EXPRESSION OF THE BUNYAMWERA VIRUS M GENOME SEGMENT GENE PRODUCTS

by

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SUMMARY

Bunyamwera virus is the prototype of both the *Bunyavirus* genus and the Bunyaviridae family, which comprises more than 300 viruses. The virus possesses a negative stranded RNA genome of three segments designated Large (L), Middle (M) and Small (S). The M segment codes for the two virion glycoproteins, G1 and G2, and a non-structural polyprotein, termed NSm. The mature gene products are derived from a polyprotein precursor which is probably cotranslationally cleaved as it has not been detected in virus-infected cells. Genetic studies have indicated that the M segment gene products are involved in many of important biological attributes of the virus, including determinants of virulence, neutralisation and haemagglutination. The M segment RNA of bunyaviruses has a different gene organisation from those of *Phlebovirus* genus in having NSm in the middle of the precursor, whereas some members of the *Phlebovirus* genus have NSm at the beginning of the precursor. The Uukuniemi group of phleboviruses and members of the *Hantavirus* genus have no NSm. A common feature among members of the Bunyaviridae is maturation at the smooth surfaced membranes of the Golgi complex. The glycoproteins encoded by the M segment RNA are thought to be the main determinants of this feature. It was therefore of interest to express the Bunyamwera virus M RNA segment in order to study the biogenesis of the glycoproteins and NSm, and with the long-term view of studying and targetting of these proteins to the Golgi.

The overall aims of this work were to express the M RNA segment of Bunyamwera virus from a cloned cDNA; to determine the cellular residence of NSm; and to investigate the transport of the proteins from the endoplasmic reticulum to the Golgi complex. No immunological reagents specific for NSm were available, and two approaches were utilized in the production of specific antisera to NSm. One involved synthesis of fusion proteins with β-galactosidase using the

pUEX3 vector system, and the second synthesis of branching peptides to selected sequences of NSm. Rabbits were immunized separately with these immunogens and the antisera characterised by enzyme-linked immunosorbent assays and radioimmunoprecipitations. Antisera to fusion proteins failed to recognise NSm in immunoprecipitations. However, immune sera raised against the branching peptides recognised NSm specifically in Bunyamwera virus-infected cells by radioimmunoprecipitation.

The antipeptide sera were used in indirect immunofluorescence experiments to determine that NSm localized to the Golgi complex in virus-infected cells; this was confirmed by dual staining with rhodamine conjugated wheat germ agglutinin which predominantly stains the Golgi complex.

For *in vitro* translation of mRNA synthesized from Bunyamwera virus cDNA, the full length cDNA was cloned in the pT7T3 transcription vector under the control of the T3 RNA promoter. Full length and various truncated transcripts were made using T3 RNA polymerase. Attempts to express the transcripts *in vitro* were unsuccessful in both rabbit reticulocyte lysate and in wheat germ cell free translation systems. However, a transcript representing nucleotides 1352 to 4001 of the M segment mRNA, with an internal in-frame AUG at nucleotides 1430-1432, was translated *in vitro* in the rabbit reticulocyte lysate system to yield a 90K protein which was immunoprecipitated by antivirus serum. These experiments suggested that sequences at the 5' end of the M segment mRNA inhibited translation *in vitro*.

Since translation *in vitro* appeared to be unsuitable in the study of the biogenesis of the M RNA gene products, attempts were made to express the proteins using recombinant vaccinia viruses. The cDNA was cloned in the vaccinia virus transfer vector vTF7-5 under control of the T7 promoter. Recombinant viruses were obtained, as demonstrated by dot

blot and Southern blot analyses, and one designated recBUN M. In cells dually infected with recBUN M and vTF7-3, a recombinant vaccinia virus, which expresses T7 RNA polymerase, G1, G2 and NSm were synthesised. These proteins were demonstrated to be antigenically authentic by immunoprecipitation reactions with anti-BUN serum, which recognises both G1 and G2, and the antipeptide serum to NSm.

Plasmids containing full length M segment cDNA and fragments representing G1 (nucleotides 1352-4458), G2 (nucleotides 1-941) and NSm (nucleotides 963-1466) under the control of the bacteriophage T7 RNA promoter were made. The genes were transiently expressed by transfecting plasmid DNA into cells infected with vTF7-3. The G1 glycoprotein was detected in cells transfected with pT7T318UBUNM and was precipitated with virus antiserum. Cells transfected with pT7T318U/G1 also synthesised G1, which co-migrated with G1 made by the virus, and was also immunoprecipitated by antivirion serum. The G2 glycoprotein was synthesised in cells transfected with pTF7-5/G2 and detected by immunofluorescence with anti-virion serum. NSm was also synthesised and detected by immunofluorescence using branched antipeptide sera to NSm. Studies on glycosylation of G1 using tunicamycin (an inhibitor of glycosylation) revealed that the glycoprotein was properly glycosylated when synthesised in dual vaccinia virus infected cells, and also when transiently expressed.

Immunofluorescence studies of cells dually infected with the recombinant vaccinia virus demonstrated that G1, G2 and NSm localised to the Golgi complex. When transiently expressed individually, G2 and NSm were localised to the Golgi complex. G1 on its own however remained in the endoplasmic reticulum. When G2 was co-expressed with G1, both transported to the Golgi complex. Some G1 was expressed on the cell surface, but no G2 was detectable on the surface.

This is the first report about the expression of the middle RNA genome segment for any member of the *Bunyavirus* genus, and provides the basis for study of the very actively researched area of intracellular protein transport.

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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'-deoxycytosine-5'-triphosphate
ddGTP	2',3'-deoxyguanosine-5'-triphosphate
ddTTP	2',3'-deoxythymidine-5'-triphosphate
DI	defective interfering
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
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	multiplicity of infection
Mr	relative molecular mass
mRNA	messenger riobnucleic acid
N-terminal	amino-terminal
NP40	Nonidet P40
nt	nucleotides
OD	optical density
ORF	open reading frame
^{32}P	phosphorus-32-radioisotope
PBS	phosphate buffered saline
PEG6000	polyethylene glycol 6000
pfu	plaque forming units
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
^{35}S	sulphur-35-radioisotope
SDS	sodium dodecyl sulphate
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/w	weight/weight (ratio)
HI	Heamagglutination Inhibition
NT	Neutralization

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
TSW	tomato spotted wilt
VSV	vesicular stomatitis virus
UUK	Uukuniemi

Amino Acid Symbols

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

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INTRODUCTION

BUNYAVIRIDAE

Taxonomy and classification

Bunyaviruses belong to the family Bunyaviridae, and form the largest and most diverse grouping of arthropod-borne viruses. They also form the largest family of RNA viruses and include about 350 distinguishable viral serotypes, subtypes and varieties (Bishop, 1990; Gonzalez-Scarano and Nathanson, 1990; Schmaljohn and Patterson, 1990; Elliott, 1990; Bouloy, 1991). The number of confirmed or putative members of the family Bunyaviridae continues to increase (Pringle, 1991). The assignment of viruses to the family was made primarily because of their structural features, including a trisegmented genome of negative polarity and a similar protein-coding pattern within each genome segment. Many viruses of the family are transmitted by arthropods and can replicate in both vertebrates and invertebrates (Bouloy, 1991). However, it has recently been demonstrated that members of the family do not exclusively infect and replicate in invertebrates and vertebrates but can infect and grow in plants, as exemplified by the tomato spotted wilt virus, TSW (Milne and Francki, 1984; de Haan *et al*, 1989, 1990).

Currently five genera within the family have been defined. They are:

Bunyavirus genus - Prototype: Bunyamwera (BUN) virus
Phlebovirus genus - Prototype: Sandfly fever Sicilian (SFS) virus
Nairovirus genus - Prototype: Crimean-Congo haemorrhagic fever (CCHF) virus
Hantavirus genus - Prototype: Hantaan (HTN) virus
Tospovirus genus - Prototype: Tomato spotted wilt (TSW) virus

The distinctive features of the viruses in the family Bunyaviridae as defined by Bishop and Shope (1979) and Pringle (1991) are the following:

1 Tripartite structure of the genome and the negative sense of the linear single-stranded virion RNA.

2 Subdivision of the family predominantly on the basis of coding strategy into five distinct genera which embrace subgeneric groupings of viruses with varying degrees of serological relationships.

3 Ambisense encoding of a putative non-structural protein gene in the S genome segment in viruses of the *Phlebovirus* and *Tospovirus* genera. This was also observed in the former *Uukuvirus* genus which now has been amalgamated with the *Phlebovirus* genus to become the *Phlebovirus* genus.

4 Enveloped pleiomorphic particles with four virus-specified proteins: the putative polymerase protein (L), cysteine rich precursor derived envelope glycoproteins (G1 and G2, plus G3 in the *Nairovirus* genus), and the nucleoprotein (N).

5 Three circular nucleocapsids, comprising in viruses of the *Bunyavirus* genus about 2100 molecules of N and 25 molecules of L per particle, all enveloped by a lipid envelope containing approximately 600 molecules of glycoproteins G1 and G2.

6 Absence of a matrix protein.

7 Maturation by budding from membranes of the Golgi apparatus.

8 Close association with specific vertebrate hosts and specific arthropod vectors, with the exception of the tospoviruses, which are transmitted by thrips, and the hantaviruses which do not seem to be arthropod-borne.

9 Temporal separation of internal and envelope protein synthesis.

10 Priming of mRNA synthesis by a cap transfer mechanism analogous to that of influenza virus, except that it occurs in the cytoplasm, and absence of 3'-polyadenylation of mRNA.

11 Insensitivity to actinomycin-D and alpha-amanitin, and variable dependence on the presence of an intact functional nucleus.

12 Limitation of gene exchange to reassortment of genome subunits between closely related viruses within serogroups.

Classification and serological relationships

Serological tests have been the main instruments used to study antigenic variance and relatedness of the more than 300 members of the Bunyaviridae family (Karabatsos, 1985). Classification of these viruses into genera and serogroups has most commonly been established by use of complement fixation, haemagglutination inhibition, indirect immunofluorescent antibody and various forms of neutralization tests in mice and cell cultures (Hunt and Calisher, 1979; Bishop, 1985a).

A member of a given serogroup is neutralized by homologous antisera and with varying degrees by heterologous antisera specific to other members of the serogroup. Usually members of one serogroup are not neutralized by antisera raised against members of another serogroup

(Bishop, 1985a). Viruses within a given serogroup that are easily distinguished by serological tests are referred to as types. Those that are only slightly different by standard serological assays are subtypes and those that show very slight but significant, repeatable cross-reactions are varieties or, if hardly distinguishable, strains (Calisher, 1983; Shope, 1985). Haemagglutination and neutralization assays detect epitopes on the viral glycoproteins whereas complement fixation antibodies are directed to the nucleocapsid proteins (Shope, 1985; Bishop, and Beaty, 1988). Complement fixation assays have been used to place viruses into serogroups while haemagglutination and neutralization tests have been used to divide members of the serogroups further into types and subtypes. This observation does not hold for members of the *Phlebovirus* genus because of the lesser conservation of the N protein than the glycoproteins G1 and G2 (Shope, 1985; Pifat *et al*, 1988) and probably *Tospovirus* genus (Law and Moyer, 1990).

The separation of viruses into dfferent genera by use of immunological techniques has also been supported by the more powerful genome sequence comparisons (Clerx-van-Haaster *et al*, 1982; Eshita and Bishop, 1984; Ihara *et al*, 1985; Collett *et al*, 1985; Lees *et al*, 1986; Schmaljohn *et al*, 1987; Ronnholm and Pettersson, 1987; Grady *et al*, 1987; Kakach *et al*, 1988; Pardigon *et al*, 1988; Simons, 1990). Such sequence comparisons, replication strategies and protein homologies of the N and NS of phleboviruses and uukuviruses showed genetic relatedness and led to the merger of the previously different genera (Simons *et al*, 1990). It is also hoped that use of monoclonal antibodies will greatly help in sorting out the many inconsistencies and errors based on conventional serology (Kingsford, 1991).

Classification of viruses in the Bunyaviridae family has been no small matter considering the number of viruses involved. Classification into genera seems reasonable for the most part but, when it comes to determining serogroups,

complexes, virus species, subtypes, variants, varieties and strains, it is very difficult in many instances to know whether the term used to describe a given isolate is indeed correct (Calisher *et al*, 1988c, Calisher, 1988). The current classification of members and candidate members of the family Bunyaviridae is listed below (Francki *et al*, 1991). Possible members of the family have been considered on the basis of morphologic (electron microscopy) studies (Beaty and Calisher, 1991).

Unassigned viruses: For these viruses, apart from data from electron microscopy, no studies have been reported on the structure and replication of the viruses and no serologic relationship has been demonstrated between these candidate members and any of the recognised members of the family (Bishop, 1990).

The list of members of the family Bunyaviridae, as reported by the International Committee on Taxonomy of Viruses, 1991, is as follows:

Bunyavirus genus

ANOPHELES A GROUP: Anopheles A, Las Maloyas, Lukuni, Tacaiuma, Trombetas, Virgin River, CoAr3624, CoAr1071, CoAr3627, ColAn57389, SpAr2317, H32580.

ANOPHELES B GROUP: Anopheles B, Boraceia.

BAKAU GROUP: Bakau, Ketapang, Nola, Tanjong Rabok, Telok Forest.

BUNYAMWERA GROUP: Anhembi, Batai, Birao, Bozo, Bunyamwera, Cache Valley, Fort Sherman, Germiston, Guaroa, Iaco, Ilesha, Kairi, Lokern, Macaua, Maguari, Main Drain, Mboke, Ngari, Northway, Playas, Santa Rosa, Shokwe, Sororoca, Taiassui, Tansaw, Tlacotalpan, Tucunduba, Wyeomyia, Xingu, AG83-1746, BeAr328208, CbaAr426.

BWAMBA GROUP: Bwamba, Pongola.

C GROUP: Apeu, Bruconha, Caraparu, Gumbo Limbo, Itaqui, Madrid, Marituba, Murutucu, Nepuyo, Oriboca, Ossa, Restan, Vinces, 63U11.

CALIFORNIA GROUP: California encephalitis, Inkoo, Jamestown Canyon, Keystone, La Crosse, Melao, San Angelo, Serra do Navio, snowshoe hare, South River, Tahyna, trivittatus, AG83-497.

CAPIM GROUP: Acara, Benevides, Benfica, Bushbush, Capim, Guajara, Juan Diaz, Moriche, GU71u344, GU71u350.

GAMBOA GROUP: Alajuela, Brus Laguna, Gamboa, Pueblo Viejo, San Juan, 75V-2621, 78V-2441, 75V-2374.

GUAMA GROUP: Ananindeua, Bertioga, Bimiti, Cananeia, Catu, Guama, Guaratuba, Itimirim, Mahogany Hammock, Mirim, Moju, Timboteua.

KOONGOL GROUP: Koongol, Wongal.

MINATITLAN GROUP: Minatitlan, Palestina.

NYANDO GROUP: Nyando, Eret-147.

OLIFANTSVLEI GROUP: Bobia, Botambi, Olifantsvei, Oubi, Dabakala.

PATOIS GROUP: Abras, Babahoyo, Estero Real, Pahayokee, Patois, Shark River, Zegla.

SIMBU GROUP: Aino, Akabane, Buttonwillow, Douglas, Facey's Paddock, Ingwavuma, Inini, Kaikalur, Manzanilla, Mermet, Oropouche, Para, Peaton, Sabo, Sango, Sathuperi, Shamonda, Shuni, Simbu, Thimiri, Tinaroo, Utinga, Utive, YABA-7.

TETE GROUP: Bahig, Batama, Matruh, Tete, Tsuruse.

TURLOCK GROUP: Lednice, Turlock, Umbre, Yaba-1.

UNGROUPED VIRUSES: Kaeng Khoi, Leanyer, Mojui dos Campos, Termeil.

Hantavirus genus

HANTAAN GROUP: Hantaan, Leaky, Seoul, Prospect Hill, Puumala, Thottapalayam.

Nairovirus genus

CRIMEAN-CONGO HAEMORRHAGIC FEVER GROUP: Crimean-Congo haemorrhagic fever, Hazara, Khasan.

DERA GHAZI KHAN GROUP: Abu Hammad, Abu Mina, Dera Ghazi Khan, Kao Shuan, Pathum Thani, Pretoria.

HUGHES GROUP: Farallon, Fraser Point, Great Saltee, Hughes, Puffin Island, Punta Salinas, Raza, Sapphire II, Soldado Zirqa.

NAIROBI SHEEP DISEASE GROUP: Dugbe, Nairobi sheep disease.

QALYUB GROUP: Bandia, Omo, Qalyub.

SAKHALIN GROUP: Avalon, Clo Mor, Kachemak Bay, Paramushir, Sakhalin, Taggert, Tillmook.

THIAFORA GROUP: Evre, Thiafora.

Phlebovirus genus

SANDFLY FEVER GROUP: Sandfly fever Naples complex, Karimabad, Sandfly fever Naples, Tehran, Toscana; Bajaru complex: Bujaru, Munguba; Candiru complex: Alenquer, Candiru, Itaituba, Nique, Oriximina, Turuna; Chillibre complex: Cacao, Chillibre; Frijoles complex: Frijoles, Joa; Punta Toro complex: Buenaventura, Punta Toro; Rift Valley fever complex: Belterra, Icoaraci, Rift Valley fever; Salehebad complex: Arbia, Salehabad; No complex assigned: Aguacate, Anhanga, Arboledas, Arumowot, Caimito, Chagres, Corfou, Gabek Forest, Gordil, Itaporanga, Odrenisrou, Pacui, Rio Grande, Saint-Floris, Sandfly fever Sicillian, Urucuri.

UUKUNIEMI GROUP: Grand Arbaud, Manawa, Murre, Oceanside, Ponteves, Precarious Point, St. Abbs Head, Uukuniemi, Zialiv, Terpeniya, EgAn1825-61, Fin V-707, RML 105355.

Other possible members of the family Bunyaviridae

BHANJA GROUP: Bhanja, Forecariah, Kismayo.

KAISODI GROUP: Kaisodi, Lanjan, Silverwater.

MAPPUTTA GROUP: Gan Gan, Mapputta, Maprik, Trubanaman.

OKALO GROUP:; Okola, Tanga.

RESISTENCIA GROUP: Antequera, barranqueras, Resistencia.

UPOLU GROUP: Aransas Bay, Upolu.

YOGUE GROUP: Yogue, Kasokero.

UNGROUPED VIRUSES: Bangui, Batken, Belem, Belmont, Bobaya, Caddo Canyon, Chim, Enseada, Issyk-Kul (Keterah), Kowanyama, Lone Star, Pacora, Razdan, Salanga, Santarem, Sunday Canyon, Tai, Tamdy, Tataguine, Wanowrie, Witwatersrand, Yacaaba.

VIRUS PARTICLE

Morphology

Viruses of the family Bunyaviridae show similar morphological features (Horzinek, 1975; Murphy *et al*, 1968, 1973; Pettersson and Von Bonsdorff, 1987). Classical negative staining showed particle pleiomorphism (Obijeski *et al*, 1976a). However, recent technology involving use of electron cryomicroscopy showed uniform spherical shapes for La Crosse virus ranging from 75 nm to 115 nm in diameter (Talmon *et al*, 1987). This method also preserved structural details of the virion in its native state and revealed 10 nm-long regular spikes and a 4 nm-thick bilayer structure.

Composition

The chemical composition for members of the Bunyaviridae has not been determined except for a member of the *Phlebovirus* genus, Uukuniemi virus (UUK). UUK particles were found to consist of 7% carbohydrate, 33% lipid, 58% protein and 2% RNA (Pettersson, 1975).

The virion particles of the family are composed of nucleocapsids containing three different RNA species, designated Large (L), Middle (M) and Small (S), in association with the nucleocapsid protein (N) and the virion polymerase (L). The nucloecapsids are packaged inside a lipid envelope during budding which occurs at the internal cellular membranes of the Golgi apparatus (Lyons and Heyduk, 1973; Murphy *et al*, 1973; Kuismanen *et al*, 1982; Smith and Pifat, 1982; Booth *et al*, 1991). In addition, all Bunyaviridae have two glycoproteins designated G1 and G2 except in some nairoviruses which may have three glycoproteins (Foulke *et al*, 1981; Watret and Elliott, 1985). For some nairoviruses a G1 protein has not been

detected and the G2 has only been detected for members of the Qalyub and CCHF serogroups (Clerx *et al*, 1981; Booth *et al*, 1991).

Sedimentation coefficients and buoyant densities

Sedimentation coefficients ($S_{20,w}$) for various members of the family have been reported: BUN (Kascsak and Lyons, 1977), CCHF (Donets and Chumakov, 1975), RVF (Polson and Levitt, 1963), UUK (Pettersson *et al*, 1971), TSW (Joubert *et al*, 1974). The reported sedimentation coefficient values range from 350S to 500S (Francki *et al*, 1991).

Buoyant densities range from 1.17 to 1.19g/cm^3 and such buoyant densities were shown for BUN (Kascsak and Lyons, 1977), California encephalitis virus (Goldman *et al*, 1977; White, 1975), CCHF (Chumakov and Donets, 1975), UUK (von Bonsdorff and Pettersson, 1975). The average buoyant density in caesium chloride is 1.2g/cm^3 (Obijeski and Murphy, 1977).

GENOME STRUCTURE AND ORGANISATION

Sizes of the genome RNA segments

Sizes of the genomic RNA segments were determined from the electrophoretic mobilities of the RNA in agarose or polyacrylamide gels or for some segments, by nucleotide sequence determinations of cloned cDNA (Elliott *et al*, 1991). The pattern of RNA segment size is distinctive for bunyaviruses, nairoviruses, and tospoviruses. There is less difference between the pattern of RNAs of hantaviruses and phleboviruses. The sizes of RNA segments for each genus of the Bunyaviridae are presented in Table 1.1.

The 3' and 5' RNA sequences

When observed under the electron microscope, the nucleocapsids of members of Bunyaviridae were seen to form circular structures (Pettersson and von Bonsdorff, 1975; Sarnso *et al*, 1975; Obijeski *et al*, 1976b). This observation is explained by the complementarity of the 3' and the 5' termini of the RNA segments which accounts for the formation of circular and/or panhandle forms of the RNAs seen by the electron microscope (Bouloy *et al*, 1973, 1974; Samso *et al*, 1976; Hewlett *et al*, 1977; Pardigon *et al*, 1982). The terminal 11 bases of the three BUN RNAs are conserved and complementary except for positions 9 and -9 and the following 20 or so bases show segment specific complementarity (Elliott, 1990). These are shown in Fig 1.1. The mismatch at positions 9 and -9 is found in all bunyavirus RNAs sequenced. However, this mismatch has not been observed in the complementary sequence termini of the phlebovirus M and S segments nor the hantavirus M and S segments, although a mismatch in positions 12 and -12 was repoted for Hantan virus M in the terminal 18 complementary nucleotides (Yoo and Kang, 1987a).

Table 1.1 Sizes of the genome RNA segments of the Bunyaviridae[a]

RNA segment	Bunyaviridae genus					Tomato spotted wilt virus
	Bunyavirus	*Hantavirus*	*Nairovirus*	*Phlebovirus*	*Uukuvirus*	
L[b]	8100-9400	6500-8500	11000-14400	6500-8200	6500-8500	7900-8200
M[b]	5400-6900	4200-5700	4400-5700	3300-6600	3000-3900	5000-5400
S[b]	850-1500	1800-2300	1760-2050	1200-2400	1200-1900	2900-3400
L[c]	BUN: 6875					
M[c]	BUN: 4458	HTN: 3616		PT: 4300	UUK: 3231	
	GER: 4534	NE: 3682		RVF: 3885		
	LAC: 4526	SR-11: 3651				
	SSH: 4527					
S[c]	BUN: 961	HTN: 1696	DUG: 1712	PT: 1904	UUK: 1720	TSW: 2916
	GER: 980	NE: 1785		SFS: 1746		
	MAG: 945	PH: 1675				
	LAC: 984	SR-11: 1769				
	SSH: 982					
	Aino: 850					

[a] Sizes given in nucleotides
[b] Estimates from electrophoresis of RNAs
[c] Nucleotide sequence data from cloned cDNAs

(From Elliott *et al*, 1991)

Figure 1.1 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 represent nucleotides which are conserved on a segment specific basis in all available sequenced bunyavirus RNA segments.

(From Elliott, 1990)

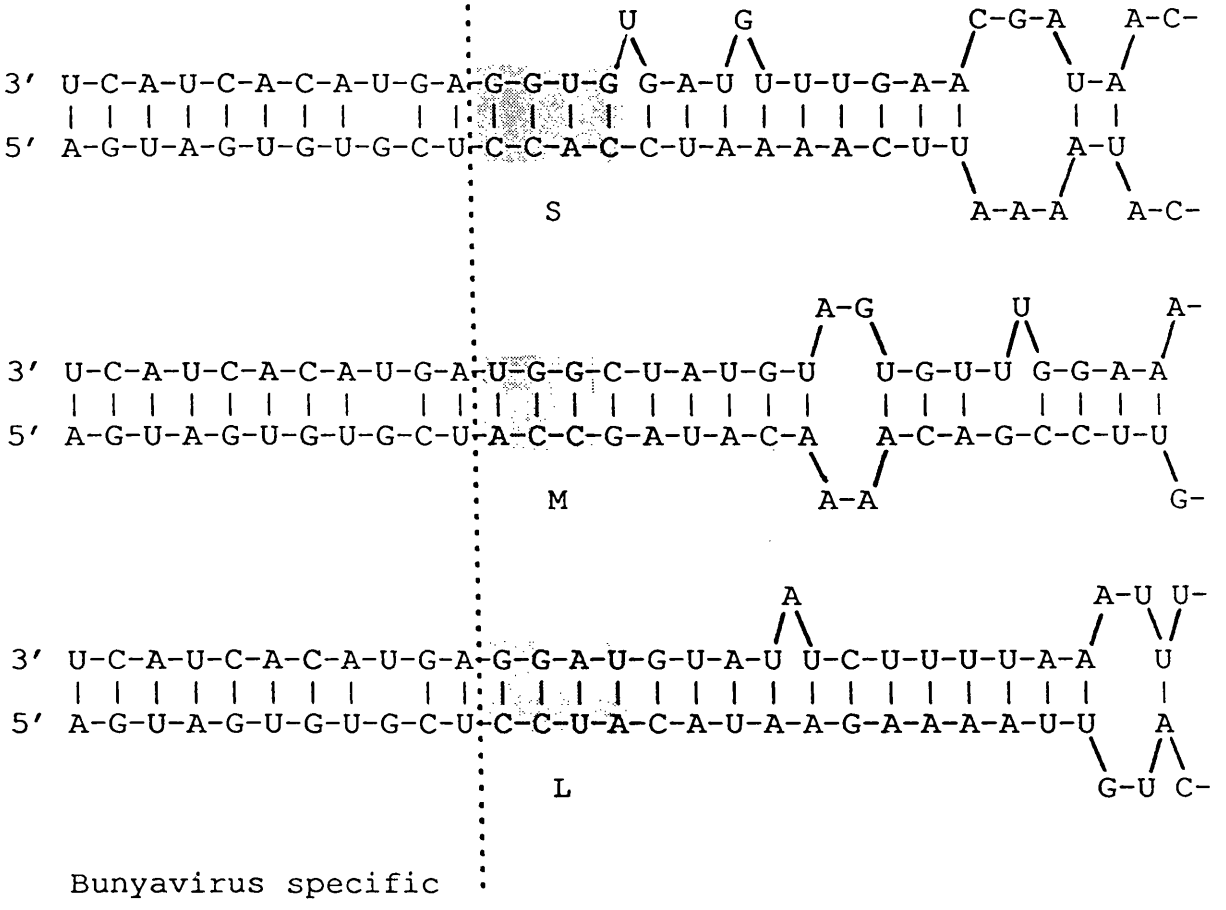

3′ U-C-A-U-C-A-C-A-U-G-A-G-G-U-G G-A-U U-U-U-G-A-A
5′ A-G-U-A-G-U-G-U-G-C-U-C-C-A-C-C-U-A-A-A-A-C-U-U
S
3′ U-C-A-U-C-A-C-A-U-G-A-U-G-G-C-U-A-U-G-U U-G-U-U G-G-A-A
5′ A-G-U-A-G-U-G-U-G-C-U-A-C-C-G-A-U-A-C-A A-C-A-G-C-C-U-U
M
3′ U-C-A-U-C-A-C-A-U-G-A-G-G-A-U-G-U-A-U U-C-U-U-U-U-A-A U
5′ A-G-U-A-G-U-G-U-G-C-U-C-C-U-A-C-A-U-A-A-G-A-A-A-A-U-U A
L
Bunyavirus specific

Figure 1.1

It has been suggested that the conserved terminal sequences of the RNAs are important for encapsidation by the N protein as the genomic (-) and full-length viral complementary (+) RNA are encapsidated, whereas viral mRNA, which has 5'-terminal extensions and is shorter at the 3' end, is not (Raju and Kolakofsky, 1986b). However, the same authors found a small amount of mRNA-like transcripts late in infection that were encapsidated and full-length (+) molecules with 5' extensions that were not encapsidated. They concluded that nucleocapsid recognition sequences were at the 5' end of the RNA. Raju and Kolakofsky (1989) also showed that full-length (-) and (+) strands are base-paired in the viral nucleocapsid and they emphasised the importance of unmatched and mismatched bases within the double-stranded regions of RNA for recognition by proteins (reviewed in Wickens and Dahlberg, 1987).

The consensus 3' and 5' terminal nucleotide sequences of the Bunyaviridae genome RNA segments are shown in Table 1.2.

Bunyavirus genus

The complete genome sequence of BUN, the prototype of the family Bunyaviridae, has been determined (Lees *et al*, 1986; Elliott, 1989a, b). This is the only member of the Bunyavirus genus whose complete sequence has been reported (Elliott *et al*, 1991). The genome comprises 12294 nucleotides.

The BUN L RNA segment is 6875 nucleotides long and has a base composition of 66.7% A+U residues; this is richer in A+U residues than the M (61.0%) or the S (58.2%) segments. Only limited sequence information is available for the L RNA segments of other bunyaviruses. Some sequences from the L RNA segment of snowshoe hare virus (SSH) and La Crosse

Table 1.2 Terminal nucleotide sequences of the L, M and S genome segments of representative members of the Bunyaviridae

Genus	Virus	Gene Segment	3' terminus[a]	5' terminus[b]
Bunyavirus	La Crosse	S	3' UCAUCACAUGAGGUG	5' AGUAGUGUGCUCCAC
		M	3' UCAUCACAUGAUGGU	5' AGUAGUGUGCUACCA
		L	3' UCAUCACAUGAGGAU	
Hantavirus	Hantaan	S	3' AUCAUCAUCUGAGGG	5' UAGUAGUAUGCUCCC
		M	3' AUCAUCAUCUGAGGC	5' UAGUAGUAGACACCG
		L	3' AUCAUCAUCUGAGGG	
Nairovirus	Qalyub	S	3' AGAGAUUCUGCCUGC	
		M	3' AGAGAUUCUUUAUGA	
		L	3' AGAGAUUCUUUAAUU	
Phlebovirus	Rift Valley Fever	S	3' UGUGUUUCGG	
		M	3' UGUGUUUCUGCCACGU	5' ACACAAAGACCGGUG
		L	3' UGUGUUUCUG	
Uukuvirus	Uukuniemi	S	3' UGUGUUUCUGGAGGUU	
		M	3' UGUGUUUCUGCCGAUU	5' ACACAAAGACGGCUA
		L	3' UGUGUUUCUGGAGUUG	

[a] 3' terminal sequences were obtained by direct sequencing of virion RNAs or by analysis of cDNA clones. Sequences identical on all three genome segments are underlined.
[b] 5' terminal sequences were determined by sequence analysis of cDNA.

(From Schmaljohn and Patterson, 1990)

virus (LAC) (Clerx-van Haaster *et al*, 1982a; Hacker *et al*, 1990) have been reported. Comparison of the sequences shows about 40% homology with the BUN L RNA.

Complete nucleotide sequences of four bunyavirus M segments are available from viruses of two serogroups; the Bunyamwera serogroup and the California serogroup (Table 1.3). The segments are similar in size (4458-4534 bases, base composition 61.0-63.2% A+U) and an economy in the use of RNA to encode protein (Elliott *et al*, 1991).

Nucleotide sequence homology is greater between the M segments of viruses of the same serogroup (e.g. SSH virus and LAC virus 79%; Grady *et al*, 1987) than between viruses in different serogroups (e.g. SSH virus and BUN virus, 55%, Lees *et al*, 1986).

Sequences of the S segment of six members of the *Bunyavirus* genus have been reported, representing the Bunyamwera, California and Simbu serogroups (Table 1.4). The sizes range from 850 to 984 nucleotides in length with a base composition of 55.9-59.9% A+U residues.

Hantavirus genus

The *Hantavirus* genus was included into the family Bunyaviridae as a result of the analysis of the virus RNA which showed similarities with other members of the family (Schmaljohn and Dalrymple, 1983). Nucleotide sequences have been reported for the M and/or the S genome segments of four serologically distinct hantaviruses: Hantaan (HTN) virus, Sapporo rat (SR-II) virus, nephropathia epidemica (NE) virus (Hallnas strain), and Prospect Hill (PH) virus. L segment sequences of HTN, NE and Seoul viruses have also been reported.

Table 1.3 Comparison of the M RNA segments of four bunyaviruses

Serogroup	Virus	RNA				Gene Products		
		Number of nucleotides				Number of amino acids	Number of potential glycosylation sites	
		Total	5'nc	3'nc	% A+U		G1	G2
Bunyamwera	BUN	4458	56	103	61.0	1433	2	2
	GER	4534	56	104	63.2	1437	2	2
California	LAC	4526	61	141	62.0	1441	2	2
	SSH	4527	61	142	61.0	1441	2	2

(From Elliott *et al*, 1991)

Table 1.4 Comparison of the S RNA segments of six bunyaviruses.

Serogroup	Virus	RNA				Gene products	
		Number of nucleotides				Number of amino acids	
		Total	5'nc	3'nc	% A+U	N	NSs
Bunyamwera	BUN	961	85	174	58.2	233	101
	GER	980	87	194	57.6	233	109
	MAG	945	73	170	59.9	233	101
California	LAC	984	81	196	58.8	235	92
	SSH	982	79	198	56.3	235	92
Simbu	Aino	850	34	117	55.9	233	91

(From Elliott *et al*, 1991)

In common with other members of the Bunyaviridae family, the L, M and S segments show consensus sequences at the 3' termini which are distinct from viruses of the other four genera (Schmaljohn and Dalrymple, 1983, 1984; Schmaljohn *et al*, 1985). Complementary 5' terminal sequences were identified by analyses of cDNA copies of viral genomes (Schmaljohn *et al*, 1986a, 1987b; Giebel *et al*, 1989; Stohwasser *et al*, 1990; Arikawa *et al*, 1990; Parrington and Kang, 1990), and this allows noncovalent circularization of the individual genome segments forming stable, base-paired, panhandle structures.

Nairovirus genus

The L RNA segment of members of the *Nairovirus* genus is much larger (almost twice the size) than L RNAs of other genera. Some estimates of the size of the M segment indicate that this is also larger in the *Nairovirus* genus than in other genera (Clerx and Bishop, 1981; Clerx *et al*, 1981; Watret and Elliott, 1985; Ward *et al*, 1990).

Ward *et al* (1990) reported the sequence of the S segment of Dugbe virus. The segment is 1712 nucleotides long and has a base composition of 30.6% A, 20.1%C, 23.4% G and 25.9% U. Sixteen of the 17 terminal nucleotides at the 3' and 5' ends are complementary. No homologies were found with other S RNA segments of the other genera in Bunyaviridae.

Phlebovirus genus

Molecular genetic analysis of phleboviruses and uukuviruses revealed ancestral relationship and led to the unification of the previously separate genera (Elliott *et al*, 1991; Francki *et al*, 1991). The nucleotide sequence for the L RNA segment has been determined for RVF (Muller *et al*, 1991) and UUK (Elliott *et al*, 1992). Sequences for the M segment are available for Punta Toro (PT), RVF and UUK

viruses (Ihara *et al*, 1985b; Collett *et al*, 1985; Rounholm and Pettersson, 1987). Sequence analyses of the S segment have also been reported for PT, SFS and UUK (Ihara *et al*, 1984; Marriott *et al*, 1989; Simons *et al*, 1990).

Tospovirus genus

The genome of TSW, the prototype of the genus, consists of three linear RNA molecules of 2916 nucleotides for S RNA, about 5000 nucleotides for M RNA and 8897 nucleotides for the L RNA. Analysis of the terminal sequences of TSW RNA showed some difference from those of all members of the Bunyaviridae. Strikingly the termini of the TSW RNAs showed considerable sequence homology to that of RNA segment 3 of Thogoto virus and segment 2 of Dhori virus, which are members of the Orthomyxoviridae (de Haan, 1991, PhD Thesis). This may reflect ancentral relationships between TSW and some members of the Orthomyxoviridae. The 3' end sequence is conserved and complementary to the 5' end (de Haan *et al*, 1989).

GENE EXPRESSION STRATEGIES

The coding properties of the genome segments for different genera of members of the Bunyaviridae family are quite similar, despite the differences in relative sizes of the corresponding subunits. Generally, the coding assignments of the different segments are:

1 The L RNA segment codes for the L protein.

2 The M RNA segment codes for two glycoproteins, G1 and G2, in the form of a precursor polypeptide which is then cleaved cotranslationally to give mature polypeptides.

3 The S RNA segment encodes the nucleocapsid protein N.

The L, M and S RNA segments of bunyaviruses and hantaviruses are of negative polarity, i.e. the viral genome segments have to be transcribed into positive sense mRNA for protein synthesis. However, the S RNA segments of phleboviruses and tospoviruses resemble the S RNA segment of arenaviruses, i.e. a linear single-stranded structure of covalently-linked negative- and positive-sense molecules from which separate mRNAs are made. This kind of arrangment is referred to as being ambisense.

The L segment

The bunyavirus L RNA codes for the L protein (Elliott, 1989b; Endres *et al*, 1989). There is a single AUG-initiated open reading frame in the BUN L segment viral complementary RNA flanked by 5' and 3' noncoding regions of 50 and 108 bases respectively. The open reading frame (ORF) contains 2239 codons. There is a short ORF of 63 codons which is present in the negative-sense strand, but no gene product has been identified for it (Elliott, 1989b).

Sequence analysis of the cDNA of the L RNA segment of HTN (Schmaljohn, 1990) showed a similar coding strategy to that of the L segment of BUN described above. The sequence analysis of the RVF phlebovirus shows a single long ORF coding for 2149 amino acids (243.6 Kd) in the viral-complementary sequence. The 5' and 3' non-coding regions in the viral-complementary RNA sequences are 18 and 141 nucleotides in length, respectively. There is the potential for a short gene product (3.5 Kd) coded in the 5' region of the viral RNA sequence, suggesting that the L RNA, like the RVF S RNA, may have an ambisense coding strategy. This remains to be confirmed by identification of a gene product (Muller *et al*, 1992). No sequence reports have been made for the L segment of nairoviruses but it is assumed that similar strategies are employed. The L RNA segment of TSW, has been determined (de Haan *et al*, 1991) and an ORF starting with an AUG at base 34 and extending to an UAA

stopcodon at position 8659. The non-coding regions of the complementary RNA are 33 bases at the 5' end and 235 bases at the 3' end. A representation of the coding strategy of the L segment is shown in Fig 1.2.

The M segment

Bunyavirus genus

Four M RNA segments of members of the bunyavirus genus have been sequenced for SSH, LAC, GER and BUN. All have a single ORF of 1433-1441 amino acids which represents a polypeptide precursor to the two virion glycoproteins, G1 (Mr 108,000-120,000) and G2 (Mr 29,000-41,000), and a nonstructural protein NSm (Mr 15,000-18,000; Gentsch and Bishop, 1979; Fuller and Bishop, 1982; Elliott, 1985). The putative precursor has not been detected in virus infected cells (Pennington *et al*, 1977; Lees *et al*, 1986). It has not been possible to translate mRNA derived from the M RNA segment *in vitro* (Abraham and Pattnaik, 1983; Elliott, 1985) although it has been possible to express phlebovirus glycoproteins using similar conditions. No reports have been made about the expression of bunyavirus M segment from cloned cDNAs in both prokaryotic and eukaryotic systems.

Phlebovirus genus

The most extensively studied M RNA segments of the phlebovirus genus are those of the RVF and UUK. The M segment of RVF has 3,885 nucleotides with a coding potential of 133kd in the main ORF of the cRNA (Battles and Dalrymple, 1988; Collett *et al*, 1985). The M segment mRNA of RVF has a 3' terminal truncation of about 100 nucleotides as compared to the genomic RNA. There is also an extension beyond the 5' genomic end by 12-14 nucleotides of heterogeneous sequence. Amino-terminal sequence analysis of purified G1 and G2 with respect to cRNA showed a coding potential of about 17kd between the first AUG translation initiation

Figure 1.2 Coding strategies of Bunyaviridae genomes.

Thin lines: the genomic RNAs; arrows: mRNAs, : 5' end; : 3' end; solid boxes: gene products (not to scale).

(From Elliott *et al*, 1991).

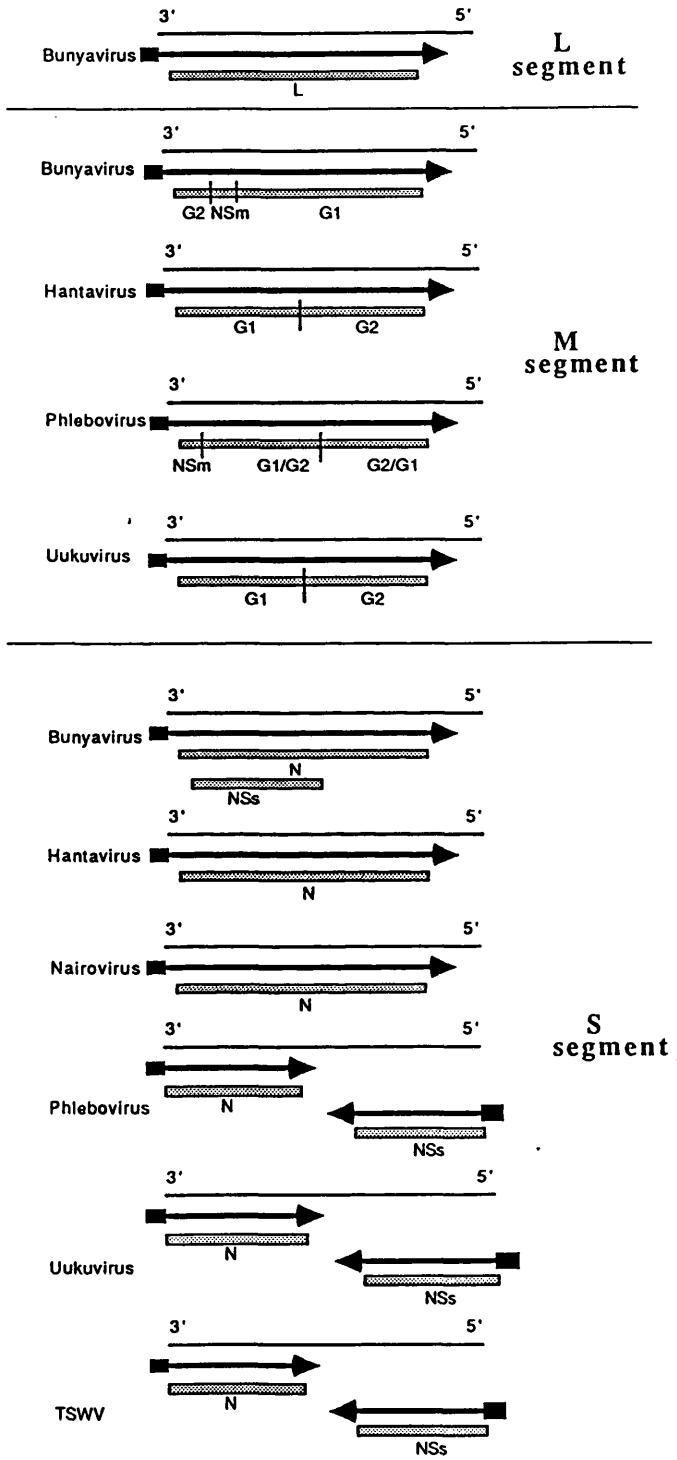

3'
5'
Bunyavirus
L
L segment
G2 NSm
G1
Hantavirus
G1
G2
M segment
Phlebovirus
NSm
G1/G2
G2/G1
Uukuvirus
N
NSs
Nairovirus
S segment
TSWV

Figure 1.2

codon and the amino terminus of G2 (Collett, 1985, 1986). This region, referred to as preglycoprotein or nonstructural M (NSm), has five in-frame AUG codons which come before G2. *In vitro* translation of an RNA transcript of cDNA containing the five initiation codons gave rise to a polypeptide of 133 Kd which, when translated in the presence of microsomal membranes, yielded four proteins. Apart from the two glycoproteins, G1 and G2, two other polypeptides of 78 Kd and 14 Kd were observed. Alteration of the initiation codons and expression in vaccinia virus systems indicated involvement of independent initiation at each of the first and second AUG codons yielding primary translation products which are co-translationally processed (Kakach *et al*, 1989). Initiation at the first AUG of the ORF yielded the 78 Kd protein representing a fusion between the preglycoprotein region and the G2 glycoprotein coding sequences, and the 78 Kd protein probably represents the unprocessed polyprotein of NSm and G2 (Kakach *et al*, 1988; Suzich and Collett, 1988). Translation from the second in-frame AUG, which is 37 amino acids downstream from the first, yielded the 14 Kd protein which consists only of the preglycoprotein sequences. There is a common glycosylation site at position 285 which is not utilized in the 14 Kd protein but is used in the 78 Kd protein (Kakach *et al*, 1989). G1 appears to be produced by internal initiation downstream of the preglycoprotein region (Suzich *et al*, 1990).

The terminal complementary 3' and 5' sequences of the M segments of UUK are identical for 10-15 nucleotides to those of RVF (Collett *et al*, 1985; Parker and Hewlett, 1981). Although the former *Uukuvirus* genus is now included in the *Phlebovirus* genus, sequence analysis of UUK M segment revealed a coding strategy different from that of other phleboviruses. There are common features like encoding G1 and G2 proteins and cotranslational cleavage of a polyprotein precursor (Ronnholm and Pettersson, 1987; Ulmanen *et al*, 1981). However, the uukuviruses do not have a preglycoprotein coding region and there is only one

initiation AUG codon which is 17 amino acids upstream of the amino terminus of G1. The G1 of UUK appears similar to the RVF 78kd protein.

Hantavirus genus

The M genome segment of HTN consists of 3,616 nucleotides and has a continuous ORF in the cRNA that encodes G1 and G2. The M segment does not appear to encode NSm. There is a 17 amino acid leader sequence between the first AUG codon and the amino terminus of G1. A second in-frame AUG codon which is located nine amino acids upstream of the amino terminus of G1 apparently is not used for initiation. This was demonstrated by removing the first initiation codon and expressing the cDNA in vaccinia or baculovirus systems (Schmaljohn *et al*, 1989). There is a region of 34 amino acids between G1 and G2 and 19 of these showed characteristics that constitute a signal sequence for G2 (Schmaljohn *et al*, 1987b).

Nairovirus genus

There is currently no information concerning the M segment coding strategy of nairoviruses. The M RNA of different nairoviruses range from 4,400 to 6,300 bases (Clerx and Bishop, 1981; Clerx *et al*, 1981; Watret and Elliott, 1985b). Analysis of nairovirus glycoproteins show molecular size estimates of Mr 72,000-84,000 for G1 and Mr 30,000-45,000 for G2 (Clerx and Bishop, 1981; Clerx *et al*, 1981; Cash, 1985; Watret and Elliott, 1985b). Three virion glycoproteins have been observed for Hazara virus with Mr of 84,000, 45,000 and 30,000 (Foulke *et al*, 1981). Reports have been made about non-structural glycoprotein precursors which were shown to be related to the structural glycoproteins by pulse-chase experiments (Clerx and Bishop, 1981; Cash, 1985; Watret and Elliott, 1985b). Details about the cleavage events or interrelationships between the identified proteins are not available.

Tospovirus genus

The expression strategies of the M RNA segments of representative viruses is shown in Fig 1.2.

The S segment

Bunyavirus genus

The S RNA segment for members of the *Bunyavirus* genus encode two proteins, the nucleocapsid N protein (Mr 19,000-26,000) and a nonstructural protein, NSs (Mr 10,000-12,000). These proteins are encoded in overlapping reading frames, i.e. proteins are synthesised from the same nucleotide sequences but in different reading frames (Gentsch and Bishop, 1978; Cash *et al*, 1979; Bishop *et al*, 1982; Fuller and Bishop, 1982; Akashi and Bishop, 1983; Cabradilla *et al*, 1983; Fuller *et al*, 1983; Akashi *et al*, 1984; Bouloy *et al*, 1984; Elliott, 1985; Gerbaud *et al*, 1987b; Elliott and McGregor, 1989). Only one S segment mRNA species has been described for bunyaviruses SSH, LAC and GER (Bouloy *et al*, 1984; Eshita *et al*, 1985; Patterson and Kolakofsky, 1984) and it appears therefore that the N and NSs proteins are made from the same mRNA species, the result of alternative initiation of translation (Bishop *et al*, 1983b; Elliott and McGregor, 1989). The S segments of GER and MAG bunyaviruses contain an additional ORF downstream of, and in the same frame as, NSs (Gerbaud *et al*, 1987b; Elliott and McGregor, 1989). The product of this third ORF has been translated *in vitro* from cloned cDNA but the protein has not been observed *in vivo* (Elliott and McGregor, 1989).

Phlebovirus genus

The coding strategy of the S segments of phleboviruses is different from those of bunyaviruses, nairoviruses and hantaviruses but appear to be similar to tospoviruses.

Cloning and sequence analysis and cell-free translation studies have revealed the existence of two ORFs for PT (Ihara *et al*, 1984), SFS (Marriott *et al*, 1989), Toscana, RVF (Giorgi *et al*, 1991) and UUK (Simons *et al*, 1990). One ORF is present in the viral complementary sense RNA and codes for the N protein; the other ORF is present in the virion sense RNA and codes for the NSs protein. This strategy of encoding two proteins from non-overlapping regions of virion-sense and anti-virion-sense RNA is referred to as ambisense and had been identified previously in the S segment of arenaviruses (Bishop, 1986). The N and NSs proteins are encoded by two subgenomic mRNAs of opposite polarity.

Hantavirus genus

Members of the *Hantavirus* genus apparently do not encode any NSs polypeptide. There is only one continuous ORF in the HTN S cRNA that encodes N. The N protein is translated from a virus complementary-sense messenger RNA which is indistinguishable in size from genomic length S RNA (Schmaljohn *et al*, 1986a, 1987a). The HTN N protein, which is 48-kD, is much larger than those of bunyaviruses and phleboviruses.

Nairovirus genus

The nucleotide sequence of the S RNA segment of Dugbe (DUG) virus has been determined (Ward *et al*, 1990). The S RNA of DUG virus is 1712 nucleotides in length and contains one large ORF of 1326 nucleotides. The ORF is flanked by 3' and 5' non-coding regions of 49 and 337 nucleotides, respectively. The identity of the protein encoded by the ORF was demonstrated when monoclonal antibodies to the DUG virus N protein precipitated a fusion protein of β-galactosidase and the DUG virus ORF. The S segment of DUG virus mRNA was found to be essentially full-length, and similar to that of hantaviruses

(Schmaljohn *et al*, 1986). No evidence was found for the overlapping reading frame coding strategy of bunyaviruses and the ambisense coding strategy of phleboviruses.

Tospovirus genus

De Haan *et al* (1990) reported the nucleotide sequence of the S RNA segment of TSW. The RNA is 2916 nucleotides long and analysis of the genome revealed two major reading frames. The ORF on the viral RNA strand starts with an AUG codon at position 88 and ends at a UAA stop codon at position 1481, which is capable of coding for a protein of 465 amino acids. The ORF on the cRNA strand starts with an AUG codon at position 2763 and ends at the UGA codon at position 1989 (numbered from the 5' end of the viral strand). It encodes a protein of 259 amino acids. *In vitro* translation of this second ORF from a cloned cDNA showed that it was the nucleocapsid protein, N, as it co-migrated with authentic N viral protein and reacted with antiserum raised against purified nucleocapsids. Two subgenomic mRNAs were detected by northern blots. It was deduced that TSW S RNA segment had an ambisense arrangement (de Haan *et al*, 1990).

The coding strategies of the S genome segments of the family Bunyaviridae are shown in Fig 1.2.

VIRAL PROTEINS AND FUNCTION

Most of the viruses of the Bunyaviridae family are composed of four structural proteins designated L, N, G1 and G2. The L and N proteins are associated with RNA, forming nucleocapsids. G1 and G2 are located at the surface and form spikes observable in the electron microscope. Apart from their structural proteins, bunyaviruses and some phleboviruses have two nonstructural proteins, designated NSm and NSs; uukuviruses of the *Phlebovirus* genus express one nonstructural protein, NSs. These nonstructural

proteins have not been reported for members of the other genera (Bouloy, 1991). Proteins encoded by the Bunyaviridae are shown in Table 1.5.

The L protein

The L protein is thought to be the virion-associated transcriptase. In the *Bunyavirus* genus transcriptase activity has been detected in detergent disrupted preparations of Lumbo (Bouloy and Hannoun, 1976), LAC (Patterson *et al*, 1984), GER (Gerbaud *et al*, 1987a). Schmaljohn and Dalrymple (1983) found RNA-dependent RNA polymerase activity associated with purified HTN virions. Jin and Elliott (1991) demonstrated the polymerase activity of the L protein by synthesizing BUN RNA using the L protein made by recombinant vaccinia virus. There have yet to be similar reports on the polymerase activity of the other genera.

The M segment proteins

The middle RNA segment codes for two glycoproteins, G1 and G2, and, depending on the genus, a nonstructural protein, NSm. Apart from their structural role the glycoproteins show biological functions including neutralising epitopes, an acid pH-dependent fusion activity, and are involved in attachment to cellular receptors. They are also responsible for the induction of immune responses resulting in the production of haemagglutinating and neutralizing antibodies. The glycoproteins have been shown to be the main determinants of virulence in mice (Shope *et al*, 1981; Gonzalez-Scarano *et al*, 1985a; Janssen *et al*, 1986; Endres *et al*, 1990) and for infection of mosquitoes (Beaty *et al*, 1981, 1982; Sundin *et al*, 1987).

Neutralising epitopes have been mapped to only G1 of the bunyaviruses (Gonzalez-Scarano *et al*, 1982; Kingsford *et al*, 1983; Kingsford and Boucquey, 1990; Grady *et al*,

Table 1.5 Structural and non-structural proteins[a]

	Structural			Non-structural		
Genus	L	G1	G2	N	NSs	NSm
Bunyavirus	180-200 (259)	100-125	29-40	19-27 (10.5-11)	7-12	11-16
Hantavirus	180-200 (246.5)	68-72	54-60	50-52 (48-49)	None	None
Nairovirus[b]	180-200	72-84	80-40	48-54 (49)	None	?
Phlebovirus	180-200	55-70	50-60	28 (27-28)	29 (29-36)	14 and 78[c] or 30[d] (17[c] - 30[d])
Uukuvirus	180-200	70-75	65-70	25 (28)	30 (32)	None
Tomato spotted wilt	*200*	*78*	*58*	*29 (29)*	*50 (52)*	?

[a] Size is expressed in kilodaltons, estimated from analyses in sodium dodecyl sulfate-polyacrylamide gels. When available, values in parentheses represent the size predicted from sequence data.

[b] In the nairovirus Hazara three glycoproteins have been described (Foulke *et al*, 1981) for Rift Valley fever virus[c] and for Punta Toro virus[d].

(From Bouloy, 1991)

1983) whereas they have been found in both G1 and G2 of RVF (Keegan and Collett, 1986; Battles and Dalyrymple, 1988), PT (Pifat and Smith, 1987; Pifat *et al*, 1988) and HTN (Yamanishi *et al*, 1984; Dantas *et al*, 1986; Arikawa *et al*, 1989). The ability of the glycoprotein to induce immunity and protection has been reported for the G2 of RVF (Collett *et al*, 1987; Dalrymple *et al*, 1989; Schmaljohn *et al*, 1989) and for both G1 and G2 of HTN (Pensiero *et al*, 1988; Schmaljohn *et al*, 1990).

Acid-dependent fusion activity has been demonstrated in LAC and HTN virions. This activity is thought to be important in the attachment of virions to cells in the early stages of infection. For LAC bunyavirus it has been suggested that the G2 glycoprotein is actually responsible for cell fusion (Pobjecky *et al*, 1989) although it is G1 that has been observed to undergo a conformational change at the pH of fusion (Gonzalez-Scarano, 1985; Gonzalez-Scarano *et al*, 1985b). Protease treatment of LAC caused a dramatic effect on the ability of the virus to attach to and infect vertebrate and invertebrate cells. Treatment of LAC with protease decreased the ability to attach to and infect vertebrate cells by 77%, while attachment to mosquito cells increased to as much as 306% (Ludwig *et al*, 1989). The same authors observed that treatment with protease was a prerequisite for detectable attachment of virus to mosquito midguts, suggesting that protease treatment of LAC may play an important role in attachment to, and subsequent infection of, mosquito midguts in nature. On the basis of their observation, Ludwig *et al* (1989) proposed a model for infection of mosquitoes by LAC. Earlier work had suggested that for vertebrates G1 is the glycoprotein responsible for attachment to cells, either by fusion or through interactions with specific virus receptor proteins (Kingsford and Hill, 1981; Janssen *et al*, 1984; Gonzalez-Scarano *et al*, 1984; Gonzalez-Scarano, 1985). Thus, Ludwig *et al* (1989) hypothesise that when LAC enters the mosquito midgut after feeding on vertebrate hosts, the virus would be

unable to bind directly to epithelial cells because G1 receptor proteins may not be present. The binding of virus to midgut occurs after mosquito secretory proteases cleave the G1 protein, exposing regions on G2 which allow virus to fuse to cells or attach to specific receptor proteins. This was based on the fact that proteases are present in mosquito midguts immediately following a blood meal.

Glycosylation

The envelope glycoproteins of all members of the family Bunyaviridae analysed to date possess N-linked oligosaccharide side chains (Cash *et al*, 1980; Pesonen *et al*, 1982; Schmaljohn *et al*, 1986). Glycosylation is initiated by *en bloc* transfer of preformed oligosaccharide chains from an oligosaccharide pyrophosphryldolichol intermediate to an asparagine residue in the amino acid sequence, Asn-X-Ser/Thr, in the newly synthesized polypeptide chain (Hubbard and Ivatt, 1981; Klenk and Rott, 1980). Sugar residues are then modified by enzymes in the endoplasmic reticulum (ER) and the Golgi complex as the glycoproteins translocate through these organelles (Kornfeld and Kornfeld, 1985).

Modification of oligosaccharide in the ER and Golgi complex

High mannose type oligosaccharide side chains, which are the primary sugar residues attached in the ER, are specifically cleaved to remove glucose and some mannose by endoglycosidase H. After removal of their residues, the side chains are converted to complex sugar chains by attachment of the peripheral sugars in the Golgi complex and the resulting mature forms of sugar become resistant to endoglycosidase H. This phenomenon can be used to study the transport of membrane glycoproteins between the ER and the Golgi complex. Kuismanen (1984) showed that the G1 and G2 glycoproteins of UUK acquired endoglycosidase H resistance with a half-life of 45 min and 90-150 min, respectively.

Similar times were observed for PT (Chen *et al*, 1991; Matsuoka *et al*, 1988) and the G1 glycoprotein of LAC (Madoff and Lenard, 1982). However, the G glycoprotein of vesicular stomatitis virus (VSV) and the influenza virus haemagglutinin (HA) acquired endo H resistance much more rapidly, in about 15-20 min after synthesis (Copeland *et al*, 1988; Doms *et al*, 1987; Strous and Lodish, 1980). From this, it appears that the bunyavirus glycoproteins translocate rather slowly.

AMINO ACID SEQUENCES AND GENE ORDER OF THE M SEGMENT PROTEINS

Bunyavirus genus

The amino acid sequences of four bunyaviruses M segments (BUN, GER, LAC and SSH) have been determined and analysis of the amino acid sequences derived from the coding nucleotide sequences of the four segments reveal a notable conservation of cysteine residues (Elliott, 1990) and are shown in Fig 1.3.

The gene order of the bunyavirus M segment was determined by Fazakerley *et al* (1988) using direct amino acid sequence analysis and specific peptide antibodies as 5' G2-NSm-G1 3' in the viral complementary RNA. The following points can be observed when data on SSH are used and extrapolated to BUN, GER and LAC. There is a putative signal sequence of 13 to 21 residues at the amino terminus of the precursor. The G2 proteins show an overall amino acid homology of about 66%. The positions of N-linked glycosylation sites As-Xxx-Ser/Thr are strictly conserved. Cleavage at the carboxy terminus of G2 occurs after a conserved arginine residue. NSm shows about 50% conservation of residues. Prediction of the amino terminus of G1 by alignment with SSH is not clear because of low sequence homology in this area but appears to occur after a conserved alanine residue. The G1 proteins exhibit the

Figure 1.3 Alignment of the glycoprotein precursors of SSH, LAC, BUN and GER viruses.

The amino and carboxy termini of G2 and amino terminus of G1, as determined for SSH (Fazakerley *et al*, 1988) are indicated. The exact boundaries of NSm are not known. N-linked glycosylation sites conserved in all four proteins are indicated by ● and in two proteins (serogroup conserved) ⊖ or ⊖ . There is a potential glycosylation site at position 89 in GER which is not conserved.

(From Elliott, 1990).

