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A Study of the Genetics and Growth of a  
Tripartite RNA virus using ts Mutants

by

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A thesis submitted to the University of Warwick,  
Department of Biological Sciences for the degree  
of Doctor of Philosophy.

March 1987.

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## DECLARATION

Some of the work in Results Sections 1(a - c), and 3,  
was presented at "The Biology of Negative Strand Viruses",  
Robinson College, Cambridge, September 15 - 20, 1985.

I declare that all the work  
described in this thesis is my own.  
John Hampton.

## Abbreviations

A	Adenine
BHK	Baby hamster kidney-21 line of tissue culture cells
BS-C-1	African green monkey kidney line of tissue culture cells
C	Cytosine
CCCP	Carbonylcyanide <i>m</i> -chlorophenylhydrazone
cDNA	complementary DNA
cpm	Counts per minute
dATP	2' deoxyadenosine 5' triphosphate
dCTP	2' deoxycytidine 5' triphosphate
dGTP	2' deoxyguanosine 5' triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
eop	Efficiency of plating
FTIC	Fluoromycin isothiocyanate
G	Guanine
GDM	Glasgow modification of Eagle's medium
moi	Multiplicity of infection
mRNA	Messenger RNA
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pfu	Plaque forming unit
PMSF	Phenylmethylsulfonyl fluoride
psi	Pounds per square inch
RNA	Ribonucleic acid
rpm	Revolutions per minute

SDS	Sodium dodecyl sulphate
T	Thymine
TEMED	N,N,N',N'-tetramethylethylenediamine
TLCK	N-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
ts	Temperature sensitive
ts <sup>+</sup>	Wild type with respect to temperature sensitivity
TPP	Thymine 5' triphosphate
U	Uridine
v/v	Volume/volume
w/v	Weight/volume

### Summary

The growth characteristics of Maguari virus and Bunyamwera virus of the family Bunyaviridae were investigated using is mutants and revertants. By genetic analysis involving recombination by segment reassortment between viruses, it was possible to assign Group I is mutants to the S RNA, Group II to the M RNA and Group III to the L RNA segments. Representatives of each of these groups of mutants showed different phenotypes, which presumably reflect the effects of the is lesions on the different virus functions encoded by each genome segment.

It was found that Maguari virus dominates over Bunyamwera virus in mixed infections and this probably accounts for the predominance of Maguari genotype isolates from genetic crosses. Examination of a mixed reassortant clone indicated that multiple copies of at least the S RNA segment can exist in a virus preparation in a more intimate relationship than a simple mixture of two populations of viruses.

Analysis of Group II revertants with altered G1 protein electrophoretic mobility provided information on the glycosylation of wild type G1 protein. It was found that the growth of these revertants in tissue culture was not defective despite the gross changes in a major structural protein. However, it was found that antiserum which neutralised the wild type virus failed to neutralise infectivity of one of the revertants.

INTRODUCTION

## Foreword

The work presented here is an analysis of a tripartite RNA virus, using mutants and genetic recombination to study the biological functions of the genome segments. These viruses can undergo recombination with each other by reshuffling of their genome segments during mixed infections. Analysis of the recombinants so formed was used to study the genetics and growth of these viruses. The effects of temperature sensitive mutations in relationship to their location in specific segments on replication of a virus was studied. Using these techniques, many interesting characteristics of these viruses were analysed and are presented in this thesis.

The viruses used are members of the large family of arthropod borne, negative strand, RNA viruses - the Bunyaviridae. Initially I shall outline the taxonomy of the family and the position of the viruses used within the group, and then give a brief description of the natural history of these particular viruses. This is followed by a review of the literature concerning the growth and genetics of the viruses of the family.

# II. General Characteristics and Taxonomy of the Bunyaviridae

The family Bunyaviridae is divided into four genera (Matthews, 1982) with a fifth genus proposed (Schmaljohn & Dalrymple, 1983). The genera are serologically unrelated but have molecular, biochemical and morphological features in common, allowing definition of the family (Porterfield et al., 1976; Matthews, 1982). The virions are spherical and enveloped, 90-100nm in diameter, containing the negative sense, single stranded RNA genome in three segments; L (large), M (medium) and S (small). Virions develop in the cytoplasm of infected cells and mature by budding into smooth surfaced vesicles in the Golgi region, or nearby. Replication is not blocked specifically by inhibitors of DNA transcription. The envelope contains at least one virus specified glycoprotein. Three size classes of ribonucleoprotein complex can be extracted from disrupted particles. Transcriptase activity is associated with the virions.

The four genera are further subdivided into antigenic groups - serogroups - the premise of such a group being that intragroup reactions result from shared antigens. Thus the degrees of relatedness of viruses by serological parameters are ascertained. Subdivision of the family into genera reflects both the antigenic relationships between the groups, and the molecular differences that have been observed between selected members of the genera (Bishop et al., 1980). The four genera are bunyavirus, phlebovirus, nukuvirus and hantavirus. The fifth proposed genus - hantavirus - differs from the four accepted genera in that no arthropod vector



has been identified for the member viruses: therefore, this genus will not be considered further here.

Each genus can be defined by the observed range of characteristics which are shared by its member viruses. The taxonomy of the family has been recently reviewed by Bishop(1985) and only a brief description of each genus is given here.

#### I1a. *Bunyavirus* genus.

The name of the genus is derived from the place where the type species, *Bunyamwera virus*, was first isolated, in the *Bunyamwera* region of the Semiliki Forest in Uganda (Smithburn, 1946). This is the largest genus of the family with at least 150 named viruses distributed between 16 serogroups. According to Newton et al. (1983), the four viruses of the Mapputta serogroup should also be included in the genus, making it even larger. The serogroups are outlined in Table 1. The shared molecular characteristics of the genus are described in Table 2.

The bunyaviruses are transmitted primarily by mosquitoes but occasionally by other haematophagus arthropods - biting midges, sandflies, ticks and horseflies (Berge, 1975). Some of the viruses have been shown to be capable of transovarial and venereal transmission in mosquito populations (Crane et al., 1977; Grimstad & Haramis, 1984; Thompson & Beatty, 1978).

#### I1b. *Phlebovirus* genus.

The name of the genus is derived from the phlebotomines - or sandflies - which are the major vectors of these viruses

Table 1.

## Serogroups of Bunyavirus genus.

Serogroup	Number of Members	Serogroup	Number of Members.
Anopheles A	12	Guama	12
Anopheles B	2	Koongol	2
Bunyamvera	26	Minatitlan	2
Bwamba	2	Olifantavlei	3
C	16	Patois	6
California encephalitis	15	Simbu	25
Capim	10	Tete	5
Gamboa	7	Turlock	5

Table 2.

## Structural Characteristics of Bunyaviruses.

RNA - sizes		Structural proteins - sizes	
L	$2.7 - 3.1 \times 10^6$	L	$145 - 200 \times 10^3$
M	$1.8 - 2.8 \times 10^6$	G1	$108 - 120 \times 10^3$
S	$0.28 - 0.5 \times 10^6$	G2	$29 - 41 \times 10^3$
		N	$19 - 25 \times 10^3$

(Berge, 1975). The genus contains 27 viruses in 8 serogroups, shown in Table 3. 11 viruses are assigned to the genus but are not serologically related to viruses in the serogroups and are 'unassigned' (Bishop, 1985). Table 4 shows the general structural characteristics of members of the genus.

The viruses infect various vertebrate species and are transmitted primarily by sandflies, although occasionally other arthropods are vectors - mosquitoes and biting midges. Transovarial transmission in a sandfly has been demonstrated (Endris et al., 1983). The type species of the genus is Sandfly fever (Sicilian) virus - SFS virus (Berge, 1975).

#### Iic. Uukuvirus genus.

The name of the genus is taken from the place in Finland where the type species, Uukuniemi virus, was first isolated (Berge, 1975). Table 5 shows that the genus is regarded as a single serogroup with at least 7 member viruses. The structural characteristics of these viruses are outlined in Table 6.

The viruses infect various vertebrates and have been shown to be transmitted by ticks (Berge, 1975).

#### Iid. Nairovirus genus.

The name of the genus is derived from the first isolated member of the genus, Nairobi sheep disease virus, although the type species is Crimean-Congo haemorrhagic fever virus. Table 7 shows the 6 serogroups which contain 29 member viruses. Table 8 shows the structural characteristics of these viruses.

Table 3.

Serogroups of Phlebovirus genus.

Serogroup	Number of Members	Serogroup	Number of Members
Sandfly fever Naples	4	Frijoles	2
Bujaru	3	Rift Valley fever	4
Candiru	8	Salehabad	2
Chilibre	2	Sandfly fever Sicilian	2
Unassigned	11		

Table 4.

Structural Characteristics of Phleboviruses.

RNA - sizes		Structural proteins - sizes	
L	$2.6 - 2.8 \times 10^6$	L	$145 - 200 \times 10^3$
M	$1.8 - 2.2 \times 10^6$	G1	$55 - 70 \times 10^3$
S	$0.7 - 0.8 \times 10^6$	G2	$50 - 60 \times 10^3$
		N	$20 - 30 \times 10^3$

Table 5.

Serogroup of Uukuvirus

Serogroup	Number of Members
Uukuniemi	7

Table 6.

Structural Characteristics of Uukuviruses.

RNA - sizes		Structural proteins - sizes	
L	$2.0 - 2.5 \times 10^6$	L	$180 - 200 \times 10^3$
M	$1.0 - 1.3 \times 10^6$	G1	$70 - 75 \times 10^3$
S	$0.4 - 0.6 \times 10^6$	G2	$65 - 70 \times 10^3$
		N	$20 - 25 \times 10^3$

Table 7.

## Serogroups of Nairovirus genus.

Serogroup	Number of Members
Crimean-Congo	
haemorrhagic fever	3
Dera Ghazi Khan	6
Hughes	8
Nairobi sheep disease	3
Qalyub	3
Sakhalin	6

Table 8.

## Structural Characteristics of Nairoviruses.

RNA - sizes		Structural proteins - sizes	
L	$4.1 - 4.9 \times 10^5$	L	$145 - 200 \times 10^3$
M	$1.5 - 1.9 \times 10^6$	G1	$72 - 84 \times 10^3$
S	$0.6 - 0.7 \times 10^6$	G2	$30 - 40 \times 10^3$
		M	$48 - 54 \times 10^3$

The viruses infect various vertebrates and are transmitted mainly by ticks but occasionally by mosquitoes and biting midges.

Iie. Possible members of the family.

There are 27 viruses considered to be possible members of the family but which have not been assigned to genera. 15 of these are arranged in 5 serogroups (Bishop, 1985) as shown in Table 9.

Iif. The complexity of arbovirus transmission.

Members of the *Bunyaviridae* (apart from the hantavirus genus) are all arboviruses and are generally considered to have complex biological associations with their arthropod hosts, which include ticks, mosquitoes, sandflies, biting midges and horseflies. The best studied virus-arthropod relationships are those involving mosquitoes. Usually, an adult female has a lifelong infection following ingestion of a blood meal from an infected vertebrate. Sometimes transovarial transmission to offspring, and venereal transmission from infected females to males and also from transovarially infected males to females, can occur. The complexity of factors affecting mosquito infection and subsequent ability to transmit infection is described by Hardy et al. (1983). Sandflies (which are vectors of phleboviruses) are also capable of transovarial transmission (Endris et al., 1983) and there is a suggestion of transovarial transmission of an *ukuvirus* in ticks (Nuttall et al., 1981).

The interrelationships of a susceptible vertebrate population with an infected arthropod vector, capable of transmitting that

Table 9.

Possible Members of the Family.

Serogroup	Number of Members.
Bakau	2
Kalodi	3
Hantaan	4
Maputta	4
Yogue	2
Unassigned	12



infection, are subject to a variety of factors such as climatic variation, overwintering of infected adult arthropods, overwintering of infected eggs and larvae, hibernation of viraemic vertebrates, migration of vertebrates (both birds and mammals), changes in land drainage and vegetation, all of which have effects on the interactions of the vector population with the vertebrate host population. As some arthropods can effectively transmit a virus through their own population independent of vertebrate involvement, other than as a source of initial infection, the vector population can act as a reservoir of infection. Modulation of a virus in a vector population is potentially very important. Interactions between two different viruses in the same vector population can occur, with the possibility of the generation of novel recombinant genotypes of different pathogenicity from either of the two original viruses (Beaty et al., 1981; Beaty et al., 1983). Not all arboviruses are transovarially or venereally transmitted between arthropods, and how these viruses are maintained in an environment is less well understood. In order to fully appreciate the biology of a virus, ecological factors need to be determined and the influence of all the different facets of the environment will play a role in the selection of a given genotype best suited to that environment.

## I2 Natural History of the Two Viruses Used for this Study,

### Bunyamwera Virus and Maguari Virus.

Both Bunyamwera and Maguari viruses are members of the Bunyamwera serogroup of the bunyavirus genus. The arthropod vectors for these viruses are mosquitoes and both viruses have been isolated from these insects.

Bunyamwera virus is prevalent in Africa - in certain areas, up to 80% of human inhabitants have been found to be seropositive (Berge, 1975). Virus isolation and serological surveys have identified the distribution of Bunyamwera virus to be throughout the African continent, and there is also serological evidence of its presence in South America (Berge, 1975).

Maguari virus was first isolated in 1957 from a pool of mixed mosquitoes collected in the Utinga Forest, a watershed forest, in Brazil (Causey *et al.*, 1961). Virus isolations from mosquitoes and various mammals, and serological data, indicate that Maguari virus is distributed throughout South America. Serological studies have indicated that the virus frequently infects humans and large mammals, notably horses, although the virus itself has not been isolated from humans (Berge, 1975; Spence *et al.*, 1968). Maguari virus is commonly isolated from mosquitoes and in a recent study it was the most frequently isolated arthropod-borne virus from mosquitoes in Ecuador (Calisher *et al.*, 1983).

Both Bunyamwera virus and Maguari virus have been associated serologically with naturally occurring human infections (Theiler

& Downs, 1973). Bunyamwera virus can cause disease in humans resulting in fever, prostration, stiffness of the neck, and various other symptoms (Kokernot et al., 1958; Berge, 1975), and there are reports of laboratory acquired infections.

As both viruses have the ability to infect humans, it is important to exercise caution in the handling of them - especially in work involving recombination.

## 13 Virus Structure

Bunyavirus structure has been well reviewed by Bishop and Shope (1979) and most of the information has come from studies of the California encephalitis and Bunyamwera serogroups of the bunyavirus genus, and Uukuniemi virus of the uukuvirus genus. Electron microscopic studies have shown that representatives of the whole family are structurally very similar. A recent study (Martin *et al.*, 1985) indicates that in spite of general structural similarities, members of each genus have morphologically distinct surface structures - as determined by negative stain electron microscopy of glutaraldehyde fixed viruses.

The virus particles are spherical, with a buoyant density in sucrose of  $1.17 - 1.19 \text{ g cm}^{-3}$ . Surface projections of 5 - 10nm with a central hole are present: in the bunyavirus genus, the arrangement appears to be irregular (Obijeski *et al.*, 1976); in phleboviruses and nairoviruses the arrangements are regular but indistinct - no central hole has been observed in the projections of nairoviruses (Martin *et al.*, 1985); the arrangement in Uukuniemi virus is an icosahedral lattice, which is distinct from the other members of the family (von Bonsdorff & Pettersson, 1975; Martin *et al.*, 1985).

The genome of the viruses consists of three segments of single stranded RNA arranged in three distinct nucleocapsids. These are named in terms of size as L (large), M (medium), and S (small). For Lumbo and LaCrosse viruses of the bunyavirus genus, and

Uukuniemi virus of the uukuvirus genus, the RNA segments have been shown to be arranged as non-covalently closed circles (Bishop & Shope, 1979). The nucleocapsids each contain a single species of viral RNA, N polypeptide (the nucleocapsid protein), and minor quantities of L polypeptide, thought to be a viral transcriptase (Bishop et al., 1980).

Virion-associated transcriptase activity has been shown to be associated with various members of the family: Uukuniemi virus (Ranki & Pettersson, 1975), LaCrosse virus (Patterson et al., 1984), Lumbo virus (Bouloy & Hannoun, 1974), and Germiston virus (Bouloy et al., 1984), which suggests that the genomes of the viruses in the family are of opposite polarity to mRNA and so the Bunyaviridae is regarded as a family of negative strand viruses.

The virus envelope contains two glycoproteins - G1 and G2 - in approximately equal numbers of molecules (Bishop et al., 1980). The functions of these proteins have been studied and will be discussed later.

### 13a. Organisation of genomic RNA.

As mentioned, the three genome segments of bunyaviruses are single stranded (Bishop & Shope, 1979). The 5' terminal nucleotides of the viral RNA species have not been found to be capped or methylated, and is pppAp for snowshoe hare (Gentsch et al., 1977), LaCrosse (Obijeski et al., 1976), and Uukuniemi (Pettersson et al., 1977) viruses. No 3' polyadenosine tract has been found

on the viral RNA of Lumboc, Ukuniemi, snowshoe hare or LaCrosse viruses (Bouloy et al., 1974; Pettersson et al., 1977; Clewley et al., 1977). The RNA species of bunyaviruses can be isolated from virions as non-covalently closed circles. The circularisation is thought to involve inverted complementary sequences at the 3' and 5' ends of the RNA segments, which result in hydrogen bonding and the formation of panhandle structures (Pardigon et al., 1982).

There is a high level of terminal sequence conservation between viruses of the same genus - usually involving 10 - 15 nucleotides. This is thought to reflect the importance of terminal hydrogen bonding, which may be involved in nucleocapsid formation and morphogenesis (Pardigon et al., 1982; van Haaster et al., 1982; Clerx-van Haaster & Bishop, 1980). It may also be a means by which genomic RNA can be functionally distinguished from mRNAs and may also represent preservation of polymerase binding sites. The various genera have evolved different terminal sequences. If these are indeed initiation sites for virus transcriptases, the viruses have probably evolved genus - specific enzymes (Strauss & Strauss, 1983).

Oligonucleotide fingerprinting analyses have shown that each of the three size classes of RNA (for a given virus) has a unique nucleotide sequence, which suggests that each contains separate genetic information, rather than representing degradation of a large molecule (Bishop & Shope, 1979).

### I3a 1 Molar ratios of species of RNA.

The molar ratios obtained for the three RNA species of various bunyaviruses are highly variable - even with different preparations of the same virus (Bishop & Shope, 1979). Generally, S RNA is the most abundant (McPhee & Westaway, 1981; Kascsak & Lyons, 1977). It has been reported that S RNA is found in even larger amounts in preparations of defective Bunyamwera virus (Kascsak & Lyons, 1978). There is also a report of a ratio of L : M : S of 1 : 4-6 : 2 for Uukuniemi virus (Pettersson & Kaariainen, 1973). Thus it is not known how many copies of each RNA segment are present in each infectious virus particle.

### I3b Sequence analyses.

#### I3b 1 S RNA.

Sequence analyses of the S RNA of LaCrosse and snowshoe hare viruses (California encephalitis serogroup: bunyavirus genus) have shown that two gene products - N (nucleocapsid protein) and NSs (a nonstructural protein) - are encoded in overlapping reading frames in viral complementary mRNA, as the viral RNA is of negative sense (Akaishi & Bishop, 1985; Bishop *et al.*, 1982; Fuller & Bishop, 1982; Fuller *et al.*, 1983; Cabradilla *et al.*, 1983; Clerx-van Haaster *et al.*, 1982). Sequence analysis of a phlebovirus, Punta Toro, has indicated a different coding strategy from the bunyavirus genus (Ihara *et al.*, 1984). N protein is encoded in a viral complementary mRNA, and a presumptive NSs polypeptide is encoded in a different, viral sense mRNA. Thus it

would appear that phleboviruses have an "ambisense" coding strategy for the S RNA, a term which was first used to describe the coding strategy of the S RNA of Arenaviruses (Auperin *et al.*, 1984). However, the S RNAs of bunyaviruses and arenaviruses are not related: the Arenaviridae have only two segments of genomic RNA, originally thought to be of negative sense, and their S RNA codes for two envelope glycoproteins in addition to a nucleocapsid protein (Bishop *et al.*, 1981).

Comparison of the sequences of the S RNA segments of three closely related viruses of the California encephalitis serogroup (bunyavirus genus) identified very few substitutions in the coding region of the S RNA, and this is thought to reflect constraints imposed on variation by the existence of overlapping reading frames (Clerx-van Haaster *et al.*, 1982).

### I3b 2 M RNA.

The sequences of the M RNA segments of Bunyamwera and snowshoe hare bunyaviruses (Lees *et al.*, 1986; Eshta & Bishop, 1984), and Rift Valley fever and Punta Toro phleboviruses (Collett *et al.*, 1985; Ihara *et al.*, 1985) have all shown that there is only one, large open reading frame - in a viral complementary sense - which is capable of encoding G1 and G2 and a nonstructural protein NSs in one mRNA. Genetic studies had previously shown that these polypeptides were all encoded by the M RNA segment (Bishop & Shope, 1979). For Bunyamwera virus, the single open reading frame in



the viral complementary sense is flanked by non-coding regions, longer at the 3' than at the 5' terminus (Lees *et al.*, 1986). Also there are only a few possible asparagine - linked glycosylation sites; the sequence of snowshoe hare M RNA also indicates only a small number of glycosylation sites (Eshita & Bishop, 1984).

There is a conservation of cysteine residues - 66 out of 70 - in the M RNA complementary sequences of snowshoe hare and Bunyamwera viruses, and an overall 43% homology between the amino acid sequences for the products from these two sequences. This suggests an evolutionary relationship and a preservation of structural features of the encoded gene products between two apparently distantly related viruses (Lees *et al.*, 1986).

The gene order for G1, G2 and NSm is not known for either Bunyamwera or snowshoe hare bunyaviruses.

The sequences of the M RNA segments of Punta Toro and Rift Valley fever phleboviruses indicate a similar coding strategy to that used by the bunyavirus genus - namely a single large open reading frame in a viral complementary RNA, which encodes two glycoproteins G1 and G2, and probably also a nonstructural protein NSm (Ihara *et al.*, 1985; Collett *et al.*, 1985).

The order of G1 and G2 on both these sequences has been determined; on the viral RNA the order is 3' G1 - G2 for Punta Toro virus and 3' G2 - G1 for Rift Valley fever virus. However, it should be made clear that G1 and G2 are named solely in terms of size and not function (Bishop *et al.*, 1980), the larger protein

being named G1. The two sequences are related to each other (Dhara et al., 1985): Punta Toro G1 and Rift Valley fever G2 show 35% amino acid homology, and Punta Toro G2 and Rift Valley fever G1 show 49% amino acid homology. The implication is that Punta Toro G1 and Rift Valley fever G2 may be functionally equivalent, and similarly for Punta Toro G2 and Rift Valley fever G1. However, the authors do not give the percentage homology between the G1 proteins of the two viruses or the G2 proteins of the two viruses, therefore the evidence is hardly conclusive. No primary homology was seen when the two phlebovirus sequences were compared with that of the bunyavirus snowshoe hare.

A computer generated model for the secondary structure of the viral M RNA of Rift Valley Fever virus (Collett et al., 1985) revealed a prominent, energetically stable, base paired, stem structure. It was proposed that this may be important for viral polymerase recognition - consequently the secondary structure may be important in replication and transcription.

### I3b 3. L RNA.

There are no complete sequence data for the L RNA of any bunyavirus. Analysis of the termini of some L RNAs has shown conservation and complementarity as described earlier. Analysis of LeCrosne and snowshoe hare viruses' L RNAs (Clerx-van Haaster et al., 1982) has indicated that the first 3' UAC triplet (AUG in the mRNA) does not initiate an extensive open reading frame in the

viral complementary RNA but that a second UAC does appear to do so.  
Apart from this, few other results have been reported on the L RNA  
sequence.

#### I4 Virus Replication

##### I4a Adsorption and entry.

Since the viral glycoproteins G1 and G2 project from the viral envelope, they are the most exposed portion of a virus. As such they are the first elements to come into contact both with potential host cells and the host's immune system. Studies of G1 and G2 have implicated the involvement of G1 in attachment of virions to cells (Kingsford & Hill, 1980). However, the mechanisms of entry, and whether only one or both glycoproteins are involved, are not known.

##### I4b RNA synthesis: primary transcription.

As previously mentioned, at least some bunyavirus preparations have a virion associated RNA dependent RNA polymerase. With other families of negative strand RNA viruses, which have a similar enzyme activity, treatment of virus infected cells with inhibitors of protein synthesis inhibits replication at an early stage (Strauss & Strauss, 1983). In the presence of such inhibitors, the only detectable virus synthetic process is the production of small amounts of RNA by the virion transcriptase (Wagner, 1975; Chopin & Compans, 1975). This is defined as primary transcription. In order to progress to the stage of amplified RNA synthesis - secondary transcription - protein synthesis is required. On reaching this stage, continued RNA synthesis at the amplified rate is independent of protein synthesis (Scholtissek & Rott, 1970; Robinson, 1971). Families of negative strand viruses show transcription characteristics of this type: Orthomyxoviridae

(Bean & Simpson, 1973), Ehabdoviridae (Marcus et al., 1971), and Paramyxoviridae (Robinson, 1971).

Several members of the Bunyaviridae have been examined in terms of transcription, but the data are conflicting. Primary transcription as defined above, using inhibitors of protein synthesis, was first reported for Bunyamwera virus in 1977 (Kascsak & Lyons). Subsequently, two separate studies with snowshoe hare virus also showed that primary transcription occurs (Vezza et al., 1983; Eshita et al., 1985). Similarly, a study of the unrelated phlebovirus Punta Toro, also indicated that primary RNA synthesis occurs in the presence of inhibitors of protein synthesis (Ihara et al., 1985). However, work with LaCrosse virus - which is so closely related to snowshoe hare virus that it has been described as a variety (Bishop & Shope, 1979) - has indicated that no RNA synthesis occurs in the presence of inhibitors of protein synthesis (Patterson & Kolakofsky, 1984). A similar result had been obtained earlier with Bunyamwera virus by Abraham and Pattanaik (1983) who also showed that Akabane virus (Simbu serogroup: bunyavirus genus) differed from the typical properties of negative strand viruses in that amplified (secondary) transcription was also sensitive to inhibitors of protein synthesis. Recently, Bellocq et al. (1985) reported that protein synthesis was required for the production of long transcripts by LaCrosse virus, both in vivo and in vitro.

Whether the apparent conflict results from practical differences

in the methods used for analysis, or whether some bunyaviruses do indeed have a requirement for host protein synthesis in order to make long primary transcripts, is as yet unresolved.

#### I4c RNA synthesis: production of mRNA.

Recent work on the transcription processes of bunyaviruses has revealed that the polymerase activity of these viruses which serves to transcribe mRNA is remarkably similar to that of influenza virus (Patterson *et al.*, 1984; Bishop *et al.*, 1983). Influenza virus uses a virion, methylated cap - dependent endonuclease to provide primers for mRNA synthesis, by removal of the cap structure and 10 - 15 nucleotides from preformed host cell mRNA molecules in the nuclei of infected cells (Bouloy *et al.*, 1980; Krug, 1981; Plotch *et al.*, 1981; Penn & Mahy, 1984). Bunyaviruses have been shown to grow in enucleated cells and so (unlike influenza virus) do not have a nuclear dependence (Goldman *et al.*, 1977; Patterson *et al.*, 1983); presumably the bunyavirus endonuclease uses preformed host mRNA species present in the cytoplasm as substrates to provide the primers at the 5' ends. As a consequence of using such a primer for mRNA synthesis, there is considerable 5' terminal sequence heterogeneity between different mRNA transcripts from the same genome segment.

Analysis of bunyavirus S RNA mRNA species has established that the messages are shorter than the genomic S RNA (Dehita *et al.*, 1985; Bouloy *et al.*, 1984; Patterson *et al.*, 1984; Bishop *et al.*, 1983;

Fuller et al., 1983; Cash et al., 1979). The studies have been performed with members of the California encephalitis serogroup viruses of the bunyavirus genus. The 5' end of the mRNA was found to be complementary to the 3' end of the viral RNA until the penultimate base. The 5' end of the mRNA was then extended beyond the 3' end of the genome and this additional sequence was heterogeneous - presumably as a consequence of the origin of the primer. Both the S RNA and M RNA mRNA molecules are subgenomic (Rahita et al., 1985) and for snowshoe hare virus, lack 100 nucleotides (S RNA transcripts) or 60 nucleotides (M RNA transcripts) at the 3' end.

One consequence of using a primer for the subgenomic messages is that mRNA molecules will not contain the end complementarity seen with genomic RNA. This also means that there must be at least two distinct types of transcription processes: the primer dependent mRNA transcription, and a process which results in an exact complementary copy ("antigenome") of the genomic RNA species which would be a replicative intermediate for the synthesis of full length genomic RNA progeny molecules. As yet there is no information on this latter transcription process.

As mentioned, the coding strategy of the S RNA of bunyaviruses is of overlapping reading frames in the same viral complementary sense, encoding two distinct gene products. The presence of these overlapping reading frames means either one species of mRNA is translated into both products on separate occasions, or two distinct mRNA molecules are transcribed from the same template. It appears

that bunyaviruses use the former, that is, a single species of subgenomic mRNA is translated to give N and NSs (Clerx-van Haaster et al., 1982). As more N polypeptide than NSs is detected in infected cells, presumably this mRNA is translated so that signals which result in the formation of N are obeyed more frequently than the signals which result in NSs. The mechanism of this control is not known.

The situation is different for phleboviruses. As the S RNA is ambisense, two separate mRNA molecules must be synthesised: one - for N protein - is viral complementary, and the other - for the putative NSs polypeptide - is virus sense. There is much less of the NSs mRNA than either S viral RNA or N mRNA, which indicates that the synthesis of the two messages are regulated independently (Thara et al., 1985). As NSs polypeptide cannot be synthesised until RNA replication has begun - antigenome is required as template for its synthesis - it does not seem likely that NSs is involved in the initiation of RNA replication - at least for phleboviruses.

#### I4d Protein synthesis

Four structural proteins, L, G1, G2 and N, are generally seen in cells infected with bunyaviruses. No evidence of any polypeptide processing involving proteolytic cleavage has been seen, although sequencing studies of the M RNA (see I3b 2) and in vitro translation of mRNA from the M RNA of Uukuniemi virus (Ulmänen et al., 1981) have indicated that at least the two glycoproteins



G1 and G2 are derived from a common precursor. The failure to detect a precursor in infected cells indicates that the cleavages involved are very rapid and probably cotranslational.

The four structural proteins appear to be synthesised in two stages: an early phase, involving the synthesis of N and L (N has been detected as early as two hours after infection), and a late phase, involving G1 and G2. The synthesis of N and L peaks and declines earlier than G1 and G2 (Pennington *et al.*, 1977; McPhee & Westaway, 1981; Cash, 1982; Struthers *et al.*, 1984).

At least two virus - coded nonstructural proteins have been detected in cells infected with members of the bunyavirus genus, and genetic studies have determined that one - NS<sub>m</sub> - is encoded by the M RNA segment, and the other - NS<sub>s</sub> - by the S RNA segment (McPhee & Westaway, 1981; Fuller & Bishop, 1982; Short *et al.*, 1982; Elliott, 1985). Nonstructural proteins have been described for viruses from the other three genera: Ukuniemi and St. Abb's Head ukuviruses (Ulmann *et al.*, 1981; Watret *et al.*, 1984), Karimabad and Rift Valley fever phleboviruses (Smith & Pifat, 1982; Struthers *et al.*, 1984) and Qalyub and Clo Morairoviruses (Clerx & Bishop, 1981; Watret *et al.*, 1984). A third nonstructural protein has been reported for several bunyaviruses (Elliott, 1985; Bouloy *et al.*, 1984; Fuller & Bishop, 1982) and this protein appears to be related to N protein. It is not clear whether it is a result of degradation of N or a translation product from another transcript of the S RNA in the same reading frame as N (Elliott, 1985). Also, at least some of the nonstructural proteins observed for

nairoviruses have been shown to be precursors of the viral glycoproteins (Clerx & Bishop, 1981; Watret & Elliott, 1985). The temporal synthesis of NSs has been seen to follow that of the M protein and the synthesis of NSs follows that of G1 (Watret et al., 1984; Short et al., 1982).

Generally, bunyaviruses cause an inhibition of host cell protein synthesis (in mammalian cell lines) 5 - 7 hours after infection, dependent on the multiplicity of infection used (Pennington et al., 1977; Bishop & Shope, 1979) but the virus function responsible for this phenomenon has not been identified.

No modifications such as sulphation or phosphorylation of virus proteins have been observed, except for a report of phosphorylation of a nucleus - associated viral nonstructural protein for the phlebovirus Rift Valley fever virus (Struthers et al., 1984).

#### 1.4 The glycoproteins G1 and G2.

The glycosylation of proteins in a number of bunyaviruses has been well studied and the characteristics of the glycoproteins of viruses from different genera appear to be similar. The glycosylation of these proteins can be inhibited by tunicamycin (Maloff & Lemard, 1982; Cash, 1982) which indicates that glycosylation requires the action of a lipid - linked intermediate. Tunicamycin inhibits the addition of N-acetyl glucosamine to dolichol phosphate, which is the first step in the formation of N-linked carbohydrates (Tkacz & Lampen, 1975). After addition of this core oligosaccharide,

the carbohydrate attached to the polypeptide is processed to greater or lesser extents in the Golgi apparatus. Treatment of Uukuniemi virus - infected cells with tunicamycin causes the unglycosylated glycoproteins to accumulate in the endoplasmic reticulum, which indicates that glycosylation is necessary for transport to the Golgi apparatus (Kuismanen *et al.*, 1984). Carbohydrate moieties which are not extensively processed in the Golgi are termed oligomannosyl carbohydrates, and contain only two types of sugar - N-acetyl glucosamine and mannose. Complex carbohydrates may be substituted and processed so as to contain fucose, lactosamine, sialic acid or galactose residues (Stanley, 1984). Whether a glycoprotein contains highly processed oligosaccharides or not can be assessed by the susceptibility of the carbohydrates of the glycoprotein to hydrolysis by endo  $\beta$ -N acetylglucosaminidase F (endo-H) (Tarentino *et al.*, 1974). Complex carbohydrates which terminate in sialic acid or galactose residues are resistant to hydrolysis.

A study of LeCrosse G1 protein indicates that this glycoprotein acquires complex carbohydrates (and consequently becomes endo-H resistant) by a process that occurs in the Golgi at a rate resembling maturation of secreted proteins (Madoff & Lenard, 1982). A more detailed study of both G1 and G2 of Uukuniemi virus has shown that mature G1 contains only endo-H resistant carbohydrate, whereas G2 is mainly endo-H sensitive (Kuismanen, 1984). It is generally considered that the time for acquisition of endo-H resistance

reflects the transport time from the endoplasmic reticulum to the Golgi apparatus for a particular protein (Strous & Lodish, 1980). As for LaCrosse virus, this study (Kuismanen, 1984) with Uukuniemi virus showed that the kinetics of maturation of G1 and G2 with respect to endo-H resistance, was slower than for the glycoproteins of many other virus families, indicating a slower transport from the endoplasmic reticulum to the Golgi.

