EXPRESSION OF THE BUNYAMWERA VIRUS POLYMERASE GENE

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Summary

Bunyamwera virus is the prototype of the family Bunyaviridae and has a tripartite negative-sense RNA genome. The largest RNA segment (L) contains 6875 nucleotides and encodes a high molecular weight protein (259K) also termed L. The L protein is presumed to be the virion-associated transcriptase or RNA polymerase, although direct proof of this is lacking. The large size of the L protein suggests it is multifunctional and most probably catalyzes initiation, elongation, and termination of RNA synthesis and perhaps also plays a role in events which generate the host-derived primers for transcription, such as cap-binding and endonuclease activities. With the long-term aim of defining the functional domains within the bunyavirus L protein, the available cloned cDNAs were exploited to establish systems in which the L protein can be expressed.

As a prelude to characterizing the expressed L protein, monospecific antisera to portions of the L protein fused to ß-galactosidase were prepared. Five fusion proteins were expressed using the bacterial expression vector pUEX, and two antisera raised against the C and N termini of the L protein specifically recognized the L protein.

A full-length cDNA to the L gene of Bunyamwera virus was constructed from the existing cDNA subclones and synthetic oligonucleotides. The assembled L gene cDNA was subsequently cloned into various expression vectors and several expression systems were explored in order to obtain a biologically active L protein for domain mapping.

The full-length cDNA to the L gene was cloned into two vaccinia virus expression systems. In the first, the L gene is under control of vaccinia virus p7.5 promoter by using the plasmid transfer vector pSC11; in the second, the L gene is under control of the bacteriophage T7 ø10 promoter by using the plasmid transfer vector pTF7-5, and expression of the L gene requires coinfection with a second recombinant vaccinia virus vTF7-3 which synthesizes T7 RNA polymerase. Both systems expressed a protein which is the same size as the Bunyamwera virus L protein and was recognized by the monospecific L antisera. The L protein was also transiently expressed by transfection of vTF7-3 infected cells with recombinant plasmid DNAs which contained the cDNA for the L gene under control of the T7 promoter.

Expression of the L protein in a cell free system (rabbit reticulocyte lysate) by translation of an *in vitro* transcribed L gene was performed. Minor amounts of an L sized protein were synthesized, but many prematurely terminated polypeptides were also produced.

The L gene was also cloned under the T7 promoter in a bacterial expression vector (pET8c) to express the L protein in *E. coli*. However, no L protein was expressed by either IPTG induction or infection of the culture with a bacteriophage (CE6) to provide the T7 RNA polymerase.

To test the functionality of the L protein expressed by the recombinant vaccinia viruses, three assay systems were explored: complementation of bunyavirus ts L gene mutants; *in vitro* transcription assay; and the ability of the expressed L protein to replicate an RNA template in infected cultured cells.

The transcriptase activity of the expressed L protein present in cytoplasmic extracts from the recombinant vaccinia virus infected cells was assayed *in vitro* using Bunyamwera virus intracellular nucleocapsids as templates. However, the activity of the authentic L protein which was associated with the RNP was stimulated by mock infected cell extracts. Thus, the intracellular nucleocapsid was not an appropriate template for this assay. The attempts to complement bunyavirus ts mutants led to noninterpretable results. Efforts t reconstitute a synthetic RNA template containing the 5' and 3' end sequences of the Bunyamwera virus S RNA were also made. Linearized recombinant plasmid pUC-BUNS292 containing bases 1-99 and 768-961 nucleotides of the S RNA under control of the T7 promoter, was transfected into vTF7-3 infected cells, and positive-sense RNA should have been transcribed. The cells also expressed N protein, either transiently or from Bumyamwera virus infection, to encapsidate any short S-like RNA. However, it was observed that the transfected linearized shorter S cDNA was not transcribed by the T7 RNA polymerase in vTF7-3 infected cells.

The L protein, expressed from either of the two vaccinia virus systems or transiently expressed, was demonstrated to be functional by its capability of replicating viral S RNA in a nucleocapsid (RNP) transfection assay. Recombinant vaccinia virus-infected cells were transfected with purified Bunyamwera virus nucleocapsids, and subsequently total cellular RNA was analyzed by Northern (RNA) blotting or the cells were labelled with ^{35}S-methionine to analyze viral protein synthesis. No Bunyamwera virus RNA or proteins were detected in the cells transfected with the RNP alone, but in cells which had previously been infected with recombinant vaccinia viruses or had been transfected with recombinant plasmid with the L gene under control of the T7 promoter, Bunyamwera virus S RNA of both polarities was detected. Bunyamwera virus N protein was also detected in the transfected cells which had the L protein expressed. These results indicated that the expressed L protein had replicated the S RNA supplied by the transfected RNP and hence the newly synthesized positive-sense S RNA was translated into the N protein. The functionality of the recombinant L protein was further confirmed by demonstrating its ability to replicate the S RNA of DI particles generated from Bunyamwera virus persistently infected mosquito cells.

Site-directed mutagenesis of the L gene cDNA was performed to begin mapping the RNA polymerase domain within the L protein, and some preliminary data are presented in this thesis. Four conserved amino acids in the four predicted RNA polymerase motifs were chosen for specific amino acid substitution. Sixteen L mutants involving amino acids substitution of the conserved aspartic acid (D) residue at position 1037 and the D residue at position 1165 of the L protein were obtained. One mutant (L-A-1037, with substitution by alanine) was shown to exhibit RNA polymerase activity in the RNP transfection assay. All the other substitution mutants showed either no or much reduced activity in this assay. A deletion mutant, which was generated by deleting 166 amino acid residues at the C-terminus of the L, lost activity in the RNP assay, indicating that the intactness of the L is important for its functionality.

The work described in this thesis directly proves that the L protein encoded by the largest RNA segment of a bunyavirus is the viral RNA polymerase. The functional assay system developed will be useful for further probing the protein requirements for bunyavirus RNA replication and for understanding the mechanism of viral RNA genome replication. Site-directed mutagenesis of the L protein could identify possible targets for developing antiviral chemotherapy in the future.

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Contents

List of Tables and Figures

Abbreviations

aa	amino acid
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney 21, clone 13 cells
BUdR	5-bromodeoxyuridine
BSA	bovine serum albumin
C	cytosine
Ci	curie
C-terminal	carboxy terminal
COOH	carboxy
CTP	cytosine-5'-triphosphate
CV-1	African green monkey kidney CV-1 cell line
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dCTPαS	2'-deoxynucleotide-5'-O-(1-thiotriphosphate)
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	dATP, dCTP, dGTP, and dTTP
ddATP	2', 3'-dideoxyadenosine-5'-triphosphate
ddCTP	2', 3'-dideoxycytosine-5'-triphosphate
ddGTP	2', 3'-dideoxyguanosine-5'-triphosphate
ddTTP	2', 3'-dideoxythymidine-5'-triphosphate
DI	defective interfering
DNA	deoxyribonulceic acid
ds	double-stranded
DNase	deoxyribonuclease
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
g	grams
G	guanine
GMEM	Glasgow modification of Eagle's medium
GTP	guanosine-5'-triphosphate
ITP	inosine-5'-triphosphate
K	kilodalton(s)
kb	kilobase(s)
hr	hour(s)

M	molar
mg	milligrams
min	minute
ml	millilitre
mM	millimolar
moi	mutltiplicity of infection
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
N-terminal	amino-terminal
NP40	Nonidet P40
nt	nucleotides
PEG6000	polyethylene glycol 6000
pfu	plaque forming units
OD	optical density
ORF	open reading frame
^{32}P	phosphorus-32-radioisotope
PBS	phosphate buffered saline
pfu	plaque forming units
RNA	ribonucleic acid
RNP	nucleocapsid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
^{35}S	sulphur-35-radioisotope
SDS	sodium dodecyl sulphate
ss	single-strand
TK	thymidine kinase
Tris	tris (hydroxymethyl) aminomethane
ts	temperature sensitive
TTP	thymidine-5'-triphosphate
UV	ultraviolet
V	volts
µg	micrograms
µl	microlitres
v/v	volume/volume (ratio)
W	watts
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

wt	wild type

Virus abbreviations:

BUN	Bunyamwera
DUG	Dugbe
GER	Germiston
HTN	Hantaan
LAC	La Crosse
MAG	Maguari
NE	nephropathia epidemica
PH	Prospect Hill
PT	Punta Toro
RVF	Rift Valley fever
SR	Sapporo rat
SSF	Sicilian sandfly fever
SSH	snowshoe hare
TOS	Toscana
TSW	tomato spotted wilt
VSV	vesicular stomatitis virus
UUK	Uukuniemi

Amino acid symbols:

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

Chapter 1

Review of the literature

1.1. Introduction

The Bunyaviridae is the largest known family of animal viruses. More than 300 viruses, which are characterized by a tripartite negative-strand or ambisense RNA genome and are mostly arthropod-borne (Bishop and Shope, 1979), are classified into this family (Karabatsos, 1985). Some viruses in the family are of medical and veterinary significance, causing severe or fatal infections in human and animals. Examples of serious human diseases caused by the Bunyaviridae are Rift Valley fever, Crimean-Congo haemorrhagic fever, California encephalitis and haemorrhagic fever with renal syndrome (Table 1.2). In addition, the Bunyaviridae also display some unique features in aspects of their molecular biology such as genome structures and genome expression strategies, which make them of interest to study. Our knowledge of the molecular biology of the Bunyaviridae is limited and not balanced: the Bunyaviridae has such a large diversity in serology, pathogenicity and biological properties that some genera have attracted more attention than others.

Bunyamwera virus is the prototype virus of the family and is the first member whose genome sequence has been completely determined (Lees *et al.*, 1986; Elliott, 1989a and b). The research project presented in this thesis concerns expression of the Bunyamwera virus L gene and analysis of its role in RNA synthesis. In this chapter, a general overview of the Bunyaviridae is provided, and the virus gene expression, RNA synthesis and the polymerase protein will be discussed in more detail. Other aspects of the biology of the family, not directly related to this area, have to be dealt with only briefly.

The Bunyaviridae is classified as a family of negative-strand RNA viruses and shares some common features with the other negative-strand RNA viruses in its replication mechanism. Negative-strand RNA viruses include six families: Arenaviridae, Bunyaviridae, Filoviridae, Orthomyxoviridae, Paramyxoviridae and Rhabdoviridae. These six families are to some extent differentiated by morphology and fall into two distinct categories in terms of genome structure (Pringle, 1991), which are summarized in Table 1.1. The Arenaviridae, Bunyaviridae and Orthomyxoviridae have segmented genomes, whereas the Filoviridae, Paramyxoviridae and Rhabdoviridae have linear undivided genomes. Studies on other groups of RNA viruses with negative-sense genomes will no doubt help our understanding of the Bunyaviridae. Therefore, references will be made to various negative-strand RNA viruses throughout the thesis.

Table 1.1 Classification of Negative-strand RNA Viruses

Family	Genus	Representative virus	Genome size (kb)	No of RNA segments
Arenaviridae	*Arenavir us*	Old world, e.g. Lassa New world, e.g. Tacaribe	10.6	2
Bunyaviridae	*Bunyavirus*	Bunyamwera		
	Hantavirus	Hantaan		
	Nairovirus	Crimean Congo haemorrhagic fever	12-17	3
	Phlebovirus	Sandfly fever		
	(Uukuvirus)	Uukuniemi		
	Tospovirus	Tomato spotted wilt		
Filoviridae	*Filovirus*	Marburg Ebola	12.7	1
Rhabdoviridae	*Vesiculovirus*	Vesicular Stomatitis	11.2	1
	Lyssavirus	Rabies	11.9	
Paramyxoviridae	*Morbillivirus*	Measles		
	Paramyxovirus	Parainfluenza virus type 3 Sendai Mumps	15.2 - 15.9	1
	Pneumovirus	Respiratory syncytial virus		
Orthomyxoviridae	*Influenzavirus*	Influenza virus type A	13.5	8
		B	14.0	8
		C	14.0	7

1.2. Bunyaviridae

1.2.1. Classification and taxonomy

The classification of the viruses in the family Bunyaviridae was originally based on their serological relationships and the data have been further supplemented and largely supported by biochemical analyses. At present, five genera are recognized within the family Bunyaviridae: *Bunyavirus, Hantavirus, Nairovirus, Phlebovirus,* (Bishop *et al.*, 1980; Schmaljohn and Dalrymple, 1983) and the recently established *Tospovirus*. The *Tospovirus* genus contains only one plant-infecting member of the Bunyaviridae, tomato spotted wilt virus (TSWV, Milne and Francki, 1984; de Haan *et al.*, 1990), and ICTV has approved its inclusion in the Bunyaviridae recently (Peters, 1991). *Uukuvirus* had been an independent genus in the family, however, most recently ICTV has approved its inclusion in *Phlebovirus* genus. In addition, at least 10 serogroups containing 28 viruses, as well as some 27 other viruses are considered to be possible members of the family (Bishop, 1990).

The classification of the Bunyaviridae is summarized in Table 1.2. The essential characteristics of viruses assigned to the Bunyaviridae, as defined by Bishop and Shope (1979) are as follows:

(1). The viruses are spherical (90-100 nm in diameter) and enveloped with glycoprotein surface projections.

(2). The virions contain three unique segments of negative sense single-stranded RNA (S, M, and L) in the form of circular ribonucleoprotein complexes (nucleocapsids) and a transcriptase enzyme.

(3). The viruses mature by budding into intracytoplasmic vesicles associated with the Golgi apparatus.

(4). The viruses have the capacity to interact genetically with certain other closely related viruses and produce recombinant viruses by genome segment reassortment.

(5). The viral RNA species have a negative-sense coding strategy except the S segment of phleboviruses and tospovirus has an ambisense strategy.

In general, viruses within a genus share complement fixation antibodies and are subdivided into different serogroups on the basis of neutralization and haemagglutination-inhibition reactions. Phleboviruses are exceptions in that the complementation fixation test is specific and the haemagglutination-inhibiton reaction is cross-reactive (Calisher & Karabatsos, 1988).

The majority of viruses in the Bunyaviridae are transmitted to vertebrate hosts by arthropods. There is no evidence for insect vector transmission for hantaviruses and it is

suggested that each hantavirus is perpetuated within a single (or a few) rodent species (LeDuc *et al.*, 1982). Hantaviruses cause asymptomatic persistent infections of rodents, and rodent-rodent, and rodent-human infections are caused by aerosolized rodent excretions. Human diseases have been associated with representatives of all genera except *Tospovirus*.

Table 1.2 Classification of the Bunyaviridae

Genus	No. of serogroups	No. of viruses	Examples of viruses associated with human disease	Principal arthropod vectors
Bunyavirus	16	156	La Crosse California encephalitis Tahyna Jamestown Canyon Oropouche	Mosquitoes Gnats
Hantavirus	1	9	Hantaan Puumula Seoul	None (rodents)
Nairovirus	6	32	Crimean-Congo haemorrhagic fever	Ticks
Phlebovirus	1 (8)*	39	Sandfly fevers Toscana Rift Valley fever	Sandflies Gnats
*(Uukuvirus)***	1	12	none	Ticks
Tospovirus	1	1	none	Thrips

* Phleboviruses comprise a single serogroup (Phlebotomus fever) divided into eight antigenic complexes. ** *Uukuvirus* is now included in *Phlebovirus* genus. This table is modified from Elliott (1990).

1.2.2. Virus morphology

Bunyaviridae particles in general are spherical, 80-120 nm in diameter and display surface glycoprotein projections of 5-10 nm which are embedded in a lipid bilayered envelope apparently 5 nm thick as observed under the electron microscope. There are some morphological varieties among viruses in all genera and unique external features have been described for representatives of each genus. The surface structures of uukuvirus are defined by clustered glycoproteins which form hollow cylindrical morphological units (von Bonsdorff & Pettersson, 1975). Bunyaviruses have distinct,

regular sharply defined round surface structures about 10 nm in diameter (Martin *et al.*, 1985). However, the surface structure of nairoviruses exhibits less distinct, small morphological surface units (Donets *et al.*, 1977). Hantaviruses sometimes display an unusual gridlike pattern on their surfaces and elongated particles (110-210nm long) are often observed (Hung *et al.*, 1985). Phleboviruses exhibit knotlike morphological units with no distinct pattern (Martin *et al.*, 1985). It is possible that the morphological differences between genera reflect altered interaction of the stain with the surface proteins, or different arrangement of the surface proteins on the particle.

The chemical composition of virus has been determined only for Uukuniemi virus and found to be about 2% RNA, 58% protein, 33% lipid and 7% carbohydrate (Obijeski & Murphy, 1977). The virus particle of the Bunyaviridae contains four structural proteins (G1, G2, L and N) and the RNA genome.

1.2.3. RNA genome structure

The genome of the Bunyaviridae comprises three unique single stranded RNA segments (L, M and S) of negative or ambisense polarity.

Each RNA genome segment is intimately associated with the N (2100 molecules per particle) and the L (25 molecules per particle) proteins to form a nucleocapsid (Obijeski *et al.*, 1976). The nucleocapsids can be released from virions by disruption with nonionic detergents and purified by CsCl gradient centrifugation. All three nucleocapsids species are sufficiently stable in the high salt solution to band at a buoyant density of 1.31 g/ml in CsCl, whereas mRNAs are pelleted (Obijeski *et al.*, 1976). The buoyant density of the Bunyaviridae nucleocapsids in CsCl is very similar to those of rhabdoviruses and paramyxoviruses, as well as tobacco mosaic virus. The association of RNA with the N protein renders the nucleocapsids resistant to RNase digestion to some extent.

The three RNA segments are designated on the basis of their size class (summarized in Table 1.3) and are usually not present in equimolar amounts, the S species usually predominates (Obijeski *et al.*, 1976; Gentsch *et al.*, 1977). As can be seen from Table 1.3, the S segments of bunyaviruses are considerably smaller than those of other genera; the L RNA segments of nairoviruses are much larger than the others; differences of the M RNA segments among the genera are relatively less apparent.

When viewed in the electron microscope, all three RNA segments were found to be circular (Petterson & von Bonsdorff, 1975; Samso *et al.*, 1975). Panhandle structures of varying lengths have been observed in RNA preparations of Uukuniemi virus (Hewlett *et al.*, 1977) and Germiston virus (Pardigon *et al.*, 1982), in addition to circular forms. Such circular or panhandle structures can be rendered linear under denaturing conditions (Hewlett *et al.*, 1977). It was found that genomic RNA contains free 5' termini (pppA)

and 3' hydroxyl ends (Obijeski *et al.*, 1976), indicating that the circular RNA species are not closed by covalent linkage between the 3' and 5' ends. More recently, psoralen cross-linking studies on nucleocapsids showed that the ends of the RNA within nucleocapsids are capable of base pairing to form dsRNA, as they are in the naked RNA (Raju and Kolakofsky, 1989). Therefore, it appears that in the nucleocapsids the N protein must have been locally displaced from the RNA to allow RNA molecules to base pair.

Table 1.3 Sizes of RNA segments of the Bunyaviridae

Genus	L	M	S
Bunyavirus	6,875	4,458-4,534	850-984
Hantavirus	6,530-6,550	3,616-3,682	1,696
Nairovirus	11,000-14,000*	4,400-6,300*	1,712
Phlebovirus	6,500-8,200*	3,884-4,330	1,690-1,904
(Uukuvirus)	6,423	3,231	1,720
Tospovirus	8,897	5,000-5,400	2,916

* The length of the RNA segments were estimated by gel analysis. Others were determined by nucleotide sequence analysis.

The 3' and 5' terminal sequences of the individual genome segments are complementary, which presumably accounts for the observation of circular and/or panhandle forms of the RNA segments. The terminal 9-15 nucleotides are conserved within each genus (Elliott, 1990); the conserved sequences of bunyaviruses and hantaviruses are similar and those of phleboviruses and uukuviruses are identical (Table 1.4). The terminal base-paired structures may have functional roles in viral RNA synthesis and will be discussed later.

1.2.4. Replication cycle

The replication cycle of Bunyaviridae involves virus attaching and entering cells, genome transcription and translation, genome replication and virus maturation.

Table 1.4. 3'-terminal nucleotide sequences of Bunyaviridae RNA segments

Genus	Virus	Genome segment	3'terminus
Bunyavirus	Bunyamwera	S	UCAUCACAUGAGGU
		M	UCAUCACAUGAUGG
		L	UCAUCACAUGAGGA
Hantavirus	Hantaan	S	AUCAUCAUCUGAGGG
		M	AUCAUCAUCUGAGGC
		L	AUCAUCAUCUGAGGG
Nairovirus	Qalyub	S	AGAGAUUCUGCCUGC
		M	AGAGAUUCUUUAUGA
		L	AGAGAUUCUUUAAUU
Phlebovirus	Rift Vally fever	S	UGUGUUUCGG
		M	UGUGUUUCUG
		L	UGUGUUUCUG
(Uukuvirus)	Uukuniemi	S	UGUGUUUCUGGA
		M	UGUGUUUCUGCC
		L	UGUGUUUCUGGA

1.2.4.1. Attachment and entry

The life cycles of viruses in the Bunyaviridae family can involve replication alternately in vertebrates and arthropods. The nature of the Bunyaviridae receptor(s) either in vertebrate or in invertebrate is not known. It is assumed that the external glycoproteins on virions mediate virus attachment to host cell receptors. James and Millican (1986) isolated some virus mutants by repeated passages of Bunyamwera virus through mosquito cells and it was found that those mutants were better adapted to replicate in the invertebrate cells than the initial virus. Meanwhile, these mutants lost their reactivity to G1 specific monoclonal antibodies raised against the parental virus, suggesting that the relative resistance of insect cell-passaged virus to neutralizing antibodies results from genetic changes not post-translational modification of viral proteins. Treatment of virions with trypsin or pronase, which removed most of G1, renders the virus completely noninfectious in vertebrate cells (Kingsford & Hill, 1981), suggesting that G1 is involved in the attachment process. Ludwig *et al.* (1989) demonstrated that G2 was required for efficient attachment and infection of mosquito cells. When they treated La Crosse virus with pronase and trypsin to remove G1 but leaving G2 intact, it was found that enzyme treated virions had less infectivity in

vertebrate cells, but the infectivity was either unaffected or increased in invertebrate cells. They proposed that the major determinants for attachment to mosquito cells are on G2 and are usually masked by G1, and digestion of G1 by proteases in the insect midgut renders efficient attachment and infection of mosquito midgut cells. This also explains the molecular basis for midgut infection barriers and species susceptibility to arbovirus infection in nature.

Fusion of infected cells at acidic pH values has been reported for viruses in the Bunyaviridae (Gonzalez-Scarano *et al.*, 1985) as well as numerous other enveloped viruses. The membrane fusing activities of these viruses become manifest only upon exposures to acid pH, which appears to induce a conformational change in the virus surface components. Viral mutants with a defective fusion function have been selected and it appears that G1 protein of bunyavirus mediates such fusion (Gonzalez-Scarano *et al.*, 1985). However, it was suggested that the large hydrophobic domain in the bunyavirus G2 (residues 187-245) was responsible for fusion activity and the role of G1 concerned with the cell surface receptor rather than directly with the lipid bilayer (Pobjecky *et al.*, 1989). The molecular mechanism involved in bunyavirus mediated cell fusion has yet to be elucidated.

1.2.4.2. Transcription and translation

After uncoating of the viral genome, transcription of a negative-strand virus RNA genome to mRNA is believed to occur by the virion associated RNA polymerase. Transcriptase activity has been detected in detergent-disrupted virion preparations of several members of the Bunyaviridae (Ranki & Pettersson, 1975; Bouloy & Hannoun, 1976; Schmaljohn & Dalrymple, 1983; Patterson *et al.*, 1984; Gerbaud *et al.*, 1987a). The features of the Bunyaviridae RNA synthesis are outlined below and will be discussed more extensively in section 1.5.

It is observed that viral mRNA is not polyadenylated; it is 60-100 nucleotides shorter at 3' end than the full length genome; there is a host derived capped methylated primer present at 5' end of the mRNA (for review, see Elliott *et al*, 1991; Kolakofsky & Hacker, 1991). Bunyavirus transcription is not affected by actinomycin D or α-amanitin (Vezza *et al.*, 1979) and takes place in the cytoplasm of infected cells (Rossier *et al.*, 1986; Kolakofsky *et al.*, 1987). For some viruses, continual protein synthesis is required for mRNA synthesis (Abraham & Pattnaik, 1983; Patterson & Kolakofsky, 1984).

Viral polypeptides are synthesized shortly after infection although the relative rate of synthesis varies with the virus, host-cell type and multiplicity of infection (McPhee & Westaway, 1981; Struthers *et al.*, 1984; Watret *et al.*, 1985). At a high multiplicity of

infection, N and NSs proteins of RVF phlebovirus can be detected as early as 2 hr of postinfection and the glycoprotein shortly afterwards (Parker *et al.*, 1984). Hantaviruses and uukuviruses exhibit a slower rate of protein synthesis and the viral proteins can not be detected until about 6 hours after infection.

During translational process, some of the viral polypeptides undergo several maturation changes such as cotranslational cleavage, glycosylation of the glycoprotein, and in the case of RVFV, phosphorylation of the NSs protein (Struthers & Swanepoel, 1982; Struthers *et al.*, 1984).

1.2.4.3. Genome replication

For negative-strand RNA viruses, the change from primary transcription to genome replication requires a switch from mRNA synthesis to the synthesis of full length antigenomic RNA and then viral genomic RNA. Both genomic and antigenomic RNA are encapsidated by the N proteins, in contrast to mRNA which is naked. Therefore, it appears that the N protein may play an important role in genome replication.

The factors involved in the switch from primary transcription to genome replication have not been defined for any member of the Bunyaviridae family. Presumably, some viral factor(s) is required to signal a suppression of the transcription termination signal responsible for generation of truncated mRNA and also to prevent the addition of host derived primers to the 5' termini of antigenomic RNA. It is speculated that the NSs proteins (where they exist) might be involved in the switch through their interaction with the N protein and/or the viral RNA polymerase (Schmaljohn & Patterson, 1990).

1.2.4.4. Virus maturation

A feature of the Bunyaviridae is virus maturation which occurs by a budding process at intracellular smooth membranes, principally in the Golgi complex (Kuismanen *et al.*, 1982, 1984; Smith & Pifat, 1982). However, in one report, virus maturation was observed at the cell surface of RVFV infected primary rat hepatocytes (Anderson and Smith, 1987). Viruses in the Bunyaviridae family lack a matrix (M) protein which is responsible for bridging the gap between the integral viral envelope proteins and nucleocapsids of other negative-strand RNA viruses. Therefore, the early events of assembly must include an interaction between viral nucleocapsids and glycoproteins, which have been observed at smooth membrane vesicles, and predominantly at membranes in or adjacent to Golgi complex (Smith & Pifat, 1982).

The Golgi-targeting property of the Bunyaviridae glycoproteins has been shown for Uukuniemi, Rift Valley fever, Hantaan and Bunyamwera viruses to be independent of viral replication and other viral gene products (Gahmberg *et al.*,1986; Wasmonen *et al.*,

1988; Pensiero *et al.*, 1988; Nakitare & Elliott, unpublished data). These observations suggest that Golgi localization and/or retention signals are contained within the viral glycoproteins themselves, but these have not yet been delineated.

1.2.5. Genetics of Bunyaviridae

1.2.5.1. Genome reassortment

As with other segmented genome viruses, the tripartite genomes of the Bunyaviridae can exchange RNA segments resulting in reassortant viruses. Reassortant bunyaviruses have been isolated from mosquitoes collected in nature (Klimas *et al.*, 1981), and in mosquitoes which have been dually infected via intrathroacic inoculation, simultaneous feeding or interrupted feeding (Beaty *et al.*, 1981; Beaty *et al.*, 1985; Beaty & Bishop, 1988; Chandler *et al.*, 1990). Furthermore, Bunyaviridae reassortment has also been demonstrated *in vitro* and only occurs between closely related viruses, not between viruses in different genera, nor between viruses in different serogroups of the same genus. Even within a serogroup some viruses appear to be incompatible with each other (Gentsch & Bishop, 1976; Gentsch *et al.*, 1977, 1979; Iroegbu & Pringle, 1981; Ozden & Hannoun, 1978, 1980; Rozhon *et al.*, 1981; Pringle *et al.*, 1984; Janssen *et al.*, 1986).

Reassortment in nature may be of significance as a means of evolution for viruses with segmented genomes. Genome reassortment has been used to assign the viral proteins to genome segments, which will be discussed in section 1.4.

1.2.5.2. ts mutants

Genetic studies of the Bunyaviridae family have been largely due to isolation of temperature sensitive (ts) mutants, mostly derived by chemical mutagen induction and a few spontaneous mutants. More than 200 ts mutants of 10 bunyaviruses and Uukuniemi virus have been produced (Gentsch *et al.*, 1979; Bishop, 1979; Ozden & Hannoun, 1978, 1980; Iroegbu & Pringle, 1981; Gahmberg, 1984). It is an anomaly that despite the tripartite nature of the Bunyaviridae genome, the vast majority fall into two recombinant/reassortment groups, except for a single mutant, Maguari virus ts 23 which defines a third group (Iroegbu & Pringle, 1981). Recently, the S RNA segment of Maguari virus ts 23 has been sequenced (Pritlove & Elliott, unpublished data) and a point mutation which would cause amino acid substitution in both N and NSs proteins was found. Thus in contrast to the suggestion of Murphy & Pringle (1987), Maguari virus ts 23 might be assigned to the S segment though more investigation is needed to confirm this. Since the RNA template of negative-strand RNA viruses is in a nucleocapsid form,

mutation of the N protein might affect its RNA binding function and further affect virus genome replication. Further, the ORFs of the N and NSs overlap so that in the overlapping region a mutation yielding a ts protein for one may concomitantly produce an inactive (lethal) protein for the other (Bishop, 1990). These might be the reason why the S mutants have not been isolated for most viruses in the family. In addition, Maguari virus ts 23 mutant shows very poor growth characteristics at the permissive temperature. Group I and II have been assigned to M segment and L segment respectively by recombination studies (Iroegbu & Pringle, 1981; Bishop, 1979). One of the bunyavirus ts mutant in Group II, BUN ts 7 has a faster migrating G1 in SDS-polyacrylamide gels. The plaque morphology and plaque size of progeny recombinants derived from recombination of parental ts mutants are determined by ts mutants in Group I, whereas plaque opacity is taken from Group II. Group II mutants of Bunyamwera virus have been assigned to the M segment, Group I mutants are presumed to have defect in the L segment (Pringle, personal communication). On the other hand, Group II ts mutants in California encephalitis serogroup are located in the L RNA segment and Group I ts mutants are in the M RNA segment, which have been confirmed by RNase fingerprinting and *in vitro* transcripts assay of the mutants (Vezza *et al.*, 1979).

Homologous and heterologous recombination among the ts mutants have been performed and in most combinations, heterologous recombination is less efficient than homologous recombination (Iroegbu and Pringle, 1981). There is no genetic barrier to the exchange of genetic material between Batai, Bunyamwera and Maguari viruses *in vitro*. Complementation between Bunyamwera ts mutants and those of La Crosse virus (California encephalitis group) have been unsuccessful (Iroegbu and Pringle, 1981) and the molecular basis for this restriction has not been established.

1.2.6. Persistent infection and defective interfering particles

Viruses in all genera, except the *Hantavirus* genus, are capable of alternately replicating in vertebrates and arthropods. In general, viruses are cytopathogenic in their vertebrate hosts whereas persistent infection is readily established in their invertebrate host. Persistent infections can be established *in vitro* in mosquito cell cultures. Mosquito cells persistently infected with Bunyamwera, La Crosse, Marituba bunyaviruses and Toscana phlebovirus showed no differences in their metabolic activities, but continued to shed infectious virus (Newton *et al.*, 1981; Nicoletti & Verani, 1985; Carvalho *et al.*, 1986; Elliott & Wilkie, 1986; Rossier *et al.*, 1988; Scallan & Elliott, 1991). Features of bunyavirus persistent infection include (Elliott & Wilkie, 1986): (1) both viral antigens and RNA can be detected in the persistent infected cells; (2) the cells are resistant to

superinfection by homologous or closely related viruses; (3) the cells continue to shed infectious virus after prolonged passages; (4) the persistent state is accompanied by genetic and phenotypic changes in the viruses, including plaque morphology, ts phenotype and generation of defective interfering particles (DI).

The molecular mechanisms involved in the establishment and maintenance of persistent infection have not been fully elucidated. However, some possibilities have been suggested. In La Crosse virus (LAC) infected mosquito cells, viral mRNA is encapsidated by the N protein late in infection, thereby rendering the mRNA untranslatable (Hacker *et al.*, 1989). Therefore, it is suggested that the self-limiting nature of the infection of mosquito cells is related to the control of the N protein translation (Kolakofsky and Hacker, 1991, Hacker *et al.*, 1989). Generation of DI particles may also be involved in persistent infection. DI particles from Bunyamwera virus infected carrier cultures appear to contain only S segment RNA (Elliott & Wilkie, 1986; Scallan and Elliott, 1991). More typical DI RNAs, derived from the L RNA segment, have been observed in Germiston bunyavirus-infected cells (Cunningham & Szilaggi, 1987). Recently, Scallan and Elliott (1991) reported defective RNAs derived from the L RNA segment in Bunyamwera virus persistently infected mosquito cells but these RNAs were not packaged into virus particles. Another possible mechanism of persistent infection might be because the viral transcriptase and endonuclease activity of the L protein is less active in arthropod than in mammalian cells (Schmaljohn & Patterson, 1990).

1.3. Viral proteins and protein function

1.3.1. Structural proteins

Four structural proteins are present in the Bunyaviridae virions: two internal proteins, the N (nucleoprotein) and the L proteins which are associated with the RNA segments to form nucleocapsids, and two external glycoproteins, termed G1 and G2, which are inserted into the virus membrane. Some nairoviruses may contain three glycoproteins (Foulke *et al.*, 1981).

1.3.1.1. Glycoproteins G1 and G2

Glycoproteins G1 and G2 are named according to their protein size. Both proteins appear to be located on the external side of the lipid membrane as evidenced by their sensitivity to protease digestion. The protease treated virus preparations had spike-less particles and showed decreased infectivity (Obijeski *et al.*, 1976). Various analyses have been undertaken to characterize the structural features of the glycoproteins: direct amino

acid sequencing analysis, specific peptide antibodies mapping (Fazakerley *et al.*, 1988), nucleotide sequence determinations of cloned cDNAs and gene expression analysis.

Some structural features of bunyavirus glycoproteins have been revealed by comparison of the glycoprotein precursors of four bunyaviruses, snowshoe hare (SSH), La Crosse (LAC), Bunyamwera and Germiston viruses (Lees *et al.*, 1986; Elliott, 1990). G1 and G2 are rich in cysteine residues (Elliott, 1990) which have the potential of forming disulfide bonds and a very complex tertiary structure. G1 has a single hydrophobic domain close to its carboxy terminus which is proposed as a transmembrane and anchor domain. An overall amino acid homology of about 40% is revealed among these four G1 molecules, with more similarity in the carboxy half than the amino half of the molecule. It is suggested that G1 is oriented with its amino terminus external to the viral envelope and its carboxy terminus internal to the envelope. The amino acids in the N-terminus of G1 is more variable, indicating the external amino half of G1 may determine the antigenicity of the individual viruses in the *Bunyavirus* genus. G2 has higher amino acid homology of about 66%. The hydropathy profiles of the four polypeptides shows that G2 has a rather long single hydrophobic sequence (residues 187-245) constituting about 20% of the molecule. The large hydrophobic region is longer than a single transmembrane domain and is followed by a highly charged stop-transfer sequence (residue 246-252). The orientation of G2 relative to the viral envelope is not known yet (Fazakerley *et al.*, 1988).

Bunyavirus glycoproteins have few oligosaccharide side-chains (Cash *et al.*, 1980; Lees *et al.*, 1986; Pardigon *et al.*, 1988). The glycans are mostly of the endoglycosidase H complex type (Madoff & Lenard, 1982). Some endo H-sensitive high-mannose and some small endo H-sensitive intermediate type glycans are also found. Four predicted N-linked glycosylation sites have been found to be conserved in glycoproteins in the form of Asn-Xxx-Ser/Thr. Two are strictly conserved in G2, a single strictly conserved site in G1 and a second conserved between viruses in the same serogroup of the four bunyaviruses compared (Elliott, 1990). When Bunyamwera virus infected BHK cells were labelled with ^{35}S-methionine or ^{35}S-cysteine in the presence of the glycosylation inhibitor tunycamycin, the G1 showed a faster migrating ability (Lees *et al.*, 1986). Both G1 and G2 of Inkoo bunyavirus can be labelled with sugar precursors e.g., ^{3}H-mannose, which has not been found in the unglycosylated N protein (Pesonen *et al.*, 1982). The glycoproteins G1 and G2 have been found to relate with viral virulence, neutralization, haemaglutination and cell fusion (for review, see Elliott, 1990).

The G1 and G2 proteins of Uukuniemi virus are present in nearly equimolar amounts in purified virions and may form a dimer to compose the structural units on the virus surface (Pettersson and Bonsdorff, 1987). Virion structural units are arranged in a T=12, P=3 icosahedral surface lattice as viewed by negative staining and freeze-etching,

implying that the surface is formed by 110 hexamers and 12 pentamers, i.e. 720 structure units.

1.3.1.2. The N protein

The nucleoprotein (N protein) is found to be associated with the RNA genome both in the virion and infected cells. By analysis of the distribution of ^{3}H-labelled LAC virus RNA species after oxidation and reduction with ^{3}H-sodium borohydride, the approximate number of N protein molecules per RNA strand of LAC bunyavirus has been estimated by Obijeski *et al.* (1976). It was calculated that 1209 N protein molecules are associated with the L RNA segment, 750 N with the M and 167 N with the S segment. Thus, in the nucleocapsids of bunyavirus, one N protein would associate with 6 nucleotides.

The N proteins of viruses in the *Bunyavirus, Phlebovirus,* and *Tospovirus* genera are of similar size ranging from 19K to 30K. Nairoviruses and hantaviruses have larger N proteins of M_r 48K-54K (Watret and Elliott, 1985a; Elliott, 1989b). The N protein sequences of six viruses of *Bunyavirus* genus have been compared (Elliott, 1989b): Bunyamwera (Elliott, 1989b), Maguari (Elliott & McGregor, 1989) and Germiston (Gerbaud, 1987a, b) of Bunyamwera serogroup, snowshoe hare (Bishop *et al.*, 1982) and La Crosse viruses (Akashi & Bishop, 1983; Cabradilla *et al.*, 1983) of the California serogroup and Aino virus of Simbu serogroup (Akashi *et al.*, 1984). The six way alignment of the N proteins shows that the N protein has overall 40% similarity between serogroups and 80% or greater similarity within a serogroup. Certain regions of the N protein are well conserved, particularly between residues 62-102, residues 123-169 and the carboxy-terminal 15 residues. The conserved regions may reflect their functional significance, presumably binding to the viral RNA, and may also account for the cross-reactivity throughout the *Bunyavirus* genus (Shope,1985).

1.3.1.3. The L protein

The large (L) protein of the Bunyaviridae is also associated with RNA genome and is considered to be the viral RNA polymerase. The transcriptase activity of the L protein was first detected in purified Lumbo bunyavirus preparations by Bouloy and Hannoun (1976) and further confirmed in a number of other bunyaviruses (Gerbaud *et al.*, 1987a; Patterson *et al.*, 1984). Patterson *et al.* (1984) demonstrated that LAC virion associated polymerase synthesized (+) transcripts *in vitro* and the polymerase activity can be stimulated by dinucleotides (e.g. ApG), cap analogues (e.g. mGpppAm) and natural mRNAs (e.g. alfalfa mosaic virus RNA 4). An endonuclease activity which cleaved methylated capped mRNAs *in vitro* was also detected. Their results imply that the L

protein present in the virion is responsible for the polymerase and endonuclease activities, although more direct evidence is lacking.

The L proteins of the viruses in the Bunyaviridae are very similar in size with M_r of more than 200K. The L protein of Bunyamwera virus has M_r 259K and the L protein of hantaviruses has M_r 247K. Considering such a large size of the L protein, some other functions may also be associated with it and it may be a multifunctional protein. The L protein has been implicated in some biological properties of the virus, such as virulence which was demonstrated by the studies of ts mutants (Beaty *et al.*, 1981; Rozhon *et al.*, 1981) and reassortant viruses (Janssen *et al.*, 1986).

1.3.2. Virus-coded nonstructural proteins

Two nonstructural proteins have been found in cells infected with some members of Bunyaviridae and are designated as NSs and NSm according to their coding strategies (see later). NSs proteins have been detected in bunyavirus, phlebovirus, tospovirus (TSWV) and uukuvirus infected cells, whereas NSm proteins have only been identified in bunyavirus and phlebovirus infected cells.

1.3.2.1 NSs

The NSs protein detected in bunyavirus infected cells has a molecular weight of 10K-13K (Fuller and Bishop, 1982; Short *et al.*, 1982; Bouloy *et al.*, 1984; Elliott, 1985; Watret *et al.*, 1985; Elliott & McGregor, 1989). Phleboviruses induce the synthesis of an NSs protein of M_r 29K-32K (Struthers and Swanepoel, 1982; Watret *et al.*, 1985; Overton *et al.*, 1987). The NSs protein encoded by TSWV is significantly larger and has M_r 52.4K (Kormelink *et al.*, 1991).

Conflicting data have been reported on the presence of nonstructural proteins in virus particles. Overton *et al.* (1987) reported that the NSs protein can be detected in purified virion and intracellular nucleocapsid preparations of PT phlebovirus. However, Simons *et al.* (1990) could not demonstrate NSs in highly purified Uukuniemi virions. Kormelink et al. (1991) also reported that NSs protein of TSWV can not be detected in purified virions by Western blot analysis. Thus, the specific association of NSs protein with the nucleocapsid structure needs more extensive investigation. Struthers *et al.* (1984) clearly showed that the NSs of RVFV is phosphorylated, which is the only case of a Bunyaviridae specific phosphoprotein reported to date. The other unique feature of RVFV NSs is that it is present in the nuclei of the infected cells, which might be responsible for the formation of the intracellular inclusions observed in RVFV infected cells (Struthers and Swanepoel, 1982). The NSs protein of TSWV was demonstrated to be dispersed throughout the cytoplasm or associated with fibres in infected cells using an

immunogold labelling technique. The amount of NSs protein has also been observed to be related with the severity of disease symptoms induced (Kormelink *et al.*, 1991). NSs has been suggested to be involved in the viral RNA synthesis process (Bishop, 1985) and its function remains to be elucidated.

1.3.2.2. NSm

The NSm proteins have been detected in bunyavirus and phlebovirus infected cells. The NSm proteins of bunyavirus are of M_r 11K-16K (Fuller and Bishop, 1982; Elliott, 1985; Lees *et al.*, 1986). Inspection of the amino acid sequence of four bunyavirus NSm proteins (Elliott, 1990) shows that NSm is highly hydrophobic, suggesting it is a membrane-associated protein.

In the *Phlebovirus* genus, the NSm of Punta Toro (PT) virus has different molecular weight (30K) to that of RVFV (14K). In addition to the 14K nonstructural protein, a 78K protein was also detected in RVFV infected cells (Kakach *et al.*, 1988). Subsequent studies (Wasmonen *et al.*, 1988) indicated that the 78K protein is the precursor polypeptide of preglycoprotein and G2.

Hydrophobic profile analysis of NSm implied that it may function as a signal peptide during translation and processing of the glycoproteins (Lees *et al.*, 1986). However, using vaccinia virus expression system, the 78K and 14K proteins of RVFV do not appear to be necessary for glycoprotein synthesis, processing, modification or Golgi distribution. Thus, the role of NSm in the virus life cycle remains to be established.

1.4. Gene expression strategies

The coding relationship between the genomic RNA segments and the polypeptide gene products has been established through genetic and biochemical studies of the viruses. As segmented RNA genome, members of Bunyaviridae have the capacity to produce recombinant viruses by genome reassortment. By comparison of the polypeptide profiles of parental and recombinant viruses, the N protein was mapped to the S RNA segment (Gentsch and Bishop, 1978); the glycoproteins (G1 and G2) were mapped to the M RNA segment (Gentsch and Bishop, 1979); the L protein was mapped to the L RNA segment (Endres *et al.*, 1989; Elliott, 1989a); and the nonstructural proteins NSs to the S segment and NSm to the M segment (Elliott, 1985; Fuller *et al.*, 1982).

These results have been confirmed by virus RNA genome nucleotide sequencing data which have also revealed the expression strategies of the individual genome segments. As the Bunyaviridae family contains such a large numbers of viruses (>300), there is significant diversity of viruses in genome structures and expression strategies.

Based on the available information, the coding strategies of the Bunyaviridae genomes will be discussed in more detail regarding individual cases.

1.4.1. L RNA segment

It has been assumed that the L RNA segment encodes the L protein which functions as the viral polymerase. Although the L protein has been observed in representative viruses of all genera, direct evidence has only been obtained recently (Elliott, 1989a; Endres *et al.*, 1989; Antic *et al.*, 1991; Schmaljohn, 1990; Stohwasser *et al.*, 1991). By making genome reassortment viruses in conjugation with viral protein migrating analyses, Endres *et al.* (1989) mapped the L protein of viruses in California serogroup to the L RNA segment. By a similar approach, Elliott (1989a) assigned the L protein of Bunyamwera virus to the L RNA segment.

The sequence data of the L RNA segments have been available for the Bunyamwera virus (Elliott, 1989a), hantaviruses (Antic *et al.*, 1991; Schmaljohn, 1990; Stohwasser *et al.*, 1991), TSWV (de Haan *et al.*, 1991), and Uukuniemi virus (Elliott *et al.*, unpublished data). A long single open reading frame is identified in the viral complementary RNA with potential of encoding a protein of M_r >200K (Table 1.5 and Fig. 1.1). The L RNA segments and the encoded L proteins are very similar in size except that the nairovirus L segment is considerably larger (about twice size of other L RNA segments). Therefore, it appears that the coding potential of the nairovirus L RNA segment is grossly under utilized; sequence data are needed to elucidate its coding potential. All the L segments are rich in A and U residues (53-66.7%) and show economical use of the genomic RNA: 97.7-98.86% nucleotides of the segment are used to encode the L protein. AUG-initiated ORFs in both (-) and (+) sense of the L segment have been screened and, in addition to the unique L ORF in the (-) sense, some short ORFs have been found in the (+) sense of Bunyamwera and Hantaan viruses (Elliott, 1989a; Schmaljohn, 1990). The longest ORF in (+) sense of Bunyamwera virus L segment is in position of nucleotides 6772-6383 and has 129 codons with potential of encoding a protein of M_r 14.7K. In Hantaan virus L segment the similar ORF (6302-5637) which could encode a polypeptide of 24 K was detected. However, no homology could be detected between the polypeptides encoded by these two ORFs (Schmaljohn, 1990). Preliminary data indicate that these proteins are not present in the virus infected cells, nor has the subgenomic transcript originating from these ORFs been detected by Northern blotting analysis (Elliott, 1989a). A small ORF has also been reported at the 3' end of the L segment of Tacaribe arenavirus and the encoded 11K protein can be detected in the virus infected cells. This protein contains a potential metal binding domain and has been identified as a "zinc finger" protein (Iapalucci *et al.*, 1989). "Zinc finger" sequences are generally rich in Cys residues, and

Table 1.5 Complete nucleotide sequences of Bunyaviridae L genome segments

Genus	Virus	Nucleotides			Proteins		Reference
		Length	A+U (%)	Coding region	No of aa	Mr	
Bunyavirus	Bunyamwera	6875	66.7	51-6767 (97.7%)	2238	258.6	Elliott, 1989b
Hantavirus	Hantaan 76-118	6530	63.0	38-6494 (98.8%)	2150	246.5	Schmaljohn, 1990
	Seoul 80-39	6530	62.5	37-6490 (98.8%)	2150	246.7	Antic et al., 1991
	Nephropathia epidemica	6550	63.8	73-6504 (98.7%)	2156	246.0	Stohwasser et al., 1991
Tospovirus	tomato spotted wilt	8897	66.5	63-8831 (98.5%)	2875	331.5	de Haan et al., 1991
(Uukuvirus)	Uukuniemi	6423	53.0	17-6328 (98.5%)	2103	241.0	Elliott et al., unpublished

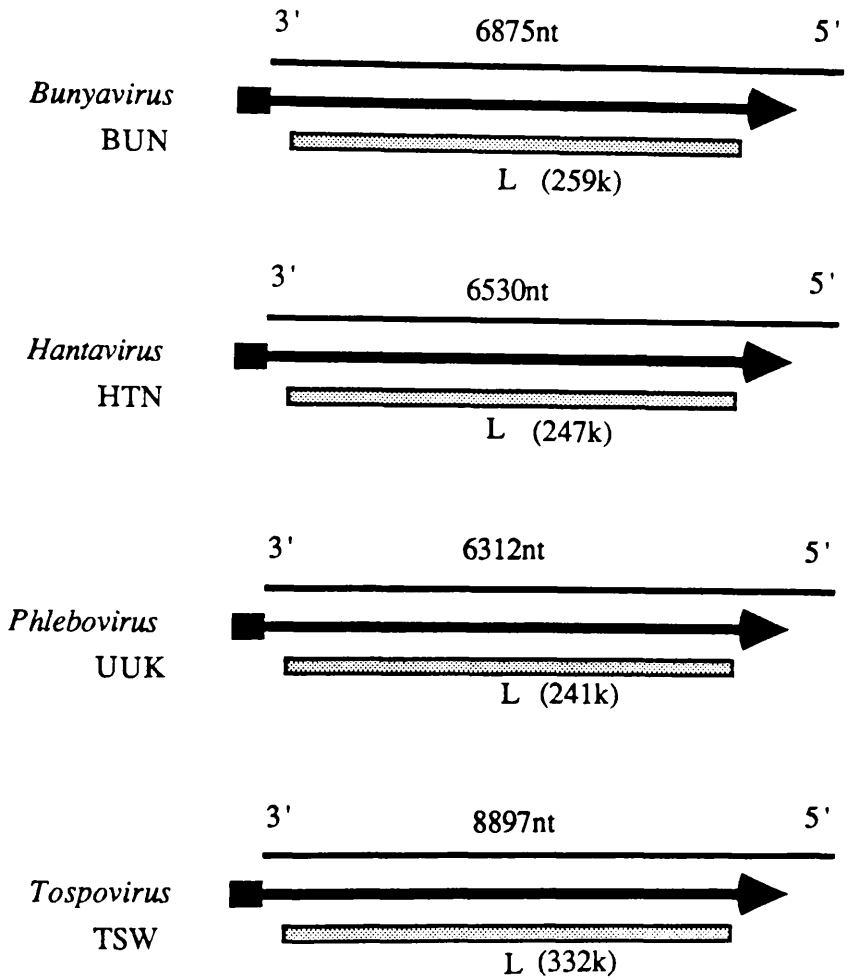


Fig.1.1. Gene expression strategy of the Bunyaviridae L RNA segment. Genomic RNAs are represented by thin lines, mRNAs by arrows (5' end and 3' ends are indicated) and gene products are designated by stippled boxes (not to scale). This figure is from Elliott (1991).

are well known for their role in the recognition of specific sequences in dsDNA (Berg, 1988) and specific recognition of RNA as found in moloney murine leukemia virus (Gorelick *et al.*, 1988). Therefore, the significance of the ORFs in the viral sense L RNA segment requires to be investigated in more detail.

1.4.2. M RNA segment

The complete nucleotide sequence data of M RNA segments representing four genera in the family are available and are summarized in Table 1.6. The M RNA segments of all the viruses in the Bunyaviridae family encode glycoproteins G1 and G2. In addition, bunyaviruses and phleboviruses M segments also encode nonstructural protein(s) even though their gene orders are different. The gene order of the bunyaviruses M RNA segment is 5' G2-NSm-G1 3', hantaviruses and uukuviruses have

the gene order of 5' G1-G2 3', and phleboviruses have 5' NSm-G1/G2-G2/G1 3' (Fig 1.2). Thus, the gene expression strategies of viruses in each genus are apparently different and are discussed individually as follows:

Bunyavirus: The bunyavirus M segment encodes, in the viral complementary RNA, two virion glycoproteins G1 (M_r 100K-120K) and G2 (M_r 29K-41K), and a nonstructural protein termed NSm (M_r 11K-18K, Gentsch and Bishop, 1979; Fuller and Bishop, 1982; Elliott, 1985).

The M segments of bunyaviruses have very similar length, ranging from 4458 to 4534 nucleotides, and have a single open reading frame of 1433-1441 amino acids (Elliott, 1990). The precursor polypeptide corresponding to this ORF has not been detected in infected cells. Attempts to translate bunyavirus M segment specific mRNA *in vitro* have also been unsuccessful (Abraham & Pattnaik, 1983; Elliott, 1985). The M RNA segment of Bunyamwera virus has been cloned into vaccinia virus, and G1 and NSm proteins have been produced (G2 was not labelled well enough for detection), and no precursor protein can be detected (Nakitare and Elliott, unpublished data). Thus, it appears that the polypeptide cleavage is probably a cotranslational event (Pennington *et al.*, 1977; Lees *et al.*, 1986).

Using direct amino acid sequencing and specific peptide antibodies, Fazakerly *et al.* (1988) were able to determine the gene order of SSH M segment to be 5' G2-NSm-G1 3' in the complementary RNA. The polypeptide sequences of the other three bunyavirus M segment precursor proteins have been aligned with that of SSH by Elliott (1990), which revealed a putative signal peptide of 13-21 residues at the amino terminus of the precursor. G2 is generated by cleavage after a conserved Arg residue; cleavage of G1 from NSm may occur after a common Ala residue; there is a major trypsin sensitive site in G1 around residue 761 (Fazakerley *et al.*, 1988; Gonzalez-Scarano, 1985).

Hantavirus: Hantavirus M segment encodes two virion associated glycoproteins G1 (68K-72K) and G2 (56K-57K) with the gene order of 5' G1-G2 3'(Fig.1.2). There is an 18-residue signal peptide preceding G1 and a small peptide of M_r 5.9K encoded between G1 and G2. The 5.9K peptide has not been detected in the virus infected cells and may only act as a signal sequence for G2 (Schmaljohn *et al.*, 1987).

The M RNA segments of the three sequenced hantaviruses are very similar in size (3616-3682 nt). Two potential translation initiation codons are present in the sequence preceding the coding region for the G1 protein. The first AUG is conserved in the ORFs of all three hantaviruses and its exclusive use is also supported by the presence of more favourable flanking sequences. It is 18 codons away from the amino terminus of HTN G1 and 16 codons from that of SR-11 G1. This 16-18 amino acid stretch would constitute an efficient signal sequence (von Heijne, 1983; Schmaljohn *et al.*, 1987) as in the case of bunyaviruses.

Table 1.6 Complete nucleotide sequences of Bunyaviridae M genome segments

Genus	Serogroup	virus	Nucleotides				Encoded proteins	Reference
			length (nt)	5'	3'	A+U (%)		
Bunyavirus	Bunyamwera	BUN	4458	56	103	61.0	G1(110K), G2(32K), NSm(16K)	Lees et al., 1986
		GER	4534	58	164	63.2	G1(110K), G2(32K), NSm(16K)	Pardigon et al., 1988
	California	LAC	4526	61	141	62.0	G1(108K), G2(32K), NSm(12K)	Grady et al., 1987
		SSH	4527	61	142	61.0	G1(109K), G2(32K), NSm(11K)	Eshita & Bishop, 1984
Hantavirus	Hantaan	HTN 76-118	3616	40	168	60.7	G1(70K), G2(55K)	Schmaljohn et al., 1987 Yoo and Kang, 1987
		SR-11	3651	46	203	61.0	G1(70K), G2(56K)	Arikawa et al.,1990
		NE	3682	40	195	61.0	G1(70K), G2(56K)	Giebel et al., 1989
Phlebovirus	Phlebotomus fever	RVF	3884	20	284	54.5	G1(62K), G2(55K), NSm(14K), 78K	Collett et al., 1985
		PT	4330	16	372	60.3	G1(61K), G2(55K), NSm(30K)	Ihara et al., 1985b
(Uukuvirus)	Uukuniemi	UUK	3231	17	187	52.2	G1(72K), G2(67K)	Ronnholm & Petterson, 1987

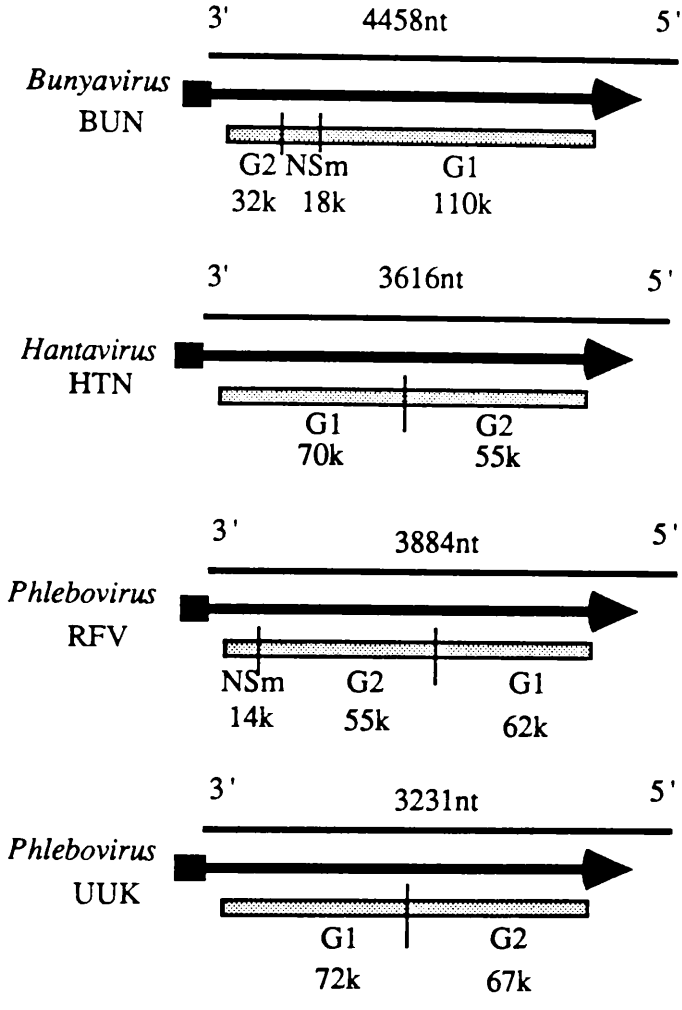


Fig. 1.2. Coding strategies of the Bunyaviridae M genome segment. Genomic RNAs are represented by thin lines, mRNAs by arrows (5' end and 3' ends are indicated) and gene products are designated by stippled boxes (not to scale). This figure is from Elliott (1991).

No precursor protein has been detected in hantavirus infected cells, suggesting either very rapid post-translational cleavage or co-translational cleavage event. The exact cleavage site has not been mapped, neither have the factors affecting the cleavage. The G2 protein may also be synthesized through an internal initiation codon which precedes sequences encoding the G2 proteins of HTN virus and SR-11 virus (Schmaljohn *et al.*, 1987; Arikawa *et al.*, 1990). This AUG can be efficiently used to produce HTN G2 by both recombinant baculovirus and vaccinia virus expression systems (Schmaljohn *et al.*, 1989, 1990), though its use in Hantaan virus infected cells remains to be determined.

Phlebovirus: The M segment of phlebovirus encodes G1, G2 and NSm, with NSm preceding G1 or G2. The M segment of two phleboviruses, Punta Toro (PT) virus

and Rift Valley fever virus (RVFV), have been sequenced and are 4330 bases and 3884 bases in length respectively. The gene order is 5' NSm-G1-G2 3' for PT virus and 5' NSm-G2-G1 3' for RVFV M segment. The G1 protein of PT virus shows 35% similarity with the G2 protein of RVFV, and the G2 protein of PT virus has 49% similarity with the G1 protein of RVFV (Ihara *et al.*, 1985b). This reflects the nomenclature system for the glycoproteins, whereby the slowest migrating protein on SDS-polyacrylamide gel is designated as G1. The sizes of nonstructural proteins encoded by PT virus and RVFV are significantly different. PT virus M segment encodes NSm of M_T 30K (Ihara *et al.*, 1985b), whereas RVFV M segment encodes a 14K protein as well as a 78K protein (Fig.1.3), all of which have been detected in virus infected cells.

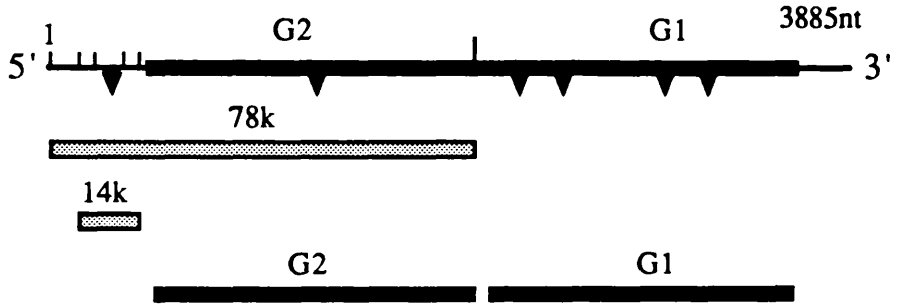


Fig. 1.3. Expression strategy of RVFV M RNA segment. At the top is shown the RNA with the coding region indicated. AUG codons are shown as I and N-linked glycosylation sites as ψ. The four protein products detected in infected cells (solid boxes - structural protein, stippled boxes - nonstructural proteins) are indicated below. This figure is modified from Elliott (1990).

The large precursor protein has not been detected in phlebovirus infected cells. A common cleavage event by an alanine-specific protease may be used by phlebovirus to cleave G1 and G2 (Ihara *et al.*, 1985b), as happens in the cleavage of the bunyavirus glycoprotein precursor to generate G1. The 78K protein translated from RVFV M segment probably contains an unprocessed polyprotein of NSm and G2 sequences. It is unclear why the cleavage site between the 14K (NSm) and G2 is not used in the 78K protein. It is speculated that glycosylation may have some role in the cleavage of this site, as it is shown that the 78K protein is glycosylated at both the preglycoprotein site which is not utilized in the 14K protein and the site that it shares with glycoprotein G2 (Kakach *et al.*, 1989).

The coding capacity and gene expression strategy of phlebovirus M RNA segment is very complicated and has been studied in detail in the case of RVFV (Fig. 1.3). There are five inframe AUG codons upstream of the amino terminus of the first glycoprotein (G2). Four proteins G1, G2 and two additional products, a glycosylated 78K protein and a nonglycosylated 14K protein (Kakach *et al.*, 1989; Wasmonen *et al.*, 1988) are

derived from the single large ORF. The sequences represented in the 78K protein begin from the first AUG of the ORF and extend to the carboxy end of G2. The 14K protein initiates at the second AUG (37 amino acids downstream from the first AUG) and contains only pre-G2 sequence (Suzich & Collett, 1988; Suzich *et al.*, 1990). Efficient synthesis of G2 requires a translation start site within the preglycoprotein region and appears to use the second AUG codon as the 14K protein. By site-directed mutagenesis and recombinant vaccinia virus expression analyses, Suzich *et al.* (1990) found that about half the production of the G1 was synthesized by a mechanism independent of all in-phase methionine codons in the preglycoprotein region and suggested that an internal translation initiation may be responsible for this. Translation initiation at the first AUG results in utilization of the glycosylation site at position 285 in the 78K protein which is not used in the 14K protein and the lack of cleavage at the junction of the preglycoprotein and G2 of the 78K protein. Therefore, the sequence between AUG codon 1 and 2 is implicated in exerting a profound influence on subsequent protein glycosylation and proteolytic processing.

The M segment sequence of Uukuniemi virus has been determined. It is 3231 bases long and encodes G1 and G2 in a single ORF on the viral complementary RNA.

A large precursor protein (110K) has been detected by translation *in vitro* of M segment specific mRNA extracted from infected cells, and the *in vitro* produced 110K polypeptide was cleaved roughly in the middle in the presence of microsomal membranes to generate G1 and G2 (Ulmanen *et al.*, 1981). The cleavage site between G1 and G2 and the factors affecting the cleavage (presumably microsome associated) have not been determined.

The gene order of the uukuvirus M segment is 5' G1-G2 3' and no preglycoprotein coding region has been found. The amino terminus of G1 is located 17 amino acids downstream of the AUG (Ronnholm & Pettersson, 1987). Both G1 and G2 are preceded by stretches of predominantly hydrophobic amino acids which might act as signal sequences.

1.4.3. S RNA segment

The expression strategies of S RNA segment have been examined in detail because of the availability of more sequence data and are summarized in Fig. 1.4 and Table 1.7.

The S segments of bunyaviruses are very similar in size (850-961 bases in length) and the coded proteins N (233-235 aa) and NSs (91-101 aa) are also very similar. N (25K) and NSs (10K-12K) are encoded in overlapping reading frames in the complementary RNA. A single mRNA species appears to be used for translation of resulting from alternative initiation of translation (Bishop *et al.*, 1982;

Elliott & McGregor, 1989). There also appears a third AUG-initiated ORF(ORF3) in the S RNA segments of Germiston and Maguari viruses which is in the same frame as and downstream of the NSs ORF. ORF3 of Maguari virus can be translated *in vitro* into a 9.3 K protein from mRNA transcribed from its cDNA, but this protein has not been found in virus infected cells and its significance is questionable (Elliott & McGregor, 1989).

The S RNA segment expression strategies of *Hantavirus* and *Nairovirus* are very similar. The segments of both viruses have similar length, 1675 to 1785 nucleotides for hantaviruses and 1712 nucleotides for Dugbe nairovirus. There is a single ORF encoding the N protein (48-49K) in the viral complementary RNA (for references, see Table 1.7). No evidence for an NSs encoded by the S RNA has been found. Interestingly, a small ORF which could code for a 5.9K polypeptide was identified in the same reading frame immediately following the termination codon of two strains of Hantaan viruses but not in SR-11, NE and PH viruses and Dugbe nairovirus (Schmaljohn *et al.*, 1986; Ward *et al.*, 1990). This 5.9K protein has not been detected in virus infected cells and the reading frame is not conserved in the S segments of the four sequenced hantaviruses. Therefore, its significance remains to be determined but it does not appear to be equivalent to the NSs protein of bunyaviruses.

The S segment of phleboviruses and tospovirus uses a novel ambisense expression strategy (for references, see Table 1.7). The S RNA segments of phleboviruses are very similar in length (1690-1746 bases) and are about twice the size of the bunyavirus S segment. Thus, they have more than sufficient potential coding information for both N (27K-28.5K) and NSs (30K-32k) proteins. The S segment of TWSV is even larger (2916 bases long) and also encodes both N (28.8K) and NSs (54.2K) proteins. The N protein of these viruses is encoded in a complementary plus sense RNA corresponding to the 3' half of the genomic S RNA, whereas NSs is encoded in the 5' half of the genomic RNA (Fig.1.4). The N and NSs proteins are translated from separate subgenomic mRNAs (Ihara *et al.*, 1984, 1985a). This ambisense strategy of encoding two proteins from non overlapping regions of virion sense and anti-virion sense RNAs has also been identified in the S and L segments of arenaviruses. Another unique feature of these three different viruses is the presence of an intergenic region (IR) between the ORFs for N and NSs. However, the lengths of the IR are dramatically different, ranging from 62 to 508 nucleotides (Table 1.7). PT virus S segment IR is A+U rich and can potentially form an energetically stable hairpin structure involving 200 nucleotides (Emery and Bishop, 1987), and such an RNA secondary structure might be involved in transcription termination process. The IRs of Uukuniemi virus and TSWV are also A+U rich and are predicted to form a stem-loop structure (Simons *et al.*, 1991; De Haan *et al.*, 1990). In

contrast, the S RNA of SSF virus has a predominantly C-rich IR and cannot be folded into a base-paired structure (Marriott *et al.*, 1989).

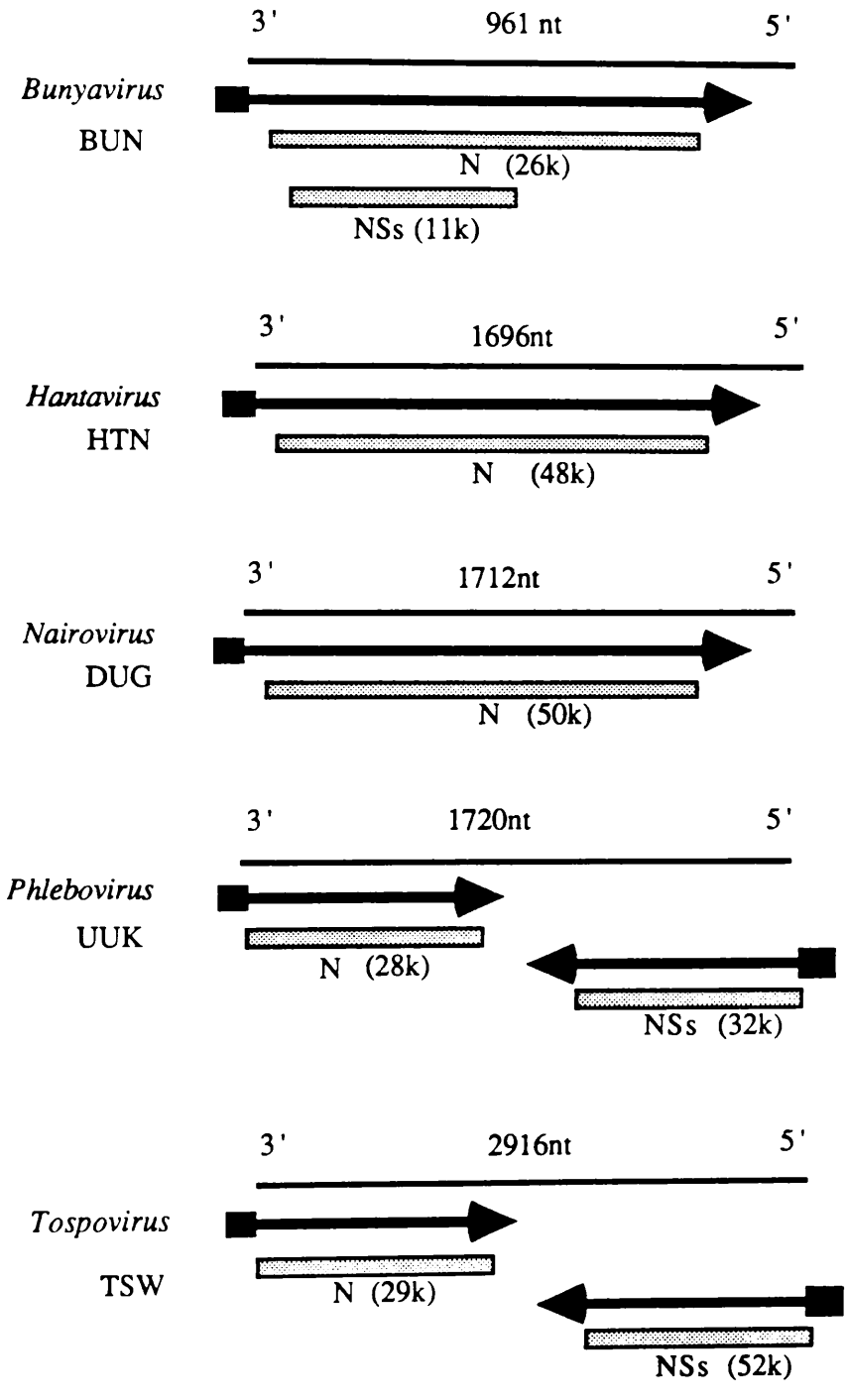


Fig. 1.4. Coding strategies of the Bunyaviridae S genome segment. Genomic RNAs are represented by thin lines, mRNAs by arrows (5' end and 3' ends are indicated) and gene products are designated by stippled boxes (not to scale). This figure is from Elliott (1991).

