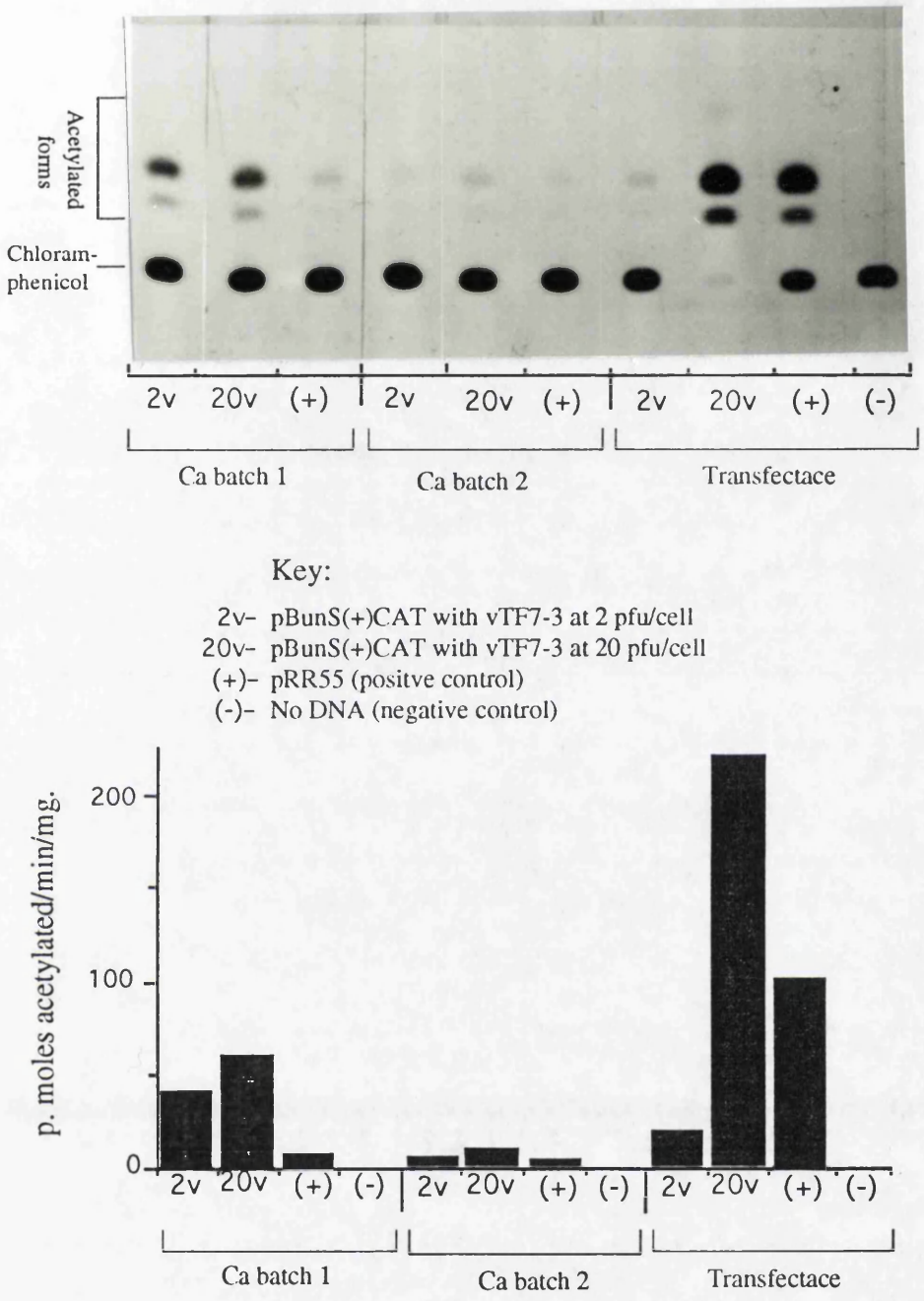


Figure 5.5 Transfection conditions for expression of pBunS(+)CAT. BHK monolayers were transfected using either of two batches of calcium chloride or Transfectace™ as described in the text. CAT assays were performed on cell extracts (top) and activity expressed, after correcting for protein concentrations of samples determined by Bradford assay, as picomoles chloramphenicol acetylated/min/mg protein (graph). (The autoradiograph of the TLC plate (top) was cut up and rearranged for photography to simplify interpretation—the results presented were obtained under identical conditions at the same time)

5μg circular pBunS(+)CAT DNA was lipofected into BHK cells infected with vTF7-3 at 0, 1, 3, 5, 7.5 or 10 pfu/cell. In addition, 10 or 3μg of linearised and dephosphorylated plasmid was transfected into cells infected with vTF7-3 at 10 pfu/cell. CAT activity was detected in all cases except in the absence of vTF7-3 (Figure 5.6). In general the linearised plasmid DNA produced lower levels of CAT than circular plasmid. This may be due to less efficient uptake of linear DNA, compared to supercoiled plasmids, by cells. Another possibility was that the circular plasmids produced concatameric RNA transcripts by the T7 polymerase (known to be a highly processive enzyme) repeatedly transcribing around the plasmid. This would effectively increase the copy number of the transcript. To result in the higher CAT levels observed, the concatameric transcript would have to be capable of being translated by internal initiation. An alternative explanation is that the shorter transcripts derived from linearised template were more rapidly degraded from their 3' ends than longer transcripts produced from the circular templates.

For vTF7-3 infections, a slight increase followed by a slight decrease of activity was observed with increasing multiplicity used (Figure 5.6). From these results, 5 pfu/cell was adopted for use in further experiments. Any increased expression at higher multiplicities was felt to be outweighed by the increased cpe observed.

5.3 Expression of pBunS(+)CAT in bunyavirus infected cells.

The 30 and 29 terminal 5' and 3' nucleotides of the transcript expected from pBunS(+)CAT are identical to the respective termini of the Bunyamwera virus S segment cRNA. Although the genuine S segment has much longer non-coding regions (87 and 185 nt respectively for the 5' and 3' ends of cRNA), the 30 and 29 terminal nucleotides include at least the sequences which are conserved between all segments of all bunyaviruses (see Figure 1.9.1) and so would be expected to include the promoter and packaging signals. Under the correct conditions, the RNA transcribed from pBunS(+)CAT, might be recognised and replicated by Bunyamwera virus proteins.

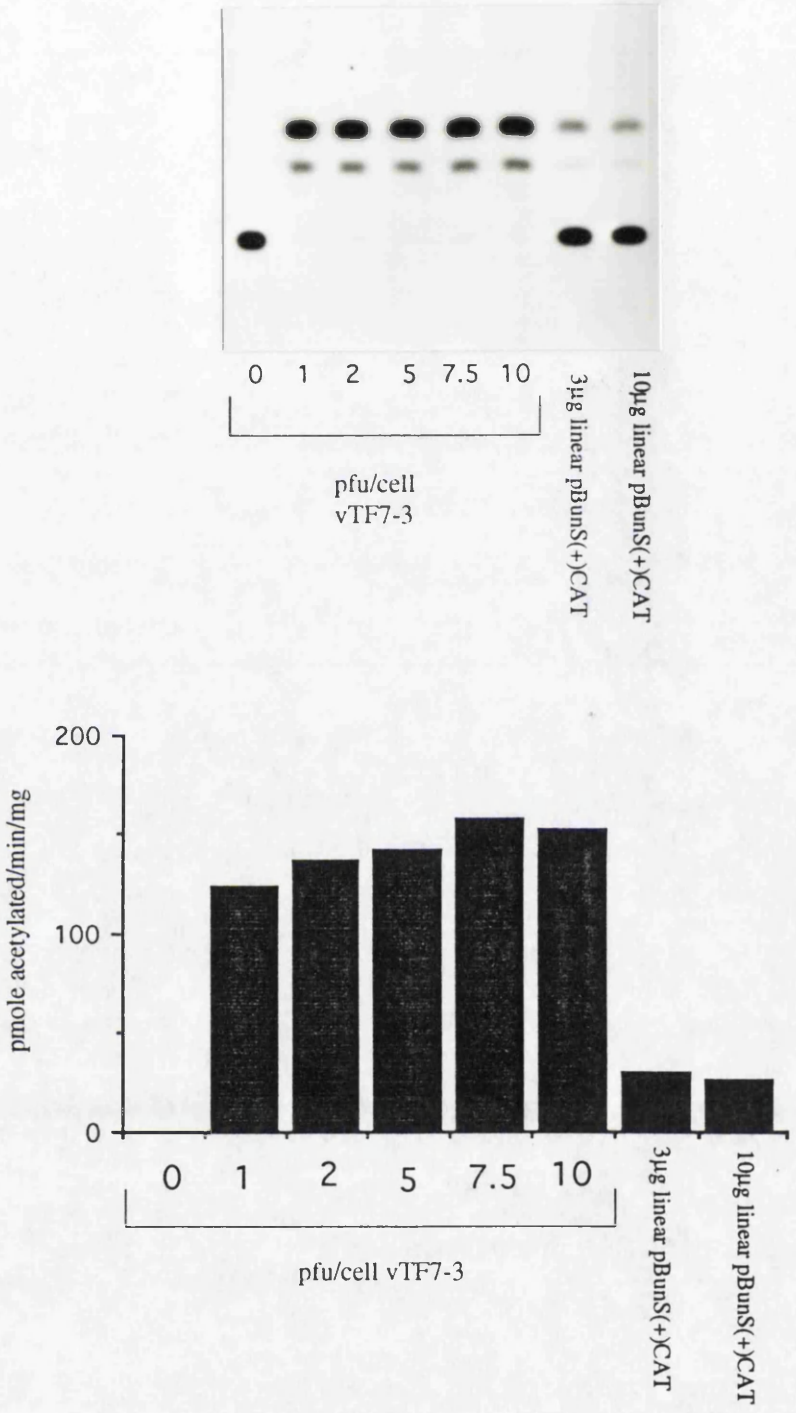


Figure 5.6 vTF7-3 dependent CAT expression. BHK cells were transfected with 3µg pBunS(+)CAT after infection of vTF7-3 at varying multiplicities to determine the optimum conditions for expression of the CAT transcript by T7 RNA polymerase encoded by the recombinant vaccinia virus. 3 or 10µg of linearised and dephosphorylated pBunS(+)CAT was transfected into cells infected with vTF7-3 at 10pfu/cell. These show reduced CAT activity compared with the transfection of circular plasmids.

This possibility was investigated by transfecting linearised pBunS(+)CAT into cells infected with both vTF7-3 and Bunyamwera virus. To increase the chances of the Bunyamwera virus infection being at the correct stage for recognition of the synthetic RNA, the Bunyamwera virus was added to cells either immediately preceding (-1 hour), or 12 hours before, transfection and infection with vTF7-3. Supernatants were harvested at 24, 48 or 72 hours post transfection and examined to see if any of the synthetic RNA had being packaged by progeny virions.

The levels of CAT activity in the transfected/infected cell sheets are shown graphically in Figure 5.7. Infection of cells with Bunyamwera virus clearly reduces the level of CAT activity (compare the second and third lanes to the first lane for each of the four panels of the graph). This reduction might have been due to encapsidation of the CAT RNA by N protein or to a non-specific reduction of non-bunyavirus expression (Figure 3.3 demonstrates the marked shut off of host cell and vaccinia virus gene expression in the presence of Bunyamwera virus). When supernatants from these transfections were used to infect fresh BHK cells, which were then assayed for CAT activity (to see if RNP containing CAT RNA had been packaged into virions), there was no evidence of Bunyamwera virus specific CAT activity. In fact, carry over of plasmid DNA, vTF7-3 and (presumably) transfection reagent, resulted in higher detectable CAT activity than those also infected with Bunyamwera virus (see Figure 5.7). Similar inhibition of CAT activity was observed when Bunyamwera virus infected cells were transfected with control plasmid pRR55 which contains no bunyavirus sequence—suggesting a non-specific shut off by Bunyamwera virus. It is assumed that if the synthetic RNA were recognised and encapsidated by N protein that it would then be recognised as a 'bunyavirus gene' and not be down regulated. This proposal could only be confirmed by achieving rescue of the RNA. No evidence of rescue occured here and efforts were directed to the second reporter constuct which is designed to produce CAT activity only if replicated by a bunyavirus. However, the use of the positive stranded CAT construct had at least allowed assessment of suitable transfection and vTF7-3 infection conditions.

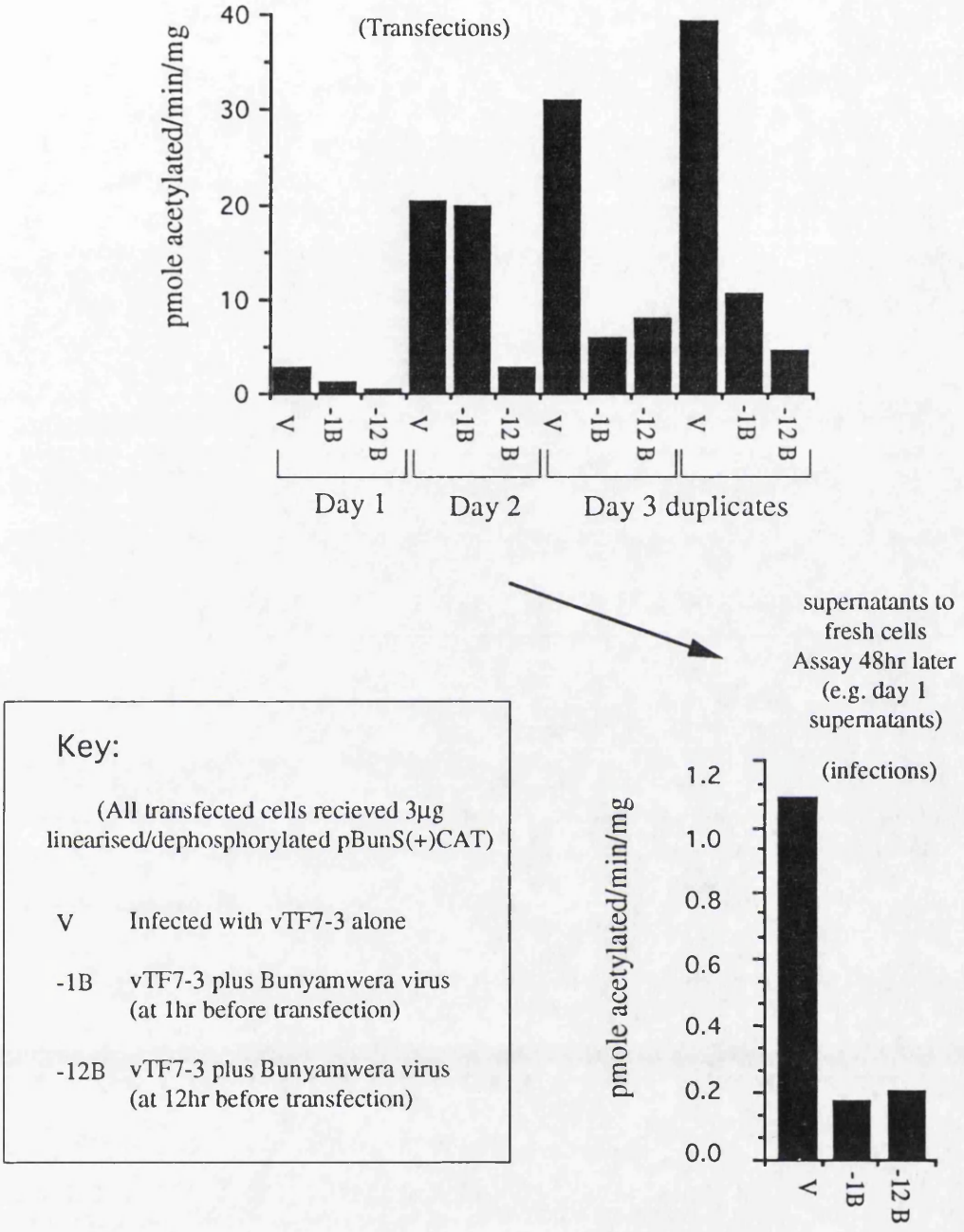


Figure 5.7 Expression of pBunS(+)CAT in the presence of replicating Bunyamwera virus. Transfections (top graph): BHK cells were infected with vTF7-3 at 5 pfu/cell to supply T7 RNA polymerase. In addition some cells were infected with Bunyamwera virus (10 pfu/cell) either immediately (-1hr) or 12 hours prior to transfection of all cells with 3µg linearised and dephosphorylated pBunS(+)CAT. After 24, 48 or 72 hours, cell sheets were harvested and assayed for CAT activity. In all cases Bunyamwera virus infection appeared to significantly reduce the level of CAT detected. Supernatants were retained and used to infect fresh cells (bottom graph). Activity was observed following addition of supernatant fluids from the transfected cells, to fresh cells which were then harvested 48 hr later. The activity for vTF7-3 infection alone was strongest and could be due only to carry over from transfection. Samples also infected with Bunyamwera virus showed markedly reduced activity.

5.4 Negative stranded Bunyamwera virus S segment / CAT RNA.

The initial breakthrough in negative strand RNA virus engineering was facilitated by a simple reporter system for detecting influenza virus dependent replication (Luytjes *et al* 1989; see Section 1.8.5). A chimeric RNA containing an anti-sense copy of the CAT open reading frame flanked by negative stranded (vRNA) influenza virus termini was constructed. Being of negative polarity, the plasmid derived RNA could not be translated to give CAT enzyme unless it was first copied to give a positive stranded (mRNA) transcript. Since RNA templated transcription does not normally occur in eukaryote cells, this copying step could only occur if the chimeric RNA was recognised by influenza virus proteins. Having a sensitive and absolute reporter system for the influenza virus dependent copying step (i.e. assaying for the presence of CAT enzyme), it was possible to define conditions for associating viral proteins with the chimeric RNA, to reconstitute a synthetic RNP and replicate it using a wild type influenza helper virus.

To apply the reporter system to Bunyamwera virus, assuming the transcription and translation signals lie in the non-coding regions of viral segments, it was decided to base the construct on the most highly expressed segment (the S segment) to increase the levels of reporter gene expression.

5.4.1 Design of pBunS(-)CAT

The precise design for the chimeric construct was based on the following criteria:

i) Transcription by T7 RNA polymerase should give a negative stranded (vRNA) transcript containing an antisense copy of CAT such that translation competent mRNA can only be made if the transcript was copied by bunyavirus proteins. T7 RNA polymerase was chosen as it would permit both *in vitro* and *in vivo* (by vTF7-3 infection) transcription from a defined and stringent promoter.

ii) The termini of the chimeric RNA should contain the same sequences as the Bunyamwera virus S segment with terminal bases exactly mimicking viral segments. This would require precise initiation and termination of the T7 transcript.

iii) Since the *cis* signals required for bunyavirus transcription, translation, encapsidation by N protein and packaging into virions are not clearly defined, as

much as possible of the non-coding S segment sequence should be present. The ideal would be an exact replacement of the N protein open reading frame with CAT coding sequence.

