GENETIC AND BIOLOGICAL STUDIES ON LABORATORY-DERIVED AND NATURAL ISOLATES OF ROSS RIVER VIRUS

BY

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STATEMENT

All the work reported in this thesis
was carried out by myself.

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ABSTRACT

This thesis is divided into seven Chapters. The first Chapter presents introductory information, Chapters 2-6 present experimental results and Chapter 7 represents the conclusion.

Chapter 2 describes the location of neutralisation epitopes in the primary amino acid sequence of the envelope glycoprotein E2 of the prototype T48 strain of the alphavirus Ross River virus (RRV). Using four neutralising monoclonal antibodies (mabs) T1E7, T10C9, NB3C4 and T1B1, fourteen neutralisation-resistant variants of RRV T48 were isolated (frequency of isolation, ~10^{-5}). Sequencing of the structural protein genes showed that point mutations had occurred in the E2 gene of all variants; in each case these resulted in a predicted non-conservative amino acid substitution in E2. No mutations were detected in the genes coding for E1, E3, C or the 6K proteins except in one variant where a silent mutation occurred in the E1 gene. Variants resistant to mab T10C9 were altered at residue 216 of E2, T1E7 variants were altered at residues 246, 248 or 251, NB3C4 variants at residue 234 and T1B1 variants at residue 246. Thus three neutralisation epitopes were defined around amino acids 216 (epitope I), 234 (epitope II) and 246-251 (epitope III). Cross-neutralisation tests of the mab-resistant variants showed that variants altered in one epitope were neutralised by mab interacting with a different epitope.

The three neutralisation epitopes lie between the only two predicted asparagine-linked glycosylation sites in the

primary amino acid sequence of E2. Comparison of the primary amino acid sequences of RRV, SFV and SIN in this region of E2 showed that there are short sequence stretches which are conserved between the three alphaviruses. The amino acids which were altered in the mab-resistant variants were located within regions which were not conserved between RRV, SFV and SIN or were immediately adjacent to these regions. Examination of the hydropathy profile of RRV E2 showed that epitope I was located in a moderately hydrophilic domain of E2 whereas epitopes II and III were in two major hydrophilic domains which were conserved as to position and hydrophilicity profile between RRV, SFV and SIN.

Multi-epitope variants with amino acid changes in all three epitopes were isolated by sequential selection with three different mabs. Sequencing of two such variants showed that a change in epitope I led to a restriction on the type of change that could be introduced in epitopes II and III indicating that the three epitopes may be conformationally connected.

RRV T48 is highly virulent for young mice whereas another natural isolate, RRV NB5092, is avirulent. Sequence comparisons of mab-resistant variants of the two strains showed that the location of the three neutralisation epitopes was same for each strain.

In Chapter 3 the biological properties of the mab-resistant RRV T48 variants were examined in cultured cells and in mice. There were no significant differences between RRV T48 and epitope I and II variants in growth, or in RNA or protein synthesis in BHK cells. However epitope III variants showed a

slight retardation of growth, and of RNA and protein synthesis in BHK cells compared to RRV T48 together with an altered rate of "penetration" as measured by the rate of acquisition of neutralisation resistance of the infecting virus to polyclonal antibody. No variants altered in epitopes I or II showed changes in virulence (measured as LD_{50} and the average survival time, AST, of infected mice) compared with RRV T48. However, three of seven epitope III variants examined had slightly reduced virulence for week-old mice. An hypothesis relating alterations in E2 to changed biological properties of the virus is presented.

During the course of sequencing the 26S RNA of RRV T48, Dalgarno _et_ _al_. (1983) obtained evidence for the presence, in the virus stock, of a mutant with a 21-nucleotide deletion in the E2 gene. Subsequently RRV dE2, the predicted deletion mutant, was isolated from the stock and the mutation confirmed by sequencing the appropriate region of E2 (L. Dalgarno and S.G. Faragher, unpublished data). In Chapter 4 we examine certain aspects of the biological properties of this mutant with particular emphasis on its virulence and growth in mice. In day-old mice RRV dE2 produced less severe symptoms than did RRV T48. The mutant had a slight increase in LD_{50} and AST compared to RRV T48 indicating a slight attenuation. RRV dE2 grew to peak titers which were approximately 3-4 log units lower in blood, brain and hind leg muscle tissues of day-old mice compared to RRV T48. Although there were no differences in the LD_{50} and the AST values for RRV T48 between the day-old and the week-old mice, RRV dE2 was apparently totally avirulent

in week-old mice. Thus the mutant induced no detectable symptoms even when injected at high doses (~8x10^6 PFU). In week-old mice, peak RRV dE2 titers in hind leg muscle, brain and blood, respectively, were 2,5, and 5 log units lower than the corresponding titers for RRV T48. Peak RRV T48 titers in blood (~10^{10} PFU/ml) and in hind leg muscle (~10^{12} PFU/g) were similar in day-old and week-old mice, however peak RRV T48 titers were ~3 log units lower in the brain of week-old mice than in day-old mice. Similarly peak RRV dE2 titers in blood (~10^5 PFU/ml) and in hind leg muscle (~10^8 PFU/g) were similar in day-old and in week-old mice whereas peak titers in brain were ~3 log units lower in week-old mice compared to day-old mice. Since the major difference in RRV dE2 titers between week-old and day-old mice, and between RRV dE2 and RRV T48 titers in week-old mice was in brain tissue it appears that the outcome of RRV infection was probably determined by the extent of replication in neural tissue.

In Chapter 5 we attempt to define genetic and biological changes associated with the attenuation of RRV T48 virulence for mice which occurs on passaging the virus in cultured cells. Attenuated variants of RRV T48 were obtained with 12 serial passages in BHK cells. In week-old mice the passaged-variants showed 4-30 times greater LD_{50}s and about two times greater ASTs than RRV T48. Growth of one of four such attenuated variants was examined in week-old mice. There was no significant difference between the titers of RRV T48 and the variant in hind leg muscle tissue, although the variant reached titers which were ~2 log units less than RRV T48 in blood and

brain tissue suggesting a change in tissue tropism. In BHK cells, attenuated variants showed more rapid "penetration" than RRV T48. No differences were detected in viral growth rates and protein synthesis when comparing variants and RRV T48 in BHK cells, although rates of AMD-resistant RNA synthesis in cells infected with variants were approximately three times greater than those in RRV T48-infected cells. No mutations were detected in the structural protein genes of the variants. The non-structural protein genes were not sequenced.

Chapter 6 examines the evolution of the envelope glycoprotein E2, the neutralisation antigen, during the epidemic spread of RRV in a non-immune human population in the South Pacific during 1979-81. Sequencing revealed that only limited evolution took place in the E2 gene during the epidemic. There were only two nucleotide changes selected in the E2 gene as the virus moved from Fiji to American Samoa and to the Cook Islands. One of these resulted in an amino acid change (Thr→Ala) at residue 219 in epitope I.

Previous studies of Faragher _et al_. (1985a) based on HaeIII and TaqI restriction digest analysis suggested that strains of RRV isolated in the South Pacific epidemic were closely related to an RRV isolate obtained from mosquitoes in the Narran Lake area of New South Wales. Sequence studies on the E2 gene of these isolates indicated that the Narran Lake isolate of RRV was closely related, but not identical, to the South Pacific RRV isolates.

During a recent outbreak of polyarthritis in Rockhampton, patients showed a similar unusual disease pattern

to that seen in the South Pacific outbreak. Comparison of the E2 gene sequences of RRV from the two areas showed that RRV isolates obtained from humans in Rockhampton were closely related, but not identical, to RRV isolates obtained from humans in the South Pacific.

In the final part of Chapter 6 we have examined, for a small number of natural RRV isolates, the positions of predicted amino acid differences in E2. Comparing within this group and with RRV T48, a total of eight amino acid differences were detected in the various RRV isolates. Half of these were located in the domain containing the neutralisation epitopes which comprises only ~15% of the E2 protein.

PUBLICATIONS FROM THIS THESIS

Journal article

Vrati S., Faragher S.G., Weir R.C. and Dalgarno L. (1986) Ross River virus mutant with a deletion in the E2 gene: Properties of virion, virus specific macromolecule synthesis and attenuation of virulence for mice. Virology 151, 222-232

Papers presented

Meek A.D., Vrati S., Faragher S.G., Burness A.T.H., Pardoe I., Boulton R.W., Weir R.C. and Dalgarno L. (1984) Virulence determinants of Ross River virus. (presented at the sixth International Congress of Virology held in Sendai)

Vrati S., Fernon C., Dalgarno L. and Weir R.C. (1986) Identification of antigenic determinants involved in neutralisation of the alphavirus, Ross River virus. (presented at the Australian Society for Microbiology annual conference held in Melbourne)

Kerr P., Vrati S., Fernon C., Fitzgerald S., Tregear G.W., Dalgarno L. and Weir R.C. (1986) Identification of neutralisation epitopes of an alphavirus (Ross River virus) and studies with synthetic peptides. (accepted for presentation at the Vaccine Meeting '86 to be held in Lorne, Victoria in October 1986)

ABBREVIATIONS

AMD	actinomycin-D
AST	average survival time
BHK cells	baby hamster kidney cells
CBA	competitive binding assay
cpe	cytopathic effects
d	day/s
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EMEM	Eagle's minimal essential medium
EPA	epidemic polyarthritis
EV	extracellular virus
GMEM	Glasgow modified Eagle's BHK medium
HBSS	Hank's balanced salt solution
HI	haemagglutination inhibition
hr	hour/s
i.p.	intra-peritoneal
K	thousand
mab	monoclonal antibody
moi	multiplicity of infection
mol. wt.	molecular weight
PBS	phosphate buffered saline
PFU	plaque forming unit/s
pi	post-infection
poly(A)	polyadenylic acid
PRNT	plaque reduction neutralisation test
s.c.	sub-cutaneous
SDS	sodium dodecyl sulphate
Vero	African green monkey kidney cells

Abbreviations for alphaviruses are given in Table 1.1

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Alphaviruses and their medical significance

Alphaviruses, previously known as group A arboviruses, are classified as a genus of the family Togaviridae. Sindbis (SIN) is the prototype alphavirus (Westaway et al., 1985). Alphaviruses are arboviruses; they are maintained in nature principally by transmission between susceptible vertebrate hosts via blood-feeding arthropods. These viruses possess the ability to multiply in both vertebrate and invertebrate hosts and in cell cultures derived from them. Alphaviruses are almost exclusively mosquito-borne, and are found in tropical and temperate regions of the world (Chamberlain, 1980).

There are 25 recognised alphaviruses (Westaway et al., 1985) which have been classified serologically into complexes, types and subtypes (Calisher et al., 1980; Chamberlain, 1980; Porterfield, 1980). Calisher et al. (1980) have listed six complexes within the alphavirus genus: the Eastern equine encephalitis virus (EEE), Middleburg virus (MID), Ndumu virus (NDU), Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEE) and Western equine encephalitis virus (WEE) complexes. The first three complexes contain a single virus. Each of the other three includes several viruses which are antigenically closely related but are serologically distinguishable (Table 1.1). Ross River virus (RRV), the subject of this thesis, belongs to the SFV complex.

Of the 25 alphaviruses, 16 are proven or suspected agents of disease in humans or domestic animals (Shope, 1980;

Table 1.1 **Serological classification of alphaviruses.**

Complex	Species	Subtypes
Eastern equine encephalitis (EEE)	EEE	
Middleburg (MID)	MID	
Ndumu (NDU)	NDU	
Semliki Forest (SFV)	SFV	
	Chikungunya (CHIK)	CHIK
		O'Nyong Nyong (ONN)
	Getah (GET)	GET
		Sagiyama (SAG)
		Bebaru (BEB)
		Ross River (RRV)
	Mayaro (MAY)	MAY
		Una (UNA)
Venezuelan equine encephalitis (VEE)	VEE	VEE
		Everglades (EVE)
		Mucambo (MUC)
		Pixuna (PIX)
		Cabassou (CAB)
Western equine encephalitis (WEE)	WEE	
	Highlands J (HJ)	
	Fort Morgan (FM)	
	Sindbis (SIN)	SIN
		Whataroa (WHA)
		Kyzylagach (KZL)
	Aura (AURA)	

From Calisher et al. (1980).

Monath and Trent, 1981). There are two major types of disease syndrome induced by alphavirus infections. Encephalitis is associated with infections by EEE, VEE, WEE and SFV whereas arthritis, fever and rash are associated with infections by chikungunya (CHIK), O'Nyong-Nyong (ONN), Mayaro (MAY) and RRV. CHIK and ONN cause the most significant alphavirus disease of humans and have been responsible for epidemics in Asia and Africa affecting millions of people (Shope, 1980).

RRV-induced epidemic polyarthritis (EPA) has occurred in eastern Australia at regular intervals since the syndrome was first described in 1928 (Nimmo, 1928). Every year several hundred cases of EPA occur in Australia; however during epidemics the number reaches several thousand (Marshall and Miles, 1984). Symptoms of EPA include rash, joint and muscle pain with or without mild fever (Anderson and French, 1957; Mataika, 1981; Mudge and Aaskov, 1983). The disease is reported mainly in adults and is rare in children (Marshall and Miles, 1984). Based on serological surveys, Aaskov et al. (1981a) reported a subclinical to clinical ratio of about 50:1 for RRV infection in humans in Fitzroy and Far North divisions of Queensland.

RRV has Australia-wide distribution (Mudge and Aaskov, 1983). About 50% of human sera collected in Queensland during 1957-64 had detectable RRV antibodies (Doherty et al., 1966). Sera from about 13% of the population in New South Wales contained RRV antibodies in 1981-82 (Boughton et al., 1984). Until 1979 RRV activity was limited to Australia, New Guinea

and the Solomon Islands (Tesh _et_ _al_., 1975, Kanamitsu _et_ _al_., 1979). However, during 1979-81 RRV spread to a non-immune population in the South Pacific islands (Aaskov _et_ _al_., 1981b; Rosen _et_ _al_., 1981; Tesh _et_ _al_., 1981; Fauran and Le Conidec, 1982). EPA was first reported in Fiji in April 1979. During the RRV epidemic in Fiji an estimated 300,000-500,000 people, representing about 50-80% of the island's population, were infected. In August 1979 the disease spread to American Samoa where about half the population was affected. Then in February 1980 EPA was reported from the Cook Islands (for a review, see Marshall and Miles, 1984).

RNA viruses may evolve rapidly as demonstrated for poliovirus which underwent 1-2% nucleotide substitutions during a one-year epidemic in a non-immune population (Nottay _et_ _al_., 1981). No information exists on the evolution, at the nucleotide sequence level, of an alphavirus during an epidemic. The defined sequential spread of RRV in a non-immune population in the South Pacific provides an opportunity to investigate the evolution of an alphavirus during an epidemic. This is examined in a section of this thesis.

There are no vaccines available for humans against any medically important alphavirus. An experimental vaccine for CHIK has been developed and tested in limited fashion, but is not available commercially (Craven, 1984). However, vaccines against EEE, VEE and WEE are currently in use in horses. A live virus vaccine against VEE uses a tissue culture passaged equine-attenuated strain (TC-83) of VEE. This vaccine is not

ideal, for vaccine virus has been isolated from mosquitoes suggesting a high viraemia in some horses. Formalin-inactivated vaccines are being used against EEE and WEE, but vaccination needs to be repeated every year (for a review see Gibbs, 1976).

The lack of alphavirus vaccines for humans is partly due to the only sporadic epidemics of these viruses in recent years. However, the RRV epidemic in the South Pacific indicates that under the right circumstances alphaviruses can pose serious problems. Information on the structure and location of the neutralising epitopes in alphavirus proteins may be useful in designing control measures against these viruses. In a section of this thesis we determine the location of RRV neutralising epitopes in the primary amino acid sequence of the structural proteins.

1.2 Structure of the alphavirus virion

The most detailed structural studies of alphaviruses have been on SIN and SFV. In electron micrographs, negatively stained alphaviruses appear as spherical particles of about 50-55 nm diameter, to which 7 nm long spikes are attached in radial orientation (Figure 1.1). In thin sections an icosahedral nucleocapsid surrounded by a lipid bilayer can be visualised (see Murphy, 1980).

The alphavirus nucleocapsid consists of about 300 molecules of capsid protein (mol. wt. ~30K) complexed with

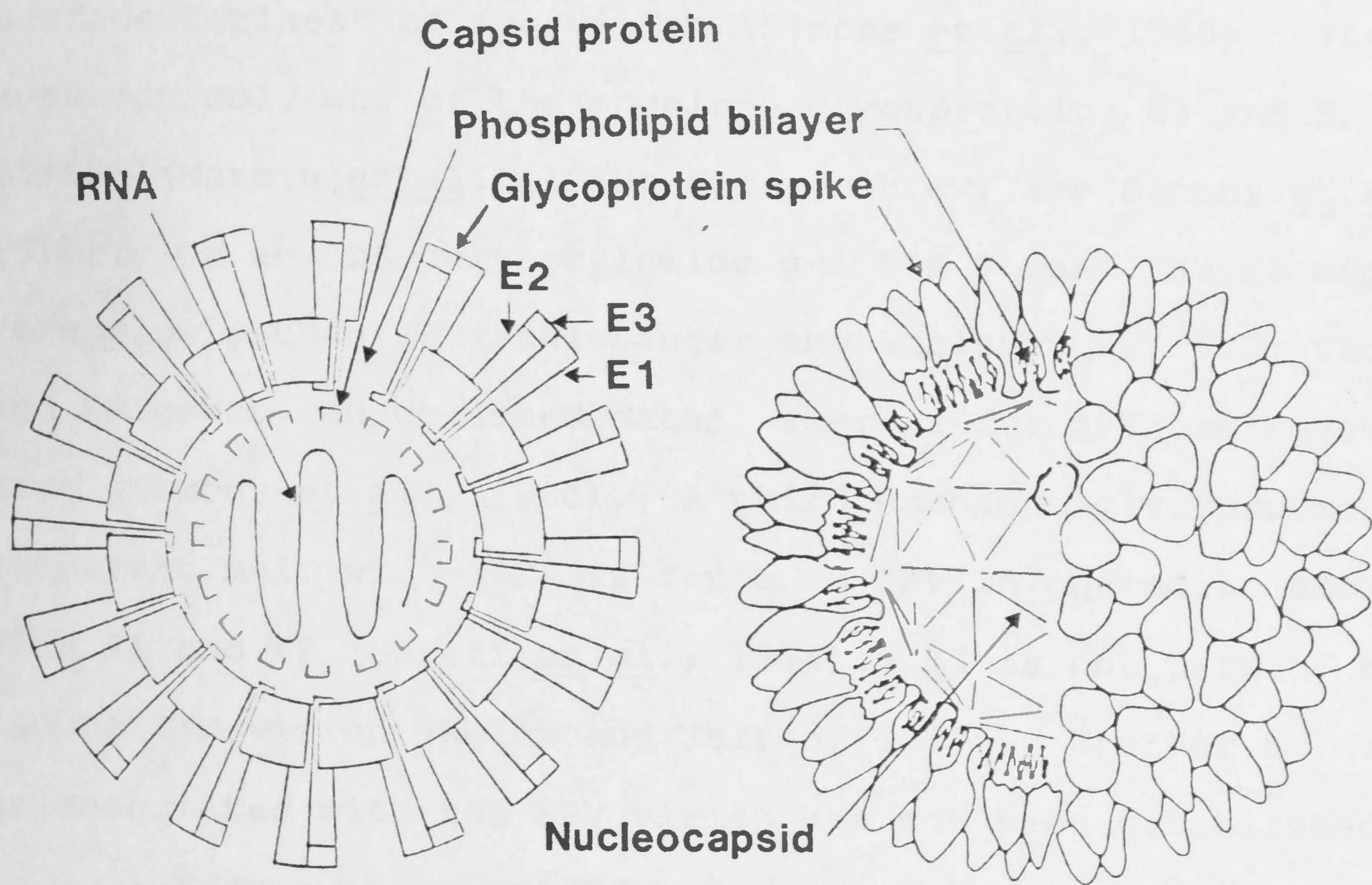


Figure 1.1 Diagrammatic representation of the alphavirus virion.
(From Faragher, 1982 and McCarthy and Harrison, 1977)

single stranded 49S genomic RNA (Friedman, 1968; Scheele and Pfefferkorn, 1969; Söderlund, 1973). This is surrounded by an envelope of host derived plasma membrane (Strauss and Strauss, 1977; Murphy 1980). The envelope membrane contains phosholipids together with two or three (E1, E2 and in the case of SFV, E3) glycosylated viral proteins which constitute the surface "spikes" of the virion (Simons et al., 1980). The apparent mol. wt. of the envelope glycoproteins E1 and E2 is 45-59K (Martin et al., 1979; for a review, see Simons et al., 1980). On an SDS-polyacrylamide gel SIN E1 and RRV E1 migrate more slowly than E2 (Schlesinger and Schlesinger 1972; Short and Dalgarno, unpublished data) whereas for SFV the reverse is true (Simons et al., 1980). A third envelope glycoprotein, E3 (apparent mol. wt. ~10K) is found in SFV in equimolar amounts with E1 and E2 (Garoff et al., 1974). E3 is not part of the mature SIN virion (Welch and Sefton, 1979). Whether or not E3 is associated with the RRV virion has not been established.

Sefton et al. (1973) showed that when SIN is iodinated enzymatically using lactoperoxidase, E2 is more iodinated than E1, suggesting that E2 may be more exposed on the virion surface than E1. This may not be the case, however, since the extent of iodination will depend upon the number of tyrosine residues exposed to the outer environment in the three dimensional structure.

Treatment of intact SFV virions or of detergent solubilised glycoproteins with bifunctional reagents leads to the covalent cross linking of E1 and E2 (Ziemiecki and Garoff,

1978). Similar observation were made for SIN (Rice and Strauss, 1982a). Since such bifunctional reagents are capable of establishing cross links within a distance of about 11 angstroms (Bickle et al., 1972) it was suggested that E1 and E2 may exist as heterodimers in SFV and SIN (Ziemiecki and Garoff, 1978; Rice and Strauss, 1982a). Kielian and Helenius (1985) have demonstrated that in SFV E1 and E2 interact with each other to form stable heterodimers; the interactions are concentrated in or near the transmembrane domains of the two proteins (see Section 1.5). It has been observed that binding of anti-E1 or anti-E2 antibodies to Triton X100 solubilised E1-E2 complexes can induce rearrangement leading to their disruption (Ziemiecki and Garoff, 1978; Rice and Strauss, 1982a).

1.3 The alphavirus genome

The alphaviruses contain a single stranded RNA genome of positive polarity which can serve as mRNA in infected cells. Expression of the genome does not require a virus-associated polymerase (reviewed in Kennedy, 1980a).

SIN is the only alphavirus for which the complete genome sequence has been determined; the genome is 11,703 nucleotides in length excluding the poly(A) tail, with a calculated mol. wt. of 4×10^6 (Strauss et al., 1984) and a sedimentation coefficient of ~49S (Kennedy, 1980a).

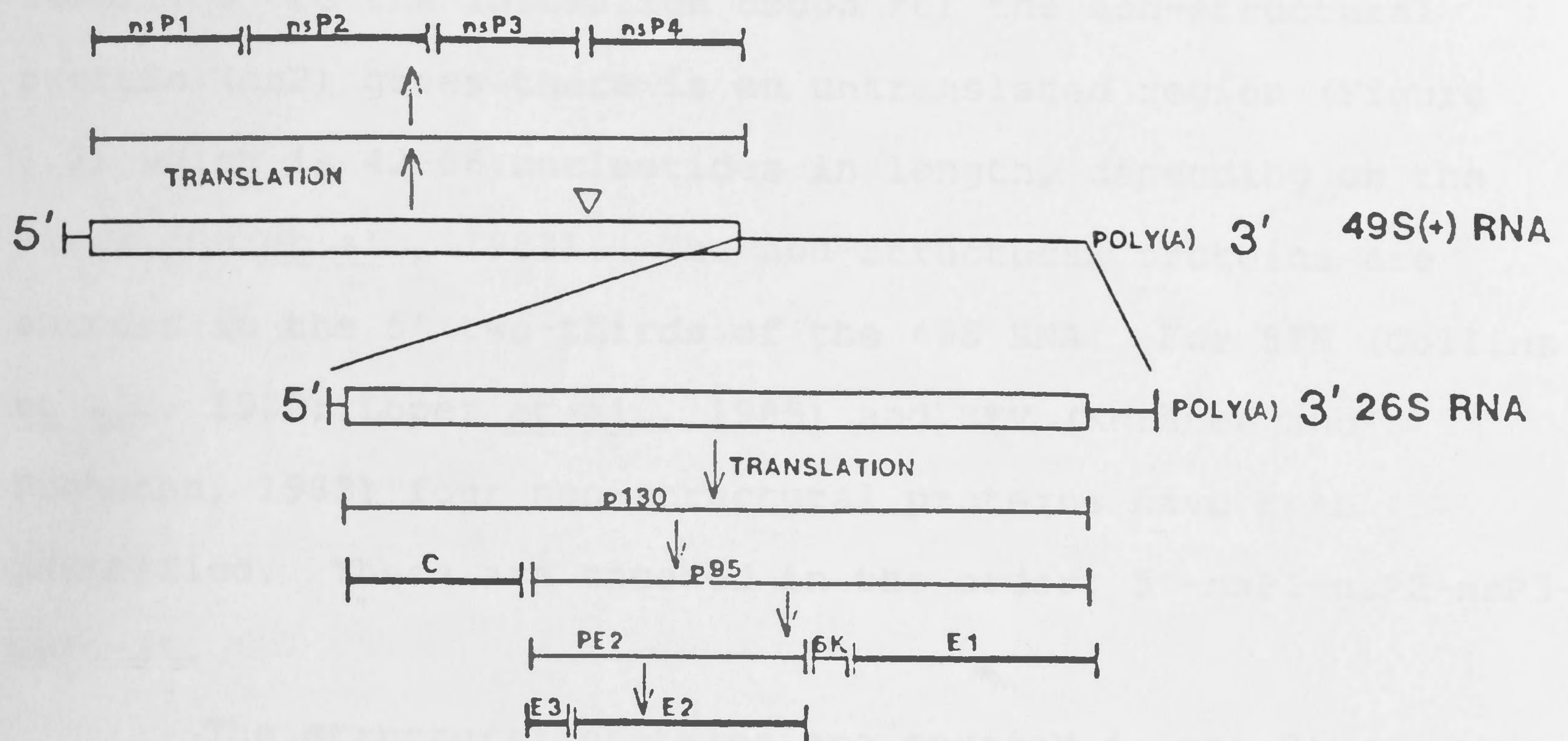


Figure 1.2 **Alphavirus genome and replication strategy.**
(from Meek, 1986)

The figure is a schematic representation of the alphavirus genome, and the translation and processing of the proteins. Untranslated regions of the genome are shown as a single line, and the translated regions as an open box. The subgenomic 26S RNA is expanded below using the same convention. The final protein products are indicated with heavy lines. For the structural proteins the complete processing is shown. For the non-structural proteins the initial polyprotein and the final products only are shown. The opal codon following the nsP3 gene in SIN is indicated by ▽.

In common with many eukaryotic mRNAs, the 5'-end of the alphavirus genome is capped and methylated (Dubin et al., 1979). 5' to the initiation codon for the non-structural protein (nsP) genes there is an untranslated region (Figure 1.2) which is 42-86 nucleotides in length, depending on the virus (Ou et al., 1983). The non-structural proteins are encoded in the 5' two-thirds of the 49S RNA. For SIN (Collins et al., 1982; Lopez et al., 1985) and SFV (Keränen and Ruohonen, 1983) four non-structural proteins have been identified. These are encoded in the order: 5'-nsP1-nsP2-nsP3-nsP4-3'.

The structural proteins are encoded in the 3' one-third of the 49S RNA in the order: 5'-C-E3-E2-6K-E1-3' in SIN (Rice and Strauss, 1981), SFV (Garoff et al., 1980a,b) and RRV (Dalgarno et al., 1983). Between the structural protein genes and the non-structural protein genes there is a short untranslated region (the "junction region") which varies in length between alphaviruses from 47-50 nucleotides (Ou et al., 1982a).

The alphaviruses also have a 3'- untranslated region which, depending upon the virus, is 264-524 nucleotides in length and contains repeat sequence blocks (Ou et al., 1981, 1982b; Dalgarno et al., 1983). The RRV T48 3'- untranslated region contains four repeats of around 50 nucleotides each. Interestingly, the 3'- proximal repeat sequence is conserved in all geographic isolates of RRV examined. However, 5' to this region deletions, insertions, sequence rearrangements and

single nucleotide substitutions are observed between RRV isolates (Faragher and Dalgarno, 1986). These workers suggested that the conserved 3'-proximal sequence block has an important role in virus replication.

At the 3' terminus the alphavirus genome contains a poly(A) tract of 50-120 nucleotides (Eaton et al., 1972; Johnston and Bose 1972a,b; Clegg and Kennedy, 1974). The biological significance of this poly(A) tail is unknown, but it may play a role in the stability, translation or replication of the RNA (Kennedy, 1980a).

1.4 **Alphavirus replication**

Alphaviruses replicate in the cytoplasm of vertebrate or invertebrate cells over a temperature range of 20-41°C (Pfefferkorn and Shapiro, 1974; Follet et al., 1975; Strauss and Strauss, 1977). At 37°C the one-step growth curve is complete 6 to 10 hr post infection (pi) (Dulbecco and Vogt, 1954; Rubin et al., 1955; Veckenstedt and Wagner, 1973). Multiplication of alphaviruses in vertebrate cells usually causes cytopathic effects and the host cell dies 10-12 hr pi at 37°C (Hardy and Brown, 1961; Acheson and Tamm, 1967; Erlandson et al., 1967). In a cytolytic infection, up to 20,000 progeny particles are released from the cell into the medium (Pfefferkorn and Shapiro, 1974; Tuomi et al., 1975; Strauss and Strauss, 1977). In Aedes albopictus (mosquito) cells alphaviruses establish a persistent infection and do not

generally produce cytopathic effects (Raghow *et al*., 1973; Davey and Dalgarno, 1974; for a review see Stollar, 1980). The various steps involved in alphavirus replication are reviewed below.

Entry of virus into the cell. Initiation of infection of vertebrate cells by alphaviruses is thought to be by adsorption to specific cellular receptors through the virus envelope glycoproteins (Fries and Helenius, 1979; Marsh and Helenius, 1980; for reviews see Choppin and Scheid, 1980; Dimmock, 1982). However little is known about the cellular receptor or the receptor binding site on the virus (the anti-receptor site). Although binding studies have shown that histocompatibility antigens can bind SFV (Helenius *et al*., 1978), SFV grows in cells lacking histocompatibility antigens showing that they are not necessary for SFV attachment (Oldstone *et al*., 1980).

It is not clear whether E1, E2 or both are involved in virus attachment to the cell. However Baric *et al*. (1981) have presented evidence that E2 plays a role in penetration. These workers, following 11 serial passages of SIN in BHK cells, isolated an attenuated mutant which had an increased "penetration rate" measured as the rate of acquisition of resistance by infecting virus to neutralisation by polyclonal antibody during infection of BHK cells. The mutant had altered reactivity to neutralising anti-E2 mabs; an epitope in E2 was identified as a region important in the process of

"penetration" since penetration revertants of attenuated SIN also reverted with respect to reactivity with anti-E2 mabs (Olmsted et al., 1986).

After attachment, SFV and SIN are internalised by adsorptive endocytosis into coated vesicles and transferred to prelysosomal endocytotic vesicles or endosomes where the acid pH triggers the fusion of viral and endosomal membranes, releasing the nucleocapsid into the cytoplasm (Helenius et al., 1980; Marsh et al., 1983; Cassel et al., 1984).

The fusion activity probably resides in E1, since purified E1 causes hemolysis in vitro due to membrane fusion (Chanas et al., 1982). The low pH in endosomes may induce a conformational change in E1 such that a fusion domain (see Section 1.5) is exposed; this could insert into a neighbouring lipid bilayer to initiate fusion and release the nucleocapsid into the cytoplasm (Garoff et al., 1980a). The 49S RNA is released from the viral nucleocapsid by an unknown mechanism.

Translation of 49S RNA. The released 49S RNA acts as an mRNA for the synthesis of the non-structural proteins during the first 3 hr of infection (Friedman and Grimley, 1969; Scheele and Pfefferkorn, 1969; Wengler and Wengler, 1975). The four non-structural proteins (nsP1-4) are derived by cleavage of a precursor polyprotein (Keränen and Ruohonen, 1983; Strauss et al., 1984). In SIN, translation of nsP4 is controlled by an opal (UGA) termination codon at the 3′ end of the nsP3 gene. Occasional read through of this codon allows synthesis of a

polyprotein containing nsP4 (Strauss et al., 1984). SFV, however, does not have this opal codon (C.M. Rice, personal communication). Although the precise functions of the non-structural proteins are unknown, they must include activities controlling recognition of replicase binding sites for minus- and plus-strand 49S RNA synthesis and the 26S RNA synthesis. They may also include a virus specific protease to process the polyprotein (Strauss and Strauss, 1983).

Viral RNA synthesis. Plus-strand 49S RNA is copied by the viral polymerase to form minus-strand 49S RNA. It has been proposed that minus-strand synthesis is initiated by the replicase binding to a conserved 19 nucleotide sequence adjacent to the poly(A) tail (Ou et al., 1981). Minus-strand 49S RNA is made only in small amounts and its synthesis ceases early in infection as the polymerase enzymes are short lived (Sawicki and Sawicki, 1980). The encapsidation of the plus-strand 49S RNA may also reduce the availability of template 49S RNA for minus-strand synthesis.

Minus-strand 49S RNA acts as template for synthesis of both plus-strand 49S RNA and subgenomic 26S RNA. The 3'- end of the minus strand 49S RNA must contain a replicase binding site for the synthesis of plus-strand 49S RNA although the sequence involved has not been identified (Ou et al., 1983). Initiation of transcription of 26S RNA from minus-strand 49S RNA is probably by binding of transcriptase to a conserved 21 nucleotide sequence in the "junction region" (Ou et al.,

1982a). This sequence is not homologous to that at the 3′-end, suggesting that different enzymes function for synthesis of 26S RNA and minus-strand 49S RNA.

The molar ratio of 49S to 26S RNA synthesis varies between 0.2 and 0.8 depending upon the virus and cell system used (see Kennedy, 1980b). Since much of the 49S RNA enters nucleocapsids and is unavailable for translation, up to 90% of viral messenger RNA in infected cells may be 26S RNA (Kennedy 1980b; Strauss and Strauss, 1983).

Translation and processing of structural proteins. The subgenomic 26S RNA is translated in a single long open reading frame as a precursor polyprotein (p130) which is then processed by proteolytic enzymes to form viral structural proteins (Figure 1.2) (Schlesinger and Kääriäinen, 1980). The C protein is probably cleaved from the nascent polyprotein by an autoproteolytic activity (Hahn et al., 1985) and remains transiently associated with the 60S ribosomal subunit before complexing with 49S RNA to form nucleocapsids (Söderlund and Ulmanen, 1977). The remaining polypeptide (p95) contains E2 and E1, together with their respective signal sequences (E3 and the 6K proteins). Removal of the capsid protein from p130 exposes the signal sequence at the N-terminus of E3, which results in the integration of p95 into the endoplasmic reticulum (Wirth et al., 1977). Cleavage of the precursor of E2 (PE2, containing E2 and E3) from p95, possibly by signalase (Rice and Strauss, 1981), exposes a signal sequence in the 6K

protein which inserts the 6K protein and E1 into the endoplasmic reticulum (Hashimoto _et al_., 1981). Signalase may cleave the 6K protein from the E1-6K complex (Rice and Strauss, 1981). In SIN replication both E3 and the 6K proteins are released into the culture medium (Welch and Sefton, 1979) whereas for SFV the 6K protein is released into the medium (Welch and Sefton, 1980) while E3 is incorporated into the mature virion (Garoff _et al_., 1974).

Asparagine-linked glycosylation of envelope proteins takes place by host cell enzymes in the lumen of the endoplasmic reticulum during their synthesis (Burge and Huang, 1970; Grimes and Burge, 1971; Keegstra _et al_., 1975; Schlesinger _et al_., 1976; for a review see Schlesinger and Kääriäinen, 1980). The tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X is any amino acid, serves as the recognition sequence for asparagine-linked glycosylation (Mayne _et al_., 1985) which involves the _en bloc_ transfer of a mannose and N-acetylglucosamine oligosaccharide (the "simple" carbohydrate chain) from a dolichol pyrophosphate intermediate to the potential attachment site in the envelope protein (Waechter and Lennarz, 1976). Trimming of these carbohydrate chains and addition of different glycosides to form a "complex" type chain occurs during transport through the Golgi apparatus (Mayne _et al_., 1985). Fatty acid acylation of E1 and E2 also probably occurs during transport through the Golgi apparatus (Schmidt and Schlesinger, 1980).

Assembly, maturation and release. The virus envelope proteins are probably transported to the plasma membrane from the Golgi apparatus by membrane vesicles which fuse with the cell surface (Garoff *et al*., 1982). Cleavage of PE2 by a cellular protease may occur during the transport (Garoff *et al*., 1982). Virus RNA and capsid protein aggregate to form nucleocapsids in the cytoplasm; these migrate to and associate with the cytoplasmic side of the host cell membrane which has been modified by the insertion of virus-specified proteins destined to become the glycoproteins of the viral envelope. Envelopment of the nucleocapsid in the modified membrane is followed by budding which results in release of virus from the cell (reviewed by Brown, 1980).

Effect of viral replication on the host cell. Replication of alphaviruses in vertebrate cells is generally cytolytic. Host DNA, RNA and protein synthesis are shut down and cell division is inhibited, leading to cell death (Lust, 1966; Mussgay *et al*., 1970; Atkins, 1976; Wengler and Wengler, 1976; Wengler, 1980).

Various mechanisms have been proposed for host protein synthesis inhibition during alphavirus infection. SIN infection of chick cells leads to an increase in intracellular Na^+ concentration and a decrease in intracellular K^+ concentration. This favours viral mRNA translation and inhibits translation of the great majority of cellular mRNAs (Garry *et al*., 1979a,b). Van Steeg *et al*. (1984) showed that

SFV capsid protein can interfere specifically with binding of host mRNAs into initiation complexes thus causing the shut down of host protein synthesis in neuroblastoma cells. It is not known whether these different mechanisms operate only in the different cells examined or whether both mechanisms operate simultaneously.

1.5 Functional domains in alphavirus envelope proteins

The complete amino acid sequences of the envelope proteins of three alphaviruses, SFV (Garoff _et al_., 1980a), SIN (Rice and Strauss, 1981) and RRV (Dalgarno _et al_., 1983) have been deduced from the sequence of the 26S RNA which encodes these proteins. Comparison of the deduced amino acid sequences of RRV, SFV and SIN structural proteins has identified a number of domains which are conserved either as to amino acid sequence, hydropathy characteristics or in the position of predicted asparagine-linked glycosylation sites (Dalgarno _et al_., 1983). Since SIN, SFV and RRV are thought to have evolved by divergence from a common ancestor (Bell _et al_., 1984) this conservation of sequence in the face of evolution is thought to reflect the functional importance of these regions. Thus different domains in the envelope proteins have been predicted to be associated with various important biological functions (Dalgarno _et al_., 1983).

A section of this thesis examines aspects of the biological role of RRV E2. This involves an examination of the

biological properties of a number of RRV mutants altered in E2. In order to assist in interpreting the results we describe below the important features of different domains of alphavirus E2. Since in virions E2 and E1 exist as heterodimers, it is possible that they constitute a functional dimer. We, therefore, also review features of various domains in E1.

Glycoprotein E2. The amino acid sequence of E2 deduced from the nucleotide sequence of the 26S RNA predicts that E2 is 423 amino acids long for SIN (Rice and Strauss, 1981) and 422 amino acids long for SFV (Garoff *et al*., 1980a) and RRV (Dalgarno *et al*., 1983). Predicted amino acid sequence homology between RRV E2 and SFV E2 is 69%; it is 42% between RRV E2 and SIN E2 (Dalgarno *et al*., 1983). The overall degree of amino acid sequence conservation in E2 is less than that in E1 (see below). This together with the fact that E2 carries type specific neutralisation activity (Dalrymple *et al*., 1976) suggests an evolutionary function of E2 in generating strain diversity (Dalgarno *et al*., 1983).

E2 has a conserved hydrophobic membrane-spanning domain (residues 364-389) near the C-terminus which is poorly conserved at the amino acid sequence level (Dalgarno *et al*., 1983). This domain contains covalently attached palmitic acid residues (Schmidt *et al*., 1979; Rice *et al*., 1982b) the precise position and role of which is unknown.

C-terminal to the transmembrane domain is a stretch of approximately 30 hydrophobic amino acids which form the

cytoplasmic domain. Amino acid sequence conservation is higher in the cytoplasmic domain than in the transmembrane domain (Dalgarno _et_ _al_., 1983). This domain may interact with the capsid during maturation (Simons _et_ _al_., 1980; Brown, 1980; Rice and Strauss, 1981; Strauss and Strauss, 1985).

In RRV, SFV and SIN, E2 has two potential attachment sites for asparagine-linked oligosaccharides (Dalgarno _et_ _al_., 1983; Mayne _et_ _al_., 1985). The first of these (at Asn 196 for SIN and Asn 200 for SFV and RRV) is conserved as to position. The second site is not conserved as to position since it is at Asn 262 in SFV and RRV and at Asn 318 in SIN (Dalgarno _et_ _al_., 1983). For SIN the first site is always linked to a "complex" carbohydrate whereas the second may be linked to a "simple" or "complex" carbohydrate depending upon the host cell (Mayne _et_ _al_., 1985). The conservation of the location of the first site and the conservation of the nature of the carbohydrate attached to it may suggest the importance of the first glycosylation site in virus maturation (Mayne _et_ _al_., 1985). Datema _et_ _al_., (1984) showed that in SIN infection cleavage of PE2 to E2 is inhibited by the presence of a glucosidase inhibitor which prevents processing to "complex" chains, thus indicating the importance of a "complex" carbohydrate chain at the first site in virus maturation.

Amino acids 1-363 constitute the external domain of E2 which does not contain any strongly conserved sequences. Since parts of the external domain will be exposed to the outer environment this may contain amino acid sequences involved in

virus-cell interaction. Baric et al. (1981) showed that SIN E2 is involved in penetration (Section 1.4). Thus E2 may contain the receptor binding site. However the region of E2 involved in penetration or the location of receptor binding site has not been identified.

The E2 protein contains antigenic sites which induce and react with neutralising antibodies (Dalrymple et al., 1976; Schmaljohn et al., 1983; Boere et al., 1984; Stanley et al., 1985; Stec et al., 1986). However the location of neutralising antigenic site(s) has not been determined in the primary amino acid sequence of E2 for any alphavirus. In this thesis we determine the location of RRV neutralisation epitopes in the primary amino acid sequence of E2.

There are reports in literature of alphavirus mutants with large deletions in E2 (Bracha and Schlesinger, 1978; Leone et al., 1980). However the precise location of these deletions and their effect on biological properties of the mutants are not known. A mutant of RRV T48 has been isolated which, on the basis of nucleotide sequencing, has been predicted to have a deletion of seven amino acids between residues 55-61 in E2 (Dalgarno et al., 1983; S.G. Faragher, unpublished data). In a subsequent Chapter of this thesis we examine the effect of this deletion on the biological properties of the virus in an attempt to understand the biological role of E2.

Glycoprotein E1. E1 is predicted to be 430 amino acids long for SIN (Rice and Strauss, 1981) and 438 amino acids long

for SFV (Garoff et al., 1980a) and RRV (Dalgarno et al., 1983). Amino acid homology between RRV E1 and SFV E1 is 79%; for RRV and SIN the corresponding figure is 51% (Dalgarno et al., 1983).

Towards the N-terminus of E1 there is a stretch of hydrophobic amino acids (residues 80-96) which is highly conserved between RRV, SFV and SIN and which may be responsible for the fusion activity of the virus and of E1 (Garoff et al., 1980a, Rice and Strauss 1981, Dalgarno et al., 1983). This domain lies within a larger conserved region (residues 45-108).

The transmembrane domain of E1 (residues 413-436 in RRV T48) is strongly hydrophobic and is close to the C-terminus; it is not well conserved between alphaviruses at the sequence level (Dalgarno et al., 1983). This domain is followed by two C-terminal arginine residues which are believed to represent a small cytoplasmic "stop transfer" signal (Rice et al., 1982b). The transmembrane domain contains covalently attached palmitic acid residues (Schmidt et al., 1979; Rice et al., 1982b); the location of the attachment site is unknown. Schmidt and Lambrecht (1985) have suggested an involvement of protein-bound fatty acids in the induction of membrane fusion by SFV.

E1 has one or two asparagine-linked glycosylation sites depending upon the virus. SIN, SFV and RRV all have a "complex" carbohydrate chain attached to Asn 139 (SIN) or Asn 141 (SFV, RRV) of E1 and thus the "complex" chain site is conserved as to position. SIN has a second, "simple" carbohydrate chain at Asn 245 (Mayne et al., 1985; Strauss and

Strauss, 1985). Since the location of the first site is conserved it appears that this may be essential for virus maturation (Mayne et al., 1985).

E1 contains antigenic sites which induce haemagglutination inhibiting (HI) antibodies (Dalrymple et al., 1976; Chanas et al., 1982; Schmaljohn et al., 1983; Boere et al., 1984; Stanley et al., 1985; Stec et al., 1986). However the location of these sites is yet to be identified.

This view of the biological function of E2 and E1 may be complicated if the two proteins exist as a functional unit, in which case alteration(s) in either protein may affect the function(s) attributed to another protein.

1.6 **Antigenic analysis of alphavirus envelope proteins**

One of the first reports on the antigenic structure of alphaviruses was that of Pederson and Eddy (1974) on the antigenic characterisation of VEE structural proteins. They showed that rabbit antiserum against VEE E2 neutralised virus infectivity in plaque reduction neutralisation tests (PRNT) and inhibited haemagglutination; anti-E1 serum did not possess any significant HI activity or neutralising activity. Dalrymple et al. (1976) observed that polyclonal rabbit antiserum raised against purified SIN E1 inhibited haemagglutination but did not neutralise virus infectivity in PRNT; rabbit anti-E2 serum neutralised SIN infectivity in PRNT and lacked HI activity.

Helenius et al. (1976) showed that antibodies against SFV E1 had HI activity. Thus in general antibodies to E2 neutralise virus infectivity and antibodies against E1 inhibit haemagglutination. It should be noted that the antigenic properties of VEE, where both neutralisation and haemagglutination activities are located in E2, are different from that of SIN and SFV.

Monoclonal antibodies (mabs) have been extensively used in studying virus antigenic structure (for a review see Yewdell and Gerhard, 1981). Competitive binding assays (CBAs) have been used to provide information on the topographic arrangement of the antigenic sites in a protein. CBAs have limitations, however, as they do not discriminate between two independent but physically close epitopes since binding of a mab to an epitope may sterically hinder the binding of another mab to a physically close epitope, thus defining the two discrete epitopes in the same antigenic site.

A number of spatially distinct antigenic sites on E1 and E2 of alphaviruses have been demonstrated by CBAs. Thus for SIN, Schmaljohn et al. (1983) defined five sites on purified E1 and one site on purified E2. However, in the SIN virion only one site on each of E1 and E2 could be detected indicating that four out of the five SIN E1 sites are masked in intact virion; mab binding to either of these two sites neutralised virus infectivity (Schmaljohn et al., 1983). More recently, the same group of workers with a further anti-E2 mab defined an additional neutralisation site on SIN E2, thus

giving a total of two neutralisation sites on E2 and one on E1 (Stec *et al*., 1986). The existence of a neutralisation site on SIN E1 has also been demonstrated by Chanas *et al*. (1982).

For SFV, using intact purified virus in CBAs Boere *et al*. (1984) demonstrated a total of 11 antigenic sites, six on E1 and five on E2; mabs to three of the sites on E1 and two on E2 neutralised virus infectivity. Using a similar method Roehrig *et al*. (1982) defined seven antigenic sites for VEE, four on E1 and three on E2; one site on E2 was involved in neutralisation. For RRV, C. Fernon and R.C. Weir (unpublished data) have detected at least two antigenic sites on E2, mabs to one of these sites were neutralising; no anti-E1 mab neutralised virus infectivity.

Thus it appears that both anti-E1 and anti-E2 mabs can neutralise alphavirus infectivity. However, the number of neutralisation sites and their distribution on E1 and E2 is different between different alphaviruses. The difference could be partly explained in terms of the different number of mabs used to study the antigenic structure of different alphaviruses (e.g. Stec *et al*., 1986).

Although the studies based on CBAs have provided information on the topographic arrangement of the antigenic sites in alphavirus structural proteins, there is no information on their structure and location in the primary sequence of the proteins. In a Chapter of this thesis we investigate the location of neutralising epitopes in the RRV structural proteins.

1.7 Alphavirus virulence and the role of the structural proteins

The virulence of a virus is a measure of its ability to cause disease in the host (Mims and White, 1984); the term refers to those properties of a virus which lead to serious disease symptoms in the host by overcoming the host defences. Therefore virulence is a compound of the reactions of host and virus. The question of what properties of a virus determine its degree of virulence is important in developing effective control measures against the virus. In general, rapid growth of the virus is considered important in determining whether a virus will overcome the host's immune response and fully express its "inherent virulence" (Schlesinger, 1980).

Our understanding of virulence determinants is most advanced for myxo-, paramyxo- and reoviruses. For these viruses, growth rates and tissue tropisms are determined, at least in part, by differences in envelope proteins which are involved in receptor recognition, virus attachment and penetration, and membrane fusion to initiate the infection (Homma and Oguchi, 1973; Nagai et al., 1976; Gething et al., 1978; Bosch et al., 1979; Huang et al., 1980; Fields and Green, 1982; Webster et al., 1982; Webster et al., 1986).

A number of approaches have been used to investigate virulence determinants of alphaviruses at the molecular level. These have involved a comparison of the growth of virulent and

avirulent strains in cultured cells and in mice, a brief review of which is presented below.

Comparison of virulent and avirulent alphaviruses in cultured cells. Atkins (1983) examined the replication of mouse-virulent and avirulent SFV isolates in BHK cells where the avirulent strain grew to higher titers than the virulent strain although the virulent SFV made more virus-specific RNA than the avirulent strain. The avirulent strain grew poorly compared to the virulent SFV in C1300 cells of neuronal origin. This was consistent with the observation that the avirulent strain did not infect neurons in mice.

A major problem in comparing such naturally occurring isolates is that many mutations unrelated to virulence may have accumulated during the separate evolution of the two isolates. More useful comparisons can be made using clonally related variants of different levels of virulence.

Mecham and Trent (1983) compared the replication of the equine-virulent TRD strain of VEE with the avirulent vaccine strain TC-83 derived from TRD by 83 tissue culture passages. In Vero cells the two strains had similar latent periods, although TC-83 grew more slowly than the TRD strain for first 12 hr; at later stages the two reached similar titers. The TRD strain made more viral RNA than TC-83 and caused more rapid shut down of host protein synthesis. Mecham and Trent (1983) suggested that reduced virus replication rates in cultured cells may be related to the reduced virulence in equines; they

suggested that changes may have occurred in sections of genome controlling RNA replication although genetic differences between the two strains were not examined.