Table 1.7 Complete nucleotide sequences of Bunyaviridae S genome segments

Genus	Serogroup	Virus	Nucleotides					Encoded proteins		Reference
			length	5'nc	3'nc	A+U (%)	IR	N (aa)	NSs (aa)	
Bunyavirus	Bunyamwera	BUN	961	85	174	58.2		233	101	Elliott, 1989b
		GER	980	87	194	57.6		233	109	Gerbaud, 1987b
		MAG	945	73	170	59.9		233	101	Elliott & McGregor, 1989
	California	LAC	984	81	198	58.8		235	92	Akashi & Bishop, 1983 Cabradilla et al., 1983
		SSH	982	79	198	56.3		235	92	Bishop et al., 1982
	Simbu	Aino	850	34	117	55.9		233	92	Akashi et al., 1984
Hantavirus	Hantaan	HTN76-118	1696	36	370	57.1		429	-	Schmaljohn et al., 1986
		SR-11	1769	42	437			429	-	Arikawa et al., 1990
		NE	1785	42	453	56.9		433	-	Stohwasser et al., 1990
		PH	1675	42	331	57.4		434	-	Parrington & Kang, 1990
Nairovirus	Nairoibi sheep	DUG	1712	50	340	56.5		442	-	Ward et al., 1990
Phlebovirus	Phlebotomus fever	RVF(ZH-548)	1690	38 (c)	34(v)	50.9	82	245	265	Giorgi et al., 1991
		TOS	1869	38 (c)	56(v)	52.9	62	253	316	Giorgi et al., 1991
		PT	1904	28 (c)	34(v)	60.4	360	250	243	Ihara et al., 1984
		SSF	1746	41 (c)	20(v)	53.2	141	219	268	Marriott et al., 1989
(Uukuvirus)	Uukuniemi	UUK	1720	34 (c)	25(v)	50.0	74	254	273	Simons et al., 1990
Tospovirus		TSW	2916	153 (c)	87(v)	64.5	508	259	465	De Haan et al., 1990

(v): viral sense; (c): viral complementary-sense .

1.5. RNA synthesis

All negative-strand RNA viruses require active transcription of their genomic RNA following penetration into a susceptible host cell as a prerequisite for replication of their genomes and the Bunyaviridae is no exception. The minus-strand genomes are templates for two kinds of RNA synthesis, that of mRNAs and antigenomes. Following translation of the primary mRNA transcripts, genome replication and amplification by secondary transcription of mRNA then occur. Antigenomic RNA is encapsidated by the N protein and acts as a replicative intermediate, whereas mRNA is naked which ensures its translational properties. The RNA synthesis process of viruses in Bunyaviridae is not fully understood. Data on mRNA synthesis are mainly based on the studies of *Bunyavirus* genus and little is known about the genome replication process.

1.5.1. RNA templates

The functional template for both transcription and replication of all the RNA viruses with negative-strand genome is a ribonucleoprotein complex (nucleocapsid) with RNA completely encapsidated by the N protein. The genome segments (L, M and S) of bunyaviruses are found as viral nucleocapsids both in virions and in infected cells which have been described in section 1.2.3.

The first 11 bases of 5' and 3' end structures of the three segments of bunyaviruses are strictly conserved and can form panhandle structure by base pairing. Recently, Raju & Kolakofsky (1989) have shown that the ends of the full-length (-) and (+) strands are base-paired in the viral nucleocapsid. They reiterated the importance of unmatched and mismatched bases within double strand regions of RNA for recognition by proteins (Wickens & Dahlberg, 1987). They stressed that the ends of the (-) and (+) RNAs would have significantly different structures as the G:U pairs in genomic RNA are allowed pairs but the corresponding A:C pairs in antigenome would break the helical stack. Therefore, it is speculated that the G:U pairs in genomic and A:C pairs in antigenomic RNA can be recognised by proteins differently. The ability of proteins to distinguish these structures may be the basis by which genomes and antigenomes are differently transcribed, and genomic RNA packaged into virions.

It has been realized that the larger the segment, the longer and stronger the panhandle structure (Kolakofsky and Hacker, 1991). The longer segment has also less mRNA and anti-mRNA made per template (Hacker *et al.*, 1990), presumably because it is relatively more difficult to open the longer end structure for mRNA synthesis. In fact, the amounts of the three RNA species are not equimolar and S is usually the predominant

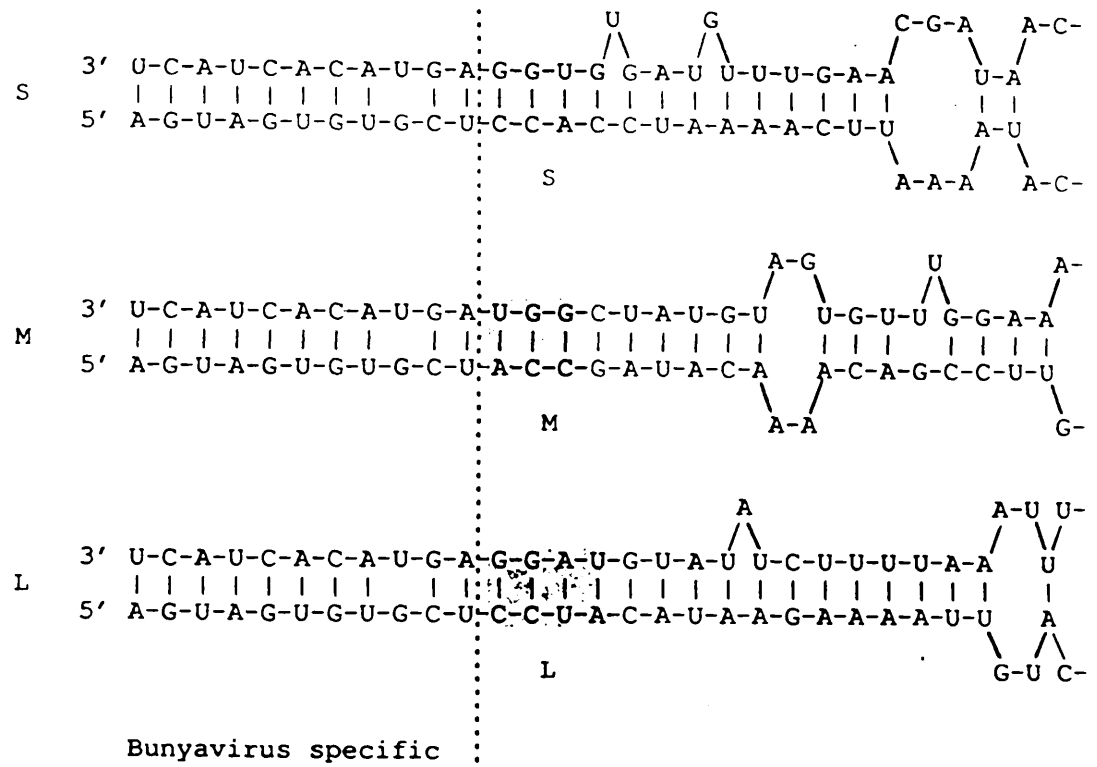


Fig. 1.5. Complementary sequences and possible base-paired structures between the 3' and 5' termini of Bunyamwera virus genomic RNA segments. The terminal 11 nucleotides are conserved in all genome segments; shaded regions, nucleotides which are conserved on a segment specific basis in all available sequenced bunyavirus RNA segments. This figure is from Elliott *et al.* (1991).

species. In attempts to measure the relative amounts of the three mRNA species, Rossier *et al.* (1988) used slot blot hybridization. mRNA from LAC virus infected BHK cells was pelleted through a CsCl gradient and hybridized to riboprobes specific for each segment. The riboprobes were of similar length (400-600 nt) to minimize differences in their annealing characteristics. It was found that the S mRNAs (5 per S genome segment) were approximately 10-fold more abundant than M mRNAs (0.5 per M genome) and 100-fold more abundant than L mRNAs (0.05 per L genome). Therefore, the three genome segments appear to transcribe their mRNAs at very different rates depending on the requirements for their individual gene products. The exact mechanism regulating the transcription of each of the three genome segments is not known yet.

The mechanism by which the N proteins encapsidate viral RNAs is not fully understood yet. Recently, Raju and Kolakofsky (1987) have observed two unusual transcripts in LAC virus-infected BHK cells. By Northern blot and primer extension analyses of S RNA species, two S mRNA-like species were detected in the banded

intracellular nucleocapsid preparations. One had nucleotides 1-886 of the S RNA, which terminated to the mRNA termination site. The other had nucleotides (-) 15-983, which had the 3' genomic end but contained host-derived primers at 5' end. Since both were encapsidated with the N protein, it was suggested that the encapsidation site is located at the 5' end of the genome and antigenome. Later, Hacker *et al.*(1989) reported that in LAC bunyavirus infected mosquito cells, the S mRNAs of LAC were encapsidated by the N protein. Analysis of the micrococcal nuclease sensitivity of encapsidated S RNA showed that the nontemplated primers were also encapsidated. On the other hand, little or no cellular mRNA was found to be even partially assembled. It appears that nucleocapsid assembly occurs bidirectionally and the N protein recognizes a specific viral sequence or structure at the 5' end of mRNA. Therefore, it can be concluded that the assembly site is initiated from the conserved 5' ends of the genome and antigenome chains.

The RNA binding site of the N protein has not been investigated. Inspection of the charge distribution along the amino acid sequence of the N proteins revealed that the N proteins of phleboviruses possess a 70 residue region with distinctly basic character preceded by a 30 residues acidic amino-terminal domain (Elliott *et al.*, 1991). This stretch of basic amino acid region may contain the RNA binding site.

1.5.2. Initiation

The length of mRNA is different from the genomic or antigenomic RNA. It is about 100 bases shorter at 3' end and has extra bases at 5' end. Plus-sense antigenomic RNA is a full length copy of the genome, which is encapsidated with the N protein and acts as a replicative intermediate. The initiation of mRNA and antigenomic RNA synthesis is different and will be discussed separately.

1.5.2.1. Initiation of mRNA synthesis

Sequence analyses of the 5' end of virus mRNA have been undertaken in order to determine the transcription initiation mechanism and the data have been available for five viruses in the family: LAC (Patterson & Kolakofsky, 1984), SSH (Bishop *et al.*, 1983; Eshita *et al.*, 1985), GER (Bouloy *et al.*, 1990; Gerbaud *et al.*, 1987a) of *Bunyavirus* genus, UUK (Simons & Pettersson, 1991) and PT (Collett, 1986; Ihara *et al.*, 1985a) of *Phlebovirus* genus. All the viral mRNA species analysed possess a 12-18 nt long heterogeneous nonviral sequence which may be derived from host mRNAs and used to prime mRNA syntheses, a mechanism remarkably similar to that of influenza virus (for a review, see Krug, 1981, Krug *et al.*, 1989). The speculation has been supported by further analysis of polymerase activity of a purified LAC virion preparation (Patterson *et*

al., 1984). It was demonstrated that the LAC virion associated polymerase activity can be stimulated by oligonucleotides such as (A)nG which can be incorporated into the *in vitro* synthesized transcripts, cap analogs such as mGpppG and natural mRNAs such as alfalfa mosaic virus RNA4 (ALMV RNA4). An endonuclease activity was also detected in purified LAC virions and 12-18 nucleotides can be cleaved from the cap group of ALMV RNA4. This endonuclease activity may be responsible for the mRNA instability in mammalian cells induced by bunyavirus infection (Raju and Kolakofsky, 1988). Again, analogous to the influenza virion endonuclease, the LAC endonuclease does not cleave unmethylated ALMV RNA4. The capped sequences present in the LAC virus S mRNA could be specifically selected by anti-cap antibodies (Hacker *et al.*, 1990). Thus, these data strongly suggest that bunyaviruses initiate the synthesis of their mRNAs by capturing the 5' end sequence of a host mRNA and using it as a primer.

The influenza virus genome is transcribed in the nucleus of the host cell; the primers required for influenza virus RNA transcription initiation are cleaved from the cellular mRNA precursors and influenza virus RNA needs to undergo maturation processes, such as splicing and polyadenylation which are catalyzed by host nuclear enzymes (Krug *et al.*, 1989). In contrast, bunyaviruses do not have spliced mRNAs and appear to replicate solely in the cytoplasm (Rossier *et al.*, 1986). It was reported that California encephalitis bunyavirus could produce progeny in enucleated cells (Goldman *et al.*, 1977). By pulse-labelling of infected cells for various times and determination of the amount of the labelled S mRNA in both the cytoplasmic and nuclear fractions, Rossier *et al.* (1986) were able to examine the cellular site of LAC virus S mRNA synthesis. They demonstrated that the majority of the pulselabelled LAC S mRNA (85-87%) were detected in the cytoplasmic fraction and suggested that LAC virus S genome transcription appeared to be in the cytoplasm. Moreover, unlike influenza virus, bunyavirus replication is not affected by actinomycin D, a drug which inhibits DNA dependent-RNA polymerase such as host cell RNA polymerase II (Obijeski & Murphy, 1977). It is therefore believed that bunyaviruses acquire the primers needed for transcription initiation from a cytoplasmic pool rather than from newly synthesized nuclear transcripts.

Bouloy *et al.* (1990) found that the primers present at the 5' end of the M and S Germiston mRNA had a high C and G content which was never less than 50% (Fig.1.6). With one exception, the penultimate base at position -1 was either U or C, with U being the preferred base (13 cases of 18), indicating that the nucleolytic activity of the endonuclease of bunyavirus should be U or C specific. This observation was also confirmed for the 5' ends of the N mRNAs and 5' ends of the NSs mRNAs of Uukuniemi virus (Simons & Pettersson, 1991).

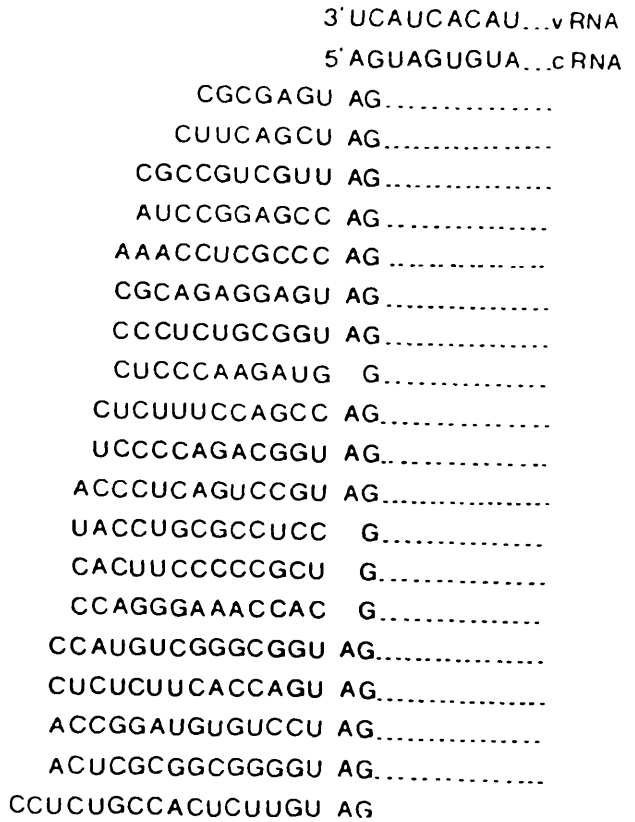


Fig.1.6. Sequences of the 5' region of several S mRNA molecules of Germiston virus. Nineteen recombinant plasmids with S-specific inserts were sequenced and the nonviral sequence of the 5' end of the insert is indicated. The sequence of the 3' end of the virion RNA and its copy representing the 5' end of the mRNA are shown on the top lines. This figure is from Bouloy *et al.* (1990).

Recently, studies on Tacaribe arenavirus mRNA end structures (Garcin & Kolakofsky, 1990) reveal that a nontemplated heterogeneous capped base is also present at the 5' ends of the N mRNAs of Tacaribe virus. Therefore, it appears that all the three negative-strand RNA virus families with segmented genomes, Arenaviridae, Bunyaviridae and Orthomyxoviridae, employ a similar initiation mechanism by using specifically capped RNA fragments derived from host cell RNAs.

The promoter sequence in the influenza virus genome has been investigated by an *in vitro* transcription assay of reconstituted nucleocapsid templates containing only the 3' end of genomic RNA. The evidence suggested that the promoter of influenza virus RNA lay within 15 nucleotides of 3'-terminus (Parvin *et al.*, 1989). Yamanaka *et al.* (1991) further defined the important promoter signal of influenza virus to reside at position of 6-14 of the 3' terminus using a transfection system with a synthetic genetically engineered RNA. The chloramphenicol acetyltransferase (CAT) gene was inserted between the non coding region sequences of the influenza virus RNA genome segment 8 and negative sense CAT RNA was synthesized *in vitro* by T7 RNA polymerase. This RNA was then complexed with influenza viral RNA polymerase and NP proteins and introduced into cells infected with a helper virus. Transcription of the engineered RNA was monitored by CAT assay. Site-directed mutagenesis of the terminal sequences was performed and it was found that mutations of the 3' terminal base hardly affected transcription, whereas mutations at positions 6-14 from the 3' end significantly reduced transcription, suggesting that the 6-14 bases of the 3' end of the influenza virus RNA are important in the promoter sequence. It is most likely that the promoter sequence of bunyavirus is also located within the conserved end sequence.

It has been considered that viral mRNA synthesis only initiates on a genome sense RNA template. Recently, Hacker *et al.* (1990) reported that mRNA-like transcripts were also synthesized from antigenome templates of LAC virus as examined by Northern blotting using strand-specific riboprobes and primer extension analysis. These mRNA-like transcripts were thus termed as anti-mRNA and were characterized by possession of a similar range of capped nontemplated sequences at their 5' ends which were precipitated by anti-cap antibodies. Therefore, the initiation of anti-mRNA is probably the same as that for mRNA, and the viral RNA polymerase may recognize the 3' end sequences of antigenomes to synthesize anti-mRNA. However, the role of these anti-mRNAs during virus infection is unclear.

1.5.2.2. Initiation of genome replication

Genomes and antigenomes initiate with ATP at the precise 3' end of the templates (Obijeski *et al.*, 1980), clearly by a different mechanism to that of mRNA synthesis. It is known that protein synthesis is required for bunyavirus replication to occur, although it is not determined which viral proteins are required. Since both genomes and antigenomes are found only as nucleocapsids and the sites for the N protein assembly appear to start at the 5' ends of these chains, it is logical to speculate that RNA synthesis and assembly take place concurrently (Kolakofsky and Hacker, 1991). It appears that the presence of assembling N protein participates in the initiation of genome replication. The switch between transcription and replication is most likely to occur at the level of chain initiation though the factors controlling this switch are not well understood. In addition to the N protein, the most likely candidates are modification of the L protein, or possibly the NSs protein where it exists.

The NS protein (now referred to as P protein) of VSV has been proposed to play an important role in the switch between transcription and replication of VSV (Wertz *et al.*, 1987). Three functional domains of VSV NS protein have been identified (Banerjee, 1987). The template binding domain is located in the carboxy-terminal basic region (Gill *et al.*, 1986); the L protein binding domain which is close to the template binding domain is directly involved in binding with the L protein to form the active RNA polymerase complex (Chattopadhyay & Banerjee, 1987a, b); and an ancillary domain at the N terminus might be responsible for the chain elongation (Chattopadhyay & Banerjee, 1988). It appears that the NS protein of VSV regulates replication by controlling the availability of the N protein, thereby controlling the balance between RNA replication and transcription (Wertz *et al.*, 1987). More detailed studies of the NSs protein of bunyaviruses are required to elucidate its function in the RNA synthesis process and to see whether it functions similarly to the VSV NS protein.

1.5.3. Termination

The mRNAs of viruses in the Bunyaviridae are about 60-120 nucleotides shorter than full-length transcripts and there appears to be a termination site(s) near the 5' end of the genome to prevent the polymerase reading through. A few termination sites have been mapped for some of the viruses by analyses of the 3' end sequences of viral mRNA species (Patterson & Kolakofsky, 1984; Eshita *et al.*, 1985; Emery & Bishop, 1987; Bouloy *et al.*, 1990; Simons & Pettersson, 1991). It has been found that all the stop signals are usually located in a sequence rich in Us and contain a G residue. RVF phlebovirus is an exception in that the proposed termination site is C rich (Collett, 1986).

Germiston M and S mRNA have transcription stop sequence of UUUUUGUUU and UUUGUU respectively (Bouloy *et al.*,1990), LAC virus S mRNA has GUUUUU and Bunyamwera virus M mRNA has the sequence GUUUUUUG after the translational stop codon. These U rich sequences are similar to the termination signal of influenza viruses which also consists of a stretch of 5-7 uridine residues (Hay *et al.*, 1977; Krug *et al.*, 1989). Because other similar U rich sequences are also present in the virus genome, especially upstream from the termination site, it is possible that flanking sequences are also involved to enable the transcriptase to recognize the termination signal (Bouloy *et al.*, 1990). Further mapping studies are required to delineate these in detail.

For phleboviruses having an ambisense expression strategy, the internal repeat regions of RNA genome may be involved in the terminal signal recognition. Northern blot analysis of PT virus infected cellular mRNA with ^{32}P-labelled synthetic oligonucleotide probes has enabled the 3' termini of both the N and NSs mRNA species to be mapped to a common region of the PT S RNA segment genome between nucleotides 977 and 1017 (Emery and Bishop, 1987). Secondary structure analysis of the intergenic region reveals an energetically stable A:U rich hairpin structure extending from nucleotide 886 to 1090 with its peak at 996 which correlates with the region involved in termination of mRNA transcription. However, some phleboviruses such as sandfly fever Sicilian virus have a C-rich intergenic region which can not be folded into a large base paired structure (Marriott *et al.*, 1989). Most recently, Simons & Petterson (1991) reported that the 3' ends of the N and NSs mRNAs of Uukuniemi virus overlap each other by about 100 nucleotides. The 3' end of the NSs mRNA extends into the coding sequence of the N mRNA, whereas the N mRNA is terminated just prior to the stop codon of NSs. Therefore, the role of the intergenic region in the termination of phlebovirus mRNA transcription remains to be determined.

An important consequence of the missing sequence at the end of the mRNAs has been speculated to have some significant role in translation process. Unlike the antigenomic and genomic RNA, the ends of mRNA are no longer complementary and can not form circular or panhandle structures which might interfere with their translation (Kolakofsky and Hacker, 1991).

1.5.4. Elongation: translational requirement of mRNA synthesis

Conflicting data concerning the requirement for ongoing host protein synthesis of virus primary transcription have been reported in the *Bunyavirus* genus. Protein synthesis inhibitors have been found to have no effect on primary transcription in other negative-strand RNA virus families such as the Orthomyxoviridae (Bean & Simpson,

1973), Rhabdoviridae (Marcus *et al.*, 1971) and Paramyxoviridae (Robinson, 1971). Very low levels of primary transcripts have been detected in Bunyamwera or snowshoe hare virus infected cells in the presence of drugs that inhibit host cell protein synthesis (Kascsak & Lyons, 1977; Vezza *et al.*, 1979). Using cDNA hybridization probes for analysis of the mRNA species in snowshoe hare virus infected cells which had been treated with puromycin, only S mRNA was detected (Eshita *et al.*, 1985). Furthermore, *in vitro* studies showed that full-length S transcripts of Germiston virus could be obtained in an *in vitro* transcription system (Gerbaud *et al.*, 1987a), although Germiston S segment mRNA synthesis was inhibited in cell culture by either anisomycin or cycloheximide.

Some evidences indicate that bunyavirus mRNA synthesis is sensitive to drugs that disrupt protein synthesis. This finding was first reported for Bunyamwera virus (Abraham & Pattnaik, 1983) and Akabane bunyavirus (Pattnaik & Abraham, 1983), and has been confirmed for LAC (Raju & Kolakofsky, 1986b) and Germiston virus (Gerbaud *et al.*, 1987a). These results have no precedence among other negative-strand RNA viruses and are contradictory to the results mentioned above.

A detailed examination of the translational requirement of bunyavirus mRNA synthesis has thus been performed on the S segment of LAC virus in both *in vitro* and *in vivo* systems (Raju & Kolakofsky, 1986a; Bellocq *et al.*, 1987; Raju *et al.*, 1989). In the absence of a reticulocyte lysate, transcription *in vitro* from the S segment genome by the virion associated polymerase produced two incomplete transcripts, 110 and 205 nucleotides in length, whereas full length S mRNA (900 nt) was predominantly made when the lysate was added. The drugs puromycin, cycloheximide and pactamycin, were then added to the system to test their effect on LAC transcription. It appears that all these drugs caused S transcription to terminate prematurely at the defined sites. The data suggest that the requirement for ongoing protein synthesis for productive transcription *in vitro* is not at the level of chain initiation but at the level of the nascent RNA chain elongation beyond the termination sites (Bellocq *et al.*, 1987). Further studies indicate that the translational requirement for complete LAC mRNA synthesis is cell-type dependent (Raju *et al.*, 1989). Total cellular mRNAs from LAC virus infected cells which had been exposed to various translational inhibitors for 4 hours were analysed by solution hybridization to strand specific riboprobes which represent three different regions of LAC S RNA. The hybrids were further assayed by RNase mapping techniques. Small amounts of shorter S plus-strand RNA (approximately 154 nt in length) were found in virus infected BHK cells. These shorter transcripts were also detected in HEL and Vero mammalian cells infected with LAC virus in the presence of protein synthesis inhibitors. On the other hand, in the mosquito cell line, C6/36, infected with the virus in the presence of cycloheximide, virus RNAs were found to resist RNase

digestion. Surprisingly, in the mosquito cells, secondary transcription of S RNA of LAC was not affected by the drug either.

Using either BHK or C6/36 cell extracts for reconstitution studies *in vitro* Raju *et al.* (1989) suggested that the translational requirement was due to a factor(s) present in BHK, but not in C6/36 cells. When uninfected BHK cell extracts were added to the *in vitro* transcription system programmed with virus genomes prepared from LAC virus infected mosquito cells, the extract recreated the translational requirement of mRNA synthesis. Further, by pelleting mRNA prepared from LAC virus infected cells through a sucrose cushion to remove cellular protein components, the ability of S mRNA transcription to read through the termination sites was increased.

Since premature termination of LAC S mRNA synthesis takes place efficiently when purified virions alone were used (Bellocq *et al.*, 1987), it is unlikely that a host termination factor is involved in the *in vitro* system. Thus, the possible role of RNA:RNA interactions in transcription premature termination was then proposed (Kolakofsky *et al.*, 1987). It had been reported that substitution of inosine for guanosine would weaken possible RNA:RNA interactions (Lee & Yanofsky, 1977). When GTP was progressively replaced with ITP in the absence of reticulocyte lysate in the LAC virion polymerase reaction, the synthesis of complete S mRNA became more predominant (Kolakofsky *et al.*, 1987). BrUTP incorporation has been suggested to strengthen base pairing and its incorporation into the nascent LAC S mRNA chain counteracted the effect induced by inosine incorporation. Thus, it is possible that the nascent chain can interact with its template cause premature termination and therefore, concurrent translation of the nascent chain is required to break these interactions.

Based on the observations described above, Kolakofsky *et al.* (1987) have proposed a model to explain the curious coupling of translation to the primary transcription. The model suggests that interaction between the nascent chain and its template causes the polymerase to pause and terminate, thus generating the incomplete transcripts. In the presence of concomitant protein synthesis, ribosomes moving along the nascent mRNA behind the polymerase prevent the mRNA from hybridizing to its template and enable the polymerase to read through the major termination sites (Bellocq & Kolakofsky, 1987; Kolakofsky *et al.*, 1987). Such a translational requirement mechanism would then be similar to that in certain bacterial systems (Platt, 1986; von Hippel *et al.*, 1984), whereby ribosomes prevent RNA:RNA interactions and thus premature termination. Whether this hypothesis only applies to LAC virus or to all the viruses in the family remains a question and investigation of more primary transcription process of all three RNA segments of the viruses in the family should help clarify this issue. A functional analysis of viral RNA polymerase from molecularly cloned and expressed L gene segment is also required to explain this.

1.6. Viral RNA polymerases

RNA polymerase is the key enzyme for RNA viruses to transcribe and replicate their RNA genomes. Most RNA viruses replicate outside the nucleus of the host cells and thus are independent of cellular replication functions. Some positive-strand RNA viruses, such as picornaviruses, have evolved to encode the RNA polymerase in their genomic sense RNA which can be translated right after entering the cells. The negative-strand RNA viruses, on the other hand, due to their genomic RNAs being unable to directly translate this unique RNA polymerase, have to package the enzyme within the virion during viral morphogenesis. The virion associated RNA polymerase, in turn, plays a unique role in initiating infection by carrying out primary transcription of the genome when the virus infects a cell. Except orthomyxoviruses, all negative-strand RNA viruses encode a large (L) polymerase protein in their complementary RNA, with similar molecular weight of >200 K (Fig.1.7 and Table 1.8), despite the diversity of their genomes. The polymerase of orthomyxoviruses comprises three proteins, PB1, PB2 and PA, which are encoded by three different segments. However, the combined length (6915 bases) of these three polymerase genes is very similar to the L gene of other negative-strand RNA viruses and the combined molecular weight of the three P proteins is 246-285 K (Lamb, 1989) which is also very similar to that of the L proteins.

The polymerase of negative-strand RNA viruses is unique in that it can not directly utilize the naked RNA as its template. Rather, it recognizes the viral genome only in the context of the nucleocapsid core. The N protein confers a helical structure on the nucleocapsid and is essential for the recognition of the genomic template RNA by the virus polymerase. By virtue of its large size and its presence in very low amounts in the virion of the L protein, the majority of the enzymatic activities involved in transcription and replication are thought to reside in the L protein and hence this large protein is presumably multifunctional.

Through genetic and biochemical studies, several enzyme activities have been found to be associated with the L protein of vesicular stomatitis virus (VSV), such as viral mRNA capping (Banerjee, 1987), methyltransferase (Hercyk *et al.*, 1988), poly(A) polymerase (Hunt *et al.*, 1984) and protein kinase (Sanchez *et al.*, 1985) activities. For VSV transcription, the L protein alone is unable to perform RNA synthesis and the phosphorylated NS protein plays a crucial role in the transcription process by complexing with the L protein to maintain its functional conformation (Banerjee, 1987). It is not known if the L protein of bunyaviruses performs all the polymerase functions or if some other protein is also required.

To map the various functional domains within the L protein, it is necessary to determine the relationship between its primary structure and its role in RNA transcription and replication. The determination of the complete nucleotide sequence of the L RNA segment represents the first important step for analysis of the virus polymerase function and further elucidation of the mechanism of RNA transcription and replication.

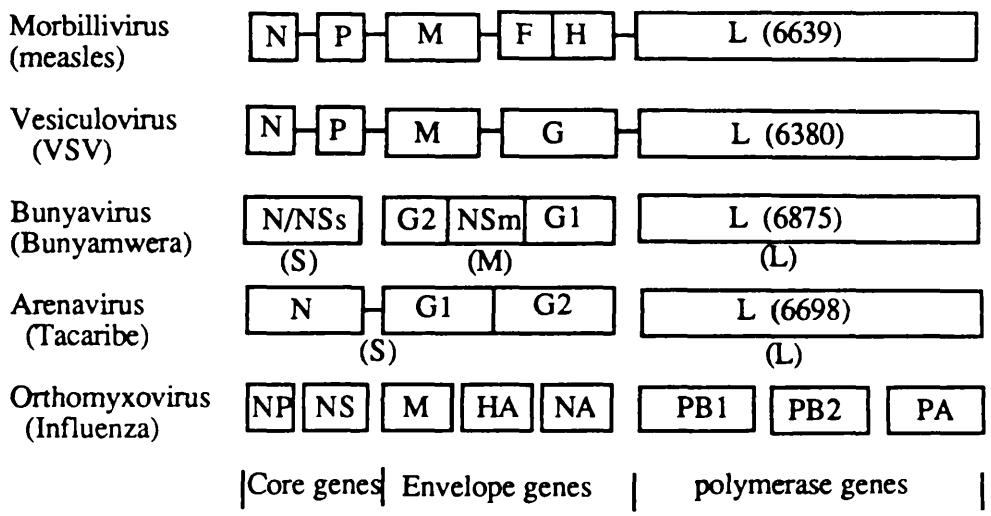


Fig.1.7. Gene alignment of the genomes of negative-strand RNA viruses. This figure diagram is modified from Pringle (1991) and Peters (1991).

1.6.1 Sequence analysis of the L gene

The complete nucleotide sequence of the L segment has been determined for six viruses in the Bunyaviridae: Bunyamwera (Elliott, 1989a) of the *Bunyavirus* genus, Hantaan (Schmaljohn, 1990), Seoul 80-39 (Antic *et al.*, 1991) and nephropathia epidemica viruses (Stohwasser *et al.*, 1991) of the *Hantavirus* genus, TSWV (de Haan *et al.*, 1991) of the *Tospovirus* genus and Uukuniemi virus (Elliott *et al*, unpublished data) of the *Phlebovirus* genus.

The properties of the amino acids in the predicted L protein of NE virus have been studied and the NE L protein displays an acidic character at its carboxy terminus, and within this acidic domain, a stretch of 80 amino acids (residue 1948-2027) is mainly composed of four hydrophilic amino acids. In addition, there is a slightly acidic cluster in the central region and close to it exists a strongly basic domain between residues 900-1000 (Stohwasser *et al.*, 1991).

Table 1.8. Comparison of the polymerase genes and deduced proteins of negative-strand RNA viruses

Family	Virus	Polymerase Gene (nt)	Deduced Protein	
			Residues	M_r (kDa)
Paramyxoviridae	RSV	6575	2165	250
	MEV	6639	2183	248
	PIV3	6755	2233	256
	NDV	6704	2204	279
	SeV	6800	2228	253
Rhabdoviridae	RaV	6475	2142	244
	VSV	6380	2109	241
Arenaviridae	LCMV	6677	2210	254
	TV	6698	2210	252
Bunyaviridae	BUN	6875	2238	259
	HTN	6530	2150	247
	SEO	6530	2150	247
	NE	6550	2156	246
	UUK	6423	2103	241
	TSW	8897	2876	331
Orthomyxoviridae	Influ A	2320 (PB1)	757	87
		2320 (PB2)	759	88
		2211 (PA)	716	82

RSV: Respiratory syncytial virus (Stec et al., 1991),
MEV: Measles virus (Blumberg et al., 1988),
PIV3: Parainfluenza virus (Galinski et al., 1988),
NDV: Newcastle disease virus (Yusoff et al., 1987),
SeV: Sendai virus (Shioda et al., 1986),
RaV: Rabies virus (Tordo et al., 1988),
VSV: Vesicular stomatitis virus (Schubert et al., 1984),
LCMV: Lymphocytic choriomeningitis virus (Salvato et al., 1989),
TV: Tacaribe virus (Iapalucci et al., 1989),
BUN: Bunyamwera virus (Elliott, 1989b),
HTN: Hantaan virus (Schmaljohn, 1990),
SEO: Seoul 80-39 virus (Antic et al., 1990),
NE: Nephropathia epidemica virus (Stohwasser et al., 1991),
UUK: Uukuniemi virus (Elliott et al., unpublished)
TSW: Tomato spotted wilt virus (de Haan et al., 1991),
Influ A: Influenza A virus (Lamb, 1989).

Comparison of the deduced amino acid sequences from NE hantavirus and Bunyamwera virus L proteins revealed a high degree of diversity with overall amino acid identity of only 17%, even though three clusters of 30-40% amino acid identity can be detected between these two L genomes (Stohwasser *et al.*, 1991). This is not so

surprising as the Bunyaviridae is such a diverse family. No other complete bunyavirus L segment has been reported, though Clerk-van Haaster *et al.* (1982) have sequenced 200 bases at the 3' termini of the L RNA segments from LAC and SSH viruses of California serogroup of the *Bunyavirus* genus. Elliott (1989a) compared the amino acid sequences of these ORFs with the N-terminal residues of the Bunyamwera virus L protein and found that these predicted proteins are strongly homologous, with 29/46 residues (63%) being identical, suggesting that the L protein may have a high degree of conservation within the genus.

Most recently the L protein of the Seoul 80-39 hantavirus has been compared with the L protein of other negative-strand RNA viruses, which revealed 44% homology with part of the Bunyamwera virus L protein and a very weak homology (17%) with the influenza virus PB1 protein (Antic *et al.*, 1991). The same comparison has also been made for the NE L protein by Stohwasser *et al.* (1991). The highest degree of similarity was found in the region of BUN L (1070-1286), NE L (1017-1242) and PB1 (356-561), showing a total of 38% homology. Several clusters of higher homology within these central regions which contains the polymerase motifs were also evident. de Haan *et al.* (1991) revealed a significant amino acid sequence homology in an internal region (approximately 1000 amino acids long) between the L proteins of Bunyamwera virus and TSWV. However, homology between TSWV and Hantaan virus is lower and restricted to a shorter internal stretch of about 200 to 250 residues long. Comparisons of RNA polymerases from TSWV, Bunyamwera virus, Hantaan and influenza viruses also reveal the presence of amino acids sequence motifs that are present in all RNA polymerase proteins.

1.6.2. Sequence relationships of RNA-dependent RNA polymerases

By extensive and sophisticated computer analysis of 80 RNA-dependent polymerases from diverse sources of positive, negative and double-strand RNA viruses, as well as RNA dependent DNA polymerases encoded by retroid elements (reverse transcriptase), Poch *et al.* (1989) have identified four motifs in the same linear arrangement in all the polymerase proteins. The four motifs span a region of 120-210 amino acids which contains four strictly and 18 conservatively maintained amino acids. Each motif has invariant amino acid(s) roughly centred between the conserved amino acids (Fig.1.8). Motif C (S/GDD) corresponds to the YGDD span previously described by Kamer and Argos (1984) as being conserved among many RNA polymerases. However, more extensive analysis of Poch *et al.* reveals that only the DD dipeptide is universally maintained and may be a key functional domain in RNA-dependent

polymerases. One important exception is that all the polymerase proteins of unsegmented negative-strand RNA viruses have GDN in motif C. In contrast, all the segmented negative-strand RNA viruses analysed, such as orthomyxoviruses and arenaviruses, have SDD in this motif. The only other instance of the DN dipeptide is in the putative reverse transcriptase of a *Chlamydomonas* species. The difference in the S and the second D between segmented (SDD) and unsegmented (GDN) RNA polymerases might represent important elements of the active site different for these two virus groups (Poch *et al.*, 1989).

The linearity of the polymerase motifs in all these divergent RNA polymerase molecules suggests they may cooperate to form an ordered functional domain after folding of the protein. Secondary structure predictions suggest that the invariant amino acids are in, or close to, tight turns and perhaps the orientation of these residues is crucial for template recognition in a catalytic event. The predicted domains of RNA polymerases given by Poch *et al.* can be dissected in fine detail by site-directed mutagenesis of the polymerase proteins.

Site directed mutagenesis experiments have recently been performed on a region of the reverse transcriptase of HIV1 encompassing the four conserved motifs (Larder *et al.*, 1987). Within all the mutated amino acids, just two mutations totally destroyed the polymerase activity. They involve the invariant D residue of motif A and the first invariant D residue of motif C. Within the other amino acids mutated, drastic loss of activity was observed when the Y residue (position 4 of motif C) was mutated, while the other mutations (D and A in position 12 and 13 of motif A respectively, and G in position 11 of motif C) had limited effects. These site-directed mutagenesis experiments were not exhaustive and in particular do not involve the invariant G residue in motif B and the K in motif D. Hizi *et al.* (1988 and 1989) reported that insertion of amino acids at position 3 or 10 of motif B induced the loss of reverse transcriptase activity of the HIV enzyme. As far as motif C is concerned, the key functional role of this motif is further highlighted by mutation experiments within the replicase of a plus-strand RNA virus, the Qß bacteriophage, in which substitution of the G of the YGDD sequence by A, S, M, or V residues totally destroyed the activity (Inokuchi and Hirashima, 1987).

More recently, Poch *et al.* (1990) made a more extensive comparison of five polymerases (L proteins) of rhabdoviruses and paramyxoviruses. The five L proteins exhibit a high degree of homology along most of their length, with strongly invariant amino acids embedded in 6 conserved blocks separated by variable regions. The relative frequency of G (15.3%) and W (3.8%) residues among the invariant amino acids is remarkably higher (2.5- to 3-fold) than their average abundance in the L protein, suggesting that these residues are especially important for the L protein. In addition to the possible active polymerase sites, some other functional sites which may assure the

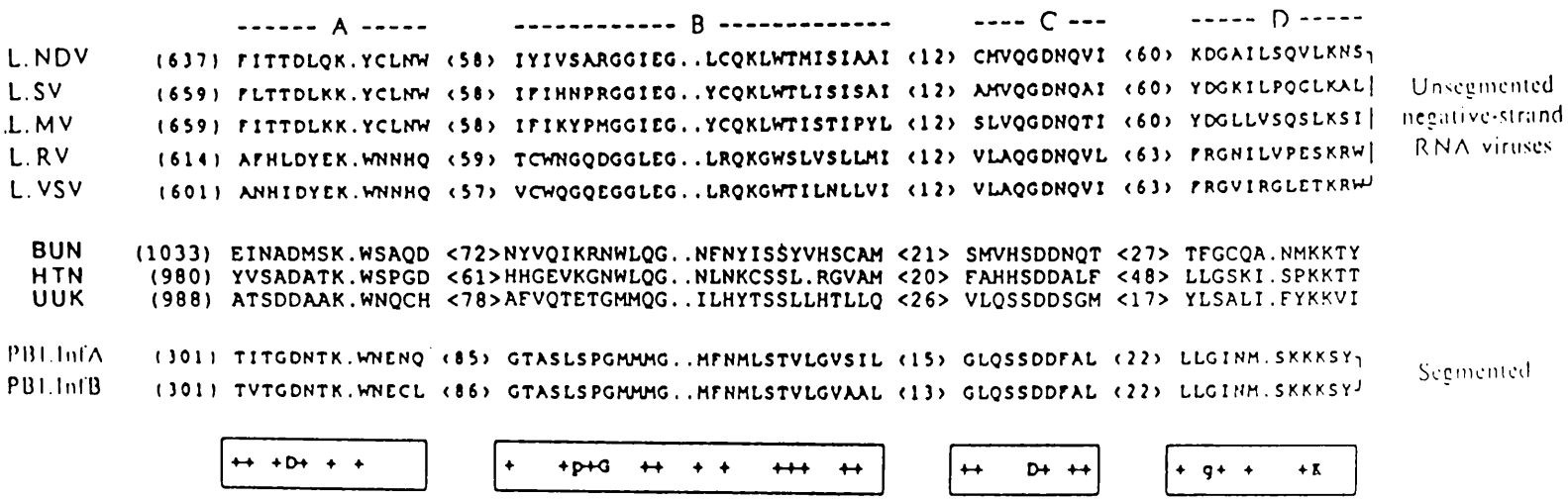


Fig.1.8. Four consecutive sequence motifs shared by RNA dependent polymerases of unsegmented and segmented negative-strand RNA viruses. In boxes at the bottom of the figure capital letters, small letters and pluses indicate invariant, nearly invariant and conservatively maintained residues, respectively. This figure is modified from Poch et al. (1990) by R. M. Elliott.

polymerase activity are also tentatively identified. One of the conserved blocks, which contains basic and hydrophobic amino acids constituting a positively charged region, has been suggested as the template recognition site of the L protein. Two substrate dNTP-binding sites have been experimentally located in the Moloney murine leukaemia virus reverse transcriptase, targeted within the residues flanking the putative site for polymerization (Basu *et al.*, 1988). In the L proteins sequenced, there are many invariant K residues surrounding the active site GDN which may be involved in dNTP binding.

Stec *et al.* (1991) determined the L gene sequence of human respiratory syncytial virus (RSV) and compared the L protein of RSV to that of four other paramyxoviruses and two rhabdoviruses by data base search, dot matrix alignments and other alignment methods. Although homology was detected among all the L proteins compared, the five L proteins of paramyxoviruses were more closely related to each other than to those of the rhabdoviruses, indicating the evolutionary relationship between the two families.

The RNA polymerase motifs identified by Poch *et al.* have also been detected in the L protein of the Bunyaviridae family (Fig. 1.8). Elliott (1989a) compared the Bunyamwera virus L protein with several L proteins of nonsegmented negative-strand RNA viruses and influenza virus PB1, PB2 and PA proteins, and revealed an area (residues 950-1220) of weak homology with part of the influenza virus PB1 protein. PB1 has been identified as the polypeptide responsible for RNA polymerization (Braam *et al.*, 1983; Krug *et al.*, 1989). This area contains the four proposed polymerase motifs between residue 1030-1210. The N residue at position 1119 of the Bunyamwera virus L protein aligns with the invariant G residue in motif B. It is not clear if this N is an important residue in polymerization activity or the G residue at position 1123 serves as the similar function as the invariant G residue in motif B.

Despite the similar size of the L proteins between Bunyaviridae and nonsegmented RNA viruses, the L proteins of Bunyaviridae have invariant amino acids SDD in motif C instead of GDN. This further confirmed the observation of Poch *et al.* (1989) that polymerase proteins of all the segmented RNA viruses have common invariant amino acids SDD in motif C (Fig.1. 8).

1.6.3. Functional analysis of RNA polymerase

Although sequence analysis has revealed the RNA polymerase motifs in the L protein, the functional domains of the bunyavirus L protein involved in initiation (cap recognition and endonuclease activity), elongation and termination of RNA transcription and in the replication process are entirely undetermined.

To map the various domains within the L proteins, it is essential to obtain biologically active RNA polymerase proteins and suitable RNA templates for the assay. It has been possible to purify functional RNA polymerase of some negative-strand RNA viruses from virion or intracellular nucleocapsids. More recently, with the molecular cloning of the RNA polymerase genes in several virus families, the recombinant polymerase proteins have proved to be useful in analyzing polymerase functions. Therefore in this section, analyses developed to dissect the polymerase functions of other negative-strand RNA viruses will be discussed so that some possible approaches can be exploited to delineate the polymerase protein of bunyaviruses.

1.6.3.1. RNA polymerase from virus

Various efforts have been made to purify RNA polymerase proteins either from virus infected cells or purified virions. It is known that the RNA polymerase of VSV is comprised of the L and NS proteins which are associated with the nucleocapsids. The method for isolating and purifying these two protein molecules from the purified virions has been reported by De & Banerjee (1984). VSV was disrupted with 1.85% Triton X-100 in the presence of 0.4 M NaCl to release the RNP, and the L and NS proteins were released from the RNP by 0.8 M NaCl treatment. The preparation was centrifuged through a 30% glycerol and 100% glycerol noncontinuous gradient to separate the L and NS proteins from N-templates. The N-templates were pelleted on the 100% glycerol cushion and the released L and NS proteins were recovered from the top of the tube. The NS protein was further separated from the L protein by phosphocellulose chromatography purification, since the NS protein does not bind to the column and therefore can be recovered free of the L protein. The L and NS proteins purified by the above method were biologically active since they specifically transcribed the VSV N-template when added to the reconstituted transcription assay (De & Banerjee, 1984; 1985). In heterogeneous reconstitution reactions *in vitro*, the purified L and NS proteins of VSV(NJ) did not transcribe N-RNA template purified from VSV(IND), but mRNA synthesis ensured when purified homologous NS (IND) was added (De & Banerjee, 1984). This suggests that specific interaction of homologous L and NS proteins with the N-template is important in RNA synthesis.

Influenza virus RNA polymerase contains three P proteins (PB1, PB2 and PA) which are associated together as P-protein complex on the nucleocapsids. Parvin *et al.*(1989) reported that the influenza virus polymerase proteins can be purified from virions. Purified influenza virions (2-3 mg) were disrupted by incubation in the buffer containing 1.5% Triton X-100, 10 mg/ml lysolecithin, 5% glycerol and 1.5 mM DTT. The RNP cores were fractionated on 30-70% (w/v) glycerol step gradient followed by a

second gradient consisting of CsCl and glycerol. The polymerase proteins were recovered from the region of the gradient correlating with 1.5 to 2.0 M CsCl and dialyzed against DTT and glycerol containing buffer. The polymerase preparation was shown to be free of viral RNA and used to copy short synthetic RNA templates. The synthetic RNA templates, which were transcribed from cDNA corresponding to the 3'-terminal sequences of genomic RNA, were efficiently copied by the purified polymerase. The polymerase was also shown to be able to transcribe full-length influenza virus genome *in vitro*. Unexpectedly, the virion-derived polymerase preparation copied complementary RNA templates inefficiently. Thus, it is speculated that the influenza virus polymerase may be modified in cells during infection to recognize the slightly different promoter sequence at the 3' terminus of positive sense RNA. This speculation was confirmed by Akkina *et al.* (1991) who reported that, in influenza A virus infected cells, there exist two heterogeneous forms of the PB1 (85K and 70K) and PA (60K and 62K) proteins. These additional proteins were only associated with intracellular RNP and could not be detected in purified virus. Peptide mapping analysis revealed they are subsets of their normal counterparts and might participate in virus replication in infected cells.

Szewczyk *et al.* (1988) reported reconstitution of the influenza virus transcriptase simply by renaturing the NP and P-proteins by *E. coli* thioredoxin. The proteins in virion nucleocapsids were separated by SDS polyacrylamide gel electrophoresis and blotted onto a polyvinylidine difluoride membrane. Individual protein bands (PB1, PB2, PA and NP) were eluted from the membrane with Triton X-100 at pH 9.0. The four proteins were then mixed with purified influenza virion RNA in the presence of *E. coli* reduced thioredoxin to renature the proteins. In the presence of a primer, the renatured RNA polymerase thus synthesized plus-strand RNA products which were apparently of relatively large size (900-1800 nt in length). The data indicate that NP is able to bind to naked vRNA to form a functional template and the three P proteins form a functional enzyme complex to initiate and elongate mRNA chains. This is a potentially exciting result which may become a simple practical means to obtain reasonable amounts of active RNA polymerase.

The approach for purification of the Sendai virus L protein was developed by Einberger *et al.* (1990) using hydroxylapatite column chromatography of SDS-solubilized Sendai virus proteins. Purified Sendai virions (60 mg) were disrupted with 3% SDS and the soluble fraction was applied to a hydroxylapatite column. The L protein was completely separated from all other viral and cellular proteins by eluting with linear gradient (0.01-0.5 M) of sodium phosphate buffer. The SDS was removed from the L protein preparation by dialysis and acetone precipitation. The L protein was then renatured by dialyzing against a DTT containing buffer and further purified by

immunoprecipitation. The L protein preparation was shown to contain kinase enzyme activity, although the polymerase assay was not performed.

1.6.3.2. RNA polymerase expressed from cDNA

Functional RNA polymerase has been expressed from the recombinant DNA for some members of negative-strand RNA viruses: VSV (Schubert *et al.*, 1985; Pattnaik and Weitz, 1990), influenza virus (Krystal *et al.*, 1986), Sendai virus (Gotoh *et al.*, 1989) and measles virus (Ballart *et al.*, 1990). But it has not been possible to purify the expressed RNA polymerase proteins of negative-strand RNA viruses for *in vitro* transcriptase assays. Therefore, alternative assays have been developed, such as complementation of a *ts* mutant and replication of a suitable template in *vivo* using the expressed RNA polymerase.

The VSV L gene cDNA was inserted into the simian virus 40 transient expression vector pJC119 and the 241 K L protein expressed in the recombinant plasmid transfected COS cells (Schubert *et al.*, 1985). The expressed VSV L protein was found to be biologically active, since it efficiently complemented and rescued VSV temperature sensitive (ts) polymerase mutants at the nonpermissive temperature. It appeared that the efficiency of complementation was dependent on the level of the L protein expressed, as it was observed that the cells with high levels of L protein expression never showed immunofluorescent staining for the N protein, whereas cells with low levels of the L protein did. Schubert *et al.* (1985) suggest that high levels of the L protein or the L message may arrest transcription or replication of the ts mutants. The observation was further confirmed by Meier *et al.* (1987). It was found that high levels of the L protein expression not only greatly inhibited complementation, but also inhibited wild type virus replication. These results suggest that the requirement of the L protein in transcription and/or replication is catalytic. It also suggests that the unsegmented RNA viruses could have evolved in a manner such that the location of the L gene at the extreme 5' end of the genome RNA is transcribed least frequently, yielding low quantities of L protein in infected cells (Fig.1.6). Banerjee (1987) revealed that the truncated L proteins expressed in COS cells did not show their functionality. Therefore, truncation mutation of the L on the function of the L protein has yet to be determined.

Using a similar approach, Krystal *et al.* (1986) tried to test the ability of cells expressing influenza virus RNA polymerase proteins to complement ts mutants at the nonpermissive temperature. Instead of employing a transient expression system, they constructed transformed cell lines expressing the three P proteins. Complementation was determined by analysis of virus protein synthesis and infectious virus yield in ts mutant infected transformed cell lines at the nonpermissive temperature. Two cell lines

expressing all three P proteins were isolated. Both cell lines complemented the growth of PB2 ts mutants, one of them also complemented PA ts mutants, but none of them complemented PB1 ts mutants at the nonpermissive temperature. The complementation for each ts mutant varied (3- to 328-fold) for the two cell lines and the reason was not clear. However, these complementation data show that the presence in a single cell of all three P-proteins produced a synergetic effect and the three P-proteins may have formed a functional complex in the cells. That the three P-proteins form a complex in the absence of other virus proteins has been reported by Angelo *et al.* (1987) using recombinant baculoviruses.

Recently, Pattnaik and Wertz (1990) have made more exciting progress by showing that VSV proteins expressed from vectors containing cloned virus cDNAs support replication and amplification of VSV DI particle RNAs. They used a recombinant vaccinia virus-T7 RNA polymerase expression system developed by Fuerst *et al.* (1986, 1987) to synthesize individual VSV proteins (N, NS and L) in cells. Plasmid DNAs that contain cDNA copies of the VSV genes downstream of the T7 RNA polymerase promoter were transfected into cells infected with a recombinant vaccinia virus which produces T7 RNA polymerase. In that way, they were able to examine the ability of VSV proteins, individually and in combination, to support DI particle RNA replication. Rapid efficient replication and amplification of DI particle RNA only occurred in cells expressing the N, NS and L proteins of VSV. Omission of any one of the three viral proteins abrogated the replication. They demonstrated that both (+) and (-) sense DI particle RNA were synthesized and the newly synthesized DI RNA was encapsidated by the N protein supplemented from plasmid DNA transfection, as judged by immunoprecipitation of ^{3}H-uridine labelled nucleocapsids. The data strongly suggest that functional proteins expressed from the input templates were assembled with newly synthesized RNAs to form active nucleocapsid templates that were able to undergo multiple rounds of replication, and the expressed proteins (L and NS) function as the polymerase to transcribe and replicate the DI RNA.

Pattnaik and Wertz (1991) further exploited this replication system and successfully expressed all five VSV proteins (N, NS, L, M and G) simultaneously when cells were cotransfected with five recombinant plasmids. When the cells expressing all the five VSV proteins were superinfected with the DI particles, DI particle replication, assembly and budding were observed and furthermore, infectious DI particles were released into the culture fluids. The released DI particles were confirmed by their infectivity in the presence of a helper virus and their morphological appearance viewed by electron microscopy. Omission of either M or G protein expression resulted in no DI particle budding. The system established by these workers provides a powerful approach for

detailed structural and functional analysis of the virus gene products in each step of the viral replicative cycle .

For Sendai virus, the polymerase proteins (L and P/C) have also been expressed by a recombinant vaccinia virus system and virus ribonucleoprotein (RNP) was used as templates for the polymerase assay (Gotoh *et al.*, 1989). It is known that the Sendai virus L and P/C proteins are associated with the RNP as observed by immuno-electron microscopy assay (Portner *et al.*, 1988), but they can be easily dissociated from the RNP during the preparation of the RNP and the RNP is usually noninfectious. Gotoh *et al.* prepared RNP from purified virions by NP-40 disruption and recovered them by pelleting onto a 46% (w/w) CsCl cushion through a 2 ml layer of 50% glycerol. The RNP preparation was then transfected into cells infected with recombinant vaccinia viruses expressing the L and P proteins. Culture fluids from the transfected cells were analysed for the Sendai virus yields and it was revealed that the infectious Sendai virus was rescued from the transfected RNP. A recombinant vaccinia virus with the L gene under control of the vaccinia virus TK promoter, which expressed less L protein was far more efficient (500-fold) in rescuing Sendai virus than that with the L gene under control of the P7.5 promoter (12-fold) which expressed more L protein. This result is consistent with the report of Meier *et al.* (1987) on high level expression of VSV L protein inhibiting VSV infection.

From the recombinant RNA polymerase studies described above, it can be seen that in the systems involving complementation of virus ts mutants (Meier *et al.*, 1987; Krystal *et al.*, 1986) or rescue of infectious virus from RNP (Gotoh *et al.*, 1989), the level of the polymerase protein is crucial. In most cases, overexpression of the L protein shows less activity or even an inhibitory effect. However, the system used by Pattnaik and Wertz (1990 and 1991) which only involved DI particles does not appear to have the similar effect.

1.6.3.3. Reconstitution of RNA templates

In vitro encapsidation of genomic RNA by the N protein with subsequent RNA transcription should help unravel the role of the L protein and eventually the mechanism of RNA transcription in general. The difficulty in generating biologically active nucleocapsids of negative-strand RNA viruses has been a major stumbling block for detailed genetic analysis of this group of viruses. The nucleocapsids assembled *in vitro* should satisfy the following criteria as proposed by Mirakhur and Peluso (1988): (1) the RNP is resistant to RNase degradation; (2) the nucleocapsids band at the appropriate density in CsCl, and display the correct sedimentation rate in sucrose gradients; and (3) the viral N protein is found on the nucleocapsids. Only recently, synthetic transcripts

representing the genomic RNAs of influenza virus (Luytjes *et al.*, 1989), and short synthetic templates representing the end sequences of VSV (Moyer *et al.*, 1991), have been assembled into biologically active nucleocapsids. These accomplishments hold great promise as experimental systems for genetic manipulation of negative-strand RNA virus genomes.

For VSV, the use of DI particles has been crucial, as the DI particle has smaller size (2.2Kb) and can be easily separated and purified by gradients centrifugation from the wild type virus. Mirakhur and Peluso (1988) extracted RNA from the purified DI particles and incubated this RNA with VSV infected cytoplasmic extracts which contained the N protein to encapsidate the RNA molecules. The reassembled RNP was shown to have the same CsCl buoyant density as the authentic RNP, was resistant to RNase digestion, and was capable of replicating in cells infected with a helper virus.

More recently, synthetic VSV nucleocapsids have been assembled by Moyer *et al.* (1991) and shown to be a functional template by an *in vitro* transcription assay. Various length cDNAs representing the 3' end of the VSV genome were cloned under the SP6 RNA polymerase transcription start site and *in vitro* transcribed RNA encapsidated with N protein which had been purified from VSV virions. Optimal encapsidation occurred when the concentration of the N protein stock was less than 300 μg/ml. It appeared that as few as 19 nucleotides from the 5' end of positive-strand RNA allowed maximal encapsidation and 5' terminal 10 nucleotides allowed partial (50%) encapsidation. The synthetic nucleocapsids resisted RNase digestion and had a buoyant density typical of wild type VSV nucleocapsids. The *in vitro* encapsidation produced synthetic VSV nucleocapsids were specifically transcribed by the viral RNA polymerase and the products are of the correct positive strand sense with the proper 5' nucleotides. It was also found that some other factors present in the infected cell extracts, probably poly(A), were also needed in the transcription assay, possibly by associating with the N protein to prevent its aggregation.

Synthetic influenza virus segment 8 RNP have been successfully reconstituted *in vitro* (Yamanaka *et al.*, 1990) and were structurally similar to the authentic RNP since they had the same RNase V1-sensitive sites as authentic virus RNP. In a similar way, the influenza virus segment 8 RNA synthesized from its cDNA *in vitro* was encapsidated with the NP protein purified from virions. More recently, Yamanaka *et al.* (1991) reported that the *in vitro* reconstituted RNAs were functional templates which could be transcribed by viral RNA polymerase *in vivo* upon transfection of these templates into the cells. These data suggest that the NP proteins can encapsidate the presynthesized RNA and the method could be very useful for reconstituting RNA templates of other negative-strand RNA viruses.

The success of making full length synthetic virus RNA nucleocapsids was claimed by Ballart *et al.* (1990). A full length measles virus cDNA (15894 nucleotides in length) was cloned under control of T3 or T7 promoters so that both (+) and (-) sense RNA can be transcribed respectively. The recombinant plasmids were linearized just downstream of the cDNA, and 'committed' transcripts were made by incubation with T3 or T7 RNA polymerase and just two ribonucleoside triphosphates. The complexes were microinjected into the helper cells to continue the transcription of the 'committed' complexes using the cellular NTP pool. The helper cell line was obtained by cocultivation of brain tissue from a SSPE patient with cells persistently infected with measles virus. The cells support efficient propagation of the resident genome without releasing infections virus because of defects in the envelope genes. It appears that after microinjection, transcription of the 'committed' complexes continued and the input measles virus genome was replicated by the virus RNA polymerase present in the helper cells. Infectious virus was recovered from the supernatant of the helper cells and contained specific silent mutations which had been introduced into the cDNA by site-directed mutagenesis, indicating that the released virus genome was replicated from the input cDNA. The production of the infectious measles virus indicates that the N protein available in the helper cells cotranslationally or posttranslationally encapsidated the nascent measles virus RNA to produce the functional nucleocapsids. However, this paper has been retracted recently (J. Virol. 65:5656, 1991) and the approach of making infectious virus by microinjection is thus questionable.

1.7. Aims of the project

The L genome segment of Bunyamwera virus encodes the L protein, which is presumed to be the viral polymerase protein. The large size of the L protein suggests it is multifunctional and might be involved in initiation, elongation, and termination of RNA synthesis. With the long-term aim of defining the functional domains within the L protein, it is required to establish a system to attack these areas. Systems used for analysing RNA polymerase proteins of other negative-strand RNA viruses, recently establised by other workers, have been described in section 1.6. In this project, I considered to exploit the available cloned cDNAs to establish systems in which the functionally active L protein can be expressed. Thus the aims of the project were proposed as follows:

(1) To construct a full-length cDNA to the L gene of Bunyamwera virus from the existing cDNA subclones;

(2) To express the L protein in various systems;

(3) To produce monospecific antisera against the L protein in order to characterize the antigenicity of the expressed L protein;

(4) To assay the RNA polymerase function of the L protein.

Chapter 2

Materials and methods

2.1. Materials

1. Chemicals

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

Ampicillin (Penbritin, Beecham Research Labs. Ltd.); Ammonium persulphate and N,N,N'N' tetramethyl ethylenediamine (TEMED, Bio-Rad Labs. Ltd); En^{3}hance autoradiography enhancer (Du Pont UK Ltd.); Formamide (Fluka Chemicals Ltd.); Absolute alcohol 100 (James Burough (FAD) Ltd); Boric acid and caesium chloride (Koch-Light Labs.); Acetic acid (glacial), chloroform, glycerol and hydrochloric acid (May and Baker Ltd.); Ribonucleoside 5'-triphosphates, ultra-pure dNTP set, 'universal' and T7 promoter sequencing primers, diguanosine triphosphate sodium salt (m^7GpppG) and Sephadex G-50 (Pharmacia LKB Ltd.).

2. Miscellaneous Materials

Dialysis membrane (Medical International Ltd.)

Nitrocellulose BA85 (Schleicher and Schuell)

RNasin (Promega Corp.)

Tissue culture plastics (Gibco Ltd. and Sterilin Ltd.)

Trypsin (Gibco Ltd.)

X-ray film for autoradiography (Kodak Ltd.)

3. Enzymes

Restriction endonuclease enzymes, DNA polymerase I, and T4 DNA ligase (Bethesda Research Labs. or Boehringer Mamhein); calf intestinal phosphatase (Boehringer Mannhein); T4 polynucleotide kinase (New England Biolabs.) and T7 DNA polymerase (Pharmacia LKB Ltd.)

4. Radiochemicals

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

^{35}S-L-methionine	approximately 800 Ci/mmol (15 μCi/μl)
5'-α-^{32}P-dNTPs	3,000 Ci/mmol (10 μCi/μl)
5'-α-^{35}S-dATP	> 1,000 Ci/mmol (10 μCi/μl)
5'-α-^{32}P-GTP	400 Ci/mmol (10 μCi/μl)

5. Synthetic oligonucleotides

Synthetic oligonuceotides were produced using a Biosearch model 8600 DNA synthesiser by Dr. J. McLauchlan of this Institute.

6. Plasmids

Cloning vectors pUC18/pUC19, pTZ18/pTZ19, M13mp18/M13mp19 (Yanisch-Perron *et al.*, 1985) and pUC118/pUC119 (Vieira and Messing, 1987) were supplied by Dr. R.M. Elliott. Vaccinia virus transfer vactor pTF7-5 was obtained from Dr. B. Moss (Lab. of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA), and pSC11 obtained from Dr. L. Whitton formerly of this Institute. Bacterial T7 expression vector pET8c was obtained from Dr. C. Preston of this Institute. The pUEX bacterial expression vector series were from Dr. D. P. Leader (Dept. of Biochemistry, University of Glasgow).

Recombinant plasmids containing cDNA derived from the Bunyamwera virus L segment, cloned into pBR322 have been described by Elliott (1989a). Recombinant plasmids containing full length Bunyamwera virus S segment in pUC9 and pUC18 were constructed by A. McGregor; Recombinant plasmids containing Bunyamwera virus S

segment derived cDNA in bacteriophage vectors M13mp18 and M13mp19 were supplied by M. Scallan of this Institute.

7. Bacterial and bacteriophage strains

E. coli strain DH5 (*supE44 hsdR17 recA1 endA1 gryA96 thi-1relA1*, Hanahan, 1983) was the usual host for the propagation of many of the recombinant plasmids. *E. coli* strain XL-1 (*supE44 hsdR17 recA1 endA1 gryA46 thirelA1 lac⁻ F⁻*; Bullock *at al.*, 1987) was used for the growth of phagemids. *E. coli* strain TG1(*supE hsdΔ5thiΔ[lac-proAB] F'[traD36 proAB⁺ lacIq lacZΔM15]*) was used for the growth of bacteriophage M13mp18 or M13mp19. *E. coli* strain LE392.23 (*supE44 supF58 hsdR514 galK2 galT22 metB1 tryR55 lacY1*) was used for the growth of bacteriophage CE6. *E. coli* strain LE392.23 and the strains described below were obtained from Dr. W. Studier (Studier *et al.*,1990; Biology Dept. Brookhaven, National Lab. NY, USA). *E. coli* strains HMS174 (*recA1 hsd R rif*) and BL21 (*F⁻hsdS gal;* Studier & Moffatt, 1986) were used as hosts for bacterial T7 expression system of recombinant plasmids. The lysogens BL21(DE3) and HMS174(DE3) contain a single chromosomal copy of the gene for T7 RNA polymerase under control of the lac UV5 promoter. The *E. coli* strains HMS174(DE3)pLysS, HMS174(DE3)pLysE, BL21(DE3)pLysS and BL21(DE3)pLysE have plasmid pLysS or pLysE in the cells. Plasmid pLysS contains a fragment which has the lysozyme gene cloned under the tet promoter of pACYC184, the fragment has a ø3.8 promoter for T7 RNA polymerase immediately following the lysozyme gene, whereas pLysS has the fragment cloned in the opposite orientation.

Bacteriophage CE6 obtained from Studier is a lambda derivative that carries the gene for T7 RNA polymerase under control of the phage P_L and P_I promoters and also has the cI857 thermolabile repressor and the sam^7 lysis mutation. Bacteriophage M13KO7 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).

8. Bacterial culture media

The following bacterial culture media were used:

L-Broth (LB): 10 g NaCl, 10 g Bactopeptone, 15 g yeast extract per litre.
L-Broth agar: L-Broth plus 1.5% (w/v) agar.
2 YT broth: 5 g NaCl, 16 g Bactopeptone, 10 g yeast extract per litre.
M9 minimal medium: 0.5 g NaCl, 3 g KH_2PO_4, 6 g Na_2HPO_4, 4 g glucose, 1 ml 1 M $MgSO_4$ per litre.

Ampicillin was added at 100 µg/ml into the medium where appropriate.

9. Cells and tissue culture media

BHK-21, C13 cells, a continuous cell line derived from baby hamster kidneys (MacPherson and Stoker, 1962), were supplied by the Cytology Unit of this Institute, and used for growing Bunyamwera virus in Glasgow modified Eagle's medium supplemented with 10% new born calf serum (10% GMEM, Stoker and McPherson, 1961). GMEM was supplied as a 10 x liquid concentrate. This was diluted in distilled water and supplemented with 5 mM L-glutamine, 2.75 g/l $NaHCO_3$, 100 unit/ml penicillin, 100 µg/ml streptomycin and 10% tryptose phosphate broth. CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium containing 5% foetal calf serum (5% DMEM). TK^-143, Human thymidine kinase deficient (TK^-) 143 cells (Rhim *et al.*, 1975) were grown in DMEM supplemented with 5% foetal calf serum and 25 µg 5-bromodeoxyuridine per ml.

10. 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 BS-C-1 cells by Prof. C. R. Pringle in this Institute. The temperature sensitive mutants of Bunyamwera virus (ts5) and Maguari virus (ts7) were isolated by C. U. Iroegbu (Iroegbu and Pringle, 1981). The snowshoe hare bunyavirus ts mutants were obtained from Prof. D. H. L. Bishop (NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.).

vTF7-3, a recombinant vaccinia virus which expresses 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.