5.4.2 Transcript 5' end tailoring

At the transcription start site, T7 RNA polymerase usually initiates (+1) on a guanosine residue immediately downstream of the tightly conserved 17bp promoter. In bacteriophage T7 the next two transcribed nucleotides are also G (Dunn & Studier 1983; Hamm *et al* 1990). For all bunyavirus segments, the 5' terminal nucleotide is an adenine residue followed by guanosine then uridine. Influenza virus cRNA transcripts, which have the same first five 5' nucleotides as bunyavirus RNA, had been generated by other workers using T7 RNA polymerase. Two groups assessed initiation on an A residue by demonstrating incorporation of ^{32}P supplied as gamma labelled ATP (Luytjes *et al* 1989; Seong and Brownlee 1992a). The required sequences were therefore expected to be obtainable by replacing the usual G triplet with viral sequence (see Figure 5.8). Since the first four nucleotides are identical to those used by influenza virus and since T7 influenza virus transcripts had been successfully replicated by viral proteins, no attempt was made to map the 5' end of the transcripts produced from the Bunyamwera virus construct.

5.4.3 Transcript 3' end tailoring.

For the Bunyamwera virus S segment plasmid constructs described in Chapter 3, the 3' end had been defined by run off transcription from a linearised plasmid. The blunt-ended run-off DNA end was generated by using mung bean nuclease to remove the 5' single-stranded extension remaining after XbaI digestion of an appropriately positioned site (Figure 5.2). While this approach is adequate in principle, the precise conditions for nuclease digestion were difficult to control and monitor, and it was felt preferable to simplify this step in the new construct. For T7 RNA polymerase, a run-off end which is blunt or 3' recessed is preferable to reduce the possibility of the enzyme transferring template strands and extending the transcript with complementary bases (Promega 1991). This limited the means of producing template with correct end sequence since for a 3' recessed cutting restriction enzyme, most of the enzyme recognition sequence would remain in the 5' overhang and would be transcribed by the polymerase. A search of available restriction enzyme recognition sites ruled out a fortuitous match of an enzyme site

to the terminal sequences required and so a different approach was sought. Attention was turned to restriction enzymes which have separated cleavage and recognition sites. BbsI was chosen as it does not cut the vector nor planned insert and its non-palindromic recognition site could be inverted and placed down stream of the insert to cleave a 5' overhang ending at the precise final template base required (see Figure 5.9)

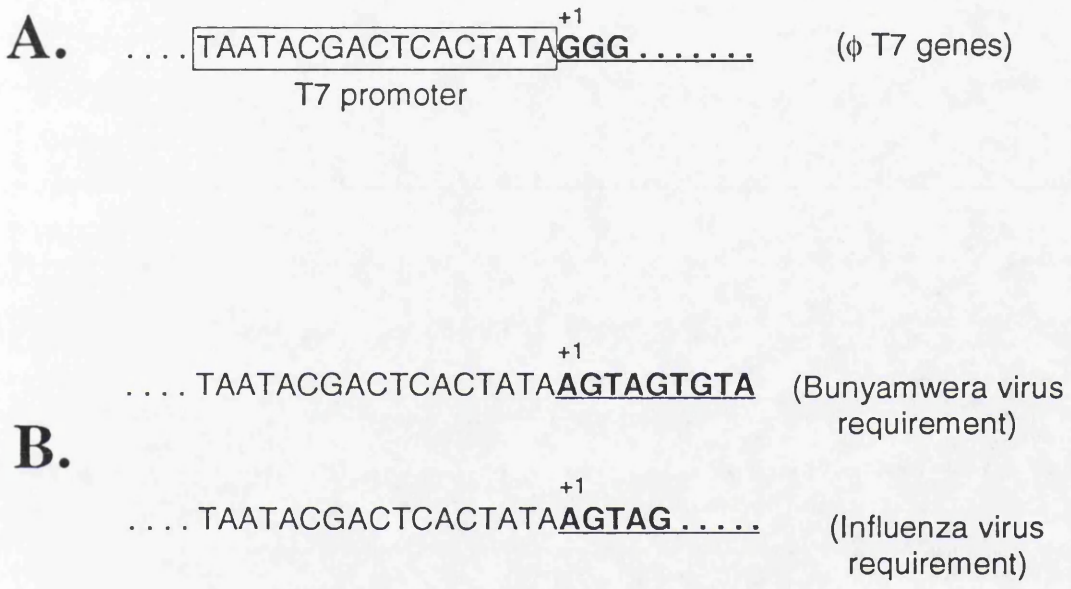


Figure 5.8 T7 promoter initiation and bunyavirus cDNA requirements. The 17nt core promoter is written 5'–3' top strand. Sequences transcribed into RNA are in bold type with the 1st transcript base shown labelled +1. A shows the optimum initiation nucleotides—three G residues, as found in bacteriophage T7 (Dunn & Studier 1983; Hamm *et al* 1990). B shows the alternative bases required for transcription of bunyavirus or influenza virus-like transcripts. These are common for the first five nucleotides and influenza virus transcripts produced in this way have been successfully replicated by influenza virus (Luytjes *et al* 1989 suggesting that the arrangement should function to produce bunyavirus transcripts initiating on the correct nucleotide.

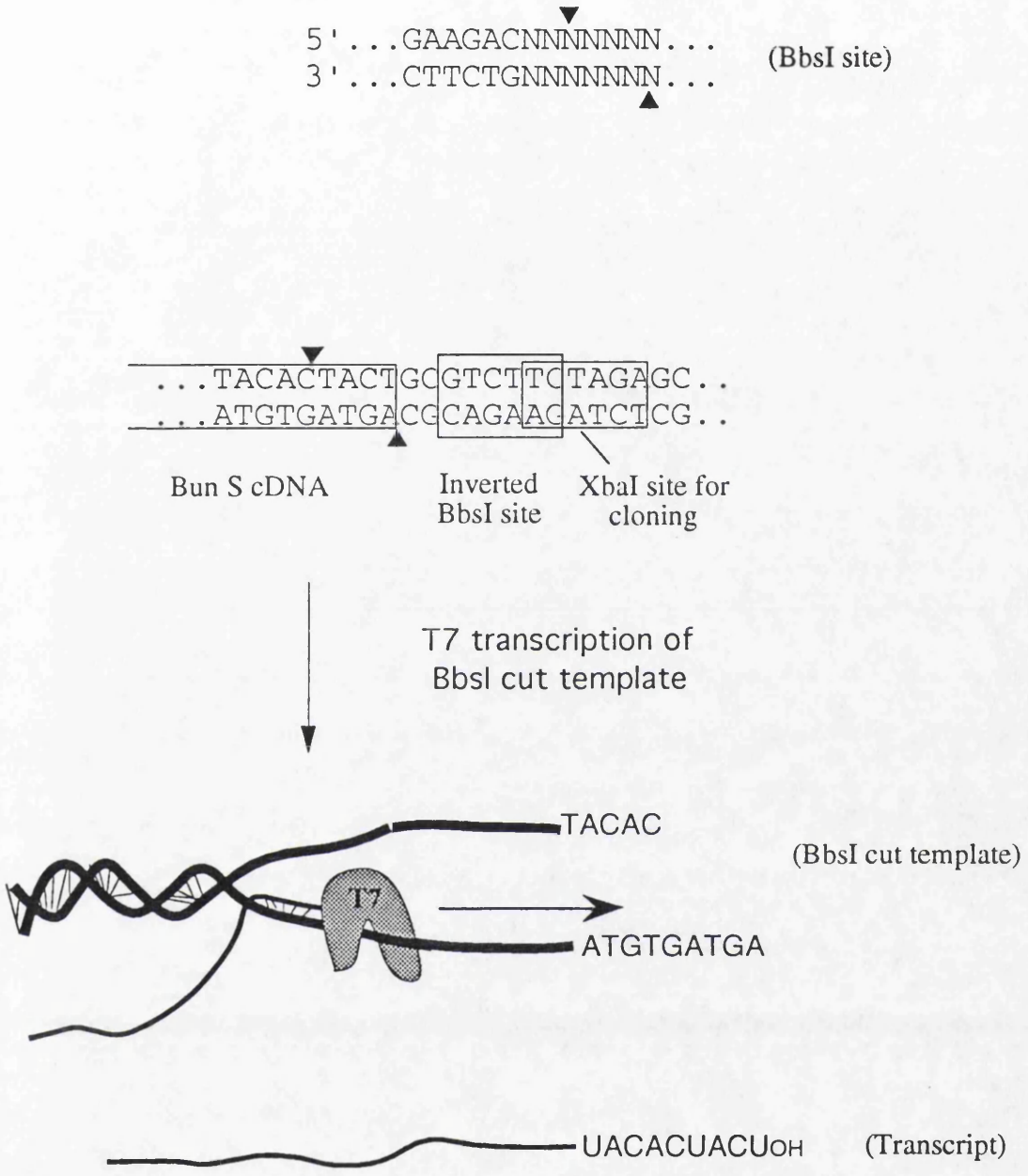


Figure 5.9 BbsI restriction site for run off transcription. Because the recognition site is separated from the sequence-independent cleavage site, the non-palindromic site of BbsI could be positioned to produce the required cDNA terminus with a 5' overhang. Transcription from a template prepared by a single cleavage reaction gives a transcript with the exact 3' end sequence required for bunyavirus vRNA. The sequence shown, including the XbaI site for fragment cloning were used for the pBunS(-)CAT construct described in this chapter.

5.5 Construction of pBunS(-)CAT

The negative stranded CAT/Bun S construct was synthesised using existing DNA constructs and four oligonucleotides. Two oligonucleotides were used to recombine the CAT open reading frame into the S segment at the start and stop codons for N protein, thereby replacing it. The other two oligonucleotides introduced the T7 promoter and BbsI restriction site for run off transcription. A multi step PCR procedure was employed for the *in vitro* recombination of the component fragments at the precise positions required (see Figure 5.10). To lessen the likelihood of accumulating mutations during repeated amplification cycles, two precautions were taken:

i) Taq DNA polymerase was replaced with Vent™ thermostable polymerase (New England Biolabs) as this enzyme has a higher fidelity due to its 3'–5' exonuclease 'proof-reading' activity which Taq lacks (Gelfand 1989; Mattila *et al* 1991).

ii) For each step, several template concentrations were employed and each amplified for different numbers of cycles. The reaction which had undergone the fewest rounds of amplification, while still giving a clean product on gels, was used for subsequent steps. In this way the total number of cycles employed, and therefore the number of opportunities for mutation to arise, was kept to a minimum.

The first step was to amplify the CAT open reading frame with oligonucleotides to tail the CAT sequence with short regions identical to those flanking the N protein open reading frame in the S segment. This step also removed the Tn9 flanking sequence. This stage was achieved by only eight cycles of PCR (Figure 5.11). Next, in separate reactions, each terminus of the S segment was recombined with the Bun-tailed CAT fragment using the relevant terminal primer (which included T7 promoter or BbsI site) together with the distal CAT primer. This generated two overlapping fragments both containing the CAT coding sequence recombined with either the 3' or 5' non coding S segment sequences together with the relevant terminal modifications (Figure 5.12). These fragments were recombined by mixing and amplifying with the segment terminal primers (Figure 5.13). The whole fragment was digested with XbaI (one site, adjacent to the BbsI site) and ligated as a blunt ended/XbaI fragment into XbaI and SmaI digested pUC118. The final fragment had under gone a maximium of 40 PCR rounds with the S segment regions only being subjected to 32 rounds. Sequencing of the termini into the CAT open reading frame, showed that the recombination was correct and that no point mutations had occured.

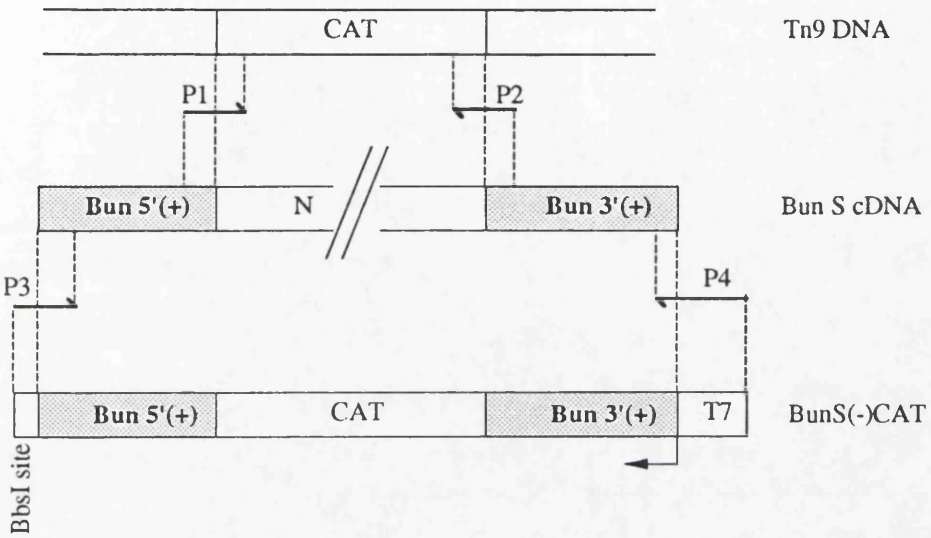


Figure 5.10 Templates and primers for construction of pBunS(-)CAT.The relationship between the primers used (P1 to P4) and the DNA templates is shown. Primers are drawn as lines with half arrow heads at their 3' ends. P1 and P2 are designed to amplify the CAT open reading frame from a fragment of transposon Tn9 and tail it with short regions of Bunyamwera virus S segment immediately adjacent to the N protein coding region. The sequences of the primers are as follows: **P1**: 5' GGTCATTAAAGGCTCTTTAATGGA GAAAAAAATCACTGG —underlined sequence is Bunyamwera virus S segment cDNA nucleotides 67–85 which immediately preceed the N protein open reading frame. The remainder of the sequence is the first 20 nucleotides of the CAT open reading frame starting with the ATG initiation codon. **P2**: 5' GCCCGATTAAAAATGCATCCCTGCTTACGCCCCGCCCTGCC ACT —underlined sequence is the complement of Bunyamwera virus S segment cDNA nucleotides 787–811 which immediately follow the N protein open reading frame. The remainder of the sequence is the complement of the last 20 nucleotides of the CAT open reading frame starting with the complement of the stop codon. **P3**: 5' GCTCTAGAAGACGCAGTAGTGT ACTCCACAC —Underlined sequence is the first 17 nucleotides of Bunyamwera virus S segment cDNA. The remainder of the sequence codes for a XbaI cloning site and an inverted BbsI site to allow cleavage at the viral cDNA terminus for run-off transcription (see Figure 5.9). **P4**: 5' CGGAATTCTAATACGACTCACTATAAGTAGTGTGCTCCACCT —Underlined sequence is the complement of the last 17 nucleotides of the Bunyamwera virus S segment cDNA. This is immediately preceeded by a T7 promoter (17 nucleotides ending TATA) and an EcoRI restriction site. See Figures 5.11 to 5.13 for construction steps.

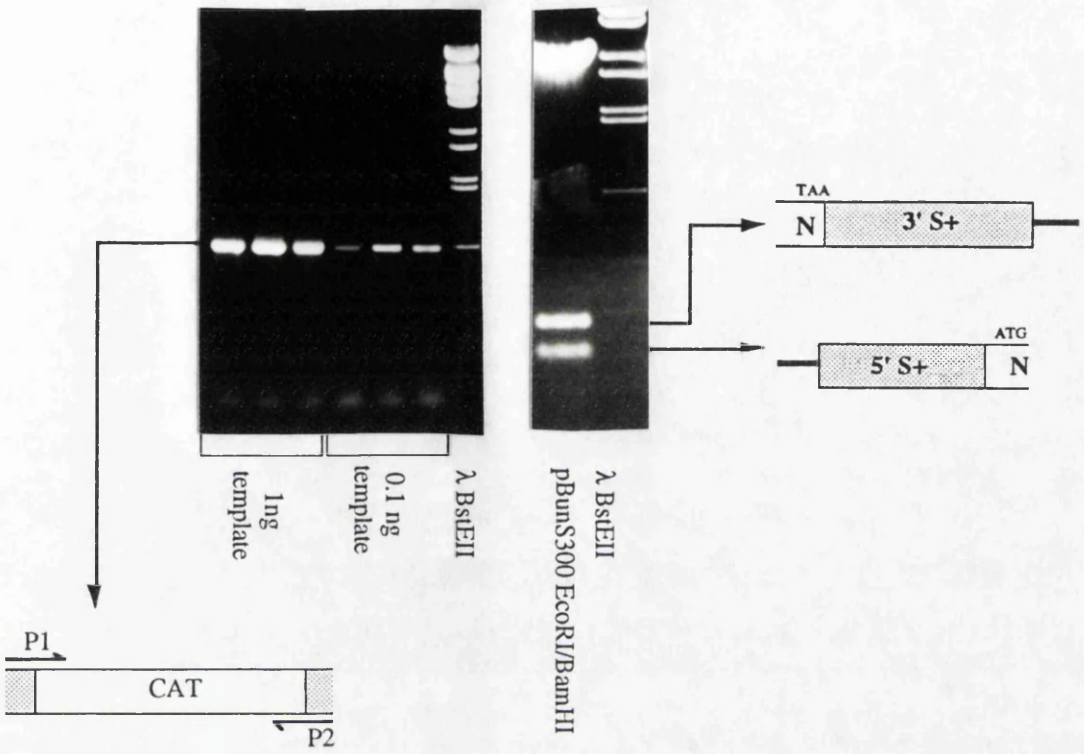


Figure 5.11 Construction of pBunS(-)CAT. Figure shows the generation of three DNA fragments used in the construction of pBunS(-)CAT. A fragment containing the CAT gene tailed with short Bunyamwera virus S segment sequences which usually lie immediately adjacent to the N protein open reading frame, was generated from Tn9 DNA by PCR using primers P1 and P2 (see Figure 5.10 for primer sequences). The left gel shows the DNA after eight amplification cycles starting with 1 or 0.1ng Tn9 DNA. Bunyamwera virus S segment cDNA termini containing both complete non-coding regions and a few codons at either end of the N protein open reading frame, were generated by restriction digest (right gel) of pBunS300, an engineered cDNA in which most of the N protein coding region has been deleted but the start and stop translation codons remain (A. McGregor & R.M. Elliott personal communication). Figures 5.12 and 5.13 show the use of these three fragments to produce pBunS(-)CAT.