Baric et al. (1981) compared growth and RNA synthesis in BHK cells of mouse-virulent SIN and its BHK cell passaged derivative (see Section 1.4) which was attenuated for mice. The attenuated SIN "penetrated" (for definition see Section 1.4) faster, had a reduced latent period and grew to higher titers than virulent SIN in BHK cells. Attenuated SIN had a peak viral RNA synthesis rate twice that of virulent SIN in BHK cells. This difference in RNA synthesis rates did not co-vary with virulence since the virulent revertant of attenuated SIN maintained a higher rate of RNA synthesis in BHK cells (Olmsted et al., 1984). However, rapid "penetration" in BHK cells was correlated to attenuation in mice and an alteration in neutralisation epitope(s) in E2 was associated with the changed "penetration" and virulence (Olmsted et al., 1986). It should be noted that a change in the sequence of E2, E1 or even C protein could affect the conformation of an epitope in E2. However the change in attenuated SIN was not localised to a specific protein by sequencing.

Meek (1986) compared growth and RNA synthesis in BHK cells, of mouse-avirulent RRV NB5092 and clonally related variants of markedly enhanced virulence derived from it by ten serial passages in mice. The virulent variants did not grow more rapidly in BHK cells than RRV NB5092 but did have higher RNA synthesis rates. Meek (1986) showed that one or two amino

acid changes had occurred in E2 of seven out of these eight virulence variants. Thus, in five variants, single amino acid changes occurred at positions 212, 232 or 251; in two variants there were two amino acid changes in each at positions 27 and 172, and at 72 and 234. No changes were found in E1, E3, C, the 6K protein or in the 3'- untranslated region of the 26S RNA of two of the variants sequenced. No extensive sequence data was obtained for the non-structural protein genes of the variants although changes in RNA synthesis rates suggested that mutations had occurred in regions of genome controlling RNA replication.

Comparison of virulent and avirulent alphaviruses in mice. The mouse has been the most common laboratory host for alphavirus studies *in vivo* (Shope and Sather, 1979). The virulence of a virus can be measured in terms of lethal dose (LD_{50}), the average survival time (AST) of the infected mice, and the time of onset and severity of disease symptoms.

In most cases alphavirus infection of mice is neurotropic, i.e. neuronal tissue is the primary site for virus replication, e.g. SFV (Atkins *et al*., 1985). Comparison in mice of SFV mutants of different virulence levels shows that mutants of reduced virulence have reduced replication in brain tissue (Pusztai *et al*., 1971; Bradish and Allner, 1972; Fleming, 1977; Barrett *et al*., 1980). Reduced growth in brain also correlates with attenuation of virulence of WEE (Takayama and Nakano, 1975) and VEE (Krieger *et al*., 1979) for mice.

RRV infection of mice is myotropic, the hind leg muscle being the primary site of virus replication (Murphy et al., 1973; Seay et al., 1981). Taylor (1972) compared the growth of mouse-virulent RRV T48 and of mouse-avirulent RRV NB5092 in different tissues of day-old mice. The highest titers for both strains were reached in hind leg muscle although RRV NB5092 grew more slowly and to lower titers than RRV T48 in blood, brain and muscle tissues of mice (Taylor, 1972, Mims et al., 1973; Murphy et al., 1973). Recently, Meek (1986) has reported on the growth in mice of clonally related virulent variants of RRV NB5092 altered in E2 (see above); he observed that the virulent variants attained a significantly higher titer in brain tissue than did avirulent RRV NB5092.

The studies described in this Section suggest that alphavirus structural proteins are one of the important determinants of virulence. However, in no case was the crucial genetic difference between virulent and avirulent strains established conclusively. A start towards investigating the relationship, if any, between changes in alphavirus structural proteins and virulence was made by Meek (1986). However, in these studies unidentified genetic differences in the non-structural protein genes may have been responsible for, or have contributed to virulence enhancement. To investigate the role of E2 as a virulence determinant we examine in this thesis the virulence and growth characteristics of mab-resistant variants of RRV T48 with defined point mutations in E2. In another section we examine the biological properties of an RRV T48

mutant with a seven amino acid deletion in E2 (see Section 1.5). Since these mutants were altered, we believe, only in E2 a change in their biological properties could more meaningfully be related to the known genetic change.

1.8 Genetic and the biological variants of RRV in nature

It has been demonstrated that in nature antigenically distinct RRV strains exist which differ in their mouse virulence (Woodroofe et al., 1977). For example, RRV NB5092 and RRV T48, isolated from mosquitoes at Nelson Bay in coastal New South Wales and at Townsville in northern Queensland respectively, had marked differences in their mouse virulence. Thus RRV T48 killed near 100% of day-old, one week- and two week-old mice; in contrast, RRV NB5092 had only a 24% mortality rate for day-old mice and caused no disease symptoms in week-old mice (Gard et al., 1973; Taylor and Marshall 1975a). It is thought that these two RRV antigenic types are enzootic to their respective regions and have evolved in isolation from a common ancestral virus (Woodroofe et al., 1977; Marshall et al., 1980). Recently, neutralising mabs have also demonstrated antigenic differences between RRV isolates from different geographical locations in Australia and the South Pacific (C. Fernon and R.C. Weir, unpublished data).

Further evidence for the existence of genetically different, enzootic strains of RRV has been presented by Faragher et al. (1985a) who compared TaqI and HaeIII restriction digest profiles of cDNA to the genomic RNA of 15

isolates of RRV from eastern Australia and the Pacific islands. They grouped the isolates into three genetic types on the basis of similarities in restriction digest profiles. RRV T48 and RRV NB5092 were placed in genetic types I and III respectively. RRV isolates from human EPA cases in Fiji, American Samoa and the Cook Islands showed identical restriction digest profiles (Faragher et al., 1985a). Thus it was concluded that a single RRV genetic type was involved in the outbreaks of EPA in the South Pacific islands. Interestingly, RRV NL26301 isolated from mosquitoes collected at Narran Lake in northern New South Wales, appeared to be closely related to the Pacific isolates and all were placed in genetic type II. Faragher et al. (1985a) estimated that nucleotide sequence divergence between the strains within a genetic type was <1.5% and was 1.5-5.0% between genetic types. Nucleotide sequencing of the 26S RNA of RRV T48 (genetic type I) and RRV NB5092 (genetic type III) has shown that the sequence divergence between these two strains is 2.3% (Meek, 1986).

Although isolated frequently from mosquito pools in Australia, RRV had not been isolated from an EPA patient until the South Pacific epidemic of 1979-81 (Aaskov et al., 1981b; Rosen et al., 1981; Tesh et al., 1981; Fauran and Le Conidec, 1982). Recently RRV was isolated for the first time from Australian EPA patients in Rockhampton (Aaskov et al., 1985). Interestingly an important common feature of the Pacific and Rockhampton EPA cases was that the patients did not have detectable RRV antibody when the first symptoms of disease

appeared (Rosen et al., 1981; Aaskov et al., 1985). This was in contrast to previous Australian cases where sera had RRV antibodies when symptoms of EPA first appeared (Clarke et al., 1973). This leads to several questions. Firstly, was virus isolable from human serum because of the changed properties (e.g. more rapid growth) resulting in viraemia before the appearance of antibody, or was isolation a result of a change in viral immunogenicity resulting in a delayed or poor immune response ? Secondly, are the Pacific and Rockhampton RRV isolates genetically similar, and what is the relationship between them and endemic RRV isolates ? And thirdly, do the isolates from the Pacific and Rockhampton represent a strain of RRV which is particularly virulent to humans ? Aspects of these questions will be addressed in a subsequent Chapter of this thesis.

1.9 Outline of the thesis

A major aim of the studies described in this thesis is to locate the neutralisation antigenic sites of RRV in the primary amino acid sequence of the viral structural proteins. Chapter 2 describes (i) the isolation of variants of RRV T48 and RRV NB5092 resistant to neutralising mabs, and (ii) sequencing studies on the structural protein genes of these variants to locate the neutralisation epitopes.

Since the RRV T48 mab-resistant variants had point mutations in E2 their biological properties were examined to

determine the effect of these changes on the biological properties of the virus. Chapter 3 describes studies on the growth of these RRV variants in cultured cells together with studies on their virulence and growth in mice.

RRV dE2, a mutant of RRV T48, has a predicted seven amino acid deletion in E2. This provided an opportunity to examine the effect of a major change in E2 on the biological properties of the virus. Chapter 4 compares the growth properties of RRV dE2 and RRV T48 in cultured cells together with comparative studies on the virulence and growth of the two in mice.

Chapter 5 uses another approach to understand the role of viral structural proteins in RRV virulence. Attenuated variants of RRV T48 were isolated by serial passage in BHK cells as described for SIN by Baric et al. (1981). The biological properties of the variants were examined in cultured cells and in mice and attempts made to locate changes in the structural protein genes.

Chapter 6 describes sequence studies on the E2 gene of RRV isolates from humans obtained at various stages of the epidemic of polyarthritis in the non-immune population in the South Pacific. Studies were directed at examining the extent and location of evolutionary changes in the E2 gene. Chapter 6 also describes the genetic relationship between the Pacific isolates, those obtained from human EPA patients in Australia and a number of enzootic strains obtained from mosquitoes in Australia. To this end, sequence studies on the E2 gene of these various isolates were carried out.

CHAPTER 2

LOCATION OF NEUTRALISATION EPITOPES IN RRV STRUCTURAL PROTEINS

2.1 INTRODUCTION

Competitive binding assays with mabs have been used to study the neutralisation antigenic sites of a number of alphaviruses (Chapter 1). However there is no published information on the precise location of these sites in viral structural proteins. The work described in this Chapter aims at locating the neutralisation epitopes of RRV in the primary amino acid sequence of the structural proteins.

A number of mabs have been generated against RRV, a small number of which neutralise the virus (R.C. Weir, unpublished results). These mabs were used to locate the neutralisation epitopes of RRV using the following strategy. RRV variants were selected which were resistant to neutralisation by individual mabs. The nucleotide sequence of the genes encoding the structural proteins of the variants was determined by the dideoxy method of Sanger _et al_. (1977) using synthetic oligodeoxynucleotide primers based on the RRV T48 26S RNA sequence (Dalgarno _et al_., 1983). The sequence of the variants was compared with that of the parental virus and the amino acid substitution(s) responsible for the mab resistant phenotype of the variants identified by comparing the deduced amino acid sequence of the structural proteins of the variants and the parental virus. The structural and functional relationship between different epitopes, identified in this way, was investigated by selecting and sequencing RRV antigenic

variants resistant to two or three mabs. Properties of the domain harbouring the neutralising epitopes were examined by comparing the primary amino acid sequences of RRV, SFV and SIN in the appropriate region. The hydropathy profile and the predicted secondary structure of the protein containing the neutralisation epitopes were generated to examine the structural features of the domain containing the neutralisation epitopes.

2.2 MATERIALS AND METHODS

2.2.1 Virus

The prototype T48 strain of RRV (Berge, 1975) was from the Yale Arbovirus Research Unit. The virus had been passaged ten times in mice when received. It was subjected to two plaque purification steps to remove a small plaque former (see Chapter 4). RRV NB5092 was from Dr. I.D. Marshall (John Curtin School of Medical Research, The Australian National University). The virus had been isolated from a pool of 50 *Aedes vigilax* mosquitoes collected at Nelson Bay, NSW in 1969 (Gard *et al*., 1973) and had been plaque purified twice by A.D. Meek before use. Working stocks of virus were tissue culture supernatants derived by growth in baby hamster kidney (BHK) cells infected at low multiplicity (~0.01).

2.2.2 Cell culture

BHK cells were propagated in Glasgow Modified Eagle's BHK medium (GMEM) supplemented with 8% heat-inactivated bovine serum. African green monkey kidney (Vero) cells were grown in Medium 199-Lactalbumin hydrolysate (M199-LAH; Commonwealth Serum Laboratories, Australia) supplemented with 10% heat-inactivated bovine serum as previously described (Davey and Dalgarno, 1974). Cells were maintained at 36°C in 5% CO_2/95% air.

2.2.3 Infection of cells

Cells were seeded on 35 mm plastic dishes (TC grade, Corning Technical Product, USA). Infecting virus was diluted in Hank's balanced salt solution (HBSS). Mock infections were with HBSS. Adsorption was for 1 hr at room temperature in the dark. Excess inoculum was removed by washing the cells with phosphate buffered saline (PBS). Zero time was taken as the time of addition of the inoculum to the cells. Following infection, medium was added and cells were incubated at 36°C in an atmosphere of 5% CO_2/95% air.

2.2.4 Plaque assay

Virus was assayed in duplicate by plaque formation on Vero cells in 6-well plastic trays (TC grade, Linbro Scientific Inc., USA). Virus (0.1 ml) was adsorbed for 60 min and

overlayed with 3.5 ml of overlay (Medium 199-LAH, 20 mM Hepes, 0.2% DEAE-Dextran, 2% foetal calf serum, 8% bovine serum, 1% agar). The trays were incubated at 36°C in 5% CO_2/95% air. After 2 days, 1.5 ml of staining medium (0.2% each of agar and neutral red in distilled water) was added to each well and plates incubated overnight before counting the plaques.

2.2.5 **Virus purification**

Virus for enzyme linked immunosorbent assay (ELISA) and RNA extraction was purified as described by Martin et al. (1979). Briefly, BHK cell monolayers in roller bottles were infected at a moi ~0.25. Infected cell supernatants were harvested at 24 hr pi and virus was precipitated with 8% polyethylene glycol, followed by resuspension in 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.4), 0.001 M EDTA. Virus was purified by sucrose gradient centrifugation and stored in sucrose at -70°C. Purified virus in sucrose was diluted appropriately in PBS for use in ELISA and immunoprecipitation. However, the virus was pelleted by centrifugation for RNA extraction (see Section 2.2.13).

2.2.6 **Preparation of amino acid labelled virus**

Confluent monolayers of BHK cells in roller bottles were infected (moi ~1). After adsorption cells were incubated with 100 ml Eagle's minimal essential medium (EMEM) containing one-tenth the normal concentration of amino acids and 10 μCi/ml

[^{3}H]-amino acid mixture (Amersham) at 37°C. The culture supernatant was harvested at 24 hr pi and virus purified as above.

2.2.7 **Production of hybridomas secreting anti-RRV monoclonal antibodies (Fazekas de St.Groth and Scheidegger (1980)**

Briefly, 6-week old female Balb/C mice were injected intra-peritoneally (i.p.) with ~10^5 PFU of purified virus in 0.1 ml gelatin saline (0.5% gelatin in PBS). Six weeks later, immunised mice were boosted with ~$3x10^7$ PFU of virus injected i.p. Two weeks later, 4 days before the cell fusion, mice were again boosted with ~$3x10^7$ PFU of virus injected intravenously. Approximately $5x10^7$ mouse myeloma cells (X63.Ag8.653) were fused with ~$3x10^7$ spleen cells from an immunised mouse using 50% polyethylene glycol. Fused cells were pelleted and resuspended in RPMI 1640 medium (Gibco; supplemented with 15% heat-inactivated foetal calf serum, 200 mM L-glutamine, 100 mM sodium pyruvate, 0.6% penicillin, 1.0% streptomycin and 0.05 mM 2-mercaptoethanol) mixed with HAT (final concentration: 0.1 mM hypoxanthine, 0.4 μM aminopterine and 0.016 mM thymidine). Cells were distributed in 96-well tissue culture plates and cultivated in RPMI 1640-HAT for two weeks to select hybridomas. This was followed by the cultivation in RPMI 1640 medium (supplemented as above) in presence of HT (final concentration: 0.1 mM hypoxanthine, 0.016 mM thymidine) for one week. By this stage successfully fused cells had grown to about 50-80%

confluency in the well. After an initial ELISA screening (see below), cells from those wells producing anti-RRV antibodies were subcultured and grown in 24-well plates. After an ELISA screening of the 24-well plates anti-RRV antibody producing cells were subcultured and grown in larger amounts in tissue culture bottles. Hybridomas producing anti-RRV antibodies were cloned by limiting dilution. Clones arising from a single cell were grown in tissue culture and the supernatant screened for RRV neutralising activity by plaque reduction neutralisation tests (PRNT, see below). Hybridomas secreting neutralising mabs were injected i.p. in pristane-primed-mice where they grew as ascites. The ascitic fluid was harvested and centrifuged to obtain supernatant containing mab which was stored at -20°C.

2.2.8 **Detection of anti-RRV antibody activity by ELISA (R.C. Weir, personal communication)**

Approximately 10^7 PFU of sucrose gradient purified RRV T48, appropriately diluted in PBS + 0.02% sodium azide, was used to coat 96-well polyvinyl chloride microtiter plates (25 µl/well). Plates were incubated in a humid atmosphere at 37°C for 2 hr. They were stored at 4°C until required (for up to one week). Before use, liquid from the wells was aspirated and the wells washed (x3) with 240 µl PBS containing 0.5% bovine serum albumin (or PBS containing 5% dried commercial defatted powdered milk) and 0.02% sodium azide. Hybridoma supernatant (25 µl) was added to each well and the plates incubated at

37°C. Polyclonal RRV-antiserum and RPMI 1640 respectively were used as positive and negative controls. After 2 hr, supernatant was removed and the wells washed (x3) with PBS containing 0.05% Tween-20 and 0.02% sodium azide. Urease-conjugated rabbit anti-mouse immunoglobulin (CSL, Australia) diluted in PBS containing 0.5% bovine serum albumin (or 5% dried milk powder), 0.05% Tween-20 and 0.02% sodium azide was added (25 µl) and the plates incubated at 37°C for 30 min. Conjugate was removed and plates washed (x6) with PBS containing 0.05% Tween-20 and 0.02% sodium azide followed by six washings with distilled water. Urea substrate (CSL, Australia) was added (50 µl/well) and plates incubated at 37°C for 30 min. Positive (purple colour) and negative (yellow colour) anti-RRV antibody activity was detected visually. For determination of end point titers, 10-fold dilutions of culture supernatant or ascitic fluid containing the mab were used. The highest dilution giving a positive reaction was taken as the titer of the mab.

2.2.9 **Plaque reduction neutralisation test (PRNT)**

Virus (100 µl; ~200 PFU) was incubated with an equal volume of ascitic fluid containing mab (diluted 1:10 in HBSS) in an Eppendorf tube at 37°C for 1 hr and assayed for infectivity on Vero monolayers (Section 2.2.4). HBSS was used in place of mab in controls.

2.2.10 **Production of polyclonal RRV antiserum**

Polyclonal antiserum to RRV T48 was produced as ascitic fluid in Balb/C mice by Dr. L. Dalgarno using the method of Tikasingh et al. (1966).

2.2.11 **Protein specificity of monoclonal antibodies reacting with intact virus (R.C. Weir, personal communication)**

[^{3}H]-amino acid labelled purified RRV T48 (10 µl, ~400 cpm) was incubated with an equal volume of undiluted mab in 0.5 ml Eppendorf tubes at 37°C. After 1 hr, 3 µl of GTNE buffer (200 mM glycine, 50 mM Tris, 100 mM NaCl, 1 mM EDTA; pH 7.4) containing 1% NP40 (Sigma) was added. After 10 min, 25 µl of 50% Protein-A Sepharose (Pharmacia) in GTNE buffer was added and the tubes incubated at room temperature for 1 hr. The tubes were centrifuged and the pellet washed (x3) with 250 µl GTNE buffer containing 1% NP40. The pellets were dissolved in 30 µl of electrophoresis sample buffer (Laemmli, 1970) containing mercaptoethanol and analysed by electrophoresis on a 10-20% gradient polyacrylamide slab gel (King and Laemmli, 1971). Radiolabelling was detected by impregnating gels with PPO, drying under vacuum, and exposing at -70°C to pre-flashed Fuji Medical X-ray film (Bonner and Laskey, 1974; Laskey and Mills, 1975).

2.2.12 Selection of RRV variants resistant to neutralisation by monoclonal antibodies

Approximately 5 x 10^6 PFU of RRV T48 or RRV NB5092 in 100 µl of HBSS was incubated (37°C, 1 hr) with an equal volume of ascitic fluid containing mab (diluted 1:10 in HBSS). The mixture was diluted in HBSS (x10) and used to infect Vero monolayers. After 1 hr adsorption overlay was added and the plates incubated at 36°C for 3 days. Virus which escaped neutralisation formed normal size plaques. No unusually small or large plaques were seen. Unstained plaques were picked, grown in BHK cells and tested for resistance to neutralisation by the selecting mab. The resistant virus was further plaque purified and grown in BHK cells to prepare seed stocks of the variants.

2.2.13 Extraction of virion RNA for sequencing (Ou et al., 1981)

Virus from peak gradient fractions (Section 2.2.5) was pelleted by centrifugation in an SW41 rotor at 36,000 rpm for 3 hr and resuspended in 0.01 M Tris-HCl (pH 7.4), 0.06 M NaCl, 0.001 M EDTA and 1% SDS. Virion RNA was extracted once with phenol and twice with chloroform/isoamyl alcohol (99:1, v/v), and precipitated 3 times with ethanol. RNA was suspended at a concentration of 2 pmole/7 µl (1.14 µg/µl in 5 mM Tris-HCl, pH 8.2, + 2.5 mM EDTA) and stored at -70°C. RNA concentration was

determined by spectrophotometry. The yield was 10-18 µg RNA/roller bottle.

2.2.14 Extraction of RNA for sequencing from RRV infected BHK cells (Shine and Dalgarno, 1973)

Dideoxy sequencing of RRV RNA using specific primers could be carried out with high molecular weight RNA from virus-infected BHK cells (A.D. Meek, personal communication). For RNA extraction BHK cell monolayers in 140 mm glass petri dishes were infected with virus (moi ~1). At 17 hr pi nucleic acids were extracted with a mixture of sodium p-amino salicylate and phenol/m-cresol/8-hydroxyquinoline. After extraction, nucleic acids were precipitated with ethanol and dissolved in 0.45 ml water. TNE buffer (50 µl; 0.12 M Tris-HCl, pH 7.4, 1.2 M NaCl, 0.01M EDTA) was added followed by 0.5 ml of 4 M LiCl to remove DNA and low mol. wt. RNA. After vigorous vortexing high mol. wt. (viral and ribosomal) RNA was pelleted by centrifugation. After a further LiCl precipitation, two ethanol precipitations of RNA in the presence of 0.3 M sodium acetate (pH 5.6) were performed. RNA was finally suspended at a concentration of 11.4 µg/µl (i.e. ten times the concentration of virion RNA, above) in 5 mM Tris-HCl (pH 8.2) containing 2.5 mM EDTA and stored at -70°C. The RNA concentration was determined by spectrophotometry. The RNA yield from one petri dish was 400-600 µg.

2.2.15 Dideoxy sequencing of viral RNA

The method of Sanger et al. (1977) as described by Faragher and Dalgarno (1986) was used. RRV T48 or RRV NB5092 RNA was sequenced in parallel when sequencing RRV variants. Occasionally a cross band, corresponding to a single nucleotide, was seen in all four lanes of the sequence ladder. If this was present in the same position in the RRV T48 or RRV NB5092 sequence ladder the nucleotide was considered to be the same as in RRV T48 (Dalgarno et al., 1983) or RRV NB5092 (S.G. Faragher and A.D. Meek, unpublished data) respectively, otherwise it was recorded as an unidentified nucleotide (N). Details of the method are described below.

Reaction mixture. Reaction mixtures contained in a final volume of 10 µl, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithriothreitol (Sigma), 50 mM dATP, dTTP and dGTP, 2.5 µM dCTP (all dNTPs from Boehringer-Mannheim), 10 µCi of [α-^{32}P] dCTP (3000 Ci/mmole; Amersham), either 10 µM ddATP, 5 µM ddTTP, 2 µM ddGTP or 0.25 µM ddCTP (all ddNTPs from Boehringer-Mannheim), 1 µl primer-template mix (see below) and 12 units of AMV reverse transcriptase (Molecular Genetic Resources, USA). Incubation was at 0° for 20 min, followed by 60 min at 42.5°C. One microlitre of a solution which was 0.5 mM in all dNTPs was then added and incubation continued at 42.5° for 20 min. Reactions were stopped with 4 volumes of formamide-dye mix (96% deionised formamide; 25 mM Tris-borate;

0.5 mM Na_2 EDTA; xylene cyanol and bromophenol blue dyes), followed by heating at 90° for 2 min.

Electrophoresis. Reaction products were separated on 80 cm long, 0.4 mm thick 5% polyacrylamide gels (acrylamide:bisacrylamide, 19:1). Gels were exposed at -20°C to Fuji RX medical X-ray film.

Primer-template mix. 3.5 µl of virion RNA (1 pmole) or infected cell RNA (11.4 µg of total RNA which contained approximately 1 pmole of virion RNA) was mixed with 1 pmole of primer (in 1 µl, see below) and incubated for 5 min at 56° or for 90 sec at 80°C. The mixture was quick cooled and KCl (0.5 µl; 0.8M) added.

The concentration of primer (diluted in 5 mM Tris-HCl, pH 7.4, 0.05 mM EDTA) was generally 1 pmole/µl which gave a molar ratio in the primer-template mix of 1:1 when virion RNA was the template. With primers E2/3559 and E2/1635 (Table 2.1) a ratio of 10:1 improved the quality of the sequence ladder.

Primers. Synthetic 15- or 17-residue oligodeoxynucleotide primers complementary to appropriate regions of RRV T48 26S RNA (Dalgarno *et al*., 1983) were from Biotechnology Research Enterprises, S.A. Pty. Ltd., Adelaide, South Australia (Table 2.1). Computer searches of RRV T48 26S RNA were conducted to check that the primers would not prime at a second site. These were done by A.D. Meek and S.G. Faragher

Table 2.1 **Synthetic oligonucleotide primers used to sequence RRV antigenic variants.**

Primer*	Nucleotide sequence	Priming site in RRV T48 26S RNA†
C/14	5'-GGTGTAACCATAGTA-3'	141-155
C/587	5'-AGGCGTCGGACTTCA-3'	587-601
E3/963	5'-TTGTCTTCCAGCATC-3'	963-977
E2/1455	5'-GGUGGGUAGAGAGAAGU-3'	1455-1471
E2/1635	5'-TTGTACCTGATAGTC-3'	1635-1649
E2/1990	5'-GATGATGCGCTCAGA-3'	1990-2004
E2/2350	5'-GGTTTTGTTCTCGTC-3'	2350-2364
E1/2755	5'-TCCACCCCACATGAA-3'	2755-2769
E1/3181	5'-GTATGGCACATGCAC-3'	3181-3195
E1/3559	5'-CGCCGTGGAAAAGTG-3'	3559-3573
3'UT/4024	5'-TCTATTTGTCTACTT-3'	4024-4038

*The region in 26S RNA which contains the priming site is indicated; this is followed by the position of the first nucleotide of the priming site in RRV T48 26S RNA (Dalgarno _et al_., 1983).

†Stretch of nucleotides in RRV T48 26S RNA to which the primer is complementary.

using the SEQUENCE program on the VAX 11/750 computer at the Research School of Biological Sciences, ANU.

2.2.16 Hydropathy analysis of E2

Hydropathy profiles of RRV, SFV and SIN E2 protein published by Dalgarno et al. (1983) together with that for RRV NB5092 using sequence data from S.G. Faragher and A.D. Meek (unpublished data), were generated on a VAX 11/750 computer using a program based on the algorithm of Kyte and Doolittle (1982). A seven residue moving segment was used to scan the sequences.

2.2.17 Secondary structure prediction of RRV T48 E2

This was performed on the above computer using a program based on the algorithm of Chou and Fasman (1978, 1979).

2.3 RESULTS

2.3.1 Production of hybridomas secreting neutralising monoclonal antibodies

Hybridomas secreting anti-RRV mabs were produced by fusion of RRV-immunised mouse spleen cells with X63.Ag8.653 mouse myeloma cells (Methods). Three separate fusions performed by Dr. R.C. Weir were with spleen cells from mice immunised with RRV T48; one used cells from mice immunised with RRV

Table 2.2 **Characterisation of anti-RRV neutralising monoclonal antibodies.**

Virus used to immunise mice	Fusion number	Monoclonal antibody isolated*	ELISA titer of ascitic fluid†	Protein specificity††
RRV T48	RW-1	T1E7	10^{-5}	E2
	RW-2	T1D11	10^{-5}	E2
	RW-3	T10C9	10^{-4}	E2
	RW-3	T4D2	10^{-4}	E2
	SV-1	T1B1	10^{-4}	E2
RRV NB5092	RW-4	NB3C4	10^{-4}	E2

*The virus against which the monoclonal antibody was isolated is indicated by T (RRV T48) or NB (RRV NB5092); this is followed by the well number from which the hybridoma was isolated.

†Against RRV T48.

††Determined by immunoblotting of SDS-disrupted RRV T48 (C. Fernon and R.C. Weir, unpublished data) except for T1B1 where the specificity was determined by antibody binding to the purified and intact RRV T48 in an immunoprecipitation test.

NB5092. From the four fusions, five hybridomas secreting RRV neutralising mabs were obtained. Neutralising mabs T1E7, T10C9, T1D11 and T4D2 (for nomenclature see legend, Table 2.2) were produced against RRV T48; NB3C4 was produced against RRV NB5092. These mabs were provided by Dr. Weir as mouse ascitic fluids. All these mabs belonged to the IgG class and bound to RRV T48 E2 in immunoblotting experiments using SDS-disrupted RRV T48 (C. Fernon and R.C. Weir, unpublished data).

From one fusion reaction, performed by the author using spleen cells from an RRV T48 immunised mouse, 14 hybridomas producing anti-RRV mabs were isolated. Of these, one clone, T1B1, produced a neutralising mab. T1B1 was subcloned and injected into a pristane-primed Balb/C mouse to obtain ascitic fluid. Mab T1B1 and the other neutralising mabs described above bound to E2 in purified intact RRV T48 in immunoprecipitation tests (data not shown).

All the antibodies described above neutralised both RRV T48 and RRV NB5092 infectivity in PRNT although there were differences in the reactivity patterns (see Section 2.3.7). The ELISA titers of the mab stocks used in the present study are shown in Table 2.2.

2.3.2 Isolation of RRV T48 variants resistant to neutralisation by monoclonal antibodies

In order to locate the epitopes in E2 to which the neutralising mabs bound, RRV T48 variants resistant to

Table 2.3 **Isolation of neutralisation resistant variants of RRV T48.**

Monoclonal antibody used for selection	Frequency of isolation of variants*	Variants isolated†
T1E7	1.0×10^{-5}	TV1, TV2, TV3, TV5
T10C9	0.8×10^{-5}	TV31, TV33, TV35, TV42
NB3C4	1.4×10^{-5}	TV61, TV62, TV63, TV64
T1B1	0.8×10^{-5}	TV51, TV59

*An average of three independent determinations for each antibody.

†TV indicates an RRV T48 variant.

neutralisation by mabs T1E7, T10C9, T1D11, T4D2, T1B1 and NB3C4 were selected.

For each selection approximately 5 x 10^6 PFU of RRV T48 was incubated with neutralising mab at 37°C for 1 hr. Antigenic variants were selected by plaque formation on Vero cell monolayers. With mabs T1E7, T10C9, T1B1 and NB3C4 the isolation frequency of the variants was 0.8 x 10^{-5} - 1.5 x 10^{-5} (Table 2.3). These variants were stable through 2-3 cycles of growth in BHK cells, although no experiments specifically designed to test for reversion were performed. With mabs T1D11 and T4D2, apparently neutralisation-resistant virus appeared at frequencies of 10^{-3} and 10^{-2} respectively but after a growth cycle in BHK cells virus was not resistant to the selecting mab. These mabs were not used in further studies.

A total of 14 stable antigenic variants of RRV T48 were isolated which were resistant to neutralisation by the selecting mab (Table 2.3). Four variants, TV1, TV2, TV3 and TV5 were isolated using mab T1E7. Variants TV31, TV33, TV35 and TV42 were isolated with mab T10C9. Two variants, TV51 and TV59, were selected with mab T1B1. Variants TV61, TV62, TV63 and TV64 were isolated using mab NB3C4. All variants were neutralised by polyclonal RRV T48 antiserum, confirming that they were isolates of RRV (data not shown).

2.3.3 Nucleotide sequences of the structural protein genes of RRV T48 antigenic variants

The structural protein genes of the RRV T48 antigenic

Table 2.4 **Comparison of the sequence of the structural protein genes of RRV T48 used in this study with the published sequence (Dalgarno et al., 1983).**

Nucleotide*			Amino acid		
Position in 26S RNA†	RRV T48 (Dalgarno et al., 1983)	RRV T48 used in this study	Protein/ Amino acid††	RRV T48 (Dalgarno et al., 1983)	RRV T48 used in this study
1135	N	U	E2/ 29	?	Tyr
1174	C	U	E2/ 42	Pro	Ser
1293	C	U			
1365	Y	U			
1547	A	G	E2/166	Glu	Gly
2272	C	U	E2/408	Pro	Ser
2391	P	A			
3200	C	A	E1/235	Pro	Glu
3971	Y	U			

*N=unidentified nucleotide; Y=pyrimidine; P=purine.

†Numbered from the 5′-end of RRV T48 26S RNA (Dalgarno et al., 1983).

††Numbered from the N-terminus of each protein (Dalgarno et al., 1983).

variants were sequenced in order to identify the genetic changes responsible for the neutralisation resistant phenotype. Sequencing was by the dideoxy-chain termination method using virion RNA as template and synthetic oligodeoxynucleotide primers (Table 2.1) based on the RRV T48 sequence (Dalgarno et al., 1983).

For all 14 RRV T48 variants, the genes coding for E1, E2, E3 and the 6K proteins were sequenced. In addition, the capsid protein genes of TV1, TV42, TV51 and TV61 were sequenced. These four were chosen as representatives of the four groups of variants resistant to each of the selecting mabs. To facilitate comparison of sequence ladders, RRV T48 RNA was sequenced in parallel in each set of reactions. In the course of sequencing RRV T48, four ambiguities in the published sequence (Dalgarno et al., 1983) were resolved and five differences from the published sequence observed (Table 2.4). Four amino acid differences from the published sequence are predicted. These are in E2 at residues 42, 166 and 408, and in E1 at residue 235 (Table 2.4). These differences may reflect errors in the published sequence or mutations in the RRV T48 stock.

Sequence studies on T10C9-resistant variants. Four variants (TV31, TV33, TV35 and TV42) selected with T10C9 were sequenced. All variants had a single, identical nucleotide change (C→U) in the E2 gene at position 1697 (Figure 2.1) which led to a predicted non-conservative Thr→Ile substitution at

Table 2.5 **Mutations in monoclonal antibody resistant variants of RRV T48.**

Monoclonal antibody used for selection	Variant	Mutation and position†	Amino acid substitution and position††
T10C9	TV31	ACC→AUC (1697)	Thr→Ile* (216)
	TV33	ACC→AUC (1697)	Thr→Ile* (216)
		ACG→ACA (3462)	No change
	TV35	ACC→AUC (1697)	Thr→Ile* (216)
	TV42	ACC→AUC (1697)	Thr→Ile* (216)
NB3C4	TV61	AAA→CAA (1750)	Lys→Glu* (234)
	TV62	AAA→AUA (1751)	Lys→Ile* (234)
	TV63	AAA→AAU (1752)	Lys→Asn* (234)
	TV64	AAA→GAA (1750)	Lys→Glu* (234)
T1E7	TV1	GAU→AAU (1786)	Asp→Asn* (246)
	TV2	AGG→AGC (1800)	Arg→Ser* (251)
	TV3	ACA→CCA (1792)	Thr→Pro* (248)
	TV5	GAU→GUU (1787)	Asp→Val* (246)
T1B1	TV51	GAU→AAU (1786)	Asp→Asn* (246)
	TV59	GAU→AAU (1786)	Asp→Asn* (246)

†Numbered from the 5′-end of RRV T48 26S RNA (Dalgarno et al., 1983).

††Numbered from N-terminus of E2 (Dalgarno et al., 1983).

*Non-conservative change; the following changes are designated as conservative: Arg=Lys, Ser=Thr, Asp=Glu, Gln=Asn, Val=Leu=Ile=Met, Ala=Gly, Ala=Val, Tyr=Phe.

Figure 2.1 **Sequence ladders for RRV T48 variants selected with T10C9.**

Virion RNA from purified virus was sequenced using primer E2/1990 (Table 2.1). The position of the changed nucleotide in the cDNA sequence ladder is indicated by an arrow.

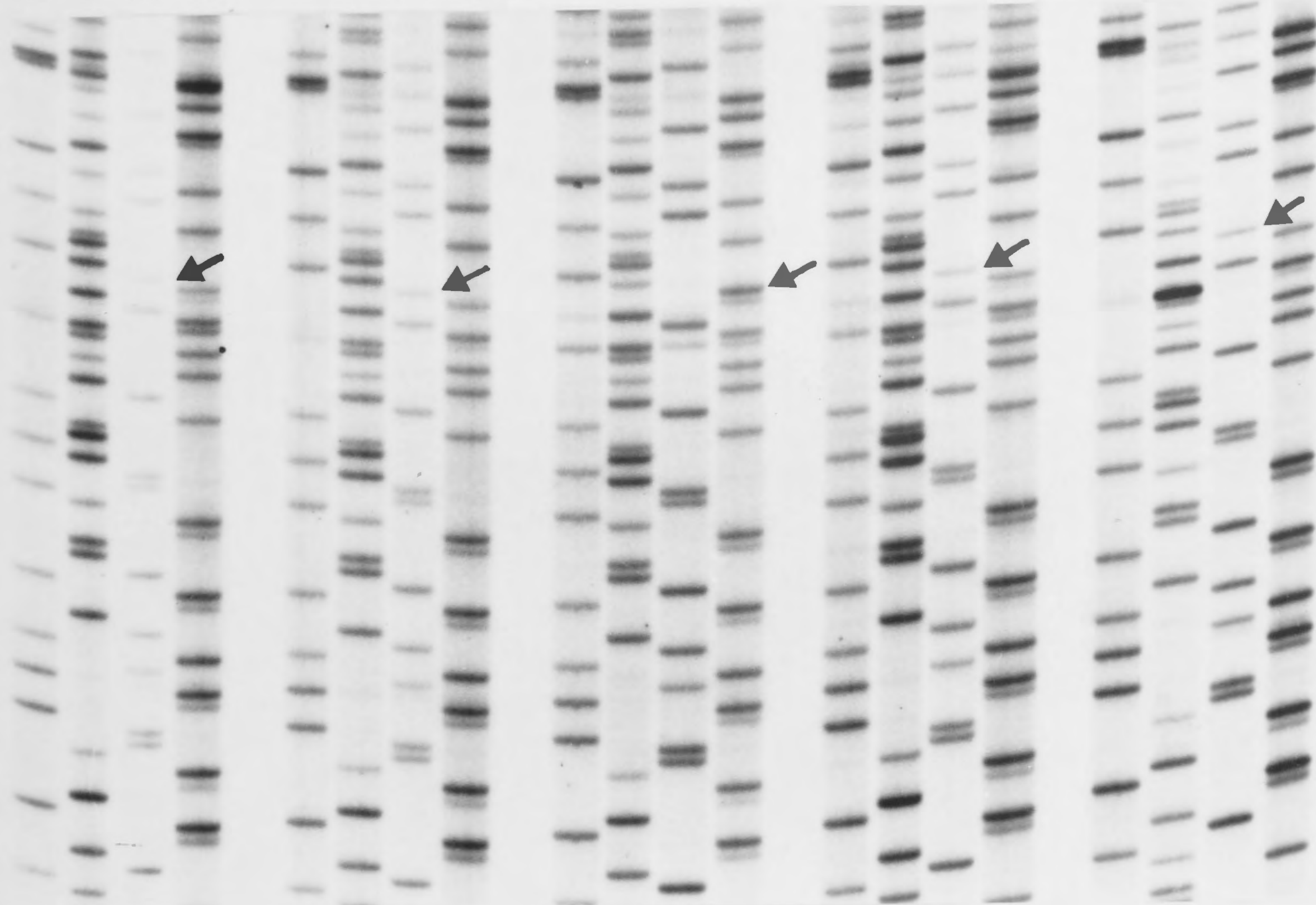
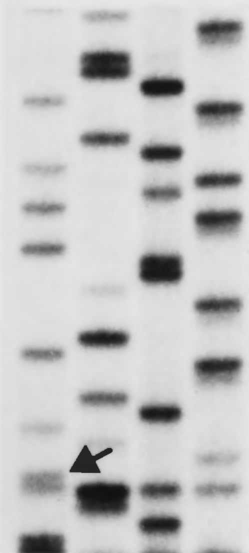
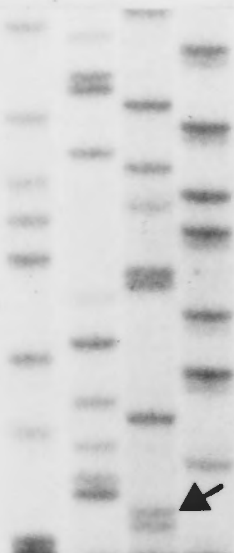
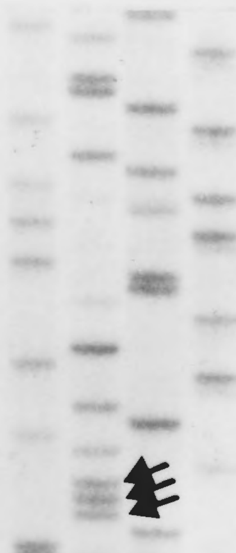

TV 31
TV 33
RRV T48
TV 35
TV 42
C T A G
C T A G
C T A G
C T A G
C T A G

Figure 2.2 **Sequence ladders for RRV T48 variants selected with NB3C4.**

Virion RNA from purified virus was sequenced using primer E2/1990 (Table 2.1). The position of the changed nucleotide in the cDNA sequence ladder is indicated by an arrow.


TV64
C T A G


TV63
C T A G


RRV T48
C T A G

TV62
C T A G

TV61
C T A G

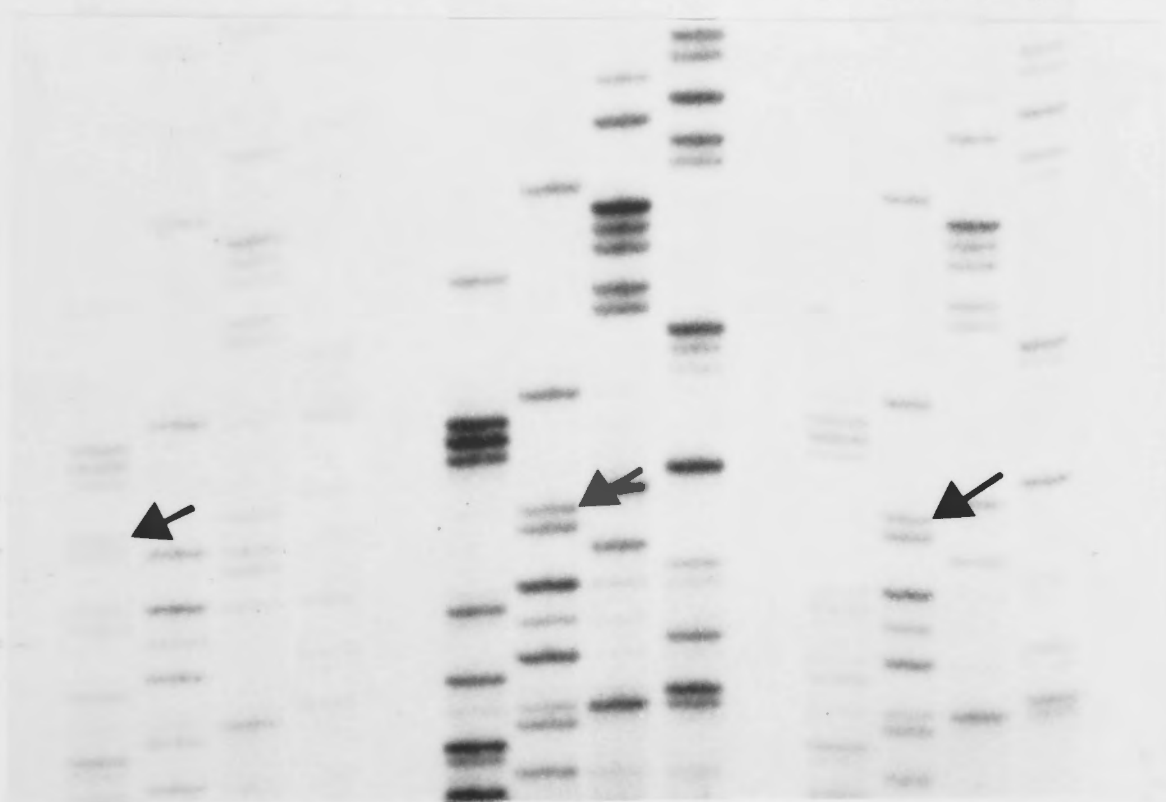
residue 216 in E2 (Table 2.5). No changes were found in the E1, 6K or E3 genes of any of the variants except for TV33 where an additional nucleotide substitution (G→A) was found at position 3462 in the E1 gene (data not shown). This was a silent, third position change. For TV42 the C protein gene was also sequenced which showed no difference from that of RRV T48.

Sequence studies on NB3C4-resistant variants. Four variants of RRV T48 (TV61, TV62, TV63 and TV64) selected with NB3C4 were sequenced. All variants had single nucleotide substitutions in the E2 gene. The substitutions were non-identical but all involved the same codon (AAA). They were at positions 1750, 1751, 1752 and 1750 in TV61, TV62, TV63 and TV64 respectively (Figure 2.2). They each resulted in a different amino acid change in E2 at position 234. The amino acid substitutions were non-conservative and were Lys→Gln in TV61, Lys→Ile in TV62, Lys→Asn in TV63 and Lys→Glu in TV64 (Table 2.5). None of the variants had mutations in genes coding for E1, 6K and E3. No mutation was detected in the C gene of TV61.

Sequence studies on T1E7-resistant variants. Variants TV1, TV2, TV3 and TV5 resistant to T1E7 were sequenced. Single nucleotide substitutions were detected at positions 1786, 1800, 1792 and 1787 in the E2 gene of TV1, TV2, TV3 and TV5 respectively (sequence ladders not shown). In each case this led to a different predicted amino acid substitution in E2

Figure 2.3 **Sequence ladders for RRV T48 variants selected with T1B1.**

Virion RNA from purified virus was sequenced using primer E2/1990 (Table 2.1). The position of the changed nucleotide in the cDNA sequence ladder is indicated by an arrow.


RRV T48
TV51
TV59
C T A G
C T A G
C T A G

(Table 2.5). In TV1 the change was Asp→Asn at position 246. TV2 was changed at position 251 (Arg→Ser). In TV3 the change was at position 248 (Thr→Pro). In TV5 the change was Asp→Val at residue 246. All these amino acid substitutions were non-conservative (Table 2.5). There were no changes found in the E1, 6K or E3 genes of any variant or in the C gene of TV1.

The Asp→Asn substitution in TV1 gives rise to a tripeptide sequence, Asn-Gln-Thr, potentially involved in asparagine-linked glycosylation (Chapter 1). We predict that TV1 E2 may be glycosylated at position 246.

<u>Sequence studies on T1B1-resistant variants</u>. Both TV51 and TV52 had identical mutations in the E2 gene at position 1786 (Figure 2.3). This resulted in an Asp→Asn substitution in E2 at residue 246 (Table 2.5). The nucleotide change and amino acid substitution in these variants was similar to that found in TV1 (Table 2.5) and it could be predicted that these variants may also be glycosylated at amino acid 246 in E2. These variants had no change in their E1, 6K or E3 genes or in the C gene of TV51.

In summary, in all mab-resistant variants of RRV T48 single nucleotide substitutions had taken place in the E2 gene. In each case this led to a single predicted amino acid substitution in E2. No mutations were found in the E1, 6K, E3 or C genes of any variant except TV35 where a single nucleotide mutation was found in E1. This, however, did not result in an

amino acid change. All amino acid substitutions were non-conservative and were located within a limited stretch of 36 amino acids (216-251). Interestingly, this stretch lies between the two predicted asparagine-linked glycosylation sites in E2 at residues 200 and 262 (Dalgarno et al., 1983). T10C9-resistant variants had an amino acid substitution at residue 216; NB3C4-resistant variants at residue 234; T1E7-resistant variants at residues 246, 248 or 251; and T1B1-resistant variants had substitutions at amino acid 246 in E2, as in the T1E7 variants. Thus three neutralising epitopes in the primary amino acid sequence of E2 have been defined around amino acids 216 (T10C9 epitope), 234 (NB3C4 epitope) and 246-251 (T1E7 and T1B1 epitope).

2.3.4 Attempts to select multi-epitope antigenic variants of RRV T48 with two mabs used concurrently

The question of whether the epitopes identified above were in functionally the same or different antigenic areas was examined by estimating the isolation frequency of RRV T48 variants when two mabs were used in combination. Given that the isolation frequency of antigenic variants altered in a single epitope is $\sim10^{-5}$ (Table 2.3), and that these variants are point mutants, we reasoned that a variant with mutations in two functionally distinct epitopes would be isolated at a frequency equal to the product of the two mutation rates, that is $\sim10^{-10}$ (Yewdell et al., 1979; Portner et al., 1980).

No variants could be isolated from ~2 x 10^7 PFU of RRV T48 when mabs T1E7 and NB3C4, T1E7 and T10C9 or NB3C4 and T10C9 were used concurrently. This indicates that the frequency of variants altered in a combination of these two epitopes is <2 x 10^{-7}. However, when mabs T1E7 and T1B1 were used concurrently, variants could be isolated at a frequency of 1.8 x 10^{-5} (data not shown).

We conclude that the epitopes defined by T10C9 and NB3C4 are distinct, they will be referred to below as epitopes I and II respectively; T1E7 and T1B1 define an overlapping, if not identical, epitope which will be referred to as epitope III.

2.3.5 Cross-neutralisation of antigenic variants of RRV T48 by monoclonal antibodies

To determine whether a point mutation in one epitope grossly affects the neutralisation function of others, cross-neutralisation of RRV T48 antigenic variants with the non-selecting mabs was examined. TV5 and TV51 (both epitope III variants), TV61 (epitope II variant) and TV42 (epitope I variant) were chosen as representatives of each group of variants. Their neutralisation by mabs T1E7, T10C9, T1B1 and NB3C4 was examined in PRNTs (Table 2.6).

All variants were sensitive to neutralisation by non-selecting mabs except that TV5 (resistant to T1E7) was resistant to T1B1, and TV51 (resistant to T1B1) was resistant

Table 2.6 **Cross neutralisation of RRV T48 antigenic variants by monoclonal antibodies.**

Antibody used	Neutralisation*			
	Variant TV5	Variant TV51	Variant TV42	Variant TV61
T1E7	0	0	100	100
T10C9	100	100	0	100
NB3C4	100	100	100	0
T1B1	0	0	100	100
Polyclonal RRV T48 antiserum	100	100	100	100

Approximately 200 PFU of virus was incubated with undiluted antibody at 37°C for 1 hr and plated on Vero cell monolayers for plaque assay. HBSS was used in place of antibody in controls.

*Per cent neutralisation of virus infectivity.

to T1E7. All variants were neutralised by polyclonal RRV T48 antiserum.

These results confirmed that epitopes defined by T1E7 and T1B1 were overlapping and demonstrated that using this method of analysis, the three epitopes were independent in their neutralisation function since a mutation in one epitope did not grossly affect the neutralisation of virus by mab recognising a different epitope.

2.3.6 Isolation of RRV T48 antigenic variants resistant to neutralisation by two or three mabs using sequential selection

In an attempt to explore the relationship between the epitopes defined above, antigenic variants resistant to two or three mabs were sequentially selected with mabs directed to different epitopes. It was argued that if the epitopes were totally independent (i.e. an amino acid change in one having no influence on the conformation of others), an amino acid substitution in one epitope should not change the frequency of isolation of variants on selection with a second mab, the location of the amino acid change or the nature of the substitution.