11. Antiserum

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

12. Commonly used solutions

Agarose gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol.

10 x EM buffer: 0.05 M sodium borate, 0.1 M sodium sulfate, 0.01 M EDTA, 0.15 M boric acid.

2 x RNA gel sample buffer: 20% glycerol, 0.05% bromophenol blue, 2 x EM buffer.

Carrier DNA: 10 mg/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 nonfat milk in water and stored at -20°C.

NTE: 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

PBS: 170 mM NaCl, 3.4 mM KCl, 10 mM HPO_4, 1.8 mM KH_2PO_4, pH 7.2, 6.8 mM $CaCl_2$ and 4.9 mM $MgCl_2$.

Protein dissociation mix: 100 mM Tris-HCl, pH 6.8, 200 mM ß-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol.

RNase A solution: 10 mg/ml pancreatic RNase A in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, heated at 100°C for 15 min, cooled and stored at -20°C.

SM: 0.1M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, 0.01% gelatin.

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

20 x SSC: 3 M NaCl, 3 M trisodium citrate.

20 x SSPE: 3 M NaCl, 0.18 M NaH_2PO_4, 20 mM EDTA, pH 7.4.

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

TBS^+ buffer: 25 mM Tris-HCl, pH 7.4, 135 mM NaCl, 50 mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$.

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

TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

10 x Tris-glycine: 0.25 M Tris, 2.5 M glycine, 1% SDS, pH 8.3.

X-Gal: 20 mg 5-bromo-4-chloro-3-indolyl-ß-D-galactoside in 1ml dimethylformamide, stored at -20°C.

2.2. Methods

1. Preparation of plasmid DNA

1.1. Growth of bacterial cultures

A single colony of the appropriate *E. coli* strain was picked from an agar plate and inoculated into 10 ml of 2 x YT broth plus antibiotics, if necessary, and shaken overnight at 37°C.

1.2. Large scale preparation and purification of plasmid DNA.

The propagation and purification of supercoiled plasmid DNA on caesium chloride/ethidium bromide gradients were essentially as described by Maniatis *et al.* (1982). An overnight culture (5 ml) was inoculated into a 2-litre flask containing 400 ml of L-broth (with 100 μg/ml of ampicillin where appropriate) and shaken at 37°C for 20 hr. The bacterial cells were pelleted by centrifugation at 6000 rpm (Sorval GS3 rotor) for 20 min at 4°C, washed by resuspending in 10 ml of TE and repelleted in the SS34 rotor at 6000 rpm for 5 min 4°C. The pellet was resuspended in 8 ml of 50 mM glucose, 250 mM Tris-HCl, pH8.0 and 12 mM EDTA containing 5 μg/ml lysozyme. The mixture was incubated at RT for 5 min before the addition of 16 ml of freshly made 0.2 N NaOH, 1% SDS to allow cell lysis. Following 10 min incubation on ice, 12 ml of ice cold 5M potassium acetate, pH4.8, was added to precipitate DNA and the mixture was incubated on ice for a further 10 min. After centrifugation at 18,000 rpm (Sorval SS34 rotor) for 20 min at 4°C, the supernatant was decanted into Corex tubes, the plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol at RT for 15 min, and centrifuged at 12,000 rpm for 30 min at 15°C. The DNA pellet was washed with 70% ethanol, dried and resuspended in a total volume of 10 ml TE, pH8.0. Ten g of caesium chloride was added to the DNA solution to give a final density of 1.55 to 1.60 g/ml. One ml of a 10 mg/ml solution of ethidium bromide was then added and the solution transferred to a Ti 50 heat-sealable centrifuge tube. An isopycinic caesium chloride gradient was formed by centrifugation at 40,000 rpm (Beckman Ti 65 or 50 rotor) for 36 hr at 15°C. The plasmid DNA was identified as a red band in which the ethidium bromide intercalates with the DNA α-helix. The plasmid DNA was carefully removed with a syringe through the side of the tube.

Ethidium bromide was removed by successive extractions (usually four times) with an equal volume of isoamyl alcohol equilibrated with 5 M NaCl. The DNA was then

dialysed against TE (pH 8.0) at 4°C overnight, precipitated with 2 volumes of ethanol, dissolved in water and stored at -20°C. The concentration of the plasmid DNA preparations were determined by measuring their absorbance at 260nm, assuming OD_{260} of 1=50 µg/ml with a 1 cm pathlength cuvette.

1.3. Small scale preparation of plasmid DNA

Single colonies of transformed *E. coli* strain DH-5 were picked with a cocktail stick into 2 ml of 2 x YT broth containing 100 µg/ml of ampicillin. After shaking at 37°C overnight, bacterial cells (1.4 ml of culture) were pelleted in 1.5 ml Eppendorf tubes for 1 min and the supernatant removed by aspiration. The bacterial cell pellet was resuspended in 100 µl of an ice-cold solution of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA and the cells were lysed by the addition of 200 µl of 0.2 N NaOH, 1% SDS at RT for 5 min; 150 µl of 5 M potassium acetate (pH 4.8) was added to the tubes and incubated for a further 5 min. After centrifugation for 5 min, the supernatant was extracted with phenol/chloroform and plasmid DNA ethanol precipitated. The DNA pellet was washed with 70% ethanol, dried under vacuum and dissolved in 40 µl water.

2. Restriction enzyme digestion of DNA

DNA was digested with the appropriate restriction enzyme (normally 1 unit/µg DNA) in commercial restriction enzyme buffer (BRL or Boehringer Mannheim). For the analysis of small scale plasmid DNA preparation, 5 µl of DNA was digested in a 10 µl volume containing 20 µg/ml RNase A for 1 hr at 37°C and DNA fragments separated by eletrophoresis on an agarose gel.

3. Electrophoresis of DNA

3.1. Non-denaturing agarose gels

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

3.2. Denaturing polyacrylamide gels

Polyacrylamide gel electrophoresis was as described by Maniatis *et al.* (1982). Vertical slab gels (257 x 165 x 1 nm) comprised 8% or 12% polyacrylamide sequencing

gel mix containing 8 M urea in 1 x TBE. Polymerization was catalysed by the addition of ammonium persulphate 0.1% (w/v) and TEMED 0.01% (v/v). Samples were loaded in 5% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol and elecrophoresed at 200-300 V in 1 x TBE buffer until the dye fronts had migrated the required distances.

4. Purification of DNA fragments from agarose gels

4.1. Electroelution

Plasmid DNA was cleaved with the appropriate restriction enzyme(s) and the fragments fractionated by electrophoresis on a 1% agarose TBE gel. The DNA bands were visualised under long wave UV light and the required DNA band excised from the gel. The gel slice was placed in a dialysis bag with 0.2-0.5 ml 0.5 x TBE and immersed in a minimal volume of 0.5 x TBE in an electrophoresis tank. After electrophoresis at 250 V for 2hr, the current was briefly reversed to release the DNA from the wall of the dialysis bag. The DNA solution was extracted with phenol/chloroform until protein contamination was not visible at the interface, and the DNA was recovered by ethanol precipitation from the aqueous phase. The DNA precipitates were pelleted by centrifugation for 10 min, washed with 70% ethanol, lyophilised and resuspended in 50 µl water.

4.2. Silica matrix adsorption

A commercial kit, Geneclean (BIO 101 Inc, La Jolla, CA), which contains a specially formulated silica matrix called glassmilk that binds DNA specifically in the presence of sodium iodide (NaI), was used for recovering of DNA fragments from agarose gels (Vogelstein & Gillespie, 1979). An agarose gel slice (in TAE buffer) containing the DNA of interest was mixed with 3 volumes of NaI solution and the tube was incubated at 45-55°C for 5 min until the agarose was completely dissolved. Glassmilk was added at concentration of 5 µl/5 µg of DNA and the mixture was incubated at 4°C for 5 min to allow the DNA to bind to the glassmilk. Following a short spin (about 5 seconds), the pellet was washed three times with NEW wash solution (which contains Tris, EDTA, NaCl, ethanol) by repeated suspension and pelleting. The DNA was eluted from the glassmilk by resuspending the pellet in 20 µl water and incubating at 45-55°C for 1-2 min. After a short centrifugation, the DNA solution was removed and stored at -20°C.

5. DNA cloning

5.1. End repair and DNA ligation

DNA fragments (0.1 µg) were ligated with vector DNA (0.01-0.05 µg), which had been digested with appropriate restriction enzymes, in a volume of 10 µl containing 0.1 M Tris-HCl, pH7.4, 50 mM $MgCl_2$,1 mM spermidine, 1 mM ATP, 100 µg/ml BSA and 1 unit of T4 DNA ligase at 16°C for 16 to 24 hr.

For blunt end DNA ligation, DNA fragments with sticky ends were converted to blunt ends by incubating the DNA fragment (up to 1 µg) in reaction buffer containing 33 mM Tris. OAc, pH7.9, 66 mM KOAc, 10 mM $MgCl_2$, 0.5mM DTT, 0.1 mg/ml BSA, 0.2 mM of each dNTP, 0.1 mM ATP and 3 units of T4 DNA polymerase at 37°C for 30 min. After extraction with phenol/chloroform, the DNA was resuspended in 20 µl of NT buffer with 0.2mM dNTP, and incubated with 1 unit of Klenow fragment of DNA polymerase I at 20°C for 30 min. The DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in 20 µl of water. Ligation was carried out as above except that the ATP concentration was reduced to 0.05 mM.

5.2. Preparation of competent bacterial cells

A 0.5 ml aliquot of an overnight bacterial culture was diluted into 50 ml of 2 x YT broth in a 250-ml flask. The culture was shaken at 37°C for approximately 2 hr until the OD_{550} reached 0.2. The bacterial cells were harvested by centrifugation at 3000 rpm for 10 min. The cell pellet was gently resuspended in 10 ml of 0.1 M $CaCl_2$ and placed on ice for at least 20 min. The suspension was centrifuged at 3000 rpm for 5 min and the cell pellet resuspended in 3 ml of 0.1 M $CaCl_2$. These competent cells were used directly for transformation or could be stored at 4°C for up to 24 hr.

5.3. Transformation

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

6. Purification of synthetic oligonucleotides

Synthetic oligonucleotides were produced by the phosphoram idite method and supplied in ammonium hydroxide which was heated to 55°C for 5 hr to remove the base protecting groups, followed by lyophylisation overnight to ensure removal of the ammonia. The oligonucleotide pellet was dissolved in 0.2 ml of water and loaded in 10% glycerol onto an 8% or 12% polyacrylamide gel containing 8 M urea. After electrophoresis, the oligonucleotides were viewed under UV light as a dark shadow against a fluorescent thin-layer chromatography plate. The gel slice containing the major full length oligonucleotide species was excised with a razor blade and eluted in 1-2 ml of water at 42°C overnight with shaking. The supernatant was transferred to a Spin-x centrifuge unit (COSTAR) which contains a 0.45 µm cellulose acetate filter in a microfuge tube. Acrylamide gel pieces were removed by centrifugation for 2 min in a microcentrifuge. The oligonucleotides were precipitated with 3 volumes of ethanol in the presence of 0.2 M NaCl at -20°C overnight. The oligonucleotide pellet was washed with 70% ethanol, dried and dissolved in water. The concentration of oligonucleotide was quantitated by spectrophotometry, assuming that an OD_{260} nm of one=20 µg/ml with a 1 cm pathlengh cuvette.

7. Preparation of DNA templates for sequencing

7.1. Preparation of ssDNA templates from M13 bacteriophage derived vectors

Two ml aliquots of a 1:100 dilution of an overnight culture of *E. coli* strain TG1 in 2 x YT broth were dispensed into universal bottles. Each universal was inoculated with a colourless M13 phage plaque picked with a sterile cocktail stick and shaken at 37°C for 5 hr. One and half ml of culture were transferred to a microcentrifuge tube and centrifuged for 5 min at RT. The supernatant was removed to a fresh tube and 250 µl of 20% PEG/2.5 M NaCl solution added and incubated at RT for 15 min. After centrifugation for 5 min, the supernatant was removed by aspiration using a drawn-out Pasteur pipette. All the remaining traces of PEG were removed by centrifugation and aspiration. The M13 phage pellet was resuspended in 200 µl of TE buffer and extracted with 100 µl of TE saturated phenol. The upper phase was transferred to a fresh tube, the DNA was ethanol precipitated and dissolved in 25 µl of water.

7.2. Preparation of denatured dsDNA templates

Forty µl of small scale prepared DNA (10-20 µg) was digested with RNAase A at 20 µg/ml at 37°C for 30 min. Ten µl of 2 M NaOH was added and the DNA was denatured at RT for 10 min. After extraction with phenol/chloroform, the DNA was ethanol precipitated, dried and resuspended in 20 µl of water. Ten µl (5-10 µg) of DNA was used for one set of sequencing reactions.

8. Dideoxynucleotide sequencing

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

Ten µl of DNA (about 2-10 µg), either single stranded or denatured double stranded DNA, was mixed with 2 µl of sequencing oligonucleotide primer (0.8 µM) and 2 µl of annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The annealing reaction was carried out at 60°C for 10 min followed by incubation at RT for at least 10 min. The labelling reaction was performed at RT for 5 min: 6 µl of enzyme premix (5 µCi of α-^{32}P-dATP at 3000 Ci/mmol, or α-^{35}S-dATP at >1000Ci/mmol, 0.57 µM dGTP, dCTP, dTTP and 3 units of T7 DNA polymerase) was added to the annealed DNA/premix mix. Two and half µl of termination mix (150 µM each of the 4 dNTPs, 15 µM one ddNTP) was pipetted into the corresponding well of a 96-well microtitre plate labelled as 'G', 'A', 'T', 'C'; the plate had been prewarmed at 37°C for 1 min. Four and half µl of the labelling reaction mix were then added and mixed with each of the four sequencing termination mixes by centrifugation at 1000 rpm for 1 min. The plate was incubated at 37°C for 5 min; 5 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each well, and the plate was heated at 80°C for 2 min immediately before loading onto a 6% sequencing gel.

The 6% sequencing gel mix was made by dissolving 480 g urea, 57 g acrylamide, 3 g bisacrylamide in 800 ml of distilled water and deionised by stirring with Amberlite for 30 min. The solution was filtered through a Whatman 3MM filter paper, 100 ml of 10 x TBE was then added and the volume was adjusted to 1 litre and stored at 4°C. The BRL model S2 electrophoresis apparatus (44.5 x 42.2 x 21.6 cm) was used to run sequencing reaction samples. One pair of glass plates (one is 2 cm shorter) were siliconized and assembled with one spacer (0.4 mm thick) on each side. Seventy ml 6% sequencing gel mix was used for one gel and poured beween 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 (2-2.5 µl) were loaded into the appropriate well of the gel. Electrophoresis was performed at 60 watts for 2-4 hr and one of the plates was carefully removed from the gel; the gel was transferred to a supporting sheet of Whatman 3 MM filter paper, covered with "cling film" and dried using a vacuum gel drier. The cling film was removed and the gel was exposed to X-ray film for several hours for ^{32}P labelled DNA, and1-3 days for ^{35}S labelled DNA.

9. Extraction of total cellular RNA

The extraction of total cellular RNA from virus infected or uninfected cell monolayers was by a modification of the method described by Chomcznski and Sacchi (1987). Cell monolayers in 35 mm dishes were washed once with PBS and lysed in 0.5 ml GSCN solution (4.2 M guandinium thiocyanate, 0.5% sarcosine, 100 mM 2-mercaptoethanol, 0.33% Antifoam A emulsion, 50 mM Tris-HCl, pH 7.5, 2.0 mM EDTA). The lysate was then transferred to a microfuge tube. Fifty µl of 2 M NaOAc, pH4.0, 500 µl of water saturated phenol, and 100 µl chloroform/isoamylalcohol (49:1) were added to the tube. The tube was vortexed for 10 seconds, incubated on ice for 15 min and centrifuged for 10 min. The RNA containing aqueous phase was precipitated by an equal volume of isopropanol at -20°C for 60 min followed by centrifugation for 10 min at RT. The RNA pellet was resuspended in 300 µl of GSCN solution, mixed with 300 µl isopropanol and reprecipitated at -20°C for 60 min. After centrifugation for 10 min, the pellet was dissolved in 200 µl of water by heating at 60°C for 5 min and extracted with an equal volume of a 4:1 mixture of chloroform and butan-2-ol. The RNA aqueous phase was precipitated with three volumes of ethanol in the presence of 0.3 M NaOAc. After centrifugation for 10 min, the RNA pellet was washed with 70% ethanol, dried, dissolved in water and stored at -70°C.

10. Electrophoresis of RNA

RNA samples were separated on an agarose gel containing 5 mM methylmercuric hydroxide, as described by Perbal (1988). Electrophoresis was performed on a horizontal slab gel (14 x 11 x 0.5 cm) of 1% or 1.2% agarose containing 1 x EM buffer (5 mM sodium borate, 10 mM sodium sulphate, 1 mM EDTA, 15 mM boric acid) and 5 mM methylmercuric hydroxide. 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 2 x 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 V

for 4 hr until the bromophenol blue had migrated to the bottom of the gel. All manipulations involving methylmercuric hydroxide were performed under a fume hood.

11. Northern transfer of RNA

RNA samples separated on an agarose gel containing 5 mM methylmercuric hydroxide weretransferred to a 0.2 µm nitrocellulose membrane by the standard method (Southern, 1975; Maniatis *et al.*, 1982). 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 nitrocellulose membrane which had been prewetted with 20 x SSC. The nitrocellulose was then covered by a stack of blotting pads (BRL) about 10 cm high and a weight (500g). Transfer was performed overnight, the nitrocellulose membrane was then dried in air and baked under vacuum for 2 hr at 80°C before hybridization.

12. Extraction of total cellular DNA

Cell monolayers in a 24-well Linbro tray were washed once with PBS and lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.6% SDS, 500 µg/ml proteinase K) in a 37°C incubator for three hours. The lysate was transferred to microfuge tubes, NaCl was added to 0.375 M and the lysate extracted with phenol/chloroform (49:1). The DNA containing aqueous phase was precipitated with 2 volumes of ethanol. The DNA pellet was washed with 70% ethanol, dried and resuspended in 100 µl of TE buffer. RNA was removed by digesting with 0.25 µg/ml RNAase A and 50 units/ml RNAase T1 at 37°C for 30 min. After extraction with phenol/chloroform, the DNA was reprecipitated with ethanol, dissolved in water and stored at -20°C.

13. Southern transfer of DNA

DNA restriction fragments separated in a TBE agarose gel weredenatured in 1.5 M NaCl, 0.5 M NaOH for 1 hr at RT with gentle agitation and neutralised with 1 M Tris-HCl, pH8.0, 1.5 M NaCl for 1 hr. The gel was transferred to a 0.45 µm nitrocellulose membrane by the same method described for the Northern transfer of RNA (Southern, 1975; Maniatis *et al.*, 1982).

14. Hybridization of radiolabelled probes to nucleic acids immobilized on nitrocellulose filters

When ^{32}P-labelled cDNA was used as a probe, the nitrocellulose filter was prehybridized with 5 x SSPE, 0.5% non-fat milk, 0.2 mg/ml carrier DNA (salmon sperm DNA), 50% formamide, in a heat sealable bag at 42°C for 2-4 hr. The solution was then replaced by hybridization solution containing 5 x SSPE, 0.1% milk, 0.2 mg/ml carrier DNA, 50% formamide. ^{32}P labelled cDNA probes were denatured by boiling at 100°C for 5 min, quickly chilled on ice and added to the hybridization bag. The bag was sealed, submerged in a water bath and the hybridization was performed at 42°C with agitation for 12 to 18 hr.

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

After hybridization, the filter was removed from the bag and immediately submerged in a tray containing 200 ml of 2 x SSC and 1% SDS. The filter was washed at RT for 30 min with constant agitation with one change of the wash solution. The filter was further washed with 0.1% SSC and 1% SDS for 30 min at RT. 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.

15. Radiolabelling of nucleic acid as hybridization probes

15.1. Nick translation labelling of DNA

Plasmid DNA was labelled with ^{32}P *in vitro* by nick translation as described by Maniatis *et al.* (1982). Plasmid DNA (0.2 - 0.5 µg) in a 50 µl reaction volume, comprising NT buffer (100 mM Tris-HCl, pH7.5, 10 mM $MgSO_4$, 1 mM DTT, 0.05 mg/ml BSA), 10 µCi of α-^{32}P-dATP, 10 µCi of α-^{32}P-dTTP, 20 mM each of the four unlabelled dNTPs, 1 unit of *E. coli* DNA polymerase I and 10^{-3}µg/ml of DNase I, was incubated at RT for 1 hr. 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 siliconized glass wool plug. Orange G dye was added to the reaction as an indicator, the DNA was applied to the column followed by elution with

water. The eluted DNA was collected before the orange G reached the bottom of the column. The probes were heated at 90-100°C for 2 min before hybridization.

15.2. Riboprobes from *in vitro* transcription

Recombinant plasmid DNA with a cDNA insert under the control of the T7 promoter was linearized by digestion with an appropriate restriction enzyme and the DNA transcribed using T7 RNA polymerase (Promega Biotech) by the method of Nielsen and Shapiro (1986). One µg of the linearized DNA was added to the *in vitro* transcription reaction (total volume 20 µl) which contained 40 mM Tris-HCl, pH 8.0, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine·HCl, 5 mM DTT, 0.5 mM ATP, GTP, UTP, 1.2 µM CTP, 50 µCi/ml α-^{32}P-CTP (400Ci/mmol), 40 units of RNasin, 25 units of T7 RNA polymerase. The reaction was incubated at 37°C for 60 min followed by the addition of 1 µl of RQ DNase I (1 µg/µl) to remove the DNA template; the incubation was continued for 15 min at 37°C and the reaction mix was extracted with phenol/chroloform. The RNA aqueous phase was precipitated by 3 volumes of ethanol in the presence of 1 M ammonium acetate (NH_4OAc), dried and the labelled RNA pellet was dissolved in 50 µl water; it was either used immediately or stored at -70°C.

15.3. Random primer labelling of DNA

Random primer labelling of DNA with ^{32}P was based on the method developed by Feinberg and Vogelstein (1983 and 1984), in which a mixture of random hexanucleotides was used to prime DNA synthesis *in vitro* from a double stranded DNA template. Recombinant plasmids containing cDNA derived from Bunyamwera virus S segment, cloned into M13 bacteriophage vectors, were obtained from M. Scallan of this laboratory and used to make strand specific DNA probes with the multiprime DNA labelling kit (Amersham LKB Ltd.). Five µl of DNA (25 ng), denatured at 95-100°C for 2 min and chilled on ice. To this denatured DNA, the following reagents were added: 5 µl of 5 x labelling buffer, 5 µl each of dGTP and dCTP, and 5 µl of primer, 5 µl of each α-^{32}P-dATP and α-^{32}P-dTTP, 5 units of Klenow enzyme. The reaction was incubated at 37°C for 30 min and unincorporated label was removed by gel filtration. The eluted labelled DNA was denatured at 95-100°C for 2 min and rapidly chilled on ice. The probes were used directly in hybridization reactions or stored at -20°C for later use.

16. Metabolic labelling of intracellular proteins

Monolayers of cells in 35 mm dishes were radiolabelled with ^{35}S-methionine in 1 ml of PBS (100 µCi/ml) for 2 hr at 37°C. The radioactive solution was then removed, the monolayers washed with cold PBS and the cells lysed in 200 µl of protein

dissociation mix (0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue). Cell lysates were boiled for 5 min before loading onto an SDS containing polyacrylamide gel (see method 18).

17. Immunoprecipitation

Cell monolayers were radiolabelled with ^{35}S-methionine (50 µCi/ml) in 1 ml methionine free minimal essential medium for 2 to 16 hr. The cells were washed with cold PBS and 1 ml of cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.02% SDS) was added to each dish to lyse the cells. The dishes were incubated on ice for 30 min and the solution was transferred to microfuge tubes. Nuclei and other cellular debris were removed by centrifugation for 5 min and the lysate was precleared by incubation with 10 µl of preimmune serum for 30 min at 4°C followed by a further 30 min incubation at 4°C with 50 µl of a 10% suspension of protein A-Sepharose. The protein A-Sepharose beads were removed by centrifugation and 0.5 ml of the supernatant were reacted with 5 µl of antiserum for 4-16 hr at 4°C on a mixing wheel. Immmune complexes were collected by incubation with 50 µl of protein A-Sepharose for 30 min at 4°C and pelleted by centrifugation for 30 seconds. The pellet was washed three times with 0.5 M LiCl/0.1 M Tris-HCl, pH 8.0, resuspended in 50 µl of protein dissociation mix and boiled for 5 min prior to electrophoresis on an SDS polyacrylamide gel.

18. SDS-polyacrylamide gel electrophoresis of proteins

Proteins were fractionated on SDS-containing polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). The resolving gel contained 10% or 12% polyacrylamide in which the acrylamide was cross linked with N, N'-methylene bisacrylamide in a ratio of 75:1 (w/w) in resolving gel buffer (0.373 M Tris-HCl, pH 8.8, 0.1% SDS). Ammonium persulphate and TEMED were added to a final concentration of 0.1% (v/v) to polymerize the gel. The gel was overlaid with butan-2-ol to ensure a smooth interface on polymerization. The butanol was removed once the gel had polymerized and the interface washed once with unpolymerized stacking gel solution. The stacking gel solution contained 3.8% polyacrylamide, 62.5 mM Tris-HCl, pH 6.8, 0.1% SDS and was layered on top of the resolving gel once the ammonium persulphate and TEMED had been added to the solution. Protein samples were boiled for 5 min and separated by electrophoresis in tank buffer (53 mM Tris, 53 mM glycine, 0.1% SDS) at 40 mA per gel 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 min followed by destaining with gel fix (7%

acetic acid, 20% methanol) or processed for fluorography. For fluorography, the gel was treated with gel fix for 30-60 min, soaked in En^{2}hance (Dupont, Boston, USA) for 30 min and rehydrated in several changes of water for 30 min. The gel was then dried under vacuum and placed in contact with Kodak-X-Omat S film at -70°C for at least 18 hr.

19. Production of antisera against the Bunyamwera virus L protein

19.1. Expression of portions of the L protein fused to ß-galactosidase

The pUEX series of vectors (Bressan and Stanley, 1987) were used to express *lac* Z gene fusion proteins. These are bacterial expression vectors which can give controlled expression in many *E. coli* strains as a result of the cI857 thermolabile repressor gene located in the plasmid. The Bunyamwera virus L cDNA fragments representing different regions of the L protein were cloned into pUEX1 or pUEX3 and transformed into *E. coli* strain DH5 cells. The recombinant plasmid DNAs were analysed by DNA restriction enzyme digestion and DNA sequencing. Single bacterial colonies were inoculated into 10 ml of 2 YT broth containing 100 µg/ml of ampicillin and shaken at 30°C overnight. One ml of the overnight bacterial culture was diluted to 100 ml with 2 x YT broth containing ampicillin at 100 µg/ml and incubated at 30°C for approximately 3-4 hr at 30°C until the OD_{550} reached 0.2. The culture was transferred to a 42°C water bath for 20 min and then incubated at 42°C with shaking for a further 2 hr.

The bacterial cells were harvested by centrifugation of the culture at 3,000 rpm in the Sorval SS34 rotor for 10 min. The pellet was resuspended in 4 ml of lysozyme solution (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 15% of sucrose, 0.1 mg/ml of lysozyme) and incubated on ice for 40 min. The cells were lysed by incubation with 5 ml of 0.2% Triton X-100, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 5 min on ice. The inclusion bodies were pelleted by centrifugation at 10,000 rpm for 15 min in the Sorval SS34 rotor and resuspended in 2.5 ml of 8 M urea, 2% ß-mercaptoethanol. An equal volume of protein dissociation mix was added and boiled for 5 min before the proteins being fractionated on SDS-polyacrylamide gels.

The fusion proteins were partially purified by separating on 10% polyacrylamide-SDS gels. The proteins were visualized by staining with 0.05% Coomassie brilliant blue in water for 10 min followed by destaining in water for 10-20 min. The fusion protein band, which was the major band visible, was excised from the gel with a scapel and stored at -20°C before further processing.

19.2. Immunization of rabbits

The SDS-polyacrylamide gel slices containing the fusion proteins were macerated by passing through a 20-ml syringe a few times, then through a 21 gauge needle several times until the gel pieces were small enough to pass through the needle easily. An equal volume of water was added and the suspension, which contained about 0.1 mg of fusion protein, was injected subcutaneously into New Zealand white rabbits. The rabbits were boosted three times at 10 days intervals with the same amounts of fusion proteins and the sera were tested by immunoprecipitation and Western blotting before the final bleed.

20. Western blotting

Proteins separated on an SDS-polyacrylamide gel were transferred to 0.45 μm nitrocellulose filter by electroblotting using a LKB2117-250 NovaBlot electrophoretic transfer unit at 0.8 mA/cm^2 gel area for 1 hr in transfer solution (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol) according to the manufacturer's instructions.

The nitrocellulose filter was air dried and excess protein binding sites were blocked by floating the membrane on TBST buffer (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.05% Tween 20) containing 1% BSA at RT for 30 mim with agitation. The blocking solution was replaced with TBST containing the appropriate dilution of primary antibody (1:50 - 1:200 for polyclonal antisera) and incubated at RT for 30 min. The membrane was washed three times for 15 min with TBST buffer, reacted with 1:2500 diluted anti-IgG horseradish peroxidase conjugate at RT for 30 min followed by three 5 min washes with TBST. The membrane was then transferred to the colour development solution which was made by adding 50 μl of 100 mg/ml 4-chloro-1-naphthol to 2 ml methanol and mixing with 8 ml of TBS (10 mM Tris-HCl, pH8.0, 150 mM NaCl) and 35 μl of 30% hydrogen peroxide. When the colour had developed to the desired intensity, the reaction was stopped by rinsing the membrane in water for a few minutes. The membrane was dried in air and photographed.

21. Bunyamwera virus growth and titration

BHK-21, clone 13, cells (MacPherson and Stoker, 1962) were useed for growing Bunyamwera virus stocks and titration. Confluent BHK-21 cells grown in 10% GMEM in 80 oz 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 5,000g for 20 min. The clarified fluids were aliquoted and stored at -70°C.

Virus was titrated by plaque assay (Iroegbu and Pringle, 1981). 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 0.1 ml of diluted virus was added to each dish. The dishes were incubated at 31°C or 37°C for 1 hr to allow the virus to absorb 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 monolayers were fixed with 1% glutaraldehyde in PBS for a few hours, and plaques were counted after Giemsa staining.

22. Purification of Bunyamwera virus

Clarified Bunyamwera virus infected tissue culture fluids were stirred with 23 g/l NaCl and 70 g/l PEG 6,000 for 4 hr at 4°C (Obijeski *et al.*, 1976). The PEG precipitate was collected by centrifugation at 8,000 rpm at 4°C for 20 min in the GSA rotor. The crude virus pellet was resuspended in 1 x NTE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and sonicated for 1 min before centrifuging out any aggregates at 2,000 rpm for 5 min at 4°C. The crude virus supernatant was layered onto potassium tartrate/glycerol gradients. The gradients comprised 5.6 ml of 50% (w/w) potassium tartrate and 6.4 ml of 30% (v/v) glycerol in TST41 tubes. The tubes were centrifuged at 39,000 rpm for 150 min at 4°C. The virus band was removed with a syringe through the side of the tubes, diluted with 1 M NaCl, 10 mM Tris-HCl, pH 7.5 and pelleted through a 3 ml cusion of 25% sucrose in 1 M NaCl, 10 mM Tris-HCL, pH 7.5 at 40,000 rpm for 60 min at 4°C in the TST41 rotor. The virus pellet was then resuspended in 1 M NaCl, 10 mM Tris, pH 7.5 (0.5 ml per five roller bottles), loaded onto a 13 ml 20-60% (w/v) sucrose gradient in 1 M NaCl, 10 mM Tris-HCl, pH 7.5 and centrifuged at 24,000 rpm overnight at 4°C in the TST41 rotor. The virus band was collected and stored in 10% glycerol at -70°C.

23. Preparation of Bunyamwera virus nucleocapsids

The method was based on Leppert *et al.* (1979). Bunyamwera virus infected cells were scraped into the medium with a rubber policeman and pelleted by centrifugation at 3,000 rpm for 30 min in a 50-ml Falcon tube. The cell pellet was suspended in cold lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.6% NP-40, 2 ml per roller bottle of infected cells) and incubated on ice for 5 min. The suspension was vortexed for 2 min to break the cell membranes. Nuclei and cell debris were removed by centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was made to contain 6 mM EDTA and 3-4 ml of extract was loaded onto a CsCl gradient. The gradient was made of 6 ml of 20-40% (w/w) CsCl overlaid with 2.0 ml of 5% (v/v) sucrose in 50 mM NaCl, 25 mM Tris-

HCl, pH 7.5, in a TST41 tube. The nucleocapsids were separated by centrifugation at 32,000 rpm overnight at 12°C in the TST41 rotor. The nucleocapsids band which was visible in the middle of the gradient was harvested with a syringe through the side of the tube and diluted with NTE buffer three fold. The nucleocapsids were then pelleted at 40,000 rpm for 2 hr at 4°C in the TST41 rotor, resuspended in NTE buffer (0.2 ml per roller bottle) and stored at -70°C.

24. Expression of the Bunyamwera virus L protein by T7 RNA polymerase in *E. coli*

The plasmid vector pET-8c was used for cloning and expressing the target DNAs under the control of T7 promoter (Studier *et al.*, 1990). Recombinant plasmids containing Bunyamwera virus L cDNA were transformed and established in different *E. coli* strains: BL21; BL21(DE3); BL21(DE3)plysS and BL21(DE3)plysE, or the equivalent set of HMS174.

24.1. Expression by IPTG induction

For recombinant plasmids established in *E. coli* strains BL21(DE3) and HMS174(DE3) which contain lysogen DE3 to provide T7 RNA polymerase or in these strains containing pLysS or pLysE, expression was induced by IPTG. A fresh overnight culture was grown from a single colony obtained from a freshly incubated plate. The overnight culture was diluted 1:100 in LB broth and shaken at 37°C in the presence of 50 μg/ml of ampicillin (25 μg chloramphenicol/ml was also added if the bacterial strain had the pLysS or pLysE genotype) and the IPTG was added to final concentration of 0.4 mM when the culture had reached an OD_{550} of 0.6-1. After induction for 2-3 hr, the bacterial cells were pelleted by centrifugation at 3,000 rpm for 5 min. The bacterial pellet was resuspended in protein dissociation mix and boiled for 5-10 min before loading onto an SDS polyacrylamide gel .

24.2. Expression by CE6 phage infection

An alternative way to deliver T7 RNA polymerase to the cell was by infection with CE6 phage for the expression of recombinant plasmids in *E. coli* strain BL21 or HMS174. Bacteriophage CE6 is a lambda derivative that carries the gene for T7 RNA polymerase under the control of the phage P_L and P_I promoters; it also has the cI857 thermolabile repressor and the $S_{am}7$ lysis mutation (Studier and Moffatt, 1986).

CE6 phage was grown in host strain *E. coli* LE392-23 by adding a single plaque to 50 ml of 2 x YT broth in a 125-ml flask and shaken at 37°C for 6 hr. The bacterial cultures were made 0.5 M in NaCl and centrifuged at 10,000 rpm for 10 min at 4°C in the

SS34 rotor. The phage was further purified by CsCl gradient centrifugation (Maniatis *et al.*, 1982). The supernatant containing CE6 phage was treated with DNAase I and RNAase A at 1 µg/ml for 30 min at RT. Forty g PEG6000 and 14.5 g NaCl were added to the 50 ml phage supernatant and stirred at 4°C for least 60 min. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. The phage pellet was gently resuspended in SM solution (0.1 M NaCl, 10mM $MgSO_4$, 50 mM Tris-HCl, pH7.5, 0.1% gelatin) and extracted with phenol/chloroform to remove the PEG and cell debris. CsCl was added to 0.75 g/ml, an isopycinic caesium chloride was formed by centrifugion at 38,000 rpm at 4°C for 24 hr in the Beckman Ti 50 or Ti 60 rotor. The phage band was harvested with a syringe and the purified phage were stored in the CsCl solution at 4°C. Dilutions were made in SM solution, and the titre of the CE6 phage was determined by plaque assay. One hundred µl of appropriate dilutions of CE6 phage was mixed with 200 µl of fresh overnight host *E. coli* strain LE392.23 in 4 ml of top agar at 42°C and poured onto LB agar plates. After overnight incubation at 37°C, plaques were counted for the various phage dilutions.

E. coli strain BL21 or HMS174 transformants were grown in LB broth with ampicillin at 50 µg/ml until the cultures reached an OD_{550} of 0.3, then glucose was added to a final concentration of 4 mg/ml. After an additional 1-2 hr of growth, $MgSO_4$ was added to a final concentration of 10 mM and CE6 phage added to 10^9 pfu/ml, giving a multiplicity of 5 pfu/cell. Cells were harvested 2-3 hr after infection, the bacterial cells were pelleted by centrifugation and the proteins separated on a 10% polyacrylamide-SDS gel. Gels were stained with Coomassie brilliant blue to visualize the expressed proteins.

To monitor protein synthesis by radiolabelling, cells were pelleted after induction and resuspended in M9 minimal medium containing ^{35}S-methionine (50 µCi/ml) and incubated at 37°C for 2h. The proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

25. *In vitro* transcription and translation of recombinant plasmids

The bacterial expression vector pTZ contains a T7 promoter and a full lengh Bunyamwera virus L cDNA was cloned under control of this promoter. The recombinant plasmid DNA was linearized at the 3' end of the coding sequence with an appropriate restriction enzy me, and the linearized DNA was phenol/chloroform extracted and ethanol precipitated. The DNA concentration was adjusted to 1 µg/µl.

In vitro transcription was performed in a total volume of 50 µl containing 1 µg of linearized DNA, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermine, 5 mM DTT, 0.4 mM each of ATP, CTP, GTP, UTP, 40 units of RNasin, 25 units of T7 RNA

polymerase and in some reactions, 0.5 mM m^7GpppG (Sutton and Boothroyd, 1986). The reaction was incubated at 37°C for 1 hr, and the DNA template was removed by incubating with 1 unit RQ DNase I for 15 min at 37°C. The RNA transcripts were extracted with phenol/chloroform and precipitated with 3 volumes of ethanol on dry ice for 15 min. The RNA pellet was dissolved in 10 µl of water and used immediately or stored at -70°C.

RNA transcripts were translated *in vitro* using the New England Nuclear (NEN) reticulocyte lysate translation system or Promega's reticulocyte lysate system according to the manufacturer's instructions. A typical reaction (25 µl) using the NEN system was 2 µl of RNA, 10 µl of rabbit reticulocyte lysate, 5 µl of ^{35}S-methionine, 5.5 µl in *vitro* translation cocktail, 2 µl 1 M KOAc, 0.5 µl 32.5 mM $MgOAc_2$. The reaction was incubated at 37°C for 90 min. For the Promega system, 2 µl of RNA, 12.5 µl of reticulocyte lysate, 4 µl of ^{35}S-methionine, 1 µl of -methionine mix and 1 µl RNasin were added together and incubated at 31°C for 60 min. The *in vitro* translated protein products were analysed by electrophoresis on an SDS-polyacrylamide gel directly or after immunoprecipitation.

26. Construction of recombinant vaccinia viruses

Two plasmid transfer vectors pTF7-5 (Fuerst *et al.*, 1987) and pSC11 (Chakrabarti *et al.*, 1985) obtained from B. Moss and L. Whitton respectively were used to make recombinant vaccinia viruses. pTF7-5 contains a T7 promoter and flanking TK sequences of vaccinia virus. pSC11 has cloning sites downstream of the vaccinia virus early and late promoter P7.5, a lacZ gene under the control of vaccinia virus later promoter P11, and also the flanking TK sequences. The cDNAs of interest were cloned dowstream of T7 promoter of pTF7-5 or under P7.5 promoter of pSC11, and the correct orientation of the insert determined by restriction endonuclease digestion and confirmed by nucleotide sequencing.

Recombinant vaccinia viruses were prepared essentially as described by Mackett *et al.* (1985). Confluent monolayers of CV-1 cells in 35mm dishes were infected with vaccinia virus (WR strain) at a multiplicity of 0.05 pfu/cell at 37°C for 1h. The virus inoculum was removed, the monolayer washed once with TBS^+ buffer (25 mM Tris-HCl, pH 7.4, 135 mM NaCl, 50 mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$) and then transfected with 5 µg of plasmid DNA by the calcium phosphate method. DNA coprecipitates were formed by incubating DNA in 250 µl of HEPES buffered saline (140 mM NaCl, 5 mM KCl, 0.75 mM $NaHPO_4$, 6 mM dextrose, 25 mM Hepes, pH 7.05) and 125 mM $CaCl_2$ at RT for 30 min. The DNA was added to the infected monolayer and the dishes incubated at RT for 30 min. Two ml of 5% DMEM were added and the

dishes incubated at 37°C for 3-4 hr. The medium was changed and the incubation was continued for a further 48 hr. The transfected cells were scraped into the medium, virus released by three cycles of freeze-thawing (dry ice/37°C) and sonication for 5min. Recombinant vaccinia viruses were isolated by plaque assay.

TK^- recombinant viruses were selected by growth in TK^- 143 cells in the presence of 25 µg/ml BUdR (Mackett *et al.*, 1985). The progeny viruses produced by transfection were plaqued on TK^- 143 cells using a 1% low melting point agarose overlay containing 25 µg/ml BUdR and incubated at 37°C for 2 days. The plaques were stained by neutral red and for pSC11 derivatives, neutral red was replaced by 300 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; BRL). The plaques were picked with a pasteur pipette and added to 200 µl of 5% DMEM; half of the plaque was used to infect a TK^- 143 cell monolayer in 24-well Linbro tray. Virus was adsorbed at 37°C for 60 min and the inoculum removed. 5% DMEM supplemented with 25 µg/ml BUdR was added and the tray incubated at 37°C for 2 days. The cells were lysed with 100 µl of 0.5 M NaOH and 1% SDS at RT for 20 min. To the lysed cells, 50 µl of neutralization buffer (1 M NaCl, 0.3 M Na·citrate, 0.5 M Tris-HCl, pH 8.0, 1 M HCl) was added and the solution was further diluted with 100 µl of distilled water. Half the amount of DNA was applied to a nitrocellulose filter using a 96-well filtration dot blotting apparatus and the filter probed with ^{32}P-labelled Bunyamwera virus L gene specific cDNA. After dot blot filter hybridization screening, plaques positive for the L gene were purified by three rounds of plaque-to-plaque passage. Finally, the genomes of the recombinant viruses were analysed by restriction enzyme digestion and Southern blotting as described in methods 2 and 13.

27. Preparation and purification of recombinant vaccinia virus stocks

Recombinant vaccinia viruses were grown in CV-1 cells and purified as described by Mackett at al. (1985). Half of the progeny virus released from a small monolayer culture (25 cm^2) was diluted to 16 ml with PBS and used to infect 8 large flasks (150 cm^2) of CV-1 cells. Following virus adsorption at 37°C for 1 hr, 40 ml 5% DMEM was added and the flasks were incubated at 37°C for 48 hr.

The infected cells were scraped into the medium and the cells were pelleted by centrifugation at 3,000 rpm for 5 min. The pellet from each flask was resuspended in 2 ml of 10 mM Tris-HCl, pH 9.0. After three cycles of freeze-thawing, the nuclei were removed by centrifugation at 750g for 5 min. The supernatant was then incubated with 0.1 volume of trypsin (2.5 mg/ml) at 37°C for 30 min with frequent vortexing. The mixture was layered onto an equal volume of 36% (w/v) sucrose in 10 mM Tris-HCl,

pH9.0 in a TST41 tube and centrifuged at 12,000 rpm for 80 min at 4°C. The virus pellet was resuspended in 2 ml of 1 mM Tris-HCl, pH9.0 and incubated with 0.1 volume of 2.5 mg/ml trypsin at 37°C for 30 min. Two ml of this virus suspension were then overlaid onto a continuous sucrose gradient (15-40% sucrose in 1 mM Tris-HCl, pH9.0) in a TST41 tube and centrifuged at 12,000 rpm for 40 min at 4°C. The virus band was collected with a syringe, diluted with 1 mM Tris-HCl, pH9.0, and pelleted at 13,000 rpm for 60 min at 4°C. The virus pellet was resuspended in 1 mM Tris-HCl, pH9.0 and stored at -20°C. The titre of the virus was determined by plaque assay.

28. Complementation assay

Bunyamwera virus temperature sensitive (ts) mutants BUN ts5, Maguari virus ts mutant MAG ts7 and snowshoe hare virus (SSH) ts mutants were used for complememtation assay. The recombinant Bunyamwera virus L protein expressed from recombinant vaccinia viruses was assayed for its ability to complement ts mutants with a defective L protein. CV-1 cells in 35 mm dishes were infected with the ts mutants at multiplicity of 1 pfu/cell. Viruses were allowed to adsorb at RT for 40 min in 0.5 ml of medium. The inocula were removed and 2 ml of prewarmed 5% DMEM added. The dishes were incubated at the nonpermissive temperature 39.8°C (SSH) or 38.5°C (BUN ts5 and MAG ts7) for 2 hr, the cells were infected with recombinant vaccinia viruses vSC11-BUNL or vTF7-5-BUNL with vTF7-3 at multiplicity of 5 pfu/cell. Adsorption was performed at 31°C for 60 min, the infected cell monolayers were washed three times with 40°C warmed PBS, and 2 ml of prewarmed 5% MEM was added. Incubation was continued at the nonpermissive temperature for 1 or 2 days. The culture fluids were harvested and the titres of bunyavirus were determined by plaque assay at 31°C. As the culture fluids contained both vaccinia viruses and bunyaviruses, 250 μg/ml of phosphonoacetic acid was added to the agarose overlay to inhibit vaccinia virus growth without interfering with bunyavirus plaquing.

29. *In vitro* transcriptase assay of the Bunyamwera virus L protein

The polymerase activity of the L protein from either detergent disrupted purified virions or CsCl gradient purified intracellular nucleocapsids was assayed *in vitro*. The activity of the L protein expressed from recombinant vaccinia viruses were tested by adding infected cytoplasmic extracts to the reaction to investigate any stimulation of transcriptase activity. The extracts were prepared by a modification of the method described by Peluso and Moyer (1983). Mock or virus infected cell monolayers were washed twice with 50 mM Hepes, pH 8.1, 0.1 M KCl, 6 mM $MgCl_2$, 1 mM DTT and

the cell membranes were penetrated with 200 µg/ml lysolecithin in the same buffer for 1-2 min. Following a gentle wash with buffer, the monolayers were scraped into 200 µl of buffer containing 10 µg/ml of actinomycin D. The suspension was pipetted up and down 15 to 20 times followed by centrifugation for 2 min to remove the nuclei and cell debris. The resulting supernatant (cytoplasmic extract) was either used immediately or stored at -70°C in 10% glycerol.

The transcription reaction was performed in a total volume of 25 µl containing 50 mM Hepes, pH 8.1, 0.1 M KCl, 6 mM $MgCl_2$, 1 mM DTT, 40 units /ml of RNasin, 1 mM each of ATP, UTP, CTP, 0.01 mM GTP, 2.5 µl of a-^{32}P-GTP, 2.5 µl of nucleocapsids (prepared by the method described in method 23), or 2.5 µl of virions (prepared by the method described in method 22) disrupted with 1% NP-40 and 10 µl of cell extract. The reaction was incubated at 30°C for 2 hr and stopped by the addition of 50 µl of 10 mM Hepes, pH 8.1, 1% SDS, 1 mM EDTA, 100 mM NaCl. The reaction was phenol/chloroform extracted and ethanol precipitated. Half of the sample was loaded onto a 1% agarose gel containing 5 mM methylmercuric hydroxide. After electrophoresis, the gel was dried and the radiolabelled RNA products were visualized by autoradiography.

30. Nucleocapsid transfection assay

The nucleocapsids purified from Bunyamwera virus infected cells (method 23) were used as RNA templates and delivered into recombinant vaccinia virus infected cells to analyse the RNA polymerase function of the recombinant L protein. The nucleocapsids were transfected into the recombinant vaccinia viruses infected cells by the calcium phosphate method (Sambrook *et al.*, 1990). CV-1 cells (10^6 per 35 mm dish) were infected with recombinant vaccinia viruses at a multiplicity of 5 pfu/cell for 1hr at 37°C, and then washed once with TBS^+ buffer. The nucleocapsids transfection mix was added and comprised, in a total volume of 250 µl, purified nucleocapsids (10 µl), 140mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO_4 \cdot 2H_2O$, 6 mM dextrose, 25 mM HEPES, pH 7.05 and 125 mM $CaCl_2$. After 30 min incubation at RT with occasional shaking, 2 ml of 5% DMEM was added and incubated at 37°C for 3-4 hr. The supernatant was removed and the cells were shocked with TBS^+ containing 10% glycerol for 1-2 min. After two further washes with TBS^+, 2ml of 5% DMEM was added and the incubation continued at 37°C for 20 hr. Cell monolayers were then either radiolabelled with ^{35}S-methionine to analyse the protein synthesis, or total cellular RNA extracted for Northern blotting analysis.

31. Site-directed mutagenesis

31.1. Preparation of DNA templates for mutagenesis

A cDNA fragment containing the DNA region to be mutated was isolated, cloned into pUC119 phagemid vector (Vieira and Messing, 1987) and transformed into *E. coli* strain XL-1 cells. A single colony was picked and inoculated into 2 ml of 2 x YT broth containing 70 µg/ml of ampicillin and $5x10^7$ pfu/ml of helper phage M13KO7 to make single stranded DNA templates. Following 1-2 hr shaking at 37°C, kanamycin was added to 70 µg/ml and incubation was continued for 14-20 hr.

Bacterial cells were pelleted by centrifugation for 5 min in a microcentrifuge, 1.25 ml of the supernatant were transferred to a fresh tube, mixed with 200 µl of 20% PEG/2.5M NaCl and incubated at RT for 15 min followed by centrifugation for 5 min. The supernatant was removed by aspiration and following a brief spin, all the residual supernatant was carefully removed. The PEG precipitation was repeated by resuspending the pellet in 0.5 ml of TE buffer and mixing with 100 µl of 20% PEG/2.5 M NaCl. The phage pellet was resuspended in 300 µl of 0.3 M NaOAc and extracted with phenol/chloroform. DNA was precipitated by adding 0.8 ml of ethanol and incubated on dry ice for 10 min. After centrifugation, the pellet was washed with 70% ethanol, dried and resuspended in 25 µl of water. Ten aliquots of DNA were combined, quantitated by OD at 260 nm and the DNA concentration was adjusted to 1 µg/µl.

31.2. Oligonucleotide-directed *in vitro* mutagenesis

The Eckstein method (Nakamaye and Eckstein, 1986; Sayers, *et al.*, 1988) was used for oligonucleotide directed mutagenesis. The method involves a strand specific selection step which eliminates the unwanted non-mutant DNA sequence. Selective removal of the nonmutant strand is made possible by the incorporation of a thionucleotide (e.g. dCTPαS) into the mutant strand during *in vitro* synthesis. As certain restriction enzymes (e.g. Nci I) can not cleave phosphorothioate substituted DNA, single strand nicks are generated in DNA containing one phosphorothioate and one non-phosphorothioate strand. Such nicks present sites for exonuclease III to digest away most of the non-mutant strand. This avoids host mediated repair which can remove the mutant sequence resulting in poor yields of mutated DNA. The commercial oligonucleotide-directed *in vitro* mutagenesis kit (version 2, Amersham LKB Ltd.) was used for the mutagenesis reaction according to the manufacturer's instruction.

The synthetic oligonucleotides were phosphorylated by incubating 100 pmol of oligonucleotide with 5 units of polynucleotide kinase in a total volume of 30 µl containing

1 mM ATP, 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 7 mM DTT at 37°C for 10 min. The enzyme was inactivated by heating at 70°C for 10 min. The phosphorylated oligonucleotide was used immediately or stored at -20°C. Phosphorylated oligonucleotide (1.75 µl) was annealed to 2 µg of DNA template in a total volume of 8.5 µl by heating at 70°C for 3 min followed by incubating at 37°C for 30 min.

The extension reaction was carried out by replacing dCTP with dCTPαS in the presence of 3 units of Klenow fragment and T4 DNA ligase at 16°C for 16-20 hr. Sodium chloride was then added to final concentration of 0.5 mM and the solution filtered through 2 sheets of nitrocellulose filters by centrifugation at 1,500 rpm for 10 min. The filters were washed by centrifuging 100 µl of 0.5 M NaCl through the filters and the filtrates pooled. The combined filtrates were ethanol precipitated and half of the DNA was digested with 4 units of Nci I at 37°C for 90 min followed by incubating with 50 units of exonuclease III at 37°C for 20 min. The enzymes were inactivated by heating at 70°C for 15 min. The gapped DNA was repaired by polymerization and ligation in the presence of the 4 dNTPs, ATP, $MgCl_2$, 1.25 units of DNA polymerase I and 1.5 units of T4 DNA ligase at 16°C for 3 hr. Five to twenty µl of the reaction were used to transform *E. coli* strain XL-1 cells which were plated on agar plates containing 100 µg/ml of ampicillin. Single colonies were picked, grown in the presence of helper phage M13KO7 and single stranded DNA prepared for sequencing. The bacterial pellets were saved for preparing dsDNA for subcloning the desired mutant DNA fragments back into plasmids containing the Bunyamwera virus full length L gene cDNA.

32. Computing

Computer analysis of DNA and protein sequence were performed on the Micro VAX 11 and PDP11/14 computers in this Institute, using primarily the University of Wisconsin Genetics Computer Group package.

Chapter 3

Production of monospecific antisera to the L protein

3.1. Introduction

Virus specific antibodies are a principle means of defence against infection and have also been used as important reagents in the serological classification of viruses and in the study of aspects of virus replication. An antiserum against purified Bunyamwera virus had been raised in rabbits by Watret (1985) in this Institute. However, the production of monospecific antibodies against the L protein was required for the future characterization of recombinant L protein molecules. The recent determination of the complete nucleotide sequence of the Bunyamwera virus L genome segment from cloned cDNA (Elliott, 1989a) made it possible to produce L protein specific antisera by a genetic engineering approach.

To obtain the L protein antigen, I expressed five fusion proteins in *E. coli*. Out of five antisera raised against the fusion proteins, I obtained two potent monospecific antisera against both the N- and C-termini of the L protein.

3.2. Construction of recombinant plasmids containing portions of the L gene cDNA in pUEX

To obtain suitable L protein antigens, the pUEX bacterial expression vector series, constructed by Bressan and Stanley (1987), was used (Fig.3.2). The pUEX vectors, transformed into DH5 cells, are capable of expressing cro-ß-galactosidase fusion proteins at high levels when induced by temperature shift to 42 ℃. It had been reported previously that it was difficult to express a protein fragments larger than 130 residues in the pEX

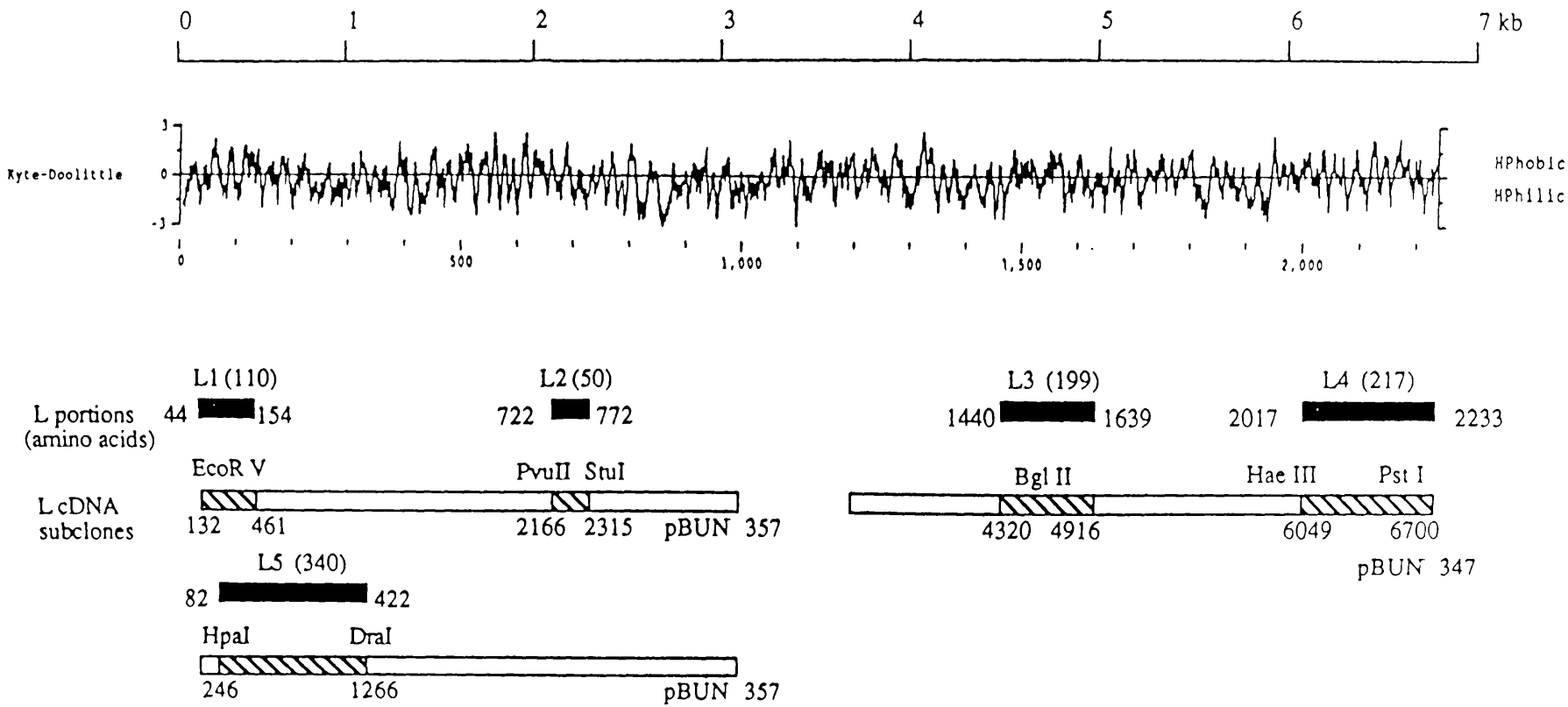


Fig. 3.1. Portions of the L protein used to make fusions with β-galactosidase. The hydropathy profile of the L protein is shown on the top. The positions and length of the L protein portions, and the length of the Bunyamwera virus L gene cDNA fragments cloned in pUEX vectors are shown in the lower part. Restriction enzyme sites used to remove the cDNA fragments from the L gene cDNA subclones and the length of the L portions are indicated.

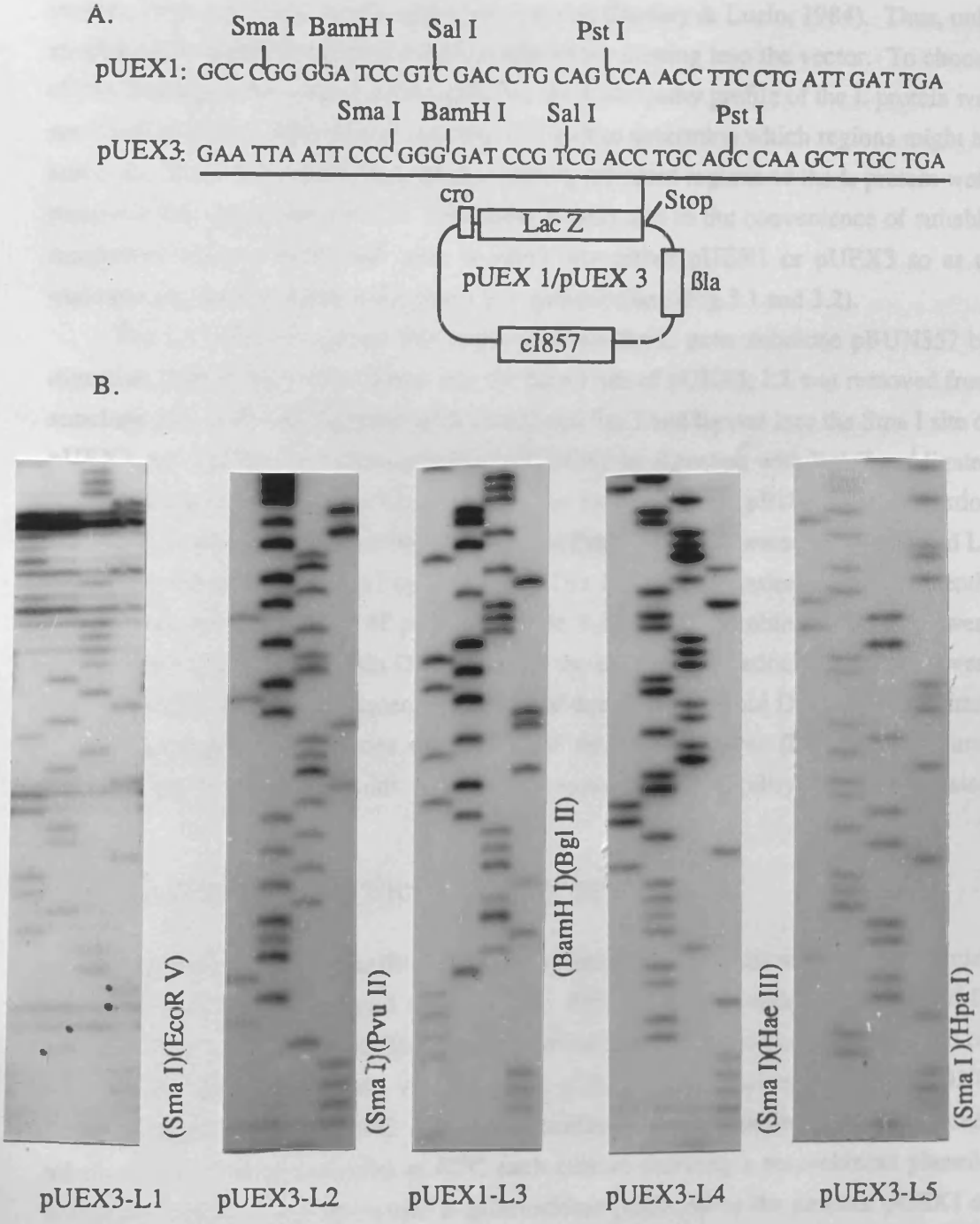


Fig.3.2. Nucleotide sequence analyses of pUEX-L constructs. Panel A: Map of plasmid pUEX1/3 showing the unique restriction enzyme sites and reading frames of ß-galactosidase through the cloning linker. 'Stop' is the sequence containing stop codons in all reading frames. Panel B: Portions of sequencing gels showing the correct sequence across the insertion point. The alkaline denatured DNA derived from pUEX-L1 to pUEX-L5 was sequenced by the dideoxy chain termination method and the order of the lanes is G, A, T, and C.

vectors, from which the pUEX series was derived (Stanley & Luzio, 1984). Thus, only small L gene cDNA fragments were considered for cloning into the vector. To choose cDNA fragments for making fusion proteins, the hydropathy profile of the L protein was analysed (Fig.3.1). However, it was still difficult to determine which regions might be antigenic. Thus, five cDNA fragments spanning different regions of the L protein were removed from the L gene cDNA subclones, largely due to the convenience of suitable restriction enzyme sites, and were inserted into either pUEX1 or pUEX3 so as to maintain the reading frame when fused to ß-galactosidase (Fig.3.1 and 3.2).

The L1 cDNA fragment was removed from the L gene subclone pBUN357 by digestion with EcoR V and ligated into the Sma I site of pUEX3; L2 was removed from subclone pBUN357 by digestion with Pvu II and Stu I and ligated into the Sma I site of pUEX3; L3 was removed from subclone pBUN347 by digestion with Bgl II and ligated into the BamH I site of pUEX1; L4 was taken from subclone pBUN347 by digestion with Hae III and Pst I and inserted between the Sma I and Pst I sites of pUEX1; and L5 was removed from pBUN347 by Hpa I and Dra I double digestion and subsequently cloned into the Sma I site of pUEX3 (Table 3.1). The recombinant plasmids were transformed into *E. coli* strain DH5 cells and the correct orientation of the inserts were determined by nucleotide sequence analysis of denatured plasmid DNA using a primer complementary to nucleotides 4101-4121 of the pUEX vector (Fig.3.2). Cultures carrying recombinant plasmids were then assayed for their ability to express fusion proteins.

3.3. Expression of the L fusion proteins

Cultures of *E. coli* transformed with recombinant plasmids were grown to mid-logarithmic phase at 30°C and transferred to 42°C for 2 hr to induce synthesis of ß-galactosidase fusion proteins. The protein profiles of total cell lysates were analysed on 10% polyacrylamide-SDS gels. A comparison of the profiles obtained at 30°C and 42°C (Fig.3.3) showed that at 30°C no ß-galactosidase fusion proteins were produced, whereas after 2 hr of induction at 42°C each culture carrying a recombinant plasmid induced a fusion protein larger than ß-galactosidase produced by the parental pUEX1 or pUEX3. The migration differences of the fusion proteins correlated closely with those predicted by the size of each inserted L gene cDNA fragment. In one case (pUEX1-L3, Fig.3.3. lane 8), a protein that comigrated with ß-galactosidase was also produced during the induction period, which may have resulted from specific degradation of the L3 fusion protein product.

The level of the fusion proteins expressed was similar to that of the ß-galactosidase produced by pUEX1 or pUEX3, and was high enough for purification. Large scale bacterial cultures (100 ml) were induced at 42 °C, the cells collected by centrifugation and the insoluble fusion proteins were separated from bacterial soluble proteins (see chapter 2). The fusion proteins were partially purified by electrophoresis on 10% polyacrylamide-SDS preparative gels as described in chapter 2, and a aliquot of each preparation was analysed on a 10% polyacrylamide-SDS gel. As can be seen from Fig.3.4, the fusion proteins composed the major protein components of the preparations. The quantity of each fusion protein was estimated by comparison with known amounts of standard ß-galactosidase protein by Coomassie blue staining of the gel. Two rabbits were immunized with each fusion protein following the schedule given in chapter 2.

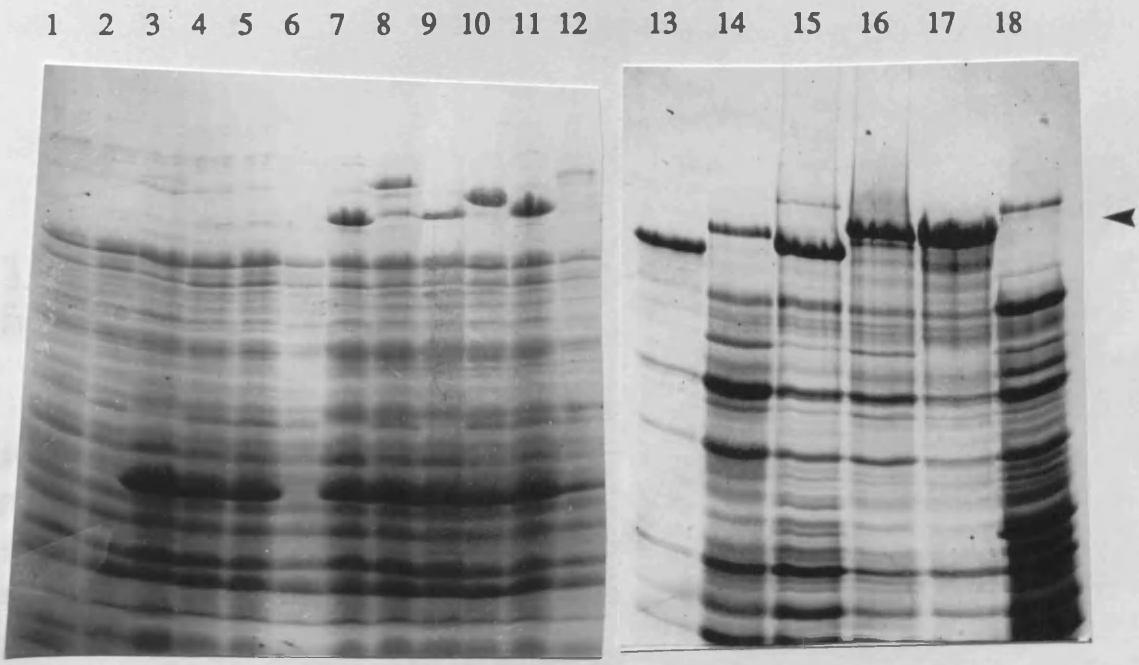


Fig.3.3. Fusion protein expression in *E. coli* transformed with recombinant plasmids. Cultures were grown at 30 °C (lanes 1-6) and induced at 42 °C for 2 hr (lanes 7-18), and the bacteria were collected by centrifugation. Proteins were separated on 10% polyacrylamide-SDS gels and stained with Coomassie brilliant blue: pUEX1 (lanes 1, and 7); pUEX1-L3 (lanes 2, 8 and 16); pUEX3 (lanes 3, 9 and 13); pUEX3-L1 (lanes 4, 10 and 14); pUEX3-L2 (lanes 5, 11 and 15); pUEX3-L4 (lanes 6, 12 and 17) and pUEX3-L5 (lane 18).

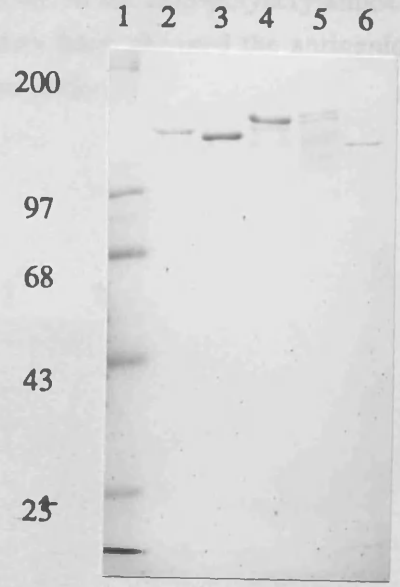


Fig.3.4. Partially purified fusion proteins analysed on a 10% polyacrylamide-SDS gel. L1-L4 fusion proteins are shown in lanes 2-5. Lane 1 is protein size markers and lane 6 shows purified ß-galactosidase.