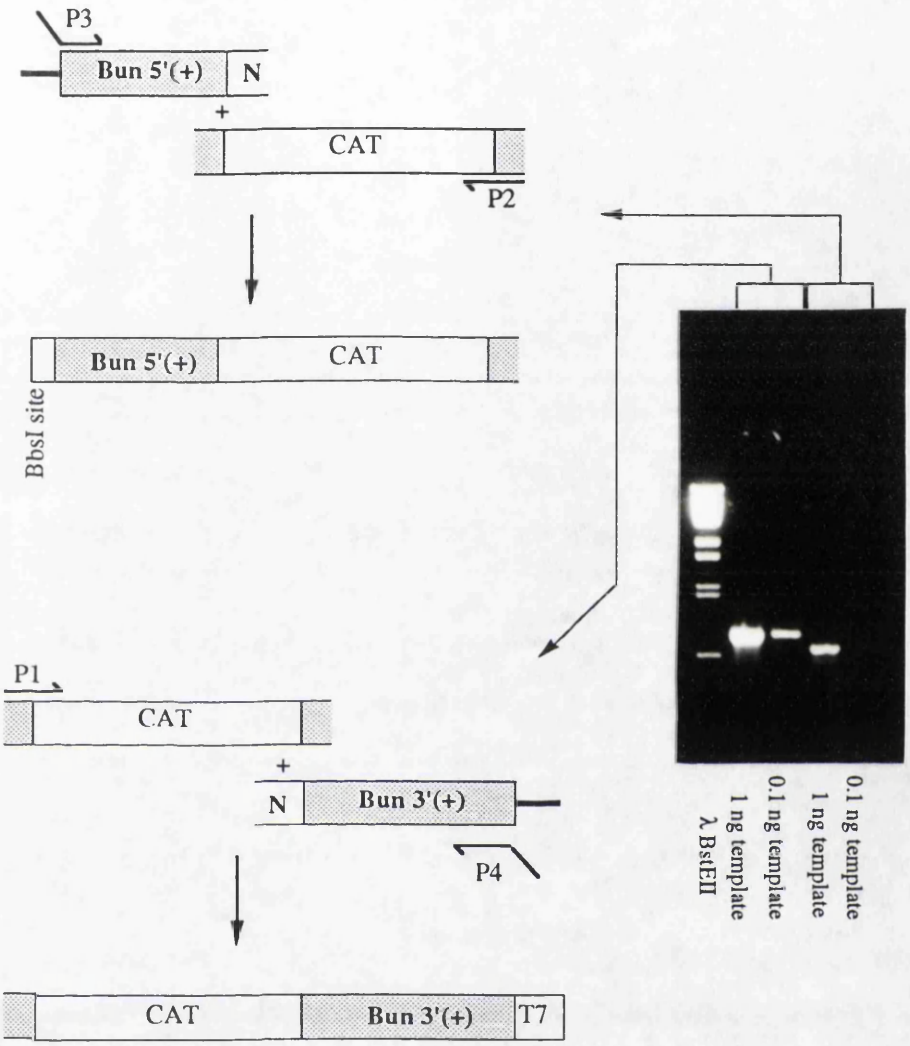


Figure 5.12 *In vitro* recombination of CAT gene DNA with Bunyamwera virus S segment cDNA termini. The Bun-tailed CAT fragment from Figure 5.11 was recombined with the Bunyamwera virus S segment non-coding regions in separate PCR reactions to give two over lapping fragments. The primers used also introduced T7 promoter or BbsI restriction site into the relevant fragment (see Figure 5.10 for primer sequences). The gel shows recombination and amplification of the two fragments after 12 PCR rounds. Figure 5.13 shows final recombination of the two fragments generated here to give pBunS(-)CAT.

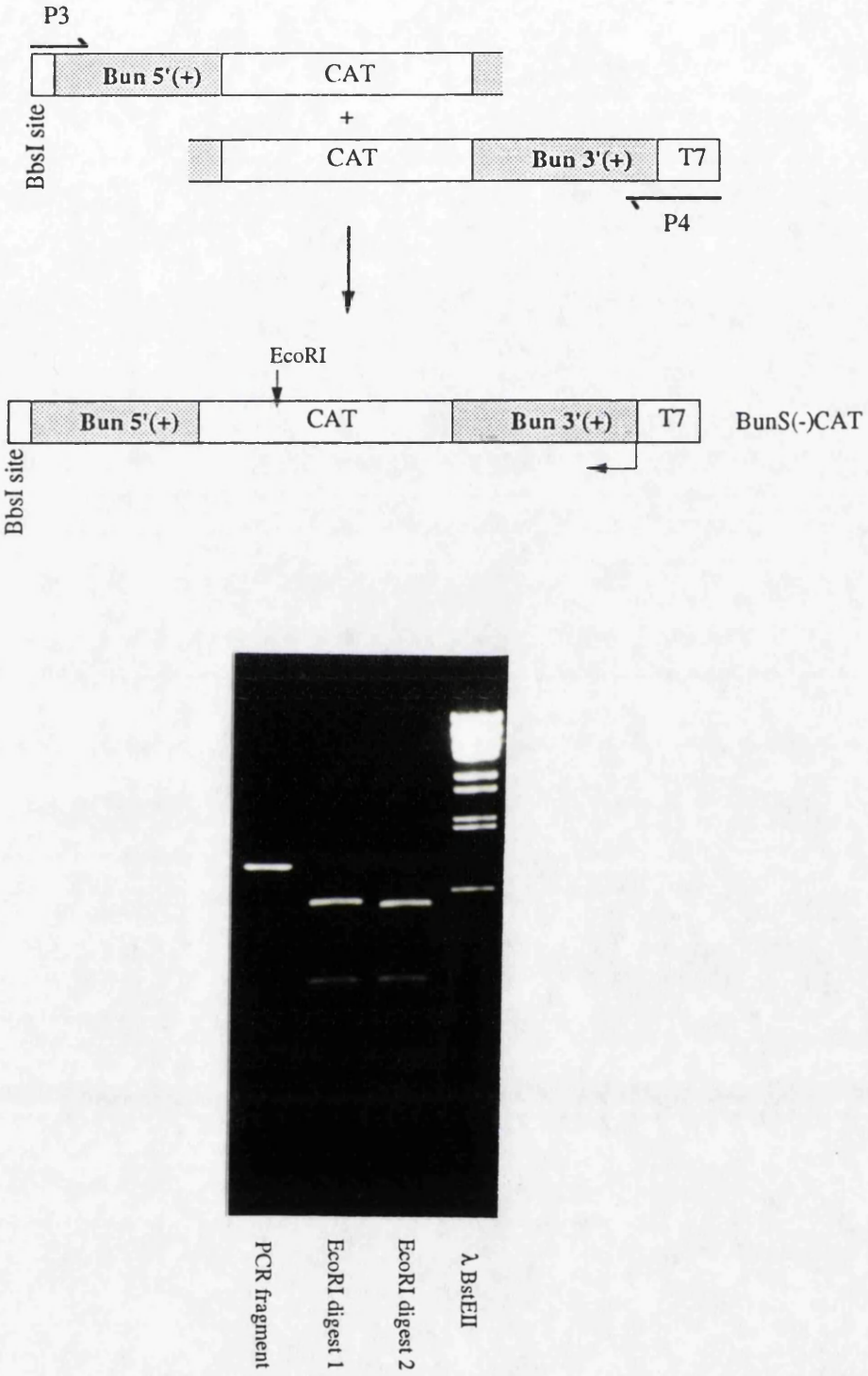


Figure 5.13 Final recombination to produce pBunS(-)CAT. The overlapping fragments generated in figure 5.12 were mixed and recombined by PCR using primers P3 and P4. The gel shows the final PCR product and EcoRI digestion pattern EcoRI digestion was done to ensure, prior to cloning, that the correct fragment was amplified since any contaminating Bunyamwera virus S segment cDNA would be efficiently amplified by the primers used and the expected size would be very similar—no EcoRI site is present in BunS cDNA. The intact fragment was purified from the gel digested with XbaI (site adjacent to BbSI site for cloning purposes), 5' phosphorylated and cloned to SmaI and XbaI cut pUC118.

5.6 Attempt to demonstrate bunyavirus-dependent replication of pBunS(-)CAT RNA

BbsI cleaved template DNA was transfected into BHK cells infected with vTF7-3 and Bunyamwera virus to see if the negative stranded RNA transcribed by T7 RNA polymerase could be used as template for bunyavirus-dependent transcription. In the absence of coinfecting Bunyamwera virus, vTF7-3 should produce negative stranded transcripts incapable of being translated to give CAT activity. Only if Bunyamwera virus recognised and replicated the transcript would CAT activity be detected. As a positive control, pBunS/CAT(+)DNA was transfected into vTF7-3-infected cells. The results of the transfections/infections are shown in Figure 5.14.

It was immediately apparent that the pBunS(-)CAT was not behaving as expected since CAT activity was observed when the construct was transfected into cells infected with vTF7-3 alone. In this situation, only negative stranded transcripts would be expected from the T7 promoter. The 'no DNA' and 'no virus' controls were negative indicating that the activity was dependent on the presence of both pBunS(-)CAT and vTF7-3. To investigate whether the activity was dependent on the expression of T7 RNA polymerase, by vTF7-3, or of some other activity of vaccinia virus, pBunS(-)CAT was transfected into cells infected with either vTF7-3 or non-recombinant vaccinia virus (WR strain). pBunS(+)CAT, which is expected to produce CAT activity dependent on T7 RNA polymerase, was also included as a control. Figure 5.15 shows that for pBunS(+)CAT, activity is dependent on vTF7-3 with only very low level acetylation occuring in the presence of only the WR strain of vaccinia virus. In contrast, strong activity was detected on transfection of pBunS(-) CAT into cells infected with either vTF7-3 or vaccinia virus WR strain. This demonstrated that the RNA giving rise to CAT activity from pBunS(-)CAT was due to a usual product of vaccinia virus infection and was not dependent on the presence of T7 RNA polymerase.

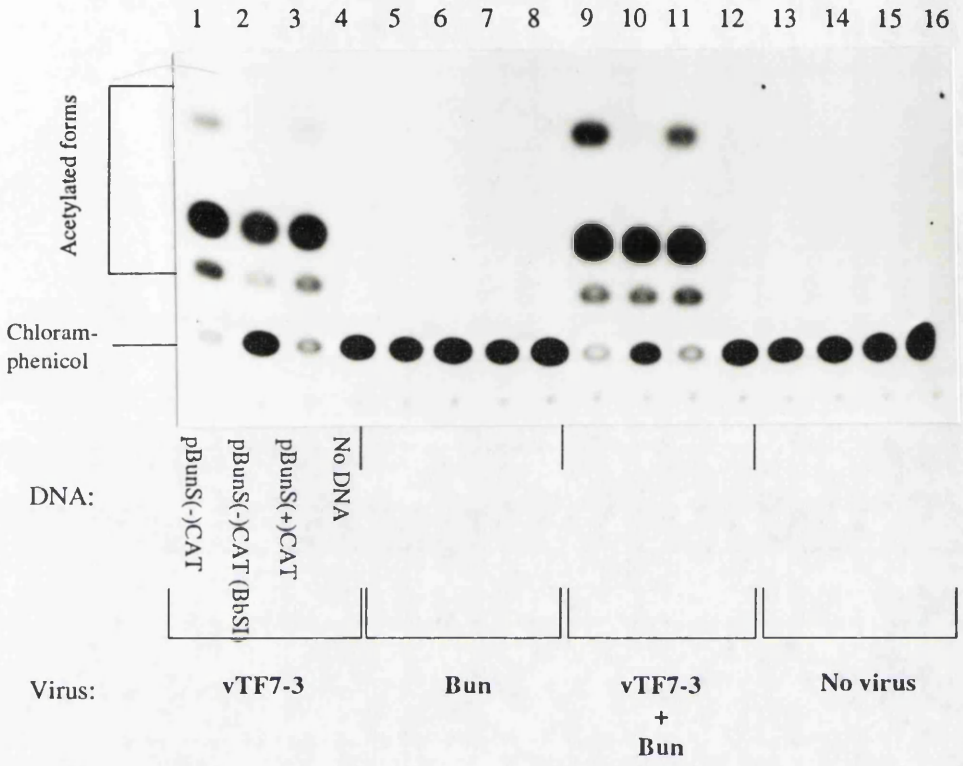


Figure 5.14 *In vivo* expression of pBunS(-)CAT. BHK cells were transfected with uncut (lanes 1, 5, 9 and 13) or BbsI linearised (lanes 2, 6, 10 and 14) pBunS(-)CAT or with pBunS(+)CAT (lanes 3, 7, 11 and 15). Cells were also infected with vTF7-3 (lanes 1–4), Bunyamwera virus (lanes 5–8), vTF7-3 and Bunyamwera virus (lanes 9–12) or no virus (lanes 13–16). The experiment was designed to see if the negative stranded T7 transcript expected during vTF7-3 infection could be replicated by Bunyamwera virus proteins and produce positive stranded RNA which could be translated to give CAT enzyme. While the no virus and Bunyamwera virus alone controls were negative as expected, activity from pBunS(-)CAT was detected, dependent on vTF7-3 infection and independent of co-infection with Bunyamwera virus.

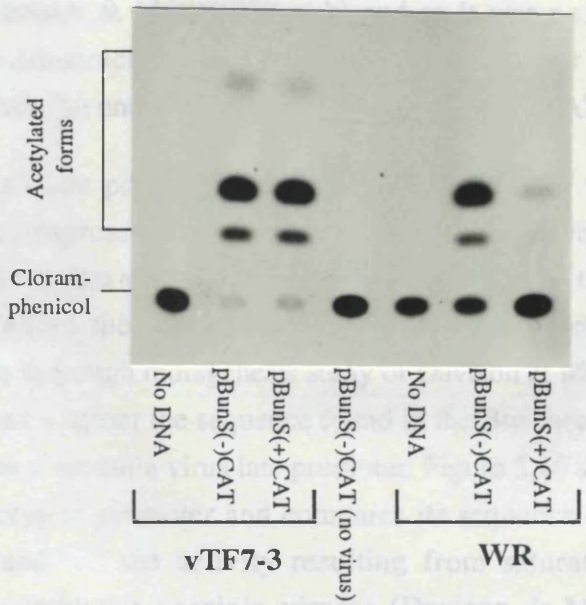


Figure 5.15 Expression of CAT from pBunS(-)CAT by vaccinia virus. BHK cells were transfected with either pBunS(+)CAT, which expresses CAT in the presence of T7 polymerase supplied by vTF7-3, or pBunS(-)CAT from which T7 transcripts are anti sense to CAT mRNA. Infection with vTF7-3 (lanes 1-3) resulted in CAT expression from pBunS(+)CAT as expected (lane 3). CAT activity was also detected in vTF7-3 infected cells transfected with pBunS(-)CAT (lane 2)). Transfection in the absence of vaccinia virus (lane 4) resulted in no detectable CAT activity while infection with wild type (WR strain) vaccinia virus (lanes 5–7) resulted in strong expression of CAT from pBunS(-)CAT (lane 6) demonstrating that a vaccinia virus protein (not T7 RNA polymerase which is only present during vTF7-3 infection) is the cause of the observed activity. Very low level CAT activity was also detected for pBunS(+)CAT in the presence of WR strain vaccinia virus although the activity was negligible compared to vTF7-3 dependent expression as expected. This suggested a cryptic vaccinia virus promoter was present in pBunS(-)CAT.

Vaccinia virus gene expression is temporally controlled from two types of promoter (early or late, with constitutively expressed genes having both types of promoter). Both early and late vaccinia virus promoters have been well characterised (Davison & Moss 1989 a, b) and so it was possible to examine sequences in the construct to see if a cryptic vaccinia virus promoter existed which might explain the unexpected expression of pBunS(-)CAT.

The vaccinia virus late promoter contains a TAAAT motif which if mutated drastically reduces expression from it (Davison & Moss 1989b). This motif was found in the Bunyamwera virus S segment cDNA upstream of the N protein open reading frame. While the flanking sequences differed from those found in vaccinia virus, the thorough mutagenesis study of Davison & Moss (1989b) made it possible to assess whether the sequence found in the Bunyamwera virus cDNA would likely act as a vaccinia virus late promoter. Figure 5.16 shows the position of the potential cryptic promoter and compares its sequence with the vaccinia virus promoter and the activity resulting from saturation mutagenesis performed in recombinant vaccinia viruses (Davison & Moss 1989b). For vaccinia virus, mutations to the sequence flanking the critical TAAAT motif produce either an increase or decrease of gene expression. When the sequences found flanking the TAAAT motif of the Bunyamwera virus cDNA were applied to the findings of the mutagenesis study (stars on relevant columns in the graph of Figure 5.16) only one nucleotide would be expected to result in significantly decreased promoter activity while four are associated with greatly enhanced expression (3–4 fold stimulation). The mutagenesis data was compiled from the effects of single point mutations but Davison & Moss (1989b) also tested activity of the combined predicted optimum flanking sequences and found activity increased 6-fold which was greater than any of the component single site alterations. This suggests that the effect of the single unfavourable nucleotide in the Bunyamwera virus cDNA sequence (the C residue immediately 3' to the TAAAT motif—which still produces ~30% activity of wild type), might easily be compensated for by the four extremely favourable bases present at other positions (the 'activity product' of each is ~60-fold greater than wild type vaccinia virus sequence).

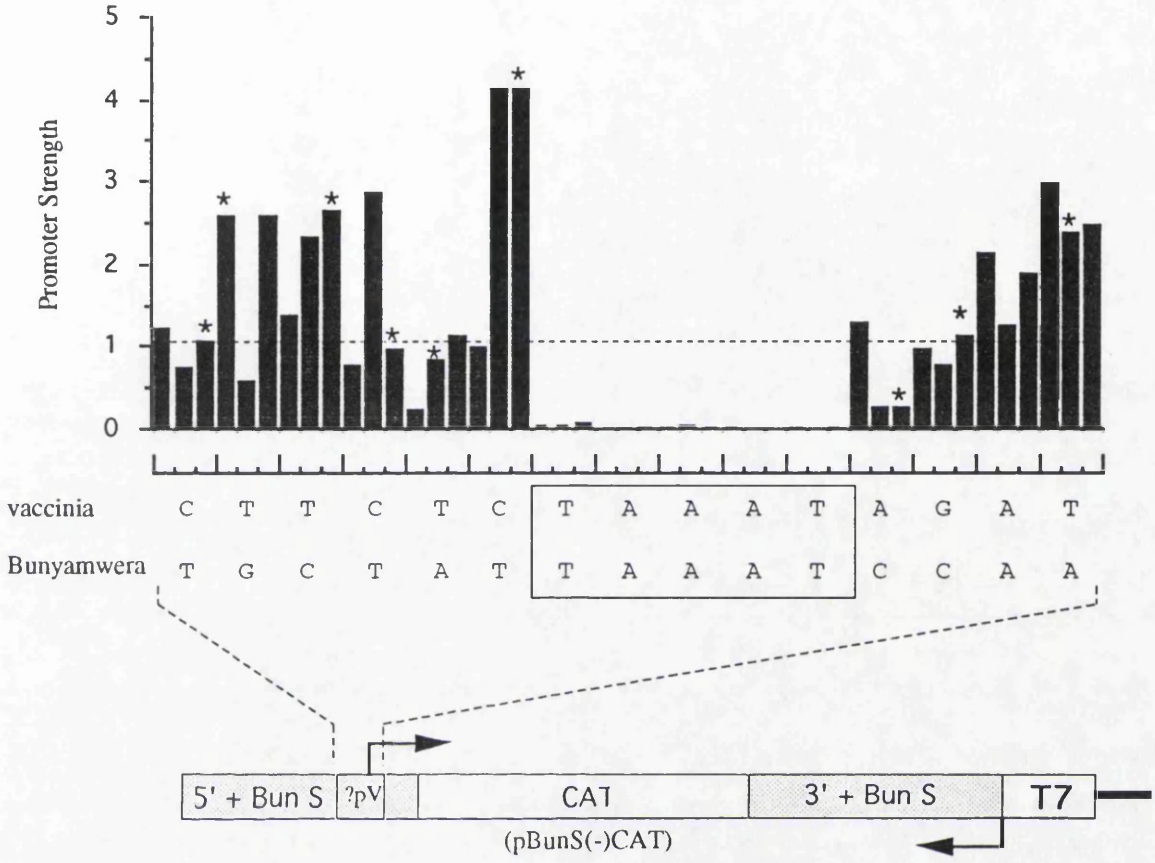


Figure 5.16 Cryptic vaccinia virus promoter in Bunyamwera virus cDNA. After vaccinia virus dependent CAT expression was detected from pBunS(-)CAT (see Figure 5.15), examination of the construct sequence revealed a potential cryptic vaccinia virus promoter in the 5' non-translated region of the Bunyamwera virus S segment cDNA (?pV on construct map). Transcripts initiating in this region would be expected to contain translation competent CAT sequences. The Bunyamwera virus sequence contains the critical TAAAT motif found in the vaccinia virus 28kD late promoter (boxed sequence in line up of vaccinia and Bunyamwera virus sequences). The Bar chart shows the effect of saturation mutatagenesis of the promoter or flanking nucleotides in vaccinia virus recombinants expressing β galactosidase from the 28kD promoter (Davison & Moss 1989b). The basal level for wild type promoter is 1 (dotted line) while the activity associated with each of the other three possible nucleotides (in the order GATC with the wild type base left out) at each position, is shown as vertical bars. Mutation of the TAAAT motif drastically reduces activity while alteration of flanking sequences either increases or decreases activity (bars extending above or below the basal level dotted line respectively). Flanking mutations equivalent to substitution with corresponding Bunyamwera virus nucleotides are marked with stars at the top of the relevant column.

