Molecular and immunological analysis of EHV-1 and -4 infections

Peter John Cox ©

A thesis submitted to the University of Glasgow in part fulfilment of the requirements for a Ph.D.

The research described in this thesis was carried out in the Department of Veterinary Pathology, University of Glasgow Veterinary School.

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Declaration

The studies presented in this thesis were carried out in the Department of Veterinary Pathology, University of Glasgow between October 1991 and April 1995. All the work is my own, without exception. I confirm that no part of this thesis has been, or is being, submitted for any other academic award.

PETER JOHN COX

FEBRUARY 1999

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Dedication

To my wife, Deborah, and my two sons, Daniel and Matthew. I have taken so much of the time that should have been ours together, and you have given without complaint. For this reason and others I consider this thesis as much yours as it is mine. Unfortunately it falls short of repaying the great debt that I owe you all. I love you all very much.

Abbreviations

AA or aa	=	amino acid	mg	=	milligram
APS	=	ammonium persulfate	mins.	=	minutes
bp	=	base pairs	ul	=	microlitre
BPB	=	bromophenol blue	ml	=	millilitre
°C	=	degrees centigrade	uM	=	micromolar
CCV	=	channel catfish virus	mM	=	millimolar
CFT	=	complement fixation test	mmol	=	millimoles
Ci	=	curies	MOI	=	multiplicity of infection
CMI	=	cell mediated immunity	msec.	=	milliseconds
CNS	=	central nervous system	MW	=	molecular weight
CPE	=	cytopathic effect	ng	=	nanogrammes
d	=	distilled	nm	=	nanometres
dATP	=	deoxyadenine triphosphate	O.D	=	optical density
dCTP	=	deoxycytosine triphosphate	ORF	=	open reading frame
dGTP	=	deoxyguanine triphosphate	PAGE	=	polyacrylamide gel electrophoresis
dTTP	=	deoxythymidine triphosphate	PBS(T)	=	phosphate buffered saline (tween)
dNTP	=	deoxynucleotide triphosphate	PCR	=	polymerase chain reaction
DNA	=	deoxyribonucleic acid	pmol	=	picomoles
dpi	=	days post infection	PRV	=	pseudorabies virus
ds	=	double stranded	R	=	repeat
EBV	=	epstein-barr virus	RFLP	=	restriction fragment length polymorphism
EDTA	=	ethylenediaminetetra-acetic acid	RNA	=	ribonucleic acid
EHV	=	equine herpesvirus	rpm	=	revolutionper minute
ELISA	=	enzyme linked immunoassay	rt	=	reverse transcriptase
FCS	=	foetal calf serum	RTR	=	right TR
g	=	grams	SDS	=	sodium dodecylsulphate
GST	=	glutathione s-transferase	ss	=	single stranded
HCMV	=	human cytomegalovirus	SSCP	=	ss conformation polymorphism
HHV	=	human herpesvirus	TBE	=	tris-borate EDTA
hrs	=	hours	TBS(T)	=	tris buffered saline (tween)
HSV	=	herpes simplex virus	TCID	=	tissue culture infectious dose
HV	=	herpesvirus	TE	=	tris-EDTA
HVS	=	HV samurai	TEMED	=	N,N,N',N' Tetramethylene ethylene diamine
IF	=	immunofluorescence	TIF	=	trans-inducing factor
IIF	=	indirect IF	TR	=	terminal repeat
λ	=	lambda	UV	=	ultraviolet
IPTG	=	isopropyl β-D-thiogalactoside	V	=	volts

IR	=	inverted repeat
K	=	kilodaltons
Kbp	=	kilobases
KV	=	kilovolts
L	=	litre
L-broth	=	luria broth
LTR	=	left TR
M	=	molar
mA	=	milliamperes
Mab	=	monoclonal antibody
MCMV	=	Murine CMV
MEM	=	minimal essiential medium
uF	=	microFarads
ug	=	micrograms
VHS	=	virion host shutoff
VN	=	virus neutralisation
x	=	times
X-gal	=	5 Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Abstract

The equine pathogens EHV-1 and -4 exert dramatic detrimental effects upon the equine population. Infection with either virus can directly or indirectly lead to the death of valuable animals although the outcome of EHV-4 infection is usually less severe than that of EHV-1 infection. The main consequences of EHV-1 associated outbreaks include abortion and/or paralysis. EHV-1 induced paralytic disease occurs less frequently than abortigenic disease but can result in the direct or indirect loss of animals of any age or sex. It is because of this that the paralytic form is most feared since it may lead to the loss of established sporting or stud animals. Clinically apparent EHV-4 infections are quite common whereas episodes associated with EHV-1 are less frequent. The epidemiology of EHV-1 and 4 is inextricably linked from the point of view of disease management since the initial stages of infection with either virus are similar but result in dramatically different consequences.

The objective of the research carried out in this thesis was to identify molecular components of EHV-1 and -4 with a view to the development of diagnostic systems for these viruses.

The identification of an EHV-1 paralysis inducing strain-specific marker would allow the epidemiological monitoring of such strains in the circulating strain population and possibly allow the identification of animals most at risk from this potentially devastating condition. Chapter 3 describes the search for such a marker in EHV-1 glycoprotein C (gC). This search was initiated by obtaining partial DNA sequence data from the gC gene of a number of different strains of EHV-1 with different disease associations. The enigmatic nature of this data necessitated further investigation, described in chapter 4, of the nature of the gC gene and the encoded gC protein from certain EHV-1 isolates.

The ability to distinguish EHV-1 from EHV-4 infected animals on the basis of serology has been shown to be of epidemiological and clinical value, especially given the need for rapid identification and management of EHV-1 infected horses against a background of relatively prevalent EHV-4 infection. Few antibody capture reagents have been forthcoming that would be useful for such type-specific diagnostic serology. This may seem suprising from a simplified perspective given the wealth of data generated in the past 20 years on EHV-1 and -4 glycoproteins which are the main targets of the equine humoral immune response to these viruses. Chapter 5 describes the utilisation of this glycoprotein data to identify potentially useful peptides. These peptides were subsequently assessed by Pepscan ELISA using sera from naturally and experimentally infected horses in order to identify peptides or combinations of peptides that would be potentially useful type-specific diagnostic antibody-capture reagents.

Regions of the EHV-1 and -4 glycoprotein G (gG) homologues have previously been shown to be valuable serological type-specific diagnostic reagents. Chapter 6 describes the identification and the assessment of the diagnostic value of peptides representing epitopes in these regions.

"The Truth is out there"

Opening credits of The X files.

Chapter One

A General Introduction to the Herpesviridae, EHV-1 and EHV-4

1.1 A SUMMARY OF HERPESVIRUS CHARACTERISTICS

1.1.1 STRUCTURE OF A TYPICAL HERPESVIRUS VIRION

All herpesviruses have very similar morphology (see figure 1.1). The size of the herpesvirus particle was shown to range from 120-300nm in diameter as determined by electron microscopic techniques (Roizman and Furlong, 1974). Considering the virion from the outside in, the first entities encountered are heterogeneous spike-like structures formed by viral glycoproteins and possibly other virus encoded proteins that contain membrane spanning domains (MacLean *et al.*, 1991; Baines *et al.*, 1991). These structures extend from the surface of the virus particle and are rooted in the viral envelope (Stannard *et al.*, 1987). The envelope, a lipid bilayer derived from host cell membranes, is separated from the virus nucleocapsid by the tegument, a proteinaceous layer that is not well defined. Until recently variations in the size and shape of each virion were attributed to differences in the envelope and tegument; however, refinements of electron microscopic procedures for visualising virus particles have shown these structures to possess greater uniformity than originally thought (Rixon, 1993). The nucleocapsid consists of protein structures called capsomeres which are arranged to give the nucleocapsid a distinct icosahedral shape (Schrag *et al.*, 1989; Baker *et al.*, 1990). The icosahedron contains the virus nucleic acid, a large linear dsDNA molecule. Although still contentious most recent evidence (Puvion-Dutilleul *et al.*, 1987; Booy *et al.*, 1991) suggests that for HSV-1 at least, the viral genome fills the capsid interior and is most likely not structurally associated with any protein as purported by earlier studies (Furlong *et al.*, 1972).

Variations of this basic structure, the most notable being light particles (Szilágyi and Cunningham, 1991), can arise during the life-cycle of some herpesviruses but these generally do not interfere with the identification of a herpesvirus.

Once a virus has been tentatively identified as a herpesvirus attempts are made to place it in one of the herpesvirus sub-families.

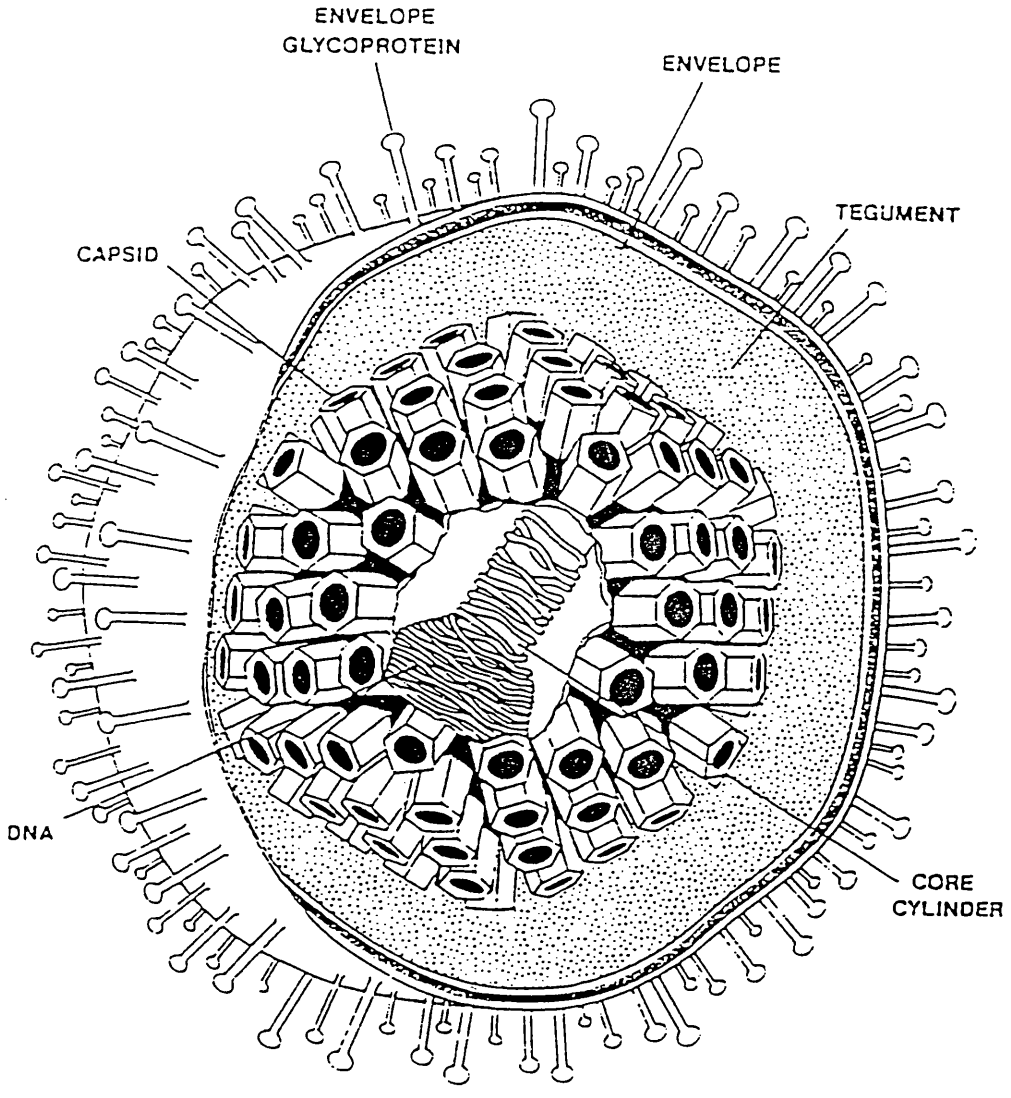


Figure 1.1 Schematic representation of a herpesvirus virion. (Kindly provided by Prof. D.E. Onions)

1.1.2 HERPESVIRUS CLASSIFICATION

In 1995 herpesvirus-like DNA was discovered in homosexual men with Karposis sarcoma and infectious virus subsequently isolated (Renne *et al.,* 1996). This highly publicised discovery represents the eighth herpesvirus identified in humans thus far. It is likely that more will be discovered which will add to the long list of this virus family, the *Herpesviridae* , that affects many different species (Roizman and Baines, 1991). Given the potential enormity of this family which has over 100 members, separating them into well defined groups would be of academic, medical and environmental importance.

Classification remains a formidable task, the perfect system being one that allows a new member to be rapidly classified on the basis of genetic and biological relatedness to well characterised members of the *Herpesviridae.* A system was devised that divided the *Herpesviridae* into three sub-families namely the α, β, and γ *herpesvirinae* (see table 1.1) (Roizman, 1982). A herpesvirus is defined as a member of one of the sub-families mainly on the basis of its *in vitro* and *in vivo* growth characteristics. The availability of viral DNA sequence may also aid in classification. Although the classification system is essentially quite simple complications can arise in that a virus, an example being Marek's disease virus (MDV), may have the growth characteristics of a γ herpesvirus while DNA sequence information and genome organisation may indicate an α herpesvirus classification (Buckmaster *et al.,* 1988). Such anomalies reinforce the idea that a reductionist classification system would not suffice in that the concerted action of genes in giving rise to a given characteristic must be considered e.g. with reference to MDV, divergent groups of genes in γ and α herpesviruses have similar phenotypic effects.

1.1.3 PROPERTIES OF HERPESVIRUS GENOMES

In a herpesvirus virion the viral genome consists of a single linear double stranded DNA molecule of molecular weight 80-150 x 10^6 daltons. Herpesvirus DNAs range in size and percentage GC base composition from approximately 120 to 235 kilobase pairs (Kbp) and 32 to 75% respectively. Technological advances of

Table 1.1: Properties of the subfamilies of the herpesviridae. (Roizman, 1990; Roizman and Baines, 1991). Each genera is written in italics and examples of viruses from each genera are given in brackets.

SUBFAMILY	HOST RANGE	IN-VITRO PROPERTIES	SITES OF LATENCY	GENERA
Alphaherpesvirinae	Variable	Relatively quick growth and spread in cell-culture which generally results in cell destruction	Sensory ganglia and cells of lymphoid origin	*Simplexvirus* (HSV-1) *Varicellovirus* (VZV, EHV-1, EHV-4)
Betaherpesvirinae	Restricted	Slow growth and spread in culture which can result in the swelling of cells to form a cytomegalia	Wide variety of tissues including lymphoreticular cells, secretory glands, kidneys etc.	*Cytomegalovirus* (HCMV) *Muromegalovirus* (MCMV) *Roselovirus* (HHV-6)
Gammaherpesvirinae	Restricted to hosts or species of the same order	Replicate in lymphoblastoid cells and some may multiply in certain types of fibroblastic or epithelial cells	Lymphoid tissue	*Lymphocryptovirus* (EBV) *Rhadinovirus* (HVS)

the last fifteen years have made the task of completely sequencing herpesvirus DNAs possible. As alluded to earlier, partial sequence data has aided in classification in that it has been possible to calculate evolutionary relationships among herpesviruses by comparing specific protein sequences. The availability of more extensive data has seen the application of complex mathematical methods for comparing herpesvirus DNA sequences. The application of such methods have yielded interesting results with regard to the anomalies of the biological classification system (see Karlin *et al.,* 1994).

An alternative method for separating herpesviruses into distinct groups is based on the structure of the viral nucleic acid (Roizman, 1990). There are six types or groups of herpesvirus genomes labelled A through to F which differ from each other with respect to the presence, type, number, size, and position of repeat sequence elements (see figure 1.2).

The structure of a type A genome consists of a large sequence from one terminus directly repeated at the other terminus. Group B genomes differ from those of group A in that the group B terminal sequence is shorter and is directly repeated a variable number of times at the genomic termini. Group C genomes are typified by multiple reiterations of one set of sequences in the same orientation at both ends of the genome. Tandem reiterations of other sets of sequences are found within the type C genome. Group D genomes consist of two covalently linked long (L) and short (S) segments. L consists of a unique sequence (U_L) and S is a unique sequence (U_S) bordered by inverted repeats, an internal repeat (IR_S) and a terminal repeat (TR_S). The L segment is generally in fixed orientation whereas the S segment can invert relative to L which gives rise to two isomeric forms of the genome. Type E genomes comprise of sequences from both ends of the genome that are repeated in an inverted orientation and juxtaposed internally. The L and S segments formed by this genome arrangment are flanked by inverted repeats and therefore can invert relative to each other giving rise to four potential isomers of the genome. One other genome type has been identified that is totally dissimilar from the other types in that it consists of a unique long sequence bordered by non-identical terminal sequences that have no internal counterparts.

One of the most interesting aspects of herpesvirus genome structure is the presence of these repeat regions. It is because of recombination within and between

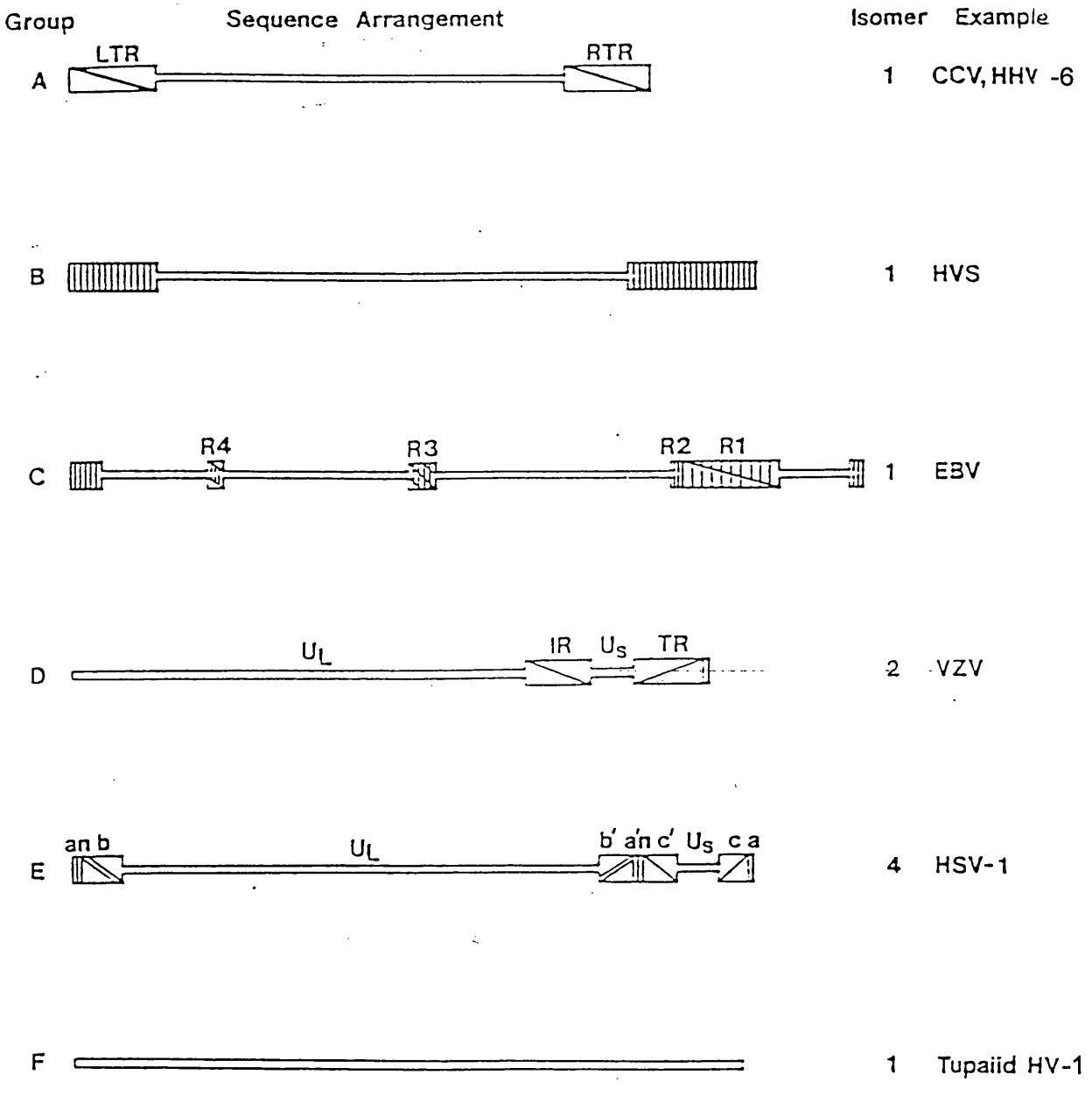


Figure 1.2 Different types of herpesvirus genome structures (adapted from Roizman, 1990).

these regions that the structure and size of the genome of a given virus varies within a population. Whether these repeat driven changes have any functional significance remains to be seen.

1.1.4 THE MULTIPLICATION PROCESS OF AN α-HERPESVIRUS

The mechanisms via which HSV-1 infects and multiplies within a cell (see figure 1.3) are being studied in detail. Much of what is known can loosely be extended to the other viruses in its sub-family, including EHV-1 and -4.

The attachment and entry of HSV-1 into host cells involves the virus envelope glycoproteins. The virus first binds to heparin sulphate moieties on the surface of a potential host cell (WuDunn and Spear, 1989; Shieh *et al.,* 1992). This function is mediated by the viral glycoprotein gC and possibly independently by gB (Herold *et al.,* 1991 and 1994). This attachment is reversible.

The formation of a stable attachment of the virus to its host cell is probably linked to the viral entry process. Evidence is accumulating for the interaction of gD with a cell surface receptor to initiate irreversible attachment. Recently potential candidate cell surface receptors were identified (Montgomery *et al.,* 1996; Geraghty *et al.,* 1998). gD, gB, gH and gL have all been implicated in the associated penetration process. Two processes, namely receptor mediated endocytosis and membrane fusion, were proposed as methods of viral entry into the cell but the balance of evidence suggests that only the latter results in a productive viral infection (Campadelli-Fiume *et al.,* 1988; Wittels and Spear, 1991).

The fusion process results in the loss of the viral envelope allowing the release of the nucleocapsid and associated tegument proteins into the cell. Host protein synthesis is switched off via degradation of mRNA by the virion host shut-off (VHS) protein which normally resides in the tegument. Interestingly this shut-off does not occur in EHV-1 infected cells although the virus does encode a functional VHS protein (Feng *et al.,* 1996). The capsid and tegument proteins are transported to the nucleus into which the viral DNA is released where it circularises via ligation of the terminal repeats. The α-TIF, a viral encoded transcripitional activator contained in the tegument, induces with the aid of host co-factors

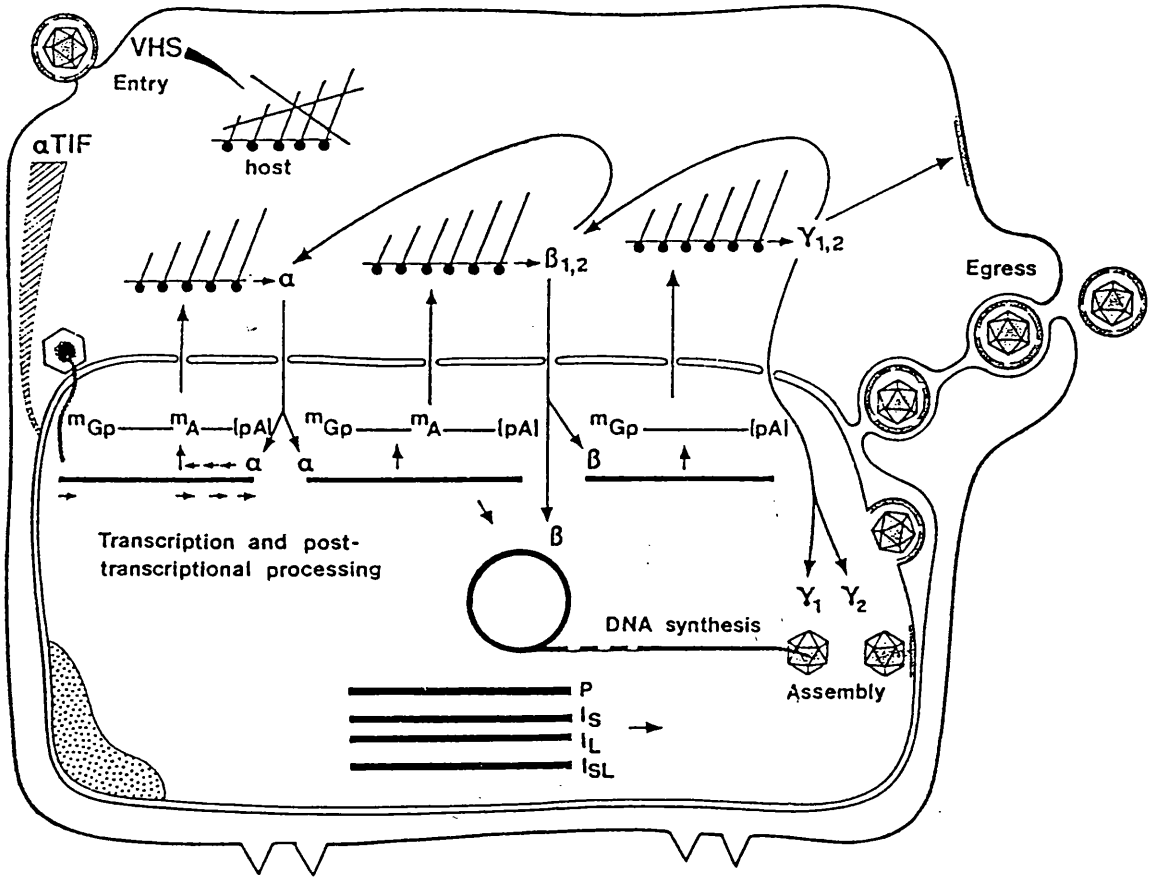


Figure 1.3 Life-cycle of an α herpesvirus. (Adapted from Fenner *et al.*, 1987)

(McKnight *et al.*, 1987) the first round of viral gene expression (Campbell *et al.*, 1984). Viral genes are expressed in an ordered fashion depending mainly on when they are required during the lytic-cycle (Honess and Roizman, 1974).

α-TIF induces the first round of transcription of viral genes namely the α or immediately early genes. The protein products of these genes are involved in either self-regulation or induce subsequent rounds of transcription which result in the production of the β / early and γ / late genes. β proteins are required for the synthesis and replication of viral DNA. The late genes which encode mainly structural proteins can be further subdivided into γ_1 and γ_2 genes in that DNA replication is a prerequisite for the initiation of γ_2 but not γ_1 gene transcription.

In the nucleus replication products i.e new virus genomes are packaged into recently constructed capsids. The capsids bud from the inner nuclear membrane where the newly synthesized precursor glycoproteins have been inserted and are subsequently transported to the cell surface. The transportation pathway is unclear but probably involves some interaction with the golgi-apparatus where the precursor glycoproteins are most likely glycosylated. A recent report suggests that the extracellular virions have envelopes which differ from the nuclear membranes with respect to phospholipid composition. The envelope phospholipid composition reflects either a golgi or plasma membrane origin (Van Genderen *et al.*, 1994).

Release of the virus from the cell is thought to involve a number of virus glycoproteins including gD, gH and gK.

1.2 EHV-1 and EHV-4

Eight distinct herpesvirus have been isolated from members of the Equidae. These viruses have been designated EHV-1 to -8 (See table 1.2). Not all these viruses have a definitive disease association. Given the detrimental effects of EHV-1 and -4 associated diseases particularly with regard to profitable industry based on the equids, it is not surprising that these equine herpesviruses have been singled out for much attention by the virology research community.

The following is a review of the aspects of the biology of EHV-1 and -4 which are relevant to this thesis and is intentionally an incomplete review. Reference to other herpesviruses will be made where insufficient information is available regarding the aforementioned EHVs.

1.2.1 THE DISEASE SPECTRUM OF EHV-1 AND -4

Historically EHV-1 and -4 were considered to be a single entity, equine rhinopneumonitis virus (Dimock and Edwards, 1936). Subsequently serological and clinical observations led to the conclusion that this virus was not a single entity but two sub-types of the same virus, namely EHV-1 sub-types 1 and 2 (Burrows and Goodridge, 1973). Finally, in 1981, the results of a number of restriction enzyme analysis studies prompted Studdert to suggest that these viruses should be considered as distinct entities.

The most frequently quoted reason for studying these viruses is their detrimental impact on industries associated with the equid. Horses assembled in relatively large groups frequently succumb to EHV-1 and -4 infections. The outcome of an infection with either virus cannot be accurately predicted but may lead to death under certain conditions. Both viruses are respiratory pathogens but generally differ with regard to the diseases they cause.

Infection by EHV-1 and -4 is normally contracted by the inhalation and to a lesser extent ingestion of material contaminated with virus. Common sources of infectious material include the secretions of infected in-contact animals, aborted

EHV	CLASSIFICATION	DISEASE ASSOCIATION
2	Biological properties of β herpesviruses but is distinct γ herpesvirus on the basis of DNA sequence (Telford *et al*., 1993).	Foal upper respiratory tract disease (Pálfi *et al*., 1978). Mild neonatal rhinitis and conjunctivitis (Gleeson and Studdert 1977)
3	α herpesvirus	Veneral disease equine coital exanthema (ECE) (Bryans and Allen, 1973).
5	Biological properties of β herpesviruses but is distinct γ herpesvirus on the basis of DNA sequence (Telford *et al*., 1993).	None
6	α herpesvirus	Causes lesions resembling ECE in donkeys (Burrows, 1973).
7	β herpesvirus	None
8	α herpesvirus	None

Table 1.2: EHVs other than EHV-1 and -4.

foetuses and material i.e. fomites e.g. bedding that has been directly or in-directly in-contact with affected animals. It has also been proposed that infection may also occur from within as a result of reactivation of latent virus. Both viruses replicate in the respiratory epithelium and infection can spread via the lymphatic system to local lymph nodes. EHV-4 causes a relatively mild respiratory infection, the risk associated with this virus being that it may make affected animals, particularly foals, susceptible to potentially life threatening secondary infections. Affected competition animals are not allowed to compete which has obvious financial consequences. In young horses EHV-1 respiratory infection can have extremely serious consequences.

EHV-1 establishes a viraemic infection. The virus multiplies in the local lymph nodes (Prickett, 1970) where it most likely initiates a cell associated viraemia, the vehicle by which it spreads to other tissues (Scott *et al.,* 1983). The consequences of EHV-1 associated viraemia are multiple.

Experimental infections have shown that EHV-1 can replicate at a number of sites within the uterus of a pregnant mare (Smith *et al.,* 1993). Pregnant mares can abort as a result of EHV-1 infection of the placenta and foetus or the placenta alone, the latter giving rise to abortion of a virus negative foetus (Smith *et al.,* 1992). The devastating effects of the abortigenic potential of EHV-1 can be seen on stud-farms with a large population of pregnant mares, where after the initial index case of EHV-1 infection practically all the mares sequentially abort in what is referred to as an abortion storm (Allen and Bryans, 1986). EHV-1 induced abortion can occur at any time during the gestation period. In some cases abortion does not occur but the foals are still-born or born alive but die due to complications arising from a severe EHV-1 infection (Dixon *et al*. 1978). Isolated incidents of abortion are not uncommon.

EHV-1 infected horses regardless of gender can develop paralytic symptoms. Animals can recover from mild cases of paralysis but in some instances horses that are severely affected have to be euthanased. In some cases of EHV-1 induced paralysis, the eye can also be affected (Platt *et al.,* 1980). Apart from paralysis of the eye direct tissue damage has been observed in the retina and cornea of EHV-1 affected animals (Slater *et al.,* 1992). Pathological examination indicates that

paralytic symptoms are due to damage of the blood vessels supplying the affected areas of the CNS (Edington *et al.,* 1986).

Although EHV-1 infection results in pathology of a number of different body organs the culmination of a number of studies indicates a unifying pathological mechanism with regard to abortion and neurological disease elicited by certain EHV-1 strains (Edington *et al.,* 1991; Smith *et al.,* 1992 and 1993). Virus induced damage of endothelial cells lining blood vessels supplying the nervous system and uterus seems to be a major contributory factor in the pathogenesis of EHV-1. It is unclear if endothelial cell damage is directly or indirectly a result of virus replication in these cells. This mechanism of CNS induced damage is atypical of other alphaherpesviruses which normally multiply within the CNS thus inducing disease. There is very little evidence for productive replication of EHV-1 in the CNS of the horse although reactivatable latent virus has been demonstrated in the trigeminal ganglion (Slater *et al.,* 1994).

It has been assumed that EHV-4 does spread from the site of infection, however the virus has been associated with isolated cases of abortion (Allen and Bryans, and references therein 1986) and paralysis (Meyer *et al*. 1987). EHV-4 was recently isolated from endothelial cells and synovial epithelium of a pony foal suffering from pneumonia (Blunden *et al*. 1995). Furthermore EHV-4 has also been demonstrated as a latent presence in widely disseminated tissues including the CNS (Edington *et al.,* 1994; Borchers *et al.,* 1997). It seems likely that under favourable conditions infection of horses with certain strains of EHV-4 may result in a viraemia.

1.3 THE MOLECULAR BIOLOGY OF EHV-1 AND -4

1.3.1 VIRION AND GENOME STRUCTURE

The virions of EHV-1 and -4 are as that described for a typical herpesvirus (O'Callaghan *et al.,* 1978; Baker *et al.,* 1990).

Restriction endonuclease analysis played a central role in the elucidation of the structures of the genomes of these viruses and led to the conclusion that they were distinct species and not sub-types of the same virus which confused many early scientific studies of EHV-1 and -4 (Sabine *et al.,* 1981; Studdert *et al.,* 1981; Turtinen *et al.,* 1981). Both viruses have a group D genome structure (see figure 1.2). Extensive restriction enzyme maps have been constructed for three EHV-1 and two EHV-4 strains (Whalley *et al.,* 1981; Henry *et al.,* 1981; Baumann *et al.,* 1986; Cullinane *et al.,* 1988; Nagesha *et al.,* 1992; Kirisawa *et al.,* 1993a).

The entire DNA sequences of the AB-4 strain of EHV-1 and EHV-4 strain NS80567 were determined. The EHV-1 genome is 150,224bp in size and the G+C content is 56.7% (Telford *et al.,* 1992). The EHV-4 genome is 145,597 bp in size (Telford *et al.,* 1998). Partial sequence data is available for other strains of EHV-1 and EHV-4. These data have proved extremely valuable and attempts are now being made to identify the function of the products of the genes sequenced and their contributions to the viruses life cycles and pathogenicity.

1.3.2 VIRION GLYCOPROTEINS

The virions of EHV-1 and -4 contain at least twelve glycoproteins. The presence of glycoproteins in these viruses was initially demonstrated by glycoprotein specific staining techniques and by utilising tritiated glucosamine as a sugar source for virus infected cells which results in the metabolic labelling of sugar-containing proteins within the cells (Turtinen *et al.,* 1981; Turtinen and Allen, 1982; Meredith *et al.,* 1989; Crabb and Studdert, 1990). Two different EHV glycoprotein nomenclature systems were proposed the first based on glycoprotein electrophoretic mobility in SDS polyacrylamide gels and the second on the apparent molecular

weight of the glycoproteins (Turtinen and Allen, 1982; Meredith *et al.,* 1989). Both systems have not been universally accepted since they are prone to misinterpretation based on technical differences between laboratories studying these viruses and their glycoproteins (see table 1.3). The advent of molecular techniques has allowed a direct comparison of glycoprotein genes between HSV-1, other α herpesviruses and EHV-1 and -4. Subsequently the HSV glycoprotein nomenclature system is now commonly employed, whenever possible, for the glycoproteins of EHV-1 and -4.

Five approaches have been utilised in order to identify the genes encoding some of these glycoproteins.

1. Glycoprotein specific monoclonal antibodies (Mabs) have been used to probe λgt11 EHV-1 and -4 genomic DNA expression libraries in order to identify clones containing partial sequence data (Allen and Yeargen, 1987; Whittaker *et al.,* 1991; Cullinane *et al.,* 1993; Wilson *et al.,* 1994). An alternative yet similar approach is to clone part of a suspected glycoprotein gene into a bacterial expression vector and subsequently develop an antiserum against the bacterially expressed protein which should then react with one of the virion glycoproteins (Crabb *et al.,* 1992).

2. On the basis of colinearity between EHV-1 and -4 and utilising glycoprotein genes from other α herpesviruses, namely HSV-1, as low stringency probes to screen EHV-1 and -4 genomic DNA plasmid libraries, it has been possible to identify EHV glycoprotein genes (Riggio *et al.,* 1989; Bonass *et al.,* 1990; Nicolson *et al.,* 1990; Nicolson and Onions, 1990).

3. Sequencing of relatively large regions of virus genome and subsequent comparison of identified ORFs with glycoprotein gene homologues of other herpesviruses (Audonnet *et al.,* 1990; Elton *et al.,* 1991a and b; Telford *et al.,* 1992 and 1998). Those genes thought to encode potential glycoproteins but with no homology to existing glycoproteins can be examined at the amino acid level for the presence of sequence traits common to herpesvirus glycoproteins, namely a N-terminal signal sequence, a membrane spanning domain and glycosylation sites.

4. Deletion of a specific gene from the viral genome and the subsequent absence of a specific glycoprotein from the mutant virus (Sun *et al.,* 1994).

Table 1.3: EHV-1 and -4 glycoprotein nomenclature systems: Details of virion glycoproteins of EHV-1 and EHV-4 for American, British and Australian isolates as identified by different research groups. (A) Allen *et al* (1992); (B) Meredith *et al* (1989) and (C) Crabb and Studdert, (1990); Crabb *et al* (1991) and (1992); Drummer *et al* (1998). The nomenclatures (a) Turtinen *et al* (1992) and (b) Meredith *et al* (1989) are used to describe the glycoproteins. The * represents the component of the disulphide-linked complex. The molecular weights in respect of glycoproteins identified by group (C) are presented as such (Kindly provided by Dr. P.C. Sharma).

EHV-1			HSV Homologue	EHV-4		
A[a]	B[b]	C		A	B	C
gp2	gp300	250K	Unknown	gp(2) (190-240K)	gp300gp260 gp220	gp270K
gp10 Region						
(a) 120k	gp118	110K	gH (not definitive)	gp10 (124K) Not characterised		
(b) 115k			VP14		gp138	113K
(c) 110k	gp108*	127K	gB		gp112*	127K
gp13 (96k)	gp88	87K	gC	gp13 (110K)	gp92	67K
gp14 (90k)	gp76*	78K	gB	gp14 (b) (87K)	gp74*	77K
gp18a (58-60k)	gp60	60K	gD	gp18a (61K)	gp61*	56K
gp18b (58-60k)	gp58*	58K	gB	dimeric form unknown	gp59	62K
gp21/22a	gp45	49K	Unknown	gp21/22a	gp45	ND
ND	ND	ND	gI	ND	ND	ND
ND	ND	ND	gE	ND	ND	ND
ND	ND	66K, 10-12K	gG	ND	ND	68K, 12K

5. None of the aforementioned approaches definitively link a particular protein with a particular gene unlike the fifth and final approach which requires the purification of the glycoprotein of interest followed by N-terminal protein sequencing of the purified product in order to relate amino acid sequence to DNA sequence (Wellington *et al.,* 1996a).

The available glycoprotein gene sequences have been exploited mainly for the purposes of vaccine development and as yet very little is known of the functional significance of their gene products.

gC

The gC gene homologues sequences of three strains of EHV-1 (Allen and Coogle, 1988; Telford *et al.,* 1992; B.S. Crabb, Personal communication) and two strains of EHV-4 (Nicolson and Onions, 1990; Telford *et al.,* 1998) have been determined. All genes have partial homology with their HSV-1 counterpart. The first EHV-1 gC gene to be sequenced was initially identified using method one above (Allen and Yeargen, 1987). The EHV-4 homologues were identified on the basis of colinearity between the EHV-1 and -4 genomes (Nicolson and Onions, 1990) or by sequencing the entire EHV-4 genome (Telford *et al.,* 1998).

gC of HSV-1 is a multifunctional envelope glycoprotein. It is involved in the adsorption of virus to host cell heparin sulphate moeities and is known to bind the C3b component of the complement cascade system when expressed on the surface of infected cells and on the virion envelope (Herold *et al.,* 1991; Hidaka *et al.,* 1991; Friedman *et al.,* 1984). No evidence as yet exists for the participation of the gC homologues of EHV-1 and -4 in the adsorption process, although some anti-gC Mabs against the EHV-1 homologue were shown to neutralise viral infectivity but only in the presence of complement (Sinclair *et al.,* 1989). Furthermore unpublished data suggests that heparin may have a limited role in the adsorption of wild-type strains of EHV-1 to cells in culture suggesting that the role of the EHV-1 gC homologue in adsorption may be somewhat different from that of its HSV-1 counterpart (see Sugahara *et al.,* 1994). EHV-1 infected bovine cells express a complement binding activity (Bielefeldt-Ohmann and Babiuk, 1988). That anti-EHV-1

and -4 gC Mabs can inhibit the agglutination of equine complement coated erythrocytes is indirect evidence of the complement binding activity of these glycoproteins. Direct evidence for the complement binding activity of gC of EHV-1 and -4 was obtained by showing that gC purified from EHV infected cell lysates could bind C3 and cells transfected with a plasmid encoding gC under the control of a eukaryotic promoter could bind complement (Huemer *et al.,* 1995).

gD

An identical situation exists between gD and gC as regards the availability of EHV-1 and -4 sequence information (Audonnet *et al.,* 1990; Elton *et al.,* 1991b; Flowers *et al.,* 1991; Whalley *et al.,* 1991; Telford *et al.,* 1992; Cullinane *et al.,* 1993; Telford *et al* ., 1998).

The gD homologues of other α-herpesviruses are the focus of much attention because they play an essential role in the infectivity process. Antibodies against a peptide derived from the EHV-1 gD homologue were shown to neutralise virus infectivity in the absence of complement (Flowers and O'Callaghan, 1992). Anti-gD Mabs inhibit penetration but not adsorption of EHV-1 thus showing that EHV-1 gD functions in a similar fashion to other gD homologues (Whittaker *et al.,* 1992a). The HSV-1 gD homologue is also thought to be involved in virion egress from infected cells and the ability of the virus to spread from cell to cell without release into the extracellular environment. These properties are probably mediated in part by the viruses ability to cause cell-cell fusion, a process known to involve gD (Noble *et al.,* 1983). No evidence to date suggests that EHV-1 gD is involved in cell fusion elicited by EHV-1 infection although this glycoprotein does promote cell fusion when expressed in cell lines (Wellington *et al.,* 1996b).

Little is known about the EHV-4 gD homologue.

gB

The gB homologue of EHV-1 is structurally similar to that of other herpesviruses with the exception of HSV-1 in that it is cleaved at two sites

(Wellington *et al.,* 1996a) to form a heterodimeric complex of processed proteins all initially derived from cleavage of precursor gB (Meredith *et al.,* 1989). The amino acid sequence of the EHV-4 gB homologue derived from the gene sequence predicts that it would be processed in a similar fashion (Riggio *et al.,* 1989).

gB of HSV-1 is involved in the penetration process (Cai *et al.,* 1988). Neubauer and colleagues (1997) have demonstrated a role for the EHV-1 gB homologue in penetration and cell-cell spread. Also anti-EHV-1 gB Mabs have been shown to inhibit cell-cell fusion thus suggesting a possible function for this glycoprotein in cell to cell spread and egress (Wellington *et al.,* 1996c).

gE and gI

The gene homologues of these glycoproteins have been identified for EHV-1 and -4 (Cullinane *et al.,* 1988; Audonnet *et al.,* 1990; Elton *et al.,* 1991a; Telford *et al.,* 1992 and 1998). These glycoproteins function together to form Fc receptors for immunoglobulin G (IgG) complexes, and gE alone can act as a receptor for IgG in HSV-1 infected cells (Dubin *et al.,* 1990). EHV-1 infected bovine cells bind the Fc portion of immunoglobulins (Bielefeldt-Ohmann and Babiuk, 1988).

VP13/14

The VP13/14 and VP14 glycoproteins of HSV-1 and -2 respectively are located in the tegument and for HSV-1 at least may have some involvement in gene control. An anti-EHV-1 gp10 Mab was shown to cross-react with VP13/14 and VP14. This Mab was used to isolate the gene encoding an EHV-4 VP13/14 homologue (Whittaker *et al.,* 1991). The EHV-1 homologue has also been sequenced (Telford *et al.,* 1992).

gp2 (gp300)

This glycoprotein was initially characterised by Whittaker *et al* (1992b). It differed from other previously characterised herpesvirus glycoproteins in that it contained a large amount of O-linked carbohydrate as opposed to N-linkages although it was also modified in this way (Whittaker *et al.,* 1990). Also tentative evidence suggested that it may cross the membrane a number of times. The EHV-1 gene encoding this glycoprotein was identified as the HSV-1 UL32 gene homologue and was confirmed by an anti-EHV-1 gp2 Mab which recognised a procaryotic fusion protein expressed from this gene. However controversy has arisen in that another gene the EHV-1 homologue of HSV-1 gene US5 has been proposed to encode gp2 (Sun *et al.,* 1994).

Both studies have found common ground with regard to the possible role of gp2 in promoting cell to cell fusion.

It is now accepted that gp2 is encoded by gene 71 of EHV-1 (Wellington *et al.,* 1996c). Apart from a role in inducing cell fusion deletion mutant studies also suggested a role for this glycoprotein in virus maturation and envelopment. The EHV-4 gene homologue has been characterised (Nagesha *et al.,* 1993).

gM

The EHV-1 gM homologue is encoded by gene 52. In contrast to the HSV-1 homologue gM of EHV-1 is a major constituent of the envelope of EHV-1. It has been proposed that this glycoprotein may also span the membrane a number of times (Pilling *et al.,* 1994). Mutation of the gene has demonstrated a potential role for the EHV-1 gM homologue in virus penetration and cell-cell spread but it is not an ion channel as first suggested by Pilling and coworkers (1994) (Osterrieder *et al.,* 1996). An EHV-4 homologue has recently been identified (Telford *et al.,* 1998).

gG

The DNA sequences of the EHV-1 and -4 gG homologues have been determined (Crabb *et al.,* 1992; Telford *et al.,* 1992; Telford *et al.,* 1998). Their functions remain to be elucidated, however like the PRV homologue, both the EHV-1 and -4 gG homologues are secreted (Crabb *et al.,* 1992; Drummer *et al.,* 1998).

gH, gL, and gK

Sequencing of the EHV-1 genome has revealed genes with homology to HSV-1 gH, gL and gK (Robertson *et al.,* 1991; Telford *et al.,* 1992; Zhao *et al.,* 1992). The EHV-4 gH (Nicolson *et al.,* 1990; Telford *et al.,* 1998), gL and gK (Telford *et al.,* 1998) gene homologues have also been sequenced. Very little information regarding the protein products of these genes is available.

1.3.3 IMMUNITY TO EHV-1 and EHV-4

Immunity to EHV-1 and -4 generally does not last very long and horses may be reinfected every three to six months. Young horses are infected by 1 year of age and tend to be particularly susceptible to reinfection (Allen and Bryans, 1986).

The envelope glycoproteins of EHV-1 and -4 are obvious targets for the host humoral immune response. A number of studies using a variety of approaches have identified gp2, gC, gB, gD, gp21/22a and possibly the gp10 complex to be targets (Allen and Bryans, 1986; Crabb *et al.,* 1991; Ahmed *et al.,* 1993). Ostlund and co-workers (1992) have shown that gB followed by gC are the major target antigens for the equine humoral immune response. Antibodies to gG have also been detected in EHV-1 and -4 infected horses as well as experimentally infected animals (Crabb *et al.,* 1992; Crabb and Studdert, 1993). There is also preliminary evidence that the EHV-1 membrane glycoproteins are targets for cell-mediated immune responses (CMI) (Bridges *et al.,* 1988).

The sensitivity of new-born foals to infection could be due to several factors including their tendency not to develop sufficient levels of circulating anti-virus antibodies (Coignoul *et al.,* 1984a and references therein) and the insufficient protection conferred by the passive immunity provided by the mares colostrum (Higgins *et al.,* 1987). In contrast, as outlined previously, older animals tend to develop good adaptive responses to EHV glycoproteins and other virion polypeptides which can neutralise virus infectivity *in vitro*. One of the most perplexing questions in EHV-1 and -4 biology is why, when given a good adaptive response, animals are reinfected with such frequency.

The answer may come from studies of innate immunity to EHVs which were initiated in the late 1970s. It was demonstrated that foals could evolve a CMI response to infection; however EHV-4 infection can result in defective neutrophil function in young horses (Coignoul *et al.,* 1984b). Subsequent studies illustrated that EHV-1 infection could derail a number of aspects of the innate immune response (Bridges and Edington. 1986; Hannant *et al.,* 1991; Lunn *et al*. 1991). Recent data has suggested that elevated levels of the cytokine TGF-β in EHV-1 affected animals may be responsible for the immunosuppressive effect of that virus (Charan *et al.,* 1997). It remains to be seen exactly how the viruses elicit these alterations in immune function and if these changes contribute to the frequency at which horses are reinfected.

1.3.4 STRAIN VARIATION

The ability to detect differences between strains of either EHV-1 or -4 is of great epidemiological importance for two reasons. Firstly it should allow the assessment of the contribution of infection with particular strains to the morbidity and mortality rates in a given equine population. Secondly it may allow strains to be grouped according to their pathogenic potential. Such information may ultimately lead to the elucidation of factors which determine virus pathogenicity and allow the determination of the best treatment or preventative measures for a given horse population.

Phenotypic/growth characteristics and protein structure

EHV growing in cell culture showed inconsistent variation in plaque size and morphology (Holmes *et al.*, 1979).

Patel *et al* (1982) noted that abortigenic and paralytic isolates have different *in vivo* cellular tropisms. These differences may not necessarily reflect inherent differences between the strains in that the study involved observations of natural infections and therefore host factors may be involved.

Minor undesignated differences in the protein profiles of different strains of EHV-1 and -4 were identified (Turtinen *et al.*, 1981).

Epitope content

Initial attempts to serologically characterise strains of EHV-1 and -4 involved the use of crude polyclonal antiserum. The serum neutralisation tests utilised were prone to variation and although inter-strain antigenic variation was purported to be detected the data were considered unreliable (Allen and Bryans, 1986 and references therein).

In the ensuing years the development of monoclonal antibody (Mab) technology had a major impact on serology. Consequently a number of studies aimed at characterising EHV specific Mabs and their utilisation in diagnostic tests revealed potential antigenic variation between EHV strains. The reactivity of different strains of EHV-1 and -4 with pools of Mabs was shown to vary in enzyme immunofiltration assays and indirect immunofluorescence (IIF) tests (Yeargen *et al.*, 1985). Edington noted what were termed as inconsistent differences in reactivity of EHV strains with specific Mabs in IIF tests (Edington *et al.*, 1987). Both enzyme immunofiltration assays and IIF tests rely on detection of cell surface expression of virus proteins and therefore the aforementioned results are not necessarily indicative of strain specific antigenic variation.

An extensive study revealed that 72 strains of EHV-1 differed from each other with respect to anti-gC Mab reactivities in direct ELISAs (Allen *et al.*, 1988). Japanese strains of EHV-1 differed with regard to neutralisation by Mabs in the

presence of complement (Shimizu *et al.,* 1989). Sinclair and co-workers noted variability in the reactivity of EHV-4 strains with a Mab against a virion polypeptide and an anti-gC Mab in immunofluorescence assays (Sinclair *et al.,* 1989; Sinclair and Mumford, 1992).

Genome variation

Restriction fragment length polymorphism (RFLP) analysis has revealed variation in the genomic structure of EHV-1 and -4 strains (Sabine *et al.,* 1981; Studdert *et al.,* 1981; Allen *et al.,* 1983a). Earlier RFLP-based studies may have been compromised by the fact that many of the strains analysed were passaged in cells that were not of equine origin. Subsequent studies revealed that handling the viruses in this way could alter their genomic structure (Allen *et al.,* 1983b; Studdert *et al.,* 1983; Studdert *et al.,* 1986; Meyer *et al.,* 1987; Sugahara *et al.,* 1994). The most extensive RFLP analyses were performed on strains of EHV-1 and -4 by Allen and co-workers (1983a). That study confirmed earlier ambiguous findings that both viruses could be placed into defined groups on the basis of loss or gain of restriction enzyme sites. It was demonstrated that greater genomic variability existed between EHV-4 strains relative to that of EHV-1 (Allen *et al.,* 1983a).

The epidemiological value of RFLP analysis was illustrated in two separate studies where in one it was possible to identify which were the dominant abortigenic strains in Kentucky (Allen *et al.,* 1985), and in the second it was possible to distinguish an abortigenic from a paralytic isolate during a single outbreak on the same farm (Studdert *et al.,* 1984). However, it must be stated that the latter study did not indicate whether the RFLPs identified were located in the repeat regions of the EHV-1 genome which can be inherently unstable.

The availability of the genomic map of a variety of EHV-1 strains and the entire DNA sequence of one EHV-1 strain should allow the retrospective localisation of these variable regions. Indeed recent studies involving Australian, Japanese, and British isolates revealed that many RFLPs of EHV-1 isolates are due to differences in the number of copies of repeated regions located at a number of

sites in the EHV-1 genome (Studdert *et al.,* 1992; Kirisawa *et al.,* 1993a; Bonass *et al.,* 1994; Matsumura *et al.,* 1994; Binns *et al.,* 1994; McCann *et al.,* 1995). These studies revealed that the genomes of the EHV-1 strains examined were quite conserved. However the latter RFLP study (McCann *et al.,* 1995) using restriction enzymes with 4bp recognition sites was able to separate EHV-1 strains into defined groups of epidemiological use, with the added bonus that all paralytic isolates resided in a single group.

Although RFLP analysis reflects variation at the DNA sequence level it cannot be expected to reveal all sequence variation between strains of EHV-1 and -4. In the paper describing the EHV-1 strain AB4 DNA sequence, variation was detected in different coding and non-coding regions between strain AB4 and other strains sequenced (Telford *et al.,* 1992). Similarly a comparison of the gene sequences from EHV-4 strains reveals inter-strain sequence variation (Telford *et al.,* 1998). It is worth noting that a few of the EHV-1 and -4 strains from which sequence data has been derived were passaged several times in non-equine cells.

Another study suggested sequence variation between EHV strains could affect detection of specific virus genes via PCR. Sharma and colleagues (1992) noted that the failure of some EHV-4 isolates and possibly some EHV-1 isolates to be detected by a gC gene specific PCR may be due to sequence variation across the primer sites in some isolates. Again it should be noted that the EHV-4 strain from which the primer sequences were derived i.e 1942, was passaged in non-equine cells.

1.3.5 DIAGNOSIS

After the initial clinical diagnosis of an EHV-1 or 4 infection confirmatory tests involving virus isolation, complement fixation (CF) tests and/or IIF are routinely employed. Recent advances in immunological and molecular techniques have resulted in a plethora of diagnostic methods for these viruses which may circumvent some of the disadvantages of older tests.

Immunological methods

Antibody to EHV-1 and -4 is routinely measured by CF, immunoflourescence (IF), and virus neutralisation (VN). A measurable rise in antibody titre as determined by CF is thought to be a sign of recent EHV infection. These tests suffer from three main disadvantages:

1. changes in antibody titre may be slow and not necessarily measurable, especially in older horses which have experienced a number of prior infections. McCartan and co-workers (1995) also demonstrated that foals infected with a highly virulent strain of EHV-1 did not develop significant increases in antibody titre,

2. the tests do not necessarily distinguish between EHV-1 and -4 infections, and

3. depending on the reagents used antigenic variants may not be detected (Thomson *et al.,* 1976).

The two latter disadvantages were overcome by employing pools of type-specific Mabs to detect either virus in IIF or enzyme immunofiltration assays (Yeargen *et al.,* 1985). A disadvantage of these types of tests is the requirement that the virus be grown and its antigens expressed in cell culture which may be time consuming or impossible.

An antigen capture system using uncharacterised Mabs was used to detect virus antigen in nasal swabs. The test although not particularly sensitive was fast and did not rely on the presence of infectious virus. Strain variation was not a problem with respect to EHV-1 detection but only a limited number of samples were examined (Sinclair and Mumford, 1992).

A recombinant GST fusion protein derived from EHV-1 gB was used as an antibody capture system in an ELISA. The recombinant protein contained an immunodominant domain and cross-reacted with serum from EHV-4 infected animals (Sinclair *et al.,* 1993a). Crabb and co-workers (1992 and 1995) used a similar system to express recombinant proteins derived from gG of both viruses which were of value in serologically distinguishing EHV-1 from EHV-4 infected horses. Retrospective analysis of EHV-1 infection in the Australian horse population

revealed the epidemiological value of this test (Crabb and Studdert, 1993). However its clinical usefulness remains to be determined especially in countries where widespread anti-EHV-1 vaccination of thoroughbred horses takes place. GST fusion proteins containing truncated forms of EHV-1 gC were also demonstrated to have diagnostic potential not unlike that of gG (Crabb and Studdert, 1995).

An immunoperoxidase assay was used to detect viral antigen in wax-embedded tissues (Whitwell *et al.,* 1992). This test is particularly useful for post-mortem diagnosis especially in cases where it is difficult to isolate the virus e.g from the CNS (Whitwell and Blunden, 1992).

A radial immunodiffusion enzyme assay was proposed as a potential field test for EHV-1 antibody by Gradil and Joo (1988).

Molecular methods

The first generation of molecular diagnostic techniques for detection of EHVs are not applicable to routine diagnosis because of the specialised techniques involved.

Morris and Field (1988) used cloned fragments of EHV-1 DNA to detect virus DNA in Southern and dot blots of tissue and cellular DNA. They could detect >1 genome/cell. Cross reaction between cellular DNA and the probes, particularly with dot blot analysis, could lead to erroneous results. The authors suggested that it might be possible to estimate the concentration of viral load and to detect the form of the viral DNA with their system.