The antibiotic monensin interferes with the transport of material from the Golgi apparatus to the plasma membrane (Tartakoff, 1983). Madoff and Lenard (1982) found that monensin caused an accumulation of LaCrosse virus G1 of intermediate endo-H resistance in infected cells, in which only some of the carbohydrate sites had been processed to endo-H resistance. This suggests that the drug is acting by inhibiting specific carbohydrate processing reactions rather than simply blocking the transport of G1 from one compartment to another.

Analysis of glycoproteins has established that generally they contain a highly hydrophobic amino terminus which is responsible for insertion of the nascent polypeptide into a membrane. This occurs by recognition of the membrane by the hydrophobic sequence, insertion, and establishment of a membrane - ribosome junction. The hydrophobic signal sequence is then proteolytically removed from the nascent protein (Gheah, 1980). There is no direct evidence of such a signal peptide in bunyavirus glycoproteins, but analyses of the M RNA sequences have identified regions of highly hydrophobic amino acids which may serve this function (see 13b 2).

#### I4f Morphogenesis.

Several studies have shown that the Golgi apparatus is central to the replication of viruses of the Bunyaviridae, not only as it is the organelle involved in the processing and saturation of the envelope glycoproteins, but as a means of transport to the extracellular environment (Bishop & Shope, 1979). The morphogenesis of the Bunyaviridae is different in this respect from all other enveloped viruses: some mature at the plasma membrane of infected cells - for example, alphaviruses, orthomyxoviruses, paramyxoviruses and rhabdoviruses (Kärrlén & Soderlund, 1978; Compans & Choppin, 1971; Knudson, 1973); some in the rough endoplasmic reticulum - for example, coronaviruses (McIntosh, 1974); and some in the nuclear membrane - for example, herpesviruses (Cook & Stevens, 1970). The budding of bunyaviruses occurs exclusively on smooth internal membranes - predominantly those in or adjacent to the Golgi apparatus (Bishop & Shope, 1979; Smith & Pifat, 1982).

The best studied system is Uukuniemi virus. Recent work with temperature sensitive (*ts*) mutants of this virus (Gahberg, 1984; Gahberg *et al.*, 1986) has indicated that either one or both glycoproteins is Golgi specific and is probably responsible for the morphological changes of proliferation and vacuolisation characteristically seen in infected cells (Kuismanen *et al.*, 1982; Smith & Pifat, 1982). Information from immunofluorescence studies has shown that N protein associates closely with the cytoplasmic face of smooth surfaced vesicles of the Golgi in which are inserted the glycoproteins, and that this association is probably a result of

direct interaction of the nucleocapsids with the cytoplasmic part of one or both glycoproteins (Kuusimäen *et al.*, 1982).

In all studies with bunyaviruses, negligible amounts of glycoprotein are transported to the plasma membrane, and release is almost certainly a result of exocytosis, from the fusion of vesicles containing virions with the plasma membrane (Smith & Pifat, 1982). This indicates that the role of the plasma membrane in bunyavirus morphogenesis is passive. The absence of viral antigen on the plasma membrane of infected cells may allow such cells to avoid detection by the immune system.

The morphogenesis of the Bunyaviridae is unique in that the Golgi is apparently the site of virion assembly and maturation and probably plays a key role in virion release. Despite many differences between the viruses of the four genera, it appears that the mechanisms of morphogenesis are similar.

#### I4g The roles of the glycoproteins G1 and G2.

Viral glycoproteins are known to be important determinants of disease processes (Crumpaker, 1980) and to have a major role in the infectious process and the induction of a specific antiviral response. It has been established that G1 protein of members of the bunyavirus genus elicits antibodies capable of neutralising virus infectivity (Gentsch *et al.*, 1980; Kingsford & Hill, 1983; Gonzalez-Scarano *et al.*, 1982).

It has been shown that G1 undergoes a conformational change - at least for LaCrosse virus-- thought to be related to the

virus - membrane fusion function (Gonzalez-Scarano, 1985). Fusion is a function characteristic of enveloped viruses as it is necessary for entry of virion genetic material into host cells - this may occur at the cytoplasmic membrane, or in vesicles following endocytosis - and is also necessary for the formation of progeny virions in discrete membrane - bound particles. However, it is not known whether the change in G1 mediates fusion directly, but the isolation of a G1 monoclonal selected variant - defective in fusion - indicates the importance of G1 in fusion. G2 does not appear to be very immunogenic and most studies have been performed with polyclonal or monoclonal antibodies to G1. Consequently, no specific function has as yet been assigned to G2.

Studies involving proteolytic cleavage of intact virus have established that G1 of LaCrosse virus is cleaved into only two products by trypsin and chymotrypsin and that the other virus proteins are unaffected (Kingsford & Hill, 1981; Kingsford & Hill, 1983). This cleavage of G1 affects virus attachment to cells and neutralisation. Whether this is a result of removal of sites involved in these functions, or whether the effects of cleavage affect distant sites - possibly even involving G2 - remains to be ascertained.

Genetic studies of California encephalitis serogroup viruses have shown that the M RNA segment is a major determinant of pathogenicity (Shope *et al.*, 1981) and also determines the ability of a virus to establish an infection in, and be transmitted by, a

mosquito vector (Beatty et al., 1981; Beatty et al., 1981). Also, a variant of LaCrosse virus selected with a G1 specific monoclonal antibody shows an altered, avirulent phenotype, suggesting that G1 specifically is an important determinant of pathogenicity (Gonzalez-Scaramo et al., 1985).



# 15a RNA as the genetic material.

Viruses are the only known category of genetic systems which can have RNA as their genetic material. In fact, RNA viruses of eukaryotes greatly outnumber DNA viruses (Holland et al., 1982). The reason for the success of the group must be a consequence, at least in part, of the nature of their genetic material. Comparison of the sizes of the genomes of DNA and RNA viruses has shown that RNA genomes tend to be much smaller than DNA (Reanney, 1982). This constraint on the size of the molecules containing genetic information is thought to result from several forces. The error rate in DNA - that is, the rate of mismatch per nucleotide incorporated - is usually quoted as about  $10^{-6}$  -  $10^{-11}$ . This observed error rate is a result of mistakes introduced by polymerase enzymes and their correction by proofreading mechanisms in the cell. The observed error rate for the replication of RNA is between  $10^{-3}$  and  $10^{-4}$  errors per nucleotide incorporated (Holland et al., 1982; Reanney, 1984). The error rate of DNA polymerase enzymes per se is not dissimilar to the observed error rate for the replication of RNA, but as the replication of DNA involves mechanisms by which errors are identified and corrected, the observed difference is such that RNA genomes are about a million times more error prone than DNA genomes.

As errors introduced by polymerases are thought to occur at random, the smaller the molecule, the fewer the number of errors per replication event. RNA is also exposed to different

chemical pressure from RNA. It is more labile than DNA to hydrolysis and, as a single stranded molecule, is susceptible to degradation by ribonucleases present in the cytoplasm. For these reasons the smaller a molecule, the longer its half-life in the intracellular environment (Reaney, 1984).

The high error rate for the replication of RNA viruses means that for a virus with an RNA genome length of  $10^4$  nucleotides, each replication event will produce replica molecules which differ from the template by an average of one nucleotide. As most virus genomes are bigger than this, in a population of viruses a spectrum of sequences is present. Thus, the concepts of an RNA virus sequence - genotype - and its biological features - phenotype - are in fact the average characteristics of a population of viruses. The degree to which changes can accumulate is restricted in a number of ways. The genome must still be functional both in terms of packaging for assembly into virus particles, and the subsequent ability to initiate and complete replication. An exception to this is a particular class of mutants known as defective interfering viruses which are discussed later (15f).

Thus the variations arising from the high mutation rate are subject to selection. Changes in the environment can allow rapid changes to occur in a virus population by selection for mutant characteristics which are already present in the population, albeit at a low level.

#### I5b Segmented genomes.

As mentioned, the genome size of RNA viruses is restricted as a result of using RNA as the vehicle of genetic information. One way in which genetic information can be protected is by dividing the information into smaller molecules. This has the effect of reducing the probability of errors per molecule. Also, assembly of virions now requires the interactions of several different molecules of genetic material in order to assemble a fully competent genome, and these interactions may provide a crude means of proof-reading. Molecules of incorrect nucleotide sequence or topology - which is a consequence of the sequence - may be rejected (Reaney, 1982). Indeed, the topology of RNA molecules can be very complex (Kim et al., 1974) and is undoubtedly relevant to the replication of RNA viruses.

#### I5c Overlapping reading frames.

One way of further reducing the size of a genome is by using overlapping reading frames, in which more than one gene product is encoded by one nucleotide sequence in different reading frames. As all the encoded proteins must be able to function adequately, the amount of change which can be tolerated in such a sequence is reduced. A change in the sequence may not have a deleterious effect on one protein but may abolish the function of another, thereby limiting the amount of variation which can be tolerated. Overlapping reading frames pose the additional problems of gene regulation and independent expression of the encoded products but

has the advantage of compacting information into smaller molecules.

#### I5d Recombination.

Segmented genomes have the ability to undergo reassortment when more than one virus infects a cell. This allows mixing of genetic information so that new combinations of genetic material, which are fit for the environment and for replication, can emerge. Recombination of this type was the first to be described for an RNA virus - influenza - in 1949 (Burnet & Lind.). This reassortment is a kind of recombination as there is exchange of information between two genetic units. Recombination in DNA is an intramolecular event. There is evidence that intramolecular recombination can occur in single stranded RNA viruses - at least in the picornaviruses (Hirst, 1962; Ledinko, 1963; Pringle, 1965; Cooper, 1977; King et al., 1982; Agol et al., 1985) and also in coronaviruses (Lai et al., 1985). It has been suggested that such intergenic recombination may be essentially redundant in RNA viruses as the high inherent mutation rate allows appreciable variation to occur within a virus population (Holland et al., 1982).

#### I5e Temperature sensitive - ts - mutants.

Genetic studies often involve the use of conditional lethal mutants - usually temperature sensitive (ts). The principal aim of isolating and studying such mutants is to use them to investigate the function and mode of replication of a virus genome. Such mutants are phenotypically different from 'wild type' virus: the

mutants are unable to replicate at a high temperature - the non-permissive temperature - at which wild type, or  $ts^+$ , virus is able to replicate and produce infectious virus. All studies on the phenotypic behaviour of mutants must be related to the behaviour of non-mutant  $ts^+$  virus. As mentioned, such a standard phenotype is an average expression of all the individuals in a population but the same is also true of a mutant phenotype, and so comparisons can be made.

Genetic studies of viruses with segmented genomes have been able to exploit the phenomenon of recombination by segment reassortment to define the functions of the gene products encoded by the various segments. The use of ts mutants is a means by which genome segments can be followed, or marked, by the ts phenotype in reassortants. With influenza viruses (Mahy, 1983) and reoviruses (Fields, 1981), this method has allowed much information to be obtained on the function of various gene products.

#### 15f Defective interfering viruses.

It has been shown that repeated passage of viruses at high multiplicity leads to the accumulation of a particular sort of mutant: defective interfering virus. These mutants are defective in replication and require the presence of non-mutant or "standard" virus. In vesicular stomatitis virus, they lack up to 90% of the standard virus genome and interfere with the replication of standard virus, greatly reducing the yield of infectious virions

(Struass & Strauss, 1983). Deletion of the genome to a greater or lesser extent means that defective interfering viruses are subgenomic deletion mutants. It has been found that their shortened genomes retain one or both termini of the original RNA molecule presumably because they are enzyme recognition sites and may be involved in virus packaging. The mode of generation of the truncated genome is uncertain. In the case of vesicular stomatitis virus it is thought to involve strand switching by a polymerase during replication (Holland *et al.*, 1982).

The defective interfering particles of influenza virus arise from internal deletions of one of the polymerase genes. The particles contain a combination of standard viral genome segments and defective polymerase segments which are responsible for interference (Nayak & Sivasubramanian, 1983). It is proposed that the mechanism by which influenza virus defective interfering RNAs are formed is a result of the formation of secondary structures in a template RNA during transcription which could result in the production of truncated transcripts by the polymerase.

It has been noted that negative strand viruses, and especially segmented genome viruses, are likely to generate defective interfering virus (Huang, 1973): for example, influenza viruses (Nayak, 1980); arenaviruses (Gisenez & Compans, 1980); and bunyaviruses (Kocsak & Lyons, 1978). It has been suggested, at least for influenza virus, that the unequal amounts of the various RNA segments observed in virus preparations may reflect the presence of defective interfering viruses.

# 15g The genetics of bunyaviruses.

Genetic analyses of bunyaviruses have confirmed that recombination occurs at high frequency by segment reassortment during mixed infections (Bishop, 1969; Bishop & Shope, 1979) both with homologous virus and in certain combinations of different viruses. If two is mutants of the same virus can recombine by reassortment to produce ts<sup>+</sup> recombinant viruses, the two viruses are defined as being in distinct recombination groups. Viruses in the same recombination group are unable to recombine with each other to produce ts<sup>+</sup> virus. The existence of more than one recombination group suggests that the genetic information of a virus is contained in more than one segment of RNA (Gentsch et al., 1977). As bunyaviruses have three segments of RNA it was expected that three recombination groups of is mutants could be isolated. However, only two recombination groups have been found: Batai, Bunyamwera and Germiston of the Bunyamwera serogroup (Iroegbu & Pringle, 1981; Ozden & Hannoun, 1980); Guaroa, LaCrosse, Lumbo, snowshoe hare, Tahyna and Trivittatus of the California encephalitis serogroup (Bishop, 1979; Bishop et al., 1981; Ozden & Hannoun, 1978); Ukumemi virus of the uukuvirus genus (Gahnberg, 1984). There is only one case in which the three predicted recombination groups have been identified: Maguari virus of the Bunyamwera serogroup (Pringle & Iroegbu, 1982), and there is only one representative of this group identified so far. This mutant shows a phenotype of host range restriction - in tissue culture cells - which has not

been seen for any other Maguari virus mutant, or any mutants of Batai, Bunyamwera, LaCrosse, Lumboc, snowshoe hare, Tahyna or Trivittatus viruses (Pringle & Iroegbu, 1982). It has been suggested that this host restriction may account for the rarity of Group III mutants, although it may be that lesions in the genome segment involved are simply more often lethal.

Complementation between pairs of mutants from different recombination groups has been shown to occur with many of the ts mutants, including snowshoe hare virus (Gentsch & Bishop, 1976), Lumboc and Germiston viruses (Ozden & Hannoun, 1978; 1980), and Ukuniemi virus (Gahmberg, 1984). Generally, complementation between mutants in the same recombination groups has not been observed (Bishop & Shope, 1979; Ozden & Hannoun, 1978; 1980; Gahmberg, 1984). Complementation has been reported between two Group I mutants of Maguari virus (Iroegbu & Pringle, 1981a,b) which indicates that the genome segment carrying the Group I lesion codes for two distinct virus functions. Since it is known that bunyaviruses encode four structural proteins and at least two nonstructural proteins, complementation between mutants of the same recombination group would be expected for at least some groups as at least one of the three RNA segments must code for more than one polypeptide. The reason for this general lack of complementation is not known.

#### 15h Restrictions on recombination in bunyaviruses.

When two viruses with tripartite genomes recombine, the



potential number of genotypes which can be produced is  $2^3$  including the two parental genotypes. Gentsch et al., (1980) reported heterologous recombination between pairs of various members of the California encephalitis serogroup, but one virus - Guarca - did not reassort with any of the viruses tested. Also, not all possible genotypes were isolated for any of the pairs of viruses. Later, the missing genotypes of recombinants between two of the viruses, LaCrosse virus and snowshoe hare virus, were obtained by crossing a reassortant with a LaCrosse Group I is mutant, indicating that these gene combinations can interact viably (Boshon et al., 1981) in the California encephalitis serogroup. Similarly, it has been reported that there are restrictions on the genotypes produced by recombination of Bunyamwera virus with other members of the Bunyamwera serogroup. Reassortants between the L and S RNA segments were difficult to isolate which suggested that they may be genetically linked. However, this link could be broken when heterologous reassortant viruses were used as parents, indicating that the L, M and S RNA segments of the Bunyamwera serogroup viruses studied are not incompatible (Pringle et al., 1984a,b).

Not only did Guarca virus fail to recombine with any members of the California encephalitis serogroup, but it has not been found to recombine with members of the Bunyamwera serogroup (Elliott et al., 1984; Gentsch et al., 1980). Two Bunyamwera serogroup viruses also failed to recombine with other members of the Bunyamwera serogroup (Elliott et al., 1984). Also, despite many attempts to isolate reassortants between viruses of the Bunyamwera serogroup

and viruses of the California encephalitis serogroup, no recombination has been observed (Elliott et al., 1984; Bishop et al., 1981). No recombination has been observed between a Simbu serogroup virus, Sathuperi, and either Bunyamwera or California encephalitis serogroups. (Elliott et al., 1984). These results indicate that recombination by genome segment reassortment rarely or never occurs between viruses of different serogroups, and there may be restrictions on recombination even within serogroups. One consequence of this in terms of analysis of the viruses is that the recombination groups of the mutants of viruses in different serogroups cannot be compared directly, and so the assignment of these recombination groups to genome segments can only be considered within the confines of each serogroup.

#### I51 Information gained from genetic analyses of bunyaviruses.

The use of recombinants has allowed definition of the functions of some of the bunyavirus RNA segments, which have subsequently been supported by studies of the sequences of the genome segments (see I3b). It was found that the M RNA of California encephalitis serogroup viruses coded for the two glycoproteins (Gentsch & Bishop, 1979); the products of this M RNA were shown to be major determinants of pathogenicity (Roshon et al., 1981; Shope et al., 1981); the M RNA products were shown to determine the ability of viruses to infect and be transmitted by mosquitoes (Beatty et al., 1981a,b); and the M RNA products were also shown to include the neutralisation antigen (Gentsch et al., 1980). By similar genetic analysis it was

shown that S RNA encodes the N protein (Bishop & Shope, 1979) and the S RNA products may be involved in the dissemination of California encephalitis serogroup viruses from the gut of the arthropod vector (Beatty et al., 1982). It appears that mutations in the L RNA - at least in California encephalitis serogroup - can attenuate virus pathogenicity (Boshon et al., 1981), although it has not been possible to identify what virus polypeptides are encoded by this segment, although the L protein is an obvious candidate (Bishop & Shope, 1979).

It has been determined that Group I and Group II ts mutants of the California encephalitis serogroup are associated specifically with lesions in the M and L RNA segments respectively (Gentsch et al., 1977). The Bunyamwera serogroup Group II ts mutants have been shown to assign to the M RNA (Pringle & Iroegbu, 1982), but unequivocal assignments of the Group I mutants and the Group III mutant have not been made. As mentioned, "coordinate segregation" of the L and S RNA segments during recombination had been observed for the Bunyamwera serogroup (Pringle et al., 1984); also, no ts<sup>+</sup> reassortants between crosses of the Group III mutant with a heterologous Group I Bunyamwera virus ts mutant could be isolated. This prevented the assignment of the three groups of Maguari virus mutants to the three genome segments. No assignment of the two recombination groups of Uukuniemi virus ts mutants to RNA segments has yet been made.

15j In vivo recombination with bunyaviruses.

Most studies on reassortment of bunyaviruses have been performed in tissue culture systems. However, there is evidence from the bunyavirus genus that reassortment is not a laboratory contrived event and may occur naturally. Two viruses of the Patois serogroup - Shark River and Pahayokee - have almost identical L and S RNA segments, but different M RNA segments, suggesting that they may represent naturally occurring reassortant viruses (Uehijima et al., 1981). There is also some evidence that reassortment can occur by dual infection of mosquitoes with both LaCrosse and snowshoe hare viruses (Beatty et al., 1981b) although it is not clear in this study whether reassortants were formed before transmission to a vertebrate host, or in the vertebrate itself.

The ability of reassortment to take place in nature is dependent on the ecology of each virus. Limitations of geography, and the use of different arthropod and vertebrate hosts, will serve to prevent recombination between viruses in the wild which may reassort freely in laboratory conditions. The absence of reassortment between heterologous viruses in nature does not preclude the possibility of reassortment between strains of the same virus, and heterologous recombination may be a very rare event. The role of recombination in these viruses, in the light of the high inherent mutation rate may not be very significant in terms of the evolution of these viruses, especially as there are considerable restrictions - both logistical and biological - on reassortment between any two

bunyaviruses. The segmented genome may have evolved in order to reduce variation rather than to enhance it. The ability of two viruses to reassort may simply reflect similarity of growth characteristics and compatibility of gene products as a result of a common ancestor. As each virus evolves further, independently, their ability to reassort will presumably decrease.

The work presented here is a study of bunyaviruses using ts mutants and revertants in an attempt to increase the understanding of bunyavirus replication. As discussed, there is only one virus for which the expected three recombination groups of ts mutants have been isolated and that is the bunyavirus Maguari virus. A part of this study involved analysis of a representative of each of the three groups of mutants and they are referred to as Mag ts 7(I), Mag ts 8(II), and Mag ts 23 (III) (recombination groups I, II, and III respectively). The ts nature of these mutants has been established and some genetic studies of recombination with these mutants have been published (Iroegbu & Pringle, 1981; Pringle & Iroegbu, 1982; Elliott et al., 1984; Pringle et al., 1984<sup>a</sup>). The specific aims of this study were to study the effects of these mutations on the growth of Maguari virus and thereby to provide more information on the growth characteristics, protein synthesis and protein processing of these viruses, and to interpret these observations in the light of the location of these mutations in the virus genome segments. A better understanding of the reassortment characteristics and the organisation of the genome of these viruses was sought. The materials and methods used to attain these objectives, and the results obtained, are described in the following sections.

M A T E R I A L S

## Materials

### 1 Antisera.

Rabbit polyclonal antiserum against Bunyamwera virus (Watret et al., 1985) was a kind gift from Dr. R.M.Elliott, M.R.C. Virology Unit, Glasgow. Hamster polyclonal antiserum against Mequari virus was a kind gift from Dr. J.S.Porterfield, Sir William Dunn School of Pathology, Oxford.

Fluorescein isothiocyanate (FITC) conjugated goat-anti-rabbit immunoglobulin was obtained from Miles Laboratories Ltd..

### 2 Autoradiography.

Ex X-ray film was obtained from Fuji Photofilm Co. Ltd.. For  $^{32}\text{P}$  radiochemicals, Dupont Cronex Lightning - Plus P.A. intensifying screens were used.

### 3 Bacterial culture media.

Bacto-agar and tryptone were obtained from Difco Laboratories Ltd.. Yeast extract was obtained from Oxoid Ltd..

Tetracycline and chloramphenicol were obtained from Sigma Chemical Co. Ltd..

### 4 Bacterium.

Escherichia coli strain HB 101 was a kind gift from Dr. A.J. Easton, University of Warwick.

### 5 Chemicals.

All chemicals were obtained from RIE Ltd. of Analar grade,



unless otherwise stated.

Methanol was of standard laboratory reagent grade.

Hydrochloric acid, trichloroacetic acid, glacial acetic acid, sodium citrate and amyl alcohol were obtained from May & Baker Ltd.. Tris was obtained from Sigma Chemical Co. Ltd..

#### 6 Dot hybridisation.

The hybri-dot manifold was obtained from BRL Inc..

Nitrocellulose was obtained from Schleicher & Schuell GmbH.

Formamide was obtained from Fisons and Formaldehyde from Fluka of puriss grade. Ficoll 400 and Sephadex G50 were obtained from Pharmacia Ltd.. Polyvinylpyrrolidone, bovine serum albumin, salmon sperm DNA and deoxynucleotide triphosphates were obtained from Sigma Chemical Co. Ltd..

#### 7 Drugs used to analyse protein processing.

Tunicamycin, monensin, canavanine,  $N_\alpha$ -p-Tosyl-L-lysine chloromethyl ketone (TICK),  $N$ -tosyl-L-phenylalanine chloromethyl ketone (TPCK) and carbonylcyanide  $m$ -chlorophenylhydrazine (OCCP) were all obtained from Sigma Chemical Co. Ltd..

Tunicamycin, OCCP and monensin were dissolved in 50 %  $v/v$  ethanol to give 1000 fold concentrates. TPCK and TICK were dissolved in 50 %  $v/v$  dimethylsulphoxide as 1000 fold concentrates. Canavanine was dissolved in water to give a 1000 fold stock.

8 Enzymes.

Escherichia coli DNA polymerase I was obtained from Amerham International Ltd.. Deoxyribonuclease was from Worthington Inc..

Trypsin (TPCK treated), chymotrypsin (TLCK treated).

Staphylococcus aureus V8 protease and endoproteinase arg-C were obtained from Sigma Chemical Co. Ltd.. Endoproteinase lys-C was from ECL Ltd..

9 Filter paper.

3MM filter paper and grade 1 2.5cm diameter discs were obtained from Whatman Ltd..

10 Immunofluorescence.

16mm diameter round coverslips were obtained from Chance-Propper Ltd., and acetone was from May & Baker Ltd..

11 Immunoprecipitation.

Immunoprecipitin (10% w/v formalin fixed Staphylococcus aureus cells) was obtained from ERL Inc..

Sodium deoxycholate and phenylmethylsulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co. Ltd., and sodium dodecyl sulphate (SDS) was from Fisons.

12 In vitro translation

Rabbit reticulocyte lysate and canine pancreatic microsomal membranes were obtained from Amerham International Ltd..

13 Photomicrography.

For fluorescent microscopy, a Zeiss fluorescent microscope with Kodak Tri-X film was used. For visible light microscopy, a Leitz microscope with Kodak Panatomic X film was used.

14 Plasmids.

All recombinant plasmids were a generous gift from Dr. R.M. Elliott, M.R.C. Virology Unit, Glasgow.

15 Plasmid preparation.

Lysosyme and ethidium bromide were supplied by Sigma Chemical Co. Ltd..

Caesium chloride was obtained from Fisons.

16 Polyacrylamide gels.

i. Components.

Acrylamide, sodium dodecyl sulphate (SDS) and glycine were obtained from Fisons. Ammonium persulphate was supplied by BRL Inc.. N N'-methylene bis acrylamide was obtained from Eastman Kodak Co.. N,N,N'-N-tetramethylethylenediamine (TEMED) was obtained from Sigma Chemical Co. Ltd..

ii. Processing materials.

Coomassie brilliant blue and molecular weight marker kit MW - SDS - 200 were supplied by Sigma Chemical Co. Ltd.. Amplify was from Amersham International Ltd.. The gel drier used was a Dual Temperature Slab Gel Drier from Riorad Ltd..

17 Radiochemicals.

L-( $^{35}\text{S}$ ) methionine, specific activity of 1000 Curies  $\text{mmol}^{-1}$  was obtained from Amersham International Ltd.. ( $\alpha$ - $^{32}\text{P}$ ) deoxycytidine 5'-triphosphate, specific activity of 3000 Curies  $\text{mmol}^{-1}$  was obtained from WEN Ltd..

18 Scintillation counter.

A Rackbeta model 1216 supplied by LKB Ltd. was used.

19 Scintillation fluid.

Ready solv EF from Beckman RIIIC Ltd. was used.

20 Spectrophotometer.

The spectrophotometer used was a Unicam SP500 series 2.

21 Tissue culture.

i. Cells.

BHK - 21 cells were originally from Flow Laboratories Ltd., and BS-C-1 cells were originally obtained from Dr. J. Williams, M.R.C. Virology Unit, Glasgow.

ii. Media.

All tissue culture media were obtained from Flow Laboratories Ltd..

New born and foetal calf serum were supplied by Gibco Ltd.. Benzylpenicillin - used at 100 000 units per litre, and streptomycin - used at 0.1g per litre, were obtained from Glaxo Ltd..

Growth medium was the Glasgow modification of Eagle's medium (GMEM) supplemented with 5% v/v new born calf serum and antibiotics as above. Maintenance medium was GMEM supplemented with 1.5% v/v new born calf serum and antibiotics as above.

Agarose for overlay medium was of electrophoresis grade type II from Sigma Chemical Co. Ltd., and was used at 0.6% w/v in maintenance medium.

The diluent for viruses was phosphate buffered saline (PBS).

iii. Plastic ware.

Multiwell dishes - 12 well and 6 well dishes - were supplied by Costar. 3cm and 5cm diameter Petri dishes and 25cm<sup>2</sup> and 75cm<sup>2</sup> flasks were obtained from Bunc Intermed.

## 22 Viruses.

All bunyaviruses were obtained originally from the Vector-borne Disease Laboratory, Centre for Disease Control, Fort Collins, Colorado, U.S.A. and were provided as clonal stocks by Professor C.R. Pringle, University of Warwick (see Iroegbu & Pringle, 1981).

The original bunyavirus wild-type viruses, ts mutants and electrophoretic mutants were provided by Professor Pringle as infected - tissue culture supernatants. The wild-type viruses used were Maguari virus and Bunyamwera virus. Three ts mutants of Maguari virus were used, each representing one reassortment group of ts mutants: Mag ts 7 (Group I); Mag ts 8 (Group II); and Mag ts 23 (Group III). Two Bunyamwera virus mutants were also used: Bun ts 5 (I) and Bun ts 7 (II). These mutants have been described previously (Iroegbu & Pringle, 1981; Pringle & Iroegbu, 1982).

Two mutants isolated by Professor Pringle from Mag ts 8 (II) were also used: Revertant 1, originally named 81/167/65, and Revertant 2, originally named 81/232/15. Both were obtained after passage of Mag ts 8(II) in BEK cells at the nonpermissive temperature of 38°C (although in the case of Revertant 2 the temperature fluctuated between 37°C and 39°C during the four days of incubation) and plaqueing of these infected cell supernatants in order to isolate virus clones.

23 Virus plaque assays.

Crystal violet was obtained from Sigma Chemical Co. Ltd..

Glutaraldehyde was from BDE Ltd. supplied as a 50% v/v solution.

## METHODS

#### Methods A: Virological Methods.

##### A1 Virus Stocks.

###### a Preparation.

For the preparation of virus stocks from cloned viruses, the inoculum containing the agarose plug and eluted virus (see Method A3) was poured onto BEK cell monolayers in 25cm<sup>2</sup> flasks, and incubated at 31°C for one hour for virus adsorption. 10ml of maintenance medium was then placed on the cells and incubation continued until extensive cytopathic effect was apparent (3 - 4 days). The infected cell supernatant medium was removed and stored at -70°C.

###### b Maintenance.

BEK cell monolayers in 1 litre glass bottles (about  $3.5 \times 10^7$  cells) were inoculated with 1ml of inoculum containing about  $3 \times 10^5$  pfu, so that the moi was 0.01pfu per cell. This was incubated at the appropriate temperature - 31°C for ts virus, 37°C for ts<sup>+</sup> virus - for 30 minutes for virus adsorption. 40ml of maintenance medium was added to the bottles, and the infected monolayers were incubated at the appropriate temperature until extensive cytopathic effect was seen (3 - 5 days). The medium was then removed and dispensed into 3ml aliquots for storage at -70°C.

##### A2 Assay of viruses by plaque formation.

Ten-fold serial dilutions in PBS were made of the tissue culture fluid to be assayed. The minimum volume used for these dilutions was 1ml. Appropriate volumes of these dilutions were inoculated onto 90% confluent cell monolayers (0.1ml for 12-well



dishes 2.2cm in diameter; 0.2ml for 6-well dishes 3.5cm in diameter; 0.4ml for 5cm dishes). The virus inoculum was adsorbed to the cells at 31°C or 38°C in a humidified 5% CO<sub>2</sub> atmosphere for one hour. The agarose overlay medium was prepared by melting a volume of sterile 1.2% w/v agarose, cooled to 45°C, and mixing it with an equal volume of double strength maintenance medium, also at 45°C. At the end of the adsorption period, this agarose overlay medium was placed over the monolayers to a depth of 0.5cm and allowed to set at room temperature - typically ten minutes. The dishes were then incubated at 31°C or 38°C for four days.

If crystal violet staining of the cell monolayers was required, at the end of the incubation period, the agarose medium was flooded with a solution of 1% v/v glutaraldehyde in PBS, and incubated at room temperature for a minimum of four hours to fix the cells. The agarose medium was then flicked off the monolayers with a spatula and the cell sheet covered with a solution of 0.1% w/v crystal violet staining solution. This was made by twenty-fold dilution of a stock of 2% w/v crystal violet dissolved in 20% v/v ethanol. After staining for ten minutes, excess stain was rinsed off and the dishes drained on absorbent paper. Plaques could be easily seen as holes in a violet cell sheet, and the titre of the virus preparation assayed could be calculated.

#### A3 Cloning of bunyaviruses by propagation from single plaques.

For isolation of virus clones from a virus stock, a sample of the stock was diluted and inoculated onto HEK cells in 5cm dishes, overlaid with medium containing 0.6% w/v agarose, and incubated at 31°C or 38°C. Twelve hours before the end of the incubation period, a further 0.5cm depth of overlay medium, this time containing 0.002% w/v neutral red (diluted from 0.1% w/v stock solution) was placed on top of the existing medium. When set, the plates were incubated as before for the remaining twelve hours. Plaques could then be seen as clear unstained areas in a background of stained cells.

For virus cloning, only well separated plaques were chosen. These were removed by pushing a Pasteur pipette through the agarose overlay directly over the plaque and thereby removing a plug of agarose containing virus. The plug was then ejected into a vial containing 1ml of growth medium and stored at +4°C for two hours, or stored at -70°C until required. The whole of this medium containing eluted virus and the agarose plug was then used to inoculate a HEK cell monolayer in a 25cm<sup>2</sup> flask as described (Method A1).

#### A4 Genetic crosses between two bunyaviruses.

The titres of the two preparations to be used were determined by plaque assay as described (Method A2). Three inocula were then prepared, each containing the same number of pfu. One contained equal amounts of both viruses and was used for the cross, the other two contained only one virus of the pair, as controls

for the cross. The volumes of all three inocula were made equal by the addition of maintenance medium. These three inocula were then used to infect three confluent monolayers of HK cells in 25cm<sup>2</sup> flasks. The flasks were incubated at 31°C for one hour for virus adsorption. Each monolayer was then washed five times with 5ml of warmed (31°C) maintenance medium to remove unadsorbed virus, and the cells were incubated at 31°C with 10ml of maintenance medium until extensive cytopathic effect was seen - usually 3 - 4 days. The tissue culture fluid containing virus released by the infected cells was removed and stored at -70°C.

Methods B: Analysis of Clones from Genetic Crosses.

B1 Radiolabelling of bunyavirus proteins.

HEK cell monolayers in 6-well dishes were inoculated with 0.2ml of virus, to give a moi of about ~~50pfu~~ per cell, or were mock-infected with 0.2ml of PBS. The cells were then incubated at 31°C for one hour for adsorption of virus. 2ml of maintenance medium was added to each well and the infected cells incubated at 31°C usually for 17 hours. Sometimes adsorption and incubation were carried out at 38°C. The medium was then removed from the monolayers and the cells rinsed once with methionine-free GMEM (not supplemented with serum or antibiotics) and overlaid with 1ml of this medium for 1 hour at the incubation temperature. At the end of this "pretreatment" period, the medium was removed and replaced with 100 $\mu$ l of methionine-free GMEM containing 50 $\mu$ Ci ml<sup>-1</sup> of <sup>35</sup>S-methionine. The cells were radiolabelled in this medium for one hour, so the time of the pulse labelling was 18 - 19 hours after infection. This time was generally used as G1 and M proteins were found to label well and there was relatively little host cell protein-synthesis at this point. G2 did not label well with <sup>35</sup>S-methionine and was only visible after immunoprecipitation. If the time of the pulse was to be different from 18 - 19 hours, the cells were pretreated as described for one hour before the required time and then pulsed for one hour.