5.7 CAT reporter constructs : summary

A construct, pBunS(+)CAT, was assembled which gives a Bunyamwera virus S segment cRNA-like transcript containing a copy of the bacterial reporter gene CAT. This construct was used in transfection experiments to determine suitable expression conditions. Transfection using Transfectace™, a comercially available liposome preparation, resulted in higher levels of expression than obtained by conventional calcium phosphate mediated transfection. Various multiplicities of infection with vTF7-3 were examined to supply T7 RNA polymerase *in vivo* while keeping vaccinia virus-induced cpe to a minimum: 5pfu/cell was found to permit efficient expression of CAT from the reporter construct.

Packaging of the synthetic reporter RNA into Bunyamwera virus particles could not be demonstrated. Carry-over in supernatants of transfected cells of DNA, vTF7-3 and transfection facilitator resulted in significant transfer of activity from cells not infected with Bunyamwera virus. Where Bunyamwera virus was present, CAT activity was significantly diminished. The higher CAT levels found in the absence of Bunyamwera virus prevented identification of any Bunyamwera virus specific activity.

A second construct was built, pBunS(-)CAT, which was designed to produce CAT activity only if T7 transcribed RNA could be copied by Bunyamwera virus proteins. Transfection of this construct into vTF7-3 infected cells produced CAT activity even in the absence of Bunyamwera virus. This unpredicted activity was found to be due to a vaccinia virus protein other than T7 RNA polymerase present in the recombinant vTF7-3, since it could also be induced by infection with non recombinant (WR strain) vaccinia virus. Comparison of the construct sequence with the well characterised promoter sequences of vaccinia virus indicated a potential vaccinia virus late promoter-like sequence in the 5' non-coding region of the Bunyamwera virus S segment cDNA, just upstream of the protein coding sequences. This sequence is thought to act as a cryptic promoter producing positive stranded (mRNA like) transcripts from pBunS(-)CAT in the presence of vTF7-3 and so account for the unexpected CAT activity.

The potential cryptic promoter sequence was also present in the whole segment constructs investigated in Chapters 3 and 4 and so would be expected to have given rise to incorrect RNA transcripts in these earlier studies and may therefore have been partly responsible for the failure of this approach.

Chapter 6: *In vitro* RNP reconstitution

As an alternative to transcribing RNA *in vivo* in the presence of replicating helper virus, attempts were made to assemble RNP *in vitro* using viral proteins and synthetic RNA. Transfection into virus infected cells of RNP reconstituted *in vitro* may allow Bunyamwera virus specific replication of synthetic RNA. This was the approach used for the rescue of synthetic RNA by influenza virus (Luytjes *et al* 1989). For Bunyamwera virus, the ability to reconstitute RNP *in vitro* may prove particularly useful since it has been shown previously that RNP can be transfected into cells and replicated by L protein expressed by a recombinant vaccinia virus (Jin & Elliott 1991). This may allow expression of synthetic RNP without the need for a replicating helper Bunyamwera virus and so may ultimately allow rescue of whole virus by *in vitro* transcription of cDNAs corresponding to all three genome segments.

6.1 CsCl density gradient analysis to assess bunyavirus RNP reconstitution.

As discussed in Section 1.9, bunyavirus nucleocapsids are stable in high salt concentrations and can be isolated from infected cell lysates or disrupted virions by centrifugation through preformed CsCl gradients. In contrast, the RNP of orthomyxoviruses dissociate into protein and RNA components under these conditions, while for other negative strand RNA viruses such as members of the *Rhabdoviridae* and *Paramyxoviridae* RNP stability in high salt concentrations is also observed. RNP bands at a density of 1.31g/ml while RNA pellets (>1.4g/ml) and free protein is found as a diffuse band towards the top of the gradient. For a TST41 rotor centrifuge tube, a 6ml preformed gradient containing 20–40% (w/w) CsCl overlayed with 5ml sample/low salt buffer will give densities of ~1.45g/ml–1.1g/ml over the original 6ml volume. Following centrifugation to equilibrium, the RNP bands at the centre of the gradient (see Figure 6.1). For Bunyamwera virus sufficient intracellular RNP is present in the cell monolayer of one or two large (175 cm^2) flasks to allow visual detection.

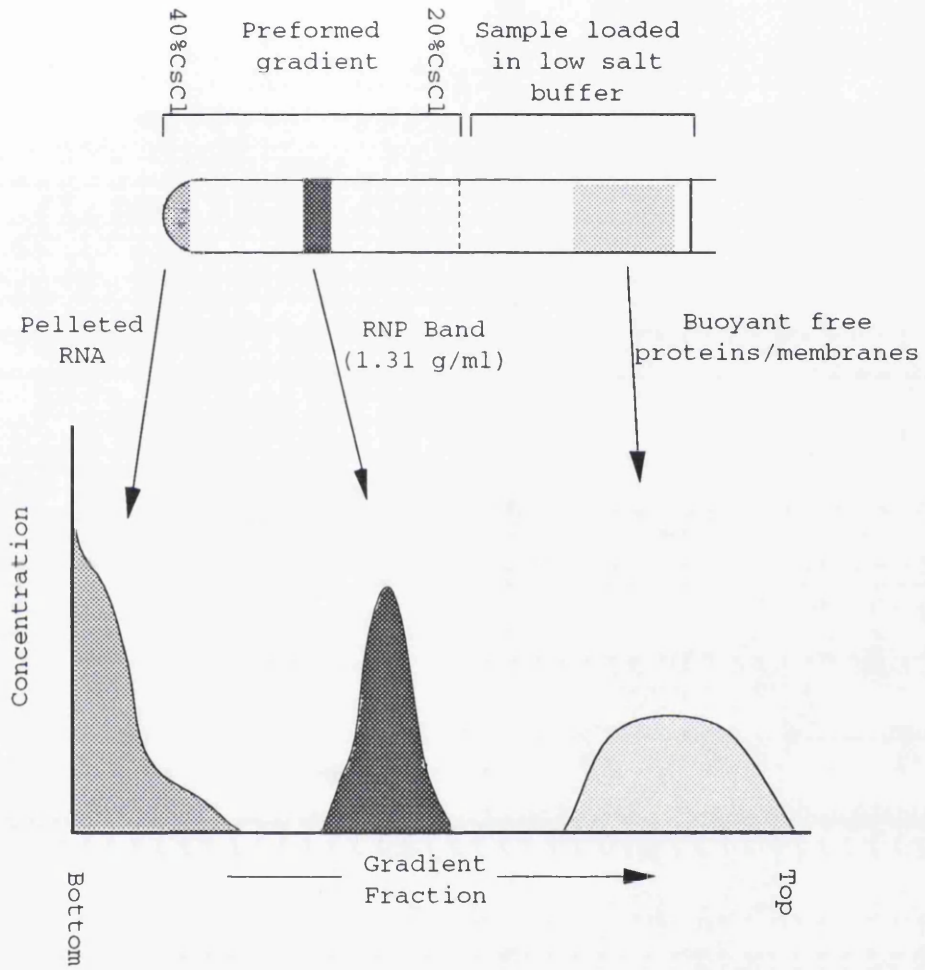


Figure 6.1 Banding of Bunyamwera virus RNP in CsCl density gradients. Diagramatic representation of the distribution of protein and RNA following centrifugation through continuous preformed 20–40% (w/w) CsCl density gradients. Cellular and viral mRNA pellets while RNA encapsidated as RNP bands at a characteristic density below free proteins and small molecules. The top portion represents a TST41 gradient tube in which an infected cell lysate has been loaded and separated into protein, RNA and RNP components. The graph shows the type of profile found by quantifying protein and RNA in fractions of the gradient.

Smaller amounts of RNP can be detected, by scintillation counting of gradient fractions, if either their RNA or protein component is radioactively labelled. The density of each fraction can be determined by refractive index measurement which is related to concentration, and therefore density, of a given salt solution.

For reconstitution experiments labelled RNA was transcribed *in vitro* by including either $\alpha^{32}P$ or $^{35}S(\alpha S)$ labelled NTP. When applied to gradients as naked RNA, the label was found at the bottom of the gradient (RNA pellet) and also at the top of the gradient (unincorporated label) depending on the degree of purification used to separate the RNA from small molecules. Successful formation of an RNP would result in detecting radioactivity in an intermediate band of characteristic density (1.31g/ml). CsCl gradients could therefore be used as a useful indicator of RNP formation.

6.2 Attempted encapsidation during ongoing translation of N protein.

One possible source of N protein for reconstitution studies is from *in vitro* translation of mRNA transcribed from recombinant plasmid. pTF/BunN (Chapter 3) can be transcribed and translated *in vitro* to form Bunyamwera virus N and NSs proteins. The RNA produced from pTF/BunN has heterologous sequences flanking the N protein open reading frame and so would not be expected to be specifically encapsidated by N protein. RNA transcribed from pBunS(-)CAT has termini identical to Bunyamwera virus S segment and is therefore thought to contain encapsidation signals for N protein recognition.

As an alternative to incubating RNA with preformed protein, attempts were made to encapsidate pBunS(-)CAT RNA by transcribing it while N protein was being translated from preformed pTF/BunN RNA. A requirement for rescue of reovirus RNA was that the RNA was first translated in a rabbit reticulocyte lysate (Roner *et al* 1990). Protein synthesis was shown to be required although nuclease analysis suggested that encapsidation of RNA by newly translated proteins did not occur. Nevertheless, for bunyaviruses encapsidation by newly translated proteins might occur by allowing polymerisation of N protein onto nascent RNA—as is expected to occur *in vivo* for bunyaviruses which replicate entirely in the cytoplasm. To investigate this possibility, a rabbit reticulocyte lysate was used to translate N protein from preformed RNA and was supplemented with a T7 transcription reaction containing pBunS(-)CAT template to see whether the nascent RNA could be efficiently encapsidated by newly translated N protein.

N protein was labelled with ^{35}S methionine and a control reaction lacking the pBunS(-) CAT template was included (reactions performed by E. Dunn and R.M. Elliott).

The products of these reactions were analysed on CsCl density gradients to see if any ^{35}S labelled protein had banded lower down the gradient than the diffuse banding expected for free protein. Figure 6.2 shows the distribution of radioactivity for fractions of each gradient and demonstrates no clear difference in the presence or absence of pBunS(-)CAT template suggesting that no RNP was formed. Parallel gradients showed RNP to band in fractions 12, 13 and 14 which are well separated from fraction 20 and onwards where rising counts, due to the less dense free protein and label, are observed. Therefore in this experiment, no RNP formation was observed when pBunS(-)CAT RNA was transcribed in a reticulocyte lysate in which Bunyamwera virus N protein was being translated from preformed RNA.

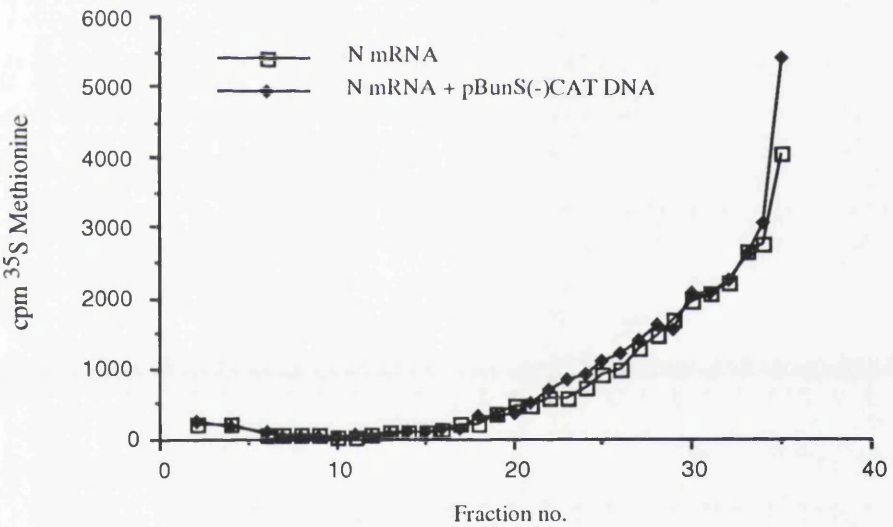


Figure 6.2 Attempted encapsidation during N protein synthesis. *In vitro* transcribed BunN mRNA was translated in a rabbit reticulocyte lysate containing a transcription mix to synthesise RNA from pBunS(-)CAT RNA in the presence of ongoing N protein translation (N mRNA + pBunS(-)CAT DNA). A control reaction contained equivalent components but lacked pBunS(-)CAT DNA template (N mRNA). Proteins were labelled with ^{35}S methionine. The products of both reactions were analysed by CsCl gradient centrifugation. Scintillation counting of fractions was used to determine the distribution of label in the gradient. In a parallel gradient RNP was shown to band in fractions 12, 13 and 14. No peak of counts is seen in this region suggesting no RNP was formed. The increasing counts seen towards the top of the gradient (fraction 20 onwards) are as expected for free protein and unincorporated label.

6.3 Reconstitution using denatured RNP as a protein source.

For influenza virus, reconstitution of synthetic RNPs had been achieved by dissociating RNP in high salt concentrations and physically separating protein and RNA before mixing the protein with fresh, synthetic RNA. Because of their relative instability in high salt solutions, influenza virus RNP could be dissociated and their components separated simply by centrifugation through preformed CsCl/glycerol gradients (Privin *et al* 1989). Clearly this approach would not be applicable to the RNPs of bunyaviruses which are stable in high salt concentrations. For VSV nucleocapsids the technique was successfully modified by including in the gradients 3M guanidinium chloride, a strongly denaturing salt. Gradient fractions were analysed by SDS PAGE to identify those containing denatured N protein which were then pooled. After dialysis to remove the denaturant, new RNP complexes could be formed by incubating the renatured protein with synthetic RNA (Moyer *et al* 1991).

Guanidinium denatured Bunyamwera virus nucleocapsids were examined to see if the N protein could be renatured and used for *in vitro* RNP reconstitution. CsCl gradient purified RNP was recentrifuged through CsCl gradients containing 3M guanidinium thiocyanate. Reduced CsCl concentrations were used to maintain the required density range. Suitable adjustment was determined empirically by weighing measured volumes of solutions formed by mixing three volumes of 4M guanidinium thiocyanate with 1 volume of CsCl solution. The resultant density range would result in intact RNP banding near the bottom with free protein occuring further up the tube. These gradients were sufficiently denaturing to allow separation of N protein from the RNA as judged by the appearance of a visible protein band well above the position expected for RNP. The visible band was shown to contain N protein by SDS PAGE analysis. Denatured N protein was mixed with labelled *in vitro* transcribed RNA and the guanidinium denaturant removed by dialysis. It was hoped that removal of the denaturant would allow renaturation of the N protein onto the synthetic RNA. CsCl gradient analysis of the renaturation reactions revealed that the label was concentrated in the pellet suggesting that no RNA had banded as a RNP complex. Visually, the even suspension of denatured protein formed an irregular precipitate on removal of the denaturant suggesting that the protein formed aggregates (data not shown). Attempts to prevent aggregation involved performing the renaturation in larger volumes in the hope that the N protein would bind to RNA before self-aggregating. Using N protein denatured by guanidinium thiocyanate, no

conditions were found which enabled banding of synthetic RNA as RNP when analysed by CsCl gradients.

6.4 Reconstitution of influenza virus RNPs using micrococcal nuclease treated cores as protein source.

In addition to the use of CsCl gradient purified proteins for reconstituting influenza virus RNP, protein derived from native RNP by nuclease digestion has been successfully used (Seong & Brownlee 1992a). Detergent-disrupted virus was treated with micrococcal nuclease (MCN) to degrade influenza virus RNA while leaving functional proteins. MCN requires calcium ions for activity and so can be inactivated by chelating the calcium ions with EGTA. After MCN inactivation, the nuclease-treated influenza virus 'cores' were incubated with short influenza virus-like RNA transcripts synthesised *in vitro*. The use of EGTA as a chelating agent allowed the presence of free magnesium ions for subsequent transcription assays since, unlike EDTA, EGTA specifically chelates Ca^{2+} ions while leaving Mg^{2+} free in solution. Analysis showed that the synthetic RNA was recognised and used as template for transcription by the influenza virus proteins. The MCN-treated cores were also used to assemble synthetic RNP using a chimeric influenza-like RNA containing an antisense copy of the CAT gene, which could be expressed *in vivo* following transfection into cells.

6.5 Preparation and nuclease treatment of Bunyamwera virus RNP

For influenza virus, MCN treated cores were prepared from purified virus grown in eggs. Since Bunyamwera virus does not grow to very high titre in tissue culture and does not grow in eggs, the best yields of purified RNP are obtained from infected cells by CsCl gradient centrifugation.

Intracellular RNPs were prepared by centrifuging infected cell lysates to equilibrium through preformed 20–40% (w/w) CsCl gradients (see Chapter 2). The visible RNP band was harvested and dialysed against 10mM Tris HCl (pH7.4), 10mM NaCl and 1mM EDTA. Typically final preps contained 50–500ng/µl protein (measured by Bradford assay) depending on the amount of cell extract loaded to the gradient. On Coomassie blue-stained SDS PAGE gels, the N protein was the predominant band although low levels of other contaminating proteins were usually observed (Figure 6.3).

Prior to MCN digestion, samples were redialysed in 50mM Tris (pH8), 50mM NaCl and 1mM $CaCl_2$ to remove the EDTA (which would inhibit the Ca^{2+} dependent MCN) and to slightly raise the pH to the optimum level for MCN. Reaction mixes were assembled each containing 10µg (protein content) RNP and varying amounts of MCN. These were incubated at 37°C or 25°C for three hours to determine conditions which degraded viral RNA. Analysis of N protein in MCN treated RNPs by Coomassie blue stained SDS PAGE (Figure 6.3), demonstrated that the N protein remained intact by the treatment. For RNA analysis, reactions were halted by phenol extraction and the RNA precipitated, denatured in glyoxal and electrophoresed through 1% agarose for Northern transfer. Hybridisation was with a labelled S segment RNA, transcribed *in vitro* from appropriate cDNA constructs, as a probe. Figure 6.4 shows the effect of MCN treatment on the S segment RNA. The signal for naked RNA is abolished by 0.05units MCN in 30 minutes. For RNP, the band representing intact S segment RNA is replaced by a smear of smaller fragments of heterogeneous size following MCN digestion. This smear probably represents smaller protected fragments. For binding studies, 25°C was adopted for MCN digestion. The reaction was scaled up and digestion of the endogenous RNA to smaller protected fragments confirmed by Northern analysis for each batch.