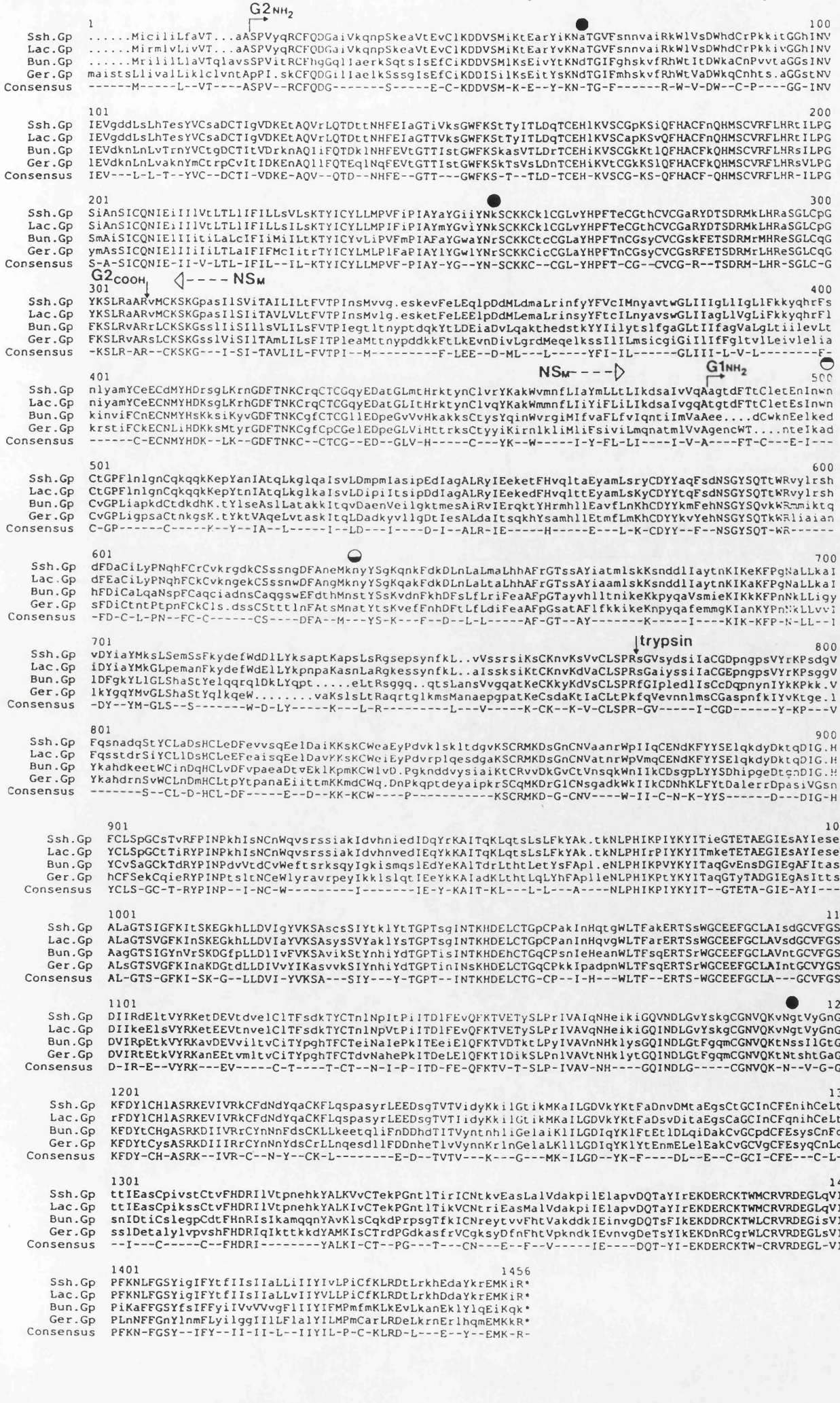

G2NH2
1
100
Ssh.Gp .....MiciliLfaVT...aASPVyqRCFQDGaiVkqnpSkeaVtEvClKDDVSMiKtEarYiKNaTGVFsnnvaiRkWlVsDWhdCrPkkitGGhINV
Lac.Gp .....MirmlvLivVT...aASPVyqRCFQDGaiVkqnpSkeaVtEvClKDDVSMiKtEarYvKNaTGVFsnnvaiRkWlVsDWhdCrPkkivGGhINV
Bun.Gp .....MrililLlaVTqlavsSPVitRCFhgGqlIaerkSqtsIsEfCiKDDVSMlKsEivYtKNdTGIFghskvfRhWtItDWkaCnPvvtaGGsINV
Ger.Gp maistsLlivalLiklclvntApPI.skCFQDGilIaelkSssgIsEfCiKDDISilKsEitYsKNdTGIFmhskvfRhWtVaDWkqCnhts.aGGstNV
Consensus ------M-----L--VT----ASPV--RCFQDG-------S-----E-C-KDDVSM-K-E--Y-KN-TG-F------R-W-V-DW--C-P----GG-INV
101
200
Ssh.Gp IEVgddLsLhTesYVCsaDCTIgVDKEtAQVrLQTDttNHFEIaGTiVksGWFKStTyITLDqTCEHlKVSCGpKSiQFHACFnQHMSCVRFLHRtILPG
Lac.Gp IEVgddLsLhTesYVCsaDCTIgVDKEtAQVrLQTDttNHFEIaGTTVksGWFKStTyITLDqTCEHlKVSCapKSvQFHACFnQHMSCVRFLHRtILPG
Bun.Gp IEVdknLnLvTrnYVCtgDCTItVDrknAQliFQTDklNHFEVtGTTIstGWFKSkasVTLDrTCEHiKVSCGkKtlQFHACFkQHMSCVRFLHRsILPG
Ger.Gp lEVdknLnLvaknYmCtrpCvItIDKEnAQllFQTEqlNqFEVtGTTIstGWFKSkTsVsLDnTCEHiKVtCGkKSlQFHACFkQHMSCVRFLHRsVLPG
Consensus IEV---L-L-T--YVC--DCTI-VDKE-AQV--QTD--NHFE--GTT---GWFKS-T--TLD-TCEH-KVSCG-KS-QFHACF-QHMSCVRFLHR-ILPG
201
300
Ssh.Gp SiAnSICQNIEiIIlVtLTLlIFILLsVLsKTYICYLLMPVFiPIAYaYGiiYNkSCKKCklCGLvYHPFTeCGthCVCGaRYDTSDRMkLHRaSGLCpG
Lac.Gp SiAnSICQNIEiIIlVtLTLlIFILLsILsKTYICYLLMPIFiPIAYmYGviYNkSCKKCklCGLvYHPFTeCGthCVCGaRYDTSDRMkLHRaSGLCpG
Bun.Gp SmAiSICQNIElIIitiLaLcIFIiMiILtKTYICYvLiPVFmPIAFaYGwaYNrSCKKCtcCGLaYHPFTnCGsyCVCGskFETSDRMrMHReSGLCqG
Ger.Gp ymAsSICQNIElIIiIiLTLaIFIFMcIitrTYICYLMLPlFaPIAYlYGwlYNrSCKKCicCGLaYHPFTnCGsyCVCGsRFETSDRMrLHReSGLCqG
Consensus S-A-SICQNIE-II-V-LTL-IFIL--IL-KTYICYLLMPVF-PIAY-YG--YN-SCKKC--CGL-YHPFT-CG--CVCG-R--TSDRM-LHR-SGLC-G
G2COOH
NSM
301
400
Ssh.Gp YKSLRaARvMCKSKGpasIlSViTAILILtFVTPInsMvvg.eskevFeLEqlpDdMLdmaLrinfyYFVcIMnyavtwGLIIIgLlIgLlFkkyqhrFs
Lac.Gp YKSLRaARvMCKSKGpasIlSIiTAVLVLtFVTPInsMvlg.esketFeLEElpDdMLemaLrinsyYFtcILnyavswGLIIagLlVgLiFkkyqhrFl
Bun.Gp FKSLRvARrLCKSKGsslIiSIllsVLILsFVTPIegtltnyptdqkYtLDEiaDvLqakthedstkYYIilytslfgaGLtIIfagVaLgLtiilevLt
Ger.Gp FKSLRvARsLCKSKGsslViSIlTAmLILsFITPleaMttnypddkkFtLkEvnDivLgrdMeqelkssIlILmsicgiGiIlIfFgltvlLeivlelia
Consensus -KSLR-AR--CKSKG---I-SI-TAVLIL-FVTPI--M---------F-LEE--D-ML---L-----YFI-IL------GLIII-L-V-L--------F-
NSM
G1NH2
401
500
Ssh.Gp nlyamYCeECdMYHDrsgLKrnGDFTNKCrqCTCGqyEDatGLmtHrktynClvrYKakWvmnfLIaYmLLtLIkdsaIvVqAagtdFTtCletEnInwn
Lac.Gp niyamYCeECNMYHDKsgLKrhGDFTNKCrqCTCGqyEDatGLItHrktynClvqYKakWmmnfLIiYiFLiLIkdsaIvgqAtgtdFTtCletEsInwn
Bun.Gp kinviFCnECNMYHsKksiKyvGDFTNKCgfCTCGllEDpeGvVvHkakksCtysYqinWvrgiMIfvaFLfvIqntiImVaAee....dCwknEelked
Ger.Gp krstiFCkECNLiHDKksMtyrGDFTNKCgfCpCGelEDpeGLViHttrksCtyyiKirnlkliMliFsiviLmqnatmlVvAgencWT....nteIkad
Consensus ------C-ECNMYHDK--LK--GDFTNKC--CTCG--ED--GLV-H-----C---YK--W-----I-Y-FL-LI----I-V-A----FT-C---E-I---
501
600
Ssh.Gp CtGPFlnlgnCqkqqkKepYanIAtqLkglqaIsvLDmpmIasipEdIagALRyIEeketFHvqltaEyamLsryCDYYaqFsdNSGYSQTtWRvylrsh
Lac.Gp CtGPFlnlgnCqkqqkKepYtnIAtqLkglkaIsvLDipiItsipDdIagALRyIEekedFHvqlttEyamLsKyCDYYtqFsdNSGYSQTtWRvylrsh
Bun.Gp CvGPLiapkdCtdkdhK.tYlseAslLatakkItqvDaenVeilgktmesAiRvIErqktYHrmhllEavfLnKhCDYYkmFehNSGYSQvkWRmmiktq
Ger.Gp krstiFCkECNLiHDKksMtyrGDFTNKCgfCpCGelEDpeGLViHttrksCtyyiKirnlkliMliFsiviLmqnatmlVvAgencWT....nteIkad
Consensus CvGPLigpsaCtnkgsK.tYktVAqeLvtaskItqLDadkyvllgDtIesALdaItsqkhYsamhllEtmfLmKhCDYYkvYehNSGYSQTkWRliaian
C-GP------C-----K--Y--IA--L-----I--LD---I----D-I--ALR-IE-----H-----E---L-K-CDYY--F--NSGYSQT-WR------
601
700
Ssh.Gp dFDaCiLyPNqhFCrCvkrgdkCSssngDFAneMknyYSgKqnkFdkDLnLaLmaLhhAFrGTssAYiatmlskKsnddlIaytnKIKeKFPgNaLLkaI
Lac.Gp dFEaCiLyPNqhFCkCvkngekCSssnwDFAngMknyYSgKqakFdkDLnLaLtaLhhAFrGTssAYiaamlskKsnddlIaytnKIKaKFPgNaLLkaI
Bun.Gp hFDiCaLqaNspFCaqciadnsCaqgswEFdthMnstYSsKvdnFkhDFsLfLriFeaAFpGTayvhlltnikeKkpyqaVsmieKIKkKFPnNkLLigy
Ger.Gp sFDiCtntPtpnFCkCls.dssCStttlnFAtsMnatYtsKvefFnhDFtLfLdiFeaAFpGsatAFlfkkikeKnpyqafemmgKIanKYPnNkLLvvI
Consensus -FD-C-L-PN--FC-C------CS----DFA--M---YS-K---F--D--L-L-----AF-GT--AY-------K-----I----KIK-KFP-N-LL--I
701
trypsin
800
Ssh.Gp vDYiaYMksLSemSsFkydefWdDlLYksaptKapsLsRgsepsynfkL..vVssrsiKsCKnvKsVvCLSPRsGVsydsiIaCGDpngpsVYrKPsdgV
Lac.Gp iDYiaYMkGLpemanFkydefWdElLYkpnpaKasnLaRgkessynfkL..aIssksiKtCKnvKdVaCLSPRsGaiyssiIaCGEpngpsVYrKPsggV
Bun.Gp lDFgkYLlGLShaStYelqqrqlDkLYqpt.....eLtRsggq..qtsLansVvgqatKeCKkyKdVsCLSPRfGIpledlIsCcDqpnynIYkKPkk.V
Ger.Gp lkYgqYMvGLShaStYqlkqeW.........vaKslsLtRaqrtglkmsManaepgpatKeCsdaKtIaCLtPkfqVevnnlmsCGaspnfkIYvKtge.l
Consensus -DY--YM-GLS--S-------W-D-LY-----K---L-R---------L---V-----K-CK--K-V-CLSPR-GV-----I-CGD------Y-KP---V
801
900
Ssh.Gp FqsnadqStYCLaDsHCLeDFevvsqEelDaiKKsKCWeaEyPdvklskltdgvKSCRMKDsGnCNVaanrWpIIqCENdKFYYSElqkdyDktqDIG.H
Lac.Gp FqsstdrSiYCLlDsHCLeEFeaisqEelDavKKsKCWeiEyPdvrplqesdgaKSCRMKDsGnCNVatnrWpVmqCENdKFYYSElqkdyDktqDIG.H
Bun.Gp YkahdkeetWCinDqHCLvDFvpaeaDtvEklKpmKCWlvD.Pgknddvysiai KtCRvvDkGvCtVnsqkWnIIkCDsgpLYYSDhipgeDtgnDIG.H
Ger.Gp YkahdrnSvWCLnDmHCLtpYtpanaEiittmKKmdCWq.DnPkqptdeyaipkrSCqMKDrGlCNsgadkWkIIkCDNhKLFYtDalerrDpasiVGsn
Consensus -------S--CL-D-HCL-DF-----E--D--KK-KCW----P----------KSCRMKD-G-CNV----W-II-C-N-K-YYS------D---DIG-H
901
1000
Ssh.Gp FCLSpGCsTvRFPINPkhIsNCnWqvsrssiakIdvhniedIDqYrKAITqKLqtsLsLFkYAk.tkNLPHIKPIYKYITieGTETAEGIEsAYIeseIP
Lac.Gp YCLSpGCtTiRYPINPkhIsNCnWqvsrssiakIdvhnvedIEqYkKAITqKLqtsLsLFkYAk.tkNLPHIrPIYKYITmkeTETAEGIEsAYIeseVP
Bun.Gp YCvSaGCkTdRYPINPdvVtdCvWeftsrksqyIgkismqslEdYeKAlTdrLthtLetYsFApl.eNLPHIKPVYKYITaqGvEnsDGIEgAFItasIP
Ger.Gp hCFSekCqieRYPINPtsltNCeWlyravrpeyIkklslqtIEeYkKAIadKLthtLqLYhFAplleNLPHIKPtYKYITaqGTyTADGIEgAsIttsIP
Consensus YCLS-GC-T-RYPINP--I-NC-W---------I-------IE-Y-KAIT-KL---L-L---A----NLPHIKPIYKYIT--GTETA-GIE-AYI---IP
1001
1100
Ssh.Gp ALaGTSIGFKItSKEGkhLLDVIgYVKSAscsSIYtklYtTGPTsgINTKHDELCTGpCPakInHqtgWLTFakERTSsWGCEEFGCLAIsdGCVFGSC(
Lac.Gp ALaGTSVGFKInSKEGkhLLDVIaYVKSAsysSVYaklYsTGPTsgINTKHDELCTGpCPanInHqvgWLTFarERTSsWGCEEFGCLAVsdGCVFGSC(
Bun.Gp AagGTSIGYnVrSKDGfpLLDlIvFVKSAvikStYnhiYdTGPTisINTKHDEhCTGqCPsnIeHeanWLTFsqERTSrWGCEEFGCLAVntGCVFGSCC
Ger.Gp ALsGTSVGFKInaKDGtdLLDIVvYIKasvvkSIYnhiYdTGPTinINsKHDELCTGqCPkkIpadpnWLTFsqERTSrWGCEEFGCLAIntGCVYGSCQ
Consensus AL-GTS-GFKI-SK-G--LLDVI-YVKSA---SIY---Y-TGPT--INTKHDELCTG-CP--I-H---WLTF--ERTS-WGCEEFGCLA---GCVFGSCQ
1101
1200
Ssh.Gp DIIRdEltVYRKetDEVtdvelClTFsdkTYCTnlNpItPiITDlFEvQFKTVETySLPrIVAIqNHeikiGQVNDLGvYskgCGNVQKvNgtVyGnGvP
Lac.Gp DIIkeElsVYRKetEEVtnvelClTFsdkTYCTnlNpVtPiITDlFEvQFKTVETySLPrIVAVqNHeikiGQINDLGvYskgCGNVQKvNgtVyGnGvP
Bun.Gp DVIRpEtkVYRKavDEVviltvCiTYpghTFCTeiNalePkITEeiElQFKTVDTktLPyIVAVnNHklysGQINDLGtFgqmCGNVQKtNssIlGtGtP
Ger.Gp DVIRtEtkVYRKanEEtvmltvCiTYpghTFCTdvNahePkITDeLElQFKTIDikSLPnlVAVtNHklytGQINDLGtFgqmCGNVQKtNtshtGaGtP
Consensus D-IR-E--VYRK---EV-----C-T----T-CT--N-I-P-ITD-FE-QFKTV-T-SLP-IVAV-NH----GQINDLG-----CGNVQK-N--V-G-G-P
1201
1300
Ssh.Gp KFDYlCHlASRKEVIVRkCFdNdYqaCKFLqspasyrLEEDsgTVTVidyKkilGtikMKaILGDVkYKtFaDnvDMtaEgsCtGCInCFEnihCeLtlh
Lac.Gp rFDYlCHlASRKEVIVRkCFdNdYqaCKFLqspasyrLEEDsgTVTIidyKkilGtikMKaILGDVkYKtFaDsvDitaEgsCaGCInCFqnihCeLtlh
Bun.Gp KFDYtCHgASRKDIIVRrCYnNnFdsCKLLkeetqliFnDDhdTITVyntnhliGelaiKlILGDIqYKlFtEtlDLqiDakCvGCpdCFEsysCnFqiv
Ger.Gp KFDYtCysASRKDIIIRrCYnNnYdsCrLLnqesdllFDDnheTlvVynnKrlnGelaLKlILGDIqYKlYtEnmELelEakCvGCVgCFEsyqCnLqit
Consensus KFDY-CH-ASRK--IVR-C--N-Y--CK-L--------E-D--TVTV---K---G---MK-ILGD--YK-F----DL--E--C-GCI-CFE---C-L---
1301
1400
Ssh.Gp ttIEasCpivstCtvFHDRIlVtpnehkYALKVvCTekPGntlTirICNtkvEasLalVdakpilElapvDQTaYIrEKDERCKTWMCRVRDEGLqVILE
Lac.Gp ttIEasCpikssCtvFHDRIlVtpnehkYALKIvCTekPGntlTikVCNtriEasMalVdakpiIElapvDQTaYIrEKDERCKTWMCRVRDEGLqVILE
Bun.Gp snIDtiCslegpCdtFHnRIsIkamqqnYAvKlsCqkdPrpsgTfkICNreytvvFhtVakddkIEinvgDQTsFIkEKDDRCKTWLCRVRDEGisVIFE
Ger.Gp sslDetalylvpvshFHDRIqIkttkkdYAMKIsCTrdPGdkasfrVCgksyDfnFhtVpkndkIEvnvgDeTsYIkEKDnRCgrWLCRVRDEGLsVIFE
Consensus --I---C-----C--FHDRI--------YALKI-CT--PG---T---CN---E--F--V-----IE----DQT-YI-EKDERCKTW-CRVRDEGL-VI-E
1401
1456
Ssh.Gp PFKNLFGSYigIFYtfIIsIIaLLiIIYIvLPiCfKLRDtLrkhEdaYkrEMKiR*
Lac.Gp PFKNLFGSYigIFYtfIIsIIaLLvIIYVLLPiCfKLRDtLrkhDdaYkrEMKiR*
Bun.Gp PiKaFFGSYfsIFFyiIVvVVvgFlIIYIFMPmfmKLkEvLkanEklYlqEiKqk*
Ger.Gp PLnNFFGnYlnmFLyilggIIlLFlalYILMPmCarLRDeLkrnErlhqmEMKkR*
Consensus PFKN-FGSY--IFY--II-II-L--IIYIL-P-C-KLRD-L---E--Y--EMK-R-

Figure 1.3

lowest homology (about 40%) and similarity is more enhanced in the carboxy half than the amino half of the molecule. There is one strictly-conserved N-glycosylation site whereas a second site is conserved between viruses of the same serogroup (Elliott, 1990). The G1 protein is susceptible to trypsin digestion (Kingsford and Hill, 1983; Gonzalez-Scarano, 1985). Fazakerley *et al* (1988) determined the major trypsin site by limited trypsin digestion of the G1 of SSH coupled with amino-terminus sequencing of the fragment from the trypsin digestion. The trypsin sensitive site is also conserved.

Hydropathy profiles of BUN, GEM, SSH and LAC M proteins

The hydropathy profiles of the M segment proteins of the four viruses are very similar (Elliott, 1990) and are shown in Fig 1.4.

The G2 protein has a rather long hydrophobic sequence (residues 187 to 245) followed by a charged stop-transfer sequence. The orientation of G2 in relation to the membrane has not been determined (Elliott, 1990). The G1 protein has more characteristics of a typical membrane glycoprotein than G2. There is a carboxy-terminal transmembrane and anchor sequence and this evidence together with the data from trypsin digestion orientate G1 with its amino terminus external to the membrane (Fazakerley *et al*, 1988). Neither the function nor the intracellular location of NSm are known; examination of the amino acid sequence and hydropathy profile suggest that it is a membrane bound protein. Information from the amino acid homologies, gene order and hydropathy profiles demonstrate the evolutionary relatedness of the glycoprotein species of viruses from the two serogroups of the bunyavirus, i.e. the Bunyamwera serogroup and the California serogroup and antigenic differences that differentiate individual viruses may be located in external (least

Figure 1.4 Hydropathy profiles of (a) SSH, (b) LAC, (c) BUN and (d) GER virus glycoprotein precursors.

The boundaries of G2, NSm and G1 are indicated. H designates the hydrophobic domain in G2, suggested to be involved in fusion.

(From Elliott, 1990).

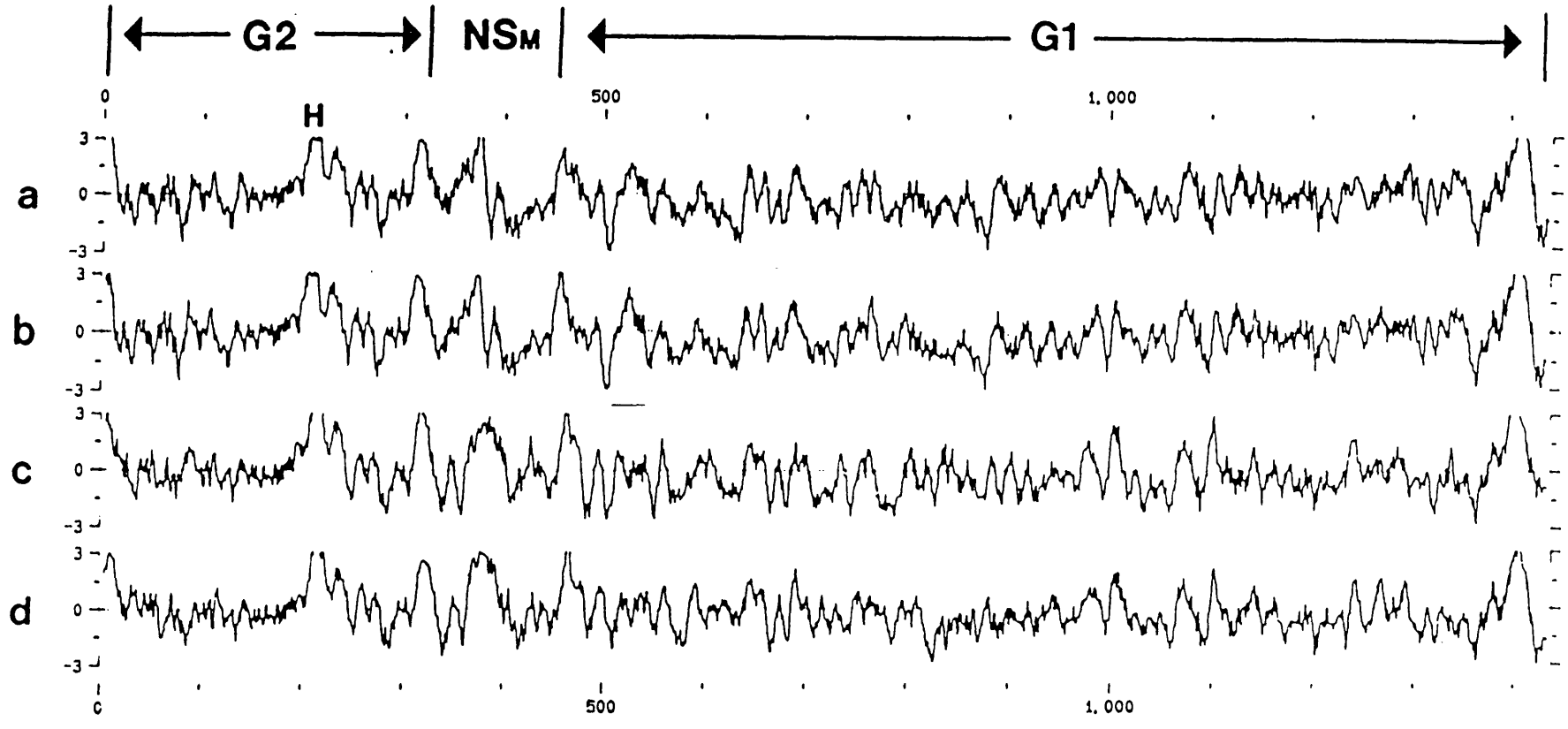


Figure 1.4

homologous) amino half of G1. The proposed topology of the bunyavirus M segment proteins in relation to membranes is illustrated in Fig 1.5.

The Hantavirus genus

The G1 and G2 proteins of hantaviruses are similar to those of other members of the Bunyaviridae family in being both glycosylated and rich in cysteine residues. Amino acid sequences have been determined for three members of the genus (HTN, NE and SR-11) for both G1 and G2. Hydropathy plots of the predicted amino acids of HTN, NE and SR-11 are almost superimposable, even if the overall amino acid sequence homologies of only 43% for G1 and 55% for G2 exist among the three viruses. When differences due to conservative substitutions are taken into account the amino acid sequence homologies show 58% and 71% for G1 and G2, respectively (Arikawa *et al*, 1990). Deduced amino acid sequences encoded in the SR-11 virus M segment ORF are more closely related to those of HTN virus (78% identical) than those of NE virus (53% identical). These amino acid sequence homologies reflect the closer serological relationship of HTN and SR-11 viruses compared to HTN and NE viruses (Schmaljohn *et al*, 1985). Two isolates from patients with Korean haemorrhagic fever showed homologies in excess of 98% with HTN, indicating the indistinguishable serological characteristics of those isolates (Schmaljohn *et al*, 1988).

The amino-terminal residues of G1 for both HTN and SR-11 is leucine. The amino-terminal residues for G2 were found to be serine and alanine for HTN and SR-11, respectively.

There are five asparagine-linked glycosylation sites in the G1 proteins of SR-11 and HTN viruses. The first four sites are conserved. Three of the four glycosylation sites are also maintained in the NE G1 protein.

Figure 1.5 Gene organisation of M genome segments of bunyaviruses and proposed topology of their protein products.

The ORF for the precursor protein is shown with hydrophobic regions (■) and known amino termini (↱) and carboxy termini (↰) of G1 and G2 glycoproteins. The predicted transmembrane topology of proteins based on the hydrophobicity profiles is also shown. Arrows: polypeptide cleavage sites.

(From Matsuoka *et al*, 1991).

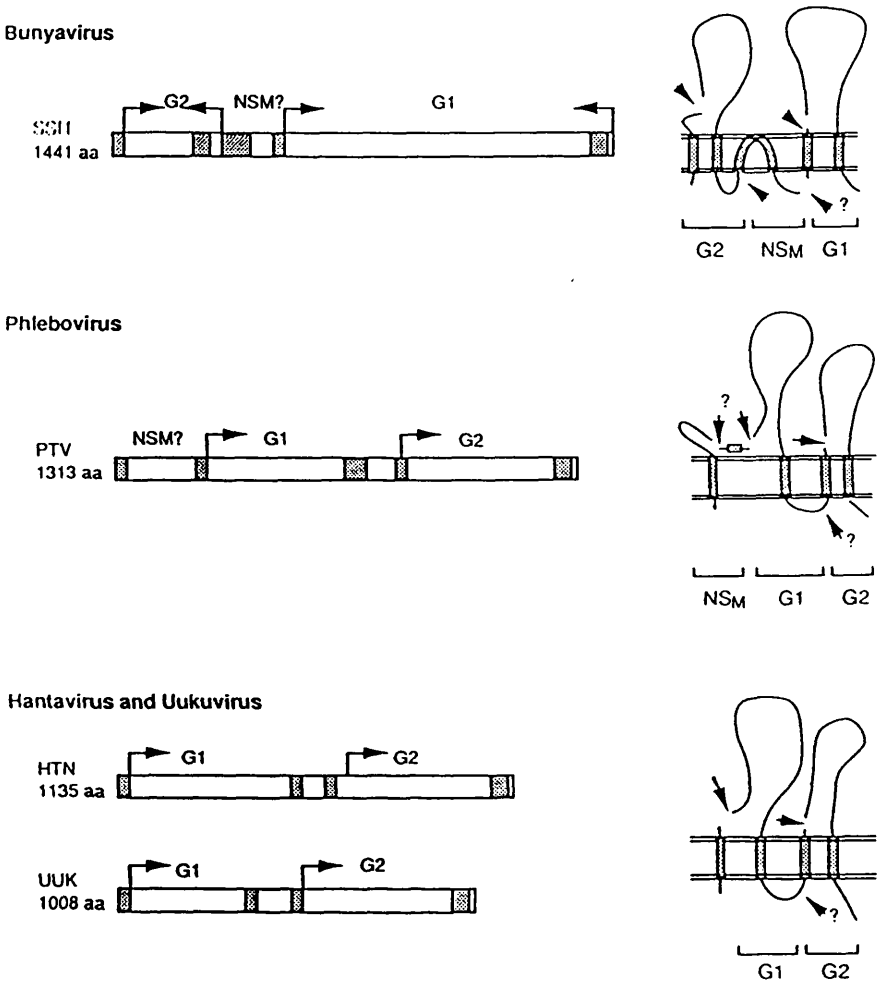

Bunyavirus
G2
NSM?
G1
SSH
1441 aa
?
G2
NSM
G1
Phlebovirus
NSM?
G1
G2
PTV
1313 aa
?
?
NSM
G1
G2
Hantavirus and Uukuvirus
G1
G2
HTN
1135 aa
G1
G2
UUK
1008 aa
?
G1
G2

Figure 1.5

Hydropathy profiles of hantavirus M segment proteins

The hydropathy plots of the predicted amino acids within the middle segment ORFs of HTN, NE and SR-11 viruses are nearly superimposible (Elliott *et al*, 1991) and are shown in Fig 1.6.

The Phlebovirus genus

Amino acid sequence comparisons of three members of the *Phlebovirus* genus have been made for PT, RVF and UUK. The proteins are rich in cysteine residues (5-7%) and of these 70% are conserved among the three viruses (Ihara *et al*, 1985b; Ronnholm and Pettersson, 1987). This was suggestive of the important role the cysteine residues played in the structure of the protein and also indicated the close relatedness of the viruses. This observation contributed towards the data that necessitated the inclusion of the former *Uukuvirus* genus into the *Phlebovirus* genus.

Within the unique ORF present in the viral complementary-sense RNA, which codes for a polypeptide precursor, the gene order for RVF has been determined as NSm-G2-G1, for PT NSm-G1-G2 and G1-G2 for UUK. The uukuvirus group lacks the nonstructural protein found in the phlebovirus group within the genus. The difference in the gene order of PT and RVF merely reflects the relative size difference between the two glycoproteins as measured on polypeptide gels, with G2 being the faster migrating.

Hydropathy plots of PT, RVF and UUK

Hydropathy plots of the three viruses, i.e. PT, RVF and UUK, are strikingly similar. Preceeding each glycoprotein is a stretch of 17-20 hydrophobic amino acids that may represent a signal or membrane insertion sequence. There is an additional hydrophobic region near the carboxy-terminal

Figure 1.6 Hydropathy plots of the M segment ORFs of hantaviruses.

The hydropathic profiles of the deduced amino acids of the M segment ORFs of HTN, SR-11, and NE viruses are displayed. Where protein sequence information is available, vertical lines are added to represent the position of the amino terminus of the indicated proteins. In all cases, the mature glycoprotein coding sequences are preceeded by a hydrophobic signal sequence (black box). Similarly, at or near the carboxy terminus of the first glycoprotein, possible hydrophobic transmembrane anchor sequences are identified (shaded boxes). Asterisks represent potential asparagine-linked glycosylation sites.

(From Elliott *et al*, 1991).

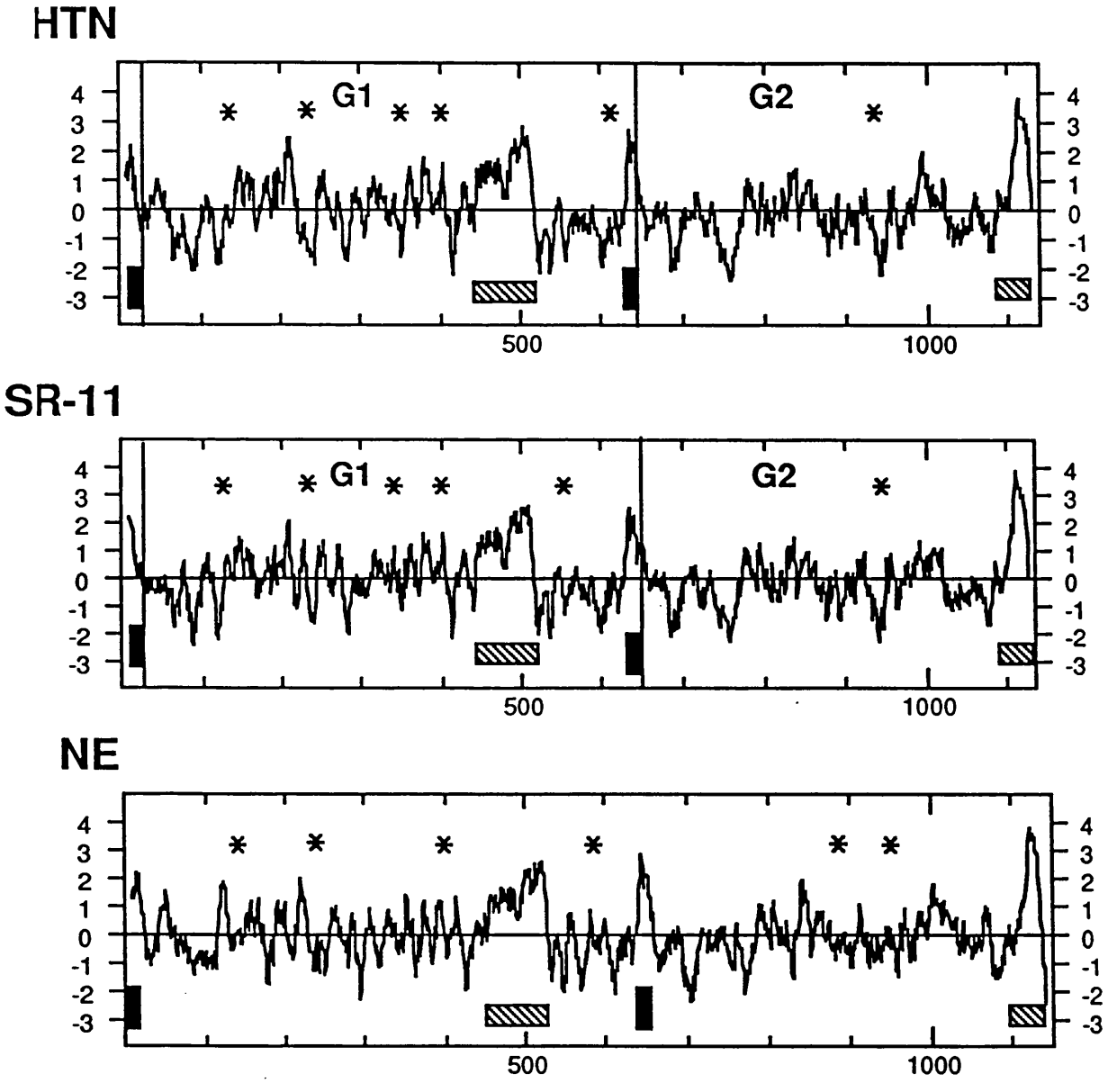

HTN
G1
G2
4
3
2
1
0
-1
-2
-3
500
1000
SR-11
G1
G2
4
3
2
1
0
-1
-2
-3
500
1000
NE
4
3
2
1
0
-1
-2
-3
500
1000

Figure 1.6

end of both G1 and G2 which may represent transmembrane anchor sequences. The profiles are different from those of the bunyaviruses but the carboxy hydrophobic end of G2 (PT), G1 (RVF) and G2 UUK are similar to the G1 of the bunyaviruses (Fig 1.7).

There is a single site for N-linked glycan attachment in RVF G2 and four sites in PT G1 and UUK G1. The second glycoprotein has four sites for each virus.

Nairovirus M segment proteins

In the absence of sequence data, not much is known about the M segment proteins of nairoviruses but it is presumed that they will have common features with other members of the Bunyaviridae family, like richness in cysteine residues and glycosylation sites.

Tospovirus M proteins

The M RNA segment of TMW has not been sequenced but it is presumed it encodes the 78K and 58K proteins which are glycosylated and represent the G1 and G2 glycoproteins, respectively (Mohamed *et al*, 1973).

The S segment proteins

The bunyavirus S segment encodes the N protein (Mr 19K to 26K) and a nonstructural protein NS_s (Mr 10K to 12K). The nucleotide sequences of six bunyavirus S segments have been determined and range in size from 850 to 984 bases. The six segments which represent three serogroups in the *Bunyavirus* genus have 40% sequence similarity overall, whereas the N proteins from the same serogroup show 80% similarity (Elliott, 1989a). Some regions of the N protein are strictly conserved between all six sequences. The conserved regions may be important in binding to the viral RNA, although homology to RNA binding proteins (Chen *et al*,

Figure 1.7 Hydropathy plots of the M segment ORFs of phleboviruses.

The hydropathic profile of the amino acid sequence of the M segment ORF of PT, RVF, and UUK viruses are displayed. Vertical lines represent the position of the amino terminus of the indicated proteins. Pre-G is the protein coding region preceeding that of the first envelope glycoprotein. In all cases, the mature glycoprotein coding sequences are preceeded by a hydrophobic signal sequence (black box). Similarly, towards the carboxy end of the first glycoprotein and at the end of the second, there appear hydrophobic putative transmembrane anchor sequences (shaded boxes). Asterisks demonstrate potential asparagine-linked glycosylation sites.

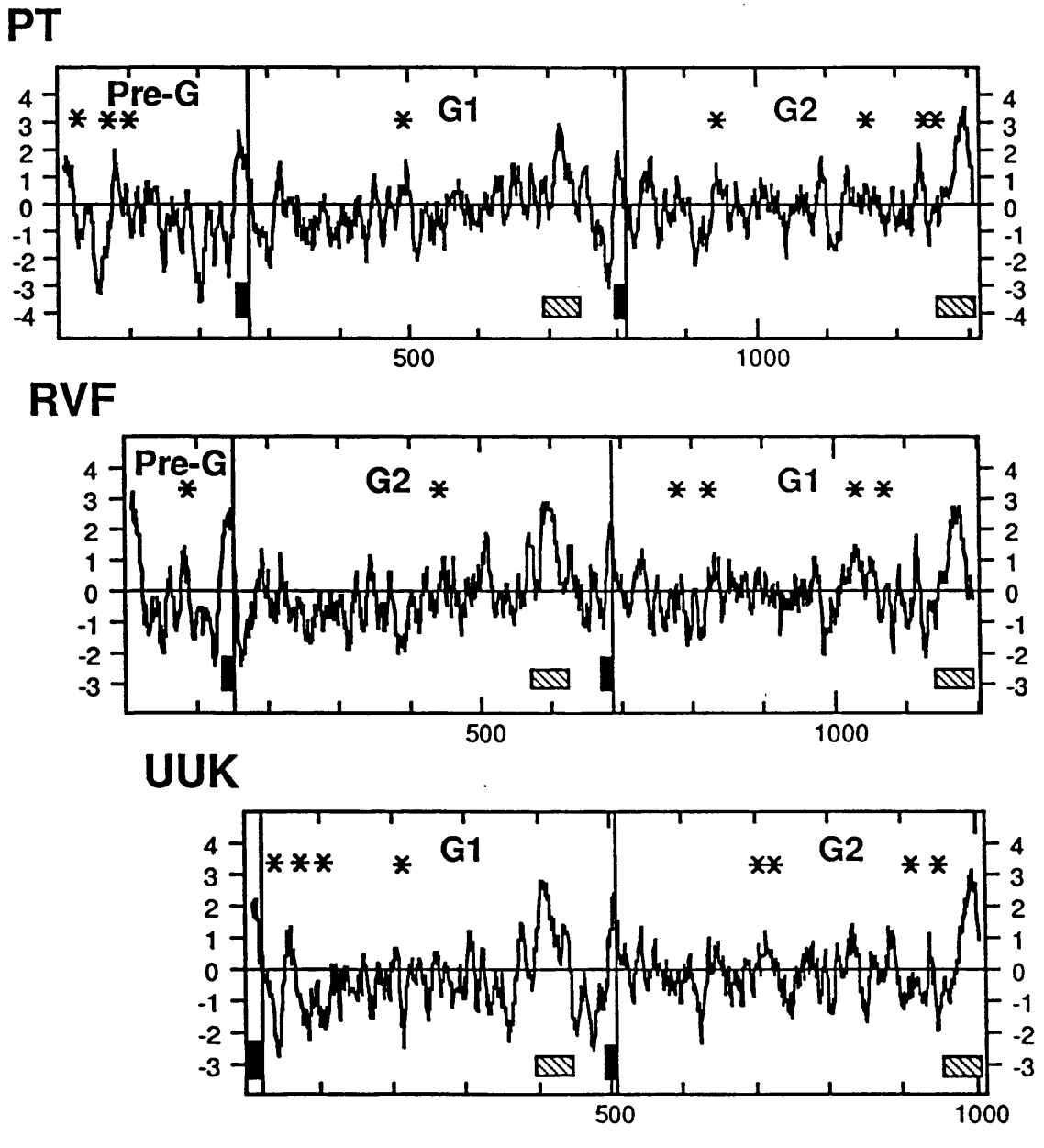


Figure 1.7

1989; Query *et al*, 1989) is not obvious. They may also be important in eliciting complement-fixing antibodies since the N is the complement fixing antigen. The N protein synthesised in large amounts, e.g. via baculovirus expression systems, will be invaluable for diagnostic use for specifying a genus, since complement fixation cross-reactivity does not occur beyond a genus.

In the *Hantavirus* genus, the N protein is about 48.1K predicted Mr. It seems that no nonstructural protein is synthesised or coded for in this genus.

The N protein is an important cross-reactive antigen in immunoprecipitation reactions between different antigenic groups (Sheshberadaran *et al*, 1988) which probably reflects areas of sequence conservation on the S segment detected by cross-hybridisation studies (Schmaljohn *et al*, 1987b).

The nairovirus N protein is similar in size to that of hantavirus (Mr. 48K to 52K). It is the major viral protein detected in infected cells (David-West, 1974; Clerx and Bishop, 1981; Clerx *et al*, 1981; Foulke *et al*, 1981; Cash, 1985; Watret and Elliott, 1985b; Watret *et al*, 1985).

In the *Phlebovirus* genus the S RNA codes for two proteins, N and NS_s, of predicted Mr of 24-30K and 29-31K, respectively. The NS_s protein has not been observed in cells infected with some members of the genus. The NS_s could be seen in cells infected with RVF, KAR and SSF but not in cells infected with PT. The intracellular location of this protein also varied as NS_s of RVF was observed in the nuclei of infected cells (Struthers and Swanepoel, 1982) whereas NS_s of KAR remained entirely in the cytoplasm (Smith and Pifat, 1982). NS_s of PT has been found to be specifically associated with nucleocapsids (Overton *et al*, 1987).

BIOLOGY AND EPIDEMIOLOGY

Disease

Most of the more than 300 members of the family Bunyaviridae rarely infect humans or domestic animals (Gonzalez-Scarano and Nathanson, 1990). However, there are members in the family of considerable medical and economic importance. The full pathogenic importance of the viruses may not be fully appreciated, especially in the so-called third world countries, where diagnostic technologies are less advanced and in some cases non-existent. Inappropriate diagnosis often places infections caused by members of the Bunyaviridae and other arboviruses under the 'great umbrella' of malaria and are often treated as such (Downs, 1975; Shope, 1985). RVF, California encephalitis virus, CCHF and HTN are important human pathogens. TSWV, which infects up to 400 species from 50 different plant families, both monocotyledons and dicotyledons, cause an enormous economical loss due to its wide geographical distribution. Selected Bunyaviruses that cause disease in humans or domestic animals are shown in Table 1.6.

Transmission and perpetuation

The ecological patterns of different bunyaviruses are quite diverse between different genera (Gonzalez-Scarano and Nathanson, 1990). Bunyaviruses, nairoviruses and phleboviruses are transmitted between vertebrate hosts by a wide variety of haematophageous vectors, including culicine and anopheline mosquitoes, argasid and ixodid ticks, biting midges and sandflies. Virus genera are typically associated with a given vector taxon (Beaty and Calisher, 1991): bunyaviruses are principally transmitted by mosquitoes, nairoviruses and the uukuvirus group of phleboviruses by ticks and the phlebovirus group of phleboviruses by sandflies. Tospoviruses are transmitted by thrips and hantaviruses, which are maintained by persistent

Table 1.6 Selected bunyaviruses that cause disease in humans or domestic animals

Genus, serogroup	Virus	Host	Disease	Frequency
Bunyavirus				
California	La Crosse	Human	Encephalitis	Endemic
	Snowshoe hare	Human	Encephalitis	Rare
	California encephalitis	Human	Encephalitis	Rare
	Jamestown Canyon	Human	Encephalitis	Sporadic
	Tahyna	Human	Influenzalike	Endemic
Simbu	Oropouche	Human	Acute fever	Epidemic
	Akabane	Ungulates	Fetal malformations	Endemic
Phlebovirus				
RVF	RVF	Sheep	Hepatitis	Epidemic
		Human	Influenzalike	Epidemic
Sandfly fever	Sandfly fever			
	Naples	Human	Acute fever	Endemic
	Sicilian	Human	Acute fever	Endemic
Hantavirus				
Hantaan	Hantaan	Human	HFRS	Endemic
	Seoul	Human	HFRS	Endemic
	Puumala	Human	Renal syndrome	Endemic
Nairovirus				
NSD	NSD	Sheep	Hepatitis	Endemic
CCHF	CCHF	Human	Hemorrhagic fever	Sporadic

Ref: F. Gonzalez-Scarano *et al*, 1991.

infection in rodents, by aerosolised excreta of rodents. Reservoir host for members of the Bunyaviridae are mainly mammals and birds.

Geographic distribution

The Bunyaviridae are found worldwide. Restriction of a virus to a vertebrate-vector pairing with defined geographic distribution may lead to natural isolation and therefore genetic stability in divergent evolution (Calisher and Karabatsos, 1988). For example, no Bunyamwera serogroup virus has been isolated in Australia whereas Batai virus, which is maintained in birds, has been isolated in Asia and Europe - probably because of migrating birds.

Vertebrate host-virus interactions

Reservoir hosts are usually not untowardly affected by arboviral infection, probably because they have evolved a relative benign host-parasite relationship over time. In contrast, tangential hosts may experience considerable morbidity and mortality when infected with the virus (Beaty and Calisher, 1991). Nosocomial infections have been observed with CCHF, RVF and HTN viruses.

Vector-virus interactions

Like other arboviruses, bunyaviruses are transmitted either biologically or mechanically by vectors. Biological transmission involves ingestion of the virus by the vector after feeding on a viraemic vertebrate host. After the vector becomes infected the virus replicates in the tissues and organs. The virus may then be transmitted when the vector seeks another blood meal. This occurs after an extrinsic period, i.e. the period between after digestion and when the vector is competent to transmit the virus.

In mechanical transmission, the vectors do not need to become infected by the virus but the virus is carried on the mouth parts. For transmission to occur the vector must feed upon a host in time before the virus becomes inactivated.

In both cases the process by which the virus enters a vector and exits is quite complex and involves multiple infection and dissemination barriers (Hardy, 1988; Kramer *et al*, 1981; Beaty and Bishop, 1988). The usual sequence of events in the extrinsic incubation period modulated by temperature (Turell, 1989) is: (a) infection of vector midgut cells, (b) replication in these cells, (c) escape into the haemolymph, and (d) dissemination to an infection of epidemiologically significant target organs. Infection of salivary glands permits transmission to vertebrates and infection of ovaries permits transovarial transmission to progeny.

It is the accepted view that arbovirus infection is not detrimental to natural vectors in contrast to vertebrate hosts, where sometimes dramatic effects occur. However, it has been shown that some effect associated with arboviral infection of vectors takes place (Beaty *et al*, 1980; Grimstad *et al*, 1980; Weaver *et al*, 1988).

PROTEIN TARGETTING

Overview

It is a generally accepted view that proteins which are not retained at their site of synthesis have signals that direct their intracellular transport, their folding and interaction with other cell components once they reach their destination. These signals may be divided into four groups:

1 *Routing signals* operate at the earliest stages of protein targetting and direct the specific interaction between targetted polypeptides and the surface of the first membrane

with which they come into contact. Routing signals may be located in any part of the polypeptide and they need not be cleaved off during the early stages of translocation. If they are proteolytically processed, then the initial form of the polypeptide is the precursor and the processed polypeptide is the mature form.

2 *Sorting signals* direct proteins into different branches of a targetting pathway or redirect them once they have reached their initial destination.

3 *Retention signals* prevent proteins from passing along the entire length of targetting pathway or from being released from organelles to which they have been targetted.

4 *Membrane topology - stop transfer signals* involve routing signals which are not cleaved off and remain embedded in the membrane. These are then referred to as membrane anchor sequences. Those that halt the translocation of the polypeptide through the membrane are termed stop transfer signals.

Bunyavirus protein transport

One of the characteristics of the members of the Bunyaviridae family is that they mature by budding at smooth-surfaced membranes close to the nucleus (Lyons and Heyduk, 1973; von Bonsdorff *et al*, 1970). Kuismanen *et al* (1982) showed by immunological cytochemical and immunoelectron microscopic techniques that this region corresponds to the Golgi complex. Budding at the Golgi membranes was also observed by Murphy *et al* (1973) by direct electron microscopy.

Bunyavirus glycoproteins accumulate at the membranes of the Golgi apparatus before virus assembly and it has been suggested that the glycoproteins serve to direct other structural components to the site of maturation (Matsuoka

et al, 1991). Bunyavirus glycoproteins expressed from cloned cDNAs are retained in the Golgi complex and behave like resident proteins of the Golgi complex. Studies with recombinant glycoproteins also revealed that the glycoproteins are targetted to the Golgi complex in the absence of other viral components, structural or nonstructural (Matsuoka *et al*, 1988; Pensiero *et al*, 1988; Wasmoen *et al*, 1988).

The location and funtional roles of other protein products of the M segment are not clear.

Possible mechanisms for intracellular transport and localization

Some proteins remain in the ER while others are exported to the Golgi complex, most of which are subsequently distributed to various organelles. Two mechanisms have been demonstrated for retention in the ER. Resident ER proteins like disulphide isomerase contain the same carboxy terminal tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL). Removal of the KDEL sequence resulted in the protein being secreted and addition of the sequence to secretory proteins caused them to accumulate in the ER (Munro and Pelham, 1987; Pelham, 1988). Another mechanism to control the exit of proteins from the ER has been described as possession of transport competency - this involves correct folding and assembly of proteins. Mutations introduced into viral glycoproteins were reported to block or slow export of proteins from the ER (Rose and Doms, 1988).

It has also been observed that for some proteins including influenza HA and VSV G protein, assembly into oligomers is required for transport from the ER (Copeland *et al*, 1986; Doms *et al*, 1988; Gething *et al*, 1986; Hurtley and Helenius, 1989; Kreis and Lodish, 1986). Failure to assemble into an oligomer resulted in ER retention, without

the requirement for a specific signal. In virions, the G1 and G2 glycoproteins of UUK exist as heterodimers (Persson *et al*, 1989). Protein products from recombinant vaccinia viruses containing only either G1 or G2 coding sequence of PT failed to exit the ER (Chen *et al*, 1991). The same was observed for HTN glycoproteins (Ruusala *et al*, 1992).

The question of whether the glycoproteins of bunyaviruses contain a Golgi retention signal is unclear, and if they had a retention signal, is it present on both G1 and G2 or just on one of them? Are extra sequences such as the NSm coding sequence involved in retention of glycoproteins in the Golgi? The requirement of an NSm protein in Golgi retention was ruled out for phlebovirus: recombinant vaccinia viruses with truncated 5' ends, hence lacking NSm, were synthesised, processed and transported to the Golgi complex and stably retained in the Golgi membranes (Matsuoka *et al*, 1988; Wasmoen *et al*, 1988).

VIRUS REPLICATION CYCLE

The main stages of replication are as follows:

Attachment and entry

The early events in the infection process of members of the Bunyaviridae are not well understood (Schmaljohn and Patterson, 1991), but, in common with other enveloped viruses, one or both of the integral viral glycoproteins mediate attachment to host-cell receptors. Obijeski *et al* (1976b) showed that when LAC was treated with proteolytic enzymes, the virus particles became 'spikeless' and this resulted in a five-log reduction in infectivity as compared to the untreated virions. Using polyclonal and monoclonal antibodies to block infection or haemagglutinating activity and with limited digestion with bromelain or pronase which degrade part of G1, it has been concluded that G1 is the more actively involved glycoprotein in binding to host cells

(Gonzalez-Scarano *et al*, 1982; Grady *et al*, 1983; Kingsford and Hill, 1981; Kingsford *et al*, 1983). However, neutralising and haemagglutination inhibition sites have been found on both the G1 and G2 proteins of HTN (Arikawa *et al*, 1989; Dantas *et al*, 1986).

Fusion, which is pH-dependent, is thought to be part of the early events in the infection process, especially at the point of translocation of RNA and proteins into the host cell cytoplasm. Marsh and Helenius (1980) described entry of Semliki Forest virus into cells by endocytosis via coated vesicles. These endosomes were shown by Tycko and Maxfied (1982) to be acidified and acidification triggered release of the nucleocapsid into the cell cytoplasm. A similar series of events was observed for RVF in which viral particles appeared to enter cells in phagocytic vacuoles by electron microscopy (Ellis *et al*, 1988). It is assumed the same events occur for other members of the family Bunyaviridae.

Genome replication

In negative strand viruses, the change from primary transcription to replication needs a switch from mRNA synthesis to synthesis of full-length cRNA templates and then vRNA. These steps are schematically illustrated in Fig 1.8 using S RNA as an example.

The events involved in making the switch from mRNA synthesis to synthesis of full length cRNA templates and vRNA are not understood. However, there must be some signal to suppress the termination signal responsible for generation of truncated mRNA and also to prevent the addition of capped and methylated structures to the 5' termini of the cRNAs (Schmaljohn and Patterson (1991). It is known that continuous protein synthesis is necessary for replication of the genome as translational inhibitors such as cycloheximide interfere with this process. The actual protein that may be

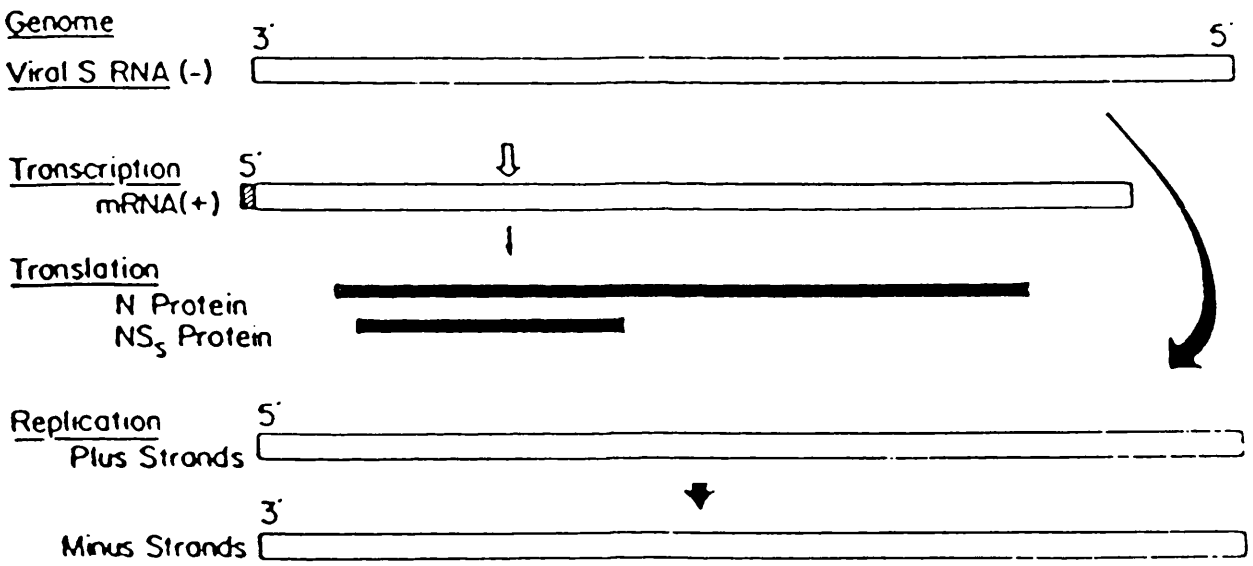


Figure 1.8. Bunyavirus S RNA coding, transcription and replication strategies, from Bishop D.H.L.,1990.

important in the switch from mRNA synthesis to replication is not known but a candidate could be nonstructural protein NSs, in those viruses which encode it.

Viral assembly and release

Although assembly of the bunyaviruses occurs by budding at the smooth membrane vesicles of the Golgi apparatus (Murphy *et al*, 1973; Kuismanen *et al*, 1982; Ellis *et al*, 1988), reports of budding at the plasma membrane and possibly the ER have been made. When RVF was grown in primary hepatocyte cultures, growth kinetics were comparable to those seen in other permissive cell lines but maturation was associated with budding at cellular surface membranes in addition to the Golgi and ER membranes (Anderson and Smith (1987). Maturation at the plasma membrane was also reported by Madoff and Lenard (1982) for LAC in BHK 21 cells.

The actual mechanisms of budding are not understood but it has been suggested that budding involves a transmembrane interaction between membrane glycoproteins and other components of the virus in the cytoplasm, followed by pinching off from the membrane surface (Lenard and Compans, 1974). The importance of the glycoproteins in the process of maturation has been demonstated by use of glycoproteins expressed via vaccinia virus or baculovirus. The G1 and G2 of HTN, RVF and PT viruses translocated to the Golgi in the absence of other viral glycoproteins (Pensiero *et al*, 1988; Matsuoka *et al*, 1988; Wasmoen *et al*, 1988). Studies on *ts* mutants of UUK defective in virus maturation also showed that G1 and G2 are localized in the Golgi even if virion maturation failed to occur (Gahmberg, 1984; Gamberg *et al*, 1986a). Smith and Pifat (1982) suggested that transmembranal recognition between the viral glycoproteins and the nucleoproteins induced budding.

Budding is followed by the virions acquiring their lipid bilayer from the host cell membranes as most other

host cell membrane proteins are excluded from the viral particles. The viral particles are transported in vesicles to the plasma membrane where fusion of the vesicles and the cellular plasma membrane occurs and the release of the viral particles takes place, presumably by normal exocytosis.

Effects of viral replication on host cells

The c.p.e. observed in cultured cells infected with members of the family Bunyaviridae vary considerably, depending on the virus and the type of host cell. Viruses of all genera except the Hantavirus genus alternatively replicate in vertebrates and arthropods. The viruses are cytolytic for their vertebrate hosts but not their invertebrate hosts (James and Millican, 1986; Lyons and Heyduck, 1973). In infections of mammals, there appears to be both organ and cell tropism. For example, bunyaviruses like LAC appear to be neurotropic (Parsonson and MacPhee, 1985) whereas the RVF phlebovirus is primarily hepatotropic (Anderson and Smith, 1987; Anderson *et al*, 1987; Easterday, 1965; Peters *et al*, 1988). The HTN hantavirus replicates and remains persistent in rodent lungs (Lee *et al*, 1981). The mechanism for this apparent tropism is not well understood but Sundin *et al* (1987) working with LAC variant and revertant viruses suggested that the specificity resided in the G1 glycoprotein and probably at the level of attachment to host cells.

Bunyaviruses and phleboviruses have been shown to cause a reduction in host-cell protein synthesis which increases with progression of infection (Pennington *et al*, 1977, for BUN; Madoff and Lenard, 1982, for LAC; Parker *et al*, 1984, for RVFV). Similar reduction in host-cell protein synthesis has not been observed in UUK infected cells (Pettersson, 1974; Ulmanen *et al*, 1981), nor in Dugbe nairovirus infected cells (Cash, 1985). Some host protein synthesis reduction was, however, observed when *Xenopus laevis* cell line was infected with Clo Mor, a Scottish nairovirus and St Abb's

Head phlebovirus (Watret *et al*, 1985). On the other hand, hantaviruses have not been demonstrated to cause reduction in host protein synthesis (L H Elliott *et al*, 1984; Schmaljohn and Dalrymple, 1984), but often establish persistent, non-cytolytic infections in susceptible mammalian host cells. This observation is consistent with the documented non-pathogenic persistence in their natural rodent hosts (Lee *et al*, 1981).

GENETICS OF BUNYAVIRIDAE

Evolution of Bunyaviridae

The plethora of viruses and the multiplicity of serogroups that comprise the family Bunyaviridae suggest that viruses in this family have considerable evolutionary potential (Beaty and Calisher, 1991). This evolutionary potential will result in viruses with altered virulence and other characteristics like new viral phenotypes capable of infecting new vector species. New viruses of potentially significant epidemiologic consequences may also result (Beaty *et al*, 1988). Natural host vectors provide many opportunities for the evolution of the Bunyaviridae by intramolecular changes in the viral genome, such as point mutations, sequence deletions, inversions, etc (genetic drift) and RNA segment reassortment (genetic shift).

Genetic drift

Most of the life cycle of bunyaviruses, nairoviruses and phleboviruses occurs in the vector host. If transovarial transmission occurs, viruses may persist through generations of infected arthropods without being transmitted to the vertebrate host. As with other negative-strand RNA viruses, such conditions will enhance nucleotide sequence deletions, inversions and point mutations (Holland *et al*, 1982). This was explained in part by the absence of proof-reading exonucleases and the high error rate of RNA

polymerases (Holland *et al*, 1982). For members of Bunyaviridae, it has been shown that no two isolates of LAC recovered from nature had identical genome segments, as demonstrated by oligonucleotide fingerprints (Bishop and Beaty, 1988). This was true for LAC isolates obtained at the same place at the same time, the same place at different times, and different places at different times. Low-passage SSH virus isolates from mosquitoes exhibited different fingerprints (Beaty and Calisher, 1991). In contrast, SSH and LAC viruses derived from the original prototype and maintained by lytic passage in different laboratories for many years had identical oligonucleotide fingerprints (Beaty and Calisher, 1991). Genetic stability has also been demonstrated during long-term persistent infections of vectors with some bunyaviruses in the laboratory (Bilsel *et al*, 1988; Baldridge *et al*, 1989). This involved passaging of plaque purified viruses transovarially for two generations in vectors and horizontally to mice during each generation. Examination of oligonucleotide fingerprints at each stage showed them to be identical. This state of affairs indicated that genetic instability occurs more frequently in nature.

Genetic shift

The segmented genome of the Bunyaviridae provides the chance for virus evolution to occur through RNA segment reassortment during dual infection of cells. High frequency reassortment of members of the family have been documented to occur *in vitro*, *in vivo* and in nature. In the laboratory reassortment has been shown to be restricted to viruses within a genus, and even within a genus reassortment was confined or only possible among some closely related members within serogroups (Gentsch and Bishop, 1976; Gentsch *et al*, 1977b, 1979; Ozden and Hannoun, 1980; Iroegbu and Pringle, 1981; Rozhon *et al*, 1981; Pringle *et al*, 1984; Pringle and Iroegbu, 1982; R M Elliott *et al*, 1984; Janssen *et al*, 1986; Bishop, 1988). Klimas *et al* (1981) showed by genotypic

analysis of natural isolates of LAC that RNA segment reassortment occured between topotypes of LAC and Shark River and that Pahayokee virus may be a reassortment of LAC and Shark River viruses (Bishop and Shope, 1979). Shope (1985) analysed seven group C viruses from the Utinga Forest in Brazil. These viruses segregated into three groups by HI and NT tests which assay for the gene products of the M RNA segment. However, the viruses segregated into three alternative groups by CF tests, which assay mainly the gene products of the S RNA segment. This suggested that reassortment of RNA segments was occuring between the group C viruses. For example, two of the group C virus, Caraparu and Itaqui, share vector and vertebrate hosts and are identical by CF tests, but differ in their HI and NT, i.e. one cannot induce HI or NT antibodies to the other in the vertebrate host. This meant that their M RNA segment gene products differ.

Although reassortment is typically restricted to viruses from a given serogroup, suggesting the serogroups constitute gene pools that are evolving divergently, serologic relationships have been observed between different groups. Whitman and Shope (1962) found serologic relationship between Guaroa virus and the California group viruses by NT test and to the Bunyamwera group viruses by CF.