3.4. Characterization of the antisera raised against the L fusion proteins

Antisera raised against portions of the Bunyamwera virus L protein fused with ß-galactosidase were characterized by Western blotting and immunoprecipitation. The L protein present in intracellular nucleocapsids, which were purified from Bunyamwera virus infected cells by CsCl gradient centrifugation (see chapter 2), were used as the antigen for Western blotting. The nucleocapsid proteins were separated by electrophoresis on a 10% polyacrylamide-SDS gel and the L and N proteins were detected by Coomassie brilliant blue staining (Fig.3.5A). Of the antisera raised against four fusion proteins (L1-4) which were initially screened, two antisera which were made against the fusion proteins produced in pUEX1-L3 and pUEX3-L4 transformed bacterial cells reacted with the L protein (Fig.3.5B. lanes 3-4). Anti-L1 and anti-L2 did not react with the L protein (lanes 1-2). In an independent assay, the anti-L4 serum reacted with the L protein bound on the nitrocellulose filter strongly (lane 6), which is not seen with the preimmune serum (lane 5).

As the L protein separated on the SDS-polyacrylamide gels is a denatured antigen and because denaturation may have changed the antigenicity and hence affected its reactivity with the sera, I then performed immunoprecipitation assays to test each serum.

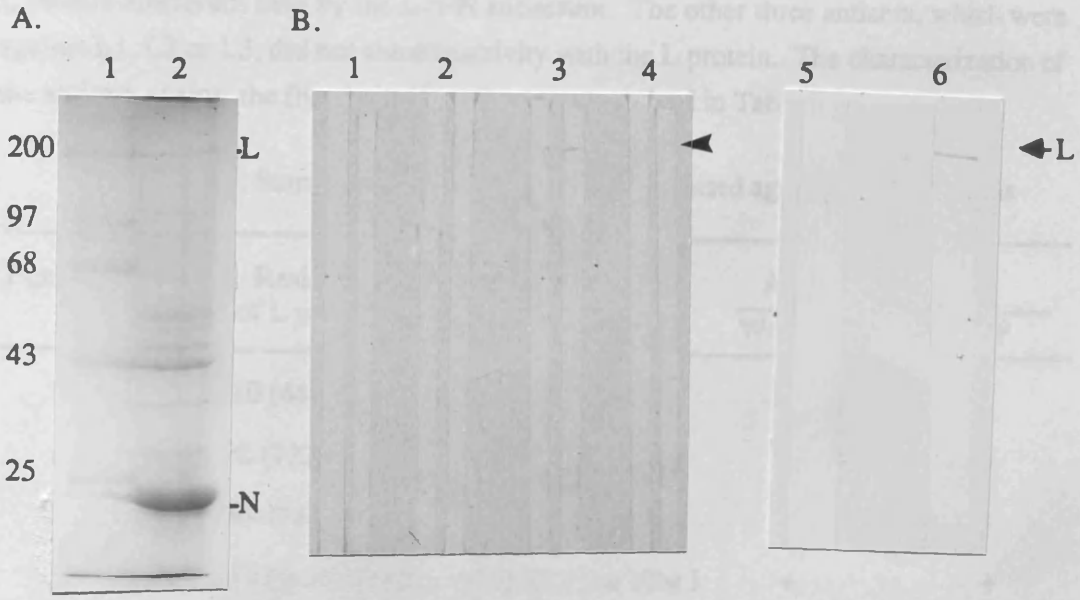


Fig.3.5. Immunodetection of the Bunyamwera virus L protein by Western blotting. **A.** Analysis of Bunyamwera virus nucleocapsids. Bunyamwera virus nucleocapsids were purified from the infected BHK cells by CsCl gradient centrifugation, separated on a 10% polyacrylamide-SDS gel and stained with Coomasie brilliant blue (lane 2). The positions of the L and N proteins are indicated and the protein size markers are shown in lane 1. **B.** Characterization of the test sera response to the L protein by Western blot assay. Bunyamwera virus nucleocapsids were separated on a 10% polyacrylamide-SDS gel and transferred to a nitrocellulose filter. The strips of the filters were incubated with 1:50 diluted anti-L1 (lane 1), anti-L2 (lane 2), anti-L3 (lane 3), anti-L4 (lanes 4 and 6), or preimmune serum (lane 5), the bound antibodies were detected by their reaction with goat anti-rabbit antibody conjugated with horseradish peroxide (1:80 dilution) which produced a blue-black colour by reaction with Chloronaphthol. The L protein derived from intracellular nucleocapsids was specifically detected by anti-L3 (lane 3) and anti-L4 (lanes 4 and 6), but not by anti-L1 (lane 1), anti-L2 (lane 2) or preimmune serum (lane 5).

^{35}S-methionine labelled Bunyamwera virus infected cell extracts were reacted with each individual serum followed by analyses on a 10% polyacrylamide-SDS gel. As shown in Fig.3.6, two sera raised against the L4 and L5 fusion proteins, which represent the C- and N-termini of the L protein, specifically precipitated the L protein; no reaction was observed with the preimmune serum control. More L protein was precipitated by anti-C L protein antiserum than by the anti-N antiserum. The other three antisera, which were against L1, L2 or L3, did not show reactivity with the L protein. The characterization of the antisera against the five fusion proteins is summarized in Table 3.1.

Table 3.1. Summary of the L protein antisera raised against fusion proteins

Fusion protein	Residues of L protein	Cloning site in pUEX	Antiserum reactivity	
			Western Blotting	IP
L1	110 (44-154)	pUEX3-Sma I	-	-
L2	50 (722-772)	pUEX3-Sma I	-	-
L3	199 (1440-1639)	pUEX1-BamH I	+	-
L4	217 (2017-2233)	pUEX3-Sma I/Pst I	+	+
L5	340 (82-422)	pUEX3-Sma I	ND	+

ND: not determined

It was noted that in Fig.3.6, a protein migrating slower than the G2 protein was also immunoprecipitated by both positive antisera against the L protein and this band was not clearly shown in the lane which was precipitated with anti-Bunyamwera virus serum. The origin of this protein band is unclear and might be a nonspecific precipitation, which has never been seen on other occasions. Minor amount of the N protein was also precipitated by the L specific antisera. The more L protein precipitated, the more N protein could be seen (Fig.3.6). As the cytoplasmic extracts were prepared for immunoprecipitat ion under essentially nondenaturing conditions, the antisera against the L protein might have reacted with the L associated with the nucleocapsid and thus precipitated the nucleocapsid-associated N protein. The precipitation of the L protein by anti-Bunyamwera virus serum may have resulted from precipitation of the N protein on the nucleocapsid with which the L is associated.

The two positive antisera against either the N- or C-terminus of the L protein were also characterized by their reactivity with an N-terminal half L protein which was

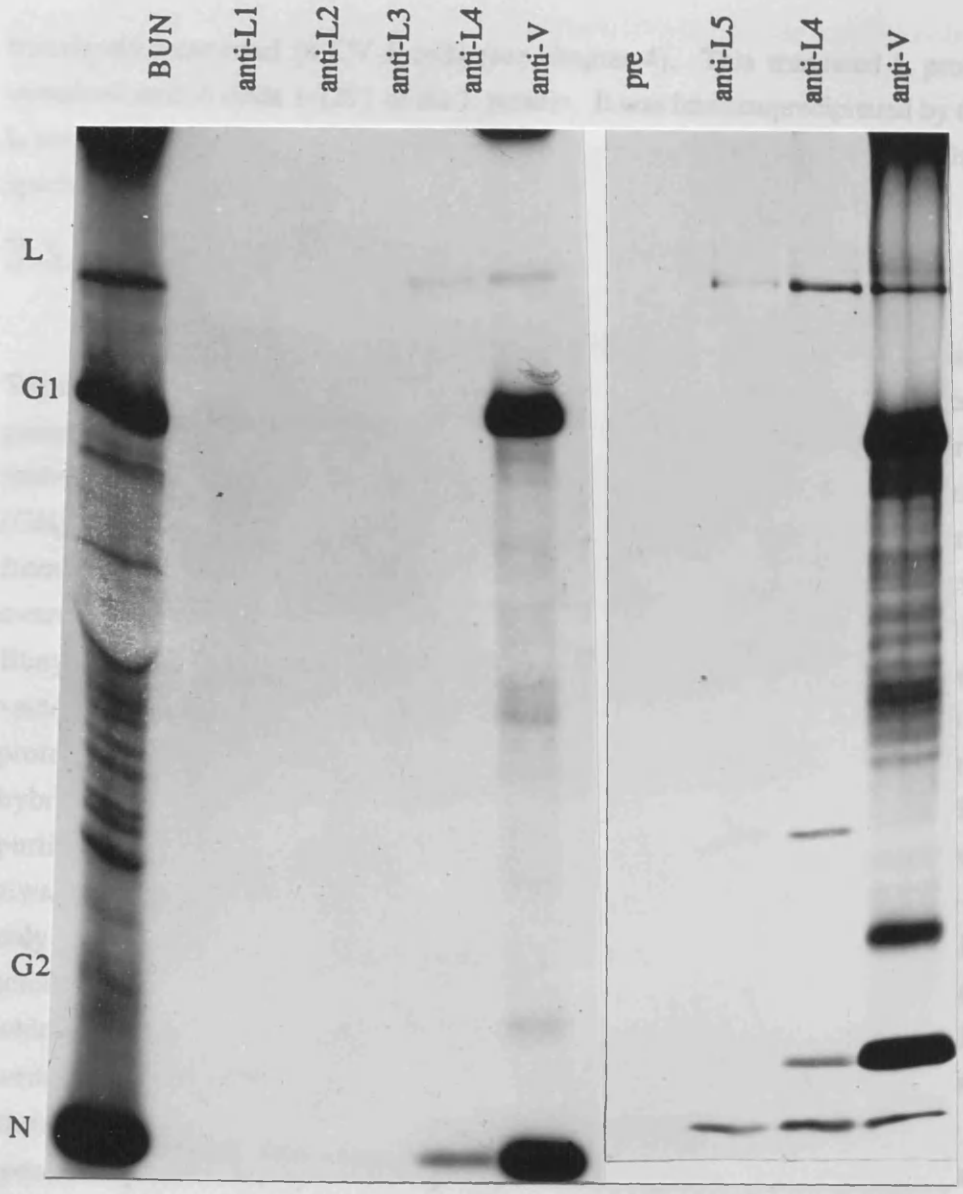


Fig.3.6. Characterization of the test sera response to the Bunyamwera virus L protein by immunoprecipitation. Bunyamwera virus infected cells were labelled for 4 hr at 16 hr postinfection and cytoplasmic extracts were immunoprecipitated with sera against fusion proteins (anti-L1 to anti-L5) and anti-Bunyamwera virus (anti-v) or preimmune serum (pre) as indicated, followed by electrophoresis on 10% polyacrylamide-SDS gels. Two antisera (anti-L4 and anti-L5) precipitated the L protein; no reaction was seen with the preimmune serum. More L protein was precipitated by the anti-L4 antiserum. Minor amounts of N protein were also precipitated by these two antisera with more N precipitated by anti-L4. Antiserum against L1, L2 or L3 did not react with the L protein. Lane BUN is Bunyamwera virus infected cells examined without immunoprecipitation. Lanes anti-v are immunoprecipitation of Bunyamwera virus infected cell extracts with the anti-Bunyamwera virus serum and the Bunyamwera virus proteins L, G1, G2, and N are indicated; these lanes were overexposed to visualize the L protein.

transiently expressed in CV-1 cells (see chapter 4). This truncated L protein only contained amino acids 1-1271 of the L protein. It was immunoprecipitated by the anti-N L protein antiserum but not by the anti-C L protein antiserum (Fig.3.7), confirming the specificity of the two L protein antisera.

3.5. Discussion

Hitherto, specific antisera to the Bunyamwera virus L protein were unavailable. The major reason was the lack of suitable amounts of antigen because there is very little L protein present within each virus particle. It has been estimated that there are 25 molecules of L protein compared to 2100 molecules of N protein in each virus particle (Obijeski *et al.*, 1976). Therefore, it is very difficult to get enough L protein antigen from the virus to elicit antibodies. Expression of the protein via a bacterial vector could overcome the problem, but it has not been possible to express the full-length Bunyamwera virus L protein in *E. coli* (see chapter 4). Thus, the bacterial expression vector series pUEX were used for expression of fusion proteins, with portions of the L protein fused to bacterial ß-galactosidase. ß-galactosidase confers insolubility on the hybrid protein and thus protects the protein against proteolysis and facilitates its purification (Stanley & Luzio, 1984). As expected however, fusion proteins are not always successful immunogens. Initially I expressed four fusion proteins (L1-L4), but only one antiserum, that raised against the C-terminal part of the L protein (anti-L4), was screened positive by immunoprecipitation. As it would be useful to obtain an antibody which specifically reacted with the N-terminus of the L protein for future work, I then went on to clone a bigger fragment removed from the 5' end of a cDNA subclone. The antiserum raised against this fusion protein (L5) recognized the virus L protein specifically as tested by immunoprecipitation analysis. Thus, two antisera which represent the anti-C and anti-N termini of the L protein have been characterized.

Western blotting and immunoprecipitation assays were used to test the antisera since these two methods use different forms of antigen. The fusion protein immunogens were made as the denatured form by electrophoresis on SDS-polyacrylamide gels. The antigen in a Western blotting assay is presented as the denatured form and the antibodies should recognize the protein more efficiently. Therefore, the antiserum against fusion protein L3 showed its reaction with the L protein by Western blotting (Fig.3.5), but failed to recognize the L protein by immunoprecipitation (Fig.3.6). The antisera against fusion protein L1 and L2 did not react with the L protein in either assay; whether this was

because of the relatively small size of the fragment fused to ß-galactosidase or because the regions were not antigenic is not known.

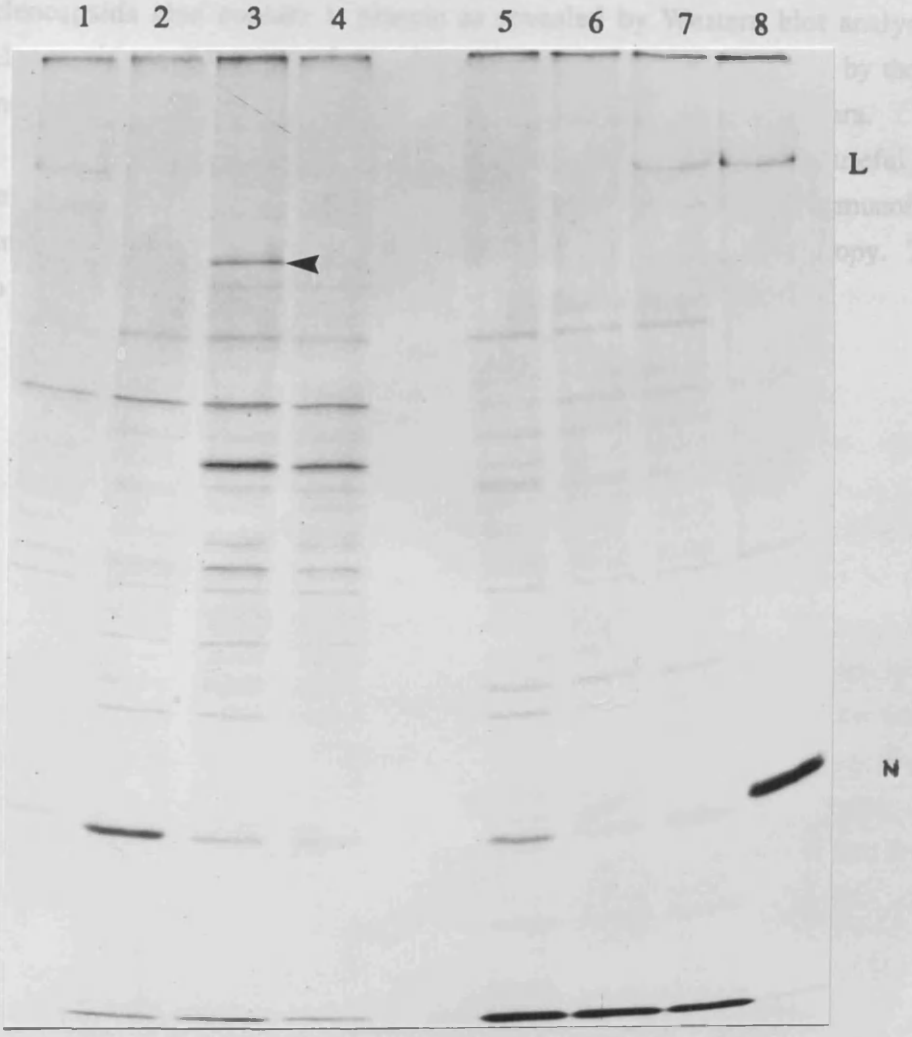


Fig.3.7. Reactivity of the anti-C and anti-N terminal L protein antisera with a C-terminal truncated L protein by immunoprecipitation. The N terminal half of the L protein which contained residues 1-1271 (lanes 3 and 6) and full-length L protein (lanes 4 and 7) were transiently expressed in CV-1 cells (see chapter 4) and the proteins were labelled with ^{35}S-methionine. The cytoplasmic extracts were immunoprecipitated with the anti-N (lanes 1-4) or anti-C (lanes 5-8) specific L protein antisera followed by electrophoresis on a 12% polyacrylamide-SDS gel. Immunoprecipitation of Bunyamwera virus infected cytoplasmic extracts (Lanes 1 and 8) and cell extracts without transient expression (lanes 2 and 5) by either sera are also shown. The N terminal half of the L protien was only precipitated by the anti-N terminus of L protein antiserum but not by the anti-C terminus of L protein antiserum.

The specific reaction of the antisera with the Bunyamwera virus L protein provides further evidence that the L RNA genome segment encodes the L protein, which had previously been determined by genome reassortment experiments (Endres *et al.*, 1989; Elliott, 1989a). This work also demonstrated that in addition to the N protein, nucleocapsids also contain L protein as revealed by Western blot analysis of the nucleocapsids purified from the Bunyamwera virus infected cells, and by the fact that some N protein was also precipitated by the monospecific L protein antisera.

The monospecific antisera raised against the L protein should be useful probes in determining the localization of the L protein in infected cells, e.g. by immunofluorecent staining, and in virus nucleocapsids, e.g. by immune electron microscopy. They will also be helpful for the functional analysis of the L protein.

Chapter 4

Expression of the Bunyamwera virus L protein

4.1. Introduction

The L protein of Bunyamwera virus is presumed to be the virion associated transcriptase or RNA polymerase, but direct proof of this is lacking. Little is known about its role in viral RNA synthesis and it is considered that the L protein is multifunctional. Expressing the L protein from cloned cDNA would be the first important step towards defining the functional domains within the bunyavirus L protein.

Over the past decade several systems for heterogeneous gene expression have been established. Each system has its own advantages and disadvantages over the others. To obtain biologically functional L protein, it was considered prudent to evaluate more than one expression system. Three systems were tried to express the L protein: *in vitro* translation of *in vitro* synthesized mRNA; the bacterial T7 expression system described by Studier *et al.* (1990); and the construction of recombinant vaccinia viruses.

4.2. Construction of a full-length cDNA to the Bunyamwera virus L gene.

An intact copy of the Bunyamwera virus L gene coding region was constructed from existing cDNA clones (Elliott, 1989a) and synthetic oligonucleotides. The major part of the gene was built from the unique Hind III site just downstream of the ATG initiation codon for the L protein by using three subgenomic cDNA clones (Fig.4.1A). A 1.67 kb Hind III-Sph I fragment isolated from pBUN357 and a 2.07 kb Sph I-Pst I fragment isolated from pBUN367 were ligated together with Hind III- and Pst I-doubly digested pUC18 DNA. The resulting plasmid, pUC18 357/367, was digested with Pst I, treated with alkaline phosphatase, and then ligated to a 3.06 kb Pst I fragment isolated

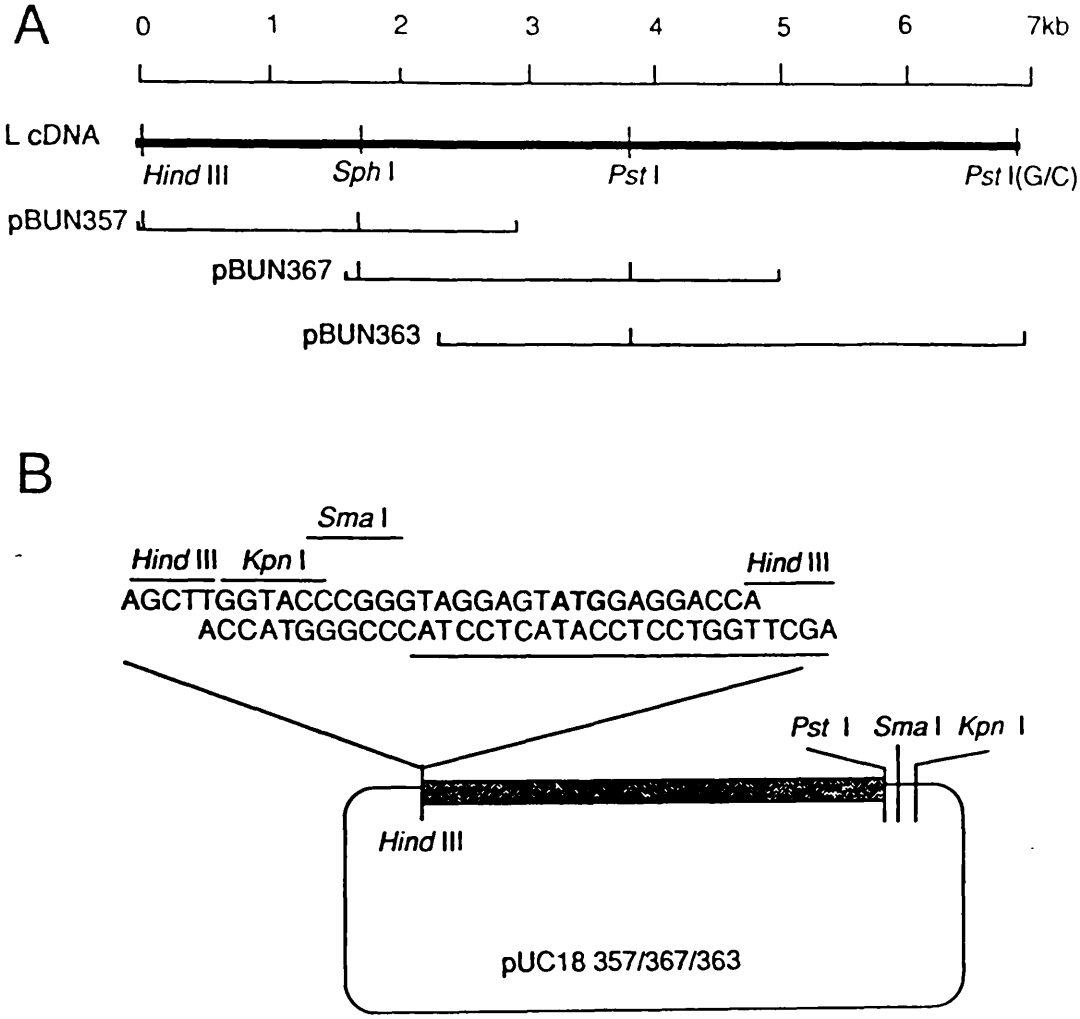


Fig.4.1. Construction of a full-length Bunyamwera virus L gene cDNA. **A.** Relationship of the three subgenomic cDNA clones and relevant restriction enzyme sites used in the construction. Pst I(G/C) indicates a Pst I site created by GC tailing during the initial cDNA cloning of the L RNA. The major part of the L gene coding region comprised the Hind III-Sph I fragment of pBUN357, the Sph I-Pst I fragment of pBUN367, and the Pst I-Pst I fragment of pBUN363. **B.** Insertion of complementary synthetic oligonucleotides, containing Hind III, Kpn I, and Sma I restriction enzyme sites and nucleotides 43 to 65 of the L gene cDNA (underlined), including the ATG translational start codon, to complete the L gene coding sequence. The shaded box represents the L gene cDNA (not to scale), and relevant restriction sites at the 3' end of the insert are indicated.

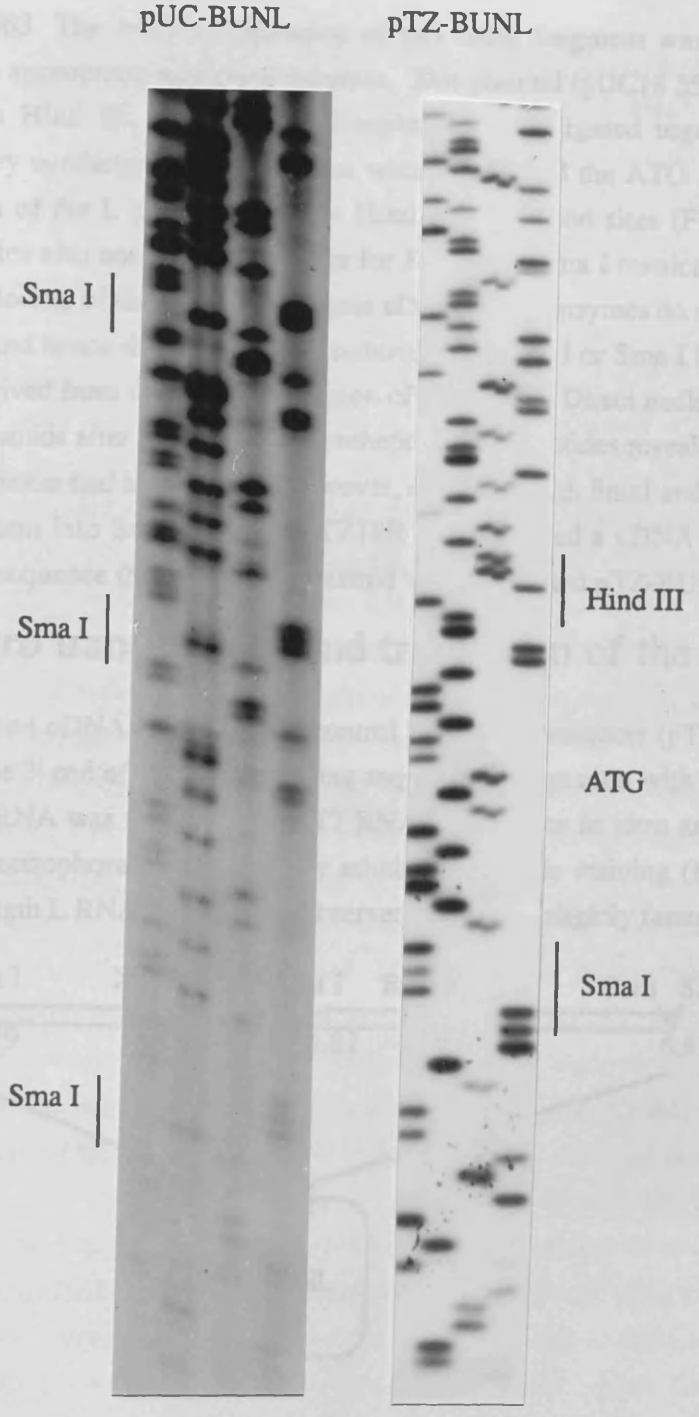


Fig.4.2. Dideoxy chain termination sequencing gels of alkali-denatured DNA derived from pUC-BUNL and pTZ-BUNL, showing multiple copies of linkers were introduced in pUC-BUNL and the correct sequence after subcloning Sma I digested insert into pTZ18R to give pTZ-BUNL. The order of the lanes is G, A, T, and C. Positions of Sma I and Hind III restriction enzyme sites and the ATG translational start of the L protein are indicated.

from pBUN363. The correct orientation of this latter fragment was confirmed by digestion with appropriate restriction enzymes. This plasmid (pUC18 357/367/363) was digested with Hind III, treated with phosphatase, and ligated together with two complementary synthetic oligonucleotides which contained the ATG start codon and missing bases of the L gene, flanked by Hind III restriction sites (Fig. 4.1B). The oligonucleotides also contained sequences for Kpn I and Sma I restriction enzymes to facilitate subcloning of the full-length L gene cDNA: these enzymes do not cleave the L gene cDNA, and hence the gene may be removed as a Kpn I or Sma I fragment, the 3' sites being derived from the polylinker region of the vector. Direct nucleotide sequence analysis of plasmids after ligation of the synthetic oligonucleotides revealed that multiple copies of the linker had been cloned; however, digestion with SmaI and ligation of the 6.83 kb fragment into Sma I cleaved pTZ18R DNA yielded a cDNA containing the correct 5' end sequence (Fig.4.2). This plasmid was designated pTZ-BUNL.

4.3. *In vitro* transcription and translation of the L gene

The L gene cDNA cloned under control of the T7 promoter (pTZ-BUNL) was linearized at the 3' end of the L gene coding sequence by digestion with Sal I (Fig.4.3). Full length L RNA was transcribed by T7 RNA polymerase *in vitro* and analysed by agarose gel electrophoresis followed by ethidium bromide staining (Fig.4.4A). The 6.83 kb full-length L RNA (lane 2) was observed to migrate slightly faster than the 6.6

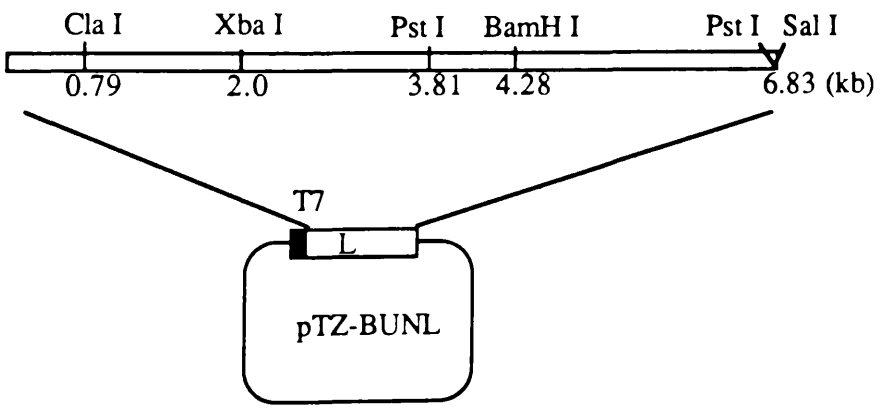


Fig.4.3. Positions of the restriction enzyme sites used to linearize the L gene cDNA cloned under control of the T7 promoter in PTZ-BUNL. The linearized pTZ-BUNL was transcribed *in vitro* to synthesize mRNA by T7 RNA polymerase for *in vitro* translation. The 3.81 kb RNA transcribed from a recombinant plasmid which has nucleotides 41-3810 of the L gene sequence cloned under control of the T7 promoter followed by a T7 terminator in pTF7-5 (see later).

kb DNA size marker which probably reflects the migration difference between DNA and RNA species. The 6.83 kb RNA band appeared rather smeared, which might have been due to degradation of the RNA or because some incomplete transcripts were made during the *in vitro* transcription. This RNA was then translated in a message dependent reticulocyte lysate (NEN). No full-length L protein was detected; a number of smaller polypeptides were seen below the position where L should migrate (Fig.4.4B. lane 3). Therefore, RNA transcripts truncated from the 3' end of the L were made to examine the possible translational attenuation of the L gene. Digestion of pTZ-BUNL with Cla I, Xba I and BamH I produced RNAs of 0.87 kb, 2.0 kb and 4.28 kb in length respectively (Fig.4.4A. lanes 7-5). The digestion of pTZ-BUNL with Xba I was not complete and a bigger RNA band was also seen (lane 6). Translation of these RNAs was performed and only the 0.87 kb RNA (Fig.4.4B. lane 4) synthesized a protein of the expected size (34.8K); the other two RNAs (Fig.4.4B. lanes 5 and 6) produced proteins shorter than the predicted species of 80K and 170K. These experiments indicated that most probably correct translation initiation of the L protein occurred, but no full-length L protein could be synthesized *in vitro*.

Since translational activity of reticulocyte lysates produced by different companies might be different, I then repeated the L gene *in vitro* translation experiment using the Amersham reticulocyte lysate. As can be seen from Fig.4.5, both the 0.87 kb (lane 1) and the 3.8 kb (lane 2) RNA transcripts were translated to give proteins which correlated with their estimated size (34.7K and 150K). These polypeptides were also immunoprecipitated by a mixture of the monospecific antisera against the C- and N-termini of the L protein (lanes 5 and 6). No L protein was clearly detected in the *in vitro* translation reaction which was supplemented with the 6.83 kb full-length L RNA (lane 3), but a protein of the same size as the authentic L was detected in an independent experiment (Fig.4.5. right panel). However, the level of this L sized protein was not high enough to be immunoprecipitated by the antisera against the N- or C-termini of the L protein. Many smaller polypeptides were made during the *in vitro* translation of the full length L gene and some of them were precipitated by the antiserum against the N-terminus of the L protein. The smaller proteins produced were considered to be premature termination products of the *in vitro* translation of the L messenger RNA or else possibly resulted from degradation of larger polypeptides. The experiment suggested that there was a strong translational attenuation during the *in vitro* translation of the L.

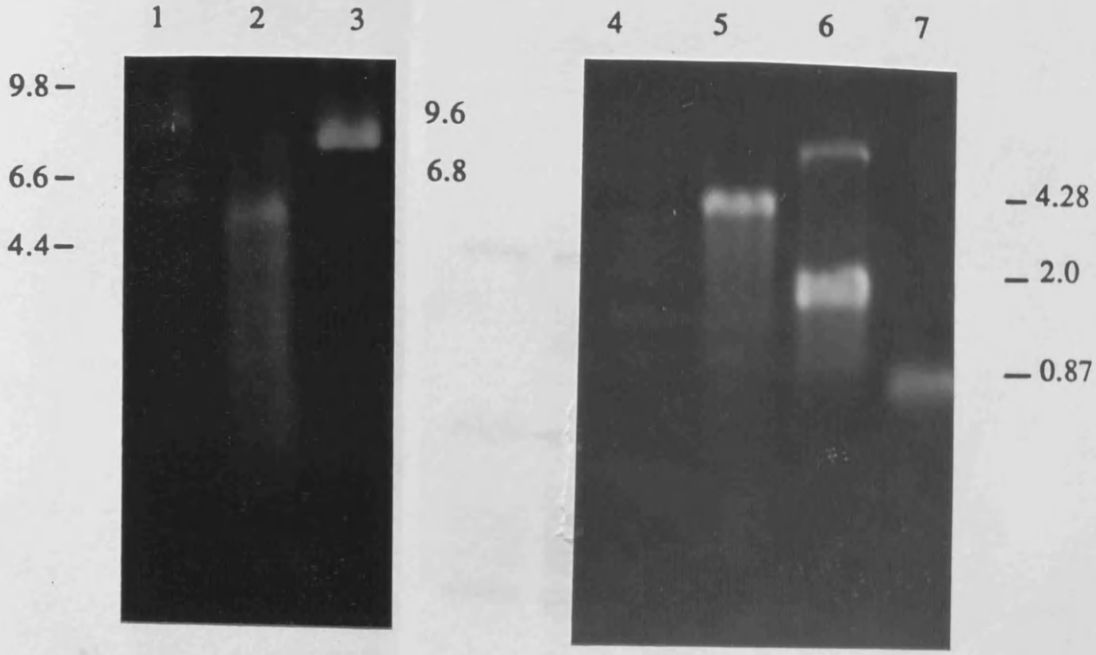


Fig.4.4A. Agarose gel analysis of the *in vitro* synthesized RNA. About 10% of the RNA synthesized *in vitro* by T7 RNA polymerase (see chapter 2) was fractionated on a 1% agarose gel containing 5 mM methylmercuric hydroxide and stained with ethidium bromide. The Sal I linearized 9.6 kb pTZ-BUNL DNA (lane 3) and the 6.83 kb full length L RNA (lane 2) were visualized in the gel. Lane 1 shows the DNA size markers which are designated on the side of the gel. Lane 4 shows 1kb ladder DNA size markers. The 4.28 kb RNA generated by transcription of pTZ-BUNL linearized with BamH I (lane 5), 2.0 kb RNA from Xba I linearized pTZ-BUNL (lane 6) [the bigger band was probably derived from uncut plasmid], 0.87 kb RNA transcribed from Cla I linearized pTZ-BUNL (lane 7) were clearly seen in the gel. It appeared that the bigger the RNA size, the more smearing of the RNA band occured, indicating some degradation of the RNA occurred or incomplete transcripts were produced.

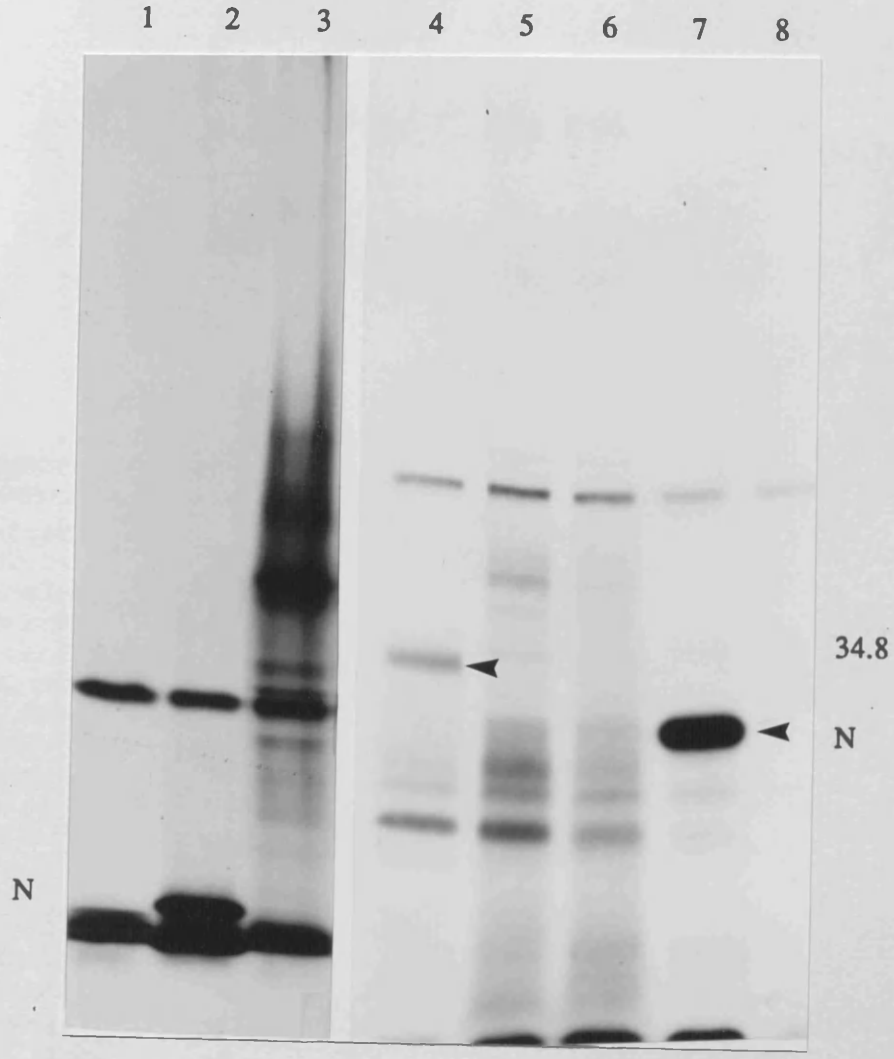
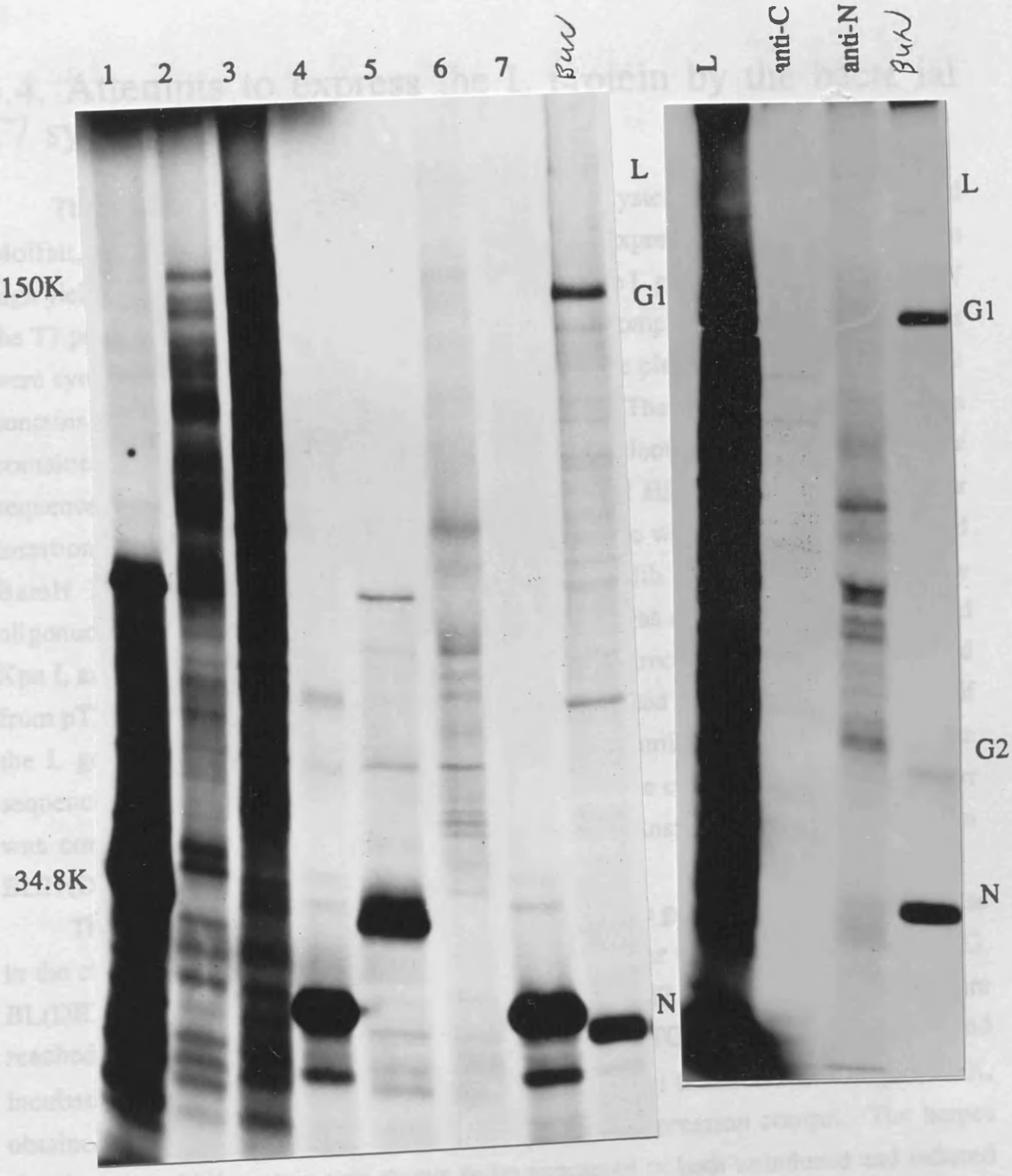


Fig.4.4B. *In vitro* translation of RNA transcripts in the NEN reticlulocyte lysate. The *in vitro* transcribed RNA was translated in the NEN reticulocyte lysate as described in chapter 2. The ^{35}S-methionine labelled proteins were analysed in a 10% polyacrylamide-SDS gel (left) or 12% polyacrylamide-SDS gel (right). The full-length 6.83 kb RNA (lane 3) did not produce a protein of 259K but some smaller polypeptides were observed. The 0.87 kb RNA produced a protein of the expected size which was about 34.8K (lane 4), whereas the 2.0 kb RNA (lane 5) and 4.28 kb RNA (lane 6) did not synthesize proteins of the expected size (80K and 170K). Lanes 2 and 7 were *in vitro* translation reactions supplemented with the Maguari S RNA *in vitro* transcribed from pTZ-MAGS (Elliott and McGregor, 1989), which was translated into the N protein as a system control. Lanes 1 and 8 show products of reactions which had no RNA added.

Fig.4.5. *In vitro* translation of the L gene in the Amersham reticulocyte lysate. *In vitro* tanslations performed according to the manufacturer's instructions as described in chapter 2 and two independent experiments are shown in this figure. The ^{35}S-methionine labelled *in vitro* synthesized proteins were assayed directly on the gel (lanes 1-4 and L), or after immunoprecipitation with a mixture of anti-N and anti-C L protein specific antisera (lanes 5-6) or with anti-Bunyamwera virus antiserum (lane 7) or with the serum indicated. The 0.87 kb RNA (lane 1) was translated into a protein which correlated with the estimated size (34.8K) and also a larger protein which was probably translated from RNA derived from partially digested DNA; both were precipitated by the L protein specific antisera. The 3.81 kb RNA (lane 2) synthesized a protein of the expected size (150 K) which was also precipitated by the mixed antisera against the L protein (lane 6). Lane 4 is Maguari S RNA control with the N protein synthesized and precipitated by the anti-Bunyamwera virus antiserun (lane 7). Lane BUN shows the Bunyamwera virus protein L, G1, and N markers. Note that the mobility of the Maguari virus N protein is slower than the Bunyamera virus N protein. Translation of the full-length L RNA did not show production of a full length L protein in lane 3 of the left gel, but it was observed in the right gel that a protein of the same size as the L protein produced in Bunyamwera virus infected cells was detected, although it was not shown to be immunoprecipitated by the anti-C or anti-N L protein antiserum.


1
2
3
4
5
6
7
Bun
150K
34.8K
L
G1
N
L
anti-C
anti-N
Bun
L
G1
G2
N

4.4. Attempts to express the L protein by the bacterial T7 system.

The bacterial T7 RNA polymerase expression system in *E. coli* (Studier and Moffatt, 1986; Studier *et al.*, 1990) has been used to express a number of proteins in high yields. To construct a recombinant plasmid with the L gene cDNA under control of the T7 promoter suitable for expression in *E. coli*, two complementary oligonucleotides were synthesized for inserting the L gene cDNA into the plasmid vector pET8c, which contains the T7 promoter followed by a T7 terminator. The synthetic oligonucleotides contained the ATG start codon of the L protein, nucleotides 43-65 of the L gene sequence between the ATG and the Hind III site, and Hind III and Kpn I sites for insertion of the L gene cDNA (Fig.4.6). Plasmid pET8c was digested with Nco I and BamH I, treated with phosphatase and ligated with the two complementary oligonucleotides. The resulting plasmid pET8c(H/K) was digested with Hind III and Kpn I, and ligated with the Hind III and Kpn I cDNA fragment which had been removed from pTZ-BUNL; the recombinant plasmid was designated pET-BUNL. The 5' end of the L gene sequence inserted into the vector was examined by dideoxynucleotide sequencing of the alkaline denatured pET-BUNL and the correct ligation of the insert was confirmed (Fig.4.6B). pET-BUNL was then transformed into *E. coli* strain BL21(DE3) for analysis of expression.

The lysogen BL(DE3) contains a single copy of the gene for T7 RNA polymerase in the chromosome under control of the lacUV5 promoter which is inducible by IPTG. BL(DE3) transformed with pET-BUNL was grown at 37 °C for 5 hr until the culture reached an OD_{600} of 0.6-1. To induce expression, IPTG was added to 0.4 mM and incubation was continued for 2 hr at 37 °C. BL21(DE3) transformed with pET-65K, obtained from Dr. C. Preston, was used as positive expression control. The herpes simplex virus 65K protein was shown to be expressed in both uninduced and induced (Fig.4.7a), indicating that some T7 RNA polymerase was synthesized in uninduced cultures. No L protein was synthesized in BL21(DE3) cells transformed with pET-BUNL (b). As it was possible that the basal level of T7 RNA polymerase in BL21(DE3) cells may have induced L protein expression which might have been toxic to the cells, *E. coli* strains BL21(DE3)pLysS and BL21(DE3)pLysE were transformed with pET-BUNL and then used for the L expression. BL21(DE3)pLysE carries a plasmid containing the lysozyme gene cloned under the tet promoter and BL21(DE3) pLysS has the lysozyme gene cloned in the plasmid in the opposite orientation and accumulates

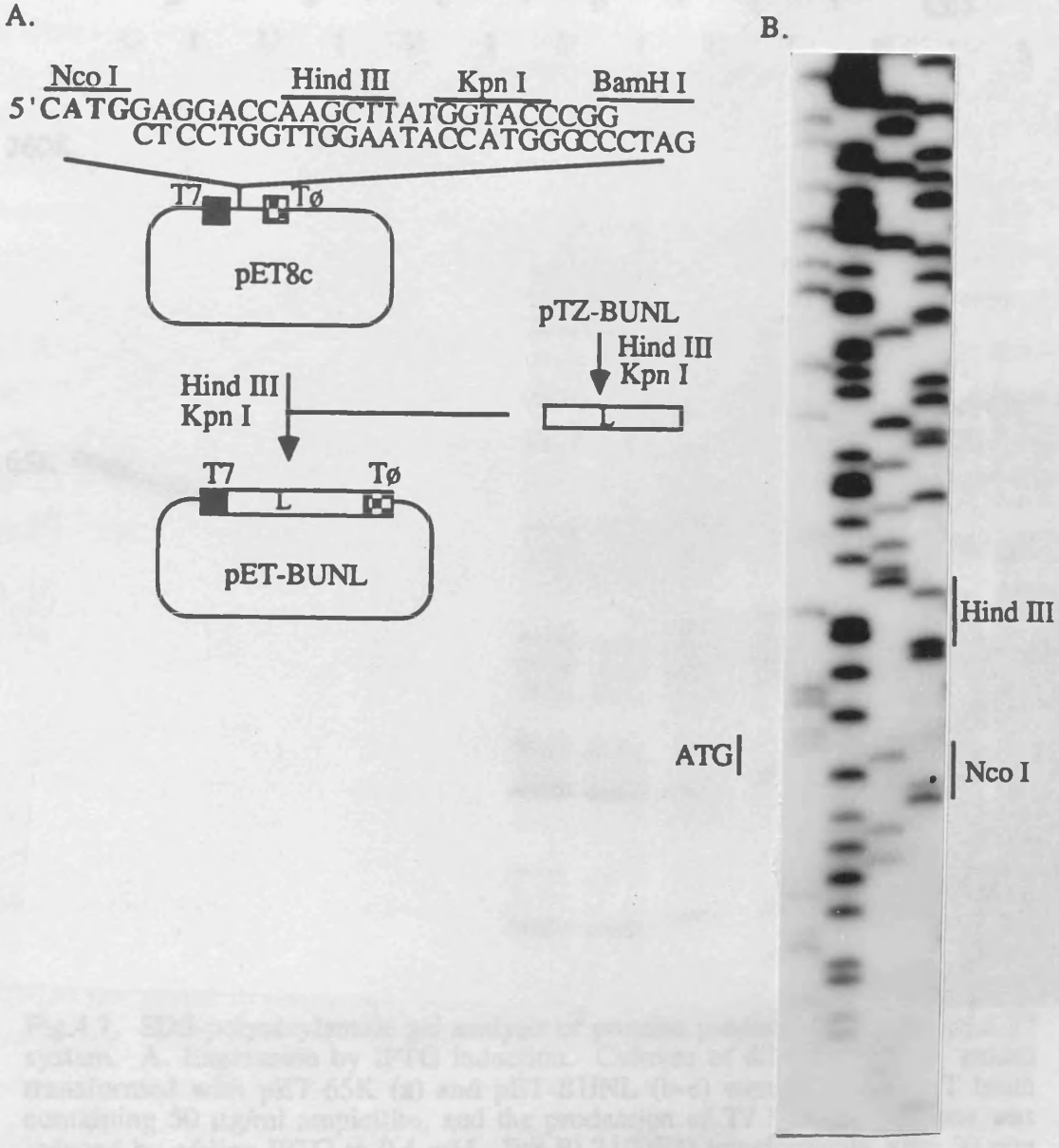


Fig.4.6. Construction of the recombinant plasmid for expression of the L protein in the bacterial T7 system. **A.** Insertion of a complementary synthetic oligonucleotide linker between the Nco I and BamH I sites of the vector pET8c. The linker contained the ATG translational start codon of the L protein in the Nco I site, nucleotides 43-65 of the L gene cDNA, and Hind III and Kpn I sites for insertion of the L gene fragment. The resulting vector pET8c(H/K) was digested with Hind III and Kpn I and ligated with the L gene cDNA (not to scale) which was removed from pTZ-BUNL by Hind III and Kpn I double digestion, and the recombinant plasmid was designated pET-BUNL. The restriction enzyme sites created in the linker and the ATG translational start site are indicated. **B.** Dideoxy chain termination sequencing gel of pET-BUNL, showing the correct sequence inserted by the L cDNA. The orders of the lanes is G, A, T, and C. The positions of the ATG start codon and Hind III site are indicated.

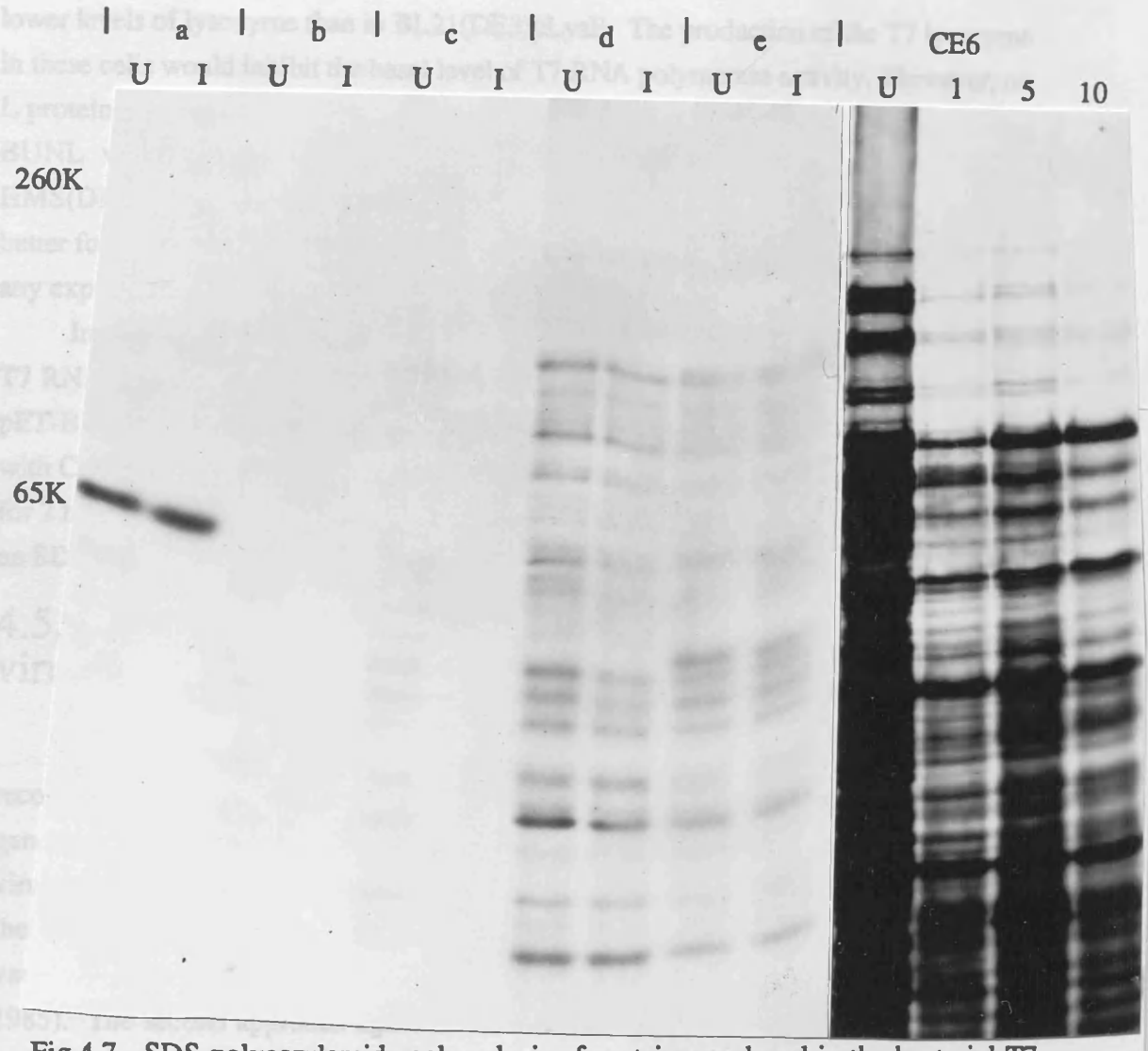


Fig.4.7. SDS-polyacrylamide gel analysis of proteins produced in the bacterial T7 system. A. Expression by IPTG induction. Cultures of different *E. coli* strains transformed with pET-65K (a) and pET-BUNL (b-e) were grown in YT broth containing 50 µg/ml ampicillin, and the production of T7 RNA polymerase was induced by adding IPTG to 0.4 mM. For BL21(DE3) transformants, after 30 min induction rifampicin was added to the cultures at 200 µg/ml for 10 min to inhibit host protein synthesis. Bacterial cells were collected by centrifugation and an amount equal to 100 µl of culture were radiolabelled with ^{35}S-methionine (20 µCi/ml) in PBS for 60 min in the presence of IPTG. The labelled proteins were analysed by gel electrophoresis. The cultures of *E. coli* were BL21(DE3) (a and b), HMS174(DE3) (c), HMS174(DE3)pLysS (d) or HMS174(DE3)pLysE (e) and were uninduced (U) or induced (I) by IPTG. The 65K protein was produced in both uninduced and induced *E. coli* cells containing pET-65K (a), but no L protein was observed in cells containing pET-BUNL (b-e).

B. CE6 phage directed expression. Cultures of HMS174 carrying pET-BUNL were grown in M9 medium containing 50 µg/ml ampicillin and infected with bacteriophage CE6 at moi of 1, 5, or 10 pfu/cell. Immediately after 2 hr of infection, 100 µl of the cultures were radiolabelled with ^{35}S-methionine (20 µCi/ml) for 20 min at 37 °C. The cells were collected by centrifugation and the proteins subjected to electrophoresis in a 10% polyacrylamide-SDS gel. No L protein was detected.

lower levels of lysozyme than in BL21(DE3)pLysE. The production of the T7 lysozyme in these cells would inhibit the basal level of T7 RNA polymerase activity. However, no L protein was detected in these cells transformed with pET-BUNL. Meanwhile, pET-BUNL was also transformed into *E. coli* K12 strains HMS174, HMS(DE3), HMS(DE3)pLysS and HMS(DE3)pLysE to examine whether these strains would be better for L expression. Again, none of these cells established with pET-BUNL showed any expression of the L protein (Fig.4.7c-e).

In further experiments, attempts were made to express the L protein by providing T7 RNA polymerase through CE6 phage infection of HMS174 cells transformed with pET-BUNL. The cells were grown at 37 °C overnight in M9 medium and then infected with CE6 phage at moi of 1, 5, or 10 pfu/cell, followed by continued incubation at 37 °C for 2 hr. The infected cells were labelled with ^{35}S-methionine and proteins separated on an SDS-polyacrylamide gel (Fig.4.7). However, no L protein was detected in the gel.

4.5. Expression of the L protein by recombinant vaccinia viruses

Two approaches were used to express the Bunyamwera virus L protein by recombinant vaccinia viruses. The first employed the classical method in which the L gene was inserted in lieu of the vaccinia virus TK gene, under control of the vaccinia virus P7.5 promoter, by using the plasmid transfer vector pSC11; this vector includes the *E. coli* ß-galactosidase gene under control of the P11 promoter so that recombinant vaccinia viruses produce blue plaques when stained with X-Gal (Chakrabarti *et al.*, 1985). The second approach again used the TK locus as the site for recombination of the L gene, but in this case the L gene was under control of the bacteriophage T7 promoter and terminator by using the transfer vector pTF7-5 (Fuerst *et al.*, 1987). Expression of the Bunyamwera virus gene should occur only when T7 RNA polymerase, supplied by a second, coinfecting recombinant vaccinia virus, vTF7-3, was present in the cell (Fuerst *et al.*, 1987; 1986).

4.5.1. Construction and characterization of recombinant vaccinia virus vSC11-BUNL

The Bunyamwera virus L gene was removed from pTZ-BUNL by digestion with Sma I and cloned into Sma I site in pSC11 (Fig.4.8). The correct orientation of the insert was ascertained by restriction enzyme digestion and the recombinant plasmid was designated pSC11-BUNL. pSC11-BUNL was then recombined by standard procedures

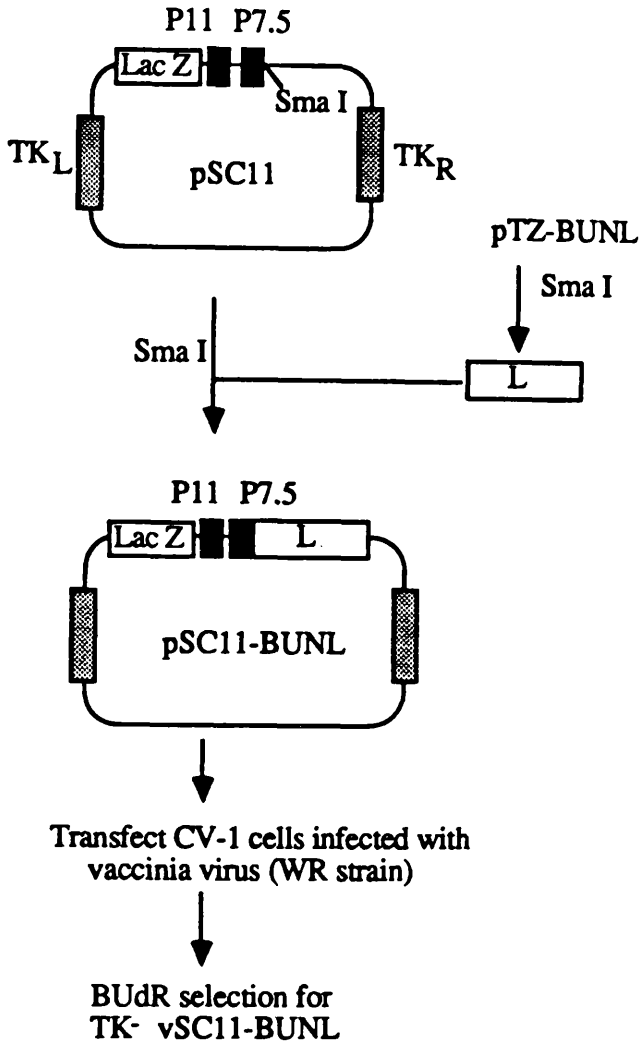


Fig.4.8. Construction of recombinant plasmid pSC11-BUNL for making recombinant vaccinia virus vSC11-BUNL. A 6.83 kb Sma I fragment (not to scale) containing the entire L gene coding sequence was removed from pTZ-BUNL and inserted into the unique Sma I site of the vaccinia virus transfer vector pSC11. The L gene is under control of the vaccinia virus P7.5 promoter and the chimaeric gene is flanked by the left (TK_L) and right (TK_R) vaccinia virus TK gene sequences. Plasmid DNA was transfected into CV-1 cells infected with wild type vaccinia virus (WR strain) and TK^- recombinants were selected by plaque isolation in TK^- cells in the presence of BUdR.

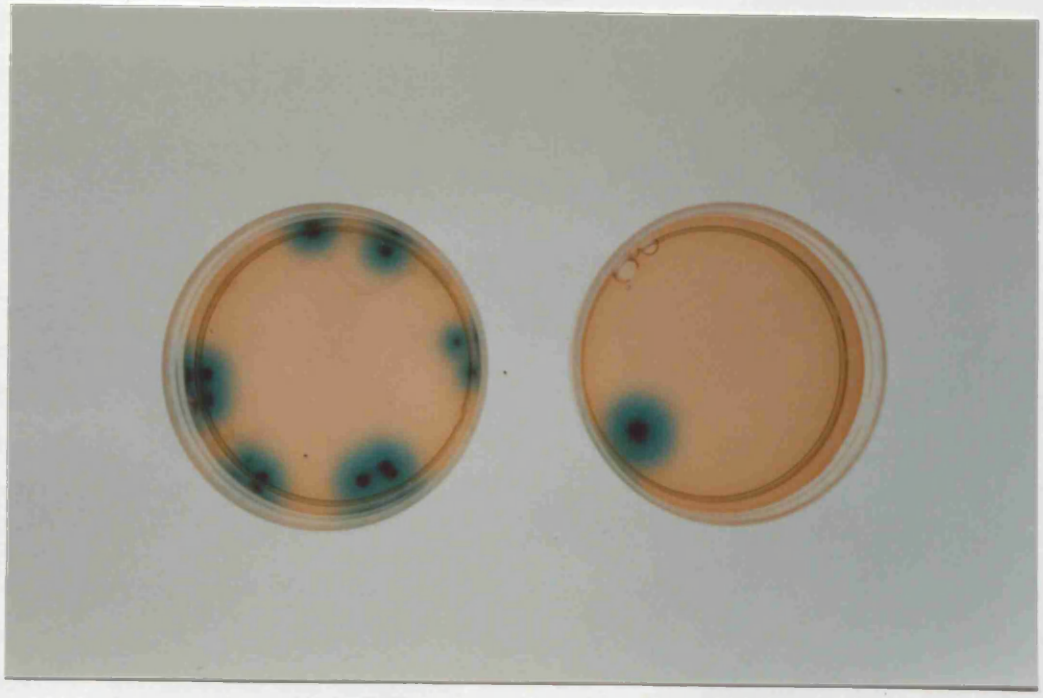


Fig.4.9. Plaque selection of TK$^-$ recombinant vaccinia virus vSC11-BUNL, showing the blue plaques produced in TK$^-$ cells after staining with X-Gal.

(see chapter 2) into the TK locus of vaccinia virus WR strain, and TK$^-$ viruses were selected by plaque assay on TK$^-$ 143 cells in the presence of 5-bromodeoxyuridine (BUdR). TK$^-$ blue plaques were picked after addition of X-Gal to the overlay (Fig.4.9). The TK$^-$ plaques were grown up in TK$^-$ 143 cells and the cells were lysed and total cellular DNA dot blotted onto a nitrocellulose filter. Hybridization with an L gene cDNA probe revealed a possible signal with DNA from a blue plaque (Fig.4.10A. lane 1), but not in a colourless plaque (lane 2). The blue plaque was then further plaque-to-plaque purified, and finally confirmed by analysis of the restriction enzyme profile of vaccinia virus DNA. Total cellular DNA extracted from the vaccinia virus infected cells (Fig.4.10B. lanes 1 and 3) and plasmid pSC11-BUNL DNA (lanes 2 and 4) was digested with BamH I or EcoR I, fractionated on an agarose gel, transferred to a nitrocellulose filter, and hybridized to ^{32}P-labelled L gene cDNA. The 2.55 kb and 4.28 kb BamH I L cDNA restriction fragments were detected for both vSC11-BUNL and pSC11-BUNL DNA (lanes 1-2); the 3.7 kb, 1.5 kb and 0.9 kb EcoR I fragments were present in each pair of DNAs (lanes 3-4), indicating that the L gene cDNA was recombined into the vaccinia virus. The recombinant virus obtained was then designated vSC11-BUNL.

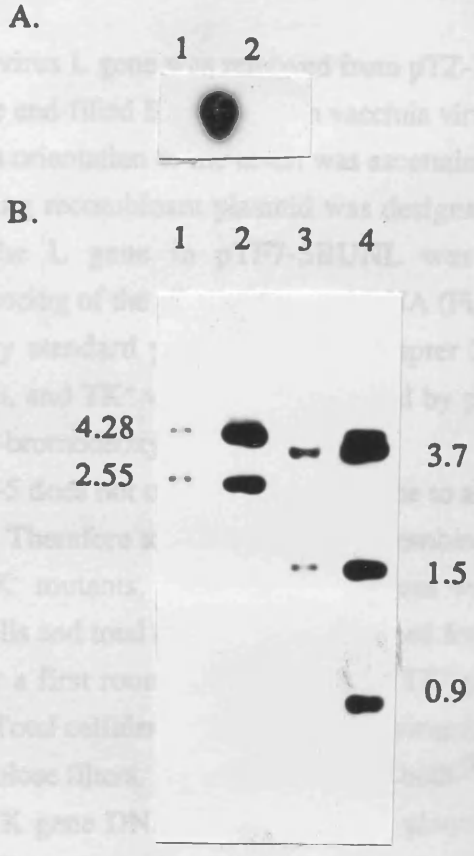


Fig.4.10. Characterization of recombinant vaccinia virus vSC11-BUNL. **A.** DNA dot blot analysis. A TK$^-$ blue plaque (lane 1) and a colourless TK$^-$ plaque (lane 2) were used to infect small monolayer cultures of TK$^-$ cells and after 2 days the cells were lysed and total cellular DNAs prepared. The DNA was dot blotted onto a nitrocellulose filter and hybridized with ^{32}P-labelled pTZ-BUNL DNA. An L gene signal was detected in the DNA from the blue plaque infected cells (lane 1), but not in the colourless plaque infected cells (lane 2). **B.** Southern blot analysis. DNA was extracted from the recombinant vaccinia virus (vSC11-BUNL) infected cells and digested with either BamH I (lane 1) or EcoR I (lane 3), transferred to nitrocellulose filter, and hybridized with ^{32}P-labelled pTZ-BUNL DNA. Plasmid pSC11-BUNL DNA was also digested with either BamH I (lane 2) or EcoR I (lane 4). The L cDNA 4.28 and 2.55 kb BamH I fragment was detected in both vSC11-BUNL infected cellular DNA and plasmid pSC11-BUNL DNA (lanes 1 and 2). The L cDNA 3.7 kb, 1.5 kb and 0.9 kb EcoR I fragments were also present for both DNAs (lanes 3 and 4).

The 0.9 kb EcoR I fragment was visible in the original film.

4.5.2. Construction and characterization of recombinant vaccinia virus vTF7-5BUNL

The Bunyamwera virus L gene was removed from pTZ-BUNL by digestion with Sma I and cloned into the end-filled BamH I site in vaccinia virus transfer vector pTF7-5 (Fig.4.11A). The correct orientation of the insert was ascertained by restriction enzyme digestion and the resulting recombinant plasmid was designated pTF7-5BUNL. The correct insertion of the L gene in pTF7-5BUNL was further examined by dideoxynucleotide sequencing of the alkali denatured DNA (Fig.4.11B). pTF7-5BUNL was then recombined by standard procedures (see chapter 2) into the TK locus of vaccinia virus WR strain, and TK^- viruses were selected by plaque assay on TK^- 143 cells in the presence of 5-bromodeoxyuridine.