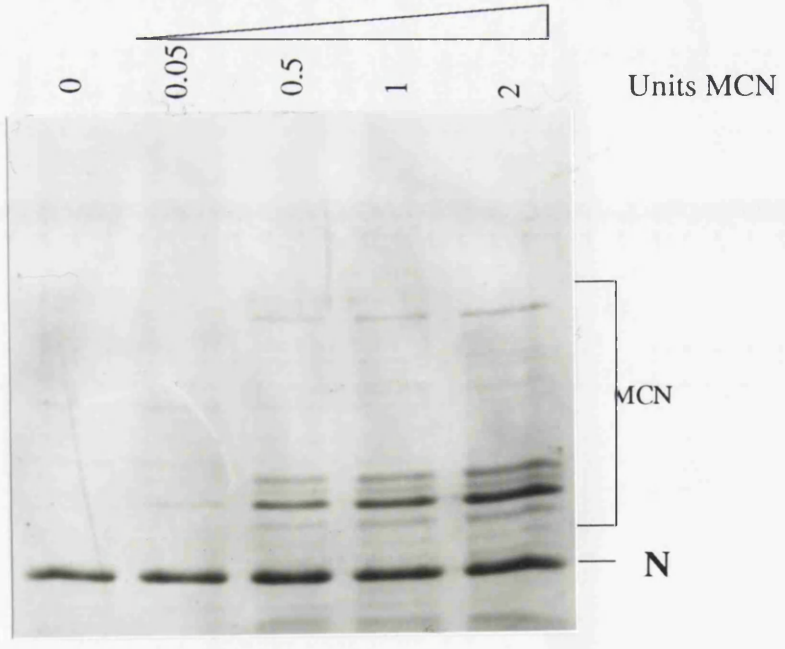


Figure 6.3 N protein analysis after micrococcal nuclease digestion of RNP. RNP was digested with MCN as described for Figure 6.4. Following digestion, N protein was examined by SDS PAGE and Coomassie blue staining. No evidence of degradation of the N protein was observed. These reactions were performed at 25°C.

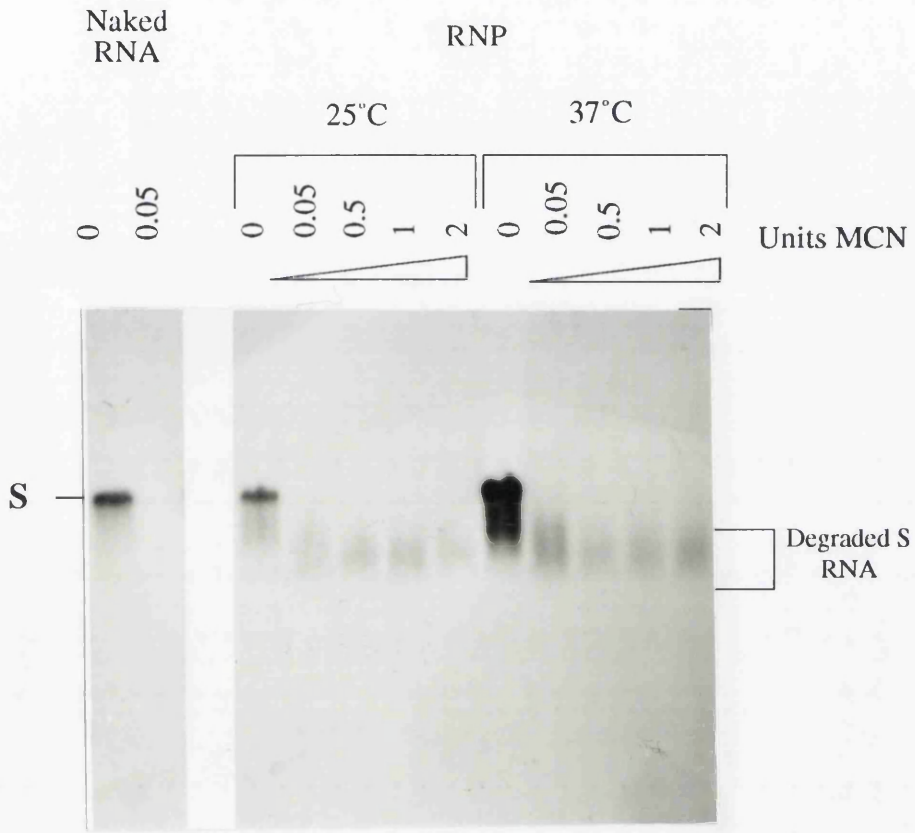


Figure 6.4 Degradation of Bunyamwera virus S segment RNA by micrococcal nuclease digestion of RNP. RNP preps (10µg protein content) were digested with various amounts of micrococcal nuclease in the presence of 1mM $CaCl_2$ at pH 8 for 3hr at 25°C or 37°C. Degradation products were purified by phenol extraction and ethanol precipitation. Samples were denatured in glyoxal, electrophoresed through 1% agarose and blotted to nylon for Northern analysis using ^{32}P labelled riboprobes transcribed *in vitro* (S segment + and - strand probes). The left panel shows complete degradation of naked S segment RNA in 30min using 0.05 units MCN. For RNP, the S segment RNA is degraded to smaller fragments for all conditions tested. Increasing amounts of MCN produced slightly increased degradation. At 37°C a weak signal corresponding to intact S RNA was also observed at all MCN concentrations while no full length band was seen at 25°C.

6.6 Bunyamwera virus RNP reconstitution

After MCN digestion, EGTA was added to a final concentration of 5mM to chelate the Ca^{2+} ions required for MCN activity and so prevent degradation of added synthetic RNA. Following the protocol of Seong and Brownlee (1992a) for influenza virus RNP reconstitution, *in vitro* transcribed Bunyamwera virus-like RNA was incubated with MCN treated RNP at 30°C for 1hr.

To assess binding, reactions containing labelled RNA were applied to preformed 20–40% CsCl gradients and centrifuged to equilibrium (32,000 rpm for 16Hr at 12°C in TST41 rotor). Gradients were then fractionated from the bottom and portions of each fraction were analysed for radiolabel content (by liquid scintillation counting) and density (by refractometry). Figure 6.5 shows reactions analysed in this way containing 1µg protein and 0.5–1.5µg labelled RNA. For each, a peak was observed in fractions 7–10 which correspond to densities 1.3–1.35g/ml. No equivalent peak was found following analysis of a gradient loaded with labelled RNA alone (no incubation with MCN treated RNP). These results suggest that proteins in the MCN-treated RNP associated with the labelled RNA to produce RNP of similar buoyant density to that expected for native bunyavirus RNP.

6.7 Controls.

To investigate whether the observed banding of *in vitro* transcribed RNA incubated with MCN treated RNP was due to formation of a Bunyamwera virus-like RNP, a number of control incubations were performed and analysed. Naked RNA alone failed to band (graph B, Figure 6.5)—demonstrating that some component of the MCN treated RNP was responsible for the banding. Components of the MCN/RNP mixture were tested to see if any gave rise to artefactual binding. Native RNP, not treated with MCN, was examined to see if the binding was due to a contaminating protein. The same experiment would address the possibility that the banding was due to the synthetic RNA binding to the protected Bunyamwera virus RNA detected by Northern analysis of the MCN treated RNP (Figure 6.4) since if this occurred, intact RNP would also be expected to bind the synthetic RNA.

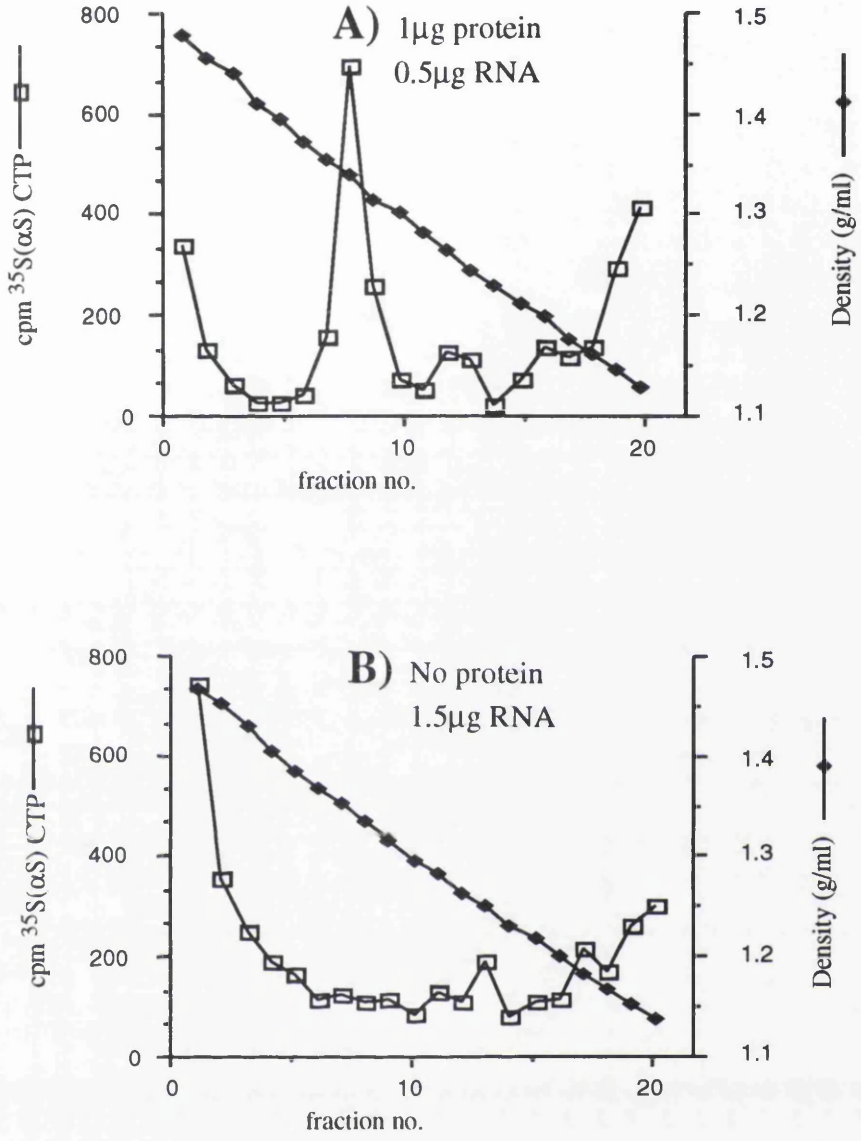


Figure 6.5 CsCl gradient analysis of *in vitro* reconstituted RNP. α^{35}S-Labelled RNA transcribed *in vitro* from pBunS(-)CAT was incubated for 30 minutes with MCN treated RNP (1µg protein). A control reaction contained no MCN-treated RNP. Reactions were analysed by CsCl gradient centrifugation as described in the text. The radiolabel content of each fraction was determined by liquid scintillation counting and the density determined by refractometry. Naked RNA (graph **B**), pelleted while analysis of reactions which included MCN treated RNP (graphs **A** and **C&D** next page) revealed a peak of radioactivity in the region of the gradient where RNP is expected to band. (Figure continued next page).

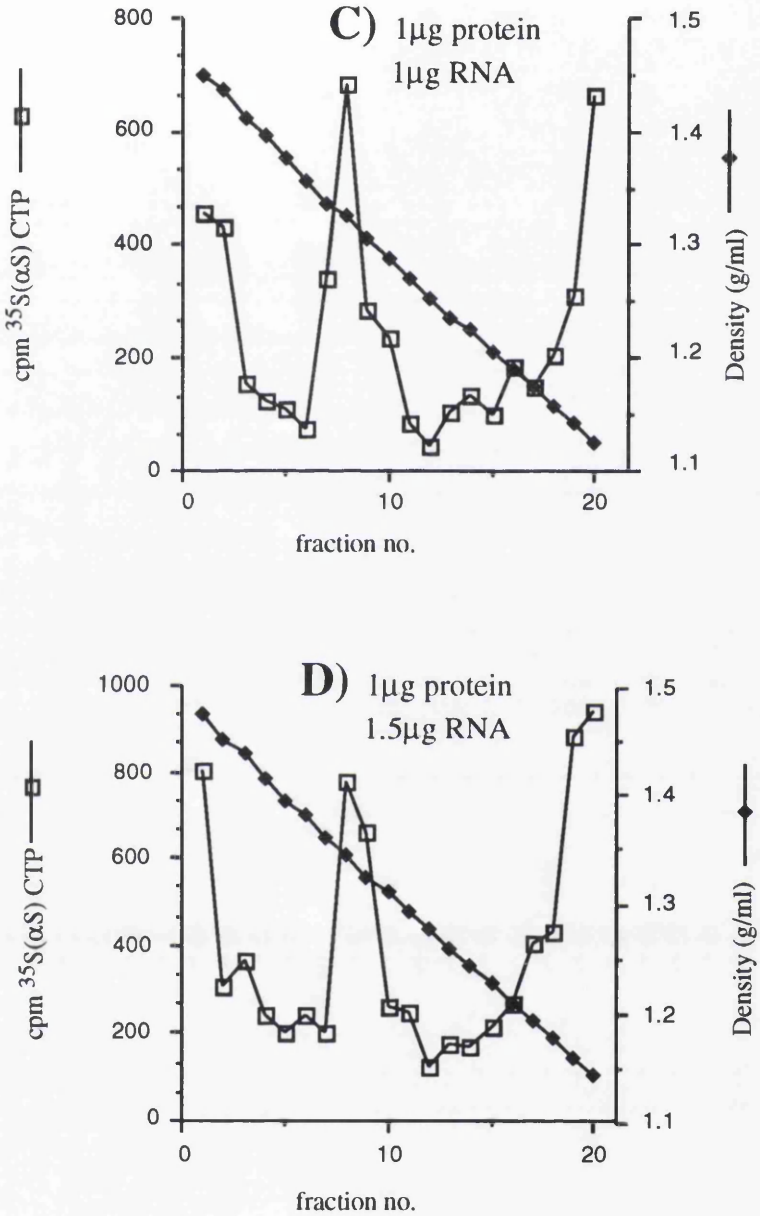


Figure 6.5 continued.

Figure 6.6 compares the gradient profiles of labelled RNA incubated with MCN treated or native Bunyamwera virus RNP. All components are exactly duplicated except for the absence of MCN in the native RNP reaction. Some radiolabel was detected in gradient fractions where RNA incubated with native RNP was analysed. The distribution of these was clearly different to that found when the incubation was with MCN treated RNP, which gave a strong peak as observed previously. This suggests that neither a contaminating cellular protein nor intact fragments of native RNP was responsible for the binding.

A formal possibility exists that a component of cellular origin is responsible for the banding of the synthetic RNA but that it requires MCN digestion to be activated. This possibility could be tested by nuclease treating fractions of a gradient loaded with uninfected cell extract and testing to see if these can produce banding of *in vitro* transcribed RNA. This control was not done and it is questionable as to whether a minor contaminant could be present in sufficient quantities to account for the degree of density shift and the amount of RNA banded.

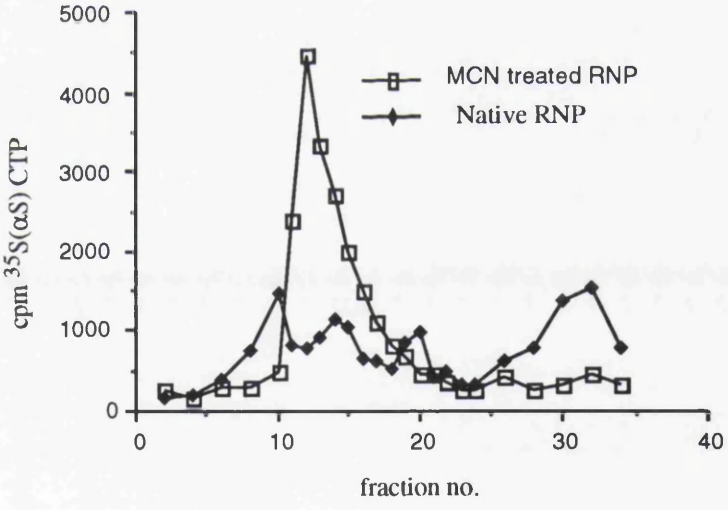


Figure 6.6 Micrococcal nuclease digestion is required for *in vitro* RNP reconstitution. To test if intact Bunyamwera virus RNP or contaminating proteins in the RNP prep were responsible for formation of the RNP complex containing synthetic RNA, CsCl gradient profiles were compared for reconstitution reactions performed using native or MCN digested RNP. The graph shows that Banding of the synthetic RNA in CsCl gradients is dependent on the RNP used for reconstitution having been digested with MCN.

The native RNP control did not include micrococcal nuclease which might itself bind RNA to cause a density shift. This possibility was tested by incubating MCN with the synthetic RNA in the presence of 5mM EGTA to prevent the nuclease degrading the RNA. The distribution of label in fractions of gradients loaded with this mixture revealed a peak at a similar position as that found when MCN treated RNP was used. This suggested that the micrococcal nuclease itself, or a contaminant present in it, can bind RNA sufficiently stably to produce a density shift in CsCl gradients (graph A, Figure 6.7). However, this experiment had used at least a 10-fold excess of MCN and the amount of RNA banded was negligible when compared to RNA incubated with MCN-treated RNP (graph B, Figure 6.7). The binding observed for MCN treated RNP incubated with synthetic RNA is not therefore due to binding of MCN to the RNA.

Together these data suggest that the MCN-treated RNP can associate with *in vitro* transcribed Bunyamwera virus-like RNA to form RNP complexes which are stable in high salt and which band at a density similar to native bunyavirus RNPs.

6.8 Co-transcriptional encapsidation.

For *in vivo* rescue of synthetic influenza virus RNP (Enami & Palese 1991), co-transcriptional encapsidation was found to be more efficient than the previous method of mixing pre-synthesised RNA with CsCl-purified proteins. This involved supplementing the T7 polymerase *in vitro* transcription reaction with the CsCl-purified proteins thus providing opportunity for nascent RNA to be encapsidated as it was being transcribed. Protein obtained by nuclease treatment of influenza virus RNP also functioned in co-transcriptional encapsidation and improved the efficiency of rescue following transfection into influenza virus infected cells (Seong & Brownlee 1992a; B. Seong personal communication).