High frequency reassortment of members of the Bunyaviridae occurs *in vitro* in vertebrate cell cultures but has not been detected *in vivo* in vertebrates (Bishop and Beaty, 1988). Lack of reassortment may be due to the ephemeral nature of infection in reservoir hosts, the relatively low-level viraemia, and the production of antibody, all of which limit the chance that two viruses infect the same cell (Beaty and Calisher, 1991). High-frequency reassortment has not been demonstrated in vectors. There appears to be only a small window of time for dual oral infection to occur in arthropod vectors. Within two to

three days after ingestion of one virus, mosquitoes become resistant to superinfection with a second closely related virus (Beaty *et al*, 1985; Sundin and Beaty, 1988). However, some mosquitoes may preclude this interference when the defensive behaviour of the host interrupts the mosquito during engorgement. The vector will engorge on an alternative host (Beaty *et al*, 1988). The ability to engorge from two hosts raises the potential for infection by different viruses and could permit dual infection and subsequent segment reassortment.

Temperature-sensitive mutants

Some 200 temperature sensitive mutants have been isolated from 11 viruses in the family Bunyaviridae. Ten of these viruses belong to two serogroups, California and Bunyamwera of the *Bunyavirus* genus. The other one belongs to the uukuvirus group of the *Phlebovirus* genus (Pringle, 1991). The majority of these mutants were generated by chemical mutagenesis using either 5-fluorouracil, 5-azacytidine or N-methyl-N'-nitrosoguanidine (Gentsch *et al*, 1977b, 1979; Bishop, 1979; Ozden *et al*, 1978, 1980; Iroegbu and Pringle, 1981; Pringle and Iroegbu, 1982; Gahmberg, 1984). A significant feature of these mutants has been the recovery of mutants assignable to two reassortment groups rather than the three expected from the tripartite structure of the genome. Maguari virus of the Bunyamwera serogroup is the exception. One mutant of the 46 *ts* mutants isolated from mutagenized wild type Maguari virus, mutant *ts*MAG23(III), recombined with all members of panels of mutants representing reassortment groups I and II and two putative double I+II mutants, thus identifying the third reassortment group (Pringle and Iroegbu, 1982). Unlike group I and II mutants, *ts*MAG23(III showed marked host cell dependence as evidenced by at least 100-fold less plaque-forming ability on BS-C-1 cells and 1,000-fold lower yields, compared to wild type.

Temperature-sensitive mutants have been important tools in the investigation of gene function, definition of genetic basis of virulence and the delineation of the extent of reassortment and the limits of gene exchange.

At the moment assignments of the group I, II and III lesions are mapped to lesions in the S RNA, M RNA and L RNA, respectively. This is true for the Bunyamwera serogroup in the Bunyavirus genus but not the California serogroup in the same genus, where apparently no N gene mutations and many L gene mutations have been observed (Pringle, 1991).

INTERFERENCE AND PERSISTENT INFECTION

Defective interfering particles

Most animal viruses can establish persistent infections. One of the mechanisms of initiation and regulation of persistent infections involves defective-interfering (DI) particles: a class of animal virus mutants that share a number of common properties. These are the inability to propagate in the absence of a helper virus (defectiveness), the ability to be complemented by helper virus and therefore multiply in the presence of helper virus, the ability to decrease the yield of the wild type virus (interference), and the ability to increase their proportion of the yield from cells co-infected with wild-type virus (enrichment) (Huang and Baltimore, 1977).

The defectiveness of DI particles indicates that they have a lesion in some essential gene which appears to be a deletion, but there exist possibilities for point mutations or gene rearrangements. Interference by DI particles is specific against the standard virus from which a particular DI particle arose or a serologically closely-related virus. The ability of host cells to support interference by DI particles varies but host functions that control these variations are not known (Huang and Baltimore, 1977).

DI particles of negative-strand viruses

For most of the negative-strand RNA viruses, DI particles appear to be identical to the standard virus in their protein composition and overall morphological characteristics. Most of the information has come from vesicular stomatitis virus and Sendai virus. By use of velocity sedimentation and hybridisation, it was shown that the DI RNA of VSV and Sendai virus was a deleted form of the standard RNA (Huang and Wagner, 1966a; Brown *et al*, 1967; Schaffer *et al*, 1968; Kingsbury *et al*, 1970). It was also shown, by RNA-RNA annealing, that a given DI particle contains a specific portion of the standard virus genome and not random pieces encapsidated into mature DI particles (Schincariol and Howatson, 1972; Stamminger and Lazzarini, 1974). Hybridisation studies of DI genomes from purified VSV DI-particles with mRNA fractions obtained from VSV-infected extracts and with mRNA synthesised *in vitro* (Leamson and Reichmann, 1974; Stamminger and Lazzarini, 1974; Schnitzlein and Reichmann, 1976; Adler and Banerjee, 1976; Roy and Bishop, 1972) showed that DI RNAs originated from the 5' end of the standard virus genome. However, a DI particle isolate of VSV (Indiana strain) was reported to have a deletion in the 3' end and exhibited other unique characteristics like heterotypic interference with VSV New Jersey strain (Prevec and Kang, 1970; Schnitzlein and Reichmann, 1976). Additional evidence showed that some DI particles had internal genome deletions with the intact 3' and 5' termini of standard VSV (Perrault and Semler, 1979). DI particles from VSV could sometimes contain (-) strands (Roy and Bishop, 1972; Stamminger and Lazzarini, 1974) and sometimes unlinked (+) and (-) strands (Roy *et al*, 1973; Leamson and Reichmann, 1974; Reichmann *et al*, 1974; Schnitzlein and Reichmann, 1976; Perrault and Leavitt, 1977b).

The RNA from influenza DI particles differed from those described for VSV and Sendai virus. It seemed that the DI particles lost, partially or completely, the largest segment of viral RNA (Duesberg, 1968; Choppin and Pons, 1970). However, other data suggested that influenza virus did not always lose a particular segment (Bean and Simpson, 1976; Nayak *et al*, 1978) but there appeared to be a relative reduction of one or more of the four largest viral RNA segments.

In the Bunyaviridae family, the existence of DI particles and their role in persistent infections has not been directly demonstrated. The difficulty to characterise such mutants may be due to the frequent inability to recover subunit RNA in equimolar amounts from purified virus (Elliott, 1990). Kascsak and Lyons (1978) reported generation of particles which interfered with homologous virus when BUN was passaged at high multiplicity in BHK cells and these particles were shown to contain the S segment-sized RNA. Elliott and Wilkie (1986) reported that particles which contained only the S segment sequences were shed from *Aedes albopictus* cells persistently infected with BUN and these particles also interfered with growth of standard virus. Later Cunningham and Szilagyi (1987) demonstrated that the interfering particles for GER grown in BHK cells had defects in the L-segment. More recently, Scallan and Elliott (1992) demonstrated that defective RNAs from persistently infected C6/36 cells (Igarashi, 1978) with BUN had subgenomic (defective) components that originated from the L RNA segment by northern blotting. Patel and Elliott (1992) characterised five BUN variants that produced small plaques on BHK and mouse L cells. They showed that progeny of these variants reduced the titre of standard coinfected virus by several hundred-fold. Analysis of cloned cDNA to the RNA of these particles revealed internal deletions in the L segment ranging from 72% to 77% of the L RNA segment. The 5'- and 3'-terminal sequences were

retained (Patel and Elliott, 1992). The same authors could readily generate defective L and M segments in mouse L cells but not in BHK-21 cells.

Persistent infection

Apart from the suggested involvement of defective interfering particles in the establishment and maintenance of persistent infections in cells, other factors including interferon, mutations in the standard virus and inherent resistence of the cell to a particular virus may have a role (Huang and Baltimore, 1970). Traditionally, persistent viral infections have been categorised into two groups. One in which the infectious virus is present and can be recovered biologically has been referred to as chronic. Infectious chronic viral infections are exemplified by hepatitis B virus in humans (Marion and Robinson, 1983) and lympocytic choriomeningitis virus (LCMV) in mice (Buchmeier *et al*, 1980; Lehmann-Grube *et al*, 1983). The other group of persistent infections involve those in which the genome is present but infectious virus is not produced except during intermittent episodes of reactivation and are referred to as latent infections. The best known in this group are the herpesviruses (Roizman and Sears, 1987; Stevens, 1989). This kind of classification is not fool-proof as it is becoming apparent that the site of infection may be the determinant of whether a given type of virus will be in a latent or chronic productive state. This was demonstrated by hepatitis B virus which was known to produce chronic infections in hepatocytes but could also cause latent infection in lymphocytes (Korba *et al*, 1988).

Although the actual mechanisms involved in the initiation and maintenance of persistent infections are not well understood, a necessary prerequisite appears to be an inhibition of the rate of virus multiplication in the early stages of infection, i.e. the virus should not be overtly cytolytic and must be able to regulate its lytic potential

(Ahmed and Stevens, 1990). This is not a problem for viruses that cause non-lytic productive infections, for example the arenaviruses which survive in nature as lifelong chronic infections of their rodent hosts (Howard, 1986; Lehmann-Grube, 1984; Rawls *et al*, 1981). Lytic viruses can get around this problem by regulating their gene expression, i.e. by restricted expression of virus proteins or by generation of viral variants that are less cytolytic or interfere with growth of the wild type virus (Ahmed and Stevens, 1990).

Another important component of viral continuance involves evasion of immunologic surveillance. This may happen by changes in the structure or expression of the surface viral glycoproteins by which the viruses can avoid recognition by humoural antibodies or by changes in any viral protein, structural or non-structural, which may be targets for T-cell recognition. Antigenic variation is another effective means of escape from neutralising antibodies. This is exemplified by the antigenic shift and drift amoung influenza viruses (Palese and Young, 1982; Webster *et al*, 1982) which although does not establish persistent infections in individuals, contibutes to the persistence of the virus in the population.

Members of the Bunyaviridae, in common with other arboviruses, do not cause serious disease in their invertebrate hosts. Instead, a non-cytocidal, self-limiting infection that leads to persistent infection ensues. The existence of persistent infections in vertebrate hosts probably occurs although not widely documented. Persistent infections have been established in mosquito cell lines with BUN, LAC and Marituba bunyaviruses and Toscana phlebovirus. The mosquito cells showed no differences in their metabolic activities from the uninfected ones and continued to shed infectious virus (Newton *et al*, 1981; Nicoletti and Verani, 1985; Carvalho *et al*, 1986; Elliott and Wilkie, 1986; Rossier *et al*, 1988; Scallan and Elliott, 1992). Apart from

establishment of persistent infections in mosquito cells, reports have been made of establishment of persistent infections for Dugbe virus in pig kidney cells (David-West and Porterfield, 1974) and Toscana virus in Vero cells (Verani *et al*, 1984).

When BUN (Newton *et al*, 1981; Elliott and Wilkie, 1986) and LAC (Rossier *et al*, 1988) were examined under identical conditions in BHK and C6/36 cells it was clearly observed that macromolecules accumulated more slowly in mosquito than in mammalian cells. It was also demonstrated that infection in mosquito cells still remained asymptomatic even when the intracellular concentration of viral products reached the same levels as those in mammalian cell infection. Except for the L mRNA, which remained close to or below the level of detection in mosquito cells, other viral RNAs accumulated linearly until about 24 hours post infection then decreased slowly over the following several days (Rossier *et al*, 1988). Scallan and Elliott (1992) reported that viral L, M and S RNAs were detectable up to 72 hours post infection. In BHK cells they observed more L and M segment RNA relative to the S segment RNA, whereas in mosquito cells there appeared to be more S segment RNA than L and M segment RNAs. There was also marked inhibition of host protein synthesis in BHK cells compared to the mosquito cells.

Bunyavirus RNA synthesis

Like other negative strand RNA viruses, replication of the BUN genome requires that the infecting genome be first transcribed into mRNA by the virion-associated transcriptase. Most studies on RNA synthesis for members of the Bunyaviridae family have concerned transcription processes leading to the synthesis of mRNA. Little is known about the synthesis of genomic or antigenomic RNA molecules (Bouloy, 1991). The negative strand genome is the template for both mRNA and antigenome (the replicative intermediates) synthesis. Events leading to the synthesis of mRNA and

which do not require concurrent protein synthesis are termed primary transcription, whereas secondary transcription occurs after the primary mRNA is translated, and genome replication is accompanied by amplification of mRNA transcription (Elliott, 1990).

mRNA molecules

For the bunyaviruses (Bishop *et al*, 1983b; Patterson and Kolakofsky, 1984; Bouloy *et al*, 1984, 1990; Eshita *et al*, 1985; Cunningham and Szilagyi, 1987; Gerbaud *et al*, 1987a), phleboviruses (Ihara *et al*, 1985a; Collett, 1986; Emery and Bishop, 19867; Marriot *et al*, 1989) and hantaviruses (Schmaljohn *et al*, 1987a) which have been studied, all mRNAs are subgenomic, do not contain a poly A tail at their 3' ends (as demonstrated by their inability to bind to oligo dT-cellulose), have 10- to 18-base heterogenous non-virus coded extensions at the 5' ends and are capped.

These non-viral sequences have been implicated as initiation primers in transcription and similarities have been drawn with influenza viruses, i.e. they utilize cellular sequences in a 'cap-snatch' mechanism to prime mRNA synthesis (Krug *et al*, 1987). Purified LAC virions were shown to have polymerase activity which was stimulated by oligonucleotides of the A(n)G series and cap structures (mGppp A). Primer extension studies of the polymerase products showed that the oligonucleotides were acting as primers. Addition of natural mRNAs, for example alfafa mosaic virus RNA 4, also stimulated polymerase activity (Patterson *et al*, 1984). That the intracellular mRNAs of LAC are indeed capped was demonstrated by Hacker *et al* (1990) using specific selection by anti-cap antibodies. The difference in initiation between bunyaviruses and influenza virus appears to be their site of action. Bunyavirus mRNAs are synthesised in the cytoplasm, whereas those of influenza virus are synthesised in the nucleus (Kolakofsky and Hacker,

1991). This has been demonstrated by the observation that transcription of influenza virus is inhibited by actinomycin D and α-amanitin, whereas bunyavirus transcription is not affected by these drugs.

The suggested mechanism for bunyavirus mRNA initiation (Kolakofsky and Hacker, 1991) would be:

1 The viral polymerase, presumably attached to the 3' end of the genome template, binds to the cap group of a host mRNA, either directly or via a host cell cap-binding protein.

2 The polymerase cleaves this chain 10-18 nucleotides downstream of the cap, depending on the particular sequence of the host mRNA, leaving a 3' hydroxyl group on the capped fragment.

3 The 3' base of the fragment is then aligned on the 3' base of the template (this need not be by base pairing; Bouloy, 1990) and utilised as a primer for viral mRNA synthesis).

The 3' ends of mRNA do not extend to the end of their templates. The mRNAs are about 60 to 100 nucleotides shorter than full length transcripts. This is important because it makes the formation of circular and panhandle structures via the complementary terminal sequences difficult: such structures may interfere with translation. When the template sequences corresponding to the 3' ends of six mRNAs were mapped by nuclease digestion, two different but related consensus sequences were identified. The LAC S (Patterson and Kolakofsky, 1984) and L (Hacker *et al*, 1990) and GER M mRNAs (Bouloy *et al*, 1990) all ended within the sequence 3' GUUUUU 5'. The SSH (Ihara *et al*, 1985) and RVF (Collett, 1986) mRNAs ended within the sequence 3' ACCCC 5'. It has been suggested that these consensus sequences probably are signals for mRNA termination. The mRNAs are

not polyadenylated, but could form stem-loop structures just upstream of their 3' ends. These stem-loop structures may substitute for the poly (A) tail in conferring mRNA stability (Kolakofsky and Hacker, 1991).

In the S segments of phleboviruses (Ihara *et al*, 1984; Simons *et al*, 1990) and tospoviruses (de Haan *et al*, 1990) which are ambisense, two subgenomic mRNAs of opposite polarity are transcribed (Elliott *et al*, 1991). The N mRNA is transcribed from the 3' end of the genome, and the NSm mRNA from the 3' end of the antigenome. The open reading frames of these transcripts do not overlap but are separated by an intergenic region which, except for SFS (Marriott *et al*, 1989), has the potential to form a strong stem-loop structure. It is assumed that stem-loop structures also form within the nucleocapsids and probably play a role in terminating the mRNAs.

Transcriptional requirement for full length mRNA synthesis

In all negative-strand RNA virus infected cells, addition of drugs which inhibit protein synthesis, such as cycloheximide and puromycin, also blocks genome replication, and has been shown for members of Bunyaviridae family (Patterson and Kolakofsky, 1984). A difference between other negative-strand viruses and the Bunyaviridae is that inhibition of protein synthesis also stops mRNA synthesis in many mammalian cell types. This was observed in BHK cells for BUN (Abraham and Pattnaik; and Abraham, 1983), LAC (Raju and Kolakofsky, 1986), GER (Gerbaud *et al*, 1987). Contradictory results, i.e. lack of translational requirement, were reported for SSH (Vezza *et al*, 1979; Eshita *et al*, 1985). Resolution of this issue has not been helped by observations in Kolakofsky's laboratory which indicated that SSH has the same translational requirement as LAC, at least in their line of BHK cells (unpublished data, cited in Kolakofsky and Hacker, 1991). Earlier, Gerbaud *et al* (1987a) reported that full-length S transcripts of GER

could be obtained in an *in vitro* transcription system, although the synthesis of transcripts was inhibited in cell culture by anisomycin or cycloheximide. It was also reported that there was hardly any translational requirement for LAC mRNA synthesis in C6/36 mosquito cells (Raju *et al*, 1989). It seems that the translational requirement is cell type dependent.

Detergent disrupted bunyavirus preparations can synthesize RNA *in vitro*, as reported by Bouloy and Hannoun (1976), Gerbaud *et al* (1987), Patterson *et al* (1984), Ranki and Patterson (1975) and Schmaljohn and Dalrymple (1983), which is paradoxical for translational requirement. However, detailed examination of LAC transcripts made *in vitro* showed that almost all of the S RNAs were found to have terminated prematurely at nucleotide 175; addition of protein synthesis inhibitors did not change the situation. Full-length S mRNA, terminating at nucleotide 886, could be obtained only when rabbit reticulocyte lysate, active in translation, was added to the transcription mixture. Examination of the sequence at position 175 and the mature mRNA termination site at position 886 suggested that the sequence 5'YAAAAAT(A)GCAG3' is involved in transcription termination (Raju and Kolakofsky, 1987).

It was concluded that addition of cycloheximide to the coupled transcription/translation system had no effect on mRNA transcripts and the effect of rabbit reticulocyte lysates in elongation of the chain was to prevent the polymerase from terminating prematurely. Bellocq and Kolakofsky (1987) showed that the polymerase could be induced to read through the termination site at nucleotide 886 even in the absence of ongoing protein synthesis. This was done by progressively replacing the guanosines of the nascent chain with inosine. It was revealed during these studies that position 175 was only the first and probably the strongest of several sites where the polymerase would terminate prematurely in the absence of

translation. When the uridines were replaced with bromo-uridine, an opposite effect was obtained, i.e. there was no premature termination of the chain and this suggested that premature termination was due to base pairing of the nascent chain and the template and that concurrent translation of the nascent chain was required to break these interactions. In the presence of concomitant protein synthesis, ribosomes moving along the nascent mRNA behind the polymerase prevent the mRNA from hybridising to its template and hence the polymerase reads through (Bellocq and Kolakofsky, 1987; Kolakofsky *et al*, 1987). This theory, however, does not explain the *in vitro* full-length S mRNA synthesis of GER in the absence of active rabbit reticulocyte lysates, reported by Gerbaud *et al* (1987).

For the ambisense S RNA of phlebovirus, four types of S segment-specific RNA were identified in cells infected with PT; these were genomic RNA, full length viral complementary RNA, N mRNA and NSs mRNA. In the presence of protein inhibitors only newly synthesised N mRNA was detected, and could be translated *in vitro* to yield the N protein (Ihara *et al*, 1985a). This result showed that NSs mRNA requires the onset of RNA replication to yield the full length viral complementary RNA which then acts as a template for NSs mRNA synthesis. Also, the synthesis of NSs is regulated independently to that of N mRNA, showing that the NSs protein is not involved in the early stages of viral complementary RNA synthesis (Bishop, 1986).

There appears to be no ongoing protein synthesis requirement for mRNA synthesis in virus infected mosquito cells, as the case is for BHK cells. To examine whether the difference is due to a host protein or a host modification of a viral component, reconstitution experiments were carried out. Addition of uninfected mosquito cell extracts of BHK genomes in an *in vitro* system had no effect but addition of uninfected BHK extracts to mosquito cell genomes both stimulated mRNA initiation and recreated the

translational requirement for efficient read-through of nucleotide 175 (Raju *et al*, 1989). This suggested that the translational requirement was due to a factor(s) present in BHK, but not in C6/36, cells. When, during the same experiments, BHK-derived genomes of LAC were washed by pelleting through sucrose cushions, the translational requirement was abolished and the polymerase was able to read through nucleotide 175. The translational requirement therefore seems to be due to a host component.

AIMS OF THE PROJECT

1 To express BUN M RNA from a cloned cDNA.

2 To clone and express individual gene products of BUN M RNA.

3 To characterise the translocation of the G1 and G2 glycoproteins and NSm.

4 To determine the cellular localization of NSm.

Experiments done after completion of this thesis by Dr R.M. Elliott showed that BUN G2 aggregates on heating (see Figures 6.1 and 6.2), thus explaining the failure to detect G2 during the course of my work. This does not invalidate any of the experiments or conclusions reported here.

MATERIALS AND METHODS

MATERIALS

Cells

BHK-21, clone 13 (BHK-c13)

BHK-c13 is a continuous cell line derived from baby hamster kidneys (MacPherson and Stoker, 1962). The cells were supplied by the Cytology Unit, Institute of Virology, Glasgow and maintained in the Glasgow modification of Eagle's Minimum Essential Medium (Stoker and MacPherson, 1961), supplemented with new born calf serum (10%), tryptose phosphate broth (10%), L-glutamine (5mM), penicillin (100U/ml) and streptomycin (100µg/ml).

CV-1 cells

CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin.

TK⁻ 143 cells

TK⁻ 143, human thymidine kinase deficient (TK⁻) cells (Rhim *et al*, 1975) were maintained in DMEM, supplemented with 5% foetal calf serum and 25µg/ml 5-bromodeoxyuridine.

HeLa T4⁺ cells

HeLa T4⁺ cells were obtained from the MRC AIDS Directed Reagent Project, repository reference number: ADP019. The cells were maintained in DMEM, supplemented with 10% foetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin. HeLa T4⁺ cells contain the CD4 gene and support infection by HIV and induction of syncytia (Maddon *et al*, 1986). The cells are relatively resistant to the cytopathic effects of vaccinia virus (Matsuoka *et al*, 1988).

Viruses

Bunyamwera virus, obtained originally as a suckling mouse brain homogenate from Dr N Karobatsos, Vector-borne Diseases Laboratory, Centers for Disease Control, Fort Collins, Colorado, USA, was cloned by three sequential plaque isolations from single plaques on BSC-1 (African Green Monkey Kidney) cell monolayers by Dr C R Pringle, formerly of the Institute of Virology, Glasgow (Watret *et al*, 1985).

Vaccinia virus, WR strain, was obtained from Dr A Patel of the Institute of Virology, Glasgow and the recombinant vaccinia virus vTF7-3, expressing bacteriophage T7 RNA polymerase (Fuerst *et al*, 1986) was obtained from Dr B Moss (National Institutes of Health, USA).

Antiserum

Antiserum to purified Bunyamwera virus was produced by intramuscular injection of rabbits with viral protein in complete Freund's adjuvant at weekly intervals for three weeks, followed by a boost four weeks later and bled two weeks after the final inoculation (Watret *et al*, 1985).

Enzymes

The following endonucleases were used: PstI, Bam HI, Hind III, EcoRI, SalI, SmaI, PvuII, NcoI, BglII, HpaI, XbaI, BclI, DraI, HaeII, HaeIII. All were products of Bethesda Research Laboratories, Boehringer Mannhein, or New England Biolabs.

Other enzymes used were T3 RNA polymerase, T7 RNA polymerase, T7 DNA polymerase (Pharmacia); DNA polymerase I, T4 DNA ligase (Bethesda Research Labs); calf

alkaline intestinal phosphatase (Boehringer Mannhein); trypsin (Gibco Ltd); DNase I, RNase A (Promega Corp); lysozyme (Sigma).

Plasmids

The cloning and transcription vectors used were pTZ18U, pTZ19U, pT7T318U, pT7T319U, and were from Pharmacia LKB Biotechnology. Vaccinia virus transfer vector pTF7.5 was obtained from Dr B Moss. The pUEX vector was obtained from Dr D P Leader (Department of Biochemistry, University of Glasgow).

The full length M segment cDNA (pBUN205) was cloned in pUC9 by Dr R M Elliott (Institute of Virology, Glasgow). The recombinant plasmid, pTZBUNS, carrying the S segment cDNA in pTZ18R under control of the T7 promoter was constructed by A McGregor (Institute of Virology, Glasgow).

Bacterial strains and culture media

The bacterial strains used in transformation experiments and in the propagation of vector and recombinant plasmids were *E.coli* DH5α (F^- ø8odlacZ M15, recA1, endA1, gyr A96, thi-1, hsdR17 (r^-k, m_k+), supE44, relA1, deoR, (lacZYA-argF) U169 and DH5 (F,- end A1, hsdR17 (r^-k, m_k+), supE44, thi-1, Γ^-, recA1, gyrA96, relA1. Ecoli JM83, 4 (F,-, ara, (lac-proAB), rpsL, ø8odlacZ M15 was used in the growth of pBUN205. These were products of BRL. *E.coli* HMS174 (recA1 hsd R rif^r) and BL21 (F^- hsdS gal) were hosts for the expression of NSm fusion proteins using the pUEX3 vector and were obtained from Dr F W Studier (Brookhaven National Laboratory, USA).

The bacteria were grown and maintained in either: L-broth (LB): 10g NaCl, 10g bactopeptone, 15g yeast extract per litre; or 2YT broth: 5g NaCl, 16g bactopeptone, 10g yeast extract per litre. L-broth agar (for plates) consisted of L-broth plus 1.5% (W/V) agar.

Chemicals and Reagents

Chemicals and reagents were products of BDH, Poole, England, except:

Absolute ethanol, Hayman Ltd, formerly James Burroughs.
Dimethyl sulphoxide, Koch-light Labs.
Caesium chloride, Melford Laboratories Ltd.
Ammonium persulphate - electrophoresis purity reagent, Biorad Laboratories, Richmond CA, USA.
TEMED (N, N, N', N'-tetramethylethylenediamine), electrophoresis purity reagent, Biorad Laboratories, Richmond CA, USA.
Agarose (genetic technology grade), FMC Bioproducts, Rockland, USA.
Goat anti-rabbit IgG fluorescein isothiocyanate conjugate, Sigma Chemicals Ltd.
Tunicamycin, Sigma Chemicals Ltd.
Agarose (low gelling temperature), Miles Labs.
Glacial acetic acid, May and Baker.
EN³hance, DuPont Ltd.
Coomasie Brilliant Blue
HindIII digested Γ DNA, Pharmacia.
Chloroform, May and Baker, Ltd.
Ampicillin (Penbrittin), Beecham Research Labs Ltd.
Methyl mercuric hydroxide, Lancaster Synthesis Ltd, Morecambe, England.
ABTS (2, 2'-Azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid), Diammonium salt, Sigma Chemical Co Ltd.
Brefeldin A (δ-4-dihydroxy-2-[6-hydroxy-1-heptenyl]-4-cyclopentanecrotonic acid Γ-lactone), Sigma Chemical Co Ltd.
Glycerol, May and Baker.
Formamide, Fluka Chemicals Ltd.
Staphylococcus aureus (10%W/V), formalin fixed, Bethesda Research Laboratories.
Sephadex G-50, Pharmacia LKB Ltd.

Amberlite MB-1 (mixed bed resin ion exchanger), Sigma Chemical Co Ltd.

Ribonucleoside 5'-triphosphates, dNTPs, 'Universal' and T7 promoter sequencing primers, methyl diguanosine triphosphate sodium salt (m^7GpppG), Pharmacia LKB Ltd.

Wheat germ agglutinin, Sigma Chemical Co Ltd.

Wheat germ agglutinin, Rhodamine isothiocyanate conjugate, Sigma Chemical Co Ltd.

RNasin, Promega Corp.

Donkey anti-rabbit IgG peroxidase, Sigma Chemical Co Ltd.

Radiochemicals

Radiochemicals were supplied by Amersham International plc, except for Tran^{35}S-label, which was obtained from ICN Biochemicals, Inc.

^{35}S-L-methionine	*about 800mCi/mmole*
Tran^{35}S-label (70% ^{35}S-L-Methionine, 15% ^{35}S-L-Cysteines	*approximately 1,100/mmole*
α-^{32}P-dATP	*3,000 Ci/mmole*
α-^{32}P-dCTP	*3,000 Ci/mmole*
α-^{32}P-dGTP	*3,000 Ci/mmole*
α-^{32}P-dTTP	*3,000 Ci/mmole*

Other materials

Rabbit reticulocyte lysates, NEN Research Products.

Nitrocellulose BA85, Schleicher and Schuell.

Dialysis tubings, Medical International Ltd.

Tissue culture flasks and dishes, Gibco Ltd; Sterilin Ltd.

Water (sterile, distilled), Media lab, Institute of Virology, Glasgow.

Trypsin (0.25%), Media lab, Institute of Virology, Glasgow.

Photographic films, Kodak Ltd.

Microtitre plates for ELISA, Immulon-2, flat-bottomed, Dynatech Inc, Chantilly, Virginia, USA.

Repelcote, BDH.

Commonly used solutions

DNA preparation solution I (50mM glucose, 25mM TrisHCl pH. 8.0, 10mM EDTA).

DNA preparation solution II (0.2M NaOH, 1% SDS).

DNA preparation solution III (5M potassium acetate pH. 4.8), (3M with respect to potassium and 5M with respect to acetate).

PBS (170mM NaCl, 3.4mM KCl, 10mM K_2HPO4, 1.8mM KH_2PO4, 6.8mM $CaCl_2$, 4.9mM $MgCl_2$), supplied by the Media lab, Institute of Virology, Glasgow.

NTE (100mM NaCl, 10mM Tris-HCl p.H. 7.5, 1mM EDTA).

TE (10mM Tris HCl, pH 7.5, 1mM EDTA).

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

20 x SSC (3M NaCl, 3M trisodium citrate).

20 x SSPE (3M NaCl, 0.18M NaH_2PO4, 20mM EDTA, pH. 7.4).

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

50 x Denhart's solution (1% polyvinylpyrrolidone, 1% BSA Pentex fraction V, 1% Ficoll).

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

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

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

Carrier DNA (10mg/ml salmon sperm DNA).

5% milk (dried skimmed milk (w/v in water).

10 x Tris-glycine (0.25M Tris HCl, 2.5M glycine, 1% SDS, pH. 8.3).

RNase A solution (10mg/ml pancreatic RNase A in 10mM Tris HCl, pH. 7.5, 15mM NaCl), prepared by heating to 100°C for 15 minutes, aliquots stored at -20°C.

Sequencing gel mix (8% acrylamide/bisacrylamide 57:3, 1 x TBE).

Agarose gel loading buffer (0.25% bromophenol blue, 0.25% Xylene cyanol, 30% glycerol).

X-gal (20mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide).

METHODS

Growth of cells and viruses

Bunyamwera virus

$1x10^8$ BHK-21 cells were seeded in burrlers ($850cm^2$ growth area) and grown at 37°C in GMEM in a 5% carbon dioxide atmosphere.

Confluent monolayers of cells were infected with BUN at an m.o.i. of 0.01 pfu/cell in 5ml GMEM containing 5% NBCS and allowed to absorb for one hour at 31°C. A further 25ml of medium was added and incubation continued at 31°C for up to five days or until the cytopathic effect was extensive.

Similar conditions were employed for the growth of BUN and Maguari virus temperature sensitive mutants except that they were grown in $175cm^2$ flasks .

The culture fluids from virus infected cells were harvested and clarified at 3,000 rpm for 10 min at 4°C in 50ml Falcon tubes. The supernatant was aliquoted and stored at -70°C.

Titration of Bunyamwera virus stocks

Growth medium was removed from confluent monolayers of BHK-21 cells in 50mm petri dishes and ten-fold serially-diluted virus stocks in PBS containing 2% NBCS (200µl) was absorbed for one hour at 31°C. Overlay medium (Eagle's supplemented with 2% NBCS and containing 0.6% agarose) was added to each dish and the dishes were incubated at 31°C for four to five days. Cidex, containing 2% glutaraldehyde, was equilibrated with an equal volume of PBS and used to fix the monolayers for two hours or overnight. Plaques were counted after staining with Giemsa.

Vaccinia virus

Vaccinia viruses (wild type strain WR and recombinant vTF7-3) were grown and propagated in CV-1 cells, essentially by the method of Mackett *et al*, 1985. Infected cell monolayers, on showing extensive c.p.e., were scraped into the medium, pelleted by centrifugation at 3,000 r.p.m. for five min at 4°C and the pellet resuspended in 2ml of 10mM Tris-HCl, pH 9.0, for each 175cm² flask. The cell suspension was subjected to three cycles of freeze-thaw or ultrasonication until the cells were completely disrupted. The nuclei were removed by centrifugation at 750g for 5 min. The supernatant was incubated with 1/10 volume of trypsin (2.5mg/ml) at 37°C for 30 min with frequent vortexing. The resultant material was layered onto an equal volume of 36% sucrose (W/V) in 10mM Tris-HCl, pH 9.0 in a Sorvall AH 627 tube. The virus and some debris were pelletted at 14,000 r.p.m. for 80 min at 4°C and resuspended in Tris-HCl pH 9.0. These partially purified virus preparations were assayed by plaque titration and used in all experiments involving vaccinia virus.

Bacterial cell cultures

Seed stocks of bacteria were made from overnight cultures which were prepared by inoculating 10ml of 2YT medium with a single bacterial colony and grown at 37°C with shaking until saturation. An equal volume of 80% (V/V) glycerol was added and the aliquots kept at -20°C or -70°C.

Large scale preparation of plasmid DNA

The procedure was essentially that described by Maniatis *et al* (1982). 10ml 2YT containing the appropriate antibiotic (ampicillin 100µg/ml or tetracycline 10µg/ml) were inoculated with 10µl of bacterial seed culture and grown overnight with shaking. 5ml of this was inoculated

into a 2 litre conical flask containing 400ml of 2YT plus antibiotic. This was grown overnight at 37°C with shaking. The bacterial cells were pellet ed at 5,000 r.p.m. in a GS3 Sorvall rotor for 10 min at 4°C. The pellet was vortexed briefly to loosen it and then resuspended in 8ml of DNA preparation solution I containing 5mg/ml lysozyme. The mixture was transferred into an SS34 plastic centrifuge tube and left to stand at room temperature for 5 min. Eighteen ml of DNA preparation solution II was added, the tube covered by parafilm and mixed by gently inverting the tube a few times and left to stand on ice for 10 min. Twelve ml of ice cold potassium acetate pH 4.8 was added and the contents mixed by sharply inverting the tube a few times and placed on ice for another 10 min.

The contents were centrifuged at 18.000 r.p.m. for 20 min in SS34 Sorvall rotor at 4°C. The supernatant was transferred to 30ml Corex tubes and DNA was precipitated by addition of 0.6vol of isopropanol and left to stand at room temperature for 15 min. The DNA was recovered by centrifugation at 10,000g in SS34 Sorvall rotor for 20 min at room temperature. The pellet was washed with 70% ethanol, repelleted and dried briefly under vacuum.

Purification of plasmid DNA on caesium chloride gradient

The DNA pellet was dissolved in a total volume of 8ml of TE (pH 8.0) or water. 1g of solid caesium chloride was added for every ml and gently vortexed until the salt dissolved. Ethidium bromide 0.8ml (10mg/ml in H_2O) was added for every 10ml of caesium chloride solution. The solution was transferred to a heat sealable Beckman tube for Ti 50 or Ti 65 rotor. The contents were centrifuged at 45,000 r.p.m. for 36 hours at 15°C. Supercoiled DNA was harvested by a gauge 18 hypodermic needle inserted in the side of the tube. Ethidium bromide was removed by extracting with isoamyl alcohol equilibrated with 5M NaCl,

until the pink colour disappeared. The DNA solution was then dialysed against several changes of TE (pH 8.0) and recovered by ethanol precipitation.

Alternative large scale purification of plasmid DNA

A quicker procedure for the purification of plasmid DNA did not involve isopycnic centrifugation on caesium chloride gradients. After the addition of potassium acetate and harvesting of the supernatant, RNase A was added to the supernatant to a final concentration of 20µg/ml. The mixture was incubated at 37°C for 30 minutes and extracted twice with an equal volume of phenol/chloroform in 50ml Falcon tubes with a 1 minute vortex and 5 minute centrifugation between the extractions. The aqueous phase was transferred to Corex tubes and two volumes of absolute alcohol added. The contents were placed on dry ice for 10 min, centrifuged for 15 minutes at 10,000 r.p.m. in an SS34 Sorvall rotor and the pellet dissolved in 1.6ml H_2O. Then 0.4ml of 4M NaCl and 2ml 20% polythene glycol (PEG) was added and incubated on ice for one hour. The DNA was collected by centrifugation at 10,000 r.p.m. for 15 minutes and the pellet washed in 70% ethanol, recentrifuged for 5 minutes, dried and dissolved in 500µl H_2O.

Small scale preparation of DNA (minipreps)

Three ml of 2YT medium containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated overnight with shaking. About half of the bacterial suspension was transferred into an Eppendorf tube and the rest kept at 4°C. The contents in the Eppendorf tube were centrifuged at 13,000 r.p.m. for one minute and resuspended in solution I and let to stand at room temperature for 5 minutes. Freshly prepared solution II (200µl) was added and the contents mixed by inverting the tubes rapidly a few times and placed on ice for 5 minutes.

Then potassium acetate 150µl pH 4.8 was added, the cap of the tube closed and vortexed briefly. The mixture was stored on ice for 5 minutes and then centrifuged at 13,000 r.p.m. for 5 minutes. The supernatant was extracted once with phenol/chloroform and centrifuged for 2 minutes. The aqueous phase was transferred to a fresh tube, the DNA ethanol precipitated, washed in 70% ethanol, dried and dissolved in 50µl H_2O.

Quantitation of DNA

DNA was quantitated spectrophotometrically in quartz microcuvettes on a Cecil S95 double beam ultraviolet spectrophotometer at the wavelength of 260nm. It was assumed that an optical density of one corresponded to a DNA concentration of 50µg/ml.

Transformation of bacterial cells with plasmid DNA

Cells were made competent using the method of Dagert and Ehrlich (1979). An overnight culture was diluted 1:100 in 50ml 2YT broth and grown to mid Log phase (about two hours) at 37°C. The cells were chilled in an ice bath for 10 minutes and pelleted by centrifugation at 3,000 r.p.m. for seven and a half minutes in sterile 50ml Falcon tubes; the cells were resuspended in ice cold (100mM) calcium chloride. The cells were chilled in an ice bath for at least 20 minutes, pelleted as before and suspended in 3ml of cold 100mM calcium chloride.

Plasmid DNA [0.5µg in 50µl of TE or H_2O, or 10µl of ligation reaction] was mixed with 200µl of competent cells and incubated on ice for one hour. The tranformation mixture was heat-shocked at 42°C for two minutes and then chilled on ice for 5 minutes. 2YT medium (500µl) was added to the mixture, incubated at 37°C for one hour and then plated out on agar plates containing appropriate antibiotics. The plates were incubated at 37°C overnight.

Restriction endonuclease digestion

The optimal conditions for digestion were set up according to the manufacturers' data sheets. The amount of DNA digested was typically 1-2µg in the appropriate buffer and for about 2 hours at 37°C. Sometimes double digests, i.e. using two enzymes on the same DNA sample, were carried out.

Electrophoresis of DNA

Electrophoresis of DNA digested fragments was done in either non-denaturing or denaturing conditions in 1% or 0.8% agarose. The agarose was melted in electrophoresis buffer in a microwave, cooled and poured in preparative or analytical tanks (minigels).

Non-denaturing agarose gels

Electrophoresis was carried out on horizontal slab gels (minigels or preparative gels) in 1X TBE containing 0.5µg/ml ethidium bromide. DNA samples were mixed with 1/5th volume loading buffer and applied into preformed wells. Electrophoresis was performed at 70V for one hour in minigels and 150V for 4 hours or 30V overnight in preparative gels.

Denaturing agarose gels

Electrophoresis was carried out on horizontal slab gels. Agarose was melted in 1X EM buffer and cooled to about 60°C. 1M stock solution methylmercuric hydroxide was added to the melted agarose to a final concentration of 5mM. After the agar had solidified and the comb removed, the wells were filled with 1X EM buffer. An equal volume of RNA sample was mixed with 2X sample buffer and to each sample 1µl of 0.1M methyl mercury solution was added. Samples were loaded and run under nonsubmarine conditions at 60V.

The gel was soaked in 200ml of 0.05M ammonium sulphate and 20µl of ethidium bromide solution (10mg/ml in H_2O) added. The gel was kept in ethidium bromide solution for 20 minutes and then photographed under UV light.

Recovery of DNA from agarose gels

Two methods for the recovery of DNA from agarose gels were employed: electroelution and adsorption on a silica matrix.

Electroelution

After electrophoresis the gel was placed on "Clingfilm" and visualised by use of a long wavelength ultraviolet transilluminator. The agarose containing the DNA of interest was excised and transferred into a dialysis bag containing 0.5X TBE so that the TBE buffer just covered the gel. The ends of the dialysis tubing were sealed, the gel slice placed on one side of the tubing and electrophoresis carried out in 0.5 x TBE, which just covered the tubing, at 150V for 3 hours. The polarity was reversed for 2 minutes to release DNA from the wall of the dialysis tubing, the buffer was saved and the tubing was washed with a small quantity of buffer. The buffers were pooled and the DNA extracted with phenol/chloroform, ethanol precipitated, dried under vacuum and dissolved in 40µl H_2O.

Silica Matrix adsorption

The principles of the procedure are based on the data of Vogelstein and Gillespie (1979). The materials came in a commercial kit known as GENECLEAN II which contained silica particles in suspension and referred to by the manufacturer as GLASSMILK; sodium iodide, NEW WASH and TBE modifier were also supplied in the kit.

The DNA band was excised from the agarose gel in TBE buffer and transferred into an Eppendorf tube. The weight of the agarose slice was determined and 3 volumes of NaI and half volume of TBE modifier added. The contents were incubated at 45-55°C to dissolve the agarose. GLASSMILK was added to the solution at concentration of 5µl per 5µg of DNA. The mixture was incubated on ice for 5 minutes with occasional mixing and pelleted by centrifugation at 13,500 r.p.m. for 5 seconds. The pellet was washed three times with NEW WASH and the DNA eluted into 20µl of H_2O by incubating at 45-55°C for 5 minutes. The silica particles were pelleted, the supernatant containing DNA saved and kept at -20°C.

Visualisation and photography of DNA

Ethidium bromide intercalates with DNA enabling it to be visualised by fluorescence (Brunk and Simpson, 1977). Electrophoresis in agarose gels was performed in 1 x TBE containing ethidium bromide. Short wavelength illumination at 280nm was used to detect DNA in analytical gels and longwave illumination at 365nm was used for preparative gels. This was to minimise nicking and dimerization which occurs more frequently in the short wave region.

Gels were placed on a transilluminator and photographed on a Polaroid camera through a red filter.

DNA cloning

Cohesive end ligations

Plasmid vector DNA (0.01-0.05µg), which had been digested by the appropriate endonuclease and dephosphorylated with 1U calf alkaline intestinal phosphase, was ligated with DNA fragments (0.1µg) cut with the same endonucleases in a reaction mixture of 20µl. The mixture contained 0.1M Tris-HCl, pH 7.4, 50mM $MgCl_2$, 1mM

spermidine, 1mM ATP, 100µg/ml BSA. The mixture was incubated at room temperature overnight in the presence of 1U of T4 DNA ligase.

Blunt end ligations

DNA fragments with 5' overlapping cohesive ends were converted to blunt ends by using the polymerising activity of the Klenow fragment of *E.coli* DNA polymerase I. One microgram of fragments were mixed with 2mM dNTPs in NT buffer. Two units of the Klenow fragment of DNA polymerase I was added and incubated for 30 min at room temperature. The DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in 20µl H_2O.

Restriction fragments with protruding 3' ends were made blunt-ended by using the 3' exonuclease activity of bacteriophage T4 DNA polymerase. The reaction included 1µg of the DNA fragment, 2mM dNTPs, in T4 polymerase buffer (33mM Tris-OAc, pH 7.9, 66mM KOAc, 100mM MgOAC, 100mg/ml BSA, 0.5mM DTT).

Typically 4U of DNA ligase was used in the ligation mixes.

Dideoxynucleotide sequencing of plasmid DNA

The procedure was based on the dideoxynucleotide chain termination method described by Sanger *et al* (1977). The sequencing kit was supplied by Pharmacia LKB and contained the following components:

Components

'A' mix-Short & Long:	ddATP in solution with dATP, dCTP, dGTP and dTTP.
'C' mix-Short & Long:	ddCTP in solution with dATP, dCTP, dGTP and dTTP.
'G' mix-Short & Long:	ddGTP in solution with dATP, dCTP, dGTP and dTTP.
'T' mix-Short & Long:	ddTTP in solution with dATP, dCTP, dGTP and dTTP.
T7 DNA polymerase:	In buffered glycerol solution.
Enzyme dilution buffer:	A buffered solution containing glycerol, BSA and DTT.
Universal primer:	5'-d[GTAAAACGACGGCCAGT]-3' in aqueous solution, 0.80 µM (4.44 µg/ml).
Annealing buffer:	A buffered solution containing $MgCl_2$ and DTT.
Labelling mix:	dCTP, dGTP and dTTP in solution.
Stop solution:	Deionised formamide solution containing EDTA, xylene cyanol and bromophenol blue.

Preparation of template

Double stranded DNA template (2µg) was denatured by incubation with 0.2M NaOH in a volume of 10µl at room temperature for 10 minutes. Three µl of 3M NaOAc (pH 4.5) and 7µl of distilled H_2O were added. Absolute ethanol (60µl) was added to the mixture and incubated on dry ice for 15 minutes. The precipitated DNA was collected by centrifugation at 13,500 r.p.m. for 10 minutes, washed in 70% ethanol, recentrifuged for 10 minutes and dried briefly under vacuum. The pellet was redissolved in 10µl of distilled water and kept at -20°C until use.

The template (10µl), prepared as described above, was annealed to the Universal primer (0.8M) by mixing 2µl of primer, 2µl of annealing buffer (200mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl) and incubating the contents at 37°C for 20 minutes in an Eppendorf tube. The tube and contents were then left to stand at room temperature for at least 10 minutes.

Labelling reaction

To the tube containing the annealed template were added 3µl labelling mix (0.57µM dGTP, dCTP, dTTP), 10µCi α-^{32}P-dATP and 2µl T7 DNA polymerase (3 units). The components were mixed by gentle agitation, collected at the bottom of the microfuge tube by a brief centrifugation and incubated at room temperature for 5 minutes. Meanwhile, 2.5µl of sequencing mixes (150µM of the 4 dNTPs, 15µM one ddNTP) was dispensed into microfuge tubes pre-labelled 'A', 'C', 'G' and 'T'. The tubes were prewarmed at 37°C for 2 minutes. After the labelling reaction had proceeded for 5 minutes, 4.5µl of this reaction was transferred into each of the prewarmed tubes, using a fresh pipette tip for each transfer. The contents were mixed by gentle agitation collected on the bottom of the tubes and incubated at 37°C for 5 minutes. Stop solution (5µl) containing 95% formamide, 20mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF was added to each tube, mixed and the samples heated at 80°C on a heating block for 2 minutes just before loading.

Sequencing gels

The 8% sequencing mix was made by dissolving 288g urea, 45.6g acrylamide, 2.4g bisacrylamide in 500ml distilled water. Amberlite (20g) was added and the mixture stirred for 30 minutes. The solution was filtered through a Whatman 3MM filter, 60ml of 10 x TBE added, the solution adjusted to 600ml with deionised water and kept at 4°C.

The BRL Model S2 electrophoresis apparatus was used. Glass plates were silicon treated with 'Repelcote' (2% dimethyldichlorosilane in 1,1,1-trichloroethane) and assembled with 0.4mm spacers. The sides of the glass plates were sealed with Sylglas clear tape. Seventy ml of 8% sequencing mix were mixed with 800µl ammonium persulphate (10%) and 100µl TEMED and carefully poured between the two plates. The flat side of the sharkstooth comb was inserted to about 3mm below the shorter plate and polymerisation allowed to take place at room temperature. The comb was removed after polymerisation (as judged by the contents that remained in the beaker) the gel assembly secured on the electrophoretic apparatus, the sharkstooth comb put into place so that the teeth just made contact with the gel. 1X TBE was used as the running buffer and electrophoresis was carried out at 80 W after loading 2µl of each sequence reaction, until the bromophenol blue marker was about 1cm from the bottom of the gel. One of the glass plates was carefully removed and the gel put on Whatman 3mm filter paper as a support by gently pressing the paper on the gel and peeling it off the second glass plate. The gel was covered with "Clingfilm" and dried at 80°C under vacuum for about one hour. The "Clingfilm" was removed and the gel exposed to X-ray film overnight.

Extraction of total cellular DNA

The procedure used was that described by Mackett *et al* (1985). CV-1 monolayers, infected with putative BUN M segment cDNA containing vaccinia virus recombinants, were washed once with cold PBS, scraped into 1ml PBS and pelleted at 13,500 r.p.m. for one minute. The cells were resuspended in lysis buffer (50mM Tris-HCl, pH 7.8, 1mM EDTA and 30% sucrose, 1% SDS and 100mM 2-mercaptoethanol). After incubation on ice for 30°C, Proteinase K was added to a final concentration of 500µg/ml and digestion carried out at 37°C. Total nucleic acids were extracted with phenol saturated with 1M Tris-HCl pH 7.5. The aqueous phase was

removed and re-extracted with chloroform: isoamyl alcohol (24:1). One tenth volume of 5M NaCl and 2.5 vol ethanol were added and left to precipitate at -20°C overnight. The contents were centrifuged at 13,500 r.p.m. for 5 minutes, and dried under vacuum. The pellet was redissolved in 10mM Tris-HCl and digested at 37°C with 0.25µg/ml RNase A for 30 minutes, phenol/chloroform extracted, ethanol precipitated and dissolved in 20µl H_2O.

Southern transfer of DNA

Purified plasmid or total cellular extracted DNAs were digested with appropriate restriction enzymes and the fragments separated by electrophoresis in agarose gels in TBE. The gel was transferred to a baking tray and the DNA denatured by soaking the gel in several volumes of 1.5M NaCl and 0.5M NaOH for one hour at room temperature with constant shaking. The gel was neutralized by soaking in several volumes of 1M Tris-HCl (pH 8.0) and 1.5M NaCl for one hour at room temperature, again with constant shaking. A piece of Whatman 3MM paper was wrapped around a glass plate and placed in a large baking dish on a support. The dish was filled with 10 x SSC to almost one third, with wetting of the Whatman paper. Air bubbles in the 3MM paper were removed by smoothing out with a 10ml pipette.

The gel was placed on the damp 3MM paper such that the original underside was now uppermost. Nitrocellulose filter paper was cut using a scapel to about 1-2mm larger than the gel in both dimensions. The nitrocellulose paper was wetted by floating it on the surface of 2 x SSC until wet completely and then immersed in the same solution for 3 minutes. The wet nitrocellulose paper was placed on top of the gel, air bubbles removed between the gel and filter and two wet pieces of Whatman 3MM paper placed on top of the nitrocellulose paper. A stack of blotting paper (BRL)

was placed on the 3MM filter paper and a weight of 500g. Transfer of the DNA from the gel onto the nitrocellulose paper was allowed to proceed overnight.

The nitrocellulose paper was peeled off the gel and soaked in 6 x SSC at room temperature for 5 minutes. Excess fluid was allowed to drain off and the gel dried at room temperature on 3MM paper and then baked for two hours between two 3MM sheets at 80°C under vacuum.

Nick translation labelling of DNA probes

Plasmid DNA (200ng) in a reaction volume of 50µl made up of nick translation buffer (100mM Tris-HCl, pH 7.5, 10mM MgSO4, 1mM DDT, 50µg/ml BSA), 10µCi each α-^{32}P-dATP, α-^{32}P-dCTP, α-^{32}P-dGTP and α-^{32}P-dTTP, 2mM of each of the four unlabelled dNTPs, one unit of DNA polymerase I, and two units DNase I was incubated for one hour at room temperature. The nick-translated DNA was separated from the unincorporated dNTPs by gel filteration chromatography in a Sephadex G-50 column made in a 1-ml syringe plugged with siliconised glass wool.

Orange G dye was added to the reaction mixture, which was applied to the column and eluted with distilled water. The eluted DNA was collected in a microfuge tube and collection was stopped just before the orange G marker reached the bottom of the column. The probes were heated at 100°C for 2 minutes before hybridization.

Hybridization of radiolabelled probes to DNA immobilised on nitrocellular filters

The baked nitrocellulose filter was placed in a heat-sealable plastic bag to which a pre-hybridization mixture (5 x SSPE, 50% formamide, 0.5% skimmed milk, 0.2mg/ml carrier DNA [salmon sperm DNA]) in a 20ml volume was added. The bag was heat-sealed and the contents prehybridized at

42°C for 4 hours in a flat bottomed plastic box. The nitrocellulose paper was transferred to a fresh heat-sealable bag and a reaction mixture similar in composition to the pre-hybridization mixture was added. The nick-translation-labelled plasmid DNA probe was added, the bag sealed and hybridized at 42°C with shaking overnight.

After removal of the filter from the hybridization bag, it was submerged in 2 x SSC and 0.5% SDS, and washed with agitation for 30 minutes at room temperature. The filter was then transferred to another tray containing 0.1% SSC and 0.1% SDS and washed for a further 30 minutes. The filter was dried on a sheet of Whatman 3MM filter, placed on a support, wrapped in "Clingfilm" and exposed to X-ray film at -70°C.

DNA dot blot hybridization

Cells from a 24 well Limbro plate were lysed with 100µl lysis solution (0.5M NaOH, 1% SDS) at room temperature for 20 minutes. To the lysed cells, 50µl of neutralization buffer (1M NaCl, 0.3M Na citrate, 0.5M Tris-HCl pH 8.0, 1M HCl) was added and the mixture was further diluted with the addition of 100µl of distilled water. One hundred and twenty microlitres of each sample was applied onto nitrocellulose filter paper (Schleicher and Schuell, 0.2µm pore size) using a Schleicher and Schuell Minifold II and vacuum. The nitrocellulose filter was baked at 80°C for 2 hours under vacuum and then hybridized with nick-translated DNA probes.

Radiolabelling of intracellular proteins

Virus infected or uninfected cell monolayers in 35mm dishes were radiolabelled with ^{35}S-methionine or ^{35}S-methionine/cysteine (100µCi/ml) in 1ml PBS for two hours, or in Eagle's medium minus methionine for 4 hours at 37°C or at 31°C. The fluid containing the radioactive

material was removed and the monolayers washed with ice cold PBS. The cells were dissociated in a protein dissociation mix (0.125M Tris-HCl, pH 6.8, 4% SDS, 10% 2-merceptoethanol, 20% glycerol, 0.1% bromophenol blue). The contents on the plate were scraped with the plunger of a 1ml syringe and transferred into microfuge tubes, boiled for 5 minutes and kept at -20°C.

Radioimmunoprecipitation

Infected or uninfected cells were labelled as described above and after washing with cold PBS, the cells were scraped into PBS. One tenth was used to prepare total cell lysates as described above and the remaining nine tenths were processed for immunoprecipitation by pelleting the cells for 1 minute at 13,500 r.p.m. and lysing the cells in 800µl of lysis buffer (0.05M Tris-HCl pH 8.0, 0.15M NaCl, 0.5% Nonidet-P40). The contents were vortexed briefly and incubated on ice for 30 minutes with occasional vortexing. The nuclei and other debris were removed by centrifugation at 13,500 r.p.m. for 5 minutes. The supernatant was transferred to a fresh tube, incubated with pre-immune sera for 4 hours at 4°C on a turning wheel mixer and precleared by incubation with 50µl of 10% formalin-fixed *Staphylococcus aureus* for 30 minutes on the wheel mixer at 4°C. The bacteria were removed by centrifugation in a microfuge for 1 minute and aliquots of immune sera added and incubated at 4°C overnight. One hundred microlitres of *Staphylococcus aureus* suspension was added to each sample and incubation continued for another 30 minutes. The immune complexes were collected by centrifugation for one minute and washed alternately with TBS, TBS with 2.5M KCl, and two final washes of TBS + 0.1% SDS; and then resuspended in protein dissociation mix, boiled for 5 minutes and stored at -20°C.

SDS-polyacrylamide gel electrophoresis of proteins

Fractionation of proteins was carried out on polyacrylamide gels in the presence of 0.1% SDS using the discontinous buffer system described by Laemmli (1970). Resolving gels contained 10, 12 or 15% acrylamide crosslinked with N', N'-methylene bisacrylamide in the ratio of 75:1 in resolving gel buffer (0.375M Tris-HCl, pH 8.8, 0.1% SDS). For polymerisation ammonium persulphate was added to a final concentration of 0.1% (V/V), and TEMED was added to a final concentration of 0.01% (V/V). After pouring the gel between the sealed glass plates, it was overlaid with butanol to form a flat surface and exclude air. When the gel had polymerized the butanol was removed and the gel top was washed with deionised water. The stacking gel solution containing 3.8% polyacrylamide, 62.5mM Tris-HCl, pH 6.8 and 0.1% SDS was layered on the resolving gel after addition of ammonium persulphate and TEMED, and a well-former comb put into place. After polymerisation of the stacking gel, wells were washed out with deionised water to remove any unpolymerised acrylamide. The gel-glass plate assembly was secured on the electrophoretic tank, running buffer (53mM Tris-HCl 53mM glycine, 0.1% SDS) was put in the tank reservoirs and electrophoresis performed at 35mA for the small tanks or 60mA for the large tanks until the bromophenol blue tracking dye was a few milimetres from the bottom of the gel.

Gels were removed from the glass plates, immersed in fixer (7% acetic acid, 20% methanol in water) for 30-60 minutes, stained with 0.2% Coomassie brilliant blue for 30 minutes and destained in gel fixer or processed for fluorography. For the latter the gel was soaked in En^{3}Hance (Dupont) for 30-60 minutes and washed for 30 minutes against several changes of water. The gels were

dried down under vacuum using Bio-Rad gel driers and exposed to Kodak-X-Omat film at -70°C overnight or until the protein bands were satisfactorily intense.

Western blotting

Infected and uninfected cell proteins were dissociated in protein dissociation mix, boiled and fractionated on an SDS-polyacrylamide gel and transferred to a 0.45µm nitrocellulose filter by electroblotting using an LKB 2117-250 NovaBlot electrophoretic transfer system. Electroblotting was carried out at a current of 0.8mA/cm² of gel in a continuous buffer system (39mM glycine, 48mM Tris-HCl, 0.0375% SDS (W/V) 20% methanol (V/V) in H_2O). This solution was used both for the anode and the cathode. The assembly of the unit and filter stacks was done as described by the manufacturer.

After electroblotting the filter was air dried, cut into strips and excess protein sites blocked by submerging the membrane into TBST buffer (10mM Tris-HCl, pH 8.0, containing 150mM NaCl, 0.05% Tween 20, 1% BSA) for 30 minutes at room temperature with shaking. The blocking solution was replaced with TBST containing the appropriate dilution of primary antibody and incubated for 30 minutes. The unbound antibody was removed by washing three times in TBST, 5 minutes per wash. The TBST was replaced with anti-IgG horseradish peroxidase conjugate (1:2,500 dilution in the same buffer) for 30 minutes and the membrane washed 3 times again in the same buffer. The membrane was dried on filter paper and transferred to a solution containing the peroxidase substrate (4-chloro-1-naphthol). This was prepared by diluting stock solution (100mg/ml in methanol): 50µl of the stock was added to 2ml of methanol and brought to 10ml with TBS (10mM Tris-HCl pH 8.0, 150mM NaCl). To this was added 35µl of a 30% solution hydrogen peroxide.

The colour reaction was stopped by rinsing the filter with deionised water for several minutes. The membrane was air dried on filter paper and photographed.

In vitro transcription and translation of recombinant plasmids.

Transcription

The transcription vectors pTZ18U or 19U and pT7T318U or 19U or pTF7-5 were employed for transcription under the control of either the T3 or T7 RNA promoters. Run-off transcripts were synthesized using T3 RNA polymerase or T7 RNA polymerase in the presence or absence of cap analogues after linearizing with an appropriate restriction endonuclease.

The transcription reaction was carried out in a volume of 50µl containing transcription buffer (40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl), 10mM DTT, 0.5mM rNTPs (ATP, CTP, GTP, UTP), 40U RNasin, 20U T7 RNA polymerase. To make capped RNAs 0.5mM m^7GpppG was also added. The reaction was incubated for one hour and 45 minutes at 37°C. 1U of DNase I was then added and the reaction continued for another 15 minutes. RNA transcripts were extracted with phenol/chloroform, ethanol precipitated, dissolved in 10µl H_2O, and stored at -70°C until used.

Translation in vitro

In vitro translation was performed using the New England Nuclear (Dupont) rabbit reticulocyte system. A premix was made containing 5µl ^{35}S-methionine, 5.5µl translation cocktail, 2µl 1M potassium acetate and 0.5µl 32.5mM magnesium acetate. One microgram RNA in 2µl H_2O and 10µl lysate were added to the premix and incubated at 37°C for 90 minutes. Twenty five microlitres of protein

dissociation mix was added and the mixture boiled for five minutes and loaded on gels for SDS-polyacrylamide electrophoresis.

Construction of recombinant vaccinia viruses

The vaccinia virus transfer vector pTF7-5 (Fuerst *et al*, 1987) was used to make recombinant viruses. The plasmid contains the T7 (ø10) promoter and terminator which are flanked by thymidine kinase (TK) sequences of vaccinia virus to enable homologous recombination. The promoter and terminator are joined by a synthetic linker (CGGGATCCGG) which generates a unique BamHI site in the plasmid. The cDNAs of interest were cloned into the plasmid using this unique BamHI site.

The protocol used for generation of recombinant viruses was that described by Mackett *et al* (1985). Monolayers of CV-1 cells in 35mm dishes were infected with vaccinia virus (WR strain) at a m.o.i. of 0.05 pfu/cell and absorbed at 37°C for one hour. The virus inoculum was removed and the monolayer washed twice with serum free medium. One microgram of plasmid DNA was mixed with 200µl of HEPES buffered saline (140mM NaCl, 5mM KCl, 0.75mM NaHP40, 6mM dextrose, 25mM Hepes pH 7.05). The DNA was precipitated by the addition of calcium chloride to a final concentration of 125mM and incubated at room temperature for 30 minutes. The DNA precipitate was added to the infected monolayers and incubation continued for another 30 minutes at room temperature. Two millilitres DMEM containing 5% FCS was added to the dishes and incubated at 37°C for 3½ hours. The medium was changed and incubation continued for 48 hours at which time cells were scraped into the medium and the virus released by freeze-thawing three times.

The selection of TK$^-$ recombinants was done by growing the progeny virus from the transfection in TK$^-$143 cells

overlaid with 1% low gelling temperature agarose containing 25µg/ml BUdR at 37°C until plaques were visible when stained with neutral red. The plaques were picked using a pasteur pipette and put in 200µl of DMEM containing 5% FCS. The mixture was freeze-thawed a few times on dry ice/37°C with some vortexing and half the material used to infect TK$^-$143 in 24 well Limbro plates and incubated at 37°C for 2-3 days. The medium was removed and cells processed for dot blot or Southern blot analysis. Positive samples were amplified by growing the remaining half of the transfection progeny in 25cm^2 flasks, virus was released by freeze thawing and grown in 175cm^2 flasks. Eight infected flasks were used to prepare virus stocks in CV-1 cells and purified as described above.