Precisely the same procedure used for selection of variants resistant to a single mab was used. Thus to obtain variants resistant to mabs T10C9 and NB3C4, variant TV42 (resistant to T10C9, Table 2.3) was incubated with NB3C4. From

this, variants TV131, TV145 and TV147 were isolated. To obtain variants resistant to mab T10C9, NB3C4 and T1E7, variant TV147 was incubated with T1E7 leading to the isolation of TV161 and TV162. These variants were resistant to T1B1 also. All the variants isolated were neutralised by polyclonal anti-RRV antiserum.

For selection of variants resistant to a single mab, ~5 x 10^6 PFU of RRV T48 was normally incubated with the mab. In this way approximately 50 antibody resistant plaques were obtained giving an isolation frequency of ~10^{-5}. To isolate variants resistant to two mabs, it was necessary to incubate ~1.4 x 10^7 PFU of TV42 with NB3C4 in order to obtain 25 plaques giving an isolation frequency of ~1.8 x 10^{-6}, a five-fold reduction in frequency. When ~7.6 x 10^7 PFU of TV147 were incubated with T1E7 to isolate variants resistant to three mabs, 23 plaques appeared giving an isolation frequency of ~3 x 10^{-7}, a 30-fold reduction over the isolation frequency of variants resistant to a single mab (Table 2.3). Although the isolation frequencies of variants resistant to two or three mabs are based on a single set of observations, we believe that they are significantly different from the isolation frequency of variants resistant to a single mab.

Sequence studies on multi-epitope antigenic variants of RRV T48. The segment of the E2 gene of multi-epitope antigenic variants coding for the amino acids between the two predicted glycosylation sites of E2 was sequenced. Variants

Figure 2.4 **Sequence ladders for multi-epitope variants of RRV T48.**

RNA extracted from virus infected BHK cells was sequenced using primer E2/1990 (Table 2.1). The positions of the changed nucleotides in the cDNA sequence ladder are indicated by arrows.

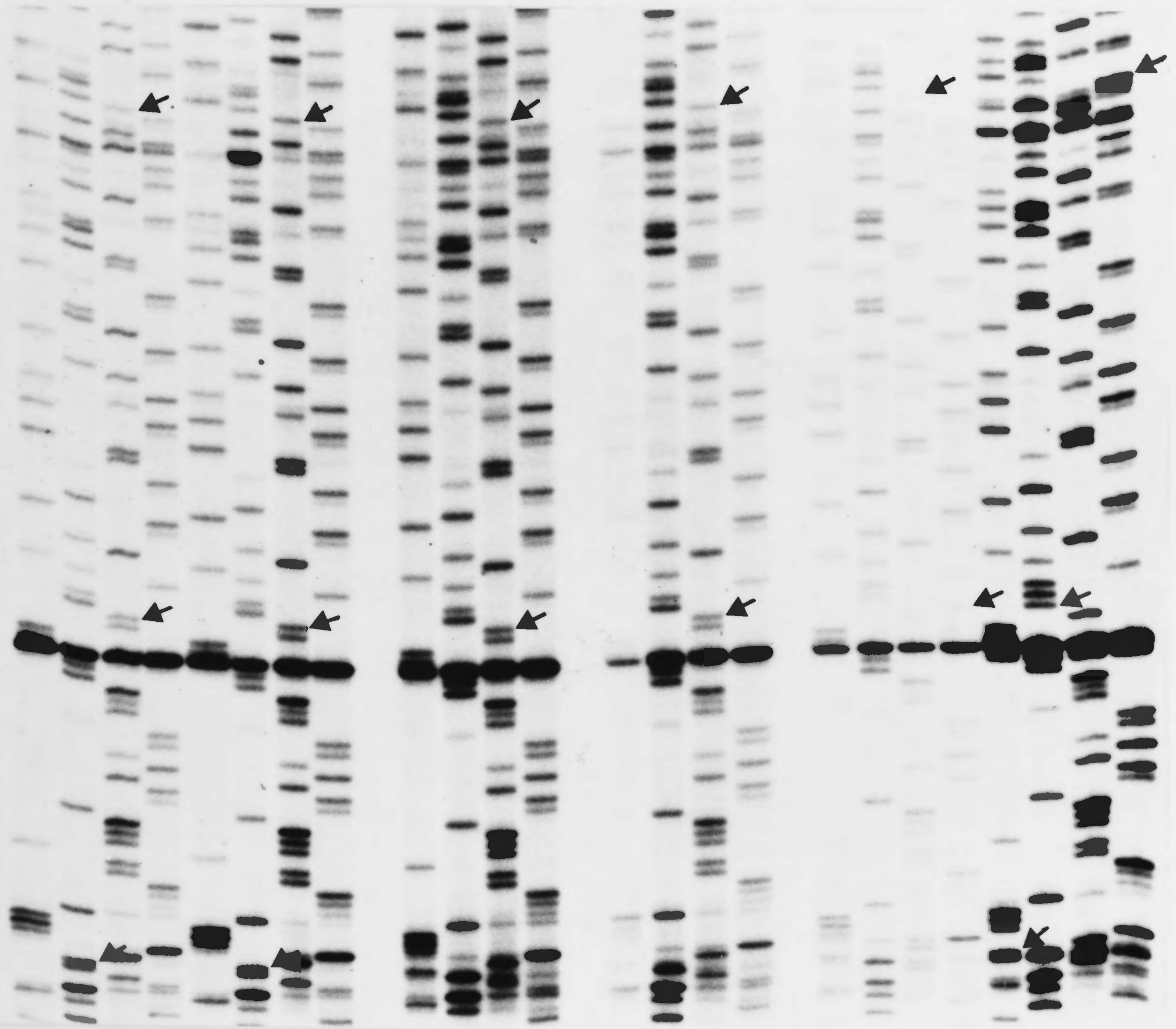

TV161 TV162 TV147 TV131 TV145 RRVT48
C T A G C T A G C T A G C T A G C T A G C T A G

Table 2.7 **Mutations in RRV T48 variants resistant to neutralisation by two or three monoclonal antibodies.**

Monoclonal antibodies used for selection	Variant	Mutation and position†	Amino acid substitution and position††
T10C9 and NB3C4	TV131	ACC→AUC (1697) AAA→AAU (1752)	Thr→Ile (216) Lys→Asn (234)
	TV145	ACC→AUC (1697) AAA→AAC (1752)	Thr→Ile (216) Lys→Asn (234)
	TV147	ACC→AUC (1697) AAA→AAU (1752)	Thr→Ile (216) Lys→Asn (234)
T10C9, NB3C4 and T1E7	TV161	ACC→AUC (1697) AAA→AAU (1752) GAU→AAU (1786)	Thr→Ile (216) Lys→Asn (234) Asp→Asn (246)
	TV162	ACC→AUC (1697) AAA→AAU (1752) GAU→AAU (1786)	Thr→Ile (216) Lys→Asn (234) Asp→Asn (246)

†Numbered from the 5′-end of RRV T48 26S RNA (Dalgarno et al., 1983).

††Numbered from N-terminus of E2 (Dalgarno et al., 1983).

TV131, TV145 and TV147 each had two nucleotide substitutions at positions 1697 and 1752 (Figure 2.4) which resulted in predicted amino acid changes at residues 216 (Thr→Ile) and 234 (Lys→Asn) in E2 (Table 2.7). These variants were resistant to T10C9 and NB3C4, the nucleotide changes and amino acid substitutions were identical to those found in the corresponding single step variants of T10C9 (e.g. TV31) and NB3C4 (e.g. TV63) (Table 2.5). Variants TV161 and TV162 had three nucleotide changes at positions 1697, 1752 and 1786 (Figure 2.4) leading to predicted amino acid substitutions at residues 216 (Thr→Ile), 234 (Lys→Asn) and 246 (Asp→Asn) in E2 (Table 2.7). The variants were resistant to T10C9, NB3C4 and T1E7 and the mutations were identical to those found in single step variants selected with T10C9 (e.g. TV31), NB3C4 (e.g. TV63) or T1E7 (e.g. TV1) (Table 2.5). The Asp→Asn change in these variants gave rise to a potential asparagine-linked glycosylation site as in TV1 (Section 2.3.3). It could be predicted that E2 of TV161 and TV162 has an additional potential glycosylation site.

In summary, multi-epitope antigenic variants were isolated at a significantly lower frequency than were the single step variants. Variants resistant to two mabs (double variants) had two predicted amino acid changes and variants resistant to three mabs (triple variants) had three predicted changes in E2. There were identical amino acid substitutions in all three double variants sequenced. Similarly, in both the

triple variants the amino acid substitutions were identical. The location and nature of amino acid changes was identical to those in the single step variants suggesting that a change in one epitope did not significantly affect the conformation of another epitope.

2.3.7 Identification of neutralisation epitopes of RRV NB5092

Boere et al. (1986) showed that the mouse virulent and the avirulent strains of SFV could be distinguished by neutralising mabs. Further, mouse-virulent RRV T48 and the avirulent RRV NB5092 could be distinguished by mab T1E7 which reacted to RRV NB5092 with 1000-fold lower neutralising titers than with RRV T48 (C. Fernon, personal communication). These observations prompted us to investigate the neutralisation epitopes of RRV NB5092. The procedures used were as described above for RRV T48.

Isolation of RRV NB5092 antigenic variants. Since mabs T1E7, T10C9 and T1B1, all raised against RRV T48, effectively neutralised RRV NB5092 infectivity, these together with NB3C4 were used to isolate neutralisation resistant variants of RRV NB5092. Twelve stable variants were isolated (Table 2.8). Variants NV111, NV113, NV115 and NV117 were selected with T10C9; variants NV101, NV103, NV104 and NV105 with NB3C4; variants NV151 and NV152 with T1E7 and variants NV153 and NV154

Table 2.8 **Isolation of neutralisation resistant variants of RRV NB5092.**

Monoclonal antibody used for selection	Frequency of isolation of variants*	Variants isolated†
T1E7	0.4×10^{-5}	NV151, NV152
T10C9	0.8×10^{-5}	NV111, NV113, NV115, NV117
NB3C4	1.6×10^{-5}	NV101, NV103, NV104, NV105
T1B1	0.5×10^{-5}	NV153, NV154

*An average of three independent determinations for each antibody.

†NV indicates an RRV NB5092 variant.

with T1B1. The isolation frequency of RRV NB5092 antigenic variants was 0.4×10^{-5} - 1.6×10^{-5} (Table 2.8), similar to that found for RRV T48 variants.

Sequence studies on the E2 gene of antigenic variants of RRV NB5092. Variants were sequenced in the region of the E2 gene encoding the amino acids between the two potential glycosylation sites at residues 200 and 262 (S.G. Faragher and A.D. Meek, unpublished data) using primer E2/1990 (Table 2.1) and virus-infected BHK cell RNA as template (Methods). The nucleotide substitutions detected and the predicted amino acid changes are listed in Table 2.9.

The four variants selected with T10C9 (NV111, NV113, NV115 and NV117) were each changed at nucleotide 1694 (sequence ladders not shown) leading to a Thr→Ile substitution at amino acid 216 (Table 2.9). Nucleotide 1694 is in the same relative position in the coding region of RRV NB5092 26S RNA as is nucleotide 1697 in RRV T48 (see Table 2.3) due to a three nucleotide deletion in the 5′-untranslated region of the 26S RNA sequence of RRV NB5092 by comparison with RRV T48 26S RNA (S.G. Faragher and A.D. Meek, unpublished data). Thus the changes in T10C9 variants of RRV NB5092 and RRV T48 were identical.

Of the four variants selected with NB3C4 (Table 2.8), three (NV101, NV104 and NV105) were changed at nucleotide 1747 (Figure 2.5) leading to a Lys→Glu substitution at residue 234 (Table 2.9). This substitution was identical to that detected

Table 2.9 **Mutations in monoclonal antibody resistant variants of RRV NB5092.**

Monoclonal antibody used for selection	Variant	Mutation and position†	Amino acid substitution and position††
T10C9	NV111	ACC→AUC (1694)	Thr→Ile* (216)
	NV113	ACC→AUC (1694)	Thr→Ile* (216)
	NV115	ACC→AUC (1694)	Thr→Ile* (216)
	NV117	ACC→AUC (1694)	Thr→Ile* (216)
NB3C4	NV101	AAA→GAA (1747)	Lys→Glu* (234)
	NV103	CAU→CGU (1742)	His→Arg* (232)
	NV104	AAA→GAA (1747)	Lys→Glu* (234)
	NV105	AAA→GAA (1747)	Lys→Glu* (234)
T1E7	NV151	?‡	?
	NV152	?	?
T1B1	NV153	?	?
	NV154	?	?

†Numbered from the 5′-end of RRV NB5092 26S RNA (S.G. Faragher and A.D. Meek, unpublished data).

††Numbered from N-terminus of E2 (S.G. Faragher and A.D. Meek, unpublished data).

*Non-conservative change (see legend to Table 2.5)

‡Mutations that could not be identified (see Text)

Figure 2.5 **Sequence ladders for RRV NB5092 variants selected with NB3C4.**

RNA extracted from virus infected BHK cells was sequenced using primer E2/1990 (Table 2.1). The position of the changed nucleotide in the cDNA sequence ladder is indicated by an arrow.

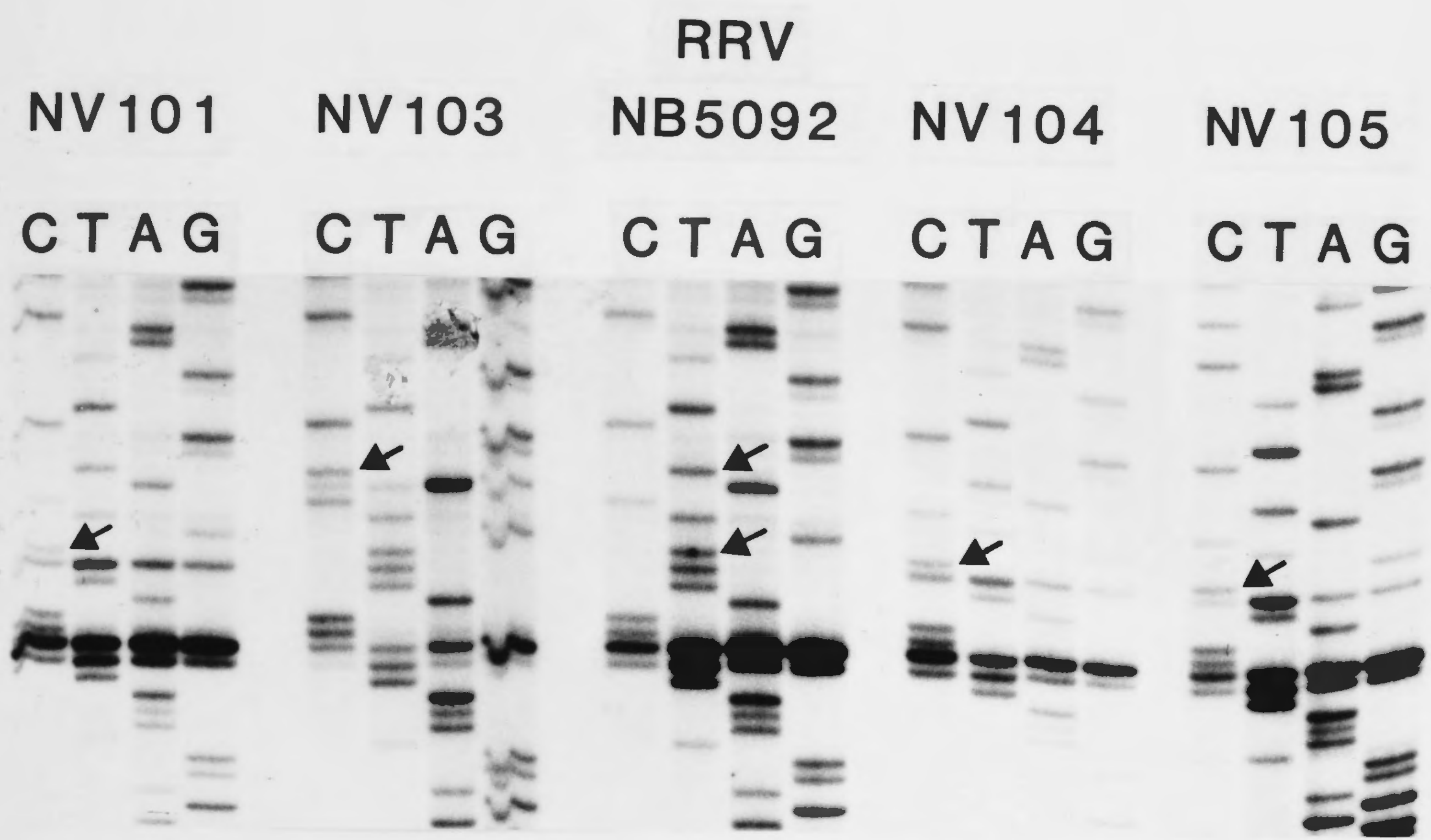
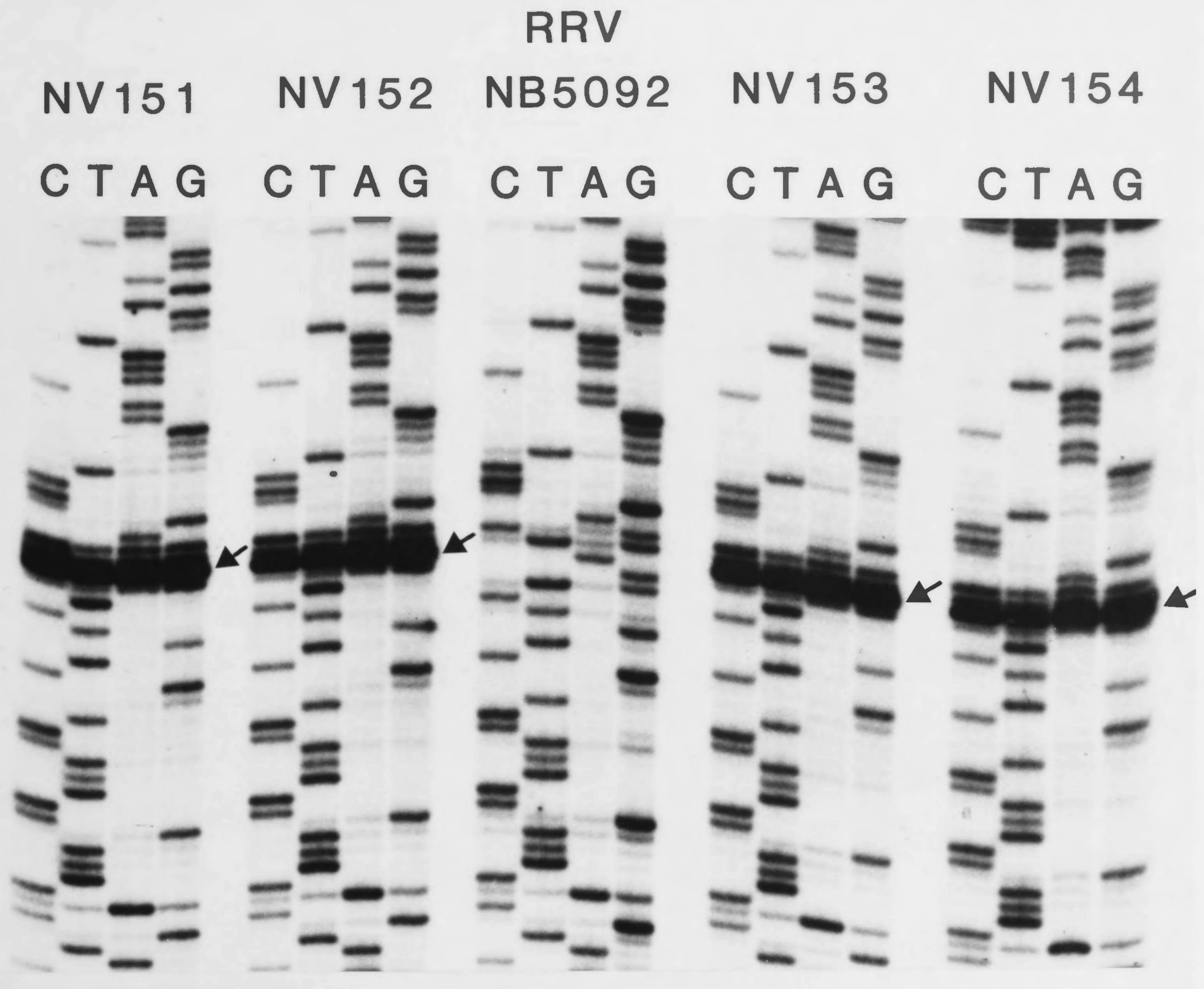

RRV
NV101
NV103
NB5092
NV104
NV105
C T A G
C T A G
C T A G
C T A G
C T A G

Figure 2.6 **Sequence ladders for RRV NB5092 variants selected with T1E7 or T1B1.**

RNA extracted from virus infected BHK cells was sequenced using primer E2/1990 (Table 2.1). Cross bands in all four lanes of the sequence ladders of the antigenic variants are indicated by an arrow and are at positions 1783-1786 of RRV NB5092 26S RNA (Faragher and Meek, unpublished data)


RRV
NV151
NV152
NB5092
NV153
NV154
C T A G
C T A G
C T A G
C T A G
C T A G

in TV64, an RRV T48 variant selected with mab NB3C4. Variant NV103 had a substitution at nucleotide 1742 (Figure 2.5) leading to a His→Arg change at residue 232.

Variants selected with T1E7 (NV151 and NV152) or T1B1 (NV153, NV154) showed cross bands in all four lanes of the sequence ladder at nucleotides 1783-1786 (Figure 2.6). As these were not seen for RRV NB5092, sequence differences between RRV NB5092 and the antigenic variants may have occurred at nucleotide(s) 1783-1786. Substitutions at these nucleotides would lead to changes at amino acids 246 and 247.

In summary T10C9-resistant variants of RRV NB5092 had a predicted amino acid substitution at residue 216, NB3C4-resistant variants had predicted substitutions at residues 232 and 234 and T1E7- or T1B1-resistant variants possibly had changes at residues 246 or 247 or both in E2. These were the same amino acids as were identified in neutralisation epitopes of RRV T48. From these data we conclude that the location of the neutralisation epitopes recognised by mabs T10C9, T1E7, T1B1 and NB3C4 is conserved between the virulent T48 and the avirulent NB5092 strains of RRV.

2.3.8 Location of amino acid change in RRV antigenic variants in predicted secondary structure of E2

To examine whether the domain of E2 containing the neutralisation epitopes (the "neutralisation domain") had any unique structural features we have generated a predicted

Figure 2.7 **Location of amino acid changes in RRV antigenic variants on predicted secondary structure map of E2.**

The primary amino acid sequence of RRV T48 E2 is shown. Below the sequence, the secondary structure predicted by the method of Chou and Fasman (1978, 1979) is shown: a, α-helix; b, β-sheet; C, random coil; T, reverse turn. The position of amino acid changes in RRV antigenic variants is indicated by ▼. The predicted glycosylation sites are marked with asterisks. Amino acids are numbered from N-terminus of RRV T48 E2 (Dalgarno *et al.*, 1983).

```
SVTEHFNVYKATRPYLAYCADCGDGYFCYSPVAIEKIRDEASDGMLKIQV   (50)
aaaabbbbbbbbbbbbbbbbbTTTTCCTTTTaaaaaaaaaTTTTbbbbbb

SAQIGLDKAGTHAHTKIRYIAGHDVQESKRDSLRVYTSAACSIHGTMGHF   (100)
bbbbbbTTTTaaaaaaaaaaaaaaaaaaTTTTbbbbbaaaaaaCCbbbbb

IVAHCPPGDYLKVSFEDANSHVKACKVQYKHDPLPVGREKFVVRPHFGVE   (150)
bbbbbTTTTaaaaaaaTTTTbbbbbbbbbbbTTTTaaaaaaaaTTTTaaa

LPCTSYQLTTAPTDEGIDMHTPPDIPDRTLLSQTAGNVKITAGGRTIRYN   (200)
aaTTTTbbbbbTTTTaaaaaaTTTTCCbbbbbbTTTTbbbTTTTbbbbbb

CTCGRDNVGTTSTDKTINTCKIDQCHAAVTSHDKWQFTSPFVPRADQTAR   (250)
TTTTTTTTCCCTTTTCCTTTTCTTTTaaaaaaTTTTbbbbbbaaaaaaaT

RGKVHVPFPLTNVTCRVPLARAPDVTYGKKEVTLRLHPDHPTLFSYRSLG   (300)
TTTbbbbbbbbbbbbbbbaaaaTTTTTTTTaaaaaaTTTTCCCCTTTTCC

AEPHPYEEWVDKFSERIIPVTEEGIEYQWGNNPPVRLWAQLTTEGKPHGW   (350)
CCCTTTTaaaTTTTbbbbbbbbbbbbbbTTTTCbbbbbbbbTTTTTTTTT

PHEIIQYYYGLYPAATIAAVSGASLMALLTLAATCCMLATARRKCLTPYA   (400)
TTTbbbbbbbaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaabbbTTTT

LTPGAVVSLTLGLLCCAPRANA                               (422)
CTTTTbbbbbbbbbbbbCCCCC
```

secondary structure map of RRV T48 E2 (Figure 2.7) using the program of Chou and Fasman (1978, 1979). The percentage of residues predicted to be in each conformation is α-helix, 29%; β-sheet, 33%; reverse turn, 31%; and random coil, 7%. The cytoplasmic domain (residues 392-422) contains residues predominantly in predicted β-sheet. The transmembrane domain (residues 364-391) is predicted to have an α-helical conformation. The external domain (residues 1-364) has alternating segments of short α-helix and β-sheet with frequent reverse turns, suggesting a globular structure. In the external domain, 31% of amino acids are in predicted reverse turns. Compared to this, 42% of amino acids in the "neutralisation domain" are in reverse turns. We predict therefore that the "neutralisation domain" has no unique structural features except for a slightly greater occurrence of reverse turns, expected for the surface region of a protein.

2.3.9 Summary

Antigenic variants of RRV resistant to neutralisation by mabs were isolated at a frequency of $\sim 10^{-5}$. All the variants had point mutations leading to non-conservative amino acid changes in E2. No mutations were found in the E1 gene except for a silent mutation in TV35. RRV variants selected with mab T10C9 had a predicted change at amino acid 216. Variants selected with NB3C4 had changed at amino acids 232 or 234 and variants selected with T1E7 had changed at amino acids

246, 248 or 251. Variants selected with mab T1B1 had changed at amino acid 246. Thus three neutralising epitopes were defined around amino acids 216 (epitope I), 232-234 (epitope II) and 246-251 (epitope III). In cross-neutralisation tests, mab-resistant variants were neutralised by the non-selecting mab interacting with a different epitope. Attempts to isolate multi-epitope variants from ~2×10^7 PFU of RRV T48 failed using two mabs belonging to two different epitopes concurrently. However, multi-epitope variants could be isolated using a sequential selection with different mabs. The location of the three neutralisation epitopes was conserved between the virulent T48 and the avirulent NB5092 strains of RRV.

2.4 DISCUSSION

2.4.1 Neutralising monoclonal antibodies

A total of six anti-RRV neutralising mabs were used in the present study. All interacted with E2 in immunoprecipitation or immunoblotting experiments (C. Fernon and R.C. Weir, unpublished data), consistent with the report of Dalrymple *et al*. (1976) that antiserum prepared against purified SIN E2 neutralises virus infectivity *in vitro*, while anti-E1 serum is more effective in HI and does not neutralise virus. However, more recently E1 has been implicated in neutralisation by the isolation of anti-E1 mabs which neutralise SIN (Chanas *et al*., 1982) or SFV (Boere *et al*.,

1984) infectivity. While the available evidence favours E2 as the major neutralisation antigen in alphaviruses, a role for E1 must be considered. Since E1 and E2 are closely associated to form heterodimers in the virion (Rice and Strauss, 1982a), binding of certain mabs to E1 may sterically hinder virus binding to the cell by blocking sites on E2 resulting in neutralisation of infectivity. An alternative explanation is that binding of certain mabs to E1 affects the conformation of E2 such that the virus is rendered non-infectious. Up till now no anti-E1 mab that neutralises RRV infectivity has been isolated. However, it is likely that the spectrum of mabs isolated against RRV is incomplete and that with more fusions neutralising anti-E1 mabs could be obtained. Thus it is not clear whether E1 is involved in RRV neutralisation.

2.4.2 Isolation of RRV antigenic variants

The frequency of isolation of RRV antigenic variants was around 10^{-5} for each of the four mabs, similar to that observed for SIN ($10^{-3.5}$-10^{-5}; Stec *et al*., 1986). The frequency of mab resistant variants of other RNA viruses is reported as 10^{-3}-10^{-5} for Sendai, 10^{-5} for influenza and vesicular stomatitis virus (Portner *et al*., 1980) and 10^{-3}-10^{-5} for polio virus (Minor *et al*., 1983). Multi-epitope variants of RRV T48 were, however, isolated at a lower frequency than the single step variants. This is perhaps connected to the observation that only one type of amino acid substitution

(Lys→Asn, Table 2.7) was detected in epitope II of the three double variants sequenced as compared to four different substitutions in each of the four single step variants altered in epitope II (Table 2.5). Similarly in both the triple variants sequenced, epitope III showed only one type of amino acid substitution (Asp→Asn, Table 2.7), whereas the four single step variants (altered in epitope III) selected with T1E7 showed four different amino acid substitutions (Table 2.5). Although we have not sequenced a sufficient number of double and triple variants to conclusively demonstrate that there is greater restriction on the type of alteration that can be accommodated once the antigenic domain is altered, our results suggest that this is a possible explanation for the lower frequency of isolation of multi-epitope variants.

In considering the results presented above, an important question relates to the "independence" of each of the variants obtained using a single mab. All the variants selected with mabs T1E7 had different nucleotide substitutions indicating that they were derived from separate, independent mutations. A similar observation was made for the NB3C4 variants. However all the T10C9-resistant variants of both RRV T48 and RRV NB5092 had the same nucleotide change (Tables 2.5 and 2.9). Similarly, both RRV T48 variants selected with T1B1 had the same mutation as each other. Since all variants were isolated using a similar procedure and the independent origin of T1E7 and NB3C4 variants has been demonstrated, it is likely that each T10C9 and T1B1 variant arose independently. This

conclusion could be extended to the multi-epitope variants also. The independent origin of variants is also supported by the presence of a silent mutation in the E1 gene of one of the T10C9 variants (TV33), although this may have arisen after selection for mab resistance. Since all epitope I variants had the same nucleotide change as opposed to the variety of substitutions seen in epitope II and epitope III variants, this may suggest that the maintenance of a particular conformation in epitope I is more important for virus infectivity than in epitopes II and III.

2.4.3 <u>Neutralisation epitopes of RRV</u>

Sequencing of RRV antigenic variants defined three neutralising epitopes around amino acids 216 (epitope I), 232-234 (epitope II) and 246-251 (epitope III) in E2. These epitopes may constitute part of a neutralisation domain in E2. It is postulated that interaction of antibodies with the neutralisation domain results in neutralisation of virus infectivity. Various approaches used to examine the relationship of the three epitopes established that they could act independently in neutralisation. This conclusion was further strengthened by the observation that the mab-resistant variants of RRV T48 did not bind to the selecting mab, and the substitution in an epitope did not affect the binding of a mab which recognised a different epitope (P. Kerr, personal communication). These epitopes were, however, conformationally connected since multi-epitope antigenic variants were isolated

at a significantly lower frequency and with a restricted type of amino acid substitution compared to the single step variants (above). This is perhaps not surprising as the epitopes are located approximately 15 amino acids apart in the primary amino acid sequence. No neutralisation epitopes were found in E1. With the help of more neutralising mabs it is likely that more epitopes involved in RRV neutralisation will be identified.

A crucial question is whether the domain in E2 which contains the identified neutralising epitopes ("neutralisation domain") represents the immunodominant region in vivo. CBAs have shown that all of the presently available anti-RRV neutralising mabs compete with each other and therefore cluster in a single region in E2 (C. Fernon and R.C. Weir, personal communication). Since these mabs were isolated from five independent fusions, it is likely that the "neutralisation domain" identified is the immunodominant region. The observation that polyclonal antiserum raised against RRV T48 is significantly less effective in neutralising TV161 (altered in all three epitopes) than RRV T48 also suggests that the three epitopes represent the immunodominant site (P. Kerr, personal communication). However, to establish with certainty whether or not this is the case, information regarding the amounts, avidity and epitope specificity of different antibodies to RRV in sera of infected animals would be required. An approach to partially satisfying this goal could be made by the method of Vandepol èt al. (1986) who identified the immunodominant region in the surface protein of vesicular stomatitis virus by

passaging it in the presence of polyclonal antibody. After 80 such passages in BHK cells, Vandepol et al. (1986) selected a mutant which had 10 amino acid changes in the surface protein, half of which occurred in a 13 amino acid stretch which had already been identified using mabs; this region was considered as the immunodominant region on the surface protein of the virus.

Using CBAs different workers have reported different numbers of neutralisation sites on E1 and E2 of SIN and SFV (Chapter 1). It is difficult to compare the number of neutralising sites for different alphaviruses with that for RRV for two reasons. First, the number of sites defined could be influenced by the number of mabs used (e.g. Stec et al., 1986; see Chapter 1); second, there are no reports for SIN or SFV on the molecular relationship of the epitopes in an antigenic site as defined by CBAs.

Multiple independent sites for neutralisation have been identified by CBA and sequencing studies in the surface proteins of rabies (Wiktor and Koprowski, 1980), influenza (Wiley et al., 1981; Wiley et al., 1984), parainfluenza (Yewdell and Gerhard, 1982), polio (Hogel et al., 1985) and the common cold viruses (Rossmann et al., 1985). Thus for influenza A, four neutralisation sites have been located in the three dimensional structure of the haemagglutinin -the neutralisation antigen. Sites A and B are around residues 140-146 and 187-196 respectively. Residues 52-54 and 275, 277 and 278 constitute site C, while site D is around residues 201-220. In polio

virus and the common cold virus three and four neutralising sites, respectively, have been identified. However, their structure is more complex since three different coat proteins (VP1-3) constitute the neutralisation antigen. Thus in polio virus type 3, site 1 comprises amino acids 89-100 of VP1, site 2 involves amino acids 164-172 of VP2 whereas site 3 consists of amino acids 286-290 of VP1 and amino acids 58, 59, 77 and 79 of VP3 (Minor, 1986). In the common cold virus, neutralisation site 1 consists of amino acids 91 and 95 of VP1, site 2 consists of amino acids 83, 85, 138 and 139 of VP1, site 3 consists of amino acids 136 and 158-162 of VP2 and amino acid 210 of VP1 whereas site 4 consists of amino acids 72-78 and 203 of VP3 and amino acid 287 of VP1 (Rossmann et al., 1985). Thus antigenic sites in these viruses are "discontinuous" such that amino acids from distant parts of the linear sequence contribute. Similarly, amino acids that are closely linked in the linear sequence can participate in different sites. In all these viruses, although the neutralisation sites were widely separated in the primary amino acid sequence, they were clustered in one region in the three dimensional structure of the virus (Wiley et al., 1981; Hogel et al., 1985; Rossmann et al., 1985). It is possible that the three neutralising epitopes in RRV E2 are "continuous" since (i) in all the mab resistant variants amino acid changes in an epitope were located within a span of no more than five amino acids in the linear sequence, and (ii) mabs bound to denatured and reduced E2 in immunoblotting tests. A conclusive picture, however, can

only be obtained by examining the location of the neutralisation sites in the three dimensional structure of the virus.

2.4.4 Comparison of the primary amino acid sequences of E2 of different alphaviruses in the region containing RRV neutralisation epitopes

The domain containing RRV neutralisation epitopes is located between the two predicted glycosylation sites in E2 which are separated by 62 amino acids. To examine the features of this domain we have compared the amino acid sequences of E2 for RRV T48, RRV NB5092, SFV and SIN in this region (Figure 2.8). In this region there is 74.6% amino acid sequence homology between RRV T48 and SFV, and 37.5% between RRV T48 and SIN. For E2 as a whole there is 69% amino acid sequence homology between RRV T48 and SFV, and 42% between RRV T48 and SIN (Dalgarno *et al*., 1983). Thus at the amino acid sequence level, the "neutralisation domain" is conserved no more or less than E2 as a whole. All four cysteine residues in the "neutralisation domain" are conserved between RRV, SFV and SIN. However, this is not unique to this domain since all the 12 cysteine residues in the external domain of RRV T48 E2 are conserved between RRV, SFV and SIN (see Dalgarno *et al*., 1983). The "neutralisation domain" has, within it, short stretches of amino acids which are conserved between RRV and SFV. The sites of change in the neutralisation resistant variants are in the

Figure 2.8 **Location of amino acid changes in RRV antigenic variants.**

The amino acid sequence of glycoprotein E2 of RRV T48, RRV NB5092, SFV and SIN between the two predicted glycosylation sites are shown. Data for RRV T48, RRV NB5092, SFV and SIN are from Dalgarno et al. (1983), S.G. Faragher and A.D. Meek (unpublished data), Garoff et al. (1980b) and Rice and Strauss (1981) respectively. The single letter amino acid code is used: A=Ala, C=Cys, D=Asp, E=Glu, F=Phe, G=Gly, H=His, I=Ile, K=Lys, L=Leu, M=Met, N=Asn, P=Pro, Q=Gln, R=Arg, S=Ser, T=Thr, V=Val, W=Trp, Y=Tyr. A gap has been introduced to maximise the homology between the sequences. Overlined amino acids in RRV NB5092, SFV or SIN are identical to corresponding amino acids in the RRV T48 sequence. The positions of the predicted glycosylation sites are indicated by asterisks. Conserved cysteine residues are shaded. The position of amino acid changes in RRV variants selected with mabs are indicated as follows:
▼, T10C9; ▽, NB3C4; ●, T1E7; and ○, T1B1.

```
            200            216                  232 234       246 248  251       262
RRV T48     TIRYNCTCGRDNVGTTSTDKTINTC KIDQCHAAVTSHDKWQFTSPFVPRADQTARRGKVHVPFPLTNVTC
RRV NB5092  TIRYNCTCGRDNVGTTSTDKTINTC KIDQCHAAVTSHDKWQFTSPFVPRADQTARKGKVHVPFPLTNVTC
SFV         KVKYNCTCGTGNVGTTNSDMTINTC LIEQCHVSVTDHKKWQFNSPFVPRADEPARKGKVHIPFPLDNITC
SIN         NITYECKCGDYKTGTVSTRTEITGC TAIKQCVAYKSDQTKWVFNSPDLIRHDDHTAQGKLHLPFKLIPSTC
```

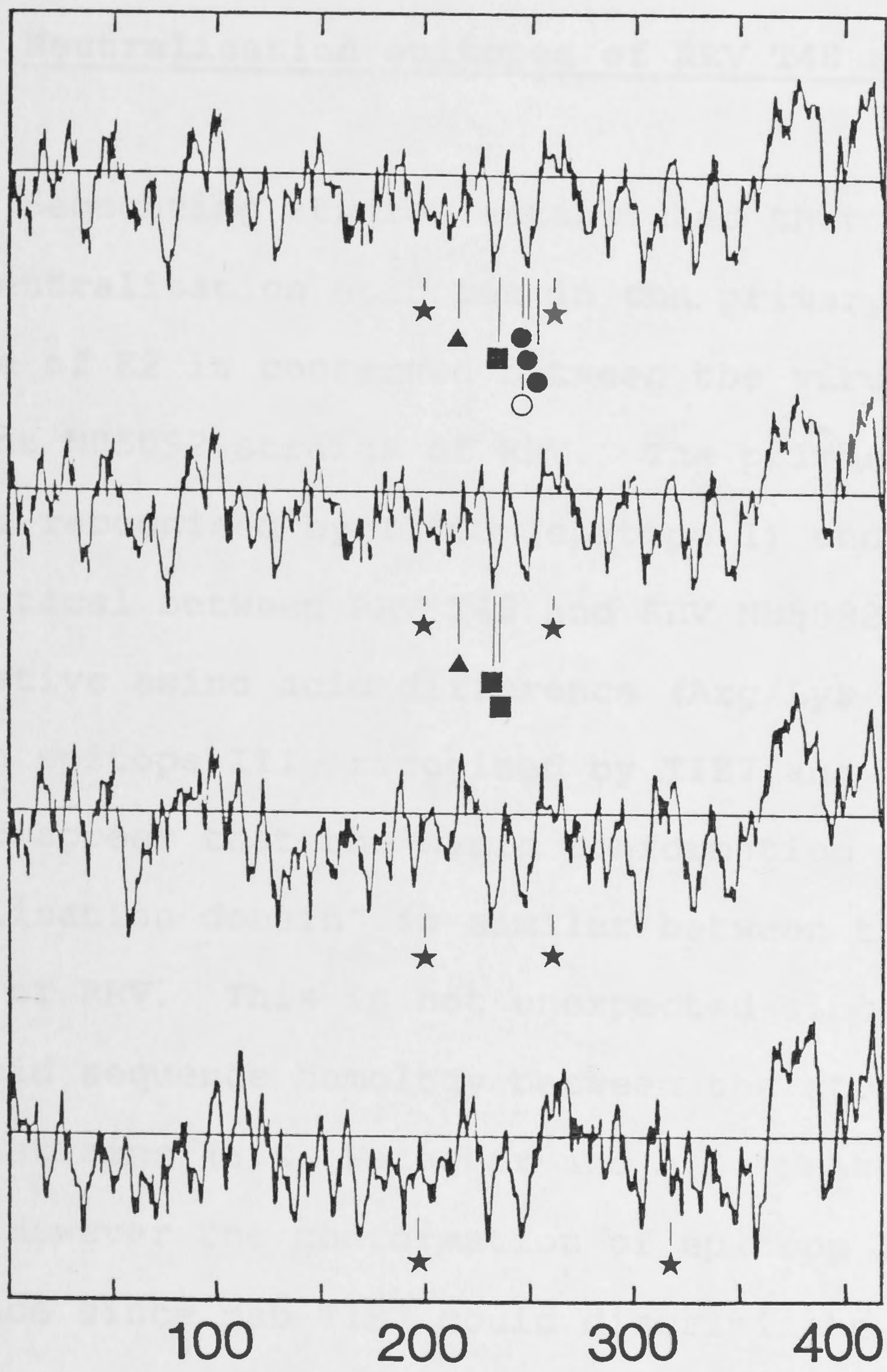
non-conserved regions or are immediately adjacent to them. This would suggest that the regions which are not conserved between RRV and SFV and which change in RRV antigenic variants include type specific determinants. Thus the sequence of neutralisation epitopes will be different in RRV and SFV, consistent with the type specific nature of alphavirus neutralisation sites (Strauss and Strauss, 1985). It may be useful to compare the amino acid sequence of Getah virus in the region of the "neutralisation domain" since RRV is serologically more closely related to Getah than to SFV (Calisher et al., 1980).

2.4.5 Comparison of the hydropathy profiles of E2 of different alphaviruses

To examine the hydrophilicity of the "neutralisation domain" the hydropathy profiles of E2 for RRV T48, SFV and SIN derived by Dalgarno et al. (1983) have been replotted together with that for RRV NB5092 and the position of amino acid changes in RRV antigenic variants indicated (Figure 2.9). There are two major hydrophilic regions conserved between RRV, SFV and SIN; both of these lie within the "neutralisation domain". Amino acid changes in RRV variants resistant to mabs T1E7 or NB3C4 occur in these regions and thus epitopes II and III are located in strongly hydrophilic segments of E2. However, change in T10C9-variants, and thus epitope I, is located in a moderately hydrophilic region. Since there are predicted

Figure 2.9 **Location in hydropathy profile of predicted amino acid changes in RRV antigenic variants.**

The hydropathy profiles of E2 of RRV T48, SFV and SIN derived by Dalgarno _et al_. (1983) using the program of Kyte and Doolittle (1982) have been replotted together with that for RRV NB5092 using sequence data of Meek (1986). The degree of hydrophobicity increases with the distance above the horizontal line, hydrophilicity increases with distance below the line. The search length was seven amino acids. The positions of predicted asparagine-linked glycosylation sites are marked with asterisks. The positions of the predicted amino acid changes in RRV variants selected with mab are as follows: ▲,T10C9; ■,NB3C4; ●,T1E7; ○,T1B1. Numbering of amino acids is from the N-terminus of E2 (Dalgarno _et al_., 1983)


RRV T48
RRV NB5092
SFV
SIN
100
200
300
400

glycosylation sites around these epitopes (Dalgarno et al., 1983) this could further increase the hydrophilicity of the "neutralisation domain", and contribute to its location on the exposed surface of E2 (Chothia, 1976).

2.4.6 Neutralisation epitopes of RRV T48 and RRV NB5092

Sequencing studies established that the location of the three neutralisation epitopes in the primary amino acid sequence of E2 is conserved between the virulent T48 and the avirulent NB5092 strains of RRV. The primary structure of epitopes recognised by T10C9 (epitope I) and NB3C4 (epitope II) was identical between RRV T48 and RRV NB5092; however a single conservative amino acid difference (Arg/Lys at residue 251) was found in epitope III recogised by T1E7 and T1B1 (Figure 2.8). It would appear that the basic conformation of the "neutralisation domain" is similar between the T48 and NB5092 strains of RRV. This is not unexpected since there is ~99% amino acid sequence homology between the structural proteins of the two strains (S.G. Faragher and A.D. Meek, unpublished data). However the conformation of epitope III had a slight difference since mab T1E7 could discriminate between RRV T48 and RRV NB5092 in PRNT (see Section 2.3.7). It is possible that this was a result of the single amino acid difference in epitope III of the two strains although a change elsewhere in E2 or even in E1 could affect the conformation of epitope III.

CHAPTER 3

BIOLOGICAL STUDIES ON RRV ANTIGENIC VARIANTS

3.1 INTRODUCTION

In the previous Chapter antigenic variants of RRV T48 were isolated which were resistant to neutralisation by mabs. These variants had single amino acid substitutions in E2. Similarly selected antigenic variants of many viruses have been shown to express altered virulence. Thus antigenic variants of reovirus type 3 selected with neutralising mabs were avirulent for mice (Spriggs and Fields, 1982). Some of the mab-resistant variants of rabies virus were avirulent for mice (Coloun *et al*., 1982; Coloun *et al*., 1983; Seif *et al*., 1985). Love *et al*. (1985) isolated a mab-resistant variant of mumps virus which had reduced virulence in suckling hamsters. Thus changes in neutralisation epitopes may affect the virulence of a virus.

In addition, there are three sets of data which suggest that changes in neutralisation epitopes in E2 may lead to alterations in the biological properties of an alphavirus. First, Meek (1986) showed that when avirulent RRV NB5092 is serially passaged in infant mice to enhance virulence, single or double amino acid substitutions occur in E2, many of which map to the neutralisation epitopes identified in the previous Chapter, although the possibility that changes occurred in non-structural proteins was not excluded. Second, Baric *et al*. (1981) isolated a mutant of SIN which penetrated BHK cells rapidly *in vitro*; the mutant was avirulent for mice and was altered in neutralisation epitope(s) in E2 as judged by its reactivity with anti-E2 neutralising mabs (Olmsted *et al*.,

1986). Third, Stanley et al. (1985) isolated a neurovirulent SIN mutant by passaging the virus in mouse brain; the mutant was altered in neutralisation epitopes in E2 as demonstrated by altered reactivity against anti-E2 neutralising mabs.

Considered together, these data gave reason to examine the effect of mutations in neutralisation epitopes on the biological properties of RRV T48 in cultured cells and in mice, the results of which are presented in this Chapter. In the first set of experiments, the replication of RRV variants was examined in BHK cells where the kinetics of growth, and the patterns of RNA and protein synthesis were compared with those of RRV T48. To compare the biological properties of RRV antigenic variants in mice, their virulence for day-old and week-old mice was assayed in terms of the viral lethal dose (LD_{50}) and the average survival time (AST) of the infected mice. Since differences in virulence have been correlated with altered tissue tropisms and with altered growth rates in the host (Chapter 1), it was of interest to study growth rates and tissue distribution of the antigenic variants in mice.

3.2 MATERIALS AND METHODS

3.2.1 Virus and cell cultures

RRV T48 and the antigenic variants have been described in Chapter 2. BHK and Vero cells were as described previously (Section 2.2.2).

3.2.2 Infection of cells and plaque assay

Cells were infected as described in Section 2.2.3. Virus titer was measured by plaque assay on Vero or BHK cell monolayers (Section 2.2.4). Titers on Vero cells are expressed as PFU whereas titers on BHK cells are expressed as BHK PFU. Titers on BHK cells were approximately 10 times higher than on Vero cells.

3.2.3 Production of anti-RRV immune ascitic fluid

This has been described in Section 2.2.10.

3.2.4 Rate of acquisition of resistance by infecting virus to neutralisation by added polyclonal antibody (Baric et al., 1981)

To measure the rates of virus "penetration" into the cells, BHK cell monolayers in 60 mm dishes were infected with ~100 BHK PFU of virus and incubated with intermittent shaking at 36°C. At various times (see text) the inoculum was removed by aspiration and 0.5 ml of polyclonal anti-RRV immune ascitic fluid (diluted 1:10 in HBSS) was added. After 10 min at 36°C the ascitic fluid was aspirated, the cell monolayers washed (x2) with PBS and overlayed for plaque development (Section 2.2.4). The number of plaques obtained after a 60 min adsorption, PBS wash, but no immune ascitic fluid treatment was

taken as 100%. The number of plaques obtained in this manner was equal to that found using the normal plaque assay conditions where inoculum is not removed by washing. Each assay was performed in triplicate.

3.2.5 **Time-course of viral RNA synthesis in infected cells (Newton et al., 1981)**

At designated times infected BHK cell monolayers in 35 mm plastic dishes were washed with PBS and incubated for 2 hr at 36°C with 0.5 ml of EMEM containing 10 μCi/ml [5-^{3}H]-uridine (New England Nuclear) and 5 μg/ml actinomycin-D (AMD; Merck Sharp & Dohme). After labelling, monolayers were washed twice with PBS and dissociated in 200 μl of 1% SDS. Duplicate samples from ~2 x 10^5 cells were dried on Gelman type AE glass fibre discs, and washed with 5% trichloroacetic acid (5 changes over 1 hr), followed by ethanol (3 changes over 10 min). Dried discs were counted in toluene/PPO scintillation fluid.

3.2.6 **Radiolabelling of viral polypeptides and polyacrylamide gel electrophoresis (Short et al., 1982)**

Confluent BHK cell monolayers in 35 mm plastic dishes were infected with virus (moi~1) or mock-infected with HBSS. Cells were labelled at various times post-infection at 36°C for 2 hr periods in 0.35 ml of EMEM containing one-tenth the normal level of amino acids and 100 μCi/ml of [^{3}H]-amino acid mixture

(New England Nuclear). Monolayers were washed (x2) with PBS and dissociated in 200 µl of 1% SDS. Cell extracts were electrophoresed on a 10-20% gradient polyacrylamide gel (Section 2.2.11).