At the end of the pulse, the medium was removed and the cells were rinsed in 1ml of PBS per well. If radiolabelled cell lysates were required, the cells were lysed in 150 $\mu$ l of gel sample buffer (Method B2) and placed in tubes in a boiling

water bath for five minutes. For immunoprecipitation the cells were suspended in PBS and treated as described in Method C1.

## B2 Polyacrylamide gel electrophoresis (PAGE) of bunyavirus proteins.

The polyacrylamide gel system used throughout was the discontinuous (Omstein, 1964; Davis, 1964), SDS-dissociating (Laemmli, 1970) system. 5% stacking gels with single concentration 7 or 10% resolving gels were used, in which the ratio of acrylamide:bisacrylamide was 30:0.75. The composition of the stacking gel was:

5% w/v Acrylamide:bisacrylamide 30:0.75  
0.25M Tris HCl pH 6.7  
0.1% w/v SDS  
0.1% w/v Ammonium persulphate from stock  
10% w/v made fresh  
0.005% w/v TEMED

The composition of the resolving gels was:

7 or 10% w/v Acrylamide:bisacrylamide 30:0.75  
0.4M Tris HCl pH 8.9  
0.1% w/v SDS  
0.1% w/v Ammonium persulphate - as above  
0.005% w/v TEMED

The resolving gel was poured between two glass plates - one was notched and had "rabbit ears" - sealed with greased silicone rubber tubing, to give a gel of dimensions 18cm long x 17cm broad x 1.5mm thick. A layer of 0.4M Tris HCl pH 8.9, 0.1% w/v SDS buffer (the same composition as that in the gel mix)

was carefully layered on top of the resolving mix before it had set, to obtain a flat top on the gel. The gel was left overnight at room temperature. Half an hour before commencing electrophoresis, the layer of buffer was poured off the gel and the area above the gel wiped dry with filter paper. The stacking gel mix was poured into this clean, dry slot, and a 15-tooth comb - the same thickness as the gel - was inserted between the glass plates into the stacking gel mix to form the sample wells. When the stacking gel had set, the comb was carefully removed and the wells rinsed free of unpolymerised gel solution with 0.25M Tris HCl pH 6.7, 0.1% w/v SDS buffer. The gel was then fitted into the apparatus for electrophoresis and the reservoirs filled with running buffer. The running buffer was 0.6% w/v Trizma, 0.4% w/v Glycine, 0.1% w/v SDS. Samples of proteins of 35 $\mu$ l in sample buffer were loaded into the wells of the stacking gel, and 35 $\mu$ l of sample buffer only was placed in the wells adjacent to the first and last sample wells, to reduce distortion. The composition of the sample buffer was:

30% v/v	Glycerol
15% v/v	2-mercaptoethanol
0.15M	Tris HCl pH 6.7
0.6% w/v	SDS
0.015% w/v	Bromophenol blue

Electrophoresis was carried out at 40mA at +4°C (cathode connected to the upper reservoir) for 6 hours for 10% gels and 5 hours for 7% gels.

After electrophoresis, the gel was removed from between the glass plates and placed in Fixing Solution (50% methanol, 7% glacial acetic acid). 250ml of this was placed in a large box so that the gel was not folded and could float freely. If Coomassie blue staining was required, 0.2% w/v Coomassie brilliant blue was dissolved in the fixing solution. The gel was gently agitated for 30 minutes (2 hours for stained gels). Stained gels were then transferred to Destaining Solution (30% v/v methanol, 7% v/v glacial acetic acid) for six hours, the solution being changed every two hours until the desired level of destaining was obtained. Gels were then dried onto trimmed pieces of 3MM filter paper for storage and autoradiography. The dried gels were exposed to X-ray film at room temperature for 1 - 14 days.

If fluorography was required to reduce the time of exposure to X-ray film, fixed gels were soaked in Amplify for 15 - 30 minutes, according to the manufacturers' instructions, before drying. The fluor-impregnated, dried gel was then exposed to X-ray film at  $-70^{\circ}\text{C}$  for 1 - 10 days.

B) Large scale preparation of recombinant plasmids.

\* Transformation of Escherichia coli HB101 with recombinant plasmid.

10ml of L Broth (10g tryptone, 5g yeast extract, 5g NaCl per litre  $\text{H}_2\text{O}$ , sterilised by autoclaving at  $121^{\circ}\text{C}$  for 20 minutes) was inoculated with a portion of a colony of E. coli HB101 and incubated at  $37^{\circ}\text{C}$  overnight. This turbid broth was then diluted

1/100 with 100ml of L Broth warmed to 37°C, and incubated at 37°C with agitation until OD<sub>550</sub> was 0.5 (typically 2.5 - 3 hours). 20ml of the culture was then incubated on ice for ten minutes and the cells pelleted by 800 x g (MSE Chilsipin, 2,500rpm) for ten minutes at +4°C. The supernatant was discarded and the cells resuspended in 10ml of ice-cold 0.1M MgCl<sub>2</sub> and the cells pelleted again as before. The supernatant was discarded and the cells resuspended in 1ml of ice-cold CaCl<sub>2</sub> and incubated on ice for 30 - 45 minutes. A solution containing 1ng of the recombinant plasmid required in 90µl of water was prepared and to this was added 10µl of 1M CaCl<sub>2</sub> and 200µl of the now competent bacteria. This mixture was incubated on ice for 30 minutes, transferred to a water bath at 42°C, and incubated again on ice for a further 30 minutes. Ten-fold serial dilutions in water were made and 100µl samples spread on warm, surface-dried L Agar plates containing tetracycline at 10µg ml<sup>-1</sup> (L Agar was 10g tryptone, 5g yeast extract, 10g NaCl, and 15g Bacto agar per litre H<sub>2</sub>O, sterilised by 121°C for 20 minutes in an autoclave: tetracycline diluted from a fresh 10mg ml<sup>-1</sup> aqueous solution and added to sterile L Agar at 45°C just before plates were poured). The plates were incubated at 37°C for 24 - 48 hours. Antibiotic-resistant colonies were then subcultured onto L Agar + tetracycline plates and then broth cultures (L Broth containing tetracycline at 10µg ml<sup>-1</sup>) were grown for storage and large scale plasmid preparation. For storage, overnight broth cultures were made 15% v/v for glycerol at the end of incubation and stored at



-20°C in 1ml aliquots.

b Plasmid preparation.

5ml of an overnight broth culture (containing tetracycline) of transformed E. coli was used to inoculate 500ml of warm (37°C) L Broth containing tetracycline in a 2l conical flask. The culture was incubated at 37°C with agitation until OD<sub>650</sub> was 0.5 (usually 6 - 7 hours). Chloramphenicol was dissolved in ethanol at a concentration of 85mg ml<sup>-1</sup> and 0.2ml was added to the 500ml culture so the final concentration was 34µg ml<sup>-1</sup>. The culture was incubated at 37°C overnight with gentle agitation.

The following day the culture was transferred to two 250ml centrifuge pots and the bacteria pelleted by 600 x g ( MSE HS25, 6 x 250 rotor, 2,000rpm) for 20 minutes. The supernatants were discarded and the 500ml culture's worth of cells resuspended in 8.4ml of Solution 1 (25% w/v sucrose, 50mM Tris HCl pH 8). This was transferred to a 500ml conical flask on ice. 1.4ml of Lysozyme solution (10mg ml<sup>-1</sup> lysozyme in 50mM Tris HCl pH 8 made fresh) was added and the flask contents mixed gently on ice for 5 minutes. 4.6ml of 0.25M EDTA was then added slowly and the flask incubated on ice with continuous gentle mixing for 10 minutes. 9.6ml of Lysis Mix (2% v/v Triton X-100, 62.5mM EDTA, 50mM Tris HCl pH 8) was added and the mixture transferred to a 50ml Oakridge tube. Chromosomal DNA was pelleted by 18,600 x g (MSE HS18, 8 x 50 rotor, 16,000rpm) at +4°C for one hour, and removed from the tube with a Pasteur

pipette, the end of which had been shaped into a hook by heating in a flame. The supernatant - still in the tube - was made 25ml with water and 25g of CsCl was added to the solution. 1-25ml of ethidium bromide at  $5\text{mg ml}^{-1}$  was added and the solution mixed thoroughly. This was then centrifuged at  $82,800 \times g$  (Beckman L8, SW27 rotor, 25,000rpm) at  $23^{\circ}\text{C}$  for one hour. The fatty pellicle on the top of the solution was removed with a spatula and the remaining supernatant poured into Beckman Quick-Seal vertical rotor tubes and any pelleted material was discarded. The tubes were centrifuged at  $225,000 \times g$  (Beckman L8, VFi 50 rotor, 45,000rpm) at  $23^{\circ}\text{C}$  for 18 hours. The resulting isopycnic gradients were observed and the plasmid-containing, ethidium bromide-stained band, clearly visible in daylight, was removed with a wide-bore syringe needle and 5ml syringe. The ethidium bromide was removed by extracting the aqueous solution five times with equal volumes of amyl alcohol. The plasmid solution was then dialysed overnight in 10mM Tris HCl pH 8, 1mM EDTA at  $+4^{\circ}\text{C}$ , to remove CsCl. The dialysate was then ethanol precipitated by making the solution 0.3M for sodium acetate and adding 2.5 volumes of cold ( $-20^{\circ}\text{C}$ ) ethanol. The tube was incubated at  $-70^{\circ}\text{C}$  for at least three hours. The plasmid DNA was pelleted by  $18,600 \times g$  (MSE HS18, 8 x 50 rotor, 16,000rpm) at  $+4^{\circ}\text{C}$  for one hour. The pellet was washed in 70% v/v ethanol and again in 100% v/v ethanol and desiccated. The dried pellet was dissolved in 0.5ml of sterile distilled water and the concentration of DNA determined by  $\text{OD}_{260}$  of the diluted samples.

#### B4 Nick translation of recombinant plasmids as probes for Bunyamwera virus RNA.

The recombinant plasmids used, which hybridise specifically to certain segments of Bunyamwera virus RNA and do not hybridise with Maguari virus RNA, have been described by Pringle et al. (1984b). The L RNA probe used was pHUS 84, and the M RNA probe was pHUS 133. The plasmids were prepared as described in Method B3, and radiolabelled for use as probes by "nick translation" (Maniatis et al., 1982).

1 $\mu$ g of DNA was used per 50 $\mu$ l reaction the composition of which was:

1 $\mu$ g plasmid DNA  
1 x Polymerase I Buffer\*  
10 $\mu$ Mole dATP (dCTP and TTP)  
2 $\mu$ g ml<sup>-1</sup> Deoxyribonuclease (diluted from 1mg ml<sup>-1</sup>  
stock before use)  
10 $\mu$ Ci <sup>32</sup>P dCTP  
4 units E. coli DNA polymerase I

(\* 10 x Polymerase I Buffer: 0.5M Tris HCl pH 9, 50mM MgCl<sub>2</sub>, 110mM 2-mercaptoethanol, stored in 0.1ml aliquots at -20°C). The reactions were incubated for 1.5 - 2 hours in a water bath at 15°C. At the end of this reaction period, the contents of each tube were loaded onto separate 6ml columns of G50 Sephadex in water, in plastic disposable pipettes. The eluent used was water. Twenty 300 $\mu$ l fractions were collected from each column and counted in a scintillation counter programmed for <sup>3</sup>H emission, without scintillant (Cerenkov counting).

The peak fractions were pooled and the remaining fractions discarded. This probe was then heated in a boiling water bath for ten minutes to denature the DNA, and rapidly cooled in ice-cold water to reduce the extent of rehybridisation.

B5      Dot hybridisation with bunyavirus RNA.

a      Preparation of RNA samples.

The method used was adapted from that of White and Bancroft (1982). BHK cell monolayers in 5cm dishes were inoculated with 0.4ml of virus preparations to give a moi of  $1 - 5$  pfu per cell ( $5 \times 10^6$  cells per dish infected with about  $10^7$  pfu of virus). After adsorption at  $31^\circ\text{C}$  for one hour, the cells were overlaid with 3ml of maintenance medium and incubated at  $31^\circ\text{C}$  for two days. The medium was removed and the monolayers rinsed with 1ml of PBS. The cells were scraped into 0.5ml of PBS with a piece of rubber held in a pair of forceps, and the suspended cells transferred to an Eppendorf tube. The cells were then pelleted by centrifugation at  $10,000 \times g$  (Anderman microfuge) for 30 seconds. The medium was removed and the cells were resuspended in 45 $\mu$ l of ice-cold 10mM Tris HCl pH 7, 1mM EDTA. 5 $\mu$ l of 2% v/v Nonidet P40 was added and the suspension mixed and incubated on ice for 5 minutes. A further 5 $\mu$ l of 5% v/v Nonidet P40 was added and the suspension mixed before centrifugation at  $10,000 \times g$  (Anderman microfuge) for 2.5 minutes. 50 $\mu$ l of the supernatant - which contained the cytoplasmic RNA - was transferred to a sterile tube containing 30 $\mu$ l of 20 x SSC (175.3g NaCl, 88.2g sodium citrate per litre  $\text{H}_2\text{O}$ ), 20 $\mu$ l

formaldehyde. The pelleted nuclei and cell debris were discarded. The contents of the tube were mixed and incubated at 60°C for 15 minutes before storage at -70°C, or dilution and application to nitrocellulose filters.

b Application to nitrocellulose filters.

Rectangles of nitrocellulose, 12cm x 9 cm, were soaked in sterile distilled water and then transferred to 20 x SSC for 5 minutes, and placed in the Hybridot manifold according to the manufacturers' instructions. The 100 $\mu$ l formaldehyde-denatured RNA samples were diluted in 1ml of 20 x SSC and applied in duplicate to the nitrocellulose in the manifold with suction from a water trap pump. When all the samples had passed through the nitrocellulose the vacuum was released and the filter placed on absorbent paper to dry. The filter was then placed between two pieces of 3MM filter paper and baked in a vacuum oven at 80°C for 2 hours.

c Hybridisation to radiolabelled probe.

The methods used follow the procedures described by Maniatis et al., 1982. Each filter was placed in a polythene freezer bag with 20ml of prehybridisation buffer (50% v/v formamide, 3 x SSC, 1 x Denhardt's solution, 150 $\mu$ g ml<sup>-1</sup> denatured salmon sperm tRNA; 50 x Denhardt's solution was 1% w/v polyvinylpyrrolidone, 1% w/v ficoll 400, 1% w/v bovine serum albumin). Care was taken to exclude air from the package and the bag was sealed with an electric heat sealer. The bag was placed in a water-filled box in a water bath at 42°C for 24 hours.

Each sealed bag was cut open and the prehybridisation

buffer poured out. This was replaced with 10ml of hybridisation buffer containing the denatured radiolabelled probe (5 x SSC, 50 % v/v formamide) - about  $3 \times 10^5$  cpm ml<sup>-1</sup>. Each bag was then resealed, again taking care to exclude air, and replaced in the water bath as before at 42°C for 24 - 48 hours.

d Washing procedure.

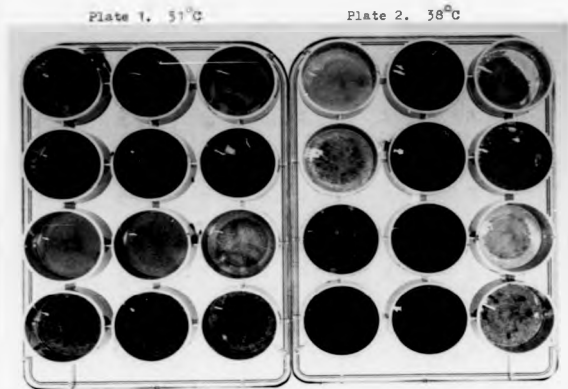
After hybridisation the bag was opened and the filter removed. Filters were either washed together in pairs or singly; no more than two filters were washed together. The filters were placed in 250ml of 2 x SSC in a box so the filters floated freely, and gently agitated at room temperature. After ten minutes the washing medium was discarded and replaced with a further 250ml of 2 x SSC for another ten minute wash. The filters were then transferred to a box in a water bath at 65°C containing 500ml of 1 x SSC, 0.1% w/v SDS for one hour without agitation. The filters were removed and dried on absorbent paper. The dried filters were then placed on 3MM filter paper and held in position with adhesive labels. This was then covered with cling-film and exposed to X-ray film with an intensifying screen for 24 - 48 hours.

B6 Determination of temperature sensitive (ts) phenotype of viruses.

Hundred-fold dilutions -  $10^{-2}$  and  $10^{-4}$  - were made of virus stocks using PBS as diluent. 0.1ml of both dilutions were used to inoculate separate HEp cell monolayers in 12-well dishes. Dishes were inoculated in duplicate with two dilutions

FIGURE 1

Assessment of ts phenotype by plaque production at 31°C and 38°C.



Pattern of inoculation:

dilution

virus

$10^{-2}$

Bun ts<sup>+</sup> Clone 1 Clone 3

$10^{-4}$

" " "

$10^{-2}$

Mag ts 7 Clone 2 Clone 4

$10^{-4}$

" " "

Deductions: Bun, ts<sup>+</sup>; Mag, ts: Clone 1, ts: Clone 2, ts:

Clone 3, ts<sup>+</sup>; Clone 4, ts<sup>+</sup>.

of each of 6 virus samples per dish. One dish of each pair was incubated at 31°C and the other at 38°C for adsorption, and incubation after covering the cells with overlay medium as described previously (Method A2). The cells were incubated for 4 days and then fixed with glutaraldehyde and stained with crystal violet (Method A2), and the plaques formed by each virus at both temperatures counted and the titres at the two temperatures calculated. Viruses which produced plaques at 31°C and 38°C such that the efficiency of plating quotient 'eop'

$$\frac{\text{titre of virus at } 38^{\circ}\text{C}}{\text{titre of virus at } 31^{\circ}\text{C}} = \text{eop}$$

was less than 0.01 were recorded as ts. Those viruses with an eop above this threshold were recorded as ts<sup>+</sup>. Known ts and ts<sup>+</sup> virus samples were included in each series of ts phenotype determination experiments as controls. It was usually found that ts viruses had an eop of 0.001 or less and ts<sup>+</sup> viruses an eop of 0.5 or more. Figure 1 shows a typical pair of plates us for ts assessment.

#### B7 Basis for inference of genotype.

The techniques used to determine the genotype of virus clones were those described by Pringle et al. (1984b). Dot hybridisation using Bunyamwera virus specific cDNA clones (Method B5) was used to ascertain the origins of the L and M RNA segments, and PAGE of <sup>35</sup>S-methionine radiolabelled virus proteins (Methods B1 and B2) was used to indicate the



origins of S and M RNA from the phenotypes of the gene products N and G1. Thus two methods were used to determine the origin of the M RNA - directly by hybridisation with a cDNA probe, and indirectly by the electrophoretic mobilities of the G1 proteins of Maguari and Bunyamwera viruses.

Figure 2 shows an example of this genotyping method. Panels A and B are from dot hybridisation of two replica nitrocellulose filters carrying RNA from cells infected with various reassortant clones and the two parental viruses.

Each RNA preparation was loaded in duplicate. Filter A was hybridised with a probe specific for Bunyamwera L RNA (pBUN 84) and where RNA of this type is present a dot is seen. Filter B was hybridised with a probe specific for Bunyamwera M RNA (pBUN 133) and where RNA of this type is present a dot is seen. In both cases, absence of hybridisation on an RNA sample is an indication of the presence of Maguari specific RNA. As can be seen, the duplicate preparations give an identical result. However, the pattern of spots between the two filters is different indicating the presence of L/M RNA reassortants. Panel C shows a PAGE autoradiograph for the proteins of the viruses used in Panels A and B. Tracks 1 and 6 are the parental controls and tracks 3 and 4 show M/S RNA reassortment as Bunyamwera G1 (M RNA product) is present with Maguari N (S RNA gene product).

It would have been more convenient and less time consuming if only one technique could have been used to genotype the viruses. However, the L proteins (L RNA gene product) of

Figure 2.

Determination of genotype by a combination of dot hybridisation  
and PAGE of virus proteins.

Dot hybridisation:

Arrangement of samples (duplicate)

1	2	3	4
5	6	7	

1 = Mag ts  
2 = Bun ts<sup>+</sup>  
3 = Clone 1  
4 = Clone 2  
5 = Clone 3  
6 = Clone 4  
7 = Bun ts<sup>+</sup>

Result

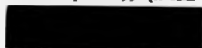
Interpretation

Panel A: pBUN 84 (L RNA probe)



-	+	+	-	-	+	+
-	-	+	+	+	+	

Panel B: pBUN 133 (M RNA probe)



-	-	+	+	-	-	+	+
+	+	+	+	+	+		

Dot hybridisation result

L and M RNA genotype

L M

Clone 1 Mag Mag

Clone 2 Bun Bun

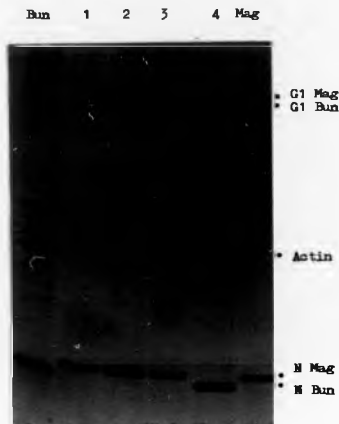
Clone 3 Mag Bun

Clone 4 Bun Bun

Figure 2 continued..

PAGE of virus proteins radiolabelled at 18 hours after infection.

Panel C



PAGE result

	G1	N
Clone 1	Mag	Mag
Clone 2	Bun	Mag
Clone 3	Bun	Mag
Clone 4	Bun	Bun

Inferred genotypes

	L	M	S
Clone 1	Mag	Mag	Mag
Clone 2	Bun	Bun	Mag
Clone 3	Mag	Bun	Mag
Clone 4	Bun	Bun	Bun

Maguari and Bunyamwera virus are indistinguishable in terms of electrophoretic mobility under conditions in which differences in the mobilities of the G1 proteins and the N proteins are resolved. Also, no S RNA probes have been found which will discriminate between Maguari and Bunyamwera RNA and they have all been found to cross-hybridise. One probe - which contains a full length cDNA of Bunyamwera S RNA except for 9 bases at the 5' end - did appear to hybridise less to Maguari RNA than to Bunyamwera RNA. However, for the purposes of genotyping even a small amount of cross-hybridisation could lead to ambiguous or false results. Consequently both dot hybridisation and protein electrophoresis had to be used to genotype clones from genetic crosses.

Both techniques have limitations which need to be considered in the interpretation of information. The preparation of proteins involved radiolabelling for a 1 hour pulse (Method B1), so only those proteins which were synthesised at that time, and which contained methionine would be detected. Thus, only the expressed proteins rather than the genetic material itself was analysed. A pulse from 18 - 19 hours after infection was found to label G1 and N proteins well, and also coincided with a high degree of inhibition of host protein synthesis, facilitating the detection of viral proteins.

In the dot hybridisation technique, a positive result indicated the presence of Bunyamwera virus RNA. A negative result was taken to indicate the presence of Maguari virus RNA, although several circumstances could lead to such a result: failure to infect the cells; faulty application

to the nitrocellulose membrane; and extraction of RNA from cells when insufficient virus-specific RNA was present. However, under the conditions used wild type Bunyamwera virus always gave a positive result and Maguari virus was always negative. In clones in which the protein data indicated a mixture of the two parent viruses from a cross, the L RNA genotype is shown as ?B as only the Bunyamwera L RNA could be detected with the dot hybridisation information, and the presence of Maguari L RNA is presumed.

# Methods C: Characterisation of Viruses.

## C1 Immunoprecipitation of bunyavirus proteins.

BHK cells were radiolabelled as usual (Method B1) in 6-well dishes, except that  $100\mu\text{Ci ml}^{-1}$  of  $^{35}\text{S}$ -methionine was used. At the end of the labelling period, the monolayers were rinsed with 1ml of PBS and then the cells from each well were scraped into 0.5ml of PBS with pieces of rubber held in a pair of forceps. The suspended cell preparations were transferred to Eppendorf tubes and the cells pelleted by centrifugation at  $10,000 \times g$  (Anderman microfuge) for 30 seconds. The cells were then resuspended in 100 $\mu\text{l}$  of Lysis Buffer (0.15M NaCl, 10mM Tris HCl pH 7.4, 1mM PMSP, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, and 0.1% w/v SDS) and incubated on ice for 30 minutes. The tube contents were then mixed briefly and the nuclei and debris pelleted by centrifugation at  $10,000 \times g$  (Anderman microfuge) for 5 minutes. 70 $\mu\text{l}$  of each supernatant was removed to another Eppendorf tube containing 10 $\mu\text{l}$  of normal rabbit serum and 20 $\mu\text{l}$  of Immunoprecipitin (a suspension of formalin fixed Staphylococcus aureus cells) and incubated on ice for 30 minutes. This was to remove any radiolabelled proteins which were not bunyavirus specific but which might bind to factors present in the Immunoprecipitin or serum. The tubes were then centrifuged at  $10,000 \times g$  (Anderman microfuge) for 1 minute to pellet the Immunoprecipitin and other precipitable material. 2 $\mu\text{l}$  of Bunyamwera antiserum (for origin see Materials section) was then added to the supernatant from each tube and the pellet discarded. Thus the antiserum was diluted 1/50. Only Bunyamwera virus antiserum was used for

immunoprecipitation as better results were obtained - even with Maguari virus proteins - than with the Maguari virus antiserum. The tubes were then incubated overnight at  $+4^{\circ}\text{C}$ . 25 $\mu\text{l}$  of Immnoprecipitin was then added to each tube and incubated on ice for 30 minutes. The tubes were then centrifuged for 30 seconds at 10,000  $\times g$  (Anderman microfuge). The supernatant was discarded and the pelleted material washed once in Lysis Buffer and twice in Wash Buffer (0.5M LiCl, 0.1M Tris HCl pH 8.5); the volume used for each wash was 200 $\mu\text{l}$ . The pellet was finally resuspended in 40 $\mu\text{l}$  of gel sample buffer (Method B2) and the tubes incubated in a boiling water bath for 5 minutes. The tubes were centrifuged at 10,000  $\times g$  (Anderman microfuge) for 30 seconds to pellet the Immnoprecipitin, and 35 $\mu\text{l}$  of each sample was loaded onto a polyacrylamide gel as described in Method B2.

## C2 Indirect immunofluorescence of cultured cells.

For each sample,  $1 \times 10^6$  BEK cells were pelleted in an Eppendorf tube at 10,000  $\times g$  (Anderman microfuge) for 30 seconds. The cells were resuspended in 0.1ml of virus inoculum, or mock infected with 0.1ml of growth medium, such that the moi was about 0.5pfu per cell. The cell suspensions were placed in incubators at  $31^{\circ}\text{C}$  or  $38^{\circ}\text{C}$  for adsorption of virus for 1 hour. The volume in each tube was then made up to 1ml with growth medium and the contents of each tube emptied into 3cm dishes containing two clean sterile round coverslips - tapped down firmly - and 2ml of growth medium. After adding the cells.

the coverslips were again tapped down to prevent them floating, and the dishes were incubated at 31°C or 38°C, as for adsorption.

Twentyfour hours after infection, the coverslips in the dishes were rinsed with 1ml of PBS and carefully removed. Each coverslip was drained by touching it to absorbent paper and then placed in cold (stored at -20°C) acetone in a glass Petri dish on the bench for ten minutes, to fix the cells. The coverslips were then removed and air-dried.

Anti-Bunyamvera virus polyclonal rabbit serum was diluted 1/50 with PBS. The fixed dried coverslips were placed in the wells of a 12-well dish - the side with the cells attached uppermost - and 0.1ml of the diluted antiserum was placed on each coverslip. The dish was then incubated at 37°C in a humidified incubator for 45 minutes. The fluid was then carefully removed and each coverslip rinsed in 1ml of PBS for 5 minutes. Goat-anti-rabbit FITC conjugated immunoglobulin was diluted 1/50 in PBS and 0.1ml placed on each coverslip. The dish was incubated at 37°C for a further 45 minutes. Again, the fluid was removed and each coverslip rinsed in 1ml of PBS for 5 minutes, and 1ml of distilled water for 5 minutes. Each coverslip was removed from the dish, drained of excess water, and placed cells-down on a drop of 80% v/v glycerol, 20% v/v PBS on a microscope slide for examination in ultra-violet light with a Zeiss fluorescence microscope.



C3 Temperature shift experiments with ts mutants.

BHK cell monolayers in 6-well dishes were inoculated with 0.2ml of virus to give a moi of 10pfu per cell. Virus was adsorbed at 31°C or 38°C for one hour. Unadsorbed virus was removed by rinsing the monolayers twice with maintenance medium warmed to the temperature used for adsorption. 2ml of medium, warmed as for rinsing, was then placed on the cells and the monolayers incubated at the appropriate temperature. At the required time after infection, a dish was transferred from 31°C to 38°C or vice versa for the temperature shift, and all the dishes were incubated until 24 hours after infection. The medium was removed from each monolayer and assayed for virus as described (Method A2). To control these experiments, a wild type ts<sup>+</sup> virus was included in the series of temperature shifts, and for each virus used samples incubated at 31°C or 38°C constantly were included.

C4 Use of inhibitory drugs.

Stock solutions of the drugs were prepared as described in the Materials section. Cells were infected or mock-infected as described in Method B1 and the drugs were added to the methionine-free medium at the concentrations stated for one hour pretreatment prior to radiolabelling. If only 30 minutes pretreatment with a drug was required, the cells were placed in methionine-free medium for 30 minutes followed by 30 minutes of methionine-free medium plus drug. A total of one hour methionine starvation was always used. The drug concentration was then maintained in the labelling medium.

C5 Dilution series for the analysis of clone 13.48.

Ten-fold dilution series in PBS of stock virus preparations of titre  $10^8 - 10^9$  pfu per ml were prepared, and 0.2ml of these dilutions was used to inoculate BHK cells in 5cm dishes. The cells were incubated at  $31^{\circ}\text{C}$  for one hour for adsorption and then overlaid with 3ml of maintenance medium. Incubation continued at  $31^{\circ}\text{C}$  until extensive cytopathic effect was seen, or six days after infection, whichever was the sooner. At that time, the medium was removed from the monolayers and stored at  $-70^{\circ}\text{C}$  or used immediately to inoculate BHK cells in 6-wall dishes for radiolabelling of virus proteins at 18 - 19 hours after infection as usual (Method B1). Radiolabelled proteins were analysed by PAGE (Method B2). The medium obtained after growth from the dilutions was also used to inoculate BHK cells for cytoplasmic RNA preparations and analysis by dot blot hybridisation (Method B5).

C6 In vitro translation.

BHK cell monolayers in  $75\text{cm}^2$  flasks were inoculated with 0.8ml of virus - or mock-infected with PBS - at a moi of about 1pfu per cell. The inoculum was adsorbed for one hour at  $31^{\circ}\text{C}$  and the cells were then overlaid with 20ml of maintenance medium and incubated at  $31^{\circ}\text{C}$  for two days. The cells were removed from the flasks (using versene and trypsin in the standard cell subculturing procedure) and pelleted in plastic universal bottles at  $500 \times g$  (MSE Chilspin, 2,000rpm) for 5 minutes at  $+4^{\circ}\text{C}$ . The cells were rinsed in 5ml of ice-cold

PBS, pelleted as before, and resuspended in 4.5ml of ice-cold 10mM Tris HCl pH 7, 1mM EDTA, and 0.25ml of 5% w/v Nonidet P40 was added. The tubes were incubated on ice for 5 minutes and a further 0.25ml of 5% w/v Nonidet P40 was added. Nuclei and debris were pelleted by 1,200 x g (MSE Chilsplin, 3,000rpm) for 15 minutes at +4°C. The supernatants were extracted three times with equal volumes (5ml) of water-saturated distilled phenol, to remove protein, and then extracted four times with 5ml of diethyl ether to remove any phenol. 0.5ml of sodium acetate (3M) to give a final concentration of 0.3M was added to each preparation, and the solutions transferred to Oakridge tubes. 20ml of cold (-20°C) ethanol was mixed with the tube contents and the tubes stored overnight at -70°C.

The precipitated RNA was pelleted by 16,300 x g (MSE HS18, 8 x 50 rotor, 15,000rpm) for 30 minutes at +4°C. The pellets were rinsed in 70% w/v ethanol and again in 100% ethanol before desiccation. The dried pellets were each dissolved in 0.4ml of sterile distilled water and a sample diluted to determine the concentration of the RNA by optical density at 260nm and 280nm. If the  $OD_{280}$  was greater than half the  $OD_{260}$  - an indication of the presence of protein - the RNA preparation was extracted with phenol and ether again as before. 40pl of 3M sodium acetate (final concentration 0.3M) was added to each tube and 1ml of cold ethanol was mixed with the contents. The tubes were stored at -20°C until required.

For use, the RNA was pelleted from the ethanol mixture by 10,000 x g (Anderman microfuge) for 5 minutes, washed with

70% v/v ethanol followed by 100% ethanol, desiccated and redissolved in 20 $\mu$ l of sterile distilled water and adjusted to a concentration of 5 $\mu$ g ml<sup>-1</sup>. 250 $\mu$ Ci of <sup>35</sup>S-methionine was desiccated and redissolved in 100 $\mu$ l of rabbit reticulocyte lysate. Each reaction was made up in tubes on ice to contain 10 $\mu$ l of lysate + radiolabelled methionine, 10 $\mu$ l of canine pancreatic microsomes in buffer (or microsome buffer only), and 2 $\mu$ l of RNA at 5 $\mu$ g ml<sup>-1</sup> (or water). The tubes were incubated in a water bath at 37°C for one hour and then placed on ice.

5 $\mu$ l of each translation reaction was added to 20 $\mu$ l of gel sample buffer (Method B2) and the remaining 17 $\mu$ l was immunoprecipitated with anti-Banyasera serum (Method C1) after first adjusting the volume to 50 $\mu$ l with immunoprecipitation lysis buffer.

#### C7 Proteolysis in polyacrylamide gels.

<sup>35</sup>S-methionine radiolabelled cell lysates of infected BHK cells were prepared as usual (Method B1) except that 500 $\mu$ Ci ml<sup>-1</sup> of label was used. 40 $\mu$ l samples of the lysates were loaded on a 10% polyacrylamide gel and electrophoresis carried out as usual (Method B2). At the end of electrophoresis, one of the glass plates was removed and the gel on the other glass plate was covered with cling-film. A sheet of X-ray film was taped over the gel (and wrapped so as to exclude light) and placed at +4°C overnight. The film was then developed and used as a template to cut out pieces of the gel which contained the radiolabelled proteins of interest. Each gel piece was placed

in 0.5ml of 0.25M Tris HCl pH 6.7, 0.1% w/v SDS, at room temperature for 15 minutes and either used immediately or stored at  $-20^{\circ}\text{C}$ .