A rapid method for isolating whole cellular DNA and subsequent analysis with labelled virus DNA allowed the detection and screening of a large number of isolates for RFLPs. The presence of replication intermediates could confuse results (Chowdhury *et al.,* 1986a).

In situ hybridisation with non-radioactive probes could detect EHV-1 DNA directly in infected tissue. This approach could not detect viral DNA in the CNS of horses with paralytic symptoms (Schmidt *et al*. 1994). This may be due to the

insensitivity of the technique or the fact that the study was not extensive since antigen was detected in the CNS in separate studies using different techniques.

The second generation of molecular diagnostic techniques are based on the polymerase chain reaction (PCR). These because of their sensitivity, speed, and relatively simple applicability may replace virus isolation as the standard diagnostic method for the EHVs and other viruses. However, it must be stressed that if PCR is to become the gold standard in diagnosis extreme care will have to be taken during its execution to avoid contamination.

A number of groups have favourably compared PCR and virus isolation with regard to sensitivity (Ballagi-Pordány *et al.,* 1990; Sharma *et al.,* 1992). The technique has been used to detect the virus in nasal secretions and a variety of tissues. A single tube PCR has been developed that can distinguish between EHV-1 and -4 (Kirisawa *et al.,* 1993b). Others have used the extremely sensitive technique of nested PCR to detect latent EHV-1 and EHV-4 in the CNS and lymphoid tissue (Welch *et al.,* 1992; Borchers and Slater, 1994; Slater *et al.,* 1994; Borchers *et al.,* 1997). PCR diagnosis may have limitations relative to virus isolation under certain circumstances. Firstly the small starting sample used for PCR may result in insensitivity especially if the clinical sample has a relatively large volume and is dilute in terms of virus concentration. Secondly PCR does not necessarily detect infectious virus which would require further specialisation of the procedure and thus make it less attractive as a clinical diagnostic tool.

1.3.6 LATENCY

Recent studies using PCR have definitively demonstrated latent EHV-1 and -4 infections in lymphoid tissue and to a lesser extent in the peripheral blood leukocytes (PBL) and trigeminal ganglion (TG) of ponies (Welch *et al.,* 1992). Subsequent studies demonstrated that the trigeminal ganglion is also a major site of EHV-1 and EHV-4 latency (Slater *et al.,* 1994; Borchers *et al.,* 1997). The authors of the latter study suggest that for EHV-1 at least the trigeminal ganglion is a more important source of reactivatable virus (Slater *et al.,* 1994). It must be stressed however that there were some key differences between both studies, in

particular the presence and potential role of EHV-2 in reactivating EHV-1 or -4 from cells of lymphoid origin. A survey of healthy animals at slaughter demonstrated a very high prevalence of latent EHV-1, -2 and -4 in both lymphoid tissue and to a lesser extent the trigeminal ganglion (Edington *et al.,* 1994). Indeed the presence of latency associated transcripts has been detected in PBLs but not the TG of horses with latent EHV-1 (Chesters *et al.,* 1997). However Borchers and colleagues (1997) utilised RT-PCR to demonstrate the presence of the EHV-4 gB transcript in some of the TG latently infected with EHV-4. It remains to be seen if the gB transcript actually is a product of virus reactivation and expression of late genes, low-level late gene transcription during latency or a left-over residual transcript from a lytic infection.

Latency is a source of great concern for the prevention and treatment of herpesvirus diseases, not least the EHVs. Induction of immunosuppression by the use of high doses of corticosteroids was initially used to demonstrate the presence of reactivatable latent/persistent EHV-1 and -4 infections (Edington *et al.,* 1985; Browning *et al.,* 1988). Previous to this circumstantial evidence suggested that as with other herpesviruses stress could lead to reactivation (Burrows and Goodridge, 1978). Thus latency represents a potentially important epidemiological source of infection which is made all the more serious by the findings that reactivated virus may be shed from the host in the absence of viraemia and overt clinical symptoms (Browning *et al.,* 1988; Slater *et al.,* 1994).

Chapter Two

Materials and Methods

2.1 MATERIALS

2.1.1 VIRUSES AND MAMMALIAN CELLS

Strains of EHV-1 utilised in this thesis were kindly provided by Prof. G. Allen (University of Kentucky, Lexington, USA) and Dr. M. Binns (Animal Health Trust, Newmarket, UK). EHV-1 strain AB4 was generously supplied by Dr. A. Davison of the Institute of Virology, Glasgow.

NBL-6 cells, a primary cell line derived from equine dermal cells were purchased from ICN Flow, High Wycombe, Buckinghamshire.

2.1.2 BACTERIA, NUCLEIC ACIDS, AND MOLECULAR WEIGHT MARKERS

E.coli strains JM109 and Invitrogen α F' were obtained from Promega, UK and Invitrogen, UK respectively.

Bluescript M13+ and lambda/HindIII, pUC19/TaqI, pUC19/Sau3AI molecular weight markers were obtained from Stratagene, UK.

High range protein molecular weight marker was supplied by Promega.

A BamHI library of EHV-1 strain HVS25A in pBR322 was kindly provided by Dr. J.M. Whalley (Macquarie University, Australia).

2.1.3 CHEMICALS AND EQUIPMENT

Chemicals and equipment for molecular biology were obtained from B.D.H chemicals Ltd, Sigma Chemical Co., Promega, LKB Pharmacia, or Applied Biosystems.

Radiochemicals were purchased from Amersham or ICN.

Immunochemicals were obtained from Dynatech labs. Ltd., Dako Ltd., or Novabiochem.

2.1.4 GROWTH MEDIA AND VESSELS

Medium and supplements for the propagation of mammalian cell lines were supplied by Gibco BRL Ltd. Tissue culture flasks were purchased from Costar.

Bacteriological media was expertly prepared by the Bacteriology section of the Department of Veterinary Pathology, University of Glasgow Veterinary School. Petri dishes were obtained from Sterilin Ltd.

2.2 METHODS

The following general techniques were an integral part of the experiments described in subsequent chapters of this thesis.

2.2.1 MAMMALIAN CELL CULTURE

Propagation of NBL-6 cells

Medium was decanted from the vessel in which the monolayer of cells was growing. A sufficient amount of 1x trypsin-EDTA was added to the vessel such that the monolayer was completely covered. The trypsin was removed and an identical volume of 1x trypsin-EDTA was added to the cells and left in contact with the monolayer until the cells had detached from the surface of the culture vessel. The trypsin was then inactivated by adding an equal volume of cell growth medium (table 2.1). Trypsinised cells were removed to a 20ml or 50ml conical centrifuge tube and centrifuged at 1000rpm for 5 mins. in a Beckman GPR centrifuge. The supernatant was removed and the cell pellet resuspended in cell growth medium. Appropriate fractions of the cells were added to new culture vessels as was a volume of cell growth medium such that the monolayer was covered by $1cm^2$ of medium. The new cell cultures were then placed in a humidified atmosphere containing 5% CO_2 at 37°C. Confluent monolayers were attained in approximately four days.

2.2.2 BASIC VIROLOGICAL TECHNIQUES

Estimation of virus titre

A 50% tissue culture infective dose ($TCID_{50}$) assay was used to determine virus titre. 200ul of the virus stock to be titrated was added to 1.8ml of virus titration medium (see table 2.1). 200ul of this dilution was transferred to another tube containing 1.8ml of virus titration medium. Ten fold serial dilutions of the virus stock down to 10^{-8} were made in this way. 1ml of each of these dilutions was transferred to separate tubes containing 2.2ml of cell growth medium, therefore the virus was also diluted from $10^{-1.5}$ down to $10^{-8.5}$. A 96 well flat bottomed microtitre dish was seeded by adding 150ul of NBL6 cells at a concentration of 10^5 cells/ ml of medium to each well. 100ul of each dilution was added to separate wells and this was done in quadruplicate i.e. four wells per dilution. The dish was covered and placed at 37°C in a humidified incubator containing 5% CO_2. The plates were monitored over a period of five days for cytopathic effects (CPE) of the virus. A score representing the number of wells per dilution exhibiting CPE was assigned to each dilution and the $TCID_{50}$ calculated using the Kärber formula (Kärber, 1931).

Preparation of virus stocks

Strains of EHV-1 were supplied either as tissue culture supernatants or in a freeze dried form. Those which were freeze dried were reconstituted by resuspension in 0.5ml of sterile distilled water. These master virus stocks were then aliquoted into sterile 1.5ml eppendorf tubes and stored at -70°C. Secondary virus stocks were prepared by infecting a monolayer of NBL6 cells in a $75cm^2$ flask with primary virus stocks at a M.O.I of 0.005 $TCID_{50}$ units per cell. The cells were monitored daily until 100% CPE was evident at which point the medium covering each monolayer was decanted into separate 30ml centrifuge tubes and clarified by spinning in a JA 21 rotor at 8000g for 10 mins. in a Beckman J2-21 centrifuge. The supernatants from each tube were aliquoted and stored as previously described for the master virus stocks.

A number of precautions were employed in the preparation of the virus stocks in order to prevent contamination of individual virus strains with other strains or with nucleic acids routinely manipulated within the laboratory. Only one virus strain was handled in a laminar flow safety cabinet at any given time. All instruments and surfaces were swabbed with 70% ethanol and left for 10 mins. prior to the handling of another virus. During this 10 min. period a sentinel control i.e an open microcentrifuge tube containing 20ul of H_2O was placed in the safety cabinet. The H_2O was then analysed by PCR (see section 2.2.5) in order to detect potential aerosol contamination. After the safety cabinet and instruments had been cleaned new latex gloves and disposable laboratory aprons were put on. Finally all viruses were aliquoted with positive displacement pipettes.

2.2.3 BACTERIOLOGICAL METHODS

Transformation of bacterial cells

There are many methods available for introducing DNA into bacteria–however commercially competent cells were routinely used. If commercially available competent bacteria were unobtainable for transformation then bacterial cells were made competent by electroporation.

One litre of L-broth was innoculated with 10ml of an overnight culture of the strain of E.coli to be transformed. The diluted culture was incubated at 37°C with vigourous shaking until the $O.D_{600}$ was between 0.5 and 1.00. The culture was placed in JA-15 centrifuge buckets and chilled on ice for 15 minutes. The cells were harvested by centrifugation at 4000g for 15 minutes in a pre-cooled centrifuge. As much as possible of the medium was removed and the cells resuspended in 1 litre of cooled sterile water and centrifuged as before. The water was removed and the cells resuspended in 500ml of cold water and again centrifuged as before. The cells were resuspended in 20ml of cold 10% glycerol and centrifuged. Finally the cells were resuspended in 2ml of cold 10% glycerol. At this stage the cells were snap frozen in a dry ice ethanol bath and stored in aliquots at -70°C or used directly for electrotransformation.

DNA which was to be transformed into bacterial cells by electroporation was first precipitated, washed twice with 70% ethanol, and resuspended in Milli-Q reverse endo-osmosis grade water in order to reduce the salt concentration of the DNA sample.

Cells if frozen were thawed on ice. 40ul of the cells were mixed with no more than 4ul of DNA in a polypropylene tube and left on ice for at least a minute. The Biorad gene pulser apparatus was set at 25uF and 2.5kV. The pulse controller also from Biorad was set at 200 ohms. The DNA and cells were placed between the electrodes at the bottom of a 0.2cm electroporation cuvette which had been prechilled on ice. Excess moisture was wiped from the cuvette prior to placing it in a safety chamber slide which was then pushed into the chamber such that the cuvette electrodes made contact with the electrodes of the gene-pulser. A pulse with a time constant of 4-5msec was delivered to the cells and DNA. The cuvette was removed from the chamber and the cells immediately resuspended in 1ml of cold L-broth. The cell suspension was transferred to a 15ml polypropylene tube and then incubated for 1 hour at 37^{o}C with vigorous shaking. 50ul, 100ul, and 200ul aliquots of cells were plated on medium selective for the transformed phenotype. In most cases the phenotype was ampicillin resistance encoded by plasmid cloning vectors routinely transformed into the bacteria. Transformations were therefore plated on to L-agar containing 50ug/ml of ampicillin and left to grow overnight at 37^{o}C.

Storage of bacterial clones

Bacteria containing plasmid clones were grown overnight at 37^{o}C with vigorous shaking in L-broth containing 50ug/ml of ampicillin. 1ml of the overnight culture was mixed with 1ml of 25% glycerol solution made up in L-broth. The mixture was vortexed and aliquoted into eppendorf tubes. The tubes were snap frozen in a dry ice ethanol bath and then immediately transferred to -70^{o}C where they were stored until required.

2.2.4 PURIFICATION AND SEPARATION OF NUCLEIC ACIDS

Small scale preparation of plasmid DNA

Plasmids were isolated from E.coli using a method described by Jones and Schofield (1990).

3ml of L-broth containing 50ug of ampicillin per ml was inoculated with a single colony harbouring the plasmid of interest, and incubated at 37°C overnight in an orbital incubator shaking at 225rpm. The next day 1.5ml of the overnight culture was decanted into an eppendorf tube and spun at 14,000rpm for 2min. The clarified broth was carefully and completely removed from the bacterial pellet. The pellet was resuspended in 150ul of solution one (table 2.1). Freshly prepared solution two (300ul) (table 2.1) was then added, and the contents of the tube gently mixed by inversion before placing it on ice for 5 minutes. Solution three (table 2.1) (225ul) was then added, the contents mixed as before, and then placed on ice for a further 5 minutes. The precipitate formed was pelleted by centrifugation at 14,000rpm for 5 minutes. The clarified supernatant was removed to a clean tube and the pellet discarded. An equal volume of absolute alcohol was added to the supernatant which was then briefly shaken prior to centrifugation at 14,000rpm for 5 minutes. The ethanol was then removed and the pellet washed with 70% ethanol before a final centrifugation at 14,000rpm for 2 minutes. The supernatant was discarded and the pellet was dried briefly in a vacuum dessicator. The pellet was resuspended in 30ul of sterile distilled water.

Direct purification of PCR products

The Promega Magic™ PCR DNA purification system was used for purification of PCR products directly from PCR reaction mixes. The protocol recommended by the manufacturer is outlined here.

The PCR reaction was brought to a final volume of 300ul with H_2O and 100ul of direct purification buffer was then added. After the addition of 1ml of the purification resin, the complete mixture was vortexed. The mixture was then loaded

into a 2ml syringe that was attached to a magic minicolumn. The syringe plunger was inserted and the liquid was slowly pushed through the minicolumn. The minicolumn was then washed with 2ml of 80% isopropanol. Residual isopropanol was removed by spinning the minicolumn at 14,000rpm for 20 seconds before leaving it to air dry at room temperature for 10 minutes. 50ul of H_2O was placed on the resin bed in the minicolumn and left for at least 1 minute. The nucleic acid eluted into the H_2O was removed from the minicolumn by centrifugation at 14,000rpm for 30 seconds and collected in a microcentrifuge tube.

PCR products purified in this way were suitable for most molecular manipulations.

Agarose gel electrophoresis of nucleic acids

Routine molecular manipulations of nucleic acids were visualised by horizontal agarose gel electrophoresis. The concentration of agarose and the dimensions of the gel used were determined by the size range and, to a lesser extent, the concentration of nucleic acids being separated.

The desired quantity of electrophoresis grade agarose was dissolved in 1xTBE (table 2.2) by heating in a microwave oven. When all the agarose had dissolved ethidium bromide was added to a concentration of 0.5ug/ml. The agarose was allowed to cool to approximately 60°C with periodic agitation. Preformed moulding trays were used to cast the gel slabs, some of which had open sides which required sealing with autoclave tape. Cooled agarose was poured into cleaned trays. Combs were inserted into their slots on the trays and bubbles removed using a pipette tip. The gel was left undisturbed to solidify after which it was submerged in 1xTBE in the electrophoresis chamber. The combs were carefully removed. 6x loading dye (table 2.2) was diluted with nucleic acid samples to 1x and the samples were then loaded into the wells formed by the combs. A molecular weight marker was also loaded into one of the wells. A voltage was then applied to the chamber such that the nucleic acids migrated through the longest portion of the gel towards the anode electrode. Gels were run for 1.5 to 3hrs at 50-80mA such that

sufficient separation of the nucleic acids was achieved. Gels were then viewed under a UV transilluminator and a photograph taken if desired.

The concentration of electrophoresed DNA was estimated by comparing the intensity of the DNA band of interest with a band of similar size in the molecular weight markers which were normally run in parallel with the DNA of interest. Since a known concentration of M.W marker is electrophoresed the quantity of the DNA in the sample can be extrapolated from this.

Purification of DNA from agarose gels

Three different methods of purification methods were used:

1. The Promega Magic prep system.

2. The Bio 101 geneclean system.

3. The Pharmacia sephaglass bandprep system.

Although all systems were routinely used the latter was preferable since it gave the most consistent results in my hands. Subsequently it is described here in more detail. Detailed protocols for the other methods can be found in the manufacturer's literature.

The band of interest was cut from the agarose after electrophoresis with a sterile scapula and transferred to a microcentrifuge tube. A pipette tip was used to mash the agarose plug into smaller pieces. The weight of the excised agarose plug was estimated and 1ul of gel solubilizer/mg of agarose was added to the plug. After vigourously vortexing the contents of the tube it was placed at 60^{o}C for 5-10 minutes in order to melt the agarose. 3ul of sephaglass was then added and the tube gently agitated for 5 minutes. The tube was spun at high speed for 1 minute in a microcentrifuge and the supernatant carefully removed so that the sephaglass pellet was undisturbed. The pellet was resuspended in 200ul of wash buffer and centrifuged as before. This was repeated twice in order to clean the pellet. The tube was inverted over a paper towel and left to dry for 10 minutes. The pellet was resuspended in 15ul of elution buffer and the tube agitated for 5 minutes.

After centrifugation for 1 minute the supernatant was removed to a clean tube and the pellet subjected to an identical elution process. The supernatants were pooled and the quantity of recovered DNA estimated by agarose gel electrophoresis of a small aliquot.

Denaturing polyacrylamide gel electrophoresis

The products of sequencing reactions described in the next section are separated by denaturing PAGE. The LKB macrophor system was employed for the assembly and operation of denaturing PAGE. A detailed description of this system is described in the manufacturer's (Pharmacia) literature. The following is a general outline of the protocol.

The plates, combs, spacers and wedges were cleaned with detergent followed by 70% alcohol in order to ensure the removal of as much dust as possible. The thermostatic plate was then treated with repel silane and the back plate with bind silane. The spacers, wedges, and thermostatic plate were assembled together. A denaturing gel solution (table 2.2) containing 6% acrylamide was freshly prepared. After addition of TEMED and 10% APS to the acrylamide the back plate was brought into contact with the spacers and thermostatic plate and the gel poured according to the manufacturer's instructions. The combs were inserted to the desired level and the gel left to polymerise for 15 minutes.

The gel sandwich was clipped in a vertical position to the macrophor apparatus such that the back plate was flush against the buffer reservoirs and the thermostatic plate tubing was then attached to the thermostatic circulator. The buffer chambers were filled with 1xTBE and the combs were carefully removed. Excess gel was trimmed away from the top of the wells formed by the combs. Unpolymerised acrylamide was flushed from the wells and the entire apparatus was checked for leaks prior to application of a voltage. The circulator was switched on and set such that the gel was maintained at a constant temperature of 55^{o}C. A constant voltage of 2000V was applied and the gel allowed to pre-run for 15-30 minutes before loading samples.

Sequencing reactions were placed at 75°C for 3 minutes prior to placing them on ice. Reactions were then quickly loaded into respective wells and electrophoresed at 2000V for 1.5-3 hours. After electrophoresis the system was dismantled and the plates eased apart. The gel adhering to the plate previously treated with bind-silane was fixed in 10% acetic acid /10% methanol, dried, and exposed overnight to autoradiographic film. The film was developed in an X-omat automatic developer.

Elution of oligonucleotides

Oligonucleotides were synthesised on an in-house DNA synthesizer and provided on a column. The following is the protocol used for elution of an oligonucleotide from a column and removal of protecting side chains from an oligonucleotide.

2ml of 30% ammonia solution were drawn into a 3ml syringe. This syringe was attached to one end of the column and an empty syringe to the other end. The column was flushed four times over a period of one hour by repeatedly pushing the ammonia back and forth through the column with the syringes. When flushing was not in progress the ammonia solution was left in contact with the column. The ammonia solution was aliquoted into screw cap microcentrifuge tubes after the final flush. The caps were screwed tightly on to the tubes and sealed with parafilm. Side chains on the oligo were removed by incubating the eluted oligonucleotide overnight at 55°C.

The deprotected oligonucleotide was then placed on ice for ten minutes. One tenth the volume of 5M NaCl and 2.5 volumes of ethanol were added and the oligonucleotide precipitated at -70°C for 10 minutes. The precipitated DNA was collected by centrifugation at 14,000rpm for 15 minutes and washed with 70% ethanol. The DNA was resuspended in a total volume of 1ml of sterile dH_2O.

The concentration of the purified oligonucleotide was estimated by UV spectrophotometry. The oligonucleotide was diluted 1/100 in a total volume of 500ul of dH_2O and the O.D of this dilution measured at 260nm. The absorbancy value was multiplied by a factor of 2 to give the concentration of oligonucleotide in ug/ul.

2.2.5 ENZYMATIC MANIPULATIONS OF NUCLEIC ACIDS

Restriction enzyme digestion of DNA

Most digestions were carried out in a total volume of 20ul. The required amount of DNA was added to a microcentrifuge tube. Water was added such that the final reaction volume was 20ul when all the reactants were present. One tenth the volume of the appropriate 10x restriction enzyme buffer was added. In the case of plasmid preparations (see section 2.5) 0.5ul of RNase one was added. The contents of the tube were mixed and briefly centrifuged. Restriction enzyme(s) (5-10 units) were added and the contents of the tube mixed and spun as before. Digestions were incubated for 1-2 hrs at the appropriate temperature after which time one fifth of the reaction was examined by agarose gel electrophoresis. If digestion was not complete more restriction enzyme was added and the reaction was left to incubate overnight.

Ligation of plasmid DNA with insert DNA

Plasmid DNA which had been suitably modified for cloning was mixed with insert DNA at a molar ratio of 1:100. 50-200ng of plasmid DNA was normally used. 10x ligation buffer (1ul) was added and the entire reaction volume was brought to 9ul with water. The contents of the tube were mixed and centrifuged then left on ice for 5 mins. T4 DNA ligase (10 units) was added and the contents of the tube mixed as before. The ligation was incubated at 12^{o}C overnight. Part of the ligation reaction was transformed into commercially competent E.coli Invitrogen α F' cells according to the manufacturer's instructions.

Nucleotide sequence analysis

The DNA sequences of plasmid DNAs were determined using the dideoxy chain termination method (Sanger *et al.*, 1977).

Single stranded DNA (ssDNA) was prepared from plasmid DNA by incubating 9ul of plasmid prepared as outlined in section 2.2.4 with 1ul of 2M NaOH for 15 mins at 37°C. After neutralisation with 3ul of solution three (table 2.1) 80ul of 100% ethanol was added and the ssDNA precipitated at -70°C for 10 mins. ssDNA was collected by centrifugation, washed with 75% ethanol, and vacuum dried. The DNA was sequenced with either the Sequenase or T7 DNA polymerase kits. The latter is described in more detail here since it was the preferred DNA sequencing system, however both are essentially the same.

ssDNA was resuspended in 10ul of H_2O. 2ul of primer at 5pmol/ul was added to the ssDNA as was 2ul annealing buffer. This annealing reaction was incubated at 37°C for 15 mins.

The annealed primer/template complex was radioactively labelled by the addition of 3ul of labelling mix, 1ul of [a-^{35}S]dCTP (370MBq/ml), and 2ul of diluted enzyme containing 3.2 units of T7 DNA polymerase. The labelling reaction was allowed to proceed for 5 minutes at room temperature. As the labelling reaction proceeded 2.5ul of each of the four chain termination mixes were aliquoted and prewarmed to 37°C. The labelling reaction was terminated by adding 4.5ul aliquots of it to each of the four termination mixes which were then left at 37°C for 5 minutes. The termination reactions were stopped by adding 5ul of stop solution. Sequencing reactions were analysed by denaturing PAGE (section 2.2.4).

PCR amplification

Saiki *et al* (1988) described the amplification of specific DNA sequences from a complex and heterogenous biological sample by the PCR. In this thesis much of the PCR was performed on clarified virus particles.

All PCR reaction mixes were prepared outside the laboratory in an EHV free area. Perkin Elmer geneamp kits were the source of the main reaction components. A reaction typically contained 1xPCR reaction buffer, 1.5mM $MgCl_2$, 50pmoles of each of the primers, 50uM of each of the dNTPs, and 2 units of amplitaq DNA polymerase in a total volume of 40ul. 10ul of virus containing approximately 10^3 $TCID_{50}$ units of virus was boiled for 10 minutes in order to release viral DNA

from the particles. Virus was added to the reaction mix which was then overlayed with mineral oil. The assembled PCRs were subjected to the following thermal cycle on a Hybaid thermal block; 95°C for 30 seconds, 48°C for 10 seconds, and 72°C for 60 seconds, repeated 25 times and followed by 72°C for 5 minutes. Variations in any of these parameters are alluded to throughout the thesis.

When the cycles were completed the overlay was removed and an aliquot of the PCR examined by agarose gel electrophoresis.

Because of the extreme sensitivity of this technique great effort was made to reduce the possibility of contamination. As referred to earlier, reaction mixes were prepared in a separate laboratory free from potential sources of contaminaton. Positive displacement pipettes were routinely used and gloves were changed between samples. Since reaction mixes were prepared in bulk a primer control was included which consisted of virus/target free water being added to one of the PCRs prior to the addition of virus to the others. A positive primer control PCR would indicate inadvertent contamination of reaction mixes with target DNA during preparation. Sentinel controls were included when multiple samples were being processed for PCR. These controls consisted of an aliquot of virus free water, one for each sample being processed. They were handled between each sample and the next in a manner identical to the manipulation of each sample before, during, and after the PCR. Positive sentinel control PCRs indicated cross contamination of samples. In general positive controls were avoided unless PCRs failed. The target in this case was normally 10ng of plasmid cloned target DNA.

Radiolabelling of DNA

The chosen method for labelling DNA was the random priming method developed by Feinberg and Vogelstein (1983). The Pharmacia Oligolabelling kit which incorporated this method was used.

25-50ng of DNA was heat denatured at 100°C for 2-3 minutes and then placed on ice for a further 2 minutes. The following was added to the denatured DNA–10ul of reagent mix, 5ul of a-P^{32}dCTP(3000Ci/mmol), and sterile dH_2O such that the final reaction volume was 50ul. 1ul of Klenow fragment of E.coli

DNA polymerase I was added and the reaction mixed and centrifuged briefly. The labelling was allowed to proceed at 37°C for 60 minutes. The labelled DNA was used directly as a probe (see section 2.2.6) without any further purification.

2.2.6 SOUTHERN TRANSFER AND HYBRIDISATION ANALYSIS OF DNA

Southern transfer of DNA

DNA was transferred to nylon membranes by a modification of a method first described by Southern (1975).

After the desired degree of separation of DNA was achieved by agarose gel electrophoresis the nucleic acid was denatured *in situ* by immersing the gel in a solution of 0.4M NaOH/0.6M NaCl for 30 minutes. The membrane was soaked in this solution and placed on a glass plate. All air bubbles were removed from between the plate and membrane before the gel was placed on the membrane. Air bubbles were removed from between the gel and membrane. Prior to preparing the membrane and gel the following assembly was constructed. A glass plate was placed on an even surface. A wad of tissue paper was placed on the glass plate. Finally 5 pieces of 3mm whatman paper of slightly larger surface area than the gel was placed on the tissue. The gel and membrane were allowed to slide carefully from the glass plate on to the whatman paper such that no air bubbles became trapped between the membrane and whatman paper. The glass plate was then placed on top of the gel and a 200g weight placed on the glass plate. This assembly was left overnight such that the DNA was drawn down onto the membrane by capillary action.

The filter was neutralised by rinsing it extensively in 50mM Sodium Phosphate buffer (pH 6.5) (table 2.2). The membrane was placed briefly on 3mm whatman paper to remove excess liquid and then allowed to air dry. The membrane was wrapped in aluminium foil and baked at 80°C for 2 hours in order to fix the DNA to the membrane.

Hybridisation of radiolabelled DNA to membrane bound DNA

QuickHyb solution from Stratagene was used as the hybridisation medium since it has the advantage of allowing quick hybridisations to be performed.

QuickHyb solution (4ml) was placed in a hybridisation bottle and heated to 68°C in a hybridisation oven. The membrane containing the fixed DNA was rinsed in deionised H_2O, placed in the bottle containing the QuickHyb and prehybridised at 68°C for 20 minutes.

Radiolabelled probe (5ul) was added to 100ul of salmon sperm DNA (10mg/ml) and boiled for 2 minutes. 1ml of the prehybridisation solution was removed from the bottle and the denatured probe mixed with it before returning it to the bottle. Hybridisation was allowed to proceed at 68°C for 1 hour.

After hybridisation the membrane was removed from the bottle and rinsed with 2xSSC buffer containing 0.2%SDS. The bottle was also rinsed with the same buffer in order to remove the hybridisation solution. The membrane was returned to the bottle and washed twice for 15 minutes at room temperature with the aforementioned buffer. If required a high stringency wash for 30 minutes at 60°C with 0.1xSSC buffer containing 0.1%SDS was performed.

The membrane was wrapped in saran wrap and exposed to an autoradiographic film with an intensifying screen at -80°C overnight. The film was developed in an X-omat automatic processor and reexposed if necessary.

Chapter Two

Tables

REAGENTS FOR GROWTH AND PROPAGATION OF ANIMAL CELLS, VIRUSES AND BACTERIA		
Cell growth medium	**Virus titration medium**	**L-broth**
MEM containing 10% FCS, 2mM Glutamine, 1x MEM non-essential amino acids, Pencillin/Streptomycin and fungizone.	Identical to cell growth medium except FCS is at a concentration of 2%.	20g Tryptone 20g NaCl 10g Yeast extract Add dH_2O to 2l Adjusted to pH 7.0 with NaOH. Autoclaved.
Ampicillin 0.5g of ampicillin dissolved in 10ml of sterile dH_2O and filter sterilized through a 0.2 micron filter, aliquoted and stored at -20°C.		

SOLUTIONS FOR PLASMID MINIPREPARATIONS		
Solution one	**Solution two**	**Solution three**
25mM Tris HCL pH 8.0 10mM EDTA 50mM Glucose The Tris HCL/ EDTA solution was autoclaved, the required amount of glucose was added and the solution filter sterilised with a 0.2 micron filter.	This solution was prepared fresh by mixing equal volumes of 0.4M NaOH and 2% SDS.	60ml 5M Potassium Acetate 11.5ml glacial acetic acid 28.5ml sterile dH_2O.

Table 2.1: Solutions for cell culture and plasmid minipreps.

ELECTROPHORESIS REAGENTS		
10x TBE	**6x Loading dye**	**Sequencing gel mix**
216g Tris base 110g Boric acid 100ml 0.5M EDTA Added dH_2O to 2l	0.25% BPB 0.25% Xylene cyanol FF 30% Glycerol	8.8ml 38% Acrylamide:2% Bisacrylamide 25.2g Urea 6ml 10x TBE When the Urea had dissolved the solution was brought to 60ml with dH_2O

SOUTHERN BLOT ANALYSIS SOLUTIONS	
50mM Sodium Phosphate pH 6.5	**20xSSC**
4.45g Na_2HPO_4 in 800ml Adjusted to pH 6.0 with conc. H_3PO_4 Added dH_2O to 1l	173g NaCl 88.2g Sodium Citrate, trisodium salt. Adjusted pH to 7.0 with HCl. Added dH_2O to 1l

Table 2.2: Solutions for electrophoresis and Southern blotting.

Chapter Three

Inter-strain sequence analysis of the N-terminal coding region of the gC gene of EHV-1

3.1 INTRODUCTION

Chapter one described the disease manifestations associated with EHV-1 infection of the horse. Of the possible outcomes of EHV-1 induced pathology, paralytic disease remains one of the most enigmatic as the following summary of research in this area will demonstrate.

Saxegaard (1966) was the first to isolate EHV-1 from a horse with paralysis. However, this study and others (Thorsen and Little, 1975; Charlton *et al.,* 1976; Little and Thorsen, 1976; Thein, 1981; Meyer *et al.,* 1987) describing successful virus isolation from the CNS may have been compromised by the fact that CNS samples were derived from viraemic animals and therefore may have been contaminated at slaughter by virus from the blood vessels supplying the CNS (Platt *et al.,* 1980). Furthermore, it has not been possible to reproduce paralytic disease by direct innoculation of the virus into the CNS (Prickett and Bryans, 1969), in contrast to results obtained via systemic infection which resulted in the fulfilment of Koch's postulates in that EHV-1 is an aetiological agent of paralytic disease in the horse (Jackson and Kendrick, 1971; Jackson *et al.,* 1977). Subsequent studies have demonstrated viral antigen, and to a lesser extent infectious virus, to be present in a few vascular endothelial cells within the CNS of affected animals (Jackson *et al.,* 1977; Edington *et al.,* 1986; Whitwell and Blunden, 1992; Blunden and Whitwell, 1993).

Pathological examination of experimentally induced and natural EHV-1 associated paresis in the equid has demonstrated that damage to the vasculature of the blood vessels supplying the CNS is the most common feature (Jackson *et al.,* 1977; Platt *et al.,* 1980; Edington *et al.,* 1986; Whitwell and Blunden, 1992; Blunden and Whitwell, 1993). This may be accompanied by thrombosis and is thought to lead ultimately to oxygen starvation of the surrounding tissues (Platt *et al.,* 1980; Edington *et al.,* 1986). Although direct damage to nervous tissue, which can vary in extent and location, is seen post-mortem (Allen and Bryans, 1986 and references therein) it is always associated with vasculitis and is most likely preceeded by it (Jackson *et al.,* 1977).

Outbreaks of EHV-1 associated paresis can occur in isolation or can be associated with outbreaks of abortigenic or respiratory disease (Allen and Bryans, 1986). Given the relatively rare and sporadic occurrence of the disease two theories have been postulated as to how and why it happens. The first theory suggests the existence of a spectrum of virulence in the natural population of circulating strains of EHV-1 i.e highly virulent strains which may not be common induce high rates of abortion and severe neurological symptoms, while those at the other end of the virulence scale rarely cause abortion or paralysis and are mainly associated with respiratory disease. The second theory purports that host factors may determine a given horse's or horse population's predisposition to paralytic disease regardless of the infecting virus (Allen and Bryans, 1986; Meyer *et al.,* 1987).

Two studies have suggested that mares that have recently given birth present symptoms of greater severity relative to other animals in a given outbreak (Greenwood and Simpson, 1980; McCartan *et al.,* 1995). Jackson *et al.,* (1977) suggested that in mares pregnancy may be a predisposing factor to EHV-1 induced paralytic disease. Hormonal immunosuppression was not considered to be a contributory factor since all of the affected mares were at an advanced stage of pregnancy when immunosuppressive hormones return to pre-pregnancy levels (Jackson *et al.,* 1977). Indeed evidence suggests that the immune system plays an active role in neurological disease associated with EHV-1. Inflammation of affected areas in the CNS led Jackson and Kendrick (1971) to propose an immunopathological mechanism. Previous exposure to virus is thought to result in a faster and potentially stronger response to reinfection which might predispose immunologically experienced animals to neurological symptoms (Dinter and Klingeborn, 1976). Of relevance to this theory is the demonstration that some newborn foals with CNS pathology do not present with clinical signs of disease (Whitwell and Blunden, 1992) and that SPF foals demonstrated clinical manifestations only after reactivation of latent virus (Gibson *et al.,* 1992a). Conversely, neurological disease has been demonstrated in foals aged two to nineteen weeks. In this study mild symptoms of disease were seen in five out of twenty five foals. Most foals tested positive for EHV-1 neutralising antibodies (Greenwood and Simpson, 1980).

Failure to isolate virus from affected tissue has been attributed to the presence of high levels of antibodies leading to the suggestion that the lesions were a consequence of immune complex deposition (Jackson *et al.,* 1977; Platt *et al.,* 1980). Immune complexes were demonstrated in animals with EHV-1 associated paresis but were detected at high levels only after the onset of pathological changes (Edington *et al.,* 1986). Indeed the role of immune factors in the development of CNS disease remains unclear in that it is yet to be unequivocally established whether or not they are a cause or an effect.

Further evidence for the involvement of host factors in the development of CNS disturbances came from the singular isolation of EHV-4 from the brain of an animal that had neurological disease (Meyer *et al.,* 1987). It may have been that the animal was unusually susceptible to neurological disease or that the virus in this case was a highly virulent strain of EHV-4, or conversely that the virus in question had nothing to do with the clinical symptoms.

The relatively rare occurrence of EHV-1 induced neurological disease has led to the suggestion that the ability to induce paralytic disease is a property of some strains of EHV-1 but not of others. Potential experimental support for this assumption came from a single study in which RFLP analysis distinguished between an isolate from a horse with paralysis and an isolate from a mare which aborted during an outbreak of EHV-1 disease on the same farm (Studdert *et al.*, 1984). This study however cannot be considered as definitive evidence for the existence of specifically neurovirulent strains since (1) one isolate may have been reactivated from latency by the superinfecting virus and may have had nothing to do with the observed pathologies, or (2) the infecting virus may have altered its genomic structure on *in vivo* passage thus giving rise to the different isolate or (3) the infecting inoculum may have contained more than one strain of virus. The two isolates differed with respect to the mobility of certain restriction fragments with at least two of the restriction enzymes utilised. This would suggest that the differences between the isolates noted within this study were confined to regions containing repetitive elements. Matsumara and colleagues (1994) demonstrated that repetitive regions were unstable on *in vivo* passage. One study has distinguished between two abortigenic isolates from the same farm on the basis of RFLP analysis. Two of the polymorphic sites used to distinguish the isolates resided in reiterated

sequences. The authors suggest that recrudescence of a latent strain or mixed inoculums may account for the observed differences between the isolates (McCann *et al.*, 1995).

A singular study has given credence to the possibility that a spectrum of pathogenicity may indeed exist in the natural EHV-1 population. This study demonstrated that a low-passage EHV-1 isolate had reduced pathogenic potential in that it has not been associated with paralytic disease and has a relatively weak abortigenic potential (Mumford *et al.*, 1994). The isolate, V592, was isolated from a foetus during an abortion storm and passaged twice on equine embryonic lung cells prior to analysis. This isolate is not endotheliotropic and does not induce the same level of non-specific immunosuppression in ponies as that induced by more virulent strains of EHV-1 (Hannant *et al.*, 1991). Obviously, more EHV-1 isolates would have to be assessed in this way i.e in the equid, in order to extend these findings.

There is evidence to suggest that wild-type HSV-1 isolates have different neuropathogenic abilities in animal model systems. Obviously the neuropathogenic potential cannot be assessed in the natural host and it is difficult to draw parallels between the natural situation and animal models. A similar situation exists with respect to EHV-1 in that EHV-1 strains which are neurovirulent in available animal models are not necessarily so in the horse and vice-versa (Chowdhury *et al.*, 1986b; Palfi and Christensen, 1995). Van Woensel *et al*. (1995) have developed a reliable mouse model system based on EHV-1 induced weight loss in mice for determining the pathogenic potential of a given virus in the horse. Whether it is also a reliable indicator of pathogenic potential with regard to the CNS remains to be seen.

A clear picture does not emerge from the data regarding EHV-1 induced paralysis. The research described in this chapter concentrated on the possibility of identifying a marker which could distinguish between paralysis-inducing and non-paralysis-inducing strains of EHV-1. In contrast to HSV-1 and other α herpesviruses it has not been possible to consistently demonstrate productive viral replication in the CNS of affected horses or animal models. Certainly studies of EHV-1 latency and reactivation would suggest that this virus is indeed neurotropic and has the ability to multiply in the CNS, but pathological examination of animals

with EHV-1 induced paralytic syndrome, as summarised in the preceding paragraphs, suggests that EHV-1 neurovirulence is not as a direct result of viral multiplication in neuronal tissue. It follows that EHV-1 is not an example of classic α herpesvirus neurovirulence, therefore focusing on previously identified HSV/PRV neurovirulence factors was considered inappropriate for this study.

This chapter describes the DNA sequence analysis of the gC gene of isolates associated with neurological disease and potentially non-neuropathogenic isolates of EHV-1.

An anti-EHV-1 gC monoclonal antibody was initially used to locate the region encoding the gC gene in a λgt11 EHV-1 expression library (Allen and Yeargen, 1987). Detailed analysis of this region led to the elucidation of the coding sequence of the EHV-1 gC gene (Allen and Coogle, 1988). The open reading frame (ORF) contains 1404bp which encode a protein containing 468 amino acids. However the predicted size of this protein i.e. 50.8K does not correlate with the 87-96K protein recognised by anti-gC Mabs. Indeed further analysis of the translated gene indicated that the protein would undergo extensive post-translational modifications. Hydropathic analysis of the amino acid sequence illustrated features typical of a membrane inserted glycoprotein. These features include (1) a signal sequence contained within the first 30 amino acids followed by (2) a region 397 amino acids long of extended hydrophobic and hydrophilic regions corresponding to the transmembrane domain, which contains 9 potential N-linked glycosylation sites, (3) a short region containing a potential hydrophobic membrane insertion site and (4) a positively charged cytoplasmic tail.

A comparison of EHV-1 and -4 gC protein sequences indicated that the positions of 9 glycosylation sites and 6 cysteine residues were conserved. However a comparison of all the herpesvirus gC sequences that have been determined to date revealed that it is quite an evolutionary divergent protein (see figure 3.1) (Nicolson and Onions, 1990 and references therein). The level of homology between the EHV-1 and -4 gC homologues is 79% (Nicolson and Onions, 1990). At the onset of this study, gC was considered to be second most divergent glycoprotein of EHV-1 and -4, gG being the first (Crabb *et al.,* 1992). Indeed Allen and co-workers (1988) extended the scope of this divergence by suggesting that 85% of the gC epitopes on EHV-1 and -4 also show sequence divergence. The divergent

N-terminus of the EHV-1 gC homologue was shown to contain an immunodominant region in addition to six other distinct epitope containing regions.

The aforementioned data, together with the high degree of interstrain antigenic variation identified in EHV-1 gC, led Allen *et al* (1988) to propose that this glycoprotein "contributes to the adaption of the particular EHV-1 subtype (EHV-1) to its unique ecological niche within the horse". Thus the N-terminal coding region of the EHV-1 gC gene became the focus of this study as it was thought that if gC did indeed reflect the unique internal environments that EHV-1 encountered within the horse, then determination of interstrain sequence variation, particularly within the N-terminal coding region of the gC gene, might reveal markers of a given strains pathogenicity since strains of differing pathogenicity may have experienced different internal environments.

Other reasons led to the supposition that the gC gene may contain a potential EHV-1 neurovirulence marker.

In vivo observations suggested that neuropathogenic EHV-1 isolates may preferentially infect endothelial cells of blood vessels supplying the CNS of affected animals. gC might be a determinant of endotheliotropism since the gC homologue of HSV-1 may determine the ability of certain HSV-1 strains to infect the apical surface of bi-polar cells *in vitro* (Sears *et al.,* 1991; Griffiths *et al.,* 1998). Of relevance to this is the finding that the gC homologue of PRV can determine whether or not PRV can infect a given cell type (Zsak *et al.,* 1992). Limited variation was detected in the protein and DNA sequences of the gC homologue of epidemiological distant strains of PRV (Ishikawa *et al.,* 1996) and HSV-2 (Terhune *et al.,* 1998) however these differences could not be correlated with differences in pathogenicity.

HSV-1 has been implicated as a potential co-factor in the development of atherosclerosis in humans, a condition which ultimately leads to thrombosis in the affected blood vessels. gC may focus the complement and coagulation cascades at affected areas and so ultimately lead to thrombosis (For a review see Hajjar, 1991). The pathology of EHV-1 induced CNS disease is thought to involve thrombosis in the blood vessels supplying the nervous system.

As referred to earlier, Allen *et al*. (1988) described antigenic variation in gC of EHV-1 strains but no linkage between antigenic variation and neurovirulence was observed. It is unlikely that Mab typing will detect all possible inter-strain variations in gC and therefore a potential marker may not be identified. As a result the DNA sequencing approach may be considered superior to RFLP and Mab typing analysis because all possible variations in the region analysed will be detected. The nucleotide sequence of the entire gC gene of four EHV-1 strains AB-4 (Telford *et al.,* 1992), KyA (Matsumura *et al.,* 1993), T431 (Allen and Coogle. 1988; Guo *et al.,* 1989) and HVS25A (B. Crabb. Personal communication) has been determined independently by other groups and has been shown to contain DNA sequence variation (see figure 3.2). KyA is relatively divergent from the other strains but this is most probably due to the passage of the virus in non-equine cells. HVS25A differs from AB-4 by at least two nucleotides, one of which results in a conserved amino acid change; however the passage history of this virus is unclear, although it is known that it is a low passage isolate. T431 differs from AB-4 at two nucleotide positions but neither of these differences results in an amino acid change.

3.2 EXPERIMENTAL APPROACH

So as to generate comparitive sequence data for the gC gene of EHV-1 strains a PCR assay was developed in order to amplify the first 700bp of the N-terminal coding region of the EHV-1 gC gene. After surmounting initial difficulties PCR products were obtained for nine EHV-1 isolates. A TA-cloning vector was generated for the purposes of subcloning the PCR products for sequencing analysis. At least three cloned PCR products were sequenced for each strain. All strains except T431 had identical DNA sequences. The entire gC gene of one strain, T373, was sequenced using a similar PCR based strategy in order to determine if any C-terminal sequence variation could explain anomalies concerning that strain.

A stringent control system was applied throughout this study to guard against the possibility of contamination at the different stages of virus preparation for and during PCR.

3.3 MATERIALS

3.3.1 VIRUS ISOLATES

The virus isolates utilised in this chapter were kindly provided by Professor George Allen and Dr. Matthew Binns of the University of Kentucky, U.S.A and the Animal Health Trust, U.K. respectively (see table 3.1).

3.3.2 OLIGONUCLEOTIDE PRIMERS

PCR primers were obtained from Applied Biosystems, U.K. and sent directly to Dr. E. Hall, Stirlingshire for the preparation of master mixes in an EHV-1 free area described in chapter 2 section 2.2.5. Sequencing primers were synthesized at the MRC retrovirus laboratory, University of Glasgow Vet School by Mr. T McPherson. See table 3.2 for a list of the primers and their sequence. The approximate position and orientation of these primers in the EHV-1 gC is shown in figure 3.3.

3.3.3 RECIPES

IPTG

A 50mM solution of IPTG was prepared in water and this was added to molten L-agar at a final concentration of 0.5mM.

X-Gal

A 40mg/ml solution of X-Gal in DMF was prepared and added to molten L-agar at a final concentration of 40ug/ul.

3.4 METHODS

3.4.1 PCR AMPLIFICATION OF EHV-1 ISOLATES

In order to amplify the N-terminal coding region of the gC gene of EHV-1 isolates two oligonucleotide primers PC-3 and PC-5 (fig 3.3 and Table 3.2) were obtained. Optimal amplification conditions were initially established using a plasmid containing the EHV-1 HVS25A gC gene as starting template. The thermocycling parameters for these primers were 25 cycles of denaturation at 94°C for 20 secs., annealing at 48°C for 20 secs., and extension at 72°C for 60 secs. A final extension at 72°C for 5 mins. was also included. Each reaction contained 2.0 units of Taq DNA polymerase. All sentinel controls associated with the growth and preparation of these isolates for PCR (see chapter 2 sections 2.2.2 and 2.2.5) were amplified with primers PC-3 and PC-5. Likewise a control containing 10^6 uninfected cells used for the propagation of viruses was subjected to PCR analysis. Primers PC-5 and PC-7 were utilised to amplify the entire gC coding sequence, using identical thermocycling parameters as for PC-5 and PC-3 with the exception that the extension times were increased to 90 secs (fig. 3.3 and table 3.2). The products of three independent amplifications for each virus were then pooled and precipitated prior to analysis by agarose gel electrophoresis.

3.4.2 AGAROSE GEL ELECTROPHORESIS AND PURIFICATION OF PCR PRODUCTS

PCR products were separated on 0.7% agarose gels or LMP gels. Individual bands of the correct size were excised from the gels with a clean scalpel blade and the DNA extracted from the gel segment using geneclean or sephaglass band prep. kits. The PCR products were eluted in 30ul of T.E or water. The yield of nucleic acid was estimated spectrophotometrically prior to storage of the purified PCR product at -20°C until it would be required for further analysis.

3.4.3 CONSTRUCTION OF A TA CLONING VECTOR

Bluescript M13+ vector was modified according to the method of Marchuck *et al* (1991), to generate a T-tailed vector for the direct cloning of PCR products. 5ug of vector were digested to completion with EcoRV. The digest was phenol chloroform extracted and the nucleic acid precipitated with ethanol. The blunt-ended vector was resuspended in a PCR reaction containing 1X PCR buffer, 1.5mM $MgCl_2$, 2mM dTTP, and 5 units of amplitaq enzyme. The reaction was incubated at 70°C for 2 hours, after which time the modified vector was phenol extracted and precipitated. The vector was resuspended in a final volume of water such that the nucleic acid concentration was 20ng/ul. Aliquots of the vector named pBSTA were stored at -20°C.

3.4.4 CLONING AND SEQUENCING OF PCR PRODUCTS

PCR products were ligated with pBSTA and transformed into commercially competent Invitrogen α-F' cells or electrocompetent JM109 cells. Clones were selected by plating the transformations on LB agar containing IPTG, X-Gal, and 50ug of ampicillin per ml of agar.

Plasmid DNA was extracted from white colonies and the presence of a cloned PCR product verified by digesting the plasmids with EcoRI and HindIII which flank the cloning site.

At least three cloned PCR products derived from each virus were sequenced. Potential discrepancies between the sequences were resolved by sequencing a fourth clone. Cloned PCR products derived using primers PC-3 and -5 were sequenced with primers PC-3, -5, -4, and -6 (figure 3.3 and table 3.2). The cloned PCR products derived from PC-5 and -7 were sequenced with primers PC-5, -7, -3, -4, -6, -8, -9 and -10 in order to derive sequence data across the entire cloned PCR product.

3.4.5 SOUTHERN BLOT ANALYSIS OF PCR CONTROLS

All control PCRs were precipitated, resuspended in 10ul of water, and run on 0.7% agarose gels along with 1pg and 0.1pg of purified PCR products. The gel was first stained with ethidium bromide and viewed under UV. DNA was transferred to a nylon membrane and then probed with a PCR product labelled with ^{32}P-dATP by random priming. The membrane was exposed overnight to autoradiographic film at -80^{o}C with an intensifying screen in place.

3.5 RESULTS

3.5.1 AMPLIFICATION OF THE gC GENE OF NINE EHV-1 ISOLATES

The amplification parameters for PCR using primers PC-3 and -5 were initially established using plasmid DNAs as targets. However, when these conditions were employed for the PCR with virus as a source of template, a number of problems were encountered which are illustrated in figure 3.4.

No amplification products were detected with either the Animal Health Trust (figure 3.4 lane 5) or University of Kentucky (figure 3.4 lane 9) virus isolates when using one unit of Taq polymerase in PCRs. Increasing Taq polymerase to two units per reaction resulted in products for AHT isolates (figure 3.4 lane 7) but not for those supplied by the University of Kentucky (figure 3.4 lane 11). A distinct difference between the isolates supplied was that those obtained from Kentucky were freeze dried while those from the AHT were supplied as tissue culture supernatants. Secondary stocks of all isolates were made as described in chapter two, and when used as sources of target template successful amplification occurred at the elevated enzyme concentration (figure 3.4 lanes 13 and 15). A PCR product of approximately 700bp corresponding to the N-terminal coding region of the gC gene was obtained for each isolate.

Isolate T373 was used as a template for PCR amplification with primers PC-5 and -7 under the conditions established for primers PC-3 and -5, with the exception that a longer extension time was employed with regard to the expected size of the PCR product. A product of approximately 1.5kbp was obtained (figure 3.5 lanes 5 and 7).

3.5.2 CLONING AND SEQUENCING OF PCR PRODUCTS

Initial attempts were made to sequence dsPCR products directly using a number of previously published techniques; however in my hands, these methods proved inconsistent and unsuitable for comparative sequence analysis (data not

shown). It was decided to adopt a more conventional approach of cloning and sequencing.

Bluescript M13+ was modified for cloning PCR products and proved quite successful in that excellent cloning efficiencies with low backgrounds were obtained. The vector remained stable for two years after which time the cloning efficiency was spontaneously reduced, possibly because of the loss of the T tails. All PCR products were cloned with this vector (figure 3.5 lanes 8 and 9) with the exception of that derived from strain 82244 which was cloned using the Invitrogen TA cloning kit according to the manufacturer's (Invitrogen) protocol.

Sequencing of the plasmid cloned PCR products revealed that all isolates had gC N-terminal coding sequences identical to EHV-1 AB-4 (table 3.3) with the exception of strain T431, the sequence of which was identical to that obtained by Allen and Coogle (1988) (see figure 3.2). The sequence of the PCR product representing the entire gC gene of T373 was identical to that of strain AB-4.

In the case of four strains T373, T632, AHT50592 and AHT91320 at least one mutation was detected, relative to the consensus sequence for each strain, in a plasmid cloned PCR product (table 3.4 and figure 3.6). Initially these were assumed to be PCR artifacts on the basis that a fourth clone sequenced through the area thought to contain the mutation was identical to EHV-1 AB-4. In the case of T373 a further 6 clones were sequenced in the region of interest and were shown to have an AB-4 like sequence in this region (table 3.4).

3.5.3 SOUTHERN BLOT ANALYSIS OF EXPERIMENTAL CONTROLS

All the controls involved in the preparation of the viruses for PCR were initially PCR amplified using primers PC-3 and -5. The PCRs, when run on ethidium bromide stained gels, revealed no contaminating template in these controls. Southern blot analysis of these gels detected the presence of as little as 0.1-1 pg of PCR product in the positive control lanes, whereas all the preparative control lanes were negative (figure 3.7).

Chapter Three

Figures and Tables

Figure 3.1: Alignment of selected α-herpesvirus gC amino acid sequences. gC homologues were aligned using a clustal program. Each virus is indicated at the beginning of each row of the corresponding gC amino acid sequence and the number of the last amino acid in each row is indicated at the end of each row. Amino acids are represented by single letters. Regions of 100% identity are boxed.