3.2.7 **Estimation of viral virulence for mice**

The lethal dose (LD_{50}) of the virus and the average survival time (AST) of infected mice were used to define the virulence of RRV T48 and the antigenic variants. Mice of either sex from an outbred line (Walter and Eliza Hall Institute; WEHI) were used. For LD_{50} determinations 30 µl samples of ten-fold virus dilutions in HBSS were injected i.p. into day- or week-old mice, using 8-10 litter-mates per dilution. LD_{50}s were calculated according to Reed and Muench (1938). Clinical signs and mortality were recorded daily for two weeks. Subclinical infections were assayed in mice surviving to the 14th day by challenging with 10^5 PFU of RRV T48 injected i.p. Mice were held for a further two weeks and the absence of clinical signs was taken as evidence of prior infection. The AST represents the arithmetic mean for all mice dying during the course of a titration regardless of dose, since for most RRV strains the time of death was independent of dose (Taylor and Marshall, 1975).

3.2.8 Virus growth in mice

WEHI mice of either sex were injected i.p. with ~100 PFU of virus. Groups of three mice were killed at each time point by cervical dislocation; tissues were removed and stored at -70°C. Blood was collected from the heart using heparinised capillary tubes (Sherwood Medical Industries, USA) and diluted 1:10 in ice-cold HBSS before freezing at -70°. For virus assay, tissues were homogenised as a suspension in ice-cold HBSS using a teflon-glass homogeniser attached to a Virtis homogeniser.

3.3 RESULTS

3.3.1 Growth kinetics of RRV T48 antigenic variants in BHK cells

The growth of the mab-resistant antigenic variants was compared to that of RRV T48 to assess whether a change in neutralisation epitope was accompanied by a change in viral replication which could be detected in cultured cells. BHK monolayers were infected with the variants or RRV T48 (moi~1) and incubated in GMEM at 36°C. Growth samples were removed at intervals and the extracellular virus (EV) titer assayed.

Growth kinetics of T10C9-resistant variants. The growth of variants TV31, TV33, TV35 and TV42 (epitope I variants) was

Figure 3.1 **Growth kinetics of T10C9-resistant variants of RRV T48 in BHK cells.**

BHK cell monolayers were infected with the variant or with RRV T48 (moi~1) and incubated at 36°C. At intervals samples were removed, diluted into HBSS, and stored at -70°C. EV titers were assayed in duplicate.

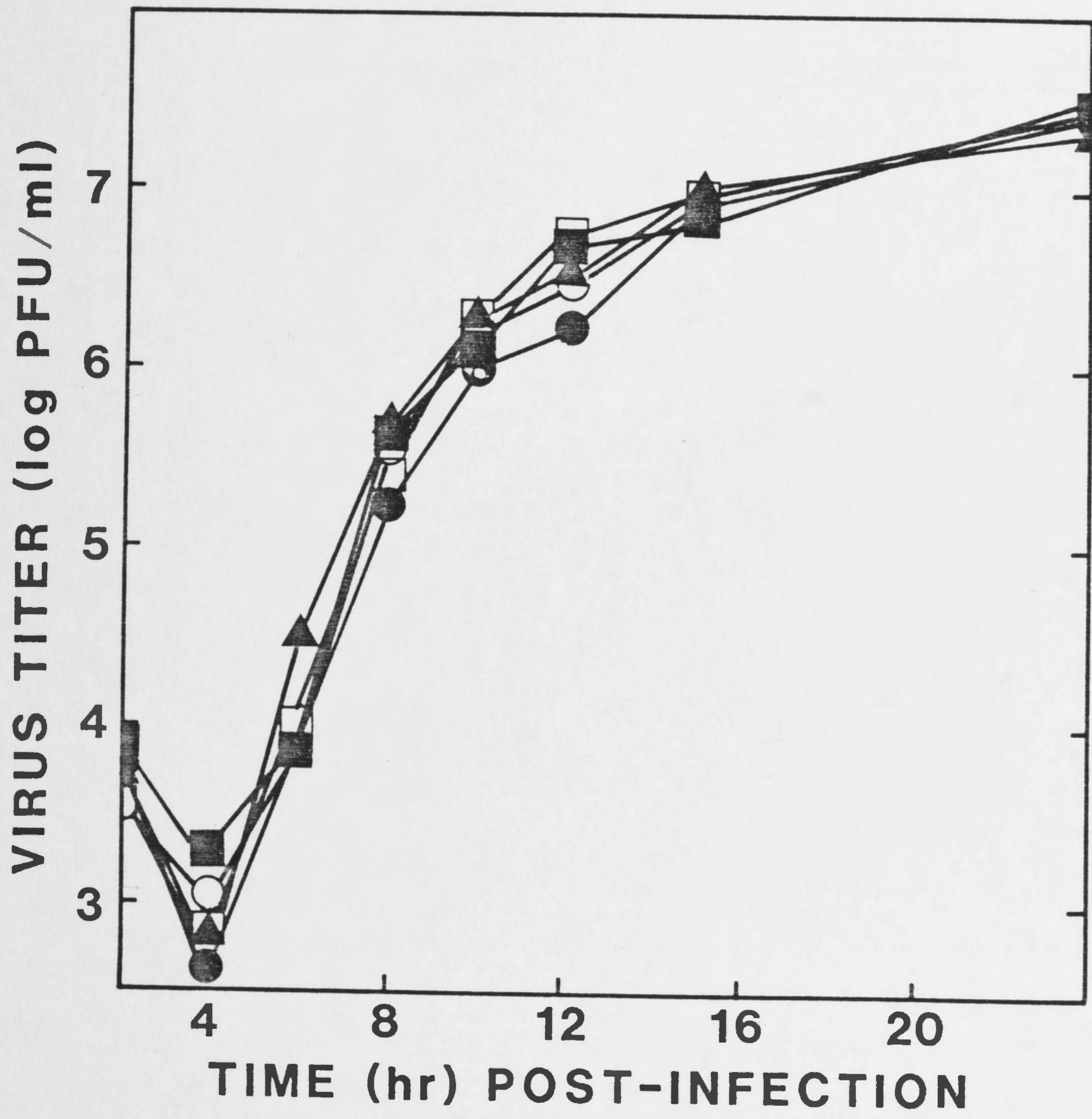
EV titer of RRV T48, (●).

EV titer of TV31, (○).

EV titer of TV33, (■).

EV titer of TV35, (□).

EV titer of TV42, (▲).

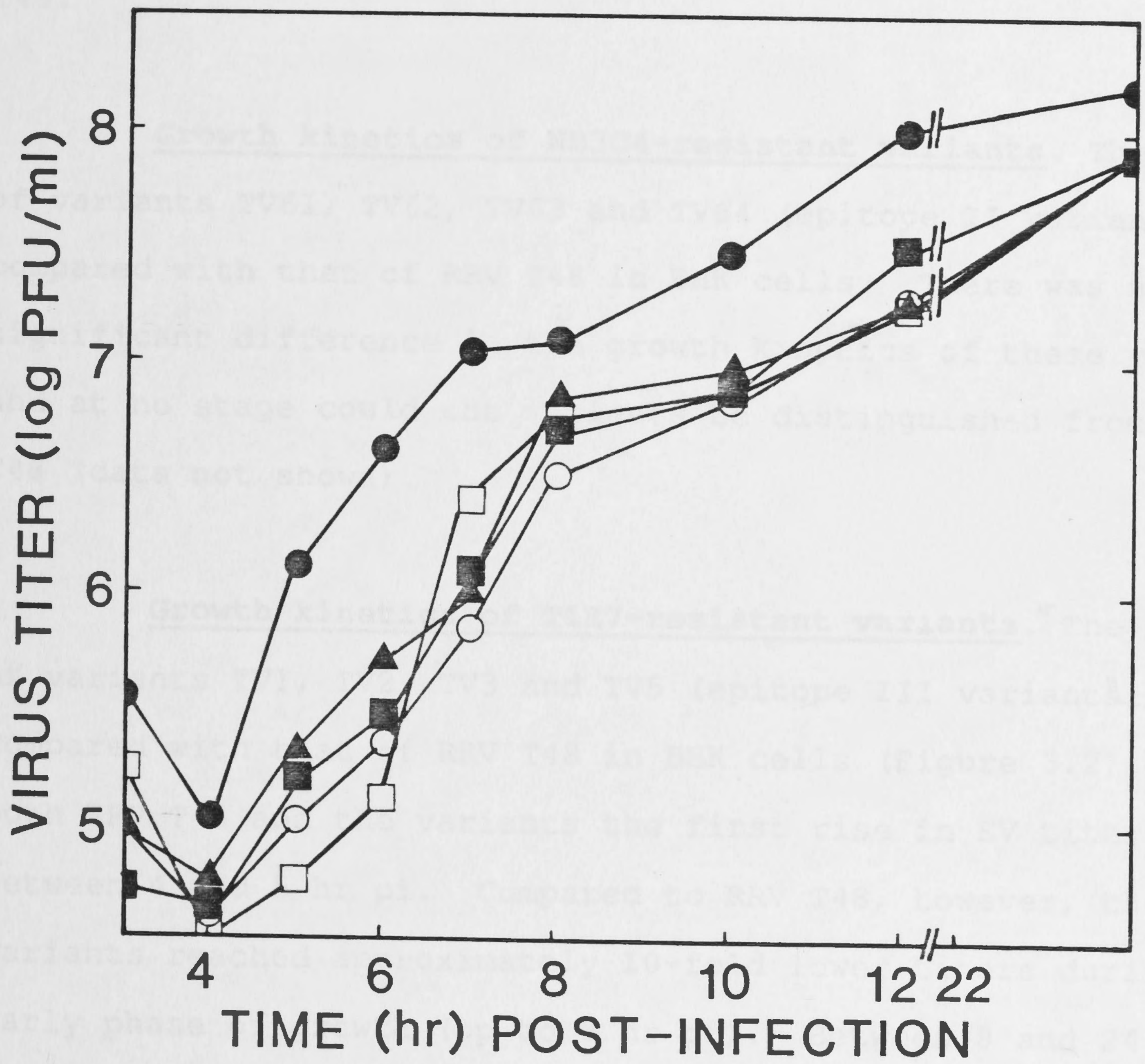


VIRUS TITER (log PFU/ml)
7
6
5
4
3
4
8
12
16
20
TIME (hr) POST-INFECTION

Figure 3.2 **Growth kinetics of T1E7-resistant variants of RRV T48 in BHK cells.**

BHK cell monolayers were infected with the variant or with RRV T48 (moi~1.5) and incubated at 36°C. At intervals samples were removed, diluted into HBSS, and stored at -70°C. EV titers were assayed in duplicate.

EV titer of RRV T48, (●).
EV titer of TV1, (▲).
EV titer of TV2, (■).
EV titer of TV3, (○).
EV titer of TV5, (□).


VIRUS TITER (log PFU/ml)
8
7
6
5
4
6
8
10
12
22
TIME (hr) POST-INFECTION

compared with that of RRV T48 in BHK cells (Figure 3.1). For RRV T48 the first rise in EV titer was between 4 and 6 hr pi. The peak titer (~10^7PFU/ml) was at 24 hr pi. The growth kinetics of all T10C9 resistant variants were similar to each other and were not significantly different from that of RRV T48.

Growth kinetics of NB3C4-resistant variants. The growth of variants TV61, TV62, TV63 and TV64 (epitope II variants) was compared with that of RRV T48 in BHK cells. There was no significant difference in the growth kinetics of these variants and at no stage could the variants be distinguished from RRV T48 (data not shown).

Growth kinetics of T1E7-resistant variants. The growth of variants TV1, TV2, TV3 and TV5 (epitope III variants) was compared with that of RRV T48 in BHK cells (Figure 3.2). For both RRV T48 and the variants the first rise in EV titer was between 4 and 5 hr pi. Compared to RRV T48, however, the T1E7 variants reached approximately 10-fold lower titers during the early phase of growth (up to 8 hr pi). Between 8 and 24 hr pi the differences in titer became less marked and at 24 hr pi, variants and RRV T48 had attained similar titers (~10^8 PFU/ml). At no stage were the growth kinetics of the variants significantly different from each other. Similar results were obtained in three separate experiments.

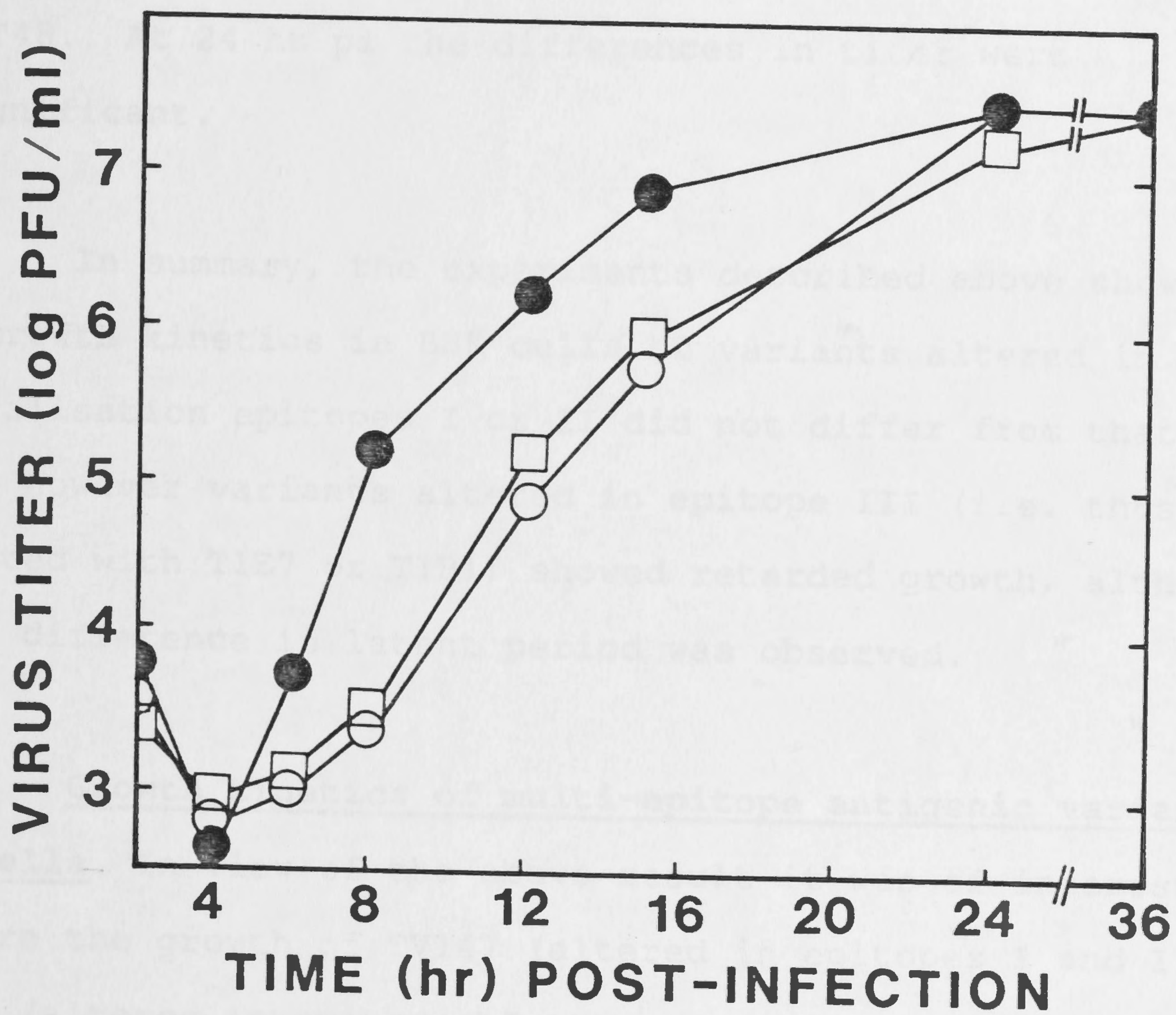
Figure 3.3 **Growth kinetics of T1B1-resistant variants of RRV T48 in BHK cells.**

BHK cell monolayers were infected with the variant or with RRV T48 (moi~1) and incubated at 36°C. At intervals samples were removed, diluted into HBSS, and stored at -70°C. EV titers were assayed in duplicate.

EV titer of RRV T48, (●).

EV titer of TV51, (○).

EV titer of TV59, (□).


VIRUS TITER (log PFU/ml)
7
6
5
4
3
4
8
12
16
20
24
36
TIME (hr) POST-INFECTION

Growth kinetics of T1B1-resistant variants. The growth of TV51 and TV59 (epitope III variants) was compared with that of RRV T48 in BHK cells (Figure 3.3). For both RRV T48 and the variants the first rise in titer was at 4-6 hr pi. However, the variants grew more slowly than RRV T48 and at 16 hr pi the EV titers of the variants were ~1 log unit less than that of RRV T48. At 24 hr pi the differences in titer were insignificant.

In summary, the experiments described above showed that the growth kinetics in BHK cells of variants altered in neutralisation epitopes I or II did not differ from that of RRV T48. However variants altered in epitope III (i.e. those selected with T1E7 or T1B1) showed retarded growth, although no clear difference in latent period was observed.

Growth kinetics of multi-epitope antigenic variants in BHK cells. In view of the above result it was of interest to compare the growth of TV147 (altered in epitopes I and II) and TV161 (altered in epitopes I, II and III) with that of RRV T48. Growth was compared in BHK cells at an moi of ~1 (Figure 3.4). Variant TV147 showed no significant differences from RRV T48 at any stage of growth. However TV161 grew far more slowly than RRV T48 although the latent periods were apparently similar. At 24 hr pi there was no significant difference in titers of TV161 and RRV T48.

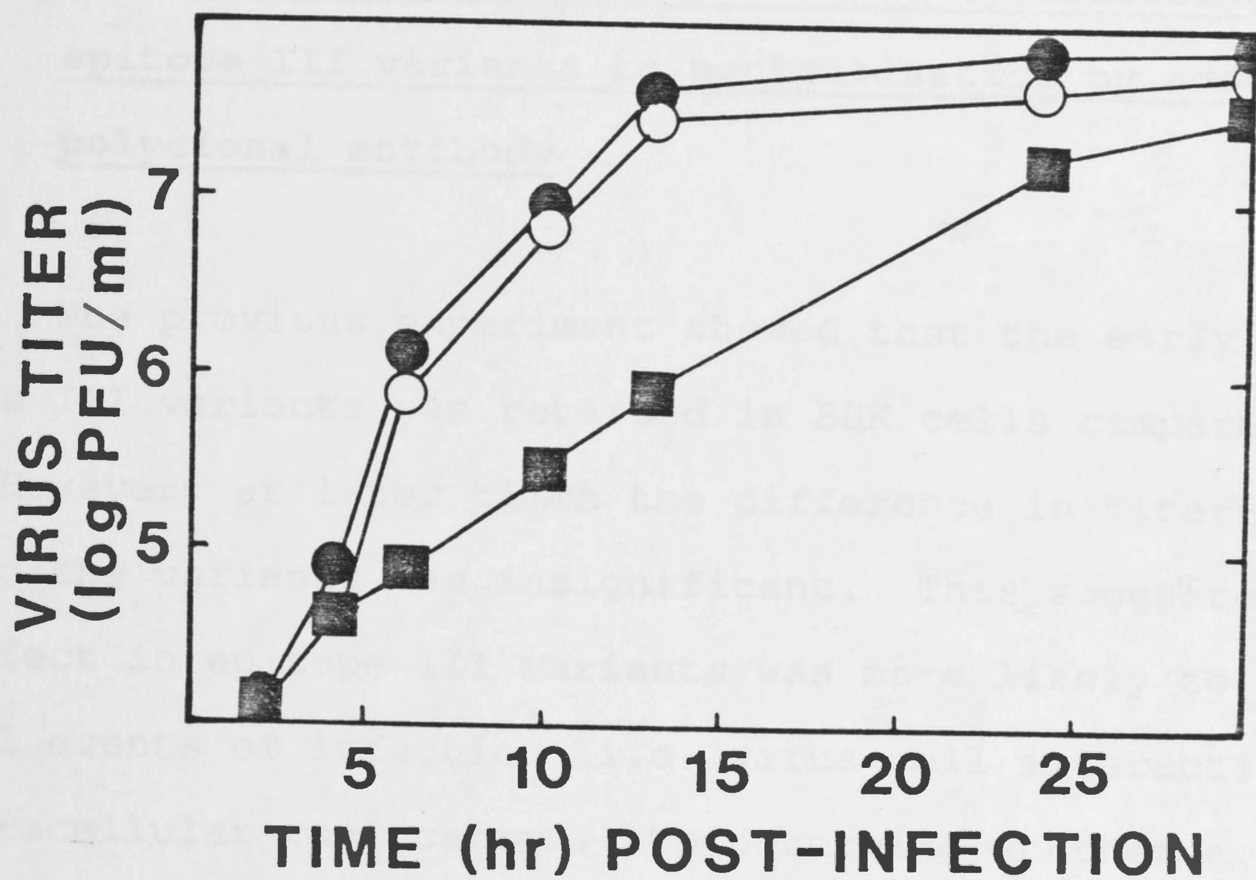
Figure 3.4 **Growth kinetics of multi-epitope variants of RRV T48 in BHK cells.**

BHK cell monolayers were infected with variant or with RRV T48 (moi~1) and incubated at 36°C. At intervals samples were removed, diluted into HBSS, and stored at -70°C. EV titers were assayed in duplicate.

EV titer of RRV T48, (●).

EV titer of TV147, (○).

EV titer of TV161, (■).


VIRUS TITER
(log PFU/ml)
7
6
5
5
10
15
20
25
TIME (hr) POST-INFECTION

We conclude that variants with mutation(s) in neutralisation epitopes I or II or both I and II showed no difference in their growth kinetics in BHK cells when compared with RRV T48. However, variants with a mutation in epitope III (TV1, TV2, TV3, TV5, TV51, TV59 and TV161) showed retarded growth in BHK cells.

3.3.2 **Rate of acquisition of resistance by infecting epitope III variants to neutralisation by added polyclonal antibody**

The previous experiment showed that the early growth of epitope III variants was retarded in BHK cells compared to RRV T48. However, at later times the difference in titers of RRV T48 and the variants was insignificant. This suggested that the defect in epitope III variants was more likely to be in the initial events of infection (i.e. virus-cell interaction) than in intracellular replication. To determine whether a lesion in virus-cell interaction of the epitope III variants existed we have compared the rates of "penetration" of the antigenic variants and RRV T48 in terms of the rate of acquisition of resistance by infecting virus to neutralisation by added polyclonal antibody (Baric *et al*., 1981).

The rate of "penetration" measured in this way was compared for RRV T48, TV1, TV3 and TV161 (Figure 3.5). After 20 min incubation, <5% of RRV T48 had acquired resistance to neutralisation although 10-20% of the variants had become

Figure 3.5 **Rate of acquisition of neutralisation resistance by epitope III variants to added polyclonal antibodies.**

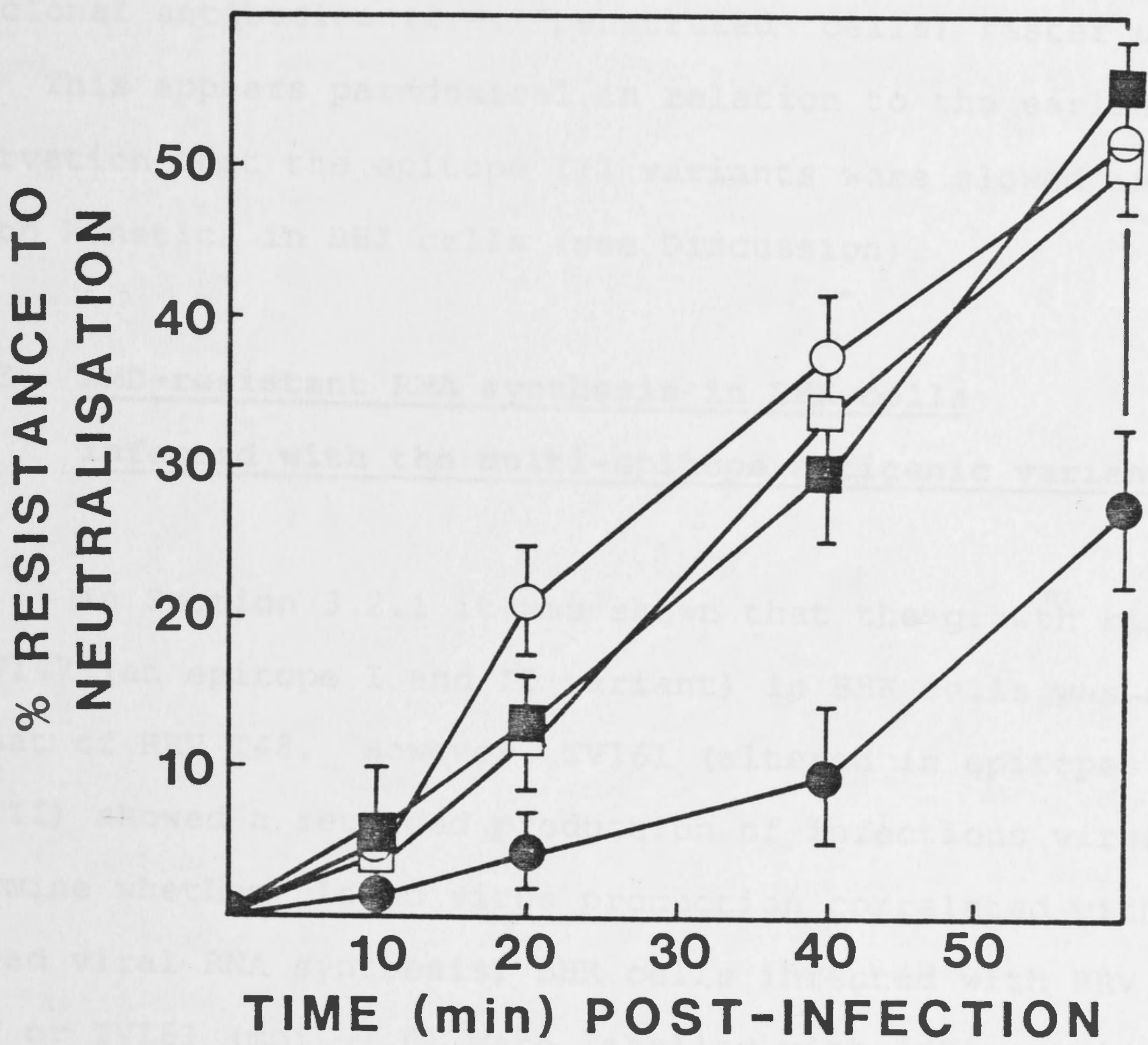
BHK cell monolayers in 60 mm dishes were infected with ~100 BHK PFU of RRV T48 or the variants. At the designated times, the inoculum was removed and 0.5 ml of polyclonal anti-RRV T48 immune ascitic fluid (diluted 1:10 in HBSS) was added. After 10 min at 36°C the antibody was removed, the monolayer washed (x2) with PBS and overlayed for plaque development. The number of plaques obtained after a 60 min adsorption, PBS wash, but no antibody treatment was taken as 100% for each virus. Each assay was done in triplicate and the average values are plotted with error bars showing the standard error of mean.

RRV T48, (●).

TV1, (○).

TV3, (□).

TV161, (■).


% RESISTANCE TO
NEUTRALISATION
50
40
30
20
10
10
20
30
40
50
TIME (min) POST-INFECTION

resistant. At 60 min the corresponding figures for RRV T48 and variants were 28% and 50-55% respectively. For SIN, Baric *et al*. (1981) reported that by 20 and 60 min respectively ~8% and 40% of virus had acquired resistance to neutralisation.

These results showed that in infecting BHK cells, the epitope III variants acquired resistance to neutralisation by polyclonal antibodies (i.e. "penetrated" cells) faster than RRV T48. This appears paradoxical in relation to the earlier observation that the epitope III variants were slowed in their growth kinetics in BHK cells (see Discussion).

3.3.3 AMD-resistant RNA synthesis in BHK cells infected with the multi-epitope antigenic variants

In Section 3.2.1 it was shown that the growth kinetics of TV147 (an epitope I and II variant) in BHK cells was similar to that of RRV T48. However, TV161 (altered in epitopes I, II and III) showed a retarded production of infectious virus. To determine whether slowed virus production correlated with delayed viral RNA synthesis, BHK cells infected with RRV T48, TV147 or TV161 (moi ~1.0) were labelled with [^{3}H]-uridine (with or without AMD) at different times and acid precipitable radioactivity determined (Figure 3.6).

For RRV T48 and TV147 a significant increase in the rate of viral RNA synthesis took place between 8 and 10 hr pi. This was not observed in TV161 infected cells. Peak viral RNA synthesis in both RRV T48- and TV147-infected cells was at 12

Figure 3.6 **AMD-resistant RNA synthesis in BHK cells infected with RRV T48 or with multi-epitope variants.**

BHK cell monolayers were infected with RRV T48 or with the variants (moi~1), or mock-infected with HBSS, and incubated in GMEM + 8% bovine serum. At intervals, monolayers were labelled for two hour periods with EMEM containing 10 µCi/ml of [5-^{3}H]-uridine (times indicated in the Figure are the middle of labelling periods) and incubated at 36°C. Infected cells were labelled in the presence of 5 µg/ml AMD, and mock-infected cells in the absence or presence of 5 µg/ml of AMD. Monolayers were dissociated in 200 µl of 1% SDS and the acid-precipitable radioactivity in 20 µl of the resulting extracts, representing ~2 X 10^{5} cell equivalents, determined.

Incorporation of [^{3}H]-uridine in:

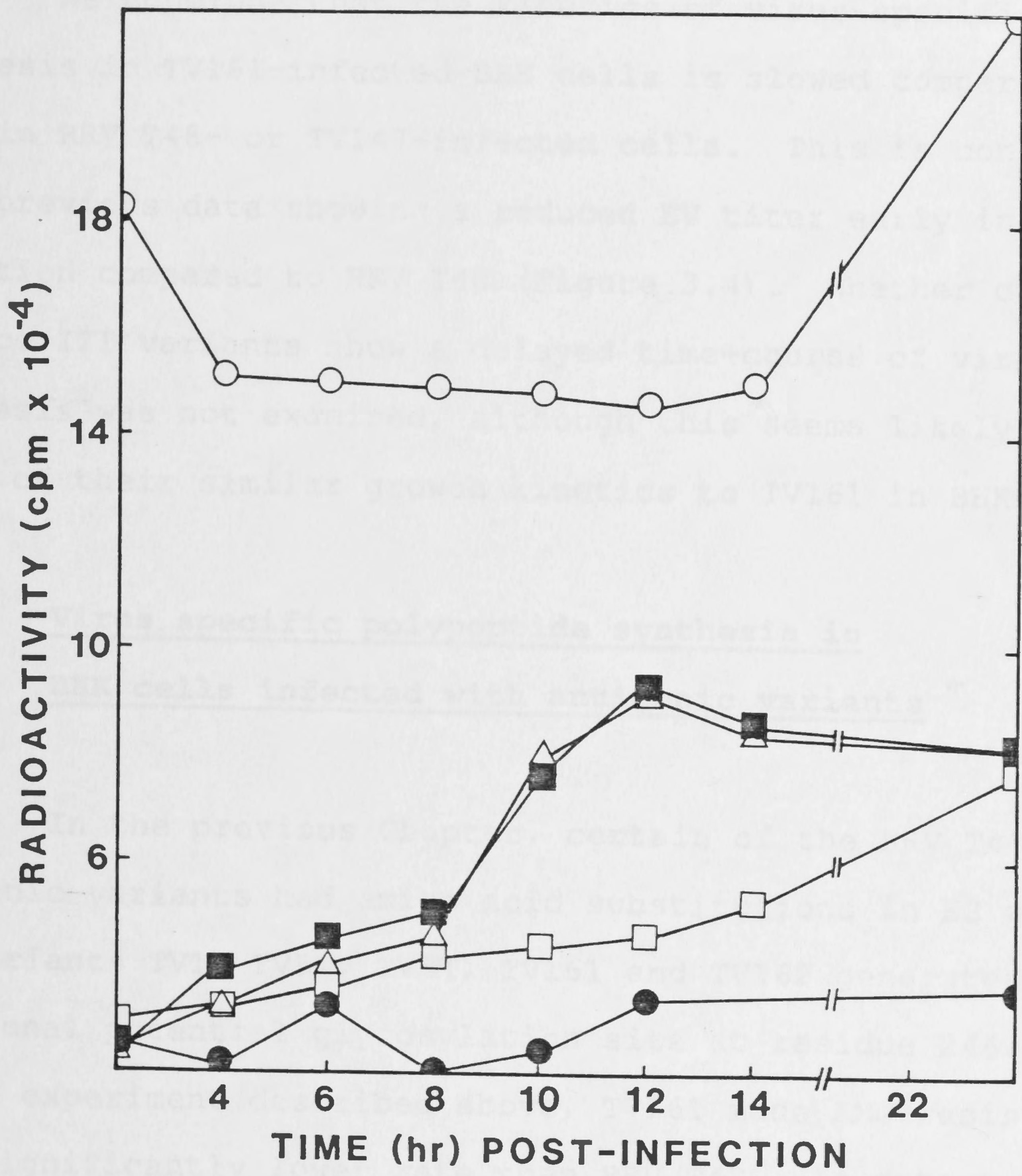
-mock infected cells without AMD, (○).

-mock infected cells with AMD, (●).

-RRV T48 infected cells, (■).

-TV147 infected cells, (△).

-TV161 infected cells, (□).


RADIOACTIVITY (cpm x 10-4)
18
14
10
6
4
6
8
10
12
14
22
TIME (hr) POST-INFECTION

hr pi. The time of peak viral RNA synthesis in TV161-infected cells could not be determined from this experiment. At 24 hr pi, cells infected with RRV T48, TV147 and TV161 showed the same rates of virus specific RNA synthesis.

We conclude that the kinetics of virus-specific RNA synthesis in TV161-infected BHK cells is slowed compared to that in RRV T48- or TV147-infected cells. This is consistent with previous data showing a reduced EV titer early in TV161 infection compared to RRV T48 (Figure 3.4). Whether other epitope III variants show a delayed time-course of viral RNA synthesis was not examined, although this seems likely on the basis of their similar growth kinetics to TV161 in BHK cells.

3.3.4 <u>**Virus specific polypeptide synthesis in BHK cells infected with antigenic variants**</u>

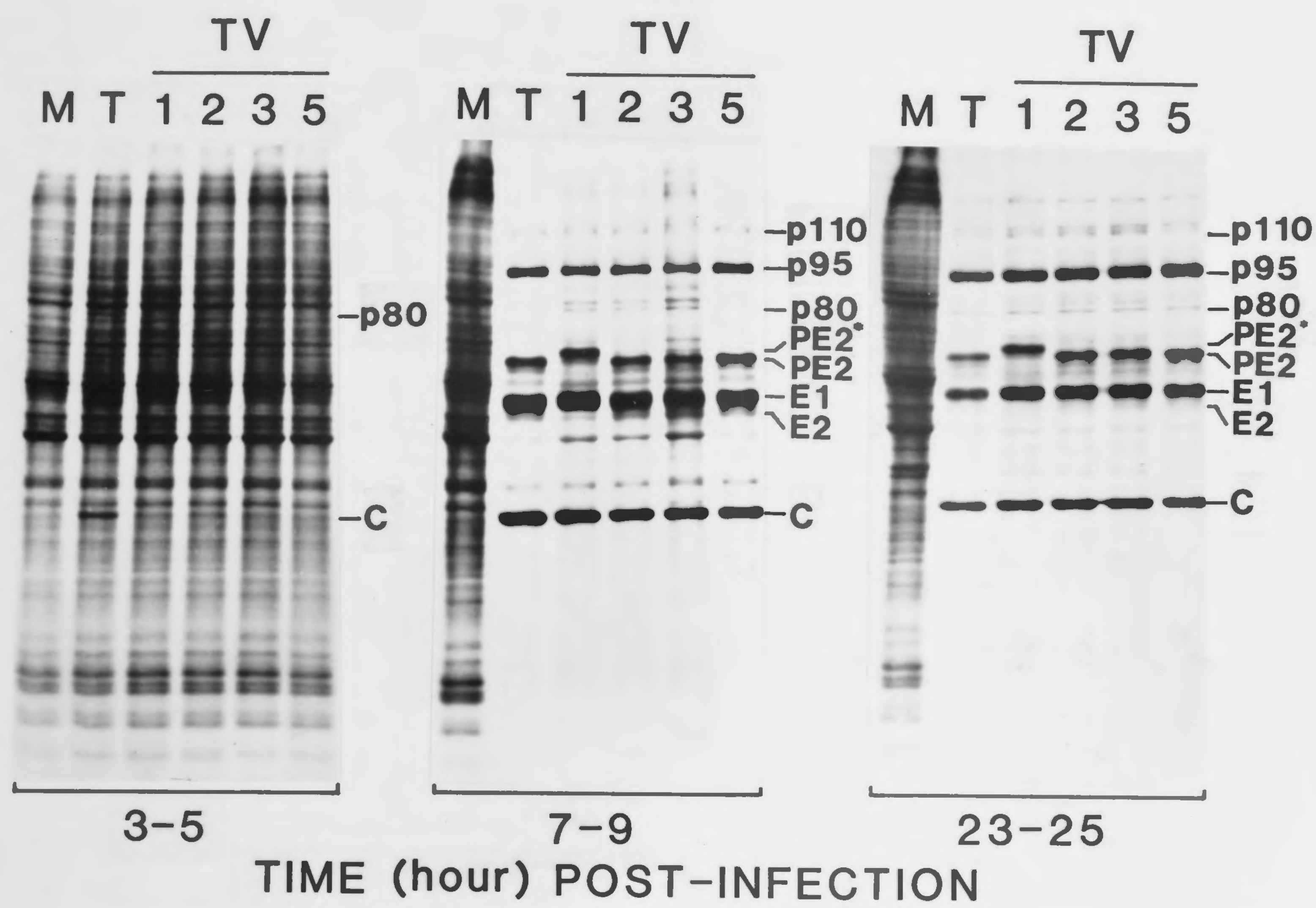
In the previous Chapter, certain of the RRV T48 antigenic variants had amino acid substitutions in E2 which, for variants TV1, TV51, TV59, TV161 and TV162 generated an additional potential glycosylation site at residue 246. Also, in the experiment described above, TV161 made AMD-resistant RNA at a significantly lower rate than RRV T48. To determine whether these differences resulted in changes in the kinetics of viral protein synthesis, in protein processing (e.g. cleavage) or in apparent molecular weight, the synthesis of viral polypeptides by RRV T48 and the above variants was examined in BHK cells. BHK monolayers were infected (moi ~1)

and labelled with [^{3}H]-amino acids for 2 hr periods at 3, 7 and 23 hr pi. Cell extracts were analysed on 10-20% gradient polyacrylamide gels. For RRV T48, eight infection-specific proteins were detected: p110, p95, p80, PE2, E1, E2, C and E3. The identity of the structural proteins (E1, E2, C) and of p95, and PE2 has been established previously (Martin et al., 1979; Short and Dalgarno, unpublished results). E3 is described in Chapter 4 of this thesis. p110 and p80 have not been described. Since these proteins were detected up to 23 hr pi, and the synthesis of the non-structural proteins of other alphaviruses declines rapidly at 4-5 hr pi (Lachmi and Kääriäinen, 1977; Schlesinger and Kääriäinen, 1980), it is probable that p110 and p80 are precursors of structural proteins.

Epitope III variants. Viral polypeptide synthesis by variants TV1, TV2, TV3, TV5 (selected with T1E7) and TV51 (selected with T1B1) was compared with that of RRV T48 (Figure 3.7). At 3-5 hr pi, T48-infected BHK cells synthesised only two virus specific polypeptides: p80 and C; none of the variants showed virus specific polypeptide synthesis at this time (data for TV51 not shown). At 7-9 and 23-25 hr pi, cells infected with T48 or with variants showed labelling of p110, p95, p80, PE2, E1, E2 and C; with longer exposure E3 was detected (data not shown). The mobilities of corresponding viral proteins were identical in all instances except in the case of TV1 (Figure 3.7) and TV51 (data not shown) where PE2 migrated more slowly than the corresponding band in RRV T48-infected cells.

Figure 3.7 **Viral polypeptide synthesis in BHK cells infected with T1E7-resistant variants of RRV T48.**

BHK cell monolayers were infected with RRV T48 (T), TV1, TV2, TV3 or TV5, or were mock-infected (M) with HBSS, and incubated in GMEM+8% bovine serum at 36°C. At indicated times monolayers were labelled for 2 hr with EMEM containing one-tenth the normal concentration of amino acids and 10 μCi/ml [^{3}H]-amino acids. Monolayers were washed (x2) with PBS and dissociated in 200 μl of 1% SDS and the cell extracts were electrophoresed on 10-20% gradient polyacrylamide gels. The gels were fluorographed and exposed to Kodak X-Omat S X-ray film at -70°C for 6 days. Positions of viral polypeptides are indicated.



TV
M T 1 2 3 5
-p80
-C
3-5
TV
M T 1 2 3 5
-p110
-p95
-p80
PE2*
PE2
-E1
E2
-C
7-9
TV
M T 1 2 3 5
-p110
-p95
-p80
PE2*
PE2
-E1
E2
-C
23-25
TIME (hour) POST-INFECTION

Designated PE2*, it had an apparent mol. wt. of 64K compared to 62K for RRV T48 PE2. This may be due to additional glycosylation since E2 of these variants was predicted to have an additional site for asparagine-linked glycosylation (Chapter 2). Interestingly, no differences were seen in the migration of intracellular E2 or E3 (which are produced by cleavage of PE2). Whether E2 in purified virions of these variants has additional glycosylation is not known.

No differences in shut-down of host protein synthesis were apparent at any time post-infection when cells infected with RRV T48 and variants were compared.

In summary, variants altered in epitope III showed delayed protein synthesis in BHK cells compared to RRV T48. Variants TV1 and TV51 made a slightly higher molecular weight form of PE2 than did RRV T48, consistent with the earlier prediction of an additional glycosylation site in E2.

Epitope I and epitope II variants. Viral polypeptide synthesis in BHK cells infected with TV31, TV33, TV35, TV42 (T10C9-resistant variants altered in epitope I) and TV61, TV62, TV63, TV64 (NB3C4-resistant variants altered in epitope II) was compared with that in RRV T48-infected cells. At 3-5 hr pi BHK cells infected with the variants or with RRV T48 showed p80 and C (data not shown). At 7-9 and 23-25 hr pi eight virus specific polypeptides were seen in cells infected with RRV T48 or with variants. These were p110, p95, p80, PE2, E1, E2, C and E3 as observed previously. Thus there were no differences

in the kinetics of viral protein synthesis by epitope I and epitope II variants compared to RRV T48, consistent with previous data showing similar growth rates in BHK cells. No differences were seen in the mobilities of viral proteins between the variants and RRV T48.

Multi-epitope variants. Viral polypeptide synthesis was compared in BHK cells infected with RRV T48, TV147 (altered in epitopes I and II) or TV161 (altered in epitopes I, II and III). At 3-5 hr pi cells infected with RRV T48 or with TV147 showed labelling of p80 and C. At this stage no virus specific proteins were detected in TV161-infected cells. At 7-9 and 23-25 hr pi, both RRV T48- and TV147-infected BHK cells showed the same eight virus specific polypeptides as noted above (data not shown). TV161-infected cells also showed the same proteins at the same relative position with the exception of PE2 which migrated more slowly than RRV T48 PE2 (data not shown). The slow migration of PE2 may be due to the predicted additional glycosylation site in TV161 E2 (Chapter 2) although no differences were seen in the migration of E2 or E3. There were no apparent differences in shut down of host protein synthesis between RRV T48-infected and the variant-infected cells.

In summary, T10C9 variants (altered in epitope I), NB3C4 variants (altered in epitope II) and TV147 (altered in epitopes I and II) showed no differences from RRV T48 in the kinetics of synthesis or pattern of processing of viral

polypeptides in infected BHK cells. However, all variants with a mutation in epitope III showed delayed synthesis of viral polypeptides in infected BHK cells. This was consistent with the delayed time course of viral RNA synthesis and the lower initial EV titers in BHK cells infected with epitope III variants. TV1, TV51 and TV161 made a higher mol. wt. form of PE2 although no differences were seen in migration of E2 or E3 of these variants compared with RRV T48.

3.3.5 **Virulence of RRV antigenic variants for mice**

It has previously been shown that mab-resistant variants of many viruses have altered virulence for their experimental host (see Introduction). This together with the observation that serial passaging of avirulent RRV NB5092 generated virulent virus altered around the neutralisation epitopes (Meek, 1986) prompted us to examine the virulence of RRV T48 antigenic variants in mice.

Day-old and week-old mice were injected i.p. with serial dilutions of the virus stocks. The onset and severity of symptoms and the number of deaths were recorded daily. The LD_{50} of virus stocks and the AST of infected mice were calculated to define the virulence of the virus. Several determinations were carried out for each variant and comparisons were made with results for RRV T48 using one way analysis of variance and t-tests.

Table 3.1 **Virulence of RRV T48 antigenic variants for day-old mice.**

Virus	Altered epitope	LD_{50}* (Vero PFU)		AST* (days)	
RRV T48	—	0.047	(3)	4.93	(4)
TV31	I	0.055	(3)	5.53	(3)
TV33	I	0.065	(3)	5.20	(3)
TV35	I	0.056	(3)	5.12	(3)
TV42	I	0.057	(3)	5.63	(3)
TV1	III	0.022	(3)	5.20	(4)
TV2	III	0.058	(3)	4.20	(3)
TV3	III	0.019	(3)	4.68	(4)
TV5	III	0.056	(3)	5.43	(3)

*Figures presented are an average of a number of separate determinations (shown in brackets).

The virulence parameters of variants were compared with those of RRV T48 using one-way analysis of variance and the t-tests. The LD_{50} and the AST values of variants did not differ significantly from those of RRV T48.

Virulence in day-old mice. RRV T48 was highly virulent for day-old mice with an LD_{50} of 0.047 PFU (Table 3.1); infected mice showed severe hind leg paralysis and had an AST of 4.93 days. Variants TV31, TV33, TV35 and TV42 (epitope I variants) and TV2 and TV5 (epitope III variants) had slightly higher LD_{50}s than RRV T48, although the differences were not statistically significant. Compared to RRV T48, variants TV1 and TV3 had slightly reduced LD_{50}s, though differences were not significant statistically. Thus none of the epitope I or epitope III variants showed a significant difference in LD_{50} or AST when compared with RRV T48 (Table 3.1). No differences were observed in the time of onset or severity of symptoms in mice infected with the variants or RRV T48.

Virulence in week-old mice. For RRV T48 the LD_{50} and AST did not differ significantly between day-old and week-old mice (Tables 3.1 and 3.2). Further, no differences were observed in the time of onset or the severity of symptoms between RRV T48 infected day-old and week-old mice. The LD_{50}s and the AST values for epitope I, II and III variants showed slight differences from those for RRV T48 although the differences were not statistically significant except for TV2, TV5 and TV161 (Table 3.2). TV2 and TV5 were slightly less virulent than RRV T48 ($p<0.05$) with LD_{50}s of 0.206 and 0.275 PFU respectively compared to 0.048 PFU for RRV T48. The ASTs of mice infected with TV2 and TV5 did not differ significantly from those of mice infected with RRV T48. However for TV161-

Table 3.2 **Virulence of RRV T48 antigenic variants for week-old mice.**

Virus	Altered epitope	Amino acid change and position in E2	LD_{50}* (Vero PFU)	AST* (days)
RRV T48	-	-	0.048 (6)	5.92 (4)
TV31	I	Thr→Ile (216)	0.056 (3)	6.04 (4)
TV33	I	Thr→Ile (216)	0.055 (3)	5.38 (3)
TV35	I	Thr→Ile (216)	0.068 (3)	6.20 (6)
TV42	I	Thr→Ile (216)	0.075 (4)	5.81 (6)
TV61	II	Lys→Gly (234)	0.039 (3)	6.12 (3)
TV62	II	Lys→Ile (234)	0.045 (3)	6.43 (3)
TV63	II	Lys→Asn (234)	0.043 (3)	5.93 (3)
TV64	II	Lys→Glu (234)	0.055 (3)	6.72 (3)
TV1	III	Asp→Asn (246)	0.031 (3)	7.08 (5)
TV2	III	Arg→Ser (251)	**0.206** (3)	5.96 (5)
TV3	III	Thr→Pro (248)	0.028 (5)	6.60 (3)
TV5	III	Asp→Val (246)	**0.275** (3)	4.84 (3)
TV51	III	Asp→Asn (246)	0.050 (3)	6.84 (3)
TV59	III	Asp→Asn (246)	0.050 (3)	6.27 (3)
TV131	I and II	Thr→Ile (216) Lys→Asn (234)	0.049 (3)	6.00 (3)
TV147	I and II	Thr→Ile (216) Lys→Asn (234)	0.053 (3)	5.80 (3)
TV161	I, II and III	Thr→Ile (216) Lys→Asn (234) Asp→Asn (246)	0.079 (3)	**8.64** (3)

*Figures presented are an average of a number of separate determinations (shown in brackets).

The virulence parameters of variants were compared with those of RRV T48 using one-way analysis of variance and the t-tests. Virulence parameters of variants did not show a significant difference from those of RRV T48 except where shown in bold type. Thus, LD_{50}s of TV2 and TV5 and the AST of TV161 showed a significant difference ($p<0.05$) from the respective virulence parameters of RRV T48.

infected mice there was a significant increase in AST (8.64 days) over that seen for mice infected with RRV T48 ($p<0.05$). No significant difference in LD_{50} or in the time of onset and severity of symptoms was noted for TV161 compared with RRV T48.

In summary, none of the epitope I, epitope II or epitopes I and II variants showed a significant difference in virulence when compared with RRV T48 in day-old or week-old mice. Only variants TV2 and TV5 (altered in epitope III), and TV161 (the only triple variant checked which was altered in epitopes I, II and III), showed any change in virulence; these variants were slightly attenuated in week-old mice. However, the other four epitope III variants showed no significant change in virulence compared to RRV T48.

3.3.6 Growth of antigenic variants in week-old mice

Since certain epitope III variants showed a small reduction in virulence for week-old mice, the growth of these variants in week old mice, together with that of other epitope I and epitope III variants of unchanged virulence was compared with RRV T48. The kinetics of viral growth, the maximum titers attained and the tissue distribution of virus was examined. The tissues selected for examination were hind leg muscle, brain and blood. Hind leg muscle was chosen as it is the major site of RRV replication in mice (Chapter 1). Brain was chosen as the extent of RRV replication in neuronal tissue may determine the severity of symptoms and death.

Figure 3.8 **Growth of RRV antigenic variants in mice.**

Week-old WEHI mice were injected i.p. with 100 PFU of virus. Mice were killed and tissues removed at 24 hr intervals. Virus titers were assayed in blood, brain and hind leg muscle. Each time point represents the average of determinations made in three mice. Error bars are shown where the error is greater than 0.2 log units.