The second gel - in which proteolysis was carried out - was a 7% polyacrylamide gel with a 5% stacking gel of composition outlined in Method B2, except that the stacking gel was 6cm long (consequently, the resolving gel was 4cm shorter than usual). The rinsed wells of the stacking gel were filled with stacking buffer and the gel pieces pushed into the wells with a microspatula so that they lay horizontally. Only alternate wells were used and only one gel piece per well. 10 $\mu$ l of 0.25M Tris HCl pH 6.7, 0.1% w/v SDS and 20% v/v glycerol was put on top of each gel slice, and then 10 $\mu$ l of the same solution containing 0.01% w/v bromophenol blue and protease at the concentration stated. The reservoirs of the gel tank were then carefully filled with electrophoresis buffer and electrophoresis carried out until the dye front was two thirds down the stacking gel. At this point the current was switched off for 30 minutes to allow proteolysis to occur. This method is based on that described by Cleveland *et al.*, 1977. Electrophoresis was then resumed and continued as usual. The gel was then fixed and treated with Amplify for fluorography before being dried and exposed to X-ray film for 5 - 10 days.

#### CB Neutralisation of virus infectivity with antiserum.

The antiserum to be used was diluted in PBS to the concentration required and 50 $\mu$ l of this was mixed with 50 $\mu$ l of the

virus sample to be tested. The tubes were incubated at  $+4^{\circ}\text{C}$  overnight and the contents were then gently mixed before dilution in PBS for analysis by plaque assay (Method A2). As controls, 50 $\mu\text{l}$  of PBS alone was mixed with 50 $\mu\text{l}$  of virus for each virus tested and incubated and assayed as for the test samples.

C9            One step growth curves.

BHK cell monolayers in 12-well dishes ( $5 \times 10^5$  cells per monolayer) were inoculated with 0.1ml of virus at a moi of 10pfu per cell. Virus was adsorbed at  $31^{\circ}\text{C}$  or  $38^{\circ}\text{C}$  for one hour. Each virus preparation was inoculated in duplicate for each time point and a separate dish was used for each time. The infected cell monolayers were rinsed twice with 1ml of maintenance medium and overlaid with 1ml of that medium for incubation at the relevant temperature. At the required time after infection, the medium was removed from each well for assay of virus released from the infected cells by plaque formation (Method A2). The monolayers were then rinsed twice in 1ml of PBS and the cells scraped into 1ml of PBS with pieces of sterile rubber held in flame-sterilised forceps. These cell suspensions were transferred to sterile bottles and frozen at  $-70^{\circ}\text{C}$ . They were then thawed and assayed for cell-associated virus, also by plaque formation. Any samples not used immediately were stored at  $-70^{\circ}\text{C}$ .

RESULTS AND DISCUSSION

Results Section 1: Assignment of ts Lesions to RNA Segments.

(a - c)

In a study of the biological effects of mutations it is useful to know what part of the genome carries the lesion. Thus a genetic approach was used to identify the genome segments bearing the ts lesions for a representative of each of the three recombination groups of Maguari virus ts mutants (Introduction 5g). The analysis was by means of genetic crosses with a heterologous virus, Bunyamwera virus, as described in Method A4. Recombinant clones were identified by a combination of two genotyping techniques (described in Method B7) using polypeptide mobility to identify gene products of the S RNA (N protein) and M RNA (G1 protein) which were distinct for the two viruses used in each cross. The identities of the L and M RNA were determined using dot hybridisation analysis of infected cell RNA with Bunyamwera virus-reactive cDNA probes specific for L and M RNAs. The origin of the M RNA for any given clone by the two methods was always in agreement, as expected, and as has been observed previously (Pringle et al., 1985). The ts phenotype of each clone was then determined as described in Method B6 and the effect of the recombinant genotype on the ts phenotype of the clones was used to identify the genome segment bearing the ts lesion for each of the three groups of mutants.

## 1a Assignment of group I to the S RNA segment.

Genetic crosses between Mag ts 7 (I) and Bunyamwera virus ts<sup>+</sup> were performed and the genotypes and ts phenotypes of



unselected progeny clones were determined.

Cross 1: Mag ts 7 (I) x Bunyamvera  $ts^+$ .

Table 10 shows the clones isolated from Cross 1. Despite inoculation with equal amounts of the two parent viruses, only clones of Maguari virus genotype were isolated. Two clones (1.10 and 1.15) were isolated which were of Maguari virus genotype but  $ts^+$  phenotype, and had a protein like EV: in place of G1. Isolates of this kind are described further in Results Section 3. As no reassortants between the parents Mag ts 7 (I) and Bunyamvera  $ts^+$  were isolated, the cross was repeated.

Cross 2: Mag ts 7(I) x Bunyamvera  $ts^+$ .

The clones isolated from Cross 2 are shown in Table 11, and as for Cross 1 most of the clones were of Maguari virus genotype. One clone - 11.14 - of inferred genotype Mag / Bun / Bun and  $ts^+$  phenotype indicated that the ts lesion was not associated with the L RNA, because the  $ts^+$  phenotype was produced when L RNA of Mag ts 7(I) was reassorted with M and S RNA from  $ts^+$  Bunyamvera virus. The mixed isolate 11.7 was analysed by subcloning in order to isolate further reassortants and the clones are shown in Table 12. Figure 3 shows autoradiographs of a protein gel and dot hybridisation filters used in this analysis and includes one of the clones of genotype Mag / Bun / Mag and ts phenotype, isolated as a subclone of 11.7. This indicates that the ts lesion is not associated with Maguari ts 7 M RNA.

Thus the ts phenotype of Mag ts 7(I) is associated specifically with the presence of Maguari S RNA. When this segment is replaced by the S RNA of Bunyamvera  $ts^+$  virus - as

Table 10

Cross 1: Analysis of Unselected Progeny Clones from

Mag ts 7(I) x Bun ts<sup>+</sup>.

Type of Clone	Inferred Genotype	Number of isolate.
Parental 1	M / M / M <u>ts</u>	47
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	0
Reassortant		0
Mutant 1	X / M / M <u>ts</u>	Clone 1.44 1
Mutant 2	G1 replaced by EV1 M / M / M <u>ts</u> <sup>+</sup>	Clones 1.10 and 1.15 2
Total		50

Table 11

Cross 2: Analysis of Unselected Progeny Clones from

Mag ts 7(I) x Bun ts<sup>+</sup>.

Type of Clone	Inferred Genotype	Number of isolates
Parental 1	M / M / M <u>ts</u>	48
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	0
Reassortant	M / B / B <u>ts</u> <sup>+</sup>	Clone 11.14 1
Mixture	72/MB/MB <u>ts</u> <sup>+</sup>	Clone 11.7 1
Total		50

in the clone Mag / Bun / Bun - as detected by the presence of N protein, an S RNA gene product, the phenotype of the virus is ts<sup>+</sup>. When only the M RNA is replaced - as in the clone Mag / Bun / Mag - the phenotype of the virus remains ts. Cross 3: 1.10 x Bunyamwera ts<sup>+</sup>.

Cross 3 was carried out in order to obtain reassortants between Bunyamwera ts<sup>+</sup> and 1.10 - which was isolated as a Mcguari virus genotype clone but with EV1 replacing G1 from Cross 1. This was to determine which RNA segment was responsible for the replacement of G1 by EV1. As shown in Table 13, only clones indistinguishable from 1.10 in genotype and phenotype - both ts<sup>+</sup> and with G1 replaced by EV1 - were isolated. This was in spite of equal input of the parent viruses in the cross.

Discussion: Assignment of the Group I ts lesion to the S RNA segment.

These genetic crosses involving Mag ts 7(I) with Bunyamwera virus yielded clones which allowed assignment of the ts lesion to the S RNA (Tables 11 and 13). Clones of genetic types Mag / Bun / Bun ts<sup>+</sup> and Mag / Bun / Mag ts were isolated, and as the only genome segment which differs in origin between these two genetic types is the S RNA, it appears that the ts phenotype is associated with the S RNA segment derived from the Mag ts 7(I) parent. The genotypes of such reassortant clones were determined by a combination of protein mobility data and direct analysis of the L and M RNA by dot hybridisation. This assignment confirms the tentative assignment of Group I

Table 12

Subclones of 11.7, a mixed isolate from Cross 2 (Mag ts 7(I) x Bun ts<sup>+</sup>)

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M <u>ts</u>	0
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	5
Reassortant 1	M / B / B <u>ts</u> <sup>+</sup>	41
Reassortant 2	M / B / M <u>ts</u>	2
Mixtures	?B/ MB/ MB <u>ts</u> <sup>+</sup>	2
	Total	50

Table 13

Cross 3: Analysis of Unselected Progeny Clones from 1.10 (Mag genotype: G1 replaced by EV1) x Bun ts<sup>+</sup>.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M <u>ts</u> <sup>+</sup>	50
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	0
Reassortant		0
	Total	50

Figure 3

Dot hybridisation and PAGE of subclones of 11.7, a mixed isolate  
from Cross 2 (Mag ts 7(i) x Bun ts<sup>+</sup>).

Dot hybridisation:

Arrangement of samples (duplicate).

1	2		
3	4	5	6

1 = Bun ts<sup>+</sup>

2 = Mag ts

3 = 11.7.13

4 = 11.7.14

5 = 11.7.15

6 = 11.7.16

Result

Panel A: pBUN 84 (L RNA probe)

• •

Interpretation

+	+	-	-		
-	-	-	-	-	-

Panel B: pBUN 133 (M RNA probe)

• •

• • • • • • • •

+	+	-	-		
+	+	+	+	+	+

Dot hybridisation result

L and M RNA genotype

L M

Clone 11.7.13 Mag Bun

11.7.14 Mag Bun

11.7.15 Mag Bun

11.7.16 Mag Bun

Figure 3 continued.

PAGE of virus proteins radiolabelled at 18 hours after infection. HEK cell lysates, 10% polyacrylamide gel, 4 day exposure.

1 2 3 4 5



• G1 Mag  
• G1 Bun

• Actin

• N Mag  
• N Bun

1 = Mag ts 7(I)

2 = 11.7.13

3 = 11.7.14

4 = 11.7.15

5 = 11.7.16

PAGE result

	G1	N
11.7.13	Bun	Bun
11.7.14	Bun	Mag
11.7.15	Bun	Bun
11.7.16	Bun	Bun

Inferred genotypes

	L	M	S
11.7.13	Mag	Bun	Bun <u>ts</u> <sup>+</sup>
11.7.14	Mag	Bun	Mag <u>ts</u>
11.7.15	Mag	Bun	Bun <u>ts</u> <sup>+</sup>
11.7.16	Mag	Bun	Bun <u>ts</u> <sup>+</sup>

Maguari virus mutants to the S RNA segment by Iroegbu and Pringle (1981) which was based on protein electrophoretic mobility data alone, at which time the origin of the L RNA was not determined.

The S RNA segment is believed to code for two gene products - M and NSs - encoded in overlapping reading frames (Introduction 3b). Group I of the Maguari virus mutants represents 12 out of 42 mutants isolated by Iroegbu and Pringle (1981) and this is the second largest group of ts mutants they isolated from this virus. This is in contrast to the results for the California encephalitis serogroup of bunyaviruses, in which neither of the two groups of ts mutants isolated was assigned to the S RNA by oligonucleotide fingerprinting of a few selected reassortants (Gentsch et al., 1977; Gentsch & Bishop, 1979; Gentsch et al., 1980). It was suggested that the fact that S RNA encoded two proteins in overlapping reading frames (Fuller et al., 1983; Akashi & Bishop, 1983) may provide an explanation for the paucity of ts mutants in S RNA, as these mutations would be more likely to be lethal since two proteins may be affected. They also pointed out that S RNA only accounts for one tenth of the total genome size (bunyavirus genus). The overlapping reading frames combined with the smaller target size of the S RNA compared with the larger M and L segments, has been put forward as a possible explanation for the absence of ts mutants assigning to the S RNA for the California encephalitis serogroup (Gentsch et al., 1977). However, S RNA is about one quarter the size of M and this could make little contribution to the low frequency

of isolation. Also, if the reason for the low frequency of isolation is due as they suggest to the presence of overlapping reading frames in the S RNA of California encephalitis serogroup viruses, then the high frequency of isolation in the Bunyamwera serogroup implies that this is not the case for the Bunyamwera serogroup.

Although there is no evidence to suppose that the coding strategy of the S RNA of Bunyamwera serogroup viruses is radically different from that of the California encephalitis serogroup viruses, members of the two serogroups are genetically quite different as indicated by the failure of viruses from the two serogroups to reassort (Introduction 5b). This may account for the difference in frequency of isolation of mutants in the S RNA for the California encephalitis serogroup and the Bunyamwera serogroup. Presumably, either mutations in the California encephalitis serogroup S RNAs are more often lethal, or they are not detected and isolated in plaque formation assays, because if S RNA mutants in the California encephalitis serogroup do not plaque, they would not be recovered.

The assignment of the ts lesion to the S RNA was made from experiments using a Maguari virus mutant, and since it is known that Group I and Group II ts mutants of Bunyamwera virus and Batai virus are equivalent to I and II of Maguari virus as determined by recombination analyses (Iroegbu & Pringle, 1981), it can be concluded that the lesions in the Group I ts mutants of the Bunyamwera serogroup lie in the S RNA segment.



The failure of Cross 1 and Cross 3 to yield any reassortant clones or even any clones of the Bunyamwera ts<sup>+</sup> virus parent among the progeny is a phenomenon which occurred throughout these genetic crosses, and probably results from suppression of the growth of Bunyamwera virus by Maguari virus during mixed infections. This is discussed in greater detail in Results Section 2.

1b Assignment of Group II to M RNA segment.

A genetic cross between Mag ts 8(II) and Bunyamwera virus ts<sup>+</sup> was performed and the genotypes and ts phenotypes of unselected progeny clones were determined.

Cross 4: Mag ts 8(II) x Bunyamwera ts<sup>+</sup>.

Cross 4 yielded reassortant genotypes which identified Mag ts 8(II) M RNA as the segment bearing the ts lesion, as indicated in Table 14. As was observed with Cross 1 and Cross 2 involving Mag ts 7(I) the majority of clones isolated were of the Maguari virus parental genotype and no Bunyamwera genotype clones were isolated. Four clones of Maguari virus genotype but ts<sup>+</sup> phenotype were isolated and presumably represent reversion of the ts phenotype of the Maguari virus parent during the cross, as the parental Mag ts 8(II) stock used as inoculum for this cross had an eop (31°C and 38°C) of less than

Table 14

Cross 4: Analysis of Unselected Progeny Clones from

Mag ts 8(II) x Bun ts<sup>+</sup>.

Type of Clone	Inferred genotype	Number of Isolates
Parental 1	M / M / M <u>ts</u>	19 (including 15.32)
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	0
Reassortant	B / M / M <u>ts</u>	1
Mixture 1	M / M / MB <u>ts</u>	1 clone 14.38
Mixture 2	MB / MB / MB <u>ts</u> <sup>+</sup>	2
Revertant	M / M / M <u>ts</u> <sup>+</sup>	4
Total		27

Table 15

Subclones of 15.48 M / M / MB ts from Cross 4.

Inferred genotype	Number of Isolates
(including 15.48.6) M / M / M <u>ts</u>	3
(including 15.48.1) M / M / B <u>ts</u>	2
M / M / MB <u>ts</u>	4
Total	9

Figure 4

Dot hybridisation and PAGE of clones from Cross 4 (Mag is 8(II)  
x Run ts<sup>+</sup>).

Dot hybridisation

Arrangement of samples (duplicate)

1	2	3	4
5	6	7	8

1 = 15.18

2 = 15.1

3 = 15.2

4 = 15.48

5 = 15.17

6 = 15.19

7 = 15.20

8 = 15.32

Result

Panel A: pBUN 84 (L RNA probe)



Interpretation

+	+	-	-	-	-
+	+	-	-	-	-

Panel B: pBUN 155 (M RNA probe)



+	+	-	-	-	-
-	-	-	-	-	-

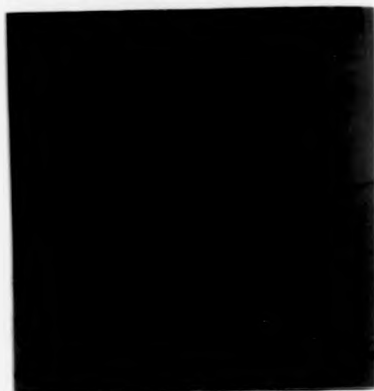
Dot hybridisation result

L and M RNA genotype

	L	M
Clone 15.18	Run	Run
15.1	Mag	Mag
15.2	Mag	Mag
15.48	Mag	Mag
15.17	Run	Mag
15.19	Mag	Mag
15.20	Mag	Mag
15.32	Mag	Mag

Figure 4 continued.

1 2 3 4 5 6 7 8 9 10



G1 Mag

G1 Bun

EV2

Actin

N Mag

N Bun

PAGE of BEK cell lysates radiolabelled by pulse labelling from 18 - 19 hours after infection. 10% polyacrylamide gel, exposed for 3 days.

PAGE result

Inferred genotypes

track	G1	M	L	M	S
1 = Bun ts <sup>+</sup>	Bun	Bun			
2 = Mag ts <sup>+</sup> 8	Mag	Mag			
3 = 13.18	N/B	N/B	13.18	? Bun M B	M B
4 = 13.1	Mag	Mag	13.1	Mag	Mag
5 = 13.2	Mag	Mag	13.2	Mag	Mag
6 = 13.48	Mag	N/B	13.48	Mag	Mag
7 = 13.17	Mag	Mag	13.17	Bun	Mag
8 = 13.19	Mag	Mag	13.19	Mag	Mag
9 = 13.20	?	Mag Bun	13.20	Mag	Mag
10 = 13.32	EV2	Mag	13.32	Mag	Mag

0-001.

Clone 13.32 - indicated in Table 14 as of Maguari virus genotype and ts phenotype - had G1 protein replaced by a polypeptide of greater electrophoretic mobility equivalent to EV2 (discussed in Results Section 3). This is shown in Figure 4. On further analysis, 13.32 was found to contain Maguari G1 in addition to EV2-like protein and this may account for the ts phenotype of this isolate, as changes in the electrophoretic mobility of G1 of Mag ts 8(II) are usually associated with reversion to ts<sup>+</sup> phenotype.

The mixed clone 13.48 (Table 14 and Figure 4) differed from the mixed clones isolated previously in that it was only mixed for the M proteins. Other mixed isolates also had both G1 proteins and were presumed to also contain both L RNA segments. 13.48 was analysed by subcloning and the result is shown in Table 15. Figure 5 shows an autoradiograph of a gel used to ascertain the origins of G1 and M proteins for these clones. All nine clones were of ts phenotype and four of the nine clones still exhibited the mixed M protein phenotype, three were of Maguari virus genotype and two were of recombinant Mag / Mag / Bun genotype. Further analysis of this interesting clone 13.48 is described in Results Section 2.

The isolation of recombinant clones of genotype Mag / Mag / Bun (Figure 5) and with ts phenotype, and Bun / Mag / Mag (Figure 4) also of ts phenotype from a cross of Mag ts 8(II) with Bunyamvera virus ts<sup>+</sup> indicate that the Group II ts lesion is associated specifically with the M RNA segment.

Figure 5

PAGE of subclones of 13.48 - M / M / MB - from Cross 4  
( Mag ts (II) x Bun ts<sup>+</sup> ).

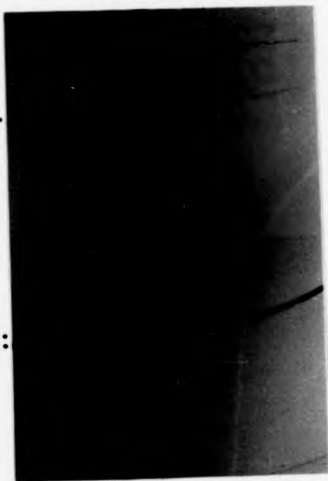
1	13.48.1	M / M / B
2	13.48.2	M / M / MB
3	13.48.3	M / M / B
4	13.48.4	M / M / MB
5	13.48.5	M / M / MB
6	13.48.6	M / M / M
7	13.48.7	M / M / MB
8	13.48.8	M / M / M
9	13.48.9	M / M / M
10	Mag <u>ts</u> B(II)	

HEK cell lysates radiolabelled by pulse labelling from  
18 - 19 hours after infection.  
10% polyacrylamide SDS gel exposed without fluorography  
for 3 days.

1 2 3 4 5 6 7 8 9 10

Mag G1 \*

Mag N .  
Bun N .



Discussion: Confirmation of the assignment of the Group II ts lesion to the M RNA segment.

The isolation of clones of genotypes Bm / Mag / Mag ts and Mag / Mag / Bm ts from Cross 4 indicates that the ts lesion is associated with the M RNA segment. This is in agreement with previous information as described by Iroegbu and Pringle (1981), and Pringle et al. (1984). Group II mutants were the most commonly isolated ts mutants in the Bunyamvera serogroup by Iroegbu and Pringle (1981) and mutants in the California encephalitis serogroup have been described which assign to the M RNA (Gentsch & Bishop, 1976; Bishop, 1979). The M RNA segment encodes two structural glycoproteins and a nonstructural protein all in a single open reading frame (Introduction 3b). The method used to genotype reassortants in this study involved the use of two procedures to analyse the M RNA, and the results from both methods were without exception in agreement. The assignment of the Group II ts lesions to the M RNA in the Bunyamvera serogroup means that the functions of the products of this segment can be studied by analysis of these mutants. It is not known as yet which of the gene products of the M RNA is affected by the Mag ts S(II) lesion, although the phenomenon of reversion of Mag ts S(II) clones to ts<sup>+</sup> phenotype in association with altered G1 protein electrophoretic mobility (Results Section 3) may implicate the G1 gene as the site of the lesion. However, the possibility of intergenic effects cannot be ruled out at present.



1c Assignment of Group III to the L RNA segment.

Cross 5: Mag ts 23(III) x Bunyamwera ts<sup>+</sup>.

A genetic cross between Mag ts 23(III) and Bunyamwera virus ts<sup>+</sup> was performed, and the genotypes and phenotypes of the unselected progeny isolated are shown in Table 16. Generally, the ts<sup>+</sup> phenotype was coincident with the presence of the Bunyamwera L RNA segment, and ts clones were presumed to have Maguari L RNA as indicated by negative dot hybridisation results. Five of the fifty isolates were found to be of Maguari virus genotype, but were of ts<sup>+</sup> phenotype, which was not in agreement with the hypothesis of the Mag ts 23(III) lesion lying in the L RNA. Group I and Group II mutants had been assigned to the S and M RNA segments respectively, so further crosses were done to confirm the assignment of the Group III lesion to the L RNA.

For these crosses, each parental virus was plaque purified and passaged only once in BEK cells (following the method outlined in Method A3) to reduce the amount of variation in the parent stocks as this may have been responsible for the isolates from Cross 5 which did not support the hypothesis of the ts lesion lying in the L RNA segment.

Cross 6: Mag ts 23(III) x Bun ts 5(I).

Cross 6 between Mag ts 23(III) and a Bunyamwera virus Group I ts mutant Bun ts 5(I) was performed. In this cross, a new strategy was tried in order to select for recombinant genotypes that would provide information as to which of the genome segments was or were responsible for the ts phenotype of Mag ts 23(III). As discussed (Results Section 1a), the Group I mutants of

Table 16

Cross 5: Analysis of Unselected Progeny from

Mag ts 23(III) x Bun ts<sup>+</sup>.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M <u>ts</u>	27
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	6
Reassortant 1	M / B / B <u>ts</u>	3
Reassortant 2	B / M / M <u>ts</u> <sup>+</sup>	4
Mixtures	?B/ MB/ MB <u>ts</u> <sup>+</sup>	2
Revertants	M / M / M <u>ts</u> <sup>+</sup>	5
Revertant/ Reassortants	M / B / B <u>ts</u> <sup>+</sup>	3
	Total	50

Maguari virus and Bunyamwera virus are equivalent and therefore it can be assumed that the mutant Bun ts 5(I) bears a ts lesion in the S RNA. Therefore, in a cross such as this, ts<sup>+</sup> reassortants would be isolated at the nonpermissive temperature for both mutants if Mag ts 23(III) has the ts mutation in a segment other than the S RNA.

Cross 6 was performed as usual (Method A4) at 31°C but on this occasion the progeny were plated at 38°C, thereby selecting for recombinant clones as neither parent was capable of plaque production at 38°C. As shown in Table 17, no plaques were recovered although undiluted culture fluid was plated. This suggests that no plaque-forming reassortants were formed or that they were generated at very low frequency. This agrees with the previous experiments of Fringle et al. (1985).

Cross 7: Mag ts 23(III) x Bun ts 7(II).

Bun ts 7(II) is a Group II Bunyamwera virus mutant and so from the work with Mag ts 8(II) (Results Section 1b) it is reasonable to assume that the ts lesion is associated with the M RNA segment. Cross 7 between Bun ts 7(II) and Mag ts 23(III) was performed as for Cross 6 in order to isolate recombinant viruses by using selection at the nonpermissive temperature of 38°C, at which neither parent was able to produce plaques. As shown in Table 18, no plaques were recovered from the cross at 38°C, again indicating that no ts<sup>+</sup> progeny virus had been formed or only at very low frequency. It has been noted previously that a cross between Mag ts 23(III) and a Group II

Table 17

Cross 6: Mag ts 23(III) x Bun ts 5 (I). Progeny selected by plating at 38°C.

Virus Preparation	pfu per ml 31°C	pfu per ml 38°C
Mag <u>ts</u> 23(III)	$1 \times 10^7$	less than $10^2$
Bun <u>ts</u> 5 (I)	$2 \times 10^7$	less than $10^2$
Cross 6	$1.2 \times 10^7$	none recovered

Table 18

Cross 7: Mag ts 23(III) x Bun ts 7(II). Progeny selected by plating at 38°C.

Virus Preparation	pfu per ml 31°C	pfu per ml 38°C
Mag <u>ts</u> 23(III)	$1 \times 10^7$	less than $10^2$
Bun <u>ts</u> 7(II)	$1 \times 10^7$	less than $10^2$
Cross 7	$1.2 \times 10^7$	none recovered

Table 19

Cross 8: Mag ts 7(I) x Bun ts 7(II). Progeny selected by plating at 38°C

Virus Preparation	pfu per ml 31°C	pfu per ml 38°C
Mag <u>ts</u> 7(I)	$1 \times 10^7$	less than $10^1$
Bun <u>ts</u> 7(II)	$3 \times 10^6$	less than $10^1$
Cross 8	$5 \times 10^6$	$1.5 \times 10^2$

Bunyamvera ts virus can yield  $ts^+$  recombinants (Pringle et al., 1985). Perhaps reassortants would be isolated if a battery of Group II mutants was tested and also more extensive plating of the whole volume of tissue culture fluid obtained from such crosses would increase the chance of isolating reassortants if they occur at very low frequency.

Cross 8: Mag ts 7(I) x Bun ts 7(II)

Cross 9: Mag ts 8(II) x Bun ts 5(I).

Cross 8 and Cross 9 were performed as control experiments for Cross 6 and Cross 7 to determine whether  $ts^+$  virus could be recovered when the Bunyamvera ts mutants were crossed with Maguari virus mutants other than Mag ts 23(III). As shown in Tables 19 and 20, a low level of  $ts^+$  virus was detected in both these crosses, a result which had been obtained earlier (Iroegbu & Pringle, 1981; Pringle et al., 1984). The cop of both parents in each cross (see Tables 19 and 20) is less than  $10^{-5}$  for each virus indicating that  $ts^+$  virus detected in the crosses was indeed due to reassortants rather than reversion of the parents, although the plaques were not isolated and analysed further. These data indicate that  $ts^+$  viruses can be recovered from Group I x Group II heterologous crosses of Maguari and Bunyamvera virus ts mutants and therefore the failure to recover  $ts^+$  virus in such crosses involving Mag ts 23(III) is presumably a consequence of the biology of the Group III mutant and its failure to interact with the two Bunyamvera virus ts mutants tested in such a way as to produce  $ts^+$  progeny at a detectable frequency.

Table 20

Cross 9: Mag ts 8(II) x Bm ts 5(I). Progeny selected by plating at 38°C.

Virus Preparation	pfu per ml 31°C	pfu per ml 38°C
Mag <u>ts</u> 8(II)	$5 \times 10^6$	less than $10^1$
Bm <u>ts</u> 5(I)	$1 \times 10^6$	less than $10^1$
Cross 9	$4 \times 10^6$	$1.9 \times 10^2$

Table 21

Cross 10: Analysis of Unselected Progeny from

Mag ts 23(III) x Bm ts<sup>+</sup>.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M <u>ts</u>	10
Parental 2	B / B / B <u>ts</u> <sup>+</sup>	6
Reassortant 1	M / M / B <u>ts</u>	3
Reassortant 2	B / M / B <u>ts</u> <sup>+</sup>	1
Mixture	1B/ MB/ MB <u>ts</u> <sup>+</sup>	1
	Total	21

Cross 10: Mag ts 25(III) x Banyamwera ts<sup>+</sup>.

As no reassortant clones had been isolated between Mag ts 25(III) and Banyamwera ts viruses, Cross 5 was repeated but on this occasion, plaque-purified low-passage viruses were used (as mentioned earlier). Cross 10 was performed at 31°C and unselected progeny were isolated from plaques produced at 31°C. As shown in Table 21, four reassortant clones were isolated: three clones of genotype Mag / Mag / Bun and ts phenotype, showing that the ts lesion is associated with either the L or M RNA segments, and one clone of genotype Bun / Mag / Bun and ts<sup>+</sup> phenotype. Autoradiographs of the M protein data and the dot hybridisation filters are shown in Figure 6. These reassortants implicate L RNA as the site of the Group III ts lesion. In contrast to the results obtained for Cross 5, no revertants of Mag ts 25(III) to ts<sup>+</sup> phenotype were isolated - either as parental types or recombinant viruses, which had previously prevented assignment of the Group III lesion to a genome segment. Presumably the use of plaque-purified, low-passage virus stocks as parents for the cross had reduced the chance of isolating aberrant viruses.

Discussion: Assignment of the Group III lesion to the L RNA segment.

As has been shown, Group I and Group II ts mutants of Maguari virus can be assigned to the S and M RNA segments respectively (Results Sections 1a and 1b). As Maguari virus has a tripartite genome it would be expected that a third

Figure 6

Dot hybridisation and PAGE of clones from Cross 10 (Mag ts 23 (III)

x Bun ts<sup>+</sup>).

Dot hybridisation:

Arrangement of samples (duplicate)

Mag <u>ts</u>	Bun <u>ts</u> <sup>+</sup>		
Clone 1	Clone 2	Clone 3	Clone 4

Panel A: pBUN 84 (L RNA probe)



Panel B: pBUN 133 (M RNA probe)



Dot hybridisation result

L and M RNA genotype

	L	M
Clone 1	Mag	Mag
Clone 2	Mag	Mag
Clone 3	Mag	Mag
Clone 4	Bun	Mag

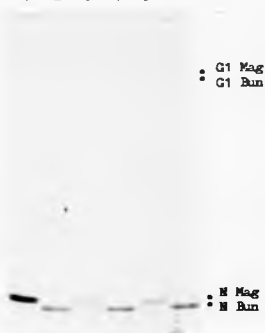


Figure 6 continued.

PAGE of HX cell lysates radiolabelled from 18 - 19 hours after infection. 10% polyacrylamide gel, exposed for 2 days.

Panel C

1 2 3 4 5 6



1 = Mag ta 25(III)

2 = Clone 1

3 = Clone 5

4 = Clone 2

5 = Clone 6

6 = Clone 4

• G1 Mag  
• G1 Bun

• M Mag  
• M Bun

PAGE result

	G1	M
Clone 1	Mag Bun	
Clone 2	Mag Bun	
Clone 4	Mag Bun	
Clone 5	M/B	M/B
Clone 6	Mag	Mag

Inferred genotypes

	L	M	S
Clone 1	Mag	Mag	Bun <u>ta</u>
Clone 2	Mag	Mag	Bun <u>ta</u>
Clone 4	Bun	Mag	Bun <u>ta</u> <sup>+</sup>

recombination group would assign to the remaining L RNA segment. The data from Cross 5 and Cross 10 show that this is indeed the case. However, the assignment of the Group III mutant was not as straightforward as the assignment of Group I and Group II and several problems unique to Mag ts 23(III) were encountered. Mag ts 23(III) may be more prone to produce mutants which can give rise to apparently conflicting results as was observed in Cross 5. In this cross, several clones were isolated which confirmed the assignment of the ts lesion to the L RNA segment, but eight of the fifty clones analysed did not (three isolates of Mag / Bun / Bun ts<sup>+</sup> and five of Mag / Mag / Mag ts<sup>+</sup>). However, when Cross 5 was repeated using plaque-purified, low-passage virus stocks as parents in Cross 10, no such viruses were found. This could happen if Mag ts 23(III) tended to produce mutant viruses during growth which are able to undergo reassortment to produce revertant and revertant recombinant clones.

In crosses involving Mag ts 7 (I) and Mag ts 8(II) (Results Sections 1a and 1b), revertants were isolated but these were not detected as reassortants and so did not prevent assignment of the ts lesions to genome segments. Also, when attempts were made to select for reassortants between Mag ts 23(III) and Bunyamwera virus by crossing with ts mutants of Bunyamwera virus and selecting at the nonpermissive temperature, no viruses were isolated (Cross 6 and Cross 7). In contrast, Mag ts 7(I) and Mag ts 8(II) were able to produce plaque-forming ts<sup>+</sup> viruses when crossed with the same Bunyamwera virus ts mutants (Cross 8

and Cross 9) under the same conditions, albeit at a low level. Perhaps Mag ts 23(III) is more restricted in its ability to reassort with Bunyamwera virus than either Mag ts 7(I) or Mag ts 8(II) - at least under conditions of selection for reassortants. When no temperature selection was used and Mag ts 23(III) was crossed with wild type Bunyamwera virus (Cross 5 and Cross 10) reassortants were isolated, some of which were of ts<sup>+</sup> phenotype. Why Mag ts 23(III) was able to produce ts<sup>+</sup> reassortants at 31°C but not at 38°C is not known. Maybe it is due to the effect of the Mag ts 23(III) lesion on the growth of the virus such that not only is it unable to replicate itself at the nonpermissive temperature, but neither can another virus with a different ts lesion in another genome segment affect its replication. It has been reported that ts<sup>+</sup> reassortants can be isolated by selection from a cross between a Group II Bunyamwera ts mutant and Mag ts 23(III) (Pringle et al., 1985) so possibly crosses using different mutants may yield reassortants under selection conditions.

The assignment of Mag ts 23(III) to the L RNA indicates that such Group III L RNA ts mutants - at least in the Bunyamwera serogroup - are very rare. As described (Introduction 3), it has been proposed (by exclusion) although not proven, that the L RNA encodes a virion transcriptase. Mutants affecting the transcriptase genes of other negative strand RNA viruses such as rhabdoviruses (Pringle, 1978) and influenza viruses (Mahy, 1985) are not rare and are well represented so it is unexpected to find only one L RNA ts mutant for the Bunyamwera serogroup

viruses. The two groups of ts mutants in the California encephalitis serogroup have been assigned to the M and L RNA segments and so in this case, the missing group of ts mutants would presumably assign to the S RNA. It may be relevant to note that the assignments of the ts mutants of the two different serogroups of bunyaviruses mentioned have been performed using different techniques - the assignments of the California encephalitis serogroup is based on the oligonucleotide fingerprints of small numbers of clones, whereas with the Bunyamwera serogroup mutants, data have been obtained from nonselective analyses of large numbers of progeny using PAGE and dot hybridisation. Whether this may account for the apparent discrepancy between two closely related groups of viruses or whether a real difference in the distribution of viable mutants exists between the groups, remains to be determined.