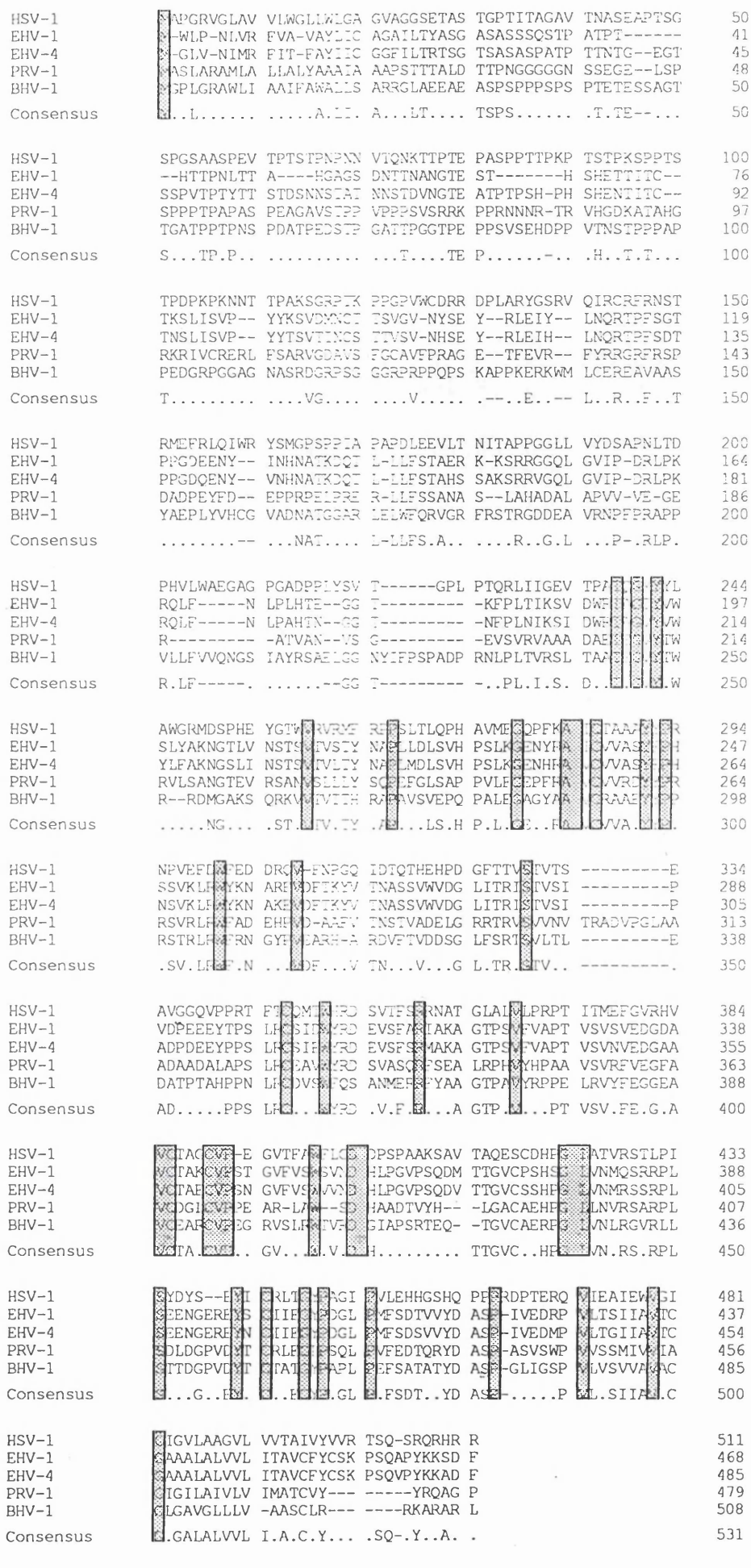
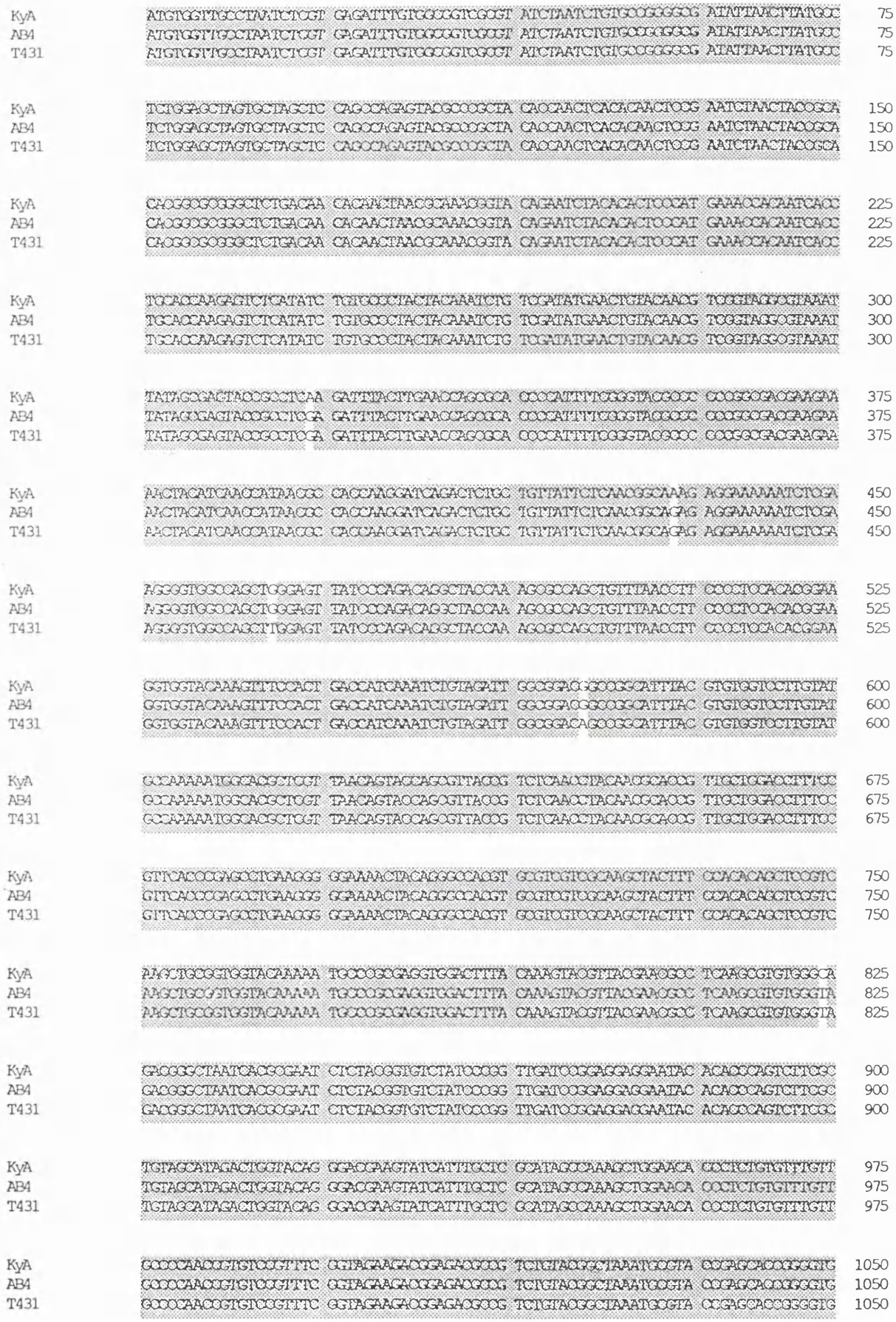
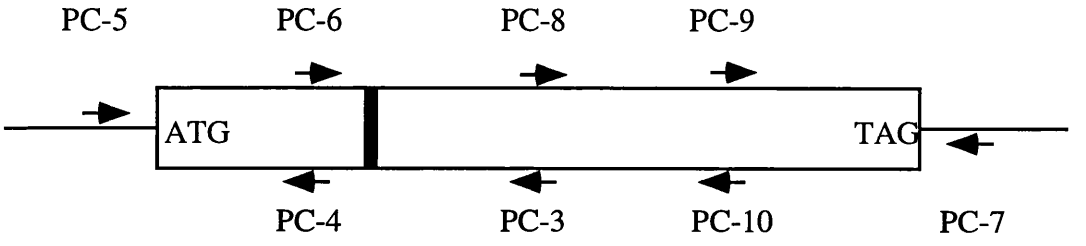


Figure 3.2: Alignment of the published gC DNA sequences of three EHV-1 strains. The EHV-1 strain is indicated at the beginning of each row of its corresponding gC DNA sequence. Nucleotides are represented by single letters and the number of the last nucleotide in each row is indicated at the end of each row. Regions of 100% identity are shaded.


KyA
AB4
T431



→ = Forward primer

← = Reverse primer

— = non-coding region

□ = gC coding region

■ = linear antigenic domain (Allen *et al*., 1992)

ATG = start codon (124)

TAG = stop codon (1531)

Figure 3.3: Schematic representation of the EHV-1 gC gene indicating the approximate position of various primers

Figure 3.4: PCR analysis of virus isolates with primers PC-3 and PC-5. Negative image of an ethidium bromide stained 1% agarose gel electrophoresis analysis of PCR reactions containing various virus isolates/stocks and different Taq polymerase concentrations. Lanes **(1)** λ HindIII/EcoRI M.W marker. Molecular weights are given on the left hand side of the gel. **(2)** Example of a negative control. **(3)** Example of a sentinel control. **(5)** Primary stock AHT82244 with 1 unit of Taq. **(7)** Primary stock AHT82244 with 2 units of Taq. **(9)** Primary stock T632 with 1 unit of Taq. **(11)** Primary stock of T632 with 2 units of Taq. **(13)** Secondary stock T632 with 2 units of Taq. **(15)** Secondary stock AHT 82244 with 2 units of Taq. **(19)** and **(20)** 1 pg of positive control plasmid DNA.

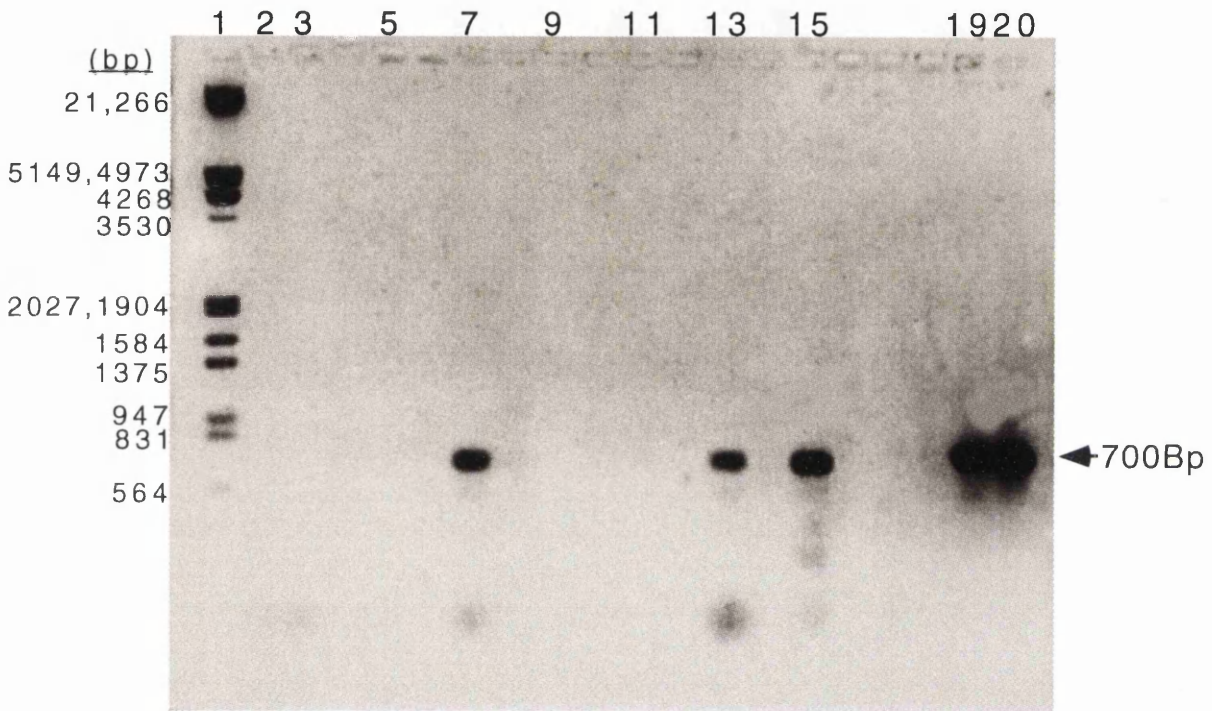

1 2 3 5 7 9 11 13 15 19 20
(bp)
21,266
5149,4973
4268
3530
2027,1904
1584
1375
947
831
564
700Bp

Figure 3.5: TA cloning of PCR product generated with primers PC-5 and PC-7. Negative image of an ethidium bromide stained 0.7% agarose gel. **Lanes (1)** λ HindIII/puc19 TaqI/puc19 Sau3A I M.W marker. Molecular weights of selected bands are listed on the left hand side of the gel. **(2)** Negative control i.e. PCR with no template **(5)** PCR with PC-5 and -7 using T373 as template. **(7)** Purified T373 PCR product. **(8)** pBSTAT373-1.5 linearised with EcoRI. **(9)** pBSTAT373-1.5 digested with HindIII and EcoRI. No samples loaded in lanes **(3)**, **(4)** and **(6)**.

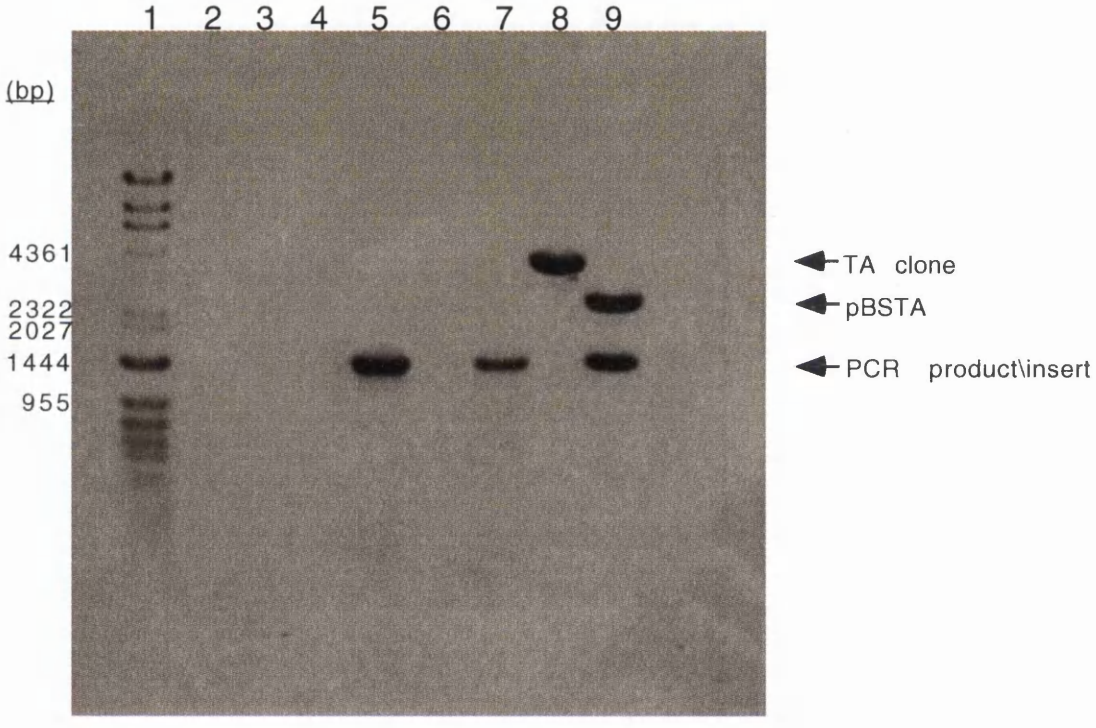

1 2 3 4 5 6 7 8 9
(bp)
4361
2322
2027
1444
955
TA clone
pBSTA
PCR product\insert

Figure 3.6 DNA sequence of cloned mutant PCR products: Autoradiographs of sequencing gels illustrating the DNA sequence of non-mutant (First four lanes of each autorad from left to right) and mutant (last four lanes of each autorad from left to right) PCR products derived from **(A)** T373 **(B)** T373 **(C)** AHT50592 **(D)** T632 and **(E)** AHT91320. The location of the mutated sequences on the autoradiograph and the type of nucleotide change are indicated on the right hand side of each autoradiograph. The number in brackets indicates the position of each mutation in the EHV-1 gC coding sequence (Allen and Coogle. 1988).

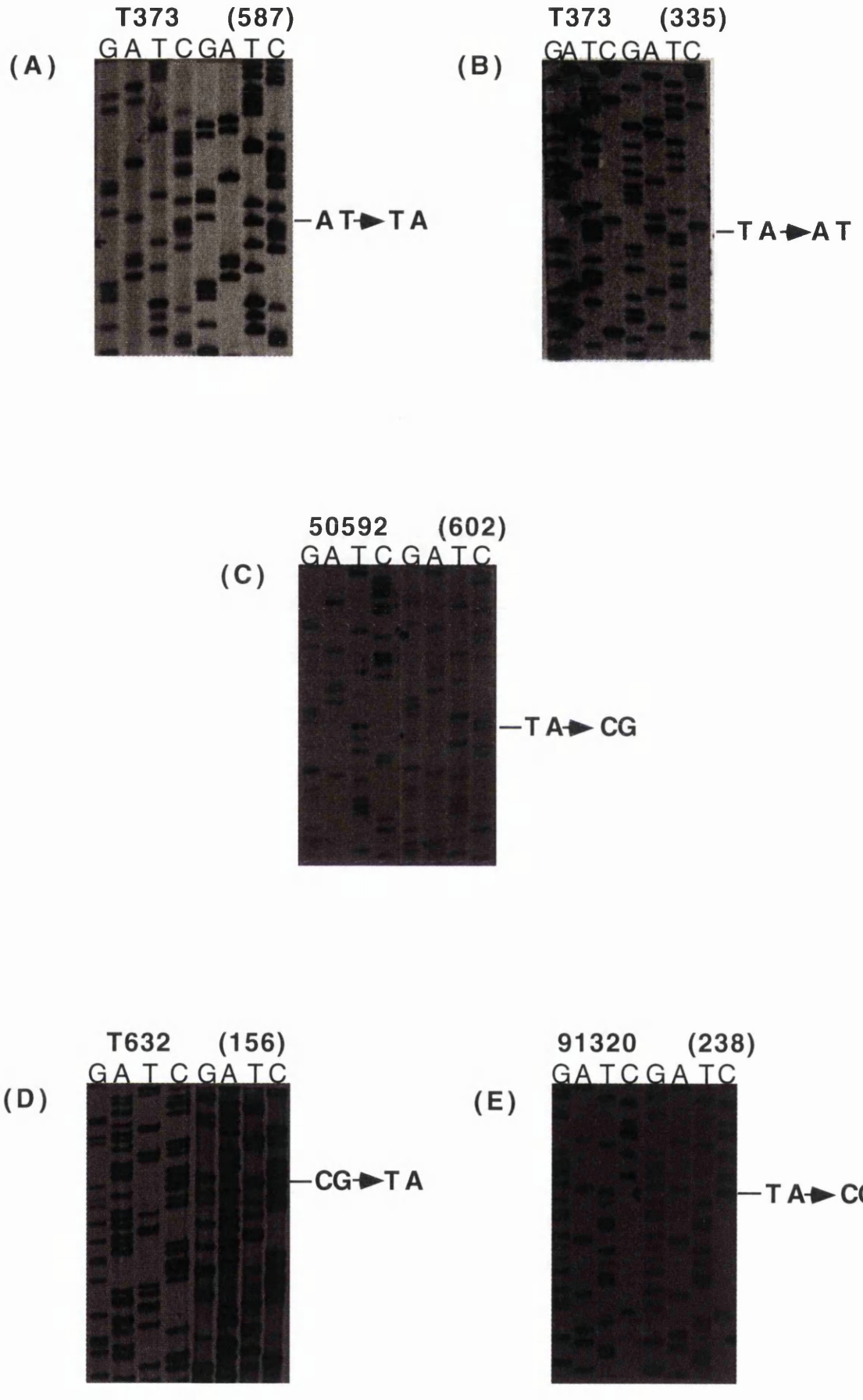
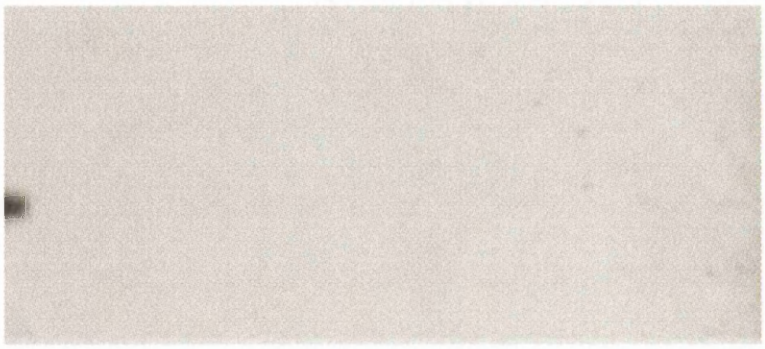

T373 (587)
G A T C G A T C
(A)
A T ► T A
T373 (335)
G A T C G A T C
(B)
T A ► A T
50592 (602)
G A T C G A T C
(C)
T A ► C G
T632 (156)
G A T C G A T C
(D)
C G ► T A
91320 (238)
G A T C G A T C
(E)
T A ► C G

Figure 3.7: Southern blot and hybridisation analysis of PCR controls: Two examples of autoradiographs showing the signals obtained from varying quantities of PCR product and thus the sensitivity of the probe used for hybridisation. These positive controls were loaded and electrophoresed on the left hand side of the gel which was then subjected to Southern transfer and hybridisation analysis. All other lanes contained a selection of either negative or sentinel controls from PCR analysis of the various isolates. All were negative in that no signal was detected.


1pg PCR product

0.1pg PCR product

Isolate	Observed clinical manifestations	Supplier	Citations
T431	Abortigenic	GA	Allen *et al.*,1988
T373	Abortigenic	GA	Allen *et al.*,1988
T654	Abortigenic	GA	None
T672	Paralytic	GA	Binns *et al.*, 1994
T632	Abortigenic/Paralytic	GA	None
T629	Paralytic	GA	Binns *et al.*, 1994
AHT91320	Paralytic	MB	McCann *et al.*, 1995
AHT82244	Paralytic	MB	McCann *et al.*, 1995
AHT50592	Abortigenic	MB	Mumford *et al.*, 1987 Hannant *et al.*, 1991 Mumford *et al.*, 1994 McCann *et al.*, 1995

Table 3.1: List of EHV-1 isolates analysed, their disease association and the supplier. GA= Professor G. Allen and MB= Dr. M. Binns. The column headed citations lists references containing details of experiments involving these isolates.

Primer	Sequence	Location (Numbered according to Allen and Coogle 1988)	Application
PC-3	5'-gacggtaacgctggtac-3'	765-749	PCR and sequencing
PC-4	5'-aatggggtgcgctggtt-3'	470-454	sequencing
PC-5	5'-tctccgaccagtggagtt-3'	61-78	PCR and sequencing
PC-6	5'-aaccagcgcaccccatt-3'	454-470	sequencing
PC-7	5'-aggttgtttgggggaga-3'	1557-1541	PCR and sequencing
PC-8	5'-gtaccagcgttaccgtc-3'	749-765	sequencing
PC-9	5'-cagtcttcgctgtagca-3'	1114-1130	sequencing
PC-10	5'-tgctacagcgaagactg-3'	1130-1114	sequencing

Table 3.2: Details of primers used for sequencing and PCR

Isolate	Origin	Associated disease syndrome	gC Sequence (1-700)
T431	California 1980	Foetal isolate from isolated abortion case	As per Allen and Coogle (1988)
T373	Kentucky 1982	Foetal isolate from abortion storm	As per AB-4 (Telford *et al*., 1992)
T654	Kentucky 1986	Foetal isolate from isolated abortion case	As per AB-4
T672	Texas 1986	Buffy coat isolate from paralytic outbreak	As per AB-4
T632	Austria 1983	Isolate from an epizootic of abortion/paralysis	As per AB-4
T629	California 1985	Isolate from cerebrospinal fluid of isolated case of paralysis	As per AB-4
AHT91320	U.K 1989	Multiple paralysis	As per AB-4
AHT82244	U.K 1988	Multiple paralysis	As per AB-4
AHT50592	U.K 1985	Abortion	As per AB-4

Table 3.3: Description of isolates and their determined gC N-terminal DNA sequence.

Isolate	No. of mutant clones	No. of AB-4 like clones	Nucleotide change	Amino acid change
T373	1*	9	T→A (587)	Leu→Gln
T373	1*	3	A→T (335)	Glu→Val
AHT50592	1	3	A→G (602)	Asp→Gly
AHT91320	1	3	A→G (238)	Thr→Ala
T632	1	3	G→A (156)	Ala→Ala

Table 3.4: Details of the cloned mutant PCR products and the isolates from which they were derived. *One clone that contains two point mutations.

3.6 DISCUSSION

All virus isolates initially proved to be unsuitable sources of PCR template when 1 unit of Taq polymerase was used in the PCR to amplify the N-terminal coding region of the EHV-1 gC gene. Raising the concentration of Taq polymerase to 2 units/reaction resulted in successful PCR amplification from AHT but not from University of Kentucky isolates. Since the latter were supplied as freeze-dried stocks it was assumed that some aspect of the freeze-drying process introduced elements to these stocks that were inhibitory to the PCR process regardless of the concentration of Taq polymerase utilised. Secondary stocks for both the AHT and University of Kentucky isolates proved to be suitable sources of PCR template at elevated Taq polymerase concentration. Retrospective examination of supplied stock virus titres may explain unsuccessful amplification at lower enzyme concentrations. All isolates supplied had titres ranging from 10^3-10^4 infectious virus particles/ml, implying that between 10-100 virus particles were used as templates in each PCR if the genome contribution of non-infectious viruses is ignored. This may have been insufficient template to produce a visible PCR product at the lower polymerase concentrations. All secondary stocks had titres ranging from 10^5-10^6 infectious particles/ml. As these stocks were not subjected to PCR at the lower enzyme concentration it was not demonstrated that low virus titre was the reason for unsuccessful PCR amplifications at lower enzyme concentrations.

The sequencing of the N-terminal coding region of the gC gene of nine isolates revealed that they all had identical amino-acid and, with the exception of T431, DNA sequences. Although the study cannot be considered extensive since only nine isolates were analysed, the sequence conservation observed is surprising in that all isolates are epidemiologically distant from each other with regard to origin, disease symptoms, or time. The results are believed to be accurate for two reasons. Firstly, a stringent control regime was in operation and this revealed that no contamination occurred during the preparation of viruses for PCR. Secondly, isolate T431 can be considered as an internal control because it was known to contain sequence variation in relation to strain AB-4 which was detected in this study.

Obviously the work of Allen and colleagues (1988), their study detecting a very high degree of inter-strain antigenic variation in EHV-1 gC, is in direct conflict with this study. Likewise, complement dependant neutralising anti-gC Mabs detected antigenic variation in EHV-1 isolates from Japan (Shimizu *et al.,* 1989). Similar studies detected inter-strain antigenic variation in the gC homologue of PRV and HSV-1 (Pereira *et al.,* 1982; Ben-Porat *et al .,* 1986). Studies of HSV-2 glycoproteins other than gC also revealed limited inter-strain antigenic variation (Bhattarakosol *et al.,* 1990; Bhattarakosol *et al.,* 1991). However, Bhattarakosol and co-workers (1990) noted that with the ELISA assay they employed to determine antigenic variation, the optical density values inexplicably varied between strains over a broad range of values which were independent of the viral protein concentration present in the ELISA. In addition, each of the aforementioned PRV and HSV studies analysed strains that were grown in cells derived from animals which are not the natural host of the virus under study. Changes in the coding region of the EHV-1 gC gene homologue have been observed after passage of the virus in cells which were not of host origin (Matsumura *et al.,* 1993; Sugahara *et al.,* 1994). However other studies (Kirisawa *et al.,* 1994; McCann *et al.,* 1995) noted that strains of EHV-1 were quite stable in terms of genomic structure after *in vitro* passage and concluded that the instability observed by others may depend on the cell type utilised for virus propagation (Kirisawa *et al.,* 1994).

It is likely that these variations in the epitope content of glycoproteins are due to inter-strain sequence variations in the encoding genes. Indeed, this has been demonstrated for HSV-1 and members of the other families of the herpesviridae. On the other hand, there is evidence accumulating that the EHV-1 genome is extremely stable and that the majority of inter-strain genomic variation is not due to genetic mutation but rather to differences in copy number of repetitive sequences (Allen *et al.,* 1983a; Studdert *et al.,* 1992; Kirisawa *et al.,* 1993b; Binns *et al.,* 1994; Bonass *et al..,* 1994; Matsumura *et al.,* 1994). Studdert *et al* (1992) demonstrated that there was no antigenic variation in Australian EHV-1 isolates which were found to diverge at the genomic level. The Mabs used in the Australian study had also been used in the studies of Allen *et al* (1988). No antigenic variation in gC was noted among a small number of English isolates (Sinclair *et al.,* 1989). It might be argued that these studies are insensitive,

epidemiologically different, or not sufficiently extensive to detect the same level of variation as that detected in the study of Allen *et al* (1988).

A consideration of the biology of EHV-1 does not resolve the apparent disparity of the studies described above. gC has been shown to be a major target of the equine humoral immune response (Allen *et al.,* 1992; Ostlund *et al.,* 1992; Ahmed *et al.,* 1993). While many of the epitopes in the gC molecule are neutralising *in vitro* (Allen *et al.,* 1988; Sinclair *et al.,* 1989) it must be considered that EHV-1 can circumvent a neutralising response *in vivo* (Bryans. 1969; Allen and Bryans. 1986). A number of studies have demonstrated a role for gC in protecting against or reducing the severity of EHV-1 disease in animal models (Guo *et al.,* 1989; Stokes *et al.,* 1989; Guo *et al.,* 1990). These facts favour the archetypal concept of immunological pressure or selection resulting in antigenic variation in a pathogen, in this case EHV-1. Opposition to this concept comes from the possibility that EHV-1 can evade the immune system of the horse thus avoiding immunological pressure. Firstly, the virus spreads systemically in a cell-associated form, potentially avoiding the initial efforts of the host's immune system to control its spread. Secondly, evidence suggests that the virus can suppress aspects of the cell mediated immune response which may also indirectly affect the humoral response , although this latter aspect has not been demonstrated. Thirdly, the host becomes susceptible to reinfection every six months, a fact especially true of younger horses, thereby indicating an ineffective immune response or ineffective immunological memory. Conversely it could be argued that reinfection may be as a result of an antigenic variant. Finally, the virus can establish a latent infection which protects it from the immune system and may be a source of rapid reinfection from within. In truth it is difficult to interpret how the complex biology of EHV-1 induced disease could affect antigenic variation in particular and strain variation in general. Antigenic and strain variation results from random and spontaneous mutation. The importance of any variation will depend on the advantages or disadvantages that result from it e.g. a variant may bypass a neutralising CMI response and reach the CNS in sufficient quantities so as to cause paralytic disease. Although this may be initially advantageous for that particular strain it may result in an increase in host mortality, ultimately leading to the disappearance of that strain from the circulating strain population. Smith and Inglis (1987 and references therein) have suggested that for any virus strain variation is affected by

a complex interplay of factors contributed by both the virus and host, the effects of which are difficult to predict.

Probably the most perplexing aspect of this study is that in contrast to the Mab typing studies performed by Allen *et al* (1988) inter-strain sequence variation was not detected, with the exception of T431, in the N-terminal coding region of gC. Mab typing detected antigenic variation in all the isolates analysed in that study which suggests that there is inter-strain sequence variation. A number of these isolates were assessed in this sequencing study yet no variation was detected. A possible explanation for this could be that the PCR/sequencing strategy adopted failed to detect variation because it is located in the C-terminal sequence. In an attempt to verify this hypothesis, and therefore resolve the discrepancies between the studies, further investigation of strain T373 was instigated. Strain T373 did not bind Mab 26A5 even though the sequence data suggested that the linear gC epitope that it recognises was intact in T373. It was proposed that sequence variation in the C-terminal region would result in the masking of this linear epitope of strain T373, possibly through altered conformation of gC. Site-directed mutagenesis studies have demonstrated that changing amino acids not directly involved in the formation of this epitope can reduce the efficiency of recognition by the Mab (Coogle *et al* , 1992). Nevertheless this theory was later proven wrong as the entire amino acid sequence of gC of T373 is identical to that of T431 which is reactive with Mab 26A5.

In light of the study presented here a number of mechanisms other than genetic mutation of epitopes might be proposed in order to explain the differential reactivity of Mabs with gC of different strains. Carbohydrates can affect the reactivity of antisera to some glycoproteins (Alexander and Elder. 1984). Sugar moieties can have either a positive or negative effect on the antigenicity of peptide epitopes in gC of HSV-1 (Sjöblom *et al.,* 1987). Variable glycosylation patterns of different strains might give rise to antigenic variation as suggested by Olofsson *et al* (1990). The tissues in which a virus has replicated can influence the glycosylation of viral proteins and thus give rise to antigenic variation (Datema *et al.,* 1987). EHV-4 gC molecular weight varies when grown in different cell lines (Crabb *et al.,* 1991). Of significance is the fact that the reactivity of anti-gC Mabs does not differ between EHV-4 grown in the different cell lines, although the Mab

reactivity was determined by different methods for the different virus preparations. Another possible mechanism of antigenic variation is the association of an invariant protein with one that is widely divergent between different strains. Envelope glycoproteins of HCMV are known to form heterologous complexes with each other (see Spaete *et al.,* 1994 and references therein). The same is true of HSV-1 for which it has also been found that some envelope glycoproteins associate with tegument proteins, with the notable exception of gC (Zhu and Courtney. 1994). It is not known if these associations are sufficiently intimate for one protein to affect the antigenicity of another.

Plasmid cloned PCR products which differed from the consensus AB-4-like sequence were obtained for four of the nine viruses analysed. These were initially considered to be Taq polymerase generated point mutations on the basis that three other cloned PCR products obtained from each virus had gC sequences identical to that of AB-4. Indeed these mutations may not have been cloned and sequenced if proof-reading thermostable enzymes such as Vent or PFU DNA polymerases had been used for PCR. These enzymes increase the fidelity of amplification. Furthermore, the cloning/sequencing strategy adopted results in the sampling of a small fraction of the PCR products generated increasing the chances of identifying such Taq induced mutations.

However, given that every effort was made to reduce the potential for errors generated by Taq, the surprising sequence bias toward AB-4 in this study and the direct conflict with that of Allen's, the possibility that these PCR products were derived from a minor variant virus in an AB-4 like population i.e. these strains were mixed virus populations, was given further consideration. That a given isolate may consist of more than one strain has been long established for RNA viruses of plants and animals, and is known as the quasi-species hypothesis (reviewed in Domingo, 1989). The mutation rates of RNA viruses are significantly higher than those of DNA viruses therefore DNA virus isolates are generally more homogeneous, although it can be inferred from studies with neutralising Mabs that some DNA virus isolates, including herpesvirus isolates/strains, are potentially mixed virus populations (reviewed in Smith and Inglis, 1987).

The potential that one of the isolates in this study consisted of a mixed virus population was given further credence as one of the cloned mutant PCR products

derived from strain T373 contained a mutation (at position 587 in the gC DNA sequence) which lay in close proximity to the region encoding (at position 561-579 in the gC DNA sequence) the epitope recognised by Mab 26A5 (Allen *et al.*, 1992). Sequencing a total of 10 cloned PCR products derived from isolate T373 indicated that the mutant PCR product represented less than 10% of the total number of PCR products cloned. Did the mutant PCR product represent the first 700bp of the gC gene of the T373 isolate originally analysed by Allen and Coogle (1988) and if so, how did the virus containing the AB-4 sequence become the predominant strain in this isolate? An AB-4 like strain may have arisen spontaneously in the T373 stock on passage in cell culture or via contamination of isolates. All strains were passaged no more than 3-10 times in equine cells. It remains to be seen how this would contribute to the spontaneous appearance and domination of an AB-4 like virus, although extensive passage in non-equine cells can induce changes in the gC gene (Sugahara *et al.*, 1994) and Mumford and co-workers (1994) have shown that plaque purification in non-equine cells followed by multiple (12) passages in equine cells resulted in the production of a strain of EHV-1 with a markedly different pathogenic potential to that of the parent strain.

As alluded to earlier, all controls were negative using the highly sensitive combination of PCR and southern blot analysis. Contamination from without within the confines of this study was considered unlikely on this basis since these controls included uninfected cells which were used to propagate the viruses and sentinel controls used in the preparation of these viruses before and during PCR. However, the possibility that the viruses were contaminated prior to the onset of this study could not be excluded. Since these possibilities could not be totally discounted and given their gravity in relation to the data described in this chapter, further investigations of the cloned mutant T373 PCR product and of a number of hypotheses introduced in this discussion were carried out as described in chapter 4.

Obviously this study failed to detect any inter-strain sequence variation other than that previously published or any marker for neurovirulence in gC of EHV-1. The reasons for choosing gC must be addressed in the light of these findings.

The N-terminal region of gC was shown to be highly divergent between α herpesviruses. This, together with the existence of inter-strain antigenic variation in gC of EHV-1, led to the proposal that this gene should contain sequence variation

that may reflect the internal host environment that a given strain has encountered. That inter-strain antigenic variation may not necessarily reflect sequence variation has already been addressed. At a superficial level inter-virus sequence divergence does not automatically imply inter-strain divergence, although a singular study has demonstrated this phenomenon in gL of HSV-1 (Novotny *et al.,* 1996). Deeper examination of this concept of divergence is required e.g. two closely related viruses such as EHV-1 and -4, having relatively similar biology and infecting the same host but with markedly different clinical sequellae, have highly divergent gC N-termini so why is the same not true of EHV-1 strains? Ignoring the essential contribution of evolution to EHV-1 and -4 divergence a comparison of the parasite-host relationships of both EHV-1 and -4 reveals significant differences. Comparing the host-parasite relationship of different strains of EHV-1 would at first glance also reveal significant differences. However the factors that lead to these differences are not clearly understood. It is emerging that the majority of EHV-1 strains follow the same course of infection regardless of the clinical outcome, and therefore different strains of EHV-1 are not exposed to significantly different internal environments which may explain the lack of sequence variation detected in this study. Furthermore, Terhune and colleagues (1998) have demonstrated that for HSV-2, replication in different organs does not lead to sequence variation in three glycoprotein genes gC, gB and gD.

The properties of endotheliotropism and induction of thrombosis, which were among the reasons for choosing gC in relation to EHV-1 induced paralysis at the onset of this study, are not necessarily hallmarks of EHV-1 induced paralysis alone but are also relevant to the pathology of EHV-1 induced abortion (Edington *et al.,* 1991; Smith *et al.,* 1992 and 1993).

Indirect evidence for the involvement of EHV-1 gC in neurovirulence came from functional studies of the HSV-1 gC homologue. In retrospect, there is also evidence to suggest that the HSV-1 homologue may have conserved functions *in vivo*, and therefore at least some sequence conservation that may also be applicable to gC of EHV-1 which seems to be completely conserved, at least in the N-terminus, according to the study presented herein. gC of HSV-1 and the PRV homologue bind virus to heparin on the surface of cells, may have a role in penetration/egress, and bind complement. The relative importance of gC in the two

former processes cannot be truly assessed since other viral proteins have similar functions e.g gB (Herold *et al.,* 1991 and 1994) or also participate in these processes. As a result, it cannot be inferred that some sequence constraints are placed upon wt HSV-1 strains with regard to these functions. Indeed for PRV it was demonstrated that a number of distinct sites in gC could bind to heparin independently and exclusively of each other (Flynn and Ryan. 1996). The situation is different with regard to complement binding in that this function was shown to be conserved in low passage clinical isolates of HSV-1 (Friedman *et al.,* 1986) and essential in protecting HSV-1 during the initial stages of infection. The areas not involved in binding C3b have been identified for HSV-1 and -2. These areas may be under less pressure to conserve sequence although there is a possibility that they are involved in other functions which require conservation e.g in HSV-1, AA 23-123 are not essential for complement binding but are important in binding properidin, a serum protein that stabilises the C3 convertase of the complement cascade system (Hung *et al.,* 1994). The overall conformation of gC of HSV-1 may be important in binding complement components (Friedman *et al.,* 1986). Furthermore, some of the antigenic sites on HSV-1 gC overlap sites involved in complement binding (Friedman *et al.,* 1986) implying that there may be indirect conservation of antigens through conservation of function. These potential conservation pressures on gC of HSV-1 may also apply to the EHV-1 homologue since it has also been demonstrated to bind complement (Huemer *et al.,* 1995). On the other hand, Huemer and co-workers (1995) have demonstrated that attenuated strains of EHV-1 with sequence variation in the gC gene as determined by RFLP analysis were able to elicit complement binding in infected cells.

The multiple outcomes of EHV-1 infection of the horse are no exception to the rule of the multifactorial nature of disease. For other α herpesviruses the viral factors, and to a lesser extent, the host factors contributing to disease are being identified. Unfortunately this data is not directly transferable to EHV-1 since its pathogenic mechanisms seem to be unique among α herpesviruses although they share many other properties. Furthermore the equid and the thoroughbred horse population in particular, through selective breeding, may have a limited gene pool compared to the hosts of other well studied herpesviruses. Therefore the apparent disparities between this study and others may be a reflection of the peculiar position that EHV-1 occupies within the α herpesvirinae.

Chapter Four

Mutant PCR products or mixed virus populations? An investigation

4.1 INTRODUCTION

Chapter three left many interesting questions unanswered, particularly with regard to isolate T373 and the cloned mutant PCR products.

Mutations in the cloned PCR products could (1) be a consequence of the intrinsic error prone DNA synthesis of the Taq polymerase during PCR or (2) indicate the existence of isolates of EHV-1 that are mixed virus populations, the cloned mutant PCR products representing the N-terminal DNA sequence of the gC gene of minor strains in an isolate consisting mainly of strains with an AB-4 like gC DNA sequence. A mutant clone derived from a PCR with isolate T373 had two mutations, one of which mapped very close to the coding sequence for the epitope recognised by Mab26A5, previously demonstrated to be unreactive with this strain. This led to the hypothesis that this PCR product may have been derived from the isolate initially analysed by Allen which did not react with Mab26A5. Therefore T373 and possibly the other three isolates from which mutant PCR products were derived may be mixed virus populations. The possibility that mixing arose through contamination was considered unlikely because of the extreme care taken in the preparation of these viruses for PCR and the fact that they were supplied by two different sources. Mixed virus populations are a natural phenomenon and an important consideration for clinicians and basic researchers trying to develop effective ways of managing EHV-1 associated diseases.

Allen *et al* (1983b) was the first to report the isolation of two genetically distinct strains from a single EHV-1 isolate by plaque purification in equine cells. The genetic alteration was located in the repeat regions of the viral genome. It is widely accepted that the terminal and internal repeat regions are the source of most of the genetic variation identified between EHV-1 strains and by their nature are quite unstable. Analysis of virus isolations, taken at different time points during the course of an infection of a horse with a strain of EHV-1 which was not plaque purified, demonstrated that the viruses isolated differed from each other in these repeat regions (Matsumura *et al.,* 1994). Two isolates from an outbreak of EHV-1 associated abortion were differentiated on the basis of RFLP analysis of PCR

products (McCann *et al.,* 1995). It remains to be unequivocally demonstrated whether the initial inoculums were mixed virus populations or if the variants arose through *in vitro* or *in vivo* selection of offspring from the infecting virus. Indeed, Matsumura and co-workers (1994) have suggested that work on PRV (Mengeling *et al.,* 1983; Wathen and Pirtle. 1984) indicates that the latter possibility is more likely.

Plaque purification followed by multiple passages in equine cells of a pathogenic isolate resulted in the isolation of a strain that had lost some of its pathogenic potential (Mumford *et al.,* 1994; Gibson *et al.,* 1992b). A comparison of this isolate with its pathogenic co-isolates has not been made in order to elucidate the potential mechanism resulting in altered pathogenicity. A separate study which may have some bearing is the demonstration that some strains consist of at least two viruses, one of which lacks a BamH1 site residing in a coding region of the viral genome (Bonass *et al.,* 1994). It is not clear whether this is a naturally occurring phenomenon since the viruses were propagated in non-equine cells.

It must be reiterated that for any of the aforementioned studies it is not clear if the infecting strain was a mixed virus population or if it spontaneously changed on *in vivo* or *in vitro* passage thus giving rise to a mixed virus population or completely new progeny. Whichever is the case, it seems certain that for the cited examples mixing of the virus population occurred at some stage during the infection process, possibly through the selection of an already existent minor population or the generation of new variant virus progeny. The factors which affect the generation of variant viruses have not been clearly defined for EHV-1. Closer examination of the studies mentioned in previous paragraphs reveals a role for external and EHV-1 encoded factors, definition of which will be of importance in the study of mixed virus populations in particular and strain variation in general.

Passage of EHV-1 and -4 in non-equine cells is well recognised as a means of generating variant viruses. Exposing a virus to a "foreign" environment forces the virus to change, possibly in order to adapt to the new environment. It is not known how strong these forces are, to what extent the virus is actually adapting and what their relevance is to the natural situation. Studdert *et al* (1986) demonstrated that 8-12 passages in Hamster cells were required in order to generate a deletion of approximately 800bp in the Us region of the EHV-1 genome which

was quite stable on multiple passages in equine cells. Extensive passage (77) of EHV-1 in bovine cells resulted in two amino acid changes in gC (Sugahara *et al.*, 1994). In these studies it is not clear at what stage i.e. passage number, these changes started to occur. However, Bonass and colleagues (1994) suggest that only three plaque purifications in rabbit cells were needed to elicit a loss of a restriction site in some of the virus genomes in a given population. Further multiple passages in rabbit cells resulted in the progressive loss of the site in all of the viral genomes. These studies would suggest that passage in non-equine cells has great influence in selecting EHV-1 variants, but a single study has suggested that the genomes of EHV-1 strains are quite stable on *in vitro* passage in non-equine cells but may depend on the cell type utilised for *in vitro* passage (Kirisawa *et al.*, 1994). All of the aformentioned examples of passaging in non-equine cells are unique in that some of the changes in the viral genome involved, or potentially involved, changes in coding regions, so it seems likely that the viruses were adapting to their environment. However, it must be stressed that the majority of identified changes induced on passaging in non-equine cells involved alterations to the copy number of repetitive elements or reiterated sequences, some of which are located in coding regions. In contrast, the viral genome seems to be highly stable on *in vitro* passage in equine cells. As referred to earlier, Mumford and co-workers (1994) demonstrated that plaque purification in non-equine cells followed by multiple passages in equine cells resulted in the isolation of a strain which was distinct from the parent virus in terms of pathogenicity. The origin of the strain with altered pathogenicity is unknown in that it may have been present in the original inoculum implying that the parent strain was a mixed virus population or that it may have arisen during replication of the parent strain. Whatever the altered strain's origin some unknown aspect of the handling of the parent virus resulted in its selection. The relevance of such *in vitro* changes to the natural situation was established by the demonstration that *in vivo* passage of EHV-1 resulted in the selection or generation of variants with alterations in repetitive/reiterated sequences (Matsumura *et al.*, 1994, Binns *et al.*, 1994). Such variants were detected at 14 days but not at 8 days post-infection (Matsumura *et al.*, 1994). The selective pressures, if any, that elicit these changes are not known.

Variation within a given herpesvirus population can also occur through spontaneous mutation, rearrangement or recombination between different strains.

Rearrangement of the repetitive elements of EHV-1 has been addressed already; however spontaneous mutation, rearrangement and recombination have not been studied to the same extent for EHV-1 as for HSV-1. The existence of mixed virus populations and the occurrence of spontaneous mutation of HSV-1 strains can be inferred from the selection of neutralising Mab resistance strains from within a non-resistant background strain population (Holland *et al.,* 1983; Kousoulas *et al.,* 1984; Marlin *et al.,* 1985; Marlin *et al.,* 1986). The frequency with which these resistant strains occurred in the virus population varied over a wide range from approx. 10^{-2} to 10^{-7}. The higher value closely correlates with the observed relative mutation rate of a transgene inserted into the HSV-1 genome. Unlike the Mab resistant phenotype, mutation of this transgene conferred no known advantageous phenotype on the recombinant virus, and therefore the relative mutation rate determined by this technique is probably a true reflection of the natural *in vitro* mutation rate of the virus. A number of studies have also demonstrated that viral encoded enzymes involved in nucleic acid metabolism e.g DNA polymerase and thymidine kinase, can affect the relative mutation rate (Pyles and Thompson. 1994 and references therein).

Given the possibility that some of the strains used in this study may have been mixed virus populations a variety of methods were employed to explore this possibility. Isolate T373 and the mutant PCR product cloned from it became a focus of attention because they offered a number of potential avenues of investigation. If it could be proven that the mutant PCR product did indeed represent the isolate initially analysed (Allen *et al.,* 1988) then Mab 26A5 could be used to remove the background reactive isolate from the original isolate by a series of panning procedures. The purified original isolate could then be re-analysed by DNA sequencing and Mab typing ELISA. For the other viruses this immunological approach was not an option since it could not be proven that the mutations in the PCR products derived from these isolates could affect an antigenic site recognised by a Mab. Bonass and colleagues (1994) fortuitously identified mixed virus populations by a combined approach of RFLP and southern blot analysis. This was not considered to be an experimental option, mainly because the mutations in the PCR products did not give rise to any RFLPs but also because of the minor concern that the sensitivity of this combined approach for detecting mixed virus populations had not been assessed.

PCR based techniques are at the core of mutation detection strategies in the broad areas of eukaryotic, prokaryotic and virus genetics. Two areas in particular, namely the genetics of cancer and RNA viruses, required the development of methods to detect mutant genomes in a large background of non-mutant genomes. A number of PCR based strategies were specifically applied in these areas. Examination of sequence patterns obtained from directly sequenced PCR products could, by virtue of parallel bands in at least two lanes of the sequencing gel, reveal the presence of at least two PCR product populations and therefore two genome populations. This was not considered an option for the purposes of this thesis because of the difficulties referred to in chapter three with regard to direct sequencing. Another option was the exhaustive cloning and sequencing of PCR products from a given PCR reaction, employed without success in chapter three and in this chapter in order to identify further clones derived from T373 with the same point mutation as the initial cloned mutant PCR product isolated for that particular isolate. The random nature of choosing clones for sequence analysis and the added complication of Taq-induced errors can be overcome by screening a library of cloned PCR products by oligonucleotide specific hybridisation with oligonucleotides that are specific for the mutant of interest (Grötzinger and Will; 1992). This method will also give a reasonably accurate estimation of the level of a mutant genome in a particular genome population, and thus allow the prediction of whether or not a given mutant arose as a result of error prone DNA synthesis by the Taq polymerase during the PCR. This technique was not employed because it necessitated that large representative PCR product libraries be constructed for each isolate, and that highly optimised hybridisation conditions be developed for each mutant specific oligonucleotide probe to be used. The method chosen was Single-Strand Conformational Polymorphism (SSCP) analysis, initially developed by Orita and co-workers (1989) and subsequently used to detect mutant cancer cells against a non-malignant cell background in tissue biopsies (Suzuki *et al.,* 1990). The technique is based on the fact that migration of ssDNA molecules of the same size through polyacrylamide gels is dependent on their conformation, which in turn is dependent on base composition. Nucleic acids of the same size but differing at a single base pair can be distinguished from each other on the basis of different mobilities in polyacrylamide gels if a number of conditions are fulfilled. These conditions include the size of the nucleic acid, the location and type of base

changes and the PAGE parameters. The refinements and the conditions necessary for optimal operation of the technique are reviewed by Yap and McGee (1994).

4.2 EXPERIMENTAL APPROACH

The majority of experiments described in this chapter centre around T373 and the cloned PCR products derived from this isolate as described in chapter 3. For the purposes of this chapter the T373 derived PCR product which was identical to the AB-4 gC DNA sequence is referred to as the non-mutant PCR product, whereas the cloned PCR product that contained the two point mutations is referred to as the mutant PCR product.

One of the mutations in the mutant PCR product results in an amino acid change in the vicinity of the epitope recognised by Mab26A5. Initial experiments were designed which involved Mab typing of Mab26A5 using the pepscan system and bacterial expression of proteins encoded by the mutant and non-mutant PCR products. The purpose of these experiments was to determine the role of the wild-type residue in binding Mab26A5 to its epitope and to discover whether the mutation of this residue encoded by the mutant PCR product altered the reactivity of the Mab with its epitope.

Mutant PCR products were obtained for four isolates as described in chapter 3. SSCP analysis was employed in order to determine if these mutations represented actual variant viruses i.e that the four isolates were mixed virus populations. The sensitivity of the SSCP technique as utilised herein for the detection of mixed virus populations was determined by artificially mixing two strains which were known to differ from each other in the region encompassed by a specific PCR primer pair.

Subsequently the experimental focus shifted to the actual T373 virus isolate. In order to unequivocally prove that the original T373 isolate was not inadvertantly contaminated during the course of this thesis a new isolate was requested and approximately 300bp of the gC gene sequenced, inclusive of the region encoding the Mab26A5 epitope. All experiments suggested that strain T373 had an AB-4 like gC DNA sequence/T431 like gC amino acid sequence. As a result, Mab typing of strains T373 and T431 with Mab26A5 was performed in order to determine if inter-strain differences in Mab reactivity are a result of a mechanism other than

genetic mutation as discussed in chapter 3 section 3.6. Finally, a further check for contamination of strain T373 was made by analysing the restriction pattern of isolated viral DNA in order to determine if it was identical to that determined for this isolate by the supplier.

4.3 MATERIALS

4.3.1 ANTIBODIES

Table 4.1 lists the antibodies used in the experimental work described in this chapter with a brief description of each. The anti-EHV gC antibodies were kindly provided by Prof. G. Allen of the University of Kentucky, U.S.A and Dr. D. Meredith of the University of Leeds, U.K. Those obtained from Prof. Allen were ascitic fluids which had been lyophilised and were therefore resuspended in 2 ml of water. The antibody from Leeds had not been lyophilised. All antibodies were divided into small aliquots and stored at -20^{o}C. A working stock of each anti-EHV gC antibody containing 0.1% Sodium Azide was maintained at 4^{o}C to avoid repeated freezing and thawing.

4.3.2 SSCP PRIMERS

Details of the primers used for SSCP analysis are given in table 4.2. These primers were obtained from Oswell DNA services, Edinburgh, U.K.

4.3.3 SOLUTIONS FOR SDS-PAGE

See table 4.3.

4.3.4 SOLUTIONS FOR WESTERN BLOTTING/ELISA

Coupling/coating Buffer: 10mM $NaHCO_3$, 1mM EGTA (pH 9.5).

Tris Buffered Saline (1x TBS): 0.15M NaCL, 50mM Tris HCL (pH 7.6). Tween was added at a concentration of 0.5% vol/vol to give TBST.

Blocking solution: TBST supplemented with Marvel and goat serum at concentrations of 2% weight/vol and 3% vol/vol respectively. This was made up fresh on the day of the western blot.

Phosphate buffered saline: See chapter 5.

Also see table 4.3.

4.3.5 SOLUTIONS FOR SSCP ANALYSIS

Denaturing Buffer: 500mM NaOH, 10mM EDTA.

Loading Dye: 0.5% w/v bromophenol blue and 0.5% w/v xylene cyanol in deionized formamide.

Mutation detection enhancement (MDEE) Gels: These were prepared with and without glycerol according to the manufacturer's (Hoefer Scientific, U.K.) instructions.

4.4 METHODS

4.4.1 MAPPING OF THE EPITOPE RECOGNISED BY MAB26A5

The procedure used for mapping epitopes was the Pepscan system which is described in detail in chapter five. Mab26A5 was assayed at a dilution of 1/1000. The anti-mouse γ chain specific peroxidase conjugate was used at a dilution of 1/5000. Only the EHV-1 gC domain I (see chapter 5) series of peptides were probed with Mab26A5 since this series contains the epitope recognised by Mab26A5 (Allen *et al.,* 1992).

4.4.2 CLONING AND EXPRESSION OF T373 MUTANT AND NON-MUTANT PCR PRODUCTS IN THE pGEX BACTERIAL EXPRESSION SYSTEM

The mutant and non-mutant PCR products were amplified using primers CEX-1 5-GGATCCATGTGGTTGCCTAATCTCGT-3 (CEX-1 contains a BamHI recognition site linked to the first 20bp of the EHV-1 gC coding region) and PC-7 (see chapter 3 table 3.2). The products were subsequently cloned into the pBSTA cloning vector. Clones were digested with BamHI and EcoRI and the insert directionally sub-cloned into the pGEX-2T expression vector. pGEX ligations were transformed into JM109 cells. The presence of mutant and non-mutant PCR products in the correct reading frame in pGEX-2T was confirmed by DNA sequence analysis. Mutant and non-mutant clones with the correct sequence and orientation in pGEX-2T were designated pGEX-gCM and pGEX-gCNM respectively.

Single bacterial colonies containing either pGEX-2T, pGEX-gCM or pGEX-gCNM plasmids were grown overnight in 10ml of LB-Amp. 500ul of each overnight culture were inoculated into 4.5ml of LB-Amp and grown for 2.5hrs at 37°C. The cultures were split in two and IPTG added to one half at a concentration of 0.5mM in order to induce expression of GST or GST fusion proteins. All cultures were incubated at 37°C for a further 2hrs.

4.4.3 PREPARATION OF PROTEIN EXTRACTS AND SDS-PAGE

1.5ml of each culture was centrifuged at 14000rpm for 2 minutes and the supernatant aspirated completely. Pellets were washed by resuspension in 1ml of water. The bacteria were pelleted as before and resuspended in 100ul of sample loading buffer by vortexing. Partial denaturation of protein and shearing of high M.W. DNA was achieved by drawing the samples repeatedly through a small gauge needle. Complete denaturation was obtained by boiling the samples for 2 minutes. Samples were centrifuged for 5 minutes and the supernatants i.e the protein extracts, removed for electrophoresis.

An SDS-PAGE gel was prepared as follows. Spacers, a comb and plates were cleaned and assembled for gel pouring. Running gel was poured to within 1.5cm of the bottom of the comb. 200ul of water-saturated butanol was added so that it formed a relatively smooth layer on top of the running gel which was then allowed to polymerise. After polymerisation the butanol layer was washed away with water. The stacking gel was then poured on top of the running gel. The comb was removed when the stacking gel had polymerised and the gel attached to the buffer chambers. Wells were washed with running buffer and 20ul of each protein extract was loaded. A well containing Protein M.W. markers was also included. Electrophoresis was set at 50V and the gel allowed to run until the dye front had reached the bottom of the gel.

After electrophoresis the glass plates were separated and the gel removed from the plates for visualisation of protein bands. The gel was gently agitated in a solution of Coomassie Blue G for one hour. The gel was then destained over a period of 3 hours with frequent changes of the destaining solution. When the desired resolution of destaining had been obtained the gel was dried overnight between 2 pieces of acetate paper and then photographed.

4.4.4 WESTERN BLOT ANALYSIS OF EXPRESSED PROTEINS

Protein extracts of GST, non-mutant gC GST fusion and mutant gC GST fusion were run in parallel on a SDS-PAGE gel as described in the previous section. The Biorad trans-blot electrophoretic transfer cell was used to blot the gel

on to 0.45um nitrocellulose paper in accordance with the manufacturer's instructions. Transfer was allowed to proceed overnight at 30V with a current limit of 100mA and at a temperature of 4°C.

After transfer was completed the nitrocellulose paper was cut into strips, each strip containing blots of the aforementioned protein extracts. The strips were then incubated in blocking solution for 3hrs before being washed once in TBST. One strip was incubated in blocking solution containing either Mab 26A5 or 56F11 at a dilution of 1/5000 for 1hr and then washed 3 times in TBST. Each strip was then incubated in blocking solution containing goat anti-mouse γ-chain specific alkaline phosphatase conjugate at a dilution of 1/10,000 for 1hr. The strips were washed 5 times in TBST and excess liquid removed by briefly placing each strip on whatman filter paper. The strips were incubated in Promega protoblot substrate solution in the dark until bands indicating positive reactivity of the Mabs with the fusion proteins were clearly visible on the membranes.

4.4.5 SSCP ANALYSIS

PCR was performed on viruses and virus mixtures in order to give a product of approximately 150bp in size, which has been shown to be ideal for SSCP analysis. The primers specific for each virus or virus mixture are listed in table 4.2. The approximate position of these primers relative to the PCR product point mutations in the gC gene is represented in figure 4.1. PCR was performed on isolate T431 diluted in isolate T632 in order to assess the sensitivity of the method for detecting mixed virus populations. The concentration of reactants was standard and the amplification conditions were 25 cycles of 95°C for 10secs, 50°C for 10secs and 72°C for 20secs. The specificity and the concentration of the PCR products were analysed by gel electrophoresis in a 3% metaphor gel.

The LKB macrophor system described in chapter 2 section 2.2.4 was used for the preparation and running of SSCP gels. Two MDEE gels were prepared, one with 5% glycerol and one without. The macrophor system was assembled and the thermostatic pump set to 15°C. 1ul of denaturation buffer was added to 9ul of PCR reaction and incubated at 42°C for 10mins. 1ul of SSCP loading buffer was then

added before loading 6ul of each denatured PCR product onto each gel. Undenatured PCR products were also loaded. Electrophoresis was conducted at a constant power of 8W for at least 14hrs. After electrophoresis the plates were separated and the plates with the attached gels were immersed in an aqueous ethidium bromide solution (0.5ug/ml) for 10-15mins and then photographed.