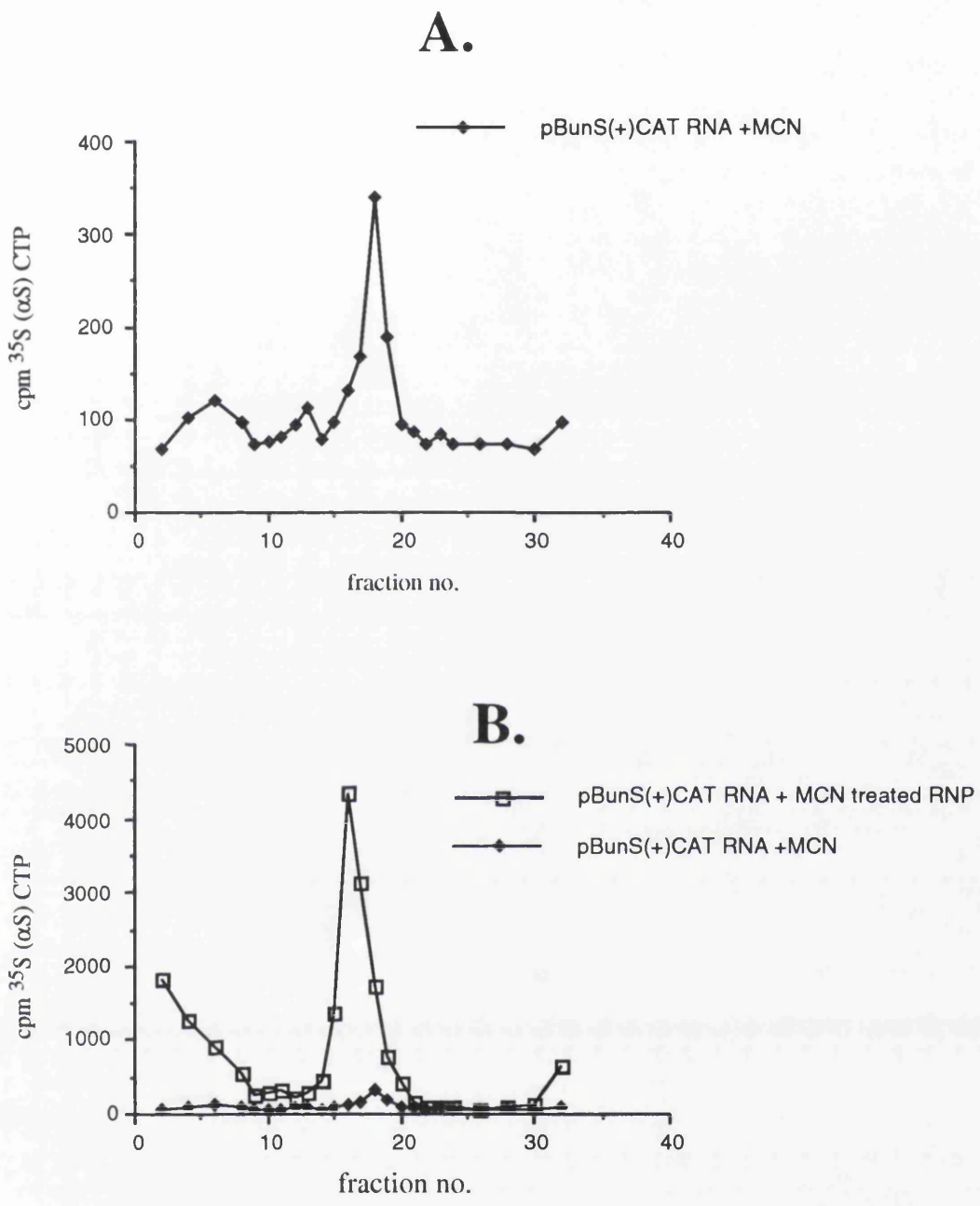


Figure 6.7 RNA incubated with micrococcal nuclease bands in CsCl gradients. RNA was incubated with 5 units of micrococcal nuclease in the presence of 5mM EGTA to prevent degradation of the RNA by the calcium-dependent enzyme (pBunS(+)CAT RNA + MCN). When analysed on a 20–40% CsCl gradient, radioactivity was detected in fractions around the middle of the gradient (**A**). *In vitro* reconstituted RNP (pBunS(+)CAT RNA + MCN treated RNP), containing identical RNA but incubated with MCN-treated RNP (MCN content <0.5 units), was analysed on a parallel gradient and found to produce a much greater peak (**B**). The y-axis scale of the two graphs is different—graph **B** shows the two traces superimposed on the same scale.

For influenza virus, short (15 minute) transcription reactions, in the presence of NP and polymerase proteins, had been shown to allow maximium rescue (Enami & Palese 1991). Longer incubation reduced the efficiency of *in vivo* replication of the RNP. The protocol developed for influenza virus was investigated to see if MCN digested Bunyamwera virus RNP could co-transcriptionally encapsidate RNA. 15µg MCN treated RNP was added to an *in vitro* T7 RNA polymerase transcription reaction containing pBunS(-)CAT DNA template. The reaction contained $^{35}S(\alpha S)$ CTP and a reduced concentration of CTP to allow efficient labelling of RNA. Transcription was halted after 15 minutes by chilling on ice and adding EDTA to 2mM final concentration. The reactions were centrifuged through 20–40% CsCl density gradients to determine if the labelled RNA had been encapsidated by N protein to form a RNP.

Figure 6.8 compares the distribution of radiolabel in gradients loaded with the products of a coupled transcription/encapsidation reaction. The two control traces are of gradients loaded with identical rections except for omission of either template DNA or nuclease treated RNP ('DNA only' and 'protein only' controls).

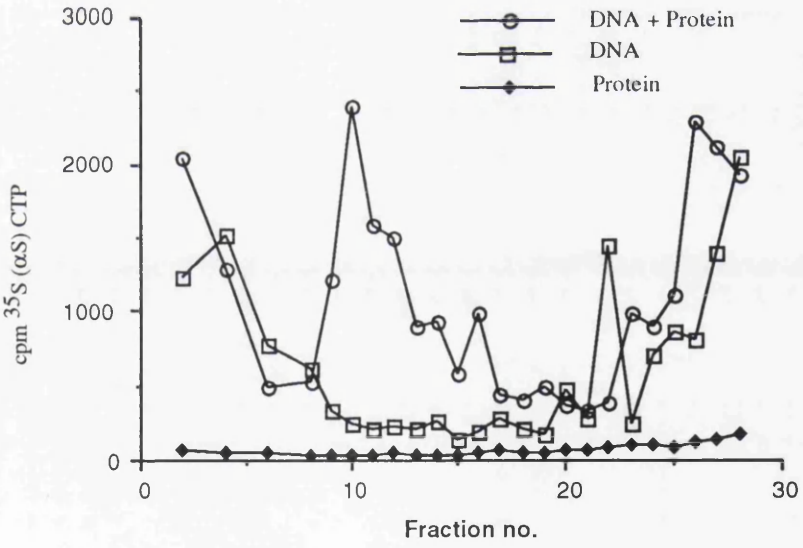


Figure 6.8 Co-transcriptional encapsidation of pBunS(-)CAT RNA. *In vitro* transcription from pBunS(-)CAT DNA template was performed in the presence of MCN treated RNP to see if the newly transcribed RNA could be encapsidated in N protein. Control reactions were identical except for the omission of either MCN treated RNP (DNA only) or template DNA (protein only). The graph compares the gradient profiles of the three reactions. The reaction in which pBunS(-)CAT was transcribed in the presence of MCN treated RNP (DNA+protein) shows a peak corresponding to RNA banded in fractions 8–14 as observed previously for RNP reconstituted *in vitro* by incubating preformed RNA with MCN treated RNP. No equivalent peak is seen for either of the control reactions.

When pBunS(-)CAT was transcribed in the presence of MCN-treated RNP, gradient analysis revealed a peak corresponding to banded RNA. The distribution was broader than previously observed for reconstitution by incubating preformed RNA with MCN treated RNP and may reflect partially encapsidated transcripts. No peak of radioactivity was detected in the position expected for RNP in gradients loaded with reactions performed in the absence of either MCN treated RNP or template DNA.

6.9 Transfection studies.

Numerous attempts were made to detect Bunyamwera virus specific CAT activity in cells transfected with *in vitro* reconstituted RNP containing pBunS(-)CAT RNA. Various multiplicities of Bunyamwera virus and several independent virus stocks were used as helper and transfection was performed using different amounts of reconstituted RNP and at varying times post infection. The CAT assays were sufficiently sensitive to detect extremely weak background acetylation in extracts from uninfected cells. No Bunyamwera virus specific activity was detected. Controls to assess transfection efficiency of the protein /RNA complexes were not routinely undertaken. Such controls involved performing the RNP transfection assay developed for study of a recombinant vaccinia virus expressing the Bunyamwera virus L protein (Jin & Elliott 1991) and required Northern analysis of test and several internal control transfections. Three transfection methods were used: Lipofection using Transfectace™ according to the manufacturers (BRL) instructions; calcium phosphate mediated transfection; and DEAE dextran transfection. The first two methods have confirmed applicability to transfection of RNA/protein complexes as judged by their use in the RNP transfection assay (H. Jin & R.M. Elliott personal communication), while the dextran method has been used in this context for influenza virus RNP transfection (Luytjes *et al* 1989).

In addition three different cell lines were used: BHK cells and CV1 cells both support bunyavirus replication and both are suitable for transfection of RNPs (H. Jin personal communication), and 293 cells which gave consistently good transfection efficiencies for influenza virus RNP rescue. None of these cell lines enabled detection of Bunyamwera virus specific expression of the synthetic RNP.

6.10 Efficiency of reconstitution

Since the free radiolabel remaining after ethanol precipitation of the *in vitro* transcribed RNA remains in solution at the top of the gradients, the ratio of counts in the banded and pelleted fractions gives an estimate of the proportion of RNA encapsidated. Gradients were analysed for reactions containing 1µg pBunS(+)CAT RNA reconstituted with 5 or 20µg MCN treated RNP (Figure 6.9). Counts were summed for six fractions centred on the peak, with counts from 3 of each of the flanking fractions subtracted as background. After correction for fraction volume, the total banded counts were compared to the counts present in the pellet which represent naked RNA. 66% and 78% of the counts were found banded for the 5µg and 20µg MCN/RNP reconstitution respectively. This value does not account for RNA lost by degradation which appeared to be different in each case as the total counts were $7{\cdot}4 \times 10^4$ and $1{\cdot}28 \times 10^5$ cpm respectively. As a total of the starting activity of the precipitated transcription reaction, <5% of the counts were accounted for by the summed pellet and banded fractions. This may reflect degradation to single nucleotide although the difference is probably largely due to co-precipitation of unincorporated label by the single ethanol precipitation used to prepare the RNA. The activity of transcripts purified away from free label by electrophoresis was ~10% of the apparent activity measured by direct counting of the precipitated transcription mix.

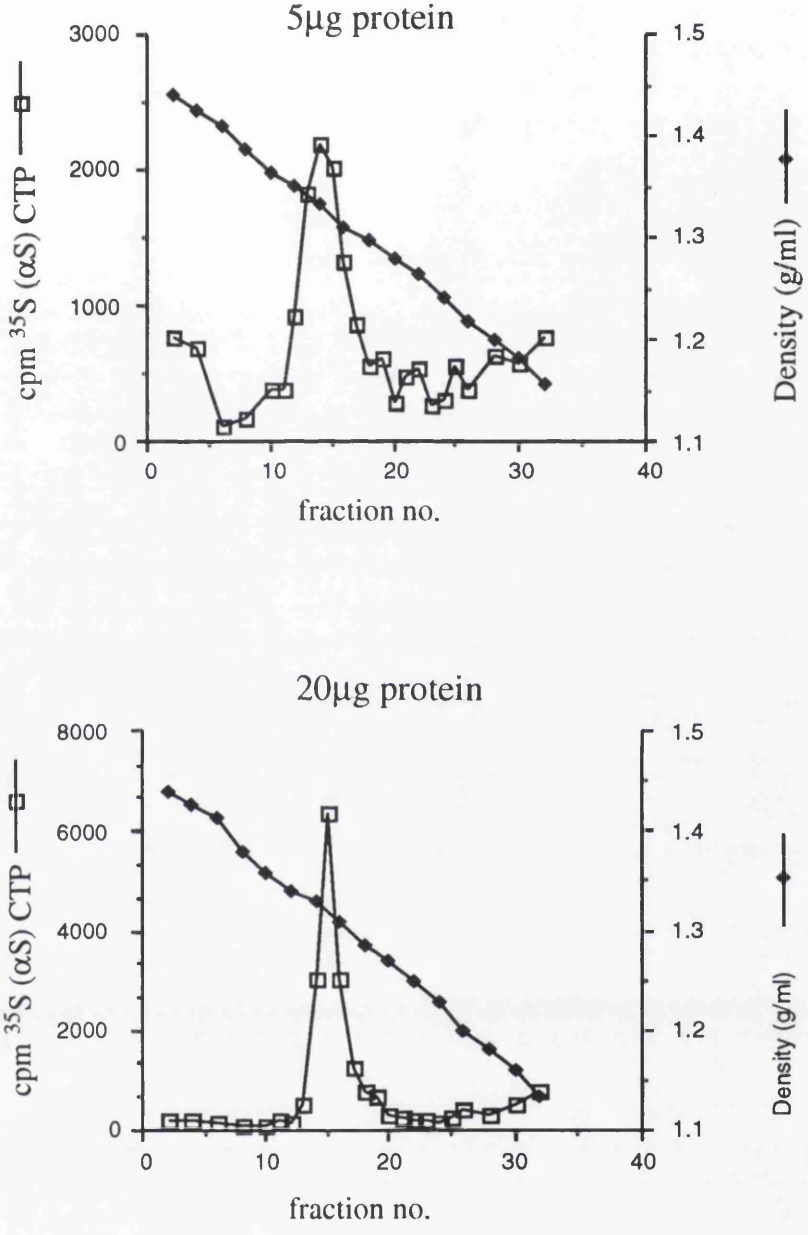


Figure 6.9 Binding of RNA to 5 or 20µg MCN treated RNP. 1µg pBunS(+)CAT RNA was incubated with 5 or 20µg MCN treated RNP and binding assessed by CsCl gradient analysis. Counts for six fractions containing the peak were summed and corrected for background by subtracting counts for three fractions either side of the peak. After correcting for fraction volume, total banded RNA was compared to pelleted (unencapsidated) RNA. In the 5µg reconstitution 66% of the combined banded/pelleted counts were found to be banded while in the 20µg reaction 78% of counts were banded.

6.11 Loss of binding to purified RNA

The products of *in vitro* transcription reactions were phenol extracted and precipitated prior to use in binding studies. Since a single strong band was present when examined on ethidium bromide-stained gels, no further purification was routinely used. For transfection experiments the maximum ratio of correct transcript was desirable and so gels containing electrophoresed labelled RNA were fractionated and radioactivity measured to assess the size distribution of RNA present. Significant quenching was observed, presumably due to the presence of agarose but counting of gel blocks of equal proportions allowed comparison to be made. Surprisingly, considering the apparantly clean band seen by ethidium bromide staining, only ~10–25% of the counts were found in the band. Significant quantities of radioactivity were detected both above and below the sharp visible band which probably represented transcripts from a low proportion of circular templates and premature termination or degradation products.

Full length RNA was purified by cutting the sharp band from agarose gels and retrieving the RNA by binding to silica matrix in the presence of high salt using a commercially available kit (RNaid™). When RNA purified in this way was incubated with micrococcal nuclease-treated RNP and analysed by CsCl gradient centrifugation, the RNA failed to band. Several factors could have contributed to the loss of binding activity and an experiment was performed to investigate some of these.

Several differences would be expected between the content of gel purified RNA and directly precipitated RNA. The binding activity of directly precipitated RNA was used as a positive control and compared with the binding ability of RNA receiving one of the following treatments:

1) RNaid™ purified bulk RNA—i.e. no gel purification (was binding ability destroyed as a consequence of being treated with the kit reagents?).

2). RNaid™ purified from agarose gel in the absence of ethidium bromide (did the agarose gel electrophoresis have an effect on binding ability?).

3) Gel purified in the presence of ethidium bromide and then RNaid™ purified (did ethidium bromide carry over occur and interfere with binding?).

4) Free labelled NTP used in place of RNA in the binding reaction (was the binding artefactual and caused by free label which would be lost during RNaid™ purification).

5) Gel purified in the presence of ethidium bromide and RNaid™ purified but binding carried out in 10 or 1mM DTT (included as a precaution since in the original reconstitution when binding inhibition had been noted, 1mM DTT was used while all earlier studies had included 10mM DTT).

Binding was only observed in one sample—the bulk RNA which had not been treated with the RNaid™ kit (figure 6.10). When free label was used in place of RNA in the reconstitution incubation, no banding of counts was observed demonstrating that RNA and not NTP band the MCN treated RNP. All other samples which failed to band in CsCl gradients had been purified by RNaid™.

A pair of reactions differing only in use or not of RNaid™ showed that this method of purification was responsible for loss of binding ('bulk RNA' and 'RNaided bulk RNA', Figure 6.10). Possible explanations include that the RNaid™ treatment either left behind some inhibitory component or removed some essential factor required for binding. Losses of RNA were not the cause since gel analysis before and after purification demonstrated good recovery. RNA purified by the kit is suitable for routine translation and enzyme manipulations of RNA and so carry over of some inhibitory compound (whose effect was complete—no binding at all was observed) would be surprising. One possibility which remains to be tested is that free NTP, which would be removed by silica matrix purification, is required for binding.

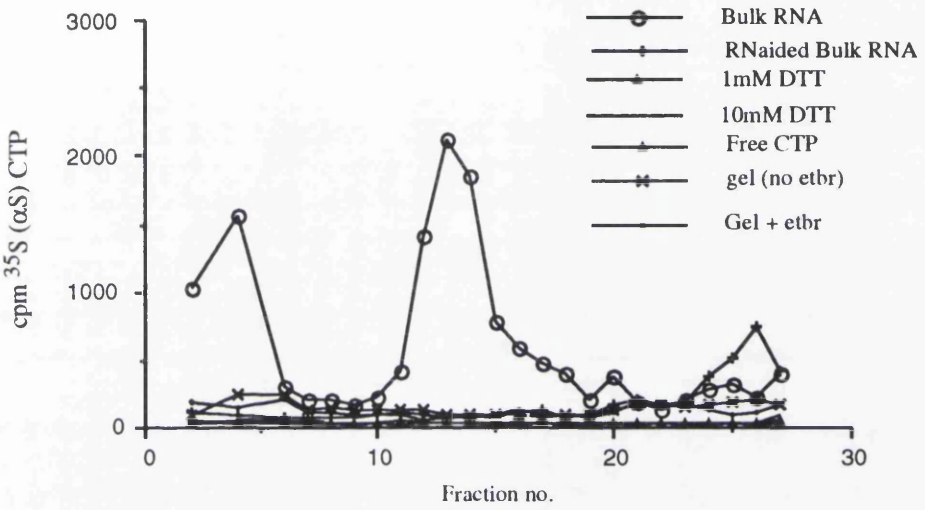


Figure 6.10 Loss of binding of purified RNA to MCN treated RNP. *In vitro* RNP reconstitution was attempted by incubating MCN treated RNP with RNA which had received one of the following treatments: (Bulk RNA)—Transcription reaction phenol extracted, ethanol precipitated, dissolved in water and used directly; (RNaided Bulk RNA)—Same as 'Bulk RNA' but additionally purified by silica matrix binding using commercially available reagents; (1mM DTT)—as 'Gel+etbr' with reconstitution reaction containing 1mM DTT; (10mM DTT)—as '1mM DTT but reconstitution included 10mM DTT; (Free CTP)—No RNA, reconstitution with labelled CTP replacing RNA; (gel (no etbr))—Transcription reaction electrophoresed through 1% agarose, RNA band located by staining a duplicate lane with ethidium bromide, excised and purified by binding to silica matrix; (Gel + etbr)—same except RNA band visualised by direct staining with ethidium bromide. The reactions were analysed on CsCl gradients. Only one reaction gave a peak in the gradient expected for RNP formation. With the exception of the 'Free CTP' reaction (which also gave no peak), all other reactions contained RNA which had been purified by binding to silica matrix.

6.12 Anomalous transcription from pBunS(-)CAT

As an alternative to generating run off transcripts from BbsI linearised pBunS(-)CAT, transcription template was generated by PCR using a primer containing a T7 promoter and a primer to generate the exact viral cDNA terminus to allow run off transcription to yield viral like transcripts. When examined on non-denaturing gels, transcripts from the PCR generated template migrated slower than the transcript seen for BbsI cleaved pBunS(-)CAT. If gels were overloaded with pBunS(-)CAT RNA, in addition to the major RNA band, a minor band of slower mobility could be detected which had a similar mobility to that of the transcript from PCR generated template (see Figure 6.11).