Table 22 summarises the reassortants isolated and the subsequent assignments of the ts lesions to genome segments for the three mutants studied.

Table 22

Summary of assignment of each ts mutant to a genome segment.

Group	Reassortant	Cross from which	Assignment
		<u>Assigned</u>	
I	M / B / B <u>ts</u> <sup>+</sup>	2	S RNA
I	M / B / M <u>ts</u>	2	S RNA
II	B / M' / M <u>ts</u>	4	M RNA
II	M / M / MB <u>ts</u>	4	M RNA
II	M / M / B <u>ts</u>	4	M RNA
III	M / B / B <u>ts</u>	5	L RNA
III	B / M / M <u>ts</u> <sup>+</sup>	5	L RNA
III	M / M / B <u>ts</u>	10	L RNA
III	B / M / B <u>ts</u> <sup>+</sup>	10	L RNA

Results Section 1: Phenotypic properties of mutants  
(d - f) representing the three recombination  
groups of Maguari virus.

The phenotypic properties of the three ts mutants were compared to each other and those of wild type Maguari virus, to examine the effects of ts lesions in each of the three genome segments on the growth of the virus.

1d Protein synthesis by the ts mutants at the nonpermissive temperature.

Time-course analysis.

The ability of the three Maguari virus ts mutants to induce virus protein synthesis at 38°C was determined as follows. HEK cells were infected with virus and incubated at 31°C or 38°C for various times before radiolabelling for one hour as usual (Method R2). The samples were radiolabelled and processed for immunoprecipitation with antiserum raised to Bunyamwera virus (Method C1). As a heterologous antiserum was used - the viruses were all Maguari viruses - it was important to determine whether the antiserum reacted with Maguari virus proteins in a similar way to the reaction with homologous Bunyamwera virus proteins, and that the amount of protein precipitated was a function of protein concentration.

To test for a quantitative relationship, <sup>35</sup>S-methionine radiolabelled cell lysates were prepared for each of the two viruses as usual (Method C1) after inoculation of HEK cells with approximately the same number of pfu in each case. Rather than adding antiserum directly to these lysates, a two-fold

dilution series of each sample was made using immunoprecipitation lysis buffer as the diluent, and then a constant amount of antiserum (1 $\mu$ l to 50 $\mu$ l of diluted lysate) was added to each tube. All the samples were then processed as usual for immunoprecipitation and a polyacrylamide gel of the electrophoresed proteins was obtained. An autoradiograph of this is shown in Figure 7. As can be seen, N protein was the most heavily labelled virus protein and therefore the easiest to isolate accurately. From this gel after autoradiography, slices were cut which corresponded to the bands of N protein seen on the autoradiograph. These were dissolved in scintillation fluid for quantitation of  $^{35}\text{S}$ -radioactivity by scintillation counting. Table 23 and Figure 8 show the result obtained when pieces corresponding to the N proteins of Maguari virus and Bunyamwera virus were analysed. As can be seen in Figure 8, straight lines were obtained for both viruses indicating that the amount of radioactivity present in a gel slice is proportional to the amount of protein present in that sample. Assuming that the amount of radioactive label incorporated into a protein is related to its rate of synthesis and therefore its concentration, it can be assumed that the amount of that protein immunoprecipitated is directly proportional to the amount of that protein present in the lysate for both Maguari virus and Bunyamwera virus N proteins. As the intensity of a band seen on an autoradiograph is a function of the amount of radioactivity present in the gel, it is reasonable to assume the intensity of a band of

Figure 7

Immunoprecipitation of serial dilutions of Maguari and Bunyamwera virus proteins shown by PAGE.

Cells radiolabelled at 18 - 19 hours after infection.

Immunoprecipitated with anti-Bunyamwera serum.

10% polyacrylamide gel fluorographed with Amplify, exposed 5 days.

- 1 nonprecipitated, uninfected BHK lysate
- 2 immunoprecipitate of uninfected BHK cells

- 3 1/1
- 4 1/2
- 5 1/4
- 6 1/8
- 7 1/16
- 8 1/32

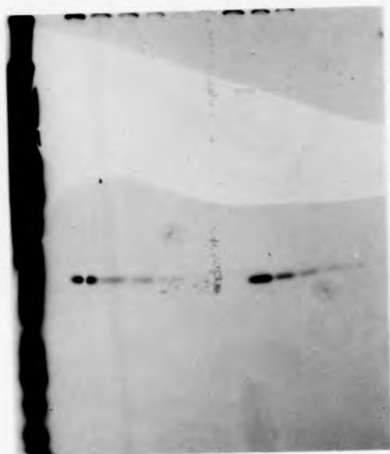
immunoprecipitates of diluted  
Maguari virus infected lysates

- 9 1/1
- 10 1/2
- 11 1/4
- 12 1/8
- 13 1/16
- 14 1/32

immunoprecipitates of diluted  
Bunyamwera virus infected lysates



1 2 3 4 5 6 7 8 9 10 11 12 13 14



Mag G1  
: Bun G1

• Actin

Mag N  
: Bun N

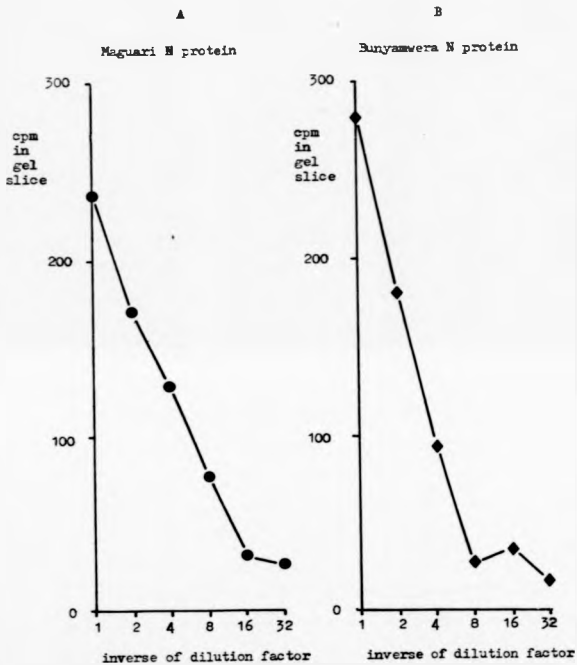
Table 23

<sup>35</sup>S cpm of gel slices from N proteins of Maguari and Bunyamwera viruses after dilution and immunoprecipitation.

Maguari virus		Bunyamwera virus	
Dilution	cpm	Dilution	cpm
1 / 1	235	1 / 1	280
1 / 2	170	1 / 2	182
1 / 4	129	1 / 4	94
1 / 8	77	1 / 8	28
1 / 16	32	1 / 16	36
1 / 32	27	1 / 32	18

Figure 8

The effect of dilution on immunoprecipitation of cpm incorporated into Maguari and Bunyamwera virus N proteins.



Maguari virus or Bunyamwera virus N protein immunoprecipitated with anti-Bunyamwera virus serum is related to the amount of N protein present in the original lysate.

In part of the investigation into the ability of the Maguari virus ts mutants to synthesize virus proteins at the nonpermissive temperature, radiolabelled cell lysates were immunoprecipitated with the Bunyamwera virus antiserum after incubation of infected cells at the permissive ( $31^{\circ}\text{C}$ ) and the nonpermissive ( $38^{\circ}\text{C}$ ) temperatures, and analysed on 10% polyacrylamide gels. To control for the presence of revertant ts<sup>+</sup> virus in the ts mutant preparations which would give rise to spurious results, samples which were not radiolabelled were incubated at  $31^{\circ}\text{C}$  and  $38^{\circ}\text{C}$  for each virus with 1ml of maintenance medium over the infected cell monolayers for 24 hours. The medium was then removed and assayed for virus as described (Method A2).

In these control assays, Maguari virus wild type always gave titres of  $10^6 - 10^7$  pfu per ml for both  $31^{\circ}\text{C}$  and  $38^{\circ}\text{C}$  samples - that is, an eop of 0.5 - 1. Mag ts 7(I), Mag ts 8(II) and Mag ts 23(III) always gave titres such that the eop was 0.001 or less, and so these viruses were shown to be ts in these experiments.

The autoradiograph shown in Figure 9 indicates that wild type Maguari virus produces significant amounts of N and G1 proteins at both temperatures. The rate of synthesis of these proteins at  $38^{\circ}\text{C}$  is apparently faster than at  $31^{\circ}\text{C}$ , with the maximum amount of virus protein at  $38^{\circ}\text{C}$  appearing at 4 - 5 hours after infection, compared with a maximum at  $31^{\circ}\text{C}$  at

Figure 9

Protein synthesis by wild type Maguari virus at 31°C and 38°C.

PAGE of immunoprecipitated proteins.

1 2 3 4 5 6



1	31°C	4 hours
2	38°C	
3	31°C	8 hours
4	38°C	
5	31°C	16 hours
6	38°C	

• G1

• N

Proteins radiolabelled for 1 hour from time-point indicated.  
Immunoprecipitated with anti-Bunyamvera serum. 10% polyacrylamide  
gel fluorographed with Amplify and exposed 5 days.

8 - 9 hours after infection.

Figure 10 shows that Mag ts 7(I) produces detectable but small amounts of N and G1 proteins at 38°C compared to the level seen at 31°C. The peak amount of N and G1 detected is seen in a pulse from 16 - 17 hours after infection, whereas the amounts of N and G1 produced at 8 - 9 hours and 16 - 17 hours after infection at 38°C are relatively similar. This is in contrast to the observation for wild type virus at 38°C, when there was a peak amount of virus protein fairly early in infection and this decreased rapidly.

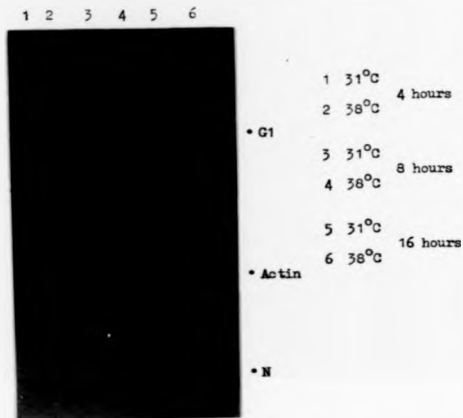
Perhaps the protein synthesis seen with Mag ts 7(I) at 38°C was due to revertant ts<sup>+</sup> virus present in the stock. However, this is unlikely because the eop of the stocks used in these experiments was 0.001 or less. This was determined from plates of cells inoculated with the stock virus preparations used for the protein synthesis experiments and incubated at 31°C or 38°C for 24 hours before assay. Under these conditions, any revertants present in a preparation would have been amplified at 38°C but the eop was still 0.001 or less. Thus a moi of less than  $5 \times 10^{-3}$  pfu per cell of ts<sup>+</sup> virus was used for the ts viruses, which would not give detectable protein synthesis by this method. Consequently, the protein synthesis seen by Mag ts 7(I) probably represents protein synthesis by the ts virus at 38°C.

Figure 11 shows that Mag ts 8(II) does direct virus protein synthesis of N and G1 proteins at both 31°C and 38°C. With this mutant, as with the wild type virus, onset of virus

Figure 10

Protein synthesis by Mag 1a 7(I) at 31°C and 38°C.

PAGE of immunoprecipitated proteins.



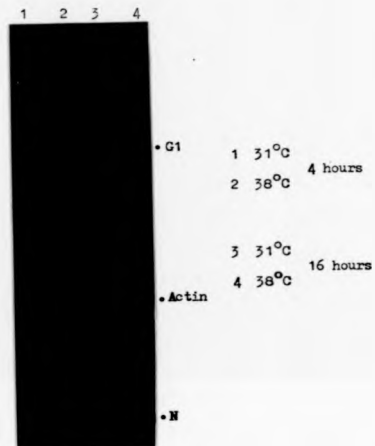
Proteins radiolabelled for 1 hour from time-point indicated.

Immunoprecipitated with anti-Bunyamvera serum. 10% polyacrylamide  
gel fluorographed with Amplify and exposed 3 days.

Figure 11

Protein synthesis by Mag ta 8(II) at 31°C and 38°C.

PAGE of immunoprecipitated proteins.



Proteins radiolabelled for 1 hour from time-point indicated.

Immunoprecipitated with anti-Bunyamvera serum. 10% polyacrylamide gel fluorographed with Amplify and exposed 2 days.



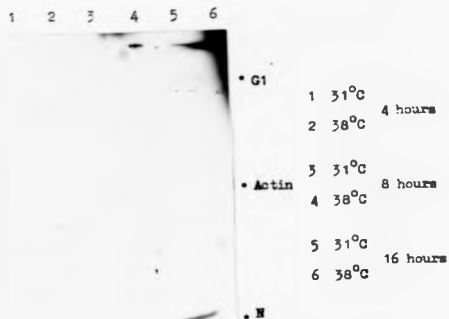
protein synthesis occurred sooner at 38°C than at 31°C. Maximum protein synthesis was then seen later at 31°C.

Figure 12 shows the time-course with Mag ts 23(III). N protein can be seen in the immunoprecipitated sample at 16 - 17 hours after infection at 31°C. No virus proteins can be seen in any of the 38°C samples. The reason N protein appeared late in infection at 31°C is that the titre of the virus inoculum used was such that the moi was only 1pfu per cell rather than the desired 10pfu per cell. By comparison with the wild type virus (Figure 9), if this mutant had induced virus protein synthesis at 38°C, it would have been expected that N protein would appear at 38°C before 31°C. As the information obtained in this experiment was inconclusive, the experiment was repeated. Figure 13 shows an autoradiograph of PAGE of immunoprecipitated samples from a time-course of protein synthesis at 31°C and 38°C for both the wild type Maguari virus and Mag ts 23(III). The moi for the wild type virus was 10pfu per cell and for Mag ts 23(III) 0.1 - 1 pfu per cell (Mag ts 23(III) consistently produced low titre stocks). At 7 - 8 hours after infection, Maguari ts<sup>+</sup> had already passed the peak rate of protein synthesis at 38°C, and as before, the peak rate of synthesis at 31°C occurred later at 16 - 17 hours after infection. Mag ts 23(III) only produced N and G1 proteins at 31°C at 24 - 25 hours after infection and no virus protein was detected at 38°C. Thus it appears that mutant Mag ts 23(III) is defective in protein synthesis at 38°C.

Figure 12

Protein synthesis by Mag in 23(III) at 31°C and 38°C.

PAGE of immunoprecipitated proteins.



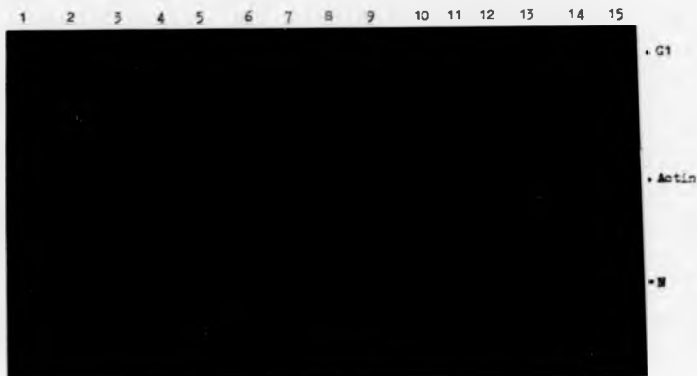
Proteins radiolabelled for 1 hour from time-point indicated.

Immunoprecipitated with anti-Bunyavirus serum. 10% polyacrylamide gel fluorographed with Amplify and exposed 3 days.

Figure 13

Protein synthesis by wild type Maguari virus and Mag ts 25(III) at 31°C and 38°C

PAGE of immunoprecipitated proteins.



1 - 7 Mag ts<sup>+</sup>

1	31°C	
2	38°C	7 hours
3	31°C	
4	38°C	16 hours
5	31°C	
6	38°C	20 hours
7	31°C	24 hours

8 - 15 Mag ts 25(III)

8	31°C	
9	38°C	7 hours
10	31°C	
11	38°C	16 hours
12	31°C	
13	38°C	20 hours
14	31°C	
15	38°C	24 hours

Proteins radiolabelled for 1 hour from time-point indicated.

Immunoprecipitated with anti-Bunyavirus serum. 10% polyacrylamide gel fluorographed with Amplify and exposed 5 days.

Indirect immunofluorescence of HEK cells infected with ts mutants.

Figure 14 shows the effect of incubation at 31°C and 38°C on indirect immunofluorescence seen with HEK cells mock-infected and virus infected, using anti-Rubymvera virus serum and acetone fixed cells (Method C2). The viruses used were wild type Maguari virus, Mag ts 7(I), Mag ts 8(II) and Mag ts 23(III).

14A shows fluorescence of mock-infected cells and there is no specific reaction at either temperature. This indicates that any fluorescence seen with virus-infected cells is a consequence of virus-specific antigen.

14B shows the result obtained with cells infected with wild type Maguari virus. At both temperatures a high level of fluorescence can be seen, mainly in the cytoplasm of infected cells. Not all the cells in the two fields of view shown are fluorescent and this reflects the low moi of 1pfu per cell that was used. Thus as expected, wild type Maguari virus produced sufficient antigen in infected cells at both temperatures to be detected by indirect immunofluorescence.

14C shows the result obtained with Mag ts 7(I)-infected cells. At 31°C a specific reaction was seen but at 38°C no fluorescence was evident. This suggests that the level of expression of virus antigen produced by Mag ts 7(I)-infected HEK cells at that temperature was not detectable by this procedure.

14D shows the result obtained with HEK cells infected with Mag ts 8(II). At both temperatures there was specific

Figure 14

Indirect immunofluorescence of mock-infected and virus-infected HEK  
cells cultured at 31°C and 38°C.

- Panel A mock-infected  
Panel B Maguari virus wild type  
Panel C Mag ts 7(I)  
Panel D Mag ts 8(II)  
Panel E Mag ts 23(III)

Acetone fixed at 24 hours after infection.

Anti-Bunyamwera virus serum (rabbit) with FITC conjugated  
goat-anti-rabbit serum.

14 Panel A: mock-infected cells.

Illumination

white light

uv light

temperature of  
incubation



31°C

38°C

14 Panel B: Maguari virus wild type.

Illumination

temperature of  
incubation

white light

uv light

31°C

38°C



Panel for 38°C, UV light  
printed in reverse

14 Panel C: Mag to 7(I).

Illumination

white light

uv light

temperature of  
incubation



31°C

38°C



14 Panel D: Mag ~~10~~ 8(II).

Illumination

temperature of white light  
incubation

uv light

31°C

38°C



14 Panel E: Mag to 23(III).

Illumination

white light

uv light

temperature of  
incubation



31°C

38°C

fluorescence in the cytoplasm. With the polyclonal antiserum used, no difference in the distribution of virus antigen between Mag is 8(II) and the wild type virus (14B) at either temperature could be seen.

14B shows the result obtained with Mag is 23(III)-infected 293 cells. At 31°C there was a specific reaction but no reaction was seen at the higher temperature, indicating the level of virus antigen was too small to be detected by this means.

The immunofluorescence data presented here complement the information obtained for protein synthesis by these mutants using the time-course method. Mag is 7(I), which had been seen to synthesize a very low level of virus protein at 38°C did not give any detectable fluorescence at 38°C. Mag is 8(II), which produced a high level of protein at 38°C was similar to the wild type virus as fluorescence was seen at 38°C. Mag is 23(III) which did not synthesize any virus protein at 38°C, was fluorescence negative at that temperature.

In all cases in which positive fluorescence was seen (either at 31°C or at 38°C), the distribution was mainly cytoplasmic. This is expected as the site of bunyavirus replication is the cytoplasm with no known nuclear involvement (Introduction 4). On the pattern of fluorescence alone, the mutants were indistinguishable from each other and the wild type virus indicating no gross perturbation of the protein expression mechanisms. More subtle differences may be detected by the use of monoclonal antibodies for various virus proteins at both temperatures.

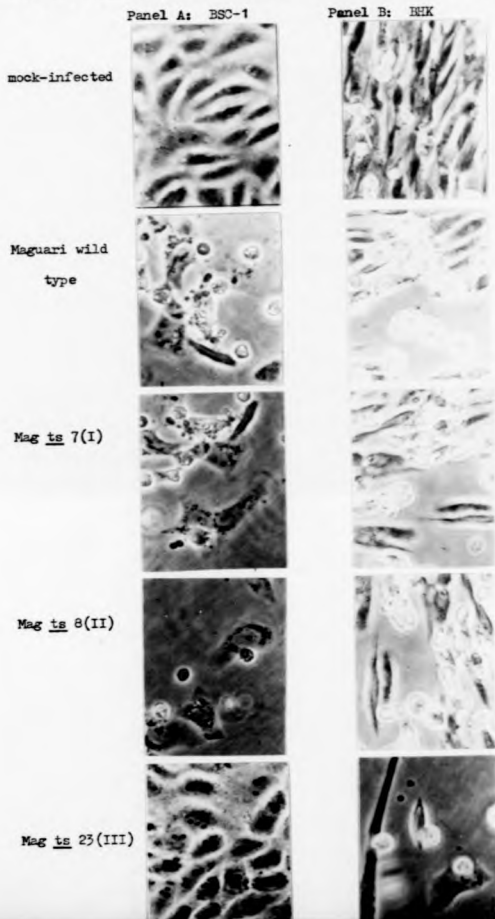
1e Host restriction in Mag ts 23(III).

It has been reported previously that Mag ts 23(III) shows host restriction in BSC-1 cells (Pringle & Iroegbu, 1982), but no such restriction was seen with either of the other two groups of mutants in the Bunyamwera serogroup or with wild type viruses. This phenomenon was examined by looking for cytopathic effect of Maguari virus mutants on BHK and BSC-1 cells. BHK and BSC-1 cell monolayers in 5cm dishes were inoculated with virus or mock-infected with PBS at a moi of 5pfu per cell. The dishes were incubated at 31°C with 5ml of maintenance medium over the infected cells for five days. Figure 15 shows that wild type Maguari virus, Mag ts 7(I) and Mag ts 8(II) caused extensive cytopathic effect with the BSC-1 cells, with granulation, rounding up and detachment from the culture surface, and these viruses were similar to each other in their effects. Mag ts 23(III) however, had very little obvious effect on the appearance of the cells although there is some evidence of granulation. The effects of all four viruses on BHK cells in the same experimental conditions were similar, with extensive cell destruction and detachment.

This result confirms the earlier report that Mag ts 23(III) is unusual in its failure to produce substantial cytopathic effect on BSC-1 cells (Pringle & Iroegbu, 1982). Whether this is a direct consequence of the lesion responsible for the ts phenotype or whether it is due to other mutations is not known.

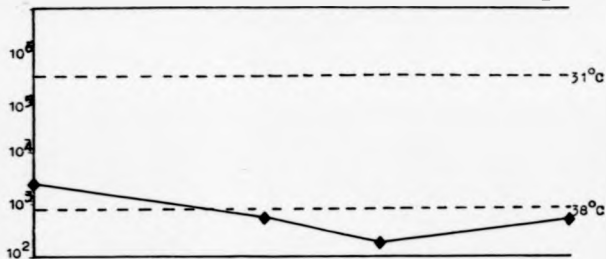
Figure 15

Effects of viruses on BSC-1 and HEK cells cultured at 31°C for 5 days.



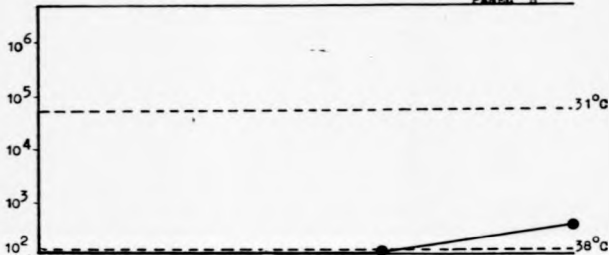


PANEL A

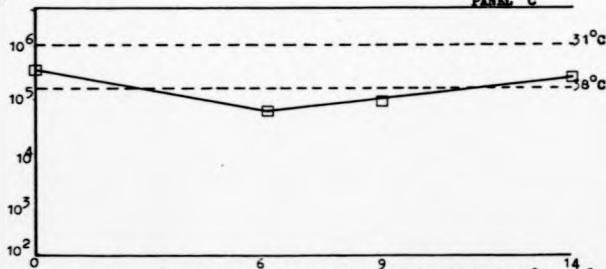


PANEL B

$\frac{Y}{\text{axes}}$   
pfu  
ml<sup>-1</sup>  
at  
24h  
after  
infection.



PANEL C



Hours after infection when plates transferred from 31° to 38°.

Figure 16

Temperature shift from 31°C to 38°C; Maguari virus ts<sup>+</sup>,

Mag ts 7(I), and Mag ts 8(II).

Panel A: Mag ts 8(II) ◆

Panel B: Mag ts 7(I) ●

Panel C: Mag ts<sup>+</sup> □

- - - - Broken lines indicate control titres from plates  
held at 31°C or 38°C as above, for the duration  
of the incubation period.

———— Solid lines indicate virus titres from the medium  
of plates transferred to 38°C during the incubation  
period, and assayed at 24 hours after infection.



time. Figure 17 shows the effect of this on the level of virus released by wild type virus. Mag ts 7(I) and Mag ts 8(II).

The wild type Maguari virus was slightly affected by temperature shifts of this nature, and the control level of virus production at 38°C was about 1 log<sub>10</sub> unit lower than the level at 31°C. However, the titres obtained with shifts both up and down do not vary much from the control levels. The changes in temperature do not appear to have had an effect on the amount of virus released by the end of the 24 hour incubation period.

The Mag ts 7(I) mutant was affected by a shift from 31°C to 38°C up to 14 hours after infection during a 24 hour incubation period. Under these conditions, no virus was detected in the tissue culture fluids - except from dishes shifted at 14 hours after infection. At this time, the titre of virus found (at the end of the 24 hour incubation period) was 2 log<sub>10</sub> units lower than that found in the 31°C control sample -  $5 \times 10^2$  pfu per ml compared with  $7 \times 10^4$  pfu per ml. Mag ts 7(I) was much less affected by a shift down from 38°C to 31°C and relatively high titres were found in the supernatants ( $6 \times 10^4$  pfu per ml), despite the fact that the ts virus-infected cells were incubated at the nonpermissive temperature for up to 14 hours after infection. The control plates held at 38°C for the duration of the incubation had barely detectable virus in the supernatants. Thus the effect of shifting the Mag ts 7(I)-infected cells from the nonpermissive temperature to the permissive temperature up to 14 hours after

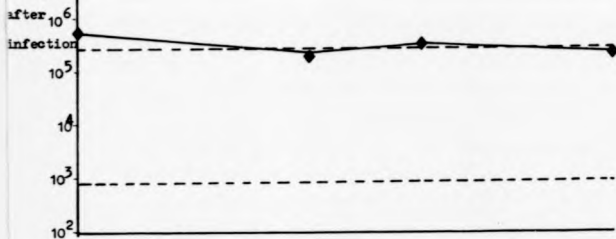
y - axes

pfu ml<sup>-1</sup>

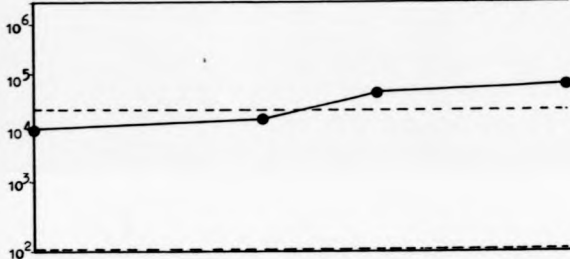
at

24h

PANEL A



PANEL B



PANEL C

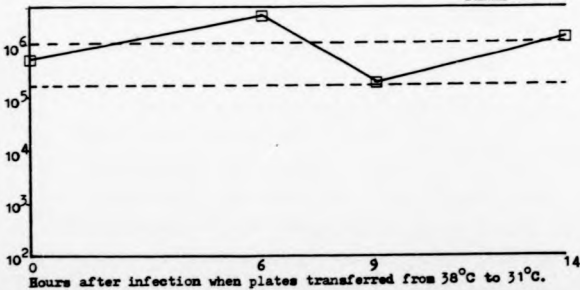


Figure 17

Temperature shift from 38°C to 31°C; Maguari virus ts<sup>+</sup>,  
Mag ts 7(I), and Mag ts 8(II).

Panel A: Mag ts 8(II) ◆

Panel B: Mag ts 7(I) ●

Panel C: Mag ts<sup>+</sup> □

- - - - Broken lines indicate control titres from plates  
held at 31°C or 38°C as shown, for the duration  
of the incubation period.

———— Solid lines indicate virus titres from the medium  
of plates transferred to 38°C during the incubation  
period, and assayed at 24 hours after infection.

infection apparently results in virus being released into the medium at levels comparable to those found with infected cells incubated at the permissive temperature throughout the incubation period.

These results indicate that the mutation in Mag ts 7(I) which prevents it from growing at 38°C can be overcome if the infection progresses at the permissive temperature for the first 14 hours of infection before incubation at the nonpermissive temperature. Incubation at 38°C earlier in the infection cycle blocks growth. Also, the virus is capable of replicating at 31°C even if initially incubated at 38°C for as long as 14 hours showing that the ability of Mag ts 7(I) to grow at the permissive temperature is not abolished by incubation at the nonpermissive temperature. It seems that after infection, incubation at 31°C for as long as 14 hours allows a product to be made or a process to be carried out which is otherwise prevented by incubation at 38°C. For this reason incubation initially at 31°C for less than 9 - 14 hours after infection before transfer to 38°C, prevents the virus producing infectious plaque-forming virions.

The growth of mutant Mag ts 8(II) was affected similarly by changes in the incubation temperature in these shift experiments. The control plates incubated at one temperature throughout the incubation period show a high titre of virus released at 31°C ( $4 \times 10^5$  pfu per ml) and a low but detectable level of virus ( $8.4 \times 10^2$  pfu per ml) at 38°C. The effect of transfer from 31°C to 38°C, up to and including 14 hours after infection, was that the amount of virus released was

depressed to around that from infected cells incubated at 38°C continuously. The effect of shift down from 38°C to 31°C up to 14 hours after infection was that titres of virus were obtained ( $3 \times 10^5$  pfu per ml) which were similar to the titres obtained from infected cells incubated at 31°C throughout ( $2.7 \times 10^5$  pfu per ml). As for Mag ts 7(I) incubation at 31°C for as long as 14 hours prior to incubation at 38°C allowed the temperature sensitive stage of the replication cycle to be passed and the virus was then able to complete replication at 38°C.

Temperature shift with Mag ts 23(III).

Figure 18 shows curves obtained for Mag ts 23(III) in temperature shift experiments and compares curves from two separate occasions for both shift up and shift down experiments. (The virus counts for these graphs are presented in Appendix 3). Overall, more virus was produced from the shift down plates than from the shift up. This shows that the virus is not inactivated by incubation at 38°C, at least early in infection. Presumably, synthesis of a ts product at 31°C is responsible for the depression in the amount of virus released in the shift up experiments. However, the level of depression seen was only 1 - 2  $\log_{10}$  units, which is much less than the difference between the control curves.

The curves shown for Mag ts 7(I) and Mag ts 8(II) (Figures 16 and 17) were consistent between three repeats of the experiments. Inconsistency was only seen with Mag ts 23(III). The failure to obtain a clear effect of such temperature shift

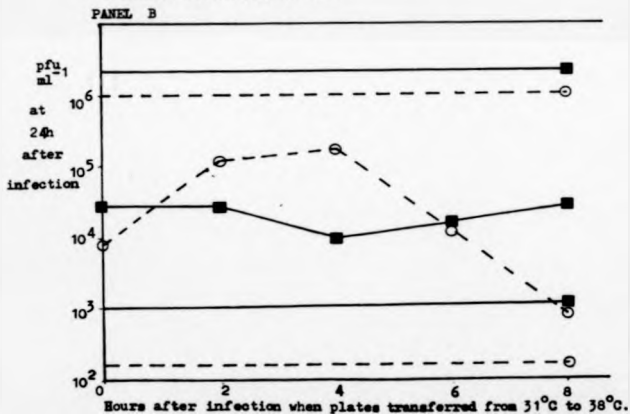
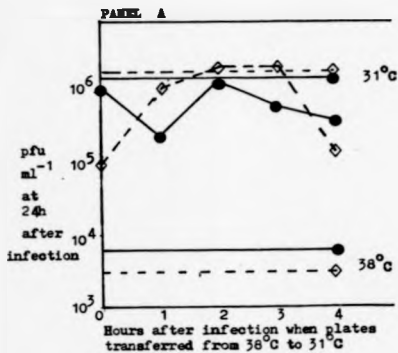


Figure 18

Temperature shift with Mag ts 23(III): comparison of different experiments.

Panel A.

Shift down from  $38^{\circ}\text{C}$  to  $31^{\circ}\text{C}$

◇ and ● are two separate experiments.

Control lines from plates held at  $31^{\circ}\text{C}$  or  $38^{\circ}\text{C}$  for the duration of the incubation period are shown for each experiment.

Panel B.

Shift up from  $31^{\circ}\text{C}$  to  $38^{\circ}\text{C}$ .

○ and ■ are two separate experiments.

Control lines from plates held at  $31^{\circ}\text{C}$  or  $38^{\circ}\text{C}$  for the duration of the incubation period are shown for each experiment.

experiments for Mag ts 25(III) perhaps indicates that this mutant is more fastidious in its growth temperature requirements than Mag ts 7(I) or Mag ts 8(II) and as pointed out the method did not ensure a rigorous temperature control. Perhaps a clear result, indicating at what stage in the infectious cycle Mag ts 25(III) is temperature sensitive could be obtained by using water baths to speed cooling and warming up times - but even so, temperature fluctuations would still occur and may be sufficient to produce variable results.

Thus, as shown in Table 24, the three ts mutants are not only different from the wild type Maguari virus in their growth restriction at 38°C, but they are also different from each other. Further experiments with different mutants from each of the recombination groups are required to determine whether these properties are characteristic of each group or are merely the characteristics of the individual mutants.

#### Discussion: Phenotypic effects of the Mag ts 7(I) lesion.

As shown, Mag ts 7(I) (S RNA associated lesion) is capable of a reduced level of virus protein synthesis at the nonpermissive temperature and that level is too low to be detected by indirect immunofluorescence. Perhaps the lesion affects amplification (as a very low constant level of protein synthesis was seen at 38°C) of virus components, or even synthesis of genomic RNA,



Table 24

Phenotypic Properties of Maguari virus wild type, Mag ts 7(I).

Mag ts 8(II) and Mag ts 23(III).

Phenotype	Mag ts <sup>+</sup>	Mag ts 7 (I)	Mag ts 8 (II)	Mag ts 23 (III)
ecp less than 0.001 at 31 & 38°C	+	-	-	-
Growth restriction after shift-up up to 14 hours	-	+	+	±
Growth restriction after shift-down up to 14 hours	-	-	-	±
Immunofluorescence at 38°C at 24 hours	+	-	+	-
Protein synthesis at 38°C by time-course	+	±	+	-
ecp in EEC-1 cells	++++	++++	++++	+

both of which are required for the formation of progeny virions. Direct analysis of the kinetics of RNA synthesis by this mutant at both temperatures will provide further information on the synthetic abilities of this virus - perhaps making use of the Bunyamwera cRNA probes which do not discriminate between Maguari and Bunyamwera virus RNA. Also there are now available some monoclonal antibodies specific for the N protein - a product of the S RNA - of Bunyamwera virus. However, monoclonal antibody provided by R. K. Elliott did not react with Maguari virus proteins (data not shown). As the Group I ts mutants of Maguari virus and Bunyamwera virus are in the same recombination group, these reagents could provide useful information on the fate of the N protein and the consequences of Group I ts lesions in other Group I mutants.