Transient expression of transfected plasmid DNAs in vTF7-3 infected cells

The procedure used is essentially that described by Rose *et al* (1991). CV-1 cells, grown to near confluency in 35mm dishes, were infected with vTF7-3, a recombinant vaccinia virus that encodes T7 RNA polymerase, at a m.o.i. of 10 pfu per cell at 37°C for 30 minutes in 0.5ml OptiMEM. Cationic liposomes were prepared (by Dr R M Elliott) by mixing one milligram of PtdEtn (dioleoylL-α-phosphatidylethanolamine) and 0.4mg DDAB (dimethyldioctadecyl ammonium bromide). The solution was evaporated to dryness in a SpeedVac concentrator, resuspended in 10ml sterile deionized water, sonicated until the solution became almost clear and kept at 4°C. Fifteen microlitres of liposomes were added to 1ml of OptiMEM followed by addition of 2.5µg of plasmid DNA. The mixture was added to infected cells and incubated at 37°C for 3 hours in a CO_2 incubator. At 3 hours post-transfection, 1ml of DMEM containing 5% FCS was added to the transfection mixture. At 4 hours post-transfection cells were processed for immunofluorescence or radioimmuno-precipitation analyses.

Indirect immunofluorescence

BHK-21 cells, CV-1 or HeLaT4$^+$ cells were seeded and grown to subconfluency on 18 x 18mm sterile coverslips in 35mm dishes at 37°C. The cells were infected with 5 pfu per cell in the case of BUN for one hour at 31°C or 10 pfu per cell with recombinant vaccinia viruses for 30 minutes at 37°C. The inocula were removed and growth medium added to the dishes. Cells were fixed at 8 hours post infection for vaccinia virus transient and dual expression systems and the following day for BUN infected cells.

The growth medium was removed from the dishes by aspiration using a pasteur pipette and the cells were washed once in PBS. The PBS was removed and the cells were fixed in 3.7% formaldehyde for 30 minutes at room temperature for the detection of surface antigens and in 3.7% formaldehyde followed by treatment for 30 min at room temperature with 0.5% NP40 for detection of internal antigens. The fixative was removed by washing twice in PBS (5 minutes per wash with shaking).

The primary antibody was diluted 1:100 in PBS and adsorbed on methanol-fixed cells overnight at 37°C (the same cells as those to be used in the experiments). The adsorbed antiserum was clarified by centrifugation at 10,000 rpm for 5 minutes. 50µl was layered onto the coverslips and incubated for 30 minutes at room temperature. The cells were washed in PBS four times, 5 min per wash with shaking. Goat antirabbit IgG fluorescein isothiocyanate conjugate diluted 1:100 in PBS, was spun at 13,500 rpm for 5 min and 50µl layered onto the coverslips. After incubation at room temperature for 30 min, the cells were washed four times as before. To stain the Golgi complex, wheat germ agglutinin rhodamine isothiocyanate conjugate, diluted 1:100 and spun at

13,500 rpm, was layered on the coverslips and incubated for another 30 minutes at room temperature, followed by washing four times in PBS.

The coverslips were immersed a few times in de-ionised water to remove as much PBS as possible and then mounted on microscope slides in CITIFLOR, an anti-fading agent (City University, London). The coverslips were sealed with nail varnish and the cells were examined and photographed on a Nikon Microphot-SA fluorescence microscope using the blue filter at a wavelength of 480nm for fluorescein and green filter at the wavelength of 546nm for rhodamine. Photographs were taken on Kodak Ektachrome 400 day film for colour slides and Kodak Plus-X Pan film for black and white prints.

Production of antiserum to the NSm protein

Two approaches were used to raise antibodies to the NSm protein. One was by synthesis of β-galactosidase fusion proteins and the other was by synthesis of branched peptides to be used as antigens.

β-galactosidase-NSm fusion proteins

The pUEX3 plasmid vector (Bressan and Stanley, 1987) was used in the cloning and expression of LacZ gene fusion proteins. This vector contains the bacteriophage Γ cro sequences, *E.coli* LacZ sequences and a CI857 thermolabile repressor gene.

The NSm protein is encoded from nucleotide 963 to 1485. A DNA fragment containing NSm sequences was obtained by digesting the BUN M segment cDNA with DraI which cuts at nucleotide 941 and HaeIII which cuts at nucleotide 1535. Both enzymes generate blunt end fragments. The NSm fragment was cloned into the SmaI site of pUEX3, as shown in Fig 3.1. Recombinant plasmids were analysed for

orientation of the insert by restriction enzyme digestion and plasmid DNA sequencing. Cultures of the correct recombinants were grown overnight at 30°C, then diluted 1:20 in 2YT containing ampicillin 100µg/ml and incubated at 30°C until the $O.D._{550}$ was about 0.2 (about 4 hours). The culture was then taken to a 42°C waterbath for 20 minutes and then incubated at 42°C for 2 hours on a shaker.

The bacterial cells were pelleted at 3,000 rpm in a bench centrifuge for 10 minutes and the pellet resuspended in 4ml lysozyme solution (50mM Tris-HCl, pH 8.0, 50mM EDTA, 15% sucrose, 0.1mg/ml lysozyme) and incubated on ice for 30 minutes. Six millilitres of detergent mix (1% Triton X-100, 0.5% deoxycholate, 0.1M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA) and kept on ice for 5 minutes. The mixture was pelleted by centrifugation at 10,000 rpm in SS34 rotor for 10 minutes, the pellet resuspended in 1.5ml 8M urea, 2% β-mercaptoethanol and an equal volume of protein dissociation mix added. The mixture was boiled for 10 minutes and the proteins were fractionated on 10% SDS-polyacrylamide gels, with commercially available β-galactosidase run on the same gels as a marker. The protein bands were stained with 0.05% Coomassie brilliant blue in de-ionised water for 10 minutes and destained in water for 30 minutes. The fusion band, which was larger than β-galactosidase, was excised from the gel with a clean scapel and the gel slice macerated.

The gel pieces containing the fusion protein was passed through a 10ml syringe a few times. A 18 gauge needle was attached to the syringe and the gel pieces were forced through the needle until they could pass through easily. An equal volume of sterile water was added and a mixture which contained about 100µg of protein injected subcutaneously into New Zealand white rabbits. The rabbits were boosted three times at 10 day intervals with the same

dose of protein and bled 10 days after the last boost. Sera were tested by radioimmunoprecipitation and enzyme-linked immunosorbent assays to branched particles.

Branched peptides

Two branched peptides, M245 and M246, spanning codons 349 to 364 and 417 to 430 respectively, were synthesised by A Owsianka, Institute of Virology, using continuous flow Fmoc chemistry (Atheron *et al*, 1979; Sheppard, 1983) using a Novabiochem peptide synthesizer. Peptides were synthesized directly onto a branched lysine core (Peptide and Protein Research, Reading, UK) to generate a peptide with eight identical branches (Possnett *et al*, 1988; Tam, 1988). The lysine core was synthesized onto an alanine residue coupled via an acid-labile bond to the resin using Fmoc-Lys (Fmoc) pentafluorophenyl ester.

After synthesis, side chain protecting groups were removed by treatment with 95% trifluoroacetic acid (5%) in water for peptide M245, and 95% trifluoroacetic acid (5%) in ethylmethylsulphide for peptide M246, which contained a methionine, using standard procedures. The amino acid compositions of the branched peptides were determined by amino acid analysis (Cambridge Research Biochemicals, Cambridge, UK) and were of the expected composition. The branched peptides are schematically illustrated in Fig 3.4.

Generation of antipeptide sera

The branched peptides were dissolved in water and emulsified in an equal volume of Freund's complete adjuvant (Gibco) for the first injection and in Freund's incomplete adjuvant for subsequent injections. New Zealand white rabbits were immunised on days 0, 10, 30 and 40 with 100µg or 300µg of peptides 245 and 246. There was a preterminal bleed on day 35 and final bleed on day 50.

Enzyme-linked immunosorbent assay

To determine the titres of antipeptide sera from immunized rabbits an ELISA was carried out in microtitre plates. The branched peptides were diluted in PBS to a concentration of 50µg per ml. Wells were precoated with 100µl of the peptide solution using Immunolon 2 (Dynatech) plates, overnight at 4°C. The peptide solution was removed and the unbound sites blocked with 1% BSA in PBS for one hour at 37°C. The plates were washed three times in PBS containing 0.02% Tween 20 (PBST) and incubated with 100µl in PBST for one hour at 37°C. The plates were washed 5 times in PBST and incubated with 100µl of donkey antirabbit IgG horseradish peroxidase diluted 1:4,000. The plates were washed another 5 times in PBST and reacted with a 0.5mg/ml solution of the substrate ABTS (2', 2-azino-bis (3-ethylbenzthazoline-6-sulphonic acid) in 0.1M citrate phosphate buffer, pH 4.0, containing 0.01% hydrogen peroxidase. The plates were incubated at room temperature for 30 minutes and read on a Titertek Multiscan plate reader at 405nm.

RESULTS

SECTION ONE

PRODUCTION OF ANTISERA TO THE NON-STRUCTURAL PROTEIN NSm

In addition to the two structural glycoproteins, G1 and G2, the middle RNA segment of BUN also encodes a non-structural protein termed NSm. The cellular residence and function of this protein are unknown. There was a need therefore to raise antisera specific for NSm, with the view of using these to uncover the intracellular localisation of NSm in BUN infected cells and in cells expressing NSm via recombinant vaccinia viruses. If NSm were identifiable in a given cell organelle/s this may help to delineate the role of NSm in viral morphogenesis. Another question to be looked into was whether NSm translocates by itself to these organelle/s or whether the presence of other BUN proteins is required.

Two approaches were designed: one involved synthesis of fusion proteins of NSm with β-galactosidase using the pUEX3 vectors, and the second was to synthesize branched peptides to selected sequences of the NSm protein. Rabbits would then be immunized with these in order to obtain antisera that reacted with NSm in a specific manner.

Construction of pUEX3/NSm plasmid

The sequences encompassing BUN NSm were predicted by extrapolation from those of SSH (Fazakerley *et al*, 1989; Elliott, 1990). The amino terminus is predicted to be at codon 300 (nucleotide 963) and the carboxy terminus at codon 477 (nucleotide 1485).

The pUEX3 vector, which contains the lacZ gene under control of the cro promoter, was linearised with SmaI and ligated with the NSm fragment such that the NSm gene was in

frame with the β-galactosidase coding sequence, as shown in Fig 3.1. *E.coli* strain DH5α cells were transformed with the ligation mixture and recombinant plasmids were analysed by restriction enzyme digestion with HpaI: this would yield three fragments if the insert was present and two fragments if there was only vector DNA. Out of 24 colonies picked, ten yielded plasmids containing the insert. Of these three had the insert in the correct orientation with respect to the LacZ gene as demonstrated by plasmid dideoxy DNA sequencing (fig 3.2) using a primer (GATTGGTGGCGACGACTCCT) complementary to nucleotides 4101-4121 of the pUEX3 vector (obtained from Dr Hong Jin, Institute of Virology).

Expression of the β-galactosidase-NSm fusion protein

Overnight cultures of *E.coli* cells carrying pUEX3/NSm recombinants were diluted 1:20 in 2YT medium and grown for four hours at 30°C. The cultures were then transferred to a 42°C shaker and incubated for two hours to induce synthesis of the fusion protein. As can be seen in Fig 3.3, lanes 1 to 3, no β-galactosidase or β-galactosidase-NSm fusion protein expression was detected when cells carrying pUEX3, or pUEX3/NSm fusion 1 and 2 were grown at 30°C. When the same cells were grown at 42°C, β-galactosidase was expressed, as shown in lane 4. In lanes 5 and 6 two new proteins, which were absent in the uninduced cultures, were seen: one of similar size to β-galactosidase and one larger, which had the expected size for the β-galactosidase/NSm fusion protein. The level of expression of β-galactosidase was much higher than that of the fusion protein. A commercial preparation of β-galactosidase protein was included as a marker in lane 7. The induced β-galactosidase was slightly larger than native β-galactosidase because pUEX3 makes a cro LacZ fusion protein.

Figure 3.1 Construction of pUEX3/NSm.

A BUN M cDNA fragment encoding all of NSm sequences was cleaved from pTF7-5M by digesting with DraI which cuts at nucleotide 941 and HaeIII which cuts at nucleotide 1535. The blunt ended fragment was ligated into the SmaI site of the β-galactosidase fusion protein vector pUEX3.

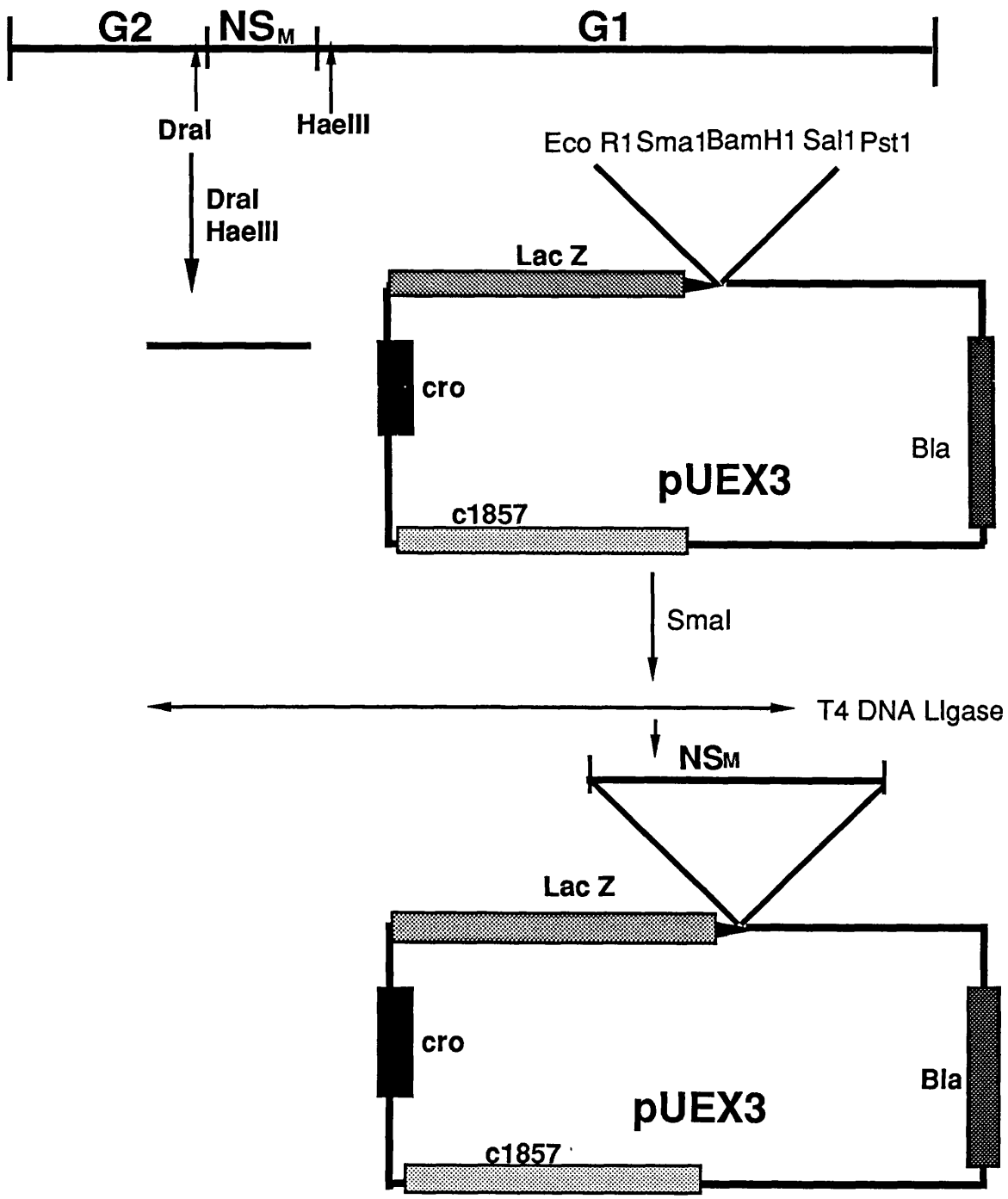

G2
NSM
G1
DraI
HaeIII
DraI
HaeIII
Eco R1 Sma1 BamH1 Sal1 Pst1
Lac Z
cro
Bla
pUEX3
c1857
SmaI
T4 DNA Ligase
NSM
Lac Z
cro
Bla
pUEX3
c1857

Figure 3.1

Figure 3.2 Determination of orientation of the NSm coding region in pUEX3/NSm.

Putative pUEX recombinants containing NSm sequences were sequenced using a primer complementary to nucleotide 4101-4121 of the pUEX3 vector. The sequence was determined by use of the T7 DNA polymerase, as described in the Methods section. Tracks are GATC from left to right. The SmaI/DraI fusion and BUN M gene nucleotides from position 942 are indicated.

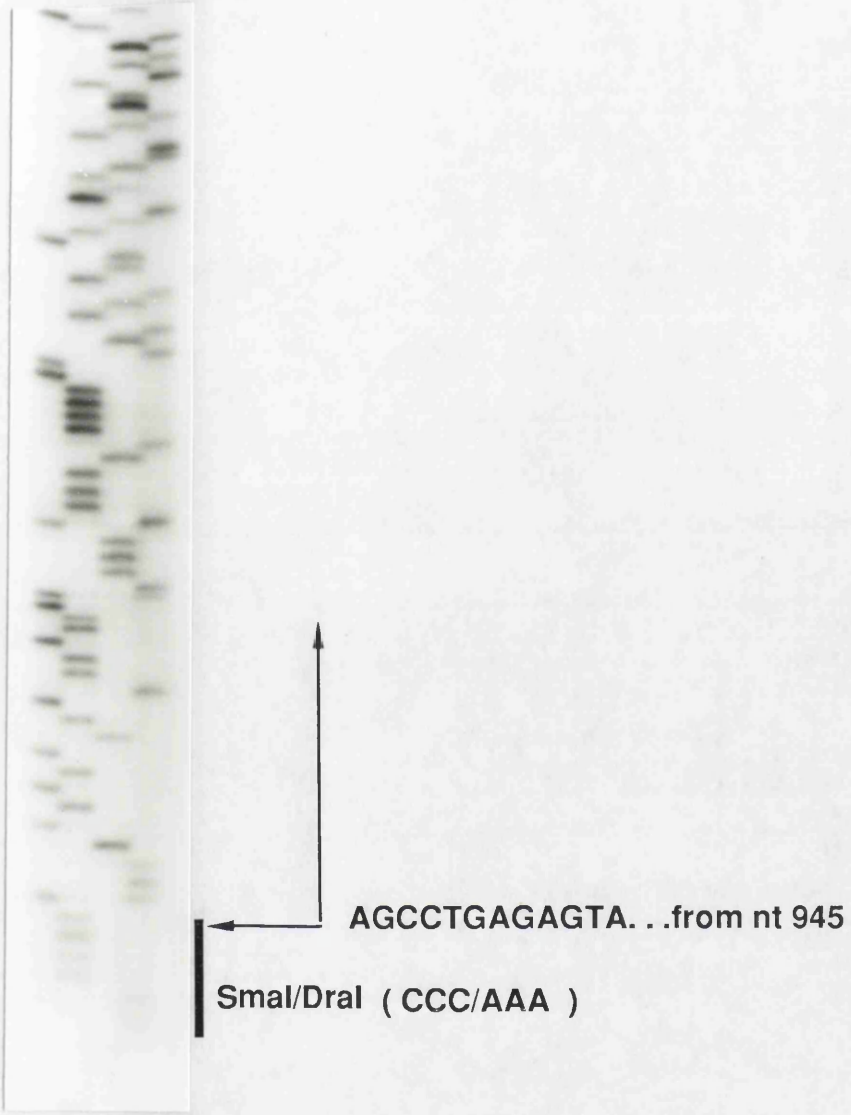


Figure 3.2

Figure 3.3 Expression of β-galactosidase/NSm fusion proteins.

E. coli DH5α cells were transformed with pUEX3 and pUEX3/NSm recombinant plasmids. Transformed cultures were maintained at 30°C and shifted to 42°C for 2 hr to induce synthesis of fusion proteins (see Methods). Controls were kept at 30°C. The cells from both the induced and the uninduced cultures were pelleted, dissolved in protein dissociation buffer and electrophoresed on a 10% polyacrylamide gel. The gel was then stained with Coomassie brilliant blue. Lane 1 represents DH5α cells transformed with pUEX3 and grown at 30°C. Lanes 2 and 3 represent cultures of recombinant pUEX3/NSm plasmids 1 and 2 at 30°C. Lane 4 is the induced pUEX3 transformed culture and expression of cro-β-galactosidase is evident. In lanes 5 and 6, which represent cultures transformed by recombinant pUEX3/NSm plasmids 1 and 2, after induction at 42°C respectively, two extra bands which are absent in uninduced cultures can be seen. The lower band is most likely cro-β-galactosidase; it is slightly larger than the commercial β-galactosidase marker shown in lane 7, because it is a cro-β-galactosidase fusion protein. The upper band is of the size expected for a fusion of cro-β-galactosidase and NSm.

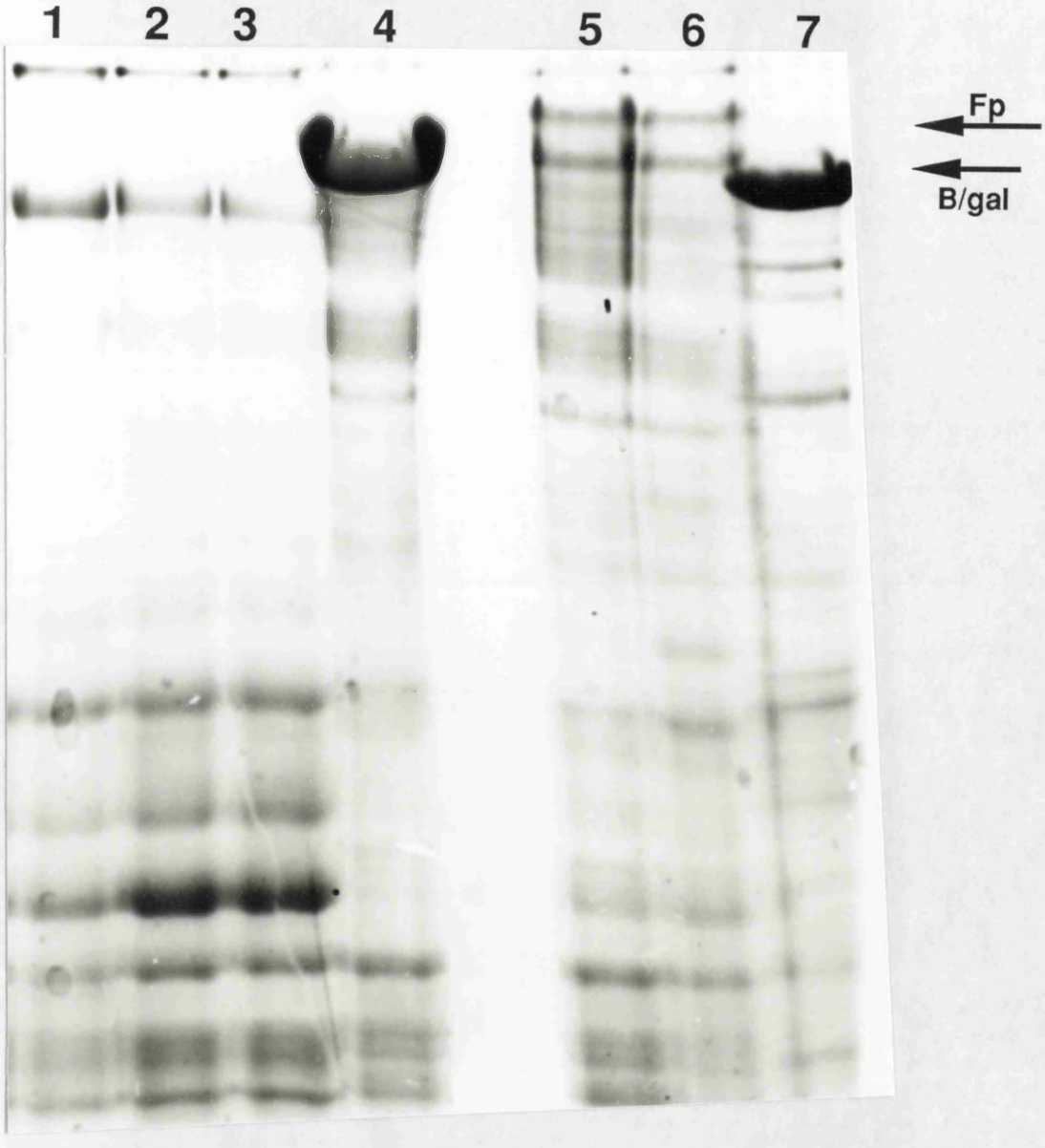


Figure 3.3

Synthesis of branched peptides to NSm

Two branched peptides, M245 and M246, were synthesised as described in the Methods Section. Branched peptide M245 and M246 corresponded to amino acids 349 to 364 and 417 to 430, respectively, and are shown schematically in Fig 3.4A.

The amino acid sequence for branched peptide M245 is:

$$(TDQKYTLDEIADVLQA)_8KA$$

and for M246:

$$(NMYHSKKSIKYVGD)_8KA$$

The branching tree of the peptides is shown in Fig 3.4B.

After synthesis, the branched peptides were analysed for their amino acid content and the results for branched peptide M245 is shown in Table 3.1A and peptide M246 in Table 3.1B.

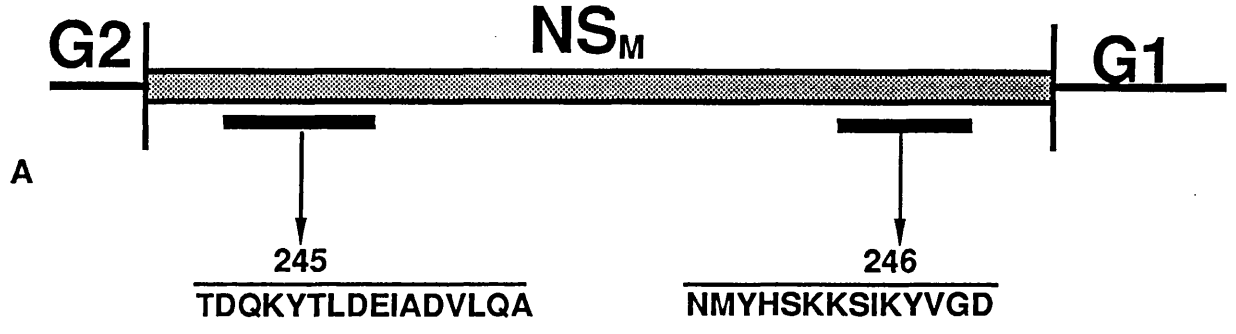
Characterisation of antisera to fusion proteins

Two New Zealand white rabbits codenamed 1/2 and 2/2 were immunized subcutaneously with macerated polyacrylamide gel slices containing the fusion proteins, as described in the Methods Section. After the final bleed, sera were characterized by radioimmunoprecipitation assay of BUN infected cell lysates and by an enzyme linked immunosorbent assay to the branched peptides from the NSm coding region. In radioimmunoprecipitation experiments the sera from rabbits inoculated with the fusion proteins of β-galactosidase/NSm failed to react with NSm in BUN infected cell lysates or in lysates of cells infected with recombinant vaccinia viruses containing the M segment cDNA (data not shown).

Figure 3.4 Branched peptides M245 and M246.

Branched peptides M245 and M246, spanning codons 349 to 364 and 417 to 430 of the BUN M segment ORF, respectively, and of amino acid sequences shown in Fig 3.4A were synthesised by the flow Fmoc chemistry. The peptides were synthesised onto a branched lysine core which was coupled onto an alanine residue generating eight identical branches, as shown in Fig 3.4B. X indicates the position of either peptide M245 or M246.

Branched Peptide Antisera to NS_M



Branched Peptide Tree

B

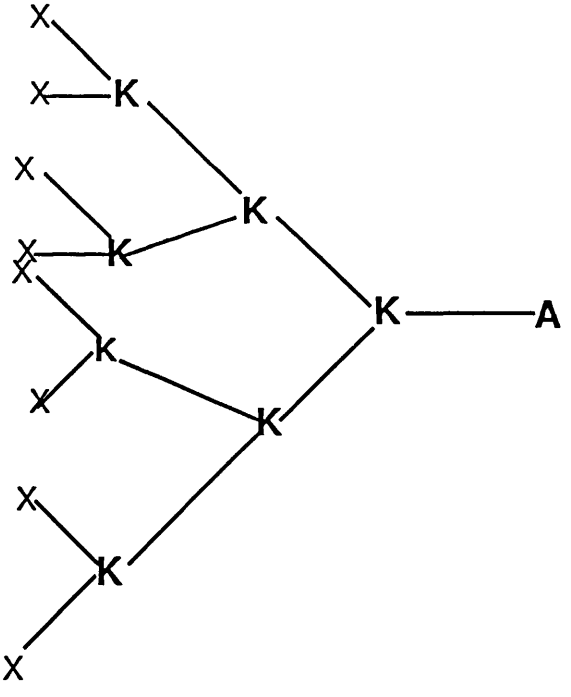


Figure 3.4

Table 3.1 Analysis of amino acid composition for branched peptide M245 (Table 3.1A) and M246 (Table 3.1B).

The analysis was carried out to determine the concentration of amino acids and also the ratio of the theoretical composition and the obtained composition.

Table 3.1A

AMINO ACID ANALYSIS OF PEPTIDE M245
$(TDQKYTLDEIADVLQA)_8K_7A$

	conc. nmoles/ml	ratio	expected ratio
ASX N	292.290	2.84	3
THR T	156.238	1.52	2
GLX Q	334.731	3.26	3
ALA A	249.068	2.42	2.13
VAL V	95.567	0.93	1
ILE I	97.492	0.95	1
TYR Y	98.016	0.95	1
LYS K	204.241	1.99	1.8
LEU L	220.126	2.14	2

Table 3.1B

AMINO ACID ANALYSIS OF PEPTIDE M246
$(NMYHSKKSIKYVGD)_8K_7A$

	conc. nmoles/ml	ratio	expected ratio
ASY N	146.770	2.05	2
SER S	121.805	1.70	2
GLY G	90.261	1.26	1
VAL V	84.981	1.19	1
MET M	69.226	0.97	1
ILE I	83.686	1.17	1
TYR Y	143.262	2.00	2
HIS H	72.865	1.01	1
LYS K	301.620	4.26	3.88

The two antisera were tested for their ability to recognise branched peptide M245 in an enzyme-linked immunosorbent assay. Antiserum 1/2 showed distinct difference between the pre-bleed and the terminal bleed sera (Fig 3.5A). Although the optical density values for antiserum 2/2 were high compared to those of 1/2, there was no difference between the pre-bleed and the terminal bleed (Fig 3.5B). The sera raised from fusion proteins were inferior in terms of specificity compared to those raised from branched peptides, as will be shown in the next section. The sera were not used for detection of NSm in cells.

Characterization of antisera to branched peptides

Branched peptide M245 was used to immunize three rabbits, designated 1/8, 2/8 and 3/8, at quantities of 100µg, 300µg and 1,000µg per inoculation, respectively. Branched peptide M246 was used to immunize rabbits designated 4/8 and 5/8 at 100µg and 300µg per inoculation, respectively. In all cases immunization was via the intramuscular route on the hind legs. The advantage of using branched peptides is that it is not necessary to couple the peptide to a carrier protein or other surface. The amounts of peptide were varied since the effective dose to generate antisera of good avidity and specificity was unknown.

After the terminal bleed blood was incubated at 37°C for 30 min and then at 4°C overnight. The serum was separated from the clot and aliquots were kept at 4°C for immediate use or at -20°C for long-term storage.

The antisera were characterized by their ability to react with NSm in BUN-infected cell lysates or in recombinant vaccinia virus (expressing the full length M

Figure 3.5 Enzyme-linked immunosorbent assay using antiserum to the β-gal/NSm fusion protein from rabbits 1/2 and 2/2.

Microtitre plates were coated with branched peptide M245, as described in the Methods section. The vertical axis represents the optical density values at 405 nm and the horizontal axis represents the Ln 2 dilution of double antiserum. The open and full-boxed graphs show the readings of pre-bleed and terminal bleed, respectively. Fig 3.5A represents readings of sera from rabbit 1/2 and Fig 3.5B represents readings of sera from rabbit 2/2.

Figure 3.5A

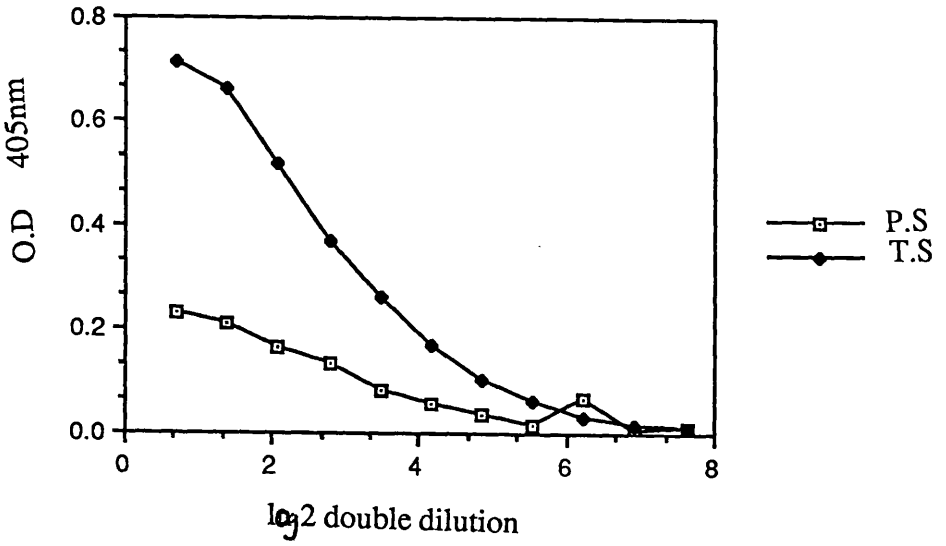


Figure 3.5B

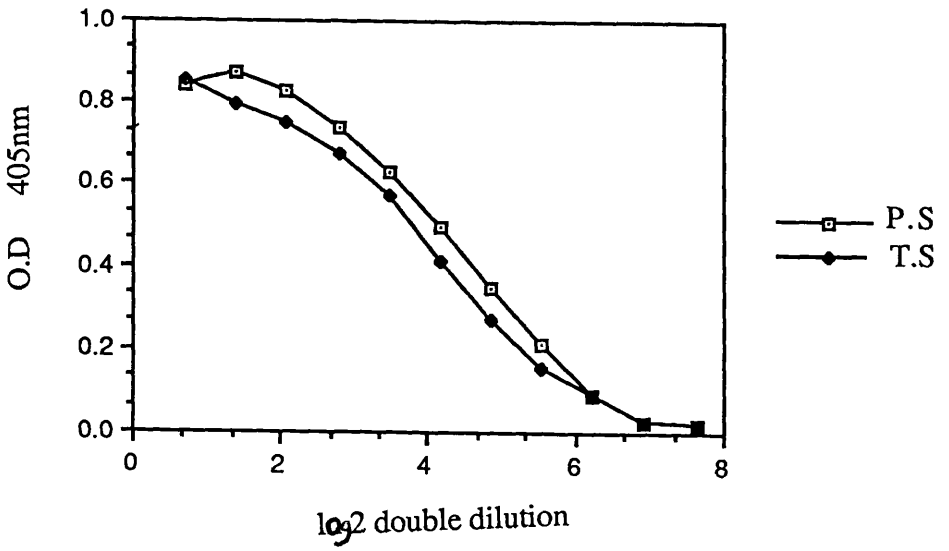


Figure 3.5

Figure 3.6 Enzyme-linked immunosorbent assay of antisera from rabbits 1/8 and 2/8 to branched peptides.

Microtitre plates were coated with branched peptide M245, as described in the Methods section. Rabbit 1/8 was immunized with 100µg of branched peptide M245, whereas rabbit 2/8 was immunized with 300µg of branched peptide M245. The curves with a dot in the box represent the prebleed sera, the curves with the full diamond respresent the pre-terminal bleed and the other curves represent the terminal bleed. Fig 3.6A represents data of sera from rabbit 1/8 and Fig 3.6B data of sera from rabbit 2/8.

Figure 3.6A

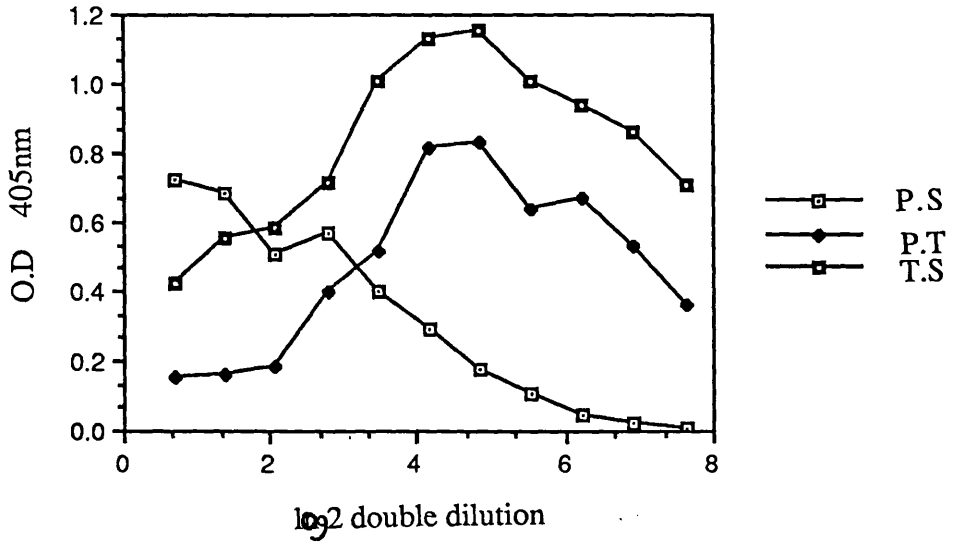


Figure 3.6B

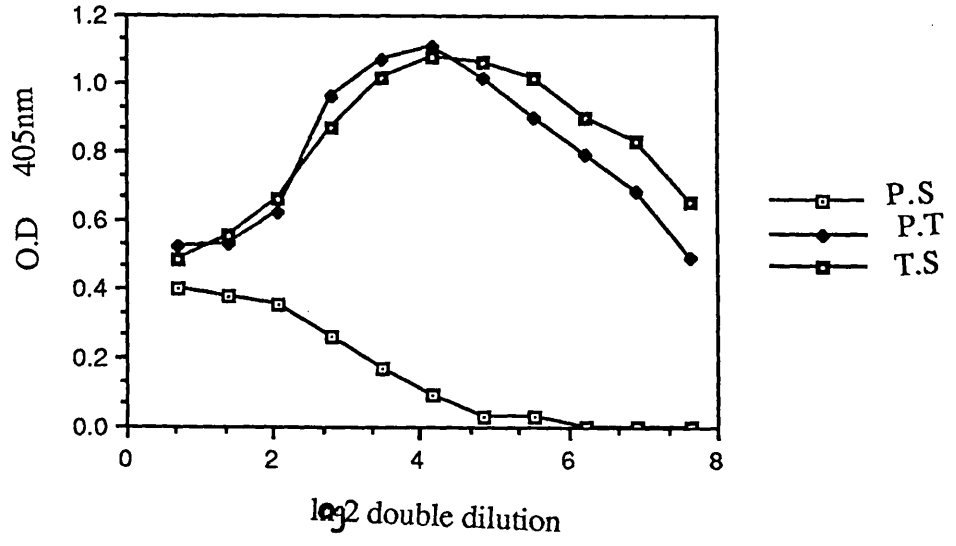


Figure 3.6

segment) infected cell lysates by radioimmunoprecipitation reactions, and some antisera were characterized by enzyme-linked immunosorbent assays.

Antisera 1/8 and 2/8 were characterised by ELISA. Microtitre plates coated with 5µg of branched peptide 245 per well were incubated with appropriate dilutions of sera, as described in the Methods section (Fig 3.6)

In radioimmunoprecipitations for detection of NSm in BUN infected cell lysates, 20µl preimmune serum were incubated with lysates in a 800µl reaction for 4 hr at 4°C and precleared by incubation with 50µl of Protein A bearing *Staphylococcus aureus* (10%) for 30 min at 4°C followed by centrifugation at 13,500 rpm for 1 min. The supernatant was transferred to a fresh tube, 5µl of the terminal bleed were added and incubated at 4°C overnight on a rotary mixer. The mixture was reacted with 100µl of *S. aureus* for 30 min at 4°C and prepared for polyacrylamide gel electrophoresis, as described in the Methods section.

As can be seen in Fig 3.7, immune sera from rabbits 1/8, 2/8, 3/8 and 4/8 immunoprecipitated NSm, but the preimmune sera did not react. The G1 and other host bands can also be seen but these are present in both preimmune and immune sera. The 2/8 antiserum appeared to recognise NSm more strongly than the other three and this antiserum was used in further experiments.

Infected cells were processed for indirect immunofluorescence, as described in the Methods Section. After the last wash, goat anti-rabbit IgG fluorescein isothiocyanate 50µl (diluted 1:100 in PBS and microfuged at 13,500 rpm for 5 min) was overlaid on the coverslips and incubated for 30 min at room temperature. The coverslips were washed four times in PBS and overlaid with 50µl of wheat germ agglutinin rhodamine isothiocyanate conjugate (diluted 1:100 in PBS) and incubated at room temperature for

Figure 3.7 Characterisation of antipeptide sera to NSm.

BHK-21 cells infected with BUN were labelled at 24 hrs for 4 hrs with ^{35}S-methionine. After removal of nuclei and other debris by centrifugation at 13,500 rpm for 5 min, cell lysates were pre-cleared by incubation with pre-bleed sera for 4 hrs followed by incubation with *Staphylococcus aureus* Protein A for 30 min and centrifuged at 13,500 rpm for 1 min. The pre-cleared lysates were incubated with antisera to branched peptide, M245 represented by 1/8, 2/8 and 3/8 and to branched peptide 246 represented by 4/8 and 5/8. P indicates pre-bleed and T indicates terminal bleed sera. The dissociated proteins were fractionated on 15% polyacrylamide gels.

Lane 1 is ^{35}S-methionine labelled mock infected whole cell lysate; lane 2 is BUN infected whole cell lysate, L, G1, N and NSm can be observed as indicated. Lane 3 is BUN infected cell lysate precipitated with pre-immune serum 1/8P. Lane 4 is BUN infected cell lysate precipitated with immune serum 1/8T. Lanes 5, 7, 9 and 11 are BUN infected cell lysates precipitated with 2/8P, 3/8P, 4/8P and 5/8P, respectively. Lanes 6, 8, 10 and 12 represent BUN cell lysates precipitated with 2/8T, 3/8T, 4/8T and 5/8T, respectively. It can be observed that except for branched peptide 5/8T, all immune branched peptide antisera recognised NSm. Other proteins were also precipitated but they are present in both the pre-immune and immune precipitations.

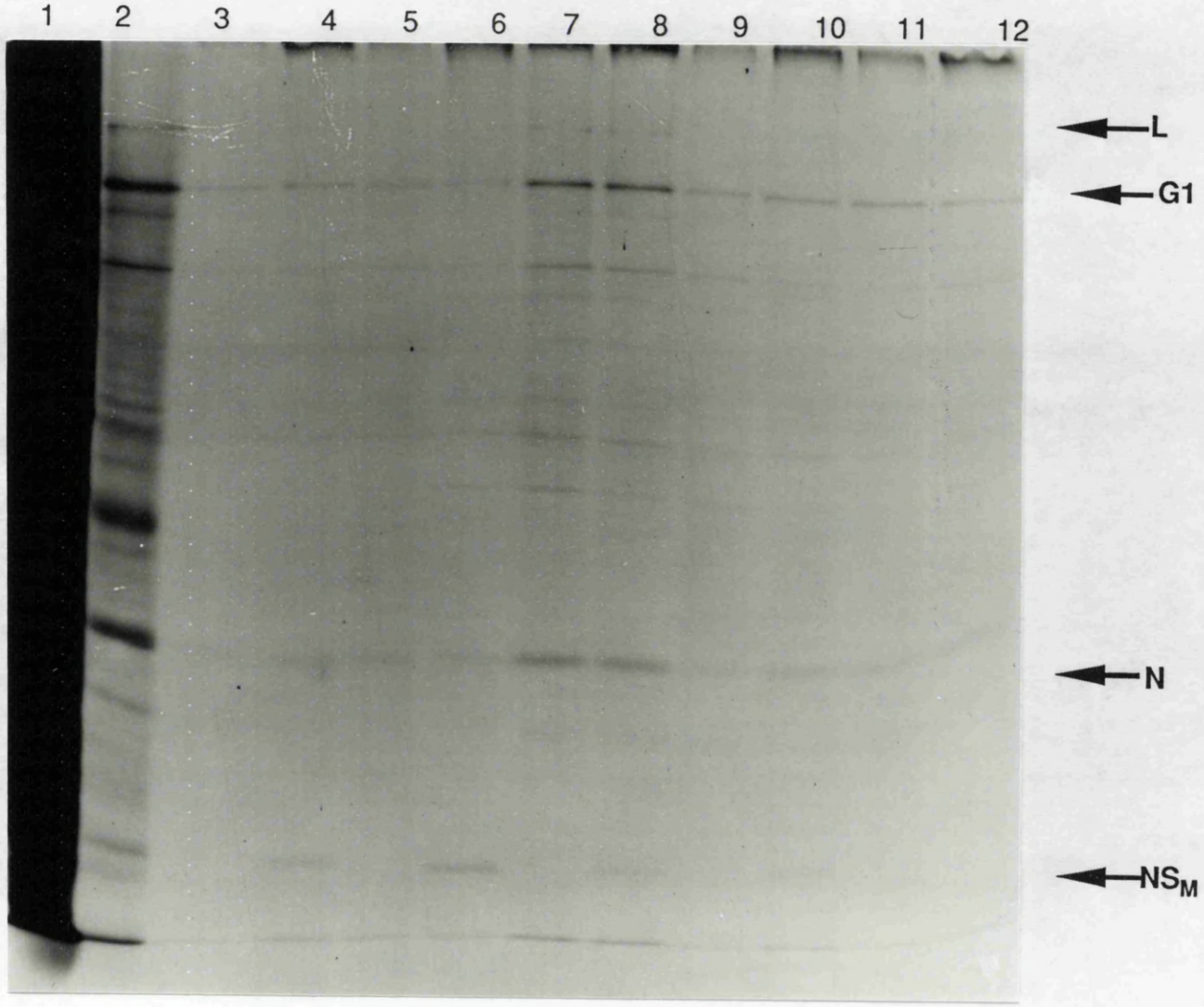


Figure 3.7

30 min then washe four times in PBS. The coverslips were immersed in de-ionised water for a few times and then mounted on microscope slides in CITIFLOR and examined on Nikon Microphat-SA fluorescence microscope, as described in the Materials section.

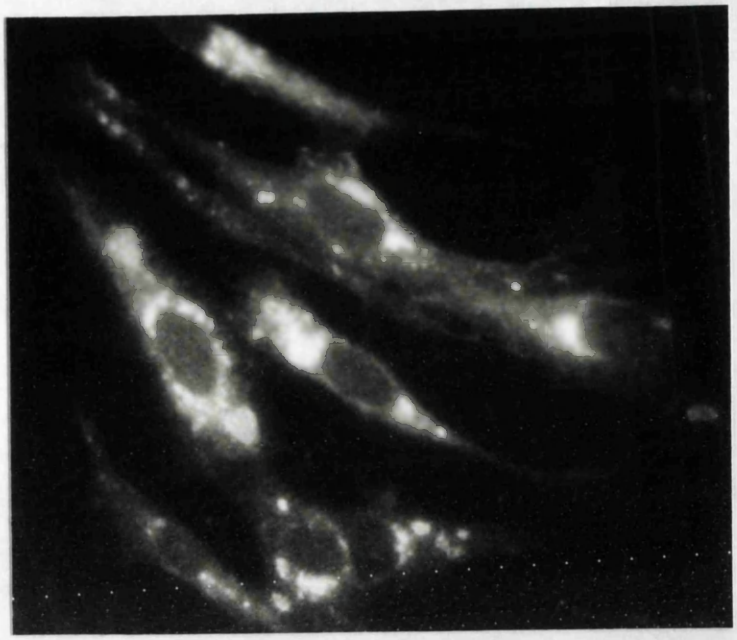
Fig 3.8A shows BUN infected cells labelled with rhodamine conjugated wheatgerm agglutinin. The pattern of staining is perinuclear and characteristic of the Golgi complex. Fig 3.8B shows the same cells as in Fig 3.8A processed for indirect immunofluorescence with branched antipeptide 2/8 and IgG conjugated fluorescein isothiocyanate. The NSm is clearly detectable and there is a striking co-localisation of NSm with the rhodamine stained Golgi complex. Figure 3.8C are BUN-infected BHK-21 cells processed for indirect immunofluorescence using branched antipeptide serum 2/8.

In summary, β-galactosidase-NSm fusion proteins were made and used to immunize rabbits to produce antisera to NSm. However, the sera produced from fusion proteins were not good enough for detection of NSm. On the other hand, sera produced using branched peptides to selected sequences in the NSm coding region recognised NSm in radioimmunoprecipitation and immunofluorescence. The branched antipeptide sera helped to demonstrate by immuno-fluorescence that NSm in BUN-infected cells localises in the Golgi complex.

Figure 3.8 Detection of NSm in BUN infected BHK-21 cells.

Cells were infected with BUN and were fixed with 3.7% formaldehyde and permeabilized with 0.5% NP40 at 24 hrs post infection. The cells were processed for indirect immunofluorescence using branched antipeptide antiserum 2/8 and fluorescein isothiocyanate conjugated antirabbit IgG. The cells were also treated with wheat germ agglutinin conjugated to rhodamine isothiocyanate. Fig 3.8A shows cells treated with rhodamine wheat germ agglutinin as viewed through the red filter at 546nm and shows the cellular distribution of the Golgi complex. Fig 3.8B shows the same BUN infected cells treated with branched antipeptide antiserum 2/8 and fluorescein isothiocyanate and viewed at 480nm. The pattern of distribution of NSm in Fig 3.8B coincides with the pattern of distribution of NSm in Fig 3.8A. Fig 3.8C is mock infected cells treated with branched antipeptide antiserum.

A



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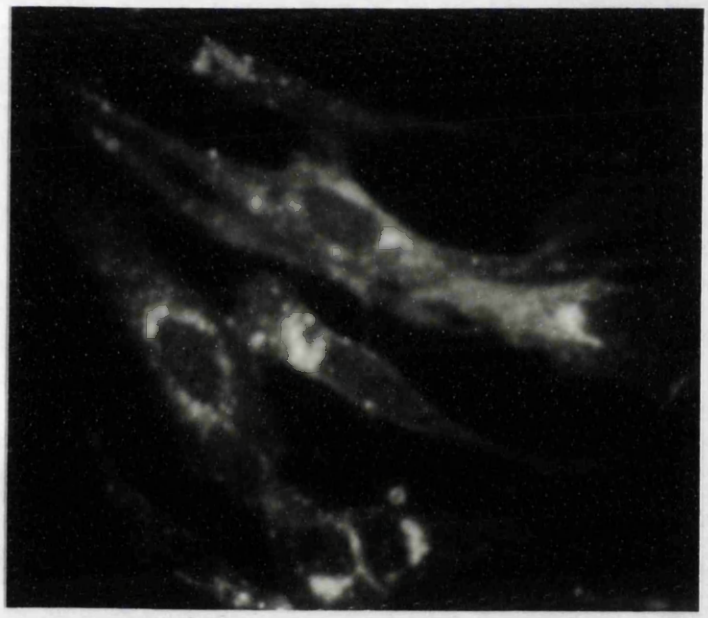


C

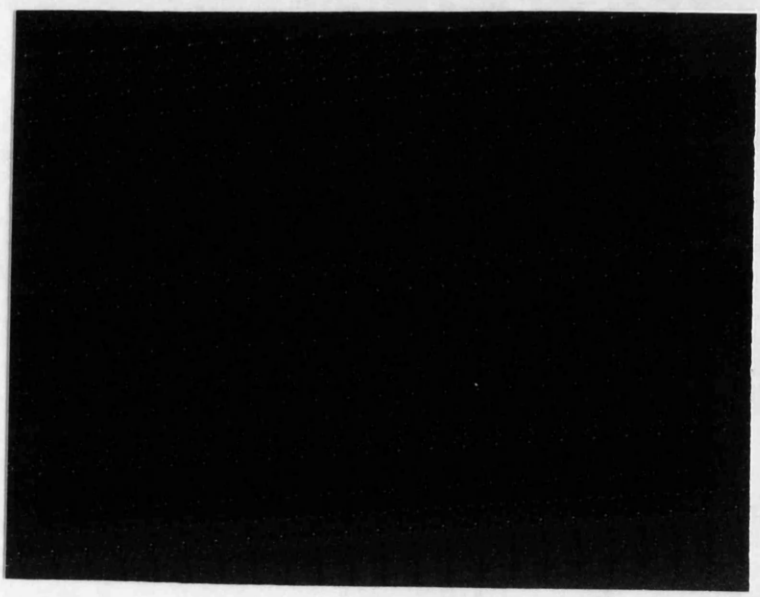


Figure 3.8

Restriction maps and BUN M sequence in back of thesis.

SECTION TWO

EXPRESSION OF THE BUN MIDDLE RNA SEGMENT

The BUN middle RNA segment encodes two glycoproteins G1 and G2 and a non-structural protein referred to as NSm. These proteins are thought to be derived from a polypeptide precursor which is probably processed co-translationally to give rise to the mature products. The polypeptide precursor has not been detected in virus infected cells, which suggests that cleavage is a cotranslational event (Pennington *et al*, 1977; Lees *et al*, 1986). Attempts to translate BUN M RNA specific mRNA *in vitro* were unsuccessful (Abraham and Pattnaik, 1983; Elliott, 1985) though the reasons for this are unclear. However, it has been possible to translate *in vitro* M segment mRNA of UUK isolated from infected cells (Ulmanen *et al*, 1981) and mRNA of RVF transcribed from recombinant plasmid DNA (Suzich and Collett, 1988).

One of the aims of this project was to express the middle RNA segment gene products from a cloned complementary DNA (cDNA). The cDNA had been made by J. F. Lees (Lees *et al*, 1986) and cloned in to pUC9, and referred to as pBUN205. The full length cDNA is 4458 base pairs long with 56 non-coding base pairs at the 5' end and 103 non-coding base pairs at the 3' end. It has an ATG initiation codon at position 57-59 and a TGA termination codon at position 4356-4358.

Two expression strategies were employed: *in vitro* translation in rabbit reticulocyte or wheatgerm cell free systems, and expression in mammalian cells using recombinant vaccinia virus.

Attempts to translate in vitro BUN M segment mRNA

Translation of M segment mRNA in cell free systems in the presence or absence of canine pancreatic microsomal membranes would give an indication as to whether the primary translation product was cleaved cotranslationally or post-translationally. This would be revealed by the presence of a precursor uncleaved polypeptide or mature processed products in polyacrylamide gels.

Since the failure to translate BUN M mRNA isolated from infected cells (Lees *et al*, 1986) may have been due to the low amounts synthesised in cells, an attempt was made to translate BUN M mRNA produced from a cloned BUN M cDNA. The full length M cDNA was cloned under control of bacteriophage T3 or T7 promoters in plasmid vectors, in order to synthesise BUN M mRNA *in vitro* by run-off transcription.

Construction of pTZ BUN M

The pTZ18U and pTZ19U transcription vectors have a T7 RNA promoter and are represented in Fig 4.1.

The pBUN205 plasmid DNA was obtained from Dr R. M. Elliott. The BUN M cDNA insert was removed from pBUN205 by digestion with PstI. pTZ18U or pTZ19U DNA was linearised with PstI and dephosphorylated with calf alkaline intestinal phosphatase at 37°C for 30 min. The insert was ligated with the vector overnight at room temperature using T4 DNA ligase. *E.coli* JM101 or DH5α were transformed with the ligation mixture, plated out on agar plates and incubated at 37°C overnight. The colonies were analysed by miniprep, as described in the Methods Section. It was apparent that there was contaminating intact pBUN205 in the ligation mixtures. This problem was overcome by digesting pBUN205 with both PstI and HaeII to prepare the M cDNA insert. HaeII cuts three times in the pUC9 backbone but

Figure 4.1 Schematic representation of the pTZ18U, pTZ19U, pT7T318U and pT7T319U transcription vectors.

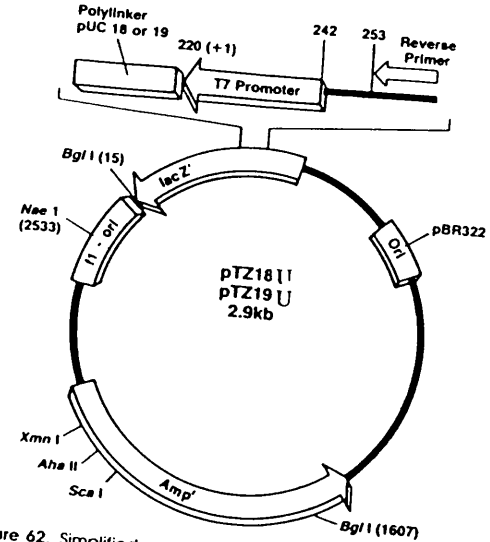


Figure 62. Simplified restriction map of pTZ vectors (courtesy of Pharmacia).

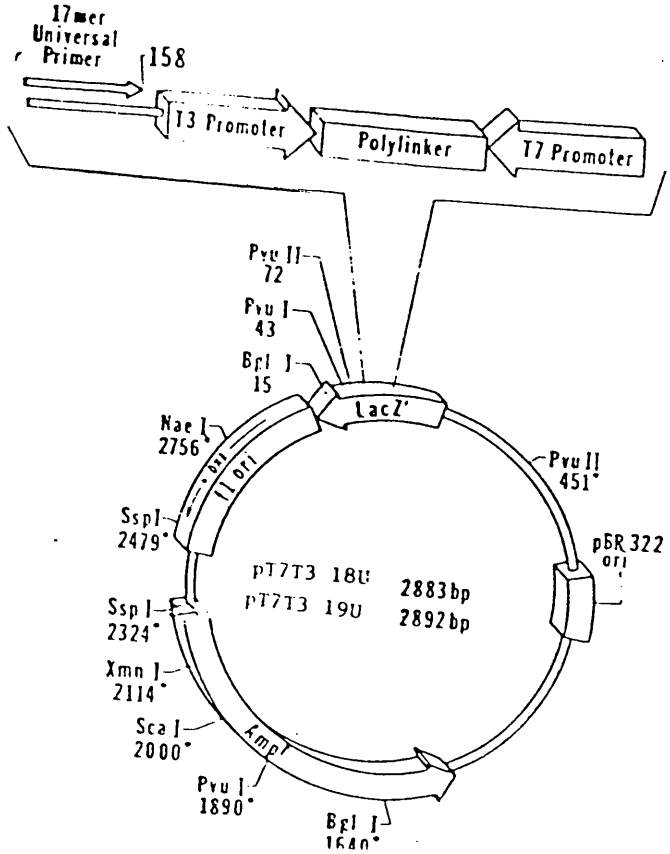


Figure 4.1

there are no sites in the BUN M cDNA. The insert was ligated with vector and 21 out of 70 colonies analysed (30%) contained recombinant plasmids, as demonstrated by digestion with PstI which gave two fragments of ~4.5kb and 2.7kb. The orientation of insert was determined by digesting with HindIII which would yield fragments of 6.7kb and 0.3kb if the M cDNA had been inserted in the wrong orientation relative to the T7 RNA promoter or about 4.3kb and 2.9kb if the cDNA had been inserted correctly. All recombinant plasmids obtained had inserts in the wrong orientation to the T7 RNA promoter. The experiment was repeated several times and similar results were obtained.

It was decided to use a dual promoter transcription vector, pT7T3, which has T7 and a T3 RNA promoters on either side of the polylinker (Fig 4.2). This was an attractive vector because the actual orientation did not matter as either the T7 or T3 RNA polymerase could be utilized to synthesise the message-sense RNA transcripts.

Construction of pT7T3 BUN M and in vitro transcription and translation of M segment mRNA

The insert and vector were prepared essentially in the same way as described for the pTZ vectors using HaeII and PstI digestion. After ligation, 45 minipreps were analysed. Six had inserts, as revealed by digestion with PstI and all were inserted such that message-sense RNA would be transcribed from the T3 RNA promoter (orientation determined by digestion with HindIII). The orientation was further confirmed by plasmid dideoxy nucleotide sequencing, as shown in Fig 4. 3.

RNA transcripts were made by run-off transcription using T3 RNA polymerase. The plasmid was cut either with HindIII at nucleotide 4326, or NcoI at nucleotide 3608, or BglII at nucleotide 2800, or PvuII at nucleotide 2467, or HpaI at nucleotide 1211. Transcripts were analysed in

Figure 4.2 Construction of pT7T3 BUN M.

The full length BUN M cDNA was cleaved from plasmid pBUN 205 by digestion with PstI. The vector sequences in pBUN205 were cleaved with HaeII to facilitate purification of the M cDNA fragment. The insert was cloned into the PstI site of the pT7T3 vector.