4.4.6 SEQUENCE ANALYSIS OF NEW T373 SAMPLE

A cell culture supernatant of cells infected with T373 (kindly provided by Prof. George Allen) was sent from Kentucky and this was subjected to PCR, the PCR product cloned and sequenced as described in chapter 3 section 3.4.4, without prior passage in cells at Glasgow Vet School. 10 clones were sequenced through the regions where the point mutations were originally localised in one of the cloned PCR products derived from the first T373 isolate supplied.

4.4.7 MAB-TYPING ELISA

A clarified stock of each virus strain to be typed was prepared from ten $175cm^2$ flasks infected 48hrs previously at a M.O.I of one. Partially purified antigen preparations were made by centrifuging the clarified stocks at 13K for 90mins. The pellets were resuspended in 6ml of coating buffer, a small sample being retained for titration of virus. A checker board ELISA, the two variable factors being antigen/virus concentration and Mab concentration, was performed in duplicate as follows.

100ul of virus, or dilutions thereof, in coating buffer was added to the wells of 96 well microtitre plates (Immulon I, Dynatech LTD., U.K). Control wells containing coating buffer without virus were included for each Mab to be assayed. Virus was left to adsorb overnight at 4°C. All wells were then blocked for 1hr at room temperature with 200ul of coating buffer containing 5% v/v goat serum. The plates were then washed 3 times with PBST. 100ul of Mabs diluted in PBST containing 20% goat serum was added to the wells. After 1hr the wells were washed 3 times with PBST and 100ul of goat anti-mouse γ chain specific

peroxidase conjugate diluted 1/5000 was added to each well. After 1hr the wells were washed 5 times with PBST and 100ul of TMB substrate added. The substrate reaction was allowed to incubate for 30mins after which time the O.D495 was determined.

4.4.8 ISOLATION OF VIRUS DNA

An antigen preparation of each virus was made as described in section 4.4.7. However the virus pellet was resuspended in 400µl of T.E instead of coating buffer to which 50ul of Proteinase K (50mg/ml) and 50ul of 10% SDS were added. The resuspended virions were lysed by incubation at 37°C for 30mins minimum. The following steps were performed with wide bore pipette tips and all additions, centrifugations and mixing performed with due care to prevent shearing of the released virus DNA. The lysates were phenol, phenol/chloroform and chloroform extracted. Half a volume of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol were then added to precipitate the virus DNA. The DNA was spooled on a pipette tip and washed in 70% ethanol before being allowed to dry briefly at room temperature and resuspended in water.

Virus DNA was digested with BamH1 for 3hrs and the digest electrophoretically analysed on a 0.6% agarose gel.

4.5 RESULTS

4.5.1 EPITOPE MAP OF MAB 26A5

As can be seen from figure 4.2 the results obtained with the Pepscan system are identical to those obtained by Allen *et al* (1992) using a slightly different approach. KKSRRGGQ which shows peak reactivity with Mab 26A5 contains the minimal number of amino acids required for Mab 26A5 reactivity within the limits of the assay used.

4.5.2 PROTEIN EXPRESSION AND WESTERN BLOT ANALYSIS

Expression from both mutant and non-mutant PCR products derived from T373 in the pGEX GST fusion system resulted in the production of fusion proteins of the expected size i.e. 80K (see figure 4.3). Western blot analysis revealed that both these fusion proteins reacted with Mabs 26A5 and 56F11 (see figure 4.4) whereas the GST protein did not. Mab 56F11 was included as a positive control in that it was known to react with an epitope encoded by strain T373. This Mab recognises a linear EHV-1 gC (aa) epitope distinct from that recognised by Mab 26A5 (Allen *et al.,* 1988; Allen *et al.,* 1992).

4.5.3 SSCP ANALYSIS

SSCP analysis can detect single base changes in nucleic acid fragments of the same size. It is evident from figure 4.5 that the bands observed are single stranded for two reasons. Firstly, the intensities of the observed bands are quite weak, indicative of the weak intercalation of ethidium bromide by ss or partially denatured DNA. Secondly, the dsPCR products are approximately 150bp (see figure 4.6) in size and are run off the SSCP gel (see figure 4.5 and lanes 16, 17 and 18) whereas the denatured PCR products electrophorese at greater than 310bp, the slower migration again indicative of ssDNA.

Mobility shifts in the PCR products derived from EHV-1 HVS25A and the plasmid containing the mutant PCR product derived from T373 (see figure 4.7a lanes 1 and 8 respectively) were detected in SSCP gels that did not contain glycerol. However a SSCP for T431 was not detected, therefore the sensitivity of the method could not be ascertained with the parameters employed. The addition of 5% glycerol facilitated the detection of mobility shifts in all PCR products known to contain point mutations, including T431 (see figure 4.7b lane 2). SSCP analysis could detect virus at a level of 10% of the total virus population but not at 1% for the given viruses and mutations (see figure 4.7b lanes 2 to 6). This closely approximates the value of 5% reported for other types of mixed populations (Enomoto *et al.,* 1994). No minor bands corresponding to the mutant PCR product derived from T373 could be detected in the SSCP analysis of either the old or new stocks of T373 virus (compare lane 8 with lanes 9 and 10 figure 4.7b). Similarly, no minor populations were detected in the SSCP analysis of PCR products derived from strains 91320 and T632, although minor bands had been uncovered by photography (see figure 4.5 lanes 12 and 14 compared to 11 and 13 respectively) but were considered artifactual since they could not be seen if the gel was examined with the naked eye.

4.5.4 SEQUENCING ANALYSIS OF THE NEW SUPPLY OF ISOLATE T373

Partial sequence analysis of the 10 cloned PCR products derived from the new supply of T373 revealed that none contained the mutations initially identified in one of the PCR products derived from the original supply of T373. This was also confirmed by SSCP analysis (compare lanes 8 and 10 figure 4.7b).

4.5.5 MAB TYPING OF STRAINS T431 AND T373

T373 (original supply) and T431 show no significant difference in reactivity as determined by ELISA with Mab 26A5, regardless of the dilution of antigen or Mab. Both strains react with the conformation-dependant Mab 14H7 and neither react with the EHV-4 specific Mab 3C4B (see table 4.4). None of the Mabs used

in the assay reacted with wells that did not contain virus. These results can only be considered to be preliminary for two of reasons.

- The concentration of protein in each well was unknown although the titre of each virus was of the same order of magnitude.
- Although a negative control Mab was included i.e the EHV-4 specific antibody 3C4B which did not react with either EHV-1 strain, no virus negative control i.e EHV-4 antigen/virus for the EHV-1 specific Mabs was included.

4.5.6 RESTRICTION ENZYME ANALYSIS OF STRAINS T431 AND T373

T373 (original supply) has a BamHI restriction profile characteristic of a 1B electropherotype whereas that of T431 is 1P (see figure 4.8). This result agrees with Allen *et al* (1988). Although it is not clear from figure 4.8 the new supply of T373 also had a 1B electropherotype.

Chapter Four

Figures and Tables

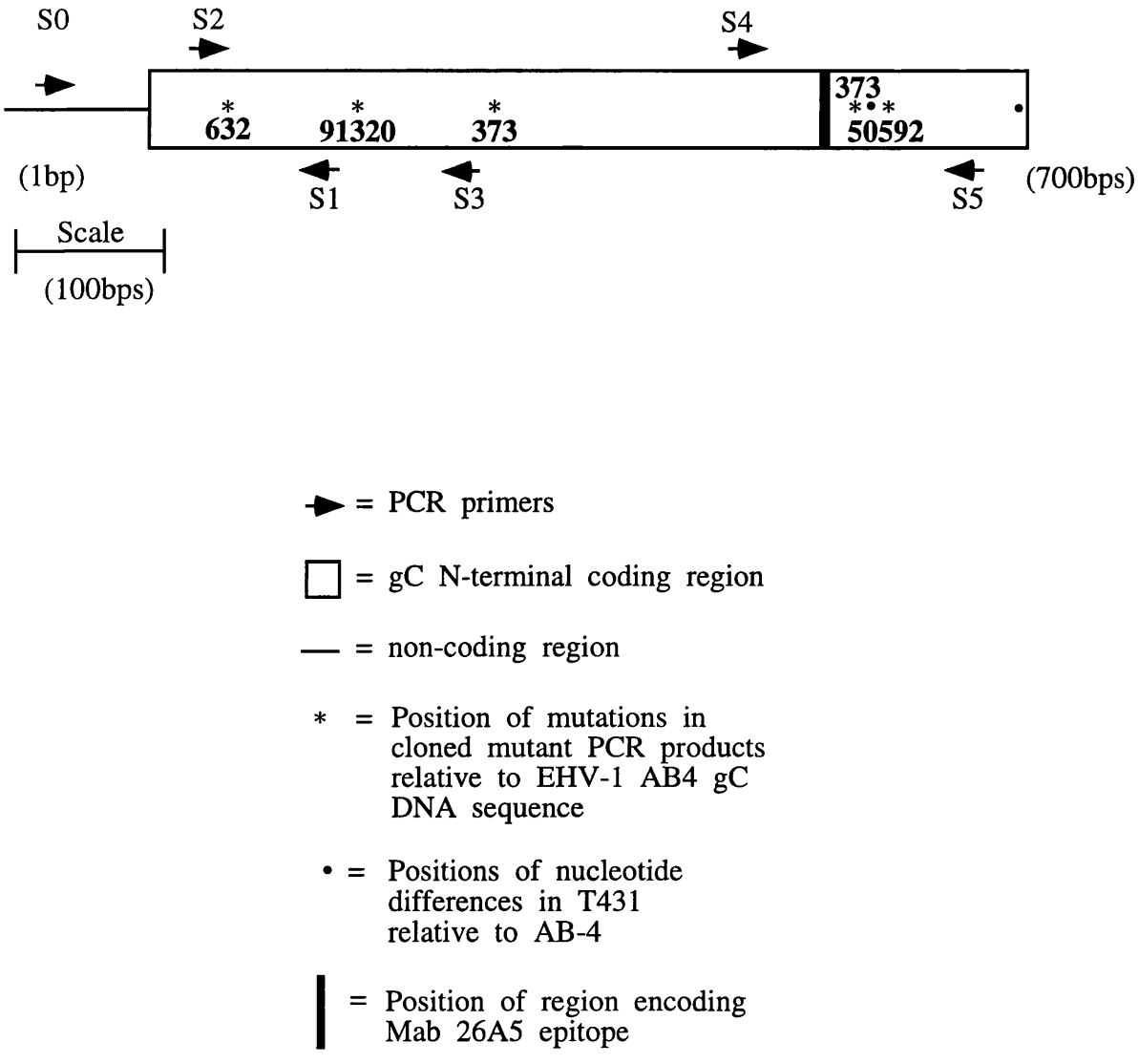
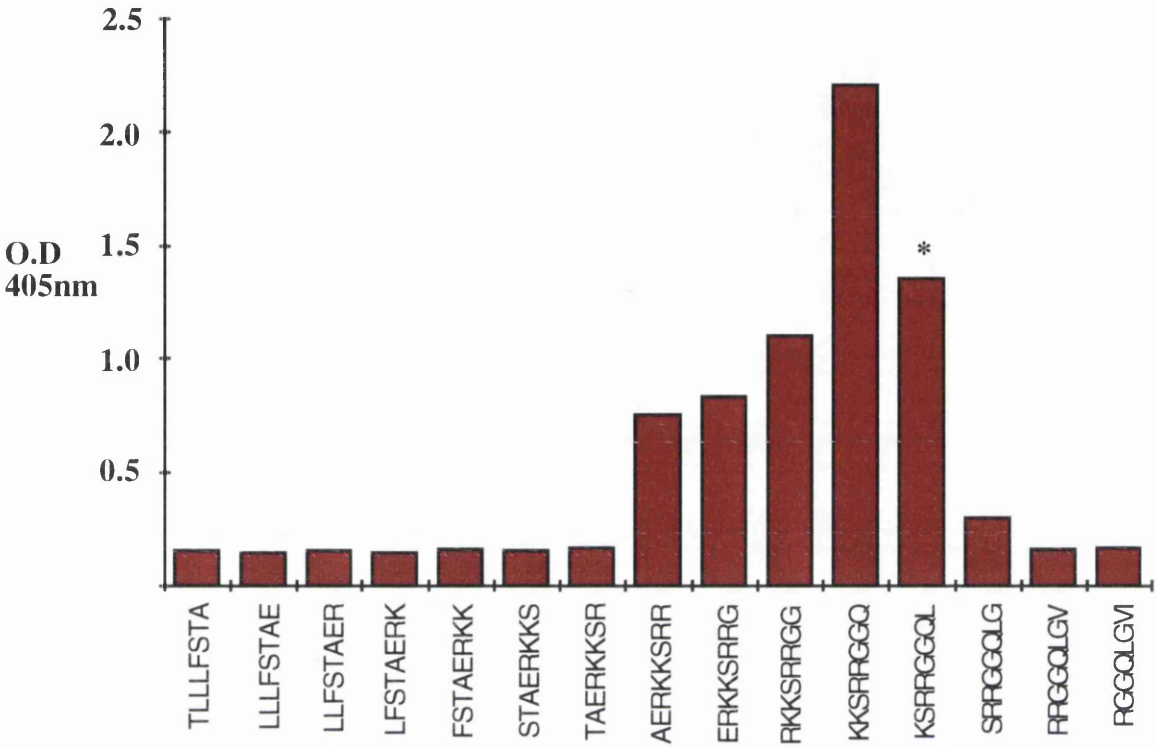


Figure 4.1: Position of SSCP primers.
Schematic representation of the first 700bp of the EHV-1 AB-4 gC gene showing the position of point mutations relative to that of SSCP primers. The isolates from which each mutant PCR product was derived is given in bold.

Figure 4.2: Mapping of the epitope recognised by Mab 26A5 by pepscan. (A) Graphical representation of the reactivity of Mab 26A5 with EHV-1 gC domain one pepscan peptides. The sequence of each peptide is given on the horizontal axis. The asteriks indicates the reactivity of the first peptide containing the amino acid that would be changed as a result of the expression of the mutation in the T373 mutant PCR product. (B) Nucleotide 587 in the T373 mutant PCR product results in an amino acid change in the proximity of the core epitope recognised by Mab 26A5. The core epitope and the affected amino acid are represented in bold and relief face respectively.

(A)



(B)

AER**KKSRR**GGQLGVI **Non-mutant**

Mutation of nucleotide (587) as in the T373 mutant clone

AER**KKSRR**GGQQGVI **Mutant**

Figure 4.3: Expression of EHV-1 gC in a bacterial expression system. Coomassie blue stained denaturing polyacrylamide gel containing in **lanes (1)** and **(8)** Promega high range protein M.W markers (partially degraded), **(2)** protein extract from uninduced JM109 pGEX-2T, **(3)** protein extract from induced JM109 pGEX-2T, **(4)** protein extract from uninduced JM109 pGEX-gCNM, **(5)** protein extract from induced JM109 pGEX-gCNM, **(6)** protein extract from uninduced JM109 pGEX-gCM and **(7)** protein extract from induced JM109 pGEX-gCM. The position of the GST protein and the GST-gC fusion proteins and the size of the molecular weight markers are indicated with arrows

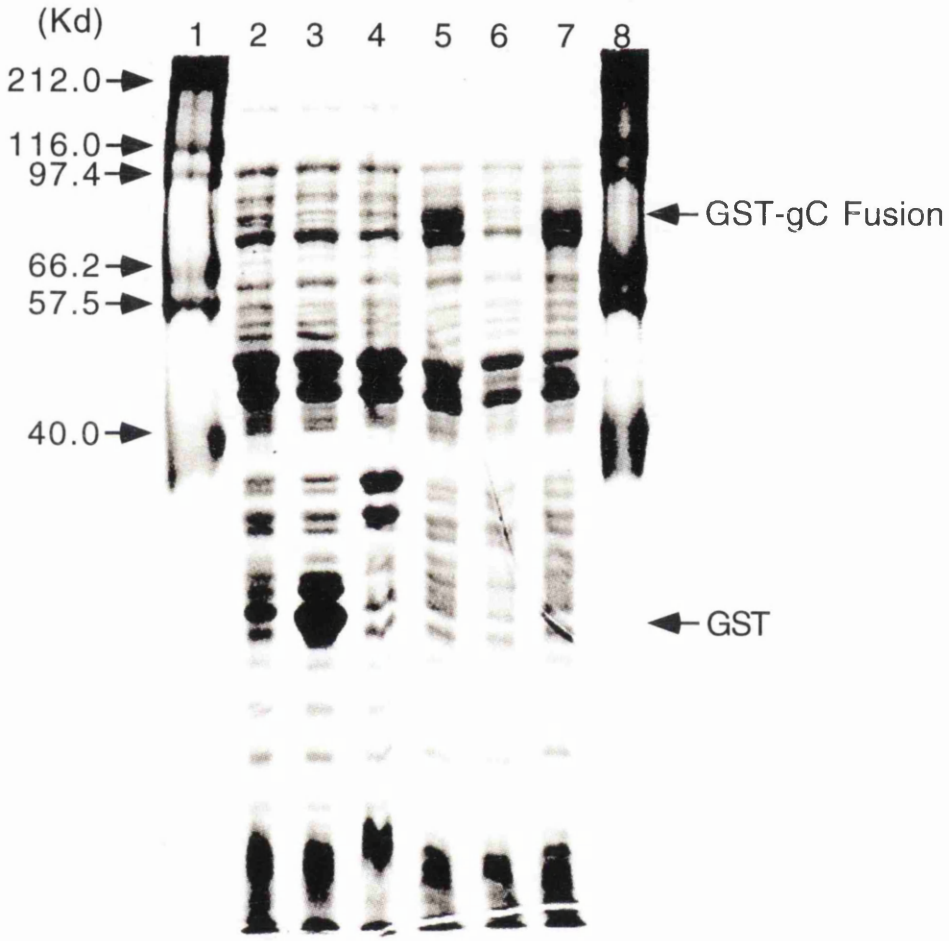

(Kd)
1
2
3
4
5
6
7
8
212.0
116.0
97.4
66.2
57.5
40.0
GST-gC Fusion
GST

Figure 4.4: Western blot analysis of GST-gC fusion proteins. Membranes containing immobilised fusion proteins were probed with **(A)** Mab 26A5 and **(B)** Mab 56F11. **Lanes (1)** GST protein, **(2)** GST-gCNM fusion protein and **(3)** GST-gCM fusion protein. The positions of reactive full length fusion proteins are indicated by arrows.

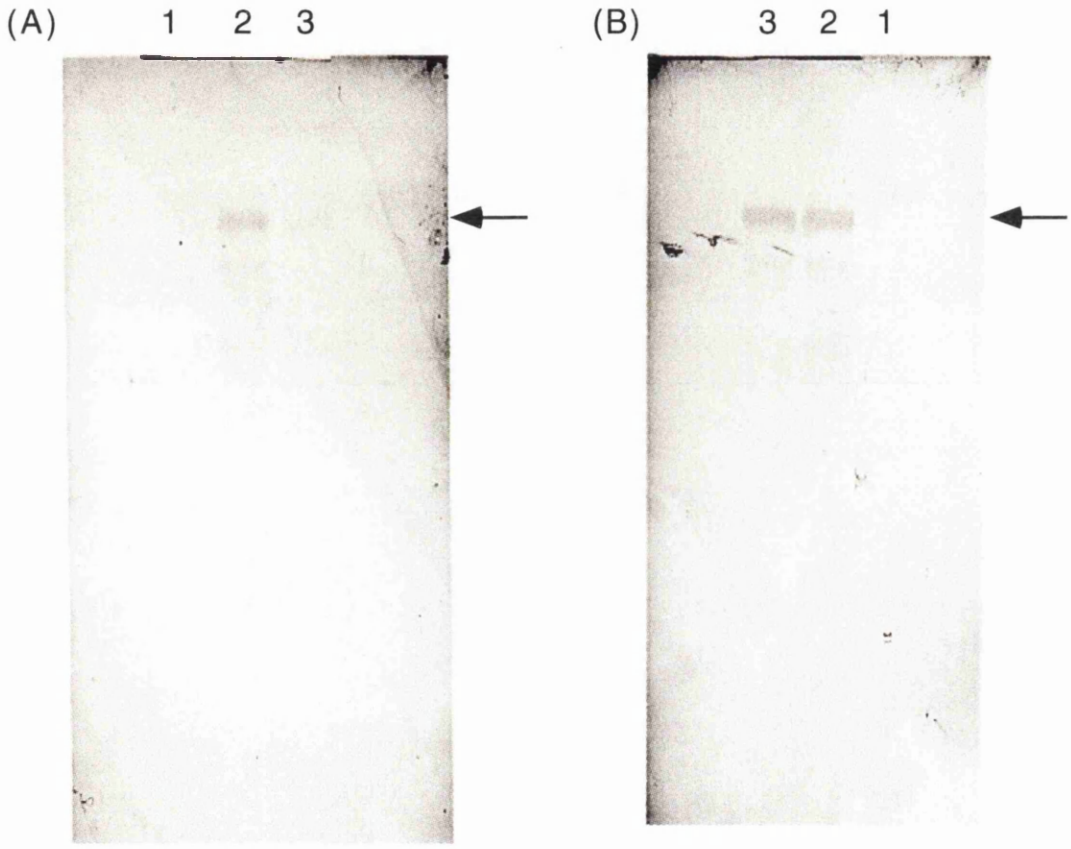

(A)
1 2 3
(B)
3 2 1

Figure 4.5: The entire SSCP gel. An ethidium bromide stained SSCP gel containing 5% glycerol. The four bands in lane **(15)** correspond to the four largest bands of the PhiX HaeIII M.W marker. Note the absence of bands in lanes **(16), (17)** and **(18)** where the undenatured PCR products were loaded. **(1)** Denatured PCR products from plasmid HVS25A gC(Primers S4+S5), **(2)** T431 10^4PFU(Primers S4+S5), **(3)** T431 5X10^3PFU:T632 5X10^3PFU(Primers S4+S5), **(4)** T431 10^3PFU:T632 10^4PFU(Primers S4+S5), **(5)** T431 10^2PFU:T632 10^4PFU(Primers S4+S5), **(6)** T632 10^4PFU(Primers S4+S5), **(7)** AHT50592(Primers S4+S5), **(8)** Mutant T373 clone(Primers S4+S5), **(9)** T373 old stock(Primers S4+S5) and **(10)** T373 new stock(Primers S4+S5). Note the position of potential minor bands in lanes **(12)** and **(14)** containing denatured PCR products from AHT91320(Primers S2+S3) and T632(Primers S0+S1) respectively. **(11)** T431(Primers S2+S3) and **(12)** T431(Primers S0+S1)

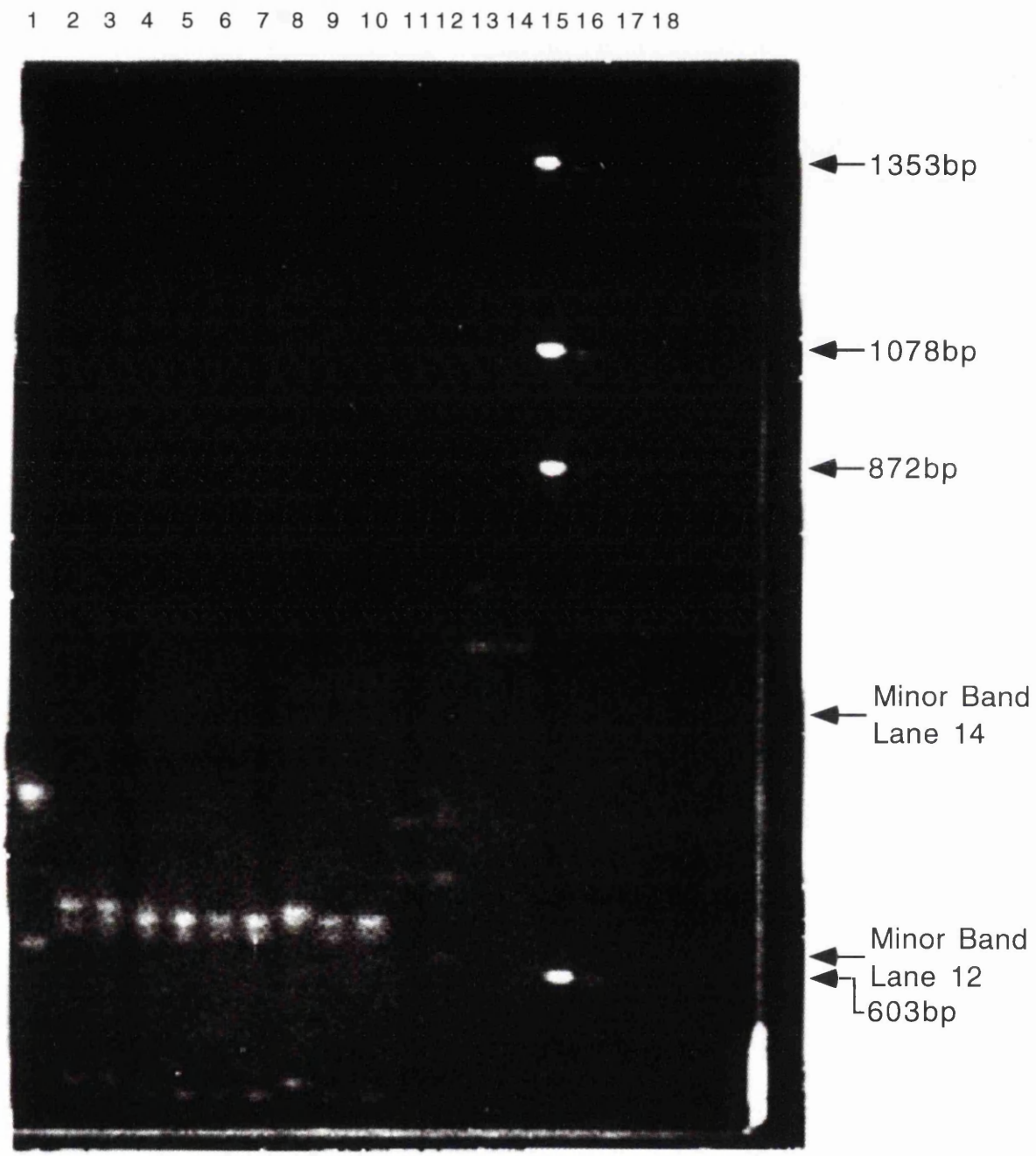

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1353bp
1078bp
872bp
Minor Band
Lane 14
Minor Band
Lane 12
603bp

Figure 4.6: PCR amplification of isolates/plasmids for SSCP analysis. An ethidium bromide stained 3% Metaphor gel containing in **lanes** **(1)** λ HindIII DNA M.W marker, **(2)** T632; (Primers S0+S1), **(3)** T431 (Primers S0+S1), **(4)** Negative control (Primers S0+S1), **(5)** AHT91320 (Primers S2+S3), **(6)** T431 (Primers S2+S3), **(7)** Negative control (Primers S2+S3), **(8)** Plasmid containing HVS25A gC gene homologue (Primers S4+S5), **(9)** 10^4PFU of T632 (Primers S4+S5), **(10)** $5x10^3$PFU of T632:$5X10^3$PFU of T431 (Primers S4+S5), **(11)** 10^4PFU of T632:10^3PFU of T431 (Primers S4+S5), **(12)** 10^4PFU of T632:10^2PFU of T431 (Primers S4+S5), **(13)** 10^4PFU of T431 (Primers S4+S5), **(14)** AHT50592 (Primers S4+S5), **(15)** T373 old stock (Primers S4+S5), **(16)** T373 new stock (Primers S4+S5), **(17)** T373 mutant clone (Primers S4+S5), **(18)** Negative control (Primers S4+S5) and **(19)** PhiX HaeIII DNA M.W markers.

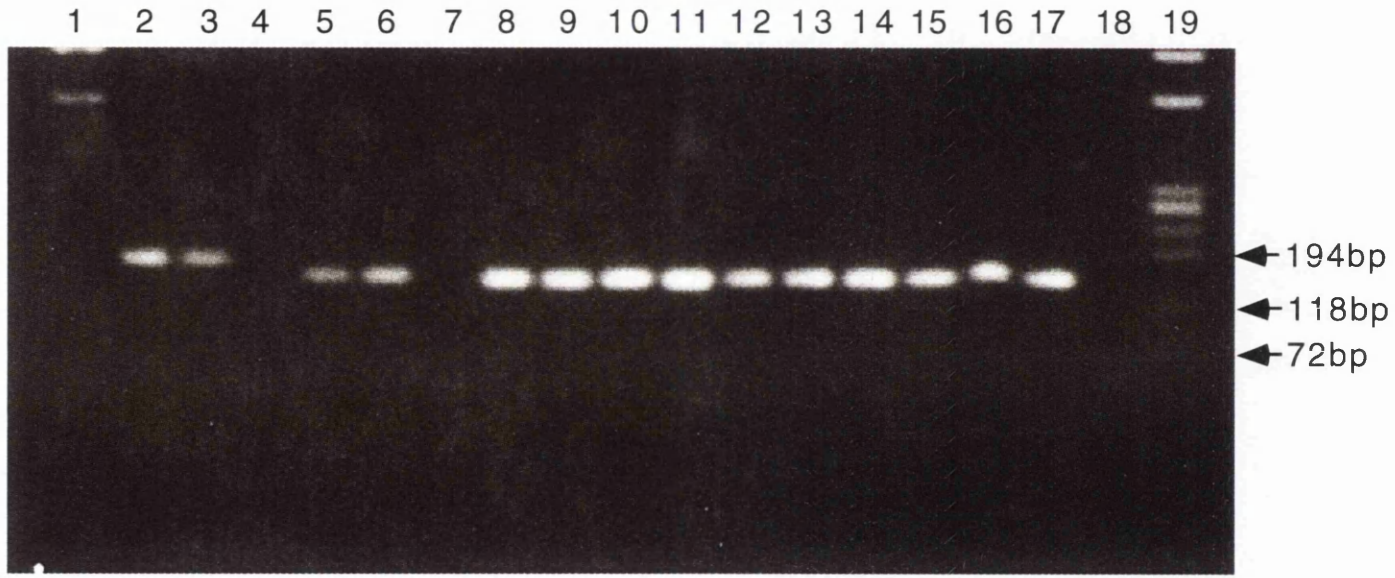

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
194bp
118bp
72bp

Figure 4.7: The effect of glycerol on and sensitivity of SSCP analysis. **(A)** Ethidium bromide stained SSCP gel without glycerol. The contents of each lane are the same as those in (B). Note the lack of mobility shift for the denatured T431 PCR product in lanes **(2)**, **(3)** and **(4)** relative to that obtained in the same lanes in (B). However mobility shifts are seen for denatured PCR products derived from the HVS25A plasmid and the T373 mutant clone lanes **(1)** and **(8)** respectively.

(B) Ethidium bromide stained SSCP gel containing glycerol. This figure contains a close-up photograph of lanes **(1)** to **(10)** of the gel in figure 4.5. Some SSCP bands are highlighted by arrows on the left hand side of the photograph labelled with the source of PCR template and the lane number(s) in brackets. Denatured PCR products from **(1)** plasmid containing EHV-1 HVS25A gC gene (Primers S4+S5), **(2)** T431 10^4PFU(Primers S4+S5), **(3)** T431 $5X10^3$PFU:T632 $5X10^3$PFU(Primers S4+S5), **(4)** T431 10^3PFU:T632 10^4PFU(Primers S4+S5), **(5)** T431 10^2PFU:T632 10^4PFU(Primers S4+S5), **(6)** T632 10^4PFU(Primers S4+S5), **(7)** AHT50592(Primers S4+S5), **(8)** Mutant T373 clone(Primers S4+S5), **(9)** T373 old stock(Primers S4+S5) and **(10)** T373 new stock(Primers S4+S5).

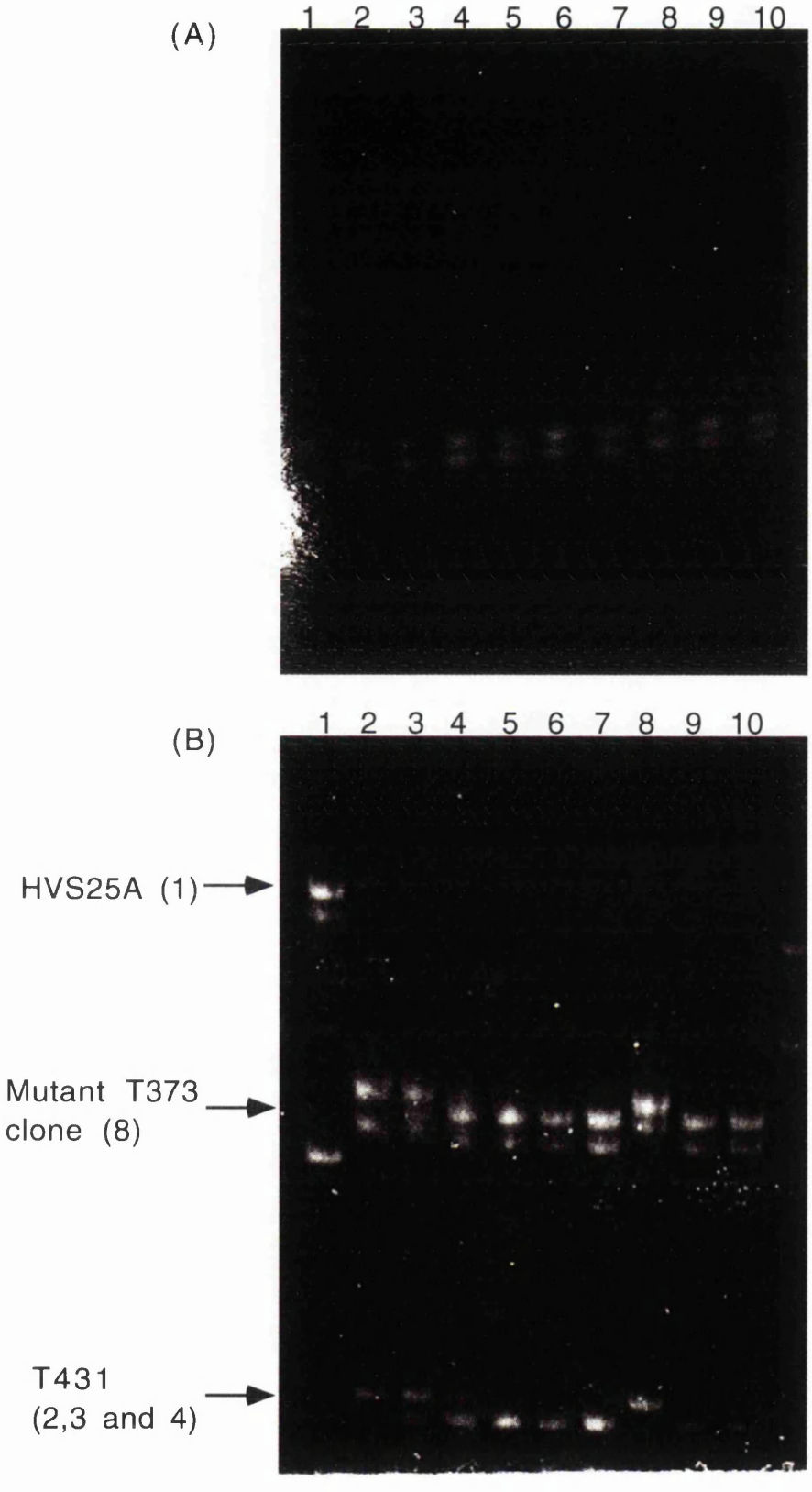
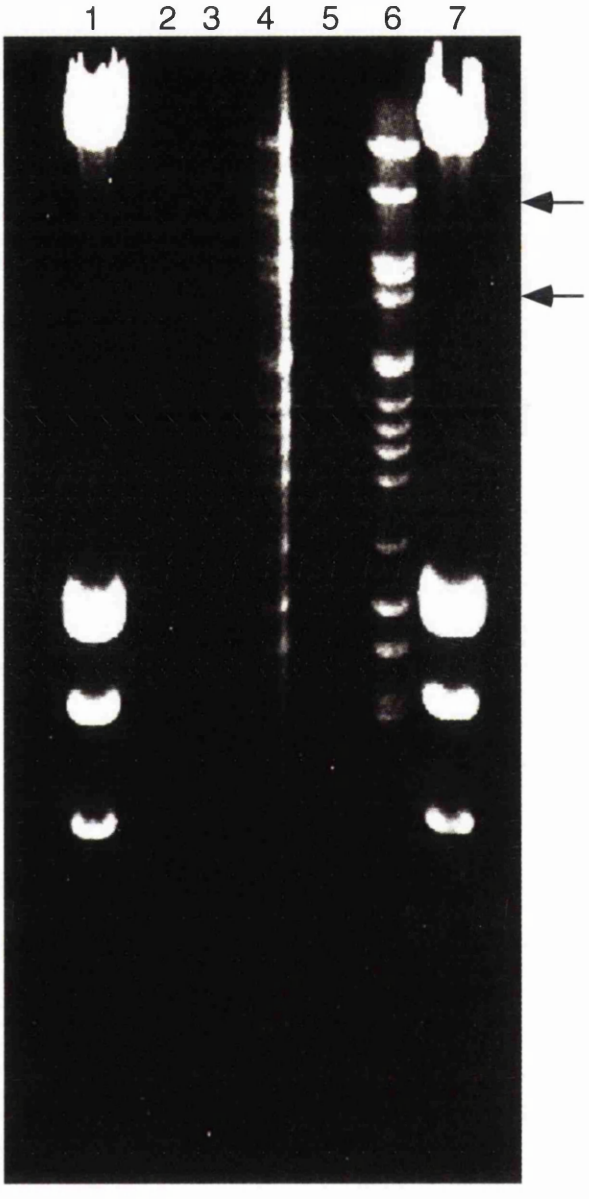

(A)
1 2 3 4 5 6 7 8 9 10
(B)
1 2 3 4 5 6 7 8 9 10
HVS25A (1)
Mutant T373
clone (8)
T431
(2,3 and 4)

Figure 4.8: BamHI fingerprints of T431 and the T373 stocks. Lanes (1) λ HindIII DNA M.W marker, **(4)** T373 old stock DNA preparation BamHI, **(5)** T373 new stock DNA preparation BamHI, **(6)** T431 DNA preparation BamHI and **(7)** λ HindIII DNA M.W marker. Arrows indicate the differences between the 1B and 1P electropherotypes. All other lanes are empty.


1
2
3
4
5
6
7

ANTIBODY	NATURE	WESTERN BLOT	WHOLE VIRUS ELISA	DESCRIPTION	SUPPLIER
26A5	Monoclonal	+	+	Specific for EHV-1 gC reacting with a linear epitope 147-KKSRRG-152 (Allen *et al*., 1992)	G. Allen
56F11	Monoclonal	+	+	Specific for EHV-1 gC reacting with a linear epitope 40-PTHTTPNLTTAHG AGSDNTT-59 (Allen *et al*., 1992)	G.Allen
14H7	Monoclonal	-	+	Specific for EHV-1 gC reacting with a conformation-dependant epitope	G. Allen
3C4B	Ascitic fluid from a hybridoma that was not monocloned	+	ND	Specific for EHV-4 gC	D. Meredith
CONJUGATE	Polyclonal	NA	NA	Anti-mouse IgG (γ-chain specific) peroxidase conjugate	Sigma, UK.

Table 4.1: Characteristics of antibodies referred to in this chapter. (+)= reactive. (-)= not reactive. ND= experiment not performed. NA= parameters not applicable to particular antibody.

Primer	Sequence	Location (Allen and Coogle. 1988)	Isolate\plasmid to be analysed by SSCP analysis
S0	5'-gacccttggtgcatgg-3'	32-48	T632, T431
S1	5'-ctagctccagaggcataa-3'	209-192	As for S0
S2	5'-gtatctaatctgtgccgg-3'	162-179	AHT91320, T431
S3	5'-taccgtttgcgttagttgt-3'	313-295	As for S2
S4	5'-cataacgccaccccggat-3'	511-528	T632, T431, AHT50592, T373, plasmid HVS25AgC, and Mutant T373 clone
S5	5'-actttgtaccaccttccg-3'	661-644	As for S4

Table 4.2: Details of the PCR primers used for SSCP analysis.

SOLUTIONS FOR SDS-PAGE AND WESTERN BLOTTING			
Stock Acrylamide		**SDS-PAGE Running Gel**	
Acrylamide	29g	Stock acrylamide	10ml
methylene-bisacrylamide	1g	1M TrisHCL (pH. 8.8)	11.2ml
dH_2O	to 100ml	dH_2O	8.5ml
		10% SDS	0.3ml
		10% APS	75ul
		TEMED	20ul
SDS-PAGE Stacking Gel		**SDS-PAGE Running Buffer**	
Stock acrylamide	1.7ml	Glycine	14.4g
1M TrisHCL (pH. 6.8)	1.2ml	Tris base	3.1g
dH_2O	7.0ml	SDS	1.0g
10% SDS	0.1ml	dH_2O	to 1l
10% APS	75ul		
TEMED	8ul		
SDS-PAGE Sample Buffer		**Coomassie Blue Staining Solution**	
2.5% SDS		Coomassie blue	0.5g
2.5% β-mercaptoethanol		Methanol	300ml
0.1M TrisHCL (pH 6.8)		Glacial Acetic Acid	100ml
10% Glycerol		dH_2O	to 1l
0.01% Bromophenol blue			
Destaining Solution		**Transfer Buffer**	
Methanol	300ml	Tris Base	3.03g
Glacial Acetic Acid	100ml	Glycine	14.4g
dH_2O	600ml	Methanol	200ml
		dH_2O	to 1l

Table 4.3: Solutions for SDS-PAGE and western blotting.

Antibody	Virus	Dilutions of virus	Mab Dilutions and O.D readings (Each column contains duplicates)		
			1/100	1/1000	1/10000
Mab 26A5	T373	Neat	0.901 0.967	0.219 0.186	0.094 0.084
		1/10	1.262 1.089	0.212 0.232	0.101 0.105
		1/100	0.596 0.614	0.203 0.222	0.108 0.109
	T431	Neat	0.823 0.864	0.218 0.222	0.106 0.085
		1/10	0.905 0.906	0.231 0.235	0.112 0.103
		1/100	0.610 0.651	0.170 0.214	0.135 0.118
	No virus	Neat	0.070 0.073	0.078 0.078	0.072 0.071
Mab 14H7	T373	Neat	0.832 0.846	0.247 0.216	0.126 0.119
		1/10	0.809 0.847	0.260 0.251	0.127 0.124
		1/100	0.507 0.596	0.271 0.252	0.167 0.163
	T431	Neat	0.802 0.824	0.262 0.267	0.153 0.134
		1/10	0.892 0.879	0.276 0.281	0.153 0.142
		1/100	0.603 0.644	0.319 0.304	0.166 0.176
	No Virus	Neat	0.070 0.077	0.086 0.079	0.074 0.075
Ab C4B	T373	Neat	0.076 0.086	0.082 0.085	0.075 0.066
		1/10	0.088 0.079	0.081 0.080	0.078 0.067
		1/100	0.078 0.081	0.081 0.80	0.070 0.069
	T431	Neat	0.073 0.072	0.064 0.072	0.068 0.058
		1/10	0.077 0.080	0.066 0.072	0.067 0.059
		1/100	0.089 0.084	0.072 0.072	0.073 0.064
	No Virus	Neat	0.072 0.082	0.088 0.089	0.077 0.069

Table 4.4: Results of antibody typing ELISA.

4.6 DISCUSSION

The experiments described here represent a systematic attempt to solve two confounding results from the study described in chapter 3, namely the high proportion of cloned mutant PCR products and the disparity between the results reported here and those of Allen *et al* (1988), especially in relation to strain T373. Since both problems are not mutually exclusive, particularly with regard to T373, rigorous experimentation was centred around the mutant PCR product derived from this virus.

Pepscan analysis revealed that the amino acid changed by one of the mutations in the T373 PCR product was not essential for optimal binding of Mab 26A5 to its epitope. It was, however, sufficiently close to the core epitope such that mutation of this residue may hinder sterically or otherwise binding of this Mab. To investigate this possibility the mutant and non-mutant PCR products derived from T373 were expressed in a bacterial system and their reactivity with Mab 26A5 assessed by western blotting. Both expressed proteins were reactive with Mab 26A5. This could not be taken as direct proof that the mutant PCR product was not derived from the isolate initially analysed by Allen and co-workers (1988), since analysis was performed with a prokaryotic version of the proteins under denaturing conditions. Although not performed, a more informative experiment would be the expression of the PCR products in a eukaryotic system followed by immunofluorescent analysis with Mab 26A5 and conformation-dependant anti-EHV-1 gC Mabs (Coogle *et al.,* 1992). Such an experiment would take into account the effects of conformation and of the other mutation in the PCR product derived from T373 which also results in an amino acid change.

Given this result it was thought prudent not to proceed with the panning experiment referred to in the introduction, although it might have been informative.

SSCP analysis was performed on all isolates from which a mutant PCR product was derived to directly assess the possibility that these viruses consisted of a mixed population of strains. The three mutations tested i.e those in gC of T431, HVS25A and the mutant T373 PCR product, were detected by SSCP analysis. One

interesting aspect of SSCP analysis of the gC gene was the presence of three SSCP bands, in the absence of a dsDNA band, in all SSCP profiles associated with primers S4 and S5. SSCP profiles should generally contain three bands, the dsDNA and the two single strands. Intermediate bands however have been reported which are thought to correspond to partially denatured DNA. Given the high GC base composition of the gC gene it is not suprising that these intermediates would arise.

While there was no definitive evidence of mixed populations the sensitivity of the assay may have been increased by radioactive labelling of the PCR products, although this has not been found to be the case (Enomoto *et al.,* 1994). This approach, through the provision of a permanent record of the DNA bands on a gel, would have allowed the definitive demonstration of the presence or absence of SSCP bands especially in relation to the artifacts seen for T632 and 91320 SSCP profiles.

Although great effort was made to reduce any possibility of contamination of viruses, another sample of T373 was requested and sequenced to determine whether or not the original isolate was unwittingly contaminated during the course of this study. The fact that the new supply of T373 had a gC DNA sequence identical to AB-4 within the region analysed suggested that this was not the case. However, it must be said that it was assumed that the cloned mutant PCR product potentially represented the original isolate analysed by Allen, and therefore only the 400-500bp of DNA sequence encompassing the region encoding the epitope of Mab26A5 was determined for the new isolate.

Mab typing experiments were then performed to determine whether it was possible that T373 may be unreactive with Mab26A5 as determined by Allen *et al* (1988) although the sequencing data in chapter 3 suggested that it should react. This experiment was performed in order to see if antigenic variation of some EHV-1 strains is mediated by some means other than genetic mutation of the antigen encoding gene as discussed in chapter 3. The preliminary Mab typing experiment performed here suggested that T373 does indeed react with Mab 26A5. This directly disagrees with the results of Allen *et al* (1988). Two potentially significant differences exist between the Mab typing described here and that performed by Allen and colleagues (1988). Firstly, although strains in both studies were

propagated in equine dermal cells, Allen *et al* (1988) used KyED cells whereas this study used NBL6 cells. Secondly, this study used crude antigen preparations whereas Allen *et al* (1988) used purified virus particles. Of interest is a report that found differences in the glycoprotein content of different strains of HSV-1, and between virus infected cell preparations and purified particles (Al-Ahdal, 1993). It is not known if these differences were of significance so as to affect the assessment of epitope content.

Finally, restriction enzyme analysis revealed that the T373 strain analysed has a 1B electropherotype and therefore it is likely that the same strain was used in both studies.

Unfortunately the results described here do not resolve the disparity between the DNA sequence analysis of this study and the Mab typing study of Allen *et al* (1988). Further meticulous experimentation would be necessary to find a solution to this enigma, which was beyond the scope of this thesis.

Ultimately the possibility that, as first thought, these mutations are due to misincorporation by Taq DNA polymerase during the PCR must be addressed. The PCR conditions described in chapter 3 i.e. low equimolar concentrations of dNTPs, low number of cycles, and reduced cycling time would generally favour a high fidelity PCR (Ling *et al.,* 1991) with a few noted exceptions (for a review of factors affecting PCR fidelity see Eckert and Kunkel, 1991). These exceptions include:

- The 10-fold lower than recommended number of starting template i.e 10^4 virus particles versus 10^5 genome copies. However it is probable that this is an underestimation of virus genome copies present in the PCR since only live virus is counted by the titration methods employed.

- A two fold greater concentration of Taq was used for virus PCRs. The effect of Taq concentration on PCR fidelity has not been documented. Higher concentrations could result in the reduction of the effective concentration of both template and dNTPs in the PCR which could potentially affect fidelity.

- Finally the addition of virus growth medium to the PCR at a 1/5 dilution may affect the pH of the reaction and thus fidelity.

Although the reported mutation rate of Taq polymerase varies widely, the rate of mutations derived from T373 at least approximates with some of the reports (Eckert and Kunkel. 1990; Gelfand and White. 1990) i.e 2 mutations out of a total of approximately 16,000bp sequenced, assuming that no other mutations occurred in the regions not sequenced in the 10 cloned PCR products derived from T373. It is possible that if more clones were sequenced for each virus mutations would be identified according to the calculation of the error rate.

Only two of the mutations analysed here i.e. those derived from 50592 and 91320 are of the type most easily extended by Taq (Kwok *et al.,* 1990). No hotspots for PCR induced mutations were recognised since the five identified mutations were distributed across the gC encoding sequence. No mutations were identified in the area of the non-coding region that was sequenced but more extensive sequencing would have to be performed to discount this possibility.

In conclusion we cannot prove whether the mutations in the cloned PCR products were derived from actual viruses or whether they were PCR induced.

Although the discord between the study described in chapter three and that of Allen and co-workers (1988) remains unresolved this chapter revealed two pertinent points. Firstly, preliminary data does indeed indicate that strain T373 encodes an intact Mab26A5 reactive epitope and secondly, on the basis of RFLP and further sequencing analysis, the aforementioned strain was not contaminated during the course of these studies. This data supports the accuracy of the study described in chapter three.

Chapter Five

Serological analysis of EHV-1 and -4 infections with the Pepscan system

5.1 INTRODUCTION

Chapter one summarised the available methods for distinguishing between horses infected with either EHV-1 or -4. The majority of these tests rely on the direct detection of the viral antigen or nucleic acid. The major drawbacks of many of these techniques are that they are slow in yielding results, insufficiently sensitive or require very specialised techniques or equipment which are not within the capabilities of many clinical diagnostic laboratories.

Serological methods e.g complement fixation (CF) and virus/serum neutralisation (VN) tests are commonplace in the EHV diagnostic laboratory. These methods are not sufficiently discriminatory between EHV-1 and -4 and so require further confirmation by direct detection methods e.g virus isolation or indirect immunofluorescence (IIF), that require virus to be grown. Ironically VN may have been a more discriminatory tool for the reasons outlined as follows. Fitzpatrick and Studdert (1984) in a study which was later corroborated by Edington and Bridges (1990) demonstrated that anti-EHV-4 serum from SPF foals could neutralise both virus types but that SPF foal anti-EHV-1 serum could only neutralise EHV-1. Unfortunately naturally infected animals experience frequent EHV-4 infections, some of which are subclinical which would preclude the use of the aforementioned one-way virus neutralisation in relation to EHV-1 infections as a type-specific diagnostic test.

It is worth reiterating the other disadvantages associated with these serological tests, namely CF, VN and direct immunofluorescence (IF) as outlined in chapter one. Firstly further confirmation of these tests are required. Secondly paired serum samples are normally required from an affected animal. These samples may not always demonstrate an increase in antibody titre indicative of a recent infection as determined by the aforementioned tests. Thirdly the tests are particularly susceptible to problems associated with reagent variability in that a sample may be positive with one set of reagents but not another. Such problems may also relate to difficulties in detecting antibodies associated with variant strains (Thomson *et al.*, 1976).

The ideal immunological assay would detect EHV type-specific antibodies in the serum of an infected animal. The assay could be tailored to detect the quantity or isotype of the type-specific antibodies in order to rapidly diagnose a recent infection. The development of such a test would first require the identification of a suitable antibody capture reagent which in this case would be type-specific epitopes from EHV-1 and -4. Obviously the means to initially identify such reagents would be to utilise serum from naturally or experimentally infected animals.

Serum from naturally and experimentally infected animals has been shown to cross-react with glycoproteins from both virus types, regardless of the virus with which the animal's immune system was initially challenged (Allen and Bryans. 1986; Crabb and Studdert. 1990; Crabb *et al.,* 1991; Ahmed *et al.,* 1993; McCartan *et al.,* 1995). Crabb and colleagues (1991) cross-absorbed EHV-1 antisera with EHV-4 to remove cross-reacting antibody and demonstrated that all glycoproteins which normally elicit an immune response in affected animals were recognised by type-specific antibodies. Not all glycoproteins were reactive to the same extent with type-specific equine antibodies, and indeed the extent to which a given glycoprotein was recognised differed between serum from an experimental and natural infection. To date the antigenic sites or epitopes recognised by these type-specific equine antibodies have not been characterised with the possible exception of those reactive with gC (Crabb and Studdert. 1995). Furthermore gG, a glycoprotein not previously considered, was shown to be truly type-specific in that neither the EHV-1 nor -4 homologues contain epitopes which cross-react with monospecific EHV-4 or -1 antiserum respectively (Crabb *et al.,* 1992; Crabb and Studdert. 1993). Crabb and coworkers (1992) also demonstrated that for EHV-4 a large glycoprotein of 250K, which like the gG EHV-4 homologue is secreted in cell culture, may also be purely type-specific in terms of recognition by the equine immune system. Zheng *et al* (1995) have demonstrated that a glycoprotein in the EHV-4 virion, with a molecular weight corresponding to that of gp2, is recognised by potentially monospecific sera from animals naturally infected with EHV-4. These sera do not react with a similar glycoprotein in the EHV-1 virion. The former and latter studies did not consider gp2 to be the identity of the glycoproteins on the basis of slight differences in size between gp2 and the secreted glycoprotein and the lack of reactivity of anti-gp2 Mabs with the high M.W. glycoprotein respectively. Interestingly Crabb and coworkers (1991) demonstrated that much of

the type-specific antibodies in a mare naturally infected with EHV-1 were reactive with gp2 and, furthermore, it has been recently shown that this glycoprotein is the second most divergent EHV-1 and -4 glycoprotein (Telford *et al.*, 1998).

The linear antigenic sites on the EHV-1 and -4 gG homologues which elicit type-specific responses were delineated and demonstrated to be of sero-epidemiological value (Crabb *et al.*, 1993; Crabb *et al.*, 1995; Drummer *et al.*, 1995).

Efforts have been made to identify antigenic sites and/or epitopes of EHV glycoproteins because of the value of this information in the development of diagnostics and vaccines. A number of techniques are available for this purpose and have been applied to EHV diagnosis. The application of some of these techniques continue to aid the characterisation of some of the epitopes type-specifically recognised by the EHV - affected equine immune system. This should allow the physical separation of cross-reactive from type-specific epitopes, an obvious prerequisite in the development of a type-specific serological assay.

The development and application of Mabs against EHV-1, particularly the work performed by Allen's group, has achieved much in extending knowledge of the EHVs in general. These Mabs have been used to probe λgt11 expression libraries of both EHV-1 and -4 in order to identify genes and the epitopes they encode (Allen and Yeargen. 1987; Whittaker *et al.*, 1991; Cullinane *et al.*, 1993). Wilson and colleagues (1994) have taken this approach a step further by screening an EHV-4 expression library with serum from naturally infected equids in order to identify naturally important epitopes. Serum from convalescent animals identified recombinant phage expressing regions of the EHV-4 gB and gG genes. Indeed a number of groups have developed Mabs against EHV-1 and -4, some of which are type-specific. The relative importance of the epitopes recognised by these Mabs in the equine immune response to the EHVs has been assessed by competitive ELISAs where the horse serum, if reactive with a given epitope, will competitively inhibit the binding of a Mab specific for that epitope (Ostlund *et al.*, 1992; Sinclair *et al.*, 1993b). Ostlund and colleagues (1992) demonstrated that for gC, three out of five previously characterised Mab binding domains (Allen *et al.*, 1992) were either only weakly recognised by horse sera, four weeks post an experimental EHV-1 infection, or were not recognised at all. The two EHV-1 gC Mab binding

domains that were marginally recognised were conformation-dependant. Conversely for gB, three out of five previously identified Mab binding domains were recognised, two strongly and one moderately. All three domains contain linear epitopes on the basis of western blot reactivity (Allen *et al.,* 1992). An increase in the antibody titre between paired acute and convalescent serum samples to an epitope recognised by a conformation-dependant cross-reactive anti-gC Mab, developed by Sinclair and co-workers (1993b), was detected in six out of seven horses naturally infected with either EHV-1 or -4. This epitope was conformation-dependant whereas an anti-gC Mab which recognised a linear epitope was not competitively inhibited by naturally infected horse sera.

All or portions of specific EHV glycoprotein genes have been expressed in bacteria as GST fusion proteins. Antigenic sites in the EHV-1 and -4 gG homologues (Crabb and Studdert. 1993), EHV-1 gB (Sinclair *et al.,* 1993a) and EHV-1 gC (Crabb and Studdert. 1995) have been characterised in this way and their diagnostic potential assessed. The C-terminus of the EHV-1 and -4 gG homologues contain only type-specific epitopes (Crabb and Studdert. 1993) and these have been exploited for the development of an invaluable diagnostic test as alluded to earlier. A GST fusion protein containing the first 50 amino acids of EHV-1 gB cross-reacts with serum from EHV-1 and -4 affected animals. Reactivity with EHV-4 antisera was 10-fold less than that with EHV-1. Although it cannot be used as a type-specific diagnostic reagent the authors have shown that when used as an antibody capture reagent in an end-point titration ELISA, the fusion protein consistently demonstrated recent infection with either virus when acute and convalescent serum samples were compared (Sinclair *et al.,* 1993a). Crabb and Studdert (1995) demonstrated the presence of cross-reactive epitopes in amino acids 107-275 of EHV-1 gC, and pointed to the existence of potentially useful EHV-1 gC type-specific epitopes in amino acids 152-275 on the basis of analysing the somewhat fortuitous occurrence of breakdown products of a gC-GST fusion protein.