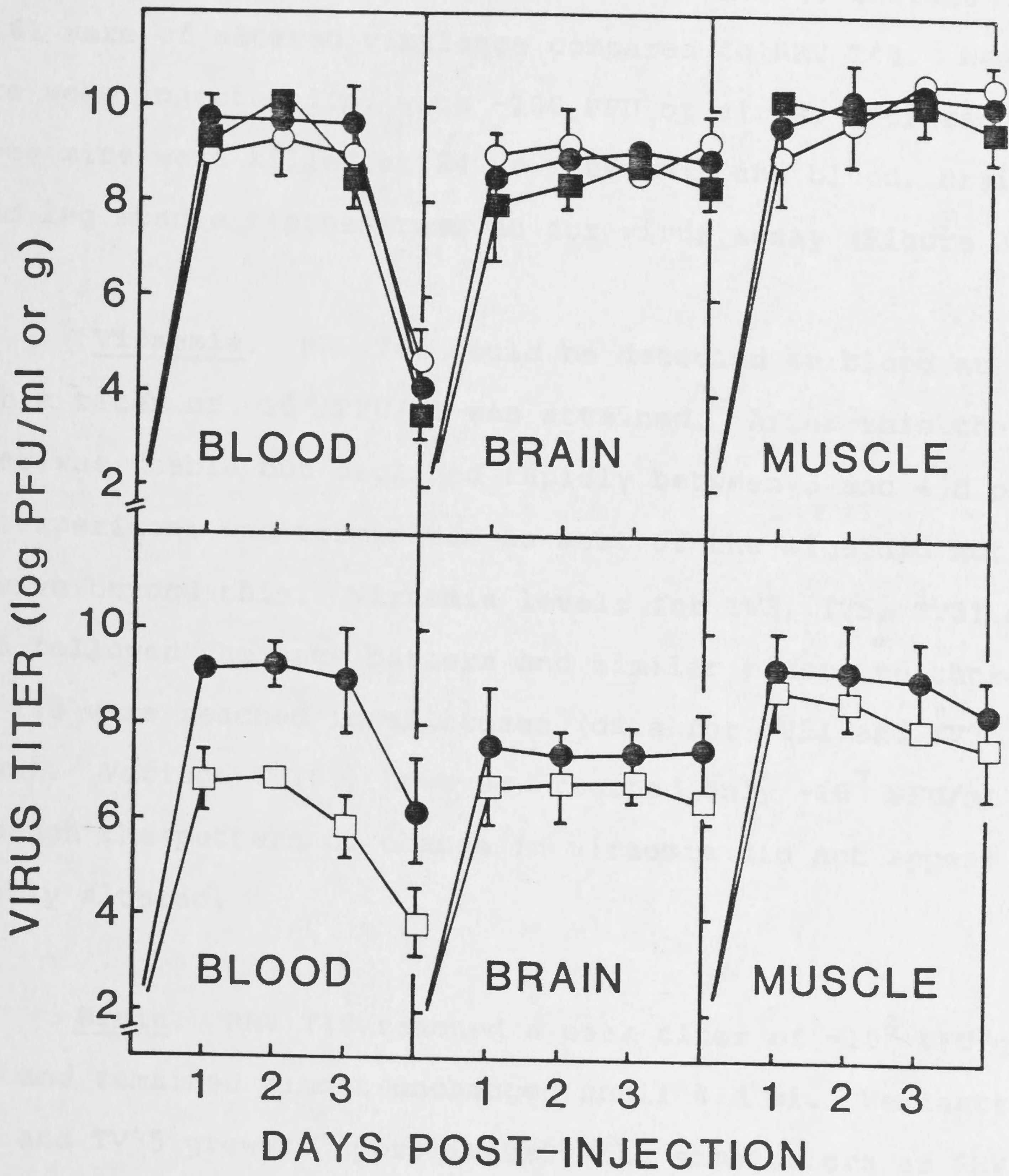
The growth of RRV T48, TV3 and TV5 was compared in one experiment (upper half of the Figure). In another experiment the growth of RRV T48 and TV161 was compared (lower half of the Figure).

RRV T48 titer, (●).

TV3 titer, (○).

TV5 titer, (■).

TV161 titer, (□).


VIRUS TITER (log PFU/ml or g)
10
8
6
4
2
BLOOD
BRAIN
MUSCLE
10
8
6
4
2
BLOOD
BRAIN
MUSCLE
1
2
3
1
2
3
1
2
3
DAYS POST-INJECTION

The variants examined were TV3 and TV5 (altered in epitope III), TV31 and TV35 (altered in epitope I) and TV161 (altered in epitopes I, II and III). Variants TV3, TV31 and TV35 had a similar virulence to that of RRV T48 whereas TV5 and TV161 were of altered virulence compared to RRV T48. Week-old mice were injected i.p. with ~100 PFU of virus. For each virus three mice were killed at 24 hr intervals and blood, brain and hind leg muscle tissues removed for virus assay (Figure 3.8).

Viraemia. RRV T48 could be detected in blood at 1 d pi when a titer of ~10^9 PFU/ml was attained. After this the virus titer was stable but declined rapidly between 3 and 4 d pi when the experiment was terminated as most of the mice did not survive beyond this. Viraemia levels for TV3, TV5, TV31 and TV35 followed the same pattern and similar titers to those of RRV T48 were reached in all cases (data for TV31 and TV35 not shown). Variant TV161, however, reached only ~10^7 PFU/ml although the pattern of change in viraemia did not appear to be greatly altered.

Brain. RRV T48 reached a peak titer of ~10^8 PFU/g at 1 d pi and remained almost unchanged until 4 d pi. Variants TV3, TV31 and TV35 grew to approximately the same titers as RRV T48 (data for TV31 and TV35 not shown). Compared to RRV T48, variants TV5 and TV161 grew to slightly lower titers at each time point, although the differences were not statistically significant.

Hind leg muscle. RRV T48 attained a peak titer of ~10^9 PFU/g at 1 d pi after which the titer did not change significantly up to 4 d pi. Variants TV3, TV5, TV31 and TV35 attained similar peak titers and showed a similar pattern of growth which did not differ significantly from that of RRV T48 (data for TV31 and TV35 not shown). Compared to RRV T48, TV161 grew to slightly lower titers; this difference was consistent throughout the experiment although not significant statistically.

In summary, none of the antigenic variants could be distinguished from RRV T48 in their growth characteristics in week-old mice except TV161 which reached ~100-fold lower titers in blood compared to RRV T48. Variant TV5, which had a ~4-fold higher LD_{50} than RRV T48, did not show a significant difference in viral growth or in peak titers when compared with RRV T48.

3.4 DISCUSSION

The aim of the studies described in this Chapter was to examine the effect of the previously determined amino acid substitution(s) in E2 on the biological properties of RRV T48 antigenic variants in cultured cells and in mice. The results obtained show that variants altered in epitopes I or II could not be distinguished from RRV T48 in their kinetics of growth, protein synthesis and RNA synthesis in BHK cells. However all epitope III variants examined showed retarded growth and delayed RNA and protein synthesis in BHK cells.

An interesting parallel to the biological properties of the variants in BHK cells was seen in mice. Antigenic variants altered in epitopes I or II showed no differences in virulence or growth in mice. However, three epitope III variants (TV2, TV5 and TV161) out of seven examined showed slightly reduced virulence for week old mice. Further, TV161 produced lower levels of viraemia in mice than did RRV T48.

From the results observed in cultured cells it seems likely that the changes seen in mice did not result from altered recognition of the variants by the immune system. This follows from two considerations. First, the week-old mice used in the present studies are considered to be immunologically immature (Murgita and Wigzell, 1981); and second, any antibody produced in mice would be polyclonal and able to neutralise the antigenic variants effectively. Hence, changes in biological properties of epitope III variants suggest some important function of this epitope besides that of reacting with neutralising antibody.

3.4.1 Involvement of the "neutralisation domain" in attachment/entry of virus in cultured cells

Antigenic variants altered in epitopes I or II, or in both I and II had similar kinetics of growth and of RNA and protein synthesis to RRV T48 in BHK cells. However variants altered in epitope III (TV1, TV2, TV3, TV5, TV51 and TV52) and TV161 (altered in epitopes I, II and III) showed retarded

growth in BHK cells. Consistent with this, epitope III variants showed a delayed synthesis of viral RNA and protein in BHK cells. However no obvious defect in RNA replication or in protein synthesis was seen. These experiments suggest that epitope III variants had a lesion in viral attachment/entry, a conclusion somewhat strengthened by the observation that epitope III variants showed an altered rate of acquisition of resistance to neutralisation by antibody compared to RRV T48. It should be noted first however that control experiments with epitope I and epitope II variants designed to determine their "penetration" rates were not carried out. Secondly, the alteration in rate of "penetration" was in the direction of an increase rather than a decrease for TV1, TV3 and TV161. Further studies are required to clarify these issues.

Studies with picornaviruses and rotaviruses suggest that the neutralisation antigenic sites of these viruses are involved in virus-cell interaction. Thus one of the three neutralisation sites on VP1, the major outer capsid protein of foot-and-mouth disease virus, is involved in virus attachment to bovine kidney cells since mabs to this site inhibit virus attachment to cells whereas mabs to other sites do not (Baxt et al., 1984). In similar type of studies Emini et al. (1983) suggested that one of the seven neutralising epitopes on polio virus type I is involved in virus attachment to cells. More recently Sabara et al. (1985) have demonstrated that the receptor binding site of simian rotavirus is located in the major glycoprotein in a domain which contains the neutralisation antigen.

3.4.2 Relationship between changes in the "neutralisation domain" and changes in virulence of antigenic variants for mice

In order to examine the role of the neutralisation epitopes in determining viral virulence, the virulence of the antigenic variants was determined in mice. None of the antigenic variants showed any differences in virulence from RRV T48, except for TV2, TV5 and TV161 which were slightly attenuated for week-old mice. None of the variants showed significantly increased virulence. Interestingly, TV2 and TV5, which showed a small increase in LD_{50} (~4 fold), did not show a significant increase in AST. Variant TV161 showed a small, but significant increase in AST, although it did not show a significant change in LD_{50}. This suggests that these two virulence parameters do not inevitably co-vary.

It is noteworthy that the three variants which showed attenuation all had a mutation in epitope III (Table 3.2). Variant TV2 had an Arg→Ser substitution at residue 251 while TV5 had an Asp→Val substitution at residue 246. However an Asp→Asn substitution at residue 246 in TV1 did not result in a significant virulence change. The explanation for this possibly lies in the nature of the amino acid changes. Variant TV161 had changes in all three epitopes: Thr→Ile at residue 216, Lys→Asn at residue 234 and Asp→Asn at amino acid 246. Variant TV1 also had an Asp→Asn substitution at residue 246 in

E2; however, its virulence was unchanged. It is possible that the attenuation seen for TV161 was a cumulative effect of multiple mutations since none of the equivalent single changes altered the virulence of the corresponding variants.

Our results are consistent with the findings of Meek (1986) who showed that single amino acid substitutions in RRV NB5092 E2 accompanied altered virulence, although the extent of change was much greater than that seen in the present study. In the present study, single amino acid substitutions in TV2 and TV5 resulted in approximately four-fold increase in LD_{50} whereas in RRV NB5092 variants the difference was ~10^6 times (Meek, 1986). This may have resulted from the positive selection pressure applied in the studies of Meek (1986) which would select for amino acid mutations enhancing viral growth and thus virulence. However, in our case the mutants were selected for their neutralisation resistance and not for a change in virulence. This may also explain why only a proportion of epitope III variants showed changes in biological properties. It should also be noted that changes leading to enhanced virulence may have occurred in the non-structural protein genes of RRV NB5092 variants of Meek (1986) (Chapter 1).

When the titers of the antigenic variants were measured in different tissues of infected mice, no significant differences were observed except that TV161 produced a lower viraemia than RRV T48. This may be related to the longer AST

of mice infected with TV161. TV5 which had a four-fold higher LD_{50} than RRV T48 showed slightly lower titers in mouse brain than RRV T48 although the differences were not statistically significant. A change in cell tropism of the variants could affect virulence and this may not have been reflected in gross titers measured in experiments carried out in this Chapter.

From these results, it is clear that only changes in epitope III altered the virulence of the virus and that even within this epitope, only certain amino acid substitutions were capable of affecting virulence. Thus TV1 and TV3 (altered at amino acids 246 and 248 respectively) did not show a change in virulence although TV5 (altered at amino acid 246) had reduced virulence. Similar observations have been made with rabies virus. Seif _et al_. (1985) isolated 58 mab-neutralisation resistant variants of rabies virus using 4 different mabs which bound to a single antigenic site. They found that only variants altered at amino acid 333 of the glycoprotein were attenuated; others changed at amino acids 330, 336 or 338 did not show any change in their virulence. They suggested that arginine 333 was probably within the region of the glycoprotein which interacted with the host cell receptor. Similarly for mumps virus only one out of four neutralisation resistant variants isolated with a single mab had altered virulence (Löve _et al_., 1985); the amino acid changes which took place in the different variants were not identified.

3.4.3 **Biological role of the "neutralisation domain" in E2**

Studies with cultured cells and with mice described in this Chapter suggest a role for epitope III in some aspect of viral attachment/entry. This is consistent with recent studies of Meek (1986) who showed that virulent mutants obtained by passaging avirulent RRV NB5092 in mice had changes at amino acids 212, 232, 234 and 251 which were located in neutralisation epitopes in E2. As RRV NB5092 was passaged in neonatal mice where the immune system is immature, it is unlikely that the mutations were selected as a result of antibody pressure. Since the virulent mutants of RRV NB5092 grew to higher titers in the blood and brain of mice it may be that mutants were selected which were better able to interact with the cellular receptor. Meek (1986) proposed that amino acids at which substitutions occurred during passaging were involved in receptor binding. In SIN, a region of E2 involved in neutralisation is involved in virus attachment and entry in BHK cells (Baric _et al_., 1981; Olmsted _et al_., 1986).

Based on these studies we propose that both receptor binding site and neutralisation epitopes are in the same domain of E2. Thus a change in a neutralisation epitope could alter the conformation of the receptor binding site leading to an altered recognition of cellular receptor. This could result in a change in cell/tissue tropism or in an altered rate of attachment/entry of virus to the cell which may affect the establishment and spread of the infection. Such changes could

affect growth rate and the virulence of the virus (Chapter 1).

If this model is correct it appears that the three epitopes of the "neutralisation domain" are not involved in virus-antibody interaction and in virus-cell interaction equally since epitopes I, II and III were all involved in virus-antibody interaction whereas only epitope III appears to be involved in virus-cell interaction. However in RRV NB5092 variants, virulence-associated changes occurred in all three neutralisation epitopes (Meek, 1986). This may suggest that the nature of amino acid change, rather than its position in the neutralisation epitope, is important in affecting virus-cell interaction.

The model described above is similar to the situation proposed for influenza virus (Wilson et al., 1981; Wiley et al., 1981) and the common cold virus (Rossmann et al., 1985) where a pocket of conserved amino acids representing the receptor binding site is surrounded by neutralisation epitopes. Mutants of swine influenza virus which differ in their capacity to replicate in chicken embryo cells, Madin-Darby kidney cells and the respiratory tract of swine (Kilbourne et al., 1981) can be distinguished by neutralising mabs (Kilbourne et al., 1983). A single amino acid difference at residue 155 is responsible for these differences; this amino acid is located in the antigenic area immediately adjacent to the proposed receptor binding site (Both et al., 1983).

3.4.4 **Mechanism of RRV neutralisation**

No published information exists which explains the mechanism of alphavirus neutralisation although different mechanisms have been proposed for neutralisation of different viruses. Thus neutralising antibodies to foot-and-mouth disease virus (Baxt et al., 1984) and bovine rotavirus (Sabara et al., 1985) inhibit virus-cell interaction. However virus bound to neutralising antibodies may still bind to host cells. Thus neutralised rabbit poxvirus (Joklik, 1964) and poliovirus (Mandel, 1967) bind to HeLa cells. Emini et al. (1983) showed that binding of mab to six out of seven neutralisation epitopes of poliovirus did not inhibit virus interaction with HeLa cells, although mab to one epitope did inhibit virus binding to the cells. Similarly influenza A virus neutralised with polyclonal antibody or with mab binds to a variety of cell lines (see Dimmock, 1984). Another mechanism proposes that antibody binding to virus induces conformational changes in viral structure, thus rendering the virus non-infectious. This is based on the observation that binding of neutralising antibody to poliovirus (Mandel, 1976) or to bovine rotavirus (Carthew, 1976) changes the isoelectric point of the virus, indicating a conformational change in the protein structure.

It would appear that different viruses may have different mechanisms of neutralisation and that different mechanisms may operate for neutralisation of the same virus

with different antibodies. Since we have proposed that the neutralisation epitopes and the receptor binding site are in the same domain of RRV E2, it is possible that binding of neutralising antibodies inhibits virus binding to cellular receptor, thus neutralising the virus infectivity. This model will require a multi-hit mechanism of virus neutralisation since a large number of E2 molecules and thus a number of receptor binding sites are available on the virion surface (Della-Porta and Westaway, 1977). Again, this may be one of several mechanisms involved in alphavirus neutralisation.

CHAPTER 4

BIOLOGICAL STUDIES ON A MUTANT OF RRV T48 CARRYING A DELETION IN THE E2 GENE

4.1 INTRODUCTION

In the course of the nucleotide sequence determination of the 26S subgenomic RNA of RRV T48, Dalgarno *et al*. (1983) obtained evidence which predicted the existence, in the virus stock, of a mutant with a deletion of 21 nucleotides in the E2 gene. Subsequently a mutant which formed uniformly small plaques was detected in the stock. The mutant was plaque purified twice (L. Dalgarno, unpublished data) and its genome sequenced in the appropriate region (S.G. Faragher, unpublished data). This showed that the small plaque mutant had the predicted deletion in a region of the E2 gene coding for amino acids 55-61. The mutant was referred to as RRV dE2. Comparison of TaqI and HaeIII restriction digest profiles of cDNAs to virion RNA showed no difference between RRV T48 and RRV dE2 except for the deletion in E2 of RRV dE2 (Faragher, 1982), although a possibility of a point mutation(s) could not be ruled out. The existence of this genetically defined mutant provided an opportunity to investigate the effect of the deletion in E2, the neutralisation antigen, on the biological properties of the virus.

Preliminary studies of Faragher (1982) indicated that RRV dE2 could not be distinguished from RRV T48 in single cycle growth in BHK cells or in neutralisation tests using polyclonal antiserum. The mutant was, however, more thermolabile at 50°C and made more RNA in BHK cells than RRV T48. In addition, RRV dE2 shut down host protein synthesis in BHK cells faster than did RRV T48.

These preliminary observations were indicative of significant changes in the structure and replication of RRV dE2 which were worthy of further investigation. In this Chapter we examine the structural proteins of RRV dE2 and RRV T48 and compare their biological properties in cultured cells. We also compare the virulence and growth of RRV dE2 in mice with that of RRV T48.

4.2 MATERIALS AND METHODS

4.2.1 Virus, cells and plaque assay

These have been described in Sections 2.2.1, 2.2.2 and 2.2.3.

4.2.2 Rate of acquisition of resistance by infecting virus to neutralisation by added polyclonal antibody (Baric et al., 1981)

This has been described in Section 3.2.3.

4.2.3 Preparation of primary mouse cell cultures

Two to three day-old outbred WEHI mice of either sex were killed and rinsed with ethanol and PBS. Skin and thigh muscles were removed, chopped and digested with ~100 units of crude collagenase (Worthington Biochemical Co., USA; diluted in

HBSS) per g of tissue at $37^{o}C$ for 30-60 min. After standing, the supernatant was removed and seived through a fine stainless steel mesh into 2.5 ml of heat-inactivated foetal calf serum. Cells were pelleted by slow centrifugation (400 g) for 5 min and resuspended in GMEM supplemented with 8% bovine serum and 2% foetal calf serum. Cells were seeded in 35 mm plastic dishes at a density of $2x10^{6}$/dish and incubated at $36^{o}C$ in a humidified atmosphere of 5% carbon dioxide and 95% air. About 90% confluent monolayers were obtained after 24 hr.

4.2.4 Labelling of virus specific polypeptides

Virus specific proteins in infected BHK cells were labelled with [^{3}H]-amino acids (Section 3.2.6).

4.2.5 Purification of virus

Virus was purified by polyethylene glycol precipitation and sucrose gradient centrifugation (Section 2.2.5).

4.2.6 Polyacrylamide gel electrophoresis of proteins

This has been described in Section 3.2.6.

4.2.7 Estimation of rates of RNA synthesis

Rates of AMD-resistant RNA synthesis were measured as described in Section 3.2.5.

4.2.8 Extraction of RNA from virus infected BHK cells

RNA was extracted by the phenol/cresol extraction method (Section 2.2.14).

4.2.9 Estimation of viral virulence for mice

Virulence was estimated in terms of the LD_{50} of the virus and the AST of infected mice (Section 3.2.7).

4.2.10 Growth of virus in mice

Virus growth in mice was examined in blood, brain and hind leg muscle tissues (Section 3.2.8).

4.3 RESULTS

4.3.1 Growth of RRV dE2 and RRV T48 in BHK cells

Since certain RRV T48 variants with amino acid alterations in E2 showed retarded growth in BHK cells (Chapter 3) it was of interest to compare the growth of RRV dE2 and RRV T48 in cell culture. Although the preliminary studies of Faragher (1982) had shown no significant differences in EV titers for the two viruses when BHK cells were infected at a moi~1, a more sensitive test would be to infect cells at a lower moi. Under these conditions virus would grow in multiple cycles and any

difference in growth would be amplified. Therefore growth of RRV dE2 and RRV T48 was examined at moi~1 and 0.001 in BHK cells.

At moi~1, the first increase in RRV T48 titer took place between 3 and 6 hr pi. A maximum titer of ~10^7 PFU/ml was reached at 12 hr pi. The growth kinetics and maximum titer of RRV dE2 were similar to those of RRV T48 (data not shown). At moi~0.001 the growth kinetics of RRV dE2 and RRV T48 were also similar, although both viruses grew more slowly than at a moi~1 (data not shown). The first rise in titer of both viruses was between 6 and 9 hr pi and both reached the maximum titer (~10^8 PFU/ml) at 36 hr pi. Thus RRV dE2 could not be distinguished from RRV T48 by growth in BHK cells. Similar results were obtained in Vero cells (data not shown).

4.3.2 Rate of acquisition of resistance by infecting RRV T48 and RRV dE2 to neutralisation by added polyclonal antibody

In Chapter 3 it was shown that certain RRV T48 variants altered in E2 had an altered rate of "penetration" as judged by the rate of acquisition of neutralisation resistance by virus to polyclonal antibody in tissue culture cells. It was of interest to investigate whether RRV dE2, with a structural perturbation in a different region of E2, was affected in the same way.

Vero monolayers were inoculated with ~100 PFU of virus and incubated at 36°C for 10, 20, 30, 40 and 60 min followed by addition of polyclonal antibody (diluted x10 in HBSS) for 10 min. After washing to remove excess antibody, overlay medium was added and plates incubated for plaque development. Each assay was carried out in triplicate. RRV dE2 and RRV T48 gave virtually identical profiles of acquisition of neutralisation resistance (data not shown).

4.3.3 Temperature sensitivity of plaque formation

Since for some alphaviruses small plaque mutants are temperature sensitive (Peleg, 1971), plaque formation and plaque morphology of RRV dE2 and RRV T48 were compared over a range of temperatures (28, 30, 32, 34, 35, 36, 37, 39 and 41°C) (Table 4.1).

For both RRV T48 and RRV dE2 maximum plaque number and maximum plaque size was at 36°C; RRV T48 and RRV dE2 made plaques of diameter ~3-4 mm and ~1-2 mm respectively. There was a decrease in the efficiency of plaque formation by both viruses above 36°C and a more marked decrease below 30°C. No differential change in the efficiency of plaque formation or in plaque size was observed for the two viruses as the temperature was altered. Thus the small plaque morphology of RRV dE2 was not due to the temperature sensitivity of plaque formation.

Table 4.1 **Temperature sensitivity of plaque formation by RRV dE2 and RRV T48.**

Temperature degrees C	RRV T48 Number of plaques*	RRV T48 Diameter (mm)	RRV dE2 Number of plaques*	RRV dE2 Diameter (mm)
28	0	-	0	-
30	122	<1	143	<1
32	136	1-2	134	<1-1
34	131	2-3	137	1-2
35	117	2-3	147	1-2
36	CL†	3-4	CL†	1-2
37	65	2-3	77	1-2
39	33	1-3	24	1-2
41	0	-	0	-

* per 6-well Linbro plate.

† CL, confluent lysis of the monolayer.

4.3.4 Structural proteins of RRV dE2

The seven amino acid deletion in E2 predicted from nucleotide sequencing (Dalgarno et al., 1983; S.G. Faragher, unpublished data) could lead to an altered pattern of processing or glycosylation of the structural proteins or to changes in the assembly of these proteins in the mature virion. Experiments were carried out to test this and to examine whether the deletion in E2 resulted in a detectable alteration in its electrophoretic mobility.

To examine the polypeptides in virions, [^{3}H]-amino acid labelled RRV T48 and RRV dE2 were purified in parallel, dissociated in 1% SDS and electrophoresed on a 10-20% gradient polyacrylamide gel (Figure 4.1). In both RRV T48 and RRV dE2 only three polypeptides (E1, E2 and C) were detected. No obvious differences were seen in the molar ratios of the three proteins in either virus. The E2 of RRV dE2 migrated with a slightly greater mobility than did E2 of RRV T48.

It is not known whether RRV virions contain E3 (Chapter 1). Further, since in SFV, E3 is found associated with E1 and E2, a deletion in E2 of RRV dE2 may affect its interaction with E3 if E3 were present in RRV virions. To determine whether E3 was present in RRV T48 and in RRV dE2, labelled RRV T48, RRV dE2 and SFV were purified in parallel and the viral proteins examined on a polyacrylamide gel (Figure 4.2). Both RRV T48 and RRV dE2 virions contained trace amounts of a protein of nominal mol. wt. 13,000 which had a slightly lower mobility than SFV E3

Figure 4.1 **Structural proteins of RRV dE2 and RRV T48.**

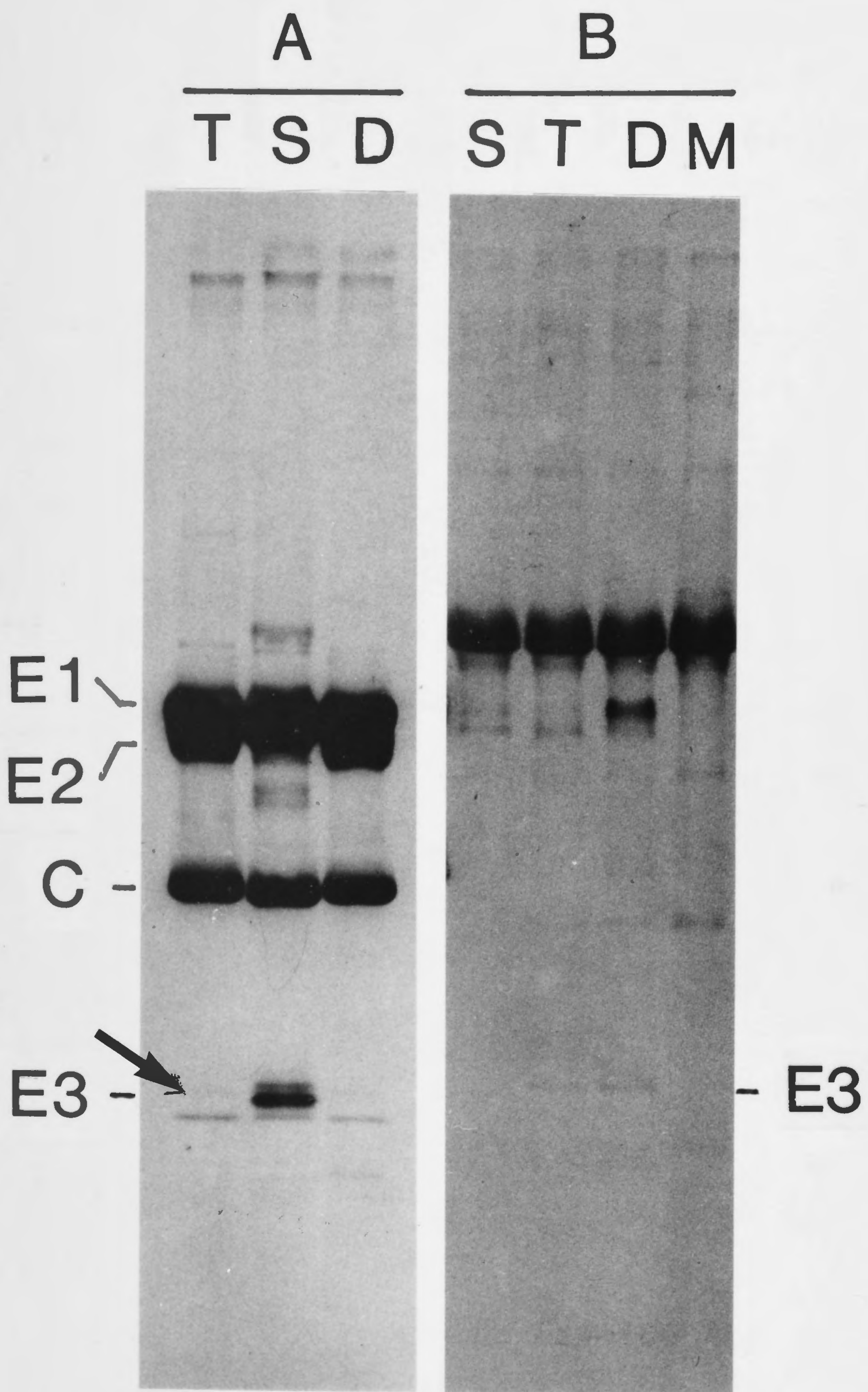
[^{3}H]-amino acid labelled RRV T48 and RRV dE2 were purified in parallel. Virus was disrupted in 1% SDS and electrophoresed on a 10-20% gradient polyacrylamide gel. The gel was fluorographed and exposed to X-ray film for a week at -70°C. The positions of E1, E2 and C are indicated.
T, RRV T48; D, RRV dE2.

D
T
E1
E2
C

Figure 4.2 **Structural proteins of RRV and SFV.**

A. [^{3}H]-amino acid labelled RRV T48, RRV dE2 and SFV were prepared and purified in parallel. Virus was disrupted in 1% SDS, electrophoresed on a 10-20% gradient polyacrylamide gel and fluorographed. The film was overexposed to allow detection of RRV E3 (see arrow). The positions of E1, E2, C and E3 are indicated. T, RRV T48; S, SFV; D, RRV dE2.

B. Extracellular tissue culture fluid from which the virus had been removed by centrifugation was mixed with sample buffer containing SDS and electrophoresed on a separate polyacrylamide gel as above. The film was exposed for six weeks. Tissue culture fluids from mock infected cells (M), RRV T48 infected cells (T), SFV infected cells (S) and RRV dE2 infected cells (D) were examined. The position of E3 is indicated.


A
B
T S D
S T D M
E1
E2
C
E3
E3

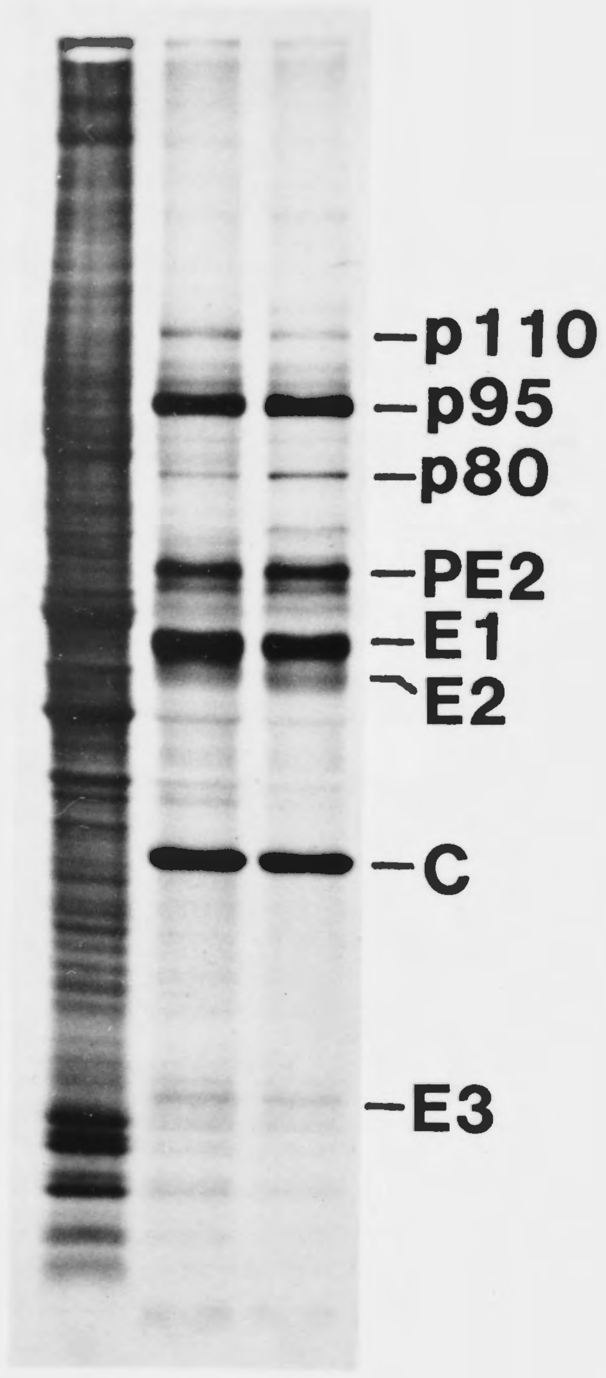
(see arrow in Figure 4.2A). A protein of similar mol. wt. was present in the corresponding extracellular fluids (Figure 4.2B) and this is presumably RRV E3. SFV virions contained E3 and no E3 was detected in SFV tissue culture fluid. Thus, as for SIN (Welch and Sefton, 1979), RRV E3 appears not to be stable in virions and is released into the culture medium.

To investigate the processing of RRV dE2 viral polypeptides, virus-specific proteins in infected BHK cells were labelled with [^{3}H]-amino acids, dissociated in 1% SDS and electrophoresed on a 10-20% gradient SDS polyacrylamide gel (Figure 4.3). In both RRV T48- and RRV dE2-infected BHK cells the following eight virus specific polypeptides were detected: p110, p95, p80, PE2, E1, E2, C and E3. No differences were observed in the migration of these polypeptides between RRV T48- and RRV dE2-infected cells except for PE2 and E2 of RRV dE2 which migrated slightly faster than the corresponding proteins in RRV T48 infection.

These experiments provided no indication of differences in the processing and assembly of the structural polypeptides between RRV T48 and RRV dE2. The altered mobility of E2 from RRV dE2 virions and of E2 and PE2 in RRV dE2-infected cell extracts could be due to a reduced mol. wt. and changed conformation owing to the deletion of the seven amino acids. As E3 in RRV dE2-infected cells had the same mobility as E3 of RRV T48, it can be concluded that there was no alteration in the cleavage of PE2 to yield E2 and E3 in RRV dE2 infection.

Figure 4.3 **Viral proteins in RRV infected BHK cells.**

BHK cells were infected (moi~3) with RRV T48 (T), RRV dE2 (D) or mock infected with HBSS (M) and incubated for 2 hr from 23 hr pi in EMEM containing one-tenth the normal amino acid concentration and 25 µCi/ml of $[^3H]$-amino acids. The cell monolayers were dissolved in 1% SDS and electrophoresed on a 10-20% gradient polyacrylamide gel. The gel was fluorographed and exposed to X-ray film for a week at -70^oC. The position of viral proteins is indicated.


M
T
D
p110
p95
p80
PE2
E1
E2
C
E3

4.3.5 Thermal instability of RRV dE2

Studies on mutants of SIN have shown that many mutants which are altered in structural proteins are thermolabile (Strauss *et al*., 1976). Similarly, Faragher (1982) found that RRV dE2 is relatively heat sensitive at 50°C. However Faragher (1982) did not investigate the mechanism responsible for the loss of virus infectivity. We have made an attempt to determine whether heat induced any structural changes in RRV dE2 virions which might correlate with the loss of infectivity at 50°C.

Samples of RRV T48 and RRV dE2 were incubated at 50°C. As described by Faragher (1982) RRV dE2 infectivity decreased sharply with time (data not shown). Thus within 40 min the RRV dE2 titer had been reduced by more than 4 log units, whereas the RRV T48 titer was reduced by less than one log unit.

To detect aggregation or dissociation of virions at 50°C, the sedimentation properties of heated and unheated RRV T48 and of heated and unheated RRV dE2 were compared. [^{3}H]-amino acid labelled purified RRV T48 and RRV dE2 were incubated separately at 50°C for 40 min followed by sedimentation on sucrose gradients (Figure 4.4). For the unheated control samples (kept on ice) sharp radioactivity peaks of infectious virus were obtained. Only a small fraction (~4%) of the radioactivity was recovered from the bottom of the gradient. When heated RRV T48 was sedimented, approximately 18%

Figure 4.4 **Effect of heat on the sedimentation properties of RRV T48 and RRV dE2 virions.**

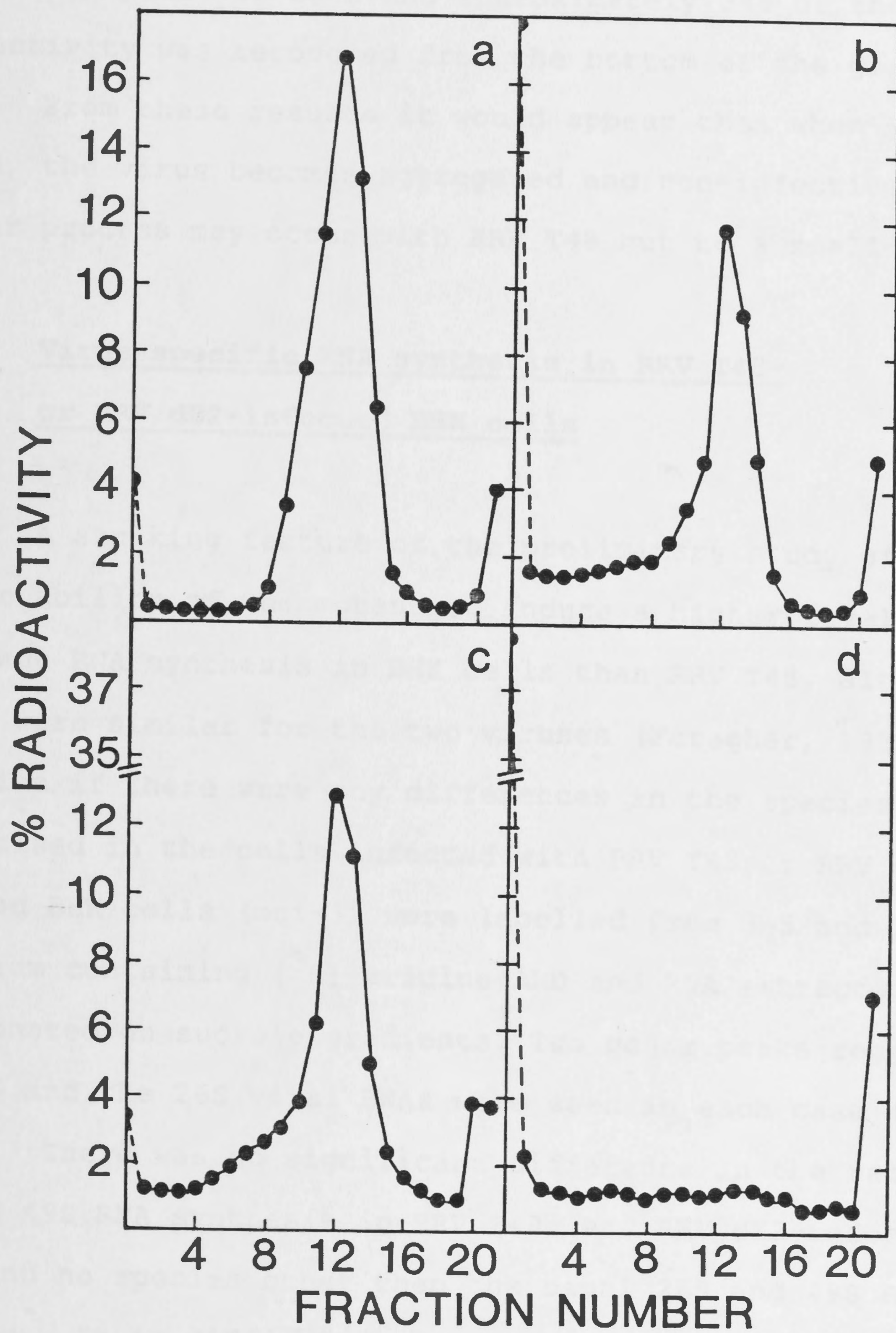
[^{3}H]-amino acid labelled purified RRV T48 (~88,000 cpm) or RRV dE2 (~85,000 cpm) were incubated separately in HBSS for 40 min at 50°C. Controls were kept on ice. The virus was sedimented on a 10-30% sucrose gradient (in NTE buffer; 0.12 M NaCl, 0.012 M Tris pH 7.5, 0.001 M EDTA, 0.1% bovine serum albumin). Fractions were collected and counted for radioactivity. The per cent recovery of total radioactivity applied to the gradient is indicated. Radioactivity which sedimented to the bottom of the tube was recovered by dissolving the pellet in distilled water.

a, unheated RRV T48;

b, heated RRV T48;

c, unheated RRV dE2;

d, heated RRV dE2.



a
b
c
d
% RADIOACTIVITY
16
14
12
10
8
6
4
2
37
35
12
10
8
6
4
2
4
8
12
16
20
4
8
12
16
20
FRACTION NUMBER

of the radioactivity pelleted. However, when heated RRV dE2 was sedimented, no radioactivity peak corresponding to the infectious virus was seen and approximately 38% of the radioactivity was recovered from the bottom of the gradient.

From these results it would appear that when RRV dE2 is heated, the virus becomes aggregated and non-infectious. A similar process may occur with RRV T48 but to a smaller extent.

4.3.6 Virus specific RNA synthesis in RRV T48- or RRV dE2-infected BHK cells

A striking feature of the preliminary study of RRV dE2 was the ability of the mutant to induce a higher level of AMD-resistant RNA synthesis in BHK cells than RRV T48, although EV titers were similar for the two viruses (Faragher, 1982). To determine if there were any differences in the species of RNA synthesised in the cells infected with RRV T48 or RRV dE2, infected BHK cells (moi~3) were labelled from 3-5 and 7-9 hr pi in medium containing [^{3}H]-uridine+AMD and RNA extracted and fractionated on sucrose gradients. Two major peaks representing the 49S and the 26S viral RNAs were seen in each case (data not shown). There was no significant difference in the ratio of 26S and 49S RNA synthesis in RRV T48- and RRV dE2-infected BHK cells and no species other than the usual 26S and 49S RNAs were detected. It is difficult to envisage how a deletion in a structural protein could affect the rate of RNA synthesis. This may indicate a lesion in a non-structural protein, however it was not investigated.

4.3.7 Virulence of RRV dE2 and RRV T48 for mice

Since some of the RRV antigenic variants changed in E2 had altered virulence (Chapter 3) it was of interest to determine if the deletion in E2 led to a change in the virulence of RRV dE2. The virulence of RRV dE2 and RRV T48 were compared in day-old and week-old mice by measuring the LD_{50} of the virus and the AST of infected mice (Table 4.2).

RRV T48 was highly virulent for day-old mice with an LD_{50} of 0.047 PFU; mice injected with low doses of virus showed severe hind leg paralysis and died with an AST of 4.93 days. RRV dE2 had a slightly greater LD_{50} (0.066 PFU) and the infected mice survived significantly longer (AST 7.46 days) than those infected with RRV T48. Hind leg paralysis was less severe in day-old mice infected with RRV dE2 than in those infected with RRV T48.

The LD_{50} of RRV T48 and the AST of infected mice did not differ significantly between day-old and week-old mice. In contrast, RRV dE2 was completely avirulent in week-old mice. The virus did not kill mice even at high doses (~8 X 10^6 PFU injected i.p.) and elicited no clinical symptoms. Two weeks after RRV dE2 injection, mice were challenged with a high dose (~10^6 PFU) of RRV T48. None of the mice showed any disease symptoms and all survived the challenge, thus demonstrating previous RRV dE2 infection and its protecting power against the virulent virus. From these results it was concluded that RRV dE2 was slightly less virulent than RRV T48 in day-old mice

Table 4.2 **Virulence of RRV dE2 and RRV T48 for mice.**

Age of mice	Virus	LD_{50}* (PFU)		AST* (days)	
One day	RRV T48	0.047	(3)	4.93	(4)
	RRV dE2	0.066	(3)	7.46	(3)
One week	RRV T48	0.048	(6)	5.92	(4)
	RRV dE2	$>8\times10^6$	(3)	NDR†	(3)

*Figures presented are an average of a number of separate determinations (shown in brackets).

†NDR = no deaths recorded.

Virulence parameters for RRV T48 and RRV dE2 were compared using one-way analysis of variance and t-tests. The LD_{50} values for RRV dE2 differ significantly from those of RRV T48 in both day-old and week-old mice ($p<0.05$). The AST values of day-old mice infected with RRV T48 or RRV dE2 are significantly different ($p<0.05$).

but, unlike RRV T48, was avirulent for week-old mice.

4.3.8 <u>Growth of RRV T48 and RRV dE2 in mice</u>

To determine whether the differences in RRV T48 and RRV dE2 virulence correlated with their growth and tissue distribution, virus growth was compared in day-old and week-old mice. Virus titers were examined in blood, brain and hind leg muscle tissues. Day-old or week-old mice were injected subcutaneously (s.c.) with ~100 PFU of RRV T48 or RRV dE2. Three mice infected with each virus were killed at 24 hr intervals, appropriate tissues removed and assayed individually for virus titers (Figure 4.5).

Growth in day-old mice. In blood, RRV T48 attained a peak titer of $\sim10^{10}$ PFU/ml at 2 d pi after which it declined; at 4 d pi RRV T48 could not be detected. The peak RRV dE2 titer in blood was ~4 log units lower than for RRV T48 and at 3 d pi RRV dE2 was not detected.

In brain, RRV T48 attained a peak titer of $\sim10^{11}$ PFU/g at 2 d pi after which the titer declined until 4 d pi. The peak RRV dE2 titer of $\sim10^{7}$ PFU/g was at 2 d pi after which it declined gradually to about 10^{5} PFU/g at 4 d pi. As no RRV T48 infected mice survived beyond this time point, the experiment was stopped. RRV dE2-infected mice survived at least 6 d pi.

The highest titers for both RRV T48 and RRV dE2 were

Figure 4.5 **Growth of RRV T48 and RRV dE2 in mice.**

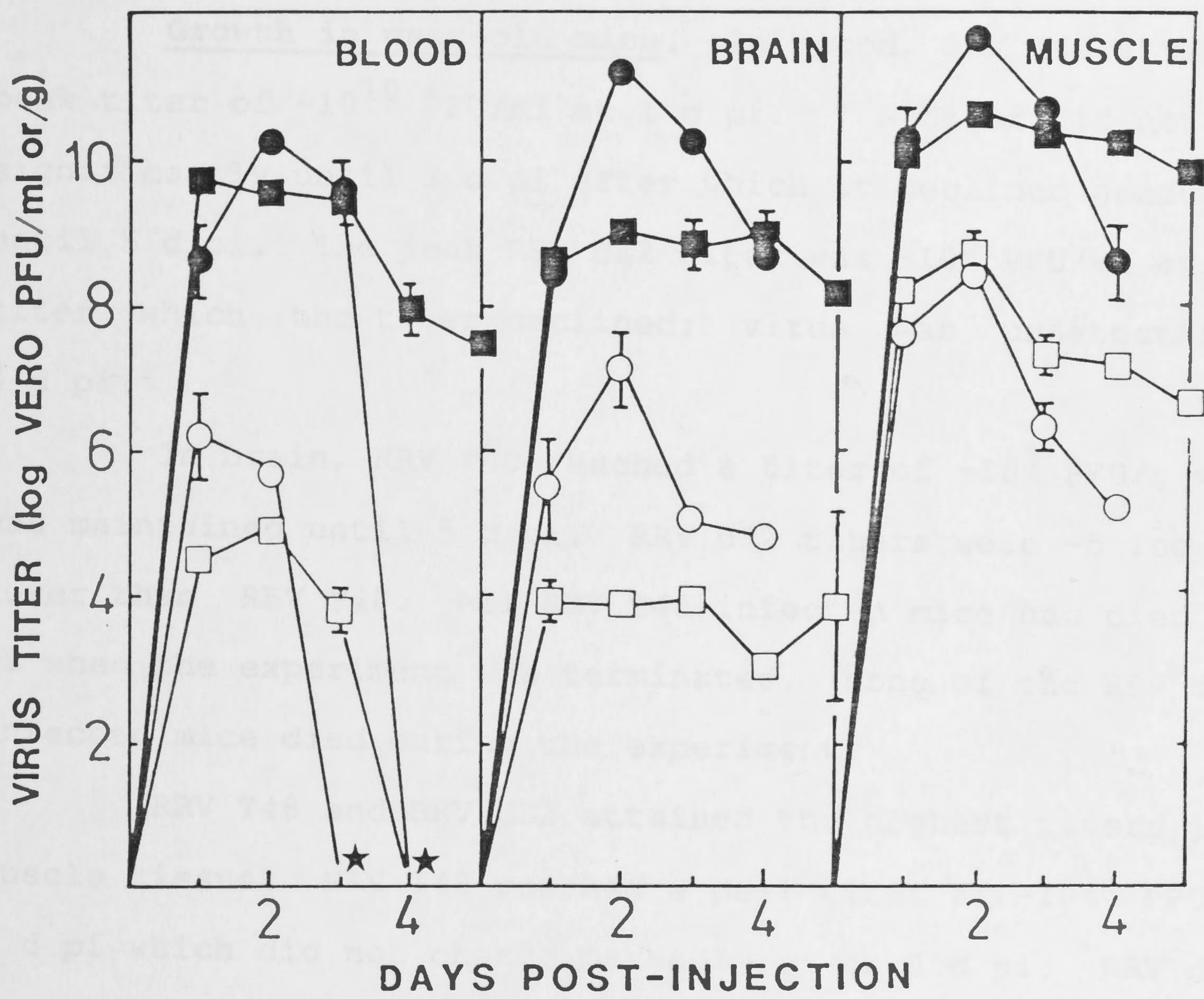
WEHI mice were injected s.c. with 100 PFU of virus. Mice were killed and tissues removed at 24 hr intervals. Virus titers were assayed in blood, brain and hind leg muscles. Each time point represents the average of separate determinations made in three mice. Error bars are shown where the error is greater than 0.2 log units. Titers of less than 100 PFU/ml are indicated by an asterisk.

RRV T48 titers in day-old mice, (●);

RRV dE2 titers in day-old mice, (○);

RRV T48 titers in week-old mice, (■);

RRV dE2 titers in week-old mice, (□);


BLOOD
BRAIN
MUSCLE
VIRUS TITER (log VERO PFU/ml or/g)
10
8
6
4
2
2
4
2
4
2
4
DAYS POST-INJECTION

reached in muscle. RRV T48 grew to a peak titer of ~10^{12} PFU/g at 2 d pi. RRV dE2 attained a peak titer of only ~10^8 PFU/g. After 2 d pi RRV dE2 titers declined gradually until 4 d pi when the experiment was stopped.

Growth in week-old mice. In blood, RRV T48 attained a peak titer of ~10^{10} PFU/ml at 1 d pi. The titer did not change significantly until 3 d pi after which it declined gradually until 5 d pi. The peak RRV dE2 titer was ~10^5 PFU/ml at 2 d pi after which the titer declined; virus was undetectable at 4 d pi.

In brain, RRV T48 reached a titer of ~10^9 PFU/g which was maintained until 5 d pi. RRV dE2 titers were ~5 log units lower than RRV T48. All RRV T48-infected mice had died at 6 d pi when the experiment was terminated. None of the RRV dE2-infected mice died during the experiment.

RRV T48 and RRV dE2 attained the highest titers in muscle tissue. RRV T48 reached a peak titer of ~10^{11} PFU/g at 2 d pi which did not change markedly up to 5 d pi. RRV dE2 grew to titers which were about 2 log units lower than RRV T48 titers.