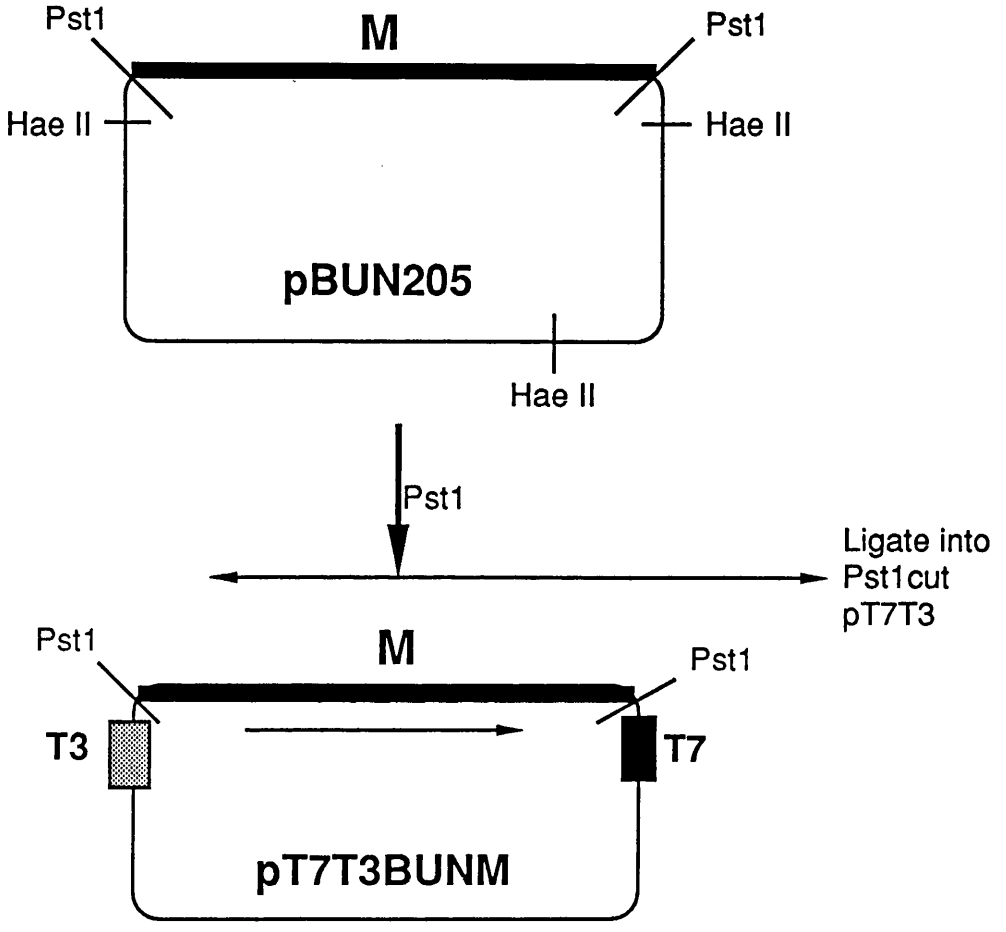
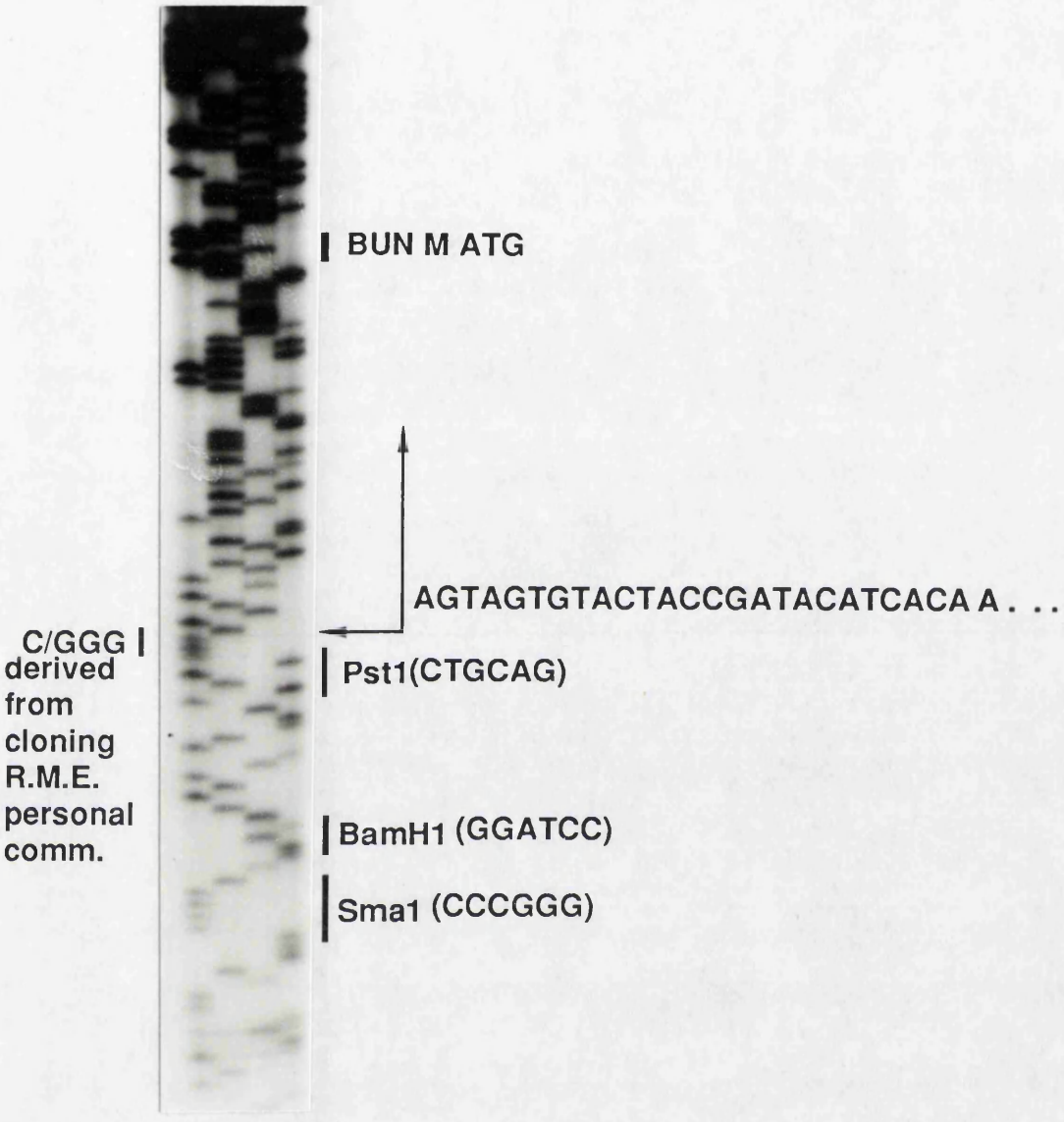

Pst1
M
Pst1
Hae II
Hae II
pBUN205
Hae II
Pst1
Ligate into
Pst1cut
pT7T3
Pst1
M
Pst1
T3
T7
pT7T3BUNM

Figure 4.2

Figure 4.3 Plasmid dideoxy sequence of pT7T3 BUN M.

Single stranded DNA template was generated by incubation with 0.2M NaOH. The template was annealed with the Universal primer and labelling reactions carried out by T7 DNA polymerase, as described in the Methods section. Track are GATC from left to right. The Pst I site and start (AGTAGT) of the BUN M segment cDNA are indicated.



Plasmid DNA dideoxy sequence of pT7T3BUNM

Figure 4.3

denaturing methyl mercuric hydroxide agarose gels, stained with ethidium bromide, and were of the expected sizes (Fig 4.4).

With the possibility that structures in the non-coding region at the 5' end of the mRNA might affect translation, another construct of pT7T3 BUN M was made from pBUN 204 which has a deletion from nucleotide 1 to 32 in the 5' end. The M cDNA of pBUN 204 also inserted such that mRNA was transcribed from the T3 promoter. Run-off transcripts were made with truncations at the 3' end similar to those of pT7T3 BUN M of the pBUN 205 origin. The transcripts were analysed on the same gel as that for pT7T3 BUN M 205, as shown in Fig 4.4.

The transcripts were translated *in vitro* in rabbit reticulocyte and wheatgerm systems under various conditions. Plasmid DNAs were linearised with HindIII and either capped or uncapped transcripts were synthesised (see Methods). The transcripts were used to programme wheat germ or rabbit reticulocyte lysates in the presence or absence of microsomal membranes (Fig 4.5). A MAG S RNA message, which was known to translate efficiently, was included as a positive control (Elliott and McGregor, 1989). The MAG N protein was synthesised in rabbit reticulocyte lysate system, lane 2, and in wheatgerm system, lane 8. Capped transcripts derived from pT7T3 BUN M 205 and 204 in lanes 3 and 4, respectively, failed to translate. When membranes were added to capped transcripts, no translation products were observed (lanes 5 and 6). Attempts to translate the transcripts in wheatgerm was also unsuccessful (lanes 9 and 10).

Truncated transcripts from plasmids cut with HindIII, NcoI, BglII, PvuII and HpaI were also not translated in the rabbit reticulocyte system, as shown in Fig 4.6. MAG and BUN S messages were translated under the same conditions to yield N protein. This suggested that the inability to

Figure 4.4 *In vitro* transcription of pT7T3 BUN 205 and pT7T3 BUN 204.

Run-off transcripts were made using T3 RNA polymerase after truncations were made on the cDNA with HindIII at nucleotide 4326, NcoI at nucleotide 3608, BglII at nucleotide 2800, PvuII at nucleotide 2467 and HpaI at nucleotide 1211. Transcripts were analysed in denaturing methyl mercuric hydroxide agarose gels and stained with ethidium bromide. The MAG S cDNA was included as a positive transcription control (last lane). The restriction map showing sites used to make truncations are shown in this Figure.

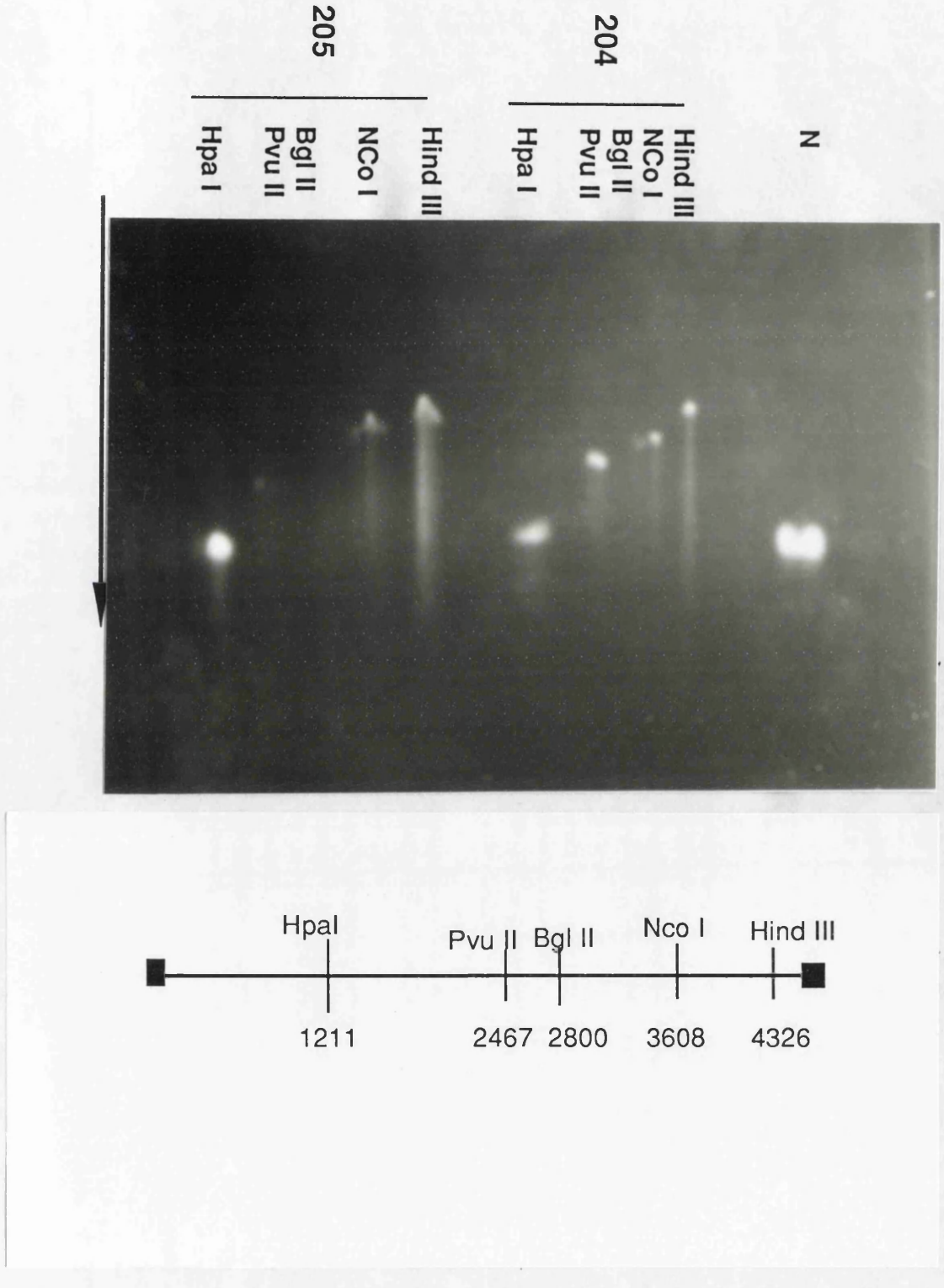

205
204
Hpa I
Bgl II
Pvu II
NCo I
Hind III
Hpa I
Bgl II
Pvu II
NCo I
Hind III
N
HpaI
Pvu II
Bgl II
Nco I
Hind III
1211
2467
2800
3608
4326

Figure 4.4

Figure 4.5 *In vitro* translation of pT7T3BUNM derived RNA.

Plasmids pT7T3BUN205 and pT7T3BUN204 were linearised with Hind III and run-off transcripts made using T7 RNA polymerase. Transcripts were translated in rabbit reticuloycte lysate and wheat germ in the presence (lanes 5 and 6) or absence (lanes 3 and 4) of microsomal membranes and labelled with ^{35}S-methionine. Lane 1 is mock-infected BHK cell proteins; lane 2 is rabbit reticulocyte lysates programmed with MAG S RNA. Lanes 3 and 4 are lysates programmed with capped RNAs derived from pT7T3BUN205 and pT7T3BUN204, respectively. Lanes 5 and 6 are lysates programmed with capped RNAs derived from pT7T3BUN205 and pT7T3BUN204, respectively, in the presence of microsomal membranes. Lane 7 is lysates to which only water was added. Lane 8 is wheat germ programmed with MAG S RNA. Lanes 9 and 10 are wheat germ programmed with RNAs derived from pT7T3BUN205 and pT7T3BUN204, respectively.

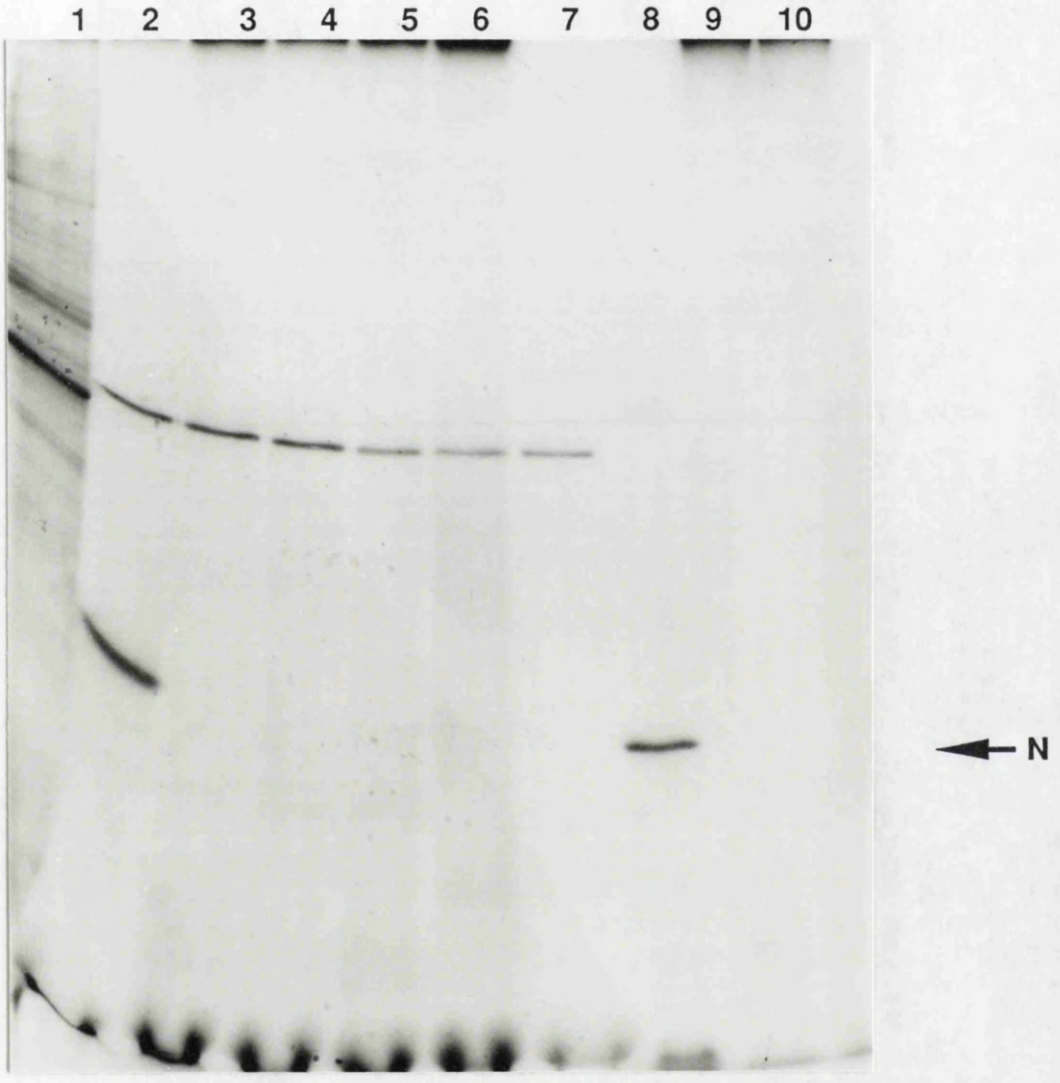


Figure 4.5

Figure 4.6 *In vitro* translation of mRNAs transcribed from pT7T3BUN205 and pT7T3BUN204.

Plasmids pT7T3BUN205 and pT7T3BUN204 were linearised with Hind III at nucleotide 4326, NcoI at nucleotide 3608, BglII at nucleotide 2800, PvuII at nucleotide 2467 or HpaI at nucleotide 1211. Run-off transcripts were made as illustrated in Fig 4.4 and translated in rabbit reticulocyte lysates. Track 1 is mock-infected BHK-21 cell proteins; tracks 2 and 3 are rabbit reticulocyte lysates programmed with MAG and BUN S RNAs, respectively. Tracks 4, 5, 6, 7 and 8 are rabbit lysates programmed with RNAs derived from pT7T3BUN205 linearised with HindIII, NcoI, BglII, PvuII and HpaI, respectively. Tracks 9, 10, 11, 12 and 13 are lysates programmed with RNAs derived from pT7T3BUN204 linearised with HindIII, NcoI, BglII, PvuII and HpaI, respectively.

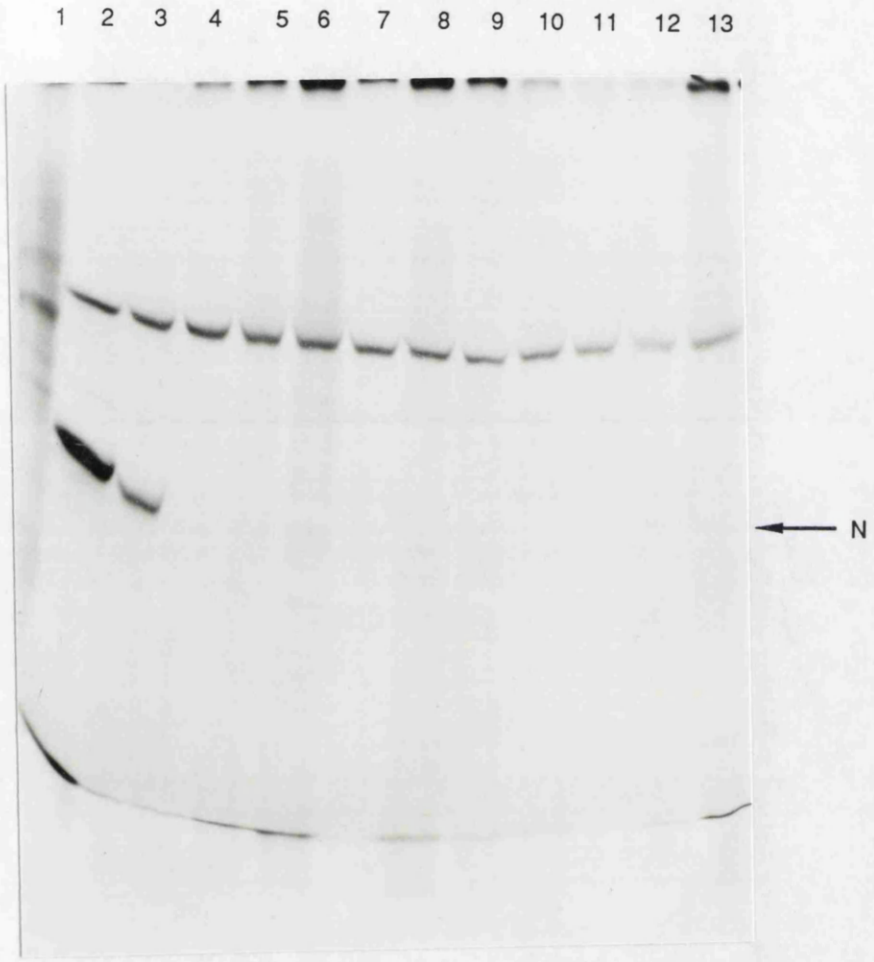


Figure 4.6

translate BUN M transcripts was caused by intrinsic factors in the M RNA segment. The addition of cap analogues did not improve translatability in the rabbit reticulocyte system.

Construction of pT7T3 BUN M BamHI and in vitro expression

An attempt was made to translate *in vitro* M segment RNA derived from the internal BamHI fragment, i.e. bases 1352-4001. This was to test the suspicion that the failure to translate full length mRNA may have been due to the sequences at the 5' end especially in the G2 region.

The BUN M cDNA fragment from nucleotide 1352 to 4001 has an in frame ATG at nucleotides 1430-1432. The fragment was inserted into the BamHI site of the pT7T319U vector. Determination of orientation of insertion indicated that all recombinants would make message-sense RNA from the T3 RNA promoter.

Run-off transcripts were synthesized, after linearising the plasmid with HindIII, or PstI using T3 RNA polymerase. Transcripts were translated in a rabbit reticulocyte system in the presence or absence of dog pancreatic microsomal membranes, as described in the Methods section. As can be seen in Fig 4.7, a protein of about 90K was made in the reactions programmed with the synthetic RNAs and this protein (which was of the size expected) was not seen in the control in which water had been added instead of RNA.

Half of the material was subjected to radioimmunoprecipitation using a polyclonal antiserum to BUN. As can be seen in Fig 4.8, the 90K protein was specifically recognised by the antiserum showing that the protein was related to the BUN M RNA segment gene products.

In vitro translation of BUN M RNA was not satisfactory for the study of the biogenesis of the G1 and G2 glycoproteins and NSm. Full length RNAs were not translated

Figure 4.7 *In vitro* translation of the BUN M BamHI internal fragment.

Transcripts were made from HindIII and PstI linearised plasmid containing the internal BUN M BamHI fragment and translated in a rabbit reticulocyte lysate system. Lane 1 represents rabbit reticulocyte lysates to which only water was added. Lanes 2 and 3 are lysates programmed with RNA derived from plasmid pT7T3 containing the internal BUN M BamHI cDNA linearised with HindIII in the absence or presence of microsomal membranes, respectively. Lanes 4 and 5 are lysates programmed with RNA derived from a plasmid pT7T3 containing the BamHI cDNA linearised with PstI in the absence or presence of microsomal membranes, respectively.

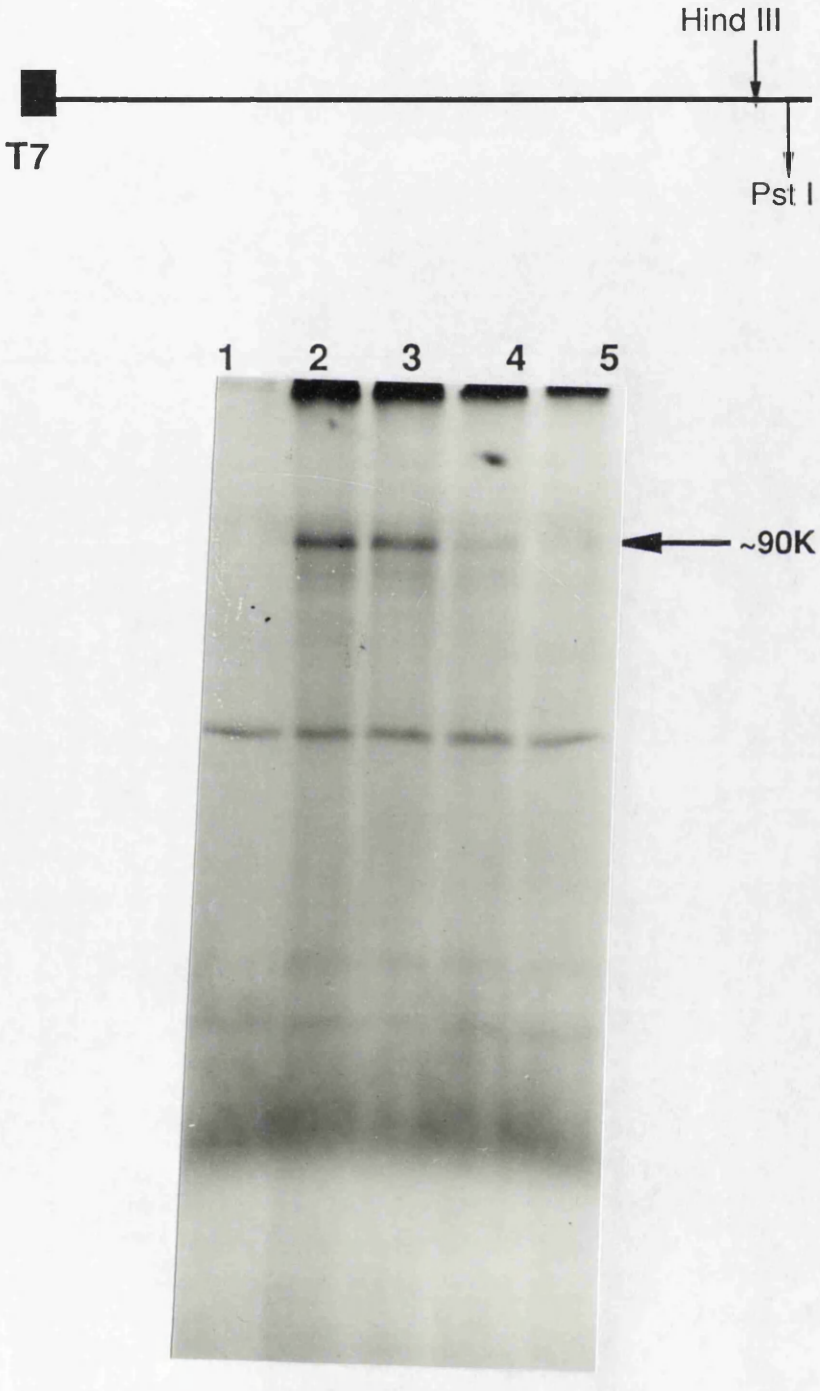


Figure 4.7

whereas an internal RNA sequence yielded a protein which was recognised by a BUN antiserum. This suggests that sequences at the 5' end of the full length mRNA may inhibit translation *in vitro*, but this has not been investigated further. It was therefore decided to attempt to express BUN M RNA gene products *in vivo* using the vaccinia virus expression system.

Expression of the BUN M RNA gene products by recombinant vaccinia viruses

The system used was that described by Fuerst *et al* (1987). This involved the construction of a recombinant vaccinia virus containing the BUN M gene flanked by bacteriophage T7 promoter and terminator sequences. Expression of the target gene would occur when cells were co-infected with a second recombinant vaccinia virus which contains the bacteriophage T7 RNA polymerase gene under the control of a vaccinia virus promoter.

Construction of pTF7-5 BUN M

pTF7-5 is a vaccinia virus transfer vector which has bacteriophage T7 RNA promoter and terminator sequences flanked by vaccinia virus TK sequences for recombination in the TK locus of the vaccinia virus genome. The vector was prepared by cutting with BamHI and filling in the 5' overhangs using the Klenow fragment of DNA polymerase I.

The BUN M insert was obtained by digesting pBUN205 with Pst I and Hae II. The PstI 3' overhanging ends were filled in using T4 DNA polymerase. The blunt-ended vector and insert DNAs were ligated using T4 DNA ligase at room temperature overnight. *E.coli* DH5α cells were transformed with the ligation mixture, as described in Methods, and miniprep analyses performed on overnight cultures. The presence of the insert was tested for by digestion with Hind III. One hundred and ninety minipreps were performed

and none of the colonies picked carried a recombinant plasmid. Repeated efforts of ligations, refilling and religating failed to give rise to recombinant molecules.

An alternative approach was therefore attempted, starting with an internally deleted fragment of the BUN M cDNA derived from pT7T3pM11d13. This construct contains M segment sequences from position 32 to the BamHI site at 1352 and from the BamHI site at position 4001 to the end of the M segment cDNA (Fig 4.9)

The insert from pT7T3pm11d13 was removed by PstI digestion, blunt-ended using T4 DNA polymerase and ligated with BamHI cut and Klenow filled in pTF7-5 DNA (Fig 4.9). DH5α cells were transformed with the ligation mixtures and plated on agar plates. Twenty four colonies were picked and one had the insert with the correct orientation relative to the T7 promoter, as shown by digestion with HindIII. The orientation of the insert was confirmed by plasmid dideoxy DNA sequencing, as shown in Fig 4.10. The construct was further digested with BamHI which showed the presence of only one BamHI site, i.e. in the BUN M cDNA.

This clone, pTF7-5/d13M, was digested with BamHI so that the 2.7kb missing fragment of BUN M cDNA (bases 1353-4000) could be inserted. Forty eight colonies were picked after ligation of pTF7-5/d13M with the 2.7kb BamHI fragment. All had the 2.7kb fragment insert but in opposite orientation to the promoter. This apparent persistence of the M segment fragments against the bacteriophage T7 RNA promoter could not be explained and was at best frustrating.

However, careful reexamination of the plates revealed some tiny colonies that were less than one third of a millimetre in diameter. Twenty four of these were picked and prepared for miniprep analyses. Twenty one of them had inserts and fifteen had the M cDNA correctly assembled. One

Figure 4.8 Immunoprecipitation of the translation products of the internal BamHl fragment of BUN M.

The translation products were immunoprecipitated with anti-BUN sera, as described in the Methods section. Lanes 1 and 3 are lysates programmed with RNA derived from plasmid pT7T3 containing the internal BUN M BamHI cDNA fragment linearised with HindIII in the absence or presence of microsomal membranes, respectively. Lanes 5 and 7 are lysates programmed with RNA derived from the plasmid linearised with PstI in the absence or presence of microsomal membranes. Lanes 2, 4, 6 and 8 are immunoprecipitates of translation products in lanes 1, 3, 5 and 7, respectively. Lane 9 is the immunoprecipitate of protein from lysates to which only water was added.

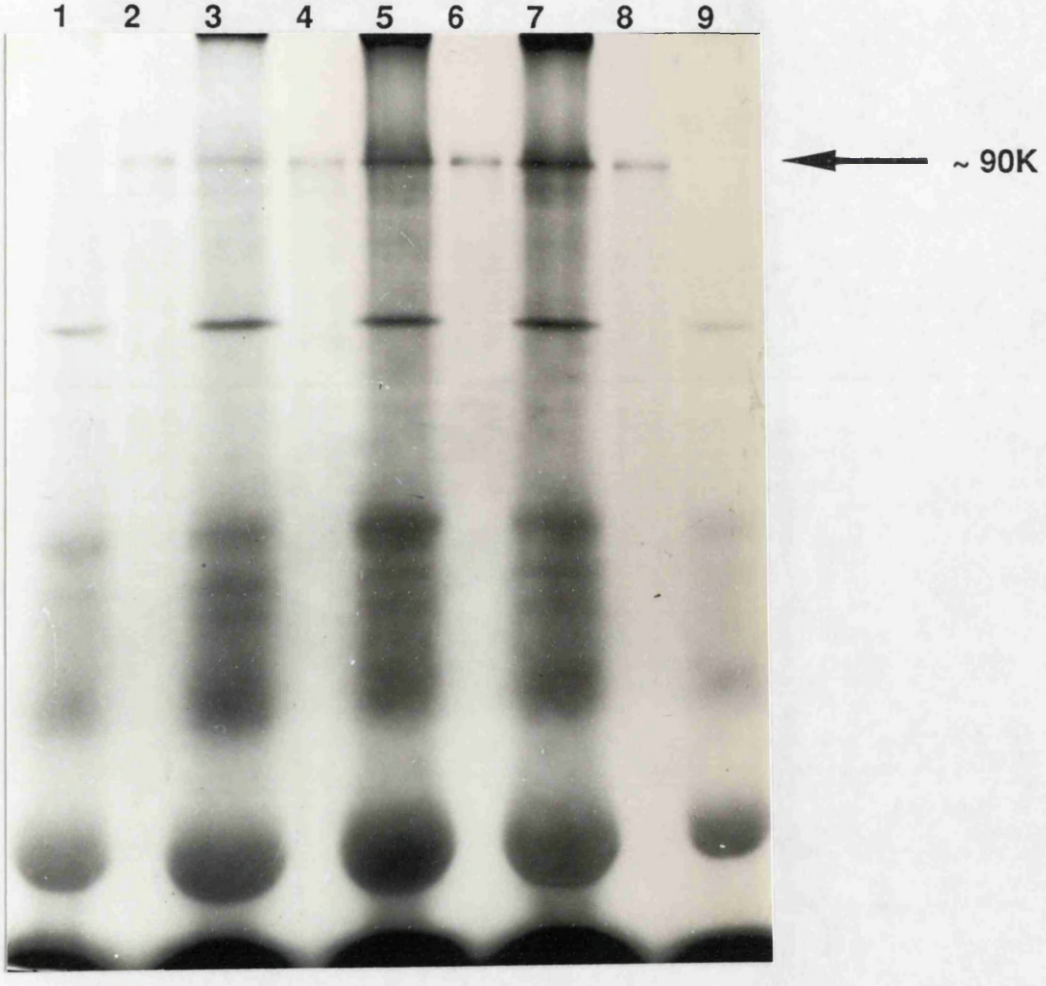


Figure 4.8

Figure 4.9 Construction of pTF7-5 BUN M.

A BUN M cDNA with a deletion from nucleotide 1352 to nucleotide 4001 was obtained by digesting pT7T3pm11d13 with PstI. The PstI 3' overhang ends were filled in with T4 DNA polymerase. The fragment was cloned into Klenow filled-in BamHI site of the pTF7-5 vector. The construct was linearised with BamHI and ligated with the missing BamHI 2.7kb BUN M fragment.

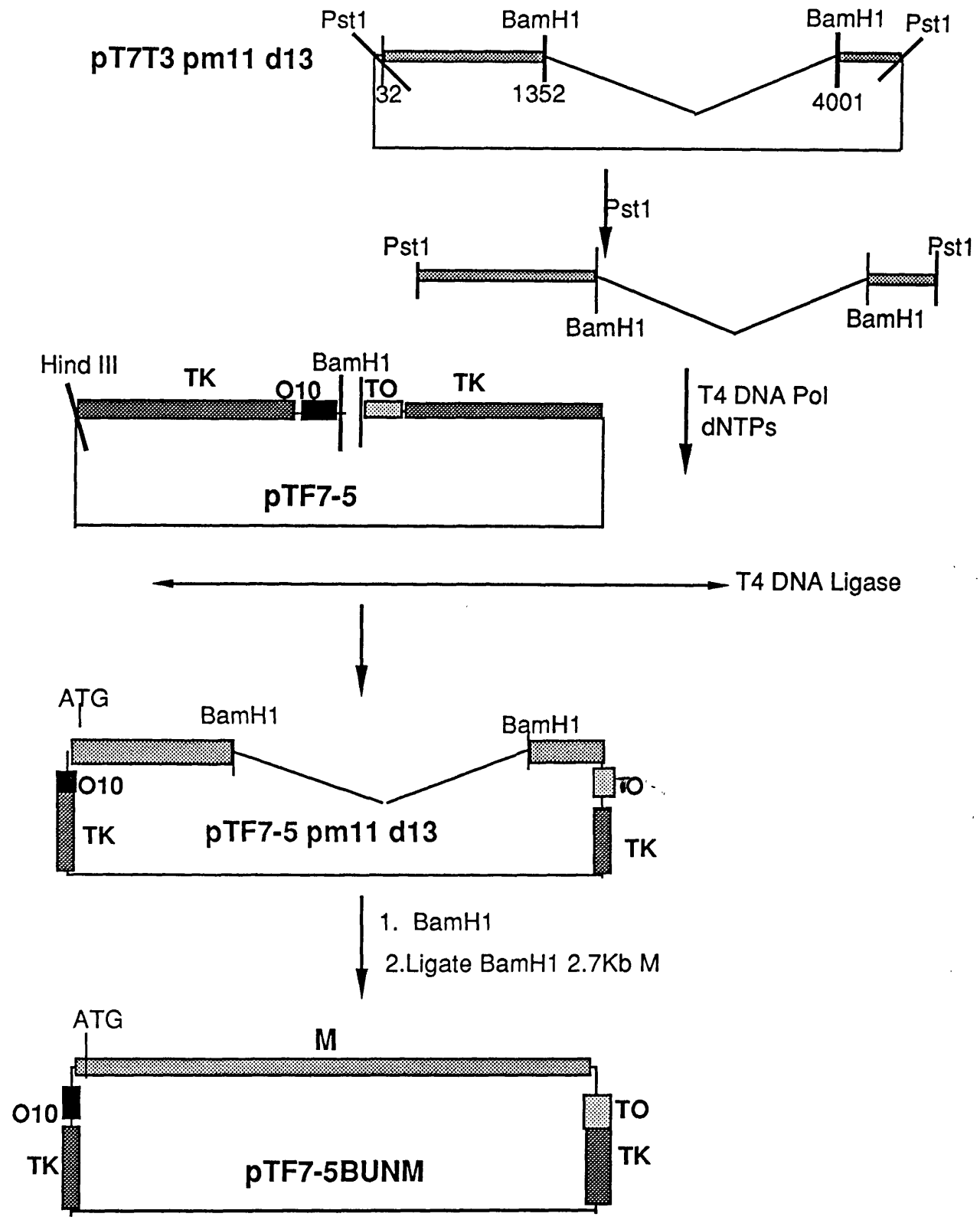

pT7T3 pm11 d13
Pst1
BamH1
BamH1
Pst1
32
1352
4001
Pst1
Pst1
Pst1
BamH1
BamH1
Hind III
TK
BamH1
O10
TO
TK
pTF7-5
T4 DNA Pol
dNTPs
T4 DNA Ligase
ATG
BamH1
BamH1
O10
TO
TK
pTF7-5 pm11 d13
TK
1. BamH1
2.Ligate BamH1 2.7Kb M
ATG
M
O10
TO
TK
TK
pTF7-5BUNM

Figure 4.9

Figure 4.10 Plasmid DNA dideoxy sequence of pTF7-5/pm11d13.

The sequence was determined by the T7 DNA polymerase, as described in the Methods section. This showed that the 5' end of BUN M cDNA had been placed under the control of the bacteriophage T7 RNA promoter. Tracks are GATC from left to right.

Plasmid DNA dideoxy sequence of pTF7-5/d13

Figure 4.10

clone was designated pTF7-5 BUN M, and grown in bulk for recombination experiments with vaccinia virus.

Generation and characterisation of vaccinia virus BUN M recombinants

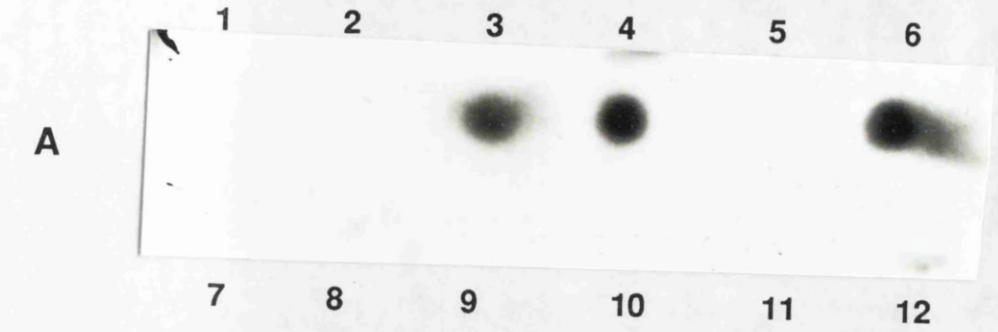
Recombinant viruses were prepared by infecting CV-1 cells with vaccinia virus strain WR and transfecting the infected cells with calcium phosphate precipitated pTF7-5 BUN M plasmid DNA. Selection of recombinants was made by plaque assay on TK$^-$ cells in the presence of BUdR, as described in the Methods Section. Plaques were picked and grown in TK$^-$ cells in 24 well Limbro plates in the presence of BUdR. Plaques which exhibited c.p.e. were regrown in TK$^-$ cells in the presence of BUdR and total cellular DNA was isolated for dot blot analysis on nitrocellulose filters. Replicate filters were hybridised with ^{32}P-labelled pBUN205 DNA or with ^{32}P-labelled pTF7-5 DNA, as shown in Fig 4.11. Out of the three isolates which appeared to be recombinants, numbers 4 and 6 were subjected to Southern analysis by digesting with BamHI and EcoRI, then running the samples on agarose gels and transferring the DNA to nitrocellulose filters. The filters were hybridised with ^{32}P-labelled pT7T3 BUN M DNA and the result is shown in Fig 4.12. The 2.7kb BamHI and 2.16kb EcoRI internal fragments could clearly be seen, indicating that recombination had occurred. Recombinant viruses referred to as recBUN M were grown in bulk and semi-purified, as described in the Methods Section.

Characterization of proteins synthesised by recombinant vaccinia virus

In order to investigate whether the positive recombinant viruses, as demonstrated by dot blot and Southern blot analyses, synthesised the gene products of the M segment of BUN, CV-1 cells were infected with both recBUN M and vTF7-3 (dual vaccinia virus). Infected cells

Figure 4.11 Characterization of vaccinia virus BUN M recombinants by dot blot hybridization.

Putative recombinant vaccinia viruses that exhibited c.p.e. in TK$^-$ 143 cells in the presence of BUdR were further grown in TK$^-$ 143 cells. Total cellular DNA was isolated and transferred onto nitrocellulose filters. The filters were hybridized either with ^{32}P-labelled pBUN205, as shown in Fig 4.11A, or with ^{32}P-labelled pTF7-5, as shown in Fig 4.11B. Lack of hybridization in samples 1, 7-12 with the pTF7-5 probe was probably due to a failure during sample preparation.



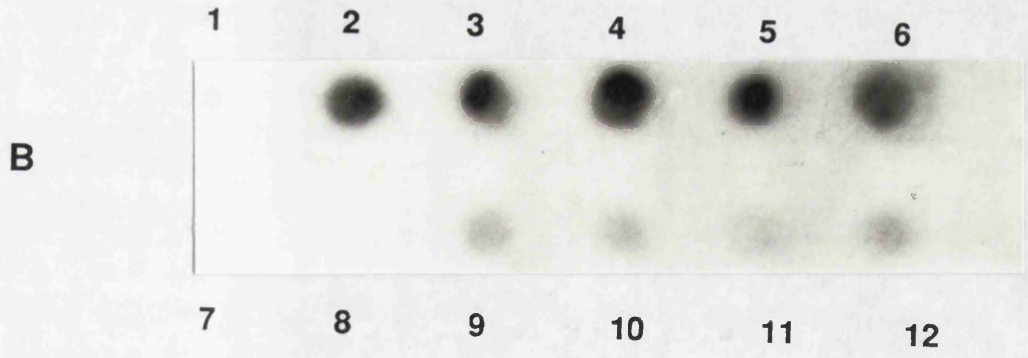


Figure 4.11

Figure 4.12 Southern blot analysis of recombinant vaccinia virus recBUNM.

Putative vaccinia virus recombinants containing BUN M cDNA, designated 2, 4, 5, 6 and 9, which exhibited c.p.e. in TK-143 cells in the presence of BudR were analysed by Southern blotting of DNA extracted from infected CV1 cells. The DNA was digested with BamHI or EcoRI, separated in a 0.8% agarose gel and transferred to a nitrocellulose filter. The filter was hybridized with ^{32}P-labelled pT7T3BUNM DNA. Lanes 1 and 7 are pBUN205 digested with BamHI and ECoRI, respectively, as markers. Lanes 3 and 5 represent samples 4 and 6 cut with BamHI and lanes 9 and 11 are the same samples digested with EcoRI. These two samples are the ones that gave a positive signal in the dot blot experiments (Fig 4.11). The difference in migration between the 2.7kb BamHI fragment and the ECoRI 2.149kb of BUN M can clearly be seen in both samples. This result confirms the dot blot result that samples 4 and 6 contained recombinant vaccinia virus.

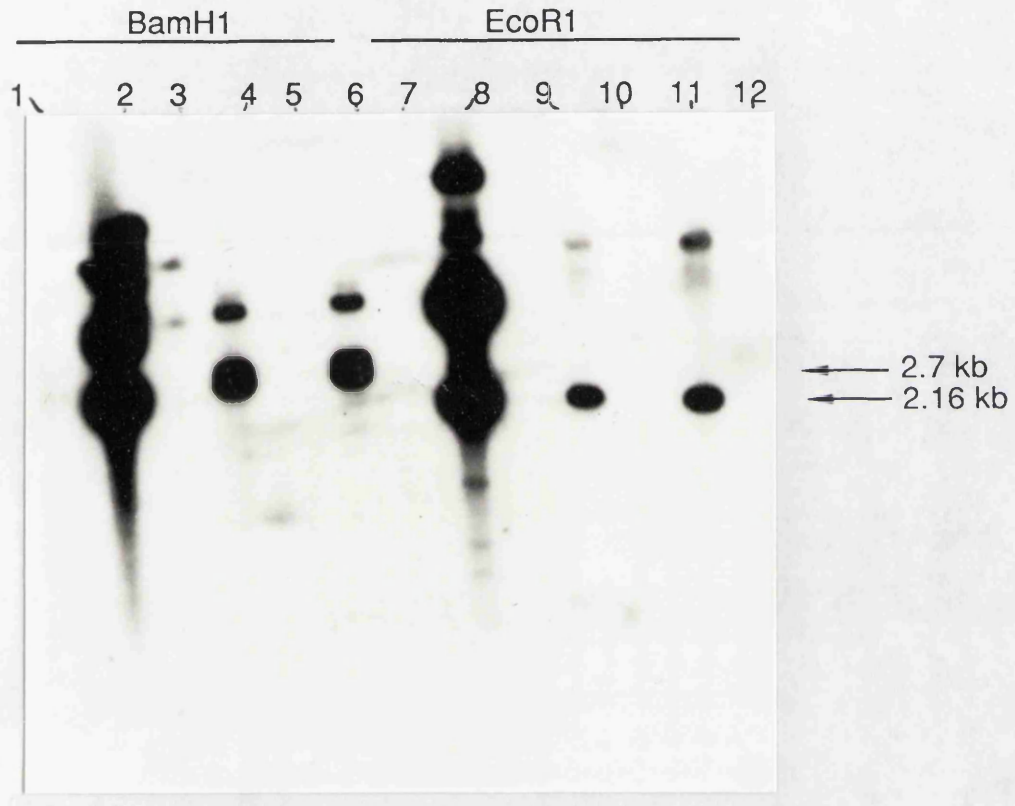


Figure 4.12

were labelled with ^{35}S-methionine from 14 to 18h post infection and cell extracts analysed by SDS-polyacrylamide gel electrophoresis.

The optimum multiplicity of infection was determined by infecting monolayers at multiplicities of 1, 5 and 10 pfu/cell of each vaccinia virus. It was observed that infecting cells at multiplicities of 10 for each recombinant vaccinia virus was ideal because at this level of infection, most of the host protein synthesis was shut off and a sharp protein band that co-migrated with authentic G1 protein could be seen, as in Fig 4.13. This protein band was absent in cell lysates separately infected with recBUN M or vTF7-3 viruses. Better separation was observed when 10% gels were used, as shown in Fig 4.14. Note that in this experiment G2 was not detected (G2 has previously been reported to be variably detected in BUN-infected cells; Elliott, 1985) and NSm would have travelled with the dye front on this gel. It was also observed that the intensity of the T7 polymerase band diminished in the dually infected cells while other vaccinia virus proteins remained relatively the same.

To determine the authenticity of the proteins synthesized, fractionated proteins in a polyacrylamide gel were electrotransferred onto nitrocellulose filters and analysed by Western blotting. The anti-BUN serum recognised the G1 and N proteins in the BUN-infected cell lysates (Fig 4.15, lane 2) and a protein that co-migrated with the authentic G1 protein in the dual vaccinia infected virus cell lysates (lane 3). A similar protein was absent in cells individually infected with recBUN M and vTF7-3 recombinant vaccinia viruses (lanes 4 and 5, respectively).

To further confirm the authenticity of G1 and to detect NSm, radiolabelled cytoplasmic extracts were prepared from dual vaccinia virus, BUN, or mock-infected cells and reacted with anti-BUN, anti-peptide or preimmune sera, and analysed in a 15% polyacrylamide gel (Fig 4.16). G1 was precipitated

Figure 4.13 Determination of optimal multiplicity of infection for the dual recombinant vaccinia viruses expressing the M segment.

CV-1 cells were mock infected, infected with BUN at pfu of 5/cell and variously infected with 1, 5, 10 pfu/cell of each recBUN M and vTF7-3. Cells were labelled with ^{35}S-methionine and fractionated on a 12% polyacrylamide gel. Lane 1 represents mock infected cells, lane 2 is BUN infected cells, lanes 4 and 7 are vTF7-3 and recBUN M recombinant vaccinia viruses, respectively. Lanes 5, 6 and 3 are dually infected cells with vaccinia viruses at 1, 5 and 10 pfu/cell, respectively. The level of expression of G1 at 1 and 5 pfu/cell looked similar. Expression of G1 was reduced at 10 pfu/cell but the host background was also reduced.

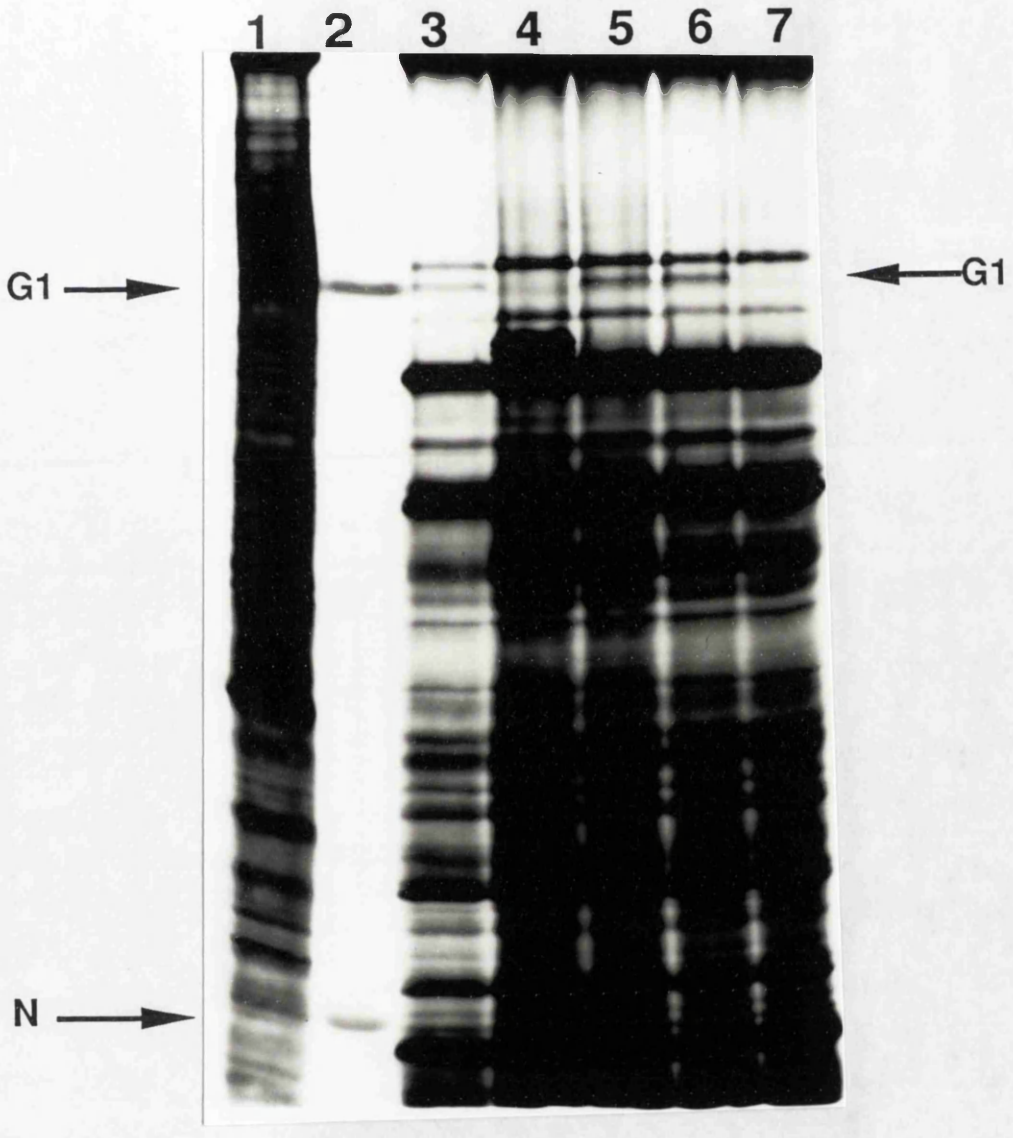


Figure 4.13

Figure 4.14 Expression of M gene in recombinant vaccinia virus.

CV-1 cells were infected with recBUN M and vTF7-3 at multiplicities of 10 pfu/cell, dually or individually. The cells were also mock infected or infected with BUN at 5 pfu/cell. The infected cells were labelled with ^{35}S-methionine in 35 mm dishes with 100µCi/dish at 24 hr post infection for 4 hrs. The cells were dissociated in protein dissociation mix, boiled and run in a 10% polyacrylamide gel. Lane 1 is the mock infected cell lysate. Lane 2 is the BUN infected, cell lysates, G1 and N proteins can clearly be identified. Lane 3 is the dual recBUN M/vTF7-3 vaccinia virus infected cell lysate and a protein that co-migrates with authentic BUN G1 can be seen. This protein is absent from the individually recBUN M and vTF7-3 infected cells, which are represented in lanes 4 and 5, respectively.

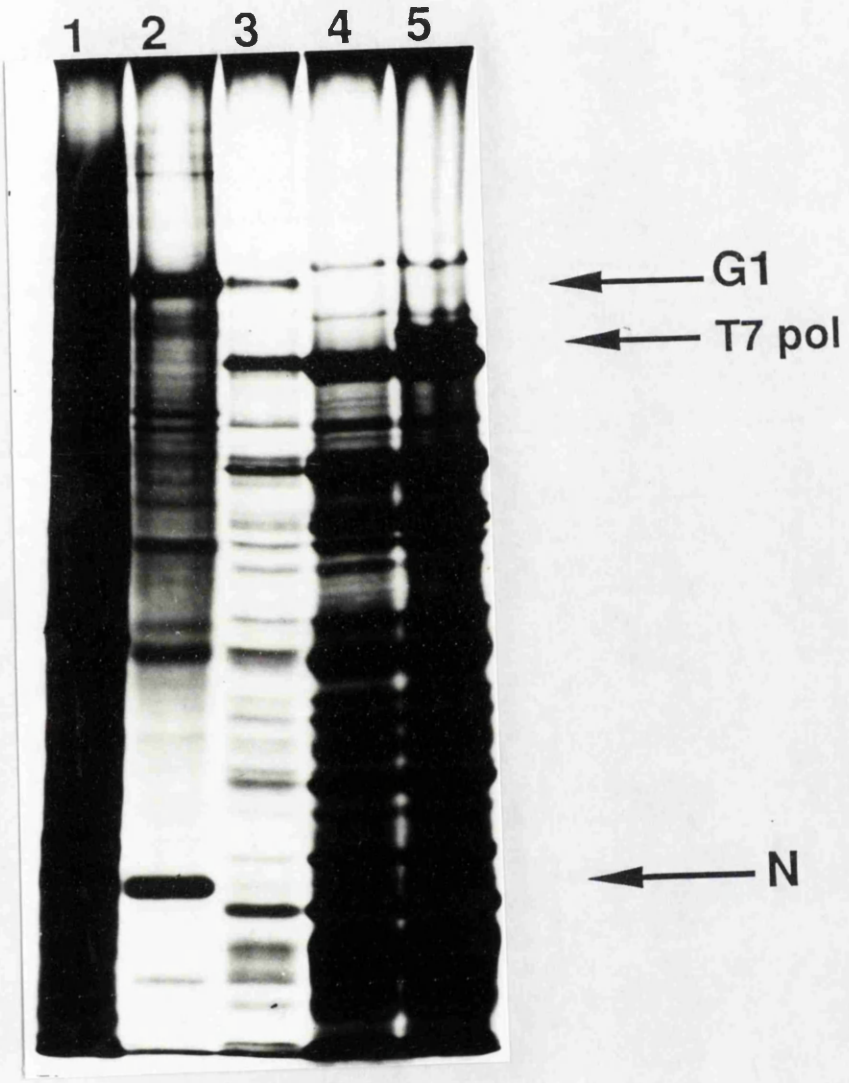


Figure 4.14

Figure 4.15 Western blot analysis of proteins synthesised in recBUN M and vTF7-3 recombinant dual expression system.

CV-1 cells were infected with BUN at 5 pfu/cell, with recBUN M and vTF7-3 at 10 pfu of each per cell and with recBUN M and vTF7-3 individually at 10 pfu/cell. Infected cells were harvested at 24 hrs post infection, lysates run in a 12% polyacrylamide gel and electrotransferred on a 0.45µm nitrocellulose filter. The filter was reacted with rabbit anti-BUN serum at a dilution of 1:100 for 30 min. The proteins were visualised by incubating the filter with anti-IgG horseradish peroxidase diluted 1:2,500 and reacted with 4-chloro-1-naphthol substrate. Lane 1 is mock infected cell lysate. Lane 2 is BUN infected cell lysate and G1 and N can clearly be seen. Lane 3 is the dual recombinant recBUN M and vTF7-3 infected cell lysate, and a protein that co-migrates with authentic BUN G1 can be seen. This protein is absent in lanes 4 and 5 which are recM BUN M and vTF7-3 infected cell lysates, respectively.

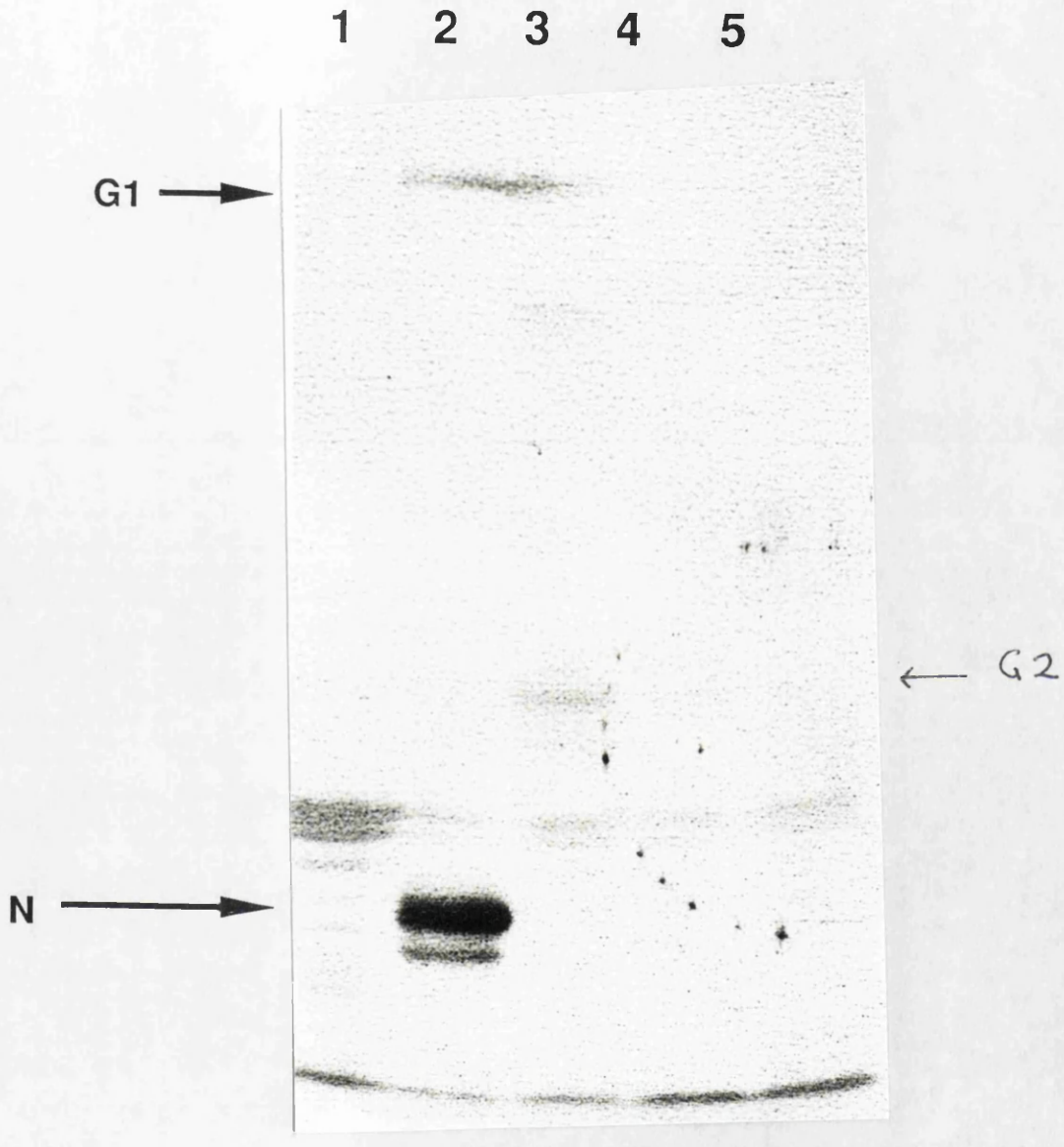


Figure 4.15

Figure 4.16 Radioimmunoprecipitation of recombinant vaccinia virus infected cells.

CV-1 cells were infected with BUN or dual recombinant vaccinia viruses recBUNM and vTF7-3. Cell lysates were precipitated with polyclonal anti-BUN serum or branched antipeptide sera M245 or M246. Lane 1 is mock infected whole cell lysate, lane 2 is mock infected cell lysate reacted with anti-BUN serum. Lane 3 is the whole cell lysate of BUN infected cells, G1, N and NSm can be identified. Lane 4 is the BUN infected cell lysate immunoprecipitated with anti-virion serum; G1 and N are recognised by the antiserum. Lane 5 is BUN infected cell lysate reacted with branched antipeptide serum M245; NSm is clearly precipitated by the antipeptide serum. Lane 6 is dual vaccinia virus lysates precipitated by anti-BUN serum, G1 is identifiable. Lane 7 is the dual vaccinia virus infected cell lysate. Lanes 8 and 10 are dual vaccinia virus infected cell lysates reacted with pre-bleed of M245 and M246, respectively. Lanes 9 and 11 are dual vaccinia virus infected cell lysates with the immune sera of M245 and M246, respectively; NSm is precipitated with the immune sera and not the non-immune.

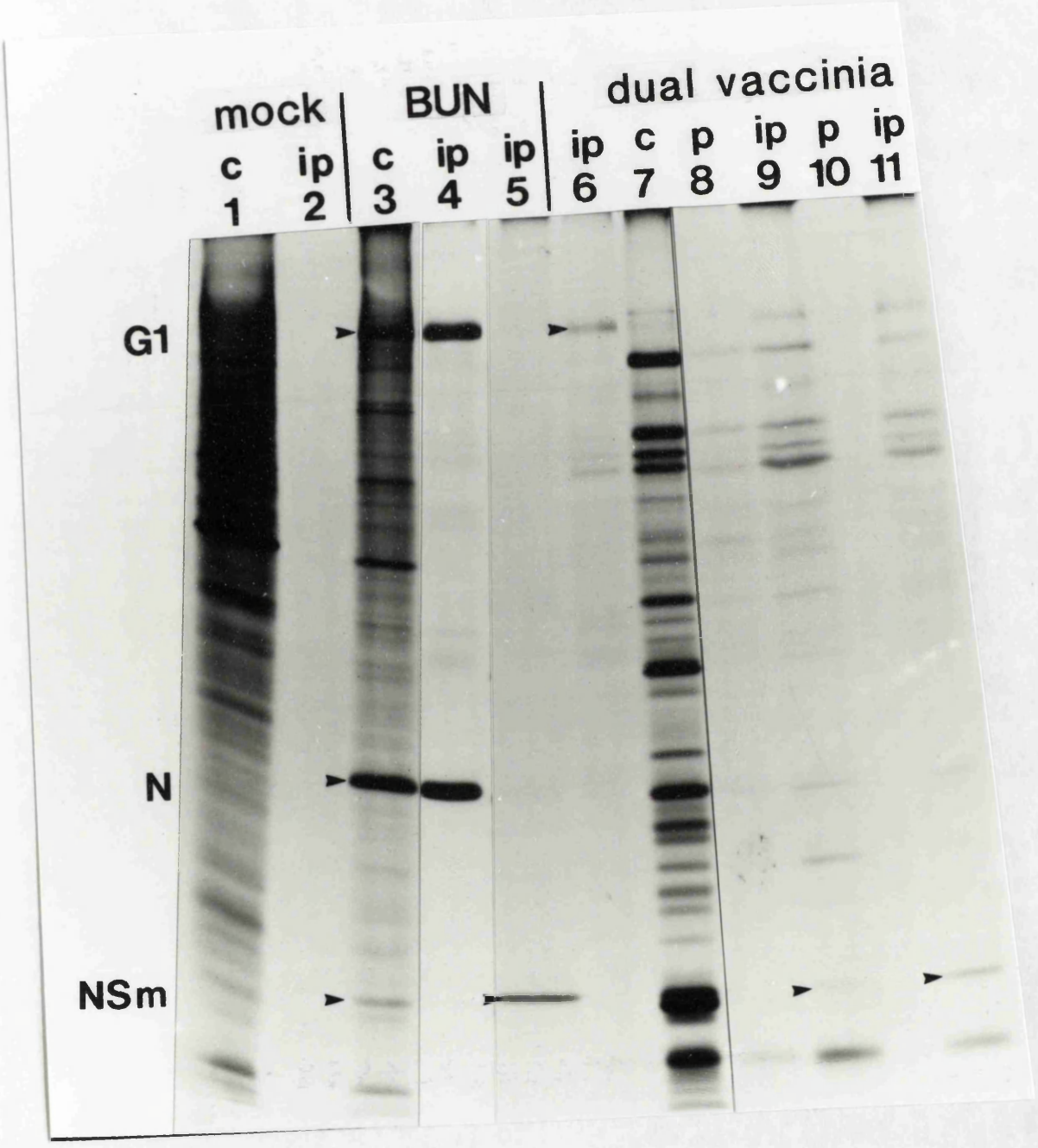

mock
BUN
dual vaccinia
c 1
ip 2
c 3
ip 4
ip 5
ip 6
c 7
p 8
ip 9
p 10
ip 11
G1
N
NSm

Figure 4.16

from dual vaccinia virus infected cells by anti-BUN serum (lane 6) but G2 was not detected in either recombinant vaccinia virus or BUN-infected cells (lanes 4 and 6). The anti-peptide sera precipitated NSm from dual vaccinia virus infected cell extracts (peptide M245, lane 9 and M246, lane 11). There was a marked background precipitation of mainly vaccinia virus proteins. However, comparison with the pattern given by the preimmune serum (lanes 8 and 10) suggests that the precipitation of NSm is specific to the immune serum. Some G1 was also precipitated from the recombinant vaccinia virus infected cells by the NSm serum; this was not observed using the BUN infected cell lysate (lane 5). This result demonstrated that the polyprotein precursor was made and properly processed.