Discussion: Phenotypic effects of the Mag ts 8(II) lesion.

Mag ts 8(II) (N RNA associated lesion) was found to be capable of apparently normal levels of protein synthesis at 38°C when compared with wild type Maguari virus, and sufficient antigen was produced to be detected by indirect immunofluorescence of cells infected at 38°C. In spite of the production of high levels of virus protein at 38°C and a normal distribution of those proteins - as far as could be seen with a polyclonal antiserum - infectious virus is not produced at that temperature. Perhaps the ts lesion of Mag ts 8(II) affects the processes

of virion assembly and release, possibly as a result of defects in the ability of proteins to interact normally. Other processes such as synthesis of virion RNA may be affected. As M RNA encodes two structural proteins, defects in the interactions of either of these could easily lead to a block in assembly and release of virions, despite high levels of virus protein synthesis. The tendency of Mag ts 8(II) to give rise to revertants of altered G1 electrophoretic mobility (see Results Section 3) suggests that the lesion may lie in the G1 coding region of the M RNA.

Discussion: Phenotypic effects of the Mag ts 25(III) lesion.

The mutant Mag ts 25(III) (L RNA associated lesion) generally failed to grow to such high titres as wild type Maguari virus, Mag ts 7(I) and Mag ts 8(II) (typically  $10^6$  pfu per ml compared with  $10^7$  -  $10^8$  pfu per ml) and unusually, was host restricted in that it did not produce extensive cytopathic effect in BSC-1 cells, although it did in RHK cells. Host restriction in other ts mutants of negative strand viruses such as vesicular stomatitis virus and influenza virus, has been associated with mutations in the polymerase functions (Pringle, 1978; Simpson et al., 1979; Szilagyi & Pringle, 1975; Israel, 1980). Pringle and Iroegbu (1982) suggested that if host restriction in Bunyamvera serogroup ts mutants is associated with polymerase functions (presumed to reside in

the L RNA), this may explain the rarity of isolation of Group III mutants as they may generally be unable to grow in systems which depend on plaque formation.

No virus protein synthesis could be detected for this mutant at 38°C and consequently the immunofluorescence result was negative. As a result of the low titres obtained for this virus, high moi for protein synthesis time-course studies could not be achieved and this probably accounts for the late appearance of virus proteins synthesized by Mag ts 23(III) at 31°C compared with wild type virus (Figure 13).

If the lesion in this mutant does affect polymerase functions, it might be expected that protein synthesis would be abolished as mRNA synthesis is usually a function of such a negative strand virus polymerase. Analysis of the ability of Mag ts 23(III) to synthesize RNA at the nonpermissive temperature would be useful in defining the point at which the virus is temperature sensitive, and would help to identify the gene functions involved.

Results Section 2: Analysis of a Clone Expressing Both  
Maguari and Bunyamwera Virus N Proteins.

Mixed clones were repeatedly isolated from genetic crosses (Results Section 1a - c) and one was examined in greater detail. Clone 13.48 of inferred genotype Mag / Mag / Mag-Bun and ts phenotype was isolated from Cross 4 of Mag ts 8(II) x Bunyamwera virus. This clone was selected for further study as it appeared to be mixed only for the S RNA segment. Subcloning of 13.48 described earlier in Table 15, had shown that subclones of the same mixed N protein phenotype as 13.48 itself were isolated at high frequency (4/9) suggesting an intimate mixture of the two S RNA segments.

2a Dilution series experiments with 13.48 using 13.48.1  
and 13.48.6 as controls.

To examine 13.48 further, a series of experiments involving dilution of the virus was performed. Two subclones of 13.48 were used as controls - 13.48.1 of genotype Mag / Mag / Bun, and 13.48.6 of genotype Mag / Mag / Mag. If 13.48 was a mixture of two different viruses of the genotypes of these two subclones, dilution might enhance differences in the proportions of the viruses present. The procedure used is described in Method C5 and involved ten-fold serial dilution of stock virus preparations of titres of  $5 \times 10^8$  pfu per ml. As the virus concentration in the dilutions was then too low to be detected by protein radiolabelling of infected cells and only low moi would occur, 0.2ml samples from the dilutions

were used to inoculate BHK cells in 5cm dishes and incubated at 31°C until extensive cytopathic effect was seen, or 6 days after infection. Consequently the moi for these virus growth procedures would have been of the order of  $10^{-2}$  pfu per cell for  $10^{-3}$  dilution and  $10^{-6}$  pfu per cell for the  $10^{-7}$  dilution. Thus, the more dilute the original virus inoculum, more rounds of replication would occur to produce significant cytopathic effect. By this procedure, viruses present in the dilutions would be amplified by growth in BHK cells and so a titre sufficient for analysis of the virus proteins by radiolabelling ( $10^6 - 10^7$  pfu per ml) would be obtained. The infected cell media produced by this process were then used as inocula for protein labelling and on one occasion for the preparation of cytoplasmic RNA for dot hybridisation analysis.

Figure 19 shows the result obtained with a ten-fold dilution series of 13.48, and Figure 20 shows the result for the control. This control consisted of equal amounts of pfu of 13.48.1 and 13.48.6 which were mixed before dilution, to give a titre equal to that used for 13.48, and then treated exactly as 13.48. Figure 19 of 13.48 shows that both N proteins were radiolabelled throughout the dilutions, but there was an increasing reduction in the radiolabelling of Bunyamwera N protein relative to Maguari N protein. At  $10^{-7}$ , the last sample in which virus proteins were detected, both N proteins were labelled similarly. No virus protein was detected at  $10^{-8}$  as presumably the virus had been diluted to extinction. The control (Figure 20) showed very little labelling of Bunyamwera

Figure 19

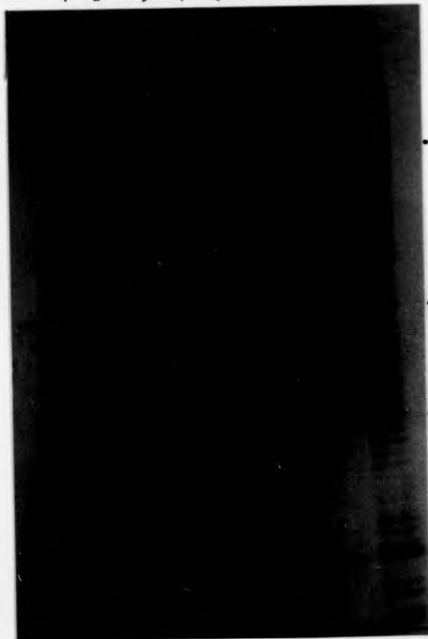
PAGE of 13.48 - M / M / MB - after dilution and culturing  
of virus from the dilution series.

- 1 Mock infected BEK cells
- 2 13.48
- 3  $10^{-2}$  sample
- 4  $10^{-3}$  sample
- 5  $10^{-4}$  sample
- 6  $10^{-5}$  sample
- 7  $10^{-6}$  sample
- 8  $10^{-7}$  sample
- 9  $10^{-8}$  sample

BEK cell lysates radiolabelled by pulse labelling from  
18 - 19 hours after infection.

10% polyacrylamide SDS gel exposed without fluorography  
for 3 days.

1 2 3 4 5 6 7 8 9



• Mag G1

• Actin

• Mag N

• Run N



Figure 20

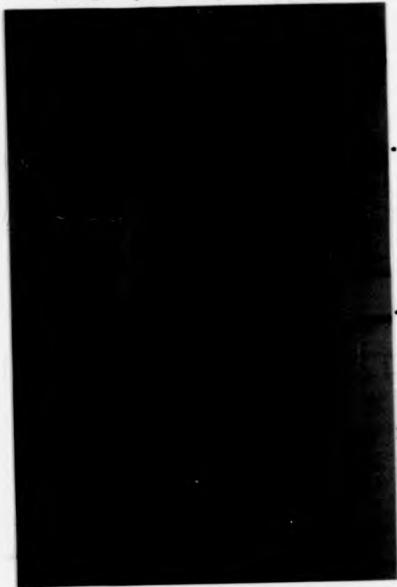
PAGE of a mixture of 13.48.1 - M / M / B - plus 13.48.6 - M / M / M - after dilution and culturing of virus from the dilution series.

- 1 Mock infected HEK cells
- 2 13.48.1 M / M / B
- 3 13.48.6 M / M / M
- 4  $10^{-3}$  sample
- 5  $10^{-4}$  sample
- 6  $10^{-5}$  sample
- 7  $10^{-6}$  sample
- 8  $10^{-7}$  sample
- 9  $10^{-8}$  sample

HEK cell lysates radiolabelled by pulse labelling from  
18 - 19 hours after infection.

10% polyacrylamide SDS gel exposed without fluorography  
for 2 days.

1 2 3 4 5 6 7 8 9



• Mag G1

• Actin

• Mag N  
• Run N

N protein at  $10^{-3}$ , less at  $10^{-4}$  and then none until the  $10^{-7}$  preparation when the Maguari N protein disappeared and only Bunyamwera N protein was labelled. As with 13.48, no virus protein was seen in the sample derived from the  $10^{-8}$  dilution. It is important to note that the amounts of the two N proteins produced by 13.48.1 (Bunyamwera N) and 13.48.6 (Maguari N) when grown alone and with a moi of 10 pfu per cell (Figure 20, tracks 1 and 2) were equivalent in amount and there was no apparent difference in the rates of synthesis of the two proteins.

2b            Dilution series experiments with Maguari and Bunyamwera viruses.

As both 13.48.1 and 13.48.6 were derived by subcloning from 13.48, it was of interest to find out whether similar results could be seen with the two parental viruses. Thus, wild type Maguari and Bunyamwera viruses were mixed and diluted as for 13.48 (Method C5), and the virus present in these dilutions amplified to provide material for radiolabelling as described. Figure 21 shows that a similar phenomenon to that seen for the mixture of 13.48.1 + 13.48.6 (Figure 20) was observed. In this case, Bunyamwera virus proteins were not seen until the  $10^{-7}$  dilution sample in which no Maguari virus protein were seen. As this experiment involved Bunyamwera virus, dot hybridisation could be used to look for the presence of Bunyamwera L and M RNA segments. This had not been possible with 13.48 and its subclones as only Bunyamwera S RNA was

Figure 21

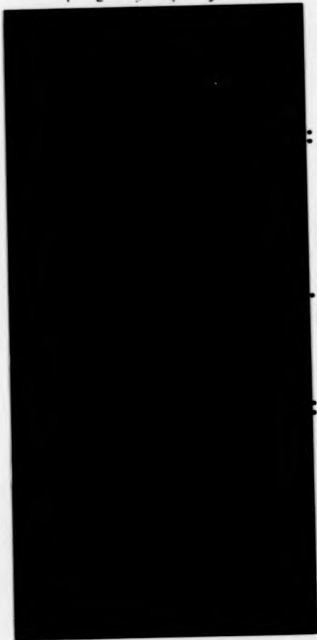
PAGE of a mixture of Maguari virus and Bunyamwera virus after dilution and culturing of virus from the dilution series.

- 1 Maguari ts<sup>+</sup>
- 2 10<sup>-3</sup> sample
- 3 10<sup>-4</sup> sample
- 4 10<sup>-5</sup> sample
- 5 10<sup>-6</sup> sample
- 6 10<sup>-7</sup> sample

HKV cell lysates radiolabelled by pulse labelling from 18 - 19 hours after infection.

10% polyacrylamide SDS gel exposed without fluorography for 3 days.

1 2 3 4 5 6



Mag G1  
Run G1

Actin

Mag N  
Run N

present for which there are no discriminating cDNA probes.

Figure 22 shows the result obtained when cytoplasmic extracts prepared from the amplified samples were analysed with pBUN 84(L RNA) and pBUN 133(M RNA). Bunyamvera L and M RNA were only detected from the  $10^{-7}$  dilution sample of the dilution series. The absence of detectable Bunyamvera RNA at  $10^{-5}$  -  $10^{-6}$  coincided with the absence of detectable labelled Bunyamvera N and G1 proteins at these dilutions.

The two mixed preparations - 13.48.1 + 13.48.6, and Maguari + Bunyamvera virus - behaved in a similar way in these experiments. 13.48 was different from them in that at  $10^{-7}$  both N proteins were labelled despite a reduction in the labelling of Bunyamvera N protein up to that sample. In these experiments, virus amplification from serial dilutions by growth in cells was used and so different moi would have been used in inoculating the cells to obtain the samples for radiolabelling. Any differences in the growth rate between Maguari and Bunyamvera virus would have been exaggerated by this procedure. However, Iroegbu (1982) found no detectable difference in the growth rates of the two viruses as determined by one step growth curves in HEK cells at  $31^{\circ}\text{C}$  which was the temperature of incubation in these dilution experiments.

## 2c The effect of infecting cells with different proportions of Maguari and Bunyamvera viruses.

In order to find out if there were any effects arising from different multiplicities of infection inherent

Figure 22

Dot hybridisation analysis of samples derived from a mixture of

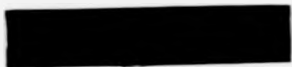
Mequari virus and Bunyamvera virus.

Samples obtained after passage in HEK cells of dilution indicated.

Arrangement of samples (duplicate)

Sam ts <sup>+</sup>	Mag ts <sup>+</sup>	10 <sup>-5</sup>	10 <sup>-4</sup>
10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	

Panel A: pHEM 84 (L RNA probe)



Panel B: pHEM 133 (M RNA probe)

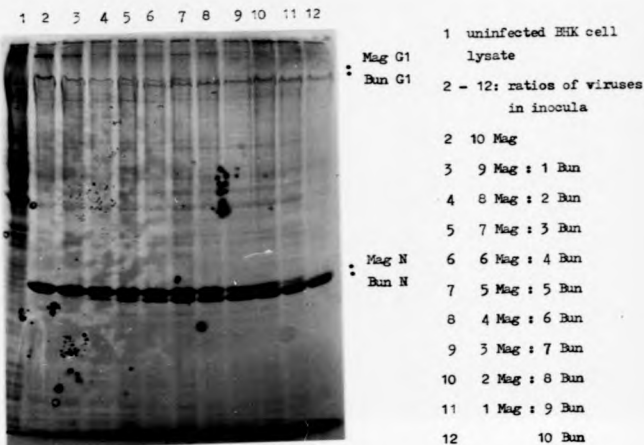


in the experiments described in 2a and 2b, the following experiment was done. A preparation of each of the two wild type viruses was diluted with growth medium so that the concentration for each virus in terms of pfu was the same. Eleven mixtures were then prepared in which different proportions of Maguari and Bunyamwera virus were combined so that the final volume was identical in each case and consequently each mixture contained the same number of pfu. The virus concentration chosen -  $1 \times 10^8$  pfu per ml - was such that a moi of 10pfu per cell could be achieved when 0.2ml of these mixtures was used to inoculate HEK cells -  $2 \times 10^6$  cells per dish - for protein labelling without any need for amplification of virus by passage in HEK cells (as had been used earlier with the dilution series samples). An autoradiograph of the gel is shown in Figure 23 and from this gel pieces corresponding to Maguari and Bunyamwera N proteins were cut out and counted for  $^{35}\text{S}$ -radioactivity in scintillation fluid. A graph of these figures is shown in Figure 24 and they are tabulated in Table 25. The graph shows that proportionately less radioactivity was incorporated into Bunyamwera N protein than Maguari N protein when mixtures of the same relative proportions of virus are compared. As indicated in Table 25, when 80% of the inoculating pfu are Maguari virus, 90% of the cpm incorporated into N proteins is of Maguari N protein and only 10% rather than 20% of the cpm is present in Bunyamwera N protein. Similarly, when only 10% of the inoculum is Maguari, 30% of the N protein cpm are found in the Maguari N protein. With 100% inoculum for each virus, similar cpm is incorporated into each N protein. The



Figure 23

PAGE of proteins radiolabelled in HEK cells after high multiplicity mixed infections with Maguari and Bunyamwera viruses.



HEK cell lysates radiolabelled at 18 - 19 hours after infection.  
10% polyacrylamide gel exposed 4 days.

Table 25

Proportions of cpm in Maguari and Bunyamwera N proteins after high multiplicity mixed infections.

Ratio Mag: Bun in inoculum	Mag N cpm	Bun N cpm	% N cpm in Mag N	% N cpm in Bun N
10 : 0	5850	-	100	0
9 : 1	5550	260	96	4
8 : 2	5240	570	90	10
7 : 3	4550	830	85	15
6 : 4	4080	1320	76	24
5 : 5	3310	1925	63	37
4 : 6	2760	2210	56	44
3 : 7	1870	2250	45	55
2 : 8	1650	2800	37	63
1 : 9	1400	3200	30	70
0 : 10	-	5370	0	100

Figure 24

Relative proportions of cpm incorporated into Maguari and  
Bunyawera N proteins in mixed infections.

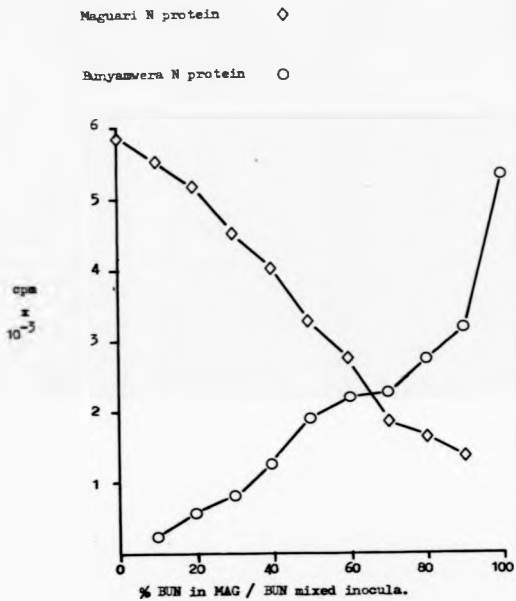


figure obtained at 100% for Bunyamwera virus is 91% of that obtained for Maguari virus (presumably the 9% difference is due to experimental error) and even considering this variability it is still apparent that the Maguari N protein is dominating over expression of the Bunyamwera N protein. Figure 23, which shows the autoradiograph of the gel from which these numbers were derived, indicates that a similar pattern occurs with the G1 proteins but these were too close together to be cut out with accuracy. Thus it appears that Maguari virus interferes slightly with the expression of Bunyamwera virus proteins - even in high multiplicity infections.

**Discussion: Interference of Maguari virus with Bunyamwera virus.**

The results described, obtained from examining mixtures of viruses and the "mixed clone" 13.48, show that Maguari virus can interfere with the growth of Bunyamwera virus. In the experiments involving dilution series of mixtures (Figures 20, 21 and 22) Bunyamwera proteins (N and G1) and RNA segments (L and M) were only detected at high levels in the preparations derived from the end of the dilution series. The dominant expression of Maguari components over Bunyamwera components was observed consistently in these experiments. Also, the experiment using high multiplicity mixed infections (Results Section 2c) shows that an initial period of replication of the mixture is not necessary for the phenomenon of Maguari virus dominance to occur. The inhibition of Bunyamwera virus in

Bunyamvera/Maguari virus mixed infections is consistent with the predominance of Maguari virus parental clones isolated from genetic crosses and the paucity of Bunyamvera virus clones (Results Section 1a - c).

A possible explanation for the suppression of Bunyamvera by Maguari virus could be differences in the kinetics of replication of the two viruses. If Maguari virus replicated faster than Bunyamvera virus, it would be preferentially amplified in the experiments in which dilution samples were passaged in BEK cells before analysis. As mentioned, Iroegbu (1982) showed that there was no detectable difference between the growth curves of Maguari and Bunyamvera viruses in BEK cells although this method would not detect slight differences.

The behaviour of 13.48 was different from that of the virus mixtures. When the virus was diluted to extinction, expression of both Maguari and Bunyamvera N proteins was seen although the amount of Bunyamvera N relative to Maguari N protein progressively decreased up to that point. The experiments with mixtures of Maguari virus genotypes with either Bunyamvera virus or recombinant virus of Mag / Mag / Bun genotype indicate that Maguari virus genotypes tend to dominate. There is a certain similarity between these results and those obtained with 13.48 although there are important differences as indicated.

As Maguari virus genotypes tend to dominate over Bunyamvera virus in the experiments in which mixtures are grown together, it is reasonable to propose that the more rounds of replication a mixed inoculum undergoes, the greater the likelihood of

extent of dominance by the Maguari virus genotype. Following on from that, the lower the moi of such a mixture, the greater the number of rounds of replication and hence the lower the proportion of Bunyamwera products relative to Maguari products. Maybe this is what has occurred in these experiments involving dilution of mixtures of viruses as there is a progressive reduction in the amount of Bunyamwera virus products with increasing dilution (and therefore decreasing moi). At the dilution end points, only a very small number of pfu will have been inoculated onto the BHK cells and if they were all of one genotype no suppression could occur and either only Maguari genotype or the Bunyamwera virus or the reassortant would be produced. In the experiments with the virus mixture dilution series, this may be what has happened as the only virus products detected at the end points were of Bunyamwera type, although no or progressively reduced amounts of those products had been seen up to that sample.

It seems likely that the phenomenon of suppression occurs at the level of at least mRNA transcription, as Figure 22 shows no detectable Bunyamwera RNA in the  $10^{-5}$  -  $10^{-6}$  samples. The dot hybridisation method detects both positive and negative sense RNA and does not discriminate between them, as cDNA probes are used. As viral mRNA tends to be most abundant in infected cells, it is probably hybridisation with this which is shown by this method. The Bunyamwera genome segments may be replicated but expression of the gene products may be suppressed. Strand-specific probes would allow this

possibility to be investigated.

Discussion: 13.48 is not a simple mixture of viruses.

The results described for 13.4<sup>P</sup> were unique as large amounts of both N proteins were present at the extinction point, and also the degree of reduction in Bunyamvera N protein relative to Maguari N protein throughout the dilution series was less pronounced than for the mixtures. Clones of three types can be isolated from 13.48: Maguari virus genotype, Mag / Mag / Bun reassortant and Mag / Mag / Mag-Bun reassortant and from the small number of isolates examined (Results Section 1b, Table 15), the proportion of the Mag / Mag / Mag-Bun reassortant was greater than either of the other two, suggesting that this genotype may be fairly stable. Thus three different genotypes possibly in different proportions are present in 13.48 and therefore in the dilution series. As they are probably present in different proportions, at the extinction point it is likely that all the infecting virus will be of the type present in the highest proportion in the original virus preparation. If that was Mag / Mag / Mag-Bun then perhaps this could allow both S RNA segments to be propagated and expressed together and thereby give rise to the result seen. Although this Mag / Mag / Mag-Bun reassortant may give rise to both Mag / Mag / Mag and Mag / Mag / Bun genotypes among the progeny, if it tended to produce more of itself than either of the other two progeny types, then insufficient

Maguari virus genotype might be present which could otherwise interfere with the replication of virus containing Bunyamwera S RNA segments. This might also account for the less pronounced suppression of Bunyamwera N protein seen in the dilution series compared with that observed for the virus mixtures. This suggests that the Mag / Mag / Mag-Bun genotype is unique and different from a simple mixture of Maguari virus genotype and a reassortant and is a much more complex association of the genome segments.

It has been reported by Elliott and Wilkie (1985) that defective Bunyamwera virus, in which only S RNA can be detected, can be formed in a mosquito cell line (Aedes albopictus C6/36). Clone 13.48 may represent a mixture of standard Maguari virus and such a defective Bunyamwera virus. However, 13.48 and its subclones all grew to high titres ( $10^7 - 10^8$  pfa per ml) which indicates that defective interfering virus is not present in large amounts, or that it does not interfere with heterologous Maguari virus. Alternatively, 13.48 may represent a heterozygote in which two different forms of the same genes (the S RNA of Maguari and Bunyamwera viruses) are present. Also, it is interesting to note that isolates of mixed phenotypes for G1 and N proteins were made from most of the crosses which yielded reassortants described in Results Section 1a - c (Crosses 2, 4, 5 and 10), involving all three Maguari virus ta mutants. It was presumed that these were simple mixtures of two or more viruses but further investigation may possibly indicate a more complex situation - as was found for 13.48 -



for some of these isolates.

There is an example of heterozygosity in a negative strand segmented genome RNA virus. Romanowski and Bishop (1983) reported that genetic crosses of certain Arenaviruses can give rise to genetically diploid viruses, but these heterozygotes were found to be unstable. The molar ratios of the two RNA segments of Arenaviruses - like bunyviruses (Introduction 3a) - are often far from equal (Murphy & Whitfield, 1975). Maybe bunyviruses also produce virions containing multiple copies of some viral nucleocapsids and this can only be detected when heterologous genome segments are present.

Results Section 3: Properties of Group II Revertants  
of altered G1 electrophoretic mobility.

It has been observed that reversion of Maguari virus Group II ts mutants to ts<sup>+</sup> phenotype is almost always accompanied by a change in the electrophoretic mobility of the G1 protein - electrophoretic variation (EV) (Elliott *et al.*, 1984; Watret *et al.*, 1985; Pringle, unpublished observations). These variants in G1 appeared to fall into two size classes, referred to here as EV1 and EV2. No such variants were seen with revertants of Group I or Group III mutants. For this reason it has been suggested that reversion of the ts phenotype occurs by intragenic suppression.

The viruses Revertant 1 and Revertant 2 were isolated as revertants of Mag ts 8(II) to ts<sup>+</sup> phenotype as described in Materials 22. It had been noted that the electrophoretic mobility of the G1 protein was substantially altered - by different amounts - in these two mutants, and that they were representative of the two size classes of electrophoretic variants.

Figure 25 shows the polypeptides of Revertant 1 and Revertant 2 in comparison with wild type Maguari virus and Mag ts 8(II). On this occasion it was not possible to visualize G1 of Mag ts 8(II) but as shown in Appendix 4 the G1 protein produced by Mag ts 8(II) is indistinguishable from that of wild type virus by PAGE. From the gel shown in Figure 25 it was deduced that wild type Maguari virus G1 (and therefore G1 of Mag ts 8(II) ) has apparent molecular weight 110,000 Mwt; Revertant 1 has a protein EV1

Figure 25

PAGE of radiolabelled proteins to compare Maguari virus wild type, Mag ts 8(II), Revertant 1 and Revertant 2.

- 1 Mock infected HEK cells
- 2 Maguari ts<sup>+</sup>
- 3 Mag ts 8(II)
- 4 Revertant 1
- 5 Revertant 2
- 6 Revertant 1

Molecular weights determined by Coomassie blue staining of molecular weight markers.

HEK cell lysates radiolabelled by pulse labelling from 18 - 19 hours after infection.

10% polyacrylamide SDS gel exposed without fluorography for 4 days.

Mwt.

1 2 3 4 5 6

116 K ▶

97 K ▶

66 K ▶

45 K ▶

29 K ▶

• L protein

• G1

• EV1

• EV2

• Actin

• M

of 80,000 Mwt; Revertant 2 has a protein EV2 of 60,000 Mwt. The ts lesion in Mag ts S(II) has been assigned to the genome segment encoding G1 (Results Section 1b), so the nature of the changes in G1 associated with reversion in these mutants was examined.

There are several possible mechanisms which could result in a reduction in the molecular weight of such a gene product. These include deletion of nucleotide sequences from the genome, synthesis of a truncated mRNA from a full length gene, premature termination of mRNA translation and modified processing of the protein once synthesized.

### 3a Analysis of glycosylation.

The mode and extent of glycosylation of G1, EV1 and EV2 were examined using various inhibitory drugs (as described in Method C9) to determine whether the difference in molecular weight of the three proteins was a consequence of differences in the amount of carbohydrate attached to a core protein. Figure 26 shows the effect of monensin at  $10^{-6}M$ . Another drug CCCP (see Materials 7), which also inhibits processes occurring in the Golgi (Morrison et al., 1985), at a concentration of  $3 \times 10^{-6}M$  gave the same result as shown in Appendix 5. As neither of these drugs affected the electrophoretic mobilities of G1, EV1 or EV2, and as the drugs inhibit processes occurring in the Golgi rather than the endoplasmic reticulum

Figure 26

PAGE of immunoprecipitated proteins to show the effects of tunicamycin and monensin on Maguari virus wild type, Revertant 1 and Revertant 2.

1	2	3	Mock infected HEK cells
4	5	6	Maguari ts <sup>+</sup>
7	8	9	Revertant 1
10	11	12	Revertant 2

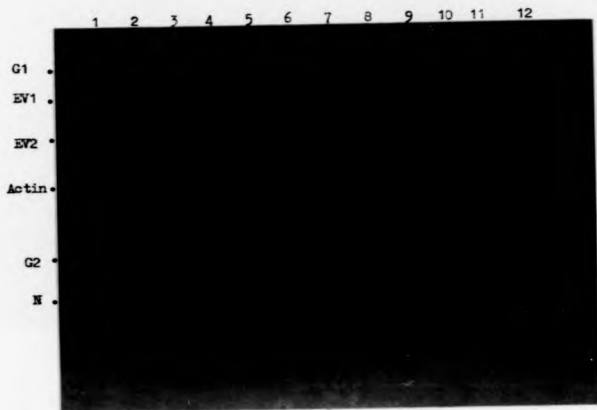
2, 5, 8 & 11 Tunicamycin @  $5 \mu\text{g ml}^{-1}$

3, 6, 9 & 12 Monensin @  $10^{-6}\text{M}$

Cells pretreated with drugs as indicated for 0-5 hours before radiolabelling at 18 - 19 hours after infection.

Immunoprecipitated with antiBunyavirus serum.

10% polyacrylamide SDS gel fluorographed with Amplify and exposed for 10 days.



(introduction 4), it was concluded that the carbohydrate components were not O-linked. Figure 26 also shows the effect of tunicamycin and this is shown more clearly in Figure 27 in which a 7% polyacrylamide gel was used to obtain better resolution of the high molecular weight proteins. Tunicamycin had a significant effect on the molecular weights of G1, EV1 and EV2, and also on G2 (shown in Panel B). The drug was incubated with the infected cells for only 1.5 hours in total (0.5 hour during pretreatment and 1 hour during radiolabelling), at a concentration of  $5\mu\text{g ml}^{-1}$ , so both glycosylated and unglycosylated forms of the glycoproteins are seen. The mobility difference in the case of G1 is equivalent to approximately 6,000 Mwt; the differences in the cases of EV1 and EV2 are 4,000 Mwt and 2,000 Mwt respectively. The difference in the case of G2 is about 2,000 Mwt. This indicates that the carbohydrate on all these glycoproteins is N-linked and that the difference in molecular weight between G1, EV1 and EV2 is not a consequence of the carbohydrate component although they are glycosylated to different extents.

**Discussion: Glycosylation of G1, EV1 and EV2.**

The extent and mode of glycosylation of Maguari G1, EV1 and EV2 were examined in order to determine whether differences in the carbohydrate moieties were responsible for the differences in molecular weight between the three proteins. Mutations affecting the glycosylation of the proteins of other negative



Figure 27

Effect of tunicamycin on Maguari virus wild type, Revertant 1 and Revertant 2: PAGE of immunoprecipitated proteins.

Panel A .

1 & 2 Maguari ts<sup>+</sup>

3 & 4 Revertant 1

5 & 6 Revertant 2

2, 4 & 6 Tunicamycin @  $5 \mu\text{g ml}^{-1}$

Panel B.

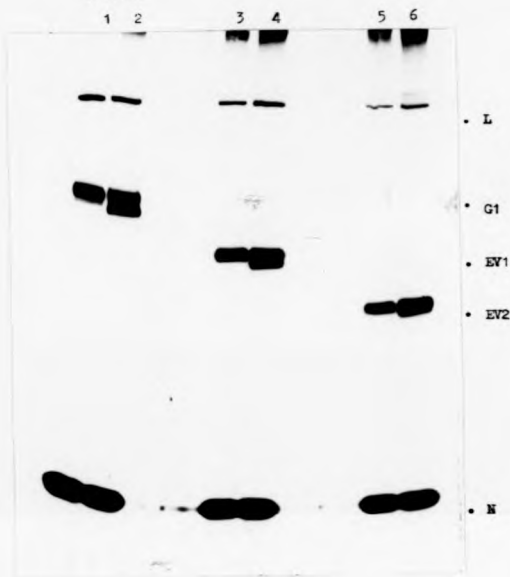
Longer exposure of the lower part of Panel A.

Cells pretreated with tunicamycin for 0.5 hours before radiolabelling at 18 - 19 hours after infection.

Immunoprecipitated with antiBunyavirus serum.

7% polyacrylamide SDS gel fluorographed with Amplify and exposed for 4 days (panel A) and 10 days (panel B).

Panel A



Panel B



sense RNA viruses have been reported - for example, rabies virus (Wunner *et al.*, 1985), vesicular stomatitis virus (Zilberstein *et al.*, 1980) and influenza A viruses (Palese, 1978; Lohmeyer & Klank, 1979). For some virus glycoproteins, such as G protein of respiratory syncytial virus, it has been shown that the carbohydrate moiety can account for up to 50% of the molecular weight of the mature glycoprotein (Gruber & Levine, 1985). Thus, it was possible that Revertant 1 and Revertant 2 represented mutants in the glycosylation process. The results presented, however, indicate that this is not the explanation of the extreme differences in molecular weight associated with these mutations.

As for most virus glycoproteins, the carbohydrate moieties were found to be only N-linked and using inhibitors, no evidence of O-linkage - which would take place in the Golgi - was seen (Figure 26). In the presence of tunicamycin, which inhibits N-linked glycosylation, Maguari G1 was seen to increase in mobility equivalent to a loss of 6,000 Mwt. EV1 by an amount equivalent to 4,000Mwt and EV2 by 2,000 Mwt. The G2 proteins in all three cases were identical and lost 2,000 Mwt in the presence of the drug (Figure 27). Thus, the G1 of Maguari virus appears to have three times as much carbohydrate as EV1, and EV1 has twice as much as EV2. However, these differences are not sufficient to account for the total difference in molecular weight between the three glycoproteins.

It is interesting to note that the core oligosaccharides indicated by Stanley (1984) as involved in glycoprotein biosynthesis

are of the order of 2,000 - 3,000 Mwt. The carbohydrate on G1 and G2 totals 8,000 Mwt; if the component on G2 is considered to represent "one unit" of carbohydrate (that is 2,000 Mwt), a total of 4 units of carbohydrate are present. In the same way, EV1 has 2 units and EV2 has 1 unit. This implies that the polyprotein precursor encoded by Maguari M RNA has at least 4 occupied glycosylation sites. Lees *et al.* (1986) have identified 4 possible glycosylation sites in Bunyamwera M RNA message, and Vorndam and Trent (1979) have identified 3 - 6 possible sites in viruses of the California encephalitis serogroup. Comparison of the nucleotide sequences of the M RNA of Bunyamwera and LaCrosse viruses (Lees *et al.* 1986) showed that there was considerable conservation of glycosylation sites between the two viruses, so there is likely to be great similarity in the glycosylation sites of two more closely related viruses such as Bunyamwera virus and Maguari virus. The data presented here suggest that the 4 glycosylation sites identified for Bunyamwera virus by sequence analysis of the M RNA may all be utilised.