The plasmid pTF7-5 does not contain the LacZ gene to allow for colour selection of recombinant plaques. Therefore to screen for true recombinant TK^- vaccinia viruses against spontaneous TK^- mutants, about 80 TK^- plaques were picked. The plaques were grown up in TK^- cells and total cellular DNA screened for the L gene signal by dot bot hybridization. After a first round of screening, 10 TK^- plaques were picked and replicated in TK^- cells. Total cellular DNA from the 10 virus infected monolayers were dot blotted onto nitrocellulose filters. Hybridization with both ^{32}P-labelled L gene DNA and the vaccinia virus TK gene DNA revealed that six plaques contained the L gene sequence (Fig.4.12A). Thus, these six plaques were used to infect TK^- cells and the presence of the L gene was confirmed by another round of dot blot analysis (Fig.4.12B). Finally, these vaccinia virus DNAs were characterized by Southern blot analysis. DNAs extracted from vaccinia virus infected cells were digested with Pst I and fractionated in an agarose gel (Fig.4.12C). Hybridization of the DNAs with ^{32}P-labelled L gene cDNA showed that all of the six viruses contained an L gene cDNA 3 kb Pst I fragment, indicating that the L gene was recombined into the vaccinia virus. The resulting recombinant vaccinia virus was designated vTF7-5BUNL.

Further characterization of recombinant vaccinia virus vTF7-5BUNL by Southern blot analysis is shown in Fig.4.13. pTF7-5BUNL (lanes 3 and 6), vTF7-5BUNL (lanes 1 and 4), and vaccinia virus WR strain (lanes 2 and 5) DNAs were analysed using EcoR V and EcoR I digestion followed by Southern blotting. It was shown that no hybridization occurred with the WR strain DNA, whereas the pattern of EcoR V bands (3.0 kb and a bigger DNA) for vTF7-5BUNL and pTF7-5BUNL was the same; and the pattern of EcoR I bands was also consistent for the expected structure of a recombinant vaccinia virus vTF7-5BUNL.

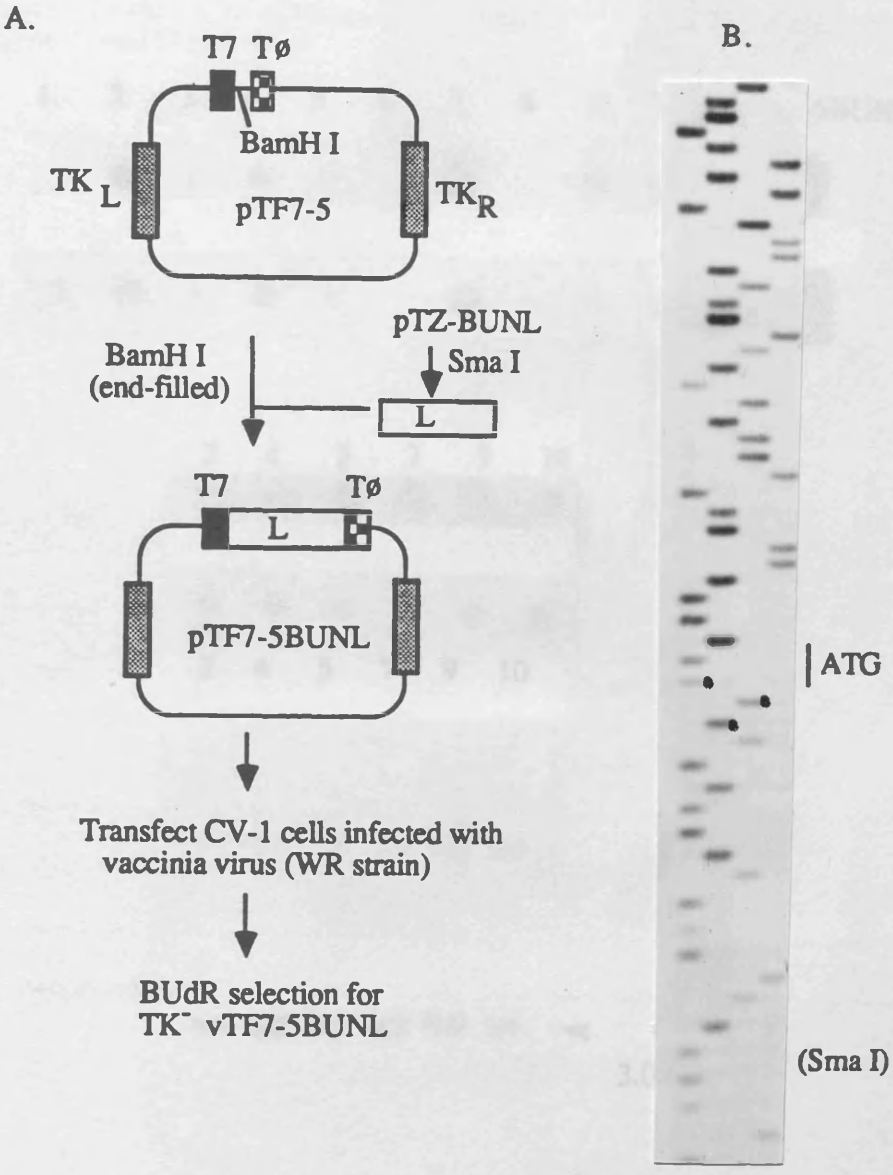


Fig.4.11. Construction of recombinant plasmid pTF7-5BUNL for making recombinant vaccinia virus vTF7-5BUNL. **A.** Insertion of the L gene cDNA into vaccinia virus transfer vector pTF7-5. A 6.83 kb Sma I fragment containing the entire L gene coding sequence (not to scale) was inserted into the end-filled BamH I site of PTF7-5. The recombinant plasmid pTF7-5BUNL has the L gene under control of the bacteriophage T7 promoter and T7 terminator. The chimaeric gene is also flanked by vaccinia virus TK gene sequences. The plasmid was transfected into CV-1 cells which had been infected with wild type vaccinia virus (WR strain). TK^- recombinants (vTF7-5BUNL) were selected by plaque isolation in TK^- cells in the presence of BUdR. **B.** Dideoxynucleotide chain termination sequencing gel, showing the correct 5' end sequence of the L gene inserted into the filled BamH I site in pTF7-5. The insertion site and the ATG start codon of the L protein are indicated.

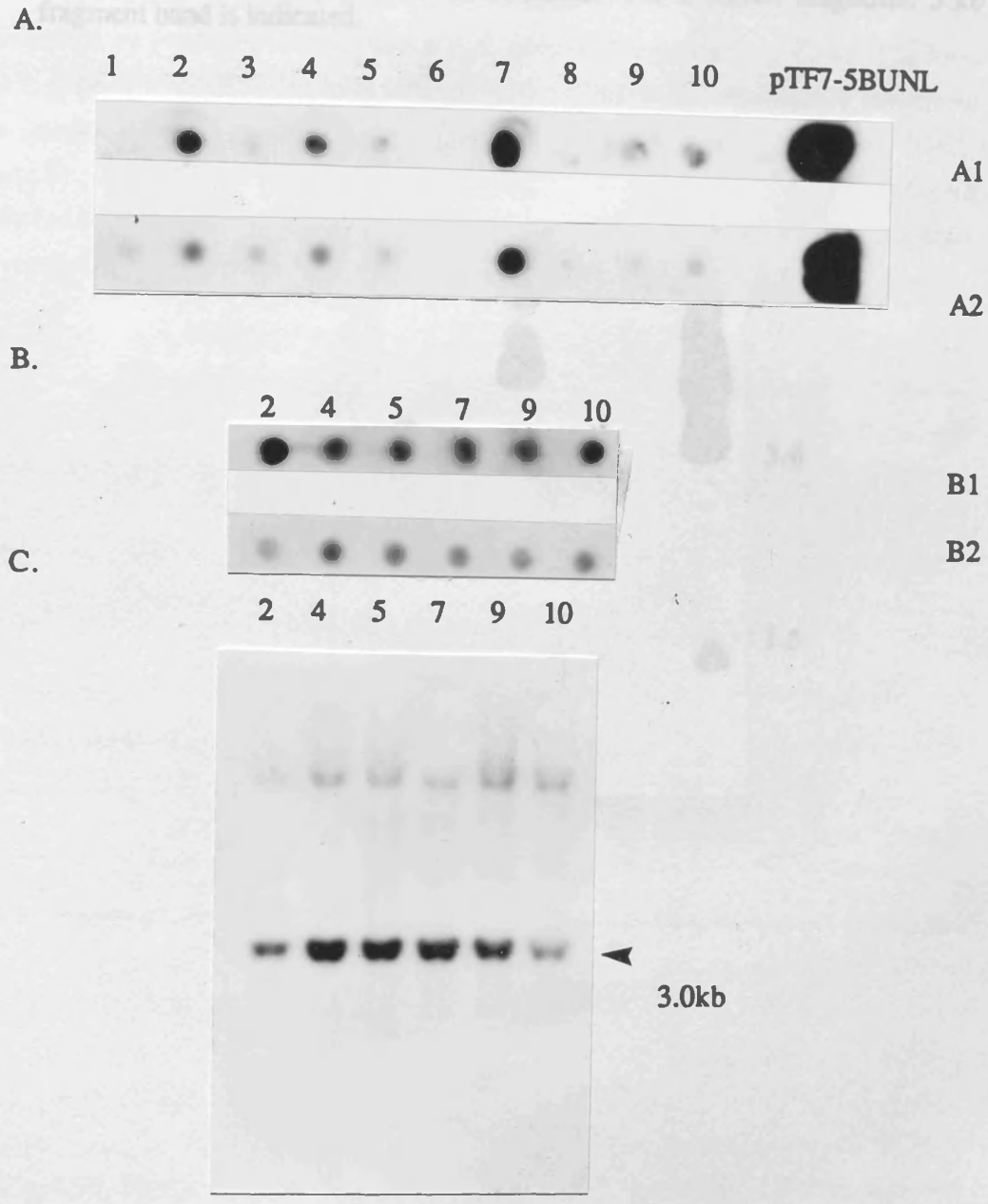


Fig.4.12. Screening of recombinant vaccinia virus vTF7-5BUNL. **A.** and **B.** DNA dot blot analysis. Total cellular DNA from small monolayer cultures infected with TK^- vaccinia virus plaques (1-10) were dot blotted onto nitrocellulose filters and the DNA probed with ^{32}P-labelled pTZ-BUNL DNA (A1) or ^{32}P-labelled pTF7-5 DNA for detection of the TK gene (A2). Six plaques which showed strong signals on both blots (2, 4, 5, 7, 9, and 10) were amplified in TK^- cells and the DNA dot blotted onto nitrocellulose filters for another round of screening. The blots were either hybridized with ^{32}P-labelled pTZ-BUNL (B1) or ^{32}P-labelled pTF7-5 (B2). The L gene was shown to be recombined into the vaccinia viruses. **C.** Southern blot analysis. DNAs were extracted from the six recombinant vaccinia virus infected cells and digested with Pst I. After fractionation on a 1% agarose gel, the DNAs were transferred to a nitrocellulose filter, and hybridized with ^{32}P-labelled pTZ-BUNL DNA. All the

viruses had the same pattern of DNA bands. The L cDNA diagnostic 3 kb Pst I fragment band is indicated.

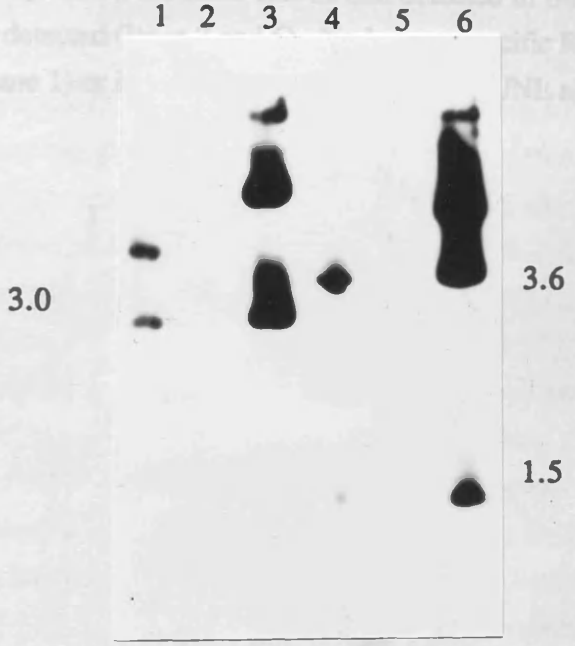


Fig.4.13. Characterization of recombinant vaccinia virus vTF7-5BUNL by Southern blot analysis. DNA from cells infected with vTF7-5BUNL (lanes 1 and 4) or from the transfer vector pTF7-5BUNL (lanes 3 and 6) was digested with either EcoR V (lanes 1 to 3) or EcoR I (lanes 4-6), fractionated on an agarose gel and transferred to nitrocellulose. Hybridization with ^{32}P-labelled pTZ-BUNL DNA indicated the expected genetic structure for vTF7-5BUNL; no hybridization occurred with the vaccinia virus parental DNA (lanes 2 and 5). The size of the L gene cDNA restriction fragments is designated on the side of the gel.

Analysis of RNA synthesized in recombinant vaccinia virus-infected cells was performed by Northern blotting using an L gene cDNA probe (Fig.4.14). The level of the L gene-specific RNA in cells infected with vSC11-BUNL was barely detectable by the standard procedure (lane 5). In cells dually infected with vTF7-3 and vTF7-5BUNL (lane 3) , an RNA transcript about the same size as that detected in Bunyamwera virus-infected cells was easily detected (lanes 2 and 6). No L gene-specific RNA was detected in mock-infected cells (lane 1) or in cells infected with vTF7-5BUNL alone (lane 4).

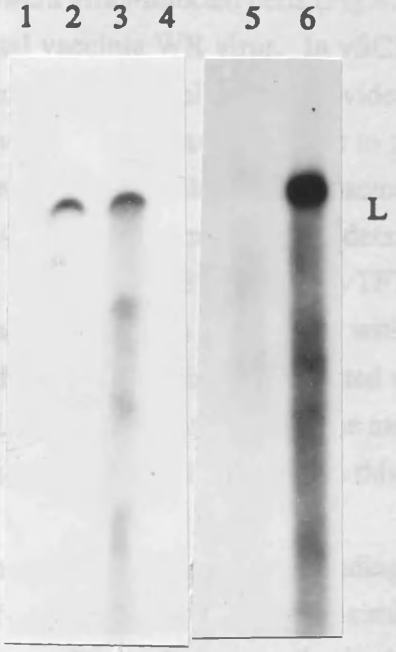


Fig.4.14. Northern blot analysis of vSC11-BUNL and vTF7-5BUNL infected cell RNA. Total cellular RNA was extracted from mock-infected cells (lane 1), or from cells infected with Bunyamwera virus (lanes 2 and 6), vTF7-5BUNL and vTF7-3 (lane 3), vTF7-5BUNL alone (lane 4), or vSC11-BUNL (lane 5), and fractionated on a 1% agarose gel containing methyl mercuric hydroxide. After transfer to nitrocellulose filters, hybridization was performed with ^{32}P-labelled pTZ-BUNL DNA. An L segment specific RNA, similar in size to that observed in the Bunyamwera virus infected cells (lane 2), was detected in the cells infected with vTF7-5BUNL and vTF7-3 (lane 3). No hybridization was observed in mock-infected (lane 1) or vTF7-5BUNL singly infected cells (lane 4). Barely detectable L RNA was observed in vSC11-BUNL infected cells (lane 5).

4.5.3. Analyses of proteins synthesized by recombinant vaccinia viruses.

To investigate the proteins made by the recombinant vaccinia viruses, CV-1 cells were infected with the various recombinants at moi of 5 pfu/cell and pulse-labelled at 22 to 24 hr postinfection with ^{35}S-methionine; the cell lysates were fractionated on SDS-polyacrylamide gels (Figs.4.15 and 4.16). The cells infected with vSC11-BUNL produced a high molecular weight protein of the same electrophoretic mobility as the L protein identified in Bunyamwera virus-infected cells (Fig.4.15), which was not seen in cells infected with the parental vaccinia WR virus. In vSC11-BUNL infected cells, a band corresponding to ß-galactosidase was also clearly evident and comigrated with the G1 protein of Bunyamwera virus. Other bands specific to the vSC11-BUNL infected lysate were seen migrating between the positions of ß-galactosidase and the L protein, of which one (labelled X in Fig.4.15) is considered in more detail later.

Cells dually infected with vTF7-5BUNL and vTF7-3 (expressing T7 RNA polymerase) also contained a protein which comigrated with the Bunyamwera virus L protein (Fig.4.16). This band was not seen in cells infected with either virus singly. In cells infected with vTF7-5BUNL only, a band having the same mobility as the X band seen in vSC11-BUNL infected cells was observed, but this band was not seen in the dually infected cells.

Thus, both systems synthesized a protein corresponding in size to the L protein; in the T7 system, the amount of the expressed protein was similar to that in Bunyamwera virus-infected cells, whereas more L protein was synthesized by vSC11-BUNL.

Expression of the L protein by the T7 system was further studied by varying the multiplicity of infection of both vTF7-3 and vTF7-5BUNL (Fig.4.17). It was observed that the level of the L protein was increased at moi of 5 pfu/cell of both vTF7-3 and vTF7-5BUNL (lane 14) as compared to 1 pfu/cell of each vaccinia virus (lane 13) and decreased at moi of 20 pfu/cell (lane 17). This result suggests that infection with 5 pfu/cell of vTF7-3 was sufficient to supply adequate T7 RNA polymerase for maximal expression. Increasing the amount of T7 RNA polymerase produced by vTF7-3 did not appear to significantly enhance the level of the L protein synthesized. Since less vaccinia virus proteins, especially the T7 RNA polymerase, were produced in dual vaccinia virus infected cells (lanes 13-18) compared with singly vTF7-3 infected cells (lanes 1-6), it was possible that higher moi of vaccinia virus inhibited the protein synthesis ability of the cells.

To confirm the identity of the protein made in the recombinant vaccinia virus-infected cells, immunoprecipitation experiments were performed with the monospecific

Fig.4.15. Protein synthesis in recombinant vaccinia virus vSC11-BUNL infected cells. CV-1 cells were mock infected or infected with the WR strain of vaccinia virus, the recombinant vSC11-BUNL, or with Bunyamwera virus (BUN) as indicated. The cells were labelled with ^{35}S-methionine at 22 to 24 h after infection and the proteins separated by electrophoresis on a 10% acrylamide-SDS gel. The Bunyamwera virus proteins L, G1, and N are designated on the right, and a band corresponding to L protein is observed in the vSC11-BUNL lane but not in the parental vaccinia virus lane. The position of the X protein (see text) is also noted. vSC11-BUNL synthesizes ß-galactosidase which has a mobility similar to that of G1.

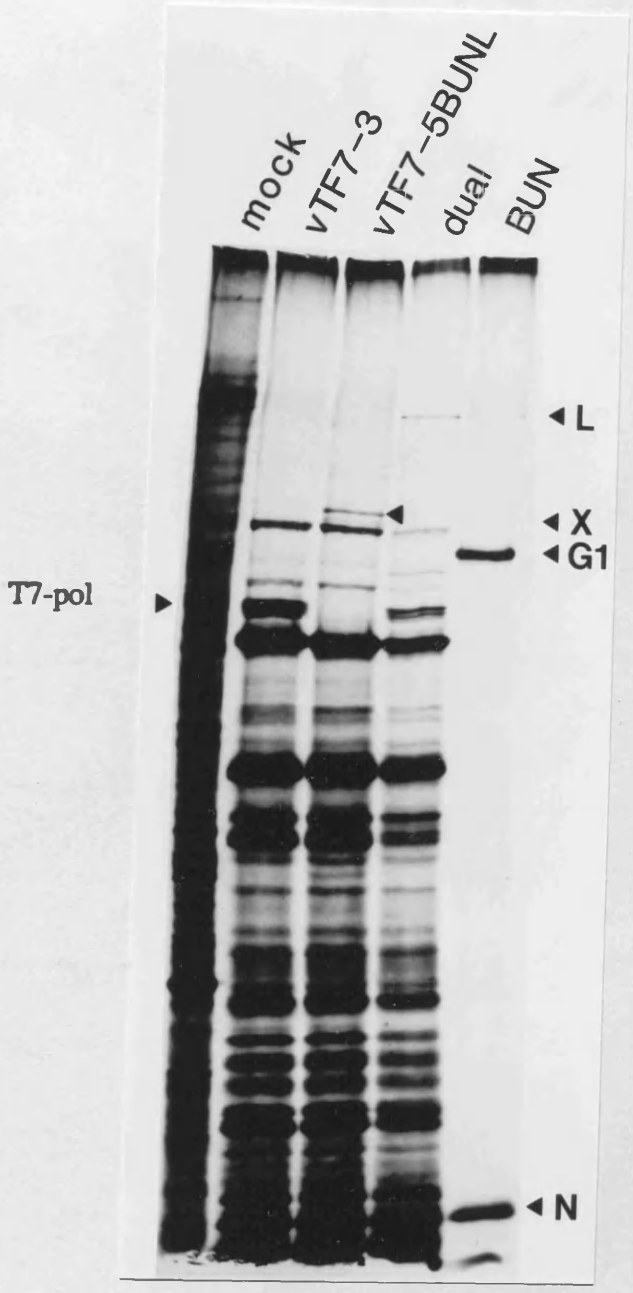
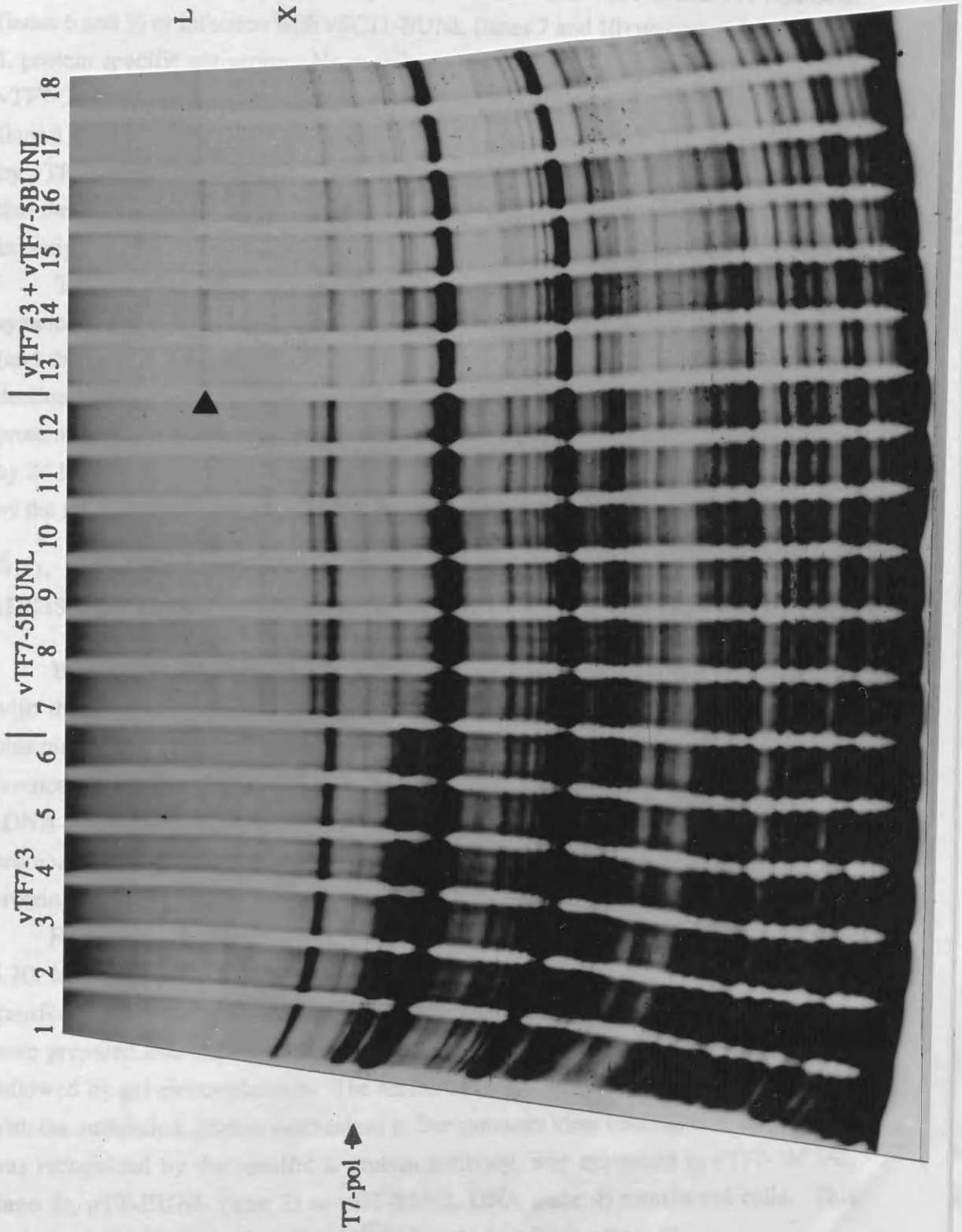


Fig.4.16. Protein synthesis in recombinant vaccinia viruses vTF7-5-BUNL/vTF7-3 infected cells. CV-1 cells were mock infected or infected with vTF7-3, vTF7-5BUNL, vTF7-3 and vTF7-5BUNL (dual), or Bunyamwera virus (BUN) as indicated. The cells were labelled with ^{35}S-methionine at 22-24 hr after infection and the proteins separated on a 10% acrylamide-SDS gel. The Bunyamwera virus L, G1, and N proteins are indicated, and a band corresponding to L protein is observed in the dual-infected lane. L protein is not seen in cells infected with vTF7-5BUNL alone, but the X protein is present. The position of T7 RNA polymerase, synthesized by vTF7-3, is indicated.

Fig.4.17. Expression of the L protein by recombinant vaccinia viruses vTF7-3/vTF7-5BUNL at different multiplicity of infection. CV-1 cells were infected with vTF7-3 (lanes 1-6), vTF7-5BUNL (lanes 7-12), or vTF7-3 and vTF7-5BUNL (lanes 13-18) at moi of 1, 5, 10, 15, 20, or 25 pfu/cell. The cells were labelled with ^{35}S-methionine for 2 hr at 20 hr of post infection and lysates analysed by electrophoresis on a 10% polyacrylamide-SDS gel. The L protein was detected in the dually infected lanes. The level of the L protein increased at moi of 5 pfu/cell of each vTF7-3 and vTF7-5BUNL (lane 14), and declined at moi 20 pfu/cell (lane 17). The X protein is also seen in the vTF7-5BUNL singly infected cells (lanes 7-12) and the position of T7 RNA polymerase is indicated.


1 2 vTF7-3 3 4 5 6 vTF7-5BUNL 7 8 9 10 11 12 vTF7-3 + vTF7-5BUNL 13 14 15 16 17 18
L
X
T7-pol

antiserum against the C-terminus of the L protein. As shown in Fig.4.18 the protein, which was similar in length to the L protein observed in Bunyamwera virus infected cells (lanes 2 and 3), synthesized by dual infection with vTF7-3 and vTF7-5BUNL (lanes 6 and 9) or infection with vSC11-BUNL (lanes 7 and 10) was precipitated by the L protein specific antiserum. No proteins were precipitated from cells infected with vTF7-3 alone (lanes 4 and 8), and no proteins were precipitated by preimmune serum (lane 1). Of interest is the X protein, which was immunoprecipitated from cells infected by vTF7-5BUNL alone (lane 5) and by vSC11-BUNL (lane 7). This result suggests that the X protein is a carboxy terminal fragment of the L protein, but its genesis was not investigated.

The time course of the L protein synthesis by the two different vaccinia virus systems was compared (Fig.4.19). In cells infected with vSC11-BUNL, the L protein (and the X protein) could be detected at 6-8 hr postinfection and the synthesis of L declined by 24 hr of postinfection. In contrast, using the dual vaccinia virus system L protein could not be detected before 10 hr postinfection and its synthesis was greater by 24 hr of postinfection. Therefore, the optimal times for expression of the L protein by the two vaccinia virus systems were different.

4.6. Transient expression of the L protein by plasmid transfection

Bacteriophage T7 RNA polymerase, expressed in the cytoplasm of cells infected with the recombinant vaccinia virus vTF7-3, can be used to transcribe transfected plasmid DNAs that contain target genes placed downstream of T7 RNA polymerase promoter (Fuerst *et al.*, 1986, 1987). As described above the Bunyamwera virus L gene cDNA had been cloned into different plasmid vectors under the T7 RNA polymerase promoter: pET-BUNL, pTZ-BUNL, and pTF7-5BUNL. Transient expression of the L protein by these recombinant plasmids were then investigated.

Recombinant plasmid DNAs pET-BUNL, pTZ-BUNL, or pTF7-5BUNL (Fig. 4.20. lanes 2-4) were transfected into vTF7-3 infected-CV-1 cells and after 6 hr of transfection the cells were labelled with ^{35}S-methionine for 4 hr. Cytoplasmic extracts were prepared and immunoprecipitated with the anti-C terminus L protein antiserum followed by gel electrophoresis. The results showed that a protein, which comigrated with the authentic L protein synthesized in Bunyamwera virus infected cells (lane 1) and was recognized by the specific L protein antibody, was expressed in pTF7-5BUNL (lane 2), pTZ-BUNL (lane 3) or pET-BUNL DNA (lane 4) transfected cells. This protein was not observed in cells without plasmid transfection (lane 5).

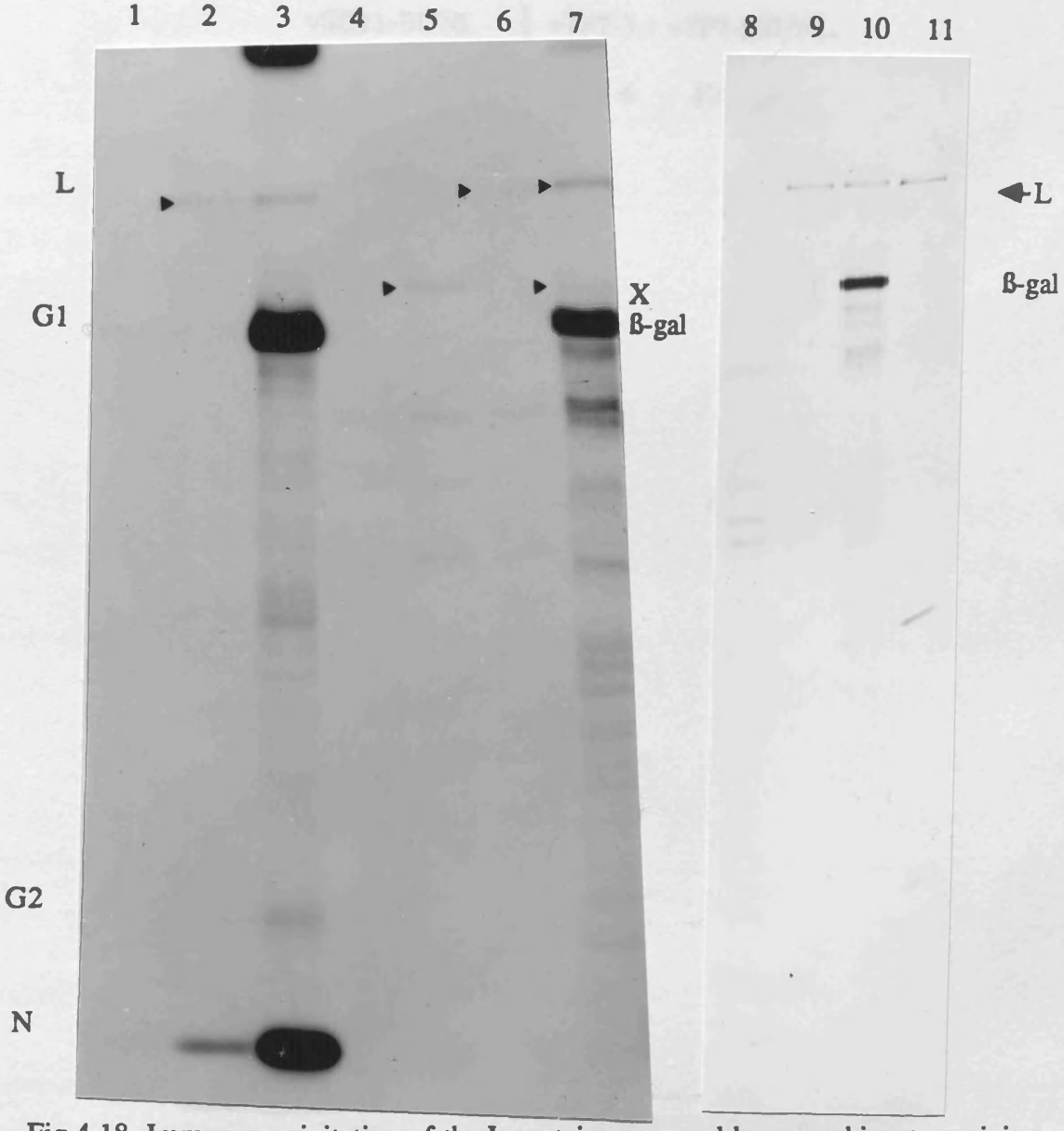


Fig.4.18. Immunoprecipitation of the L protein expressed by recombinant vaccinia virus. Two independent experiments are shown in this figure. Recombinant vaccinia virus infected cells were labelled with ^{35}S-methionine for 2 hr at 22 to 24 hr postinfection, and cell lysates were prepared for immunoprecipitation. The samples were precipitated with preimmune serum (lane 1) or a monospecific L protein antiserum (anti-C) in lanes 2 and 4 to 11 or with an anti-Bunyamwera virus serum in lane 3. Cells were infected with Bunyamwera virus (lanes 1-3 and 11), vTF7-3 (lanes 4 and 8), vTF7-5BUNL (lane 5), vTF7-3 and vTF7-5BUNL (lanes 6 and 9), or vSC11-BUNL (lanes 7 and 10). Lane 3 shows Bunyamwera virus protein markers L, G1, G2, and N. The monospecific L protein antiserum precipitated L protein and minor amounts of N protein from Bunyamwera virus-infected cells (lane 2) but not from vTF7-3-infected cells (lane 4). L protein was precipitated from cells dually infected with vTF7-3 and vTF7-5BUNL (lanes 6 and 9) or with vSC11-BUNL (lanes 7 and 10). The fusion protein antibody also precipitated ß-galactosidase from vSC11-BUNL infected cells. The X protein was precipitated by this antibody from cells infected by vTF7-5BUNL alone (lane 5) or vSC11-BUNL (lane 7). No proteins were precipitated by preimmune serum (lane 1).

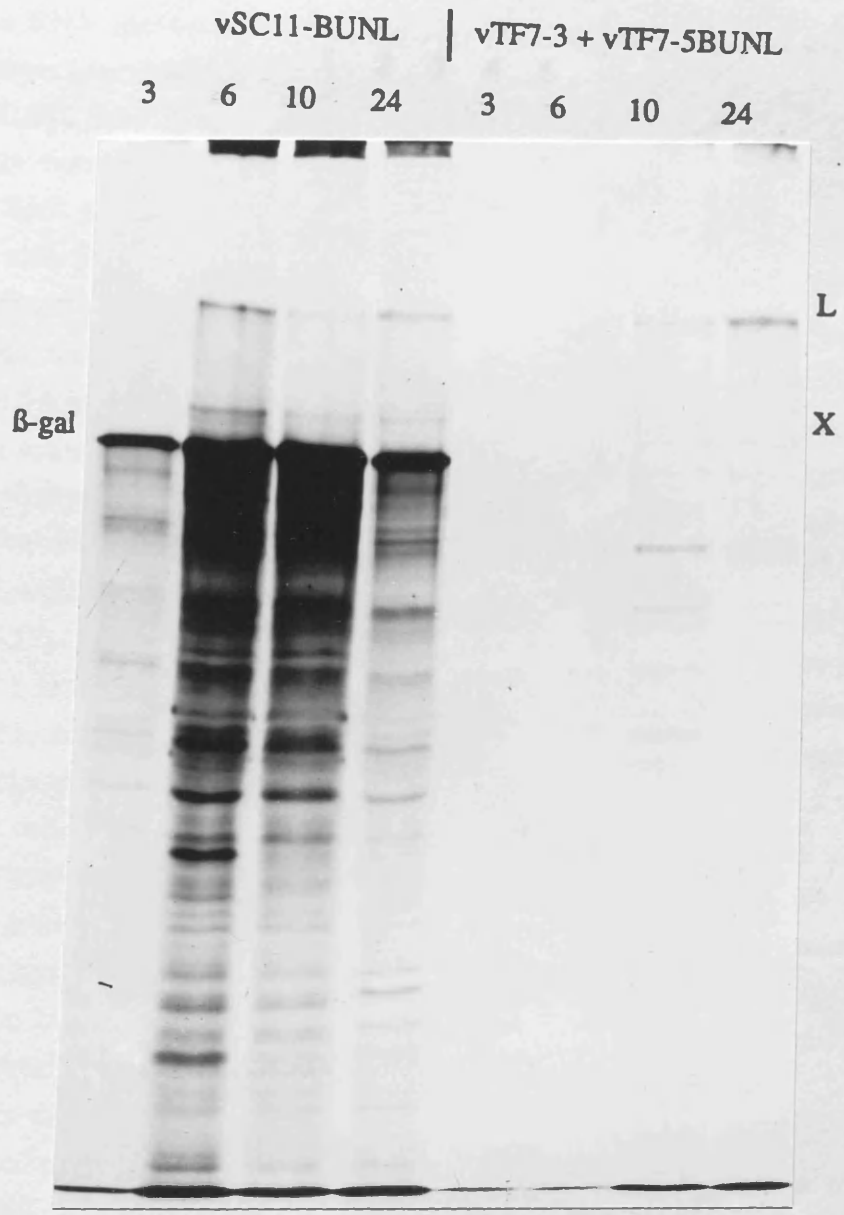


Fig.4.19. Comparison of the time course of the L protein synthesis by the different recombinant vaccinia viruses. Cells were infected with either vTF7-5BUNL and vTF7-3 or with vSC11-BUNL, radiolabelled with ^{35}S-methionine for 2 hr at the indicated times (hr) of post infection, and cell extracts prepared for immunoprecipitation with the monospecific L antiserum (anti-C). The positions of L, X and ß-galactosidase proteins are indicated. L protein expression by vSC11-BUNL was detected by 6 hr postinfection, whereas L protein could not easily seen before 10 hr with the T7 system. Some vaccinia virus proteins were also precipitated in vSC11-BUNL infected cells in this experiment.

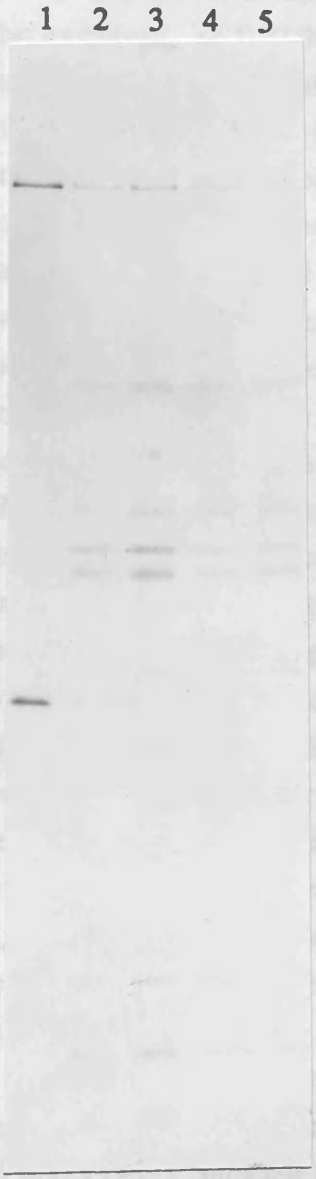


Fig.4.20. Transient expression of the L protein. Subconfluent monolayers of CV-1 cells in 35 mm dishes were infected with vTF7-3 at moi of 5 pfu/cell (lanes 2-5) and then transfected with 20 μg recombinant plasmid DNA pTF7-5BUNL (lane 2), pTZ-BUNL (lane 3), pET-BUNL (lane 4), or were not transfected (lane 5). Lane 1 is the extract from cells infected with Bunyamwera virus. The cells were labelled with ^{35}S-methionine for 16 hr at 6 hr post transfection. Cytoplasmic extracts were prepared, and the proteins were immunoprecipitated with a specific L protein antiserum (anti-C) and analysed by electrophoresis in a 12% polyacrylamide-SDS gel. A protein which was similar in size to the L observed in Bunyamwera virus infected cells (lane 1) was detected in cells transfected with each of the plasmids (lanes 2-4).

The RNA species in vTF7-3 infected cells which had been transfected with recombinant plasmids were also examined. pET-BUNL DNA (Fig.4.21. lane 1) and pTF7-5BUNL (lane 2) were transfected into vTF7-3 infected CV-1 cells, total cellular RNA was extracted and Northern blotted with ^{32}P-labelled L gene cDNA. An L specific RNA transcript about the same size as that observed in Bunyamwera virus-infected cells (lane6) or Bunyamwera virus nucleocapsids (lane 5) and vaccinia virus dually infected cells (lane 4) was detected in the plasmid transfected cells (lanes 1 and 2), which was not seen in cells without plasmid transfection (lane 3). However, no L specific RNA was detected in pTZ-BUNL transfected cells (data not shown).

The amount of the transiently expressed L protein did not increase significantly as the multiplicity of vTF7-3 infection was increased from 5 to 20 pfu/cell and it appeared that an adequate amount of T7 RNA polymerase was synthesized from vTF7-3 at moi of 5 pfu/cell, which correlated with the result of recombinant vaccinia virus dual infection (see Fig.4.17). In order to examine the quantitative relationship between the level of the expressed L protein and the amount of the recombinant plasmid DNA transfected, CV-1 cells were infected with vTF7-3 at moi of 5 pfu/cell and subsequently transfected with various amounts (10 μg, 20 μg, and 30 μg) of pTF7-5BUNL DNA (Fig. 4.22A, lanes 1-3). The cells were labelled with ^{35}S-methionine for 16 hr after 6 hr of transfection and cytoplasmic extracts were immunoprecipitated with the monospecific L protein antiserum (anti-C) followed by gel electrophoresis. The result showed that transfection with the increased amounts of pTF7-5BUNL resulted in the synthesis of the increased levels of the L protein. Some of the vaccinia virus proteins, smaller than the L protein, were also precipitated in this experiment (Fig.4.22A).

I then determined how soon after DNA transfection the L protein could be detected, and monitored the time course of L protein synthesis by transient expression. pTF7-5BUNL was transfected into vTF7-3 infected CV-1 cells and the cells were radiolabelled with ^{35}S-methionine for 2 hr at the indicated times shown in Fig.4.22B. Cytoplasmic extracts were immunoprecipitated with the antiserum against the C-terminus of the L protein. The L protein was shown to be synthesized as early as 4-6 hr after the DNA was first added to the cells. The level of the L protein decreased at 11 hr post-transfection and was hardly detectable at 22-24 hr. This result indicates that the transient expression of the L protein occurs much earlier than in the cells dually infected with vTF7-3 and vTF7-5BUNL recombinant vaccinia viruses.

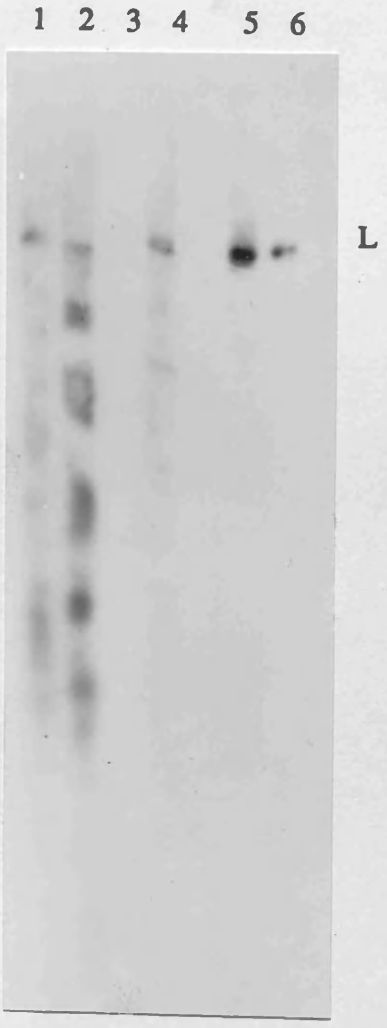


Fig.4.21. Northern blot analysis of the RNA transcribed from plasmid vectors. Total cellular RNA was extracted from vTF7-3 infected cells which were transfected with pET-BUNL (lane 1), pTF7-5BUNL (lane 2), or were not transfected (lane 3), or infected with vTF7-5BUNL (lane 4). Lane 5 is RNA extracted from purified intracellular nucleocapsids and Lane 6 is RNA extracted from Bunyamwera virus infected cells. The RNA was fractionated on a 1% agarose gel containing 5 mM methyl mercuric hydroxyde and transferred to nitrocellulose. Hybridization with ^{32}P-labelled L gene cDNA showed that an RNA species, similar in size to that observed in the Bunyamwera virus infected cells (lane 6) and dual vaccinia virus infected cells (lane 4), was detected in both pET-BUNL and pTF7-5BUNL transfected cells (lanes 1 and 2), but not in cells without plasmid transfection.

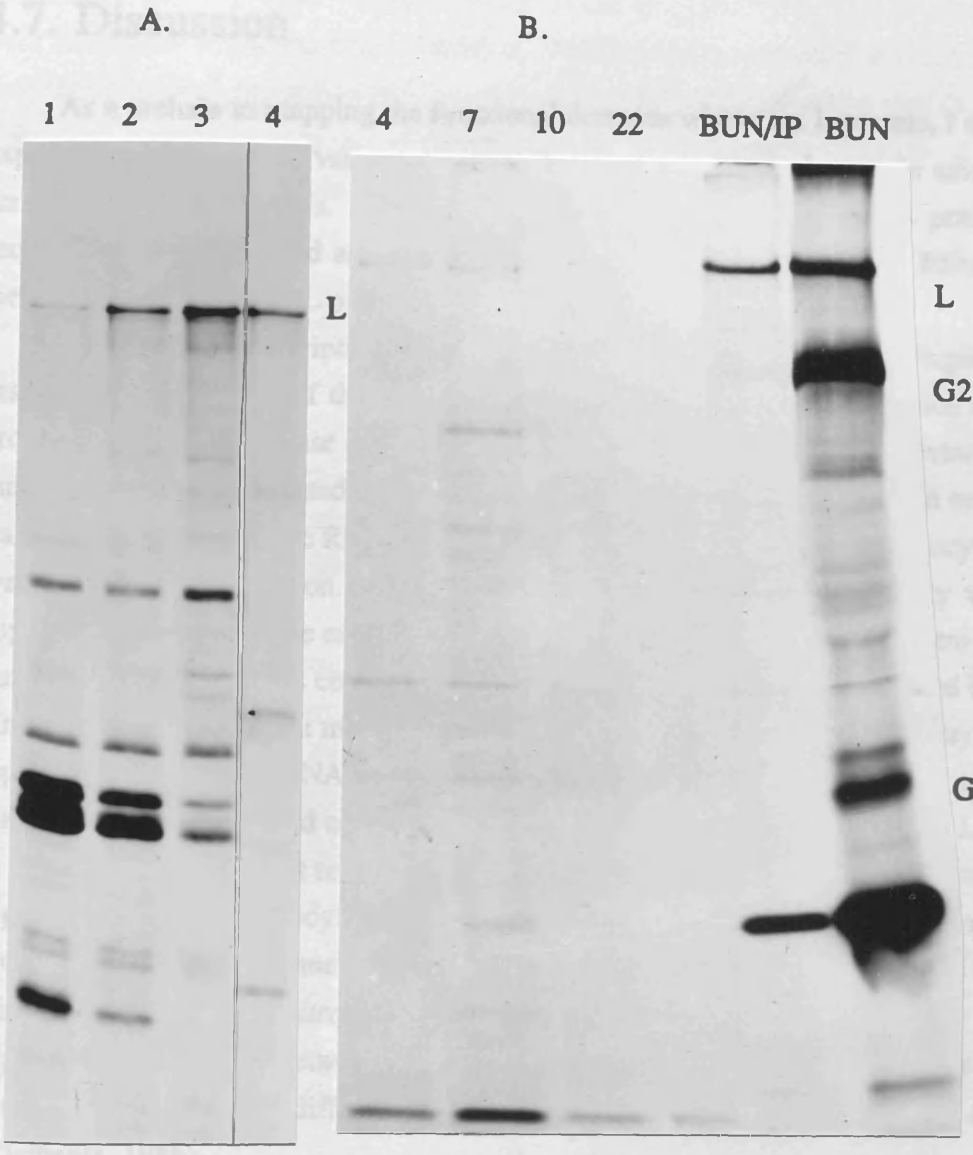


Fig.4.22. Characterization of the transient expression system for expression of the L protein. A. Dose response of the L protein synthesis. CV-1 cells were infected with vTF7-3 at moi of 5 pfu/cell and transfected with 10 μg (lane 1), 20 μg (lane 2), or 30 μg (lane 3) pTF7-5BUNL DNA. The cells were labelled overnight at 6 hr post transfection and cytoplasmic extracts were precipitated with a monospecific L protein antiserum (anti-C). Increased levels of the L protein were produced following transfection of increased amount of pTF7-5BUNL DNA. Some vaccinia virus proteins were also precipitated. Lane 4 is the immunoprecipitation of the Bunyamwera virus infected cells with the L protein antiserum. B. Time course of the L protein expression. CV-1 cells were infected with vTF7-3 at moi of 5 pfu/cell and transfected with 20 μg pTF7-5BUNL DNA. Cells were labelled for 2 hr at the indicated times post transfection. The cytoplasmic extracts were immunoprecipitated with the L protein antiserum (anti-C) followed by electrophoresis on a 10% polyacrylamide-SDS gel. Lane BUN/IP is Bunyamwera virus infected cell extract immunoprecipitated with the L protein antiserum. Lane BUN shows Bunyamwera virus proteins L, G1, G2, and N, which are designated on the right. L protein expression was detected by 4 hr of posttransfection, but declined as early as 10 hr.

4.7. Discussion

As a prelude to mapping the functional domains within the L protein, I sought to express the L protein in various expression systems to obtain L protein suitable for further functional analysis. Firstly a cDNA containing the full-length L gene coding sequence was constructed and subcloned into various plasmid vectors. Efforts were then made to express the L protein in various systems.

(1). *In vitro* transcription and translation of the L gene could be a simple way to assay the functionality of the L protein *in vitro* by coupling the translation of the L protein with its transcriptase activity analysis. Patterson *et al.* (1984) demonstrated LAC bunyavirus virion-associated RNA polymerase and endonuclease activites in an *in vitro* transcription assay and the RNA synthesis ability was increased when reticulocyte lysate was added to the reaction. Since the *in vitro* system is saturated only with the supplemented mRNA, the expressed protein could be assayed directly *in vitro* without purification. Thus it was considered that if the L protein could be expressed in the *in vitro* translation system, it might be possible to analyse the polymerase activity of L by replication of a suitable RNA template or to assay the endonuclease activity by analysing the processing of a capped mRNA. However, initial attempts to translate the L protein in a messenger dependent reticulocyte lysate were not successful. The experiment was repeated using a reticulocyte lysate which was produced by a different company; a protein which was the same size as the L protein was detected. However the level of this protein produced *in vitro* was very low and could not be precipitated by the specific L protein antiserum. Whether this protein really was L is therefore questionable. These results also reflect the difficulty of translating a protein larger than 60K *in vitro* (Clements, 1986).

(2). The bacterial T7 expression system established by Studier and his colleagues (1986 and 1990) is a practical means to obtain large amounts of protein expressed from the cloned cDNA in *E. coli*. However, considering the large size of the Bunyamwera virus L protein, it is probably very difficult to synthesize it in bacterial system. Efforts were made to use this system for the expression of the L protein, but the data provided here showed that the L protein was not produced in the bacterial T7 system.

(3). Vaccinia virus is a useful vector for expressing genes within the cytoplasm of eukaryotic cells and recombinant vaccinia viruses have been used to synthesize a number of biologically active proteins and further to analyse successfully the structure-function relations of protein molecules in various fields (Moss, 1991). Therefore, I cloned the Bunyamwera virus L gene cDNA into two vaccinia virus transfer vectors to make recombinant vaccinia viruses. Two vaccinia virus expression systems, the "constitutive"

system and the "inducible" vaccinia virus/bacteriophage T7 promoter vector system, were chosen for the expression experiment. These two systems were used in parallel in case "constitutive" expression of L by the vaccinia virus P7.5 promoter was detrimental to vaccinia virus replication: it was reported that the VSV M protein was toxic to vaccinia virus in making a recombinant based on pSC11 (Li *et al.*, 1988). The use of the T7 RNA polymerase system would have overcome this problem. In fact both systems expressed full length antigenically authentic Bunyamwera virus L protein. Apparently more L was synthesized using vSC11-BUNL than with vTF7-3 and vTF7-5BUNL, even though the level of L mRNA was considerably higher in cells infected with the latter. In their characterization of the vaccinia virus-T7 polymerase system, Fuerst and Moss (1989) also observed much higher levels of mRNA compared to the level of protein made, and suggested that the apparent inefficient translation of the T7 polymerase transcribed RNAs was because only 5-10% contained terminal cap structures.

In addition to full-length L, other proteins apparently specific to the recombinant vaccinia viruses were observed (Figs.4.15-4.19). In particular, the protein designated X is worthy of mention as this was immunoprecipitated by the monospecific L antibody, suggesting it to be a carboxy-terminal fragment of L. Although the genesis of this protein has not been investigated, this protein may be the translation product of an RNA transcribed by the vaccinia virus polymerase recognising a cryptic vaccinia virus-like promoter sequence in the L cDNA. This is not unreasonable as the L cDNA is relatively AT-rich (67%; Elliott, 1989a) and so are vaccinia virus promoters (Davison and Moss, 1989a and 1989b); vaccinia virus late promoters contain the highly conserved sequence TAAAT (Davison and Moss, 1989b) and this sequence is found 49 times in the L cDNA (considering both strands).

(4). In addition to expressing the L protein by the two recombinant vaccinia viruses, the L was also synthesized by transient expression. The L gene cDNA, under control of the T7 promoter in different plasmids, such as pTZ18R, pET8c, and pTF7-5, was transfected into cells infected with a recombinant vaccinia virus vTF7-3 expressing the T7 RNA polymerase, and L protein was synthesized. In contrast to pTF7-5 and pET8c vectors, pTZ18R does not have the T7 terminator sequence and no L specific RNA was detected in pTZ-BUNL transfected cells. However, it appeared that there must be some functional L RNA transcripts made in the pTZ-BUNL transfected cells which were used to translate the 259 K L protein. Expression of the L protein by plasmid transfection may be very useful for future assays. The transient expression system may have the advantage for expressing large numbers of L gene constructs so that the functionality of a number of the mutated L genes could be screened directly

without having to recombine into vaccinia virus. It may also be useful in that the levels of the L protein expressed can be controlled by the amount of the plasmid DNA transfected. The ability of controlling the protein expression level in the transfected cells may provide flexibility in defining the requirements for optimal RNA replication in functional analyses of the L protein, since it has been reported that high levels of expressed L protein are inhibitory to RNA replication in some other negative-strand RNA viruses, such as Sendai virus and VSV (Gotoh *et al.*, 1989; Meier *et al.*, 1987). The transient expression system may also be used to determine the requirements for individual virus proteins in the virus replication processes.

In summary, a protein which is similar in size to the Bunyamwera virus L protein was synthesized in an *in vitro* translation system; L could not be expressed from the bacterial T7 system; antigenically authentic L protein has been successfully produced in vaccinia virus systems, either through recombinant vaccinia viruses or by transfection of the plasmids containing the L gene cDNA under the T7 promoter into the T7 RNA polymerase producing cells.

Chapter 5

Functional analysis of the L protein expressed by recombinant vaccinia viruses

5.1. Introduction

The L protein is a multifunctional protein. It is presumed to have RNA-dependent RNA polymerase activity and some other functions, such as endonuclease activity (Patterson *et al.*, 1984). Study of the L protein of bunyavirus has been limited and mostly confined to the virion-associated enzyme protein. To date, attempts to synthesize the large polymerase protein of other negative-strand RNA viruses *in vitro* in a suitable form have not been very successful, and since it is difficult to obtain a functional synthetic RNA template (in a nucleocapsid form) for the functional assays of the expressed protein *in vitro*, This has hindered the advancement of our understanding of the L protein and hence the viral RNA synthesis process. Thus, alternative *in vitro* systems have to be developed.

In the previous chapter, I demonstrated that the full-length L gene cDNA was assembled, and that antigenically authentic 259K L protein was expressed by recombinant vaccinia viruses and by the transfected plasmid system. In order for the expressed L protein to be useful for domain mapping, it was necessary to demonstrate that the expressed L protein had RNA synthesis ability.

To determine the funtionality of the L protein expressed from the recombinant vaccinia virus, several assay systems were investigated: complemen tation of bunyavirus L gene ts mutants; *in vitro* transcription assays; and the ability of the L to replicate an RNA template *in vivo*.

5.2 Complementation of bunyavirus ts mutants

On discussion I recognized that the difference between the yields of Bunyamwera virus at 31 °C and 38.5 °C was too great to allow critical conclusions to be drawn from the complementation data.

Complemen tation of ts mutants with defects in the L gene by the L protein expressed from recombinant vaccinia viruses was first explored. It was suggested that the Group I ts mutants of Bunyamwera virus may be L mutants (C. R. Pringle, personal communication). The ts mutants BUN ts5 (Bunyamwera virus) and MAG ts7 (Maguari virus) were chosen to be the candidates for the experiment because they grew relatively better than the other Group I ts mutants at the permissive temperature (31 °C). It was considered that if the L protein was functional, superinfection with recombinant vaccinia viruses should increase the released virus yield from the ts mutant infected cells at the nonpermissive temperature.

Before carrying out the complementation experiment, I examined whether vaccinia virus infection would interfere with bunyavirus growth. CV-1 cells were infected with vaccinia virus WR strain at 5 pfu/cell and incubated at 31 °C for 60 min, the cells were washed once with the medium (5% DMEM) and superinfected with wild type Bunyamwera virus at 0, 2, 10, or 20 pfu/cell at 37 °C for 30 min. The cells were then washed twice with medium and further incubated at either 31 °C or 38.5 °C for 24 hr. The supernatants were harvested and Bunyamwera virus yields were assayed by plaque titration in the presence of phosphonoacetic acid, which is inhibitory to vaccinia virus

Table 5.1. Effect of vaccinia virus infection on Bunyamwera virus growth

BUN (wt) (pfu/cell)	WR	Bunyamwera virus yields	
		31°C ($\times 10^8$)	38.5°C ($\times 10^6$)
5	0	1.0	4.6
5	2	2.0	1.9
5	10	1.1	0.9
5	20	1.2	1.1

CV-1 cells were infected with each virus at moi indicated and the parallel dishes were incubated at the permissive (31 °C) or the restrictive temperature (38.5 °C) for the bunyavirus ts mutants. After 48 hr of infection, the culture fluids were titrated for the Bunyamwera virus yields in the presence of 250 μg/ml phosphonoacetic acid.

growth but allows Bunyawera virus to form plaques. As can be seen from Table 5.1, vaccinia virus showed little interference on Bunyamwera virus replication. The titre of Bunyamwera virus did not vary significantly in the absence or presence of vaccinia virus WR strain when the cells were incubated at the same temperature. However, it was about 100 fold lower when incubated at 38.5 °C than that at 31 °C, which might be because some of the released virus became noninfectious during the incubation or some slight temperature sensitivity of the Bunyamwera virus. Using vaccinia viruses carrying the L gene, similar results were also observed.

The protocol of the complementation assay is shown in Fig.5.1, and the result of a typical complementation assay is shown in Table 5.2. For bunyavirus ts mutants, BUN ts5 and MAG ts7, no significant difference was observed between the virus yields released from CV-1 cells infected with ts mutants alone and the cells superinfected with recombinant vaccinia viruses expressing the L protein. Thus, the L protein expressed from the recombinant vaccinia viruses did not show ability to complement these two ts mutants. The more well-characterized snowshoe hare (SSH) bunyavirus ts mutants, obtained from Prof. DHL Bishop, were also used for the complementation experiment. The incubation of the CV-1 cells infected with SSH ts mutants and recombinant vaccinia viruses was carried at 39.5 °C for 2 days. However, again no complementation of these ts mutants by the L protein was demonstrated (data not shown).

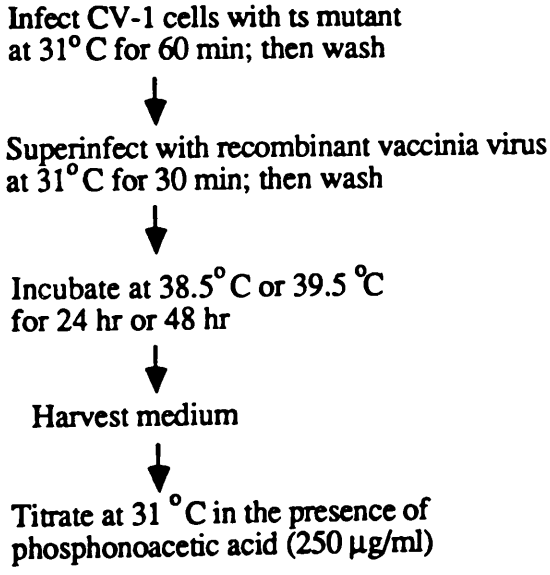


Fig.5.1. Protocol of the L gene complementation assay.

Table 5.2. Complementation assay

Virus	Virus yields	
	BUN ts5 (x 10^3)	MAG ts7 (x 10^2)
Mutant	7.5	1.25
+ vTF7-5BUNL	6.7	3.5
+ vTF7-5BUNL/vTF7-3	3.7	3.0
+ vSC11-BUNL	4.2	2.5

The moi of each virus was 5pfu/cell and the released virus yields of ts mutants titrated by plaque assay at 31 °C in the presence of 250 ug/ml of phosphonoacetic acid.

5.3. *In vitro* transcriptase analysis of the L protein

The functional RNA template for RNA synthesis of negative-strand RNA viruses is not naked RNA, but the nucleocapsid or RNP i.e. RNA complexed with the N protein. To date, we do not have a method to reconstruct this template *in vitro*. Therefore, Bunyamwera virus nucleocapsids were purified from infected BHK cells to determine if they could be used as the templates for *in vitro* transcriptase activity assay of the L protein. As a prerequisite, the polymerase activity associated with the RNP was examined by *in vitro* transcription of the preparation; the polymerase activity in virions was used as a comparison. Intracellular nucleocapsids were purified by CsCl gradient centrifugation, and Bunyamwera virus was purified and disrupted with NP-40 by the method described in Chapter 2. The RNP or the detergent disrupted virions were added to the *in vitro* transcription reaction and incubated in the presence of ^{32}P-GTP at 30 °C for 60 min under the conditions described in Chapter 2. The *in vitro* synthesized, labelled RNA was analysed by electrophoresis in a 1% agarose gel containing 5 mM methylmercuric hydroxide. As can be seen from Fig.5.2, the virion associated RNA polymerase activity was much higher than that of the RNP preparation and the L, M, and S RNA species were clearly observed.

Since lower polymerase activity in the RNP preparation was detected, it was thought possible that the RNP could be used as templates by the supplemented L protein in cytoplasmic extracts prepared from lysolecithin permeabilized cells infected with the recombinant vaccinia viruses. To ensure that the lysolecithin method (see chapter 2) would release the L protein efficiently, immunoprecipitation of the L protein with the L

Fig.5.2. Agarose gel analysis of the Bunyamwera virus RNA polymerase products. Bunyamwera virus (lane 1) and nucleocapsids (lane 2) were purified and polymerase reactions were carried out in the presence of ^{32}P-GTP under the condition described in chapter 2. RNA labelled with ^{32}P-GTP during the *in vitro* reaction was resolved in a 1% agarose gel containing 5 mM methylmercuric hydroxide. The L, M, and S RNA species are clearly seen in the detergent-disrupted virion programmed reaction (lane 1) but are barely detected in RNP programmed reaction (lane 2).

Methylmercuric hydroxide would minimize possiblility that the labelled bands were hybrids between template and ^{32}P-labelled partial products.

specific antiserum was performed. As shown in Fig.5.3A, the L protein was released efficiently. Immunoprecipitation of the cytoplasmic extracts infected with vSC11-BUNL (lane 3) or vTF7-3 and vTF7-5BUNL (lane 2) detected L protein, but not in cells infected with vTF7-3 alone (lane 1). More L was produced in vSC11-BUNL infected cells (lane 3). Unlabelled extracts were prepared and added to the RNP programmed *in vitro* transcription reaction in the presence of ^{32}P-GTP, the RNA products were fractionated on a 1% agarose gel containing 5 mM methylmercuric hydroxide, and the labelled RNA visualized after exposure to X ray film. It was observed that the vTF7-3 infected cell extract slightly stimulated the RNP associated RNA polymerase activity and the labelled S, M, and L RNA species were detected (Fig.5.3B. lane 2) as compared to the reaction without supplementation (lane 1). The L protein present in vTF7-3 and vTF7-5BUNL (lane 3) infected cell extract did not increase polymerase activity significantly. However, it appeared that slightly more RNAs were synthesized *in vitro* by the addition of vSC11-BUNL infected cytoplasmic extract (lane 4), which had more L protein produced as detected by immunoprecipitation (Fig.5.3A. lane 3). It is also possible that cytoplasmic extract prepared from vSC11-BUNL infected cells had some other protein components which stimulated the RNA polymerase. More RNA was made in the reaction supplemented with Bunyamwera virus infected cell extract (lane 5), which presumeably also contained active virus RNA templates and the L protein. The above results indicated that it was difficult to determine the functionality of the L protein by an *in vitro* transcription assay using the intracellular RNP as templates.

5.4. Attempts to reconstruct a synthetic RNA for replication assay

The template recognized by the Bunyamwera virus L protein is the RNA encapsidated with the N protein. Attempts to encapsidate a synthetic RNA in cultured cells were made to synthesize this kind of template. To obtain the N protein for encapsidation, the Bunyamwera virus S cDNA, cloned under control of the T7 promoter in pTF7-5 (pTF7-5BUNS), obtained from A. McGregor, was used for transient expression of the N protein. pTF7-5BUNS DNA was transfected into vTF7-3 infected cells and the cells were radiolabelled with ^{35}S-methionine for 4 hr at 6 hr after transfection to examine the N protein transiently expressed. Cytoplasmic extracts were immunoprecipitated with anti-Bunyamwera virus antiserum and the proteins analysed by gel electrophoresis. As can be seen from Fig.5.4A, the N protein was expressed from the transfected pTF7-5BUNS DNA; the more recombinant plasmid transfected, the more

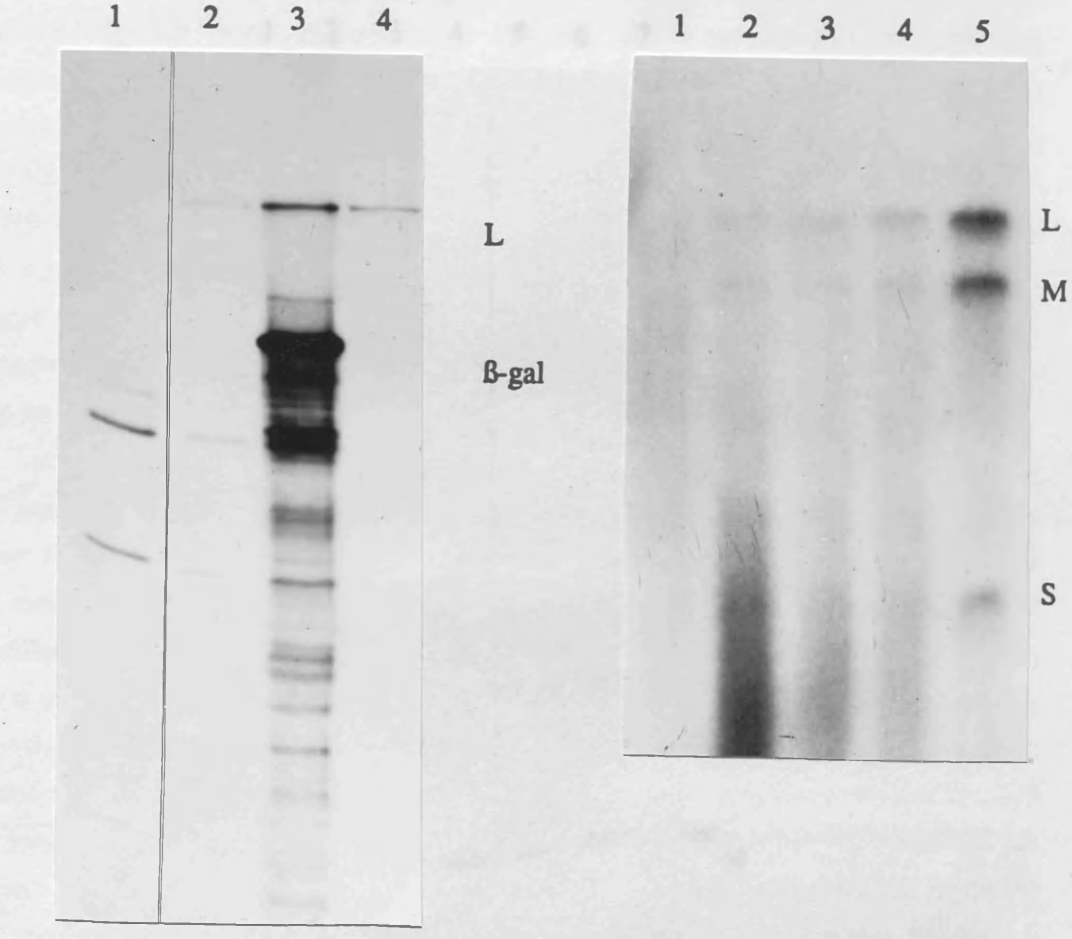


Fig.5.3. Analysis of the transcriptase activity of the L protein expressed from recombinant vaccinia viruses. **A.** Analysis of the L protein synthesis in recombinant vaccinia virus infected cytoplasmic extracts prepared by the lysolecithin permeabilization method. CV-1 cells were infected with vTF7-3 (lane 1), vTF7-5BUNL and vTF7-3 (lane 2), vSC11-BUNL (lane 3), or Bunyamwera virus (lane 4) and radiolabelled with ^{35}S-methionine at 20-22 hr post infection. The cells were then treated with lysolecithin and the extracts immunoprecipitated with the monospecific L protein antiserum (anti-C). The L protein was observed in extracts from cells infected with vTF7-5 and vTF7-5BUNL (lane 2) or vSC11-BUNL, but not in vTF7-3 infected cells. **B.** *In vitro* ^{32}P-labelled RNA synthesized from purified RNP template (lane 1) and supplemented with unlabelled cytoplasmic extracts from cells infected with vTF7-3 (lane 2), vTF7-3 and vTF7-5BUNL (lane 3), vSC11-BUNL (lane 4), or Bunyamwera virus (lane 5). Very weak transcriptase activity was detected in the reaction without supplementation with cytoplasmic extract (lane 1); more ^{32}P-labelled L, M, and S RNA were detected in other reactions supplemented with cytoplasmic extracts. The vSC11-BUNL (lane 4) infected cytoplasmic extract had slightly more activity in stimulating the RNA synthesis than vTF7-3 (lane 2) or dual vaccinia virus (lane 3) infected extracts. Much stronger activity was seen in the reaction supplemented with Bunyamwera virus infected cell extract (lane 5).