Transient expression of M, G1, G2 and NSm genes of Bunyamwera virus

An alternative expression system, which obviates the need to make a recombinant vaccinia virus, is that described by Fuerst *et al* (1986; 1987) which involves transfection of cells with T7 promoter plasmid DNA and infection with the recombinant vaccinia virus which synthesises bacteriophage T7 RNA polymerase. Constructs of the full length M segment cDNA and its truncations representing G1, G2 and NSm were made by placing the genes under the control of the bacteriophage T7 promoter.

Plasmid DNA containing a particular BUN M cDNA was introduced into the cytoplasm of cells infected with vaccinia virus vTF7-3 which synthesises T7 RNA polymerase, as described in the Methods Section. Cells were radiolabelled at 4 hr post-transfection with ^{35}S-methionine for 4 hr or processed for indirect immunofluorescence at 8 hr post-transfection.

Construction and transient expression of the full length M cDNA under control of T7 RNA promoter

The M segment cDNA, under the control of the T3 promoter in the pT7T319U transcription vector, was removed by restriction enzmye digestion with SmaI and PstI. The fragments were separated in preparative 1% agarose gel. The gel slice with the fragment of interest was excised and the DNA purified by the silica matrix adsorption and elution method as described in the Methods Section. A pT7T318U SmaI/PstI vector was prepared and the fragments ligated using T4 DNA ligase (Fig 4.17). This "forced cloning" placed the M cDNA under control of the T7 promoter.

A construct with insert in the correct orientation was transiently expressed in CV-1 cells and proteins synthesised were characterised by radioimmunoprecipitation. As can be seen in Fig 4.18, the G1 glycoprotein transiently expressed from the full length M cDNA was recognised by anti BUN serum (lane 6). The transiently expressed G1 also co-migrated with authentic G1 immunoprecipitated in BUN infected cell extracts (lane 4). The G2 glycoprotein could not be seen for reasons already mentioned above. The NSm protein would have come off the gel and cannot, of course, be recognised by the anti BUN serum in immunoprecipitation reactions.

Construction of pT7T3/G1 and transient expression of G1

The predicted sequence of the G1 gene has the amino terminal end at nucleotide 1485 and carboxy end at nucleotide 4358. A fragment containing all G1 coding sequences would have been obtained by digesting the BUN M cDNA with BamHI and PstI which cut at nucleotides 1352 and 4458, but there is another BamHI site at nucleotide 4001. Hence, pT7T319U was digested with BamHI and SalI and ligated with BamHI/SalI BUN M cDNA fragment from bases 1352 to 1641. This construct was then used to insert the rest of the cDNA

Figure 4.17 Construction of BUN M cDNA under the control of the T7 RNA promoter.

The BUN M cDNA which previously was under the control of the T3 promoter in the pT7T319U vector was removed by digestion with SmaI and PstI and ligated into the SmaI/PstI site of pT7T318U vector.

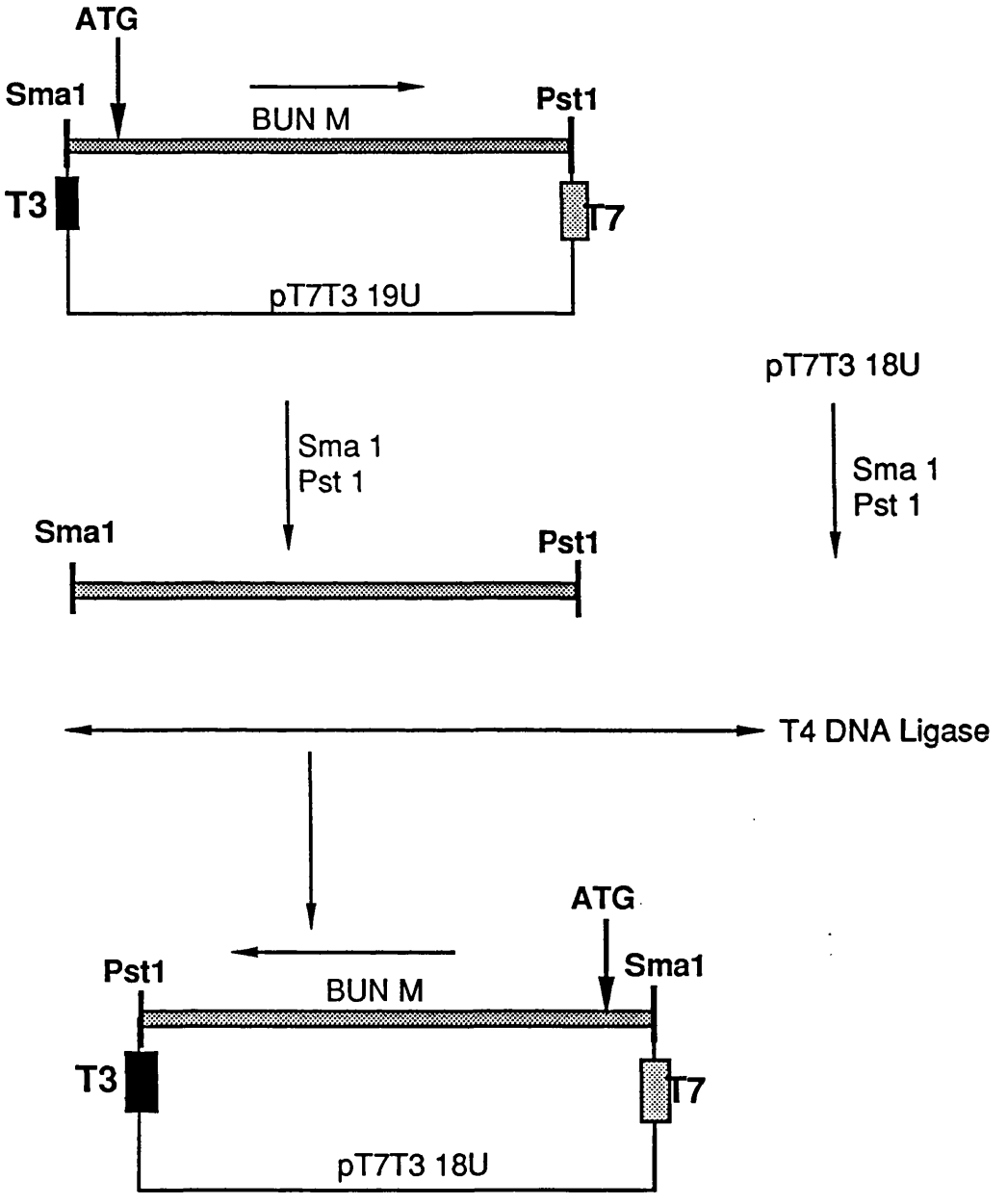

ATG
Sma1
BUN M
Pst1
T3
T7
pT7T3 19U
pT7T3 18U
Sma 1
Pst 1
Sma1
Pst1
Sma 1
Pst 1
T4 DNA Ligase
ATG
Pst1
BUN M
Sma1
T3
T7
pT7T3 18U

Figure 4.17

Figure 4.18 Transient expression of the full length BUN M cDNA.

The full length BUN M cDNA under the control of the bacteriophage T7 RNA promoter was transfected into CV-1 cells infected with the vTF7-3 vaccinia virus. Cells were labelled 4 hr post transfection with ^{35}S-methionine for 4 hr and analysed by immunoprecipitation using anti-BUN serum. Lane 1 is mock infected whole cell lysate, lane 2 is mock infected cell lysate reacted with anti-BUN serum. Lane 3 is BUN infected whole cell lysate, lane 4 is BUN-infected cell lysate precipitated with anti-BUN serum; G1 and N can be clearly identified. Lane 5 is the whole cell lysate of transfected cells and lane 6 is the immunoprecipitate of material from lane 5; the G1 glycoprotein is identifiable.

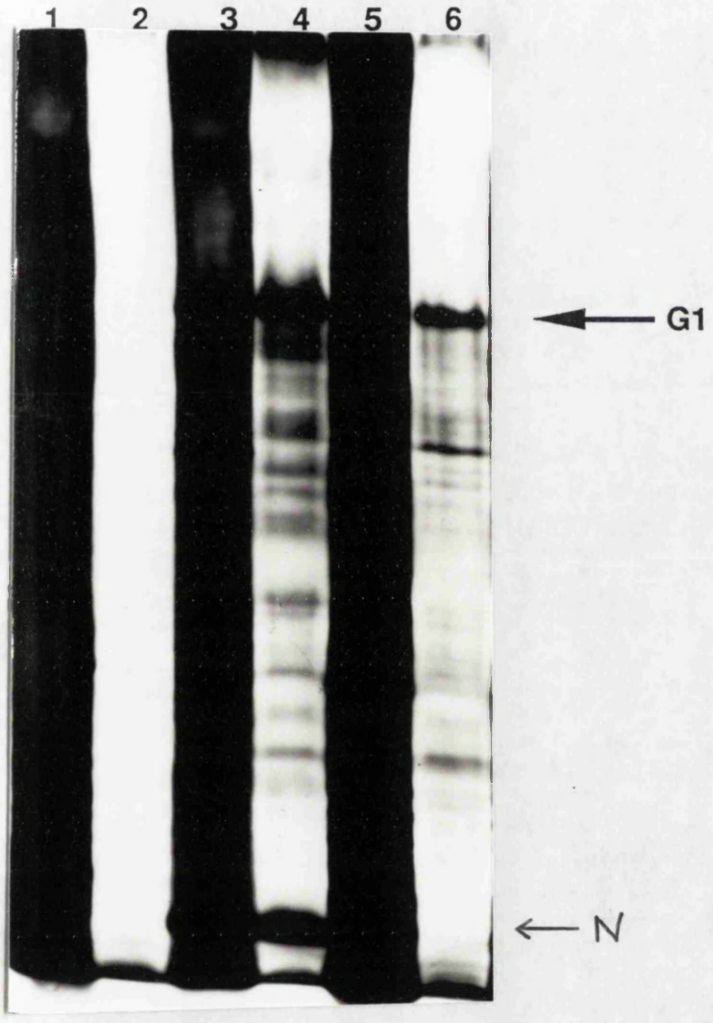


Figure 4.18

which encodes G1, i.e. from SalI (1641) to PstI (4458) with an in-frame ATG at bases 1430-1432 (Fig 4.19).

pT7T318U DNA was cut with SmaI and PstI and a SmaI/PstI G1 fragment (bases 1352-4001) from pT7T319U BUN M was obtained and ligated with the vector. This enabled the G1 fragment to be placed under the control of the T7 RNA promote in pT7T318U (Fig 4.19). The G1 protein was efficiently expressed, as shown in Fig 4.20, lane 8. The protein was precipitated by the anti-virion serum and co-migrated with G1 made by the virus (lane 4) and by recombinant dual vaccinia virus (lane 6).

A time course of the transient expression of G1 was carried out and samples prepared for radioimmuno-precipitation at 4, 6, 9 and 12 hrs post infection. The highest amounts of G1 were observed at 4hr and decreased thereafter (Fig 4.21).

The pT7T318U/G1 construct with a deletion from nucleotide 4326 (made by digestion with Hind III and religating) was transiently expressed, as described above (Fig 4.23, lane 8). The protein is slightly smaller than G1 made by BUN (lane 4) transiently expressed (G1, lane 6).

Effect of tunicamycin on glycosylation of G1

CV-1 cells in 33mm dishes were infected in duplicate with BUN at 5pfu/cell or with dual vaccinia viruses at 10pfu/cell each. The G1 glycoprotein was also transiently expressed via vTF7-3 plasmid transfection, as described above. After absorption of BUN and dual vaccinia viruses and after transfection with the G1 construct under control of T7 RNA promoter, Eagle's medium containing 5µg/ml of tunicamycin (a glycosylation inhibitor) was added to one set of dishes. The dishes were incubated at 31°C for BUN and 37°C for vaccinia viruses. The medium was removed the following day and cell lysates processed for radio-

Figure 4.19 Construction of G1 under the control of T7 RNA promoter in pT7T318U.

A BamHI/SalI fragment of BUN M cDNA (nt 1352-1641) was ligated into BamHI/SalI linearised pT7T3 19U. This construct was linearised with SalI/PstI and ligated with the SalI/PstI (nt 1642-4656) fragment of BUN M cDNA. The BUN M insert was removed by digestion with SmaI/PstI and cloned into the SmaI/PstI site of pT7T318U, so that G1 mRNA would be transcribed from the T7 promoter.

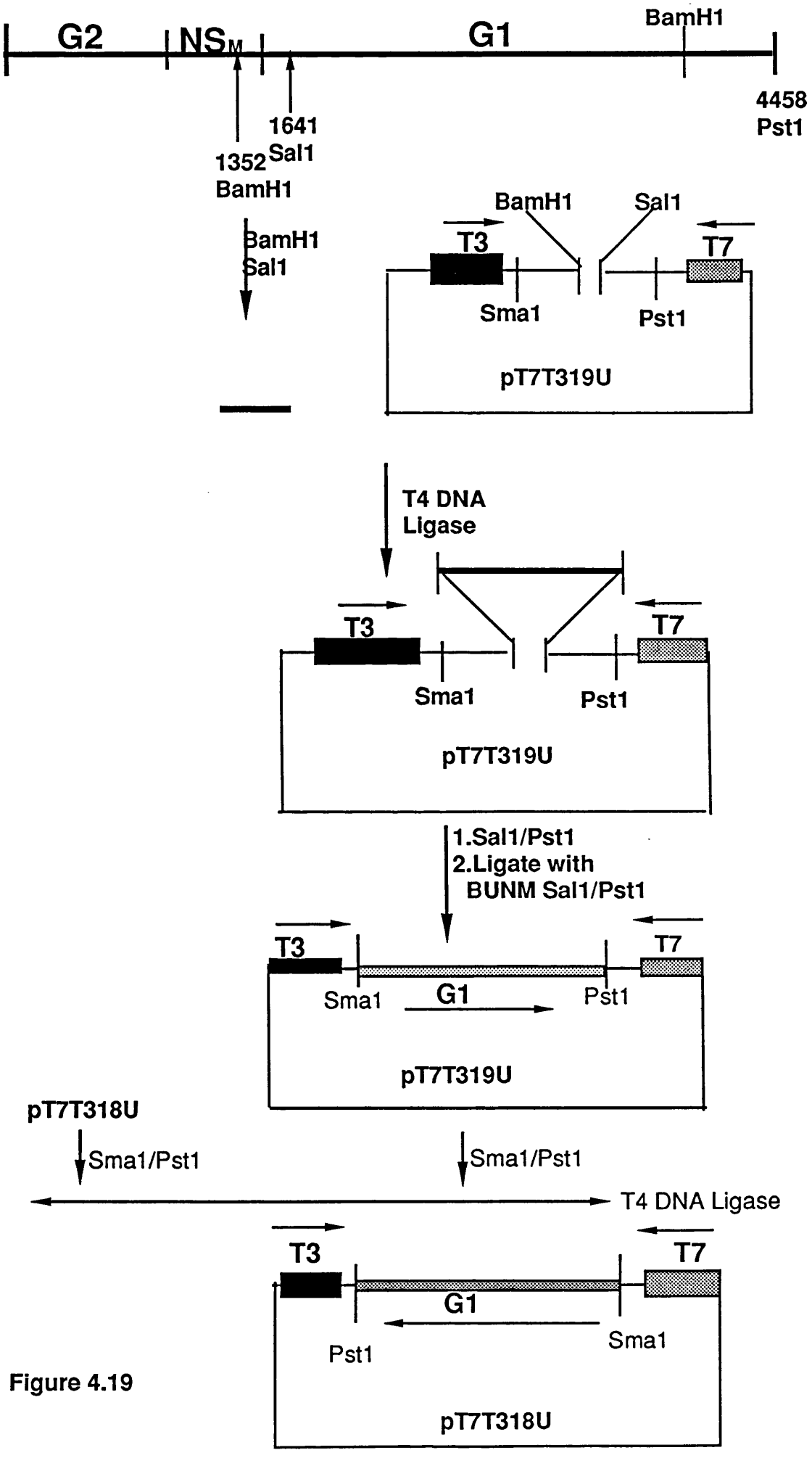

G2
NSM
G1
BamH1
4458
Pst1
1641
Sal1
1352
BamH1
BamH1
Sal1
BamH1
Sal1
T3
T7
Sma1
Pst1
pT7T319U
T4 DNA
Ligase
T3
T7
Sma1
Pst1
pT7T319U
1.Sal1/Pst1
2.Ligate with
BUNM Sal1/Pst1
T3
T7
Sma1
G1
Pst1
pT7T319U
pT7T318U
Sma1/Pst1
Sma1/Pst1
T4 DNA Ligase
T3
T7
G1
Pst1
Sma1
pT7T318U

Figure 4.19

Figure 4.20 Transient expression of G1.

CV-1 cells in 35 mm dishes were infected with recombinant vaccinia virus vTF7-3 at MOI of 10 pfu/cell. The infected cells were transfected with plasmid pT7T318UBUNG1 and labelled with ^{35}S-methionine at 4 hr post transfection for 4 hr. One tenth of the cells were dissociated in protein dissociation mix and the remainder was processed for immunoprecipitation with anti-BUN serum. Samples were analysed in a 10% polyacrylamide gel. Whole cell lysates are shown of mock (lane 1), BUN (lane 3), dual vaccinia virus infected (lane 5), vTF7-3 infected and transfected (lane 7), and vTF7-3 infected (lane 9) cells. Immuno-precipitates are shown of of mock (lane 2), BUN (lane 4), dual vaccinia virus infected (lane 6), vTF7-3 infected and transfected (lane 8), and vTF7-3 infected (lane 10) cell lysates. It can be seen that a protein which co-migrated with G1 protein in BUN infected and dual vaccinia virus infected cells was also synthesised in the transiently expressed system for G1 and this protein was recognised by BUN antiserum (lane 8).

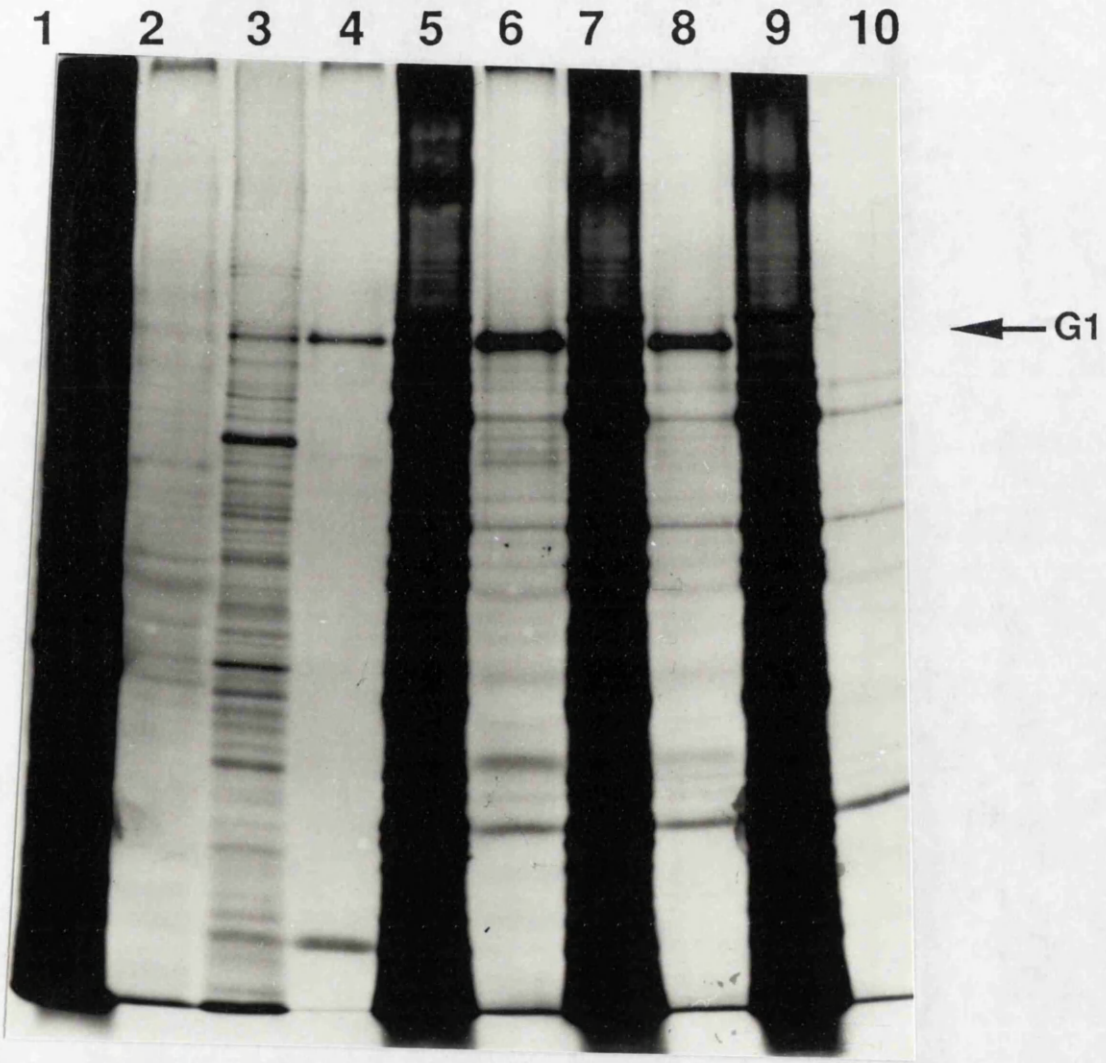


Figure 4.20

Figure 4.21 Time course of transiently expressed G1.

Near confluent CV-1 cells in 35 mm dishes were infected with vaccinia virus vTF7-3 for 30 mins in OptiMEM and transfected with 2.5µg of the plasmid construct pT7T318UBUNG1. Cells were labelled with ^{35}S-methionine at 4, 6 and 12 hrs for 4 hrs. One tenth of the cells were dissociated in protein dissociation mix and the remainder was processed for radioimmunoprecipitation reactions, as described in the Methods section. Labelled and immunoprecipitated cell lysates were run in 10% polyacrylamide gel and the gel processed for autoradiography. Lanes 1, 3 and 5 are whole cell extracts labelled at 4, 6 and 12 hrs, respectively. Lanes 2, 4 and 6 are the corresponding immunoprecipitated cell lysates using the anti-BUN polyclonal serum. The G1 glycoprotein was detected at 4 hrs post transfection and the rate of synthesis decreased after that. Lane 7 is the vaccinia virus vTF7-3 infected cell lysate and lane 8 is the immunoprecipitate of vTF7-3 infected cells with anti-BUN serum.

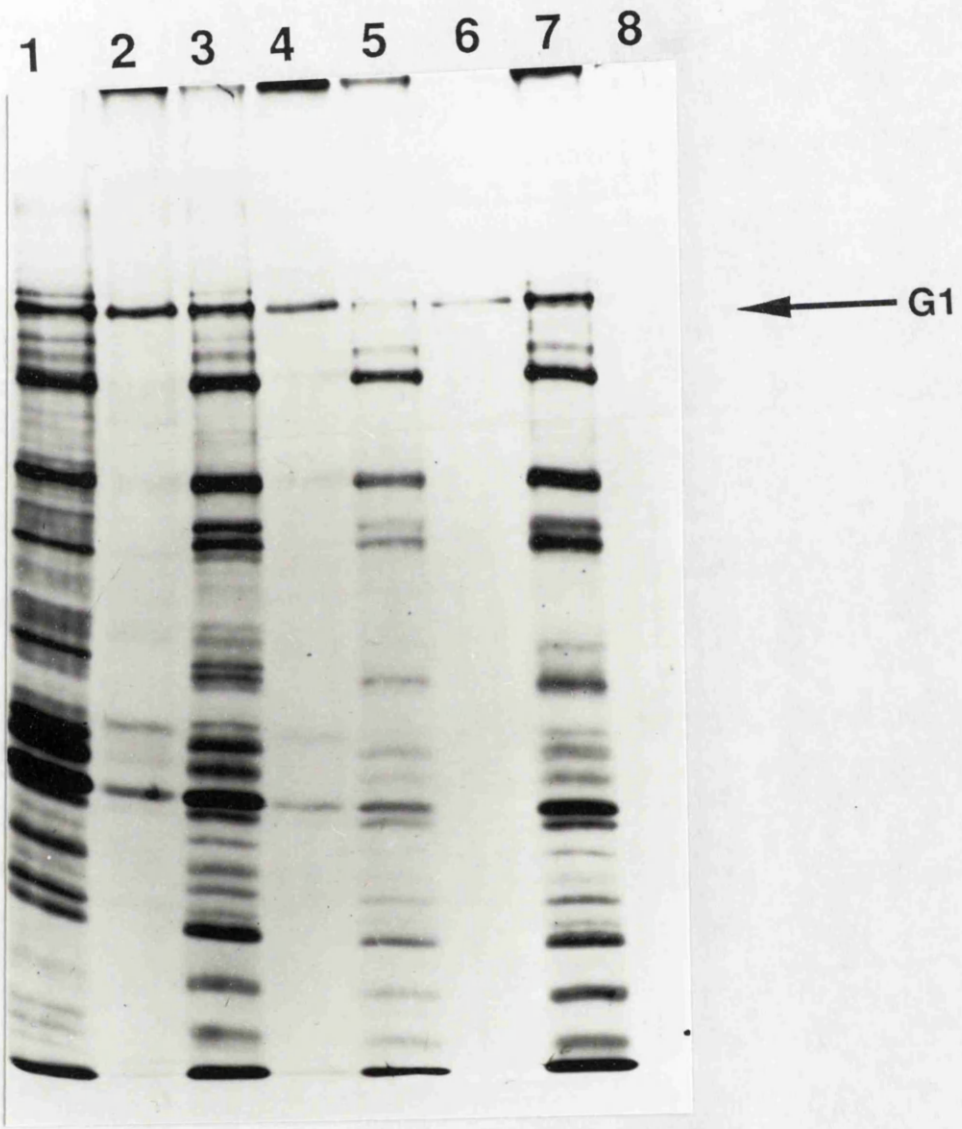


Figure 4.21

immunoprecipitation using the anti-BUN serum (Fig 4.22). The G1 glycoprotein synthesized by dual vaccinia virus (lane 4), or transiently expressed (lane 5), co-migrated with the BUN synthesized G1 (lane 3). There was no similar protein synthesized in mock-infected cell lysates (lane 2).

In the presence of tunicamycin a faster migrating form of G1, $G1_0$, was immunoprecipitated from BUN infected (lane 7), dual vaccinia virus infected (lane 8) and transfected (lane 9) cell lysates. This result shows that G1 expressed by dual recombinant vaccinia viruses or by the transient expression system was properly glycosylated, as demonstrated by the identical size with BUN synthesised G1.

Construction of pTF7-5/G2 and transient expression G2

The first attempt to clone an M cDNA fragment representing G2 was by digesting pT7T3pm11d11 with BamHI which cuts at a site at the 5' end of the M cDNA in the polylinker and with BclI which cuts at nucleotide 991 in the M cDNA. This fragment would then be cloned into the BamHI site of pTF7-5 by sticky-end ligations as the BclI site will ligate into a BamHI site, although neither site would be recreated after ligation. Problems were encountered when I tried to cut the cDNA with BclI. This was explained by the possible effect of dam methylase which introduces methyl groups at the N^6 position of adenine in the sequence ^{5}'GATC^3' (Hattman *et al*, 1978). This renders the enzyme BclI unable to cut its recognition site TGATCA (Maniatis *et al*, 1982). Attempts were made to digest pT7T3/pm11d11 grown in *E.Coli* GM48 cells, which are dam$^-$, but only partial digestion could be obtained even with prolonged incubation.

The strategy to obtain a G2 M cDNA fragment to clone in pTF7-5 was changed and involved cutting pT7T3/p11d11 with SmaI which is at the 5' end of G2 in the polylinker and DraI which cuts at nucleotide 941 and eight amino acids short of

Figure 4.22 Glycosylation of the G1 protein.

CV-1 cells in 35 mm dishes were mock-infected, infected with BUN at 5 pfu/cell, or infected with dual vaccinia viruses at 10 pfu/cell each. The G1 glycoprotein was also transiently expressed via the vTF7-3 vaccinia system. Two dishes were prepared for each infection. After absorption Eagles medium without methionine was added to one set of dishes and contained 5µg/ml of tunicamycin with 100µCi per dish of ^{35}S-methionine. The other set contained ^{35}S-methionine but no tunicamycin. The dishes were incubated at 31°C for BUN infected cells and at 37°C for vaccinia viruses. The medium was removed the following day and 10% of the cells were dissociated in protein dissociation mix and the remainder was prepared for radioimmunoprecipitations using anti-BUN serum. Samples were analysed in 10% polyacrylamide gel. Track 1 is whole mock-infected cell lysate with tunicamycin. Tracks 2 and 6 are immunoprecipitates (IPs) of mock infected lysates and mock infected lysates with tunicamycin, respectively. Tracks 3 and 7 are BUN infected lysates and BUN infected lysates with tunicamycin, respectively. Tracks 4 and 8 are dual vaccinia virus infected and dual vaccinia virus infected with tunicamycin, respectively. Tracks 5 and 9 are transiently expressed G1 and transiently expressed G1 in the presence of tunicamycin, respectively. The BUN antiserum recognised the G1 glycoprotein in both tunicamycin and tunicamycin untreated samples. The G1 protein was the same size whether expressed by BUN, dual vaccinia virus, or transiently expressed. The G1 protein in tunicamycin treated samples (G1$_o$) migrated faster than in untreated samples.

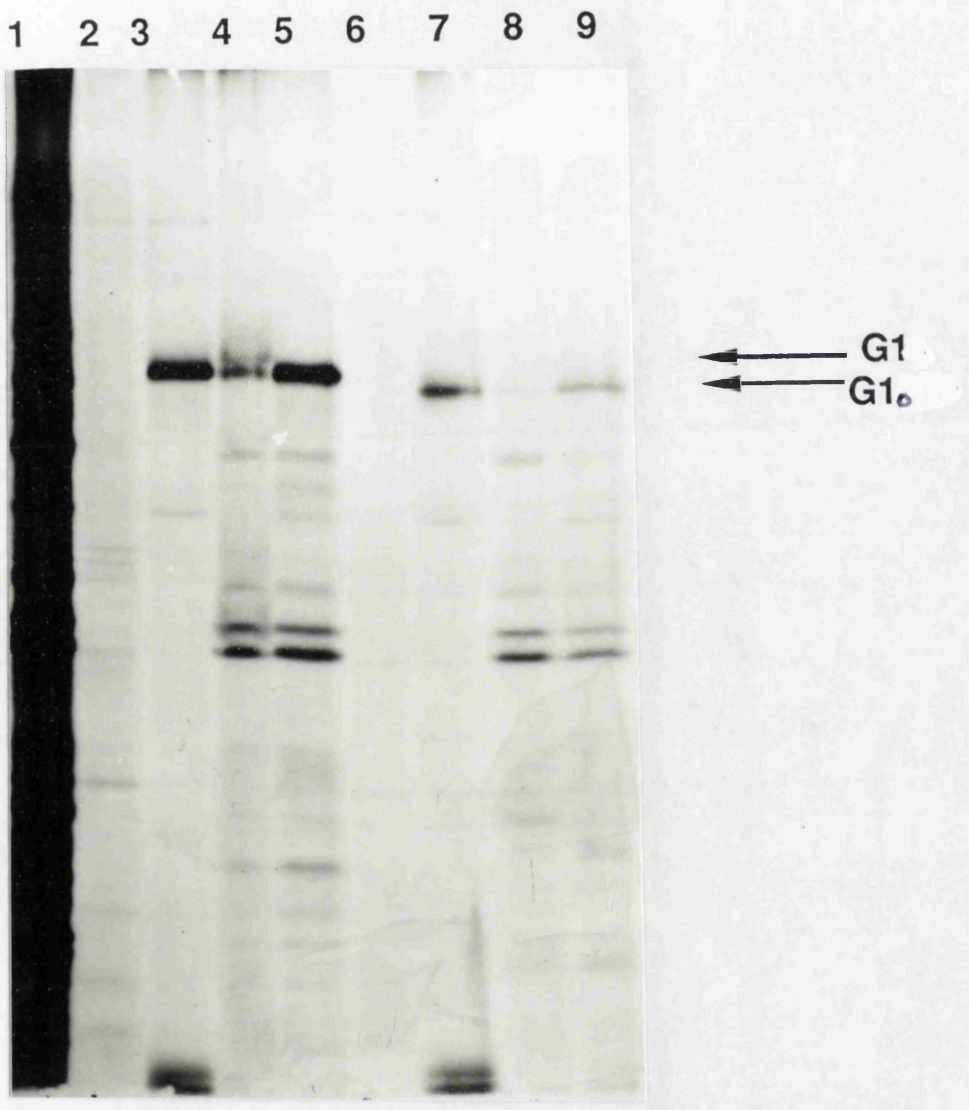


Figure 4.22

Figure 4.23 Transient expression of G1 with a deletion at the carboxy-terminus.

The pT7T318U plasmid containing G1 specific sequences under control of T7 RNA promoter was digested with HindIII which cuts at nucleotide 4326 in the G1 gene and in the vector polylinker, and religated. The deleted G1 was transiently expressed and labelled with ^{35}S-methionine, as described in the Methods section. Lane 1 is whole mock infected CV-1 cell lysate. Lanes 2, 4, 6, 8 and 10 are immunoprecipitates using anti-BUN serum of mock infected, BUN infected, transiently expressed G1, transiently expressed truncated G1 and vTF7-3 infected cell lysates, respectively. Lanes 3, 5, 7 and 9 represent whole cell lysates of BUN infected, G1 expressed, truncated G1 expressed and vTF7-3 infected, respectively. The truncated G1 in lanes 7 and 8 is slightly smaller than the full length G1 in lanes 3, 4, 5 and 6.

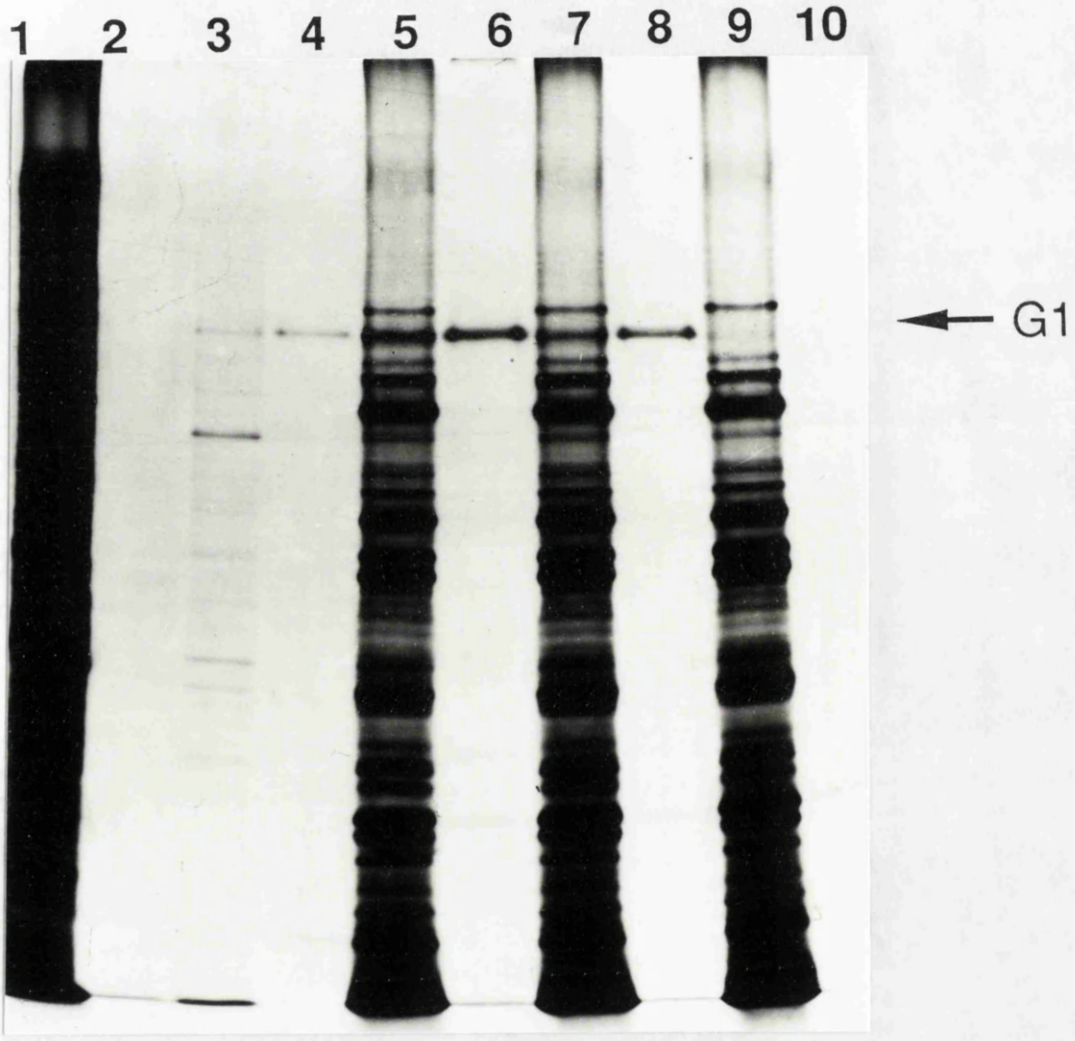


Figure 4.23

the predicted boundary of G2 and NSm. This fragment was purified by electrophoresis and electroelution in agarose gel followed by phenol/chloroform extraction and ethanol precipitation. The G2 fragment was ligated into filled-in BamHI site of pTF7-5 (Fig 4.24). Miniprep analyses were performed to demonostrate presence of insert by restriction digestion with EcoRI which would give a pattern of two bands (116 and 5115 base pairs) in agarose gels. Candidates with inserts were further digested with HindIII and EcoRI in order to determine the orientation of insertion. The correct orientation would give fragments of 3.464, 0.861 and 0.116 kb and the wrong orientation would give 1.554, 2.771 and 0.116 kb fragments. Out of 36 colonies picked, nine had inserts but only one had inserted in the right orientation as demonstrated by HindIII digestion and plasmid dideoxy DNA seqencing (Fig 4.25).

Recombinant vaccinia viruses were made with G2 as shown by DNA dot blots (Fig 4.26) but it was difficult to demonstrate its expression as radioimmunoprecipitations consistently failed to show G2 even in BUN infected cell lysates. The G2 fragment in pTF7.5 under the control of the T7 RNA promoter was transiently expressed using the vaccinia vTF7-3 system and expressed products analysed by indirect immunofluorescence, using the BUN polyclonal antiserum. Fluorescence was observed in transfected cells (Fig 4.27A) and not in mock-infected (Fig 4.27B), showing that G2 was actually synthesised. This was interesting as the same antiserum failed to detect G2 in radioimmunoprecipitations of BUN infected cell lysates and in recombinant dual vaccinia viruses expression systems.

The construct containing NSm with its own ATG was made by PCR and obtained from Dr Richard Elliott. Details of construction and expression of NSm under the control of the T7 RNA promoter are included in the next chapter.

Figure 4.24 Construction of pTF7-5/G2.

The fragment containing most of the sequences of G2 was obtained by digesting pT7T3pm11d11 with SmaI which cuts to the 5' end of G2 in the vector polylinker and DraI which cuts at nucleotide 941 in BUN M cDNA. The fragment was ligated into the Klenow filled-in BamHI site of pTF7-5.

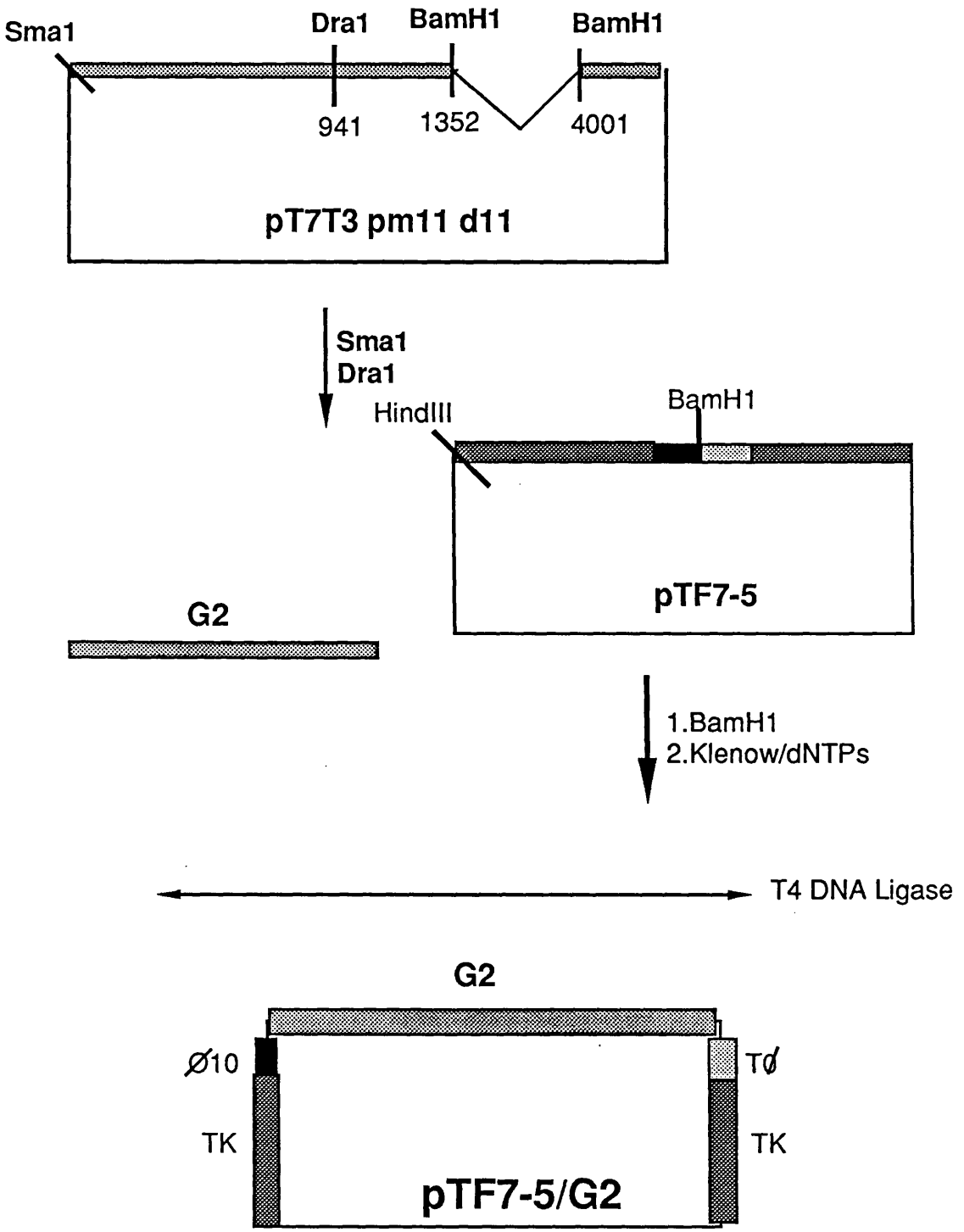

Sma1
Dra1
BamH1
BamH1
941
1352
4001
pT7T3 pm11 d11
Sma1
Dra1
HindIII
BamH1
pTF7-5
G2
1.BamH1
2.Klenow/dNTPs
T4 DNA Ligase
G2
Ø10
TØ
TK
TK
pTF7-5/G2

Figure 4.24

Figure 4.25 Determination of orientation of insertion of G2 in pTF7-5.

The orientation of G2 under the control of the bacteriophage T7 RNA promoter in the pTF7-5 vaccinia virus transfer vector was determined by plasmid dideoxy sequencing using T7 DNA polymerase, as described in the Methods section. Tracks are GATC from left to right.

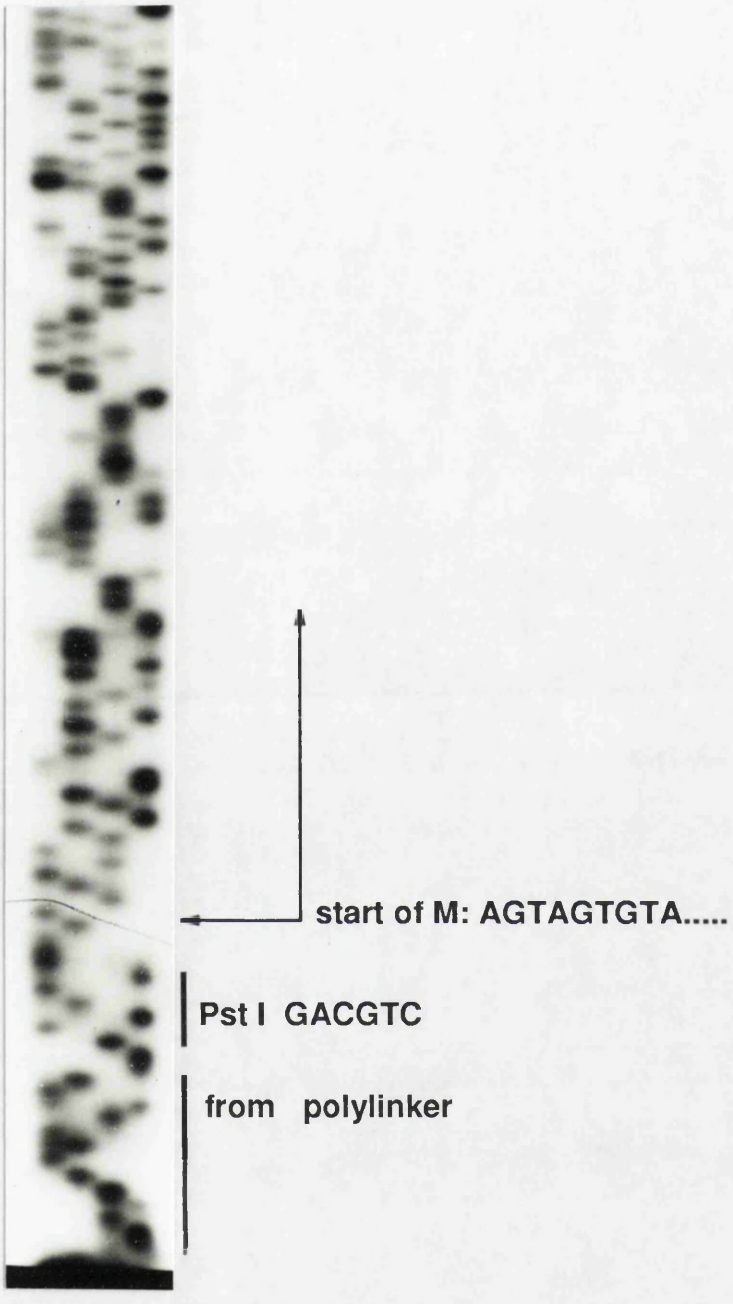
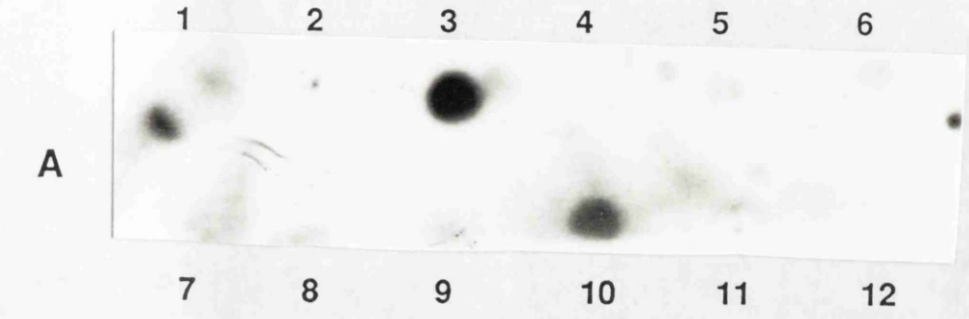


Figure 4.25

Figure 4.26 Characterisation of vaccinia virus recombinants containing G2 by dot blot hybridization.

Total cellular DNA was extracted from cells infected with putative recombinants (which had exhibited c.p.e. in TK-143 cells in the presence of BUdR) and analysed by dot blot hybridization. Fig 4.26A shows DNA transferred onto nitrocellulose paper and probed by ^{32}P-labelled pT7T3BUNM DNA. Fig 4.26B shows the same samples probed by ^{32}P-labelled pTF7-5 DNA. Plaques 3 and 10 gave a positive signal.



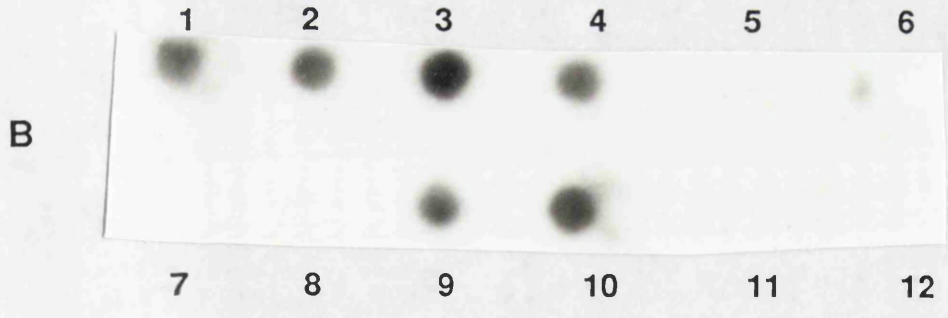
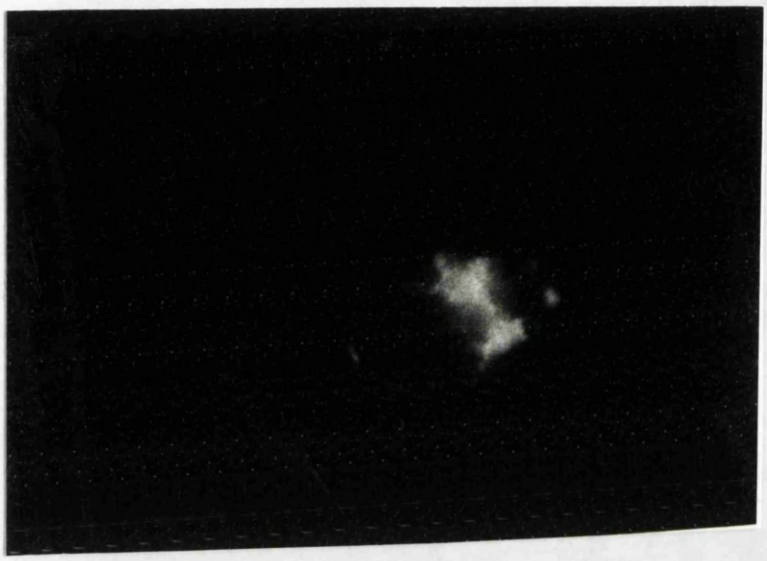


Figure 4.26

Figure 4.27 Detection of transiently expressed G2.

HeLa $T4^+$ cells infected with vTF7-3 were transfected with pTF7-5/G2 plasmid DNA and processed for immunofluorescence using anti-BUN serum, as described in the Methods section. Fig 4.27A shows staining of cells transfected with pTF7-5/G2, and Fig 4.27B are cells infected with vTF7-3 alone.

A



B

Figure 4.27

Results from this section show that the BUN M RNA can be translated *in vitro* if some sequences at the 5' end are deleted. It was possible to express full length M segment RNA in dual vaccinia virus and in transient expression systems. The G1, G2 and NSm proteins could also be expressed individually in transient expression systems.

SECTION THREE

INTRACELLULAR LOCALIZATION OF PROTEINS ENCODED BY BUN M RNA SEGMENT

In order to study the intracellular transport and targetting of the G1 and G2 glycoproteins and NSm to the Golgi complex, permeabilized infected cells were stained with specific antisera and fluorescein isothiocyanate conjugated sheep anti-rabbit IgG, counterstained with rhodamine-conjugated wheat germ agglutinin (WGA) which predominantly stains the Golgi complex. Fluorescence studies were done on cells infected with vTF7-3 and recBUN M, which express the full length BUN M cDNA, and on cells expressing G1, G2 and NSm individually in the transient system, described by Fuerst *et al* (1986).

Localisation of proteins in dual vaccinia virus expression system

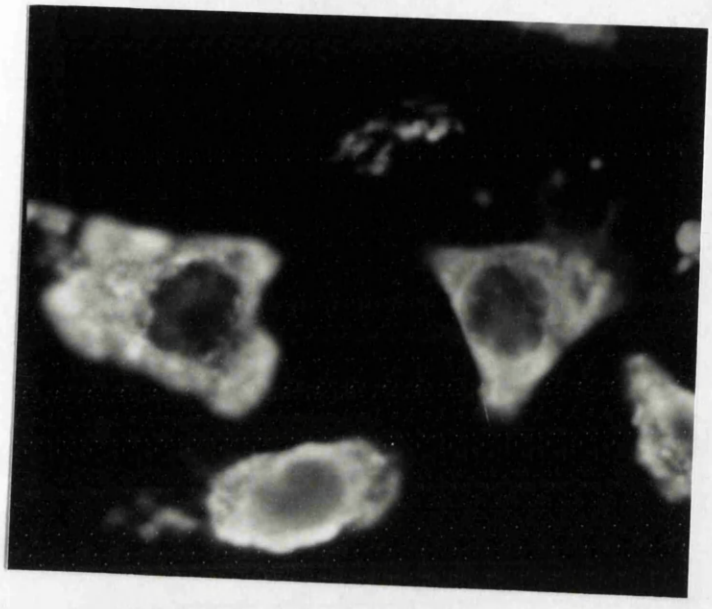
Subconfluent monolayers of HeLa T4$^+$ cells were co-infected with the recombinant vaccinia virus containing the full length M cDNA and vTF7-3, the vaccinia virus which expresses T7 RNA polymerase, as detailed in Materials and Methods. Cells were fixed with 3.7% formaldehyde and processed for indirect immunofluorescence using the anti-BUN serum which recognises both G1 and G2. Cells were also stained with the antipeptide serum to NSm. Controls included BUN, vTF7-3 and mock infected monolayers. The pattern of immunofluorescent staining of BUN-infected HeLa T4$^+$ cells (Fig 5.1A) was cytoplasmic but not localised particularly to the Golgi region of the cells. This was probably because at this relatively late time after infection (8 hpi) the cytoplasm contained mature virions in the Golgi-derived membrane vesicles en route to being released. Similar observations have been made on HeLa T4$^+$ cells infected with PT (Matsuoka *et al*, 1988). Fig 5.1B shows the pattern of staining by WGA in uninfected HeLa T4$^+$

Figure 5.1

Fig 5.1A shows fluorescence of HeLa $T4^+$ cells infected with BUN. HeLa $T4^+$ cells were processed for indirect fluorescence at 8hr pi with anti-BUN serum and fluorescein isothiocyanate conjugated anti-rabbit IgG, as already described. The fluorescence is generally cytoplasmic.

Fig 5.1B shows the fluorescence pattern of HeLa $T4^+$ cells after direct labelling with rhodamine conjugated wheat germ agglutinin. The cells were viewed and photographed using the red filter at wavelength 546nm. The fluorescence pattern is perinuclear and localises in the Golgi complex.

A



B

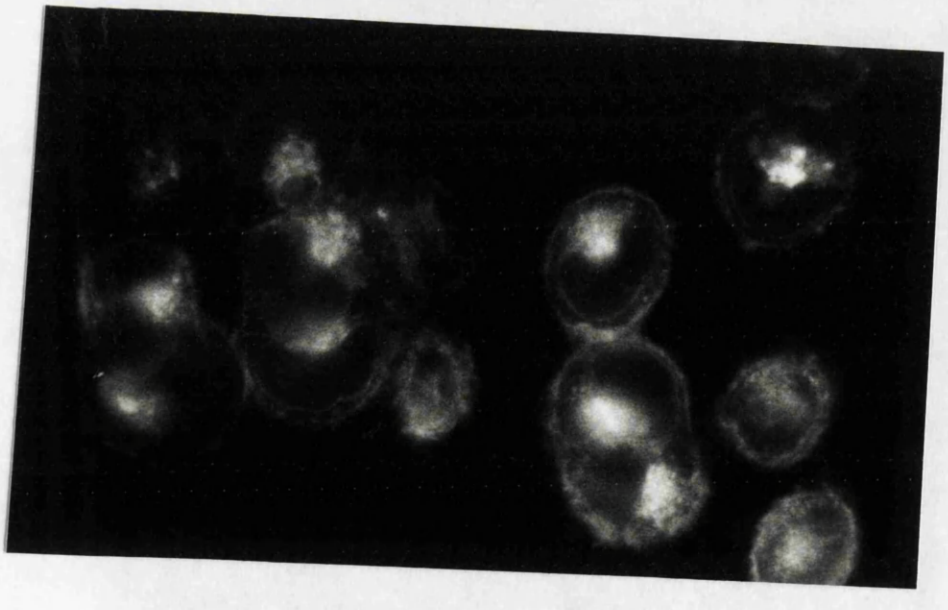


Figure 5.1

cells, and is typical for the perinuclear pattern associated with the Golgi complex. In the dual vaccinia virus infected cells (Fig 5.2A) there was strong perinuclear fluorescence indicating the localisation of the expressed proteins to the Golgi complex. No fluorescence was detected in MI cells or in cells infected with recombinant vaccinia virus vTF7-3 alone (Figs 5.2B and 5.2C, respectively).

The branched antipeptide sera to NSm recognised NSm in recombinant dual infected cells. The pattern of staining was perinuclear (Fig 5.3A) and coincided with the staining pattern given by rhodamine conjugated WGA (Fig 5.3B).

Localisation of G1

pT7T318U containing the G1 coding sequences under the control of the T7 promoter (Fig 4.19) was used to express G1 transiently, as described above. Transfected cells were processed for indirect immunofluorescence using anti-BUN serum as the primary antibody. The G1 glycoprotein was efficiently expressed, as can be seen in Fig 5.4A. The distribution of fluorescence was generally cytoplasmic and not particularly localized to the Golgi. This pattern of staining is similar to that reported for the ER (Lippincott-Schwartz *et al*, 1989).

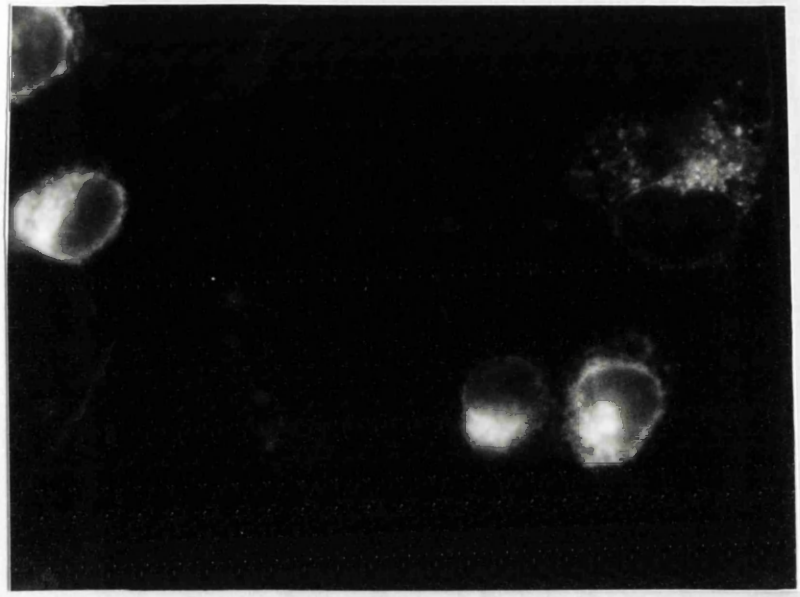
Localisation of G2

The G2 coding sequences were cloned in the vaccinia virus transfer vector pTF7-5 under the control of the bacteriophage ø10 promoter and øTO terminator (Fig 4.24). I was not confident that I would actually observe G2 specific immunofluorescence, since previous reports indicated that G2 was difficult to detect by immunoprecipitation in cell lysates (Elliott, 1985; Watret, 1985). However, the result shown in Fig 5.4B indicated that G2 was synthesised, as cells were stained by anti-BUN serum. The distribution of G2 in cells was perinuclear similar to that of NSm in BUN

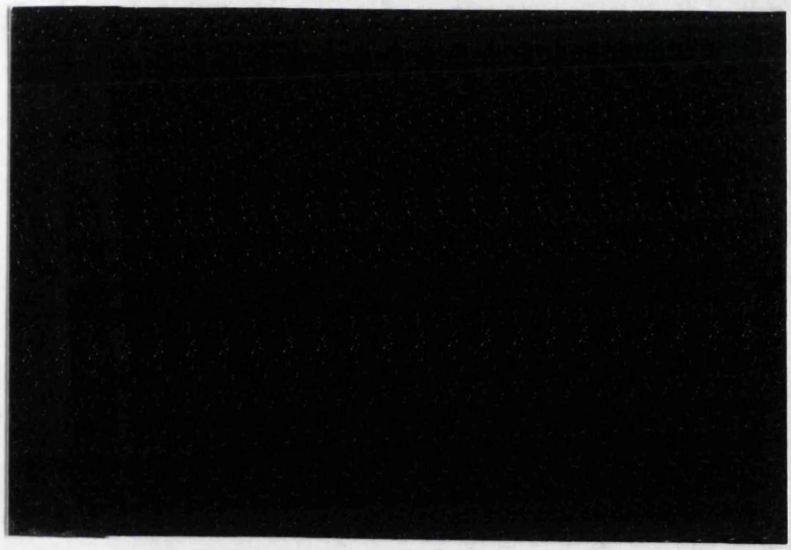
Figure 5.2 Localisation of proteins in cells infected with recBUN M and vTF7-3 recombinant vaccinia viruses.

HeLa T4$^+$ cells grown on coverslips were infected with recBUN M and vTF7-3 viruses at 10 pfu/cell each. Cells were fixed at 8 hr post infection and processed for indirect immunofluorescence using anti-BUN serum, as described in the Methods section. Fig 5.2A shows the fluorescence pattern in dual vaccinia virus infected cells. Fig 5.2B shows mock infected cells processed for indirect fluorescence using the same materials as cells in fig 5.2A. Fig 5.2C are cells which were infected with recBUN M alone and processed for indirect fluorescence, as above.