Immunofluorescence analysis, with conformation dependant Mabs, of mutants of the EHV-1 gC homologue generated by PCR based site-directed mutagenesis and expressed in eucaryotic cells allowed the complex analysis of conformational epitopes to be addressed (Coogle *et al.,* 1992).

Linear antigenic domains of EHV-1 gC and gB have also been defined using a strategy whereby multiple overlapping peptides representing the primary structure of these glycoproteins were synthesized, and their reactivity with Mabs and naturally infected animal sera assessed by ELISA (Allen *et al.,* 1992; Ostlund *et al.,* 1992). Pepscan analysis of EHV-1 gB and EHV-1 gC with nested sets of peptides 20 amino acids in length and overlapping by 10 amino acids, representing the entire amino acid sequence of these glycoproteins, was performed. Linear epitopes on gC, corresponding to amino acids 140-159 and 40-59, and on gB, corresponding to amino acids 106-125, were identified when glycoprotein specific Mabs were used as probes. The gC epitopes were not strongly recognised by convalescent equine sera whereas the gB epitope was. Furthermore an epitope corresponding to amino acids 306-325 which was not recognised by gB specific Mabs was identified using convalescent equine sera as probe.

Comparison of the EHV-1 gD sequence with that of HSV-1 led to the identification of a region homologous to a HSV-1 neutralising epitope. Subsequent development of anti-serum to a peptide representing this region of EHV-1 gD i.e. amino acids 4-22, confirmed the hypothesis that this region of EHV-1 also contains a complement independent neutralising epitope (Flowers and O'Callaghan. 1992). Anti-serum raised against a peptide representing amino acids 267-285 of EHV-1 gD was non-neutralising *in vitro* but did react with virion gD.

5.2 EXPERIMENTAL APPROACH

Many of the studies referred to in the introduction to this chapter did not examine the responses of naturally/experimentally infected horses to the linear antigenic domains identified, or did not specifically localise the antigenic sites under study. This chapter describes attempts to localise more precisely previously described linear antigenic domains using sera from experimentally and naturally infected equids and thus assess their importance, if any, in natural infection. The amino acid sequence of many EHV-1 and -4 glycoproteins has been elucidated and this data was used to identify regions which were potentially antigenic and/or variable between EHV-1 and -4 that could also be included in these experiments. The ultimate aim of this work was to identify peptides that could be utilised in a type-specific serological assay.

The approach used was to obtain overlapping peptides, synthesized by the Geysen method (Geysen *et al.,* 1984 and 1987) representing the regions of interest. The Geysen method involves the synthesis of peptides attached to a solid support i.e a pin made of polystyrene material. The advantages of the system are:

1. very small peptides can be synthesized on the surface of the pins at very high effective concentrations without problems of solubility,

2. The peptides are synthesized such that they are accessible to antibody and

3. antibody can be removed from the peptides and the pins reused a number of times without affecting the integrity of the peptides.

5.3 MATERIALS

5.3.1 PEPTIDES

94 peptides were ordered from Cambridge Research Biochemicals, Cheshire, U.K. Peptides were synthesized onto a solid support or pin according to the method of Geysen and colleagues (1984). A block of peptides was constructed by inserting 96 pins into a rectangular plastic platform in an arrangement such that the head of each pin could be inserted into the well of a 96 well microtitre plate (see figure 5.1a and b). The peptides were synthesized onto the head of each pin. A positive and negative control peptide was synthesized in parallel with every batch of 94 peptides and tested in-house by the company to quality control peptide synthesis. The 94 peptides ordered were synthesized in duplicate therefore two blocks of peptides were supplied. Each block consisted of 47 test peptides in duplicate and a positive and negative control (see figure 5.1b for the arrangement of pins containing positive and negative control peptides and duplicate test peptides). All peptides were acetylated at the N-terminus and linked through the C-terminus to the polystyrene support via a spacer region (see figure 5.1c).

The details of the 94 peptides synthesized are given in table 5.1.

5.3.2 SERA AND ANTIBODIES

Field sera from horses associated with EHV-1 and -4 outbreaks (see table 5.2) were generously supplied by Dr. R. Sinclair of the Animal health Trust, Newmarket, U.K. and Dr. A. Cullinane of the Irish Equine Centre, Co. Kildare, Rep. of Ireland. Primary sera and plasma samples from experimentally infected specific pathogen free foals were a gift from Dr. J. Gibson, Cambridge University, U.K (see table 5.3).

A control murine monoclonal antibody, which reacts with the positive but not the negative control peptide referred to in section 5.3.1, was obtained from Cambridge Research Biochemicals, Cheshire, U.K. The lyophilised antibody was

reconstituted in 500ul of sterile distilled water. 3ml of PBS were added to the reconstituted antibody and this working stock solution was stored at 4°C.

Goat anti-Horse IgG (γ chain specific) Peroxidase conjugate obtained from Dynatech Ltd., U.K. was developed by Kirkgaard-Perry, the Netherlands and maintained at 4°C.

Rabbit anti-Horse IgG (whole molecule) Peroxidase conjugate was obtained from Sigma, U.K. and stored at 4°C.

Goat anti-Mouse IgG (γ chain specific) Peroxidase conjugate was obtained from Sigma, U.K. and stored at 4°C.

5.3.3 RECIPES

See table 5.4.

5.3.4 PLASTICWARE

Immulon I microtitre dishes were obtained from Dynatech Ltd. These plates were used throughout the pepscan experiments.

5.4 METHODS

5.4.1 CRITERIA FOR CHOOSING PEPTIDES

Peptides were chosen from regions of EHV-1 and -4 glycoproteins associated with:

(1) antigenicity as determined by reactivity of said regions with Mabs or experimentally/naturally infected animal sera.

(2) potential antigenicity as predicted by computer algorithms.

(3) divergent sequence relative to the heterologous virus type.

EHV-1 and 4 gC peptides

Domain One: Peptides encompassing this region in EHV-1 were reactive with horse sera (Ostlund *et al.*, 1992). This region was proposed for extensive pepscan analysis, mainly for two reasons. (1) the degree of EHV-1 vs. 4 sequence divergence in this domain (see figure 5.2) and (2) the fact that an epitope for a type specific Mab (26A5) was localised in this region (Allen *et al.*, 1992). 27 peptides 8aa in length and overlapping by 7aa encompassing the epitope recognised by Mab26A5 were chosen. The homologous region of EHV-4 was similarly analysed as peptides from this region were immunogenic in hamsters (Stokes *et al.*, 1991) (Table 5.1).

Domain Five: This domain was shown to be weakly antigenic in the horse and potentially type specifc (Ostlund *et al.*, 1992; Allen *et al.*, 1992). Three peptides 12aa in length and overlapping by 5aa were chosen to represent the EHV-1 sequence.

EHV-1 and -4 gB peptides

AHT Domain: The corresponding EHV-1 and -4 regions were represented by 8mers overlapping by 7. These regions are quite divergent (figure 5.3) and for EHV-4 at least, are potentially antigenic (figure 5.4). Furthermore, EHV-1 fusion proteins encompassing this region react with both EHV-1 and -4 infected animal sera (Sinclair *et al.*, 1993a).

Misc peptides: 8mers overlapping by 4 were chosen to represent the EHV-1 gB sequence (aa255-270) and the homologous EHV-4 region. This region is divergent (figure 5.3) but is not predicted to be antigenic (figure 5.4). Single peptides representing gB of EHV-1 (aa512-520) and -4 (aa508-514) were included as this region is quite divergent and potentially antigenic. A peptide from EHV-1 gB (aa526-537) was also included for pin system analysis since it was potentially antigenic (figure 5.4).

Miscellaneous gH peptides

12mers overlapping by 6 representing amino acids 16-39 and 22-45 of EHV-1 and -4 respectively were included given the low identity between the EHV-1 and -4 sequences in this region (see figure 5.5). These regions were not predicted to be antigenic. Single peptides representing EHV-1 and -4 gH aa274-285 and 282-293 were selected on the basis of their potential antigenicity (see figure 5.6)

Miscellaneous gp10 peptides

Three peptides representing isolated divergent regions of EHV-1 gp10 were included (see figure 5.7).

gD peptides

Flowers and O'Callaghan (1992) demonstrated that an EHV-1 peptide homologous to a HSV-1 gD neutralising domain injected into rabbits induced anti-

EHV-1 neutralising antibodies. Another peptide from EHV-1 gD did not induce neutralising antibodies in immunised animals but did induce gD reactive antibodies. The EHV-1 and homologous EHV-4 regions are each represented by three 12mers overlapping by five for pepscan analysis (see table 5.1 and figure 5.8).

Note added in proof

All the EHV-4 peptides used in this chapter were aligned with translations of the recently published DNA sequences of the respective glycoproteins from EHV-4 strain NS80567 (Telford *et al.*, 1998). All alignments revealed 100% identity between the peptide and glycoprotein amino acid sequences (Data not shown).

5.4.2 PREPARATION AND HANDLING OF ANTI-SERA

Sodium azide 1% w/v was added to equine serum which was divided into aliquots and stored at -70°C until required. A working stock of serum was stored at 4°C. Serum was centrifuged at 14000rpm for 5 minutes before use in an ELISA. This procedure removed any serum constituents which may have precipitated during long-term storage of the sera, and may have interfered with the ELISA assay.

5.4.3 DERIVATION OF SERUM SAMPLES FROM PLASMA

Equine plasma was stored at -70°C until required. After thawing the plasma, thrombin was slowly added at a concentration of 50units/ml of plasma. The plasma was gently agitated during addition of thrombin and this agitation was maintained for 30 minutes after addition. The clot formed in the plasma was removed by centrifugation at 14000rpm for 10 minutes. Sodium Azide was added to the retained supernatant at a concentration of 0.1% w/v and the serum stored in small aliquots at -70°C. A working aliquot was kept at 4°C and handled according to section 5.4.2.

5.4.4 ELISA ANALYSIS WITH THE PIN TECHNOLOGY SYSTEM

The pins were used according to the manufacturer's instructions but with some minor modifications. All incubation steps were carried out in lids of pipette tip boxes (one block of pins/lid) unless stated otherwise. All washes were performed in enzyme boxes (one block of pins/box).

Blocking step

Non-specific binding sites on each of the two blocks of pins were blocked by incubating each block in 30ml of pre-coat buffer for 1 hour at room temperature on a rotary platform, set at a speed such that the buffer repeatedly washed completely over the pin heads.

Primary antibody incubation

The pins were then removed from the pre-coat buffer momentarily and horse serum was added to the pre-coat buffer to give a dilution of 1/1000 (unless otherwise stated). At this stage the control peptide pins were removed from the blocks and placed in the wells of a microtitre dish that contained 175ul of the control antibody. The blocks containing the experimental peptide pins were returned to the diluted serum. The primary antibody incubation step was performed overnight at 4^{o}C with gentle agitation.

Washes

The control pins were reattached to the blocks and excess diluted anti-serum/antibody was flicked from the pins which were then washed four times. A single wash involved agitating the pins in 500mls of wash buffer for 10 minutes. Clean buffer was used for each wash.

Conjugate reaction

Following removal of excess wash buffer the control pins were again removed from the blocks and placed in the wells of a microtitre dish containing 175ul of the working solution of Goat anti-Mouse IgG (γ chain specific) Peroxidase conjugate. Blocks containing the experimental pins were placed in the lids of pipette tip boxes containing 30ml of a Rabbit anti-Horse IgG (whole

molecule) Peroxidase conjugate diluted 1/5000 in conjugate diluent. The pins were gently agitated in the diluted conjugates for 1 hour at room temperature.

Substrate reaction

Each block of pins was washed as described and in the meantime 200ul of fresh substrate were added to each well of two 96 well microtitre plates. The plates were marked according to the block of pins that was to be inserted into them. Each block was positioned over the corresponding plate so that each of the 96 pins were immersed in substrate in each of the 96 wells. The orientation/position of each well and the pin it contained was noted relative to the position of the wells containing the control pins. The reaction was incubated for 45 minutes. The reaction was stopped by removing the pins from the wells. The microtitre plates were agitated for a further 10 minutes to ensure complete mixing of the colour that had developed in the substrate. The $O.D_{405nm}$ of each well was measured using an automatic plate reader.

Results were processed with particular attention being paid to the results for each duplicate of a peptide and graphs drawn using a Microsoft Excel 4.0 package.

5.4.5 REMOVAL OF PIN/PEPTIDE BOUND ANTIBODY

Standard procedure

A sonication bath was rinsed with distilled water. 4 litres of antibody removal buffer was placed in the bath and allowed to reach a temperature of 60°C. After the substrate reaction was complete (see section 5.4.4) the pins were immersed in the buffer and sonicated for 15 minutes (unless otherwise stated) and then rinsed twice with distilled water, pre-heated to 60°C, for 30 seconds. The pins were then placed in a bath of distilled water initially at a temperature of 60°C for at least 30

minutes with agitation. Excess water was removed from the pins which were then immersed in boiling methanol for 15 seconds. The pins were air dried for 15 minutes after which time they were either re-used or stored at 4°C in an air-tight container containing silica powder to absorb excess moisture.

During the course of this thesis two other antibody removal procedures were adopted.

Universal buffer procedure

The first involved incubation of the pins in universal buffer for 10 minutes at room temperature prior to following the above procedure. The low pH of this buffer is effective at disrupting antibody-antigen interactions in the pin system (Davis *et al.*, 1993).

Urea based procedure

The second was essentially the same as the manufacturer's procedure except that 8M Urea containing 1% v/v of 1-mercaptoethanol was used instead of antibody removal buffer. This is an effective procedure for denaturing proteins attached to the pin-bound peptides which remain intact (G. Tribbick, Chiron Mimitopes, Australia., Personal communication).

5.4.6 CONTROL REACTIONS

The effectiveness of the pin-bound antibody removal procedure and that of the binding of conjugate in the absence of primary antibody was tested by performing an ELISA on the pins, using the aforementioned procedure but omitting the primary antibody incubation step. Two conjugates, a Goat anti-Horse IgG (γ chain specific) peroxidase conjugate and a Rabbit anti-Horse IgG (whole molecule) peroxidase conjugate, both diluted 1/5000 were assayed in this manner prior to assaying sera with the pin system.

5.5 RESULTS

5.5.1 CONJUGATE CONTROL REACTION

A goat anti-equine γ chain specific peroxidase conjugate was shown to react weakly with some peptides in the absence of primary antibody. The peptides recognised by the conjugate were PASTQSAK, ASTQSAKT and STQSAKTV from the EHV-4 gB AHT domain series (see figure 5.9). Rabbit anti-horse IgG(whole molecule) and goat anti-mouse γ chain specific horseradish peroxidase conjugates did not react with these EHV-4 gB AHT domain peptides or any of the other peptides. These conjugates were therefore routinely used in the Pin system ELISA assays described.

5.5.2 REMOVAL OF PIN BOUND ANTIBODY

Initially two blocks of peptides were obtained from the manufacturer. These blocks were initially assayed with serum 94538 at a dilution of 1/100. A conjugate control reaction performed post 94538 indicated that the peptide reactive antibodies in this serum were not removed by the manufacturer's antibody removal protocol. The manufacturer's parent company (Chiron Mimitopes, Australia) indicated that the serum had stuck to the pins, possibly because of concentrated antibodies of very high affinity in the very low dilution of serum utilised. The manufacturer's protocol recommends a dilution of 1/1000 for naturally infected animal sera. Standard antibody removal buffer was replaced by 8M Urea in the antibody removal protocol on the parent company's recommendation, however this also failed to remove the serum. A search of the literature revealed that pins could be subjected to a low pH buffer in order to elute reactive antibodies from the pins without having any apparent adverse effects on the peptides (Davis *et al.*, 1993). Acid elution in association with the standard antibody removal procedure was attempted without success. Extending the length of any of the procedures up to one hour was equally unsuccessful. A second set of blocks (which differed from the first set of blocks in terms of peptide content) were ordered and assayed with serum 94538 at a dilution of 1/5000. Although this is the manufacturer's recommended dilution for

hyperimmune animal sera, serum 94538 stuck to the new set of pins. In order to define conditions for the removal of this serum the old set of pins were sonicated for three hours using standard antibody removal buffer. A conjugate control reaction indicated that the O.D values of all 94538 reactive peptides had dropped by 30%. On this basis the old pins were sonicated for 10 hours. The temperature of the antibody removal buffer at the end of this extended sonication period was 80°C. A conjugate control reaction determined that the O.D values had returned to those seen for the conjugate control performed prior to the use of any sera. The new blocks of pins were subjected to sonication for 10 hours in standard antibody removal buffer which resulted in the removal of serum 94538 reactivities from these pins. Table (5.5) summarises the main steps taken to remove serum 94538 and their efficacy. Although no serum was stuck to the control pins these were also treated in the same way in order to ascertain if any of the aforementioned procedures could be potentially damaging to the peptides. After each variation of the antibody removal procedures the reactivities of the control antibody with (1) the control pins which were subjected to the altered antibody removal procedure and (2) control pins subjected to the standard procedure only were compared and found to be identical.

The new blocks of pins were used for all subsequent experiments during the course of which all other sera and control antibodies were removed by the manufacturer's recommended protocol.

5.5.3 REACTIVITY OF CONTROL ANTIBODY WITH CONTROL PEPTIDES

The $O.D_{405nm}$ values for the reactivity of the control antibody with the positive and negative control peptides were in the region of 1.100 and 0.15 respectively. There was no significant inter-assay variation in these values between assays performed at different times.

5.5.4 REACTIVITY OF EQUINE SERUM WITH PIN BOUND PEPTIDES

SPF foal preinfection sera

All preinfection sera were taken from animals 2 days prior to primary infection. Preinfection serum (Foal identity unknown) which was obtained directly from Cambridge did not react with any of the peptides. All O.D values for this serum were less than 0.25, the average being 0.17.

F12 and F13 preinfection serum extracted from plasma at Glasgow Vet school reacted strongly and specifically with some of the peptides (see figure 5.10). These assays were repeated with the same results. A conjugate control was performed between these repetitions and proved negative, suggesting that the reactivities were a property of the sera being analysed and not a residual reactivity remaining on the pins after prior analyses.

Subsequent experiments with the pin system were performed with serum samples from experimentally or naturally infected animals which had been obtained directly from the sources listed in section 5.3.2 and were not derived from plasma at Glasgow Vet school by the procedure described in section 5.4.3.

Background reactivity for immune sera

Sera having an O.D value greater than 0.5 with a given peptide were generally considered to be reactive with that peptide.

EHV-1 gC domain one (137-170) Figure 5.11

At least three different antigenic regions corresponding to peptides 3-7, 10-15 and 18-23 were defined by probing these peptides with serum from naturally and experimentally infected horses. This is most clearly illustrated by the reactivity profile of foal 6. However, all sera did not have the same epitope specificity within these regions and it could be said that all peptides, with the exception of

peptides 1 and 2, gave an above background reactivity with at least some of the sera. Anti-EHV-4 sera tended to exhibit consistently better reactivities especially from peptide 17 onwards, the sequences of which are 100% homologous between EHV-1 and -4 (Nicolson and Onions, 1990). It is notable that F12 and 14 sera reacted, albeit weakly, with peptide 12 which contains an epitope recognised by an EHV-1 type-specific Mab (Allen *et al.,* 1992). This is of interest since both animals had been infected with EHV-4 and at the time when serum samples were collected had never been infected with EHV-1. Serum 94538 reacted strongly with this epitope. EHV-4 had been isolated from horse 94538 at the time of serum sampling. Sera from the same outbreaks/experimental infections generally gave comparable results e.g 21196 vs 22460 vs 21546. However there were a few notable exceptions, especially with regard to sera associated with EHV-1 e.g 32474 vs 30645. It is noteworthy that the two latter sera reacted with single peptides as did 84568. Horse 7 and 84694 did not react with any of these peptides which, in the case of 84694 could have been due to the dilution factor of 1/2500 used.

EHV-4 gC domain one (154-187) Figure 5.12

The results of this peptide series were generally similar to that of the EHV-1 series. Overall three regions were recognised peptides 2-5, 7-15 and 18-23. SPF 12 reacted with peptides in all three of these regions. Most of the sera with the exceptions of 84694 (dilution factor), 84568 and 32474 reacted with at least one of these regions. All of the aforementioned sera are derived from animals associated with outbreaks of EHV-1. A comparison of the SPF foal sera shows that F12, but not F14, both of which had been infected with EHV-4, reacted with some of peptides 2-5 whereas sera from EHV-1 associated foals did not show appreciable reactivity. However two factors are worth noting with regard to the reactivity of field sera with peptides 2-5. EHV-1 associated field sera 84890 and 30645 reacted with peptides 2-5 whereas EHV-4 associated field sera 22460, 94538 and horse 7 did not react with these peptides. Of interest is the reaction of Horse 7, which was thought to have only a potential association with EHV-4 infection, with this series. This serum reacted with peptides 9-11 in the EHV-4 gC domain one series which align with the region of EHV-1 gC that contains an epitope for an EHV-1 specific Mab. Horse 7 serum did not react with the EHV-1 gC domain

one peptides. This situation is similar to that of sera 84568 which reacts with the EHV-1 series but not the EHV-4 gC domain one series in this region. 30645 again reacts with a single peptide homologous to the 30645 reactive peptide in the EHV-1 series. The O.D reading for the EHV-4 peptide is greater than that for the EHV-1 peptide.

EHV-1 gB AHT domain (95-112) Figure 5.13

It is difficult to discern peaks of reactivity with this peptide series e.g. 21196, 32474, 84568 and 30645. This is due mainly to either high background activity e.g. 21196 or marginal peak reactivities e.g. 84568 and 30645. All the SPF foals seemed to recognise some of the peptides no. 8-11. Sera 21546, 21196 and 22460 are from the same outbreak and reacted with some of these peptides. EHV-1 associated sera from the same outbreak i.e. 32474 and 30645 reacted with peptide 6, albeit marginally. Peptides 1-2 and 5-11 were recognised by some sera e.g. 84890 and 21196 and 32474, 21546 and 21196 respectively.

EHV-4 gB AHT domain (93-108) Figure 5.14

Peaks were most frequently obtained with peptides 4-7. All SPF sera reacted with some of the peptides no. 4-7. An EHV-1 associated serum 84568 reacted strongly with peptide 7. Peptides 2-3 and 8-9 were not as frequently recognised as peptides 4-7. A comparison of sera from the same outbreaks revealed the following. Serum 21546 reacted with peptides from 2-3, 4-7 and 8-9 whereas serum 21196 reacted with peptides 3 and 9, and 22460 reacted with peptide 6. Serum 30645 reacted with peptides 2-3 and 6-9 whereas serum 32474 did not react appreciably with any of the peptides in this series.

Miscellaneous gH peptides Figure 5.15

All sera with the exceptions of 94538, 32474, and 84694 show similar reactivities with these peptides. 84694 did not react with any of the miscellaneous

gH peptides whereas sera 32474 and 94538 did not react with any of the peptides in the EHV-4 gH aa16-39 series. The reactivities of all reactive sera with the EHV-1 gH aa16-39 peptides were practically identical in that peptide 2 of this series was consistently recognised. On the other hand reactivities with the homologous EHV-4 region were not as consistent among the reactive field sera whereas the SPF foal sera had similar reactivity patterns with this peptide series. Three sera, namely 94538, F13, and F12, reacted with both EHV-4 gH peptide 282-293 and its EHV-1 homologue. EHV-4 sera from the same outbreak had identical reactivity patterns with the three different peptide series but of different intensities i.e. peak O.D for 21196>21546>22460. EHV-1 sera from the same outbreak differed in their reactivity with the first two gH peptide series but do not react with peptides representing regions 274-285 and 282-293 of EHV-1 and -4 gH respectively.

Miscellaneous peptides Figure 5.16

EHV-1 gC domain 5 peptides: Four EHV-1 associated sera i.e 30645, 84890, F13 and F6 were reactive with these peptides. Other sera from different horses from the same outbreaks/infections were not reactive. Two EHV-4 associated sera from the SPF foals were weakly reactive.

EHV-1 gp10 peptides: Sera from Foals 6, 12, 14 and horse 30645 reacted with these peptides whereas other animals had a borderline reactivity or were not reactive.

gB peptides (1)255-270 and (4)252-267: Sera 94538 and F12 reacted quite strongly with at least one peptide from each of the EHV-1 and -4 series. F14 and 84890 reacted with only one peptide in the EHV-4 series. Some sera e.g 30645 and 21196 had borderline reactivities with both series.

gB peptides (1)511-520 and (4)507-514: Two EHV-1 associated sera i.e 84558 and 84694 reacted specifically with the EHV-1 peptide. EHV-4 sera 94538 and F12 reacted with both EHV-1 and -4 peptides. 21196 reacted with the EHV-4 peptide only whereas 21546 had a borderline reactivity with the EHV-4 peptide but reacted strongly with EHV-1 peptide.

EHV-1 gB peptide (526-537): Serum 21196 reacted with this peptide. Other sera had borderline reactivity with this peptide.

EHV-1 gD neutralising and non-neutralising domain peptides and the EHV-4 homologues Figure 5.17

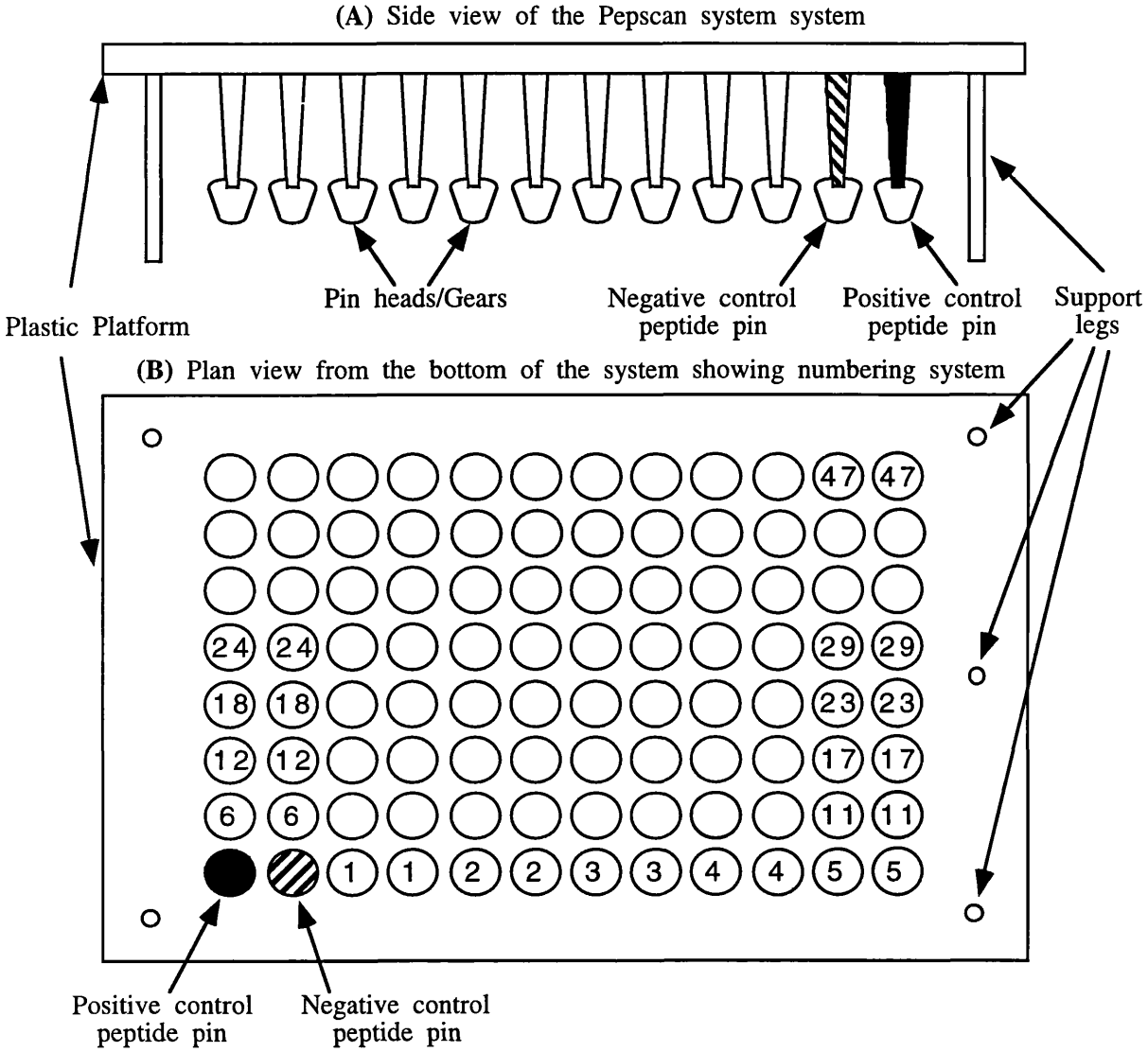
Seven of the sera did not react with the neutralising domain peptides. Of those that did react, most reacted with both EHV-1 and -4 peptides (e.g. 32474, 94538 F13, and F12). Serum 84694 is interesting in that although it is EHV-1 associated it reacted with an EHV-4 peptide. All EHV-4 sera from the same outbreak had marginal reactivity with these peptides. A comparison of sera from the same EHV-1 outbreak reveals that serum 32474 reacted with peptide 2 from the EHV-1 and -4 gD neutralising domain peptides whereas 30654 did not react with any of these peptides. Although F13 was infected with EHV-1 its sera had antibodies against the EHV-4 peptide homologous to the EHV-1 neutralising domain. This is interesting since it has been demonstrated that sera from EHV-1 infected foals do not neutralise EHV-4 *in vitro* (Fitzpatrick and Studdert 1984; Edington and Bridges 1990).

Both F6 and F13 reacted with peptide 2 of the EHV-1 gD non-neutralising domain. Both foals had been infected with EHV-1. The EHV-4 associated foals F12 and F14 had only marginal reactivity with these peptides. Of the EHV-4 associated field sera only those from the same outbreak i.e. 21196, 22460 and 21546 reacted with the EHV-1 gD non-neutralising peptides and with a specificity (peptide 1) different to that of the EHV-1 associated SPF foals (peptide 2). Some EHV-1 associated field sera e.g. 32474 and 84694 did not react with these peptides. All the SPF foal sera with the possible exception of F13 reacted strongly with peptide 2 from the EHV-4 gD non-neutralising domain. Some EHV-4 associated field sera e.g. horse 7 and 21546 reacted with EHV-4 peptide 2; however sera 21196 and 22460 had only marginal reactivity and 94538 did not react with either peptide 1 or 2. Three EHV-1 associated field sera 84890, 84568 and 30645 reacted strongly with peptide 1 but not peptide 2 from the EHV-4 neutralising domain.

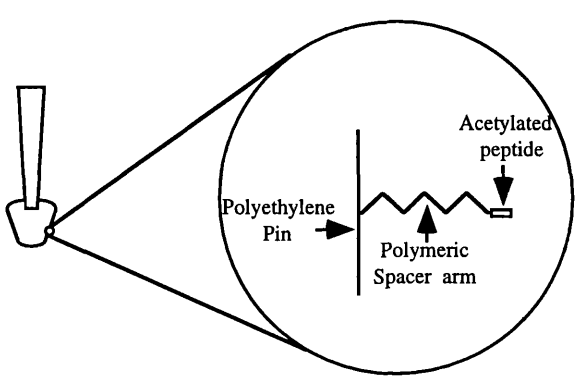
Chapter Five

Figures and Tables

Figure 5.1: Schematic representation of the Pin/Pepscan system.



(C) Magnification showing the Pin surface environment interface



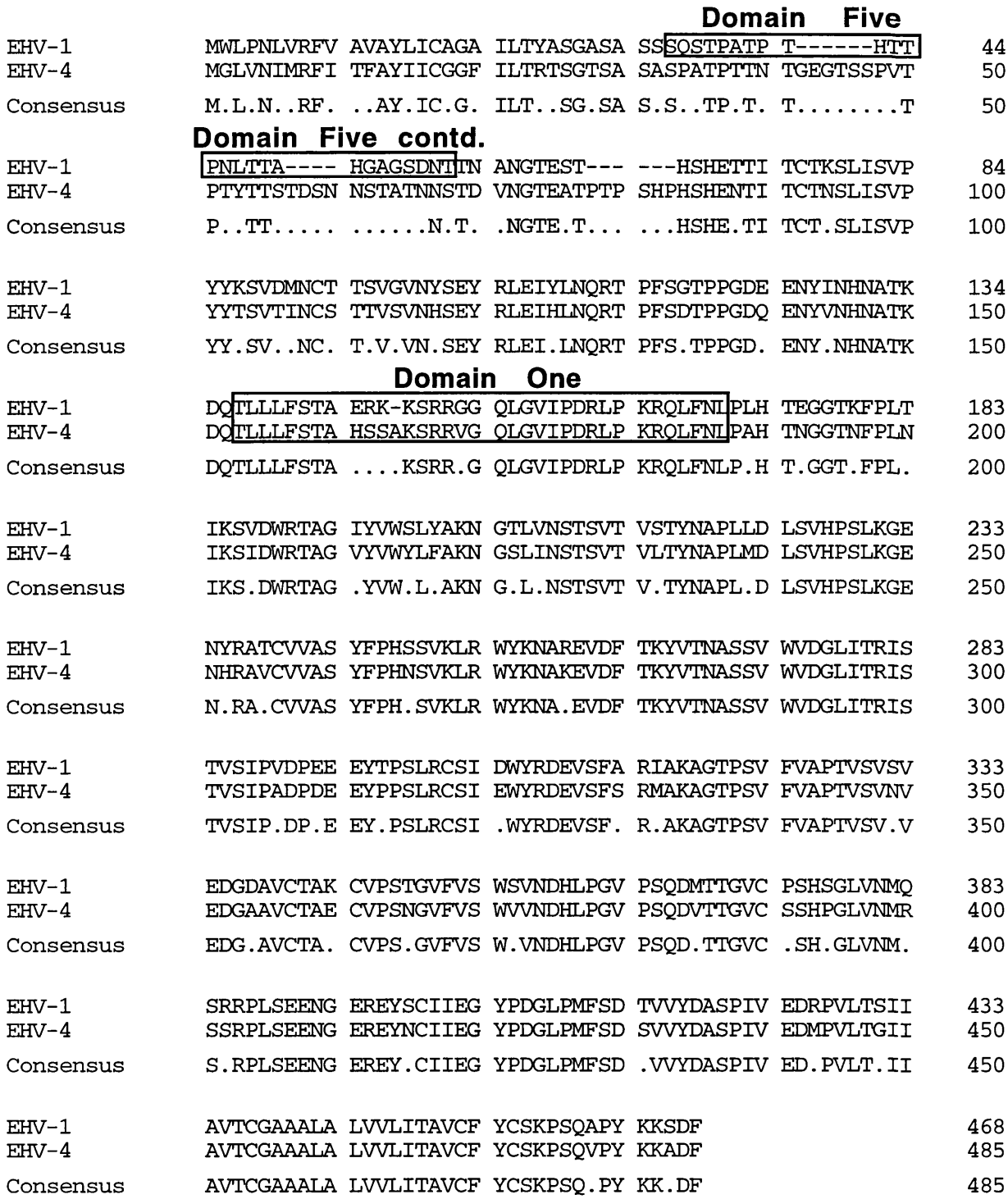
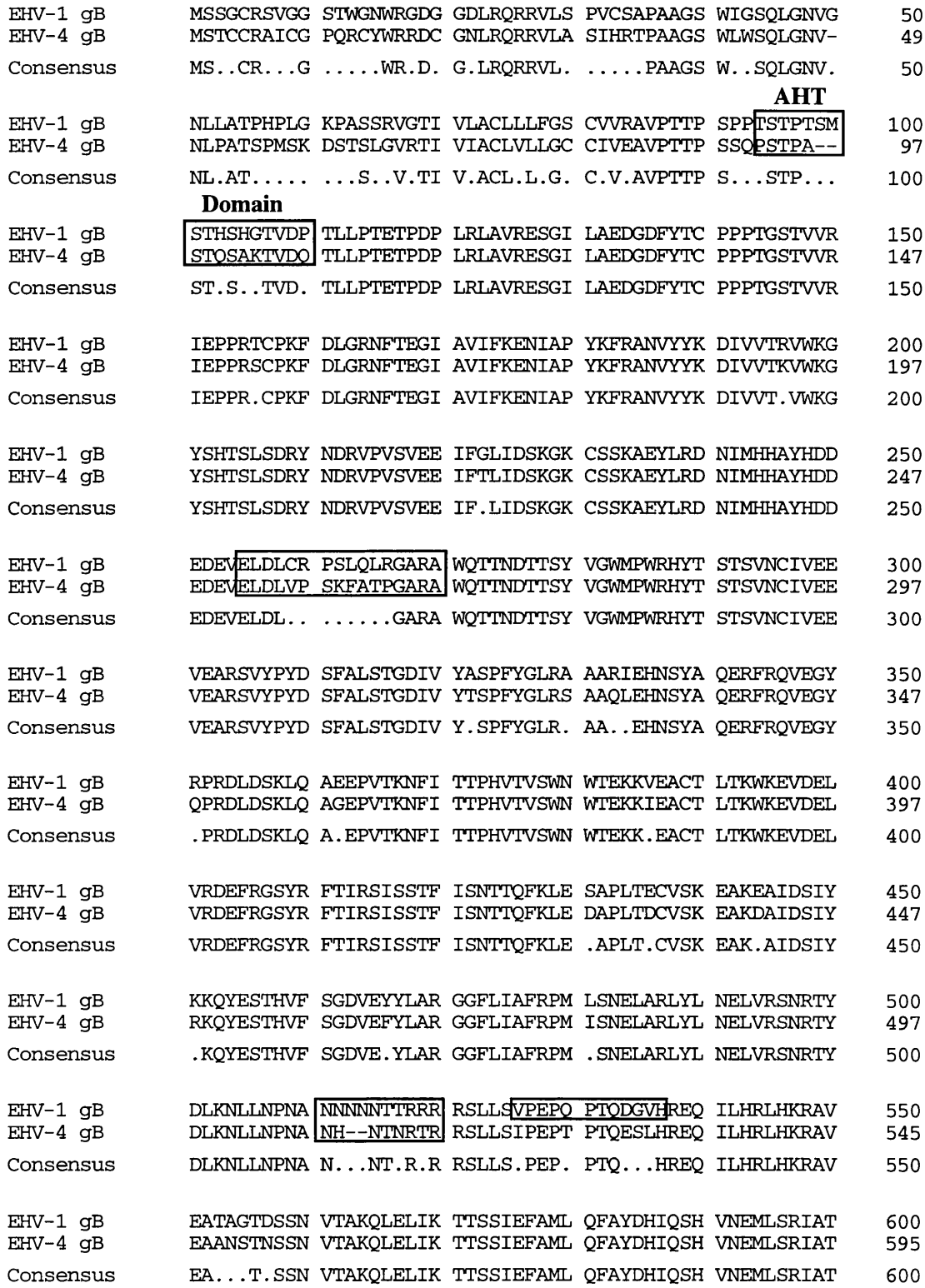


Figure 5.2: Alignment of EHV-1 and -4 gC amino acid sequences. Boxes outline regions chosen for pepscan analysis.




```
EHV-1 gB      MSSGCRSVGG STWGNWRGDG GDLRQRRVLS PVCSAPAAGS WIGSQLGNVG   50
EHV-4 gB      MSTCCRAICG PQRCYWRRDC GNLRQRRVLA SIHRTPAAGS WLWSQLGNV-   49
Consensus     MS..CR...G .....WR.D. G.LRQRRVL. .....PAAGS W..SQLGNV.   50

EHV-1 gB      NLLATPHPLG KPASSRVGTI VLACLLLFGS CVVRAVPTTP SPPTSTPTSM  100
EHV-4 gB      NLPATSPMSK DSTSLGVRTI VIACLVLLGC CIVEAVPTTP SSQPSTPA--   97
Consensus     NL.AT..... ...S..V.TI V.ACL.L.G. C.V.AVPTTP S...STP...  100

EHV-1 gB      STHSHGTVDP TLLPTETPDP LRLAVRESGI LAEDGDFYTC PPPTGSTVVR  150
EHV-4 gB      STQSAKTVDQ TLLPTETPDP LRLAVRESGI LAEDGDFYTC PPPTGSTVVR  147
Consensus     ST.S..TVD. TLLPTETPDP LRLAVRESGI LAEDGDFYTC PPPTGSTVVR  150

EHV-1 gB      IEPPRTCPKF DLGRNFTEGI AVIFKENIAP YKFRANVYYK DIVVTRVWKG  200
EHV-4 gB      IEPPRSCPKF DLGRNFTEGI AVIFKENIAP YKFRANVYYK DIVVTKVWKG  197
Consensus     IEPPR.CPKF DLGRNFTEGI AVIFKENIAP YKFRANVYYK DIVVT.VWKG  200

EHV-1 gB      YSHTSLSDRY NDRVPVSVEE IFGLIDSKGK CSSKAEYLRD NIMHHAYHDD  250
EHV-4 gB      YSHTSLSDRY NDRVPVSVEE IFTLIDSKGK CSSKAEYLRD NIMHHAYHDD  247
Consensus     YSHTSLSDRY NDRVPVSVEE IF.LIDSKGK CSSKAEYLRD NIMHHAYHDD  250

EHV-1 gB      EDEVELDLCR PSLQLRGARA WQTTNDTTSY VGWMPWRHYT STSVNCIVEE  300
EHV-4 gB      EDEVELDLVP SKFATPGARA WQTTNDTTSY VGWMPWRHYT STSVNCIVEE  297
Consensus     EDEVELDL.. ......GARA WQTTNDTTSY VGWMPWRHYT STSVNCIVEE  300

EHV-1 gB      VEARSVYPYD SFALSTGDIV YASPFYGLRA AARIEHNSYA QERFRQVEGY  350
EHV-4 gB      VEARSVYPYD SFALSTGDIV YTSPFYGLRS AAQLEHNSYA QERFRQVEGY  347
Consensus     VEARSVYPYD SFALSTGDIV Y.SPFYGLR. AA..EHNSYA QERFRQVEGY  350

EHV-1 gB      RPRDLDSKLQ AEEPVTKNFI TTPHVTVSWN WTEKKVEACT LTKWKEVDEL  400
EHV-4 gB      QPRDLDSKLQ AGEPVTKNFI TTPHVTVSWN WTEKKIEACT LTKWKEVDEL  397
Consensus     .PRDLDSKLQ A.EPVTKNFI TTPHVTVSWN WTEKK.EACT LTKWKEVDEL  400

EHV-1 gB      VRDEFRGSYR FTIRSISSTF ISNTTQFKLE SAPLTECVSK EAKEAIDSIY  450
EHV-4 gB      VRDEFRGSYR FTIRSISSTF ISNTTQFKLE DAPLTDCVSK EAKDAIDSIY  447
Consensus     VRDEFRGSYR FTIRSISSTF ISNTTQFKLE .APLT.CVSK EAK.AIDSIY  450

EHV-1 gB      KKQYESTHVF SGDVEYYLAR GGFLIAFRPM LSNELARLYL NELVRSNRTY  500
EHV-4 gB      RKQYESTHVF SGDVEFYLAR GGFLIAFRPM ISNELARLYL NELVRSNRTY  497
Consensus     .KQYESTHVF SGDVE.YLAR GGFLIAFRPM .SNELARLYL NELVRSNRTY  500

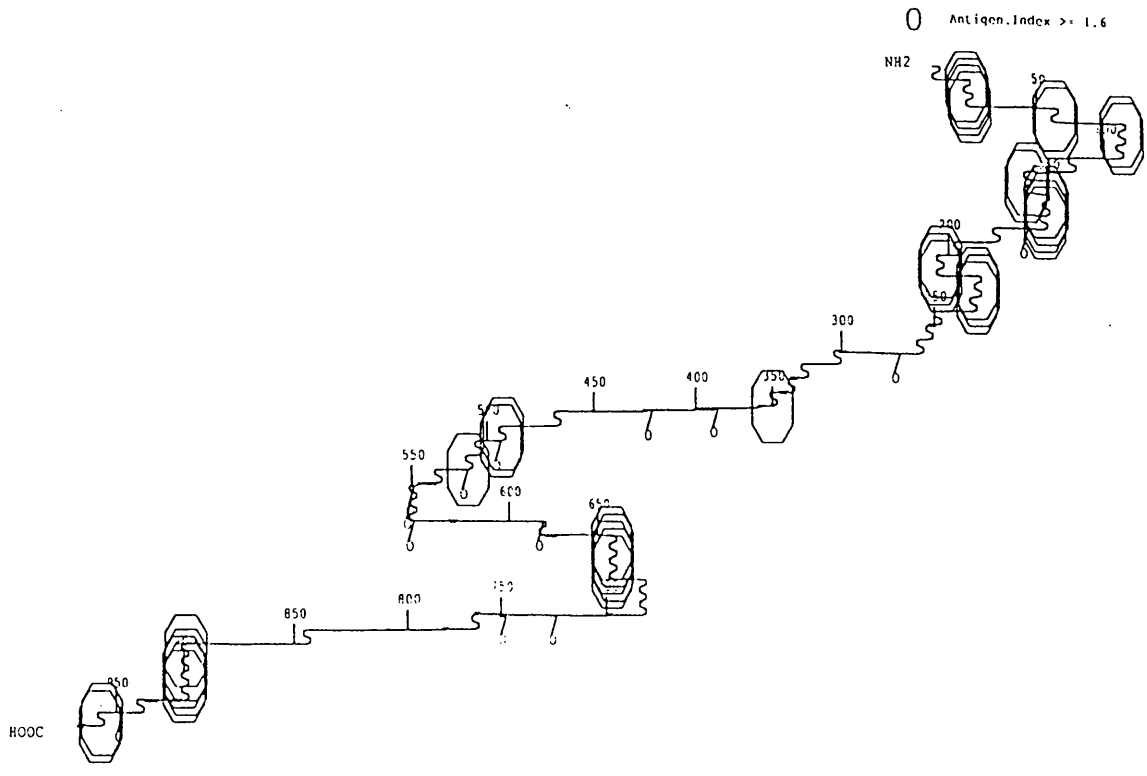
EHV-1 gB      DLKNLLNPNA NNNNNTTRRR RSLLSVPEPQ PTQDGVHREQ ILHRLHKRAV  550
EHV-4 gB      DLKNLLNPNA NH--NTNRTR RSLLSIPEPT PTQESLHREQ ILHRLHKRAV  545
Consensus     DLKNLLNPNA N...NT.R.R RSLLS.PEP. PTQ...HREQ ILHRLHKRAV  550

EHV-1 gB      EATAGTDSSN VTAKQLELIK TTSSIEFAML QFAYDHIQSH VNEMLSRIAT  600
EHV-4 gB      EAANSTNSSN VTAKQLELIK TTSSIEFAML QFAYDHIQSH VNEMLSRIAT  595
Consensus     EA...T.SSN VTAKQLELIK TTSSIEFAML QFAYDHIQSH VNEMLSRIAT  600
```

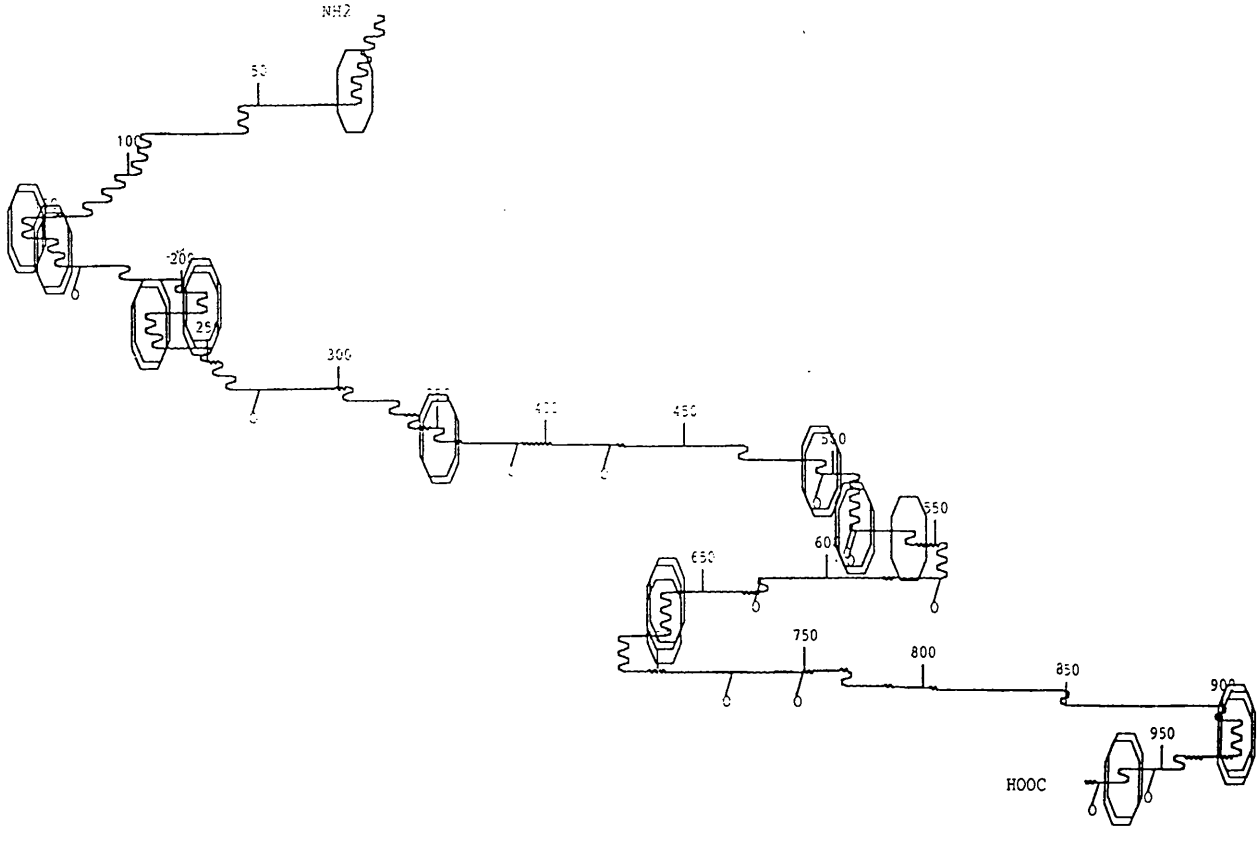
Figure 5.3: Alignment of EHV-1 (1-600) and -4 (1-595) gB amino acid sequences. Boxed regions were chosen for pepscan analysis.

Figure 5.4: Chou-Fasman prediction of antigenic regions of (A) EHV-4 and (B) -1 gB homologues. Regions surrounded by hexagons are potentially antigenic.