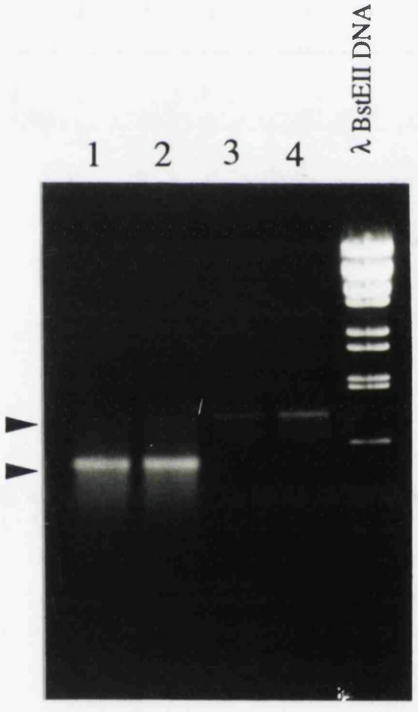


Figure 6.11 *In vitro* transcription from BbsI cleaved pBunS(-)CAT and from template prepared by PCR. A 1% agarose gel stained with ethidium bromide revealed a unique faint band resulting from transcription using PCR generated template (lanes 3 & 4). Transcription from BbsI-cleaved pBunS(-)CAT (lanes 1 & 2) produced a major species of faster mobility (lower arrow) and also a minor band of similar mobility to that observed for transcripts from PCR generated template (upper arrow).

Comparison of the mobility of the PCR generated template with a BstEII-digested phage λ size ladder on an agarose gel, revealed it to be the size expected for the construct insert. Since the PCR fragment had been amplified from pBunS(-)CAT DNA, this too must have insert of correct size. Therefore the appearance of the fast major band in the RNA transcribed from pBunS(-)CAT is not as expected. Either of two possibilities may account for the extra band, both of which can be tested experimentally. Firstly, during BbsI digestion of the template, a second shorter template might be generated either by contaminating enzyme or star activity (no BbsI site is present in CAT or Bunyamwera virus S segment cDNA and, at least for the 180 nt at the run off end of the construct, no point mutations had occufed which might have introduced a site). This possibility could be tested by digesting the PCR generated template with BbsI prior to transcription (no sites present) and checking the size of the resultant transcript. Appearance of the faster migrating transcript would indicate that an aberrant cleavage of the DNA had occured and resulted in a run off transcription end closer to the promoter.

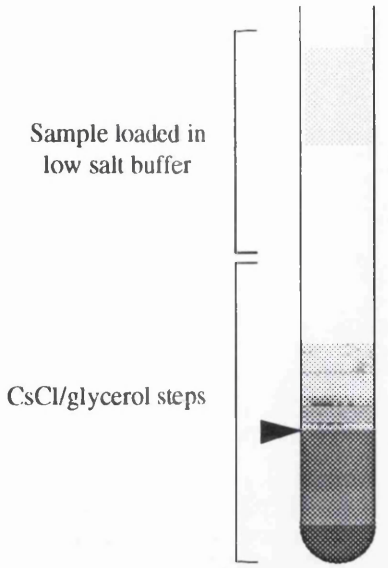
A second possibility is that a relatively minor degradation of the BbsI-generated run off transcription end results in a transcript which, although only slightly shorter than expected, had a markedly different mobility when electrophoresed under non denaturing conditions, due to a difference in secondary structure. This might be feasible if template degradation had resulted in loss of 3' terminal sequences involved in pan handle formation. The two species observed then would represent circular (pan handle containing) molecules of slow mobility and linear transcripts of almost the same size but significantly faster mobility due to the absence of the pan handle. Denaturing gel electrophoresis would lessen the difference in band mobilities by destroying the hydrogen bonding responsible for pan handle formation and so any difference would then reflect size difference.

If the major fast-mobility transcript from pBunS(-)CAT is incorrect this would explain the inability to detect Bunyamwera virus specific CAT expression of transfected RNPs reconstituted *in vitro* using RNA transcribed from this construct.

6.13 L protein activity in MCN treated RNP.

For influenza virus, MCN treated cores were used to reconstitute short synthetic templates which were transcribed by endogenous polymerase complex (Seong & Brownlee 1992a). This property was exploited to assess promoter activity from mutated synthetic RNA templates *in vitro* (Seong & Brownlee 1992b). While in principle endogenous polymerase activity of reconstituted RNP may not be essential for *in vivo* replication in the presence of a helper virus, such activity would be required for *in vitro* transcription studies and would allow extremely useful studies to be conducted for bunyaviruses whose promoters have yet to be formally defined. As a preliminary step to developing such technology, MCN treated Bunyamwera virus RNP was tested for evidence of endogenous L protein activity.

CsCl gradient purified native RNP are known to be reduced in L protein activity presumably due to effects of the high salt gradients (H. Jin personal communication). To decrease any denaturing effect of the CsCl, gradients were redesigned by including glycerol and reducing CsCl concentrations. A discontinuous gradient was established by overlaying steps of decreasing density CsCl/glycerol as shown in Figure 6.12. The steps were designed such that RNP (density = 1.31g/ml) would band at an interface between two of the fractions. Centrifugation of such gradients containing low molecular weight dyes suggested that little diffusion or density changes occured and that RNP could be harvested at the predicted interface. Coomassie blue stained SDS PAGE gels showed a strong band of the size expected for N protein in material harvested from the expected region of the gradient. Following MCN digestion, RNP purified from the combined CsCl/glycerol gradient was shown to be capable of binding *in vitro* transcribed RNA. RNP prepared in this way contained slightly more contaminating proteins than in those harvested from continuous CsCl gradients.



Glycerol concentration (%w/w)	CsCl concentration (%w/w)	measured density (g/ml @ 25°C)	step volume (ml)
50%	0%	1.13	1.5
50%	10%	1.23	1
40%	20%	1.30	0.5
50%	20%	1.35	2
0%	40%	1.42	1

Figure 6.12 Combined CsCl/ glycerol gradient for isolation of transcriptionally active Bunyamwera virus RNP. In order to reduce losses of, or damage to, the associated L protein, a gradient with reduced CsCl concentrations was designed to achieve sufficient density to band RNP. The step gradient was established using mixtures of CsCl and glycerol. The density of each mixture was determined by weighing measured volumes of solution. The choice of steps was arbitrary except for the 1.3g/ml layer which was designed to allow harvesting of RNP (1.31g/ml) at the lower interface (arrow head).

To test for evidence of L protein activity in the MCN digested RNP, a transcription mix was added (Final concentrations: 1mM ATP, 0.5mM GTP and CTP, 150μM UTP—including α^{32}P UTP at final activity 60Ci/mmole, 50mM Tris HCl pH 7.8, 50mM KCl, 10mM NaCl, 1mM DTT and 5mM $MgCl_2$.) For influenza virus MCN-treated cores, the endogenous fragments of RNA remaining after nuclease digestion were shown to act as transcription template in the absence of added synthetic RNA (B. Seong personal communication). Therefore the protected RNA fragments observed by Northern analysis of MCN-treated Bunyamwera virus RNP might be expected also to act as transcription templates. Reactions were performed at 25°C, 30°C or 37°C in the presence or absence of dinucleotide ApG which is known to slightly stimulate transcription from bunyavirus RNP. Figure 6.13 shows the products of the reactions separated on a 20% acrylamide denaturing gel. A major band is seen in all reactions. Increasing the temperature reduced the strength of the signal while presence of ApG increased the signal. The effects of temperature and ApG are consistent with the findings for similar analysis of influenza virus MCN treated cores and with the known properties of bunyavirus *in vitro* transcription assays.

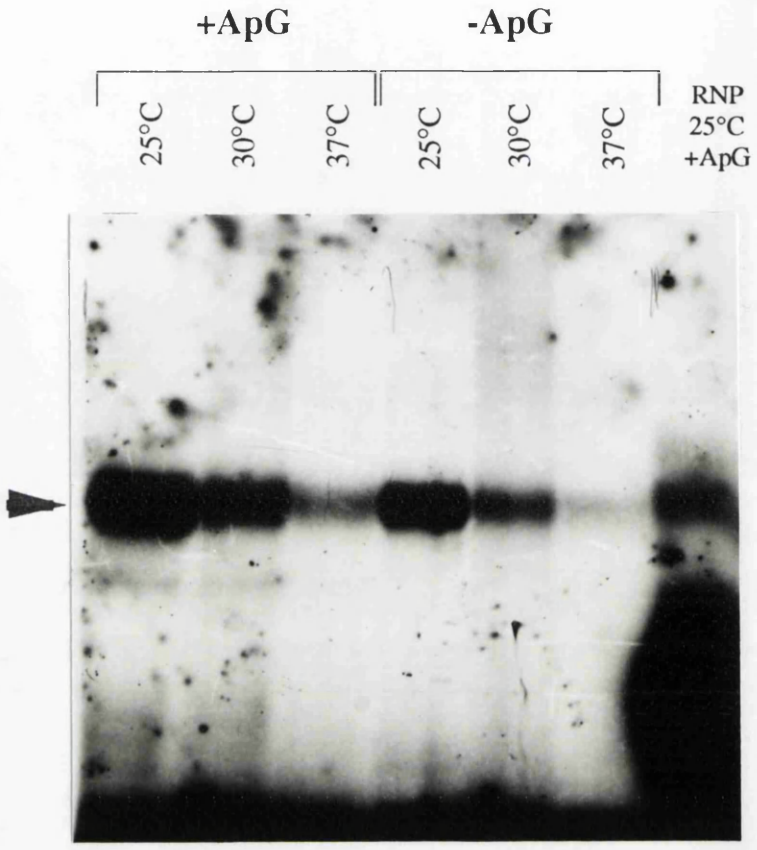


Figure 6.13 Endogenous transcription from MCN treated RNP. *In vitro* transcription reactions were performed at 25˚C, 30˚C and 37˚C in the presence or absence of 1mM ApG, for 20 hours. Products were phenol extracted, ethanol precipitated, denatured in 90% formamide and electrophoresed through 20% acrylamide containing 7M urea as denaturant. The α-^{32}P UTP labelled products were visualised by autoradiography of the dried gel. A major product (arrow) is seen for all reactions. The strength of the signal decreases for reactions performed at successively higher temperatures and at each temperature is stronger when ApG was present in the reaction.

The necessary analysis to demonstrate that the signals observed are genuine transcription products and not artefacts remain to be conducted. Presuming the signals do represent transcripts from digested fragments of RNA, it is interesting to note that a single major band is present. Transcription from randomly cleaved RNA templates would be expected to produce a ladder of products. Interestingly, at the same position in the native RNP control, there is a faint signal of the same mobility suggesting a tendency for transcripts to terminate at this point. The signal for native RNP is much weaker than that for MCN treated RNP transcribed under the same conditions and with equivalent N protein content (compare the first and last lanes of Figure 6.13). Also present from the intact RNP templated

reaction was a ladder of shorter products (on lesser exposure a ladder could be seen in place of the blurred signal in the lower part of the last lane of Figure 6.13).
Although the gel did not include size markers, the major band was estimated to represent a transcript ~22nt long by fitting the mobilities of the ladder observed for intact RNP to logarithmic graph paper—the best line of migration distance to integer logarithm gave a gradient and axis intercept similar to that obtained by plotting the migration of the bromophenol blue and xylene cyanol dyes to their predicted co migration sizes.

The estimated length coincides with the predicted length of the pan handle of the S segment. It is difficult to judge whether this coincidence is significant especially considering that the pan handle lengths for the three segments are all different and a 20% gel should easily resolve single base differences.

While promising, these results are extremely preliminary and formal demonstration that the signal represents a genuine transcript has still to be attempted.

6.14 Summary of *in vitro* reconstitution studies and future work

The data presented in this chapter, while far from complete, suggests that *in vitro* reconstitution of Bunyamwera virus RNP may be feasible. The gradient analysis of *in vitro* reconstituted RNP provides preliminary evidence that encapsidation of synthetic Bunyamwera virus-like RNA can occur *in vitro* using micrococcal nuclease treated RNP as a protein source. Density measurement of gradient fractions containing reconstituted RNP are consistent with the density expected for native RNP although direct comparison with labelled native RNP has yet to be conducted.

Further work is needed to clarify whether BbsI digestion of the pBunS(-)CAT construct produces the correct run off transcription end or whether aberrant cleavage occurs. This can be readily tested as suggested, by similar digestion of PCR generated template. If necesary the transcripion template could be generated by PCR routinely, by optimising cycling conditions to enable maximum yield of correct product. Once it is established that the correct RNA can be transcribed, transfection of *in vitro* reconstituted RNP into cells infected with Bunyamwera virus, or the L protein expressing recombinant vaccinia virus (Jin & Elliott 1991), can be re investigated.

Chapter 7: Future studies.

7.1 *In vitro* RNP reconstitution.

As discussed in Section 6.12, before further transfection studies are undertaken to attempt to rescue *in vitro* reconstituted RNP, further work is needed to clarify whether BbsI digestion of the pBunS(-)CAT construct produces the correct run off transcription end or whether aberrant cleavage occurs. This can be readily tested as suggested, by similar digestion of PCR generated template. If necesary the transcription template could be generated by PCR routinely, by optimising cycling conditions to enable maximum yield of correct product. Once it is established that the correct RNA can be transcribed, transfection of *in vitro* reconstituted RNP containing CAT reporter RNA into cells infected with Bunyamwera virus, or the L protein expressing recombinant vaccinia virus (Jin & Elliott 1991), can be reinvestigated.

The possibility that micrococcal nuclease digested Bunyamwera virus RNP retains L protein activity (Section 6.13) can be tested further by attempting to replicate a short model RNA *in vitro*, according to the protocol developed for influenza virus of Seong and Brownlee (1992a). This involves incubating the MCN treated RNP with a short, *in vitro* transcribed, RNA which has 3' terminal nucleotides identical to the 3' terminus of vRNA and a 5' portion which is of different sequence to bunyavirus RNA. Radiolabelled transcripts produced in this reaction can be characterised by RNase T1 mapping to see if they are indeed the complement of the added model template and were therefore produced by RNA-templated transcription.

Oligodeoxynucleotides for production of a suitable RNA template have been designed and synthesised to enable this experiment to be conducted. Seong and Brownlee(1992a) used partial duplex DNA to synthesise template RNA from a T7 promoter in the duplex region. The oligonucleotides designed to adapt this methodology to produce a Bunyamwera virus vRNA-like model template are shown in Figure 7.1. *In vitro* T7 RNA polymerase transcription would be used to produce unlabelled template RNA. Since the T7 system produces transcripts of varying lengths, gel purification of the full length template RNA may be desirable in the first instance although for the influenza virus system, size purification was not necessary (B. Seong personal communication). The model RNA would then be reconstituted with MCN treated Bunyamwera virus RNP (Section 6.5 and 6.6), and a transcription mixture added which included α-^{32}P CTP (Section 6.13). The oligonucleotides used to produce the template RNA are designed such that a CTP-labelled transcript produced by Bunyamwera virus L protein using the model RNA as a template, would have a characteristic RNase T1 digestion pattern. This would enable model RNA-templated transcripts to be distinguished from transcripts originating from endogenous RNA (see Section 6.13). In addition to short unlabelled fragments, RNase T1 (which cleaves 3' to G residues in RNA) digestion would produce radiolabelled fragments 8, 6, 4 and 3 nucleotides long respectively (see Figure 7.1). The 3mer product (CCC) is templated by the first three 5' nucleotides of the T7 transcript which may be heterogeneous (B. Seong personal communication) and so this fragment may be less abundant than the others and mono- and di nucleotide fragments may be present.

The success of such an experiment would permit detailed promoter analysis by comparing transcription levels from model RNAs carrying defined mutations. This method was used to assess the activity of synthetic influenza virus promoters in a systematic mutagenesis study (Seong & Brownlee 1992b). In addition to providing the first details of the promoter requirements for bunyavirus transcription, a successful outcome would also provide clear evidence that reconstitution was occuring and that therefore transfection studies using RNP containing reporter RNA should, in principle, succeed.

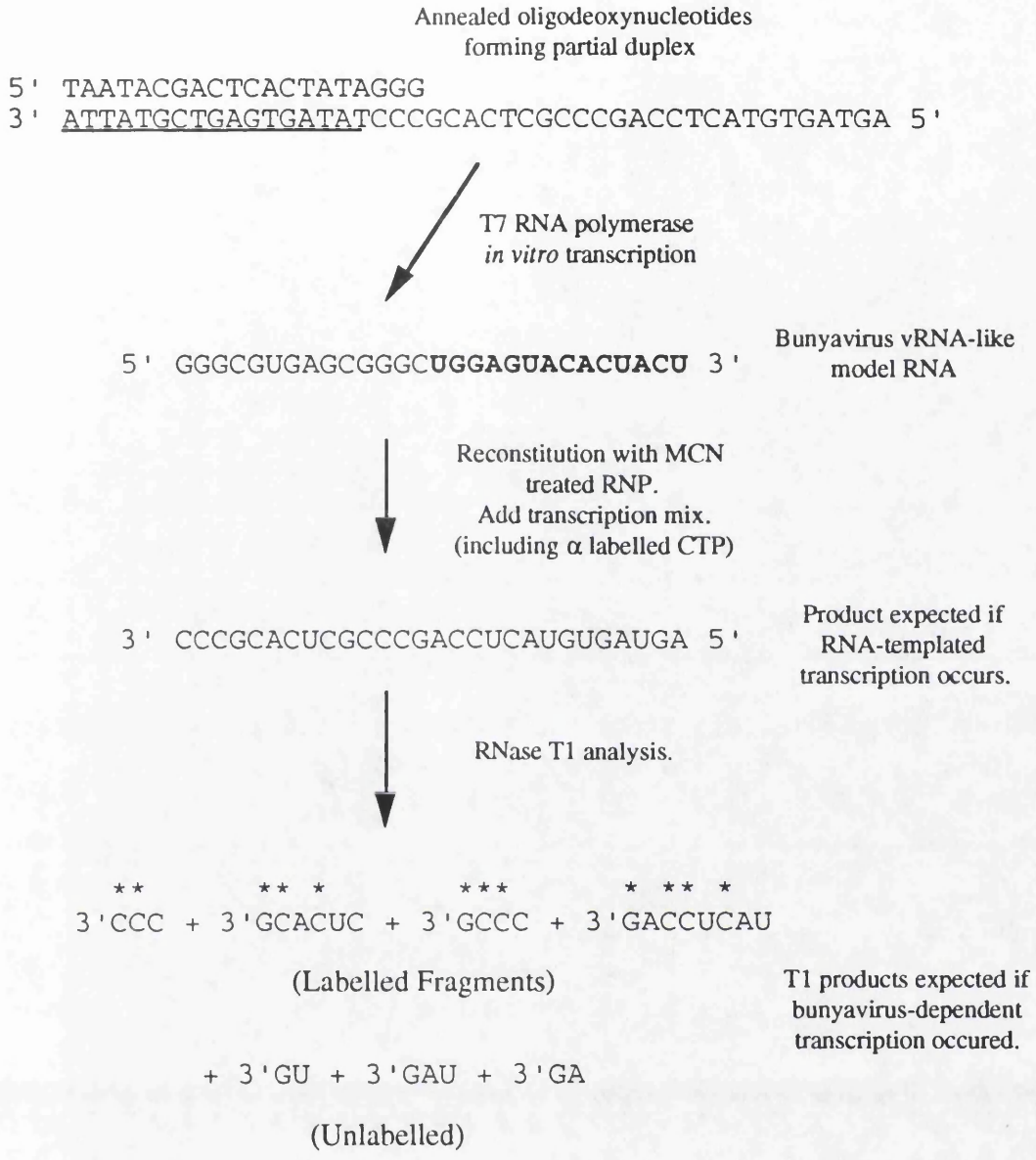


Figure 7.1 *In vitro* reconstitution and transcription of bunyavirus model RNA. Two oligodeoxynucleotides (top) are annealed to make a template for T7 transcription from the T7 promoter (underlined) to yield a bunyavirus 3' terminal vRNA-like model RNA. This model RNA (with bunyavirus sequences shown in bold type) is reconstituted with MCN treated RNP and a transcription mix added including α-^{32}P CTP. Analysis of the resulting labelled products using RNase T1 (which cleaves 3' of G residues) produces radiolabelled fragments of 8, 6, 4 and 3 nucleotides in length (positions where radioactive phosphate may occur are marked with an asterisk—MCN cleaves leaving a 3' phosphate hence the transfer of label where a C residue was 3' to a target G residue).