A



B



C

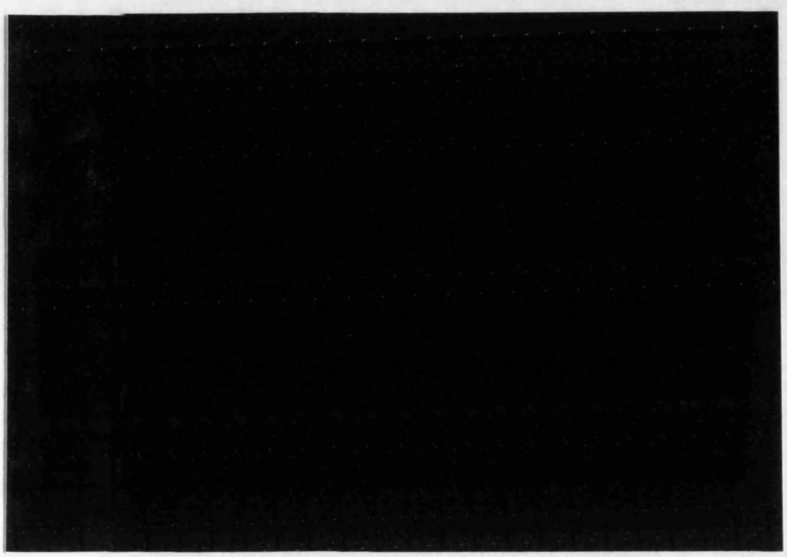


Figure 5.2

Figure 5.3 Intracellular localisation of NSm expressed from dual recBUNM and vTF7-3 recombinant vaccinia viruses.

Cells were dually infected with recBUNM and vTF7-3 vaccinia viruses at a MOI of 10 each and processed for indirect immunofluorescence with antipeptide serum to NSm and fluorescein conjugated anti-rabbit IgG (Fig 5.2A). The same cells as in Fig 5.2A were directly labelled with rhodamine conjugated wheatgerm agglutinin (Fig 5.2B). There is striking co-localization of NSm in Fig 5.2A and the Golgi complex in Fig 5.2B. Fig 5.2C is mock infected cells treated with antipeptide serum and fluorescein conjugate.

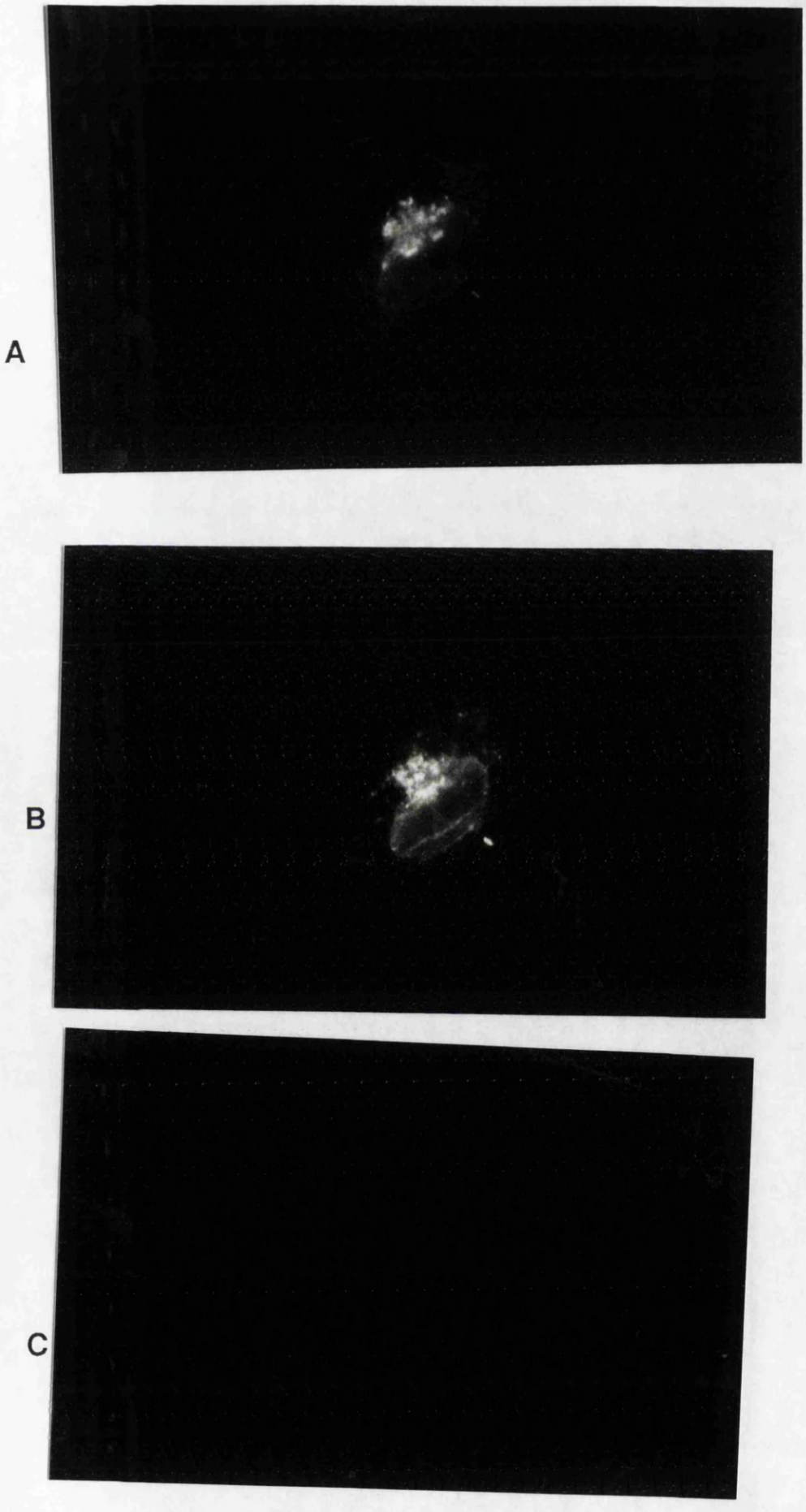


Figure 5.3

Figure 5.4 Intracellular localisation of the G1 and G2 glycoproteins.

HeLa T4$^+$ cells grown to subconfluency on coverslips were infected with the vT7-3 recombinant vaccinia virus at 10 pfu/cell for 30 min. The infected cells were transfected with pT7T318U containing G1 and pTF7-5 containing G2, both under the control of the T7 RNA promoter. At 8 hr post transfection cells were fixed in 3.7% formaldehyde and processed for indirect fluorescence using the BUN antiserum, as described in the Materials section. Fig 5.4A shows the fluorescence pattern in the pT7T3 18U/G1 transfected cells. Fig 5.4B are cells transfected with pTF7-5/G2 and Fig 5.4C are cells infected with vTF7-3 only.

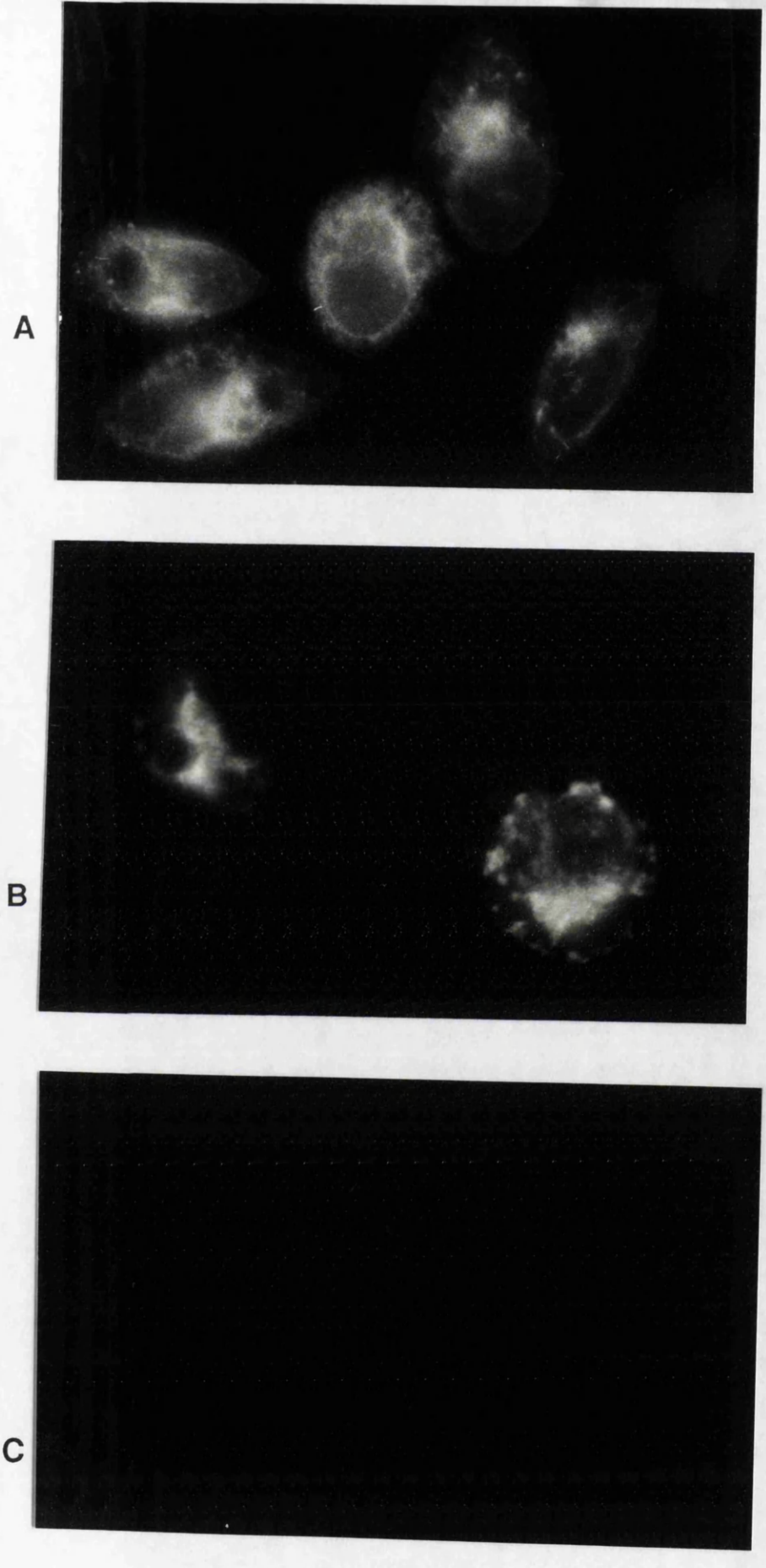


Figure 5.4

and vaccinia dual recombinant infected cells, and distinct from the pattern given by G1 alone (Fig 5.4A). This is a Golgi pattern staining and this suggested that G2 glycoprotein was competent to transport from the ER to the Golgi complex. No staining was observed in cells infected with vTF7-3 alone (Fig 5.4C). This result also demonstrates that G1 and G2 glycoproteins can be expressed independently.

Translocation of NSm

From results described in Section One regarding the localisation of NSm in BUN-infected cells and results on the localisation of NSm in dual vaccinia virus infected cells, it became apparent that NSm localises in the Golgi complex. I wanted to test whether NSm by itself, *i.e.* in the absence of other virus coded proteins, had the ability to translocate from the ER to the Golgi complex. This was made possible by transient expression of an NSm specific plasmid pTZNSm.

pTZNSm contains the NSm coding region cloned under control of the T7 promoter in pTZ18U and was provided by Dr Richard Elliott. The NSm coding sequence was obtained using the polymerase chain reaction to amplify the appropriate region of pBUN204 DNA, using the synthetic oligonucleotide primers (NSm1) 5'GCTGCCCGGGCATGAGGCTTTGCAAAT and (NSm2) 5' CGATCCCGGGTCATGCGACCATTATAATTGTAT. NSm1 is complementary to BUN M gene bases 963-982 (plus strand) and introduces an in-frame ATG to initiate translation as well as a SmaI restriction site. NSm2 is complementary to BUN M gene bases 1485-1466 (minus strand) and introduces a TGA stop codon and SmaI restriction site. pTZNSm produces a T7 RNA polymerase driven transcript which encodes amino acids 303-476 of the BUN M gene polyprotein.

HeLa T4$^+$ cells were infected with vTF7-3 and transfected with pTZ18NSm, as described above. Cells were fixed with formaldehyde and permeabilised with NP40. The

Figure 5.5 Intracellular location of NSm and N.

HeLa T4$^+$ cells infected with vTF7-3 were transfected with pTZ18NSm, containing NSm sequences under control of the T7 RNA promoter. The construct was transiently expressed, as described in the Methods section, and processed for indirect immunofluorescence using antiserum to the branched peptide. Fig 5.5A shows fluorescein staining of transfected cells and Fig 5.5B are the same cells directly stained with rhodamine wheat germ agglutinin. The staining pattern in Fig 5.5A and B is perinuclear and consistent with the Golgi complex. The cellular distribution of N is shown in Fig 5.5. The pattern is generalised cytoplasmic, suggestive of ER localisation.

A

B

C

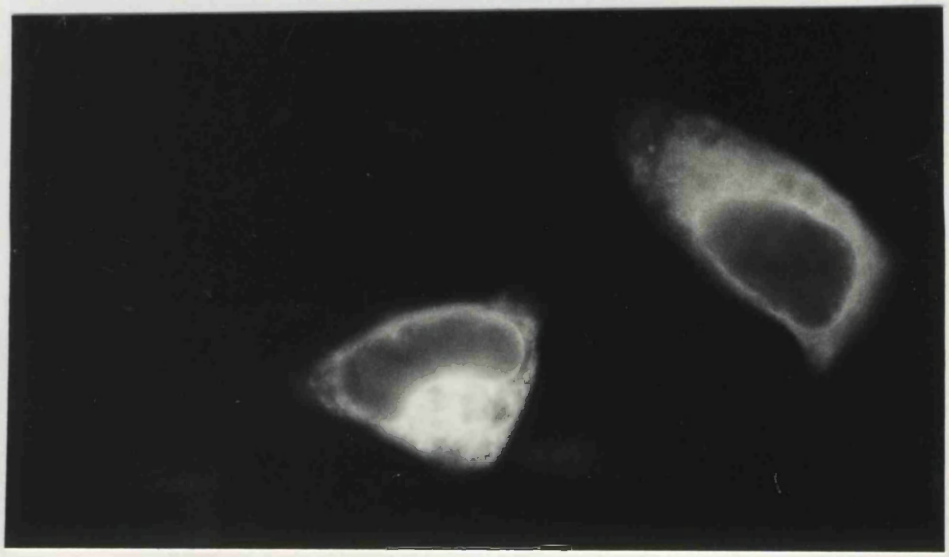
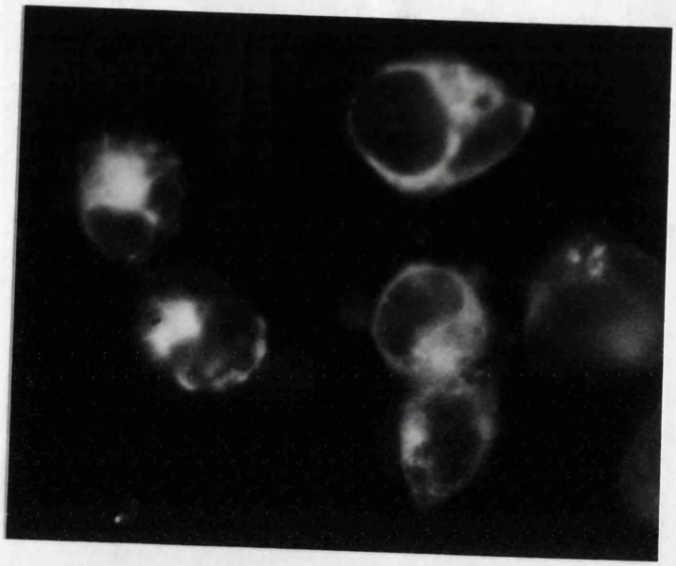


Figure 5.5

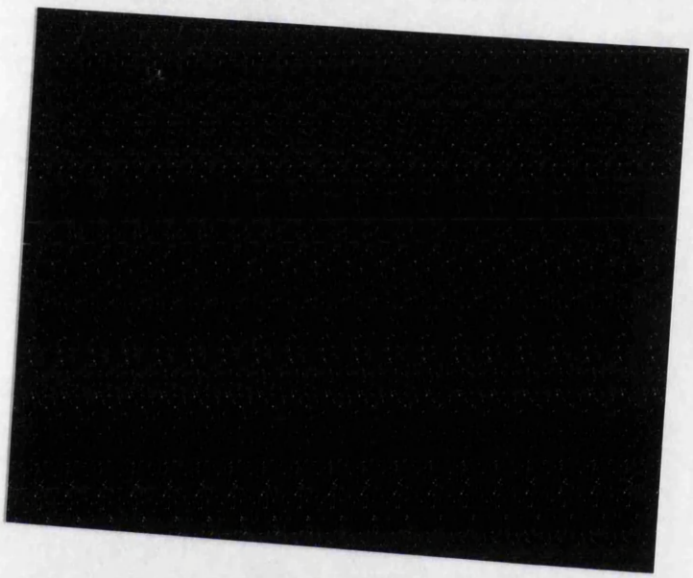
Figure 5.6 Intracellular localisation of co-expressed G1 and G2.

The G1 and G2 glycoproteins were transiently expressed in cells infected by vTF7-3 by transfecting plasmid DNAs pT7T3G1 and pTF7-5G2. The cells were treated with medium containing 50µg/ml cycloheximide at 6 hr post transfection and fixed 6 hr thereafter in 3.7% formaldehyde. The cells were processed for indirect immunofluorescence using anti-BUN serum, as already described. Fig 5.9A shows cells in which G1 and G2 were co-expressed and treated with cycloheximide. Figs 5.9B and C are mock infected and vTF7-3 infected cells, respectively.

A



B



C

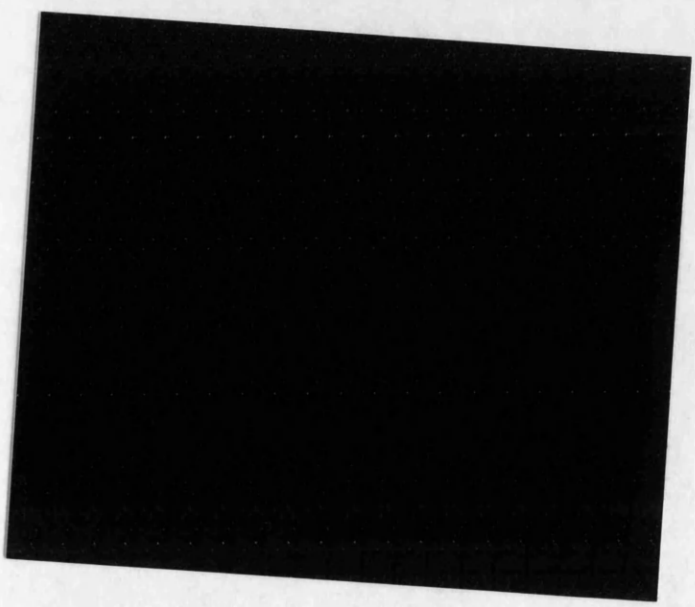


Figure 5.6

location of NSm was assessed with the antipeptide sera to NSm. The same cells were treated with rhodamine conjugated WGA. Fig 5.5A represents cells transiently expressing NSm stained with the antipeptide serum and Fig 5.5B shows the same cells directly stained with rhodamine conjugated WGA. There is a striking co-localisation of the fluorescence patterns. Since wheatgerm agglutinin stains the Golgi complex, this result shows that NSm expressed by itself is capable of translocating to the Golgi in the absence of other BUN proteins. Because of the availability of a plasmid containing the S RNA, which expresses the N protein, the cellular localisation of N was investigated using anti-virion serum. The N protein exhibited a generalised cytoplasmic pattern like G1 (Fig 5.4A).

Co-expression of G1 and G2

It has been reported that co-expression of the G1 and G2 glycoproteins of HTN is necessary for targetting of both glycoproteins to the Golgi complex (Ruusala *et al*, 1992). Since my results shown above suggested that G2 expressed on its own had the ability to translocate to the Golgi complex whereas G1 remained in the ER, I sought to determine whether co-expression of G2 and G1 would enable G1 to shift into the Golgi from the ER.

Subconfluent monolayers of HeLa T4^{+} cells were infected with vTF7-3 and transfected with equal amounts of G1 and G2 containing plasmids, and then processed for indirect immuno-fluorescence. BUN antiserum was used as the primary antibody. As can be observed in Fig 5.6A, the pattern of staining was characteristically that of the Golgi, and distinct from pattern observed when G1 was expressed alone (Fig 5.4A). It could be argued that since the pattern of staining is G2 characteristic and since the primary antibody recognises both G1 and G2, the cells may be expressing only G2 and not G1. This is improbable since, in my experience, G1 is the more efficiently expressed than G2. The relative

intensity of fluorescence in G2 expressed alone was usually lower than G1. The way to solve this problem is by use of monoclonal antibodies that specifically identify either G1 or G2. However, there have been no reports about making monoclonal antibodies specific to G2 for any member of the *Bunyavirus* genus.

Effect of cycloheximide

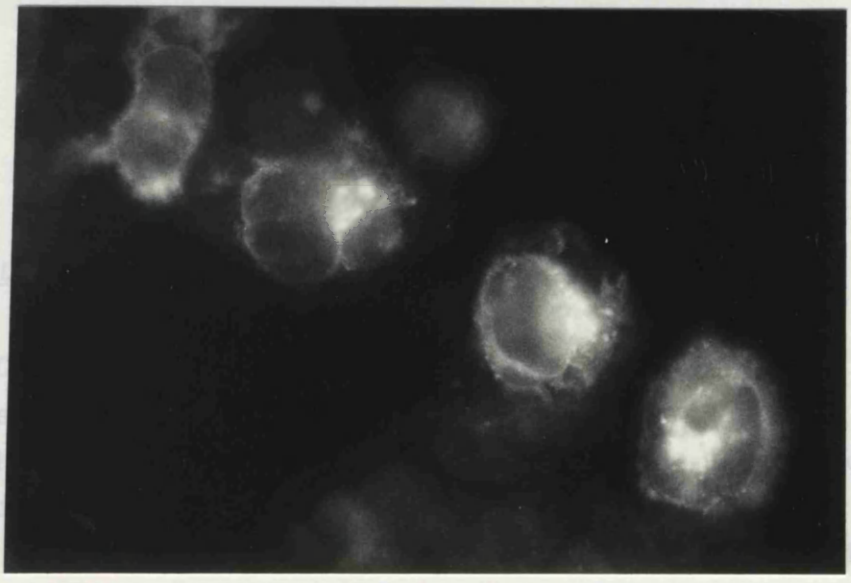
The transport of proteins from the ER to the Golgi complex was investigated in the presence of cycloheximide (a protein synthesis inhibitor). Cells infected with the full length BUN M, vaccinia virus recBUN M and vTF7-3 or cells expressing G1, G2 and NSm separately by the transient system were treated with cycloheximide for 3 hrs before fixation for immunofluorescence. Since the half-life for translocation of the Bunyaviridae glycoproteins is about 45 mins (Kuismanen, 1984; Gahmberg *et al*, 1986a) these conditions would ensure that no newly synthesised protein would be detected by immunofluorescence in the ER and also determine if previously synthesised proteins were transported out of the Golgi complex.

Effect of cycloheximide on dual vaccinia virus infected cells

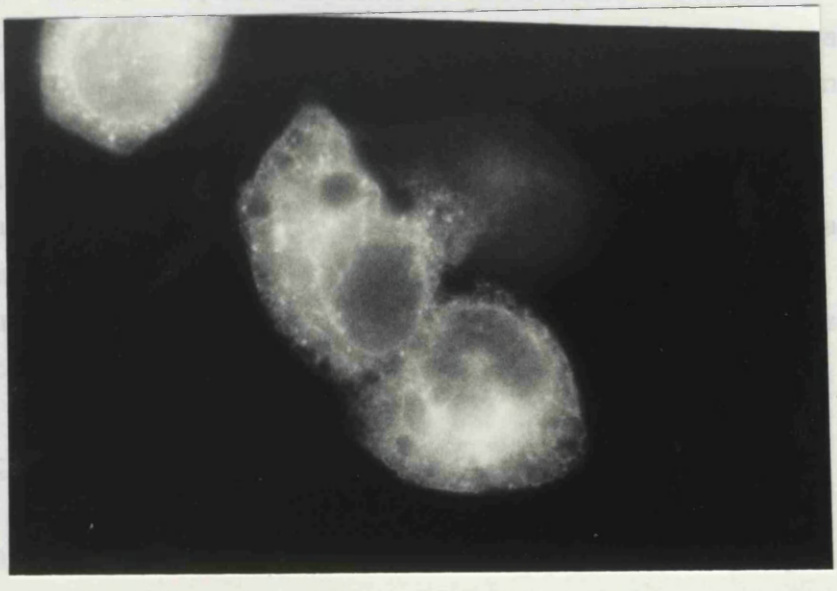
Cells were infected with recBUN M and vTF7-3 at m.o.i. of ten. The medium was replaced with warm medium containing 50µg/ml cycloheximide at 12 hrs post infection for 3 hrs, and then the cells were processed for indirect immunofluorescence. As can be seen from Fig 5.7A, the pattern of staining of the Golgi complex was sharper compared to that of the untreated dual infected cells in Fig 5.2A. This is because all the proteins had translocated from the ER giving a better definition of the Golgi complex.

Figure 5.7 Intracellular localisation and effect of cycloheximide and Brefeldin A on proteins expressed by dual infection with recBUN M and vT7-3 vaccinia viruses.

HeLa $T4^+$ cells on coverslips were dually infected with recBUN M and vT7-3 at 10 pfu/cell. At 8 hr cycloheximide (50µg/ml) or Brefeldin A (10µg/ml) was added to the medium and incubation continued for a further 3 hr. The cells were fixed in 3.7% fomaldehyde and processed for indirect immunofluorescence, as described in the Methods section. Fig 5.6A shows the dual infected cells treated with cycloheximide. The pattern is perinuclear. Fig 5.6B are cells treated with Brefeldin A. Fig 5C are mock infected cells. The pattern in Fig 5.6B is generalised cytoplasmic and consistent with the ER pattern.



A



B

C

Figure 5.7

Effect of cycloheximide on G1, G2

Cells were transfected with respective plasmids expressing G1 and G2 individually and G1 and G2 together. At 4 hrs post transfection the medium was replaced with one containing cycloheximide at 50µg/ml and incubation continued for 3 hrs. In Fig 5.8A, it can be observed that the pattern for G1 after cycloheximide treatment remained generally cytoplasmic and similar to that of untreated cells (Fig 5.4A). This means that the G1 glycoprotein continued to reside in the ER during 3 hr of protein synthesis inhibition, i.e. no transport out of the ER to the Golgi. The pattern of staining for G2 remained the same as prior to treatment with cycloheximide, *i.e.* still remained perinuclear, as shown in Fig 5.8B. Fig 5.8C shows mock-infected cells treated with cycloheximide.

Effects of brefeldin A

Brefeldin A (BFA) is a 13-membered macrocyclic lactone isolated from fungi with antibacterial activity which has also been reported to inhibit transport of proteins out of the ER (Fujiwara *et al*, 1988; Misumi *et al*, 1986; Doms *et al*, 1989; Chon *et al*, 1991; Lippincott-Schwartz *et al*, 1988; 1990). In addition to retention of proteins in the ER, BFA also causes disassembly of the Golgi apparatus and, on prolonged treatment, dilation of the ER (Fujiwara *et al*, 1988). The drug's action is most dramatic on resident and itinerant proteins in the cis and medial Golgi cisternae (Doms *et al*, 1989; Lippincott-Schwartz *et al*, 1989).

In this section of my study on the transport of proteins encoded by the M segment of BUN from the ER to the Golgi apparatus, I sought to determine whether the BUN glycoproteins G1 and G2 are recyclable back to the Golgi apparatus or are unaffected by treatment with BFA. If the

Figure 5.8 Intracellular localisation and effect of cycloheximide on transiently expressed G1 and G2.

HeLa T4$^+$ cells were infected with vT7-3 recombinant vaccinia virus for 30 min and then transfected with plasmid pT7T3 18U containing the G1 coding sequences or plasmid pTF7-5 containing the G2 coding sequences under control of the T7 RNA promoter. The medium was replaced with that containing 50µg/ml of cycloheximide at 6 hr post transfection and incubation continued for another 3 hr. Cells were processed for indirect immunofluorescence using anti-BUN serum, as already described. Fig 5.8A shows the fluorescence pattern of G1 after treatment with cycloheximide. Figs 5.8B shows the fluorescence pattern of G2 after treatment with cycloheximide. Fig 5.8C are mock infected cells treated with cycloheximide.

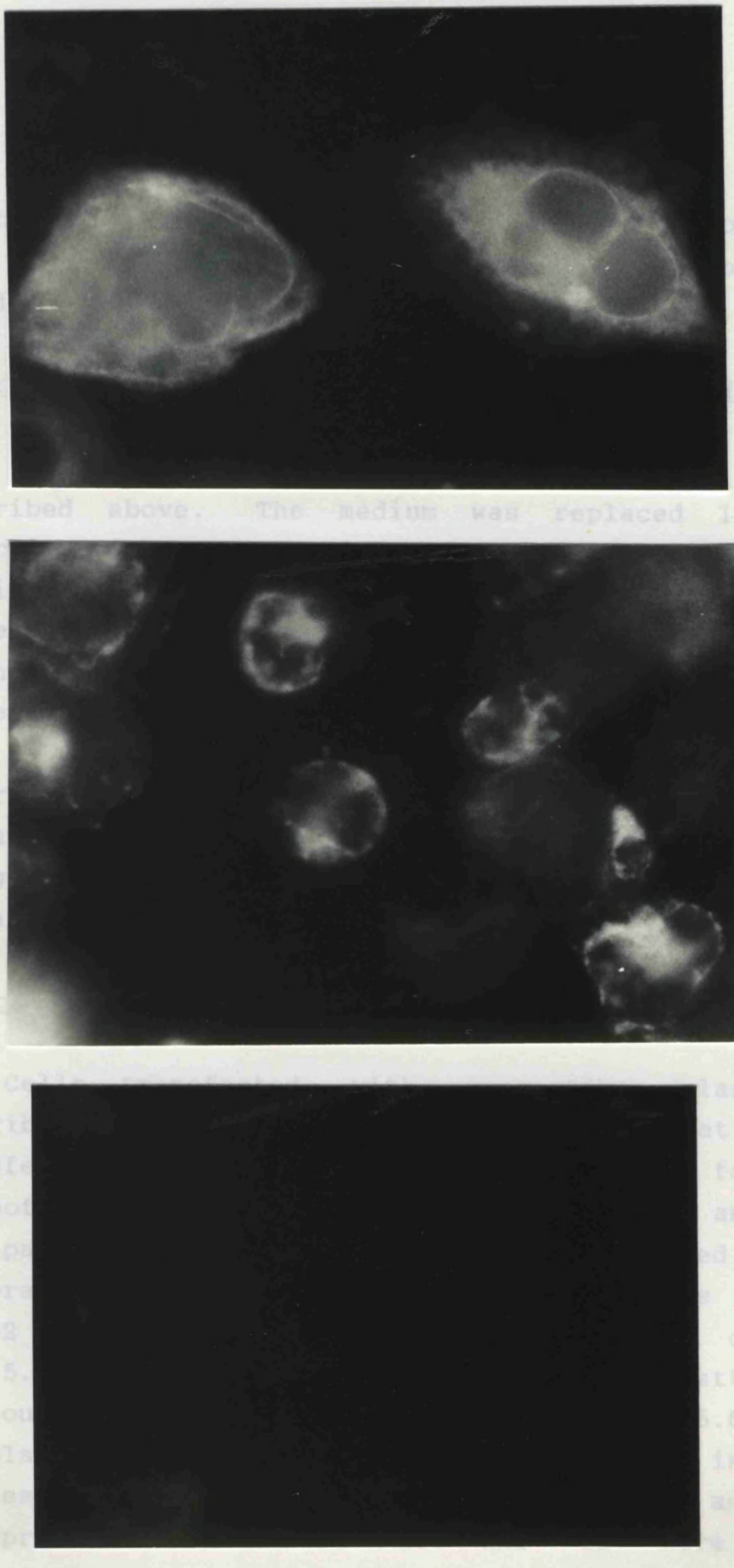


Figure 5.8

glycoproteins are not returned to the ER, this would suggest that they translocate into the trans-cisternae of the Golgi complex.

Effect of BFA in dual vaccinia virus infected cells

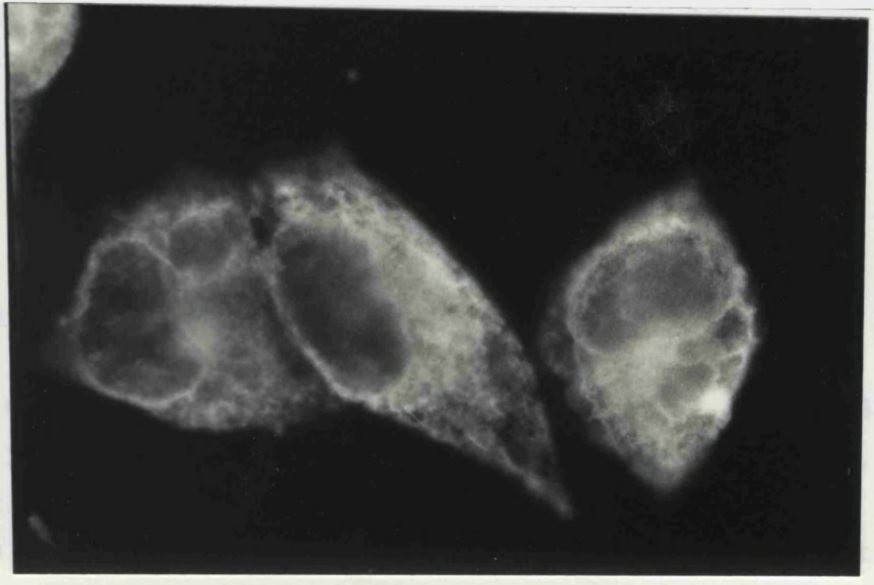
Cells were infected with recBUN M and vTF7-3, as described above. The medium was replaced 12 hrs post infection with one containing 10µg/ml BFA and incubation continued for a further 3 hrs, before the cells were processed for immunofluorescent staining. The pattern of staining (Fig 5.7B) was very different from that observed in untreated dually infected cells in Fig 5.2A or those treated with cycloheximide in Fig 5.7A. The fluorescence was observable throughout the cytoplasm, suggestive of the ER staining, and that the glycoproteins G1 and G2 which are recognised by the antiserum translocated into the cis or medial cisternae and not the trans cisternae of the Golgi.

Effect of BFA on G1, G2 and G1/G2

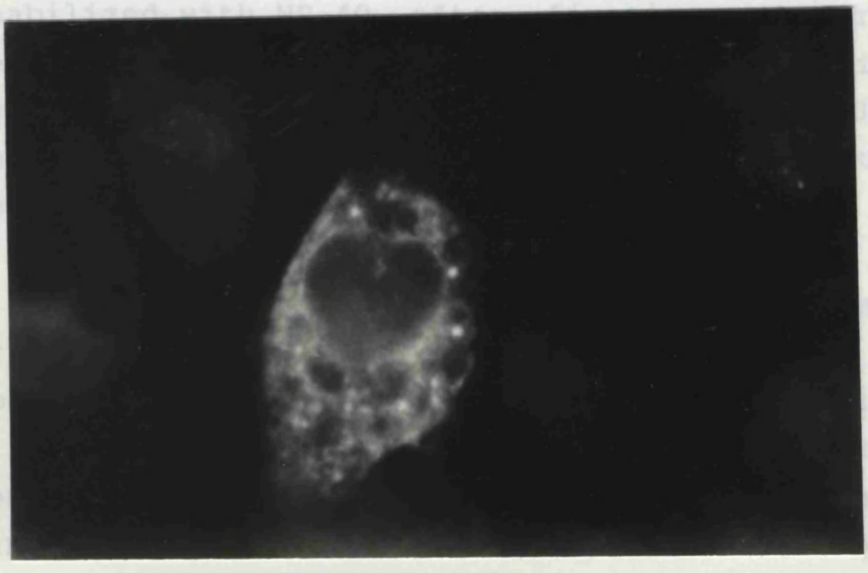
Cells transfected with respective plasmids, as described above, were treated with 10µg/ml BFA at 4 hrs post transfection for 3 hrs. Cells were processed for indirect immunofluorescence, as described in Materials and Methods. The pattern of fluorescence for G1 remained the same (generally cytoplasmic) as in untreated cells (Fig 5.9A). The G2 pattern changed from perinuclear to cytoplasmic (Fig 5.9B). The co-expressed G1 and G2 pattern which previously was perinuclear, as in Fig 5.6A, became cytoplasmic (Fig 5.9C). It was noted that the intensity of fluorescence was higher in cells where G1 and G2 were co-expressed than where G1 and G2 were expressed individually. The reasons for this are not known.

Figure 5.9 Intracellular localization and the effect of BFA on transiently expressed G1, G2 and co-expressed G1/G2.

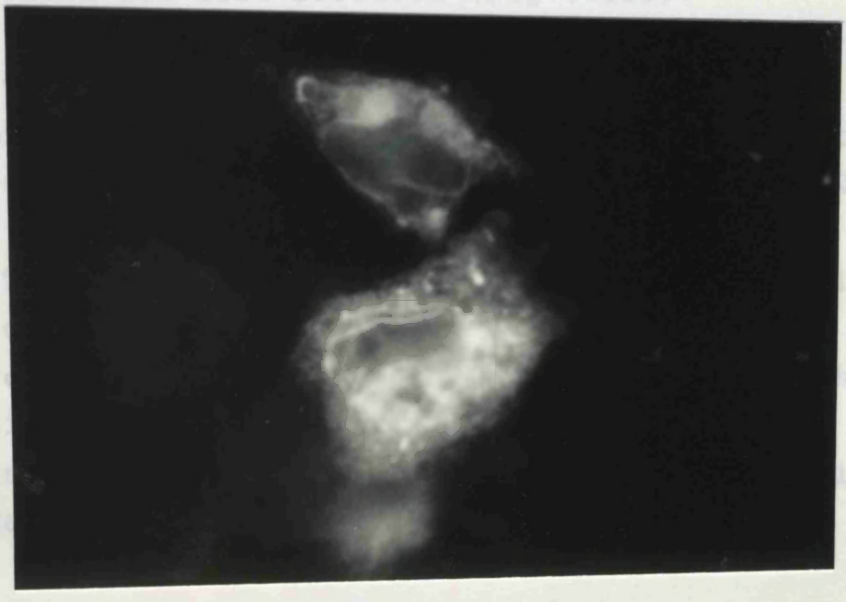
HeLa T4$^+$ cells were transfected with plasmid pT7T318U containing G1 coding sequences, plasmid pTF7-5 containing G2 coding sequences or co-transfected with the G1 and G2 containing plasmids. The cells were treated with 10µg/ml BFA at 6 hr post transfection and incubation continued for a further 3 hr. Cells were fixed in formaldehyde and processed for indirect immunofluorescence, as described in the Materials section. Fig 5.9A shows the staining pattern of transiently expressed G1 in cells treated with BFA. Fig 5.9B shows the fluorescent pattern of G2 after treatment with BFA. Fig 5.9C is the distribution of co-expressed G1 and G2 after treatment with BFA.



A



B



C

Figure 5.9

Surface expression of G1, G2 and proteins

Cells dually infected with recBUN M/vTF7-3, infected with BUN or transfected with plasmids carrying G1, G1 with a deletion from nucleotide 4326 to 4458, G2 and N genes in cells infected with vTF7-3 were prepared. Two sets of cells were prepared for each expression. One set was permeabilized with NP-40 after fixation with formaldehyde, while the other set remained unpermeabilized. All preparations were processed for indirect immunofluorescence using anti-BUN serum and IgG fluorescein isothiocyanate conjugate.

In BUN infected cells (Fig 5.10A) the internal distribution of fluorescence was observed all over the cytoplasm and also on the surface of unpermeabilised cells (Fig 5.10B). This indicated that the virion particles were present both inside the cell and on the surface. In dual vaccinia virus infected cells, internal fluorescence was seen mainly in the Golgi region (Fig 5.11A) and some surface expression was also detected (Fig 5.11B).

The G1 glycoprotein exhibited a generalised cytoplasmic pattern indicative of the ER (Fig 5.12A), but there was no appreciable fluorescence on the surface (Fig 5.12B).

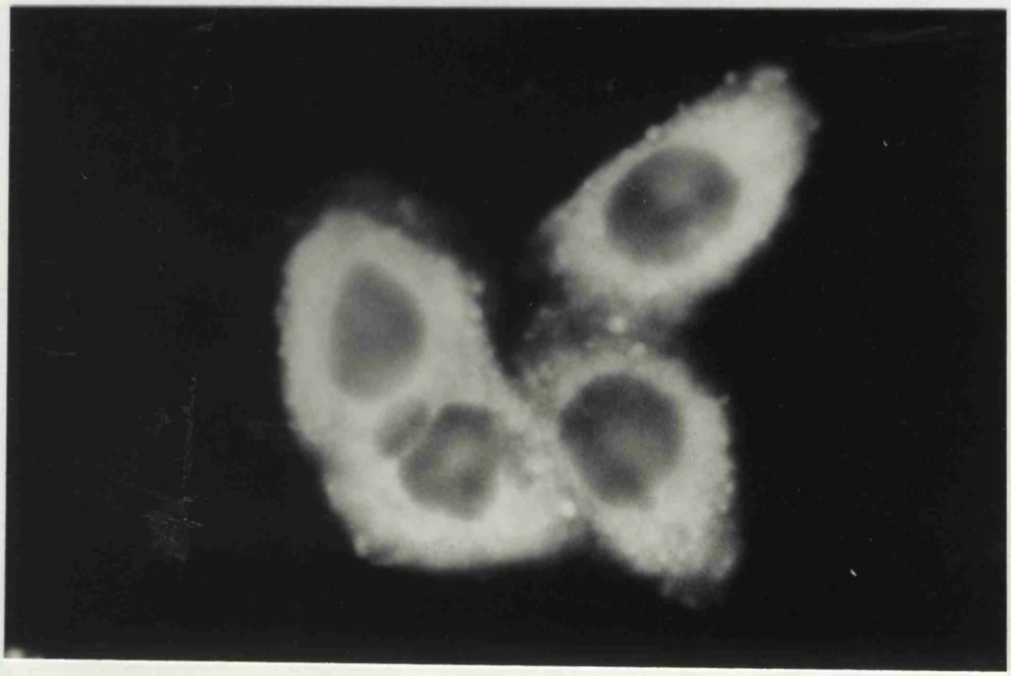
The truncated form of G1, lacking ten amino acids at the carboxy terminus, had an internal ER pattern of fluorescence (Fig 5.13A) and a marked and sharp surface staining (Fig 5.13B). This result suggests that the carboxy terminal end of G1 may have a membrane anchoring function and may also have ER retention capacity.

The G2 glycoprotein internal pattern was perinuclear (Fig 5.14A) and there was no surface fluorescence (Fig 5.14B).

Figure 5.10 Surface and internal distribution of fluorescence in BUN-infected HeLa T4^{+} cells.

HeLa T4^{+} cells on coverslips were infected with BUN at 5 pfu/cell and fixed in 3.7% formaldehyde at 12 hr. One set of infected cells were permeabilized with 0.5% NP40 and the other left intact. Both sets of cells were processed for indirect fluorescence using anti-BUN serum. Fig 5.14A shows the pattern of fluorescence in permeabilised HeLa T4^{+} cells. Fig 5.14B is the pattern of unpermeabilized cells, indicating surface distribution.

A



B

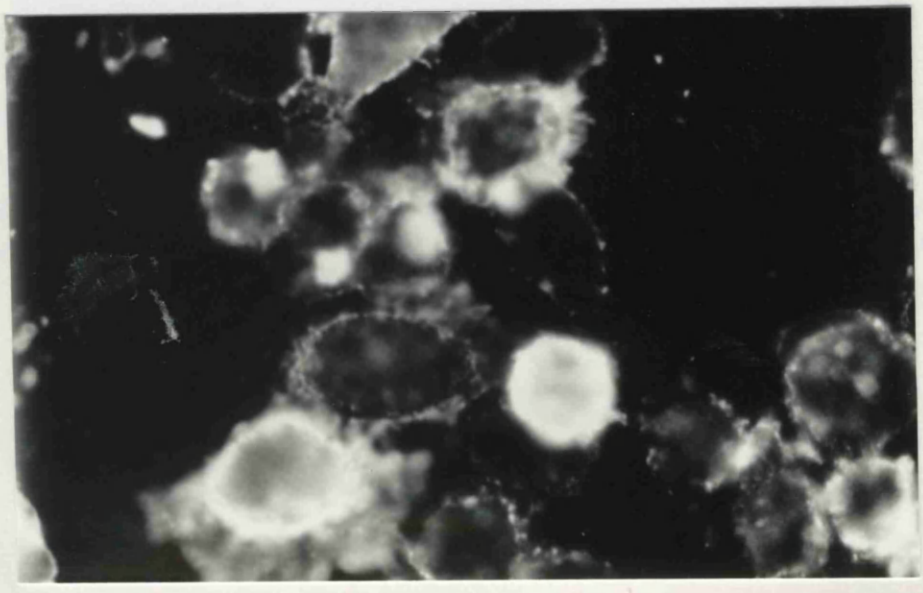
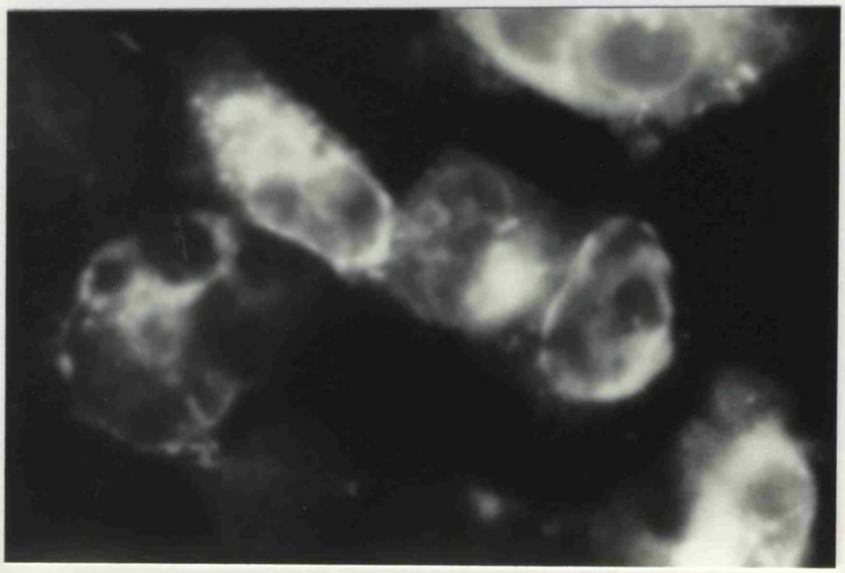


Figure 5.10

Figure 5.11 Surface and internal distribution of proteins in cells dually infected with recBUN M and vT7-3 vaccinia viruses.

HeLa T4$^+$ cells were infected with recBUN M and vT7-3, as described in the legend of Fig 5.2. Cells were fixed in 3.7% fomaldehyde at 8 hr post infection. One set of cells were permeabilized with 0.5% NP40 and both sets of cells were processed for immunofluorescence using anti-BUN serum. Fig 5.15A shows the staining pattern of the permeabilized HeLa T4$^+$ cells. Fig 5.15B is the pattern of unpermeabilized cells.

A



B

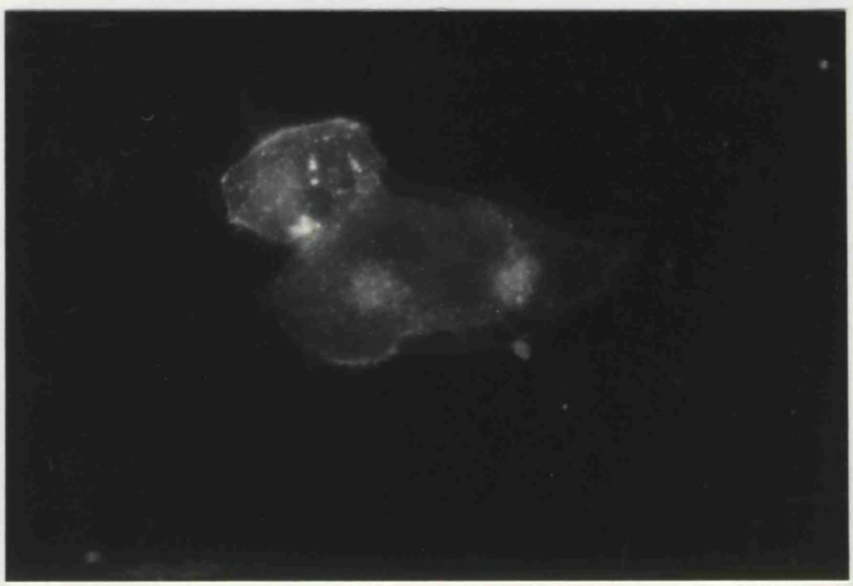
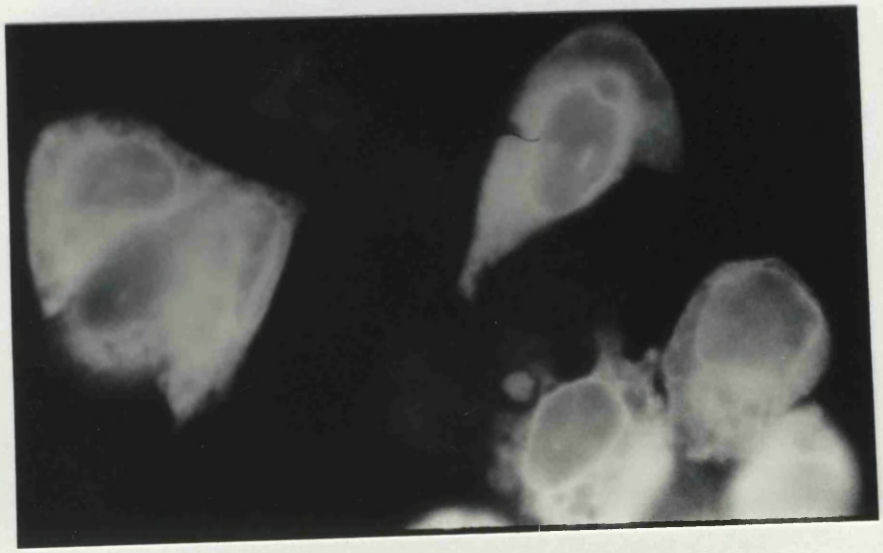


Figure 5.11

Figure 5.12 Internal and surface distribution of G1.

HeLa $T4^+$ cells transiently expressing G1, as described in the Methods section, were fixed in 3.7% formaldehyde. One set was permeabilized with 0.5% NP40 and both sets were processed for indirect immunofluorescence using anti-BUN serum. Fig 5.12A shows the internal fluorescence while Fig 5.12B shows the surface fluorescence.

A



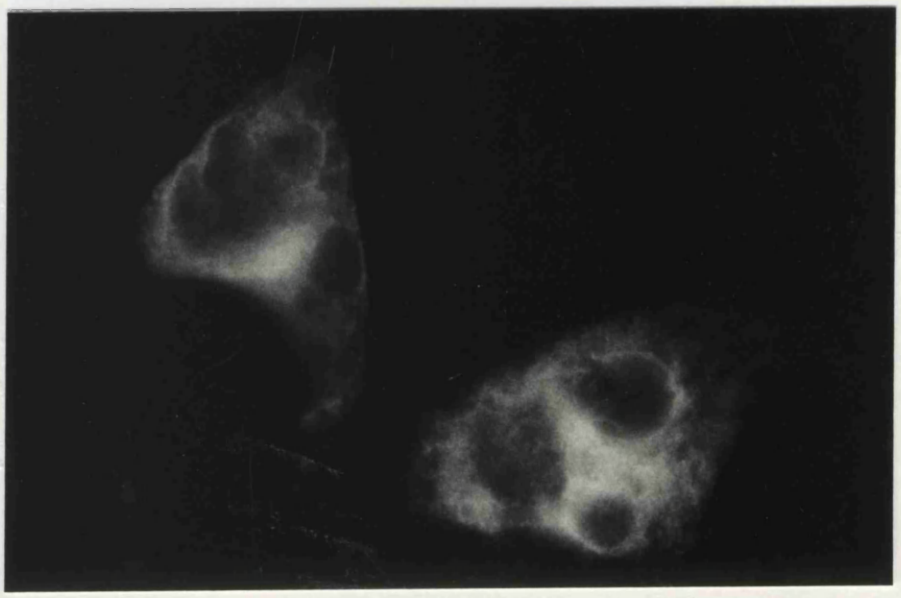
B

Figure 5.12

Figure 5.13 Internal and surface distribution of G1 with C-terminal deletion.

The cells were fixed in formaldehyde and one set permeabilized. The cells were processed of indirect immunofluorescence with anti-BUN serum. Fig 5.13A shows the fluorescence pattern in permeabilized cells. Fig 5.13B is the pattern in unpermeabilized cells, indicating surface fluorescence.

A



B

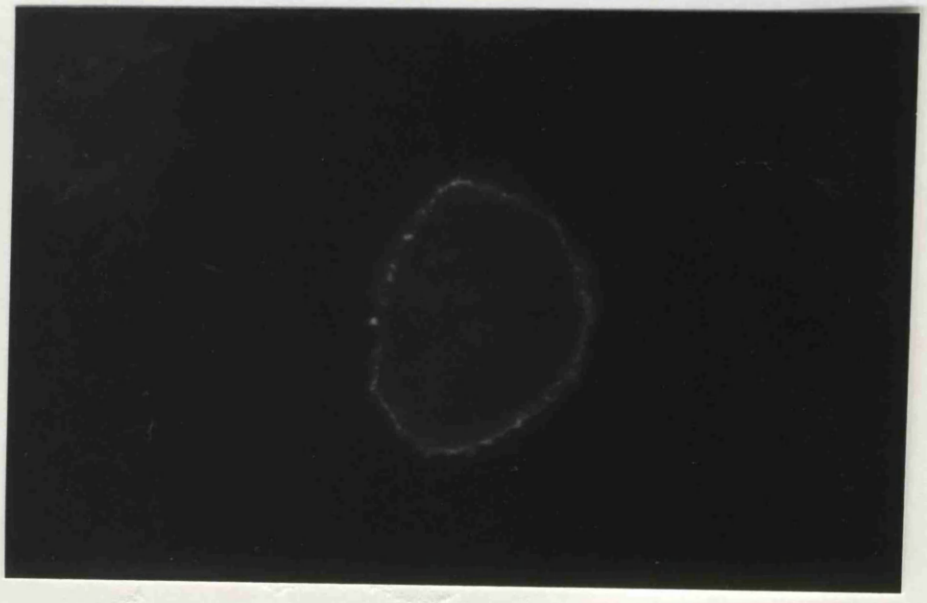


Figure 5.13

Figure 5.14 Internal and surface distribution of G2.

HeLa $T4^+$ cells transiently expressing G2 were fixed in 3,7% formaldehyde. One set was permeabilized with 0.5% NP40 and both sets were processed for indirect immunofluorescence using the anti-BUN serum. Fig 5.14A shows G2 with its characteristic Golgi complex staining pattern. Fig 5.14B shows the fluorescence distribution of G2.

A

B

Figure 5.14

Conclusions drawn from results in this Section are:

1 G2 and NSm are competent to transport independently from the ER to the Golgi complex.

2 The G1 glycoprotein becomes competent to transport into the Golgi from the ER when co-expressed with G2 from different plasmids and from the full-length cDNA.

3 The internal staining pattern of G1 and the N protein are similar, and generally cytoplasmic

4 BUN-infected cells showed fluorescence both internally and on the surface.

5 G1 and G2 hardly showed expression on the cell surface but some fluorescence was observed in dual vaccinia virus infected cells.

6 Deletion of the carboxy end of G1 led to internal fluorescence to show on the surface.

DISCUSSION

The aims of this project were to: express the BUN M RNA segment from a cloned cDNA; clone and express individual gene products of BUN M RNA; characterise the translocation of the G1 and G2 glycoproteins and NSm from the ER; and determine the cellular localization of NSm. These have largely been achieved by demonstrating the success of: (1) expression of a BUN M RNA fragment from nucleotide 1452 to 4001 *in vitro* and expression of the full length BUN M RNA from cloned cDNAs; (2) expression of the G1 and G2 glycoproteins and NSm individually; (3) production of antisera using branched peptides to NSm and using these to demonstrate the cellular localisation of NSm to be the Golgi complex; (4) demonstration that both G2 and NSm are capable of translation to, and retention in, the Golgi complex in the absence of other viral proteins; and (5) co-expression of G1 and G2, resulting in G1 (which alone was retained in the ER) being transported to the Golgi complex.

The results will be discussed under the following subtititles: Production of antiserum to NSm; Expression of BUN M RNA gene products; Intracellular transport and localization of BUN M gene products.

Raising site specific antisera to NSm

All members of the *Bunyavirus* genus encode a nonstructural protein, NSm, which was mapped to the M RNA segment (Elliott, 1985; Fuller and Bishop, 1982). The fact that NSm is encoded by the BUN M RNA segment was further demonstrated from nucleotide sequence analysis of BUN M RNA which revealed that in addition to the G1 and G2 glycoproteins, the M segment had the capacity to encode a further protein of 16K which corresponded to NSm (Lees *et al*, 1986).

The function of NSm is unknown and before this work was accomplished its cellular residence was also unknown. Demonstrating the cellular residence of NSm would serve as an important step towards delineating the role of NSm in virus replication and morphogenesis. To facilitate this aim, an antiserum that would react specifically to NSm was required. One possibility to produce an antiserum to NSm was to fractionate virus infected cell lysates in polyacrylamide gels and use those as immunogens. There would be a technical problem of efficiently isolating NSm from the background of host proteins and also the fact that NSm is present in relatively small amounts.

I employed two approaches to raise antisera to NSm. One involved the use of β-galactosidase/NSm fusion proteins and the other involved raising antisera to synthetic peptides to pre-determined sequences in the NSm coding region. Fusion proteins are becoming increasingly common as sources of immunogens for producing antibodies to proteins that were previously non-immunogenic and to proteins whose amino acid sequences have been deduced from existing nucleotide sequences. β-galactosidase (β-gal) has been utilized as a carrier protein more than other proteins because of the establishment of its amino acid sequence (Fowler and Zabin, 1978) and the fact that its antigenic sites recognised by T cell repertoires have been worked out (Krzych *et al*, 1982). β-gal fusion proteins have been shown to elicit production of antibodies to foreign proteins which are located at both the amino and carboxy termini of the β-gal moiety (Shunau *et al*, 1980; Broekhuijsen *et al*, 1986, 1987; Winther *et al*, 1986; Spindler *et al*, 1984). The system has advantages which include the relative ease of constructing appropriate vectors and the large amounts of protein that can be produced. A further advantage of using fusion proteins with β-gal is that the β-gal moiety can be used as a means of identifying the protein either immunologically by use of antibodies to β-gal or

biochemically by use of substrates such as O-nitrophenyl-β-D-galactoside (ONPG), as described by Miller, 1972.

A potential disadvantage of fusion proteins is that they are predominantly present in an insoluble form of the cell lysate (Ausubel *et al*, 1990). However, insolubility of the protein may be advantageous if the purification step uses SDS-PAGE (Rio *et al*, 1986; Spindler *et al*, 1984). The insolubility of fusion proteins may have an added advantage in protecting them from degradation by host cell proteases.

Construction of the cro-β-gal-NSm gene was facilitated by inserting the DraI (nucleotide 941) and Hae III (nucleotide 1541) fragment of the BUN M cDNA. The CI857 thermolabile repressor gene was functional and allowed control of expression of the β-gal/NSm fusion proteins. *E.coli* DH5α cells tranformed with pUEX3/NSm and pUEX3 plasmids did not synthesise fusion proteins nor β-gal at 30°C (Fig 3.3, lanes 1-3) but a 30°C to 42°C temperature shift resulted in β-gal (lane 4) and β-gal/NSm fusion proteins (lanes 5 and 6, uppermost band) being synthesised. The lower band observed in lane 5 and 6 is most likely cro-β-gal; it does not quite correspond with the induced cro-β-gal in lane 4 which appears slightly larger because of the greater expression and overloading on the gel. The lower band does not exactly co-migrate with the commercial β-gal marker because it is a cro-β-gal fusion protein.

The upper band which was of the expected size for cro-β-gal/NSm fusion protein was synthesised in much less amounts than cro-β-gal. This may be attributed to poor translation, toxicity to the cells, or protein instability introduced by the presence of NSm sequences. Proteolysis may be another factor leading to reduction in observed amounts, but this was ruled out as no new smaller bands which would suggest degradation could be observed in the induced samples.

The fusion protein band was excised from the polyacrylamide gel, macerated and used as the immunogen. The polyacrylamide which is non-toxic to animals confers a 'slow-release' effect of the cro-β-gal/NSm into the lymphoid system of rabbits, thus providing an adjuvant effect. The antiserum raised using fusion proteins failed to recognise NSm in BUN-infected cell lysates by radioimmuno-precipitation. This may be a result of the heavily hydrophobic nature of NSm which made the NSm protein inaccessible to the rabbit immune system. In an ELISA system fusion antiserum 1/2 recognised peptide M245 weakly, as shown by differences in the OD values at 405 nm of preserum and terminal sera (Fig 3.5A). There was no difference between preserum and terminal serum for fusion antiserum 2/2 (Fig 3.5B). This may be attributed to the rabbit having been exposed to cross-reacting antigens to NSm or defective blocking of unoccupied sites on the microtitre plates. The latter was ruled out because the plates come from the same batch as for antiserum 1/2 and repeated assays gave similar results. This could also be as a result of individual variation among rabbits leading to production of non-specific immunocomplexes.

Since good fusion antisera to NSm could not be made with β-gal, attention was switched to producing antipeptide sera to NSm. Studies since 1980 have shown that synthetic peptides can induce antibodies reactive with their cognate sequences in the native protein (Sela and Arnon, 1981; Lerner, 1982). Site specific antipeptide antibodies have been shown to be useful laboratory reagents for confirming *de novo* proteins from recombinant DNA, exploring biosynthetic pathways and precursors, probing structural functions and to orient proteins in cellular compartments and subcellular organelles. They are also useful in the study of the chemistry and structure of the antigen-antibody union, e.g. chemistry of virus neutralisation, and for production of passive immunoprophylaxis (Lerner, 1984).

Unlike antibodies to native proteins which are dependent on secondary structure, Green *et al* (1983), using influenza virus haemagglutinin as a model, demonstrated that reactivity of antipeptide antibodies is independent of secondary structure or location in the molecule. In cases involving peptides for eliciting antipeptide antibodies, it was necessary to couple the peptide to a carrier protein such as keyhole limpet haemocyanin. This resulted in a number of problems including immune response to the carrier (Posnett *et al*, 1988; Tam, 1988). These authors synthesised peptides by the multiple antigen peptide system which resulted in branched peptides. McLean *et al* (1991) demonstrated that branched peptides were superior to single peptides coupled to a carrier when it came to antigen presentation to the immune system.

In my study branched peptides to two predetermined amino acid sequences in the NSm coding region, as shown in Fig 3.4B, were utilised. Two regions were chosen because of the possibility that one peptide might miss intermediates in a processing cascade when the part of the protein to which the antibody is directed is removed and also because if antisera to more than one region of a protein detect the same products in cells, the certainty of a reaction of immunological specificity will be ensured (Lerner, 1984). Regions containing hydrophilic amino acids were chosen in the peptides to increase the likelihood of solubility and enable the peptides to be antibody accessible.

The antibodies to both branched peptides M245 and M246 recognised NSm in radioimmunoprecipitations, as shown in Fig 3.7. There is recognition of other proteins but this was nonspecific as both pre- and immune sera recognised these proteins. The G1 glycoprotein was also recognised by the immune sera but not preimmune sera in dual vaccinia virus expressed cell lysates. This probably is due to the

presence of vaccinia virus proteins because immune branched antipeptide M245 precipitated only NSm in BUN infected cell lysates (Fig 4.16, lane 5).

In an ELISA assay for branched antipeptide sera 1/8 and 2/8 reacting with peptide M245, there was good specificity and a high ELISA titre (Fig 3.6). It was also revealed that immunising rabbits with 300µg of branched peptide lead to a better immune response than with 100µg per inoculum. This was shown by the OD values at 405nm of preterminal sera 2/8 (300µg) exhibiting a profile similar to the terminal sera 1/8 (100µg), i.e. higher antibody levels were achieved in a shorter time (35 days) with 300µg compared with 50 days for 100µg.