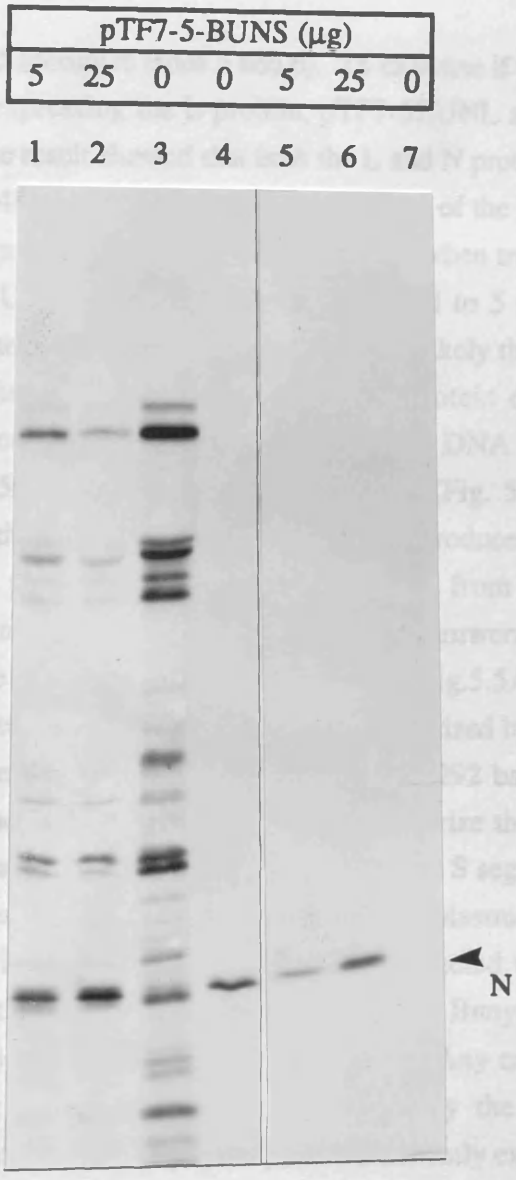


Fig.5.4A. SDS-polyacrylamide gel analysis of transiently expressed Bunyamwera virus N protein. CV-1 cells were infected with vTF7-3 (lanes 1-3 and 5-7) at moi of 5 pfu /cell and transfected with 5 μg or 25 μg pTF7-5BUNS DNA (lanes 1, 2, 5 and 6) as indicated. The cells were labelled with ^{35}S-methionine and aliquots run on the gel (lanes 1-3) or the labelled extracts were prepared for immunoprecipitation with anti-Bunyamwera virus antiserum (lanes 5-7). The N protein was only clearly shown by immunoprecipitation (lanes 5-6), and more N was made in cells transfected with 25 μg pTF7-5BUNS DNA (lane 6) compared to 5 μg DNA (lane 5). The L and G1 proteins were not seen in lane 4 - immunoprecipitation of Bunyamwera virus infected cell extract with anti-Bunyamwera virus antiserum as the gel was only exposed for 4 hr, but were seen on longer exposures.

N protein was produced (compare lanes 5 and 6). To examine if the N protein could also be expressed in cells expressing the L protein, pTF7-5BUNL and pTF7-5BUNS were transfected together; the result showed that both the L and N proteins could be expressed simultaneously (Fig.5.4B. lanes 7-9). The dose response of the N protein expression to the amount of the plasmid transfected was less apparent when transfecting 10 μg, 20 μg or 30 μg of pTF7-5BUNL DNA (B. 4-6) as compared to 5 μg or 25 μg of pTF7-5BUNS DNA transfected (Fig. 5.4A. lanes 5-6). It was likely that 10 μg pTF7-5BUNS DNA had saturated the expression system. The L protein dose response was not obvious when transfected together with pTF7-5BUNS DNA (Fig. 5.4B. lanes 7-9) compared with pTF7-5BUNL DNA transfection alone (Fig. 5.4B. lanes 1-3), which might be due to competition for the T7 RNA polymerase produced by vTF7-3.

The recombinant plasmid pUC-BUNS292 obtained from A. McGregor, contains the 3' and 5' terminal untranslated sequences of the Bunyamwera virus S RNA segment but has the most of the internal coding region deleted (Fig.5.5A). The end sequences were considered to contain the (promoter) sequence recognized by the RNA polymerase, and should be replicated by the RNA polymerase. The 292 base S cDNA was under control of the T7 promoter; digestion with Xba I to linearize the plasmid would allow synthesis of an RNA transcript with the exact 3' end of the S segment. CV-1 cells were infected with vTF7-3 and transfected with the linearized plasmid to synthesize the 292 base long (+) sense S RNA. The cells were also transfected with pTF7-5BUNS for transient expression of the N protein, or were infected with Bunyamwera virus to supply the N protein for encapsidation of the 292 base S RNA. Any encapsidated 292 base S RNA should then act as a template for replication by the L protein present in Bunyamwera virus infected cells or by the L protein transiently expressed in the cells.

pUC-BUNS292 DNA linearized by the digestion with Xba I was transfected into vTF7-3 infected cells which had also been transfected with pTF7-5BUNL and pTF7-5BUNS (Fig.5.5. lane 2) or infected with Bunyamwera virus (lane 3). Total cellular RNA was extracted and fractionated in a 1% agarose gel containing 5 mM methylmercuric hydroxide, and transferred to a nitrocellulose filter (B) or the RNA was dot blotted onto a nitrocellulose filter directly (C). However, hybridization with ^{32}P-labelled S gene cDNA could not detect S RNA of 292 bases in vTF7-3 infected cells (lane 4), indicating that transcription of pUC-BUNS292 by T7 RNA polymerase did not occur. The S RNA produced in pTF7-5BUNL transfected cells appeared as a doublet. The reason for this is not clear.

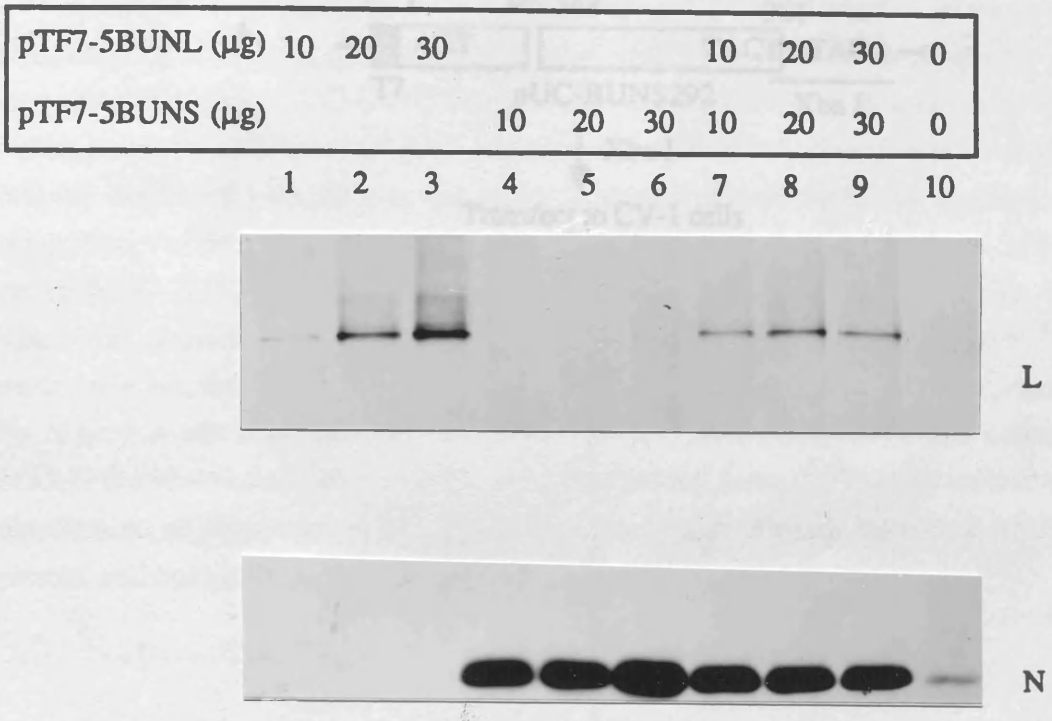


Fig.5.4B. Transient expression of the N protein together with the L protein. CV-1 cells were infected with vTF7-3 (lanes 1-10) and transfected with different amounts of pTF7-5BUNL or pTF7-5BUNS DNA as shown above the lanes. The cells were labelled with ^{35}S-methionine and immunoprecipitated with anti-Bunyamwera virus antiserum (lanes 4-10) or the anti-C terminal of the L specific antiserum (lanes 1-3 and 7-9). The L and N proteins were expressed individually (lanes 1-3 or 4-6) or simultaneously (lanes 7-10) in the cells transfected with recombinant plasmids, but were not detected in cells without plasmid transfection (lane 10). The dose response of the L protein was obvious in lanes 1-3, but not evident in lanes 7-9 which had also been transfected with pTF7-5BUNS DNA. The N protein dose response was not obvious in either singly transfected cells (lanes 4-6) or when transfected together with pTF7-5BUNL DNA (lanes 7-9). The top of the gel was exposed longer (48 hr) than the lower part of the gel (4 hr) to visualize the expressed L protein.

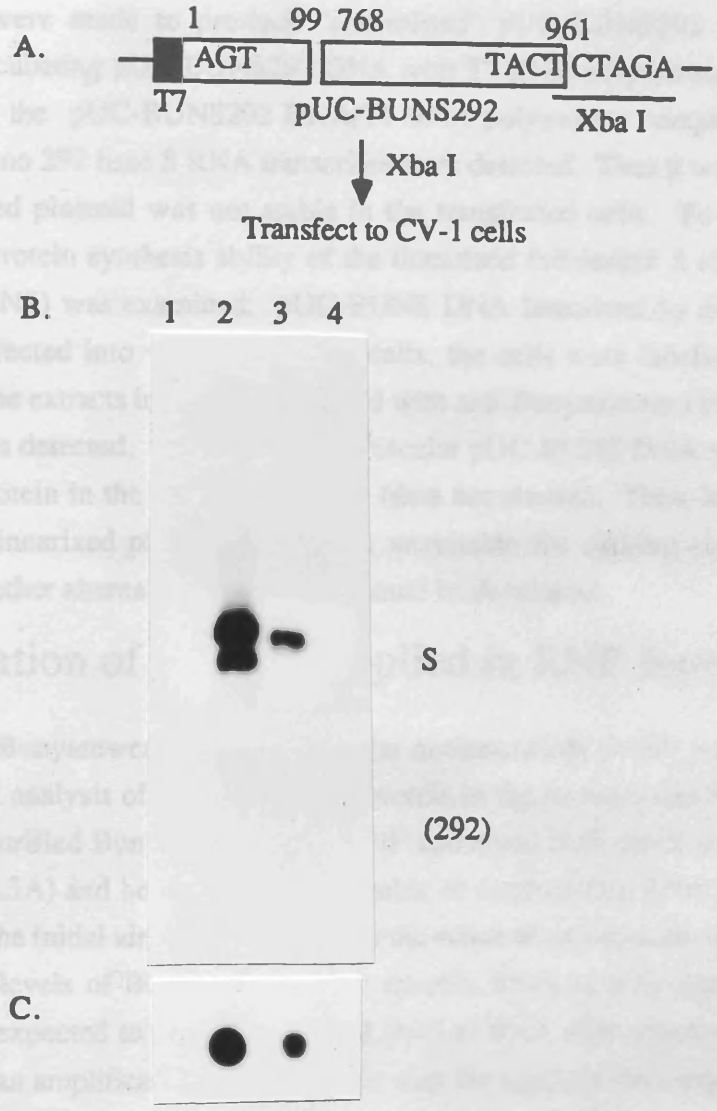


Fig.5.5. Attempts to replicate a synthetic Bunyamwera virus S RNA by recombinant L protein. A. A map of the internally deleted Bunyamwera virus S cDNA plasmid (pUC-BUNS292) which was used to make a synthetic RNA template. Bunyamwera virus S segment nucleotides 99 to 768 had been removed from the full-length S cDNA, which had been cloned under control of the T7 promoter in plasmid pUC9. Digestion with Xba I linearized pUC-BUNS292 and produced the exact 3' end sequence of the S segment. (B) Northern blot and (C) dot blot analysis of RNA extracted from cells transfected with linearized plasmid pUC9-BUNS292. The cells were mock-infected (lane 1), infected with vTF7-3 (lanes 2-4) following transfection with pTF7-5BUNS and pTF7-5BUNL (lane 2), or infection with Bunyamwera virus (lane 3). Total cellular RNA was extracted, fractionated on a 1% agarose gel containing 5 mM methylmercuric hydroxyde and transferred to a nitrocellulose filter (B), or dot blotted onto a nitrocellulose filter (C). Hybridization with ^{32}P-labelled pTZ-BUNS did not detect the synthesis of a 292 base RNA. The 961 base full-length S RNA was observed in Bunyamwera virus infected cells (lane 3); a doublet of S RNA bands were observed in pTF7-5BUNS transfected cells (lane 2).

Attempts were made to produce "committed" pUC-BUNS292 transcription complexes by incubating pUC-BUNS292 DNA with T7 RNA polymerase *in vitro* and then transfecting the pUC-BUNS292 DNA/T7 RNA polymerase complex into cells. Again, however, no 292 base S RNA transcripts were detected. Thus it was considered that the linearized plasmid was not stable in the transfected cells. To confirm this suggestion, the protein synthesis ability of the linearized full-length S cDNA in pUC vector (pUC-BUNS) was examined. pUC-BUNS DNA linearized by digestion with Xba I was transfected into vTF7-3 infected cells, the cells were labelled with ^{35}S-methionine and the extracts immunoprecipitated with anti-Bunyamwera virus antiserum. No N protein was detected. However, intact circular pUC-BUNS DNA was shown to express the N protein in the same experiment (data not shown). Thus, it seemed that transfection of linearized plasmid DNA was unsuitable for making either RNA or protein, and that other alternative approaches should be developed.

5.5. Replication of S RNA supplied in RNP form

The use of Bunyamwera virus intracellular nucleocapsids (RNP) was considered for the functional analysis of the expressed L protein in the recombinant vaccinia virus infected cells. Purified Bunyamwera virus RNP contained both the N and L proteins (Chapter 3, Fig.3.5A) and hence should be capable of synthesizing RNA if transfected into cells. Thus the initial aim was to determine the effect of recombinant vaccinia virus infection on the levels of Bunyamwera virus-specific RNA in cells transfected with purified RNP. I expected to see a background level of RNA after transfection of RNP and hoped to see an amplification after infection with the vaccinia virus expressing the L protein.

The protocol for the RNP transfection assay using the calcium phosphate transfection method, is shown in Fig.5.6. Preliminary dot blot analysis of RNA extracted from the RNP transfected CV-1 cells indicated that no Bunyamwera virus-specific S RNAs could be detected in either mock infected cells (Fig.5.7. lane 1) or cells infected with vTF7-3 (lane 4) at 24 hr after transfection. However, in the RNP transfected cells which had been infected with vTF7-3 and vTF7-5BUNL (lane 2) or infected with vSC11-BUNL (lane 3), or infected with vTF7-3 and transfected with pTF7-5BUNL (lane 5), Bunyamwera virus S RNA signals were clearly detected. These data indicated that the L protein expressed by the recombinant vaccinia viruses, or by the transfected plasmid pTF7-5BUNL DNA, had amplified the S RNA introduced into the cells through RNP transfection.

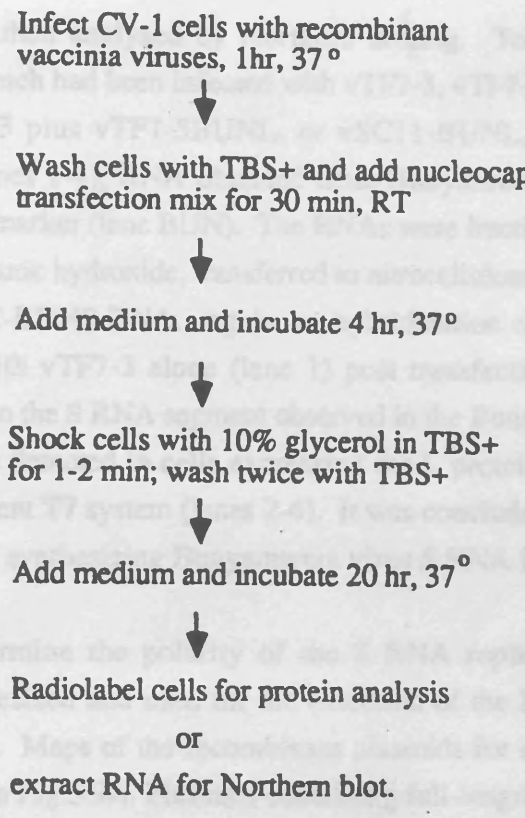


Fig.5.6. Protocol of the nucleocapsid transfection assay. (TBS+ = 25 mM Tris-HCl, pH 7.4, 135 mM NaCl, 50 mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$).

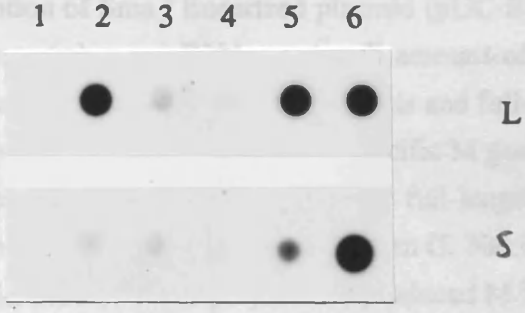


Fig.5.7. Dot blot analysis of RNA from cells transfected with Bunyamwera virus nucleocapsids following infection with recombinant vaccinia viruses. CV-1 cells were mock-infected (lane 1), or infected with vTF7-3 and vTF7-5BUNL (lane 2), vSC11-BUNL (lane 3), vTF7-3 (lane 4-6) and transfected with pTF7-5BUNL (lane 5) or transfected with pTF7-5BUNL and pTF7-5BUNS (lane 6). Twenty hours later total cellular RNA was extracted, dot blotted onto nitrocellulose filters and hybridized with ^{32}P-labelled pUC-BUNL DNA, or ^{32}P-labelled pTZ-BUNS DNA. S RNA signals were detected in cells expressing L (lanes 2, 3 and 5), but were not observed in mock (lane 1) or vTF7-3 (lane 4) infected cells.

The RNA was then analysed by Northern bloting. Total cellular RNA was extracted from cells which had been infected with vTF7-3, vTF7-3 plus transfected with pTF7-5BUNL, vTF7-3 plus vTF7-5BUNL, or vSC11-BUNL, and then transfected with RNP (Fig.5.8. lanes 1-4); RNA obtained from Bunyamwera virus-infected cells was used as a specific marker (lane BUN). The RNAs were fractionated on agarose gels containing methylmercuric hydroxide, transferred to nitrocellulose filters, and hybridized with ^{32}P-labelled pUC-BUNS DNA. Again, no hybridization was detected with RNA from cells infected with vTF7-3 alone (lane 1) post transfection; however, specific signals corresponding to the S RNA segment observed in the Bunyamwera virus infected cells (lane BUN) were detected in cells expressing the L protein either from vaccinia viruses or by the transient T7 system (lanes 2-4). It was concluded that the expressed L protein was capable of synthesizing Bunyamwera virus S RNA in the RNP transfected cells.

In order to determine the polarity of the S RNA replicated, strand-specific riboprobes were synthesized and used for the detection of the RNA amplified by the recombinant L protein. Maps of the recombinant plasmids for making S and M RNA riboprobes are shown in Fig.5.9A. Plasmids containing full-length S cDNAs, which had been cloned under control of the T7 promoter in pUC9 in either direction, were obtained from A. McGregor. Digestion with Xba I linearized the plasmid (pUC-BUNS+) and the RNA synthesized by T7 RNA polymerase *in vitro* should only hybridize to (-) sense RNA; *in vitro* transcription of Sma I linearized plasmid (pUC-BUNS-) should produce RNA which only detects (+) sense RNA. Small amounts of ^{32}P-GTP labelled S riboprobes were analysed by agarose gel electrophoresis and full-length both (-) and (+) S detecting riboprobes were observed (Fig.5.9B). Specific M gene riboprobes were also synthesized, using a recombinant plamid containing the full-length M gene under control of T7 and T3 promoters (pT3T7-BUNM) obtained from G. Nakitare. Transcription of Sma I linearized plasmid with T7 RNA polymerase produced M RNA which detects (+) M RNA, and transcription of Hind III linearized plasmid produced M RNA which would hybridize to the (-) M RNA. A mixture of the S and M probes was then used to detect either positive- or negative-sense RNA species. L segment probes were not included, as these would have detected the recombinant vaccinia virus-derived L transcript. No hybridization

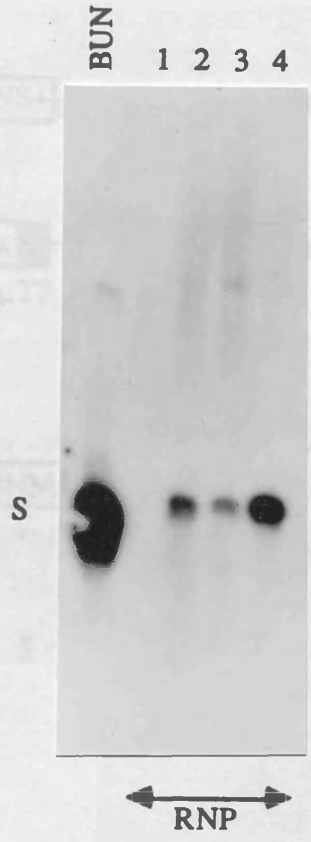


Fig.5.8. Northern blot analysis of RNA from cells transfected with RNP following infection with recombinant vaccinia viruses. CV-1 cells were infected with Bunyamwera virus (lane BUN), vTF7-3 (lane 1), vTF7-3 and transfected with pTF7-5BUNL (lane 2), vTF7-3 and vTF7-5BUNL (lane 3), vSC11-BUNL (lane 4), and subsequently transfected with RNP (lanes 1-4). Total cellular RNA was extracted at 25 hr post transfection, separated on a 1% agarose gel containing 5 mM methyl mercuric hydroxide, transferred to nitrocellulose filters and hybridized with ^{32}P-labelled pUC-BUNS DNA. The S RNA signal was detected in the RNP transfected cells which expressed the L protein (lanes 2-4), but not in RNP transfected cells which had been infected with vTF7-3 alone (lane 1).

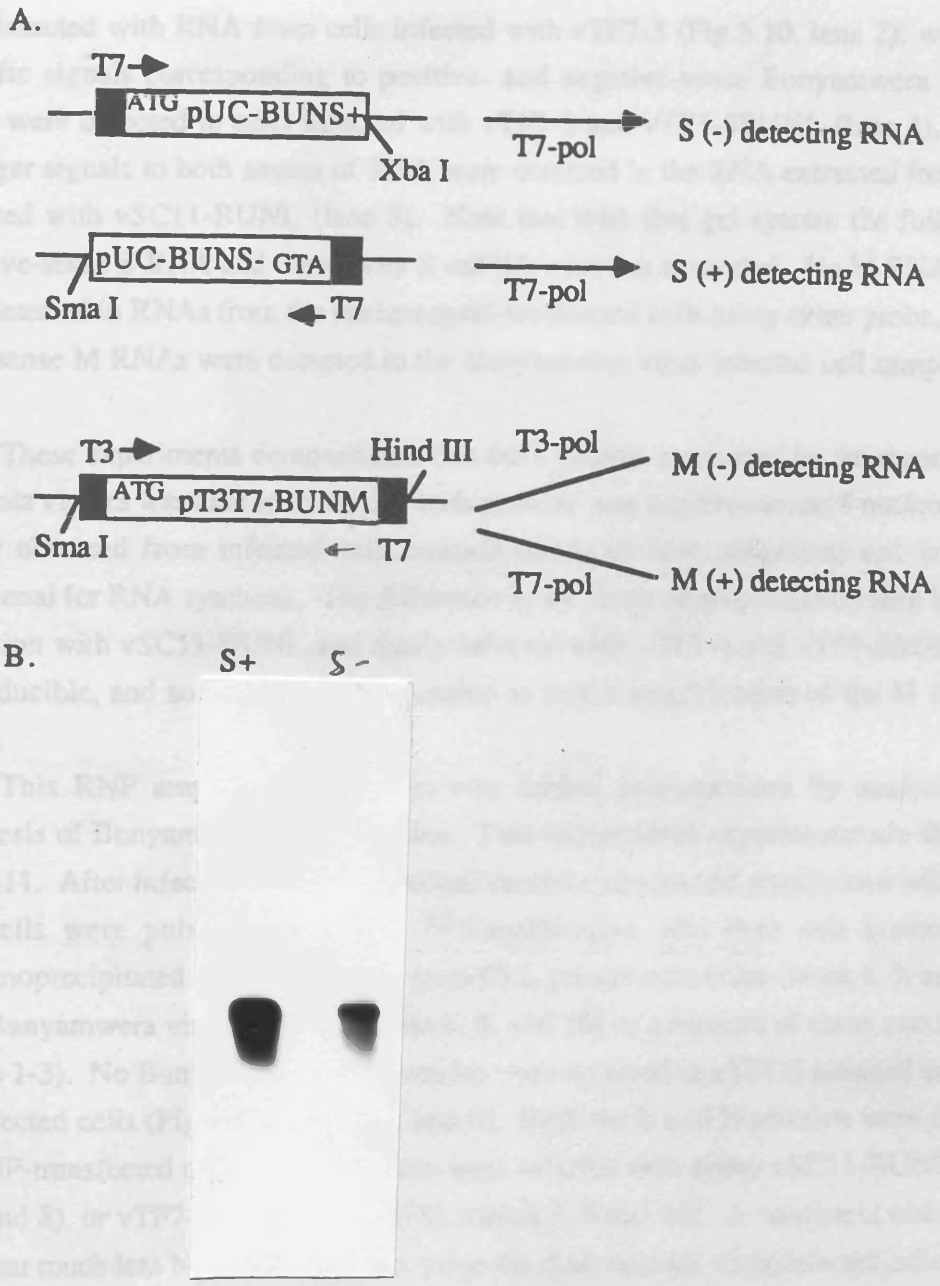


Fig.5.9. *In vitro* synthesis of ^{32}P-GTP labelled riboprobes. A. The Bunyamwera virus S cDNA was cloned under the control of the T7 promoter in either direction in pUC9. The recombinant plasmid DNA was linearized by the enzymes indicated and subjected to *in vitro* transcription to produce full-length ^{32}P-labelled riboprobes for detection of (+) or (-) strand S RNA. The Bunyamwera virus M cDNA cloned in pT3T7 vector (pT3T7-BUNM) was used for making M gene specific riboprobes. The recombinant plasmid was linearized by the enzymes indicated and transcription with T3 RNA polymerase produced a riboprobe detecting (-) sense M RNA. Transcription of the linearized pT7T3-BUNM with T7 RNA polymerase produced a riboprobe detecting (+) sense M RNA. B. ^{32}P-labelled S riboprobes detecting (+) or (-) strand S RNA were transcribed *in vitro* using T7 RNA polymerase and aliquots were analysed on an agarose gel containing 5 mM methylmercuric hydroxide, showing that the full-length S riboprobes of both senses were made.

was detected with RNA from cells infected with vTF7-3 (Fig.5.10. lane 2); weak but specific signals corresponding to positive- and negative-sense Bunyamwera virus S RNA were detected in cells infected with vTF7-3 and vTF7-5BUNL (lane 4). Much stronger signals to both senses of RNA were detected in the RNA extracted from cells infected with vSC11-BUNL (lane 3). Note that with this gel system the full-length positive-sense S RNA and the shorter S mRNA were not separated. No M RNA signal was detected in RNAs from the nucleocapsid-transfected cells using either probe, though both sense M RNAs were detected in the Bunyamwera virus-infected cell sample (lane 1).

These experiments demonstrated that the L protein expressed by the recombinant vaccinia viruses was able to transcribe both positive- and negative-sense S nucleocapsids (RNP obtained from infected cells contain RNAs of both polarities) and was thus functional for RNA synthesis. The difference in the levels of amplification seen between infection with vSC11-BUNL and dually infected with vTF7-3 and vTF7-5BUNL was reproducible, and so far I have been unable to detect amplification of the M segment RNA.

This RNP amplification system was further characterized by analyzing the synthesis of Bunyamwera virus proteins. Two independent experiments are shown in Fig.5.11. After infection with recombinant vaccinia viruses and transfection with RNP, the cells were pulse-labelled with ^{35}S-methionine, and then cell lysates were immunoprecipitated with the specific (anti-C) L protein antiserum (lanes 5, 7, and 9) or anti-Bunyamwera virus antibody (lanes 6, 8, and 10) or a mixture of these two antisera (lanes 1-3). No Bunyamwera virus proteins were detected in vTF7-3 infected and RNP transfected cells (Fig.5.11. lanes 1, 5 and 6). Both the L and N proteins were detected in RNP-transfected cells which had also been infected with either vSC11-BUNL (lanes 2, 7 and 8), or vTF7-3 and vTF7-5BUNL (lanes 3, 9 and 10). A consistent observation was that much less N protein was detected in the dual vaccinia virus-infected cells than in cells infected with vSC11-BUNL. It was also observed that minor amounts of N protein were detected in vSC11-BUNL infected cells which had been transfected with RNP and immunoprecipitated by the antiserum against the L protein (lane 7). This suggested that some of the newly synthesized N protein might have encapsidated the amplified S RNA and formed nucleocapsid structures in the RNP transfected cells, or that the N had complexed with the L protein. Further experiments are required to confirm this suggestion. The major glycoprotein G1 was not detected in the RNP-transfected cells but was observed in the Bunyamwera virus-infected control (lanes 4 and 11). The

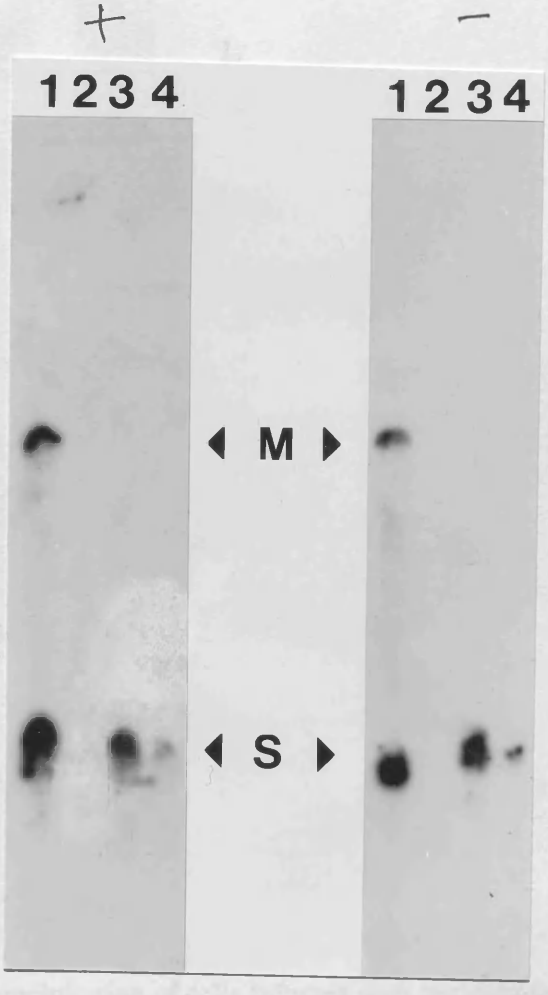


Fig. 5.10. Northern blot analysis of RNA from RNP transfected cells following infection with recombinant vaccinia virus using strand specific riboprobes. CV-1 cells were infected with recombinant vaccinia virus vTF7-3 (lane 2), vSC11-BUNL (lane 3) or both vTF7-3 and vTF7-BUNL (lane 4). The vaccinia virus infected cells were subsequently transfected with RNP and total RNA was extracted and separated on an agarose gel containing methylmercuric hydroxide. The filters were hybridized with a mixture of full length ^{32}P-labelled riboprobes specific for the M and S segment (+) or (-) strand RNAs as indicated. The positions of the M and S segment RNAs are indicated, using the Bunyamwera virus infected cell sample as markers (lane 1). Both (+) and (-) sense S segment RNAs were detected in RNP transfected cells which expressed the L protein (lanes 3 and 4), but not in cells which had been infected with vTF7-3 alone (lane 2).

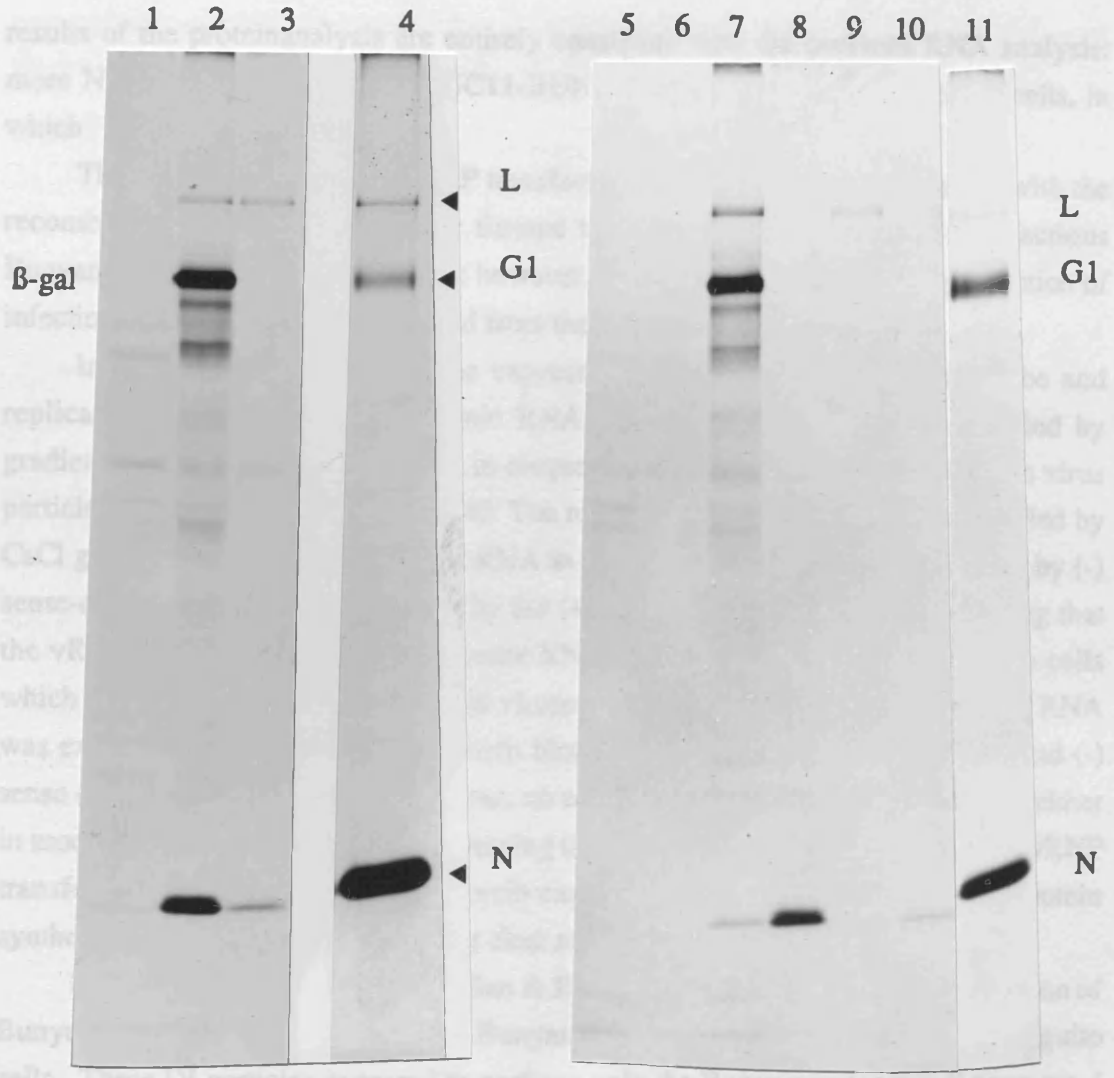


Fig.5.11. Immunoprecipitation of cells infected with recombinant vaccinia viruses and transfected with Bunyamwera virus nucleocapsids. Two independent experiments are shown in the figure. CV-1 cells were infected with vTF7-3 (lanes 1, 5 and 6), vSC11-BUNL (lanes 2, 7 and 8), vTF7-3 and vTF7-5BUNL (lanes 3, 9 and 10), or Bunyamwera virus (lanes 4 and 11). The cells were then transfected with Bunyamwera virus nucleocapsids (lanes 1-3 and 5-10) and radiolabelled cell extracts prepared 20 hr later. The cytoplasmic extracts were precipitated with a mixture of the monospecific L and Bunyamwera virus antisera (lanes 1-4), or with the L specific antiserum (lanes 5, 7, and 9) or anti-Bunyamwera virus antiserum (lanes 6, 8, 10 and 11). The positions of the Bunyamwera virus proteins L, G1 and N are indicated in lanes 4 and 11, which were cut from one side of the same gels as the other lanes to prepare the figure. The N protein was detected in cells expressing the L protein from recombinant vaccinia viruses and transfected with Bunyamwera virus nucleocapsids (lanes 2, 3, 8 and 10) by immunoprecipitation of anti-Bunyamwera virus antiserum. Minor amounts of N protein were also detected in vSC11-BUNL infected and RNP transfected cells by immunoprecipitation with the (anti-C) L specific antiserum (lane 7).

results of the proteinanalysis are entirely consistent with the previous RNA analysis: more N protein was detected in vSC11-BUNL infected and RNP transfected cells, in which more S RNA was made.

The supernatants from the RNP transfected cells which had been infected with the recombinant vaccinia viruses were titrated by plaque assay to examine if infectious Bunyamwera virus could be rescued; however, I could not demonstrate the production of infectious Bunyamwera virus rescued from the transfected RNP.

In order to define whether the expressed L protein was able to transcribe and replicate Bunyamwera virus genomic RNA, Bunyamwera virions were purified by gradient centrifugation as described in chapter 2 and the RNP was released from virus particles by disruption with 1% NP-40. The released RNP (vRNP) was then purified by CsCl gradient centrifugation. The S RNA in the vRNP preparation was detected by (-) sense-detecting S riboprobe, but not by the (+) sense-detecting riboprobe, showing that the vRNP only contained negative-sense RNA. The vRNP was transfected into cells which had been infected with vaccinia viruses expressing the L, and total cellular RNA was extracted and analysed by Northern blot. The blots were probed with (+) and (-) sense detecting S riboprobes. However, no replication of the vRNP was detected either in mock-infected cells or in cells expressing the L protein. Protein analysis of the vRNP transfected cells which had the L protein expressed also failed to detect the N protein synthesis. The reasons for this are not clear and will be discussed later.

Elliott & Wilkie (1986) and Scallan & Elliott (1991) had observed the generation of Bunyamwera virus DI particles from Bunyamwera virus persistently infected mosquito cells. These DI particles appeared to package only the Bunyamwera virus S segment. I considered to exploit these DI particles to further characterize the functionality of the L protein. BHK cells were infected with DI particles (Fig.5.12. lanes 1-3) and superinfected with vTF7-3 and vTF7-5BUNL (lane 2) or vSC11-BUNL (lane 3). Total cellular RNA was extracted after 24 hr of infection, separated on 1% agarose gels, transferred to nitrocellulose filters and hybridized with ^{32}P-labelled L gene cDNA (left panel) or ^{32}P-labelled S gene cDNA (right panel). No L RNA signal was detected in cells infected only with DI particles (left, lane 1) and no S RNA was observed either (right, lane 1). However, significant S specific signal was detected in cells infected with vSC11-BUNL (right, lane 3) and relatively less S was detected in dual vaccinia viruses infected cells (right, lane 2). This was entirely consistent with the RNP amplification data which has been described previously. More L RNA was synthesized in dual vaccinia virus infected cells (left, lane 2) than in vSC11-BUNL infected cells (lane 3). These results further confirmed that the recombinant L protein expressed from

recombinant vaccinia viruses was functional by showing its ability to replicate or amplify Bunyamwera virus S RNA in DI particles.

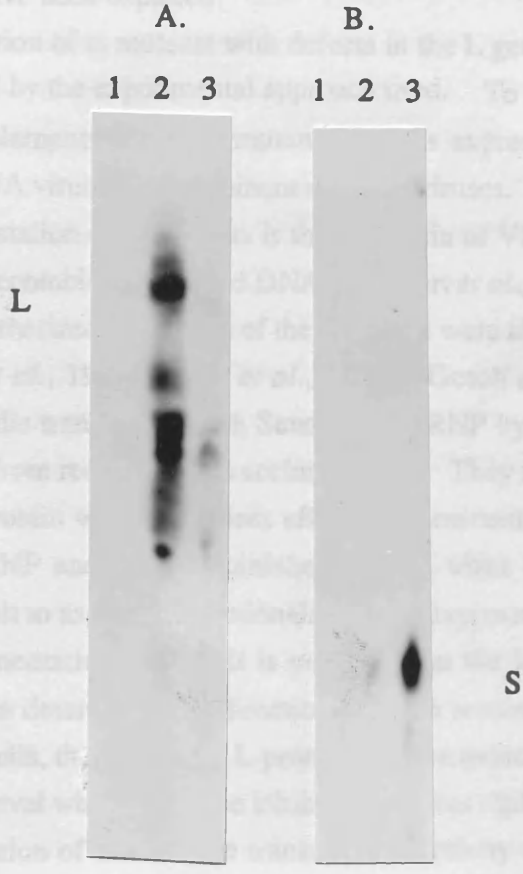


Fig.5.12. Replication of Bunyamwera virus DI particle RNA by the expressed L protein. BHK cells were infected with DI particles generated from Bunyamwera virus persistently infected mosquito cells, followed by superinfection with vTF7-3 and vTF7-5BUNL (lane 2) or vSC11-BUNL (lane 3). Total cellular RNA was extracted at 24 hr after infection and fractionated on agarose gels containing 5 mM methylmercuric hydroxide, transferred to nitrocellulose filters and hybridized with ^{32}P-labelled pTZ-BUNL DNA (panel A) or ^{32}P-labelled pTZ-BUNS DNA (panel B). No L RNA was detected in DI infected cells alone (Panel A, lane 1). The S RNA of the DI preparation was detected in cells expressing the L protein from the recombinant vaccinia viruses (Panel B, lanes 2 and 3), but was not observed in the DI infected BHK cells (lane 1).

5.6. Discussion

To test the funtionality of the L protein expressed from recombinant vaccinia viruses, three systems have been explored:

(1). Complementation of ts mutants with defects in the L gene by the expressed L protein was invalidated by the experimental approach used. To date, there have been no reports on the complementation of ts mutants in cells expressing the polymerase of any negative-strand RNA virus by recombinant vaccinia viruses. The only report which concerns the complementation of ts mutants is the L protein of VSV expressed in COS cells by transfection of recombinant plasmid DNA (Schubert *et al.*, 1985). It was shown that only cells which synthesized low levels of the L protein were able to complement the ts mutants (Schubert *et al.*, 1985; Meier *et al.*, 1987). Gotoh *et al.* (1989) rescued infectious virus from cells transfected with Sendai virus RNP by supplying the L and P/C proteins expressed from recombinant vaccinia viruses. They found that high levels of the Sendai virus L protein were much less efficient in rescuing the infectious virus from the transfected RNP and even diminished Sendai virus replication. Thus it appeares that it is difficult to assay the functionality of the expressed Bunyamwera virus L protein by a complementation assay. It is possible that the level of the L protein expressed is important in determining its functionality. In recombinant vaccinia virus and ts mutant infected cells, the expressed L protein and the defective L together might accumulate to a protein level which might be inhibitory to virus replication.

(2). The investigation of the *in vitro* transcriptase activity of the recombinant L protein was not successful, mainly due to the lack of a suitable synthetic RNA template. Transcriptase activity was demonstrated in the detergent-disrupted Bunyamwera virion preparation but only weak activity in the RNP preparation. However, the RNA polymerase activity was increased when the vTF7-3 infected cytoplasmic extract was added to the RNP programmed *in vitro* transcription. This could be due to stimulation by cellular primers used for priming bunyavirus mRNA synthesis, as it has been reported for the LAC (Patterson *et al.*, 1984), since the L protein is still associated with nucleocapsids after the purification procedure. Although it was observed that the vSC11-BUNL infected cytoplasmic extract, which contained more expressed L protein (Fig.5.3A. lane 3), showed higher activity in stimulating the RNA polymerase reaction (Fig.5.3B. lane 4), it could not be demonstrated that the increased enzyme activity was due to the L protein present in the extract. Since it is difficult to differentiate the activity of the authentic L protein with that of the expressed L protein, a synthetic nucleocapsid devoid of the L protein is needed for performing this experiment.

(3). A nucleocapsid transfection assay was then developed based on a previous report by Gotoh *et al.* (1989) who studied Sendai virus. They showed that they could enhance the yield of Sendai virus obtained from transfected nucleocapsids by supplying Sendai virus L and P/C gene products through recombinant vaccinia viruses. Here, I demonstrated that the Bunyamwera virus L protein expressed by the vaccinia virus systems, as well as by the transient expression system, was able to amplify markedly the level of S segment RNA in CV-1 cells. After transfection of cells with the Bunyamwera virus intracellular nucleocapsids, which contained both negative- and positive-sense genomes, no viral RNA was detected. This result was somewhat surprising, since the preparations of nucleocapsids contain the N and L proteins, and were expected to be capable of initiating RNA synthesis. However the *in vitro* transcription assay (Fig. 5.2) showed the nucleocapsids contained minimal enzymatic activity. The transfected nucleocapsids do provide an appropriate template for the synthesis of both polarities of at least the S segment RNA by the recombinant L protein. Thus far it has not been possible to demonstrate synthesis of the M segment RNA. Whether this reflects a relative lower abundance of the M segment, as the RNA extracted from nucleocapsid is rarely equimolar and S usually predominants (Rossier *et al.*, 1988), a lower efficiency of transfection of the M segment RNP or a lower stability of the M segment RNP is not known. Using defective interfering (DI) particles generated from Bunyamwera virus persistently infected mosquito cells, I was able to confirm the functionality of the expressed L protein by showing its ability to replicate the DI particle RNA.

The expressed L protein has transcriptase activity as the synthesis of S RNA of both polarities was observed, and the positive-sense S RNA was translated to the N protein. It is not clear whether the recombinant L protein is also able to replicate the virus genomic RNA template since the intracellular RNP contains the RNA of both polarities. It was not possible to determine if the negative-sense S RNA was transcribed from the positive-sense RNA in the RNP preparation or from the newly replicated positive-sense RNA. Since RNP were purified from the infected cells and might have associated with some other proteins, it is unclear if the L protein alone performed the polymerase function or whether some other protein components contained in the nucleocapsid also participated. This should have been clarified by using nucleocapsids (vRNP) purified from virions which only contained the negative-sense viral genomic RNA. However, attempts to replicate vRNP have not been successful. As this experiment was only performed twice, it is unclear if the failure was due to some aspects of the technique or if the purified virion RNP lacked some protein components required for the RNA synthesis. The role played by the NSs protein, which has been detected in

infected cells, is not known yet. Overton *et al.* (1987) reported that the NSs protein of PT phlebovirus was detected in the purified intracellular RNP and virion preparations. However, it is not known if the NSs of Bunyamwera virus is also associated with Bunyamwera virus RNP, and hence might have some role in viral RNA synthesis. In order to analyse the individual viral protein requirements for Bunyamwera virus RNA replication, a reconstituted system should be established using synthetic RNA templates and individually defined virus proteins.

The association of the N protein translated from the newly synthesized positive S RNA with the S RNA has not been further investigated due to the time limitations. However, it was observed that immunoprecipitation with the L protein specific antiserum of extracts of vSC11-BUNL infected cells which had been transfected with RNP showed that minor amounts of the N protein were immunoprecipitated (Fig.5.11). It was previously noted that some of the N protein in Bunyamwera virus infected cells was immunoprecipitated by the L protein antisera (see chapter 3). It is possible therefore that the N protein synthesized in the RNP transfected cells was able to associate with the newly replicated RNA and form a nucleocapsid structure. Alternatively, newly synthesized N protein could have complexed with the L protein.

Since no M segment RNA was produced and no M RNA gene products were synthesized, it was not possible to demonstrate the production of infectious Bunyamwera virus. In contrast, Gotoch *et al.* (1989) working on the unsegmented Sendai virus were able to rescue infectious virus from transfected RNP. Notwithstanding this point, the system described above should be exploitable to dissect the functions and functional domains of the complex L protein encoded by Bunyamwera virus.

Chapter 6

Site-specific mutagenesis of the L protein

6.1. Introduction

The recent identification of four conserved motifs among the RNA dependent RNA polymerase encoding elements (Poch *et al.*, 1989) provides possible targets for mutagenesis of the RNA polymerase proteins. Although the functions of the conserved regions in the putative RNA polymerase proteins are not well understood, they are predicted to be well-ordered and may have a structural role in the polymerization activity. By sequence analysis of the Bunyamwera virus L protein, Elliott (1989a and personal communication) identified the polymerase motifs in the central region of the L protein, which are embeded in a large domain of 190 amino acids (see Fig.1.8 in chapter 1).

In the previous chapter, I demonstrated that the L protein expressed by recombinant vaccinia virus had polymerase function in an RNP transfection assay. It was thus possible to probe the RNA polymerase domain within the multifunctional L protein by site-directed mutagenesis experiments. To study the functional role of the conserved amino acids in the predicted motifs in relation to RNA polymerase activity of the L protein, the conserved amino acids in the predicted RNA polymerase domain of the L protein were selected to make specific amino acid substitutions by site-directed mutagenesis of the L gene cDNA. The L gene mutants were then expressed and their functionality was determined by the RNP transfection assay.

6.2. Generation of the defined amino acid substitutions in the L protein

The predicted RNA polymerase domain of the L protein is located in the central region of the L protein between amino acid residues 1020 and 1210 (see Fig.1.8 in

chapter 1). The four conserved amino acid residues in each motif were selected as the initial targets for amino acid substitution. The positions of these residues in the L protein and their possible substitutions are shown in Fig.6.1. The sequence alignment data indicated that the L protein of Bunyamwera virus shares a feature of RNA polymerase proteins of the segmented RNA viruses which have the SDD span in motif C, whereas the similar sized L proteins of nonsegmented RNA viruses have GDN at this position. On the other hand, the RNA polymerase proteins of plus-strand RNA viruses have the conserved GDD span in this motif (Poch *et al.*, 1989 and 1990). Therefore the initial attempt was to determine if changing the SDD span to SDN in the Bunyamwera virus L protein was detrimental to the polymerase function of the L. The second D (1165) residue was thus selected as the target for making specific mutagenesis in motif C. It was also of interest to know the effect of the substitution of this D with a chemically opposite amino acid (R) or with a residue of neutral property (A) on the functionality of the L protein. Based on these considerations, a degenerate oligonucleotide (Table 6.1) was designed to cover the three desired changes so that by one mutagenesis reaction, all the three substitutions should be obtained. This degenerate oligonucleotide should also produce 5 additional amino acid substitutions.

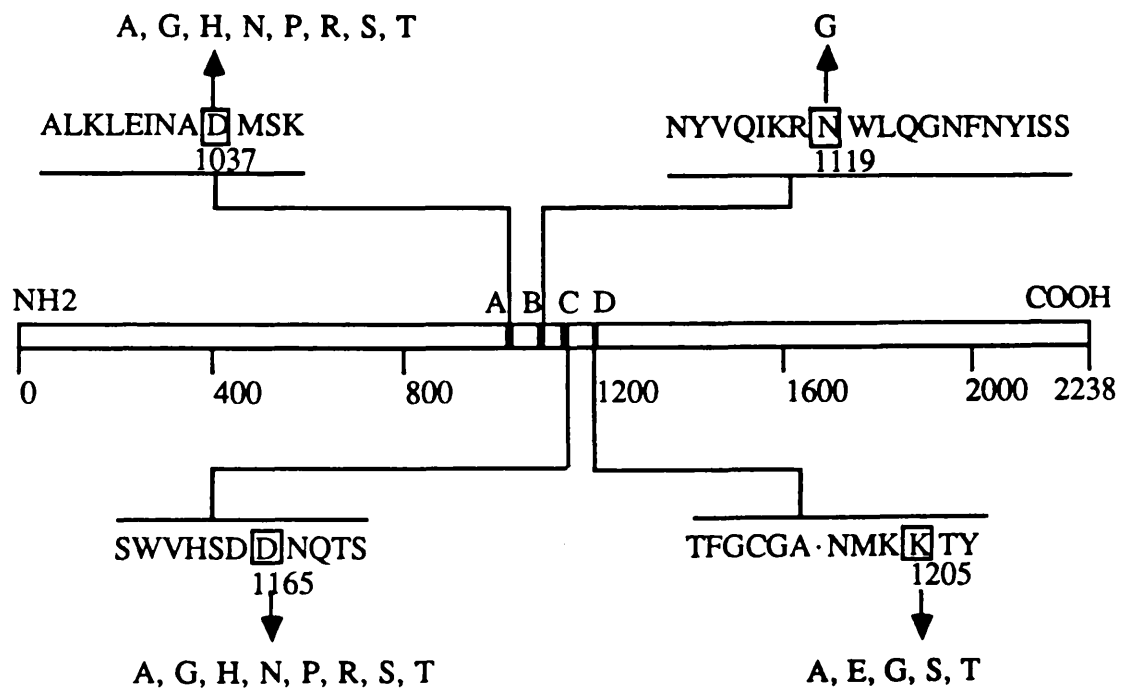


Fig.6.1. Positions of mutations in the polymerase motifs in the L protein. The L protein is represented as a linear molecule with amino acid residue numbers as indicated below. Polymerase motifs A, B, C, and D are shown as solid bands with expanded sequences in the single-letter code for amino acids. Single amino acid substitutions to be introduced in the regions are indicated.

The most conserved D (1037) residue in motif A was also chosen for substitution with three chemically different amino acids using a degenerate oligonucleotide (Table 6.1), which should also produce 5 additional amino acid substitutions. The K (1205) residue in motif D was chosen for substitution with an A residue and four other residues using a degenerate oligonucleotide (Table 6.1). The N residue at position 1119 in the L protein was found to align best with the most conserved G residue in motif B which had been identified in all the other RNA polymerase proteins analysed to date (Poch *et al.*, 1989). The importance of this N was also addressed by its conservation in the L proteins of Hantaan virus and TSWV (de Haan *et al.*, 1991). It was not known if this N (1119) residue would be an important amino acid in motif B of the Bunyamwera virus L protein. Hence I determined to substitute this N with a G residue to clarify this experimentally.

Table 6.1. Synthetic oligonucleotides used for introducing the specific changes in the L

Position	Sequence of oligonucleotide
D-1037	5'-GGAAATAAATGCAG[A/C]A[C/G]CATGTCAAAATG-3'
N-1119	5'-CCAAATTAAAAGAGGCTGGCTC-3'
D-1165	5'-GGTGCATTCAGATG[A/C]A[C/G]C AATCAAAC-3'
K-1205	5'-GGCAAACATGAAAA[G]A[C/G]A[T]ACATATATTAC-3'

Degenerate positions are shown in bracket.

The scheme for site-directed mutagenesis of the L gene cDNA is shown in Fig.6.2. To make site-specific mutations in the L, it was considered that a small cDNA fragment containing the RNA polymerase motifs should be cloned into a vector to make single stranded DNA for the mutagenesis reactions. This fragment should also be easily cloned back into the full-length cDNA after mutagenesis. By searching of the restriction enzyme sites in the L cDNA, the EcoR I (nt 2877) to Pst I (nt 3811) fragment was convenient for removal from the L gene cDNA, but it would be difficult to clone this fragment back into the L gene cDNA in the right position after mutagensis because of three other EcoR I sites in the L. Therefore, I decided to make a silent mutation first to introduce a unique Xho I site into the L cDNA at the position of nucleotide 3145 so that the resulting Xho I/Pst I fragment of the L could be replaced with the mutated fragment in a one step ligation.

Recombinant plasmid pTZ-BUNL was digested with EcoR I and Pst I, and the 1 kb EcoR I/Pst I fragment was isolated and inserted between the EcoR I and Pst I sites of M13mp19 to give MP19-L(E/P). Mutagenesis was performed using Eckstein's method

(chapter 2) and the DNA samples in each mutagenesis reaction were monitored by agarose gel analysis (Fig.6.3). The final DNA reaction sample (S4) was transformed into *E. coli* TG1 cells and dsDNA was prepared, digested with Xho I and Pst I and analysed by agarose gel electrophoresis. Plasmids containing the 0.6 kb Xho I/Pst I fragment were obtained and the correct sequence of the 0.6 kb insert was confirmed by dideoxynucleotide termination sequencing using a primer complementary to nucleotides 3079-3097 of the L gene. The EcoR I/Pst I fragment containing the Xho I site was then removed from MP19-L(E/P) and recloned into pTF7-5BUNL (Fig.6.2). The same Xho I containing fragment was also inserted into pUC119 [pUC119-L(E/P)] for other site-directed mutagenesis reactions since it was found that pUC119 was much better than M13mp19 for making template DNA for oligonucleotide-directed mutagenesis and for recovering the dsDNA fragment.

Four oligonucleotide-directed mutagenesis reactions were performed using pUC119-L(E/P). The DNAs were transformed into *E. coli* DH5 cells and single stranded DNA templates were prepared (see chapter 2). The mutants were screened by sequence analysis which involved termination with only ddATP and/or ddGTP. Fig.6.4 is an example of a screening gel showing location of mutations by a half set of sequencing reactions which were terminated with ddGTP and ddATP. All of the 8 possible amino acid substitutions of 1037 D residue in motif A and 1165 D residue in motif C, as well as one substitution of 1119 N residue in motif B, were obtained. These mutations were confirmed by the full set of sequence reaction analysis (Figs.6.5-6.7). The mutant (L-G-1119) was found later to have a one base deletion in the oligonucleotide used for the site-directed mutagenesis, which changed the protein reading frame (Fig.6.6). The mutagenesis reaction to change 1205 K residue in motif D was unsuccessful. This most conserved K residue resides in a sequence containing 7 A bases. About 120 colonies were screened by A-track sequencing analysis; two deletion mutants in the region were obtained (Fig.6.8) but none of the DNAs contained the desired mutations. It appeared that the degenerate oligonucleotide was unsuitable for the mutagenesis in this A-rich region. The designations of the obtained mutations in motifs A, B, and C are summarized in Table 6.2.

Each mutated Xho I/Pst I fragment was then removed from pUC119-L(E/P) to substitute the wild type Xho I/Pst I fragment in pTF7-5BUNL, which had been digested with Xho I and partially digested with Pst I (Fig.6.2). The Pst I site at the 3' end of the cDNA created by GC tailing during the initial cloning of the L RNA was apparently more resistant to digestion, so that only a one step cloning procedure was required.

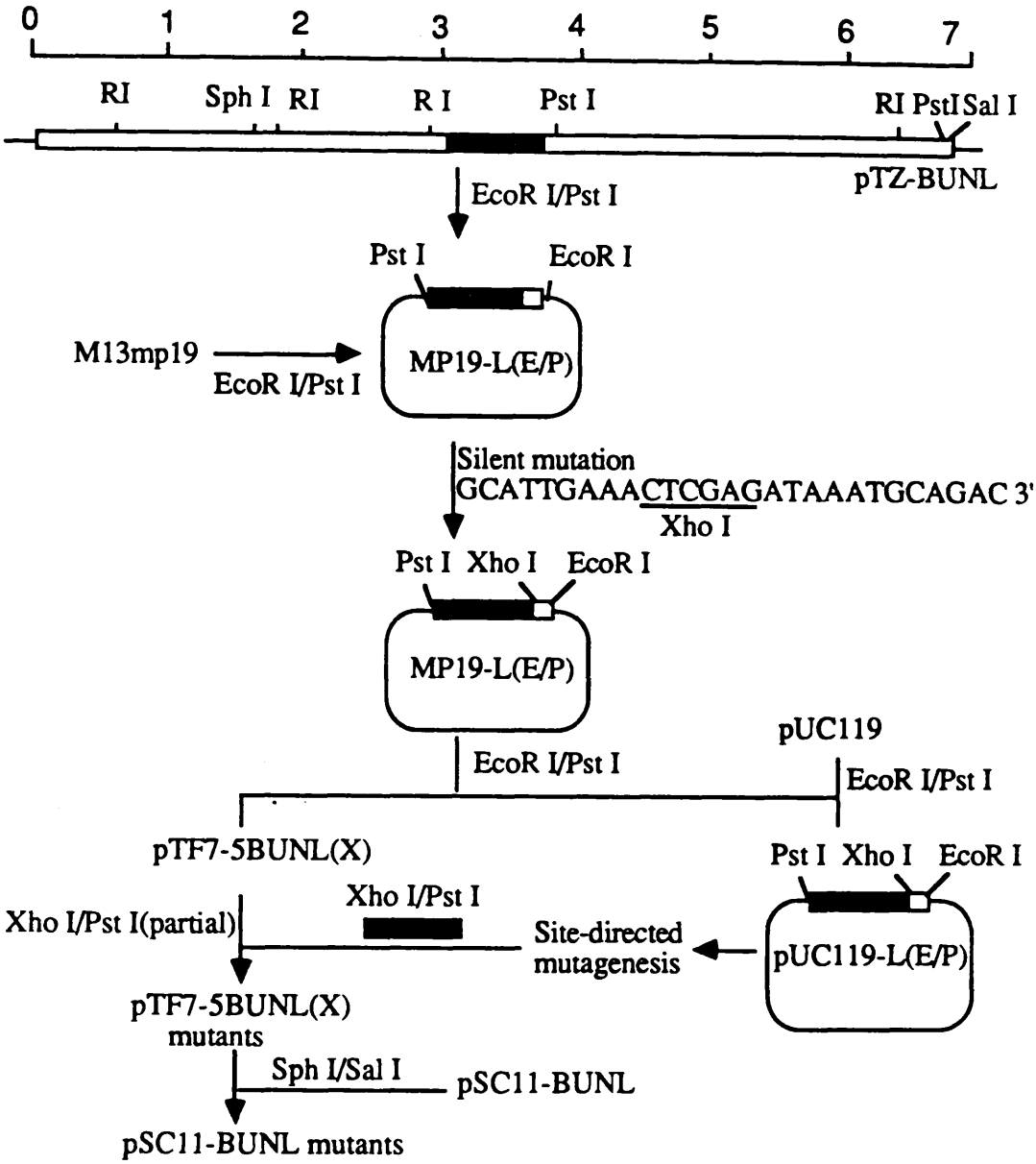


Fig.6.2. Scheme for the generation of site-specific mutations in the L gene cDNA. pTZ-BUNL was digested with Pst I and EcoR I, the 1 kb EcoR I/Pst I fragment cloned into similarly digested M13mp19 and the resulting construct designated MP19-L(E/P). Site directed mutagenesis was performed using the oligonucleotide shown in the figure to create a Xho I site, which does not affect the amino acid sequence of the L protein. The EcoR I/Pst I fragment carrying the Xho I site (nt 3145) was then inserted into EcoR I and Pst I digested pUC119 for the required mutagenesis reactions. This fragment was also used to replace the EcoR I/Pst I fragment in pTF7-5BUNL by a series of subclonings for future use. Mutagenesis was carried out on pUC119-L(E/P) using Eckstein's method (see chapter 2). The mutated Xho I/Pst I fragment was then ligated with the Xho I and Pst I partially digested pTF7-5BUNL DNA from which the 0.6 kb wild type Xho I/Pst I fragment had been removed. To insert the mutated L gene cDNA into the vaccinia virus transfer vector pSC11, pSC11-BUNL was digested with Sph I and Sal I, and the L cDNA Sph I/Sal I fragment in pSC11-BUNL was replaced with the mutated restriction fragment from pTF7-5BUNL mutants.

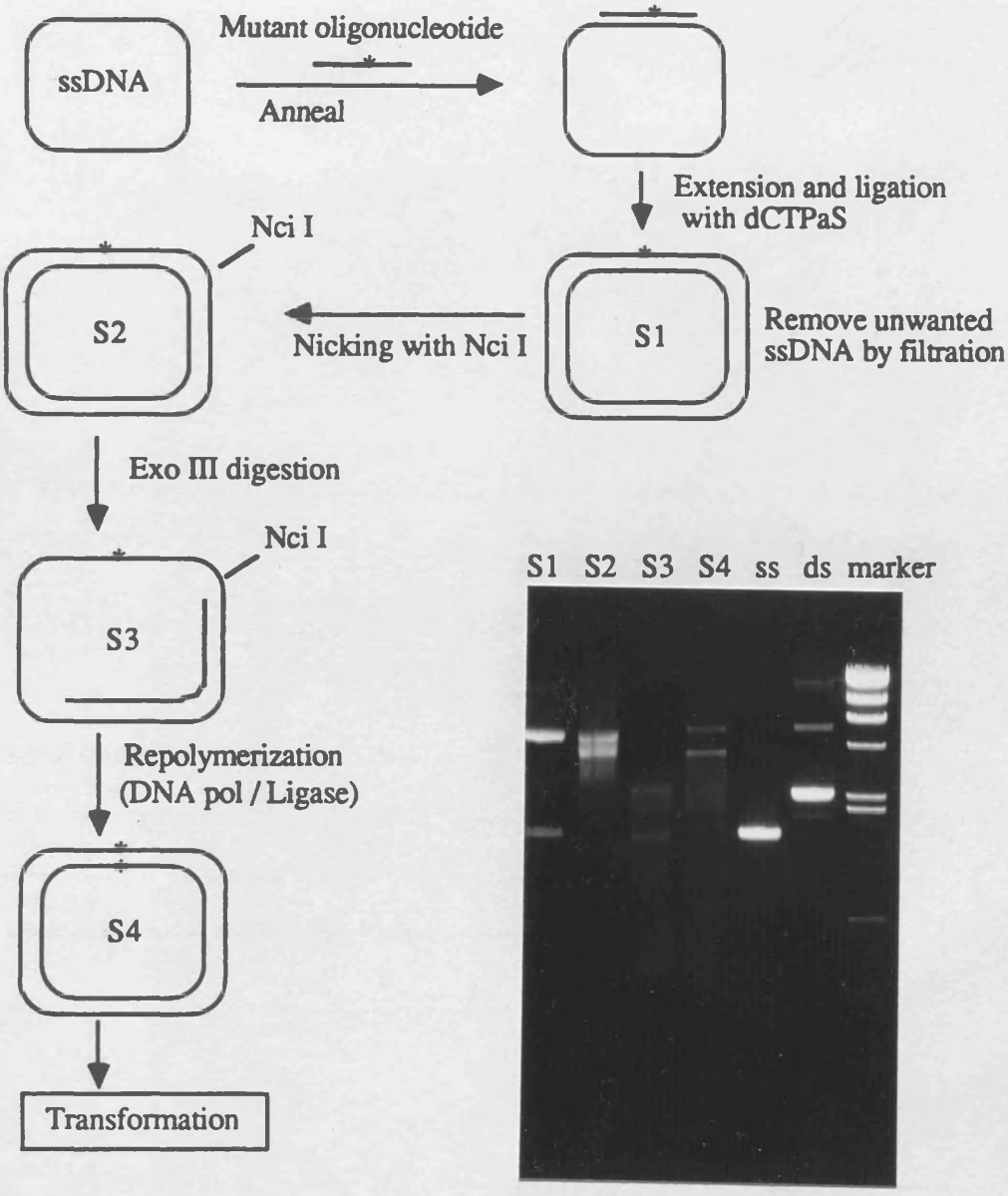


Fig.6.3. Protocol for making site-directed mutagenesis by Eckstein's mutagenesis method. The mutant oligonucleotide was annealed with the single stranded DNA template MP19-L(E/P) or pUC119-L(E/P) and the polymerization reaction was performed at 16 °C overnight. The samples (S1) were filtered to remove unwanted remaining ssDNA and the dsDNA was nicked by digestion with Nco I. The nicked DNA (S2) was further digested with Exo III (S3), which was then repolymerized by DNA polymerase I and ligated by T4 DNA ligase (S4). The DNA (S4) was then transformed into *E. coli* TG1 [MP19-L(E/P)] or DH5 [pUC119-L(E/P)] cells and the desired mutants were screened by dideoxynucleotide termination sequence analysis. The DNA samples (S1, S2, S3, and S4) taken during each step of the mutagenesis protocol were analysed by electrophoresis on an agarose gel. The single stranded and double stranded DNA of pUC119-L(E/P) and the Hind III digested lambda DNA size markers are indicated.