7.2 *In vivo* Transcription of cDNA.

Two difficulties were identified with the use of the vTF7-3 T7 polymerase expression system for *in vivo* transcription from bunyavirus cDNA (Chapters 3 and 5). The first of these was that transcripts produced were not of unique length, possibly due to recircularisation of transfected linear cDNA templates (Section 3.7). The second problem was that transcripts were occuring from Bunyamwera virus S segment cDNA constructs which were thought to be originating from a cryptic vaccinia virus late promoter (Section 5.6). Both these problems can be addressed and further attempts be made to transcribe and replicate plasmid derived RNA *in vivo*. The suitability of the vTF7-3 expression system for this type of work has recently been demonstrated by the production of VSV DI particles entirely from cDNA (Pattnaik *et al* 1992).

The cryptic vaccinia virus promoter can be readily mutated to a non-functional form. The extensive promoter-mutagenesis study of Davison and Moss (1989b) demonstrated that mutation of any residue in the TAAAT motif of the promoter results in dramatic reduction of promoter activity (see Figure 5.16 Section 5.6). The choice of bases to mutate would be based on sequence comparison of the relevant region of the Bunyamwera virus S segment with other bunyavirus S segments. Figure 7.2 shows sequence comparison of this region between Bunyamwera and Maguari virus S segment cDNAs. Despite an overall 83% identity between the two S segments, the region of the Bunyamwera virus S segment cDNA which contains the cryptic vaccinia promoter is less conserved. Therefore, it may prove possible to mutate this region of non-coding RNA without seriously affecting any function it might have. Based on the Maguari virus sequence, suitable alteration might be achieved by mutating the Bun TAAAT to Mag CAGAT. Confirmation that the mutations had reduced the promoter strength to negligible levels could be directly obtained by transfecting pBunS(-)CAT DNA (suitably mutated) into cells infected with WR strain vaccinia virus (see Figure 5.15; Section 5.6) where CAT activity should be absent or only barely detectable if promoter activity has been destroyed.

5'...CTATTAAATCCAAC...3' Bun S (49–62)

5'...CAATCAGATCCAAC...3' Mag S (50–63)

Figure 7.2 Sequence comparison between Bunyamwera and Maguari virus S segment cDNA in the region of the cryptic vaccinia virus promoter. Top strand shows Bunyamwera virus S segment cDNA nucleotides 49–62 which contains a motif resebling the core of a vaccinia virus late promoter (underlined). Bottom strand shows the equivalent region of Maguari virus cDNA demonstrating that this region is not highly conserved between bunyaviruses.

The problem of generating transcripts *in vivo*, which have precise 3' termini, was recently overcome by incorporating ribozyme sequences down stream of the required transcript produced using the vTF7-3 vaccinia virus T7 polymerase expression system (Ball 1992). This system was applied to produce VSV defective interfering (DI) particles which originated entirely from cDNA clones (Pattnaik *et al* 1992). It had been shown previously that VSV DI particles could be replicated when infected into cells expressing the L, N, and NS proteins from plasmids using the vTF7-3/T7 polymerase system (Pattnaik & Wertz 1990). If the M and G proteins were also expressed, again from T7 promoter plasmids, the replicated DI genomes could be packaged into DI particles which budded from cells (Pattnaik & Wertz 1992). In these experiments the VSV-like template was a genuine DI nucleocapsid but the system was developed further to allow replication, encapsidation and packaging into DI particles, of RNA transcribed *in vivo* from transfected cDNA (Pattnaik *et al* 1992). To achieve this, a T7 transcription plasmid was constructed which contained a VSV DI cDNA followed by DNA coding for the hepatitis delta virus (HDV) antigenomic ribozyme, followed by a terminator of T7 RNA polymerase transcription. T7 transcripts from this vector contained DI RNA sequences upstream of the HDV ribozyme and transcribed sequence from the T7 terminator. The ribozyme was positioned such that autocatalytic cleavage released DI genome RNA with the exact 3' terminus required. When the DI genome RNA was transcribed *in vivo* by T7 RNA polymerase supplied by VTF7-3 and in the presence of all five VSV gene products produced from cotransfected T7 expression vectors, the RNA was encapsidated, replicated and packaged into budding VSV DI particles. This impressive achievement demonstrates that the VTF7-3 expression system can be used to produce *in vivo* transcripts with precisely defined termini.

Pattnaik *et al.* (1992) also mapped the transcription start sites of the DI RNA produced from T7 promoters. One construct examined had the three G residues (required for optimal transcription) following the TATA of the T7 promoter (see Section 5.4.2 and Figure 5.8). In vTF7-3 infected cells this construct produced transcripts which initiated predominantly on the first G residue or the A residue immediately 5' to it. T7 DI transcripts having four or two non-DI nucleotides at their 5' termini, but with the correct 3' termini, were efficiently replicated whenVSV proteins were expressed. The extra nucleotides were removed on further replication of the DI RNA by VSV proteins. For existing Bunyamwera virus S segment cDNA reporter constructs (chapter 5) the G residues have been replaced by the first viral nucleotides 5'AGT (Figure 5.8). Although influenza virus like T7 transcripts, which require the same 5' nucleotides (Section 5.4.2) have been replicated by helper virus, replacement of the G residues with AGT may lead to inefficient production of transcripts initiating 5' AGT (D. Kolakofsky personal communication). Since the VSV work suggests that additional 5' nucleotide can be tolerated, future Bunyamwera virus S segment constructs could include G residues between the TATA of the T7 promoter and the 5' terminus of the viral cDNA. Two other modifications of the VSV DI transcript were apparent (Pattnak *et al.* 1992). Firstly, a proportion of the T7 transcripts had one extra 5' terminal nucleotide not coded for in the cDNA construct. This was thought to be due to a guanylyl transferase coded for by vaccinia virus as an extra 5' nucleotide was also observed on natural DI genomes replicated in the presence of vaccinia virus but not when the DI particle was infected alone. The second modification was a 2', 3'-cyclic phosphate group on the 3' terminal nucleotide of the DI RNA resulting from the ribozyme cleavage mechanism. Although viral RNA normally has a hydroxyl group at its 3' terminus, if present, the 2', 3'-cyclic phosphate did not prevent recognition and replication of the plasmid derived RNA.

The vTF7-3 transcription system, used in conjunction with the HDV antigenomic ribozyme, may therefore be of use for producing bunyavirus-like RNA *in vivo* and replicating it using recombinant viral proteins. Full length cDNA clones of all three Bunyamwera virus gene segments are available (R.M. Elliott personal communication) and these could be modified to include the required ribozyme and termination sequences. The Bunyamwera virus S segment CAT construct (Chapter 5) could also be modified and tested to see if CAT activity, dependent on infection with Bunyamwera virus, can be demonstrated.

The HDV antigenomic ribozyme cleaves 5' to its recognition sequence and therefore can be used to produce RNA terminating with any 3' nucleotides (Perrotta & Been 1991; Pattnaik *et al* 1992). As a first step towards modifying existing constructs, two overlapping oligonucleotides were synthesised. These were designed to produce, following annealing and extension by DNA polymerase, a double stranded fragment containing the ribozyme sequence (see Figure 7.3). The upstream end of the ribozyme DNA (5' top strand in Figure 7.3) starts 5' GGG... which are nucleotides 1, 2 and 3 of the ribozyme. This end could be ligated to a SmaI cut vector to recreate a Sma I site into which sequences coding the required RNA could be cloned as a blunt ended fragment. RNA transcribed would then be automatically cleaved immediately 5' to the first G residue and so produce an RNA with a 3' terminus corresponding exactly to the blunt ended fragment inserted into the Sma I cleaved vector. The fragment could also be used to modify existing constructs. pUCBunS(-)CAT (Chapter 5) can be cleaved with Bbs I and end-filled to produce a blunt ended linear vector into which the blunt ribozyme fragment can be cloned. Resultant plasmids with the correctly oriented ribozyme can be cleaved at the unique BamHI site immediately downstream of the ribozyme and ligated to a BamHI T7 RNA polymerase terminator obtained by digestion of a pET T7 expression plasmid (Promega).

The final construct should, when transfected into cells infected with vTF7-3, produce a transcript ~1100 nucleotides long which is autocatalytically cleaved to give an RNA molecule with a 3' end identical to the Bunyamwera virus S segment (Fig. 7.4). A vRNA-like transcript produced in this way carrying an antisense CAT gene should only produce CAT activity if it is first recognised and replicated by bunyavirus proteins. These proteins can be supplied either by infection with Bunyamwera virus or as recombinant proteins expressed from cDNAs. Active Bunyamwera virus L protein can be expressed either from a recombinant vaccinia virus (Jin 1991; Jin & Elliott 1991) or from transfected plasmids (Jin & Elliott 1992). N protein can be supplied by transfection of pTF/BunN as discussed in Chapter 3. Together the N and L proteins should be sufficient for replication and expression of the plasmid-derived RNA. Co-expression of the M segment glycoproteins (G. Nakitare & R.M. Elliott personal communication) may allow budding of virus-like particles. The rescue of VSV DI particles from cDNA (Pattnaik *et al* 1992) provides an impressive precedent and demonstrates the suitability of the vTF7-3/ribozyme system for this type of work. In principle, the application of this technology to bunyaviruses should be possible as the number of viral-protein expression constructs required is less

than for VSV. Successful replication of synthetic RNA might rapidly lead to rescue of a bunyavirus entirely from cDNA. Full length cDNAs exist for all three genome segments of Bunyamwera virus and these could be modified to produce precisely terminated RNAs by the inclusion of ribozyme sequences. The availability of T7 based expression constructs for all viral gene products makes it unnecessary to use a bunyavirus to supply viral proteins and so no background of helper virus would be present during rescue experiments. Such technology should enable precisely defined viable mutants to be generated routinely even when the mutants are partially growth restricted.

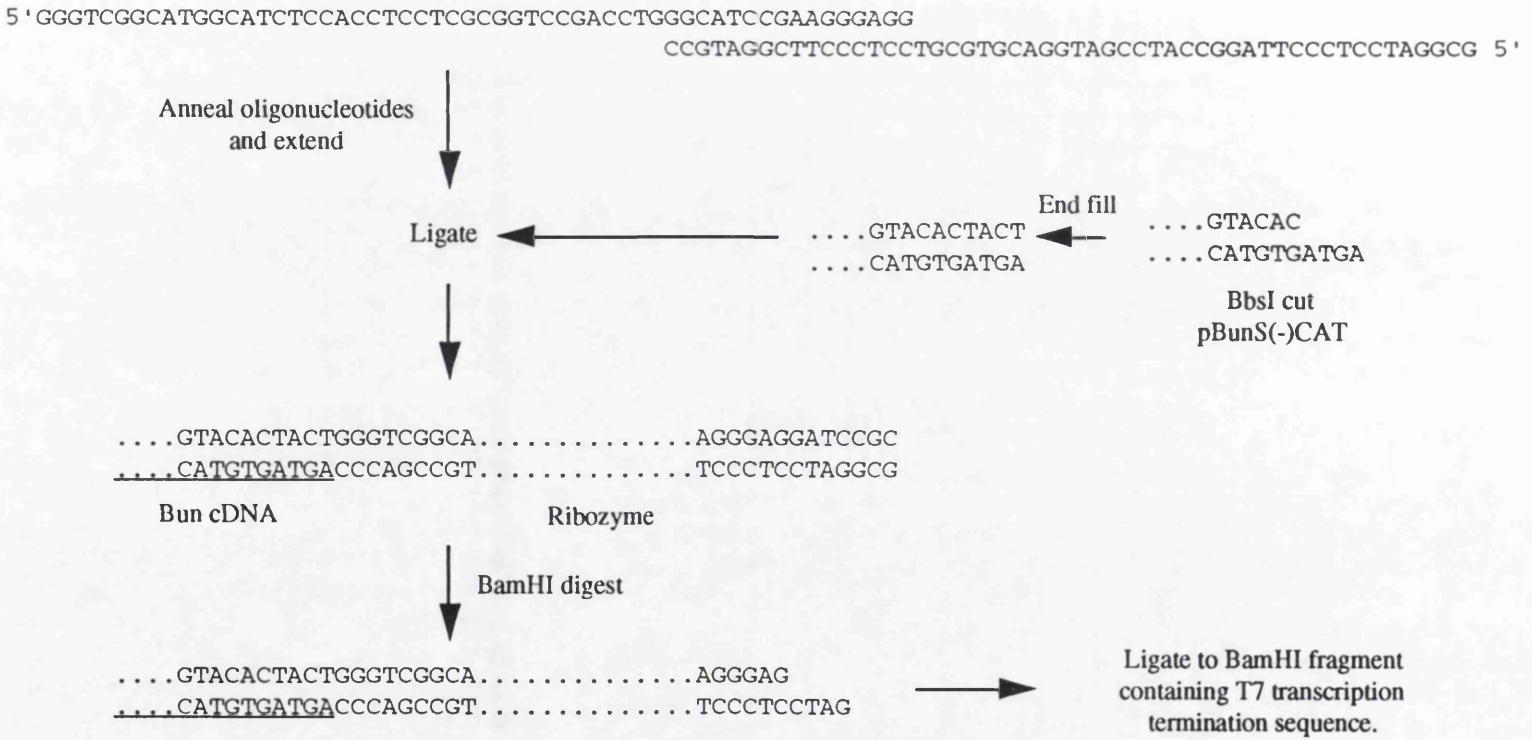


Figure 7.3 Incorporation of ribozyme sequences into pBunS(-)CAT. Two overlapping oligonucleotides (top) were synthesised to produce, following annealing and extension by DNA polymerase, a double-stranded DNA fragment containing sequences corresponding to the hepatitis delta virus antigenomic ribozyme. The ribozyme DNA fragment can be ligated directly to BbsI linearised/end-filled pBunS(-)CAT to form the required junction for specific cleavage of transcripts after the last viral nucleotides (underlined). A BamHI site is included in the ribozyme DNA to allow ligation of a transcription terminator. See Figure 7.4 for final construct map.

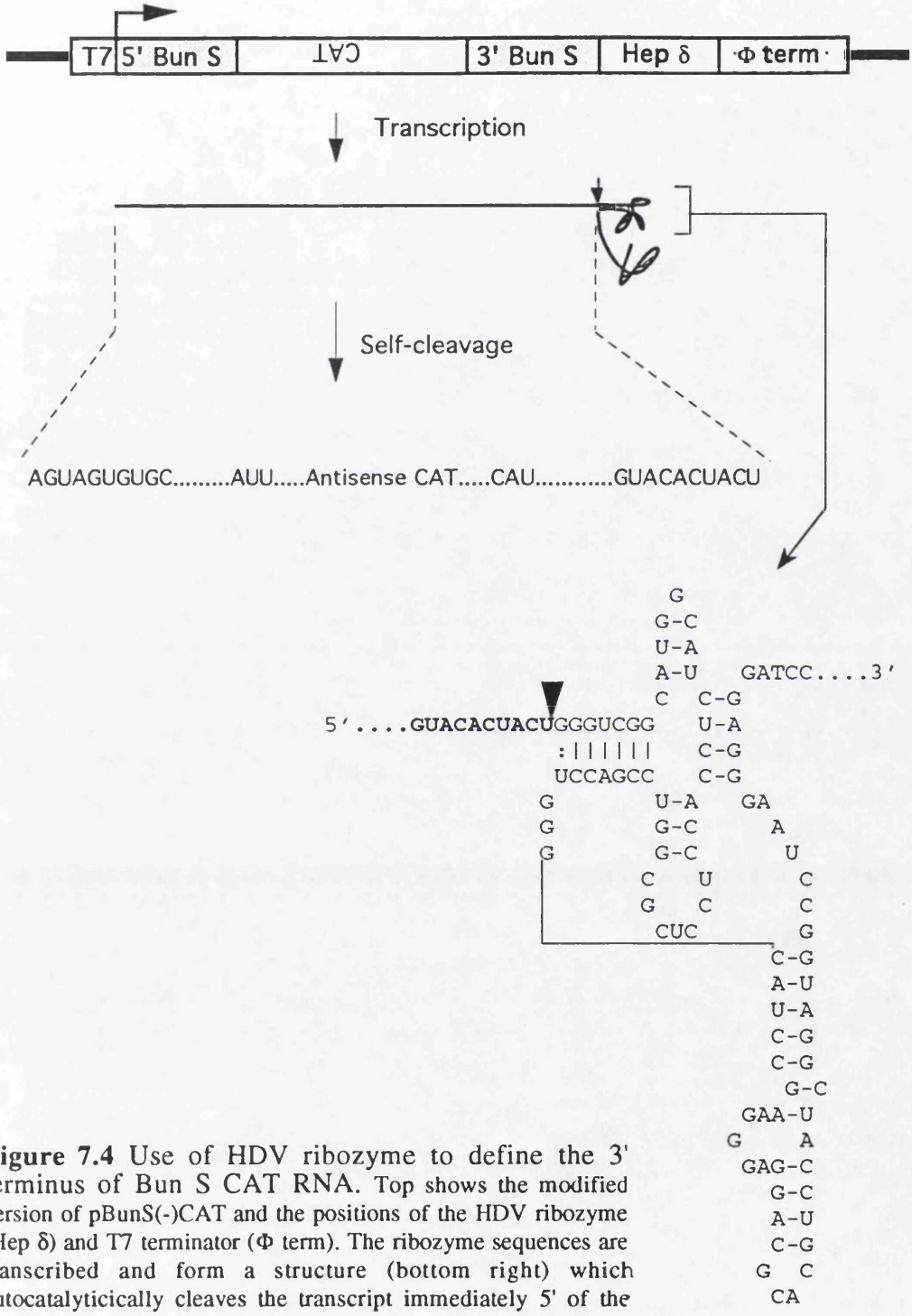


Figure 7.4 Use of HDV ribozyme to define the 3' terminus of Bun S CAT RNA. Top shows the modified version of pBunS(-)CAT and the positions of the HDV ribozyme (Hep δ) and T7 terminator (Φ term). The ribozyme sequences are transcribed and form a structure (bottom right) which autocatalyticically cleaves the transcript immediately 5' of the GGG triplet (arrow head) to give the required 3' bunyavirus RNA terminus (bold type).

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