In summary, for both viruses maximum titers in all tissues examined were at 1-2 d pi in mice of both age groups. The highest titers were in muscle. RRV dE2 grew to markedly lower titers than RRV T48 in all tissues of both one day-old and week-old mice. The greatest difference in the titers of

the two viruses (~5 log units) was in the brain tissue of week-old mice.

4.3.9 Growth of RRV T48 and RRV dE2 in primary mouse cells

To determine whether the differences in titers of RRV T48 and RRV dE2 seen in mice were due to an intrinsic difference in ability of the two viruses to replicate in mouse cells, their growth was compared in primary mouse muscle and skin cell cultures. These were chosen as the hind leg muscle is the major site for RRV replication (Chapter 1) and epidermal tissue is likely to be the initial site of replication in a natural RRV infection. Primary mouse brain cells were difficult to culture (R.W. Boulton, personal communication) and were not used in these studies.

In muscle cells infected at an moi ~1, RRV T48 grew slowly and reached a maximum titer (~10^6 PFU/ml) at 72 hr pi (Figure 4.6). RRV dE2 showed similar growth kinetics. At 48 hr pi, massive cytopathic effects (cpe) became visible in both RRV T48 and RRV dE2 infected cells.

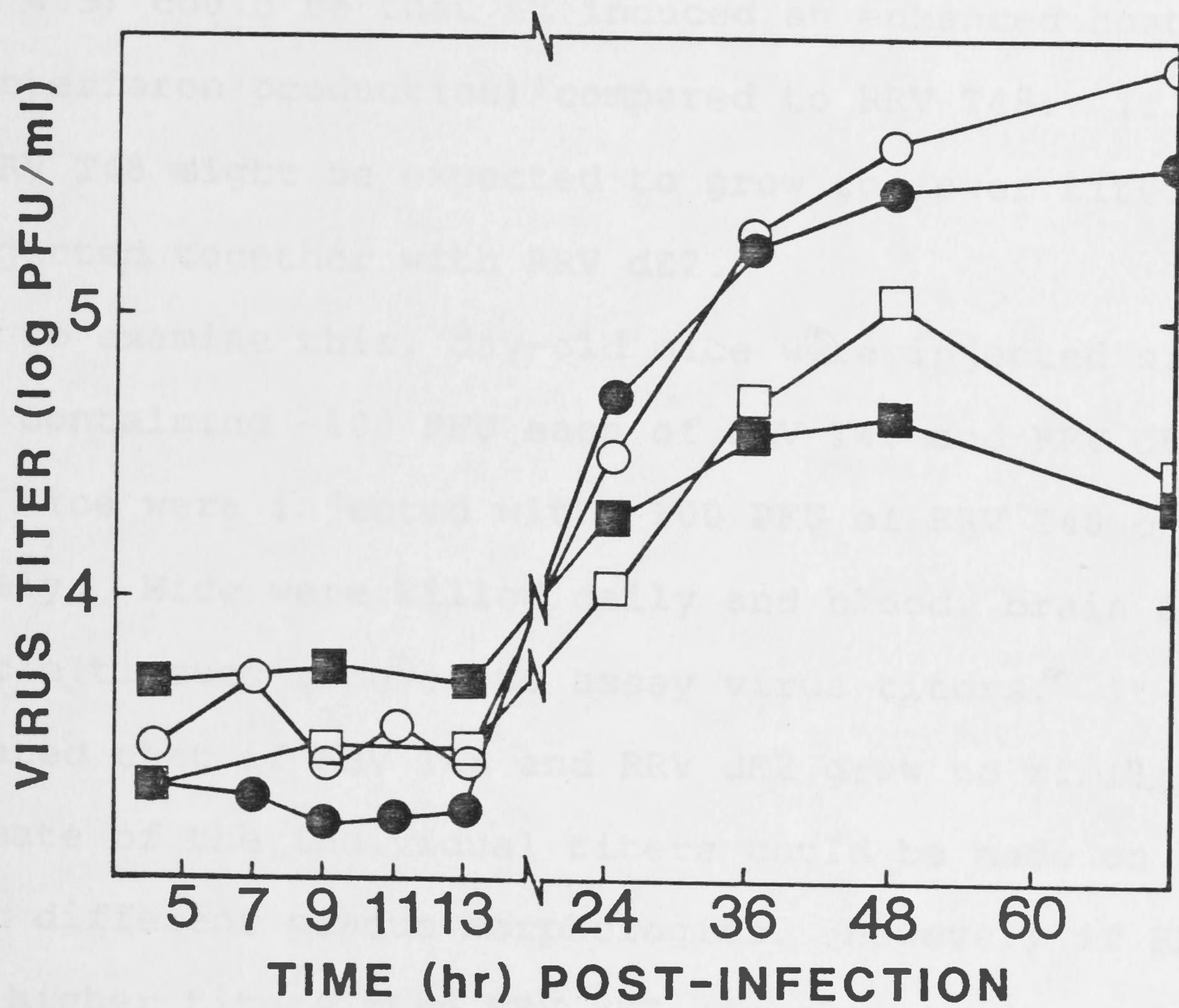
Both RRV strains grew poorly in skin cells infected at an moi ~1. The peak titer for each was ~10^5 PFU/ml at 48 hr pi (Figure 4.6). Skin cells infected with RRV T48 showed no cpe during the experiment. However, cpe was visible at 48 hr pi in cells infected with RRV dE2.

It can be concluded that RRV T48 and RRV dE2 did not differ in their growth in primary mouse muscle cells or skin

Figure 4.6 **Growth of RRV T48 and RRV dE2 in primary mouse cells.**

Primary mouse muscle or skin cell monolayers were infected (moi~1) with virus and incubated at 36° C in GMEM + 10% serum. At intervals samples were removed from the culture medium, diluted 10-fold into HBSS and stored at -70° C. EV titers were assayed by plaque formation on Vero cell monolayers.

RRV T48 titers in muscle cells, (●);
RRV dE2 titers in muscle cells, (○);
RRV T48 titers in skin cell, (■);
RRV dE2 titers in skin cells, (□).


VIRUS TITER (log PFU/ml)
5
4
5
7
9
11
13
24
36
48
60
TIME (hr) POST-INFECTION

cell cultures. However, it is possible that cultured mouse cells are not fully representative of the tissue in vivo.

4.3.10 Mixed infection of mice with RRV T48 and RRV dE2

One reason for the reduced RRV dE2 titers seen in mice (Figure 4.5) could be that it induced an enhanced host response (e.g. interferon production) compared to RRV T48. If this were true, RRV T48 might be expected to grow to lower titers in mice when injected together with RRV dE2.

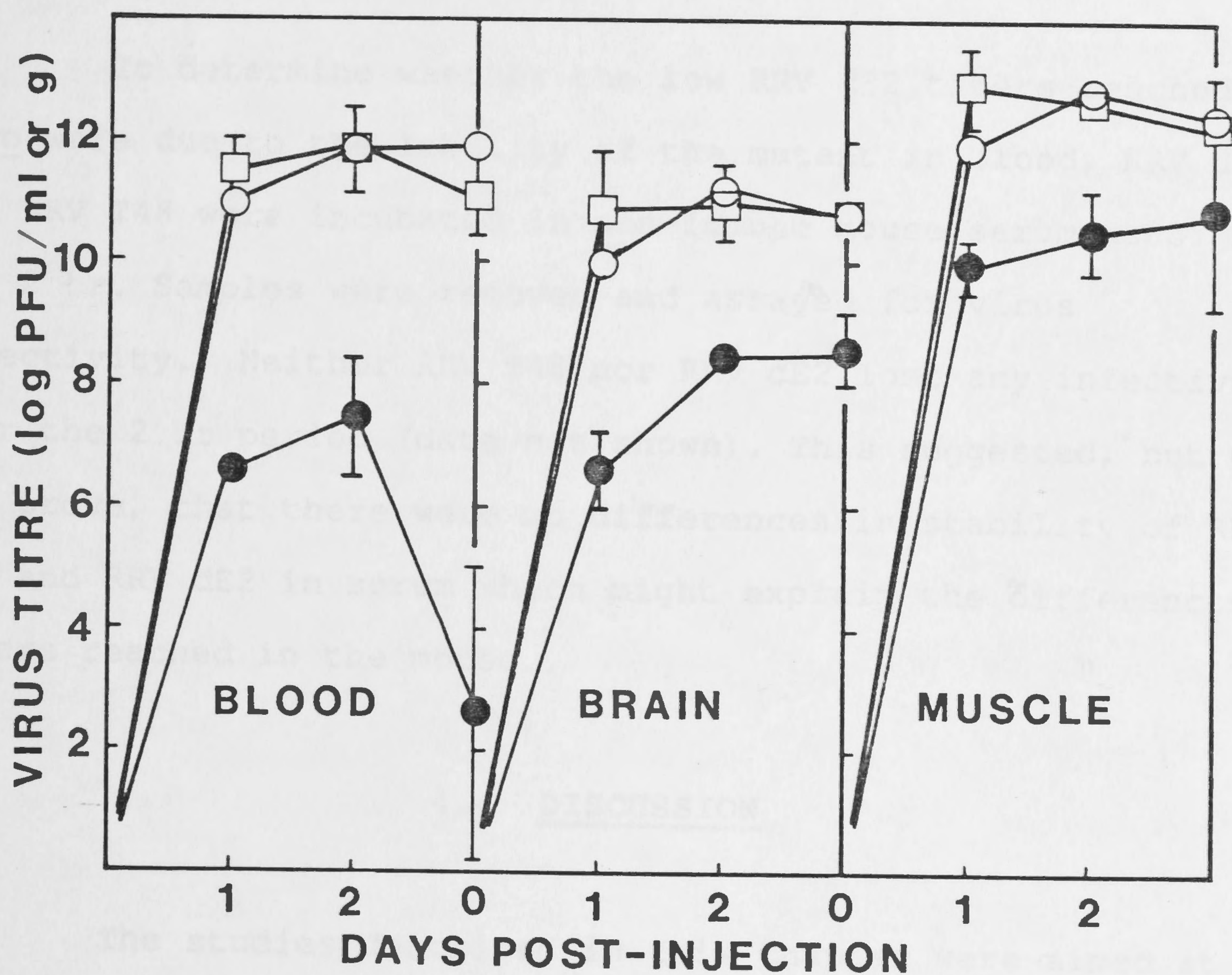
To examine this, day-old mice were injected s.c. with a mixture containing ~100 PFU each of RRV T48 and RRV dE2. Control mice were injected with ~100 PFU of RRV T48 or RRV dE2 separately. Mice were killed daily and blood, brain and hind leg muscle tissues removed to assay virus titers. It was anticipated that if RRV T48 and RRV dE2 grew to similar levels, an estimate of the individual titers could be made on the basis of their different plaque morphologies. However, if RRV T48 grew to higher titers than RRV dE2 (as above), it would not be possible to assay RRV dE2 titers as the larger RRV T48 plaques would overgrow the smaller plaques of RRV dE2.

Figure 4.7 shows that in control mice RRV dE2 grew to lower titers than RRV T48, as earlier. In mice injected with both viruses, RRV T48 grew to high titers as in control mice. As RRV dE2 could not be detected by plaque assay in any of the samples from mice infected with both viruses simultaneously, it would appear that RRV dE2 grew to far lower titers than RRV

Figure 4.7 **Growth of RRV T48 and RRV dE2 in mice during mixed infection.**

Week-old WEHI mice were injected s.c. with a mixture containing 100 PFU each of RRV T48 and RRV dE2. The control mice were injected subcutaneously with 100 PFU of RRV T48 or RRV dE2. Virus in blood, brain and hindleg muscles was assayed at various time intervals. Each time point represents the average of determinations made in three mice. Error bars are shown where errors are more than 0.2 log units.

RRV T48 titers in control mice, (○);
RRV dE2 titers in control mice, (●);
RRV T48 titers in mice infected
with both RRV T48 and RRV dE2, (□).


VIRUS TITRE (log PFU/ml or g)
12
10
8
6
4
2
BLOOD
BRAIN
MUSCLE
1
2
0
1
2
0
1
2
DAYS POST-INJECTION

T48. Although it was not possible to come to an unequivocal conclusion about the growth of RRV dE2 for this reason, the possibility that the low RRV dE2 titers were due to some host response that did not affect RRV T48 seems unlikely.

4.3.11 Stability of RRV T48 and RRV dE2 in mouse serum

To determine whether the low RRV dE2 titers reached *in vivo* were due to the lability of the mutant in blood, RRV dE2 and RRV T48 were incubated in non-immune mouse serum at 37°C for 2 hr. Samples were removed and assayed for virus infectivity. Neither RRV T48 nor RRV dE2 lost any infectivity over the 2 hr period (data not shown). This suggested, but did not prove, that there were no differences in stability of RRV T48 and RRV dE2 in serum which might explain the differences in titers reached in the mouse.

4.4 DISCUSSION

The studies described in this Chapter were aimed at examining the effects of a deletion in E2 on the biological properties of RRV T48. There have been previous reports of alphavirus mutants with deletions in the major glycoproteins (Bracha and Schlesinger, 1978, Leone *et al*., 1980). However, in no case was a precise identification of the deletion made or its effect on the biological properties of the virus examined in an experimental animal. Bracha and Schlesinger (1978)

isolated a SIN variant which had lost approximately 30 amino acids from the N-terminus of E2 as a result of an aberrant proteolytic cleavage of the precursor PE2. The variant did not differ from the parental virus in plaque morphology or single step growth kinetics in BHK cells. Leone et al. (1980) isolated a SIN mutant in which E1 had lost approximately 70 amino acids. The mutant retained the capacity for haemagglutination and did not differ significantly in its structure and growth in cultured cells from wild type virus.

The fact that large deletions in the major alphavirus envelope proteins can be tolerated without eliminating viral infectivity may seem surprising as there is strong size conservation and amino acid sequence homology in E2 between RRV, SFV and SIN (Dalgarno et al., 1983). Although RRV dE2 is viable under laboratory conditions, the effects of the deletion on virus survival in the field are not known. However, the data on RRV dE2-viraemia in mice suggests that natural transmission of the deletion mutant by mosquito vectors would be unlikely.

4.4.1 Virulence and growth of RRV dE2 in mice

RRV dE2 was only slightly less virulent than RRV T48 in day-old mice, but it grew to far lower titers (~3-4 log units) than RRV T48. Significantly, although the virulence of RRV T48 did not differ between day-old and week-old mice, RRV dE2 was avirulent for week-old mice. There was no significant

difference in peak RRV T48 titers in the blood or hind leg muscle of day-old and week-old mice. A similar conclusion was reached for RRV dE2. However, differences between RRV dE2 and RRV T48 titers in brain were greater in week-old mice than in day-old mice. Although no experiments examining virus distribution in different parts of brain were done, it seems possible that the avirulence of RRV dE2 for week-old mice was due to a changed cell tropism within the brain. Fields and Green (1982) have reported that mutants of reovirus type 3 changed in structural proteins had altered tissue tropism in mice.

The lower RRV dE2 titers in mice were probably not due to a defect in intracellular events in viral replication as RRV dE2 showed no differences in growth in BHK cells or in primary mouse cells when compared with RRV T48. Nor did the poor growth of RRV dE2 appear to be the result of enhanced interferon induction since when RRV dE2 and RRV T48 were used to coinfect mice, the growth of RRV T48 was similar to that obtained when it alone was used for infection. The differences in titer were probably not the result of different degrees of inactivation of the two viruses in mice as there was no difference in the rate of loss of infectivity of the two viruses after a two hr incubation in mouse serum at 37°C. Further, it is unlikely that the low RRV dE2 titers in mice resulted from an enhanced specific immune response to RRV dE2 since this would need to occur shortly after infection and be specific for RRV dE2. However polyclonal antibody does not

distinguish RRV T48 and RRV dE2 in neutralisation tests (Faragher, 1982).

It has recently been shown that the E2-specific monoclonal antibody NB3C4 neutralises RRV dE2 infectivity 1000 times less efficiently than RRV T48 infectivity (R.C. Weir, personal communication). This indicates that changes have occurred in the conformation of the "neutralisation domain" in RRV dE2 which may be involved in virus entry (Chapter 3). It is possible that the different growth profiles of RRV dE2 and RRV T48 in mice are due to alterations in the entry of the virus resulting from conformational changes in E2. These changes appear to have induced greater effects on replication in mice than on patterns of growth in cultured cells. This is surprising but consistent with results from Baric et al. (1981) and Olmsted et al. (1984). Baric et al. (1981) isolated a rapidly growing mutant of SIN by selecting for virus released early in infection of BHK cells. The variant had an increased rate of "penetration" (see Chapter 1) into BHK cells (but not into other cell types), and from neutralisation studies with mabs was probably altered in E2. Olmsted et al. (1984) showed that virus selected in this way was of reduced virulence for mice and that rapid penetration into BHK cells was strongly correlated with attenuation in mice. Baric et al. (1981) concluded that the class of cellular receptors active in SIN uptake by BHK cells is of low specificity compared with that involved in penetration in mouse cells .

The attenuation of virulence of RRV dE2, together with the protection of RRV dE2-immunised mice against lethal challenge with RRV T48, suggests that selection or generation of deletion variants may provide a useful route to stable (non-reverting) virus variants suitable for use as safe alphavirus vaccines.

4.4.2 RRV pathogenesis in mice

It has previously been argued that RRV pathogenesis in infant mice is not typical of alphaviruses since it does not involve early and rapid replication in neural tissues but is accompanied by extensive replication in non-neural tissues, particularly skeletal (hind leg) muscle (Mims *et al.*, 1973; Murphy *et al.*, 1973). By contrast SFV produces widespread infection and necrosis in the central nervous system and death takes place before there is time for the evolution of the pathological changes seen with RRV (Mims *et al.*, 1973). Murphy *et al.* (1973) proposed that the muscle lesions produced by RRV in newborn mice explain the paralysis seen in RRV infection since the type of involvement of brain and spinal cord which could account for this paralysis was not seen.

Using the same outbred strain of mice as that used by Murphy *et al.* (1973) we have shown in this Chapter that the loss of virulence of RRV dE2 in week-old mice is accompanied by a sharp reduction in virus replication in brain tissue (Figure 4.5) by comparison with that seen in day-old mice. In Chapter

5 of this thesis we show that an attenuated variant of RRV T48 obtained by serial passage in BHK cells grows to lower titers in mouse brain compared to RRV T48, although no differences were seen in the hind leg muscle titers. Recently, Meek (1986) showed that virulent variants of RRV NB5092 obtained by serial passage in mice, grow to higher titers in brain with little change in hind leg muscle of day-old mice. Furthermore, Seay and Wolinsky (1982) and Kingston (1983) have reported that RRV-induced central nervous system demyelination occurs in a way which suggests a causal relationship with paralysis. Considered together these data suggest that RRV replication in brain tissue may be a more important determinant of viral virulence than the extent of replication in hind leg muscle tissue.

CHAPTER 5

STUDIES ON ATTENUATED VARIANTS OF RRV T48

5.1 INTRODUCTION

In the previous Chapters it was shown that certain point mutations or a deletion in E2 resulted in attenuation of RRV virulence for mice. Thus E2 protein is one of the determinants of RRV virulence. In this Chapter we examine whether attenuation of RRV virulence achieved by virus passaging in tissue culture cells is accompanied by change(s) in E2. This was prompted by the observation of Baric et al. (1981) who isolated a mouse-attenuated variant of SIN by serial passage in BHK cells. The variant was changed in neutralisation epitope(s) of E2 as judged by its reactivity pattern with anti-E2 neutralising mabs, although the change(s) in the structural protein was not identified by sequencing (Olmsted et al., 1986).

We describe here the isolation of attenuated variants of RRV T48 by serial passage in BHK cells using the method of Baric et al. (1981). The virulence of the resulting passaged virus is measured in mice to determine the degree of attenuation. Growth of one such attenuated variant in mice is investigated to examine whether changes occurred in tissue tropism on virulence attenuation. The properties of the attenuated variants are examined in BHK cells to identify any changes which may be associated with virulence attenuation in mice. Finally the nucleotide sequence of the structural protein genes of the variants is determined and compared with the parental RRV T48 sequence to identify any mutation(s) accompanying attenuation.

5.2 MATERIALS AND METHODS

5.2.1 Virus, cells and plaque assay

These have been described previously (Chapter 2).

5.2.2 Serial passage of RRV T48 in BHK cells (Baric et al., 1981)

A BHK monolayer was infected with RRV T48 (moi ~40); adsorption was at 36°C for 1 hr. The monolayer was washed (x2) with PBS and incubated with GMEM + 8% bovine serum at 36°C. Virus was harvested 5.5 hr after the end of the adsorption period and used undiluted for the next passage. This process was carried out eleven times and virus plaque purified from the 12th passage.

5.2.3 Estimation of viral virulence for mice

LD_{50}s and ASTs were determined in week-old mice as in Section 3.2.7. The clinical dose (CD_{50}) was the dose required to produce clinical symptoms (hind leg paralysis) in 50% of the mice inoculated (Reed and Muench, 1938). One-way analysis of variance and t-tests were performed to compare the virulence parameters of RRV T48 and the passaged variants.

5.2.4 Rate of AMD-resistant RNA synthesis

Rates were determined as described in Section 3.2.5.

5.2.5 Rate of acquisition of resistance by infecting virus to neutralisation by added polyclonal antibody

The method has been described in Section 3.2.4.

5.2.6 Extraction of infected cell RNA and sequencing of viral RNA

The procedures were as described in Sections 2.2.14 and 2.2.15.

5.3 RESULTS

5.3.1 Serial passage of RRV T48 in BHK cells

In an attempt to generate attenuated variants of RRV T48, a BHK monolayer was infected at a multiplicity of ~40 and progeny virus was harvested at 6.5 hr pi. The harvest was used undiluted as inoculum for next passage. Serial passage was continued blind in this way for 12 passages. The titers of RRV at the different passage levels indicate that from the second passage onwards BHK cells were infected at a multiplicity of ~1 (Table 5.1). Six plaque purified isolates (RRV P12/1-6) were

Table 5.1 **Virus titers at different passage levels during serial passage of RRV T48 in BHK cells.**

Passage level	Titer (PFU/ml)*
1	2.0×10^6
3	2.8×10^6
5	4.1×10^6
7	2.0×10^6
9	2.2×10^6
11	1.7×10^6
12	1.0×10^6

*at 6.5 hr pi.

obtained from the 12th passage material (see Table 5.2 for nomenclature).

5.3.2 **Virulence of BHK-passaged variants for week-old mice**

In previous Chapters it was observed that virulence changes were more pronounced in week-old mice than in day-old mice. Therefore virulence of RRV P12/2, RRV P12/4, RRV P12/5 and RRV P12/6 was determined in week-old mice and compared with that of RRV T48. The LD_{50}s of the variants and ASTs of infected mice are shown in Table 5.2.

RRV T48 was highly virulent (LD_{50} = 0.05 PFU) and infected mice showed severe hind leg paralysis at 3-4 d pi. All RRV T48-infected mice showing paralysis died eventually (LD_{50}:CD_{50} ratio = 1:1) with an AST of 5.9 days. The virulence of all the passaged variants was significantly lower than that of RRV T48 ($p<0.05$). The LD_{50}s for RRV P12/2, RRV P12/4 and RRV P12/5 were 0.23, 0.63 and 0.83 PFU respectively which did not differ significantly from each other. However, the LD_{50} of RRV P12/6 was 1.48 PFU which was significantly different from that of the other isolates assayed ($p<0.05$).

As for mice infected with RRV T48, mice infected with the passaged variants also showed hind leg paralysis at 3-4 d pi; however the AST (~10 days) was approximately twice that of mice infected with RRV T48 ($p<0.05$). Thus the passaged variants had a wider range of LD_{50} values (5-30 times) than of AST values.

Table 5.2 **Virulence of BHK passaged RRV variants for week-old mice.**

Virus†	LD_{50} (PFU)*	AST (days)*
RRV T48	0.05 (6)	5.9 (4)
RRV P12/2	0.23 (3)	10.2 (3)
RRV P12/4	0.63 (3)	9.7 (3)
RRV P12/5	0.83 (3)	9.1 (3)
RRV P12/6	1.48 (3)	10.1 (3)

† Nomenclature: RRV P12/2 indicates RRV clone 2 derived from 12th passage material.

* Figures are the average of a number of separate determinations (shown in brackets). The LD_{50}s and the ASTs of all the passaged variants were significantly different from those of RRV T48 ($p<0.05$). The LD_{50}s of RRV P12/2, 4 and 5 did not differ significantly from each other, although they were significantly different from that of RRV P12/6 ($p<0.05$). The ASTs of the passaged variants did not differ significantly from each other.

A significant number of variants infected mice showing paralysis recovered within 7-10 days after the symptoms were first seen. A rough estimate of LD_{50}:CD_{50} ratio was 10:1 to 100:1, although a precise estimate of CD_{50} could not be made due to a shortage of mice.

We conclude that attenuation of virulence occurred on serial passage of RRV T48 in BHK cells and that RRV P12/6 was the most attenuated of the four passaged variants examined.

5.3.3 Growth of RRV P12/6 in mice

To determine whether attenuation correlated with changes in growth kinetics, titers reached and tissue distribution of virus in mice, the growth of RRV P12/6 and RRV T48 was compared in week-old mice (Figure 5.1).

The results obtained with RRV T48 were similar to those reported previously. In blood, RRV T48 could be detected at 1 d pi. The peak titer (~10^{10} PFU/ml) was reached at 2 d pi. The titer declined slightly at 3 d pi and was followed by a marked reduction at 4 and 5 d pi. No mice injected with RRV T48 survived beyond 5 d when the experiment was stopped. RRV P12/6 was also found in blood by 1 d pi. However, the peak titer (also at 2 d pi) was ~2 log units lower than that of RRV T48. Subsequently, the RRV P12/6 titer declined and was <100 PFU/ml at 5 d pi.

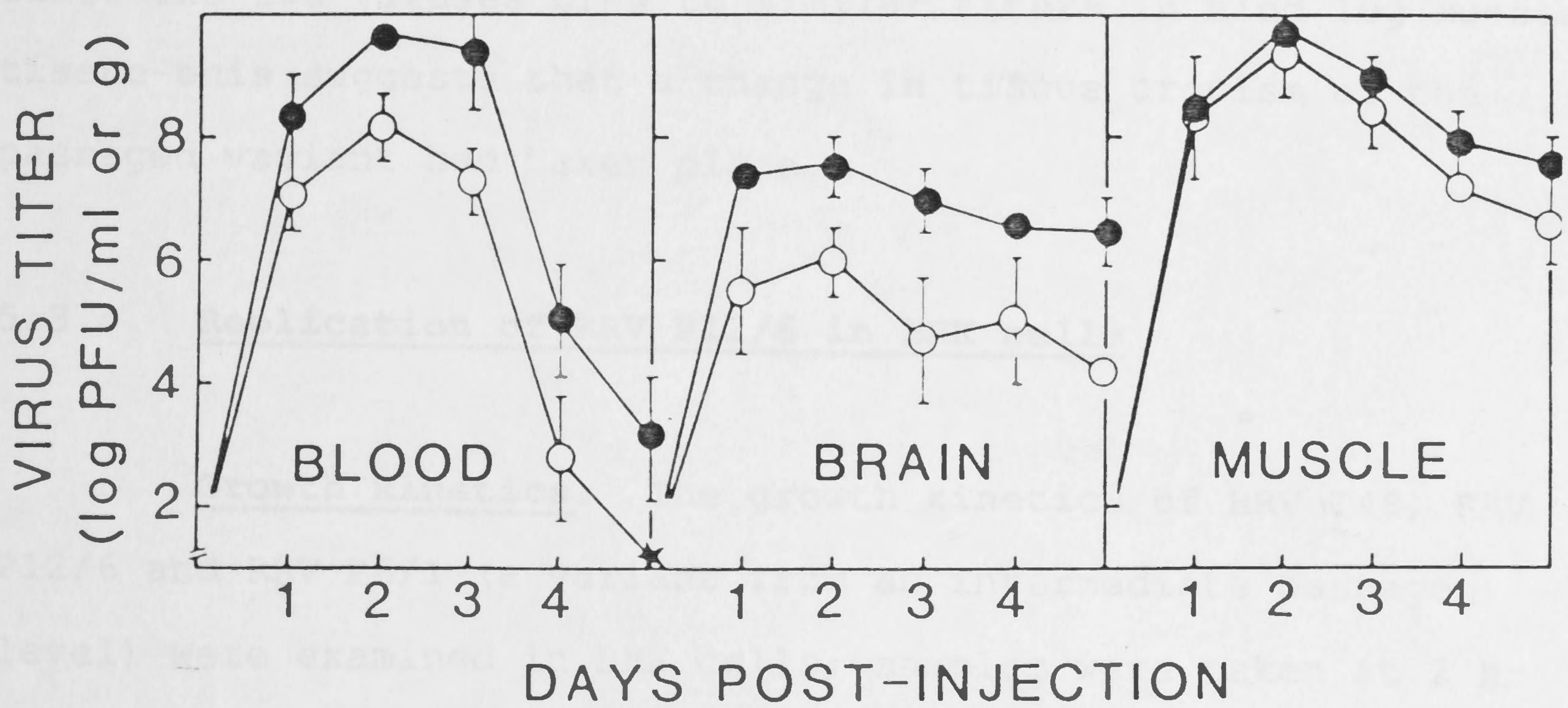
In brain, RRV T48 attained a peak titer of ~10^8 PFU/g at 2 d pi after which it declined slowly. The peak titer of

Figure 5.1 **Growth of RRV T48 and RRV P12/6 in mice.**

Week-old WEHI mice were injected i.p. with 100 PFU of virus. Mice were killed and tissues removed at 24 hr intervals. Virus titers were assayed in blood, brain and hind leg muscle. Each time point represents an average of determinations made in three mice. Error bars are shown where the error is greater than 0.2 log units. Titer less than 100 PFU/ml is indicated by an asterisk.

RRV T48 titers, (●).

RRV P12/6 titer, (○).


VIRUS TITER
(log PFU/ml or g)
8
6
4
2
BLOOD
BRAIN
MUSCLE
1
2
3
4
DAYS POST-INJECTION

RRV P12/6 was ~10^6 PFU/g and was reached at 2 d pi after which there was a gradual decline.

In hind leg muscle, RRV T48 reached a peak titer of ~10^{10} PFU/g at 2 d pi which then declined until 5 d pi. RRV P12/6 reached roughly the same titer (10^{10} PFU/g) at 2 d pi. Thus no significant difference was observed in hind leg muscle titers of RRV P12/6 and RRV T48.

In summary, RRV P12/6 reached markedly lower titers in the blood and brain tissues of week-old mice than did RRV T48. Since the two viruses grew to similar titers in hind leg muscle tissue this suggests that a change in tissue tropism of the passaged variant had taken place.

5.3.4 Replication of RRV P12/6 in BHK cells

Growth kinetics. The growth kinetics of RRV T48, RRV P12/6 and RRV P6/1 (a variant from an intermediate passage level) were examined in BHK cells; samples were taken at 2 hr intervals. For all three viruses the first increase in EV titer was at 2-4 hr pi (data not shown). At 12 hr pi both variants and RRV T48 had reached a titer of ~10^7 PFU/ml. Thus no significant difference was observed between RRV T48 and the variants in growth kinetics in BHK cells.

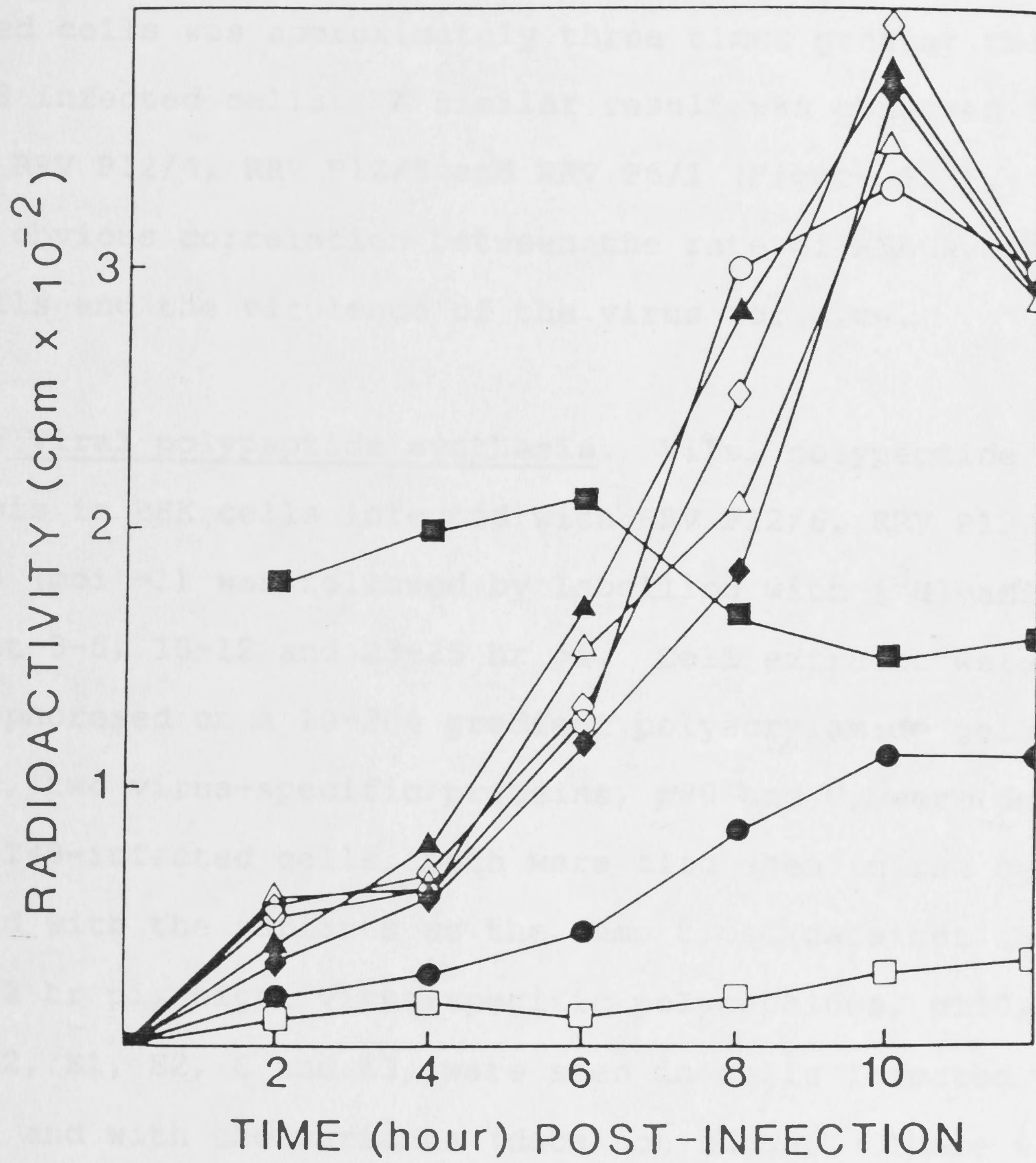
RNA synthesis. Rates of viral RNA synthesis in RRV T48- and RRV P12/6-infected BHK cells were compared by measuring the incorporation of [^{3}H]-uridine (Figure 5.2).

Figure 5.2 **AMD-resistant RNA synthesis in BHK cells infected with RRV T48 or with passaged variants.**

BHK cell monolayers were infected with RRV T48 or with variants (moi~1), or mock-infected with HBSS, and incubated in GMEM + 8% bovine serum. At intervals, monolayers were labelled for two hour periods with EMEM containing 10 µCi/ml of [5-^{3}H]-uridine (indicated in the Figure are the middle of labelling periods) and incubated at 36°C. Infected cells were labelled in the presence of 5 µg/ml AMD; mock-infected cells were labelled in the absence or presence of 5 µg/ml of AMD. Monolayers were dissociated in 200 µl of 1% SDS and the acid-precipitable radioactivity in 20 µl of the resulting extracts (representing ~2 x 10^5 cell equivalents) determined.

Incorporation of [^{3}H]-uridine in:

- -mock infected cells without AMD, (■).
- -mock infected cells with AMD, (□).
- -RRV T48 infected cells, (●).
- -RRV P6/1 infected cells, (△).
- -RRV P12/2 infected cells, (○).
- -RRV P12/4 infected cells, (▲).
- -RRV P12/5 infected cells, (◊).
- -RRV P12/6 infected cells, (♦).



RADIOACTIVITY (cpm x 10^2)
1
2
3
2
4
6
8
10
TIME (hour) POST-INFECTION

Viral RNA synthesis was detected at 2 hr pi for both viruses, although the rate was higher in RRV P12/6 infected-cells compared to that in RRV T48 infected-cells. Peak RNA synthesis was at 10 hr pi in both cases, however the rate in RRV P12/6 infected cells was approximately three times greater than in RRV T48 infected cells. A similar result was observed for RRV P12/1, RRV P12/4, RRV P12/5 and RRV P6/1 (Figure 5.2). There was no obvious correlation between the rate of RNA synthesis in BHK cells and the virulence of the virus for mice.

Viral polypeptide synthesis. Viral polypeptide synthesis in BHK cells infected with RRV P12/6, RRV P12/5 and RRV T48 (moi ~1) was followed by labelling with [^{3}H]-amino acids at 3-5, 10-12 and 23-25 hr pi. Cell extracts were electrophoresed on a 10-20% gradient polyacrylamide gel. At 3-5 hr pi, two virus-specific proteins, p80 and C, were detected in RRV T48-infected cells; both were also seen in the cells infected with the variants at the same time (data not shown). At 10-12 hr pi, eight virus-specific polypeptides, p110, p95, p80, PE2, E1, E2, C and E3, were seen in cells infected with RRV T48 and with the variants (data not shown). There was no difference in shut down of host protein synthesis or in the mobilities of virus-specific proteins between RRV T48 and the variants. A similar conclusion was drawn from labelling at 23-25 hr pi. Thus there was no difference in the time course of viral polypeptide synthesis or in shut down of the host protein synthesis between cells infected with RRV T48 or with variants

during the time periods examined in this experiment.

Rate of acquisition of resistance by infecting RRV P12/6 to neutralisation by added polyclonal antibody. Baric *et al*. (1981) observed that the BHK-passaged variants of SIN showed a rapid "penetration" in BHK cells as judged by the rate of acquisition of resistance of the infecting virus to neutralisation by polyclonal antibody. We have examined the rate of "penetration" of RRV P12/6, RRV P12/5 and RRV T48 in BHK cells using the method of Baric *et al*. (1981). BHK monolayers were inoculated with ~100 BHK PFU of virus and incubated at 36°C for 10, 20, 30, 40 and 60 min followed by addition of polyclonal antibody for 10 min. After washing to remove excess antibody, overlay medium was added and plates incubated for plaque development. Figure 5.3 shows the rate of acquisition of neutralisation resistance by RRV P12/6, RRV P12/5 and RRV T48. By 20 min, ~5% of RRV T48 and RRV P12/6 had acquired resistance to neutralisation; by comparison the figure was ~20% for RRV P12/5. By 40 min ~10% of RRV T48 had acquired resistance to neutralisation. Corresponding figures for RRV P12/6 and RRV P12/5 were ~20% and ~35% respectively. At 60 min, ~25% of RRV T48 and ~50% of RRV P12/5 and RRV P12/6 had acquired neutralisation resistance. The results for RRV T48 were similar to those reported by Baric *et al*. (1981) for SIN. We conclude that passaged variants "penetrated" BHK cells more rapidly than did RRV T48.

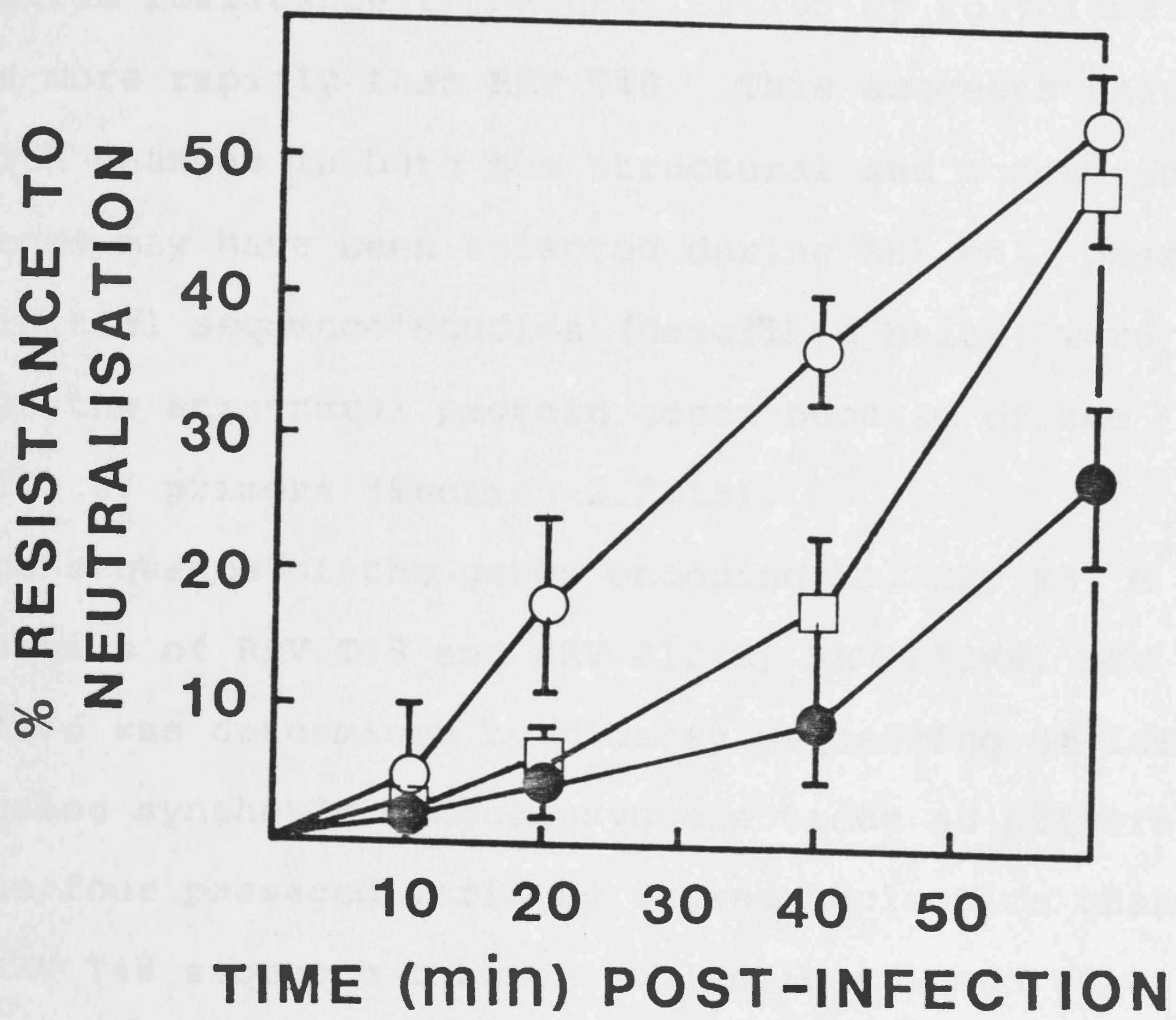
Figure 5.3 **Rate of acquisition of neutralisation resistance by passaged variants to added polyclonal antibodies.**

BHK cell monolayers in 60 mm dishes were infected with ~100 BHK PFU of RRV T48 or variants. At the designated times, the inoculum was removed and 0.5 ml of polyclonal anti-RRV T48 immune ascitic fluid (diluted 1:10 in HBSS) was added. After 10 min at 36°C the antibody was removed, the monolayer washed (x2) with PBS and overlayed for plaque development. The number of plaques obtained after a 60 min adsorption, PBS wash, but no antibody treatment was taken as 100% for each virus. Each assay was done in triplicate and the average values are plotted with error bars showing the standard error of mean.

RRV T48, (●).

RRV P12/5, (○).

RRV P12/6, (□).


% RESISTANCE TO NEUTRALISATION
10
20
30
40
50
10
20
30
40
50
TIME (min) POST-INFECTION

5.3.5 **Sequence studies on the structural protein genes of passaged variants**

The two most striking observations described above are (i) that RRV P12/2,4,5 and 6 had significantly higher RNA synthetic rates than RRV T48, and (ii) that RRV P12/5 and RRV P12/6 acquired resistance to neutralisation by polyclonal antibodies more rapidly than RRV T48. This suggests that mutants with changes in both the structural and non-structural protein genes may have been selected during BHK cell passage. However, initial sequence studies (described below) were directed at the structural protein genes because of the availability of primers (Section 2.2.15).

The sequence of the genes encoding E1, E2, E3, C and the 6K proteins of RRV T48 and RRV P12/2, RRV P12/4, RRV P12/5 and RRV P12/6 was determined by dideoxy sequencing of infected cell RNA using synthetic oligodeoxynucleotides as primers. None of the four passaged variants showed nucleotide changes from the RRV T48 sequence between nucleotides 1 and 4010 of the 26S RNA (Dalgarno et al., 1983) (data not shown). There were, however, 156 positions out of ~4000 at which the nucleotide could not be identified due to cross bands in all 4 lanes of the sequence ladder. These positions were randomly distributed throughout the region sequenced. At these positions the RRV T48 sequence ladder also showed cross bands suggesting that there was no sequence difference at these positions although the possibility of mutation/s in these positions could not be

ruled out completely. Positions at which mutations occurred in the mab-resistant variants of RRV T48 (Chapter 2) were sequenced satisfactorily, however. Sequencing of the non-structural protein genes was not attempted due to time limitations.

5.4 DISCUSSION

Serial passage of virus in tissue culture cells has often been employed to attenuate virulence (Olmsted *et al*., 1984). Consistent with this, serial passage of mouse virulent RRV T48 in BHK cells resulted in selection of variants which had reduced virulence for week-old mice. The LD_{50}s of variants obtained after 12 BHK passages were 5-30 times greater than RRV T48 and the ASTs of mice infected with the passaged variants were about twice those of RRV T48 infected mice. Thus the change in virulence was greater than that seen in mab-resistant variants of RRV T48 where only about a four-fold change in LD_{50} was observed (Chapter 3). Results described in this Chapter are consistent with those of Baric *et al*. (1981) who reported that the BHK passaged SIN was attenuated in mice, although they did not report the extent of attenuation or the method of measurement. In this context it is of interest that with ten serial passages of mouse-avirulent RRV NB5092 in mice, variants were selected in which the LD_{50} had changed ~10^6-fold (Meek, 1986).

In the present study, all RRV T48 infected mice which developed paralysis died eventually, thus giving a $LD_{50}:CD_{50}$ ratio of one, consistent with levels previously reported for virulent virus (Taylor, 1972; Meek, 1986). However a significant number of mice infected with the passaged variants recovered after showing paralysis. The $LD_{50}:CD_{50}$ ratio was between 10:1 and 100:1 indicating an attenuation of virulence. The significantly different levels of virulence of passaged variants plaque purified from the same passage level (Table 5.2) indicate that virus obtained after passaging was heterogeneous in virulence. Taylor and Marshall (1975) and Meek (1986) who passaged RRV NB5092 in mice to enhance virulence also observed sub-populations of different virulence in passaged virus stock.

Since the virus was plaque purified before passaging it seems likely that attenuated virus appeared spontaneously during passaging rather than resulting from the amplification of pre-existing mutants in the starting virus stock. As the virus was harvested, during passaging, at 6.5 hr pi, soon after the end of the latent period (Martin et al., 1979) it is likely that the rapid replication in BHK cells was the selection pressure for the variants. In fact all the passaged variants synthesised RNA at a significantly higher rate than RRV T48, although this did not confer any growth advantage on the passaged variants in terms of the EV titers. This is analogous to the situation in RRV dE2 which synthesised RNA at a significantly higher rate than RRV T48 in BHK cells, although

the EV titers of the two viruses did not differ (Chapter 4). Our results showing increased rates of RNA synthesis by RRV T48 passaged variants are similar to those reported by Baric et al. (1981) where the maximal rate of RNA synthesis by attenuated SIN in BHK cells was twice that of the parental virus. However, in contrast to our findings, attenuated SIN had a one hour shorter latent period and one log unit higher titers than the parental virus throughout the growth cycle in BHK cells (Baric et al., 1981). A change in the rate of viral RNA synthesis may also be expected to affect the kinetics of protein synthesis. However, no differences were seen in the kinetics of viral protein synthesis between BHK cells infected with RRV T48 or with the passaged variants during the limited time periods examined in the experiment. A comprehensive study of the kinetics of protein synthesis by the passaged variants needs to be carried out by labelling the infected cells more frequently.

The growth of RRV P12/6, the most attenuated of all the passaged variants examined, was compared with that of RRV T48 in week-old mice. Although the two viruses grew to similar titers in hind leg muscle, RRV P12/6 grew to peak titers in brain and blood which were ~2 log units lower than RRV T48, suggesting a change in tissue tropism. This is consistent with the previous conclusion (Chapter 4) that virus replication in the brain is more critical for RRV virulence than its replication in hind leg muscle.

During infection of BHK cells, passaged variants of RRV T48 acquired resistance to neutralisation more rapidly than did RRV T48. Thus by 60 min ~50% of the RRV P12/5 or RRV P12/6 had acquired resistance to neutralisation compared to ~25% for RRV T48. This observation is consistent with the findings of Baric et al. (1981) although the rates were much faster than seen in present case; thus after 60 min ~100% of the BHK passaged, attenuated SIN had acquired resistance to neutralisation whereas corresponding figure for parental SIN was ~40%. We have not explored the question of whether the change in rate of acquisition of neutralisation resistance is due to a faster attachment or a faster penetration of virus. Baric et al. (1981), however, concluded that the passaged SIN penetrated the cells faster since there was no difference in attachment of the passaged SIN and the parental virus.

Baric et al. (1981) and Olmsted et al. (1986) concluded that change(s) had occurred in neutralisation epitope(s) of E2 in the BHK passaged, attenuated SIN. However, mutation(s) in the structural protein gene(s) were not identified by sequencing. In our case an apparent change in tissue tropism and the change in "penetration" rate suggest that change(s) have taken place in the envelope proteins of RRV T48 during passaging in BHK cells. Surprisingly, no mutations could be detected in the genes coding for E1, E2, E3, C or the 6K proteins of RRV P12/2,4,5 and 6, although the possibility could not be ruled out rigorously due to the problem of cross bands. Mabs could be employed to explore the possibility that changes in conformation of envelope proteins of the virus had occurred.

Since BHK-passaged variants of RRV T48 synthesised RNA at a significantly higher rate than RRV T48, it seems likely that mutations have also occurred in the sections of the genome controlling viral RNA synthesis. The variants, therefore, could be useful in identifying important regions of the non-structural protein gene(s) involved in viral RNA replication. Sequencing of the non-structural protein genes would be a necessary step in this direction.

CHAPTER 6

SEQUENCE STUDIES ON THE E2 GENE OF NATURAL ISOLATES OF RRV

6.1 INTRODUCTION

RNA viruses have high potential for evolution due to the high frequency of mutations in their genome (Holland *et al*., 1982). A high rate of evolution has been demonstrated by RNA fingerprinting for polio virus which underwent 1-2% nucleotide substitution over a period of a one-year epidemic in a non-immune population (Nottay *et al*., 1981). However, there is little comparable quantitative information on the evolution of an alphavirus. This is due in part to the lack of isolates from well-defined epidemics where the temporal spread of the virus could clearly be demonstrated. The EPA outbreak in a non-immune population in the South Pacific (Chapter 1), therefore, provided a unique opportunity to study the evolution of an alphavirus. A start on this was made by Faragher *et al*. (1985a) who compared the HaeIII and TaqI restriction digest profiles of RRV isolates obtained from the South Pacific during the course of the epidemic. The isolates showed essentially identical profiles indicating that sequence evolution, if it occurred at all, had been extremely limited.