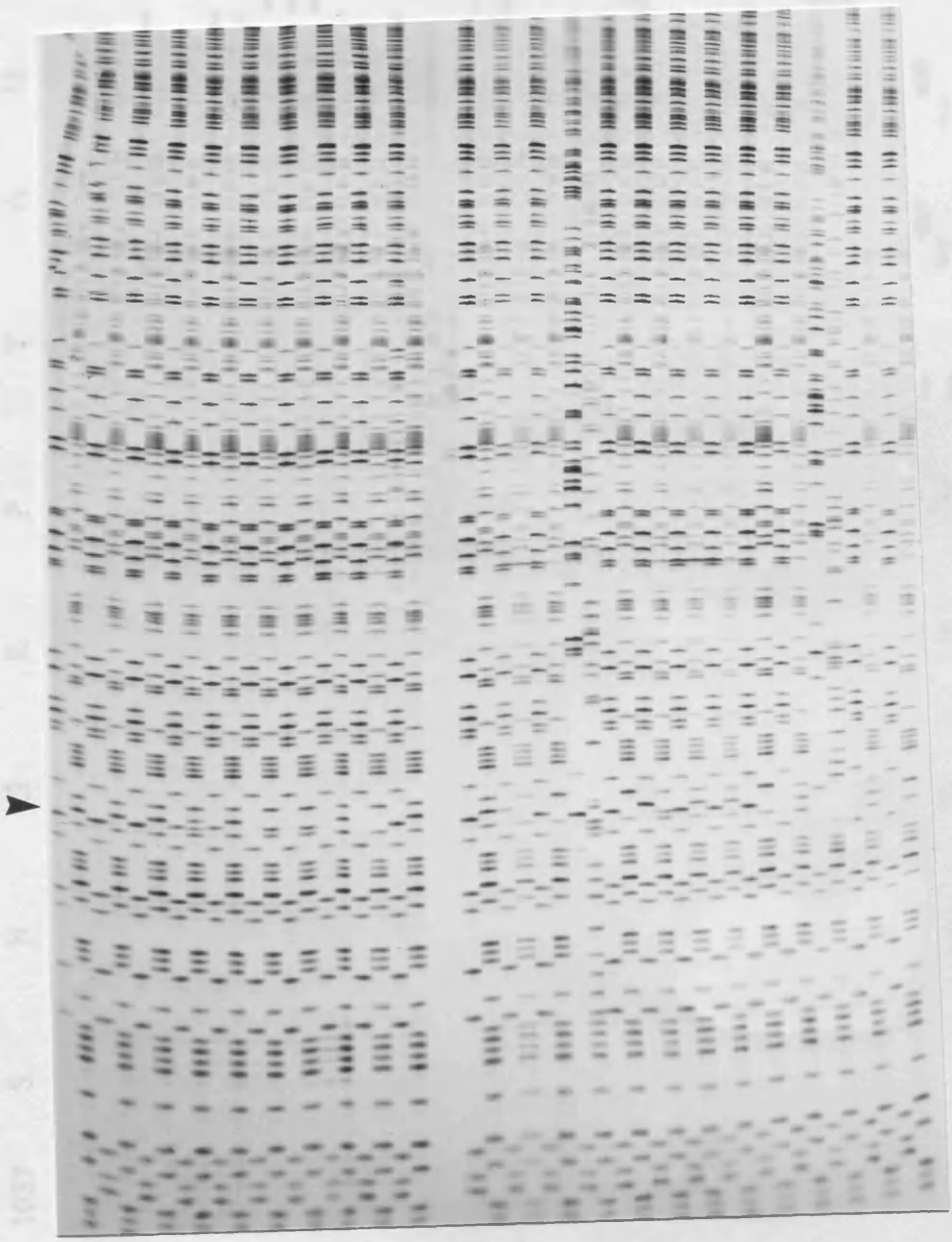


Fig.6.4. An example of a screening gel showing the identification of mutations by a half set sequencing reactions which were terminated with ddGTP and ddATP. The order of the lanes is G and A, and the position of mutations is indicated.

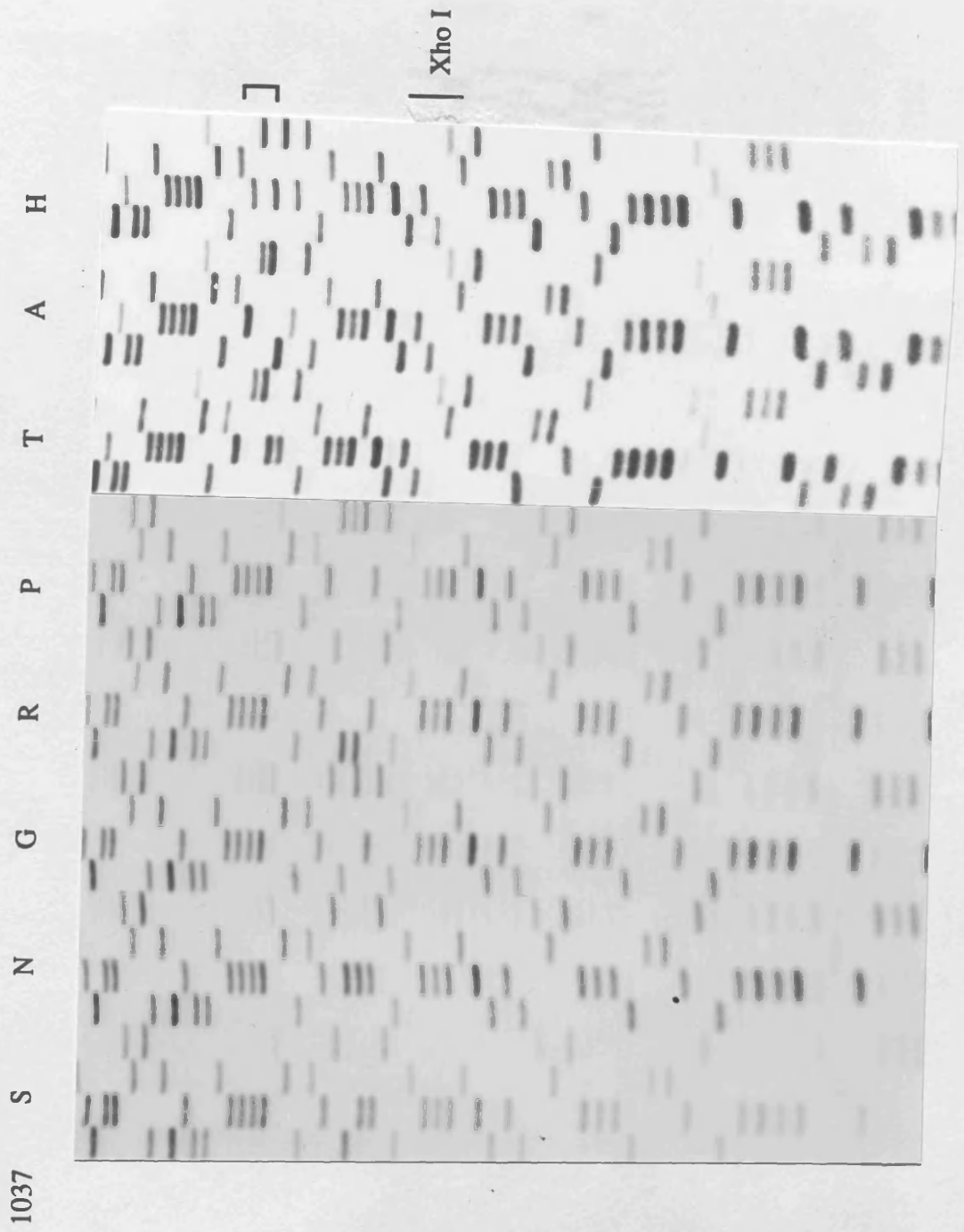


Fig.6.5. Sequencing gel showing the mutations in motif A. The changed nucleotide sequence of each mutant, the position of the mutations and the created Xho I site by silent mutation are indicated. The order of the lanes is G, A, T, and C.

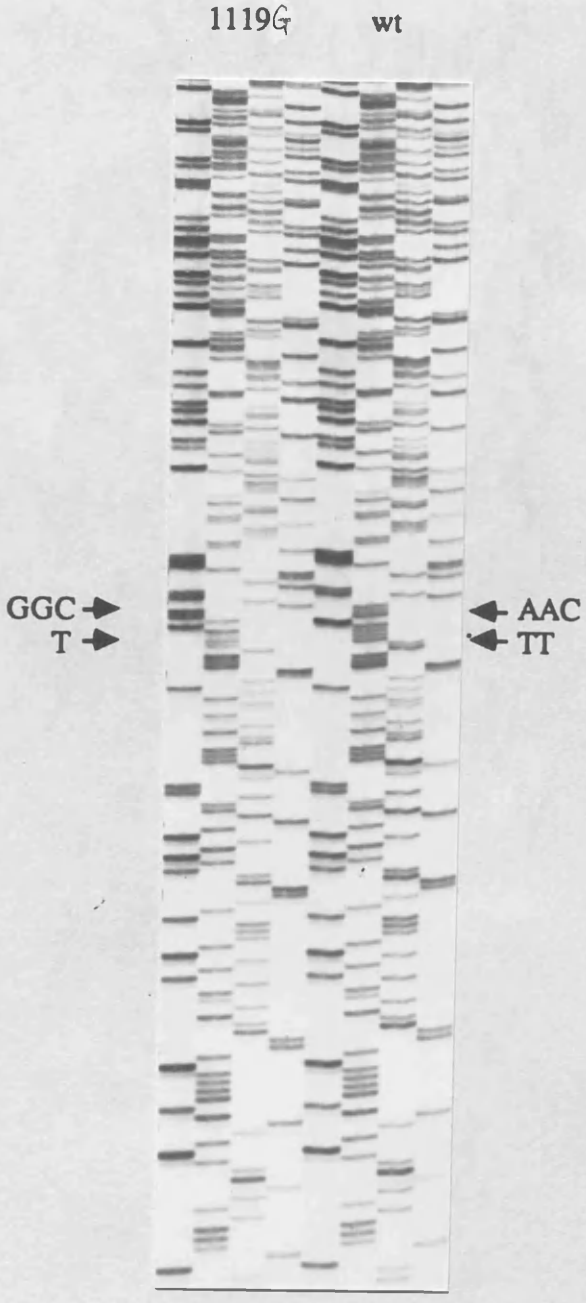


Fig.6.6. Sequencing gel showing the mutations in motif B. The mutated nucleotide sequence (GGC) and the wild type nucleotides (AAC) are indicated. The positions of the mutations and the one base deletion in the synthetic oligonucleotide are also indicated. The order of the lanes is G, A, T, and C.

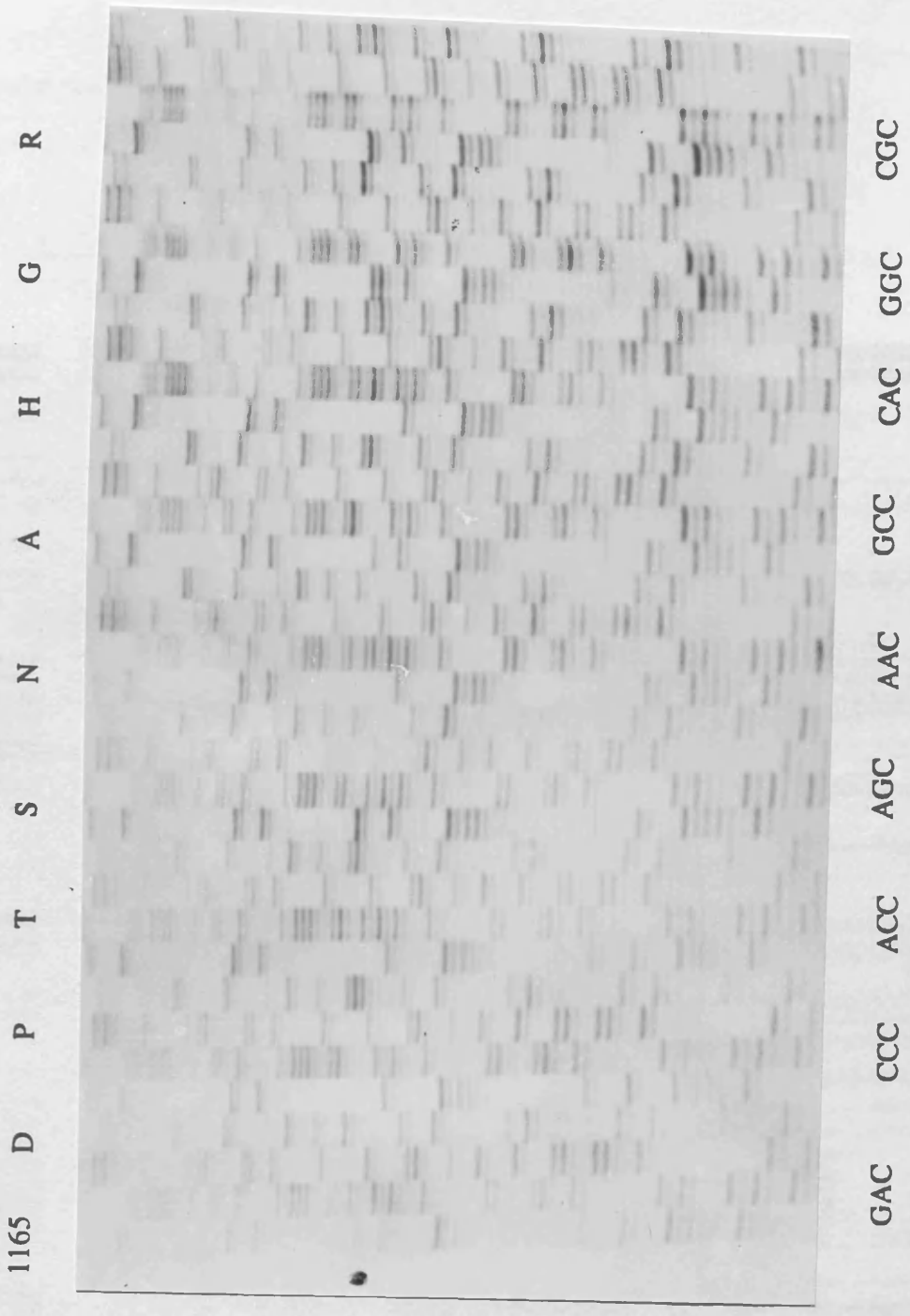


Fig.6.7. Sequencing gel showing the mutations in motif C. The mutated nucleotide sequence of each mutant and the position of mutations are indicated. The order of the lanes is G, A, T, and C.

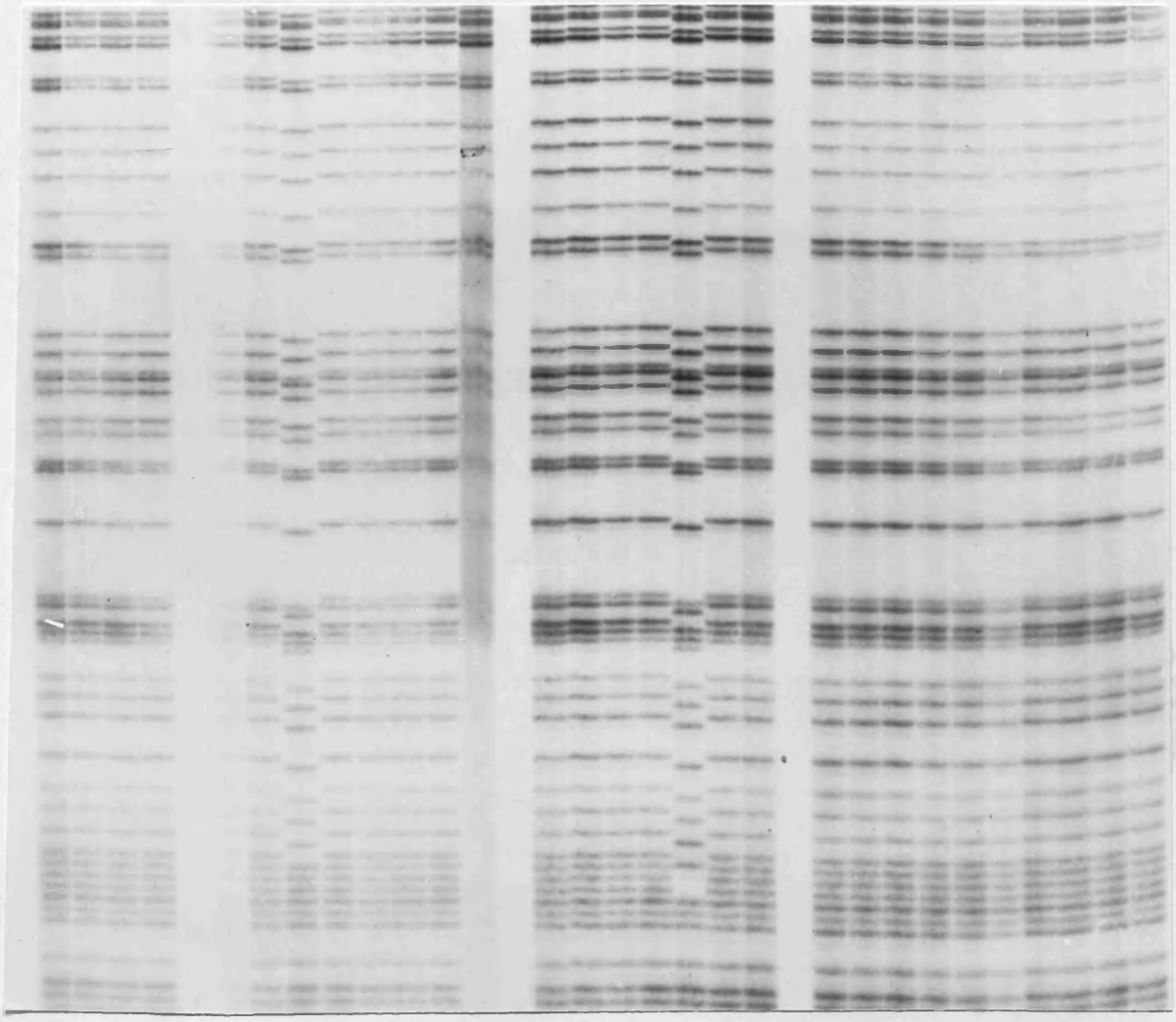


Fig.6.8. Sequencing gel showing the mutations in motif D. Single stranded DNA templates were prepared and the sequencing reactions terminated with only ddATP to determine the changes among the 7 As (as indicated) in the mutagenesis region. Only two deletion mutants were detected and none of the desired changes were obtained.

The pTF7-5BUNL mutants obtained were further confirmed by direct nucleotide sequence analysis using alkaline denatured plasmid DNAs.

Table 6.2. Mutations in RNA polymerase motifs of the L protein

Designation	Nucleic acid sequence	Amino acid sequence
Mutagenesis in Motif A:		
L-D-1037 (wt)	GAC GAC ATG	ADN
L-A-1037*	GCC	A
L-G-1037	GGC	G
L-H-1037	CAC	H
L-N-1037*	AAC	N
L-P-1037	CCC	P
L-R-1037*	CGC	R
L-S-1037	AGC	S
L-T-1037	ACC	T
Mutagenesis in Motif B:		
L-N-1119 (wt)	AGA AAC TGG	RNW
L-G-1119*	GGC	G
Mutagenesis in Motif C:		
L-D-1165 (wt)	TCA GAT GAC	SDD
L-A-1165*	GCC	A
L-G-1165	GGC	G
L-H-1165	CAC	H
L-N-1165*	AAC	N
L-P-1165	CCC	P
L-R-1165*	CGC	R
L-S-1165	AGC	S
L-T-1165	ACC	T

The mutated L genes indicated with * were cloned into recombinant vaccinia viruses.

6.3. Expression of the L protein mutants.

In the previous analysis of the L protein by the RNP transfection assay, it was consistently observed that the L protein expressed by vSC11-BUNL had greater activity. Therefore, the six initially designed mutations in the L (1037 D and 1165 D replaced with A, N, or R, and 1119 G to N substitution) were cloned into the vaccinia virus transfer vector pSC11 to make recombinant vaccinia viruses. These pTF7-5BUNL mutants were digested with the Sph I and Sal I, and the mutated Sph I/Sal I fragment was ligated with Sph I and Sal I digested pSC11-BUNL DNA from which the wild type Sph I/Sal I fragment had been removed. The resulting pSC11-BUNL mutant DNAs were then transfected into vaccinia virus WR strain infected CV-1 cells to generate recombinant vaccinia virus vSC11-BUNL mutants (see chapter 2). TK^- blue plaques were obtained after staining with X-Gal and the recombinants were characterized by Southern blot analysis. The DNAs extracted from blue plaque infected TK^- cells were fractionated on a 1% agarose gel and transferred to nitrocellulose. Hybridization with ^{32}P-labelled pTZ-BUNL DNA indicated that all the vSC11-BUNL mutants had the expected genetic structures (Fig.6.9A).

To determine the effect of the amino acid substitution on the L protein activity, the expression of the L protein mutants were first analysed. CV-1 cells were infected with vSC11-BUNL mutants and labelled with ^{35}S-methionine overnight after 6 hr of infection. Cell extracts were prepared and immunoprecipitated with the L specific antisera (anti-C plus anti-N) followed by gel electrophoresis (Fig.6.9B). Except for L-G-1119, which had a one base deletion, all the mutants synthesized an antigenically authentic protein having the same size as the L protein observed in Bunyamwera virus infected cells.

Expression of the other nine L protein mutants generated, which had been cloned into pTF7-5 under control of the T7 promoter, was analysed by transfecting plasmid DNA into vTF7-3 infected cells and then labelling with ^{35}S-methionine (Fig.6.10). Cell extracts were prepared and immunoprecipitated with the L specific antisera (anti-C plus anti-N) followed by gel electrophoresis. L protein was detected in pTF7-5BUNL mutants (L-G-1037, L-H-1037, L-P-1037, L-S-1037, L-T-1037, L-G-1165, L-P-1165, L-S-1165, and L-T-1165) transfected cells; no L was seen in the cells infected with vTF7-3 alone (lane 0). The pTF7-5BUNL mutant with 1119 N substituted with G produced a polypeptide about the half size of the L in the transfected cells due to a base deletion in the synthetic oligonucleotide which resulted in the early termination of the translation.

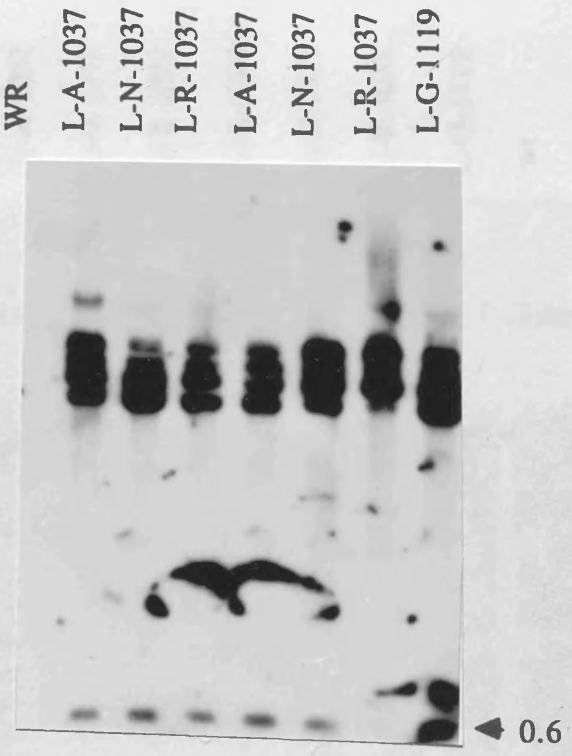


Fig.6.9. Characterization of recombinant vaccinia virus vSC11-BUNL mutants. A. Southern blot analysis. The seven mutated L cDNA fragments(L-A-1037, L-N-1037, L-R-1037, L-A-1165, L-N-1165, L-R-1165, L-G-1119) were cloned into pSC11 and recombinant vaccinia virus vSC11-BUNL mutants were selected (see chapter 2). CV-1 cells were infected with vSC11-BUNL mutants and total cellular DNA extracted at 24 hr postinfection. The DNA was digested with Xho I and Pst I, fractionated on a 1% agarose gel and transferred to a nitrocellulose filter. Hybridization with ^{32}P-labelled pTZ-BUNL showed that all the mutants had the expected genetic structure, which is not seen in the parental (WR) virus. The position of the 0.6 kb Xho I/Pst I fragment band is indicated.

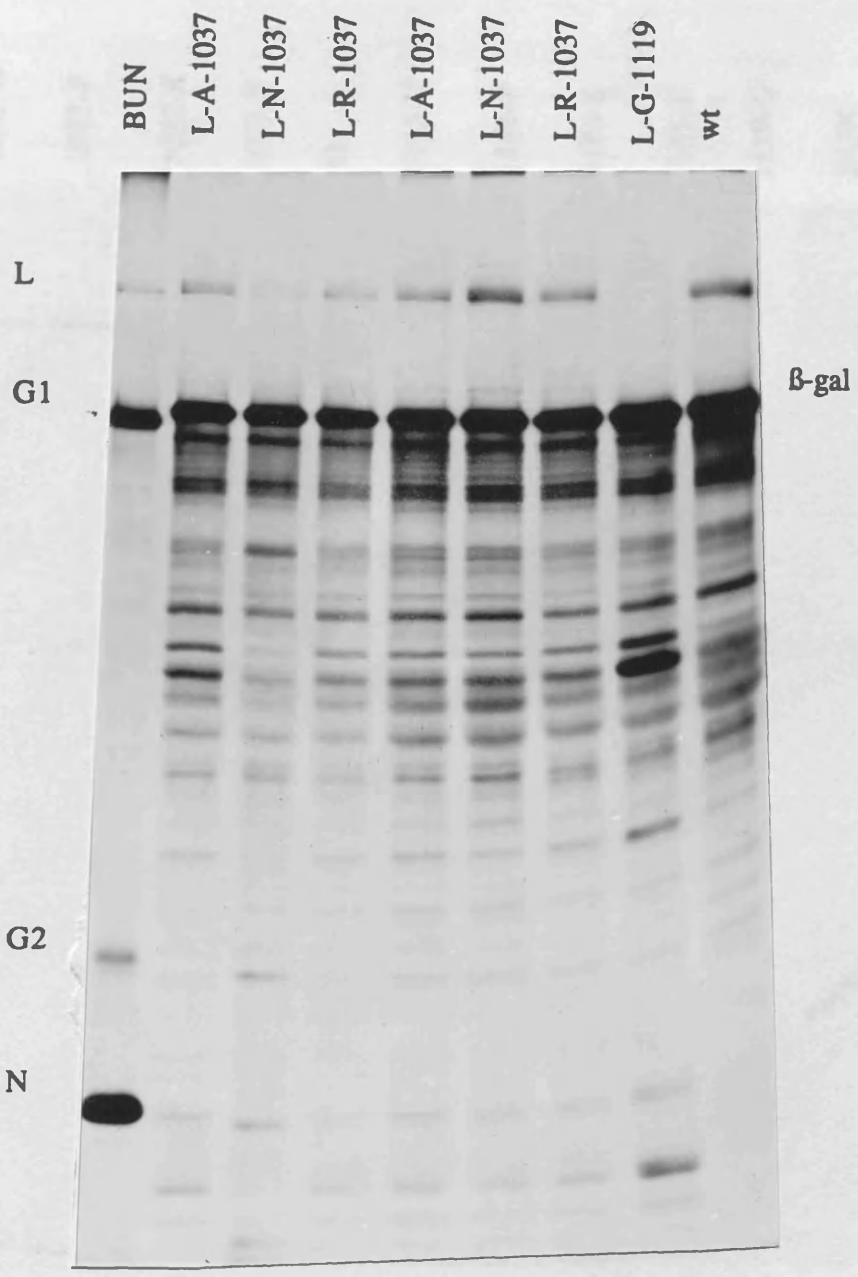


Fig.6.9 **B**. Expression of L protein by vSC11-BUNL mutants. CV-1 cells were infected with vSC11-BUNL mutants and the cells labelled with ^{35}S-methionine overnight after 6 hr of infection. Cytoplasmic extracts were immunoprecipitated with the specific antisera against the L protein (anti-C plus anti-N) followed by electrophoresis in a 12% polyacrylamide-SDS gel. Except for L-G-1119, L protein was detected in each recombinant vaccinia virus vSC11-BUNL mutant and vSC11-BUNL (wt) infected cells. The positions of L, G1, G2, N, and ß-galactosidase are indicated.

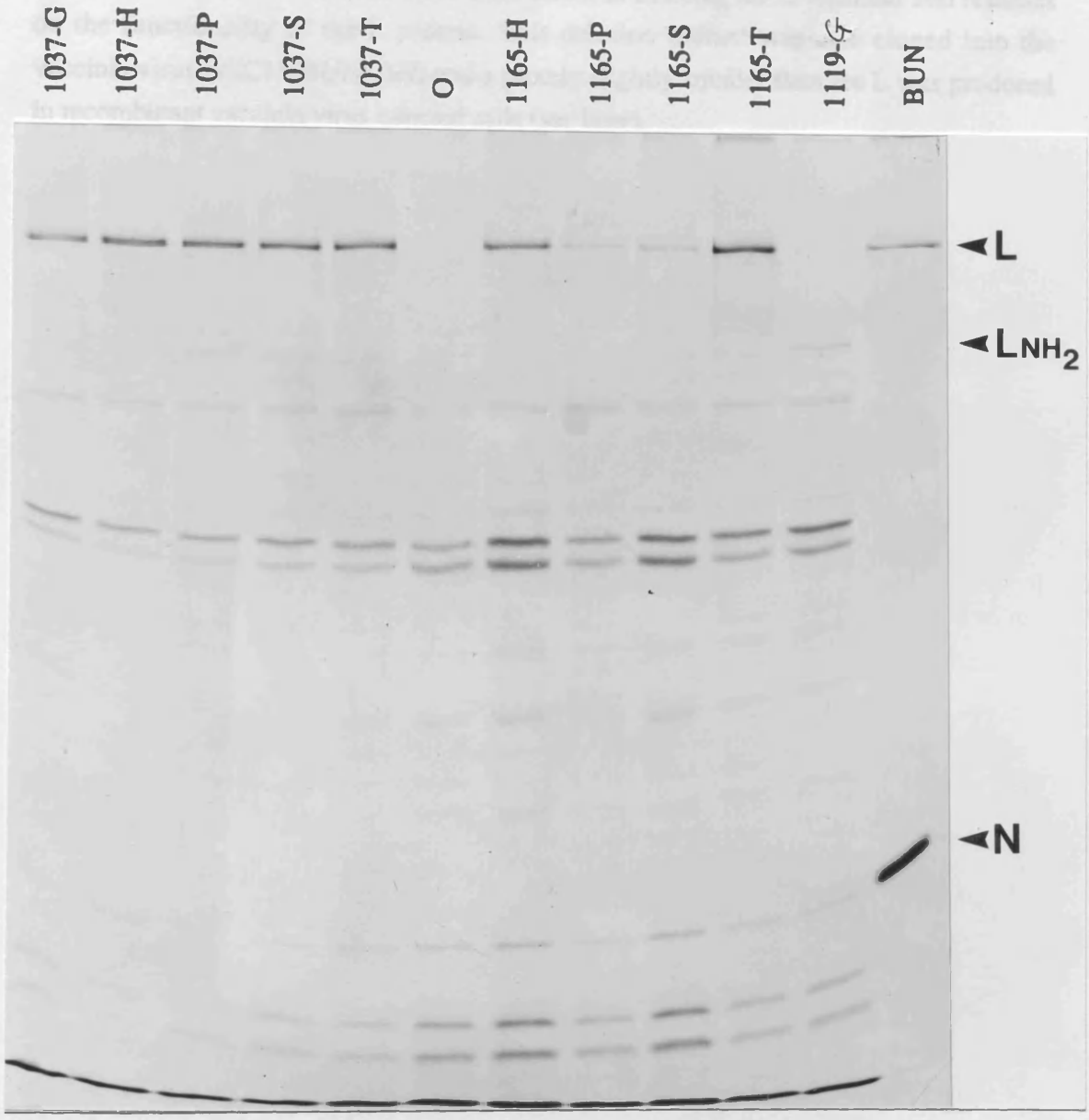


Fig.6.10. Transient expression of the mutant L proteins by transfection of the pTF7-5BUNL mutant DNAs. The mutant pTF7-5BUNL DNAs (20 μg) were transfected into vTF7-3 infected CV-1 cells and the cells labelled with ^{35}S-methionine overnight after 6 hr of transfection. Cytoplasmic extracts were immunoprecipitated with the specific L protein antisera (anti-C and anti-N). Except for L-G-1119, the L protein was expressed in cells transfected with each mutant pTF7-5BUNL DNA as indicated above the lanes. L-G-1119 had a one base deletion in the oligonucleotide used for mutagenesis and thus created a premature termination, producing a polypeptide which is about the half size of the L protein (indicated as LNH2). Lane 0 is the cells without plasmid transfection.

I also made a deletion mutant, which had the 3' end Nde I fragment (nt 6021-6519) of the L cDNA removed, to determine the effect of deleting the C-terminal 166 residues on the functionality of the L protein. This deletion mutant was also cloned into the vaccinia virus (vSC11-BUNLΔN) and a protein slightly smaller than the L was produced in recombinant vaccinia virus infected cells (see later).

6.4. Functional analysis of the mutant L proteins

The functionality of the L protein mutants were then assayed by the Bunyamwera virus RNP transfection assay as described in chapter 5. Both N protein synthesis and the S RNA amplification were analysed in the RNP transfected cells, which also had the L protein expressed, to determine the functionality of the mutants.

The functionality of the six vSC11-BUNL mutants was firstly determined by protein analysis. RNP were transfected into the cells which had been infected with the six vSC11-BUNL mutants and the cells were labelled with ^{35}S-methionine overnight after 6 hr of transfection. Immunoprecipitation of the extracts with a mixture of anti-Bunyamwera virus antiserum and the specific antiserum against the L protein revealed that the N protein was detected in the vSC11-BUNL-A 1037 mutant infected and RNP transfected cells (Fig.6.11A), indicating that this mutant maintained the RNA polymerase activity. The other two substitutions in 1037 D residue by R or N in the L protein reduced the enzyme activity. The substitution of 1165 D in motif B with N, A or R made greatly reduced level of N. The activity of vSC11-BUNL-A 1037 mutant was also confirmed by Northern blot analysis. Total cellular RNA was extracted from the RNP transfected cells (Fig.6.11B. lanes 1-3) which had been mock-infected (lane 1), infected with vSC11-BUNL-A 1037 mutant (lane 2), or vSC11-BUNL(wt) (lane 3), and fractionated on an agarose gel containing methylmercuric hydroxyde. Hybridization with ^{32}P-labelled pTF7-5BUNS DNA showed that the S specific RNA signal was detected in vSC11-BUNL-A 1037 mutant (lane 2) and vSC11-BUNL(wt) (lane 3) infected cells, but not in the mock-infected cells (lane 1). Preliminary Northern blot analysis of RNA from the other 5 vSC11-BUNL mutants (L-R-1037, L-N-1037, L-A-1165, L-R-1165, and L-N-1165) infected cells did not show S RNA amplification, indicating that these 5 substitutions in the L reduced . the RNA polymerase activity.

Some pTF7-5BUNL mutants with mutations in motif B and C were also assayed by the RNP transfection assay. pTF7-5BUNL mutant DNAs (L-G-1037, L-H-1037, L-P-1037, L-S-1037, L-T-1037, L-A-1165, L-H-1165, L-P-1165, L-S-1165, or L-T-1165), together with the RNP, were transfected into vTF7-3 infected CV-1 cells and the cells

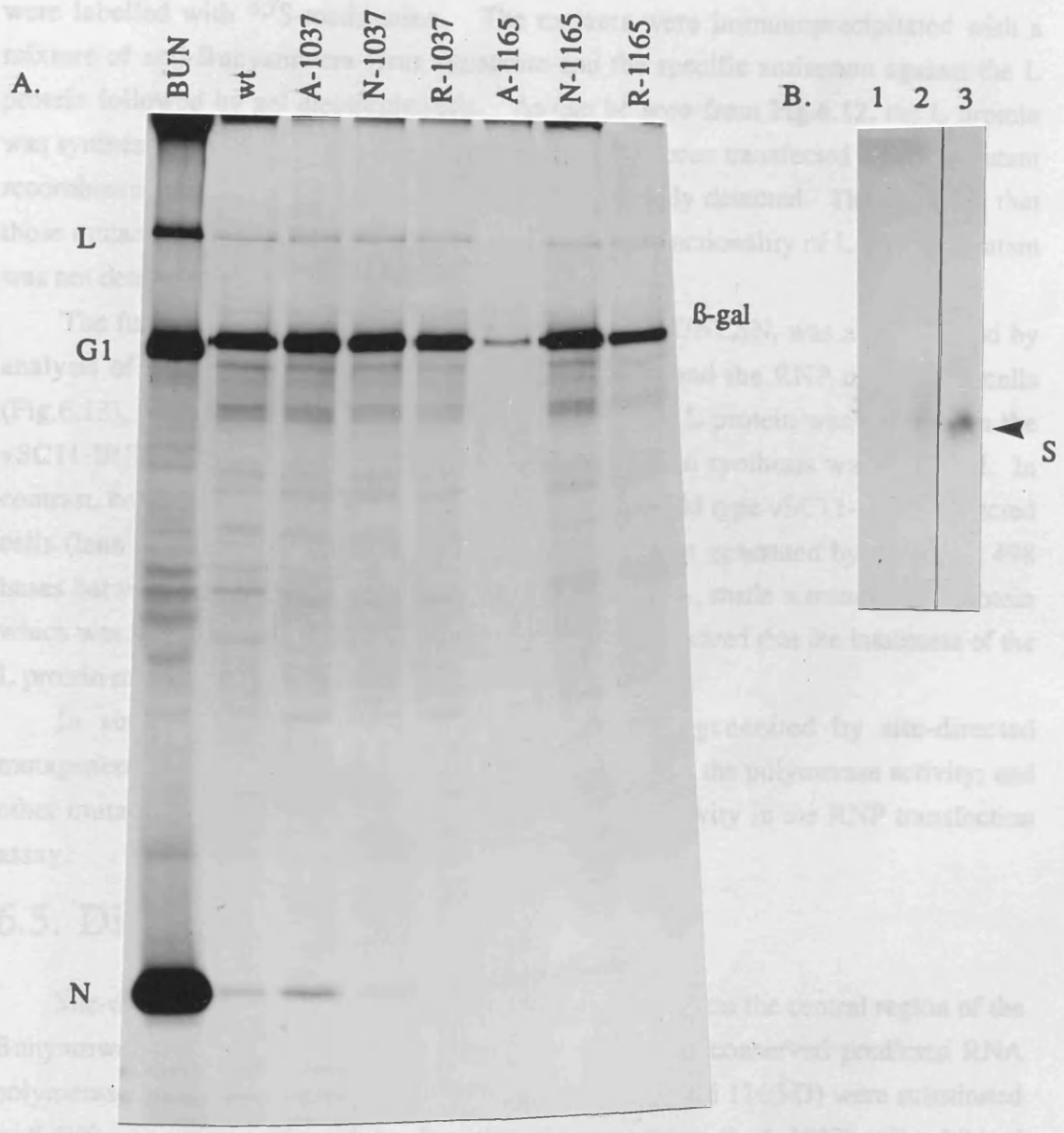


Fig.6.11. Functional analysis of the L proteins expressed by vSC11-BUNL mutants. A. Immunoprecipitation analysis of the proteins synthesized in cells infected with vSC11-BUNL mutants following transfection with the Bunyamwera virus RNP. The transfected cells which had been infected with the vSC11-BUNL mutants as indicated were labelled with ^{35}S-methionine overnight after 6 hr of transfection. Cytoplasmic extracts were immunoprecipitated with a mixture of anti-Bunyamwera virus antiserum and the specific antiserum against the L protein (anti-C). The N protein was detected in the cells infected with vSC11-BUNL(wt) and A-1037, but only very slightly, if at all, in the cells infected with the other mutants. Except for L-A-1165, the levels of the L protein produced in other mutants infected cells were similar to that of L-A-1037. B. Northern blot analysis of RNA from the RNP transfected cells (lanes 1-3) which had been mock-infected (lane 1), infected with vSC11-BUNL-A 1037 (lane 2), or vSC11-BUNL (lane 3). Total cellular RNA was extracted after 20 hr of transfection, fractionated on a 1% agarose gel containing methylmercuric hydroxide and transferred to nitrocellulose. Hybridization with ^{32}P-labelled pUC-BUNS DNA showed that the S signal was detected in the RNP transfected cells which had been infected with vSC11-BUNL-A 1037 (lane 2) and vSC11-BUNL (lane 3), but not in the mock infected cells (lane 1).

were labelled with ^{35}S-methionine. The extracts were immunoprecipitated with a mixture of anti-Bunyamwera virus antiserum and the specific antiserum against the L protein followed by gel electrophoresis. As can be seen from Fig.6.12, the L protein was synthesized in the RNP transfected cells which had been transfected with the mutant recombinant plasmid DNAs, but no N protein was clearly detected. This suggests that those mutants may have lost the enzyme activity. The functionality of L-G-1165 mutant was not determined.

The functionality of one deletion mutant, vSC11-BUNLΔN, was also analysed by analysis of N protein synthesis in the mutant infected and the RNP transfected cells (Fig.6.13). A protein migrating slightly faster than the L protein was detected in the vSC11-BUNLΔN infected cells (lane 1), but no N protein synthesis was observed. In contrast, both the L and N proteins were detected in the wild type vSC11-BUNL infected cells (lane 2). Thus, the C-terminal 166 residues deletion generated by removing 498 bases between nucleotides 6021 and 6519 of the L cDNA, made a truncated L protein which was inactive in the RNP transfection assay. This indicated that the intactness of the L protein may be important for its functionality.

In summary, among the 16 mutant L proteins generated by site-directed mutagenesis, one mutant L-A-1037 was shown to still have the polymerase activity; and other mutations in 1037D and 1165D of the L had no activity in the RNP transfection assay.

6.5. Discussion

Site-directed mutagenesis experiments were performed on the central region of the Bunyamwera virus L protein which encompasses the four conserved predicted RNA polymerase motifs. Two conserved amino acids (1037 D and 1165 D) were substituted by 8 different amino acid residues. Interestingly, one mutant (L-A-1037) still exhibited polymerase function in the RNP transfection assay, suggesting it may maintain the similar structure as the wild type L protein. The replacement of the 1037 D by other residues (G, H, N, P, R, S, or T) rendered the L protein largely inactive. Bordo and Argos (1991) have suggested that A substitution is a "safe" residue substitution in site-directed mutagenesis. Larder *et al.* (1987) also noted that not all conserved amino acids are essential for the enzyme function since some of the mutations they generated in HIV reverse transcriptase (RT) (D and A in position 12 and 13 of motif A, respectively) had no apparent effect on the biochemical properties of the enzyme.

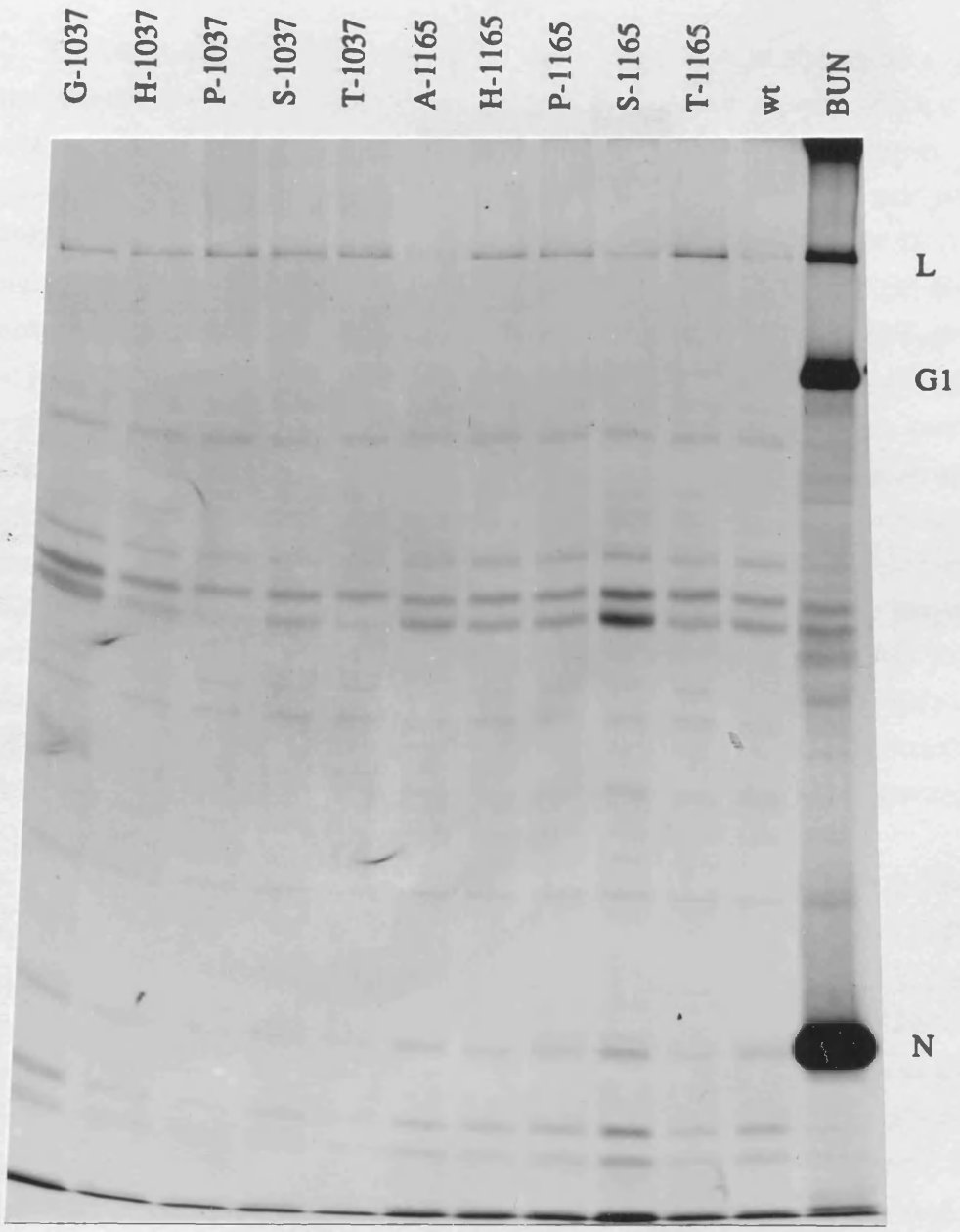


Fig.6.12. Functional analysis of the mutant L proteins by transient expression. CV-1 cells were infected with vTF7-3 at moi of 5 pfu/cell and transfected with RNP and pTF7-5BUNL mutant DNA as indicated. The cells were radiolabelled with ^{35}S-methionine overnight after 6 hr of transfection and the extracts were immunoprecipitated with a mixture of anti-Bunyamwera virus antiserum and the specific antiserum against the L protein. The vTF-7-5BUNL (wt) DNA transfected cells did not produce high levels of the N protein in this experiment. Therefore, the determination of the functionality of these mutants remained to be investigated.

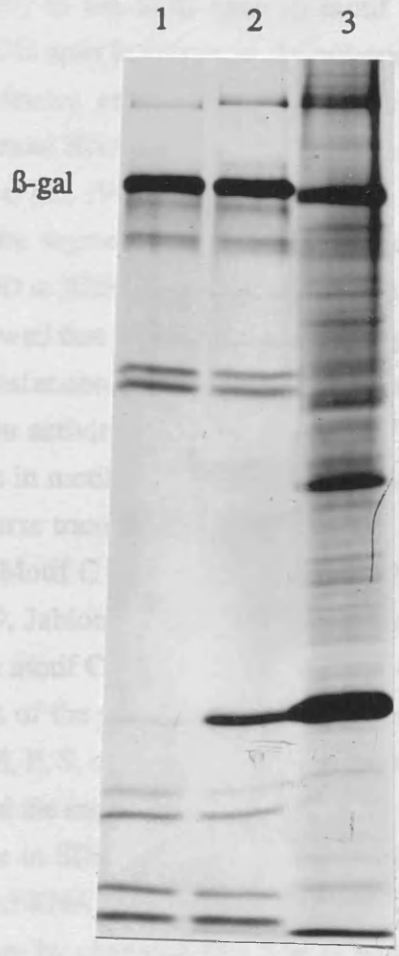


Fig.6.13. Functional analysis of a deletion L protein mutant expressed by vSC11-BUNLΔN. CV-1 cells were infected with vSC11-BUNLΔN (lane 1), vSC11-BUNL(wt) (lane 2) and transfected with RNP (lanes 1-2). The cells were labelled with ^{35}S-methionine overnight after 6 hr of transfection and the extracts were immunoprecipitated with a mixture of anti-Bunyamwera virus antiserum and the specific antiserum against the L protein (anti-C) followed by electrophoresis on a 15% polyacrylamide-SDS gel. A protein which migrated faster than the L protein was observed in vSC11-BUNLΔN infected cells, but no N protein was detected. Both L and N proteins were detected in vSC11-BUNL (wt) infected cells. The positions of L, G1, N and ß-galactosidase are indicated.

The minor band in lane 1 migrating slightly faster than N is a vaccinia virus protein.

The second D (1165) in the SDD span in motif C was chosen as a target for mutagenesis, since the SDD span is unique to the polymerase proteins of the segmented negative-strand RNA viruses analysed to date (See Fig.1.8 in chapter 1). For unsegmented negative-strand RNA viruses, the most conserved amino acid residues in motif C are GDN (Poch *et al.*, 1989, 1990). Therefore, it appeared that SDD residues might be important for the segmented RNA virus replication. My initial aim was to determine if changing SDD to SDN is detrimental to the functionality of the L protein and the preliminary result showed that the SDD to SDN change abolished the activity of the L protein in the RNP transfection assay, indicating this DD doublet is essential for maintaining the L protein activity. Larder *et al.* (1987) substituted the first D of the conserved MDD residues in motif C with H in the reverse transcriptase of HIV and the resulting mutant lost reverse transcriptase activity. This suggests the importance of the DD doublet in motif C. Motif C has been suggested to be at or near the enzyme active centre (Poch *et al.*, 1989, Jablonski *et al.*, 1991). Jablonski *et al.* (1991) studied the conserved YGDD span in motif C of the poliovirus RNA-dependent RNA polymerase by site-directed mutagenesis of the poliovirus cDNA. They replaced the G residue in the YGDD span with A, C, M, P, S, or V and found that the mutant RNA polymerase with A or S substitution, exhibited the enzymatic activity *in vitro*.

The S (1163) residue in SDD span is only conserved in the polymerase proteins of segmented negative-strand RNA viruses; it would be of interest to determine the role of this S residue in the future by changing this S to G which is conserved in polymerase proteins of plus-strand RNA viruses and unsegmented negative-strand RNA viruses. Attempts to change the 1119N with G residue were not successful due to a one base deletion in the synthetic oligonucleotide (Fig.6.6). Since this N residue is conserved in the L protein of Hantaan virus (Schmaljohn, 1990) and TSWV (de Haan *et al.*, 1991) and aligned with the most conserved G residue in motif B which has been detected in all the other RNA polymerase proteins analysed to date (Poch *et al.*, 1989 and 1990), the experiment should be repeated in the future to asses the role of this conserved N residue.

One deletion mutant which had 166 residues deleted at the C-terminus of the L lost polymerase activity, indicating the deletion could have destroyed the secondary structure of the protein or affected other functions of the L protein. This implies that the intactness of the L protein is required for its functionality in the RNP transfection assay.

More site-directed mutagenesis is obviously necessary to study the functional role of the conserved amino acids systematically. The amino acids adjacent to the four most conserved amino acids have been selected as the targets for making more L mutants and

the oligonucleotides have been synthesized. However, due to time limitations on the project, these further mutations have not yet been done. For the functional analysis of the mutants, it is also important to develop a more sensitive assay. This might have to await success in reconstituting synthetic RNA templates so that it would be possible to measure the polymerase activity of the generated L mutants more precisely. It would also be very useful if the desired L gene mutants could be introduced into an infectious virus genome to investigate their role in virus replication more fully.

Chapter 7

Outlook

The aims of the project have been achieved by demonstrating the success of: (1) construction of a cDNA representing the full-length Bunyamwera virus L gene (chapter 4); (2) expression of the L protein by vaccinia virus systems and exploration of the L gene expression in other systems (chapter 4); (3) production of monospecific antisera against the L protein in order to identify the antigenicity of the expressed L protein (chapter 3); (4) demonstration of the RNA polymerase activity of the expressed L protein (chapter 5). In addition, preliminary data on mapping the polymerase domain within the multifunctional L protein by site-directed mutagenesis of the L cDNA were presented in chapter 6.

Expression of functional L protein of Bunyamwera virus provides a very useful vehicle for detailed dissection of the functions of the different viral proteins involved in transcription and replication. A similar type of expression system has been described by Pattnaik & Wertz (1990) working with VSV, which has enabled them to identify the protein requirements for replication of the genome of a VSV DI particle. They transfected plasmids containing cDNAs for the L, N and NS genes of VSV, under control of the T7 promoter, into VSV DI infected cells which had also been infected with vTF7-3 (producing T7 RNA polymerase). Replication and amplification of the genome of DI particle were entirely dependent on the expression of the L, N and NS proteins driven by vaccinia virus-T7 polymerase; omission of any one of the proteins abolished replication. Furthermore, the degree of RNA replication depended on the relative ratios of the proteins; maximal levels were achieved at molar ratios of L:N:NS of 1:200:200, and were eight- to ten-fold higher than those achieved by wild type VSV as a helper virus.

The Bunyamwera virus RNP transfection assay developed in this project indicates that the L protein alone is required for transcription of the S RNA supplied from the

transfected RNP. Note that unlike the DI particle of VSV, which lacks genetic information (including the N gene) necessary for its genes to be replicated, the transfected Bunyamwera virus RNP contain intact viral genomes. Thus the N protein was synthesized from the plus-sense S RNA which had been transcribed by the expressed L protein in the RNP transfected cells, and hence it is difficult to assess the role of the N protein in the RNA transcription process. Similarly, the Bunyamwera virus DI particles also contained full length S RNA and were replicated by the expressed L protein. A synthetic RNA template is therefore a must for future experiments aimed at identifying the protein requirements for bunyavirus RNA transcription or replication.

The construction of synthetic RNA templates of influenza virus has been reported recently (Luytjes *et al.*, 1989; Parvin *et al.*, 1989; Enami *et al.*, 1990). Parvin *et al.* (1989) constructed synthetic RNA templates which were transcribed from plasmid-encoded sequences and encapsidated with NP protein purified from virions. The synthetic templates, which contained 3'-terminal sequences of genomic RNA could be transcribed by the purified authentic influenza virus polymerase; sequences not specific for influenza virus were not copied. Using this system, they were able to demonstrate that the promoter sequence of influenza virus lies within the 15 nucleotides at the 3'-terminus. Similarly, Moyer *et al.* (1991) also assembled functional RNA templates of VSV by encapsidating *in vitro* synthesized sequences representing the 5' end of both the negative- and positive-strand VSV genome RNAs with purified N protein. The assembled nucleocapsids could function *in vitro* as templates for transcription by the VSV RNA polymerase. They showed that as few as 19 nucleotides from the 5' end of VSV RNA allowed maximal encapsidation.

Luytjes *et al.* (1989) exploited the system developed by Parvin *et al.* (1989) to package a foreign gene into influenza virions and to support transcription of a synthetic influenza virus RNA. A plasmid was constructed containing the chloramphenicol acetyltransferase (CAT) gene flanked by the 3'- and 5'-terminal untranslated sequences from influenza virus genome segment 8. RNA was synthesized *in vitro* using T7 RNA polymerase, equivalent to the negative strand of the CAT gene, and this was mixed with the influenza virus polymerase preparation and then transfected into MDCK cells. Following superinfection with a helper virus, CAT activity was detected in the transfected cell extracts. In addition, the recombinant influenza virus gene was found to be packaged into virus particles: when the supernatant from these cells was used to infect other cells, CAT activity was again detected. In this way, they have shown that the 5'-terminal 22 nucleotides and the 3'-terminal 26 nucleotides of the viral RNAs contain all the signals necessary for transcription, replication and packaging of the influenza virus RNAs. Yamanaka *et al.* (1991) reported a similar system for the analysis of the promoter structure of the influenza virus RNA genome. They confirmed that *in vitro*

synthesized RNA must be complexed with viral RNA polymerase and NP to form active templates prior to transfection.

Progress towards the propagation of negative-strand viral genomes in the absence of homologous helper virus has also been developed for influenza virus. Huang *et al.* (1990) used a vaccinia virus vector-driven system for replication of a synthetic NS-like gene. The NS-like gene contains the CAT gene (reporter gene) in place of the antisense coding regions for the NS proteins (NS1 and NS2). This was transfected into cells which had previously been infected with vTF7-3 and transfected with a mixture of plasmids expressing the three polymerase proteins (PB1, PB2 and PA), the NP protein, NS1 and/or NS2 proteins. The replication of the synthetic RNP was assayed by analyzing cells for CAT activity. By this approach, they were able to identify that the minimum subset of influenza virus proteins needed for specific replication is the three polymerase proteins (PB1, PB2 and PA) and the NP protein.

The source of N protein used for reconstituting functional synthetic nucleocapsids of VSV and influenza virus is from purified virions. However it is difficult to obtain enough N protein from a bunyavirus for *in vitro* encapsidation of a synthetic RNA, since bunyavirus yields in infected cells are low. Other alternative approaches have to be developed. The Maguari virus N protein has been successfully expressed in a recombinant baculovirus by Elliott and McGregor (1989), and has been purified to homogeneity by chromatography (McGregor and Elliott, unpublished data). If this recombinant N protein can bind to viral RNA transcripts *in vitro* and form a functional nucleocapsid structure, it would provide an alternative template for functional analysis of the RNA polymerase *in vitro* or in intact cells.

Bunyamwera virus DI particles generated from persistently infected mosquito cells (Elliott & Wilkie, 1986; Scallan & Elliott, 1991) were appropriate templates for the polymerase activity assay as described in chapter 5. More work is required to characterize these DI particles in order to fully understand their protein composition and structure of the defective genome. It may be possible to rescue Bunyamwera virus DI particles by expressing all the viral structural proteins (L, G1, G2, and N) of Bunyamwera virus from cloned cDNA. Similar work has been achieved for the DI particle of VSV (Pattnaik & Wertz, 1991). The DI particles of VSV were successfully replicated, assembled and budded from the cells expressing all five VSV proteins (L, N, NS, G, and M) from the vaccinia virus/T7 system. Omission of either M or G protein expression resulted in no DI particle budding.

The protein requirements for Bunyamwera virus RNA replication have not been defined due to the lack of a synthetic RNA template. The function of the NSs protein encoded by the S segment is not known yet. Expression of the NSs protein by a cloned cDNA should help to unravel its role in the RNA synthesis process. It is known that the

NS protein of VSV is required for replication of the VSV genome (Pattnaik & Wertz, 1990; 1991). However, using a similar system, the NS1 and NS2 proteins of influenza virus were shown not to be essential in the replication of a NS-like synthetic RNA (Huang *et al.*, 1990). As has been discussed in chapter 5, the protein components in the transfected RNP were not characterized and it is not known if minor amounts of NSs were also present in the RNP preparations. The expressed L protein has not been shown to replicate the S RNA from the transfected vRNP; therefore it is possible that the expression of NSs cDNA in the vRNP transfected cells might aid the L protein activity. The NSs protein of bunyavirus has been suggested to play a role in the switch from viral transcription to genome replication (Bishop, 1985), which should be studied in the future.

The study of negative-strand RNA viruses in general has suffered from the lack of a method for recovering viable virus from molecularly cloned cDNA copies of genomic sequences to allow genetic manipulation of the virus genomes. Such systems have been available for positive-strand RNA viruses, such as picornavirus (Omata *et al.*, 1984; Mizutani & Colonno, 1985) for some time. For positive-strand RNA viruses, the RNAs from full-length cDNAs are infectious upon transfection, and no additional factors or proteins are required. In contrast, negative sense RNA genomes have to be first transcribed into mRNA by the viral specific RNA polymerase. This difficulty has been overcome for influenza virus (Luytjes *et al.*, 1989; Enami *et al.*, 1990), which represents a significant step towards genetic manipulation of negative-strand RNA viruses.

Enami *et al.* (1990) succeeded in rescuing infectious influenza virus by transfecting cells with RNAs derived from specific cDNAs. They constructed a recombinant plasmid containing the complete NA gene of influenza WSN virus downstream of a T3 promoter to make a faithful RNA copy identical in length to the genomic NA RNA. This RNA was then incubated with purified RNA polymerase proteins and the complexes transfected into MDBK cells. Superinfection with helper WSN-HK virus resulted in the release of virus containing the WSN NA gene. The use of helper virus WSN-HK was important in this experiment to provide a strong selection for the NA rescued viruses, because WSN-HK virus cannot form plaques in MDBK cells in the absence of protease due to the defect in its NA gene. When five point mutations were introduced into the WSN NA gene by cassette mutagenesis of the plasmid DNA, the desired mutations were revealed in the NA gene of the rescued viruses. The transfection efficiency of influenza virus transfectants has been much improved by coupling the *in vitro* transcription of cDNA with the actual assembly of the RNP to allow cDNA-derived RNA to be introduced into the genome of influenza virus more efficiently (Emini and Palese, 1991). More recently, Muster *et al.* (1991) demonstrated that a chimaeric

influenza virus could be produced by transfecting engineered RNA into MDBK cells infected with the helper virus. The hybrid influenza A/B virus gene contained the coding region of the A/WSN NA flanked by terminal sequences derived from influenza B/Lee virus; and this virus had reduced virulence in mice. The recognition of the end sequences derived from influenza B virus by the influenza A virus RNA polymerase was less efficient, suggesting that the end sequences may be responsible for the altered phenotype of the chimearic virus.

For measles virus, Ballart *et al.* (1990) claimed to have rescued infectious virus entirely from cloned cDNA by microinjecting committed cDNA/RNA polymerase complexes into the helper virus infected cells. However there are doubts on the validity of this claim and a follow up report has been retracted (*J. Virol.* 65:5656, 1991).

It is also worthwhile to mention the approach established by Roner *et al.* (1990) in establishing a productive infection by transfecting reovirus RNA. Reovirus contains 10 segments of double-strand RNA ranging in length from 196 to 3916 nucleotides and the virion also contains a transcriptase complex. The reovirus genome has been shown to be noninfectious by simply transfecting the 10 virus RNA species into the cells. The conditions described by Roner *et al.* are complex: the optimal transfection mixture comprised ssRNA (plus-strand) and ds RNA of serotype 3 (ST3) as well as reticulocyte lysate in which all 10 reovirus ST3 mRNAs had been translated. Eight hours after the introduction of this mixture into mouse L929 fibroblasts by the "Lipofectin" transfection method, the cells were superinfected with a helper virus, ST2, and virus was harvested 23 hr later. ST2 helper virus only forms plaques after 10-12 days of incubation whereas ST3 forms plaques in as short as 2 days, which provided selection for ST3 progeny virus. The yield of ST3 virus from the transfected RNAs was 0.1-0.2 pfu/cell and contained virus with only a homogeneous ST3 genome type. Reassortants were generated when a mixture of ST2 and ST3 dsRNAs, together with reticulocyte lysate primed with both ST2 and ST3 ds RNAs, were translated. Although the roles of the various components in the transfection mixture are not understood, this advancement may provide a useful approach for making infectious Bunyamwera virus entirely from the cloned cDNAs. The three RNA segments of Bunyamwera virus have been cloned and full length cDNAs are available; efforts could be made by transfecting the *in vitro* transcribed three RNA species (S, M, and L) together with the *in vitro* produced virus proteins (L, N, G1, G2, NSs and NSm) and assaying for the production of the infectious virus.

By transfecting the purified Bunyamwera virus RNP into the cells expressing the L protein, I was not able to detect infectious virus recovered from the transfected RNP (chapter 5). In contrast, Gotoh *et al.* (1989) were able to rescue infectious Sendai virus from cells transfected with RNP by supplying the Sendai virus L and P/C gene products

expressed from recombinant vaccinia viruses. Since M RNA amplification and M RNA gene products were not detected, it was speculated that the efficiency of transfecting M RNA (and L RNA) segment nucleocapsids might be lower. Unlike Sendai virus which has unsegmented RNA genome, Bunyamwera virus has three RNA segments and it is therefore more difficult to transfect all three nucleocapsids into a single cell. More investigation is required to overcome this.

The L protein is presumed to have endonuclease activity, which has been indicated by the *in vitro* transcription assay (Patterson *et al.*, 1984) and by analysis of viral mRNA species in infected cells (Bishop *et al.*, 1983; Eshita *et al.*, 1985; Collett, 1986; Bouloy *et al.*, 1990). The endonuclease activity is considered to be involved in a cap-snatching initiation of viral mRNA synthesis. The identification of the endonuclease activity and further mapping of its domain in the L protein should provide more information on the viral RNA synthesis mechanism. It may be difficult to use the L protein expressed from recombinant vaccinia virus for the endonuclease assay, since a similar activity might be present in some of the vaccinia virus proteins. In chapter 4, a protein which was the same size as the Bunyamwera virus L protein was produced *in vitro* by translation of an L gene mRNA. Due to time limitations this system has not been further explored. It would be worthwhile characterizing further the *in vitro* synthesized protein and to assay for polymerase activity. *In vitro* transcription reactions could be performed using the *in vitro* synthesized L protein and a synthetic RNA template to detect cap endonuclease-primed RNA synthesis. A similar assay system using authentic RNA polymerase protein has been described for influenza virus which employs a similar initiation mechanism for viral RNA synthesis (Parvin *et al.*, 1989). It would also be possible to incubate the expressed L protein with a capped mRNA to determine specific digestion of the cap containing sequence.

The agarose gel system used for detecting the S RNA synthesis in the RNP transfected cells did not separate the full-length S RNA and the shorter S mRNA. This should be overcome by using an alternative gel system. The 5' end sequences of the amplified S RNA could be analysed by a PCR technique to determine if the positive-sense S mRNA contains host derived primers. This should help us to understand the viral RNA initiation mechanism in an artificial system.

In summary, the results presented in this thesis demonstrated that the Bunyamwera virus L protein is an RNA polymerase, which opens a new approach for further structural and functional studies. These may also provide some insights into the mechanism of the RNA synthesis process, such as identification of the promoter sequences in the viral RNA, endonuclease function of the L and further protein requirements for viral RNA synthesis. Preliminary data on mapping the RNA polymerase domain of the L protein presented in this thesis indicated the importance of

some conserved amino acids in the predicted polymerase motifs. It would be very important to incorporate the L gene mutants into an infectious virus and determine their functions in virus replication. More mutagenesis of the L gene cDNA is required to identify the crucial amino acids within the enzyme active site of the polymerization reaction, which may provide possible targets for antiviral chemotherapy. Based on the results achieved in this project some work of immediate interest, which has been discused as above, is summarized as follows:

(1) reconstitution of RNA templates for RNA polymerase assay;
(2) determination of the protein components involved in bunyavirus genome replication using a synthetic RNA template;
(3) rescue of an infectious virus from cloned cDNA;
(4) domain mapping of the L protein: continuation of the RNA polymerase domain mapping and establishment of an endonuclease assay for identification of the endonuclease region in the L protein.

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Expression of Functional Bunyamwera Virus L Protein by Recombinant Vaccinia Viruses

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A cDNA containing the complete coding sequence of the Bunyamwera virus (family *Bunyaviridae*) L genome segment has been constructed and cloned into two recombinant vaccinia virus expression systems. In the first, the L gene is under control of vaccinia virus *P7.5* promoter; in the second, the L gene is under control of the bacteriophage T7 ϕ10 promoter, and expression of the L gene requires coinfection with a second recombinant vaccinia virus which synthesizes T7 RNA polymerase. Both systems express a protein which is the same size as the Bunyamwera virus L protein and is recognized by a monospecific L antiserum. The expressed L protein was shown to be functional in synthesizing Bunyamwera virus RNA in a nucleocapsid transfection assay: recombinant vaccinia virus-infected cells were transfected with purified Bunyamwera virus nucleocapsids, and subsequently, total cellular RNA was analyzed by Northern (RNA) blotting. No Bunyamwera virus RNA was detected in control transfections, but in cells which had previously been infected with recombinant vaccinia viruses expressing the L protein, both positive- and negative-sense Bunyamwera virus S segment RNA was detected. The suitability of this system to delineate functional domains within the Bunyamwera virus L protein is discussed.

Bunyamwera virus is the prototype of the family *Bunyaviridae* (for a review, see reference 11) and has a tripartite negative-sense RNA genome. The three genome segments have been cloned as cDNA, and their complete sequences have been determined; the smallest segment, S, is 961 nucleotides in length and encodes the nucleocapsid protein N and a nonstructural protein termed NSs (9); the middle-sized segment, M, is 4,458 bases long and encodes the two virion glycoproteins (G1 and G2) and a second nonstructural protein called NSm (22); and the largest RNA segment, L, which contains 6,875 nucleotides, encodes a high-molecular-weight protein also termed L (10). The L protein (259,000 Da) is presumed to be the virion-associated transcriptase or RNA polymerase, though direct proof of this is lacking. The Bunyamwera virus L protein does not show extended regions of homology with other negative-strand virus transcriptases (10) but does contain the polymerase motifs identified in all RNA-dependent RNA polymerases (29).

Transcriptase activity has been detected in detergent-disrupted preparations of a number of bunyaviruses (2, 16, 27), though the activity was low compared with that of other negative-strand viruses, such as vesicular stomatitis virus, which has hampered detailed biochemical analyses. However, Patterson et al. (27) demonstrated that La Crosse bunyavirus polymerase was stimulated by dinucleotides (e.g., ApG), cap analogs (e.g., mGpppAm), and natural mRNAs (e.g., alfalfa mosaic virus RNA 4) and detected an endonuclease activity which cleaved methylated capped mRNAs in vitro. These results are consistent with the notion that bunyavirus mRNA synthesis is primed by RNA sequences cannibalized from host cell capped mRNAs; further evidence stems from the observation that heterogeneous nonviral sequences are present at the 5′ ends of bunyavirus mRNAs (1, 3, 12, 18, 28). Thus, bunyavirus transcription resembles that of influenza virus (for a review, see reference 21), but in contrast to that of influenza virus, bunyavirus transcription is not inhibited by actinomycin D or α-amanitin (32) and occurs in the cell cytoplasm (20, 31). Additionally, whereas influenza viruses employ three proteins (the P proteins) to catalyze the various enzymatic events in transcription and replication, bunyaviruses most probably use just one, the L protein.

With the long-term aim of defining the functional domains within the bunyavirus L protein, we have exploited the available cloned cDNAs to establish a system in which the L protein is expressed by recombinant vaccinia viruses. We show here that the expressed protein is functional by demonstrating its ability to amplify an appropriate RNA template, supplied in the form of purified nucleocapsids.

MATERIALS AND METHODS

Viruses and cells. Bunyamwera virus was grown in BHK cells as described previously (33). Vaccinia virus WR strain was obtained from A. Patel, and the recombinant vTF7-3, which expresses T7 RNA polymerase (15), was obtained from B. Moss. Vaccinia viruses were grown in CV-1 cells and purified as described by Mackett et al. (25). CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum. Human thymidine kinase-negative (TK^-) 143 cells were grown in DMEM supplemented with 5% fetal calf serum and 25 μg of 5-bromodeoxyuridine per ml.