(A)



(B)



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EHV-1 gH        MLQPYRKMLI FAVVTVAFAM AVWSTPVPAT PSG------V GNATWANNS-     43
EHV-4 gH        MSQPYLKIAI LVAATIVSAI PVWTTPVSTS PPQQTKLHYV GNGTWVHNNT     50
Consensus       M.QPY.K..I ....T...A. .VW.TPV... P........V GN.TW..N..     50

EHV-1 gH        FNITRYDKIT MGQVYSNTSN SPIFFVVISE RNFRIVNTPL GASVFWIPKG     93
EHV-4 gH        FNVTRYDRIT MEPVYNNNLS STTFFVAISE RNFRTVNTPL GASVFWILKS    100
Consensus       FN.TRYD.IT M..VY.N... S..FFV.ISE RNFR.VNTPL GASVFWI.K.    100

EHV-1 gH        AMNPPQHQPC VANGPEPGDP RGPCVNSTVS LLFNENVEPF LMSKNLLEFE    143
EHV-4 gH        ALNPPKHQPC IANVPEPGDP RGPCVNSTVS LFFNDNLEPF LMTKNLLEFE    150
Consensus       A.NPP.HQPC .AN.PEPGDP RGPCVNSTVS L.FN.N.EPF LM.KNLLEFE    150

EHV-1 gH        VLPDTYITGW TFERSKTATT KSNPVGVVLS PPRGSPSANT TIRDDGGPKK    193
EHV-4 gH        VLPDNYITGW TFERSKTVAT KGNPVGVVLS PPRTSPDVNN TIRDDGTPKQ    200
Consensus       VLPD.YITGW TFERSKT..T K.NPVGVVLS PPR.SP..N. TIRDDG.PK.    200

EHV-1 gH        PLSIIDEYTT LVADLQNFTM TLTYISPFAA VWPIEAFQTG ITVMGCDTTQ    243
EHV-4 gH        HLSIIDEHTT FVLDLQNFTK TLTYISPFAA VWPITAFHAG ITVMGCDTTQ    250
Consensus       .LSIIDE.TT .V.DLQNFT. TLTYISPFAA VWPI.AF..G ITVMGCDTTQ    250

EHV-1 gH        VVAYLGHGFM GLQISSVNNP PLEMIVVPND VSARILNRRP SRLRLEPPGP    293
EHV-4 gH        AIAYLGNGFM GLQISSVNNP PLEMIVAPND VRARIVNRLP PRRRLEPPGP    300
Consensus       ..AYLG.GFM GLQISSVNNP PLEMIV.PND V.ARI.NR.P .R.RLEPPGP    300

EHV-1 gH        HAGPIYKVYV LSDGNFYLGH GMSRISREVA AYPEESLDYR YHLSLANLDT    343
EHV-4 gH        YAGPIYKVYV LSDGNFYLGH GMSKISREVA AYPEESLDYR YHLSLANLDT    350
Consensus       .AGPIYKVYV LSDGNFYLGH GMS.ISREVA AYPEESLDYR YHLSLANLDT    350

EHV-1 gH        LAMLAELSSG KSTDVSYYMY RIVARLAVAT FSLAEVIRLS DYMLLQEAID    393
EHV-4 gH        LAMLAELSSG KSKDVSYYLY RIIARLAVAT FSLAEVIRLS DYMLLQEAID    400
Consensus       LAMLAELSSG KS.DVSYY.Y RI.ARLAVAT FSLAEVIRLS DYMLLQEAID    400

EHV-1 gH        VDMNLRLIVP LVMKYAAGGA ADSSYTSSDV AMDQFDVAQS QIEKIVSDIN    443
EHV-4 gH        VDINLRLIVP LVMKYAAGGT ADSSYTSSDV AMDQFEVAQA QIEKIVADIN    450
Consensus       VD.NLRLIVP LVMKYAAGG. ADSSYTSSDV AMDQF.VAQ. QIEKIV.DIN    450

EHV-1 gH        VEAELRKPMY EHRSLLRSVY AYSRKPLPNA VALADRLILA MYKEAIKDRI    493
EHV-4 gH        IENELRKPMY EHRSLLKSVY AYSRKPLPNA VSFANRLITA MYKEAIKDRI    500
Consensus       .E.ELRKPMY EHRSLL.SVY AYSRKPLPNA V..A.RLI.A MYKEAIKDRI    500

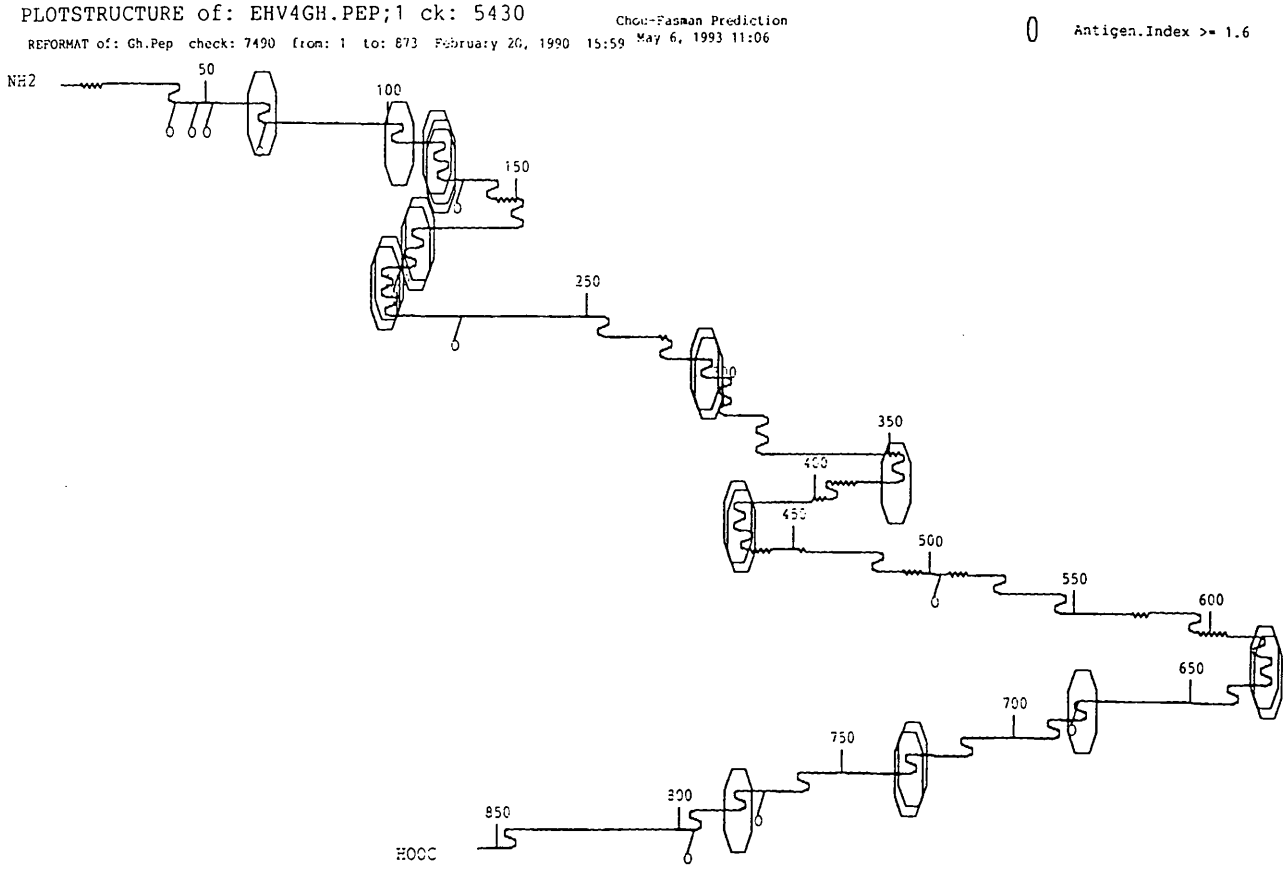
EHV-1 gH        TWNSTMREVL FFAVGAAAGS HVILTDEPEP GAPAHKDASL FLSLNRNILL    543
EHV-4 gH        TWNSTMREVL FFAVGAAAGS HVILTDGPDL GLHAHKDSSM FLSLNRNILL    550
Consensus       TWNSTMREVL FFAVGAAAGS HVILTD.P.. G..AHKD.S. FLSLNRNILL    550

EHV-1 gH        LCTAMCTASH AVSAGLKLEE VMAGLVAGGV QFSLLEVFSP CMASTRFDLA    593
EHV-4 gH        LCTAMCTASH AVSAGVKLEE VMAGLIAGGV QFSLLEVFSP CMASARFDLA    600
Consensus       LCTAMCTASH AVSAG.KLEE VMAGL.AGGV QFSLLEVFSP CMAS.RFDLA    600
```

Figure 5.5: Alignment of EHV-1 and -4 gH amino acid sequences. Boxed regions were chosen for pepscan analysis.

Figure 5.6: Chou-Fasman prediction of antigenic regions of (A) EHV-4 and (B) -1 gH homologues. Regions surrounded by hexagons are potentially antigenic.

(A)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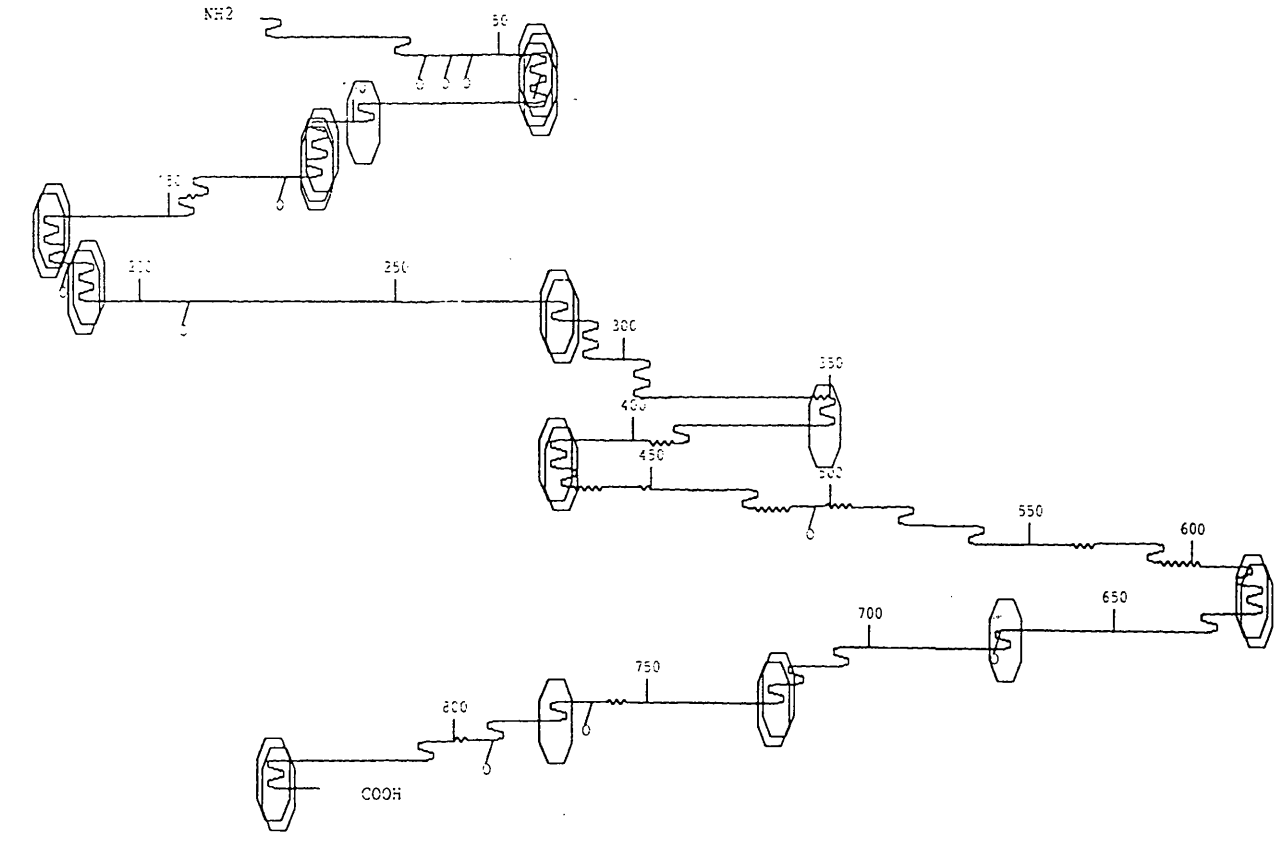
Chou-Fasman Prediction
May 6, 1993 11:06
0 Antigen.Index >= 1.6
NH2
50
100
150
250
350
400
450
500
550
600
650
700
750
800
850
HOOC

(B)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Chou-Fasman Prediction
May 6, 1993 10:35
0 Antigen.Index >= 1.6
NH2
50
250
300
350
400
450
550
600
650
700
750
800
COOH

```
EHV-1 gp10      MDQHHGARGG APIRRPRRSI ESRSHPFRAT GNTQRTYSTP RLSYRDGLSG     50
EHV-4 gp10      MDQHHGVRGG APIRRPRRSI ETRSHPFRAA GNTQRTYSTP RLSYRDGLSG     50
Consensus       MDQHHG.RGG APIRRPRRSI E.RSHPFRA. GNTQRTYSTP RLSYRDGLSG     50

EHV-1 gp10      RTASRDPQEQ ASNQDESSNP STSNAQQSTS FWGYLRRVFS DDVPAQPQAP    100
EHV-4 gp10      RASSLEPGGQ AHDQNESSTQ STSNNQPSTS FWGYLRRVFS DDAPAQPQAP    100
Consensus       R..S..P..Q A..Q.ESS.. STSN.Q.STS FWGYLRRVFS DD.PAQPQAP    100

EHV-1 gp10      RPRADFAPPA GEESSSEEEE EEGPAQAPLD EEDQLMYADQ YSVGDSSDEN    150
EHV-4 gp10      RSRADFAPPP EEDSSSEEED EEGPSQAPLD EEDQLMYADQ YSVGNSSDDN    150
Consensus       R.RADFAPP. .E.SSSEEE. EEGP.QAPLD EEDQLMYADQ YSVG.SSD.N    150

EHV-1 gp10      DEEEDPRLGS DYPTSAESSE YHDHGEMVAG AGAESESETD IDAEEEEEDD    200
EHV-4 gp10      -EEDYLQPEV EYPTSAESGE YHNSG-MFAE EEPESESESD M-----ENYE    193
Consensus       .EE.......  .YPTSAES.E YH..G.M.A. ...ESESE.D ......E...    200

EHV-1 gp10      EDDEDDMEVI RDESYRLPRT WLDKSIRLMD EALAQSSELS KAITKSTRSL    250
EHV-4 gp10      TYEENDTEVI SDDSHRLTRT WLDRSIRLMD DALAQSSEIS KAITKSTRRL    243
Consensus       ...E.D.EVI .D.S.RL.RT WLD.SIRLMD .ALAQSSE.S KAITKSTR.L    250

EHV-1 gp10      YDSQFAPGGR GYTQTATPSR RLVQLSRAGM YDSDKIVMTG DYMEVDDDPD    300
EHV-4 gp10      YDSQFTPGGR GYKQTETPSQ RLVHLSRAGM YDSDEIVMTG DYMEVDDDPN    293
Consensus       YDSQF.PGGR GY.QT.TPS. RLV.LSRAGM YDSD.IVMTG DYMEVDDDP.    300

EHV-1 gp10      SAYQSWVRAI RHPLAMNPSW EETISNHTNP SFSTDIDYDI DELIEKNLAR    350
EHV-4 gp10      SAYQSWVRAI HHPVAMNPSW EETISNHTNT SFSADIDYDI DELIEMNLAR    343
Consensus       SAYQSWVRAI .HP.AMNPSW EETISNHTN. SFS.DIDYDI DELIE.NLAR    350

EHV-1 gp10      TPPVFEGLLD SAEFFYKLPM LYTYATITQD EAYEERLAWS NTQALHGHEQ    400
EHV-4 gp10      TPPVFEGLLD SADFFYRLPM LYTYATITQD EAYEERQAWS NTQALHGHEQ    393
Consensus       TPPVFEGLLD SA.FFY.LPM LYTYATITQD EAYEER.AWS NTQALHGHEQ    400

EHV-1 gp10      SSWQALLVYY SRGGMYVSPT QEPRGIWRRA LKQAMALQLK MCVLGLSDVV    450
EHV-4 gp10      SSWPALVSDY SKGGMYVSPT QEPRGIWRRA LKQAMALQLK LCVLGLTEFV    443
Consensus       SSW.AL...Y S.GGMYVSPT QEPRGIWRRA LKQAMALQLK .CVLGL...V    450

EHV-1 gp10      TKQNATHHHA AVTFLVDALL RTARNCYLAS RLLVFAWERR RETGAKRPAE    500
EHV-4 gp10      TKRELTQHHS AVTFLVDSLL RTAKNCYLAS RLLVFAWERR RETGVRRPAE    493
Consensus       TK...T.HH. AVTFLVD.LL RTA.NCYLAS RLLVFAWERR RETG..RPAE    500

EHV-1 gp10      PLIALSGVTL LQPLPPEVSE LLEQRTFDIG LRTPNSAVFR AFFGSLVYWA    550
EHV-4 gp10      PLIALSGVTL LQPLPPEVSE LLEQRTFDIG LRTPQSGVFR AFFGPLVYWA    543
Consensus       PLIALSGVTL LQPLPPEVSE LLEQRTFDIG LRTP.S.VFR AFFG.LVYWA    550

EHV-1 gp10      ELRLALRDPA SINCRYVGFH LQTSEIYLLA RAHSASPGYT KEELVAMEAI    600
EHV-4 gp10      ELRRALRDPA AINCRYVGFH LQTSEIYLLA RAHSASPGYT KEELVAMEAT    593
Consensus       ELR.ALRDPA .INCRYVGFH LQTSEIYLLA RAHSASPGYT KEELVAMEA.    600
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Figure 5.7: Alignment of the first 600AA of the EHV-1 and -4 gp10 homologues. Boxed regions were chosen for pepscan analysis.

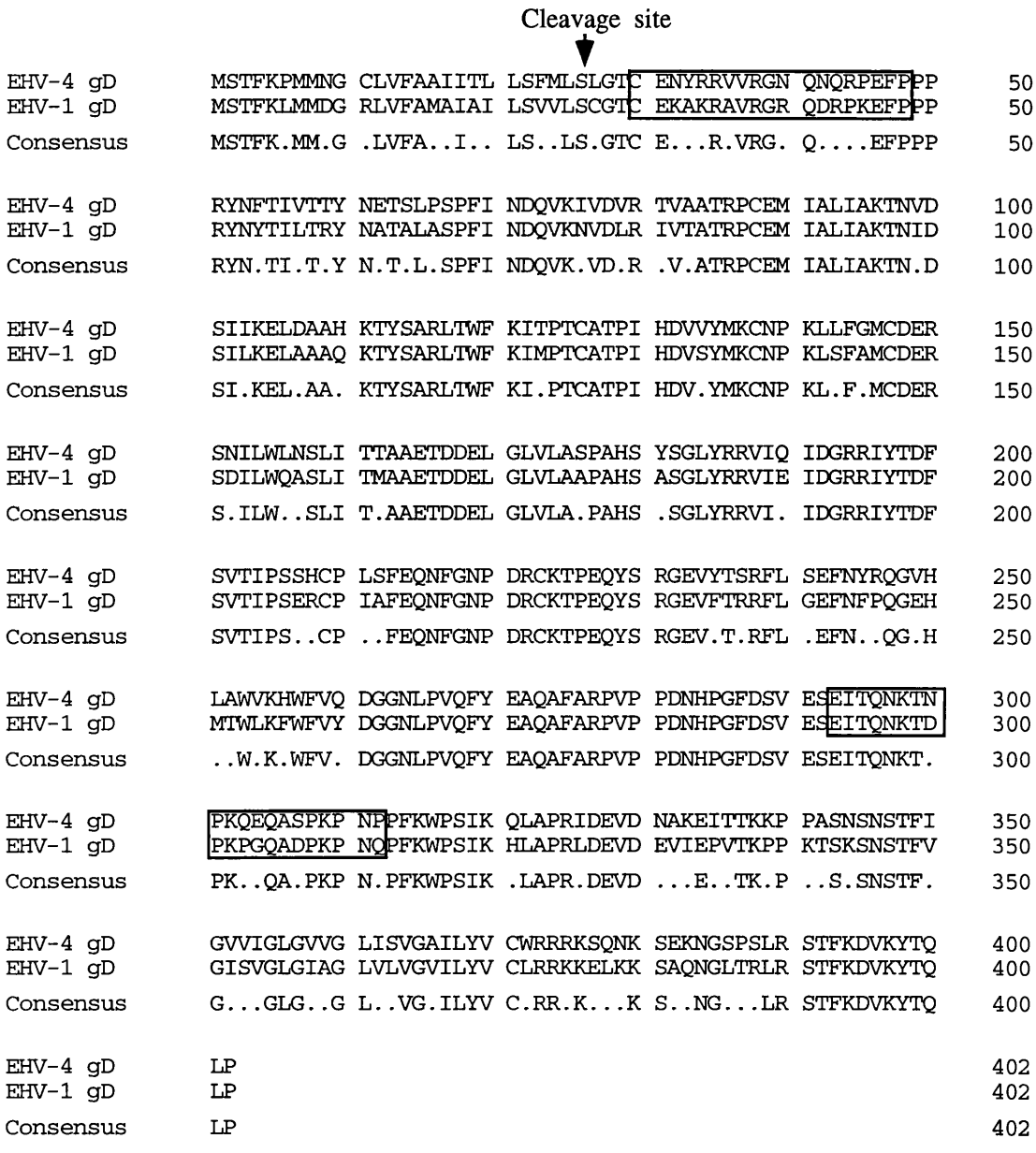
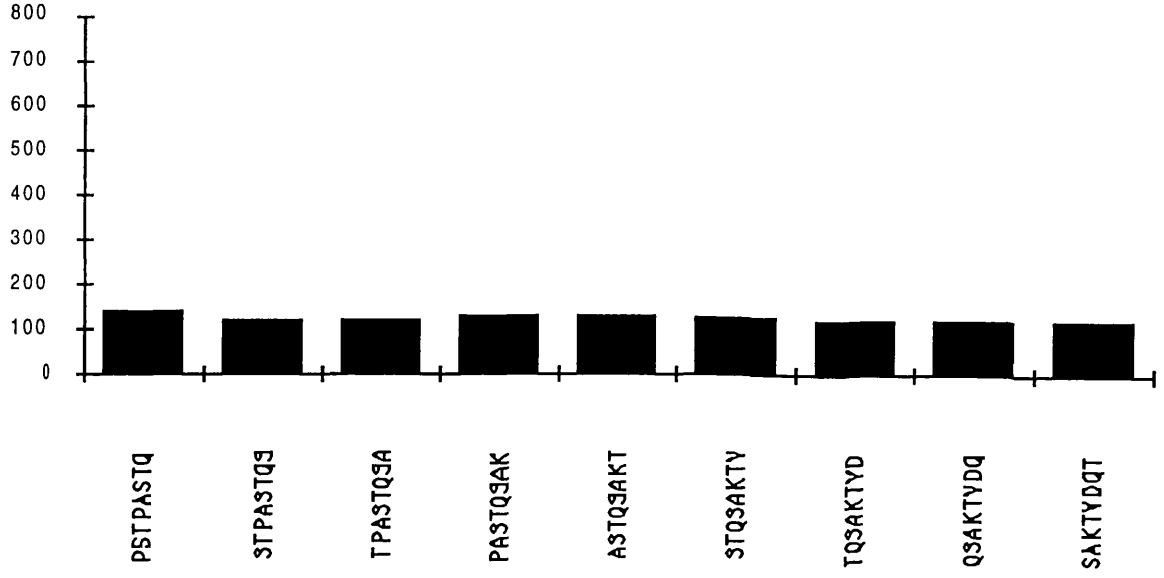


Figure 5.8: Alignment of the EHV-1 and -4 gD homologues.
Boxed regions were chosen for pepscan analysis. Peptides representing these regions were numbered from the proposed cleavage site.

(A) Rabbit anti-horse IgG H+L chain specific HRP conjugate.



(B) Goat anti-horse γ-chain specific HRP conjugate.

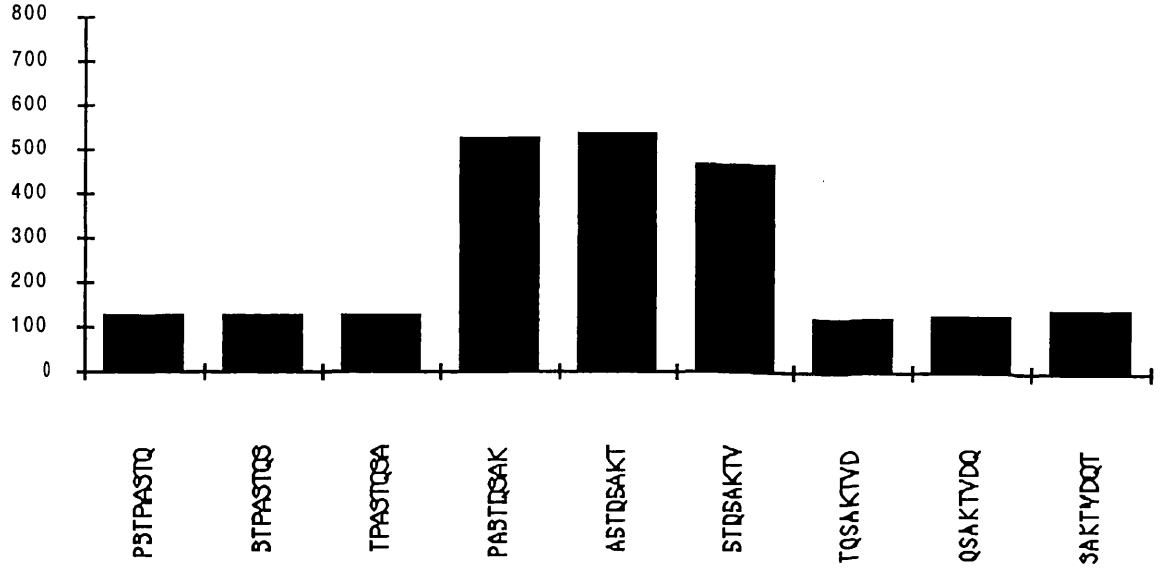


Figure 5.9: Conjugate control reactivities with the EHV-4 gB AHT domain peptides. The peptides in this series are listed on the horizontal axes. The vertical axis are labelled $O.D_{405nm}$ x 1000.

Figure 5.10: Reactivity of Preimmune SPF foal 12 and 13 plasma derived sera with pepscan peptides. (A+H) EHV-1 gC domain one peptides, (B+I) EHV-4 gC domain one peptides, (C+J) EHV-1 gB AHT domain peptides, (D+K) EHV-4 AHT domain peptides, (E+L) Miscellaneous gH peptides, (F+M) Miscellaneous gB, gp10 and gC peptides and (G+N) EHV-1 and -4 gD neutralising and non-neutralising domain peptides. The maximum O.D reading and the unit length of the vertical axis for each peptide series is given at the top and towards the bottom of each vertical axis respectively. Reactivities of preimmune SPF foal 12 and 13 are illustrated in graphs A to G and H to N respectively.

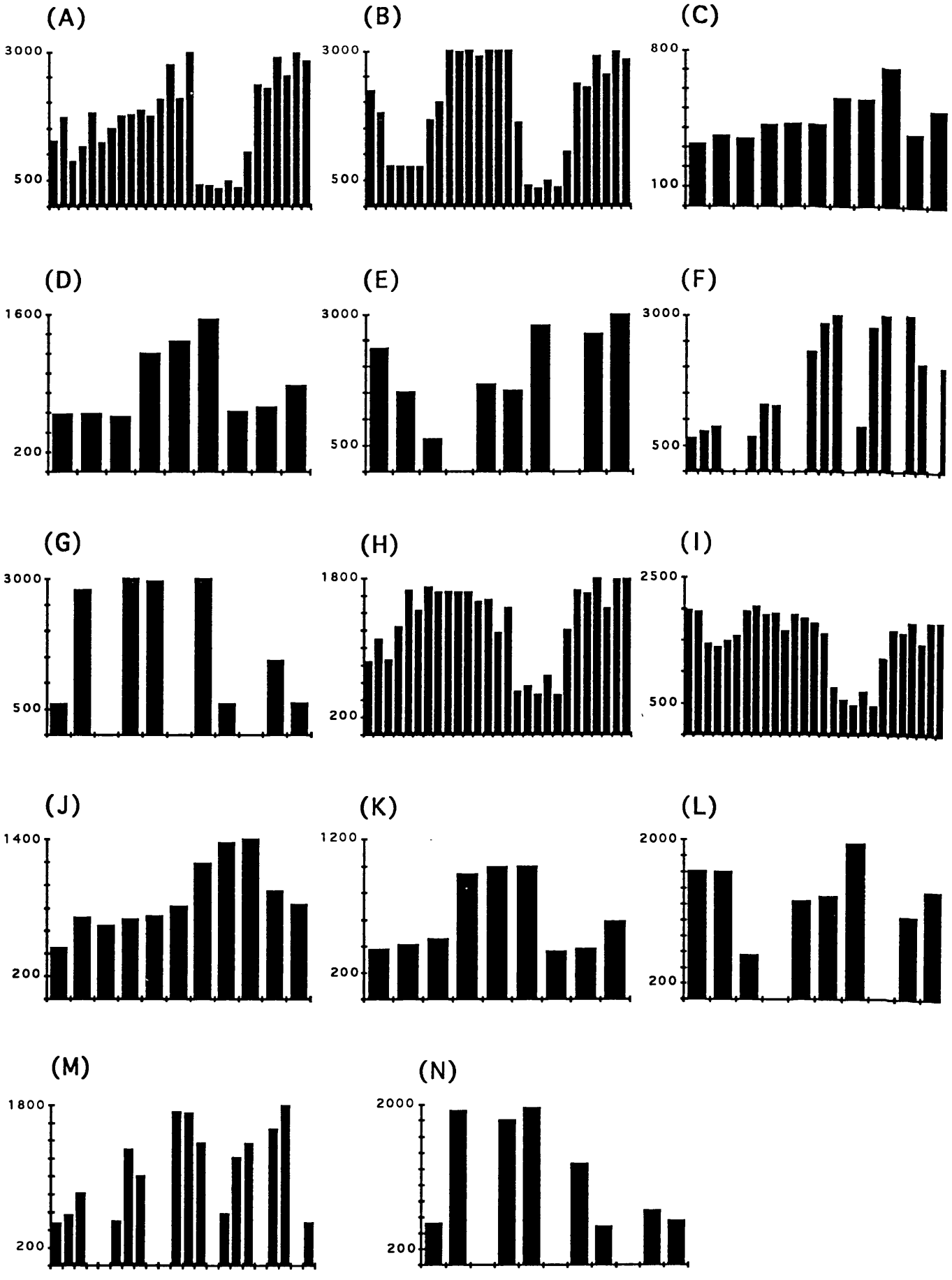

(A)
3000
500
(B)
3000
500
(C)
800
100
(D)
1600
200
(E)
3000
500
(F)
3000
500
(G)
3000
500
(H)
1800
200
(I)
2500
500
(J)
1400
200
(K)
1200
200
(L)
2000
200
(M)
1800
200
(N)
2000
200

Figure 5.11: Reactivity of sera with EHV-1 gC domain one. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

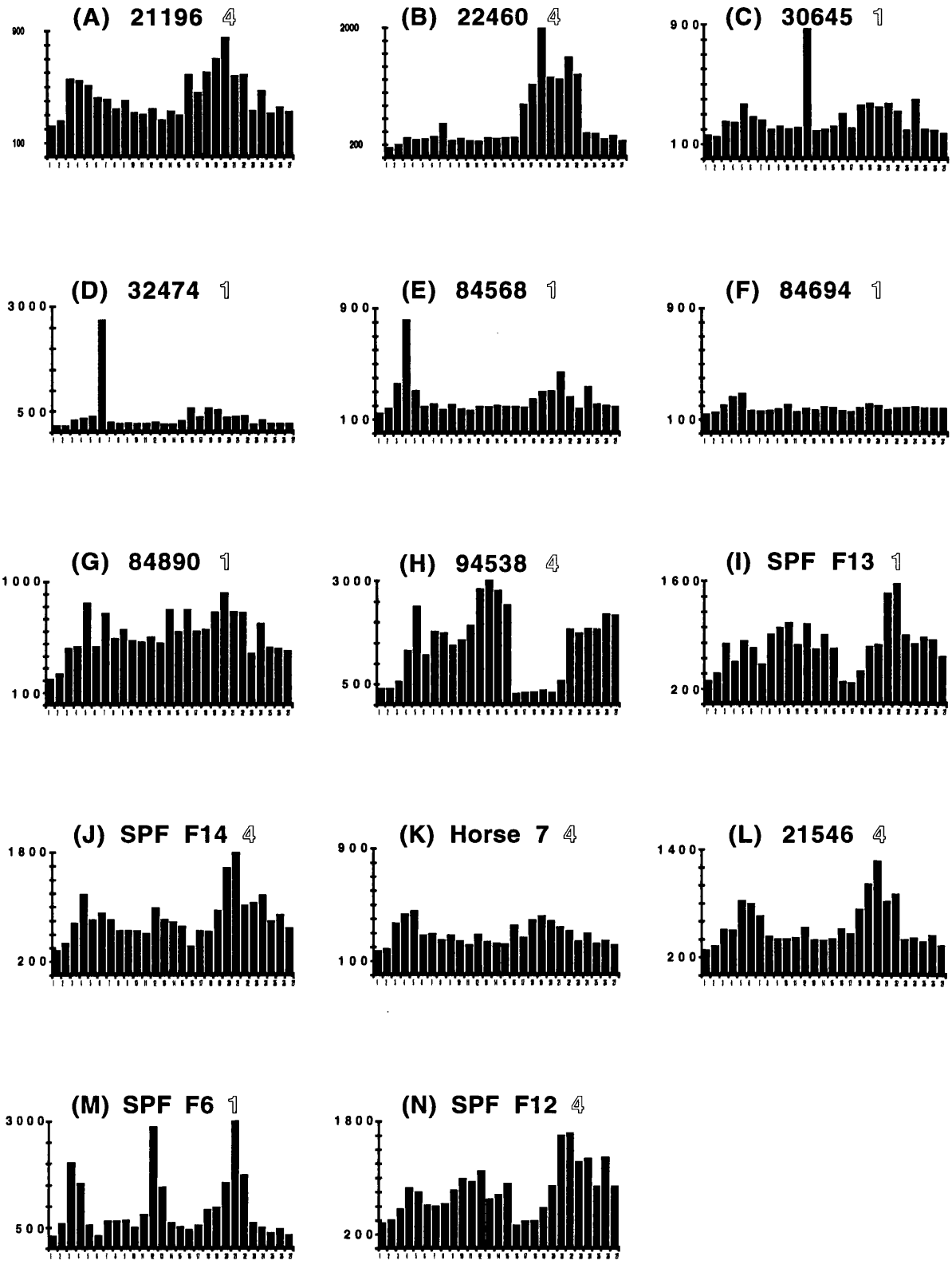
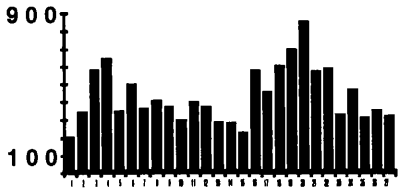
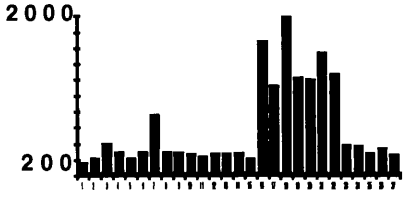

(A) 21196 4
900
100
(B) 22460 4
2000
200
(C) 30645 1
900
100
(D) 32474 1
3000
500
(E) 84568 1
900
100
(F) 84694 1
900
100
(G) 84890 1
1000
100
(H) 94538 4
3000
500
(I) SPF F13 1
1600
200
(J) SPF F14 4
1800
200
(K) Horse 7 4
900
100
(L) 21546 4
1400
200
(M) SPF F6 1
3000
500
(N) SPF F12 4
1800
200

Figure 5.12: Reactivity of sera with EHV-4 gC domain one. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

(A) 21196 4



(B) 22460 4



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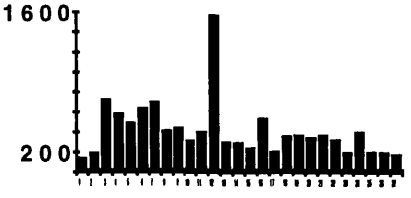
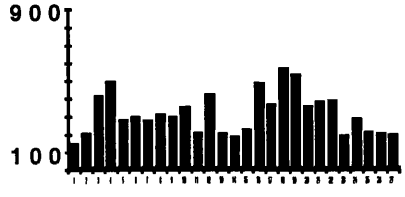
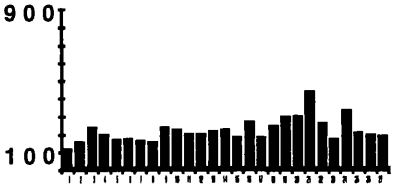


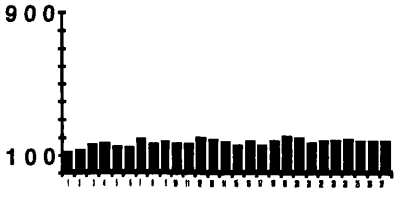
(D) 32474 1



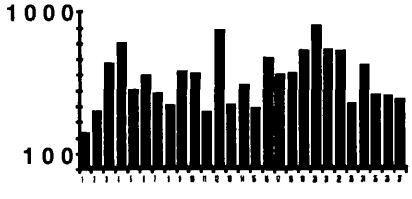
(E) 84568 1



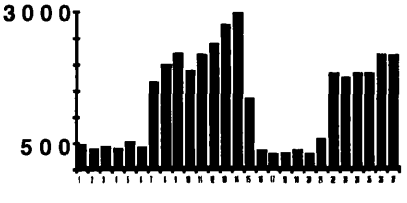
(F) 84694 1



(G) 84890 1



(H) 94538 4



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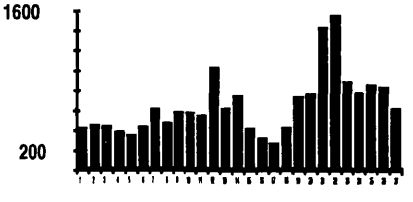
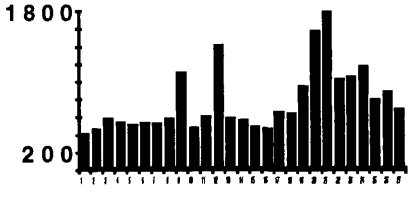
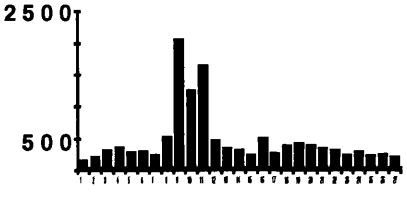


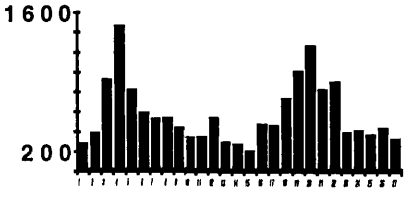
(J) SPF F14 4



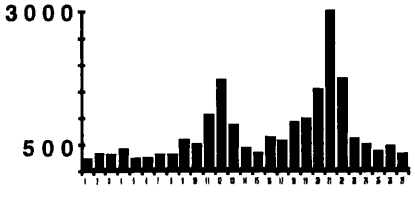
(K) Horse 7 4



(L) 21546 4



(M) SPF F6 1



(N) SPF F12 4

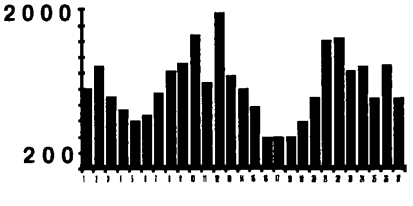
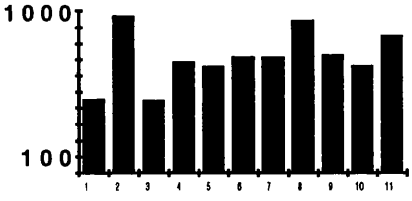
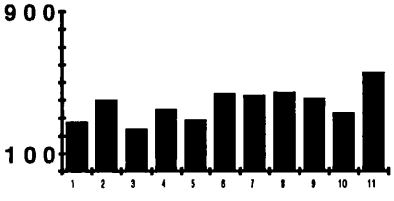


Figure 5.13: Reactivity of sera with EHV-1 gB AHT domain. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

(A) 21196 4



(B) 22460 4



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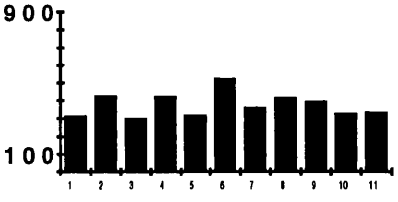
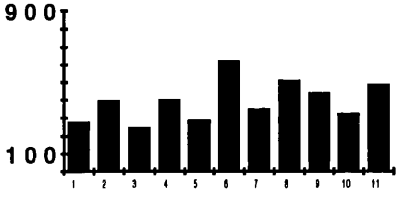
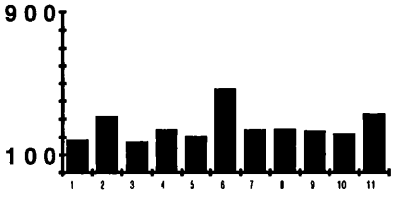


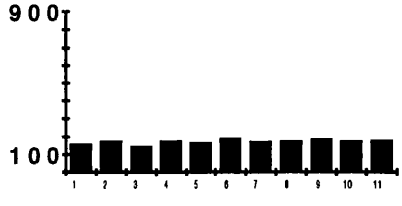
(D) 32474 1



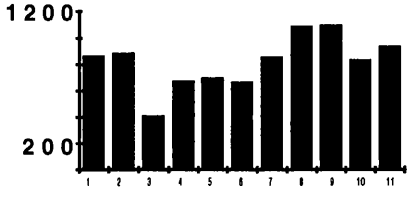
(E) 84568 1



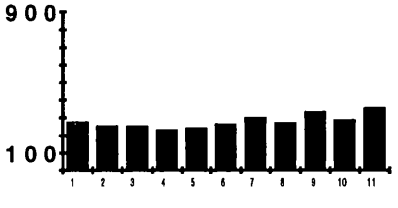
(F) 84694 1



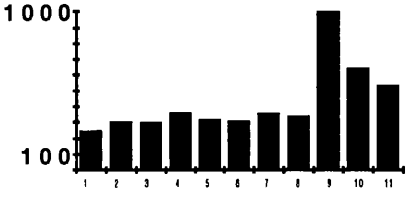
(G) 84890 1



(H) 94538 4



(I) SPF F13 1



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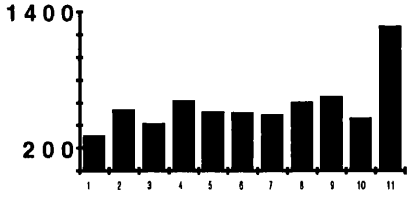
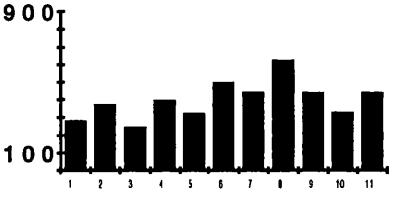


(K) Horse 7 4



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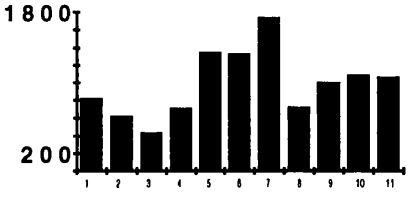

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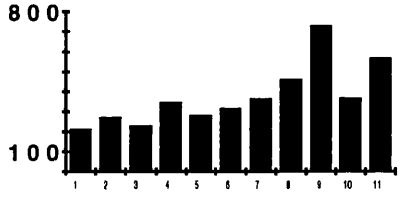


(N) SPF F12 4

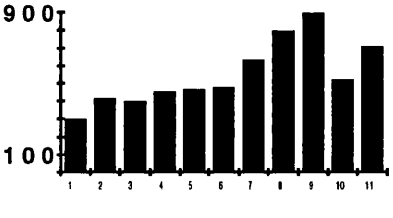
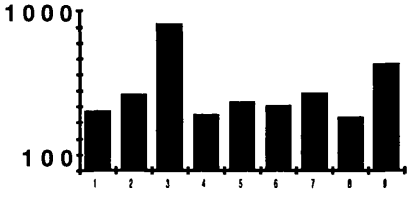
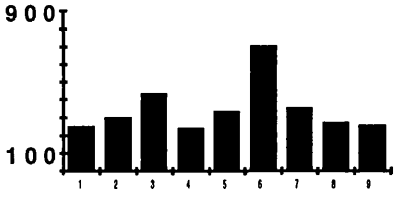


Figure 5.14: Reactivity of sera with EHV-4 gB AHT domain. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

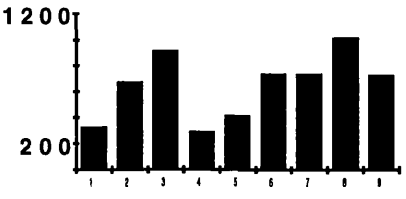
(A) 21196 4



(B) 22460 4



(C) 30645 1



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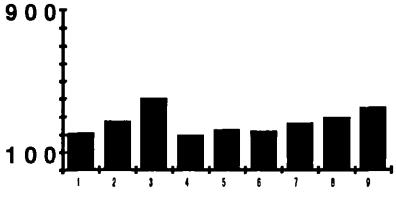
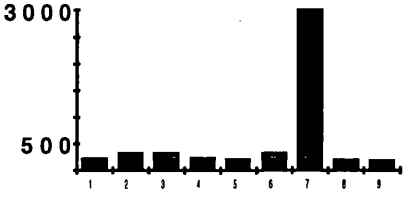


(E) 84568 1



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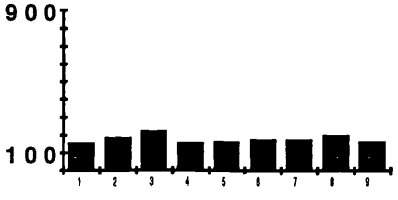
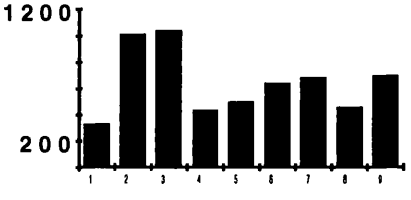
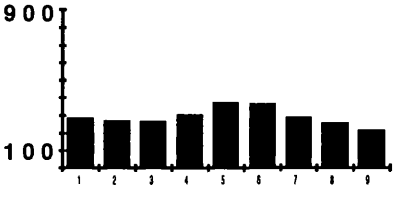


(G) 84890 1



(H) 94538 4



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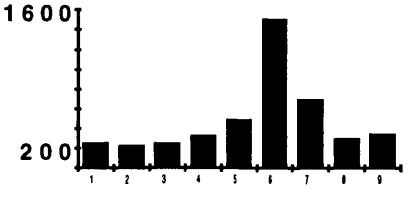

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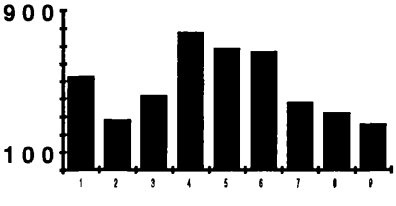
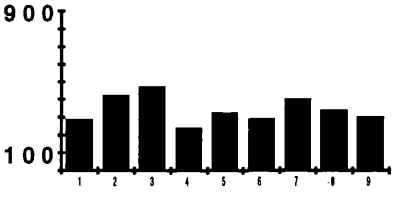
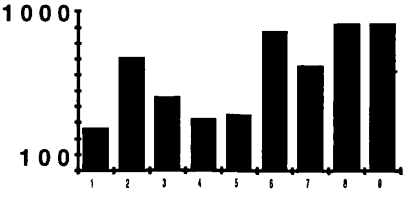


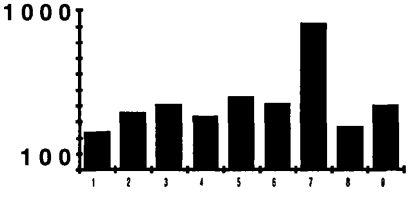
(K) Horse 7 4



(L) 21546 4



(M) SPF F6 1



(N) SPF F12 4

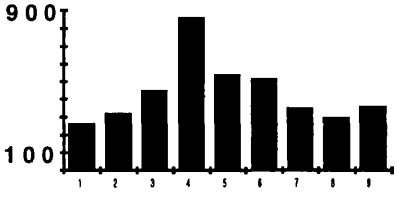


Figure 5.15: Reactivity of sera with miscellaneous gH peptides. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers where applicable. Graphs A and B show the identity of the peptide series on the horizontal axes. The positions of these identified series/peptides are conserved in the other graphs which are not labelled in this manner. Juxtaposed EHV-1 and -4 reactivities are labelled at the top of each bar with a number indicating the virus type. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

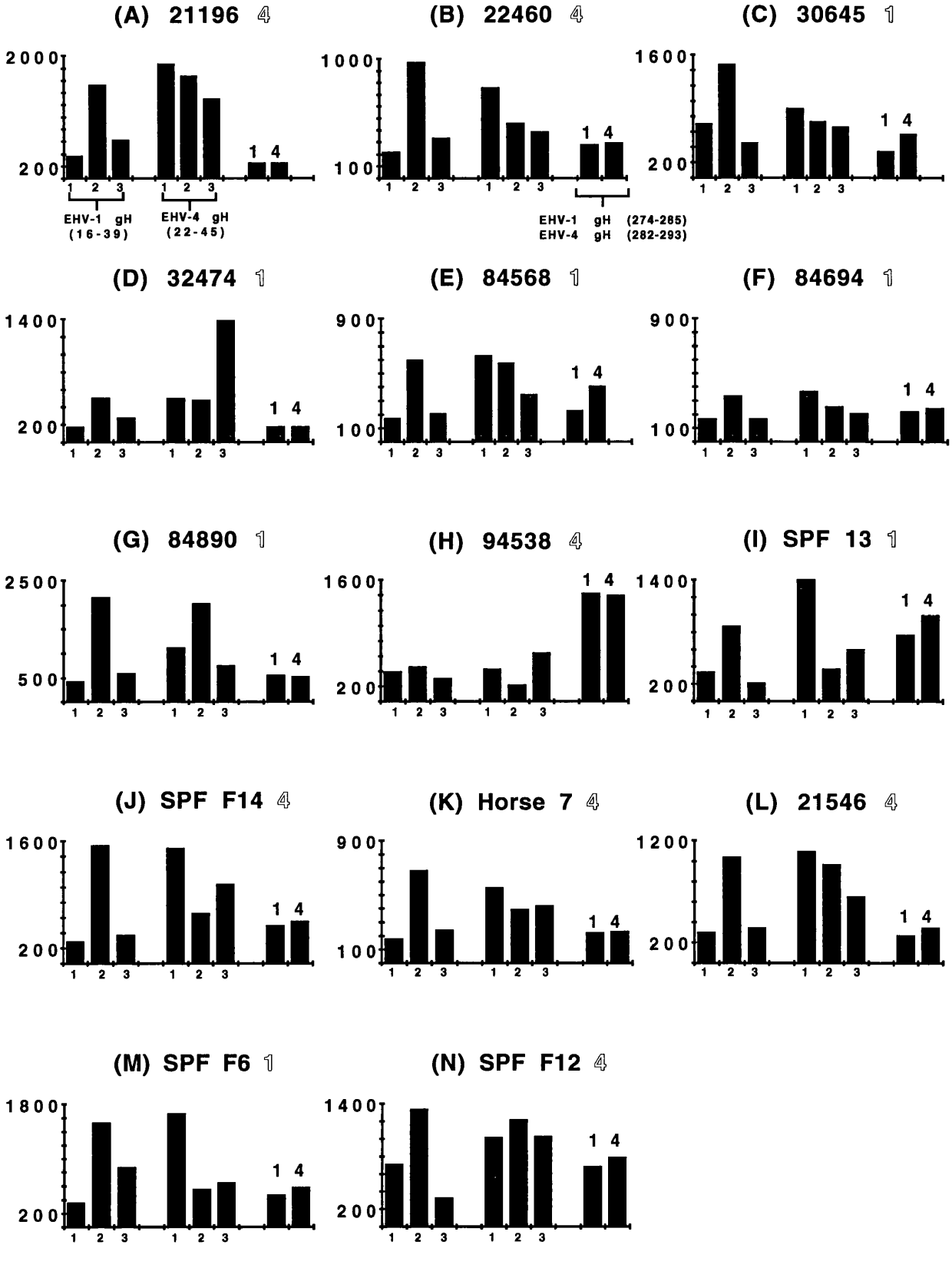

(A) 21196 4
2000
200
1 2 3 1 2 3 1 4
EHV-1 gH (16-39)
EHV-4 gH (22-45)
(B) 22460 4
1000
100
1 2 3 1 2 3 1 4
EHV-1 gH (274-285)
EHV-4 gH (282-293)
(C) 30645 1
1600
200
1 2 3 1 2 3 1 4
(D) 32474 1
1400
200
1 2 3 1 2 3 1 4
(E) 84568 1
900
100
1 2 3 1 2 3 1 4
(F) 84694 1
900
100
1 2 3 1 2 3 1 4
(G) 84890 1
2500
500
1 2 3 1 2 3 1 4
(H) 94538 4
1600
200
1 2 3 1 2 3 1 4
(I) SPF 13 1
1400
200
1 2 3 1 2 3 1 4
(J) SPF F14 4
1600
200
1 2 3 1 2 3 1 4
(K) Horse 7 4
900
100
1 2 3 1 2 3 1 4
(L) 21546 4
1200
200
1 2 3 1 2 3 1 4
(M) SPF F6 1
1800
200
1 2 3 1 2 3 1 4
(N) SPF F12 4
1400
200
1 2 3 1 2 3 1 4

Figure 5.16: Reactivity of sera with miscellaneous gC, gp10 and gB peptides. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled with the pepscan numbers where applicable. Graphs A through to F show the identity of the peptide series. The positions of these identified series/peptides are conserved in the other graphs which are not labelled in this manner. Juxtaposed EHV-1 and -4 reactivities are labelled at the top of each bar with a number indicating the virus type. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

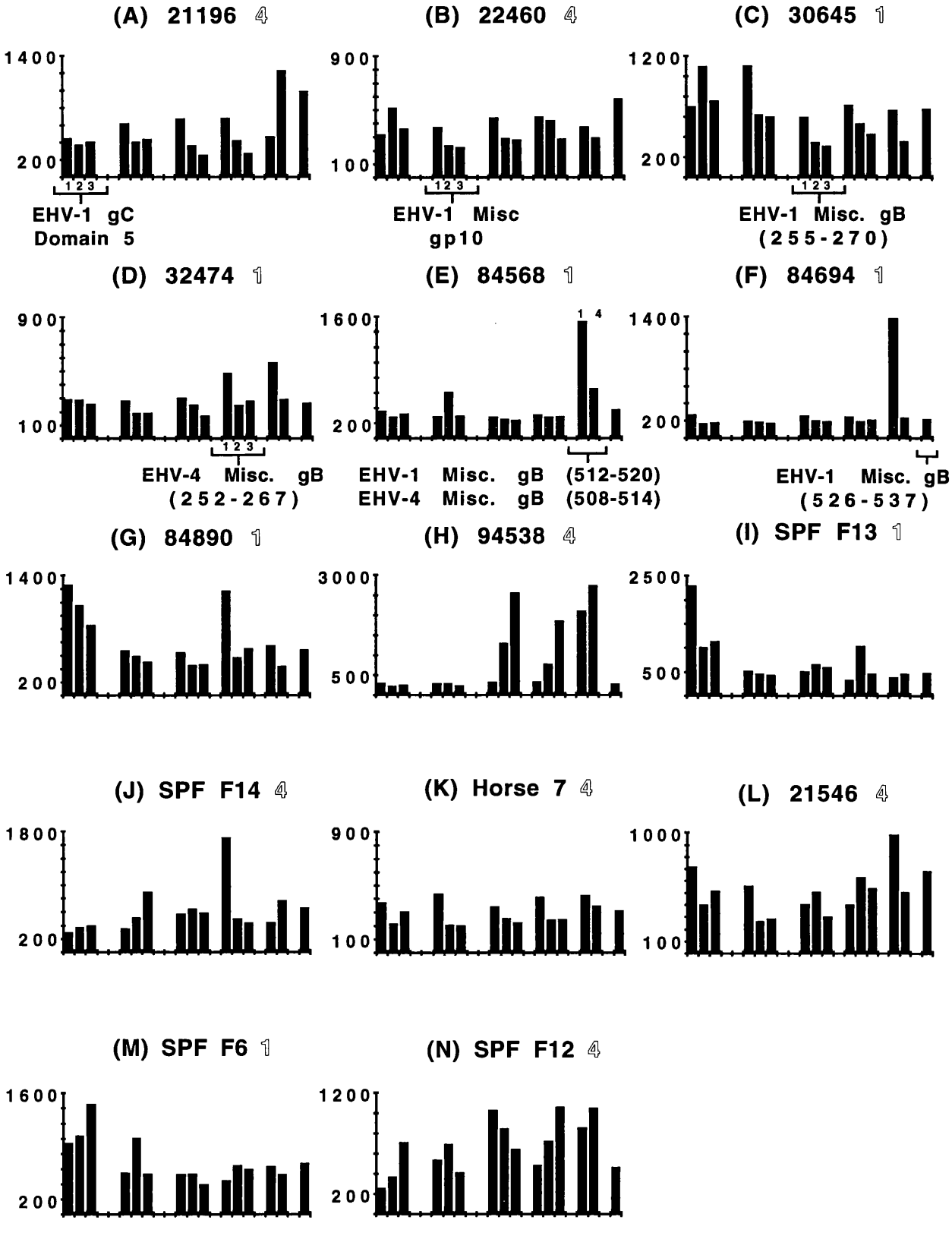

(A) 21196 4
1400
200
1 2 3
EHV-1 gC
Domain 5
(B) 22460 4
900
100
1 2 3
EHV-1 Misc
gp10
(C) 30645 1
1200
200
1 2 3
EHV-1 Misc. gB
(255-270)
(D) 32474 1
900
100
1 2 3
EHV-4 Misc. gB
(252-267)
(E) 84568 1
1600
200
1 4
EHV-1 Misc. gB (512-520)
EHV-4 Misc. gB (508-514)
(F) 84694 1
1400
200
EHV-1 Misc. gB
(526-537)
(G) 84890 1
1400
200
(H) 94538 4
3000
500
(I) SPF F13 1
2500
500
(J) SPF F14 4
1800
200
(K) Horse 7 4
900
100
(L) 21546 4
1000
100
(M) SPF F6 1
1600
200
(N) SPF F12 4
1200
200

Figure 5.17: Reactivity of sera with gD neutralising and non-neutralising domain peptides. Serum numbers are in bold face and the associated virus types in relief face. The horizontal axes are labelled showing the positions of the different peptides and their pepscan numbers. EHV-1 and -4 reactivities are labelled at the top of each bar with a number indicating the virus type. Note the differences in scale of the vertical axis ($O.D_{405nm}$ x1000) for each graph.

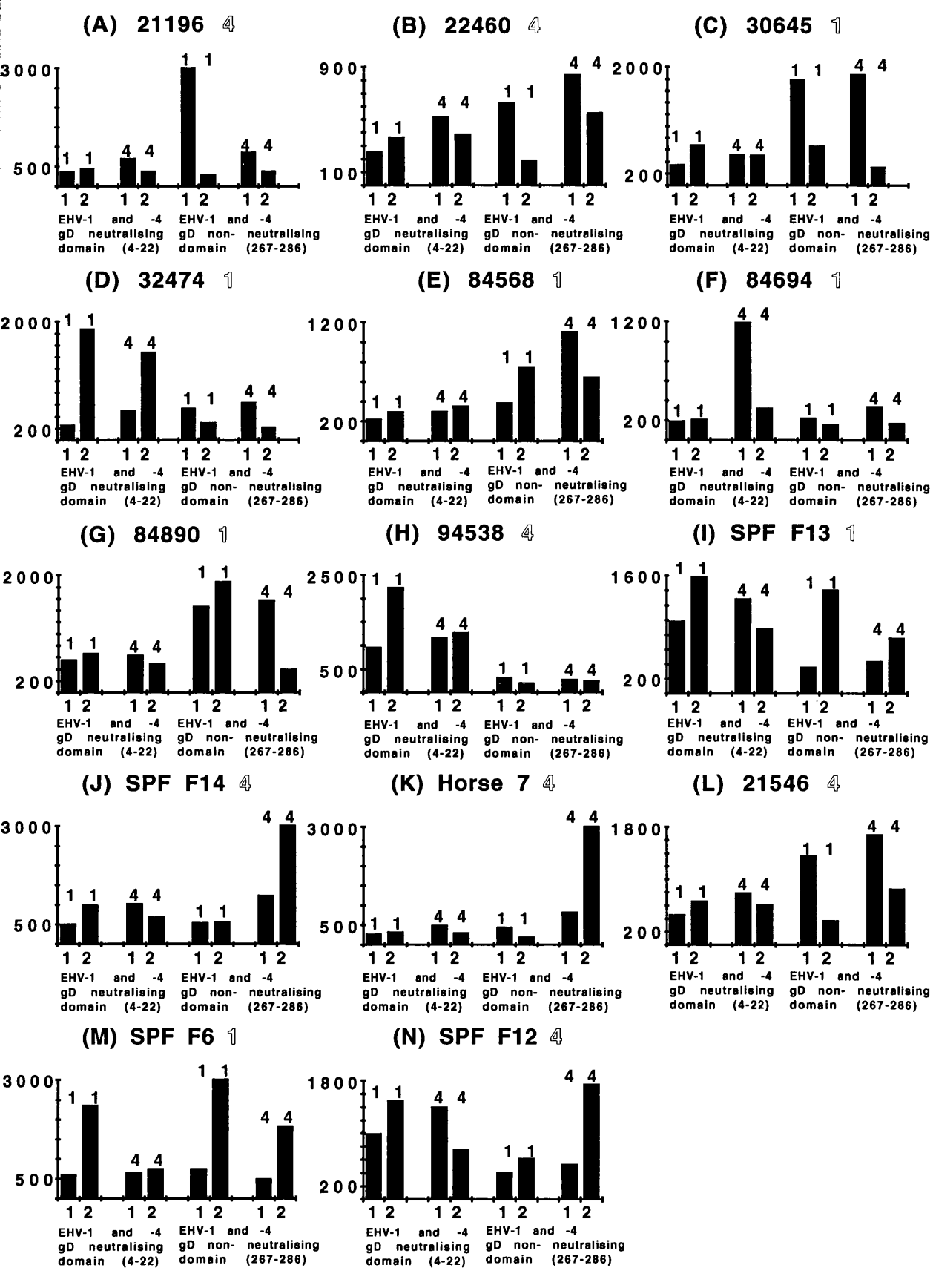

(A) 21196 4
(B) 22460 4
(C) 30645 1
(D) 32474 1
(E) 84568 1
(F) 84694 1
(G) 84890 1
(H) 94538 4
(I) SPF F13 1
(J) SPF F14 4
(K) Horse 7 4
(L) 21546 4
(M) SPF F6 1
(N) SPF F12 4
EHV-1 and -4 gD neutralising domain (4-22)
EHV-1 and -4 gD non- neutralising domain (267-286)

Table 5.1: Details of peptides chosen for pepscan analysis. PSN refers to the pepscan number used to label, where applicable, the horizontal axis of the graphs presented in the results section of this chapter. The position in the native glycoprotein sequence of the first and last amino acid in each peptide series is indicated by a number at the beginning and end of each peptide series. NA indicates that there is only one peptide in this series.