Using branched peptide serum M245 in immunofluorescence studies, the usefulness of branched anti-peptide sera to NSm was demonstrated when the residence of NSm in virus infected cells was determined to be the Golgi complex. This was shown by co-localisation of NSm using fluorescein isothiocyanate and rhodamine conjugated wheat germ agglutinin which predominantly stains the Golgi complex (Fig 3.8). This is the first demonstration of the residence of NSm for any member of the family Bunyaviridae. Since maturation of members of the Bunyaviridae family takes place in the Golgi complex, NSm is most likely to have an important role in viral morphogenesis. The heavily hydrophobic NSm probably provides the surface over which virion assembly occurs, but the exact role of NSm is an aspect for future research.

Expression of BUN M RNA segment

Previous attempts to translate mRNA derived from the M RNA segment of BUN *in vitro* were unsuccessful (Abraham and Pattnaik, 1983; Elliott, 1985). Yet, similar approaches by Ulmanen *et al* (1981) resulted in the translation of UUK M mRNA which yielded a polyprotein precursor to the G1 and G2

glycoproteins. The reasons for this difference are not known and one of the aims of my project was to express the BUN M RNA segment from a cloned cDNA. This is because the levels of mRNA made *in vitro* are higher than the levels isolated from BUN infected cells. This was also a challenge because no reports of expression of the M RNA segment from a cloned cDNA had been made for any member of the *Bunyavirus* genus, although the first sequence in the genus was reported for SSH in 1984 (Eshita and Bishop, 1984).

The initial approach was to clone the BUN M cDNA in a suitable transcription vector and translate the mRNA in rabbit reticulocyte lysate or wheat germ *in vitro* translation systems. Whether the processing (cleavage) of the putative polyprotein precursor was a co-translational or post-translational event would be demonstrated by the presence or absence of mature proteins when canine pancreatic microsomal membranes were added to the translation reaction.

The transcription vectors used were those of the pTZ series which have a T7 RNA promoter. It was expected to be a straightforward cloning exercise as the BUN M cDNA insert would easily be obtained by digestion of pBUN205 with PstI and cloning the fragment into the PstI site of the pTZ vectors. Repeated efforts however failed to place the BUN M cDNA under the control of the T7 RNA promoter in order to make message-sense transcripts - as revealed by determination of orientation using restriction enzyme digestion. The cDNA was inserted the wrong way in all cases analysed. This observation suggested that inserts under the T7 promoter were being selected against by the bacteria, causing deletions in the plasmids or that the BUN M cDNA under the T7 promoter was toxic to bacteria. The strategy was then changed to employ the pT7T3 transcription vector which has both the T7 and T3 RNA promoters on either side of the polylinker. Analysis of recombinant plasmids again showed that the BUN M cDNA inserted against the T7 promoter

in all cases but was under the control of the T3 promoter. This observation confirmed the one made above that transformed bacteria selected against the BUN M cDNA under T7 promoter control and apparently the bacteria were tolerant to BUN M cDNA under T3 RNA promoter control. The reason for toxicity could be due to the presence of the lacZ promoter in the pUEX3 vector. When attempts were made to express full length cystic fibrosis transmembrane conductase regulator (CFTR) cDNA in *E.coli*, deleterious effects were observed (Gregory *et al*, 1990). The problem was circumvented by assembling the full-length CFTA open reading frame in a vector containing a low copy number of DNA replication.

Run-off transcripts of full length and truncated cDNA made using T3 RNA polymerase revealed that mRNAs of expected sizes were made, as shown in Fig 4.4. These transcripts were made in roughly the same amounts as the control MAG S mRNA which is known to be translatable *in vitro* (Elliott and McGregor, 1989). Attempts to translate the BUN M mRNAs alongside the MAG S transcripts *in vitro* were unsuccessful in both rabbit reticulocyte lysate and wheat germ systems. Failure to translate could be as a result of poor initiation of translation. However, production of capped transcripts did not result in translation. It was thought that elements at the 5' end may be responsible for inhibiting translation. To test this theory, a BamHI fragment from nucleotide 1352 to 4001 with an ATG at nucleotides 1430-1432 was cloned into the pT7T3 vector and run-off transcripts made using the T3 RNA polymerase. These transcripts represent some carboxy aa459-1315 terminal sequences of NSm and most of G1. Interestingly, a translational product of about 90K which was recognised by the BUN antiserum in radio-immunoprecipitation was made, as shown in Figs 4.7 and 4.8. This result suggests that sequences in the 5' 1350 nucleotides of the middle RNA segment do affect initiation of translation.

The use of *in vitro* translation of BUN M mRNA transcripts made from cDNA was therefore unsuitable for the study of the biogenesis of the BUN M RNA gene products. The strategy was shifted to the use of vaccinia virus which has become widely used as an expression vector. Vaccinia virus as an expression vector has many advantages because it encodes an entire transcription system including capping, methylating and polyadenylating enzymes. Also, as a member of the poxvirus family, replication occurs within the cytoplasm rather than the nucleus making nuclear transport and processing unnecessary (Moss, 1990). Many proteins have been expressed using this system, including BUN polymerase gene (Jin and Elliott, 1991) in this laboratory.

Attempts to clone the full length BUN M cDNA in the vaccinia virus transfer vector vTF7-5 were unsuccessful. This could have been due to the usually more difficult blunt end ligations or some intrinsic properties within the BUN M gene. Li *et al* (1988) reported difficulties in obtaining a recombinant M cDNA gene of VSV that could be expressed in vaccinia. They also observed that plaques that formed had the M gene inserted in the reverse non-expressible orientation. In my case, the strategy for cloning BUN M in vTF7-5 involved starting with a truncated BUN M cDNA with a large deletion from nucleotide 1352-4001. The full length BUN M segment was then reconstructed by ligating in the BamHI fragment from nucleotide 1352-4001 at the BamHI site at position 1352. However, this turned out to be a more difficult exercise than expected. All the colonies that were picked and analysed had the internal BamHI fragment inserted against the bacteriophage ø10 promoter. Re-examination of plates revealed the presence of tiny colonies of about one third of a milimetre in diameter. These tiny colonies carried plasmids in which the internal fragment inserted in the correct orientation. This suggested that some sequences in the insert, which have some sequences of NSm and most of G1, were toxic to bacteria. The

small colony size was a useful phenotype for identifying plasmids that had inserts in the correct orientation.

Recombination of pTF7-5 BUN M with vaccinia virus was achieved by use of calcium phosphate precipitation. Among the first 12 plaques picked, three recombined correctly, as demonstrated by dot blot hybridisation (Fig 4.11) and as shown by Southern analysis (Fig 4.12). Recombinant vaccinia virus with BUN M expressed authentic G1 when co-infected with recombinant vaccinia virus vTF7-5 which expresses the T7 RNA polymerase (Fig 4.14), and also in radioimmunoprecipitation analysis, as shown in Fig 4.16, lane 6. Dual vaccinia virus expression also synthesised NSm and this was confirmed in a radioimmunoprecipitation experiment using branched antipeptide serum to NSm, as shown in Fig 4.16, lanes 9 and 11. Because G2 is not reliably seen even in virus infected cells (Elliott, 1985; Watret, 1985), this result showed that the polyprotein precursor of the M segment RNA was synthesised and properly processed. This is the first report about the expression of the middle RNA gene which encodes the G1, G2 glycoproteins and NSm for a member of the *Bunyavirus* genus of which BUN is the prototype. The dual vaccinia virus system designed by Fuerst *et al* (1985) efficiently expressed the BUN M gene.

To facilitate expression of individual BUN M proteins, the transient expression system described by Fuerst (1986) using the T7 RNA polymerase was utilized. The system has an advantage over the dual vaccinia virus system in that isolation of recombinant vaccinia viruses is not necessary. Constructs were made by placing full length M, G1, G2 and NSm genes under the control of the T7 promoter. Introduction of plasmids into cells was achieved by lipofection, using cationic liposomes to transfect the DNA.

Construction of the full length BUN M under the T7 promoter involved excising it from pT7T319UBUNM by SmaI and PstI digestion and cloning it into the pT7T318U SmaI-PstI

site (Fig 4.17). The full length BUN M cDNA under the control of the T7 promoter was expressed in CV-1 cells and the G1 glycoprotein, which co-migrated with authentic G1 in BUN infected cells, was immunoprecipitated by anti-BUN serum, as shown in Fig 4.18. The G2 glycoprotein could still not be detected for the reasons described above.

The construction of G1 involved several steps dictated mainly by the availability of restriction enzymes. These included ligating a BamHI-SalI fragment in a BamHI/SalI cut pT7T319U vector and then filling in with a SalI and PstI BUN M fragment (Fig 4.19). Ideally the BamHI site at 1352 and the PstI site at 4458 would have been the fragment to use but there is another BamHI site at nucleotide 4001. The fragment contains an in-frame ATG at position 1430-1432 in the C-terminal end of NSm (Fig 4.19) and as this was the 5' most ATG, it was hoped that this would be used to initiate translation. The G1 glycoprotein was efficiently expressed in CV-1 cells and in similar amounts to G1 expressed by the dual vaccinia virus system, as shown in Fig 4.20. There is another inframe ATG just six base pairs downstream of the first ATG and it remains to be determined which one is actually involved in initiation. The G1 glycoprotein was also transiently expressed in a time course experiment, as shown in Fig 4.21. Maximal level of expression occurred at 4-6 hr post tranfection. Expression of G1 from this internal AUG concurred with the result observed in the *in vitro* expression, and raised the possibility of internal initiation *in vivo*. However, whether this occurs in nature is unknown, but may explain inpart why apparently more G1 than G2 is seen in BUN infected cells. Similar observations and suggestions have been made for the expression of HTN glycoproteins (Pensiero and Hay, 1992). Jackson (1988) described how picornaviruses broke the rules by not conforming to the scanning ribosome model developed by Kozak (1986). The model suggested that ribosomes initiate only by binding to the 5' end followed by linear scanning of the mRNA. This was as a result of work by Pelletier and

Sonenberg (1988), which demonstrated that poliovirus which is not naturally capped is translated by internal initiation.

Work was also done to determine whether the G1 protein synthesised by dual vaccinia viruses and in transiently expressed systems was properly glycosylated. This was done by use of tunicamycin which prevents glycosylation of the lipid bound sugar intermediates needed for protein glycosylation (Rothman and Lodish, 1977). Cells infected with BUN, dual vaccinia viruses recBUN M and vTF7-3 and transfected with pT7T318U/G1 were either treated or not treated with tunicamycin. The cell lysates were immuno-precipitated with anti-virion serum and proteins analysed by electrophoresis in polyacrylamide gel (Fig 4.22). The G1 protein in treated cells migrated faster than G1 in untreated cells. This result indicated that the G1 glycoprotein was properly glycosylated in both dual vaccinia virus and transient expression systems.

As a step towards functional studies of the G1 glycoprotein, a deletion at the C-terminal end was made by digestion with Hind III which cuts at nucleotide 4326 and in the polylinker of pT7T3. The deleted plasmid was then religated to itself. The translational terminator signal is present in the pT7T3 sequences. The deleted region (aa 1424-1434) in the G1 glycoprotein contains properties of a membrane anchoring sequences (Lees *et al*, 1986). After expression the deleted form was slightly smaller than the full length G1 (Fig 4.23).

The construct containing the G2 coding sequences was made by excising the fragment from pT7T3 BUN M with SmaI and DraI and ligating it into the Klenow filled-in BamHI site of vTF7-5 vector (Fig 4.24). The ideal combination of restriction enzymes would have been SmaI and BclI because DraI cuts the M gene eight amino acids short of the full length G2. BclI however failed to cut efficiently due to

Figure 6.1 Effect of sample preparation on detection of G2.

Mock-infected or BUN infected BHK cells were labelled with ^{35}S-methionine at 24 hpi and cell lysates prepared in protein dissociation buffer (see Methods). The samples were either not heated (lanes 1 and 5), heated at 37°C for 5 min (lanes 2 and 6), heated at 100°C for 1 min (lanes 3 and 7), or heated at 100°C for 5 min (lanes 4 and 8), before electrophoresis on a 12% gel. Lanes 1 to 4 are mock-infected cell lysates, and 5 to 8 BUN infected cell lysates. The positions of BUN proteins L, G1, G2 and N are indicated. This figure was provided by Dr Richard Elliott.

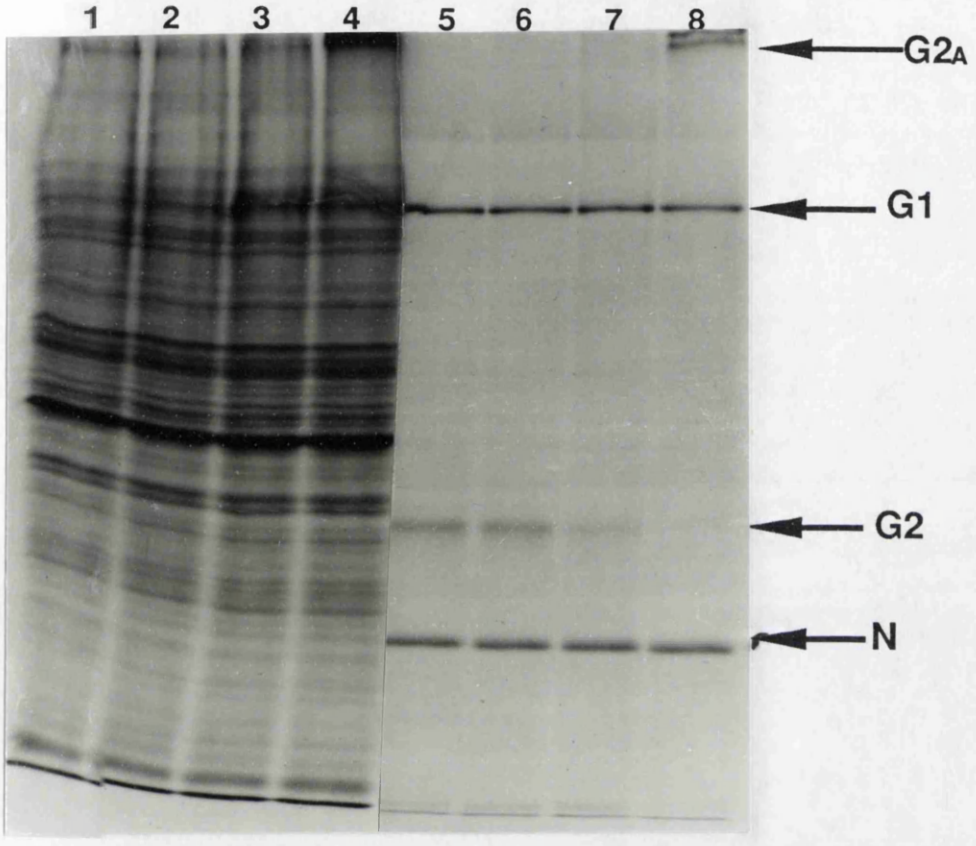


Figure 6.1

the dam methylase effect. Propagation of the plasmid in JM 48 bacteria which are dam$^-$ resulted in only partial digestion. Recombinant vaccinia virus with G2 was made but its expression could not be demonstrated. Difficulties in observing G2 were reported by Elliott (1985) and Watret *et al* (1985).

However, when I transiently expressed G2 and immunofluorescence for detection using anti-BUN serum, a positive signal indicating synthesis of G2, as shown in Fig 4.27, was observed. This observation was interesting and puzzling. It was then probable that the problem with detecting G2 labelled with ^{35}S-methionine was a technical one. Among the aspects tested was the method of preparing samples for polyacrylamide gel electrophoresis (experiment done by Dr Richard Elliott). BHK cells were infected with BUN at 10 pfu/cell, radiolabelled for 2 hr at 24 hr post infection (a time when inhibition of host protein synthesis would be marked; Scallan and Elliott, 1992), and lysed in the protein dissociation mix defined in the Methods section. The lysate was divided into four aliquots which were either left at room temperature, heated at 37°C for 5 min, boiled for 1 min or boiled for 5 min before loading samples on the gel. As can be seen from Fig 6.1, the sample boiled for 1 min (lane 7) clearly shows less G2 than the unheated (lane 5), or heated at 37°C (lane 6). G2 could not be detected in the samples boiled for 5 min, but virion specific proteins at the top of lane 8 indicate that G2 aggregates on heating and probably stays at the top of the gel, and that is a plausible reason why G2 could not be detected in cells previously boiled for 5 min. Another possibility would be degradation, but this could not be demonstrated as some smaller bands would be expected but were not seen. This result makes safe my observation that G2 was actually made and detectable by immunofluorescence when transiently expressed. This is because all immunofluorescence manipulations were carried out at room temperature.

Figure 6.2 Detection of G2 expressed in recombinant vaccinia virus infected cells.

HeLa T4$^+$ cells were mock-infected (lane 1), infected with BUN (lane 2), recBUN M and vTF7-3 (lanes 3 and 4), recBUN M alone (lane 5), or vTF7-3 alone (lane 6). Cells were labelled with ^{35}S-methionine at 18-20 hr post infection and cell lysates reacted with anti-BUN serum. The immune precipitated proteins were dissolved in protein dissociation buffer, but were not heated before electrophoresis on 12% polyacrylamide gel. Positions of BUN proteins L, G1, G2 and N are indicated. This figure was provided by Dr Richard Elliott.

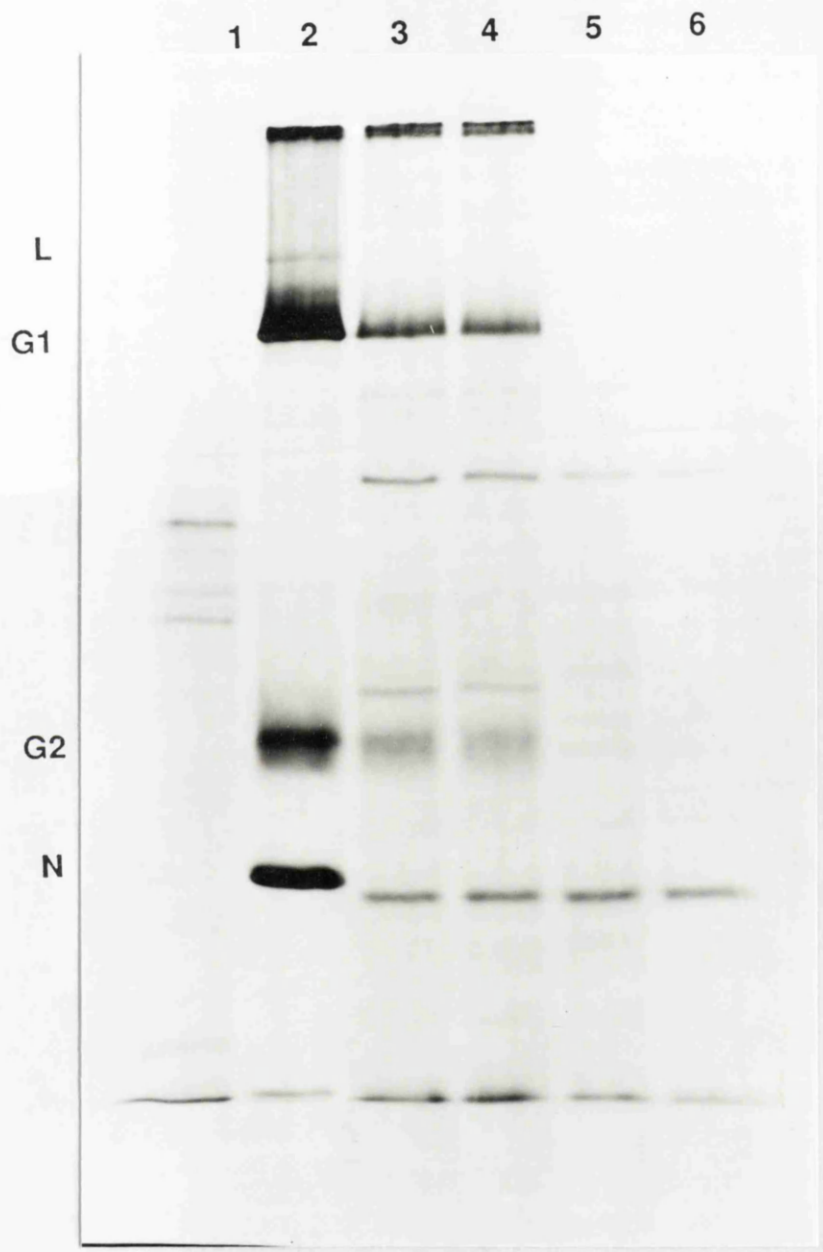


Figure 6.2

The detection of G2 strengthens the work I perfomed using recombinant vaccinia viruses in that (a) the precursor is properly translated and processed, and (b) the immunofluorescence detected by transient expression of a plasmid containing only G2 sequences appears valid.

Further experiments by Dr Richard Elliott showed that G2 was made and detectable by immunoprecipitation in dual vaccinia virus infected cells (Fig 6.2). Lane 1 is immunoprecipitation of MI, lane 2 immunoprecipitation of BUN infected cells, lanes 3 and 4 are dual vaccinia infected cells and lanes 5 and 6 are BUN recM AND vTF7-3 infected cell lysates, respectively. This result, coupled with the result in Fig 4.16, in which G1 was recognised by anti-virion sera and NSm by branched antipeptide sera, confirms that the polypeptide precursor of the middle RNA segment was made and properly processed.

Intracellular localisation of BUN M RNA gene products

Apart from their similarity in general structure which characterizes the members of the Bunyaviridae, the site of virion maturation in the Golgi cisternae is another distinctive feature of the family. The site of virion maturation was originally used as a criterion for the creation of the family (Murphy *et al*, 1973). It is now known however that other groups of viruses, including the corona and pox, also mature and bud at the Golgi complex (Pettersson *et al*, 1991).

The maturation and budding at the Golgi complex is thought to be determined by targetting of one or several of the glycoproteins to the compartment. This view was supported by the observation by Kuismanen *et al* (1984, 1985, 1988) that both the glycoproteins of UUK accumulated in the Golgi complex. Further work by Matsuoka *et al* (1988) for PT and Wasmoen *et al* (1988) for RVF also showed that the glycoproteins translocated to the Golgi complex in virus infected cells when expressed by recombinant vaccinia viruses. The same reports were made for HTN glycoproteins where G1 and G2 expressed from a vaccinia virus vector accumulated in the Golgi complex (Pensiero *et al*, 1988).

I was interested to understand how the proteins encoded by the BUN M RNA segment translocate from the ER to the Golgi complex. This was made possible by transiently expressing truncated BUN M genes that represented G1, G2 and NSm. The fate of translated products was tracked by indirect immunofluorescence using anti-BUN polyclonal antibodies for G1 and G2 and the anti-peptide antisera to NSm. Immunofluorescence was also performed on proteins expressed from the full length cDNA. The fluorescence pattern was compared with that of wheat germ agglutinin conjugated to rhodamine which predominantly stains the Golgi complex, as shown in Fig 5.1B. The pattern is distinctively perinuclear.

In cells expressing proteins in the dual vaccinia virus system, the pattern of staining using the anti-virion sera was similar to that of wheat germ agglutinin. When the cells were treated with cycloheximide, the pattern was similar but a sharper and better defined perinuclear staining, as shown in Figs 5.2A, 5.7A. This better resolution was caused by inhibition of protein synthesis, thus allowing the proteins already synthesised to transport to their destination. Treatment of cells with BFA for 3 hr before fixation resulted in redistribution of fluorescence from the Golgi pattern to the generalised cytoplasmic pattern which is characteristic of the ER (Fig 5.7B). This result suggested that the G1 and G2 glycoproteins which are recognised by anti-BUN sera reside in either the cis or medial cisternae and not the trans cisternae, which is not affected by BFA treatment (Lippincott-Schwartz *et al*, 1989).

When G1 was expressed alone, it failed to translocate to the Golgi complex even in the presence of cycloheximide (Figs 5.4A, 5.8A). Treatment of BFA had no effect on the intracellular distribution of G1 (Fig 5.9A). G1 by itself then is incompetent to transport out of the ER to the Golgi complex. On the other hand, the distribution of G2 was perinuclear and similar to that of full length dual vaccinia

expressed proteins (Fig 5.4B). Treatment of cells expressing G2 with cycloheximide improved the perinuclear definition whereas BFA had a dramatic effect of changing perinuclear Golgi pattern to the ER pattern, as shown in Fig 5.9B. This result suggested that the G2 glycoprotein has intrinsic information to translocate to the Golgi complex from the ER, and the observation is in agreement with the postulation made by Lees *et al* (1986) from the deduced amino acid sequence of BUN M RNA. The first 17 amino acids which are hydrophobic overall and which are at the amino terminal end of G2 have characteristics of a signal sequence. When the sequences of BUN, SSH, LAC and L74 a LAC variant were aligned some conservation of amino acids was observed especially at amino acid 17 for BUN and 14 for the others, where a serine is conserved. The predicted cleavage site was just before the conserved serine, as shown in Fig 6.3.

Pensiero and Hay (1992) reported that co-expression of G1 and G2 of HTN was necessary for G2 to translocate to the Golgi complex. A similar report has been made for UUK by Ronnholm (1992) where G1 expressed alone transported to the Golgi complex. However, when G1 and G2 were co-expressed, both glycoproteins translocated to the Golgi complex. It should be mentioned at this point that G1 of HTN and UUK are at the amino terminal half of their respective precursors, whereas G2 occupies this position in the BUN precursor.

It was therefore of interest to see if G2 of BUN would rescue G1 and transport it out of the ER. The G1 and G2 glycoproteins were co-expressed, as described above. The pattern of fluorescence was perinuclear in cells co-transfected with G1 and G2 cDNAs, suggesting that both G1 and G2 had translocated to the Golgi (Fig 5.6A). BFA reversed the Golgi pattern to the ER pattern, as shown in Fig 5.9C. It can be argued that the perinuclear pattern observed in the co-transfected cells is due to the expression of G2 alone but this is discounted, because in my

Figure 6.3 Putative signal peptide cleavage sites and conserved tripeptides in the M gene proteins of BUN, SSH, LAC and LAC L74 variant virus.

Sequence data from (a) Lees *et al* (1986); (b) Eshita and Bishop (1984); and (c) Clerx van-Haaster *et al* (1982).

Virus	Putative Signal Peptide												Predicted Cleavage Site	
	1											16	17	
Bunyamwera[a]	Met	Arg	Ile	Leu	Ile	Leu	Leu	Leu	Ala	Val	Thr	Gln	Leu	Ala	Val	Ser	Ser Pro Val	Ile Thr Arg Cys Phe His Gly ...
Snowshoe hare[b,c]				Met	Ile	Cys	Ile	Leu	Ile	Leu	Phe	Ala	Val	Thr	Ala	Ala	Ser Pro Val	Tyr Gln Arg Cys Phe Gin Asp ...
La Crosse[c]				Met	Ile	Cys	Ile	Leu	Val	Leu	Ile	Thr	Val	Ala	Ala	Ala	Ser Pro Val	Tyr Gin Arg Cys Phe Gln Asp ...
L74[c]				Met	Ile	Arg	Met	Leu	Val	Leu	Ile	Val	Val	Thr	Ala	Ala	Ser Pro Val	Tyr Gln Arg Cys Phe Gln Asp ...
				1								13	14					

Figure 6.3 Putative signal peptide cleavage sites and conserved tripeptides in the M gene proteins of BUN, SSH, LAC and LAC L74.

[a] Lees et al, 1986

[b] Eshita and Bishop, 1984

[c] Clerx van-Haaster et al, 1982

(Modified from Lees et al, 1986)

experience G1 is the more efficiently expressed protein than G2 and the intensity of fluorescence in co-transfected cells is higher, as can be seen in Fig 5.9C, than in individually transfected cells. The best way to resolve this problem is to utilise monoclonal antibodies. Although no reports have been made about the synthesis of monoclonal antibodies to G2 for any member of the *Bunyavirus* genus, monoclonal antibodies to G1 alone will suffice to show that G1 has been rescued by G2 to transport into the Golgi when both are co-expressed. Alternatively, specific peptide antisera could be produced. Another way would be to carry out subcellular fractionation after radiolabelling and then analyse fractions in polyacrylamide gels.

Transient expression of NSm and detection by branched antipeptide sera in immunofluorescence studies revealed that NSm by itself is competent to translocate to the Golgi complex. It remains to be seen what effect co-expression of G1 with NSm will have on the transport competency of G1 to the Golgi complex. It was reported for PT, however, that deletion of NSm had no effect on the transport of G1 and G2 to the Golgi (Chen *et al*, 1991). If NSm has no role in the translocation process then the dual vaccinia virus expression result also confirms that G2 is necessary to enable G1 to translocate to the Golgi complex. The mechanism by which G1 and G2 interact in order to translocate from the ER to the Golgi complex needs to be worked out.

Work was also done to determine whether the G1 and G2 glycoproteins and the nucleocapsid protein N expressed on the cell surface in HeLa T4$^+$ cells. Controls included cells infected with BUN. BUN infected cells showed the presence of the virus both internally (Fig 5.10A) and on the surface (Fig 5.10B). Internal fluorescence would be the total of G1 and G2 packaged into virions and G1 and G2 unpackaged and

present in the ER and the Golgi complex. The surface fluorescence presumably represents virus particles which are exiting from the plasma membrane.

There was some surface fluorescence for the glycoproteins expressed from recombinant dual vaccinia virus. Although the cells in Fig 5.11B do not show extensive vaccinia virus cpe, the fluorescence may actually be due to early vaccinia virus cpe presenting the G1 and G2 synthesising factories to the surface, enabling their detection by the anti-BUN serum. G1 transiently expressed showed its characteristic ER pattern when detected internally (Fig 5.12A). There was very little, although detectable amount, of G1 on the surface of cells. Madoff and Lenard (1982) demonstrated that LAC G1 accumulated inside infected cells, although some was detectable on the cell surface by pulse-chase experiments. They also demonstrated that the population of LAC G1 on the cell surface resembled that in extracellular virions in being fully endo-H resistant, a property that is generally considered to represent complete processing through the Golgi apparatus. It remains to be seen if BUN G1 detected on the surface when expressed individually is endo-H resistant, as results in Section Three of this thesis indicated that G1 expressed on its own could not transport to the Golgi.

A candidate to study the endo-H resistance of G1 found on the cell surface exists in the form of BUN G1 with a deletion of 10 amino acids at the carboxy end. When transiently expressed in cells the truncated G1 showed internal fluorescence pattern of the ER, as shown in Fig 5.13A, which is similar to the full length G1. Surface fluorescence was however better defined and prominent (Fig 5.13B) than in the full length G1. This result supports the predicted structure of G1 from nucleotide sequence which indicated presence of a terminal anchor sequence at the carboxy terminal end (Lees *et al*, 1986).

Unlike G1, the G2 glycoprotein showed perinuclear staining as in Fig 5.14A and no detectable surface expression. This suggests that the G2 has the signal to transport from the ER to the Golgi and also information to retain into the Golgi, and retention of G1 in the Golgi is the function of G2.

The internal fluorescence of the N protein was cytoplasmic and similar to that of G1. It is suggested that the N protein also needs the G2 glycoprotein to enable it to reach the station of virus maturation, which is the Golgi apparatus, but work needs to be done to demonstrate this.

FUTURE PROSPECTS

Expression of the middle RNA genome segment of Bunyamwera virus provides an important basis for detailed analysis of functions of G1 and G2 glycoproteins and NSm. This is especially so when the individual proteins have been cloned and expressed. Areas of immediate interest are:

1 Assigning the membrane fusion function to either G1 or G2 by independently expressing them at the pH of fusion.

2 Using site-directed mutagenesis and gene deletions to determine domains in the glycoproteins responsible for membrane anchorage.

3 Determining the amino acid sequences on G2 and NSm that are necessary for translocation from the ER to the Golgi. This would be done by use of mutagenesis and construction of chimaeric proteins, e.g. with plasma targetted glycoprotein like VSV G.

4 In the longer term, determining the function of NSm, by introducing changes in the virus by site-directed mutagenesis and gene deletions. This, however, presents a problem, because the RNAs of negative stranded viruses are non-infectious, compared with the positive stranded viruses whose RNAs are infectious.

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BUNYAMWERA VIRUS M GENE

Met Arg Ile Leu Ile Leu Leu Leu Ala Val Thr Gln Leu Ala Val Ser 16
AGTAGTGTACTACCGATACATCACAAACCTTTCAGAGACACATCTTTATTTCCAAG ATG AGA ATT CTA ATA CTG CTT TTA GCA GTC ACT CAA CTG GCT GTG AGT 104

Ser Pro Val Ile Thr Arg Cys Phe His Gly Gly Gln Leu Ile Ala Glu Arg Lys Ser Gln Thr Ser Ile Ser Glu Phe Cys Ile Lys Asp 46
AGC CCA GTT ATC ACT AGA TGC TTT CAT GGT GGG CAA CTG ATT GCA GAA AGG AAA TCC CAA ACA TCG ATT TCA GAA TTC TGC ATT AAA GAT 194

Asp Val Ser Met Leu Lys Ser Glu Ile Val Tyr Thr Lys Asn Asp Thr Gly Ile Phe Gly His Ser Lys Val Phe Arg His Trp Thr Ile 76
GAC GTT TCT ATG TTA AAA TCA GAG ATT GTC TAC ACA AAA AAT GAT ACT GGG ATT TTT GGC CAC AGT AAA GTG TTT CGT CAC TGG ACG ATC 284

Thr Asp Trp Lys Ala Cys Asn Pro Val Val Thr Ala Gly Gly Ser Ile Asn Val Ile Glu Val Asp Lys Asn Leu Asn Leu Val Thr Arg 106
ACA GAC TGG AAA GCA TGC AAC CCT GTT GTT ACG GCC GGT GGT AGT ATA AAT GTT ATA GAG GTT GAT AAA AAT CTA AAC CTT GTA ACT AGA 374

Asn Tyr Val Cys Thr Gly Asp Cys Thr Ile Thr Val Asp Arg Lys Asn Ala Gln Ile Ile Phe Gln Thr Asp Lys Leu Asn His Phe Glu 136
AAT TAT GTG TGC ACA GGG GAT TGC ACT ATA ACA GTT GAT AGG AAA AAT GCC CAA ATT ATA TTT CAG ACA GAC AAA CTT AAT CAT TTT GAA 464

Val Thr Gly Thr Thr Ile Ser Thr Gly Trp Phe Lys Ser Lys Ala Ser Val Thr Leu Asp Arg Thr Cys Glu His Ile Lys Val Ser Cys 166
GTG ACA GGA ACT ACT ATC AGC ACT GGC TGG TTT AAG TCT AAA GCA TCT GTT ACT CTC GAT AGA ACA TGT GAA CAT ATA AAA GTA AGC TGT 554

Gly Lys Lys Thr Leu Gln Phe His Ala Cys Phe Lys Gln His Met Ser Cys Val Arg Phe Leu His Arg Ser Ile Leu Pro Gly Ser Met 196
GGA AAG AAA ACA TTA CAA TTC CAT GCT TGC TTT AAG CAA CAC ATG TCT TGT GTT CGA TTC TTA CAC AGG AGC ATA CTA CCA GGG TCA ATG 644

Ala Ile Ser Ile Cys Gln Asn Ile Glu Leu Ile Ile Ile Thr Ile Leu Ala Leu Cys Ile Phe Ile Ile Met Ile Ile Leu Thr Lys Thr 226
GCA ATT TCG ATC TGC CAA AAT ATT GAG CTG ATT ATA ATA ACA ATA TTG GCA TTA TGT ATA TTT ATA ATT ATG ATA ATC TTA ACA AAA ACA 734

Tyr Ile Cys Tyr Val Leu Ile Pro Val Phe Met Pro Ile Ala Phe Ala Tyr Gly Trp Ala Tyr Asn Arg Ser Cys Lys Lys Cys Thr Cys 256
TAC ATT TGC TAC GTG TTG ATC CCA GTG TTT ATG CCC ATA GCT TTT GCT TAT GGA TGG GCG TAC AAC AGG AGT TGT AAG AAA TGC ACT TGT 824

Cys Gly Leu Ala Tyr His Pro Phe Thr Asn Cys Gly Ser Tyr Cys Val Cys Gly Ser Lys Phe Glu Thr Ser Asp Arg Met Arg Met His 286
TGC GGA TTG GCA TAT CAT CCC TTT ACA AAC TGT GGC TCA TAT TGT GTC TGT GGC TCC AAA TTT GAA ACA TCG GAC AGA ATG AGG ATG CAC 914

Arg Glu Ser Gly Leu Cys Gln Gly Phe Lys Ser Leu Arg Val Ala Arg Arg Leu Cys Lys Ser Lys Gly Ser Ser Leu Ile Ile Ser Ile 316
CGA GAA TCA GGG TTA TGT CAG GGT TTT AAA AGC CTG AGA GTA GCA AGA AGG CTT TGC AAA TCA AAA GGC TCA TCA TTG ATC ATA TCC ATC 1004

Leu Leu Ser Val Leu Ile Leu Ser Phe Val Thr Pro Ile Glu Gly Thr Leu Thr Asn Tyr Pro Thr Asp Gln Lys Tyr Thr Leu Asp Glu 346
TTA CTC TCA GTG CTG ATT CTA TCC TTT GTA ACA CCC ATA GAA GGG ACT CTC ACA AAC TAC CCT ACT GAT CAG AAA TAT ACT TTA GAT GAG 1094

Ile Ala Asp Val Leu Gln Ala Lys Thr His Glu Asp Ser Thr Lys Tyr Tyr Ile Ile Leu Tyr Thr Ser Leu Phe Gly Ala Gly Leu Thr 376
ATA GCA GAT GTC CTT CAA GCT AAA ACA CAT GAA GAT TCT ACA AAA TAC TAT ATA ATA TTA TAT ACA TCA CTC TTC GGT GCA GGT CTG ACC 1184

Ile Ile Phe Ala Gly Val Ala Leu Gly Leu Thr Ile Ile Leu Glu Val Leu Thr Lys Ile Asn Val Ile Phe Cys Asn Glu Cys Asn Met 406
ATC ATT TTT GCA GGA GTA GCA TTG GGG TTA ACA ATT ATA CTA GAA GTA TTA ACC AAG ATT AAT GTT ATT TTT TGC AAC GAA TGC AAC ATG 1274

Tyr His Ser Lys Lys Ser Ile Lys Tyr Val Gly Asp Phe Thr Asn Lys Cys Gly Phe Cys Thr Cys Gly Leu Leu Glu Asp Pro Glu Gly 436
TAC CAT AGC AAA AAA TCA ATC AAA TAT GTA GGT GAT TTC ACT AAT AAA TGT GGG TTT TGT ACG TGT GGG CTG CTA GAG GAT CCA GAG GGT 1364

Val Val Val His Lys Ala Lys Lys Ser Cys Thr Tyr Ser Tyr Gln Ile Asn Trp Val Arg Gly Ile Met Ile Phe Val Ala Phe Leu Phe 466
GTT GTT GTA CAT AAA GCT AAA AAA TCC TGT ACA TAT TCA TAT CAG ATT AAC TGG GTC AGA GGC ATC ATG ATA TTT GTT GCC TTT TTA TTT 1454

Val Ile Gln Asn Thr Ile Ile Met Val Ala Ala Glu Glu Asp Cys Trp Lys Asn Glu Glu Leu Lys Glu Asp Cys Val Gly Pro Leu Ile 496
GTA ATA CAG AAT ACA ATT ATA ATG GTC GCA GCA GAA GAA GAC TGC TGG AAA AAT GAA GAA TTA AAA GAA GAT TGT GTA GGG CCT TTA ATT 1544

Ala Pro Lys Asp Cys Thr Asp Lys Asp His Lys Thr Tyr Leu Ser Glu Ala Ser Leu Leu Ala Thr Ala Lys Lys Ile Thr Gln Val Asp 526
GCA CCT AAG GAT TGT ACT GAT AAA GAC CAT AAA ACC TAC TTG AGT GAG GCT TCA TTG TTA GCA ACA GCA AAG AAA ATA ACT CAG GTG GAT 1634

Ala Glu Asn Val Glu Ile Leu Gly Lys Thr Met Glu Ser Ala Ile Arg Val Ile Glu Arg Gln Lys Thr Tyr His Arg Met His Leu Leu 556
GCT GAA AAT GTG GAG ATA TTG GGG AAA ACT ATG GAA TCA GCA ATT AGA GTA ATT GAA AGA CAG AAG ACA TAC CAC AGA ATG CAC TTG CTT 1724

Glu Ala Val Phe Leu Asn Lys His Cys Asp Tyr Tyr Lys Met Phe Glu His Asn Ser Gly Tyr Ser Gln Val Lys Trp Arg Met Met Ile 586
GAG GCA GTA TTT CTA AAT AAG CAC TGC GAT TAT TAT AAA ATG TTT GAA CAT AAC AGT GGA TAT TCC CAA GTA AAG TGG AGG ATG ATG ATA 1814

Lys Thr Gln His Phe Asp Ile Cys Ala Leu Gln Ala Asn Ser Pro Phe Cys Ala Gln Cys Ile Ala Asp Asn Ser Cys Ala Gln Gly Ser 616
AAA ACA CAA CAC TTT GAT ATC TGT GCC TTA CAA GCA AAT AGC CCG TTT TGT GCT CAG TGC ATT GCT GAC AAT TCT TGT GCG CAA GGT TCT 1904

Trp Glu Phe Asp Thr His Met Asn Ser Thr Tyr Ser Ser Lys Val Asp Asn Phe Lys His Asp Phe Ser Leu Phe Leu Arg Ile Phe Glu 646
TGG GAA TTT GAT ACA CAT ATG AAC TCC ACA TAT TCA AGT AAA GTC GAC AAT TTT AAA CAT GAC TTC TCT CTA TTC CTC AGA ATC TTT GAA 1994

Ala Ala Phe Pro Gly Thr Ala Tyr Val His Leu Leu Thr Asn Ile Lys Glu Lys Lys Lys Pro Tyr Gln Ala Val Ser Met Ile Glu Lys Ile 676
GCA GCA TTT CCA GGC ACT GCT TAT GTT CAC TTG CTA ACA AAT ATA AAA GAA AAG AAG CCC TAT CAG GCA GTC AGC ATG ATT GAG AAA ATA 2084

Lys Lys Lys Phe Pro Asn Asn Lys Leu Leu Ile Gly Tyr Leu Asp Phe Gly Lys Tyr Leu Leu Gly Leu Ser His Ala Ser Thr Tyr Glu 706
AAG AAG AAG TTT CCG AAT AAT AAA CTG CTT ATT GGA TAT TTA GAT TTT GGC AAG TAC TTG CTA GGC TTA AGT CAT GCA AGC ACA TAC GAG 2174

Leu Gln Gln Arg Gln Leu Asp Lys Leu Tyr Gln Pro Thr Glu Leu Thr Arg Ser Gly Gly Gln Gln Thr Ser Leu Ala Asn Ser Val Val 736
TTG CAA CAG AGA CAA CTA GAT AAG TTA TAT CAG CCA ACA GAA TTA ACC CGA TCT GGT GGT CAA CAA ACA TCA TTA GCA AAT TCT GTA GTA 2264

Gly Gln Ala Thr Lys Glu Cys Lys Lys Tyr Lys Asp Val Ser Cys Leu Ser Pro Arg Phe Gly Ile Pro Leu Glu Asp Leu Ile Ser Cys 766
GGT CAA GCA ACG AAA GAA TGT AAA AAG TAC AAA GAT GTT AGT TGC TTA AGC CCA AGA TTT GGA ATT CCG CTG GAA GAT TTA ATA AGC TGC 2354

Cys Asp Gln Pro Asn Tyr Asn Ile Tyr Lys Lys Pro Lys Lys Val Tyr Lys Ala His Asp Lys Glu Glu Thr Trp Cys Ile Asn Asp Gln 796
TGT GAC CAA CCA AAT TAC AAT ATT TAT AAA AAG CCA AAA AAA GTC TAC AAA GCT CAT GAC AAA GAA GAA ACA TGG TGC ATT AAT GAT CAG 2444

His Cys Leu Val Asp Phe Val Pro Ala Glu Ala Asp Thr Val Glu Lys Leu Lys Pro Met Lys Cys Trp Leu Val Asp Pro Gly Lys Asn 826
CAT TGC CTA GTA GAC TTT GTC CCA GCT GAA GCC GAT ACT GTA GAA AAA TTG AAA CCT ATG AAA TGT TGG TTA GTT GAC CCT GGC AAG AAT 2534

Asp Asp Val Tyr Ser Ile Ala Ile Lys Thr Cys Arg Val Val Asp Lys Gly Val Cys Thr Val Asn Ser Gln Lys Trp Asn Ile Ile Lys 856
GAT GAT GTC TAC TCT ATT GCA ATA AAA ACA TGT AGA GTG GTT GAT AAG GGA GTT TGT ACT GTT AAT TCA CAA AAA TGG AAT ATA ATC AAA 2624

Cys Asp Ser Gly Pro Leu Tyr Tyr Ser Asp His Ile Pro Gly Glu Asp Thr Gly Asn Asp Ile Gly His Tyr Cys Val Ser Ala Gly Cys 886
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Lys Thr Asp Arg Tyr Pro Ile Asn Pro Asp Val Val Thr Asp Cys Val Trp Glu Phe Thr Ser Arg Lys Ser Gln Tyr Ile Gly Lys Ile 916
AAA ACT GAC AGA TAC CCA ATT AAT CCT GAT GTT GTT ACT GAC TGT GTG TGG GAA TTT ACT TCT AGG AAA TCA CAA TAT ATA GGC AAG ATC 2804

Ser Met Gln Ser Leu Glu Asp Tyr Glu Lys Ala Leu Thr Asp Arg Leu Thr His Thr Leu Glu Thr Tyr Ser Phe Ala Pro Leu Glu Asn 946
TCC ATG CAG TCT CTT GAA GAT TAC GAA AAG GCT TTA ACT GAC AGA TTG ACC CAT ACC TTG GAA ACC TAT AGT TTT GCC CCG TTA GAA AAT 2894

Leu Pro His Ile Lys Pro Val Tyr Lys Tyr Ile Thr Ala Gln Gly Val Glu Asn Ser Asp Gly Ile Glu Gly Ala Phe Ile Thr Ala Ser 976
CTC CCG CAT ATA AAA CCA GTT TAC AAA TAT ATT ACT GCA CAA GGA GTC GAA AAC TCA GAC GGT ATA GAA GGG GCA TTC ATA ACA GCC AGT 2984

Ile Pro Ala Ala Gly Gly Thr Ser Ile Gly Tyr Asn Val Arg Ser Lys Asp Gly Phe Pro Leu Leu Asp Leu Ile Val Phe Val Lys Ser 1006
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Ala Val Ile Lys Ser Thr Tyr Asn His Ile Tyr Asp Thr Gly Pro Thr Ile Ser Ile Asn Thr Lys His Asp Glu His Cys Thr Gly Gln 1036
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Cys Pro Ser Asn Ile Glu His Glu Ala Asn Trp Leu Thr Phe Ser Gln Glu Arg Thr Ser Arg Trp Gly Cys Glu Glu Phe Gly Cys Leu 1066
TGT CCA AGC AAT ATA GAA CAT GAG GCT AAC TGG TTG ACA TTT TCA CAA GAA AGA ACT AGC AGA TGG GGA TGC GAA GAG TTT GGG TGC TTG 3254

Ala Val Asn Thr Gly Cys Val Phe Gly Ser Cys Gln Asp Val Ile Arg Pro Glu Thr Lys Val Tyr Arg Lys Ala Val Asp Glu Val Val 1096
GCT GTC AAC ACA GGT TGT GTG TTT GGG TCT TGT CAA GAT GTA ATT AGA CCA GAA ACA AAA GTT TAC AGG AAA GCT GTA GAT GAA GTT GTT 3344

Ile Leu Thr Val Cys Ile Thr Tyr Pro Gly His Thr Phe Cys Thr Glu Ile Asn Ala Ile Glu Pro Lys Ile Thr Glu Glu Ile Glu Leu 1126
ATT TTA ACA GTT TGT ATT ACA TAT CCA GGA CAC ACT TTT TGC ACA GAA ATT AAT GCC ATA GAG CCA AAA ATA ACG GAA GAA ATT GAA CTC 3434

Gln Phe Lys Thr Val Asp Thr Lys Thr Leu Pro Tyr Ile Val Ala Val Asn Asn His Lys Leu Tyr Ser Gly Gln Ile Asn Asp Leu Gly 1156
CAG TTT AAA ACA GTT GAC ACG AAA ACA CTA CCA TAT ATA GTA GCC GTA AAC AAT CAT AAA CTT TAT AGT GGT CAG ATA AAT GAT TTA GGG 3524

Thr Phe Gly Gln Met Cys Gly Asn Val Gln Lys Thr Asn Ser Ser Ile Leu Gly Thr Gly Thr Pro Lys Phe Asp Tyr Thr Cys His Gly 1186
ACA TTT GGG CAA ATG TGC GGA AAT GTC CAA AAA ACA AAC AGC AGC ATT TTA GGA ACT GGG ACA CCA AAA TTT GAT TAT ACT TGC CAT GGT 3614

Ala Ser Arg Lys Asp Ile Ile Val Arg Arg Cys Tyr Asn Asn Asn Phe Asp Ser Cys Lys Leu Leu Lys Glu Glu Thr Gln Leu Ile Phe 1216
GCT AGT AGG AAG GAT ATC ATT GTT AGG AGA TGC TAT AAT AAC AAT TTT GAC TCC TGC AAA CTT CTA AAG GAA GAA ACA CAG CTT ATA TTT 3704

Asn Asp Asp His Asp Thr Ile Thr Val Tyr Asn Thr Asn His Leu Ile Gly Glu Leu Ala Ile Lys Leu Ile Leu Gly Asp Ile Gln Tyr 1246
AAT GAT GAC CAT GAT ACA ATA ACT GTT TAT AAT ACA AAT CAC TTA ATT GGT GAG TTA GCT ATA AAA TTG ATA TTA GGG GAT ATC CAG TAT 3794

Lys Leu Phe Thr Glu Thr Leu Asp Leu Gln Ile Asp Ala Lys Cys Val Gly Cys Pro Asp Cys Phe Glu Ser Tyr Ser Cys Asn Phe Gln 1276
AAA TTA TTC ACA GAA ACA TTG GAC CTC CAG ATT GAT GCG AAA TGT GTT GGC TGC CCT GAT TGC TTT GAG AGC TAT TCC TGC AAT TTC CAA 3884

Ile Val Ser Asn Ile Asp Thr Ile Cys Ser Leu Glu Gly Pro Cys Asp Thr Phe His Asn Arg Ile Ser Ile Lys Ala Met Gln Gln Asn 1306
ATA GTA TCA AAT ATA GAC ACA ATC TGC AGC CTT GAA GGG CCT TGT GAC ACA TTC CAT AAT AGG ATC TCA ATT AAA GCA ATG CAG CAA AAT 3974

Tyr Ala Val Lys Leu Ser Cys Gln Lys Asp Pro Arg Pro Ser Gly Thr Phe Lys Ile Cys Asn Arg Glu Tyr Thr Val Val Phe His Thr 1336
TAT GCT GTA AAA CTC TCC TGC CAG AAG GAT CCA AGA CCA TCA GGG ACT TTT AAA ATT TGC AAC AGG GAA TAT ACT GTT GTC TTC CAT ACA 4064

Val Ala Lys Asp Asp Lys Ile Glu Ile Asn Val Gly Asp Gln Thr Ser Phe Ile Lys Glu Lys Asp Asp Arg Cys Lys Thr Trp Leu Cys 1366
GTA GCA AAA GAT GAC AAA ATA GAA ATA AAT GTT GGA GAC CAG ACT TCA TTC ATT AAA GAG AAA GAT GAC AGG TGC AAA ACA TGG CTG TGT 4154

Arg Val Arg Asp Glu Gly Ile Ser Val Ile Phe Glu Pro Ile Lys Ala Phe Phe Gly Ser Tyr Phe Ser Ile Phe Phe Tyr Ile Ile Val 1396
AGG GTC AGA GAT GAA GGT ATT AGT GTA ATT TTT GAA CCA ATT AAA GCC TTC TTT GGG AGT TAT TTC AGC ATC TTC TTC TAC ATA ATT GTT 4244

Val Val Val Val Gly Phe Leu Ile Ile Tyr Ile Phe Met Pro Met Phe Met Lys Leu Lys Glu Val Leu Lys Ala Asn Glu Lys Leu Tyr 1426
GTT GTA GTA GTG GGA TTT TTA ATA ATA TAT ATT TTT ATG CCA ATG TTT ATG AAG TTA AAG GAA GTG TTG AAA GCA AAT GAG AAG CTT TAC 4334

Leu Gln Glu Ile Lys Gln Lys --- 1434
TTG CAA GAA ATT AAG CAA AAG TGA AAATTAGACGGTTATTAATTCATTGTTAAATACATTCAAAATTCATATTGACACATTGTGTCAAAAAACAAGGCTGTTTTGTTATCGG 4446

TAGCACACTACT 4458

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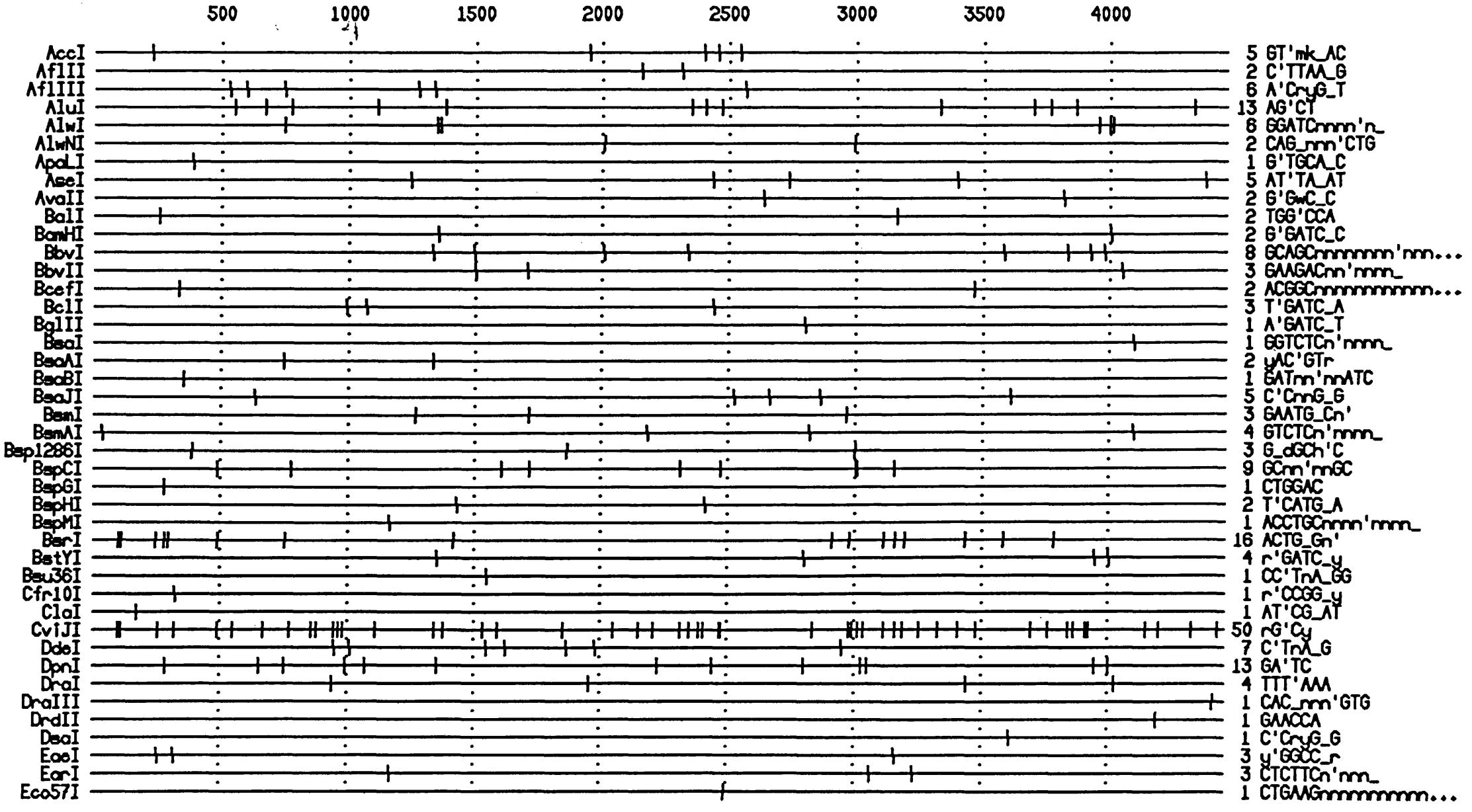
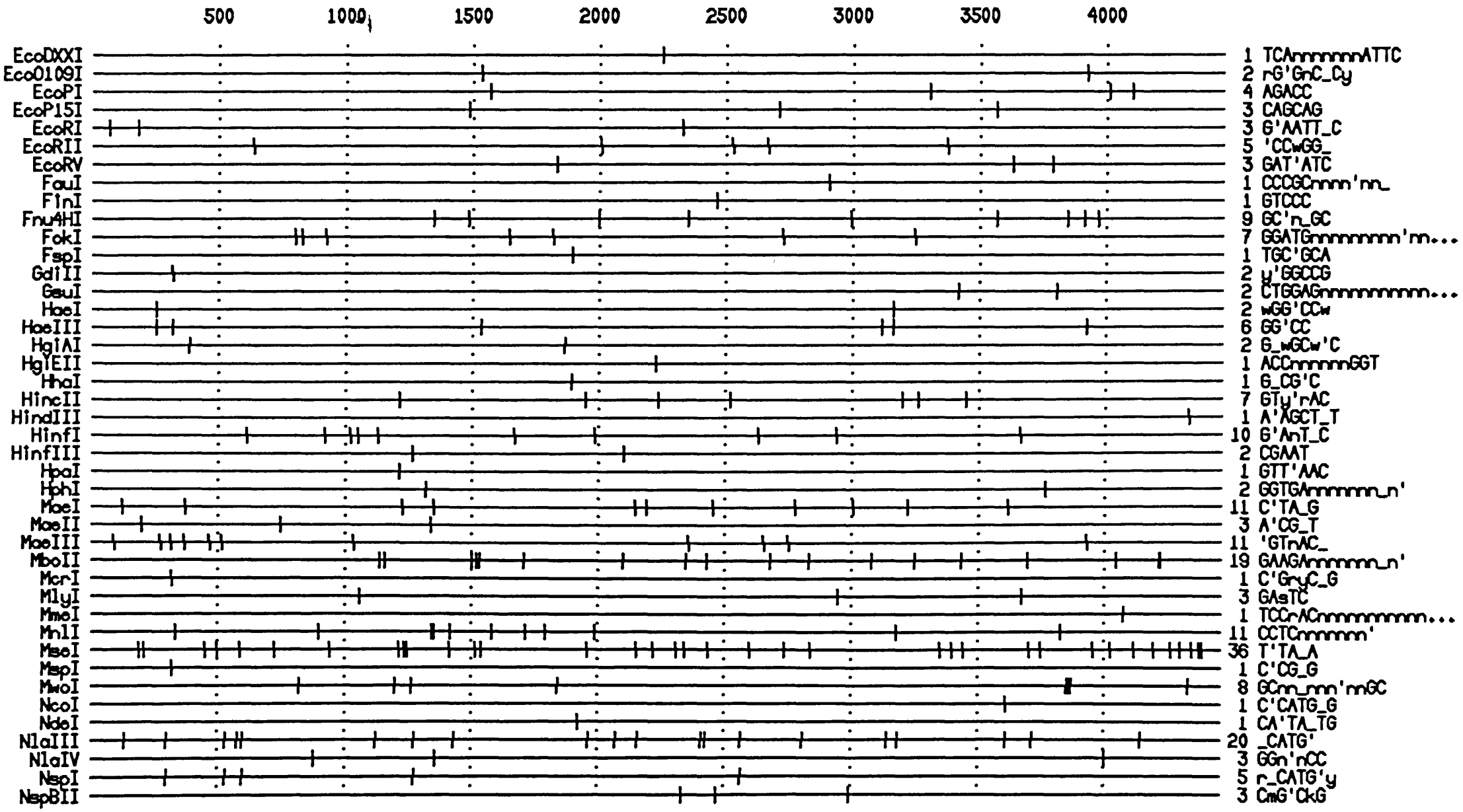
Enzyme Data: Enzyme.Dat



(Linear) MAPPLOT of: Bun.M ck: 7274, 1 to: 4458 August 28, 1992 15:25.

Enzyme Data: Enzyme.Dat



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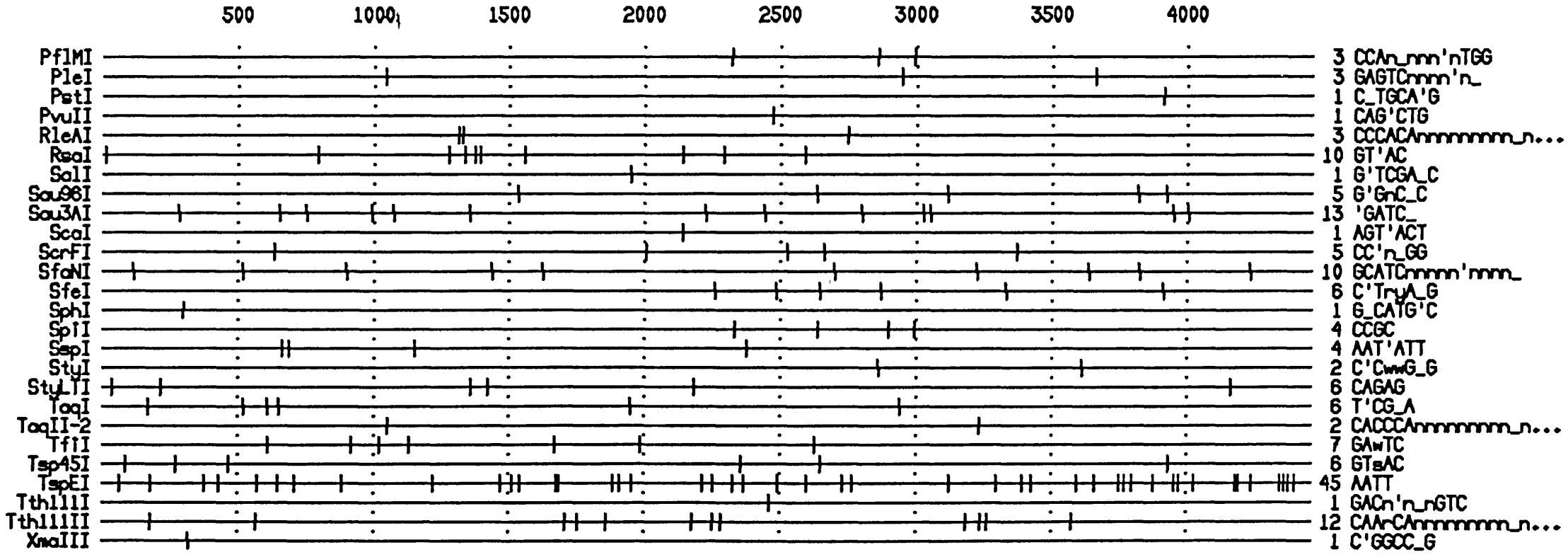
Enzyme Data: Enzyme.Dat



Enzymes that do not cut:

AatII	AgeI	AhaII	ApaI	AvaI	AvrII	BanI	BanII	BcgI	BglI	Bpu10I	BstI
BspMII	BsaHII	BstBI	BstEII	BstXI	CfrAI	DrdI	Ec1I	Eco47III	EcoAI	EcoBI	EcoDI
EcoEI	EcoKI	EcoNI	EcoR124I	EcoR124/3I	EspI	Esp3I	FseI	HaeII	HgaI	KpnI	
MfeI	MluI	NaeI	NarI	Nc1I	NheI	NotI	NruI	Ns1I	PmlI	PpuMI	PshAI
PvuI	RsrII	SacII	Sf1I	SgrAI	SmaI	SnaI	SnaBI	SpeI	Spl1	SstI	StuI
StySBI	StySJI	StySPI	StySQI	TaqII-1	ThaI	Uba1105I	Uba1108I	XbaI	XcmI	XhoI	XmnI

Enzymes excluded; MinCuts: 1 MaxCuts: 350000

NONE

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