Plasmids. Recombinant plasmids containing cDNA derived from the Bunyamwera virus L RNA segment, cloned into pBR322, were described by Elliott (10). The vaccinia virus transfer vectors pTF7-5 (13) and pSC11 (5) were obtained from B. Moss and L. Whitton, respectively.

The procedures for cloning in plasmids, transformation of bacteria (*Escherichia coli* DH5; Bethesda Research Laboratories), and purification by CsCl-ethidium bromide centrifugation were as described by Maniatis et al. (26).

The construction of the full-length cDNA (pTZBUNL) is described in the Results section. Enzymes involved were obtained from Bethesda Research Laboratories, New En-

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gland Biolabs, or Boehringer-Mannheim and used according to the manufacturers' recommendations.

Construction and characterization of recombinant vaccinia virus. A 6.8-kb *Sma*I fragment containing the complete L gene coding sequence was isolated from pTZBUNL and cloned into the *Sma*I site of pSC11 or the filled-in *Bam*HI site of pTF7-5, and the correct orientation of the insert was determined by restriction endonuclease analysis and nucleotide sequencing.

Recombinant vaccinia viruses were prepared essentially as described by Mackett et al. (25): 80 to 90% confluent monolayers of CV-1 cells in 35-mm dishes were infected with the WR strain of vaccinia virus (multiplicity of infection, 0.05) and then transfected with 5 μg of plasmid DNA by the calcium phosphate method. TK^- recombinants were isolated by plaque assay of transfected cell lysates on TK^- 143 cells by using a 1% low-melting-point agarose gel overlay containing 25 μg of 5-bromodeoxyuridine per ml, and for pSC11 derivatives, 300 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; BRL) per ml. The plaques were screened by filter hybridization (25) with ^{32}P-labelled pTZBUNL DNA, and plaques positive for the L gene were purified by three rounds of plaque-to-plaque passage. Finally, the genomes of the recombinant viruses were analyzed by restriction enzyme digestion and Southern blotting.

Radiolabelling, immunoprecipitation, and polyacrylamide gel electrophoresis. Monolayers of cells in 50-mm dishes were radiolabelled with [^{35}S]methionine (75 μCi/ml), and extracts were prepared for immunoprecipitation or polyacrylamide gel electrophoresis essentially as described previously (33). Immunoprecipitation employed either an antiserum prepared against purified Bunyamwera virus (33) or a chimeric protein comprising the carboxy-terminal 217 amino acids of the L protein fused in-frame to bacterial β-galactosidase (19) by using the pUEX expression system (4). Radiolabelled proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Watret et al. (33).

Extraction of cellular RNA and Northern (RNA) blotting. Total cellular RNA was extracted from phosphate-buffered saline-washed cells by the acid phenol-guanidinium thiocyanate procedure of Chomczynski and Sacchi (6). The RNAs were fractionated on 1% agarose gels containing 5 mM methyl mercuric hydroxide and transferred to nitrocellulose filters, as described by Maniatis et al. (26). The filters were hybridized with ^{32}P-labelled Bunyamwera virus-specific cDNA or riboprobes, washed, and exposed to X-ray film according to standard procedures (26).

Nucleocapsid isolation and transfection assay. Intracellular nucleocapsids were prepared by CsCl gradient fractionation of cytoplasmic extracts of Bunyamwera virus-infected BHK cells by the method of Leppert et al. (23). The nucleocapsid band was recovered with a syringe, diluted with NTE (0.1 M NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA), and pelleted at 40,000 rpm for 2 h at 4°C with the TST41 rotor. The preparation from 6×10^8 cells was resuspended in 0.5 ml of NTE buffer and used either immediately or after storage at −70°C in the presence of 10% glycerol.

For the transfection assay, CV-1 cells (10^6 per 35-mm dish) were infected with recombinant vaccinia virus at a multiplicity of 5 PFU per cell for 1 h at 37°C and then washed once with TBS^+ (25 mM Tris-HCl [pH 7.4], 135 mM NaCl, 50 mM KCl, 0.01% $CaCl_2$, 0.01% $MgCl_2$). The nucleocapsid transfection mix was then added and comprised, in a volume of 250 μl, purified nucleocapsids (10 μl), 140 mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO_4 \cdot 2H_2O$, 6 mM dextrose, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (HEPES; pH 7.05), and 125 mM $CaCl_2$. After 30 min at room temperature, 2 ml of DMEM containing 5% fetal calf serum (5% DMEM) was added, and incubation was continued at 37°C for 4 h. The supernatant fluid was then removed, and the cells were shocked with TBS^+ containing 10% glycerol for 1 to 2 min. After two further washes with TBS^+, 2 ml of 5% DMEM was added, and incubation was continued at 37°C for 20 h. Cell monolayers were then radiolabelled with [^{35}S]methionine, or total cellular RNA was extracted as described above.

RESULTS

Construction of a full-length cDNA of the Bunyamwera virus L gene. An intact copy of the Bunyamwera virus L gene coding region was constructed from existing cDNA clones and synthetic oligonucleotides. The major part of the gene was built from the unique *Hin*dIII site just downstream of the ATG initiation codon for the L protein by using three subgenomic cDNA clones (Fig. 1A) (10). A 1.67-kb *Hin*dIII-*Sph*I fragment isolated from pBUN357 and a 2.07-kb *Sph*I-*Pst*I fragment from pBUN367 were ligated together with *Hin*dIII- and *Pst*I-doubly digested pUC18 DNA. The resulting plasmid, pUC18 357/367, was digested with *Pst*I, treated with alkaline phosphatase, and then ligated to a 3.06-kb *Pst*I fragment isolated from pBUN363. The correct orientation of this latter fragment was confirmed by digestion with appropriate restriction enzymes. This plasmid (pUC18 357/367/363) was digested with *Hin*dIII, treated with phosphatase, and ligated together with two complementary synthetic oligonucleotides which contained the ATG start codon and missing bases of the L gene, flanked by *Hin*dIII restriction sites (Fig. 1B). The oligonucleotides also contained sequences for *Kpn*I and *Sma*I restriction enzymes to facilitate subcloning of the full-length L gene cDNA: these enzymes do not cleave the L gene cDNA, and hence the gene may be removed as a *Kpn*I or *Sma*I fragment, the 3′ sites being derived from the polylinker region of the vector. Direct nucleotide sequence analysis of plasmids after ligation of the synthetic oligonucleotides revealed that multiple copies of the linker had been cloned; however, digestion with *Sma*I and ligation of the 6.8-kb fragment into *Sma*I-cleaved pTZ18R DNA yielded a cDNA containing the correct 5′ end sequence (Fig. 1C). This plasmid was designated pTZBUNL.

Construction and characterization of recombinant vaccinia viruses. Two approaches were used to express the Bunyamwera L protein by recombinant vaccinia viruses. The first employed the classical method in which the L gene was inserted in lieu of the vaccinia virus TK gene, under control of the vaccinia virus *P*7.5 promoter, by using the plasmid transfer vector pSC11; this vector includes the *E. coli* β-galactosidase gene under control of the *P*11 promoter so that recombinant vaccinia viruses produce blue plaques when stained with X-Gal (5). The second approach again used the TK locus as the site for recombination of the L gene, but in this case, the L gene was under control of the bacteriophage T7 φ10 promoter and terminator by using the transfer vector pTF7-5 (13). Expression of the Bunyamwera virus gene should occur only when T7 RNA polymerase, supplied by a second, coinfecting recombinant vaccinia virus, vTF7-3, was present in the cell (13, 15).

The Bunyamwera virus L gene was removed from pTZBUNL by digestion with *Sma*I and ligated into the unique *Sma*I site in pSC11 or the filled-in *Bam*HI site in

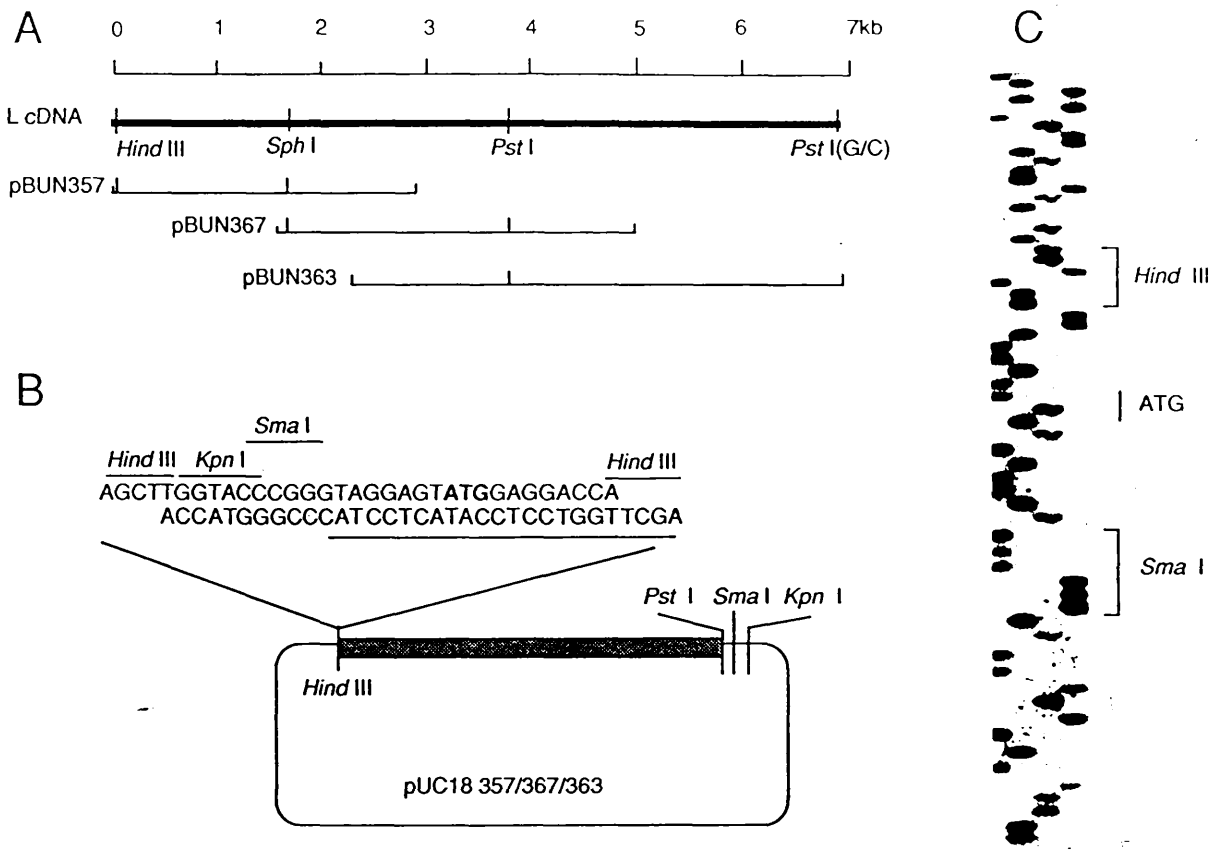

FIG. 1. Construction of a full-length Bunyamwera virus L gene cDNA. (A) Relationship of the three subgenomic cDNA clones and relevant restriction enzyme sites used in the construction. *Pst*I(G/C) indicates a *Pst*I site created by GC tailing during the initial cDNA cloning of the L RNA (10). The major part of the L gene coding region comprised the *Hin*dIII-*Sph*I fragment of pBUN357, the *Sph*I-*Pst*I fragment of pBUN367, and the *Pst*I-*Pst*I fragment of pBUN363. (B) Insertion of complementary synthetic oligonucleotides, containing *Hin*dIII, *Kpn*I, and *Sma*I restriction enzyme sites and nucleotides 43 to 65 of the L gene cDNA (underlined), including the ATG translational start codon, to complete the L gene coding sequence. The shaded box represents the L gene cDNA (not to scale), and relevant restriction sites at the 3' end of the insert are indicated. (C) Dideoxy chain termination sequencing gel of alkali-denatured DNA derived from pTZBUNL, showing the correct sequence inserted by the synthetic oligonucleotides. The order of the lanes is G, A, T, and C. Positions of the *Sma*I and *Hin*dIII restriction enzyme sites and the ATG translational start of the L protein are indicated.

pTF7-5. The correct orientation of the insert was ascertained by restriction enzyme digestion and confirmed by nucleotide sequencing. The recombinant plasmids were then recombined by standard procedures into the TK locus of vaccinia virus WR strain (see Materials and Methods), and TK^- viruses were selected by plaque assay on TK^- 143 cells in the presence of 5-bromodeoxyuridine; in the case of the pSC11-derived recombinants, blue plaques were picked after staining with X-Gal. Recombinant viruses were identified initially by hybridization with an L gene cDNA probe, then further plaque-to-plaque purified, and finally, confirmed by analysis of the restriction enzyme profile of vaccinia virus DNA (Fig. 2A and B). Recombinant viruses were obtained from both systems and designated vSC11-BUNL and vTF7-5BUNL. In Fig. 2A, pSC11-BUNL and vSC11-BUNL DNAs were digested with *Bam*HI or *Eco*RI, fractionated on agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labelled L gene cDNA. The same pattern of bands was detected for each pair of DNAs. Figure 2B shows analysis of pTF7-5BUNL, vTF7-5BUNL, and vaccinia virus (WR strain) DNA using *Eco*RV and *Eco*RI by Southern blotting; no hybridization occurred with the WR strain DNA, whereas the pattern of bands for vTF7-5BUNL was consistent for the expected structure of a recombinant containing the L gene.

Analysis of RNA synthesized in recombinant vaccinia virus-infected cells was performed by Northern blotting. The level of L gene-specific RNA in cells infected with vSC11-BUNL was barely detectable by our standard procedure (data not shown). However, in cells dually infected with vTF7-3 and vTF7-5BUNL, an RNA transcript about the same size as that detected in Bunyamwera virus-infected cells was easily detected (Fig. 2C, lanes 2 and 3). No L gene-specific RNA was detected in mock-infected cells (lane 1) or in cells infected with vTF7-5BUNL alone (lane 4).

Analyses of proteins synthesized by recombinant vaccinia viruses. To investigate the proteins made by the recombinant vaccinia viruses, CV-1 cells were infected with the various recombinants and pulse-labelled at 22 to 24 h postinfection with [^{35}S]methionine; the cell lysates were fractionated on SDS-containing polyacrylamide gels (Fig. 3). Cells infected with vSC11-BUNL produced a high-molecular-weight protein of the same electrophoretic mobility as the L protein identified in Bunyamwera virus-infected cells (Fig. 3A),

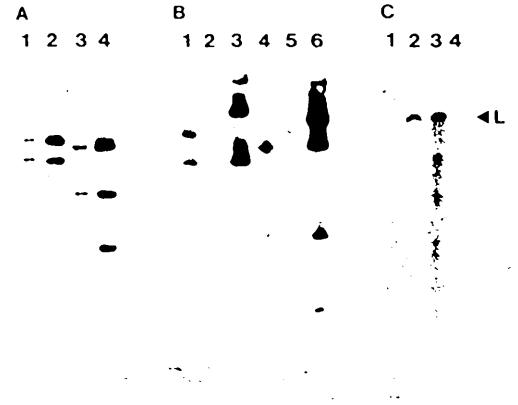

FIG. 2. Characterization of recombinant vaccinia virus. (A) Southern blot analysis of vSC11-BUNL. DNA was extracted from recombinant vaccinia virus-infected cells (25) and digested with *Bam*HI (lane 1) or *Eco*RI (lane 3). The transfer vector plasmid DNA, pSC11-BUNL, was also digested with either *Bam*HI (lane 2) or *Eco*RI (lane 4). After fractionation on agarose gels and transfer to nitrocellulose, the filters were hybridized with ^{32}P-labelled pTZBUNL DNA. The pattern of bands is similar for both DNAs with each enzyme. (B) Southern blot analysis of vTF7-5BUNL. DNA from cells infected with vTF7-5BUNL (lanes 1 and 4) or vaccinia virus WR strain (lanes 2 and 5) or from the transfer vector pTF7-5BUNL (lanes 3 and 6) were digested with either *Eco*RV (lanes 1 to 3) or *Eco*RI (lanes 4 to 6), fractionated on agarose gels, and transferred to nitrocellulose. Hybridization with ^{32}P-labelled pTZBUNL DNA indicated the expected genetic structure for vTF7-5BUNL; no hybridization occurred with the vaccinia virus parental DNA (lanes 2 and 5). (C) Northern blot analysis of infected cell RNA. Total cellular RNA was extracted from mock-infected cells (lane 1), Bunyamwera virus-infected cells (lane 2), cells infected with vTF7-5BUNL and vTF7-3 (lane 3), or cells infected with vTF7-5BUNL alone (lane 4) and fractionated on a 1% agarose gel containing methyl mercuric hydroxide. After transfer to a nitrocellulose filter, hybridization was performed with ^{32}P-labelled pTZBUNL DNA. An L segment-specific RNA, similar in size to that observed in the Bunyamwera virus-infected cells, was detected in the cells infected with vTF7-5BUNL and vTF7-3. No hybridization was observed in mock-infected or vTF7-5BUNL singly infected cells.

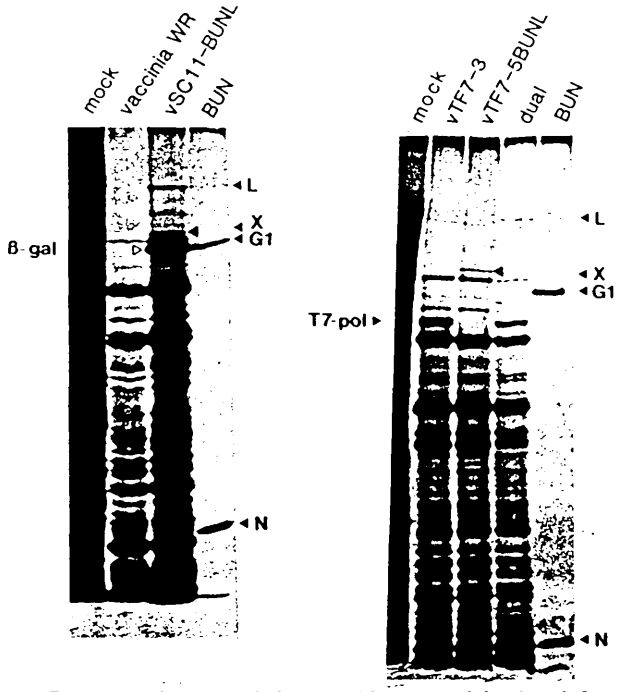

FIG. 3. Protein synthesis in recombinant vaccinia virus-infected cells. CV-1 cells were labelled with [^{35}S]methionine at 22 to 24 h after infection. (Left panel) Cells were mock infected or infected with the WR strain of vaccinia virus, the recombinant vSC11-BUNL, or with Bunyamwera virus (BUN) as indicated. The Bunyamwera virus proteins L, G1, and N are designated on the right, and a band corresponding to L protein is observed in the vSC11-BUNL lane but not in the parental vaccinia virus lane. The position of the X protein (see text) is also noted. vSC11-BUNL synthesizes β-galactosidase, which has the same mobility as G1. (Right panel) Cells were mock infected or infected with vTF7-3, vTF7-5BUNL, vTF7-3 and vTF7-5BUNL (dual), or Bunyamwera virus (BUN). The Bunyamwera virus L, G1, and N proteins are indicated, and a band corresponding to L protein is observed in the dual-infected lane. L protein is not seen in cells infected with vTF7-5BUNL alone, but the X protein is present. The position of T7 RNA polymerase, synthesized by vTF7-3, is indicated.

which was not seen in cells infected with the parental vaccinia WR virus. In vSC11-BUNL-infected cells, a band corresponding to β-galactosidase was also clearly evident and comigrated with the G1 protein of Bunyamwera virus. Other bands specific to the vSC11-BUNL-infected lysate were seen migrating between the positions of β-galactosidase and the L protein, of which one (labelled "X" in Fig. 3A) is considered in more detail later.

Cells dually infected with vTF7-3 (expressing T7 RNA polymerase) and vTF7-5BUNL also contained a protein which comigrated with the Bunyamwera virus L protein (Fig. 3B). This band was not seen in cells infected with either virus singly. In cells infected with vTF7-5BUNL only, a band having the same mobility as the X band seen in vSC11-BUNL-infected cells was observed, but this band was not seen in the dually infected cells. Thus, both systems synthesize a protein corresponding in size to the L protein; in the T7 system, the amount of the expressed protein was similar to that in Bunyamwera virus-infected cells, whereas considerably more L protein was synthesized by vSC11-BUNL.

To confirm the identity of the protein made in the recombinant vaccinia virus-infected cells, immunoprecipitation experiments were performed with a monospecific antiserum to the carboxy-terminal 217 amino acids of the Bunyamwera virus L protein (19). As shown in Fig. 4, the proteins synthesized by dual infection with vTF7-3 and vTF7-5BUNL (lane 6) and with vSC11-BUNL (lane 7) were precipitated by the L protein-specific serum; this serum recognizes authentic L protein made in Bunyamwera virus-infected cells (compare lanes 2 and 3). No proteins were precipitated by preimmune serum (lane 1) or cells infected with vTF7-3 alone (lane 4). Of interest is the X protein, which was immunoprecipitated from cells infected by vTF7-5BUNL alone (lane 5) and by vSC11-BUNL (lane 7). This result suggests that the X protein is a carboxy-terminal fragment of the L protein, but its genesis has not been investigated.

Functional assay of the recombinant vaccinia virus L protein. The above experiments indicated that antigenically authentic Bunyamwera virus L protein was produced by the recombinant vaccinia viruses, but in order for this protein to be useful for mapping domains within the L protein, it was necessary to demonstrate that the expressed L protein had

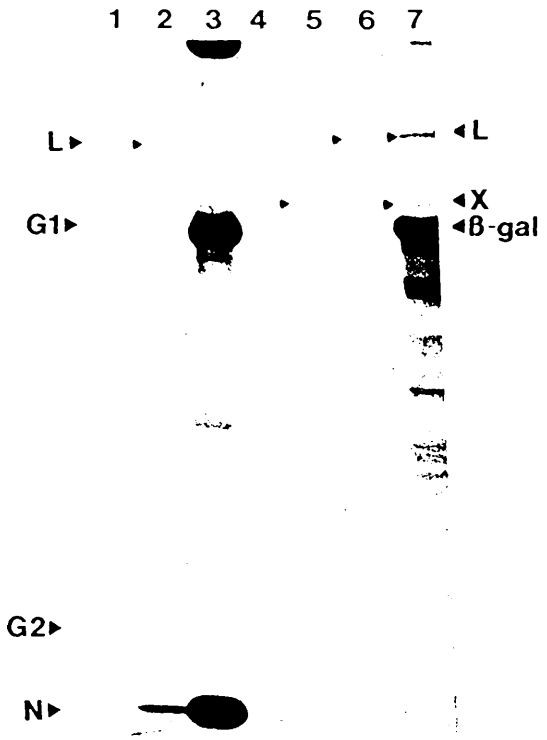

FIG. 4. Immunoprecipitation of the recombinant vaccinia virus-expressed L protein. Infected cells were labelled with [^{35}S]methionine at 22 to 24 h postinfection, and cell lysates were prepared for immunoprecipitation. The samples were precipitated with preimmune serum (lane 1) or a monospecific L protein antibody (β-galactosidase fusion with the 217 carboxy-terminal amino acids of L protein) in lanes 2 and 4 to 7 or with an anti-Bunyamwera virus serum in lane 3. Cells were infected with Bunyamwera virus (lanes 1, 2, and 3), vTF7-3 (lane 4), vTF7-5BUNL (lane 5), vTF7-3 and vTF7-5BUNL (lane 6), or vSC11-BUNL (lane 7). Lane 3 shows Bunyamwera virus protein markers L, G1, G2, and N. The monospecific L protein antiserum precipitates L protein and minor amounts of N protein (19) from Bunyamwera virus-infected cells (lane 2) but not from vTF7-3-infected cells (lane 4). L protein was precipitated from cells dually infected with vTF7-3 and vTF7-5BUNL (lane 6) or with vSC11-BUNL (lane 7). The fusion protein antibody also precipitates β-galactosidase from vSC11-BUNL-infected cells. The X protein is also precipitated by this antibody from cells infected by vTF7-5BUNL alone (lane 5) or vSC11-BUNL (lane 7). No proteins were precipitated by preimmune serum (lane 1).

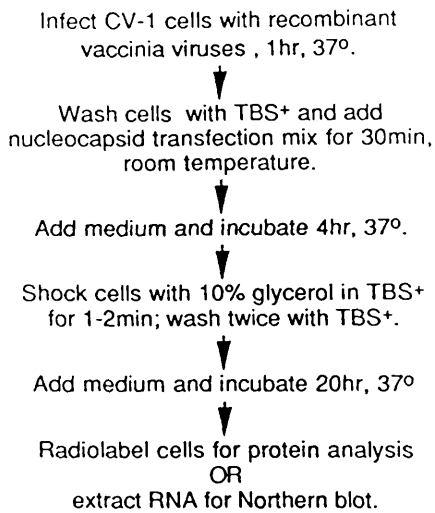

FIG. 5. Protocol of the nucleocapsid transfection assay.

RNA synthesis capability. The template for RNA synthesis employed by negative-strand viruses is the nucleocapsid, i.e., viral RNA complexed with viral proteins. At present, we do not have a method to reconstitute this template in vitro, but we can purify nucleocapsids from Bunyamwera virus-infected cells by cesium chloride centrifugation by established procedures (23, 30). Purified Bunyamwera virus nucleocapsids contain N and L proteins (19) and therefore should be capable of synthesizing RNA if transfected into cells. Thus, our initial aim was to determine the effect of recombinant vaccinia virus infection on the levels of Bunyamwera virus-specific RNA in cells transfected with purified nucleocapsids. We expected to see a background level of RNA after transfection of nucleocapsids and hoped to see an amplification after infection with the vaccinia viruses expressing L protein. In fact, preliminary experiments indicated that no Bunyamwera virus-specific RNAs could be detected in cells 24 h after transfection with nucleocapsids (data not shown). Undaunted by this result, we proceeded with the experiment using recombinant vaccinia viruses according to the scheme outlined in Fig. 5, and the results are shown in Fig. 6. Total cellular RNA was extracted from cells which had been infected with vTF7-3, vTF7-3 plus vTF7-5BUNL, or vSC11-BUNL and transfected with nucleocapsids; RNA was obtained from Bunyamwera virus-infected cells to act as specific markers. The RNAs were fractionated on agarose gels containing methyl mercuric hydroxide, transferred to nitrocellulose filters, and hybridized with strand-specific probes derived from cloned full-length Bunyamwera virus M and S segment cDNAs to detect either positive- or negative-sense RNA species. L segment probes were not included, as these would have detected the vaccinia virus-derived transcripts, at least in the case of vTF7-5BUNL. No hybridization was detected with RNA from cells infected with vTF7-3 (Fig. 6, lane 2), whereas weak but specific signals corresponding to positive- and negative-sense Bunyamwera virus S RNA were detected from cells infected with vTF7-3 and vTF7-5BUNL (lane 4). Much stronger signals to the positive- and negative-sense S RNAs were detected in the RNA extracted from cells infected with vSC11-BUNL (lane 3). Note that with this gel system, the full-length positive-sense S RNA and the shorter S mRNA are not separated. No M RNA signal was detected in RNAs from the nucleocapsid-transfected cells by using either probe, though both positive- and negative-sense M RNAs were detected in the Bunyamwera virus-infected cell sample (lane 1). These experiments demonstrate that the L protein expressed by the recombinant vaccinia viruses is able to transcribe both positive- and negative-sense S nucleocapsids (nucleocapsids obtained from infected cells contain RNAs of both polarities) and is thus functional in RNA synthesis. The difference in the levels of amplification seen between infection with vSC11-BUNL and dual infection with vTF7-3 and vTF7-5BUNL is reproducible, and so far we have been unable to detect amplification of the M segment RNA.

We have further characterized this nucleocapsid amplification system by analyzing the synthesis of Bunyamwera virus proteins (Fig. 7). After infection with recombinant

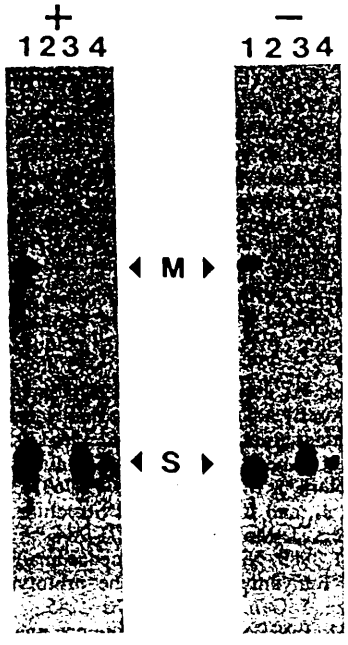

FIG. 6. Northern blot analysis of RNA from cells transfected with Bunyamwera virus nucleocapsids after infection with recombinant vaccinia viruses. CV-1 cells were infected with Bunyamwera virus (lanes 1), vTF7-3 (lanes 2), vSC11-BUNL (lanes 3), or both vTF7-3 and vTF7-5BUNL (lanes 4). The vaccinia virus-infected cells were subsequently transfected with Bunyamwera virus nucleocapsids by the calcium phosphate method (lanes 2 to 4). Twenty hours later, total cellular RNA was extracted, separated in agarose gels containing methyl mercuric hydroxide, transferred to nitrocellulose filters, and hybridized with a mixture of full-length ^{32}P-labelled riboprobes specific for the M and S segment positive (+) or negative (−) strand RNAs as indicated. The positions of the M and S segment RNAs are indicated by using the Bunyamwera virus-infected cell sample as markers (lanes 1). Both positive- and negative-sense S segment RNAs could be detected in nucleocapsid-transfected cells which expressed the L protein (lanes 3 and 4), but no Bunyamwera virus RNA was detected in transfected cells which had been infected with vTF7-3 alone (lane 2).

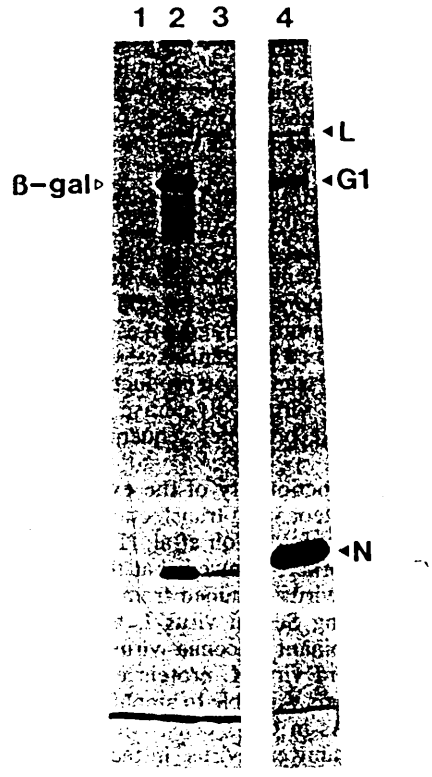

FIG. 7. Immunoprecipitation of cells infected with recombinant vaccinia viruses and transfected with Bunyamwera virus nucleocapsids. CV-1 cells were infected with vTF7-3 (lane 1), vSC11-BUNL (lane 2), both vTF7-3 and vTF7-5BUNL (lane 3), or Bunyamwera virus (lane 4). Cells infected with the vaccinia viruses were then transfected with Bunyamwera virus nucleocapsids (lanes 1 to 3), and radiolabelled cell lysates were prepared 20 h later. The lysates were precipitated with a mixture of the monospecific L protein and Bunyamwera virus antisera. The positions of the Bunyamwera virus proteins L, G1, and N from the control cells are shown in lane 4, which was cut from one side of the same gel as the other tracks to prepare the figure. The N protein was detected in cells expressing L protein from vaccinia viruses and transfected with Bunyamwera virus nucleocapsids (lanes 2 and 3) but not in cells infected with vTF7-3 alone prior to nucleocapsid transfection (lane 1).

vaccinia viruses and transfection with nucleocapsids, the cells were pulse-labelled with [^{35}S]methionine, and then cell lysates were immunoprecipitated with a mixture of the monospecific L protein antiserum (19) and an antiserum prepared against purified Bunyamwera virus (33). No Bunyamwera virus proteins were detected in vTF7-3-infected, nucleocapsid-transfected cells (Fig. 7, lane 1). Both L and N proteins were detected in nucleocapsid-transfected cells which had also been infected with either vSC11-BUNL (lane 2) or vTF7-3 and vTF7-5BUNL (lane 3). Much less N protein was detected in the dual vaccinia virus-infected cells than in cells infected with vSC11-BUNL. The major glycoprotein G1 was not detected in the nucleocapsid-transfected cells but was observed in the Bunyamwera virus-infected control (lane 4). The results of the protein analysis are entirely consistent with the previous RNA analyses.

DISCUSSION

As with other negative-strand viruses, transcription and replication of the bunyavirus genome involve the L protein. The large size of the L protein suggests it is multifunctional and most probably catalyzes initiation, elongation, and termination of RNA synthesis and perhaps also plays a role in events which generate the host-derived primers for transcription, such as cap-binding and endonuclease activities. As a prelude to mapping the individual domains within the L protein, we sought to express functional L protein in a eukaryotic vector, namely vaccinia virus. In this article, we have described the reconstruction of a cDNA containing the complete L gene coding region and its subcloning into two vaccinia virus expression systems. We chose to investigate both systems in parallel in case constitutive expression of the L gene by the vaccinia virus *P7.5* promoter was detrimental to vaccinia virus replication; e.g., Li et al. (24) reported that the vesicular stomatitis virus M protein was toxic to vaccinia virus when they attempted to make a recombinant based on pSC11. The use of the T7 RNA polymerase system would have overcome this problem. In fact, both systems expressed full-length antigenically authentic Bunyamwera virus L protein. Apparently, more L protein was synthesized with vSC11-BUNL than with vTF7-3 and vTF7-5BUNL, even though the levels of L protein mRNA were considerably higher in cells infected

with the latter. In their characterization of the vaccinia virus-T7 polymerase system Fuerst and Moss (14) also observed much higher levels of mRNA compared with the level of protein made and suggested that the apparent inefficient translation of the T7 polymerase-transcribed RNAs was because only 5 to 10% contained terminal cap structures.

In addition to full-length L protein, other proteins apparently specific for the recombinant vaccinia viruses were observed (Fig. 3 and 4). In particular, the protein designated X is worthy of mention because this was immunoprecipitated by the monospecific L protein antibody, suggesting it to be a carboxy-terminal fragment of L protein. Although we have not established the genesis of this protein, we suggest that it may be the translation product of an RNA transcribed by the vaccinia virus polymerase recognizing a cryptic vaccinia virus-like promoter sequence (7, 8) in the L gene cDNA.

To test the functionality of the expressed L protein, we developed a nucleocapsid transfection assay on the basis of a previous report by Gotoh et al. (17), who studied Sendai virus. These workers showed that they could enhance the yield of Sendai virus obtained from transfected nucleocapsids by supplying Sendai virus L and P/C gene products through recombinant vaccinia viruses. We demonstrated that Bunyamwera virus L protein expressed by both vaccinia virus systems was able to amplify markedly the level of S segment RNAs in CV-1 cells. After transfection of cells with the Bunyamwera virus intracellular nucleocapsids, which contain both negative- and positive-sense genomes, we could not detect any virus RNA. This result was somewhat surprising; since our preparations of nucleocapsids contain the N and L proteins (19), we expected these to be capable of initiating RNA synthesis. We suspect therefore that the L protein must become damaged during the nucleocapsid purification scheme. However, the transfected nucleocapsids do provide an appropriate template for the synthesis of both polarities of at least the S segment RNA. Thus far we have been unable to demonstrate synthesis of M segment RNA. Whether this reflects a relative lower abundance (RNA extracted from nucleocapsid is rarely equimolar, and usually S predominates [11]), lower efficiency of transfection or lower stability of M segment nucleocapsids is not known, and experiments to address this question are in progress. Since no M segment RNA is produced and no M RNA gene products are synthesized, we have been unable to demonstrate the production of infectious Bunyamwera virus, in contrast to the results of Gotoh et al. (17) using the unsegmented Sendai virus. Notwithstanding this point, we have described a system which we think will be exploitable to dissect the functions and functional domains of the complex L protein encoded by Bunyamwera virus.

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  51  N  N  V  L  L  D  E  I  I  L  D  V  V  P  G  V  N  L  L  N  Y  N  I  P  N  V  T  P  D  N  Y  I  W  D
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  85   G  H  F  L  I  I  L  D  Y  K  V  S  V  G  N  D  S  S  E  I  T  Y  K  K  Y  T  S  L  I  L  P  V  M
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1201  AAGCGGTTAAGATTGGCAATGCACTTGTGTTATGGGAACAACAATTCATCTTAGCAAACGACTTATTTAAAAATCAAGAAAGGCAGAAGTTCATGAAAAA

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 451  L  M  A  A  R  T  M  V  N  K  N  K  A  L  L  A  K  D  N  T  L  Q  D  L  H  P  I  I  M  Q  Y  A  S  E
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 651  E  P  S  R  Y  M  I  M  N  S  L  A  I  S  S  H  V  R  D  Y  I  A  E  K  F  S  P  Y  T  K  T  L  F  S
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 685   V  Y  M  V  N  L  I  K  R  G  C  A  S  A  N  E  Q  S  S  K  I  Q  L  R  N  I  Y  L  S  D  Y  D  I
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 718  T  Q  K  G  V  N  D  G  R  N  L  D  S  I  W  F  P  G  K  V  N  L  K  E  Y  I  N  Q  I  Y  L  P  F
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 851  R  E  I  K  D  K  E  T  E  K  S  K  K  S  T  E  K  F  D  K  K  F  R  L  S  N  P  L  F  L  E  D  E  E
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 885   A  N  L  E  V  Q  H  C  N  Y  R  A  L  I  Q  K  I  P  N  Y  K  D  Y  I  S  V  K  V  F  D  R  L  Y
2701  AAGCCAATCTTGAAGTTCAACATTGCAATTATAGGGCTCTGATACAAAAAATTCCTAATTATAAAGACTATATTTCAGTAAAGGTGTTTGATCGTCTATA
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 918  E  L  L  K  N  G  V  L  T  D  K  P  F  I  E  L  A  M  E  M  M  K  N  H  K  E  F  S  F  T  F  F  N
2801  TGAGTTGCTAAAAAATGGAGTCTTAACAGACAAACCTTTCATTGAATTAGCTATGGAAATGATGAAGAATCACAAGGAATTCTCTTTCACATTCTTTAAT

 951  K  G  Q  K  T  A  K  D  R  E  I  F  V  G  E  F  E  A  K  M  C  M  Y  V  V  E  R  I  S  K  E  R  C  K
2901  AAGGGCCAAAAGACAGCTAAAGATAGAGAGATATTCGTTGGAGAATTTGAAGCTAAAATGTGTATGTATGTAGTGGAGAGAATATCAAAAGAGAGATGTA

 985  L  N  T  D  E  M  I  S  E  P  G  D  S  K  L  K  I  L  E  K  K  A  E  E  E  I  R  Y  I  V  E  R  T
3001  AGCTGAACACGGACGAGATGATAAGTGAACCAGGTGATTCAAAATTGAAAATATTGGAAAAGAAAGCAGAAGAGGAAATCCGCTATATTGTTGAGAGAAC

1018  K  D  S  I  I  K  G  D  P  S  K  A  L  K  L  E  I  N  A  D  M  S  K  W  S  A  Q  D  V  F  Y  K  Y
3101  AAAAGACAGTATAATTAAAGGAGACCCATCAAAAGCATTGAAACTGGAAATAAATGCAGACATGTCAAAATGGAGTGCTCAAGATGTATTTTACAAATAT

1051  F  W  L  I  A  M  D  P  I  L  Y  P  A  E  K  T  R  I  L  Y  F  M  C  N  Y  M  Q  K  L  L  I  L  P  D
3201  TTTTGGCTGATAGCAATGGACCCTATACTTTATCCAGCAGAAAAAAACACGTATTCTGTATTTTATGTGCAATTATATGCAAAAACTATTAATACTTCCAG

1085  D  L  I  A  N  I  L  D  Q  K  R  P  Y  N  D  D  L  I  L  E  M  T  N  G  L  N  Y  N  Y  V  Q  I  K
3301  ATGATTTAATTGCAAATATCTTAGATCAGAAAAGACCTTATAATGATGATTTGATCCTTGAGATGACTAATGGTCTAAATTATAATTATGTCCAAATTAA

1118  R  N  W  L  Q  G  N  F  N  Y  I  S  S  Y  V  H  S  C  A  M  L  V  Y  K  D  I  L  K  E  C  M  K  L
3401  AAGAAACTGGCTCCAGGGCAATTTCAATTACATTTCTAGTTATGTGCATAGTTGTGCAATGCTTGTTTACAAAGATATCCTCAAAGAATGTATGAAGTTA

1151  L  D  G  D  C  L  I  N  S  M  V  H  S  D  D  N  Q  T  S  L  A  I  I  Q  N  K  V  S  D  Q  I  V  I  Q
3501  CTAGACGGAGACTGCTTGATTAACTCAATGGTGCATTCAGATGACAATCAAACATCGTTAGCAATTATCCAAAATAAAGTCTCTGATCAAATAGTAATTC

1185  Y  A  A  N  T  F  E  S  V  C  L  T  F  G  C  Q  A  N  M  K  K  T  Y  I  T  H  T  C  K  E  F  V  S
3601  AATATGCAGCAAACACATTTGAGTCTGTTTGTTTGACATTTGGATGTCAGGCAAACATGAAAAAAACATATATTACTCATACATGCAAAGAATTTGTCTC

1218  L  F  N  L  H  G  E  P  L  S  V  F  G  R  F  L  L  P  S  V  G  D  C  A  Y  I  G  P  Y  E  D  L  A
3701  ACTTTTCAATTTACATGGAGAACCACTATCTGTCTTTGGCAGATTTTTATTGCCTAGTGTAGGTGATTGTGCTTACATTGGGCCATATGAAGATTTAGCC

1251  S  R  L  S  A  A  Q  Q  S  L  K  H  G  C  P  P  S  L  V  W  L  A  I  S  C  S  H  W  I  T  F  F  T  Y
3801  AGCCGTTTATCTGCAGCACAACAGAGCTTAAAGCATGGGTGCCCTCCAAGTCTAGTTTGGTTAGCAATAAGCTGTAGCCACTGGATAACATTTTTCACTT

1285  N  M  L  D  D  Q  I  N  A  P  Q  Q  H  L  P  F  N  N  R  K  E  I  P  V  E  L  N  G  Y  L  N  A  P
3901  ACAACATGCTGGATGATCAAATTAATGCACCACAGCAGCATTTGCCATTCAATAATCGGAAGGAAATACCAGTTGAGTTAAATGGGTACTTGAATGCACC

1318  L  Y  L  I  A  L  V  G  L  E  A  G  N  L  W  F  L  I  N  I  L  K  K  L  V  P  L  D  K  Q  K  E  T
4001  ATTATATTTGATAGCATTAGTCGGCTTGGAAGCTGGGAACTTATGGTTTTTAATAAATATATTGAAAAAATTGGTGCCATTGGATAAACAGAAAGAAACT

1351  I  Q  S  Q  C  L  H  L  C  N  S  I  D  K  L  T  E  S  E  K  F  K  L  K  I  L  R  Y  L  T  L  D  T  E
4101  ATACAAAGCCAATGTTTACACTTATGCAATTCAATTGATAAGCTGACAGAATCAGAAAAGTTCAAATTAAAAATATTGAGATATCTTACTCTTGACACTG

1385  M  S  V  D  N  N  M  G  E  T  S  D  M  R  S  R  S  L  L  T  P  R  K  F  T  T  L  G  S  L  N  K  L
4201  AGATGTCAGTTGATAACAACATGGGAGAAACAAGTGATATGCGAAGTAGATCACTCTTAACGCCTCGCAAATTTACAACATTGGGATCCTTAAATAAACT

1418  V  S  Y  N  D  F  R  S  S  L  D  D  Q  R  F  T  D  N  L  N  F  M  L  N  N  P  E  L  L  V  T  K  G
4301  AGTTTCCTATAATGACTTTAGATCTTCTTTAGACGACCAGAGATTTACTGATAATTTGAACTTCATGCTTAATAACCCAGAATTGTTAGTTACTAAAGGT

1451  E  N  K  E  Q  F  M  Q  S  V  L  F  R  Y  N  S  K  R  F  K  E  S  L  S  I  Q  N  P  A  Q  L  F  I  E
4401  GAAAATAAAGAGCAGTTCATGCAATCTGTCCTTTTCAGATATAATTCAAAAAGATTTAAAGAAAGCCTTTCCATCCAAAAGCCAGCACAATTATTTATTG

1485  Q  I  L  F  S  H  K  P  I  I  D  Y  S  S  I  F  D  K  L  T  S  L  A  E  A  D  I  I  E  E  L  P  E
4501  AGCAGATACTGTTTTCCCATAAACCAATCATAGATTACAGCAGTATATTTGATAAATTAACCTCACTTGCAGAAGCGGATATCATTGAAGAGCTACCAGA

1518  I  I  G  R  V  T  F  P  Q  A  Y  Q  M  I  N  R  D  I  G  Q  L  P  L  D  I  D  D  I  K  L  I  F  R
4601  GATCATTGGAAGAGTTACATTTCCTCAGGCATACCAGATGATAAATAGAGATATTGGCCAACTACCTTTAGATATAGATGATATTAAGTTAATATTCCGG

1551  Y  C  I  L  N  D  P  L  M  I  T  A  A  N  T  S  L  L  C  V  K  G  T  P  Q  D  R  T  G  L  S  A  S  Q
4701  TATTGTATATTGAATGATCCACTAATGATCACAGCTGCAAACACTTCCTTATTATGTGTTAAAGGAACACCACAAGATAGAACTGGCCTCAGTGCAAGTC

1585  M  P  E  F  R  N  M  K  L  I  H  H  S  P  A  L  V  L  K  A  F  S  K  G  T  S  D  I  P  G  A  D  P
4801  AAATGCCTGAATTTAGAAATATGAAACTTATTCACCATTCCCCTGCTCTAGTTCTTAAGGCGTTTAGCAAAGGGACATCAGATATTCCTGGGGCTGATCC

1618  I  E  L  E  K  D  L  H  H  L  N  E  F  V  E  T  T  A  I  K  E  K  I  L  H  N  I  D  N  P  P  K  H
4901  TATAGAATTGGAAAAAGATCTTCATCACTTAAATGAATTTGTTGAAACAACAGCAATTAAAGAAAAGATTTTGCACAACATAGACAATCCTCCTAAGCAT

1651  L  I  G  N  E  I  L  I  Y  R  I  R  E  M  T  K  L  Y  Q  V  C  Y  D  Y  V  K  S  T  E  H  K  V  K  I
5001  TTAATAGGGAATGAAATCCTAATTTATAGAATCAGAGAGATGACCAAACTCTATCAGGTTTGTTATGATTATGTTAAATCTACAGAGCATAAGGTTAAAA

1685  F  I  L  P  M  K  S  Y  T  A  I  D  F  C  T  L  I  Q  G  N  T  I  S  D  N  K  W  Y  T  M  H  Y  L
5101  TATTTATATTACCAATGAAATCTTATACTGCAATTGACTTTTGCACATTGATTCAGGGCAACACTATCTCTGATAATAAATGGTACACAATGCATTATTT

1718  K  Q  I  A  S  G  S  I  K  G  N  I  V  T  T  S  T  S  E  Q  I  I  A  N  E  C  F  R  V  L  C  H  F
5201  AAAACAGATTGCTAGCGGATCTATCAAAGGGAATATAGTAACAACTAGTACAAGCGAGCAAATAATAGCAAATGAGTGTTTTAGAGTGCTCTGCCACTTT

1751  A  D  S  F  V  E  E  A  S  R  L  S  F  I  N  E  V  L  D  N  F  T  Y  K  N  I  S  V  N  S  L  F  N  T
5301  GCTGATTCCTTTGTGGAAGAGGCAAGCAGATTGAGCTTTATTAATGAAGTTCTTGATAATTTCACATATAAAAACATAAGTGTTAACTCCTTATTTAACA
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1785   L  L  A  S  T  T  R  L  D  F  I  P  L  L  F  R  L  K  V  L  T  Q  T  D  L  N  R  F  D  A  L  K  T
5401   CTCTATTAGCCAGCACTACAAGGTTAGACTTTATTCCTCTATTATTTAGACTCAAAGTTTTAACTCAGACAGATTTAAATAGATTTGATGCCCTTAAAAC

1818   N  E  R  V  S  W  N  N  W  Q  T  N  R  S  L  N  S  G  L  I  D  L  T  I  S  G  Y  L  R  S  I  R  V
5501   TAATGAAAGAGTTTCATGGAATAACTGGCAGACAAACCGTTCCTTAAATTCAGGTCTGATTGATTTGACAATATCCGGCTATTTAAGATCAATAAGGGTT

1851   V  G  E  D  N  K  L  K  I  A  E  L  T  I  P  N  F  Y  P  N  T  V  F  H  A  G  N  K  L  L  N  S  R  H
5601   GTGGGGGAAGATAATAAACTCAAAATTGCTGAACTAACAATACCTAATTTCTATCCAAATACAGTGTTCCATGCAGGGAACAAACTTCTAAATTCTAGAC

1885   G  L  K  F  E  Y  M  E  E  I  V  L  D  E  K  Y  N  Y  Y  I  T  Y  Q  K  K  R  A  H  I  Y  T  Y  Q
5701   ATGGATTAAAATTTGAATACATGGAGGAAATTGTTCTAGATGAAAAATATAACTATTATATAACATACCAAAAAAAGAGGGCTCATATTTATACATATCA

1918   V  S  T  I  E  H  I  L  R  R  N  N  E  G  L  Q  S  R  G  P  R  Y  N  K  M  V  P  V  C  P  V  V  L
5801   AGTATCTACAATAGAACATATTTTGAGAAGGAATAATGAAGGATTACAATCCAGAGGCCCTAGGTATAACAAAATGGTTCCTGTCTGTCCAGTTGTTTTA

1951   S  V  R  D  E  L  F  R  M  S  L  E  N  V  F  S  L  N  M  T  N  F  S  M  S  R  L  F  V  S  P  D  E  V
5901   AGTGTCAGGGATGAATTATTTAGAATGTCTCTAGAAAATGTTTTTAGTTTAAACATGACAAACTTTAGCATGTCTAGATTATTTGTTTCACCTGACGAAG

1985   A  T  V  K  K  A  H  M  S  K  M  M  F  F  S  G  P  T  I  K  A  G  I  I  N  L  T  S  L  M  R  T  Q
6001   TTGCTACTGTAAAGAAAGCTCATATGTCCAAAATGATGTTCTTTTCCGGGCCAACAATAAAAGCAGGAATTATTAATTTAACATCTTTAATGAGGACCCA

2018   E  L  L  T  L  N  Y  D  N  L  C  K  S  S  I  V  P  F  C  R  I  L  E  C  N  G  D  E  Q  G  E  L  I
6101   AGAGCTTTTAACATTGAATTATGATAATCTATGCAAATCTAGCATTGTCCCGTTTTGTAGAATATTAGAATGTAATGGCGATGAGCAAGGAGAACTAATA

2051   F  L  S  D  E  V  M  D  F  T  I  S  E  E  I  E  S  M  P  L  F  T  I  R  Y  Q  K  R  G  T  E  I  M  T
6201   TTTCTTTCAGATGAAGTCATGGATTTCACAATTTCTGAGGAGATAGAATCTATGCCATTATTTACAATAAGGTATCAGAAAAGAGGTACTGAAATTATGA

2085   Y  K  N  A  I  M  K  L  V  S  A  G  V  D  E  I  K  E  V  F  D  F  S  K  Q  G  F  Y  S  K  K  N  L
6301   CTTATAAAAATGCTATAATGAAGTTAGTTTCAGCAGGGGTAGATGAGATCAAAGAAGTTTTTGATTTTTCAAAACAAGGGTTCTATTCAAAGAAAAACTT

2118   G  I  I  N  T  I  C  S  I  I  N  I  L  E  T  N  E  W  S  T  I  L  Y  N  S  F  H  I  A  M  L  L  E
6401   AGGTATAATAAATACAATTTGTTCTATAATAAATATACTAGAGACAAATGAGTGGTCCACAATTCTATACAATTCCTTCCATATAGCAATGTTATTAGAG

2151   S  M  D  R  E  F  H  M  F  T  L  P  E  A  F  F  I  N  V  A  G  G  V  V  N  W  T  K  L  L  K  F  I  K
6501   TCTATGGATCGAGAATTCCATATGTTCACATTACCCGAAGCCTTTTTCATAAATGTGGCAGGTGGTGTTGTTAATTGGACTAAGCTGCTAAAATTTATAA

2185   S  L  P  V  I  E  Q  E  P  W  S  M  M  M  S  R  F  V  E  K  T  V  Y  L  I  E  R  E  M  N  K  D  V
6601   AGTCATTGCCAGTGATAGAGCAAGAGCCTTGGTCAATGATGATGTCAAGATTTGTAGAAAAAACTGTGTATTTGATAGAAAGAGAAATGAACAAAGATGT

2218   D  F  T  D  F  L  D  E  L  E  F  S  S  G  K  S  L  F  T  F  F      2238

6701   TGACTTCACTGATTTCTTAGATGAGTTAGAATTTAGTTCAGGAAAGTCTCTATTTACCTTTTTCTGAAACATATCTTCATTGGTTTATTTAATTGGACAT

6801   TCCAAAAGCACTATGTGGCAAAAATGATAACAGCATTCAAAAAAGTACAATTTTCTTATGTAGGAGCACACTACT  6875
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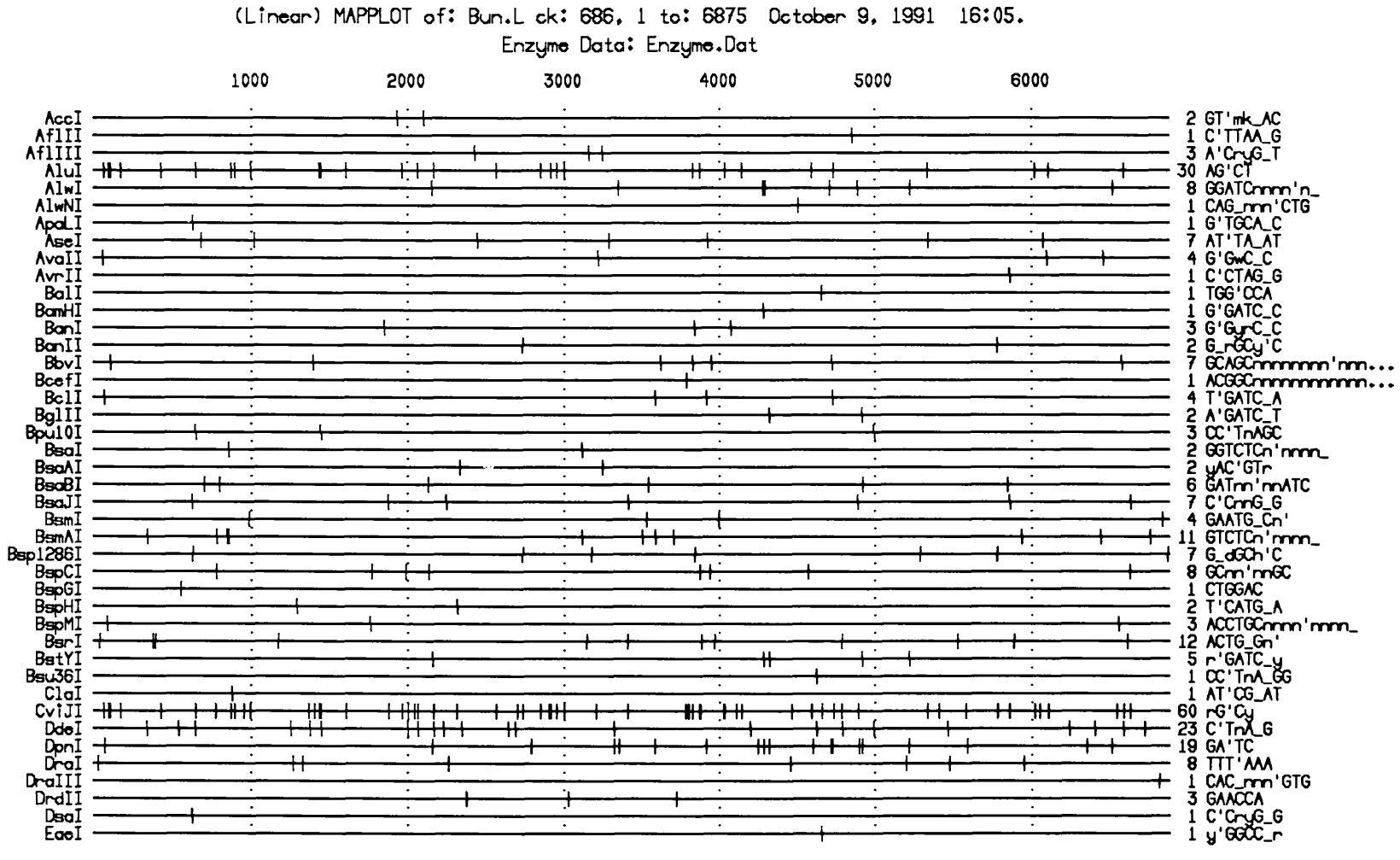
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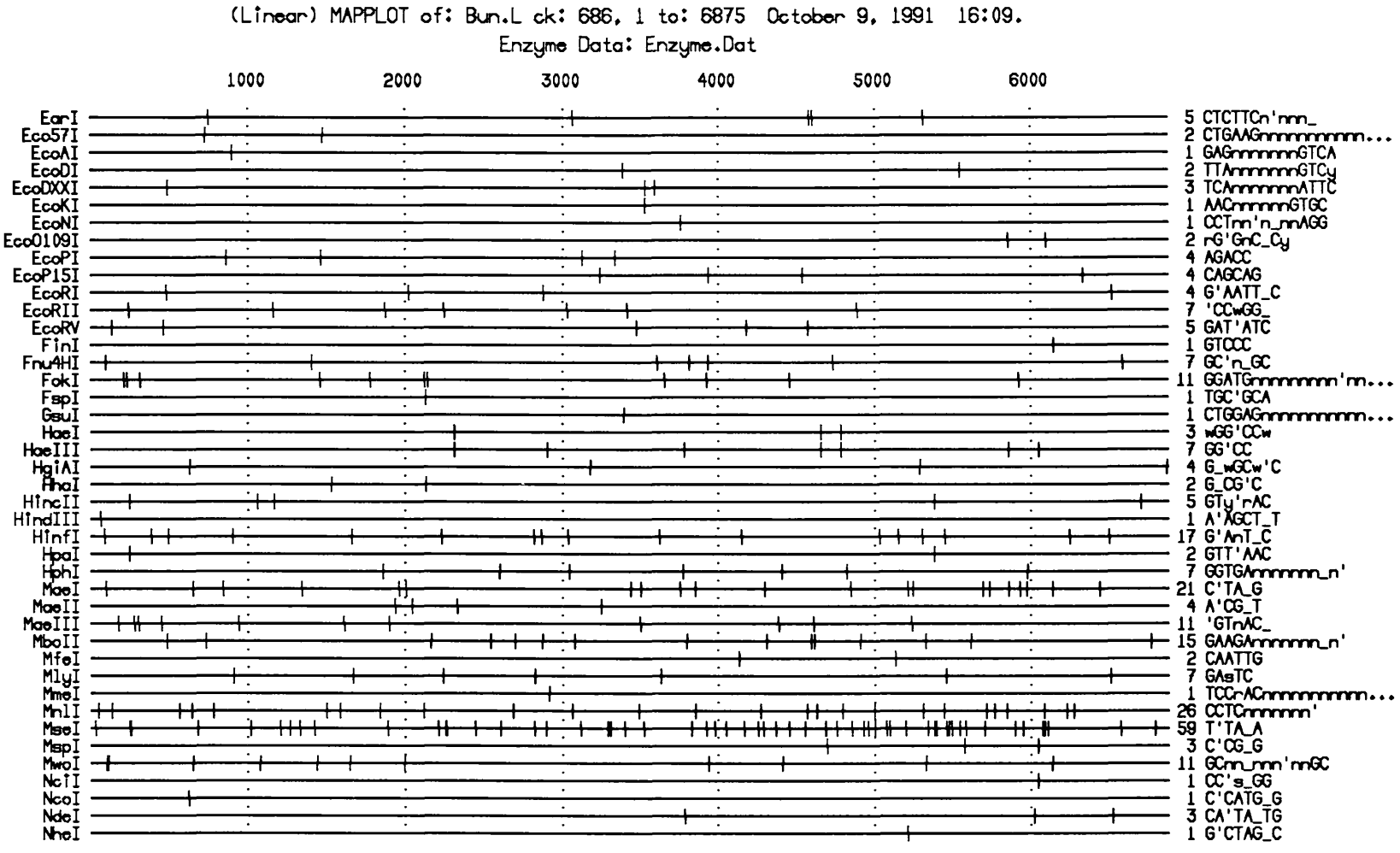
2000
3000
4000
5000
6000
AccI 2 GT'mk_AC
AflII 1 C'TTAA_G
AflIII 3 A'CryG_T
AluI 30 AG'CT
AlwI 8 GGATCnnnn'n_
AlwNI 1 CAG_nnn'CTG
ApaLI 1 G'TGCA_C
AseI 7 AT'TA_AT
AvaII 4 G'GwC_C
AvrII 1 C'CTAG_G
BalI 1 TGG'CCA
BamHI 1 G'GATC_C
BanI 3 G'GyrC_C
BanII 2 G_rGCy'C
BbvI 7 GCAGCnnnnnnnn'nnn...
BcefI 1 ACGGCnnnnnnnnnnnn...
BclI 4 T'GATC_A
BglII 2 A'GATC_T
BpulOI 3 CC'TnAGC
BsaI 2 GGTCTCn'nnnn_
BsaAI 2 yAC'GTr
BsaBI 6 GATnn'nnATC
BsaJI 7 C'CnnG_G
BsmI 4 GAATG_Cn'
BsmAI 11 GTCTCn'nnnn_
Bsp1286I 7 G_dGCh'C
BspCI 8 GCnn'nnGC
BspGI 1 CTGGAC
BspHI 2 T'CATG_A
BspMI 3 ACCTGCnnnn'nnnn_
BsrI 12 ACTG_Gn'
BstYI 5 r'GATC_y
Bsu36I 1 CC'TnA_GG
ClaI 1 AT'CG_AT
CviJI 60 rG'Cy
DdeI 23 C'TnA_G
DpnI 19 GA'TC
DraI 8 TTT'AAA
DraIII 1 CAC_nnn'GTG
DrdII 3 GAACCA
DsaI 1 C'CryG_G
EaeI 1 y'GGCC_r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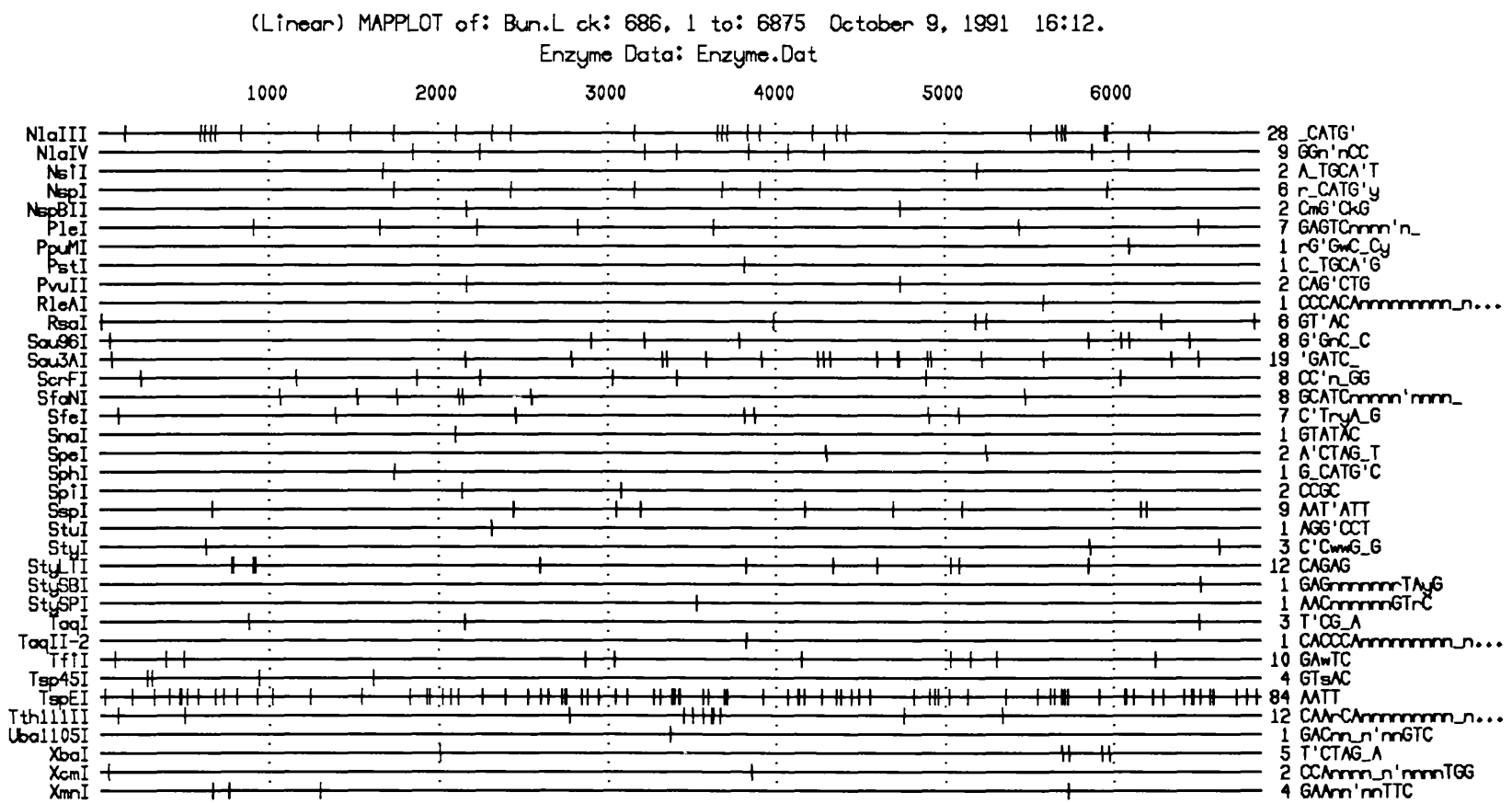
6000
EarI 5 CTCTTCn'nnn_
Eco57I 2 CTGAAGnnnnnnnnnnn...
EcoAI 1 GAGnnnnnnGTCA
EcoDI 2 TTAnnnnnnGTCy
EcoDXXI 3 TCAnnnnnnnATTC
EcoKI 1 AACnnnnnnGTGC
EcoNI 1 CCTnn'n_nnAGG
Eco0109I 2 rG'GnC_Cy
EcoPI 4 AGACC
EcoP15I 4 CAGCAG
EcoRI 4 G'AATT_C
EcoRII 7 'CCwGG_
EcoRV 5 GAT'ATC
FinI 1 GTCCC
Fnu4HI 7 GC'n_GC
FokI 11 GGATGnnnnnnnnn'nn...
FspI 1 TGC'GCA
GsuI 1 CTGGAGnnnnnnnnnnn...
HaeI 3 wGG'CCw
HaeIII 7 GG'CC
HgiAI 4 G_wGCw'C
HhaI 2 G_CG'C
HincII 5 GTy'rAC
HindIII 1 A'AGCT_T
HinfI 17 G'AnT_C
HpaI 2 GTT'AAC
HphI 7 GGTGAnnnnnnn_n'
MaeI 21 C'TA_G
MaeII 4 A'CG_T
MaeIII 11 'GTnAC_
MboII 15 GAAGAnnnnnnn_n'
MfeI 2 CAATTG
MlyI 7 GAstTC
MmeI 1 TCCnACnnnnnnnnnnn...
MnlI 26 CCTCnnnnnnn'
MseI 59 T'TA_A
MspI 3 C'CG_G
MwoI 11 GCnn_nnn'nnGC
NciI 1 CC's_GG
NcoI 1 C'CATG_G
NdeI 3 CA'TA_TG
NheI 1 G'CTAG_C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6000
NlaIII 28 _CATG'
NlaIV 9 GGn'nCC
NsiI 2 A_TGCA'T
NspI 6 r_CATG'y
NspBII 2 CmG'CkG
PleI 7 GAGTCnnnn'n_
PpuMI 1 rG'GwC_Cy
PstI 1 C_TGCA'G
PvuII 2 CAG'CTG
RleAI 1 CCCACAnnnnnnnnn_n...
RsaI 6 GT'AC
Sau96I 8 G'GnC_C
Sau3AI 19 'GATC_
ScrFI 8 CC'n_GG
SfaNI 8 GCATCnnnnn'nnnn_
SfeI 7 C'TryA_G
SnaI 1 GTATAC
SpeI 2 A'CTAG_T
SphI 1 G_CATG'C
SpiI 2 CCGC
SspI 9 AAT'ATT
StuI 1 AGG'CCT
StyI 3 C'CwwG_G
StyLTI 12 CAGAG
StySBI 1 GAGnnnnnnnTAyG
StySPI 1 AACnnnnnnGTrC
TaqI 3 T'CG_A
TaqII-2 1 CACCCAnnnnnnnnn_n...
TfiI 10 GAwTC
Tsp45I 4 GTsAC
TspEI 84 AATT
Tth111II 12 CAArCAnnnnnnnnn_n...
Uba1105I 1 GACnn_n'nnGTC
XbaI 5 T'CTAG_A
XcmI 2 CCAnnnn_n'nnnnTGG
XmnI 4 GAAnn'nnTTC

Enzymes that do not cut:

AatII	AgeI	AhaII	ApaI	AvaI	BbvII	BcgI	BglI	BsiI	BspMII	BssHII	BstBI
BstEII	BstXI	Cfr10I	CfrAI	DrdI	EciI	Eco47III	EcoBI	EcoEI	EcoR124I	EcoR124/3I	
EspI	Esp3I	FauI	FseI	GdiII	HaeII	HgaI	HgiEII	HinfIII	KpnI	McrI	MluI
NaeI	NarI	NotI	NruI	PflMI	PmlI	PshAI	PvuI	RsrII	SacII	SalI	ScaI
SfiI	SgrAI	SmaI	SnaBI	SplI	SstI	StySJI	StySQI	TaqII-1	ThaI	Tth111I	Uba1108I
					XhoI	XmaIII					

Enzymes excluded: MinCuts: 1 MaxCuts: 350000

NONE