PSN	EHV-1 gC Domain 1	Virus
1	137-TLLLFSTA	
2	LLLFSTAE	
3	LLFSTAER	
4	LFSTAERK	
5	FSTAERKK	
6	STAERKKS	
7	TAERKKSR	
8	AERKKSRR	
9	ERKKSRRG	EHV-1
10	RKKSRRGG	
11	KKSRRGGQ	
12	KSRRGGQL	
13	SRRGGQLG	
14	RRGGQLGV	
15	RGGQLGVI	
16	GGQLGVIP	
17	GQLGVIPD	
18	QLGVIPDR	
19	LGVIPDRL	
20	GVIPDRLP	
21	VIPDRLPK	
22	IPDRLPKR	1/4
23	PDRLPKRQ	
24	DRLPKRQL	
25	RLPKRQLF	
26	LPKRQLFN	
27	PKRQLFNL-170	

PSN	EHV-4 gC Domain 1	Virus
1	154-LLLFSTAH	
2	LLFSTAHS	
3	LFSTAHSS	
4	FSTAHSSA	
5	STAHSSAK	
6	TAHSSAKS	
7	AHSSAKSR	
8	HSSAKSRR	EHV-4
9	SSAKSRRV	
10	SAKSRRVG	
11	AKSRRVGQ	
12	KSRRVGQL	
13	SRRVGQLG	
14	RRVGQLGV	
15	RVGQLGVI	
16	VGQLGVIP-187	

PSN	EHV-4 gB Dom. AHT	Virus
1	93-PSTPASTQ	
2	STPASTQS	
3	TPASTQSA	
4	PASTQSAK	EHV-4
5	ASTQSAKT	
6	STQSAKTV	
7	TQSAKTVD	
8	QSAKTVDQ	
9	SAKTVDQT-108	

PSN	EHV-1 gB Dom. AHT	Virus
1	95-STPTSMST	
2	TPTSMSTH	
3	PTSMSTHS	
4	TSMSTHSH	
5	SMSTHSHG	EHV-1
6	MSTHSHGT	
7	STHSHGTV	
8	THSHGTVD	
9	HSHGTVDP	
10	SHGTVDPT	
11	HGTVDPTL-112	

PSN	EHV-1/4 misc gH	Virus
1	16-VAFAMAVWSTPV	
2	VWSTPVPATPSG	EHV-1
3	PATPSGVGNATW-39	
1	22-VWTTPVSTSPPQ	
2	STSPPQQTKLHY	EHV-4
3	QTKLHYVGNGTW-45	
NA	274-RARIVNRLPPRR-285	EHV-1
NA	282-SARILNRRPSRL-293	EHV-4

PSN	EHV-1 gC Domain 5	Virus
1	33-SQSTPATPTHTT	
2	PTHTTPNLTTAH	EHV-1
3	LTTAHGAGSDNT-58	

PSN	EHV-1 misc gp10	Virus
1	52-TASRDPQEQASN-63	
2	150-NDEEEDPRLGSD-161	EHV-1
3	192-DAEEEEEDDEDD-203	

PSN	EHV-1/4 misc gB	Virus
1	255-ELDLCRPS	
2	CRPSLQLR	EHV-1
3	LQLRGARA-270	
1	252-ELDLVPSK	
2	VPSKFATP	EHV-4
3	FATPGARA-267	

NA	512-NNNNTTRRR-520	EHV-1
NA	508-HNTNRTR-514	EHV-4
NA	526-VPEPQPTQDGVH-537	EHV-1

PSN	EHV-1/4 neut gD	Virus
1	4-CEKAKRAVRGRQ	EHV-1
2	VRGRQDRPKEFP-22	
1	4-CENYRRVVRGNQ	EHV-4
2	VRGNQNQRPEFP-22	

PSN	EHV-1/4 non-neut gD	Virus
1	267-EITQNKTDPKPG	EHV-1
2	PKPGQADPKPNQ-286	
1	267-EITQNKTNPKQE	EHV-4
2	PKQEQASPKPNP-286	

Serum	CF Titre*	Description	Supplier
21196	(1) 5 (4) 10	**EHV-4:** Convalescent serum from a horse in-contact with a mare with EHV-4 respiratory disease	AHT
21546	(1) 40 (4) 160	**EHV-4:** cohort serum of 21196,22460 and 21179	AHT
22460	(1) 640 (4) 640	**EHV-4:** cohort serum of 21196, 21179 and 21546	AHT
94538	No details	**EHV-4:** serum from a horse from which EHV-4 was isolated.	IEC
Horse 7	(1) 5 (4) 40	**EHV-4:** presumed exposure to EHV-4.	IEC
30645	(1) 160 (4) 80	**EHV-1:** convalescent serum from a horse in-contact with a mare which aborted an EHV-1 positive foetus.	AHT
32474	(1) 640 (4) 320	**EHV-1:** cohort serum of 30645.	AHT
84890	High titre No details	**EHV-1:** serum from horse which had been vaccinated after contact with horses with EHV-1 induced paralysis.	IEC
84568	(1) 80: (4) 40	**EHV-1:** No details.	IEC
84694	(1) 80: (4) 20	**EHV-1:** No details.	IEC

Table 5.2: Details of naturally infected animal sera. *In some cases the precise CF titre was not given by the supplier. In the CF titre column the numbers in brackets refer to EHV-1 or -4. AHT= the Animal Health Trust, Newmarket, UK. IEC= the Irish Equine Centre, Kildare, Ireland.

Serum	Titre	Description	Supplier
SPF Foal 6	No details	**EHV-1:** serum taken from animal 102 days after primary experimental infection with EHV-1.	CU
SPF Foal 13	No details	**EHV-1:** serum taken from animal 82 days after primary experimental infection with EHV-1	CU
SPF Foal 12	No details	**EHV-4:** serum taken from animal 82 days after primary experimental infection with EHV-4	CU
SPF Foal 14	No details	**EHV-4:** serum taken from animal 82 days after primary experimental infection with EHV-4	CU
Preimmune sample	No details	**Negative:** serum taken from SPF foal (Identity unknown) 2 days before been experimentally infected for the first time.	CU
SPF foal 12+13	No details	**Negative:** serum derived at Glasgow vet school from plasma taken from animals at Cambridge 2 days prior to their first contact with either EHV-1 or -4.	CU

Table 5.3: Details of sera from experimentally infected SPF foals. CU= Cambridge University veterinary college.

10X PBS

Na_2HPO_4	42.8g
$NaH_2PO_4.2H_2O$	15.6g
NaCl	340g

The salts were dissolved in 4l of hot dH_2O. The solution was allowed to cool to room temperature and the pH adjusted to 7.2 with 50% NaOH.

Conjugate diluent

1X PBS
1% v/v sheep serum
0.1% w/v Casein
0.1% Tween 20

Universal Buffer

1M Citric Acid
1M Phosphoric Acid
1M NaOH
0.1M HCL
57mM Orthoboric Acid

The above is the final concentration of buffer components mixed in water. The pH of the buffer was 2.5.

Precoat buffer

1X PBS
0.1% v/v Tween 20
0.1% w/v Sodium Azide
2% w/v BSA

Wash buffer

1X PBS
0.5% v/v Tween 20

Substrate

One phosphate-citrate buffer tablet (Sigma) was dissolved in 100ml of water yielding a 0.05M phosphate citrate buffer, pH 5.0. One ABTS tablet (Sigma) was dissolved in 100ml of this buffer. 25ul of 30% H_2O_2 was added immediately prior to use.

Ab removal buffer

Na_2HPO_4	1.07kg
$NaH_2PO_4.2H_2O$	390g
SDS	250g

The above components were dissolved in 5l of hot dH_2O. The temperature was allowed to equilibrate to 60°C at which point the pH of the solution was adjusted to 7.2 with 50% NaOH. This solution was brought to a volume of 20l with dH_2O providing sufficient buffer for 5 removal procedures. 5ml of 2-mercaptoethanol was added to 5l of the buffer immediately prior to use.

Table 5.4: Solutions for the Pin system.

Removal Procedure	% reduction in 94538 signal	Post reactivity of control pins
Normal	0%	100%
8M Urea	0%	100%
Universal buffer	0%	100%
Normal + 3hrs.	30%	100%
Normal + 10hrs.	100%	100%

Table 5.5: Antibody removal procedures and their effectiveness.

5.6 DISCUSSION

The Pin system was chosen because it allows a large number of potentially antigenic sites to be characterised in detail with regard to their epitope content without the problems associated with the use of small free peptides. However the system proved to be problematic.

Firstly, a goat anti-equine IgG (γ chain specific) peroxidase conjugate reacted weakly with some of the EHV-4 gB AHT domain peptides. This serum was prepared from pooled immune goat sera. It is possible that some of the animals utilised to prepare the pool could have been infected with EHV since cross-species EHV infection has been documented (Montali *et al.,* 1985; Rebhun *et al.,* 1988). Two other potential alternatives are anti-goat herpesvirus serum cross-reacting with EHV epitopes or a fortuitous reaction of the goat sera with EHV epitopes. However this conjugate was used routinely without problem for ELISA analysis of gG peptides (see chapter 6).

The second major problem to be encountered with this system involved the apparent inability of the recommended serum removal procedure to remove reactive antibodies in serum 94538 from the pin system. The recalcitrance of this serum seems to defy established facts about protein chemistry. The harsh regimes necessary to remove this serum cannot simply be justified by the presence of high affinity antibodies in the serum, although similar problems have been encountered in removing high affinity antibodies from affinity columns. Throughout the initial extremely harsh and extended removal procedures the reactivity pattern of the serum with the different epitopes was maintained i.e. peptides either side of a peak reactivity in an extended series still maintained the same O.D after the various antibody removal procedures initially employed. These peptides only contain part of the core epitope, therefore it is assumed that antibodies within the serum would not have the same affinity for them as for the core epitope. On the basis of conservation of these non-core epitope-antibody interactions after the different removal procedures, one could surmise that irreversible binding was a general property of the serum and not confined to specific high affinity antibody-paratope interactions. This suggests that the inability to remove these antibodies was not due

to their affinity, assuming that non-core epitope peptides were not also reacting with high affinity antibodies that may have been present in the serum in low but detectable amounts. Furthermore serum 94538 was assayed at a dilution of 1/5000 which was recommended for hyperimmune animal sera that may contain high affinity antibodies. An alternative explanation for the irreversible binding of the serum may have been the presence of highly reactive chemical groups that were used in the synthesis of the peptides and which had not been sufficiently blocked by the manufacturer of the pins and by the blocking reagents used herein for ELISA with the Pin system. The high concentration of amine groups in the serum available for covalent reaction with the aforementioned groups on the pins, relative to the low concentration in the highly dilute positive control Mab, may explain why irreversible binding of this serum was detected whereas the Mab seemed to be efficiently removed. Be that as it may it still remains to be understood why the serum survived the harsh denaturing antibody removal conditions. The supplier of this serum did not indicate anything unusual about this serum or the animal it was derived from, indeed other sera obtained from the same supplier were easily removed from the pins.

Given the harsh treatment of the pin-bound peptides it must be considered whether or not the peptides suffered damage which may have affected subsequent analysis of sera. The control peptides were not noticeably damaged by the treatment regimes. Chapter 3 contains the reactivity profile of Mab 26A5 with the EHV-1 gC domain one series. This result, as already stated, compares favourably with previously published data suggesting that the core epitope recognised by this Mab survived the antibody removal regime. Finally duplicate peptides consistently gave similar results. It seems likely that the peptides were not detectably damaged. On the other hand, given the high density of the peptides on each pin it is possible that a percentage of the peptides were damaged and that the degree of damage was not detected by the procedures employed here, but may have ultimately affected the useful life of the pins. Another possibility is that some peptides may be more liable to damage and would remain undetected if they had not previously been identified as being antigenic.

The third problem with the utilisation of the pin system involved the reactivities of preinfection sera derived from plasma at Glasgow that were similar in

strength and specificity to the reactivities of immune sera. Plasma samples were brought from Cambridge but had been labelled on the day of sampling i.e. prior to infection of any animals involved in the SPF foal study and before transport to Glasgow. It is therefore unlikely that these preinfection samples had been confused with post-infection plasma samples. Furthermore these samples were negative for EHV-1 and -4 antibody in a gG ELISA (see chapter 6), however the senstivity of this assay was not determined. These sera did however react marginally with peptide 15 (Stokes *et al.*, 1991) representing the EHV-4 amino acid sequence homologous to the EHV-1 gC domain one epitope (see chapter 6). Preinfection serum supplied directly by Cambridge did not react with any of the Pin system peptides. It was not determined if the Glasgow and Cambridge serum derivation procedures differed significantly. The possibility exists that the plasma was not completely coagulated using the Glasgow procedure since it was assumed that the plasma had already being citriated prior to its arrival in Glasgow. That the plasma had been citriated may not have been the case, therefore plasma proteins may have still been present in the serum prepared at Glasgow in sufficient concentrations to interfere with the pin system ELISA.

The fourth problem concerned the decision of what was to be considered the background for these ELISA assays. If the maximun O.D value obtained with preinfection serum supplied by Cambridge was considered background then many of the immune sera would have been shown to react with most of the peptides. There was a wide variation between different sera in terms of background reactivity with specific peptide series and between different peptide series with a given serum. These differences were probably due to the different anamnestic responses within each serum tested. Since these assays were mainly qualitative in nature an O.D value of $\leq$ 0.5 units was therefore empirically considered to be background reactivity.

Allen and co-workers (1992) characterised a linear epitope in an area they designated EHV-1 gC domain one. This study agrees with theirs in that not all EHV-1 infected horses recognised this epitope. They defined this epitope as being type-specific but this study found that both naturally and experimentally EHV-4 infected horses react with this epitope i.e peptide 12 representing amino acids 148-155 of EHV-1 gC. The reactivity of sera from horses naturally infected with EHV-

4 could be due to a prior EHV-1 infection, however this does not explain the reactivity of EHV-4 infected SPF foal sera. Crabb and Studdert (1995) found that fusion proteins containing this region reacted with both anti-EHV-1 and -4 serum. Likewise, Stokes *et al* (1991) found that anti-peptide antibodies to the homologous EHV-4 region were not type-specific but this may be due to other epitopes which are cross-reactive contained within the peptide used for immunisation in that study. Furthermore, the SPF foals infected with EHV-1 show reactivity with peptide 15 (Stokes *et al.,* 1991), which contains the EHV-4 amino acid sequence homologous to the EHV-1 domain one epitope used in the aforementioned study (see chapter 6). The apparent disparity between this study and that of Allen's can be justified by clarification of the definition of a type-specific epitope e.g the epitope is type-specific with regard to murine Mabs but is not with regard to serum from infected animals. The most obvious explanation for the lack of specificity in the equine sera is the polyclonality of the sera which probably contain antibodies with relaxed specificities which can recognise both homologues of this epitope.

Both the EHV-1 and -4 gC domain one regions contain previously uncharacterised epitopes although they may represent the epitopes recently localised in these regions, as alluded to earlier (Crabb and Studdert. 1995). One enigmatic aspect is that these epitopes were not identified by a previous study (Allen *et al.,* 1992). The entire EHV-1 gC amino acid sequence was represented by a set of overlapping peptides 20 amino acids in length. Each peptide overlapped the next peptide in the series by 10 amino acids. It is possible that this context in which the peptides were presented did not represent all serologically reactive linear epitopes e.g. it is likely that the first peptide in the series represented amino acids 1-20 of the EHV-1 gC sequence; however if Allen and colleagues started the peptide series at any of the amino acids from 2-20 then each series would contain 20mer peptides of different sequence. The context of the peptide series has been shown to be an important consideration when designing pepscan studies. Peptides with the maximun amount of overlap are likely to represent all linear epitopes in a given amino acid sequence (Geysen *et al.,* 1987).

Allen and co-workers (1992) however did suggest that this region may contain multiple overlapping epitopes and certainly the clustered reactivity of some serum samples around groups of peptides suggests that this might be the case.

Pepscan analysis of this region with shorter overlapping peptides with maximum overlap between subsequent peptides i.e each peptide in the series differs from the subsequent peptide by one amino acid, as in this study, should unveil the existence of overlapping epitopes.

Peptides 17-27 in the EHV-1 gC domain one series represent a region, aa153-170, which has an identical counterpart, aa170-187, in the EHV-4 gC amino acid sequence. Peptides from this region are the most frequently recognised by the EHV-1 and -4 associated sera tested. It would be interesting to assess the neutralising ability of antibodies directed against this region in order to determine what role, if any, these epitopes play in the relationship of both viruses with their host.

Peptides 2, 3 and 4 from the EHV-4 gC domain one sequence (aa155-162) could be reactive with potentially EHV-4 type-specific antibodies since these peptides are not recognised by EHV-1 associated SPF foal sera. Peptide 2 differs at only 2 amino acids when compared to the homologous EHV-1 peptide. Dolter *et al* (1992) demonstrated by site-directed mutagenesis of HSV-1 or -2 gC homologues that single amino acid changes result in recognition of the virus by type-specific Mabs against the other virus type. Therefore a peptide which differs at just two amino acids between the virus types might be recognised by type-specific antibodies However the potential diagnostic usefulness of these peptides is questionable given that some EHV-1 associated sera react with it, possibly because of prior contact with EHV-4 and more importantly, not all EHV-4 associated sera react with peptide 2.

The results obtained with both EHV gB series of peptides are extremely difficult to interpret. Since gB was demonstrated to be the major target of the equine humoral immune response (Allen *et al.,* 1992), it is possible that this region is saturated with linear epitopes which are recognised with varying specificities by different sera that could cause difficulties in defining peak reactivities in reactive sera.

The peptides included under the umbrella title of miscellaneous gH peptides were included in this analysis because they were potentially antigenic on the basis of computer predictions. The peptides corresponding to regions 16-39 and 22-45 of

EHV-1 and -4 gH homologues respectively are adjacent to the signal sequence cleavage site. These peptides contain previously unidentified linear gH epitopes and therefore merit further investigation. Peptides which are most homologous between the virus types are the most frequently recognised — however there is one notable exception i.e 32474 which, although associated with an EHV-1 infection, reacts with an EHV-4 peptide which contains very little intertypic homology. This may be due to a recent exposure to EHV-4. Why the EHV-1 series seems to be more readily recognised than its EHV-4 counterpart could be due to many reasons, the most likely being that the EHV-4 epitope was fragmented or misrepresented by the chosen context of the peptide series or that it was less immunogenic than the EHV-1 epitope.

The other two peptides in this group corresponding to amino acids 274-285 and 282-293 in EHV-1 and -4 gH homologues respectively have previously been shown to induce anti-EHV-1 and -4 antibodies when injected into hamsters (Onions *et al.,* 1992). In this study only one serum from a naturally infected animal, 94538, reacted significantly with these peptides. SPF foals 12 and 13 also showed appreciable reactivity. As with the hamster study, where anti-EHV-4 peptide antibodies reacted with both virus types, the horse serum recognised both virus peptide homologues which, when given the level of homology, is not suprising.

Reactivity with the EHV-1 gC domain five peptides is confined mainly to sera derived from horses associated with EHV-1 outbreaks, although some associated with EHV-4 show weak reactivity. Mabs reacting with this epitope have been shown to be type-specific (Allen *et al.,* 1992). Some sera show reactivity with the variety of peptides chosen from EHV-1 gp10 and from EHV-1 and -4 gB. The EHV-4 gB peptide (aa508-514) does not react with EHV-1 associated sera, however not all EHV-4 sera recognise this peptide. These potentially represent previously unidentified linear epitopes.

The EHV-1 neutralising and non-neutralising peptides were previously shown to be antigenic in mice by Flowers and O'Callaghan (1992). Here these regions and their EHV-4 homologues are also shown to be immunogenic in some horses. Unfortunately, it was not possible to correlate the presence of neutralising antibodies with clinical symptoms or time of sampling. Notably, there is a striking consistency with which homologous peptides are recognised and this might explain

why some SPF foals e.g. 12 and 13, recognise epitopes from a virus that has not been encountered before. However it must be said that some sera e.g. F6, that recognise one peptide do not recognise the homologous peptide from the other virus. EHV-1 infected SPF foals do not contain EHV-4 neutralising antibodies yet F13 recognises an EHV-4 peptide homologous to a peptide from EHV-1 gD that induces EHV-1 neutralising antibodies in mice. It is possible that the antibodies reacting with the EHV-4 peptides are not neutralising i.e. aa4-22 of EHV-4 gD do not represent an EHV-4 neutralising domain. There is only 50% homology between the EHV-1 and -4 in this region.

Peptide 2 of the EHV-1 gD non-neutralising domain reacts with the EHV-1 SPF sera but has only marginal reactivity with EHV-4 sera. This peptide represents amino acids 275-286 of EHV-1 gD and has 75% homology with the peptide representing EHV-4 gD amino acids 275-286. It is possible that this epitope binds to EHV-1 type-specific antibodies in equine sera. Its diagnostic potential may be limited since EHV-4 field sera from the same outbreak recognise a partially overlapping peptide and not all EHV-1 associated sera react with peptide 2 of the EHV-1 gD non-neutralising domain.

Overall, it remains to be seen if the analysis of the pin peptides with sera from naturally and experimentally infected animals identifed any peptide or peptide combination which could be useful for distinguishing between EHV-1 and -4 infected horses. Certainly the peptides from the EHV-4 gC domain one series, the EHV-4 gB peptide (aa508-514) and the EHV-1 gD non-neutralising domain peptide series merit further investigation. The use of slightly larger peptides containing the EHV-4 gC domain one peptides and a more thorough pepscan analysis of the EHV-1 gD non-neutralising domain may result in the identification of peptides in each case that may be recognised by more sera and that still retain their type-specificity in relation to SPF foal sera.

That more potentially useful peptides were not identified may be due mainly to the fact that no consistent relationship could be found between infection with a given virus and reactivity with a given peptide. A number of factors could have contributed to this, namely the pre-EHV infection history of the naturally infected animals, the strain of the pre- and present infecting viruses, and qualitatively or quantitatively different immunological responses of different animals. The

involvement of some of these factors in the unresponsiveness of the host to some epitopes has been suggested (Sinclair *et al.,* 1993b).

Differences in the epitope content of different strains and also strain variation in the ability to augment the immune response may have been contributory factors. The extent of strain variation in the EHV virus population has been the subject of discussion elsewhere in this thesis — suffice to say that it has not been unequivocally proven that sufficient variation exists in the virus population to elicit differences between the immune responses of animals infected with different strains of the same virus. Consistent with this view in this study is the differences seen between SPF foals infected with the same virus and between animals from the same natural outbreak, although the latter may be affected by pre-infection history and by the fact that more than one strain may be present during an outbreak.

Insufficient data exists to comment on the effects of prior EHV infection on the initial aim of this study. Such data if it did exist would probably be extremely difficult to interpret in relation to reactivity with type-specific epitopes given the relative frequency of infection of horses, especially with regard to EHV-4.

It has been demonstrated that different horses respond differently to the same antigen but these studies were examining all or nothing responses or responses to a given epitope (Ostlund *et al.,* 1992; Bodo *et al.,* 1994). In this study responses to peptides overlapping epitopes were examined, and differences between animals infected with the same virus varied from differences with regard to epitope fine specificity to recognition or lack of recognition of an epitope. The latter could have been due to the insufficient overlap between peptides such that the epitope recognised by an animal's serum was not adequately represented. This is especially true for gH, gB, gp10 and gD peptides. An alternative explanation may reside in studies with the pepscan system which have shown that closely related strains of mice and rabbits can differ with regard to the gross and fine specificity of epitopes that they recognise. Such differences are determined to some extent by genetic factors (Schwab *et al.,* 1993; Su and Caldwell. 1993; Cason *et al.,* 1993; Qu *et al.,* 1994). A similar situation might therefore extend to the thorough-bred horse population. However, Ostlund *et al* (1992) have suggested that variation between horses in response to a given epitope of EHV-1 is not due to similar factors e.g genetic, implicated in the aforementioned strain differences, but rather is an inherent

property of the immune response of the equine population in general to EHV-1 infection.

Although this study did not yield a desirable conclusion with respect to identification of type-specific linear epitopes, it did reveal valuable information with regard to previously identified epitopes and points to the existence of epitopes previously unidentified.

Chapter Six

Evaluation of peptides representing EHV-1 and -4 gG epitopes as type-specific diagnostic reagents

6.1 INTRODUCTION

Of the 12 known herpes simplex virus glycoproteins only one, the HSV-2 gG homologue, is known to be secreted. Likewise the PRV homologue gX is also secreted. Furthermore of all the HSV glycoproteins the intertypic differences in electrophoretic mobility and amino acid homology are greatest for the gG homologues.

A glycoprotein of 63K was initially identified in the cell culture supernatant of EHV-4 infected cells. This glycoprotein was detected by monospecific anti-EHV-4 sera from SPF foals but not by monospecific sera from EHV-1 infected SPF foals. This glycoprotein was assumed to be the EHV-4 gG homologue since the only α herpesvirus glycoprotein homologues known to be secreted are HSV-2 gG and the PRV homologue gX. The gG gene was located in the Us region of the EHV-4 genome, the DNA sequence determined and shown to have limited homology with the PRV gX gene. The entire gene was expressed as a prokaryotic fusion protein that, like the secreted glycoprotein, reacted with EHV-4 but not EHV-1 monospecific antisera (Crabb *et al.,* 1992).

Comparison of the HSV-1 and -2 gG homologues revealed that the amino acid identity between the glycoproteins is 45%. HSV-2 gG contains a unique region of 526 amino acids whereas HSV-1 has 42 unique amino acids in the same region (McGeoch *et al.,* 1987). These glycoproteins have formed the basis for the development of a number of different serological assays to distinguish HSV-1 and -2 infections in humans (reviewed by Bergström and Trybala, 1996).

Likewise Crabb and Studdert (1993) compared translations of the EHV-4 and -1 (Telford *et al.,* 1992) gG genes and found an overall identity of 56%. However comparison of a divergent region in the C-terminal region of the EHV-1 and -4 gG homologues revealed a level of identity of 21% (see figure 6.1). These regions representing amino acids 288-350 and 287-382 in EHV-1 and -4 gG repectively were expressed as prokaryotic fusion proteins and shown to react monospecifically with SPF foal sera. The power of these fusion proteins as antibody capture reagents for the serological analysis of EHV-1 and -4 epidemiology was

demonstrated by a number of studies performed by Studdert's group. Firstly, the presence of EHV-1 in the Australian equine population 10 years prior to the first confirmed case of EHV-1 associated abortion was inferred from the demonstration of EHV-1 type-specific antibodies in a few horses at that time by a single-dilution type-specific ELISA with the aforementioned antibody capture reagents (Crabb and Studdert, 1993). This ELISA consistently detected the presence of EHV-1 antibodies after natural and experimental infections with very few exceptions. The long-term persistence of equine anti-EHV-4 gG antibodies relative to EHV-1 antibodies was thought to reflect the relative rates of reinfection/reactivation of each virus in the equine population (Crabb *et al.,* 1995). Finally, a singular study demonstrated the clinical usefulness of the gG assay in that it was used to separate EHV-1 antibody positive from antibody negative mares at the onset of an abortion storm which resulted in a significant reduction in the number of abortions in the ensuing storm (Drummer *et al.,* 1995).

The initial observations of Crabb and co-workers (1992) may not have been possible but for the availability of monospecific antisera. The sources of such sera are maternal colostrum deprived specific pathogen free (SPF) foals. EHV-1 free ponies were initially developed by Fitzpatrick and Studdert (1984). These animals offer the potential to study EHV-1 and -4 infections without the complications or doubt associated with the interpretation of data from non-SPF equids in which the effects of preinfection history are difficult to define. Studies performed with SPF foals and the reagents thus derived/developed continue to clarify and extend data concerning the immunology, latency/reactivation, diagnosis, pathogenicity and treatment of EHV-1 and to a lesser extent EHV-4.

SPF foals are an invaluable source of unquestionably monospecific antisera, the usefulness of which has already been discussed for the identification of type-specific epitopes.

The humoral immune response to EHV-1 in SPF foals has received much attention.

Two independent studies have demonstrated that antibodies of the IgM class are the first detected after EHV-1 infection in SPF foals and reach a peak 10-18 days post infection (dpi) after which time IgM levels steadily decrease (Chong and

Duffus, 1992; Gibson *et al.,* 1992a). IgG levels peak later than IgM levels i.e. after 26d.p.i and remain at very high levels. Secondary EHV-1 infection of EHV-1 infected animals 61d.p.i did not result in changes in the levels of IgG or IgM detected pre-secondary infection. Virus neutralising (VN) and complement fixing (CF) antibodies rise in parallel with IgG levels (Gibson *et al.,* 1992a). However levels of CF antibodies will begin to fall 60d.p.i (Tewari *et al.,* 1993) unless restimulated by secondary infection (Gibson *et al.,* 1992a; Tewari *et al.,* 1993). Generally all foals in these studies had very little cross reactive anti-EHV-4 antibodies, as determined by VN, CF or western blotting.

A singular study of a primary EHV-4 infection of one SPF foal demonstrated both weak CF and VN responses. However when this animal was infected with EHV-1 84 days later there was a concomitant rise in CF and VN titres to both virus types. Glycoproteins were only weakly labelled in western blots with sera obtained 15d.p.i with either EHV-1 or -4. Positive western blot staining was detected 82d.p.i and there was significant levels of cross reactive antibodies in the EHV-4 sera relative to the EHV-1 sera, as determined by western blotting (Tewari *et al.,* 1993).

The SPF foal system has been central to the demonstration of changes in aspects of the cellular immune response to EHV-1 infection. Deficits in neutrophil function (Chong and Duffus, 1992) and levels (Gibson *et al.,* 1992a) have been observed. Lunn and colleagues (1991) have demonstrated a drop in the equine lymphocyte population that contains the cytotoxic and suppressor T lymphocytes upon EHV-1 infection of SPF foals. These factors may partly contribute to the non-specific immunosuppression seen following EHV-1 infection.

SPF foals exhibit most of the clinical signs indicative of a recent EHV-1 infection. EHV-1 associated abortigenic disease has not been demonstrated for obvious reasons. Furthermore, the paralysis associated with EHV-1 infection has not been demonstrated in SPF foals, although pathological changes in the retina which may be related to the neurological manifestation of EHV-1 disease were detected in some animals but only after secondary infection caused by virus reactivation (Gibson *et al.,* 1992a).

Although the SPF foal system will continue to be of value a number of questions concerning the real value of this system remain as yet unanswered. Firstly it may be an oversimplification of the natural situation in that most naturally infected animals have the added complication of previous EHV infections. Indeed controversy has arisen, as referred to in chapter one, with regard to the study of latency in SPF foals vs. animals that may have been or may be infected with a number of EHVs. Secondly the system suffers from the not insurmountable problems of being unable to reproduce the two most important aspects of EHV-1 induced disease, namely abortion and paralysis.

6.2 EXPERIMENTAL APPROACH

This chapter describes experiments designed to identify peptides which represent and further delineate the EHV-1 and -4 gG epitopes localised by Crabb and Studdert (1993). The identification of such peptides would preclude the need for synthesis of fusion proteins. These peptides would allow the assessment of the EHV-1 and -4 gG epitopes with sera from naturally infected horses from the U.K. and Rep. of Ireland, countries that significantly differ from Australia in terms of the epidemiology of EHV-1 and to a lesser extent EHV-4.

Two sets of overlapping peptides of 20 amino acids in length spanning the EHV-1 and -4 gG type-specific domains identified by Crabb and Studdert (1993) were chosen. The overlap between subsequent peptides in each series was 10 amino acids.

Two peptides from EHV-4 gC were also included in this study. Hamsters immunized with these peptides were shown to contain antibodies to both EHV-1 and -4 (Stokes *et al.*, 1991). One of these peptides, peptide 15, was recognised by sera from naturally and experimentally infected horses whereas the other peptide, peptide 14, was not reactive with the limited number of sera tested (Unpublished observations). Peptides 15 and 14 were therefore included in this study as it was thought that these peptides might represent positive and negative peptide controls, respectively.

All peptides were probed with SPF foal and naturally infected animal sera. SPF foals were subjected to a primary and secondary EHV-1 infection or a primary EHV-4 followed by a secondary EHV-1 infection. Serum samples were from defined days post primary or secondary infection. These samples allowed the EHV-1 and -4 gG peptides to be assessed under conditions which had not been clearly addressed by the studies performed by Studdert's group.

6.3 MATERIALS

6.3.1 SERA AND PLASMA

The sera and antibodies listed in tables 6.1 and 6.2 were obtained from the same sources as listed in Chapter 5 section 5.3.

6.3.2 PEPTIDES

The peptides illustrated in figure 6.2 were synthesised by Alta Biosciences, University of Birmingham as free acids. Peptides were supplied lyophilised.

6.3.3 REAGENTS AND PLASTICWARE

See chapter 4 for recipes for coupling/coating buffer and TBS.

Superblock in TBS was obtained from Pierce Chemical Co., UK.

ELISA wash buffer was TBS containing 0.5% Tween 20.

The ELISA substrate was stabilised TMB from Sigma.

Immulon 1 and 2 microtitre dishes were obtained from Dynatech LTD., U.K.

6.4 METHODS

6.4.1 SOLUBILISATION OF FREE PEPTIDES

Free peptides were supplied in solid form by the manufacturer. 500ul of sterile distilled water was added to each peptide. Solutions containing insoluble peptides were incubated at 55°C for 30 minutes and agitated from time to time. Those peptides which were water insoluble were subjected to the solubilisation procedure outlined in the applied biosystem's flow chart for peptide solubilisation (Figure 6.3)

All peptides were brought to a final volume of 1.0ml with water.

6.4.2 ESTIMATION OF PEPTIDE CONCENTRATION

Peptide concentration was estimated by two methods. Peptide gC 15 had been produced in milligram quantities so it was possible to prepare from it a standard solution containing a precise amount of peptide.

All peptides were diluted 1/100 in 500ul of water and the $O.D_{280nm}$ measured with a UV spectrophotometer which had been calibrated with water.

The Pierce BCA protein assay kit was also used to assess peptide solubility. The standard protocol provided by the manufacturer was followed.

Peptide gC15 was used as the standard and serial dilutions ranging in concentration from 100ug-0.1ug/ml were made. Neat and 1/500 dilutions of all other peptides were assayed. Reactions were carried out in a 96 well microtitre plate. 200ul of freshly prepared working reagent was added to 10ul of each peptide and the dilutions thereof. A blank consisting of water was also included. The plate was then incubated at 37°C for 30 minutes with gentle agitation. At the end of the incubation period the plate was equilibrated to room temperature and the $O.D_{562nm}$ of each well measured.

6.4.3 PEPTIDE ELISA

0.5ul of each peptide ≥500ng in 100ul of coating buffer was added to each well of immulon 2 microtitre plates. This was performed in duplicate for each peptide. Plates were incubated overnight at 4°C.

After washing the plates three times with wash buffer using an automatic plate washer, blocking was performed with Superblock in TBS according to the manufacturer's instructions. The plates were washed once as before.

Equine serum was diluted 1/100 in Superblock containing 0.5% tween 20 and 100ul was added to each well. Plates were then incubated at room temperature for 1 hour.

After washing the plates 6 times 100ul of goat anti-equine IgG (γ chain specific) peroxidase conjugate diluted 1/5000 in Superblock containing 0.5% tween 20 was added to each well and the plates incubated for 1 hour at room temperature.

The plates were washed 6 times. 100ul of TMB substrate was then added to each well and the substrate reaction was allowed to proceed for 15-45 minutes at room temperature with gentle agitation. The $O.D_{650nm}$ of each well was monitored over a 45 minute period with an automatic plate reader. Each serum was analysed twice in this manner. The results were processed and graphs drawn with the aid of a Microsoft Excel 2.0 package.

6.5 RESULTS

6.5.1 SOLUBILISATION AND ESTIMATION OF CONCENTRATION OF FREE gG PEPTIDES

Peptides showed a varying degree of solubility as determined visually. In some cases an insoluble residue remained even after taking all possible steps to effect solubilisation. All peptides gave an absorbancy reading at U.V. 280nm, equal to or better than the standard. A similar result was obtained with the BCA protein assay kit although there was not a direct correlation between the two tests. On this basis it was assumed that the concentration of each peptide was ≥ 1ug/ul (see table 6.3).

6.5.2 REACTIVITY OF SPF FOAL SERA WITH gG PEPTIDES

Although there was excellent agreement between duplicate values of each peptides reactivity with a given serum and between ELISA analysis of each sera performed on different days, there was a wide variation between different sera in terms of background reactivity. As with the pepscan analysis described in Chapter 5, preimmune SPF foal sera could not be used as a background standard as this would imply that most immune sera analysed were reactive with all peptides. Secondly, although gC peptide 14 was considered to be a negative peptide control one serum actually reacted with this peptide. These differences were probably due to the different anamnestic responses within each serum tested and in this respect some preimmune SPF foal sera showed appreciable reactivity with gC peptide 15. The majority of data interpretation is thus qualitative and quantitative comparisons of different sera are tentative.

F11 and F6 (see figure 6.4): On day 0 i.e. 2 days prior to primary infection, foal 11 did not react with any of the gG peptide series, although it did give an above background reactivity with the gC peptide 15 control. On day 11, post EHV-1 infection, it is obvious that the animal reacted with gC peptide 15 but it is very difficult to discern any specific reaction with the gG peptide series

despite an increase in background reactivity. During the course of these animal experiments F11 was replaced by F6. 102 days after primary infection i.e. 2 days before secondary infection, F6 exhibited a strong reaction with the EHV-1 peptide series only. 26 days after secondary infection with EHV-1 F6 still reacted strongly with the EHV-1 series. However there was also an increase in the reactivity of this serum with some of the peptides from the EHV-4 gG series.

F13 (see figure 6.5). This foal had a day 0 reaction similar to that of F11. 79 days after primary infection with EHV-1 i.e 2 days before secondary infection, F13 reacted specifically with peptides of the EHV-1 series. 2 days after secondary infection with the same strain of EHV-1 there seemed to be a 30 to 50% drop in the reactivity with the reactive EHV-1 gG peptides and with gC peptide 15. 26 days after secondary infection reactivity increased although, as with foal 6, there was also an increase in the non-specific background reactivity with the EHV-4 series.

F12 (see figure 6.6): This foal had a typical day 0 reaction. 79 days after primary EHV-4 infection or 2 days before secondary infection with EHV-1 it reacted with the EHV-4 peptide series only. 4 days after the 79th day post primary infection and 2 days after an EHV-1 challenge there was a significant drop in reactivity with these peptides. 10 days later the reactivity with these peptides had increased but there was no reactivity with the EHV-1 series, indicative of a recent EHV-1 infection. 26 days after secondary infection there was again a slight decrease in the reactivity with the EHV-4 peptide series and again no reactivity with the EHV-1 gG series.

F14 (see figure 6.7): This foal had a day 0 reaction almost identical to that of F11, F13 and F12. 79 days after primary EHV-4 infection and 2 days before secondary infection with EHV-1, F14 reacted with peptides from the EHV-4 series but also reacted with one peptide from the EHV-1 series. 12 days after secondary infection with EHV-1 this foal still showed reactivity with the EHV-4 series which seemed stronger than the reactivity of the serum sampled 14 days earlier. A similar increase in reactivity with the EHV-1 peptide 2 was also seen. This foal did not demonstrate any reactivity similar to that of other animals experimentally infected with EHV-1 in this study.

6.5.3 REACTIVITY OF FIELD SERA WITH gG PEPTIDES

22460 (see figure 6.8): This serum showed a reactivity typical of an animal which had a prior EHV-4 infection. The serum was derived from an animal in-contact with a mare suffering from respiratory symptoms indicative of an EHV-4 infection.

21196 (see figure 6.8): This horse was a cohort of 22460. Its serum reacted similarly to that derived from 22460. Interestingly, this serum also reacted with gC peptide 14 which was considered to be a negative control.

21179 (see figure 6.8): This serum was from an animal in-contact with the same mare as 21196 and 22460. It reacted with the EHV-4 gG peptide series but also with EHV-1 gG peptide 7, an interesting result considering its C.F. titre is greater for EHV-1 than -4.

21546 (see figure 6.8): This serum does not react with any of the gG peptides but does react with gC peptide 15. It is a serum from an animal in-contact with EHV-4 respiratory diease.

94538 (see figure 6.8): This serum was derived from a horse from which EHV-4 had been isolated. It shows a reactivity profile indicative of contact with EHV-4.

30645 (see figure 6.9): This serum was obtained from a horse in-contact with a mare which had aborted, the likely cause being EHV-1. The serum reacted strongly with peptide 6 indicative of contact with EHV-1. However, unlike the SPF foals, it did not react with peptide 3 in the EHV-1 gG peptide series. It also demonstrated a strong anti-EHV 4-like reactivity.

32474 (see figure 6.9): This horse was in-contact with the same mare as 30645. It had a higher C.F titre than 30645. It seemed to have a slightly different EHV-1 peptide specificity than 30645 and like 30645 reacted with the EHV-4 peptide series.

84568 and 84890 (see figure 6.9): These horses were in-contact with horses suffering from the neurological form of EHV-1 disease. After confirmation of EHV-1 infection all horses were vaccinated with Pneumabort. Although both

horses show evidence of contact with EHV-4 it is very difficult to discern if the sera reacted with the EHV-1 gG peptide series. 84568 also does not react strongly , if at all, with gC peptide 15 which is in agreement with the EHV-4 gC pepscan results for this serum (see chapter 5).

84694 (see figure 6.10): This animal was associated with EHV-1 but, like 84588 and 84890, it is difficult to decide if its serum reacted with EHV-1 peptides although it reacted strongly with the EHV-4 gG peptide series. This sera reacts with gC peptide 15 in contrast to the pepscan results for this serum. However, a lower dilution (1/100) of this serum was used for assaying gC peptide 15 than was used for pepscan (1/2500).

83451A and 83643C (see figure 6.10): These are paired acute and convalescent serum samples from a horse in-contact with animals suffering from EHV-1 induced neurological symptoms. The acute sample had a low C.F titre whereas the convalescent sample had a high titre. Both samples gave a similar reactivity pattern which was comparitively weak. There was no discernible pattern indicative of EHV-1 infection although both samples were weakly reactive with peptide 2, not unlike SPF foal 14.

Chapter Six

Figures and Tables

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EHV-4 gG        MLAVGATLCL LSFLTGATGR LAPDDLCYAE PRKTGPMPRS KPKHQPLLFE     50
EHV-1 gG        MLTVLAALSL LSLLTSATGR LAPDELCYAE PRRTGSPPNT QPERPPVIFE     50
Consensus       ML.V.A.L.L LS.LT.ATGR LAPD.LCYAE PR.TG..P.. .P...P..FE     50

EHV-4 gG        APKVALTAES KGCQLILLDP PIDMGYRLED KINASIAWFF DFGNCRMPIA    100
EHV-1 gG        PPTIAIKAES KGCELILLDP PIDVSYRRED KVNASIAWFF DFGACRMPIA    100
Consensus       .P..A..AES KGC.LILLDP PID..YR.ED K.NASIAWFF DFG.CRMPIA    100

EHV-4 gG        YREYYDCVGN AIPSPETCDG YSFTLVKTEG VVEFTIVNMS LLLQPGIYDS    150
EHV-1 gG        YREYYGCIGN AVPSPETCDA YSFTLIRTEG IVEFTIVNMS LLFQPGIYDS    150
Consensus       YREYY.C.GN A.PSPETCD. YSFTL..TEG .VEFTIVNMS LL.QPGIYDS    150

EHV-4 gG        GSFIYSALLD MDVLTGRVIL NVENDTNYPC GMTHGLTADG NINVDET-TH    199
EHV-1 gG        GNFIYSVLLD YHIFTGRVTL EVEKDTNYPC GMIHGLTAYG NINVDETMDN    200
Consensus       G.FIYS.LLD ....TGRV.L .VE.DTNYPC GM.HGLTA.G NINVDET...    200

EHV-4 gG        TTPHPRAVGC FPELINFDAW ENVTFEEMGI PDPNSFLDDE SDYPNTMDCY    249
EHV-1 gG        ASPHPRAVGC FPEPIDNEAW ANVTFTELGI PDPNSFLDDE GDYPNISDCH    250
Consensus       ..PHPRAVGC FPE.I...AW .NVTF.E.GI PDPNSFLDDE .DYPN..DC.    250

EHV-4 gG        SWDLYTYPKS LKQAEGPQTL LIGAVGLRIL AQAWKFVENE TYSQ-HTRTY    298
EHV-1 gG        SWESYTYPNT LRQATGPQTL LVGAVGLRIL AQAWKFVGDE TYDTIRAEAK    300
Consensus       SW..YTYP.. L.QA.GPQTL L.GAVGLRIL AQAWKFV..E TY........    300

EHV-4 gG        TRDAKEVDVT QPSPVQADSV LAKKRTSMKN NPIYSEGKPH AKPFSTIDSI    348
EHV-1 gG        NLETHVPSSA AESSLENQST QEESNSPEVA HLRSVNSDDS THTGGASNGI    350
Consensus       .......... ..S.....S. .......... .......... .........I    350

EHV-4 gG        HTEGMKNNPV YSESLMLNVQ HSDSITTGGV LHGLQD--CD NQL-KTVYI-    394
EHV-1 gG        QDCDSQLKTV YACLALIGLG TCAMIGLIVY ICVLRSKLSS RNFSRAQNVK    400
Consensus       .........V Y......... ....I..... ...L...... ..........    400