During the course of the polio virus epidemic (above) most of the mutations accumulated in the VP1 gene (Nottay *et al*., 1981) which codes for the viral surface protein containing neutralisation antigenic sites (Minor *et al*., 1983). For alphaviruses E2 has been implicated in strain differentiation and virus evolution (Dalgarno *et al*., 1983) and a domain in E2 has been identified which contains neutralisation epitopes

(Chapter 2). In the light of these observations the E2 gene of RRV isolates from the South Pacific was sequenced to investigate virus evolution at the nucleotide level.

In previous unpublished studies A.T.H. Burness, I. Pardoe and L. Dalgarno determined the sequence of the E2 gene of RRV isolates from Fiji (F9073), American Samoa (AS213970) and the Cook Islands (CI215398) between nucleotides 1124 and 1439 which code for the stretch of E2 between amino acids 25 and 129. They found no difference between the Pacific isolates in this region. However, their data covers only one-fourth of the E2 gene and does not cover the "neutralisation domain". In this Chapter we completely sequence the E2 gene of a number of Pacific isolates obtained from human cases at various stages during the outbreak in order to determine (i) the rate of genetic change in the E2 gene during the spread of RRV in a non-immune population, and (ii) whether any detected changes mapped to the domain previously identified as having a role in attachment/ penetration and in recognition of neutralising antibodies (Chapters 2 and 3).

Another question which arises relates to the relationship between virus causing the outbreak in the South Pacific and the various RRV genetic types and sub-types identified in Australia. The studies of Faragher _et al_. (1985a) showed that the South Pacific isolates of RRV were closely related to the Australian RRV isolates NL26301, SC18008 and BH38006 respectively, which were obtained from mosquitoes collected at Narran Lake, Sandy Camp and Barmah Forest, all in

New South Wales. In this Chapter we have determined the E2 sequence of RRV NL26301 and compared it with that of the South Pacific RRV isolates to examine their relationship at the nucleotide sequence level.

RRV could not be isolated from human EPA patients in Australia until recently when Aaskov et al. (1985) described the isolation of RRV CO and RRV MC from two patients in Rockhampton in Queensland. These patients showed a similar disease pattern to that seen in EPA patients in the South Pacific (Aaskov et al., 1985) which were different from that seen in the previous EPA cases in Australia (Rosen et al., 1981; Aaskov et al., 1985). It was therefore of interest to determine the genetic relationship between the Rockhampton isolates and those from the South Pacific. Also, the relationship between the Rockhampton isolates and other Australian isolates from non-human sources is investigated. The results of these studies are described in this Chapter.

It has been argued that RRV exists in Australia in a number of ecological niches, where, over an extended period of time independent strain differentiation has occurred (Faragher et al., 1985a). Thus based on similarities and differences in HaeIII and TaqI restriction digest profiles of viral RNA Faragher et al. (1985a) grouped the different geographical isolates of RRV into three genetic types, I, II and III (Chapter 1). However, strain specific differences between various isolates are not known. This Chapter describes an attempt to explore strain related differences in RRV by

comparing the E2 sequences of a small number of different natural isolates of the virus.

6.2 MATERIALS AND METHODS

6.2.1 Virus and cells

Virus isolates used in this study are listed in Table 6.1. Culture supernatants from infected BHK cells served as stock virus. BHK cells were grown in GMEM (Section 2.2.2).

6.2.2 Extraction of high molecular weight RNA from virus infected BHK cells and sequencing the E2 gene of RRV isolates

High molecular weight RNA extracted from virus infected BHK cells (Section 2.2.14) was the template for dideoxy sequencing using primers 6K/2350, E2/1990, E2/1635 and E2/1455 (Table 2.1). Virus was not plaque purified and the sequence obtained, therefore, represents that of the majority of virus population present in the stock. When sequencing RRV isolates, RRV T48 RNA was sequenced in parallel. Approximately 50 cross bands were seen which were evenly distributed throughout the 1266 nucleotides long E2 gene. In most instances the cross band was present in the same position in the RRV T48 sequence ladder also, in which case the nucleotide was considered to be the same as that in RRV T48 (Dalgarno *et al*., 1983), otherwise it was recorded as an unidentified nucleotide (N).

Table 6.1 **RRV isolates used in this study.**

Isolate	Area of isolation	Year	Source	Genetic type*	Passage history	Supplier	Reference
T48	Townsville, Qld.	1959	*Aedes vigilax*	I	~10 passages in mice, plaque purified	Dr. R.A. Shope Yale Arbovirus Research Unit	Doherty *et al.*, (1963)
PB629	Lake Poomah, Vic.	1974	*Culex annulirostris*	I	1-2 suckling mouse brain passages	Dr. I.D. Marshall JCSMR, ANU	Marshall *et al.*, (1982)
GG2227	Gol Gol, N.S.W.	1974	*Culex annulirostris*	I			
NL26301	Narran Lake, N.S.W.	1981	*Anopheles amictus*	II			
BH38091	Barmah Forest, Vic.	1983	*Aedes sagax*	ND	C6-36 cells/mice	Dr. I.D. Marshall	-
F213978	Fiji	1979	Human serum	II	2 passages in *Toxorhynchites amboinensis* mosquitoes	Dr. L. Rosen University of Hawaii	Aaskov *et al.*, (1981)
AS213970	American Samoa	1979	Human serum	II			Tesh *et al.*, (1981)
CI215398	Cook Islands	1980	Human Serum	II			Rosen *et al.*, (1981)
CO	Rockhampton, Qld.	1983	Human serum	ND	C6-36 cells	Dr. I.D. Marshall	Aaskov *et al.*, (1985)
MC	Rockhampton, Qld.	1983	Human serum	ND	C6-36 cells		

*From Faragher *et al.* (1985a); ND=Not determined.

Virus was not plaque purified unless indicated in Table or Text.

6.3 RESULTS

6.3.1 Sequence of the E2 gene of RRV isolates from human EPA cases in the South Pacific

During 1979-80 RRV spread sequentially in the non-immune population of Fiji, American Samoa and the Cook Islands in the South Pacific. To examine the evolution of the RRV E2 gene during this spread, the E2 gene sequence of virus isolated from human patients in different islands was determined. The isolates sequenced were RRV F213978, isolated in Fiji in 1979; RRV AS213970, isolated in American Samoa in 1979; and RRV CI215398, isolated in the Cook Islands in 1980.

Figure 6.1 shows the nucleotide sequence of the E2 gene of RRV F213978 and the deduced amino acid sequence colisted with that of RRV T48 (see below). The E2 sequences of RRV AS213970 and RRV CI215398 were identical to each other and are shown as a single sequence in Figure 6.2.

The RRV AS213970/RRV CI215398 sequence showed a single nucleotide difference from that of RRV F213978. At position 1705 an A in RRV F213978 RNA was replaced by a G in RRV AS213970 and RRV CI215398 RNAs (Figure 6.3, Table 6.2). This change led to a predicted non-conservative amino acid change (Thr→Ala) at position 219 in E2 (Table 6.3). Interestingly this change was in epitope I of E2 (Chapter 2).

Compared to RRV T48 there were 37 nucleotide differences in the E2 gene of RRV F213978 (Figure 6.1, Table

Figure 6.1 **Nucleotide sequence of RRV F213978 E2 gene and the deduced amino acid sequence of E2.**

Nucleotides are numbered from the 5'-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno _et al_., 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide

X=unidentified amino acid

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48   SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu  (40)
          AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGGACGGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG  (1170)
                   |         |         |         |         |         |         |         |         |         |         |
RRV FIJI  ------------------------------------------------------------------------------------------------------------------------
          -----------------------------------------A------NNNN----------U--C------------------------------------------------------

          AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg  (80)
          GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA  (1290)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------Leu---------------------------------------
          --------U--------U------------------------------------------------------------C-----------------------------------------

          AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer  (120)
          GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA  (1410)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------------------------C--------------------U--------------C-----A--------------------------U--------------G

          HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr  (160)
          CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA  (1530)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------------------------Asn------------------------------------------------------------------------------------
          ---------------------------------A-U--------------------------------------A-----------U--------------------------------G

          AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn  (200)
          GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU  (1650)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------Glu------------------------------------------------------------------------------------------------------
          ----------------A---U--------U------------------------------------------------------------------------------------------

          CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro  (240)
          UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA  (1770)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------------------------------------------Tyr------------------------------------------------------------------
          ---------------------------------------------------U-------U------------------------------------------------------------

          PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys  (280)
          UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG  (1890)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------Lys---------------------------------------------------------------------------------------
          -------------------------------AA--------A--------G------C--------------------------------------------------------------

          GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal  (320)
          GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG  (2010)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          --------------------------------------------------------------U--------------------A------------------------------------

          ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly  (360)
          ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA  (2130)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          --------------------------------------------------G---------------------------------------------------------------------

          LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla  (400)
          CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC  (2250)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------U--------------------------------------------G--------------------------------------------------G------

          LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                      (422)
          UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                      (2316)
                   |         |         |         |         |         |
          ---------------------Pro------------------------------------------
          C--------------------C-A--------------------------------------U---
```

Figure 6.2 **Nucleotide sequence of RRV AS213970 and RRV CI215398 E2 genes and the deduced amino acid sequences of E2.**

Nucleotides are numbered from the 5'-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno et al., 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48         SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu  (40)
                AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGGACGGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG  (1170)
                         |         |         |         |         |         |         |         |         |         |         |
RRV AS/ RRV CI  ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------------A------NNNN----------U--C------------------------------------------------------

                AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg  (80)
                GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA  (1290)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------Leu---------------------------------------
                --------U--------U------------------------------------------------------------C-----------------------------------------

                AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer  (120)
                GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA  (1410)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------C--------------------U--------------C-----A--------------------------U--------------G

                HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr  (160)
                CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA  (1530)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------------------------Asn------------------------------------------------------------------------------------
                ---------------------------------A-U--------------------------------------A-----------U--------------------------------G

                AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn  (200)
                GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU  (1650)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------Glu------------------------------------------------------------------------------------------------------
                ----------------A---U--------U------------------------------------------------------------------------------------------

                CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro  (240)
                UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA  (1770)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------------------------------------------TyrAla---------------------------------------------------------------
                ---------------------------------------------------U--G----U------------------------------------------------------------

                PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys  (280)
                UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG  (1890)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------Lys---------------------------------------------------------------------------------------
                ------------------------------AA---------A--------G------C--------------------------------------------------------------

                GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal  (320)
                GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG  (2010)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                --------------------------------------------------------------U--------------------A------------------------------------

                ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly  (360)
                ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA  (2130)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                --------------------------------------------------G---------------------------------------------------------------------

                LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla  (400)
                CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC  (2250)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------U--------------------------------------------G--------------------------------------------------G------

                LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                        (422)
                UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                        (2316)
                         |         |         |         |         |         |
                ---------------------Pro------------------------------------------
                C--------------------C-A--------------------------------------U---
```

Figure 6.3 **Sequence ladders for RRV T48, RRV F213978, RRV AS213970 and RRV CI215398.**

RNA extracted from virus infected BHK cells was sequenced using primer E2/1990 (Table 2.1). The changed nucleotide in cDNA sequence ladder is indicated by an arrow.

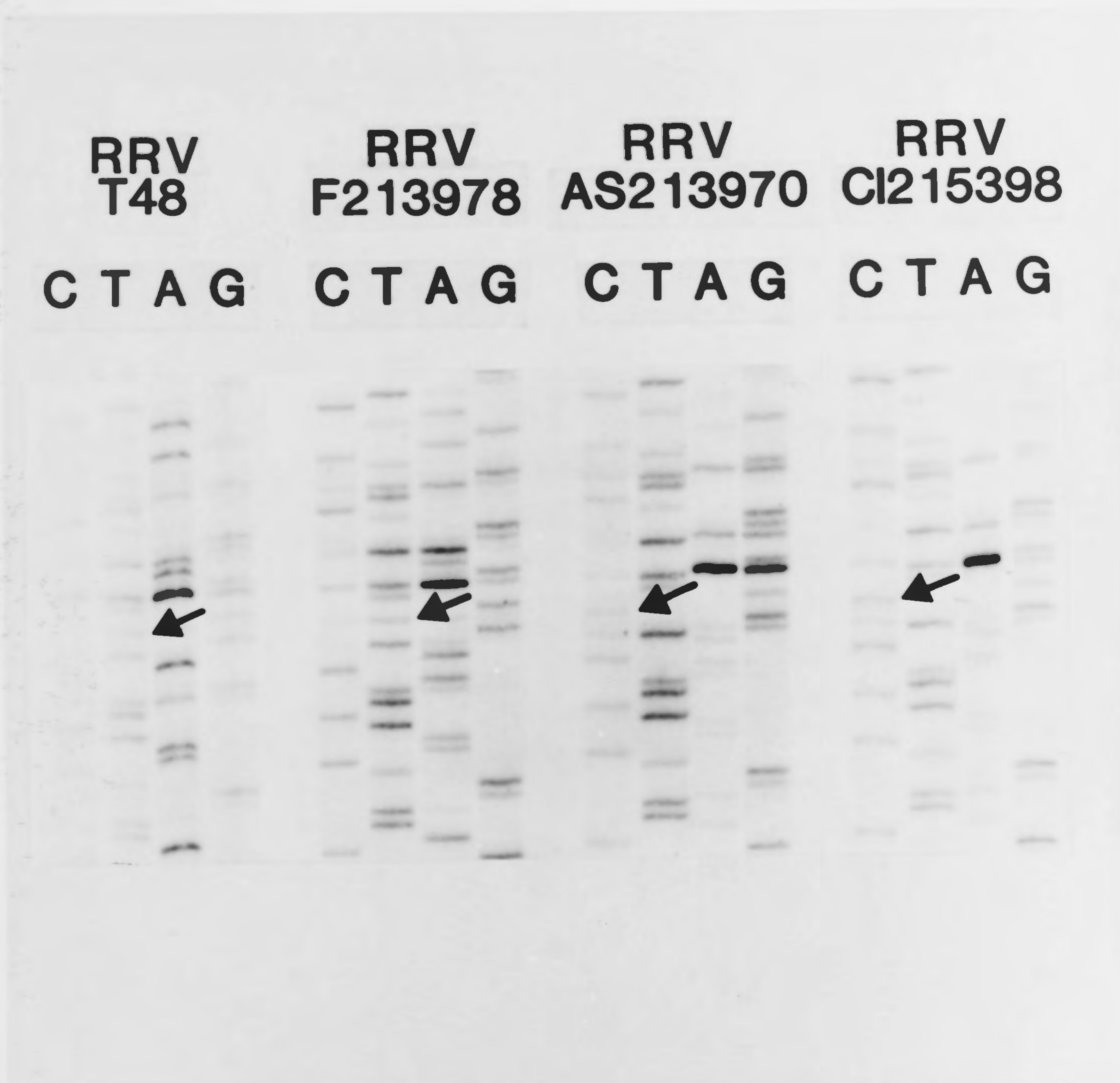

RRV
T48
RRV
F213978
RRV
AS213970
RRV
CI215398
C T A G
C T A G
C T A G
C T A G

Table 6.2 **Sequence differences between E2 gene of RRV natural isolates and RRV T48.**

Nucleotide position†	RRV T48	RRV isolate*								
		NL	F	AS	CI	MC	CO	PB	BH	GG
1092	G	A	A	A	A	A	A			
1113	C	U	U	U	U	U	U			
1116	U	C	C	C	C	C	C			
1170	G								A	A
1179	C	U	U	U	U	U	U			
1188	C	U	U	U	U	U	U			
1221	C							U	U	U
1249	A	C	C	C	C	C	C	C	C	C
1326	U	C	C	C	C	C	C			
1347	C	U	U	U	U	U	U			
1353	C							U	U	U
1362	U	C	C	C	C	C	C			
1368	G	N	A	A	A	A	A			
1371	A							G	G	G
1395	C	U	U	U	U	U	U			
1410	A	G	G	G	G	G	G			
1444	G	N	A	A	A	A	A			
1446	C	U	U	U	U	U	U			
1485	C	A	A	A	A	A	A			
1497	A	U	U	U	U	U	U			
1501	C								U	U
1527	A							G	G	G
1530	A	G	G	G	G	G	G			
1547	G	A	A	A	A	A	A	A	A	A
1551	C	U	U	U	U	U	U			
1560	C	U	U	U	U	U	U			
1702	A	U	U	U	U					
1705	A	N		G	G	N	N			
1710	C	U	U	U	U	U	U			
1794	A							G	G	G
1802	G	A	A	A	A	A	A			
1803	G	A	A	A	A	A	A			
1808	A								G	G
1812	G	A	A	A	A	A	A			
1821	A	G	G	G	G	G	G			
1828	U	C	C	C	C	C	C			
1953	C		U	U	U					
1974	G	A	A	A	A	A	A			
2061	A	G	G	G	G	G	G			
2106	U							C	C	C
2148	C	U	U	U	U	U	U			
2193	A	G	G	G	G	G	G			
2244	A	G	G	G	G	G	G			
2251	U	C	C	C	C	C	C			
2272	U	C	C	C	C	C	C			
2274	G	A	A	A	A	A	A			
2313	C	U	U	U	U	U	U			

† Numbered from 5'-end of RRV T48 26S RNA (Dalgarno et al., 1983).

* RRV isolates are indicated as follows: NL, RRV NL26301; F, RRV F213978; AS, RRV AS213970; CI, RRV CI215398; MC, RRV MC; CO, RRV CO; PB, RRV PB629; BH, RRV BH38091; GG, RRV GG2227. N=unidentified nucleotide, not considered when comparing the isolates; blank space=same nucleotide as in RRV T48.

Table 6.3 <u>**Predicted amino acid differences between E2 of RRV natural isolates and RRV T48.**</u>

Position of amino acid†	RRV T48	RRV isolate* NL	F	AS	CI	MC	CO	PB	BH	GG	Nature of amino acid change††
67	Ile	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	C
132	Asp	X	Asn	Asn	Asn	Asn	Asn				NC
166	Gly	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	NC
218	Asn	Tyr	Tyr	Tyr	Tyr						NC
219	Thr	X		Ala	Ala	X	X				NC
251	Arg	Lys	Lys	Lys	Lys	Lys	Lys				C
253	Lys								Arg	Arg	C
408	Ser	Pro	Pro	Pro	Pro	Pro	Pro				NC

† Numbered from N-terminus of E2 (Dalgarno <u>et</u> <u>al</u>., 1983).

* RRV isolates are as in Table 6.2.
X=uncertain amino acid, not considered for comparing the isolates; blank space=same amino acid as in RRV T48.

†† C=conservative, NC=non-conservative; see legend to Table 2.5.

6.2) which did not cluster in any specific region. They resulted in 6 predicted amino acid differences between RRV T48 and RRV F213978 at positions 67 (Ile/Leu), 132 (Asp/Asn), 166 (Gly/Glu), 218 (Asn/Tyr), 251 (Arg/Lys) and 408 (Ser/Pro) in E2 (Table 6.3). For RRV AS213970 and RRV CI215398 the same differences were detected but there was an additional predicted amino acid change at position 219 (Thr/Ala)(Table 6.3). Thus differences at amino acids 218 and 219, and at 251 between any of the Pacific isolates and RRV T48 were in the neutralisation epitopes I and III respectively.

6.3.2 Sequence of the E2 gene of RRV obtained from mosquitoes collected at Narran Lake in 1981

On the basis of HaeIII restriction digest profiles of cDNA to viral RNA Faragher et al. (1985a) showed that RRV NL26301 isolated from mosquitoes collected at Narran Lake in 1981, was closely related, if not identical, to the South Pacific isolates of RRV. To examine the relationship between these isolates more closely, the E2 gene of RRV NL26301 was sequenced.

Figure 6.4 shows the E2 sequence of RRV NL26301. There was a single nucleotide difference between RRV NL26301 and any of the South Pacific RRV isolates (Table 6.2). This was a silent change at position 1953. We conclude that the Pacific isolates are not identical to RRV NL26301 although they are closely related in their E2 gene sequence.

Figure 6.4 **Nucleotide sequence of RRV NL26301 E2 gene and the deduced amino acid sequence of E2.**

Nucleotides are numbered from the 5′-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno *et al.*, 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide

X=unidentified amino acid

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48   SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu   (40)
          AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGGACGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG   (1170)
                   |         |         |         |         |         |         |         |         |         |         |
RRV NL    ------------------------------------------------------------------------------------------------------------------------
          ------------------------------------------A------NNNN----------U--C-----------------------------------------------------

          AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg   (80)
          GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA   (1290)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------Leu---------------------------------------
          --------U--------U------------------------------------------------------------C-----------------------------------------

          AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer   (120)
          GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA   (1410)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------------------------C---------------------U--------------C-----N-------------------------U--------------G

          HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr   (160)
          CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA   (1530)
                   |         |         |         |         |         |         |         |         |         |         |
          --------------------------------- X ------------------------------------------------------------------------------------
          ---------------------------------N-U---------------------------------------A-----------U-------------------------------G

          AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn   (200)
          GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU   (1650)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------Glu------------------------------------------------------------------------------------------------------
          ----------------A---U--------U------------------------------------------------------------------------------------------

          CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro   (240)
          UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA   (1770)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------------------------------------------Tyr X ---------------------------------------------------------------
          ---------------------------------------------------U--N----U------------------------------------------------------------

          PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys   (280)
          UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG   (1890)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------Lys---------------------------------------------------------------------------------------
          -------------------------------AA--------A--------G------C--------------------------------------------------------------

          GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal   (320)
          GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG   (2010)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          ------------------------------------------------------------------------------------A-----------------------------------

          ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly   (360)
          ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA   (2130)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          ---------------------------------------------------G--------------------------------------------------------------------

          LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla   (400)
          CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC   (2250)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------U---------------------------------------------G--------------------------------------------------G-----

          LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                         (422)
          UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                         (2316)
                   |         |         |         |         |         |
          ---------------------Pro------------------------------------------
          C--------------------C-A--------------------------------------U---
```

There were 37 nucleotide differences between RRV NL26301 and RRV T48 E2 genes (Figure 6.4, Table 6.2). These resulted in amino acid differences at positions 67 (Ile/Leu), 166 (Gly/Glu), 218 (Asn/Tyr), 251 (Arg/Lys) and 408 (Ser/Pro) (Table 6.3). The amino acids at positions 132 and 219 could not be identified due to cross bands in the sequence ladder at the first codon nucleotide for these amino acids. However, the absence of cross bands in the RRV T48 sequence ladder at corresponding positions suggested nucleotide differences and hence amino acid differences.

6.3.3 Sequence of the E2 gene of RRV isolates obtained from mosquitoes collected in the Murray valley in 1974 and 1983

As part of a preliminary examination of the genetic relationship between different RRV isolates we have sequenced the E2 genes of three isolates obtained from mosquitoes collected during RRV epidemics in the Murray valley: RRV PB629 (from Lake Poomah near Swan Hill) and RRV GG2227 (from Gol Gol near Mildura), both isolated in 1974; and RRV BH38091 (from Barmah Forest near Echuca) isolated in 1983 (Table 6.1).

Figure 6.5 presents the E2 sequence of RRV PB629. The E2 sequences of RRV GG2227 and RRV BH38091 were identical to each other and are listed as a single sequence in Figure 6.6. The E2 sequences of RRV GG2227/RRV BH38091 differed from that of RRV PB629 at nucleotides 1170, 1501 and 1808 (Table 6.2) one

Figure 6.5 **Nucleotide sequence of RRV PB629 E2 gene and the deduced amino acid sequence of E2.**

Nucleotides are numbered from the 5'-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno _et al_., 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48   SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu     (40)
          AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGGACGGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG   (1170)
                   |         |         |         |         |         |         |         |         |         |         |
RRV PB    ------------------------------------------------------------------------------------------------------------------------
          -------------------------------------------------NNNN-------------------------------------------------------------------

          AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg     (80)
          GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA   (1290)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------Leu---------------------------------------
          --------------------------------------------------U---------------------------C-----------------------------------------

          AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer    (120)
          GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA   (1410)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          --------------------------------------------------------------U-----------------G---------------------------------------

          HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr    (160)
          CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA   (1530)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          --------------------------------------------------------------------------------------------------------------------G---

          AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn    (200)
          GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU   (1650)
                   |         |         |         |         |         |         |         |         |         |         |
          ---------------Glu------------------------------------------------------------------------------------------------------
          ----------------A-------------------------------------------------------------------------------------------------------

          CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro    (240)
          UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA   (1770)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          ------------------------------------------------------------------------------------------------------------------------

          PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys    (280)
          UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG   (1890)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------------G------------------------------------------------------------------------------------------------

          GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal    (320)
          GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG   (2010)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          ------------------------------------------------------------------------------------------------------------------------

          ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly    (360)
          ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA   (2130)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          -----------------------------------------------------------------------------------------------C------------------------

          LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla    (400)
          CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC   (2250)
                   |         |         |         |         |         |         |         |         |         |         |
          ------------------------------------------------------------------------------------------------------------------------
          ------------------------------------------------------------------------------------------------------------------------

          LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                          (422)
          UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                         (2316)
                   |         |         |         |         |         |
          ------------------------------------------------------------------
          ------------------------------------------------------------------
```

Figure 6.6 **Nucleotide sequence of RRV BH38091 and RRV GG2227 E2 genes and the deduced amino acid sequences of E2.**

Nucleotides are numbered from the 5′-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno _et_ _al_., 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48         SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu   (40)
                AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGACGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG (1170)
                         |         |         |         |         |         |         |         |         |         |         |
RRV BH/ RRV GG  ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------------------NNNN--------------------------------------------------------------------A

                AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg   (80)
                GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA (1290)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------Leu---------------------------------------
                -------------------------------------------------U----------------------------C-----------------------------------------

                AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer  (120)
                GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA (1410)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                --------------------------------------------------------------U-----------------G---------------------------------------

                HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr  (160)
                CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA (1530)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------U-------------------------G---

                AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn  (200)
                GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU (1650)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------Glu------------------------------------------------------------------------------------------------------
                ----------------A-------------------------------------------------------------------------------------------------------

                CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro  (240)
                UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA (1770)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------

                PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys  (280)
                UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG (1890)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------Arg---------------------------------------------------------------------------------
                -----------------------G-------------G----------------------------------------------------------------------------------

                GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal  (320)
                GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG (2010)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------

                ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly  (360)
                ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA (2130)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------------------------------------------------------------------C------------------------

                LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla  (400)
                CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC (2250)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------

                LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                        (422)
                UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                       (2316)
                         |         |         |         |         |         |
                ------------------------------------------------------------------
                ------------------------------------------------------------------
```

of which resulted in a single conservative amino acid difference (Lys/Arg) at residue 219 (Table 6.3). It can be concluded that the two 1974 strains were not identical and that RRV GG2227 or a close relative was present in the region (as RRV BH38091) in 1983.

When the RRV PB629 E2 gene sequence was compared with that of RRV T48, seven nucleotide differences were found (Figure 6.5, Table 6.2) two of which resulted in amino acid differences at positions 67 (Ile/Leu) and 166 (Gly/Glu) in E2 (Table 6.3). Compared to RRV T48, RRV BH38091 and RRV GG2227 had 10 nucleotide differences in the E2 gene (Figure 6.7, Table 6.2). These resulted in amino acid differences at positions 67 (Ile/Leu), 166 (Gly/Glu) and 253 (Lys/Arg) in E2 (Table 6.3). From comparing the E2 nucleotide sequences we conclude that the three Murray valley isolates of RRV are closely related to the prototype T48 strain with < 1% nucleotide sequence divergence and hence are of the same genetic type (Faragher et al., 1985a), provided the genetic divergence is no greatly different in other parts of the genome.

6.3.4 Sequence of the E2 gene of RRV isolates obtained from human EPA cases in Rockhampton in 1983

To examine the relationship between RRV isolated from human EPA patients in Australia and the South Pacific, the E2 genes of RRV CO and RRV MC were sequenced (Figure 6.7).

The two isolates were identical to each other in their

Figure 6.7 **Nucleotide sequence of RRV CO and RRV MC E2 genes and the deduced amino acid sequences of E2.**

Nucleotides are numbered from the 5′-end of RRV T48 26S RNA and amino acids from the N-terminus of E2 (Dalgarno et al., 1983). Identical nucleotides and amino acids to the top sequence are shown by a broken line.

N=unidentified nucleotide
X=unidentified amino acid

Nucleotides between position 1099-1102 could not be identified due to compression.

```
RRV T48         SerValThrGluHisPheAsnValTyrLysAlaThrArgProTyrLeuAlaTyrCysAlaAspCysGlyAspGlyTyrPheCysTyrSerProValAlaIleGluLysIleArgAspGlu  (40)
                AGUGUAACAGAGCACUUCAAUGUGUAUAAGGCUACUAGACCGUACUUAGCGUAUUGCGCUGACUGUGGGGACGGGUACUUCUGCUAUAGCCCAGUUGCUAUCGAGAAGAUCCGAGAUGAG  (1170)
                         |         |         |         |         |         |         |         |         |         |         |
RRV CO/ RRV MC  ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------------A------NNNN----------U--C------------------------------------------------------

                AlaSerAspGlyMetLeuLysIleGlnValSerAlaGlnIleGlyLeuAspLysAlaGlyThrHisAlaHisThrLysIleArgTyrMetAlaGlyHisAspValGlnGluSerLysArg  (80)
                GCGUCUGACGGCAUGCUCAAGAUCCAAGUCUCCGCCCAAAUAGGUCUGGACAAGGCAGGUACCCACGCCCACACGAAGAUCCGAUAUAUGGCUGGUCAUGAUGUUCAGGAAUCUAAGAGA  (1290)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                --------U--------U-----------------+------------------------------------------Leu---------------------------------------
                ------------------------------------------------------------------------------C-----------------------------------------

                AspSerLeuArgValTyrThrSerAlaAlaCysSerIleHisGlyThrMetGlyHisPheIleValAlaHisCysProProGlyAspTyrLeuLysValSerPheGluAspAlaAspSer  (120)
                GAUUCCUUGAGGGUGUACACGUCCGCAGCGUGCUCUAUACAUGGGACGAUGGGACACUUCAUCGUCGCACAUUGUCCGCCAGGCGACUACCUCAAGGUUUCGUUCGAGGACGCAGAUUCA  (1410)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------C--------------------U--------------C-----A--------------------------U--------------G

                HisValLysAlaCysLysValGlnTyrLysHisAspProLeuProValGlyArgGluLysPheValValArgProHisPheGlyValGluLeuProCysThrSerTyrGlnLeuThrThr  (160)
                CACGUGAAGGCAUGUAAGGUCCAAUACAAGCACGACCCAUUGCCGGUGGGUAGAGAGAAGUUCGUGGUUAGACCCCACUUUGGCGUAGAGCUGCCAUGCACCUCAUACCAGCUGACAACA  (1530)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------------------------Asn------------------------------------------------------------------------------------
                ---------------------------------A-U--------------------------------------A-----------U--------------------------------G

                AlaProThrAspGluGlyIleAspMetHisThrProProAspIleProAspArgThrLeuLeuSerGlnThrAlaGlyAsnValLysIleThrAlaGlyGlyArgThrIleArgTyrAsn  (200)
                GCUCCCACCGACGAGGGGAUCGACAUGCACACACCGCCAGAUAUACCGGAUCGCACCCUGCUAUCACAGACGGCGGGCAACGUCAAAAUAACAGCAGGCGGCAGGACUAUCAGGUACAAU  (1650)
                         |         |         |         |         |         |         |         |         |         |         |
                ---------------Glu------------------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------
                ----------------A---U--------U------------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------

                CysThrCysGlyArgAspAsnValGlyThrThrSerThrAspLysThrIleAsnThrCysLysIleAspGlnCysHisAlaAlaValThrSerHisAspLysTrpGlnPheThrSerPro  (240)
                UGUACCUGUGGCCGUGACAACGUAGGCACUACCAGUACUGACAAGACCAUCAACACAUGCAAGAUUGACCAAUGCCAUGCUGCCGUUACCAGCCAUGACAAAUGGCAAUUUACCUCUCCA  (1770)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------ X ---------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------
                ------------------------------------------------------N----U------------------------------------------------------------

                PheValProArgAlaAspGlnThrAlaArgArgGlyLysValHisValProPheProLeuThrAsnValThrCysArgValProLeuAlaArgAlaProAspValThrTyrGlyLysLys  (280)
                UUUGUUCCCAGGGCUGAUCAGACAGCUAGGAGGGGCAAAGUGCAUGUUCCAUUCCCUUUGACUAACGUCACCUGCCGAGUGCCGUUGGCUCGAGCGCCGGAUGUCACCUAUGGUAAGAAG  (1890)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -------------------------------Lys--------------------------------------------------------------------------------------
                ------------------------------------------------------------------------------------------------------------------------
                -------------------------------AA--------A--------G-----C---------------------------------------------------------------

                GluValThrLeuArgLeuHisProAspHisProThrLeuPheSerTyrArgSerLeuGlyAlaGluProHisProTyrGluGluTrpValAspLysPheSerGluArgIleIleProVal  (320)
                GAGGUGACCCUGAGAUUACACCCAGAUCAUCCGACGCUCUUCUCCUAUAGGAGUUUAGGAGCCGAACCGCACCCGUACGAGGAGUGGGUUGACAAGUUCUCUGAGCGCAUCAUCCCAGUG  (2010)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------------------------------------------------------------------------A------------------------------------

                ThrGluGluGlyIleGluTyrGlnTrpGlyAsnAsnProProValArgLeuTrpAlaGlnLeuThrThrGluGlyLysProHisGlyTrpProHisGluIleIleGlnTyrTyrTyrGly  (360)
                ACGGAAGAAGGGAUUGAGUACCAGUGGGGCAACAACCCGCCGGUCCGCCUAUGGGCGCAACUGACGACCGAGGGCAAACCCCAUGGCUGGCCACAUGAAAUCAUUCAGUACUAUUAUGGA  (2130)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                --------------------------------------------------G---------------------------------------------------------------------

                LeuTyrProAlaAlaThrIleAlaAlaValSerGlyAlaSerLeuMetAlaLeuLeuThrLeuAlaAlaThrCysCysMetLeuAlaThrAlaArgArgLysCysLeuThrProTyrAla  (400)
                CUAUACCCCGCCGCCACCAUUGCCGCAGUAUCCGGGGCGAGUCUGAUGGCCCUCCUAACUCUAGCGGCCACAUGCUGCAUGCUGGCCACCGCGAGGAGAAAGUGCCUAACACCAUACGCC  (2250)
                         |         |         |         |         |         |         |         |         |         |         |
                ------------------------------------------------------------------------------------------------------------------------
                -----------------U--------------------------------------------G--------------------------------------------------G------

                LeuThrProGlyAlaValValSerLeuThrLeuGlyLeuLeuCysCysAlaProArgAlaAsnAla                                                      (422)
                UUGACGCCAGGAGCGGUGGUAUCGUUGACACUGGGGCUGCUUUGCUGCGCACCGAGGGCGAACGCA                                                      (2316)
                         |         |         |         |         |         |
                ---------------------Pro------------------------------------------
                C--------------------C-A--------------------------------------U---
```

E2 sequences and were closely related, but not identical, to the South Pacific isolates of RRV (Table 6.2). There were two nucleotide differences between RRV CO/RRV MC and the South Pacific RRV isolates at positions 1702 and 1953 (Table 6.2). One of these resulted in a predicted amino acid difference (Tyr/Asn) at position 218 (Table 6.3). Interestingly this amino acid is part of the epitope I (Chapter 2).

RRV CO and RRV MC were closely related to RRV NL26301. There was a single nucleotide difference between the two at position 1702 of the E2 gene (Table 6.2). This resulted in an amino acid difference (Tyr/Asn) at position 218 in E2 (Table 6.3).

RRV MC/RRV CO had 36 nucleotide differences from RRV T48 in the E2 gene (Table 6.2). These resulted in amino acid differences in E2 at positions 67 (Ile/Leu), 132 (Asp/Asn), 166 (Gly/Glu), 251 (Arg/Lys) and 408 (Ser/Pro) (Table 6.3). The amino acid at position 219 may also be changed, however, an identification could not be made for reason described in Section 6.3.2.

6.4 DISCUSSION

6.4.1 Evolution of RRV during epidemic spread in a non-immune population

RRV induced EPA was first reported in Fiji in 1979. In August 1979 the virus spread to American Samoa and then in

February 1980 the EPA cases were reported from the Cook Islands (Chapter 1). We have examined the evolution of RRV during this well defined spread of virus in a non-immune population. This is the first study of its kind for any alphavirus.

Comparison of the E2 gene sequences of the early (RRV F213978) and late (RRV CI215398) isolates in the epidemic showed that only a single nucleotide change had been selected over the period of a little more than a year. These results are consistent with the findings of Faragher et al. (1985a) which indicated that during the South Pacific epidemic of RRV only limited nucleotide evolution had occurred.

Since E2 is the most divergent of the structural protein genes in terms of amino acid sequence conservation between RRV, SFV and SIN (Dalgarno et al., 1983), it is perhaps surprising that only a single nucleotide substitution was selected during virus spread in the South Pacific epidemic involving hundreds of thousands of people (Chapter 1). This single nucleotide change in 1266 nucleotides long E2 gene represents 0.078% nucleotide sequence evolution. These findings are in contrast to those of Nottay et al. (1981) who studied the evolution of polio virus during an epidemic which spread through a few hundred people belonging to an unvaccinated religious community between April 1978 and June 1979. Using the RNA fingerprinting technique they estimated that during the epidemic 1-2% nucleotide changes were selected in the polio virus genome. Most of these changes were in the gene coding for the neutralisation antigen VP1 (Nottay et al., 1981).

This marked difference in evolution rates for RRV and polio virus cannot be attributed to the intrinsic genetic stability of RRV since, as an example, the E2 gene of RRV NL26301 is ~3% divergent from that of RRV T48 (Table 6.4). Further, as judged by the frequency of isolation of the mab-resistant variants (Chapter 2) the rate of mutation in RRV genome is similar to that for other RNA viruses. A likely reason for the remarkable stability of RRV genome relates to the transmission cycle which involves alternate replication in vertebrates and invertebrates. Thus a mutation which may be selected in one host is unlikely to confer an advantage in the other host. Hence an alternate replication of RRV in different hosts may provide a negative selection pressure. In contrast, polio virus is passed directly from human to human without an intermediate host. Thus a mutation could be selected positively. Consistent with this explanation, when mouse-avirulent RRV NB5092 was passaged ten times serially in mice to enhance its virulence, it accumulated a small number of mutations in the E2 gene (Meek, 1986). By contrast, alternate passages in mice and mosquitoes keep the viral virulence unchanged (Taylor and Marshall, 1975a,b).

Since RRV genome is so stable under epidemic conditions, the question arises as to how the genetic diversity which exists between RRV isolates arose. Faragher et al. (1985a) have proposed that it resulted from the interaction between virus, mosquito vector and the mammalian host in ecological isolation over long periods of time.

Table 6.4 **Per cent sequence divergence in the E2 gene between various RRV isolates.**

	PB	BH	GG	NL	F	AS	CI	CO	MC
T48	0.63	0.86	0.86	2.68	2.92	3.00	3.00	2.76	2.76
PB		0.23	0.23	3.00	3.39	3.55	3.55	3.31	3.31
BH			0.00	3.23	3.55	3.39	3.39	3.31	3.31
GG				3.23	3.55	3.39	3.39	3.31	3.31
NL					0.70	0.70	0.70	0.70	0.70
F						0.70	0.70	0.15	0.15
AS							0.00	0.15	0.15
CI								0.15	0.15
MC									0.00

RRV isolates are as in Table 6.2.

Nucleotide sequence divergence was calculated using the number of nucleotide differences between the two isolates (Table 6.2) in 1266 nucleotides long E2 gene.

When amino acid sequences of RRV F213978 and RRV AS213970/RRV CI215398 were compared, a single non-conservative change (Thr→Ala) was found at residue 219 in neutralisation epitope I of E2. This change was probably not selected as a result of antibody selection pressure for the following reasons. First, the pre-epidemic population in the South Pacific had no previous antibodies against RRV (Marshall and Miles, 1984). Second, because the blood meal taken by a mosquito will contain a titer high enough to ensure virus transmission only at the time of peak viraemia when antibody levels are relatively low, and at this stage antigenic variants selected for the resistance to antibody would not represent a significant proportion of the injested virus. And, third, any antigenic variant which may arise during virus replication will be neutralised by the polyclonal antibody made in the vertebrate host.

A possible selective pressure may be exerted by any of the hosts in the transmission cycle. The most common invertebrate vector of RRV in inland Australia is Cx. annulirostris and in coastal Australia Ae. vigilax. The later was present in unusually large numbers in Fiji during the epidemic and could have played a crucial role in establishing RRV infection in Fiji (Marshall and Miles, 1984). Although there were many species of mosquitoes active in the Cook Islands during the epidemic, only Ae. polynesiensis was shown to be transmitting RRV (Rosen et al., 1981). No information exists on the species of mosquito involved in RRV spread in

American Samoa. Thus it is possible that the amino acid change in RRV, as it moved from Fiji to American Samoa and the Cook Islands, was selected as a result of a change in mosquito vector. On the other hand, since the virus is proposed to have moved from Australia to the South Pacific (Marshall and Miles, 1984), it can be speculated that the amino acid change was selected during the process of the virus adaptation to a different human race in the Pacific and thus the difference was not seen in the Fijian RRV isolate which was isolated early in the course of the virus spread, but was observed in the later RRV isolates from American Samoa and the Cook Islands.

6.4.2 **Relationship between RRV isolates obtained from human EPA patients**

RRV was isolated from a human patient for the first time in Fiji during the 1979-80 epidemic (Aaskov *et al*., 1981b). Efforts to isolate RRV from human EPA patients in Australia had failed until 1983 when RRV (MC and CO strains) was isolated from the human serum in Rockhampton (Aaskov *et al*., 1985). In both the South Pacific and the recent Australian EPA cases the first blood sample taken soon after the appearance of disease symptoms contained no detectable anti-RRV antibodies (Rosen *et al*., 1981; Aaskov *et al*., 1985) whereas in previous Australian cases such blood samples contained RRV antibodies (Clarke *et al*., 1973). This similarity in the pattern of pathogenesis in EPA patients in the Pacific and Rockhampton suggested an involvement of the

same or closely related RRV strains in the two epidemics. Sequencing studies showed that RRV CO and RRV MC isolated from two patients in Rockhampton were identical to each other in their E2 and were closely related to the South Pacific isolates of RRV. Thus there was only a single amino acid difference between RRV CO/RRV MC and the South Pacific RRV isolates.

Since RRV CO and RRV MC were closely related to the South Pacific RRV isolates which belonged to genetic type II of RRV (Faragher _et al_., 1985a) it appears that the only viruses isolated from human EPA cases so far belong to genetic type II. An obvious question is whether only genetic type II virus is pathogenic in humans. The answer is almost certainly no, since RRV belonging to genetic type III has frequently been isolated from mosquitoes in Nelson Bay area during several epidemics (Gard _et al_., 1973). Also, RRV PB629 and RRV GG2227 which belong to type I were isolated in the Murray valley region during epidemic years. Although the virus was isolated from mosquitoes and could not be isolated from humans (Clarke _et al_., 1973) it seems probable that it was involved in human pathogenesis.

Since there was a marked difference in the RRV pathogenesis in Australia (before the Rockhampton cases) and the South Pacific (see above) Rosen _et al_. (1981) raised the question of whether the virus isolated in the Pacific was different in its biology from that previously involved in EPA in Australia. Conceivably the virus grew faster, with symptoms

appearing before antibody was produced. Alternatively, the virus was less immunogenic thus inducing antibody poorly. Compared to all non-human RRV isolates examined in the present studies, the South Pacific isolates had differences in the "neutralisation domain" in E2. Such differences could affect viral immunogenicity and growth since this domain has been implicated in virus attachment/entry and virulence (Chapter 3). In this context it is of interest that RRV isolates obtained from different geographical locations in Australia induce host responses to different degrees as judged by relative antibody titers in mice (I.D. Marshall, personal communication).

6.4.3 Strain differentiation in RRV

There are several enzootic strains of RRV which, it is believed, have evolved in isolation over a long period of time in ecological niches (Faragher et al., 1985a). These strains cannot be distinguished by polyclonal antibody in neutralisation tests, although kinetic haemagglutination tests can distinguish some of them, e.g. RRV T48 and RRV NB5092 (Woodroofe et al., 1977). However, certain mabs can distinguish many RRV strains in neutralisation tests. Thus mab T1E7 neutralised RRV T48 with 100-fold greater titer compared to the South Pacific isolates (C. Fernon, personal communication). This indicated that the small differences in neutralisation epitope(s) between different RRV strains were significant. The interesting question, therefore, is whether

Figure 6.8 **Location of amino acid differences in E2 of various natural isolates of RRV.**

Figure is a co-listing of amino acid sequences of E2 of RRV T48, SFV and SIN (from Dalgarno et al., 1983). Gaps have been introduced to maximise homology between sequences. Asparagine-linked potential glycosylation sites are shaded. Overlined amino acids in the SFV or SIN sequence are identical to corresponding amino acids in RRV T48 sequence. Sites of amino acid differences in various natural isolates of RRV used in the studies in this Chapter are indicated by ▼. These were at amino acids 67, 132, 166, 218, 219, 251, 253 or 408 in various RRV isolates (Table 6.3). The putative hydrophobic transmembrane domain is underlined by dots. Amino acid sequence data for SFV and SIN are from Garoff et al. (1980b) and Rice and Strauss (1981) respectively.