### 3b Pulse-chase analysis.

Pulse-chase experiments were carried out to determine whether EV1 and EV2 were formed as a result of processing of G1 after synthesis. BHK cells infected with Maguari virus wild type, Revertant 1, or mock-infected with PBS, were radiolabelled with  $^{35}\text{S}$ -methionine and chased with unlabelled

methionine. At 18 hours after infection cells were incubated with medium containing  $200\mu\text{Ci ml}^{-1}$  of  $^{35}\text{S}$ -methionine (four times the normal concentration) for ten minutes. During this pulse, proteins being synthesized would be labelled. The radiolabel which had then been incorporated in proteins during that pulse was then "chased" into any processed products by incubation in a medium containing ten times the normal amount of unlabelled methionine ( $3\text{mM}$  instead of  $0.3\text{mM}$ ). The monolayers were rinsed first in this medium to remove excess radiolabel and then chased for 0 minutes, 10 minutes or 20 minutes, before lysis in gel sample buffer and electrophoresis of proteins as usual (Method E2). During the chase period it was expected that the fate of the proteins heavily labelled during the pulse could be followed without interfering effects from further radio-labelled proteins.

The autoradiograph shown in Figure 28 provides no evidence of processing of G1 into either EV1 or EV2, or of EV1 into EV2, nor any suggestion of a precursor for either G1 or EV1. There is a slight increase in the electrophoretic mobilities of both G1 and EV1 during the chase period, and this may represent cleavage of the glycoprotein signal peptide and processing of the carbohydrate moiety which are both associated with the biosynthesis of glycoproteins.

Discussion: No precursors to the glycoproteins could be detected by pulse-chase analysis.

The M RNA of bunyaviruses has been shown to encode three

Figure 28

PAGE of pulse - chase radiolabelled proteins of Maguari  
virus wild type and Revertant 1.

1, 4 & 7 Maguari ts<sup>+</sup>

2, 5 & 8 Revertant 1

3, 6 & 9 Mock infected HEK cells

1, 2 & 3 0 minute chase

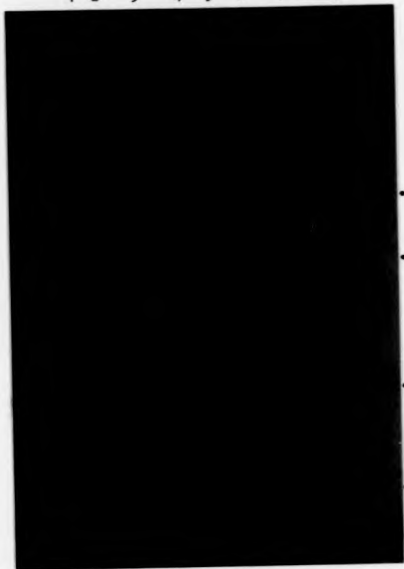
4, 5 & 6 10 minute chase

7, 8 & 9 20 minute chase

HEK cell lysates radiolabelled by 10 minute pulse at 18  
hours after infection.

7% polyacrylamide SDS gel exposed overnight without fluorography.

1 2 3 4 5 6 7 8 9



• G1

• EV1

• Actin

• N

products, G1, G2 and NSm (Introduction 3b) and the evidence obtained so far indicates that these are all synthesized from a single large open reading frame transcribed into a single large mRNA. Thus it is anticipated that a precursor would be seen in pulse-chase experiments. However, the data presented here are consistent with the results obtained by others (Introduction 4d) in that no such precursors could be identified. This general failure to detect such proteins is thought to suggest that the proteins G1, G2 and NSm are formed by cotranslational cleavage of the putative precursor and that this occurs so rapidly that it escapes detection (Cash *et al.*, 1980; McPhee & Westaway, 1981; Pennington *et al.*, 1977; Ihara *et al.*, 1985). The data shown here suggest that EV1 and presumably EV2 are formed in a similar way.

### 3c In vitro translation of RNA from Revertants 1 and 2.

Cytoplasmic RNA preparations from BHK cells mock-infected, and infected with wild type Maguari virus, Revertant 1 and Revertant 2, were used to programme in vitro translation reactions using rabbit reticulocyte lysate and canine pancreatic microsomal membranes, as described in Method C5. If the M RNAs of the three viruses encoded polypeptides different in size - either as a result of genome deletions or synthesis of truncated mRNAs or premature termination of mRNA translation - then proteins of different sizes would be made in a translation



system using reticulocyte lysate. If the differences in the size of G1, EV1 and EV2 are due to processing after translation, then it was possible that this might be detected as differences which only became apparent when the translation reaction was carried out with the addition of canine pancreatic microsomal membranes.

The in vitro translation reactions were carried out as described in Method C6 in the presence and absence of microsomes, and analysed by PAGE with and without immunoprecipitation with anti-Rubymvera virus serum. The autoradiographs of these gels are shown in Figures 29 and 30. A sample from HEK cells infected with wild type Maguari virus was included on each gel as a size indicator for viral proteins. Also included in the gels are samples from reactions to which no RNA was added in order to control for endogenous protein synthesis by the reticulocyte lysate system in the presence and absence of microsomes.

Figure 29 shows that in samples programmed with infected cell RNA and mock-infected cell RNA, a major protein the size of M protein was synthesized and no proteins of molecular weight comparable to L, G1, EV1, EV2 or G2 were seen. The presence of microsomes appeared to have a quantitative rather than a qualitative effect on translation, and has reduced the amount of radiolabelled protein rather than induced changes in the proteins synthesized. Figure 30 shows the same samples as those in Figure 29 after immunoprecipitation. The size indicator sample of an in vivo radiolabelled Maguari virus

Figure 29

In vitro translation products of RNA from Maguari virus wild type, Revertant 1 and Revertant 2 analysed by PAGE.

Reaction	Microsomes
1 Maguari ts <sup>+</sup> lysate control	-
2 no RNA	-
3 no RNA	+
4 Mag ts <sup>+</sup> RNA	-
5 Mag ts <sup>+</sup> RNA	+
6 Revertant 1 RNA	-
7 Revertant 1 RNA	+
8 Revertant 2 RNA	-
9 Revertant 2 RNA	+
10 Mock infected BHK RNA	-
11 Mock infected BHK RNA	+

7 % polyacrylamide gel fluorographed with Amplify and exposed for 3 days.

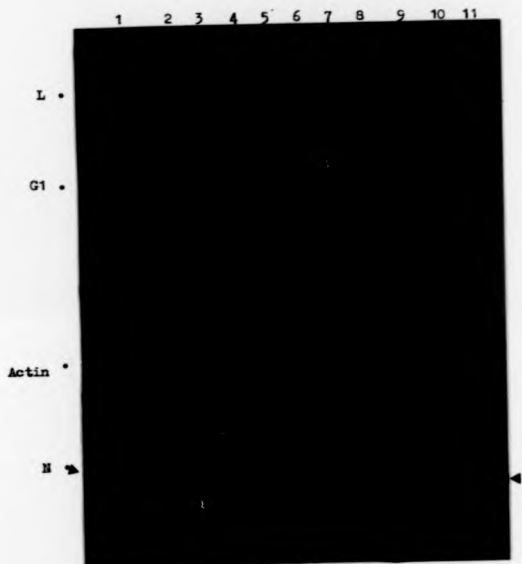


Figure 30

In vitro translation products of RNA from Maguari virus wild type, Revertant 1 and Revertant 2 analysed by PAGE of immunoprecipitated proteins.

	Reaction	Microsomes
1	Maguari ts <sup>+</sup> lysate control	
2	no RNA	-
3	no RNA	+
4	Mag ts <sup>+</sup> RNA	-
5	Mag ts <sup>+</sup> RNA	+
6	Revertant 1 RNA	-
7	Revertant 1 RNA	+
8	Revertant 2 RNA	-
9	Revertant 2 RNA	+
10	Mock infected HEK cell RNA	-
11	Mock infected HEK cell RNA	+

Immunoprecipitated with antiBunyavirus serum

7 % polyacrylamide SDS gel fluorographed with Amplify and exposed for 7 days.

1 2 3 4 5 6 7 8 9 10 11

L •

G1 •

Actin •

N •

infected cell lysate was not immunoprecipitated. The only protein which has been precipitated from the infected cell translation reactions - and not from the mock-infected or RNA free reactions - is a proteins similar in size to N protein. Thus, no M RNA products were detected in these experiments, or indeed any virus protein other than N protein.

Discussion: No M RNA products detected by in vitro translation.

The observation that an N protein-like product could be synthesized using an in vitro translation system and the failure to detect any proteins from the M RNA segment is consistent with the observations of others. Previous attempts to translate mRNA from the M RNA segment of bunyaviruses in vitro have been unsuccessful (Abraham & Pattnaik, 1983; Elliott, 1985) although Ulanen et al. (1981) were able to translate mRNA from the M RNA of the unkuvirus Uukuniemi virus, and identified a precursor of G1/G2. The reason for the failure of certain bunyavirus mRNAs to be translated in vitro is not known but may be due to secondary structures of the mRNAs - it is known that RNA molecules can have complex <sup>topographies</sup> and may therefore require specific conditions to allow translation to occur.

3d                      Analysis of G1, EV1 and EV2 by proteolysis in  
polyacrylamide gels.

Proteolysis of G1, EV1 and EV2 was performed as described in Method C7 with different proteases as indicated. As discussed (Introduction 4) it is thought that the M RNA products arise by nascent proteolytic cleavage. These experiments were performed to look for evidence of cleavage sites in G1 and EV1 when denatured in the conditions of proteolysis, which could produce proteins of the size of EV1 and EV2. The method employed is more usually used to indicate protein similarity, as related proteins give similar cleavage patterns with a given protease and dissimilar patterns are obtained with unrelated proteins (Cleveland et al., 1977). However, as the proteins considered here are of different sizes and are also glycosylated to different extents (this section, 3a) a high level of similarity in cleavage patterns would not be expected, although the proteins are probably all derived from a common protein.

Figure 31 shows the effect of Staphylococcus aureus strain V8 protease on G1, EV1 and EV2. This enzyme has produced 7 - 12 labelled fragments of different sizes from each of the three proteins but there is no suggestion of fragments of the sizes of EV1 or EV2 from G1, or of EV2 from EV1. As shown in Table 26, each protein has four fragments in common with any one other and overall three bands are shared. This suggests that the proteins are not dissimilar in origin.

The result obtained with chymotrypsin for EV1 and EV2 is shown in Figure 32. This also shows the effect of chymotrypsin

Table 26

Number of products seen when G1, EV1 and EV2 were treated with S. aureus V8 protease in PAGE.

Number of bands		Number comigrating with			
		G1	EV1	EV2	All 3
G1	7	7			
EV1	8	4	8		3
EV2	12	4	4	12	

Table 27

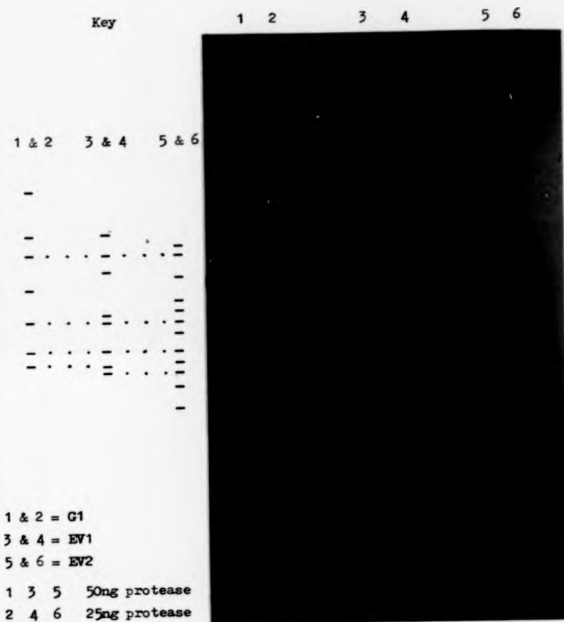
Number of products seen when H proteins of Maguari virus, Revertant 1 and Revertant 2, and EV1 and EV2 were treated with chymotrypsin in PAGE.

Number of bands		Number comigrating with			
		Mag H	Rev 1 H	Rev 2 H	All 3
Mag H	5	5			
Rev 1 H	5	5	5		5
Rev 2 H	5	5	5	5	
			EV1	EV2	Both
EV1			14		6
EV2			6	12	



Figure 31

Proteolysis in a polyacrylamide gel of Maguari G1, EV1 and  
EV2 with Staphylococcus aureus V8 protease.

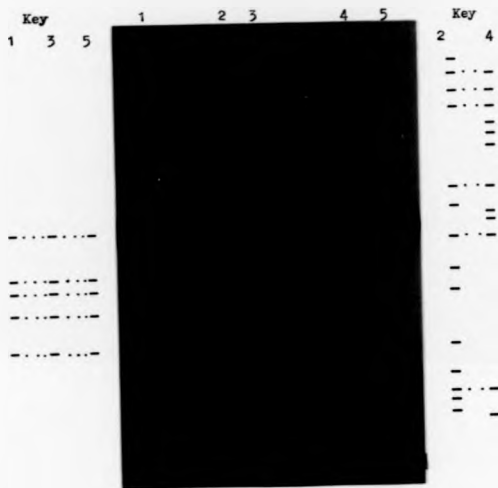


Broken lines in accompanying key indicate common bands between tracks.

10% polyacrylamide gel fluorographed with Amplify and exposed for 10 days.

Figure 32

Proteolysis in a polyacrylamide gel of EV1 and EV2, and H proteins of  
Maguari virus wild type, Revertant 1 and Revertant 2 with chymotrypsin.



1 = Maguari wild type H  
2 = EV1  
3 = Revertant 1 H  
4 = EV2  
5 = Revertant 2 H

chymotrypsin at 25 $\mu$ g per track.

Broken lines in keys indicate common bands between tracks.

10% polyacrylamide gel fluorographed with Amplify and exposed  
for 10 days.

proteolysis on the N proteins of wild type Maguari virus, Revertant 1 and Revertant 2, which are indistinguishable by PAGE of denatured, disassociated radiolabelled proteins (see Figures 25 and 26 for examples). As shown in Table 27, the cleavage patterns obtained for these N proteins are also identical and so presumably the proteins are very similar or identical. In contrast, only about half (6 out of 12 and 14) of the fragments produced with EV1 and EV2 are common and no fragments equivalent in size to EV2 were produced from EV1.

Proteolysis with trypsin was then examined and a much more limited cleavage of G1, EV1 and EV2 was produced (Figure 33, Table 28). Only 3 or 4 bands were seen for each protein and none of these was common to all three patterns. However, a major fragment the size of EV1 was produced from G1 and a fragment the size of EV2 was produced from EV1. The proteases endoproteinase lys C - shown in Figure 34, Table 29 - and endoproteinase arg C - Figure 35, Table 30 - gave very similar results to trypsin, with a fragment the size of EV1 from G1 and a fragment the size of EV2 from EV1.

Discussion: In vitro proteolysis of G1, EV1 and EV2.

Previous work has shown (Kingsford & Hill, 1983) that G1 of LaCrosse virus in intact virus particles had limited susceptibility to certain proteases, and trypsin and chymotrypsin produced large cleavage products. The proteins G1

Table 28

Number of products seen when G1, EV1 and EV2 were treated  
with trypsin in PAGE.

Number of bands		Number comigrating with			
		G1	EV1	EV2	All 3
G1	3	3			
EV1	4	1	4		0
EV2	3	0	1	3	

Table 29

Number of products seen when G1, EV1 and EV2 were treated  
with endoproteinase lys C in PAGE.

Number of bands		Number comigrating with			
		G1	EV1	EV2	All 3
G1	5	5			
EV1	4	1	4		0
EV2	3	0	1	3	

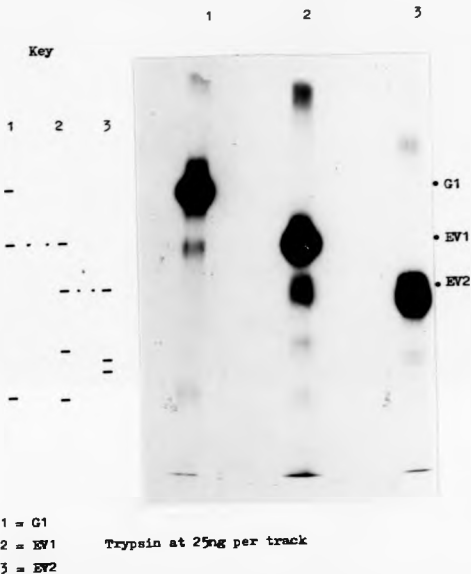
Table 30

Number of products seen when G1, EV1 and EV2 were treated  
with endoproteinase arg C in PAGE.

Number of bands		Number comigrating with			
		G1	EV1	EV2	All 3
G1	5	5			
EV1	4	1	4		0
EV2	5	0	1	5	

Figure 33

Proteolysis in a polyacrylamide gel of Maguari G1, EV1 and EV2  
with trypsin.

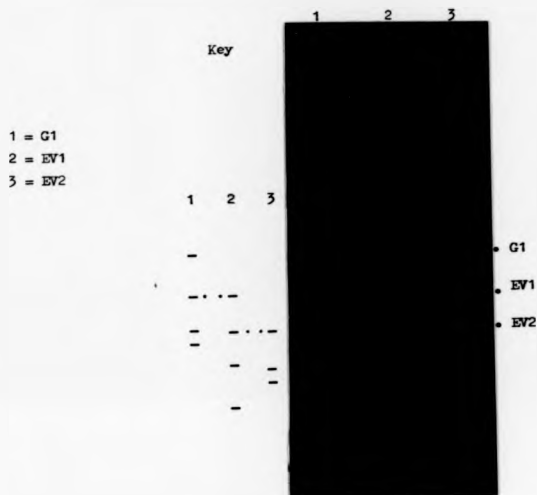


Broken lines in key indicate common bands between tracks.

7% polyacrylamide gel fluorographed with Amplify and exposed  
6 days.

Figure 34

Proteolysis in a polyacrylamide gel of Maguari G1, EV1 and  
EV2 with endoproteinase lys C.



Endoproteinase lys C at 25ng per track.

Broken lines in key indicate common bands between tracks.

7% polyacrylamide gel fluorographed with Amplify and exposed 10 days.

Proteolysis in a polyacrylamide gel of Maguari C1, EV1 and EV2 with endoproteinase arg C.



Broken lines in key indicate common bands between tracks.

7% polyacrylamide gel fluorographed with Amplify and exposed for 10 days.

(Maguari virus), EV1 and EV2 were therefore examined directly by proteolysis in polyacrylamide gels. Proteolysis with trypsin suggests that EV1 and EV2 may be formed as a result of specific proteolysis (Murphy & Pringle, 1987). Trypsin is specific for lysine and arginine residues, so proteases with a preference for either lysine or arginine were used to try to identify the amino acids involved in cleavage. However, the results obtained with these proteases (Figures 34 and 35) were similar to that obtained for trypsin. This may be because these enzymes are less specific in their action than trypsin (Neurath *et al.*, 1967) or because the cleavage involved in production of these specific fragments involves both lysine and arginine residues.

The information obtained in these experiments suggest that in the denaturing conditions of SDS/PAGE, there is a trypsin-sensitive site in G1 which can result in a polypeptide the size of EV1, and a site in EV1 which can give rise to a polypeptide the size of EV2. If the mechanism by which EV1 and EV2 are formed in vivo does involve exposure of these protease sensitive sites, it would be expected that cleavage fragments or precursor proteins would be seen in pulse-labelling and pulse-chase experiments, but under no conditions were any such polypeptides seen. This indicates that if cleavage of a mutant G1 protein is the mode by which EV1 and EV2 are formed, it occurs very rapidly - or cotranslationally - and the fragments other than EV1 and EV2 are unstable and rapidly degraded. If the fragments were relatively stable but contained no or very



few methionine residues they would not be detected by radiolabelling with  $^{35}\text{S}$ -methionine. Examination of the sequences of the closely related Rnyamwera virus M RNA (Lees *et al.*, 1986) indicates that methionine residues are scattered throughout, and so it seems unlikely that large fragments from a closely related virus would be unlabelled.

3e Radiolabelling of G1 and EV1 in the presence of the protease inhibitor TLCK.

As the results of proteolysis in polyacrylamide gels with trypsin on G1 (Maguari virus) and EV1 had indicated that proteolysis might be responsible for the formation of EV1 and EV2, attempts were made to inhibit any such cleavage in vivo using the trypsin inhibitor TLCK and the chymotrypsin inhibitor TPCK. The method used was that described in Method C4 and inhibitor freshly dissolved in 50% v/v DMSO was added to methionine-free GDM at a dilution of at least  $10^{-3}$  to give the concentration required. The infected cells were incubated with inhibitor 30 minutes before radiolabelling with  $^{35}\text{S}$ -methionine for one hour as usual. Figure 36 shows that TLCK at concentrations of  $10^{-6}$  and  $10^{-7}\text{M}$  had no detectable effect on the synthesis of G1 or EV1. The chymotrypsin inhibitor TPCK was used in identical experiments but the total level of protein synthesis was reduced so dramatically that no virus proteins could be detected even with immunoprecipitation.

Figure 36

Effect of TLCK, a trypsin inhibitor, on the synthesis of Maguari virus wild type and Revertant 1 proteins. PAGE of immunoprecipitated proteins.

1, 4 & 7	Mock infected BHK cells
2, 5 & 8	Maguari ts <sup>+</sup>
3, 6 & 9	Revertant 1
1, 2 & 3	no TLCK
4, 5 & 6	10 <sup>-6</sup> M TLCK
7, 8 & 9	10 <sup>-7</sup> M TLCK

BHK cell lysates radiolabelled at 18 - 19 hours after infection.

Pretreated for 0.5 hours before labelling with TLCK at the concentrations indicated.

Immunoprecipitated with antiBunyavirus serum.

7 % polyacrylamide SDS gel fluorographed with Amplify and exposed for 10 days

1 2 3 4 5 6 7 8 9



• G1

• EV1

• Actin

• G2

• H

Discussion: Effect of TLCK on the synthesis of G1 and EV1.

Attempts to inhibit cleavage of G1 into EV1 or of any precursor into its product via trypsin cleavages using the inhibitor TLCK were unsuccessful and so no information on the in vivo formation of EV1 was obtained.

3f The effect of canavanine, an arginine analogue, on G1, EV1 and EV2.

BHK cells in 6-well dishes were infected or mock-infected as usual and incubated in maintenance medium or medium containing canavanine at 3mM (the normal level of arginine in MEM is about 0.3mM) for 17 hours. The cells were then radiolabelled as usual (Method B1), the concentration of canavanine being maintained in the labelling medium. Figure 37 shows the effect of this treatment on Revertants 1 and 2. EV1 and EV2 were not affected by the canavanine but it is interesting to note that in the presence of the drug there is a higher background of host-cell protein synthesis, whereas in mock-infected cells the drug had no apparent effect. This suggests that canavanine may be affecting virus-directed inhibition of host protein synthesis.

As trypsin is specific for the carboxyl bonds in proteins at lysine and arginine residues, it was of interest to examine the effect of trypsin on the proteolysis of G1, EV1 and EV2 synthesized in the presence of canavanine. Figure 38 shows that G1 and EV1 radiolabelled in the presence of the drug are

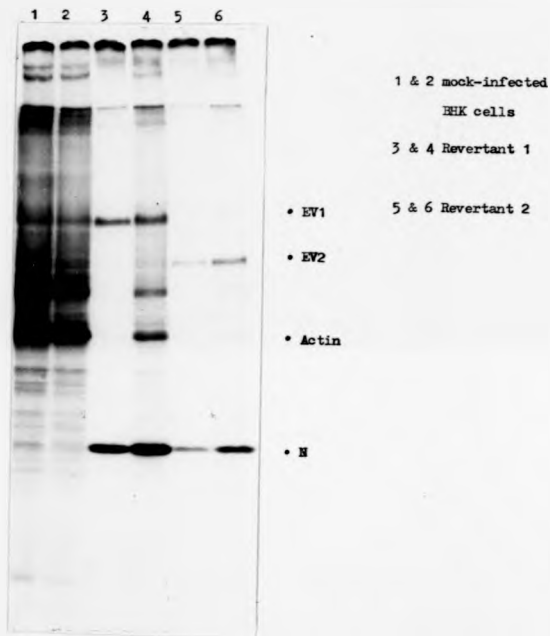
Table 31

Number of products seen when G1, EV1 and EV2 (synthesised + or - canavanine) were treated with trypsin in PAGE.

	Number of bands	Number comigrating with					
		G1	G1+c	EV1	EV1+c	EV2	EV2+c
G1	7	7					
G1 + c	7	7	7				
EV1	5	1	1	5			
EV1 + c	7	1	1	5	7		0
EV2	3	0	0	1	1	3	
EV2 + c	3	0	0	1	1	3	3

Figure 37

Effect of canavanine an arginine analogue on protein synthesis  
by Revertant 1 and Revertant 2 analysed by PAGE.



Samples 2, 4 and 6: Canavanine at 3mM throughout incubation  
and labelling. BHK cell lysates radiolabelled at 18 - 19  
hours after infection. 10% polyacrylamide gel exposed 7 days.

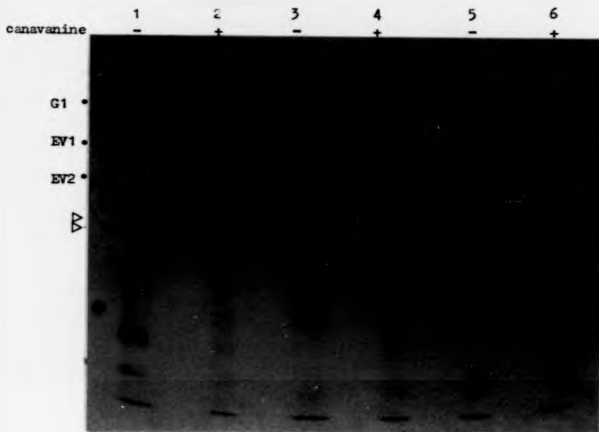
still cleaved such that G1 produces a fragment the size of EV1 and EV1 gives rise to a fragment the size of EV2. There is at least one band in the cleavage profile of EV1 which is absent in the control EV1 made in the absence of canavanine, which suggests that the drug has in some way affected the cleavage of this protein. This is also indicated in Table 31 in which it can be seen that more fragments were produced from EV1 after it had been synthesised in the presence of canavanine. This indicates that at least some canavanine had been incorporated into these proteins.

Discussion:      Synthesis of G1 and EV1 in the presence of  
canavanine.

Experiments using the arginine analogue canavanine were done to affect proteolytic reactions involving arginine residues. Proteolysis of G1, EV1 and EV2 with trypsin showed that G1 gave rise to a fragment the size of EV1 and EV1 to a fragment the size of EV2, regardless of whether the proteins had been made in the presence of the drug. A greater number of small molecular weight fragments was detected on this occasion for these proteins, probably due to more intense labelling of the proteins used (for comparison see Figures 33 and 38). The detection of a difference in the profiles obtained for EV1 indicates that canavanine was incorporated into at least EV1 but its presence did not affect cleavage into a fragment the size of EV2. This may suggest that lysine rather than arginine residues are involved in the formation of EV1 and EV2. However,

Figure 38

Proteolysis in a polyacrylamide gel of Maguari G1, EV1 and EV2 with trypsin using proteins synthesised with and without canavanine.



1 & 2    G1  
3 & 4    EV1  
5 & 6    EV2

▷ indicate products from EV1 in track 4 not present in 5.

Trypsin at 25ng per track

7% polyacrylamide gel fluorographed with Amplify and exposed for 6 days.



results obtained earlier (this section 3d) using endoproteinases lys C and arg C had indicated that the site(a) of cleavage may involve both lysine and arginine residues.

Radiolabelling of virus proteins in the presence of canavanine (Figure 37) showed that the drug caused a reduction in the level of host protein synthesis inhibition, and that normal or possibly higher levels of virus protein were made. This may indicate that the function of presumably a virus protein is affected by the presence of the analogue so that normal host-cell inhibition does not occur.

### 3g Growth curves of Maguari virus wild type, Mag ts 8(II), Revertant 1 and Revertant 2.

One step growth curves were determined as described in Method C9, to find out whether there was any difference in the growth of these revertants compared with Mag ts 8(II) from which they arose, and wild type Maguari virus. All the viruses were analysed at 31°C and the three ts<sup>+</sup> viruses were analysed at 38°C. The virus counts for all the graphs are presented in Appendix 6. Figure 39 shows the curves obtained at 31°C and shows both cell-associated and released virus. The two curves for each virus show similar kinetics of production and release. Figure 40 shows the curves for the ts<sup>+</sup> viruses at 38°C. Again the curves for the three viruses are similar. However, there is a difference between the curves obtained at the two temp-

Figure 39

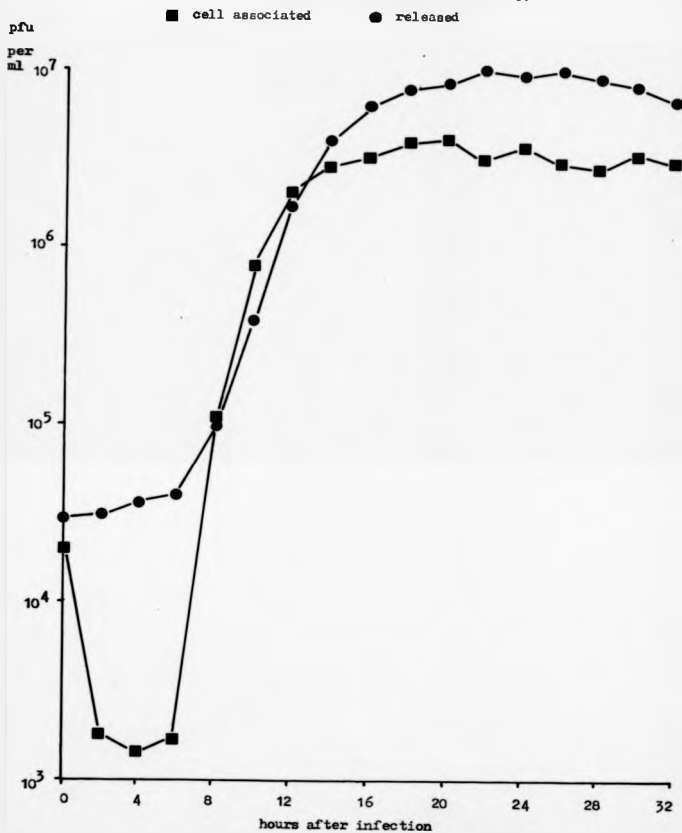
One step growth curves for Maguari virus wild type, Mag ts 8(II),  
Revertant 1 and Revertant 2 at 31°C in HEK cells.

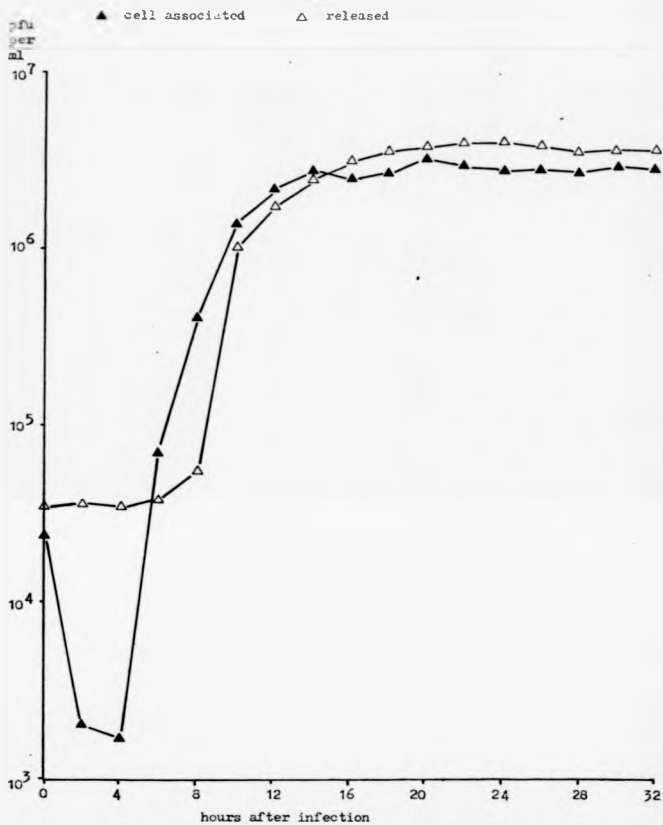
Panel A    Maguari ts<sup>+</sup>  
Panel B    Mag ts 8(II)  
Panel C    Revertant 1  
Panel D    Revertant 2

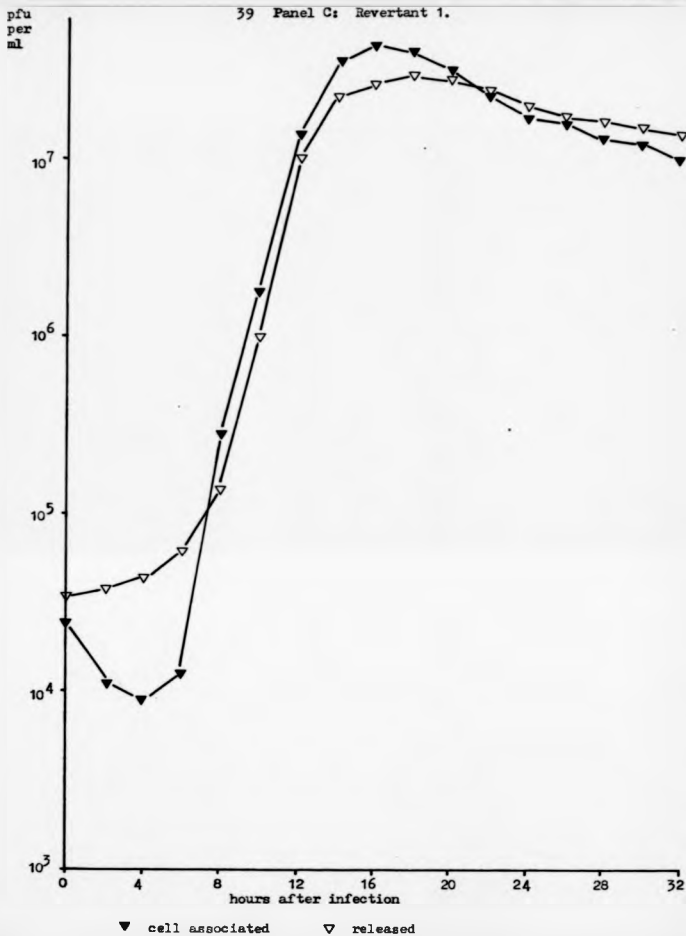
Cell associated and released virus curves as indicated for  
each panel.

moi 5pfu per cell.