EHV-4 gG        CLALIGLAHV P                                              405
EHV-1 gG        HRNYQRLEYV A                                              411
Consensus       ......L..V .                                              411
```

Figure 6.1: Alignment of EHV-1 and -4 gG homologues.
Boxed regions were chosen for pepscan

(A) EHV-1 gG PEPTIDE SERIES

1 278-RILAQAWKFVGDETYDTIRA **0**
2 GDETYDTIRAEAKNLETHV **-2**
3 EAKNLETHVPSSAAESSLEN **-2**
4 SSAAESSLENQSTQEESNS **-4**
5 QSTQEESNSPEVAHLRSVNS **-1**
6 EVAHLRSVNSDDSTHTGGAS **0**
7 DDSTHTGGASNGIQDCDSQL-357 **-3**

(B) EHV-4 gG PEPTIDE SERIES

9 277-ILAQAWKFVENETYSQHTRT **+1**
10 NETYSQHTRTYTRDAKEVDV **0**
11 YTRDAKEVDVTQPSPVQADS **-2**
12 TQPSPVQADSVLAKKRTSMK **+3**
13 VLAKKRTSMKNNPIYSEGK **+3**
14 NNPIYSEGKPHAKPFSTIDS **+1**
15 HAKPFSTIDSIHTEGMKNN **+2**
16 IHTEGMKNNPVYSESLMLNV **0**
17 VYSESLMLNVQHSDSITTGG **-1**
18 QHSDSITTGGVLHGLQDCDN-386 **-1**

(C) EHV-4 gC peptides 14 and 15 (Stokes et al., 1991)

Peptide 14: 20 37-PTTNTGEGTSSPVTPTYT-54C

Peptide 15: 21 157-FSTAHSSAKSRRVGQLGVIP-176C

Figure 6.2: Details of the gG and gC peptides.
The glycoprotein amino acid number of the first and last amino acid in each peptide series is in standard face. face. The pepscan numbers are written in relief face and the overall charge on each peptide is in bold face. The gC peptides were synthesized with an additional cysteine residue.

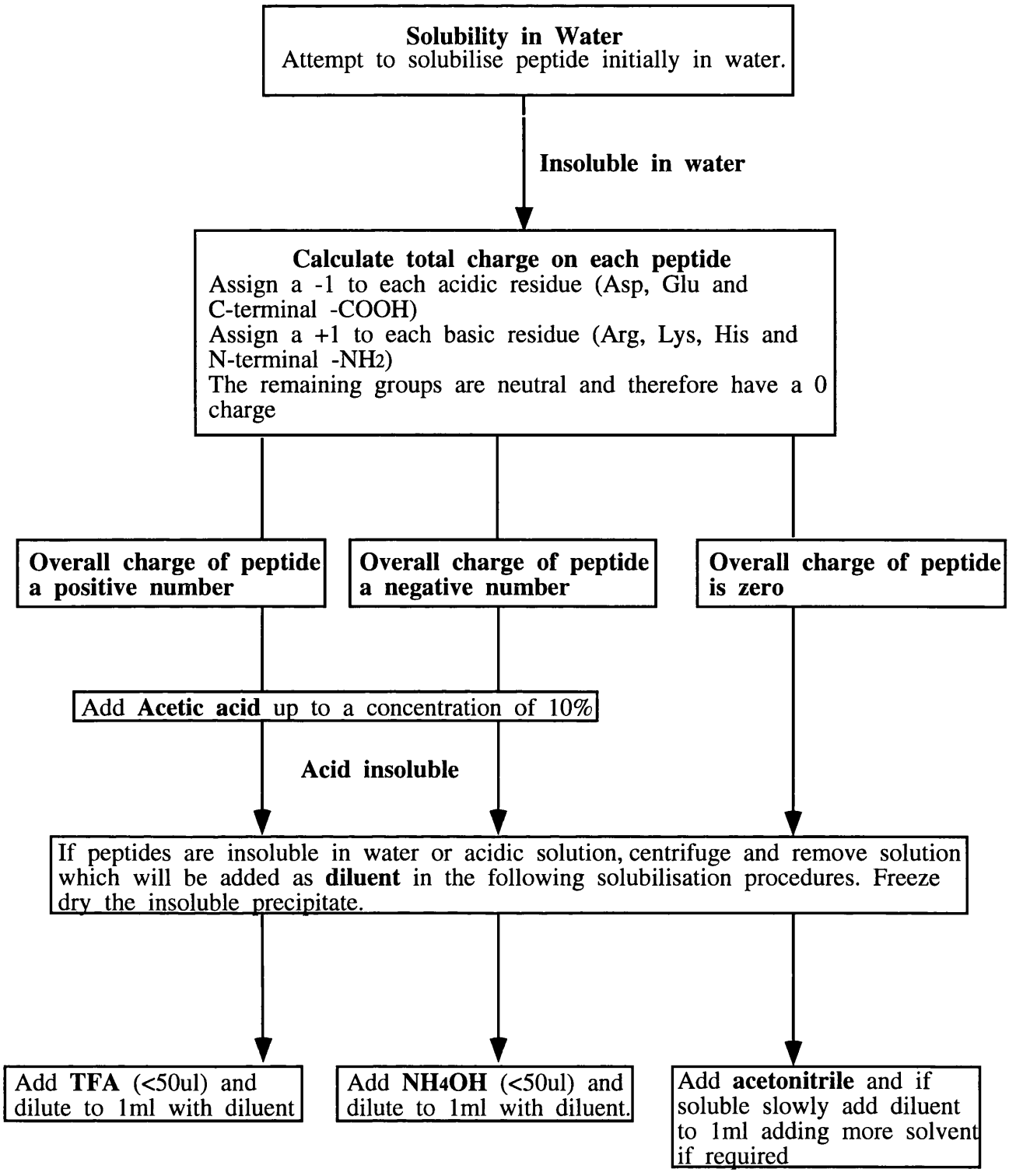
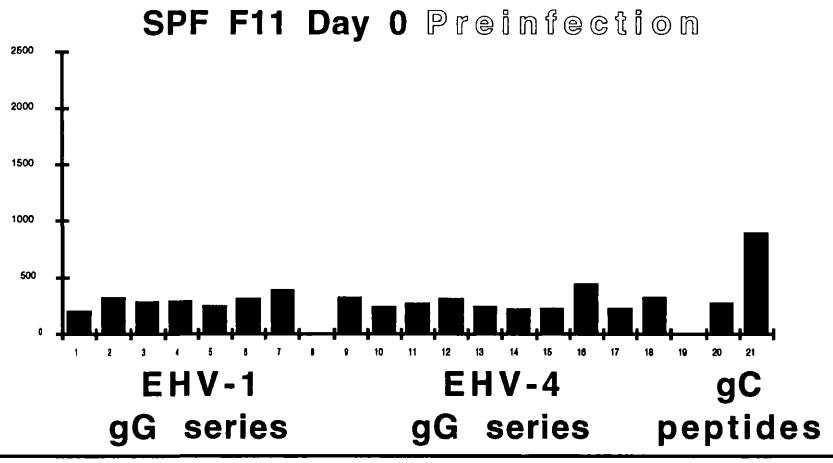


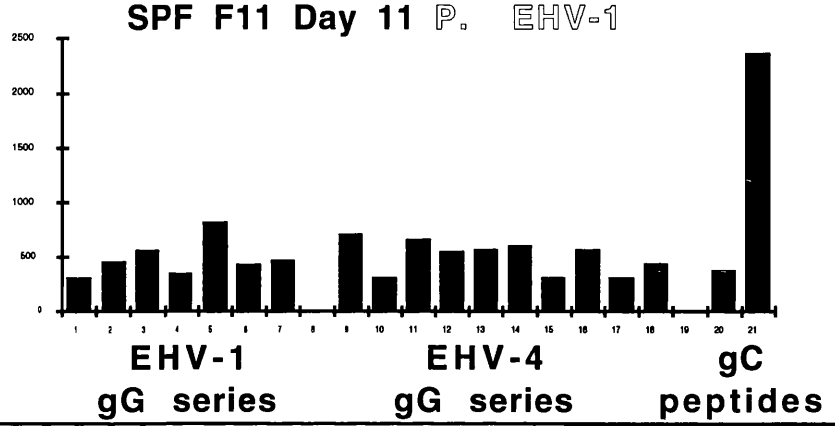
Figure 6.3: Peptide solubilisation flow chart
Adapted from the Applied Biosystems peptide synthesizer mauual

Figure 6.4: Reactivity of SPF foals 6 and 11 sera with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide. Above each graph; the foal number and the day of sampling of plasma relative to the day of primary or secondary (S) challenge are in bold followed by the virus type used for primary (P) or secondary (S) challenge in relief face. + = no. of days after, - = no. of days before infection.

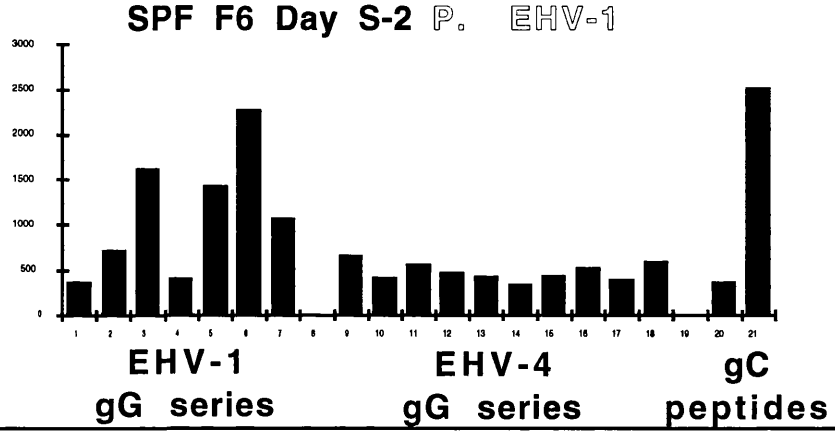
(A)


SPF F11 Day 0 Preinfection
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1
gG series
EHV-4
gG series
gC
peptides

(B)


SPF F11 Day 11 P. EHV-1
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1
gG series
EHV-4
gG series
gC
peptides

(C)


SPF F6 Day S-2 P. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1
gG series
EHV-4
gG series
gC
peptides

(D)

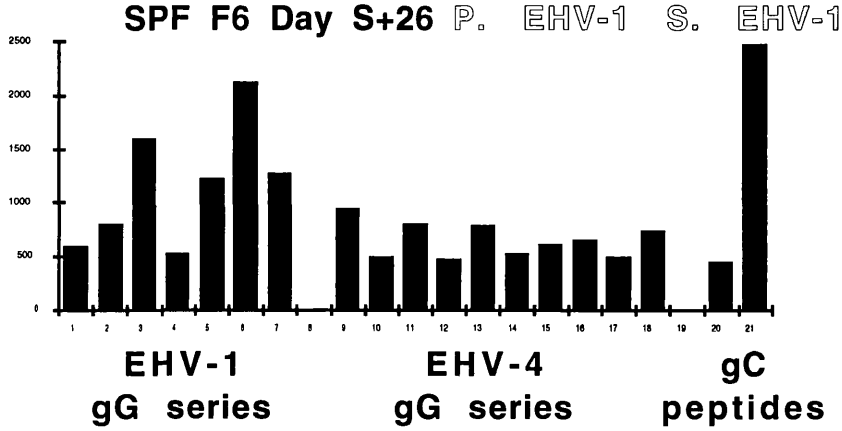
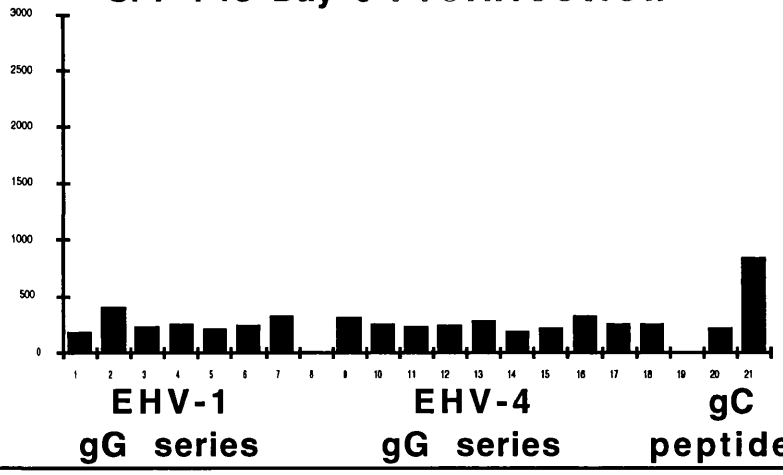
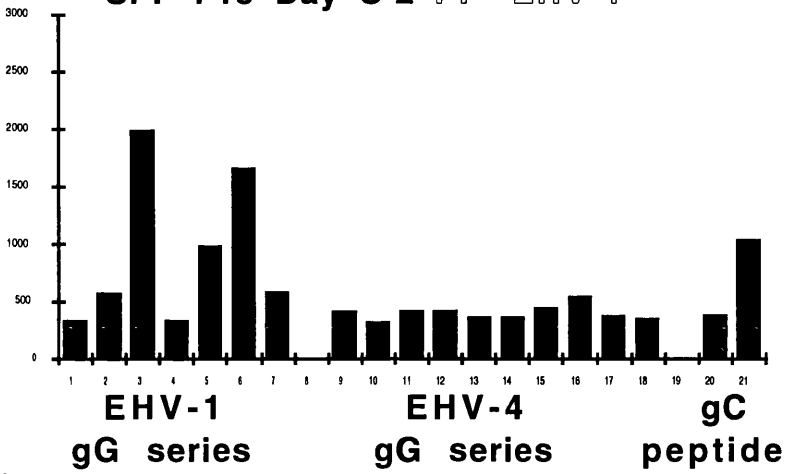

SPF F6 Day S+26 P. EHV-1 S. EHV-1
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1
gG series
EHV-4
gG series
gC
peptides

Figure 6.5: Reactivity of SPF foals 13 sera with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. Above each graph; the foal number and the day of sampling of plasma relative to the day of primary or secondary (S) challenge are in bold followed by the virus type used for primary (P) or secondary (S) challenge in relief face. + = no. of days after, - = no. of days before infection.

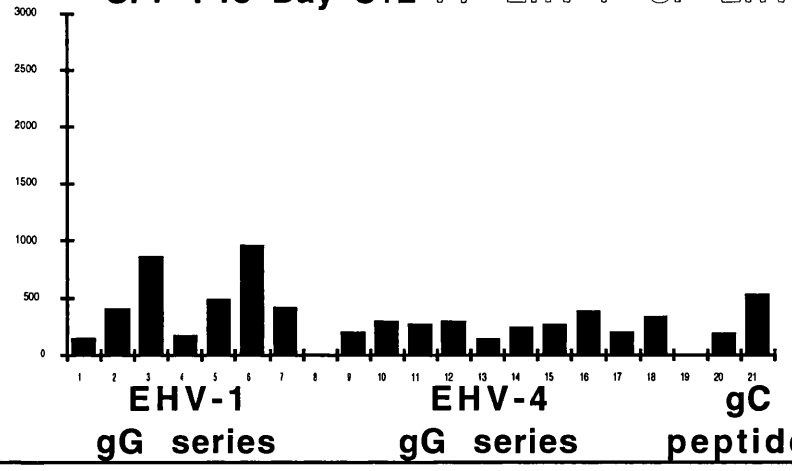
(A)


SPF F13 Day 0 Preinfection
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

(B)


SPF F13 Day S-2 P. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

(C)


SPF F13 Day S+2 P. EHV-1 S. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

(D)

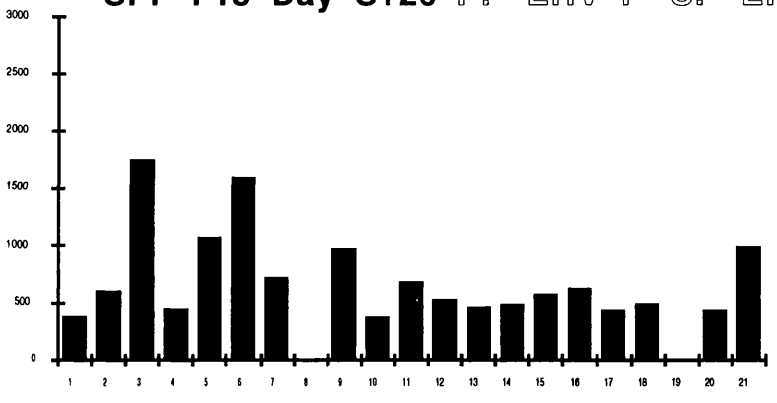
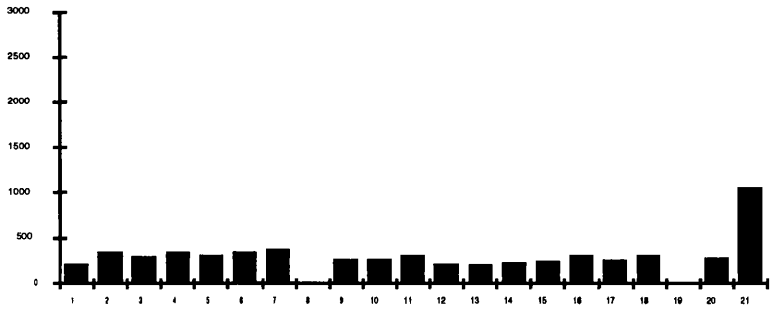

SPF F13 Day S+26 P. EHV-1 S. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Figure 6.6: Reactivity of SPF foals 12 sera with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. Above each graph; the foal number and the day of sampling of plasma relative to the day of primary or secondary (S) challenge are in bold followed by the virus type used for primary (P) or secondary (S) challenge in relief face. + = no. of days after, - = no. of days before infection.

SPF F12 Day 0 Preinfection

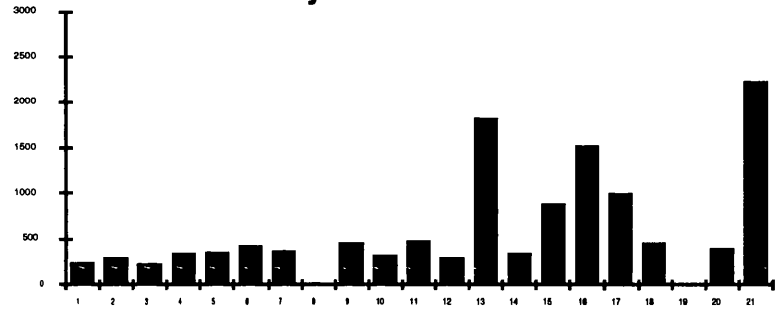
(A)



EHV-1 gG series | EHV-4 gG series | gC peptides

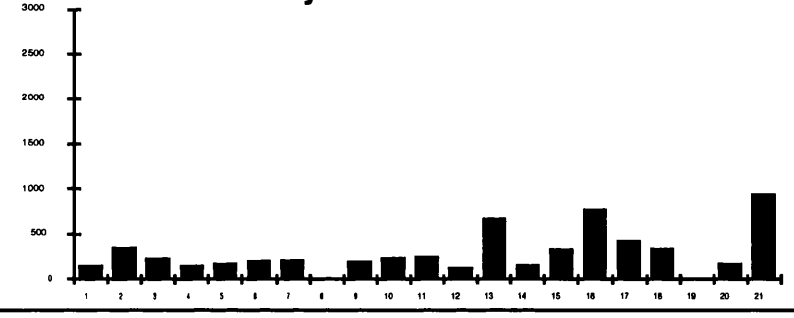
SPF F12 Day S-2 P. EHV-4

(B)



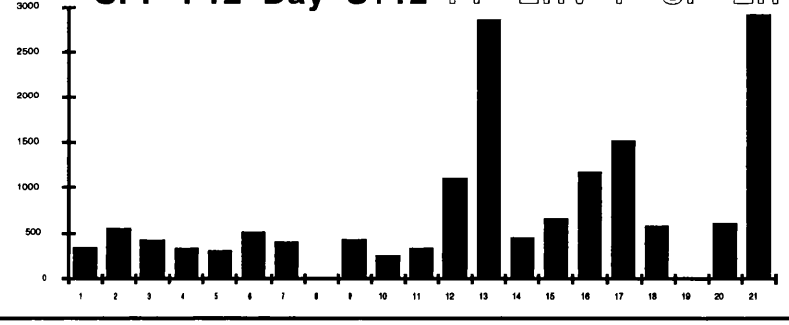
SPF F12 Day S+2 P. EHV-4 S. EHV-1

(C)



SPF F12 Day S+12 P. EHV-4 S. EHV-1

(D)



SPF F12 Day S+26 P. EHV-4 S. EHV-1

(E)

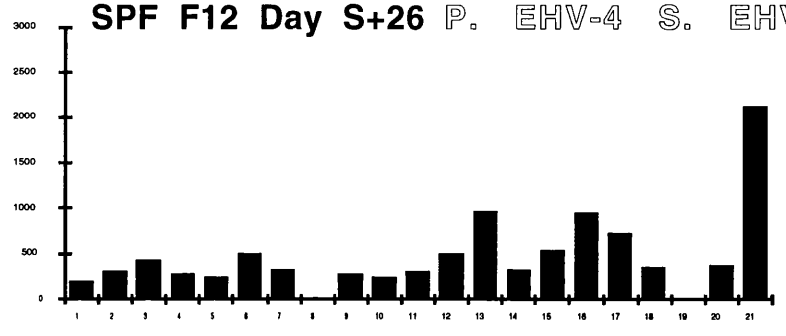
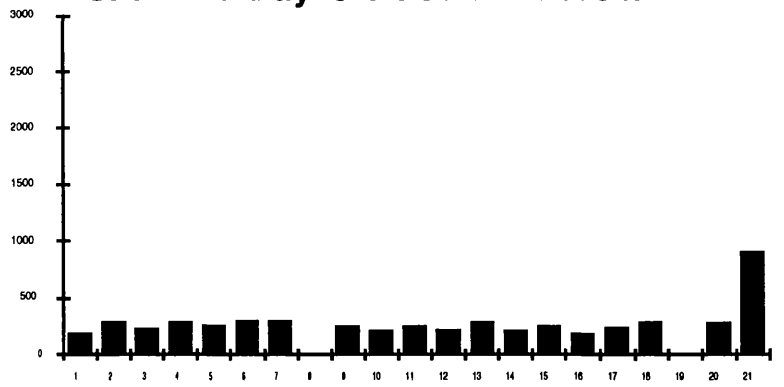
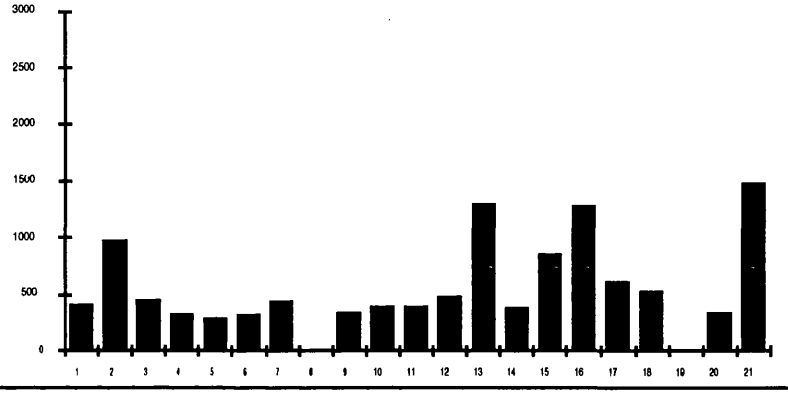


Figure 6.7: Reactivity of SPF foals 14 sera with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. Above each graph; the foal number and the day of sampling of plasma relative to the day of primary or secondary (S) challenge are in bold followed by the virus type used for primary (P) or secondary (S) challenge in relief face. + = no. of days after, - = no. of days before infection.

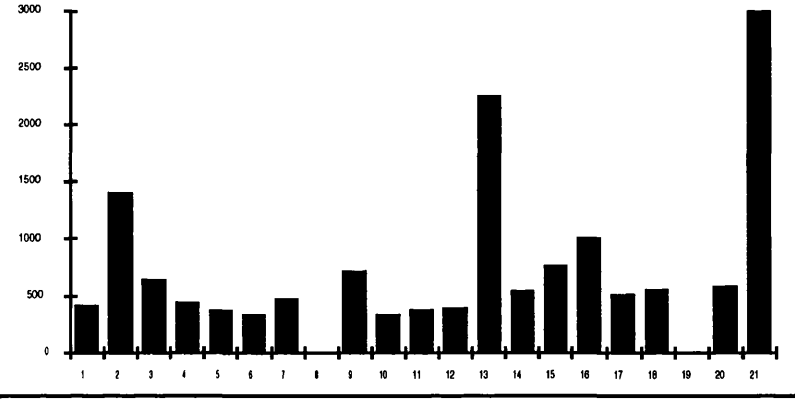
(A)


SPF F14 Day 0 Preinfection
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1
gG series
EHV-4
gG series
gC
peptides

(B)


SPF F14 Day S-2 P. EHV-4
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

(C)


SPF F14 Day S+12 P. EHV-4 S. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

(D)

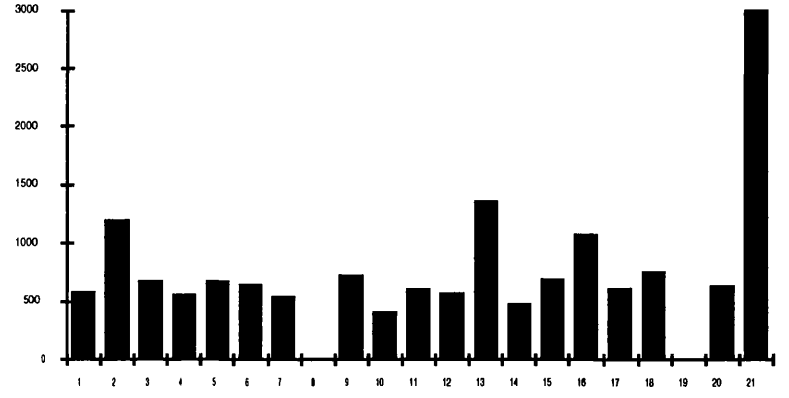
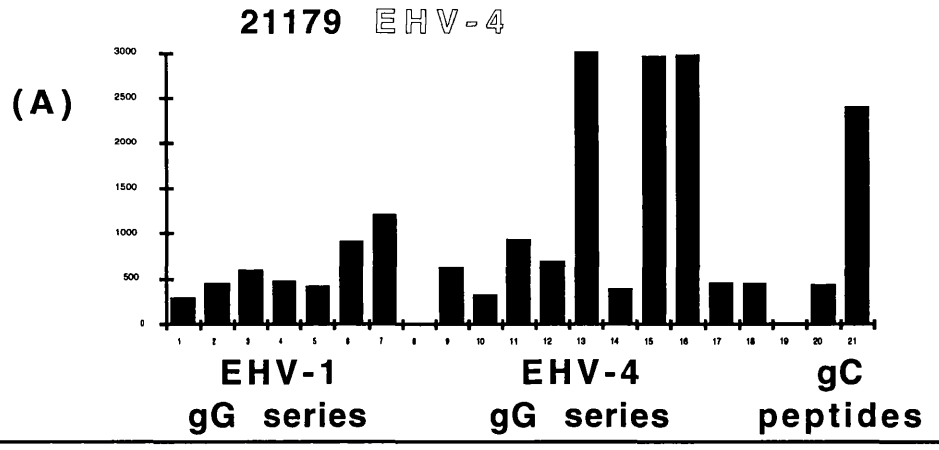
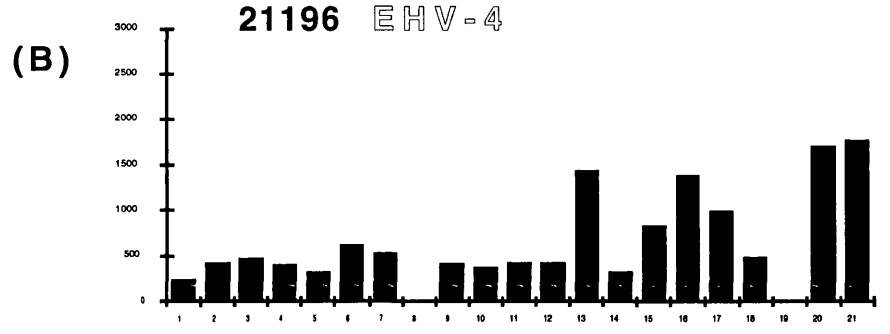
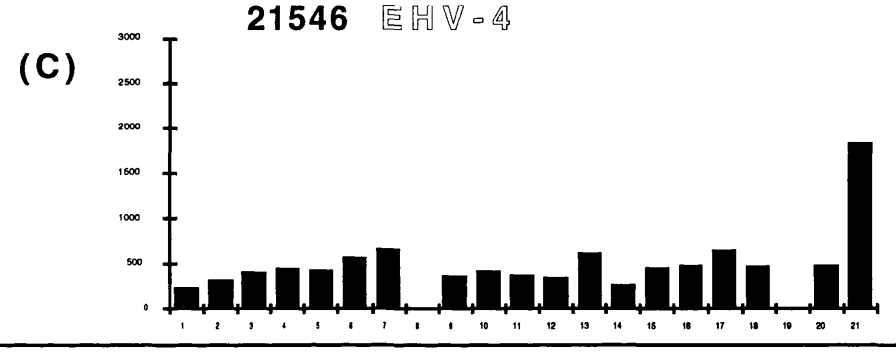
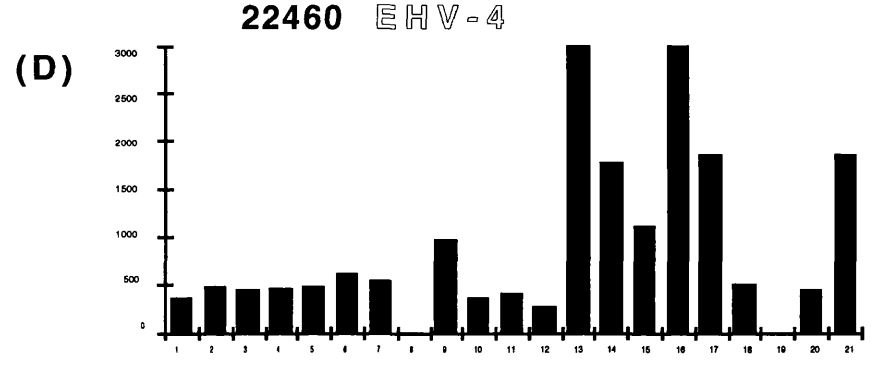

SPF F14 Day S+26 P. EHV-4 S. EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Figure 6.8: Reactivity of field sera from animals associated with EHV-4 with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. The serum number and associated virus type are positioned above each graph in bold and relief face respectively.


21179 EHV-4
(A)
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides


21196 EHV-4
(B)
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


21546 EHV-4
(C)
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


22460 EHV-4
(D)
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

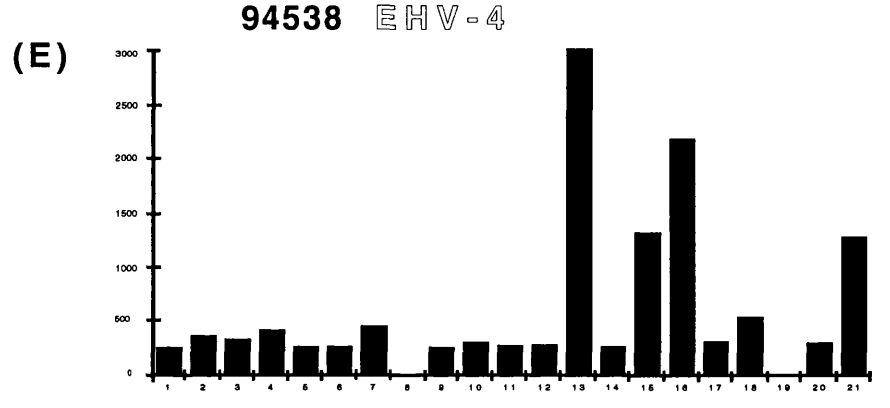
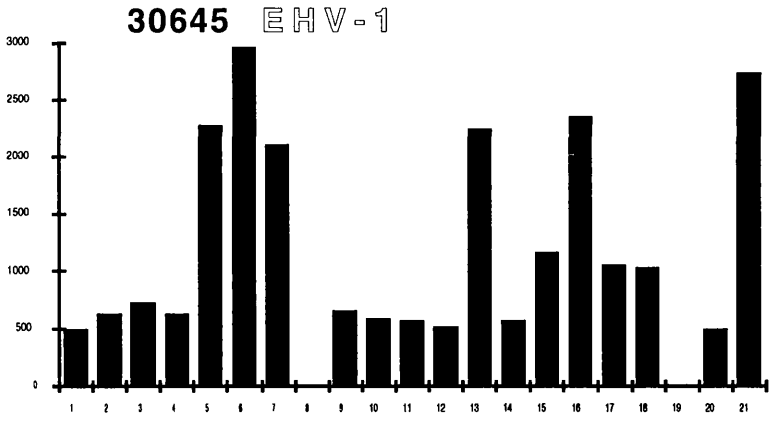

94538 EHV-4
(E)
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

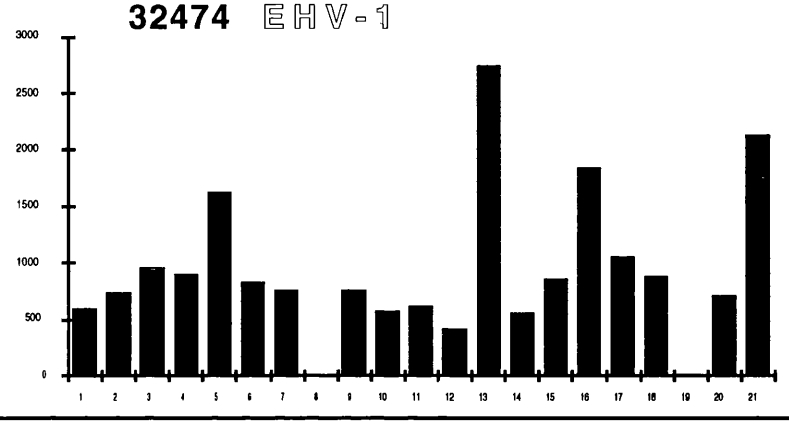
Figure 6.9: Reactivity of field sera from animals associated with EHV-1 with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. The serum number and associated virus type are positioned above each graph in bold and relief face respectively.

(A)

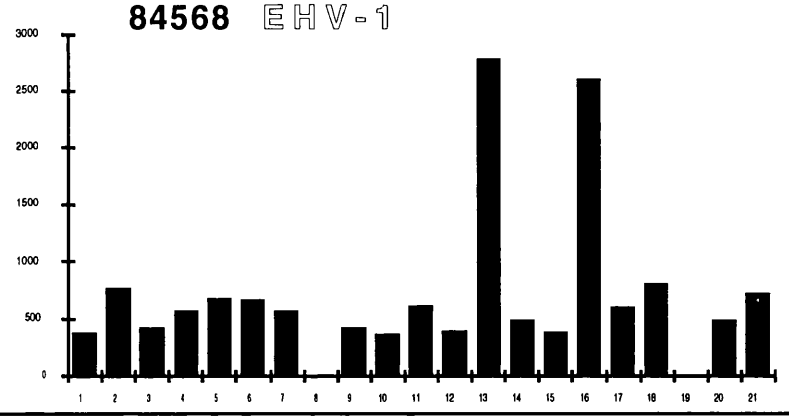


EHV-1 gG series | EHV-4 gG series | gC peptides

(B)



(C)



(D)

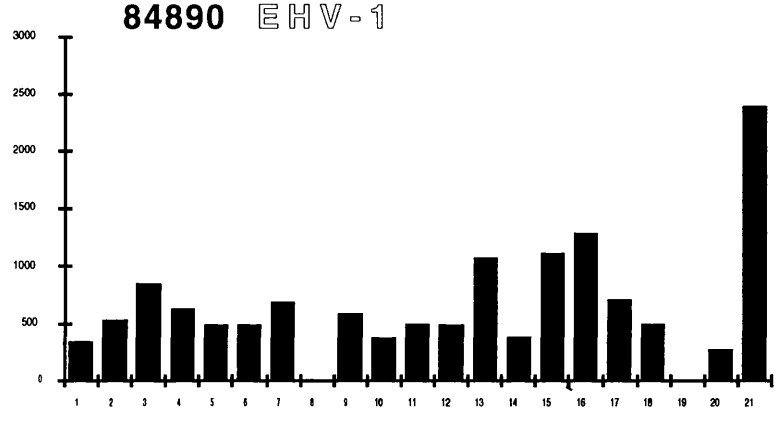
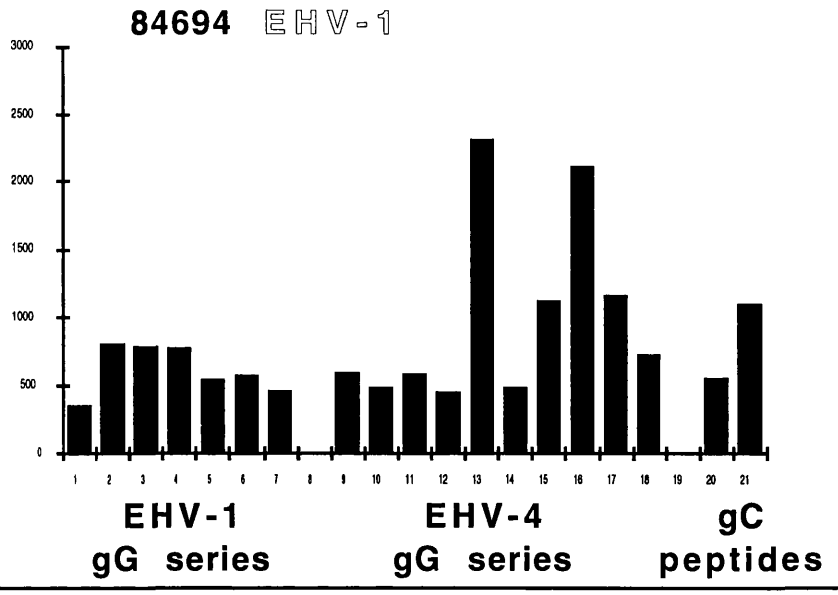
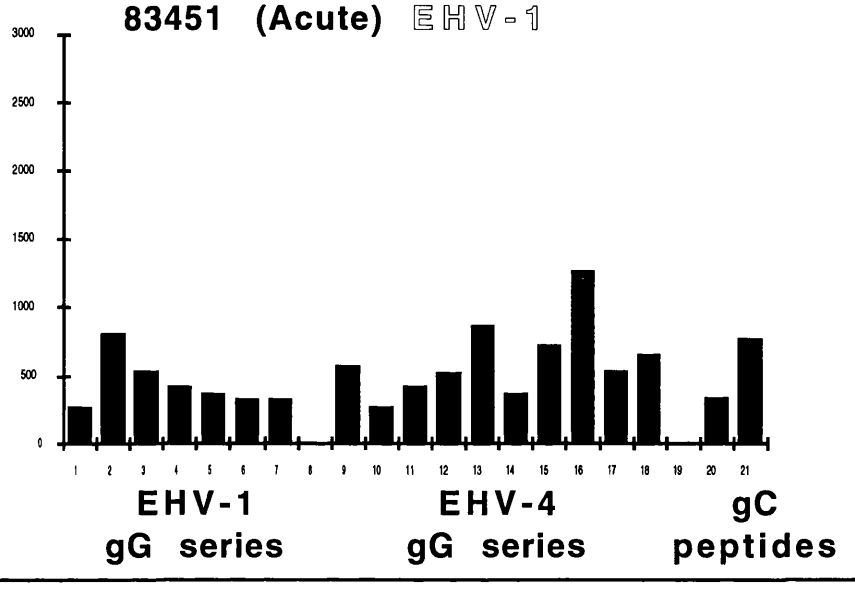


Figure 6.10: Reactivity of paired acute and convalescent field sera from animals associated with EHV-1 with gG and gC peptides. The vertical axes are labelled $O.D_{650nm}$ x1000. The horizontal axes are labelled in bold face according to the position of each peptide series and in standard face according to the pepscan number of each peptide and this labelling is conserved for all graphs. The serum number and associated virus type are positioned above each graph in bold and relief face respectively.

(A)


84694 EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

(B)


83451 (Acute) EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

(C)

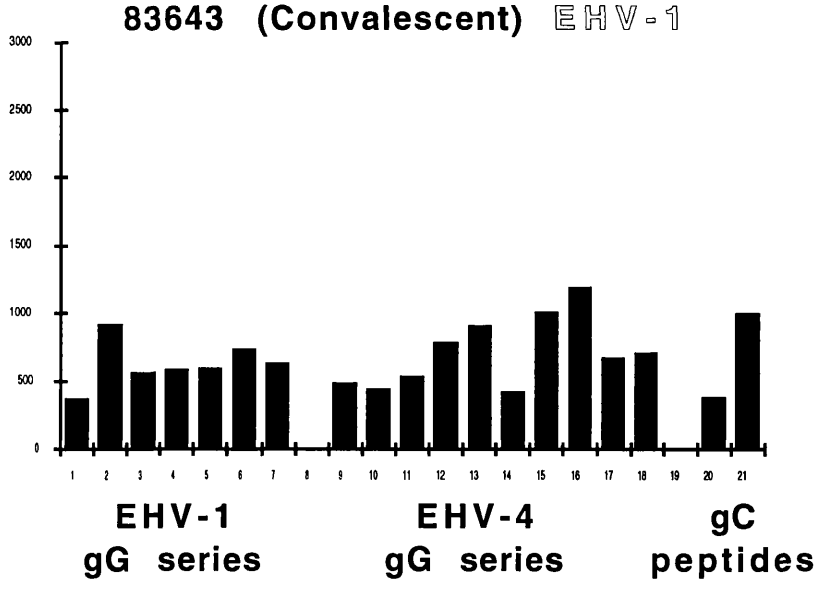

83643 (Convalescent) EHV-1
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
EHV-1 gG series
EHV-4 gG series
gC peptides

Figure 6.11: Partial sequence alignments of the gG amino acid sequences of EHV-4 strains NS80567 (Telford *et al.*, 1998) and 405/76 (Crabb *et al.*, 1992). Regions of 100% identity are given in red while those which are not homologous are given in black. The pepscan numbers of the peptides in the EHV-4 gG peptide series are in green and the relative position of each peptide in the aligned sequences is delineated by vertical blue lines. The amino acid numbers (Crabb *et al.*, 1992) are in yellow.

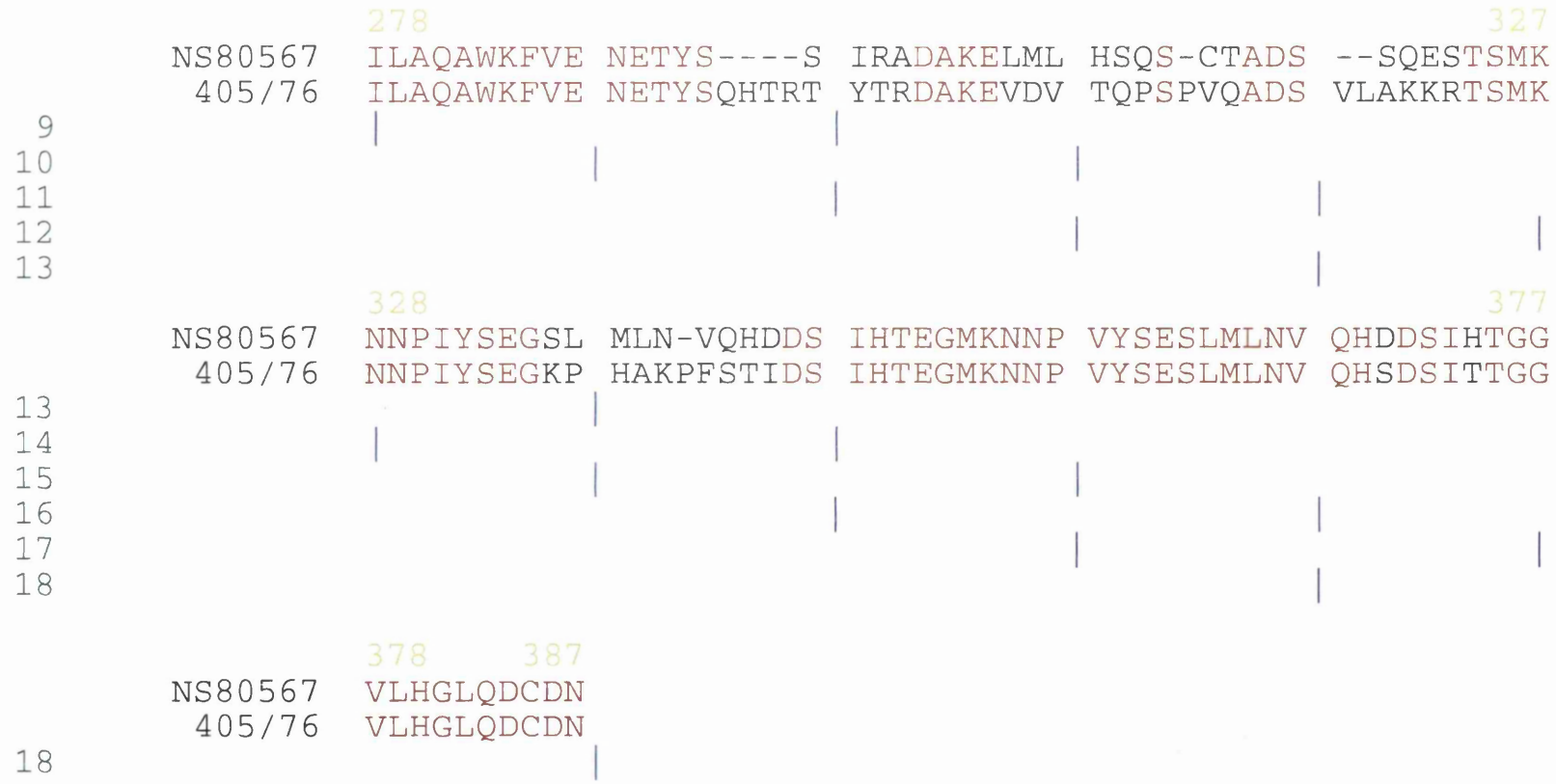

278
327
NS80567 ILAQAWKFVE NETYS----S IRADAKELML HSQS-CTADS --SQESTSMK
405/76 ILAQAWKFVE NETYSQHTRT YTRDAKEVDV TQPSPVQADS VLAKKRTSMK
9
10
11
12
13
328
377
NS80567 NNPIYSEGSL MLN-VQHDDS IHTEGMKNNP VYSESLMLNV QHDDSIHTGG
405/76 NNPIYSEGKP HAKPFSTIDS IHTEGMKNNP VYSESLMLNV QHSDSITTGG
13
14
15
16
17
18
378 387
NS80567 VLHGLQDCDN
405/76 VLHGLQDCDN
18

Table 6.1: Details of the SPF foal plasma used in this study.

SPF foal	Time course of experimental infection	Primary challenge virus	Secondary challenge virus	Samples analysed (Abbreviation)
11	Initially infected with EHV-1	**EHV-1**	**ND**	Day 0: 2 days prior to primary infection **(D0)**. Day 11: 11 days after primary infection **(D11)**
6	Initially infected with 10^7 PFU of EHV-1 strain AB-4. Received 3 dexamethasone injections (2mg/Kg) over the course of the first 3 days of infection. Foal challenged with 10^7PFU of EHV-1 strain AB-4 104 d.p.i	**EHV-1**	**EHV-1**	2 days before secondary infection **(DS-2)**. 26 days after secondary infection **(DS+26)**.
13	This foal had the same infection schedule as foal 6 with the exceptions that dexamethasone was not administered and secondary challenge commenced 84 d.p.i.	**EHV-1**	**EHV-1**	Day 0 **(D0)**. 2 days before secondary infection **(DS-2)**. 2 days after secondary infection **(DS+2)**. 26 days after secondary infection **(DS+26)**
12	Foal initially infected with 10^7PFU of EHV-4 strain MD. Challenged with 10^7PFU of EHV-1 strain AB-4 84 d.p.i.	**EHV-4**	**EHV-1**	Day 0 **(D0)**. 2 days before secondary infection **(DS-2)**. 2 days after secondary infection **(DS+2)**. 12 days after secondary infection **(DS+12)**. 26 days after secondary infection **(DS+26)**.
14	Infection schedule identical to that of foal 12	**EHV-4**	**EHV-1**	Samples from the same days as those for foal 12 were analysed.

Serum	Titre	Description	Supplier
21179	(1) 640 (4) 80	**EHV-4:** Convalescent serum from a horse in-contact with a mare with EHV-4 respiratory disease.	AHT
21196	(1) 5: (4) 10	**EHV-4:** cohort serum of 21179, 22460 and 21546.	
21546	(1) 40 (4) 160	**EHV-4:** cohort serum of 21196,22460 and 21179	AHT
22460	(1) 640 (4) 640	**EHV-4:** cohort serum of 21196, 21179 and 21546	AHT
94538	No details	**EHV-4:** serum from a horse from which EHV-4 was isolated.	IEC
30645	(1) 160 (4) 80	**EHV-1:** convalescent serum from a horse in-contact with a mare which aborted an EHV-1 positive foetus.	AHT
32474	(1) 640 (4) 320	**EHV-1:** cohort serum of 30645.	AHT
84890	High titre	**EHV-1:** serum from horse which had been vaccinated after contact with horses with EHV-1 induced paralysis.	IEC
83451A	Low titre	**EHV-1:** Acute sample from horse involved in an outbreak of EHV-1 paralysis	IEC
83643C	High titre	**EHV-1:** Convalscent sample taken from horse from which sample 83451 was derived	IEC
84568	(1) 80: (4) 40	**EHV-1:** No details.	IEC
84694	(1) 80: (4) 20	**EHV-1:** No details.	IEC

Figure 6.2: Details of sera from naturally infected animals. IEC= the Irish Equine Centre, Kildare, Ireland. AHT= the Animal Health Trust, Newmarket, UK.

Peptides Method	1	2	3	4	5	6	7	9
BCA	0.843	1.552	1.852	1.442	0.670	0.974	1.043	0.501
U.V	0.133	0.083	0.023	0.146	0.008	0.023	0.029	0.231

10	11	12	13	14	15	16	17	18	21
2.257	1.202	0.886	2.194	1.614	1.165	1.920	0.658	0.899	0.395
0.252	0.132	0.019	0.170	0.145	0.013	0.084	0.006	0.022	0.005

Table 6.3: Estimation of peptide concentration using the BCA and U.V methods. The values given are O.D readings at 280nm and 562nm for the U.V and BCA tests respectively. Column 21 corresponds to the control peptide. The numbers 1 to 21 excluding 8, 19 and 20 correspond to the pepscan number of each peptide (see figure 6.2).

6.6 DISCUSSION

This study has further delineated the epitopes identified and located by Crabb and Studdert (1993) on EHV-1 gG homologue. There are at least two regions of the EHV-1 homologue that are consistently recognised and may contain one or more epitopes. Pepscan analysis as utilised herein may allow further definition of the epitopes contained within these regions.

Telford and colleagues (1998) have recently brought to attention the errors in the DNA sequence of the EHV-4 gG gene determined by Crabb *et al* (1992), the translation of which was utilised in this thesis for the selection of peptides for analysis. An alignment of the translations of the correct and incorrect sequences demonstrates that many of the peptides supposedly representing the EHV-4 gG peptide sequence of the region under scrutiny are incorrect (see figure 6.11). The region encompassing peptides 10 to 14 of the EHV-4 gG peptide series contains the most errors. It follows that these peptides do not accurately represent the EHV-4 gG peptide sequence and may have precluded the identification of epitopes contained within the correct EHV-4 gG peptide sequence. On the other hand, relatively short stretches of amino acids are required for the formation of linear epitopes and in some cases a certain degree of variation from the consensus epitope sequence can be tolerated (Geysen *et al.*, 1987). There are short regions of consensus between the correct and incorrect sequences. Synthesis of a new EHV-4 gG peptide series based on the correct sequence would resolve these issues.

Two peptides from the EHV-4 gG peptide series were consistently reactive with anti-EHV-4 sera: peptide 16 which contains no errors and peptide 13 which contains 8 errors out of a total of 20 amino acids. Interestingly these two peptides contain a core sequence (M-K-N-N-P-V/I-Y-S-E) which could represent an epitope recognised by anti- EHV-4 sera. Phage encoding this core sequence were purified using immune horse sera from an expression library of EHV-4 DNA (Wilson *et al.*, 1994).

In agreement with the study of Crabb and Studdert (1993) it was found that with the SPF foals, responses to the primary challenge virus seemed to drop

initially on secondary challenge with the heterologous but also the homologous virus. Whether this is due to non-specific virus induced immunosuppression, as previously documented for EHV-1, remains to be proven.

The ability of the gG system as utilised here to consistently diagnose EHV-1 infection is questionable. In some experimental and naturally infected animals it was not possible to detect an expected reactivity with EHV-1 peptides. Similarly one animal associated with EHV-4 did not react with any peptides in the gG series. Two possible reasons were initially considered; firstly, the signal to noise ratio was quite low in many of these cases which may have precluded the identification of weakly positive samples and secondly, the time at which sampling occurred may not have been ideal for the detection of anti-EHV-1 reactivity. In the case of the animal associated with EHV-4 it may be reactive with a peptide which was not accurately represented by the EHV-4 gG peptide series as alluded to earlier.

With regard to the problem of signal to noise ratio, further dilution of the sera in question or monitoring of the ELISA substrate reaction as it proceeded did not reveal positivity in any samples which were negative under the standard conditions employed. Many factors have been implicated in low signal to noise ratios in antibody capture ELISAs. These include the overall charge of the antibody capture system (Pesce *et al.,* 1986; Graves. 1988a) and non-specific binding of immune complexes to the plastic surface (Cafruny *et al.,* 1986). The contribution of cationic peptides to the high background observed herein was not investigated, but it may have been prudent to include an anionic matrix coat to reduce non-specific binding of serum to cationic peptides (Graves. 1988b). Notably, the majority of peptides in the EHV-1 gG series are anionic in terms of overall charge. Of particular note in the gG ELISAs was the increased background reactivity in SPF foal sera that reacted strongly with peptides diagnostic for infection. Although supposition, this may reflect the development of immune complexes. Immune complexes have been demonstrated in animals suffering from the paralytic form of EHV-1 induced disease (Edington *et al.,* 1986). The microtitre plates used in the gG ELISAs were shown to bind immune complexes (Cafruny *et al.,* 1986). To circumvent the potential contribution of immune complexes in the equine sera diferent plates, namely immulon I, were used and Tween 20 was added to all diluted antibodies at a concentration of 0.5%, as was EDTA and EGTA, both at a

concentration of 7.5mM. These latter two precautions have been shown to reduce non-specific binding of serum proteins in ELISAs (Cafruny *et al.,* 1986; Nielsen *et al.,* 1994). None of the precautions taken made a significant difference to the overall results. A point of potential relevance was the demonstration that preimmune SPF foal sera prepared in Glasgow Vet school from plasma supplied by Cambridge Vet school reacted strongly with all pepscan peptides (see chapter 5) but not with the gG peptides. It seems likely that the procedures used in Glasgow did not efficiently remove many of the clotting factors which could have decreased the signal to noise ratio or masked potentially weak reactivity with the EHV-1 gG series. Crabb *et al* (1995) demonstrated that positive sera and plasma from a given horse gave identical results in the gG fusion protein ELISA.

Those SPF foals which were positive for EHV-1 were sampled 79 days after primary infection and unfortunately, earlier samples were not analysed which may have shed light on the problem. In this respect foal 11 was analysed 11 days post infection and foals 12 and 14 were tested 2, 12 and 26 days after secondary infection. It is possible that in the case of foal 11 and foal 12 and 14 on days 2 and 12 postinfection that these animals had not yet seroconverted. Seroconversion in general occurs 14d.p.i in EHV-1 infected SPF foals (Gibson *et al.,* 1992a; Tewari *et al.,* 1993). The majority of animals which were EHV-1 gG antibody negative at the time of diagnosis of the index case in an EHV-1 associated abortion storm had not seroconverted by day 13 post index case but were positive 54 days later (Drummer *et al.,* 1995). Seroconversion may have been detected earlier if samples were available or alternatively, these animals may not have necessarily been infected at the exact time of the index case. During an abortion storm EHV-1 antibodies were detected in mares 2 days prior to the index case but it is not known when these animals developed an active infection. Notably 7/31 in-contact mares in that outbreak remained seronegative for EHV-1 as determined by gG ELISA (Drummer *et al.,* 1995). On the other hand EHV-1 gG antibodies were detected in sera from experimentally infected animals as early as 21 days post infection (Crabb *et al.,* 1995). In the case of animals that were initially infected with EHV-4 it is possible, given the lower sensitivity of the assay for detecting EHV-1 antibodies (as determined by lower peak O.D values), that EHV-1 gG antibodies may not be detected in dual infected animals, as was suggested for gG serodiagnosis of dual HSV-1 and -2 infections in humans (Ho *et al.,* 1993).

Detection of EHV-1 antibodies in an SPF foal 3-5 months after primary EHV-4 infection, 1-2 months after secondary EHV-1 infection, was not problematic (Crabb and Studdert, 1993). In contrast a recent evaluation of different gG diagnostic systems for the herpes simplex viruses of humans has demonstrated that some individuals who were initially seropositive can become seronegative over time. There was very little agreeement between the different systems concerning an individuals shift from seropositivity to seronegativity. Furthermore, the results, as yet , have only been demonstrated for a defined group of individuals (Schmid *et al.,* 1999).

Both Crabb and Studdert (1993) and this study imply that infection with either EHV-1 or -4 can suppress the immune response to gG epitopes. Given that the responses to the EHV-1 gG peptide series were relatively weak it is possible that during the initial stages of infection the weak response is suppressed to levels not detected by ELISA. It has been suggested that the problem of weak or false positives be addressed by the utilisation of a western blot system based on gG (Crabb and Studdert. 1993).

Another valid reason which was discussed in chapter 5 in relation to the pepscan system is that the epitopes recognised by these sera are not adequately represented in the peptide set.

Some EHV-1 associated field sera did not react appreciably with the EHV-1 series. Again many of the reasons proposed for the unresponsiveness of the SPF foal sera are also applicable to the field sera. Field sera reactivity may also be influenced by the possible existence of EHV-1 gG strain variants. However, in Australia at least, this does not seem to be the case for either EHV-1 or -4, as inferred from the consistent serological reactivity of sera from different outbreaks with the gG fusion protein. This however does not preclude the possibility that specific epitopes within the fusion protein are strain specific. If there are strain-common epitopes on the fusion protein then most, if not all, strains will be detected.

Another worrying factor with regard to the use of this system as a diagnostic aid was the spurious reaction of F14 with an EHV-1 peptide which was recognised by only one EHV-1 infected animal, 83451. This may be an inherent reactivity within the serum not associated with an encounter with EHV-1. Alternatively, this

could be the converse example of the relatively rare anti-EHV-4 gG cross reactivity in anti-EHV-1 sera (Crabb *et al.*, 1995).

Many of these problems, particularly with regard to field sera, could also conceivably relate to the different epidemiology of EHV-1 in the British Isles compared to that of Australia. EHV-1 has been present in these islands significantly longer than in Australia. The immune response of horses from the British Isles could have evolved such that some animals do not recognise these linear EHV-1 gG epitopes.

The acid-test which would address many of the aforementioned problems would be to subject the sera utilised herein to the system developed by Crabb and Studdert (1993) to ascertain if the peptides do not contain the epitopes, or do not contain them in the correct context, and if the spurious reactions and unreactivities were unique to the peptide system.

This study successfully identified peptides representing EHV-1 and -4 type-specific gG epitopes. The identification of these peptides represents the first step in making the EHV-1 and -4 type-specific serological test developed by Crabb and colleagues easily available. However a number of problems must be addressed before the peptides could be employed in routine diagnosis of EHV-1 and -4 infections.

Chapter Seven

General Discussion and Concluding Remarks

7.1 DISCUSSION

The two main aims of the research described in this thesis were to identify a virus encoded marker for neurovirulence in EHV-1 isolates and a serological test that could definitively distinguish between EHV-1 and -4 infected horses. Unfortunately these aims were not realised but nonetheless the research dictates further studies and points to future directions in order to solve these perplexing challenges in the study of EHV biology.

The sequence analysis of the N-terminal coding region of the gC gene of nine epidemiological distant isolates of EHV-1 revealed total inter-strain conservation in the glycoprotein amino-acid sequence. This conflicting result was further confused by the fact that at least one isolate, namely T373, was expected to contain sequence variation on the basis of Mab typing studies (Allen *et al.,* 1988) but did not. A number of possible reasons were put forward in chapter three but were not proven by the research described in chapter four. Resolution of this issue will first require the repetition of the Mab typing of T373 as described by Allen *et al* (1988) and an extension of the gC study.

Extension of the gC study would require the analysis of a large number of EHV-1 isolates. Given the relatively low level of inter-strain variation reported in this thesis and described recently by others using RFLP analysis of different regions of the EHV-1 genome, DNA sequencing would become a cost ineffective method of defining variation. The introduction of a pre-screening method to first identify the presence of a mutation in a PCR product derived from an isolate, followed by sequencing if differences were detected, would be a more economic approach. SSCP analysis as described in chapter four is one possible approach, however it would require the amplification of the region of interest in small overlapping sections to allow an optimal mutation detection rate. Alternatively the combined use of SSCP and RFLP analysis of larger fragments is a feasible option. In recent years with the development of PCR a variety of mutation detection methods have been utilised to detect mutations in PCR products. Some of these, particularly denaturing gradient gel electrophoresis (DGGE) (Sheffield *et al.,* 1989)

and enzymatic methods (Myers *et al.*, 1985; Ganguly and Prockop. 1990; Ellis *et al.*, 1994), are well suited for the analysis of EHV-1 gene size PCR products. DGGE and the similar temperature gradient gel electrophoresis (TGGE) have not been widely adopted as mutation detection systems because of the necessity to specially adapt pre-existing electrophoresis equipment. Wider application of these techniques may be seen with the introduction of the first commercially available system from Biorad. Herpesviruses such as EHV-1 and -4 and their genes may be particularly suited to these mutation detection systems because of their relatively high GC base composition. One relatively simple system which seems to have gone unrecognised by the wider scientific community is known as low-stringency single specific primer PCR (LSSP-PCR). The system developed by Pena and co-workers (1994) involves subjecting a PCR product or DNA fragment of interest to a PCR which contains high concentrations of a single specific oligonucleotide primer and of Taq DNA polymerase and involves thermal cycling with relatively low annealing temperatures. Analysis of the PCR should reveal a complex banding pattern or fingerprint that will differ between templates with only single base differences.

The serological studies were not unlike the preceding gC study with regard to odd results, the most enigmatic being that of serum 94538. A complete chemical and physical analysis of this serum and its individual constituents may give clues as to its bizarre properties. One possible approach would be to study the interaction of this serum with appropriate antigens utilising plasmon surface resonance technology. Biosensors based on this technology allow the rapid determination of many of the parameters governing antibody antigen interactions in real time (Morgan *et al.*, 1998 and references therein), however it remains to be seen if these systems will be suitable for the detailed analysis of polyclonal antiserum.

The failure to identify a suitable diagnostic peptide or peptide combination was tempered by the identification of previously unknown linear epitopes and the confirmation that previously identified epitopes are also recognised by sera from affected animals. That these epitopes are specific for either EHV-1 or -4 will require further confirmation since the pepscan method can give rise to spurious results. Indeed all the pepscan results ideally should have been confirmed by repeating the analysis of each serum after it had been pre-adsorbed with the virus antigen with which it is associated (Schwab *et al.*, 1993). Further confirmation and

an extension of the findings of the pepscan analysis would be achieved by synthesizing a more representative set of peptides for those regions that gave extremely variable results or in the case of newly identified epitopes, the synthesis of fusion proteins encompassing the regions in which the peptides are located. These approaches might eventually lead to the detection of reactivity in animal serum previously not shown to react with the peptides derived from these regions. Furthermore the assessment of the importance of these new epitopes with regard to host control of infection would be of interest.

The gG diagnostic system is certainly of great retrospective epidemiological value. Its usefulness in a clinical laboratory remains unclear. The study described in this thesis dictates that a detailed analysis of the time course of the immune response to the EHV-1 peptides of serum from experimentally infected animals needs to be addressed. If this yields negative results then a more extensive set of peptides representing the EHV-1 region will have to be synthesized and assessed. Alternatively, proteins closely matching the native EHV-1 and -4 gG glycoproteins could be expressed and purified from the Baculovirus (Sanchez-Martinez *et al.,* 1991) or Yeast (Scorer *et al.,* 1993) based expression systems such that the type-specific conformational epitopes referred to by Crabb and Studdert (1993) might become available for assessment as clinical diagnostic tools. Diagnosis may be further enhanced if the detection of type specific IgM responses to these proteins were identified and analysed as has been described for HSV-2 (Ho *et al.,* 1992). Although not documented in this thesis one attempt was made at identifying IgM responses in SPF foals to the EHV-1 and -4 gG peptide series albeit without success.

In conclusion, there may be some overlap between the gC sequencing and the pepscan studies which may be of significance in relation to the biology of EHV-1 in particular. Horses are generally susceptible to reinfection six months after a previous episode of EHV-1 infection. The studies described here show that different horses, even those that are blood relatives and infected with the same strain, show qualitative and quantitative variability in the fine specificity of the epitopes that their serum recognises. Whether this is also true of conformational epitopes remains to be proven. However it might be postulated that given the relative weakness and inconsistency of the equine immune response to EHV-1 that, contrary to accepted

concepts for other host-virus relationships, EHV-1 is not under immunological pressure to evolve. Therefore there is little if any inter-strain variation in these viruses, gC being a case in point. Indeed the recent discovery that EHV-1 encodes a VHS protein which does not function *in vivo* (Feng *et al.,* 1996) may provide the answer as to why this is so. This discovery presents the possibility that host enzymes may still be available to correct errors made during the replication of EHV-1 DNA, thus leading to a lower virus mutation rate.

Given that the differences between strains of EHV-1 are relatively small it is likely that the viral determinants of whether a given strain will give rise to the paralytic syndrome characteristic of some EHV-1 infections are probably very elusive indeed. It is now recognised that there is probably a spectrum of pathogenicity in the natural EHV-1 and -4 strain populations. The two extremes of this spectrum, namely low and high pathogenic isolates, will probably form the basis of the most constructive means of identifying the virus determinants of pathogenicity in general and neurovirulence in particular. Molecular methods are already at hand to dissect these isolates e.g. by the deletion of specific genes and the interchange of genomic regions between isolates of well defined, albeit greatly different, pathogenic potential (Van Zijl *et al.,* 1988). The introduction of these mutant and chimeric viruses into the natural host and other model systems should yield valuable data. These approaches will most likely identify the regions of the virus genome involved in determining a viruse's pathogenicity but this will ultimately have to be extended to the general strain population. An approach similar to that outlined earlier for the extension of the gC study i.e the prescreening of regions of interest from different strains for the presence or absence of mutations would be applicable.

An alternative approach to identifying strain specific markers of neurovirulence which does not rely on prior knowledge of the genes that are potentially involved is to fingerprint a large number of isolates using the new generation of fingerprinting techniques. These techniques include the widely used random amplified polymorphic DNA (RAPD) analysis system (Williams *et al.,* 1990) and the combined RFLP/SSCP approach referred to earlier. Such systems could be used in combination with XL PCR (Barnes. 1994) to exclude regions of the virus genome which are inherently unstable i.e. the repeat regions that may provide

undue interference in such analyses. This is not to say that the repeat regions do not play an important role in the determination of a viruses pathogenicity. These areas tend to have complex transcriptional activity and also may affect viral replication.

Recent advances in DNA chip technology (for a review see Lipshutz *et al.*, 1999) might permit the identification of all types of genomic polymorphisms within the genome of any EHV-1 strain. This would involve the production of DNA chips containing the entire prototypic EHV-1 genome represented as small oligonucleotide sequences. High stringency hybridisation of these chips with a probe representing the entire genome of any strain should reveal polymorphisms within that strain relative to the prototypic virus. A comparison of the hybridisation pattern of probes generated from different strains would allow the identification of regions of the EHV-1 genome that are particularly polymorphic that have yet to be identified by current technologies.

In the age of molecular biology the power of basic techniques tends to be overlooked. An interesting yet to be published experiment would be to assess the yield of different EHV strains of varying pathogenicity from various cell types in order to achieve some idea of their relative replication rates. This alone may have a major influence on whether a virus reaches a particular tissue in sufficient quantities to exert its pathogenic effect. A more advanced version of this experiment would be to fingerprint e.g. by differential display reverse transcriptase PCR (ddRTPCR), a variety of different cell types infected by a variety of EHV isolates of varying pathogenicity to elucidate what effects, if any, different strains have on transcription in different cell types and vice-versa. The findings of this study could then be extended to the natural host via *in situ* PCR or hybridisation studies. ddRTPCR has recently been used for the preliminary characterisation of changes in cellular gene expression on infection with PRV (Hsiang *et al.*, 1996).

The viral determinants of pathogenicity have been a focus of this thesis but host factors cannot be excluded. Although there may be a spectrum of pathogenic potential in the natural virus population it is likely, given that recent studies have found little variation at the molecular level in the virus population, that the majority of viruses have roughly equal pathogenic potential and in most cases that it is the interplay between virus and host that determines the outcome of a given infection.

Two independent studies have indicated that pregnancy may predispose mares to the paralytic syndrome associated with EHV-1 infection. The factors e.g. hormonal control of immune responses, that may be at work here and how they may relate to the same syndrome in affected non-pregnant mares and stallions remain elusive. The inference of the involvement of the immune response gains further support from the idea that damage to nervous tissue may be elicited by immune effector mechanisms e.g. immune complexes. Furthermore it has been demonstrated that at least one cytokine, namely TGF-ß, is directly involved in the immunosuppression induced by EHV-1 (Charan *et al.,* 1997). It follows that some animals, that may have genome encoded anomalies in related aspects of the cytokine network, may be more or less susceptible to EHV-1 induced disease. A recent study of HSV-1 in an animal model system has suggested that the antibody response to virus has a major influence on the ensuing pathology of a HSV-1 infection. In this case the immune system controlled the spread of virus to the sites where the virus would normally cause damage rather than playing an active positive role in the generation of pathology (Mitchell and Stevens. 1996). It remains to be seen whether it is the infecting strain, the host or both that determines the effect of the immune response. Indeed, results in this thesis suggest that there are differences between the antibody response of different yet related animals infected with the same strain of virus. One possible approach to further investigate this problem would be to use expression or random peptide libraries (Cwirla *et al.,* 1990; Scott and Smith. 1990) to establish a large bank of epitopes recognised by pools of animal serum, then to utilise these banks to establish epitope profiles of sera from animals associated with different manifestations of EHV-1 infection. Incidently, this technology could also be employed to identify peptides or epitopes useful for the differentiation of EHV-1 from EHV-4 infected horses.

The advance of the human genome project has spawned many studies investigating the genetic susceptibility to infectious diseases. These studies by their very complex nature require very dense genetic maps (Dib *et al.,* 1996). The equine research community has already taken the first steps in identifying some genetic markers based on micro- and mini-satellite repeat sequences (Van Haeringen *et al.,* 1998; Coogle *et al.,* 1996 and references therein) which may eventually

provide some evidence for the involvement of genetic factors in determining the outcome of an EHV-1 infection.

Finally most of the possible avenues described above will be of no use if detailed epidemiological data is not available to complement such studies. In a recent review Powell (1992) called for a more structured approach to epidemiological studies of equine infectious diseases. He states that:

> "The recognition of the frequent multifactorial nature of disease aetiology, the significance of subclinical disease as it affects performance and the need to understand the methods by which pathogens are disseminated have increased the necessity for epidemiological studies."

This sentiment is particularly true of the studies of EHV-1 and -4 especially with regard to their involvement in the development of unusual or relatively rare diseases in the equine.

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