```
RRV                                                          SVTEHFNVYKATRPYLAYCADCGDGYFCYSPVAIEKIRDEASDGMLKIQVSAQIG   ( 55)
SFV                                                          SVSQHFNVYKATRPYIAYCADCGAGHSCHSPVAIEAVRSEATDGMLKIQFSAQIG   ( 55)
SIN                                                          SVIDGFT    LTSPYLGTCSYCHHTEPCFSPVKIEQVWDEADDNTIRIQTSAQFG   ( 52)

RRV  LDKAGTHAHTKIRYM    AGHDVQESKRDSLRVYTSAACSIHGTMGHFIVAHCPPGDYLKVSFEDADSHVKACKVQYKHDPLPVGREKFVVRPHFGVELP   (152)
SFV  IDKSDNHDYTKIRYA    DGHAIENAVRSSLKVATSGDCFVHGTMGHFILAKCPPGEFLQVSIQDTRNAVRACRIQYHHDPQPVGREKFTIRPHYGKEIP   (152)
SIN  YDQSGAASANKYRYMSLKQDHTVKEGTMDDIKISTSGPCRRLSYKGYFLLAKCPPGDSVTVSIVSS NSATSCTLARKIKPKFVGREKYDLPPVHGKKIP   (151)

RRV  CTSYQLTTAPTDEGIDMHTPPDIPDRTLLSQTAGNV KITAGGRTIRYNCTCGRDNVGTTSTDKTINTC KIDQCHAAVTSHDKWQFTSPFVPRADQTAR   (250)
SFV  CTTYQQTTAETVEEIDMHMPPDTPDRTLLSQQSGNV KITVGGKKVKYNCTCGTGNVGTTNSDMTINTC LIEQCHVSVTDHKKWQFNSPFVPRADEPAR   (250)
SIN  CTVYDRLKETTAGYITMHRPRPHAYTSYLEESSGKVYAKPPSGKNITYECKCGDYKTGTVSTRTEITGCTAIKQCVAYKSDQTKWVFNSPDLIRHDDHTA   (251)

RRV  RGKVHVPFPLTNVTCRVPLARAPDVTYGKKEVTLRLHPDHPTLFSYRSLGAEPHPYEEWVDKFSERIIPVTEEGIEYQWGNNPPVRLWAQLTTEGKPHGW   (350)
SFV  KGKVHIPFPLDNITCRVPMAREPTVIHGKREVTLHLHPDHPTLFSYRTLGEDPQYHEEWVTAAVERTIPVPVDGMEYHWGNNDPVRLWSQLTTEGKPHGW   (350)
SIN  GGKLHLPFKLIPSTCMVPVAHAPNVIHGFKHISLQLDTDHLTLLTTRRLGANPEPTTEWIVGKTVRNFTVDRDGLEYIWGNHEPVRVYAQESAPGDPHGW   (351)

RRV  PHEIIQYYYGLYPAATIAAVSGASLMALLTLAATCCMLATARRKCLTPYALTPGAVVSLTLGLLCCAPRANA                              (422)
SFV  PHQIVQYYYGLYPAATVSAVVGMSLLALISIFASCYMLVAARSKCLTPYALTPGAAVPWTLGILCCAPRAHA                              (422)
SIN  PHEIVQHYYHRHPVYTILAVASATVAMMIGVTVAVLCACKARRECLTPYALAPNAVIPTSLALLCCVRSANA                              (423)
```

the small amino acid sequence differences seen in various RRV isolates are responsible for the strain specific antigenic and biological differences. We have plotted the locations of amino acid differences in E2 between the various RRV isolates (Figure 6.8). RRV neutralisation epitopes are located in, or immediately adjacent to, the stretches of amino acids not conserved between RRV and SFV (Chapter 2). In the natural RRV isolates five out of eight differences occurred in stretches which were conserved between RRV T48 and SFV, three were in non-conserved stretches. It would appear that while RRV and SFV may be distinguished by a markedly different amino acid sequence in their neutralising epitopes, RRV strains may have minor conformational differences brought about by small change(s) within the epitope or outside in the conserved sequence in E2. This is supported by the observation that mab T1E7, raised against RRV T48, did not neutralise SFV, however, it neutralised different RRV strains, but to a different extent (see above).

Four out of eight RRV strain specific amino acid differences were found in the "neutralisation domain" which represents ~15% of E2, suggesting that the domain is particularly prone to vary in different RRV strains. Earlier we have suggested that the changes in the "neutralisation domain" may not be selected due to the antibody pressure. However, in Chapter 3 evidence was presented for the importance of this domain in virus-cell interaction. Therefore such a domain should be capable of accomodating changes necessary for

virus adaptation to a new host. It can be speculated that the changes in the "neutralisation domain" may have been selected due to host selection pressure and thus the strain specific differences may give the virus an advantage in a particular host.

6.4.4 Nucleotide sequence divergence and genetic typing

Based on similarities and differences in HaeIII and TaqI restriction digest profiles Faragher et al. (1985a) placed RRV T48, RRV GG2227, RRV PB629 in genetic type I and RRV NL26301, RRV F213978, RRV AS213970, RRV CI215398 and RRV BH38091 in genetic type II.

The sequencing studies reported here show < 1% nucleotide sequence divergence between the E2 genes of RRV T48, RRV GG2227, RRV PB629 and RRV BH38091 on the one hand and between the E2 genes of RRV F213978, RRV AS213970, RRV CI215398, RRV CO and RRV MC on the other (Table 6.4). There was, however, 2.6-3.3% sequence divergence between the two groups (Table 6.4). Thus, the genetic typing of Faragher et al. (1985a) is consistent with the results based on E2 sequences. Since RRV BH38091 showed only 0.86% sequence divergence from RRV T48 (genetic type I) and was identical to RRV GG2227 (genetic type I) in E2 we propose that it belongs to the genetic type I of Faragher et al. (1985a).

From HaeIII restriction digest profiles of virus infected BHK cell RNA, S.G. Faragher (personal communication) calculated a 5.6% nucleotide sequence divergence between RRV

T48 and the South Pacific RRV isolates. However, based on the E2 gene sequence both RRV CO/RRV MC and the South Pacific RRV isolates had ~3% sequence divergence from RRV T48. Since E2 is the most variable structural gene in alphaviruses (Dalgarno et al., 1983) this figure should be the maximum extent of sequence divergence assuming that the non-structural protein gene sequences are highly conserved due to their functional requirements. HaeIII restriction profiles "look at" the whole genome which in addition to E2 contains less divergent genes such as E1 and the non-structural genes. The 3'-untranslated region is very low in GC and thus will not "register" many bands in the restriction profile. Therefore HaeIII restriction digest analysis will be expected to give an estimate of divergence which is perhaps less than that indicated by sequencing E2 gene. However estimates of Faragher (1982) based on HaeIII restriction profiles gave figures which were significantly greater than those obtained from the E2 sequences. Meek (1986) using the purified virion RNA and the method of Faragher et al. (1985a) estimated a 2.2% nucleotide sequence divergence between RRV T48 and RRV NB5092. This was close to the 2.38% nucleotide sequence divergence as calculated by direct comparison of the 26S RNA sequences of the two strains (Meek, 1986). Thus a reason for the big difference between the estimates of S.G. Faragher (see above) and those reported in this Chapter is not clear but it may be related to the problems associated with the reproducibility of the restriction digestion analysis method when used with the infected cell RNA (Faragher et al., 1985b).

CHAPTER 7

CONCLUSION

A major aim of the studies described in this thesis was to determine the location of RRV neutralisation epitopes in the primary amino acid sequence of the relevant structural protein. Using neutralising mabs, three neutralisation epitopes were identified around amino acids 216 (epitope I), 232-234 (epitope II) and 246-251 (epitope III) in E2. The three epitopes were each located about 15 amino acids apart in a 35 amino acid long stretch between the two predicted glycosylation sites in E2. Hydropathy analysis of RRV E2 showed that while epitope I was located in a moderately hydrophilic region of E2, epitopes II and III were in the two most hydrophilic regions. These two regions were conserved as to position and hydrophilicity between RRV, SFV and SIN. The neutralisation epitopes were located in stretches of amino acids which were not conserved between RRV, SFV and SIN or were immediately adjacent to them. This indicates that the fine structure of the neutralisation epitopes is different between different alphaviruses although the conservation of all cysteine residues in RRV, SFV and SIN E2 proteins predicts that the basic structural features of the neutralisation antigen will be similar between the three alphaviruses. The location of these epitopes was the same in mouse-virulent RRV T48 and in avirulent RRV NB5092.

The relationship between the three epitopes was examined by cross-neutralisation tests which showed that the variants altered in one epitope were neutralised by mabs interacting with a different epitope thus suggesting that the three epitopes can act independently in neutralisation.

However sequencing of two multi-epitope variants, obtained by sequential selection with different mabs, showed that a change in epitope I led to a restriction on the type of changes that could be introduced in epitopes II and III indicating that the three epitopes may be conformationally connected. To conclusively establish this point, different multi-epitope variants obtained using different mabs in various combinations need to be sequenced.

For SFV and SIN, neutralisation epitopes have been found in E1 as well as in E2 (Chapter 1). In the present study no neutralisation epitopes were found in RRV E1. This may be due to the small number of mabs used in these studies. However, it appears that the three epitopes defined in RRV E2 represent the immunodominant neutralisation domain. This is reflected in the observation that all six neutralising mabs obtained from five independent fusions interact with this domain and that polyclonal antiserum raised against RRV T48 neutralises a variant altered in all three epitopes with lower efficiency compared to RRV T48 (P. Kerr, personal communication). If these three epitopes do represent the immunodominant site a synthetic peptide or fusion protein harbouring them could be used to examine its potential as a synthetic vaccine. A similar approach may be useful in developing control measures against more life threatening alphaviruses.

Another aim of the work described in this thesis was to examine aspects of the role of E2 in the process of RRV

infection. To this end the biological properties of RRV mutants were examined. RRV T48 antigenic variants which had single amino acid changes in epitopes I or II showed no significant differences in growth, or in RNA or protein synthesis in BHK cells compared to RRV T48; none of these variants showed a change in virulence or tissue tropism in mice. However, single amino acid changes in epitope III resulted in a slight retardation of growth and of RNA and protein synthesis in BHK cells. These variants "penetrated" BHK cells more rapidly than did RRV T48. Certain of the epitope III variants had a slight, but significant, increase in LD_{50} or AST for week-old mice; none of these variants showed any significant difference in growth in week-old mice when compared with RRV T48 except one variant which produced lower levels of viraemia.

Compared to the effect of the point mutations in E2 on the biological properties of RRV, a seven amino acid deletion in E2 of RRV dE2 resulted in a major alteration in virulence and tissue tropism in mice. The deletion mutant was only slightly attenuated in day-old mice but was totally avirulent for week-old mice. In week-old mice the mutant grew to titers which were significantly lower than those for RRV T48; in hind leg muscle the difference was approximately 2 log units whereas in brain the difference was ~5 log units thus indicating a change in tissue tropism.

BHK cells infected with RRV dE2 had a significantly increased rate of viral RNA synthesis than in RRV T48-infected

cells. It is difficult to envisage a mechanism which could explain how a deletion in a structural protein could affect the rate of RNA synthesis. The possibility that change(s) in the non-structural protein genes of RRV dE2 have taken place must be considered, although comparison of HaeIII and TaqI restriction profiles of cDNA to RRV dE2 and RRV T48 RNA showed no evidence of a difference other than the deletion in the E2 gene of RRV dE2 (S.G. Faragher, unpublished data).

Although the deletion in RRV dE2 was between amino acids 55-61 of E2 and was approximately 150 amino acids distant from the "neutralisation domain" in the primary amino acid sequence, this seems to have affected the conformation of this domain since mab NB3C4 gave a significantly lower neutralisation titer with RRV dE2 than with RRV T48.

Results of studies with mab-resistant variants and with the deletion mutant suggest that part or all of the domain of E2 containing the neutralisation epitopes is involved in virus-cell interaction and may be part of a receptor binding site. If this is so a change in conformation of a neutralisation epitope could alter the conformation of the receptor binding site leading to an altered recognition of the cellular receptor. This, in turn, could result in a change in cell/tissue tropism in mice or in an altered rate of attachment and penetration of virus into cells. This hypothesis is supported by the recent observation of Meek (1986) that seven out of the eight mouse-passaged virulence variants of RRV NB5092 examined had one or two amino acid changes in the

"neutralisation domain". Compared to RRV NB5092 these variants had lower LD_{50}s in mice and reached higher titers in blood and brain with little difference in hind leg muscle titers thus indicating a change in tissue tropism. However, the virulence of RRV can apparently be affected by changes elsewhere in the genome other than in the E2 gene. This was indicated by the studies reported in Chapter 5 on BHK-passaged variants of RRV T48 which had reduced virulence and altered tissue tropisms in mice although no mutations were found in the E2 gene or any other structural protein genes. Since these mutants had an increased rate of AMD-resistant RNA synthesis in BHK cells it appears likely that mutations had occurred in the non-structural protein genes although attempts to identify them by sequencing were not made. Consistent with this view virulent variants have been isolated from mouse-passaged RRV NB5092 which have no alterations in E2 but differ from parental virus in their non-structural protein genes as detected by restriction analysis of double-stranded cDNA to virion RNA (S.G. Faragher, personal communication). The site of these mutation(s) in the non-structural protein genes has not yet been identified by sequencing.

Another point which emerges from our studies on growth of RRV dE2 and BHK-passaged RRV T48 variants in mice is that a decreased replication of virus in brain correlates directly with reduced virulence. This suggests that viral replication in brain is an important determinant of RRV virulence in mice.

In the last section of this thesis the evolution of RRV E2, the neutralisation antigen, during epidemic was examined by comparing the E2 gene sequences of RRV isolates obtained during the virus spread in a non-immune population in the South Pacific. The advantage of this system was that the introduction and spread of the virus in the population has been well documented and RRV isolates obtained at different stages of the progress of the epidemic were available. These studies showed that during virus spread RRV evolved far more slowly than did polio virus during a similar spread in an unvaccinated religious community. The slower rate of RRV evolution may be attributable to a difference in the transmission cycle of the two viruses. During the RRV spread in the South Pacific a single amino acid change in E2 was the only change selected; this was in epitope I and may be related to a change in the host(s) involved in virus spread. This is consistent with our hypothesis that the "neutralisation domain" or a part of it is involved in virus-cell interaction.

During recent EPA cases in Rockhampton patients showed a pattern of disease symptoms similar to that seen during the South Pacific outbreak. Comparison of the E2 gene sequences of the Rockhampton RRV isolates with those from the South Pacific showed that the two were closely related although they were not identical.

In an attempt to explore strain related differences in RRV E2 the positions of amino acid changes were examined in a small number of RRV natural isolates. It was observed that

while the type specific sites (i.e. neutralisation epitopes) were located in stretches of amino acids which were not conserved between RRV, SFV and SIN or immediately adjacent to them, the strain related amino acid differences may frequently appear in sequences which were conserved between the three alphaviruses. Thus various strains of RRV may still be neutralised by the type specific antibodies but to a different extent owing to small differences in conformation of the epitope(s) brought about by the small change(s) in the amino acid sequence.

In summary this thesis is the first report on the location of the neutralisation epitopes of an alphavirus and on the evolution of an alphavirus neutralisation antigen during an epidemic at the nucleotide sequence level. Studies on RRV mutants were carried out to understand aspects of the biological role of E2, the neutralisation antigen. These studies suggested a role for E2 in virus-cell interaction and in determining tissue tropism and virulence although further work is required in these later areas.

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Ross River Virus Mutant with a Deletion in the E2 Gene: Properties of the Virion, Virus-Specific Macromolecule Synthesis, and Attenuation of Virulence for Mice

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A mutant of RRV T48 the prototype strain of Ross River virus has been isolated with a 21-nucleotide deletion in the gene coding for the envelope glycoprotein E2. Direct sequencing of the 26 S subgenomic RNA, together with *Hae*III and *Taq*I restriction digest analysis of cDNA to RNAs from cells infected with the mutant virus (RRV dE2) and with RRV T48, were consistent with the deletion being the only major alteration in the mutant genome. The E2 protein of RRV dE2 virions had a higher electrophoretic mobility than that of RRV T48 E2 protein. Neither RRV dE2 nor RRV T48 virions contained more than trace amounts of E3, the small envelope glycoprotein found in Semliki Forest virus. RRV dE2 generated small plaques on Vero cell monolayers; plaque formation was not temperature-sensitive between 32 and 41°. By comparison with RRV T48 the infectivity of RRV dE2 virions was thermolabile at 50°.

In BHK cells RRV dE2 grew with similar kinetics to RRV T48. Rates of synthesis of 26 S RNA and 49 S RNA were higher in cells infected with RRV dE2 than in cells infected with RRV T48. Virus-specific protein synthesis and shut-down of host protein synthesis occurred 2–3 hr earlier in RRV dE2-infected cells than in cells infected with RRV T48. Minor differences between the two viruses were observed in the profiles of virus-specific proteins generated in infected cells. In day-old mice RRV dE2 induced less severe symptoms of hind leg paralysis than did RRV T48. A small increase in LD_{50} and average survival time was observed in RRV dE2-infected mice by comparison with RRV T48 infected mice. Peak titers reached by RRV dE2 in the hind leg muscle, brain, and blood of day-old mice were 3–4 log units less than the titers reached during infection with RRV T48. In week-old mice the differences in virulence between the two strains were magnified: RRV dE2 induced no detectable symptoms even when injected at high doses (8×10^6 PFU) whereas the LD_{50} and average survival time for RRV T48 were unchanged from those in day-old mice. Peak RRV dE2 titers in hind leg muscle, brain, and blood, respectively, were 2, 5, and 5 log units less than the corresponding titers for RRV T48. Peak muscle titers reached by RRV dE2 were similar ($\sim 10^8$ PFU/g tissue) in day-old mice where lethality was high and in week-old mice where the virus was avirulent. In brain tissue, peak RRV dE2 titers were 10^7 and 10^4 PFU/g, respectively, in day-old and week-old mice. Since the corresponding figures for RRV T48 were 10^{11} and 10^9 PFU/g, the outcome of RRV infection was probably determined more by the extent of replication in neural tissues than in muscle tissues.
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INTRODUCTION

The alphavirus virion contains a single-stranded RNA molecule (49 S RNA), approximately 240 capsid protein subunits and equimolar amounts of two or three envelope glycoproteins [E1, E2, and, in the case of Semliki Forest virus (SFV), E3] which comprise the surface "spikes" of the virion (Simons *et al.*, 1980). The envelope proteins either individually or together, are required for the infectivity of the virus particle (Bose *et al.*, 1970; Igarashi *et al.*, 1970). The E1 protein is a group-specific antigenic determinant (Porterfield, 1980)

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and is responsible for hemagglutination (Dalrymple *et al.*, 1976; Helenius *et al.*, 1976), hemolysis (Yamamoto *et al.*, 1981) and probably plays a role in mediating virus fusion with intracellular membranes during penetration (Garoff *et al.*, 1980; Rice and Strauss, 1981a). The E2 protein carries antigenic determinants which recognize neutralizing antibody (Dalrymple *et al.*, 1976) and may also play a role in the early events in infection by the recognition of cell surface receptors (Baric *et al.*, 1981). The role if any of E3 is unknown.

In the course of determining the nucleotide sequence of the 26 S subgenomic RNA of the alphavirus Ross River virus (RRV), Dalgarno *et al.* (1983) obtained evidence which predicted the existence, in the virus stock, of a mutant with a deletion of 21 nucleotides in the E2 gene. For these sequence studies, viral RNA was reverse transcribed into single-stranded cDNA, 5′ end-labeled, digested with enzymes capable of cleaving single-stranded DNA (e.g., *Taq*I, *Hae*III) and sequenced by the chemical method of Maxam and Gilbert. Two *Taq*I fragments, 243 and 222 nucleotides in length, had identical 5′ and 3′ termini but differed in the presence or absence of 21 internal nucleotides. Since such a deletion mutant could provide valuable further data on the role of E2, attempts were made to characterize it further.

In the present communication we describe the isolation of the predicted mutant, report on some of the physical properties of the virion, examine the replication of the virus in tissue culture cells, and describe the growth and virulence of the virus in mice.

MATERIALS AND METHODS

Virus. The T48 strain of RRV (Berge, 1975) was obtained from the Yale Arbovirus Research Unit. The virus had been passaged approximately 10 times in mice when received. Virus was assayed by plaque formation on Vero cell monolayers (Newton *et al.*, 1981). Cell associated virus was assayed after sonication of cell pellets at 0° for 15 sec.

Cell cultures. BHK-21 cells were grown in Eagle-BHK medium and Vero cells were grown in M199-LAH medium (Newton *et al.*, 1981). Primary mouse cells were obtained by collagenase treatment of skin or muscle tissues obtained from an outbred strain of Walter and Eliza Hall Institute (WEHI) mice.

Labeling of cells. Cell- and virus-specific proteins were labeled and cell extracts prepared and electrophoresed as described by Dalgarno *et al.* (1984). Rates of viral RNA synthesis were estimated by incubation of cell monolayers with [5-^{3}H]-uridine (10 μCi/ml) for 2 hr in Eagle's minimum essential medium. When required actinomycin D was added to 5 μg/ml 10 min before isotope addition (Newton *et al.*, 1981). After labeling, monolayers were dissociated, precipitated on glass fiber disks, and counted for radioactivity (Newton *et al.*, 1981).

Purification of virus and viral RNA. RRV T48 and RRV dE2 were purified from clarified BHK tissue culture supernatant fluids (adjusted to 2.3% w/v, NaCl) by precipitation with polyethylene glycol (7% w/v; Martin *et al.*, 1979). Virion RNA was extracted from pelleted peak fractions as previously described (Ou *et al.*, 1981). Intracellular 26 S RNA was isolated from RRV dE2-infected BHK cells as described by Dalgarno *et al.* (1983).

cDNA synthesis, restriction enzyme digestion, and polyacrylamide gel electrophoresis. cDNA to virion RNA was synthesized using reverse transcriptase in the presence of short calf thymus DNA primers as previously described (Faragher *et al.*, 1985). Radiolabeled, single-stranded cDNA was digested with *Hae*III or *Taq*I and electrophoresed on polyacrylamide gels as described by Faragher *et al.* (1985).

Sequencing of viral RNA. Virion RNAs were sequenced by the dideoxy-nucleotide method using as primer a synthetic 17-residue oligodeoxynucleotide (5′ ACTTCT-CTCTACCCACC 3′) complementary to nucleotides 1455–1471 of RRV T48 26 S RNA (Dalgarno *et al.*, 1983). Reaction mixtures contained, in a final volume of 10 μl, 50 mM Tris (pH 8.3), 8 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithiothreitol, 50 μM dATP, TTP, and dGTP, 2.5 μM dCTP, 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 10 μM ddATP, 5 μM ddTTP, 2 μM ddGTP, or 0.25 μM ddCTP, 0.8 μg virion RNA, $\simeq$0.001 μg primer, and

12 units of AMV reverse transcriptase. Before addition, mixtures of template RNA and primer were heated at 56° for 5 min and quick-chilled on ice-water, and KCl was added to 80 m*M*. Incubation of reaction mixtures was at 0° for 20 min, followed by 60 min at 42.5°. One microliter of a solution which was 0.5 m*M* in all dNTPs was then added and incubation continued at 42.5° for 20 min. Reactions were stopped by addition of 4 vol of formamide–dye mix (96% deionized formamide; 25 m*M* Tris–borate; 0.5 m*M* Na_2 EDTA; xylene cyanol and bromophenol blue dyes), followed by heating at 90° for 2 min. Reaction products were separated on 80-cm-long, 0.4-mm-thick 5% polyacrylamide gels (acrylamide:bisacrylamide, 19:1). Gels were exposed at −20° to Fuji RX medical X-ray film.

Monoclonal antibodies. Neutralizing monoclonal antibodies to RRV T48 were obtained from hybridoma cells generated by fusion of spleen cells from hyperimmunized BALB/c mice with X63 myeloma cells according to the method of Fazekas de St. Groth and Scheidegger (1981).

Estimation of virulence in mice. WEHI mice of either sex were used. For LD_{50} determinations 30-μl samples of 10-fold virus dilutions in Hanks' balanced salt solution (HBSS) were inoculated intraperitoneally (ip) into 1- or 7-day-old mice, using 8–10 litter-mates per dilution. LD_{50}s were calculated according to Reed and Muench (1938). Clinical signs and mortality were recorded daily for 2 weeks. Subclinical infections were assayed in mice surviving to the 14th day by challenging with 10^5 Vero PFU of virulent RRV T48 inoculated ip. Mice were held for a further 2 weeks. The absence of clinical signs was taken as evidence of prior infection. Average survival times (AST) represent the arithmetic mean for all mice dying during the course of a titration regardless of dose, since for most RRV strains the time of death is independent of the dose (Taylor and Marshall, 1975).

Virus growth in mice. WEHI mice of either sex were injected subcutaneously with 100 Vero PFU of virus. Groups of three mice were killed at 24-hr intervals by cervical dislocation; tissues were removed and stored at −70°. Blood was collected from the heart using heparinized capillary tubes and diluted 1:10 in ice-cold HBSS before freezing at −70°. For virus assay, tissues were homogenized as a suspension in ice-cold HBSS using a Teflon–glass homogenizer.

RESULTS

Isolation of a Mutant of Ross River Virus with a 21-Nucleotide Deletion in the E2 Gene

In the course of plaque assays on the virus stock used in sequencing RRV T48 26 S RNA (Dalgarno *et al.*, 1983), uniformly small plaques (≃1.5 mm diameter) were detected on Vero cell monolayers incubated for 4 days. The small plaque former was plaque-purified twice and examined for reversion to wild-type plaque size (≃4 mm diameter). No revertants were observed in several thousand small plaques. To determine whether the small plaque former was the E2 deletion mutant predicted from previous sequencing studies (Dalgarno *et al.*, 1983), intracellular 26 S RNA from BHK cells infected with the small plaque variant and 49 S virion RNA isolated from plaque-purified RRV T48 were sequenced in the appropriate region of the E2 gene using the dideoxynucleotide method. Twenty-one nucleotides (5′GG-UCUGGACAAGGCAGGUACC-3′), representing nucleotides 1213 to 1233 of RRV T48 26 S RNA were absent from the corresponding region of the small plaque virus genome (Fig. 1). These nucleotides code for amino acids 55–61 of E2. RRV E2 is 422 amino acids long; since the predicted hydrophobic domain which anchors E2 in the membrane includes residues 354–391 and the predicted cytoplasmic "tail" is represented by residues 395–418 (Dalgarno *et al.*, 1983) it is likely that the deleted region is in the external portion of E2. It can also be noted that neither of the glycosylation sites in E2 (see below) are removed by the deletion. Since no other sequence heterogeneity was observed in RRV T48 26 S RNA (Dalgarno *et al.*, 1983) we conclude that the 21-nucleotide deletion is the only difference between RRV T48 and the mutant virus in the 26 S region of the genome, i.e., that

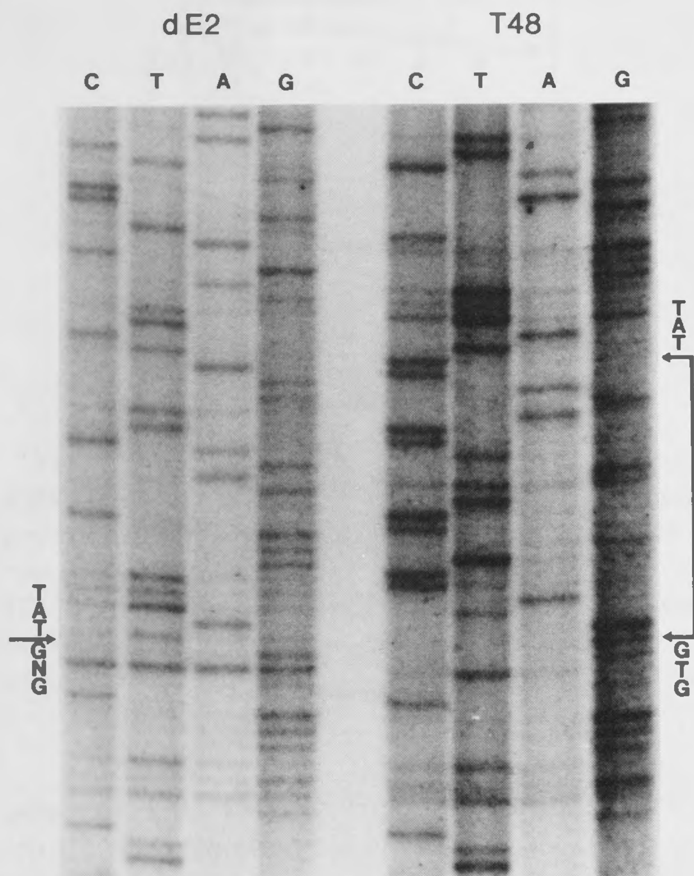

FIG. 1. Sequence of RRV dE2 cDNA in the region of the deletion. Virion RNA from purified RRV T48 or intracellular 26S RNA purified from RRV dE2-infected BHK cells was sequenced by the dideoxynucleotide method using as primer a synthetic oligodeoxynucleotide complementary to nucleotides 1455–1471 of RRV T48 26 S RNA (Dalgarno *et al.*, 1983). The 21 nucleotides enclosed between the arrows on the RRV T48 sequence are missing from the RRV dE2 sequence at the indicated position (arrow). Three nucleotides on either side of the deleted sequence are indicated. N, ambiguous nucleotide; dE2, sequence of RRV dE2 cDNA; T48, sequence of RRV T48 cDNA.

coding for the structural proteins. The mutant virus is referred to below as RRV dE2.

To determine whether RRV dE2 was altered in the non-26 S region of the genome, high molecular weight RNA was isolated from BHK cells infected with RRV T48 or with RRV dE2 or was isolated from mock-infected cells (Faragher *et al.*, 1985). RNA was transcribed into single-stranded cDNA using random calf thymus DNA primers, digested with *Taq*I or *Hae*III, and the digests were electrophoresed on a polyacrylamide gel (Faragher *et al.*, 1985).

In the *Taq*I restriction digests (Fig. 2), a cDNA fragment of approximately 243 nucleotides in the RRV T48 profiles was replaced by one of 222 nucleotides in the RRV dE2 profiles. This would be expected from the positions of the established *Taq*I re-

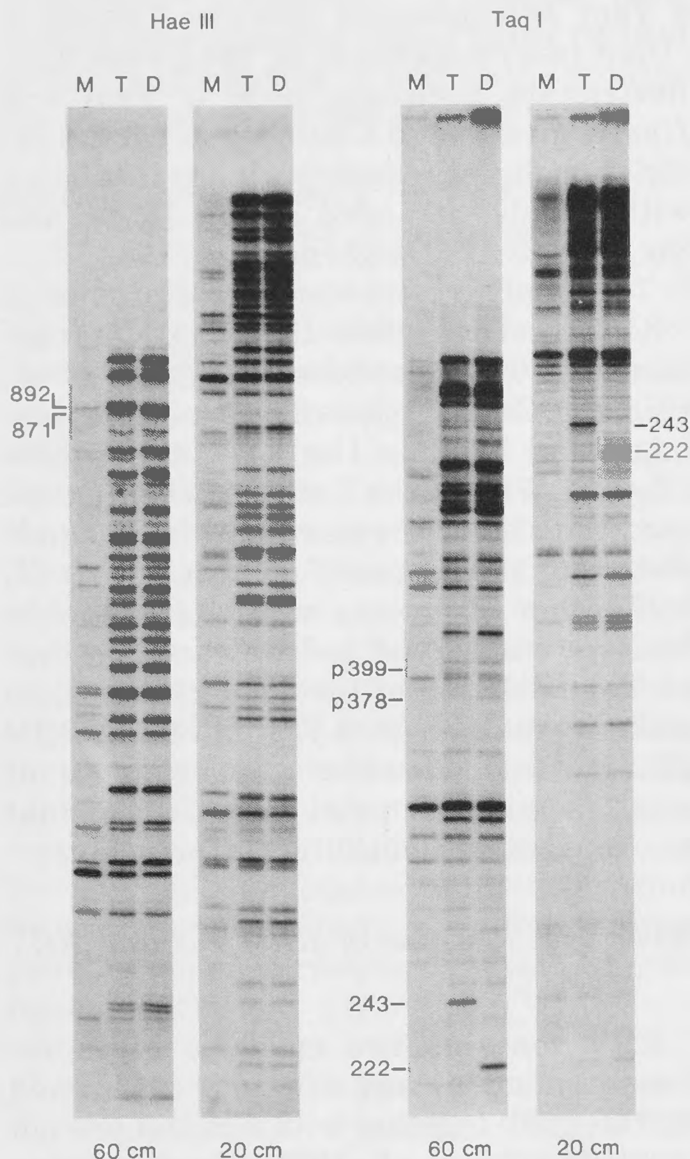

FIG. 2. *Hae*III and *Taq*I restriction digest profiles of cDNA synthesized against total RNA isolated from RRV T48-infected cells, RRV dE2-infected cells, and mock-infected cells. BHK cell monolayers were mock infected (M) or infected with wild-type RRV strain T48 (T) or deletion variant (D) (m.o.i. = 3.0). Infected-cell RNA or mock-infected cell RNA was extracted 16 hr p.i. Single-stranded cDNA, labeled with [α-^{32}P]dCTP was synthesized using random primers, and digested with *Hae*III or *Taq*I. Restriction fragments were separated on a 40-cm long 5% polyacrylamide gel on which the xylene cyanol marker was run for 60 or 20 cm, as indicated. Lengths (in nucleotides) of deletion-containing and the corresponding wild-type fragments, predicted from *Hae*III and *Taq*I restriction maps of RRV 26 S cDNA (Dalgarno *et al.*, 1983), are indicated. Estimates of fragment lengths from the known sizes of sequenced *Hae*III fragments of RRV 26 S cDNA (Dalgarno *et al.*, 1983) closely approximated the predicted lengths. Partial digestion products are denoted by "p."

striction sites in RRV T48 26 S cDNA (Dalgarno *et al.*, 1983). The fragments of 399 and 378 nucleotides (Fig. 2) probably represent partial *Taq*I digestion products resulting from occasional failure to cleave at a *Taq*I site (position 1394) in RRV 26 S cDNA (see Dalgarno *et al.*, 1983; Figs. 2, 4). Restriction nucleases such as *Taq*I and *Hae*III give rise to a number of partial restriction digest fragments when incubated with single stranded DNA (Rice and Strauss, 1981b; Dalgarno *et al.*, 1983).

The *Hae*III restriction digest profiles of RRV T48 cDNA show that a cDNA fragment of 892 nucleotides (Dalgarno *et al.*, 1983; Fig. 2) was replaced by a more rapidly migrating band in the RRV dE2 profile (Fig. 2). Within the limits of the method used the size of the new band is that predicted for the proposed mutant.

No other fragments with altered mobilities were observed and we conclude that no large deletion or insertions have taken place in the non-26 S region of the RRV dE2 genome. However the existence of small deletions or point mutations cannot be excluded without direct sequencing.

Structural Proteins of RRV T48 and RRV dE2

RRV contains two envelope glycoproteins of nominal mol wt 52,000 (gp52) and 49,000 (gp49) together with a capsid protein (p32) (Martin *et al.*, 1979). To determine which of the two RRV envelope glycoproteins is E2 we have carried out pulse–chase experiments and shown that [^{35}S]methionine from a protein of approximate mol wt 62,000 (PE2) can be "chased" into gp49 (N. J. Short and L. Dalgarno, unpublished data). We conclude that gp49 is E2.

E2 from RRV dE2 virions migrated with a slightly greater electrophoretic mobility than did E2 from RRV T48 virions (Fig. 3A). E2 and PE2 from RRV dE2-infected cells also showed slightly greater mobilities than the corresponding proteins from RRV T48-infected cells (Fig. 3B). The altered mobilities of E2 and PE2 could be due to conformational changes brought about by the deletion of seven amino acids or by an altered pattern of glycosylation. There are two potential asparagine-linked glycosylation sites in RRV E2 at amino acids 200 and 262 (Dalgarno *et al.*, 1983).

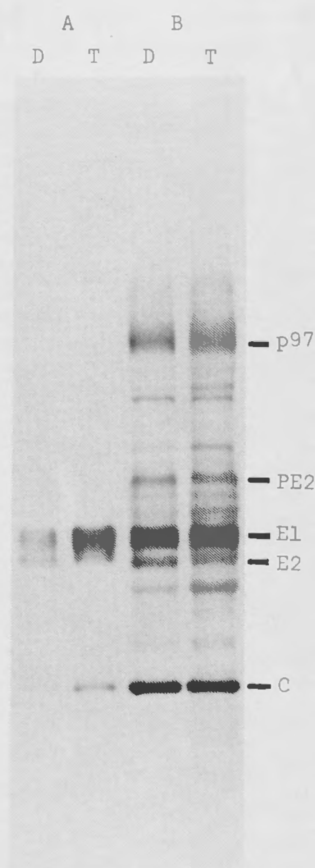

FIG. 3. RRV proteins in virions and infected cell extracts. (A) RRV T48 and RRV dE2 were purified in parallel (see under Methods). For labeling virus, cells were grown in Eagle's minimum essential medium (EMEM) containing one-tenth the normal amino acid concentration and 10 μCi/ml of ^{3}H-amino acids. Purified virus was disrupted in 1% sodium dodecyl sulfate (SDS) and electrophoresed on 10–20% gradient acrylamide gel and fluorographed. (B) BHK cells were infected (m.o.i. $\simeq$ 3) with RRV T48 or RRV dE2 and incubated in EMEM containing ^{3}H-amino acids (25 μCi/ml). At 24 hr p.i. the cell monolayer was dissolved in 1% SDS and electrophoresed as above. The position of viral proteins p97, PE2, E1, E2, and C are indicated. D, RRV dE2; T, RRV T48.

To determine whether E3 is present in RRV T48 and in RRV dE2, H^{3}-amino acid-labeled RRV T48, RRV dE2, and Semliki Forest virus were purified in parallel and the viral proteins examined on a polyacrylamide gel under reducing conditions. RRV T48 and RRV dE2 virions contained no more than trace amounts of E3; a protein of nominal molecular mass 13,000 Da was present in the corresponding extracellular fluids (data not shown). SFV virions contained E3 and no E3 was detected in SFV tissue culture fluids (data not

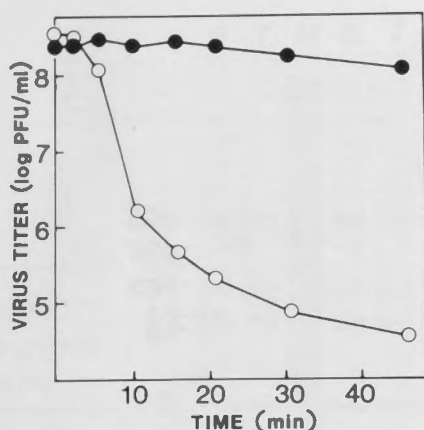

FIG. 4. Thermolability of RRV dE2, RRV dE2, or RRV T48 were incubated separately in BHK cell growth medium at 50°. At intervals samples were removed and immediately diluted 10-fold into ice-cold HBSS before assay for infectivity by plaque formation on Vero cell monolayers. ●, Titer of RRV T48; ○, titer of RRV dE2.

shown). Our results for SFV confirm previous reports (Garoff *et al.*, 1974).

Thermal Instability of RRV dE2

RRV dE2 showed a marked sensitivity to heat inactivation by comparison with RRV T48. After 30 min at 50° the titer of RRV dE2 was lowered by more than 3 log units whereas that of RRV T48 was unaffected (Fig. 4). At 37° no difference in stability between RRV T48 and RRV dE2 was detected.

Temperature Sensitivity of Plaque Formation

Plaque formation by RRV dE2 and RRV T48 was compared at 28, 30, 32, 34, 36, 37, 39, and 41°. For both RRV T48 and RRV dE2, maximum titers were recorded at 36°. There was a rapid decrease in the efficiency of plaque formation for both viruses above 36° and a more gradual decrease below 36°. No differential changes in efficiency of plaque formation or in plaque size were observed for the two viruses as the temperature was altered (data not shown).

Growth in BHK Cells and Primary Mouse Cells

The growth kinetics of RRV dE2 and RRV T48 were compared in BHK cells and in primary mouse muscle and skin cells. No significant differences were observed in the levels of extracellular virus produced in three experiments in which BHK cells were infected (m.o.i. $\simeq$ 0.001 or 1) and primary mouse cells were infected (m.o.i. $\simeq$ 3.5) (data not shown). In infected BHK cells there was no difference in the ratio of cell associated virus to extracellular virus for the two viruses (data not shown).

Rates of Penetration of RRV dE2 and RRV T48 in Vero Cell Monolayers

To compare the rates of penetration of RRV dE2 and RRV T48, Vero cell monolayers in 60-mm dishes were inoculated with approximately 100 PFU of virus and incubated at 37° for 10, 20, 30, 40, 50, and 60 min followed by the addition of polyclonal RRV immune ascitic fluid for 10 min (Baric *et al.*, 1981). After washing to remove unattached antibody, overlay was added and plates incubated for plaque development. Experiments were carried out in triplicate. RRV dE2 and RRV T48 gave virtually identical profiles of virus uptake (data not shown). In control experiments, certain mutants of RRV selected on the basis of their resistance to neutralization by monoclonal antibody, were slow in their penetration of Vero cells (S. Vrati, unpublished data).

Virus-Specific RNA Synthesis in Infected BHK Cells

Virus-specific RNA synthesis was higher in RRV dE2-infected BHK cells than in cells infected with RRV T48 (m.o.i. $\simeq$ 3). For RRV T48 and RRV dE2, virus-specific (actinomycin-resistant) RNA synthesis at the time of maximal RNA synthetic rate (8–10 hr postinfection) was 30–35% and 50–60%, respectively, of rates in mock-infected control cells in the absence of actinomycin (data not shown). Virion RNA and 26 S RNA were synthesized in the same proportions in RRV T48- and RRV dE2-infected cells (data not shown).

Polypeptide Synthesis in Infected BHK Cells

BHK cells infected with RRV dE2 or RRV T48 were labeled with ^{3}H-amino acids at various times after infection (m.o.i. $\simeq$ 2)

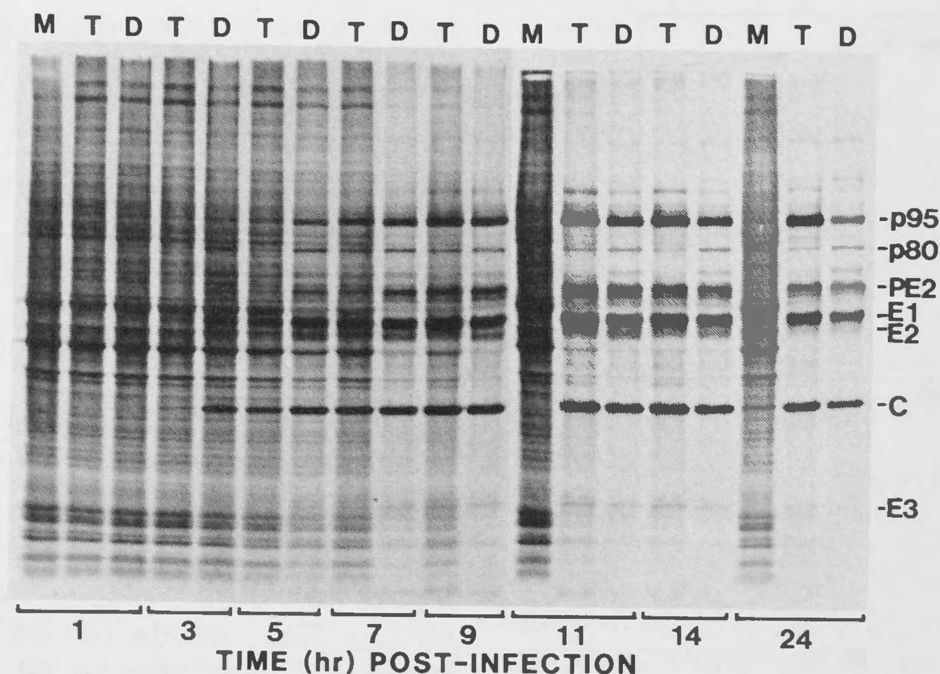

FIG. 5. Time course of polypeptide synthesis in BHK cells infected with RRV dE2 and RRV T48. BHK cell monolayers in 35-mm dishes were infected with RRV dE2 or RRV T48 (m.o.i. $\simeq 2$) or mock infected and incubated in cell growth medium. At intervals monolayers were labeled for 2 hr with 1 ml EMEM containing 100 μCi/ml of ^{3}H-amino acids and one-tenth the normal concentration of amino acids. Monlayers were disrupted in 200 μl of 1% SDS and aliquots electrophoresed on 10–20% gradient polyacrylamide slab gels followed by fluorography. Figures at tracks heads indicate extracts from mock-infected cells (M), RRV dE2-infected cells (D), and RRV T48-infected cells (T). Figures at right indicate the infection-specific polypeptides.

and cell extracts electrophoresed on 10–20% gradient acrylamide gels (Fig. 5). The following virus-specific polypeptides were identified in cells infected with RRV T48 and RRV dE2: p95, p80, p70, p62, E1, E2, C, and E3. Viral structural proteins and their precursors were labeled 2–3 hr earlier in RRV dE2-infected cells than in RRV T48-infected cells, possibly reflecting the different rates of virus-specific RNA synthesis (above). Shutdown of host protein synthesis occurred earlier in cells infected with RRV dE2 than in cells infected with RRV T48. There was no change in the pattern of processing of RRV structural proteins in cells infected with RRV dE2. However, labeling of p80 and p70, two proteins of unknown origin and function in RRV infection, was more prominent in RRV dE2-infected cells than in RRV T48-infected cells.

Discrimination between RRV dE2 and RRV T48 Using Neutralizing Monoclonal Antibodies

Five neutralizing anti-E2 monoclonal antibodies generated against RRV T48, were tested for their ability to neutralize RRV dE2 and RRV T48 in 50% plaque reduction neutralization tests. Four of them (E7, 1D11, 10C9, and 4D2) showed similar neutralizing activities (i.e., titers differed by 10-fold or less) against RRV T48 and RRV dE2. However, the antibody, 3C4, was approximately three orders of magnitude less effective against RRV dE2 than against RRV T48 (Table 1). This indicates that an epitope in E2 to which 3C4 binds

TABLE 1

PLAQUE NEUTRALIZATION OF RRV T48 AND RRV dE2 BY MONOCLONAL ANTIBODIES

Virus	Neutralization titer of monoclonal antibodies[a]				
	E7	1D11	10C9	4D2	3C4
RRV T48	4.2	4.8	3.6	4.9	4.1
RRV dE2	4.7	3.8	3.8	5.1	1.3

[a] Titer is the $\log_{10}$ dilution of monoclonal antibody resulting in 50% neutralization of virus plaque formation.

TABLE 2

VIRULENCE OF RRV T48 AND RRV dE2 FOR MICE

	RRV dE2		RRV T48	
	Day-old mice	Week-old mice	Day-old mice	Week-old mice
LD_{50} (Vero PFU)	0.066	$>8 \times 10^6$	0.046	0.051
Average survival time (days)	7.46	N.D.R.[a]	4.94	5.92

Note. Estimates of LD_{50} and average survival times, obtained as described under Materials and Methods, were assessed for statistical significance using one-way analysis of variance and *t* tests. LD_{50} values for RRV dE2 differ significantly from those of RRV T48 in both day-old mice and week-old mice ($P < 0.005$). AST values in day-old mice are significantly different ($P < 0.025$).

[a] N.D.R.: no deaths recorded.

is modified in the deletion mutant. No differences could be detected between RRV T48 and RRV dE2 in their neutralization by polyclonal immune ascitic fluid (data not shown).

Virulence of RRV dE2 and RRV T48 for Mice

RRV T48 is highly virulent for young mice (Taylor, 1972). Injection of day-old mice with low doses of virus by intracranial, intraperitoneal, or subcutaneous routes rapidly results in hind leg paralysis and death. With increasing age there is a gradual fall in the susceptibility of mice to lethal infection and RRV T48 will not kill 4-week-old mice when inoculated in large doses (Taylor, 1972).

We have compared the virulence of RRV T48 and RRV dE2 for day-old and week-old mice in terms of LD_{50}s, average survival times (AST), and severity of symptoms. In day-old mice the symptoms of hind leg paralysis were less severe for RRV dE2 than for RRV T48 in the dose range 0.02–20 PFU per mouse. In all dose ranges tested symptoms were independent of dose. LD_{50} values and ASTs were slightly greater for RRV dE2 than for RRV T48 (Table 2). In week-old mice RRV dE2 appeared to be completely avirulent: the virus elicited no symptoms and did not kill even at high doses ($\simeq 8 \times 10^6$ PFU injected ip). By contrast the virulence of RRV T48 in week-old mice was not significantly different from that in day-old mice (Table 2). We conclude that RRV dE2 is markedly less virulent than RRV T48 in week-old mice and slightly less virulent than RRV T48 in day-old mice.

Growth of RRV dE2 and RRV T48 in Mice

Day-old or week-old mice were injected subcutaneously with 100 PFU of RRV T48 or RRV dE2 and virus assayed at daily intervals in the blood, hind leg muscle, and brain tissues from each of three mice (Fig. 6). Maximum titers were at 1–2 days after injection for both age groups and were highest in muscle tissues. In day-old mice, RRV T48 reached 10^{11}–10^{12} PFU/g of muscle; the corresponding RRV dE2 titer was

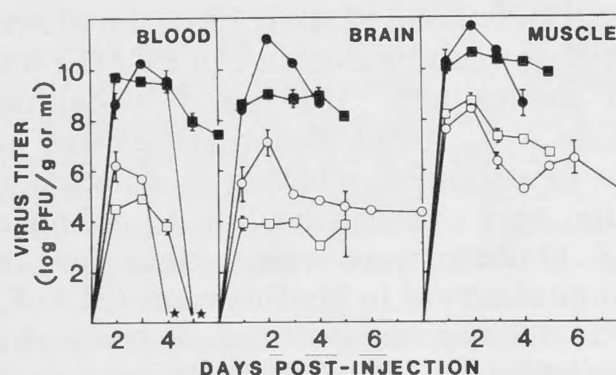

FIG. 6. RRV dE2 and RRV T48 titers in the blood, brain, and hind leg muscle of day-old and week-old mice. WEHI mice were injected subcutaneously with 100 PFU of virus and virus in the blood, brain, and hind leg muscle assayed at various times. Each time point represents the average of determinations made in three mice. Error bars are shown where the error is greater than 0.2 log units. ●, RRV T48 titers in day-old mice; ○, RRV dE2 titers in day old mice; ■, RRV T48 titers in week-old mice; □, RRV dE2 titers in week-old mice; ★, titer less than 100 PFU/ml.

10^8–10^9 PFU/g. In brain tissue RRV T48 and RRV dE2 reached approximately 10^{11} and approximately 10^7 PFU/g, respectively. In week-old mice RRV T48 and RRV dE2 reached peak muscle titers of approximately 10^{11} and 10^9 PFU/g of muscle tissue, respectively (Fig. 6). RRV T48 reached approximately 10^9 and approximately 10^{10} PFU/g, respectively, in the brain and blood of week-old mice. However, maximum RRV dE2 titers in the blood and brain tissues of week-old mice were approximately 10^5 and 10^4 PFU/g, respectively. The dramatic reduction in RRV dE2 virulence for week-old mice could be due to the decreased replication of RRV dE2 in brain tissue.

DISCUSSION

We have isolated a mutant of RRV T48 which, from nucleotide sequence determination has a deletion of seven amino acids at positions 55–61, near the N terminus of the envelope glycoprotein E2. The existence of the mutant confirms predictions made in previous studies (Dalgarno *et al.*, 1983).

There have been previous reports of alphavirus mutants with structural proteins which are altered in their electrophoretic mobilities (Bracha and Schlesinger, 1978; Cancedda *et al.*, 1981; Durbin and Stollar, 1984; Eaton, 1982; Leone *et al.*, 1980). The mobility changes have been ascribed to additional carbohydrate (Cancedda *et al.*, 1981; Durbin and Stollar, 1984), an altered site of proteolytic cleavage in PE2 (Bracha and Schlesinger, 1978) and a deletion (Leone *et al.*, 1980). Eaton (1982) was not able to establish whether a deletion or amino acid changes leading to enhanced SDS binding were responsible for the change observed in Sindbis virus (SIN) E2.

The data presented demonstrate that amino acids 55–61 of RRV E2 are not essential for infectivity. This is of interest in view of the conservation in the size and sequence of E2 between alphaviruses. RRV, SFV, and SIN E2 proteins are predicted to be 422, 422, and 423 amino acids long, respectively (Dalgarno *et al.*, 1983), and there is 69 and 42% homology between RRV E2 and the E2 proteins of SFV and SIN, respectively (Dalgarno *et al.*, 1983). The amino acids deleted in RRV dE2 are in a region of moderate homology with SFV: three of the seven deleted are identical between RRV T48 and SFV (Dalgarno *et al.*, 1983). Although amino acids 55–61 are inessential for replication under laboratory conditions, the data on RRV dE2-induced viremia in mice suggest that transmission of such deletion mutants under field conditions would be unlikely.

Since RRV dE2 showed no major replication defects in BHK cells, its failure to reach high titers in mice was probably not due to a defect in intracellular events in virus replication. Nor did the poor growth of RRV dE2 appear to be the result of enhanced interferon induction since when RRV dE2 and RRV T48 were used to coinfect mice, the growth of RRV T48 was similar to that obtained when it alone was used for infection (S. Vrati, unpublished data). The differences in titer were probably not the result of different degrees of inactivation of the two viruses *in vivo* as there was no difference in the rate of loss of infectivity of the two viruses after 2 hr incubation in mouse serum at 37° (S. Vrati, unpublished data). It is unlikely that the low RRV dE2 titers *in vivo* resulted from an enhanced specific immune response to RRV dE2 since this would need to occur shortly after infection and be specific for RRV dE2. Polyclonal antibody will not distinguish RRV T48 and RRV dE2 in neutralization tests (data not shown).

We propose that the different growth profiles of RRV dE2 and RRV T48 *in vivo* reflect alterations in penetration due to structural changes in determinants on the surface of E2. The E2-specific monoclonal antibody 3C4 neutralized RRV dE2 infectivity 1000 times less efficiently than RRV T48 infectivity. This, together with the marked reduction in thermal stability of RRV dE2 indicates that major changes have occurred in the conformation of the envelope glycoproteins of RRV dE2, particularly E2. These appear to have induced greater effects on *in vivo* replication than on patterns of growth and penetration in cultured cells, data consistent with results from Baric *et al.* (1981) and Olmsted *et al.* (1984). Baric *et al.* (1981) isolated a rapidly growing mutant of SIN by selecting for virus released early in infection of BHK cells.

The variant had an increased rate of penetration into BHK cells (but not into other cell types), and from neutralization studies with monoclonal antibodies was probably altered in E2. Olmsted *et al.* (1984) showed that virus selected in this way was of reduced virulence for mice and that rapid penetration into BHK cells was strongly correlated with attenuation in mice. Baric *et al.* (1981) concluded that the class of cellular receptors active in Sindbis virus uptake in BHK cells is of low specificity compared with that involved in penetration *in vivo.*

It has previously been argued that RRV pathogenesis in infant mice is not typical of alphaviruses since it does not involve early and rapid replication in neural tissues but, rather, is accompanied by extensive replication in nonneural tissues, particularly skeletal (hind leg) muscle (Mims *et al.*, 1973; Murphy *et al.*, 1973). By contrast SFV produces widespread infection and necrosis in the central nervous system and death takes place before there is time for the evolution of the pathological changes seen with RRV (Mims *et al.*, 1973). Murphy *et al.* (1973) proposed that the muscle lesions produced by RRV in newborn mice provide an adequate explanatory basis for the paralysis seen in RRV infection since the type of involvement of brain and spinal cord which could account for this paralysis was not seen.

Using the same outbred strain of mice as that used by Murphy *et al.* (1973) we have shown that RRV dE2 accumulates to the same level ($\simeq 10^8$ PFU/g of tissue) in the hind leg muscle of day-old and week-old mice. However, RRV dE2 is apparently totally avirulent for week-old mice although it is virulent for day-old mice (Table 2). The loss of virulence in week-old mice is accompanied by a sharp reduction in virus replication in brain tissue by comparison with that seen in day-old mice (Fig. 6). We have recently shown that, for certain RRV variants, the increase in virulence which occurs on serial passage of an avirulent strain of RRV in mice is accompanied by an increase in brain titers with little change in hind leg muscle titers (A. D. Meek, R. C. Weir, and L. Dalgarno, unpublished data). Seay and Wolinsky (1982) and Kingston (1983) have reported that RRV-induced central nervous system demyelination occurs in a way which suggests a causal relationship with paralysis. Thus RRV replication in brain tissue may be a more important determinant of viral virulence than the extent of replication in muscle.

The attenuation of virulence of RRV dE2, together with the protection of RRV dE2-immunized mice against lethal challenge with RRV T48 (S. Vrati, unpublished results), suggests that selection or generation of deletion variants may provide a useful route to stable (nonreverting) virus variants suitable for use as safe alphavirus vaccines.

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