39 Panel A: Maguari virus wild type.







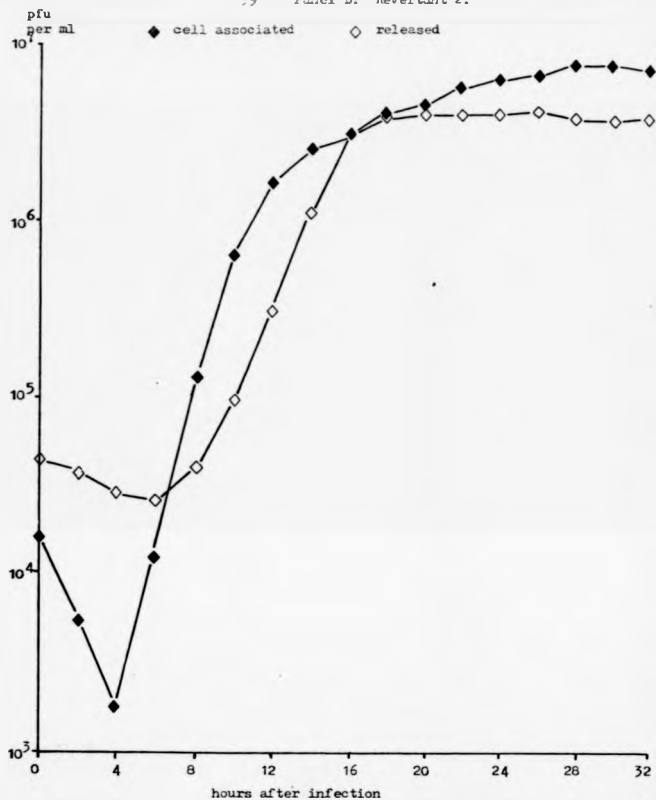


Figure 40

One step growth curves for Maguari virus wild type, Revertant 1 and Revertant 2 at 38°C in BHK cells.

Panel A Maguari ts<sup>+</sup>

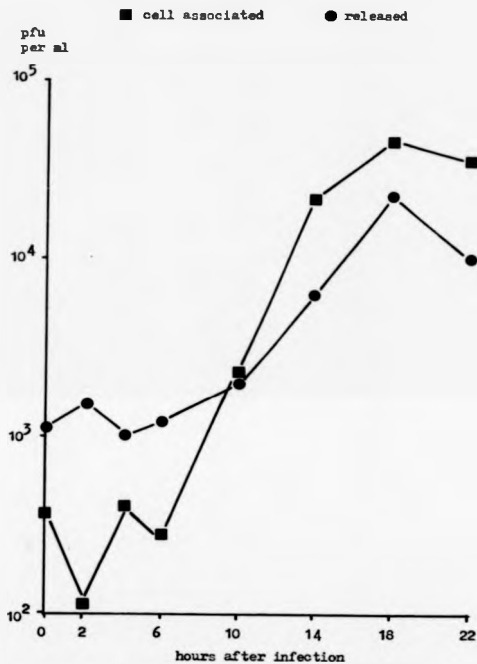
Panel B Revertant 1

Panel C Revertant 2

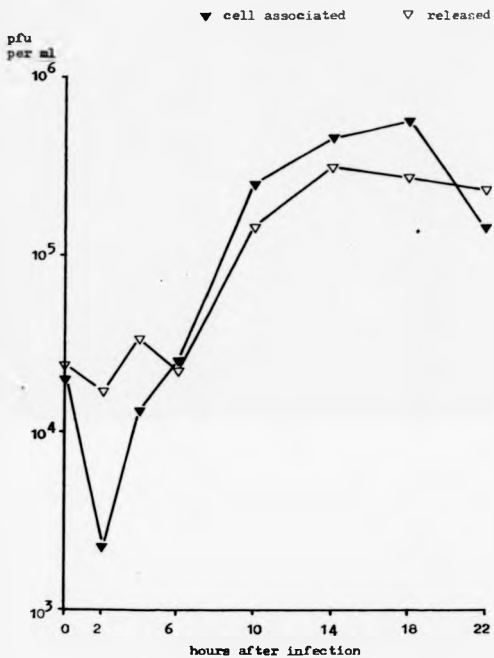
Cell associated and released virus curves as indicated for each panel.

moi 5pfu per cell.

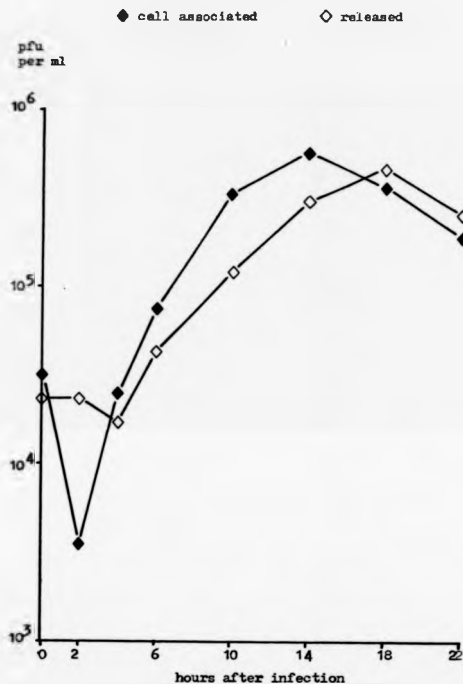
40 Panel A: Maguari virus wild type.







40 Panel C: Revertant 2.



eratures. At  $31^{\circ}\text{C}$ , much higher titres of virus were released; at  $38^{\circ}\text{C}$ , the peak titre was lower

than at  $31^{\circ}\text{C}$ . This may reflect a difference in the kinetics of virus growth at the two temperatures, with a cytotoxic effect occurring much earlier at  $38^{\circ}\text{C}$ . The phenomenon noted in Results Section 1d that virus protein synthesis was advanced at  $38^{\circ}\text{C}$  compared to  $31^{\circ}\text{C}$ , supports the suggestion of an increase in the growth rate at the higher temperature.

Discussion: Growth curves at  $31^{\circ}\text{C}$  and  $38^{\circ}\text{C}$  of Revertants 1 and 2.

The results show that Revertant 1 and Revertant 2 do not differ significantly from wild type Maguari virus in their kinetics of growth as determined by growth curves at both  $31^{\circ}\text{C}$  and  $38^{\circ}\text{C}$  and that Mag ts 8(II) was similar at  $31^{\circ}\text{C}$ . G1 protein is thought to be responsible for cell attachment - for example, Kingsford and Hill (1981) reported that cleavage of LaCrosse virions with trypsin removed a fragment from G1 with the result that virions were no longer capable of efficient attachment to host cells. As Revertant 1 and Revertant 2 appear to be able to grow normally, the moiety absent from G1 in each case does not appear to affect this function.

It was interesting to note that the growth cycle at  $38^{\circ}\text{C}$  was faster than at  $31^{\circ}\text{C}$  as it had been noted earlier that the rate of protein synthesis at  $38^{\circ}\text{C}$  by wild type Maguari virus was faster than at  $31^{\circ}\text{C}$ . The decrease in titre in the growth curves after a peak at 18 hours is in contrast to the observ-

ations at 31°C in which virus production peaked around 18 hours but then remained fairly constant (except for Revertant 1 which declined) and suggests that these viruses may be less stable at 38°C than at 31°C. If such virus inactivation was occurring this could account for the overall relatively low levels of virus detected at 38°C, rather than reduced levels of virus production.

### 3h Neutralisation of Revertants 1 and 2 by antisera.

Neutralisation assays were carried out as described in Method C8 and involved incubation of 0.05ml of a virus sample with 0.05ml of diluted antiserum (or PBS as control) overnight at +4°C. The origins of the antisera used are detailed in Materials 1. HEK cell monolayers were then inoculated with dilutions of these preparations for plaque assay. Table 32 shows the percentage of virus neutralised by dilutions of homologous anti-Maguari virus serum and Table 33 shows the percentage neutralised by a dilution of heterologous anti-Bunyamvera virus serum.

These data show that Revertant 1 was neutralised as efficiently as Maguari virus wild type and Mag ta 8(II) by both the low titre homologous serum, and the heterologous serum. Revertant 2 was not neutralised by either serum. As G1 is thought to be the major neutralisation antigen (Introduction 4g) this

Table 32

Neutralisation by Homologous Antiserum Against Wild Type

Maguari Virus.

Virus	Control Titre (PBS alone)	Test Titre (1/40 serum)	% Neutralised
Maguari ts <sup>+</sup>	$1.0 \times 10^6$	$2.4 \times 10^5$	77
Mag ts 8(II)	$3.1 \times 10^6$	$4.3 \times 10^5$	86
Revertant 1	$1.0 \times 10^6$	$1.5 \times 10^5$	85
Revertant 2	$1.0 \times 10^5$	$1.2 \times 10^5$	NIL
(1/20 serum)			
Maguari ts <sup>+</sup>	$2.7 \times 10^6$	$2.9 \times 10^5$	89
Mag ts 8(II)	$2.0 \times 10^6$	$1.6 \times 10^5$	92
Revertant 1	$2.0 \times 10^6$	$4.2 \times 10^5$	79
Revertant 2	$1.6 \times 10^5$	$2.0 \times 10^5$	NIL

Table 33

Neutralisation by Heterologous serum Against Wild Type

Bunyamvera Virus.

Virus	Control Titre (PBS alone)	Test Titre (1/50 serum)	% Neutralised
Maguari ts <sup>+</sup>	$3.4 \times 10^6$	$3.75 \times 10^5$	89
Mag ts 8(II)	$1.8 \times 10^6$	$4.8 \times 10^5$	73
Revertant 1	$3.9 \times 10^6$	$6.0 \times 10^5$	84
Revertant 2	$2.0 \times 10^5$	$2.98 \times 10^5$	NIL

suggests that the alteration resulting in EV1 does not affect neutralisation of the virus, whereas the change resulting in EV2 has abolished the ability of Maguari virus - and Bunyamvera virus - specific antisera to neutralise plaque-forming ability.

Discussion: Revertants 1 and 2 differ in their ability to be neutralised by antisera.

Kingsford and Hill (1983) noted that proteolytic cleavage of G1 in intact LaCrosse virions led to a reduction in the ability of neutralising antiserum to effect virus neutralisation. The data presented here further support this view that the absence of parts of the G1 molecule can reduce or abolish the neutralisation of the virus. It would appear that the moiety of Maguari virus G1 absent in EV1 does not affect the neutralisation epitopes (as detected with polyclonal antisera), whereas the fraction missing from EV2 abolishes the ability of antisera (specific for either Maguari virus or Bunyamvera virus) to neutralise virus infectivity. It is possible that the effect observed is not due to the change in the G1 protein which is a result of or responsible for the change in molecular weight, and could be due to other hitherto unrecognised mutations affecting G1 or even in a different protein. However as G1 is the neutralisation antigen and as the differences between G1, EV1 and EV2 in terms of molecular weight are so dramatic it is reasonable to assume that gross phenotypic differences between

them are the result of this dramatic difference rather than other possible changes. Following on from these results, it will be interesting to determine whether neutralising antiserum can be raised to Revertant 2 or whether neutralising epitopes have been lost.

These data are interesting in consideration of the observation that Revertant 2 grows as well as Revertant 1, wild type virus and Mag is 8(II) when analysed by one step growth curves. Presumably the site(s) involved in cell attachment does not require the presence of normal neutralisation epitopes to be functional, and presumably the two properties (cell attachment and neutralisation) are independent.

### 31 Genetic crosses between Revertants 1 and 2 and Bunyamwera virus.

Several attempts were made to isolate reassortants between the two revertants of Mag is 8(II) and Bunyamwera virus, to identify the genome segment causing the change in electrophoretic mobility of the G1 protein. Tables 34 and 35 show that in Cross 11 and Cross 12 between Revertant 1 and Bunyamwera virus on two separate occasions only clones of the Revertant 1 Maguari virus genotype were isolated, despite inoculation with equal concentrations of the two viruses. The same result was obtained for Revertant 2 in Cross 13 - Table 36 - and is consistent with the

Table 34

Cross 11: Analysis of Unselected Progeny from

Revertant 1 x Bm ts<sup>+</sup>, input ratio 1 : 1.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M ts <sup>+</sup>	50
Parental 2	B / B / B ts <sup>+</sup>	0
Reassortants		0
	Total	50

Table 35

Cross 12: Analysis of Unselected Progeny from

Revertant 1 x Bm ts<sup>+</sup>, input ratio 1 : 1.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M ts <sup>+</sup>	48
Parental 2	B / B / B ts <sup>+</sup>	0
Reassortants		0
	Total	48



general pattern of Maguari virus dominance over Bunyamvera virus in mixed infections, observed in Results Sections 1 and 2 and discussed in Section 2. In order to obtain clones of other than Maguari virus genotype, a ratio of 1:10 Revertant 2:Bunyamvera virus was used to infect the cells for Cross 14. When this cross was analysed, a high proportion of the clones (36 of 49) were of Bunyamvera virus genotype and the problem of suppression had been overcome. This is shown in Table 37. In an effort to isolate reassortants from this cross, one of the four mixed isolates was subcloned. It was possible that such a mixture might contain reassortants. This did indeed lead to the isolation of a reassortant - clone 15.31.10 - as indicated in Table 38 and Figure 41. In this clone, Bunyamvera N protein - and presumably S RNA - have been replaced with the Maguari N protein from Revertant 2.

Discussion: EV2 phenotype is not due to S RNA.

The only reassortant obtained from several attempts to generate reassortants from crosses of Revertant 1 or Revertant 2 with Bunyamvera virus, was of genotype Bun / Bun / Mag. The G1 protein produced by this clone is of normal Bunyamvera G1 electrophoretic mobility. Thus the formation of EV2 in Revertant 2 is not a consequence of mutation in the S RNA. More interesting would have been reassortants involving M RNA as it is expected that this is the segment bearing the mutation responsible for the altered phenotype and it would have been interesting to see

Table 36

Analysis of Unselected Progeny from Revertant 2 x Bun ts<sup>+</sup>

Cross 13 (input ratio 1 : 1).

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M ts <sup>+</sup>	49
Parental 2	B / B / B ts <sup>+</sup>	0
Reassortants		0
	Total	49

Table 37

Analysis of Unselected Progeny from Revertant 2 x Bun ts<sup>+</sup>

(Input ratio 1:10).

Cross 14.

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M ts <sup>+</sup>	9
Parental 2	B / B / B ts <sup>+</sup>	36
Mixtures	?B/ MB/ MB ts <sup>+</sup>	4
	Total	49

Table 38

Subclones of 15.31, a mixed isolate from Cross 14 (Revertant 2 x Bun ts<sup>+</sup>)

Type of Clone	Inferred Genotype	Number of Isolates
Parental 1	M / M / M ts <sup>+</sup>	2
Parental 2	B / B / B ts <sup>+</sup>	15
Reassortant 1	B / B / M ts <sup>+</sup>	1
Mixtures	?B/ MB/ MB ts <sup>+</sup>	1
	Total	19

Figure 41

Dot hybridisation and PAGE of subclones of 15.31, a mixed isolate  
from Cross 14 (Revertant 2 x Bun ts<sup>+</sup>).

Dot hybridisation:

Arrangement of samples (duplicate)

Revt. 2	Bun ts <sup>+</sup>	15.31.7
15.31.8	15.31.9	15.31.10

Panel A: pBUN 84 (L RNA probe)



Panel B: pBUN 133 (M RNA probe)



Dot hybridisation result

L and M RNA genotype

L M

15.31.7 Mag Mag

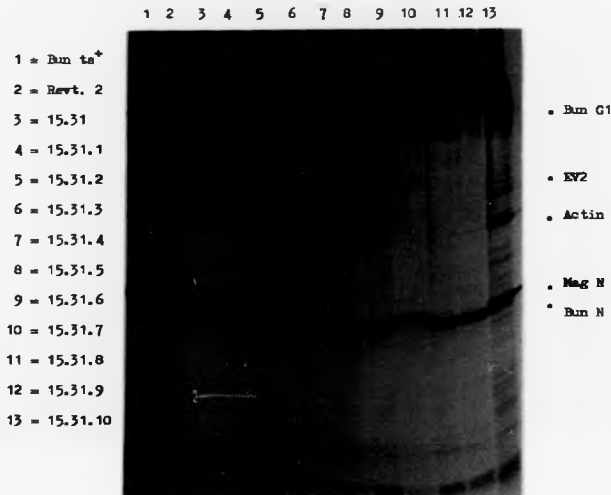
15.31.8 Bun Bun

15.31.9 Bun Bun

15.31.10 Bun Bun

Figure 41 continued.

PAGE of virus proteins radiolabelled at 18 hours after infection.



PAGE result

	G1	N
15.31.1	Bun	Bun
15.31.2	Bun	Bun
15.31.3	Bun	Bun
15.31.4	Mag	Mag
15.31.5	Bun	Bun
15.31.6	Bun	Bun
15.31.7	Mag	Mag
15.31.8	Bun	Bun
15.31.9	Bun	<u>Bun</u>
15.31.10	Bun	Mag

Inferred genotypes

	L	N	S
15.31.7	Mag	Mag	Mag
15.31.8	Bun	Bun	Bun
15.31.9	Bun	Bun	Bun
15.31.10	Bun	Bun	Mag

if the same phenotype was expressed in a different gene background.

The suppressive effect of Maguari virus over Bunyamwera virus in genetic crosses was particularly marked in these crosses involving Revertant 1 and Revertant 2. Perhaps in addition to the previously observed suppression effect, the alterations to G1 may further restrict the genotypes of viable reassortants, thereby enhancing the dominance effect.

### 3j Conclusions from analysis of Revertant 1 and Revertant 2.

Reversion of Mag to 8(II) is generally associated with alterations to the G1 protein, which may result from changes in the processing of that protein. The 4 glycosylation sites predicted for the gene products of Bunyamwera M RNA are distributed evenly throughout the sequence (Lees et al., 1986). Observations on the loss of glycosylation sites between the two size classes of revertant glycoproteins EV1 and EV2 of Maguari virus, do not suggest any possible location for G1 within the sequence of this related virus. If there had been clustering of the glycosylation sites it might have been possible to suggest a position for G1 in the sequence. As the two mutant proteins differ in the amount of carbohydrate present, possibly cleavage is occurring between glycosylation sites and moieties containing these sites may be lost and degraded. The difference

in molecular weight is much greater than the difference in the extent of glycosylation of the three proteins. Amino acid sequencing of Bunyamwera G1 protein will be required to locate it in the Bunyamwera M RNA sequence.

The data suggest that the mode of cleavage employed by Maguari virus for the formation of M RNA gene products might employ a trypsin-like protease. Whether this is virus coded or host coded remains to be determined. Further work with protease inhibitors and attempts to translate M RNA message under controlled conditions will give more information on the synthesis of these viral proteins. Also, the other possible mechanisms for the EV1 and EV2 phenotypes outlined at the beginning of this section need to be further examined as this work does not conclusively show that the mechanism is by proteolysis but is only a suggestion of a probable mechanism.

As mentioned earlier (Results Section 1a), three clones were isolated from a cross involving Mag ts 7(I) and Bunyamwera virus in which Maguari G1 was replaced by an EV1-like protein. This change was not specifically associated with reversion of the ts phenotype as two mutants were ts<sup>+</sup> and a third was ts. As Group I mutants can be assigned to the S RNA and G1 is encoded by the M RNA, it seems likely that the two clones which reverted to ts<sup>+</sup> phenotype had a second mutation which was responsible for the change in the electrophoretic mobility of G1. A reassortment experiment indicated that the S RNA did not affect the G1 phenotype. It is likely therefore that reversion of the ts phenotype of Group II mutants is usually due to a suppressor mutation within the M RNA.

## General Discussion

The data presented here show that the ts mutants of Groups I, II and III of Maguari virus, and therefore of the Bunyamwera serogroup, can be assigned to the S, M and L RNA segments respectively. In contrast to the findings of Pringle et al. (1984) (Introduction 5f) no evidence of restriction in the reassortment of the RNA segments was seen, although in each cross all possible reassortants were not recovered. This phenomenon is not unique to this study and is generally observed with such genetic crosses (for examples see Mahy, 1983 and Introduction 5b). It was found that Mag ts 7(I) and Mag ts 23(III) were in some way defective in virus protein synthesis at 38°C whereas Mag ts 8(II) was capable of producing high levels of virus protein at that temperature. Mag ts 23(III) was distinct from the other two mutants in the pattern of virus production seen with temperature shift experiments and it was unusual in that it had some degree of host restriction indicated by a great reduction in cytopathic effect seen in BSC-1 cells. The different phenotypes of the mutants presumably reflect the different locations of the ts lesions in the genome and are a consequence of perturbation of the normal functions of the sequences affected. By analysis of such changes it is possible to discover how these phenotypic effects are mediated.

Examination of a reassortant "mixed clone" (13.48) from a Group II cross (Cross 4) showed that Maguari virus can interfere with the growth of Bunyamwera virus, and tends to dominate over Bunyamwera virus. This is presumably responsible for the



consistent predominance of Maguari virus parental clones isolated from genetic crosses and the paucity of isolation of Bunyamwera virus parental clones. The data suggest that 13.48 is distinct from a simple mixture of two viruses and is a much more complex association of the genome segments.

Study of Maguari virus variants of altered G1 proteins has provided information on the nature of G1. As the M RNA products are encoded in a single open reading frame it seems probable that the individual proteins are formed as a result of cotranslational cleavage of a precursor. Therefore a mutation which results in a new cleavage site or exposes one not previously available is a mechanism by which changes in the electrophoretic mobility of G1 could occur. The data provide some evidence to support this as a mechanism by which the variants studied arose but it is not conclusive and other possibilities need to be examined in more detail. Indeed the failure to detect any cleavage fragments suggests that the mechanism by which these electrophoretic variants arise is at the level of mRNA synthesis or translation rather than post- or cotranslational modification.

The alteration to G1 in one case (Revertant 2 with EV2) abolished the ability of neutralising antiserum to neutralise virus infectivity and this indicates that the mutation in this case had affected the neutralisation epitopes. However, this virus grew normally in BEK cells, showing that the change in G1 had not had a deleterious effect on the viability of the virus. Presumably the viability in a natural environment would be affected, otherwise the wild type would have this phenotype under the pressures of natural selection.

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APPENDICES

## Appendix 1

## Company Addresses.

Amersham International Ltd.,	Amersham, Buckinghamshire, U.K..
BCE Ltd.,	Lewes, East Sussex, U.K..
BEE Ltd.,	Poole, Dorset, U.K..
Beckman RIIIC Ltd.,	High Wycombe, Buckinghamshire, U.K..
Biorad Ltd.,	Watford, Hertfordshire, U.K..
BHL Inc.,	Cambridge, U.K..
Chance-Propper Ltd.,	Warley, U.K..
Costar,	Cambridge, Mass., U.S.A..
Difco Laboratories Ltd.,	East Molesey, Surrey, U.K..
Dupont Cronex Ltd.,	Stevenage, Hertfordshire, U.K..
Eastman Kodak Co.,	Rochester, New York, U.S.A..
Fisons,	Loughborough, Leicestershire, U.K..
Flow Laboratories Ltd.,	Irvine, Ayrshire, U.K..
Fluka Ltd.,	Glossop, Derbyshire, U.K..
Fuji Photofilm Co. Ltd.,	Tokyo, Japan.
Gibco Ltd.,	Uxbridge, Middlesex, U.K..
Glaxo Ltd.,	Ware, Hertfordshire, U.K..
Kodak: see Eastman Kodak.	
LKB Ltd.,	Croydon, Surrey, U.K..
May & Baker Ltd.,	Dagenham, Kent, U.K..
Miles Laboratories Inc.,	Stoke Poges, Slough, U.K..
NEN Ltd.,	Southampton, U.K..
Nunc Intermed,	Denmark.
Oxoid Ltd.,	Basingstoke, Hampshire, U.K..
Pharmacia Ltd.,	Hounslow, U.K..
Schleicher & Schuell GmbH	East Molesey, Surrey, U.K..
Sigma Chemical Co Ltd.,	Poole, Dorset, U.K..
Whatman Ltd.,	Maidstone, Kent, U.K..
Worthington Inc.,	Illinois, U.S.A..

## Appendix 2

Virus counts for temperature shift experiments with

Mag ts 7(I) and Mag ts 8(II)

(Figures 16 and 17)

Panel A Fig. 16	Panel B Fig. 16	Panel C Fig. 16	
◆ Mag <u>ts</u> 8(II)	● Mag <u>ts</u> 7(I)	□ Mag <u>ts</u> +	
pfu per ml	pfu per ml	pfu per ml	
$3.8 \times 10^5$	$6.8 \times 10^4$	$1 \times 10^6$	31°C
$8.4 \times 10^2$	$1.2 \times 10^2$	$1.6 \times 10^5$	38°C
$2.5 \times 10^3$	$< 10^2$	$3.4 \times 10^5$	0 hours
$5.3 \times 10^2$	$< 10^2$	$5 \times 10^4$	6 "
$1.9 \times 10^2$	$< 10^2$	$8.5 \times 10^4$	9 "
$5 \times 10^2$	$4.6 \times 10^2$	$2.5 \times 10^5$	14 "

Panel A Fig. 17	Panel B Fig. 17	Panel C Fig. 17	
◆ Mag <u>ts</u> 8(II)	● Mag <u>ts</u> 7(I)	□ Mag <u>ts</u> +	
pfu per ml	pfu per ml	pfu per ml	
$2.7 \times 10^5$	$5.8 \times 10^4$	$1.1 \times 10^6$	31°C
$8 \times 10^2$	$1 \times 10^2$	$1.7 \times 10^5$	38°C
$5.6 \times 10^5$	$1.7 \times 10^4$	$5.6 \times 10^5$	0 hours
$2.15 \times 10^5$	$2.9 \times 10^4$	$3.8 \times 10^6$	6 "
$3.6 \times 10^5$	$1 \times 10^5$	$1.7 \times 10^5$	9 "
$2.3 \times 10^5$	$1.4 \times 10^5$	$1.3 \times 10^6$	14 "

## Appendix 3

Virus counts for temperature shift experiments

with Mag is 23 (III).

(Figure 18)

## Panel A

	● pfu per ml	◇ pfu per ml
31°C	$1.75 \times 10^6$	$2 \times 10^6$
38°C	$6.3 \times 10^3$	$2.95 \times 10^3$
0 hours	$1.2 \times 10^6$	$9.9 \times 10^4$
1 "	$2.3 \times 10^5$	$1.25 \times 10^6$
2 "	$1.4 \times 10^6$	$2.4 \times 10^6$
3 "	$6.7 \times 10^5$	$2.4 \times 10^6$
4 "	$3.95 \times 10^5$	$1.5 \times 10^5$

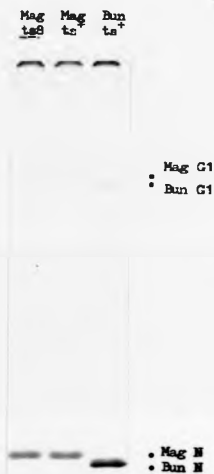
## Panel B

	■ pfu per ml	○ pfu per ml
31°C	$2.3 \times 10^6$	$1 \times 10^6$
38°C	$1 \times 10^3$	$1.6 \times 10^2$
0 hours	$2.95 \times 10^4$	$7.9 \times 10^3$
2 "	$2.9 \times 10^4$	$1.3 \times 10^5$
4 "	$1 \times 10^4$	$1.75 \times 10^5$
6 "	$1.62 \times 10^4$	$1.2 \times 10^4$
8 "	$8.7 \times 10^4$	$2.7 \times 10^2$



## Appendix 4

Mag ts 8(II) is equivalent to wild type Maguari virus by PAGE.

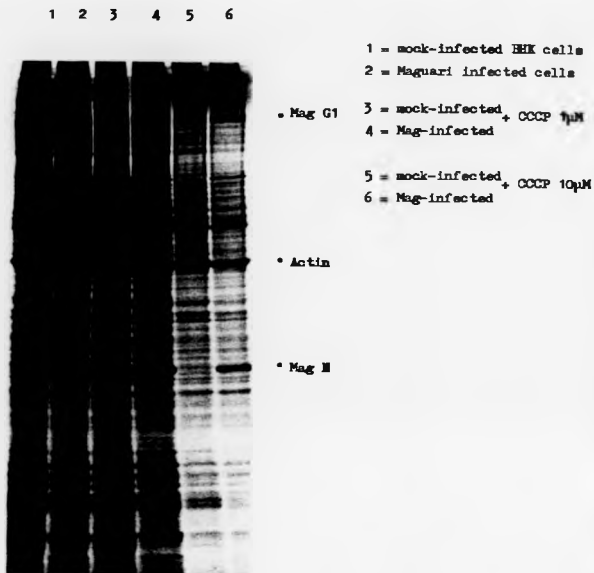


Proteins radiolabelled at 18 - 19 hours after infection.

BHK cell lysates, 10% polyacrylamide gel exposed for 7 days.

## Appendix 5

Effect of CCCP on the synthesis of Maguari virus proteins.



Proteins radiolabelled at 18 - 19 hours after infection.

CCCP at concentrations indicated from 17 hours after infection.

BHK cell lysates, 10% polyacrylamide gel exposed for 7 days.

## Appendix 6

Virus counts for Growth Curves (Figures 39 and 40).

Figure 39: Maguari virus wild type, Mag ts 8(II), Revertant 1, and Revertant 2 at 31°C. pfu per ml.

A Maguari wild type			B Mag <u>ts</u> 8(II)		
time	cell	released	cell	released	
point	associated		associated		
0 hours	$2 \times 10^4$	$2.95 \times 10^4$	$2.4 \times 10^4$	$3.5 \times 10^4$	
2	$1.75 \times 10^3$	$3.1 \times 10^4$	$2 \times 10^3$	$3.6 \times 10^4$	
4	$1.4 \times 10^3$	$3.6 \times 10^4$	$1.7 \times 10^3$	$3.5 \times 10^4$	
6	$1.65 \times 10^3$	$4 \times 10^4$	$7.2 \times 10^4$	$3.8 \times 10^4$	
8	$1.1 \times 10^5$	$1 \times 10^5$	$4 \times 10^5$	$5.6 \times 10^4$	
10	$7.56 \times 10^5$	$3.8 \times 10^5$	$1.4 \times 10^6$	$1 \times 10^6$	
12	$2 \times 10^6$	$1.7 \times 10^6$	$2.2 \times 10^6$	$1.75 \times 10^6$	
14	$2.8 \times 10^6$	$4 \times 10^6$	$2.8 \times 10^6$	$2.5 \times 10^6$	
16	$3.15 \times 10^6$	$6.3 \times 10^6$	$2.5 \times 10^6$	$3.1 \times 10^6$	
18	$3.9 \times 10^6$	$7.9 \times 10^6$	$2.7 \times 10^6$	$3.6 \times 10^6$	
20	$4 \times 10^6$	$8.5 \times 10^6$	$3.2 \times 10^6$	$3.8 \times 10^6$	
22	$3.1 \times 10^6$	$1 \times 10^7$	$2.95 \times 10^6$	$4 \times 10^6$	
24	$3.6 \times 10^6$	$9.2 \times 10^6$	$2.8 \times 10^6$	$4 \times 10^6$	
26	$2.95 \times 10^6$	$1 \times 10^7$	$2.8 \times 10^6$	$3.9 \times 10^6$	
28	$2.8 \times 10^6$	$9 \times 10^6$	$2.7 \times 10^6$	$3.6 \times 10^6$	
30	$3.2 \times 10^6$	$8 \times 10^6$	$2.9 \times 10^6$	$3.6 \times 10^6$	
32	$3 \times 10^6$	$6.8 \times 10^6$	$2.8 \times 10^6$	$3.6 \times 10^6$	

C Revertant 1			D Revertant 2		
0	$2.4 \times 10^4$	$3.6 \times 10^4$	$1.6 \times 10^4$	$4.5 \times 10^4$	
2	$1.1 \times 10^4$	$3.8 \times 10^4$	$5.6 \times 10^3$	$3.8 \times 10^4$	
4	$9 \times 10^3$	$4.5 \times 10^4$	$1.75 \times 10^3$	$2.95 \times 10^4$	
6	$1.25 \times 10^4$	$6.3 \times 10^4$	$1.25 \times 10^4$	$2.7 \times 10^4$	
8	$2.8 \times 10^5$	$1.4 \times 10^5$	$1.3 \times 10^5$	$4 \times 10^4$	
10	$1.75 \times 10^6$	$1 \times 10^6$	$6.3 \times 10^5$	$9.7 \times 10^4$	
12	$1.4 \times 10^7$	$1 \times 10^7$	$1.6 \times 10^6$	$3.1 \times 10^5$	
14	$3.6 \times 10^7$	$2.25 \times 10^7$	$2.5 \times 10^6$	$1.1 \times 10^6$	
16	$4.5 \times 10^7$	$2.7 \times 10^7$	$3.1 \times 10^6$	$3.1 \times 10^6$	
18	$4.1 \times 10^7$	$2.95 \times 10^7$	$4.1 \times 10^5$	$3.8 \times 10^6$	
20	$3.1 \times 10^7$	$2.8 \times 10^7$	$4.6 \times 10^6$	$3.9 \times 10^6$	

## Appendix 6 continued.

(Fig 39) C Revertant 1				D Revertant 2	
cell		released	cell	released	
hours	associated		associated		
22	$2.4 \times 10^7$	$2.4 \times 10^7$	$5.7 \times 10^6$	$4 \times 10^6$	
24	$2 \times 10^7$	$1.75 \times 10^7$	$6.3 \times 10^6$	$4 \times 10^6$	
26	$1.75 \times 10^7$	$1.6 \times 10^7$	$6.4 \times 10^6$	$4.1 \times 10^6$	
28	$1.65 \times 10^7$	$1.3 \times 10^7$	$7.4 \times 10^6$	$3.8 \times 10^6$	
30	$1.5 \times 10^7$	$1.2 \times 10^7$	$7.4 \times 10^6$	$3.7 \times 10^6$	
32	$1.4 \times 10^7$	$9.7 \times 10^6$	$7.2 \times 10^6$	$3.8 \times 10^6$	

Figure 40: Maguari virus wild type, Revertant 1 and Revertant 2 at  $38^\circ\text{C}$ , pfu per ml.

A Maguari wild type			B Revertant 1	
time	cell	released	cell	released
point	associated		associated	
0	$3.6 \times 10^2$	$1.1 \times 10^3$	$2 \times 10^4$	$2.4 \times 10^4$
2	$1.1 \times 10^2$	$1.5 \times 10^3$	$2.2 \times 10^3$	$1.7 \times 10^4$
4	$4 \times 10^2$	$1 \times 10^3$	$1.3 \times 10^4$	$3.4 \times 10^4$
6	$2.7 \times 10^2$	$1.2 \times 10^3$	$2.5 \times 10^4$	$2.2 \times 10^4$
10	$2.3 \times 10^3$	$2 \times 10^3$	$2.5 \times 10^5$	$1.4 \times 10^5$
14	$2.2 \times 10^4$	$6.3 \times 10^3$	$4.5 \times 10^5$	$3.1 \times 10^5$
18	$4.5 \times 10^4$	$2.3 \times 10^4$	$5.6 \times 10^5$	$2.7 \times 10^5$
22	$3.6 \times 10^4$	$1 \times 10^4$	$1.4 \times 10^5$	$2.3 \times 10^5$

C Revertant 2		
0	$3.1 \times 10^4$	$2.3 \times 10^4$
2	$3.6 \times 10^3$	$2.3 \times 10^4$
4	$2.5 \times 10^4$	$1.7 \times 10^4$
6	$7.4 \times 10^4$	$4.3 \times 10^4$
10	$3.3 \times 10^5$	$1.2 \times 10^5$
14	$5.6 \times 10^5$	$3 \times 10^5$
18	$3.6 \times 10^5$	$4.6 \times 10^5$
22	$1.9 \times 10^5$	$2.5 \times 10^5$